Review Article

Bacterial exopolysaccharides – a perception

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Microbial polysaccharides are multifunctional and can be divided into intracellular polysaccharides, structural polysaccharides and extracellular polysaccharides or exopolysaccharides (EPS). Extracellular polymeric substances (EPS), produced by both prokaryotes (eubacteria and archaebacteria) and eukaryotes (phytoplankton, fungi, and algae), have been of topical research interest. Newer approaches are carried out today to replace the traditionally used plant gums by their bacterial counterparts. The bacterial exopolysaccharides represent a wide range of chemical structures, but have not yet acquired appreciable significance. Chemically, EPS are rich in high molecular weight polysaccharides (10 to 30 kDa) and have heteropolymeric composition. They have new-fangled applications due to the unique properties they possess. Owing to this, exopolysaccharides have found multifarious applications in the food, pharmaceutical and other industries. Hence, the present article converges on bacterial exopolysaccharides.

Keywords: Applications / Bacteria / Biopolymer / Biosynthesis / Exopolysaccharide / Properties

Received: May 26, 2006; returned for modification: July 20, 2006; accepted: August 18, 2006

DOI 10.1002/jobm.200610203

Introduction

The increased demand for natural polymers for various industrial applications in recent years has led to a renewed interest in exopolysaccharide production by microorganisms. Many microorganisms have an ability to synthesize extracellular polysaccharides and excrete them out of cell either as soluble or insoluble polymers.

Bacterial biopolymers have emerged as new, industrially important polymeric materials, which are gradually proving economically at par with natural gums produced by marine algae and other plants. Various exopolysaccharides produced by bacteria have novel and unique physical characteristics and are generally referred to water-soluble gums (Morin 1998). Exopolysaccharides have found extensive applications in food, pharmaceutical and other industries. Many species of gram-positive and gram-negative bacteria, fungi and also some algae are known to produce exopolysaccharides. Considering the biodiversity of the microbial world and the number of articles published each year on new microbial exopolysaccharides (Table 1), it is astonishing to realize that only three of them (*i.e.* dextran, xanthan and gellan gums) have survived the industrial competition.

Bacterial exopolysaccharides (EPS) produced by several strains have multitudinous applications and occur in two basic forms:

1. As a capsule (capsular polysaccharide, CPS, K-antigens) where the polysaccharide is ultimately associated with the cell surface and may be covalently bound, and
2. As slime polysaccharides which are loosely bound to the cell surface.

Vanhooren and Vandamme (1998) suggested that the exopolysaccharide is bound at a limited number of discrete sites. Alternatively, only molecules of the correct length may bind to attachment sites, with larger and smaller molecules forming the slime.

Physiological role of bacterial exopolysaccharides

The precise role of the exopolysaccharide in exopolysaccharide-producing bacteria, evident in different ecological niches, is dependent on the natural environment

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Table 1. Bacterial polymers, producing strains and their application.

Organism	Polysaccharide/ biopolymer	Application
Pseudomonas aeruginosa and *Azotobacter vinelandii*	Alginate	As immobilization matrix for viable cells and enzymes, coating of roots of seedlings and plants to prevent desiccation, micro-encapsulation matrix for fertilizers, pesticides and nutrients, hypo-allergic wound-healing tissue
Acinetobacter calcoaceticus	Emulsan	-same as above-
Sphingomonas paucimobilis	Gellan	For solidifying culture media, especially for studying marine microorganisms
Streptococcus equii and *Streptococcus zooepidemicus*	Hyaluronic acid	As replacer of eye fluid in ophthalmic surgery, in artificial tear-liquid, synovial fluid replica, in wound healing, cosmetic industry (lotions, moisturizing agent)
Xanthomonas	Xanthan (E 415)	In secondary and tertiary crude-oil recovery, in paints, pesticide and detergent formulations, cosmetics, pharmaceuticals, printing inks (to control viscosity, settling and gelation), in food as thickening and stabilizing agent, often used in combination with guar gum
Acetobacter spp.	Cellulose	In human medicine as temporary artificial skin to heal burns or surgical wounds, in nutrition as natural non-digestable fibers (which can be impregnated with amino acids, vitamins and minerals), as hollow fibers or membranes for specific separation technology, as acoustic membranes in audio-visual equipment
Rhizobium meliloti and *Agrobacterium radiobacter*	Curdlan	As a gelling agent, immoblization matrix, Curdlan along with zidovudine (AZT), displays promising high antiretroviral activity (anti AIDS-drug)
Alcaligenes faecalis var. *myxogenes*	Succinoglycan	-same as above-
Leuconostoc mesenteriodes	Dextran	In veterinary medicine, in human medicine as blood plasma extender or blood flow improving agent and as cholesterol lowering agent, in separation technology, as molecular sieve and in aqueous two phase systems, as micro-carrier in tissue/cell culture (cross-linked dextran)

ment of the microorganism. Most of the functions ascribed to exopolysaccharide are of a protective nature. The ability of a microorganism to surround itself with a highly hydrated exopolysaccharide layer may provide it with protection against desiccation and predation by protozoans. Also, the presence of a gelled polysaccharide layer around the cell may have paramount effects on the diffusion properties, both into and out of the cell (Dudman 1977). For instance, cells buried within a polymer matrix would be inaccessible to antibiotics. Anionic exopolysaccharide may also bind and affect the penetration of both useful and toxic metal ions through the cell surface. This type of interaction assumes practical importance in the corrosion of metallic surfaces. The production of exopolysaccharide in the form of capsules is eminent in pathogenic bacteria, wherein the pathogenicity of an organism depends on the rate of synthesis and the amount of exopolysaccharide synthesized. Capsules enable evasion of phagocytosis. A noteworthy fact is that all capsular polysaccharides do not activate the immune system, which is due to the fact that their chemical structures may mimic the host cell surface components. The lectins (polysaccharide binding proteins secreted by the plant, *e.g.* Trifolin A) play a crucial role in the establishment of the symbiotic association between *Rhizobium* spp. and leguminous plants (Vanhooren and Vandamme 1998).

Exopolysaccharides play a principal role in the formation of cell aggregates, initiation of flocculation and similar processes. This property is vital for wastewater treatment and soil aggregation (Sutherland 2002). The presence of exopolysaccharide in adherent biofilms on inert and biological surfaces has been recognized for some time. However, the widespread incidence of these biofilms and their commercial implications in microbiological problems are as divergent as fouling of pipelines and the onset of dental caries (Vanhooren and Vandamme 1998).

