

Optimization of cultural conditions of gellan gum production from recombinant *Sphingomonas paucimobilis* ATCC 31461 and its characterization

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ABSTRACT

Microorganisms that produce exopolysaccharides have currently gained broad attention and are a subject of great concern for modern-day biotechnologists and microbiologists. *Sphingomonas paucimobilis* is a Gram-negative rod-shaped bacterium isolated from the *Elodea* plant tissue. The effect of the culture conditions on gellan gum production by recombinant *S. paucimobilis* ATCC 31461 in a stirred-type bioreactor was investigated. The products of fermentation were characterized by Fourier transform infrared (FTIR), scanning electron microscopy (SEM), thermogravimetric analysis (TGA), and differential scanning calorimetry (DSC). The FTIR spectrum revealed characteristic peaks of different functional groups in the gellan. SEM was carried out to observe the morphology of the gellan gum, while the thermal stability of the biopolymer was determined by TGA and DSC analysis. The compositional analysis of the purified gellan showed that it is a heteropolymer containing glucose, rhamnose, and glucuronic acid. The compression test of the gellan revealed its textural property. Maximum gellan gum was attained after 48 h of incubation at 30°C with a pH of 6.5, 500 rpm, and 100% dissolved oxygen. The rheology study indicated the non-newtonian nature of gellan gum. The solubility of gellan gum in different polar and non-polar solvents was also investigated. The findings in this study showed that gellan gum produced by recombinant *S. paucimobilis* ATCC 31461 has a high potential to be utilized in the pharmaceutical and food industry.

1. INTRODUCTION

Microbial exopolysaccharides are water-soluble, high molecular weight, natural, or semi-synthetic long-chain polymers. They are composed of sugar residues and are economically valuable due to their functional properties and unique structure. Microbial exopolysaccharides are secreted by microorganisms into the external environment through sugar fermentation. Gellan gum from the strain of *Sphingomonas paucimobilis*, bacterial alginates from *Azotobacter vinelandii*, xanthan from *Xanthomonas campestris*, and wellan from *Alcaligenes* are some of the widely used biopolymers which have been the subject of extensive research. Physicochemical factors such as pH, temperature, incubation time, agitation speed, medium, carbon, and nitrogen sources play a key role in the exopolysaccharides yield [1].

Gellan gum has specific characteristics such as biocompatibility, biodegradability, temperature resistance, stability at acidic conditions, rigidity, and clarity makes the polymer widely used in the food and

pharmaceutical industry. Gellan gum in its native form comprises of tetrasaccharide repeating unit of β -D-glucose, β -D-glucuronic acid, α -L-rhamnose, and acyl groups, namely, acetate and glycerate [2].

The simple media containing a carbon source, nitrogen source, and inorganic salts are used for the production of gellan gum [3]. The carbon sources such as glucose, fructose, maltose, sucrose, and mannitol play an essential role in the production yield, composition, structure, and properties of gellan gum. The choice of nitrogen sources and their concentration influences gellan broth characteristics and the flow of carbon to either biomass or product formation. The pH of the media has a strong effect on product formation and cell proliferation. The temperature has a profound influence on the activity of the enzymes involved in the production of gellan gum and microbial growth by *Sphingomonas paucimobilis* ATCC 31461 [4].

Agitation at the lower levels provides inadequate homogenous conditions, whereas at higher levels, shear forces inside the fermentation broth increase, which can lead to mechanical damage of the cells and thus affecting polysaccharide synthesis [5]. Aeration is a prerequisite for aerobic species such as *S. paucimobilis* for cellular growth and gellan production. Rho and Loung [6] observed a complete decrease in gellan gum production when the dissolved oxygen in the bioreactor was set at zero.

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During gellan gum production, the viscosity of the polymer reduces with an increase in shear rate, that is, they exhibit pseudoplastic behavior, and the rheology of the fermentation broth was found to be dependent on media components, dissolved oxygen levels, and agitation rate [7]. The water-holding characteristics of the gellan gum maintain the shape, consistency, and freshness of the food for a longer duration of time. The ability of gellan gum to solubilize in water has created various applications in the pharmaceutical and food industries [8].

Gellan gum promptly forms a gel with calcium ions, and the produced gels exhibit accurate edges and sublime shapes [9]. The textural properties and gelation of the gel samples were evaluated using compression tests. The compression behavior of the gellan gum is based on the speed of the applied compressive force [10].

This study was undertaken with the objectives to investigate the optimum medium components and culture conditions during the fermentation process for maximum gellan gum production by recombinant *S. paucimobilis* ATCC 31461. The characterization of gellan gum in terms of rheological, textural, and structural properties was also investigated.

2. MATERIALS AND METHODS

2.1. Bacterial Strains, Vectors, and Reagents

The strain *S. paucimobilis* ATCC 31461 was procured from LGC, Promochem Pvt. Ltd., Bengaluru. The primers for polymerase chain reaction (PCR) were purchased from Eurofins. DNA isolation, PCR purification, gel extraction, and plasmid isolation kits were procured from Thermo Fisher Scientific (USA). The vector pBBR122 was obtained from MoBiTec. The mediums Yeast peptone glucose (YPG), Luria-Bertani (LB), and nutrient broth (NB) were obtained from HIMEDIA India. All the chemicals and reagents used in the present study were of analytical reagent grade quality and obtained from Merck, India.

2.2. Cloning and Expression of the Gellan Gum Biosynthetic Gene Gel D

The genomic DNA from *S. paucimobilis* ATCC 31461 was isolated using the Wizard® DNA isolation kit. The primers for gene gelD encoding the protein for polymerization and export of gellan were designed using the Primer Premier Software. The PCR for gene gel D was performed using a Thermal cycler (GeneAtlas) in a total reaction volume of 50 µl comprising 1 µl of template DNA (ng), 5 µl of *Pfu* buffer with MgSO₄ (10×), 1 µl of each primer (10 µM), 2 µl of *Pfu* DNA polymerase (2.5 U/µl) (100 U), 2 µl of deoxynucleotide triphosphate (10 mM), and 38 µl of nuclease-free water. The gene gel D was amplified under the PCR conditions one cycle of initial denaturation (95°C, 2 min), followed by 30 cycles of denaturation (95°C, 30 s), annealing (57°C, 45 s), extension (72°C, 3 min), and final extension (72°C, 10 min). The amplified gel D was purified using the Gene Jet PCR purification kit. The purified gelD and pBBR122 vector were double digested using restriction enzymes NcoI and EcoRI and gel eluted by Thermo scientific GeneJET Gel extraction kit. The eluted DNA and vector were ligated using T4 DNA ligase at 16°C overnight. The ligation mixture was transformed into chemically competent *Escherichia coli* DH5α cells. The preparation of competent cells and transformation by heat shock was carried out by standard protocols described by Sambrook *et al.* [11]. The transformants were selected based on chloramphenicol resistance, and the plasmid isolation from

the transformed cells was done using the alkali lysis method [12]. The clones were confirmed by double digestion with restriction enzymes, colony PCR, and sequencing. The transformation of the recombinant plasmid (pBBR122-gelD) into *S. paucimobilis* electrocompetent cells was carried out by electroporation [13]. The recombinant protein was purified by affinity chromatography using Ni-NTA resin [14], and the purified protein was analyzed by SDS-polyacrylamide gel electrophoresis [15].

