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Original Article

Microbial Production of Xanthan Gum Using Various Agro Wastes and Molecular Characterization of Xanthan Gum Producing Isolates

O. I. Ajayi[1,2]*, T. A. Okedina[1], C. P. Onyemali[1], J. Ehiwuogu-Onyibe[1], A. D. Aina[2], O. A. Thonda[2], A. K. Lawal[1], J. A. Tutuwa[1]

[1]*Department of Biotechnology, Federal Institute of Industrial Research, Oshodi, P.M.B. 21023, Ikeja, Lagos State, Nigeria.*
[2]*Department of Microbiology, School of Science and Technology, Babcock University, Ilisan 121003 Remo, Ogun State, Nigeria*

ABSTRACT
Xanthan gum is a microbial exopolysaccharide (natural polymer) with outstanding rheological properties produced by Xanthomonas sp through fermentation. It has unique rheological properties pseudo plastic behaviour and physico-chemical characteristics; xanthan gum is extensively used as thickening or stabilizing agent emulsifier, thickener, friction and water mobility reducer in the food, cosmetics, pharmaceutical and oil recovery industries. However, the cost of producing xanthan gum is high hence, the need to source for low-cost material as agro waste as sole substrate that can be utilized in the producing of xanthan gum to reduce production cost and environmental impacted associated agro waste disposal. Xanthan gum was produced using agro waste (sugarcane bagasse, sugarcane peel and pineapple peel) via standard microbiological techniques for xanthan gum production. The xanthan gums produced were characterised using Fourier Transform Infrared spectroscopy (FTIR) and molecular characterization of isolates was carried out using 16S rRNA gene sequencing. Xanthan gum produce by Xanthomonas sp strain SFL2 using sugarcane peel had the highest yield of g/L. The FTIR characterization of the xanthan gums produced revealed functional groups such as Hydroxyl (-OH), Carbonyls (-C=O), Carbonyl (-CHO) present. The molecular characterisation of the isolates revealed their identity as Xanthomonas species The present study confirmed that xanthan gum can be produced using sugarcane bagasse, sugarcane peel and pineapple peel and the characterisation of the xanthan gum produced revealed it can be used for various industrial purposes.
Keywords: Agrowaste, Xanthan gum, Xanthomonas sp, FTIR

INTRODUCTION

Xanthan gum is produced via fermentation by *Xanthomonas* sp a microorganism. Microorganisms are an omnipresent part of the living world, be it plants, animals, algae, fungi, bacteria, or archaea, and are known to play an essential role in maintaining their structural integrity and functionality [1]. *Xanthomonas* sp belongs to genus *Pseudomandaceae* and class *Gammaproteobacteria.* It contains species causing diseases in more than 400 different plant hosts such as rice, wheat, citrus, tomato, pepper, cabbage, cassava, banana and beans. *Xanthomonas* are gram negative aerobic, short rod shaped bacterium organism that can produce xanthan gum. Examples of *Xanthomonas* sp include *Xanthomonas campestris, Xanthomonas phaseoli* and *Xanthomonas malvacearum* amongst others. *Xanthomonas* sp are mesophilic with optimal temperature at 25 - 35 ºC characterised by its two cell walls and yellow pigment. They can live in soil for over a year and spread through any movement of water including rain, irrigation and surface water. The aerobic bacterium performs a number of metabolic pathways that are uniquely dependent on the pathovar. Glycolysis, Citrate Cycle, Pentose Phosphate Pathway and it gains energy through oxidative phosphorylation, carbon fixation, methane, nitrogen and sulphur metabolism [2]. They have the ability to produce various secondary metabolites such as EPS during their growth cycle. Of all these strains, the plant pathogen, *Xanthomonas campestris* is the most widely used for the industrial production of xanthan

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based on its high yield and the high quality product suitable for many applications.