The phenomenal demand for natural polymers for various industrial applications has led to a vibrant interest in exopolysaccharide production by microorganisms. In recent years, there has been a substantial interest in the isolation and identification of new microbial polysaccharides that might have innovative uses as gelling agent, emulsifier, stabilizer or texture-enhancing agent (Sutherland 2001). Production of exopolysaccharide by a moderately halophilic bacterium has been reported by Iyer *et al.* (2001). Many bacteria, which are able to produce exopolysaccharides, have been described to produce either homo or hetero-polysaccharide. However, bacteria such as *Serratia marcescens, Aeromonas salominicida* and *Pseudomonas* sp. strain NCIB 2021 have been reported to produce two different polysaccharides (Kwon *et al.* 1994).

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Biosynthesis and assembly

Extensive progress has been made in recent years in determining the biosynthetic and genetic mechanisms involved in synthesis of exopolysaccharides. The biosynthesis of most exopolysaccharides closely resembles the process by which the bacterial cell wall polymer, peptidoglycan and lipopolysaccharide are formed (Fig. 1). Indeed, the three types of macromolecules share the characteristic of being synthesized at the cell membrane and being exported to final site external to the cytoplasmic membrane. The only exceptions are levans, alternans and dextrans, which are synthesized by an extracellular process (Vanhooren and Vandamme 1998).

Intracellular synthesis of exopolysaccharides

The enzymes involved in exopolysaccharide synthesis are located at different regions of the microbial cell and may be classified into four groups:

- Group I: The first group of enzymes is found intracellularly and is involved in many other cell metabolic processes. One of them is hexokinase, which is involved in the phosphorylation of glucose (Glc) to glucose-6-phosphate (Glc-6-P) and the second enzyme, phosphoglucomutase converts glucose-6-phosphate (Glc-6-P) to glucose-1-phosphate (Glc-1-P).
- Group II: The second group of enzyme is believed to be intracellular. One of them includes uridine diphosphate-glucose pyrophosphorylase (UDP-glucose pyrophosphorylase). This catalyses the conversion of Glc-1-P to uridine diphosphate glucose (UDP-Glc), which is the key molecule in exopolysaccharide synthesis (Fig. 2). The two roles of the sugar nucleotide include

a) the interconversion into other sugars: For example, UDP-Glc $\rightarrow$ UDP-Gal in presence of UDP-Gal-4-epimerase and/or UDP-Glc $\rightarrow$ UDP-Glucuronic acid (UDP-GlcA) in presence of UDP-Glc dehydrogenase,

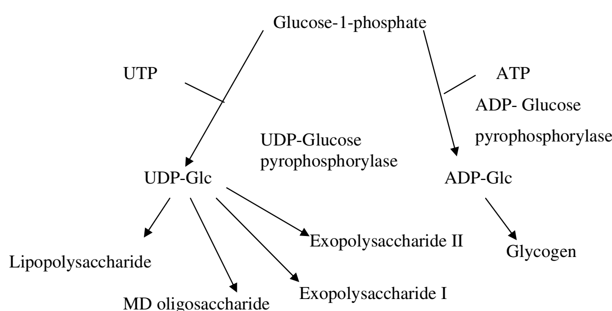

Figure 1. Routes of anabolism of glucose in gram-negative bacteria.

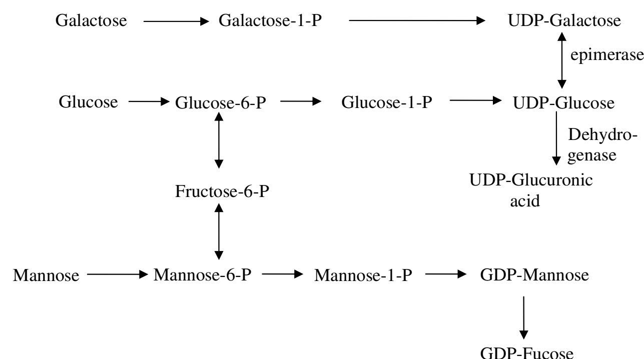

Figure 2. Catabolic systems leading to sugar nucleotide synthesis and interconversions.

b) they are a source of monosaccharide residues during exopolysaccharide synthesis.

- Group III: This group of enzymes is located in the cell periplasmic membrane and referred as glycosyl transferases. They transfer the sugar nucleotides UDP-Glc or UDP-Gal and/or UDP-GlcA to a repeating unit attached to glycosyl carrier lipid. The carrier lipid is identified as an isoprenoid alcohol, and its terminal alcohol group is attached to a monosaccharide residue through a pyrophosphate bridge.
- Group IV: This group of enzymes situated outside the cell membrane and the cell wall are presumably involved in the polymerization of the macromolecules. The exopolysaccharide is then extruded from the cell surface to form a loose slime or a well-attached polysaccharide capsule surrounding the cell (Margaritis and Pace 1985).

Many extracellular polysaccharides possess acyl groups along with activated monosaccharide components. The biosynthesis of such molecules involves a mechanism that needs activated forms of acetate, pyruvate, succinate, phosphate and sulfate. Studies on the type 54 acetylated exopolysaccharide system from *Klebsiella pneumoniae,* vividly indicate that acetyl groups are added from acetyl CoA, whereas phosphoenol pyruvate is the precursor of pyruvate (Vanhooren and Vandamme 1998). In methylated polysaccharides of both eukaryotes and prokaryotes, the methyl groups are derived from methionine or S-adenosyl methionine. It is likely that *O*-methyl sugars are introduced into exopolysaccharides at the isoprenoid lipid-linked polysaccharide level. The possible mechanism of addition of amino acids to exopolysaccharide is unclear. However, it may involve ATP, a specific transferase with Mn^{+2} as a cofactor and amino acids at the sugar nucleotide level. Phosphate groups may be derived from ATP by an appropriate phosphorylation reaction, but in many cases they are more likely to emanate from the sugar nucleo-

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tide. In exopolysaccharides, neither the source of sulfate groups nor the mechanisms of their addition have been examined. Xanthan is one of the widely studied exopolysaccharide. A series of studies on *Xanthomonas campestris* has confirmed that synthesis of xanthan (Fig. 3) followed the same pattern as in *Klebsiella pneumoniae* and other bacterial species.

Diverse mechanisms have been proposed to elucidate transport of exopolysaccharide units across the membrane. Studies show that capsular polysaccharide in *Escherichia coli* are exported at 200–400 discrete zones of adhesion or Bayer's junctions per cell (where the cytoplasmic and outer membranes come into close apposition). These sites also play a role in outer membrane biogenesis by providing a mechanism for inserting new material into the growing membrane. For another *Escherichia coli* exopolysaccharide, a 60-kilodalton periplasmic protein, porin (water-filled channel) is required for its translocation. Presumably, energy, in the form of proton motive force and/or energy-rich phosphate bonds, is needed to permit the extrusion and release of the polymer, but the mechanism has not yet been elucidated.

