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Study on the Effect of pH, Temperature and Aeration on the Cellular Growth and Xanthan Production by *Xanthomonas campestris* Using Waste Residual Molasses

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Abstract

Waste residual molasses, a non-edible portion produced during the processing of sugarcane juice for the preparation of molasses, may be an alternative low-cost renewable substrate to the pricey food-grade molasses for xanthan production. Systematic strategies were applied to improve xanthan production with a newly isolated indigenous strain *Xanthomonas campestris* originated from Tezpur, Assam. Analyses with TLC, HPLC and FTIR show that the polymer consisted mainly of glucose, galactose and glucornic acid but showed no evidence of xylose, arabinose or glycoprotein in the polysaccharide. The isolated xanthan exhibited all the required physico-chemical characteristics and were examined by using TGA, DSC, XRD and SEM. Maximum concentration of xanthan was observed after 24h of incubation of the culture media, pH 7 at 28°C with 200 rpm. The viscosity of xanthan was found to be stable over a wide range of pH, reduced with the increase in temperature and raised at the higher xanthan concentration. The results obtained in the present investigation are noteworthy for the possible xanthan production from low-cost waste residual molasses at an industrial level.

Keywords: Xanthan; pH; Temperature; Rheological properties; Stable

Introduction

Xanthan is a type of microbial polysaccharide, produced by specific bacterial sp. such as *Xanthomonas campestris*. These are generally regarded as safe (GRS) and approved for its use in food by the Food and Drug Administration (FDA) [1]. The molecular weight of xanthan is approximately around 2×10^6, but it can reach up to 13×10^6 to 50×10^6 [2]. The polymer is acidic in nature and made up of pentasaccharide subunits, forming a cellulose backbone with trisaccharide side-chains composed of mannose (β 1,4), glucuronic acid (β 1,2) and mannose attached to alternate glucose residues in the backbone by α-1,3 linkages [3]. Xanthan exhibits high pseudo-plastic character in aqueous solutions with high viscosity at low shear forces, resistant to enzymatic degradation and extremely stable over a wide range of pH (2-11), temperature (up to 9°C) and salinity (up to 15% g/l NaCl) [4]. Due to such characteristic behaviors xanthan has numerous applications such as stabilizing, viscosifying, emulsifying, thickening, and suspending agents in various fields [3-5].

The cost involvement with the fermentation medium represents a critical aspect for the commercial production of xanthan. Searching for cheaper carbon sources, in place of glucose or sucrose, might lower the cost for the xanthan production. In such circumstances, non-edible crude molasses, a waste by-product of sugarcane based industries is an interesting substrate for xanthan gum production, since it is renewable source and the by-product is produced in a large quantity during the processing of sugarcane juice. The present work aims at providing relevant scientific information about the direct use of such residual waste molasses, an abundant agro-industrial residue of sugarcane based industries, to produce xanthan. The objective of the present investigation was to produce cost effective xanthan using waste residual molasses as sole feedstock carbon source. Physico-chemical characterization of xanthan gum was studied and the effect of supplementation of nutrients, pH, temperature and agitation was evaluated to optimize the production of xanthan.

Material and Methods

Microorganisms and culture conditions

The waste residual molasses were collected from the sugarcane processing unit of Tezpur, Assam, India. The total soluble solids of sugarcane molasses were adjusted by adding water at a ratio of 3:1 (v/w). The diluted molasses was preheated at 90°C in a water bath for 15 min with continuous stirring and centrifuged at 10000 rpm for 25 min. An indigenous strain of *Xanthomonas campestris* was used throughout the study. The inoculum was prepared by transferring a single colony from the slant culture to the 100 ml of Erlenmeyer conical flasks containing 25 ml of the sterile yeast mould broth (YMB) and subsequently incubated in an orbital incubator shaker at 28 ± 1°C for 48 h at 180 rpm. Fermentation was carried out in 250 ml Erlenmeyer flask with a working volume of 100 ml consisted of waste residual molasses 175g/l; yeast extract, 5.0 gm; peptone, 10.0 gm; NaCl, 10.0 gm; and K_2HPO_4, 4.0 gm. The pH of the culture medium was adjusted to 7.0 and sterilization was done by autoclaving at 121°C for 15 min and incubated for 48 h at 28°C at 180 rpm. To determine the effect of each of the component of xanthan production, different sources of carbon, nitrogen and salts were added to the basic medium. Important parameters (pH, agitation and temperature) were also studied separately. All experiments were repeated thrice.

