ECOFRIENDLY BIOSYNTHESIS OF XANTHAN GUM BY XANTHOMONAS CAMPESTRIS.

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ABSTRACT

This work attempts to synthesize Xanthan-industrial gum using sugar molasses, Wheat bran, Rice Bran, Whey. By batch fermentation method Xanthan gum production has taken from each mentioned agro industry waste as substrate, and finally Xanthan yield calculated along with its cell mass (%) of each substrate. The characterization of Xanthan gum also been carried out using sophisticated analytical technique i.e. FT-IR ,which gives comparative analysis of Xanthan gum derived from waste with standard Xanthan gum.

Keywords: Xanthan, pseudo plasticity, viscosity, fermentation, substrate.

INTRODUCTION

Xanthan is a product from the plant pathogen Xanthomonas campestris. It has a cellulosic backbone on every second glucose residue of which a trisaccharide side chain is attached. This unusual structure confers physical properties to the polymer which are utilized in food and other industries.

Xanthan is stable at both acid and alkaline pH and forms pseudo plastic dispersion in water. Relatively low polysaccharide concentrations produce highly viscous solutions and the viscosity does not change greatly on raising the temperature. The solutions are compatible with many other ingredients in food and give good flavor release. Xanthan is also a good suspending and stabilizing agent for oil/water emulsions such as salad dressings, because of...
all these features and its inherent safety, xanthan received GRAS listing (Generally Regarded As Safe) for food use in the US after its initial discovery in the USDA laboratories in Peoria and its development by KELCO. Subsequently, the poly-saccharide received approval in the EU.

Xanthan gum is a heteropolysaccharide with a primary structure consisting of repeated pentasaccharide units formed by two glucose units, two mannose units, and one glucuronic acid unit, in the molar ratio 2.8:2.0:2.0. Its main chain consists of β-D-glucose units linked at the 1 and 4 positions. The molecular formulae of xanthan are \((C_{35}H_{49}O_{29})\)

In this present work, Xanthan gum is produced by submerged aerobic fermentation by using *Xanthomonas spp.* At the end of the fermentation, the broth contains xanthan, bacterial cells, and many other chemicals. For recovering the xanthan, the cells are usually removed first, either by filtration or centrifugation (10). Further purification may include precipitation using water-miscible nonsolvents (iso-propanol, ethanol, and acetone), addition of certain salts, and pH adjustments. After precipitation, the product is mechanically dewatered and dried.

**MATERIALS AND METHODS**

**Sample collection**

*Xanthomonas campestris* NCIM 2954 was obtained from NCL, Pune.

**Enrichment**

Enrichment of pure culture in PDB for 48 hrs. at 28°C on rotary shaker.

**Selection of medium and optimization of sugars**

Medium used for Inoculum Build up Sterile Potato Dextrose Broth. Medium for used for xanthan gum production *Garcia –Ochoa* production medium (100 ml) and *G.Pace medium.* Both G. Pace chemical medium and Garcia –Ochoa synthetic medium are complex medium which forms precipitation on whole medium sterilization. Thus, instead of whole media sterilization, different stock solutions were prepared and sterilized separately.

**Strains Used**

*X. campestris* NCIM 2954 (Obtained from (NCL).

**Fermentation process**

Inoculated a loopful of respective cultures in to 25 ml sterile PD broth. Kept it on shaker at
R.T. at 112 rpm for 5-6 hrs to prevent significant amount of gum production as this would retard the intake of essential nutrients by the cells during the growth phase.(5).

After 5-6 hrs, inoculated 10 ml (5-10 % of fermentation broth) of inoculum of respective strains into sterile Garcia-Ochoa production medium and G.Pace medium under aseptic conditions. Kept the flasks on the rotatory shakers at 112 rpm for three days. (3)

**Recovery of Xanthan Gum**

The broth obtained after 72 hrs of fermentation 6000 rpm for 20 min. The supernatant was used for precipitating xanthan gum. The pellet obtained was resuspended in distilled water and centrifuged at 4000 rpm for 10 min. The pellet thus obtained was then transferred to a pre-weighed aluminum foil cup and dried in oven for 3-4 hrs at 60°C.

