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Research Article

Microbial Cellulose Production from Bacteria Isolated from Rotten Fruit

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Microbial cellulose, an exopolysaccharide produced by bacteria, has unique structural and mechanical properties and is highly pure compared to plant cellulose. Present study represents isolation, identification, and screening of cellulose producing bacteria and further process optimization. Isolation of thirty cellulose producers was carried out from natural sources like rotten fruits and rotten vegetables. The bacterial isolates obtained from rotten pomegranate, rotten sweet potato, and rotten potato were identified as *Gluconacetobacter* sp. RV28, *Enterobacter* sp. RV11, and *Pseudomonas* sp. RV14 through morphological and biochemical analysis. Optimization studies were conducted for process parameters like inoculum density, temperature, pH, agitation, and carbon and nitrogen sources using *Gluconacetobacter* sp. RV28. The strain produced 4.7 g/L of cellulose at optimum growth conditions of temperature (30°C), pH (6.0), sucrose (2%), peptone (0.5%), and inoculum density (5%). Characterization of microbial cellulose was done by scanning electron microscopy (SEM).

1. Introduction

Microbial cellulose is an extracellular polysaccharide produced by some bacterial genera such as *Acetobacter*, *Agrobacterium*, *Gluconacetobacter*, *Rhizobium*, *Achromobacter*, *Alcaligenes*, *Aerobacter*, *Azotobacter*, *Rhizobium*, *Salmonella*, *Escherichia*, and *Sarcina*. It represents alternative to plant-derived cellulose for some specialty applications in the medical field and food and other industries [1, 2]. Members of *Gluconacetobacter* genus like *Gluconacetobacter xylinus* and *Gluconacetobacter hansenii* are the most potential producers compared to other strains. The microbial cellulose (MC) produced by *Acetobacter* strain can be used as diet food and to produce new materials for high performance speaker diaphragms, medical pads, makeup pads, paint thickeners, and artificial skin because of the unique properties of this cellulose distinct from those of plant cellulose [3, 4]. The microbial cellulose has a large specific surface area, higher water retention value, moldability, and high tensile strength compared with plant cellulose. Microbial cellulose can be produced by culturing a strain of *Acetobacter xylinum*, reclassified as the genus *Gluconacetobacter*, which is typically found on decaying fruits, vegetables, vinegar, fruit juices, and alcoholic beverages. The bacteria of this family convert ethanol to acetic acid. In the earlier studies, several attempts were made to isolate *Gluconacetobacter* sp. from fruits [5], flowers, fermented foods [6], beverages [7], and vinegar. In the current study, we aimed to isolate cellulose producing bacteria from rotten fruits and rotten vegetables. The cellulose producing strains were identified by morphological and biochemical characterization. In fact, to achieve high yield cellulose production, the culture conditions have a crucial influence, in particular factors such as carbon source, nitrogen source, temperature, pH, and agitation [1, 8]. In this work, we have investigated the influence of the culture conditions on cellulose production by *Gluconacetobacter* sp. RV28 isolated from rotten pomegranate. The effects of carbon source, nitrogen source, pH, temperature, and inoculum density were investigated. The cellulose produced from *Gluconacetobacter* sp. RV28 was characterized by scanning electron microscopy (SEM).

2. Materials and Methods

2.1. Collection of Samples. Samples of rotten fruits (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) were collected from various places of Shimoga, Davangere, and Haveri (Karnataka, India). All the samples were stored in normal saline at 4°C till further reference.

2.2. Chemicals and Reagents. All the media ingredients and biochemical test kits (KB001 and KB009) were procured from Hi-Media, Mumbai, India. Fluorescent brightener 28 was purchased from Sigma-Aldrich.

