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Innovative production of bio-cellulose using a cell-free system derived from a single cell line

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ABSTRACT

The current study was intended to produce bio-cellulose through a cell-free system developed by disrupting *Gluconacetobacter hansenii* PJK through bead-beating. Microscopic analysis indicated the complete disruption of cells (2.6×10^7 cells/mL) in 20 min that added 95.12 μg/mL protein, 1.63 mM ATP, and 1.11 mM NADH into the medium. A liquid chromatography mass spectrometry/mass spectrometry linear trap quadrupole (LC–MS/MS LTQ) Orbitrap analysis of cell-lysate confirmed the presence of all key enzymes for bio-cellulose synthesis. Under static conditions at 30 °C, microbial and cell-free systems produced 3.78 and 3.72 g/L cellulose, corresponding to 39.62 and 57.68% yield, respectively after 15 days. The improved yield based on consumed glucose indicated the superiority of cell-free system. Based on current findings and literature, we hypothesized a synthetic pathway for bio-cellulose synthesis in the cell-free system. This approach can overcome some limitations of cellulose-producing cells and offers a wider scope for synthesizing cellulose composites with bactericidal elements through in situ synthesizing approaches.

1. Introduction

Cellulose is the most abundant polysaccharide produced by both plant and microbial cells. Plant cellulose possesses impurities in the form of lignin and hemicelluloses. On the contrary, microbial cellulose represents the purest form that is produced by a class of acetic acid bacteria (Seo et al., 2014; Ul-Islam, Khan, & Park, 2012). The unique structural features and purity bestow microbial cellulose with additional advantages over plant cellulose. Furthermore, it decelerates water evaporation, and possesses high water-holding capacity, hydrophilicity, polyfunctionality, crystallinity, tensile strength, ultrafine fiber network, and moldability into three-dimensional structures (Czaja, Krystynowicz, Bielecki, & Brown, 2006; Ul-Islam et al., 2012). Moreover, microbial cellulose has numerous applications in various industries such as food, paper, acoustic membrane, and pharmacy (Phisalaphong, Suwanmajo, & Tammarate, 2008). Additionally, it has been widely used as a carrier in drug delivery systems and enzyme immobilization, wound-dressing material, and scaffold for tissue engineering (Czaja et al., 2006; Mahmoudi, Hosni, Hamdi, & Srasra, 2015).

Economically feasible production of microbial cellulose has remained a debatable issue since its first report. Its production from a chemically defined media is an expensive approach, thus limiting its applications at a commercial scale. It must be efficiently, yet cost effectively produced in high yields. Therefore, it is of immense importance to investigate alternative, cheaper media for microbial cellulose production. Several studies have reported the utilization of various carbon and nitrogen sources for its production (Kurosumi, Sasaki, Yamashita, & Nakamora, 2009; Park, Park, & Jung, 2003). The issue has been resolved to some extent by utilizing waste materials from industries. However, several other limitations associated with microbial production of cellulose lower the overall yield, thus making the process ineffective for large-scale production. Besides cellulose production, the substrate is utilized as feed, for growth and proliferation, as well as for the release of certain byproducts (Khan & Park, 2008; Park et al., 2003). In addition, the microbial cells are prone to contamination by other microorganisms, since microbial cellulose production is an aerobic process (Park, Hyun, & Jung, 2004). Furthermore, a negative strain formation takes place during shaking cultivation, which prevents the production of cellulose (Jung, Khan, Park, & Chang, 2007). These factors restrict the commercial availability of microbial cellulose, which is required in bulk for several medical and other applications. Therefore, a major goal of the microbial cellulose research

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is to improve the efficacy of the process, and enhance its production. This necessitates the development of a more advanced strategy to overcome the existing limitations, and to fulfill the market demand. In this vein, several strategies have been employed, such as conventional fermentation approaches, genetic engineering strategies, strain improvement approaches, etc. (Kojima, Seto, Tonouchi, Tsuchida, & Yoshinaga, 1997).

A cell-free system can be a possible solution to the limitations encountered by the conventional microbial cellulose production process. The cell-free cellulose can be synthesized in vitro, by conducting the enzymatic reactions involved in the natural biochemical pathway of cellulose production by microbial cells. Such a system would offer several advantages over the whole-cell system, such as extended and continuous production through immobilization, sustainable and effective regeneration of cofactors, and prevention of abnormal accumulation of intermediary metabolites (Khan et al., 2015; Khattak, Ul-Islam, et al., 2014). It can also be operated under controlled conditions, since the absence of microbial cells eliminate the possibility of interaction with the external environment. Moreover, the substrate would be effectively utilized for the desired product formation, rather than for the growth and proliferation of the microbial cells. Several approaches such as mechanical, thermal, and enzymatic cell lysis etc. have been employed for the development of cell-free system that isolate the cellular lysate by rupturing the outer envelope (Khattak, Ullah, et al., 2014). Among these, bead beating is believed to be simple, efficient, robust, economical, and applicable to nearly all kind of cells (Khattak, Ullah, et al., 2014; Griffiths et al., 2006). To date, cell-free systems have been explored for the production of various biofuels and other bioproducts (Khattak, Ullah, et al., 2014; Khattak, Ul-Islam, et al., 2014; Ullah, Khattak, Ul-Islam, Khan, & Park, 2014; Welch & Scopes, 1985). However, it has not been extensively investigated for bio-cellulose production. A preliminary study, reported long back, described a combined system (enzymes and cells) for cellulose production with very brief information on the cellulose features (Colvin, 1957). The current study reports the development of a complete cell-free system derived from a single cell line, through a simple cell-lysing approach. We characterized the system for the presence of all the required enzymes and cofactors, and evaluated it for the synthesis of bio-cellulose. Notably, the cell-free system may provide novel insights into its production mechanism(s), yield improvement, as well as other physico-mechanical properties.