Xanthan gum is one of very few microbial polysaccharides that has found multifarious applications in various food, chemical, petroleum and pharmaceutical industries as either thickeners or emulsifiers. [4,5,6,7,8]

Xanthan gum is produced via fermentation using either submerged or solid state fermentation. It can be produced using sugar such as sucrose, glucose others sources of these sugars such as agro waste (i.e. waste generated from agricultural practices) which causes environmental and public health problems. Commercially, the xanthan gum production uses glucose or sucrose as carbon source for media preparation, making the process limited by the processing costs. Mostly naturally found raw materials are utilized for industrial production of xanthan gum. Carbon source generally used are glucose, sucrose, or starch and sometimes acid whey, up to a concentration of 5% as greater concentrations could suppress both growth and final production. Due to its high cost of production a lot of alternative sources have been tired in its production. Some alternative sources have been suggested as substrate for the production of xanthan gum sources, such as cheese whey [9], whey [10,11,12] cassava serum [13], apple juice residue [14], cocoa residue [12], green coconut shell [15], sugar cane broth [16], treated tapioca pulp [17], shrimp cell [18], and glycerin derived from the biodiesel production process [19]. [17] used treated tapioca pulp as sources of nutrients for the fermentation medium to produce xanthan while [20] also evaluated the feasibility of using the waste hydrolyzate broth (green coconut shell, straw and corn cob and passion fruit peel) as a substrate for the production of xanthan gum. In their researches, $K_2H_2PO_4$, $MgSO_4$, $(NH_4)_2SO_4$, citric acid, H_3BO_3, $ZnCl_2$, $FeCl_3$, $CaCO_3$ were added to the medium and this improved the xanthan gum production but unfortunately increased its cost. Sucrose is an important sugar that has been used in the production of xanthan gum. Examples of some agricultural waste that contain sucrose include pineapple peel, sugar cane bagasse, sugarcane peel, sweet potato peel amongst others. Pineapple (*Ananas comosus*), the succulent and vibrant tropical fruit, belongs to the family *Bromeliaceae.* is a symbol of exoticism and sweetness. It contains sugar e.g. sucrose and it is readily available throughout the year. The peel is waste which has little or no use and as such it is discarded into the environment thereby constituting nuisance he pineapple peel, often considered as waste, has garnered attention for its potential applications. The pineapple peel is rich in essential nutrients, including calcium, potassium, vitamin C, carbohydrates, dietary fibre, and water, making it beneficial for the digestive system, weight management, and overall balanced nutrition. It contains significant amounts of sugars such as sucrose, glucose, and fructose, along with citric acid as the predominant organic acid. The peel also contains bromelain, a proteolytic enzyme known for its digestive properties. Studies have highlighted the pharmacological properties of pineapple peel, such as its potential anti-parasitic effects, alleviation of constipation, and benefits for individuals with irritable bowel syndrome (IBS). Its applications range from the production of vinegar, alcohol, and citric acid to the development of various food products, including squash, syrup, jelly, and pickles [21].

Sugarcane (*Saccharum officinarum L.)* is a giant tropical grass from the family *Graminaceae* whose stalk has the particular capacity to store crystallisable sugar sucrose. And its main use is in the industrial processing of rum. The sugarcane contains 75 - 85% water, 10 - 21% sucrose (non reducing sugar), 10 - 15% fibre, 0.3 - 3% reducing sugars (glucose and fructose) and other organic and inorganic compounds The liquid jaggery or semi liquid syrup is obtained during concentrating purified sugarcane juice for solid jaggery making. It contains 30 - 36% water, 40 - 60% sucrose, 15 - 25% invert sugar, calcium 0.3%, iron 8.5 - 10 mg/100mg, phosphorous 0.5 /100mg, protein 0.1/100mg and vitamin B 14/100mg . Sugarcane juice and its unrefined products are the richest source of phenolic acids, flavonoids, and different glycosides [22]. The peel and bagasse obtained after sucking its juice are waste which has little or no use and as such it is discarded into the environment. All of these wastes (sweet potato peel, sugarcane bagasse, sugarcane peel and pineapple) can be used by *Xanthomonas* sp to produce xanthan gum (exoploysaccharide) via fermentation since they all contain sucrose. The aim of the study is to utilization of low-cost material as such as agro waste to produce xanthan gum, characterized the xanthan gum produced as well as the isolates used in xanthan gum

production. This will help to reduce production cost and environmental impacted associated with agro waste disposal. FTIR characterisation of xanthan gum will help to know the function groups present and its possible application. More so, the molecular characterisation will help reveal the identity for the isolates and provide adequate information for future isolation purpose.

MATERIALS AND METHODS

Collection of isolates

Screened xanthan gum producing isolates RU1, RU3, RU4, PEAR4, BAN2, Sfl2 and Sfl3.were collected form the Biotechnology department of the Federal Institute of Industrial Research Oshodi, Lagos, Nigeria

Subcultruing of Isolates

The colonies of these isolates were purified on nutrient agar plates for use.