2.3. Isolation of Cellulose Producing Bacteria. A known weight (1 g) of each sample was taken separately and inoculated into 9 mL saline (0.9%) and serially diluted up to 10^{-6} dilution; further pour plate method was done using standard Hestrin-Schramm agar (D-glucose 20 g/L, yeast extract 5 g/L, peptone 5 g/L, disodium phosphate 2.7 g/L, citric acid 1.15 g/L, and agar 15 g/L) and incubated for 48 h at 30°C. One loopful of each isolate was inoculated into 9 mL of Hestrin-Schramm media (D-glucose 20 g/L, yeast extract 5 g/L, peptone 5 g/L, disodium phosphate 2.7 g/L, and citric acid 1.15 g/L). These tubes were incubated statically at 30°C for 7 days. After incubation, the tubes with white pellicle covering the surface of liquid medium were selected.

2.4. Screening of Cellulose Producer. All the flasks were observed for pellicle formation at air liquid interface. Those flasks with pellicle growth were selected and purified the culture by repeated streaking on HS agar plates to obtain isolated colonies. Each distinct isolate was inoculated on screening media, that is, HS agar with fluorescent brightener dye (0.02% w/v) and antifungal agent cycloheximide incubated at 30°C for 3 days. The fluorescent dye binds to the cellulose content in the organism. Cellulose producing bacterial colonies fluoresces when observed under UV light. So the fluorescent colonies were selected as cellulose producers [2].

2.5. Identification of Cellulose Producer. Bacterial isolates were identified by performing gram staining, colony morphology, motility test, and biochemical characteristics followed by carbohydrate fermentation test [9]. The strain was characterized for its biochemical properties using rapid biochemical test kit KB002 and KB009 (Hi-Media, India) according to manufactures instructions.

2.6. Detection of Cellulose Production and Quantification. The pellicle formed at the air-liquid interface of broth was treated with 1 N NaOH at 80°C for 15 minutes and then washed for about 3-4 times with distilled water; then, pellicle was neutralized with 4% acetic acid and again washed for 3-4 times with distilled water and dried in hot air oven at 60°C overnight. Then, the dry weight of cellulose was determined. Cellulose producing isolates were selected and inoculated into Hestrin-Schramm media at 30°C and incubated for 14 days. Dry weight of cellulose was quantified by using similar method as mentioned.

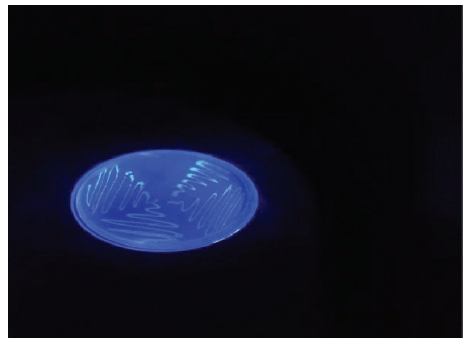

Figure 1: Fluorescent colony of *Gluconacetobacter* sp. RV28 under UV light.

2.7. Optimization of Culture Conditions. To optimize cellulose production by *Gluconacetobacter* sp. RV28, different physiological and nutritional parameters were studied such as pH, temperature, incubation period, agitation, carbon, and nitrogen sources. The experiments for optimization were carried out in triplicate and the standard error graphs were plotted. All the experiments were carried out in static condition of growth.

2.8. Scanning Electron Microscopy (SEM). The ultrafine structure of bacterial cellulose fibrils was characterized using scanning electron microscope (SEM model: JEOL Model JSM, 6390LV). Thin layers of freeze-dried cellulose were gold coated using ion sputter and coated samples were viewed and photographed at 20 k.

3. Result

3.1. Isolation and Screening of Cellulose Producing Bacteria. In the present study, thirty-six bacterial isolates were obtained from different natural sources which are found to produce cellulose. Those isolates showed fluorescence when observing their growth in screening medium under UV light. In screening media fluorescent dye binds to the cellulose content in the organism. Thus, cellulose producing bacterial colonies fluoresces when observed under UV light. So the fluorescent colonies were selected as cellulose producers (Figure 1). The isolates which obtained RV28 (rotten pomegranate), RV11 (rotten sweet potato), and RV14 (rotten potato) showed better cellulose production compared to other isolates.

3.2. Identification of Cellulose Producer. Identification of the strain was 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 *Gluconacetobacter* sp. RV28, *Pseudomonas* sp. RV14, and *Enterobacter* sp. RV11.

Table 1: Biochemical characterization for the isolates.

