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Original Article

Isolation, Screening, and Identification of Local Bacterial Isolates Producing Bio-Cellulose

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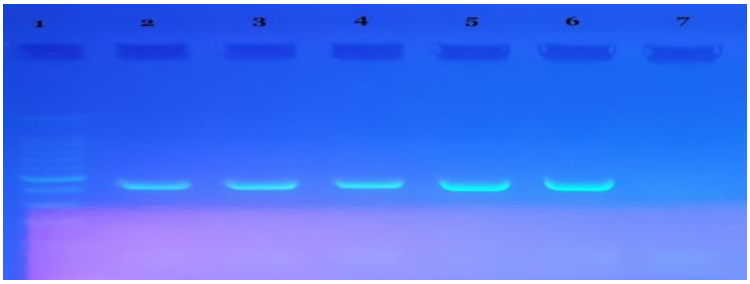
ABSTRACT

The current study aims to easily, quickly, and cheaply select the local bacterial isolate that produces bacterial cellulose. Ten samples of spoiled fruits (triplicate for each) were taken in Al-Kut City/ Wasit Province/ Iraq. A modified H-S medium was used to isolate the local bacteria from spoiled fruit samples. One-hundred and twelve bacterial isolates were obtained from all samples, where the lemon sample give the highest number of isolates, as it reached 20 isolates, while the banana sample gives the lowest number of isolates reached to 6 isolates. The two steps of screening were achieved to select the best cellulose-producing local isolate, results from the primary screening indicated that only 18 isolates appeared a clear ability to create clear zones as an indicator to produce bacterial cellulose, and the isolates TELE8, TELE11, TEKI4, and TEAP5 gave the highest clear zones of 7.5, 6.1, 6.2, and 6.5 cm, respectively. Also, the second screening step confirmed the results collected from the first step, as the results hydrogel test indicated that only 18 strains showed the ability to produce bacterial cellulose, while the other isolates were unable to do so, as the results showed that the isolate TELE8 was superior in its ability to produce bacterial cellulose than isolates TEK11, TEKI4, and TEAP5. Therefore, the local isolate TELE8 was chosen as the best isolate that produces bacterial cellulose. Thereafter, the phenotype and genotype characteristics were used to identify the TELE8 isolate; its results indicated that the isolate belongs to *Komagataeibacter xylinum* (previously named first *Acetobacter xylinum*, and then *Gluconacetobacter xylinus*). The three methods (static, shaker, and fermenter) were used to produce the bacterial cellulose by using the best local bacterial isolate Acetobacter xylinum TELE8 in HS medium. The results revealed that the highest production of BC (22.8 g/200 ml medium) was obtained when using the fermenter method, while the shaker method did not give the productivity of bacterial cellulose.

GRAPHICAL ABSTRACT



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Introduction

Cellulose is considered as one of the most prevalent polymeric compounds in nature, and it is presented in all plants and manufactured by various microorganisms. The cellulose production is currently available from plant woods only, and this is the only stable supply that can keep up with the industrial requirements for the material [1]. In addition to cellulose from plant cells, certain bacterial strains can produce the extracellular cellulose in the fibrils form attached to the bacterial cell. This cellulose known as bacterial cellulose or bio-cellulose is an extracellular polymer composed of monosaccharides or simple sugars as a substrate, such as glucose, xylose, and galactose, or from the alternative carbon sources, such as ethanol and glycerol [2]. The main sources that produce cellulose are several plants including trees, flax, cotton, and hemp. This cellulose is used in many industrial applications such as money papers, papers, and clothes. These applications are just a few products made possible by the plant cellulose. In addition to the plant sources, there are a few other sources that are important for the cellulose production, such as fungi, seaweed, and a few bacterial organisms, most importantly the aerobic non-pathogenic such as gram-negative *Komagateibacter* sp. which can produce cellulose, and *Komagateibacter xylinus* isolate previously called *Acetobacter* that is today considered the most investigated species [3, 4]. The bacterial isolates producing cellulose have been isolated from different sources, including fruits, vegetables, flowers, vinegar, and fermented beverages. In particular, spoiled fruits or their residuals have a lot of potentials to be used to produce bacterial cellulose due to their availability and low cost [5]. The macromolecular and surfactant characteristics of the bacterial cellulose are significant, making it ideal for both *in vivo* and *in vitro* medical and biological purposes. A novel biomaterial for biomedical use has been developed in the form of bacterial cellulose [6]. Among the most promising new developments in regenerative medicine is the use of bacterial cellulose, which improves cell attachment, multiplication, movement, and eventually specialization, leading to the quicker wound repair and re-epithelialization. A moist wound microenvironment, sufficient gas transfer, absorption of interstitial fluids, minimal tissue adhesion, and thermal insulation are all features of bacterial cellulose-based biomedical devices. To develop a great biomaterial, the bacterial cellulose should be designed and manufactured with a sufficiently reactive surface to enable the cellular interaction [7]. Biomaterials for organ transplantation, medication administration, wound repair, and tissue/organ recovery receive support greatly from the close association between cells and bacterial cellulose. These interactions are ideally related to the physical, chemical, and medicinal characteristics, such as the charge of the surface, wettability, topographical properties, and the existence of hydrophobic or hydrophilic substances. The overall result of new biomedical device types is decided by how cells react to bioactive molecules [8]. The goal of this study was to isolate a local bacterial cellulose-producing bacterium from spoiled fruits.

