BIOCONVERSION AND VALUE ADDITION OF MATURE COCONUT WATER AND CASHEW APPLE JUICE INTO BACTERIAL CELLULOSE USING *GLUCONACETOBACTER* sp.

By

G. GAYATHRY, M.Sc. (Agri.)
ID. No. 10-606-102

DEPARTMENT OF AGRICULTURAL MICROBIOLOGY
DIRECTORATE OF NATURAL RESOURCE MANAGEMENT
TAMIL NADU AGRICULTURAL UNIVERSITY
COIMBATORE - 641 003

2013

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Thesis submitted in part fulfilment of the requirements for the award of the Degree of ***Doctor of Philosophy (Agriculture) in Agricultural Microbiology***
to the Tamil Nadu Agricultural University, Coimbatore

By

G. GAYATHRY, M. Sc. (Agri.)
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2013

CERTIFICATE

This is to certify that the thesis entitled **"Bioconversion and value addition of mature coconut water and cashew apple juice into bacterial cellulose using *Gluconacetobacter* sp. "** submitted in partial fulfilment of the requirements for the award of the degree of **DOCTOR OF PHILOSOPHY IN AGRICULTURAL MICROBIOLOGY** to the Tamil Nadu Agricultural University, Coimbatore is a record of bonafide research work carried out by **Mrs. G. GAYATHRY** under my supervision and guidance and that no part of the thesis has been submitted for the award of any degree, diploma, fellowship or other similar titles. However, a part of the thesis work has been published in peer reviewed scientific journal of national/ international repute (copy enclosed).

Place : Coimbatore
Date :

Dr. G. GOPALASWAMY
Chairman

Approved By

Chairman: **Dr. G. GOPALASWAMY**

Members: **Dr. R. MURUGESAN**

Dr. R.VISVANATHAN

Dr. D. MALATHI

External Examiner
Date:

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(G. Gayathry)

LIST OF ABBREVIATIONS

^{o} C	:	Degree Celsius
µg	:	Microgram
eV	:	Electron Volt
µ	:	Micron
(%)	:	Percentage
lbs	:	Ponds in Seconds
h	:	Hours
ha	:	Hectare
mg	:	Milligram
min	:	Minutes
sec	:	Seconds
ml	:	Millilitre
l	:	litre
rpm	:	Revolutions per minute
cm	:	Centi meter
kg	:	Kilogram
g	:	Gram
nm	:	nanometer
N	:	Normality, Newton
M	:	Molarity
bp	:	basepairs
kDa	:	Kilo Daltons
GPa	:	Giga Pascals
MPa	:	Mega Pascals
ppm	:	Parts per million
mmol	:	milli mloles
g l^{-1}	:	Gram per litre
SI	:	Similarity Index
sq.in	:	Square inch
g^{-1}	:	per Gram
GCMS	:	Gas Chromotography Mass Spectroscopy
NIST	:	National Institute of Standards and Technology
CD	:	Critical difference
SEd	:	Standard error of deviation

pH	:	Negative logarithm of hydrogen ion concentration
EDTA	:	Ethylene diamine tetra acetic acid
RSM	:	Response surface methodology
CCD	:	Central composite design
ANOVA	:	Analysis of variance
cfu	:	Colony forming unit
α	:	alpha
v/v	:	Volume by volume
w/v	:	Weight by volume
BC	:	Bacterial Cellulose
DSC	:	Difrential Scanning Colorimeter
TGA	:	Thermogravimetric Analyser
SEM	:	Scanning Electron Microscopy
FTIR	:	Fourier Transforming Infra Red
MCW	:	Mature Coconut water
CAJ	:	Cashew Apple Juice
HS	:	Hestrin Shramn
GYC	:	Glucose Yeast extract Calcium carbonate
GYP	:	Glucose Yeast extract Peptone

ABSTRACT

BIOCONVERSION AND VALUE ADDITION OF MATURE COCONUT WATER AND CASHEW APPLE JUICE INTO BACTERIAL CELLULOSE USING *GLUCONACETOBACTER* SP.

By

G. GAYATHRY

Degree : Doctor of Philosophy in Agricultural Microbiology

Chairman : Dr. G. GOPALASWAMY

Deputy Registrar (Education),

Tamil Nadu Agricultural University

Coimbatore - 641 003.

2013

Bacterial cellulose is a good alternative to plant cellulose. It can be obtained in higher purity without any impurities such as hemicellulose, pectin and lignin. The unique gel like properties of microbial cellulose, combined with its complete indigestibility in the human intestinal tract, make this as an attractive food base.

In the present study, bacterial cellulose (BC) was produced using *Gluconacetobacter* sp. isolated from fermented sugarcane juice and designated as sju-1. The 16SrRNA sequence analysis of the isolate showed 99.02 per cent similarity to *Gluconacetobacter intermedius.* Scanning Electron Microscopy (SEM) revealed the variation in the structural properties of the cellulose fibrils with a size ranging between 128 and 207 nm. Similarly, Fourier Transform Infra Red Spectroscopy (FTIR) of cellulose microfibrils registered the transmittance peak at 3229 cm^{-1} and 2911 cm^{-1}, showing the presence of OH and CH_2 groups indicating a pure form of cellulose type Iα in the BC. The thermograms obtained from Thermo Gravimetric Analyser (TGA) and Differential Scanning Colorimeter (DSC) showed the thermal resistance of BC from *Gluconacetobacter* sp. sju-1, by registering degradation or the decomposition temperature more than 300°C.

The primary aim of the present investigation is to utilize the two sugar rich, edible bio-wastes namely Mature Coconut Water (MCW) and Cashew Apple Juice (CAJ) for

bacterial cellulose production and to develop value added products namely reinforced paper, *nata* and vinegar from BC. With this view, the effect of various process parameters and media components were optimized in the Hestrin Scramn (HS) medium. The optimum incubation time, pH, temperature, carbon, nitrogen and growth hormones for maximum cellulose production were found to be 20 days (480 h), 6.5, 31°C, fructose, yeast extract and indole acetic acid respectively. With regard to aeration or fermentation condition, dry weight of BC was 14.92±0.30 g l^{-1} with a biosynthetic yield of 74.7±0.32 per cent under static culture condition. But shaken culture recorded only 9.63±0.20 g l^{-1} dry weight of BC and the biosynthetic yield was 48.15±0.98. In general, the moisture content of BC was above 90 per cent and for making paper, BC has to be reinforced with polysaccharide additives to achieve high tensile strength paper. In this study, the HS medium was optimized by incorporating various polysaccharide additives, among which calcium alginate and lignosulfonate improved the dry weight of BC to 17.8±0.38 and 17.8±0.41 g l^{-1} respectively. From the results of texture analysis, the hardness of calcium alginate incorporated BC was 45 N which makes it highly amenable for high quality paper production rather than for edible purpose. So, with the pulp of BC obtained from calcium alginate incorporated HS medium, reinforced paper was produced with modular paper making machine at Sheshasayee Paper Boards, Erode, Tamilnadu, India. The viscosity and degree of polymerization of BC pulp were127.4 ±0.29 cP and 2074±2.91 respectively. The tensile strength and Young's modulus of BC reinforced paper was recorded to be 120 ± 2.89 MPa and 4.9 ± 0.01 GPa respectively.

Further, the use of MCW and CAJ as a basal medium for bacterial cellulose production for development of an edible dietary fibre rich product *nata*, was pretested to optimize the low and high levels of C, N and buffer sources. Media optimization was carried out to find the interaction effect of each of the individual components using a statistical based Response Surface Methodology (RSM). The model was found to be highly significant with a predicted R^2 values for the dry weight of cellulose of 0.9490 for MCW medium and 0.9417 of CAJ medium respectively. With MCW as the basal medium, the concentration of fructose (20 g l^{-1}), yeast extract (10 g l^{-1}), citric acid (1.5 g l^{-1}) and disodium hydrogen phosphate (1.1 g l^{-1}) at the end of 20th day of fermentation recorded the maximum predicted dry weight of BC with 16.74 g l^{-1} which was slightly higher than the actual experiment which recorded 16.11 g l^{-1}. With CAJ as the basal medium, concentration of fructose (4.0 g l^{-1}), yeast extract (6.0 g l^{-1}), citric acid (1.5 g l^{-1}) and disodium hydrogen phosphate (0.75 g l^{-1}) had the maximum predicted dry weight of BC with 16.24 g l^{-1} than the actual experiment which resulted in 16.22 g l^{-1}. From the results, it was confirmed that, CAJ medium recorded a significantly higher dry weight of BC, with a low input of carbon. Hence, CAJ medium was selected for further

studies pertaining to mass production in a model fermentor and testing of laxative properties of BC using animal experiment.

One of the major constraints observed during the harvest of BC grown in Erlenmeyer flasks was narrow mouth of the flasks used in the study. Hence a wide mouthed, autoclavable jar type model fermentor of 5 litre capacity was designed for mass production using CAJ medium. The studies on fermentation kinetics revealed that Specific growth rate (μ) of *Gluconacetobacter* sp. sju-1 was 0.00316 ±0.04 h^{-1} with the BC production of 1.62 ± 0.01 g $100g^{-1}$ of the substrate and 1.30 ± 1.11 g $100g^{-1}$ of cells respectively.

The quality parameters such as colour, texture, water holding capacity and dietary fiber content of BC obtained from MCW, CAJ and HS medium were compared for *nata* production. The colour of the BC from MCW and CAJ medium was pure white and yellowish respectively, but those produced from the HS medium was light brown in colour. The hardness of BC in terms of texture from both MCW and CAJ medium was very low of 07.00±1.37 and 11.00±1.24 N respectively. The BC from HS medium showed a comparatively higher content of insoluble fibre (8.50±0.35 g) followed by MCW medium (7.20±0.04 g) and CAJ medium (7.80±0.02 g). Water holding capacity, an important parameter for quality *nata* production was comparatively higher for BC from CAJ medium with 88.54±0.02 per cent.

The result of GCMS analysis of fermented broth of CAJ medium revealed the presence of eight volatile compounds dominated by thiophene-2-carboxylic acid and santolin diacetylene (24.8 per cent), 1,5-dihydroxyl-3,3-dimethyl-7-acetylaminomethyl-tricyclododecane (10.75 per cent), 3-phenyl-3-m-tolylpropionamide and 1-(2-hydroxyethylthio)-1-phenyl-2-butanone (12.33 per cent), nonane and 4-hepatanol (6.53 per cent) and 3-(4-nitrobenzoyl) cinnoline (5.8 per cent).

Coconut water vinegar, a value added product was prepared by using the spent liquor of CAJ medium after BC extraction. The spent liquor at the rate of 10 per cent was used as a mother culture for coconut water vinegar production. The resulted vinegar contained 4.8 per cent of acetic acid.

Animal experiment was conducted at KMCH College of Pharmacy, Coimbatore to test the laxative properties of BC. The effect of colloidal bacterial cellulose laxative on feed intake, water intake and faecal properties of loperamide induced constipated Sprague-Dawley (3 weeks old) rats were comparable with those of the control laxative, Isabgol (*Psyllium* sp.). This was evidenced by the increase in the number and weight of faecal pellets which was comparatively higher in colloidal bacterial cellulose laxative administered rats of 46.34±2.55 and 16.87±54 g respectively. The loperamide induced constipated rats administered with

colloidal bacterial cellulose laxative twice a day at the rate of 5.0 ml per dose significantly increased the faecal weight, which is a good indicator of laxative property of BC.

Considering the dietary fibre value, BC was processed into a jelly form called as *nata-de-coco* and *nata-de-cashew* using MCW and CAJ medium respectively. The sensory evaluation of the developed products showed the overall acceptability of 9.2 and 9.02 per cent respectively. The cost benefit ratio for the production of *nata* was worked out and it was found to be 1.05 for unprocessed *nata* and for *nata* in jelly base it was 1.97.

To conclude, bacterial cellulose of high purity can be obtained from *Gluconacetobacter* sp. sju-1 using mature coconut water and cashew apple juice at static fermentation condition without high energy and sugar input. Thereby, as an alternative production medium, MCW and CAJ can be value added into bacterial cellulose and cellulose based products.

CHAPTER I

INTRODUCTION

Cellulose is a natural, biodegradable, non-toxic polymer produced by vascular plants. Wood and cotton fibres form the major source of cellulose based products. Cellulose can also be obtained from algae (*Vallonia*), bacteria (*Acetobacter xylinum*, *A. aceti, A.acetigenum, A.hansenii*, *Sarcina, Agrobacterium, Rhizobium, Pseudomonas, Chromobacterium, Achromobacter*, *Aerobacter, Alcaligenes, Zooglea*), fungi (*Sparolegnia*) and slime moulds (*Dictyostelium discoideum*). Among the various microbial sources of cellulose, bacterial cellulose (BC) produced by *Gluconacetobacter xylinum* (formerly called as *Acetobacter xylinum*) is commercially important (Swings, 1992).

Gluconacetobacter xylinum is a non-photosynthetic, Gram negative, purple α-proteobacterium that can utilize glucose, sugars, glycerol and other organic substrates and convert them into pure cellulose (Yamada, 2000). The bacterium producing an extracellular insoluble polysaccharide was first described in 1886 by Brown. *G. xylinum* has been used as a model microorganism for basic and applied studies on bacterial cellulose, because of its ability to produce relatively high levels of polymer from a wide range of carbon and nitrogen sources. Under strict aerobic condition in static culture this bacteria forms an extracellular secretion which can form aggregated fibrils that crystallize into ribbons and assemble into a thick cellulosic mat known as a pellicle formed at the air-liquid interface of the culture medium (Bielecki *et al*., 2005).

BC is a pure aggregate without any other impurities such as hemicelluloses, pectin and lignin as that of plant cellulose. It differs from plant cellulose with respect to physical and chemical features and displays unique mechanical properties that include high crystallinity, high water holding capacity, large surface area, elasticity, mechanical strength and biocompatibility (Helenius *et al*., 2006).

The crystallographic form of the bacterial cellulose has revealed that it is made of cellulose type Iα, which is commonly seen in plant fibres. The molecular orientation of the cellulose is parallel to the direction of fibrils. This property makes it highly suitable for pulp and paper industry, especially in the manufacture of fire retardant papers, reinforced papers, parchment papers and the sheets of bacterial cellulose is expected to be useful in specialty paper grades. Further bacterial cellulose may partly replace plant fibre in the pulp and paper industry, as a green energy saving material (Sokolnicki *et al*., 2006).

Processed bacterial cellulose from coconut water is also called as *nata-de-coco* which is edible, creamy white in colour, tastes good with smooth, slippery and chewy nature, virtually digestible and totally safe (Halib *et al*., 2012). Further the bacterial cellulose has wide application in various fields such as hydrogels in agriculture, organic dietary fibre food, clarification of fruit juices, healthcare, cosmetics, clothing, baby care products, paper industry, packaging materials, pharmaceuticals, wound dressing supra absorbent papers, electronic display papers and specialty papers like archival and currency notes, audio products like speaker diaphragms, stabilizers and thickeners in paints and adhesives, filter membrane in water treatment units, nanocomposites for biomedical purpose and enzyme immobilization etc. (Hameed *et al*., 2012).

BC is relatively expensive to produce and as such it cannot replace traditional sources of cellulose. High medium costs limit the commercial production of BC. One of the most important application problem in BC usage is its low productivity. Moreover the scale of production, processing and use is relatively small because of problems associated with selection of sufficiently efficient producers and costs of culture media. In microbial fermentations, the cost of the fermentation medium can account for almost 30 per cent of the total cost. The current price of media and the low production rate of the BC limit the commercial use of the BC. Improved fermentation processes are required for a breakthrough towards an economical process for BC production. Therefore, one of the key aspects in the fermentation process is the development of a cost-effective culture medium to obtain maximum product yield. Most of the studies on the 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. The majority of studies on BC production have also used media containing complex nitrogen and vitamin sources such as yeast extract and polypeptone. However, these nutrient sources are economically unfavourable, because these substrates are very expensive. One way to reduce the cost of production is to use cheap and readily available substrates. The manufacturing cost of BC can be significantly reduced if fruit wastes, coconut water and other sugary rich wastes are used as a raw material.

Many researchers have tried to increase the productivity of bacterial cellulose using different carbon and nitrogen sources, potato process effluents, beet molasses, sugarcane molasses, corn steep liquor medium, tender coconut water medium, pine apple juices and date palm extract medium (Hong and Qiu, 2007). There is a wide potential for utilizing agricultural wastes in the production of bacterial cellulose.

India, being the tropical country there is an abundant source of coconut water and in particular at Tamilnadu, large coconut processing yards are available. It is evident from the survey among coconut growers that it is not popular in India and very little knowledge exists

about *nata-de-coco* among the coconut processors. Further in our country coconut water is thrown away in copra processing industries, in hotels, in restaurants and even from temples into ditches and onto agricultural lands causing a great pollution hazard. The Biological Oxygen Demand of waste coconut water is commonly of the order of 40,000 mg l^{-1} and may be more than double this value (Shivakumar and Vijayendra, 2006). In India, a conservative estimate of annual production of coconut water waste is in excess of 10 lakh litres. 1000 coconut yields on an average about 140 litres of coconut water. From 100 litres of matured coconut water, 20 kg of *nata* (value added product) can be produced. A factory handling 8 lakh nuts each day will produce about 1,12,000 litres of coconut water which can offer 24000 kg of *nata* (Prades *et al*., 2012).

Cashew (*Anacardium occidentale* L.), an important nut crop, is grown in India on an area of 9.23 lakh ha, which produced 22 to 25 lakh tonnes of cashew apple fruits (Shalini, 2010). Although cashew apples are highly nutritious and available in plenty, it is little used in India. In Goa, it is fermented to an alcoholic beverage called '*feni*'. Protocols for preparation of juice, jam, jelly and candy from cashew apple have been documented. However, cashew apple-based products are not produced on a commercial basis in India. Although several studies have been published on the benefits of using agro waste for industrialization, few studies are there in characterization of cashew apple of various agro fields of Tamil Nadu. Furthermore, cashew apple has no commercial value, except for its use by rural inhabitants in the production of homemade alcoholic beverages. The majority of cashew apples rot in the soil. Large quantities of fruits are wasted in lands without proper usage. Thus, under this situation the agro-waste like cashew apple left behind for natural degradation can be utilized effectively to produce bacterial cellulose. The use of cashew apple juice and residue in industrial process as low cost substrate is interesting as a way of using the cashew apple as alternative feedstock (Arindam *et al*., 2011).

In the era of declining forests, global climate changes, continuing expansion of industrialization, it is reasonable to consider the consequences of an alternative source of cellulose. The demand for bacterial cellulose outpaces the supply, largely because of lack of investment in research and development to optimize production on a large scale. Hence recycling of fruits and coconut water waste is one of the most important means of utilizing them for production of useful products and microbial technology has to be developed for such recycling.

This investigation was conducted with the following objectives.

1. To isolate and characterize cellulose producing *Gluconacetobacter sp.* from coconut, fruits, tea and coffee processing wastes.
2. To study the feasibility of bio-wastes such as mature coconut water and cashew apple juice for cellulose biopolymer production using *Gluconacetobacter sp.*
3. To optimize various process parameters for the scale up of cellulose production in making high tensile strength paper.
4. To explore the possibilities of production and characterization of value added products such as *nata* (jelly) and vinegar from microbial cellulose.

CHAPTER II

REVIEW OF LITERATURE

Cellulose is of great importance in fundamental research and industrial applications because of its widespread occurrence, uniform molecular structure and unique properties such as polyfunctionality, multichirality, hydrophilicity and biocompatibility (Stasiak and Blazejak, 2009). The cellulose industry generally utilizes wood to produce white cellulose pulp, which has been used for paper and cellulose derivative products. The utilization of locally available raw materials such as sugar cane, baggasse, banana, paddy straw etc., as an alternative source for the production of cellulose derivatives would considerably decrease the use of wood for this purpose and would also minimize deforestation. However, there's still a large amount of pollution due to the paper and cellulose industry effluents (Cheng *et al*., 2009). To address environmental issues, it is mandatory for the new technologies or redesigned products to meet new environmental criterion set by the environmental agency. Pokalwar *et al*. (2010) have indicated that microbial cellulose is a good alternative to plant cellulose though not to a wide extent, it can be used in the manufacture of high end cellulose based products.

2.1. Bacterial Cellulose (BC)

Cellulose is the major constituent of cotton (over 94%) and wood (over 50%). Cotton and wood are the major resources for all cellulose products such as paper, textiles, construction materials, cardboard, cellulose derivatives such as cellophane, rayon and cellulose acetate. Cellulose from major land plants such as forest trees and cotton is assembled from glucose which is produced in the living plant cell from photosynthesis. Even animals, fungi and bacteria can assemble cellulose, but these organisms are devoid of photosynthetic capacity and usually require glucose or some organic substrate synthesized by a photosynthetic organism to assemble their cellulose. Some bacteria can utilize methane or sulfur substrates to produce glucose and other organic substrates for cellulose (Brown *et al*., 1976). The molecular formula of bacterial cellulose $(C_6H_{10}O_5)n$ is same as that of plant cellulose, but their physical and chemical features are different. Bacterial cellulose is preferred over the plant cellulose as it can be obtained with higher purity and exhibits a higher degree of polymerization and crystallinity index. Microfibrillar structure of bacterial cellulose was first described by Muhlethaler in 1949. Fibrils of bacterial cellulose are about 100 times thinner than that of plant cellulose. Bacterial cellulose exists as a basic structure known as microfibrils, which are composed of glucan chains interlocked by hydrogen bonds so that a crystalline domain is produced. Different types of cellulose produced by bacteria were given by Chiciudean (2011).

