Production of Bacterial Cellulose and Cellulase Enzyme using Wastepaper Hydrolysate and Coconut Water as Dual Cheap Carbon Sources

V. Francis John, Dr. Ambili Mechoor, Rahna K. Rathnan and
Dr.P.A. Soloman

Abstract--- *Bacterial cellulose (BC) is a highly crystalline and mechanically stable nanopolymer, which has excellent potential as a material in many novel applications, especially if it can be produced in large amounts from an inexpensive feedstock. Traditionally, BC is produced from expensive culture media, containing glucose as carbon source and other nutrient sources resulting in very high production costs, which limits the use of this material to very high value added applications. The use of cheap carbon and nutrient sources is an interesting strategy to overcome this limitation and therefore to increase the competitiveness of this unique material. Waste papers as well as coconut water, rich source of carbon is used for economical production of BC. In this study, the possibility to combine production of BC and hydrolytic enzymes from waste was investigated. Bacterial cellulose and enzymes were produced through sequential saccharification and fermentations with the filamentous mixed fungal cultures and the bacterium Gluconacetobacter xylinus. The BC yield in paper hydrolysate based medium reached 7.2 g/l and in coconut water medium reached 8.3 g/l after 4 days of cultivation but in medium containing mixture of coconut water and paper hydrolysate the BC yield was increased to 10 g/l. The cellulase enzyme was produced from bacterial cellulose production medium and was found to be 1.02 U/ml. It was shown that waste paper as well as coconut water is a suitable raw material for production of bacterial cellulose and enzymes through sequential fermentation. The concept studied offers efficient utilization of the various waste materials and affords a possibility to combine production of two high value-added materials. (Abstract)*

Keywords--- *Bacterial cellulose, Gluconacetobacter xylinus, Paper saccharification, Coconut water, BC production spent, Mixed fungal cultures, Cellulase production*

I. INTRODUCTION

Production of high value-added materials from renewable cheap waste feedstock is an interesting possibility [1]. One such value added material is bacterial cellulose (BC), which is a nano-structured material produced by various species of bacteria. BC is mainly built up by microfibrils, which are around 2–4 nm in diameter and which in turn build up fibers with an approximate size of less than 100 nm.

Many species of bacteria, such as those in the genera Gluconacetobacter, Agrobacterium, Aerobacter, Azotobacter, Rhizobium, Sarcina, Salmonella, Enterobacter, Escherichia and several species of cyanobacteria are reported to produce extracellular cellulose [2-4]. Bacterial Cellulose has many desirable properties such as high purity (free of lignin and hemicelluloses), high crystallinity, a high degree of polymerization, a nano-structured work, a high wet tensile strength, a high water holding capacity and good biocompatibility [5].

The fine and well-ordered structure of BC offers several advantages when it is used in matrices with other materials, such as low thermal expansion and superior reinforcement [5-6]. Today, BC has reached a wide array of applications, such as health food, cosmetics, pharmaceutical and biomedical products, reinforcement of high-quality papers, diaphragms for electro-acoustic transducers, paint additives, coatings, reinforcement for optically transparent films, and proton-conducting membranes of fuel cells [7].

Bacterial cellulose (BC) has excellent potential as a material in many novel applications, especially if it can be produced in large amounts from an inexpensive feedstock. Traditionally, BC is produced from expensive culture media, containing glucose as carbon source and other nutrient sources resulting in very high production costs, which limits the use of this material to very high value added applications [8-9]. The use of cheap carbon and nutrient sources is an interesting strategy to overcome this limitation and therefore to increase the competitiveness of this unique material.

This project researches the exploration of pollution causing waste materials as cheap source for the production of this biopolymer. An advantage of using agricultural or industrial residual streams as feedstock for production of bacterial cellulose is the low cost of the raw material [9]. Thus the objectives of this were to investigate the appropriateness of paper industry waste for production of BC, and the possibility to combine the production of BC with production of hydrolytic enzymes useful for degradation of lignocellulose.

