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Isolation, Production and Characterization of the Polysaccharide "Xanthan Gum" from *Xanthomonas spp*

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A B S T R A C T

The xanthan gum is an exopolysaccharide of the microbial origin, produced by the bacteria of the *Xanthomonas* spp. In the present study *Xanthomonas* spp were obtained from cabbage leaves and lemon sample. The isolates were coded as SC1 and SC4. From various cultural, morphological and biochemical characteristics the bacteria were identified as belonging to *Xanthomonas* spp. The bacteria were then tested for production of Xanthan in the fermentation medium. Measurement of viscosity and residual sugar was carried out. The effect of different carbon sources on its production was also tested. Xanthan production reached their highest levels (In SC1 0.5 g/l and in SC4 0.45 g/l) after 120 hrs incubation, in a yeast malt medium. Sucrose acted as best carbon source for xanthan production.

Introduction

Xanthan is an important biopolymer discovered in the 1950s at the National Regional Research Laboratories (NRRL) of United States Department of Agriculture (Gils *et al.*, 2009). Under acidic and alkaline conditions the xanthan has excellent solubility and stability, it is a heteropolysaccharide (Rosalam and England, 2006). This polysaccharide is produced by the bacterium *Xanthomonas* (Kurbanoglu and Kurbanoglu, 2007). *Xanthomonas* spp. are gram negative, aerobic, straight rods with single polar flagellum. Colonies are usually yellow, smooth, butyrous or viscid (Velu *et al.*, 2016). Xanthan gum is an expolysaccharide (EPS) produced by the gram negative bacteria of the genus *Xanthomonas* through aerobic submerged fermentation (Azuaje and Sanchez, 1999).

At low concentrations gum are high soluble in water which can produce gels or highly viscous solution and gums are high molecular weight compound. There is a wide variety of substances that present the "gummy" characteristics and can be referred to as gums (Kang and Pettitt, 1993). Xanthan is composed of pentasaccharide repeating units,

containing d-glucose, d-mannose, d-glucoronic acid (at a ratio 2:2:1), acetal-linked pyruvic acid and d-acetal groups (Jansson *et al.,* 1975). The xanthan gum was liberated by FDA in 1969, allowing its use in the production of food (WHO, 1990). It is widely used in foods, toiletries, cosmetics, as water-based paints, pharmaceutical, artificial juices, sauces for salads, meat, chicken or fish, as well as for syrup and covering for ice-cream, desserts (Luvielmo and Scamparini, 2009; Nussinovitch, 1997).

Materials and Methods

Sample collection

Lemon sample was procured from local vegetables market, cabbage and cauliflower leaves showing the yellow necrotic lesions were selected for the present study were collected from fields near Surat region, Gujarat, India.

Isolation and screening of xanthan producing bacteria

The diseased leaf sample and lemon lesions were cut into small pieces soaked in 5 ml distilled water and incubated for 24 hrs. The resulting suspension was streaked onto nutrient agar plate and the plates were incubated at 30°C for 48 hrs and examined. Isolated colonies were further streaked on YCDA (Almarza and Romero, 2013) plates and incubated at 30°C for 48 hrs. The bacteria with yellow mucoid colonies were selected for further study. The isolates were coded S1, S2, S5, S7, SC1 and SC4.

Growth conditions

The inoculum was prepared from the selected isolates and inoculated in YM broth (20 g/l glucose, 3g/l yeast extract, 3g/l malt extract, and 5g/l peptone). The selected bacterial cells were grown in 100 ml inoculums medium at 37°C in shaking conditions (200 rpm) for 24 hrs (Zakeri *et al.,* 2015).

