catalysts

MDPI

Review

Microbial Spore-Based Biocatalysts: Properties, Applications and New Trends

Brana Pantelic *, Nikola Radivojevic, Ivana Aleksic, Jelena Simic and Jasmina Nikodinovic-Runic *

Institute of Molecular Genetics and Genetic Engineering, University of Belgrade, Vojvode Stepe 444a, 11221 Belgrade, Serbia

* Correspondence: brana.pantelic@imgge.bg.ac.rs (B.P.); jasmina.nikodinovic@imgge.bg.ac.rs (J.N.-R.); Tel.: +381-64-8044093 (B.P.)

Abstract

Microbial spores are increasingly recognized as multifunctional platforms for enzyme immobilization, combining natural resilience with biotechnological versatility. Their inherent structural complexity enables high enzyme load, thermal and chemical stability, and robustness to be repeatedly used under industrially relevant conditions, largely widening their application scope. This review explores the growing role of spore-based systems in biocatalysis, from naturally active spores to engineered microbial hosts capable of producing immobilized enzymes in situ. Compared to conventional immobilization techniques, spore-based strategies offer simplified workflows, reduced environmental impact, and greater sustainability. Recent innovations also extend beyond traditional applications, introducing artificial spores and incorporating spores into biocomposite materials and biosensors. These developments reflect a shift from basic enzyme stabilization research toward scalable solutions in waste remediation, polymer degradation, green chemistry, and synthetic biology. Overall, spore-enabled biocatalysis represents a modular and robust toolset for advancing industrial biotechnology and sustainable manufacturing, instrumental in achieving a circular and bioeconomy.

Keywords: biocatalysis; spore; spore display; enzyme immobilization; *Bacillus*

Academic Editor: Aniello Costantini

Received: 25 August 2025
Revised: 12 September 2025
Accepted: 16 September 2025
Published: 17 September 2025

Citation: Pantelic, B.; Radivojevic, N.; Aleksic, I.; Simic, J.; Nikodinovic-Runic, J. Microbial Spore-Based Biocatalysts: Properties, Applications and New Trends. *Catalysts* **2025**, *15*, 894. https://doi.org/10.3390/catal15090894

1. Introduction

As society increasingly pivots towards sustainable manufacturing practices in a circular and bioeconomy, the role of biocatalysis has become irreplaceable [1]. This transition, driven by the need to reduce industrial waste, lower energy consumption, and minimize environmental impact, requires innovative technologies that align with the principles of green chemistry. Biocatalysis, with its use of enzymes under mild conditions, exceptional selectivity, and inherent biodegradability [2], is uniquely positioned to meet these demands and is instrumental in achieving the ambitious goals set by initiatives such as the EU Green Deal and the UN Sustainable Development Goals.

Enzyme immobilization has long been a cornerstone strategy in industrial biocatalysis, key for enhancing the stability, longevity, and reusability of biocatalysts. Conventional ex situ techniques, which involve attaching purified enzymes to carriers like SiO_2 or synthetic polymers through methods such as adsorption or covalent binding, have been widely adopted [3,4]. However, these approaches are usually multi-step and resource-intensive, requiring costly upstream processes for enzyme production and purification that ultimately reduce the economic and environmental sustainability of a biocatalytic processes [5,6].

Seeking more sustainable and integrated solutions, microbial spores have emerged as highly adaptable, bio-based matrices for biocatalyst immobilization [7,8].

Spores are naturally resilient structures that enable certain organisms to enter a dormant state in suboptimal environmental conditions, protect the organism, and later germinate when circumstances become favorable [9]. They usually consist of dense, multilayered coats of polysaccharides and proteins that shield the mother cell's genetic material from desiccation, pH fluctuations, temperature extremes, gamma radiation, and can even shield from the harsh conditions of outer space [10]. Across the prokaryotic phylogenetic tree, spore-forming species are almost exclusively found in the Gram-positive Firmicutes and Actinobacteria phyla [11,12] and are largely absent across other lineages, with few exceptions [13]. Broadly, spores can be classified into two types: endospores, formed by asymmetric division within a mother cell and released upon lysis (e.g., *Bacillus*), or exospores, formed by outward differentiation of vegetative cells or specialized structures (e.g., *Streptomyces*) (Figure 1A). As *Bacillus* is one of the best-studied model organisms in industrial biotechnology, *Bacillus* spores have been thoroughly investigated [14]. In *Bacillus*, the spores' protective shell, called the spore coat, is a complex structure that is assembled from about 70 different proteins (mainly Cot proteins) sectioned from the cortex, the basement layer to the inner and outer coat layer, and finally the crust, while the inner part of the spore, carrying the genetic material, is referred to as the core [15]. The described coat structure is characteristic of *B. subtilis*, but some species of *Bacillus*, such as *B. megaterium* or *B. cereus*, have an additional polysaccharide layer called the exosporium [15] (Figure 1B).

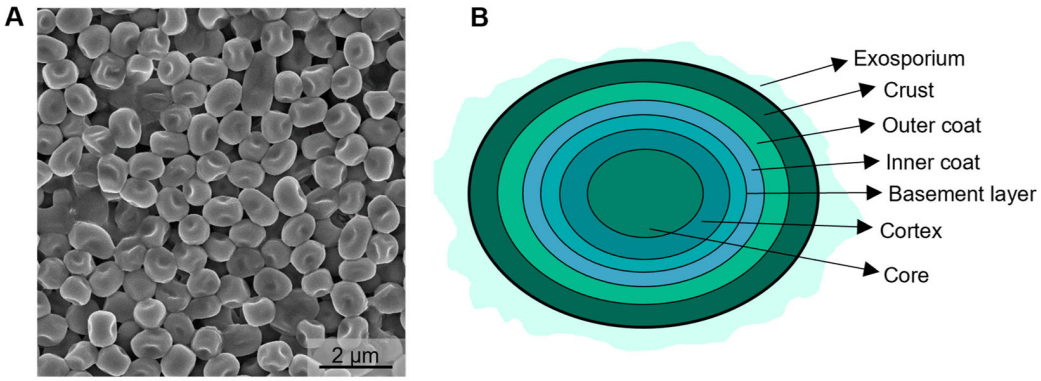

Figure 1. (**A**) Representative SEM micrography of *Streptomyces* spores (10,000× magnification); (**B**) Graphic representation of *Bacillus* spore structure (spore layers are not represented to scale).

Through adsorption, chemical conjugation, or genetic engineering, enzymes can be attached to the surface of spores to create spore-based biocatalysts [7]. Spore surfaces provide a structurally intricate 3D matrix characterized by a high surface-to-volume ratio, which facilitates efficient enzyme adsorption and entrapment [16,17]. The abundance of naturally occurring functional groups on spore surfaces eliminates the need for activating carriers in covalent immobilization, thereby reducing reliance on hazardous chemicals commonly associated with some conventional methods [17]. At the same time, the attached enzymes are protected by the spore and retain high activity, resist proteolysis and harsh process conditions, and can be readily recovered and reused [18]. Importantly, spores can be genetically engineered to display target enzymes by creating chimeric fusion proteins consisting of spore coat proteins and target enzymes [8,19]. This enables in situ production and immobilization of enzymes, effectively bypassing labor-intensive and costly purification steps. Such systems significantly lower biocatalyst production expenses by integrating synthesis and immobilization within the microbial host. Furthermore, spore-based surface display leverages native secretion and anchoring pathways, minimizing the need for harsh

reagents that could compromise enzyme structure and function [8]. In addition to the typical advantages of immobilized systems in terms of reusability and recovery, spores offer the unique potential for full biocatalyst recycling through theoretically unlimited germination and re-sporulation cycles. These features have been successfully exploited in diverse applications, ranging from antigen and therapeutic delivery in immunomodulatory formulations to the stabilization of industrially relevant biocatalysts [7,20,21]. A bibliometric analysis of publications from the last 10 years using the search terms "spore display" and "spore immobilization" indeed reveals a clear clustering of research into two main fields: antigen presentation/vaccine-oriented applications and biocatalysis-oriented applications based on the term co-occurrence network presented in Figure 2A. As the use of spores displaying different peptide antigens as vaccines is covered elsewhere [22,23], this review will focus on the applications of microbial spores in biocatalysis.

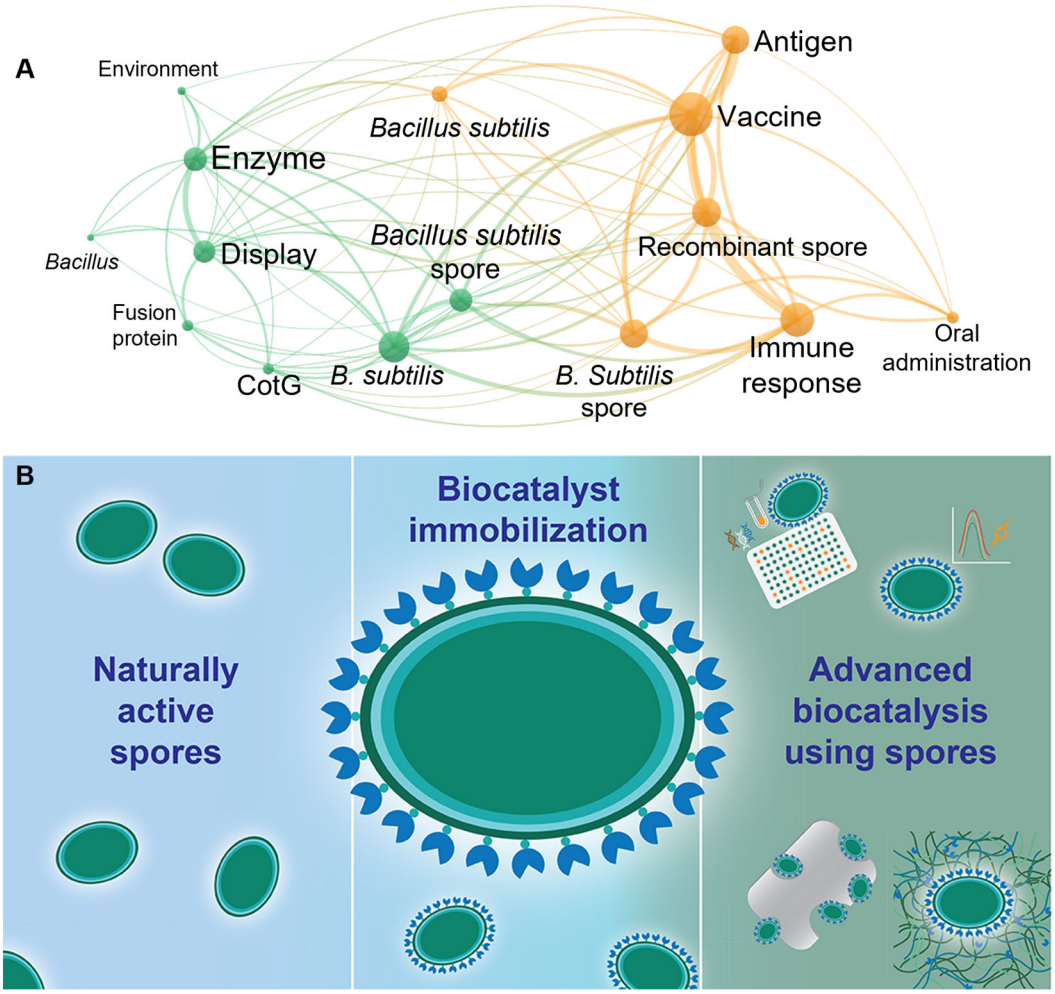

Figure 2. (**A**) Co-occurrence network visualization of terms related to publications on spore display in the last 10 years, prepared using WOSviewer [24] with green nodes indicating terms related to spore-based biocatalyst research and orange nodes representing terms related to spore-based vaccine research; (**B**) Graphic representation of the different types of spore-based biocatalysis from naturally active spores, using spores in traditional biocatalysis as enzyme carriers and advanced biocatalysis using spores.

This review explores the multifaceted role of microbial spores in biocatalysis, ranging from their natural activity as biocatalysts, such as CotA-containing spores, to their well-established use as durable carriers for immobilized enzymes in food processing, pharmaceuticals, and environmental applications. Beyond these conventional roles, recent

advances have positioned microbial spores at the forefront of innovative biotechnological strategies, including their use in directed evolution, biosensing platforms, and the development of smart living materials (Figure 2B). Furthermore, emerging methods for engineering artificially sporulated cells are expanding the potential and versatility of this unique biocatalytic system. Together, these developments underscore the growing relevance of microbial spores as robust, adaptable, and forward-looking platforms in modern biocatalysis.

2. Naturally Occurring Biocatalytically Active Spores

Bacterial spores are typically studied for their physio-ecological role in survival, transmission, and resistance to extreme environmental conditions [25]. However, beyond these well-established functions, certain spores exhibit inherent biocatalytic properties, which is an aspect that has gained increasing attention in recent years. Among these, the most comprehensively studied example is spore-coat enzyme CotA (EC:1.10.3.2), a multicopper oxidase found in the spore coat of most members of the Bacillaceae family [26–28]. Although CotA's primary biological function is to produce a pigment that protects the spore from UV radiation and oxidative agents like hydrogen peroxide [15], its versatile catalytic activity has also been harnessed for other biocatalytic applications.

CotA laccase catalyzes the oxidation of a broad range of phenolic and non-phenolic substrates. This enzymatic activity enables bacterial spores to function as naturally immobilized biocatalysts in numerous oxidative transformations, particularly in toxic dye decolorization and pollutant degradation.

