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Production of Bacterial Cellulose from Coconut Liquid Endosperm using *Acetobacter xylinum* sju-1

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Bacterial cellulose (BC) or *nata* is produced by the Gram negative bacterium *Acetobacter xylinum* at the air liquid interface of sugary rich liquid medium. In the present investigation, BC was produced from Hestrin Schramn (HS) medium and Modified Coconut Liquid Endosperm (MCLE) medium using the efficient cellulose producing isolate of sugarcane juice (sju-1) under static batch fermentation condition. The standard culture *Acetobacter xylinum* NCIM was also compared for the production of BC. Results showed that sju-1 produced BC of 14.10 and 15.40 g/l in HS medium and MCLE medium respectively after 14 days of fermentation period. NCIM 2526 produced only 11.10 and 9.80 g/ l of cellulose. The biosynthetic yield of cellulose was more in MCLE medium when compared to the HS medium and proved to be highly significant. BC obtained was highly juicy and rich in crude fibre. Water holding capacity of BC ranged from 84.44 % to 87.80% and the crude fibre content in the range 10.74 to 11.25 g. The results clearly confirmed that the coconut liquid endosperm medium could effectively be used as potential alternative to the glucose normally used in the media of BC production and the *Acetobacter xylinum* sju-1 isolate produced bacterial cellulose in quantities of commercial interest.

Key words: Bacterial Cellulose, modified coconut liquid endosperm medium, *Acetobacter xylinum* sju-1.

Bacterial cellulose (BC) produced by *Gluconacetobacter xylinum* (formerly called as *Acetobacter xylinum)* at the air-liquid interface of sugary rich medium is popularly known as *nata-de-coco*. *Nata* is derived from Latin word *nature* which means to float from fermenting liquid substrates. *A. xylinum* uses the nutrients in the medium, forms a thin slimy, transparent layer of cellulose on the surface of the medium, which thickens with age forming a thick whitish sheet after 15–20 days of fermentation under static condition. This thick sheet of cellulose is value added by cutting into cubes, washing and boiling in water and cooked in sugar syrup. *Nata* so formed is more than 90% water imbibed in cellulose and finds use in desserts, fruit cocktails and fruit jellies. It is an organic high dietary fiber food product, high in cellulose, low in fat and calories and contains no cholesterol. The cellulose is recognized by the FDA as edible and the *Gluconacetobacter* is a non-pathogenic cellulose-producing food grade bacterium (Kersters *et al*. 2006). Moreover bacterial cellulose has significant advantages over plant cellulose. Bacterial cellulose fibrils are highly amorphous and generated as a never-dried membrane in a nearly pure form without lignin and hemicelluloses as that of plant cellulose. It has more than 200 times greater surface area than isolated softwood cellulose and has a tensile strength. Many potential high value markets exist for thin film bacterial cellulose, including acoustic diaphragms, artificial skin, pulp and paper industry

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artificial blood vessels, liquid loaded medical pads, super-sorbers and specialty membranes. The production of BC is receiving great attention because of its wide application possibilities (Keshk and Sameshima 2006).

In microbial fermentations, the cost of the fermentation medium can account for almost 30% of the total cost. Medium costs limit commercial use of bacterial cellulose or *nata*. So use of alternative substrates is one way to reduce the cost of production of bacterial cellulose. Development of a cost-effective culture medium to obtain maximum product yield will solve this problem. Most of the studies on BC production by Acetobacter strains have been carried out in media containing pure sugar as carbon source, such as glucose, sucrose, fructose, mannitol, and arabitol (Jung *et al*. 2010). An alternative sugary nutrient rich substrate that can be used for fermentation is mature coconut water. Mature coconut water botanically called as coconut liquid endosperm is thrown away during copra making and becomes a significant waste. Coconut water from mature nuts is abundantly available at desiccated coconut factories, coconut milk/cream factories, and large copra processing centers. On the basis of about 140 ml of water per nut, a desiccated coconut factory utilizing 400,000 nuts per day, would have about 56,000 litres of coconut water daily. This is currently wasted, as the constituents of mature coconut water appear to be too diluted for any large-scale commercial use. In fact, coconut water from these factories has created disposal problems.

