Asian Journal of Chemistry; Vol. 34, No. 2 (2022), 453-458

ASIAN JOURNAL OF CHEMISTRY

https://doi.org/10.14233/ajchem.2022.23598

Production of Bacterial Cellulose by *Acetobacter tropicalis* Isolated from Decaying Apple Waste

LAKHVINDER KAUR[1,*] and SHACHI SHAH[2]

[1]Department of Food Science and Technology, Manav Rachna International Institute of Research and Studies, Faridabad-121004, India
[2]School of Interdisciplinary and Transdisciplinary Studies, Indira Gandhi National Open University, New Delhi-110068, India

*Corresponding author: E-mail: E-mail: lakhvinder.fahs@mriu.edu.in

Received: 28 October 2021; *Accepted*: 6 January 2022; *Published online*: 11 January 2022; AJC-20674

Fruits and vegetables have the highest wastage rates of 45% of any food. One of the recent research areas is food waste valorization as a potential alternative to the disposal of a wide range of organic waste using microorganisms as one of the strategies known as microbial valorization. Bacterial cellulose is best known microbial valorization product because of its low cost, environmentally friendly nature, renewability, nanoscale dimensions, biocompatibility and extremely high hydrophilicity. Therefore, present study focuses on the isolation, characterization and identification of cellulose producing bacteria from decaying apple waste. Cellulose producers were isolated from decaying apple waste. The bacterial isolates obtained were identified through the morphological biochemical, physiological and molecular identification. The bacterial isolates exhibited potential remediation options to biovalorize decaying fruit waste by producing value added products as well as in safe disposal of waste.

Keywords: Valorization, Biodegradable, Biocellulose, Novel microorganisms.

INTRODUCTION

Many microorganisms and plants naturally synthesize cellulose composed of extracellular polysaccharide [1]. Because of its biodegradability and environmental friendliness, it is the most widely used biopolymer. In addition to plant cellulose, many bacteria produce cellulose, which is a viable substitute source of cellulose production [2]. Gram-negative bacteria such as *Acetobacter*, *Pseudomonas aeruginosa*, *Salmonella s*pp. and *Salmonella agrobacterium* [3,4] are capable of producing cellulose. Among the several naturally occurring cellulose-producing microorganisms, the most common is *Acetobacter xylinum*, despite the fact that these species are well-known for producing cellulose. It is estimated that one *Acetobacter xylinum* cell is capable of converting 108 glucose molecules into cellulose per hour. Fermentation waste, decaying fruit and vegetable scraps, vinegar and its scraps, soil and wastewater are all common places where these bacteria can be found [5-7]. These sources yielded Gram-negative, non-pathogenic, rod-shaped, aerobic bacteria that synthesize microfibrils of cellulose and at last form a thick mass/mat in the fermenting media [8,9].

Because it lacks hemicellulose and lignin, bacterial cellulose has a higher immaculateness than plant cellulose, as well as higher water holding capacity, hydrophilicity, polymerization level, mechanical quality, crystallinity, porosity and a more pure structural fibre arrangement. This system of filaments, which is uniform, constant and nano-scalar, is what gives bacterial cellulose its improved mechanical properties. Several variables including the culture conditions, microorganisms and fermentation media have an effect on these properties. In terms of material properties, bacterial cellulose is a significant biopolymer with superior properties such as purity, high porosity, relative high penetration to fluid and gas, high water-take-up limit, rigidity and ultrafine network. In light of the above-mentioned characteristics, bacteria cellulose is the first choice as a plant cellulose option for wound dressings, antimicrobial action and artificial blood [10-12]. A wide variety of food industries used the bacterial cellulose produced as a coating, binding, thickening and emulsifying agent [1,13]. Because of bacterial cellulose's unique characteristics, the purpose of this research is to identify and describe the most effective cellulose producing bacteria from decaying fruits waste.

This is an open access journal, and articles are distributed under the terms of the Attribution 4.0 International (CC BY 4.0) License. This license lets others distribute, remix, tweak, and build upon your work, even commercially, as long as they credit the author for the original creation. You must give appropriate credit, provide a link to the license, and indicate if changes were made.

