Bacillus subtilis

Bacillus subtilis- An Overview and Applications

Bacillus subtilis is the type species of the genus *Bacillus* which is commonly found in diverse environments ranging from soil to the gastrointestinal tract of cattle and humans.

It is a Gram-positive, rod-shaped, spore-forming, and facultative anaerobe that is the most commonly isolated *Bacillus* species from environmental samples.

B. subtilis has been extensively studied as a model for cell differentiation and engineering in biotechnology. It is also known as **hay *Bacillus*** or **grass *Bacillus*** as it is widespread in different types of grasses and hay sources.

B. subtilis is the most studied Gram-positive bacterium as it is studied as a model organism for studies regarding bacterial chromosome replication and transformation. It is ubiquitous in distribution which is facilitated by the resistance of the bacteria to cold, heat, and common disinfectants.

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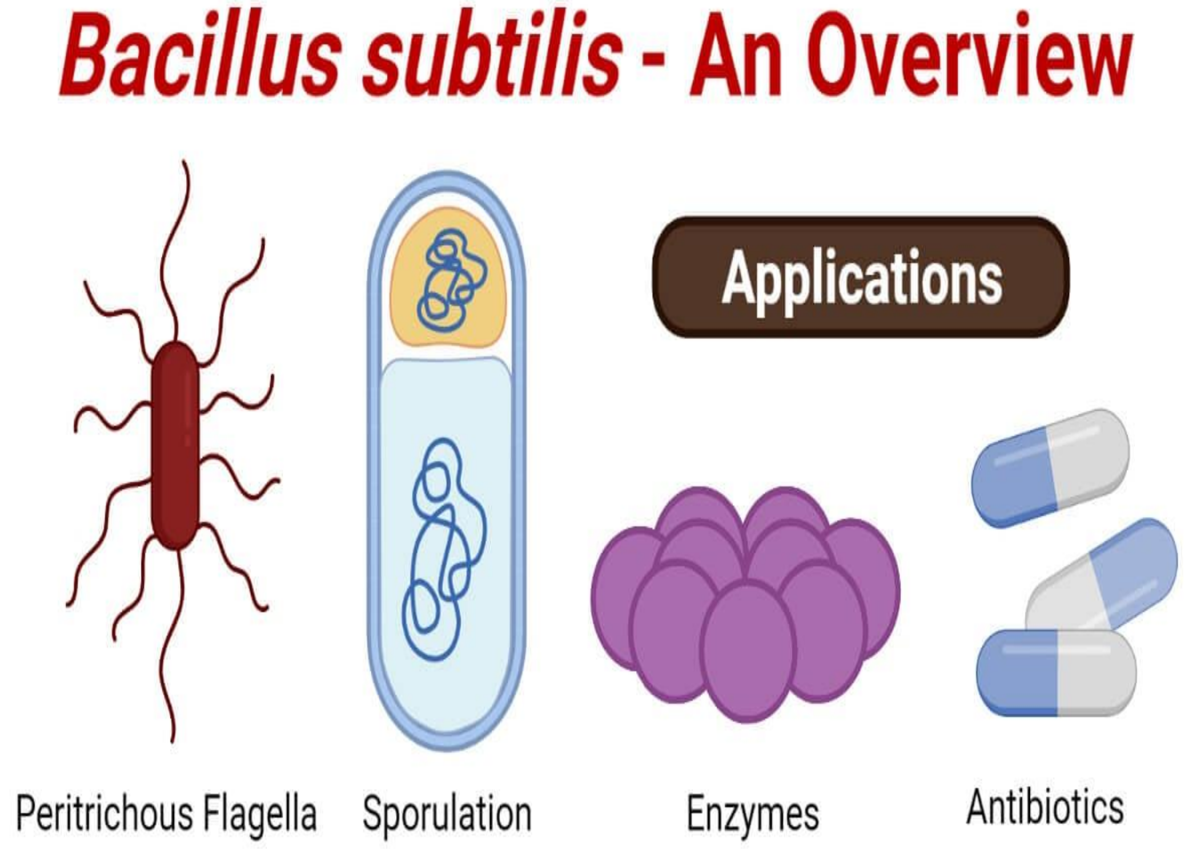

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Most *B. subtilis* species are non-pathogenic and are not associated with infections, but some strains have been associated with neoplastic diseases like fatal pneumonia and bacteremia, septicemia, and infections of necrotic axillary tumors in breast cancer. Some strains have also been implicated in foodborne illness and cases of bovine mastitis and ovine abortion.