CotA-containing spores of *B. amyloliquefaciens* exhibit strong biocatalytic activity, achieving up to 98% synthetic dye decolorization under mild thermal and alkaline conditions [27,28]. The process was further enhanced through redox mediation, light exposure, and extended contact time, with high stability at elevated temperatures and in the presence of solvents and hydrogen peroxide [29]. These spores also exhibited selective oxidation of aromatic amines and benzylic alcohols, and were effective across multiple reuse cycles when immobilized and paired with natural mediators like acetosyringone [30]. *B. licheniformis* spores demonstrate high resilience and catalytic efficiency under industrial conditions, with CotA laccase retaining activity in high salinity and solvent-rich environments, remaining stable at pH 9 and partially active at 80 °C [26]. Expanding on the resilience seen in *B. licheniformis*, spores from the marine-derived *Bacillus* sp. KC2 offers impressive catalytic stability under extreme environmental conditions. Their CotA-like laccase-maintained activity across a wider temperature range (20–70 °C), at neutral to alkaline pH, and in the presence of high salinity and organic solvents. Additionally, the spores effectively decolorized various dye types, including azo, indigo, thiazine, and triarylmethane compounds, achieving complete dye removal within 5 h [31]. As part of further improvement efforts, several studies demonstrated that spores themselves could be immobilized, as exemplified by *B. pumilus* spores immobilized on diethylethanolamine (DEAE) cellulose. The spores exhibited enhanced stability and reusability under alkaline and thermal stress. These immobilized systems maintained high decolorization efficiencies (up to 88.6%) and retained activity across multiple cycles [32], highlighting their scalability and robustness as engineered biocatalysts for long-term industrial wastewater treatment.

Clostridium difficile spores also harbor CotA homologues alongside several other redox-active enzymes (catalases, peroxiredoxins) located in the spore coat, suggesting latent oxidative capabilities yet to be fully explored [33]. Fungal spores likewise show promise. Species like *Penicillium* have been used for selective bioconversion of fatty acids and terpenes into methyl ketones or aroma compounds under biphasic or encapsulated conditions, especially strains of *P. roquefortii* [34] and *P. digitatum* [35]. While such systems are less common, they illustrate the metabolic diversity and potential of spore-based catalysts.

Naturally active spore-based biocatalysts offer a unique combination of natural immobilization, resilience, and broad substrate scope for oxidizing persistent dyes and environmental pollutants such as aromatic amines, benzylic alcohols, and phenolic compounds under industrially relevant conditions, making them strong candidates for eco-friendly wastewater treatment and green synthesis. Unfortunately, naturally active spores can only catalyze a small number of reactions in the overall biocatalytic reaction landscape. Therefore, to widen the scope of microbial spores in biocatalysis, it was essential to develop efficient engineered modular protein display systems that enable the functional integration of non-native enzymes with the inherent stability of spores (Figure 2).

3. Spore Immobilization/Display Systems for Biotechnologically Relevant Enzymes

Enzymes can be immobilized/displayed on the surface of bacterial spores using adsorption, covalent binding and genetic display (Figure 3A). Among bacterial spore display systems, *Bacillus* species are the most extensively employed. Owing to their extremely robust spores and well-established genetic manipulation techniques [36], *Bacillus* spores have been used to construct both non-recombinant and recombinant display systems [7,8]. The most exploited species include *B. subtilis, B. thuringiensis* [37], *B. megaterium* [38] and *B. polyfermenticus* [39]. Notably, *B. megaterium* and *B. polyfermenticus* were implemented in adsorption processes only, with no genetic display platforms developed. Recently, other endospore-forming bacterial species such as *Shouchella clausii* [40] and *Paenibacillus polymyxa* [41] were used in display systems. While *S. clausii* spores were used in non-recombinant display as protein-based drug delivery agents, spores of *P. polymyxa* have been employed in biocatalysis through genetic surface display. In addition to the available scientific literature, several patents describing methods for displaying proteins on *Bacillus, Clostridium*, *Brevibacillus*, *Lysinibacillus* and *Viridibacillus* species have been filed, highlighting the commercialization potential of spore-based biocatalysts (Table 1). Some of the specific uses of spore-based biocatalysts covered by patented technology are: spore-bound β-galactosidase for the food industry (KR100758209B1), biosensors (WO2022049442A1, CN109593695A) and plant growth promotion (WO2019060574A1) (Table 1). Overall, spore surface display is primarily conducted using endospore-forming species, however, other species like *Streptomyces* (Figure 1A), which are known for exospore formation, hold promising potential as candidates for spore surface display, although this area of research remains largely unexplored.

Table 1. Selection of patents covering methods for producing and using spore-based biocatalysts.

Description of Patent	Patent	Year	Application Filed by
Methods for surface display of target proteins on bacterial spores	WO2020232316A1	2019	Bayer Cropscience LP
	WO2020102642A2	2018	Bayer Cropscience LP
	WO2019099635A1	2017	Bayer Cropscience LP
	WO2016140702A1	2015	US Department of Health & Human Services
	WO2012001000A1	2010	DSM IP Assets B.V.
	WO2011160026A2	2010	Research Development Foundation
	WO2006012366A2	2004	Phyllom LLC (Piedmont, CA, USA)
	WO2005028654A1	2006	Korea Advanced Institute of Science and Technology
	WO2002046388A1	2000	Genofocus Co., Ltd. (Daejeon, Republic of Korea)
	WO2002000232A2	2000	Maxygen, Inc. (Newark, CA, USA)
	KR102173586B1	2019	Korean Agency for Defense Development

Table 1. *Cont.*

Description of Patent	Patent	Year	Application Filed by
Specific applications of spore-based biocatalysts.	WO2022049442A1	2020	3M Innovative Properties Company (St. Paul, MN, USA)
	WO2019060574A1	2017	Spogen Biotech Inc. (Saint Louis, MO, USA)
	KR101244433B1	2009	Korea Research Institute of Bioscience and Biotechnology
	KR100758209B1	2004	HLB Genex (Daejeon, Republic of Korea)
	CN109593695A	2018	Qilu University of Technology
	CN103014054A	2012	Jiangnan University

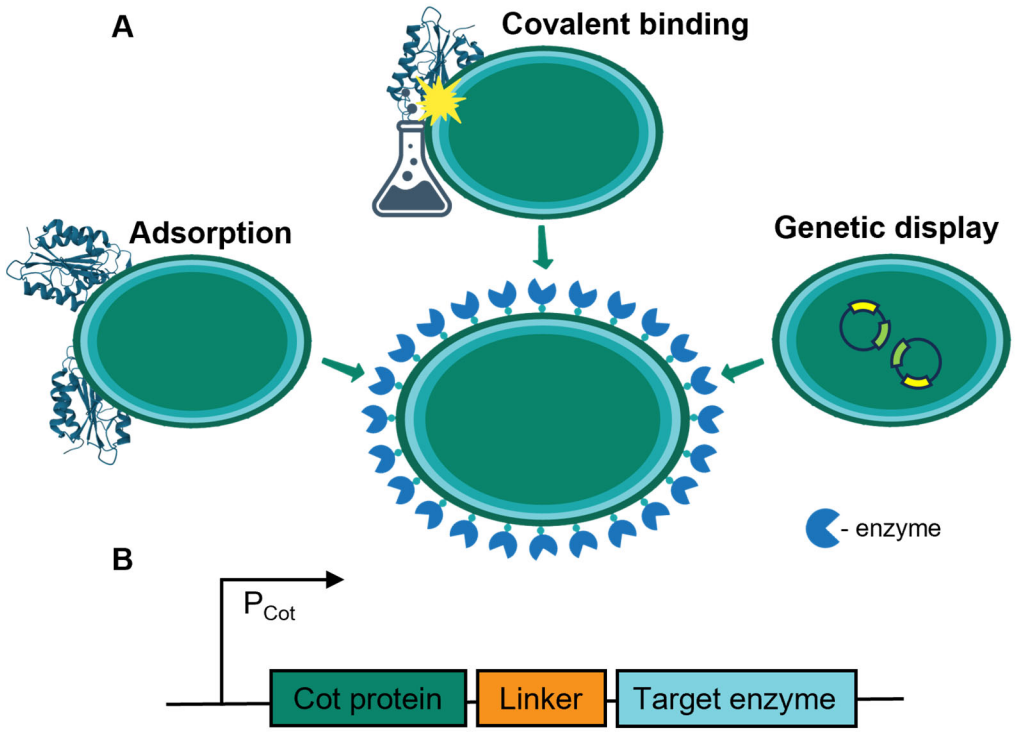

Figure 3. Strategies for enzyme immobilization on bacterial spores. (**A**) Illustration of the three primary methods for displaying enzymes on the spore surface: adsorption, covalent immobilization, and genetic display; (**B**) Schematic of an expression cassette used for genetic display consisting of a sporulation-specific promoter, spore coat (Cot) protein, a peptide linker, and the target enzyme.

3.1. Adsorption of Enzymes onto Spores

Adsorption is a non-specific, reversible, physical method of binding an enzyme to a carrier, stabilizing it and, enhancing its resistance to pH, temperature, organic solvents, and proteases [42,43]. Effective adsorption relies on strong enzyme–carrier affinity, mediated by weak Van der Waals, electrostatic, and hydrophobic forces [44,45]. Ideal carriers are inert, durable, biocompatible, microbe-resistant, and readily available, with common examples including starch, collagen, cellulose, silica gel, clay, and aluminum oxide [42,46].

Bacterial spores emerged as a bio-based alternative carriers for enzyme adsorption [17]. A key advantage of spores lies in their nature as "living systems" capable of germination, setting them apart from non-living organic or inorganic carriers while retaining properties such as high thermal stability and resistance to organic solvents, which are absent in other living carriers [47]. The structure of the spore coat, with numerous proteins and polysaccharides, provides the functional groups necessary for efficient enzyme adsorption. Since adsorption is driven by electrostatic attraction, the process is highly dependent on pH. Spore surfaces have a net negative charge, so for the strongest binding, the target enzyme

should be positively charged. Therefore, acidic conditions (below the pI of most proteins) are favored for adsorbing proteins onto spores [48]. For instance, phytase from *E. coli*, with a pI of 4.5, demonstrates the best adsorption onto *B. subtilis* spores at pH 3, where the phytase is positively charged, resulting in a strong binding to the negatively charged spore surface [39].

The 3D structure of the outermost layer of spores is another important factor for enzyme adsorption. The previously described coat structure (Figure 1B) is characteristic of *B. subtilis*, but some species of *Bacillus*, such as *B. megaterium* or *B. cereus* have an additional polysaccharide layer called the exosporium [15]. In both exosporium and non-exosporium forming species, the crust is made up mostly of the CotY protein, which self-assembles, creating a semipermeable honeycomb-like lattice structure [49]. During adsorption, enzymes embed in these structures and are consequently adsorbed onto the layer beneath. Namely, in exosporium-forming species, enzymes localize between the outer layer and exosporium, whereas in species lacking an exosporium, proteins are positioned between the outer and inner layers [50]. The spore coat is a complex structure that remains an active area of research, with recent cryo-electron tomography research of spore surfaces continuing to advance understanding of spore architecture and its role in adsorption [16].

Modification of the spore coat has also been attempted by creating recombinant strains lacking some of the coat proteins, the porosity of the spore is increased, which in turn leads to higher adsorption capacity. Sirec et al. demonstrated that the enzyme efficiency of immobilized β-galactosidase increased from 50% in wild-type spores to 80% in mutants lacking cotXYZ genes. Similarly, spores lacking CotH could adsorb 88% of red fluorescence protein from solution, compared to 68% reported for wild-type spores [50]. Although the efficiency of the enzyme adsorption onto spores was improved, the enzymes remained loosely bound, leading to increased leakage. Furthermore, in some cases, enzymes adsorbed onto mutant spores exhibit reduced stability [51]. Therefore, wild-type spores, exhibiting greater stability, reusability, and minimal leakage, are still more suitable for biocatalysis and industrial applications. Crosslinking of adsorbed proteins to the spore surface was also applied in order to prevent enzyme leakage. While adsorption is a straightforward immobilization method, the weak interactions often lead to enzyme desorption after just a few reaction cycles, causing a sharp drop in overall activity [52,53]. This issue can be partially overcome by using cross-linking agents to covalently lock the adsorbed proteins in place. For instance, by cross-linking *Staphylococcus aureus* protein A to spores using glutaraldehyde, the immobilization yield increased 4-fold compared to simple adsorption, and the system retained 60% of its activity after seven cycles, indicating minimal leakage [54]. However, even when leakage is prevented, adsorption-based methods provide little control over the orientation of the enzyme on the carrier surface. Consequently, some enzyme molecules invariably adsorb in a way that renders their catalytic site inaccessible to substrates, leading to their inactivation [52,53].

3.2. Covalent Immobilization of Enzymes onto Spores

Another prominent method of enzyme immobilization is achieved through covalent binding. Although commonly applied to inorganic and organic carriers, covalent immobilization is rarely employed in spore-based systems. Covalent immobilization of enzymes onto spores was reported for α-amylase and organophosphorus hydrolase (OPH). Immobilization of both enzymes was achieved by using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and N–hydroxysulfosuccinimide (NHS). In a two-step incubation process performed at room temperature and acidic pH, EDC activates free carboxyl groups, namely aspartate and glutamate residues on the spore surface, creating a nucleophile. NHS reacts with the nucleophile, creating an ester linkage and subsequently

the NHS ester reacts with free amino groups of the enzyme, creating amide bonds and binding the enzyme to the spore. In the case of α-amylase, covalent binding increased immobilization efficiency almost 4-fold compared to adsorption [55], while covalently bound OPH displayed increased activity retention [56]. Additionally, both enzymes immobilized in this way showed higher pH and thermal stability. The main drawback of covalent immobilization is the use of toxic EDC and NHS, raising environmental concerns [57]. Furthermore, covalent immobilization has limitations such as long, intricate incubation steps as well as low quantity of immobilized enzymes [3]. Also, similarly to adsorption methods, covalent immobilization requires prior enzyme purification, increasing waste generation and raising the cost of the process [21].

