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The Impact of Using Date Juice as a Carbon Source on Curdlan Produced by a Local Isolate of *Agrobacterium leguminum*

Yaqob S. S. Al-Rumaidh, Shayma. T. G. Al-Sahlany* & Haider. I. Ali

Department of Food Sciences, College of Agriculture, University of Basrah, Iraq

***Corresponding author email:** shayma.gddoa@uobasrah.edu.iq; **Y.S.S.**: yagoobshemall33@gmail.com; **H.I.A.**: haider.ali@uobasrah.edu.iq

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Abstract: *Agrobacterium* species are responsible for the production of the polysaccharide known as curdlan. The curdlan was produced from 10 isolates that were collected from a variety of local sources, including as agricultural soils, root nodules, and plant roots. The isolates were identified by examinations using morphological, microscopic, and biochemical tests. After testing the isolates to see which ones could produce the most curdlan, the A2 isolate finally emerged with a production capacity of 29.2 $g.L^{-1}$. According to the findings, the production of curdlan was increased using the modified medium that contained date juice at a concentration of 26.4 mL.100 mL^{-1} of the production medium. The resultant yield was 30.7 $g.L^{-1}$, which was the highest possible yield. The identification of curdlan was validated by the utilization of FTIR, NMR, and HPLC techniques. The modified medium had the capability of being utilized in the production of curdlan from agricultural waste products.

Keywords: *Agrobacterium leguminum*, Curdlan, Date juice.

Introduction

The production of curdlan takes place outside of the bacterial cell and is the result of a secondary metabolic process. It is a linear homologous microbial polysaccharide that is primarily generated by the *Agrobacterium* species through the fermentation of various carbon sources. The molecular weight of curdlan is rather high. Its basic building block is glucose. β-(1→3)-glucan is the name of the glycosidic bond that connects the individual units (Ozawa *et al*., 2021; Wu *et al*., 2022). Curdlan is a substance that is non-toxic, biodegradable, and edible. It is also regarded to be a dietary fiber, which means that it might also improve the functional properties of a variety of food products. Studies have been focused on the production of exo-polysaccharides (EPS), also known as extracellular polymeric materials, by microorganisms. This is because there has been an increase in the demand for natural polymers to be used in a variety of industrial and biotechnological applications (Watanabe *et al*., 2021).

Bacteria belonging to a variety of genera are responsible for the production of curdlan. These bacteria include *Agrobacterium* spp., *Paenibacillus polymyxa, Rhizobium radiobacter*, *Bacillus* spp., *Pediococcus parvulus,* and *Cellulomonas flavigena*. Curdlan was initially discovered by Conn in 1942 and belongs to the genus *Agrobacterium*, which is one of the most common types of bacteria that may produce it. It is a type of bacteria that is gram-negative, aerobic, and does not form spores. It is a member of the

Rhizobiaceae family, and its flagella count ranges from one to four. The colonies of these bacteria can either be single or pair, and they have the ability to infect plants and cause tumors on the roots and stems of plants (Mousavi *et al*., 2014).

The capacity of *Agrobacterium* species to make 3-ketolactose from lactose serves as the definitive test for classifying these microorganisms (Pulawska *et al*., 2012). *Agrobacterium* spp. colonies, when produced on grown-on mannitol yeast agar culture media, appear white to creamy, convex or spherical, with a rounded edge, and are often aggressive. When there are many kinds of carbohydrates present in the growth medium to serve as a source of energy and carbon, it is able to thrive on a variety of different food media. One of the forms of bacteria that produce exogenous polysaccharides is called *Agrobacterium*. These polysaccharides give the colonies a viscous appearance in the growth medium (Ortiz Martinez *et al*., 2016).

Microbial polysaccharides can be produced by bio-fermentations by using basic sources that are uncomplicated and inexpensive and coming from a variety of bacterial species. In a wide number of applications in the fields of medicine, pharmacology, and the food industry, polysaccharides are utilized as various types of thickeners, stabilizers, and anti-crystallization agents (Al-Roomi & Al-Sahlany, 2022; Mohammed & Niamah, 2022).

Curdlan has the capacity to produce gels that are rigid and elastic, and as a result, it may enhance the qualities of food products (Martinez *et al*., 2016). Curdlan gel, commonly referred to as thermal gel, is distinguished by its exceptional capacity to withstand exposure to high temperatures. Due to the fact that it is unaffected by the freezing and thawing processes, it may be used in the production of foods that call for cooling procedures throughout the manufacturing process (Verma *et al*., 2020). Dates from Iraq, known as Zuhdi, have a high concentration of sugar in their flesh. In addition to it, there is glucose that makes up 32.8% of the total, fructose that makes up 39.2%, and a significant amount of inverted sugar (Khassaf *et al*., 2019). Therefore, the purpose of the current research was to find local bacteria isolates that were capable of making polysaccharides (curdlan) from a cheap culture medium that contained date juice as a carbon source. This was done in an effort to minimize the economic production expenses of curdlan.