Characteristics test	*Enterobacter* sp. RV11	*Gluconacetobacter* sp. RV28	*Pseudomonas* sp. RV14
Gram reaction	Gram negative rods	Gram negative rods	Gram negative rods
Motility	Motile	Motile	Motile
Cellulose production	+	+	+
Cellulose yield g/L	1.9	3.1	1.2
Catalase	+	+	+
Oxidase	−	−	+
Citrate utilization	+	−	−
Indole test	+	−	−
Methyl red	+	−	−
Voges-Proskauer	−	−	−
Urease	+	−	−
H_2S production	+	−	−

Table 2: Carbohydrate fermentation test.

Carbon source	*Enterobacter* sp. RV11	*Gluconacetobacter* sp. RV28	*Pseudomonas* sp. RV14
Glucose	+	+	+
Adonitol	−	+	+
Arabinose	+	+	+
Lactose	−	+	+
Sorbitol	+	+	+
Mannitol	+	+	+
Rhamnose	+	+	+
Sucrose	+	+	+
Dextrose	+	+	+
Xylose	+	−	+
Maltose	+	+	+
Fructose	+	+	+
Galactose	+	+	+
Raffinose	+	−	+
Trehalose	+	−	+
Melibiose	+	−	+
L-Arabinose	+	+	+
Mannose	+	+	+
Sodium gluconate	+	−	−
Glycerol	+	+	+
Inositol	+	+	−
Erythritol	+	+	−
α-Methyl-D-glucoside	+	+	−
Xylitol	+	+	+
ONPG	−	−	−
Esculin hydrolysis	−	−	+
Malonate utilization	−	−	+
Sorbose	+	+	+

3.3. Detection of Cellulose. The strain *Gluconacetobacter* sp. RV28, *Pseudomonas* sp. RV14, and *Enterobacter* sp. RV11 were observed to form pellicle at air liquid interphase (Figure 3). The pellicle was treated with alkali at 80°C followed by washing with distilled water. The cellulose is resistant to this treatment and remains undissolved and is accepted as pure cellulose. Yield is quantified for each isolate and presented in Table 4.

Figure 2: Colony morphology of *Gluconacetobacter* sp., *Enterobacter* sp., and *Pseudomonas* sp.

Table 3: Cultural characterization of *Gluconacetobacter* sp. RV28.

Colony morphology	*Gluconacetobacter* sp. RV28
Configuration	Round
Margin	Entire
Elevation	Raised
Surface	Smooth, mucoid
Color	Pink
Opacity	Translucent
Motility	Motile
Cell shape	Rod
Spore formation	Negative

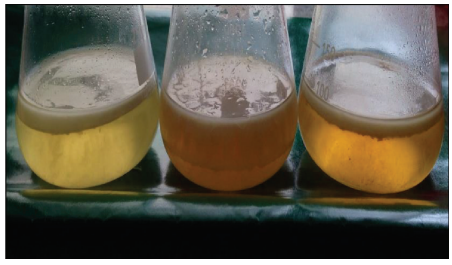

Figure 3: Pellicle formed at air liquid interface by the isolates *Gluconacetobacter* sp., *Enterobacter* sp., and *Pseudomonas* sp.

3.4. Scanning Electron Microscope. The ultrafine structure of bacteria cellulose constituted by cellulose nanofibre structure magnified at 5000 at 20 kV. Cellulose microfibrils and nanofibres were evidenced through SEM studies (Figure 4).

3.5. Effect of Inoculum Density on Cellulose Production. The inoculum volume plays an important role in cellulose production. To study the effect of inoculum size of *Gluconacetobacter* sp. RV28 inoculum size ranging from 1% to 10% (v/v) was examined for cellulose production. The results are presented in Figure 5. The cellulose production was observed in all inoculum size tested but lower and higher

