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# *Geobacillus* Bacteria: Potential Commercial Applications in Industry, Bioremediation, and Bioenergy Production

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## Abstract

The genus *Geobacillus* is represented by obligately thermophilic bacteria able to grow in the temperature range of 35–75°C. They are modest bacteria isolated from various sources on routine media such as nutrient agar. Originally classified as representatives of *Bacillus*, the species of *Geobacillus* were established in 2001 as a new genus. However, sequence similarity between all species indicates that at least some species need to be reclassified at the genus level. In addition to 16S rRNA, housekeeping genes, 16S-23S rRNA gene internal transcribed spacer, and repetitive sequences can be used in classification and identification of thermophilic bacteria. The ability to survive and grow at high temperatures as well as utilization and synthesis of a wide range of compounds makes these bacteria and their products attractive for use in various spheres: food, paper, biotechnology industries, medicine, bioremediation, etc. A broad spectrum of applications arouses increased interest in the study of physiological and biochemical characteristics and triggers emergence of new usage areas for *Geobacillus*, such as bioenergy. The growing demand for energy leads to the development of alternative technologic options. *Geobacillus* species demonstrated the ability to generate or enhance productivity of important sources of bioenergy such as ethanol, isobutanol, 2,3-butanediol, biodiesel, and biogas.

**Keywords:** *Geobacillus* bacteria, biotechnology industry, production of thermostable enzymes, food enzyme applications, bioenergy, biogas, technologies, energy efficiency

## 1. Introduction

The *Geobacillus* species are Gram-positive, aerobic or facultatively anaerobic, spore-forming, rod-shaped cells with the temperature range for growth 35–75°C (optimum at 55–65°C) (**Figure 1**). Neutrophilic bacteria multiply at pH 6.0–8.5, with optimal pH values 6.2–7.5. Most species are modest bacteria able to develop without growth factors or vitamins and to utilize n-alkanes as carbon and energy sources [1].

As obligate thermophiles, *Geobacillus* might have been expected to be found only in the warmest regions of the planet, such as equatorial deserts or naturally



**Figure 1.** Cell morphology of *Geobacillus stearothermophilus* (basonym *Bacillus stearothermophilus*) BIM B-202 from Belarusian collection of nonpathogenic microorganisms. Legend: phase-contrast microscopy, magnification 1000 $\times$ , 16 h cultivation;  $\rightarrow$  – spores.

occurring geothermal and hydrothermal springs. However, *Geobacillus* can be isolated in large numbers anywhere, even from cool soils and permanently cold ocean sediments. It was shown that these bacteria are also minor opportunistic decomposers of plant-derived organic matter, capable of rapid growth under transient thermophilic conditions, but endowed with mechanisms to survive extended time spans when growth is impossible. Nevertheless, *Geobacillus* species play only a modest role in large microbial communities. High populations of bacteria have gradually accumulated in long-term perspective due to spore formation. Adaptive features of *Geobacillus* spores ensure their mobilization in the atmosphere and transport over long distances. Their spores are suggested to remain viable for long periods of time [2]. Spores of *Bacillus* species related to geobacilli have shown resistance to heat, radiation, and chemicals [3].

Growth at high temperatures makes *Geobacillus* species promising agents in biotechnological processes. They can be sources of various thermostable enzymes, such as proteases, amylases, lipases, and pullulanases. *Geobacillus* species can also generate exopolysaccharides and bacteriocins and take part in production of biofuel and bioremediation. New applications are constantly emerging for this group of thermophilic bacteria.

## 2. Cultivation of *Geobacillus* bacteria

*Geobacillus* species are obligately thermophilic chemoorganotrophs. Temperature ranges for growth generally lie between 37 and 75°C, with optima between 55 and 65°C. They are neutrophilic bacteria growing within pH range 6.0–8.5 at optimal values 6.2–7.5. Growth factors, vitamins, NaCl, and KCl are not required; so that, most strains will grow on routine media such as nutrient agar. A wide range of substrates is utilized, including carbohydrates, organic acids, peptone, tryptone, and yeast extract. The ability to utilize hydrocarbons as carbon and energy sources is a widely distributed property in the genus [1].

Thermophiles may be obtained easily by incubating environmental or other samples in conventional cultivation media at high temperatures. A selective method for the isolation of thermophilic flat sour organisms from food was described in 1963 by Shapton and Hindes. The method used yeast-glucose-tryptone agar containing peptone (5 g), beef extract (3 g), tryptone (2.5 g), yeast extract (1 g), and glucose (1 g) diluted in distilled water (1000 ml) [4]. This procedure was used for the isolation of *Geobacillus stearothermophilus* [1]. A prototrophic strain of *G. stearothermophilus* has been shown to grow in the medium containing only glucose and mineral salts, while auxotrophic strains additionally required biotin, thiamine,

nicotinic acid, and DL-methionine. The presence of L-leucine in minimal medium necessitated the addition of L-valine; however, growth occurred in the absence of both amino acids [5]. The use of medium containing 0.5% beef extract, 0.9% soy peptone, 0.2% NaCl, 0.1% K<sub>2</sub>HPO<sub>4</sub>, and 0.075% KH<sub>2</sub>PO<sub>4</sub> resulted in 10 times higher biomass production by *G. stearothermophilus* than the application of the standard fermentation medium [6].

*Geobacillus caldodoxylosilyticus* was isolated from soil by adding 0.1–0.2 g sample to the minimal medium and incubating at 65°C for up to 24 h. After two transfers of 1 ml culture into the fresh medium, enrichments were plated on solidified minimal medium and incubated at 65°C for 24 h [7]. Further isolations were made by heating samples at 90°C for 10 min, plating on CESP agar (casitone, 15 g; yeast extract, 5 g; soytone, 3 g; peptone, 2 g; MgSO<sub>4</sub>, 0.015 g; FeCl<sub>3</sub>, 0.007 g; MnCl<sub>2</sub>·4H<sub>2</sub>O, 0.002 g; water, 1000 ml; pH, 7.2.) and incubating at 65°C for 24 h [8]. The strain *G. caldodoxylosilyticus* UTM6 demonstrated ability to reduce toxic chromium (VI) to nonharmful chromium (III). It was found to grow optimally in nutrient broth medium supplemented with 250 ppm of glucose at 55°C and pH 6.5, with the highest OD<sub>600</sub> reading of 0.910 [9].

*Geobacillus gargensis* was isolated from the upper layer of a microbial mat of Garga hot spring by serial dilutions and inoculation onto the agar medium supplemented with 15 mM sucrose: TES [N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid], 10 g; NH<sub>4</sub>Cl, 1 g; NaCl, 0.8 g; MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.2 g; CaCO<sub>3</sub> (precipitated chalk), 0.2 g; KCl, 0.1 g; K<sub>2</sub>HPO<sub>4</sub>, 0.1 g; CaCl<sub>2</sub>·2H<sub>2</sub>O, 0.02 g; yeast extract, 0.2 g; trace metal solution, 5 ml; vitamin solution, 10 ml; water to 1000 ml, pH, 7.0 [10, 11].

*Geobacillus kaustophilus* was isolated from uncooled pasteurized milk by plating on peptonized milk agar, followed by subculturing on the same medium or on nutrient agar supplemented with 1% yeast extract, 0.25% tryptophan broth, and 0.05% glucose [1]. Strains of *Geobacillus kaustophilus* are capable to grow optimally on rich media, including tryptic soy broth and Luria broth. *G. kaustophilus* A1 grown on minimal defined medium at 55°C and pH 6.5 demonstrated the ability to utilize maltose, glucose, sucrose, glycerol, fructose, galactose, citric acid, acetic acid, pyruvic acid, lactic acid, or succinic acid. Cells did not utilize dodecane, m-xylene, cellulose, oxalic acid, tartaric acid, maleic acid, propanoic acid, benzoic acid, or picolinic acid as the sole carbon sources. *G. kaustophilus* DSM7263 displayed similar characteristics, but it did not metabolize citric acid [12].

*Geobacillus thermoleovorans* was isolated by adding soil, mud, and water samples to L-salts basal medium supplemented with 0.1% n-heptadecane and incubated at 60°C for 1–2 weeks, followed by transfer from turbid cultures to fresh medium of the same composition. After several such transfers, pure cultures were obtained by streaking on plates with L-salts basal medium supplemented with 0.2% n-heptadecane and solidified with 2% agar [13, 14]. Strain *G. thermoleovorans* T80 displayed extremely specific glucose utilization leading to high growth rates, followed by extensive cell death and lysis with the onset of substrate exhaustion. The addition of extra carbon substrate did not halt the rapid death and lysis. Lytic phenomenon was observed for a range of different carbon substrates (glucose, pyruvate, acetate, n-hexadecane, and nutrient broth), as well as ammonium (the nitrogen source). Batch cultures grown at reduced initial substrate concentration, at lower temperatures, or at lower dilution rates than continuous-flow cultures exhibited lesser rates and degree of cell death and lysis [15]. Optical density of *G. thermoleovorans* DSM 5366 increased in casein digest medium supplemented solely with Ca<sup>2+</sup> or Mg<sup>2+</sup>. Na<sup>+</sup>, and to a greater extent K<sup>+</sup>, with concerted action of Ca<sup>2+</sup> or Mg<sup>2+</sup> also induced increased optical density readings of the strain [16].

*Geobacillus subterraneus* and *Geobacillus uzenensis* were isolated from serial dilutions of thermophilic hydrocarbon-oxidizing enrichment cultures derived from oil fields. The cultures were inoculated on agar medium supplemented with 0.1% n-hexadecane and incubated at 55–60°C [17]. *Geobacillus jurassicus* was isolated from oil field formation water by diluting enrichment cultures grown on the following medium (NH<sub>4</sub>Cl, 1 g; KCl, 0.1 g; KH<sub>2</sub>PO<sub>4</sub>, 0.75 g; K<sub>2</sub>HPO<sub>4</sub>, 1.4 g; MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.2 g; CaCl<sub>2</sub>·2H<sub>2</sub>O, 0.02 g; NaCl, 1.0 g; water, 1000 ml; pH 7.0) supplemented with 4% crude oil, incubated at 60°C, and plated on the same medium solidified with 2% agar [18].

*Geobacillus thermocatenulatus* was isolated from a slimy bloom at about 60°C on the inside surface of a pipe in a steam and gas thermal borehole using potato-peptone and meat-peptone media [19]. Some studies showed that *G. thermocatenulatus* strain does not hydrolyze starch and gelatine. It neither produces acid from xylose and lactose nor generates acetoin [20]. Trypticase soy agar can be used as the medium for cultivation of representatives of this species. Addition of 5 mg/l MnSO<sub>4</sub> encourages sporulation of the species [21].

