BIOPLASTIC AND BIOPOLYMER PRODUCTION

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Contents

Summary

Micro-organisms provide a source of bioplastics and biopolymers (polysaccharides) from renewable sources. Although currently considerably more expensive than plastic

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.

1. Bioplastics

1.1. 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.

1.2. 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. In species such as *Azotobacter vinelandii*, simultaneous

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

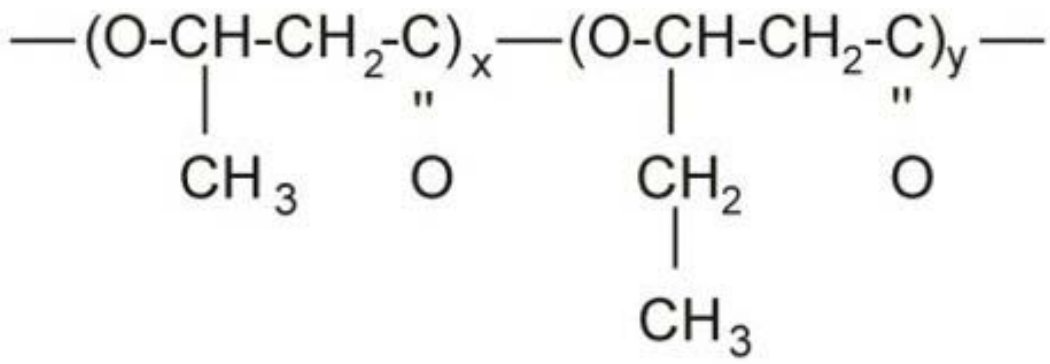

Figure 1: The typical structure of a polyhydroxyalkanoate copolymer

1.3. Biosynthesis

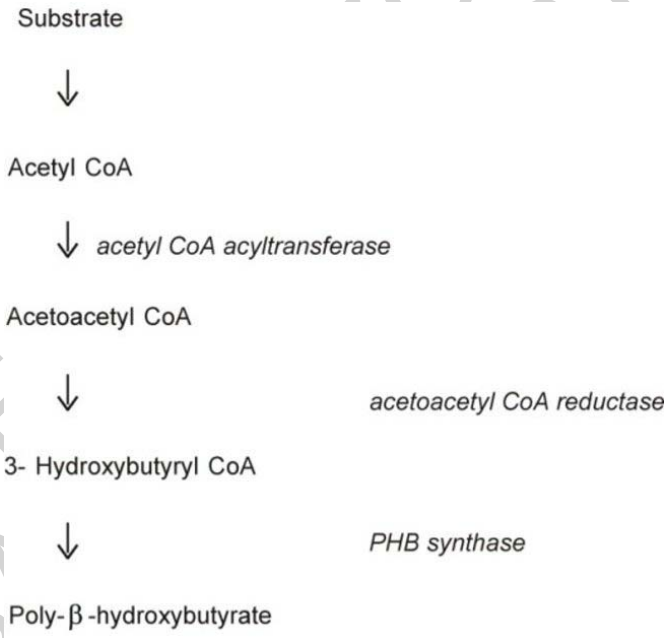

Figure 2: The synthesis of PHB

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. A slightly modified alternative pathway found in

Rhodospirillum rubrum was outlined by Steinbüchel and Füchtenbusch. 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 10^6 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.

1.4. 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 co-polymers 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 (T_m), crystallinity and glass transition temperature (T_g) 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 water-impermeable 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.

1.5. Production and Recovery

Wild-type strains of *R. eutropha* utilise fructose but not glucose. However, glucose-utilising 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. Flotation

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)

2.1. 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.	Ester	None
Propionate Rare - some	Ester	None

Escherichia coli		
Pyruvate Very common - e.g. *Klebsiella* spp.; colanic acid	Ketal	Negative
Succinate *Rhizobium* spp.; *Agrobacterium* spp.	Half ester	Negative
Inorganic acids		
Phosphate Common in some genera, Gram positive spp.		Negative
Sulphate Cyanobacteria; *Haloferax mediterranea*		Negative

Table 1: 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*.

In electron micrographs, some EPS appear to form network-like structures; in others,

macromolecular strands have been demonstrated. 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.

2.2. 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 .

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