ORIGINAL ARTICLE

Screening and Characterization of Cellulose-Producing Bacterial Strains from Decaying Fruit Waste

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ABSTRACT Bacterial cellulose is an extracellular homopolysaccharide produced from various species of micro-organisms: bacteria, fungi and slime moulds with distinctive characteristics like high purity and high degree of crystallinity, high water absorbing capacity, mechanical strength, long fiber length, and nano scale fibril dimensions. The bacterial strains producing higher yields of cellulose is too limited, therefore the present study is conducted to screen and characterize the potential bacterial strains from decaying fruit waste. Thirty-seven bacterial isolates were obtained from 12 decaying fruit waste samples collected from fruit mandis of Delhi which are found to be potential cellulose producer. These thirty seven isolates were screened for elite cellulose producer based on Gallardo scale of thickness. The isolated strain wa-2 produced cellulose pellicle with 9 mm thickness followed by wo-2 with 7 mm and wgr-2 with 6 mm pellicle thickness. The potential isolated strains were identified using morphological and biochemical analysis and further characterized using 16S rRNA gene sequence analysis. Three biocellulose producing strains were: Acetobacter tropicalis wa-2 from apple waste, *Acetobacter fabarum* wo-2 from orange waste and Acetobacter lovaniensis, wgr-2 from grapes waste.

Keywords: Biopolymer, biodegradable, biocellulose, 16S rRNA

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INTRODUCTION

Bacterial cellulose is a popular biopolymer considering its environment friendly nature. Bacterial cellulose has been developed as an alternative to plant cellulose. Cellulose from bacteria (BC) was discovered much later than plant cellulose, however it has become a very good alternative to plant one due to its simple and environmentally sensitive extraction conditions compared to extraction of it from plants [1]. The molecular formula of cellulose obtained from plant and Bacteria is the same (C6H10O5)n, despite of the fact that physical and chemical properties of bacterial cellulose are unique. BC possesses a unique 3-D threadlike structure and the multifunctional properties as high purity and high degree of crystallinity, high water absorbing capacity, mechanical strength, long fiber length and and greater tensile strength [2, 3, 4]. The distinctive properties of BC has made it to be used as a biological material in the food industry such as traditional dessert Nata de Coco [5], multifunctional food ingredients [6], as wound dressing materials [7], artificial skin, vascular grafts, scaffolds for tissue engineering [8, 9] products, as sponges to collect leaking oil and materials for absorbing toxins, Optoelectronics materials (liquid crystal displays) [10].

Biocellulose is produced from various species of microorganisms: bacteria (*Acetobacter xylinum*, *A.aceti, A.acetigenum, A.hansenii*, *Sarcina, Agrobacterium, Rhizobium, Pseudomonas, Chromobacterium, Achromobacter*, *Aerobacter, Alcaligenes, Zooglea*), fungi (*Sparolegnia*) and slime moulds (*Dictyostelium discoideum*). One such microorganism of commercial importance that can produce cellulose is *Gluconacetobacter xylinus* (formerly called as *Acetobacter xylinum,* now known as *Komagataeibacter xylinus* [11].

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Many researchers isolated *Acetobacter* species of bacteria from fruit and vegetable waste, rotten fruits, waste from vinegar industry, flowers, agri-industry waste, industrial waste water and soil [12-14]. Therefore taking above into consideration, in this study, decaying fruit waste was used to isolate cellulose producing bacteria. The isolated strains were identified through physiological, biochemical and molecular characterization.

MATERIAL AND METHODS

Sample Collection

Samples of decaying fruits wastes were collected from five different mandis (markets) of Delhi (India) in a sterile container.

Chemicals and Reagents

All the media ingredients were analytical grade and procured from Hi-Media, Sigma-Aldrich and Merck.