Table. 1. Types of cellulose produced by bacteria

S. No	Organisms	Type of cellulose produced
1.	*Acetobacter*	Extra-cellular pellicle
2.	*Achromobacter*	Fibrils
3.	*Aerobacter*	Fibrils
4.	*Agrobacterium*	Short fibrils
5.	*Alcaligenes*	Fibrils
6.	*Pseudomonas*	No distinct fibrils
7.	*Rhizobium*	Short fibrils
8.	*Sarcina*	Amorphous cellulose
9.	*Zooglea*	Not well defined
10.	*Chromobacterium*	Extracellular matrix of fibres

2.1.1. *Acetobacter* in cellulose production

Acetic acid bacteria are defined within the family Acetobacteraceae and they are characterized by their ability to oxidize ethanol to acetic acid. Yamada and Kondo (1984) divided the genus *Acetobacter* into two subgenera on the basis of differences in the ubiquinone system, the type subgenus *Acetobacter* was characterized by Q-9 quninone, and the type subgenus *Gluconacetobacter* was characterized by Q-10. Members of the subgenus *Gluconacetobacter* are divided into two groups *viz.*, the *Gluconacetobacter liquefaciens* group and the *Gluconacetobacter xylinus* group. The former group consists of the non-nitrogen fixers such as *G. liquefaciens* and *G. sacchari* and the nitrogen fixers such as *G. diazotrophicus*, *G. azotocaptans* and *G. johannae*. The latter group consists of the non-BC producers such as *G. hansenii* and *G. europaeus.* The BC producers are *G. xylinus, G. obediens*, *G. intermedius, G. nataicola* and *G. rhaeticus*. Amongst all *G. xylinus* (formerly *Acetobacter xylinum*) are the most common species. The subgenus *Gluconacetobacter* was elevated to the generic level on the basis of partial 16S rRNA sequence analysis by Kojima *et al*. (1998). The generic name *Gluconoacetobacter* was corrected to *Gluconacetobacter* in accordance with the rule 61 of the bacteriological code on the occasion of validation (International Committee on Systematic Bacteriology, 1998).

Recent classification of the acetic acid bacteria includes the genera *Acetobacter*, *Acidomonas*, *Asaia*, *Gluconacetobacter, Gluconobacter, Kozakia, Swaminathania and*

Saccharibacter. Multiple species of acetic acid bacteria are capable of incomplete oxidation of carbohydrates and alcohols to aldehydes, ketones and organic acids (Matsushita *et al*., 2005; Yamada and Yukphan, 2008). Apart from vinegar, the key metabolites produced by those microorganisms include cellulose, gluconic acid, L-ribose and dihydroxyacetone. Brenner *et al*. (2005) have reported that *Gluconacetobacter xylinum* is nature's most prolific cellulose producing bacterium. A number of synonyms that describe this organism are *Acetobacter xylinum*, *Bacterium xylinoides* and *Bacillus xylinum*. Trcek (2005) have reported that some species of *Acetobacter*, recently named as *Gluconacetobacter* are known to produce cellulose exhibiting superior features over plant cellulose. It is a Gram negative, non pathogenic, rod shaped obligate aerobic bacterium. Their sizes are in the range of 0.6 to 1.2 × 1.0 to 3.0 μm. Cells of *G. xylinum* occur individually or in pairs, chains or in small clusters. Only part of them is characterized by peritrichal ciliation which provides their motor capacity. They produce catalase, do not produce oxidase, indole nor hydrogen sulfide, and they do not fluidize gelatin. It is a model organism in biochemical and genetic investigations into mechanisms of cellulose synthesis. A typical single cell can convert up to 108 glucose molecules per hour into cellulose. In addition, they transform ethanol into acetic acid, whereas further oxidation to CO_2 and H_2O depends on the concentration of that compound in a culture medium. Some strains are also capable of oxidizing lactates to CO_2 and H_2O.

Kersters *et al*. (2006) have reported that bacterial cellulose is an exopolysaccharide produced by various species of bacteria, such as those of the genera *Gluconacetobacter, Agrobacterium*, *Aerobacter*, *Achromobacter*, *Azotobacter*, *Rhizobium*, *Sarcina* and *Salmonella*. Amongst all the cellulose producing microbes, the cellulose synthesized by *G. xylinum* is ‘‘spun’’ into the growth medium as highly crystalline ribbons with exceptional purity, free from the contaminating polysaccharides and lignin found in most plant cell walls. Electron microscopic observations showed that the cellulose produced by *G. xylinum* occurs in the form of fibres. The bacteria first secreted a structurally homogeneous slimy substance within which, after a short time, the cellulose fibers were formed.

2.2. Site and mechanism of cellulose synthesis in *G. xylinum* (Iguchi *et al*., 2000)

G. xylinum produces two forms of cellulose: (*i*) Cellulose I, the ribbon-like polymer, and (*ii*) Cellulose II, the thermodynamically more stable amorphous polymer. Microfibrillar structure of bacterial cellulose is responsible for most of its properties such as high tensile strength, higher degree of polymerization and crystallinity index.

A single bacterial cell contains a number of cellulose-synthesizing sites that are observed as a linear row of particles on the membrane surface. Each particle synthesizes a sub-elementary fibril predicted to consist of 16 glucan chains. The sub-elementary fibrils from

different particles join to give rise to the ribbon of microfibrils. Each particle contains a number of cellulose synthases (AcsAB protein) and associated proteins (AcsC and AcsD). The arrangement of the cellulose synthase and associated proteins is shown in a section of the cellulose synthesizing complex in the cytoplasmic membrane of *A. xylinum)* (Fig. 1). Cellulose synthase is a membrane protein present in the cytoplasmic membrane. The globular region of this protein faces the cytoplasm and it is this region that contains the conserved amino acid residues involved in the polymerization step. The site of cellulose synthesis and microfibril formation and arrangement is depicted in Fig. 2 and 3. The synthesis of cellulose in *G. xylinum* or any other cellulose producing organisms including plants follows two steps:

(i) Formation of β-glucan chain with polymerization of glucose units and

(ii) Assembly and crystallization of cellulose chain.

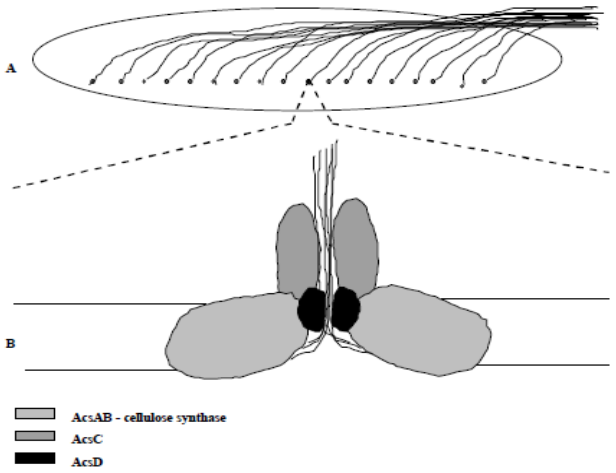

Fig. 1. Site of cellulose synthesis in *Gluconacetobacter xylinum*

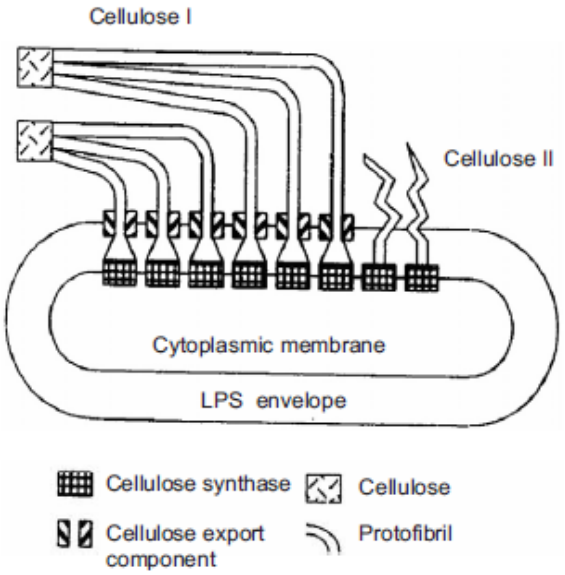

Fig. 2. Assembly of cellulose microfibrils in *Gluconacetobacter xylinum*

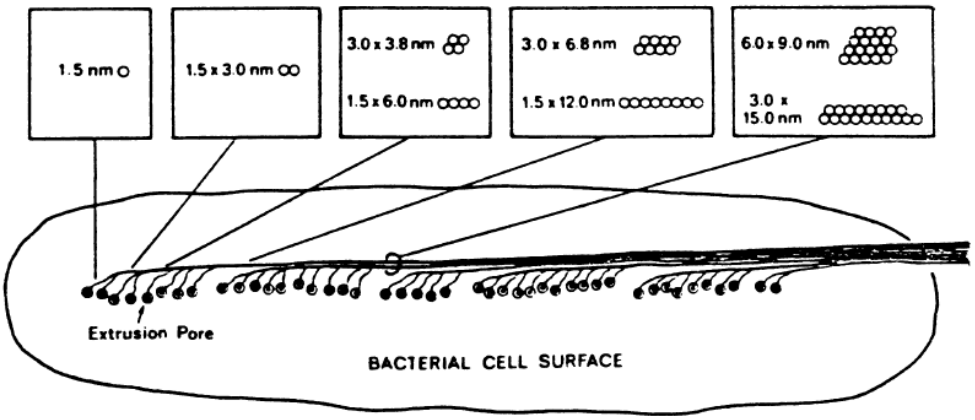

Fig. 3. Generalized model of ribbon assembly in *Gluconacetobacter xylinum* showing a possible mechanism of origin or microfibrils and separate fibrillar subunits within the ribbon. Shown in the boxes are possible packing arrangements of the 1.5 nm tactoidal aggregates.

2.3. Physiological functions of cellulose production in *Gluconacetobacter* sp.

2.3.1. Colonisation efficiency and competition

Williams and Cannon (1989) have reported that *G. xylinum* synthesize cellulose as a primary metabolite, enhanced the colonization efficiency of *G. xylinum* on the substrate and provides protection from competitors which use the same substrate as a source of nutrients.

2.3.2. Aerobic environment

Jonas and Farah (1997) have indicated that the cellulose synthesis mechanism helps the aerobic bacteria to move to the oxygen-rich surface. It has been proposed that cellulose serves to hold the bacterium in an aerobic environment probably on the surface of the decaying fruit that it uses for nutrients. Further it may have multiple functions in the growth and survival of the organism in nature. They have suggested that the bacteria immobilize themselves in such a "cage" to maintain their position between a substrate rich fruit surface and the oxygen rich air space.

2.3.3. Moisture retention

The pellicle aids in moisture retention to prevent drying of the natural substrates for *G. xylinum* while the bacterium is growing on them. Because of the viscosity and hydrophilic properties of the cellulose layer, the resistance of producing bacterial cells against unfavourable changes such as decrease in water content, variations in pH, appearance of toxic substances, pathogenic organisms, etc., in a habitat is increased, and they can further grow and develop inside the envelope. Cellulose acts as moisture absorbent pads and retains moisture so as to release into the outer environment slowly (Marci *et al*., 2006).

2.3.4. UV Protection

Ross *et al*. (1991) found that cellulose placed over bacterial cells protects them from ultraviolet radiation. As much as 23 per cent of the acetic acid bacteria cells covered with BC survived a 1 h treatment with ultraviolet irradiation. Removal of the protective polysaccharide brought about a drastic decrease in their viability (3 per cent). Klemm *et al*. (2001) have reported that the cellulose pellicle is produced to protect the cells from as a defence against sunlight and ultraviolet radiation.

2.4 Biochemistry of cellulose synthesis in *Gluconacetobacter* sp.

2.4.1. Carbon metabolism in *Gluconacetobacter* sp. (Vincent, 1999)

Gluconacetobacter xylinum follows two amphibolic pathways namely pentose phosphate cycle or the Krebs cycle coupled with gluconeogenesis. Synthesis of bacterial cellulose is a precisely and specifically regulated multi-step process, involving a large number of both individual enzymes and complexes of catalytic and regulatory proteins. The process

includes the formation of UDPGlc, which is the precursor in the formation of cellulose, followed by glucose polymerization into the β-1-4 glucan chain and a nascent chain which forms ribbon-like structure of cellulose chains formed by hundreds or even thousands of individual cellulose chains, their extrusion outside the cell, and self-assembly into fibrils. The pathway from the substrate glucose to cellulose involves a number of reactions in which glucose is first converted to glucose-6-phosphate by the enzyme glucokinase. In the second step, glucose-6-phosphate is converted to glucose-1-phosphate by the enzyme phosphoglucomutase. In the next step, glucose-1-phosphate is converted to UDP-glucose in the presence of UTP and the enzyme UDPG pyrophosphorylase. The UDP-glucose produced is used as a substrate by the enzyme cellulose synthase (Fig. 4). In *G. xylinum*, this enzyme is activated by the cyclic nucleotide, c-di-GMP. The cellulose synthase activator c-di-GMP is synthesized in *G. xylinum* by the enzyme diguanylate cyclase, and the concentration of this activator is regulated by the action of phosphodiesterases. Genes for the diguanylate cyclase and phosphodiesterases have been identified and they are organized together in more than one operon in the *G. xylinum* chromosome.

2.5. Isolation of *Gluconacetobacter* sp.

Cellulose producing *Acetobacter* has been isolated from rotting apple, citrus, custard apple, dates, guava, *Kombucha* tea, malt vinegar, mango, mulberry, oranges, radish, sapota and tomato. Faparusi (1973) had isolated *Acetobacter* sp. from palm wine and immature spadix of palm tree. *Gluconacetobacter oxydans* subsp. *suboxydans* was found on the floret of palm tree, in the tap holes and in palm sap (Faparusi, 1974). Tayama *et al*. (1985) isolated *Acetobacter* sp. NBI 1022 from fruit vinegar which contained cellulose units made of heteropolysaccharides like glucose, mannose, rhamnose and glucuronic acid. Moonmangmee *et al*. (2002) isolated *G. tropicalis* from surface culture of vinegar fermentation, producing cellulose pellicle composed of glucose, galactose and rhamnose units. Son *et al*. (2002) isolated *G. xylinum* KJ1 from decayed grapes and used for BC production using sachharogenic food wastes. Park *et al*. (2003a) had isolated sulfaguanidine resistant mutants of *Acetobacter xylinum* subsp. *sucrofermentans* from rotten apples. Kim *et al*. (2007) isolated *Gluconacetobacter* sp. RKY5 from persimmon vinegar and optimized the conditions for maximum cellulose production. Nguyen *et al.* (2008) have reported 0.28 g l^{-1} cellulose production with *Gluconacetobacter xylinum* isolated from *Kombucha* culture. The *Gluconacetobacter* strain was isolated from homemade vinegar culture and identified by 16S rRNA method by Arahal *et al*. (2008) as *Gluconacetobacter swingsii* sp. Andelib and Nuran (2009) have attempted to isolate five strains of acetic acid bacteria from wastes of vinegar fermentation. Jung *et al.* (2010) isolated *Acetobacter* sp. V6 strain from the traditionally

fermented vinegar which produced 4.16 g l^{-1} cellulose in a complex medium containing glucose as a carbon source. Wee *et al*. (2011) isolated a novel cellulose producing *Gluconacetobacter intermedius* $TF2^T$ from the liquid part of a three year ripened persimmon vinegar in Korea. Lam *et al*. (2013) isolated a cellulose producing bacterial strain BHN2 from Hanoi fresh beer and identified by 16SrDNA sequence analysis as *Gluconacetobacter intermedius.* Suwanposri *et al.* (2013) have isolated two hundred and four strains of bacterial cellulose producing strains of *Gluconacetobacter* from rotten fruits and have characterized them into seven subgroups based on partial sequencing of 16SrDNA.

2.6. Economical importance of bacterial cellulose

2.6.1. Production of *nata* - organic high fibre dietary food from bacterial cellulose

Nata is a white or creamy-yellow to pinkish, firm gelatin like substance formed by bacterial action on sugary fruit juices. *Nata-de-pina* and *nata-de-coco* are well known dessert delicacies in Phillippines (Alaban, 1962). In 1992, microbial cellulose was introduced into diet drinks in Japan. *Acetobacter* was grown along with yeast in the tea extract and sugar. This is consumed as a *kombucha* or Manchurian tea for improved health needs. *Nata* is a word derived from Latin word*'natuer'* meaning to float from fermenting coconut water or fermenting rotting fruits. It is prepared from *Acetobacter xylinum* (*xylinum* means cotton) and is widely used in food processing because of its distinctly soft texture and high fibre content. This dietary fibre have been found to be very effective in normalizing blood glucose, preventing constipation and colon cancer (Lapuz *et al*., 1967).

In Thailand, bacterial cellulose is cut into small cubes for use in desserts, health foods, salads and fruit cocktails, and is known as "*Vhun-sawan*". In most Thai factories, *A. xylinum* is cultured using the traditional static culture in a tray containing coconut water, which results in a BC sheet on the surface of the liquid medium (Hermansson, 1986). *A. xylinum* uses the nutrients in the coconut water 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 to 20 days. This thick sheet of cellulose is cut into cubes, washed and boiled in water before cooking in sugar syrup and called as *nata* (Cummings *et al*., 1992).

Nata is more than 90 per cent water imbibed in cellulose and finds use in desserts, fruit cocktails and jellies. Being microbial cellulose, *nata-de-coco* is highly hydrophilic, holding water over 100 times of its weight. Water holding capacity, a physical property is defined as the ability of a food structure to prevent water being released from the 3-dimensional structure. The water is trapped in the cellulosic matrix and is important both for food and non-food applications of cellulose. Thickness of *nata* influences the water holding capacity which in turn has a bearing on the physical textural and organoleptic properties.

Nata-de-coco has distinct but preferred textural properties like a firm chewy, soft and smooth surface (Premjet *et al*., 2012).

2.6.2. Production of acetic acid/ vinegar using *Gluconacetobacter* sp.

Acetic acid is conventionally produced from petrochemical feedstocks by oxidation of acetaldehyde, the oxidation of liquid-phase hydrocarbons or the carbonylation of methanol. Worldwide production of acetic acid is about 4 million tonnes per year, with U.S. demand alone being 1.9 million tonnes per year at a price of 600-800 US Dollars per tonne. Acetic acid can also be produced from renewable sources such as corn or biomass by fermentation. Joyeux *et al*. (1984) have indicated that the production of vinegar consisted of filling jars half full with wine, grape pomace, grinded date fruits and were then subjected to spontaneous fermentation. Vinegar was contemporarily used as a seasoning, a refreshing drink and a medicine. The capability of bacteria from the genus *Acetobacter* to ferment ethyl alcohol to acetic acid is employed in the food industry for the production of vinegar. Katikaneni and Cheryan (2002) has indicated that the fermentation of ethanol to acetic acid ($C_2H_5OH + O_2 = CH_3COOH + H_2O$) is one of the oldest known fermentation. Yamada (2000) has reported that out of the six genera, the acetic acid bacteria that are utilized for vinegar fermentation are distributed mainly in the genera *Acetobacter* and *Gluconacetobacter*. Stasiak and Blazejak (2009) have indicated that in the production of vinegar using submerged culture system, there are two types of fermenting organism. One is the 'medium acid-producing' which produce 5 to 10 per cent acetic acid, in which cellulose-less strains of *G. xylinum* play an important role. The other is the 'high acid-producing' in which 10 to 20 per cent acetic acid produced and *Acetobacter polyoxogenes* usually predominates. In the genus *Gluconacetobacter*, the vinegar producing acetic acid bacteria are phylogenetically restricted only to sub-lineage B, which is composed of *Gluconacetobacter xylinum*, *G. hansenii, G. europaeus, G. polyoxogenes*, *G. oboediens*, *G. intermedius* and *G. entanii.*

2.6.3. Production of paper and paper based products using bacterial cellulose

Biocellulose is expected to be a new biodegradable biopolymer and can replace wood cellulose and reduce consumption of wood for production of paper and fibre. The development of biocellulose in the production of fibre is becoming important in solving ecological problems. Paper produced from such a cellulose is highly elastic, resistant and more importantly, completely biodegradable. It is well suited as a reinforcing agent for paper and diverse specialty products (Johnson and Winslow, 1990)

Suwannapinunt *et al.* (2007) have reported that paper made from BC derived from static condition showed that the physical properties of a parchment paper with high wet strength and was resistant to disintegration by any chemical agents. Shah and Brown (2005)

have elucidated that BC obtained from *Acetobacter xylinum* sub sp. *sucrofermentans* sub sp. nov under agitated culture conditions could be incorporated at the wet end of paper making process to enhance the tensile strength and filler retention of the final product. Tabuchi and Baba (2005) have reported cellulose microfibrils are quite strong and resilient. When wet, the pellicles resist tearing; when dry, they have the consistency of paper. Apart from the paper industry, BC may also be applied for the electrophoretic separation of DNA. Due to an internal, three-dimensional network of microfibres, cellulose may be used for the separation of DNA fragments with sizes of even 10 to 100 pz. The strong, tough, highly pure cellulose synthesized by microorganisms is just emerging as a new industrial product with exciting new applications in medicine, foods, paper and packaging as well as potential for use in industrial separations. Bungay *et al.* (1997) have indicated that making a polymer by bioprocessing is inherently much less damaging to the environment than the petrochemical processes, and this cellulose may substitute for plastics and for special grades of paper. This would eliminate some petrochemical processing and the highly pollutional steps for pulping and bleaching of wood cellulose. Wastes that contain cellulose can be converted to sugars that are substrates for the microorganisms that make cellulose. At the Institute of Papermaking and Printing, Technical University of Lodz, Poland, complex research was undertaken by Slusarska *et al*. (2008) to develop a procedure for the modification of different papermaking semi-products with bacterial cellulose with considerable thermal stability and proved that the bacterial cellulose could be an excellent resource base for paper making.