2.3. Maintenance of Culture

The recombinant *S. paucimobilis* ATCC 31461 culture was maintained in YPG agar slants. The YPG medium contained the following composition (g/l): Glucose 30; peptone 5; yeast extract 3; NaCl 5; and agar 20. The pH of the media was adjusted to 6.5. The YPG agar slants were incubated at 30°C for 48 h. The agar slants were stored at 4°C for routine use and maintained as glycerol stocks at -80°C for long-term storage.

2.4. Inoculum Preparation

The inoculum of recombinant *S. paucimobilis* ATCC 31461 was prepared by transferring a loopful of YPG slant culture into a 1L Erlenmeyer flask containing 50 ml of YPG broth. The inoculated flask was incubated at a temperature of 30±1°C on rotary shaker at 300 rpm for 24 h.

The impact of process parameters on gellan gum production by recombinant *S. paucimobilis* ATCC 31461 was studied. The various process parameters evaluated include the effect of media, incubation time, size and age of inoculum, temperature, pH of the medium, carbon source, nitrogen source, agitation speed, dissolved oxygen, and C/N ratio. The effect of one parameter was studied at a time by keeping other parameters constant.

2.5. Optimization of Parameters

To determine the optimum biomass and gellan gum production, recombinant *S. paucimobilis* ATCC 31461 was grown in a different medium (YPG, LB, and NB) at various incubation time (12–96 h) with different incubation temperature (25–50°C), medium pH (4.0–8.0), dissolved oxygen (20–100%), and agitation speed (100–600 rpm). Various size (2–12%) and age (6–27 h) of inoculum were investigated to study their effect on gellan gum production. To determine the requirement of additional nutrients for gellan production, different carbon sources (sucrose, glucose, maltose, starch, and lactose) at a concentration of 20 g/l and nitrogen sources (urea, tryptone, peptone, sodium nitrate, ammonium sulfate, ammonium nitrate, and potassium nitrate) at 5 g/l concentration were used. The influence of carbon and nitrogen sources on gellan production was examined by varying the C:N ratios (0.5, 1, 2, 3, 4, and 5). The fermentation was performed with a fixed carbon source of glucose (2 g/100 ml) and varied nitrogen source of peptone concentration (0.25 g, 0.5 g, 1 g, 1.5 g, 2 g, and 2.5 g/100 ml).

2.6. Fermentation

Fermentations were carried out in a 6 L stirred-type bioreactor (EsedraPlus 6.0, Solari's biotechnology with SBC-08 Software) with 4 L working volume under optimized conditions. The pH was measured by combined type pre-pressurized glass-gel electrode, and dissolved oxygen was monitored using a polarographic type electrode. The temperature of the fermentor was maintained by a temperature-controlled probe. The data acquisition, monitoring, and control were

accomplished using software SBC-08. A standard inoculum was used to inoculate a 4 L production medium in the fermenter. The samples were withdrawn at regular intervals to determine biomass and gellan gum yield.

2.7. Analysis of Biomass and Isolation of Gellan Gum

The dry weight of cell biomass and recovery of gellan gum were determined by the gravimetric technique. The fermented broth was collected from the fermenter and was immersed in a boiling water bath for 15 min and cooled. The pH of the heated broth was increased to 10.0 by 2.0 M NaOH. The culture broth was then heated at 80°C in a water bath for 10min, cooled followed by the lowering of pH to 7.0 by 2M HCl. To separate the cells, the pre-treated culture broth was then subjected to centrifugation at 8000 rpm for 30 min at 4°C. The adhering polymer was removed by treating the cells with dimethyl sulfoxide. Then centrifugation was carried out at 8000 rpm for 30 min at 4°C. The cell pellet collected was dried at 80°C in a hot air oven for 24 h to obtain dry cell weight. The cell dry weight was expressed in g/l [16]. The recovery of gellan was carried out using cell-free supernatant. The polymer was then precipitated by adding three volumes of isopropyl alcohol, and the mixture was stored at 4°C for 24 h. The precipitate was collected by centrifuging at 8000 rpm for 30 min at 4°C. The gellan precipitate was then dried in a hot air oven at 80°C for 12 h to obtain a crude polymer [17].

2.8. Solubility Test and Viscosity of Gellan Gum

2 ml of organic and inorganic solvents were mixed with gellan powder and vortexed. The gellan gum was evaluated for its solubility in the following compounds: Water, acetone, chloroform, ethanol, methanol, ether, and phenol. The viscosity of the various concentrations of gellan gum (0.5, 1, 1.5, 2, 2.5, and 3.0% w/v) was determined using a Brookfield viscometer (RVDV-I Prime). The viscometer was operated at a shear rate of (20 s⁻¹–100 s⁻¹) using disk spindle No-40, and the temperature was maintained at 30°C. The viscosity was expressed in centipoises (mPa.s).

2.9. Gellan Gel Preparation

One percent gellan powder was dissolved in 100 ml of deionized distilled water with constant magnetic stirring. To obtain a homogeneous dispersion, the gellan solution was heated at 100°C for 15 min. The calcium concentrations ranging from 4 mM, 24 mM, 44 mM, 64 mM, 84 mM, and 104 mM were added to the hot gellan solution, and the temperature was progressively reduced to 50°C. The dispersions were stirred for 3 min and poured into preheated (80°C) stainless steel tubes for compression tests or into polypropylene tubes for centrifugal dehydration. The gels were then cooled in running tap water and stored for 1 day at room temperature (20°C) [18].