3.3. Genetic Display Systems for Immobilization of Enzymes onto Spores

To fully benefit from using a living system for displaying heterologous enzymes, genetic manipulation can be used to create a myriad of different biocatalysts, bypassing the need for enzyme purification prior to immobilization. Directly engineering spore-forming microorganisms to display target enzymes on the surface of spores couples the process of enzyme production and immobilization [8], thereby directly eliminating the need for enzyme purification. This enables a significant cost cut, simplifies downstream processing and drastically increases the sustainability of biocatalyst production [58]. The spore coat of *Bacillus* spores is made up of dozens of proteins (Cot proteins, CgeA, SpoI, SafA, etc.), mainly acting as structural proteins. By engineering fusion enzymes of various coat proteins and target proteins, the target protein can be directed onto the surface of the spore. The most commonly used coat proteins for surface display are structural proteins CotG, CotE, CotC and CotB [7] located in the inner and outer coat [15], while the native laccase CotA has not been used for spore display. However, more recent reports have utilized CotZ and CotY, which are parts of the spore crust. It was found that the highest spore display efficiency could be achieved with CotZ and CotY, with activity retention of over 30% [59]. Fuzion enzymes need to have a certain degree of mobility, so the link between the two proteins needs to be flexible. Short, flexible peptide linkers are used for this purpose, with the GGGGS linker being the most prevalent [60]. Additionally, to ensure the correct temporal expression, the fusion proteins need to be put under the control of a promoter that is active during sporulation, such as P_{CotG}, P_{CotYZ} and P_{CotV}. In summary, an engineered cassette for spore display needs to consist of the following components: a sporulation promoter, coat protein, linker and target protein (Figure 3B), which is either introduced as a plasmid or integrated directly into the genome of the selected strain. In these systems, apart from eliminating the need for enzyme purification, spore-displayed proteins exhibit reduced misfolding and enhanced disulfide bond formation. During sporulation, proteins encapsulate the prespore within the mother cell, eliminating transmembrane transport, thus reducing misfolding, while disulfide bond formation mechanisms are naturally present in *Bacillus* species [15,61].

The concept for spore display has been used for over two decades and has aided in various biocatalytic as well as antigen presentation endeavors. Currently, ready-to-use vectors for constructing both C- and N-terminal fusions with CotB, CotC, CotG, CotZ, CotY, CotX, CotW and CgeA are available [59,62] and numerous procedures are patent-protected as well (Table 1). Despite it being a well-established procedure, novel methods are being developed continuously. The amount of displayed protein can be controlled by using inducible promoters rather than the sporulation-activated promoters of Cot proteins. Using IPTG-inducible promoters, Nguyen et al. were able to tune the display efficiency of an array of Cot and target protein combinations [63]. Alternatively, the number of displayed proteins can be controlled by tweaking sporulation conditions. Since CotB and

CotG are more abundant in spores sporulated at 25 °C, while CotC is more prevalent at 42 °C, the concentrations of fusion proteins can be controlled by adjusting the sporulation temperature. The same concept was found to dictate adsorption efficiency as well [64]. Recently, a T7 RNA polymerase-enabled display system (TIED system) was developed for the efficient spore display. The system consists of a genetic circuit with a T7 RNA polymerase integrated into the genome of the strain under the control of a sporulation-activated promoter and fusion proteins with CotZ/Y designed to be transcribed by the polymerase. In essence, during sporulation, the heterologous RNA polymerase is expressed, driving the expression of fusion proteins, achieving ultra-high display efficiency of over 10^7 enzymes per spore [19].

Apart from conventional non-recombinant methods for immobilization and usual genetic display, the use of heterologous anchoring proteins, such as the cohesin–dockerin system, offers an alternative hybrid strategy [17,65]. These proteins lack enzymatic activity but function as structural organizers due to their strong protein-binding capability [66]. Application of this system in spore display involves genetically displaying a cohesin domain, followed by the binding of an engineered fusion dockerin domain-containing target enzyme. In solution, the cohesin and dockerin modules form a tight protein–protein interaction specifically linking the target enzyme to the spore. β-galactosidase bound in this way exhibited a 3.7-fold increase in specific activity over the genetically immobilized variant and a 13-fold increase compared to the adsorbed form [67]. This system was further optimized by using different cohesin–dockerin protein types, achieving increased activity [65].

4. Biocatalyst Stability Improvements Through Immobilization onto Spores

Spore-based systems confer remarkable stability to biocatalysts under a wide range of challenging process conditions, including extreme pH [38,40,56,68,69], wide temperature operational ranges [39,70–73], resilience to proteases [74,75], organic solvents [65,74,76], improved activity, stability and longevity upon immobilization [39,41,51,67], robustness upon recycling [55,77–81] (Table 2).

Spores provide a protective microenvironment that preserves enzyme structure and function at high temperatures. For instance, organophosphorus hydrolase covalently immobilized on *B. subtilis* spores exhibited an optimal temperature of 65 °C, a 10 °C increase compared to the free enzyme, and retained over 72% of its activity after 1 h of incubation at 90 °C [56]. Similarly, enzymes displayed via genetic fusion demonstrate exceptional heat tolerance. Thermotolerant L-arabinose isomerase displayed on *B. subtilis* spores retained over 50% of its activity after 1.5 h at 85 °C [82], while the displayed meta-cleavage product hydrolases MfphA and BphD retained more than 80% of their activity at 80 °C [81]. Even non-covalent adsorption offers significant protection, the β-galactosidase from *Alicyclobacillus acidocaldarius* adsorbed onto spores retained approximately 80% of its activity after 2 h at 75 °C, a condition under which the free enzyme was completely inactivated [51]. This pronounced thermotolerance expands the operational window for biocatalysts, enabling processes that would otherwise be unfeasible.

Spore-based biocatalysts also exhibit tolerance to a wide range of pH values, vital for processes requiring acidic or alkaline conditions. The protective effect of spores was clearly demonstrated with adsorbed β-galactosidase, which remained fully active after 24 h at pH 4, whereas the free enzyme lost all activity under the same conditions [51]. The benefits extend to highly alkaline environments as well. Covalently immobilized organophosphorus hydrolase, for example, shifted its optimal pH from 11 to 13 and showed significantly improved stability over its soluble counterpart in both acidic and

alkaline solutions [56]. Moreover, spore-displayed MfphA and BphD retained over 85% of their relative activity within a pH range of 7 to 10 [81]. Increased pH stability proved crucial for the synthesis of N-acetyl-D-neuraminic acid (Neu5Ac), where a spore-displayed aldolase retained substantial activity at pH values up to 10, facilitating efficient synthesis under alkaline conditions unfavorable for the free enzyme [69].

In addition to thermal and pH stability, the resilience of spore-immobilized enzymes in organic solvents significantly broadens their application scope, particularly in the synthesis of fine chemicals and pharmaceuticals, where substrates are often hydrophobic. For instance, Lipase B from *B. subtilis* displayed on the spore surface demonstrated stability in acetone and benzene, whereas the free enzyme was completely deactivated, the spore-displayed form retained 38% and 22% of its activity, respectively [74]. Similarly, spore-displayed haloalkane dehalogenase DhaA exhibited higher resistance and catalytic activity in the presence of methanol and diethyl ether compared to the purified enzyme [83]. The protective effect is particularly striking in biphasic systems where spore-displayed β-galactosidase retained up to 78.8% of its activity in a toluene-water mixture after 1 h, whereas the free enzyme was almost completely inactivated, retaining only 4.2% activity under the same conditions [84]. This is instrumental in integrating biocatalysis into multi-step organic syntheses, paving the way for greener and more efficient production routes for complex molecules.

Beyond abiotic stressors, the spore-displayed enzymes are protected against proteolytic degradation by the spore coat, a critical advantage for applications in complex biological media or when using crude cell lysates. Spore-displayed organophosphorus hydrolase was effectively shielded from trypsin and proteinase K, retaining significant activity while the free enzyme was almost completely degraded [56]. Likewise, spore-displayed Lipase B retained 40–68% of its activity after treatment with various proteases [74], and when spore-displayed ω-transaminase was exposed to proteases, it retained up to 40% of its initial activity [85], further confirming that the spore architecture limits the accessibility of proteases to the biocatalyst. This inherent resistance allows for the use of spore biocatalysts in environments where contaminant proteases would otherwise compromise stability.

Table 2. Diversity of enzymes immobilized on bacterial spores and stability improvements achieved.

Enzyme	Source Organism	Temperature	pH	Organic Solvents	Protease	Reusability	References
Alcohol dehydrogenase	*Bombyx mori Acetobacter pasteurianus*	✓	✓	✓		✓	[76,86]
Cellobiose dehydrogenase	*Trametes sanguinea*	✓	✓			✓	[68]
Laccase	*Streptomyces coelicolor*	✓				✓	[87]
Manganese peroxidase	*Irpex lacteus*	✓	✓			✓	[88]
Lignin peroxidase	*Phanerochaete chrysosporium*	✓				✓	[77,89]
Peroxiredoxin (Bcp1)	*Sulfolobus solfataricus*		✓				[38]
Tyrosinase	*B. megaterium*			✓		✓	[78,90]

Table 2. *Cont.*

Enzyme	Source Organism	Temperature	pH	Organic Solvents	Protease	Reusability	References
Trehalose synthase	*Pimelobacter* sp. R48	✓	✓			✓	[79]
ω-transaminase	*Vibrio fluvialis*				✓		[85]
Lipoyl synthase	*P. polymyxa*	✓				✓	[41]
Esterase	*B. subtilis* *P. polymyxa*	✓	✓	✓		✓	[41,70]
Lipase	*Thermotoga maritima*	✓	✓	✓	✓	✓	[41,71,74]
Phytase	*E.coli*	✓	✓		✓		[39,72,91]
Organophosphorus hydrolase	*Pseudomonas diminuta* *Flavobacterium* sp.	✓	✓	✓	✓	✓	[56,75]
Peptidoglycan hydrolase	*Lactobacillus rhamnosus*	✓	✓				[92]
α-amylase	*B. licheniformis*	✓	✓			✓	[55]
β-Galactosidase	*Alicyclobacillus acidocaldarius* *B. subtilis* *B. stearothermophilus* *E. coli*	✓	✓	✓	✓	✓	[51,65,67,80,84,93–95]
Exochitinase	*Paenibacillus barengoltzii*	✓	✓			✓	[73]
Bromelain	*Ananas comosus*		✓				[40]
Keratinase	*B. tequilensis*	✓	✓				[96,97]
Nitrilase	*Clostridium thermocellum* *Thermotoga maritima*	✓	✓			✓	[98–100]
Hydrolase	*E. coli* *Burkholderia cepacia*	✓	✓				[81]
Haloalkane dehalogenase	*Rhodococcus rhodochrous*	✓	✓	✓	✓		[83]
Photodecarboxylase	*Chlorella variabilis*		✓				[101]
N-acetyl-D-neuraminic acid aldolase	*E. coli*		✓				[69]
Cellobiose 2-epimerase	*Caldicellulosiruptor saccharolyticus*	✓	✓				[102]
D-psicose 3-epimerase	*Clostridium scindens*	✓					[103]
L-arabinose isomerase	*Lactobacillus brevis*	✓					[82]

5. Application of Bacterial Spores in Traditional Biocatalysis

Due to their enhanced stability and ease of manufacturing, spore-based biocatalysts have been adopted across numerous industries. Based on a search of PubMed, SciFinder, Web of Science and Scopus, we have identified 54 instances utilizing spore display for biocatalysis. These reports primarily focus on prokaryotic enzymes, however, fungal, plant and animal proteins have been successfully displayed on spores as well (Table 2). The

functional versatility of spore-based biocatalysts is demonstrated by the successful display of five of the six main enzyme classes on spores, with ligases being the only exception. Most reported examples involve displayed hydrolases (EC 3), which comprise 63%, followed by oxidoreductases (EC 1) at 19%, and fewer notable examples of isomerases (EC 5) at 8%, transferases (EC 2) at 6% and lyases (EC 4) at 4% (Figure 4A).

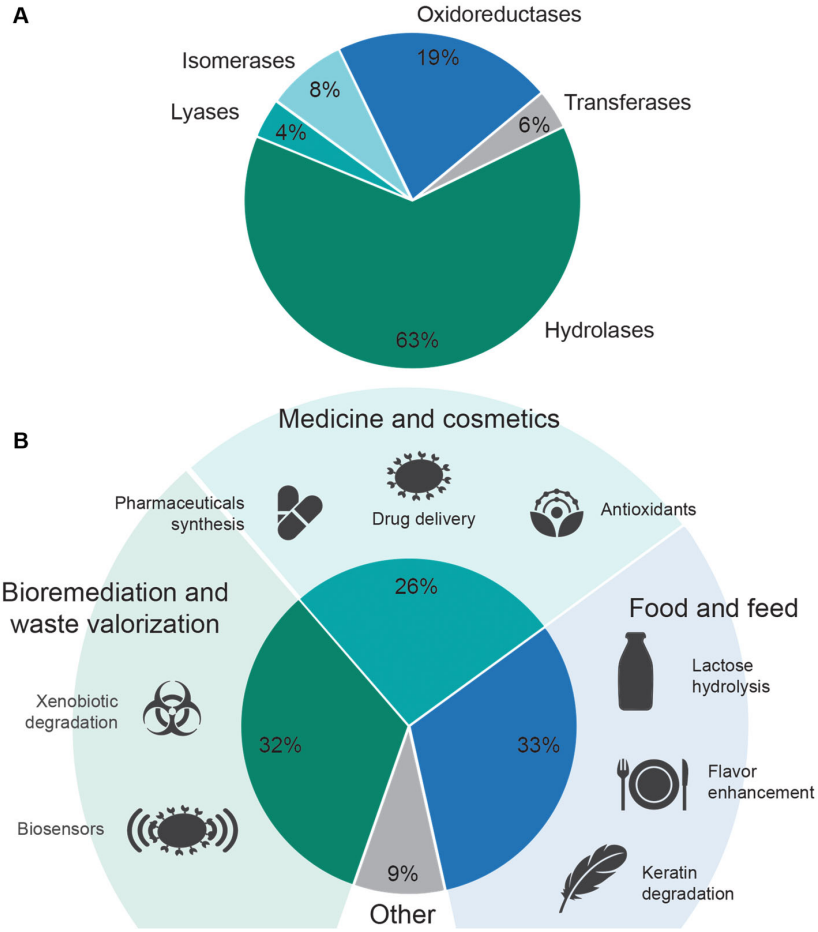

Figure 4. (**A**) Representation of different enzyme classes used to construct spore-based biocatalysts; (**B**) Representation of papers investigating the use of spore-based biocatalysts in different industries.