B. subtilis was first isolated in 1835 by Ehernberg who also named the bacterium, *Vibrio subtilis*, but it was later reclassified and named by Cohn in 1872. The species name *'subtilis'* is a Latin word that means 'slender' indicating the long rod-shaped structure of the bacteria.

B. subtilis is considered an essential industrial bacterium as it is widely used in biotechnology due to the improved expression and secretion of enzymes by the bacteria. Besides, it is also used in food industries as flavor enhancers, sweeteners, and animal feed. *B. subtilis* are further classified into two subspecies; *Bacillus subtilis* subsp. *subtilis* and *Bacillus subtilis* subsp. *spizizenii*.

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Classification of *Bacillus subtilis*

- The genus *Bacillus* belongs to the family Bacillaceae on the basis of phylogenetic analysis of 16S rRNA gene sequences.
- The family consists of *Bacillus* and 18 other genera, the majority of which are aerobic or facultatively anaerobic chemoorganotrophs.
- The genus *Bacillus* consists of more than 100 different species which are grouped into manageable and better-defined groups.
- The initial classification of *Bacillus* species was based on phenotypic, cultural, and metabolic characteristics of the bacteria.
- The grouping of species is based on the similarity of the 16S rRNA gene sequences and DNA-DNA hybridization.
- *B. subtilis* belongs to group 1 of Bacillus, and it is closely related to other species like B. licheniformis and the pathogenic group of Bacillus species including B. cereus, B. anthracis, and *B. thuringiensis*.
- Group 1 of *Bacillus* species consists of different species that are industrially important for the production of different compounds.
- The *B. subtilis* species are further classified into two subspecies; *B. subtilis* subsp. *subtilis* and *Bacillus subtilis* subsp. *spizizenii.* These subspecies cannot be distinguished on the basis of phenotypic characteristics and require genotypic analysis.
- The following is the taxonomical classification of *B. subtilis:*

Domain	Bacteria

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Phylum	Firmicutes
Class	Bacilli
Order	Bacillales
Family	Bacillaceae
Genus	*Bacillus*
Species	*B. subtilis*

Habitat of *Bacillus subtilis*

- *Bacillus* is ubiquitous in distribution and is found in various habitats throughout the world, ranging from soil to human and animal bodies.
- Most aerobic endospore-forming bacteria like *Bacillus* are saprophytes that are distributed in the natural environments in the form of spores.
- However, some might be found in animate surfaces like tissues as opportunistic or obligate pathogens.
- The most important habitat of *B. subtilis* is soils of different kinds, ranging from acid to alkaline, cold to hot, and fertile to the desert. The types of strains living in the habitats depend on the water content and deposits.
- The availability of *B. subtilis* in different environments is due to the distribution of bacterial spores in the form of aerosols.
- Besides, *B. subtilis* can survive in a wide range of different temperatures from 15°C to 55°C.
- Some variety of *B. subtilis* can be found as ubiquitous contaminants of food, water, and environments that are natural, domestic, industrial, and hospital.

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- The endospores formed by *B. subtilis* are resistant to physical and chemical agents like temperature, disinfectants, antibiotics, and toxic compounds.
- Bacterial spores are also found in high concentrations in dried foods like spices, milk powder, and other products.
- These spores are dispersed easily by the wind, which allows the spores to migrate to long distances and discover new ecological niches.
- *B. subtilis* are heterotrophic organisms that are isolated from environments with complex nutrient availability and environmental conditions.
- The occurrence of *B. subtilis* in soil and the rhizospheric area might exist in a close relationship with the plants by helping in the production of phytohormones and enhancement of root nodulation.
- Some spores of *B. subtilis* can also be found in animal surfaces like the human intestinal tract and skin surfaces and can be isolated from samples like human feces.

