HIGH VISCOSITY DIUTAN GUMS and METHODS OF PRODUCING

Field of the Invention

The present invention describes the production of a diutan polysaccharide exhibiting increased viscosity properties as compared with previously produced polysaccharide of the same type of repeating units. Such an improved diutan polysaccharide is produced through the generation of a derivative of *Sphingomonas sp.* ATCC 53159 that harbors a multicopy broad-host-range plasmid into which genes for biosynthesis of diutan polysaccharide have been cloned. The plasmid provides the capability within the host *Sphingomonas* strain to produce multiple copies of genes for such polysaccharide synthesis. In such a manner, a method of not just increased production of the target diutan polysaccharide, but also production of a diutan polysaccharide of improved physical properties (of the aforementioned higher viscosity) thereof is provided. Such a diutan polysaccharide has proven particularly useful as a possible viscosifier in oilfield applications and within cement materials. The inventive methods of production of such an improved diutan polysaccharide, as well as the novel cloned genes required to produce the improved diutan within such a method, are also encompassed within this invention. Additionally, the novel engineered *Sphingomonas* strain including the needed DNA sequence is encompassed within this invention.

Background of the Invention

Polysaccharides or gums are primarily used to thicken or gel aqueous solutions and are frequently classified into two groups: thickeners and gelling agents. Typical thickeners include starches, xanthan gum, diutan gum, welan gum, guar gum, carboxymethylcellulose, alginate, methylcellulose, gum karaya and gum tragacanth. Common gelling agents include gelatin, gellan gum, starch, alginate, pectin, carrageenan, agar and methylcellulose.

Some polysaccharides, or more particularly stated, biogums, such as xanthan, gellan, welan and diutan have been produced via fermentation from microbes for many years. Such biogums exhibit varied characteristics such as viscosity modification capabilities that have permitted their utilization in many different applications. Included within such a list are gelling agents for foods, such as confectionery jellies, jams and jellies, dessert gels, icings and dairy products, as well as components of microbiological media. Furthermore, thickening agents are utilized for myriad end-use applications to modify the viscosity of target liquids. Of particular interest is the ability of such gums to impart viscosity modification to underground and/or underwater petroleum liquids to facilitate collection thereof, although many other different possible end-uses exist (including cement production, as one example). Different biogums have been produced from different bacterial sources, such as xanthan gum, from *Xanthomonas campestris*, gellan gum, from *Sphingomonas elodea*, welan gum from *Sphingomonas sp.* ATCC 31555, and diutan gum (S-657), from *Sphingomonas sp.* ATCC 53159. Genetic modifications of such strains have been undertaken in the past to effectuate significant changes in the resultant gum materials produced through the aforementioned

fermentation procedures. Such modifications have permitted such changes as removal of acyl groups to create different gum materials exhibiting different physical properties. Generally, such genetic modifications have been of the type to either alter the composition of the target biogum ultimately through altered gene expression within the host organism, or increase the yield of the target biogum, through introduction of a plasmid that exhibits gene amplification alone (such as in U.S. Patent Nos. 5,854,034, 5,985,623, and 6,284,516, to Pollock et al. and U.S. Patent No. 6,709,845 to Pollock alone).

Diutan gum (also known as heterpolysaccharide S-657) is prepared by fermentation of strain *Sphingomonas sp.* ATCC 53159 and exhibits thickening, suspending, and stabilizing properties in aqueous solutions. Diutan generally exhibits a hexameric repeat unit consisting of four sugars in the backbone (glucose-glucuronic acid-glucose-rhamnose) and a side chain of two rhamnose residues attached to one of the glucose residues. Details of the diutan gum structure may be found in an article by Chowdhury, T. A., B. Lindberg, U. Lindquist and J. Baird, Carbohydrate Research 164 (1987) 117-122. Diutan was shown to have two acetyl substituents per repeat unit within Diltz et al., Carbohydrate Research 331 (2001) 265-270. Both of these references are hereby incorporated by reference in their entirety. Details of preparing diutan gum may be found in U.S. Pat. No. 5,175,278, which is hereby incorporated by reference in its entirety. Diutan may be produced from the *Sphingomonas* strain by utilizing standard fermentation techniques such as using carbohydrate sources (glucose, maltose, and the like, as non-limiting examples), a nitrogen source, and additional salts.

The physical characteristics imparted by such a diutan biogum in its wild-type form are desired by certain industries, particularly in terms of its viscosity modification properties and/or water retention characteristics. Unfortunately, diutan has proven difficult to produce cost effectively. Furthermore, such cost issues militate against widespread utilization of diutan currently since the degree of viscosity exhibited by such a biogum is insufficient to supplant other less expensive, but effective, biogums (such as xanthan gum, as one example). As such, it has been an established need to provide a method to produce such an effective diutan at lower cost, at the very least, and/or to provide a manner of producing a biogum of the diutan type that exhibits a significant improvement in physical properties as well. To date, the only mention of production of any types of related sphingans (without any demonstrations for diutan specifically) is in terms of higher yield (within the Pollock et al. patents mentioned above). There has been no discussion or fair suggestion of any manner of providing a method for producing an improved diutan gum of higher molecular weight that exhibits any improvement in viscosity measurements via such a production method.

Brief Description of the Invention

It has now been realized that amplification of certain novel isolated DNA sequences for diutan biosynthesis within a host *Sphingomonas* organism not only permits increased production of diutan gum therefrom, but also produces a diutan gum that exhibits increased viscosity properties. Such a novel DNA sequence (that is introduced within a host organism via any well known method, such as, without limitation, a plasmid) thus provides the desired results that have been sought after for diutan synthesis

methods. A distinct advantage of such utilization of these genes amplified on a plasmid is the relatively simple nature of incorporating such an isolated DNA sequence into diutan synthesis procedures. Another advantage is the ability to produce such higher viscosity properties for the target diutan gum, while potentially increasing the fermentation production efficiency, if necessary.