EXPERIMENTAL

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

Sample collection: Samples of decaying fruits wastes were collected from five different fruits markets of Delhi city, India in a sterile container.

Isolation of cellulose producing bacteria: Each collected sample (1 g) was transferred in 100 mL of flask of Hestrin-Schramm [14] medium; containing 0.5% yeast extract (w/v), 0.27% Na_2HPO_4 (w/v), 2.0% D-glucose (w/v), 0.5% peptone (w/v), 0.2% acetic acid (v/v), 0.12% citric acid (w/v) and 0.01% supplemented with cycloheximide (w/v) in order to restrict the contamination by fungi and yeast and thereafter incubated at 30 ºC for 120 to 168 h under static conditions. The flasks with white mass/gelatinous mat on the surface of fermenting medium were selected.

Screening of cellulose producing bacteria: A solution of 0.9% NaCl was used for serial dilution upto 10^{-6} of the flasks that had developed pellicle growth. It was then incubated for 48 h at 30 ºC or until bacterial colonies appeared on the agar plate containing GEY (glucose, ethanol, yeast extract) agar media (2.0% D-glucose, 1.0% yeast extract, 5.0% ethanol, 0.30% $CaCO_3$ and 2% agar). The plates with a clear zone around the distinctive colonies were selected for further screening. The 37 bacterial strain isolates from the mentioned waste were selected for further study.

Screening of best cellulose producing bacteria: About a loopful of the purified colonies of isolated strains were transferred to test tubes containing 10 mL of Hestrin-Schramm broth and incubated at 30 ºC for 7 days. Among the various isolates, the best cellulose producing strain was screened and selected based on the thickness of the pellicle mat formed on the surface medium. The isolates were grouped according to Gallardo scale of thickness as poor (1 mm thickness), fair (2 mm thickness), good (4 mm thickness), excellent (5 mm thickness and above). Isolates with ≥ 5 mm thickness were selected for further study [15].

Identification of cellulose producing bacteria

Biochemical and physiological: Moltility test, gram staining, colony morphology and biochemical characteristics, followed by carbohydrate fermentation test were used to identify bacterial isolates [16].

Morphological characterization (SEM examination): The morphological investigations of the cellulose produced by standard and isolated strain were characterized using scanning electron microscope (SEM) (Model: Zeiss EVO40) at 23000_X magnification. Micrograph was studied for the morphological features of the isolate [17,18].

Molecular identification of bacteria: The isolated bacterial strain wa-02 was identified using the 16S rRNA sequences analysis according to the standard method given by Yukphan *et al*. [19].

Cellulose production from *Acetobacter* wa-2: The efficiency of isolated strain wa-2 was investigated by cellulose producted on the Hestrin-Schramm medium. The pH of Hestrin-Schramm medium was adjusted to 6.0. The fermentation medium was steriled at 121 ºC for 20 min. The isolate wa-02 was cultured in Hestrin-Schramm media at 30 ºC and incubated for 14 days.

Purification of cellulose produced from *Acetobacter* wa-2: Sodium hydroxide (1 N) was used to neutralize the pellicle mat formed at the fermenting broths air-liquid interface for 15 min, then the pellicle was washed 3-4 times with distilled water and then dried in an oven at 60 ºC for overnight period. The dried mass of bacterial cellulose was then weighed and calculated.

Characterization of dried bacterial cellulose: The surface morphology and structural constitution of dried bacterial cellulose were characterized by: Fourier transforms infrared (FTIR, model (Frontier, CN-Perkin-Elmer, USA); scanning electron microscopy (SEM, Model: Zeiss EVO40). X-ray diffraction (model, PANalytical X'pert PRO, Netherlands): The crystallinity index (CrI) was calculated using the ratio of the height of the 002 peak and the height of minimum (I_{am}) between the 002 and the 110 peaks (eqn. 1).

$$\text{Crystallinity index (CrI, \%)} = \frac{I_{002} - I_{am}}{I_{002}} \times 100 \quad (1)$$

RESULTS AND DISCUSSION

The present work was undertaken in order to isolate the potential cellulose producing bacteria from decaying fruit waste and to characterize the produced cellulose.