Mature coconut water has been traditionally used in South East Asian countries, but an effective media has not been formulated for BC production. In India, Coconut water is highly underutilised and discarded as waste from coconut processing industries and hotels generating ecological problems leading to an increase in the BOD to 30000 mg. In the present study an attempt has been made to produce BC using mature coconut water. The main objective of this study is to develop a simple, relatively inexpensive, cost effective MCLE medium supplemented with yeast extract from efficient BC producing bacterial strain isolated from fermented sugarcane juice (sju-1) and compare with the reference strain *Acetobacter xylinum* NCIM 2526.

MATERIALS AND METHODS

The present investigation on the production of cellulose from coconut liquid endosperm using *Acetobacter xylinum* sju-1 was conducted at the Department of Agricultural Microbiology, Tamil Nadu Agricultural University (TNAU), Coimbatore, Tamilnadu during 2011 – 2012. *A. xylinum* was isolated from different fruits, fruit juices, sugarcane juice, flower nectar, palm sap/ neera etc., collected from the local market and university orchard. Approximately 1.0 g of fermenting portion or 1.0 ml of fermenting juice was aseptically transferred in test tubes containing 10 ml of sterilized Hestrin Schramn (HS) medium containing glucose 20 g, yeast extract 5 g, peptone 5 g, citric acid 2.7 g and disodium hydrogen phosphate 1.17 g and incubated at 30° C for 14 days. Formation of fleshy brownish mat like pellicle that is insoluble in water on the surface of the medium confirms the presence of bacterial cellulose/ *nata* producing strains in the tubes. Further phenotypic characterization was performed for the identification of the organism. Among the various isolates, the best cellulose producing isolate was screened and selected. Based on the thickness of the pellicle formed on the surface of the HS medium, the isolates were grouped as poor (1mm thickness), fair (2 mm thickness), good (4mm thickness), excellent (5 mm thickness and above) (Aydin and Aksoy, 2009). Isolates with 5 mm thickness were selected for further study. The best isolate was biochemically and morphologically characterized by following the methods given by Swings (1992).The screened isolate sju-1 was grown in HS medium and used as inoculum for BC production. The reference culture, *A. xylinum* NCIM 2526 was purchased from National Collection of Industrial Microorganisms (NCIM), Pune, India to compare the efficiency of sju-1.

Mature coconut water from coconut processing industries and hotels were collected and filtered using muslin cloth. The Total Soluble Sugar (TSS) of the coconut water was analyzed using hand refractometer and the TSS was found to be 4° Brix. To 1.0 L of coconut liquid endosperm, sucrose (~ 0.6 %) was added to bring the TSS into 10° Brix. To this 0.5 percent of yeast extract was added as nitrogen source for the growth of the microorganism, pH of the medium was adjusted to

6.0 using glacial acetic acid. Then the mixture was sterilized by autoclaving. Then it was cooled and inoculated with 10 percent of *A. xylinum* sju-1 culture inoculum. Similarly NCIM 2526 was also inoculated separately in the above said medium to compare the cellulose producing pattern of the two strains sju-1 and NCIM 2526 respectively. The inoculated media was incubated at 30°C for 14 days at static fermentation condition without disturbance. A thick layer of surface pellicle obtained from different treatments was aseptically transferred into trays, washed scrupulously with tap water and boiled for 15 min to remove the acid flavour and to kill the live bacteria.

The cleaned pellicle was subjected to physicochemical property analysis. The physical properties like weight of the cellulose (wet weight and dry weight), moisture content, Water Holding Capacity (WHC), pH and thickness were analyzed. The yield of the biosynthesis process (Y %) was calculated as Y=C/Gx100 in %, where: C is the weight of dry film in g, G is the weight of carbon source in sub-strate in g. The fresh cellulosic pellicle formed was lifted and after several washing, the excess water was dripped off completely and weighed for measuring the fresh weight (g). The moisture content (%) was determined based on the weight loss of the cut cubes when dried under vacuum for 8 hr at 25 bar pressure at 75°C. A known quantity of BC was homogenised using a homogenizer at 10000 rpm and the pH was analyzed using a digital pH meter. The thickness (mm) was measured using a digital caliper. Cellulosic mat after cutting into perfect cubes of equal dimensions was wrapped in filter paper and centrifuged at 5000g for 10 min. During centrifugation the water released was absorbed by the filter paper. The per cent ratio of the moisture in the centrifuged cellulose to the original moisture content yielded the WHC of the BC (Bielecki *et al*. 2005). The chemical properties like reducing sugar, TSS, Total Titrable Acidity (TTA), Crude fibre, Potassium, Sodium and Iron were analyzed as per the procedures given by AOAC (2002). The morphological investigations of the bacterial cells on the cellulose and the cellulose fibrils were characterized using Scanning Electron microscope (SEM) (Model: S-3400 HITACH Co., Japan). Thin layers of freeze dried cellulose were gold coated using ion sputter (Fisons Instruments, UK). The gold coated sample was viewed and photographed using SEM. Three Factorial ANOVA was performed to compare the effects of culture (t), medium (m) and their interaction effects (tm) according to the procedures given by Panse and Sukhatme (1985). Critical differences were worked out at 5% probability level and presented. The values were expressed as means of three replicate determinations.

