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Bacillus Species: Evolving Roles in Bio-Based Detergents

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Abstract: Enzymes and biosurfactants, often referred to as "green chemicals," play pivotal roles in enhancing the washing performance of bio-based detergents—a growing trend driven by environmentally conscious consumers. However, the widespread adoption of such bio-based detergents faces challenges, including high costs, limited efficiency, and the need for ongoing innovations. *Bacillus* species have long been universally acknowledged and exploited for industrial applications, and *Bacillus* spp. are largely differentiated from other microorganisms for their enzymatic applications, particularly in detergent production. Recent developments in bio-surfactant production by *Bacillus* sp. support the adoption of green detergents, and these bacterial biosurfactants are a promising source for detergent manufacturing. This article provides an overview of the current understanding of promising *Bacillus* species and their potential to advance and accelerate the production of bio-based detergents.

Keywords: bio-based detergents; proteases; α-amylase; cellulases; lipases; biosurfactants; *Bacillus* species; low-cost substrates; cold enzymes; directed evolution; CRISPR-based genetic tools; Cas9

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1. Introduction

Detergents have become an indispensable commodity in modern society, driven by factors such as population growth, increased urbanization, and industrialization, particularly in the wake of the COVID-19 pandemic. However, the growth of the detergent market risks increased adverse environmental impact through the use of non-biodegradable detergents, due to the bioaccumulation of synthetic surfactants, low biodegradability, and higher solid content, putting greater pressure on ecosystems [1].

Detergents have changed significantly over history, with incremental improvements to their ingredients for various reasons, yet surfactants remain the central component [2,3]. Traditional detergents, derived from soaps produced by saponifying fats or oils with lye (sodium or potassium), were the first natural and eco-friendly surfactants used for fabric washing [4–6]. These soaps remained the sole detergent source until 1916. During World War I, the scarcity of fats, combined with the petrochemical boom in World War II, accelerated the shift to synthetic surfactants [4]. The advantages of synthetic surfactants, such as their easy availability, low cost, and expanded application areas, have made them increasingly popular. However, synthetic surfactants mainly derived from petroleum have been the primary culprit behind environmental concerns, from petrochemical processing to the discharge of washing wastewater [1,7,8]. This has raised public awareness about the acute toxicity of detergents and their harmful effects on freshwater organisms, among other negative environmental impacts. A 2009 survey revealed that 40–64% of consumers

across Germany, France, the UK, and the USA preferred clean and green detergents [9]. This consumer pressure and changes to environmental laws have become the key drivers behind modern detergent innovations [10].

To meet growing consumer demand and comply with environmental regulations, detergent companies have adopted various approaches. Some focus on sourcing renewable ingredients, while others prioritize reducing environmental impact through compact packaging, lower wash temperatures, and minimal water consumption. Consumer response to the former has been positive; a survey found that around 75% of respondents expressed concern about detergent ingredients, favoring biodegradable options to reduce chemical and water pollution [9]. Enzymes, once considered minor additives, have gained increasing significance in recent years, cementing their role in sustainable detergent formulations [9]. While bio-based surfactants have garnered consumer support for their ecological benefits and diverse substrate availability, the market is still in its early stages, facing challenges such as technical limitations, higher costs compared to synthetic alternatives, and a shortage of skilled labor [11]. Enzymes and biosurfactants are key to bio-based laundry detergents, offering benefits like superior cleaning at lower temperatures, reduced energy use, and decreased fossil fuel dependence [9]. However, high enzyme production costs limit their availability to mature markets like Europe, the USA, and Japan [12]. As a result, consumers are demanding more affordable, higher-performance products [10,13].

Compared to plant- and animal-based sources, microbes have garnered significant attention in the enzyme and biosurfactant industries due to their scalability and cost-effectiveness [14]. Microbial production offers advantages such as rapid growth, high yields, controlled fermentation, production efficiency, scalability, raw material availability, and genetic engineering [15]. Despite these benefits, there is still room for improvement. To further lower costs, studies have explored the valorization of agro-industrial organic waste as low-cost substrates, as they represent 30–50% of the end product value [16,17]. Coproducing enzymes in a single fermentation batch using microorganisms, while optimizing enzyme proportions, offers a cost-effective solution to improve production efficiency [18]. Genetic manipulation enables the creation of microbial enzymes with enhanced properties [19], which could contribute to the invention of the next generation of smart bio-based detergents. Much research provides evidence that *Bacillus* species, in particular, offer significant potential, as they can meet both performance and cost demands, paving the way for more sustainable and effective detergent formulations [20,21].

The genus *Bacillus* has also become the dominant microbial group used in recent microbial-based cleaning products. These products, which incorporate live microbial strains as active ingredients, are increasingly adopted across various countries and regulated under legal frameworks in regions such as Europe, the United States, and Canada. Notably, several *Bacillus* species, including *B. subtilis*, *B. megaterium*, and *B. pumilus,* have been officially approved for use in cleaning formulations and evaluated for safety under Canada's Canadian Environmental Protection Act, 1999 (CEPA 1999). These strains are also classified as Risk Group 1 by the U.S. Centers for Disease Control and Prevention (CDC), indicating a low risk to human health and the environment, and many hold GRAS (Generally Recognized as Safe) status by the U.S. Food and Drug Administration (FDA). Regulatory acceptance of microbial levels up to 10^4 CFU/m^3, which may be released during product application, has further supported their commercialization. As a result, *Bacillus*-based strains have been successfully incorporated into a wide variety of cleaning products—including hard surface cleaners, odor control formulations, degreasers, and septic system treatments—offering environmentally friendly alternatives to conventional chemical-based solutions [22,23].

However, while *Bacillus* species themselves are well-accepted, the use of genetically modified microorganisms (GMMs) as live agents in microbial cleaning products remains highly restricted. Barriers such as regulatory complexity, consumer skepticism, and the prohibitive costs and lengthy timelines associated with GMM approval have limited their commercial deployment in direct-use cleaning formulations. Nonetheless, using GMMs in upstream fermentation processes strictly for metabolite production has emerged as a viable and legally permissible approach. Provided the final product is free of viable GM cells and residual recombinant DNA and meets established safety, purity, and labeling standards, GMM-derived enzymes and biosurfactants can be incorporated into detergent formulations. This strategy enables the harnessing of cutting-edge bioengineering while maintaining regulatory compliance and consumer safety, thus supporting the development of next-generation, high-performance cleaning products [23,24].

While the existing literature addresses the industrial applications of *Bacillus* sp., this review delves deeper into their specific role in the detergent industry. Focusing on *Bacillus* species, it explores their potential in producing enzymes and biosurfactants tailored for bio-based detergent formulations.

2. Biosurfactants of *Bacillus* Species in Detergents

Biosurfactants are surface-active compounds that have microbial origins, such as bacteria and yeast. According to chemical structures, there are different types of microbial biosurfactants produced by a wide range of microorganisms, but the most popular compounds are glycolipids [25,26], followed by lipopeptides and phospholipids [27]. Lipopeptides are one of the most interesting and potent classes of biosurfactants produced chiefly by *Bacillus* sp. and *Pseudomonas* sp. [28,29]. Biosurfactants are superior to their synthetic counterparts in numerous areas, such as higher biodegradation, lower toxicity, biocompatibility, and extremophilic tolerance (pH, temperature, and salt concentration), as well as a reduced carbon footprint relative to that of synthetic surfactants [26,28].

Detergents represent the largest application segment for both synthetic surfactants and biosurfactants [21], as surfactants constitute 15–40% of modern detergent formulations, serving as their most crucial component [4,28,30,31]. In 2022, the global value of surfactants was worth US dollars (USD) 41.9 billion and is expected to rise to USD 60.0 billion in 2030 (https://www.vantagemarketresearch.com/industry-report/surfactants-market-1671, accessed on 12 March 2025), with household detergents accounting for 46% of total consumption (https://www.spglobal.com/commodityinsights/en/ci/research-analysis/global-surfactants-industry.html, accessed on 12 March 2025). In contrast to the mature synthetic surfactant market, the global biosurfactants market is still in its developmental stage, as the first biosurfactants were only discovered between 1948 and 1949, during research on the production of antibiotics and hemolysin by bacteria [21]. In 2023, the biosurfactant market was estimated at USD 4.4 billion and is expected to reach USD 4.7 billion in 2024 and USD 6.71 billion by 2032 (https://www.fortunebusinessinsights.com/biosurfactants-market-102761, accessed on 12 March 2025). Despite facing intense cost competition with synthetic surfactants, the exploitation and commercial competition of biosurfactants in detergents are forecasted to increase, fueled by environmental concern and higher quality [21,28].