Materials and methods

Spoiled fruit collection

Ten samples of spoiled fruit were used to isolate the bacteria, where triplicate samples were collected from each, obtained from the local markets of the Al-Kut City/ Wasit Province/ Iraq. These ten different fruit types included Apple (*Malus domestica*), Grape (*Vitis vinifera*), *Citrus aurantium* (small), *Citrus aurantium* (big), Lemon (*Citrus limon*), Orange (*Citrus sinensis*), Pomegranate (*Punica granatum*), Kiwi (*Actinidia deliciosa*), Banana (*Musa acuminata*), and Strawberry (*Fragaria ×ananassa*).

Fruit juice preparation

100 g from each whole spoiled fruit was used to prepare the fruit juice, where each spoiled fruit was placed in a blender containing 200 ml of tap water, and then the ingredients were mixed and filtered with a cloth filter to separate the fruit residues and collect the fruit juice [9, 10].

Bacterial isolation

The modified liquid Hestrin-Schramm (HS) medium consists of (0.2% D-glucose, 0.5% peptone water, 0.5% yeast extract, 0.27% Na_2HPO4, 0.12% citric acid, 0.2% acetic acid, and 0.5% ethanol), was prepared according to the method described by [11, 12], and then the pH was adjusted to 6, and 0.01% of cycloheximide was added to reduce fungal and yeast contamination. 10 mL of each fruit juice was added to flasks containing 90 ml of HS medium, and flasks were incubated at 30 °C for 7 days in a static incubator. After the incubation period, the flasks with white granules covering the surface of the liquid medium were selected, and a loopful from each flask was cultured on plates of solid HS medium by streaking method. All plates were incubated at 30 °C for 72 hours until colonies formed, and then the single colony of bacterial isolates was isolated and purified by transferring the bacterial colonies several times by using the plates containing the solid HS medium. Thereafter, the plates containing the pure bacterial isolates were incubated at 30 °C for 72 hours, and then kept in the refrigerator until use [13, 14].

Screening of bacterial isolates

The selected bacterial isolates from the last step were tested by two methods to choose the most efficient bacterial isolate for bio-cellulose production. The first step involved measuring the diameter of clear zones, where all selected isolates were inoculated on glucose-yeast ethanol extract agar (GYEA), which consists of (2% D-glucose, 1% yeast extract, 5% ethanol, 0.3% CaCo3, and 2% agar), all plates were incubated at 30 °C for 5-7 days, and then the diameter of clear zones was measured after 4, 5, 6, and 7 days of incubation [14].The second step of the screening is called the hydrogel test, where the inoculum of bacterial isolates was prepared by using one loopful from each bacterial isolate to inoculate test tubes containing 5 mL of liquid HS medium, and then the tubes were incubated at 30 °C for 24 hours, where the inoculum of each isolate was used in the hydrogel test [15]. The hydrogel test was carried out by using test tubes containing 10 ml of liquid HS medium. After that, each tube was inoculated with 1 mL inoculum of each bacterial isolate, and then the inoculated test tubes were incubated at 30 °C for 7 days. The formation of the gel layers or bacterial cellulose is monitored from the fourth day of incubation until the seventh day, and the results are recorded [10].