Materials & Methods

Isolation

Agricultural soil, plant roots (*Vicia faba*, *Medicago sativa*, *Helianthus annuus*, *Solanum lycopersicum,* and *Vitis* spp.), and root nodules (tomato and broad bean) were some of the local sources from which *Agrobacterium* species were isolated.

Isolation media

Dissolving 10g of mannitol, 0.5 g of dipotassium phosphate (K_2HPO_4), 0.2 g of anhydrous magnesium sulfate ($MgSO_4$), 0.1 g of sodium chloride (NACl), 1g of yeast extract, 0.016 g of thymol blue, and 15 g of agar was the first step in preparing Hofer's Alkaline medium. After bringing the volume up to one liter with distilled water and adjusting the pH to 11, the medium was autoclaved at 121°C and 15 bar for 15 minutes. After being allowed to cool, the medium was poured into plates and allowed to solidify before being put to use in the process of isolating *Agrobacterium* spp. (Subbarao, 1995).

Identification of bacterial isolates

Tests including morphology, microscopy, and biochemical were utilized in order to determine the identities of the bacterial isolates that were

collected from the selective medium (Young *et al*., 2005). The capability of the isolates to produce curdlan was evaluated throughout the screening process (Shih *et al.*, 2009).

Curdlan production media

Dissolve 140 g of sucrose, 3g of ammonium chloride (NH_4CI), 1g of potassium dihydrogen phosphate (KH_2PO_4), and 0.5g of $MgSO_4$ in one liter of distilled water. After this, add 10 mL of trace elements of varying concentrations (1 $g.L^{-1}$of $FeCl_3$, 1 $g.L^{-1}$ of $CaCl_2$, 1 $g.L^{-1}$of ZnCl, and 0.03 1 $g.L^{-1}$of $CaCO_3$). After that, autoclave the mixture at 121°C for 15 minutes. Old bacteria at a concentration of 5% (186×10^8 $CFU.ml^{-1}$) were added and cultured for 120 hours in a shaking incubator at a speed of 180 rpm at a temperature of 30°C (Shih *et al*., 2009). The following concentrations of date juice were present in the production medium in place of the carbon source (sucrose): 17.6, 22.1, 26.4, 30.9, and 35.3 $ml.100\ ml^{-1}$ of production medium (Al-Roomi & Al-Sahlany, 2022).

Curdlan extraction and purification

The procedure that was developed by Mangolim *et al*. (2017) was utilized, albeit with a few modifications, in order to extract and purify the curdlan. After the fermentation process was complete, 5ml of the sample was combined with 10ml of 0.5N NaOH, and the mixture was allowed to remain at room temperature for one hour. After that, centrifugation was performed at a speed of 6000 rpm for 15 minutes, the biomass was removed, the supernatant was obtained, and the pH was adjusted to 7 utilizing 3 N HCl. Finally, the mixture was stored in the refrigerator for 16 hours. After that, the supernatant was put into dialysis bags with a cutoff of 10,000 Daltons to eliminate the salts and sugars that had dissolved in the medium. After that, it was centrifuged at 6000 rpm for 15 minutes to precipitate the curdlan and remove the solvent. The resulting curdlan was allowed to dry at 60°C for 24 hours before being weighed.

Biomass estimation

After being rinsed three times with distilled water, biomass was placed in an oven preheated to 70°C for 24 hours before being weighed.

Curdlan identification using FTIR spectrum

After combining 1mg of the sample with one hundred grams of potassium bromide, a Fourier transform infrared spectrometry (FTIR) was performed at the Polymer Research Center of the University of Basrah in order to identify the functional groups of curdlan samples that were produced using the standard medium (CP1) and the date juice medium (CP2) in order to make a comparison with the curdlan chemical standard (CS). At a wavelength of 4000-400 cm^{-1} (Mangolim *et al*., 2017).

^{1}H-NMR spectrum

At a frequency of 400.1 MHz, the nuclear magnetic spectrum of the hydrogen atom was evaluated with the assistance of a nuclear magnetic resonance (NMR) spectrometer. This analysis was performed on samples of CS, CP1, and CP2. In order to prepare the samples, 15 mg of curdlan samples were dissolved in 1mL of D_2O solvent, and then the solution was transferred to special tubes that were designed for measuring ^{1}H-NMR spectrometry (Suflet *et al*., 2015).

HPLC analysis

The produced curdlan (CP1 and CP2) as well as CS were prepared by dissolving 5 mg of curdlan in 500 µL of NaOH (0.5N). The mobile phase was comprised of dimethyl sulfoxide ($[CH_3]_2SO$), and it had a flow rate of 1 $ml.min^{-1}$. In the College of Pharmacy at the University of Basrah, an HPLC system was utilized in conjunction with a C18 column to perform a separation at a temperature of 80°C (Salah *et al*., 2011).

Statistical analysis

Completely Randomized Designs (CRD) and the analysis of variance (ANOVA) were used in the statistical analysis of the data data obtained from

experimental analysis. The least significant difference (LSD) test was used to assess the significant differences between the treatment averages at a significant threshold of 0.05. The analysis was performed using the SPSS software version 26.

