Isolation of Cellulose Producing Bacteria from Wastes of Vinegar Fermentation

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***Abstract*—Five strains of acetic acid bacteria were isolated from wastes of vinegar fermentation. Three of the strains were found to secrete cellulose. These strains were identified by several biochemical and physiological tests and the results were compared with refererence strains *Gluconacetobacter xylinus* DSM 46604 and *Gluconacetobacter hansenii* DSM 5602.**

***Index Terms*—Acetic acid bacteria, bacterial cellulose, vinegar.**

I. Introduction

Acetic acid bacteria are defined within the family *Acetobacteraceae* and they are characterized by their ability to oxidize ethanol to acetic acid [1]. Recent classification of the acetic acid bacteria includes the genera *Acetobacter*, *Acidomonas*, *Asaia*, *Gluconacetobacter*, *Gluconobacter*, *Kozakia, Swaminathania* and *Saccharibacter* [2]. Some species of *Acetobacter*, recently named as *Gluconacetobacter*, are known to produce cellulose exhibiting superior features over plant cellulose although being chemically identical. The unique features of this material such as extreme purity, high cristallinity and degree of polymerization has gained considerable commercial and scientific interest [3]-[6].

Several attempts have been made to isolate *Gluconacetobacter* sp. from fruits [7], [8], flowers, fermented foods [9], [10], beverages [11], [12] and vinegar [13]-[15]. Although many more bacteria such as *Agrobacterium, Pseudomonas, Rhizobium* and *Sarcina* have been proven to synthesize bacterial cellulose, *Gluconacetobacter* sp. are known to have the highest cellulose production capacity [3]-[6]. Cellulose yield depends not only on the microorganism but also on the production method. Conventionally, bacterial cellulose is produced either statically, or by agitation. Though agitation enhances the rate of oxygen diffusion, it causes the formation of celluloseless mutants, thus lowering the yield most of the time. It is obvious that strains or production methods with higher yields are required for mass production [16].

Several attempts have been made to isolate highly productive strains for the industrial production of bacterial cellulose [7], [17], [18]. The highest capacity reported in literature is 9.7 g cellulose·L^{-1}, which was attained by sulfaguanidine resistant mutants of *Acetobacter xylinum* subsp. *sucrofermentans* [19].

In the current study, we aimed to isolate bacteria possessing high ability to produce cellulose from wastes of vinegar fermentation. Strains were characterized by biochemical and morphological tests.

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II. Materials and Method

A. Microorganisms and Culture Conditions

Waste of vinegar fermentation was supplied by Kukrer A.S (Turkey). 1 ml samples were incubated statically at pH 6.0 and 30ºC for 7 days in Hestrin-Schramm (HS) medium (30 ml) composed of 2.0% D-glucose, 0.5% peptone, 0.5% yeast extract, 0.27% Na_2HPO_4 and 0.115 citric acid [16]. In case of microbial growth, the cultures were streaked onto HS-agar (1.5% agar) plates. The growth of the colonies were observed during incubation at 30ºC for 3 days. White to cream colonies with mucous structure were purified by repeated streaking onto agar plates.

Gluconacetobacter xylinus DSM 46604 and *Gluconacetobacter hansenii* DSM 5602 were used as the reference strains and were obtained from the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Germany).

B. Biochemical and Physiological Tests

Morphology of the cells was examined under light microscope. Gram staining was performed to select gram negative strains. The motility of cells was observed by hanging drop method.

Purified cultures were streaked onto $CaCO_3$-agar plates to confirm acid production and investigate overoxidation of acetic acid by formation and disappearance of clear zones around colonies [1]. $CaCO_3$-agar medium was composed of 0.05% D-glucose, 0.3% peptone, 0.5% yeast extract, 1.5% $CaCO_3$, 1.2% agar and 1.5%(v/v) ethanol. Microbial growth was examined during incubation at 30ºC for 2-7 days. Acid forming colonies were subjected to further biochemical tests. Catalase, oxidase and indole tests were performed using BD reagent droppers (Becton, Dickinson and Company, USA). Acid production from fructose, galactose, glucose, lactose, maltose, mannose, sucrose and xylose was investigated by using 1% of tested sugar solution as the only source of carbon. Urea broth (0.01% yeast extract, 0.91% KH_2PO_4, 0.95% Na_2HPO_4, 2.0% urea and, 0.001 % phenol red) was used to determine urea utilization. Citrate utilization was tested using citrate broth (0.1% $NH_4H_2PO_4$, 0.1% KH_2PO_4, 0.5% NaCl, 0.02% $MgSO_4$, 0.2% sodium citrate, 0.008% bromthymol blue and, 1.3% agar). Growth at temperatures 25, 30, 37 and 45ºC and at pH 3.0, 7.0 and 8.0 was tested using HS broth.