Isolation and screening of bacteria producing cellulose: In this study, 37 bacterial isolates were obtained from samples of decaying fruits wastes, which are found to produce cellulose. These isolates showed clear zone around the devel-oped 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 (Fig. 1). These 37 isolates were screened for elite cellulose producer based on Gallardo scale of thickness. The isolate strain wa-2 produced cellulose pellicle with 9 mm thickness and was graded as excellent and selected for further study.

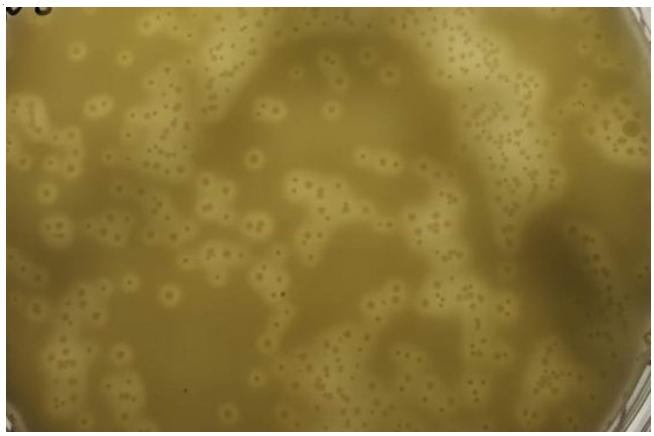

Fig. 1. Clear zones depicting presence of biocellulose producing bacteria

Identification of cellulose producing strain

Biochemical and physiological: The strain was identified through a combination of cultural, biochemical and carbo-

hydrate fermentation tests. The results were observed as white, cream-coloured, smooth, sticky, convex, dense colonies of the 37 isolates on Hestrin-Schramm agar plates after 48 h of growth. These colonies had a circular or irregular shape and an entire or undulating margin. Gram-negative rod- or short rod-shaped bacteria were found in all of the isolates and they were either single or in pairs as shown in Table-1. Oxidase, voges proskauer, indole, urease, methyl red and H_2S production tests were all found to be negative. Citrate utilization and catalase activity were found to be positive as shown in Table-1. The bacterial strain was identified as one belonging to the *Acetobacter* genus based on biochemical characteristics.

The morphological and biochemical analysis identified that the strain wa-2 is the member of *Acetobacter* sp isolated from decaying apple waste. As explained in Bergey's Manual of Determinative Bacteriology [20], *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 isolated strain wa-2 on GEY agar showed clear zones around the colonies of bacteria due to the disappearance of $CaCO_3$. Because of the production of acetic acid, which reacts with $CaCO_3$ to produce calcium acetate, $CaCO_3$ disappeared and a clear zone was formed around the growing colony.

Morphological characterization: Scanning electron microscopic view of short rods of *Acetobacter* wa-2 taken at 5000X (Fig. 2a). When an individual cell was viewed at 23000X, formation of cellulose fibrils which were radiated from the exterior of cell membrane could be vividly seen (Fig. 2b).

Molecular identification of bacteria: 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* and the phylogenetic tree was constructed using the 16S rRNA gene sequences of the isolated bacteria as shown in Fig. 3.

The selected elite strain wa-2 when analyzed for 16SrRNA, showed most similarity with *Acetobacter* genus and most closely related to *Acetobacter tropicalis* SCMA 23. A new strain of bacterium producing cellulose was isolated from a rotten fruit which was identified as *Glucon acetobacter* sp. F6 through morphological, cultural and biochemical characteristics and by 16S rDNA sequencing [21]. *Acetobacter tropicalis* has been recovered from fermented foods (palm wine and rice wine), fruits (lime, orange and guava) and coconut juice whose similarity ranges from 96.5 to 98% between the type strain of *A. tropicalis* and the type strains of other *Acetobacter* species [22].

Cellulose production from *Acetobacter* wa-2: *Acetobacter* wa-2 produced the most bacterial cellulose (8.2 g/L). Fig. 4a and 4b shows the bacterial cellulose mat and shows the cellulose pellicle, respectively.