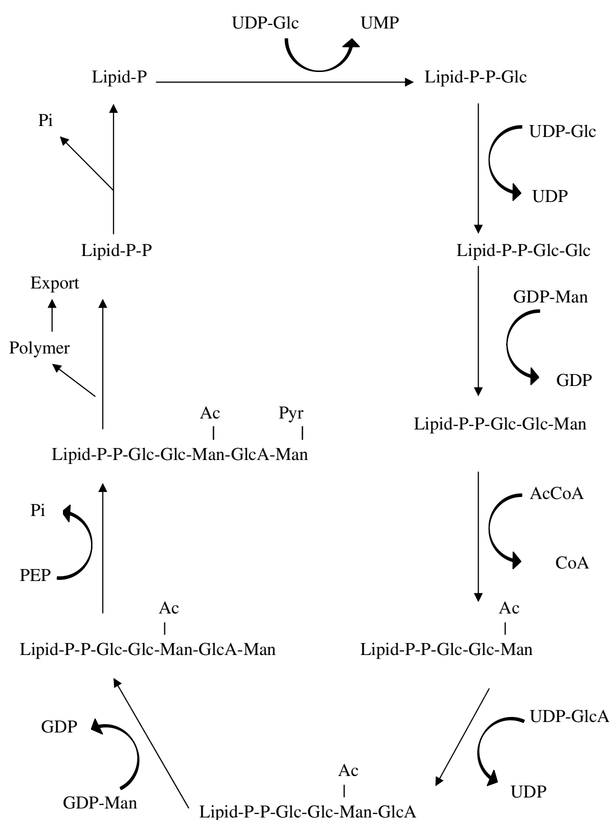

Figure 3. A scheme for xanthan synthesis.

Extracellular synthesis of exopolysaccharides

Well-known examples of exopolysaccharides that are produced extracellularly are dextran, alternan and levan. Dextran is a homopolysaccharide with varying molecular weight [15–20,000 kDa] produced by *Leuconostoc mesenteroides*. Dextransucrase is a glucosyltransferase, which transfers glucose from sucrose to the reducing end of a growing dextran chain, according to the following overall reaction:

$$\text{Sucrose} \rightarrow \text{dextran} + \text{D-fructose}$$

Formation of alternan by *Leuconostoc mesenteroides* occurs by alternansucrase and is analogous to dextran. Alternansucrase is probably a translation product of a mutant gene sequence originally coding for a dextransucrase. The alternansucrase synthesizes alternan, a glucan containing alternating α-(1 → 6) and α-(1 → 3) glycosidic linkages, according to the following reaction:

$$\text{Sucrose} \rightarrow \text{alternan} + \text{fructose}$$

Levan is a β-2,6-fructan also produced extracellularly by *Bacillus*, *Erwinia* and *Gluconobacter* spp., especially when grown on sucrose as a carbon source with the involvement of the enzyme levansucrase (Vanhooren and Vandamme 1998).

$$\text{Sucrose} \rightarrow \text{levan} + \text{D-glucose}$$

Genetics and regulation of exopolysaccharide synthesis

Synthesis of exopolysaccharide is an intracellular process involving nucleoside diphosphate sugars. This constitutes a cassette of genes, the products of which are responsible for acylation and the addition of individual sugars to isoprenoid lipid acceptors. The repeating units are polymerized on the carrier lipids and excreted into the extracellular environment. Typically a gene sequence of the order of 12–17 kb may be required depending on the complexity of the polysaccharide. Considerable similarity among the gene products from different polysaccharide-synthesizing systems has been noted. Although less is known about the polymerization and excretion mechanisms, an ABC transporter is almost certainly involved in those systems which have been best characterized (Sutherland 2001).

In case of *Xanthomonas campestris,* enzymes needed for the formation of precursors not specifically associated with exopolysaccharide production, apparently seem to be under separate control, whereas gene products solely needed for xanthan production occur in the linked group of genes (Fig. 4).

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gum B	gum C	gum D	gum E	gum F	gum H	gum I	gum J	gum K	gum L	gum M

Figure 4. The sequence of genes involved in xanthan synthesis, gum C, gum M, gum H and gum I represent the sequence of 5 monosaccharide transferases forming the pentasaccharide-repeating unit. gum L is a ketalase adding the pyruvate group while gum F is an acetylase. gum E is a polymerase while other genes are concerned with export.

Alginate synthesis in *P. aeruginosa* is also under control of a complex series of regulatory system including *algA*, *algD*, *algE*, *algC* and *algK* genes. The alginate biosynthetic gene cluster in *Azotobacter vinelandii* is organized in 3 operons as opposed to the single operon in *P. aeruginosa.* Little is known about the regulation of acetylation or epimerization, two major modifications which play a very significant role in the final properties of the bacterial alginate macromolecule. The most comprehensive studies on the genetics and biochemistry of exopolysaccharide synthesis have been made in *Rhizobium* strains, which produce succinoglycan. Both the genes and enzymes involved in succinoglycan synthesis have been fully characterized (Sutherland 2001).

Four distinct EPS gene clusters have been characterized to date in *Streptococcus thermophilus* and recent work suggests a dozen or more unique *eps* clusters may occur in this species (Broadbent *et al.* 2003). The genes responsible for exopolysaccharide (EPS) synthesis in *S. thermophilus* Sfi39 are located on a 20-kb genomic fragment. The two genes, *epsE* and *epsG*, were shown to be involved in EPS synthesis as their disruption lead to the loss of the ropy phenotype (Germond *et al*. 2001). On the other hand, *Stenotrophomonas maltophilia* WR-C, capable of forming biofilm on polystyrene and glass, has *rmlA, rmlC* and *xanB* responsible for lipopolysaccharide/exopolysaccharide production (Huang *et al.* 2006)

In some bacteria, exopolysaccharide synthesis is controlled through megaplasmids rather than the chromosome. The exopolysaccharide genes of most mesophilic lactic acid bacteria, such as *Lactobacillus casei* subsp. *cremoris* are situated on plasmids (Vanhooren and Vandamme 1998).