Cheap media consisting of coconut water and sugar from paper wastes hydrolysate can be used for bacterial polymer production. The coconut water is good source of vitamins and minerals to enhance the bacterial polymer production from

V. Francis John, Dr. Ambili Mechoor, Rahna K. Rathnan, Dept. of Biotechnology, Sahrdaya College of Engineering and Technology, Kodakara, Email id: francisjov@gmail.com

Dr.P.A. Soloman, Dept. of Chemical Engineering, Govt. Engineering College, Thrissur, Email id: pasolomon@gmail.com

ISBN 978-93-82338-83-3

industrial waste. The use of different microorganisms and enzymes for the complete utilization of these industrial wastes will produce biopolymers and enzymes which help in manufacturing of products like bioethanol.

II. MATERIALS AND METHODS

A. Isolation and identification of organism

2 ml of grape juice samples were incubated statically at pH 6.0 and 30ºC for 7 days in 60 ml of Hestrin-Schramm (HS) medium composed of 4.0% D-glucose, 1.0% peptone, 1.0% yeast extract, 0.54% Na_2HPO_4 and 0.23% citric acid [12]. After incubation, the cultures were streaked onto HS-agar plates. The growth of the colonies was observed during incubation at 30ºC for 3 days. White to cream colonies with mucous structure was purified by repeated streaking onto agar plates. 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 over oxidation of acetic acid by formation and disappearance of clear zones around colonies [2]. $CaCO_3$-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. Acid production from fructose, galactose, glucose, lactose, maltose, sucrose and xylose was investigated by using 1% of tested sugar solution as the only source of carbon. Urea broth was used to determine urea utilization. Citrate utilization was tested using citrate broth.

B. Cellulose production

Inoculum was prepared by inoculation of 10 ml test tubes containing HS broth. The tubes were incubated statically at 30°C for one week. Cellulose production was 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. The cellulose pellicles produced by acetic acid bacteria was confirmed by treating the pellicles with 0.5N NaOH. 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 [3]. Cellulose was washed three times with distilled water and dried at 105ºC.

C. Production of cellulase

Enzyme production with Penicillium citrinum SCET 3 was performed by using spent hydrolysates obtained after BC production, and with the addition of 1% (w/v) of the corresponding dried paper waste. Reference medium, which was used as a control, was based on glucose (10 g/L) with 1% (w/v) additional waste paper. Each of a series of 500-mL flasks contained 100 mL spent hydrolysate supplemented with 0.1% (w/v) tryptone, 0.05% citric acid, 2% Vogel's media [11], and 0.015% Tween 80. The flasks containing the media were autoclaved at 110°C for 30 min. The flasks were then inoculated with 5% (v/v) of a suspension of P.citrinum SCET 3 spores from a culture with glucose-based reference medium that was pre-grown at 30°C for 36 h. The cultivations were carried out at 28°C and 160 rpm for the following 15 days.

D. Enzyme activity assay

The cellulase activity of each culture was measured by determining the amount of reducing sugars liberated by using a dinitrosalicylic acid (DNS) method. One unit of FP-ase was defined as the amount of enzyme, which released µmole of reducing sugar measured as glucose per min under the assay conditions.

E. Filter paper assay

Filter paper assay (FPase) for total cellulase activity in the culture filtrate was determined according to the standard method. 0.5ml of culture filtrate as enzyme source was added to Whatman No. 1 filter paper strip (1 x 6 cm; 50 mg) immersed in 1ml of 0.5 M Sodium citrate buffer of pH 5.0. After incubation at 50 ^{0} C for 1h, the reducing sugar released was estimated by dinitrosalicylic acid (DNS) method [13]. One unit of filter paper (FPU) activity was defined as the amount of enzyme releasing 1 µmole of reducing sugar from filter paper per ml per min.

