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Effect of Environmental Factors and Carbohydrate on Gellan Gum Production

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Abstract

Submerged culture fermentation studies were carried out in batch mode for optimizing the environmental parameters and carbon source requirement by *Pseudomonas elodea* for the production of gellan gum. The maximum production of gellan gum was obtained with 16-h-old culture and 8% inoculum at 30°C and pH 7.0 after 52 h of incubation (6.0 g/L). Of the various carbon sources tested, 2% sucrose, glucose, and soluble starch yielded considerably high amounts of gellan. Studies on the concentration of various carbohydrates on gellan gum production indicated that the optimum concentration of glucose and starch was 3%, whereas for sucrose it was 4%. The addition of glucose in the medium above 3% had a detrimental effect on gellan yield. The investigation of intermediate two-step addition of glucose under identical conditions of fermentation showed an enhanced production of gellan (8.12 g/L) as compared with the control (6.0 g/L). To optimize the recovery of gellan from fermented broth, different solvents were tested for precipitation of gellan gum. Among the various solvents tested, tetrahydrofuran gave better recovery of gellan (82%) as compared with the conventional solvent isopropanol (49%).

Index Entries: Exopolysaccharide; gellan gum; *Pseudomonas elodea*; batch fermentation.

Introduction

The microbial exopolysaccharides (EPSs) are high-value polymers that have many industrial applications. These water-soluble microbial EPSs give aqueous solutions with rheologic properties that render them useful in stabilizing, thickening, and emulsifying applications in food and pharmaceutical industries *(1,2)*. Gellan gum is a relatively new complex microbial EPS composed of a repeating tetrasaccharide unit of D-glucose (D-glc), D-glucuronic acid (D-glcA), and L-rhamnose (L-rha) in the molar ratio of

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2:1:1 having the structure [→3)-β-D-Glcp (1→4)-β-D-GlcAp (1→4)-β-D- Glcp (1→4)-α-L-rhap (1→] and is substituted with L-glyceric and acetic esters *(3,4)*. It is a high molecular mass anionic heteropolysaccharide produced aerobically in high yields from the bacteria *Pseudomonas elodea*, renamed *Sphingomonas paucimobilis*, which, on deacylation, results in a change from soft elastic, thermoreversible gels to rigid, brittle gels. This polysaccharide is one of the series of eight structurally closely related bacterial polymers *(5,6)*.

In addition to its application as gelling, thickening, texturing, and stabilizing agents in various food, gellan has application as a potential agar substitute especially for thermophiles, in enzyme and cell immobilization, in gel electrophoresis, and in the controlled release of encapsulated drugs.

The characteristics and properties of gellan solutions and gels have been extensively studied, and many patents have been registered on food and biologic applications of gellan, but limited literature is available on the factors controlling the gellan yield. There are possibilities for enhancing the production of gellan gum by controlling and modifying the culture conditions such as pH, temperature *(7,8)*, growth medium composition *(9,10)*, and utilization of carbon sources *(11–13)*. The work reported herein was undertaken to understand the biochemical aspects of gellan synthesis. We have evaluated the effect of various carbohydrates and environmental factors on microbial production of gellan gum by *P. elodea*. We also studied the effect of intermediate feeding of glucose on gellan yield. The aim of this study was to optimize the cultural conditions and selection of suitable carbon source, and to establish intermediate glucose feeding strategies that enhance gellan gum production by *P. elodea*.

Materials and Methods

Organism and Growth and Maintenance of Culture

The working strain *P. elodea* was a gift from I. S. Correia, Lisbon, Portugal. It is an aerobic, Gram-negative, straight/rod-shaped bacteria. It was routinely cultured in a chemically defined YPG medium containing 0.3% yeast extract, 0.5% peptone, 0.3% NaCl, and 2% glucose, 2% agar, pH 7.0. The slants were incubated for 48 h at 30°C and stored at 4°C. The composition of the production medium was the same with an additional 1% mineral salt solution. The composition of the mineral salt solution was as follows: 1.8 g/L of $MnCl_2 \cdot 4H_2O$, 2.4 g/L of $FeSO_4 \cdot 7H_2O$, 0.283 g/L of H_3Bo_3, 0.0027 g/L of $CuCl_2$, 0.0021 g/L of $ZnCl_2$, 0.0074 g/L of $CoCl_2 \cdot 6H_2O$. The carbon source used was glucose (2%) unless otherwise mentioned, and the initial pH of the medium was maintained at 7.0 by the addition of 2 *N* NaOH.