Accordingly, this invention includes a diutan gum exhibiting an improvement in a number of different viscosity measurements. Among these are: i) an intrinsic viscosity of greater than 150, preferably higher than 155, more preferably higher than 160dL/g; ii) a sea water 3 rpm viscosity greater than 35, preferably higher than 37, more preferably higher than 40, and most preferably higher than 42 dial reading; iii) a sea water 0.3 rpm viscosity greater than 35,000, preferably higher than 39,000, more preferably higher than 40,000, and most preferably higher than 41,000 centipoise (cP); and a PEG low shear rate viscosity greater than 3500, preferably higher than 3700, more preferably higher than 3900, and most preferably higher than 4000 cP. Also, this invention encompasses a method of producing such a diutan gum, as defined in any of those terms above, through the introduction of a specific cluster of genes into a host *Sphingomonas* organism and permitting fermentation of said organism to produce a resultant diutan gum. Furthermore, this invention encompasses the specific DNA sequences and any vector (such as a plasmid) to provide multiple copies of the genes or increased expression of the genes by use of a stronger promoter, and the like. Additionally, the genetically modified strain of *Sphingomonas* containing multiple copies of the diutan biosynthetic genes defined by such unique isolated DNA sequences is also encompassed.

Such a unique isolated DNA sequence has been found to require at least one diutan biosynthetic enzyme being a DpsG polymerase. In another possible embodiment, such a diutan biosynthetic enzyme will include a DpsG polymerase and a glucose-1-phosphate thymidylyltransferase; a dTDP-6-deoxy-D-glucose-3-5-epimerase; a dTDP-D-glucose-4,6-dehydratase; and a dTDP-6-deoxy-L-mannose-dehydrogenase. In yet another possible embodiment such a diutan biosynthetic enzyme will include a DpsG polymerase and a rhamnosyl transferase IV; a beta-1,4-glucuronosyl transferase II; a glucosyl isoprenylphoaphate transferase I; and a glucosyl transferase III. In still another possible embodiment, such a diutan biosynthetic enzyme comprises a dpsG polymerase and polysaccharide export proteins dpsD, dpsC, and dpsE. In yet another possible embodiment, such a diutan biosynthetic enzyme will include a rhamnosyl transferase IV; a beta-1,4-glucuronosyl transferase II; a glucosyl isoprenylphoaphate transferase I; glucosyl transferase III; a glucose-1-phosphate thymidylyltransferase; a dTDP-6-deoxy-D-glucose-3-5-epimerase; a dTDP-D-glucose-4,6-dehydratase; and a dTDP-6-deoxy-L-mannose-dehydrogenase. Generally, the diutan biosynthetic enzyme of the inventive method and within the inventive product may be selected from the group consisting of polymerase; lyase; rhamnosyl transferase IV; beta-1,4-glucuronosyl transferase II; glucosyl transferase III; polysaccharide export protein; secretion protein; glucosyl-isoprenylphosphate transferase I; glucose-1-phosphate thymidylyltransferase; dTDP-6-deoxy-D-glucose-3-5-epimerase; dTDP-D-glucose-4,6-dehydratase; dTDP-6-deoxy-L-mannose-dehydrogenase and combinations thereof. Further encompassed within this invention then is an isolated nucleic acid molecule (in addition to DNA which may be present on the target chromosome) which encodes at least one diutan biosynthetic

enzyme as shown in SEQ ID NO: 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, and 43, or an enzyme which is at least 95 % identical to SEQ ID NO: 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, and 43.

The inventive method (as well as the products made thereby) thus concern sphingan gums, particularly diutan types, including, without limitation, S88, S60, and S657.

As noted above, the present invention is the culmination of development and realization that specific DNA sequences that are introduced in multiple copies within certain *Sphingomonas* strains can provide increased biosynthetic production of high viscosity diutan polysaccharide. The engineered bacteria containing such genes for increased production produce significantly greater amounts of diutan polysaccharide compared to non-engineered bacteria and create the aforementioned resultant high viscosity properties.

The DNA sequences that are introduced within the host organism (in any well known form, such as, again, as one non-limiting example, a plasmid) to generate the aforementioned increased production and increased viscosity properties (through what is believed, without any reliance upon any specific scientific theory, an increase in molecular weight range properties) according to the present invention may be isolated, recovered and cloned by techniques that are readily available in the art. Thereafter, the DNA is delivered into bacteria of the genus *Sphingomonas* in multiple copies (via plasmid, other known manner) or increased expression of the genes via a suitable, e.g., stronger promoter. After insertion into the target bacteria, the production of diutan can be determined by fermenting the engineered bacteria and comparing the yield in terms of

amount produced and quality produced. Increased production and viscosity increases can both be determined by comparing diutan production via the inventive method in comparison with the wild type diutan-producing strain (ATCC 53159).

Brief Description of the Drawings

FIG. 1 is a diagrammatic representation of the isolated genes for diutan gum biosynthesis. Putative or known genes are indicated. The segments inserted into different plasmids are also indicated.

FIG. 2 is a graphical representation of the improvements in intrinsic viscosity measurements achieved by such inventive diutan biogum materials.

Detailed Description of the Invention

The following terms shall be used throughout the specification in connection with the present invention and have the meaning indicated:

The term "*Sphingomonas*" is used throughout the specification to refer to strains of gram-negative bacteria from the genus *Sphingomonas*.

The term "increased producer" or "increased production" is used throughout the specification to describe engineered bacteria containing multiple copies of DNA sequences isolated from the same strain which produce significantly greater (at least about 5% more on a weight by weight basis) diutan polysaccharide compared to wild-type bacteria of the same strain.

The term "isolated" is used to describe DNA which has been removed from a microorganism and subjected to at least some degree of purification, i.e., one or more purification steps, and which can be cleaved or cut by restriction enzymes, cloned into multiple copies or inserted into plasmid vectors or otherwise inserted or incorporated into bacteria.

The term "sequence" is used to describe a specific segment of DNA which is identified by its nucleotide units. The term "inserted" is used throughout the specification to describe the process and outcome of transferring DNA segments isolated from the chromosomal DNA of a diutan-producing *Sphingomonas* strain into the *Sphingomonas* strain (via a plasmid, as one non-limiting example). Such isolated DNA may be introduced first into, again as one non-limiting possibility, the desired plasmid (here pLAFR3), by well-known techniques in the art, and then transferred, for example, by conjugation or mobilization into a recipient *Sphingomonas* bacterium. After insertion into a recipient *Sphingomonas* bacterium, the plasmid containing the relevant DNA sequence will replicate in the recipient cell to give several (at least two and usually 4-10) copies of the DNA segment necessary for increased production of high viscosity (again, believed to be high molecular weight range) diutan polysaccharide. The use of conjugation or mobilization to transfer the plasmid vectors into recipient bacteria is generally effective. Electroporation or chemical transformation of competent cells with purified DNA may also be used. Other vectors or bacteriophages can be used to transfer DNA into the host cell. Maintaining the DNA segments on plasmids (or other well known delivery vectors) in the recipient diutan-producing *Sphingomonas* is not necessary. It is routine to introduce additional copies of a DNA segment into the

bacterial chromosome so that the segments are replicated each generation by the same mechanism that replicates the bacterial DNA. Alternatively, increased expression of the genes may be achieved by using stronger promoter elements.