Physical properties

The physical properties of polysaccharides are dependent, not only on the way in which the sequences of sugars are arranged, but also the way these single polysaccharide chains associate with each other (Vanhooren and Vandamme 1998). Relationship exists between the glycoside linkage, geometry of polysaccharide and their conformation. Many microbial polysaccharides undergo transition from an ordered state at lower temperature in the presence of ions, to a disordered state at elevated temperature under low ionic environments (Nisbet *et. al.* 1984). In the case of some polymers, this represents conversion from a gel to a sol state. Slight changes may induce considerable differences in physical properties. The side-chains found on many linear polysaccharides promote conformational disorder and inhibit any ordered assembly. This results in solubility in aqueous solutions. Thus, xanthan, which possesses a cellulose backbone with trisaccharide side-chains on alternate glucose residues, has been described as a natural water-soluble cellulose derivative (Christensen 1995).

Charged residues, found on the exterior of the extended molecules, may readily promote interaction with ions and macromolecules (Chandrasekaran *et al.* 1994). This may account for the extensive binding of heavy metal cations ascribed to *Zoogloea* flocs and polysaccharide (Friedman and Dugan 1967, Kuhn and Pister 1989). Bacterial alginates resemble their algal analogs in binding calcium in preference to magnesium ions, with binding and selectivity being greatly enhanced by the removal of acetyl groups (Geddie and Sutherland 1994).

Extensive studies on polysaccharides such as xanthan have revealed some of the relationships between their carbohydrate structure, acylation and physical properties. Here, the usual ordered structure appears to be in the form of a double helix. This undergoes transition to disorder at various temperatures in the range of 50–90 °C depending on the extent and type of acylation and salt concentration of the solute. Pyruvate and acetate influence the transition by respectively stabilizing or destabilizing the ordered conformation. The degree of acylation in xanthan also affects the interactions with the plant polysaccharides to form effective synergistic gels. Acetyl groups generally stalled synergistic association and their removal yielded polymers gelling at lower concentrations. Xanthan is also unusual in that, unlike many polyanionic polysaccharides, it is soluble in its acid form at pH <3 (Sutherland 1997).

The alginate-like exopolysaccharides produced by *Pseudomonas aeruginosa* and *Azotobacter vinelandii* strains are comparable with other commercial products from marine algae. Non-acetylated alginate shows strong, selective binding with Ca^{+2} and Sr^{+2}, whereas, the acetylated alginates from *Azotobacter vinelandii* evidenced reduced binding and lack of selectivity towards Ca^{+2} and Mg^{+2} (Geddie and Sutherland 1994).

Another exopolysaccharide, in which acetylation plays a critical role in conformation and physical prop-

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erties, is XM6 from an *Enterobacter* spp. It is composed of a tetrasaccharide-repeating unit and lacks acylation. It interacts with various monovalent and divalent cations to form a gel with a very sharp sol-gel transition proximate to 30 °C. However, *K. aerogenes* type 54 exopolysaccharide possesses the same carbohydrate structure but is either acetylated on the fucosyl residue of each repeating unit or every alternate unit. This polysaccharide showed high viscosity but failed to gel. X-ray fiber diffraction indicated that both types of exopolysaccharide had similar helical structures, though XM6 was highly crystalline (Atkins *et al.* 1987).

The physical properties of a microbial exopolysaccharide are explained in terms of different rheological behaviors which include pseudoplastic flow or shear thinning, yield stress, thixotropy and viscoelasticity (Margaritis and Pace 1985).

Modified polysaccharides

Many polysaccharides exhibit useful properties when they undergo chemical modification. This usually entails alkali treatment to remove acyl groups or acid treatment. Xanthan with modified side-chains, in which, the β-D-mannosyl residues were removed by mild alkali treatment, proved capable of maintaining the ordered double standard state (Christensen *et al.* 1993a, b). Callet *et al.* (1987) demonstrated that in xanthans of the same molecular weight, neither the acetyl nor pyruvate substitutes influence dilute solution viscosity. They noted that acetyl group had a stabilizing effect on conformational transition of xanthans while pyruvate group had the opposite effect.

Synergistic gelling

Xanthan and acetan demonstrate considerable structural similarities. Both yield viscous aqueous solution and undergo a thermally reversible order-disorder transition in solution. However, the differences are conspicuous in synergistic gelling. Ross-Murphy *et al.* (1996) used a series of xanthan preparation to demonstrate that the acyl group of xanthan created a significant role in interactions with guar gum, locust bean gum (LBG) and konjac mannan. Removal of the acetyl group from xanthan enhanced gelation. Generally, xanthan formed a relatively strong network with LBG. It has been found that although native acetan does not form gel with LBG or konjac mannan, deacetylation of acetan promotes synergistic interaction with both (Ojinnaka *et al.* 1998).

Source of exopolysaccharide-producing bacteria

Microorganisms producing exopolysaccharide are found in various ecological niches. Environment offering a medium with high carbon/nitrogen ratio are known to contain microorganisms producing polysaccharides, for example, effluents from the sugar, paper or food industries as well as wastewater plants (Morin 1998).

Isolation of exopolysaccharide-producing bacteria

Exopolysaccharide producing organisms can be isolated using a complex media or a chemically defined synthetic media. These organisms produce colonies with mucoid or watery surface and thus can be detected macroscopically. Morin (1998) stated that, no direct correlation exists between morphological characteristics of colonies on solid medium and the ability of a culture to produce polysaccharides in liquid medium. Some polysaccharides might form stable complexes with water-soluble dyes such as aniline blue, which could be used as a screening tool.

Production of exopolysaccharides

Process for production of exopolysaccharide are characterized by the extreme rheology of the fermentation, product concentration, the diversity of subtle structural and conformational changes (which can occur throughout the entire process) and the discernable effect of these changes on the product's end application performance. This can be elaborated as under.

Fermentation of exopolysaccharides

The successful design of a fermentation state of the process relies on producing the product to a set specification while achieving a product concentration, yield and productivity for a set economic target. These goals can best be reached within minimum risk by establishing how the microbe's performance is controlled by its environment, in turn, how it relates to equipment design operation. The specific controls which exists at these two levels, i.e. environmental and equipment design and operation, are characteristics of exopolysaccharide fermentations.

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There is no single set of culture conditions that guarantees high exopolysaccharide yields, since organisms differ in their carbon and nitrogen source utilization, mineral requirements, temperature and pH optima, which are the critical factors for maximum exopolysaccharide production (Sutherland 1972, Williams and Wimpenny 1977). Physiological control is used to modulate the relative molecular mass (M_r), the pattern and number of residues and the degree of branching of the exopolysaccharide produced. The yield and quality of microbial exopolysaccharide are greatly affected by the nutritional and environmental conditions and an increase in polymer production is possible by manipulating the culture conditions.