Preparation of Inoculum

Inoculum was prepared in 250-mL Erlenmeyer flasks containing 50 mL of the medium. A 24-h-old liquid culture grown at 30 ± 1°C and 10% inoculum was used for production. The submerged culture method

for production of gellan gum was carried out for 72 h at 30 ± 1°C, pH 7.0 at 200 rpm in a rotary shaker.

Recovery of EPS Gellan

Samples withdrawn after appropriate incubation were analyzed for their biomass and polysaccharide concentration. The fermented broth obtained was heated in a boiling water bath for 15 min, cooled, and its pH increased to 10.0 (2 *M* NaOH). The broth was then kept in a constant-temperature water bath (80°C) for 10 min after which the pH was brought to 7.0 (2 *M* H_2SO_4). This pretreatment killed the cells, deactivated the enzymes, and enhanced the gelling properties of the polysaccharide by deacylating the polymer *(14)*. This pretreated broth was diluted with distilled water and centrifuged at 12,000*g* for 45 min to separate the cells. The cells were washed three times to remove any adhering polymer and again centrifuged. Then the cells were dried at 80°C for constant weight and weighed to estimate cell dry wt. Gellan was recovered from the supernatant by precipitation with twice the volume of isopropanol and dried at a temperature not exceeding 40°C. This partially purified sample was dissolved in 4 mL of distilled water and used for quantitative estimation of the polysaccharide. The total dry weight was determined by precipitating the whole broth with twice the volume of isopropanol and centrifuged at 12,000*g* for 45 min. The recovery of the polysaccharide (g/L) was determined by subtracting cell dry wt from total dry wt *(15)*.

Quantitative Estimation of EPS

Gravimetric Method

The addition of twice the volume of chilled isopropanol in cold cell-free supernatant quantitatively precipitated gellan. It was recovered by centrifugation and estimated as described earlier.

Calorimetric Method

Total carbohydrate concentration in the partially purified samples was determined by the phenol sulfuric acid method *(16)*. Reducing sugars was determined by dinitrosalicylic acid method *(17)* after hydrolysis of gellan gum (2 *M* HCl for 1.5 h at 100°C), glucuronic acid by the carbazole method *(18)*, and rhamnose by the Dische method *(19)*. The acetate content of the unhydrolyzed gellan gum was estimated by the hydroxamic method *(20)*. Commercial grade gellan gum (Gelrite®; Sigma) was used to prepare the standard. Total protein in the washed cell suspensions was determined by the Lowry method *(21)*.

Viscometric Method

The viscosity of the gellan gum produced was measured using a Brookfield cone/plate viscometer model RTDV-1. The temperature was maintained at 30°C, and cone size CP-41 at a shear rate of 10 s^{-1} was used. All results are depicted in centipoise.