The term "gene amplification" is used to refer to either increased copies of genes, for example by cloning the target genes on a multicopy plasmid (such as from 4 to 10 copies) or insertion of multiple copies (such as from 4 to 10) of the genes into the bacterial genome, or alternatively increased expression of genes by modification of promoter elements to increase gene expression. Both of these methods and others can result in increased amounts of the encoded proteins.

The term "biosynthesis" is used throughout the specification to describe the biological production or synthesis of diutan by *Sphingomonas* bacteria. Diutan polysaccharide is synthesized from individual carbohydrate units in a series of steps controlled by a large number of enzymes of the bacteria.

The relevant DNA sequence which is incorporated into the recipient bacteria in any selected form (such as, again, preferably, but not necessarily, plasmid form) encodes genetic information which is known to be beneficial or essential for the biosynthesis of increased production and increased molecular weight diutan polysaccharide. Additionally, though, the particular inventive DNA sequence (such as within plasmid pS8) is believed, without relying on a specific scientific theory, to induce, not just increased production, but also an increase in number of repeating units polymerized within the individual polymers of the diutan itself. As a result, it is believed that such an increase in repeating units produces the resultant high viscosity properties surprisingly

provided by the diutan gum. A molecular weight increase has been hypothesized due to measured increases in intrinsic viscosity which is related to molecular weight by a power law relationship. For a linear polymer (like diutan gum), intrinsic viscosity is thus known to be essentially proportional to molecular weight in that respect.

The isolation of the relevant DNA sequences that are the basis of this inventive method and that generate the increased viscosity diutan polysaccharide is accomplished via standard techniques and methods. Such sequences may thus be generated from a diutan-producing *Sphingomonas* strain that has been cultured using standard procedures. Extraction of the DNA can then be performed, for example, through initial centrifugation and resuspension of the bacterial cells and then subsequent elution of the DNA through purification columns. After purification is completed, the isolated DNA can be digested with restriction endonucleases and cloned into the desired plasmid or other delivery vector and subsequently transferred to a recipient strain. Other techniques as are known in the art can be used without limitation.

The cloning of DNA in the present invention relies on general techniques and methods which have become standard in the art. It is noted that any number of methods may be used to clone the DNA segments according to the present invention and the present invention is not limited, for example, to the use of plasmid cloning vectors. For example, the DNA fragments may be cloned by insertion into a bacteriophage vector.

The cloned DNA sequences can be then introduced to a *Sphingomonas* strain via a plasmid or other delivery vector. The genetically modified *Sphingomonas* strain can then be used to produce diutan by fermentation. Basically, a suitable medium for

fermentation is an aqueous medium which generally contains a source of carbon such as, for example, carbohydrates including glucose, lactose, sucrose, maltose or maltodextrins, a nitrogen source such as, for example, inorganic ammonium, inorganic nitrate, organic amino acids or proteinaceous materials such as hydrolyzed yeast, soy flour or casein, distiller's solubles or corn steep liquor, and inorganic salts. A wide variety of fermentation media will support the production of diutans according to the present invention.

Carbohydrates can be included in the fermentation broth in varying amounts but usually between about 1 and 10% by weight (preferably 2-8%) of the fermentation medium. The carbohydrates may be added prior to fermentation or alternatively, during fermentation. The amount of nitrogen may range from about 0.01% to about 0.4% by weight of the aqueous medium. A single carbon source or nitrogen source may be used, as well as mixtures of these sources. Among the inorganic salts which find use in fermenting *Sphingomonas* bacteria are salts which contain sodium, potassium, ammonium, nitrate, calcium, phosphate, sulfate, chloride, carbonate and similar ions. Trace metals such as magnesium, manganese, cobalt, iron, zinc, copper, molybdenum, iodide and borate may also be advantageously included.

The fermentation can be carried out at temperatures between about 25° and 40°C, with a temperature range of about 27° and 35°C preferred. The inoculum can be prepared by standard methods of volume scale-up, including shake flask cultures and small-scale submerged stirred fermentation. The medium for preparing the inoculum can be the same as the production medium or can be any one of several standard media well-known in the art, such as Luria broth or YM medium. More than one seed stage may be

used to obtain the desired volume for inoculation. Typical inoculation volumes range from about 0.5% to about 10% of the total final fermentation volume.

The fermentation vessel may contain an agitator to stir the contents. The vessel also may have automatic pH and foaming controls. The production medium can be added to the vessel and sterilized in place by heating. Alternatively, the carbohydrate or carbon source may be sterilized separately before addition. A previously grown seed culture can be added to the cooled medium (generally, at the preferred fermentation temperature of about 27° to about 35°C) and the stirred culture can be fermented for about 48 to about 110 hours, producing a high viscosity broth. The diutan polysaccharide can be recovered from the broth by the standard method of precipitation with an alcohol, generally isopropanol.

Preferred Embodiments of the Invention

Including Detailed Descriptions of the Drawings

The following examples are provided to illustrate the present invention. The description of the examples should not be misconstrued to limit the scope of the present invention in any way.

DNA Sequence Isolation/Plasmid Production

To undergo the initial isolation and determine the proper sequence for the inventive results described previously, a gene library of the ATCC 53159 organism was constructed as follows: Chromosomal DNA was isolated from *Sphingomonas sp.* ATCC 53159 and partially digested with *Sau3*AI restriction endonuclease. DNA fragments in the range of 15 to 50 kb were purified from an agarose gel and ligated into *Bam*HI digested cosmid cloning vector pLAFR3 (in accordance with Staskawicz, et al., "Molecular characterization of cloned avirulence genes from race 0 and race 1 of *Pseudomonas syrinae* pv. *Glycinea*", J. Bacteriology. 1987. **169**: 5789-94), isolated from *Eschericia coli* strain JZ279 (from Harding, et al., "Genetic and physical analysis of a cluster of genes essential for xanthan gum biosynthesis in *Xanthomonas campestris*", J. Bacteriology. 1987. **169**: 2854-61). Ligation reactions were packaged in λ phage particles (using Gigapack III Gold packaging extract, from Stratagene, La Jolla, CA) and transfected into Library Efficiency *E. coli* DH5αMCR cells (Life Technologies, Rockville, MD). Approximately 10,000 tetracycline resistant colonies were pooled to form the gene library. From this library, individual sequences were then isolated. The work undertaken in this instance involved the isolation of specific genes for polysaccharide biosynthesis from the *Sphingomonas* ATCC 53159 organism.

