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Microbial Production of Biopolymers and Polymer Precursors

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Abstract: Living organisms, namely, prokaryotes and eukaryotes, are able to synthesize a variety of polymers, such as nucleic acids, proteins, and other polyamides, polysaccharides, polyesters, polythioesters, polyanhydrides, polyisoprenoids, and lignin. Microorganisms provide a source of biopolymers and biopolysaccharides from renewable sources. Bacteria are capable of yielding biopolymers with properties comparable to plastics derived from petrochemicals, though more expensive. They have the additional advantage of being biodegradable. A wide range of microbial polysaccharides have been studied, and structure/function relationships for a number of these macromolecules have been determined. These biopolymers accomplish different essential and beneficial functions for the organisms. Among the biopolymers produced, many are used for various industrial applications. Currently, the biotechnological production of polymers has been mostly achieved by fermentation of microorganisms in stirred bioreactors. The biopolymers can be obtained as extracellular or intracellular compounds. Alternatively, biopolymers can also be produced by in vitro enzymatic processes. However, the largest amounts of biopolymers are still extracted from plant and animal sources. Biopolymers exhibit fascinating properties and play a major role in the food processing industry, e.g., modifying texture and other properties. Among the various biopolymers, polysaccharides and bioplastics are the most important in the food industry. This chapter will discuss the sources of polymers, their biosynthesis by different organisms, and their application in different fields. A huge variety of biopolymers, such as polysaccharides, polyesters, and polyamides, are naturally produced by microorganisms. These range from viscous solutions to plastics and their physical properties are dependent on the composition and molecular weight of the polymer. The genetic manipulation of microorganisms opens up an enormous potential for the biotechnological production of biopolymers with tailored properties suitable for high-value medical application such as tissue engineering and drug delivery.

I. BIOPLASTICS

A. Introduction

When grown under conditions where growth becomes limited through exhaustion of a key nutrient such as nitrogen or phosphorus and carbon substrate remains available, many prokaryotes can synthesise intracellular storage compounds. These act as carbon and energy reserves which can be utilised when balanced growth is resumed. One such is glycogen, while another type was initially identified as poly-β-hydroxybutyric acid (PHB). These storage compounds can represent over 50 per cent of cell dry weight under some growth conditions and are normally recognised through use of iodine and sudanophilic dyes respectively.

B. Occurrence & Composition

The occurrence of polyhydroxyalkanoic acids as storage polymers in prokaryotic cells is now known to be very widespread indeed. They are water-insoluble compounds with the general structure shown in Figure 1. Many bacteria produce an intracellular carbon and energy storage compound - poly-β-hydroxybutyric acid (PHB) - in relatively large quantities . While this property is absent from enteric species, it is widely found in Pseudomonads and related species including the plant symbiont Rhizobium and also in nitrogen-fixing Azotobacter spp.

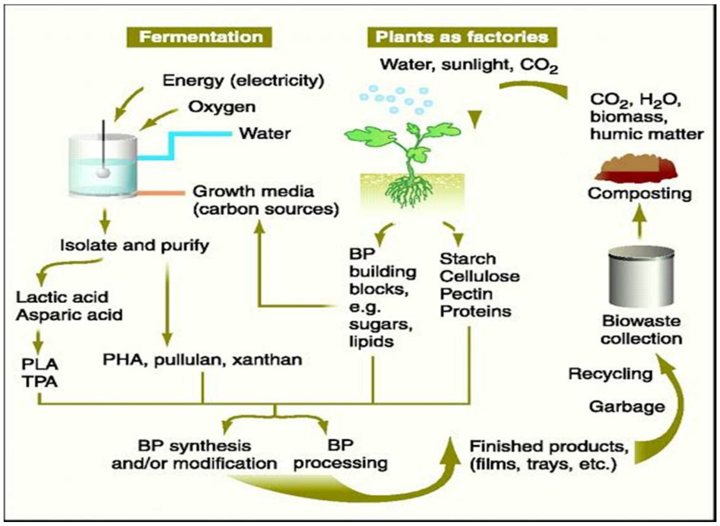
Accumulation is normally a response to unbalanced growth in the presence of excess carbon and energy source. Under appropriate conditions the polymer can amount to more than 50-80 per cent of cell dry weight. The storage product is found as granular inclusion bodies within the cytoplasm.