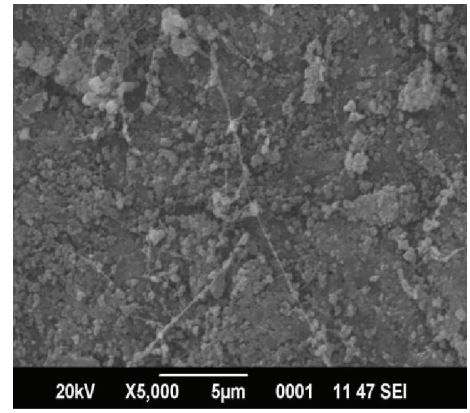

Figure 4: SEM image of cellulose produced by *Gluconacetobacter* sp.

values than 5% inoculum showed there is a decrease in cellulose production. By this experiment, we can conclude that 5% inoculum size is optimum for cellulose production and achieved 2.5 g/L yield compared to other inoculum sizes.

3.6. Effect of pH on Cellulose Production. The pH plays an important role in cell growth and cellulose production. To study the effect of pH on cellulose production, the organism was grown in medium with pH value ranging from 2 to 10. The results are presented in Figure 6. The cellulose production was observed in all pH values tested but pH range 3–7 showed better production compared to other pH values. By this investigation, we can observe that pH 6 is optimum for cellulose production and achieved 2.1 g/L which is maximum yield compared to other pH values.

3.7. Effect of Temperature on Cellulose Production. The temperature plays very important role as it directly affects cell growth and cellulose production. To study the effect of temperature on cellulose production by *Gluconacetobacter* sp. RV28 temperature, range from 20 to 45°C was examined.

Table 4: Screening of isolates for cellulose production (yield in g/L).

Sample number	Source	Isolate code	Gram reaction	Fluorescence	Yield g/L
1	Apple	RV01	Gram negative	+	0.91
2	Sweet lime	RV02	Gram negative	+	1.0
3	Apple	RV06	Gram negative	+	0.96
4	Apple	RV03	Gram negative	+	0.51
5	Apple	RV04	Gram negative	+	1.1
6	Apple	RV05	Gram negative	+	0.81
7	Ladies finger	RV20	Gram negative	+	0.75
8	Ladies finger	RV21	Gram negative	+	0.09
9	Ladies finger	RV22	Gram negative	+	0.22
10	Ladies finger	RV23	Gram negative	+	0.66
11	Ladies finger	RV24	Gram negative	+	0.08
12	Ridge guard	RV25	Gram negative	+	0.08
13	Onion	RV10	Gram negative	+	0.01
14	**Sweet potato**	**RV11**	**Gram negative**	+	**1.9**
15	Sweet potato	RV13	Gram negative	+	0.89
17	Sweet potato	RV16	Gram negative	**+**	**1**
18	Pomegranate 1	RV26	Gram negative	+	1.1
19	Pomegranate 2	RV27	Gram negative	+	0.97
20	**Pomegranate 3**	**RV28**	**Gram negative**	**+**	**3.1**
21	Apple	RV7	Gram negative	+	0.9
22	**Potato**	**RV14**	**Gram negative**	**+**	**1.2**
23	Sweet lime 1	RV30	Gram negative	+	0.9
24	Sweet lime 1a	RV31	Gram negative	+	1
25	Sweet lime 1b	RV32	Gram negative	+	0.1
26	Grape 1	RV33	Gram negative	+	0.9
27	Grape 2	RV34	Gram negative	+	0.7
28	Sweet lime 1c	RV35	Gram negative	**+**	0.3
29	Sweet potato	RV36	Gram negative	+	0.2
30	Sweet potato	RV37	Gram negative	+	0.9

The results indicated that temperatures 28–30°C favoured maximum cellulose production 1.99–2.31 g/L; the results are presented in Figure 7. The cellulose production was least at 37°C and cellulose production was not observed in the range of 40 and 45°C. By this investigation, we can conclude that optimum temperature for cellulose production is 28–30°C.

3.8. Effect of Carbon Source on Cellulose Production. The carbon is a sole source for cellulose production and cell growth. To study the effect of carbon source on cellulose production, carbon sources like maltose, mannitol, mannose, sucrose, lactose, glucose, and fructose were supplemented at 2% (w/v) in standard Hestrin-Schramm medium. The results are presented in Figure 8; the strain utilized all carbon sources tested and least percent was from lactose. The maximum cellulose production was observed in sucrose followed by mannitol giving cellulose yield of 1.58–2.35 g/L.

