Improved Production of Bacterial Cellulose From Gluconacetobacter persimmonis GH-2

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Abstract

Bacterial cellulose produced by members of the genus Gluconacetobacter displays unique properties including high mechanical strength, high water holding ability, high crystallinity, and an ultra-fine and highly pure fiber network structure. It has many potential applications in biomedical, biosensor, food, textile and other industries. A cellulose producing strain isolated from nata sample (a desert) was identified as Gluconacetobacter persimmonis (GH-2) based on biochemical studies and 16S rDNA complete sequence analysis. The strain produced 5.14 g/L of cellulose in standard medium under stationary growth conditions. For effective production of cellulose from the strain, various carbon and nitrogen sources were investigated in flask cultures under stationary conditions of incubation. Among various carbon sources glucose, fructose, sucrose, mannitol and inositol were found to be suitable for growth and cellulose production. The strain utilized a wide range of organic nitrogen sources such as peptone, casein hydrolysate, beef extract, and malt extract for growth and cellulose production. The optimum pH and temperature for BC production by the strain were observed to be 5.5 and 30°C respectively. The organism was also found to produce substantial amount of cellulose under aerated and agitated culture conditions. Batch fermentation for cellulose production using glucose as a carbon source by the strain was carried out in a laboratory-scale fermenter. The strain produced 6.71 g/L cellulose in the fermenter which was 30% higher than that of the yield under stationary conditions of growth.

Keywords: Bacterial cellulose; Gluconacetobacter; Carbon and nitrogen sources; Batch fermentation

Introduction

Many species of bacteria, such as those in the genera Gluconacetobacter (earlier classification Acetobacter), Agrobacterium, Aerobacter, Azotobacter, Rhizobium, Sarcina, Enterobacter, Escherichia, Salmonella, Klebsiella, Gluconobacter, and several species of cyanobacteria have been reported to produce extracellular cellulose (Napoli et al., 1975; Ross et al., 1991; Mathyssse et al., 1995; Romling, 2002; Jia et al., 2004; Hungund and Gupta, 2010). This cellulose from bacterial source is called bacterial cellulose (BC). Among all, the most efficient producer of cellulose is Gluconacetobacter xylinus which has been used as a model organism for the elucidation of basic features of cellulose biosynthesis. This cellulose is the same as ‘Nata de Coco’, a traditional fermented food that has been a cottage industry in the Philippines obtained from Gluconacetobacter sp. BC differs from plant cellulose with respect to chemical and physical features. BC exhibits unique properties such as high purity, high crystallinity, excellent biodegradability, large water holding ability and excellent biological affinity (Yoshinaga et al., 1997; Shoda and Sugano, 2005). With these characteristics, BC is expected to have applications as an alternative to plant cellulose or as a new biodegradable material available in food and chemical industries and in medical field. These potential applications of BC largely depend on its price and accessibility. Therefore, strains and production medium must be optimized. Since commercial exploitation of BC is limited by its yield, many researchers have tried to increase the productivity using different carbon and nitrogen sources from Gluconacetobacter xylinus (Masaoka et al., 1993; Embuscado et al., 1994; Ramana et al., 2000; Keshk and Sameshima, 2005). Most of the studies used stationary culture conditions but static culture method does not allow mass production of the BC to reduce cost. An aeration and agitation culture process may be more suitable for mass production on a commercial scale to increase productivity. Cellulose production was found to increase in some strains when they were grown in fermenters under controlled conditions. Investigations of culture conditions are essential to achieve industrial levels of cellulose production. Many researchers produced bacterial cellulose under agitated culture conditions using batch, fed-batch and continuous fermentation processes (Toyosaki et al., 1995; Kouda et al., 1997; Naritomi et al., 1998; Lee and Zao, 1999; Bae and Shoda, 2004, Bae and Shoda, 2005). The aim of the present study was to investigate potential of Gluconacetobacter persimmonis GH-2 isolated from nata sample for cellulose production under stationary and agitated culture conditions.