Growth on glutamate and mannitol agar were investigated by inoculation of agar plates composed of 1.0% glucose, 0.5% sodium glutamate, 0.1% KH_2PO_4, 0.02% $MgSO_4.7H_2O$, 0.01% KCl and, 2.0% agar for glutamate and 2.5% mannitol, 0.5% yeast extract, 0.3% peptone and 2.0% agar for mannitol. Plates were incubated statically at 30ºC for 10 days. Yeast extract peptone medium (0.3% yeast extract, 0.2% peptone and 0.002% bromthymol blue) was supplemented with 0.2% sodium salt of the corresponding substrate for the oxidation of acetate and lactate. Hydrolysis of gelatin was investigated by inoculation of nutrient gelatin tubes (0.5% peptone, 0.3% beef extract and 12% gelatin) incubated at 37ºC for 1-2 weeks. The production of dihydroxyacetone was tested by the addition of Benedict's solution to a heavily inoculated tube containing 3.0% glycerol incubated statically at 30ºC for 10 days [20]. All tests were performed three times to assure replicability of the results.

C. Cellulose Formation and Detection

Seed broth was prepared by inoculation of 10 ml test tubes containing HS broth. The tubes were incubated statically at 30°C for one week. Cultures were carried out by the addition of 1% (v/v) seed broth to the culture medium. Cellulose formation was monitored by the appearance of a white pellicle on the surface of culture broth. However, the pellicles produced by acetic acid bacteria are not essentially cellulose and thus, an additional treatment is required for confirmation of cellulose structure.

As pellicles were observed in the medium, a simple test was conducted for cellulose detection. The broth was centrifuged for 10 minutes at 4,000*g*. After washing three times with distilled water, pellicles were subjected to boiling for 15 minutes with 0.5 N NaOH. Cellulose is resistant to this treatment and thus remaining material was accepted as cellulose free from microbial cells and medium components [15]. Cellulose was washed three times with distilled water and dried at 105ºC.

III. Results and Discussion

A. Identification of Isolates

Seventeen single opaque colonies with cream to beige color and approximately 2.5 to 3 mm in diameter were observed at the fourth streak on HS-agar plates. Gram staining of the cultures proved that fourteen strains were either gram negative or gram variable, being short bacilli. Only two of these cultures were motile. Gram negative or variable cultures were transferred onto $CaCO_3$-agar plates for visualization of acid production. Only five of the colonies formed $CaCO_3$ clear zones. These colonies were selected as acetic acid bacteria and were subjected to further analysis.

Three of the strains could oxidize acetate to CO_2 and H_2O, whereas all of the isolates tested negative for lactate oxidation. Acetate oxidizers were also capable of cellulose production. The ability to oxidize both acetate and lactate is regarded as a distinctive property of *Acetobacter, Acidomonas* and *Asaia* spp. [8], [21], whereas the lack of this ability is distinctive for *Gluconobacter* genus [7], [21], [22]. Thus, two of the isolates, namely AS2 and AS9, were members of the *Gluconobacter* genus. Remaining three isolates, coded as AS6, AS7 and AS14 showed similar oxidation character with *Gluconacetobacter* genus [21], [22], which was also verified with positive response for cellulose production. The biochemical properties of the isolates capable of cellulose production and reference strains are summarized in Table 1.

TABLE 1. COMPARISON OF THE CHARACTERISTICS OF ISOLATES AND REFERENCE STRAINS

Characteristics	AS6	AS7	AS14	DSM 46604	DSM 5602
Catalase	+	+	+	+	+
Oxidase	-	-	-	-	-
Indole production	-	-	-	-	-
Brown pigmentation	-	-	-	-	-
H_2S formation	-	-	-	-	-
Urea utilization	-	-	-	-	-
Cellulose production	+	+	+	+	+
Sodium citrate utilization	-	-	-	-	±[a]
Oxidation of ethanol	+	+	+	+	+
Overoxidation of acetic acid	+	+	+	+	+
Lactate oxidation	-	-	-	+	+
Gelatin liquefaction	-	-	-	-	-
Ketogenesis of glycerol	-	+	+	+	+
Growth on mannitol agar	+	+	+	+	±
Growth on glutamate agar	-	-	w[b]	-	-
Acid production from:					
D-glucose	+	+	+	+	+
Sucrose	+	+	+	-	±
Fructose	-	w	-	-	±
Lactose	-	-	-	-	-
Galactose	+	+	w	+	±
Maltose	-	-	-	±	-
Mannose	+	+	+	+	+
Xylose	+	+	+	+	+

[a] ± :variable response.
[b] w: weak response.