2.10. Dehydration by Centrifugation

Gels were excised from polypropylene tubes, and small cylindrical gels were kept in a Gelman z-spin centrifuge filter with pore size 0.45 μ. The gels were then centrifuged at room temperature for time intervals ranging from 5 min, 20 min, 35 min, 50 min, and 65 min at 2000 rpm using a microcentrifuge. After the centrifugation, gel samples were weighed instantly. The centrifugal dehydration of the gels was characterized as the ratio (%) of gel weight after centrifugation to the original gel weight (W/W0) [19].

2.11. Constituents of Gellan Polysaccharide

The exopolysaccharide gellan gum obtained from the recombinant and wild strain was analyzed for the presence of glucose, glucuronic

acid, rhamnose, acetate, and glycerate. Different concentrations of gellan gum ranging from (0.2–1%) were selected for the study. The hydrolysis of gellan gum using 2 M HCl for 2.5 h at 100°C was carried out to estimate the chemical constituents of gellan polysaccharide. The glucose content was determined using the GOD-POD enzymatic kit, and absorbance was measured at 500 nm. The levels of rhamnose were investigated using the orcinol sulfuric acid method described by Bruckner [20]. The absorbance was measured at 420 nm using a rhamnose solution as the standard. The content of glucuronic acid was determined by the method reported by Nelly and Gustav [21], and absorbance was measured at 520nm. The concentration of acetate was estimated using the method illustrated by John and Beatrice [22], and the optical density was measured at 625nm. The glycerate content was determined by the technique portrayed by Bartlett [23].

2.12. Processing of Gellan Gum into Various Structures

The gellan gum powder was blended with distilled water at room temperature. The mixture was stirred continuously to attain a final concentration of 0.8% (w/v). It was followed by heating the solution at 90°C for 20–30 min until homogeneous dispersion of the material was obtained. Subsequently, 0.04% (w/v) of CaCl₂ was added to the gellan gum solution, and the temperature was reduced to 50°C. For gellan disc production, the solution was cast into cylindrical molds at room temperature for 2–5 min. After gel formation, the discs were incised using a borer. Gellan gum membranes were acquired by casting the solution into Petri dishes at room temperature for 5–10 min. The Petri dishes were placed in the hot air oven for 120 min at 37°C to solidify the gel. Gellan gum powder was mixed with 0.10 M NaOH solution to obtain a final concentration of 4% (w/v), followed by stirring at room temperature to produce gellan gum fibers. Gellan solution was extruded into a 20% (v/v) L-ascorbic acid solution by employing a 21 G needle under a steady flow rate of 0.2 mL/min. Gellan gum fibers were then rinsed using distilled water, pressed into cylindrical molds, and dried at 37°C overnight. For gellan gum particle production, 4% (w/v) of the gellan gum solution was extruded into a 20% (v/v) L-ascorbic acid solution using a 21 G needle under a steady flow rate of 0.8 mL/min [24].

2.13. Compression Analysis

The 1% of gellan gum dispersed in deionized distilled water was kept in a boiling water bath for 15 min at 90°C. The gellan solution was stirred continuously, and 1% calcium chloride was added. The hot gellan solution was poured into a cylindrical mold, followed by cooling in running tap water for 15 min. The gel was stored at 22°C for 24 h to form a solid gel. The effect of storage time on textural properties was examined by storing the gel specimen for 1 day and 30 days after performing compression studies. The compression analysis of the gel was carried out using universal testing machine (UTM) based on the method described by Tang *et al.* [25]. The tests were carried out at room temperature in an autograph AG-X plus model UTM interfaced with TRAPEZIUM X software. The cylindrical gellan sample was placed between an aluminum probe and a flat surface and subjected to a constant load of 10 kN. The gel was compressed at a speed of 3mm/min. At the end of each experiment, force (N), stress (N/mm²), displacement (mm), strain (%), and plot of force versus displacement curve were automatically recorded by the TRAPEZIUM X software.

2.14. Characterization Studies

The gellan was characterized by their morphology by scanning electron microscopy (SEM). The SEM image of the sample was recorded

using a SEM (Hitachi SU 6600) operated at an accelerating voltage of 15 kV, magnification of 2 μm , and a working distance of about 15 mm. The Fourier transform infrared (FTIR) spectral measurement was performed to identify the possible functional groups in gellan gum. The FTIR analysis was carried out by FTIR spectrophotometer Agilent technologies model Cary 630 operated at a resolution of 8 cm^{-1} , sample scan of 32, and scanning range of 4000–400 cm^{-1} . The thermal characterization of gellan gum was carried out by thermogravimetric analysis (TGA) (Q50 V20.13 Build 39) at a uniform heating rate of 10°C/min for a temperature range of up to 600°C. The differential scanning calorimetry (DSC) thermogram of gellan gum has been recorded using DSC (Q20 V24.11 Build 124) at a temperature range of up to 250°C with a heating rate of 10°C/min.

3. RESULTS AND DISCUSSION

3.1. Nucleotide Sequence Accession Number

The nucleotide sequence for gene gel D from *S. paucimobilis* ATCC 31461 was deposited in GenBank under the Accession number MK430034.

The biomass and gellan gum production by both wild and recombinant strain containing gelD were carried out in the laboratory fermentor. The gellan gum production was higher in the case of recombinant strain compared to the wild type. The recombinant strain containing multiple copies of gelD showed the maximum biomass (5.55 g/l) and gellan gum (12.46 g/l) yield. The wild-type strain yielded 6.49 g/l of gellan and 2.83 g/l of biomass.

3.2. Fermentation Parameters

3.2.1. Influence of medium on gellan gum production

The fermentation was carried out using different media, namely, YPG, LB, and NB, to select the optimal culture medium to obtain enhanced gellan gum production from recombinant *S. paucimobilis* ATCC 31461. The cell growth and gellan gum yield were monitored after 48 h of incubation. The effect of different media on biomass and gellan gum production is depicted in Figure 1a. Among the various media tested, cell growth, as well as gellan gum production, was found to be highest in YPG medium. YPG medium supported the maximum gellan gum and biomass production when compared to LB and NB. The result indicates that YPG being a complex medium, is ideal for biomass production as well as biosynthesis of gellan gum, and therefore it was selected as the production medium for further optimization studies on gellan gum production.