**Xanthan Gum Precipitation**

Supernatant (10 ml) was mixed 0.01 gm CaCl\textsubscript{2} powder. About 20 ml IPA was then added drop wise with constant mixing in order to precipitate xanthan. The mixture was then centrifuged at 4000 rpm for 10 min. The pellet comprising the xanthan was transferred to pre-weighed aluminum foil cup and dried in oven at 100°C till it was dried. The net weight of the gum was calculated by finding the final weight of the aluminum foil cup.

**Optimizations of carbon source i.e. sugar in selected G.Pace medium**

Glucose sugar solutions of various concentrations such as 20 gm/lit, 30 gm/lit, 40 gm/lit, 50 gm/lit were prepared and sterilized separately in separate conical flasks, and further used for inoculate in Sterile G.Pace medium without any change in any ingredient of the medium.

Four flasks containing sterile G.Pace medium supplemented with the 20 gm/lit, 30 gm/lit, 40gm/lit, 50 gm/lit of glucose concentrations. Then, all these flasks were inoculated with the selected strain of organism i.e. *X.campestris* NCIM 2954 culture. The flasks were kept on rotatory shaker for 3 days and later subjected to a recovery process.

**Use of alternate carbon source for the production of xanthan gum by batch culture method**

Use of alternate carbon source i.e. agri industry waste such as: Sugar molasses, Whey, Wheat Bran, Rice husk.
Extractions of sugar from Agriculture waste i.e. wheat bran and Rice Husk
First weighed 500 gm of wheat bran and Rice Husk .Then ground these both wastes separately in Mixer grinder. Soaked it in water overnight. Then. boiled these wastes separately in beaker for 30 min. After cooling, removed solid part manually. Then removed by filtration through suction pump. The filtrate of solubilized agricultural waste was incorporated into the culture medium as a carbon source.

Estimation of total sugar of all agro-industry waste by Phenol-sulphuric acid Method and standard graph preparation
Phenol sulfuric acid method was used to measure the total sugar in the sample. Stock solution of 200 microgram/ml of standard sugar (glucose) was prepared (For composition kindly refers to Appendix B). Then prepared various concentrations of standard sugar, (table-6), then added 5 % phenol 1 ml in each concentration, then 5 ml of conc. H₂SO₄ added in each stayed for 30 min. And finally taken absorbance at 480 nm to measure color intensity. Each sample (i.e. agro industry waste) kept as a unknown sample, which also been assayed by same way. absorbance had been taken for each sample. And finally by plotting standard graph estimated a unknown concentration of total sugar from each waste.

Estimation of reducing sugar by DNSA method and Standard graph preparation
3, 5, di-nitro salicylic acid method is used to determine reducing sugar from respective agro-industry wastes. First, stock solution of standard sugar (Maltose) 100mM was prepared (For composition kindly refer to Appendix B). Prepared various concentrations of standard sugar, added 1 ml of DNSA reagent. Boiled all the tubes for 10 min. Then cooled diluted up to 10 ml by adding 8 ml distilled water. Optical density measured at 540 nm. Each sample (i.e. agro industry waste) kept as a unknown sample, which also been assayed by same way, absorbance had been taken for each sample.

And finally by plotting standard graph estimated a unknown concentration of reducing sugar from each sample before xanthan gum production and after xanthan gum production.

Production of xanthan gum from alternate carbon sources
The same batch fermentation process is followed for the production of xanthan gum from agro-industry waste, as used in selection of medium and optimization of sugar section. And result noted in form of dry weight of xanthan gum and cell mass in gram %.
CHARACTERIZATION OF XANTHAN GUM

FT-IR spectra of the exopolysaccharides were obtained. The sample was incorporated into KBr (spectroscopic grade) and pressed into a 2 mm pellet. IR spectra were recorded in the transmittance mode from 4000 to 400 cm\(^{-1}\), using Shimadzu FT-IR 8400S model.

Observations and Results

The yield of xanthan was close to standard value 1.3g%. Xanthomonas spp. Comparison of growth and production of the respective organisms in Garcia-Ochoa medium and G.Pace medium are given below Table 1.2 and 1.3 supplemented with 40 gm/lit concentration of sugar, kept on rotatory shaker for 3 days at 112 rpm. This is optimized sugar concentration for the growth of organism as well as higher yield of xanthan gum, as we observed in Table 1.4 more than 40 gm/lit concentration doesn’t give a higher yield of xanthan gum i.e. at 50 and 60 gm/lit. Lower yield is observed shown in Graph 1.1 Total sugar from each substrate was estimated by plotting a standard graph, the unknown concentration of each waste is found form graph, and Reducing sugar from each substrate was estimated by plotting a standard graph, the unknown concentration of each waste is found form graph before xanthan gum production, obtained values are noted obtained values are noted in Table 1.5

As result shows that from sugar molasses as compare to other substrate gives a maximum xanthan yield of 1.20 gm % and less yield by whey i.e. 0.50 gm %.