Results & Discussion

Morphological and microscopic identification

A total of ten distinct plant roots, root nodules, and soil samples were analyzed in order to acquire bacterial isolates. The morphological and microscopic characteristics of the bacterial isolates were included a spherical shape, a viscous consistency, and a creamy white coloration. The bacterial strains that were isolated were all rod-shaped, Gram-negative, and motile. The findings of this investigation were in agreement with similar findings reported by Young *et al.* (2005) and Vatankhah *et al.* (2022). These researchers found that *Agrobacterium tumefaciens* isolates were gram-negative, mobile, and had creamy white colonies with regular margins that were round in shape and viscous.

The biochemical tests

The results of the biochemical tests on the 10 different isolates have been compiled in Table 1. These findings were consistent with those of Puławska *et al.* (2012), who isolated 57 colonies from the root nodules of legume plants. The results demonstrated that isolates A1, A3, and A4 belong to *Agrobacterium rhizogenes*. Phenotypic and biochemical analyses were conducted in order to determine the identity of the bacteria that were isolated, and it was determined that they were *Agro. rhizogenes*. The A2 isolate was found to belong to a type of *Agrobacterium* called *Agrobacterium leguminum*. This strain of bacteria was distinct from all other *Agrobacterium* species. It can grow at temperatures of 42°C, and all of the isolates obtained in this experiment grew in the pH range of 5–10 (Castellano-Hinojosa *et al.,* 2021). The *Agro. vitis* strain was found in the following isolates: A21, A26, A29, and A30. At 1 and 2% NaCl, these isolates were able to proliferate (Kerr & Panagopoulos, 1977). However, the A27 and A28 isolates were identified as *Agro. tumefaciens*. These findings corresponded with those of Young *et al.* (2015) and Ferdous *et al*. (2021), who demonstrated that these bacteria were positive for catalase and oxidase, 3-ketolactose synthesis, growth at 2% salt concentration, and carbohydrate fermentation, but negative for H_2S gas production.

Screening of bacterial isolates

Fig. (1) illustrates the quantity of curdlan that was produced by each of the identified bacterial isolates. The A2 bacteria that were isolated from the root nodules of the bean plant provided the maximum yield (29.2 $g.L^{-1}$), while, the A27 isolate produced the lowest yield (17.2 $g.L^{-1}$). Because bacteria produce secondary metabolites such as curdlan, raising the rate at which curdlan is synthesized result in a decrease in the amount of biomass produced by these bacteria. These findings were in concordance with Liu *et al*. (2016), who found that *Agrobacterium* sp. HX1126 had the maximum production of curdlan. Curdlan production by this isolate reached 30 $g.L^{-1}$ after being incubated at 30°C. In a other investigation, the roots of the plant were sampled in order to acquire 165 local isolates (Moselhy *et al*., 2020).

Curdlan could be produced by 35 of these isolates when they were tested. Following the completion of the screening process, two isolates, Cs5 and T1, were chosen because of their capacity to produce a significant quantity of curdlan (7.1 and 5.7 $g.L^{-1}$, respectively).

Table (1): Evaluation of bacterial isolates using biochemical tests

		Isolates									
	Test type	A1	A2	A3	A4	A21	A26	A27	A28	A29	A30
Biochemical tests	3-Ketolactose	-	+	-	-	-	-	+	+	-	-
	Litmus	+	+	+	+	+	+	+	+	+	+
	Catalase production	+	+	+	+	+	+	+	+	+	+
	Citrate utilization	+	+	+	+	+	+	+	+	+	+
	Oxidase	+	+	+	+	+	+	+	+	+	+
	Indole	-	-	-	-	-	-	-	-	-	-
	Production of gas from glucose	-	+	-	-	-	-	+	+	-	-
	Decomposition of urea	-	+	-	-	+	+	+	+	+	+
	nitrate reduction	+	+	+	+	+	+	+	+	+	+
	Voges- Proskauer	+	+	+	+	+	+	+	+	+	+
	H_2S	+	+	+	+	+	+	-	-	+	+
Growth at different temperature	25 ºC	+	+	+	+	+	+	+	+	+	+
	30 ºC	+	+	+	+	+	+	+	+	+	+
	35 ºC	-	+	-	-	-	-	+	+	-	-
	40 ºC	-	+	-	-	-	-	-	-	-	-
salt concentration	1%	+	+	+	+	+	+	+	+	+	+
	2%	-	+	-	-	+	+	+	+	+	+
	3%	-	+	-	-	-	-	-	-	-	-
pH	5	+	+	+	+	+	+	+	+	+	+
	6	+	+	+	+	+	+	+	+	+	+
	7	+	+	+	+	+	+	+	+	+	+
	8	+	+	+	+	+	+	+	+	+	+
	9	+	+	+	+	+	+	+	+	+	+
	10	+	+	+	+	+	+	+	+	+	+
fermentation of carbohydrates	fructose	+	+	+	+	+	+	+	+	+	+
	maltose	+	+	+	+	+	+	+	+	+	+
	lactose	+	+	+	+	+	+	+	+	+	+
	sorbitol	+	+	+	+	+	+	+	+	+	+
	sleeboys	+	+	+	+	+	+	+	+	+	+
	mannose	+	+	+	+	+	+	+	+	+	+
	dextrose	+	+	+	+	+	+	+	+	+	+
	arabinose	+	+	+	+	+	+	+	+	+	+
	glycerin	+	+	+	+	+	+	+	+	+	+
	xylose	+	+	+	+	+	+	+	+	+	+
	galactose	+	+	+	+	+	+	+	+	+	+
	sucrose	+	+	+	+	+	+	+	+	+	+
	glucose	+	+	+	+	+	+	+	+	+	+
	raffinose	+	+	+	+	+	+	+	+	+	+
	erthritol	+	-	+	+	-	-	-	-	-	-
	cellulose	-	-	-	-	-	-	-	-	-	-
	starch	-	-	-	-	-	-	-	-	-	-
	agar	-	-	-	-	-	-	-	-	-	-

