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Short communication

Xanthan from sulphuric acid treated tapioca pulp: Influence of acid concentration on xanthan fermentation

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ABSTRACT

Xanthan gum was produced by fermentation of sulphuric acid pre-treated tapioca pulp. Effect of sulphuric acid concentration (0.5%, 2.5% and 5.0%) on xanthan fermentation was investigated. Maximum xanthan yield (7.1 g/l) was obtained with 0.5% sulphuric acid pre-treatment. Further, increase in sulphuric acid concentration caused formation of inhibitory substance and lowered xanthan yield. The product was confirmed as xanthan using FTIR, ^{1}H NMR analyses. Viscosity was measured by Brookfield viscometer and the molecular weight was determined from the intrinsic viscosity. The results confirmed that the yield and quality of xanthan produced were strongly influenced by the acid concentration.

1. Introduction

Xanthan gum is an industrially important extracellular heteropolysaccharide produced by *Xanthomonas campesteris* through aerobic fermentation. It was only the second exopolysaccharide to be approved, after dextran, by Food and Drug Administration (FDA) as a safe food ingredient (Born, Langendorff, & Boulenguer, 2005).

As the cost of carbon source is one of the major factors contributing to the production cost, research on production of xanthan from low cost substrates is very important. Various agricultural wastes, consisting of complex polysaccharides like cellulose, hemicellulose, lignin and starch, had been tried as low cost substrates to reduce the raw material cost (Demirci, Arici, & Gumus, 2012; Faria et al., 2011; Stredansky & Conti, 1999; Woiciechowski, Soccol, Rocha, & Pandey, 2004). Bioconversion of low cost agro-wastes to value added products provides a solution to solid waste disposal problem (Demirci et al., 2012; Göksungur, Uzunoullari, & Dagbagli, 2011; Kalyanasundaram, Doble, & Gummadi, 2012). Cost of commercial substrates namely glucose and sucrose are in the order of US$ 400–600/Metric Tonne (ISMA, 2013; Wilke, 1999). On the other hand agricultural residues, generated as solid wastes in agro based industries, are costless. In fact disposal of these solid wastes properly in secured manner is a major problem faced by these industries. While a small fraction of them are sold out as a raw material for cattle feed, soil enrichment etc., rest of them is disposed off as land fills. The only cost involved in procuring these materials is the handling and transportation cost.

Bioconversion of solid waste to useful product, usually, is a two step process. In the first step, various pre-treatment techniques are employed to break down complex carbohydrates into simple reducing sugars. In the second step, desired products are obtained by fermentation of the reducing sugars (Lange, 2007; Lee, 1997; Sun & Cheng, 2002). Often, the composition of the intermediate influences the quality and quantity of the final product. Some of the intermediates formed during acid pre-treatment are inhibitory to product formation(Larsson et al., 1999). Though inhibitory compounds are not formed in enzymatic pre-treatment, owing to the cost of enzymes involved, hydrothermal and/or acid pre-treatments are preferred over enzymatic pre-treatment (Li & Zhu, 2011).In this work, sago industry tapioca pulp (SITP), an agro-industry waste from cassava (*Manihot esculenta*) was utilized as a low cost substrate for xanthan production. Cassava waste is preferred over other agro-industrial waste as a substrate in bioconversion processes due to its well-balanced nutritional composition (Sugumaran, Jothi, & Ponnusami, 2014). The sugar degradation products, like furfural and HMF, affect cell energetics (Taherzadeh, Gustafsson, Niklasson, & Liden, 2000). More over, they inhibit biological activities and damage microorganisms by various mechanisms like reducing enzyme activity, breaking down DNA, inhibiting protein formation and RNA synthesis. To the best of our knowledge, a systematic study on influence of intermediates on xanthan fermentation had not been reported earlier. This is the first report to investigate the influence of SITP hydrolysate composition and its sugar degraded products on xanthan fermentation.