Materials and Methods

All the media ingredients used in the investigation and Biochemical testing kits were purchased from HiMedia Laboratories, India. Enzymes glucose oxidase and peroxidase, ortho-dianisidine required for glucose oxidase method was procured from Sigma Aldrich.

Microorganism

Gluconacetobacter persimmonis GH-2 was isolated from nata sample (Iso-12). For the isolation of the bacterial strain, standard Hestrin-Schramm (HS) medium (Hestrin and Schramm, 1954) was employed with modifications. The medium consisted of (g/L): D-glucose, 20; yeast extract, 5; peptone, 5; disodium phosphate, 2.7;
and citric acid, 1.15; pH 6.0. An actively fermenting nata sample was obtained from Philippines. A known amount (1 g) of this sample was added into 100 mL of HS medium with 0.2% (v/v) acetic acid, 0.5% (v/v) ethanol, and 0.02% (w/v) cycloheximide and incubated at room temperature for 24 hours. From this enriched sample, serial dilutions were prepared in phosphate buffer and 0.1 mL of aliquot was spread onto the screening medium. Screening medium consisted of HS agar supplemented with 0.02% calciluor white. Calcofluor white present in the screening medium avidly binds to β-D glucans in a definable, reversible manner and cellulose producing bacterial colony fluoresces when observed under UV light (Ross et al., 1991). The fluorescing colony was subsequently subcultured into HS medium and purity was confirmed. Pure culture was identified by Aristogene Biosciences, India.

Production of cellulose under stationary conditions

100 mL of standard HS medium (pH 6.0) contained in 250 mL conical flask was inoculated with the organism. The flasks were incubated at 30°C for 14 days and observed for pellicle formation. The pellicle formed after 14 days of incubation was removed carefully, boiled in 2% NaOH solution for 30 minutes and thoroughly washed with distilled water. Drying was carried out at 70°C for 6 hours. The dry weight of the cellulose was calculated. The product formed was confirmed as cellulose by carrying out the qualitative analysis for cellulose. The sample was subjected to acetylation by acetic/nitric reagent (150 mL of 80% acetic acid and 15 mL of concentrated nitric acid) then to hydrolysis by sulfuric acid (67%). The carbohydrate content was measured by the anthrone method (Dreywood, 1946). Different carbon and nitrogen sources were used to test their effect on cellulose production by the strain. Also the effect of initial pH and incubation temperature on cellulose production by the strain was investigated.

Production of cellulose using fermenter

Fermenter: The fermenter is an in-situ sterilizable high quality fermenter system for laboratory-scale fermentation applications (Sciegenics, India). It consists of stirrer driven fermentation vessel, a PLC (Programmable Logic Controller) based measurement and control unit, and other utilities like compressor, water supply unit, steam generator, cooling unit, etc. The fermenter system consists of vessel pipe rack assembly and instrument cabinet. The vessel has a jacket ringed for heating and cooling water to flow and a variable speed agitator. Instrument also consists of manual and automatic controls for temperature, pH, dissolved oxygen (DO) and antifoam. The agitator speed and airflow can be controlled manually. It also houses temperature measurement amplifier, speed controller, pH measurement amplifier, antifoam control, dissolved oxygen (DO) amplifier, and flow controller. A schematic diagram of the fermenter system is presented in Figure 1.

Culture vessel: The culture vessel (5 L) is made up of stainless steel and has a height / diameter ratio of about 2:1. The vessel is heated and cooled via the jacket. The agitator (turbine type) enters the vessel through a mechanical seal and is provided with 2 impellers, which can be adjusted to any height. The shaft is driven by a AC motor at 220 V at 100-1000 rpm. A separate drive controller is available for adjusting the speed.

Inoculum development: A loopful of frozen stock culture was inoculated aseptically into flasks containing 25 mL of HS medium. The flasks were incubated at 30°C for 48 hours. The cells attached to the surface pellicle of the medium were removed by manual shaking to produce a cell suspension. A 10 mL of this culture was inoculated into 200 mL of sterile HS medium. The flasks were kept on a shaker incubator at 30°C for 24 hours at 125 rpm. Then the inoculum was
added aseptically using peristaltic pump into fermenter containing 2 L of standard HS medium.