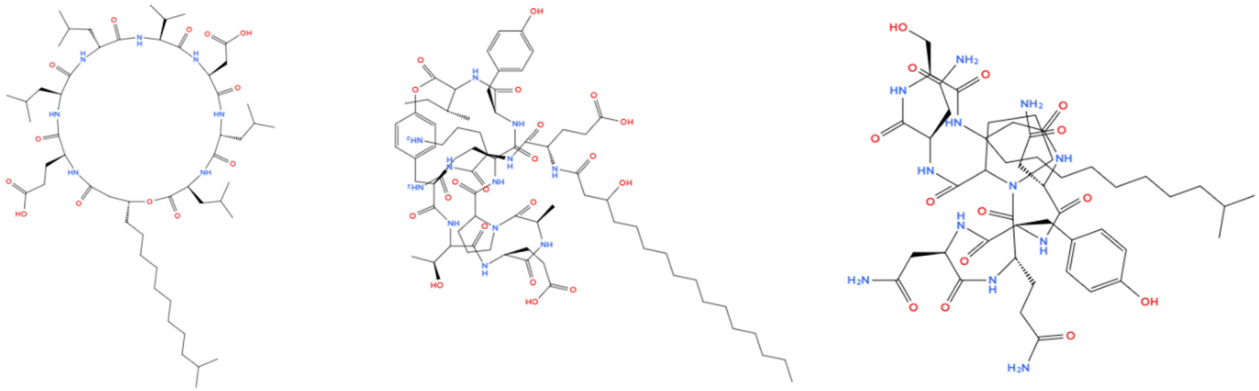
Linear alkylbenzene sulphonates (LASs) are among the most widely used synthetic anionic surfactants, primarily employed in household detergents such as laundry powders and liquids, dishwashing products, and all-purpose cleaners. Commercial LAS products are complex mixtures, typically containing homologues with alkyl chains ranging from C10 to C14. In 2005, LAS consumption in European detergent applications covered by the Human and Environmental Risk Assessment (HERA) reached approximately 350 kt,

representing over 80% of the total LAS usage in Europe, estimated at 430 kt. After use and disposal, LAS can enter the environment through direct discharge or via sewage treatment plant effluents [32]. Although LAS is considered readily biodegradable, its high volume of use can result in bioaccumulation, posing ecological risks. In contrast, biosurfactants offer a sustainable alternative due to their superior environmental profiles. They are biodegradable, exhibit low toxicity, and maintain functionality under extreme conditions such as low temperatures, high salinity, and extreme pH. Furthermore, biosurfactants typically possess significantly lower critical micelle concentrations (CMCs), often 10 to 40 times lower than those of synthetic surfactants [33], allowing them to reduce surface tension at much lower concentrations. These properties make them promising substitutes for synthetic surfactants in detergent formulations [34,35].

In detergents, biosurfactants can mimic traditional surfactants but with higher efficiency [34]. Alongside essential surface-active properties such as lowering surface tension and forming stable emulsions—key characteristics shared with conventional surfactants [35]—biosurfactants exhibit a significantly lower critical micelle concentration (CMC, the minimum concentration required for micelle formation). In general, biosurfactants exhibit CMC values 10 to 40 times lower than those of synthetic surfactants [33], meaning they require significantly lower concentrations to achieve the same surface tension reduction.

Among the three major lipopeptide biosurfactants produced by *Bacillus* strains, surfactin, fengycin, and iturin are the most widely studied. However, fengycin and iturin have higher CMC values due to their rigid cyclic peptide rings (Figure 1), which hinder micelle formation. Fengycin forms micelles at 15–18 mg/L [36] while iturin requires $\geq$25 mg/L [37,38], indicating weaker self-assembly into micelles compared to surfactin. The superior efficiency of surfactin is attributed to its cyclic structure, formed by a β-hydroxy fatty acid linked to a loop of seven amino acids: L-asparagine (Asn), L-leucine (Leu), glutamic acid (Glu), L-leucine (Leu), L-valine (Val), and two D-leucine residues [28]. These amino acids are connected via a lactone linkage, which is more flexible than the amide bonds found in fengycin and iturin. This structural flexibility makes surfactin highly dynamic, enhancing micelle formation and surface tension reduction efficiency. Combined with its low molecular weight, this property underscores surfactin's superior emulsifying and solubilizing capabilities for hydrophobic stains [29], making it an ideal ingredient in detergent formulations. Surfactin forms micelles at and above 10 mg/L (Figure 2) [37] and is the most well-known lipopeptide compatible with commercial detergents [34,39]. Even at concentrations below its CMC (7.5–10 mM, depending on buffer conditions), detergent-like permeabilization effects were observed, while complete solubilization and mixed micelle formation occurred at the CMC [38]. Additionally, structural diversity within the surfactin family—with more than 30 known variants—results from differences in amino acid and fatty acid residues. However, identical surfactin molecules are observed depending on their chiral sequence, further influencing their functional properties [29].

The surface-active properties of *Bacillus* biosurfactants as detergent ingredients have been demonstrated through several publications. *Bacillus subtilis* strain SPB1, for instance, produces a lipopeptide biosurfactant that reduces water surface tension by 34 mN/m and effectively removes hydrophobic stains, such as coffee and turmeric. When combined with a commercial detergent, it improves oil (45%) and tea (65%) removal efficiency, compared to 34% and 58% with the detergent alone [34]. Similarly, thermophilic *Bacillus subtilis* strains DM-03 and DM-04 produce biosurfactants that remain stable at 80 °C for 60 min across a pH range of 7.0–12.0. While DM-03 primarily secretes iturins and DM-04 is rich in surfactins, leading to different wash performances, the latter showed better emulsification with oils when combined with laundry detergents. However, the overall oil and blood stain removal efficiency of both strains was still lower than that of detergent alone [40].



(**A**) (**B**) (**C**)

Figure 1. Chemical structure of biosurfactants (**A**) surfactin, (**B**) fengycin, and (**C**) iturin A (https://app.molview.com/).

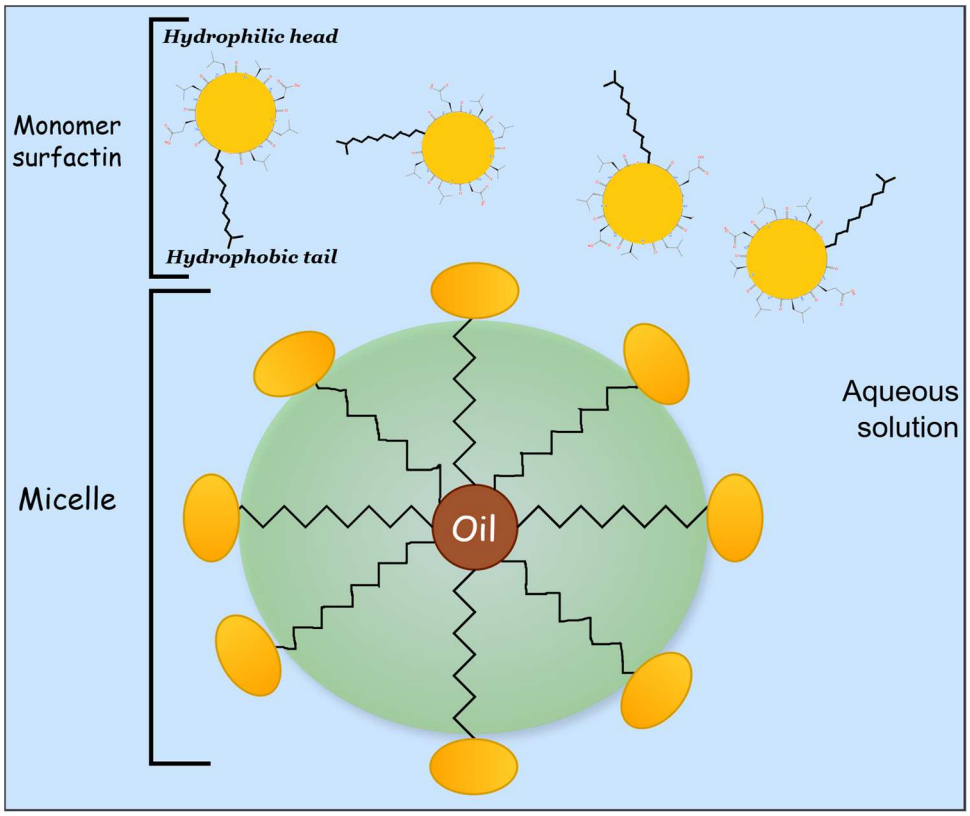


Figure 2. Micelles of surfactin.

Simultaneously with biosurfactant secretion, the co-production of stable enzymes by *Bacillus* species not only facilitates detergent formulations but also improves their economic viability in detergent applications. For example, *Bacillus subtilis* PF1 simultaneously produces proteases, amylase, and biosurfactants when grown on agro-industrial by-products, with the resulting biosurfactant maintaining stability at alkaline pH (10–11) and temperatures between 30 and 60 °C. More importantly, its combination with hydrolytic enzymes improves stain removal from cotton fabrics, outperforming SDS-based treatments [41]. Similarly, Kavuthodi and Sebastian [42] studied the simultaneous production of pectinase and biosurfactant by *B. subtilis* BKDS1 using pineapple stem extract in a 1 L fermenter, confirming its potential for scale-up.

Despite its superior performance at low concentrations, surpassing synthetic surfactants like LAS and common biosurfactants such as rhamnolipids and sophorolipids, surfactin's broader adoption in detergents remains limited, with its use largely confined to high-value sectors such as biomedicine and cosmetics. The major barrier to commercial viability lies in high production costs and low yields from wild-type *Bacillus* strains (0.1–1 g/L) (Table 1).

