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REVIEW

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Bacillus subtilis surface display technology: applications in bioprocessing and sustainable manufacturing

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Abstract

The growing demand for sustainable and eco-friendly alternatives in bioprocessing, healthcare, and manufacturing has stimulated significant interest in *Bacillus subtilis* surface display technology. This innovative platform, leveraging both spore and vegetative cell forms, provides exceptional versatility for a wide spectrum of applications, spanning from green technologies to advanced biomedical innovations. The robustness of spores and the metabolic activity of vegetative cells enable efficient enzyme immobilization, biocatalysis, and biosensor development, facilitating bioremediation, pollutant degradation, and renewable energy generation. Additionally, *B. subtilis* surface display systems have demonstrated remarkable potential in vaccine development and drug delivery, offering a cost-effective, scalable, and environmentally sustainable alternative to traditional methods. These systems can effectively present antigens or therapeutic molecules, enabling targeted drug delivery and robust immune responses. This review explores recent advancements, challenges, and opportunities in harnessing *B. subtilis* surface display technology for sustainable biomanufacturing, green innovations, and transformative biomedical applications, emphasizing its role in addressing pressing global challenges in environmental sustainability and healthcare.

Keywords *Bacillus subtilis*, Surface display technology, Sustainable biomanufacturing, Environmental biotechnology, Vaccine development

Background

The field of microbial biotechnology, which harnesses microorganisms for technological applications, has become a fundamental pillar of modern industry, driving advancements in bioprocessing, biomanufacturing, and therapeutic innovations [1]. *Bacillus subtilis* is a Gram-positive bacterium known for its adaptability and productivity. It plays a vital role as a model organism in microbial biotechnology and is extensively used in industrial applications. Its designation as Generally Recognized as Safe (GRAS) by the U.S. Food and Drug Administration (FDA) underscores its established safety profile and broad applicability. The organism's ease of genetic modification allows for targeted engineering to enhance protein expression and surface display capabilities, while its exceptional protein secretion capacity simplifies downstream processing in industrial-scale production. Furthermore, its capacity to form endospores enhances its industrial utility by enabling extended storage, transportation, and manufacturing processes. These characteristics, combined with its optimal growth properties, have established *B. subtilis* as an essential resource in industrial, agricultural, and medical biotechnology, supporting both fundamental research and commercial production [2, 3]. *B. subtilis* has proven invaluable

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as a versatile platform for generating diverse high-value products, including industrial enzymes (e.g., amylases, xylanases, etc.), biofuels (e.g., butanediol, ethanol, etc.), vaccine components (e.g., production of recombinant antigens), and other biological compounds (e.g., vitamins, antibiotics, etc.) [2, 4–6]. Its genetic plasticity enables efficient production using sustainable resources, substantially reducing reliance on finite materials and conventional energy sources. This unique combination of versatility, productivity, and environmental responsibility establishes *B. subtilis* as a crucial contributor to biotechnological advancement across multiple sectors [7].

One of the most common applications of *B. subtilis* is its use in surface display technology. Surface display technology is a molecular technique that enables the attachment of peptides and proteins to the microbial cell surfaces by utilizing natural surface proteins as anchors [8]. It can be achieved through recombinant methods, which involve genetic engineering to fuse target proteins with carrier proteins, or non-recombinant methods, which rely on natural interactions or direct binding to microbial surfaces without genetic modification [9]. Common surface display systems, including bacteriophages, yeast, prokaryotic cells, and bacterial spores, rely on specific surface proteins as anchors to ensure the stable presentation of target proteins [10]. For instance, *B. subtilis* spores utilize spore coat proteins like CotB, CotC, CotG, and CotX as anchoring proteins to efficiently display target proteins on their surface [11]. Several outer membrane proteins, including outer membrane protein A (OmpA), OmpC, and outer membrane pore protein E precursor (PhoE), have been utilized as anchors in Gram-negative bacteria for surface display [12]. Gram-positive bacteria, which lack an outer membrane, use proteins like Protein A (SpA), LysM, and other cell wall-anchored (CWA) proteins to tether proteins directly to the thick peptidoglycan layer, enhancing stability [13]. Yeast, particularly *Saccharomyces cerevisiae*, leverages surface proteins such as α-agglutinin, the Aga1-Aga2 system, and Flo1p, which enable eukaryotic post-translational modifications [14, 15]. Phage display, a widely used surface display technique, employs coat proteins like pIII and pVIII from the M13 bacteriophage to fuse and present foreign proteins efficiently [16].

Each surface display system has distinct advantages but also specific limitations. Phage display is effective for high-throughput screening but unsuitable for large-scale applications due to the need for phage propagation [17]. Bacterial surface display in Gram-negative bacteria, such as *E. coli*, faces challenges with protein transport across the membrane and stability under harsh conditions [18]. Yeast surface display supports eukaryotic protein folding and modifications but demands complex cultivation and specialized media, limiting scalability [19]. In contrast, *B. subtilis* as Gram-positive bacteria stands out as an exceptional platform for surface display applications, demonstrating remarkable versatility in both vegetative cells and spores, which makes it invaluable across diverse biotechnological applications [20]. During the vegetative phase, surface display system in *B. subtilis* enables the presentation of functional proteins, enzymes, or antigens on the cell surface through anchor proteins such as S-layer proteins and CWPs. This approach serves as a versatile biotechnological tool for diverse applications, including protein engineering, biocatalysis, bioremediation, and biosensing, offering a robust platform for both industrial and environmental processes [8, 21]. Beyond vegetative cells, *B. subtilis* spores present distinct advantages for surface display owing to their exceptional structural resilience. The spore's remarkable resilience to harsh conditions, such as heat, dehydration, and UV exposure, enhances the stability of displayed proteins, making them suitable for long-term storage and challenging environments. Furthermore, as spore coat forms intracellularly and is followed by the autolysis of the mother cell, the need for secretion and, consequently, the optimization of signal peptides for heterologous proteins is eliminated [10, 22].

In the landscape of modern biomanufacturing, *B. subtilis* surface display technology, whether on vegetative cells or spores, offers exceptional versatility across various sectors, including industrial, agricultural, and medical applications. Engineered *B. subtilis* spores and cells can be tailored to display proteins, making them ideal for applications such as biosensing, environmental remediation, and bioactive compound delivery [23, 24]. A detailed comparison of the capabilities of these systems with traditional ones is presented in Table 1, highlighting their unique contributions to sustainability-driven innovation. These systems effectively address industrial challenges such as scalability and stability, while simultaneously reducing ecological impact by minimizing dependence on non-renewable natural resources and energy-intensive processes. By bridging cutting-edge research with environmentally friendly industrial applications, these systems align with the broader goals of precision and environmental stewardship in biomanufacturing. This review comprehensively explores the applications of *B. subtilis* surface display technology across bioprocessing and biomanufacturing, highlighting its benefits, limitations, and future potential.

Table 1 Comparative advantages of *B. subtilis* spore surface display and *B. subtilis* cell surface display systems versus other display technologies for biotechnological applications

Feature	*B. subtilis* spore surface display	*B. subtilis* cell surface display	Other technologies	Ref
Protein folding efficiency	Circumvents folding issues as proteins are displayed outside the membrane	Proteins are directly expressed on the cell surface, maintaining near-native conformation	Soluble protein systems may require chaperones for correct folding	[22, 25]
Stability under harsh conditions	Highly resistant to heat, desiccation, radiation, UV, and oxidation	Moderate stability; proteins may degrade under extreme conditions	Purified proteins require stabilizers for stability	[26, 27]
Immobilization process	Proteins are naturally immobilized during sporulation	Proteins are anchored on live cells during expression, allowing dynamic interactions	Other systems (e.g., phage, yeast) require genetic engineering and optimization	[22, 28]
High-throughput screening	Suitable for screening in extreme environments due to robustness	Enables real-time functional assays for enzyme engineering	Cell-free and phage systems lack cellular context but offer flexibility	[29–31]
Economic feasibility	Cost-effective; large-scale production without special culture conditions	Cost-efficient, but requires fresh cultures for best performance	Purified protein production is costly due to purification steps	[11, 32]
Safety considerations	GRAS (Generally Recognized as Safe) status; suitable for long-term storage	Safe for live-cell vaccines and probiotics	Recombinant systems (e.g., *E. coli*) may need additional precautions	[33]
Ease of handling	Durable and storable for long periods without refrigeration	Requires continuous culture maintenance to preserve functionality	Purified proteins need cold storage and stabilizers	[11, 34]
Protein functionality	Immobilized proteins retain activity and stability in harsh conditions	Active proteins in a near-native environment for functional assays	Soluble proteins may have high activity but lack durability	[35, 36]
Dynamic vs. static applications	Suitable for static applications like vaccines or long-term catalysts	Enables dynamic applications like continuous substrate processing	Phage and yeast display offer high diversity for functional screening	[11, 37]

Mechanisms of surface display technique in *B. subtilis*

Surface display technology in *B. subtilis* offers two key approaches: 1) cell surface display, and 2) spore surface display, each tailored for distinct purposes. Cell surface display leverages the bacterial cell wall for real-time functionality during vegetative growth, while spore surface display utilizes the robust spore coat as a stable platform for long-term applications. Together, these systems position *B. subtilis* as a versatile platform, serving as both a dynamic biofactory and a robust carrier for a wide range of applications in bioprocessing, biomanufacturing, and related fields.

The cell surface display in *B. subtilis* primarily targets the vegetative cell wall, utilizing various surface proteins as display platforms, including (i) transmembrane proteins, (ii) lipoproteins, (iii) LPXTG-like proteins (LPDTS and LPDTA), and (iv) cell wall binding proteins [13]. This approach enables the presentation of target molecules on actively proliferating bacterial cells. Given that vegetative cells maintain metabolic activity, this display system facilitates dynamic applications such as continuous catalysis, biosensing operations, and precision delivery of therapeutic compounds. However, this approach remains temporary and demands carefully regulated conditions to maintain bacterial viability [38–40].