Hydrolases exhibit broad applicability across diverse fields, positioning them as highly amenable targets for optimization and functional enhancement. Spore surface display of β-galactosidase from *B. subtilis* (LacA, EC 3.2.1.23) enabled efficient lactose hydrolysis for lactose-free dairy production, while enhancing enzyme storage stability, reuse and cost-effectiveness compared to conventional formulations [95]. Acid-resistant keratinase H4 (EC 3.4.99.11) displayed on *B. subtilis* spores enhances catalytic performance under acidic and thermal stress conditions, enabling efficient feather waste conversion in fermentation-based feed production. The system supported scalable, cost-effective enzyme use for sustainable valorization [96]. Spore-bound organophosphorus hydrolase (OPH, EC 3.1.8.1), immobilized via EDC-NHS chemistry, offers a robust, reusable solution for detoxifying pesticide-contaminated water and soil. Its stability and low-cost integration into biosensors and bioreactors make it a scalable tool for sustainable environmental soil remediation [56]. Considering that hydrolases usually do not require cofactors, are single-chain enzymes and often function as extracellular enzymes, their prevalence is unsurprising. Furthermore, hydrolases are the most widely employed class of enzymes in industrial biocatalysis [104], a trend that is clearly reflected in the development of spore-based systems

and reinforced by the relatively balanced distribution of spore-based biocatalysts across the sectors illustrated in Figure 4B. While hydrolases are predominant, the utility of spore-based biocatalysts extends beyond them, as notable examples of other enzyme classes have also found successful applications across these sectors, bringing the overall distribution of spore-based biocatalysts to 32% in the food and feed industries [76,79,81,103], 32% for bioremediation and waste valorization [87] and 26% for medicine and cosmetics [38,69,90].

Within the food and feed industry, lactose hydrolysis [95], keratin degradation [96], rare sugar synthesis [103] and flavor enhancement [76] have been achieved. For instance, alcohol dehydrogenase A (AdhA, EC 1.1.1.1) from *Acetobacter pasteurianus* displayed on *B. subtilis* spores resulted in a biocatalyst with significantly enhanced ethanol tolerance. When exposed to 80% ethanol at 37 °C for 24 h, the CotC-AdhA mutant retained superior viability and enzymatic activity compared to the wild-type, demonstrating resilience under stress conditions typical of liquor fermentation, supporting flavor formation through aroma compound synthesis via esterification and Maillard reactions [76]. In the area of sweetener production, several spore-based systems have shown great promise. A thermostable L-arabinose isomerase (L-AI, EC 5.3.1.4) from *Lactobacillus brevis* was displayed on spores, enabling robust production of the rare sugar D-tagatose from D-galactose. This biocatalyst retained over 90% of its activity at temperatures between 60 and 70 °C and achieved a 79.7% conversion rate, demonstrating its potential for creating scalable, food-grade sweeteners [82]. Similarly, a spore-displayed D-psicose 3-epimerase (EC 5.1.3.30) from *Clostridium scindens* served as a thermostable biocatalyst for producing D-allulose (low-calorie sweetener increasingly used in beverages, fruit juices and confectionery) from D-fructose, retaining 82% of its activity after 30 min at 80 °C [103]. Furthermore, trehalose synthase displayed on *B. subtilis* spores achieved a 74.1% conversion rate of D-maltose to D-trehalose and maintained its efficiency (73%) for four reaction cycles [79]. These systems offer a food-grade, reusable platform that bypasses the limitations of isolated enzymes and whole-cell biocatalysis. By improving enzyme stability under harsh industrial conditions and allowing for repeated reuse, these innovations can lower production costs, minimize environmental impact, and accelerate manufacturing processes.

Bacterial spores are increasingly recognized as valuable platforms for enzyme stabilization and delivery in pharmaceutical, biomedical and cosmetic applications as well enabling the development of spore-based systems for therapeutic compound synthesis [90], antioxidant deployment [38] and mucosal delivery of bioactive agents [69]. For instance, spore-displayed tyrosinase (EC 1.14.18.1) from *B. megaterium* enabled the efficient and highly regioselective 3-OH hydroxylation of phloretin to produce 3′-hydroxyphloretin, an antioxidant with 1.5-fold stronger activity than its precursor. This system achieved complete conversion of 1 mM phloretin within 7 h and retained its functionality over seven reuse cycles, making it a promising tool for the sustainable synthesis of bioactive flavonoids for applications in metabolic health, cancer prevention, and skin protection [90]. In another application, peroxiredoxin (Bcp1, EC 1.11.1.15) from *Sulfolobus solfataricus* was adsorbed onto *B. megaterium* spores, creating a stable antioxidant biocatalyst suitable for mucosal delivery. The spore-bound enzyme demonstrated remarkable resilience in simulated gastric (pH 2) and intestinal (pH 6.8) environments, making it a promising candidate for probiotic formulations targeting gastrointestinal inflammation [38]. Furthermore, spore display has been crucial in pharmaceutical manufacturing. A CotG-fused N-acetyl-D-neuraminic acid aldolase (EC 4.1.3.3) created a pH-stable biocatalyst for synthesizing Neu5Ac, a key precursor for the antiviral drug zanamivir. The enzyme retained substantial activity at alkaline pH (up to pH 10), conditions unfavorable for soluble enzymes, offering a robust and reusable platform for scalable drug synthesis [69]. Despite the rigorous safety and reproducibility standards of the medical sector, the inherent stability of spores and

their potential for targeted functionalization position them as promising candidates for next-generation diagnostic and therapeutic platforms.

Bacterial spores have found their place in the field of environmental remediation as resilient, bio-inert platforms capable of supporting stable and reusable enzyme systems for pollutant degradation and remediation under harsh operational conditions. Spore-based biocatalysts have been successfully employed for detoxifying pesticides, decolorizing industrial dyes (discussed in Section 2) [87] and advancing sustainable wastewater and soil treatment technologies [56]. For example, small laccase (SLAC, EC 1.10.3.2) from *Streptomyces coelicolor*, when displayed on *B. subtilis* spores, enabled the efficient decolorization of indigo carmine dye, achieving 90% degradation within 5 h. This spore-based biocatalyst demonstrated exceptional thermal stability, retaining 70% of its activity after 3 h at 90 °C, and maintained functionality over eight reuse cycles without significant loss [87]. This resilience is also evident for organophosphorus hydrolase (OPH, EC 3.1.8.1), which was covalently immobilized onto *B. subtilis* spores, resulting in a robust biocatalyst for the environmental detoxification of organophosphorus pesticides [75]. The system maintained activity over multiple uses, making it suitable for integration into biosensors and bioreactors for field-scale decontamination. Its low cost, environmental safety, and reusability position spore-bound OPH as a promising tool for the sustainable remediation of pesticide-contaminated water and soil. As industrialization strains natural resources, scalable and resilient bioremediation strategies enabled by spore-based biocatalysts will play an increasing role in restoring environmental balance.

Apart from single enzyme reactions, spores have been successfully employed in cascade enzyme reactions, central in natural metabolic pathways, they represent sequential reactions where the product of one enzyme reaction is a substrate for another enzyme [105,106]. Cascade enzyme reactions exhibit significant biotechnological potential due to their versatility and ability to produce highly complex molecules [107]. However, conventional use of these reactions usually occurs as multiple consecutive, separate one-pot reactions. The process becomes less cost-efficient because of the extraction and purification of intermediates and products, resulting in low product yields [105]. An alternative to conventional multi-step cascades is a one-pot transformation system where multiple enzymes are mixed together, which in turn enhances product yields relative to single-step enzymatic processes [106]. Immobilization of enzyme cascades onto carriers, such as spores, nullifies the drawbacks of enzyme fragility while allowing easier extraction of the product [108]. For bio-based hydrocarbon production, a spore-displayed photodecarboxylase (EC 4.1.1.106) was paired with a commercially immobilized lipase, facilitating the direct conversion of lipids with a 3-fold yield increase compared to two-step reactions [101]. For environmental remediation, a dual-spore system displaying AtzA and AtzB (EC 3.8.1.8) enabled a sequential cascade for atrazine degradation, with AtzA-spores retaining over 80% activity after 45 months of storage [37,109]. Even the naturally occurring CotA laccase was used in a cascade with an engineered glyphosate oxidase to create a durable, low-cost biosensor [110]. Furthermore, by co-displaying phosphite dehydrogenase (EC 1.20.1.1) and xylose reductase (EC 1.1.1.307) on a single spore, researchers achieved tunable enzyme stoichiometry and efficient cofactor regeneration, increasing xylitol production efficiency by 30% over free enzymes [108]. Spore-enabled enzyme cascades offer a modular framework that can be tailored for both spatial organization and catalytic synergy, overcoming the limitations of conventional multi-step processes by minimizing intermediate loss and process variability. This integration is critically important for industry as it paves the way for high-yield bioconversions that are more cost-effective and sustainable, directly addressing demands for precision and scalability in the production of fuels, chemicals, and pharmaceuticals.

6. Expanding the Utility of Spores in Biocatalysis

So far, the most common way of integrating spores into biocatalytic processes is through the genetic display or adsorption of enzymes onto the spore surface, shielding the enzymes and yielding robust, recyclable biocatalysts. However, as our understanding of spores grows and spore display technology is becoming more prevalent and accessible, increasingly creative ways of using spores in biocatalysis have emerged. The applicative potential of spores was recognized in systems for high-throughput screening of enzyme libraries, enzyme-based biosensors, self-degrading plastics and smart living materials (Figure 5). The general use of spores in novel smart applications has been covered elsewhere [47], thus, here we will only focus on novel uses of spores through the lens of biocatalysis.

The formation of endospores involves the layering of proteins inside the mother cell without requiring cross-membrane transport, a process that mitigates protein misfolding issues and leverages naturally present chaperones. This, coupled with their inherent stability under harsh conditions, makes spores ideal platforms for screening in protein engineering campaigns. The spore coat laccase, CotA, was used to demonstrate this concept by screening mutant libraries in *B. subtilis* to increase the enzyme's solvent and pH stability. As spores remain viable in up to 90% DMSO, a concentration toxic to most cells, they enable high-throughput screening under extreme conditions. Taking advantage of this, a single CotA mutant with 2.38-fold increased activity in 60% DMSO was identified from a 3000-clone library, proving spores can function as platforms for high-throughput screening in extreme conditions [111]. The same system was used to evolve a CotA variant with 61-fold higher acid stability (pH 4) and a 5.3-fold higher product yield [112]. This platform was later adapted for heterologous proteins, where a CotB-fused lignin peroxidase was evolved to achieve a 196% increase in azo dye-degrading activity [89].

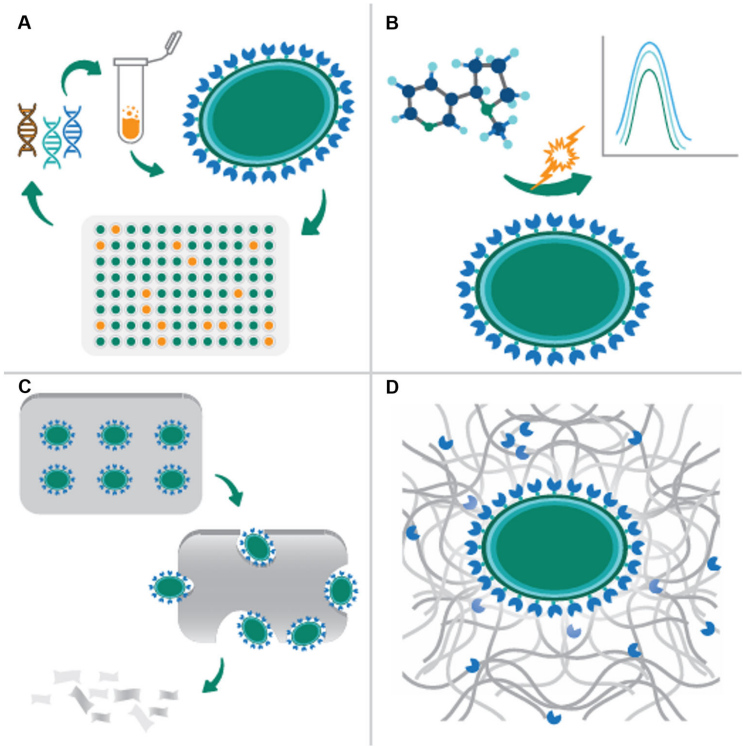

Figure 5. Innovative uses of biocatalytically active spores. (**A**) Spores as platforms for high-throughput screening; (**B**) Spore-based biological sensors (adapted from Xiong et al. [113]); (**C**) Self-degradable plastics enabled by engineered spores (adapted from Tang et al. [114]); (**D**) Integration of spores into biocatalytic hydrogels (adapted from Kawada et al. [115]).