The FT-IR spectra of the EPS from commercially available xanthan gum are shown in Graph 1.3 A broad absorption peak at 3317.34 cm\(^{-1}\) indicates the hydrogen bonded OH groups. Two peaks, one at 105.39 cm\(^{-1}\) and the other at 458.98 cm\(^{-1}\), are attributed to C-C or C-O and glycoside bending groups. A band of 2894.51 cm\(^{-1}\) is due to C-H bending from C-H\(_2\) and C-H\(_3\) vibrations, respectively. The peaks at 1602.74 cm\(^{-1}\) (COO\(^{-}\) asymmetric stretching) and 1419.51 cm\(^{-1}\) (COO\(^{-}\) symmetric stretching) are due to carboxyl group. As compared with commercially available xanthan gum FT-IR spectra, The FT-IR spectra of the EPS from rice husk are shown in Graph 1.4 A broad absorption peak at 3311.55 cm\(^{-1}\) indicates the hydrogen bonded OH groups. A band of 2839.02 cm\(^{-1}\) is due to C-H bending from C-H\(_2\) and C-H\(_3\) vibrations, respectively. The peaks at 1622.02 cm\(^{-1}\) (COO\(^{-}\) asymmetric stretching) and 1415.65 cm\(^{-1}\) (COO\(^{-}\) symmetric stretching) are due to carboxyl groups. As compared with commercially available xanthan gum FT-IR spectra, The FT-IR spectra of the EPS from sugar molasses are shown in Graph 1.5 A broad absorption peak at 3326.98 cm\(^{-1}\) indicates
the hydrogen bonded OH groups. A band of 2900.74 cm$^{-1}$ is due to C–H bending from C-H$_2$ and C-H$_3$ vibrations, respectively. The peaks at 1620.09 cm$^{-1}$ (COO$^-$ asymmetric stretching) and 1415.65 cm$^{-1}$ (COO$^-$ symmetric stretching) are due to carboxyl groups.

As compared with commercially available xanthan gum FT-IR spectra, The FT-IR spectra of the EPS from whey are shown in Graph 1.6. A broad absorption peak at 3309.62 cm$^{-1}$ indicates the hydrogen bonded OH groups. A band of 2839.02 cm$^{-1}$ is due to C–H bending from C-H$_2$ and C-H$_3$ vibrations, respectively. The peaks at 1602.74 cm$^{-1}$ (COO$^-$ asymmetric stretching) and 1415.65 cm$^{-1}$ (COO$^-$ symmetric stretching) are due to carboxyl group. As compared with commercially available xanthan gum FT-IR spectra, The FT-IR spectra of the EPS from wheat are shown in Graph 1.7. A broad absorption peak at 3292.26 cm$^{-1}$ indicates the hydrogen bonded OH groups. A band of 2837.09 cm$^{-1}$ is due to C–H bending from C-H$_2$ and C-H$_3$ vibrations, respectively. The peaks at 1602.74 cm$^{-1}$ (COO$^-$ asymmetric stretching) and 1409.87 cm$^{-1}$ (COO$^-$ symmetric stretching) are due to carboxyl groups.

As per the FT-IR spectra obtained, all shows same pattern of spectra with standard xanthan gum spectra that means recovered crude samples of xanthan were identical to standard xanthan.

**DISCUSSION**

The pure culture of *Xanthomonas campestris* NCIM 2954 shows 0.8 g/100ml of xanthan production. The yield of xanthan was closed to the standard value 1-3 g%. The final xanthan yield would also include the weight of CaCl$_2$ powder (0.1%) used for precipitation, as per FDA guidelines, IPA is recommended it as a precipitating agent. Since, CaCl$_2$ reduces the amount of IPA required; it was used for the precipitation process. The need to add salt depends on the final use of the end product, ‘Xanthan’.