Isolation of Cellulose Producing Bacteria

One gm of each sample was transferred in 100 ml of flask of Hestrin-Schramm [15] (HS) medium ; containing 2.0% D-glucose (w/v), 0.5% peptone (w/v), 0.5% yeast extract (w/v), 0.27% Na_2HPO_4 (w/v), 0.12% citric acid (w/v), 0.2% acetic acid (v/v), and 0.01% supplemented with cycloheximide (w/v) to restrict the contamination of fungal and yeast, and incubated at 30 °C for 120 to 168 h in static condition. The flasks with white pellicle/gelatinous mat on the surface of medium were selected.

Screening of Cellulose Producing Bacteria

The flasks with pellicle growth were selected and serially diluted up to 10^{-6} with 0.9% (w/v) sodium chloride solution. 0.1 ml of serially diluted sample was taken and spread on GEY (Glucose, Ethanol and Yeast extract) agar plate containing (2.0% D-glucose, 1.0% yeast extract,5% ethanol 0.3% $CaCO_3$ and 2% agar) then incubated at 30 °C for 48 h or till the colonies were produced. The plates with clear zone in the region of the colonies were selected for further fermentation. The thirty seven bacterial isolates were isolated from the above mentioned waste and selected for further investigations.

Screening of Elite Cellulose Producing Bacteria

About a loopful of the purified colonies of isolated strains were transferred to test tubes containing 10 ml of HS broth and incubated at 30 °C for 7 days. Among the various isolates, the best cellulose producing strain was screened and selected based on the thickness of the pellicle formed on the surface of the HS medium. The isolates were grouped according to Gallardo scale of thickness as poor (1mm thickness), fair (2 mm thickness), good (4mm thickness), excellent (5 mm thickness and above). Isolates with ≥5 mm thickness were selected for further study [16].

Identification of Cellulose Producing Bacteria

Biochemical and Physiological

Bacterial isolates were identified by performing gram staining, colony morphology, motility test, and biochemical characteristics followed by carbohydrate fermentation test [17].

Molecular Identification of Isolates

The isolated bacterial strains was identified using the 16S rRNA sequences analysis as per the standard method by by Yukphan *et al.* [20]. The genomic DNA of the selected bacterial isolates was extracted using DNeasy Blood and Tissue Kit (Qiagen). Two universal primers 16S Forward Primer (5'-AGAGTTTGATCCTGGCTCAG-3' and 16S Reverse Primer: (5'-TCTACGCATTTCACCGCTAC-3') were used Primers and excess nucleotides were removed from the amplified DNA using a PCR clean-up kit (Qiagen). Amplification conditions for the PCR assay was: 5 min at 94 °C to denature the DNA, followed by 30 cycles of denaturation at 94 °C for 30 sec, primer annealing at 55 °C for 10 sec and strand extension at72 °C for 30 sec. The phylogenic tree was constructed by the neighbor-joining method using "Unipro UGENE: a unified bioinformatics toolkit" after gene sequences alignments obtained using the same software. The BLAST program from the NCBI (National Center for Biotechnology Information) database was used to identify the closer related species to the bacterial strain.

Cellulose Production from Isolates

The efficiency of isolated strains were investigated by cellulose production on the Hestrin and Schramm (HS) medium. The pH of medium was adjusted to 6.0. The fermentation medium was sterilized at 121 °C for 20 min. The isolated strains were inoculated into HS media at 30 °C and incubated for 14 days.

RESULTS

Isolation and Screening of Cellulose Producing Bacteria

In the present study, thirty-seven bacterial isolates were obtained from samples of decaying fruit waste which are found to produce cellulose. These isolates showed clear zone around the developed colonies because of their ability to

produce acetic acid that dissolves $CaCO_3$ of glucose yeast extract medium and formation of zones around colonies of those isolates (Figure 1). These thirty seven isolates were screened for elite cellulose producer based on Gallardo scale of thickness. The isolate strain wa-2 produced cellulose pellicles with 9 mm thickness followed by -2 with 7mm and wgr-2 with 6 mm pellicle thickness and were graded as excellent cellulose producer.

