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Identification and Characterization of Xanthan Gum Produced from Date Juice by a Local Isolate of Bacteria *Xanthomonas campestris*

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Abstract: Xanthan gum is a microbial polysaccharide produced by the bacteria *Xanthomonas* spp. Seven local isolates of *Xanthomonas campestris* were used after microscopic and biochemical tests identified them. The isolates were subjected to a screening for xanthan production in medium broth consisting of 20 g.L-1 sucrose, 0.1 g.L-1 urea, and 1 g.L-1 K2HPO4. Isolate X1 showed the highest yield, which reached 6.26 g.l-1. The isolate was confirmed by a 16S rRNA test, and it was recorded in the gene bank with the code MZ262533. Xanthan gum was produced from date juice at a concentration of 3.5 ml (1.5 g glucose.100 ml-1 from the production medium, with the highest yield being 18.9 g.l-1. The resulting xanthan gum was identified by using FTIR, TLC and HPLC techniques, and it was the same chemical as xanthan gum. In the manufacture of xanthan gum and its usage in food, alternative media made from agricultural waste can be employed.

Keywords: Date juice, *Xanthomonas campestris*, Xanthan gum.

Introduction

Xanthan gum is one of the secondary metabolites produced and secreted outside the living cell. It is a heterogeneous microbial polysaccharide that is mainly produced from the fermentation of carbohydrates by isolates of the species belonging to the genus *Xanthomonas* (Palaniraj, & Jayaraman, 2011). Among the most important species producing this gum is *X. campestris*; xanthan gum has a high molecular weight, similar in structure to cellulose. It consists of two glucose molecules linked by a (β,1-4) glycosidic bond that stabilizes compound. Sub-chains of xanthan gum contain two molecules of mannose and one molecule of glucuronic acid alternately (Sworn, 2021).

Xanthan gum contains residues of pyruvic acid and acetyl. The amount of pyruvic acid that integrates with the gum and is a part of it varies according to the type of bacteria producing it. Usually, it ranges between 1-7.4% of the weight of the gum. The percentage of acetyl in the gum produced by *X. campestris* bacteria has reached 4.7% (Abbaszadeh *et al*., 2015).

Xanthomonas belongs to the family Xanthomonadaceae, which includes many species and genera. *X. campestris* is the typical type of this bacteria and is characterized by aerobic, Gram-negative growing at 25-35°C. It is motile due to polar flagella and can produce

many enzymes such as gelatinase and catalase. It forms a sticky mucous substance when it grows in a medium containing glucose (Kyrova *et al*., 2020).

Xanthan gum is produced by several bacterial species of the genus *Xanthomonas* under optimum conditions of temperature and pH and in the presence of a carbon source. Bacteria ferment many sugars, except lactose, to produce xanthan gum; three bacterial isolates were identified by using biochemical tests and a 16S rDNA test; two isolates belong to *X. campestris* bacteria and the third isolate belongs to *X. vesicatoria* bacteria; they were used to produce xanthan gum, which amounted to 7 and 35 mg.100 ml^{-1} of medium respectively (Rojas *et al*., 2019).

Xanthan gum is characterized by its stability and high solubility in basic and acidic solutions, so it has become one of the best polymers used in food and other industries due to its stability when there are concentrations of salts; the properties of xanthan gum are affected by its molecular weight and the amount of its acetate and pyruvic acid content; the amount of acetate and pyruvic acid varies depending on the species of the genus *Xanthomonas* used in the production of the gum and the production conditions (Espert *et al*., 2019).

In addition to being non-toxic and biodegradable, xanthan gum has been widely used in cosmetics and medicines, and in many food industries as a thickener, stabilizer, emulsifier, and gelling agent (Nejadmansouri *et al*., 2020). Xanthan gum is produced by fermenting glucose and sucrose as a carbon source, then separated and purified. Many agricultural and industrial wastes, such as molasses, whey, and date juice, are used to cut costs in the manufacturing process of xanthan gum (da Silva *et al*., 2017). Iraqi dates such as the Zahdi type are characterized by containing a high percentage of sugar, reaching more than 86.8%, with glucose accounting for 32.77%, fructose at 39.15%, and 12.70% invert sugar (sucrose) (Al-Gboori & Krepl, 2010). Due to the low cost of Iraqi dates, they can be used as a good carbon source in xanthan gum production. The current study aims to use local isolates capable of producing xanthan gum from a culture medium containing date juice as a carbon source.

Materials & Methods

Isolation sources

Xanthomonas spp. were isolated from the leaves of infected plants, including *Phoenix dactylifera, Bougainvillea, Uncaria tomentosa, Sansevieria trifasciata, Plumeria alba, Frangula alnus* and *Lawsonia inermis.* After cleaning, the leaves were enriched by immersing 1 g of these leaves in 9 ml Nutrient broth (Hi-media, India) for 24 hours at 30°C. The enriched leaves were streaked on Phyto Buffered Starch Agar Base (Hi-media, India) as selective media for these bacteria. Bacterial cultures were incubated at 28°C for 4 days (Yuen *et al.*, 1987).

Identification of bacterial isolates

The bacterial isolates obtained from the selective medium were identified according to what was mentioned in Bergey's Manual for the years 1974, 1994 and 2005. The phenotypic, microscopic and biochemical examinations (Saddler & Bradbury, 2005).