Morphology of *Bacillus subtilis*

- *Bacillus subtilis* is a Gram-positive, rod-shaped bacterium that is the type species of the genus *Bacillus*, commonly used as a model organism to describe the structure of different species of the genus.
- The cells of *B. subtilis* are Gram-positive motile rods that form ellipsoidal to cylindrical spores present centrally or paracentrally in the swollen sporangia. The spores are visible inside the dormant cell via spore staining.

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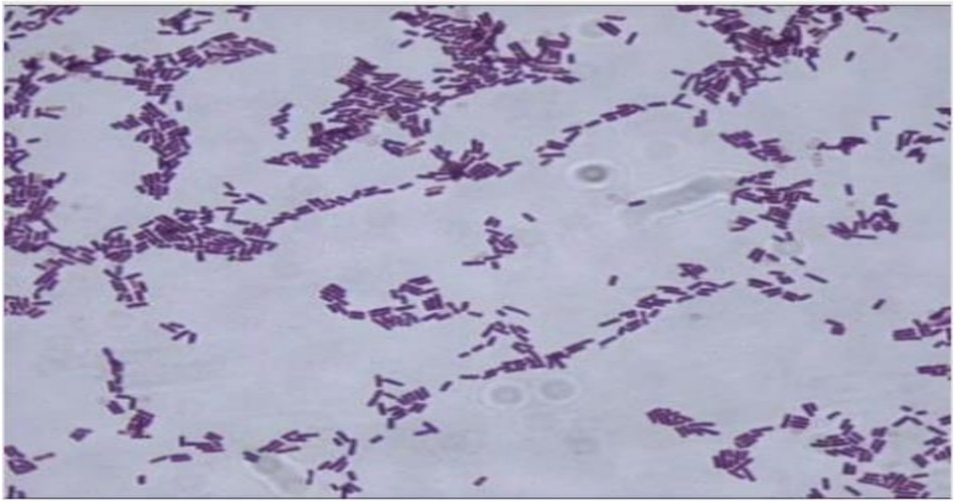

Figure: Morphology of *Bacillus subtilis*. Image Credit: Z M Hussein et al. 2019.

- A single endospore is present in a cell, and these spores are very resistant to adverse environmental conditions.
- The cells are round-ended and vary in size ranging between 0.7-0.8 μm × 2.0-3.0 μm.
- The arrangement of the cells is mostly single or in pairs; *B. subtilis* rarely form chains. The cells are motile with peritrichous flagella.
- Even though *B. subtilis* was initially considered obligate aerobe, but based on more recent findings, these are known to be facultative anaerobe.
- Most strains of *B. subtilis* are non-capsulated, but some strains produce capsules composed of polyglutamic acid or polysaccharides.
- The production of poly-γ-glutamic acid by *B. subtilis* occurs during the stationary phase of growth.
- The cell wall of *B. subtilis* cells is composed of peptidoglycan units with the most common type of linkage being meso-diaminopimelic acid.

- The cross-linkage is formed in the form of a peptide bond between the diamino acid in position 3 of one subunit and the D-alanine in position 4 of the neighboring peptide subunit.
- Underneath the cell wall is a cell membrane that is made up of lipid bilayer and protein structures that determine the fluidity of the membrane.
- The cytoplasm consists of a circular chromosome, mitochondria, and chloroplasts distributed throughout the cell.
- Filament-forming proteins are present along the longer axis of the cell that pushes the newly replicated DNA after cell division.
- The genome of *B. subtilis* is 4214810 bp long with about 4000 protein-coding genes. The G+ C content of the bacteria ranges between 40-45%.

Cultural Characteristics of *Bacillus subtilis*

- The colony morphologies of *B. subtilis* are highly variable, within and between strains which may give the appearance of a mixed culture during growth on an artificial medium.
- In spite of the diversity, the colonies of *Bacillus* species can be recognized on agar plates quite easily.
- The growth on a simple medium like nutrient agar might result in the swarming growth of the bacteria through the plate. This can be avoided by increasing the agar content of the media.
- Bacillus species usually have simple nutrient requirements which allow their growth in simple non-selective media like Nutrient Agar.
- The optimum temperature for the growth of B. subtilis is 28-30°C with a minimum temperature of 5-20°C and a maximum temperature of 45-55°C.