Identification of selected isolate

The phenotype and genotype characteristics including morphological, biochemical, and genetic tests were done to identify the more efficient local isolate for bio-cellulose production. The biochemical and morphological tests include the colony color, shape, size, colony texture, gram staining, oxidase test, catalase test, indole production, and other tests, as described in Bergey's Manual of Determinative Bacteriology [16]. Whereas, the genotype identification was carried out by extracting the whole genomic DNA of the selected isolate by using the kit of Presto TM Mini gDNA Bacteria from (Geneaid, Korea), and the kit protocol was conducted. The 16S rRNA gene in genomic DNA was amplified according to the method described by Lavasani *et al*. (2017) by using the forward primer (5-GAGGAACCTGCGTTCGATTAG-3) and reverse primer (5-TACACTGGGAATTCCACAACC-3) [17], and then the PCR products were purified, and their sequence was achieved by Source BioScience Company (Nottingham, UK). The results of sequencing were submitted to BlastN for sequence alignment and homology comparisons against the NCBI GenBank database to identify the bacterial isolate [18]. A further step of molecular analyses was achieved by a specific gene called (*bcs*A1) responsible for producing the cellulose synthase enzyme as the second step to prove that the local bacterial isolate has this gene. This step was achieved according to Lavasani *et al*. (2017) using a specific forward primer (5-TCCATATCGGGCAGCGCGTG-3) and reverse primer (5-CCCAGGAACAAGAACGCCAGC-3) [17], and then the PCR products were purified and sequenced by Source BioScience Company (Nottingham, UK). The results of sequencing were submitted to BlastN for sequence alignment and

homology comparisons against the NCBI GenBank database to investigate the presence of this gene in local isolate [18]

Bio-cellulose production by the selected isolate

Three methods were used to produce the bacterial cellulose in static conditions without aeration, in shaker conditions with 150 rpm, and in a fermenter with an aeration level of 0.5 L/min., the 200 ml of liquid HS medium with pH 5.5 was used in the three experiments, and then 5 ml of bacterial inoculum was added to each experiment under their respective conditions, and all experiments were incubated at 30 °C for 7 days of incubation. The productivity of bacterial cellulose was determined at the end of each experiment.

Results and Discussion

The results in Table 1 indicate that 112 bacterial isolates were obtained from the rotten fruit samples. Likewise, the results show a clear difference in the number of bacterial isolates obtained from each fruit sample, where the lemon sample gave the highest number of isolates, which reached 20 isolates, while the banana sample gave the lowest number of isolates as it reached 6 bacterial isolates, and this may be due to the nature of the microbial flora of each isolated sample in addition to the environmental conditions surrounding each isolated sample.

Table 1: The bacterial isolates obtained from local rotten fruit samples

No.	Samples	Sample code	The number of obtained bacterial isolates
1	Orange	OR	8
2	*Citrus aurantium* (big)	CB	9
3	*Citrus aurantium* (Small)	CS	11
4	Lemon	LE	20
5	Kiwi	KI	12
6	megranatePo	PO	14
7	Apple	AP	10
8	Grapes	GR	12
9	Strawberry	ST	10
10	Banana	BA	6
	Total bacterial isolates		112

The bacteria are considered the primary producers of pure cellulose for the food and biomedical industries, as well as for the development of bio-composite polymers and nanostructure materials, it finds broad usage due to its high purity and distinctive physicochemical properties [19-22]. According to the results in Table 1, 112 bacterial isolates were found in the samples of spoiled fruit, where the lemon sample gave the highest number of isolates, which reached 20 isolates, while the banana sample gave the lowest number of isolates as it reaching 6. Our results come in agreement with those of Rangaswamy *et al.* [23], who used the spoiled fruit waste (apple, banana, guava, grape, mango, orange, pomegranate, and sweet lime) and rotten vegetables (potato, ladies finger, onion, ridge guard, sweet potato, carrot, brinjal, and tomato), 36 isolates of cellulose-producing bacteria were obtained. It was found that the best fruit (pomegranate), (sweet potato), and (potato) showed better cellulose production compared with the other isolates. Ibrahim *et al.* obtained three local isolates of cellulose-producing bacteria from 103 isolates which were isolated from 20 samples of rotten fruits [13]. Moreover, Güzel and Akpınar [24] revealed that Komagataeibacter hansenii GA2016 was able to produce bacterial cellulose from the peels of kiwi, melon, apple, and pomegranate with the highest ratio of synthesis belonging to kiwifruit (11.53%). Whereas, Yanti *et al.* [25] thirteen strains of producing bio-cellulose were isolated from pineapple waste (pineapple core and peel) and seven of them were capable to produce bio-cellulose by using sago liquid waste substrate. In

