Review

Synthesis of the Microbial Polysaccharide Gellan from Dairy and Plant-Based Processing Coproducts

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Abstract: This review examines the production of the microbial polysaccharide gellan, synthesized by *Sphingomonas elodea*, on dairy and plant-based processing coproducts. Gellan is a water-soluble gum that structurally exists as a tetrasaccharide comprised of 20% glucuronic acid, 60% glucose and 20% rhamnose, for which various food, non-food and biomedical applications have been reported. A number of carbon and nitrogen sources have been tested to determine whether they can support bacterial gellan production, with several studies attempting to optimize gellan production by varying the culture conditions. The genetics of the biosynthesis of gellan has been explored in a number of investigations and specific genes have been identified that encode the enzymes responsible for the synthesis of this polysaccharide. Genetic mutants exhibiting overproduction of gellan have also been identified and characterized. Several dairy and plant-based processing coproducts have been screened to learn whether they can support the production of gellan in an attempt to lower the cost of synthesizing the microbial polysaccharide. Of the processing coproducts explored, soluble starch as a carbon source supported the highest gellan production by *S. elodea* grown at 30 °C. The corn processing coproducts corn steep liquor or condensed distillers solubles appear to be effective nitrogen sources for gellan production. It was concluded that further research on producing gellan using a combination of processing coproducts could be an effective solution in lowering its overall production costs.

Keywords: gellan; polysaccharide; applications; culture conditions; genetics; mutants; processing coproducts; *Sphingomonas elodea*

1. Introduction

The purpose of this review is to provide both background about the microbial polysaccharide gellan and, more specifically, to examine bacterial gellan synthesis on dairy and plant-based processing coproducts. The anionic heteropolysaccharide gellan is known to be synthesized by *Sphingomonas elodea* strain ATCC 31461 [1,2]. Although originally classified as *Pseudomonas elodea*, this strain was reclassified as a species of *Sphingomonas* [3,4]. Structurally, the water-soluble gum gellan exists as a tetrasaccharide composed of 20% glucuronic acid, 60% glucose and 20% rhamnose [5–7]. The native form of gellan has been shown to contain acetyl and L-glyceryl groups. These substituents need to be removed by an alkaline heat treatment to produce a gel. The degree of deacetylation of gellan can be directly correlated to its gel-forming ability [8]. The alkaline treatment of the native biopolymer results in a tetrasaccharide sequence that is anionic (Figure 1). As the deacetylated biopolymer cools, a double helix forms from the disordered coils which results in gelation. Gelation of the polysaccharide is enhanced when the pH of the polysaccharide becomes more acidic. An acidic pH diminishes the negative charge of the polysaccharide molecule that results in greater repulsion within the helix. The addition of monovalent cations to a gellan solution increases its rate of gelation [9–14].
Figure 1. The structure of gellan involves tetrasaccharide repeating units consisting of two molecules of D-glucose as well as one molecule each of L-rhamnose and D-glucuronic acid.

When the monovalent cations bind to the helices, they bind to interact with the carboxylate groups on the polysaccharide to diminish repulsion within the helices. The binding of the monovalent ions to the helices increases with ionic size. Gellan behaves like a normal polymer solution when low monovalent cation concentrations are present. As the monovalent cation concentration is increased, a self-supporting gel is formed. The addition of divalent cations to a gellan solution also increases its rate of gelation [9–17]. The divalent cations bind between the helices to cause gelation. High concentrations of divalent cations result in a reduction in gel strength. It has been shown that the concentration of divalent cations needs to be equivalent to the carboxylate group content of the gellan to achieve maximum gellan strength. It has been noted that to achieve maximum gel strength, a higher concentration of monovalent cations needs to be added to the polymer solution than the divalent cation concentration. The binding properties of gellan allowed a colorimetric assay to be developed using the dye toluidine blue O where the gellan assay was found to be linear up to a concentration of 0.7 g/L [18].