Fermentation medium: The following medium was used [Sugar cane molasses (30, 60, 90 g/l), KH_2PO_4 (5g/l), $MgSO_4$. $7H_2O$ (0.2g/l), citric acid (2g/l), $FeCl_3.6H_2O$ (0.002 g/l), $CaCO_3$ (0.02g/l), Glutamate (2g/l)] (Zakeri *et al.,* 2015). The medium was sterilized for 20 min at 121°C and medium initial pH was adjusted to 7. Fermentation was carried out in 250 ml Erlenmeyer flask, each of which contained 100 mL of the sterile production medium. The medium was inoculated with 5 (v/v%) of the inoculum and incubated at 37°C for 72 hrs at 200 rpm. The different isolates were inoculated in these media and after incubation the viscosity of the broth was measured. Isolates giving highest viscosity were further used for optimization studies.

Characterization and identification of bacteria

For characterization and identification of bacteria its morphological, cultural and biochemical characteristics were studied.

Cultural characteristics

The colonies were purified on nutrient agar plates for observation and examination of colonial characteristics.

Morphological characteristics

Gram staining was used to study the morphological characteristics and gram reaction.

Biochemical characteristics

Various biochemical tests were performed for the identification of the isolates like, Sugar

fermentation tests, Indol production test, Methyl red test, Voge's-Proskauer test, citrate utilization test, urea hydrolysis test, Hydrogen-sulphide production, gelatin liquefaction, catalase test and growth characters on TSI agar slant. All these media were inoculated with the loop full of culture by aseptic transfer technique or stabbing technique. The inoculated test media were incubated at 37°C for 24-48 hrs.

Effect of different parameters

Effect of different carbon sources on xanthan production

To study the effect of carbon source on xanthan production, 200 ml of YM broth was inoculated with the obtained isolates (SC1 and SC4). Different carbon sources like glucose, sucrose and molasses were used. Estimation of the residual sugar was carried out by the phenol sulphuric acid method and viscosity of the broth was measured. Later xanthan was recovered from the broth.

Effect of incubation state on xanthan production

200 ml of YM broth were inoculated with the obtained isolates. One flask kept in the static condition and the other flask shaking conditions at 37°C, 200 rpm at 120 hrs. After incubation cell free supernatant was collected and was further analyzed by estimation of residual sugar by phenol sulphuric acid method and viscosity of the broth was also measured using viscometer.

Determination of residual sugar in broth

The culture supernatant was used for the determination of sugars. Residual sugar was determined by the phenol sulphuric acid method using glucose as standard (Dubois *et al.,* 1956). In this method 1ml of 5% phenol and 5 ml of 96% sulphuric acid was added to the cell free supernatant. Mixed and incubated for 20 min at room temperature. Then the residual sugar was estimated in UV- Visible spectrophotometer at 490nm.

Viscosity

The viscosity of the xanthan solution was determined using Ostwald viscometer (Ashour *et al.,* 2000). Distilled water was used as control.

Xanthan recovery

Xanthan was extracted from the cell free supernatants. 10 ml cell free supernatant was precipitated, using two volumes of isopropanol solvent in the presence of 1% KCl salt. The mixture was kept at 4°C for 24 hour to precipitate the xanthan. Then, the supernatant was centrifuged at 6000 rpm for 30 min. Finally the obtained precipitate was dried in an oven at 60°C for 24 hour and weighed (Zakeri *et al.,* 2015).

Spectroscopy of Fourier Transform Infared (FTIR)

Fourier transform infared spectroscopic analysis was perform at the Ankleshwar Research and Analytical Infrastructure Ltd. Samples of commercial xanthan gum (standard) and produced xanthan gum (SC1 and SC4) were analysed using Fourier Transform Infrared Spectrophotometer in the spectral window of 1000- 4000 cm^{-1}.

Results and Discussion

Sample collecting site

In present study the isolation of xanthan producing bacteria, cabbage and cauliflower leaves collected from (21.1948° N, 72.9557° E) and lemon sample collected from the (21.2695° N, 72.9577° E) area of Surat region, Gujarat, India.

Screening of xanthan producing bacteria

Various samples were streaked on nutrient agar plates. 30 isolates were found and among them 11 isolates gave yellow colonies on nutrient agar plate, further these 11 colonies were streaked onto YCDA. Out of which 6 showed yellow mucoid colonies and 5 were mucoid but not yellowish.