III. RESULT AND DISCUSSION

The isolation of Gluconobacter from grape juice was done on selective media containing ethanol. Only Gluconobacter will grow in this media. 15 white to cream colonies with mucous structure were observed after incubation and they were purified by repeated streaking on to agar plates.

Three Gram negative rod shaped bacteria were observed when viewed under light microscopy. They were named as SCET 4, SECT 7 and SCET 8. These Gram negative cultures were transferred onto $CaCO_3$-agar plates for visualization of acid production. The isolate formed $CaCO_3$ clear zones. The isolates SCET 7 had given maximum clear zone. These colonies were selected as acetic acid bacteria and were subjected to further analysis. The biochemical properties of the isolates capable of cellulose production and reference strains are summarized in Table 1.

Table.1 Biochemical characteristic of bacterial isolate SCET 7

Characteristics	MTCC Culture	Isolate
Motility	+	+
Catalase	+	+
Indole	–	–
Cellulose production	+	+
Oxidation of acetate	+	+
Citrate	+	+
Acid production from		
D-glucose	+	+
Sucrose	+	+
Fructose	–	–
Lactose	–	–
Galactose	+	+

ISBN 978-93-82338-83-3 © 2013 Bonfring

Maltose		–		–
Mannose	+		+	
Xylose		+		+

The bacterial cellulose production by isolates SCET 7 and MTCC in production media was compared. The turbidity of the culture broth increased within hours of inoculation and a cellulosic film was obtained on the air-liquid interface after the 4th day of incubation in production media inoculated with SCET 7 and MTCC cultures (Figure 1). Incubation was lasted for a week and cellulose production was monitored. The pH value of the media decreased during the cultivations, from around pH 5 to around pH 3.

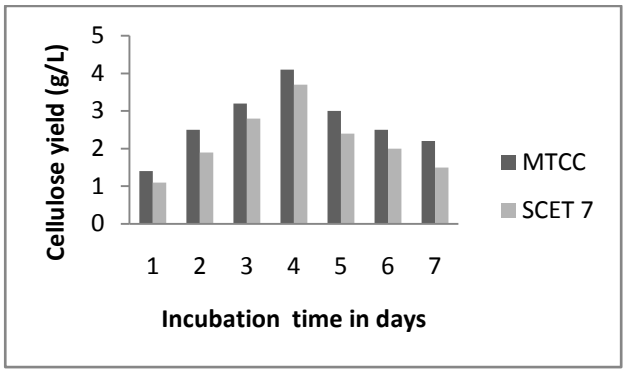

Figure 1: Comparative Study of Bacterial Cellulose Production by MTCC and SCET 7 Isolates on Cellulose Production Medium

The isolated bacterial pellet was confirmed by treating with cellulase enzyme as well as by NaOH treatment. The bacterial cellulose was used as substrate for cellulase enzyme. Bacterial cellulose and enzyme was mixed with acetate or citrate buffer and incubated at 50^0C for 1 hr. After incubation the reaction was stopped by heating for 5 min and DNS assay was carried out. The DNS assay has given positive result for reducing sugar. In this way the presence of bacterial cellulose in production media was confirmed.

The amount of sugar present in the natural carbon sources was estimated by DNS method. Carbohydrate analysis of paper hydrolysate and coconut water showed presence of 7.5 and 8.3% of total sugar respectively. Figure 2 shows the bacterial cellulose production from various natural carbon sources provided at 2% in HS medium with 2.0% peptone, 0.5% yeast extract, 0.115% citric acid, adjusted to pH 6. After 7 days of incubation the cellulose yield was calculated. The BC yield in paper hydrolysate based medium reached 7.2 g/l and in coconut water medium reached 8.3 g/l after 4 days of cultivation but in medium containing 2:2 mixture of coconut water and paper hydrolysate the BC yield was increased to 10g/l (Figure 2). In another study, BC production was undertaken from various fruit juices including orange, pineapple, apple, Japanese pear, and grape [10].