3.9. Effect of Nitrogen on Cellulose Production. The nitrogen source is required for cell growth and cellulose production. To study the effect of nitrogen source on cellulose production, the nitrogen sources like peptone, ammonium nitrate, ammonium chloride, and ammonium sulphate were supplemented at 0.5% (w/v) instead of peptone in standard medium. The results are presented in Figure 9. The maximum cellulose production was observed with peptone which gave cellulose yield of 2.15 g/L. By this investigation, we can conclude that good nitrogen source for cellulose production is peptone.

4. Discussion

Most of the earlier studies describe cellulose production by culturing a strain of *Acetobacter xylinum*, reclassified as the genus *Gluconacetobacter*, which is typically found on decaying fruits, vegetables, vinegar, fruit juices, and alcoholic beverages. The members of this family convert ethanol to acetic acid. Several attempts have been made to isolate *Gluconacetobacter* sp. from fruits [5], flowers, fermented foods [6], beverages [7], and vinegar. In the present study, we aimed to isolate bacteria possessing ability to produce higher cellulose from rotten fruits and rotten vegetables. The cellulose producing strains are identified by morphological and biochemical characterization. In the present investigation, we

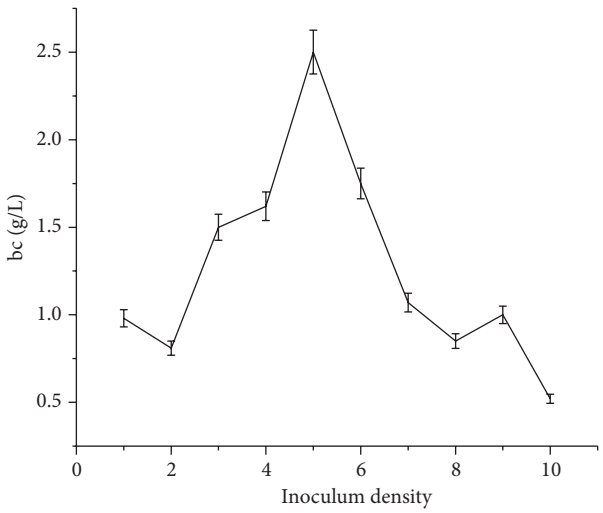

Figure 5: Effect of inoculum size on cellulose production by *Gluconacetobacter* sp.

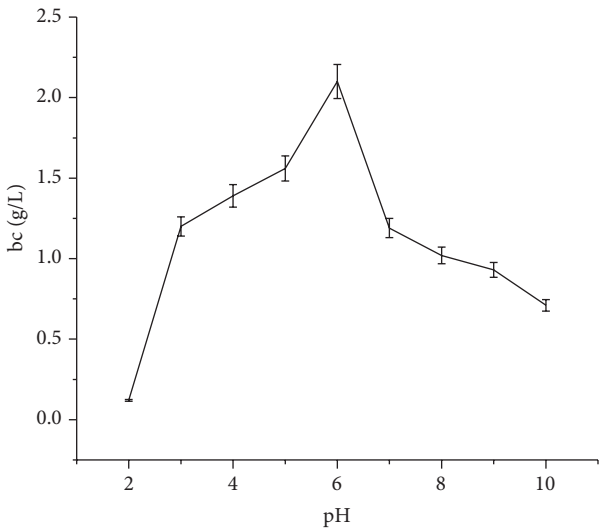

Figure 6: Effect of pH on cellulose production by *Gluconacetobacter* sp.