RESULTS AND DISCUSSION

Isolation and screening of *Acetobacter xylinum* for BC production

The isolate of fermented sugarcane juice (sju-1) developed cellulose of 12.0 mm thickness and selected as the excellent strain for BC production. Among the various isolates from different sources, *A. xylinum* isolated from fermented sugarcane juice was found to be an excellent producer of cellulose and was screened as the best isolate for the production of BC. The phenotypic characterization has revealed that it was Gram negative, non spore forming bacteria occurring singly or in pairs (Table 1). Formation of surface pellicle by *A. xylinum* sju-1 in the HS medium is illustrated in Fig 1 and Fig 2, shows the purified colonies of *A. xylinum* sju-1.

Production of Bacterial Cellulose

A thick layer of cellulosic mat was formed on the surface of the HS medium and MCLE medium by the two strains namely sju-1 and NCIM 2526. The real image of washed, boiled cellulose pellicle obtained from MCLE medium is depicted in Fig 3. *A. xylinum* sju-1 isolate produced 14.1 g/l and 15.14 g/l of cellulose with thickness accounting to 13 mm and 15 mm in HS medium and MCLE medium respectively. The sju-1 isolate produced more quantities of cellulose than NCIM 2526 which produced only 11.40 and 9.80 g/ l of cellulose in two of the media respectively (Table 2). Cellulose produced by sju-1 isolate was found to be highly significant at $p<0.05$ when compared to the cellulose produced by *A. xylinum* NCIM 2526. Earlier reports of cellulose production using NCIM 2526 using tender coconut water was found to produce only 9.5mm thickness of cellulose (Jagannath *et al*. 2008) which was lesser than the thickness of the pellicle formed by sju-1 isolate. Glucose, sucrose, fructose are the most preferred carbon sources for cellulose production. Since

coconut water being rich in all of these sugars, major and minor nutrients have greatly influenced the production of bacterial cellulose. The thickness of the cellulose pellicle formed from different treatments was found to be statistically insignificant but the biosynthetic yield was highly significant with 151.40% in the case of sju-1 isolate in MCLE medium. The results clearly indicate that sju-1 in MCLE medium has got high conversion rate of the carbon source present in the medium. The moisture content of the cellulose produced by sju-1 was 92.90% and 92.27 % in HS and MCLE medium respectively whereas cellulose from NCIM 2526 recorded a slightly higher moisture per cent of about 92.89 and 94.40 in HS and MCLE medium respectively. pH of the fermenting medium

Table 1. Phenotypic characteristics of the cellulose producing isolate sju – 1

Morphological characters	
Colonies	Circular, convex, smooth brownish white opaque colonies with entire margin
Cell Shape	Short rods
Arrangement	Singly and in pairs
Gram reaction	Negative
Spore	Negative
Biochemical tests	
Reaction to litmus milk	Negative
Indole production	Negative
Nitrate reduction	Negative
Gelatin hydrolysis	Negative
Catalase reaction	Negative
Growth in Hoyer's medium	Negative
Dehydroxyacetone from glycerol	Positive
Oxidation of ethanol to acetic acid	Positive
Production of water insoluble surface pellicle	Positive
Identification of isolate	*Acetobacter xylinum* sju-1