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Xanthan recovery

The fermented culture broth was centrifuged at 12,000 rpm for 30 min at 4°C. The culture supernatant was added with three volume of ice cold ethanol (95%, v/v) and kept overnight at 4°C to precipitate. The mixture was then centrifuged at 10,000 rpm for 30 min at 4°C to recover the precipitate. The precipitate was then washed with 95% (v/v) ethyl alcohol and dried in a hot air oven for overnight period at 45°C. The production of the biopolymers by the bacterial strain was determined gravimetrically and the average was expressed in g/l.

Analytical methods

The qualitative analysis of the isolated xanthan was done by thin layer chromatography (TLC) [6]. TLC was performed for the monosaccharide components using a mobile system consist of butanol-ethanol-water (5:5:4, v/v/v). The resultant spots on the TLC plates were visualized by spraying with a solution of 5% (v/v) sulphuric acid in ethanol followed by heating at 120°C. The hydrolyzed xanthan was dissolved in acetonitrile: water solution (75:25, v/v), filtered and analyzed by HPLC (waters 2489 UV/Visible detector). The separation was performed in isocratic mode (acetonitrile: water; 75:25, v/v), with a photodiode array detector at 254 nm using a flow rate of 1.0 ml/min on an analytical column (C18, 250×4.6 mm). The element content of the isolated xanthan was determined by an elemental analyzer (Perkin Elmer; Model PR 2400 Series II). IR spectrum of the isolated xanthan gum was recorded on a Perkin Elmer- Spectrum100 using KBr. The isolated xanthan was further characterized by ^{1}H NMR and ^{13}C spectroscopy on a Jeol ECS-400 NMR model [7].

Physical characterization

Thermo-stability of the isolated xanthan was determined using a thermo gravimetric analyzer Shimadzu TG50, Japan. The dynamic differential scanning calorimetry (DSC) experiments were conducted in N_2 atmosphere, using a Shimadzu differential scanning calorimeter (model DSC-60). X-ray diffraction study was carried out on Model-Miniflex, Rigaku at room temperature over the range of 2Θ = 2-60°. A thin xanthan film was prepared and mounted on to a stub using double sided carbon tape, coated with a thin layer of platinum using ion sputter JFC 1100 and examined under JSM 6390LV, JEOL scanning electron microscope at a magnification of 2000-10, 000 X.

Rheological characterization

The rheological behavior was determined using a Brookfield LVT Viscometer spindle no. 1 at varying shear rate range of 3-60 rpm. All rheological measurements have been carried out at 25°C. The viscosity of the aqueous solutions of xanthan samples were studied in the temperature range of 25-80°C, and at different pH values. The rpm values were converted to shear rate (sec^{-1}) using a factor (0.34) provided by the manufacturer connected to LVT Model.

Results and Discussion

Chemical characterization

The carbohydrate composition of the xanthan extracted from waste residual molasses supplemented medium composed of four neutral sugars that were separated at different relative mobility (Rf) (Figures 1a and b). The isolated xanthan contains glucose, galactose, and glucuronic acid. Since, the relative mobility of glucose and arabinose were very close as shown by TLC; further verification was done by HPLC. HPLC analysis confirmed the presence of glucose, galactose, arabinose,

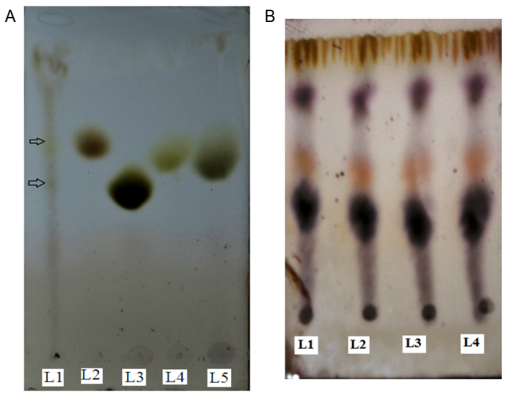

Figure 1: a) TLC plate with lane 1- acid hydrolyzed xanthan, Lane 2 galactose, Lane 3 glucose, Lane 4 lactose and Lane 5 ribose. b) TLC plate with lanes 1-4 represent replicas of isolated xanthan.