Screening and 16S rRNA test of isolate

Screened isolates belonging to *X. campestris* on the basis of their production of xanthan gum in broth media. The 16S rRNA test used to confirm the identification of the most productive isolate. The 16S rRNA test was conducted for the bacterial isolate using universal primers, F-$^{5'-}$AGTTTGATCCTGGCTCAG$^{-3'}$ R-$^{5'-}$GTTACCTTGTTTACGACTT^{-3}, the amplification program included an initial denaturation at 94℃ for 3 min, followed by 35 cycles of denaturation at 94℃ for 45 sec, annealing at 56℃ for 45 sec, extension at 72℃ for 1min and followed by a final extension at 72℃ for 7 min. After detect the PCR product by the electrophoresis method, the preparation, purification and sequencing of the PCR product was achieved at the BIONEER Company, Korea. PCR products were then exposed to treatment and re-correction before they were tested in the "BLAST program" supplied by the National Center for Biotechnology Information Service. http://www.ncbi.nlm.nih.govwhich (Loy *et al*., 2002).

Cultural condition, inoculation and incubation

The medium consisted of 20 g sucrose, 0.1 g urea, 1 g K_2HPO_4 and was used and dissolved in a litre of distilled water and the pH was adjusted to 7 (de Sousa Costa *et al*., 2014). The medium was distributed into 250 ml Erlenmeyer flask at 100 ml per flask and sterilized at 121°C for 15 minutes. After cooling, the flasks were seeded with a 5% (35×10^9 CFU.ml^{-1}) inoculum of local isolate and incubated in a shaker incubator at 175 rpm and 30°C. The carbon source (sucrose) of the production medium was replaced with the prepared date juice according to what was mentioned in Khassaf *et al*. (2019). The following concentrations (2.5, 3.5, 4.5, 5.5 and 6.5) percent of date juice, of the date juice, which are equivalent to 1.1, 1.5, 2.0, 2.4 and 2.8 g of sugar, estimated as glucose (Dubois *et al*., 1956).

Isolation and purification of xanthan gum

Following the end incubation time, centrifugation was carried out at 5500 rpm for 40 minutes. The biomass was removed and the supernatant was collected Then, isopropanol was added at a ratio of 2:1 (V: V) to precipitate the xanthan gum, then incubated at refrigerator temperature for 12 hours, then centrifuged at a speed of 7000 rpm for 30 minutes The above step was repeated again for xanthan purification and the precipitate was collected and dried at 50°C until a constant weight was achieved (Prabhakar *et al.*, 2004; Rottava *et al*., 2009).

Biomass estimation

The precipitate was washed with distilled water twice, the biomass was weighed after being dried in an oven at 60°C until the weight was fixed.

Xanthan gum identification

Infrared spectrum (FT-IR)

The described method of Soleymanpour *et al*. (2018), as followed for FTIR spectra for the reference (Xanthan chemical (XG), Sigma company), Xanthan produced from standard media (XGP1) and xanthan produced from date juice media (XGP2) were recorded after mixing 1 mg of the sample with 100 g of potassium bromide. The spectrum of effective groups of xanthan gum was observed between a

wavelength of 4000 and 400 cm^{-1} at room temperature.

Thin-layer chromatography (TLC)

Xanthan gum solutions (XG, XGP1 and XGP2) were prepared by dissolving 0.02g in 2 ml of distilled water and injecting the samples into a thin layer which was put into a glass container containing a mixture of solvents (distilled water, butanol and ethanol) (4:5:5). After the solvent mixture reached the finish line, the plate was sprayed with a reagent consisting of 5% sulfuric acid, diluted with ethanol alcohol, and moved to an oven at 120°C. Until the yellow colour of the separated spots appears, then the R*f* value was calculated by following equation (Mudoi *et al.*, 2013).

R*f* value= X/Y

Where, X= compound's distance travelled, Y= the distance that solvent front has travelled

High-performance liquid chromatography (HPLC)

Gum solutions (XG, XGP1 and XGP2) were prepared by dissolving 5 mg of each sample in 350 µL of distilled water, 500 µL acetonitrile and 20 µL DMSO. The C18 column is used for separation. The mobile phase consisted of 75:25 (V: V) of distilled water and acetonitrile, respectively, and 100 µL of formic acid at 60 °C using the HPLC device at the College of Pharmacy, University of Basrah. (Mudoi *et al.*, 2013).

Statistical analysis

The significant differences between the mean of xanthan production treatments were calculated using the Completely Randomized Designs (CRD) and the analysis table ANOVA and the least significant difference test (LSD), probability level 0.05 by using the Genstat program version 12.1 (Ocimati, *et al*., 2018).

Results & Discussions

Morphological and microscopic tests of isolates

The phenotypic and microscopic examination revealed that the colonies were creamy white, circular, sticky, rods, Gram-negative, and motile and these results were agreed with Saddler & Bradbury (2005) and Tilahun *et al*. (2020) when they identified *X. campestris* bacteria as Gram-negative, motile and rod.