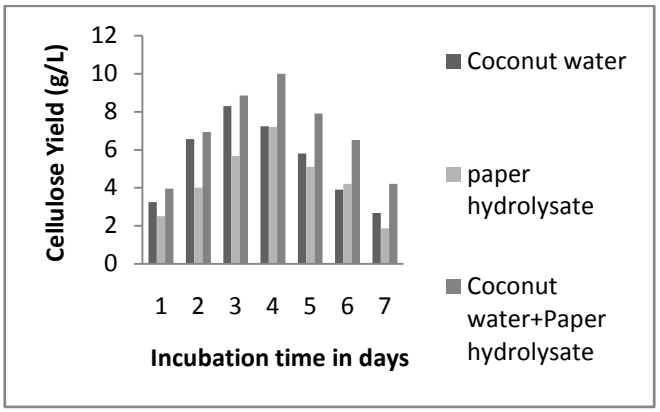

Figure 2: Bacterial Cellulose Production using Cheap Carbon Source using SCET 7 Isolates

The cellulase enzyme was produced from spent Bacterial cellulose production medium and was found to be 1.02 U/ml. Coconut water is rich in carbohydrates, proteins, and trace elements; they can be used as good nutrients for the production of food grade bacterial cellulose. Use of these cheap substances should provide economical sources of nutrients for the production of bacterial cellulose.

ACKNOWLEDGMENT

We express our sincere gratitude to Dr. Mary Thomas, Head of Chemical Engineering Deparment ,Govt. Engineering college Thrissur and management of Sahrdaya College of Engineering and Technology,Kodakara for providing all the facilities in completing this work.

REFERENCES

[1] Napoli C, Dazzo F, Hubell D (1975) Production of cellulose microfibrils by Rhizobium. Appl Microbiol 30: 123-131.
[2] Ross P, Mayer R, Benzimann M (1991) Cellulose biosynthesis and functions in bacteria. Microbiol Mol Biol Rev 55: 35-58.
[3] Hungund BS, Gupta SG (2010) Production of bacterial cellulose from Enterobacter amnigenus GH-1 isolated from rotten apple. World J Microbiol Biotechnol 26: 1823-1828.
[4] Yoshinaga F, Tonouchi N, Watanabe K (1997) Research progress in production of bacterial cellulose by aeration and agitation culture and its application as new industrial material. Biosci Biotechnol Biochem 61: 219-224.
[5] Shibazaki H, Kuga S, Onabe F, Usuda M (1993) Bacterial cellulose membrane as a separation medium. J Appl Polym Sci 50: 965-969.
[6] Klemm D, Schumann D, Udhardt U, Marsch S (2001) Bacterial synthesized cellulose artificial blood vessels for microsurgery. Prog Polym Sci 26: 1561- 1603.
[7] Thomson DN, Hamilton MA (2001) Production of bacterial cellulose from alternate feedstocks. Appl Biochem Biotechnol 93: 503-513
[8] Kurosumi A, Sasaki C, Yamashita Y, Nakamura Y (2009) Utilization of various fruit juices as carbon sources for production of bacterial cellulose by Acetobacter xylinum NBRC 13693. Carbohydrate Polymers 76: 333-335.
[9] Hungund BS, Gupta SG (2010) Improved production of bacterial cellulose from Gluconacetobacter persimmonis GH-2. Journal of Microbial & Biochemical Technology 2: 127.
[10] Embuscado ME, Marks JS, BeMiller JN (1994) Bacterial cellulose. I. Factors affecting the production of cellulose by Acetobacter xylinum. Food Hydrocoll 8: 407-418.
[11] Dreywood R (1946) Qualitative test for carbohydrate material. Ind Eng ChemAnal Ed 18: 499.
[12] T. K. Ghose.(1987). “Measurement of cellulase activities,” Pure Applied Chemistry, 59.:257–268.

ISBN 978-93-82338-83-3 © 2013 Bonfring