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RESEARCH ARTICLE

BIOCHEMICAL CHARACTERIZATION OF LOCALLY ISOLATED STRAIN PRODUCING XANTHAN GUM AND KINETIC MODELLING

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ABSTRACT

In the present investigation the biochemical characteristics of a local isolated strain producing xanthan gum was studied. The strain was isolated by serially diluting the extract of infected banana petioles. Biochemical characteristics such as gram staining, growth on asparagines medium, growth on nutrient agar, salt tolerance, starch hydrolysis, hydrogen sulfide production, oxidase test and catalase test were studied. Xanthan gum production was carried out at pH - 7, Temperature -30°C, glucose concentration – 40 g/1 and inoculums size - 10 % (v/v). The maximum xanthan gum production of 21.8 g/l was achieved at 96 hrs. The produced xanthan gum was compared with that of the commercial xanthan gum by FTIR spectra. The ability of the various growth models, namely, Logistic, Monod, Herbert, Shehata & Marr, Tessier and Haldane models in representing the batch kinetic data of the present work were analyzed, while Logistic model is best suited in describing the xanthan gum production of the present work. Substrate utilization and product formation kinetics were also studied.

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INTRODUCTION

Xanthan gum is an extracellular hetero-polysaccharide, which is produced by the aerobic fermentation of *Xanthomonas campestris* (Baird JK 1989). It was discovered in the 1950s at the Northern Regional Research Laboratories (NRRL) of the United States Department of Agriculture (Gils PS *et.al* 2009). 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-acetyl groups (Jansson PE *et.al* 1975). The annual worldwide production of xanthan gum is 30,000 tons.

Xanthan gum has excellent solubility and stability under both acidic and alkaline conditions. It is a water-soluble hetero-polysaccharide. It is used in textile printing pastes, ceramic glazes, slurry explosive formulations, and rust removers (Rosalam S *et.al* 2006). Xanthan gum is used in many applications, mainly in food industry as thickening, suspending and stabilizing agent.

It is widely used in a broad range of industries, such as in toiletries, cosmetics, as water-based paints, etc., due to its superior rheological properties and is also used as a rheological control agent in aqueous systems and as stabilizer for emulsions and suspensions. High viscosity of solutions and water solubility of the polymer have created important applications for xanthan in the petroleum industry where it is commonly used in drilling fluids and in enhanced oil recovery processes (Bradford PA *et.al* 1983).

In this present work, xanthan gum is produced by submerged aerobic fermentation by using locally isolated strain from infected banana petioles. Biochemical characteristics of locally strain were studied. At the end of fermentation, the broth contains xanthan gum, bacterial cells, and many other chemicals. For recovering the xanthan gum, the cells are usually removed first, either by filtration or centrifugation. Further purification may include precipitation using ethanol. After precipitation, the product is mechanically dewatered and dried.

MATERIALS AND METHODS

Isolation of strain and maintenance

A local strain was isolated from infected banana petioles. The diseased petiole tissues were cut into small pieces, soaked in 10ml sterile distilled water and 10^{-5} dilution of the suspension was streaked on to the agar plates (10 g/l glucose, 3 g/l yeast extract, 3 g/l malt extract, 5 g/l peptone and 20 g/l agar). The plates were incubated at 30°C for 24h.

Individual bacterial colonies developed were purified by re-plating on selected medium and stored at 4°C for further use. The inoculums was prepared in the same medium without agar and incubated for 24 h at 30°C.

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Biochemical characteristics of locally isolated strain

Gram staining reaction

The Gram-reaction of each isolate was determined following the staining procedure (Schaad NW 1988). First, thinly spread bacterial smear was prepared on a clean slide, dried in air and fixed by heating. The dried smear was flooded with crystal violet solution for one minute and washed in tap water for few seconds. It was again flooded with iodine solution for one minute and washed and blot-dried. It was then decolorized with 95% ethyl alcohol by applying drop by drop until no more color flows from the smear and washed and blot dried. Finally slides were counter stained for about 10 seconds with safranin, washed and examined under microscope using oil immersion objective. Isolates that appeared pink, Gram negative bacteria were subjected for further tests.