Such genes for polysaccharide biosynthesis are typically identified by complementation of mutants defective in polysaccharide synthesis, particularly those blocked in the first step, glycosyl transferase I. Since initially no transferase I defective mutants of ATCC 53159 were available, complementation of transferase I defective mutants of *Sphingomonas elodea* and *Xanthomonas campestris* were utilized to identify

genes for diutan polysaccharide biosynthesis. Plasmid pLAFR3 can be transferred from its *E. coli* host to other gram-negative bacteria by tri-parental conjugation using a helper plasmid that supplies IncP transfer functions (in accordance with Ditta, et al., "Broad host range DNA cloning system for gram-negative bacteria: construction of a gene bank of *Rhizobium meliloti*", Proc. Natl. Acad. Sci. 1980. **77**:7347-51.). RK2 type plasmids have an estimated copy number in *E. coli* of five to seven per chromosome (Figurski et al., "Suppression of ColE1 replication properties by the Inc P-1 plasmid RK2 in hybrid plasmids constructed *in vitro*", J. Mol. Biol. 1979 **133**: 295-318.).

The gene library of ATCC 53159 chromosomal DNA in *E. coli* was transferred into a nonmucoid mutant (GPS2) of *S. elodea* ATCC 31461, by triparental conjugation, selecting for tetracycline and streptomycin resistance. The helper plasmid used was pRK2013 (in *E. coli* strain JZ279), which contains a narrow-host-range origin of replication but exhibits trans acting functions needed to mobilize pLAFR3. Plasmid pRK2013 was not replicated in *Sphingomonas* strains. *S. elodea* ATCC 31461 produces the polysaccharide gellan. Both gellan and diutan polysaccharides have the same tetrasaccharide repeat unit, comprised of [→4)-α-L-rhamnose-(1→3)-β-D-glucose-(1→4)-β-D-glucuronic acid-(1→4)-β-D-glucose-(1→]. Diutan, however, also includes a side chain comprised of two rhamnose molecules attached to one of the glucose residues, and is modified by acetyl, whereas gellan has no side chain sugars and is modified with acetyl and glyceryl. The mutant GPS2 is defective in the first step of polysaccharide biosynthesis, i.e., transfer of glucose-1-phosphate from UDP-D-glucose to the bactoprenyl phosphate lipid carrier by glucosyl transferase I enzyme. From tetracycline selection plates, polysaccharide-producing (mucoid) colonies were isolated from a

background of non-mucoid colonies. Clones restoring polysaccharide production presumably contained the ATCC 53159 gene encoding glucosyl transferase I plus approximately 20-25 kb of adjacent DNA. Plasmid DNA was isolated from eight mucoid GPS2 transconjugants and transferred to *E. coli* strain DH5α (Life Technologies) by electroporation. The plasmids were isolated from *E. coli* to obtain sufficient DNA for double-digestion with restriction endonucleases *Hind*III/ *Eco*RI (which cut either side of the *Bam*HI restriction endonuclease site in the polylinker), to excise the insert DNA from the vector. The sizes of the insert DNA in the clones were determined by gel electrophoresis. The end sequences of several plasmids were determined by sequencing from primers specific to plasmid sequences flanking the *Bam*HI site of the vector. The sequences were analyzed by comparison to sequences in computer databases using BLASTX. Two of these plasmids, pS8 and pS6, are presented in Fig. 1. Similarly, the ATCC 53159 gene library was transferred into a rifampicin-resistant nonmucoid *X. campestris* mutant defective in transferase I (CXC109)(such as in the Harding et al. reference noted above) through triparental conjugation selecting for resistance to tetracycline and rifampicin. *X. campestris* produces xanthan polysaccharide, the synthesis of which is also initiated by transfer of glucose-1-phosphate from UDP-D-glucose to the bactoprenyl phosphate lipid carrier by transferase I enzyme (Ielpi et al., "Sequential assembly and polymerization of the polyprenol-linked pentasaccharide repeating unit of the xanthan polysaccharide in *Xanthomonas campestris*", J. Bacteriology. 1993. **175**: 2490-500). Plasmids were purified from mucoid transconjugants and the end sequences determined as described above. Two of these plasmids pX6 and pX4 are presented in Fig.1.

The S657 DNA cloned in plasmids pS8 and pX6 was completely sequenced by double-stranded shotgun sequencing at Lark Technologies Inc., (Houston, TX). These sequences were analyzed to identify the genes for diutan biosynthesis (presented in Figure 1). Gene functions were designated based on homology to other genes in databases, in particular to the published genes for biosynthesis of S-88 sphingan (such as within the aforementioned '516 Pollock et al. patent), GenBank accession number U51197 and gellan (GenBank AY217008 and AY220099). Genes were identified (Figure 1) that encoded the transferases for the four sugars of the backbone and four genes for dTDP-rhamnose synthesis. Genes for secretion of the polysaccharide were based on homology to genes for biosynthesis of other polysaccharides. Two genes encode proteins homologous to proteins involved in protein secretion. Two genes putatively encode a polymerase and a lyase. The insert in plasmid pX6 contained 17 genes including gene *dpsB* encoding transferase I (which initiates the first step in diutan synthesis), genes for secretion and four genes for dTDP-rhamnose synthesis, but lacks the genes for transferases II, III and IV and the putative genes for polymerase and lyase Plasmid pS8 contains 20 genes of the *dps* gene cluster, including genes for all four backbone sugar transferases, the four genes for dTDP-rhamnose synthesis, andgenes for secretion of the polysaccharide, including the putative genes for polymerase and lyase, but lacks the genes of unknown function, *orf6* and *orf7*. Plasmid pS6 contains genes for secretion and the four sugar transferases but does not have all genes for dTDP-rhamnose synthesis or the gene for polymerase. Plasmid pX4 contains only a small part of the *dps* region but includes the gene encoding transferase I and the four genes for dTDP-

rhamnose synthesis that were reported by Pollock et al. to be sufficient to result in an increase in production of polysaccharide in *Sphingomonas* strains.