In the United States, the source of commercial gellan production is C.P. Kelco (Atlanta, GA, USA). There are a number of commercial applications reported for this polysaccharide gum. With respect to food applications, gellan is used in confectionery jellies, fabricated foods, pie fillings and puddings, bakery icings and frostings, dairy products, fruit, milk-based and carbonated beverages as well as film or coatings for food adhesion [19–23]. With respect to non-food applications, gellan was originally proposed as a replacement for agar in microbial growth media since agar is the most economical polysaccharide gum available to be used as a solidifying agent that produces opaque gels [24–26]. Gellan has found greater use as a substitute for agar in plant tissue culture. The higher clarity of the gels at lower gellan concentrations is a marked advantage over agar. Another advantage of gellan is that it contains less impurities compared to agar. The use of gellan gels in plant tissue cultures allows tissue development to be observed more clearly than using agar. Gellan also has a number of pharmaceutical uses for drug delivery and enzyme immobilization [27–34]. With its non-toxicity, rapid gelation, ability to retain water and its biodegradability, gellan is used in oral formulations for drug delivery in capsules, beads and tablets. In ophthalmic formulations, it is used in situ gelling solutions to deliver anti-glaucoma and anti-conjunctivitis drugs [35]. Gellan is employed in nasal formulations as an in situ nasal gel to deliver a variety of pharmaceuticals. Another biomedical application of gellan is its use in wound healing where its properties have promise to serve as a carrier in tissue engineering [36–40].

2. Effect of Culture Conditions on Gellan Production by Sphingomonas elodea
2.1. Effect of Carbon Source

Relative to the carbon source, glucose-grown cells of S. paucimobilis strain ATCC 31461 were first shown to synthesize gellan [1,14]. A model developed to optimize gellan production was devised using ATCC 31461 in a simplified 3% glucose-containing medium [41]. It has been shown that maximum yield and productivity for ATCC 31461 were determined
in a medium containing 1% glucose when no nitrogen source was present [42]. The strain ATCC 31461 was also shown to synthesize gellan on sucrose, mannose, galactose, fructose or maltose as a carbon source after 72 h of growth at 30 °C [43]. It was also found that cellular biomass production by ATCC 31461 did not correlate with gellan synthesis [43]. A prior study examining the effect of carbon source concentration of glucose and sucrose noted that polysaccharide production was maximal after 52 h in a pH 7.0-buffered medium containing 3% glucose or 4% sucrose at 30 °C [44]. With respect to the optimal incubation temperature for ATCC 31461 cells grown in a glucose-containing medium, it was shown to be 30–31 °C [45]. Similarly, cellular productivity was maximum for gellan production at 31 °C after 72 h of growth in batch cultures [45]. A two-stage culture strategy was developed to improve gellan production by ATCC 31461 using sucrose as a carbon source. This strategy was based on the findings that lower sucrose concentrations and higher temperatures favored bacterial cell growth but not gellan synthesis while low cell growth was observed when higher sucrose concentrations and lower temperatures were used which favored increased gellan production. The initial stage of the process involved pulse fed batch feeding of the culture during the first 24 h. ATCC 31461 cells were cultured in a pulse fed-batch mode with an initial sucrose concentration 10 g/L at 33 °C while the second stage involved incubating the batch culture at 28 °C to promote gellan production [46]. The influence of the initial pH of a glucose-containing culture medium on gellan synthesis by ATCC 31461 was analyzed. When the initial pH of the culture medium was 6.8 to 7.4, it was observed that gellan formation was greatest after 72 h of growth at 30 °C. Biomass production by the ATCC 31461 after 72 h was highest when the initial pH of the glucose-containing medium was adjusted to 7.8 [47]. Lastly, the effect of adding the glucose analog 2-deoxy-D-glucose to ATCC 31461 cultures was examined. When 50 µg/L 2-deoxy-D-glucose was added to the ATCC 31461 culture medium after 24 h, it was observed that the highest gellan concentration (20.78 g/L) was produced. The activity of UDP-glucose pyrophosphorylase activity was inhibited while the glucosyltransferase activity was elevated. It was thought that the glucose analog greatly inhibited glycolysis while activating the biosynthetic pathway for gellan in ATCC 31461 cells [48].