Basta and El-Saied (2009) have prepared and evaluated economic flame retardant bacterial cellulose. The study confirmed that the use of glucose phosphate along with glucose favoured for maximum production of phosphate containing bacterial cellulose (PCBC). Incorporating 40 per cent of PCBC with wood pulp during paper sheet formation was found to improve kaolin retention, strength, and fire resistance properties as compared to paper sheets produced from incorporating bacterial cellulose alone. This modified BC proves to be a valuable product for the preparation of specialized flame retardant paper, in addition to its function as fillers aid. Gao *et al*. (2011) have reported that the stiffness of the paper and other physical properties can be enhanced by incorporation of bacterial cellulose to pulp used for paper production.

Ton and Le (2011) have indicated the application of bacterial cellulose in immobilisation of yeast used in wine fermentation and can increase the ethanol production rate of the immobilised yeast increased to 116.1 per cent from 91.8 per cent (control). Shanshan *et al*. (2012) developed regenerated bacterial cellulose films using N-methylmorpholine N-oxide (NMMO) as a solvent to dissolve the bacterial cellulose. The

dissolubility of native materials, molecular and supermolecular structure, morphology, thermal stability and physical properties of the films were found to be superior to that that of bacterial cellulose dissolved using cupriethylenediamine. Kulkarni *et al*. (2012) have evaluated the bacterial cellulose produced from *Acetobacter xylinum* as pharmaceutical incipient. Loss on drying, moisture sorption capacity of the bacterial cellulosic incipient was found to be highly significant than the commercially available incipient Avicel 101.

2.7. Factors affecting the production of bacterial cellulose

Major factors affecting cellulose production mainly includes growth medium, environmental conditions and formation of byproducts. Generally, medium containing high carbon to limiting nutrient ratio (often nitrogen) is favourable for polysaccharide production. Conversion of 60 to 80 per cent of the utilized carbon source into crude polymer is commonly found in high yielding polysaccharide fermentation. The optimal design of the medium is very important for the growth of a microorganism and thus stimulating the formation of products. Nutrients required for the growth of a microorganism are carbon, nitrogen, phosphorus, sulphur, potassium and magnesium salts. The fermentation medium contains carbon, nitrogen and other macro and micronutrients required for the growth of organism (Dudman, 1959). The changes in the medium components affect the growth and the product formation directly or indirectly. Secretion of exopolysaccharides is usually most noticeable when the bacteria are supplied with an abundant carbon source and minimal nitrogen source. Sometimes a complex medium supplying amino acids and vitamins is also used to enhance the cell growth and production (Makoto and Yasushi, 2005).

2.7.1. Carbon source

Many strains of *G. xylinum* are capable of producing cellulose in wide variety of substrates like glucose, lactose, sucrose, fructose, invert sugar, mannitol, xylose, ethanol and glycerol. Suitable initial carbon source concentration is within the range of 2 to100 g l^{-1}. Sugar can be added to the growing culture to maintain a certain desirable concentration for optimum cellulose production. The quantity of cellulose synthesized by the species is proportional to an increase of biomass, whereas the yield of that process is determined by the source of carbon (Embuscado *et al*., 2009). Once ethanol is the major source of carbon, then multi-stage oxidation of that substrate occurs that results in the formation of acetic acid. Once the culture medium contains glycerol, *G. xylinum* oxidizes it to dihydroxyacetone. Ishihara *et al*. (2002) used xylose as a carbon source for the production of cellulose by *A. xylinum* IFO 15606 and obtained a yield of 3.0 g l^{-1}. Slusarska *et al*. (2008) have reported that bacterial cellulose synthesized with glucose and mannitol as a carbon source showed highest degree of polymerization, but when xylose was used the average degree of polymerization decreased

substantially. Panesar *et al*. (2009) have indicated that the maximum cellulose production was obtained when mannitol was used (1.8 g l^{-1}) followed by glucose (1.5 g l^{-1}). Hungund and Gupta (2010a) have reported that inositol gave maximum cellulose production of 5.0 g l^{-1} followed by mannitol, glucose, fructose and maltose.

2.7.2. Nitrogen sources

Matsuoka *et al*. (1996) have reported that the addition of extra nitrogen favours the biomass production, but diminishes cellulose production. Further they observed that corn steep liquor along with fructose increased the cellulose production. The effect of yeast extract concentration on cell growth and cellulose production in different nitrogen sources media was studied by Yang *et al*. (1998). They have reported that yeast extract yielded maximum concentration (6.7 g l^{-1}) of cellulose. The effect of various nitrogen sources on the production of bacterial cellulose has been reported by Ramana *et al*. (2000). They have indicated that casein hydrolyzate and peptone gave yield of 5.0 g l^{-1} and 4.8 g l^{-1} of cellulose respectively. Son *et al*. (2001) has reported that the production of cellulose reduced when inorganic sources of nitrogen were used for the cellulose production using *Acetobacter* sp. A9.

2.7.3. Temperature

The optimum temperature for *G. xylinum* was reported to be in the range of 25 to 35˚C. Both *Acetobacter* and *Gluconobacter* alike were unable to grow at 37˚C. *Acetobacter* strains showed positive growth not only at 37˚C but also at 40˚C (Holt *et al*., 1994).

2.7.4. pH

Growth of *G. xylinum* was found to be inhibited at pH > 7.0. Elsewhere, it has been reported that growth of *G. xylinum* is inhibited at pH < 3.5. Accordingly, with acidgenerating substrates, such as glucose, optimal growth was obtained when the initial pH of the medium was held within 5.0 to 7.0. With alkali generating substrates of the type of a salt of an organic acid, eventual rise of the pH beyond 7.0 could not be completely prevented but was sufficiently delayed (> 60 days) when the initial pH was brought to 4.0. Increase in pH levels has been observed during growth in media containing fructose or ethanol as the carbon source, but could be held within desired limits by setting the initial pH value at 5.0. *A. xylinum* has been shown to be distinctly acid tolerable capable of growing at pH as low as 3.5 (Chung and Shyu, 1999). Verschuren *et al*. (2000) reported pH 4.0 and 5.0 to be ideal for the development of cellulose.

Jagannath *et al*. (2008) studied the effect of pH, sucrose and ammonium sulphate concentration on the production of *nata-de-coco*, a form of bacterial cellulose, by *G. xylinum*. Comparisons for physical properties like thickness, wet weight, water holding

capacity, moisture content and hardness, a textural parameter were done on *nata-de-coco* grown in tender coconut water medium supplemented with varying concentration of sucrose and ammonium sulphate at different pH values. The study showed that *G. xylinum* could effectively use sucrose as the sole carbon source in coconut water medium and cellulose production was more dependent on pH than either sucrose or ammonium sulphate concentrations. Maximum thickness of *nata* was obtained at pH 4.0 with 10 per cent sucrose and 0.5 per cent ammonium sulphate concentrations. These conditions also produced good quality *nata-de-coco* with a smooth surface and soft chewy texture.

2.7.5. Effect of various additives and precursors

Premjet *et al*. (1994) found that the addition of lignosulfonate in the Hestrin-Schramm (HS) medium remarkably enhanced the efficiency of the cellulose production from *A. xylinum* ATCC 10245. Naritomi *et al*. (2002) have reported about the co-substrates such as ethanol and organic acids (acetic, fumaric, lactic, malic, pyruvic, succinic) which stimulated cell growth and increased the amount of cellulose obtained in culture medium. Zhou *et al*. (2007) have reported that the addition of supplements to the cell culture medium like endoglucanase, pyruvate, ethanol, lactate and sodium alginate would boost bacterial cellulose production. The bacterial cellulose production by *A. xylinum* NUST4.1 carried out by adding sodium alginate (NaAlg) into the medium enhanced the yield and modified the crystalline properties of cellulose. Various water soluble polymers added into the medium are known to interfere with the aggregation of microfibrils into a normal ribbon assembly. Addition of water-soluble polymers such as xanthan, agar, polyacrylamide-co-acylic acid (PCA) and acetan can increase relative viscosity of the broth to reduce shear stress, hinder coagulation of bacterial cellulose during the cultivation to form uniform smaller pellets, which are advantageous to transfer nutrients and oxygen into bacterial cells located inside and on the surface of the cellulose matrix. Marzieh *et al*. (2010) have reported that the addition of carboxy methyl cellulose during cellulose formation increases the water holding capacity to 1000 times its dry weight and favours for increased crystallinity index of bacterial cellulose.

2.8. Production of bacterial cellulose

G. xylinum is usually cultured in static fermentation condition in a chemically undefined medium called as HS medium developed by Hestrin and Schramm (1954). The main culture conditions set for the production of BC are cultivation method, carbon source, nitrogen source, pH and temperature. Shoda and Sugano (2005) have reported that *G. xylinum* utilizes a range of carbon sources such as ethanol, fructose, galactose, glucose, glycerol, inositol, invert sugar, mannitol, methanol and sucrose. However, the bacterial cellulose yield is very low and commercial carbon sources such as glucose are relatively expensive. *G.*

xylinum could be made to grow on ethanol, acetate, succinate and L-malate. The cost of the fermentation medium account for almost 30 per cent of the total cost. Unfortunately, the current price of media and the low production rate of the BC limit the commercial use of the BC. Most of the studies on the BC production by *Acetobacter* strains have been carried out in media containing pure sugar as carbon source, such as arabitol, fructose, glucose, mannitol and sucrose The majority of studies on BC production have also used media containing complex nitrogen and vitamin sources such as yeast extract and polypeptone. These nutrient sources are economically unfavorable, because these substrates are very expensive. However, the high economic costs of carbon and nitrogen sources become a drawback for BC's industrial production and extended applications. Hence utilization of pure fructose, glucose and sucrose as carbon sources is not cost effective and the use of alternative, less expensive, carbon sources will help make BC production economical. Improved fermentation processes are required for a breakthrough toward an economical process for BC production. Therefore, one of the key aspects in the fermentation process is the development of a cost effective culture medium to obtain maximum product yield (Chawla *et al*., 2009).

2.8.1. Production of bacterial cellulose from agricultural wastes

Agriculture produces significant amount of wastes, which contain high quantities of organic matter and sugary rich compounds. The agricultural wastes produced in a particular period of the year pose potential pollution problems. Therefore, an efficient utilization of such agricultural wastes is of great importance not only for minimizing the environmental impact, but also for obtaining a higher profit. Bacterial cellulose is relatively expensive to produce, and as such is unlikely to replace traditional sources of cellulose. Relatively high cost of the production of cellulose may limit its application to high value-added products as well as speciality chemicals. Significant cost reductions are possible with improvements in fermentation efficiency and economics of scale, the lower limit of the cost of microbial cellulose being determined by the price of the raw material substrates. One way to reduce the cost of producing bacterial cellulose is to use cheap and readily available substrates (Chao *et al*., 2001).

2.8.1.1. Sugarcane molasses in bacterial cellulose production

Molasses, as runoff syrup, is a byproduct of the final crystallization stage in the process of sugar production. Because molasses is one of the most economical carbon sources, it is widely used as a substrate in microbial fermentation.

Bae and Shoda (2005) studied the production of bacterial cellulose by *A. xylinum* BPR2001 using molasses medium in a jar fermentor. They have developed an efficient cultivation method for BC production using molasses at concentration of 20 g l^{-1}. They

reported that H_2SO_4 heat treated molasses yielded 76 per cent more cellulose than the yield in HS medium. Keshk and Sameshima (2006) investigated the production of bacterial cellulose using sugarcane molasses in HS medium and indicated it to be a better carbon source than glucose for cellulose production. Premjet *et al*. (2007) added components of sugarcane molasses such as sucrose, fructose, glucose, nitrogenous compounds, non-nitrogenous acids, nucleic acids, vitamins, other carbohydrates, minerals and black colour substances individually or in combined forms into HS medium, and investigated their effect on bacterial cellulose production by *Acetobacter xylinum* ATCC 10245. They concluded that the addition of vitamins, amino acids, other carbohydrates, minerals and black colour substances to the molasses in the HS medium with a mixture of sucrose and fructose as the carbon source increased the bacterial cellulose yield. Kongruang (2008) investigated the effect of bacterial cellulose production by *A. xylinum* ATCC 10245 and concluded that the addition of vitamins, amino acids, other carbohydrates and minerals to the molasses in the HS medium increased the bacterial cellulose yield.

2.8.1.2. Beet molasses in bacterial cellulose production

Keshk *et al*. (2006) have reported that bacterial cellulose was produced from beet molasses using *Gluconacetobacter xylinus* ATCC 10245. The yield of the bacterial cellulose produced from beet molasses was higher than that of glucose as a sole carbon source. The structure of BC produced in the presence of beet molasses was studied using IR spectroscopy and X-ray diffractometry. IR spectra showed that the relative absorbance of C-O-C ether linkage (at 1120 cm^{-1}) in BC using glucose has a relatively lower value than that from molasses which indicated that BC produced from glucose had relatively higher degree of polymerization. Keshk and Sameshima (2006) has developed low cost environment friendly cellulose utilizing beet molasses as a sole cheap carbon source in HS medium using *Acetobacter pasteurianus* HBB6 and *Acetobacter lovaniensis* HBB5.

2.8.1.3. Food and process effluents in bacterial cellulose production

Food process effluents such as potato effluents, cheese, whey permeate and sugar beet raffinate have also been studied as substrates for cellulose production using *Acetobacter xylinum* ATCC 10821 and 23770 in static cultures (Thompson and Hamilton, 2001).

Moon *et al*. (2006) developed a low cost mass production technology using saccharogenic liquid medium. The saccharogenic food wastes obtained by the enzymatic saccharification of food wastes were used as a medium for production of bacterial cellulose using spherical air circulation bioreactor. Inedible substrates such as peat hydrolyzates, wood,

dextran production wastes, petrochemical wastes and products, ethanol, methanol, glycerin and ethylene glycol are often suitable for BC production.

2.8.1.4. Fruit juice and fruit wastes in bacterial cellulose production

Junsangsree *et al*. (2002) evaluated the value addition of pineapple juice by production of *nata-de-pina* using *Acetobacter xylinum* TISTR 893. The optimum substitution of pineapple juice (25 per cent) to coconut juice developed good quality bacterial cellulose. Kurosumi *et al*. (2009) developed an effective culture method to produce bacterial cellulose from fruit juices by *Acetobacter xylinum* NBRC 13693. Various fruit juices including orange, pineapple, apple, Japanese pear and grape were investigated for the possibility ofa producing bacterial cellulose from those juices.
The yield of the bacterial cellulose was increased by addition of nitrogen source to the fruit juices. In addition, it was confirmed that the orange and Japanese pear juices were suitable medium for bacterial cellulose production. The bacterial cellulose was produced from the various component of orange such as peel and squeeze residue, and the bacterial cellulose of 0.65 g (dry weight) was produced from 100 g of orange peel and the solid residue from the orange was about 17.2 g. Kumalaningsih *et al*. (2011) have developed an innovative natural dietary fibre (*nata*) made from solid waste of pineapple concentrate industry using *Acetobacter xylinum.* The developed product was 3.12 per cent rich in dietary fibre. Costa *et al*. (2011) have reported on the production of bacterial cellulose from Colombian agro wastes such as sugarcane juice and pine apple peel wastes using *Gluconacetobacter swingsii* sp.

2.8.1.4.1. Cashew apple - low cost substrate for industrial fermentation

Cashew apple or false fruit (*Anacardium occidentale* L.) a pseudo fruit formed by an enlarged peduncle contains approximately 30 per cent fermentable sugars (fructose and glucose), which can be utilized for fermentation of ethanol. Cashew apple juice normally has sufficient organic nutrients and minerals (vitamin C, calcium, iron, phosphorus, potassium and sodium) to make it suitable for ethanol production by fermentation with microorganisms. Cashew apple juice is a rich source of vitamin C and sugars and also presents functional properties such as cancer prevention and the prevention of bacteria (*Helicobacter pillori*) that causes severe gastritis, besides preserving antioxidant properties. Forty eight volatile compounds were identified in the cashew apple juice, the volatile compounds were predominantly esters (42 per cent of total compounds), followed by aldehydes (14 per cent) (Kubo *et al.,* 2006).

India is the third most producer of cashew apple in the world. Cashew is grown on the West coast (Maharashtra, Goa, Karnataka and Kerala), East coast (Tamil Nadu, Andhra

Pradesh, Orissa and West Bengal) and North-Eastern states of India. In Tamilnadu, it is cultivated at Cuddalore, Tindivanam, and Pudhukottai Districts. In recent years the economic value of cashew apple juice has increased. It can also be squeezed for fresh juice and then fermented into cashew wine, which is a very popular drink in West Africa. In parts of India, it is used to distill cashew liquor, referred to as *feni* (alcoholic drink). Fresh cashew apple juice (CAJ) and processed juice, called *cajuina*, are among the most popular natural products in Brazil and protocols for preparation of juice, jam, jelly, candy, etc., from cashew apple have been documented (Cavalcante *et al.,* 2003). Carvalho *et al.* (2007) have developed a blended beverage based on coconut water, cashew apple juice and caffeine. Costa *et al.* (2009) have developed fruit powders from cashew apple and guava (*Psidium guajava* L.) residues after extracting the juice and used as a source of vitamin C and lipid respectively.

However, cashew apple-based products are not produced on a commercial basis in India, unlike in Brazil. Blending of cashew apple waste (the residue left after production of *feni*) up to 10 per cent with cattle feed was shown to have no adverse effect on milk yield. Cashew apple contain tannins, causing astringency, and these have to be precipitated out before preparation of cashew apple juice. Procedures for removal of astringency from cashew apple by cooking in 2 to 4 per cent salt solution for 3 to 4 min, and preparation of juice, syrups and jams have been reported (Mini *et al*., 2005). According to the report of Tigressa *et al*. (2008) only 12 g $100g^{-1}$ of the total peduncle is processed. This pseudo fruit is underutilized and often fed to cattle and pigs. Honorato *et al.* (2007) have illustrated that cashew apple juice has been used as an alternative raw material for the production of various industrially important products such as surfactants, alcoholic beverages and cashew apple syrups.

2.8.1.5. Mature coconut water in bacterial cellulose production

Coconut water from mature nuts is abundantly available at desiccated coconut factories, coconut milk/cream factories and large copra processing centres. Nutrition contained in coconut water are not only micro elements, but also the macro elements such as carbon and nitrogen. In general, coconut water contains 4.7 per cent total solids, 2.6 per cent sugar, 0.55 per cent protein, 0.74 per cent fat and 0.46 per cent minerals. Types of sugars contained in coconut water are glucose, fructose and sucrose. It consists primarily of sugars, minerals, proteins and vitamins. It is slightly acidic, with pH around 5.5, transparent, non-sticky and slightly sweet taste (Matsui *et al*., 2005; Sousa *et al*., 2006). These features make the coconut water an excellent substrate for microbial growth. 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.

Bacterial cellulose is recognized by the FDA as edible and the *Acetobacter* is a non-pathogenic cellulose producing food grade bacterium. The major component of *nata* was shown to be cellulose and not dextran (Iguchi *et al*., 2000). *Nata* is considered one of the traditional holiday foods among Philippine people and is also popular in other Asian countries, including Indonesia, Japan and Taiwan, due to its distinctly soft texture and high fibre content. Over the years, it has become popular in other countries like Korea and USA. Bottled *nata-de-coco* is exported from Phillippines to Korea and USA. It is also produced for local consumption and export in Thailand, Indonesia and Sri Lanka. In Sri Lanka, it is called coconut cherry cubes. *Nata-de-coco* can also be produced from pine apple juice and such a product is called as *nata-de-pina* (Halib *et al*., 2012).

2.9. Animal experiment

Cellulose is found in abundance in nature and it is virtually found in all plant tissues and is therefore a common component of our diet. Modified celluloses such as ethyl and carboxy methyl derivatives and methyl cellulose (which may be prescribed as a laxative or as an appetite suppressant), have very different chemical properties from pure cellulose. Their substituent groups disrupt the hydrogen bonding and the resulting compounds are more soluble. Dietary cellulose is thought not to be digested in the stomach and small intestine. But 85 per cent of cellulose was recovered from ileostomy contents of the test rats. In the large intestine however, it is fermented by the microflora with the ultimate production of short chain fatty acids, hydrogen, carbon dioxide and methane (Edward, 1995).

In 1972, the US Food and Drug Administration declared cellulose to be generally recognised as safe (GRAS). In animal studies conducted by Freeman *et al*. (1978) cellulose was found to prevent colon cancer. Costerton *et al*. (1982) has illustrated that the mode of action of cellulose in the large intestine is probably related to its digestibility, because the water holding capacity of the material is very limited. Breakdown of cellulose in the colon stimulates microbial growth, while any undigested cellulose provides a surface for bacteria, which may lead to the growth of specialised subpopulations and fermentation of cellulose, which requires a complex interaction of microorganisms that eventually produces short chain fatty acids. Montgomery (1982) have indicated that cellulose is an inert material. In humans, bulk doses up to 30 g per day can be used as a laxative. In experimental animals, no reproduction toxicity was found in rats and mice after lifetime cellulose dosing in the diet.