Conversely, spore surface display involves fusing target proteins with spore coat proteins, ensuring their incorporation into spore coat during sporulation. The external spore coat exhibits remarkable durability, providing superior protection against environmental challenges including thermal stress, dehydration, and chemical exposure [9]. This characteristic makes spore display platforms particularly suitable for applications demanding long-term stability, such as vaccine administration, enzyme stabilization, or biosensing in extreme environments. Furthermore, the spore display system ensures that target proteins retain their functionality and remain accessible on the spore's outermost layer [23, 41].

Anchor proteins serve as essential components in both display systems. In spore surface display, specific proteins including CotB, CotC, and CotG are frequently utilized. These proteins naturally integrate into spore coat structure and offer excellent accessibility for engineering modifications [42]. While CotB and CotC demonstrate particular effectiveness for displaying smaller antigens or enzymes, CotG proves more suitable for presenting larger or structurally complex proteins [43]. CotX introduces additional flexibility for applications requiring enhanced structural stability [44].

For cell surface display, specialized anchor domains, such as the LysM domain and putative sortase-dependent substrate proteins (YhcR, YfkN), facilitate the anchoring of fusion proteins to the cell wall. These domains effectively maintain the displayed proteins' attachment while ensuring their functional integrity [45, 46].

The specific orientation between the target and anchor proteins significantly influences functional preservation. In N-terminal fusion configurations, the anchor protein's N-terminus connects to the target protein's C-terminus, while C-terminal fusion involves linking the anchor protein's C-terminus to the target protein's N-terminus [47].

Genetic engineering techniques play a fundamental role in optimizing these surface display systems. Surface display mechanisms in both whole-cell and spore systems rely on the fusion of target and anchor proteins for surface exposure. As shown in Fig. 1A**,** in whole-cell display systems, *B. subtilis* synthesizes fusion proteins and transports them to the cell surface using signal sequences for proper positioning. During vegetative growth, either constitutive or inducible promoters regulate fusion protein expression. One effective approach for stable surface display is the Sortase A (SrtA)-mediated system, where recombinant proteins are engineered with a C-terminal LPDTS sorting motif. SrtA enzyme recognizes and cleaves this motif, facilitating covalent linkage of the protein to the pentaglycine cross-bridge in the peptidoglycan layer. This mechanism ensures robust attachment and functional stability of biomolecules on the *B. subtilis* cell surface [47]. For *B. subtilis* spore surface display, sporulation-specific promoters regulate the expression of spore display constructs, ensuring the incorporation of target proteins into spore coat during sporulation. As illustrated in Fig. 1B, coat proteins located in the outer spore layer serve as stable anchors for target protein display. The direct fusion of target proteins with coat proteins ensures their exposure to the environment while leveraging the spore's inherent durability and resistance to harsh conditions [48].

This advanced technology enables diverse applications of *B. subtilis* in microbial cell bioprocessing and biomanufacturing. The applications include the development of next-generation vaccines, and therapeutic protein delivery systems. Additionally, *B. subtilis* surface display system plays a key role in environmental applications such as bioremediation and waste treatment, and is utilized in biosensor development for monitoring bioprocesses and ensuring process control in industrial production.

Applications in microbial cell bioprocessing

Surface display technology in *B. subtilis* has emerged as a transformative tool in microbial cell bioprocessing, demonstrating remarkable versatility across multiple industrial sectors. From facilitating enzyme immobilization in industrial catalytic processes to enabling the development of sophisticated biosensors for continuous

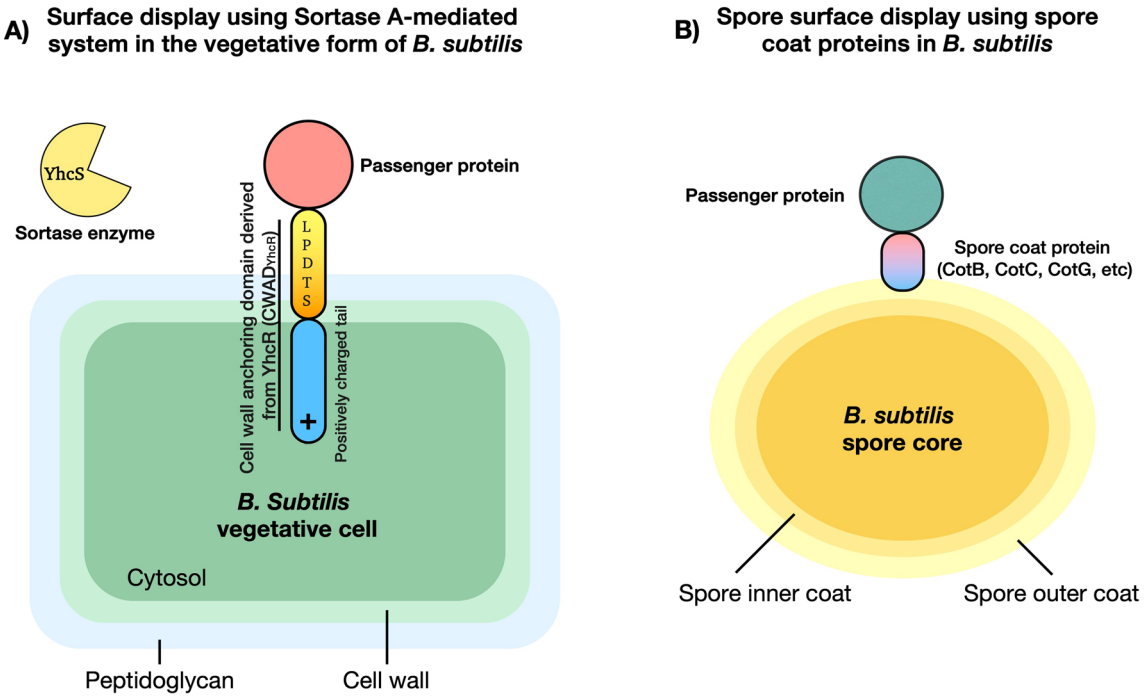

Fig. 1 **A** Mechanism of surface display using Sortase A-mediated system in the vegetative form of *B. subtilis*. This figure illustrates the use of Sortase A (SrtA) to anchor recombinant antigens or enzymes onto the cell wall of *B. subtilis*. The recombinant protein is engineered to contain a C-terminal LPDTS sorting motif recognized by SrtA. Upon cleavage of the LPDTS motif, the protein is covalently linked to the pentaglycine cross-bridge in the peptidoglycan layer. This system enables stable surface display of functional biomolecules on the vegetative cells of *B. subtilis*. Key components of the system, including SrtA, the LPDTS motif, and the peptidoglycan layer, are highlighted. **B** Mechanism of spore surface display using spore coat proteins CotB, CotC, and CotG in *B. subtilis*. The figure depicts the use of spore coat proteins as anchoring platforms for the display of antigens or enzymes on *B. subtilis* spores. Recombinant proteins are genetically fused to CotB, CotC, CotG, or CotX, which are localized on the outer layers of spore coat. CotB and CotC are primarily located in the outermost layer of spore coat, while CotG and CotX serve as scaffold proteins for stable attachment

monitoring, and addressing environmental challenges through pollutant degradation, and bioremediation, this technology offers innovative solutions to global challenges in the field of microbial biotechnology [20, 49] (see Fig. 2).

Enzyme immobilization for industrial catalysis

The implementation of enzyme immobilization techniques provides one of the most successful strategies for enzyme preparation, significantly enhancing their stability, expanding their commercial applications, and maximizing their economic potential [50]. Immobilized enzymes have found widespread uses across numerous large-scale industrial sectors, including medical, food processing, detergent manufacturing, textile production, pharmaceutical development, and water treatment facilities. The selection of suitable immobilization methodologies and carrier materials is largely determined by specific application requirements [51]. Enzyme immobilization plays a crucial role in industrial catalysis, enhancing enzyme stability, reusability, and process efficiency. Traditional enzyme immobilization methods include adsorption, entrapment, covalent attachment, and cross-linking, each with inherent advantages and limitations. Physical adsorption is simple and cost-effective but often leads to enzyme leaching, reducing long-term stability. Entrapment techniques, such as polymeric gels or microcapsules, provide a protective environment but may hinder substrate diffusion. Covalent attachment and cross-linking offer strong enzyme-support binding and improved durability but may compromise enzymatic activity due to conformational changes or steric hindrance [52–54]. The growing demand for cost-effective, safe, and cleaner immobilization materials has sparked considerable interest in microbial enzyme display systems [55].

Surface display systems in *B. subtilis* enable the anchoring of enzymes to bacterial cells or spores through fusion with specific display motifs, offering a sustainable alternative to traditional enzyme immobilization methods. This strategy not only preserves enzyme accessibility to substrates, but also provides protection against harsh conditions, such as high temperatures, extreme pH levels, or organic solvents. Additionally, the immobilization