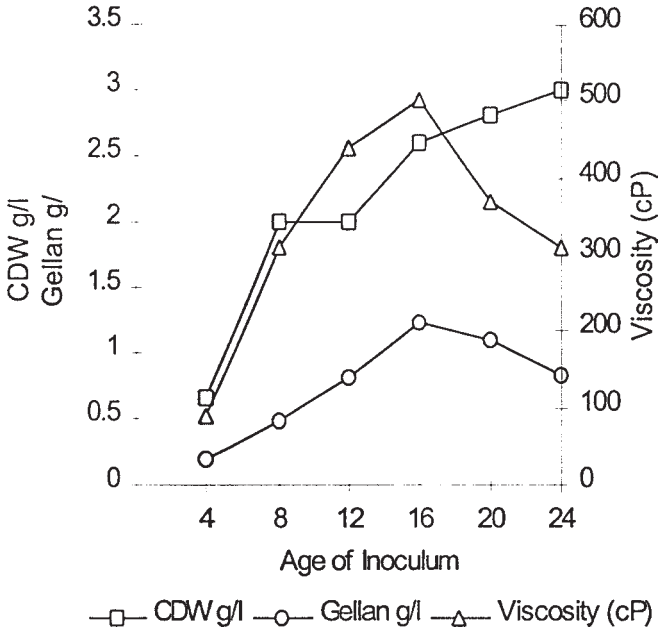

Fig. 1. Effect of age of inoculum on growth and gellan gum production. CDW, cell dry wt.

Results and Discussion

Effect of Age and Volume of Inoculum

The size and the physiologic condition of the inoculum have a profound effect on gellan synthesis by *P. elodea*. There exists variation in the use of inoculum age and inoculum volume for gellan production, as revealed in the literature. Some investigators have reported the use of 24-h-old culture with a 10% inoculum volume for optimum gellan production *(13,22)*. However, there is also a report of using 18-h-old culture with a 1% inoculum volume *(23)*. Therefore, a systematic investigation was conducted to determine the optimum age and volume percentage of inoculum required for gellan synthesis. Experiments were conducted with cultures of different ages ranging from 4 h to 24 h old and inoculum volume ranging from 2 to 14% of the medium's volume. The effect of these parameters on growth, gellan production, and viscosity are shown in Figs. 1 and 2. The maximum production of gellan was obtained with a 16-h-old culture. A reduction in gellan yield was observed with a further increase in the age of the inoculum. At lower inoculum volume, a reduced growth leading to lower production of gellan was observed. The higher yield of gellan was obtained with 8% inoculum (Fig. 2), but on further increase in inoculum volume, a reduction in the production of gellan was observed. The studies indicated that an enhanced gellan production was achieved with a 16-h-old culture with an 8% inoculum volume.

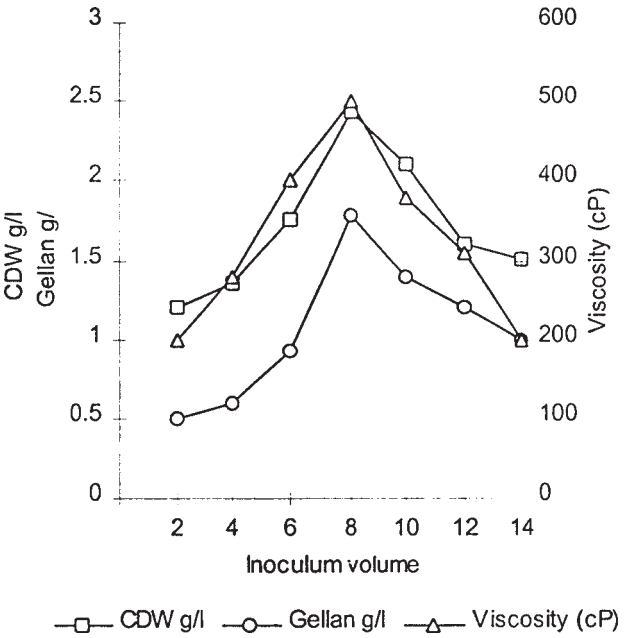

Fig. 2. Effect of inoculum volume on growth and gellan gum production. CDW, cell dry wt.