Downstream processing, accounting for 60–80% of total cost, is the major economic barrier to biosurfactant production, alongside costly substrates (~50%) [43]. This high cost is influenced by various factors such as the biosurfactant's solubility, ionic nature, and cellular localization (intracellular, extracellular, or membrane-bound). Liquid–liquid solvent extraction, commonly using organic solvents like chloroform–methanol or ethyl acetate, is widely reported but is environmentally unfriendly and cost-intensive due to the large volumes of solvents required [44]. To address this, environmentally benign alternatives are being explored, including adsorption onto activated carbon or resins, centrifugation, ion exchange chromatography, ultrafiltration, and foam fractionation. These methods enable the recovery of highly pure biosurfactants at a lower cost with reusable materials. Among them, foam fractionation is especially attractive due to its solvent-free nature. This approach enables simultaneous production and recovery of biosurfactants by continuously removing surface-active molecules adsorbed on air bubbles, which can also prevent product accumulation that inhibits biomass growth [28]. Still, the overall production cost of biosurfactants is 10–12 times higher than that of synthetic surfactants [45]. Equipment costs for downstream processing can comprise up to 76% of total capital investment, compared to just 21% for upstream processes, with the remainder being facility-dependent. As no single downstream method is sufficient, multi-step recovery strategies are required to obtain biosurfactants with various purity levels. While crude biosurfactants may suffice for environmental remediation and detergents, high-purity products for industrial applications necessitate more sophisticated separation and purification steps [44].

Additionally, low biosurfactant yields of wild-type *Bacillus* strains remain a major obstacle to commercializing bio-based detergents, but recent advances in genetic engineering have significantly improved biosurfactant production in *Bacillus* species. The *srfA* operon, which encodes the mega-enzyme surfactin synthetase, was revealed as a crucial player in biosurfactant synthesis, significantly enhancing detergent potential [29]. Building on this finding, the *srfA* gene from *Bacillus* sp. SK320—originally isolated from endosulfan-contaminated cashew plantation soil—was cloned into *E. coli*, resulting in substantially higher biosurfactant production compared to the wild-type *Bacillus* strain [28]. In a separate study, a non-producing strain, *Bacillus subtilis* 168, was subjected to extensive metabolic engineering. This included the integration of a complete *sfp* gene, reduction in competing metabolic pathways, enhancement of cellular tolerance to surfactin, increased supply of branched-chain fatty acid precursors, and redirection of acetyl-CoA flux toward surfactin biosynthesis by upregulating *srfA* transcription. These combined interventions elevated surfactin production to 12.8 g/L [46]. Moreover, genome shuffling applied to *Bacillus amyloliquefaciens* led to recombinant strains with up to a 15.7-fold increase in surfactin production compared to the wild type [47].

By contrast, rhamnolipids and sophorolipids offer a more practical and balanced profile for detergent use. Both exhibit strong surface activity (CMC ranges of 10–200 mg/L and 40–100 mg/L, respectively), along with high biodegradability and low ecotoxicity. Sophorolipids are particularly advantageous due to their high production yields, often exceeding 200 g/L and reaching over 400 g/L at commercial scale [45]. Their relatively low production cost (approximately USD 3/kg) and favorable properties have facilitated their incorporation into sanitizer and detergent formulations, especially in mild detergents

and personal care products. Rhamnolipids, although promising in terms of performance, face significant challenges in large-scale production, particularly excessive foaming during fermentation and regulatory scrutiny, owing to their microbial origin from *Pseudomonas aeruginosa*, an opportunistic human pathogen [48].

Regardless of these challenges, *Bacillus* biosurfactants are gaining industrial recognition. A notable example is the French company Lipofabrik SAS (Lesquin, France), which has developed and commercialized lipopeptide-based formulations obtained from *B. subtilis* fermentation using renewable resources [21]. These advancements indicate a growing trend toward integrating biosurfactants into commercial detergent formulations. Given their exceptional stability, synergy with enzymes, and enhanced cleaning efficacy, *Bacillus* biosurfactants hold great potential as sustainable alternatives to synthetic surfactants in the detergent industry.

Table 1. Comparative profile of synthetic and biosurfactants used in detergents.

Category	LAS	Rhamnolipids	Sophorolipids	Surfactin	Refs.
Typical Source	Chemical synthesis	*Pseudomonas aeruginosa*	*Starmerella bombicola*	*Bacillus subtilis*	[35,49]
CMC (ppm)	C10–14 433–650 C12LAS~360 C13LAS~150	10-200	40–100	~10–20	[4,32,35,37]
Surface Tension (mN/m)	Commercial BIO-SOFT® S-101 C11.3 ~ 35	~30–35	~30–40	~27	[35,50]
Biodegra-dability	97–99% (Aerobic)	High	High	High	[28,51,52]
Eco-toxicity	EC_{50} = 3.5 ppm *Dunaliella* sp.	Low	Low	Low	[28,53]
Cost	USD ~2 highly scalable	USD ~223/100 g 90% pure	USD ~3/kg	USD ~22.3/mg ≥ 98% pure	[28,45]
Concentration (g/L)	Industrial scale	39–112	>200	0.1–1 WT *Bacillus* sp.	[43,49,54,55]
Scaling	Fully commercial	Limited commercial	Fully commercial	Pre-commercial	[55,56]
Use	Household and industrial detergents	Ecodetergents, bioremediation, cosmetics	Detergents, cosmetics, skincare	Pharma., cosmetics, skincare	[35]

3. Enzymes from *Bacillus* Species in Detergents

Since Otto Röhm's patent in 1913 described the application of enzymes in the detergent industry over a century ago, steady adoption of this technique has progressed [57]. Enzymes are natural catalysts produced by living organisms that are active and stable during the washing processes but are nontoxic in discharge. Thus, they are well-accepted ingredients in a variety of existent detergent types, ranging from powder and liquid household detergents, laundry pre-spotters and stain removers, automatic dishwashing detergents, and industrial and institutional cleaners [58]. As mentioned above, surfactants are able to disperse, solubilize, and remove stains effectively with particular small dirt and liquid fatty soils. However, organic soiling arising from long polymer chains or solid fat can attach strongly to surface textiles. In such cases, the synergistic action between surfactants and enzymes enhances soil removal, enabling more efficient degradation and detachment of these stubborn residues [31]. Most recently, large numbers of alkaline enzymes, such as proteases, lipases, α-amylases, and cellulases, have been introduced in heavy-duty laundry and automatic dishwashing detergents [59]. However, the total enzyme content is low (0.2–2%) in detergent formulations, depending on whether solid or liquid forms of detergents are used; these highly effective multi-enzyme systems can facilitate the transition toward compact detergents, which bring the subsequent significant environmental savings [60].

Enzymes used in detergents must meet specific requirements. They must remain stable and efficient at a wide range of alkaline pH levels and a variety of temperatures (from low temperatures with synthetic fibers to high temperatures with cotton ones) throughout washing processes. Detergents are generally alkaline as most of the difficult-to-remove soils are more easily hydrolysed (saponified), chelated, and dispersed at alkaline pH levels. Moreover, the enzymes must be strictly compatible with the remaining detergent chemicals like surfactants, builders, bleaching agents, and other detergent enzymes, etc. The stability and compatibility of enzymes within a detergent formulation are important in determining the cleaning efficiency of the enzymes [61].

The detergent market is the single biggest consumer of enzymes, with around 25–30% of total industrial enzyme sales by 2014 [61,62]. The global enzyme market was estimated to be worth USD 14.0 billion in 2024, and revenue is projected to be USD 20.3 billion in 2030 [63]. Within this landscape, *Bacillus*-based products alone accounted for at least USD 18 billion in 2020, considering only the applications discussed in the current context. Notably, *B. subtilis*, *B. amyloliquefaciens*, and *B. licheniformis* are estimated to collectively contribute to around 50% of global industrial enzyme production, underscoring their technical and commercial significance. This dominant market share suggests that enzymes derived from *Bacillus* species are both high-performing and cost-effective compared to those produced by other microbial genera. Moreover, the consistent submission of over 650 patent documents annually since 2017 highlights ongoing innovation and substantial industrial investment in this genus [64,65].

3.1. Proteases from Bacillus Species

Proteases are the largest enzyme group across the global market and are extremely popular in many industrial sectors, including the food and feed industry, waste management, the leather industry, the chemical industry, the medical field, and the detergent industry [66]. The half-life of peptide bonds at neutral pH and 25 °C is over 100 years, and stubborn protein-based stains can become permanent when subjected to bleaching and drying processes due to oxidation and denaturation. Through proteolysis, proteases can catalyze these peptide stains in milliseconds. Thus, these enzymes serve to remove proteinaceous stains like eggs, milk, grass, blood, human sweat, etc., that strongly adhere to fabrics. Proteases were the first enzymes incorporated into detergents [57,66]. Initially, having been included as an add-on, they progressively became common ingredients in various types of detergents [67].