Biochemical tests of isolates

Table (1) shows the results of the biochemical tests of the seven isolates. All isolates showed the ability to produce catalase, gelatinase, casein hydrolysis, citrate utilization, gas production from glucose, H_2S gas production, esculin hydrolysis, resistance to the antibiotic erythromycin, grown at different temperatures and with different salt concentrations, fermentation of many sugars, as well as the production of a viscous substance in a medium containing 5% of glucose. These results agreed with Ndongo *et al*. (2018), which indicated the most important biochemical tests that confirm the belonging of bacterial isolates to the genus *Xanthomonas*. The seven isolates were given a negative result for the following tests: oxidase production, nitrate reduction, urea hydrolyze, lactose fermentation, and growth at 40°C, confirming belonging to the *Xanthomonas campestris* isolates. These results agreed with the biochemical tests mentioned by Popovic *et al*. (2013) when identifying *Xanthomonas* bacteria locally isolated from cabbage, green cauliflower and turnip plants.

Table (1): Biochemical characters of the isolates.

	source of isolates	*Sansevieria trifasciata*	*Phoenix dactylifera*	*Bougainvillea*	*Uncaria tomentosa*	*Plumeria alba*	*Frangula alnus*	*Lawsonia inermis*
	Isolate's symbol	**X1**	**X11**	**X12**	**X13**	**X15**	**X24**	**X25**
Biochemical tests	Citrate utilization	+	+	+	+	+	+	+
	Hydrolysis of gelatin	+	+	+	+	+	+	+
	Catalase production	+	+	+	+	+	+	+
	H_2s production	+	+	+	+	+	+	+
	Esculin hydrolysis	+	+	+	+	+	+	+
	Oxidase production	-	-	-	-	-	-	-
	Nitrate reduction	-	-	-	-	-	-	-
	Urea hydrolysis	-	-	-	-	-	-	-
	Growth on nutrient agar with 5% glucose	+	+	+	+	+	+	+
	Erythromycin sensitivity test	R	R	R	R	R	R	**R**
	casein hydrolysis	+	+	+	+	+	+	+
	Gas from glucose	+	+	+	+	+	+	+
Growth at temperatu	25°C	+	+	+	+	+	+	+
	30°C	+	+	+	+	+	+	+
	35°C	+	+	+	+	+	+	+
	40°C	-	-	-	-	-	-	-
NaCl tolerance	0.5%	+	+	+	+	+	+	+
	1%	+	+	+	+	+	+	+
	2%	+	+	+	+	+	+	+
	3%	+	+	+	+	+	+	+
	4%	+	+	+	+	+	+	+
	5%	+	+	+	+	+	+	+
	6%	-	-	-	-	-	-	-
fermentation of sugars	Fructose	+	+	+	+	+	+	+
	Galactose	+	+	+	+	+	+	+
	Melibiose	+	+	+	+	+	+	+
	Sucrose	+	+	+	+	+	+	+
	Glucose	+	+	+	+	+	+	+
	Mannose	+	+	+	+	+	+	+
	Lactose	-	-	-	-	-	-	-
	Cellobiose	+	+	+	+	+	+	+
	Maltose	+	+	+	+	+	+	+
	Arabinose	+	+	+	+	+	+	+
	Xylose	+	+	+	+	+	+	+

+: positive test, -: negative test, R: resistance

Screening bacterial isolates

Fig. (1) shows the amount of xanthan gum produced from the identified bacterial isolates. The highest yield was obtained in case of the isolate X1 isolated from the leaves of *Sansevieria trifasciata*, which amounted to 6.26 $g.l^{-1}$, while the lowest yield was for X15 isolate which was 5 $g.l^{-1}$

These results are consistent with Kalogiannis *et al*. (2003) who obtained different production rates of xanthan gum when using four strains belonging to *X. campestris* isolated locally from lemon trees. This showed the effect of the bacterial strain on the amount of gum produced. While Makut *et al*. (2018), studied the effect of different locally isolated strains from plant leaves (mango, orange, rice, melon and sugar cane) belonging to *X. campestris* on the production of xanthan gum, and the highest yield of gum obtained by the bacterium isolated from orange leaves was 1.25 g.l^{-1}.