**Fermentation conditions**: Batch fermentation experiments were conducted in a 5 L laboratory-scale fermenter (working volume, 2 L). The fermentation conditions set for cellulose production by *G. persimmonis* GH-2 were: temperature: 30°C, pH: 5.5, RPM: 120 and DO level of 20%. The DO concentration was kept at 20% of the saturated dissolved oxygen concentration by regulating the agitation speed and air flow rate (cascade mechanism). Inoculum was added at a concentration of 10% and the medium was agitated continuously with supply of sterile air. The set temperature was maintained by passing hot-water or chilled-water through jacket of the fermenter. The duration of fermentation was eight days. Sample was withdrawn after every 24 hours and analyzed for total viable count, residual glucose concentration, and product yield. Total viable count was performed using standard HS medium with 1.5% agar by pour plate method. Glucose estimation was done using enzyme glucose oxidase assay referring to standard graph.

**Total viable count**: Total viable count was determined as total count comprising cells entrapped in BC pellets and those suspended in the broth. Sampled culture broth was agitated and homogenized gently to loosen the associated cells from cellulose clumps. Further the sample was subjected to serial dilution using sterile phosphate buffer. Pour plate technique was used for counting and reported as colony forming units (cfu) per mL of broth sample.

**Glucose estimation**: The broth was analyzed for glucose level using glucose oxidase method (glucose assay kit, Sigma). The samples were withdrawn from the culture vessel and centrifuged at 5000 rpm for 10 minutes, and the supernatant was diluted with deionized water. Enzyme glucose oxidase catalyzes the oxidation of D glucose to D-glucal-1, 5-lactone with the formation of hydrogen peroxide; the lactone is then slowly hydrolyzed to D-gluconic acid. The oxygen liberated from hydrogen peroxide by peroxidase reacts with the O-dianisidine and oxidizes it to a red chromophore product. A fixed volume (1 mL) of diluted sample and 1 mL of oxidase-peroxidase reagent was added. The tubes were incubated at 35°C for 40 minutes. The reaction was terminated by adding 2 mL of 6 N HCl. The color intensity was read at 540 nm in a spectrophotometer. Glucose estimation was done using enzyme glucose oxidase assay referred to standard graph.

**Cellulose estimation**: Intermittent cellulose estimations were done by withdrawing the samples from the culture vessel and following the procedure as discussed earlier. The pH value was maintained using sterile 2 N HCl and 2 N NaOH. Required DO level was maintained by sparging sterile air through cartridge filter into fermentation medium. The duration of fermentation was eight days. Sample was withdrawn after every 24 hours and analyzed for total viable count, residual glucose concentration, and product yield. Total viable count was performed using standard HS medium with 1.5% agar by pour plate method. Glucose estimation was done using enzyme glucose oxidase assay referring to standard graph.

**Sample preparation for scanning electron micrograph**

Initially the microorganisms were pellet down by centrifugation of the cell suspension for 15 minutes at 10,000 rpm. These pellets were mixed with 5-10 ml of 2.5% glutaraldehyde and kept in freezer at 4°C overnight. Each suspension was centrifuged to pellet down the microorganisms and glutaraldehyde was decanted. Series of alcohol wash and were pelleted down. These pellets were washed with acetone, transferred to Eppendorf tubes, centrifuged and were pelleted down. These Eppendorf tubes were kept cap open in hot air oven at 80°C overnight.