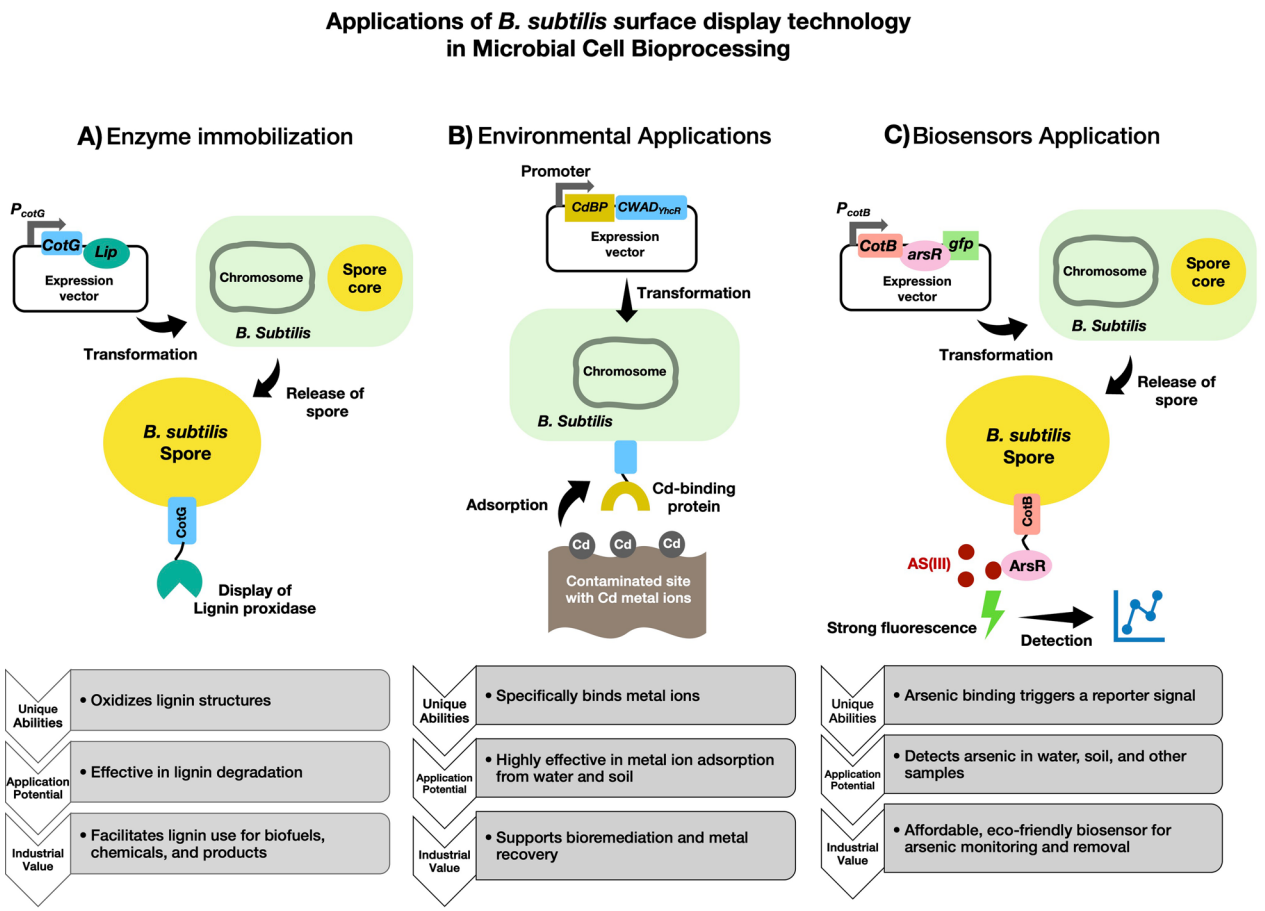

Fig. 2 Applications of *B. subtilis* surface display technology in microbial cell bioprocessing: **A** *B. subtilis* surface display technology for biocatalyst development. A specialized *E. coli–B. subtilis* shuttle expression vector, pHS-cotG-lip, was engineered for LiP display on the *B. subtilis* spore surface. The lip gene was fused to the CotG gene, an outer spore coat protein, facilitating stable anchoring of the LiP protein. The expression of the fusion construct was driven by a strong sporulation-specific promoter, ensuring optimal production during the sporulation phase. Following sporulation, the LiP protein was successfully displayed on the spore surface. **B** *B. subtilis* surface display technology for bioremediation. The first step is to select a metal-binding protein that can specifically interact with and bind to toxic metals, such as cadmium or mercury. These proteins typically have a high affinity for specific metal ions, making them ideal for bioremediation applications. The plasmid is engineered to include the cell wall anchoring domain from a putative sortase substrate, YhcR at the C-terminal of the metal-binding protein. Upon successful transformation, Sortase A recognizes and cleaves the sorting signal and catalyzes the attachment of the metal-binding protein to the bacterial cell wall. Once anchored to the surface of *B. subtilis*, the metal-binding protein binds to toxic metals, such as cadmium, from contaminated environments. This enables the bacteria to efficiently adsorb and sequester the harmful metals. **C** *B. subtilis* surface display for biosensor development. The plasmid is designed to express both the biosensor protein (such as arsenic-binding protein) and a reporter protein, GFP (green fluorescent protein). The GFP gene is included in the plasmid as a visual marker for detecting biosensor activity. The plasmid also contains a sequence for an anchoring protein, such as CotB, to ensure the surface display of the biosensor protein on *B. subtilis* spores. After the plasmid is introduced into *B. subtilis* through transformation, the cells are induced to undergo sporulation. During this process, the biosensor (arsenic-binding protein) is displayed on the surface of the spores, while GFP is expressed inside the cells. Upon exposure to arsenic(III) or another target contaminant, the biosensor protein binds to the target molecule. This binding can trigger a conformational change or activate GFP expression, making the biosensor detectable. The activation of GFP, in response to the binding of arsenic(III) or the target molecule, can be monitored using fluorescence. The intensity of the green fluorescence correlates with the amount of arsenic(III) in the sample, enabling real-time detection

of enzymes via surface display facilitates the efficient separation of biocatalysts from reaction mixtures, optimizing enzyme recovery and reuse while enhancing overall biocatalytic performance [56]. *B. subtilis* surface display-based enzyme immobilization can be applied in various bioreactor systems, including stirred-tank, packed-bed, fluidized-bed, and membrane reactors, depending on process requirements [57]. Additionally, the feasibility of continuous production is high since enzymes remain surface-bound, allowing for prolonged catalytic activity and repeated use without significant loss of efficiency. While large-scale industrial applications of cell surface display

are still limited, the rising demand for biocatalytic systems and ongoing advancements in surface display technology are likely to accelerate its adoption. This system is highly scalable from laboratory to industrial production, and enables the direct use of whole-cell biocatalysts without the need for complex purification processes, making it a more practical and cost-effective solution [56]. Given its stability, ease of production, and scalability**,** the *B. subtilis* display system offers a promising platform for biocatalysis, particularly in applications requiring robust and cost-effective enzyme immobilization. In the following, we review studies showcasing *B. subtilis* as a platform for enzyme immobilization.

Chitin, the second most prevalent polysaccharide in nature after cellulose, is a valuable source of bioactive amino-oligosaccharides derived through enzymatic degradation. The enzymes facilitating this degradation, known as chitinases, have garnered considerable interest due to their extensive industrial potential. The significant research demonstrated the successful recombinant expression and immobilization of exochitinase PbChiA, derived from *Paenibacillus barengoltzii*, onto *B. subtilis* WB800N spore surfaces using CotG as the anchoring protein. In this study, the gene encoding PbChiA was cloned into a plasmid containing the *cotG* gene promoter and expressed in *B. subtilis* WB800N. The resulting strain, *B. subtilis* WB800N pHS-CotG-Chi, displayed the exochitinase on its spore surface. The immobilized enzyme exhibited impressive thermal stability and enzymatic activity across various pH conditions, with chitinolytic activity reaching approximately 16.06 U/mL. Activity was enhanced in the presence of Ni^{2+}, Zn^{2+}, K^{+}, and EDTA. Furthermore, the enzyme retained over 70% of its activity through three cycles of reuse and 62.7% after four cycles. These results emphasize *B. subtilis*' potential as a versatile enzyme immobilization platform, delivering enhanced stability, reusability, and environmentally sustainable solutions for industrial applications [58].

Lignin peroxidase (LiP) holds significant potential for applications in lignin degradation, environmental remediation, straw-based feed production, and several other industries. However, its use is limited by the enzyme's high production cost and low stability. In a notable investigation by Li et al., a specialized *E. coli–B. subtilis* shuttle expression vector, pHS-cotG-lip, was engineered to display LiP on *B. subtilis* spore surfaces. The *lip* gene was fused to the *cotG* gene under the control of a strong promoter, ensuring its expression during sporulation. Enzymatic evaluations demonstrated that the surface-bound LiP exhibited superior performance characteristics, with optimal catalytic activity at 55 °C and pH 4.5, marking a substantial improvement over free LiP, which operated optimally at 42 °C and pH 4.0. The immobilized enzyme demonstrated remarkable thermal stability, maintaining over 30% activity after one hour at 70 °C, while free LiP completely deactivated under identical conditions. The enzyme's performance was substantially enhanced through the addition of specific compounds: Mn^{2+} increased activity by 313%, DL-lactic acid by 146%, and PEG-4000 by 265%. Furthermore, the immobilized LiP showed exceptional reusability, retaining more than 50% enzymatic activity through four successive cycles. These results highlight the industrial potential of spore surface-displayed LiP, particularly in wastewater treatment applications, offering an efficient and economical approach to enzyme immobilization. The study reinforces the effectiveness of *B. subtilis* spores as a versatile platform for enzyme display and immobilization in industrial applications [59].

A subsequent research effort successfully established a *B. subtilis* spore display platform for immobilized enzyme production. This system effectively converted lipids from various sources, including *Yarrowia lipolytica*, *Cutaneotrichosporon oleaginosus*, and olive oil, into hydrocarbons. The process utilized a bienzymatic system, combining commercial immobilized lipase with spores, either with or without heterologous lipase. Surface-displayed photodecarboxylase enzyme (CvFAP) facilitated fatty acid decarboxylation, with hydrocarbon composition varying by lipid source. Using specialized 3D printed lighting equipment, the system achieved hydrocarbon yields reaching 64.0 ± 5.6 mg/L [60].

In another significant investigation by Zhengfen Wu and colleagues, researchers developed an innovative technique for displaying cellobiose dehydrogenase (CDH) from Pycnoporus sanguineus fungus (psCDH) on *B. subtilis* spore. This innovative approach effectively addresses the challenges associated with conventional fungal expression systems, particularly their time-intensive and cost-prohibitive nature. The investigation revealed that the spore display system significantly enhanced psCDH's resilience to extreme conditions, including high temperatures (80 °C) and acidic environments (pH 3.5), demonstrating superior stability compared to its unbound form. This study also revealed that specific compounds, namely glycerol, lactic acid, and malic acid, positively influenced enzyme activity, with glycerol demonstrating the most significant enhancement, increasing enzyme activity from 16.86 ± 1.27 to 46.26 ± 3.25 U/mL. The immobilized enzyme maintained 48% of its initial activity after four consecutive reuse cycles, indicating robust enzyme recovery. This CotG-based spore display system presents an efficient solution for CDH expression, immobilization, and stability enhancement, suggesting promising applications in industrial processes [61].