Agrobacterium tumefaciens was determined to be present in the Cs5 and T1 isolates on the basis of their morphological and biochemical characteristics as well as the 16S rRNA marker (Moselhy *et al*., 2020).

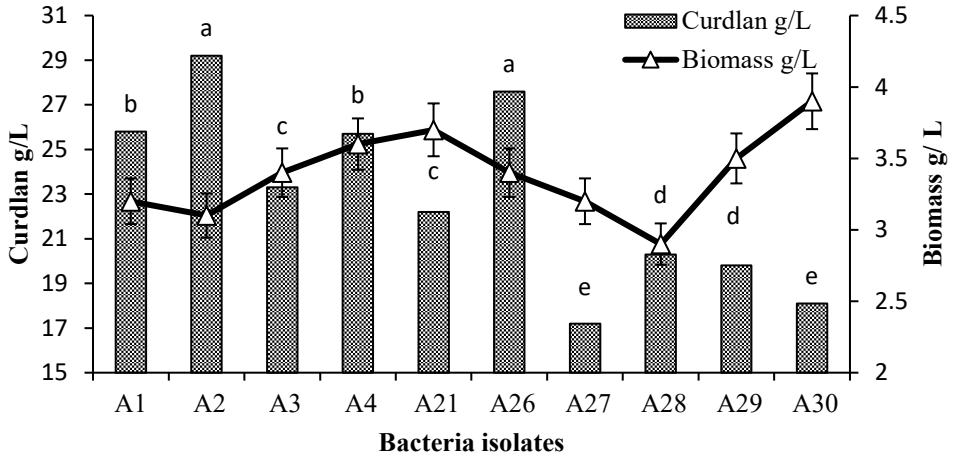

Fig. (1): A graphical presentation of the amount of curdlan that was produced as well as the biomass of the various bacterial isolates

The impact of date juice media curdlan production

The effect of using different concentrations of date juice as an alternative carbon source in the growth media on curdlan production is depicted in fig. (2). The highest curdlan yield (32.9 $g.L^{-1}$) was recorded at a concentration of 26.4 mL of date juice per 100 ml, while the biomass weight was 2.3 $g.L^{-1}$. The production dropped as the concentration of date juice increased, reaching a maximum of 28.6 $g.L^{-1}$ when there was 35.3 ml of date juice added for every 100 ml of production medium. At a concentration of 26.4 ml of date juice per 100 ml of standard medium, the statistical analysis revealed that there were significant differences between the standard medium and the date juice medium. This could be because certain types of monosaccharides, like glucose, fructose, and inverted sugars, are present, which stimulate bacteria to produce curdlan (Al-Roomi & Al-Sahlany, 2022). Additionally, the amount of biomass decreased with an increase in production, which could be because bacteria have a tendency to focus on production rather than biomass. Despite the fact that these results differed from those of Gao *et al*. (2020), they demonstrated that the optimal concentration of sugars in the production medium is 40 $g.L^{-1}$of sucrose, glucose, and fructose as a carbon source at a temperature of 30°C and a pH of 7.

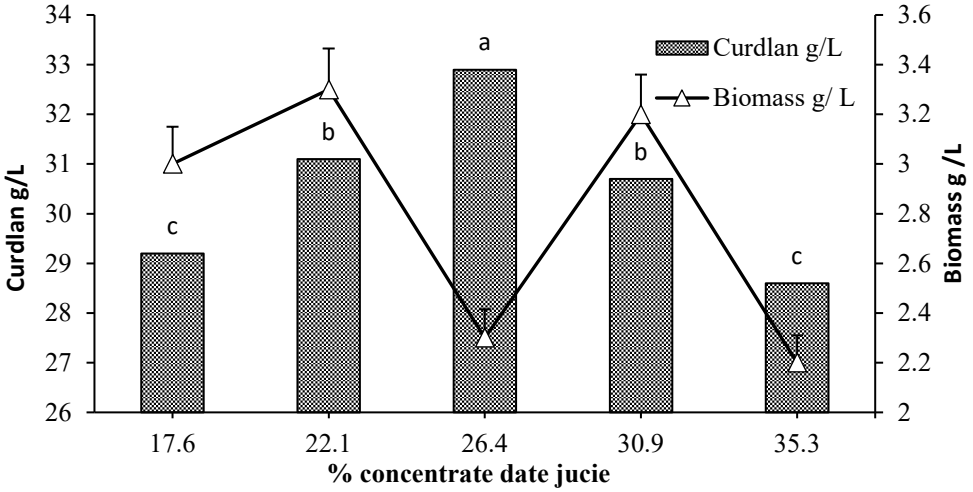

Fig. (2): A graphical presentation of the process by which curdlan was produced by *Agrobacterium leguminum* Ag003 after the bacterium was grown in a medium that contained concentrations of date juice