**Results and Discussion**

**Microorganism**

In the previous studies *Acetobacter* strains, including *Acetobacter pasteurianus*, *Acetobacter xylinus*, *Acetobacter aceti*, *Acetobacter liquefaciens*, *Acetobacter hansenii*, and *Acetobacter oxydans* are reported to produce cellulose (Ramana et al., 2000; Park et al., 2003; Jia et al., 2004). Yeo et al. (2004) have isolated cellulose producing strains, including *Gluconacetobacter persimmonis* sp. from Korean traditional Persimmon vinegar. On the basis of 16S rDNA sequences and taxonomic characteristics, they have proposed the strain as *Gluconacetobacter persimmonis* sp. nov. under the type strain KJ 145T. Embuscado et al. (1994) have reported isolation of *Gluconacetobacter xylinus* from actively fermenting *nata* sample and studied the factors affecting BC production.

*Gluconacetobacter persimmonis* GH-2 was isolated from *nata* sample and was observed to form cellulose pellicle. The cellulose formation was purely confined to air-liquid interface with the bottom portion of the medium remaining clear without any turbidity. Figure 2 and Figure 3 present the pellicle formed by the strain under
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Table 2: Biochemical characteristics.

| Tests                     | Strain GH-2 |
|---------------------------|-------------|
| Indole test               | Negative    |
| Methyl red test           | Positive    |
| Voges Proskauer test      | Negative    |
| Citrate utilization       | Negative    |
| Urease                    | Negative    |
| Nitrate reduction         | Negative    |
| Ornithine decarboxylase   | Positive    |
| Lysine decarboxylase      | Negative    |
| Cal-Collase test          | Negative    |
| H₂S production           | Negative    |

Growth in AE broth containing 4% acetic acid and 3% ethanol Negative

Table 1: Morphological characteristics on HS agar.

| Characteristics | Strain GH-2 |
|-----------------|-------------|
| Colony morphology | Strain GH-2 |
| Configuration   | Round       |
| Margin          | Entire      |
| Elevation      | Convex to flat |
| Surface         | Smooth to rough |
| Color           | Pale while |
| Opacity         | Translucent |
| Gram's reaction | Gram negative |
| Cell shape      | Thin long rod |
| Size (μm)       | 1-2 T 3' |
| Spore formation | Negative |
| Motility        | Positive |

Figure 4: Aligned sequence for Gluconacetobacter persimmonis GH-2.

| BLASTN 2.2.18 [Mar-02-2008] | Query=1-12 DNA |
|------------------------------|----------------|
| Score | E |
|-------------------|---|---|
| Gluconacetobacter_entani--v--T--J2531110 | 7533 | 0.0 |
| Gluconacetobacter_persimmonis--v--T--AB095100 | 2478 | 0.0 |
| Gluconacetobacter_kombuchae--v--T--AY684843 | 2478 | 0.0 |
| Gluconacetobacter_hansenii--v--T--AB166736 | 2478 | 0.0 |
| Gluconacetobacter_hansenii--v--N--AB166735 | 2478 | 0.0 |
| Gluconacetobacter_hansenii--v--N--AB166734 | 2478 | 0.0 |
| Gluconacetobacter_maltiacti--v--T--AB166744 | 2470 | 0.0 |

Gluconacetobacter hansenii--v--T--J2531110 2478 0.0
gluconacetobacter_persimmonis--v--N--AB095100 2478 0.0
gluconacetobacter_kombuchae--v--N--AY684843 2478 0.0
gluconacetobacter_hansenii--v--N--AB166736 2478 0.0
gluconacetobacter_hansenii--v--N--AB166735 2478 0.0
gluconacetobacter_hansenii--v--N--AB166734 2478 0.0
gluconacetobacter_maltiacti--v--N--AB166744 2470 0.0

Figure 5: Blast Results for Gluconacetobacter persimmonis GH-2.

Table 3: Effect of different carbon sources on BC production by strain GH-2.

| Sr. No. | Carbon source (2%) | BC yield (g/L) |
|---------|-------------------|----------------|
| 1       | D-glucose         | 5.14           |
| 2       | D-fructose        | 5.96           |
| 3       | Lactic acid       | 3.23           |
| 4       | Sucrose           | 4.62           |
| 5       | Maltose           | 3.04           |
| 6       | Mannitol          | 4.53           |
| 7       | Inositol          | 4.81           |
| 8       | Glycerol          | 2.47           |

Cells were cultured in the standard medium with an initial carbon-source concentration of 2% (w/v or v/v).