2.2. Influence of Nitrogen Source

The effect of a complex nitrogen source substituting for ammonium nitrate in a glucose-containing culture medium of ATCC 31461 was explored [43]. The complex nitrogen source tested was soytone (hydrolyzed soy protein), corn steep liquor, corn steep solids, ethanol stillage, peptone or tryptone [43]. In all cases, the complex nitrogen source promoted higher gellan production by ATCC 31461 in the glucose-containing medium compared to the medium containing glucose as the carbon source and ammonium nitrate as the nitrogen source [43]. Gellan production by ATCC 31461 was found to be highest in a medium containing glucose and soytone. It was also observed that soytone stimulated ATCC 31461 biomass production in the glucose-containing medium [43]. Having determined that the nitrogen source soytone enhanced gellan production by ATCC 31461, yeast extract supplementation of the glucose-containing medium was studied to learn whether the yeast extract concentration in the medium could further promote gellan production [49]. It was determined that only if the concentration of yeast extract in the glucose-containing medium was 0.1% did the polysaccharide level produced by ATCC 31461 increase significantly.

2.3. Effect of Aeration and Surfactants

The effect of aeration on gellan and cell biomass biosynthesis by ATCC 31461 was investigated. Biomass production was higher than gellan production by the bacterium as aeration and dissolved oxygen tension was elevated [50]. It was observed that 100% dissolved oxygen tension caused the gellan yield to increase to 23 g/L, with the polysaccharide being synthesized having increased viscosity and molecular weight. Another study found that high dissolved oxygen tension was not required for elevated gellan synthesis. Instead, it appeared that maximum gellan synthesis by the sphingomonad occurred when
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oxygen limitation preceded the initiation of gellan formation. It was also concluded that maximum gellan production by the bacterium did not correlate with maximum cellular biomass production [51]. It has been reported that treatment with 2–4 mM hydrogen peroxide of sphingomonad cells over a period of 12 h resulted in 36% greater gellan production than the untreated cells. In addition, cell growth was noted to be inhibited by the presence of hydrogen peroxide. The study found that oxidative stress placed upon the bacterial cells resulted in the enzymes UDP-glucose pyrophosphorylase activity and glucosyltransferase activity being elevated [52]. The increase in these enzyme activities likely accounts for the enhanced bacterial gellan production. The addition of the non-ionic surfactants Tween 80, Tween 40 and Triton X-100 to the medium of ATCC 31461 cultures were studied to determine whether they affected gellan synthesis [53]. It was observed that supplementation of each surfactant enhanced polysaccharide production by ATCC 31461 cells. A 1.2-fold increase in gellan synthesis by ATCC 31461 was noted when 0.75 g/L Triton X-100 was present in the culture medium compared to gellan production by ATCC 31461 cells grown in unsupplemented culture medium. Surfactant supplementation of the bacterial cultures also resulted in a high viscosity polysaccharide being synthesized. The same study found that the gellan yield (14.62 g/L) was highest in a 5 L laboratory fermentor incubated at 29.6 °C in a culture medium buffered at pH 6 containing 1.0 g/L Triton X-100 [53]. Gellan production by ATCC 31461 in the fermentor could be elevated by 1.9-fold with an aeration speed of 1000 rpm and a 100% dissolved oxygen tension similar to what was observed in an earlier study [50,53].