The stability and ease of genetic manipulation of spores are also beneficial for constructing biological sensors, overcoming the common issue of poor robustness in conventional biosensors [116]. For example, a glyphosate-detecting biosensor was created using a spore-bound glyphosate oxidase in a chemiluminescent cascade with the native CotA laccase. The sensor could detect glyphosate concentrations as low as 0.09 mg/L and retained activity for over 42 days, showcasing its efficiency and durability [110]. Spores are also suitable for electrical biosensors, as demonstrated by a CotE-fused nicotine oxidase used for nicotine detection. This system simplified sensor production by bypassing protein purification and offered enhanced stability, retaining over 98% activity after 14 days while resisting matrix interference in blood and urine samples [113].

Due to their unparalleled stability, spores can be integrated into processes that are incompatible with other biological entities, driving novel technologies. Recently, it was shown that spores can be incorporated into plastic materials using standard techniques like melt extrusion (at 135–170 °C), where they act as reinforcement fillers, increasing the toughness of a polyurethane (PU) thermoplastic by 45% [117]. Building on this, several reports have created self-degrading plastic materials using spores that are either naturally active against plastics, engineered to secrete degrading enzymes, or decorated with them. To enhance spore survival during processing, *B. subtilis* spores were evolved to withstand 135 °C without viability loss and were then incorporated into biodegradable PU to facilitate its degradation in compost with endogenous PU-degrading enzymes [118]. In a different study *B. subtilis* cells were engineered to express *Burkholderia cepacia* lipase, sporulated and the resulting spores were used to make poly (caprolactone) plastics (PCL). When the surface of the spore-embedded plastic was damaged, the spores could germinate and produce the plastic-degrading enzyme, enabling rapid degradation of PCL in rich media and almost halving the time needed for complete degradation in compost [114]. The protection that spores provide to enzymes displayed on their surface also permitted the incorporation of lipase-decorated spores into poly (lactic acid) (PLA) and PCL. By integrating the spores, both plastics could be completely degraded in under 48 h, proving that catalytically programmable spores could be successfully integrated into materials without the loss of enzyme activity [119].

Furthermore, spores can be integral structural components of synthetic polymers. Relying on the diol groups on spore surface glycans, phenylboronic acid polymers can interact directly with spores to produce crosslinked hydrogel structures with tunable stiffness and viscoelasticity. By decorating spores with a lipase and peroxidase, researchers created catalytically active hydrogels that are programmable, self-healing, and can reassemble via reversible covalent bonds, introducing a novel class of smart living materials [115]. Likewise, spores are compatible with 3D printing, allowing for the manufacture of complex objects containing biocatalysts. For instance, 3D-printed hydrogels containing spores were engineered as personalized wound dressings that can sense the autoinducer peptide secreted by *Staphylococcus aureus* and, in response, secrete a pathogen-specific bacteriolytic enzyme [120]. The unparalleled stability and genetic programmability of spores have enabled their successful integration as active components in advanced biotechnological systems, moving far beyond their traditional role as simple enzyme carriers. Their expanded utility highlights the transformative potential of spore-based biocatalysts in developing sustainable bio-based technologies.

7. Artificial Spores

As was stated in previous segments, using naturally occurring and engineered biocatalytically active spores offers an easy-to-implement, cost-effective and straightforward way of immobilizing biocatalysts and using them in diverse settings. However, despite

the stated advantages, such approaches still rely on the inherent stability of spores from different microorganisms and are limited to a finite number of sporulating genera. The inability of other bacterial genera to sporulate leaves untapped metabolic potential inaccessible to aid in biocatalytic reactions in various environmental conditions. Inspired by nature and in an attempt to broaden the scope of biocatalytic reactions facilitated by the use of spores, recent research has focused on generating artificial spores. Initially devised to preserve fragile cells from harsh environmental conditions and later expanded to include biocatalytic reactions, the methods for producing artificial spores revolve around nanoencapsulation of non-sporulating microorganisms in a protective shell ("cell-in-shell") using bioorganic molecules (melanin-like species, proteins and polysaccharides), synthetic polymers and metal–organic frameworks [121] (Figure 6). The cell shells allow for uninhibited cell metabolism, providing a platform for whole-cell biocatalysis with protected cells, while by integrating the enzymes into the shells themselves, a form of protein display is achieved.

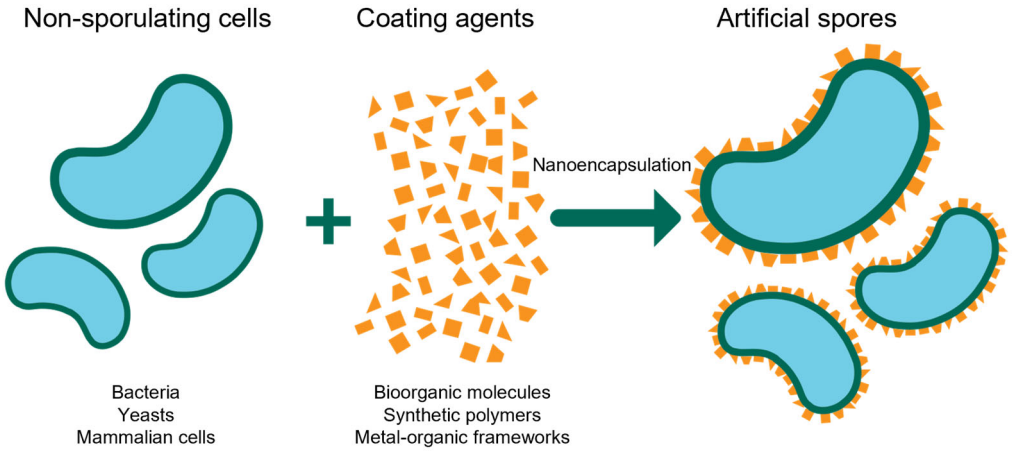

Figure 6. Schematic of the artificial sporulation process, where non-sporulating cells are coated with a protective layer to confer spore-like properties.

In a pioneering study by Iturralde et al., *E. coli* cells were coated with tannic acid–Fe^{3+} complexes, which, apart from protecting cells, provided a shell rich in galloyl groups capable of coordinating metal ions. These artificially sporulated cells were subsequently used to bind His-tagged proteins, effectively acting as metal chelate affinity carriers, offering a novel approach to extracting His-tagged enzymes from solutions. Attached amine transaminase from *Pseudomonas fluorescens*, alcohol dehydrogenase and alcohol oxidase were used to catalyze a two-step oxidative amination of diols into amino alcohols, proving the effectiveness of this system in relevant biocatalytic reactions [122]. Artificial spores also allow for systems with improved extracellular electron transfer in microbial fuel cell systems. By coating *Shewanella xiamenensis* with polydopamine, a 5.1-fold higher current and 6.1-fold higher maximum power density were achieved with minimal tradeoffs in terms of cell viability (97% viability) and proliferation, further expanding the uses of artificially sporulated cells [123].

Furthermore, the ability to precisely tune the coating process is a critical feature of artificial spores, as it significantly expands their range of potential applications. The hydrophobicity of dopamine-coated *E. coli* cells was fine-tuned by adjusting the concentration of N-oleoyl dopamine, which allowed for the creation of artificial spores with a specific water contact angle of 90°. These tailored spores were then used to form stable water-in-oil Pickering emulsions, providing an ideal environment for biocatalytic reactions at the interface. The functionalized cell surfaces not only stabilized the emulsion but also protected the encapsulated cells and enzymes from interfacial stress. This approach drasti-

cally improved product yields in cascade reactions; cells expressing alcohol dehydrogenase and *Candida antarctica* lipase B (CALB) or benzaldehyde lyase (BAL) demonstrated significantly higher yields compared to their uncoated counterparts [124]. In a related study, alkylated polyethyleneimine-coated cells engineered to overexpress CALB were used to achieve efficient polycarbonate plastic degradation in water-toluene Pickering emulsion reactions, achieving 4.5 mM of bisphenol A release after 72 h, further underlining that tunable coating allows for the creation of biocatalysts tailored for specific and challenging reaction environments [125]. Apart from hydrophobicity, cell shells can be fine-tuned in other ways as well. Additionally, artificially sporulated cells could be combined with standard chemical catalysts to perform complex chemoenzymatic reactions. By combining artificially sporulated *E. coli* cells expressing CALB and palladium nanoparticles, 350-fold higher catalytic activity was achieved [124]. Similarly, by combining anthraquinone as a photocatalyst and BAL, benzyl alcohol could be converted to benzoin with a 15-fold increase compared to using the two catalysts separately [126].

In conclusion, artificial spores are emerging as a powerful and highly versatile technology, providing solutions for whole-cell biocatalysis, interfacial reactions, and complex chemoenzymatic systems. Future work may focus on combining these synthetic platforms with naturally occurring spores to create novel hybrid materials. Such an approach could unlock additional functionalities by pairing the tunability of coating methods developed for artificial sporulation with the inherent resilience and unique surface properties of natural spores.

8. Limitations of Spore-Based Biocatalysts

Despite the significant advantages and expanding applications of spore-based biocatalysts, several challenges and limitations must be addressed to facilitate their transition from laboratory-scale research to widespread industrial implementation. One of the primary hurdles is the scalability of spore production. While high-density and cost-effective spore-yielding fermentations have been reported for unmodified spores used in agriculture as biofertilizers [127], large-scale production of spore-based biocatalysts remains unexplored. Most published studies are conducted at the lab scale, and there is a lack of detailed information on optimizing fermentation parameters for the industrial production of catalytically active spores [64]. Furthermore, comprehensive techno-economic analyses are largely absent from the current literature. While the benefits of reusability and simplified workflows are often cited [7,8], rigorous assessments of the overall process costs, including media, fermentation time, energy consumption, and downstream processing, are needed to definitively establish the economic feasibility of spore-based systems compared to established immobilization techniques.

Regulatory and public perception issues, particularly concerning genetically modified organisms (GMOs), also present a significant barrier. The use of genetically engineered spores, especially in environmental cleanup applications or applications involving direct contact with humans such as medicine and the food industry, faces stringent regulatory hurdles and potential public resistance in many regions [128,129]. While non-recombinant methods like adsorption can circumvent the GMO issue, they often come with trade-offs in terms of enzyme stability, control and cost. Finally, a lack of standardized methodologies for characterizing spore-based biocatalysts complicates direct comparisons between different studies and with other immobilization platforms. Metrics such as enzyme loading, specific activity, and immobilization yield are often reported in inconsistent units (e.g., per spore, per gram of spores, activity retention), making it difficult to benchmark performance accurately. Establishing standardized protocols for quantifying the catalytic efficiency of spore-bound enzymes is vital for providing a clear framework for evaluating new spore-

based biocatalysts. Addressing these challenges will be paramount for unlocking the full commercial potential of this biocatalytic platform.

9. Future Perspectives

As ongoing innovations continue to broaden the utility of spore-based biocatalysis beyond simple enzyme stabilization, its full potential is just starting to be realized. As was mentioned earlier, spore-based biocatalysts have already demonstrated significant potential across various industrial sectors. Their applications range from the food and feed industries for lactose hydrolysis, rare sugar synthesis, and flavor enhancement, to environmental remediation of pesticides and industrial dyes. Moreover, they have shown promise in medicine and cosmetics for the sustainable synthesis of high-value compounds like antioxidants and pharmaceutical precursors. This developing field is moving from basic research to creating advanced, programmable platforms for use in sustainable manufacturing, bioremediation, and advanced materials science [47]. The advancement of spore biocatalysis will likely be influenced by progress in three main areas: improving engineering technologies, extending applications to new fields, and increasing the variety of microbial hosts used.

The development of sophisticated expression and engineering tools, such as CRISPR-Cas systems, will facilitate quick, multiplexed changes to host genomes, optimizing sporulation, enhancing anchor protein expression, and removing unwanted native enzymes, therefore enabling improved accuracy and effectiveness of spore display systems. Additionally, creating multi-enzyme cascades on a single spore surface is a major goal. Although current systems have successfully shown two-enzyme cascades for cofactor regeneration or sequential reactions [108,109], the next step is to develop more complex, spatially arranged synthetic pathways. It will be crucial to achieve the right balance of enzymes and reduce the diffusion of intermediate products. Hybrid methods, like the cohesin–dockerin system that permits adjustable enzyme ratios, provide a promising model for designing these complex "spore factories" capable of carrying out multiple synthesis steps in one self-contained unit.

With materials science, nanomanufacturing, biomaterial and bioprocess engineering advancements, the uses of spore biocatalysts will grow more ambitious. Integrating spores into smart living materials is a particularly exciting possibility. Drawing from existing examples of self-degrading plastics [119] and programmable hydrogels [115], future materials could be designed with spores that can detect environmental signals like a pollutant or a specific pH level and activate a catalytic reaction in response. Such self-sufficient systems could transform industries from personalized medicine, such as smart wound dressings that deliver medication [120], to environmental monitoring. In the field of bioremediation, the remarkable durability of spores makes them well-suited for on-site applications. Engineered spores could be released directly into contaminated soil or water to break down stubborn pollutants like microplastics or PFAS. Another promising direction is the development of hybrid systems that integrate biological catalysis with traditional chemical catalysts to perform complex chemoenzymatic reactions unachievable by either system alone. As was demonstrated by pairing spore-based biocatalysts or protected whole cells with metal nanoparticles [124], it is possible to unite the intricate biosynthetic capabilities of living systems with the unique reactivity of chemical catalysts in a single, robust catalytic unit.