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was done for the strain, phylogenetic analysis and blast results showed that the isolate phylogenetic to genus Gluconacetobacter. aligned sequences, blast results and Phylogenetic tree for the strain are shown in Figure 4, Figure 5 and Figure 6 respectively. Referring to Bergey’s Manual of Systematic Bacteriology, G. entani has exclusive requirement for acetic acid and grows well in AE broth containing 4% acetic acid and 3% ethanol. GH-2 was unable to grow in AE broth with 4% acetic acid and 3% ethanol, hence classified as G. persimmonis.

Effect of various carbon and nitrogen sources

To investigate the effect of carbon sources on the production of BC, various carbon sources were provided at 2% (w/v or v/v) instead of glucose in standard medium. Table 3 shows the results for BC production when different carbon sources were provided. The strain effectively utilized glucose, fructose, sucrose, mannitol, and inositol for BC production, giving maximum BC yield with fructose. Effect of different levels of fructose on BC production was investigated and highest BC yield of 6.29 g/L was observed at 3% (w/v) of fructose (Figure 7). Different authors reported use of different carbon and nitrogen sources for BC production. Most preferred carbon sources for BC biosynthesis are glucose and fructose. Yoshinaga et al. (1997) have investigated enzyme activities for cellulose biosynthesis pathway in a new isolate Acetobacter xylinum subsp. sucrofermentans BPR 2001 by using glucose and fructose as carbon sources. They found that, the activity of phosphoglucose isomerase differed, depending on the

stationary conditions of growth and its scanning electron micrograph respectively. Strain GH-2 was found to be gram-negative, non-spore-forming, and motile. After incubation on HS agar medium for 5 days, the colonies were pale white, smooth to rough, concave, opaque, and approximately 3-4 mm in diameter. The strain was biochemically characterized using RAPID Biochemical Test Kit KB 002 and KB 009 (HiMedia, India) according to manufacturer’s instructions. The morphological and biochemical characteristics of the strain are shown in Table 1 and Table 2 respectively. The strain carried out carbohydrate fermentation for lactose, xylose, maltose, fructose, dextrose, galactose, raffinose, trehalose, sucrose, l-arabinose, mannose, inulin, glycerol, inositol, sorbitol, mannitol, adonitol, rhamnose, cellobiose, and xyitol but could not ferment melezitose, ONPG, esculin, citrate and malonate. A 16S rDNA sequence analysis
carbon source, that UDPG pyro-phosphorylase activity was high in the cellulose-producing *Acetobacter*, and that for the uptake of fructose, a phosphotransferase system exists in addition to the hexokinase system. This might have resulted in increased cellulose production for our isolate when grown on fructose. Various nitrogen sources were also screened separately to assess their effects on cellulose production. Among all, beef extract was the best nitrogen source (Table 4). Effect of different levels of beef extract on BC production was similarly investigated. Highest BC yield of 5.89 g/L was observed at 0.6% (w/v) of beef extract (Figure 8). Usually organic nitrogen sources are the preferred ones for effective BC production (Ross et al., 1991; Embuscado et al., 1994; Ramana et al., 2000; Hungund and Gupta, 2010).

**Effect of initial pH and temperature values**

The effects of different temperatures (20-40°C, unit increase of 2°C) were investigated using standard medium. The optimum temperature for cellulose production was found to be 30°C. The effect of initial pH on cellulose production was investigated in the range of 3-9 with unit increase of 0.5. The strain was found to produce cellulose over wide range of pH from 4-7 with optimum at pH 5.5. It is generally accepted that the optimal pH range for cellulose production by *Gluconacetobacter xylinus* is 4-7 (Ross et al., 1991).