Molecular characterisation of xanthan gum producing isolates

Molecular characterization of xanthan gum producing isolate was carried out by the method described by [23].

DNA extraction

100 µL of nutrient broth containing 24 hours old culture of the isolate was dispensed into a microcentrifuge tube and 500µL of the Lysis Buffer was added. It was vortex and incubated at 56 ºC for 10 minutes. It was then centrifuge at 10,000 rpm for 1 minute. After spinning 200 µL of absolute ethanol was added to the tube the mixture was the transferred the mixture into the spin column and centrifuge at 10,000 rpm for 30 seconds. The flow-through was discarded and the collection tube was blotted on a tissue paper.500 µL of wash buffer was added to the spin column. It was then centrifuged at 10,000 rpm for 30 seconds. The flow-through was discarded and the collection tube was blotted on a tissue paper. 500 µL of wash buffer 2 was added to the spin column and centrifuge at 10,000 rpm for 1 minute. The flow-through was discarded and the collection tube was blotted on a tissue paper. It was then centrifuged (spin column) again at 12,000 to 14,000 rpm for 3 minutes to remove all traces of ethanol. The Spin column into was added into another microcentrifuge tube.50 µL Elution Buffer or nuclease-free water was added to the centre of the column and incubated at room temperature for 1 to 2 minutes. It was then centrifuge at 10,000 rpm for 1 min to elute the DNA .The DNA was stored at -80 °C till further use.

Polymerase chain reaction amplified the 16SrRNA gene (27F and 1492R). Polymerase chain reaction was carried out to amplify the 16SrRNA gene of the bacteria using the primer pair 27F- 5′-AGAGTTTGATCCTGGCT CAG -3’, and 1492R 5’-GGTTACCTTGTTACGACTT -3’. The PCR reaction was carried out using the Solis Biodyne 5X HOT FIREPol Blend Master mix. PCR was performed in 25 µL of a reaction mixture, and the reaction concentration was brought down from 5X concentration to 1X concentration containing 1X Blend Master mix buffer Buffer (Solis Biodyne), 1.5 mM $MgCl_2$, 200 µM of each deoxynucleoside triphosphates (dNTP)(Solis Biodyne), 25pMol of each primer (BIOMERS, Germany), 2 unit of Hot FIREPol DNA polymerase (Solis Biodyne), Proofreading Enzyme, 5µL of the extracted DNA, and sterile distilled water was used to make up the reaction mixture. Thermal cycling was conducted in an Eppendorf Vapo protect thermal cycler (Nexus Series) for an initial denaturation of 95 °C for 15 minutes followed by 35 amplification cycles of 30 seconds at 95 °C; 1 minute at 61 °C and 1 minute 30 seconds at 72 °C. This was followed by a final extension step of 10 minutes at 72 °C.

The amplified product was separated on a 1.5% agarose gel and electrophoresis was carried out at 80 V for 1 hour 30 minutes. After electrophoresis, DNA bands were visualized by ethidium bromide staining. 100 bp DNA ladder (Solis Biodyne) was used as DNA molecular weight marker. The PCR product was then sequenced to obtain the molecular composition of each isolates at Epoch Life Sciences United States of America.

Production and extraction of xanthan gum

The fermentation broth was prepared using potassiumdihydrogen phosphate [KH_2PO_4 (5 g/L)], magnesiumsulphatehexahydrate [$MgSO_4.7H_2O$ (0.2 g/L)], citric acid [(2 g/L)], ferric chloride [$FeCl_3.6H_2O$ (0.002 g/L)], calcium carbonate [$CaCO_3$ (0.02 g/L)], glutamate (2 g/L)]. Various agro waste (carbon sources) such as sugar cane bagasse, sugarcane peel and pineapple peel and yeast extract were used [24]. pH was adjusted to 7.0 with 1 M Sodium hydroxide solution before sterilization using autoclaving at 121 °C for 15 minutes. Briefly, freshly prepared inoculum (10%) of *Xanthomonas* sp culture was grown in best