addition, Andritsou *et al.* [26] reported that *K. sucrofermentans* DSM 15973 was able to produce bacterial cellulose from open-market-discarded oranges, grapefruits, and lemon with a high concentration of cellulose from Grapefruit and orange juices (6.7 and 6.1 gm/L, respectively). Furthermore, Raiszadeh-Jahromi *et al.* [27] found that the bacterium; *Komagataeibacter xylinus* PTCC 1734, was efficient in the synthesis of bacterial cellulose from a combination of date syrup, cheese whey, and ascorbic acid with the highest synthesis of bacterial cellulose on day 10 of bacterial cultivation.

Bio-cellulose production

Two steps of screening were used to determine the ability of 112 bacterial isolates which were previously isolated from 10 samples of spoiled fruit. Table 2 shows that only 18 bacterial isolates out of a total of 112 isolates revealed a clear ability to form a clear zone in the test medium after 5, 6, and 7 days of incubation, while none of the isolates were able to give a clear zone after 4 days of incubation. The results also indicate a significant difference in the diameters of the clear zones, where the highest diameter of the clear zone was 7.5 cm for the bacterial isolate TELE8, while the lowest diameter of the transparent area was 4 cm for the isolate TECB2 after 7 days of incubation.

The results from the second step of screening as described in Table 3 also indicate that only the same 18 isolates can produce bacterial cellulose, while the other isolates do not. The results of the hydrogel test further show that these bacterial isolates appeared the ability to produce bacterial cellulose with a clear difference according to the studied incubation periods, where the results show that only TELE8 isolate can produce cellulose after 4 days of incubation, while only three isolates called TELE11, TEKI4, and TEAP5 were able to produce cellulose.

Table 2: The ability of bacterial isolates to produce bacterial cellulose using the clear zone method as the indicator

Isolates code	Source of isolation	Diameter of a clear zone (cm) after			
		4 days	5 days	6 days	7 days
TECB2	*Citrus aurantium* (big)	0	2	2.5	4
TELE1	Lemon	0	3.5	4.6	5.35.3
TELE4	Lemon	0	3.2	3.9	4.8
TELE8	Lemon	0	4.3	5.7	7.5
TELE11	Lemon	0	3.1	4.6	6.1
TELE14	Lemon	0	2.8	3	3.5
TELE19	Lemon	0	3	3.9	5.1
TEKI2	Kiwi	0	3.1	4.3	5.2
TEKI4	Kiwi	0	3.4	4.2	6.2
TEKI9	Kiwi	0	2	3.1	4.2
TEPO8	Pomegranate	0	3.6	4.3	5.6
TEAP2	Apple	0	3.1	4.4	5.5
TEAP5	Apple	0	3.3	4.5	6.5
TEGR6	Grapes	0	3.5	4	4.8
TEGR9	Grapes	0	3.6	4.6	5.1
TEST3	Strawberry	0	3.1	4.2	5.2
TEST8	Strawberry	0	3.3	4.6	5.1
TEBA1	banana	0	2.8	4.3	4.9
94 bacterial isolates indicate the negative results		-	-	-	-

Table 3: The ability of bacterial isolates to produce bacterial cellulose during the periods of incubation by using the hydrogel method as the indicator

Isolate code	Source	The ability of isolates to produce BC after			
		4 days	5 days	6 days	7 days
TECB2	Citrus aurantium(big)				+
TELE1	Lemon			+	
TELE4	Lemon			+	
TELE8	Lemon	+			
TELE11	Lemon		+		
TELE14	Lemon				+
TELE19	Lemon				+
TEKI2	Kiwi			+	
TEKI4	Kiwi		+		
TEKI9	Kiwi				+
TEPO8	Pomegranate			+	
TEAP2	Apple			+	
TEAP5	Apple		+		
TEGR6	Grapes				+
TEGR9	Grapes			+	
TEST3	Strawberry			+	
TEST8	Strawberry				+
TEBA1	banana			+	
94 bacterial isolates		-	-	-	-