Identification of Selected Bacterial Strains

Identification of the strains were based on cultural characterization, biochemical characterization, and carbohydrate fermentation tests and results were tabulated (Tables 1, 2, and 3) (Figure 2). On the basis of biochemical characteristics, bacterial strains were identified as *Acetobacter tropicalis* wa-2 from apple waste, *Acetobacter fabarum* wo-2 from orange waste and *Acetobacter* wgr-2 from grapes waste *respectively*. The results showed that colonies of the selected isolates on HS agar plates after 48-hr growth were white/milky white, smooth, viscous, convex, with circular or irregular shape and entire or undulating margin. All the isolates were Gram-negative, rod-shaped or short rod and found to occurr singly or in pairs as depicted in Table 1. They showed negative results for oxidase, Voges Proskauer, indole, urease, Methyl red and H_2S production. But positive results showed for citrate utilization and catalase

Figure 1: Clear Zones Depicting Presence of Biocellulose Producing Bacteria in (a) Apple Waste (b) Orange Waste (c) Grapes Waste

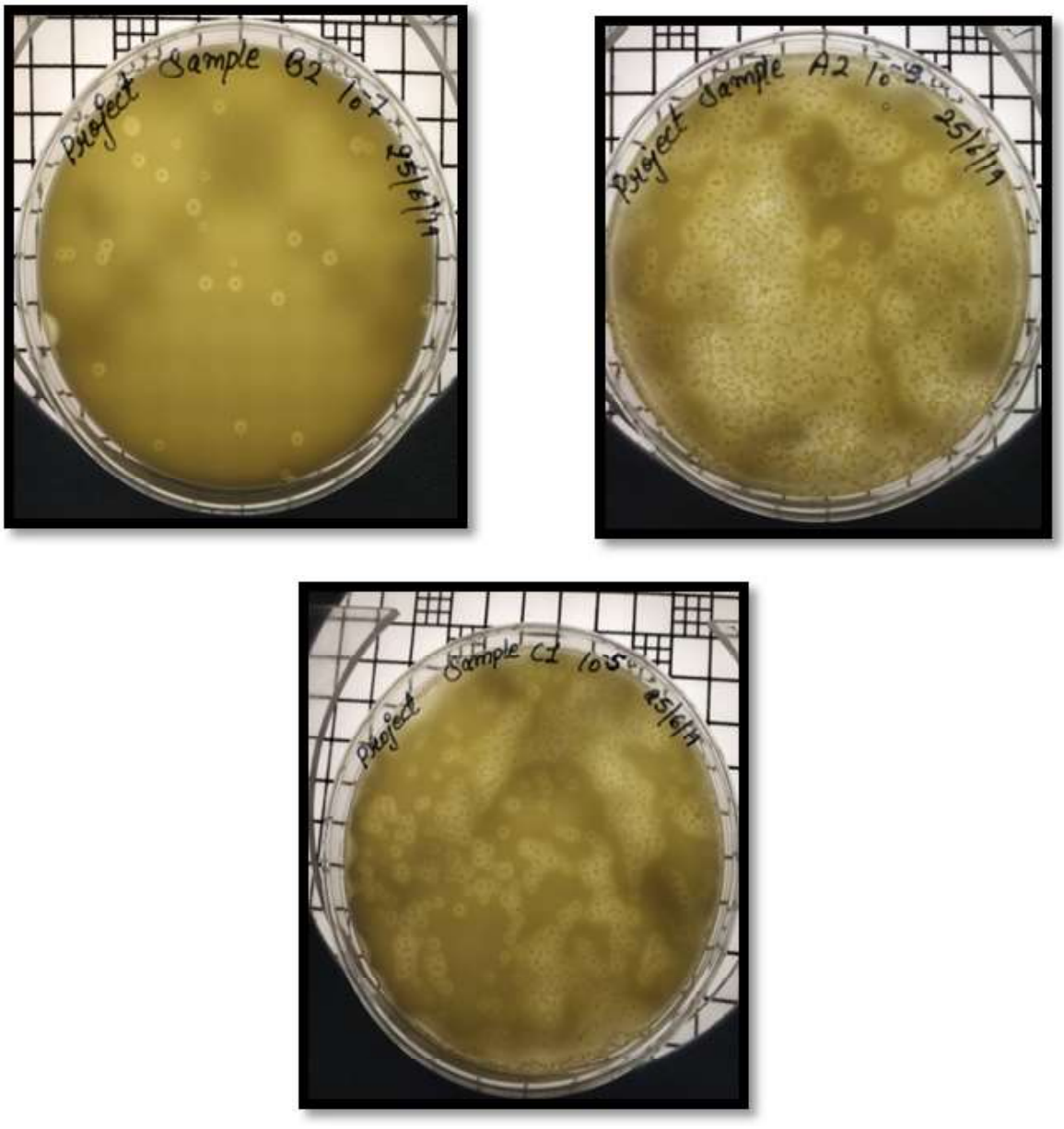