Growth on Asparagine medium

All Gram-negative isolates were allowed to grow on Asparagine medium (Asparagine, 0.5g; KH_2PO_4, 0.1g; $MgSO_4.7H_2O$, 0.2g; KNO_3, 0.5g; $CaCl_2$, 0.1g; NaCl, 0.1g and agar, 12-15g (for plates) in 1 liter distilled water with PH 7 and autoclaved at 121 ^oC for 15 minutes) at 28 ^oC for 48-72 hours without any other carbon and nitrogen sources (Dye DW *et.al* 1980). This is used as a diagnostic test for *Xanthomonas* as they are not able to grow on it while others like yellow *Enterobacteriaceae* and many *Pseudomonads* can grow on it. The growth of the bacteria on Asparagine agar plates and broth was recorded and those isolates that were unable to grow on the medium were taken for further tests. In all cases, uninoculated medium was taken as negative control. Isolates that grew on Asparagine but which formerly showed yellowish mucoid growth and found grown alone on YPSA medium initially were included in further tests.

Growth on Nutrient agar with 5% Glucose

Each isolate was streaked on nutrient agar with 5% glucose (Nutrient agar, 23g; 5% Glucose in 1 liter distilled water with PH 7 and autoclaved at 121 ^oC for 15 minutes) and incubated at 28 ^oC for 48-72 hours. Mucoid and yellow colony growth on this medium is one of the characteristics that differentiate *Xanthomonas campestris* from other *Xanthomonas* species (Bradbury JF 1984). Therefore the growth and colony color of each isolate was recorded.

Salt Tolerance

Isolates were inoculated to nutrient broth with 0%, 1%, 2%, 3%, 4% and 5% NaCl concentration to evaluate their salt tolerance (Hayward AC 1964). Inoculated salt free (0%) nutrient broth was used as positive control and uninoculated broth of each salt concentration was used as negative control and the presence or absence of growth was recorded.

Starch hydrolysis

The isolates were streaked on starch agar medium (starch soluble, 20g; Peptone, 5g; Beef extract, 3g; agar, 15g in 1 liter distilled water with PH 7 and autoclaved at 121 ^oC for 15 minutes) to evaluate their ability to hydrolyze starch (amylase production). The plates were incubated at 28 ^oC and for 2-3 days starch hydrolysis was observed by flooding the plates with Gram's iodine solution for 30 seconds. The appearance of clear zone around the line of growth of each isolate indicated starch hydrolysis (Aneja KR 1996).

Hydrogen sulfide production

The isolates were evaluated for H_2S production using Sulphide Indole Motility (SIM) agar medium (Peptone, 30g; Beef extract, 3g; Ferrous ammonium sulfate, 0.2g; Sodium thiosulphate, 0.025g and Agar, 3g in 1 L distilled water autoclaved at 121 ^oC for 15 minutes). The isolates were inoculated by stabbing and incubated at 28 ^oC for 48-72 hours. The presence of black coloration along the line of stab inoculation was recorded as positive for H_2S production (Aneja KR 1996).

Oxidase Test

A small loopful of each bacterial isolate was rubbed on a filter paper with drops of 1X (W/V) aqueous N, N, N, N- tetra methyl -p-Phenylenediamine dihydrochloride solution. Isolates which developed purple color within 10 seconds were taken as positive, purple color in 10-60 seconds were taken as slow positive and those with no color for more than 60 seconds taken as negative to oxidase test (Dickey R S *et.al* 1988).

Catalase Test

Few drops of 3% hydrogen peroxide was added on the surface of 48 hours old culture of each isolate on YPSA medium and bubble formation was recorded as positive for catalase activity (Dickey RS *et.al* 1988).

Production media and culture conditions

The production of Xanthan gum was carried out in 500ml Erlenmeyer flasks with 100ml medium containing (g/l) : glucose 40, yeast extract 3, peptone 5, KH_2PO_4 5, $MgSO_4.7H_2O$ 0.2, $(NH4)_2SO_4$ 2.7, citric acid 2, H_3BO_3 0.06, $ZnCl_2$ 0.06 and $CaCO_3$ 0.02. The initial pH of the medium was adjusted to 7.0 with 1 N NaOH and incubated for 4 days at 30°C in an orbital shaker.

Fermentations

Experiments were carried out in 500ml Erlenmeyer flasks containing 90ml of medium and 10 ml of the inoculums (grown for 24 h on MGYP medium). Production medium was incubated for 4 days at 30°C in an orbital shaker at 200 rpm (constant agitation was maintained throughout the experiments) and a constant airflow rate of 1 vvm was maintained.