Tables 2 and 3 present two screening steps used to determine the ability of bacterial isolates to produce the bacterial cellulose by using the clear zone and hydrogel method as the indicator. A large number of researchers were able to isolate bacterial isolates that can produce bacterial cellulose, as they were screening by using several methods, where Yanti *et al.* [25], Ibrahim *et al.* [13], and Singh *et al.* [14] used clear zone method, also Alemam *et al.* [10] used clear zone and the hydrogel method, while Rangaswamy *et al.* [23] used the Fluorescent colony, the fluorescent dye binds to the cellulose content in the organism. The cellulose-producing bacterial colonies are fluoresced when observed under the UV light, so the fluorescent colonies were selected as cellulose producers.

Identification of local isolate

Table 4 indicates the results of the colonies and cells morphology, and also the results of biochemical tests that were conducted on the local isolate TELE8 with the highest productivity, where the results reveal that the TELE8 isolate can be identified as *Komagataeibacter xylinum* (previously named first *Acetobacter xylinum* and then *Gluconacetobacter xylinus*).

The results from the genotype test by using universal primers to detect the 16S rRNA in the whole genomic DNA of local bacterial isolate TELE8, the electrophoresis results of the amplified and purified PCR product was displayed in Figure 1. There is a single band in lanes 2 to 6 that has about 436 pb compared with the DNA ladder in lane 1. Also, the genotype test indicates that the sequence of PCR product from the local isolate TELE8 has appeared a high similarity reached to 100% with the sequence of a registered strain called *Komagataeibacter xylinus* NBRC 11664, as depicted in Figure 2. Therefore, this local isolate TELE8 can be classified as *Komagataeibacter xylinus*.

Table 4: Morphological characteristics and biochemical tests of most active bacterial isolate; TELE8

Morphological and biochemical characteristics of the selected bacterial isolate (TELE8)	Results
Gram reaction	Gram-negative
Colony shape	Circular
Colony color	Light golden
Texture	Mucous
Surface	Smooth
Cell shape	Rod (bacilli)
Colony elevation	Convex
Colony margin	Entire
Catalase test	+
Oxidase test	-
Citrate test	-
Urease test	-
Indole test	-
TSI (triple sugar iron agar)	-
Growth in the presence of 0.35% acetic acid (pH=3.5)	+

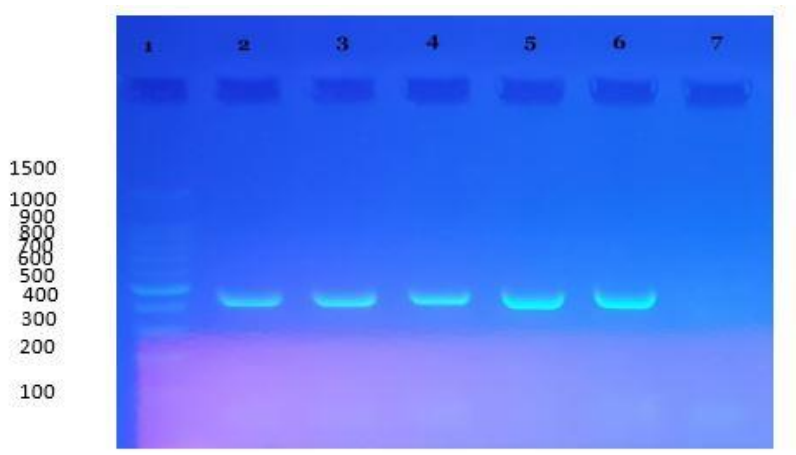

Figure 1: Gel electrophoresis of 16 S gene PCR product of *Komagataeibacter xylinus*. 2% agarose gel, 100 v for 50 min. Lane 1 indicates molecular marker, lanes 2-6, positive shows a single band of about 436, and lane 7 demonstrates the negative control

The second step of the molecular analysis to detect the bcsA1 gene in the local isolation is illustrated in Figure 3, where it is observed that there is a single band in lanes 2 to 6 that represents the PCR products after amplification and purification.