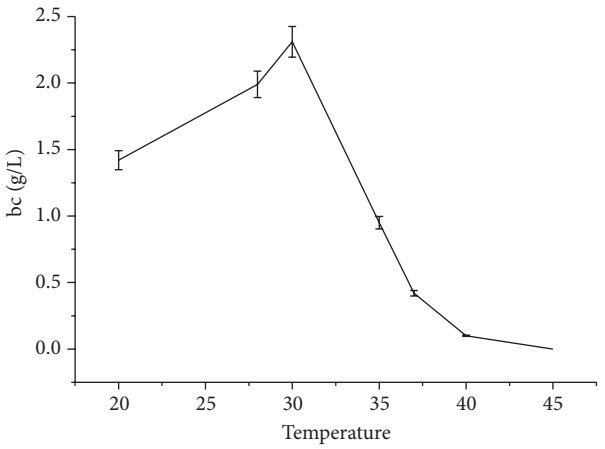

Figure 7: Effect of temperature on cellulose production by *Gluconacetobacter* sp.

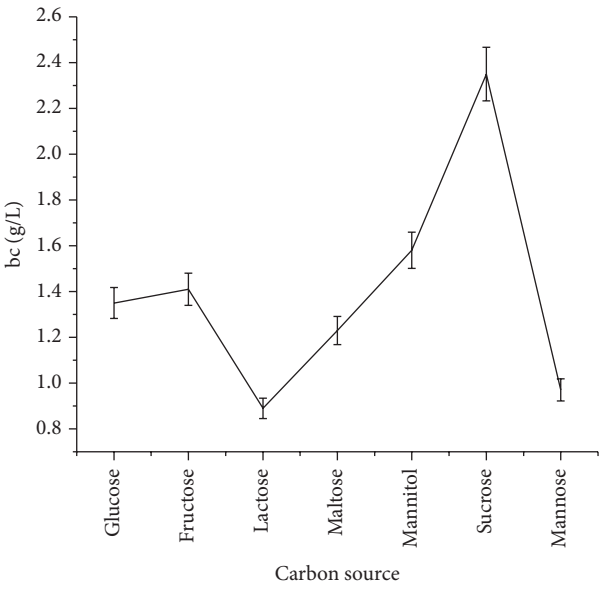

Figure 8: Effect of carbon source on cellulose production by *Gluconacetobacter* sp.

report the potent cellulose producer *Gluconacetobacter* sp. RV28 isolated from rotten pomegranate. The previous studies describe cellulose synthesis as part of primary metabolism which is observed in bacterial species such as *Acetobacter xylinum* [10], *Rhizobium leguminosarum* [11, 12], *Klebsiella pneumoniae* [13], *Sarcina ventricle* [10], *Agrobacterium tumefaciens* [14], *Salmonella typhimurium* [15], *Escherichia coli* and *Enterobacter* [2, 15], and cyanobacteria [16]. Schramm and Hestrin identified optimal growth conditions for cellulose production [17]. Similarly in this work several attempts were made to isolate cellulose producer and isolated *Pseudomonas* sp. RV14, *Enterobacter* sp. RV11, and *Gluconacetobacter* sp. RV28. Among all the genera, the *Gluconacetobacter* genus stands out due to its ability to synthesize and extrude copious amounts of highly pure ribbons of cellulose. In the present study, we isolated most prominent model organism, that is, *Gluconacetobacter* sp. RV28 from rotten pomegranate. The highest yield of 4.7 g/L was achieved from the isolate *Gluconacetobacter* sp. RV28 in optimized medium. Previous studies prove that *Acetobacter xylinum* a Gram negative, obligate aerobic bacterium has been considered for several years, as an archetype for cellulose synthesis-related studies. A single cell can polymerize 200,000 glucose molecules per second [18], which are extruded in the form of a 100 nm wide, flat ribbon of cellulose along the longitudinal axis of the cell [19] which remain attached to the cells during cell division [20].

In this study to screen cellulose producing bacteria we added fluorescent brightener dye/calcofluor white in the screening medium. The colonies were observed to be fluorescing when observed under UV light. Calcofluor white present in the screening medium avidly binds to β-D glucans