However, many of these compounds represent in relatively small amounts or because of their short chain lengths or other properties are unsuitable as potential bioplastics. Among species synthesising PHB and PHV are some Archae including Haloferax mediterranea. These halophilic bacteria might present advantages for production as their culture requirements of salinity and relatively high temperature provide little opportunity for growth of contaminants.

C. Bioplastic and Biopolymer Production

Ian W. Sutherland ©Encyclopedia of Life Support Systems (EOLSS) production of large amounts of exopolysaccharide diverts substrate to alternative products and makes recovery of PHB difficult. Development of high-yielding mutant strains resulted in conversion rates of 65 per cent for PHB and eventual PHA yields of 71 per cent dry weight Figure 1: The typical structure of a polyhydroxyalkanoate copolymer

Biosynthesis Figure 2:



D. Biopolymers Synthesis and Application

The synthesis of PHB Biosynthesis of PHB is much simpler than the formation of most polysaccharides, as only 3 enzymes are normally involved - β-thioketolase, acetoacetylCoA reductase and PHB synthase (Figure 2). The genes involved have been studied in Ralstonia eutropha (formerly designated Alcaligenes eutrophus) and Pseudomonas oleovorans as well as other species. Genetic control of the process is also relatively simple; the 3 genes involved are organised in an operon to form a sequence of 3 open reading frames and these can be transferred relatively easily to other bacterial species and to yield transgenic plant species.

The PHA synthase gene products from different bacteria have been grouped into 3 classes varying in their constitutive proteins and their substrate specificity. Recent studies using recombinant strains of E.coli into which the PHA biosynthesis genes from R. eutropha had been inserted, yielded PHB with mass of 3-11 x 106 daltons. The mechanical properties of films from this product were improved by stretching over 400 per cent. The enzymes for PHB synthesis are normally constitutive and regulation of synthesis appears to occur at the enzyme level.

E. Products

Bioplastics are thermoplastic compounds which, unlike products of the petrochemical industry, are biodegradable. They have the further advantage that they can be produced from renewable resources. They are normally highly crystalline, optically active and possess piezoelecetric properties. ICI (Zeneca) put considerable effort into development of bioplastics and soon found that PHB did not have all the properties they wanted. However they discovered that it was possible to synthesise linear co-polymers containing poly-β-hydroxybutyric acid and hydroxyvaleric acid. Such random copolymers were formed by R. eutropha with glucose and propionic acid as substrates. Typically polymers consisted of 12-20 per cent hydroxyvalerate and approximately 300000 Da. The melting point of the co-polymers decreases with increasing hydroxyvaleric acid content. The melting point of PHB is close to 175^{o} C whereas those of PHAs are lower. Unlike PHB which is brittle, the copolymers are elastic. PHA are thermoplastic polymers and they become highly viscous at temperatures above their melting point, thus rendering them mouldable. The melting point (Tm), crystallinity and glass transition temperature (Tg) depend on the composition of the product. The commercialised product 'Biopol' has been used to form biodegradable plastic bottles, together with golf tees, disposable razors and other products. However the high cost of the product in relation to chemically synthesised plastics has led to closure of production. Alternative applications which have been proposed, are as waterimpermeable coatings for biodegradable packaging, as temporary plates and pegs in repair of bone injuries etc. , but it is unclear whether a market exists under the current economic climate.

F. Production and Recovery

Wild-type strains of R. eutropha utilise fructose but not glucose. However, glucoseutilising strains have been developed for use in the commercial production of 'Biopol'. PHA-producing bacteria can be grown on a large scale to high cell densities in stirred tank fermenters using glucose, sucrose or molasses as carbon source. Fed-batch systems have been preferred to continuous processes and yields of 70-80 per cent PHA have been reported for R. eutropha grown in a mineral salts medium on glucose supplemented with propionate as the sole carbon sources. Cell densities were of the order of 100g. dry weight litre-1. Following cell harvesting by centrifugation or by flocculation, the PHA can be recovered by the use of surfactants and hypochlorite to lyse the cells and release the intracellular product. Although hypochlorite provided a suitable laboratory procedure it can lead to some degradation of the product.