Production of xanthan gum

The obtained 6 yellow mucoid colonies were inoculated in the fermentation media and incubated at 37°C for 72 hrs at 200 rpm. After incubation the media were centrifuged at 5000 rpm for 30 min and cell free supernatant was collected, viscosity was measured using viscometer. Distilled water was also measured by viscometer considered as a blank reading. Blank reading was 300.67 sec and it was used for the calculation of the viscosity of the broth.

By measuring the viscosity of the broth the isolates showing highest viscosity SC1 and SC4 were selected from cabbage and lemon samples. These two isolates were further studied for the effect of different carbon sources and incubation conditions for xanthan production (Table 1).

Characterization and identification of xanthan producing bacteria

The isolates were tested for their morphological characteristics and cultural characteristics. The colony characteristics on nutrient agar plate, showed circular, yellow colonies, Small/ intermediate/ large colonies with entire/ irregular edge after 48 hours incubation were observed (Table 2).

Biochemical characteristics

Identification of the organisms was carried out by various biochemical tests (Table 3). According to Bergey‘s Manual of Determinative Bacteriology.

From the cultural, morphological and biochemical characteristics both organisms were identified as belonging to *Xanthomonas* spp. by standard microbiological procedures.

Effect of different parameters

Sugar estimation

Sugar was estimated by phenol sulphuric acid method using glucose as a standard.

Effect of different carbon sources on xanthan production

Glucose

SC1 and SC4 were inoculated in glucose at (2%) concentration in production medium and viscosity of the broth was also measured. Glucose used as source of carbon and residual sugar estimated by phenol sulphuric method and extraction of xanthan was also done. The viscosity and residual sugar was determined at different time intervals. The highest viscosity obtained was at 120 hrs. Viscosity of the broth in SC1 and SC4 was 2.80 g/cm^3 and 2.74 g/cm^3 respectively. And residual sugar in SC1 and SC4 was 0.243 mg/ml and 0.228 respectively.

In our study xanthan was extracted at 120 hrs and xanthan yield in SC1 and SC4 was 0.4 g/l and 0.30 g/l. Cadmus *et al.,* (1978) reported that highest viscosity was 7000 cP for defined media using 2.5% glucose as carbon source. Palaniraj and Jayaraman (2011) reported that maximum xanthan production (14.744 g/l.) was obtained when glucose was used as carbon source (Table 4 and 5).

Sucrose

SC1 and SC4 were inoculated in sucrose (2%) containing production medium and viscosity

of the broth was measured. Sucrose was used as a source of carbon and residual sugar was estimated by phenol sulphuric acid method and xanthan was extracted.

The viscosity and residual sugar was determined at different time intervals. The highest viscosity was obtained at 120 hrs. In SC1 and SC4 yield viscosity was 3.04 g/cm^3 and 3.00 g/cm^3 respectively and residual sugar in SC1 and SC4 was 0.219 mg/ml and 0.224 mg/ml respectively.

In our experiment xanthan was extracted at 120 hrs and yield in SC1 and SC4 was 0.5 g/l and 0.45 g/l. This result was in agreement with Kassim, (2011) who reported that *X. campestris* produced 6.8 g/l xanthan when sucrose used as a carbon source. Souw and Demain, (1979) also found that *X.campestris* NRRL B1459 gave higher producer of xanthan and high viscosity was obtained in sucrose 15000 cP. Saied *et al.,* (2002) reported that sucrose gave the highest yield (11.99 g/l). Kawahara and Obata, (1998) who stated that, maximum xanthan production was obtained when sucrose was used as a carbon source using *X. campestris* NRRL-B 1459.