Furthermore, investigating other spore-forming organisms could lead to new capabilities and platforms designed for particular uses. For instance, *Streptomyces* species, known for producing a wide variety of secondary metabolites and forming exospores [130], offer a strong alternative. Engineering *Streptomyces* exospores could result in systems that combine effective biocatalysis with the simultaneous creation of valuable chemicals, antibiotics, or

other bioactive substances. Exploring the metabolic capabilities of other extremophilic or specially adapted spore-formers will further expand the range of biocatalytic tools, offering customized solutions for increasingly specific industrial needs. Ultimately, these ongoing developments highlight the significant potential of spores. By combining principles from synthetic biology, materials science, and biocatalysis, spore-based platforms are transforming from basic enzyme carriers into highly advanced and adaptable tools. Their continued progress will be key to tackling major sustainability issues and fostering a more resilient and efficient bioeconomy.

Author Contributions: Conceptualization, Visualization, Writing—original draft, Writing—review and editing, B.P.; Data curation, Visualization, Writing—original draft, N.R.; Writing—original draft, I.A.; Writing—original draft, J.S.; Conceptualization, Writing—review and editing, Supervision, J.N.-R. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Ministry of Science, Technological Development and Innovation of the Republic of Serbia, grant number 451-03-136/2025-03/200042 and the European Union's Horizon Europe call HORIZON-WIDERA-2023-ACCESS-02 under Grant Agreement No 101159570 (Twinn4MicroUp).

Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

AdhA	Alcohol dehydrogenase A
BAL	Benzaldehyde lyase
Bcp1	Peroxiredoxin
CALB	*Candida antartica* lipase B
DEAE	N,N-Diethylethanolamine
DMSO	Dimethyl sulfoxide
EC	Enzyme Commission
EDC	1-ethyl-3-(3-dimethylaminopropyl) carboniimide hydrochloride
IPTG	Isopropyl β-D-1-thiogalactopyranoside
LacA	β-galactosidase from *B. subtilis*
L-AI	L-arabinose isomerase
Neu5Ac	N-acetyl-D-neuraminic acid
NHS	N-hydroxysulfosuccinimide
OPH	Organophosphorus hydrolase
PCL	Poly(caprolactone)
PLA	Poly(lactic acid)
PTDH	Phosphite dehydrogenase
PU	Polyurethane
SLAC	Small laccase from *Streptomyces coelicolor*
TIED	T7 RNA polymerase enabled displayed system
XR	Xylose reductase

References

1. Intasian, P.; Prakinee, K.; Phintha, A.; Trisrivirat, D.; Weeranoppanant, N.; Wongnate, T.; Chaiyen, P. Enzymes, in vivo biocatalysis, and metabolic engineering for enabling a circular economy and sustainability. *Chem. Rev.* **2021**, *121*, 10367–10451. [CrossRef] [PubMed]
2. Yi, D.; Bayer, T.; Badenhorst, C.P.; Wu, S.; Doerr, M.; Höhne, M.; Bornscheuer, U.T. Recent trends in biocatalysis. *Chem. Soc. Rev.* **2021**, *50*, 8003–8049. [CrossRef] [PubMed]
3. Maghraby, Y.R.; El-Shabasy, R.M.; Ibrahim, A.H.; Azzazy, H.M.E.-S. Enzyme immobilization technologies and industrial applications. *ACS Omega* **2023**, *8*, 5184–5196. [CrossRef]

4. Bornscheuer, U.T.; Huisman, G.; Kazlauskas, R.; Lutz, S.; Moore, J.; Robins, K. Engineering the third wave of biocatalysis. *Nature* **2012**, *485*, 185–194. [CrossRef]
5. Fasim, A.; More, V.S.; More, S.S. Large-scale production of enzymes for biotechnology uses. *Curr. Opin. Biotechnol.* **2021**, *69*, 68–76. [CrossRef]
6. Sharma, V.; Tsai, M.-L.; Nargotra, P.; Chen, C.-W.; Kuo, C.-H.; Sun, P.-P.; Dong, C.-D. Agro-industrial food waste as a low-cost substrate for sustainable production of industrial enzymes: A critical review. *Catalysts* **2022**, *12*, 1373. [CrossRef]
7. Lin, P.; Yuan, H.; Du, J.; Liu, K.; Liu, H.; Wang, T. Progress in research and application development of surface display technology using *Bacillus subtilis* spores. *Appl. Microbiol. Biotechnol.* **2020**, *104*, 2319–2331. [CrossRef]
8. Bahrulolum, H.; Ahmadian, G. *Bacillus subtilis* surface display technology: Applications in bioprocessing and sustainable manufacturing. *Biotechnol. Biofuels Bioprod.* **2025**, *18*, 34. [CrossRef]
9. Paul, C.; Filippidou, S.; Jamil, I.; Kooli, W.; House, G.L.; Estoppey, A.; Hayoz, M.; Junier, T.; Palmieri, F.; Wunderlin, T. Bacterial spores, from ecology to biotechnology. *Adv. Appl. Microbiol.* **2019**, *106*, 79–111.
10. Horneck, G.; Rettberg, P.; Reitz, G.; Wehner, J.; Eschweiler, U.; Strauch, K.; Panitz, C.; Starke, V.; Baumstark-Khan, C. Protection of bacterial spores in space, a contribution to the discussion on panspermia. *Orig. Life Evol. Biosph.* **2001**, *31*, 527–547. [CrossRef] [PubMed]
11. Hutchison, E.A.; Miller, D.A.; Angert, E.R. Sporulation in bacteria: Beyond the standard model. In *The Bacterial Spore: From Molecules to Systems*; ASM Press: Washington, DC, USA, 2016; pp. 87–102.
12. Brbić, M.; Piškorec, M.; Vidulin, V.; Kriško, A.; Šmuc, T.; Supek, F. The landscape of microbial phenotypic traits and associated genes. *Nucleic Acids Res.* **2016**, *44*, 10074–10090. [CrossRef]
13. Tocheva, E.I.; Ortega, D.R.; Jensen, G.J. Sporulation, bacterial cell envelopes and the origin of life. *Nat. Rev. Microbiol.* **2016**, *14*, 535–542. [CrossRef]
14. Mahmoodi, A.; Farinas, E.T. Applications of *Bacillus subtilis* protein display for medicine, catalysis, environmental remediation, and protein engineering. *Microorganisms* **2024**, *12*, 97. [CrossRef]
15. McKenney, P.T.; Driks, A.; Eichenberger, P. The *Bacillus subtilis* endospore: Assembly and functions of the multilayered coat. *Nat. Rev. Microbiol.* **2013**, *11*, 33–44. [CrossRef]
16. Bauda, E.; Gallet, B.; Moravcova, J.; Effantin, G.; Chan, H.; Novacek, J.; Jouneau, P.-H.; Rodrigues, C.D.; Schoehn, G.; Moriscot, C. Ultrastructure of macromolecular assemblies contributing to bacterial spore resistance revealed by in situ cryo-electron tomography. *Nat. Commun.* **2024**, *15*, 1376. [CrossRef] [PubMed]
17. Ricca, E.; Baccigalupi, L.; Isticato, R. Spore-adsorption: Mechanism and applications of a non-recombinant display system. *Biotechnol. Adv.* **2021**, *47*, 107693. [CrossRef] [PubMed]
18. Zhang, X.; Al-Dossary, A.; Hussain, M.; Setlow, P.; Li, J. Applications of *Bacillus subtilis* spores in biotechnology and advanced materials. *Appl. Environ. Microbiol.* **2020**, *86*, e01096-20. [CrossRef] [PubMed]
19. Hui, Y.; Cui, Z.; Sim, S. Stress-tolerant, recyclable, and renewable biocatalyst platform enabled by engineered bacterial spores. *ACS Synth. Biol.* **2022**, *11*, 2857–2868. [CrossRef]
20. Mohsin, M.Z.; Omer, R.; Huang, J.; Mohsin, A.; Guo, M.; Qian, J.; Zhuang, Y. Advances in engineered *Bacillus subtilis* biofilms and spores, and their applications in bioremediation, biocatalysis, and biomaterials. *Synth. Syst. Biotechnol.* **2021**, *6*, 180–191. [CrossRef]
21. Lozančić, M.; Sk. Hossain, A.; Mrša, V.; Teparić, R. Surface display—An alternative to classic enzyme immobilization. *Catalysts* **2019**, *9*, 728. [CrossRef]
22. Bahrulolum, H.; Beyranvand, P.; Ahmadian, G. Harnessing *Bacillus subtilis* spore surface display (BSSD) technology for mucosal vaccines and drug delivery: Innovations in respiratory virus immunization. *Drugs Drug Candidates* **2024**, *3*, 774–795. [CrossRef]
23. Oggioni, M.R.; Ciabattini, A.; Cuppone, A.M.; Pozzi, G. *Bacillus* spores for vaccine delivery. *Vaccine* **2003**, *21*, S96–S101. [CrossRef]
24. Arruda, H.; Silva, E.R.; Lessa, M.; Proença Jr, D.; Bartholo, R. VOSviewer and bibliometrix. *J. Med. Libr. Assoc.* **2022**, *110*, 392. [CrossRef]
25. Leggett, M.J.; McDonnell, G.; Denyer, S.P.; Setlow, P.; Maillard, J.Y. Bacterial spore structures and their protective role in biocide resistance. *J. Appl. Microbiol.* **2012**, *113*, 485–498. [CrossRef]
26. Lu, L.; Zhao, M.; Wang, T.-N.; Zhao, L.-Y.; Du, M.-H.; Li, T.-L.; Li, D.-B. Characterization and dye decolorization ability of an alkaline resistant and organic solvents tolerant laccase from *Bacillus licheniformis* LS04. *Bioresour. Technol.* **2012**, *115*, 35–40. [CrossRef]
27. El-Bendary, M.A.; Ezzat, S.M.; Ewais, E.A.; Al-Zalama, M.A. Optimization of spore laccase production by *Bacillus amyloliquefaciens* isolated from wastewater and its potential in green biodecolorization of synthetic textile dyes. *Prep. Biochem. Biotechnol.* **2021**, *51*, 16–27. [CrossRef]
28. El-Bendary, M.A.; Ewais, E.A.; Ezzat, S.M.; Al-Zalama, M.A. Process optimization of the bio-decolorization of textile dyes by spore-bound laccase of *Bacillus amyloliquefaciens*. *Microbiology* **2019**, *13*, 23–33.