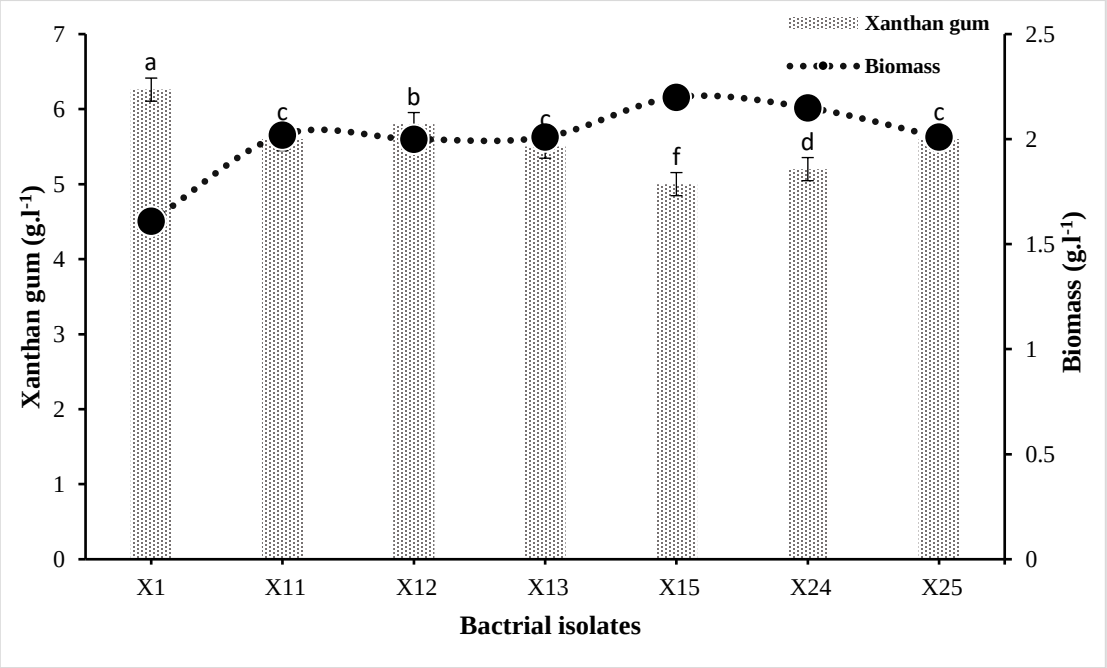

Fig. (1): Biomass dry weight and produced xanthan *by X. campestris* isolates

16S rRNA test for the most productive bacterial isolate.

The 16S rRNA test was conducted to confirm the identity of the most productive isolate of *X. campestris*. Fig. (2) explains the results of PCR amplification. The bacteria isolate showed a bright band at 1500 bp. Then the results showed the sequence of nitrogenous bases in the blast program of the National Center for Biotechnology Information (NCBI), which matched the local isolate 100% with the *X. campestris* MAFF 106181X strain (Fig. 3). The isolate has been recorded in the Japanese GenBank as *X. campestris* XCB1 and encoded with the code MZ262533, https://www.ncbi.nlm.nih.gov/nuccore/MZ262533.

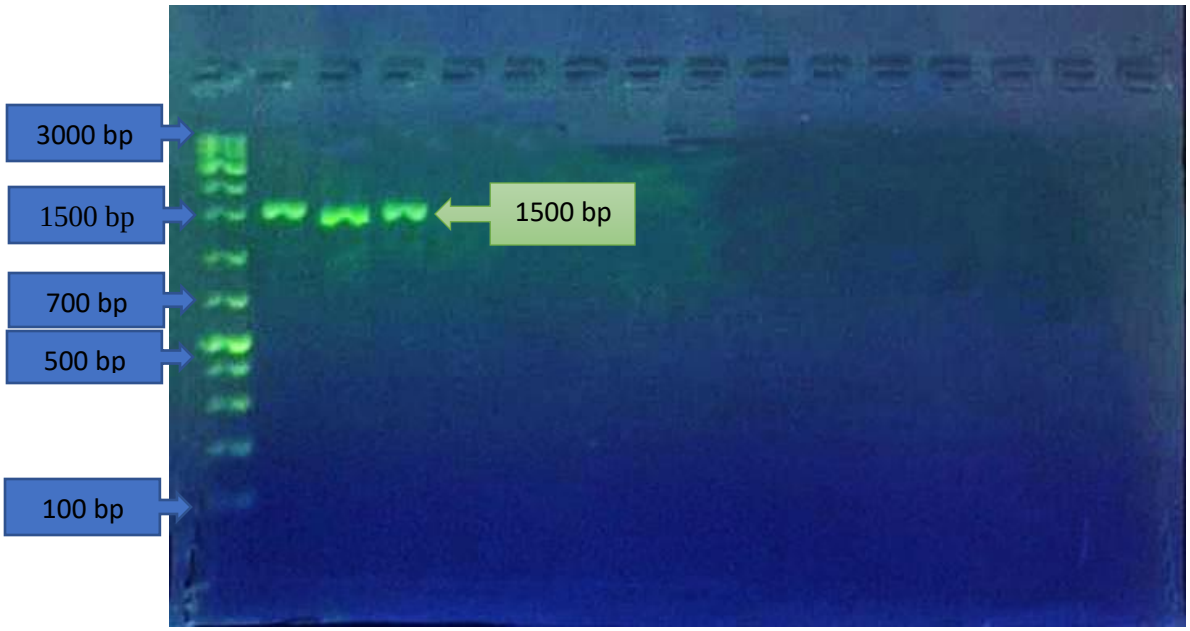

Fig. (2): Electrophoresis of the gene responsible for testing 16S rRNA for the X1 isolate on 1.5% agarose gel.