II. BIOPLASTIC AND BIOPOLYMER PRODUCTION

Ian W. Sutherland ©Encyclopedia of Life Support Systems (EOLSS) processes have also been used to separate the PHA from soluble intracellular products. Alternatively, solvent extraction has been proposed. This can only be effectively achieved if the cell mass has first been dried by spray drying or by lyophilisation, thus adding to the cost of the process. Large amounts of solvent are needed as concentrated solutions of PHA are highly viscous. Chloroform or methylene chloride can dissolve most PHA products but pretreatment of the cells may be required to achieve maximum extraction efficiency. The actual yield and composition of the product depend on the substrate regime chosen. Choi and Lee observed initial low polymer content of cells due to the high residual content of propionic acid in the medium. By altering the feed strategy, use of acetic acid induction and oleic acid addition, P(3HB-co-3HV)-polymer yields of 78 per cent wt. and productivity of 2.88 g l-1 h-1 were obtained. 2. Biopolymers (Polysaccharides)

A. Introduction

A large number of micro-organisms produce exopolysaccharides. The structures of many of these polymers have now been accurately determined. Some chemical structures have also been correlated with the physical functions of EPS. In several bacteria of medical significance, including Streptococcus pneumoniae, Escherichia coli and Klebsiella aerogenes, systematic studies on a large number of different serotypes have also determined the relationship between serological specificity and chemical structure. Other bacterial groups have been studied because of either their pathogenicity or their symbiotic interactions with plants and the roles which polysaccharides play in such processes. Although large numbers of microbial polysaccharides are potentially available, relatively few have been commercially developed. There are many reasons for this: the microbial source may be pathogenic; production costs may be very high; product quality may be difficult to maintain and to guarantee; the product may not achieve regulatory acceptability or (very commonly) there is no market niche. Despite such problems, several EPS are now recognised products of biotechnology. Several more may be developed in the next few years, especially as we look to renewable resources for alternatives to several chemical products. Substituent Occurrence Linkage Charge conferrred Organic Acids Acetate Very common - e.g. Klebsiella spp.; colanic acid Ester None Glycerate Sphingomonas elodea Ester Negative Hydroxybutanoate Rhizobium trifolii; R. leguminosarum, etc.

Non-Carbohydrate Substituents of Exopolysaccharides Microbial homopolysaccharides are mainly neutral glucans, although levans (fructans) may also have potential uses. The majority of heteropolysaccharides are polyanionic due to the presence of uronic acids; alternatively, or additionally, charge can be conferred by the presence of organic or inorganic substituents. These include pyruvate ketals or succinyl half-esters, phosphate and (relatively rarely) sulphate groups. Further intriguing properties may be conferred by the presence of O-acetyl groups which are of widespread occurrence. Almost all microbial heteropolysaccharides are composed of regular repeating units varying in size from disaccharides to octasaccharides. These frequently contain one mole of a uronic acid. D-glucuronic acid is most common but some heteropolysaccharides contain D-galacturonic acid. D-Mannuronic acid is found in bacterial alginates and a few other polysaccharides, as is L-guluronic acid. Rare aminouronic acids are also found. Very occasionally, two uronic acids are present in a repeat unit. The proposed uniformity of the repeat units is mainly based on chemical studies and some irregularities may possibly be found, especially in polymers composed of larger and more complex repeat units. Short side-chains, varying from one to four sugars in length may be present. Bacterial alginates, linear molecules composed only of uronic acids, lack any regular repeat unit. They are heteropolysaccharides composed of D-mannuronic and L-guluronic acids in an irregular linear structure. Various acyl groups and other substituents may be found in exopolysaccharides in addition to a wide range of monosaccharides (Table 1). The most common substituents, ester-linked O-acetyl groups, do not confer any charge on the macromolecule but may greatly affect the physical characteristics of aqueous solutions. Pyruvate ketals are also common and they confer a negative charge due to the free carboxylic acid group.