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2. Materials and method

2.1. Microorganism and inoculum preparation

Xanthamonas campestris (NCIM 2954) was obtained from National Collection of Industrial Microorganisms (NCIM), Pune, India. The microorganism was maintained on MGYP agar (Hi-media) slant containing malt extract 3 g/l; glucose 10 g/l; yeast extract 3 g/l; peptone 5 g/l with 2% agar adjusted to pH 7 as recommended by NCIM. It was allowed to grow for 24 h at 30 °C and then stored at 4 °C. This was sub-cultured every two weeks. The cells from freshly prepared agar slant were inoculated into the broth medium containing MGYP in 250 ml conical flask and this was used as the inoculum for fermentation. 24 h culture was used as seed for the fermentation process.

2.2. Substrate preparation and characterization

Sun dried SITP was obtained from a nearby sago industry at Salem, India. It was ground to fine size and the powder was size separated using sieve shaker. Particles of mesh size BSS # −25 + 30 were collected and stored for further use. It was then dried in hot air oven at 60 °C overnight to remove residual moisture and then stored in air-tight container at room temperature. The substrate (100 g/l) was hydrolyzed at 121 °C for 20 min with 0.5%, 2.5% and 5% (w/v) sulphuric acid. The hydrolysate was filtered using syringe filter with 0.45 μm polypropylene membrane to remove suspended particles. The hydrolysates were labelled as hydrolysate H1, H2 and H3, respectively.

Dextrose equivalent (DE) of hydrolysate was determined by Lane and Eynon constant titre method (Lane & Eynon, 1923) (Supporting file). Its composition was analyzed by high performance liquid chromatography (HPLC). Hi-plex H column, 7.7 × 300 mm (Agilent Technologies) was used to estimate the monosaccharide composition of hydrolysate at 65 °C with 5 mM of H_2SO_4 as eluent at a flow rate of 0.6 ml/min. Byproducts of pretreated SITP hydrolysate, especially HMF and furfural were analyzed using C-18 column in HPLC with UV detector at 280 nm. The solid residue was dried at 105 °C and analyzed for its starch content according to Hodge and Hofreiter (1962) and hemicellulose (Goering & Vansoest, 1975) and cellulose content according to Updegraff (1969) (Supporting file). Crude fibre, moisture and ash contents were estimated according to Maynard method and AOAC, 2000 (AOAC, 2005; Maynard, 1970).

2.3. Fermentation

Untreated SITP and the filtered hydrolysates H1, H2 and H3 with 30% initial reducing sugar were used as carbon source. The other media constituents were (in g/l) KH_2PO_4 – 5; yeast extract – 5; citric acid – 2; $MgSO_4{\cdot}7H_2O$ – 0.2; $CaCO_3$ – 0.02 and H_3BO_3 – 0.006. The media pH was adjusted to 6.8. Batch fermentation was carried out with 100 ml of production media in 250 ml conical flask as triplicates. It was inoculated with 5% *Xanthamonas campestris* (10^7 CFU/ml). The flasks were incubated at 28 °C for 72 h in refrigerated rotary shaker at 200 rpm. Samples withdrawn from the fermentation broth after 72 h were autoclaved first and then centrifuged at 10,000 rpm, 30 °C for 20 min. Ice-cold acetone at 10 °C was added to the supernatant in the ratio of 2:1 (v/v) and the mixture was left for 24 h for precipitation of xanthan. In order to ensure purity the precipitate was re-suspended in water and then filtered through filtration system (Make & Model; GE healthcare, MidGee Systems, CFP-1E-MM01A cartridge, pore size 100 nm and surface area 16 cm^2). Purified xanthan was precipitated from retentate as mentioned above by adding ice-cold acetone. The precipitate was then was dried in hot air oven at 105 °C for 6 h. The product was characterized using FT-IR and proton NMR.

Table 1
Composition of untreated and acid treated sago industry tapioca pulp hydrolysate.