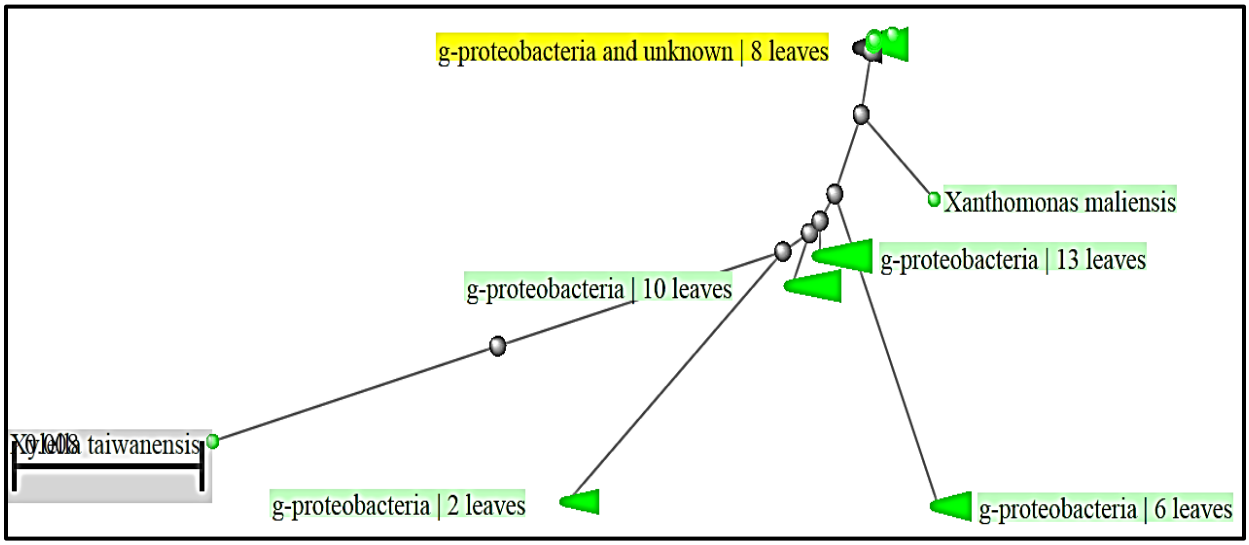

Fig. (3): The genetic tree of *X. campestris* XCB1 bacteria.

Production of xanthan gum from date juice

Fig. (4) shows the effect of using different concentrations of sugar in the form of date juice on xanthan gum production using *X. campestris* XCB1. The highest gum yield when using a concentration of 3.5 ml of date juice (1.5 g glucose100.ml^{-1}) of the production medium was 18.9 g.l^{-1}, and the weight of the biomass was1.35 g.l^{-1}. The

production of xanthan gum decreased as the concentration of date juice increased, reaching 8.61 g.l^{-1} at a concentration of 6.5 ml of date juice (2.8 g glucose.100ml^{-1}) of the production medium. The statistical analysis showed significant difference between the standard medium and the date juice medium at a concentration of 3.5 ml (1.5 g glucose.100ml^{-1}) of medium. The is may be due to the presence of monosaccharides such as glucose and fructose and invert sugar. These substances stimulate bacteria to produce xanthan gum. The amount of biomass has decreased as gum production has increased, possibly due to the bacteria's proclivity to produce gum rather than biomass.

The findings corroborated those of Khosravi-Darani *et al.* (2011), who discovered that the best concentration of date juice as a carbon source in the production medium was 4 ml /100 ml of the production medium and the resulting xanthan yield was 11.30 g.l^{-1} , while Salah *et al*. (2011), reported the best concentration of date juice when was used as a substitute for the carbon source in the production medium was at a concentration of 6 ml (5.16 g sugar.100ml^{-1}), as the yield of the xanthan gum was 24.5 g.l^{-1}.

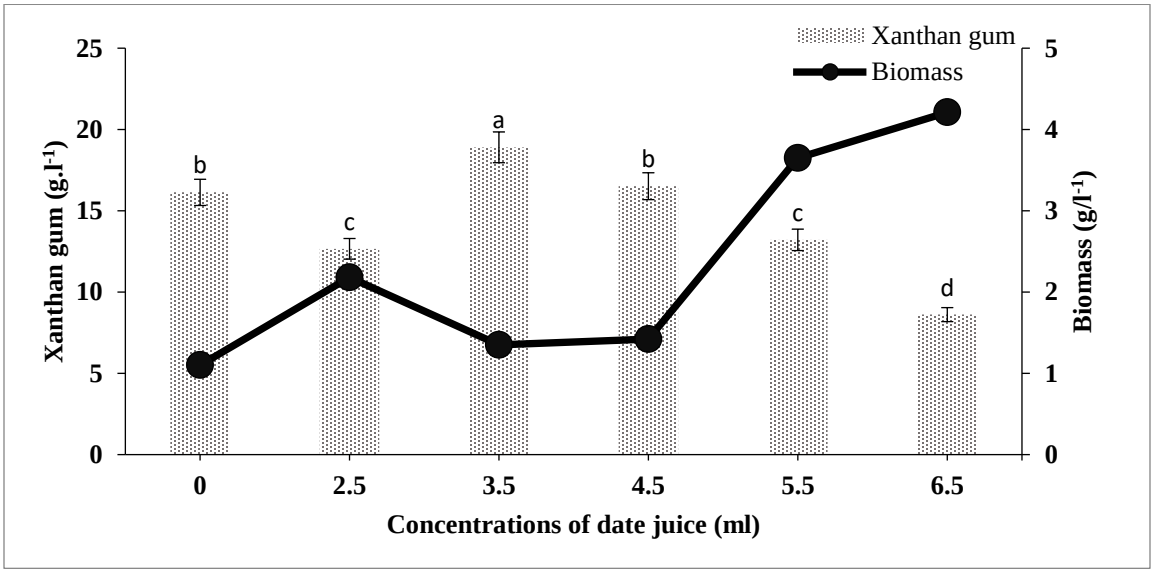

Fig. (4): Production of xanthan gum by isolate using different concentration of concentrations sugar in the form of date juice.