Although the very first enzymatic detergent proposed by Otto Röhm in 1913 included crude pancreatic proteases, the efficacy of enzymes was not widely recognized until the 1960s, with the advent of microbial proteases extracted from *Bacillus* spp. [68]. Microbes became the chief protease producers, and *Bacillus* spp. represented the most important strains for alkaline protease production [18]. In 1960, Novo industry A/S, one of the most well-known enzyme manufacturers, marketed a trade product called BIOTEX related to subtilisin from *B. licheniformis* [57].

Based on the structure of active sites and proteolytic mechanism, proteases were classified into seven groups: the serine- (EC 3.4.21), cysteine- (EC 3.4.22), aspartic-(EC3.4.23), metallo- (EC 3.4.24), threonine peptidases (EC 3.4.25), glutamic peptidases (currently included in EC 3.4.23) and asparagine peptide lyases (EC 4.3.2). Among those, while metalloproteases become inactive because of the loss of their metal cofactors by chelating agents, and thiol (or cysteine) proteases can be oxidized by the bleaching agents, alkaline serine endopeptidases are the most suitable in detergents because of their high stability in alkaline conditions and resistance to oxidizing chemicals [15,69].

Many studies have looked at the suitability of alkaline proteases produced by *Bacillus* spp. as detergent ingredients. *Bacillus clausii* KSM-K16 and *Bacillus* sp. *strain* KSM-KP43 were successfully used to produce alkaline proteases in bulk, which were then introduced into laundry cleansers [70]. Nadeem et al. (2013) [71] purified serine proteases produced by mutant *B. licheniformis* UV-9 to homogeneity and characterized them to elucidate this additive's precise properties. The serine proteases could also maintain a high level of their relative activity regardless of the addition of common inhibitors, metal ions, surfactants, and oxidants, but were found to be sensitive to PMSF, DFP, and SDS [71]. The essential characteristics of proteases produced by *Bacillus licheniformis* NH1 have also been investigated. The enzyme suffers little from non-ionic surfactants, like Tween 20 and Triton X-100 [72]. A greater effect was observed with the addition of oxidants and bleaching agents. This study hypothesized that Ca^{2+} ions help to maintain the enzyme's structural configuration, thus enabling it to remain stable at high temperatures (over 65 °C) [73]. Recently, *Bacillus pumilus* MP 27, isolated from marine water, has emerged as a promising candidate. This strain produced a thermophilic protease that is stable over a broad range of temperatures, from 10 to 70 °C, tolerating pH values as high as 11. The enzyme's stability was measured after adding Triton X-100 as a surfactant, and Tide as a commercial detergent [74]. Protease from *Bacillus* sp. APR-4 was active at pH 9.0 and tolerant to temperatures up to 80 °C. The enzyme showed high resistance toward bleaching and oxidizing agents (sodium hypochlorite) and commercial detergents (Fena®, Farishta®) [75]. Finally, *Bacillus cereus* BM1, *Bacillus clausii* Sm3, and *Bacillus licheniformis* ALW1 were examined as potential candidates for the detergent industry [76,77]. In the current market, several *Bacillus* proteases are already being used in detergents, as shown in Table 2.

Table 2. Commercial protease products used in detergents [78,79].

Commercial Name	Specificity	Producer	Origin	Working Temperature
Alcalase®	Serine endopeptidase (Subtilisin A)	Novozymes	*Bacillus licheniformis*	Between 50 and 75 °C
Durazym®	Subtilisin	Novozymes	mutant *Bacillus* sp.	
Everlase™	Subtilisin A	Novozymes	mutant *Bacillus* sp.	
Savinase®	Serine endopeptidase (Subtilisin A)	Novozymes	mutant *Bacillus* sp.	
Esperase®	Serine endopeptidase (Subtilisin A)	Novozymes	*B. halodurans*	
Neutrase®	Metalloprotease	Novozymes	*B. amyloliquefaciens*	
Protamex™	Protease	Novozymes	*Bacillus* sp.	
Purafect® Prime	Subtilisin	Genencor Intl	*Bacillus lentus*	Between 20 and 40 °C
Properase®	Protease	Genencor Intl	*Bacillus clausii*	

3.2. α-Amylases from Bacillus Species

With the amylase enzymatic system, α-amylase has gained greater attention than β-amylase and γ-amylase due to its potential applications, especially in industrial sectors, ranging from the food and beverage, fermentation, paper, textile, and pharmaceutical industries [80,81]. The enzyme, α-amylase (1,4-α-d-glucan glucanohydrolase [E.C. 3.2.1.1]), is an extracellular enzyme that breaks down 1,4-α-d-glycosidic starch linkages at random to release short-chain carbohydrates. α-amylase is the second most significant position in the global enzyme market (accounting for 25–33% of the total market value), second only to proteases [82], of which, the global value in 2022 was USD 1.84 billion (https://www.persistencemarketresearch.com/market-research/alpha-amylase-market.asp, accessed on 12 March 2025) preceded by USD 2 billion of proteases

(https://www.futuremarketinsights.com/reports/protease-market, accessed on 12 March 2025). In addition to its substantial market share, α-amylase is also highly demanded across industries, particularly in detergents, where it is historically the second most utilized enzyme after serine proteases [83] and is present in about 90% of modern liquid detergents [80,84]. α-amylase converts starchy foods such as spaghetti, pasta, potatoes, gravy, custards, chocolate, etc., into water-soluble products (oligosaccharides and dextrins) for easy removal. Moreover, α-amylase is also responsible for the anti-adhesion of suspended soils [15]. Microbial α-amylase possess characteristics that are well suited to detergents, and both *Bacillus* species and *Aspergillus* species are the chief suppliers for alkaline and thermostable α-amylase [80,85].

Several findings have indicated that adding Ca^{2+} stimulates most bacterial amylases because Ca^{2+} plays an important role as an essential cofactor for these enzymes [81]. Hence, amylase is generally considered a metalloenzyme. Analysis of α-amylase's three-dimensional structure reveals that Ca^{2+} is bonded to two of its three domains and plays a role in maintaining the solid tertiary structure of the enzyme, resulting in stable amylolytic activity [80]. However, the stability of enzymes and the availability of Ca^{2+} could be threatened when combined with builders as calcium-chelating agents. These chemicals act to soften hard water and enhance the performance of liquid detergents. Therefore, the search for Ca-independent α-amylase to boost the quality of detergent formulations is ongoing [86]. In addition, like other metalloenzymes, amylase is inhibited by the presence of EDTA, which induces a need to find novel amylase-producing strains.

Bacillus α-amylase has been investigated by many authors. Remarkably, the *Bacillus subtilis* strain AS-S01a was isolated from a soil sample and produced an alkaline α-amylase that does not require Ca^{2+} for allosteric activation. This purified enzyme was most active at 55 °C and pH 9, and the existing activity remained when treated with EDTA (2 mM) (a chelating agent), 1% Triton X-100, Tween 20, and Tween 80 (non-ionic surfactants). The α-amylase from strain AS-S01a also exhibited 69–100% stability at 30 °C and 37 °C toward commercial detergents, such as Surf excel® and Wheel®, Tide® and Ariel®, Henko®, Fena Ultra®, Safed® and Ujala® [86]. A partially purified α-amylase obtained from *Bacillus* sp. strain TSCVKK remained stable at the pH range of 6–9.5, and enzymatic hydrolysis was enhanced by mixing 1% soluble starch plus 5 mM $CaCl_2$ at 55 °C, while inhibition was observed by 8 M urea and 5 mM EDTA. Other factors tested, such as SDS (an anionic surfactant), Triton X-100, Tween 20, Tween 40, and Tween 80, had only a slight effect on the original activity [87]. *Bacillus cereus* strain GA6 was able to synthesize cold-active α-amylase, which was stable at the lower temperature of 4–37 °C, pH ranging from 7 to 11. However, the cold-active amylase was denatured by Fe^{2+}, Zn, $CuSO_4$, and H_2O_2. Unlike amylase secreted by other strains, which were stimulated by Ca^{2+}, the amylase from *Bacillus cereus* strain GA6 still showed good quality in the presence of EDTA, as well as Urea and SDS [88]. The enzyme was compatible with commercial detergents (e.g., Tide and Ghari detergents), thus proving its potential application in this field [89].

Numerous commercial *Bacillus*-derived amylases are used in detergents, namely BAN® (*Bacillus amyloliquefaciens)*, Stainzyme® (mutant *Bacillus licheniformis*), Duramyl, Maxamyl (*Bacillus* sp.), and Solvay amylase [15], Termamyl®, and Takaterm (*B. licheniformis*) [90,91].