Table 1: Report on the Self Analysis of Health Care Practices/Awareness of the Respondents

Colony Morphology	Wa-2	Wo-2	Wgr-2
Configuration	Round	Round	Round
Margin	Entire	Entire	Undulated
Elevation	Raised	Raised	Flat
Surface	Smooth, mucoid	Smooth	Smooth
Color	Milky white	white	Milky white
Opacity	Translucent	Translucent	Translucent
Motility	Motile	Motile	Motile
Cell shape	Rod	Rod	Rod
Spore formation	Negative	Negative	Negative

Table 2: Biochemical Characterization for the Isolates

Characteristics Test	Wa-2	Wo-2	Wgr-2
Gram reaction	Gram negative rods	Gram negative rods	Gram negative rods
Motility	Motile	Motile	Motile
Cellulose production	+	+	+
Cellulose yield g/L	6.62	3.3	2.9
Catalase	+	+	+
Oxidase	–	–	–
Citrate utilization	+	+	+
Indole test	–	–	–
Methyl red	–	–	–
Voges-Proskauer	–	–	–
Urease	–	–	–
H_2S production	–	–	–

Table 3: Carbohydrate Fermentation Test

Carbon Source	Wa-2	Wo-2	Wgr-2
Glucose	+	+	+
Malic acid	+	+	+
Sorbitol	+	+	+
Mannitol	-	-	-
Sucrose	+	+	+
Fructose	+	+	+
Lactose	–	–	-

Table 3 (Cont.)

Maltose	–	–	-
Fructose	+	+	+
Galactose	+	+	+
Mannose	+	+	+
Glycerol	+	+	+
Rhamnose	+	+	+
Xylose	+	+	+

Figure 2: Phylogenetic Tree of Acetobacter Tropicalis Based on the 16S rRNA Sequence