Effect of pH, Temperature, and Incubation Time

pH and temperature greatly affect the growth of the microorganism and the activity of the enzymes involved in the production of any metabolite. Different incubation temperatures ranging from 28 to 37°C were studied to determine the most suitable temperature for gellan production. At 30°C a maximum yield of gellan was obtained (2.7 g/L). A further increase in incubation temperature caused a decrease in gellan production whereas optimum pH was 6.5, giving enhanced yield (4.2 g/L) of gellan (Figs. 3 and 4). The time course of fermentation in terms of cell growth, viscosity, and gellan synthesis by *P. elodea* in batch mode at pH 6.5, 30°C, and 200 rpm was studied. The results indicate that lower production of gellan was observed up to 28 h of fermentation (Fig. 5). After 30 h there was a steady increase in the production of gellan, becoming maximum at 52 h of fermentation, and 90% of glucose was consumed. Gellan synthesis was found to be partially growth related. However, gellan production was also continued at stationary phase, but at a slower rate than that obtained during exponential growth phase. We now know that all the factors that limit the bacterial growth will also limit the production rate of gellan. A further increase in incubation time results in a decrease in the production of gellan, which can be attributed to the formation of a highly viscous slime layer of the polymer around the cells preventing nutrient and oxygen transport to the microbial cells, leading to reduced production of gellan gum.

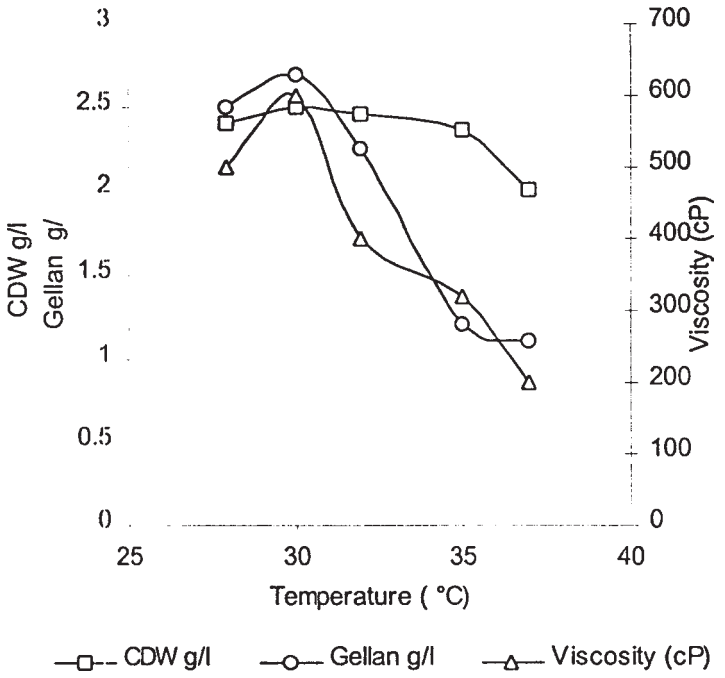

Fig. 3. Effect of temperature on growth and gellan gum production. CDW, cell dry wt.

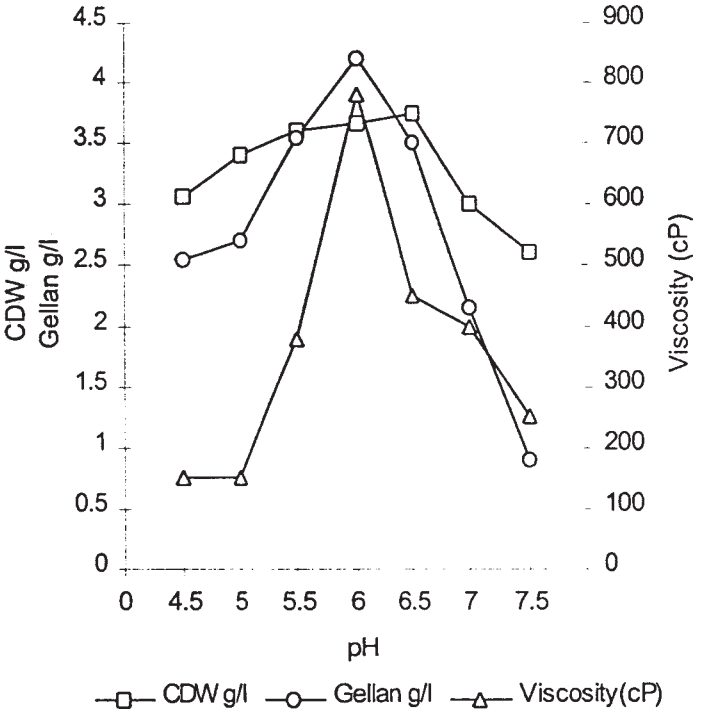

Fig. 4. Effect of pH on growth and gellan gum production. CDW, cell dry wt.