Identification of Xanthan gum

FTIR spectrometer of xanthan gum

Fig. (5) depicts the results of comparing xanthan gum produced from the standard medium and date juice XGP1 and XGP2 to the chemical xanthan gum XG using infrared spectroscopy technology that detects the vibrational frequencies of the functional groups present in the produced gum and XG. At the wavelength of 3406-3437 cm^{-1}, a spectral absorption band appears, which is connected to hydroxyl group fluctuation, whereas the band at 2926-2933 cm^{-1}

corresponds to C-H and CHO groups. At 1732-1743 cm^{-1}, a definite peak formed, which belongs to the acetate groups. While the C=O groups of pyruvate appear at wavelengths of 1616-1622 cm^{-1}, the -COO groups appear at wavelengths of 1409-1417 cm^{-1}, and the CO group appears at wavelength 1234-1259 cm^{-1}, and its peak at wavelength of 1051-1062 cm^{-1} is due to the C-O-C ether bond (Table 2). The slight differences in peaks that appear at close wavelengths may be due to changes in the chemical composition of the gum that result from the effects of different *Xanthomonas* strains, production conditions, type of growth medium and carbon source used. (Miranda *et al*., 2020). Infrared technology was used by Barua *et al*. (2016) to detect active groups of hydroxyls, carboxylic and carbonyl groups, as well as acetate groups, in xanthan gum.

Table (2): locations of the active groups of XG, XGP1 and XGP2 in the FTIR spectrometer.

Effective group	XG	XGP1	XGP2
-OH	3437	3414	3406
C-H and CHO	2926	2931	2933
-C=O stretching of the acetate group	1732	1734	1743
C=O	1616	1616	1622
-COO groups	1417	1409	1417
CO of COC	1253	1259	1234
C-O-C stretching from ether linkages	1051	1062	1053

These results agree Rojas *et al*. (2019) when identifying the active groups in xanthan gum. A peak appeared for hydroxyl groups at wavelength 3410 cm^{-1}, while wavelength 2935 cm^{-1} was due to CH_2 and CH_3 groups, and the peak that appeared at wavelength 1060 cm^{-1} was due to the C-ether bond. The wavelength of the effective groups of xanthan gum when analyzed by infrared spectroscopy, was 3400-3450 cm^{-1}, with a return to the hydroxyl groups, while the groups of CH and CHO peaks appeared at the wavelength of 2850-2950 cm^{-1}, and the effective group C=O appeared at the wavelength of 1700-1600 cm^{-1} (Pawlicka *et al*., 2019).

