

Microbial Biotechnology

Review



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**Introduction**

**Microbial biotechnology** is an exceptionally dynamic and exciting sector of the biomedical sciences that is unique in the breath and diversity of products and services it provides/will provide and to which it contributes, such as disease prevention and therapy, diagnostics, agriculture and horticulture, food provision and nutrition, energy production, production of chemicals and materials, water and waste treatment, recycling, acquisition of and adding value to natural resources, environmental monitoring, forensics, sustainable practices, etc. The development of rapid and affordable genomics technologies and accompanying bioinformatic tools, of systems and synthetic biology approaches, single cell techniques, and of high resolution analytical and imaging instruments, has provided new impulses to the field and opened new avenues of application, some of which, such as microbiome engineering, bioenergy and bioelectric applications, the use of microbial toxins for therapy and cosmetic applications, etc., promise to revolutionize our lives in a manner similar to that ushered in by the development of computers, the Internet and smart phones. The extent of present and future enrichment of human endeavour, prosperity and well-being to be brought about by microbial biotechnology, as well as its contribution to solutions to fundamental problems we and planet Earth are facing – the Grand Challenges – is only now beginning to be appreciated. The Editors and Friends of Microbial Biotechnology consider that this is an opportune moment to strategically analyse the immediate future of the field of microbial biotechnology. Luminaries in the field have therefore been invited to define where, in the context of Grand Challenges, we aspire to be in 2020, to articulate which obstacles lie in the way, and to suggest how these may be circumvented: in other words – to propose a road map for dealing with the obstacles and arriving at the goals set for 2020. These pieces make up this Special Issue entitled Microbial Biotechnology-2020. We believe that this Special Issue will make interesting immediate reading for researchers in the field and serve as a useful guide over the next few years.

**Microbial Inoculation for Sustainable Development**

Population growth and industrialization has put significant pressure on global ecosystems. Currently, 39% of terrestrial biomes are affected by intensive land use or settlements. Urbanization often takes place on previously cultivated land and to produce more food, farmers tend to intensify, using agrochemicals that include a variety of structurally different compounds. This is especially predominant in developing countries.

Fifty years ago, the “green revolution” was launched, combining high-yielding cultivars, inorganic fertilizers, and pesticides to foster food production. The impacts were great, albeit, today, to maintain healthy environments, new technologies need to be applied, including microbial inoculations. These can either replace or reduce agrochemicals, and clean areas heavily affected by pollution. Although green revolution and industrialization generally made human life easier and improved world's economy, effluents from industry also affected the health of soil, water and atmosphere leading to overall environmental degradation. Heavy metal contamination from industries poses deleterious effects not only on soil fertility and plant growth but also a serious serious threat to vulnerable human health. The over-use of ecosystems services is also alarming. While the use of regulating services, such as air quality, erosion control, and water purification as well as provisioning services, such as pollination and genetic resources strongly increase, their conditions steadily decrease. It is estimated that 22 million hectares of soil are adversely affected by chemical contamination worldwide, mostly in Europe, but also in Asia. The contamination and degradation of ecosystems by industrial pollutants is an emerging problem in the twenty-first century. There are several approaches, which can be used, on sustainable basis to meet food requirements without compromising environmental health. Among these, use of microbial products is pivotal to ensuring food security in changing climate. Microbial approaches can successfully be used for sustainable agricultural development. These microbes can enhance plant growth by improving nutrient availability to crop plants through various mechanisms thus decrease the dependence on chemical approaches.

Microorganisms as Plant Growth Promoters

Plants are entirely dependent upon soil microorganisms to utilize soils as a growth medium, and the synergy between both is important for their survival. The rhizosphere, the region of soil surrounding the roots, has the greatest concentration of microorganisms. Root exudates dictate the microbial communities. Manipulating the rhizosphere, changes microbial diversity and could improve plant performance by influencing water dynamics and enzyme activities. A wide range of microscopic organisms inhabits the rhizosphere: bacteria, algae, fungi, protozoa and actinomycetes. Of these, bacteria is the most abundant and important group of microorganisms regarding plant growth and productivity. They either live freely in rhizosphere, or in inter and intracellular spaces of root tissues, forming symbiotic associations with plants. Fungi play an important role in organic matter decomposition, and therefore nutrient cycling. Among soil fungi, arbuscular mycorrhizal fungi (AMF) are the most important and widely studied group as potential biofertilizers and biopesticides.