Components	Untreated (%)	Acid hydrolysis (%)		
		0.5% H_2SO_4	2.5% H_2SO_4	5% H_2SO_4
DE (no unit)	–	12 ± 1	57 ± 3	71 ± 4
Maltodextrin	–	21.3 ± 1.1	3.1 ± 0.27	–
Glucose	–	5.8 ± 0.39	45.8 ± 4.1	54.1 ± 2.6
Xylose	–	0.8 ± 0.02	11.3 ± 0.97	13.4 ± 0.89
Arabinose	–	0.6 ± 0.04	2.9 ± 0.15	2.1 ± 0.099
Galactose	–	0.6 ± 0.03	8.1 ± 0.47	7.3 ± 0.23
Furfural	–	0.2 ± 0.009	0.4 ± 0.03	1.5 ± 0.07
HMF	–	0.2 ± 0.009	1.3 ± 0.06	2.4 ± 0.008
Acetic acid	–	0.07 ± 0.007	0.9 ± 0.04	1.7 ± 0.011
Formic acid	–	0.03 ± 0.002	0.06 ± 0.003	0.1 ± 0.008
Starch	57.8 ± 3.2	42.4 ± 3.1	3.4 ± 0.011	1.9 ± 0.087
Cellulose	18.1 ± 0.7	17.4 ± 1.5	14.4 ± 0.93	8.7 ± 0.27
Hemicellulose	8.6 ± 0.5	4.7 ± 0.23	1.4 ± 0.082	1.2 ± 0.075
Ash	2.3 ± 0.1	2 ± 0.17	2.1 ± 0.11	1.8 ± 0.077
Protein	1.2 ± 0.08	0.67 ± 0.04	0.4 ± 0.003	0.02 ± .001
Moisture	10.2 ± 0.7	2.5 ± 0.12	2.7 ± 0.16	2.1 ± 0.16

2.4. Determination of intrinsic viscosity and molecular weight

Viscosity of xanthan was measured using Brookfield DV (II) LDV Viscometer. The intrinsic viscosity of the polymer [η] was obtained from the *y* intercept of the plot η_{sp}/C versus xanthan concentration C (mg/l).

$$[\eta] = \lim_{C \to 0} \frac{\eta_{sp}}{C} \quad (1)$$

Here η is the measured viscosity and η_0 is viscosity of the solvent and η_{sp} is $((\eta - \eta_0)/(\eta \cdot \eta_0))$.

Molecular weight of xanthan was determined from the intrinsic viscosity using the Mark–Houwink equation (Casas, Santos, & Garcıa-Ochoa, 2000) given below:

$$[\eta] = KM^a \quad (2)$$

where M is the molecular weight of polymer and 'K' and 'a' are Mark–Houwink constants ($K = 1.7 \times 10^{-4}$ and $a = 1.14$ (Milas, Rinaudo, & Tlnland, 1985)).

2.5. Characterization of xanthan gum

Xanthan was characterized using FT-IR spectroscopy and proton nuclear magnetic resonance (^{1}H NMR) spectroscopy. Fourier transform infrared spectra were obtained using Perkin Elmer RX I model spectrophotometer. Sample was prepared by KBr pellet method and scanned from 400 to 4000 cm^{-1}. ^{1}H NMR was done using Avance Bruker 500 MHz spectrometer by dissolving xanthan in D_2O at 80 °C.

3. Result and discussion

3.1. Composition of intermediates

Table 1 summarizes the composition of raw STIP, treated SITP and its hydrolysate. Typically, raw SITP contains 10% moisture, 58% starch and traces of free reducing sugars and proteins. When higher acid concentration was used for pre-treatment, DE and glucose content increased. Increasing acid concentration resulted in decrease in starch, cellulose, hemicellulose and maltodextrin contents of tapioca pulp. These observations indicated that increase in acid concentration favoured hydrolysis of the SITP. However, it can also be noted that pre-treatment resulted in formation of inhibitory