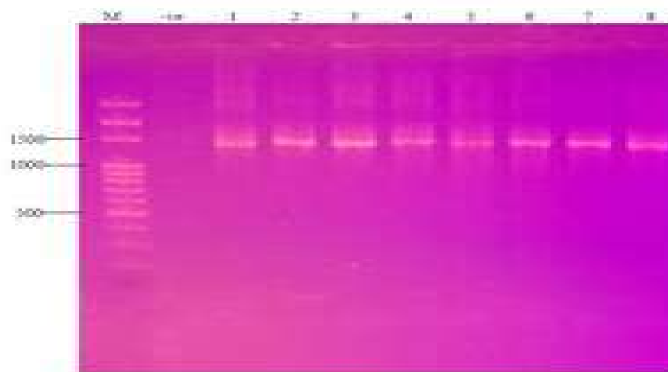
agro-waste at 35 ºC for 72 hours under static condition. The suspension was subsequently centrifuged (12000 × g for 15 minutes) to separate the biomass and further treated with tri-chloro acetic acid to removing the protein moieties. The xanthan gum was precipitated using ice cold ethanol (thrice the volume), centrifuged (19200 × g for 15 minutes) and the resultant crude xanthan gum was dissolved in Milli-Q water. Finally, the crude xanthan gum was subjected to dialysis and freeze dried for further physico-chemical analysis [25].

Characterisation of xanthan gum

Fourier Transform Infrared Spectroscopy (FTIR)

The functional group analysis of synthesized xanthan gum was carried out using FTIR as described by [26]. The technique involves the use of Fourier Transform Infrared Spectroscopy (FT-IR) Shimadzu 9200S spectroscope. Dried powder of xanthan gum was used for the analysis. It was fused into Potassium Bromate (KBr) and pressed into pellet under pressure of the FT- IR Shimadzu 9200S spectroscope. The transmittance mode used during analysis of the xanthan gum was 4000 to 400 cm^{-1}. The results were compared to commercial xanthan gum for similarities.

RESULTS AND DISCUSSIONS



Lane M represent 1500 bp molecular ladder, lane 1- RU 1, lane 2- RU3, lane 3 - RU4, lane 4 -PEAR4, lane 5- BAN2, lane 6-Sfl2 and lane 7-Sfl3.

Figure 1: Agarose gel electrophoresis of the 16s rRNA gene of the Xanthomonas sp.

Molecular characterisation of the selected isolates was done using a PCR based method. DNA was isolated and primers were used to amplify the target region after which gel electrophoresis was carried out. The PCR product was then sequenced to obtain the molecular +composition of each isolates at Epoch Life Sciences United States of America.

Agarose gel electrophoresis of the 16s rRNA gene of the *Xanthomonas* sp isolates is shown in figure 4.1. Lane M containing molecular ladder while lanes 1,2,3,4,5,6,7,8 contained isolates RU 1, RU3, RU4, SPEAR1, PEAR4, BAN2, Sfl2 and sfl3 respectively. The agarose gel electrophoresis revealed the base pairs of the *Xanthomonas* sp isolates RU 1, RU3, RU4, SPEAR1, PEAR4, BAN2, SFL2 and SFL3 to be base pair 1500 bp and this is line with earlier works by [26] which also showed the 16srRNA gel electrophoresis of *Xanthomonas* sp isolates as 1500 bp.

Table 1: Molecular identity of Xanthomonas sp isolates with their accession number

Organism code	Identity in NCBI Database	Accession Number
Sfl2	*Xanthomonas* sp. strain Sfl2	PP992848.1
Sfl3	*Xanthomonas* sp. strain Sfl3	PP992849.1
RU1	*Xanthomonas* sp. strain RU1	PP992850.1
RU4	*Xanthomonas* sp. strain RU4	PP992851.1
BAN2	*Xanthomonas* sp. strain BAN2	PP992852.1
PEAR4	*Xanthomonas* sp. strain PEAR4	PP992853.1
RU3	*Xanthomonas* sp. strain RU3	PP992854.1

NCBI -National Center for Biotechnology

Table 1 showed a summary of the molecularly characterised isolates with their codes, identity and accession number in the National Centre for Biotechnology (NCBI) database. Sfl2 was identified as *Xanthomonas* sp. strain Sfl2 with accession number PP992848.1, Sfl3 was identified as *Xanthomonas* sp. strain Sfl3 with accession number PP992849.1, RU 1 was identified

as *Xanthomonas* sp. strain RU1 with accession number PP992850.1, RU 4 was identified as *Xanthomonas* sp. strain RU4 with accession number PP992851.1, BAN 2 was identified as *Xanthomonas* sp. strain BAN2 with accession number PP992852.1, PERA4 was identified as *Xanthomonas* sp. strain PEAR4 with accession number PP992853.1 and RU3 was identified as *Xanthomonas* sp. strain RU3 with accession number PP992854.1