Biological Nitrogen Fixation

Atmospheric N is reduced to plant available form though natural or artificial means. When done artificially, N2 is reduced to ammonia via the Haber–Bosch process, in which natural gas (CH4) and N2 are converted to reduced forms of N at high temperature and pressure. In nature, N2 reduction is performed by N-fixing microorganisms that use the nitrogenase enzyme to reduce N2 to ammonia. This biological nitrogen fixation (BNF) is responsible for two-thirds of the total fixed N worldwide. The microbes performing BNF can be generally categorized as symbiotic, associative symbiotic, and free-living. However, a number of free-living N fixing bacteria, such as Azotobacter, Gluconoacetobacter, and Azospirillum spp. are present in nature and fix N for plants. The highest proportion of BNF is performed by symbiotic N2 fixers, i.e., rhizobia, which make symbiotic associations with the roots of leguminous plants. The establishment of symbiotic association involves a complex mechanism and exchange of chemical signals between host plant and symbionts i.e., rhizobia, leading to the formation of root knots, also called nodules. These develop from the swelling of cortical cells that host rhizobia as intracellular symbionts. PGPR other than rhizobia also have the nitrogenase enzyme and can fix N in non-leguminous plants, such as diazotrophs, which are capable of forming non-obligate interactions with host plants other than legumes. Nitrogenase is a two-component metallo-enzyme that consists of an iron (Fe) protein (dinitrogenase reductase) and molybdenum (Mo)-Fe protein (dinitrogenase). For nitrogenase complex to function, both components should be present. During N fixation, the Fe protein receives electrons with high reducing power from a low redox donor, such as reduced ferredoxin (Fd) or flavodoxin, and is reduced itself. The reduced Fe protein passes its electrons to Mo-Fe protein and becomes oxidized. This complex chain of reactions requires significant metabolic energy to reduce N2. The genes involved are nif genes, which are present both in symbiotic and free-living microorganisms. Inoculation of legumes with rhizobia of a specific cross inoculation group can be helpful to improving the nodulating capability of crops under field conditions.

**Microbial metabolites in nutrition, healthcare, and agriculture**

These microscopic organisms are used in the preparation of variety of foods and used as a source of food and feed supplements. For example, amino acids are obtained from Corynebacterium, Brevibacterium and Escherichia coli; vitamins from Propionibacterium and Pseudomonas; organic acids from Aspergillus, Lactobacillus, Rhizopus, and enzymes from Aspergillus, Bacillus. Microbes have been recognized considerably for their potential in the development of bioprocess technologies for unhindered production of food products and supplements to meet increasing demand by continuously growing world population. In addition, microorganism-based methodologies do not constitute a major source of pollution, and therefore, are preferred alternative for overcoming serious environmental problems, which arise from the conventional chemical methods. According to Business Communication Company (BCC), the total global market for microbes and microbial products was estimated at nearly $143.5 billion in 2014, and is expected to reach nearly $306 billion at a compound annual growth rate (CAGR) of approximately 14.6% over the period from 2015 to 2020. New technologies to produce microbial products are replacing synthetic production processes due to technical and economical advantages. These products include nutrition supplements such as, vitamins and amino acids, organic acids, agriculturally important metabolites, enzymes, flavoring agents, coloring agents and pharmaceutical products. Healthcare was the largest end-user market for microbes and microbial products at about $100.4 billion in 2014, and expected to increase to nearly $111.5 billion by 2015, and over $187.8 billion by 2020. The large size of the healthcare market reflects the importance of microbe-based biopharmaceutical industry.