Kim J and collaborators developed a groundbreaking live immobilization methodology to enhance lipase stability in organic solvent reactions, utilizing *B. subtilis* spores as the display platform. The researchers employed *B. subtilis* CotE as an anchoring protein to display two distinct lipases, LipA and LipB, on the spore surface. Their findings revealed that the spore-displayed CotE-LipB fusion protein demonstrated superior lipolytic activity compared to the free CotE-LipA protein. Surface localization of Lipase B was confirmed through flow cytometry and protease accessibility experiments. Notably, the spore-displayed lipase maintained its activity in organic solvents including acetone and benzene, while free soluble lipase completely lost activity under identical conditions [62].

In another study by Gupta et al., a glucose-tolerant β-glucosidase (UnBgl1A) was displayed on the surface of *B. subtilis* cells using cell wall binding domain (CWB_b) of the major *B. subtilis* autolysin (CwlB) as the anchor protein and plasmid pHT01 under an IPTG-inducible promoter. Glucose-tolerant β-glucosidases have attracted significant interest in recent years for their involvement in cellulose degradation to produce bioethanol. The fusion protein was constructed with a Gly-Ser linker for enhanced folding and surface expression. Induction at 30 °C for 6 h facilitated expression, which was verified through immunofluorescence microscopy, LiCl-based protein extraction, and western blotting. Enzymatic activity was tested using p-NPG as a substrate, confirming the displayed enzyme's catalytic function, with glucose release quantified to assess cellobiose hydrolysis. These engineered cells (SD-01) were directly utilized for biocatalysis without the need for cell lysis or enzyme purification. The SD-01 cells exhibited approximately twice the catalytic activity compared to cells expressing the enzyme intracellularly (IN-01). The displayed enzyme showed comparable glucose tolerance (IC50 ~0.9 M glucose), temperature optima (~50 °C), and pH optima (~6.0) to the purified enzyme. Notably, the surface-displayed UnBgl1A retained about 50% of its activity after 4 h at 50 °C, whereas the purified enzyme completely lost its activity within the same timeframe. Furthermore, the SD-01 cells could be reused efficiently for three consecutive reactions. Supplementing a cellulase cocktail with SD-01 cells led to approximately double the glucose release from sugarcane bagasse compared to supplementation with purified UnBgl1A [39]. This study highlights the potential of *B. subtilis* surface display technology as an efficient and cost-effective platform for the commercial production of industrial enzymes.

β-Cyclodextrin (β-CD), a crucial cyclic oligosaccharide, finds extensive applications in food, environmental protection, and cosmetics. Traditionally, it is produced through enzymatic synthesis in industrial processes. However, this method faces several challenges, such as complex procedures and obstacles to implementing continuous fermentation and catalysis. In an innovative study, a biofilm-based immobilized fermentation system was developed, integrating enzyme catalysis through surface display in *B. subtilis*. In a study by Wang D and colleagues, a biofilm-based continuous catalysis system was developed by integrating biofilm formation with a surface display strategy in *B. subtilis*. The *bslA* gene, encoding the surface hydrophobic protein BslA, was selected due to its dual role in promoting biofilm formation and serving as an anchoring protein for surface display. Initially, the function of the *bslA* gene in biofilm formation was confirmed. Subsequently, β-CGTase was displayed on the *B. subtilis* cell surface using BslA as the anchoring protein. The system was further optimized by constructing a biofilm-based immobilized fermentation process on cotton fiber carriers. This approach improved enzyme stability, reduced the fermentation cycle, and enhanced biocatalytic efficiency, providing a robust platform for continuous biofilm-based biocatalysis. Compared to free-cell catalysis, the biofilm-based approach broadened the operational temperature range to 40–70 °C and the pH range to 5–7.5. Remarkably, during continuous catalysis, the system retained approximately 52% relative activity by the 13th batch, achieving a conversion rate exceeding 36%, comparable to single-batch free-cell catalysis [63]. This research offers valuable insights and practical strategies for advancing the industrial production of β-CD and other biochemicals through efficient continuous catalysis.

In conclusion, these investigations collectively highlight the remarkable potential of *B. subtilis* surface displays systems as a robust and versatile platform for enzyme display and immobilization. This innovative approach substantially improves enzyme stability, activity, and reusability under extreme conditions, creating new possibilities for industrial applications, particularly in biocatalysis and biofuel production. These findings provide valuable insights for developing more sustainable and cost-effective enzyme systems, although further research is needed to optimize and scale this technology for broader implementation.

Environmental applications

B. subtilis surface display technology presents significant potential in environmental biotechnology, especially for pollutant degradation and bioremediation. By engineering *B. subtilis* to display pollutant-degrading enzymes or binding proteins on its surface or spores, these platforms can effectively target and degrade environmental contaminants, providing a sustainable and efficient approach

to environmental cleanup. This approach enhances the biodegradation process by increasing enzyme accessibility to contaminants while also enhancing the stability and survivability of the engineered microbes in challenging environmental conditions. These attributes make *B. subtilis*-based systems a promising tool for addressing pollution and promoting environmental sustainability [64, 65]. In the following, we will explore various applications of this technology in environmental cleanup, focusing on its role in pollutant degradation, waste treatment, and bioremediation strategies for sustainable environmental management.

Organophosphorus contamination, which contributes to both terrestrial and aquatic pollution, causes acute and subacute toxicity in plants, animals, humans, and insects. Given the excessive use of these pesticides, it is crucial to develop effective solutions to address these pollution challenges in real-world environments [66]. *B. subtilis* surface display technology can be engineered to degrade organophosphorus pollutants by presenting specific enzymes on its surface, enabling efficient and targeted bioremediation. In a study by Tianyu et al., *B. subtilis* spore display systems were developed to present organophosphorus hydrolase (OPH, EC 3.1.8.1) and organophosphorus acid anhydrolase (OPAA, EC 3.1.8.2), two of the most well-characterized and potentially important enzymes for degrading organophosphates. The researchers engineered recombinant *B. subtilis* spores to display both OPH and OPAA on their surfaces, demonstrating the potential of this spore-based display system for bioremediation and enzyme immobilization applications. This study utilized *B. subtilis* DB104 as a host strain. The *cotG* gene, encoding an outer spore coat protein, was fused with OPH and OPAA to enable enzyme display on the spore surface. Enzyme display on the CotG fusion protein was confirmed by western blot, dot blot, and immunofluorescence microscopy. The activity and stability of spore-displayed enzymes were tested under various harsh conditions, such as extreme pH, temperature, and protease treatments. The results indicated that the recombinant spores retained normal sporulation and exhibited enzymatic activities of 15.81 and 10.67 U/mg of dry spores for OPH and OPAA, respectively. Additionally, the spore-displayed enzymes showed enhanced stability and greater resistance to harsh conditions compared to their free-form [67]. This spore-based technology presents a practical and efficient solution for the bioremediation of organophosphorus pollutants in diverse environmental settings, overcoming key challenges associated with conventional enzyme applications.

Halogenated compounds, commonly used in agriculture and industry as components in pesticides, solvents, and plasticizers, are among the most toxic and persistent environmental pollutants. Despite being banned by the Chemical Weapons Convention (CWC), some have been used as chemical warfare agents, causing serious health risks and environmental damage. Traditional remediation of halogenated compounds faces significant limitations, such as harsh reaction conditions and the production of toxic byproducts. Enzyme-based strategies offer a safer and more efficient alternative. For instance, haloalkane dehalogenases (HLDs) can degrade toxic compounds like sulfur mustard (HD) into harmless products. However, their practical use is hindered by sensitivity to temperature and pH [68]. To overcome these limitations, Wang et al., explored the use of *B. subtilis* spore surface display technology to enhance the stability and applicability of DhaA, a well-characterized HLD. Recombinant *B. subtilis* spores were engineered to express DhaA from *Rhodococcus rhodochrous* NCIMB 13064. Dot blot analysis showed that CotG-linker-DhaA represented 0.41% of the total spore coat proteins, and immunofluorescence confirmed its successful display on the spore surface. The displayed DhaA retained hydrolytic activity, with an activity of 1.74 U/mL towards an HD analog and a specific activity of 0.34 U/mg. This study demonstrated that DhaA displayed on *B. subtilis* spores remains enzymatically active, offering a promising solution for real-world bioremediation of environments contaminated with halogenated pollutants [69].

Heavy metal contamination from industrialization and human activities has become a major global concern, affecting water, air, and food, with serious health risks for humans and animals. Increased exposure to toxic metals such as mercury, lead, chromium, cadmium, nickel, etc., over the past century has led to widespread environmental pollution and poisoning incidents. Traditional heavy metal remediation methods are costly and often generate additional toxic waste. In contrast, microbial bioremediation has emerged as a more affordable and eco-friendly alternative [70]. An innovative solution to mitigate this issue is the use of surface-displayed proteins on microbial cells to sequester toxic metals by anchoring metal-binding peptides or proteins to the microbial surface, offering a promising bioremediation strategy. These surface-bound metal-binding proteins act as efficient biosorbents, enabling the removal of harmful metals from contaminated water and soil [71, 72]. For example, recombinant *B. subtilis* spores have been engineered for nickel ion adsorption by modifying the spore surface protein CotB to include 18 histidine residues. These recombinant spores showed significantly improved nickel-binding efficiency compared to wild-type spores, with minimal influence from pH and temperature. Additionally, nickel ions could be partially recovered through simple washing, highlighting the potential of *B. subtilis*

spores as a cost-effective and eco-friendly solution for heavy metal remediation [73].

Altogether, these studies demonstrate that *B. subtilis* surface display technology offers a robust and cost-effective solution for environmental remediation. By combining the durability of spores with enzymatic efficiency, this technology provides a sustainable alternative for detoxifying pollutants. Its stability under harsh conditions makes it a promising tool for real-world applications in environmental cleanup and ecosystem restoration.