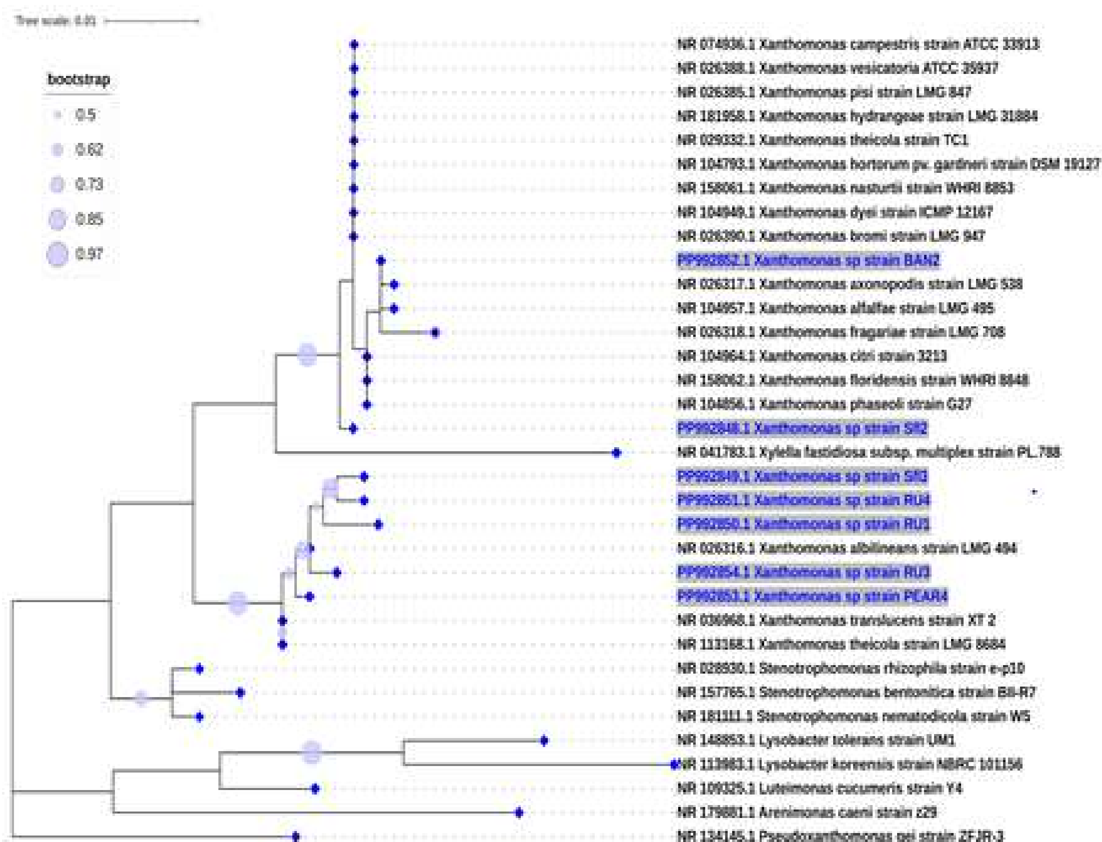


Figure 2: Phylogenetic tree of the Xanthomonas sp isolates.

Figure 2 showed the phylogenetic tree showing the evolutionary distance between the *Xanthomonas* sp isolates. *Xanthomonas* sp Sfl3 PP992849.1 isolated from sunflower is highly related to *Xanthomonas* sp strain RU4 PP992851.1 and closely related to *Xanthomonas* sp strain RU1 PP992850.1. *Xanthomonas* sp Sfl3 PP992849.1, *Xanthomonas* sp strain R4 PP992851.1 and *Xanthomonas* sp strain RU1 PP992850.1 are related to *Xanthomonas* sp strain RU3 PP992854.1 isolated from sunflower. *Xanthomonas* sp strain RU3 PP992854.1 is also related to *Xanthomonas* sp strain PEAR4 PP992853.1 isolated from pear while *Xanthomonas* sp strain BAN2 PP992852.1 isolated from banana is closely related to *Xanthomonas axonopodis* strain LMG 538 NR026317.1, *Xanthomonas alfalfa* strain LMG 495 NR 104957.1 and *Xanthomonas fragariae* strain LMG 708 NR 026318.1. *Xanthomonas* sp strain Sfl2 PP992848.1 is closely related to *Xanthomonas campestris* ATCC 33913 NR 074936.1, *Xanthomonas vesicatoria* ATCC 35937 NR 026388.1 and *Xanthomonas pisi* LMG *847NR 026385.1, Xanthomonas hydrangeae* strain *LMG 31884, Xanthomonas theicola* strain TC1, *Xanthomonas hortorum pv. gardneri strain DSM* amongst others as shown in the phylogenetic tree above.