3.3. Lipases from Bacillus Species

In terms of general commercial consumption and detergent preparations, lipases (triacylglycerol hydrolases, E.C. 3.1.1.3) are the third most important biocatalysts, after proteases and carbohydrases [92]. Lipases added to household and industrial cleansers digest fatty stains and greasy soils, including butter, margarine, fats, fat-based sauces, salad oils, soups, human sebum, or certain cosmetics [93]. The addition of lipase is beneficial as this

innovation can help replace harsh chlorine bleach, and indirectly mitigate environmental pollution from laundry effluents [94]. Lipase acts by attacking ester bonds in triacylglycerols to liberate diacylglycerols, monoacylglycerols, long-chain fatty acids, and glycerol in an aqueous solution at the lipid–water interface. In 1958, Sandra and Denuelle described the catalytic mechanism in kinetics terms as "interfacial activation," hence, the reaction does not follow Michaelis–Menten kinetics. This unique phenomenon was not found in true esterases (EC 3.1.1.1, carboxyl ester hydrolases), which act on substrates soluble in water [92,95]. Because lipases are members of the alpha/beta-hydrolase fold family, their secondary structure, with a central β-sheet surrounded by α-helices, eclipses the active site by establishing a lid. This causes the enzyme to become inactive in homogeneous aqueous environments until an oil-water interface is introduced. The catalytic center containing the triad Ser-Asp (or Glu)-His has to undergo a conformational rearrangement to allow substrates to access the active site [92].

The vast majority of lipases come from bacteria [96–98]. Various brands have successfully launched a number of lipase-based products [93]. The first commercial detergent containing fungal lipases, named Lipolase, was developed in 1988 by Novo Nordisk, and manufacturers have continued to develop upgraded versions using fungi (*Humicola lanuginose*, *Aspergillus oryzae*, e.g.). So far, bacterial lipases used in two laundry detergents, Lumafast (Genencor International) and Lipomax (Gist Brocades), were isolated from *Pseudomonas mendocina* and *Pseudomonas glumae*, respectively [93]. Despite the fact that *Bacillus* is not the dominant lipase producer for detergents, its potential has been recognized with great interest, and the ability of an array of *Bacillus* sp., e.g., *Bacillus subtilis* JPBW-9, *Bacillus licheniformis*, *Bacillus licheniformis* VSG1, *Bacillus pumilus* SG2, *Bacillus flexus* XJU-1, to synthesize detergent-compatible lipases has been studied [94,99].

Bacillus lipases possess characteristics that are valued for detergent applications [94]. Mostly, the enzymes' activity is stable in neutral to slightly alkaline media (pH = 7–9), their optimal temperature is around 45–50 °C, and they tolerate high levels of Ca^{2+}, surfactants, bleaching agents, organic solvents, and proteases. A variety of *Bacillus* species have been reported to produce alkaliphilic lipases, such as *Bacillus subtilis* DR8806, *Bacillus licheniformis*, and *Bacillus* sp. RSJ-1, *Bacillus* sp. LBN2 [99]. The lipase from *Bacillus methylotrophicus* PS3 is thermostable with optimal conditions of 55 °C and pH 7.0. The stability of the enzyme is stimulated by Mg^{2+}, Triton X-100, and organic solvents (particularly methanol) [98]. *Bacillus cereus* C7 produces lipase, which preserves its activity when in combination with commercial detergents, hydrogen peroxide, sodium hypochlorite, and trypsin [100]. *Bacillus* sp. DH4, *Bacillus* sp. RSJ-1 lipases show significant tolerance to surfactants and laundry detergents. *B. sphaericus* 205y lipase retains high activity with proteolysis [92].

3.4. Cellulases from Bacillus Species

Cellulases belong to the glycoside hydrolase family that hydrolyse β-1,4-glycoside linkages of cellulose polymers. Cellulases are a complex system with three single enzymes: endoglucanases (E.C. 3.2.1.4), exoglucanases (E.C. 3.2.1.91), and β-glucosidase (E.C. 3.2.1.21). While endoglucanases randomly cleave internal bonds in amorphous cellulose to generate new shorter chain ends, exoglucanases cleave the non-reducing and reducing ends of cellulose to produce cellobiose as major products, and β-glucosidase hydrolyzes the cellobiose into glucose [59]. Current detergent preparations usually include a cellulose cocktail. In laundry, they protect color and maintain the fabric's smoothness [101]. In contrast to other enzymes in detergent formulations, cellulase does not react directly to soils on the fabric's surfaces. Instead, it reacts with cellulose chains in the amorphous region of the fibers. The effect is not only to remove stains but also to eliminate the microfibrils

and fuzzes of well-worn cotton clothing, which gives rise to the fabric of a grayish or dull appearance [102].

Although fungal cellulases have been studied extensively, they are either acid or neutral enzymes and cannot be compatible with the detergent's alkaline medium. Thus, bacteria have been considered in manufacturing because they are an abundant source of alkaline enzymes [101]. Among microbes, the *Bacillus* genus is the most promising candidate. Cellulases from *Bacillus* sp. are usually alkalophilic and generally compatible with other detergent ingredients [16,101]. In 1987, Kao developed the first detergent cellulase produced by *Bacillus* sp., which demonstrated significantly improved washing performance. Since 1998, Genecor International has marketed an endo-cellulase product named Puradax that is extracted from alkaliphilic *Bacillus* and used in detergents [15].

Bacillus cellulases share common characteristics that confer tolerance to specific conditions of detergents. In 1988, Ito et al. [102] studied the enzymatic properties and genetics of *Bacillus* sp. KSM-635 and extended the use of this species to heavy detergents. This was the first application of *Bacillus* cellulase in a detergent. *Bacillus* cellulases usually remain stable at a pH of 8–10, a broad range of temperatures from 40 to 120 °C, and are slightly inhibited by EDTA [102]. Recent studies have demonstrated the considerable properties of *Bacillus* sp. SMIA-2 has made it another potential strain for use. This bacterium can simultaneously produce an array of enzymes, including protease, amylase, and cellulase. Thus, cellulase from this strain could withstand proteases. Cellulase from *Bacillus* sp. SMIA-2 was stable in SDS, non-ionic surfactant, and RENEX 95, Ultra Biz® detergents, but not in Triton X-100, H_2O_2, and Ariel® [103]. In contrast, cellulase produced by *Bacillus licheniformis* AMF-07 maintained its activity in the presence of Triton X-100 but was inhibited by H_2O_2. In terms of interactions with commercial detergents, the enzyme was stimulated by Dioxigene (122%), Shooma (116%), and slightly inhibited by Barf (90%), Kaf (85%), Taj, and Darya (33%) [101].

4. Recent Innovations in Harnessing *Bacillus* Species in Bio-Based Detergents

4.1. Affordable Green Detergents via Low-Cost Substrate Utilization

Despite the growing demand for enzymes and biosurfactants, the large-scale production of these organic molecules continues to pose a challenge in terms of process economics [77]. A careful design is required to narrow the gap between the necessary financial investment and industrial production because profit is always of significant concern when developing at an industrial scale [104]. Various options have been considered to reduce the price of fermentation operations, and in many cases, raw materials account for the majority of the production costs of industrial enzymes [16]. The same difficulty has been witnessed in the production of microbial biosurfactants, which increased the price of microbial biosurfactants up to USD 34 per kilogram compared to USD 1–4 per kilogram of the average price of synthetic surfactants and decelerated the growth rate of the biosurfactant market. The substrates in fermentation necessary to generate biosurfactants occupy more than 50% of the total cost [7]. One practical option is to use agro-industrial residues and by-products as media for fermentation [105]. Using these low-cost substrates also offers a variety of additional benefits, such as minimizing pollution, increasing the availability of a diverse spectrum of substrates, and being nontoxic to microorganisms. The volume of organic waste generated surges in parallel to the mounting consumption of the global population, which leads to a heavy burden on the environment and financial responsibility to waste management, unless valorization approaches can be established to circularize these residues. A total of 13×10^9 tonnes of organic by-products are estimated to be produced annually, which means that these residues represent a promising and abundant resource

for the valorization in high-value-added by-products while mitigating the environmental problems [106]. Numerous studies highlight the potential of *Bacillus* sp. fermentation using circular substrates as cost-effective alternatives. *Bacillus* sp. is a well-known industrial microorganism used in recycling diverse agricultural and agro-industrial wastes, namely molasses, cassava waste, orange peel, corn steep liquor, sugarcane bagasse, tomato waste proteins, waste sunflower oil, etc., into value-added products [41,107]. However, compared to conventional media, these waste-derived substrates often suffer from imbalanced nutrient composition, logistics, feedstock inconsistency, and feedstock availability, which must be carefully managed to achieve optimal productivity [28]. Therefore, selecting a suitable agro-industrial waste or residue for biosurfactant or enzyme production requires considering factors such as raw material availability, transportation costs, minimal or no pretreatment requirements, and the avoidance of refined feedstock supplementation [33].