Primary metabolites include amino acids, nucleotides, and fermentation end products such as ethanol and organic acids, which are considered essential for proper growth of microorganisms. Microbial synthesis is becoming the dominant and optimal process for amino acid production because of its ease to produce enantiomerically pure amino acids at low cost and ecological acceptability. Secondary metabolites are organic compounds that form at the end or near the stationary phase of growth, and are not directly associated with growth, development, and reproduction of microorganisms. These products are largely involved in healthcare activities as antimicrobial agents, antiparasitic agents, antitumor, enzyme inhibitors and immunosuppressive etc. The serendipitous discovery of antibiotic penicillin by Fleming in 1929 has drawn the interest of scientists to investigate the therapeutic role of microbial products for combating life-threatening infections. This lead to mass production of antibiotics during World War II by surface culture techniques and the period till 1960 was called as golden age of antibiotics. For the discovery and concept of antibiotics in infectious disease therapy Alexander Fleming, Howard Florey and Emst Boris Chain shared Nobel Prize in physiology/medicine in 1945. Since then, many soil as well as marine microorganisms have been explored for their inexhaustible involvement in pharmaceutical industry. The products derived from microbes are inevitably used to control and cure many infectious diseases acting as antibacterial, cholesterol lowering agents, immunosuppressants, anthelmintics and antiparasitic drugs. Secondary metabolites with activities as plant growth stimulants, herbicides and insecticides have also been reported. Some metabolites, such as Adriamycin, bleomycin, daunomycin, and mithramycin were used as antitumor compounds. In addition, secondary metabolites are also used as anesthetics, anti-inflammatory agents, anti-coagulants, anabolic, haemolytic, hypercholesteraemic and vasodilatory. Different strategies have been considered for effective and overproduction of primary metabolites, where genetic and physiological manipulations have played a significant role.

Vitamins

Vitamins are essential micronutrients required in trace amount to maintain normal physiological function of the body. These vital nutrients are not synthesized by mammals, and therefore, dietary supplement is necessary from external sources to maintain the balanced metabolism in all living organisms. Some vitamins are required as coenzymes to facilitate the biochemical reactions catalyzed by the enzymes. Vitamin K is required for normal blood clotting and also to activate receptor to facilitate transcription mechanisms in bone tissues, and to treat osteoporosis. Vitamin A is required as precursor to rhodopsin and other visual pigments, and also associated with specific gene transcription activation that facilitates growth and development. Vitamins are produced during regular metabolism of microorganisms and widely used as food additives, health supplements, and therapeutic agent etc. Vitamins are produced commercially either through direct fermentation or combined chemical and microbiological processes using appropriate microorganisms. According to a Global Strategic Business Report, the global market of vitamins is expected to reach over $9.0 billion by 2020 due to increasing health awareness and adoption of precautionary healthcare practices.

Organic acids

Organic acids are among the most versatile ingredients in food, beverages, pharmaceuticals, solvents, petrochemicals, textile, detergents, detergents, pharmaceuticals, rubber, perfumes, plastics, dyes and adhesive. In addition, they are used widely in the production of chemicals that are utilized in the automotive and construction industries. The catalytic potential of microbes is used in commercial production of several organic acids, such as acetic acid, lactic acid, gluconic acid, citric acid. Global market of organic acids was estimated at approximately $12 billion in 2014 and is expected to reach over $18 billion by 2023. Increasing demand of vinyl acetate monomer in food packaging industry and high growth of pharmaceutical industry are among major driving factors for huge growth in market. Citric acid is used for a wide range of applications in food industries, such as acidulant, flavorant, preservatives, sequestrant, emulsifiers, and buffering agent. The global market of citric acid was $2.6 billion in 2014 and is expected to reach $3.6 billion by 2020 at a CAGR of 5.5% from 2015 to 2020. Incessant growth in the production of citric acid is linked with increasing demands in food, beverages, cosmetic industries, and personal care products. About 99% production of total citric acid occurs via microbial processes using surface or submerged cultures and approximately, 70% citric acid of total production is used as an acidifier or antioxidant in food and beverage industry to preserve or enhance the flavours and aromas of fruit juices, ice cream, and marmalades. Aspergillus sp. and several other yeasts *Candida catenula*, *C. guilliermondii*, *C. tropicalis* and *Yarrowia lipolytica* are employed to produce citric acid.

Antitumor agents

Tumours are generally treated by surgical removal, radiation and chemotherapy. Surgical methods and radiation therapy are inefficient to treat metastatic cancer and therefore, chemotherapy is predominantly helpful in treatment of cancer that has spread to other parts of the body than origin site. According to World Health Organization (WHO), approximately 8.2 million people die annually from cancer, an estimated 13% of all deaths worldwide, and are expected to increase by \*70% new cases of cancer over the next two decades. Many microbial metabolites have been reported for effective anticancer properties in healthcare after the discovery of first anticancer agent, actinomycin by Wakesman and Woodruff. Approximately, 60% of the compounds with anticancer properties are derived from natural sources. Many of the chemotherapeutic agents used in cancer treatment are secondary metabolites produced by microorganisms, especially of the genus Streptomyces. The chemotherapy market is currently the fastest growing in the pharmaceutical industry, driven by the magnitude of the disease worldwide and growing understanding of potential therapeutic targets revealed by the molecular genetics assessments of cancer biology.