Fourier Transform Infrared Spectroscopy (FTIR)

The results of an analysis of the curdlan produced in both CP1 and CP2 as well as a comparison with curdlan (CS) produced chemically are presented in fig. (3). The FTIR spectroscopy, which detected the vibrational frequencies of the functional groups present in the resulting curdlan parts, revealed that a band of spectral absorption at the wavelength of 3368–3399 cm^{-1} was due to the fluctuation of hydroxyl groups. This was noticed by observing a band of spectral absorption at the wavelength of 3368–3399 cm^{-1}. A distinct peak was seen at a wavelength range of 1594–1646 cm^{-1}, which corresponded to the –C=O (carbonyl) group. In contrast, the band that spans 2929–2938 cm^{-1} was part of the C–H and –CHO families. The –COO groups were seen at a wavelength that ranged from 1423 to 1435 cm^{-1}, the –CO group was found at a wavelength that ranged from 1202 to 1279 cm^{-1}, and the peak that was observed at a wavelength range that ranged from 1079 to 1036 cm^{-1} was ascribed to the C-O-C ether bond. Popescu *et al*. (2019) shown that the existence of C-O-C bonds may be identified by scanning at the infrared spectra at the absorption of 1041 cm^{-1}. Curdlan may be identified by the use of FTIR spectrophotometry. This method identified active groups such as hydroxyls, carboxylic, and carbonyl groups in addition to acetyl groups. These results were in line with what Tao and co-workers had found (Tao *et al*., 2022). A peak of hydroxyl groups occurred at a wavelength of 3417 cm^{-1}, while the wavelength was 2920 cm^{-1} owing to the groups of CH_2 and CH_3, and the peak that appeared at 840 cm^{-1} corresponded to the carbon in the ether bond. The active groups of curdlan were able to be recognized.