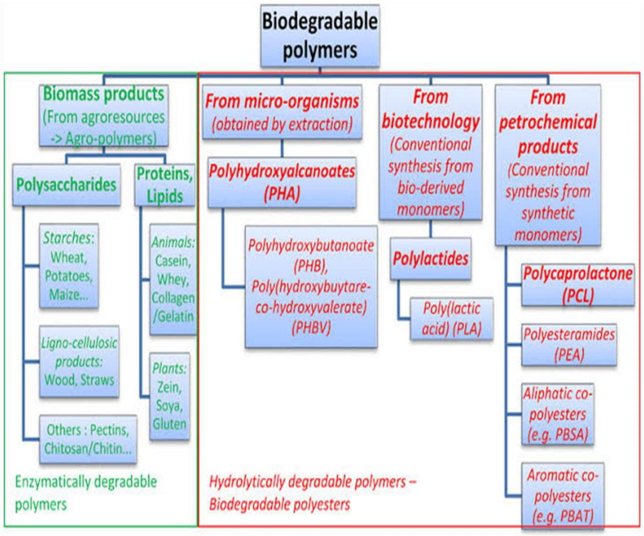
Acetyl and pyruvate groups are more common in heteropolysaccharide structures than homopolysaccharides. Succinyl half esters are found in polymers from Rhizobium species. Indeed, the succinoglycan EPS from Rhizobium and related spp. usually contain all three acyl groups. Two types of inorganic substituents may be present. Phosphate groups are relatively common in polysaccharides from Gram positive bacteria though rare in Gram negative products; sulphate groups have been found in EPS of Cyanobacteria and of halophiles including Haloferax mediterranea.

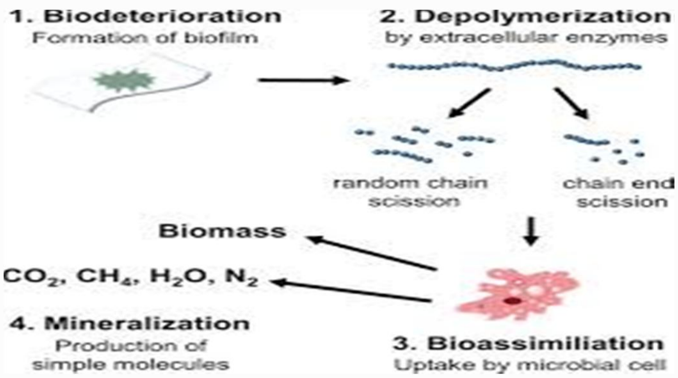
The EPS are usually highly hydrated. EPS apparently serve to protect the bacterial cells against desiccation, phage and other agents. Cellulomonas flavigena provides a rare example in which the EPS (curdlan) has been shown to function as a carbon and energy reserve.

B. Occurrence and Composition

Three types of microbial homopolysaccharide structure have been characterised:

1) linear neutral polymers composed of a single linkage type (the "mixed linkage" type of glucan found in cereal plants such as oats and barley have not been detected in micro-organisms);
2) Other homopolysaccharides are exemplified by the fungal polymer scleroglucan. These are linear polysaccharides with regular, short side-chains composed on one or two residues of the same monosaccharide as the main chain. Scleroglucan possesses tetrasaccharide repeating units due to the 1,6-α-D-glucosyl side-chains present on every third main chain residue.
3) The third type, including dextrans, are branched homopolysaccharides, as are levans (polyfructans). A few polyanionic homopolymers are also known. They include poly-D-glucuronic acid formed by a Rhizobium mutant, the 'Vi' antigen found in some species of Enterobacteriaceae and some bacterial sialic acids. Also in this category are the acetylated, poly-D-mannuronic acid polymers produced by some Pseudomonas aeruginosa mutants





The biochemistry and genetics of biosynthesis of xanthan, alginate, cellulose, cyanophycin, poly(gamma-glutamic acid), levan, hyaluronic acid, organic acids, oligosaccharides and polysaccharides, and polyhydroxyalkanoates.