3. Genetics of Gellan Biosynthesis and Mutant Isolation

3.1. Genetics of Gellan Biosynthesis

A number of investigations have explored the pathways thought to be involved in the synthesis of gellan. The enzymes thought to be involved in the pathway of gellan synthesis include phosphoglucomutase, dTDP-D-glucose pyrophosphorylase, dTDP-D-glucose 4,6-dehydratase, dTDP-4-dehydrorhamnose 3,5 epimerase, dTDP-4-dehydrorhamnose reductase, glucose-1-phosphate uridylyltransferase and UDP-D-glucose dehydrogenase (Figure 2). It has been shown that S. elodea ATCC 31461 contains a gene cluster of 18 genes. The identified genes showed a high degree of homology to the genes responsible for sphingan synthesis in Sphingomonas sp. ATCC 31554 [54]. Interestingly, the genes encoding the glycosyl transferases showed little homology. The rml gene cluster, consisting of four genes, is thought to be involved in the synthesis of dTDP-rhamnose [55]. The first gene of the gene cluster is rmlA which was shown to encode an enzyme that exhibited dTDP-glucose pyrophosphorylase following expression of the gene in Escherichia coli. Characterization of the bacterial fusion protein of the pyrophosphorylase revealed that it had a K_m of 12.0 µM for dTDP-glucose. It is known that pgmG encodes phosphoglucomutase [55]. Another study performed protoplast fusion experiments with S. elodea ATCC 31461 and the genes, rmlA and pgmG were cloned [56]. The approximate molecular weight of the rmlA protein was 50 kDa while the molecular weight of the pgmG protein was 31 kDa. The enzyme glucose-1-phosphate uridylyltransferase, encoded by ugpG, catalyzes the production of UDP-glucose and pyrophosphate from its substrates glucose-1-phosphate and UTP in S. elodea ATCC 31461 [57]. The X-ray crystal structure of the glucose-1-phosphate uridylyltransferase from ATCC 31461 has been elucidated [58]. It was reported that there is monomeric structural similarity between glucose-1-phosphate uridylyltransferase and glucose-1-phosphate thymidylyltransferase. In S. elodea ATCC 31461, the enzyme UDP-glucose dehydrogenase was shown to be encoded by ugdG gene since the dehydrogenase was synthesized when the gene was expressed recombinantly in E. coli [59]. The dehydrogenase produces UDP-D-glucuronic acid from UDP-D-glucose. The K_m values of the recombinant dehydrogenase for its substrate UDP-glucose or NAD^+ was 0.87 or 0.4 mM, respectively. The temperature optimum for the dehydrogenase was 37 °C while its pH optimum was 8.7. A regulatory gene affecting gellan synthesis by ATCC 31461 was also thought to have been identified [54]. The concentration and viscosity of gellan was noted to be affected by the products of the
three genes *gelI*, *gelM* and *gelN* [54]. With respect to the metabolism of glucose by the bacterium to synthesize gellan, the production of glucose-6-phosphate and gluconate are necessary so that they can be utilized by the Entner–Doudoroff and pentose phosphate pathways plus the enzyme glucose-6-phosphate dehydrogenase was also thought to be involved in gellan synthesis [60].

**Figure 2.** The metabolic pathways in *Sphingomonas elodea* thought to be involved in gellan synthesis [54–59]. The pathway enzymes involved include (1) phosphoglucomutase; (2) dTDP-D-glucose pyrophosphorylase; (3) dTDP-D-glucose 4,6-dehydratase; (4) dTDP-4-dehydrorhamnose 3,5 epimerase; (5) dTDP-4-dehydrorhamnose reductase; (6) glucose-1-phosphate uridylyltransferase; (7) UDP-D-glucose dehydrogenase. Abbreviations: UDP, uridine diphosphate; dTDP, thymidine diphosphate.

### 3.2. Mutant Isolation Related to Gellan Production

A mutant strain of the *S. elodea* ATCC 31461 was isolated using chemical mutagenesis and resistance to the antibiotic ampicillin [61,62]. Using glucose or corn syrup as a carbon source, the isolated mutant demonstrated enhanced gellan production [61]. Compared to its parent strain ATCC 31461, the mutant strain produced approximately double the gellan concentration on glucose or corn syrup after 48 h of growth at 30 °C, and decreased to approximately 1.4-fold higher after 72 h of growth at 30 °C. It was noted that there was no correlation between biomass production and gellan production by the mutant strain. A correlation between increased gellan production by the mutant strain on either carbon source and an increase in culture medium viscosity was observed. There was no difference in the glucuronic acid content of the gellan produced by the mutant and parent strains [61]. A problem with ATCC 31461 is that it accumulates yellow carotenoid pigments and poly-β-hydroxybutyrate which results in diminished levels of gellan [63]. Pigment removal from the polysaccharide also increases processing costs in gellan production. A
possible solution to this problem was to engineer a mutant strain that did not accumulate pigment and poly-β-hydroxybutyrate. A mutant strain was isolated where carotenoid and poly-β-hydroxybutyrate syntheses were blocked but its ability to synthesize gellan was diminished [63]. Using this double mutant strain, a further effort was employed to isolate a strain exhibiting increased gellan production. The resultant strain isolated produced approximately a 1.4-fold higher gellan concentration than ATCC 31461 after 48 h of growth at 30 °C in a sucrose-containing medium.