The isolated strain was characterized based on colony morphology, specific biochemical tests, and 16S rDNA sequence analyses. Therefore, the local bacterial isolate TELE8 was classified as Komagataeibacter xylinus TELE8, also the genotype test indicates that the sequence of PCR product from the local isolate TELE8 has appeared with a high similarity reached to 100% with the sequence of a registered strain called *Komagataeibacter xylinus* NBRC 11664. Shaheen *et al.* [28] found a strain namely, SA3.1, which was isolated from spoilage apple, the biochemical assays of the strain SA3.1 exhibited the identical characteristics of *K. hansenii* NCIMB 8746, the molecular identification was performed through amplifying of the 16s rDNA fragment (1500 bp) of K. hansenii SA3.1 by using PCR technique. The latter technique affirmed that, undoubtedly, the similarity of the examined strain was much closer to the sequence of *Komagataeibacter hansenii* NBRC 14,820 which attained 99.4%.

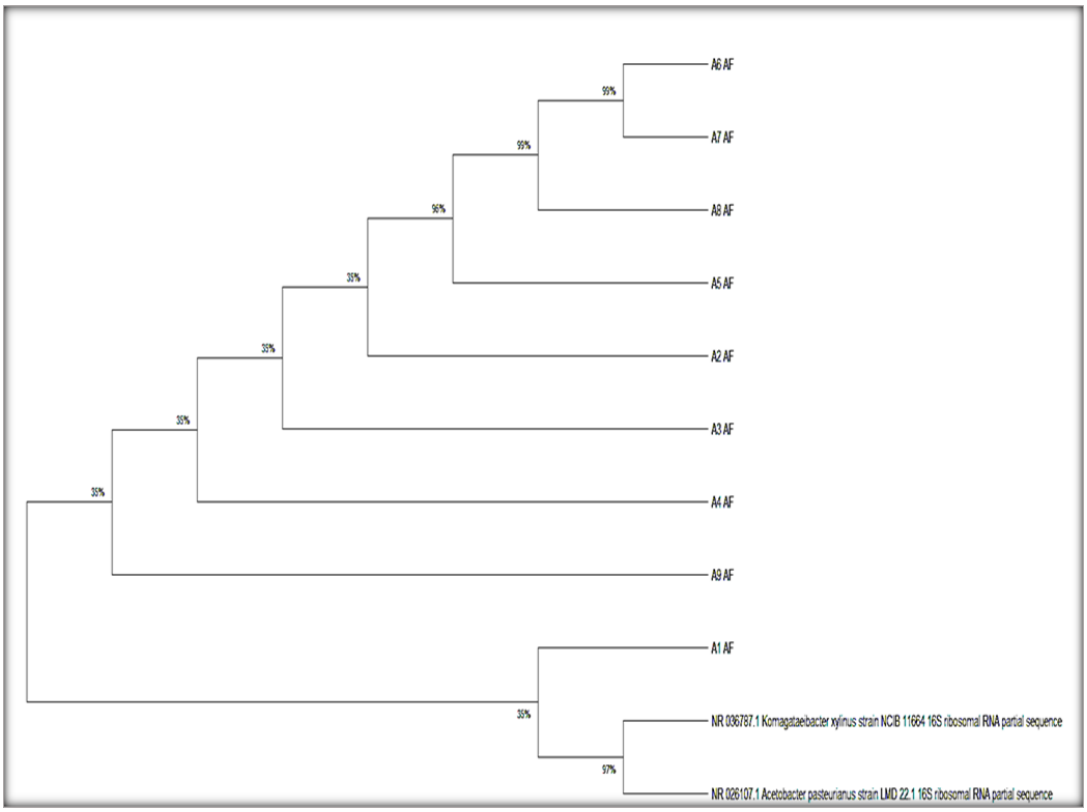

Figure 2: The phylogenetic tree constructed UPGMA algorithm

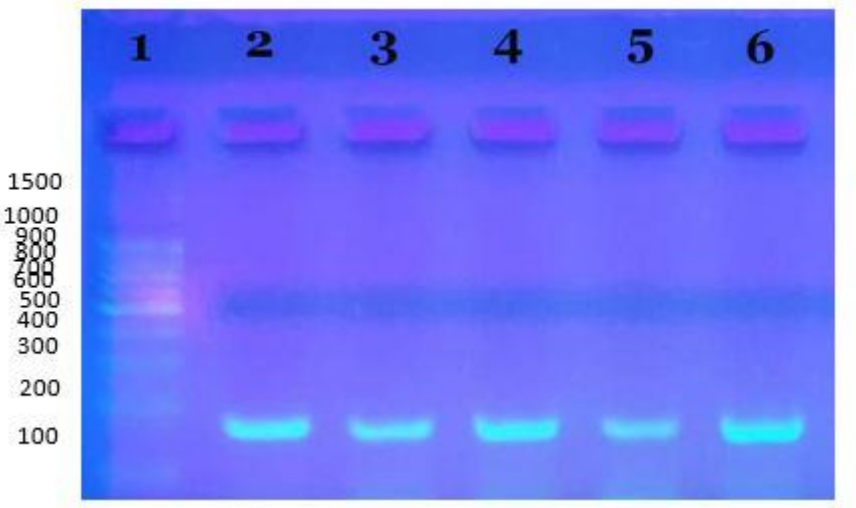

Figure 3: Gel electrophoresis of bscAI gene PCR product, 2% agarose gel, 100 v for 50 min. Lane 1 molecular marker and lanes 2-6 show a single band of about 189 bp. products of the bscAI gene

Our results are in agreement with those found by Zhang *et al.* [29], who used *G. xylinus*, recovered from kombucha, to identify this bacterium by using PCR and DNA sequencing, targeting the 16S rRNA gene and found that this bacterium belongs to the genus; *Acetobacter* (by PCR) and *Acetobacter xylinum* (by DNA sequencing). Lavasani *et al.* [17] reported that a PCR method that targeted the 16S rRNA gene of *K. xylinus*, grew on vinegar samples, was able to detect the bacterium at the genus level plus the species level, especially after DNA sequencing was done that targeted the same bacterial gene. Liu *et al.* [18] revealed that their whole genomic analysis revealed the presence of bcsAI genes in two strains of *G. xylinus* with a similar identity of about 67% of the nucleotide sequence. Moreover, Singhania *et al.* [30] reported the presence of

bcsAI genes in the DNA of *K. xylinum* when using the genomic analysis.

Bio-cellulose production by the selected isolate