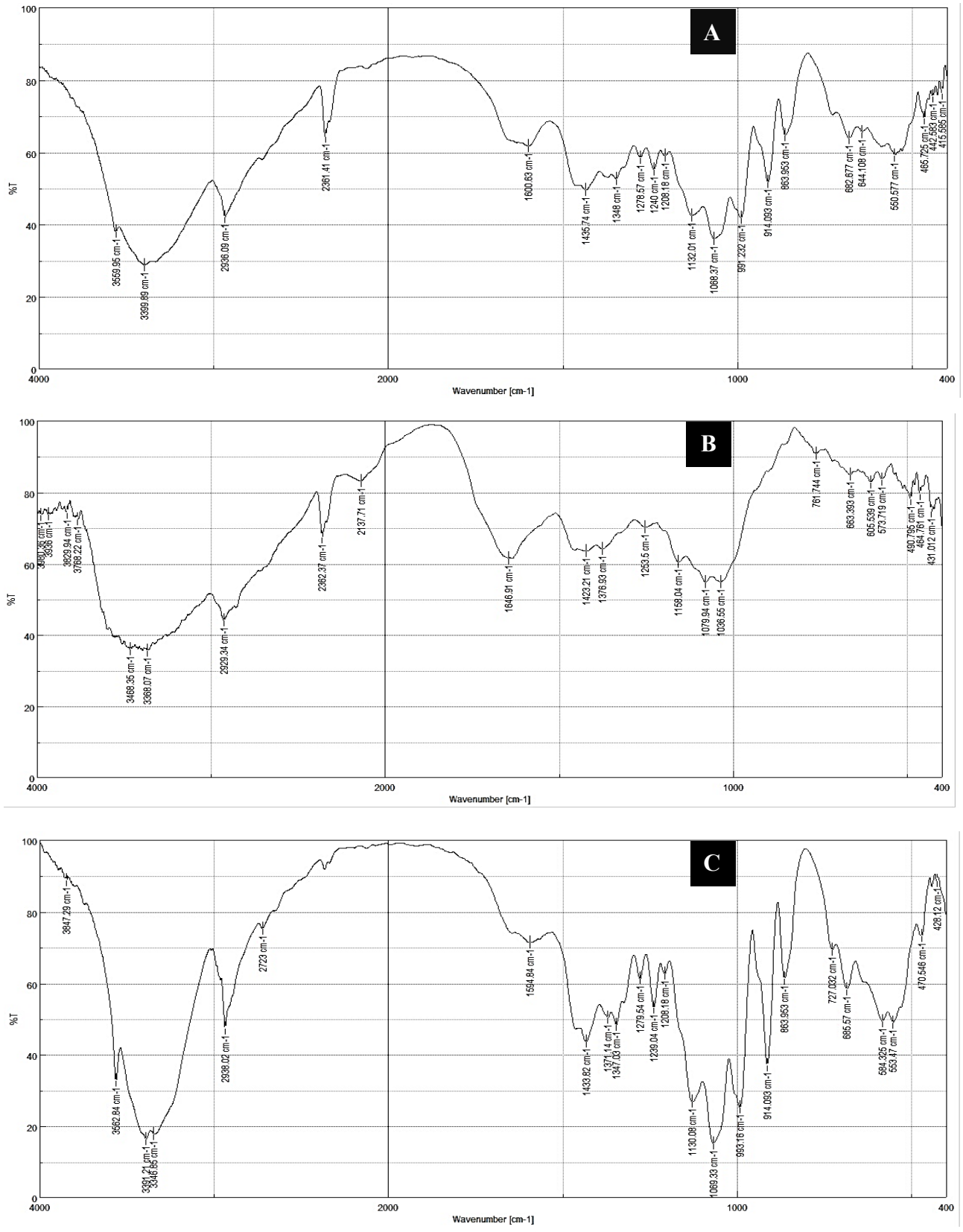

Fig. (3): The results of the FTIR analysis on the Curdlan samples: (A) the Curdlan standard (CS), (B) the standard media (CP1), and (C) the date juice medium (CP2)

Curdlan analysis by using ^{1}H-NMR

The ^{1}H-NMR analysis of the curdlan that was produced in CP1 and CP2 is presented in fig. (4), along with a comparison to the curdlan standard (CS), in order to establish the type of bonding that 1, 3-β-glucan possesses. The ^{1}H-NMR spectra provide information on the positions of the hydrogen atoms in curdlan. In the fundamental structure of glucose, which is known as the curdlan unit, there are five primary signals of hydrogen atoms. The hydrogen atom values of glucose are listed in table (2). The findings of the current study were comparable to the findings of Liang *et al*. (2017), who found that the appearances of five values of hydrogen in the curdlan were, respectively, 5.32, 4.47, 3.68, 3.44, and 3.26 when identified by ^{1}H-NMR.

Table (2): The position of the hydrogen atom in the ^{1}H-NMR spectrum of curdlan samples, the curdlan chemical (CS), the standard medium (CP1), and the date juice medium (CP2)

Hydrogen atom	CS	CP1	CP2
H1	4.48	4.48	4.47
H2	4.57	4.59	4.58
H3	4.98	4.98	4.99
H4	5.11	5.14	5.13
H5	5.40	5.44	4.42

Curdlan analysis by HPLC

Presents a comparison of the curdlan produced in the CP1 and CP2 media to the chemical curdlan (CS) that was analyzed by HPLC. Additionally, the identification of the curdlan produced in these two mediums is presented. One distinct peak that did not overlap with any other peaks showed up, which is evidence of the high purity of the curdlan that was produced (CP1 and CP2). When the absorbance of the peaks finally reached a point where they clearly converged, the values for CS, CP1, and CP2 were 131, 176, and 191 mAU, respectively. In addition, the results demonstrate that the amount of retention time for the apparent peaks was 5.92 minutes for CS, 5.95 minutes for CP1, and 5.91 minutes for CP2, respectively. This indicates that curdlan was the substance that was produced from both the standard medium and the date juice medium. According to Jaafar *et al.* (2020), who observed that one abrupt peak developed at the time of retention (5.42 minutes) of curdlan produced from glucose as a carbon source in the culture medium, these findings were in agreement with the findings of the present study. Using the HPLC method, the researchers Liu *et al.* (2016) were able to identify curdlan that was produced from YM medium by *Agrobacterium* HX1126. One distinct peak was seen throughout their investigation.