4. Gellan Synthesis on Processing Coproducts

4.1. Dairy Processing Coproduct

A number of crop processing coproducts have been tested in an effort to lower the overall cost of producing the polysaccharide gellan commercially (Table 1). One such coproduct is whey from dairy processing. A surplus of the lactose-rich whey exists and studies have examined whether whey could be utilized as a carbon source for gellan production [64,65]. One study found that whey containing 4.9% lactose was able to sustain gellan production by ATCC 31461 when tryptone served as the nitrogen source. Dilution of the whey by 1:4 resulted in the highest level of gellan production by ATCC 31461 [64]. Another investigation examining whey as a carbon source and peptone as a nitrogen source also found that dilution of the whey by 1:4 resulted in the highest level of gellan being produced by ATCC 31461 [65].

4.2. Corn Processing Coproducts

Maltose corn syrup is a processing coproduct that is synthesized from the wet milling of corn. The conversion of corn starch into corn syrup occurs using a dual acid enzyme conversion process where the level of maltose is about 42%. In a number of studies (Table 1), the maltose corn syrup was used as a carbon source while various nitrogen sources were screened to learn which combination allowed the highest gellan production by ATCC 31461 [43,45,47,48,61,66,67]. It was observed that in a corn syrup-containing medium that using corn steep liquor as a nitrogen source resulted in a higher level of gellan production by ATCC 31461 than when ATCC 31461 utilized soytone, corn steep solids, tryptone or peptone as a nitrogen source [43]. Similarly, an investigation where a glucose-containing medium in which corn steep liquor served as a nitrogen source caused a high level of gellan to be produced by strain ZJUT 1008 [68]. The advantage of using corn steep liquor as a nitrogen source in the medium is that it is more cost effective than using yeast extract or ammonium salts as a nitrogen source. Another processing coproduct is condensed corn solubles which is derived from the dry milling of corn for ethanol production [69]. One advantage of using condensed corn solubles is that the solubles contains components found in yeast extract and ammonium salts. It was determined that when the solubles was included in a 3% glucose-containing medium (pH 6.5) in a bioreactor incubated at 28 °C, gellan production by ATCC 31461 was shown to be higher on condensed corn solubles than the standard medium containing yeast extract and ammonium salts. It was concluded that condensed corn solubles as a gellan production medium substitute could substantially reduce the commercial production cost of gellan [69].
Table 1. Gellan production by strains grown on processing coproducts.

| Strain         | Carbon Source       | Nitrogen Source    | Growth Conditions | Gellan (G/L) | Reference |
|----------------|---------------------|--------------------|-------------------|--------------|-----------|
| ATCC 31461     | Maltose corn syrup  | Corn steep solids  | 72 h, 30 °C       | 2.71         | [43]      |
| ATCC 31461     | Maltose corn syrup  | Corn steep solids  | 72 h, 30 °C       | 2.98         | [43]      |
| ATCC 31461     | Maltose corn syrup  | Tryptone           | 72 h, 30 °C       | 1.93         | [43]      |
| ATCC 31461     | Maltose corn syrup  | Peptone            | 72 h, 30 °C       | 1.80         | [43]      |
| ATCC 31461     | Soluble starch      | Peptone            | 72 h, 30 °C       | 5.73         | [44]      |
| ATCC 31461     | Maltose corn syrup  | Hydrolyzed soy protein | 72 h, 30 °C | 2.24         | [60]      |
| EGP-1          | Maltose corn syrup  | Hydrolyzed soy protein | 72 h, 30 °C | 3.20         | [60]      |
| ATCC 31461     | Whey                | Tryptone           | 64 h, 28 °C       | 3.10         | [64]      |
| ATCC 31461     | Whey                | Peptone            | 72 h, 30 °C       | 7.90         | [65]      |
| ZJUT 1008      | Glucose             | Corn steep liquor  | 72 h, 30 °C       | 14.41        | [68]      |
| ATCC 31461     | Glucose             | Condensed corn solubles | 82 h, 30 °C | 13.40        | [69]      |
| ATCC 31461     | Soluble starch      | Tryptone           | 48 h, 30 °C       | 24.32        | [70]      |
| ATCC 31461     | Soluble starch      | Yeast extract/tryptophan | 48 h, 30 °C | 43.60        | [71]      |
| ATCC 31461     | Molasses            | Tryptone           | 48 h, 30 °C       | 13.81        | [72]      |
| NK 2000        | Glucose             | Soybean pomace     | 72 h, 30 °C       | 7.50         | [73]      |
| ATCC 31461     | Olive oil waste     | Yeast extract      | 63 h, 30 °C       | 6.80         | [74]      |