3.2.2. Influence of temperature on gellan gum production

Each microorganism requires an optimum temperature for its growth and activity of the enzymes involved in the synthesis of any metabolite. The effect of temperature on gellan production and bacterial growth was checked with a temperature range of 25°C–50°C and is shown in Figure 1b. The highest productivity of gellan and biomass was obtained at 30°C. Similar results were obtained by Martin and Sa-Correia [26]. However, a further increase in incubation temperature resulted in a decline in cell growth and gellan production, which might be associated with the inhibition of the enzymes involved in the production of gellan gum.

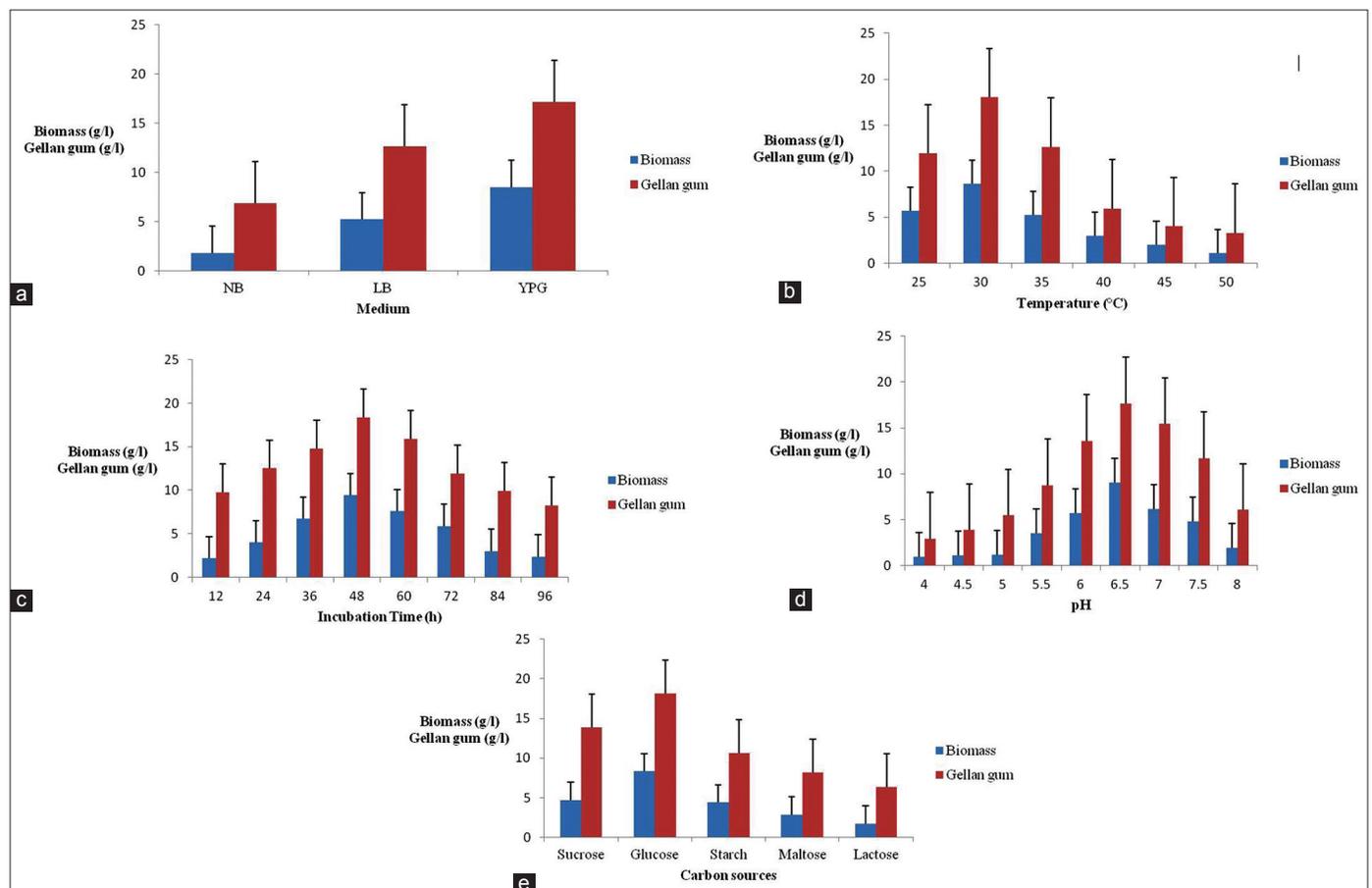


Figure 1: Effect of different parameters on biomass and gellan gum production (a) Medium (b) Temperature (c) Incubation Time (d) pH (e) Carbon sources.

3.2.3. Influence of incubation period on gellan gum production

The incubation time can be as short as a few hours or as long as days for various microorganisms. The effect of different incubation time on biomass and gellan gum production is shown in Figure 1c. In the present study, the maximum amount of gellan gum and biomass was achieved during 48 h of incubation by recombinant *Sphingomonas paucimobilis*. The minimum amount of gellan gum and biomass production was recorded during 96 h of the incubation period. The synthesis of gellan is partly growth-related, and it was found that the production of gellan gum was continued at a lesser rate during the stationary phase than in the course of the exponential phase. The reduction in gellan production with an increase in the incubation period might be due to the development of an extremely viscous slime layer of the polymer around the cells limiting the transport of oxygen and nutrient to the cells [27].

3.2.4. Influence of pH on gellan gum production

pH is one of the main factors, which have an impact on growth, cell membrane, and structure, thereby affecting nutrient uptake and gellan production. The influence of pH on biomass and gellan gum production is depicted in Figure 1d. The maximum yield of gellan and biomass was obtained when pH 6.5 was used. The decrease in gellan and biomass yield with increasing pH clearly shows that the pH of the media affects the microbial cells by interrupting the transport of nutrients and functioning of the enzymes. The results obtained by us are in accordance with other reports [28].

3.2.5. Influence of carbon sources on gellan gum production

Carbon sources are a prerequisite in culture medium for the biosynthesis of gellan as they provide the energy essential for cell growth. The effect of carbon sources on biomass and gellan gum production by recombinant *S. paucimobilis* was investigated in YPG

broth and is depicted in Figure 1e. Different carbon sources (starch, glucose, sucrose, maltose, and lactose) at a concentration of 20 g/l were used. After a fermentation period of 48 h, glucose was found to be the most suitable carbon source for biomass and gellan production. Similarly, maximum gellan yield from medium containing glucose has been reported by Wang *et al.* [29].