Strains of *Geobacillus thermodenitrificans* were isolated from soil by suspending 1 g soil sample in 5 ml of sterile distilled water and heating at 90°C for 10 min, then plating 1 ml aliquot on nutrient agar and incubating at 65°C for 24 h [22]. *G. thermodenitrificans* showing L-arabinose isomerase activity was grown in media containing 1.5% pancreatic digest of casein, 0.2% yeast extract, 0.2% beef extract, 0.2% glycerol, 0.2% K<sub>2</sub>HPO<sub>4</sub>, 0.2% KH<sub>2</sub>PO<sub>4</sub>, 0.01% MgSO<sub>4</sub>, 0.0004% D-biotin, and pH 6.8. Inocula were prepared by culturing the organisms for 10 h at 65°C and 200 rpm in a rotary shaking incubator using 250-ml flasks containing 50 ml of the above-described medium. These cultures were used to inoculate the fermenter where the mixture was incubated for 14 h at 65°C with agitation (400 rpm) and aeration (1.0 vvm) [23]. Some strains of *G. thermodenitrificans* were isolated and suspended in 100 ml of 0.1 mol/l potassium phosphate buffer solution (pH 7.0) with 1.0% (w/v) gelatinized potato starch in 500-ml conical flasks. The flasks were incubated at 50°C for 2 days, then 1 ml portions were transferred to 10 ml of 0.1 mol/l phosphate buffer (pH 7.0) and agitated for 6 h followed by suspension onto starch agar plates (10 g peptone, 5 g yeast extract, 10 g potato starch, and 15 g agar in 1 l of a 0.1 mol/l potassium phosphate buffer, pH 7.0) and incubation at 60°C for 24 h [24].

*Geobacillus thermoglucosidasius* was isolated from Japanese soil by adding 0.1 g sample to 5 ml of medium I (peptone, 5 g; meat extract, 3 g; yeast extract, 3 g; K<sub>2</sub>HPO<sub>4</sub>, 3 g; KH<sub>2</sub>PO<sub>4</sub>, 1 g; water, 1000 ml; pH 7.0) in test tubes and incubating at 65°C for 18 h, with the tubes leaning at an angle of about 10°, followed by further enrichments in tubes with the same medium and then purification on plates containing medium I solidified with 3% agar [25, 26]. Studies showed that *Geobacillus thermoglucosidasius* strains grew well and gave reproducible and comparable viable cell counts on the semi-defined agar medium (SDM) with glycerol and pyruvate as carbon sources under aerobic conditions at 70°C. SDM contains a dual carbon source (glycerol and pyruvate) and low levels of yeast extract, tryptone, and inorganic salts. The main components of the SDM are (g/l): glycerol 5.0, pyruvate 5.0, tryptone 0.2; yeast extract 0.2; citric acid 0.32; di-sodium hydrogen orthophosphate (anhydrous) 2.0; magnesium sulfate (heptahydrate) 0.4; potassium sulfate 0.3; ammonium chloride 2.0; manganese chloride (tetrahydrate) 0.003; ferric chloride 0.007; agar 15, and 1 ml of trace elements solution. SDM formulation suggests joint preparation of glycerol and pyruvate separated from the other constituents and mixing after sterilization at 121°C [27].

*Geobacillus lituanicus* was isolated using 10-fold serial dilutions of crude oil. The dilutions were inoculated onto Czapek agar and plates were incubated

aerobically at 60°C for 48 h [28]. *Geobacillus toebii* was isolated from a suspension of hay compost plated onto solid modified basal medium (polypeptone, 5 g; K<sub>2</sub>HPO<sub>4</sub>, 6 g; KH<sub>2</sub>PO<sub>4</sub>, 2 g; yeast extract, 1 g; MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.5 g; L-tyrosine, 0.5 g; agar to solidify; and deionized water, 1000 ml) and incubated at 60°C for 3 days [29]. *G. toebii* subsp. *decanicus* was cultivated in medium containing 8 g/l peptone, 4 g/l yeast extract, 2 g/l NaCl at pH 7.0 [30]. *Geobacillus vulcani* was isolated from a marine sediment sample by inoculating into Bacto marine broth and medium D and incubating aerobically for 3 days at 65°C, followed by plating positive cultures again onto Bacto marine agar [31]. *Geobacillus galactosidasius* was isolated from a compost sample and inoculated in 10 ml of enrichment media cultured within pH range 5.5–7.2 and the temperature range 60–80°C for 3 days. The enrichment growth media used were medium A containing 8 g/l peptone, 4 g/l yeast extract, and 2 g/l NaCl at pH 7.2, and medium B containing 6 g/l yeast extract and 6 g/l NaCl at pH 5.5 [32]. *Geobacillus icigianus* was isolated from sludge samples of an explosive hydrothermal spring located near the Troinoy geyser and purified on LB agar medium at 55–65°C [33].

### 3. *Geobacillus* bacteria: taxonomy and identification

Thermophilic bacterium known as *Bacillus stearothermophilus* (now typical species *Geobacillus stearothermophilus*) was discovered in 1920 [34]. For many years, geobacilli have been referred to *Bacillus* species. The development of molecular genetic methods resulted in the division of bacilli into several phylogenetically distinct genera. Group 5 including *B. stearothermophilus*, *Bacillus kaustophilus*, and *Bacillus thermoglucosidasius* formed a generic lineage distinct from *Bacillus* species [35]. In 2001, Nazina et al. proposed that the six species of bacilli, namely *B. stearothermophilus*, *B. kaustophilus*, *B. thermoglucosidasius*, *B. thermocatenuatus*, *B. thermoleovorans*, and *B. thermodenitrificans*, should be incorporated into a new genus, *Geobacillus*, along with two novel species, *Geobacillus subterraneus* and *Geobacillus uzenensis* based on 16S rRNA gene sequence analysis and a variety of physical and biochemical characteristics [17].

At present, geobacilli include about 20 species [36]. However, the lowest level of 16S rRNA gene sequence similarity between all *Geobacillus* species is around 93%, which indicates that at least some species need to be reclassified at the genus level [1]. Full-length *recN* and 16S rRNA gene sequence analysis clustered 68 isolates from the genus *Geobacillus* into 9 similarity groups. Some of these groups corresponded unambiguously to the known species, whereas the others contained two or more type strains from species with validly published names. *recN* was clearly superior to the 16S rRNA gene, with nearly an order of magnitude greater resolving power at the species-subspecies level, but the analysis was much less effective for higher taxa [37]. The availability of complete genome sequence data offers higher resolution of the phylogenetic relationships of *Geobacillus* species than the use of a single housekeeping gene. For example, the two strains of *G. kaustophilus* do not form a phylogenetically coherent monophyletic clade, while the two strains of *G. thermoleovorans* are closely related and share 99.4% nucleotide sequence identity [38]. The use of the phylogenomic metrics such as average amino acid identity (AAI), average nucleotide identity (ANI), and digital DNA-DNA hybridization (dDDH) indicated that the current genus *Geobacillus* is composed of 16 distinct genomospecies. Phylogeny constructed on the basis of the core genes demonstrated that the genus clustered into two monophyletic clades differing in terms of nucleotide base composition. The G + C content ranges for clade I and II were 48.8–53.1 and 42.1–44.4%, respectively. The *Geobacillus* species within clade II can be considered as a new genus [39].

It was shown that evolutionary conservatism of 16S rRNA leads to the case, when different bacterial strains belonging to the same species or closely related species may have identical sequences. It decreases efficiency of 16S rRNA analysis and provokes the search for alternative approaches, like comparative analysis of the nucleotide sequences of “housekeeping genes” determining the main metabolic processes. These genes have some advantages similar to those of 16S rRNA genes: universal distribution, evolutionary conservatism, and vertical inheritance. At the same time, comparative phylogenetic analysis of “housekeeping genes” may be more efficient at the lowest taxonomic levels for the following reasons: (1) the level of conservatism varies for different genes and distinctions between the nucleotide sequences may be more pronounced as compared to sequences of 16S rRNA; (2) “housekeeping genes” code for proteins allowing to better determine the frequency of synonymous substitutions and locate taxonomic position; (3) the majority of these genes are represented by a single copy. Besides the abovementioned *recN* gene, application prospects have been demonstrated for other genes. Genes encoding the  $\beta$ -subunits of a type II topoisomerase (gyrase, *gyrB*) and a type IV topoisomerase (*parE*) provided for a more precise determination of the phylogenetic position of bacteria at the species level as compared to 16S rRNA analysis of *Geobacillus* [40]. Additionally, genes *recA* and *rpoB* can be used in identification and taxonomic affiliation of *Geobacillus* species [41]. Gene *spo0A* codes for protein serving as the master regulator of the endospore formation process in the endospore-forming bacteria and can be engaged in taxonomic positioning of these bacterial groups. The gene cannot be used as the phylogenetic marker within the genus *Geobacillus*, although it was shown to be helpful for the identification of *G. thermodenitrificans*, *G. stearothermophilus*, *G. jurassicus*, and cluster *G. subterraneus*-*G. uzenensis* [42].

16S-23S rRNA gene internal transcribed spacer (ITS) separates 16S and 23S rRNA genes and may contain tRNA genes. ITS exhibits larger variations in both the length and the sequence of this region and can be more useful in identification than 16S rRNA. *Geobacillus* genus-specific primers GEOBAC allowed to amplify the DNA from 13 species. Different species could be grouped according to the number and size of the PCR products and identified on the basis of the restriction analysis of these products [43].

Repetitive DNA is also a promising tool for identification of bacteria. Usually, repetitive DNA is applied for genotyping of medically important microorganisms, but it may be employed in analysis of biotechnologically important *Geobacillus* species. In total, 33 *Geobacillus* genus-specific motifs with length over 20 nucleotides were determined: 3 were genus-specific, 15—species-specific, and 15—species cluster-specific. Motifs have been used for the construction of the genus- and species-specific (*G. thermodenitrificans* and *G. toebii*) primer pairs [44]. Rep-PCR molecular method based on the usage of outwardly facing oligonucleotide PCR primers complementary to interspersed repetitive sequences is widely applied in the characterization of different groups of bacteria. BOX, ERIC, REP, and (GTG)<sub>5</sub> are examples of evolutionarily conserved repetitive sequences. Rep-PCR fingerprinting technique using (GTG)<sub>5</sub>- and BOX-PCR has been shown as a rapid, easy-to-conduct, and reproducible tool for differentiation of thermophilic bacteria at the species, subspecies, and potentially up to the strain level, with a single-performance protocol [45]. Multilocus variable-number tandem-repeat (VNTR) analysis (MLVA) has been used to discriminate between different genotypes within species by analyzing length polymorphism of several VNTR loci, while high-resolution melt analysis (HRMA) has been shown as post-PCR method for analysis of genetic variations in PCR products using DNA-binding fluorescent dyes and a PCR machine with a highly precise temperature control. HRMA in conjunction with MLVA (MLV-HRMA) displayed a stronger discriminatory power and better reproducibility than RAPD-PCR and hence can be used for genotyping *Geobacillus* species [46].

Amplified ribosomal DNA restriction analysis (ARDRA) using *AluI* was shown to be a valuable, easy, and accurate technique for the identification of *G. stearothermophilus*. The presence of a fragment 162 bp in size and the absence of 76 and 86 bp fragments were recognized to be characteristic traits of this species. However, the potential of the method for the identification of other species of geobacilli is limited [47].

#### 4. Applications of *Geobacillus* bacteria

*Geobacillus* species are represented by thermophilic chemoorganotrophs. They are able to utilize a wide range of substrates, including hydrocarbons, and produce various metabolites of commercial use, like enzymes (proteases, lipases, and amylases), ethanol, bacteriocins, etc. Such metabolic diversity coupled to high temperature resistance makes the bacteria attractive for various applications. On the other hand, *Geobacillus* species are known to be a major cause of spoilage in canned food. The use of thermostable enzymes and biofuel production are considered in the next chapters.