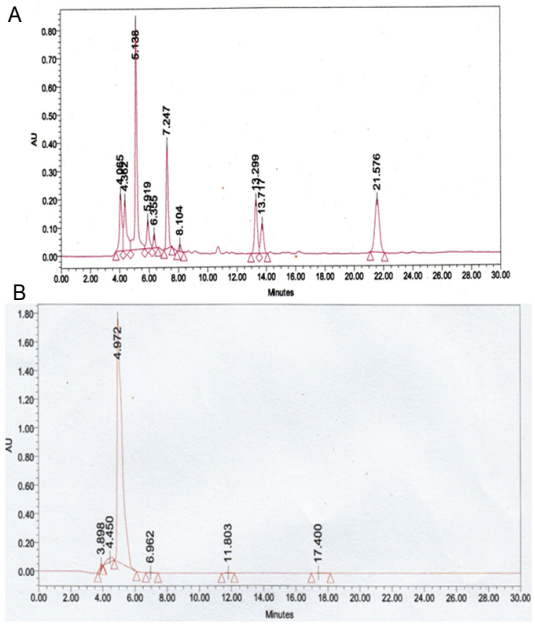

Figure 2: High performance liquid chromatographic (HPLC) spectra of (a) isolated xanthan (b) commercially purchased standard xanthan after hydrolysis with 2M trifluoroacetic acid.

glucuronic acid, and absence of xylose and rhamnose sugar (Figures 2a and 2b). Further, HPLC analysis indicated the presence of around 98.4% of glucose in the acid hydrolyzed. Lowson and Symes, Souza and Venduscolo and Silva et al. [8-10] reported the presence of rhamnose in the xanthan. Silva et al. [10] reported another type of xanthan that devoid of rhamnose. Heyraud et al. [11] reported a type of xanthan that devoid of glucornic acid. Hence, such variation in the chemical composition of xanthan depends directly on the bacteria. The results of FTIR (Figures 3a and 3b) and NMR (Figures 4a and 4b) analysis are presented in Table 1.

Physical characterization

The thermal properties of the isolated xanthan were studied at room temperature (28 ± 2°C) and are shown in Figure 5a. The first reduction in the weight occurred over the temperature range of 30-91.0°C, which

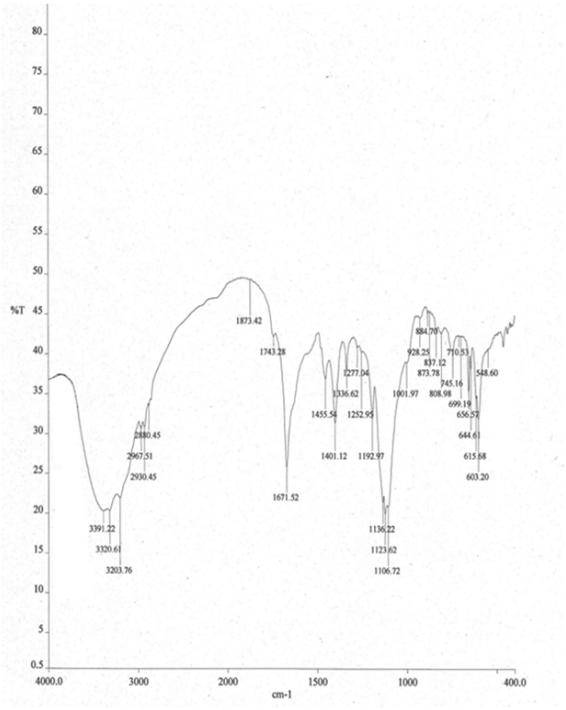

Figure 3: Fourier transforms infrared (FT-IR) spectra of (a) isolated xanthan (b) commercially purchased standard xanthan.