Biosensors for monitoring bioprocesses

Biosensors are analytical devices that convert a biological response into an electrical signal, offering several advantages, including high specificity, independence from factors like pH and temperature, and reusability. These features make biosensors increasingly valuable in diagnostic applications such as food safety, clinical testing, and environmental monitoring. Their rapid response, high specificity, ease of mass production, cost-effectiveness, and suitability for field use further enhance their growing importance. The specificity of biosensors is based on biological interactions such as, antigen–antibody binding, enzyme–substrate/cofactor reactions, receptor–ligand binding, chemical interactions, and nucleic acid hybridization, which are coupled with various transducers to enable precise detection [74, 75].

B. subtilis holds significant potential for biosensor development through cell surface display technology. Its robust productivity, high yield, and ability to display large target molecules make it an ideal platform for such applications. The cell envelope of *B. subtilis*, consisting of a plasma membrane and a thick, rigid peptidoglycan layer, provides structural stability. Surface-displayed proteins are covalently anchored to this durable cell wall, which enhances the biosensor's durability and functionality. This technology enables the presentation of receptor proteins or enzymes on the bacterial surface, allowing for detection of specific chemical or biological signals. Consequently, these biosensors can be engineered to detect a wide range of substances, from environmental pollutants to disease biomarkers, making them highly versatile for both industrial and biomedical applications. The unique structural and functional properties of *B. subtilis* significantly improve the sensitivity, efficiency, and stability of these biosensors [76].

Surface-displayed antibodies or enzymes can be utilized in biosensors for detecting pathogens or environmental contaminants in air or water. *B. subtilis* cells, displaying receptors for specific target molecules, can be incorporated into biosensor systems for rapid detection and quantification, providing continuous, real-time data on bioprocesses or environmental conditions. For example, a study introduced a generic whole-cell bacterial biosensor, named "Sposensor" which uses immobilized spores from genetically engineered *B. subtilis*. The Sposensor consists of two types of spores: reporting spores, which contain a reporter gene linked to a promoter responsive to the target compound, and control spores, which monitor cell germination and viability. To simplify on-site analysis, a one-step incubation and detection process was developed, where spores were directly incubated with a culture medium containing the target compound. The reporter protein, β-galactosidase, was used in both types of spores, and its activity was measured via a colorimetric assay. The Sposensor successfully detected two specific compounds, a metal ion (Zn^{2+}) and the peptidic antibiotic bacitracin. Due to the inherent stability and resilience of spores, the Sposensor proved to be a robust, user-friendly tool for identifying toxic compounds in environmental settings [77].

In another study, a green fluorescent protein (GFP)-based whole-cell biosensor (WCB-GFP) was developed in *B. subtilis* for the detection of arsenic (As). The system utilized a reporter gene fusion, in which the *gfpmut3a* gene was placed under the control of the promoter region of the arsenic operon (*Pars::gfpmut3a*) and carried on the extrachromosomal plasmid pAD123. This construct was transformed into *B. subtilis* 168, resulting in a strain designated as BsWCB-GFP for arsenic detection. The biosensor specifically responded to inorganic arsenic species, As(III) and As(V), while showing no activation by dimethylarsinic acid [DMA(V)], and demonstrated remarkable tolerance to arsenic toxicity. After 12 h of exposure, the biosensor exhibited lethal dose (LD) thresholds, with LD50 and LD90 values for As(III) determined to be 0.89 and 1.71 mM, respectively. Notably, dormant spores derived from BsWCB-GFP retained their arsenic detection capabilities. Upon germination, these spores could report As(III) concentrations ranging from 0.1 to 1,000 μM within 4 h. The specificity, high sensitivity, and resilience of this biosensor to toxic arsenic levels highlight its potential for environmental monitoring. The ability to detect arsenic concentrations as low as 0.1 μM makes it particularly relevant for assessing contamination in groundwater, aligning with World Health Organization (WHO) guidelines for permissible arsenic levels in drinking water [78]. This innovative biosensor system offers a promising tool for the direct detection of arsenic contamination in environmental samples.

These advancements illustrate the broad potential of *B. subtilis* surface display-based biosensors to address critical needs in environmental monitoring and public health. The robust cellular architecture of *B. subtilis*, coupled with its ability to anchor large molecules on its surface and spores, significantly enhances biosensor stability

and performance. These attributes establish *B. subtilis* as a reliable platform for biosensors designed for diagnostic and environmental applications, such as pathogen detection, pollutant monitoring, and clinical diagnostics. However, further research and optimization are necessary to expand their target range, improve sensitivity, and adapt them for large-scale deployment in various fields.

Table 2 summarizes the various applications of *B. subtilis* surface display technology in microbial cell bioprocessing, highlighting their key advantages along with examples of specific industrial and environmental uses.

Applications in biomanufacturing

B. subtilis surface display technology plays a crucial role in biomanufacturing, especially in vaccine development, therapeutic protein production, targeted delivery systems, and high-throughput screening (HTS). Recently, the engineering of *B. subtilis* as a non-invasive and heat-stable antigen delivery system has shown promising success. By displaying antigens, therapeutic proteins, or receptor molecules on *B. subtilis* surfaces, it enables efficient vaccine platforms, improves drug delivery strategies, and accelerates the discovery of new therapeutics through HTS. This versatile approach enhances production efficiency, specificity, and scalability in pharmaceutical and biomedical applications [79, 80].

Vaccine production using vegetative cells and spore-displayed antigens

One of the most promising applications of *B. subtilis* surface display technology in biomanufacturing is its use in vaccine production. The utilization of live bacteria as vaccine delivery platforms has emerged as a key strategy in advancing the development of innovative and more effective vaccines [81]. *B. subtilis* offers several advantages as both an expression host and a delivery vehicle for subunit vaccines. The use of *B. subtilis* to express antigens that can be administered either intranasally or sublingually is providing new insights in the area of mucosal vaccines. The ability to administer antigens sublingually and intranasally provides a major advantage over traditional parenteral and oral vaccination methods for targeting systemic and mucosal diseases, respectively [81]. They also possess adjuvant properties, which enhance the immunogenicity of poorly immunogenic oral antigens. In the gut, spores can germinate, triggering strong mucosal immune responses through antigen presentation by antigen-presenting cells (APCs), although cellular immune responses can also occur without germination. *B. subtilis* is cost-effective platform for vaccine production due to simple production processes that avoid the need for extensive purification, such as the removal of endotoxins required for *E. coli*-based systems. Additionally, *B. subtilis* spores are stable at ambient temperatures, eliminating the need for refrigeration and reducing vaccine storage and transportation costs. Recognized as safe (GRAS) by the FDA, *B. subtilis* is widely used as a probiotic and food additive, with established production platforms facilitating rapid scalability. The availability of advanced genetic engineering tools further enhances its utility in vaccine development. Compared to conventional platforms like viral vectors, *B. subtilis* avoids challenges such as risks of pathogenicity, pre-existing immunity, and potential tumorigenesis. These features position *B. subtilis* as a promising candidate for the development of safe, cost-effective, and thermostable vaccines, particularly for mucosal administration, with numerous antigens demonstrating promising preclinical results [13].

As illustrated in Fig. 3, antigen presentation in *B. subtilis* can be achieved through various strategies, each with unique benefits and limitations:

a. Antigen display on vegetative cells: Several strategies have been developed to display heterologous proteins on the surface of *B. subtilis* vegetative cells, utilizing covalent attachment to the peptidoglycan layer. A common approach involves the use of anchor proteins such

Table 2 Applications and benefits of *B. subtilis* surface display technology in microbial cell bioprocessing

Application	Description	Key benefits	Examples	Ref
Enzyme immobilization for industrial catalysis	Surface display of enzymes on *B. subtilis* cells or spores for stable and efficient catalysis	– Increased enzyme stability – Reusability of enzymes – Improved reaction efficiency	– Biofuel production (enzymes for polysaccharide breakdown) – Food and beverage processing	[39]
Environmental applications	Surface display of enzymes or binding proteins for degrading or sequestering environmental pollutants	– Enhanced biodegradation – Direct interaction with pollutants – Sustainable cleanup	– Bioremediation – Heavy metal removal (metal-binding proteins)	[69, 73]
Biosensors for monitoring bioprocesses	Display of receptors or enzymes for real-time detection of chemicals or biological signals	– Real-time monitoring – High sensitivity and specificity – Continuous feedback	– Pollutant detection in wastewater – Pathogen detection in food or bioprocesses	[77]