Petroleum is a complex compound consisting of hydrocarbons, a small ratio of nonhydrocarbon components, and trace metals. In order to increase oil production, traditional water flooding method is used. Heavy oil, the largest potentially recoverable petroleum energy resource, is very viscous and has a high freezing point. It is problematic to extract the residual crude oil by the conventional method, so that alternative techniques, like use of microorganisms, for enhanced oil recovery have been proposed. Microorganisms and their metabolites help to retrieve residual oil by promoting its emulsification and reducing viscosity. Since high temperature reservoirs are more difficult to exploit by this method, thermophilic bacteria are advantageous in this case. *G. stearothermophilus* A-2 shows strong surface hydrophobicity and produces a bioemulsifier. The fermentation broth of strain A-2 induced crude oil dispersion and decreased oil viscosity. Moreover, strain A-2 preferentially degraded heavy oil components and polycyclic aromatic hydrocarbons. These features make the strain an excellent candidate for enhanced microbial oil recovery from high-temperature deposits [48]. *Geobacillus* sp. ZY-10 could utilize tridecane, hexadecane, octacosane, and hexatridecane as the sole carbon sources, and the digestion rate of long-chain alkanes was lower than that of short-chain alkanes. Addition of inorganic salts and trace yeast extract led to the significantly increased concentration of short-chain alkanes and the decreased content of long-chain alkanes, suggesting that the larger hydrocarbon components in crude oil were converted into shorter-chain alkanes. Thus, strain ZY-10 proved effective for improving the mobility and upgrading quality of heavy crude oil [49]. *G. pallidus* H9 was able to grow in temperature range 45–80°C at salinity 0–15% and synthesize biosurfactant using crude oil as the sole carbon source under aerobic or anaerobic conditions. After incubation in LB medium, 20 ml bacterial suspension was transferred to 500 ml triangular flask with 10 g of sterile crude oil-containing mineral medium, and incubated at 65°C and 180 rpm for 100 days under aerobic or anaerobic conditions. The medium was boiled for 30 min in order to expel all dissolved oxygen prior to incubation under anaerobic conditions; L-cysteine and resazurin as oxygen indicators were added to the medium to final concentrations of 0.05% and 0.01 (g/l), respectively. The yields of biosurfactant were  $\approx 9.8$  and  $\approx 2.8$  g/l under aerobic and anaerobic conditions, respectively. The fractionated components and composition of the purified biosurfactant differed between aerobic (glycosides $\approx 50.3\%$ , lipids $\approx 34.5\%$ , and peptides $\approx 15.2\%$ , w/w) and anaerobic (glycosides $\approx 53.8\%$ , lipids $\approx 31.2\%$ , and

peptides ≈ 26.0%, w/w) cultures. The maximum production of biosurfactant under aerobic conditions is determined by the different electron acceptors, resulting in the different pathways of biodegradation with different reaction activation energy and generation of different catabolic enzymes. The strain H9 showed preference for utilization of medium- and long-chain alkanes (C23-C43) under aerobic conditions, and degradation of long alkanes (C33-C43) under anaerobic conditions. The evident difference in the metabolic pathways between aerobic and anaerobic degradation was possibly due to a change in redox potential during the biochemical reaction. The strain H9 and its biosurfactant are potentially promising agents intensifying microbial oil recovery, especially in high temperature and salinity oil reservoirs [50]. Besides, the ability to utilize hydrocarbons makes geobacilli indispensable in bioremediation of environment, like removal of oil spills [51].

Bacteriocins are ribosomally synthesized antimicrobial peptides or proteins, usually possessing the narrow antagonistic activity spectrum against bacterial strains closely related to the strain-producer. The activity of this group of molecules against foodborne and pathogenic bacteria opens wide opportunities for their application in medicine and food industry. Little is known about bacteriocins of thermophilic bacteria; however, *Geobacillus* species demonstrated antibacterial activity. *Geobacillus* strains isolated from the surface soil above oil deposits were active against at least 1 of 19 tested pathogenic bacteria. The derived antibacterial compounds were stable in broad temperature and pH ranges, sensitive to proteolytic enzymes, proving their proteinaceous nature. They were active against closely related thermophilic bacteria, which suggests that these substances are most likely bacteriocins [52]. *Geobacillus* species have been shown to produce antimicrobials such as antibiotics. *G. thermodenitrificans* NG80-2 synthesizes two antibiotics: geobacillin I and II. The former antibiotic is nisin analog showing antimicrobial spectrum similar to nisin A, with increased activity against *Streptococcus dysgalactiae*, one of the causative agents of bovine mastitis. Geobacillin I demonstrated increased stability compared to nisin A. Geobacillin II displayed antimicrobial activity only against *Bacillus* strains [53].

Nanomaterials are defined as engineered materials with a least one dimension in the range of 1–100 nm. They often exhibit unique and considerably modified physical, chemical, and biological properties. Nanomaterials are used in cosmetics, chemical, and food industries, construction, medicine, agriculture, production of electronic equipment and sensors, etc. [54, 55]. The available physical methods for the metal nanoparticle synthesis such as gas condensation and irradiation with ultraviolet or gamma rays usually resulted in low production rate and high expenditure. The large scale synthesis of metal nanomaterials suffers from certain drawbacks such as polydispersity and lack of stability, especially if the reduction is carried out in aqueous media. Biological synthesis of nanoparticles can be an alternative choice. The exposure of *G. stearothermophilus* cell-free extract to the metal salts leads to the formation of stable silver and gold nanoparticles in the solution. The stability of nanoparticle solution could be due to the secretion of certain reducing enzymes and capping proteins by the bacterium. Preliminary gel electrophoresis indicates that the bacterium secretes not less than seven different proteins of molecular mass ranging between 12 and 98 kDa. One or more of these proteins could be a reductase enzyme that reduces metal ions [56]. Silver nanoparticles can be produced from silver nitrate using spore extract of *G. stearothermophilus*. Cytotoxicity of nanoparticles derived from this extract toward microbial pathogens such as *Candida albicans*, *Candida glabrata*, *Streptococcus mutans*, and *Streptococcus sobrinus* was generally higher than cytotoxic effect of silver nanoparticles stemming from spore extract of *Bacillus subtilis*. Moreover, antibacterial effects significantly surpassed antifungal effects [57].

Various human activities generate wastewater containing nitrogenous compounds. In the natural environment, nitrogen removal often occurs at 20–30°C, temperatures suitable for growth of nitrifying and denitrifying bacteria. As a result, many wastewater treatment plants use mesophilic bacteria for bioremediation. Thermophilic bacteria are expected to have increased enzyme activity and stability in comparison with mesophilic ones in such applications. The thermophilic denitrifying bacterium *Geobacillus* sp. strain TDN01 showed 12 times higher specific nitrate removal rate on media with ammonia than without ammonia. The consumption rates of nitrate and succinate were proportional. The growth rates with 120 and 150 mM nitrate were only slightly lower than those with 60 mM, not leading to notable growth inhibition. The maximum denitrification rate was six times higher than that of mesophilic bacteria [58]. Organophosphonates are characterized by the presence of a stable, covalent C–P bond. One of important applications for synthetic organophosphonates is manufacturing of herbicides, such as glyphosate. Glyphosate is the most widely used nonselective herbicide worldwide. It can cause a wide range of clinical manifestations in human beings, like skin and throat irritation to hypotension, oliguria, and death [59]. Strain *G. caldoxylosilyticus* T20 could utilize a number of organophosphonates as the sole phosphorus source for growth at 60°C. During growth on glyphosate, aminomethylphosphonate release to the medium was observed [60]. Azo dyes are characterized by the presence of one or more azo bonds (–N=N–) in association with one or more aromatic systems, which may carry sulfonic acid groups. The pigments are extensively used in the dyeing and textile industries and can provoke grave problems when discharged in the environment. *G. stearothermophilus* UCP 986 was able to degrade 96–98% of the azo dye after 24 h of incubation on LB medium under aeration. The brine shrimp *Artemia salina* showed the absence of toxic metabolites during the decolorization process. However, increased concentration of the dye and vigorous agitation led to high mortality rate of the shrimp [61]. Synthetic polymers generally display strong resistance to biodegradation, causing serious pollution problems as wastes persisting in the environment for a long time. Aliphatic polyamides (nylons), like nylon 6, nylon 66, and nylon 12, are produced in large amounts and are regarded as recalcitrant to biodegradation. At 60°C, *G. thermocatenulatus* grew on 5 g/l nylon 12, decreasing its molecular weight from 41,000 to 11,000 over 20 days. The strain also degraded nylon 66 with a decline in its molecular weight from 43,000 to 17,000 in 20 days at 60°C. However, nylon 6 was not utilized [62].

The heavy metals are well-known toxicants and their determination is vital for ecological control of soils, food, and water. Cells of *G. thermoleovorans* subsp. *stromboliensis*, immobilized on Amberlite XAD-4, showed the sorption capacity of 0.0373 and 0.0557 mmol/g for Cd(II) and Ni(II), respectively. The detection limits were 0.24 µg/l for cadmium and 0.3 µg/l for nickel. The system sustained 20 operation cycles without any loss in its sorption potential and can be used for metal determination in water and food samples [63]. Bacteria may be also applied in biosorption and removal of toxic metals from aqueous solutions. Dead biomass of *G. thermodenitrificans* MTCC 8341 in the synthetic metal solutions reduced the concentration of Fe<sup>+3</sup> (91.31%), Cr<sup>+3</sup> (80.80%), Co<sup>+2</sup> (79.71%), Cu<sup>+2</sup> (57.14%), Zn<sup>+2</sup> (55.14%), Cd<sup>+2</sup> (49.02%), Ag<sup>+</sup> (43.25%), and Pb<sup>+2</sup> (36.86%) at different optimum pH values within 720 min. The strain in the industrial wastewater reduced concentrations to 43.94% for Fe<sup>+3</sup>, 39.2% for Cr<sup>+3</sup>, 35.88% for Cd<sup>+2</sup>, 18.22% for Pb<sup>+2</sup>, 13.03% for Cu<sup>+2</sup>, 11.43% for Co<sup>+2</sup>, 9.02% for Zn<sup>+2</sup>, and 7.65% for Ag<sup>+</sup> within 120 min. [64]. In turn, Cd<sup>+2</sup>, Cu<sup>+2</sup>, Co<sup>+2</sup>, and Mn<sup>+2</sup> removal at 50 mg/l concentration in 60 min by 50 mg of dry *G. thermantarcticus* cells at optimum growth temperatures was 85.4, 46.3, 43.6, and 65.1%, respectively [65]. Thus, *Geobacillus* strains may be used for disposal of heavy metals.

Thermophilic bacteria can be the sources of valuable biomolecules, like complex polysaccharides for medical application. Extracellular polysaccharide produced by strain of *G. thermodenitrificans* hinders HSV-2 replication in human peripheral blood mononuclear cells (PBMC). High levels of IFN- $\alpha$ , IL-12, IFN- $\gamma$ , TNF- $\alpha$ , and IL-18 were detected in exopolysaccharide supernatants following PBMC treatment. This effect was dose-dependent. Therefore, the immunological disorders determined by HSV-2 could be partially resolved by treatment with polysaccharide [66].