Carbon source

A wide variety of carbon sources, used to produce microbial exopolysaccharides, include sucrose, glucose, lactose, maltose, mannitol, sorbitol, whey, starch, sugar concentrates (Neosorb™, Cerelose™), methanol and C_9 to C_{16} *n*-alkanes. The type of carbon source influences the yield of exopolysaccharide (Morin, 1998). The size of the exopolysaccharide may also vary with the carbon source. For instance, alginate produced on fructose and glucose after 48 hours of growth had a maximum M_r of 500 kDa and 276 kDa, respectively (Conti *et al.* 1994). *Lactobacillus delbruckii* synthesizes different exopolysaccharides when grown on glucose or fructose (Grobben *et al.* 1996). When grown on fructose, the strain produced 25 mg l^{-1} exopolysaccharide composed of glucose and galactose in the ratio 1:2.4. When the carbon source was switched to a mixture of fructose and glucose, the exopolysaccharide production increased to 80 mg l^{-1} while the sugar composition changed to glucose, galactose and rhamnose in a ratio of 1: 7: 0.8. However, Petry *et al.* (2000), reported contradictory observations wherein different carbon sources did not influence the component sugars of exopolysaccharide produced by *Lactobacillus delbruckii.* Degeest and De Vuyst (2000) and Escalante *et al.* (1998) made similar observations in case of *Streptococcus thermophilis,* where different carbohydrates did not result in variation in exopolysaccharide composition. They reported that carbon source invariably affects the total amount of polysaccharide produced. West and Strohfus (1998) have studied the effect of different carbon sources on gellan production by *Sphingomonas paucimobilis*. According to them, a number of carbon sources including glucose and corn syrup could support gellan production. However, the ability of carbon source to produce higher cell weight did not translate into increased gellan production.

Nitrogen source

The nitrogen sources currently being used for exopolysaccharide production are ammonium sulfate, peptone, sodium nitrate, urea and yeast extract. The use of organic nitrogen sources often results in a higher specific growth rate and exopolysaccharide production, which might be due to the addition of growth factors in trace amounts (Farres *et al.* 1997). Further, some of the carbon found in the nitrogen source might serve as a substrate for exopolysaccharide production (de Souza and Sutherland 1994). This contributes to increase in the C:N ratio, thus promoting the exopolysaccharide production (Morin 1998). According to Vergas-Garcia *et al.* (2001), biomass levels were higher as the nitrogen concentration increased in the medium. However, under these conditions, exopolysaccharide synthesis showed an opposite pattern to that observed for growth. Generally, exopolysaccharide production was higher at lower nitrogen concentration. Although it has been reported that supplementation with small amounts of combined nitrogen stimulates exopolysaccharide yield (Vermani *et al.* 1997). Gorret *et al.* (2001) demonstrated that addition of yeast extract to the medium improved both growth and exopolysaccharide production by *Propionibacterium acidi-propoinici.*

Ion source

Phosphorous content influences exopolysaccharide production. In case of *Klebsiella* spp., maximum exopolysaccharide could be produced in absence of phosphate ion (Farres *et al.* 1997). Optimization of the fermentation parameters in pH-controlled, phosphate-limited, acetate-free media has led to high alginate yields upto about 7 g l^{-1} (Clementi *et al.* 1995). Omission of iron (Fe^{+3}), zinc (Zn^{+2}) and ammonium (NH_4^+) ions have no impact on the growth and exopolysaccharide production by *Lactobacillus bulgaricus.* Sugar composition of exopolysaccharide was also not affected by variation in the concentration of these ions (Grobben *et al.* 2000). Contradictory results have been reported for the iron requirement of lactic acid bacteria by Pandey *et al.* (1994). In case of *Lactobacillus casei*, addition of Mn^{+2}, alone or in combination with citrate, Ca^{+2} and SO_4^{-2}, are known to strongly stimulate exopolysaccharide production (Mozzi *et al.* 1995).

Oxygen and aeration rate

According to Lee *et al.* (2001), high aeration rate resulted in enhanced exopolysaccharide production and increased the viscosity of the culture broth in the case of marine bacterium *Hahella chejuensis.* Similar results were obtained by Yang and Liau (1998) where higher

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agitation and aeration appeared to be favorable for the formation of polysaccharide by *Ganoderma lucidum.*

Transport of monosaccharides outside the cell involves coupling to C-55 isoprenoid alcohol phosphate. Polymerization is performed by a polysaccharide polymerase outside the cell. Thus, molecular oxygen would be necessary for primary energy metabolism and also for the oxidation of sugar to the corresponding alcohol and for reoxidizing reduced pyridine nucleotides. An increase in exopolysaccharide production may result as a consequence of better availability of oxygen and nutrients (Dassy *et al.* 1991, Bayer *et al.* 1990). The use of detergents may ameliorate oxygen concentration in the exopolysaccharide containing broth. In the presence of detergents, *Xanthomonas* cells are smaller than those seen in the absence of detergents. This may lead to higher oxygen uptake rate (OUR). On the other hand, an adverse effect of high oxygen-transfer rates may be seen in the case of *Aureobasidium pullulans* (Weckner and Onken 1991) and *Fusarium solani* (Rau *et al.* 1989).

Viscosity

Change in the rheology of a medium is a direct consequence of product formation or exopolysaccharide secretion. During exopolysaccharide production, the broth develops non-Newtonian characteristics and may act as a pseudoplastic fluid where the measured viscosity decreases with increasing shear rate. This change in rheology can be caused by exopolysaccharide producing microorganisms, their behavior and product formation. This may also be due to lack of homogeneity in terms of mixing, mass, oxygen and heat transfer in the bioreactor. Under such conditions, where the exopolysaccharide producing microorganisms in non-Newtonian broth are exposed to gradients in a number of variables, the quality of the polysaccharide would be heterogeneous (*i.e.*, changes in M_r, branching and rheological properties). Viscosity of the culture broth might result from polymer concentration with negligible contributions from the cells. Here, the rheology can be used as a parameter to monitor exopolysaccharide production and quality (Morin 1998).