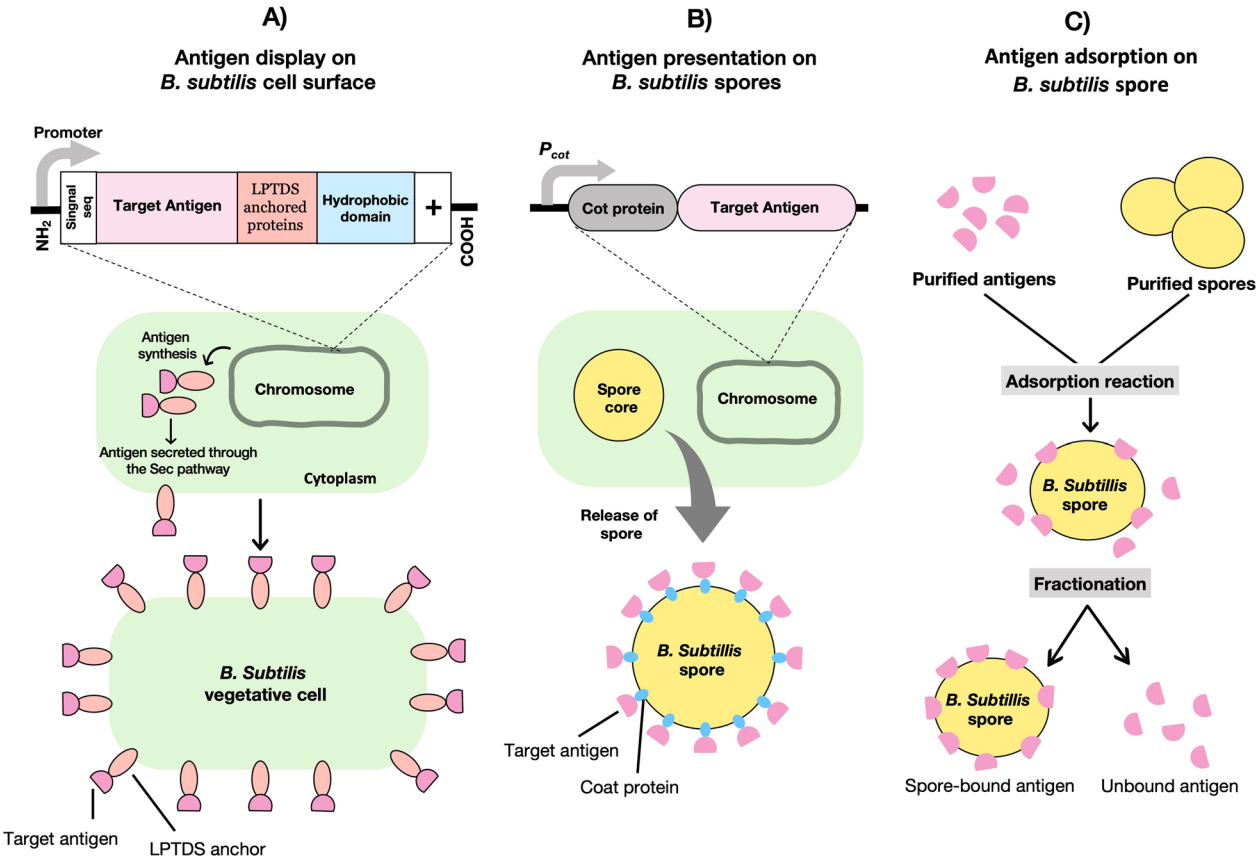

Fig. 3 **A** Antigen presentation via surface display on *B. subtilis* vegetative cells. The target antigen, often fused with the LPTDS motif, is cloned into a plasmid vector containing the gene for an anchor protein with a Sortase A recognition site, a signal sequence for proper secretion, and possibly a hydrophobic domain for membrane association. This plasmid ensures the correct expression, secretion, and stable display of the fusion protein on *B. subtilis* cells. The recombinant plasmid is introduced into *B. subtilis* cells via transformation, where the plasmid integrates into the host genome or remains episomal, depending on the vector used. Once inside the cell, the target antigen fused with the LPTDS motif is synthesized in the cytoplasm. The protein is then secreted through the Sec pathway, utilizing the signal sequence to direct the protein across the cytoplasmic membrane. In the periplasmic space, the sortase enzyme recognizes the LPTDS motif, cleaves the fusion peptide, and covalently links the target protein to the peptidoglycan layer in the bacterial cell wall, aided by any hydrophobic domains that assist in stable membrane attachment. The antigen is finally displayed on the surface of the *B. subtilis* vegetative cells, making it available for immune recognition. **B** Surface display of antigens on *B. subtilis* spore coat. The target antigen is fused with spore coat proteins such as CotB, CotC, or CotG, creating a recombinant plasmid. The plasmid contains the gene for the fusion protein, ensuring correct expression and incorporation into spore coat during sporulation. The recombinant plasmid is introduced into *B. subtilis* cells through transformation, where it integrates into the genome. During sporulation, the Cot proteins (CotB, CotC, or CotG) become incorporated into the outer spore coat, anchoring the fused antigen to the surface of the developing spore. As sporulation completes, the mature spore is released from the mother cell. The spore, now display the antigen on its surface, is resistant to environmental stressors, which enhances its stability and longevity. **C** Adsorption of antigens onto *B. subtilis* spores. This non-recombinant approach relies on the spontaneous and stable attachment of purified antigens to the outer surface of *B. subtilis* spores through chemical interactions, such as electrostatic and hydrophobic forces. This process occurs at 25 °C in an acidic reaction buffer (pH 4.0). Following the adsorption process, centrifugation is used to separate spore-bound antigens from unbound antigens

as the SrtA motif. The target antigen, often fused with the LPTDS motif, undergoes normal protein synthesis in the cytoplasm and is exported through the Sec pathway across the cytoplasmic membrane. In the periplasmic space, the sortase enzyme recognizes the sorting motif (e.g., LPTDS), cleaves it, and covalently links the antigen to the peptidoglycan via a transpeptidation reaction. This strategy ensures stable antigen presentation on the bacterial cell surface, enabling potential use in mucosal vaccine development. While effective for antigen presentation, it has limitations, as some antigens may be unsuitable for fusion or secretion [8, 82].

b. Antigen display on spores (recombinant approach): In recombinant display strategy, a gene fusion is created between DNA encoding a spore surface protein such as CotB, CotC, CotG, or CotX, and desired

antigen, with the expression regulated by the transcriptional and translational signals of the spore surface gene. This fusion is inserted into an integrative plasmid alongside an antibiotic-resistance gene cassette, and placed between two segments of a non-essential *B. subtilis* gene. The plasmid is integrated into the *B. subtilis* chromosome via a double crossover, disrupting the non-essential gene. During sporulation, the gene fusion is expressed in the mother cell, and the chimera assembles around the developing spore. At the end of sporulation, the mother cell lyses, releasing the mature spore with the antigen stably attached to its surface [83]. Spore-based vaccines offer advantages, including stability and the ability to survive the gastrointestinal tract, germinate, and replicate briefly in the intestine before excretion (spores are robust structures comprising a core, cortex, and proteinaceous coat, with essential morphogenetic proteins (e.g., SpoIVA, CotE) critical for coat formation). Their stability and ability to withstand harsh conditions make *B. subtilis* spores a versatile and efficient platform for vaccine development [84]. A major drawback of the recombinant approach is that, it relies on the genetic modification of the host, leading to the release of a recombinant microorganism into the environment. This raises significant concerns, particularly when the system is intended for human or animal applications [85].

c. Antigen adsorption on spore surfaces (non-recombinant approach): The non-recombinant display system involves directly attaching heterologous antigen to the spore surface, eliminating the need for recombinant strain construction and reducing the risk of releasing genetically modified bacteria into the environment. In this approach, spores are mixed with an antigen under controlled conditions to promote binding. The reaction typically occurs in a specific buffer to optimize adsorption. After incubation, centrifugation or another separation technique is employed to isolate spores display the antigen from any unbound spore [83]. This approach stabilizes and protects the adsorbed antigen by leveraging their interaction with spores, thereby minimizing antigen degradation and maintaining enzyme activity under unfavorable conditions [59]. An additional advantage of the non-recombinant approach is its higher efficiency compared to the recombinant spore display system, requiring up to 70 times fewer spores to deliver a comparable antigen dose. Moreover, it uniquely enables the display of multimeric antigens in their native conformation, ensuring proper receptor recognition and effective immune system activation [83].

The surface display system in all forms allows for the precise orientation and presentation of antigens to the immune system, improving their recognition and boosting the immune response. The administration of *B. subtilis*-based vaccine through oral, nasal and parenteral routes has been shown to elicit protective immune responses to the expressed antigens. These responses include both humoral and cellular immunity, with the cellular response being primarily Th1, characterized by IFN-γ production, when the heterologous antigens are displayed on the either cell wall or spore coat. Additionally, these vaccines are capable of inducing mucosal immune responses, such as the production of secretory IgA (sIgA) [86]. To date, *B. subtilis* has been engineered to display a wide variety of antigens, including those derived from viruses, bacteria, and parasites. The robust nature of *B. subtilis* spores allows them to withstand harsh conditions during storage and transit, while vegetative cells provide versatility in antigen expression and delivery [87]. Together, these features make *B. subtilis* a highly suitable candidate for vaccine production, particularly in low-resource settings. To date, numerous vaccines targeting these pathogens have been developed using this innovative technology (Table 3).

Therapeutic protein production and delivery systems

B. subtilis has emerged as a versatile and robust host for protein expression, offering an efficient and scalable platform for producing bioactive proteins in high yields, particularly in bioreactor settings. Its natural ability to secrete proteins into the surrounding environment makes it a valuable tool for various biotechnological applications. Through genetic engineering and surface display technology, *B. subtilis* can be optimized to express proteins such as antibodies, enzymes, and cytokines on the surface of bacterial cells or spores. This approach not only enhances protein production efficiency, but also simplifies downstream processing, as the expressed proteins can be directly harvested from bacterial cultures. Compared to traditional mammalian cell culture methods, this system offers a more cost-effective and scalable alternative for therapeutic protein production [117].

A study demonstrated the potential of *B. subtilis* spores as a biocontrol platform by displaying a chitinase enzyme (ChiS) from *Bacillus pumilus* on their surface. Chitinases are well-known for their ability to inhibit fungal pathogens, which pose significant threats to global agricultural productivity. The researchers used CotG, a major spore coat protein, as a carrier to anchor ChiS on the spore surface. The expression of ChiS was validated using immunofluorescence microscopy, confirming its localization on the spores. Functional assays revealed that the surface-displayed enzyme retained its activity and effectively inhibited the growth of two pathogenic fungi, *Rhizoctonia solani* and *Trichoderma harzianum*. Western blot analysis indicated partial processing of the CotG-ChiS fusion protein during the display process. Additionally,