Molasses

SC1 and SC4 were inoculated in 2% molasses containing production medium and viscosity of the broth was also measured. Molasses used as source of carbon and residual sugar estimated by phenol sulphuric method and xanthan was extracted. The viscosity and residual sugar was determined at different time intervals. The highest viscosity obtained at 120 hrs. In SC1 viscosity was 2.63 g/cm^3 and in SC4 viscosity was 2.59 g/cm^3 and remaining residual sugar in SC1 and SC4 was 0.545 mg/ml and 0.524 mg/ml respectively.

In our experiment xanthan was extracted at 120 hrs and yield in SC1 and SC4 was 0.35 g/l and 0.25 g/l. Mossavi *et al.,* (2010) reported that the yield of xanthan from molasses in his study was similar to sucrose but in our experiment sucrose was higher producer of xanthan than molasses.

Effect of incubation state on xanthan production

SC1 and SC4 were inoculated in YM broth and one flask incubated in static condition and other kept under the shaking condition at 200 rpm, 37°C. After incubation residual sugar estimated by phenol sulphuric acid and viscosity of the broth was measured and recovery of xanthan was also done after 120 hrs incubation. The viscosity and residual sugar was determined at 120 hrs. In static condition the viscosity obtained in SC1 and SC4 was 2.04 g/cm^3 and 1.97 g/cm^3 and in shaking condition the obtained viscosity was in SC1 and SC4 was 3.10 g/cm^3 and 3.07 g/cm^3.

In static condition the remaining residual sugar in SC1 and SC4 was 0.34 mg/ml and 0.39 mg/ml and in shaking condition the remaining residual sugar in SC1 and SC4 was 0.21 mg/ml and 0.29 mg/ml. Xanthan was extracted in static condition SC1 and SC4 was 0.09 g/l and in 0.06 g/l. In shaking condition xanthan yield in SC1 and SC4 was 0.35 g/l and 0.3 g/l.

In our experiments the higher production of xanthan was observed in shaking than in static condition (200 rpm). Suow and Demain, (1979) also reported that 250 rpm resulted in greater exopolysaccharide production.

Result of FT-IR

Standard

The Fourier transmission-infrared spectrum (FT-IR) is a method to detect similarities or

difference present in functional groups of compound. The functional groups present in commercial xanthan gum and produce synthesized xanthan gum were analyzed and compared (Table 6–8 and Fig. 1–9).

Table.1 Viscosity observed in a fermentation broth

Isolates No	Viscosity (gram/cubic centimeter)
S1	1.74
S2	1.02
S5	1.52
S7	1.73
SC1	2.18
SC4	2.08

Table.2 Cultural and morphological characteristic of obtained isolates SC1 and SC4

Name of medium	Colony characteristics			Gram reaction and morphology	
		SC1	SC4	SC1	SC4
sNutrient agar medium	Size	Intermediate	Small	Gram negative short rods occurring singly 	Gram negative rods occurring singly 
	Shape	Circular	Circular		
	Elevation	Convex	Convex		
	Consistency	Smooth	Moist		
	Edge	Entire	Entire		
	Opacity	Translucent	Translucent		
	Pigmentation	Yellow	Yellow		

Table.3 Biochemical characteristics of SC1 and SC4

Biochemical Test	Nutrient sucrose broth	Nutrient lactose broth	Nutrient maltose broth	Nutrient glucose broth
SC1	+	-	+	+
SC4	+	-	+	+

Biochemical Test	Indol test	MR test	V-P test	Citrate test	H_2S test	Gelatin liquefaction test	Catalase test	Urea hydrolysis
SC1	-	-	-	-	+	+	+	-
SC4	-	-	-	-	+	+	+	-

TSI agar slant	Slant	Butt	H_2S	Gas production
SC1	Alkaline	Alkaline	-	+
SC4	Alkaline	Alkaline	-	-

(+) = positive, (-) = negative

Table.4 Standard graph of glucose

Glucose (mg/ml)	Optical density (490 nm)
0	0
20	0.293
40	0.429
60	0.729
80	0.805
100	1.126