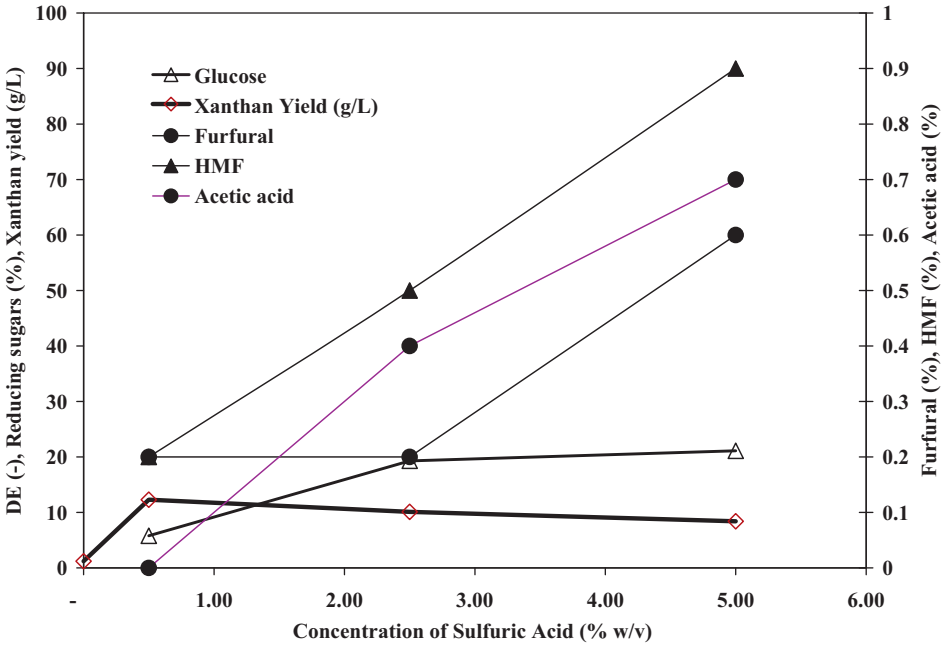

Fig. 1. Xanthan yield from raw and hydrolystes H1, H2 and H3 of sago industry waste with 30% initial reducing sugar.

compounds like aliphatic acids and furan aldehydes. Concentrations of these compounds were negligible in H1, but they were appreciably higher in the case of H2 and H3 (Tables S1 and S2). Higher acid concentration used during the pre-treatment resulted in the formation of these inhibitory compounds.

3.2. Xanthan yield

Fig. 1 shows the xanthan yield from hydrolysates H1, H2 and H3 from sago industry tapioca pulp with 30% initial reducing sugar. Xanthan yield was in the following order: H1 > H2 > H3 > RCB. Maximum xanthan yield (7.1 g/l) was achieved when 0.5% sulphuric acid was used for pre-treatment. Though the glucose content and DE of the sugar hydrolysate increased with increase in acid concentration, xanthan gum yield did not increase proportionately (Fig. 1). This was obviously due to the formation of inhibitory substances and their concentration in the hydrolysate. Though direct report on inhibitory action of HMF and furfural on *X. campesteris* is not available in literature, inhibitory action of HMF and furfural on various other microorganisms had been widely reported (Almeida, Bertilsson, Gorwa-Grauslund, Gorsich, & Lidén, 2009; Palmqvist & Hahn-Hägerdal, 2000; Taherzadeh et al., 2000).

A comparison of xanthan yield from various carbon sources reported in previous literature is given in Table 2. It can be seen from the table that sulphuric acid can be effectively used for efficient conversion of tapioca pulp to xanthan.