3.2.6. Influence of inoculum age on gellan gum production

The optimization of inoculum age is crucial as it may affect the length of the lag phase. The effect of inoculum age ranged between 6% and 27% on gellan production was studied and is depicted in Figure 2a. The maximum yield of gellan and biomass was achieved when 24 h old inoculum was used. A similar trend was observed by Lobas *et al.* [27] in *S. paucimobilis* for gellan production. The gellan yield was found to be lower when the inoculum age was increased beyond 24 h, which might be due to inefficient nutrient utilization.

3.2.7. Influence of inoculum size on gellan gum production

Optimization of inoculum size is vital in determining the gellan biosynthesis, cell morphology, growth pattern, and duration of the lag phase. The production media were inoculated with different inoculum levels (2.0–12.0%, v/v) to study the effect of inoculum size on gellan gum production and are shown in Figure 2b. The results indicated that gellan production increased with an increase in inoculum size from 2% (v/v) to 10% (v/v), and further raise in inoculum size did not surge the gellan production. The maximum gellan gum and biomass production was observed at 10% v/v of inoculum size. Similar results have been shown by other researchers [30]. A higher inoculum size resulted in the lower production of gellan due to increased cell density, which might have consumed the substrate for cell growth.

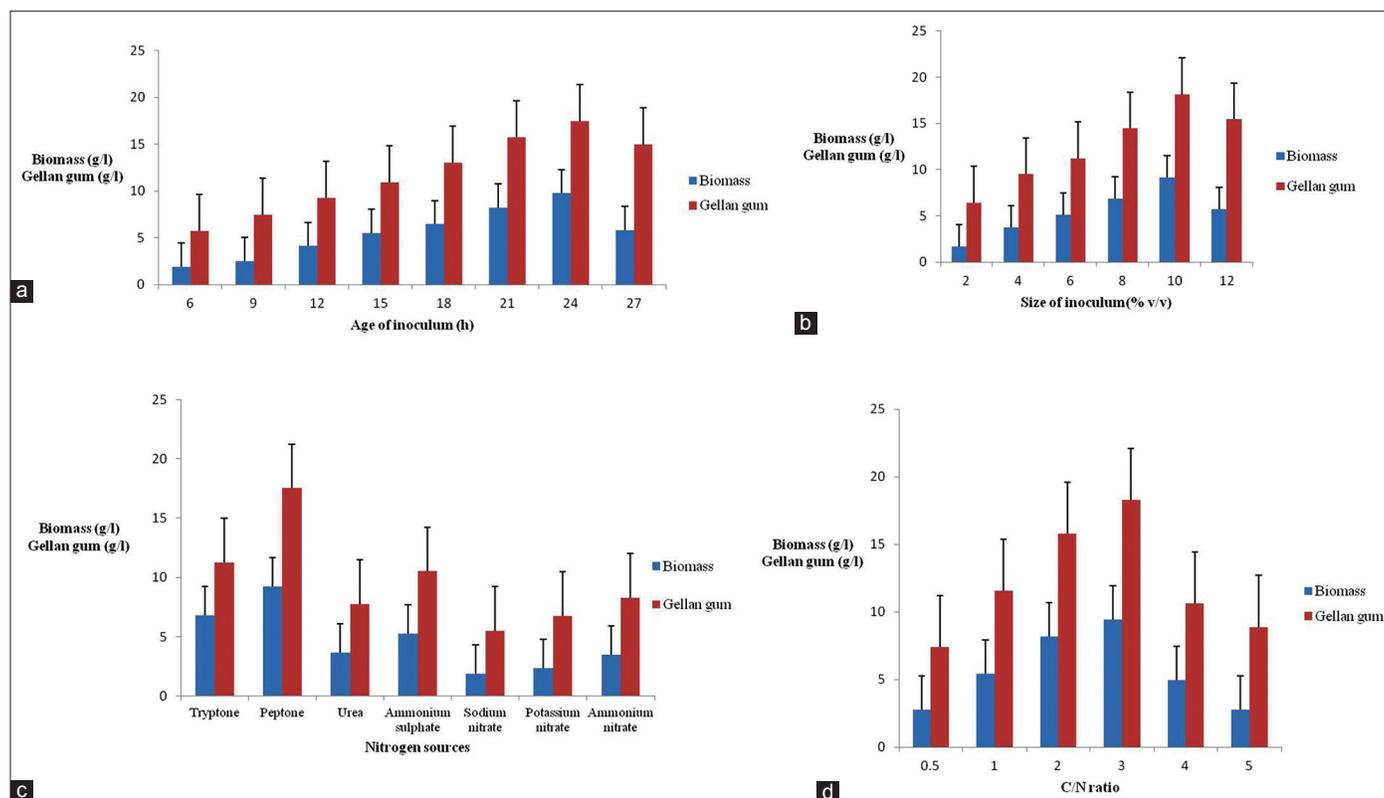


Figure 2: Effect of different parameters on biomass and gellan gum production (a) Age of inoculum (b) Size of inoculum (c) Nitrogen sources (d) C/N ratio.

3.2.8. Influence of nitrogen sources on gellan gum production

Biomass and gellan production by recombinant *S. paucimobilis* was also influenced by nitrogen sources. The effect of various organic and inorganic nitrogen sources on gellan production and cell growth was examined and is shown in Figure 2c. The result obtained from this study indicated that the best nitrogen source in terms of biomass and gellan yield was peptone. In contrast with organic nitrogen sources, inorganic nitrogen sources gave rise to comparably lessened biomass and gellan production, which is in accordance with the finding of other researchers [31]. It might be because organic nitrogen sources were quickly absorbed by the cells than the inorganic ones.

3.2.9. Influence of the C/N ratio on gellan gum production

Production of exopolysaccharides by bacteria depends on the availability of both the carbon and nitrogen sources in the culture medium. The effect of the C/N ratio on biomass and gellan production is depicted in Figure 2d. The higher C/N ratio resulted in increased production levels of gellan gum, and similar was the effect on biomass. The media containing 3 C/N ratios exhibited the highest gellan and biomass yield. Maximum secretion of exopolysaccharides was observed when mediums were provided with a minimum nitrogen source and abundant carbon source. The production of gellan, as well as biomass, decreased above 3 C/N ratio. Williams and Wimpenny [32] reported that surplus nitrogen in the medium lead to a decline in the conversion of carbon source to polysaccharide synthesis.