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- B. subtilis isolates from food samples optimally at 20-25°C, whereas that from clinical samples grow well at 35°C.
- Growth of B. subtilis can be seen within the pH range of 5.5 to 8.5, but the growth of some strains might be limited even within the said range.
- The growth of B. subtilis can occur on a minimal medium with glucose and ammonium salt as the sole sources of carbon and nitrogen, respectively.
- Most of the strains can tolerate 7% NaCl in the medium, but some can tolerate up to 10% NaCl.
- Even though *B. subtilis* are known as obligate aerobes; some restricted growth can be observed under anaerobic conditions in complex media with glucose or even nitrate.
- In liquid culture, LB broth is commonly used for the culture of *B. subtilis*. The growth is observed in the form of turbidity, and the cells begin to settle down as the growth ceases.
- The following are some cultural characteristics of *B. subtilis* in different culture media:

1. *Bacillus subtilis* in Nutrient Agar

- The colonies of *B. subtilis* on nutrient agar are round to irregular in shape. The isolates obtained from soil samples tend to form swarming growth throughout the plate.
- The size of the colonies is also variable ranging between 2-3 mm in diameter as the younger cultures tend to be larger and older colonies shrink up in size.
- The colonies have varying margins varying from undulate to fimbriate. The colonies are opaque with surfaces that are dull or even wrinkled.

- The color of the colonies is mostly white but can range between creamy and brown. Some strains produce varying pigments like creamy, yellow, orange, pink, and red to brown and black depending on the source or sample.
- These pigments are often observed in potato agar or glucose-containing agar medium.
- Strains that produce brown or black pigments were formerly called *Bacillus subtilis* var *aterrimus*, whereas those producing brownish-black pigment on tyrosine-containing media were name *B. subtilis* var *niger*.

2. *Bacillus subtilis* in Blood Agar

- *B. subtilis* form grey or white-colored colonies that are round, opaque, flat, and dry on blood agar supplemented with 5% rabbit blood.
- The colonies are medium-sized (ranging between 3-4 mm in diameter) that often dry on the surface as the culture dries out.
- Most strains of *B. subtilis* show β-hemolysis in the form of clearing of the media with the hemolysis of red blood cells. This is more common in *B. subtilis* obtained from clinical samples than from environmental samples.

3. *Bacillus subtilis* in Tryptic Soy Agar

- White to creamy colored colonies of *B. subtilis* are obtained on Tryptic Soy Agar. The colonies are circular or irregular in shape depending on the strain and the conditions for growth.
- The colonies have an irregular margin, and they are mostly flat. The surface is opaque and mucoid.
- On this agar, optimal growth occurs at 35°C under aerobic conditions. Some species may be facultatively anaerobic and might grow better in some 2% CO_2.

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Biochemical Characteristics of *Bacillus subtilis*

The biochemical characteristics of *B. subtilis* can be tabulated as follows:

S.N	Biochemical Characteristics	*Bacillus subtilis*
1.	Capsule	Most strains are non-capsulated, but some might contain a polyglutamic capsule.
2.	Shape	Rod
3.	Gram Staining	Gram-Positive
4.	Catalase	Positive (+)
5.	Oxidase	Variable
6.	Citrate	Positive (+)
7.	Methyl Red (MR)	Negative (-)
8.	Voges Proskauer (VR)	Positive (+)
9.	OF (Oxidative-Fermentative)	Facultative Heterofermentative
10.	Coagulase	Positive (+)
11.	DNase	Negative (-)
12.	Urease	Negative (-)

13.	Gas	Negative (-)
14.	H_2S	Negative (-)
15.	Hemolysis	β-hemolytic
16.	Motility	Motile with peritrichous flagella
17.	Nitrate Reduction	Positive (+)
18.	Gelatin Hydrolysis	Positive (+)
19.	Pigment Production	Positive (+)
20.	Indole	Negative (-)
21.	TSIA (Triple Sugar Iron Agar)	Alkali/Alkali (Red/ Red)
22.	Spore	Endospore-forming

Fermentation

S.N	**Substrate**	***Bacillus subtilis***
1.	Adonitol	Negative (-)
2.	Arabinose	Positive (+)
3.	Cellobiose	Positive (+)
4.	Dulcitol	Negative (-)