Strain Production

The four plasmids described above were then introduced within *Sphingomonas* strain ATCC No. 53159 by triparental conjugation as described above to form the novel S657 engineered strains (S657/pS8, S657/pS6, S657/pX6 and S657/pX4. Fermentation was followed, as described above, thereafter in order to produce a biogum material as noted below. All four plasmids had a beneficial effect on diutan productivity; however, the pS8 plasmid surprisingly also provided extremely large increases in diutan viscosity, and increase in molecular weight. The DNA sequence of pS8 (26278 bps)(DNA Sequence No. 1) is provided and the encoded genes are listed in Table 1 below, and in diagram form in Figure 1. The insert DNA in plasmid pS8 includes genes *dpsG* through *rmlD* and a portion of genes *dpsS* and *orf7*.

The following gene table is basically a list of the genes represented by the DNA sequence for insert in plasmid pS8 as provided within FIG. 1.

TABLE 1
Genes on pS8 plasmid insert

Start	End	Name	Description
2*	1054	dpsS	(partial) homologous to *gelS*
2738	1113 C	dpsG	putative polymerase
4895	2898 C	dpsR	putative lyase
5093	6031	dpsQ	putative rhamnosyl transferase IV
7082	6111 C	dpsI	unknown
7121	8167	dpsK	beta-1,4-glucuronosyl transferase II
8164	9030	dpsL	glucosyl transferase III
10467	9079 C	dpsJ	unknown
11076	12374	dpsF	unknown
12389	13306	dpsD	putative polysaccharide export protein
13341	14687	dpsC	putative polysaccharide export protein
14687	15394	dpsE	putative polysaccharide export protein
15405	16286	dpsM	putative polysaccharide export protein
16270	16968	dpsN	putative polysaccharide export protein
18454	17060 C	atrD	putative secretion protein
20637	18451 C	atrB	putative secretion protein
21229	22641	dpsB	glucosyl-isoprenylphosphate transferase I
22757	23635	rmlA	glucose-1-phosphate thymidylyltransferase
23632	24198	rmlC	dTDP-6-deoxy-D-glucose-3-5-epimerase
24202	25263	rmlB	dTDP-D-glucose-4,6-dehydratase
25263	26129	rmlD	dTDP-6-deoxy-L-mannose-dehydrogenase
26277*	26146 C	orf7	(partial) unknown function

*First in-frame codon, the start codon is not present

Diutan Production

Diutan production by the engineered plasmid-containing *Sphingomonas* S657 strains compared to the S657 wild-type strain without a plasmid was determined in three sets of fermentations run in the same liquid media in Applikon 20L fermentors, with agitation and aeration. For the plasmid containing strains, the antibiotic tetracycline at 5 mg/L was added throughout the fermentation to ensure retention of the plasmid. KOH was added as needed to control pH. Two seed stages were used with 1% to 6% inoculum transfers. Media used for fermentation contained corn syrup as carbohydrate source, an

assimilable nitrogen source and salts. Nutrients that can be used for fermentation are well known in the art and include a carbohydrate, for example, glucose, sucrose, maltose or maltodextrins, a nitrogen source, for example inorganic nitrogen as ammonium or nitrate, organic nitrogen such as amino acids, hydrolyzed yeast extract, soy protein, or corn steep liquor, and additional salts containing for example, chloride, phosphate, sulfate, calcium, copper, iron, magnesium, potassium, sodium, or zinc.

As a measure of the resultant diutan production, broth viscosity and precipitated fibers were determined. The viscosity of the fermentation broths was measured via a Brookfield viscometer run at 60 rpm with a spindle #4, and the results are shown in Table 2. At the end of the fermentation, the broths were treated with the well known introduction of glucoamylase enzyme to hydrolyze any remaining oligosaccharides from the corn syrup. The diutan gums produced were then precipitated from an aliquot of broth with two volumes of isopropyl alcohol. The fibers were collected on a filter and dried. In Table 2, the term DWY means the total precipitable dry weight yields of biogums after hydrolysis of excess oligosaccharides from corn syrups

Clearly the resultant material is in higher yield with plasmids pX4, pX6, pS6 or pS8 carrying additional copies of genes for diutan biosynthesis present therein. However, with the pS8 plasmid, there was an unexpected high increase in broth viscosity relative to the increase in dry weight yield indicating that some factor in addition to increased amount of diutan produced was affecting the viscosity.

TABLE 2

Fermentation of plasmid-containing strains

DWY

Strain	Run #1	Run #2	Run#3	av.	% Increase
S657	34.3	32.2	33.9	33.5	----
S657/pS8	37.1	35.4	35.9	36.1	8.0%
S657/pX6	38.4	37.6	33.5	36.5	9.1%
S657/pS6			37.6		12.3%
S657/pX4			36.4		8.8%

Broth Viscosity

Strain	Run #1	Run #2	Run #3	av.	% Increase
S657	5150	4950	5550	5217	----
S657/pS8	6650	6850	6850	6783	30.0%
S657/pX6	5400	6250	5125	5592	7.2%
S657/pS6			6675		28.0%
S657/pX4			5525		5.9

Clearly, there was a higher yield of resultant material with any of the four plasmids present therein, whereas the pS8 and pS6 plasmids permitted a highly unexpected increase in broth viscosity thus indicating high product quality as well. The quality , i.e. viscosity , of the resultant diutan gum products was then determined.

Diutan Rheology in Applications Tests

These diutan gum samples were then analyzed in terms of potential beneficial uses within two different areas: oilfield additives for oil recovery and cement additives for water retention and quick set-up.

The oilfield industry relies upon what is termed a "sea water viscosity" (SWV) test as an estimate of acceptable performance for gums for oil recovery. Such a test basically is an indicator of the effectiveness of a gum to increase viscosity in briny conditions of water (to replicate recovery from seabeds, for example).