3.2.10. Influence of agitation speed on gellan gum production

Agitation speed has a significant effect on the production of biomass and gellan gum by maintaining the homogeneity and also enhancing the oxygen availability for the microbes in the fermentation broth. The impact of agitation speed on biomass and gellan production

is shown in Figure 3a and b. When the agitation speed was varied from 100 to 500 rpm, the gellan gum and biomass yield increased. However, when the agitation speed was increased beyond 500 rpm, the production of biomass and gellan declined. It may be due to an increase in shear inside the fermentation broth might have affected the homogeneity of the culture broth, which, in turn, leads to reduced mass transfer of nutrients and oxygen, cell damage, and thereby affecting polymer production. A similar trend was observed by Lee *et al.* [33] in *S. paucimobilis* NK-2000 for gellan production.

3.2.11. Influence of dissolved oxygen on gellan gum production

According to Huang *et al.* [34], dissolved oxygen plays a key role in many aspects of cellular metabolism in aerobic organisms like *S. paucimobilis*. The influence of dissolved oxygen on biomass and gellan production is depicted in Figure 3c and d. The highest biomass production was obtained when DO was set at 100% (40 min), whereas the maximum gellan production was achieved under 100% DO (60 min). Gellan productivity increases at a higher DO level because dissolved oxygen acts as a driving force to enhance the oxygen uptake in the cells. These results suggest that the maintenance of a high DO level is essential for gellan formation and is consistent with the findings of Giavasis *et al.* [35].

A maximum of 20.67 g/l gellan and 9.86 g/l biomass was produced under optimized conditions.

3.3. The Solubility of Gellan Gum

The solubility analysis of gellan gum in different organic and inorganic solvents unveiled its insolubility in nearly all of the organic solvents tested. The gellan gum was found to be completely soluble in an inorganic solvent such as water and thus indicating its polar nature and

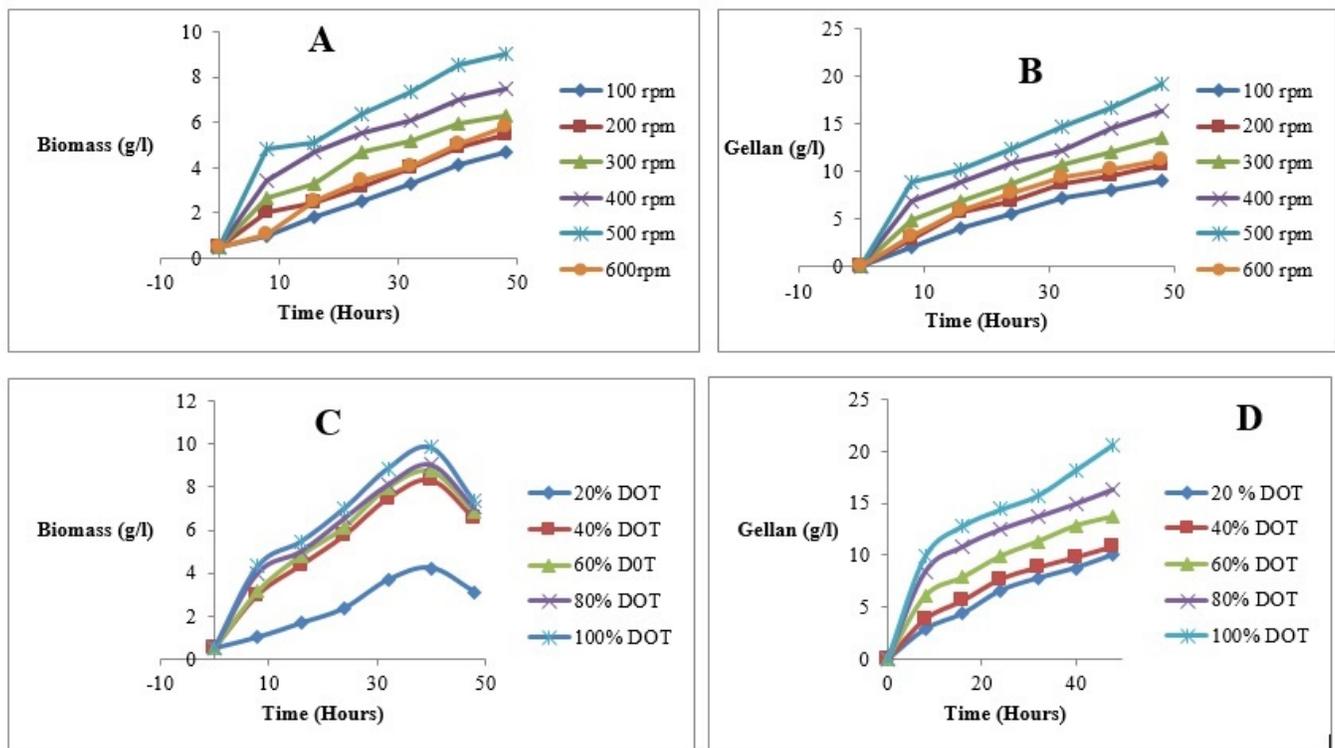


Figure 3: Effect of different parameters on biomass and gellan gum production (a) Agitation speed on biomass production (b) Agitation speed on gellan gum production (c) Dissolved oxygen on biomass production (d) Dissolved oxygen on gellan gum production

presence of hydroxyl groups in the structure of the polysaccharide. Several hydroxyl residues in the glucuronic acid monomer unit of gellan gum enhance its solubility [36].

3.4. Viscosity Measurement

The viscosity analysis of gellan gum is vital as it is useful to determine the potential applications of gellan as stabilizers, emulsifiers, thickening, and gelling agents. The viscosity of different concentration of gellan gum at 100 rpm is shown in Figure 4a. The influence of different shear rates on the viscosity of the gellan gum at various concentrations is depicted in Figure 4b. The viscosity of the gellan increased with concentration (0.5–3% w/v) at 100 rpm shear rate, indicating the non-Newtonian behavior. In the meantime, increasing shear rate from 20 to 100 rpm, the viscosity of the gellan decreased, therefore exhibiting its higher shear-thinning behavior [3].

3.5. Centrifugal Dehydration

The centrifugal dehydration is the ability of the natural gum to retain water. It is a significant criterion for gums to be used in various applications and also determines its storage stability [37]. The different reaction parameters, such as calcium concentration and reaction time, were optimized to get the sample with maximum water holding capacity. The water holding capacity of gellan gels is shown in Figure 4c. It was observed that the water holding capacity was found to increase with an increase in CaCl_2 concentration and reached maximum value at 104 mM of CaCl_2 . It could be due to gellan forms stronger gel in the presence of divalent cations and thus forming complex structural bonds with the water [38]. A large amount of water was lost from gellan gel with centrifugal time. A rapid decrease in water was observed after 10 min of centrifugation.