Table 2. Physicochemical properties of bacterial cellulose

Physicochemical characteristics	*A. xylinum* sju - 1		*A. xylinum* NCIM 2526	
	HS medium	MCLE medium	HS medium	MCLE medium
Wet weight of cellulose(g/L)	180.00±0.82	171.12±0.21	160.50±0.44	175.00±0.13
Dry weight of cellulose(g/L)	14.10±0.26	15.14±0.17	11.40±0.15	9.80±0.15
Moisture (%)	92.27±0.05	92.90±0.44	92.89±0.29	94.40±0.06
pH (final)	4.00±0.16	4.50±0.07	3.00±0.07	3.50±0.02
Thickness (mm)	13.00±0.14	15.00±0.01	9.00±0.01	12.00±0.00
Water Holding Capacity (%)	84.44±0.25	87.14±0.13	85.50±0.12	87.80±0.13
Biosynthetic yield (%)	70.05±0.27	151.40±0.14	57.00±0.03	98.00±0.08
Total Soluble Sugars (TSS) (ÚBrix)	2.00±0.15	1.00±0.33	3.00±0.07	3.00±0.05
Total Titrable Acidity (TTA) (%)	2.00±0.15	3.50±0.08	3.39±0.01	3.25±0.06
Crude fibre (g)	11.25±0.22	10.74±0.04	11.00±0.04	10.78±0.15
Sodium (mg)	45.00± 0.08	41.00±0.09	42.25±0.03	49.60±0.33
Potassium (mg)	245.00±1.50	241.50±0.02	243.14±0.02	234.12±0.15
Iron (mg)	0.08±0.01	0.07±0.01	0.14±0.00	0.12±0.00
	t	m	tm	
SED	0.211	0.117	0.422	
CD@5 %	0.418	0.232	0.837	