Hillman *et al*. (1983) have reported that doses of purified cellulose from 15 to 20 g day^{-1} given to volunteers in long term feeding studies lead to modest increases in stool

output, shortening of transit time and a fall in stool pH. Cellulon^{TM} fibre is a cellulose produced by bacterial fermentation process employing a strain of *Acetobacter aceti* subsp. *xylinum* and it most closely resembles powdered and microcrystalline cellulose. Experiments revealed that Cellulon^{TM} was not a skin and eye irritant in rabbits (Schmitt *et al*., 1991). Lee and Hwang (1997) reported that cellulose increases faecal excretion by increasing water content and bulk, and elevating viscosity, and both water soluble and insoluble cellulose increases faecal egestion. Faecal water content and volume were increased by eating fibre that does not decompose and is not digested by coliform bacilli. Lee (2002) reported that intestinal transit time was diminished following administration of food-mixed with cellulose and 5 per cent guar gum, compared with rats given normal food. Moreover, cellulose increases faecal egestion and reduces intestinal transit time, as well as reducing faecal sojourn time within intestines.

Mesomya *et al*. (2002) studied on the comparative effects of 6 different high dietary fibre diets and casein diet on protein bioavailability (protein efficiency ratio, net protein utilization, biological value and digestibility) in male Sprague - Dawley rats. The results indicated the highest dietary fibre diet (>10 per cent) showed the highest content of faeces and decreased the value of net protein utilization and digestibility.

Mesomya *et al*. (2006) reported that feeding of *nata* decreases serum lipids in human and have indicated that 40 per cent of *nata-de-coco* effectively lowered serum triglycerides in human. Hagiwara *et al*. (2010) conducted studies on the oral toxicity of fermentation derived cellulose to F344 rats and proved that bacterial cellulose did not show any adverse effect when both the sexes of rats were fed with dietary concentration upto 5 per cent level and no toxicity was observed in any of the organ of rats.

CHAPTER III

MATERIALS AND METHODS

The details of the laboratory experiments conducted and the techniques adopted in the chemical, biochemical, microbiological and molecular analyses in the present investigation are presented in this chapter.

3. General

a. Location

All the laboratory experiments were conducted in the Department of Agricultural Microbiology, Tamil Nadu Agricultural University, Coimbatore which is located at an altitude of 426.7 meters above MSL, 11° N latitude and 77° E longitude.

b. Agro climatic conditions

The mean maximum and minimum temperature during the investigation period at Coimbatore were 32.2°C and 20.8°C respectively. The mean relative humidity was 63.5 per cent and the average annual rainfall was 674.2 mm.

c. Glasswares

The glassware of Borosil grade were used in all the experiments. The glassware used for the experiments were cleaned first with soap water and then treated with 6.5 per cent chromic acid solution (100 g potassium dichromate dissolved in one litre of water with 500 ml of concentrated sulphuric acid) for 4 to 5 h, and finally washed with water. The glassware thus cleaned were rinsed once with distilled water and sterilized in an hot air oven at 180°C for 3 h before use.

d. Sterilization techniques

All glassware were sterilized in an hot air oven at 180°C for 3 h. All growth media, broth and water blanks were sterilized in an autoclave at 15 lbs pressure for 20 min. Vitamin solution, growth factors and antibiotics were filter sterilized using Sartorius 0.22 µm nitrocellulose membrane filters. Isolation, purification, inoculation and other microbiological works were carried out in a laminar air flow chamber.

e. Composition of media, reagents, buffers and solutions

The compositions of different media used were given in Annexure I.

f. Chemicals used in the study

The analytical reagent (AR) grade chemicals of M/s. BDH, E-Merck Hi-Media, Qualigens and Sigma were used for media preparation and biochemical, microbiological and molecular studies.

3.1. Flow diagram of the experimental work

The flow diagram of the entire study with regard to bacterial cellulose (BC) production and value addition of BC into cellulose based products are illustrated in Fig. 1.

3.2. Isolation of cellulose producing bacteria from fruits and juices

A total of 23 samples of semi-rotting, fermenting fruits and juices such as apple, banana, cashew apple, citrus, cocoa extract, coconut milk, coconut water, coffee extract, custard apple, dates, grapes, guava, hibiscus flower nectar, mango, palm fruit, palm vinegar, papaya, pine apple, pomegranate, sapota, sugarcane juice (different sugarcane varieties collected from Sugarcane Breeding Institute (SBI), Coimbatore), tea extract and wood apple, etc., collected from local markets of Coimbatore and in the University premises (TNAU) were used for isolation. The fruit sample and juice sample was added 1 g or 1 ml each separately to 9 ml of sterilized peptone water, mixed thoroughly and serial dilutions for each sample were done separately. Then 1.0 ml aliquots from 10^6 dilutions were taken and spread on Glucose Yeast extract Calcium carbonate (GYC) medium and incubated at 30°C for 48h (Sowden and Colvin, 1978).

3.2.1. Purification of isolates

Morphologically distinct, single colony with zone of hydrolysis on the GYC plates were picked and purified by streaking on Glucose Yeast extract Peptone (GYP) medium.

3.2.2. Screening of isolates for bacterial cellulose (BC) production in HS medium

The purified colonies obtained for each of the isolates were serially named and used for production in Hestrin Shramn (HS) medium (Hestrin and Schramn, 1954).

3.2.3. Screening of elite cellulose producing bacteria

Approximately a loopful of the purified colonies of different strains isolated from different sources were transferred to test tubes containing 10 ml of HS broth and incubated at 30°C for 8 days. Among the various isolates, the best cellulose producing strain was screened and selected based on the thickness of the pellicle formed on the surface of the HS medium. The isolates were grouped according to Gallardo scale of thickness as poor (1mm thickness), fair (2 mm thickness), good (4mm thickness), excellent (5 mm thickness and above). Iolates with ≥5 mm thickness were selected for further study (Gallardo De Jesus and Elvira, 1971).

3.2.4. Standard bacterial cultures used in the study and their source

The reference culture, *Acetobacter xylinum* NCIM 2526 was collected from National Chemical laboratory (NCL), National Collection of Industrial Microorganisms (NCIM), Pune, India to compare the efficiency of selected strains. It was maintained in sorbitol medium as instructed by NCL. For comparison with the isolates obtained from the present study, the reference culture was cultured in HS medium.

3.3. Morphological characterization of selected isolates

The best isolates were morphologically characterized by following the methods given by Carr and Passmore (1979).

3.3.1. Colony characters

The colony characters *viz.*, colour, shape, size and colony character were observed on HS medium.

3.3.2. Gram staining

The Gram's reaction of isolates was observed as per the method described by Gerhardt *et al*. (1981).

3.3.3. Microscopical analysis

Morphology of the cells was examined under phase contrast microscope at 10X and 40X. The morphological investigations of the cells formed were also 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 at 6000 X.

3.4. Biochemical characterization of selected isolates

Biochemical characteristics such as cellulose production test, oxidase, catalase, indole production, urea utilization, citrate utilization, gelatin liquefaction, oxidation of ethanol, over-oxidation of acetic acid, dihydroxyacetone production test and special tests like, cellulose solubility test and sodium hydroxide treatment test of the cultures were performed as follows.

3.4.1. Cellulose production test (Swings, 1992).

About 1.0 ml of 24 h old culture was transferred to the 9.0 ml HS medium and incubated for 3 days at 30˚C. Formation of fleshy pellicle on the air liquid interface/ surface of the medium confirms the production of cellulose.

3.4.2. Oxidase test (Collins and Lyne, 1970)

Small pieces of filter paper were soaked in 1 per cent aqueous tetra methyl-p-phenylene diamine and placed in a Petri dish. Fresh young cultures to be tested were scraped with a glass rod and rubbed on the moistened filter paper. Development of a deep violet colour after 10 sec indicated positive oxidase test where as development of a light violet or no colour indicated negative oxidase test.

3.4.3. Catalase test (Smibert and Krieg, 1981)

Cellulose producing isolates were grown on nutrient agar slants for 24 to 48 h and flooded with 0.5 ml of 3 per cent hydrogen peroxide. Rapid effervescence showed positive result for catalase test.

3.4.4. Indole production test (Gillus, 1956)

The indole production test was performed by inoculating the bacterial isolates into tryptone broth. The indole production during the growth was detected by adding Kovac's reagent (P-dimethyl amino benzaldehyde), which produced cherry red colour for positive reaction.

3.4.5. Urea utilization test (Christensen, 1946).

Urea broth (0.01 per cent yeast extract, 0.91 per cent KH_2PO_4, 0.95 per cent Na_2HPO_4, 2.0 per cent urea and, 0.001 per cent phenol red) was used to determine urea utilization. Urease test was performed on 5 ml urea broth in test tubes containing phenol red (pH 6.8) as the pH indicator. The urea broth tubes inoculated with the isolates were incubated for 24 h. The development of reddish pink colour in the broth indicated, positive reaction for the test.

3.4.6. Citrate utilization test (Simmons, 1976)

Citrate test was performed to find out the ability of the isolates to utilize or ferment citrate as the sole carbon source. The test was carried out on Simmon's citrate agar medium. Change in colour of the medium from apple green to blue was positive for the test.

3.4.7. Gelatin hydrolysis test (Lysenko, 1961)

Hydrolysis of gelatin was investigated by inoculation of the isolates into nutrient gelatin tubes (0.5 per cent peptone, 0.3 per cent beef extract and 12 per cent gelatin) incubated at 37ºC for 1-2 weeks. Liquefaction of gelatin confirms positive for the test.

3.4.8. Overoxidation of ethanol test (Brenner *et al*., 2005).

Purified cultures were streaked onto $CaCO_3$-agar plates to confirm acid production and investigate overoxidation of ethanol by formation of clear zones around colonies. $CaCO_3$-agar medium was composed of 0.05 per cent D-glucose, 0.3 per cent peptone, 0.5 per cent yeast extract, 1.5 per cent $CaCO_3$, 1.2 per cent agar and 1.5 per cent (v/v) ethanol. Microbial growth was examined during incubation at 30ºC for 7 days.

3.4.9. Overoxidation of acetic acid test (Brenner *et al*., 2005).

To the clear zones around the colonies of $CaCO_3$-agar plates, approximately 0.25 ml of bromocresol green was added slowly. Overoxidation of acetic acid present in the clear zone leads to the production of CO_2 and H_2O which indicates the change of colour of the indicator bromocresol green to yellow and confirms positive for the test.

3.4.10. Dihydroxyacetone production test (Ishikawa *et al*., 1995).

The 24 h grown cultures were inoculated into a test tube having 3.0 per cent glycerol and incubated statically at 30ºC for 10 days. After incubation the production of dihydroxyacetone (DHA) was tested by the addition of Benedict's solution to it and noticed for change of colour. DHA was confirmed by the production of blue colour precipitate upon addition of Benedict's solution and confirms positive for the test.

3.4.11. Growth in Congo red medium (Zogaj *et al*., 2001)

The purified colonies of the isolates were grown in LB plates containing Congo red dye at 40 µg ml^{-1} for 48 h. Formation of red coloured colonies indicates that cellulose produced by the bacteria has taken up the colour of the Congo red dye.

3.4.12. Cellulose solubility test (Nadia *et al*., 2012)

The water insoluble pellicle formed on the HS medium was confirmed as cellulose by solubility of the pellicle in different solvents. Ten gram of BC pellicle was separately mixed with 4 per cent of methanol, acetone and cupriethylenediamine. The solubility of the fibre in each solvent was then observed. Complete solubility in cupriethylenediamine confirms positive for the cellulose solubility test.

3.4.13. Sodium hydroxide treatment test (Son *et al*., 2002)

The broth along with the pellicle was centrifuged for 10 min at 5000 rpm. After washing three times with distilled water, pellicles were subjected to boiling for 15 min with 0.5 N NaOH. The resistance of pellicle even after boiling and bleaching of the media components confirms that the product formed was cellulose and confirmed positive for the test.

3.5. Structural confirmation of BC using Fourier Transform – Infra Red Spectroscopy (FTIR) (Smith, 1996)

FTIR spectroscopy was used to identify the chemical structure of BC and compare the structural properties with the pure Micro Crystalline Cellulose (MCC). In transmission FT-IR spectrometer, the sample (1.0 mg) was placed in between two infrared-transparent plates. The infrared beam then impinges on the sample after passing

through the plates and the per cent transmittance was recorded against wavenumbers cm^{-1}. The FTIR spectrum of the celluloses were analyzed at wave numbers of mid IR range from 4000 to 400 cm^{-1}.

3.6. Visualization of BC by Scanning Electron Microscope (SEM)

The morphological investigations of the MCC and BC were characterized using Scanning Electron Microscope (SEM) (Model: S-3400 HITACH Co., Japan). Thin layers of freeze dried celluloses were gold coated separately using ion sputter (Fisons Instruments, UK). The gold coated samples were viewed and photographed using SEM at 6000 X and 8000 X for cells and cellulose fibrils respectively. To view the cellulose fibrils production of an individual cell, it was viewed under 37000 X.

3.7. Thermal Properties of BC using Thermo Gravimetric Analyzer (TGA) and Differential Scanning Colorimeter (DSC)

In order to confirm whether the pellicle formed was of pure cellulose, the thermal properties was compared with the micro crystalline cellulose and bacterial cellulose from standard *Acetobacter xylinum* NCIM 2526 isolate.

3.7.1. Thermogravimetric analysis (TGA) (Costa *et al*., 2011)

The dynamic weight loss and the bacterial cellulosic fibre weight variation at different temperature and its thermal stability was determined using Thermo Gravimetrical Analyzer model: Perkin-Elmer (TGA Q50V20). About 5.0 mg of air dried sample was taken for testing. Heating rate was set at the rate of 20°C min^{-1} under nitrogen atmosphere at the rate of 40 ml min^{-1}. The temperature range was selected from 0 to 1000°C. The weight loss curve and its derivative weight loss percent were obtained.

3.7.2. Differential Scanning Calorimeter (DSC) (Slusarska *et al*., 2008)

The crystalline and thermal behavior of the celluloses were carried out with the help of Differential Scanning Calorimeter (DSC). The decomposition rate of cellulose was quantified using DSC Q20 V24.2 model, Germany. The thermal property was analyzed in nitrogen free atmosphere in the following conditions: sample weight 15.0 mg taken in an aluminium crucible, rate of heating = 10°C min^{-1}, temperature range of

50 to 400°C. The crystallinity (Tc) and melting temperature (Tm) were recorded as the inflection point of the increment of specific heat and as the peak value of the endothermic process in the DSC curve.

3.8. Molecular characterization of cellulose producing isolates (Navarro and Komagata, 1999)

The cellulose-producing bacteria isolated from sugarcane juice (sju-1) produced a maximum cellulose pellicle thickness of 13 mm and was selected for further study. The isolate sju-1 was subjected to 16S rRNA gene sequence analysis.

3.8.1. Isolation of total genomic DNA

The genomic DNA of selected isolate sju-1 was extracted using the method given by Melody (1997) with slight modifications. Actively grown culture of 25 ml quantity was taken in a centrifuge tube and centrifuged at 6,000 rpm for 5 min at 4ºC in a Kubota refrigerated centrifuge (Tokyo, Japan). The supernatant was removed, the pellet was suspended in 1 ml TE buffer, added with 0.5 ml of 1-butanol, vortexed well to mix with the cells (to remove extracellular materials), centrifuged at 5000 rpm for 5 min at 4ºC, the supernatant was discarded and the pellet was resuspended in 2 ml of TE buffer and centrifuged again at 5000 rpm for 5 min to remove all traces of butanol. The pellet was resuspended in 1 ml TE buffer added with 100 µl lysozyme (10 mg ml^{-1} freshly prepared) and incubated at room temperature for 5 min. After incubation, 100 µl of 10 per cent SDS and 25 µl of 100 µg ml^{-1} proteinase K were added, mixed well and incubated at 37°C for 1 h. To this 200 µl of 5 M NaCl was added and mixed well. CTAB solution in 150 µl quantity was added, mixed well and incubated at 65ºC for 10 min. The mixture was extracted with 1 ml of phenol: chloroform mixture, mixed well and centrifuged at 6000 rpm for 15 min at 4ºC. The aqueous layer was transferred carefully to a 2 ml microfuge tube and DNA was precipitated by adding 0.6 volume of ice cold isopropanol, incubated overnight at -20 ºC. The DNA was pelletized by centrifugation at 12000 rpm for 15 min at 4ºC. The pellet was washed with 70 percent ethanol, dried under vacuum for 10 min and resuspended in 50 µl of TE buffer. One µl DNAse free RNAse (10mg ml^{-l}) was also added by swirling and incubated at 37ºC for 30 min. The purity of the genomic DNA was assessed from the A260/A280 and A260/A230 extinction ratios. The DNA was stored at -20ºC for further use.

3.8.2. Agarose gel electrophoresis

Agarose gel electrophoresis was performed based on the method given by Sambrook *et al.* (1989) to check the quality of DNA and also to separate the products amplified through polymerase chain reaction. 1X TAE tank buffer in 500 ml quantity was prepared to fill the electrophoresis tank and to prepare the gel. In a separate conical flask, agarose (0.8 per cent for genomic DNA and 1.5 per cent for PCR product) was added to 1X TAE buffer, boiled till the agarose dissolved completely and cooled to lukewarm temperature. Ethidium bromide was added at the rate of 5 µl 100 ml^{-1} to agarose solution and was allowed to mix completely. It was then poured into the gel mould and the comb was placed properly, allowed to solidify for half an hour at room temperature. After solidification the comb was removed carefully. The caste gel was placed in the electrophoresis tank containing 1X TAE buffer with the well near the cathode and submerged to a depth of 1 cm. Fifteen µl of the PCR product was mixed with 3µl of 6X tracking dye (Bromo phenol blue) and mixed well by pipetting in and out for 3 times. The mixture was loaded into the wells with the help of the micropipette. Two µl of 1 kb DNA ladder (Fermentas, USA) was loaded in one of the wells as a standard marker. The cathode and anode were connected to power pack using power cord and the gel was run at a constant voltage of 60 volts. The negatively charged DNA molecules move towards the anode and get separated according to their molecular weight. The power was turned off when the marker reached the anode end and the gel was viewed in an UV transilluminator and the banding pattern was analyzed.

3.8.3. Gel documentation

After the separation of the PCR products with 1 per cent agarose gel, it was viewed and photographed using Alpha imager TM1200 documentation and analysis system.

3.8.4. Amplification of 16S rDNA (Weisberg *et al.*, 1991)

Full-length 16S rDNAs (1500 bp) were amplified from the isolates by PCR using the universal forward primer fp1 (5' AGAGTTTGATCGTGGCTCAG 3') and the universal reverse primer rp2 (5' ACGGCTACCTTGTTACCACTT 3'). The 45 µl PCR reaction mixture consisted of DNA template 50 ng, 1XTaq buffer, 0.2 mM of each of

dNTP mixture, 1 μM of each primers, 1.5mM $MgCl_2$ and 2U of Taq DNA polymerase (Bangalore Genei, India). PCR amplification was performed in a thermocycler (Eppendorf Master cycler, German) using the following conditions: initial denaturation at 95°C for 1 min, 35 cycles consisting of 94°C for 1 min (denaturation), 60°C for 1 min (annealing), 72°C for 1 min (primer extension) and final extension at 72°C for 5 min.

3.8.5. Sequencing analysis

Sequencing reactions were performed using ABI prism terminator cycle sequencing ready reaction kit and electrophoresis of the products were carried out on an Applied Biosystems (Model 3100) automated sequencer at Yaazh Xenomics, Madurai.

3.8.6. Phylogenetic analysis

A specific fragment for 16S rRNA gene-coding regions was amplified using PCR amplification. Two primers, 20F (5'-GAG TTT GAT CCT GGC TCA G-3'; positions 9-27) and 1500R (5'-GTT ACC TTG TTA CGA CTT-3'; positions 1509-1492) were used. The positions in the rRNA gene fragment were based on the *Escherichia coli* numbering system (accession number V00348). The purified 16S rRNA genes from positions 9 to 1509 (approximately 1,500 bases) were sequenced by using four primers, 27F (5'-AGA GTT TGA TCM TGG CTC AG-3'; positions 27-46), 800R (5'-TAC CAG GGT ATC TAA TCC-3'; position 800-783), 518F (5'- CCA GCA GCC GCG GTA ATA CG-3'; position 518- 537) and 1492R (5'- TAC GGY TAC CTT GTT ACG ACT T-3'. A phylogenetic tree for 1456 bases was constructed by the Neighbour-Joining (NJ) method using MEGA programme (version 5.0) after multiple alignments of the sequences obtained with CLUSTAL W (Tamura *et al*., 2007). The distance matrices for the aligned sequences were calculated by the two-parameter method. The bootstrap values at branching points in the phylogenetic tree were calculated with 1,000 replications. A 16S rRNA gene sequence similarity between the type strain of *Gluconacetobacter* sp. enlisted in the NCBI database and the selected strain (sju-1) was calculated for 1,456 bases.

3.9. Effect of culture concentration on cellulose production by sju-1

The culture concentration required for optimum cellulose production was standardized by inoculating different concentration of the 24 h grown culture sju-1 *viz.,* 5,

10, 15 and 20 per cent was inoculated into 100 ml of sterilized HS medium and incubated at static condition at 30°C for 20 days. Dry weight of the cellulose was recorded for every concentration at the end of incubation. The culture concentration at which maximum cellulose production obtained was recorded.