**Cellulose production in a fermenter**

As shown in Figure 9, the amount of BC produced in the culture vessel of the fermenter increased rapidly during 2nd, 3rd and 4th days of cultivation. This can be attributed to higher mass transfer of the nutrients and oxygen in the vessel and maintenance of optimum pH and temperature. The glucose concentration in the medium decreased sharply during first two days of cultivation (Figure 10). This decrease could be because of increased cell numbers and cellulose formation. It is generally known that the shear stress generated

| Sr. No. | Nitrogen source (0.5%) | BC yield (g/L) |
|---------|------------------------|---------------|
| 1       | Peptone                | 5.16          |
| 2       | Casein hydrolyzate     | 5.25          |
| 3       | Beef extract           | 5.05          |
| 4       | Maize extract          | 4.83          |
| 5       | Sodium nitrate         | 0.81          |
| 6       | Ammonium chloride      | 0.82          |
| 7       | Ammonium sulfate       | 0.83          |
| 8       | Potassium nitrate      | 0.77          |
| 9       | Ammonium nitrate       | 0.42          |

Table 4: Effect of different nitrogen sources on BC production by strain GH-2.

Cells were cultivated in glucose medium with an initial nitrogen-source concentration of 0.5% (w/v).
in a shaking cultivation causes Acetobacter strains to convert into Cel mutants (Schramm and Hestrin, 1954). During our fermentation trials, formation of Cel mutants was observed by streaking the culture broth onto screening medium. Cel cells fluoresced brightly and Cel cells were markedly darker. The medium became more viscous that, cellulose synthase enzyme responsible for cellulose synthesis in Gluconacetobacter is a typical membrane-anchored protein, having a molecular mass of 400-500 kDa and is tightly bound to the cytoplasmic membrane (Lin and Brown, 1989). Further, this enzyme appears to be a very unstable protein. Various authors proposed that the synthesis of cellulose in Gluconacetobacter occurs between the outer membrane and the cytoplasmic membrane by a cellulose-synthesizing enzyme complex, which is in association with pores at the surface of the bacterium. This fact may be influencing the polymerizing process for cellulose at the surface of bacterial cell. Hence, not all the members of Gluconacetobacter produce BC under agitated culture conditions. Krystynowicz et al. (2005) showed that, Cel mutants lacked two enzymes phosphoglucomutase and UDP glucose phosphorylase, which generate UDP-glucose being the substrate for cellulose synthase. Thus, genetic instability of Gluconacetobacter cells under shaking conditions made developing a stable organism for agitated cultivation, a major research interest in recent years.

**Conclusion**

In conclusion, the new cellulose producing bacterial strain has been identified as *Gluconacetobacter persimmonis*. The strain was observed to produce substantial amounts of BC both under stationary and submerged agitated conditions of growth. Thus, it is one of the rare reports of BC production under aerated agitated conditions. The yield of BC from this strain was increased from 5.14 g/L to 5.89 g/L under stationary conditions of incubation by providing a static cultivation method is inappropriate for the commercial production of BC, as static cultivation is labor intensive and requires long culture period. Hence, an attempt was made to produce cellulose from strain GH-2 under aerated agitated conditions in a fermenter. It is known that, cellulose synthase enzyme responsible for cellulose synthesis in Gluconacetobacter is a typical membrane-anchored protein, having a molecular mass of 400-500 kDa and is tightly bound to the cytoplasmic membrane (Lin and Brown, 1989). Further, this enzyme appears to be a very unstable protein. Various authors proposed that the synthesis of cellulose in Gluconacetobacter occurs between the outer membrane and the cytoplasmic membrane by a cellulose-synthesizing enzyme complex, which is in association with pores at the surface of the bacterium. This fact may be influencing the polymerizing process for cellulose at the surface of bacterial cell. Hence, not all the members of Gluconacetobacter produce BC under agitated culture conditions. Krystynowicz et al. (2005) showed that, Cel mutants lacked two enzymes phosphoglucomutase and UDP glucose phosphorylase, which generate UDP-glucose being the substrate for cellulose synthase. Thus, genetic instability of Gluconacetobacter cells under shaking conditions made developing a stable organism for agitated cultivation, a major research interest in recent years.

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