The biosurfactants, amphiphilic molecules consisting of hydrophobic and hydrophilic groups, are used in cosmetic formulas, pharmaceutical, chemical, food industries, agriculture, production of cleansers, enhanced oil recovery, and in bioremediation of oil-contaminated sites. Biosurfactant of *G. stearothermophilus* UCP 0986 grown on corn steep liquor and palm oil medium is capable of reducing the surface and interfacial water tensions to significantly lower values, and possesses excellent emulsifying and dispersion properties. The biosurfactant was isolated by precipitation of metabolic cell-free liquid with acetone 1:1 (v/v). The precipitate was allowed to stand for 24 h at 4°C, and then, it was centrifuged at 4000 rpm for 15 min at 5°C. The supernatant was discarded and the isolated biosurfactant was subjected to dialysis against deionized water for 72 h at 5°C. Properties of the compound arouse interest in terms of bioremediation of hydrophobic molecules [67].

Lactic acid is used in several industrial sectors producing food, pharmaceuticals, chemicals, and cosmetics. Around 90% of its entire production is derived from fermentation by lactic acid bacteria. However, application of thermophilic bacteria may reduce the risk of contamination and sterilization cost. *G. stearothermophilus* DSM494 generated lactic acid as the major product of anaerobic metabolism. The strain produced 37 g/l optically pure (98%) L-lactic acid in 20 h from 50 g/l raw potato starch. Smaller amounts (<7%w/v) of acetate, formate, and ethanol were also formed. Yields of lactic acid increased from 66 to 81% when potato residues from food processing were used as a starchy substrate in place of raw potato starch [68].

Non-standard application for *Geobacillus* has been demonstrated in the enhancement of cement-sand mortar properties. The *G. stearothermophilus* was chosen for the ability to resist extreme environmental factors. Bacteria at concentration  $1 \times 10^9$  CFU/ml improved the performance of cement-sand mortar in terms of compressive strength and water absorption as compared to the control mortar. The incorporation of *G. stearothermophilus* has catalyzed the occurrence of wollastonite ( $\text{CaSiO}_3$ ) capable to upgrade concrete properties by modifying its pore structure [69].

*Geobacillus* species can be used for cultivation and study of other bacteria. Many microorganisms in nature cannot be successfully cultured under artificial conditions, even in the presence of appropriate nutrients. *Symbiobacterium toebii* requires some growth-supporting factors from its partner bacterium *G. toebii* and does not show sustainable growth in artificial culture in the absence of the partner or its supernatant/cell-free extract [70]. *Geobacillus* sp. W2-10 enhances the cellulose-degrading activity of cellulolytic bacteria *Clostridium thermocellum* CTL-6 in peptone-cellulose solution medium under aerobic conditions. Cellulose degradation efficiency of filter paper and alkaline-treated wheat straw significantly increased up to 72.45 and 37.79%, respectively. The carboxymethyl cellulase activity and biomass productively of CTL-6 also rose from 0.23 U/ml and 45.1  $\mu\text{g/ml}$  up to 0.47 U/ml and 112.2  $\mu\text{g/ml}$ , respectively. In addition, coculture resulted in the accumulation of acetate and propionate up to 4.26 and 2.76 mg/ml [71].

*Geobacillus* species are able to reduce effects of osmotic stress in plants. *G. caldxylosilyticus* IRD, halophilic facultative aerobic bacterium, inoculated into 5-day-old maize cultivars prior to treatment with 350 mM NaCl for 10 days improved

maize growth and dry weight. The number of vascular bundles decreased in roots and increased in leaves upon inoculation with bacteria. In addition, the accumulation of toxic  $\text{Na}^+$  and  $\text{Cl}^-$  was much lower in treated seedlings. Proline level, stress indicator, became two to four times higher in seedlings exposed to salt without *Geobacillus* [72].

Crystalline bacterial cell surface layers (S-layers) are composed of identical proteins or glycoprotein subunits which can self-assemble into two-dimensional crystalline arrays. It has been shown that S-layers of *Geobacillus* could be used as a biological template for immobilization of molecular array and provide new approaches for nanoelectronic biosensor design [73].

Probiotics are health-promoting microbial agents. Mechanisms of probiotic action are diverse. Positive effect can be expressed as direct action on the organism, like enhancement of barrier function, metabolism, immunomodulatory effects, or action on pathogenic microorganisms via secretion of bioactive compounds and/or competition with pathogens (Figure 2). The global probiotics market reached \$31.8 and \$34.0 billion in 2014 and 2015, respectively. The market capacity is likely to expand to \$50.0 billion by 2020, growing at compound annual growth rate of 8.0% from 2015 to 2020 (Figure 3) [74]. Lactic acid bacteria are main sources of probiotics. However, it was reported that a number of bacteria such as *Bacillus* spp., *Aeromonas hydrophila*, and yeasts such as *Saccharomyces boulardii*, *Candida famata*, and *Candida parapsilosis* possess probiotic potential. *Geobacillus* species are not usually regarded as probiotic candidates, but these bacteria are able to produce a number of beneficial compounds that can favorably influence the organism or demonstrate antimicrobial features. Some bacteria show the adherence ability. Thus, *Geobacillus* species can be considered as potential sources of probiotics [75].

Due to their resistance and spore formation, geobacilli may be used as biological indicators of sterility. They are usually applied in the studies on efficiency of sterilization processes [76–78]. Finally, *Geobacillus* species can find use in engineering applications, such as directed evolution of robust variants of mesophilic proteins [79].

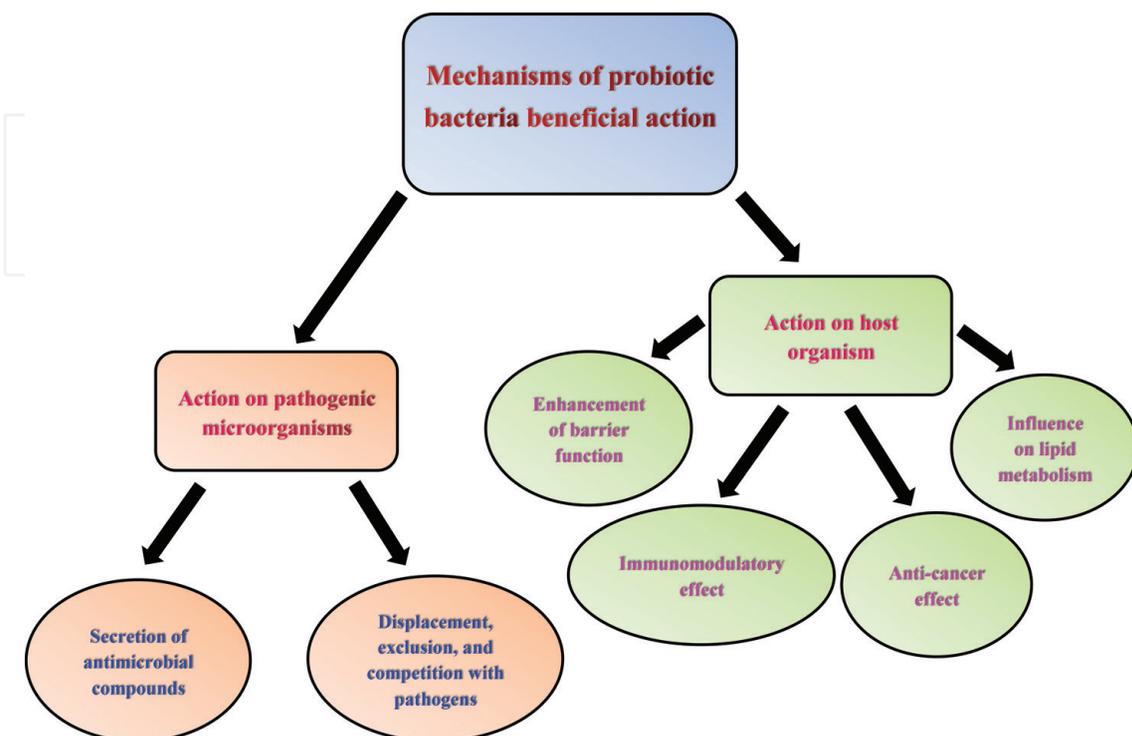
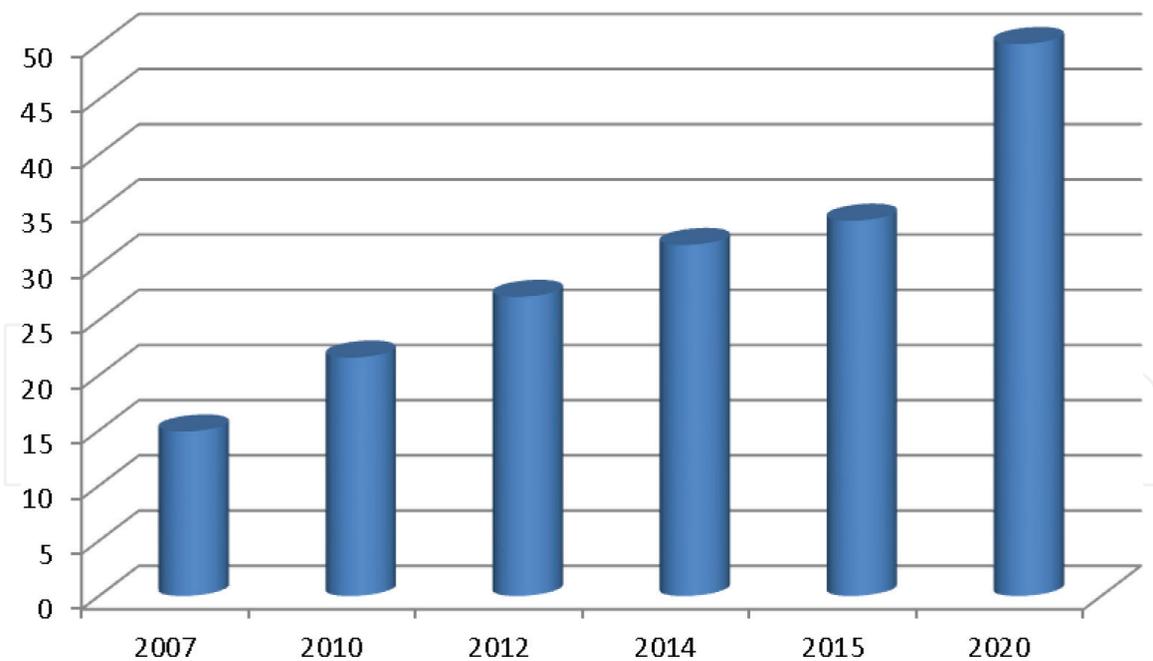


Figure 2.  
Mechanisms of beneficial action of probiotic bacteria.