3.10. Effect of incubation time and temperature on cellulose production by sju-1

The time required for maximum cellulose production was standardized by inoculating 10 ml of the culture broth containing sju-1 into 100 ml of sterilized HS medium and incubated at static condition at 30°C. Dry weight of the cellulose was recorded at the interval of 20 days. The day at which maximum cellulose production obtained was recorded. The isolate was grown in different temperatures from 25°C to 35°C at 2°C increase and the optimum temperature at which maximum cellulose production obtained was recorded.

3.11. Effect of culture conditions on the cellulose production by sju1

To optimise the culture condition, the selected strain sju-1 was cultured in two different conditions namely static culture and shaken culture at 120 rpm.

3.11.1. Static culture condition

In this method, 10 ml of 24 h old culture containing 20 x10^8 cfu ml^{-1} of the seed culture of sju-1 was transferred into 250 ml flask containing 100 ml of sterilized HS medium. After inoculation the flasks were incubated at 30°C without shaking the flask for 8 days. Similarly the standard culture *Acetobacter xylinum* NCIM 2526 was also inoculated and incubated for comparison.

3.11.2. Shaken flask culture condition

In the shaken flask culture method 10 ml of the seed culture of sju-1 was transferred into 250 ml flask containing 100 ml of sterilized HS medium. After inoculation with 24 h old cultures containing 20 x10^8 cfu ml^{-1} of *Gluconacetobacter* sp. sju-1, the flasks were incubated at 120 rpm at 30°C in an incubator shaker (Orbitek, Scigenics Biotech, India) for 8 days. Similarly the reference culture *Acetobacter xylinum* NCIM 2526 was also inoculated and incubated for comparison.

3.11.3. Measurement of yield parameters on cellulose production by sju-1

3.11.3.1.Measurement of total biomass (Kouda *et al*., 1997)

For biomass, the BC pellicle of sju-1 was added to 90 ml 0.1 M potassium acetate-acetate buffer (pH 5.0) and 10 ml of 20 per cent cellulase solution and incubated at 30°C with shaking at 100 rpm for 1 h to hydrolyze BC. Then, the solution was centrifuged at 5000 rpm for 20 min. The precipitate was washed with deionized water twice and centrifuged for 6000 rpm for 30 min at 4°C. Finally, the precipitate was dried in an oven at 80°C overnight and then weighed to determine biomass

3.11.3.2. Measurement of viable cell number (Forng *et al*., 1989)

Total viable cell number comprises the viable cells entrapped in cellulose pellicle and those suspended in the broth. The culture broth was incubated with 20 ml of cellulase (cellucast, Hi-Media) at 30°C for 1 h to hydrolyze BC pellicle and the solution was plated in HS medium. The total cell number was counted as colony forming units per ml (cfu ml^{-1}).

3.11.3.3. Determination of wet weight and dry weight of BC (George *et al*., 2005).

The precipitated BC were treated with 0.1 N NaOH solution at 80°C for 30 min to remove the bacterial cells and medium components. This NaOH treatment was repeated three times and then, the solution was centrifuged at 5000 rpm for 20 min. Then it was repeatedly washed with distilled water several times and dripped free of water. Then the wet weight was recorded. Dry weight of the purified cellulose was estimated by drying in an oven at 80°C overnight and then weighed.

3.11.3.4. Determination of moisture content of bacterial cellulose

The moisture content (per cent) was determined based on the weight loss of the bacterial cellulose using the formula

$$\text{Moisture content (\%)} = \frac{w_1 - w_2}{w_2} \times 100$$

where,

w_1 = wet weight of the bacterial cellulose (g)

w_2 = dry weight of the bacterial cellulose (g)

3.11.3.5. Estimation of biosynthetic yield

The yield of the biosynthesis process was calculated using the formula:

$$\text{Biosynthetic yield (\%)} = \frac{\text{Dry weight of the cellulose in g (C)}}{\text{Weight of the carbon source in the substrate in g (G)}} \times 100$$

3.11.3.6. Estimation of soluble polysaccharides (Dubois *et al.*, 1956)

Water soluble polysaccharides other than cellulose was estimated by phenol sulphate method. 100 µg of the cellulose pellicle was taken in 50 mM sodium acetate and added with 100 µL freshly made 5 per cent phenol in water. About 1 ml of conc. H_2SO_4 was added into the mixture and shaked well. Immediately it was vortex-mixed for several seconds. An orange colour with intensity proportional to concentration will begin to develop immediately and reach a maximum of about 2 h at 30°C. It was read against glucose standards at 500 nm.

3.12. Determination of optimum bacterial cellulose production at different growth conditions

Optimal cellulose production was measured by growing culture in HS medium under different pH (4.0,.4.5, 5.0,5.5,6.0,6.5,7.0,7.5, and 8.0), temperature (25, 27, 29, 31,33 and 35°C), carbon sources (fructose, galactose, glucose, glycerol, inositol, malic acid, mannitol, mannose, sorbitol, starch and sucrose), nitrogen sources (ammonium chloride, ammonium sulphate, ammonium carbonate, ammonium nitrate, beef extract, calcium nitrate, casein hydrolysate, malt extract, peptone, potassium nitrate, sodium nitrate and yeast extract). The effect of two growth hormones namely kinetin and Indole Acetic Acid (IAA) on optimum cellulose production were tested. The effect of different additives in cellulose production was measured by the addition of different polysaccharides such as agar, agarose, acetan, calcium alginate, chitin, chitosan, cellulose, carboxy methyl cellulose, dextran, gelatin, gellan, levan, lignosulfonate and poly ethylene glycol to the HS medium. The optimum conditions (pH, temperature, carbon, nitrogen source, additives and growth hormone) at which the culture showed higher quantities of cellulose were recorded.

3.12.1. Effect of different pH on the dry weight of bacterial cellulose

Different ranges of pH (4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0), were prepared separately using HS medium containing citrate disodium hydrogen phosphate buffer was inoculated with 24 h old cultures containing 20 $x10^8$ cfu ml^{-1} of *Gluconacetobacter* sp. sju-1 and incubated at 30°C for 20 days.

3.12.2. Effect of different temperature on the dry weight of bacterial cellulose

Effect of temperature on optimum cellulose production was tested by inoculating 24 h old cultures containing 20 $x10^8$ cfu ml^{-1} of *Gluconacetobacter* sp. sju-1 at different temperature such as 25, 27, 29, 31, 33 and 35°C in an incubator for 20 days.

3.12.3. Effect of different carbon sources on the dry weight of bacterial cellulose

The effect of different carbon sources (glucose, sucrose, fructose, galactose, rhamnose, mannose, malic acid, mannitol, sorbitol, inositol, glycerol and starch) on cellulose production were studied by varying one factor (C sources) at a time and keeping other factors constant. The carbon source of HS medium is glucose (2 per cent). Hence the different carbon sources were taken at 2 per cent of 1M of respective carbon source separately in 250 ml flasks and added with 5.0 g yeast extract, 5.0 g of peptone and citric acid 2.7 g and disodium hydrogen phosphate 1.17 g in each of the different carbon containing flask and inoculated with 24 h old cultures containing 20 $x10^8$ cfu ml^{-1} of *Gluconacetobacter* sp. sju-1 and incubated at the pre-optimised temperature of 31°C for 20 days.

3.12.4. Effect of different nitrogen sources on the dry weight of bacterial cellulose

The effect of different nitrogen sources (ammonium chloride, ammonium sulphate, ammonium carbonate, ammonium nitrate, calcium nitrate sodium nitrate, potassium nitrate, yeast extract, peptone, malt extract, beef extract and casein hydrolysate) on cellulose production was standardized by taking each nitrogen source at a concentration of 1.0 per cent with fructose and all other factors constant. Then it was inoculated with 24 h old cultures containing 20 $x10^8$ cfu ml^{-1} of *Gluconacetobacter* sp. sju-1 and incubated at 31°C for 20 days.

3.12.5. Effect of growth hormones on the dry weight of bacterial cellulose

Two growth hormones namely kinetin and indole acetic acid were tested for the effectiveness in increasing the growth of cellulose producing bacteria. Different concentration ranging from 200 ppm to 1000 ppm of two hormones were prepared in standard volumetric flaks. Accurately 1.0 ml of each growth hormone of each concentration were taken in a separate 250 ml Erlenmeyer flask. To this, 24 h old cultures containing 20 $x10^8$ cfu ml^{-1} of *Gluconacetobacter* sp. sju-1 was inoculated and incubated for 20 days at 31°C.

3.12.6. Effect of different additives on the dry weight of bacterial cellulose

Different polysaccharides such agar, agarose, acetan, calcium alginate, chitin, chitosan, cellulose, carboxy methyl cellulose, dextran, gelatin, gellan, levan, lignosulfonate and poly ethylene glycol were taken at 0.1 per cent level in separate 250 ml Erlenmeyer flasks and added with other components of pre-standardised medium. It was sterilized in an autoclave, inoculated with 24 h old cultures containing 20 $x10^8$ cfu ml^{-1} of *Gluconacetobacter* sp. sju-1 and incubated at 31°C for 20 days.

3.13. Bioconversion of Mature Coconut Water (MCW) and Cashew Apple Juice (CAJ) into bacterial cellulose using sju-1

3.13.1. Collection and preparation of Mature Coconut Water (MCW)

Mature coconut water was collected from various processing units, University canteen, hostels and from temples. They were pooled, mixed well and filtered using a muslin cloth to remove dust particles. Then it was refrigerated under 4°C for further use.

3.13.2. Collection and extraction of Cashew Apple Juice (CAJ)

Cashew apples were cut into slices and were crushed in a mixer cum grinder. The initial extract was prepared by soaking 200 g of cashew apples in 500 ml of distilled water and then mixed well with a blender for 1 min at low speed and an additional 3 min at a high speed. The homogenised extract was filtered through a double layer of cheese cloth. The juice was extracted by a juice squeezer and clarified by adding 1 per cent gelatin to remove tannin and suspended solids. The treated juice was filtered and treated with sodium or potassium meta-bisulphite to prevent the growth of microorganisms. The juice sample was collected in a jar and preserved at 4°C.

3.13.3. Proximate composition of MCW and CAJ

The mature coconut water and cashew apple juice was analyzed for the properties such as pH, Total Soluble Solids (TSS) °Brix, Total Titrable Acidity (TTA) (%), Reducing sugars (g), Protein (g), Fat (g), Potassium (mg), Sodium (mg), Calcium (mg), Magnesium (mg), Phosphorous (mg), Copper (µg) and Iron (µg) by the procedures suggested by AOAC (1990).

3.13.3.1. Determination of pH

Twenty ml of mature coconut water and cashew apple juice was taken in a 100 ml beaker separately. Forty ml of distilled water was added to the beakers and stirred well with a glass rod and allowed to stand for 30 min with intermittent stirring. pH was determined using a pH stick by immersing it in a beaker containing the samples.

3.13.3.2. Determination of Total Soluble Solids (TSS)

Total soluble solids content of a solution is determined by the index of refraction. This is measured using a refractometer, and is referred to as the degrees Brix. Brix is the term used when a refractometer equipped with a scale, based on the relationship between refractive indices at 20°C and the percentage by mass of total soluble solids of a pure aqueous sucrose solution. This tests the solids concentration of a sucrose containing solution. Sugar concentration is expressed in degrees Brix. At 20°C, the Brix is usually considered equivalent to the percentage of sucrose (sugar) in the solution (60°Brix is equivalent to a sugar content of 60 per cent). The measurement must be made at 20°C to get an accurate value.

3.13.3.3. Determination of Total Titrable Acidity (TTA)

One ml of phenolphthalein indicator was added to 200 ml of hot boiled deionized water. Five ml of juice sample was transferred volumetrically to 0.05N NaOH. The endpoint was the appearance of pink colour. TTA was calculated as follows and expressed in per cent.

$$\text{Total Titrable Acidity (\%)} = \frac{\text{(Volume of base) x (N base) x (0.075) x (100)}}{\text{Sample volume (ml)}}$$

Where,

Volume of base - Volume of NaOH used in ml

N base - Normality of NaOH.

3.13.3.4. Determination of reducing sugars

Reducing sugar content of each juice sample was determined by Nelson and Somogyi method**.** Accurately 10 g alkaline copper tartrate was dissolved in 2.5 g anhydrous sodium carbonate, 2 g sodium bicarbonate, 2.5 g potassium sodium tartrate and 20 g anhydrous sodium sulphate in 80 ml water and made up to 100 ml. 15 g of copper sulphate was dissolved in a small volume of distilled water. One drop of sulphuric acid was added into it and made up to 100 ml. Accurately 96 ml of alkaline copper tartrate was mixed with 4 ml of copper sulphate solution. Arsenomolybdate reagent was prepared by dissolving 2.5 g ammonium molybdate in 45 ml water. Accurately 2.5 ml of sulphuric acid was added and mixed well. Then 0.3 g of disodium hydrogen arsenate was added and dissolved in 25 ml water. It was mixed well and incubated at 37°C for 24 to 48 h. Standard glucose solution was mixed by dissolving 100 mg in 100 ml distilled water. Working standard was prepared by diluting 10 ml of stock 100 ml with distilled water (100 $\mu g\ ml^{-1}$).

About 100 ml of the sample was taken and sugar was extracted with hot 80 per cent ethanol twice (5 ml each time). Supernatant was collected and evaporated by keeping it on a water bath at 80ºC. To this 10ml of water was added to dissolve the sugars. Pipette out aliquots of 0.1 or 0.2 ml to separate tubes. Then 0.2, 0.4, 0.6, 0.8 and 1.0 ml of the working standard solution was pipetted out into a series of test tubes. Volume was made for both sample and standard tubes to 2 ml with distilled water. Pipette out 2ml distilled water in a separate tube to set a blank. About 1ml of alkaline copper tartrate reagent was added to each tube and placed in boiling water for 10 min. Then it was cooled and added with 1.0 ml of arsenomolybdic acid reagent to all the tubes. Then the volume was made upto 10 ml with water. The absorbance was read at 620 nm for 10 min or till the blue colour develops. From the graph drawn, amount of reducing sugars present in the sample was calculated as follows and calculated in grams.

Absorbance corresponds to 0.1ml of test = X mg of glucose

$$10 \text{ ml contains} = \frac{X}{0.1} \times 10\text{mg of glucose}$$

3.13.3.5. Determination of protein

Solution A was prepared by mixing 2.0 g of Na_2CO_3 and dissolved in 0.1 M NaOH. Solution B was prepared by taking 1.0 g of $CuSO_4.5H_2O$ dissolved in 80 ml of distilled water and made upto 100 ml. Two g of sodium potassium tartrate was taken and dissolved in 80 ml of distilled water and made up to 100 ml to get Solution C. Folin-ciocalteau reagent at a concentration of 2 N was prepared and mixed with distilled water in the ratio 1:1, before use. Lowry stock reagent was prepared by adding 49 ml of solution A to 0.5 ml of solution B and 0.5 ml of solution C. Then the reagent was mixed thoroughly.

Aliquots of (0.1 ml) each sample was pipetted out in test tubes and simultaneously 0.2, 0.4, 0.6, 0.8 and 1 ml of working standard was pipetted out into test tubes. Volume of samples and standards was made up to 2 ml with distilled water. To one ml of distilled water blank, one ml of Lowry stock reagent was added to each tube. The tubes were incubated at room temperature for 30 min and after cooling the tubes were added with 2 ml of Folin reagent. Finally, the volume was made up with distilled water to 10 ml and left for 10 min. The absorbance was recorded at 620 nm using spectrophotometer and the values were plotted in a graph against concentration. Protein content in the sample was calculated and expressed in grams.

3.13.3.6. Determination of fat

Accurately 2.0 g of sample was weighed into a filter paper thimble. The fat was extracted with petroleum ether for 6 h in a soxhlet apparatus. The extraction was done by heating on the mantle. The ether in the flask was evaporated over a water bath and then the outside wall was wiped. Finally it was transferred into an hot air oven and dried for 30 min. at 100°C, cooled in a desiccator and weighed.

$$\text{Fat content (g/ 100g)} = \frac{100 \times (X-Y)}{W}$$

where, X = weight of the flask with fat (g)

Y = weight of the flask (g)

W = weight of the sample (g)

3.13.3.7. Estimation of potassium and sodium

Five ml tri-acid extracted sample was pipetted out (digested with tri-acid mixture: nitric, sulphuric and perchloric acids in the ratio of 9:2:1) into 25 ml volumetric flask and neutralized with ammonium hydroxide (the piece of red litmus put into the flask turned blue). The volume was made up with distilled water and mixed well to obtain a homogenous solution. The concentration of potassium and sodium in the solution was determined by using flame photometer at 768 and 580 nm respectively. The concentration of potassium and sodium in the solution was deduced from the standard curve and expressed in mg per 100g.

3.13.3.8. Estimation of calcium and magnesium

The calcium and magnesium contents were estimated by Versenate method. Five ml of the extract (digested with tri-acid mixture nitric, sulphuric and perchloric acids in the ratio of 9:2:1) was taken in a porcelain basin to which 5 ml of sodium hydroxide, pinch of murexide indicator (for calcium) and 5 ml ammonium chloride - ammonium hydroxide, pinch of Eriochrome black T indicator (for magnesium) were added. The content was titrated against 0.02 N EDTA. The results on total calcium and magnesium contents were expressed in mg per 100g.

3.13.3.9. Estimation of copper and Iron

The micronutrients namely copper and iron were estimated using Atomic Absorption Spectrophotometer (AAS) About 5 ml of the extract digested with tri-acid mixture (nitric, sulphuric and perchloric acids in the ratio of 9:2:1) was fed into the atomic absorption spectrophotometer. The content of copper and iron were measured in AAS by using the wavelengths of 325 and 248 nm respectively.

3.14. Bioconversion and value addition of MCW and CAJ into bacterial cellulose and cellulose based products

3.14.1. Pre-optimisation of media components using MCW

Media components such as mature coconut water, fructose, citric acid and disodium hydrogen phosphate were pre-optimised to standardise the optimum concentration of the components. The various treatment combinations used for pre-optimisation of MCW media are presented in Table 2.

3.14.2. Pre-optimisation of media components using CAJ

Media components such as cashew apple juice, fructose, citric acid and disodium hydrogen phosphate were pre-optimised to standardise the optimum concentration of the components. The various treatment combinations used for pre-optimisation of cashew apple juice CAJ media are presented in Table 3.

3.15. Media optimisation using Response Surface Methodology (RSM)

(Bas and Boyaci, 2007)

Central Composite Design (CCD) contains an embedded factorial or fractional factorial design with center points that is augmented with a group of 'star points' that allow estimation of curvature and number of base level points 'n_0' to estimate the experimental error. A central composite design always contains twice as many star points as there are factors in the design. The star points represent new extreme values (low and high) for each factor in the design (Annexure II). The distance from the center of the design space to a factorial point is ± 1 unit for each factor and the distance from the center of the design space to a star point is $\pm \alpha$ with $| \alpha | > 1$. The precise value of 'α' depends on certain properties desired for the design and on the number of factors involved. In order to maintain rotatability, the value of 'α' depends on the number of experimental runs in the factorial portion of the central composite design.

3.15.1. Media optimization by RSM using mature coconut water (MCW) medium

RSM was used for the design of experiments for the screening of four critical media components, as independent variables. The variables were fructose, yeast extract, citric acid and disodium hydrogen phosphate. Dry weight of the cellulose (g l^{-1}) was

selected as the dependent variable. The design consists of 30 experimental trials and the experiments were conducted in a randomized fashion at two levels of concentrations (high level and low level).

The independent factors considered for optimization are Fructose concentration (FC), Yeast extract concentration (YC) Citric acid concentration (CC) and Disodium hydrogen phosphate concentration (DC). The natural, coded levels and interval of variation of the independent variables in the experimental plan for the optimization of cellulose production process is given in Appendix II. The basic points *i.e.,* low and high values for the design were selected from a pre-optimisation studies conducted using MCW as a base and other media components. According to central composite rotatable design for 4 independent factors, the total experiments to be conducted were found to be 30. Thirty experiments were performed for 4 variables with 5 levels of each variable (Appendix II).

3.15.2. Media optimization by RSM using cashew apple juice (CAJ) medium

The independent factors considered for optimization are Fructose concentration (FC), Yeast extract concentration (YC) Citric acid concentration (CC) and Disodium hydrogen phosphate concentration (DC). The natural, coded levels and interval of variation of the independent variables in the experimental plan for the optimization of cellulose production process are given in Annexure II. The basic points *i.e.*, low and high values for the design were selected from a pre-optimisation studies conducted using cashew apple juice as a base and other media components.

According to central composite rotatable design for 4 independent factors, the total experiments to be conducted were found to be 30. Thirty experiments were performed for 4 variables with 5 levels of each variable are given in Annexure II.