As we discussed earlier, those macronutrients such as carbon and nitrogen, glucose and sucrose are the most frequently used carbon sources. The concentration of carbon source affects the xanthan yield; a concentration of 2-4 % is preferred. Higher concentrations of these substrates inhibit growth. To get a maximum growth and higher yield of xanthan optimization (Use of different concentrations of sugar) is done. During this study, the selected chemical medium defined by G.Pace varied with respect to carbon source i.e. sugar...
here in this medium 2% sugar concentration is used for the growth of organism.

Analysis of these used agro industry waste in these experiments showed the higher presence of total soluble sugars (glucose and sucrose). Rice straw an agricultural by product which has sufficient amount of sugar (38%), used as an alternative energy source for microorganisms, which is renewable, efficient, safe, ideally an inexpensive and abundantly available carbon source. Cellulose is degraded to fermentable sugar through cellulase enzymes. From whey also give maximum yield if respective strain will genetically modified. Sugar molasses showed 58% of total sugar.

Due to their high level of sugar, these are suitable for the production of xanthan biogum. Previous works have demonstrated the fermentative production of xanthan gum by X. campestris using various substrates such as dates, glucose, and peach pulp. Fermentation by X. campestris using sugar molasses at 28°C and 112 rpm showed that xanthan gum was produced in satisfactory yields. Salah et al. (2010) reported the optimal conditions for xanthan.(20)

Xanthan production by Xanthomonas campestris increased with an increase in fermentation period, and reached a maximum value of 1.20 g/100 mL in sugar molasses and 1.00 g/100 mL in rice husk after 72 hrs. The results show that these agro industries waste seems to be a better source of carbon for xanthan production and is apparently, a useful source of raw material for this process.

Therefore a useful biogum from various waste material containing a good source of carb for xanthan production i.e. producing xanthan which serves to control a environmental pollution and also a reuse or utilization of waste or useless material for the production of useful product from microorganism.

To produce xanthan gum, X. campestris needs several nutrients, including micronutrients (e.g. potassium, iron, and calcium salts) and macronutrients such as carbon and nitrogen. Maintenance of pH was essential for maximum yield of xanthan gum. Biomass of Xanthomonas campestris using different carbon and nitrogen sources. The biomass was gradually increased during fermentation period due to increase in its growth.

Comparative study of FTIR reports of commercial Xanthan and newly produced Xanthan by various agro-industry waste ensures the purity of recovered product. The goal of any absorption Spectroscopy (FTIR, ultraviolet-visible ("UV-Vis") spectroscopy, etc.) is to
measure how well a sample absorbs light at each wavelength. The most straightforward way to do this, the "dispersive spectroscopy" technique, is to shine a monochromatic light beam at a sample, measure how much of the light is absorbed, and repeat for each different wavelength. Specifically, the main difference in FT-IR absorption peak for tested exopolysaccharides and commercial xanthan was the presence of a band at 1718 cm$^{-1}$ in commercial xanthan which is attributed to C=O stretching of the acetyl ester. Comparative study of FTIR reports of commercial xanthan and newly produced xanthan by batch fermentation different different agro-industry wastes ensures the purity of recovered product.

Table 1.1 Typical properties of commercial xanthan gum.

<table>
<thead>
<tr>
<th>Sr.No.</th>
<th>PROPERTY</th>
<th>VALUE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Physical status</td>
<td>Dry, cream colored powder</td>
</tr>
<tr>
<td>2</td>
<td>Moisture (%)</td>
<td>8-15</td>
</tr>
<tr>
<td>3</td>
<td>Ash (%)</td>
<td>7-12</td>
</tr>
<tr>
<td>4</td>
<td>Nitrogen (%)</td>
<td>0.3-1.0</td>
</tr>
<tr>
<td>5</td>
<td>Acetate content (%)</td>
<td>1.9-6.0</td>
</tr>
<tr>
<td>6</td>
<td>Pyruvate content (%)</td>
<td>1.0-5.7</td>
</tr>
<tr>
<td>7</td>
<td>Monovalent Salts (%)</td>
<td>3.6-14.3</td>
</tr>
<tr>
<td>8</td>
<td>Divalent Salts (%)</td>
<td>0.085-0.17</td>
</tr>
<tr>
<td>9</td>
<td>Viscosity (cP)</td>
<td>13-15</td>
</tr>
</tbody>
</table>

Table 1.2 Cell mass and Xanthan yield in G.Pace.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Dried cell mass (gm%)</th>
<th>Xanthan yield (gm %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>X.campestris NCIM 2954</td>
<td>0.1%</td>
<td>0.9%</td>
</tr>
</tbody>
</table>