Dilution rate

Fermentation, used for exopolysaccharide production, is batch or fed-batch processes, during which the exponential growth phase and the exopolysaccharide synthesis do not occur simultaneously. In batch cultures, polysaccharide synthesis takes place when the medium is depleted with one or more nutrients and it is often maximal in media with a high carbon/nitrogen ratio. Maximum levels of polysaccharides were observed at a low dilution rate of continuous cultures of *Pseudomonas, Alcaligenes* and *Klebsiella* spp. Lower dilution rate increases the residence time of microorganisms and promotes the utilization of excess carbon for the production of exopolysaccharide. At high dilution rates, the isoprenoid lipid could be insufficiently available for simultaneous synthesis of various surface polymers, including the exopolysaccharide (Sutherland 1977).

Incubation temperature

An incubation temperature below the optimum growth temperature results in greater production of exopolysaccharide (Cerning *et al.* 1992 and Gancel and Novel 1994). A low incubation temperature (32 °C) can cause reduction in growth rate and cell mass, which in turn resulted in long logarithmic phase of growth and higher viscosity as compared to high temperature (37 °C). The difference between optimal temperature for growth and exopolysaccharide production could also be a result of increased activities of enzymes involved in the synthesis of exopolysaccharide precursors. For instance, production of GDP-mannuronic acid which is a precursor of alginic acid was enhanced at a sub-optimal growth temperature (Morin 1998). Gorret *et al.* (2001) demonstrated increased exopolysaccharide production at lower temperature in case of *Propionibacterium acidipropionici*. Such results could be explained by the mechanism proposed by Sutherland (1972), *i.e.* a decrease in temperature causes a decrease in growth rate and cell wall polymer biosynthesis, making more precursors available for exopolysaccharide synthesis. Paradoxically, Garcia-Garibay and Marshall (1991) have reported increase in production of exopolysaccharide with increase in temperature in case of *Lactobacillus delbrueckii* subsp. *bulgaricus*. Pediococci used as meat starters were found to produce exopolysaccharide when grown at 15–35 °C.

Incubation pH

Numerous microorganisms produce exopolysaccharide in media buffered at neutral pH. Many of the exopolysaccharide-producers require a constant pH for maximum production of exopolysaccharide (Morin 1998). Others like *Neisseria meningitidis* produce more exopolysaccharide at acidic pH values (Morin, 1998). Exopolysaccharide produced by some meat starters were excreted when the starters were grown in culture media buffered between pH 5.2–6.5 and contained 2–4% sodium chloride (Van Beek 1997). Poor bulk mixing may result in significant pH gradients in the bioreactor, which may generate problems in fermentation monitoring and control of pH (Morin 1998). In case of *Propi-*

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onibacterium acidi-propionici, production of exopolysaccharide was possible only between pH 5.3–6.5, suggesting that regulation of the biosynthetic pathway of exopolysaccharide production may be dependent on pH (Gorret *et al.* 2001).

Age of the exopolysaccharide producing cells and growth

Bergmaier *et al.* (2002) reported linear effect of age on lactic acid concentration in case of *Lactobacillus rhamnosus* RW-9595M. Studies on effect of physiological age and state on survival of *Pseudomonas aeruginosa* have been carried out by Skaliy and Eagon (1972), wherein they noticed the vital role of exopolysaccharide. Exopolysaccharide-producing microorganisms usually reach their optimal growth within the initial 24 h of incubation, whereas, maximal exopolysaccharide production occurs in the later stages of growth (*e.g.* during the stationary phase) (Morin 1998). Other factors such as the presence of endogenous glycanolytic enzymes may affect the characteristics of the exopolysaccharide. Some polysaccharides are indeed subjected to enzyme hydrolysis during the late phase of growth. This degradation contributes to the reduction in viscosity of the culture medium. Such types of microbial enzymes (alginase) are reported in case of alginate production.

Sutherland (1982) stated that there is a competition between EPS and cell-wall polymer (peptidoglycan, teichoic acids, lipopolysaccharides) biosynthesis for the isoprenoid glycosyl lipid carriers, and consequently EPS production is not growth-associated. Petry *et al.* (2000) reported majority of the EPS production during the stationary phase for two *Lactobacillus delbrueckii* subsp. *bulgaricus* strains. In case of a batch experiment with *Alteromonas macleodii* subsp. *fijiensis*, isolated from a deep sea vent, the production of EPS began at the end of the exponential phase and continued throughout the stationary phase, reaching a value of 6.0 g (dry weight) per liter at the end of 60 h (Raguenes *et al.* 1996).

The size and form of the producing cells may vary during exopolysaccharide production. *Sclerotium glucanicum* and *Sclerotium rolfsii* tend to grow as pellets surrounded with a layer of scleroglucan, which reduces production of this polymer, possibly because of a reduced mass transfer rate to/from the cells. Similar interpretation could be derived for bacterial exopolysaccharide too.

Osmolarity

Osmolarity plays crucial role in both, growth and exopolysaccharide production. Fast-growing *Rhizobium melilotii* produces two different water-soluble exopolysaccharides, namely succinoglycan and galactoglucan, the ratio of which is influenced by the osmolarity of the culture medium. In the absence of sodium chloride in the medium, the ratio of both exopolysaccharides was 1:1. Upon increasing osmolarity of the culture medium up to 0.6 M NaCl, the proportion of sucinoglycan increased to 85% (Navrini *et al.* 1992).

Detergents

The presence of detergents influences the production of xanthan (Morin 1998). Effect of detergents such as Tween 40 (polyoxyethylene sorbitan monopalmitate) or Tween 80 (Polyoxyethylene sorbitan monooleate), CHAPS (3-[(3-cholamidopropyl) dimethyl ammonio]-1-hydroxypropane- sulfonate and Triton X-100 (nonaethylene glycol octylphenol ether) has been investigated by adding them after 24 h of growth of *Xanthomonas campestris.* An increase of 1.45 fold in xanthan production was observed at a low concentration (0.1 g l^{-1}) of Triton X-100. Here no severe foaming was observed because detergents were added after 24 h of growth when viscosity was already high. The addition of Triton X-100 improved xanthan production probably by altering the oxygen transfer rate and had no effect on the biomass production and broth viscosity. The addition of such detergents could affect factors controlling the rheological quality of xanthan such as its M_r (molecular weight). It has been suggested that, by interacting with the *Xanthomonas campestris* membrane, detergents could enhance the polymerization process or the release of xanthan (Morin 1998).

Carbon/nitrogen ratio (C/N or C : N ratio)

Carbon (C), nitrogen (N), phosphate (P) and oxygen (O) limitations are few of the factors that effect the conversion of the carbon source into polysaccharide. For instance, N, C and O limitations affected the conversion of glucose into alginate and the proportion of mannuronate to glucuronate residues in *Pseudomonas mendocina*. Exopolysaccharide production is favored by a high carbon/nitrogen ratio, where 10:1 is considered to be the most favorable for maximal exopolysaccharide production. Disappearance of nitrogen from the medium might also be a signal for exopolysaccharide synthesis, as observed for pullulan and scleroglucan (Morin 1998).