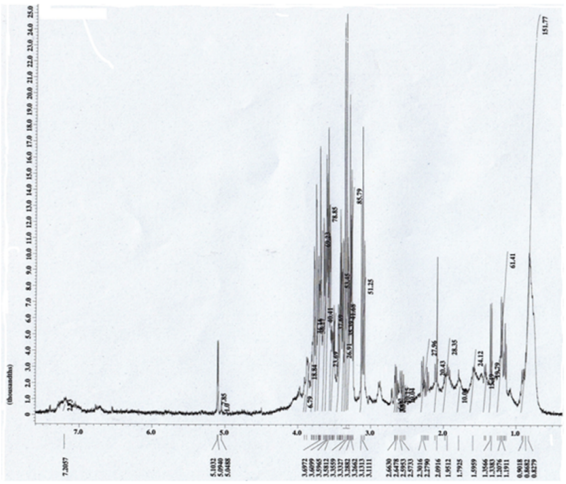

Figure 4: a) ^{1}H and b) ^{13}C NMR spectra of isolated xanthan.

might be due to the loss of residual water present in the sample. The second reduction between the temperature ranges of 215.1 and 303.2°C described the dehydration and decarboxylation of the xanthan, leads to the formation of inter and intra molecular anhydride. About 62.0% of weight loss occurred within this section of thermogram. The third decomposition stage was in the temperature range of 365.2 - 470.1°C and due to the degradation of the residual polymer. The result of differential scanning calorimetric analysis is shown in Figure 5b, the glass transition temperature (Tg) appeared at 29°C. Temperature at 130°C was determined as crystalline melting point where the compound was converted from its crystalline state to rubbery state. The melting point of the polymer was 178°C (Tm). Xanthan was found to be almost similar like that of a typical semi-crystalline amorphous material (Figure 6) and confirms the findings of DSC study. The

Spectroscopic and analytical analyses	Observation	Interference/Remark
FTIR	Band at 3391.22 cm^{-1}	-OH stretching of the hydroxyl group
	peaks between 2880.45 and 2967.51 cm^{-1}	-CH stretching of methyl and methylene groups
	1743.28cm^{-1}	-C=O stretching of the acetate group
	peaks at 615.68 cm-1 and 1455.54 cm^{-1}	-COO groups
	strong signals at 1401.12 cm-1 and 1252.95 cm^{-1}	carboxylate asymmetric stretching and -C=O acetate deformation
	absorption peaks between 928.25-745.16 cm^{-1}	β- glycoside linkages
	absorption peaks at 1671.52 cm^{-1}	pyruvate group
	absorption peaks at 1743.28 cm^{-1}	acetyl group
^{1}H NMR	1.44	pyruvate
	2.12 ppm	acetate groups
^{13}C NMR	107 to 95 ppm	quaternary C_2 of pyruvate
		C_1 carbon of glucose, galactose and glucuronic acid
	175.04 ppm	carbonyl carbons of proteins, fatty acids and exopolysaccharide
	107.8 and 75.9 ppm	carbons of the glucose and ketal group of pyruvic acid and polymeric chain of glucose

Table 1: FTIR and NMR analysis of the isolated xanthan.

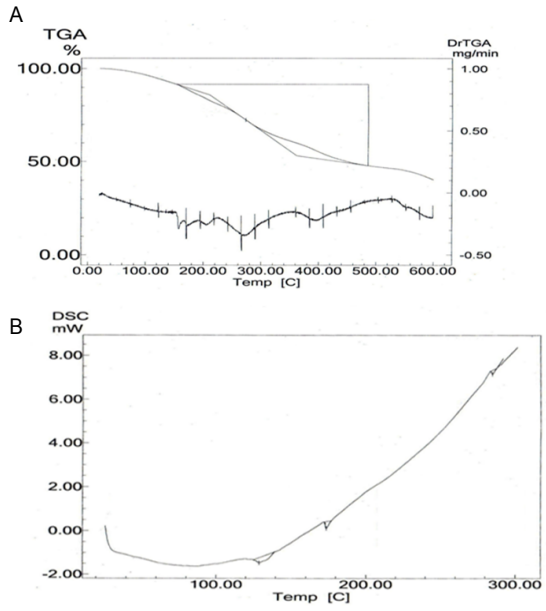

Figure 5: Typical thermograph of (a) Thermo-gravimetric analysis (TGA) and (b) Differential scanning calorimetry (DSC) of isolated xanthan.