Table 1.3 Cell mass and Xanthan yield in Garcia-ochao

<table>
<thead>
<tr>
<th>Strain</th>
<th>Dried cel mass (gm%)</th>
<th>Xanthan yield (gm %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>X.campestris NCIM 2954</td>
<td>0.15%</td>
<td>0.8%</td>
</tr>
</tbody>
</table>
Table 1.4 The effect of different sugar concentration on Xanthan yield (gm %) and cell mass (gm %).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Concentration of sugar</th>
<th>Cell mass (gm %)</th>
<th>Xanthan Yield (gm %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>X. campestris NCIM 2954</td>
<td>30 gm/lit</td>
<td>5.2%</td>
<td>8.5%</td>
</tr>
<tr>
<td></td>
<td>40 gm/lit</td>
<td>3.5%</td>
<td>9.0%</td>
</tr>
<tr>
<td></td>
<td>50 gm/lit</td>
<td>0.7%</td>
<td>1.3%</td>
</tr>
<tr>
<td></td>
<td>60 gm/lit</td>
<td>0.3%</td>
<td>1.1%</td>
</tr>
</tbody>
</table>

Graph 1.1 The effect of different sugar concentration on Xanthan yield (gm %) and cell mass (gm %)

Table 1.5 Estimation of total sugar and reducing sugar (before and after xanthan production from each agro–industry waste.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Total sugar (gm/ml)</th>
<th>Amount of Fortified sugar (grams)</th>
<th>Total sugar in final medium (4gm/ml)</th>
<th>Total reducing sugar before xanthan production (gm/ml)</th>
<th>Total reducing sugar after xanthan production (gm/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sugar molasses</td>
<td>3.29978</td>
<td>0.70022</td>
<td>4</td>
<td>0.02089</td>
<td>0.00648</td>
</tr>
<tr>
<td>Whey</td>
<td>2.17760</td>
<td>1.92240</td>
<td>4</td>
<td>0.03495</td>
<td>0.00144</td>
</tr>
<tr>
<td>Wheat bran</td>
<td>0.96840</td>
<td>3.03160</td>
<td>4</td>
<td>0.02702</td>
<td>0.00504</td>
</tr>
<tr>
<td>Rice Husk</td>
<td>0.43760</td>
<td>3.56240</td>
<td>4</td>
<td>0.01116</td>
<td>0.00288</td>
</tr>
</tbody>
</table>
Table 1.6 and Graph 1.2  Growth and Production of respective organism xanthan gum from alternate carbon sources in G.pace medium

<table>
<thead>
<tr>
<th>Strain</th>
<th>Substrates</th>
<th>Cell mass (gm%)</th>
<th>Xanthan Yield (gm %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>X.campestris NCIM 2954</td>
<td>Sugar molasses</td>
<td>0.2%</td>
<td>1.2%</td>
</tr>
<tr>
<td></td>
<td>Wheat Bran</td>
<td>0.2%</td>
<td>1.0%</td>
</tr>
<tr>
<td></td>
<td>Rice Husk</td>
<td>0.1%</td>
<td>0.9%</td>
</tr>
<tr>
<td></td>
<td>Whey</td>
<td>0.1%</td>
<td>0.5%</td>
</tr>
</tbody>
</table>

Graph 1.2  Growth and Production of respective xanthan gum from alternate carbon sources in G.pace medium

Graph 1.3 FT-IR spectra of Xanthan gum EP food grade standard.
Graph 1.5 FT-IR spectra of xanthan gum derived from agro waste Rice husk as substrate.

Graph 1.6 FT-IR spectra of xanthan gum derived from sugar industry waste sugar molasses.

Graph 1.7 FT-IR spectra of xanthan gum derived from dairy industry waste whey.
CONCLUSIONS

The *X.campestris* NCIM 2954 gives maximum xanthan in chemical medium. Thus, the strain *X.campestris* and chemical medium defined by G.Pace was further selected for optimization. The optimized sugar concentration, as optimized carbon source found to be 40 gm/lit. Xanthan gum is produced from alternate carbon sources with maximum xanthan yield in G.Pace medium by using *X.campestris* NCIM 2954.

According to the results or from obtained spectra of FT-IR ,it has been confirmed that the crude xanthan obtained from various waste substrate is same as the commercially available xanthan gum.

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