The local isolate TELE8 was used to determine the optimal method for the production of bacterial cellulose, as reported in Table 5 that the use of the fermenter method with 0.5 l/min. of aeration led to an increase in the productivity of bacterial cellulose by 35.22% compared with the productivity in the static method without aeration, while no production in the shaker method with 150 rpm, as all the above cellulose productivity experiments [23-35] were conducted under the same growing conditions.
In Table 5, the cellulose production ability of the isolated strain was compared by using three different types of cultivation conditions, where the production in static conditions was (14.64 g/ml) and in the fermenter method with 0.5 l/min (22.8 g/mL), while no production was found in the shaker method with 150 rpm. Zhang *et al.* [29] used both static and shaking to produce bacterial cellulose, where the production capacity was 7.56 ± 0.57 g/L under the static culturing conditions and 8.31 ± 0.79 g/L under shaking conditions, while Esa *et al.* [31] found that the use of the fermentation method with a rotatory shaker via 150 - 200 rpm in the presence of matured coconut water as a carbon source at 30 ˚C to produce BC from *A. xylinum* revealed a high yield of bacterial cellulose.

Table 5: Production of bacterial cellulose by *Komagataeibacter xylinus* TELE8 under three cultivation methods

Cellulose production (200 mg/mL medium)		
Static	Shaker	Fermenter
14.64	No production	22.8

Conclusion

The spoiled fruit samples are considered a good source to obtain the local bacterial isolates that produce bacterial cellulose, whereas the lemon is the best rotten fruit that can be used to obtain a large number of bacterial isolates capable of producing bacterial cellulose. The local isolate TELE8 which was isolated from the lemon and was identified as *Komagataeibacter xylinus* TELE8 gave the highest ability to produce the bacterial cellulose.

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Authors' contributions

All authors contributed to data analysis, drafting, and revising of the paper and agreed to be responsible for all the aspects of this work.

Conflict of Interest

The author declared that they have no conflict of interest.

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