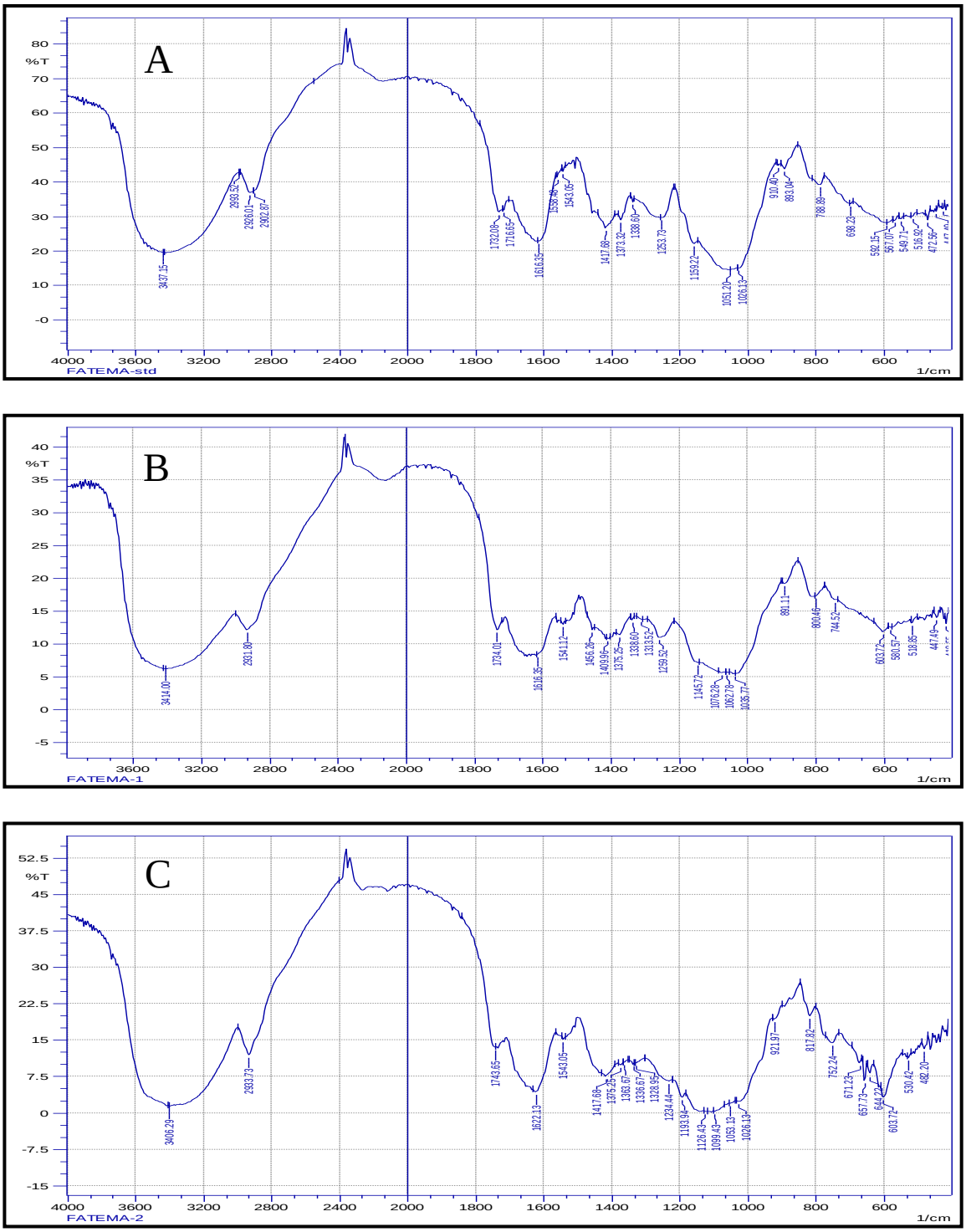

Fig. (5): FTIR analysis results for xanthan gum samples, where A=GX, B=GXP1 and C=GXP2.

Thin-layer chromatography (TLC) of xanthan gum

TLC technology was used to identify GXP1 and GXP2 compared to the chemical xanthan

gum (GX) (Fig. 6). The relative movement R*f* of the stains of GXP1 and GXP2 was equal compared with the GX. It was equal to 0.6. The absence of other samples indicates the high purity of the Xanthan gum produced from the standard medium and the date juice. Gondim *et al*. (2019) mentioned that the reason for the differences in the intensity and size of spots when diagnosing xanthan gum using the thin layer chromatography technique is that the strains of the genus *Xanthomonas* produce polymers with different viscosities. The viscosity increases with increasing gum content of mannose sugar and glucuronic acid.

High-performance liquid chromatography of xanthan gum

XGP1 and XGP2 were identified and compared with chemical xanthan gum (XG) using HPLC technology. Fig. (7) shows the results of the identification. One clear, sharp peak appeared without any other peaks, indicating the purity of the produced gum. The retention time for the apparent peaks was 2.97, 2.94 and 3.03 minutes for XGP1, XGP2 and GX, respectively. A clear convergence in absorbance between the peaks was obtained at 290, 240 and 280 mAU, respectively. This confirms that the compound produced from the standard medium and the medium of date juice by the local isolate *X. campestris* XCB1 is xanthan gum.

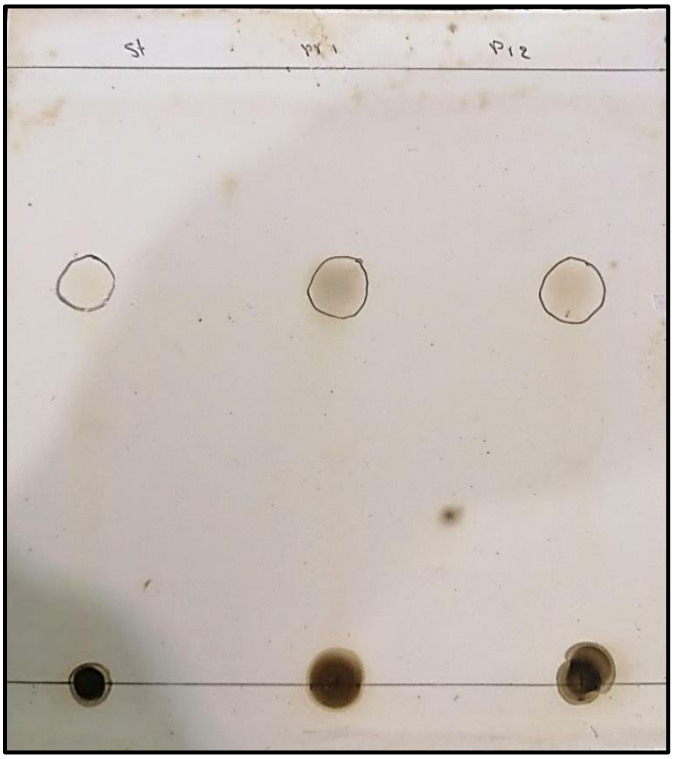

Fig. (6): Identify xanthan gum by using the thin layer chromatography technique of GX, GXP1 and GXP2