29. Peng, F.; Zheng, B.; Zhang, Y.; Faheem, A.; Chai, Y.; Jiang, T.; Chen, X.; Hu, Y. Biocatalytic oxidation of aromatic compounds by spore-based system. *ACS Sustain. Chem. Eng.* **2020**, *8*, 14159–14165. [CrossRef]
30. Lu, L.; Zhao, M.; Li, G.-F.; Li, J.; Wang, T.-N.; Li, D.-B.; Xu, T.-F. Decolorization of synthetic dyes by immobilized spore from *Bacillus amyloliquefaciens*. *Catal. Commun.* **2012**, *26*, 58–62. [CrossRef]
31. Asadi, E.; Makhdoumi, A.; Asoodeh, A. Laccase mediator system obtained from a marine spore exhibits decolorization potential in harsh environmental conditions. *Ecotoxicol. Environ. Saf.* **2020**, *191*, 110184. [CrossRef]
32. Zhou, W.; Guan, Z.-B.; Cai, Y.-J.; Chen, Y.; Zhang, N.; Liao, X.-R. Preparation and characterization of immobilized spores with laccase activity from *Bacillus pumilus* W3 on DEAE-cellulose and their application in dye decolorization. *Braz. J. Chem. Eng.* **2017**, *34*, 41–52. [CrossRef]
33. Paredes-Sabja, D.; Shen, A.; Sorg, J.A. *Clostridium difficile* spore biology: Sporulation, germination, and spore structural proteins. *Trends Microbiol.* **2014**, *22*, 406–416. [CrossRef]
34. Creuly, C.; Larroche, C.; Gros, J.-B. Bioconversion of fatty acids into methyl ketones by spores of *Penicillium roquefortii* in a water-organic solvent, two-phase system. *Enzym. Microb. Technol.* **1992**, *14*, 669–678. [CrossRef]
35. Wolken, W.; Van Der Werf, M. Geraniol biotransformation-pathway in spores of *Penicillium digitatum*. *Appl. Microbiol. Biotechnol.* **2001**, *57*, 731–737. [CrossRef]
36. Stülke, J.; Grüppen, A.; Bramkamp, M.; Pelzer, S. *Bacillus subtilis*, a swiss army knife in science and biotechnology. *J. Bacteriol.* **2023**, *205*, e00102-23. [CrossRef]
37. Hsieh, H.-Y.; Lin, C.-H.; Hsu, S.-Y.; Stewart, G.C. A *Bacillus* spore-based display system for bioremediation of atrazine. *Appl. Environ. Microbiol.* **2020**, *86*, e01230-20. [CrossRef] [PubMed]
38. Lanzilli, M.; Donadio, G.; Fusco, F.A.; Sarcinelli, C.; Limauro, D.; Ricca, E.; Isticato, R. Display of the peroxiredoxin Bcp1 of *Sulfolobus solfataricus* on probiotic spores of *Bacillus megaterium*. *New Biotechnol.* **2018**, *46*, 38–44. [CrossRef] [PubMed]
39. Cho, E.-A.; Kim, E.-J.; Pan, J.-G. Adsorption immobilization of *Escherichia coli* phytase on probiotic *Bacillus polyfermenticus* spores. *Enzym. Microb. Technol.* **2011**, *49*, 66–71. [CrossRef] [PubMed]
40. Corona, R.; Bontà, V.; Baccigalupi, L.; Ricca, E. Probiotic spores of *Shouchella clausii* SF174 and displayed bromelain show beneficial additive potential. *Int. J. Mol. Sci.* **2025**, *26*, 942. [CrossRef]
41. Zander, M.; Schmid, J.; Kabisch, J. Implementation of spore display in *Paenibacillus polymyxa* with different hydrolytic enzymes. *Microorganisms* **2024**, *12*, 1438. [CrossRef]
42. Brena, B.; González-Pombo, P.; Batista-Viera, F. Immobilization of enzymes: A literature survey. In *Immobilization of Enzymes and Cells*, 3rd ed.; Humana Press: Totowa, NJ, USA, 2013; pp. 15–31.
43. Datta, R.; Anand, S.; Moulick, A.; Baraniya, D.; Imran Pathan, S.; Rejsek, K.; Vranova, V.; Sharma, M.; Sharma, D.; Formanek, P. How enzymes are adsorbed on soil solid phase and factors limiting its activity: A Review. *Int. Agrophysics* **2017**, *31*, 287–302. [CrossRef]
44. Jesionowski, T.; Zdarta, J.; Krajewska, B. Enzyme immobilization by adsorption: A review. *Adsorption* **2014**, *20*, 801–821. [CrossRef]
45. Nguyen, H.H.; Kim, M. An overview of techniques in enzyme immobilization. *Appl. Sci. Converg. Technol.* **2017**, *26*, 157–163. [CrossRef]
46. Górecka, E.; Jastrzębska, M. Immobilization techniques and biopolymer carriers. *Biotechnol. Food Sci.* **2011**, *75*, 65–86.
47. Kummetha, L.R.; Oh, J.-J.; van der Linden, F.H.; Aubin-Tam, M.-E. Leveraging the versatile properties of bacterial spores in materials. *Trends Biotechnol.* **2025**, *43*, 812–825. [CrossRef]
48. Huang, J.-M.; Hong, H.A.; Van Tong, H.; Hoang, T.H.; Brisson, A.; Cutting, S.M. Mucosal delivery of antigens using adsorption to bacterial spores. *Vaccine* **2010**, *28*, 1021–1030. [CrossRef] [PubMed]
49. Jiang, S.; Wan, Q.; Krajcikova, D.; Tang, J.; Tzokov, S.B.; Barak, I.; Bullough, P.A. Diverse supramolecular structures formed by self-assembling proteins of the *Bacillus subtilis* spore coat. *Mol. Microbiol.* **2015**, *97*, 347–359. [CrossRef]
50. Donadio, G.; Lanzilli, M.; Sirec, T.; Ricca, E.; Isticato, R. Localization of a red fluorescence protein adsorbed on wild type and mutant spores of *Bacillus subtilis*. *Microb. Cell Factories* **2016**, *15*, 153. [CrossRef]
51. Sirec, T.; Strazzulli, A.; Isticato, R.; De Felice, M.; Moracci, M.; Ricca, E. Adsorption of β-galactosidase of *Alicyclobacillus acidocaldarius* on wild type and mutants spores of *Bacillus subtilis*. *Microb. Cell Factories* **2012**, *11*, 100. [CrossRef]
52. Thyparambil, A.A.; Wei, Y.; Latour, R.A. Experimental characterization of adsorbed protein orientation, conformation, and bioactivity. *Biointerphases* **2015**, *10*, 019002. [CrossRef]
53. Fears, K.P.; Sivaraman, B.; Powell, G.L.; Wu, Y.; Latour, R.A. Probing the conformation and orientation of adsorbed enzymes using side-chain modification. *Langmuir* **2009**, *25*, 9319–9327. [CrossRef]
54. Ghaedmohammadi, S.; Rigi, G.; Zadmard, R.; Ricca, E.; Ahmadian, G. Immobilization of bioactive protein A from *Staphylococcus aureus* (SpA) on the surface of *Bacillus subtilis* spores. *Mol. Biotechnol.* **2015**, *57*, 756–766. [CrossRef]
55. Gashtasbi, F.; Ahmadian, G.; Noghabi, K.A. New insights into the effectiveness of alpha-amylase enzyme presentation on the *Bacillus subtilis* spore surface by adsorption and covalent immobilization. *Enzym. Microb. Technol.* **2014**, *64*, 17–23. [CrossRef] [PubMed]

56. Falahati-Pour, S.; Lotfi, A.; Ahmadian, G.; Baghizadeh, A. Covalent immobilization of recombinant organophosphorus hydrolase on spores of *Bacillus subtilis*. *J. Appl. Microbiol.* **2015**, *118*, 976–988. [CrossRef] [PubMed]
57. Moshnikova, A.; Afanasyev, V.; Proussakova, O.; Chernyshov, S.; Gogvadze, V.; Beletsky, I. Cytotoxic activity of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide is underlain by DNA interchain cross-linking. *Cell. Mol. Life Sci. CMLS* **2006**, *63*, 229–234. [CrossRef]
58. Becker, M.; Lütz, S.; Rosenthal, K. Environmental assessment of enzyme production and purification. *Molecules* **2021**, *26*, 573. [CrossRef] [PubMed]
59. Bartels, J.; López Castellanos, S.N.; Radeck, J.; Mascher, T. Sporobeads: The utilization of the *Bacillus subtilis* endospore crust as a protein display platform. *ACS Synth. Biol.* **2018**, *7*, 452–461. [CrossRef]
60. Wang, H.; Wang, Y.; Yang, R. Recent progress in *Bacillus subtilis* spore-surface display: Concept, progress, and future. *Appl. Microbiol. Biotechnol.* **2017**, *101*, 933–949. [CrossRef]
61. Yang, H.; Qu, J.; Zou, W.; Shen, W.; Chen, X. An overview and future prospects of recombinant protein production in *Bacillus subtilis*. *Appl. Microbiol. Biotechnol.* **2021**, *105*, 6607–6626. [CrossRef]
62. Iwanicki, A.; Piątek, I.; Stasiłojć, M.; Grela, A.; Łęga, T.; Obuchowski, M.; Hinc, K. A system of vectors for *Bacillus subtilis* spore surface display. *Microb. Cell Factories* **2014**, *13*, 30. [CrossRef]
63. Nguyen, Q.A.; Schumann, W. Use of IPTG-inducible promoters for anchoring recombinant proteins on the *Bacillus subtilis* spore surface. *Protein Expr. Purif.* **2014**, *95*, 67–76. [CrossRef]
64. Petrillo, C.; Castaldi, S.; Lanzilli, M.; Saggese, A.; Donadio, G.; Baccigalupi, L.; Ricca, E.; Isticato, R. The temperature of growth and sporulation modulates the efficiency of spore-display in *Bacillus subtilis*. *Microb. Cell Factories* **2020**, *19*, 185. [CrossRef]
65. Wang, H.; Jiang, X.; Qian, Y.; Yin, L. Constructing an efficient *Bacillus subtilis* spore display by using Cohesin−Dockerin interactions. *Molecules* **2021**, *26*, 1186. [CrossRef]
66. Miras, I.; Schaeffer, F.; Béguin, P.; Alzari, P.M. Mapping by site-directed mutagenesis of the region responsible for cohesin−dockerin interaction on the surface of the seventh cohesin domain of *Clostridium thermocellum* CipA. *Biochemistry* **2002**, *41*, 2115–2119. [CrossRef]
67. Chen, L.; Holmes, M.; Schaefer, E.; Mulchandani, A.; Ge, X. Highly active spore biocatalyst by self-assembly of co-expressed anchoring scaffoldin and multimeric enzyme. *Biotechnol. Bioeng.* **2018**, *115*, 557–564. [CrossRef]
68. Wu, Z.; Li, P.; Chen, X.; Feng, Y.; Ma, Y.; Ni, Z.; Zhu, D.; Chen, H. Surface display system of *Bacillus subtilis*: A promising approach for improving the stability and applications of cellobiose dehydrogenase. *Protein Expr. Purif.* **2024**, *218*, 106448. [CrossRef]
69. Gao, C.; Xu, X.; Zhang, X.; Che, B.; Ma, C.; Qiu, J.; Tao, F.; Xu, P. Chemoenzymatic synthesis of N-acetyl-D-neuraminic acid from N-acetyl-D-glucosamine by using the spore surface-displayed N-acetyl-D-neuraminic acid aldolase. *Appl. Environ. Microbiol.* **2011**, *77*, 7080–7083. [CrossRef] [PubMed]
70. Chen, H.; Zhang, T.; Jia, J.; Vastermark, A.; Tian, R.; Ni, Z.; Chen, Z.; Chen, K.; Yang, S. Expression and display of a novel thermostable esterase from *Clostridium thermocellum* on the surface of *Bacillus subtilis* using the CotB anchor protein. *J. Ind. Microbiol. Biotechnol.* **2015**, *42*, 1439–1448. [CrossRef] [PubMed]
71. Chen, H.; Tian, R.; Ni, Z.; Zhang, Q.; Zhang, T.; Chen, Z.; Chen, K.; Yang, S. Surface display of the thermophilic lipase Tm1350 on the spore of *Bacillus subtilis* by the CotB anchor protein. *Extremophiles* **2015**, *19*, 799–808. [CrossRef] [PubMed]
72. Mingmongkolchai, S.; Panbangred, W. Display of *Escherichia coli* phytase on the surface of *Bacillus subtilis* spore using CotG as an anchor protein. *Appl. Biochem. Biotechnol.* **2019**, *187*, 838–855. [CrossRef]
73. Ullah, M.; Xia, Y.; Alshaya, D.S.; Han, J.; Attia, K.A.; Shah, T.A.; Chen, H. Display of bacterial Exochitanase on *Bacillus subtilis* spores improved enzyme stability and recyclability. *Molecules* **2024**, *29*, 4302. [CrossRef] [PubMed]
74. Kim, J. Surface display of lipolytic enzyme, Lipase A and Lipase B of *Bacillus subtilis* on the *Bacillus subtilis* spore. *Biotechnol. Bioprocess Eng.* **2017**, *22*, 462–468. [CrossRef]
75. Song, T.; Wang, F.; Xiong, S.; Jiang, H. Surface display of organophosphorus-degrading enzymes on the recombinant spore of *Bacillus subtilis*. *Biochem. Biophys. Res. Commun.* **2019**, *510*, 13–19. [CrossRef]
76. Yuan, Y.; Feng, F.; Chen, L.; Yao, Q.; Chen, K. Surface display of *Acetobacter pasteurianus* AdhA on *Bacillus subtilis* spores to enhance ethanol tolerance for liquor industrial potential. *Eur. Food Res. Technol.* **2014**, *238*, 285–293. [CrossRef]
77. Li, S.; He, L.; Shi, N.; Ni, Z.; Bu, Q.; Zhu, D.; Chen, H. Display of Lignin Peroxidase on the Surface of *Bacillus subtilis*. *Appl. Biochem. Biotechnol.* **2024**, *196*, 6849–6863. [CrossRef]
78. Hosseini-Abari, A.; Kim, B.G.; Lee, S.H.; Emtiazi, G.; Kim, W.; Kim, J.H. Surface display of bacterial tyrosinase on spores of *Bacillus subtilis* using CotE as an anchor protein. *J. Basic Microbiol.* **2016**, *56*, 1331–1337. [CrossRef]
79. Liu, H.; Yang, S.; Wang, X.; Wang, T. Production of trehalose with trehalose synthase expressed and displayed on the surface of *Bacillus subtilis* spores. *Microb. Cell Factories* **2019**, *18*, 100. [CrossRef]
80. Wang, H.; Yang, R.; Hua, X.; Zhang, W.; Zhao, W. An approach for lactulose production using the CotX-mediated spore-displayed β-galactosidase as a biocatalyst. *J. Microbiol. Biotechnol.* **2016**, *26*, 1267–1277. [CrossRef]