Table 3 *B. subtilis* spore-based vaccine technology for various pathogens

Pathogen type	Examples	Target antigen(s)	Anchor	Vaccine development status	Applications	Ref
Viruses	SARS-CoV-2	Receptor binding domain (RBD)	CotZ/CotY	Preclinical to early clinical stages	Prevention of respiratory and gastrointestinal viral infections	[88]
		RBD of (Wuhan-Hu-1 2019-nCov and Omicron B.1.1.529 variants) and the HR1-HR2 domain (Wuhan-Hu-1 2019-nCov) of the Spike protein	CotB/CotC			[89]
	Influenza	Influenza virus M2 protein (M2e)	CotB			[90]
	Porcine reproductive and respiratory syndrome (PRRS)	PRRSV GP5 protein	CotB			[91]
	Transmissible gastroenteritis virus (TGEV)	TGEV spike protein	CotG			[92]
	Rabies virus (RABV)	Rabies virus G protein	CotG-E			[93]
	Duck hepatitis A virus type 1 (DHAV-1)	DHAV-1 capsid protein VP1	CotB			[94]
	White Spot Syndrome Virus	VP28 protein	CotB-CotC			[95]
	Feline coronavirus (FCoV)	HR2-derived peptide (HR2P) from a serotype II feline enteric CoV (FECV)	CotB			[96]
	Enterovirus 71 (EV71)	EV71	CotB			[97]
	Grass Carp Reovirus (GCRV)	antigen Vp7 GCRV	CotB/CotC			[98]
	Nervous necrosis virus (NNV)	Major capsid protein (MCP) of RGNNV	CotC			[99]
Bacteria	*Bacillus anthracis*	Secreted protective antigen (PA)	CotB	Demonstrated efficacy in animal models	Immunity against anthrax	[100]
	Mycobacterium tuberculosis	Immunodominant secretory antigen, MPT64	CotB			[101]
	Clostridium difficile	Flagellin protein, FliD	CotB			[102]
		C-terminal domains of toxin A (A26-39; codons for Ser_{2388} to Pro_{2706}) or toxin B (B15-24; codons for Glu_{2137} to Glu_{2366}	CotB/CotG			[103]
	Clostridium tetani	C-term fragment of the tetanus toxin, TTFC	CotB			[9]
	Clostridium perfringens	C-term of alpha toxin gene, Cpa247-370 fused to the GST gene	CotB			[104]
	Helicobacter acinonychis	Urease subunit alpha, UreA	CotG/CotZ			[105]
	Helicobacter pylori	Vacuolating cytotoxin A, CagA	CgeA			[106]
	Salmonella serovar Pullorum	OmpC	CotG			[107]
	Vibrio vulnificus	Vibrio antigenic protein, OmpK	CotY			[108]
	Mannheimia haemolytica	*M. haemolytica* chimeric proteins MhCP1 and MhCP2	Non-recombinant approach			[109]

Table 3 (continued)

Pathogen type	Examples	Target antigen(s)	Anchor	Vaccine development status	Applications	Ref
Parasites	*Haemonchus contortus*	*H. contortus* glyceraldehyde-3-phosphate dehydrogenase (HcGAPDH)	CotB		Prevention of parasitic infections	[110]
	Clonorchis sinensis	*C. sinensis* tegumental protein (CsTP22.3)	CotG			[111]
		C. sinensis serpin (CsSerpin)	CotC			[112]
		C. sinensis enolase (Csenolase)	CotC			[113]
		Paramyosin of *C. sinensis* (CsPmy)	CotC			[114]
	Schistosoma japonicum	Glutathione *S*-transferase (GST) protein	CotC			[115]
	Plasmodium spp.	*P. falciparum* CSP (r*Pf*CSP)	Non-recombinant			[116]

molecular dynamics simulations suggested that fusing ChiS to CotG reduced the structural stability of the enzyme [118]. The results emphasize the potential of this platform for developing biological control strategies to combat fungal diseases in agriculture.

Furthermore, *B. subtilis* can be engineered as an effective carrier for the oral delivery of therapeutic proteins, presenting significant advantages over intravenous administration. Oral drug delivery system is the most preferred route for drug administration due to many advantages such as non-invasiveness, and patient compliance and convenience. However, achieving optimal therapeutic outcomes through oral administration, particularly for conditions like cancers, remains challenging. Many anti-cancer drugs suffer from poor water solubility, instability, and low bioavailability. To overcome these limitations, oral drug delivery systems have been developed to protect drugs from degradation by gastric acid and ensure precise release in the intestinal tract. Once released, the drugs can be efficiently absorbed, enhancing their therapeutic efficacy. *B. subtilis*, with its ability to be genetically modified, represents a promising platform for developing such protective and targeted drug delivery systems, improving the stability and bioavailability of anti-cancer therapies [119]. *B. subtilis* spores with their hydrophobic nature and large specific surface area, are another delivery system that has recently been applied for delivery of therapeutic proteins, anti-cancer drugs, cytotoxic genes, and as vectors with great potential for gene therapy [119]. *B. subtilis* spores, highly resilient survival structures formed under harsh conditions, have shown remarkable potential as drug carriers, particularly in the tumor microenvironment. Their ability to thrive under hypoxic conditions makes them highly compatible for tumor targeted therapies. They can protect drugs with poor stability and pharmacokinetics, ensuring their survival through the stomach and controlled release in the gut. These characteristics make spore-based systems a promising platform for enhancing the efficacy and precision of oral therapies [120]. *B. subtilis* spores are highly effective in loading significant quantities of hydrophobic drugs through adsorption or covalent binding. Upon germination in the intestinal tract, the spore's outer coat is broken down and degraded, facilitating the release of the loaded substances. These unique characteristics make *B. subtilis* spores an excellent oral delivery vehicle, capable of transporting a wide range of beneficial compounds to the intestinal tract. This potential has spurred significant interest in exploring their use for targeted and efficient drug delivery [121].

A novel oral drug delivery system utilizing *Bacillus* spores has shown promising potential for colon cancer treatment. Yin L et al., have designed a novel probiotic

Bacillus spore-based oral colon targeted drug carriers for the treatment of colon cancer. Curcumin (CUR) is a natural plant-derived anti-cancer agent, which can inhibit tumor cell proliferation and induce tumor cell apoptosis. Curcumin as a model drug was linked covalently to the outer coat of *Bacillus* spore (SPORE-CUR). Subsequently, folate (FA) was incorporated covalently to curcumin on spore surface (SPORE-CUR-FA). This delivery platform leverages the spores' natural resilience to harsh gastric conditions, allowing them to traverse the stomach and reach the colon. Upon germination in the colon, the spore's outer coat disintegrates, releasing the loaded drug. Investigations into the system's drug release profile, both in vitro and in vivo, demonstrated its ability to achieve targeted delivery to the colon. Pharmacokinetic analyses confirmed that the spore-based carrier significantly enhanced the oral bioavailability of curcumin. Further anti-tumor evaluations revealed that SPORE-CUR-FA effectively inhibited colon cancer cell growth, showcasing its therapeutic potential [122]. These findings underscore the efficacy and viability of *Bacillus* spore-based oral drug delivery systems as a targeted approach for cancer therapy.

In another study, Nguyen VA and colleagues developed an innovative drug delivery platform utilizing recombinant *B. subtilis* spores (SA1) engineered to express streptavidin as a fusion protein with spore coat protein CotB. These inactivated spores were designed to function as bioparticle carriers for biotinylated cetuximab, enabling precise targeting of the epidermal growth factor receptor (EGFR) on HT-29 human colorectal cancer cells. When conjugated with the chemotherapeutic agent paclitaxel, the SA1–cetuximab complex exhibited a fourfold improvement in delivery efficiency to cancer cells, as demonstrated by fluorescent labeling. This targeted delivery significantly enhanced the therapeutic efficacy of paclitaxel, reducing its IC50 value to 2.9 nM, approximately five times lower than the IC50 of water-soluble paclitaxel (14.5 nM). Moreover, treatment with the delivery system at higher concentrations (16 nM) led to the induction of polyploidy in cancer cells, indicating its ability to interfere with cell division [123]. These findings highlight the potential of SA1 spores as versatile drug carriers capable of selectively delivering chemotherapeutic agents to cancer cells through conjugation with biotinylated antibodies. This strategy offers a promising approach to improve therapeutic outcomes while minimizing off-target effects.

Expanding upon novel therapeutic delivery strategies, Tran D.M and colleagues investigated the use of nasal-spraying probiotics as a treatment for respiratory syncytial virus (RSV)-induced acute respiratory tract infections (ARTIs) in children. Their study utilized a probiotic formulation containing 5 billion *Bacillus* spores (LiveSpo Navax). Conducted as a 6-day randomized controlled clinical trial, the study included RSV-infected children (n = 40–46 per group) who were treated with either Navax or standard care. This study highlights the potential of spore-based probiotics as a novel intervention for managing viral respiratory infections in pediatric patients [124].

In a pioneering study, *Clostridium butyricum* spores were successfully utilized to carry gemcitabine-loaded mesoporous silicon nanoparticles (MGEM), forming the SPORE-MGEM system for targeted drug delivery to pancreatic tumors for the treatment of pancreatic ductal adenocarcinoma (PDAC). Leveraging the advantages of spore-based drug delivery platforms, *B. subtilis* spores, with their well-characterized stability and resilience, could serve as an alternative carrier system for chemotherapeutic agents. By leveraging the gut–pancreas axis, *B. subtilis* spores could potentially facilitate more efficient intratumoral drug delivery, improving localized drug accumulation while minimizing systemic toxicity. The versatility of *B. subtilis* in surface display systems and its ability to efficiently protect and deliver bioactive molecules suggest that it could be a promising candidate for enhancing the delivery of chemotherapy drugs like gemcitabine in PDAC treatment, similar to the success observed with *C. butyricum* spores [125]. This platform could represent a significant advancement in overcoming the challenges associated with PDAC chemotherapy, offering enhanced drug targeting and reduced side effects.

These studies highlight the remarkable potential of microbial spores, particularly *B. subtilis*, as versatile platforms for innovative therapeutic strategies. From targeted drug delivery systems in cancer treatment to combating respiratory infections, spore-based technologies demonstrate unique advantages such as stability, bioavailability, and specificity. By harnessing the gut–organ axis and surface display systems, *B. subtilis* spores offer promising strategy to overcome traditional drug delivery challenges, thereby enhancing therapeutic efficacy and minimizing adverse effects. Collectively, these findings pave the way for spore-based platforms to revolutionize biomedical applications, especially in treating challenging diseases like cancers and respiratory infections.