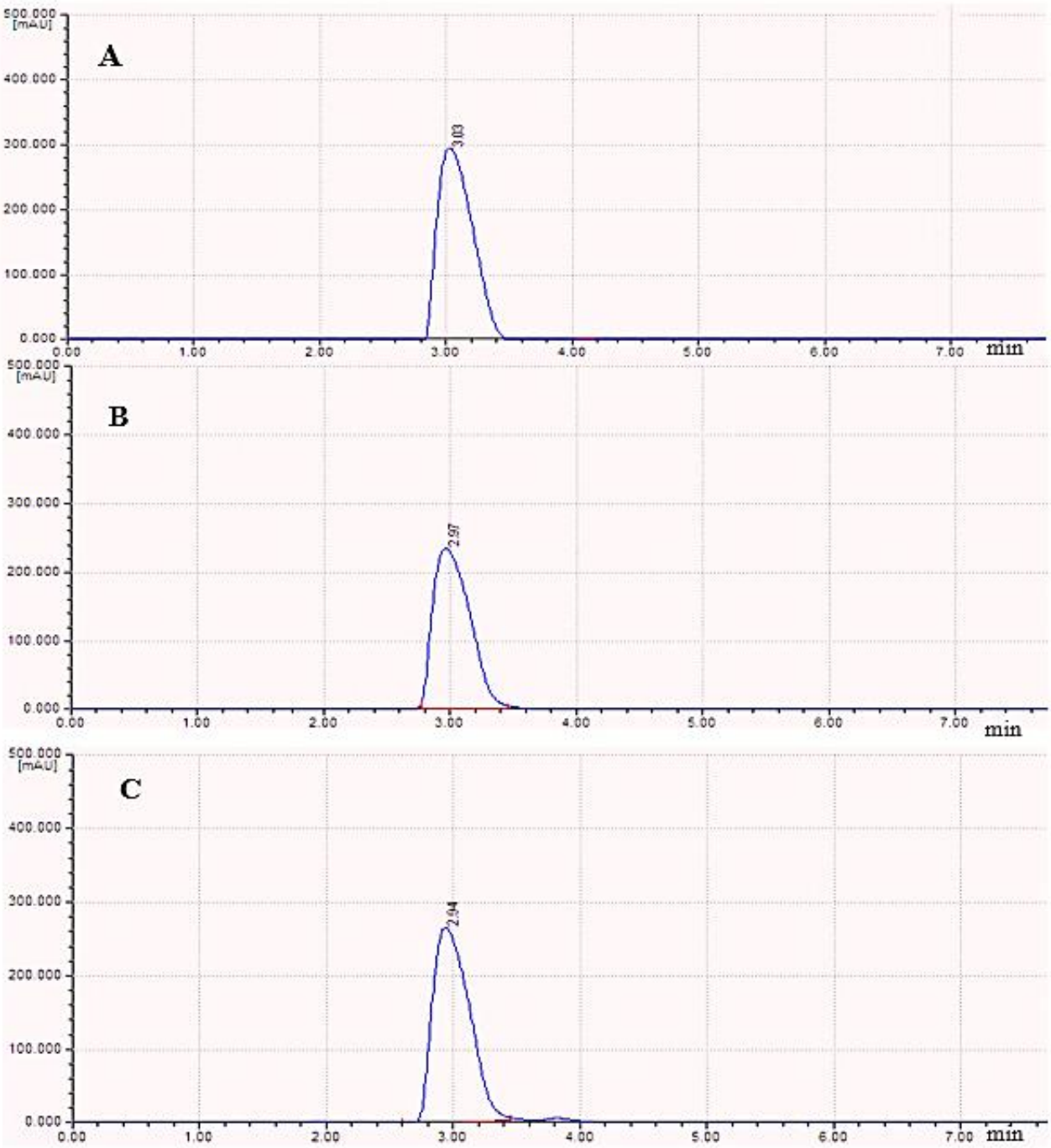

Fig. (7): HPLC analysis results for xanthan gum samples, where A= XG, B = XGP1, C = XGP2.

These results are in agreement with those reported by Salah *et al*. (2011), with a single, sharp peak at a holding time of 3.66 minutes for xanthan gum produced from a medium containing date juice. Papagianni *et al*. (2001) found that when xanthan gum was diagnosed using the HPLC technique, a single sharp peak appeared back to xanthan gum produced by *X. campestris* bacteria using a Luria-Bertani (LB) culture medium with 0.2% glucose addition.

Conclusions

Microbial polysaccharides, including xanthan gum, are secondary metabolites produced by microorganisms which have nutritional and health benefits. It can be introduced into many food industries, and the product yield varies according to the type of strain and the agricultural media used. Agro wastes such as molasses and date extract (juice) have been used to reduce the economic cost of producing xanthan gum, as well as to get rid of agricultural waste that causes environmental damage.

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Conflicts of Interest: The authors declare no conflicts of interest.

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تشخيص وتوصيف صمغ الزانثان الناتج من عصير التمر باستعمال عزلة محلية لبكتريا *Xanthomonas campestris*

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المستخلص: يعد صمغ الزانثان من السكريات المتعددة المايكروبية التي تنتج من البكتريا *Xanthomonas* spp. استعملت سبع عزلات محلية عائدة لبكتريا *Xanthomonas campestris* بعد تشخيصها باختبارات المجهرية والكيموحيوية، غربلت العزلات على وسط سائل يحتوي على 20 غم .لتر$^{-1}$ و0.1 غم .لتر$^{-1}$ يوريا و 1 غم .لتر$^{-1}$ K_2HPO_4 واظهرت العزلة X1 اعلى انتاج اذ بلغ 6.26 غم. لتر$^{-1}$ تم تأكيد العزلة باختبار 16S rRNA وسجلت في بنك الجينات بالرمز MZ262533 أنتج صمغ الزانثان من عصير التمر بتركيز 3.5 مل (1.5 غم كلوكوز) / 100 مل من وسط الانتاج اذ بلغ اعلى حاصل 18.9 غم. لتر$^{-1}$ شخص صمغ الزانثان الناتج باستعمال تقنيات FTIR و TLC وHPLC واظهر تطابقاً مع صمغ الزانثان الكيميائي. يمكن استعمال اوساط بديلة من المخلفات الزراعية في انتاج صمغ الزانثان واستعماله في الاغذية.

الكلمات المفتاحية: صمغ الزانثان، بكتريا *Xanthomonas campestris*، مستخلص التمر.