1) Xanthan Biosynthesis by Xanthomonas Bacteria

Plant-pathogenic bacteria of the genus Xanthomonas are able to produce the acidic exopolysaccharide xanthan gum. Because of its physical properties, it is widely used as a viscosifer, thickener, emulsifier or stabilizer in both food and non-food industries. Xanthan consists of pentasaccharide repeat units composed of D-glucosyl, D-mannosyl, and D-glucuronyl acid residues in a molar ratio of 2:2:1 and variable proportions of O-acetyl and pyruvyl residues. The xanthan polymer has a branched structure with a cellulose-like backbone. Synthesis originates from glucose as substrate for synthesis of the sugar nucleotides precursors UDP-glucose, UDP-glucuronate, and GDP-mannose that are required for building the pentasaccharide repeat unit. This links the synthesis of xanthan to the central carbohydrate metabolism. The repeat units are built up at undecaprenylphosphate lipid carriers that are anchored in the cytoplasmic membrane. Specific glycosyltransferases sequentially transfer the sugar moieties of the nucleotide sugar xanthan precursors to the lipid carriers. Acetyl and pyruvyl residues are added as non-carbohydrate decorations. Mature repeat units are polymerized and exported in a way resembling the Wzy-dependent polysaccharide synthesis mechanism of Enterobacteriaceae. Products of the gum gene cluster drive synthesis, polymerization, and export of the repeat unit. This review outlines aspects of the biosynthetzic pathway and genetic loci involved in xanthan biosynthesis, including the synthesis of the sugar nucleotide precursors, building of the repeat unit, as well as polymerization and export of the polymer. Comparative aspects based on recent genomic data of various Xanthomonas strains are also covered.

2) Microbial Production of Alginate: Biosynthesis and Applications

Alginate is the main representative of a family of polysaccharides that neither show branching nor repeating blocks or unit patterns and this property distinguishes it from to other polymers like xanthan or dextran. Although only consisting of two different components, β-D-mannuronic acid and its C5-epimer α-L-guluronic acid, its unique and random structural pattern has attracted a lot of scientific and commercial interest over the past decade. Besides its production by brown algae, it is only produced by the two bacterial genera Pseudomonas and Azotobacter, which played a major role in the unravelling of its biosynthesis pathway. The pathway involves the generation of the cytosolic precursor GDP-mannuronic acid its polymerization to poly-mannuronic acid while traversing the cytoplasmic membrane. In the bacterial periplasm it can undergo enzymatic modification in form of acetylation or epimerization before the polymer is finally exported through the outer membrane and released into the environment. The degree of variability in the polymer and the possibility of genetical engineering of its producing bacterial hosts have been increasingly considered as an option to tailor-make alginates as biomaterials for numerous applications. Like DNA, alginate is a highly negatively charged polymer, which in combination with its random pattern imparts material properties ranging from viscous solutions to rigid gel-like structures in the presence of divalent cations. Traditionally, the self-assembly processes of algal alginates were mainly used in biotechnology for encapsulation purposes but given the option of fine-tuning its material properties, bacterial alginates are more and more considered for the production of micro- or nanostructures suitable for medical applications.

3) Bacterial Cellulose Production: Biosynthesis and Applications

Svein Valla, Helga Ertesvåg, Naoto Tonouchi and Espen Fjaervik Gluconacetobacter xylinus (formerly Acetobacter xylinum) has for technical reasons been a favorite model system used for studies of the biochemistry and genetics of cellulose biosynthesis, even though the vast majority of the polymer is produced by green land-plants. Structurally cellulose is a simple polysaccharide, in that it consists only of one type of sugar (glucose), and the units are linearly arranged and linked together by β-1,4 linkages only. The mechanism of biosynthesis is however rather complex, partly because in native celluloses the chains are organized as highly ordered water-insoluble fibers. Currently the key genes involved in cellulose biosynthesis and regulation are known in a number of bacteria, but many details of the biochemistry of its biosynthesis are still not clear. A survey of genome sequence databases clearly indicates that a very large number of bacteria have the genes needed to produce cellulose, and this has also been experimentally confirmed for a smaller number of organisms. The biological functions of bacterial celluloses vary among species, and range from a role as a floating device to involvement in plant root adhesion and biofilm formation. In spite of the enormous abundance of cellulose in plants bacterial celluloses have also been investigated for industrial exploitations.