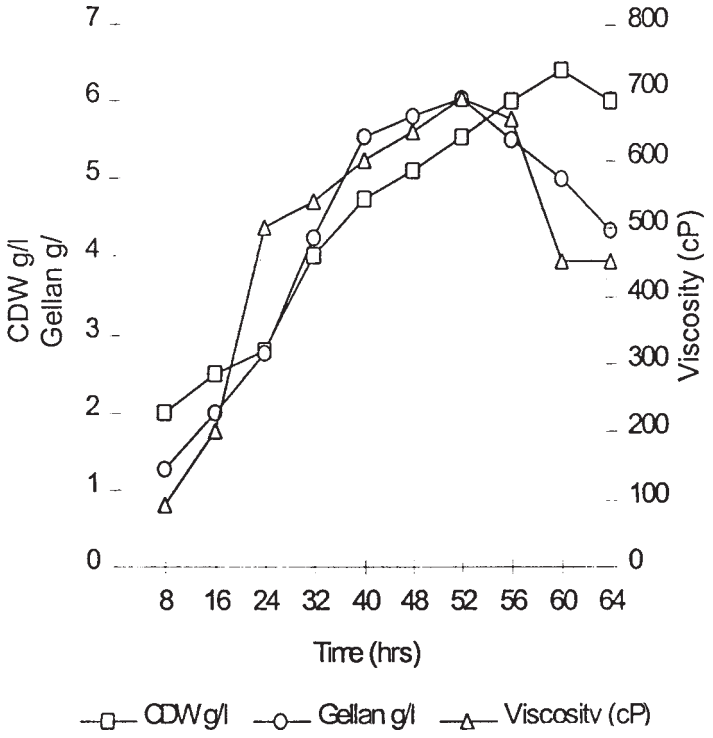

Fig. 5. Time course of fermentation for gellan production. CDW, cell dry wt.

Gellan Production with Different Carbon Sources

There are reports on the effect of various carbon sources on the synthesis of EPSs from *xanthomonas* species *(24,25)*, but no such information is available for gellan synthesis. Therefore, different carbon sources were tested for gellan synthesis—glucose, sucrose, soluble starch, maltose, lactose, xylose, mannitol, and fructose—added individually at a concentration of 2% (Fig. 6). Of the various carbon sources tested, 2% sucrose (6.4 g/L), glucose (6.06 g/L), and soluble starch (5.732 g/L), sucrose supported the maximum growth and synthesis of gellan. In the case of soluble starch, growth of the organism was greater with a reduced yield of gellan. Utilization of lactose by *P. elodea* was poor and production of polymer was also less; the possible reason for this is that the strain was not adapted for lactose utilization. There was no measurable growth of the organism and the yield of the gellan when xylose was used as the only carbon source. The effect of different concentrations of three carbon sources (glucose, sucrose, and soluble starch) on gellan synthesis was tested. It was found that sucrose supported maximum production of gellan at a 4% concentration whereas glucose and soluble starch gave higher yields at 3% (Fig. 7). Although the addition of excess sucrose had no detrimental effect on gellan production, the addition of excess glucose was inhibitory for both growth and gellan gum production.