**Figure 3.**  
Probiotics market (USD billion dollars) growth (years 2007–2020).

## 5. Production and applications of thermostable enzymes of *Geobacillus* bacteria

Pectinases or pectinolytic enzymes hydrolyze pectic substances. Pectinolytic enzymes are involved in fruit juice extraction and clarification, scouring of cotton, degumming of plant fibers, wastewater treatment, vegetable oil extraction, tea and coffee fermentations, bleaching of paper, in fabrication of poultry feed additives and alcoholic beverages, and in food industries [80]. The raw pressed juice is rich in insoluble particles mainly made up by pectic substances. It is difficult to extract juice by pressing or using other mechanical methods. The addition of pectinases decreases viscosity of the fruit juice, leading to its higher yields. These enzymes are produced by numerous bacteria, including *Geobacillus* species. The purified pectin lyase of *G. pallidus* P26 retained stability and full activity after 24 h incubation in temperature range from 40 to 50°C. Its activity decreased when the temperature increased above 70°C. However, the purified enzyme from thermophilic bacteria can keep its activity for 2 and 5 h at 80 and 70°C, respectively. In addition, the activity fell only by 50% after 24 h at 60°C. The optimal pH value for pectin lyase was 9, and it remained active between pH 5 and 11. Compared to the control, the enzyme increased juice yield from apple, banana, carrot, and peach pulps. Nevertheless, the results obtained for purified enzyme were less than those for commercial Pectinex 100 L Plus [81].

$\alpha$ -Amylase is one of the enzymes of worldwide interest in food, pharmaceutical, and fermentation industries. The moderate thermostability and  $\text{Ca}^{2+}$  requirement of  $\alpha$ -amylases limit their industrial potential. The supplementation of wheat flour with hyperthermostable and  $\text{Ca}^{2+}$ -independent  $\alpha$ -amylase of *G. thermoleovorans* NP54 accelerated the rate of fermentation and reduced the viscosity of dough, resulting in the improved volume and texture of bread, its increased shelf life and softness [82]. The same amylase used in starch saccharification produced hydrolysate containing a high proportion of maltose. As a consequence, this enzyme can find use in the manufacture of high maltose syrups consumed by the food, chemical, and pharmaceutical industries [83]. The amylase from *Geobacillus* sp. IPTN was stable over a broad range of temperatures from 40 to 120°C and pH from 5 to 10 and showed

resistance to protease. These characteristics emphasize the enzyme potential in industrial applications [84]. Engineered  $\alpha$ -amylase of *G. stearothermophilus* US100 with its significantly lower requirement for calcium ions, high resistance to thermal inactivation, to chelators, to protease, and to oxidative additives, in conjunction with well-preserved activity after storage, may be an excellent candidate for manufacturing enzyme detergent [85].

$\alpha$ -Glucosidases are hydrolases releasing  $\alpha$ -glucose from the ends of the substrates such as oligo- and polysaccharides. They are usually found in association with other amylolytic enzymes which accomplish complete degradation of starch.  $\alpha$ -Glucosidases show diversity in substrate specificity and transglycosylation activities, and such specificity differs considerably with the enzyme source. They have a number of potential applications in fundamental research, industrial starch processes,  $\alpha$ -amylase assay kits for clinical laboratories, and synthesis of oligo-, di-, and trisaccharides. The thermostable exo- $\alpha$ -1,4-glucosidase of strain *G. thermodenitrificans* F84a remained active over temperature range 35–70°C and pH range 4.5–11.0, with optimum activity at 60°C and pH 7.0. The  $\alpha$ -glucosidase hydrolyzed  $\alpha$ -1,6,  $\alpha$ -1,3, and  $\alpha$ -1,4 bonds of substrate molecules in addition to a high transglycosylation activity. The enzyme was also found to be resistant to most of the denaturing agents and inhibitors. The characteristics of  $\alpha$ -glucosidase make it a promising agent for biotechnological processes. It can be used for enzymatic synthesis of novel tri- and oligosaccharides due to high conformational stability and transglycosylation activity [86].  $\alpha$ -Glucosidase from *Geobacillus* sp. strain HTA-462 isolated from sediment of the Mariana Trench exclusively hydrolyzed  $\alpha$ -1,4-glycosidic linkages of oligosaccharides in an exo-type manner. The enzyme showed an overwhelming transglycosylation activity and glycosylated various non-sugar molecules when maltose was used as a sugar donor. It converted maltose to isomaltose.  $\alpha$ -Glucosidase is also a potential catalyst in the biosynthesis of complex carbohydrates [87].

Cellulose is the major component of plant biomass. The enzymes degrading it are applied in the textile industry for cotton softening, in the production of detergents for color care and cleaning, in the food industry for mashing, and in the pulp and paper industries for deinking, drainage promotion, and fiber modification. Thermophilic cellulases show advantages in many industrial applications because elevated processing temperatures provide for accelerated reaction rates, increased solubility of reagents and reduced contamination. Endoglucanase of *Geobacillus* sp. 70PC53 expressed an optimal activity at 65°C and pH 5.0, and it exhibited 10-fold higher specific activity than the commercially available *Trichoderma reesei* endoglucanase. The enzyme displayed activity over a broad temperature range from 45 to 75°C and good prospects in biomass conversion, detergent upgrading, paper pulping, textile manufacturing, and juice clarification [88]. The thermophilic *Geobacillus* sp. T1 is able to grow and produce cellulase efficiently on untreated wheat and barley straw as the sole carbon sources. It harbors the potential for conversion of agricultural biomass to fuels [89]. Endocellulase of *G. thermoleovorans* T4 can hydrolyze carboxymethylcellulose, phosphoric acid-swollen cellulose, Avicel, filter paper, and salicin. When the strain was grown in medium with carboxymethylcellulose, the cellulolytic enzyme activity in culture supernatants was stable up to 70°C. More than 10% of the original activity was still detectable after heating to 100°C at pH 7.0 for 1 h [90].

Lipase catalyzes hydrolysis of triglycerides and produces esters by esterification reaction. They are used in many sectors such as food, pharmaceutical, chemical, petrochemical, biodiesel, and in detergent industries. Their main application is the enantioselective synthesis of precursors of pharmaceutically active compounds and the conversion of natural fats and oils into high-value products such as cocoa,

butter, and oil enriched with omega-3 fatty acids. Thermostable lipases are characterized by inherent stability, so that they can find use in various industries and biotechnological sectors as additives to detergents, food processing aids, environmental bioremediation agents, and molecular biology tools. Lipase from *Geobacillus* sp. immobilized by surface adsorption onto silica showed maximum activity at temperature 55°C and 82.94% yield of methyl salicylate. The latter has various medicinal applications, like control of muscular pain [91]. In leather industry, the addition of hydrolytic enzymes such as lipases and proteases in the soaking step facilitates fat degradation and raises leather quality. Thermoalkaliphilic lipase from *G. thermoleovorans* DA2 is produced in high amounts using cheap substrate, such as fatty restaurant wastes, thus making the production process cost-effective. The lipase with Triton X-100 proved the best degreasing agent by lowering the total lipid content to 2.6% as compared to kerosene, commonly utilized organic solvent, (7.5%) or the sole crude enzyme (8.9%). As a result, the chemical leather process can be substituted with thermoalkaliphilic lipase treatment to upgrade the quality of leather and reduce the environmental hazards [92]. Thermostable lipase from *G. thermodenitrificans* IBRL-nra exhibited the highest stability in the presence of acetone, ethanol, and acetonitrile and showed elevated activity (220%) when pretreated with Triton X-100. It could preserve 100% of its activity in the presence of protease up to 4 h and could retain 70% of its initial activity after 24 h of incubation; hence, the enzyme can be applied in biotechnological processes and industries [93]. Lipase from *G. thermodenitrificans* nr68 has expressed great enzymatic biodeinking activity toward a laser jet printed paper, with deinkability brightness test of 55%, a value that was slightly lower than that shown by a commercial lipase (63%). Such lipases can be used in the recycling of waste paper [94]. *Geobacillus* sp. T1 lipase fused with a cellulose binding domain has a strong medium chain (C8:0 and C10:0) preference and acts weakly on C18:1 n-9 in acidolysis reactions. This enzyme could be used as a potential biocatalyst in the synthesis of structured lipids. The latter are triacylglycerols carrying particular fatty acids in certain positions of the glycerol backbone, which provide for nutritive or therapeutic purposes [95].

Pullulanases are debranching enzymes able to hydrolyze the alpha-1,6 glycosidic linkage in pullulan, starch, amylopectin, and related oligosaccharides. Pullulanase is used for the production of glucose or maltose syrups in a two-stage liquefaction and saccharification process. Temperature variation in the saccharification process causes deactivation of enzymes and therefore the increase of enzyme costs, so that application of thermostable pullulanase could decrease the industrial costs and increase the process efficiency by merging the two-step liquefaction and saccharification scheme into one stage. The enzyme from strain *Geobacillus* sp. LM14-3 showed maximum activity at 60°C and pH 6.5 with a half-life time about 56 h, which favors the potential industrial application [96].

Xylan is a major component of hemicellulose. Its complete hydrolysis requires the combined action of various enzymes, like endo-1,4- $\beta$ -xylanase,  $\beta$ -xylosidase,  $\alpha$ -arabinofuranosidase, acetylxylan esterase,  $\alpha$ -glucuronidase, and feruloyl esterase. Applications of xylanases include food, pulp and paper industry, and agriculture. The great majority of these enzymes are optimally active in the acidic or neutral pH range at temperatures up to 60°C. Since the incoming pulp for enzymatic bleaching is hot and alkaline, the use of thermostable alkaline xylanases is very attractive from economical and technical point of view. *Geobacillus* sp. 71 produces xylanase at optimum temperature 75°C and pH 8.0, but it is active over a broad pH range. The action of the enzyme on oat spelt xylan produced xylobiose and xylotetrose. Due to its characteristics, the enzyme is a promising candidate for the production of xylosaccharides in the pulp and paper and food industries [97]. Xylanase of *G. thermoleovorans* was optimally active at pH 8.5 and 80°C, and it was found to be

useful in the pulp prebleaching process, since cellulases may adversely affect the quality of the paper pulp by destroying the structure of cellulose [98]. The immobilized xylanase from thermophilic *Geobacillus* sp. TF16 displayed an increase in optimum temperature from 55 to 65°C and shift in the pH value from 6.0 to 8.5 as compared to the free enzyme. The enzyme was found to be effective in release of the reducing sugars from juice and poultry feed and oven spring in bakery [99]. The  $\beta$ -xylosidase of the extremely thermophilic *G. thermodenitrificans* is optimally active at 60°C and pH 7.0 and it catalyzes transxylosylation reactions in the presence of alcohols as acceptors. The pharmaceutically important  $\beta$ -methyl-D-xylosides could be produced using  $\beta$ -xyloside as the donor and methanol as acceptor [100]. L-Arabinosyl residues widely distributed in hemicelluloses constitute monomeric and/or oligomeric side chains on the  $\beta$ -(1  $\rightarrow$  4)-linked xylose or galactose backbones in xylans, arabinoxylans, and arabinogalactans and make up the core in arabinans forming  $\alpha$ -(1  $\rightarrow$  5)-linkages.  $\alpha$ -L-Arabinofuranosidases are enzymes hydrolyzing terminal nonreducing  $\alpha$ -L-arabinofuranosyl groups in L-arabinose-containing polysaccharides.  $\alpha$ -L-Arabinofuranosidase from *G. caldoxylolyticus* TK4 released L-arabinose from arabinan and arabinooligosaccharides. No endoarabinanase activity was detected. L-Arabinose has a sweet taste, is not readily assimilated by the body, hence it can be used as a food additive [101].