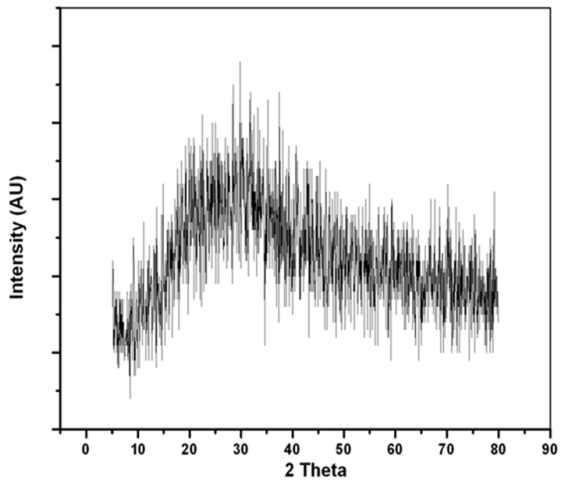

Figure 6: X-ray diffraction (XRD) spectrum of the isolated xanthan.

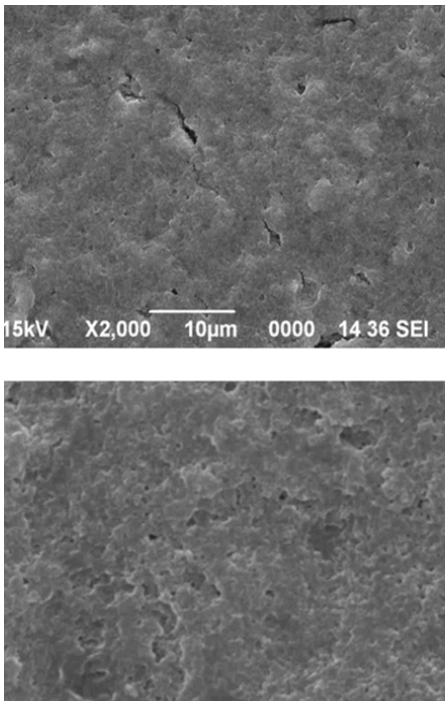

Figure 7: Scanning electron microscopic images of the isolated xanthan at a magnification of (a) 2000 X and (b) 10,000 X.

theoretical values for xanthan elemental analysis are 34.35% C, 6.45% H, and 54.62% O, respectively. The presence of nitrogen in the xanthan samples might be the due to the presence of impurities (proteins). The cross-sectional view of the dry xanthan exhibited the homogeneity of the biopolymer and shown in Figures 7a and 7b. The appearances of pores in the prepared film are due to the evaporation of water on drying. Formation of pores played a crucial role in fast swelling and de-swelling kinetics of the xanthan [12].

Optimization of xanthan production

Out of five different fermentation media [13-17], media described by Kalogiannis et al. [17] was found to be efficient on the basis of yield per liter of the medium. The results showed that the amount of xanthan produced on waste residual molasses was comparatively different with that of produce from glucose, sucrose, lactose, galactose and maltose (Figure 8). The effects of different nutritional parameters (include concentration of K_2HPO_4, NaCl, and molasses) and culture conditions (pH, temperature and aeration) on xanthan gum production are presented in Figures 9a, 9b and 9c and Figures 10a, 10b and 10c. An

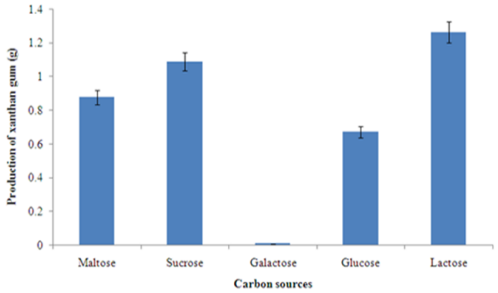

Figure 8: Effect of different carbon sources on the production of xanthan by *Xanthomonas campestris*.