The prediction of the viability of a resultant gum as a proper viscosity modifier for oil recovery purposes is generally accepted in terms of viscosity modification of a test sea water formulation. Such a "Synthetic Seawater" formulation is produced by mixing 419.53 grams of Sea Salt (ASTM D-1141-52) in 9800 grams deionized water. For the seawater viscosity test, 0.86 grams of the sample gum is added to 307.0 g Synthetic Seawater and mixed at approximately 11,500 rpm in a Fann Multimixer (Model 9B5, part number N5020) for 35 minutes. At the end of 35 minutes, the solution is cooled to approximately 26°C before the viscosity is measured. For the 3-rpm reading, the sample is placed on the Fann sample platform (Fann model 35A; Torsion spring MOC 34/35 F0.2b; Bob B1; Rotor R1) and the speed is adjusted to 3 rpm by turning the motor to low speed and setting the gearshift in the middle position. The reading is then allowed to stabilize and the shear stress value is read from the dial and recorded as the SWV 3 rpm dial reading (DR). For the 0.3-rpm reading, a Brookfield viscometer is used (Brookfield LV DV-II or DV-II viscometer, with LV-2C spindle) to measure the viscosity. The speed of the spindle is set to 0.3 rpm and the spindle is allowed to rotate at least 6 minutes

before the viscosity is recorded as the SWV-0.3 rpm reading and expressed in centipoises (cP). For cement applications, the PEG LSRV test (a low shear rate viscosity using polyethylene glycol as dispersant as outlined below) provides an indication as to effectiveness of performance of a viscosity modifier to that industry. Such a test measures the viscosity of a 0.25% solution of biogum in Standard Tap Water (STW). STW is prepared by adding 10.0 grams NaCl and 1.47 grams $CaCl_2 \bullet 2H_2O$ to 10 liters deionized water. For the viscosity measurement, 0.75 grams of biogum is added to 4.5 grams Polyethylene Glycol 200 (CAS 25322-68-3) in a 400-mL beaker and thoroughly dispersed. Then, 299 grams of STW are added to the beaker and mixed for approximately 4 hours using a low-pitched, propeller-style stirrer at 800 ± 20 rpm. After the 4-hr mixing time, the beaker is placed in a 25°C water bath and allowed to sit undisturbed for approximately 30 minutes. The viscosity is then measured using a Brookfield LV viscometer equipped with a 2.5+ torque spring (or equivalent instrument such as Model DVE 2.5+) at 3 rpm using the LV 1 spindle after allowing the spindle to rotate for 3 minutes and expressed in centipoises (cP).

The diutan samples produced above were tested in this manner; the results were as follows:

TABLE 3

Rheology of diutan from plasmid-containing strains

Strain	SWV 3 rpm DR			SWV-0.3 rpm cP		PEG LSRV cP		
	Run #1	**Run #2**	**Run #3**	**Run #1**	**Run #2**	**Run #1**	**Run #2**	**Run #3**
S657 wild-type	25	26	22	24400	28600	2820	3150	2280
S657/pS8	42	43	47	41500	38800	4720	4980	4920
S657/pX6	25	29	26	25000	29100	2860	3400	3270
S657/pS6	---	---	22	---	---	---	---	2270
S657/pX4	---	---	24.5	---	---	---	---	2950

SWV = viscosity in sea water

LSRV = low shear rate viscosity

Unexpectedly, there are definite increases in viscosity exhibited by the inventive diutan gums produced by some of the engineered plasmid-containing strains Most surprisingly, however, is that the increase in viscosity for SWV at 3 rpm for the pS8 strain is 80%, whereas the same analysis made for the pX6 strain is merely 9.6% over the wild-type results. Plasmids pS6 and pX4 had no significant increase. Likewise, the lower SWV rpm test reveals an increase of 51.5% over the wild-type for the pS8 type versus just over 2% for the pX6. Finally, the polyethylene glycol LSRV test showed that the pS8 results were in excess of 77% viscosity increase over the wild-type gum, as compared with less than 16% increase for the pX6 diutan, and 7.2% increase for pX4 and no significant increase for plasmid pS6. Again, the highly unexpected results in these terms shows the drastic improvements accorded diutan gum production via the utilization of the needed gene sequence exemplified within the pS8 plasmid, as one manner of introducing such a sequence within a target diutan-producing bacterium.

Thus, the inventive diutan produced via the introduction of pS8 exhibited surprisingly increased viscosity measurements on all three counts, particularly as compared with the wild type and pX6 plasmid-produced varieties. Thus, it was expected that such a novel diutan would function extremely well under typical oilfield conditions and within cement applications.

Fundamental Explanation for Rheology Improvement

The previous examples showed that diutan from the S657/pS8 strain showed a significant increase in rheological parameters. Such a substantial increase in the sea water and PEG low shear rate viscosity measurements thus cannot be attributed to the increase in productivity alone since the pX6 strain also exhibited similar, if not greater, yield results. Indeed, in the prior example illustrated by Table 2, the dry weight yields (alcohol precipitable matter) increased by 8.0%, while the rheological parameters increased significantly more for the S657/pS8 strain (52-80%). A fundamental study was pursued to explain why rheological improvements are obtained with strain S657/pS8 over the wild-type strain.

Intrinsic viscosity is a well known technique in polymer science to infer the molecular weight of macromolecules (C. Tanford, 1961. Physical Chemistry of Macromolecules. John Wiley & Sons, New York). The intrinsic viscosity is obtained by plotting the reduced viscosity (viscosity normalized for concentration) versus the solution concentration, and extrapolating a linear regression of the data to zero concentration (the y-intercept of the plot). Surprisingly, the resultant gums exhibited increases in intrinsic viscosity as noted below in the following table.

Five diutan samples, two from the wild-type strain (Control 1, Control 2) and three from the S657/pS8 strain (Sample 1, Sample 2, Sample 3) were evaluated for intrinsic viscosity, neutral sugars, and organic acid analyses. These samples were purified by alcohol precipitation, re-hydrated, treated with hypochlorite, treated with glucoamylase, treated with lysozyme, and finally treated with protease (in that sequential order). They were then recovered at a 4:1 CBM:Broth ratio, dried and milled. CBM is an azeotropic isopropyl alcohol/water mixture including ~82% by weight of the isopropyl alcohol.

The samples were tested for moisture content by performing the following: generally, two 0.7 gram aliquots of sample were tested using a Mettler HB 43 halogen moisture balance. The results from the two trials were then averaged and these results were utilized for moisture correction.

After obtaining the moisture data, a 0.2% solution of the gum was prepared in 0.01M NaCl on a moisture corrected basis. For these trials 200 grams total of the 0.2% solution were prepared. The gum was weighed on an analytical balance to the nearest ten thousandth and added to the water weighed to the nearest thousandth. The samples were stirred for two hours using a 2.5 inch diameter propeller mixer @ 1000 rpm in a 400 ml tall form beaker.

Following initial hydration, each sample was diluted to 0.02% using 0.01M NaCl. This was done by weighing 20 grams of the 0.2% solution into a 400 ml beaker, then adding back 180 mls of the diluent. The diluted samples were mixed for an additional 30 minutes. The final dilutions ultimately used for determining the intrinsic viscosity were

prepared from this sample. Each diutan sample was evaluated at the following concentrations: 0.004%, 0.008%, 0.010%, and 0.012%.