Determination of xanthan gum concentration

Xanthan gum was recovered from the samples by centrifugation at 10000g for 30 minutes at 4^oC. Xanthan gum in the supernatant was precipitated using ethanol (1:2 v/v). The solution was maintained at 5^oC for 24h and re-centrifuged at 10000g for 30 minutes at 4^oC. The centrifuged precipitate was collected which is to be the required product, xanthan gum (Rumelhart D *et.al* 1986). The moisture was removed from precipitate by lyophilization.

Spectroscopy of Fourier transform infrared (FTIR)

Fourier transform infrared spectroscopic analysis was performed at the Facility of Chemical Engineering at

Annamalai University, Tamil Nadu – India. Samples of commercial xanthan gum (CX) and produced xanthan gum (PX) were analyzed by operating in the spectral window from 400 to 4000 waves/cm using KBR pellets.

Kinetics and Modelling

Logistic Model

The logistic model states that the rate of growth of the cell is proportional to the cell mass concentration present at any time. When the cell mass reaches the stationary phase there is no growth and hence the rate becomes zero. The growth rate thus depends on how far the cell mass concentration is away from the stationary phase.

$$\frac{dX}{dt} = kX(X_S - X) \quad (1)$$

where, k is a constant defined as the specific growth rate. The Equation 1 implies that the growth rate increases with an increase in cell mass concentration and is independent of the substrate concentration.

Substrate Utilization Kinetics

Substrate consumption depends on the magnitude of three sink terms, the instantaneous cell mass growth rate, the instantaneous product formation rate and a cell mass maintenance function. The assumed kinetic form is a linear combination of these terms (Huang T.Y, *et.al*, 2006) and is given by,

$$\frac{dS}{dt} = \frac{1}{Y_{X/S}}\frac{dX}{dt} \quad (2)$$

This equation is used for substrate utilization kinetics. The yield coefficient of biomass ($Y_{X/S}$) is based on the substrate utilized which is defined as follows:

$$Y_{X/S} = \frac{Cell\ mass\ produced}{Substrate\ consumed} = -\frac{\Delta X}{\Delta S} = \frac{X - X_0}{S_0 - S} \quad (3)$$

These equations are rearranged and integrated with the set of initial conditions $X=X_0$ and $S= S_0$ gives,

$$S = S_0 - \left[\frac{X - X_0}{Y_{X/S}}\right] \quad (4)$$

Product Formation kinetics

Product formation kinetics can be represented by logistic incorporated Luedeking-Piret model which is represented in Equations 5 and 6 respectively.

$$\frac{dp}{dt} = \alpha\frac{dx}{dt} \quad (5)$$

$$= Y_{P/X} = \left[\frac{P - P_0}{X - X_0}\right] \quad (6)$$

Where, p – xanthan gum concentration, g/l
– Growth associated product formation constant

RESULTS AND DISCUSSION

Morphological and Biochemical Characteristics

Sample collected from infected banana plants were grown on GPYM plates. The isolate showed (Fig.1) mucoid growth with deep yellow was tested for their reaction to Gram staining and for their growth ability on asparagine agar and broth. The isolate were found to be gram negative shown in Fig.2.

Fig1 Isolated strain

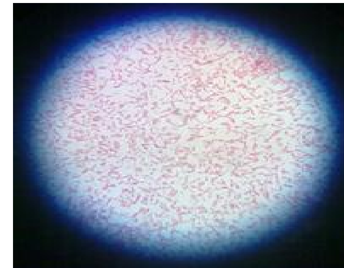

Fig2 Gram Staining Test

Fig3 Growth on Asparagine

Among the gram negative, that did not grow on asparagine medium in Fig.3. They are yellow and highly mucoid on YPSA plates and no other bacterial colonies were found grown together. The growth of the isolate on nutrient agar with 5% glucose medium, which is highly mucoid, is shown in Fig.4. For NaCl tolerance test shown in Fig.5 the isolate tolerated 1%, 2%, 3%, 4% and 5% NaCl. The strain produced H_2S gas shown in Fig.6. Oxidase reaction test was weak positive and Catalase reaction was positive shown in Fig.7. The strain hydrolyzed starch shown in Fig.8.