**Microbial chitinases: properties, current state and biotechnological applications**

Chitin is the second most abundant carbohydrate polymer in the world. It is a biopolymer made up of N-acetyl d-glucosamines that are connected via β-1,4-glycosidic linkages. Chitin exists naturally as the structural polysaccharide within the exoskeleton and the shell of crustaceans and within fungal cell walls. Biomass that contains high proportions of shellfsh, such as shrimp, crab, and krill, has been used for a broad range of applications in agriculture, food industries, and medicine. Chitin and its derivatives are also used in wastewater treatment, drug delivery, as wound healing accelerators, and as dietary fber. Moreover, chitin may be useful in regenerative medicine. Chitin is, however, challenging in regard to its isolation, solubilization, and manipulation to generate materials with desired forms and properties. Chitinases, enzymes that hydrolyze chitin, are synthesized by various microbes, including viruses, bacteria, and fungi, and by insects, higher plants, and animals. In microorganisms, chitinases are produced to allow these organisms to parasitize other fungi or nematode eggs, and the degradation of chitin results in the formation of derivative nutrient sources such as nitrogen and carbon. Chitinases can be classifed according to their catalytic mechanism into endochitinases (EC 3.2.1.14) and exochitinases. Endochitinases are characterized by their ability to randomly hydrolyze chitin along the internal chain to generate soluble oligomers of N-acetylglucosamine (GlcNAc). Exochitinases have been further classifed into two subcategories that include chitobiosidase (EC 3.2.1.29), which releases diacetylchitobiose at the non-reducing end of chitin microfbrils, and whole N-acetylglucosaminidase (EC 3.2.1.30), which cleaves N-acetylchitooligosaccharides to generate GlcNAc monomers. Considering the importance of bioconversion of the chitinous biomass and the role of chitinases in the development of novel value-added products.

* Application of chitinase in the food industry:

Production of single‑cell proteins

Certain microbes are gaining interest owing to their ability to produce single cell proteins (SCPs), which can be used as a protein dietary supplement and can replace costly conventional protein sources such as soymeal and fshmeal. As single cells, microorganisms could utilize inexpensive waste sources to allow for growth and increased biomass. Owing to their short generation time, ease of genetic engineering, and their ability to utilize a variety of substrates, microbes possess many advantages. Shellfsh waste is a rich source of chitin, CaCO3, and protein. Tom and Carroad (1981) reported that chitinases extracted from Pichia kydriavzevii and S. marcescens hydrolyzed chitin and generated single-cell proteins with yields of 45% protein and 8–11% nucleic acids. In another study, Myrothecium verrucaria and S. cerevisiae chitinolytic enzymes transformed chitin waste to single-cell proteins and ethanol. Patil and Jadhav (2014) used an enzymatic hydrolysis system where Penicillium ochrochloron MTCC 517 converted chitin to N-acetyl-d-glucosamine and Yarrowia lipolytica NCIM 3450 then further generated single-cell proteins. They found that up to 2% chitin was sufcient for achieving optimal single-cell protein yield, with a maximum yield of 65% protein and 2.9% nucleic acids from 9.4 g/L of biomass. Additionally, this yeast single-cell protein could be replaced with 50% fsh meal to induce growth of the fsh Lepidocephalus thermalis. These studies suggest that chitinases exhibit promising new properties to produce single-cell proteins for use as cheaper alternative protein sources, particularly in the context of converting marine or aquaculture waste to fshmeal.