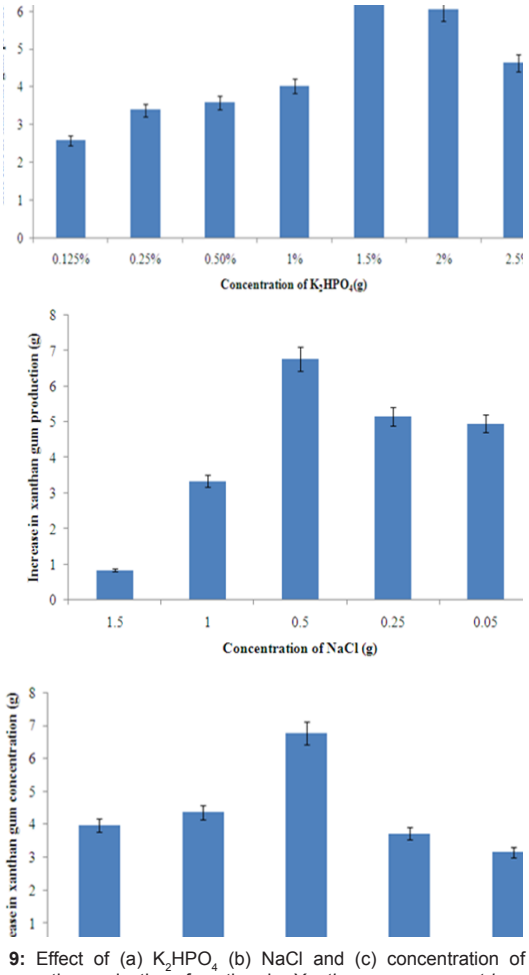

Figure 9: Effect of (a) K_2HPO_4 (b) NaCl and (c) concentration of waste molasses on the production of xanthan by *Xanthomonas campestris*.

increase on the concentration of NaCl on the medium was found to improve the xanthan production. The effect of K_2HPO_4 showed that the concentration was not significant in the range investigated. Therefore additional experiments varying the K_2HPO_4 concentrations viz. 0.125%, 0.25%, 0.5% and 1.0% (w/v) were carried out. The maximum production was 5.23 g/l at 1.5% of K_2HPO_4. Previous reports indicated that K_2HPO_4 can be useful as a buffering agent as well as a nutrient for the *X. campestris* [17,18]. Various authors have reported the use of agro-industrial wastes such as citrus waste [19], olive mill wastewaters [20], cheese whey [10] and date juice by-products [21] as carbon source for xanthan production. Hence, on the basis xanthan production per liter, use of waste residual molasses appears to be favorable

Rheological studies

The effect of xanthan concentration, pH and temperature on the rheological behavior of xanthan produced by the indigenous strain *X. camprestris* are shown in Table 2. The differences in apparent viscosity (AV) values were more evident at low concentrations of xanthan. At concentration above 0.5% (w/v) of xanthan gum attains relatively high viscosity indicating the pseudoplastic behavior in the solutions and the apparent viscosity decreased with the increase in shear rate. Such behavior is a characteristics feature shown by polymeric solutions of microbial polysaccharides with large molecular weight [22,23]. When shear stress was increased gradually, viscosity was progressively getting reduced because the aggregates of xanthan molecules bound through hydrogen bonding and polymer entanglement are gradually disrupted under the pressure of applied shear. However, on removal of shearing stress, the initial viscosity of the solution is recovered almost immediately [24]. Xanthan solutions are specific in their ability to retain their viscosity until it reaches its melting temperature. At such temperature, the viscosity sharply decreases due to a reversible molecular conformation change [24]. The effects of different temperatures on xanthan gum viscosity are shown in Table 2. Decrease in the viscosity of xanthan solution was observed with the increase in the temperature up to 80°C, though it was only a transient phenomenon but the solution returned to their original viscosity upon cooling. The appeared viscosity of the solutions decreased with increasing temperature at all pH values. To determine the function of pH, the stability of xanthan solution was checked over the pH range of 3.5-7.0 and results are presented in Table 2. The viscosity values, however, were not influenced by pH changes and did not show any linear correlation with pH values. Since xanthan is a neutral and nonionic polymer, its viscosity was independent of pH and remained stable from a pH range of 2 to 10. Such stability of the xanthan at various pH ranges may find its application in a number of food products.