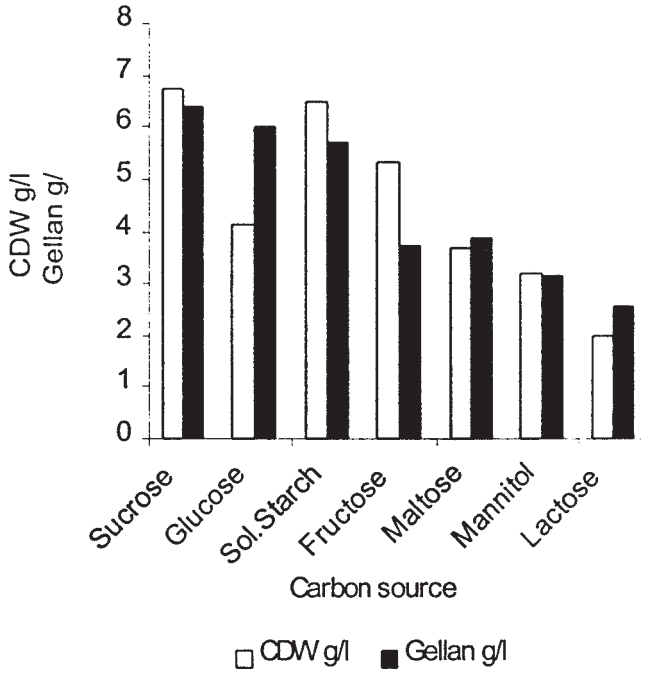

Fig. 6. Effect of different carbon sources on growth and gellan gum production. CDW, cell dry wt.

Intermediate Glucose Feeding

The experiments with intermediate glucose feeding were carried out along with two controls, C1 with 2% glucose and C2 with 4% glucose, and F1 with initial 2% glucose with intermediate feeding of 1% shortly after the exponential growth phase. At the 4% initial glucose concentration, a lower amount of gellan was produced owing to catabolic repression. A similar result was reported for xanthan gum synthesis *(25)*. An enhanced yield of gellan gum was obtained with the two-step addition of glucose at a 1% concentration shortly after the exponential growth phase and after 48 h when the glucose concentration was below 2% *(26)*. An increase in production (8.12 g/L) was observed at 72 h as compared with that of control C1 at 52 h (6.1 g/L). The results obtained are in good agreement with xanthan gum production reported by Amanullah et al. *(27)*. From the data given (Table 1) it is clear that intermediate feeding of glucose overcame the catabolic repression and supported the higher gellan production.

Isolation of Gellan Gum from Fermented Broth

The economics of gellan production lies in its efficient recovery from fermented broth. This operation is commonly performed by precipitating the EPS with low molecular weight solvents *(28,29)*. There are reports of studies on optimization of solvent for recovery of xanthan gum from fer-