Beyond substrate selection, fermentation strategies are chosen based on the substrate's nature, with solid-state fermentation (SSF) and submerged fermentation (SmF) being widely employed. While SSF is particularly efficient for fungal and yeast fermentation, it has also shown promise in certain *Bacillus* strains. For instance, *Bacillus pumilus* UFPEDA 448 yielded a higher concentration of lipopeptides under SSF when cultivated on an okara-based medium supplemented with sugarcane [108]. Similarly, the thermophilic bacterium *Bacillus* sp. BBXS-2 successfully fermented nonsterile open wheat straw as a substrate, co-producing protease and amylase, offering a cost-effective approach for detergent applications [109]. Despite SSF's advantages—such as high volumetric productivity, higher product concentrations, reduced effluent generation, and simpler fermentation equipment—its industrial scalability is hindered by challenges in downstream processing, limited oxygen transfer, difficulties in scaling up, heterogeneous substrate composition, and moisture control issues, necessitating a case-specific approach. Conversely, SmF, though requiring capital-intensive fermentation infrastructure, enables homogeneous nutrient distribution and precise control over key cultivation parameters such as temperature, pH, and dissolved oxygen [110]. For example, *Bacillus subtilis* LB1a and LB5a produced biosurfactant, protease, and amylases more effectively using cassava wastewater than with a synthetic medium. Interestingly, during bioreactor operation, the frequent occurrence of foam was identified as a contributing factor in protease recovery, as higher values of enzyme were found in foam, suggesting a simple and viable downstream process. However, as reported in the study, the enzyme yields in 3 L bioreactors were lower than those observed in flasks for both protease and amylase [111]. Thus, developing a successful large-scale fermentation process requires not only the advantages of controllable fermenter systems but also the optimization of key parameters, which play a crucial role in overcoming challenges posed by wastewater containing unwanted substances. In an experiment utilizing wheat bran and groundnut oil cake as feedstock in 600 mL and 5 L bioreactors, *Bacillus amyloliquefaciens* exhibited a linear increase in amylase production with higher aeration, agitation, and biomass levels, indicating a growth-dependent pattern of α-amylase production. These findings, supported by Syu and Chen as well as El-Tayeb, highlight the significant influence of physical and biological factors in enhancing amylase yields [112].

To date, numerous studies have investigated the use of agro-industrial residues and wastewater for the production of enzymes and biosurfactants, primarily evaluating their feasibility at the laboratory and pilot scales. These efforts have provided valuable insights into substrate characteristics, microbial compatibility, and product potential for integration into detergent formulations. However, scaling up from pilot to industrial production remains challenging and requires further technological innovation, comprehensive economic assessments, and alignment with regulatory standards to ensure process stability, consistent product quality, and commercial viability. As summarized in Table 3, the detergent indus-

try emerges as a particularly promising application area for these sustainable bio-based ingredients obtained through residual fermentation.

Table 3. Fermentation using different types of waste by *Bacillus* sp.

Types of Waste	Products	Strains	Types of Fermentation	Remarks	References
Wheat bran and rice husk as a carbon source	α-amylase	*Bacillus subtilis*	Solid-state fermentation	*B. subtilis*, isolated from hot springs. 7.3-fold higher enzyme production in wheat bran compared to rice husk	[113]
Wheat bran	α-amylase	*Bacillus cereus* MTCC 1305	Solid-state fermentation	Highest enzyme production was observed with wheat bran (94 ± 2 U/g) after 72 h	[114]
Potato starch waste as the sole carbon source	α-, β-, γ-amylase	*Bacillus amyloliquefaciens*	Shaking flasks	Using the medium containing 2% potato starch waste in shaking flasks (150 rpm) at 50 °C produced the maximum α and β-amylase after 30 h, γ-amylase after 36 h	[115]
Rice bran as a carbon source	Cellulase	*Bacillus carboniphilus* CAS 3	Shaking flasks	At initial pH 9.0, and temperature 50 °C, obtained 4040.4 U/mL of cellulose activity	[116]
Lignocellulosic wastes	Cellulase	*Bacillus halodurans* CAS 1	Shaking flasks	With an optimum pH, temperature of 9.0 and 60 °C, an extracellular halotolerant, thermoalkaline cellulase was produced	[117]
Wheat bran and lentil husk as a carbon source	Alkaline protease	*Bacillus* sp.	Solid-state fermentation	Greatest yields of 429.04 and 168.64 U/g were achieved in 0.1 M carbonate/bicarbonate buffer at pH 10	[118]
Cotton seed cake as a nitrogen source	Alkaline protease	*B. cereus* NS-2	Shaking flasks	Wheat bran supported maximal fibrinolytic protease production (148 U/mL), cotton cake enhanced the fibrinolytic protease production to 315 U/mL, and *Bacillus* protease has the ability to remove blood stains.	[119]
Waste cooking oil	Lipase	*Bacillus subtilis*	Shaking flasks	The optimal lipolytic activity was 4.96 U/mL in 84 h of fermentation	[120]
Wheat bran, banana waste, melon waste, watermelon waste, lentil husk, and rice husk as carbon sources	Lipase	*B. coagulans*	Solid-state fermentation	Melon waste supplemented with 1% olive oil was found to be the best substrate for lipase production (78.069 U/g)	[121]
Chicken feather peptone (CFP) as a nitrogen source	Lipase and amylase	*Bacillus licheniformis* 016	Shaking flasks	The optimum concentration of CFP for lipase and amylase production was determined as 5 and 6 g/L, respectively	[122]
Chicken feathers as a complex substrate of carbon and nitrogen source	Alkaline proteases and thermostable amylases	*Bacillus licheniformis* NH1	Shaking flasks	Potential application as a detergent additive	[72]
Industrial waste (feather meal, potato peel and rape seed cake)	Keratinolytic protease, amylase, and biosurfactant	*Bacillus subtilis* PF1	Shaking flasks	An overall 2.3% increase in proteases, 0.85% increase in amylase production, and 1.2% increase in biosurfactant production were achieved with optimized media.	[41]
Corn steep liquor	Biosurfactant	*Bacillus subtilis*	Shaking flasks	10% (*v*/*v*) of Corn steep liquor, with a biosurfactant production of about 1.3 g/L	[123]
Soybean oil waste	Biosurfactant (lipoprotein)	*Bacillus pseudomycoides* BS6	Liquid culture	1.2 g crude biosurfactant was extracted from 1000 mL culture broth	[26]
Cassava wastewater as an unconventional carbon source	Biosurfactant	*Bacillus subtilis* LB5a	40 L Bioreactor	An average of 25.7 g of surfactant was recovered per batch (0.68 g of surfactant/L of cassava wastewater	[124]

Table 3. *Cont.*

Types of Waste	Products	Strains	Types of Fermentation	Remarks	References
Wheat straw	Protease and amylase	*Bacillus* sp. *BBXS-2*	Solid-state fermentation	12,200 U/g and 6900 U/g dry matter for protease and amylase, respectively, after a 5-day fermentation at 45 °C, initial pH of 8.5, nonsterile open fermentation	[109]
Soybean flour and rice straw	Biosurfactant	*Bacillus amyloliquefaciens* XZ-173	Solid-state fermentation	A surfactin yield of 15.03 mg/gram dry substrate was attained in a 1000-fold scale-up fermentation in a 50 L fermenter	[125]

4.2. Energy-Saving Detergents with Cold-Adapted Microbes

Cold-active enzymes have drawn a lot of attention from the detergent industry because their unique properties allow greater energy conservation [126]. In the past, wash performance relied heavily on the level of mechanical agitation in combination with water temperature. Hot water, potentially up to 95 °C, used to be preferred for washing clothes. This caused an array of problems, such as high energy consumption and wear and tear on fabrics [30,99]. The introduction of enzyme-based detergent formulations redefined the optimal washing temperature, shifting it to a milder range of 30–60 °C—lower than traditional, heat-dependent standards. This shift stems from the fact that enzyme activity decreases markedly at higher temperatures. Nevertheless, at these reduced temperatures, effective cleaning is still achieved through the catalytic efficiency of enzymes, even without substantial thermal input [30]. The common conditions for detergent applications are an alkaline pH and a low temperature [79], and the percentage of global cold-water washing machine loads rose from 38 to 53% from 2010 to 2014 [78]. This has been achievable because of the development of cold enzymes detergent applications that are active at alkaline pH ranges with broad thermostability (5–60 °C). An array of cold-active enzymes, including protease, lipase, cellulase, amylase, mannanase, and pectate lyase, has already been incorporated into various commercial detergent formulations by leading companies such as Novozymes, DuPont, Genencor, and Jupiter. This widespread adoption underscores the undeniable industrial appeal and functional value of cold-active enzymes in enhancing detergent performance under energy-saving, low-temperature washing conditions [79].