Viscosity measurements were carried out using the Vilastic® VE System. Prior to measurements the Vilastic was calibrated with water to less than 2.0% error. The samples were measured using the Timer program @ 2 Hz, a strain of 1 and a shear rate of approximately 12 1/sec, all at a constant temperature of 23°C. Five measurements were made for each sample and averaged. The averaged viscosity data were then used to calculate the intrinsic viscosity. Figure 2 and Table 4 below provide the final results of these trials.

TABLE 4

Comparison of Diutan Based on Intrinsic Viscosity Calculations

Diutan Sample	Measured Solids	Intrinsic Viscosity
S657 Control 1	93.76	138.3
S657 Control 2	92.42	143
S657/pS8 Sample 1	91.7	170.7
S657/pS8 Sample 2	91.4	162.2
S657/pS8 Sample 3	91.94	162.8

These results indicate that the S657/pS8 strain consistently produced diutan with significantly higher intrinsic viscosity; in fact the average reduced viscosity for the inventive strains was 165.2, whereas the control was 140.7, all at similar measured solids

levels. This finding indicates that diutan produced by S657/pS8 is higher in molecular weight than the wild-type control.

Figure 2 is the graphical representation of these trends showing the consistent higher intrinsic viscosity measured at similar solids content between the control and inventive strains.

To determine if the higher viscosity diutan gum from S657/pS8 had the same composition as diutan from the wild-type strain, the composition was determined by testing for neutral sugars and organic acids. The purified sample used for intrinsic viscosity measurements were used for neutral sugar analysis. An aliquot of each purified sample was hydrolyzed to component sugars by hydrolysis with trifluoroacetic acid (100°C/~18hr). The hydrolysate neutral sugars were quantified by high-performance anion-exchange chromatography with pulsed amperometric detection. The hydrolysate organic acids were quantified by high-performance ion-exclusion chromatography with chemically suppressed conductivity detection. Table 5 summarizes the results from the neutral sugar analysis. As shown, the neutral sugar profile for the S657/pS8 strain is nearly identical with the neutral sugar profile for the S657 wild-type strain. Although both results are different from the theoretical values, these results indicate that the structure of the repeat unit of the diutan gum produced using pS8 is the same as that for wild-type and that any increase in viscosity imparted by the pS8 material is due to longer chains, meaning higher molecular weight.

TABLE 5

Neutral sugars and organic acid analysis for pS8 and wildtype (control) diutan strains

	Strain	% Rhamnose	% Glucose	% Acetate
Sample 1	S657/pS8	32	19	8.9
Sample 2	S657/pS8	32	19	8.2
Sample 3	S657/pS8	32	17	8.6
Control 1	S657 wildtype	30	18	8.6
Control 1	S657 wildtype	33	20	8.7
AVERAGE	S657/pS8	32	18.3	8.6
AVERAGE	S657 wildtype	31.5	19	8.65
THEORETICAL	---	46	30	8

The greatly improved seawater viscosity and PEG low shear rate viscosity of the diutan produced by the S657/pS8 engineered strain is thus attributable to an increase in molecular weight or length of the diutan molecule, i.e., more repeat units per molecule and not to a change in its compositon and thus not to changes in the repeat structure itself. Nor can this improved rheology be due soley to increase in amount of diutan produced. Although four plasmids, pS6, pS8, pX4, and pX6, with different portions of the cluster of genes for diutan biosynthesis cloned, were evaluated, and all showed some increase in productivity, only plasmid pS8 showed the unexpected and very high increase in rheological parameters of the recovered diutan product.

A comparison of the genes for diutan biosynthesis cloned in the tested plasmids suggests that the most likely gene to be responsible for the increase in molecular weight is the gene *dpsG*, since this gene is present in pS8 and not in the other plasmids. Gene

dpsG encodes a hydrophobic membrane protein with strong homology to other membrane proteins involved in polysaccharide synthesis. A portion of the protein has homology to proteins for polymerase, an enzyme which catalyzes the linkage of repeat units to form the high molecular weight polysaccharide. The homologous gene *gelG* in S60 has been postulated to function as a polymerase for gellan synthesis (Harding, N. E. et al. 2004. "Organization of genes required for gellan polysaccharide biosynthesis in *Sphingomonas elodea* ATCC31461". J. Ind. Microbiol. Biotech. **31**:70-82. Sa-Correia, I. et al. 2002. "Gellan gum biosynthesis in *Sphingomonas paucimobilis* ATCC 31461: Genes, enzymes and exopolysaccharide production engineering". J. Ind. Microbiol. Biotechnol. **29**: 170-176.). Homologues of *dpsG* have also been isolated from *Sphingomonas* strains ATCC 31554 and ATCC 21423 producing polysaccharides S88 and S7 (Pollock et al. U.S. Patent Nos. 5,854,034, 5,985,623 and 6,284,516, and Pollock, T. J. U.S. Patent No. 6,709,845). It is thus very likely that additional copies of the gene for polymerase may have an effect on increasing the molecular length of the diutan molecule. It cannot be ruled out that other genes in the diutan biosynthetic gene cluster may be required in combination with *dpsG* to achieve the observed increase in viscosity. Likely candidates would be the genes *dpsB, dpsL, dpsK* and *dpsQ* encoding the sugar transferases I, II, III, and IV, in particular the gene *dpsB* which encodes transferase I that adds the first sugar of the repeat unit to the lipid carrier. Other important genes may be *dpsD, dpsC* and *dpsE,* which are homologous to genes *gumB* and *gumC* that have been shown to increase the molecular weight of xanthan when amplified on a multicopy plasmid. It is possible that all genes cloned in plasmid pS8 may be required to achieve the dramatic increase in viscosity.

While the invention will be described and disclosed in connection with certain preferred embodiments and practices, it is in no way intended to limit the invention to those specific embodiments, rather it is intended to cover structural equivalents and all alternative embodiments and modifications as may be defined by the scope of the appended claims and equivalence thereto.

Deposits

The following bacterial strain was deposited with the Patent Depository at the American Type Culture Collection at 10801 University Boulevard, Manassas, Va. 20110, on October 21, 2005, pursuant to the Budapest Treaty for the International Recognition of the Deposit of Microrganisms:

Sphingomonas strain S657 with plasmid pS8.