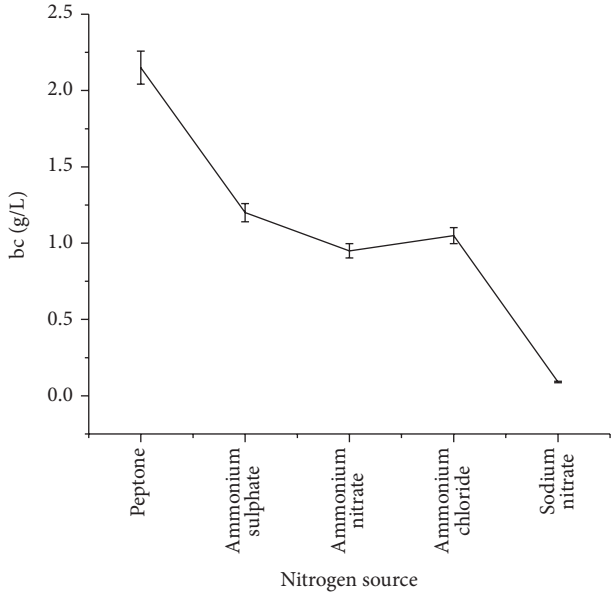

Figure 9: Effect of nitrogen source on cellulose production by *Gluconacetobacter* sp.

in a definable, reversible manner and cellulose producing bacterial colony fluoresces when observed under UV light [10, 21].

The cellulose production not only depends on strain but also depends on media ingredients and culture cultivation conditions to achieve highest production. In this study, the effects of different parameters such as inoculum density, pH, temperature, carbon, and nitrogen sources were carried out. The optimized medium contains standard Hestrin and Schramm media, that is, sucrose 2% and peptone 0.5% in the presence of yeast extract 0.5%, temperature 30°C, pH 6, and inoculum density 5% resulting in 4.7 g/L. These results prove that optimization of culture conditions is as important as that of the organism. In the present study, inoculum density cannot be studied by OD method because the strain grown media are almost transparent and pellicle formation at air liquid interphase can be observed within 48 h of incubation. Many researchers explain that, for maximum cellulose production, the total cell count is not important, and significant point is the number of cell counts in the aerobic zone that are producing cellulose [22, 23]. Most of the studies describe that the efficiency of cellulose production by *Gluconacetobacter* is dictated by carbon source availability and the accumulation of metabolic by-products that cause unfavourable growth conditions [1]. Other environmental factors such as temperature, culture type (agitated or static), oxygen diffusion, and pH also influence cellulose synthesis. The most efficient production of cellulose by *Gluconacetobacter* sp. occurs under static conditions between 28°C and 30°C [24].

The *Gluconacetobacter* sp. can synthesize cellulose from a variety of carbon sources [25]; the most efficient production of cellulose is achieved when glucose is used as the primary carbon source [26]. Different from other carbon sources, glucose can be shuttled directly into the cellulose synthesis pathway [10]. The metabolism of glucose, however, results in the accumulation of gluconate and a concurrent decline in culture pH [27]. Optimum cellulose synthesis is achieved at a pH range of 5-6. When the culture pH falls below 4 as a consequence of gluconate accumulation, cellulose synthesis declines. Once all of the glucose in the media has been oxidized, the bacteria begin to metabolize the gluconate and a gradual increase in culture pH is observed as the bacteria consume the gluconate. Cellulose synthesis and cell division resume once the pH levels climb above 4 [1]. Material properties are very important criteria linked to the structure resulting in chemical composition; arrangement of cellulose can be studied by scanning electron microscope. The ultrafine cellulose fibres showed in SEM proved that cellulose produced from *Gluconacetobacter* sp. RV28 was cellulose microfiber arrangement which in turn proves its water holding capacity. This property was stated by earlier researcher [28].

5. Conclusions

The present investigation reported isolation of cellulose producing bacteria from rotten fruit and vegetable samples. The isolates were identified as *Pseudomonas* sp. RV14, *Enterobacter* sp. RV11, and *Gluconacetobacter* sp. RV28. The yield of cellulose with respect to each organism was studied. Under optimum conditions of growth, *Gluconacetobacter* sp. RV28 achieved highest cellulose yield of 4.7 g/L. The bacterial cellulose harvested from this organism showed ultrafine microfibrils in scanning electron micrographs. These findings are significant for the continual improvement of cellulose synthesis by *Gluconacetobacter* sp. RV28 with future implications of bioengineering to produce cellulose on an industrial scale.

Conflict of Interests

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

Acknowledgments

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