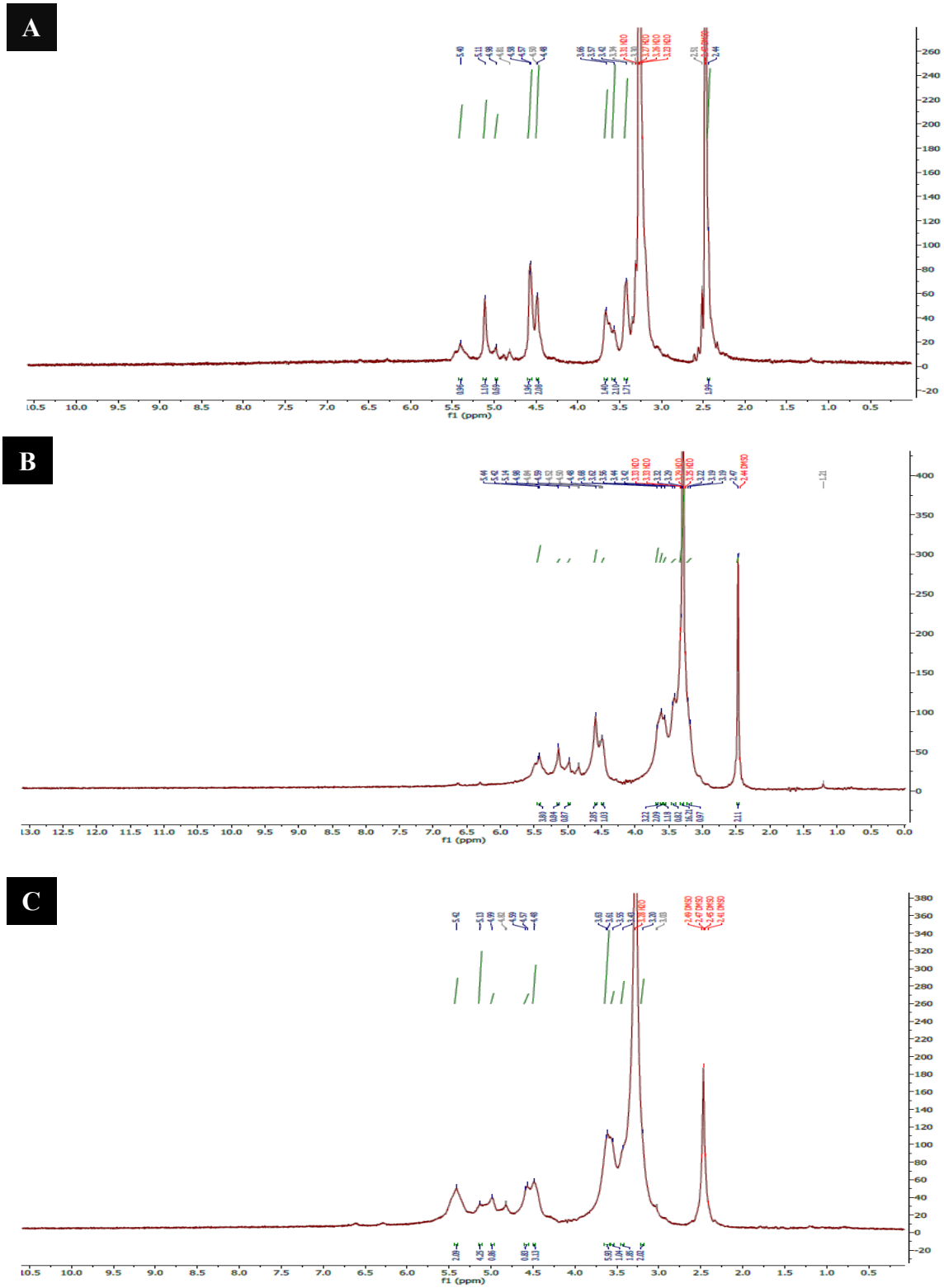

Fig. (4): ^{1}H-NMR spectra of three different types of curdlan samples: (A) the curdlan standard (CS), (B) the standard media (CP1), and (C) the date juice media (CP2)