TABLE-1
MORPHOLOGICAL AND BIOCHEMICAL CHARACTERIZATION OF wa-2 ISOLATE

Morphological		Biochemical	
Colony morphology	wa-2	Characteristics test	wa-2
Margin	Entire	Gram reaction	Gram-negative rods
Elevation	Raised	Motility	Motile
Surface	Smooth	Cellulose production	+
Colour	Milky white	Catalase	+
Opacity	Translucent	Oxidase	–
Motility	Motile	Citrate utilization	+
Cell shape	Rod	Indole test	–
Spore formation	Negative	Methyl red	–
		Voges-Proskauer	–
		Urease	–
		H_2S production	–

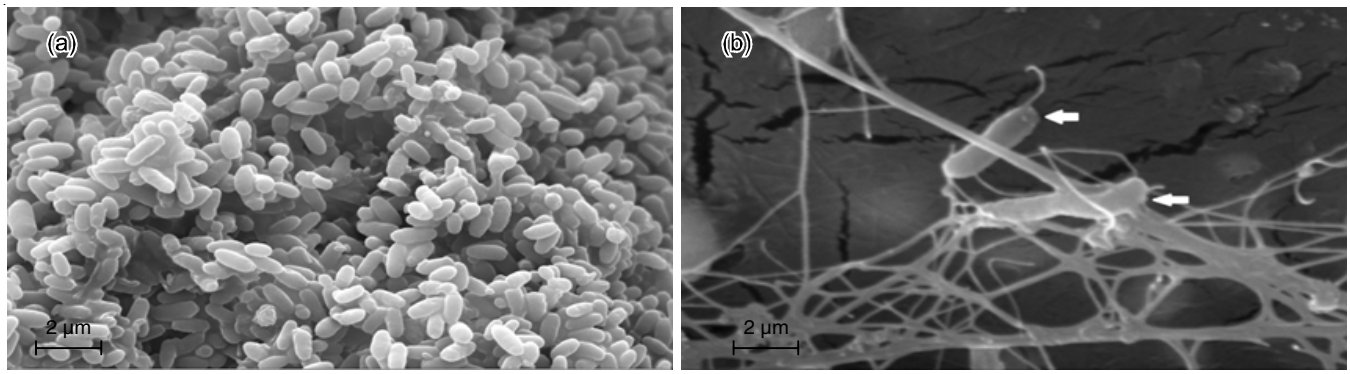

Fig. 2. SEM view of (a) short rods of isolate of wa-2 embedded in the cellulose pellicle at 5000X (b) isolate of wa-2 producing cellulose fibrils from the cell membrane

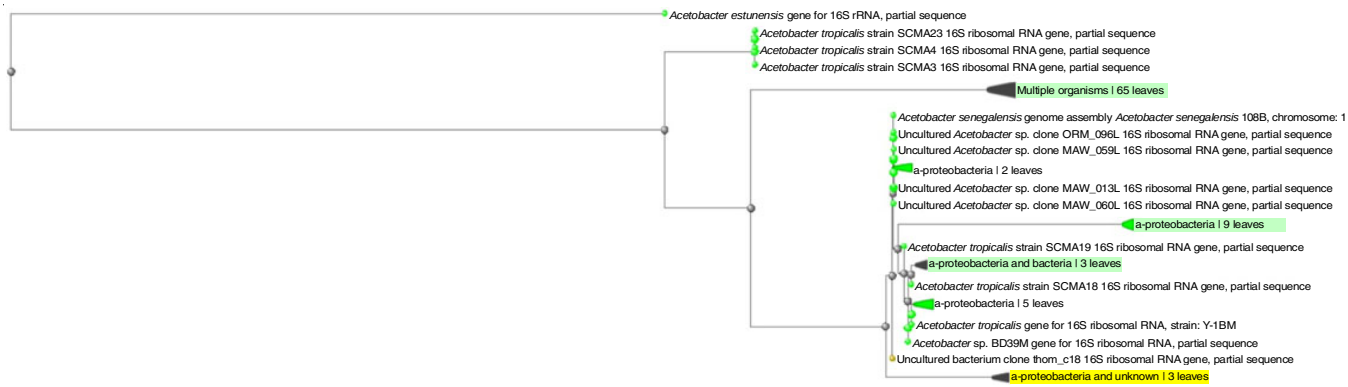

Fig. 3. Phylogenetic tree of *Acetobacter tropicalis* based on the 16S rRNA sequence