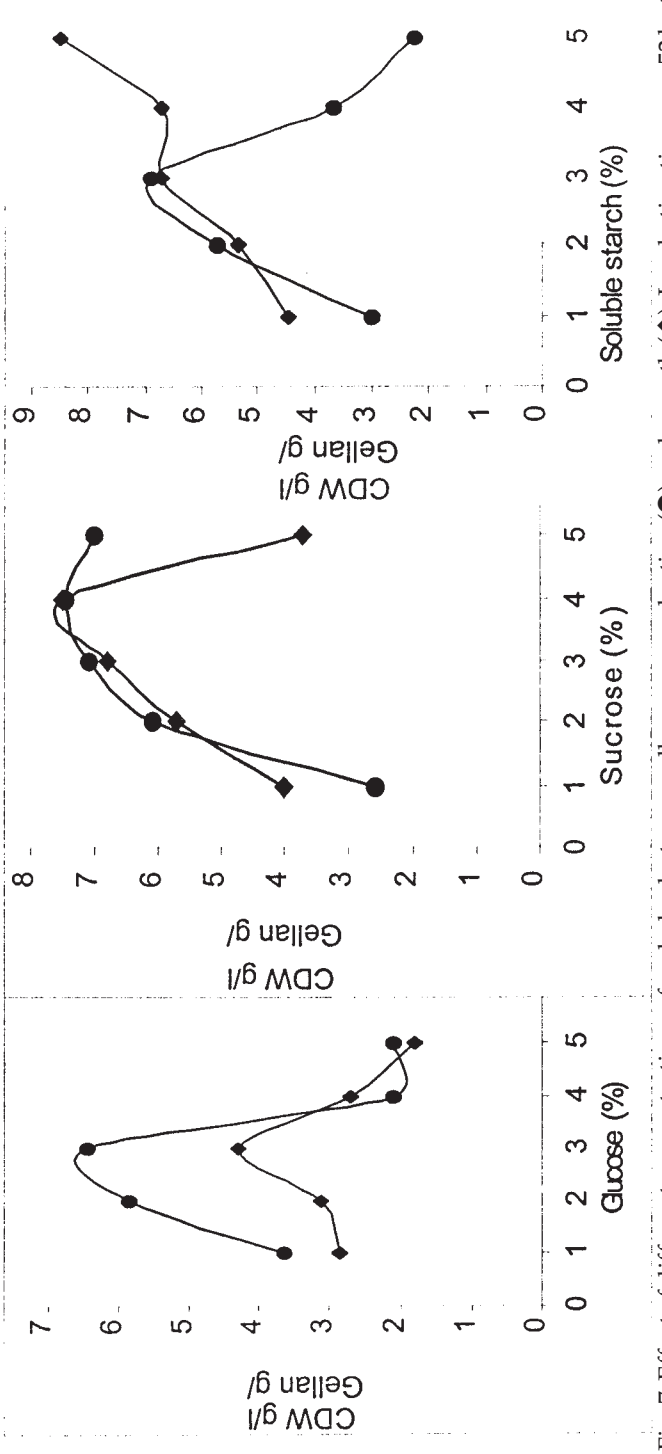

Fig. 7. Effect of different concentrations of carbohydrates on gellan gum production (●) and growth (◆). Incubation time was 52 h at 30°C. CDW, cell dry wt.

Table 1
Summary of Results with Glucose Feeding Strategy

Fermentation	Maximum gellan (g/L)	Glucose consumed (g/L)	Gellan yield (Y/g)	Fermentation time (h)	Gellan productivity (g/[L·h])
C1	6.1	18	0.28	52	0.11
C2	2.5	18	0.14	48	0.056
F1	8.12	18	0.40	72	0.11

Table 2
Percentage Recovery of Gellan from Fermented Broth with Increasing Concentration of Different Solvents

	Recovery of gellan with increasing concentration of solvents (%) (broth:solvent)			
Name of solvent	1:1	1:2	1:3	1:4
Isopropanol	33	39	49	25
Ethanol	48	61	45	40
Acetone	29	18	17	6
Tetrahydrofuran	43	82	79	17
Acetonitrile	31	38	71	15
Methanol	—	—	40	32

mented broth *(30,31)*. However, there is limited information in the literature regarding optimization of solvent for precipitation of gellan gum from fermented broth. Therefore, different solvents at various proportions were tested for maximum recovery of gellan from the fermented broth. The different low molecular weight solvents taken in increasing proportion for precipitation of gellan were as follows: acetone, acetonitrile, ethanol, isopropanol, methanol, and tetrahydrofuran. Among these tetrahydrofuran gave maximum recovery (82%) followed by ethanol (61%) in the ratio of 1:2 (broth:solvent). Both the solvents turned out to be better than isopropanol (39%) (Table 2).

Conclusion

The presence of glucose, glucuronic acid, and rhamnose in the acid-hydrolyzed solutions of EPS confirms the production of gellan gum by *P. elodea*. The optimization of various environmental factors leads to a 60% increase in the production of gellan. Here it is also concluded that utilization of glucose in batch fermentation cannot be improved by using an initial concentration of 4% owing to growth inhibition and catabolic repression. However, two-step feeding strategies with a lower initial glucose concentration of 2% offer an advantage when compared with batch

fermentation with no feeding. Further detailed investigations in this regard are in progress. Among the different carbon sources used, sucrose, glucose, and soluble starch gave considerably high yields. The 4% concentration of sucrose and 3% concentration of glucose and soluble starch supported higher yields of gellan gum. Tetrahydrofuran and ethanol gave comparatively higher recovery of the gellan gum from fermented broth than isopropanol. The results obtained showed that production of gellan gum could be greatly enhanced by operating the fermentation at optimized cultural conditions and intermediate glucose feeding strategies.

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