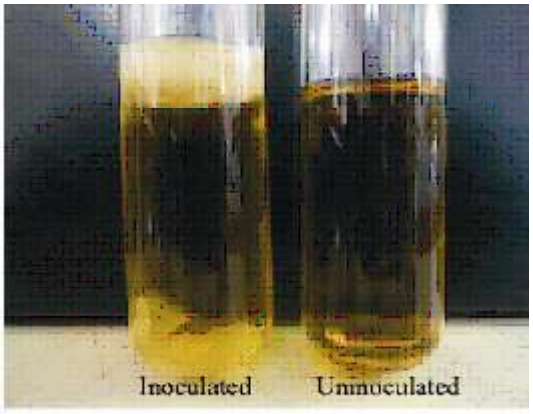

Fig. 1. Formation of cellulose pellicle on the surface of HS medium

Fig. 2. Purified colonies of *Acetobacter xylinum* sju-1

Fig. 3. Real image of cellulose pellicle obtained from MCLE medium

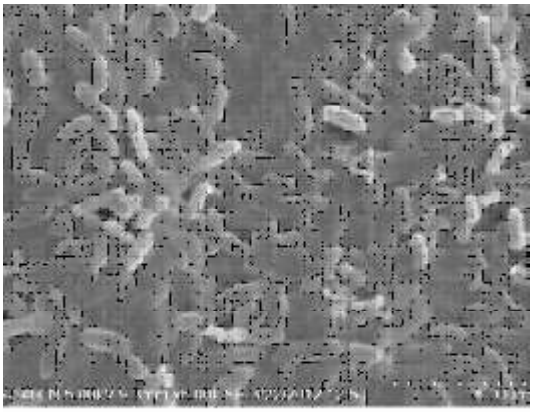

Fig. 4(a). *Acetobacter xylinum* cells on the surface of the cellulose pellicle

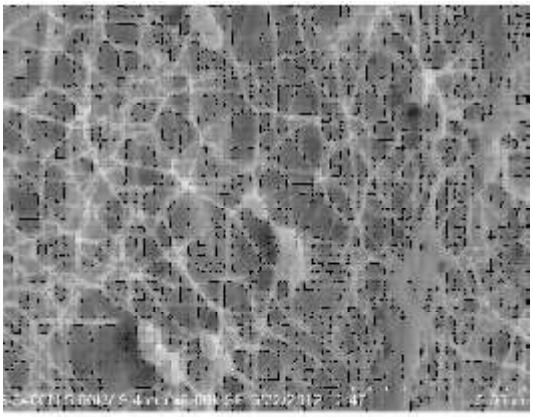

Fig. 4(b). Cellulose matrix formed by *A. xylinum* sju – 1

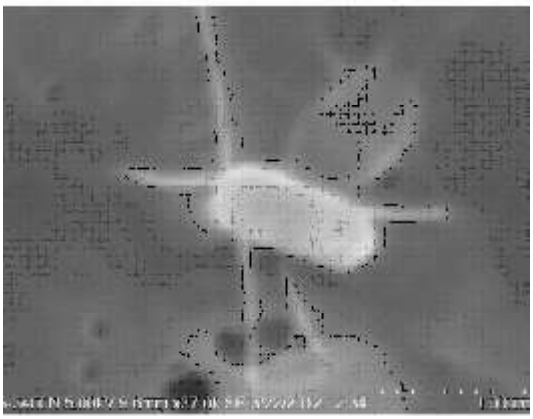

Fig. 4(c). Individual cell *of A. xylinum* sju-1 producing cellulose fibrils

Fig. 4. SEM micrographs of *Acetobacter xylinum* sju-1 cells and cellulose microfibrils

decreased from 6.0 to 4.0 by sju-1 whereas the drop in pH from 6.00 to 3.00 and 3.50 was recorded by the NCIM 2526. The drop in pH might be due to the production of acetic acid and gluconic acid, and the results clearly describes that NCIM 2526 has produced more organic acids than sju-1. Coban and Biyik (2011) has indicated that cellulose and acid production are negatively correlated. If gluconic acid production is greater the yield of cellulose is reduced. The results obtained from the present study indicate that sju-1 has produced lesser acids in comparison to the NCIM 2526. TSS has dropped from 10°Brix to 1°Brix in the MCLE medium by the sju-1 isolate whereas NCIM 2526 has recorded the TSS drop of 3°Brix which clearly indicates that the isolate sju-1 has efficiently converted the sugars present in the medium into cellulose pellicle. The TTA was comparatively lower of 2.00% in cellulose produced in HS medium by sju-1 isolate. The Water Holding Capacity (WHC) of 87.14% has very well indicated that its highly juicy in nature. The BC produced in this experiment has got higher WHC and naturally possess the characteristics of a juicy product and makes it highly suitable for food purposes especially in salads.

The crude fibre content was in the range of 10.74 g and11.25 g. Fibre content of BC clearly elucidates that the cellulose provides a good fibre rich product. According to Mesomya *et al*. (2006) *nata-de-coco* with crude fibre of 9.20 g was an organic dietary food supplement used to control serum triglycerides in hyperlipidimic patients. The results of crude fibre content of BC obtained from the current study was

1.5 to 2.0% higher than the earlier studies indicating that BC produced by sju-1 isolate was more fibrous. With respect to the mineral content, potassium was found to be in higher quantities in bacterial cellulose with 245.00 mg in HS medium by sju-1 isolate and 243.14 mg in HS medium by NCIM 2526.

Scanning Electron Microscopy

Fig 4, shows the SEM micrographs of the cellulosic pellicle formed on the MCLE medium. Fig 4a displays the short rods of *A. xylinum* occurring singly on the cellulosic fibrils, fig 4b shows the thread like cellulosic microfibrils and the bacterial cells entangled in it. Fig 4c depicts a single cell producing the cellulosic fibrils from outside of the cell membrane at 6K magnification.

This study has showed that bacterial cellulose can be derived from mature coconut water supplemented with 0.6% sucrose and 0.5 % yeast extract instead of 2.0% pure glucose, 0.5 % of yeast extract and 0.5 % peptone used in HS medium. And also the efficient cellulose producing isolate has been proved to yield cellulose in quantities of commercial interest. The present study investigated an extended approach to prepare a low cost medium using coconut liquid endosperm which is discharged as waste from coconut processing industries. Coconut water as cheap source of carbon in the fermentation media proves to be a cost effective substrate for bacterial cellulose production. Bacterial cellulose has broad prospective applications. Due to high levels of organic dietary fibre, BC/ *nata* is gaining popularity. Many health benefits like prevention of colon cancer, heart attack, and increase in blood pressure or hypertension have been attributed to *nata*. The structural and textural properties of cellulose in terms of water holding capacity and hardness have additional applications in the field of industrial immobilization and as hydrogels in dryland agriculture also.

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