Table.5 Glucose containing media on viscosity

Time (hrs)	Viscosity (gram/cubic centimeter)	
	SC1	SC4
24	1.52	1.50
48	1.75	1.77
72	2.08	2.1
96	2.38	2.22
120	2.80	2.74

Table.6 Estimation of viscosity in Sucrose containing medium

Time (hrs)	Viscosity (gram/cubic centimeter)	
	SC1	SC4
24	1.48	1.50
48	1.81	1.77
72	2.25	2.18
96	2.53	2.47
120	3.04	3.00

Table.7 Estimation of viscosity in Molasses containing medium

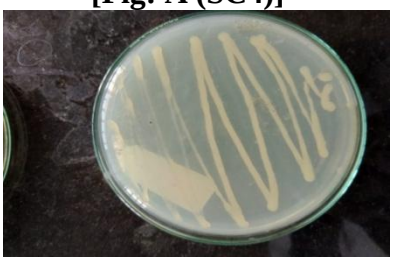
Time (hrs)	Viscosity (gram/cubic centimeter)	
	SC1	SC4
24	1.51	1.56
48	1.97	1.89
72	2.10	2.01
96	2.34	2.26
120	2.63	2.59

Table.8 Effect of incubation state on viscosity

Condition	Time (hrs)	Viscosity (gram/cubic centimeter)	
		SC1	SC4
Static	120	2.04	1.97
Shaking	120	3.10	3.06

Fig.1 Growth on YCD agar

[Fig: A (SC4)]



[Fig: B (SC1)]