Gellan synthesized by *Sphingomonas paucimobilis* is the microbial exopolysaccharide finding recently extensive use in food, pharmaceutical industries and in microbial cultivation media. Gellan lyase lowers the gellan viscosity in solutions and might broaden its current spectrum of application. However, gellan is soluble at temperatures higher than 50°C, so that industries need a thermostable gellan lyase. The enzyme from *G. stearothermophilus* 98 demonstrated maximum activity at 70°C. The thermal denaturation curve of the enzyme at 214 nm showed a highly cooperative transition with a midpoint at about 75°C. Kinetic studies indicated high affinity of the enzyme to gellan as a substrate. A single product was identified after enzyme action on gellan [102].

$\beta$ -Galactosidases catalyze hydrolysis of lactose into glucose and galactose and take part in transgalactosylation reaction that produces galactooligosaccharide.  $\beta$ -Galactosidase has been used in biopharmaceutical, food, and dairy industries to prevent crystallization of lactose, to improve sweetness, to increase the solubility of milk products, to prepare low lactose-containing food products for relatively lactose-intolerant people, and to utilize cheese whey, which would otherwise become an environmental pollutant [103]. Some *Geobacillus* species possess these enzymes. Evolved  $\beta$ -galactosidases BgaB from *G. stearothermophilus* KVE39 provide for transglycosylation of lactose into oligosaccharide characterized as 3'-galactosyl-lactose. Galactooligosaccharides are established prebiotic food ingredients used to promote the development of bifidobacteria and lactobacilli in the large intestine in order to reduce growth of pathogenic microorganisms [104].

Monosaccharides are applied as low-calorie sweeteners, bulking agents, antioxidants, glycosidase inhibitors, and nucleoside analogs. Production of monosaccharides can be catalyzed by isomerases. A recombinant mannose-6-phosphate isomerase from *G. thermodenitrificans* isomerizes aldose substrates possessing hydroxyl groups oriented in the same direction at the C2 and C3 positions such as the D- and L-forms of ribose, lyxose, talose, mannose, and allose. The enzyme was shown to catalyze the conversion of D-lyxose to D-xylulose with 38% yield after 3 h, and conversion of L-ribose to L-ribulose with 29% yield [105].

Keratin is the insoluble fibrous hard-to-degrade protein of feathers, wool, hair, including other epidermal appendages. It accounts for nearly 90–95% of feather weight, which constitutes up to 10% of the total chicken weight. The increased amounts of keratin by-products may represent a pollution problem. Keratinolytic

proteinases or keratinases are known to utilize insoluble substrates such as fibrin, keratin, elastin, and collagen, and soluble substrates such as sodium caseinate, albumin, and gelatin. These enzymes have potential outlets in biomedicine, pharmaceuticals, cosmetics, and waste bioconversion. A keratinolytic proteinase from *G. stearothermophilus* AD-11 shows optimal activity at pH 9 and 60°C and degrades keratin from wool > collagen > sodium caseinate > gelatin > and bovine serum albumin in descending order with production of high-value small peptides suitable for industrial applications [106].

Hydroxamic acids are weak organic acids. They are key pharmacophores in chemotherapeutic formulas possessing a wide spectrum of activities as growth factors, food additives, tumor inhibitors, antimicrobial, antituberculosis and antileukemic agents. Acetohydroxamic acid is a potent and irreversible inhibitor of bacterial and plant urease used in adjunctive therapy of chronic urinary infections. It also selectively shows anti-HIV activity and inhibits arachidonate 5-lipoxygenase, demonstrating potential use in the treatment of asthma. Intracellular amidase of *G. pallidus* BTP-5x MTCC 9225 showed complete conversion of acetamide to acetohydroxamic acid in 1 h at 50°C. At 65°C, the rate of reverse reaction was found to be higher. The acetamide bioconversion rate for the strain was 90–95% and 51 g powder containing 40% acetohydroxamic acid was recovered after lyophilization [107].

Chiral  $\alpha$ -hydroxy acids are used in the production of pharmaceuticals and other fine chemicals. L-Lactate dehydrogenase from the thermophilic organism *G. stearothermophilus* may be employed for the industrial synthesis of chiral  $\alpha$ -hydroxy acids. It is also possible to engineer the enzyme with enhanced activity toward the selected  $\alpha$ -keto acids, besides natural substrates [108].

Recombinant alcohol dehydrogenase from *G. stearothermophilus* takes part in the biocatalytic synthesis of  $\omega$ -oxo lauric acid methyl ester (OLAME), a key intermediate in bio-based polyamide 12 production from the corresponding long-chain alcohol. The enzyme provides for up to 23% conversion of the substrate to OLAME after 30 min. No overoxidation to the dodecanoic diacid monomethyl ester was detected. Thus, the engineered alcohol dehydrogenase is a promising biocatalyst for industrial polymer production [109].

Amidases are among the most widely used amide-hydrolyzing enzymes. They find use as catalysts in the treatment of industrial effluents containing toxic amides, in organic synthesis, and as therapeutic agents. The amidase of *G. pallidus* RAPc8 exhibited high thermal stability at 50 and 60°C, with half-lives over 5 h at both temperatures. At 70 and 80°C, the half-life values were 43 and 10 min, respectively. The enzyme catalyzed the hydrolysis of low molecular weight aliphatic amides, with D-selectivity toward lactamide. Acyl transfer reactions were demonstrated with acetamide, propionamide, isobutyramide, and acrylamide as substrates and hydroxylamine as the acyl acceptor. This amidase shows potential for application as a biocatalyst for D-selective amide hydrolysis yielding enantiomerically pure carboxylic acids and for the production of novel amides by acyl transfer [110]. Acrylamide is extensively used as a monomer in the synthesis of polyacrylamides, in dye composition, in gels for electrophoresis, in contact lenses, in food wraps, and in construction grouts. However, it displays many toxic properties such as neurotoxicity, genotoxicity, and carcinogenicity and can cause water and soil pollution. Acrylamidase from *G. thermoglucosidasius* AUT-01 is able to transform acrylamide to acrylic acid with pH and temperature optima of 6.2 and 70°C [111].

L-Nucleosides have been widely used as nucleoside-analog drugs in the treatment of severe viral diseases because they have more potent biological activities and lower toxicities than the corresponding D-nucleosides. L-Ribose is a potential starting material for the synthesis of many antiviral drugs, such as L-nucleoside

derivatives. A triple-site variant of mannose-6-phosphate isomerase from *G. thermodenitrificans* demonstrated 3.1 and 7.1 times higher specific activity and catalytic efficiency, respectively, for L-ribulose isomerization compared to the wild-type enzyme at pH 7.0 and 70°C. The triple-site variant produced 213 g/l L-ribose from 300 g/l L-ribulose by 60 min, which exceeded 4.5 times the level of the wild-type enzyme. The specific activity, catalytic efficiency, and productivity of the variant were approximately two-fold higher than those of the *Thermus thermophilus* R142N isomerase, which exhibited the highest values previously reported [112]. Recombinant thermostable enzymes purine nucleoside phosphorylase II and pyrimidine nucleoside phosphorylase from *G. stearothermophilus* B-2194 retained high activity after 20 reuses in nucleoside transglycosylation reactions at 70–75°C with yields of the target products as high as 96%. These enzymes are suitable for the production of pharmacologically important natural and modified nucleosides [113].

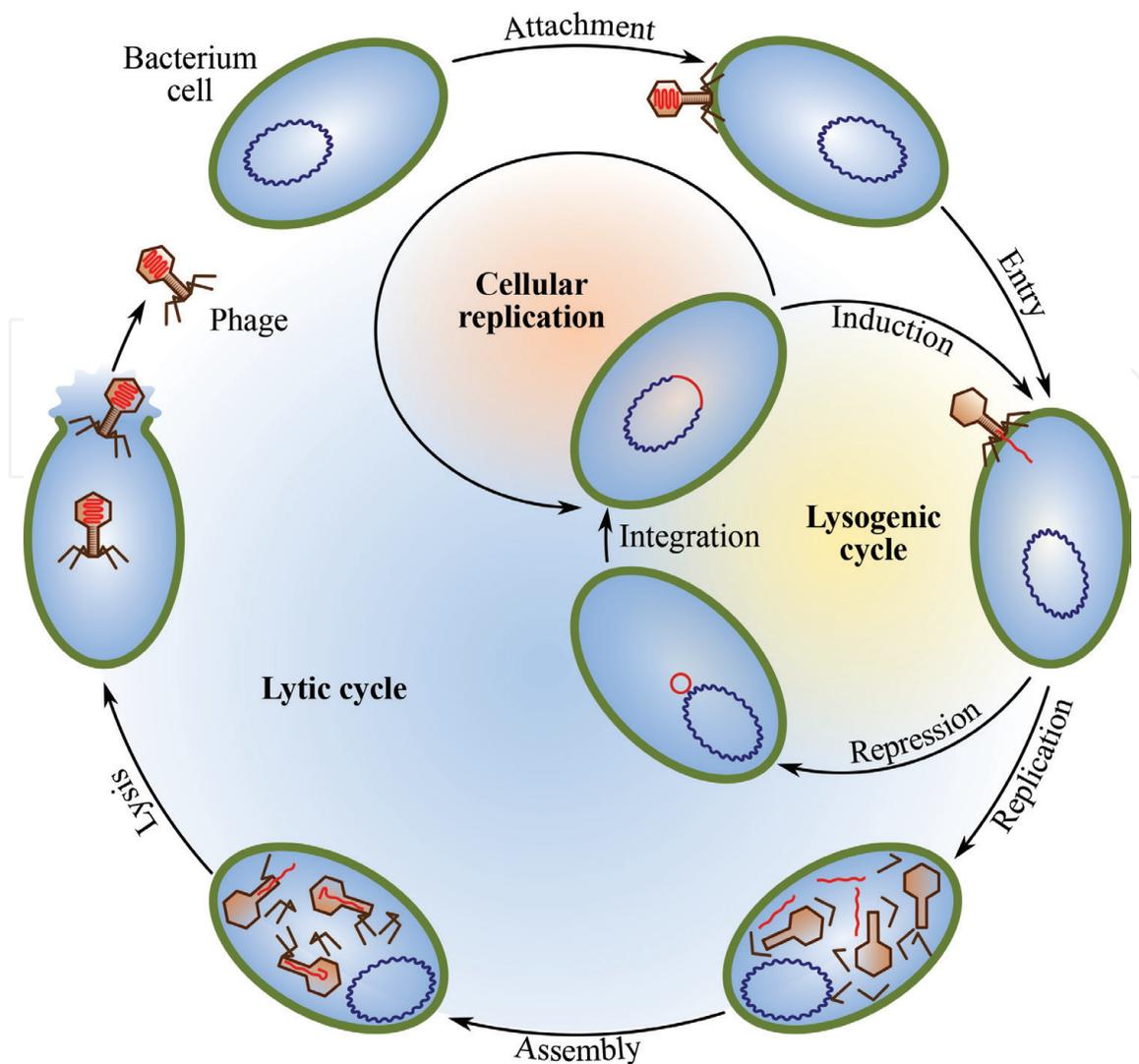
Phytic acid is the most important storage form of inositol and phosphate in plants constituting approximately 5% of the dry weight of seeds in legumes and grain cereals. Phytic acid can form complexes with proteins and ions such as magnesium, calcium, zinc, and iron and act as antinutritional factor. Addition of phytases to animal feed can be an effective strategy to decrease phosphorus contamination and increase the bioavailability of phosphorus and essential minerals to animals. After 4 h incubation, hydrolysis capacity of chitosan- and Ca-alginate immobilized phytases of *Geobacillus* sp. TF-16 for soy milk phytate was calculated as 24 and 33%, respectively. The chitosan- and Ca-alginate immobilized enzymes conserved their original activity after 8 and 6 cycles of reuse, respectively [114].