Role in drug discovery and high-throughput screening (HTS)

Microbial systems have been extensively studied and genetically engineered to address industrial requirements and solve societal challenges. However, their complexity often requires combinatorial approaches to achieve optimal results. HTS, with its advantages of miniaturization,

time efficiency, and automation, allows for the rapid and comprehensive evaluation of a wide range of experimental conditions. HTS is particularly valuable in antibacterial drug discovery, enabling the swift screening of large molecular libraries for bioactivity. Despite more than half of the antibiotics currently utilized being derived from natural products, discovering novel antibiotics has become increasingly challenging as many accessible sources have already been explored. Moreover, finding new natural sources with antibacterial properties has proven difficult, hindering progress in this field [126, 127]. Integrating bioengineering with HTS enables the automated evaluation of thousands of samples for binding activity against one or multiple targets simultaneously. By utilizing bacterial surface display, a diverse array of engineered ligands can be presented on the surface of microorganisms, significantly enhancing ligand diversity and streamlining the development process. This method enables the rapid identification and optimization of ligands with desired properties, thereby accelerating the development of novel therapeutics. This approach allows for rapid and efficient compound screening without the need for complex purification processes or expensive analytical equipment [128–130].

The use of *B. subtilis*, either as whole cells or in spore form, significantly enhances the efficiency of protein, antibody, and peptide library screening for drug discovery. By displaying diverse protein fragments, antibody variants, or peptides on the bacterial cell surface or durable spore coat, interactions with disease-specific targets, such as cancer-related receptors, viral proteins, or enzymes involved in neurodegenerative disorders, can be screened. The exceptional stability of *B. subtilis* spores further supports HTS under various conditions, making them ideal for drug discovery applications. Advanced sorting techniques, such as fluorescence- and magnetic-activated cell sorting, enable the rapid identification of candidates with strong binding affinities, facilitating the discovery of novel therapeutic and diagnostic molecules. This approach accelerates drug development by enabling efficient assessment of binding specificity and optimization of bioactive compounds for clinical applications [128, 129, 131, 132].

Supporting this approach, Fleetwood et al., demonstrated the potential of Gram-positive bacterial surface display for selecting high-affinity binding proteins from a combinatorial Nanobody library. Their study utilized *Staphylococcus carnosus* to display a library of ~107107 Nanobody variants, enabling the identification of subnanomolar affinity binders via flow cytometry. Compared to traditional phage display, bacterial display systems yielded distinct sets of high-affinity variants. Notably, some nanobody candidates identified through bacterial display exhibited fluorescence-enhancing properties when binding to green fluorescent protein (GFP), showcasing the influence of display technology on selection outcomes. These findings highlight the effectiveness of Gram-positive bacteria, including *B. subtilis*, in constructing and screening protein libraries, reinforcing their role in the discovery of novel therapeutic molecules [133].

Another study by Gupta et al., highlighted the potential of *B. subtilis* spores as an innovative platform for screening protein libraries, demonstrating their resilience and advantages over traditional systems such as Gram-negative bacteria, phages, and yeast. The researchers engineered a mutant of CotA, a laccase naturally found on spore coat, achieving a 120-fold increase in specificity for ABTS [diammonium 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate)] over SGZ (4-hydroxy-3,5-dimethoxybenzaldehyde azine). This study underscores the ability of *B. subtilis* spores to bypass protein-folding issues associated with membrane translocation, enhancing protein stability and functionality. Combined with advanced sorting techniques, such as fluorescence- and magnetic-activated cell sorting, this approach enables the rapid identification of candidates with strong binding affinities, facilitating the discovery of novel therapeutic and diagnostic molecules [134].

Overall, *B. subtilis* spores offer a versatile and robust platform for various applications in drug discovery, protein engineering, and HTS. Their stability under harsh conditions, ability to display peptides, proteins, and antibodies on the spore surface, and ease of handling make them an ideal choice for advancing biotechnological innovations.

Challenges and opportunities in *B. subtilis* surface display technique

B. subtilis surface display systems hold significant promise for biotechnology, but several challenges hinder their broader application. Although numerous surface display strategies have been developed for both the vegetative cells and spores of *B. subtilis*, most remain limited to experimental settings. Scaling up the production of target proteins for industrial applications poses critical challenges for both systems.

One of the key challenges in spore-based display systems is identifying appropriate carrier proteins, as the stability of surface-displayed proteins relies on both the protective properties of spore coat and the intrinsic stability of the displayed protein. Although the outermost spore layer offers significant protection against environmental factors such as pH shifts, temperature fluctuations, and enzymatic degradation, some surface-displayed proteins may still experience structural instability or loss

of function, especially if they are not effectively anchored or naturally stable. Therefore, choosing resilient carrier proteins and refining display strategies are essential for preserving protein activity and durability [135, 136]. Recent studies have investigated alternative strategies, including the use of *B. subtilis* OxdD, an inner-coat enzyme, as an anchoring motif. Unlike outer-coat proteins, OxdD's lower abundance and internal localization offer advantages, such as improved protection of passenger proteins from environmental exposure and reduced disruption of spore coat assembly [137]. Therefore, this approach presents a promising direction for diversifying carrier protein options and enhancing the functional potential of spore-based display platforms.

Despite significant advancements in the last two decades, *B. subtilis* spore display still faces challenges related to low expression levels of passenger proteins fused to carrier proteins on the spore surface, which limits its suitability for industrial applications. Achieving optimal expression levels is essential for maintaining functional activity without impairing spore integrity or overloading the expression system. In one study, *B. subtilis* spores were used as a display platform, with outer surface proteins CotB, CotC, and CotG serving as carriers, and amyQ-encoded α-amylase and GFPuv as passenger proteins. The translational fusions were regulated by two different IPTG-inducible promoters, allowing controlled expression of the passenger proteins. By systematically varying IPTG concentrations during sporulation, the researchers demonstrated that the amounts of fusion proteins displayed on the spore surface could be fine-tuned. Notably, the optimal IPTG concentration varied depending on the specific combination of carrier and passenger proteins, highlighting the importance of precise optimization in surface display systems [138]. This study addresses the challenge of adjustable protein expression by establishing a method for regulatable surface display. In the future, further research should focus on developing stronger promoters through genetic engineering to enhance expression efficiency and optimize surface display systems.

Considering the challenges of low expression levels in *B. subtilis* spore display, optimizing these systems also requires addressing protein stability, folding, and functionality. A critical aspect in this optimization is the design of the linker connecting the anchor and displayed proteins, as it significantly influences the structural integrity, spatial orientation, and functional activity of the fusion proteins. Rigid linkers, such as EAAAK, stabilize protein structure but can limit the mobility and flexibility of the displayed proteins, while overly flexible linkers, like GGGGS, may destabilize proteins. To address this issue, a combinatorial strategy involving both rigid and flexible linker units has been developed, with intermediate flexibility linkers (e.g., FFFRR) showing optimal performance in fusion proteins like PhoC-GFP [139]. Applying similar strategies to spore surface displays can enhance protein stability, accessibility, and functionality, opening new possibilities for applications in enzymatic biocatalysis, vaccine delivery, and biosensing. To further improve these systems, future research should focus on utilizing computational tools and HTS methods to design novel linkers. This approach could accelerate the development of robust, multifunctional spore-based systems for a variety of biotechnological applications.

The development of microbial engineering for surface protein display is often complicated by issues like protein degradation and cellular autolysis. A notable example is the Cel8A-LysM reporter, which is susceptible to extracytoplasmic proteases and autolysis during the stationary phase. To address these challenges, protease-deficient *B. subtilis* strains, including WB800, BRB07, BRB08, and BRB14, have been systematically assessed for their ability to improve protein stability. Among these, the BRB08 strain showed the most promise, offering enhanced stability of surface-displayed proteins without inducing secretion stress or impairing cell growth. Furthermore, the application of chemical protease inhibitors and a two-step procedure for constructing enzyme-coated vegetative cells significantly improved protein stability and retention [8]. These advancements highlight the potential for developing more stable and efficient surface protein display systems, with broad implications for diverse biotechnological applications. Future optimization of protease-deficient strains, combined with advanced molecular tools such as synthetic biology, could further improve the stability and efficiency of these systems, enabling their wider adoption in biotechnology and pharmaceutical industries.

The complex structure of *B. subtilis* spores, which consists of numerous distinct proteins, poses challenges for the efficient display of exogenous proteins. A primary limitation is the number of available anchoring proteins on the spore surface, which restricts both the display efficiency and the functional versatility of spore-based systems. To overcome this challenge, researchers have explored the simultaneous use of multiple anchoring proteins to enhance display capacity. By utilizing proteins such as CotC and CotG, *B. subtilis* spores can display a variety of exogenous proteins concurrently, significantly broadening their functional potential. One such recent development is the use of a spore surface display system for trehalose synthase (TreS) production. In this system, CotC and CotG were used as carriers, achieving a high efficiency with a 73% conversion rate maintained over four reuse cycles. The successful use of TreS surface

display demonstrates the advantages of multi-anchor strategies in enhancing both performance and reusability [140]. This approach highlights the potential of expanding spore-based display systems by simultaneously utilizing multiple anchoring proteins. Future research should focus on identifying and engineering additional anchoring proteins to further diversify the display options. Additionally, advanced molecular tools, including protein engineering and HTS, could optimize multi-anchor systems for a wider range of applications.

Conclusion

B. subtilis surface display, utilizing both spore and vegetative cell forms, offers a versatile platform for a wide range of biotechnological applications. Spore-based systems stand out for their robustness, long-term stability, and resistance to harsh conditions, making them ideal for enzymatic biocatalysis, environmental biosensors, mucosal vaccines, and drug delivery system. Recent innovations in linker design and multi-anchor strategies have further improved their performance. Meanwhile, vegetative cells serve as dynamic hosts for real-time therapeutic protein production, live-cell delivery, and metabolic engineering. Combining metabolic engineering with surface display enhances in vivo biocatalysis, enabling more efficient production of biochemicals, biofuels, and therapeutic compounds. Future research should focus on integrating the strengths of both spore- and cell-based systems by leveraging synthetic biology, computational modeling, and HTS. These advancements have the potential to transform bioprocessing and biomanufacturing, driving innovation in global healthcare and environmental sustainability.

Author contributions

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Declarations

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