81. Qu, Y.; Wang, J.; Zhang, Z.; Shi, S.; Li, D.; Shen, W.; Shen, E.; Zhou, J. Catalytic transformation of HODAs using an efficient meta-cleavage product hydrolase-spore surface display system. *J. Mol. Catal. B Enzym.* **2014**, *102*, 204–210. [CrossRef]
82. Guo, Q.; An, Y.; Yun, J.; Yang, M.; Magocha, T.A.; Zhu, J.; Xue, Y.; Qi, Y.; Hossain, Z.; Sun, W. Enhanced D-tagatose production by spore surface-displayed L-arabinose isomerase from isolated *Lactobacillus brevis* PC16 and biotransformation. *Bioresour. Technol.* **2018**, *247*, 940–946. [CrossRef] [PubMed]
83. Wang, F.; Song, T.; Jiang, H.; Pei, C.; Huang, Q.; Xi, H. *Bacillus subtilis* spore surface display of haloalkane dehalogenase DhaA. *Curr. Microbiol.* **2019**, *76*, 1161–1167. [CrossRef]
84. Kwon, S.J.; Jung, H.-C.; Pan, J.-G. Transgalactosylation in a water-solvent biphasic reaction system with β-galactosidase displayed on the surfaces of *Bacillus subtilis* spores. *Appl. Environ. Microbiol.* **2007**, *73*, 2251–2256. [CrossRef]
85. Hwang, B.-Y.; Kim, B.-G.; Kim, J.-H. Bacterial surface display of a co-factor containing enzyme, ω-transaminase from *Vibrio fluvialis* using the *Bacillus subtilis* spore display system. *Biosci. Biotechnol. Biochem.* **2011**, *75*, 1862–1865. [CrossRef] [PubMed]
86. Wang, N.; Chang, C.; Yao, Q.; Li, G.; Qin, L.; Chen, L.; Chen, K. Display of *Bombyx mori* alcohol dehydrogenases on the *Bacillus subtilis* spore surface to enhance enzymatic activity under adverse conditions. *PLoS ONE* **2011**, *6*, e21454. [CrossRef]
87. Kim, W.; Jeong, Y.; Back, S.; Kim, S.; Kim, J. Decolorization of textile dye by spore surface displayed small laccase for the enhanced thermal stability and robust repeated reaction. *Biotechnol. Bioprocess Eng.* **2022**, *27*, 930–937. [CrossRef] [PubMed]
88. He, L.; Ullah, M.; Naeem, M.; Ni, Z.; Feng, Y.; Shah, T.A.; Assefa, M.; Almaary, K.S.; Chen, H. *Bacillus subtilis* spore surface display enhances manganese peroxidase stability and stress resistance. *Bioresour. Bioprocess.* **2025**, *12*, 57. [CrossRef]
89. Shi, N.; Li, S.; He, L.; Feng, Y.; Saeed, M.; Ma, Y.; Ni, Z.; Zhu, D.; Chen, H. High-throughput screening and identification of lignin peroxidase based on spore surface display of *Bacillus subtilis*. *J. Sci. Food Agric.* **2025**, *105*, 2179–2189. [CrossRef]
90. Jeong, Y.; Kim, H.; Kim, B.G.; Kim, J. Regioselective hydroxylation of phloretin by tyrosinase using *Bacillus subtilis* spore display system. *Biotechnol. Bioprocess Eng.* **2025**, *30*, 377–385. [CrossRef]
91. Potot, S.; Serra, C.R.; Henriques, A.O.; Schyns, G. Display of recombinant proteins on *Bacillus subtilis* spores, using a coat-associated enzyme as the carrier. *Appl. Environ. Microbiol.* **2010**, *76*, 5926–5933. [CrossRef] [PubMed]
92. Kang, S.J.; Jun, J.S.; Moon, J.A.; Hong, K.W. Surface display of p75, a *Lactobacillus rhamnosus* GG derived protein, on *Bacillus subtilis* spores and its antibacterial activity against Listeria monocytogenes. *AMB Express* **2020**, *10*, 139. [CrossRef]
93. Hwang, B.-Y.; Pan, J.-G.; Kim, B.-G.; Kim, J.-H. Functional display of active tetrameric β-galactosidase using *Bacillus subtilis* spore display system. *J. Nanosci. Nanotechnol.* **2013**, *13*, 2313–2319. [CrossRef] [PubMed]
94. Wang, H.; Yang, R.; Hua, X.; Zhao, W.; Zhang, W. Functional display of active β-galactosidase on *Bacillus subtilis* spores using crust proteins as carriers. *Food Sci. Biotechnol.* **2015**, *24*, 1755–1759. [CrossRef]
95. Tavassoli, S.; Hinc, K.; Iwanicki, A.; Obuchowski, M.; Ahmadian, G. Investigation of spore coat display of *Bacillus subtilis* β-galactosidase for developing of whole cell biocatalyst. *Arch. Microbiol.* **2013**, *195*, 197–202. [CrossRef]
96. Yan, M.; Wang, Z.; Zhou, H.; Chen, Y.; Saeed, M.; Xu, Y.; Chen, Y.; Ni, Z.; Fang, Z.; Chen, H. Exploring the potential of spore surface-displayed keratinase for feather waste degradation using high-throughput screening. *J. Sci. Food Agric.* **2025**, *105*, 5714–5727. [CrossRef] [PubMed]
97. Wang, Z.; Yan, M.; Saeed, M.; Li, K.; Chen, Y.; Okoye, C.O.; Fang, Z.; Ni, Z.; Chen, H. The flexible linker and CotG were more effective for the spore surface display of keratinase KERQ7. *World J. Microbiol. Biotechnol.* **2024**, *40*, 35. [CrossRef]
98. Chen, H.; Zhang, T.; Sun, T.; Ni, Z.; Le, Y.; Tian, R.; Chen, Z.; Zhang, C. *Clostridium thermocellum* nitrilase expression and surface display on *Bacillus subtilis* spores. *J. Mol. Microbiol. Biotechnol.* **2015**, *25*, 381–387.
99. Chen, H.; Chen, Z.; Ni, Z.; Tian, R.; Zhang, T.; Jia, J.; Chen, K.; Yang, S. Display of *Thermotoga maritima* MSB8 nitrilase on the spore surface of *Bacillus subtilis* using out coat protein CotG as the fusion partner. *J. Mol. Catal. B Enzym.* **2016**, *123*, 73–80. [CrossRef]
100. Chen, H.; Chen, Z.; Wu, B.; Ullah, J.; Zhang, T.; Jia, J.; Wang, H.; Tan, T. Influences of various peptide linkers on the *Thermotoga maritima* MSB8 nitrilase displayed on the spore surface of *Bacillus subtilis*. *J. Mol. Microbiol. Biotechnol.* **2017**, *27*, 64–71. [CrossRef]
101. Karava, M.; Gockel, P.; Kabisch, J. *Bacillus subtilis* spore surface display of photodecarboxylase for the transformation of lipids to hydrocarbons. *Sustain. Energy Fuels* **2021**, *5*, 1727–1733. [CrossRef]
102. Gu, J.; Yang, R.; Hua, X.; Zhang, W.; Zhao, W. Adsorption-based immobilization of *C. aldicellulosiruptor* saccharolyticus cellobiose 2-epimerase on B acillus subtilis spores. *Biotechnol. Appl. Biochem.* **2015**, *62*, 237–244. [CrossRef]
103. He, W.; Jiang, B.; Mu, W.; Zhang, T. Production of D-allulose with D-psicose 3-epimerase expressed and displayed on the surface of *Bacillus subtilis* spores. *J. Agric. Food Chem.* **2016**, *64*, 7201–7207. [CrossRef] [PubMed]
104. Chapman, J.; Ismail, A.E.; Dinu, C.Z. Industrial applications of enzymes: Recent advances, techniques, and outlooks. *Catalysts* **2018**, *8*, 238. [CrossRef]
105. Hwang, E.T.; Lee, S. Multienzymatic cascade reactions via enzyme complex by immobilization. *ACS Catal.* **2019**, *9*, 4402–4425. [CrossRef]
106. Chauhan, K.; Zárate-Romero, A.; Sengar, P.; Medrano, C.; Vazquez-Duhalt, R. Catalytic kinetics considerations and molecular tools for the design of multienzymatic cascade nanoreactors. *ChemCatChem* **2021**, *13*, 3732–3748. [CrossRef]

107. Cai, X.; Huang, Y.; Zhu, C. Immobilized Multi-Enzyme/Nanozyme Biomimetic Cascade Catalysis for Biosensing Applications. *Adv. Healthc. Mater.* **2025**, *14*, 2401834. [CrossRef]
108. Chen, L.; Mulchandani, A.; Ge, X. Spore-displayed enzyme cascade with tunable stoichiometry. *Biotechnol. Prog.* **2017**, *33*, 383–389. [CrossRef] [PubMed]
109. Hsu, S.-Y.; Hsieh, H.-Y.; Stewart, G.C.; Lin, C.-H. Bioremediation of Atrazine and its Metabolite Using Multiple Enzymes Delivered by a *Bacillus thuringiensis* Spore Display System. *bioRxiv* **2023**. [CrossRef]
110. Qin, Y.; Wu, G.; Guo, Y.; Ke, D.; Yin, J.; Wang, D.; Fan, X.; Liu, Z.; Ruan, L.; Hu, Y. Engineered glyphosate oxidase coupled to spore-based chemiluminescence system for glyphosate detection. *Anal. Chim. Acta* **2020**, *1133*, 39–47. [CrossRef]
111. Jia, H.; Lee, F.S.; Farinas, E.T. *Bacillus subtilis* spore display of laccase for evolution under extreme conditions of high concentrations of organic solvent. *ACS Comb. Sci.* **2014**, *16*, 665–669. [CrossRef] [PubMed]
112. Sheng, S.; Jia, H.; Topiol, S.; Farinas, E.T. Engineering CotA laccase for acidic pH stability using *Bacillus subtilis* spore display. *J. Microbiol. Biotechnol.* **2017**, *27*, 507–513. [CrossRef]
113. Xiong, X.; Xiong, S.; Zhu, R.; Liu, S.; Gao, F.; Chen, Y. Engineering *Bacillus* Spores to Display Nicotine Oxidase: In Situ Specific and Sensitive Nicotine Detection. *ACS Sens.* **2025**, *10*, 3589–3599. [CrossRef]
114. Tang, C.; Wang, L.; Sun, J.; Chen, G.; Shen, J.; Wang, L.; Han, Y.; Luo, J.; Li, Z.; Zhang, P. Degradable living plastics programmed by engineered spores. *Nat. Chem. Biol.* **2025**, *21*, 1006–1011. [CrossRef]
115. Kawada, M.; Jo, H.; Medina, A.M.; Sim, S. Catalytic materials enabled by a programmable assembly of synthetic polymers and engineered bacterial spores. *J. Am. Chem. Soc.* **2023**, *145*, 16210–16217. [CrossRef] [PubMed]
116. Nemiwal, M.; Zhang, T.C.; Kumar, D. Enzyme immobilized nanomaterials as electrochemical biosensors for detection of biomolecules. *Enzym. Microb. Technol.* **2022**, *156*, 110006. [CrossRef]
117. Kim, H.S.; White, E.M.; Crane, G.; Patel, K.; Noh, M.H.; Rahman, M.A.; Feist, A.M.; Locklin, J.J.; Pokorski, J.K. Scalable fabrication of a tough and recyclable spore-bearing biocomposite thermoplastic polyurethane. *Chem. Eng. J.* **2025**, *505*, 159863. [CrossRef]
118. Kim, H.S.; Noh, M.H.; White, E.M.; Kandefer, M.V.; Wright, A.F.; Datta, D.; Lim, H.G.; Smiggs, E.; Locklin, J.J.; Rahman, M.A. Biocomposite thermoplastic polyurethanes containing evolved bacterial spores as living fillers to facilitate polymer disintegration. *Nat. Commun.* **2024**, *15*, 3338. [CrossRef]
119. Cui, Z.; Kawada, M.; Hui, Y.; Sim, S. Programming aliphatic polyester degradation by engineered bacterial spores. *Biomacromolecules* **2025**, *26*, 1882–1891. [CrossRef]
120. González, L.M.; Mukhitov, N.; Voigt, C.A. Resilient living materials built by printing bacterial spores. *Nat. Chem. Biol.* **2020**, *16*, 126–133. [CrossRef]
121. Rheem, H.B.; Kim, N.; Nguyen, D.T.; Baskoro, G.A.; Roh, J.H.; Lee, J.K.; Kim, B.J.; Choi, I.S. Single-Cell Nanoencapsulation: Chemical Synthesis of Artificial Cell-in-Shell Spores. *Chem. Rev.* **2025**, *125*, 6366–6396. [CrossRef] [PubMed]
122. Iturralde, M.; Ripoll, M.; Silvio, D.d.; Gallego, M.; Grajales-Hernández, D.A.; López, X.; Betancor, L.; López-Gallego, F. Artificial Spores as Multi-Functional Biocatalysts to Perform Biosynthetic Cascades. *Adv. Funct. Mater.* **2024**, *34*, 2406097. [CrossRef]
123. Liu, S.-R.; Cai, L.-F.; Wang, L.-Y.; Yi, X.-F.; Peng, Y.-J.; He, N.; Wu, X.; Wang, Y.-P. Polydopamine coating on individual cells for enhanced extracellular electron transfer. *Chem. Commun.* **2019**, *55*, 10535–10538. [CrossRef]
124. Sun, Z.; Hübner, R.; Li, J.; Wu, C. Artificially sporulated *Escherichia coli* cells as a robust cell factory for interfacial biocatalysis. *Nat. Commun.* **2022**, *13*, 3142. [CrossRef]
125. Wang, S.; Gong, Z.; Hübner, R.; Karring, H.; Wu, C. Pickering Emulsion Biocatalysis with Engineered Living Cells for Degrading Polycarbonate Plastics. *Small* **2025**, *21*, 2504376. [CrossRef] [PubMed]
126. Ning, J.; Sun, Z.; Hübner, R.; Karring, H.; Ebbesen, M.F.; Dimde, M.; Wu, C. Engineering living cells with polymers for recyclable photoenzymatic catalysis. *Nat. Catal.* **2024**, *7*, 1404–1416. [CrossRef]
127. Bolmanis, E.; Grigs, O.; Didrihsone, E.; Senkovs, M.; Nikolajeva, V. Pilot-scale production of *Bacillus subtilis* MSCL 897 spore biomass and antifungal secondary metabolites in a low-cost medium. *Biotechnol. Lett.* **2024**, *46*, 355–371. [CrossRef] [PubMed]
128. Naveen, A.K.; Sontakke, M. A review on regulatory aspects, challenges and public perception in acceptance of genetically modified foods. *Food Sci. Biotechnol.* **2024**, *33*, 791–804. [CrossRef]
129. Kauffmann, F.; Van Damme, P.; Leroux-Roels, G.; Vandermeulen, C.; Berthels, N.; Beuneu, C.; Mali, S. Clinical trials with GMO-containing vaccines in Europe: Status and regulatory framework. *Vaccine* **2019**, *37*, 6144–6153. [CrossRef] [PubMed]
130. Vojnovic, S.; Aleksic, I.; Ilic-Tomic, T.; Stevanovic, M.; Nikodinovic-Runic, J. *Bacillus* and *Streptomyces* spp. as hosts for production of industrially relevant enzymes. *Appl. Microbiol. Biotechnol.* **2024**, *108*, 185. [CrossRef]