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5.	Fructose	Positive (+)
6.	Galactose	Positive (+)
7.	Glucose	Positive (+) Facultative heterofermentative
8.	Glycerol	Positive (+)
9.	Glycogen	Positive (+)
10.	Hippurate	Negative (-)
11.	Inulin	Positive (+)
12.	Inositol	Positive (+)
13.	Lactose	Positive (+)
14.	Malonate	Positive (+)
15.	Maltose	Positive (+)
16.	Mannitol	Positive (+)
17.	Mannose	Positive (+)
18.	Melibiose	Variable
19.	Pyruvate	Negative (-)
20.	Raffinose	Positive (+)

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21.	Rhamnose	Negative (-)
22.	Ribose	Positive (+)
23.	Salicin	Positive (+)
24.	Sorbitol	Positive (+)
25.	Starch	Positive (+)
26.	Sucrose	Positive (+)
27.	Trehalose	Positive (+)
28	Xylose	Positive (+)

Enzymatic Reactions

S.N	**Enzymes**	***Bacillus subtilis***
1.	Acetoin	Positive (+)
2.	Acetate Utilization	Positive (+)
3.	Tyrosine Hydrolysis	Negative (-)
4.	Lecithinase	Negative (-)
5.	Casein Hydrolysis	Positive (+)
6.	Esculin Hydrolysis	Positive (+)

7.	Lysine	Negative (-)
8.	Ornithine Decarboxylase	Negative (-)
9.	Phenylalanine Deaminase	Negative (-)

B. subtilis can decompose pectin and polysaccharides of plant origin. Dextran and levan are formed extracellular from sucrose during carbohydrate degradation.

Virulence Factors of *Bacillus subtilis*

- *Bacillus subtilis* is ubiquitous in distribution and is found in different environmental regions like air, water, soil, and even animal and human body surfaces.
- The ability of the bacteria to bind and colonize different parts of the human and animal bodies indicates a possible virulence factor of the bacteria.
- However, different studies have indicated that the colonization of human body surfaces doesn't involve a distinct mechanism that might be involved in disease production.
- A possible virulence factor of *B. subtilis* is toxin production as the bacteria produces the enzyme lecithinase which has been shown to be involved in food poisoning.
- Besides, *B. subtilis* also produces an extracellular toxin called subtilisin which is a proteinaceous compound capable of causing allergic reactions in some individuals.
- These reactions are often observed in immunocompromised individuals when they are exposed to such toxins regularly.

- The cases of allergies and hypersensitivity reactions, including dermatitis and respiratory distress, are often observed after the use of laundry products that are made with the said toxin.
- The virulence of *B. subtilis*, as well as *B. subtilis* toxins, is relatively low and it has been suggested that the bacteria do not produce significant quantities of the enzymes or toxins.

Role of *Bacillus subtilis* in animal and plant diseases

- *Bacillus subtilis* has been isolated from different cases of bovine and ovine abortions, but it hasn't been implicated as the causative agent of such infections.
- Besides, *B. subtilis* has been associated with cases of bovine mastitis, but the number of cases of mastitis caused by *B. subtilis* is low when compared to other species.
- *B. subtilis* has also been shown to be capable of infecting and resulting in the death of 2nd instar larvae of the mosquito.
- The ability of *B. subtilis* to cause infections in insects indicates the potential of the use of *B. subtilis* as a biocontrol agent.
- *B. subtilis* is not considered a plant pathogen, but there have been some reports regarding the involvement of *B. subtilis* in the soft rot of garlic cloves.
- Based on a report, it was assumed that *B. subtilis* might be involved in the broad open cancer ulcrea in maple trees.

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- The occurrence of *B. subtilis* in both animals and plants is quite limited and is not the primary causative agent.

Industrial uses / Applications of *Bacillus subtilis*

The use of *Bacillus* species as workhorse industrial microorganisms has been done for thousands of years with application in applied microbiology.

The high growth rates of the bacteria, short fermentation cycle, and the capacity to secrete extracellular proteins and enzymes enable the use of *Bacillus subtilis* in different industries.

B. subtilis is one of the few species of *Bacillus* that is considered "Generally Regarded as Safe" (GRAS) by the Food and Drug Administration.