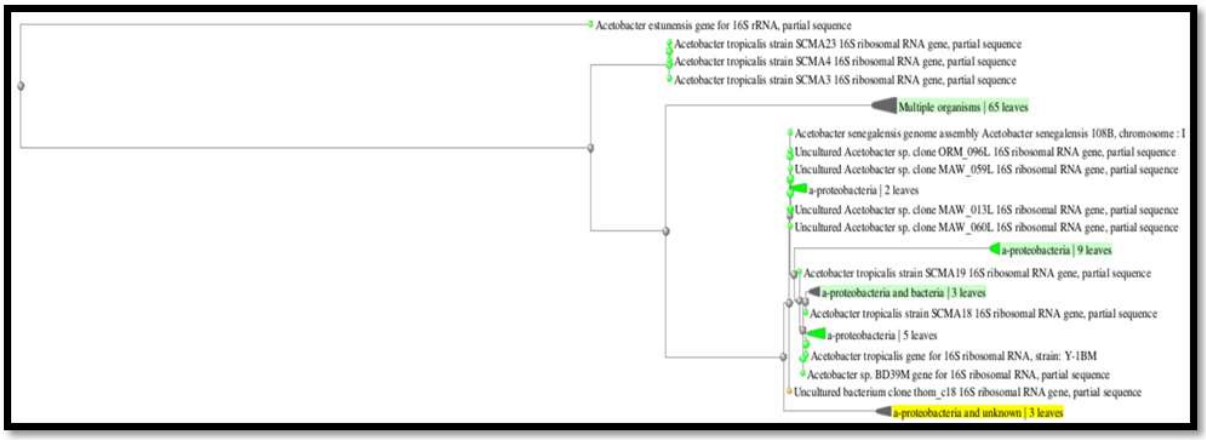
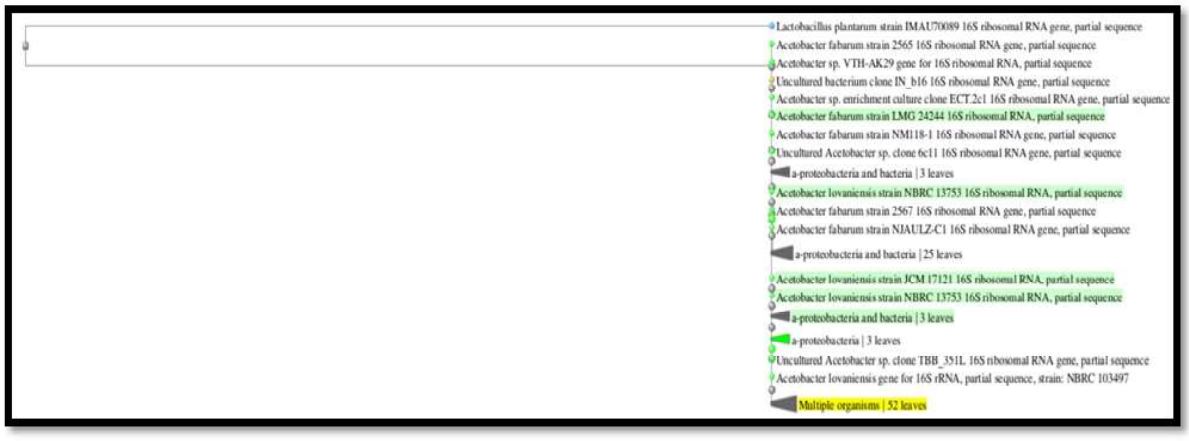


Figure 3: Phylogenetic Tree of Acetobacter Lovaniensis Based on the 16S rRNA Sequence

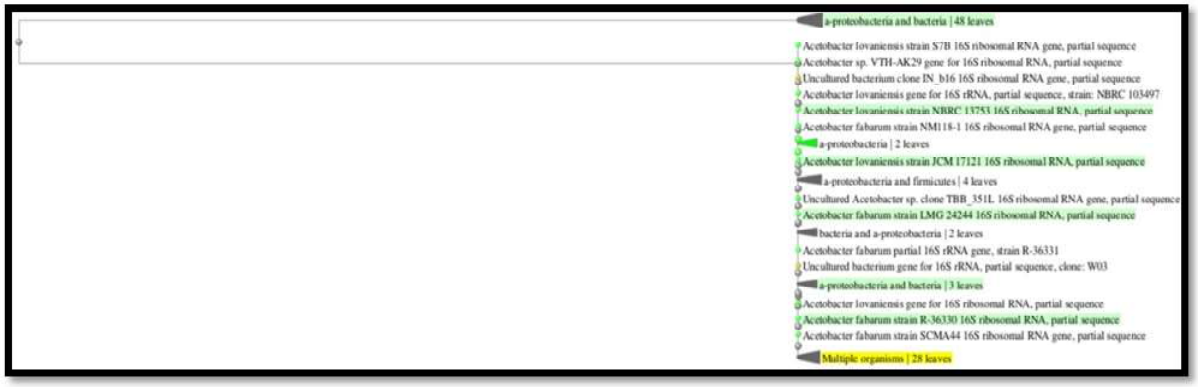


activity as indicated in Table 2. All three bacterial isolates produced acid from glucose, but no acid production from lactose, mannitol and maltose.