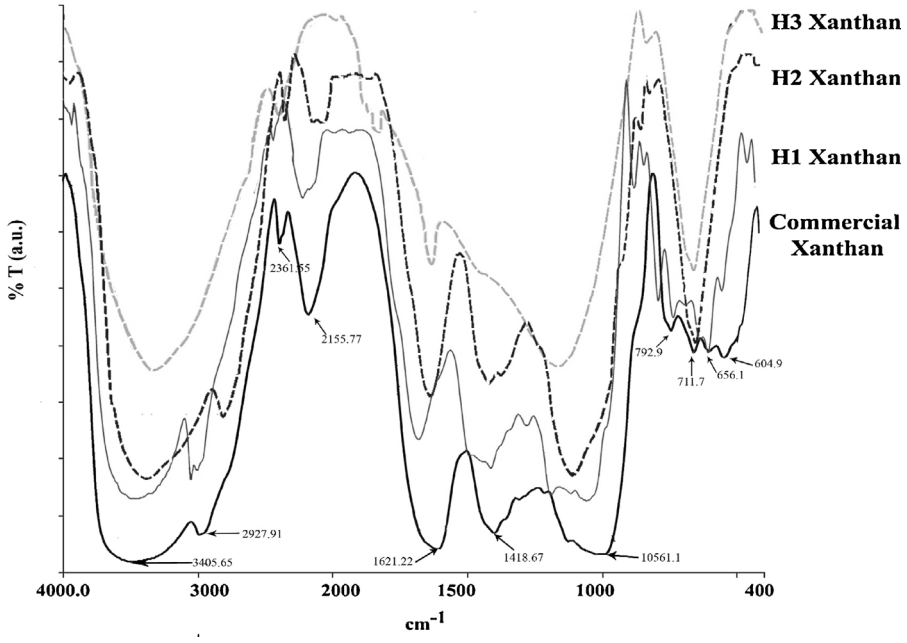

Fig. 2. FTIR spectra of xanthan gum from commercial xanthan, SITP hydrolysate H1, SITP hydrolysate H2 and SITP hydrolysate H3.

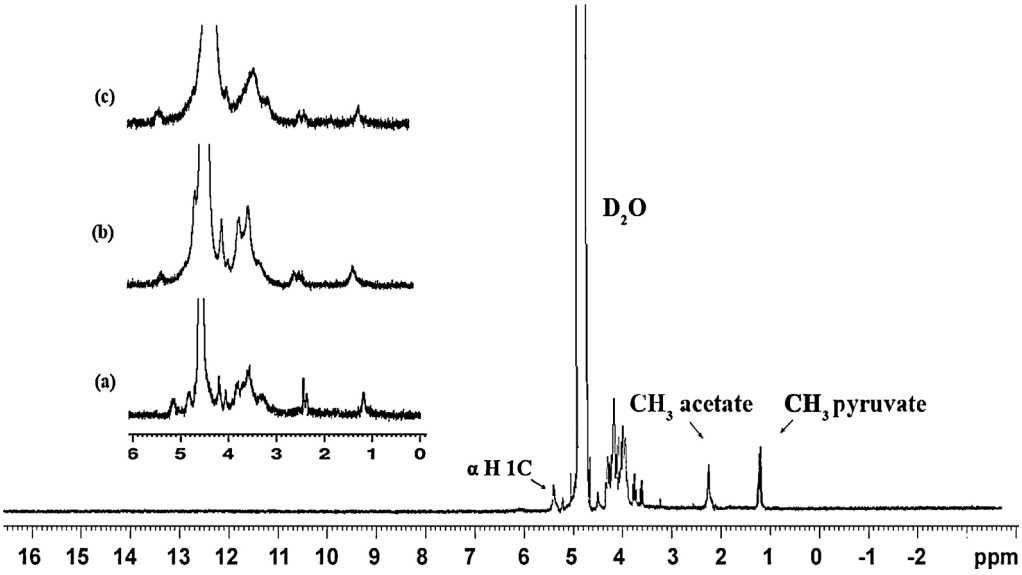

Fig. 3. ^{1}H NMR spectra of commercial xanthan with inset spectra of xanthan from (a) SITP hydrolysate H1, (b) SITP hydrolysate H2 and (c) SITP hydrolysate H3.

Table 2
Comparison of xanthan yield from various carbon sources.

Substrate	Xanthan yield (g/l)	Reference
Glucose	14.4	Rosalam and England (2006)
Sucrose	13.2	Leela and Sharma (2000)
Maltose	12.3	Leela and Sharma (2000)
0.5% Sulphuric acid treated tapioca pulp	**7.1**	This work
Soluble starch	12.1	Leela and Sharma (2000)
Date extract	11.2	Khosravi-Darani, Reyhani, NaserNejad, and Farhadi (2011)
2.5% Sulphuric acid treated tapioca pulp	**5.04**	This work
Potato starch	9.7	Leela and Sharma (2000)
5% Sulphuric acid treated tapioca pulp	**4.5**	This work
Galactose	7.1	Leela and Sharma (2000)
Raw starch based media (cassava) (mutant strain)	6.0	Kerdsup, Tantratian, Sanguandeekul, and Imjongjirak (2011)
Raw starch based media (cassava) (wild strain)	4.3	Kerdsup et al. (2011)

Bold values distinguish present results from previous literature data.