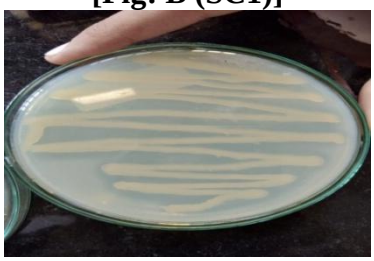


Fig.2 Glucose standard Curve

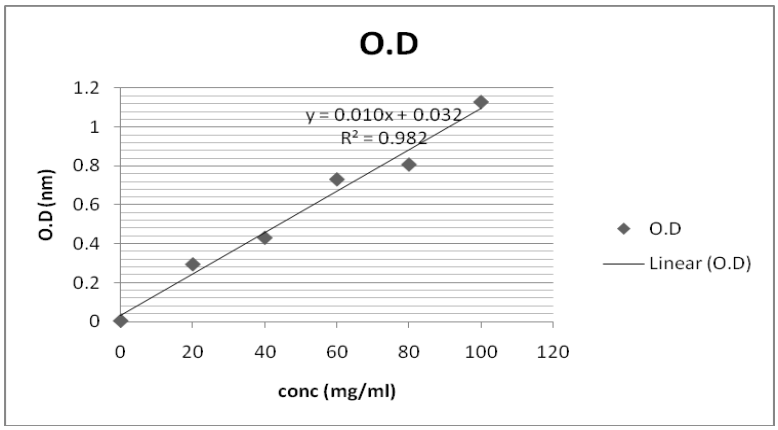


Fig.3 Estimation of residual sugar

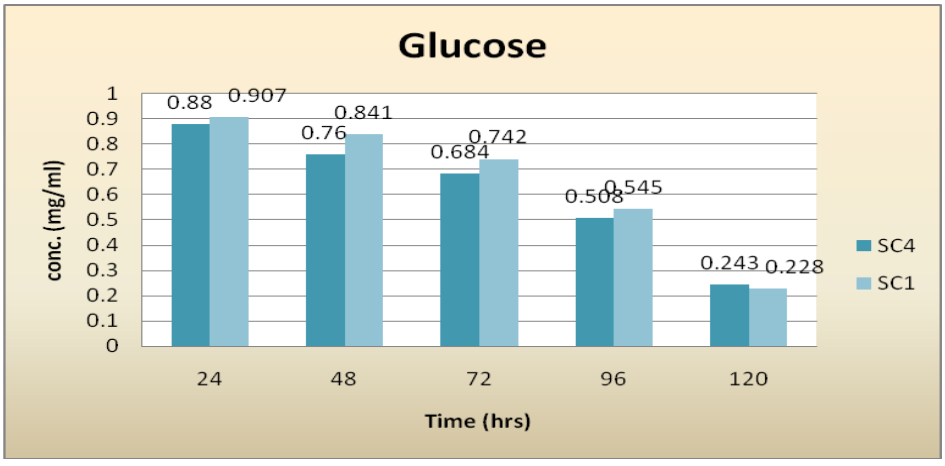


Fig.4 Estimation of residual sugar

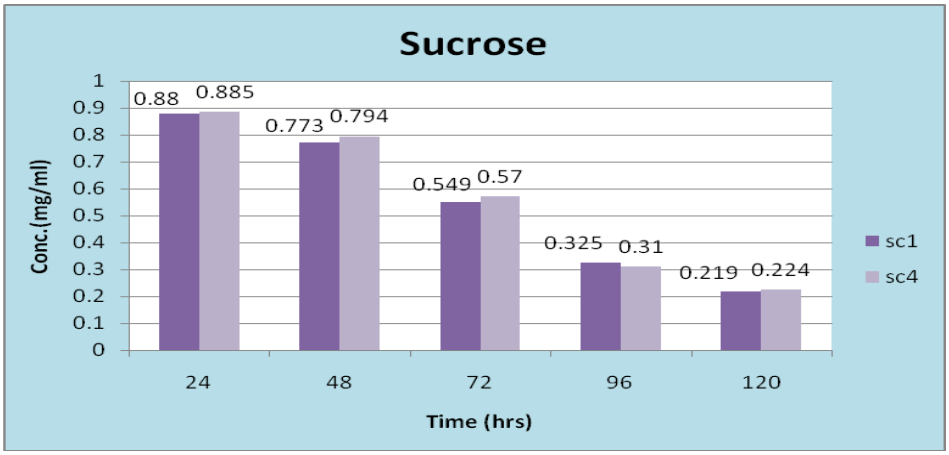


Fig.5 Estimation of residual sugar

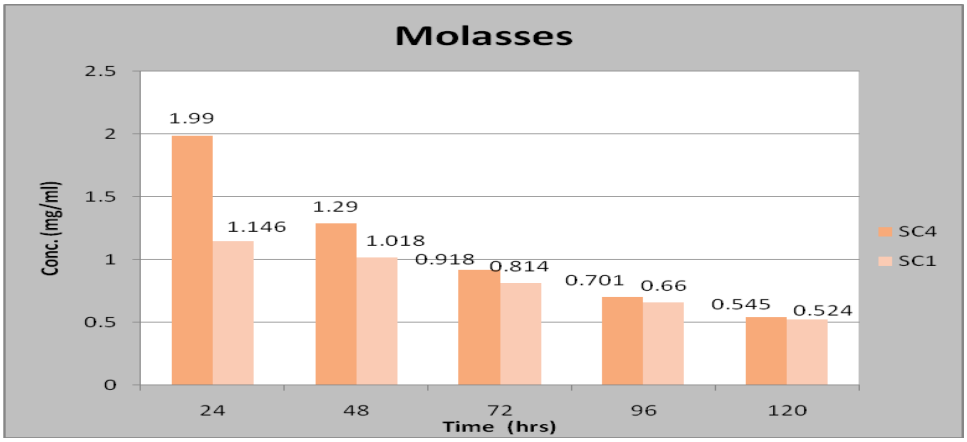


Fig.6 Effect of incubation conditions on residual sugar

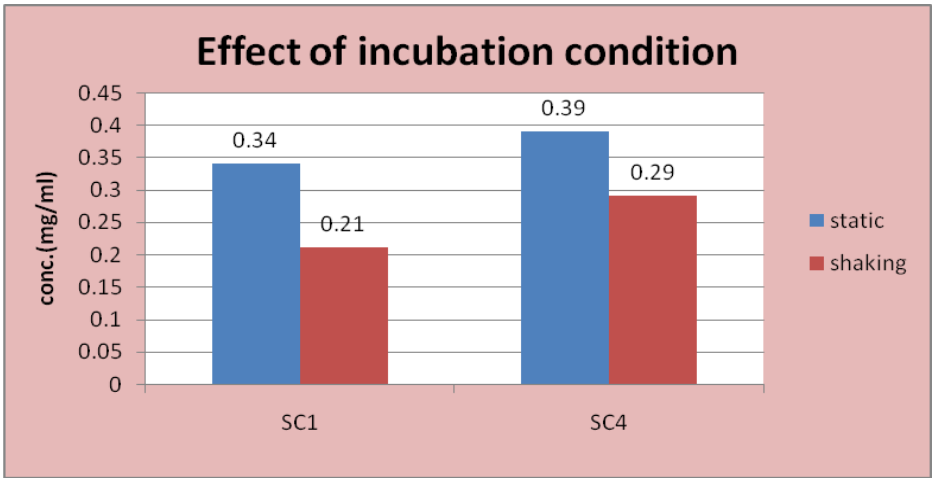


Fig.7 FT-IR spectra of standard xanthan gum

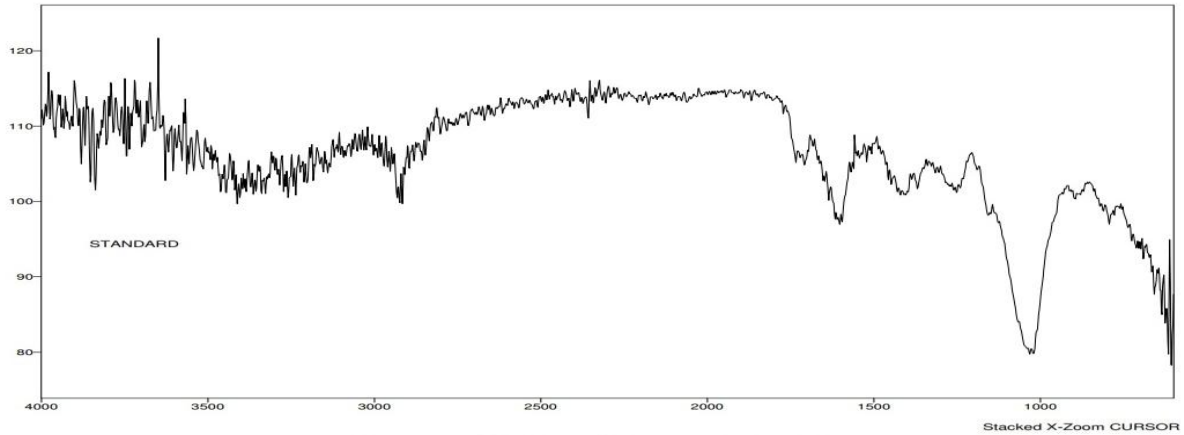


Fig.8 FT-IR spectra of produced xanthan gum from SC4

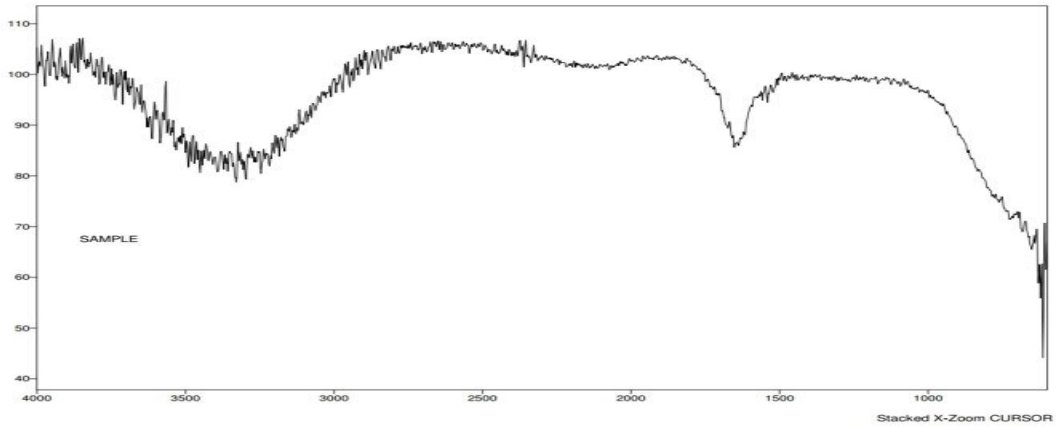
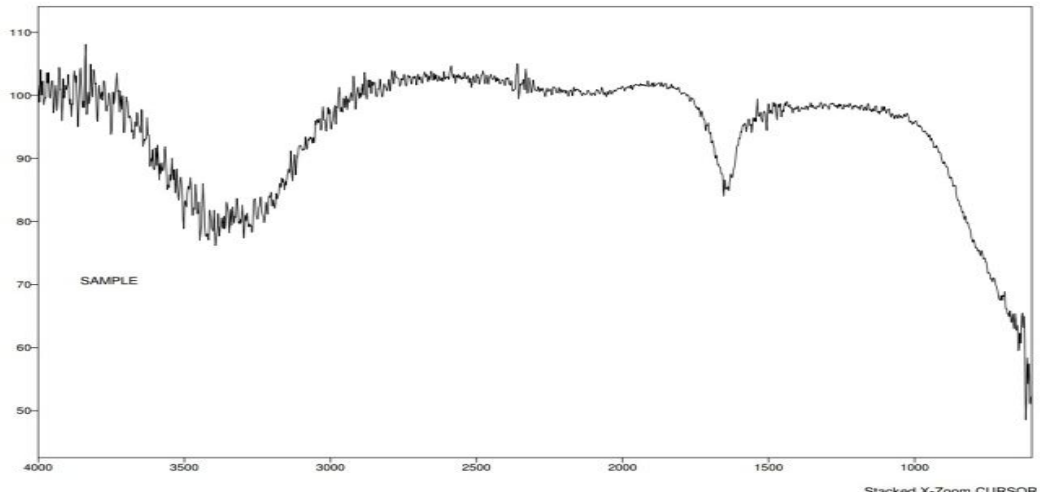


Fig.9 FT-IR spectra of produced xanthan gum from SC1