Bacterial identification by 16S rRNA gene sequence analysis

The isolates were found to be most similar to *Acetobacter* genus. The obtained 16S rRNA sequence read was identified using the Basic Local Alignment Search Tool (BLAST) as it was aligned against the National Center for Biotechnology Information (NCBI) Genbank databases. BLAST results showed that the bacterial strain showed 99% homology with the bacterial strain *Acetobacter tropicalis SCMA 23, Acetobacter fabarum and Acetobacter lovaniensis,* the phylogenetic tree was constructed using the 16S rRNA gene sequences of the isolated bacterial strains as shown in Figure 2.

Figure 4: Phylogenetic Tree of Acetobacter Fabarum Based on the 16S rRNA Sequence



DISCUSSION

Thirty seven bacterial isolates were isolated from different decaying fruits waste for cellulose production on HS (Hestrin and Schramm) medium. wa-2, wo-2 and wgr-2 isolates were selected for the characterization on the basis of Gallardo scale. The physiological and biochemical examinations were carried out as per Bergey's Manual [17], which says that *Acetobacter* strains are individual cells that are rod shape, occurring singly or in pairs or in short or long chains. Young cells are Gram negative while old cells are Gram variable. The mor-phological results for isolate *Acetobacter* wa-2 are identical with other researchers [18, 19, 20]. According to the cultural and biochemical analysis, wa-2 gave negative results for: xylose fermentation, indole, H_2S production and TSI. But gave positive results for catalase and glycerol fermentation. These results coincide with those described in Bergey's manual of determinative bacteriology [17]. The isolate wa-2, wo-2 and wgr-2 showed clear zones around the bacterial colony on GEY agar due to the disappearance of $CaCO_3$. The disappearance of $CaCO_3$ and formation of clear zone around the growing colony was due to the production of acetic acid which reacts with $CaCO_3$ and produced calcium acetate which is water soluble. Sharafi *et al.* (2008), Hanmoungjai *et al.* (2007) used the similar selection method [21, 22]. *Acetobacter* and *Gluconacetobacter* are usually selected based on the formation of clearing zone in ethanol containing glucose yeast extract calcium carbonate medium [23]. The isolated strain wa-2, wo-2 and wgr-2, when analysed for 16Sr RNA, showed most similarity with *Acetobacter* genus and most closely related to *Acetobacter tropicalis* L31. A novel and potent cellulose-producing bacterium was newly isolated from a rotten fruit and identified as *Gluconacetobacter* sp. F6 through morphological, cultural, and biochemical characteristics and by 16Sr DNA sequencing [24]. *A.* tropicalis was isolated from fermented foods (palm wine and rice wine), fruits (lime, orange, guava, coconut), and coconut juice whose similarity is in a range of 96.5 to 98.9% between the type strain of *A.* tropicalis and the type strains of other Acetobacter species [25]. Others researchers have isolated *A.* tropicalis with similarity index of 99.7% from fermented wine [26]. In Senegal *A.* tropicalis with 93.3% similarity was isolated from mango fruit [27].

CONCLUSION

In conclusion, three new biocellulose-producing bacterial strains were isolated: *Acetobacter tropicalis* wa-2 from apple waste, *Acetobacter fabarum* wo-2 from orange waste and *Acetobacter lovaniensis,* wgr-2 from grapes waste .This proves that the decaying fruit waste is a potential source for biocellulose producing strains. Further studies can be conducted on the optimization of culture conditions in order to improve the biocellulose yeild by these bacterial strains.

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