Table 2: Production of xanthan gum in g/L by various isolates using different agro waste

Isolates code	Production of xanthan gum in g/L		
	Sugarcane bagasse	Sugarcane peel	Pineapple peel
RU1	2.82	2.98	2.67
RU3	2.83	2.97	2.58
RU4	2.89	3.14	2.78

BAN2	2.76	2.85	2.53
SFL2	3.39	3.68	3.29
SFL3	3.09	3.44	2.89
PEAR4	2.58	2.67	2.37

Table 2: showed the various yield of xanthan gum produced after fermentation with various agro waste materials and various isolates. Isolate SFL2 had the highest yields of xanthan gum 3.39 g/L, 3.68 g/L and 3.29 g/L in sugarcane bagasse, sugarcane peel and pineapple peel respectively while PEAR 4 had the lowest yields of xanthan gum 2.58 g/L, 2.67 g/L and 2.37 g/L in sugarcane bagasse, sugarcane peel and pineapple peel respectively. Strains differ in their ability to utilize carbon sources such as sucrose, some strains can efficiently take up and metabolize the carbon source and channel more of it for xanthan gum production while some cannot leading to less efficient metabolic process. The reduction in the metabolic can leads to variations in growth rates and product yields of various strains as shown in the table above. Furthermore, the enzymes involved in xanthan gum synthesis may also vary between the strains such that strains with more active or stable enzymes will easily and efficiently convert substrates to xanthan gum. Nutrient uptake and metabolism vary and as such can impact also overall xanthan gum yield [27]. Different strains can also exhibit different responses to environmental conditions like pH, temperature amongst others and this can which can enhance their growth and productivity under specific conditions.

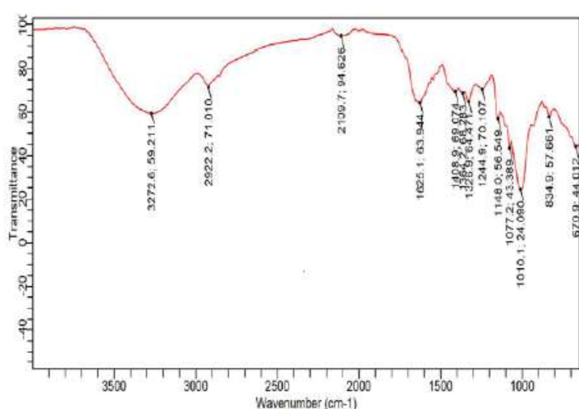


Figure 3: FTIR spectra of crude xanthan gum produced from sugarcane bagasse.

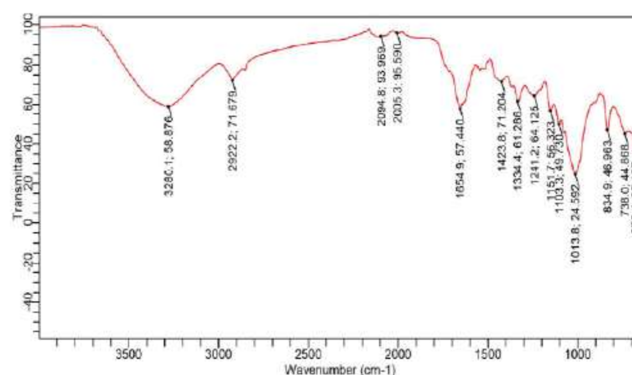


Figure 4: FTIR spectra of crude xanthan gum produced form sugarcane peel.