CLAIMS

What we claim is:

1. A diutan gum exhibiting an intrinsic viscosity of greater than 150 deciL/g

2. The diutan gum of Claim 1 exhibiting an intrinsic viscosity of greater than 155 deciL/g.

3. The diutan gum of Claim 2 exhibiting an intrinsic viscosity of greater than 160 deciL/g.

4. A diutan gum exhibiting a sea water 3 rpm viscosity greater than 35 dial reading.

5. The diutan gum of Claim 4 exhibiting a sea water 3 rpm viscosity greater than 37 dial reading.

6. The diutan gum of Claim 5 exhibiting a sea water 3 rpm viscosity greater than 40 dial reading.

7. The diutan gum of Claim 6 exhibiting a sea water 3 rpm viscosity greater than 42 dial reading.

8. A diutan gum exhibiting a sea water 0.3 rpm viscosity greater than 35,000 cp.

9. The diutan of Claim 8 exhibiting a sea water 0.3 rpm viscosity greater than 35,000 cp.

10. The diutan of Claim 9 exhibiting a sea water 0.3 rpm viscosity greater than 38,000 cp.

11. The diutan of Claim 10 exhibiting a sea water 0.3 rpm viscosity greater than 40,000 cp.

12. The diutan of Claim 11 exhibiting a sea water 0.3 rpm viscosity greater than 41,000 cp.

13. A diutan gum exhibiting a low shear rate viscosity in the presence of polyethylene glycol dispersant of greater than 3500 cp.

14. The diutan gum of Claim 13 exhibiting a low shear rate viscosity in the presence of polyethylene glycol dispersant of greater than 3700 cp.

15. The diutan gum of Claim 14 exhibiting a low shear rate viscosity in the presence of polyethylene glycol dispersant of greater than 3900 cp.

16. The diutan gum of Claim 15 exhibiting a low shear rate viscosity in the presence of polyethylene glycol dispersant of greater than 4000 cp.

17. A method of producing a diutan gum comprising:

introducing a coding sequence for at least one diutan biosynthetic enzyme into a host diutan producing *Sphingomonas* organism;

culturing the host organism under fermentation conditions, whereby the host organism produces a diutan gum which exhibits at least one of the following characteristics:

a) an intrinsic viscosity of greater than 150 dL/g;

b) a sea water 3 rpm viscosity greater than 35 dial reading;

c) a sea water 0.3 rpm viscosity greater than 35,000 centipoise; and

d) a low shear rate viscosity in the presence of polyethylene glycol dispersant of greater than 3500 centipoise.

18. The method of claim 17 wherein the at least one diutan biosynthetic enzyme is a DpsG polymerase.

19. The method of claim 17 wherein the at least one diutan biosynthetic enzyme comprises a DpsG polymerase and a glucose-1-phosphate thymidylyltransferase; a dTDP-6-deoxy-D-glucose-3-5-epimerase; a dTDP-D-glucose-4,6-dehydratase; and a dTDP-6-deoxy-L-mannose-dehydrogenase.

20. The method of claim 17 wherein the at least one diutan biosynthetic enzyme

comprises a DpsG polymerase and a rhamnosyl transferase IV; a glucosyl-isoprenylphosphate transferase I; a beta-1,4-glucuronosyl transferase II; and a glucosyl transferase III.

21. The method of claim 17 wherein the at least one diutan biosynthetic enzyme comprises a DpsG polymerase and polysaccharide export proteins DpsD, DpsC, and DpsE.

22. The method of claim 17 wherein the at least one diutan biosynthetic enzyme comprises a rhamnosyl transferase IV; a beta-1,4-glucuronosyl transferase II; glucosyl transferase III; a glucose-1-phosphate thymidylyltransferase; a glucosyl-isoprenylphosphate transferase I; a dTDP-6-deoxy-D-glucose-3-5-epimerase; a dTDP-D-glucose-4,6-dehydratase; and a dTDP-6-deoxy-L-mannose-dehydrogenase.

23. The method of claim 1 wherein the at least one diutan biosynthetic enzyme is selected from the group consisting of polymerase; lyase; rhamnosyl transferase IV; beta-1,4-glucuronosyl transferase II; glucosyl transferase III; polysaccharide export protein; secretion protein; glucosyl-isoprenylphosphate transferase I; glucose-1-phosphate thymidylyltransferase; dTDP-6-deoxy-D-glucose-3-5-epimerase; dTDP-D-glucose-4,6-dehydratase; dTDP-6-deoxy-L-mannose-dehydrogenase and combinations thereof.

24. A method of producing a sphingan gum which has a longer average polymer length, comprising:

introducing a coding sequence for at least one sphingan polymerase enzyme into a host sphingan producing *Sphingomonas* organism;

culturing the host organism under fermentation conditions, whereby the host organism produces a sphingan gum which has a longer average polymer length than that produced by the *Sphingomonas* organism prior to introduction of the coding sequence.

25. The method of claim 24 wherein the sphingan gum is S88.

26. The method of claim 24 wherein the sphingan gum is S60.

27. The method of claim 24 wherein the sphingan gum is S657.

28. An isolated nucleic acid molecule which encodes at least one diutan biosynthetic enzyme as shown in SEQ ID NO: 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, and 43, or an enzyme which is at least 95 % identical to SEQ ID NO: 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, and 43.

29. The isolated nucleic acid molecule of claim 28 which encodes a diutan polymerase.

30. The isolated nucleic acid molecule of claim 28 which encodes a diutan polymerase and a polysaccharide export protein.

31. The isolated nucleic acid molecule of claim 28 which encodes a diutan polymerase and a rhamnosyl transferase IV; a glucosyl-isoprenylphosphate transferase I; a beta-1,4-glucuronosyl transferase II; and a glucosyl transferase III.

32. The isolated nucleic acid molecule of claim 28 which encodes a diutan polymerase and a glucose-1-phosphate thymidylyltransferase; a dTDP-6-deoxy-D-glucose-3-5-epimerase; a dTDP-D-glucose-4,6-dehydratase; and a dTDP-6-deoxy-L-mannose-dehydrogenase.

33. The isolated nucleic acid molecule of claim 28 which encodes a diutan polymerase; lyase; rhamnosyl transferase IV; beta-1,4-glucuronosyl transferase II; glucosyl transferase III; polysaccharide export protein; secretion protein; glucosyl-isoprenylphosphate transferase I; glucose-1-phosphate thymidylyltransferase; dTDP-6-deoxy-D-glucose-3-5-epimerase; dTDP-D-glucose-4,6-dehydratase; and dTDP-6-deoxy-L-mannose-dehydrogenase.

34. The isolated nucleic acid molecule of claim 28 which comprises the nucleic acid sequence according to SEQ ID NO: 1.