3.6. Gelation of Gellan Gum and Processing into Different Structures

Gellan gum is used mainly for their gelation property that is the ability to gelify *in situ* within seconds and retains a large quantity of water. The gellan structure is stabilized by either monovalent or divalent cations [39]. The different factors affecting gel formation by gellan gum include the concentration of the gelling agent, pH of the media, degree of polymerization, ionic composition, and temperature. Figure 4d shows the versatility of gellan gum to attain several geometrical forms that can be used in several areas of applications. In our study, by optimizing the concentration of gellan gum, calcium ions, and temperature led to the processing of gellan gum into various structures and thereby improving its functionality. The gellan gum has been employed as an encapsulating agent and is a vital carrier for growth factors and matrices for various tissue engineering and cellular therapies [40].

3.7. Chemical Composition Analysis

The chemical composition of recombinant gellan gum was determined by estimating their monomeric components. The glucose, rhamnose, glucuronic, acetate, and glycerate content of the recombinant gellan gum are depicted in Table 1. From the results, we can infer that the gellan gum was composed predominantly of glucose with minor quantities of other sugars. Gellan gum is comprised of glucose rhamnose and glucuronic acid in the molar ratio of 2:1:1. This result is in line with previous reports of gellan gum from *S. paucimobilis* [41].

3.8. Textural Properties

The UTM equipped with a 10 KN load cell was used to examine the textural properties of the gellan gum. Using the data retrieved from compression analysis, the load versus displacement graph was

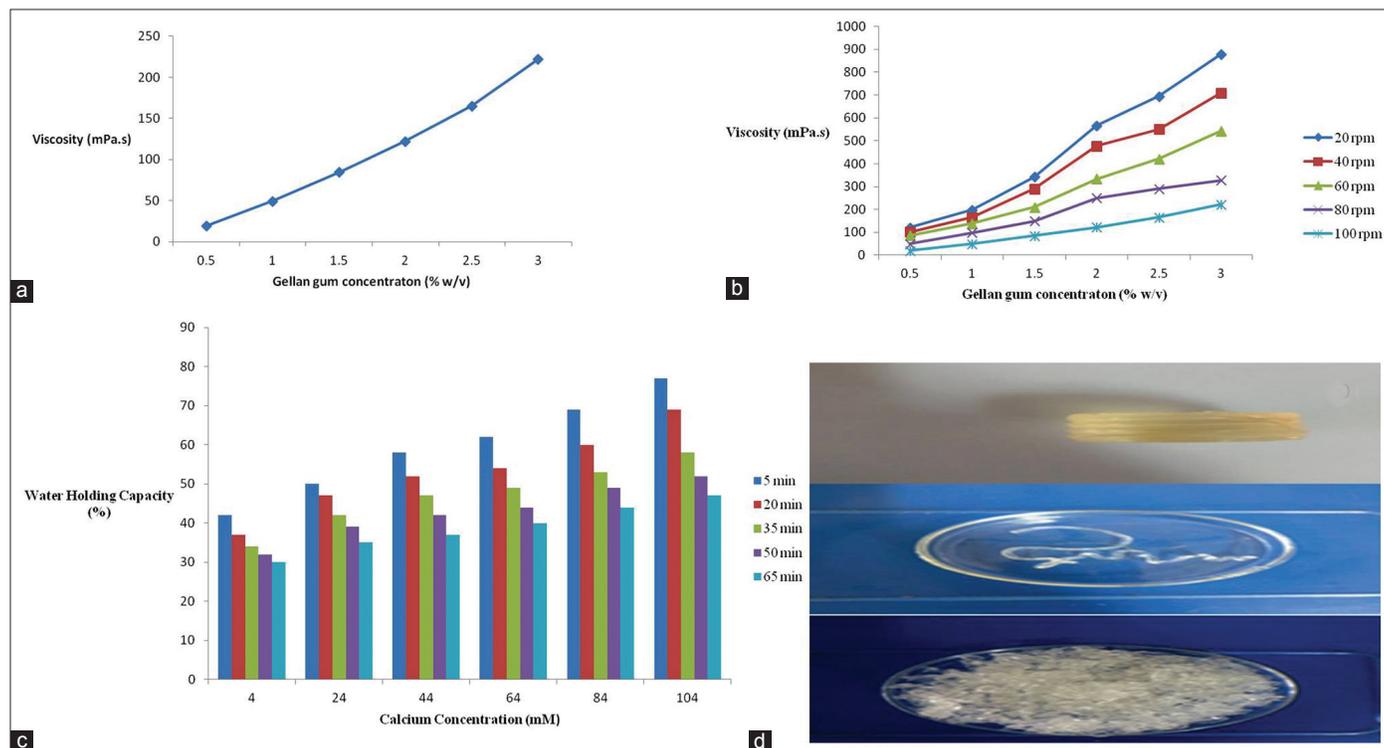


Figure 4: Viscosity Measurement and Water holding Capacity (a) Viscosity measurement of different concentration of gellan gum at 100rpm (b) The effect of different shear rates on the viscosity of the gellan gum at different concentrations (0.5%, 1%, 1.5%, 2%, 2.5% and 3.0% [w/v]) (c) Water holding capacity of gellan gels when subjected to centrifugal force (d) Processing of gellan gum into various structures.

plotted and is depicted in Figure 5a and 5b. After storing the 1% gel at an interval of 1 day, the force, displacement, stress, and the strain recorded during the compression test are 37.8005 N, 10.9315 mm, 0.00791 N/mm², and 33%, respectively. At 30 days of gellan storage, the force, displacement, stress, and the strain of gellan gel are 37.5001 N, 13.5375 mm, 0.00849 N/mm², and 41%, respectively. There was no significant difference observed in the appearance and textural properties of gellan gel after storage for 30 days. Higher gellan and calcium chloride concentration corresponds to increased compressive

strength. Tang *et al.* [42] reported that the concentration of calcium chloride above a critical value resulted in a decline in gel strength.

3.9. Surface Morphology Analysis

The SEM micrograph of the gellan gum is illustrated in Figure 6a. The surface morphology of the *gellan gum* displayed a smooth and porous surface.