Cold enzymes are mainly produced by psychrophilic microorganisms (*Archaea*, *Bacteria*, and *Eukarya*) [127]. In addition to the many different psychrophilic bacteria, there are numerous studies examining *Bacillus* species. *Bacillus subtilis* ITRCGG-3, which can produce cold-active proteases, was isolated from the Gangotri Glacier in the western Himalayas, where the temperature ranges from 2 to 5 °C in summer to below freezing in winter. The partial protease expressed by ITRCGG-3 exhibited unusual stability in the presence of SDS as a typical surfactant in detergent formulations, and its activity was even increased by Tween 80 and commercial detergents like Wheel. Furthermore, this enzyme demonstrated stability between 10 and 30 °C, pH from 9 to 11, with optimal activity at 20 °C and pH 10 [128]. A pure cold-active protease isolated from *Bacillus subtilis* WLCP1 was found to be active at pH 10 and stable at pH 7–11; its highest activity was recorded at pH 10 and 15 °C. The enzyme was also excellent at removing blood stains from fabrics [129]. A cold-active amylase from *Bacillus cereus* GA6 was active in a wide range of temperatures from 4 to 37 °C as well as the pH of 7–11, and showed maximum activity at 22 °C, pH 9. The enzyme displayed considerable potential against urea, SDS, and EDTA, and compatibility with commercial laundry detergents [130]. *Bacillus subtilis* N8 was observed for its ability to produce a cold-active, alkaline, detergent-stable α-amylase. The enzyme's optimal temperature and pH were 25 °C and 8.0, respectively. This enzyme also resisted some chemical denaturants in the detergent industry, namely SDS, EDTA, Triton X-100, and

urea [131]. Psychrophilic *Bacillus sphaericus* MTCC 7526 produced a cold-active lipase that was preferable for use in detergents. Its optimal activity was at 15 °C and pH 8.0, and its activity remained significant in the presence of acetone, DMSO, and EDTA [132]. *Bacillus* sp. strain SY-7 was isolated from the sewage of an oil-producing cold-active lipase. This lipase showed good activity at pH 4.0–10.0 and 5–50 °C, with optimal activity at pH 8.0 and a temperature of 20 °C. Moreover, the enzyme was measured for other properties pertinent to the laundry industry, such as maintaining its activity despite the presence of denaturants and commercial detergents [133].

Compared to cold enzymes, which represent a pioneering research field with some already commercialized, the term "cold-active biosurfactants" has only recently emerged in scientific literature (Table 4). This interest has been driven by the Low-Temperature Washing Campaign launched in 2013, yet research in this field remains limited [134]. While this initiative offers significant environmental benefits, particularly in the context of the ongoing energy crisis, maintaining washing efficiency at low temperatures remains a challenge. Conventional surfactants are generally less effective in cleaning at lower temperatures. Below the Krafft temperature, some surfactants crystallize, resulting in the loss of essential surface activities such as dispersion, emulsification, and critical micelle formation [135].

Cold-active biosurfactants, however, function effectively at low temperatures and can also be produced without requiring heating [134]. This valuable property aligns well with the push for low-temperature washing practices to conserve energy, offering a promising alternative to conventional surfactants. These biosurfactants originate from extreme cold environments, where microorganisms have adapted to thrive in freezing conditions. These cold-adapted microbes are capable of producing biosurfactants with low Krafft temperatures —the minimum temperature at which surfactants can form micelles-making them suitable for diverse applications, particularly in cold and harsh environments.

Despite the growing interest, studies on biosurfactant-producing psychrophiles remain limited. Research has focused on Antarctic environments [136], cold soils, sand, lake in polar regions and high altitudes [137–140], cold marine environments in Atlantic Canada [141], old seeps in the deep sea of South China [142], and cold seep sediments [143], etc. These findings indicate that cold-adapted microorganisms belong to several genera, including *Bacillus*, *Pseudomonas*, *Burkholderia*, *Sphingomonas*, *Vibrio*, *Rhodococcus*, *Alcanivorax*, *Exiguobacterium*, *Halomonas*, *Acinetobacter*, *Streptomyces*, *Janthinobacterium*, *Psychrobacter*, and *Serratia* [136,143–145].

A major drawback that must be considered is that psychrophilic microorganisms generally exhibit slow growth, making them less ideal for large-scale industrial production. This challenge is further exacerbated by their lower biosurfactant yields, doubling the difficulty of commercializing these compounds. However, promising developments have emerged from studies on certain Antarctic isolates, which have demonstrated the ability to produce biosurfactants even at 4 °C using crude oil as their sole carbon source [136]. With the increasing interest in microbial biosurfactants and their potential applications across multiple sectors, including bioremediation, gas hydrate technologies, and green detergents, further research and development efforts are expected to address these challenges [134].

Table 4. Cold-active enzymes and biosurfactants from *Bacillus* strains for detergent applications.

Bacillus Species	Culture Medium/Conditions	Growth Temp and Time	Enzyme/Biosurfactant	Enzyme Activity Characteristics	Scale	References
Bacillus sp. S1DI 10 (Himalayan Spring isolate)	Glucose–casein–peptone + salts; pH 7	20 °C; ~48 h	Cold-active metallo-protease	Optimum: 10 °C, pH 8; stable with 2% SDS and Tween-80;	Lab scale	[146]
Bacillus subtilis N8 (Turkey, alkaline soil)	Starch-based alkaline medium; 40 g/L glucose	15–25 °C; ~48 h	Cold-active α-amylase	Optimum: 25 °C, pH 8; stable pH 8–12 and 10–40 °C; resists SDS, EDTA, Triton X-100, urea	Lab scale	[131]
Bacillus cereus GA6 (Himalayan glacier)	Glycerol + ammonium acetate; pH~10	20 °C; 96 h	Cold-active α-amylase	Optimum: 22 °C, pH 9; active 4–37 °C, pH 7–11; stable with SDS, EDTA, urea; active in detergents	Lab scale	[130]
Bacillus sp. SY-7 (oil-mill sewage)	Tributyrin and olive oil broth	20 °C; 72 h	Cold-active lipase	Active 5–50 °C, pH 4–10; optimum at 20 °C, pH 8; stable in 5% SDS, detergents, metal ions	Lab scale	[133]
Bacillus subtilis SPB1 (Tunisian soil isolate)	Glucose, urea, NH_4Cl, 2% kerosene; DO control	30 °C; 48–72 h	Biosurfactant (surfactin)	Stable pH 2–9; 70 °C/1 h retention; improves detergent stain removal by 33–45%	Pilot (2.6 L bioreactor)	[34,147]

4.3. Advanced Specialty Detergents Through Protein Engineering

Thanks to advances in molecular biotechnology, many challenges in the detergent industry can now be addressed through protein engineering, in addition to genetic engineering (Table 5) [79]. The most effective strategy for producing biologically relevant molecules involves manipulating the genes encoding these molecules, coupled with approaches such as directed evolution, semi-rational design, or rational design. Among these, directed evolution is regarded as a promising platform for rapidly generating enzymes with novel properties. By leveraging random DNA manipulation, this method produces a vast pool of mutated proteins [148]. The significance of this approach was recognized by the scientific community with the awarding of the 2018 Nobel Prize in Chemistry [149].

Protein engineering has been successfully applied to *Bacillus* spp., addressing the requirement of specific criteria—thermostability, alkaline pH tolerance, and resistance to chemical oxidizing agents—for enzymes intended for use in laundry detergent formulations. Wild-type enzymes often fail to retain their functional properties under the harsh processing conditions typically encountered in such applications.

To enhance thermostability and broaden enzyme activity across a range of temperatures, a single round of random mutagenesis followed by recombination of improved variants was conducted on a mesophilic subtilisin-like protease from *Bacillus sphaericus*. This modification resulted in a 6.6-fold increase in the catalytic rate constant (*kcat*) at 10 °C and a 9.6-fold improvement in catalytic efficiency (*kcat*/*KM*) compared to the wild-type enzyme [150]. A psychrophilic enzyme named TA39 subtilisin (S39) was converted into the mesophilic subtilisin, savinase from Antarctic *Bacillus lentus* (clausii). The hybrid enzyme displayed its highest activity at 55 °C and catalyzed a wider substrate profile and showed a higher specificity toward synthetic substrates [151]. *B. gibsonii* alkaline protease (BgAP) was modified by a directed evolution campaign toward lower temperatures. After using three iterative rounds of Sequence Saturation Mutagenesis to broaden activity, one hybrid variant, MF1, was created. This variant showed greater activity at 15 °C and 100 times superior thermal resistance at 60 °C [152].

With respect to pH-dependent activity, *B. gibsonii* alkaline proteases (BgAP), which had an optimal pH of 11, underwent a post-translational autocatalytic deamidation process substituting positively charged asparagine and glutamine residues with negatively charged aspartic acid and glutamic acid, respectively. This led to a twofold increase in pH-dependent activity at pH 8.6 [153].

Bacillus subtilis DB104 was also successfully exploited as a host for a variant protease that could mediate the production of oxidative agents, such as peroxycarboxylic acid, as a side catalytic reaction [154]. The recombination of three variants at position Gly165 exhibited an effective redirection from proteolysis using a standard suc-AAPF-pNA substrate to perhydrolysis of methyl-propionate, methyl-butyrate, and methyl-pentanoate as substrates when expressed in *Bacillus subtilis* DB104 [154]. Methionine 197, located close to the active site of *B. licheniformis* amylase, was replaced with a non-sulfur-containing amino acid, which resulted in the improvement of oxidation stability and better performance in the presence of bleach. The type of mutant amylase has been employed by Glencore International and Novozyme according to two commercial products, Purafect OxAm® and Duramyl®, respectively [155,156].

Table 5. The application of protein engineering in the detergent industry.