Bacteria modulate their population density via the regulatory mechanism called quorum sensing. The latter takes part in bioluminescence, antibiosis, biofilm development, and control of expression of virulence genes. The Gram-negative bacteria use N-acylhomoserine lactone (AHL) as the quorum sensing signal. *G. caldoolosilyticus* YS-8 was found to produce AHL lactonase degrading various AHLs. This enzyme can be further used in medical applications [115].

Some enzymes of *Geobacillus* species can be engaged in biotechnologies. GeoICI from *Geobacillus* sp., a member of atypical IIS restriction endonucleases, recognizing/cleaving 5'-GCAGC(N<sub>8/12</sub>)-3' DNA sequences is highly active at elevated temperatures, up to 73°C and over a very wide salt concentration range, and hence can be applied in DNA manipulations [116]. β-Galactosidases are also objects for molecular applications in thermophiles and under anaerobic conditions. The enzyme from *G. stearothermophilus* functions both as a marker, when it cleaves thermostable dye, 3,4-cyclohexenoesculetin β-d-galactopyranoside (S-gal) to black product, and as a reporter enabling quantitative measurement by a simple colorimetric assay [117].

Aldehyde dehydrogenases are a group of diverse enzymes catalyzing the oxidation of aldehydes to carboxylic acids, using NAD<sup>+</sup> or NADP<sup>+</sup> as the coenzyme. The enzyme from *G. thermodenitrificans* NG80-2 demonstrated a broad substrate range including both aliphatic and aromatic aldehydes. It is expected to play a role in the degradation of alkanes and aromatic hydrocarbons present in crude oil. The aldehyde dehydrogenase activity was detected in the temperature range from 40 to 70°C, and in the pH range from 6.0 to 8.8. The optimum temperature was determined to be 60°C and the optimum pH 8.0. The enzyme was inactivated after incubation at 80°C [118].

Purification protocols for enzymes include several steps. The initial step is the release of the enzyme from the cell material, if the protein is not secreted by the organism into the surrounding solution. This procedure requires either a mechanical or chemical lysis of the cells. Techniques vary from gentle methods such as osmotic shock, detergent lysis, or enzymatic digestion to more vigorous methods



**Figure 4.**  
Bacteriophage lytic and lysogenic cycles.

such as homogenization in a blender, grinding with an abrasive substance or ultrasonication. The selected procedure must not damage the target enzyme, and therefore, the conditions must be optimized for each cell type and target enzyme in terms of the pH of the extraction buffer, the temperature, and the concentration of certain components of the buffer such as detergents, salts, or reducing agents. The next step after clarification is the concentration of the enzyme preparation. Dialysis can be performed prior to this procedure in order to remove salts from the cell extract. The most common concentration procedures are ammonium sulfate precipitation, ultrafiltration, and ion exchange chromatography [119]. Purification of *Geobacillus* proteins follows the same steps. Cell-free supernatant of  $\alpha$ -1,4-glucosidase-producing strain *G. thermodenitrificans* F84a was removed from the medium by centrifugation, fractionated with solid ammonium sulfate, dialyzed overnight and centrifuged to remove insoluble residues. Then, the sample was concentrated 6.5-fold by centrifugation, suspended in Tris-HCl buffer (pH 8.0) and applied to cation-exchange chromatography [86]. The culture broth with amylase-producing *Geobacillus* sp. IPTN was centrifuged; then supernatant was collected and filtered followed by ion-exchange chromatography [84]. Cells of amidase-producing *G. pallidus* RAPc8 were harvested by centrifugation and resuspended in an appropriate amount of potassium phosphate buffer. Cell lysis was achieved by freezing the cells at  $-20^{\circ}\text{C}$  overnight, thawing at room temperature and sonication for 6–10 cycles. After subsequent centrifugation, the soluble fraction was

heat-treated. Then, precipitated proteins were removed by centrifugation. Further purification was performed by gel exclusion chromatography [110].

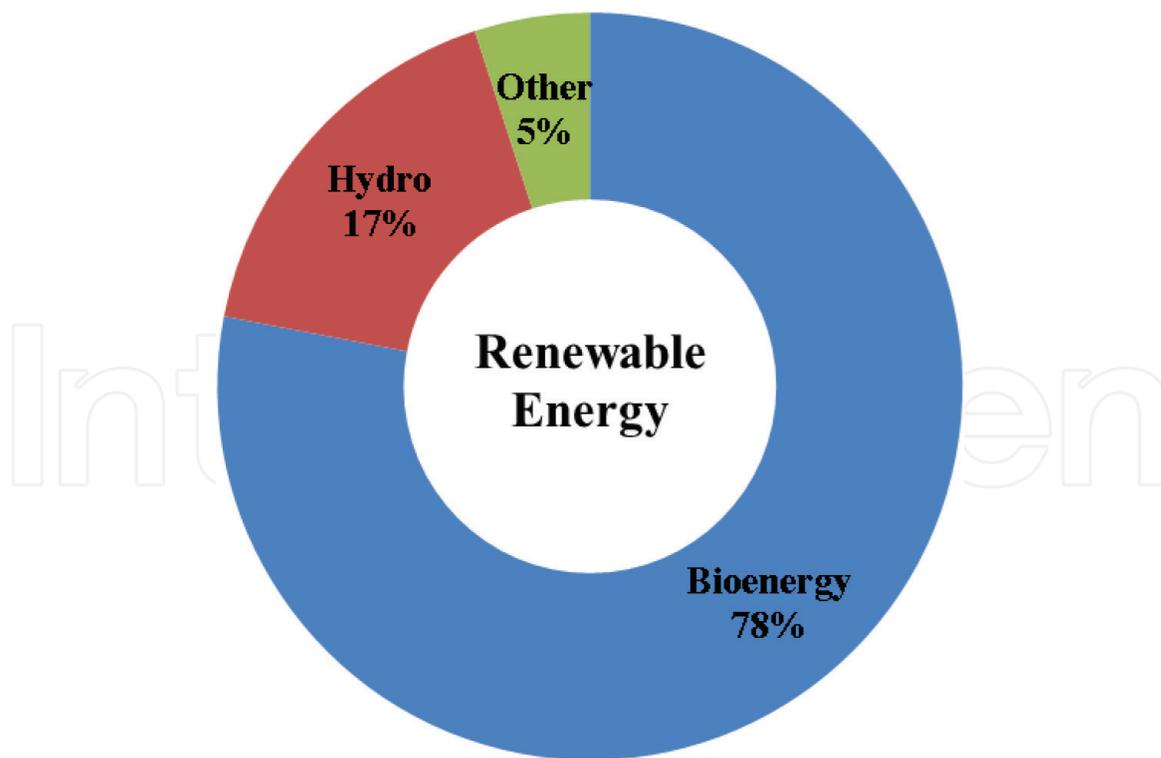
Besides bacteria themselves, their bacteriophages are also potential sources of enzymes. Viruses constitute a major component of the biosphere, playing a significant role in nutrient and energy turnover of carbon, nitrogen, and phosphorus, and producing important impact on the evolution of their hosts. Thermophilic viruses are worse studied compared to the mesophilic viruses, and the majority of them infect archaeal genera. Generally, the life cycle of bacteriophage includes several programmed steps, such as phage adsorption to host cell surface, injection of phage genomic DNA into bacterial cell, metabolic transition from host to phage, phage genome replication, phage morphogenesis, phage package, and lysis of the host (**Figure 4**). Bacteriophage-host protein interactions in high-temperature environment remain poorly understood. Nevertheless, studies are carried out, like the discovery that the host's aspartate aminotransferase, chaperone GroEL, and viral capsid protein VP371 of bacteriophage GVE2 (host *Geobacillus* sp. E263) formed a linearly interacted complex for protection of the virus reproduction in high-temperature environment [120]. Because of adaptation to extreme conditions, such bacteriophages can be used as sources of ligases, polymerases, and nucleases for biotechnological processes [121].

## 6. Bioenergy technologies: the potential of biological processes

The growing demand for energy to keep up with the industrial spurt and the rampant urbanization has created a huge shortfall, urging to resort to alternative energy options. The global bioenergy market is anticipated to reach \$246.52 billion by 2024 from \$158.39 billion in 2015. During the forecast period of 2016–2024, the global market is expected to rise at a compound annual growth rate of 4.9% [122]. Bioenergy is renewable resource derived from organic matter (biomass), i.e., all materials of biological origin not embedded in geological formations (fossilized). Biomass can be used in its original form as fuel, or be refined to different kinds of solid, gaseous, or liquid biofuels. The supply of biomass can be provided from forestry, agriculture, industrial, and municipal waste [123].

Bioenergy is divided into three broad categories: solid biomass (e.g., wood, harvesting residues), liquid biofuels (e.g., bioethanol, biodiesel), and gaseous biofuels (e.g., biogas). Bioenergy accounted for 14% of the global energy consumption in 2012 with roughly 2.6 billion people dependent on traditional biomass for power supply (**Figure 5**). USA and Brazil lead the world in production and consumption of liquid biofuels for transport accounting for almost 80% of the market. The production of all biofuels in the Americas increased from about 16 billion liters in 2000 to 79 billion liters in 2012. The use of biomass for electricity is prominent in Europe and North America—predominantly produced from forestry products and residues. The Europe and American continent contribute more than 70% of overall consumption of biomass for electricity. In 2013, 462 TWh of electricity was produced globally from biomass. The major use of biomass is household heating in rural and developing countries [123].

Bioethanol is a biodegradable, renewable energy resource which is produced from biomass through sugar fermentation and chemical process. It forms an attractive alternative to conventional fuel sources owing to its high octane value and lower greenhouse gas emissions. Bioethanol can be used as a motor fuel, fuel for power generation, feedstock for chemical industry, substrate for fuel cells and cogeneration systems, in cosmetic technology, and in manufacturing processes due to its clean burning and ready availability [124]. The global bioethanol market is projected to reach USD 68.95 billion by 2022, at a compound annual growth rate of 5.3% between 2017 and 2022 [125].



**Figure 5.**  
Global renewable energy consumption.

Ethanol produced from renewable sources by fermentation is the most promising biofuel and the starting material for various chemicals. Substrates for ethanol production can be classified into three main groups: (1) those containing considerable amounts of readily fermentable sugars (sugarcane, sugar beet, and sweet sorghum), (2) starches and fructosans (corn, potatoes, rice, wheat, and agave), and (3) cellulosics (stover, grasses, corn cobs, wood, and sugarcane bagasse) [126]. Ethanol-producing microorganisms such as *Saccharomyces cerevisiae* and *Zymomonas mobilis* lack amylases and cellulases and are unable to directly convert starch and cellulosics into ethanol. Traditionally, the starch is hydrolyzed enzymatically into fermentable sugars via liquefaction and saccharification processes prior to ethanol fermentation [82].