4.3. Soluble Starch as a Carbon Source for Gellan Production

Selected concentrations of soluble starch have been tested as a possible carbon source (Table 1) to support gellan production by ATCC 31461 [44,70,71]. When 2% soluble starch was included in a medium containing peptone as a nitrogen source, it was observed that ATCC 31461 produced 5.73 g/L gellan after 72 h at 30 °C [44]. Another study also used 2% soluble starch as a carbon source for gellan production by ATCC 31461 but tryptone was utilized as the nitrogen source in the culture medium [70]. It was reported that 4% soluble starch supported a higher level of gellan (44.2 g/L) by ATCC 31461 than if the strain was grown on 2% soluble starch [70]. It was not clear if the higher gellan concentration produced by ATCC 31461 was due to unused soluble starch also being precipitated in addition to the gellan. A third study also used soluble starch as a carbon source for the production of gellan by ATCC 31461 [71]. A statistical approach was taken to determine the optimum conditions for gellan production by ATCC 31461 on 4% soluble starch. It was determined that a concentration of 43.6 g/L of starch-free gellan was synthesized by ATCC 31461 in the presence of yeast extract, tryptophan and ADP in the culture medium after 48 h at 30 °C. This gellan concentration is substantially higher than previously reported levels of gellan produced by ATCC 31461 on processing coproducts [71]. It appeared that soluble starch could be an ideal substrate for bacterial gellan production.

4.4. Other Processing Coproducts

Another processing coproduct examined as a substrate for bacterial gellan mutant was molasses from cane processing (Table 1). A fermentor containing 3 L of culture medium containing 1.125% molasses as a carbon source and tryptone as a nitrogen source supported the production of 13.81 g/L gellan by ATCC 31461 after 48 h at 30 °C [72]. In a glucose-containing medium (Table 1), gellan production by a S. elodea strain NK2000 was investigated to determine whether soybean pomace was an effective nitrogen source [73].
Soybean pomace is a processing coproduct of the soy sauce industry. It was found that strain NK2000 produced a higher gellan concentration on soybean pomace than peptone in a bioreactor after 72 h at 30 °C. This indicated that soybean pomace could substitute for peptone in a gellan production medium which make the production of the polysaccharide more economical [73]. A processing coproduct of olive oil production is olive mill waste water. When olive mill waste water was utilized as a carbon source and yeast extract was used as a nitrogen source in the culture medium (Table 1), ATCC 31461 was shown to synthesize gellan in a fermentor [74]. After 63 h at 30 °C, ATCC 31461 produced 6.8 g/L gellan in a 15 L stirred tank bioreactor with a 10 L working volume. The addition of glycerol to the medium caused a slight increase in gellan production by ATCC 31461 after 63 h in the bioreactor [74].

From comparing the possible lower cost processing coproducts that could be utilized to commercially synthesize gellan, it appeared from the earlier studies that soluble starch as a carbon source was capable of supporting the highest level of gellan by ATCC 31461. Considering that the concentration of nitrogen source added to the medium is much lower than the carbon source concentration, soluble starch probably could have a greater impact on lowering the production cost of gellan. A production medium for gellan synthesis where soluble starch is used as the carbon source and another cheaper processing coproduct, such as corn steep liquor or condensed corn solubles, serves as the nitrogen source could be developed so that large-scale gellan production could become more economical. This may lead to a decrease in the cost of gellan which would make it competitive to the production costs of other commercially available polysaccharides.

5. Conclusions

In summary, there appears to be an opportunity to use low-value processing coproducts to synthesize the commercially available polysaccharide gellan. From an economic perspective, development of a coproduct-based process to synthesize gellan at a lower price could increase its large-scale production and likely increase the number of industrial applications for gellan. With many of the processing coproducts considered as waste products that often pollute the environment, their use in large-scale polysaccharide production would greatly reduce any impact that they have on the environment. It can be concluded that further research is necessary to identify the appropriate combination of processing coproducts for optimal microbial production of gellan.

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