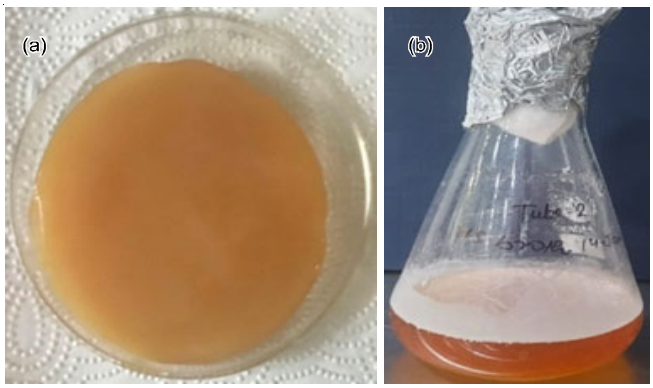

Fig. 4. (a) Bacterial cellulose by *Acetobacter* wa-2 (b) cellulose pellicle mat at air liquid interphase

Characterization of cellulose produced by isolated bacteria

Visualization of cellulose by scanning electron microscopy (SEM): The view of scanning electron microscopic of cellulose taken at 220000X is shown in Fig. 5 depicts the highly reticulated nature of bacterial cellulose with more cross links and branching pattern from *Acetobacter* wa-2 in Hestrin-Schramm media. The length and diameter of the fibrils were very thin. The structure of bacterial cellulose produced in Hestrin-Schramm medium in this study was same as of the reported results [23]. The strands are entangled results in the dense structure, which is in agreement with Sarkono *et al*. [24].

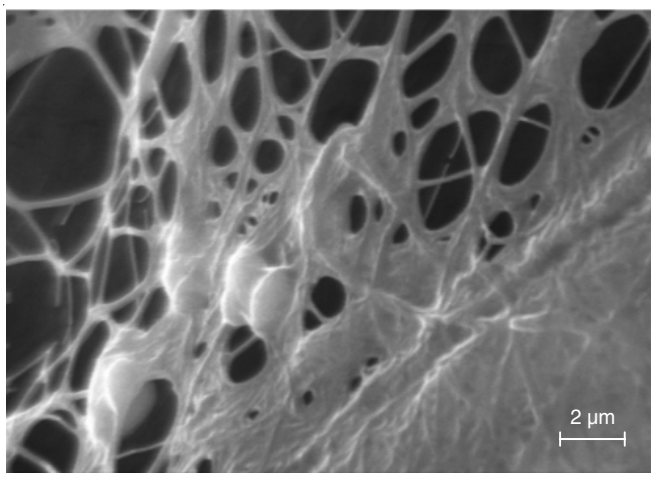

Fig. 5. Cellulose fibrils from *Acetobacter* wa-2 in Hestrin-Schramm medium

FT-IR spectrum: IR spectrum obtained for the bacterial cellulose produced from Hestrin-Schramm media proves that it is a pure form of cellulose with more of type Iα cellulose (Fig. 6). The characteristic bands of cellulose (type I) including 3340 cm^{-1} for the stretching vibration of hydroxyl groups (–OH), 2975 cm^{-1} for the asymmetric stretching vibration of methylene ($–CH_2–$), 1461.8 cm^{-1} for the asymmetric deformation vibration of methyl and methylene and 1099 and 1051 cm^{-1} for the stretching vibration of C–O–C in the sugar ring, respectively which are in agreement with the previous results [25-29].