Functional food and medicine

Chitinases can be used for a number of human healthcare applications such as the production of ophthalmic preparations with chitinases and microbiocides. Owing to the antifungal activity of chitinase, there has been an unfortunate gap in the development of a naturally occurring agent and therapy for fungal diseases. Antifungal drugs combined with essential amounts of chitinase have been used as a therapeutic treatment for various fungal infections. According to Allonsius et al, some Lactobacillus sp. strains, especially the taxa encompassing the L. casei group, can produce chitinases capable of breaking down the main polymer of the hyphal cell wall of Candida albicans. The synergistic efects of chitinase and lactic acid have been observed to enhance the growth inhibition of C. albicans, and these efects can assist in the selection of proper probiotic strains for use as potential probiotics in patients with Candida infections. However, the growth of marine Pseudoalteromonas sp. on chitin produced novel antimicrobial compounds that could lead to new bioprospecting strategies for the treatment of infectious diseases. Additionally, crude chitinase mixtures derived from S. marcescens and S. griseus possess prodrug activities and can inhibit the growth of and destroy cancer cells (MCF-7 and B11-2) in combined immunodefciency mice. Based on these fndings, the use of chitinase as a clinical test reagent should be further explored, as to the best of our knowledge, very few chitinases have been reported to exhibit cytotoxicity toward mammalian cells.

**Agriculturally important microbes in sustainable food production**

Agriculturally important microorganisms (AIMs) can influence both the efficiency of nutrient availability to crop plants and soil biodiversity, and they also regulate the interactions between plants and another harmful microflora (pathogens). Such interactions among plants and microbes have been reported to influence the physicochemical, biochemical, and microbiological properties of the soil. Moreover, root exudates (the chemicals secreted into the soil by roots that work as connecting links between roots and the soil microbial community) as signaling molecules can enhance the communication between crops and beneficial microbial agents, and consequently enhance growth, yield, and immunity in crop plants. Beneficial microbes have advanced abilities to suppress or alter the protective responses of the host plant, permitting them to epiphytically or endophytically colonize their hosts. Therefore, the exploitation and prospecting of beneficial microbial bioagents and their formulations may be an appropriate option for enhancing crop production for a rapidly growing human population. Efficient microbial communities that can offer services including plant growth promotion, nutrient use efficiency, bioremediation, and control of pests/phytopathogens at the farming level are known as ‘microbial inoculants. A profound understanding of the environmental factors influencing the viability and performance of these microbial inoculants is essential for their large-scale use in sustainable agriculture.

**Technologies for Beneficial Microorganisms Inocula Used as Biofertilizers**

Environmental issues such as freshwater pollution, energy saving, and soil erosion are forcing the farmers to introduce methods of cultivation that have a lower impact on the environment. The application of environmentaly friendly practices is promoted by voluntary certification schemes (e.g., GlobalGAP or organic farming schemes) as well as by legally binding regulations (e.g., the EU Directive 2009/128 aiming at the implementation of sustainable pest management practices). In this context, the reduced use of chemical fertilizers with increased application of organic fertilizers is considered a compulsory route to alleviate the pressure on the environment derived from agricultural practices.

Several organic fertilizers have been introduced in the recent years, which are also acting as natural stimulators of plant growth and development. A specific group of this kind of fertilizers includes products based on plant growth-promoting microorganisms (PGPM). Three major groups of microorganisms are considered beneficial to plant nutrition: arbuscular mycorrhizal fungi (AMF), plant growth-promoting rhizobacteria (PGPR), and nitrogen-fixing rhizobia, which are usually not regarded as PGPR. Microbial inoculants based on these microorganisms can be divided into different categories depending on their use, even though exact definition of these categories is still unclear. Nevertheless, the category of biofertilizer most commonly refers to products containing soil microorganisms increasing the availability and uptake of mineral nutrients for plants (like rhizobia and mycorrhizal fungi). According to the definition proposed by Vessey, biofertilizers are substances which contain living microorganisms which, when applied to seed, plant surfaces, or soil, colonize the rhizosphere or the interior of the plant, and promote growth by increasing the supply or availability of primary nutrients to the host plant. Another category of PGPM-containing products is that of phytostimulators which are generally containing auxin-producing bacteria, inducing root elongation.

The policies supporting sustainable agricultural production and extensive research that has improved the effectiveness and consistency of microbial inocula have resulted in the registration of several strains for both biocontrol and biofertilization, with mycorrhizal and PGPR preparations being marketed in several countries. Yet, a wider use of microbial inoculants, especially those acting as phytostimulators and biofertilizers, has been frequently hampered due to the variability and inconsistency of results between laboratory, greenhouse, and field studies. The reason for these discrepancies lies in the incomplete understanding of the complex relationships established between the components of the system: the plant, the microorganisms, and the environmental conditions, particularly that of soil. In addition, the lack of correct formulations and the expensive and time-consuming procedures of registration are also among the factors holding back the use of PGPM on a wider scale.

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