Fig4 Growth on Nutrient agar with 5% Glucose

Fig5 Salt Tolerance

Fig6 Hydrogen sulfide production

Fig7 Catalase test

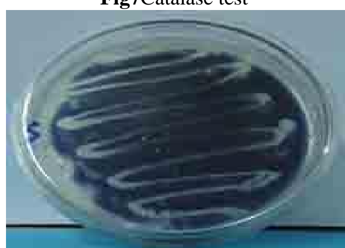

Fig8 Starch hydrolysis

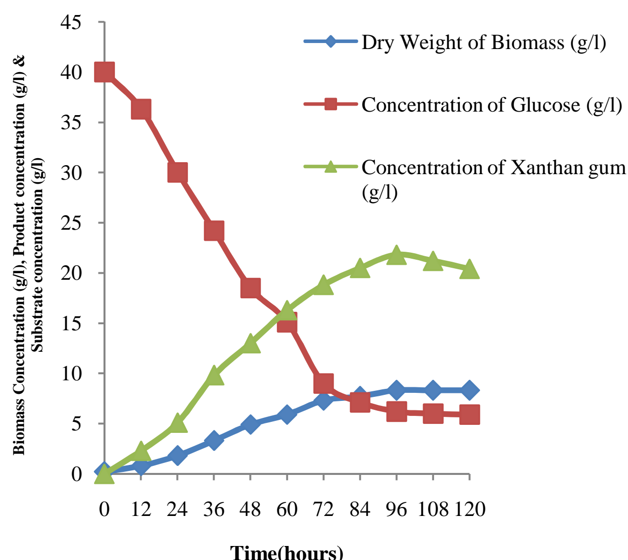

Figure 9 Effect of fermentation time on biosynthesis of Xanthan gum in batch culture

Effect of fermentation time

In order to find an optimum fermentation time for xanthan gum fermentation using glucose as substrate, the experiments were carried out for different fermentation times. The time profile of cell mass, xanthan gum and glucose concentration are presented in Fig.9. The xanthan gum concentration increases steeply up to the fermentation time of 72 h, after that, a gradual increase was observed up to a fermentation period of 96 h. After 96 h of fermentation, the declining trend of xanthan gum was observed. The same kind of tendency was observed for the cell mass. The growth rate of the biomass was slightly higher up to 60 hours of fermentation

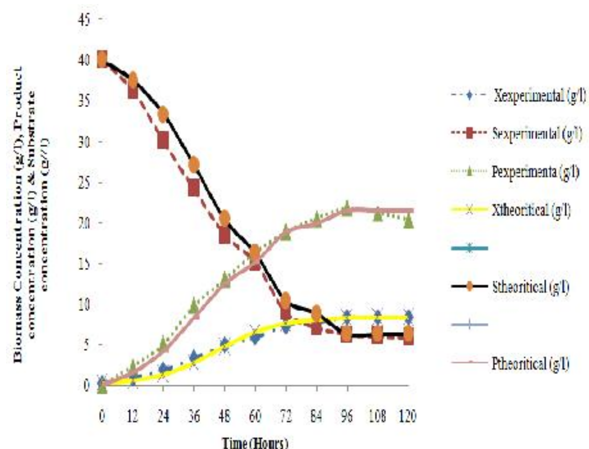

Figure 10 Comparison between experimental data (doter lines) and theoretical predictions (lines) for different substrate, product and cell mass concentration*s* of xanthan gum production using isolated strain

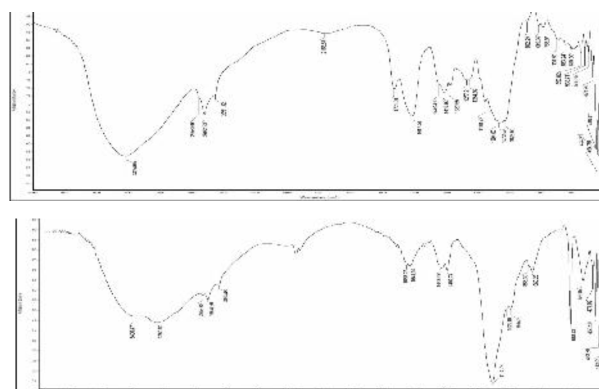

Figure 11(a) & 11 (b) FTIR spectra of commercial xanthan gum and produced Xanthan gum using local isolated strain

Table 1 Effect of fermentation time on biosynthesis of xanthan gum in batch culture

Time (hours)	Dry Weight of Biomass (g/l)	Concentration of Xanthan gum (g/l)	Concentration of Glucose (g/l)
0	0.2	0	40
12	0.8	2.3	36.3
24	1.8	5.1	30
36	3.3	9.8	24.2
48	4.9	13	18.5
60	5.9	16.3	15.1
72	7.3	18.8	9
84	7.7	20.5	7.1
96	8.3	21.8	6.2
108	8.3	21.2	6
120	8.3	20.4	5.9

when compared to the latter stages of fermentation. After 96 hours of fermentation, cell mass concentration reached constant. Over all, the highest concentration of xanthan gum (22 g/L) was obtained at a fermentation period of 96 h. Table 1 shows concentration of xanthan gum, concentration of biomass and glucose utilization at regular time intervals.