3.16. Physical properties of BC from HS, MCW and CAJ medium

3.16.2. Colour analysis of BC (Clydesdale, 1978).

Bacterial cellulose obtained from three media were subjected to colour analysis. A section of 10 g of bacterial cellulose was cut into 1 cm^3 sections to measure colour appearance. The colour parameters like lightness L*, a*, and b* values were measured by

Table 2. Pre-optimisation of Mature Coconut Water (MCW) media for bacterial cellulose production

S. No.	Treatment combinations
1.	Mature coconut water alone (1000 ml)
2.	Mature coconut water (1000 ml)+ 10 g fructose
3.	Mature coconut water (1000 ml) + 15 g fructose
4.	Mature coconut water (1000 ml)+ 20 g fructose
5.	Mature coconut water (1000 ml) + 15 g fructose + 5.0 g yeast extract
6.	Mature coconut water (1000 ml) + 15 g fructose +7.5 g yeast extract
7.	Mature coconut water (1000 ml) + 15 g fructose + 10.0 g yeast extract
8.	Mature coconut water (1000 ml)+ 15 g fructose + 10.0 g yeast extract +1.5 g citric acid
9.	Mature coconut water (1000 ml)+ 15 g fructose + 10.0 g yeast extract +2.0 g citric acid
10.	Mature coconut water (1000 ml)+ 15 g fructose + 10.0 g yeast extract +2.5 g citric acid
12.	Mature coconut water (1000 ml) + 15 g fructose + 10.0 g yeast extract +2.0 g citric acid+ 1.10 g disodium hydrogen phosphate
13.	Mature coconut water (1000 ml) + 15 g fructose + 10.0 g yeast extract +2.0 g citric acid+ 1.15 g disodium hydrogen phosphate
14.	Mature coconut water (1000 ml) + 15 g fructose + 10.0 g yeast extract +2.0 g citric acid+ 1.20 g disodium hydrogen phosphate

Table 3. Pre-optimisation of Cashew Apple Juice (CAJ) media for bacterial cellulose production

S.No.	Treatment combinations
1.	Cashew apple juice alone (250 ml)
2.	Cashew apple juice alone (500 ml)
3.	Cashew apple juice alone (750 ml)
4.	Cashew apple juice alone (1000 ml)
5.	Cashew apple juice (1000 ml) + 2.0 g fructose
6.	Cashew apple juice (1000 ml)+ 4.0 g fructose
7.	Cashew apple juice (1000 ml) + 6.0 g fructose
8.	Cashew apple juice (1000 ml) + 8.0 g fructose
9.	Cashew apple juice (1000 ml) + 4.0 g fructose + 2.0 g yeast extract
10.	Cashew apple juice (1000 ml) + 4.0 g fructose + 4.0 g yeast extract
11.	Cashew apple juice (1000 ml) + 4.0 g fructose + 6.0 g yeast extract
12.	Cashew apple juice (1000 ml)+ 4.0 g fructose + 8.0 g yeast extract
13.	Cashew apple juice (1000 ml) + 4.0 g fructose + 6.0 g yeast extract +0.5 g citric acid
14.	Cashew apple juice (1000 ml)+ 4.0 g fructose + 6.0 g yeast extract +1.0 g citric acid
15.	Cashew apple juice (1000 ml) + 4.0 g fructose + 6.0 g yeast extract +1.5 g citric acid
16.	Cashew apple juice (1000 ml) + 4.0 g fructose + 6.0 g yeast extract +2.0 g citric acid
17.	Cashew apple juice (1000 ml)+ 4.0 g fructose + 6.0 g yeast extract +1.5 g citric acid+ 0.5 g disodium hydrogen phosphate
18.	Cashew apple juice (1000 ml)+ 15 g fructose + 6.0 g yeast extract +1.5 g citric acid+ 0.75 g disodium hydrogen phosphate
19.	Cashew apple juice (1000 ml) + 15 g fructose + 6.0 g yeast extract +1.5 g citric acid+1.0 g disodium hydrogen phosphate

a Hunter Lab Colour Quest (Memmert, Germany) colorimeter with the CIELAB colour system. These values were then used to calculate Chroma or saturation (C*). L* indicates lightness, with a scale ranging from 0 (black) to 100 (white). Positives and negatives in a* represent red and green, whereas positives and negatives in b* represent yellow and blue, respectively.

3.16.3. Texture evaluation of BC (Bourne, 2002)

Bacterial celluloses, sized 7.5x7.5x1.0 cm were used after incubation in an hot air oven at 100°C for 3 h. Texture was analysed with texture analyser (TA-XT21, Germany). The instrument was set as follows. 4.0 cm distance between probe and samples, 1.0 mm sec^{-1} pretest speed, 1.0 mm sec^{-1} test speed, and 10.0 mm sec^{-1} post test speed. The compression test was run at a rate of 20 times per sample. The compression force was recorded.

3.16.4. Determination of water holding capacity of BC (Bielecki *et al*., 2005)

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 (per cent) 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 and the dry weight was calculated, thickness (mm) was tested by using a micrometer type calliper.

Cellulosic mat after cutting into perfect cubes of equal dimensions was wrapped in filter paper and centrifuged at 5000 rpm 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 water holding capacity of the BC.

3.16.6. Determination of crude fibre of BC

Crude fibre estimation was done as outlined by Chopra and Kanwar (1976). Two g of ground sample was taken in a 500 ml beaker; 200 ml of 1.25 N sulphuric acid was added and boiled for 30 min. The volume was maintained constant by replacing the water lost by evaporation. After 30 min, the mixture was filtered through a muslin cloth and washed with hot water till it was free of acid. The residue was then transferred to the

same beaker and 200 ml of 2.5 N sodium hydroxide was added. The mixture was allowed to boil for 30 min. while keeping the volume constant. The mixture was then filtered through ashless filter paper. The residue was washed with water to remove the alkali. The filter paper with the residue was transferred to a pre-weighed silica crucible (w_1). It was dried overnight at 100±5°C and weighed (w_2). This was ignited for 30 min. in a muffle furnace. After cooling, the weight (w_3) was taken. The loss in weight on ignition was worked out and the crude fibre content was calculated.

$$\text{Crude fibre content (g)} = \frac{(w_2-w_1) - (w_3- w_1)}{\text{Weight of the sample}}$$

where,

w_1= pre-weighed dish (g)

w_2 = weight of dish after drying (g)

w_3 = weight of dish after ignition and cooling (g)

3.16.7. Estimation of soluble and insoluble fibre content of BC (Johansson *et al*., 1983)

To 1.0 g of the sample, 15.0 ml of phosphate buffer (0.1M, pH 6.0) was added. Termamyl (0.1ml) was added while keeping it in water bath for 30 min with occasional stirring. The contents were cooled, 20 ml water added and pH adjusted to 1.5 with HCl (4N). Pepsin (100 mg) was added and incubated in a shaking water bath at 37°C for 1 h. The contents were again cooled, 20 ml water added and pH adjusted to 6.8 with sodium hydroxide (4M). It was then incubated by the addition of pancreatin (100 mg) for 1 h at 40°C. Finally, the contents were cooled and pH adjusted to 4.5 with HCl (4N) and filtered though a dried and weighed crucible containing 0.5 g of celite. (Drying and weighing of crucible with celite was done by adding 0.5 g of celite into the crucible and washing it with 20 ml of 95 per cent ethanol and 20 ml of acetone. It was then dried in an oven at 105°C for 30 min and weighed). The residue (insoluble fibre) retained on the crucible was washed with 20 ml of 95 per cent ethanol and 20 ml of acetone. The crucibles were dried at 105°C overnight and weight taken. To the filtrate (soluble fibre); four volumes of 95 per cent ethanol was added and kept at room temperature for 1 h for precipitation. The precipitate was filtered through dried and weighed crucible

containing celite as before and washed with 20 ml of 95 per cent ethanol and 20 ml acetone. Crucibles were dried at 105°C overnight and weighed. Blank was processed as above without sample. The crucibles were incinerated at 500°C in a muffle furnace for 6 to 8 h. Insoluble or soluble dietary fibre was calculated using the formula

$$\text{Weight after drying (g/ 100g)} = \frac{\text{Weight after incineration} - \text{blank}}{\text{Weight of the sample (g)}}$$

3.17. Studies on laxative and digestive properties of bacterial cellulose by animal experiment

3.17.1. Experimental animals and housing conditions

Animal experiments were carried out based on the ethical guidelines laid down by the committee for the purpose of control and supervision of experiments on animals by the Government of India, Ministry of Social Justice and Empowerment (Annexure III).

Three weeks old weanling male Sprague-Dawley rats, with a mean initial weight of 50-60 g, mean body weight within a group not different than 10 g and between groups not more than 5 g, were obtained from the Kovai Medical College Hospital, (KMCH College of Pharmacy, Coimbatore, Tamilnadu and maintained in the institute animal house facility. The Institute is recognized for animal studies by the ethical committee. The animals were assigned to four groups, all consisting of 6 animals of each group. The animals were housed in polycarbonate cages (six rats per cage) on soft chip bedding, which was changed thrice per week. For drinking water, tap water was provided. Rats were given free access to diet and water. They were housed in a room maintained at 25 ± 2°C with a relative humidity of 60 to 70 per cent and exposed to a light and dark cycle of 12 h duration.

3.17.2. Diet

Rats were fed with commercial diet (M/s. Amrut – laboratory animal feed, Pranav Agro Industrial Ltd.) obtained from Bangalore.

3.17.3. Colloidal bacterial cellulose laxative

The studies on laxative properties were designed as per the methods prescribed by Wintola *et al*. (2010). The test laxative, namely colloidal bacterial cellulose was orally administered at levels based on body weight of animals. To prepare BC in colloidal form, the cellulose obtained from *Gluconacetobacter* sp. sju-1 was freeze dried and made into a fine powder. To 100 ml of sterile water, 9:1 ratio of fine powder of freeze dried BC and sterilized guar gum was added to make into a colloidal form. The prepared formulation was stored in sterilized vials at room temperature. Isabgol, a commercial plant based natural laxative obtained from *Psyllium* husk was used to compare the laxative effect of colloidal bacterial cellulose laxative.

3.17.4. Development of constipation and grouping of animals

The efficacy of colloidal formulation of bacterial cellulose obtained from *Gluconacetobacter* sp. sju-1 was studied against loperamide-induced constipation in Sprague-Dawley rats. The rats were randomly divided into 4 groups. Prior to the initialisation of the experiment, constipation was induced to all the 4 groups by administration of 0.15 mg of loperamide $100g^{-1}$ of body weight twice a day for 5 days. During the development of constipation, parameters such as feed intake, water intake, faecal numbers, water content of faecal pellet and weight of faecal pellet was quantified for the normal control fed with 0.9 per cent saline and constipation induced rats for 5 days. The passage of reduced, hard and dry faecal pellets indicated constipation in the rats. Then the laxatives were administered twice a day orally to the constipated rats for the period of 7 days.

Group-I: Constipation induced rats without any laxative treatment (negative control)

Group-II: Constipation induced rats fed with Isabgol laxative twice a day at the rate of 2.5 ml per dose (positive control)

Group-III: Constipation induced rats fed with colloidal bacterial cellulose laxative twice a day at the rate of 2.5 ml per dose.

Group-IV: Constipation induced rats fed with colloidal bacterial cellulose laxative twice a day at the rate of 5.0 ml per dose.

3.17.5. Measurement of feed, water intake and faecal weight

Feed and water intake were measured for each group for 24 h period. Faeces were collected from each groups daily at 9.00 h and measured for wet weight. The dry weight was recorded after keeping it at 60°C overnight, and the moisture per cent of the faeces were calculated.

3.18. Production of *nata* in jelly base

The bacterial cellulose obtained from the mature coconut water medium and cashew apple juice medium were value added into *nata-de-coco* and *nata-de-cashew* after processing. They were processed separately. The cellulose from each of the media were cut into cubes and were boiled for 15 min to remove the acid flavour completely and to kill the bacteria if any. The water was drained completely from the cubes and cooked in sugar syrup at 72°Brix. Then it was added with 2.0 per cent of dark red colour pigment obtained from beet root extract developed from Post Harvest Technology Centre (PHTC), TNAU, Coimbatore and boiled for 5 min. Then it was packed in sterilized bottles.

To produce *nata* in a jelly form the, processed uncolored *nata* (10 g) was grounded into paste and mixed with 100 ml of mature coconut water to develop *nata-de-coco* and 100 ml of cashew apple juice to prepare *nata-de-cashew* separately. Then they were added with 50 g of boiled China grass agar, a pinch of vanilla flavour and allowed to set at room temperature. Then it was cooled in a refrigerator and subjected to sensory evaluation using 9 point hedonic scale.

3.18.1. Sensory evaluation of *nata-de-coco* and *nata-de-cashew*

Since the textural properties of *nata* derived from MCW and CAJ was found to be highly suitable for edible purpose, *nata-de-coco* and *nata-de-cashew* were developed and the properties the organoleptic properties were evaluated for *nata-de-coco* and *nata-de-cashew* using 9 point hedonic scale explained by Omojola and Adesehinwa (2007).

A total of 15 volunteers were involved. The volunteers rated the samples for colour, flavour, tenderness, texture, juiciness and overall acceptability. The acceptability of the final

product was evaluated based on the average of the overall acceptability score values using the score card given in the Annexure IV.

3.19. Designing a model fermentor for cellulose production using CAJ medium

A schematic design of laboratory scale production of cellulose growth in static condition is presented in Fig. 6. Five litre capacity TPX^{TM} polymethylpentene transparent light weight, autoclavable jar with handle was taken, The lid was designed using Galvanised Iron sheet (GI) with four holes (4 cm) placed at an equal distance and a handle at the centre. The holes were plugged with cotton plugs for aeration. Accurately 3.5 litre of cashew apple juice medium was taken and closed with the designed lid. The entire set up was sterilized by autoclaving. After sterilisation, 350 ml of seed culture was inoculated into it and incubated statically at room temperature for 20 days. Through the holes, samples can be withdrawn with a sterilised pipette and quantitative and qualitative analyses were performed.

3.19.1. Fermentation kinetics in CAJ medium (Lee and Rogers, 1983)

The growth kinetics of *Gluconacetobacter* sp. sju-1 in cashew apple juice medium under static fermentation in the pre-designed model fermentor was expressed in terms of growth rate (K, g $l^{-1}d^{-1}$), specific growth rate (μ, h^{-1}), C max = maximum BC concentration (g l^{-1}), (Qp) = g of BC produced l^{-1} h^{-1}, Yp/s = g of BC produced for every 100 gram of sugar source used in the medium, Yp/x = g of BC produced for every 100 gram of cells.

Growth rate (K, g $l^{-1}d^{-1}$) = dX/dT

Where T- days, X- cell biomass concentration (g $l^{-1}d^{-1}$)

Specific Growth Rate (μ) = (1/X) dX/dT

C max = maximum BC concentration (g l^{-1})

BC Production rate (Qp) = g of BC produced l^{-1} h^{-1} (g l^{-1} h^{-1})

Yp/s = g of BC produced for every 100 g of carbon source used.

Yp/x = g of BC produced per 100 g of cells

13.19.1.1. Estimation of viable cell number, pH changes and total sugars during fermentation

Samples were withdrawn at two day intervals upto 20 days of fermentation to quantify changes in viable cell number, pH changes and total sugars. 1.0 ml of the culture both was withdrawn to determine total viable cell count by pour plate method in HS medium. Growth curve was plotted with curve expert version 1.2 using Gombertz equation given below

Gompertz Relation: y=a*exp(-exp(b-cx))

Simultaneously 10 ml of the culture broth was pipetted aseptically in a pre-weighed and sterilized centrifuge tube and added with 90 ml of 1.0 M potassium acetate buffer (pH 5.0) and digested with 10 ml of 20 per cent cellulase at 50°C with shaking at 100 rpm for 1 hr to hydrolyze bacterial cellulose in the broth. Then it was centrifuged at 6000 rpm for 30 min at 4°C. The precipitate settled at the bottom of the centrifuge tube was dried at 80°C over night and biomass was estimated. The supernatant solution was used to analyze total sugar content by Anthrone method (AOAC, 1990). Anthrone reagent was prepared by dissolving 200 mg of Anthrone in 100 ml of ice cold 95 per cent H_2SO_4. Standard stock solution having the concentration of 1.0 mg glucose ml^{-1} was prepared by dissolving 100 mg of D-glucose in small amount of distilled water and final volume was made up to 100 ml with distilled water. About 100 ml of the stock was diluted to 100 ml with distilled water in a 100 ml volumetric flask to obtain the glucose concentration of 100 µg glucose ml^{-1} which served as working standard.

About 100 mg of each sample was taken into a boiling tube. The samples were hydrolyzed by keeping it in a water bath for 3 h with 5 ml of 2.5 N HCl and cooled to room temperature. The volume was made up with distilled water to 100 ml and centrifuged. Aliquots of (0.5 ml) each sample was pipetted out in test tubes and simultaneously 0.2, 0.4, 0.6, 0.8 and 1 ml of working standard was pipetted out in test tubes. Volume of samples and standards were made up to 1 ml with distilled water. To one ml of distilled water blank, 4 ml of Anthrone reagent was added to each tube. The tubes were kept in a boiling water bath for eight min. After cooling the tubes, the

absorbance was recorded at 630 nm using spectrophotometer and the values were plotted in a graph against concentration. From the graph drawn, the amount of total sugars was calculated and expressed in (g l^{-1}).

To measure the total amount of cellulose produced, cellulose pellicle was washed twice with distilled water and then treated with 1 per cent NaOH at 90°C for 30 min to dissolve the cells attached with the cellulose and washed twice with distilled water to remove remnant alkali and other media components. It was then dripped free of water and the wet weight was recorded. Then it was dried at 80°C over night and dry weight of the cellulose was estimated.

3.19.1.2. Preparation of crude extract of fermented broth and identification of bioactive compounds by Gas Chromatography Mass Spectrophotometer (GCMS)

The fermentation media containing the cellulosic pellicle and the broth was homogenized well in a blender homogenizer and the cellulose was allowed to sediment and settle down. The crude extract of fermented broth was obtained as per the procedures of Ibrahim *et al*. (2011). The supernatant was mixed with equal quantities of chloroform and methanol (2:1) and shaken well vigorously in a separating funnel and incubated overnight. The solvent extract were separated and used for testing the bioactive compounds secreted by the bacteria using GCMS.

The Gas Chromatography Mass Spectrometry analysis of the crude extract of fermented broth was performed using Thermo GC - Trace Ultra version: 5.0, Thermo MS DSQ II equipped with DB 5 - MS capillary standard non-polar column of 30 m dimension, ID: 0.25 mm, film: 0.25 μm, for GC-MS detection, an electron ionization system with ionization energy of 70 eV was used. Helium was used as carrier gas with a flow rate of 1.0 ml min^{-1} and oven temperature 70°C raised to 250°C at 6°C min^{-1}. The chromatographic peak identification was carried out by comparing their mass spectra with those of the bibliographic data of unknown compounds from the NIST library mass spectra database on the basis of the criterion similarity (SI)>800 (the highest value is 1000). The approximate quantification of volatile compounds was estimated by the integration of peaks on the total ion chromatogram using X calibur software, Vienna. The results are presented as the per cent peak area normalized.

3.20. Coconut water vinegar production using spent liquor/ fermented broth of cashew apple juice medium as mother culture

Fermented broth or the spent liquor left after the cellulose production in the model fermentor using cashew apple juice medium was value added into coconut water vinegar. Spent liquor was used as mother culture of acetic acid bacteria in vinegar production. Mature coconut was collected from university canteen and filtered using muslin cloth. Approximately 100 ml filtered mature coconut water was taken in 250 ml Erlenmeyer flasks. It was then adjusted to sugar level 17°Brix, mixed well and pasteurized. Sweetened coconut water was then allowed for alcoholic fermentation for one week by addition of 10 g baker's yeast and then for acetic acid fermentation by addition of spent liquor (obtained from the CAJ media after extraction of cellulosic layer) at 10 per cent level and was incubated at room temperature upto 32 h and sampling was taken at intervals of 8, 16, 24 and 32 h to measure the volatile acidity.

3.20.1. Purification of acetic acid by solvent using liquid-liquid extraction method (Katikaneni and Cheryan, 2002)

The acetic acid formed was extracted at regular intervals of 8, 16, 24 and 32 h. The acetic acid formed was extracted using an extractant, Alamine 336 and a diluent 2-ethyl-1-hexanol. Extraction of acetic acid was conducted in a 100 ml separating funnel at room temperature. About 25 ml of the fermented solution containing acetic acid was mixed with 25 ml of the organic solvent system consisting of 12.5 ml of Alamine-336 and 12.5 ml of 2-ethyl-1-hexanol (Extractant: diluent, 1:1 ratio). The funnel was shaken for 5 min, and the mixture was equilibrated for 12 h at room temperature. The mixture gets separated into two phases was centrifuged at 10000 rpm for 5 min to separate the aqueous and organic phases. The volatile acidity was estimated as described below.

3.20.1.1. Estimation of volatile acidity (Bar *et al.,* 1986)

About five ml of each organic and aqueous phase samples were added into 100 ml of deionized water. About 2-3 drops of phenolphthalein indicator was added and titrated using standard sodium hydroxide (0.1N NaOH) to a pink colour end point. The volume of NaOH used in titration was recorded and the volatile acidity was calculated and expressed in per cent of acetic acid.

$$\text{Volatile Acidity (\%)} = \frac{\text{(Volume of base) x (N base) x (0.060) x (100)}}{\text{Sample volume (ml)}}$$

Where,

Volume of base - Volume of NaOH used in ml;

N base - Normality of NaOH.

3.21. Production of bacterial cellulose paper (Suwannapinunt *et al*., 2007).

To produce reinforced paper sheets, the starter culture *Gluconacetobacter* sp.sju-1 was inoculated in 1.0 litre of sterilized modified HS medium containing the polysaccharide calcium alginate (CaAlg) (0.1 per cent) and incubated statically for 20 days at 30°C. After the incubation period, bacterial cellulose was formed as a white pellicle floating at the top of the medium. The pellicle was lifted from the medium and washed thoroughly in running tap water. Pellicle was treated in 4 per cent (w/v) sodium hydroxide solution in boiling water bath for 30 min to remove microbial cells attached to the pellicle. After that, it was washed with excess water to remove the alkali completely. Then it was pressed with a 10 tonne press unit to squeeze out water and to get the BC wet sheets. The BC wet sheets were bleached by soaking in 5 litre of 1.5 per cent H_2O_2, which was adjusted to pH 11 with 0.5 per cent NaOH, in a sealed plastic bag. The plastic bag with the BC was then shaken for 10 min and put into boiling water for 30 min or until the BC colour turned white. After bleaching, the white BC sheets were washed with tap water and pressed again with a 10 tonne press unit and air dried for 24 h to get to produce reinforced paper sheets. Reinforced paper made from wood cellulose served as the control to compare the physical and mechanical properties.