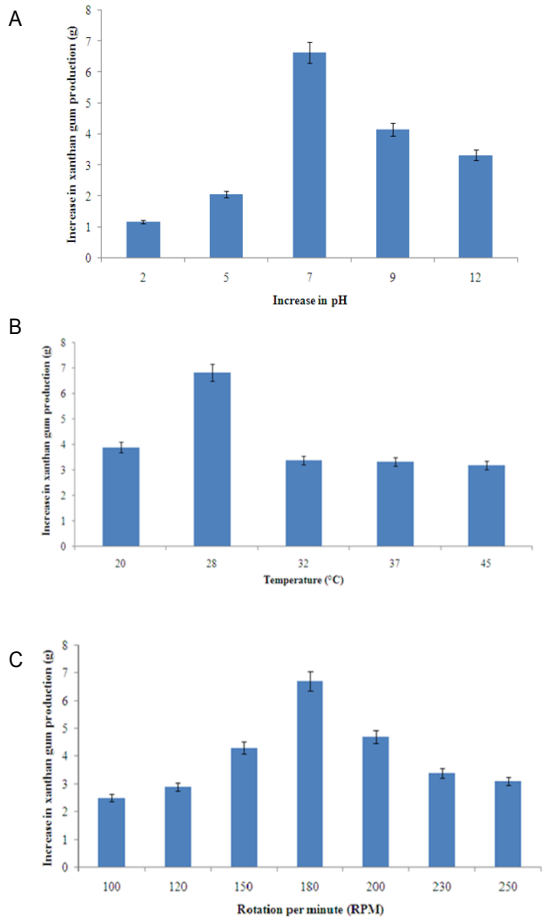

Figure 10: Effect of (a) pH (b) temperature and (c) aeration on the production of xanthan by *Xanthomonas campestris*.

Concentration of xanthan gum (%)	pH	Temperature (°C)	Viscosity (mPa. sec)
0.75	3.5	25	119 ± 0.7
		40	123 ± 0.5
		60	99 ± 0.8
		80	93 ± 0.4
	5	25	120 ± 0.8
		40	112 ± 0.6
		60	102 ± 0.7
		80	97 ± 0.0
	7	25	123.6 ± 0.5
		40	120 ± 0.6
		60	108 ± 0.4
		80	96 ± 0.2
1.5	3.5	25	137 ± 0.5
		40	133 ± 0.7
		60	127 ± 0.3
		80	100 ± 0.4
	5	25	139 ± 0.1
		40	131 ± 0.2
		60	120 ± 0.5
		80	102 ± 0.4
	7	25	150 ±0.6
		40	96 ± 0.5
		60	84 ± 0.8
		80	102 ± 0.7
3.0	3.5	25	159 ± 0.6
		40	148 ± 0.5
		60	136 ± 0.4
		80	123 ± 0.3
	5	25	154 ± 0.3
		40	152 ± 0.2
		60	138 ± 0.6
		80	119 ± 0.5
	7	25	162 ± 0.2
		40	154 ± 0.4
		60	141 ± 0.8
		80	124 ± 0.5

Table 2: Effect of xanthan gum concentration, pH and temperature on the viscosity of xanthan.

Conclusion

The potentiality of using a low-cost renewable residual molasses as a substrate for xanthan production by *X. campestris* seems to be advantageous from the view point of economy. Under optimal conditions a maximum of 5.23 g/l xanthan could be produced using such feedstock. The isolated xanthan exhibited a greater degree of pseudo plasticity as shown by the progressive reduction in the viscosity in direct response to shear. The isolated gum was nonionic in nature and stable to a wide range temperature and pH. The present study suggests the possibility of using residual waste molasses as carbon feedstock and the xanthan produced on such substrates could be useful in a variety of industrial applications.

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