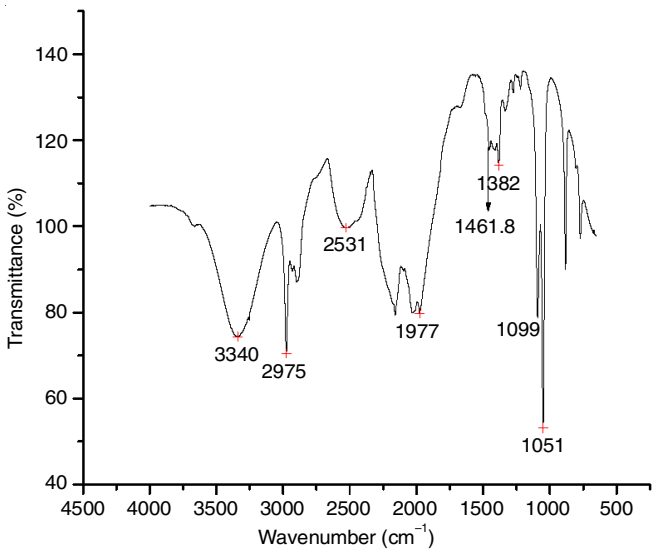

Fig. 6. FTIR spectrum of bacterial cellulose produced by *Acetobacter* wa-2

XRD pattern: *Acetobacter* wa-2-produced bacterial cellulose analyzed for diffraction pattern which revealed two distinct peaks at 2θ 14.5° and 23° (Fig. 7). It is easy to identify the cellulose 1α and 1β by looking at the diffraction peaks at 14.5° and 23°. During drying, the ribbons of cellulose preferentially orient themselves parallel to the surface of the film, resulting in the 1α and 1β unit cells having one chain and two parallel chains. Bacterial cellulose from *Acetobacter* wa-2 has a 43% crystallinity index in Hestrin-Schramm medium.

The results indicated that the decaying fruit waste present ideal conditions for the growth of cellulose producing bacterial strains. The bacteria wa-2 isolated from decaying apple waste was capable of producing cellulose onto Hestrin-Schramm

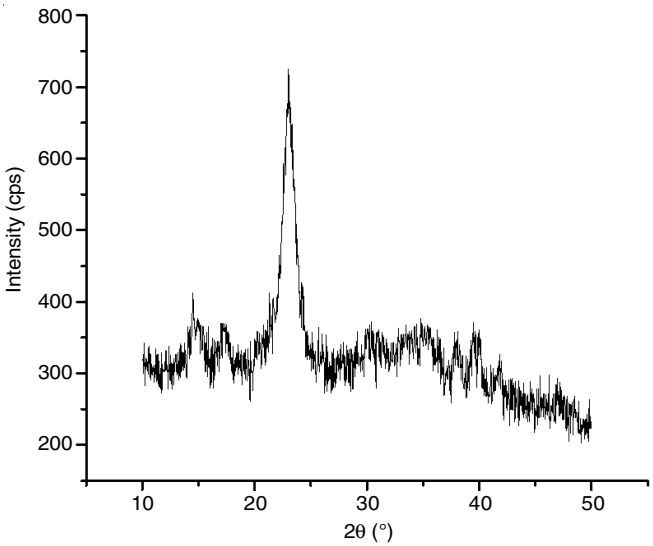

Fig. 7. X-ray diffraction pattern of bacterial cellulose produced by *Acetobacter* wa-2

media under static culture condition with 6.6 g/L (dry weight) after 14 days of incubation. Most of the researchers reported that the cellulose is produced by culturing a strain of *Acetobacter xylinum*, presently classified as the *Glucon acetobacter*, chiefly present on decaying/rotten fruits and vegetables, fruit juices, alcoholic beverages and vinegar. The *Glucon acetobacter* genus produces acetic acid from ethanol. It has been reported by researchers to isolate this genus from fruits [7], flowers, fermented foods [30], beverages [31] and vinegar. In earlier studies, bacteria like *Acetobacter xylinum* have this type of primary metabolism [8], *Rhizobium leguminosarum* [32,33], *Klebsiella pneumoniae* [34], *Sarcina ventricle* [8], *Agrobacterium tumefaciens* [35], *Salmonella typhimurium* [36], *Escherichia coli* and *Enterobacter* [37,38]. The optimum growth conditions for cellulose production were identified by Schramm & Hestrin [39].