The infrared spectra of standard xanthan and produced xanthan of SC1 and SC4 showed that the most important bands recorded were: 3400-3450 cm^{-1}: axial deformation of -OH; 2850-2950 cm^{1}: axial deformation of C-H and CHO; 1700-1600 cm^{-1}: C=O carboxylic acid; 800-600 cm^{-1}: C-Cl. The infrared spectrum of the SC1 and SC4 was quite similar to commercially available xanthan gum. This result is almost similar to (Velu *et al.*, 2016).

In conclusion, the production of xanthan gum by using vegetables samples was carried out. Several bacteria were isolated from these sample and they were then characterized by cultural and morphological characteristics and biochemical test. Obtained isolates SC1 and SC4 was identified as *Xanthomonas* spp. These isolates were further optimized for xanthan production by using different carbon sources (glucose, sucrose and molasses). Among them sucrose acted as best carbon source for the xanthan gum production. The highest viscosity and recovery was obtained in 2% sucrose medium in SC1 and SC4. Different incubation conditions revealed that shaking condition (200 rpm) showed higher production of xanthan than static conditions. As compared to SC4, SC1 gave high production of xanthan. On analysis FT-IR spectra proved a correlation value between synthesized and standard xanthan gum, indicating quite similar results with that of standard. The product can be further tested for its production on large scale to be applied in food agricultural and pharmaceutical industry.

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