Model evaluation

Various growth, substrate utilization and product formation kinetic models were analyzed using the experimental data. The models which showed maximum fit are represented in Table 2 along with the estimated parameters. The optimized model parameter values are evaluated using MATLAB coding. Comparisons are made between the experimental data and the theoretical predictions as shown in Figure 10. The regression

Table 2 Values of the kinetic parameter and regression coefficient (R^2) for xanthan gum production using isolated strain

Model	Parameter Estimation	Regression coefficient (R^2)
Logistic	k=0.082 hr^{-1}, =0.12 L/g	0.9874
Substrate utilization	$Y_{X/S} = 0.24$	
Product formation	$Y_{P/X} = 2.65$	

Table 3 Growth kinetic parameters and regression obtained for different models during xanthan gum production using isolated strain

Model	Parameter estimation	Regression coefficient (R^2)
Monod model	$\mu_m = 0.1027$, $k_s = 10.14$	0.2275
Herbert model	$\mu_m = -2.671$, $k_s = 12.87$, m = 2.773	0.2275
Shehata & marr model	$\mu_m = 0.1042$, $\mu_1 = -19.62$, $k_1 = 0.1192$, $k_2 = 0.1459$	0.3715
Tessier model	$\mu_m = 0.08059$, $T_s = 9.15$	0.2797
Haldane model	$\mu_m = 23.89$, $k_i = 6.416$ $k_s = 32.88$	0.4202

coefficient values (R^2) were estimated for the substrate concentration and are presented in Table 3. From the regression coefficient values, it is clear that the correlation between the experimental data and the theoretical predictions are good. Thus, the Logistic model represents xanthan gum production very well using isolated strain.

Spectroscopic analysis (FT-IR)

The Fourier Transform-infrared spectrum (FT-IR) is a methodology to detect similarities or differences in chemical structures of compounds. The functional groups present in commercial xanthan gum (CX) and produced xanthan gum (PX) were analyzed and compared. The region studied included all the spectral bands located in the window between the wave numbers 400 and 4000 cm^{-1}. Figure 11(a) & 11(b) shows the infrared spectra of CX and PX using locally isolated strain. The most important bands recorded in the range of 3500–500 cm^{-1} were: 3400–3450 cm^{-1}: axial deformation of –OH; 2850–2950 cm^{-1}: axial deformation of C–H (may be due to absorption of symmetrical and asymmetrical stretching of CH_3 or even groups of CH_2) and CHO; 1700–1600 cm^{-1}: axial deformation of C=O ester, acid carboxylic, aldehydes and ketones; 1430–1650 cm^{-1}: axial deformation of C=O of enols (-diketones); 1410–1430 cm^{-1}: deflection angle C–H; and 1050–1100 cm^{-1}: axial deformation of C–O) [12]. Figure 10(a) & 10(b) shows that the infrared spectrum of the CX is very similar to that obtained for the PX using local isolated strain. Based on the results obtained from FTIR, the remote polysaccharide was found to follow the same spectral behavior as the standard.

CONCLUSION

The production of xanthan gum by the locally isolated strain was carried out. The isolated strain was found to be gram negative, no growth on asparagines medium, mucoid growth on nutrient agar, tolerate salt, hydrolysis starch, produces hydrogen sulphide, weak oxidase positive and catalase positive. Under the optimized fermentation time of 96 hrs, the isolated strain produced the maximum xanthan gum of 21.8 g/l after utilizing 84.5% of substrate. The batch kinetic data obtained for substrate utilization growth kinetics and product formation and fitted into various models. The substrate utilization data best fits with substrate utilization kinetics with yield co efficient of 0.24, the growth kinetic data fits with the Logistic model with a correlation coefficient of 0.9874 and Luedeking-Piret model fits for the product formation with yield co efficient of 2.65. Functional group of the produced xanthan gum was compared with that of the commercial xanthan gum by FTIR spectra.

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