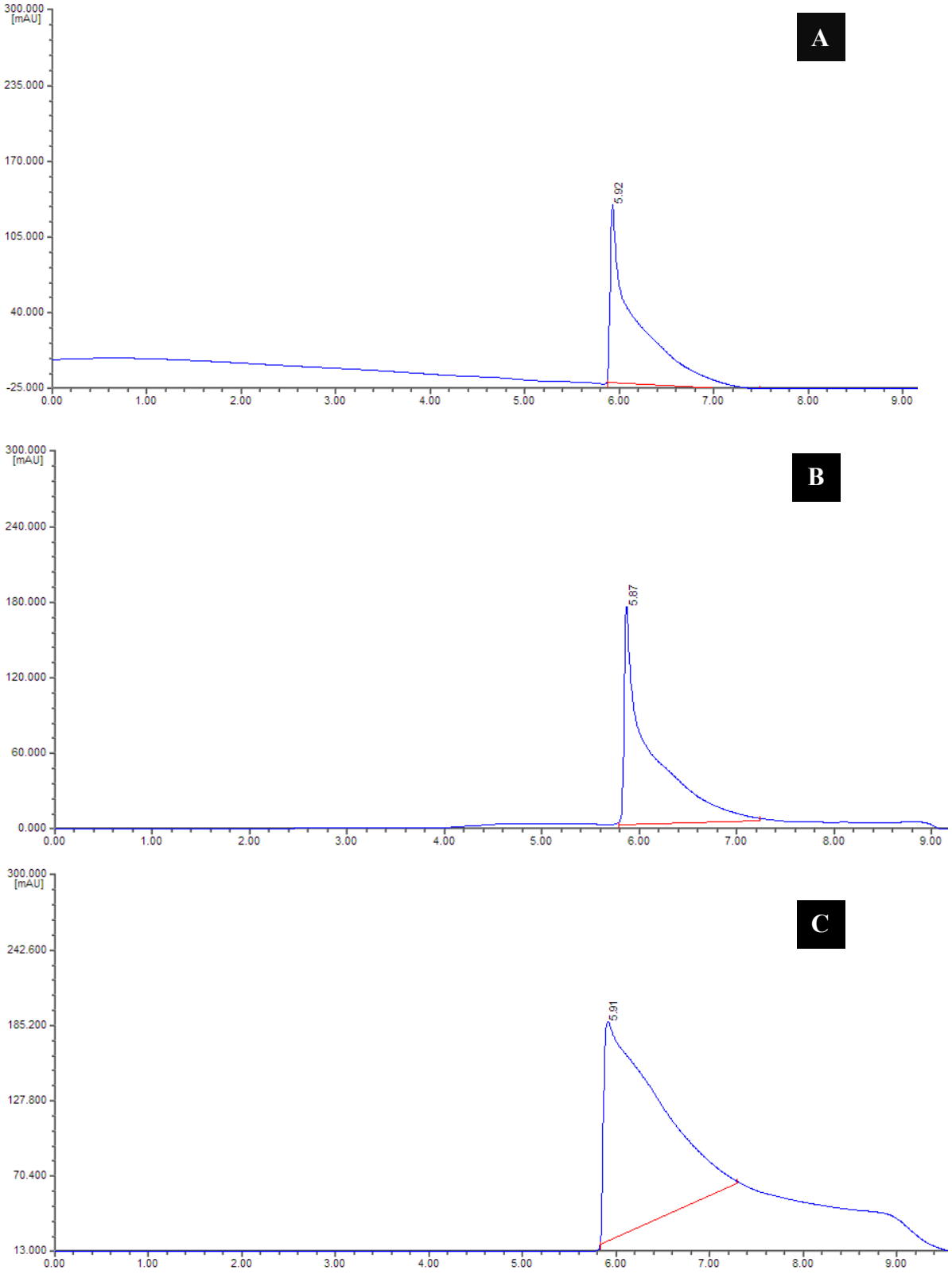

Fig. (5) : The results of employing the high-performance liquid chromatography technique to curdlan samples, (A) the curdlan standard (CS), (B) the standard media (CP1), and (C) the date juice media (CP2)

Conclusion

Curdlan, with its distinctive gel formation, limited water solubility, moderate processing conditions, indigestibility, and processing capabilities, has been the subject of research for almost fifty years. Researchers are presently investigating the substance's potential use in the food, health care, and biomedical industries. Because it possesses several functional properties, including those of a stabilizer, a fortifier, and a thickening, as well as those of a prebiotic, it contributes to the nutritional value of the product. Therefore, it is possible to implement it in a variety of different food industries. Producing curdlan at a lower cost to the economy was accomplished by utilizing agricultural byproducts as carbon sources. These included date juice, molasses, grape juice, and whey protein. In addition to the disposal of agricultural waste, which is a factor in the environmental damage. The amount of curdlan that may be produced is highly dependent on the kind of strain as well as the culture medium that is used.

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Contributions of authors

Y.S.S.: Carried out the experiment in the field and collected data.

S.T.G.: Analyzed the results statistically and contributed to the writing of the manuscript. Writing the draft of manuscript.

H.I.A.: Developed the idea and research plan and contributed to writing the manuscript and collecting sources.

All authors reviewed the manuscript.

ORCID

S.T.G. Al-Sahlany: https://orcid.org/0000-0002-0617-6870

H.I. Ali: https://orcid.org/0000-0001-5185-6093

Conflicts of interest

The authors declare that they have no conflict of interests.

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تأثير استعمال عصير التمر كمصدر للكربون على الكوردلان الناتج من العزلة المحلية لبكتريا *Agrobacterium leguminum*

يعقوب شمال شايش الرميض وشيماء ذياب جدوع السهلاني وحيدر ابراهيم علي

قسم علوم الاغذية، كلية الزراعة، جامعة البصرة، البصرة، العراق

المستخلص: يعد الكوردلان من السكريات المتعددة الميكروبية التي ينتج من البكتريا.*Agrobacterium* spp استعملت 10 عزلات لإنتاج الكوردلان تم الحصول عليها من مصادر محلية مختلفة تضمنت انواع من الترب الزراعية والعقد الجذرية وجذور النباتات شخصت العزلات اعتماداً على الفحوصات المظهرية والمجهرية واختبارات الكيميوحيوية، غربلت العزلات واظهرت العزلة A2 اعلى انتاج اذ بلغ 29.2 غم. لتر$^{-1}$. اظهرت النتائج ان الوسط البديل الحاوي على عصير التمر بتركيز 26.4 مل.100 مل$^{-1}$ من وسط الانتاج هو الافضل في انتاج الكوردلان اذ بلغ اعلى حاصل 30.7غم. لتر$^{-1}$. شخص الكوردلان الناتج من الوسط الزرعي القياسي والوسط الزرعي البديل باستعمال تقنيات FTIR و NMR وHPLC واظهر تطابقاً مع الكوردلان القياسي. لذلك يمكن استعمال اوساط محلية بديلة من المخلفات الزراعية في انتاج الكوردلان الذي يعد أحد اهم المثبتات الغذائية التي يمكن استعماله في العديد من الصناعات الغذائية.

الكلمات المفتاحية: بكتريا *Agrobacterium leguminum*، الكوردلان، عصير التمر.