Table 3
Intrinsic viscosity and molecular weight.

S. no.	Xanthan from hydrolysate	Intrinsic viscosity (10^3 ml/g)	Molecular weight (10^6 g/mol)
1	H1	2.13 ± 0.08	1.68
2	H2	1.22 ± 0.10	1.04
3	H3	0.82 ± 0.05	0.73

3.3. Characterization of xanthan

Molecular weight of the xanthan obtained from these hydrolysates were calculated using Mark–Houwink equation with constants $K = 1.7 \times 10^{-4}$ and $a = 1.14$ (Milas et al., 1985). Intrinsic viscosity $[\eta]$ for xanthan and its respective molecular weights from hydrolysates H1, H2 and H3 are shown in Table 3. It is evident that the xanthan fermentation was effective only in H1 hydrolysate. Once again this could be attributed to favourable composition of the intermediates and negligible concentrations of inhibitory substances in the H1 hydrolysate.

Fig. 2 shows the Fourier transform IR spectra of xanthan obtained from standard and H1, H2 and H3 hydrolysates. A strong broad absorption peak at 3405 cm^{-1} was observed due to hydroxyl stretching. C—H stretching was noted from the peak between 2850 and 2950 cm^{-1} which may be attributed to asymmetric and symmetric stretching of CH_2 or CH_3 and aldehyde groups. Carbonyl group was identified from the peak stretching between the wave numbers 1670 and 1820 cm^{-1}. C—H deflection angle and C—O stretching vibration were detected in the wave region of 1420–1430 cm^{-1} and 1050–1150 cm^{-1}. Peaks between 650 and 600 cm^{-1} was mainly due to vibrations of C—H bending. The FTIR spectra of the xanthan produced were matching with that of standard xanthan and were similar to the reports of previous researchers (Faria et al., 2011).

Xanthan gum obtained after fermentation was further characterized by ^{1}H NMR and the comparative result is shown in Fig. 3. Evident peak observed at 4.79 ppm was due to deutriated water used for sample preparation. The two peaks at 1.3 and 2.2 ppm correspond to acetate and pyruvate groups of the polymer. Peak at 5.2 ppm (coupling constant – 3.6) is attributed to the equatorial α-anomeric proton of mannopyranosic unit. Peak found between 4.6 (coupling constant – 1.8) and 4.8 (coupling constant – 19.8) ppm corresponds to protons of β carbon of pyranose. Peak found between 3.2 and 3.9 ppm corresponds to carbon linked to hydroxyl group and glucoronic acid, respectively (Faria et al., 2011; Rinaudo, Milas, & Lambert, 1983). The peaks of commercial xanthan match with those of xanthan prepared with H1, H2 and H3 hydrolysates. The degree of acetylation (4.9%, 5.1% and 5.1%) and degree of pyruvation (6.5%, 4.8% and 2.8%) were calculated for H1, H2 and H3 hydrolysates from ^{1}H NMR spectra. The characteristic features from Figs. 2 and 3 also confirmed the product as xanthan from STIP hydrolysate. With these initial investigations further research can be carried out to study the effect of hydrolytic products in metabolic pathway of *Xanthamonas campestris* and quality of xanthan gum.

4. Conclusion

Production of xanthan gum by fermentation of 0.5%, 2.5% and 5% sulphuric acid pre-treated tapioca pulp with 30 g/l of reducing sugar yielded 7.1 g/l, 5.04 g/l and 4.5 g/l of xanthan. The effect of acid concentration used in pre-treatment and hence composition

of hydrolysate played a significant role in yield and viscosity of xanthan. FTIR and ^{1}H NMR results of xanthan from STIP were matching with those of commercial xanthan. The results reported here confirmed that sulphuric acid pre-treatment was effective for efficient bioconversion of tapioca pulp to xanthan.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2013.11.006.

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