4) Cyanophycin: Biosynthesis and Applications

This chapter provides an overview on the synthesis, large-scale production, biodegradation and potential applications of the polyamide cyanophycin (CGP), which is a non-ribosomally synthesized compound consisting of a poly(aspartic acid) backbone with arginine residues linked to the carboxyl group of each aspartate by their amino group. In its natural host microorganisms, CGP functions as a storage polymer for nitrogen, carbon and energy. The key enzyme of CGP synthesis is represented by the CGP synthetase (CphA). The gene coding for this enzyme has been used for recombinant expression of CGP biosynthesis in various prokaryotes. Meanwhile, also transgenic eukaryotes, yeasts and plants, were enabled to synthesize the polymer in considerable amounts. Biodegradation of CGP occurs by intra- or extracellular CGPases, CphB or CphE, respectively. Degradation products of CGP are usually dipeptides which are then split to free amino acids by intracellular dipeptidases. Biotechnical interest of CGP is high; products resulting from its biodegradation could be applied in various biochemical, medical or industrial applications.

5) Biosynthesis and Application of Poly(gamma-glutamic acid)

This review chapter deals with chemistry and biosynthesis of poly-γ-glutamic acid (γ-PGA) produced by various strains of Bacillus. In addition to the production, purification and characterization of γ-PGA, an updated critical literature review on the physiological function, biochemical and chemical properties, genetic aspects and enzymology of γ-PGA metabolism (racemization, polymerization and degradation), the molecular mechanism of glutamic acid polymerization were provided. Potential applications of γ-PGA as thickener, cryoprotectant, humectant, drug carrier, biological adhesive, flocculant, or heavy metal absorbent with biodegradability in the fields of food, cosmetics, medicine, water treatments and environment were also reviewed.

6) Levan: Applications and Perspectives

Levan, a homopolysaccharide which is composed of D-fructofuranosyl residues joined by 2,6 with multiple branches by 2,1 linkages has great potential as a functional biopolymer in foods, feeds, cosmetics, and the pharmaceutical and chemical industries. Levan can be used as food or a feed additive with prebiotic and hypocholesterolemic effects. Levan is also shown to exert excellent cell-proliferating, skin moisturizing, and skin irritation-alleviating effects as a blending component in cosmetics. Levan derivatives such as sulfated, phosphated, or acetylated levans are asserted to be anti-AIDS agents. In addition, levan is used as a coating material in a drug delivery formulation. In addition, levan has a number of industrial applications such as a surfactant for household use due to its excellent surface-active properties, a glycol/levan aqueous two-phase system for the partitioning of proteins, etc. However, there are some limitations for the industrial applications of levan due to its weak chemical stability of in solution and the complex process to purify levan. Once the limitations are solved, the market for levan will gradually increase in the various fields.