3.10. FTIR Analysis of Gellan Gum

The FTIR spectra of gellan gum are shown in Figure 6b. The broad absorption band at 3309.9 cm⁻¹ was due to the OH-stretching. The peaks at 2885.0 cm⁻¹ were assigned to C-H stretching. The sharp band observed at 1599.0 cm⁻¹ and 1401.5 cm⁻¹ corresponds to asymmetric and symmetric -COO stretching. The peak at 1017.6 cm⁻¹ is due to C-O stretching for alky ether.

3.11. Thermogravimetric Studies

The thermal decomposition process of gellan gum is illustrated in Figure 6c. TGA showed weight loss of gellan gum occurred in two

Table 1: Glucose, rhamnose, glucuronic acid, acetate, and glycerate content of recombinant and wild type gellan gum.

Parameters	Recombinant type	Wild type
Glucose (M)	0.95	0.85
Rhamnose ^a	0.83	0.79
Glucuronic acid ^a	0.78	0.73
Acetate ^a	0.60	0.64
Glycerate ^a	0.81	0.76

^aUnits are in molar ratio w.r.t. glucose

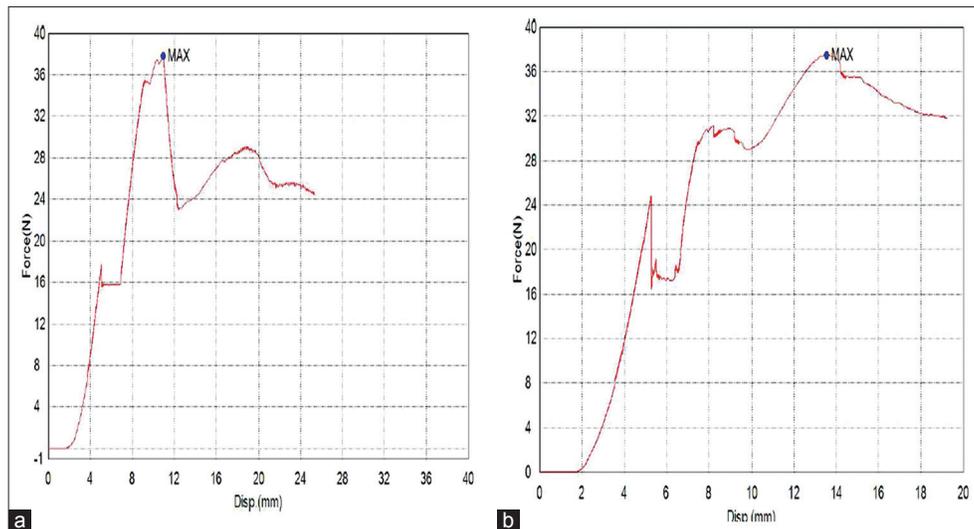


Figure 5: Compression graph (a) Force (N) Vs Displacement (mm) after 1 day incubation (b) Force (N) Vs Displacement (mm) after 30 days incubation

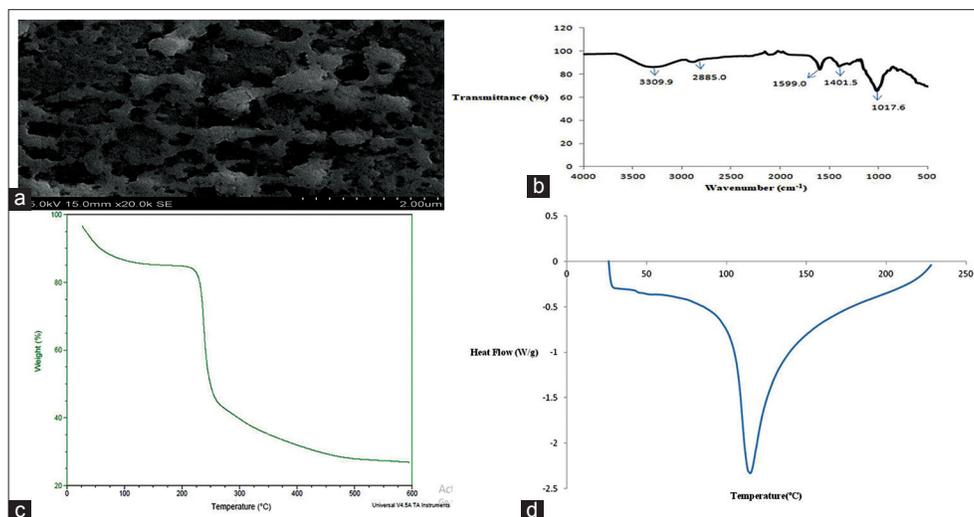


Figure 6: Characterization and thermal studies (a) Scanning electron micrograph of gellan gum (b) FT-IR spectra of gellan gum (c) TGA thermogram of gellan gum (d) DSC graph of gellan gum.

stages. The early weight loss in gellan occurred at 210°C which was due to the desorption of moisture as hydrogen bound water to the polysaccharide structure. The second stage (degradation process) occurred at 290°C, accompanied by a weight loss. The second weight loss is attributed to the decomposition of the polymer. The result suggests that gellan gum had good thermal stability [43].

3.12. DSC

The DSC Curves for gellan gum is depicted in Figure 6d. DSC thermogram of gellan showed that initial endotherm at 26°C is attributed to the loss of water, which corresponds to the hydrophilic nature of functional groups present in the gellan gum. The second endothermic transition was observed at 114°C [44].

4. CONCLUSION

The genetic engineering solves the significant aspect of increasing production yield and gellan modification. The gellan gum yield can be raised by modifying the expression of any single or group of genes. In this study, an exopolysaccharide gellan gum from recombinant *S. paucimobilis* was isolated. The effect of different physicochemical parameters on the production of gellan gum was investigated. The yield of gellan was higher at a temperature of 30°C, pH 6.5 in the presence of glucose and peptone as carbon and nitrogen source, respectively, and at a higher C/N ratio of 3. The compositional analysis revealed that the gellan gum is composed of three sugar residues, namely, glucose, rhamnose, and glucuronic acid in the molar ratio of 2:1:1. The gellan gum displayed high water holding capacity and compression property. Rheological analysis of gellan gum revealed non-Newtonian nature.

5. AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

6. FUNDING

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7. CONFLICTS OF INTEREST

The authors report no conflicts of interest in this work.

8. ETHICAL APPROVALS

This study does not involve the use of animals or human subjects.

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