3.21.1. Physical and mechanical properties of reinforced BC paper and reinforced wood cellulosic paper

3.21.1.1. Tensile strength and Young's modulus (Anonymous, 1993).

The tensile strength of the sheet was measured by a tensile tester (SGA-100, Shin Gang Co. Ltd., Korea). The sheet was cut into ribbons (5 × 30 mm) for measurement of tensile strength. The thickness of the ribbons was 100 μm, and the elongation rate was

1.0 mm min^{-1}. Young's modulus was determined from the ratio of the stress exerted on the sample to that exerted on the deformed sample.

3.21.1.2. Viscosity and Degree of Polymerization (TAPPI, 1993; 2005)

The viscosity of finely divided sample was measured as centipoises (cP) using cupriethylendiamine (CED) as a solvent and a capillary viscometer. From viscosity values, the average Degree of Polymerization (DP) of the BC sample was calculated from the following equation:

$DP^{0:905}$ = 0:75 (954 log(X)-325)

where X =TAPPI viscosity in cP.

3.21.1.2. Air penetration and castor oil penetration test (TAPPI, 1993; 2005)

Air penetration test of paper (sec $50ml^{-1}$) was the time measurement in seconds on the air resistance of approximately 1 sq.in. of a circular area of paper using a pressure differential of 1.22 KPa. Castor oil penetration test (min $0.05ml^{-1}$) was the measurement of the time required for a drop of castor oil to produce a uniform translucent spot on the underside of the test specimen by permeating through the specimen.

3.22. Cost of production of bacterial cellulose

Cost of production of both the wet bacterial cellulose and in jelly base was analysed and the Benefit Cost Ratio (BCR) was calculated.

3.23. Statistical analysis

All the data presented in tables and figures were expressed as mean ± standard deviation of the three replications. All the experiments in this study were conducted with three replicates and reported with the mean values. Statistical analysis was done as per the method of Panse and Sukhatme (1976). To study the effect of different independent parameters on all dependent variables by Response Surface Methodology (RSM) using the statistical software Design Expert Version 7.1.6, AGRES and Excel 2007.

Design Expert Version 8.0.7.1 software was used in analyzing the data, developing the regression models and plotting the 3 dimensional surface plots to study the effect of various independent variables on dependent variables by employing multiple

regression technique. The model fermentor was designed using Computer Aided Design (CAD) Software Solidworks 2012. Curve Expert version 1.2 was used to derive Gombertz growth model. All the treatments were compared at $p \leq 0.01$ and 0.05 level of significance using the critical difference (CD) test which was performed by Excel-2007. The analysis of variance (ANOVA), Standard Error (SE), and T test for dependent parameters were tabulated and the level of significance was reported.

CHAPTER IV

RESULTS

4.1. Isolation of cellulose producing bacteria from fruits and juices

The results are presented in Tables 4 and 5. From the total of twenty two samples from different fruits and juice samples tested, 141 isolates produced zone of hydrolysis in GYC medium. Amongst the different fruit sample, apple recorded maximum number of cellulose producing isolates with eight morphologically distinct colonies, producing zone of hydrolysis in GYC medium. Cashew apple showed positive for zone of hydrolysis to 11 isolates. Coffee extract and palm vinegar produced 9 morphologically distinct colonies producing zone of hydrolysis which was followed by custard apple. In pomegranate, only two isolates with clearing zone on GYC medium were obtained. About 141 morphologically distinct colonies from various fruits and juices were purified further by streak plate method. When all the purified isolates were separately grown in HS medium, out of 141 isolates that produced zone of hydrolysis, fifty nine cellulose producing isolates were obtained. Amongst them apple recorded 5 number of isolates, producing cellulose pellicle in HS medium. Cashew apple, cocoa extract, coconut milk, coconut water, coffee extract, custard apple and palm fruit produced 4 numbers of cellulose producing isolates each.

Further to check whether sugarcane varieties contains cellulose producing bacteria, high TSS containing sugarcane varieties (Co1148 , Co 2000-12, Co 2001-13, Co 2001-15, Co 86032, Co 94008, Co 94012, Co 99004, Co 99008, Co 0218, Co 8371, CoC 671, Co 99008, Co 0218 and Co 8371) were collected and used for the isolation of cellulose producers. Purified colonies of sju-1 and formation of clearing zone by sju-1 in HS and GYC medium are depicted in Plates 1 and 2. A total of 29 cellulose producers were obtained from the sugarcane juice of different varieties. Out of these, sugarcane juice from the variety Co1148 and Co 2000-12 produced three isolates each *viz*., sju-1, sju-2, sju-3 and sju-6, sju-7, sju-8 respectively. Invariably most of the sugarcane juice varieties (Co 2000-12, Co 86032, Co 99004, Co 99008, Co 0218, Co 8371, CoC 671, Co 99008, Co 0218 and Co 8371) produced two isolates of cellulose producer, while Co 2001-15, Co 94008 and Co 94012 produced only one cellulose producing isolate each.

4.1.1. Screening of isolates for bacterial cellulose production in HS medium

Screening of cellulose producers based on Gallardo scale of thickness is indicated in Table 6. The isolates app-1 and app-2 produced cellulose pellicle of 2 mm thickness while app-3, app-4, app-5, ban-1 isolates produced 0.5 mm thickness. Among the cashew apple isolates caap-4 produced cellulose of 5 mm thickness and was graded as excellent. The isolates coex-1 coex-2, cw-2, cw-3, paf-1 produced pellicle each of 6 mm thickness and were graded excellent, whereas paf-4 produced 7 mm thickness. The isolate cuap-4 produced 8 mm thickness of cellulose pellicle. Among the sugarcane juices from different varieties, sju-1 produced maximum pellicle thickness of 13 mm followed by sju-6, sju-7, sju-12, sju-16, sju-17, sju-18, sju-19 and sju-25 which formed 10 mm thickness pellicle each. Among the tea extract isolates tex-1 produced 8 mm thickness and tex-2 and tex-3 produced 4 mm thickness respectively. All the isolates from wood apple were found to be good producers with 4 mm thickness each. However, the reference culture *A. xylinum* NCIM 2526, was categorized as an excellent producer with 9 mm thickness of cellulose pellicle. From the results of Gallardo scale, it is very clear that sju-1 produced maximum thickness of cellulose pellicle and was selected for further study.

4.2. Morphological and biochemical characterization of elite cellulose producing isolates

The morphological characteristics of the isolates that were graded excellent are indicated in Table 7. Most of the morphological characteristics were identical among the isolates screened and graded as excellent producers of cellulose. Since sju-1 produced comparatively thicker cellulose pellicle (13 mm), it was selected for the analysis of biochemical characteristics and compared with reference culture *Acetobacter xylinum* NCIM 2526 and the results are presented in Table 8. Further to confirm upto the species level, molecular characterization was performed using 16S rRNA method.

4.2.1. Colony characters

The colony characters *viz.*, colour, shape and colony character were observed on HS medium. The colonies of the cellulose producing isolates that developed on the HS

agar plates under aerobic conditions were observed for its shape, colour and colony margin. Among the observations in colony colour, there existed a wide range of variations such as white, pale yellow, light cream and yellow, brownish yellow and brown. Isolates like caap-4, coex-2, cw-2 and sju-1 were milky white in colour. Exceptionally colonies of the isolates sju-2, sju-25 and sju-27 were brown in colour. Yellow coloured colonies belonged to cw-4, fln-3, sju-6 and sju-24. Four isolates namely paf-4, sju-8, sju-10, sju-12, sju-16, sju-18, sju-23 exhibited cream white colouration. The colony morphology revealed that the isolates were produced a range of surface and marginal characters.

4.2.2. Cell shape

All of the isolates screened and selected as an excellent cellulose producer were short rods and NCIM 2526 was very short rods when compared to all the isolates.

4.2.3. Microscopical analysis of isolates

The cell morphology examined under phase contrast microscope at 40X is depicted in Plate 3. Scanning electron microscopic view of sju-1 taken at different maginification is shown in Plate 4. Scanning electron microscopic view of short rods of sju-1 taken at 6000X is depicted in Plate 4a, and Plate 4b shows the cellulose fibrils viewed at 8000X which clearly exhibits a highly reticulated nature of fibrils that looks like a spider web arising from the cell membrane of the cells. When an individual cell when viewed at 37000X, formation of cellulose fibrils that are radiating from the exterior of cell membrane could be vividly seen (Plate 4c).

4.2.4. Biochemical characterization of the isolates

For the tentative identification of the selected isolate sju-1, various biochemical tests were performed. The most important and the key characteristics of formation of water insoluble pellicle was tested positive for all the isolates. Formation of cellulose pellicle by sju-1 on the surface of HS medium is shown in Plate 5. The real image of cleaned cellulose pellicle produced by the *Acetobacter xylinum* NCIM 2526 and *Gluconacetobacter* sp. sju-1 is shown in Plate 6.

All the cellulose producing isolates exhibited positive response to catalase test that was evident when production of bubbles was documented in the test media on the addition of hydrogen peroxide. The isolate tested were oxidase, gelatinase and urease negative. In general, the isolate showed no growth in citrate medium. The isolate tested negative for indole production. Formation of red coloured colonies in Congo red incorporated LB medium due to the presence of cellulose fibrils is depicted in Plate 7. Formation of dihydroxyacetone in glycerol medium was tested positive which was confirmed by the production of blue colour precipitate upon addition of Benedict's solution. Oxidation of ethanol was confirmed by the presence of clearing zone in the ethanol containing GYC medium and overoxidation of acetic acid to CO_2 and H_2O were found to be positive by the change of colour of the indicator bromocresol green to yellow. Further special tests like cellulose solubility test and sodium hydroxide treatment tests revealed that the bacterial cellulose does not dissolves in sodium hydroxide, methanol and acetone. However, it completely dissolved in cupriethylenediamine, the best well known dissolution agent for cellulose. Cellulose was resistant to the alkali treatment and the remaining material was accepted as pure cellulose free from microbial cells and media components.

4.2.5. Probable identification

Based on the morphological characteristics and biochemical tests carried out, the selected isolate sju-1 belonged to *Gluconacetobacter* sp.

4.3. Molecular characterization of cellulose producing isolate

The sequence similarity of sju-1 with other *Gluconacetobacter* strains analyzed using BLAST is presented in Table 9. The Neighbor-Joining tree of partial (341 bp) 16S rDNA sequence of *Gluconacetobacter* sp. (sju-1) obtained in this study together with selected sequences downloaded from GenBank database is shown in Fig. 7. The sequence analysis for the 16S rDNA gene of the isolate G*luconacetobacter sp.* sju-1 was analysed in BLAST + of NCBI, GenBank to find similar nucleic acid sequences. The 16S rRNA gene sequence of *Gluconacetobacter* sp. (sju-1) with 1456 bases possessed 99 per cent similarity to *G. obediens*, *G. intermedius* and *G. xylinum* and was deposited in the NCBI GenBank database under the accession number KF164613, which will be released in the

website in the month of September'2013. But when analysed in EZTAXON VERSION 2.1 to compare with the type strains of *Gluconacetobacter*, the selected isolate sju-1 had 99.02 per cent similarity to *Gluconacetobacter intermedius* sp. Further the purified culture was deposited to Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India with a strain designation of *Gluconacetobacter* sp. sju-1.

4.4. Structural characterization of bacterial cellulose using Fourier Transform Infra Red Spectroscopy (FTIR)

The IR spectrum of pure Micro Crystalline Cellulose (MCC), Bacterial Cellulose (BC) from *A. xylinum* NCIM 2526 and *Gluconacetobacter* sp. sju-1 are presented in Fig 8. IR spectrogram of MCC showing per cent transmittance peak at 3411 cm^{-1} and 2920 cm^{-1} representing OH and CH_2 grouping (Fig. 8a). The per cent transmittance peak at 1651 cm^{-1} showed the presence of carboxylic acid groups (COOH). The band at 1459 and 1432 cm^{-1} attributed to the occurrence of carbonyl group in MCC. The band at 1160 cm^{-1} and 1115 cm^{-1} showed the presence of C-O-C functionalities present. The transmittance peak of 3411 cm^{-1} indicated that MCC is in pure form consisting cellulose with more hydrogen bonding. Similar to those of the IR spectrum of MCC, BC from *A. xylinum* NCIM 2526 also registered a very closer hydrogen bonding and CH_2 grouping patterns at the per cent transmittance peak of 3374 cm^{-1} and 2690 cm^{-1} respectively (Fig. 8b). Further the per cent transmittance peak at 1650 cm^{-1} confirmed the presence of carboxylic acid groups (COOH) in bacterial cellulose also. The band at 1457 and 1449 cm^{-1} clearly attributed to the presence of carbonyl group and the band at 1161 cm^{-1} and 1110 cm^{-1} showed the prevalence of C-O-C functionalities present in BC from *A. xylinum* NCIM 2526.

IR spectrum obtained for BC produced by *Gluconacetobacter* sp. sju-1 (Fig. 8c) showed per cent transmittance peak at 3229 cm^{-1} and 2911 cm^{-1} representing OH and CH_2 grouping. Broader bands of 3229 cm^{-1} indicated the presence of more hydrogen bonding patterns. The strong per cent transmittance peak at 1644 cm^{-1} confirms the presence of carboxylic acid groups (COOH) in cellulose structure. The band at 1428 cm^{-1} attributed to the occurrence of carbonyl group in BC. The band at 1163 cm^{-1} and 1068 cm^{-1} shows the possibilities of C-O-C functionalities present in the structure of BC.

The transmittance peak nearing to 3240 cm^{-1} was achieved, indicating that cellulose type Iα was abundantly present in BC produced by *Gluconacetobacter* sp. sju-1.

4.5. Visualization of bacterial cellulose by Scanning Electron Microscopy (SEM)

The scanning electron microscopic view of celluloses taken at 8000X is illustrated in the Plate 8. Plate 8a depicts the fibrils of cellulose from Micro Crystalline Cellulose. The fibrils were thicker and uniform in nature with more of straight branches. Plates 8b and 8c depicts the highly reticulated nature of bacterial cellulose with more cross links and branching pattern with the bacterial cells attached on them. The length and diameter of the fibrils were comparatively very thinner than the MCC. The size of the microfibrils of both the bacterial celluloses ranged from 128 to 207 nm. In the case of BC fibers from *Gluconacetobacter* sp. sju-1, the fibrils are very closely and densely packed than the microfibrils of BC from *A. xylinum* NCIM 2526.

4.6. Thermo Gravimetric Analysis (TGA)

The results are illustrated in Fig. 9. In order to analyze the thermal behavior of the bacterial cellulose, the derivative weight loss of the cellulose versus temperature plots were recorded. The TG spectrum shows the derivative weight loss of cellulose for every degree rise in the temperature ranging from 0 to 600°C. Microcrystalline cellulose showed 8.38 per cent weight loss at 150°C and at 350°C a derivative weight loss of 53.87 per cent took place (Fig. 9a). For the cellulose from *A. xylinum* NCIM 2526 the loss in 50 per cent weight takes at a temperature of approximately 360°C with a derivative weight loss per cent of 50.53 and at 175°C a drop in weight of 12.58 per cent was recorded. A quick drop in weight occurred at 375°C with a derivative weight loss at this temperature was 53.87 per cent (Fig. 9b). For the cellulose from *Gluconacetobacter* sp. sju-1, a quick drop in weight at a temperature of approx. 350°C with a derivative weight loss per cent of 56.59 was registered. The TG curve shows that the maximum rate of degradation that is more than 50 per cent weight loss occurs at temperature of 350°C. Even when the temperature approaches 200°C the per cent weight loss was only 6.26 (Fig. 9c).

4.7. Differential Scanning Calorimeter (DSC)

The DSC thermograms of the thermal behavior of the celluloses from various sources are depicted in the Fig. 10. Fig. 10a shows the decomposition temperature of MCC, Fig. 10b and Fig. 10c shows the decomposition temperature of bacterial cellulose from *A. xylinum* NCIM 2526 and *Gluconacetobacter* sp. sju-1 respectively. The start of crystallization, Crystallinity temperature (Tc) and Decomposition temperature (Tm) were 65.28°C, 102.65°C and 284.66°C for MCC, 65.21°C, 89.25°C and 275.20°C for cellulose from *A. xylinum* NCIM 2526 and 62.38°C, 133.78°C and 295.85°C for cellulose from *Gluconacetobacter* sp. sju-1. From this, it is evident that sju-1 recorded higher values for all the thermal parameters compared to the other two.

4.8. Effect of culture concentration on cellulose production

The appropriate concentration of liquid broth of *Gluconacetobacter* sp. sju-1 required for optimum production of bacterial cellulose is presented in Table 10. Among the different concentrations, 10 per cent level recorded a significantly higher dry weight on the 7th day of incubation in HS medium. Further an increase in concentration from 10 to 15 per cent did not show any significant difference in the dry weight of the cellulose.

4.9. Effect of incubation time on cellulose production

The effect of incubation time on dry weight of bacterial cellulose is presented in Table 11. There was a gradual increase in the dry weight of bacterial cellulose from 2nd day (0.02 ± 0.45 g l^{-1}) to 20th day (0.02 ± 0.45 g l^{-1}) after incubation. The visible floating of the cellulose pellicle could be seen only after 3rd day of incubation. From the 4th day onwards, the production increased at an increasing rate and was found to be highly significant at 18th day which recorded dry weight of 14.97 ± 0.87 g l^{-1}. From 8th day to 10th day the dry weight doubled from 1.97 ± 0.54 g l^{-1} to 5.39 ± 0.45 g l^{-1}, increased steadily till 16th day and thereafter it remained constant till the 20th day without any significant difference. So the optimum time for maximum cellulose production was found to be 20 days (480 h).

4.10. Effect of different pH on the dry weight of cellulose

The pH of the growth medium had the direct impact on cellulose production. The influence of pH on the dry weight of bacterial cellulose formed by sju-1 are

presented in Table 12 and Fig. 11. Among the different pH levels tested, maximum dry weight of 15.7±0.36 g l^{-1} was found to occur at pH 6.5 followed by pH 6.0 and 7.0 which a recorded dry weight of cellulose 15.4±0.36 g l^{-1} and14.2±0.36 g l^{-1} respectively. Increasing or decreasing the pH above or below the near neutral level negatively affected the dry weight of cellulose. It is inferred from these experiments that the optimum pH for better cellulose production was at pH 6.5. But even in the acidic pH of 4.0, the cells were able to produce cellulose of 13.1±0.2 g l^{-1}.

4.11. Effect of different temperature on the dry weight of cellulose

The results are presented in Table 13 and Fig. 12. Among the different temperature ranges tested from 25°C, 27°C, 29°C, 31°C, 33°C and 35°C, the optimum dry weight of the cellulose was reached at 31°C. Distinct variation in the dry weight of the cellulose was observed at different temperatures. The maximum dry weight of the cellulose 14.9±0.34 g l^{-1} was observed at 31°C followed by 33°C which registered 13.2±0.30 g l^{-1}. At 25°C the dry weight of cellulose was 11.2±0.23 g l^{-1}. There was no significant difference in the dry weight of the cellulose at temperatures such as 25°C, 27°C and 29°C. But when the temperature goes beyond 35°C, the dry weight of the cellulose decreased abruptly to 10.7±0.25 g l^{-1}.

4.12. Effect of culture conditions in HS medium on cellulose production by sju-1

The cellulose yield parameters such as biomass (g l^{-1}), viable cell count (cfu ml^{-1}), pH (final), wet and dry weight of cellulose, moisture content, amount of soluble polysaccharides and biosynthetic yield are presented in Table 14.

In the case of static culture condition, the cellulose formed was of thick mat floating on the surface of the medium and the fibrils were highly uniform in nature, but in shaken culture condition, the pellicle formed as small globules and the fibrils were highly disintegrated (Plate 9). In both the culture conditions, the pellicle formation started on the 2nd day of incubation.

The static culture condition registered higher biomass (8.42±0.17 g l^{-1}), dry weight of cellulose (14.92±0.30 g l^{-1}) and less water soluble polysaccharides (0.48±0.01 g l^{-1}). The static culture condition registered a higher per cent of biosynthetic yield (74.7±0.32)

than shaken culture condition which registered only 48.15±0.98 per cent. More acidic pH (3.0±0.06), high moisture (94.6±1.92) and more water soluble polysaccharides (1.65±0.03) were registered in shaken culture condition. In respect to the total viable count, static and shaken culture condition recorded 4.2 X 10^{11} and 3.7 X 10^{11} cfu ml^{-1} respectively.

4.13. Effect of different carbon sources on the dry weight of cellulose

The data on the dry weight of cellulose in different carbon sources at equimolar concentration are presented in Table 15 and Fig. 13. In general, all the carbon sources. influenced the growth and dry weight of cellulose. Among the 12 carbon sources tested, fructose was found to be an ideal carbon source that resulted in significantly higher dry weight of cellulose (15.8±0.32 g l^{-1}) followed by glucose and sucrose which recorded 14.1±0.35 g l^{-1} and 14.6±0.34 g l^{-1} respectively. Mannitol recorded a dry weight of 12.5±0.29 g l^{-1}. Among the different carbon sources inositol and starch were the least preferred carbon source with respect to cellulose production.