Accordingly, all the selected strains were oxidase negative and catalase positive. A negative reaction was observed for indole production, citrate and urea utilization, H_2S formation and gelatin liquefaction. Strains grew weakly at 25ºC, well at 28-35ºC, but growth was negative at 45ºC. The strains grew weakly at pH 3.0, well at 4.0, 6.0 and 7.0, but growth was negative for pH 8.0. All these characteristics were in accordance with the previous literature on *Acetobacter* and *Gluconacetobacter* spp. [10], [13], [16], [18], [23]-[26]. Unlike the other isolated and reference strains; AS6 was not able to produce dihydroxyacetone from glycerol. Several contradictory data exist in literature for ketogenic activity towards glycerol. Negative results were reported by Toyosaki *et al.* [18] and Navarro and Komagata [26] for some strains of *Gluconacetobacter xylinus*, but Son *et al.* [15] and Kojima *et al.* [8] reported positive reactions for certain isolates and reference cultures. Isolated strains were all able to grow on mannitol agar, whereas only AS14 showed scant growth on glutamate agar. Even though the reference strains DSM 46604 and DSM 5602 were not able to grow on glutamate agar, Navarro and Komagata [26] had previously reported certain strains of *Gluconacetobacter xylinus*, which could exhibit positive growth on glutamate. The isolated strains could produce acids from glucose, sucrose, mannose, galactose and xylose. The strain AS7 was distinguished from others by acid production from fructose.

Comparison of the results for isolates and reference cultures showed that the strain AS6 could most likely be a species of *Gluconacetobacter xylinus* whereas the strain AS7

showed similar characteristics with *Gluconacetobacter hansenii.* The strain AS14 was also similar to *Gluconacetobacter hansenii* but further biochemical analysis is yet required.

B. Cellulose Production

The turbidity of the culture broth increased within hours of inoculation and a cellulosic film was obtained on the air-liquid interface after the first day of incubation. Incubation was lasted for a week and cellulose production was monitored.

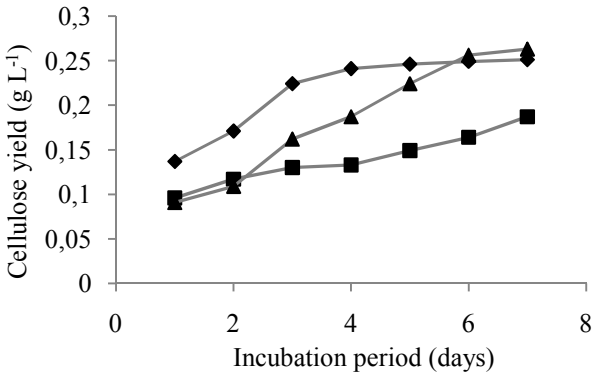

Figure 1. The production of bacterial cellulose by isolated strains (υ) AS6 (ν) AS7 (σ) AS14.

The productivity of the strains in HS medium were determined as 0.251±0.03 g L^{-1}, 0.187±0.07 g L^{-1} and 0.263 ±0.02 g L^{-1} for the isolates AS6, AS7, and AS14, respectively. Nguyen *et al.* [27] have reported 0.28±0.01 g L^{-1} cellulose production with a *Gluconacetobacter xylinus* strain isolated from *Kombucha* culture. Also, Park *et al.* [9] have reported 0.35 g L^{-1} cellulose production on the fourth day of incubation by a *Gluconacetobacter hansenii* strain isolated from rotten apple. As shown, the productivity of strains AS6, AS7 and AS14 are quite similar to the values reported in literature for *Gluconacetobacter xylinus* and *Gluconacetobacter hansenii* strains. Yet, one can improve the yield by manipulating the initial pH [15], changing the carbon or nitrogen source [27] or by improving cultivation conditions [17].

IV. Conclusion

The genus *Gluconacetobacter* and *Acetobacter* are closely related and can be isolated from similar ecological environment [1]. Several acetic acid bacteria such as *Acetobacter aceti*, *Acetobacter pasteurianus*, *Acetobacter oboediens*, *Gluconacetobacter xylinus*, *Gluconacetobacter hansenii*, *Gluconacetobacter europaeus*, *Gluconacetobacter intermedius*, *Gluconacetobacter entanii* have been isolated from vinegar [14], [16], [24], [25] by various researchers.

Three cellulose producing strains were isolated from vinegar wastes. Physiological and biochemical tests of the isolated and reference strains showed significant resemblance. Discrepancies were found in the oxidation of lactate, ketogenesis of glycerol, growth on glutamate agar and acid production from sucrose, which will be resolved by further testing of more reference strains. However, for more precise identification, other procedures including 16S rRNA sequencing, DNA-DNA similarity tests, DNA base composition and Quinone analysis are recommended.

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