2. Materials and methods

2.1. Microorganism and cell culture

Gluconacetobacter hansenii PJK (KCTC 10505BP) was grown on a basal medium agar plate containing glucose 10 g/L, yeast extract 10 g/L, peptone 7 g/L, acetic acid 1.5 mL/L, succinic acid 0.2 g/L, and agar 20 g/L, dissolved in 1.0 L of distilled water (Ul-Islam et al., 2012). The pH of the medium was adjusted to 5.0 with 1.0 N NaOH. This was then autoclaved for 15 min at 121 °C. A few colonies, from the *G. hansenii* PJK culture plate were inoculated into 100 mL of the basal media broth (pH 5.0) in a 250-mL Erlenmeyer flask, and incubated for 24 h at 30 °C under shaking conditions (150 rpm). The cell density was measured by a Neubauer-improved hemocytometer, and was found to be 4.31×10^6 cells/mL.

2.2. Development of the cell-free system

The cell-free system was developed using bead beating, as reported previously (Jeon, Yi, & Park, 2014). Briefly, a freshly prepared 50 mL culture of *G. hansenii* PJK was taken in a Becton Dickinson (BD) falcon tube, and centrifuged at 3500 rpm for 15 min. The pellet was resuspended in 5 mL of the supernatant to attain a 10× concentrated cell culture. The density of the culture rose to 2.6×10^7 cells/mL. Thereafter, equal volumes of the concentrated cell culture and sterile chilled glass beads (Sigma–Aldrich, G9268) with diameter 425–600 μm were taken in a sterilized glass vial, and vortexed for various time intervals (1, 2.5, 5, 7.5, 10, 12.5, 15, 17.5, and 20 min) to rupture the bacterial cells. Importantly, the samples were incubated on ice at regular intervals of 2.0 min, to avoid thermal denaturation of the proteins. The lysate was then collected using a sterile syringe. The cell-free lysate was passed through a Whatman® microfilter (0.45-μm pore diameter; GE Life Sciences, Pittsburgh, PA, USA) to remove cell debris, as described previously (Khattak, Ul-Islam, et al., 2014).

2.3. Microbiological analysis

The density of the *G. hansenii* PJK cells was measured by the standard Trypan Blue method using a hemocytometer, according to a previously reported procedure (Louis & Siegel, 2011). The colony forming units (CFU) on the basal medium agar plates (which were incubated at 30 °C for 24–36 h) were counted. A fluorescence microscope (JENMED, Germany) was used to examine the cell morphologies before, during, and after bead beating.

2.4. Liquid chromatography–mass spectrometry/mass spectrometry linear trap quadrupole Orbitrap (LC–MS/MS LTQ) analysis

An LC–MS/MS LTQ Orbitrap analysis of the *G. hansenii* PJK cell-free lysate was performed using a linear trap/Orbitrap (LTQ Orbitrap) hybrid mass spectrometer (Thermo Electron Corp., Bremen, Germany), according to a previously reported procedure (Yates, Cociorva, Liao, & Vlad, 2006). The trypsin digested peptide mixture was separated on-line with a Surveyor LC (Thermo Electron, San Jose, CA), using a 100 mm × 0.15 mm C18 column (Microtech Scientific, Orange, CA). The mobile phases A and B, for LC separation, were 0.1% formic acid in deionized water, and acetonitrile, respectively. The chromatography gradient was planned for a linear increase from 3% B to 40% B in 80 min, then to 60% B in 10 min, 95% B in 10 min, and 3% B in 20 min. During the analysis, the flow rate was maintained at 1 μL/min using the 80 min 10–80% acetonitrile/water gradient. Ion transmission into the linear trap and further to the Orbitrap was automatically optimized for maximum ion signal for 150–2000 m/z, using automatic gain control (AGC). The product ion spectra were collected in the information-dependent acquisition (IDA) mode, and they were analyzed by linear trap/Orbitrap (LTQ Orbitrap) hybrid mass spectrometer, using a single microscan lasting 0.3–1 s at 400 m/z. For MS/MS, precursor ions were activated using 25% normalized collision energy at the default activation q of 0.25.

2.5. Database searching

The peptide sequences that matched those in a protein sequence database were identified through a Mascot algorithm (Matrixscience, USA). The database search criteria were taxonomy, *G. hansenii* fixed modification, carboxyamido methylation at cysteine residues, variable modification, oxidation at methionine residues, a maximum allowed missed cleavage of 2, an MS tolerance of ±100 ppm, and an MS/MS tolerance of ±0.8 Da. We considered only those peptides that were obtained from trypsin digests.