Conclusion

The present investigation has confirmed that decaying fruit waste are capable producers of bacterial cellulose producing bacteria. The isolate wa-2 (from decaying apple waste from Azadpur market) is most efficient bacterial cellulose producer. The isolate wa-2 identified as *Acetobacter tropicalis* as per the results of biochemical and molecular identification based on the reported results it can be concluded that decaying fruit waste can provide bacteria for the production of cellulose in Hestrin-Schramm medium. These findings clearly shows that *Acetobacter tropicalis* mostly present in soil got mixed into decaying apple waste and was capable enough of producing bacterial cellulose. These findings are significant for the value addition of non-worthy decaying fruits wastes and improving *Acetobacter* sp. cellulose production with bioengineering in order to produce cellulose on a large scale.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

REFERENCES

1. P.R. Chawla, I.B. Bajaj and S.A. Survase, *Food Technol. Biotechnol.*, **47**, 107 (2009).
2. R.M. Brown Jr., *J. Polym. Sci. A Polym. Chem.*, **42**, 487 (2004); https://doi.org/10.1002/pola.10877
3. M. Shoda and Y. Sugano, *Biotechnol. Bioprocess Eng.*, **10**, 1 (2005); https://doi.org/10.1007/BF02931175
4. J.Y. Jung, J.K. Park and H.N. Chang, *Enzyme Microb. Technol.*, **37**, 347 (2005); https://doi.org/10.1016/j.enzmictec.2005.02.019
5. H. Toyosaki, T. Naritomi, A. Seto, M. Matsuoka, T. Tsuchida and F. Yoshinaga, *Biosci. Biotechnol. Biochem.*, **59**, 1498 (1995); https://doi.org/10.1271/bbb.59.1498
6. P. Lisdiyanti, H. Kawasaki, T. Seki, Y. Yamada, T. Uchimura and K. Komagata, *J. Gen. Appl. Microbiol.*, **47**, 119 (2001); https://doi.org/10.2323/jgam.47.119
7. F. Dellaglio, I. Cleenwerck, G.E. Felis, K. Engelbeen, D. Janssens and M. Marzotto, *Int. J. Syst. Evol. Microbiol.*, **55**, 2365 (2005); https://doi.org/10.1099/ijs.0.63301-0
8. P. Ross, R. Mayer and M. Benziman, *Microbiol. Rev.*, **55**, 35 (1991); https://doi.org/10.1128/mr.55.1.35-58.1991
9. B. McKenna, D. Mikkelsen, J. Wehr, M. Gidley and N. Menzies, *Cellulose*, **16**, 1047 (2009); https://doi.org/10.1007/s10570-009-9340-y
10. D. Klemm, D. Schumann, U. Udhardt and S. Marsch, *Prog. Polym. Sci.*, **26**, 1561 (2001); https://doi.org/10.1016/S0079-6700(01)00021-1
11. W. Czaja, A. Krystynowicz, S. Bielecki and R. Brownjr, *Biomaterials*, **27**, 145 (2006); https://doi.org/10.1016/j.biomaterials.2005.07.035
12. I.M. Ida, P. Norhayati and Z. Khairul, Eds.: M.W. Wasim and K. Prasad, Bacterial Cellulose as Secondary Metabolite: Production, Processing and Applications. In: Plant Secondary Metabolites: Biological and Therapeutic Significance, Apple Academic Press, CRC: USA, vol. 1, pp 169-200 (2016).
13. R. Jonas and L.F. Farah, *Polym. Degrad. Stab.*, **59**, 101 (1998); https://doi.org/10.1016/S0141-3910(97)00197-3
14. S. Hestrin and M. Schramm, *Biochem. J.*, **58**, 345 (1954); https://doi.org/10.1042/bj0580345
15. R.M.A. Gallardo De Jesus and T.M. Elvira, *Philipp. J. Sci.*, **100**, 41 (1971).
16. J.G. Holt, N.R. Krieg, P.H. Sneath, J.T. Staley and S.T. Williams, Bergey's Manual of Determinative Bacteriology, Ed.: 9 (2004).
17. P. Loganathan, R. Sunita, A.K. Parida and S. Nair, *J. Appl. Microbiol.*, **87**, 167 (1999); https://doi.org/10.1046/j.1365-2672.1999.00804.x
18. J. Trèek, K. Jernejc and K. Matsushita, *Extremophiles*, **11**, 627 (2007); https://doi.org/10.1007/s00792-007-0077-y
19. P. Yukphan, T. Malimas, M. Takahashi, W. Potacharoen, T. Busabun, S. Tanasupawat, Y. Nakagawa, M. Tanticharoen and Y. Yamada, *J. Gen. Appl. Microbiol.*, **50**, 189 (2004); https://doi.org/10.2323/jgam.50.189
20. D.L. Jozef, G. Monique and S. Jeans, Bergey's Manual of Systematic Bacteriology, Williams and Wilkins Publishing: USA, pp. 267-274 (1986).
21. F. Jahan, V. Kumar, G. Rawat and R.K. Saxena, *Appl. Biochem. Biotechnol.*, **167**, 1157 (2012); https://doi.org/10.1007/s12010-012-9595-x
22. P. Lisdiyanti, H. Kawasaki, T. Seki, Y. Yamada, T. Uchimura and K. Komagata, *J. Gen. Appl. Microbiol.*, **46**, 147 (2000); https://doi.org/10.2323/jgam.46.147
23. D. Lin, P. Lopez-Sanchez, R. Li and Z. Li, *Bioresour. Technol.*, **151**, 113 (2014); https://doi.org/10.1016/j.biortech.2013.10.052
24. Sarkono, S. Moeljopawiro, B. Setiaji and L. Sembiring, *Biosci. Biotechnol. Res. Asia*, **11**, 1259 (2014); https://doi.org/10.13005/bbra/1514
25. M. Ul-Islam, J.H. Ha, T. Khan and J.K. Park, *Carbohydr. Polym.*, **92**, 360 (2013); https://doi.org/10.1016/j.carbpol.2012.09.060