Strategies	Targeted Improvement	Results	References
Directed Evolution	Thermostability and substrate specificity	• Random mutagenesis on *Bacillus sphaericus* protease: increased kcat (6.6-fold at 10 °C) and kcat/KM (9.6-fold) • MF1 variant of *B. gibsonii* alkaline protease (BgAP): higher activity at 15 °C, 100-fold improved thermal resistance at 60 °C	[150,152]
Post-translational Modification	pH-dependent activity enhancement	• Deamidation of BgAP: two-fold increase in enzymatic activity at pH 8.6	[153]
Recombination/Site-directed Mutagenesis	Oxidation stability	• Recombination at Gly165 in *B. subtilis* DB104: peroxycarboxylic acid production • Methionine 197 substitution in *B. licheniformis* amylase: improved bleach resistance	[154–156]
Semi-rational/Rational Design	Mentioned as complementary to directed evolution	• Complementary strategies to refine enzyme properties	[148]

4.4. Smart Detergents for Precision Stain Removal with CRISPR

The pursuit of high-efficiency detergents remains an enduring research objective, with significant advancements in the innovation and industrial integration of bio-based ingredients. The powerful gene-editing technology known as CRISPR has enabled the precise engineering of eco-friendly molecules such as enzymes and biosurfactants, paving the way for more sustainable, effective, and environmentally friendly detergent formulations [19,157]. Notably, CRISPR-based systems derived from the Cas9 protein—such as the gene-editing CRISPR-Cas9 system and the gene-regulation platforms CRISPR activation (CRISPRa) and CRISPR interference (CRISPRi)—offer precise, versatile, and efficient approaches for enhancing microbial strains used in detergent production [19,158].

Unlike traditional genetic modification, which often depends on random mutations, CRISPR-Cas9 gene editing enables precise and efficient alterations at specific genomic sites. This technology harnesses the Cas9 enzyme system from the natural immune response of bacteria and manipulates it to function as molecular scissors that cut DNA at a specific location, guided by a single guide RNA (gRNA). Once the DNA is cut, the cell's natural repair mechanisms are triggered, facilitating simultaneously the insertion or deletion of genetic material or the replacement of faulty genes with new ones, thus enabling targeted and precise modifications [159,160]. Moreover, a modified version of this system, CRISPR-dCas9, has been repurposed for transcriptional regulation rather than gene editing. This system consists of three core components: a catalytically inactive Cas9 (dCas9) protein, a programmable single guide RNA (sgRNA) that targets promoter regions, and a transcriptional effector—either an activator (CRISPRa) or a repressor (CRISPRi). When the dCas9-sgRNA-effector complex binds to the promoter region of a target gene, it can inhibit transcription by blocking RNA polymerase binding or elongation (in the case of CRISPRi) or enhance transcription (in the case of CRISPRa) [19,158]. These systems thus allow for precise modulation of gene expression levels, offering powerful tools for the rational engineering of microbial strains tailored for smart detergent applications.

The application range of the CRISPR-based system is virtually limitless, encompassing fields such as medicine, agriculture, and industrial biotechnology, including the creation of genetically engineered organisms like bacteria, plants, and animals.

There are several strategies using this tool anticipated to be applied in the detergent industry. CRISPR array sequencing enables bacterial strain genotyping and detects past virome infections, aiding CRISPR-Cas vaccine development to prevent fermentation failures [161], which frequently cause economic losses in industrial-scale enzyme or biosurfactant production. A particularly noteworthy advantage of CRISPR-based genetic tools, especially relevant to the detergent industry, is their ability to simultaneously target and regulate multiple genes using different single guide (sg) RNAs. This enables the concurrent production of multiple targeted products, as demonstrated by CRISPRi-optimized metabolic flows in *C. glutamicum*, which facilitated the first efficient L-lysine and squalene co-synthesis by regulating pyruvate metabolism. This strategy also serves as a reference for synergistic amino acid and terpene production [162].

Additionally, by enabling expression level control of any genes of interest without altering the genomic sequence [161], CRISPR is able to control the proportion of individual enzymes in the mixture for optimization. This, in turn, enhances compatibility in detergent formulations and provides an effective solution for reducing enzyme production costs. Such versatility aligns seamlessly with the portfolios of start-up manufacturers, offering opportunities for cost savings and increased operational efficiency, like India, China [162,163]. Fehler, Kallehauge [164] demonstrated that CRISPR-dCas9 boosts α-amylase production in *B. subtilis* by 2–3-fold through the knockdown of flagellar-associated genes, with potential to further enhance enzyme production, offering promising applications in biotechnology.

Yet, harnessing CRISPR-based technologies with *Bacillus* species for green detergent production remains a relatively novel application of this technology, as it has so far been used for genome editing/regulation in only a limited number of bacterial species, particularly those with challenging transformation or recombination processes. Among the *Bacillus* species, *B. subtilis*, *B. licheniformis*, *B. megaterium,* and *B. cereus* represent a few examples that have been targeted, but for purposes other than detergent applications [19,165,166]. One rare finding by Price, Cruz [167] demonstrated the use of CRISPR-Cas9 to enhance *Bacillus subtilis* for industrial enzyme production, focusing on improving subtilisin E, a key detergent protease. In this case, the CRISPR system was first validated by knocking out the *amyE* gene, which encodes α-amylase, demonstrating successful gene editing. Next, they applied in situ modification to the *aprE* gene, which produces subtilisin E, aiming to enhance its thermostability and pH tolerance. Since the wild-type enzyme is vulnerable to detergent formulations and heat, they introduced a salt-bridge triad (Arg19-Glu271-Arg275) found in *Bacillus clausii* M-protease, known for its heat resistance. Using CRISPR-Cas9, they replaced specific residues (Gln125-Gln377-Gln381) in subtilisin E to form this stabilizing salt bridge, then tested the modified enzyme's thermostability and activity. Thus, future perspectives highlight the potential of CRISPR-based genetic tools as a simple, rapid, and effective approach for engineering to achieve sequence-specific genome editing in *Bacillus* species, tailored specifically for the detergent industry (Figure 3).

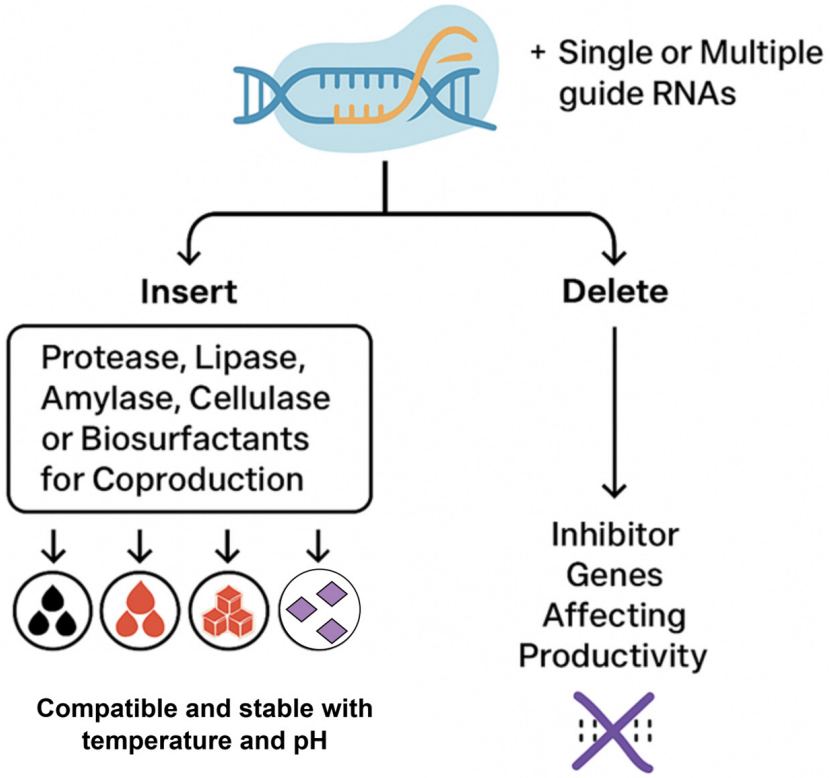


Figure 3. Potential applications of CRISPR-based genetic technologies in the detergent industry.

5. Conclusions

The increasing demand for environmentally friendly detergents has underscored the need for sustainable and efficient alternatives to conventional chemical formulations. *Bacillus* species have solidified their position as industrial microbial workhorses, demonstrating exceptional potential in enzyme and biosurfactant production for bio-based detergents. Their ability to generate a diverse array of extracellular enzymes, including proteases, α-amylase, lipases, and cellulases, and biosurfactants such as lipopeptides underscores their versatility and resilience under extreme conditions. These attributes not only enhance washing performance but also provide viable substitutes for synthetic surfactants with high toxicity. Although cost and efficiency remain significant challenges, continued research into innovative *Bacillus* strains and advanced biotechnological strategies will be essential for optimizing production and enhancing efficiency. Key approaches include optimizing fermentation processes with low-cost substrates and developing desirable "green chemicals" with novel characteristics and higher yields through gene-editing techniques, protein engineering, and the discovery of new candidates with unique features, such as cold-active biosurfactants and enzymes. While the widespread adoption of next-generation green detergents remains far off, advancements in microbial biotechnology, particularly within *Bacillus* species, will pave the way for more sustainable, high-performance detergent formulations, ultimately contributing to global environmental sustainability.

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