7) Microbial Hyaluronic Acid Biosynthesis

Hyaluronic acid (HA) is a biopolymer with valuable applications in the pharmaceutical and cosmetic industry. Currently, HA is produced commercially by either extraction from animal tissues i.e. rooster comb or bacterial fermentation. Increased concerns over the contamination of animal derived products with infectious agents have made bacterial fermentation a more desirable production system to meet future demands. The high viscosity of HA dictates low titres of 5-10 g/L, a level readily achieved through batch fermentation of Group C streptococci. Substrate cost is a minor factor for this high value polymer, hence strain and process development has focused on improving quality, in particular molecular weight. Little is known about what controls molecular weight of beta-polysaccharides such as HA. This is even true for abundant beta-polysaccharides such as chitin and cellulose. Several groups including ours have pursued various hypotheses for the past decade, but no hypothesis has captured the Mw regulation observed in bioreactors. The HA synthase is responsible for all steps in polymerisation and most likely also translocation. In vitro studies have identified several residues essential for high molecular weight and maximum molecular weight appears to be an intrinsic feature of the synthase. The actual molecular weight realised in fermentation, however, depends on fermentation conditions. In general, high molecular weight is observed under conditions with excess resources. Surprisingly, however, preliminary studies cannot relate these findings to higher levels of the UDP-sugars used in biosynthesis. Metabolic engineering and the recent advance in omics technologies are providing new opportunities. Heterologous hosts such as B. subtilis, L. lactis, and E. coli have been successfully engineered to produce HA and may prove more amenable to engineering high molecular weight HA.

8) Fermentative Production of Organic Acids for Polymer Synthesis

Polymers derived from petrochemicals are indispensable materials in mankind's daily life. Owing to the increasing environmental concerns and increasing oil price, there has recently been much interest in developing processes for the production of monomers from renewable resources. In this chapter, we review fermentative production of three and four-carbon organic acids that can be used as monomers for polymer synthesis. Microorganisms and bioprocesses employing them for the production of lactic, acrylic, succinic, fumaric, and aspartic acids are reviewed. Metabolic pathways and characteristics for the formation of these acids are detailed along with metabolic engineering strategies.

9) *Metabolic Engineering of Microorganisms for Oligosaccharide and Polysaccharide Production*

Microorganisms naturally produce a wide variety of carbohydrate molecules, yet large-scale manufacturing requires production levels much higher than the natural capacities of these organisms. Metabolic engineering efforts generate microbial strains capable of meeting the industrial demand for high synthesis levels. This chapter reviews the achievements and challenges of engineering microorganisms to produce two categories of carbohydrates: oligosaccharides and polysaccharides. As both oligosaccharide and polysaccharide synthesis are carbon and energy-intensive processes, improved production of these products require similar metabolic engineering strategies. Strategies unique to polysaccharide synthesis are also discussed. Metabolically engineered strains have successfully produced many carbohydrate products, and many unexplored strategies, made available from recent progress in systems biology, can be used to engineer even better microbial catalysts.

10) *Microbial Exopolysaccharides: Variety and Potential Applications*

Microorganisms synthesize a wide spectrum of multifunctional polysaccharides including intracellular polysaccharides, structural polysaccharides and extracellular polysaccharides or exopolysaccharides (EPS). Exopolysaccharides generally constitute of monosaccharides and some non-carbohydrate substituents (such as acetate, pyruvate, succinate, and phosphate). Owing to the wide diversity in composition, exopolysaccharides have found multifarious applications in various food and pharmaceutical industries. Many microbial EPS provide properties that are almost identical to the gums currently in use. With innovative approaches, efforts are underway to supersede the traditionally used plant and algal gums by their microbial counterparts. Moreover, considerable progress has been made in discovering and developing new microbial EPS that possess novel industrial significance. The present article accentuates on providing a glimpses of varieties and applications of microbial exopolysaccharides.

Bioplastic and Biopolymer Production - Ian W. Sutherland ©Encyclopedia of Life Support Systems (EOLSS) derived from petrochemicals, bacteria have proved capable of yielding bioplastics with comparable properties. They have the additional advantage of being biodegradable. The processes, which have been developed for their production, have been optimised and a range of products obtained. Research is currently determining the possibility of transferring the genes for synthesis of these bioplastics to plants in order to test the commercial viability of such sources. Considerable effort is also being applied to the discovery of new bacterial sources of polymers with different properties. A very wide range of microbial polysaccharides has been studied and structure/function relationships for a number of these macromolecules have been determined. Several biopolymers have gained acceptability in a wide range of applications in manufactured food technology, in other industrial applications and as useful adjuncts in oil exploration and production. Some of the biopolymers possess unique properties and are of high value. These products are generally competing against a range of established plant and algal gums, including starch. However, they are also prepared from renewable resources and are biodegradable.

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