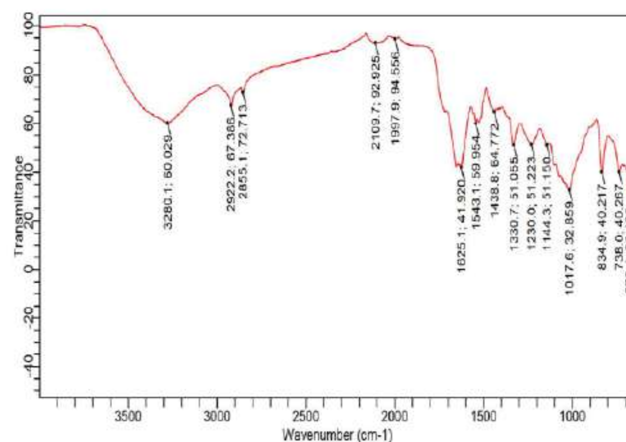


Figure 5: FTIR spectra of crude xanthan gum from pineapple peel.

The Fourier transform infrared spectroscopy was used to investigate the functional groups present as well as observe the similarities and differences in the chemical structures in the samples (crude, purified and commercial xanthan gum) as revealed in figure 3 to 5. The function groups present in all the samples were compared. The FTIR spectrum indicated peaks of various shapes ranging from strong, broad, bending and stretching each representing a functional group in the samples. The regions studied include all peaks between 400 and 4000 cm^{-1} wavenumber. The FTIR spectra generated for the xanthan gum produced using sugarcane bagasse had four peaks above 1500 cm^{-1}. The peaks were at 1625.16 cm^{-1}, 2109.79 cm^{-1}, 2922.27 cm^{-1}, and 3272.65 cm^{-1}. The peak at 1659.00 cm^{-1} indicated the presence of C=C stretching axial deformation of C=O ester, acid carboxylic aldehydes

and ketones. 2109.79 cm^{-1} showed the presence of –NCS, asymmetric and symmetric stretching of CH_3 indicating axial deformation of –C-H and –CHO was observed at 2922.27 cm^{-1} and 3272.00 cm^{-1} had the H bonded OH stretching of the hydroxyl group indicating axial deformation of –OH. The spectra obtained from the FTIR analysis of the xanthan gum produced using sugarcane peel had five peaks above 1500 cm^{-1}. The peaks 1654.91 cm^{-1}, 2005.39 cm^{-1}, 2094.89 cm^{-1}, 2922.27 cm^{-1} and 3280.15 cm^{-1}. The peak at 1654.91 cm^{-1} represent stretching of C=C axial deformation of C=O ester, acid carboxylic, aldehydes and ketones, 2005.39 cm^{-1} and 2094.89 cm^{-1} showed the presence of –NCS, the peak at 2922.27 cm^{-1} revealed the –CH_3 symmetric and asymmetric stretching indicating axial deformation of –C-H and –CHO then the stretching of the H bonded OH of the hydroxyl group was observed 3280.15 cm^{-1} indicating axial deformation of –OH. The FTIR of xanthan gum produced using pineapple peel had six significant peaks above 1500.00 cm^{-1}. The six peaks were observed at 1625.14 cm^{-1}, 1997.99 cm^{-1}, 2109.79 cm^{-1}, 2855.17 cm^{-1}, 2922.26 cm^{-1} and 3280.16 cm^{-1}.The peak at 1625.14 cm^{-1} showed indicated the presence of C=C stretching indicting axial deformation of C=O ester, acid carboxylic, aldehydes and ketones, the peak at 1997.99 cm^{-1} showed the presence of –NCS, the peak at 2109.79 cm^{-1} revealed the presence of C≡C, the peaks at 2855.17 cm^{-1}, 2922.26 cm^{-1} indicated C-H stretching and 3280.16 cm^{-1} showed the H bonded OH stretching of the hydroxyl group indicting axial deformation of –OH. The cumulative stretching and bending vibrations of the FTIR spectra of the xanthan gum samples mimic intently the structure of xanthan gum and the values (wavenumber) and function group present are similar to earlier works carried on xanthan gum by [28] showed that xanthan gum contained the hydroxyl (-OH), (-CH), aldehyde (-CHO), carbonyl (-C=O) functional groups amongst others.

Conclusion

In this study, we successfully produced xanthan gum using various *Xanthomonas* sp isolates and agro waste. *Xanthomonas* strain Sfl2 showed exceptional xanthan gum yield (36.8 g/L) in the sugarcane peel fermentation broth. The characterised xanthan gum has significant promise for industrial applications especially in sectors requiring emulsifiers or thickeners due to the functional groups present. Further research could focus on exploring genetic modifications of the *Xanthomonas* sp strains to enhance gum yield and the utilization of xanthan gum for various industrial purposes.

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