The development and exploitation of *B. subtilis* are enabled by the information that is available on the biochemistry and genetics of the organisms.

The following are the industrial application of *B. subtilis* and its products:

1. Enzymes

- It has been estimated that *Bacillus* accounts for about 50% of the total industrially important enzymes in the world.
- Among all the species of *Bacillus*, *B. subtilis* is the most important species for industrial application.
- The alkaline serine proteases (subtilisins) produced by *B. subtilis* are primarily used in the production of household detergents.
- The alkalophilic organism and enzymes can be used in heavy-duty enzyme production with high alkaline tolerance.

- Besides, neutral *B. subtilis* produce proteases that are zinc metalloproteinases and have application in milk protein modification, nitrogen control, mash extraction and chill-haze removal in brewing industries.
- The amylases can be used in a number of industrial processes like food, fermentation, textile and paper industries.
- α-amylases cleave the internal α-1,4-linkages internally to produce shorter strands of carbohydrates that can then be used in different industries.
- Similarly, β-amylases are also industrially important as they operate to remove maltose units externally.
- *B. subtilis* also produces glucose isomerases that are essential in the final stages of starch processing to sweeteners. These enzymes are important for the conversion of glucose syrups to high fructose corn syrups.
- Other enzymes like cellulases, chitinases, and tannases also have industrial importance in the paper and textile industries.
- *B. subtilis* is one of the most potent producers of alkaline proteases that have an alkaline pH range and good thermostability. Alkaline proteases are used in the production of detergents and in abating, dehairing leather, and recovery of silver from X-ray films.
- Variants of *B. subtilis* enzymes have been prepared commercially through protein engineering to produce enzymes with improved performance.

2. Antibiotics

- Different species of *B. subtilis* produce various classes of antibiotics that are effective against different Gram-positive and Gram-negative bacteria. The compounds are produced during the early stages of sporulation.

- *B. subtilis* has also been used to transport bacitracin biosynthetic gene clusters from other *Bacillus* species like *B. licheniformis* to produce the compounds more efficiently.
- *B. subtilis* produces other antimicrobial compounds like subtilin, bacilysin, subsporins, lipooligopeptides, and rhizocticins.
- A lipopeptide antibiotic surfactin is also produced by *B. subtilis* which has potential antitumoral, antiviral, antibacterial, and hypocholesterolemic activity.

3. Purine nucleotides

- Purine nucleotides and nucleosides have applications in different industries like medicine and as flavor enhancers.
- *B. subtilis* produces nucleosides by subsequent chemical phosphorylation. In some cases, mutants of *B. subtilis* are used to produce inosine which easily passes through the cell membrane into the extracellular medium.
- Besides inosine, *B. subtilis* is also used for the production of other nucleotides like guanosine, riboflavin, and folic acid.

4. Vitamins

- Some strains of *B. subtilis* can be used for the limited production of some vitamins by fermentation.
- Processes like the cloning of riboflavin, cobalamin, and biotin biosynthesis are exploited in order to produce vitamins in a commercial way.
- In *B. subtilis*, deregulation of purine synthesis and a mutation in a flavokinase-flavin adenine dinucleotide synthetase is required to produce riboflavin from *B. subtilis*.

- Recombinant DNA techniques, along with fermentation strategies, are used to develop commercially important levels of riboflavin in *B. subtilis*.

5. Poly-γ-glutamic acid

- γ-polyglutamic acid is a naturally occurring anionic homopolyamide that is composed of D- and L-glutamic acid units connected by amide linkages.
- The acid is a water-soluble, edible and biodegradable compound with application in different industries like food, cosmetic and medical areas.
- Poly-γ-glutamic acid and its derivatives are used as thickeners, humectants, cryoprotectants, drug carrier and heavy metal absorber.
- Besides, it can also be used for wastewater treatment as a biopolymer flocculent as well as an animal feed additive.

6. D-Ribose

- D-Ribose is often used as a flavour enhancer in different industrial products like cosmetics, pharmaceuticals, food and animal feed.
- It also has application in the treatment of myocardial ischemia and muscular pain.
- Several strains of *B. subtilis* can produce D-ribose via fermentation which can be increased by applying genetic engineering technology.

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