26. M. Ul-Islam, W.A. Khattak, M. Kang, S.M. Kim, T. Khan and J.K. Park, *Cellulose*, **20**, 253 (2013); https://doi.org/10.1007/s10570-012-9799-9
27. M.W. Ullah, M. Ul-Islam, S. Khan, Y. Kim and J.K. Park, *Carbohydr. Polym.*, **136**, 908 (2016); https://doi.org/10.1016/j.carbpol.2015.10.010
28. G. Gayathry and G. Gopalaswamy, *Indian J. Fiber Text. Res.*, **39**, 93 (2014).
29. H.-I. Jung, O.-M. Lee, J.-H. Jeong, Y.-D. Jeon, K.-H. Park, H.-S. Kim, W.-G. An and H.-J. Son, *J. Appl. Biochem. Biotechnol.*, **162**, 486 (2010); https://doi.org/10.1007/s12010-009-8759-9
30. J.K. Park, Y.H. Park and J.Y. Jung, *Biotechnol. Bioprocess Eng*., **8**, 83 (2003); https://doi.org/10.1007/BF02940261
31. S. Jia, H. Ou, G. Chen, D.B. Choi, K.A. Cho, M. Okabe and W.S. Cha, *Biotechnol. Bioprocess Eng.*, **9**, 166 (2004); https://doi.org/10.1007/BF02942287
32. H. Kitagawa, M. Kanamori, S. Tatezaki, T. Itoh and H. Tsuji, *Spine*, **15**, 1236 (1990); https://doi.org/10.1097/00007632-199011010-00028
33. T. Mukai, T. Toba, T. Itoh and S. Adachi, *Carbohydr. Res.*, **204**, 227 (1990); https://doi.org/10.1016/0008-6215(90)84039-W
34. M. Nomura, H. Harino and T. Itoh, *Jpn. J. Appl. Phys.*, **29**, 2456 (1990); https://doi.org/10.1143/JJAP.29.2456
35 . A.G. Matthysse, S. White and R. Lightfoot, *J. Bacteriol.*, **177**, 1069 (1995); https://doi.org/10.1128/jb.177.4.1069-1075.1995
36. Y. Hatta, M. Baba and S. Aizawa, *Acta Haematol. Jap.*, **53**, 923 (1990).
37. B.S. Hungund and S.G. Gupta, *World J. Microbiol. Biotechnol.*, **26**, 1823 (2010).
38. T.K. Ayaki, K. Fujikawa, H. Ryo, T. Itoh and S. Kondo, *Genetics*, **126**, 157 (1990); https://doi.org/10.1093/genetics/126.1.157
39. M. Schramm and S. Hestrin, *J. Gen. Microbiol.*, **11**, 123 (1954); https://doi.org/10.1099/00221287-11-1-123