Recovery of exopolysaccharides

The cost of recovery of exopolysaccharide, including concentration, isolation and purification, is a signifi-

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cant part of the total production cost. This is due to lower concentration of exopolysaccharide in the fermentation broth, the presence of contaminating solid (*e.g.* cells), solutes in the stream and the high viscosity of the fermentation liquid/broth. The objective of recovery include

a) concentration of fermentation broth to enable easy handling, microbiological stability, easy transport and storage and ready solubility;
b) purification to reduce non polymer solid (cells or salt), and to improve functional performance, color, odor or the taste of the product;
c) deactivation of undesirable contaminating enzymes (Smith and Pace 1982).

The constraint in downstream processing of exopolysaccharide lies in the separation of microbial cells from the culture broth and the degree of association of the exopolysaccharide to the microbial cells (*i.e.* as slime or a capsule).

Extraction of exopolysaccharide

a) Extraction of exopolysaccharide existing as slime: "Slime" exopolysaccharide can be isolated from microorganisms by centrifugation. The speed and duration of centrifugation depend on the nature and viscosity of the polysaccharide. When working at the laboratory scale, ultra-centrifugation may be used to remove most of the cells or their debris from the culture broth containing polysaccharides (Morin 1998). In cases where the exopolysaccharide is thermally stable, heat treatment can be used to improve the separation of microorganisms from the broth. Besides lowering the viscosity, heat treatment partially kills the cells through pasteurization and also inactivates some of the enzymes present in the broth. In case of xanthan, heat treatment enhances the viscosity, though this effect is pH dependent (Sutherland 1990).
b) Extraction of exopolysaccharide existing as a capsule: "Capsular" exopolysaccharide must first be dissociated from the cells. The selection of the method depends on the nature of the association between the cells and the polysaccharides. Centrifugation enables the separation of weakly associated capsular exopolysaccharide. As the capsular exopolysaccharide is strongly associated to the cells, more severe conditions, such as alkaline treatment (*e.g.* with sodium hydroxide), prior to centrifugation and alcohol precipitation are needed (Morin 1998).

Other drastic methods include boiling the cell suspension for 15 min in water, heating at 60 °C in saline solution, heating in a mixture of phenol water at 65 °C or sonicating the cell suspension. Autoclaving is the most frequently used treatment for releasing capsular polysaccharides from cells, but this might cause cell disruption and decrease in broth viscosity.

Precipitation and purification of exopolysaccharide

Exopolysaccharides are generally recovered by solvent precipitation of the culture broth. The polysaccharide can be precipitated from the supernatant by the addition of polar organic solvent miscible with water, such as lower alcohols or acetone. The proportion of solvent used is variable, which can be one, two or three volumes of the culture broth, although two volumes are most often used. Organic solvents permit separation by lowering the exopolysaccharide solubility in water. They may also serve to decolorize and to extract low-molecular-mass fermentation products and medium components.

During solvent precipitation, proteins and salts of the medium may also precipitate along with exopolysaccharide. Deproteination and desalting treatments may be performed if a pure exopolysaccharide is needed. A rhamnose-containing polysaccharide produced as a capsule and slime by *Klebsiella* spp. was dissociated from the cells by adjusting the pH of the broth to 4–5 with 11.32 M HCl prior to autoclaving at 121 °C for 30 min. Such treatment reduces 40% of the protein content of the supernatant containing the exopolysaccharide, thus enhancing the recovery of rhamnose (Morin 1998).

Besides currently used water-miscible organic solvents, the use of cetyltrimethylammonium bromide (CTAB) and 3,5,6-triphenyl-2,3,5,6-tetraaza bicyclo-1-hexene (commercially known as nitron) has also been reported. Recovery of about 99% has been reported using only 0.1 volume of a 10% solution of nitron in 3% acetic acid as the precipitating agent of neutral (*e.g.* hydroxypropyl starch) and acidic (*e.g.* sodium alginate) polysaccharides. Precipitation of the same polysaccharide using three volumes of propanol contributed to recovery of about 75% (Morin 1998).

The final exopolysaccharide precipitate could be harvested following centrifugation, filtration, pressing, or settling. Drying is performed under vacuum or with inert gas. The dry exopolysaccharide is milled to the desired mesh size. The final exopolysaccharide products are off-white to white, depending on their purity. Other alternatives like ultrafiltration and reverse osmosis are also available for reducing the water content of exopolysaccharide preparations (Sutherland 1990).

In case of dairy starters, milk protein such as casein must be removed prior to exopolysaccharide isolation. Here, the culture broth is digested with proteolytic

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enzymes (*e.g.* Pronase E, Protease type XIV) in the presence of 0.1% merthiolate to preclude further microbial growth. Inactivation of the enzyme is carried out using heat treatment followed by precipitation with 10% trichloroacetic acid. Cells can be removed by centrifugation and the supernatant is concentrated by ultrafiltration (molecular weight cutoff 8–10 kDa). The exopolysaccharide is recovered by ethanol precipitation.

The precipitated exopolysaccharide are recovered by centrifugation, dissolved with distilled water and dialyzed for 24 h at 4 °C to remove residual sugars or other medium components. The dialyzed exopolysaccharide is then freeze-dried and stored at 4 °C (Cerning *et al.* 1988).

Treatments after recovery of microbial exopolysaccharide

Exopolysaccharides are usually used in their native form (as produced) i.e. no modifications are carried out prior to its use. However, they may be physically and chemically treated or modified. These treatments affect the rheological or physical properties of polysaccharides. Dextrans of varying molecular mass can be obtained by hydrolysis of high molecular mass dextran at elevated temperature with hydrochloric acid (Sutherland 1990).

Gellan, treated with mild alkali (pH 10, with sodium hydroxide or potassium hydroxide) to deacetylate it, formed a clear and brittle gel (Kang *et al.* 1982). A rhamnose-containing exopolysaccharide has been claimed to harbor emulsifying activity and to enhance microbial cell surface hydrophobicity due to which it could find application in cosmetics. Methyl-pentose-containing polysaccharides, when used as source of rare sugars such as fucose and rhamnose, are subjected to hydrolysis with 2N sulfuric acid under reflux at 100 °C followed by neutralization with barium hydroxide. Biotechnology is a splendid way to modify exopolysaccharide structure to achieve changes in functional properties (Morin 1998).