Biodiesel is a mixture of long-chain monoalkylic esters of fatty acids obtained from renewable resources, to be used in diesel engines, alone or blended with diesel oil. The global biodiesel market size was estimated as USD 28.04 billion in 2016 and is likely to reach USD 54.8 billion by 2025. The market is expected to expand at 7.3% compound annual growth rate owing to increasing demand for biodiesel as automobile fuel with eco-friendly characteristics. The automotive fuel segment accounts for over 75% of the market [127]. The raw materials for biodiesel production are vegetable oils, animal fats, and short-chain alcohols. Biodiesel is produced by transesterification reaction. This chemical reaction converts vegetable oil or animal fat into a mixture of esters of the fatty acids that make up oil or fat. Biodiesel is derived by purification of the mixture of fatty acid methyl esters. Transesterification can be basic, acidic, or enzymatic. In the latter case, lipases are used [128].

Global isobutanol market demand was estimated at 552.4 kilo tons in 2014. It is used as a raw material for the production of various chemicals, including cleaners and coating solvents, isobutyl esters, extractants for pharmaceuticals, textiles, polish additive, gasoline admixture, agricultural products, and biofuel. The global isobutanol market is expected to reach USD 1.18 billion by 2022. Synthetic isobutanol was the largest product segment accounting for 58.1% of total market volume in 2014. Bio-based isobutanol is estimated to witness the highest growth of 7.0%

from 2015 to 2022 [129]. Isobutanol can be a better biofuel than ethanol due to its higher energy capacity and lower hygroscopicity. Furthermore, the branched-chain structure of isobutanol gives a higher octane number than the isomeric *n*-butanol. Bacteria have been shown to produce this compound, but isobutanol demonstrated toxicity to cells, forcing to use more resistant microorganisms [130].

Biogas, a mixture of methane and carbon dioxide, is produced from the methanogenic decomposition of organic waste under anaerobic conditions. Between 2009 and 2015, the number of biogas plants in Europe increased significantly from around 6000 to nearly 17,000. Total European Union biogas primary energy production in 2014 was estimated at 14.9 Mtoe, up 6.6% from the previous year. About 57 TWh of EU electricity was produced from biogas in 2014, up 9% from 2013. However, biogas share is only 1.9% of the total electricity generation. USA has been slower in launching biogas plants, with around 2200 in operation, of which the majority are run at wastewater treatment facilities [131].

*Geobacillus* species have been shown to produce or stimulate synthesis of some biofuels. Nevertheless, investigations of *Geobacillus* role in these processes started recently.

## 7. *Geobacillus* bacteria in bioenergy technologies and applications

The demand for energy production is at an all-time high level. Fossil materials, like coal and oil, are important fuels; however, they are considered as non-renewable resources raising serious environmental concerns. Bioenergy is the alternative source of fuels. Multiple approaches are currently explored for the use of microorganisms in the production of biofuel (alcohols, hydrogen, biodiesel, and biogas) from various starting materials. *Geobacillus* species able to synthesize a wide range of enzymes and resistant to high temperatures, allowing to minimize the risk of contamination and reduce energy consumption for product separation and fermenter cooling, appear excellent agents for bioenergy production.

Ethanol can be produced through fermentation by various strains of mesophilic bacteria, yeasts, and fungi. Thermophiles are able to utilize a wide range of sugars at high temperature converting them into ethanol. Most organisms in this class do not naturally carry out homoethanol fermentation and do not naturally exhibit high product tolerance, demanding additional metabolic engineering [132]. Thermophilic bacterium *G. thermoglucosidasius* M10EXG fermented a range of C5 (e.g., xylose) and C6 sugars (e.g., glucose) and was tolerant to high ethanol concentrations (10%, v/v). The carbon flux during microaerobic growth was directed to ethanol, L-lactate (>99% optical purity), acetate, and formate. Under fully anaerobic conditions, the strain was involved in a mixed acid fermentation process, achieving a maximum ethanol yield of  $0.38 \pm 0.07$  mol/mol glucose [133]. Strains of *G. thermoglucosidasius* have been engineered to divert their fermentative carbon flux from a mixed acid pathway to that where ethanol becomes the major product. The triple mutant TM242 generated ethanol from glucose at yields higher than 90% of the theoretical value 0.51 g/g with productivity 2.85 g/l/h. In addition, it was particularly rapid in the metabolism of cellobiose (productivity 3.2 g/l/h) [134]. Bacterial enzymes can be used in ethanol production. About 20 and 30% raw pearl millet starch exposure to the above-mentioned hyperthermostable and  $\text{Ca}^{2+}$ -independent  $\alpha$ -amylase of *G. thermoleovorans* NP54 for 3 h resulted in the sugar yields of 68 and 55.8%, respectively. Upon subsequent treatment with amylopullulanase of *G. thermoleovorans* NP33 for 4 h, 85 and 80% starch saccharification rates were attained from 20 and 30% raw starch, respectively. Saccharification was further enhanced to 98 and 92.4%, respectively, when the hydrolysate was

treated with glucoamylase of *Thermomucor indicae-sedaticae* for 12 h. Following fermentation of reducing sugars in the hydrolysates, ethanol production levels by *S. cerevisiae* were 35.40 and 28.0 g/l [82].

As mentioned above, biodiesel is produced via transesterification reaction, and in some cases, lipases are used. Lipase from *G. thermodenitrificans* AV-5 showed molecular weight 50 kDa, temperature and pH optima, 65°C and pH 9.0, respectively, and was able to efficiently convert waste cooking oil and coconut oil to biodiesel with yields 76 and 45.5%, respectively [135]. The enzymatic reaction system enables to utilize low-grade and low-cost feedstock with high free fatty acid or water content. It reduces the amount of alkali wastewater that requires treatment and promotes easy recovery of the main product and the by-product glycerol. In addition, the biocatalyst is biodegradable and when immobilized can be reused for many production cycles and can lower the operation cost of the process. However, the major drawbacks of the enzymatic system are relatively high biocatalyst cost and its limited stability in the presence of high methanol concentrations. In order to achieve complete conversion of one oil molar equivalent to fatty acid methyl ester, three molar equivalents of methanol are necessary. Nevertheless, in reaction systems with more than 1.5 methanol equivalents, the methanol is not completely dissolved and its droplets stay in the mixture leading to enzyme unfolding and inactivation. The triple mutant lipase of *G. stearothermophilus* T6 showed a half-life value of 324 min in the presence of 70% methanol, which reflects 87-fold enhanced stability as compared to the wild type. This variant also exhibited an improved biodiesel yield from waste chicken oil when compared to commercial Lipolase 100L<sup>®</sup> and Novozyme<sup>®</sup> CALB [136]. The recombinant lipase from *G. stearothermophilus* G3 immobilized on the aminated silica gel can be used as a biocatalyst for the preparation of fatty acid methyl esters from vegetable oils. The optimum reaction parameters allowed to produce fatty acid methyl esters with 40–43% yield within 96 h [137].

Isobutanol biosynthesis shares intermediates with the valine biosynthesis pathway, which exists in most microorganisms, including *Geobacillus*. Engineered *G. thermoglucosidasius* variant produced 3.3 g/l of isobutanol from glucose and 0.6 g/l of isobutanol from cellobiose by 48 h at 50°C, demonstrating stable isobutanol generation in recombinant bacteria at an elevated temperature [138]. The other *G. thermoglucosidasius* culture was able to produce isobutanol in amounts around 50 mg/l via the conversion of isobutyryl-CoA to isobutyraldehyde by aldehyde dehydrogenase and from isobutyraldehyde to isobutanol by alcohol dehydrogenase. It was observed that supplementing the growth medium with an intermediate of the valine biosynthesis pathway, 2-ketoisovalerate, resulted in increased isobutanol titers [139].

Acetoin is widely used in food processing, flavoring, cosmetic formulation and chemical synthesis, while its reduced form, 2,3-butanediol, compares favorably with ethanol (29,100 J/g) and methanol (22,100 J/g) in heating value (27,200 J/g), so that 2,3-butanediol can be used as a liquid fuel or fuel additive. Its dehydration yields the industrial solvent methyl ethyl ketone, which can be hydrogenated to high octane isomers, suitable for high-quality aviation fuels. Acetoin and 2,3-butanediol are currently fermented below 40°C using mesophilic strains, but the processes often suffer from bacterial contamination. The application of thermophilic strains is able to meet the challenge. *Geobacillus* strain XT15 generated 7.7 g/l of acetoin and 14.5 g/l of 2,3-butanediol when incubated with corn steep liquor as a nitrogen source at 55°C. Acetoin, 2,3-butanediol, and their derivatives accounted for about 96% of total volatile products, while organic acids and other metabolites were minor by-products [140].

Due to its high organic content, sewage sludge is used as a substrate for anaerobic digestion to recover biogas. Anaerobic digestion of organic waste material is an effective technology for both waste disposal and energy generation. The addition of sludge with aerobic thermophilic (AT) bacteria (closely related to *G. thermodenitrificans*) to methanogenic sludge enhanced the production of biogas. The optimum added volume and the pretreatment temperature of the AT sludge for optimum biogas production were 5% and 65°C. The AT sludge inoculated with the AT seed sludge (mass of sludge containing populations of microorganisms) improved biogas production by 2.2 times. The addition of bacterial culture reduced volatile solids by 21%, which was higher than 12.6% achieved with the sewage sludge addition. The bacteria enhanced biogas production more than AT seed sludge [141]. The increased biogas production from anaerobic digestion of sewage sludge can be caused by the protease activity [142].

Microbial fuel cells (MFCs), another power-generating system, utilize bacteria acting as living catalysts to convert organic substrates into electricity. MFCs lack viability in most applications and demand optimization. Recently, it has been shown that *Geobacillus* species can be used for energy generation. However, *Geobacillus* strain could not produce current in the absence of an exogenous electron shuttle [143].

## 8. Conclusion

The *Geobacillus* species are represented by Gram-positive, aerobic or facultatively anaerobic, spore-forming, rod-shaped cells. The bacteria able to survive and grow at high temperatures can be found everywhere. At present, *Geobacillus* genus comprises about 20 species, but they are quite heterogeneous and require reclassification.

Owing to the ability of geobacilli to utilize and to produce a wide range of substances, coupled with resistance to high temperatures, they are considered as promising agents for many biological processes. *Geobacillus* genus is a source of a vast array of thermostable enzymes: amylases, lipases, pectinases,  $\beta$ -galactosidases, endonucleases, etc. Applications of bacteria and their enzymes range from food industry and medicine to molecular biology and bioremediation. New investigations concerning *Geobacillus* characteristics and spheres of use are carried out every year.

The growing demand for energy urges researchers and manufacturers to resort to alternative options. Bioenergy is the attractive source of fuels. It accounted for 14% of the global energy consumption in 2012. Multiple approaches are currently being probed to use microorganisms in the production of various fuels from diverse materials. *Geobacillus* genus demonstrated ability to produce or stimulate synthesis of some biofuels. Generation of biofuels coupled with resistance to high temperatures allows to enlist *Geobacillus* bacteria as potential candidates for bioenergy projects.

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