Applications of exopolysaccharides

Exopolysaccharides find their applications in various fields ranging from medicinal applications to being used as a source of monosaccharides. They are enlisted below:

1. Medicinal applications: Antitumor, antiviral and immunostimulant activities of polysaccharides produced by marine *Vibrio* and *Pseudomonas* have been reported by Okutani (1984 1992). A low molecular weight heparin-like exopolysaccharide exhibiting anticoagulant property has been isolated from *Alteromonas infernus*, obtained from deep-sea hydrothermal vents (Colliec *et al.* 2001). Clavan, a L-fucose containing polysaccharide has a potential application in preventing tumor cell colonization of the lung, in controlling the formation of white blood cells, in the treatment of the rheumatoid arthritis, in the synthesis of antigens for antibody production and in cosmeceuticals as skin moisturizing agent (Vanhooren and Vandamme 2000).
2. Gelling agent: Gelrite, obtained from *Pseudomonas* spp., is a new gelling polysaccharide with good thermal stability and clarity. It has been reported that gelrite is superior to agar (Lin and Casida 1984). It forms a brittle, firm and optically clear gel upon deacetylation using mild alkali (Kang *et al.* 1982).
3. Emulsifiers: Surfactants and emulsifiers from bacterial sources have attracted attention because of their biodegradability and possible production from renewable resources. Emulsan produced by *Acinetobacter calcoaceticus* RAG-1 has been commercialized (Rosenberg *et al.* 1979). A viscous exopolysaccharide has also been isolated from *Sphingomonas paucimobilis*. This polysaccharide stabilized emulsions more effectively than other commercial gums such as arabic, tragacanth, karaya and xanthan (Ashtaputre and Shah 1995). Apart for Emulsan, an exopolysaccharide produced by a marine bacteria, is reported to form stable emulsions with a number of hydrocarbons. This exopolysaccharide proved to be more efficient than the commercially available emulsifiers (Iyer *et al.* 2006).
4. Heavy metal removal: Contamination of the environment by heavy metals is of growing concern because of the health risks posed to humanity and animals. Cell bound polysaccharide produced by marine bacterium, *Zooglea* sp., has been reported to adsorb metal ions like chromium, lead and iron in solutions (Kong *et al.* 1998). Biosorption of heavy metals by *Enterobacter cloaceae* is reported by Iyer *et al.* (2004, 2005a).
5. Enhanced oil recovery: The *in situ* production of xanthan-like polysaccharide in the oil-bearing strata has been suggested as a means of aiding tertiary oil recovery (Wells 1977). *Volcaniella eurihalina* F2-7 is known to synthesize an exopolysaccharide, the rheological properties of which are stable to pH and inorganic salts, which makes it a suitable candidate for enhanced oil recovery (Calvo *et al.* 1995). Exopolysaccharide produced by *Enterobacter cloaceae* has been reported to have good viscosity even at high tem-

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perature, which makes it a probable candidate for microbial enhanced oil recovery (Iyer *et al.* 2005b).

6. Source of monosaccharides: Certain bacterial extracellular homo- and hetero-polysaccharides are source materials to obtain unusual but valuable monosaccharide constituents *e.g.* L-fucose, L-rhamnose, L-altrose, D-mannose etc. which are otherwise difficult to obtain because chemical synthesis or extraction from plant or animal tissues is laborious, expensive and often in scant supply. *Clavibacter* species produce clavan, which is rich in D-fucose (Vanhooren and Vandamme 2000). The exopolysaccharide (containing 18.9% w/w L-fucose) produced by *Klebsiella pneumoniae* is a source of L-fucose (Vanhooren and Vandamme 1998). Iyer *et al.* (2005c) reported the production of exopolysaccharide by *Enterobacter cloaceae* which is also rich in fucose.

New products

"Could we expect enterprising products", is an upbeat question to be explored at this junction of time. Widespread screening of exotic and native environments is being carried out, which have yielded novel bacteria. It is likely that many more new products from bacteria will be developed and commercialized. Obtaining food approval for many of these would be a costly and time-consuming business, whether the exopolysaccharide is from natural sources or results from a genetic manipulation. However, search for novel properties like improved gelation, might lead us to serendipitous discovery of new products for non-food use.

Poly-D-glucuronic acid has several attributes of alginates and might prove to be applicable for similar purposes (Courtois *et al.* 1993). Like alginates, this polymer could form a thermostable gel in the presence of Ca^{+2}. It also formed a thermoreversible gel with monovalent ions at higher concentrations. The use of extracellular poly-D-mannuronosyl–C-5-epimerase found in many alginate-synthesizing bacteria may permit tailoring of the composition of alginates of bacterial or algal origin to yield novel physical characteristics (Sutherland 2001). A novel rhizobial exopolysaccharide, composed of disaccharide repeating units of D-glucose and D-glucuronic acid, has been reported by Guentas *et al.* (2000).

Examination of numerous isolates from deep-sea hydrothermal vents revealed a few polymers with interesting properties such as gelation or high viscosity in aqueous solution (Rougeaux *et al.* 1996). One such exopolysaccharide having viscosity of the same order of magnitude as that of xanthan is a bacterial polysaccharide of industrial interest, produced by *Alteromonas macleodii* sub sp. *fijiensis*, containing glucose, mannose, pyruvated mannose and galactose along with galacturonic acid and glucuronic acid. This unusual exopolysaccharide produced during the stationary phase, has interesting biological properties and has application in cosmetics (Raguenes *et al.* 1996 and Cambon-Bonavita *et al.* 2002).

In order to improve the industrial production of exopolysaccharide, it is necessary to follow either of the following criteria or a combination of them:

- Increasing the rate of exopolysaccharide synthesis
- Modifying exopolysaccharide as per requirement during or after production
- Preventing alteration of exopolysaccharide by unwanted enzymes
- Altering the surface properties of the exopolysaccharide-producing microorganism in order to promote cell separation during downstream processing
- Transferring genetic determinants of exopolysaccharide to more efficient host producers

The polymers already in the market scenario (plant, algal and microbial) appear quite adequate for most applications and industrial demand. Nonetheless, the look for greener technologies will probably augment the use of bacterial exopolysaccharide for industrial applications. Thus, the use of bacteria as renewable resource for the production of biopolymers can be envisaged. The present knowledge of bacterial exopolysaccharides suggests that these polymers may cover a broad range of complex chemical structures and consequently different properties. Moreover, it is reasonable to anticipate that exopolysaccharides from newer bacteria would provide ample opportunities for newer industrial avenues and have chattels different from those already available.

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