4.14. Effect of different nitrogen sources on the dry weight of cellulose

As many as 12 nitrogen sources were used in the study and the results of dry weight of cellulose are presented in Table 16 and Fig. 14. In general, organic sources of nitrogen supported better cellulose production than inorganic nitrogen sources. Among the nitrogen sources, yeast extract was found to be the most preferred nitrogen source with the significant dry weight of 15.1±0.37 g l^{-1} followed by peptone, malt extract, potassium nitrate which supported the dry weight of 14.1±0.74, 13.4±0.38 and 13.1±0.33 g l^{-1} respectively. Casein hydrolysate (12.9±0.31 g l^{-1}) and ammonium chloride (12.0±0.23 g l^{-1}) recorded no significant difference in the dry weight of cellulose. Ammonium carbonate, beef extract and calcium nitrate recorded almost nearer values of 11.2±0.55, 11.9±0.45 and 11.5±0.35 g l^{-1} respectively. Among the different nitrogen sources, sodium nitrate registered a lowest dry weight of 9.81±0.35 g l^{-1}.

4.15. Effect of growth hormones on cellulose production

The results are presented in Table 17. Between the two growth hormones namely kinetin and indole acetic acid (IAA) tested to check the influence on cellulose production

by sju-1, no significant difference was noticed. However, IAA showed comparatively higher cellulose production of 16.9±0.02 g l^{-1} at 800 ppm than kinetin which recorded cellulose production of 15.4±0.07 g l^{-1} at the same concentration. At the concentration of 200 ppm, kinetin recorded a dry weight of cellulose of 14.1±0.03 g l^{-1} and IAA recorded a 1 per cent increased yield of 15.1±0.03 g l^{-1}. At higher concentration of 1000 ppm kinetin recorded a dry weight of 15.8±0.01 g $l^{-1}$and IAA recorded 16.4±0.12 g l^{-1}.

4.16. Effect of different additives on the dry weight of cellulose

The effect of 15 different additives at 0.1 per cent concentration on the production of cellulose was analyzed and presented in the Table 18 and Plate 10. The maximum dry weight of cellulose was achieved in medium supplemented with calcium alginate and lignosulfonate of 17.8±0.38 g l^{-1} and 17.8±0.41 g l^{-1} followed by polyethylene glycol which recorded 17.0±0.39 g l^{-1}. Addition of agar, agarose, carboxy methyl cellulose, dextran and sodium alginate registered recorded 14.7±0.30, 14.5±0.29, 14.7±0.30, 14.1±0.49 and14.2±0.73g l^{-1} respectively. Additives like acetan, chitin, chitosan, gelatin and levan recorded 15.4±0.92, 15.9±0.56, 15.5±0.32, 15.7±0.32 and 15.2±0.20 g l^{-1} respectively. Only 11.1±0.23 g l^{-1} of dry weight of cellulose was recorded by the additive cellulose.

4.17. Physicochemical properties of Mature Coconut Water (MCW) and Cashew Apple Juice (CAJ)

The physicochemical properties *viz.,* pH, total soluble solids, total titrable acidity, reducing sugars, protein, fat, phosphorus, potassium, sodium, calcium, magnesium, copper and iron of mature coconut water and cashew apple juice were analyzed and presented in Table 19. The pH of two substrates namely MCW and CAJ, were acidic in nature with a pH of 5.2±0.01 and 4.29±0.12 respectively. In general, CAJ registered higher proximate contents in most cases than MCW with TSS (12.9 ± 0.37°Brix), TTA (0.27±0.01 per cent), reducing sugar (11.3±0.33 g/ 100g), protein (3.32±0.10 g/ 100g), fat (0.50±0.01 g/ 100g), phosphorous (211.2±6.14 mg/ 100g) and magnesium (178.2±0.14 mg/ 100g). However, MCW recorded higher potassium content (247.0±0.02 mg/ 100g) while CAJ registered 69.5±0.61 mg/ 100g. Both the copper and iron content were comparatively higher of 26.0±0.15 and 79.0±0.35 µg/ 100g in MCW.

4.18. Pre-optimization of MCW and CAJ as a basal medium for the production of bacterial cellulose

The data on pre-optimisation of MCW and CAJ media are presented in Tables 20 and 21. The use of MCW and CAJ as a basal medium for bacterial cellulose was pretested to optimize the low and high levels of C, N and buffer sources. By testing the various C and N sources, it was standardized that fructose and yeast extract proved to be an appropriate source for optimum dry weight of cellulose. Further two important buffer sources namely citric acid and disodium hydrogen phosphate were equally found to significantly contribute to the dry weight of cellulose. So, MCW and CAJ can be value added and converted into an end product of high economic value. With this view, MCW and CAJ juices were collected, filtered and used separately as a basal medium for the production of cellulose. To 1000 ml of the basal media like MCW and CAJ, fructose, yeast extract, citric acid and disodium hydrogen phosphate were added at different combinations and the dry weight of cellulose was recorded and presented.

Since the TSS of CAJ is 12.9 ± 0.37°Brix, CAJ alone was tested different concentration of 25 (250 ml), 50 (500 ml), 75 (750 ml) and 100 (1000 ml) per cent for cellulose production without any fructose amendment. The formation of thick cellulose pellicle was observed both in 75 and 100 per cent. However, dry weight of cellulose was comparatively higher in 100 per cent level with 2.50 g l^{-1} (Plate 11).

The optimum MCW and fructose combinations were 1000 ml and 15 g and the dry weight of cellulose recorded the maximum of 7.50 g l^{-1}. When tested for the optimum nitrogen level, 10.0 g of yeast extract gave higher yield of 12.74 g l^{-1} than yeast extract level of 5.0 g which recorded 11.7 g l^{-1}. Citric acid influenced for two per cent increase in the dry weight of cellulose (14.77 g l^{-1}) when used at the concentration of 2.0 g. When added with phosphate source namely disodium hydrogen phosphate the yield reached the maximum of 16.12 g l^{-1}. The optimum concentration of all the sources were found to be 1000 ml MCW, 15 g fructose, 10 g yeast extract, 2.0 citric acid, 1.15 g of disodium hydrogen phosphate.

Comparison of cellulose in three different medium namely HS medium, mature coconut water (1000 ml) + 15 g of fructose medium and cashew apple juice (1000 ml) +

4 g of fructose alone is depicted in Plate 12.Yeast extract level of 6.0 g along the fructose and cashew apple juice recorded the maximum production of cellulose with the dry weight of 14.57 g l^{-1}. About 1.5 g citric acid along with fructose and yeast extract recorded the dry weight of 15.84 g l^{-1}. Optimum level of disodium hydrogen phosphate was 0.5 g to 1.0 g and there was no significant difference in the dry weight of cellulose between the combinations (16.00 to 16.11 g l^{-1} respectively). In the case of CAJ, the optimum concentration of all the sources were found to be 1000 ml CAJ, 4 g fructose, 6 g yeast extract, 1.5 citric acid, 0.5 g of disodium hydrogen phosphate.

With this knowledge on low and high levels of each component, the interaction effect of two particular components was optimized using statistical based Response Surface Methodology (RSM).

4.19. Response Surface Methodology (RSM) based Optimization of MCW medium for maximum cellulose production

The results of optimization of MCW medium using RSM are presented in Fig. 15. Four level central composite design and the experimental responses of the dependent variable (dry weight of bacterial cellulose) are listed in Table 22. Second order polynomial equation was used to correlate the independent process variables, with bacterial cellulose production. The second order polynomial coefficient for each term of the equation was determined through multiple regression analysis using the Design Expert 8.0.7.1. The data obtained were used to develop models in which each dependent variable was obtained as the sum of the contributions of the independent variables through second order and interaction terms.

The results were analyzed by using ANOVA and shown in Table 23. The model F-value of 19.92 for bacterial cellulose production using MCW implies that the model is significant since the Model P-value (Prob > F) was very low (0.0001). Model F-value was calculated as ratio of mean square regression and mean square residual. The P values were used as a tool to check the significance of each of the coefficients, which in turn, are necessary to understand the pattern of the mutual interactions between the test variables. The smaller the magnitude of the P, the more significant was the corresponding coefficient. Values of P less than 0.05 indicate model terms were significant.

The regression coefficient estimates and the corresponding P values (Table 24) suggested that among the variables used in the cellulose production, A (Fructose concentration), B (Yeast extract concentration), C (Citric acid concentration), D (Disodium hydrogen phosphate concentration (A * B (Fructose concentration * Yeast extract concentration), A * C (Fructose concentration * Citric acid), A * D (Fructose concentration * Disodium hydrogen phosphate concentration), B * C (Yeast extract concentration * Citric acid concentration), B * D (Yeast extract concentration * Disodium hydrogen phosphate concentration), C * D (Citric acid concentration * Disodium hydrogen phosphate concentration), A^2 (Fructose * Fructose), B^2 (Yeast extract concentration * Yeast extract concentration), C^2 (Citric acid concentration * citric acid concentration) and D^2 (Disodium hydrogen phosphate concentration * Disodium hydrogen phosphate concentration) were significant model terms. Fructose concentration ($P<0.0001$) had the largest effect in dry weight of cellulose, followed by interaction of fructose and yeast extract, fructose and citric acid, yeast extract and disodium hydrogen phosphate ($P<0.0001$). The corresponding second-order response model that was found after analysis for the regression was,

Final equation in terms of coded factors for dry weight of bacterial cellulose

Dry weight of bacterial cellulose = +13.77 + 0.82 * A - 0.26 * B - 0.26 * C + 0.18 * D + 0.77 * A * B - 0.48 * A * C - 0.60* A * D + 0.39 * B * C + 1.18 * B * D - 0.07 * C * D - 0.00 * A^2 + 0.15 * B^2 + 0.09 * C^2 +0.04 * D^2

Final equation in terms of actual factors for dry weight of bacterial cellulose

Dry weight of bacterial cellulose = + 70.40 + 2.82 * fructose concentration - 12.88 * yeast extract concentration + 1.88 * citric acid concentration - 59.19 * disodium hydrogen phosphate concentration + 0.06 * fructose concentration * yeast extract concentration - 0.19 * fructose concentration * citric acid concentration - 2.39 * fructose concentration * disodium hydrogen phosphate concentration + 0.31 * yeast extract concentration * citric acid concentration +9.44 * yeast extract concentration * disodium hydrogen phosphate concentration - 2.925 * citric acid concentration * disodium hydrogen phosphate concentration + 1.12 E - 0.04 * fructose concentration^2 + 0.024 * yeast extract concentration^2 + 0.376 * citric acid concentration^2 +14.62 * disodium hydrogen phosphate concentration^2

Model P value (Prob > F) was very low (0.0001). The fit of the model was also expressed by the coefficient of determination R^2 0.9490 indicating that 94.90 per cent of the variability in the response could be explained by the model for maximum cellulose production using MCW as a base substrate. The predicted R^2 values of 0.9490 are in agreement with adjusted R^2 value of 0.9013. The closer the R^2 value 1, the better the model was fit enough to experimental data, the less was the distance between the predicted and the observed values (Table 25).

Graphical representations of 3D response surfaces in Fig. 15 visualize the effect of fructose, yeast extract, citric acid and disodium hydrogen phosphate concentration on dry weight of bacterial cellulose using MCW as the basal medium. Fig. 15a showed the interaction between fructose and yeast extract concentration. Dry weight of the cellulose gradually increases with increase in fructose concentration of 20 g l^{-1} along with yeast extract at 10 g l^{-1} which had high interaction effect on the dry weight of cellulose (16.74 g l^{-1}). The effect of yeast extract (10 g l^{-1}) and citric acid concentration (1.5 g l^{-1}) on cellulose dry weight exhibited no significant effect on the yield (Fig.15d). Accordingly, the optimal level of the 4 parameters as obtained from the maximum point of the polynomial model was estimated using the design expert 8.0.7.1 software. Fructose concentration (20 g l^{-1}), yeast extract concentration (10 g l^{-1}), citric acid (1.5 g l^{-1}) and disodium hydrogen phosphate (1.1 g l^{-1}) had the maximum predicted dry weight of cellulose of 16.74 g l^{-1}.

4.20. Verification of optimum conditions for BC production using MCW medium

To confirm the combination of parameters predicted by using Design Expert software for maximum cellulose production, cross verification was done on cellulose production efficiency in 100 ml Erlenmeyer flasks with all the possible and predicted set of parameters. The parameters include physical component like fructose concentration (FC), yeast extract concentration (YC), citric acid concentration (CC) and disodium hydrogen phosphate concentration necessary for performing the cellulose production. At the end of 20th day of incubation, all the combinations were measured for its dry weight. Earlier it was predicted that at the combination of fructose (20 g l^{-1}), yeast extract (10 g l^{-1}), citric acid (1.5 g l^{-1}) and disodium hydrogen phosphate (1.1 g l^{-1}) in the basal

media MCW at the end of 20th day of fermentation the maximum dry weight obtained was 16.74 g l^{-1} for MCW. Interestingly the predicted values were with a good correlation with the actual values from the experiment as 16.11 g l^{-1}.

4.21. Response Surface Methodology (RSM) based optimization of CAJ medium for maximum cellulose production

The results of optimization of cashew apple juice for maximum cellulose production using RSM are presented in Table 26 and Fig. 16. The combined effect of 4 independent variables (Fructose Concentration (FC), Yeast extract Concentration (YC) Citric acid Concentration (CC) and Disodium hydrogen phosphate Concentration (DC) on bacterial cellulose production was analyzed using 30 experiments of central composite design. Four level central composite design and the experimental responses of the dependent variable (cellulose yield) are listed in Table 26.

The results were analyzed by using ANOVA, *i.e.* analysis of variance suitable for the experimental design. The results are shown in Table 27. The model F-value of 17.30 for dry weight of cellulose using CAJ implies that the model is significant. Model F-value was calculated as ratio of mean square regression and mean square residual. Model P-value (Prob > F) was very low (0.0001). The P values were used as a tool to check the significance of each of the coefficients, which in turn, are necessary to understand the pattern of the mutual interactions between the test variables. The smaller the magnitude of the P, the more significant was the corresponding coefficient. Values of P less than 0.05 indicate model terms were significant.

The regression coefficients estimates and the corresponding P values (Table 28) suggested that among the variables used in the cellulose production, A (fructose concentration), B (yeast extract concentration), C (citric acid concentration), D (disodium hydrogen phosphate concentration (A * B (fructose * yeast extract concentration), A * C (fructose concentration * citric acid concentration), A * D (fructose concentration * Disodium hydrogen phosphate concentration), B * C (yeast extract concentration * citric acid concentration), B * D (yeast extract concentration * disodium hydrogen phosphate concentration), C * D (citric acid concentration * disodium hydrogen phosphate concentration), A^2 (fructose * fructose), B^2 (yeast extract concentration * yeast extract

concentration), C^2 (citric acid concentration * citric acid concentration) and D^2 (disodium hydrogen phosphate concentration * disodium hydrogen phosphate concentration) were significant model terms. Fructose concentration ($P<0.0001$) had the largest effect in dry weight of bacterial cellulose, followed by yeast extract concentration with a P value of 0.08, The corresponding second-order response model that was found after analysis for the regression was,

Final equation in terms of coded factors for dry weight of bacterial cellulose

Dry weight of bacterial cellulose = +16.24 + 1.86 * A + 0.32 * B + 0.19 * C - 0.27 * D + 0.048 * A * B - 0.41 * A * C + 0.50* A * D - 0.28 * B * C + 0.28 * B * D – 0.24 * C * D – 1.34 * A^2 – 1.19 * B^2 - 0.27 * C^2 - 0.39 * D^2

Final equation in terms of actual factors for dry weight of bacterial cellulose

Dry weight of bacterial cellulose = - 5.94 + 3.09 * fructose concentration + 3.48 * yeast extract concentration + 3.37 * citric acid concentration + 2.484 * Disodium hydrogen phosphate concentration + 0.01 * fructose concentration * yeast extract concentration - 0.20 * fructose concentration * citric acid concentration + 1.00 * fructose concentration * disodium hydrogen phosphate concentration - 0.13 * yeast extract concentration * citric acid concentration + 0.56 * yeast extract concentration * disodium hydrogen phosphate concentration - 0.97 * citric acid concentration * disodium hydrogen phosphate concentration - 0.33 * fructose concentration^2 - 0.29870 * yeast extract concentration^2 - 0.26 * citric acid concentration^2 - 6.31 * disodium hydrogen phosphate concentration^2

Model P value (Prob > F) was very low (0.0001). The fit of the model was also expressed by the coefficient of determination R^2, which was found to be 0.9417 indicating that 94.17 per cent of the variability in the response could be explained by the model for dry weight of bacterial cellulose using CAJ. The predicted R^2 values for dry weight of bacterial cellulose using CAJ was 0.9417 and had reasonable agreement with adjusted R^2 values 0.9872. The closer the R^2 value was 1, the better the model was fit enough to experimental data, the less was the distance between the predicted and the observed values (Table 29).

Graphical representations of 3D response surfaces are shown in Fig. 16 visualizes the effect of fructose concentration, yeast extract concentration, citric acid concentration and disodium hydrogen phosphate on dry weight of bacterial cellulose using CAJ. Fig. 16a showed the interaction between fructose and dry weight of cellulose gradually increases with increase in fructose concentration and at the concentration of 4 g l^{-1} along with other optimum media components in the basal media containing CAJ, the dry weight of the cellulose was highly significant. The interaction effect of citric and fructose was also found to increase the dry weight of cellulose (Fig. 16b). There was no interaction effect between the citric acid and disodium hydrogen phosphate levels and is indicated in the Fig. 16f.

Accordingly, the optimal level of the 4 parameters obtained from the maximum point of the polynomial model was estimated using the design expert 8.0.7.1 software. Fructose concentration (4.0 g l^{-1}), Yeast extract concentration (6.0 g l^{-1}), Citric acid (1.5 g l^{-1}) and Disodium hydrogen phosphate (0.75 gl^{-1}) had the maximum predicted dry weight of cellulose with 16.24 g l^{-1}, whereas the actual experiment resulted in 16.22 g l^{-1}.

4.22. Verification of optimum conditions for BC production using CAJ medium

To confirm the combinations of parameters predicted by using Design Expert software for maximum cellulose production, cross verification was done on cellulose production efficiency in 100 ml Erlenmeyer flasks with all the possible and predicted set of parameters (Plate 13). The parameters include physical component like Fructose concentration (FC), Yeast extract concentration (YC) Citric acid concentration (CC) and Disodium hydrogen phosphate concentration necessary for performing the cellulose production. At the end of 20th day of incubation, all the combinations were measured for its dry weight. Earlier it was predicted that at the combination of fructose concentration of 4.0 g l^{-1}), yeast extract concentration of 6.0 g l^{-1}, citric acid concentration of 1.5 g l^{-1} and disodium hydrogen phosphate concentration of 0.75 g l^{-1}, the predicted dry weight of cellulose was 16.24 g l^{-1}, whereas the actual experiment resulted in 16.22 g l^{-1} with a good correlation.

4.23. Pilot scale bacterial cellulose production using model fermentor (5 litre capacity)

4.23.1. Fermentation kinetics

Once the predicted and actual values of cellulose production were standardized using RSM, a pilot scale bacterial cellulose production was carried out in a 5 litre capacity model fermentor containing cashew apple juice medium. The real image of the model fermentor showing the development of cellulose pellicle is depicted in Plate 14. In the model fermentor designed, the culture showed good growth with improved production and harvest friendly due to the use of wide mouthed container. Further after the extraction of the bacterial cellulose, the fermented spent liquor was used as inoculum for vinegar production process. The selected isolate, *Gluconacetobacter* sp. sju-1 was used as a mother culture for the pilot scale fermentation studies by adopting the already optimized parameters. The growth of sju-1 and cellulose production were analyzed by periodic withdrawal of samples to find out the correlation between the cell growth and cellulose production and other kinetic parameters. The results were tabulated in Table 30.

4.23.2. Changes in viable cell count, pH and total sugars of CAJ medium in model fermentor during the course of fermentation

The results are presented in Table 31 and Fig. 17. The population increase with a gradual decrease in pH was noticed upto 16th day of inoculation. Initially the population was 0.002 x 10^{11}cfu ml^{-1}. It doubled on the second day of incubation where it reached 0.011 x 10^{11} cfu ml^{-1}, and at this point there was no significant difference in the pH where it reduced from 6.5 to 6.3, but on the 6th day of incubation when the population reached 4.21 x 10^{11} cfu ml^{-1}, the pH decreased to 6.0. Further as the day progress, on the 10th day the population reached to 25.21 x 10^{11} cfu ml^{-1} from 13.24 x 10^{11} cfu ml^{-1} of the 8th day. By the 16th day of incubation, the population reached the maximum of 34.12 x 10^{11} cfu ml^{-1} and the pH dropped to 4.5. Thereafter the population reached its stationary phase with the population of 31.22 x 10^{11} and 33.14 x 10^{11} cfu ml^{-1} on 18th and 20th day respectively. And finally on the 20th day the pH became very acidic and reached 3.5.

With respect to total sugars, initially the concentration was 18.00 g l^{-1} (Fig. 18) From the 2nd to the 6th day the total sugar content dropped from 16.14 to 10.88 g l^{-1} but

on the 8th day, the total sugar content dropped to 9.14 g l^{-1}. On the 16th day when the population was at the maximum, the total sugar content dropped to 2.18 g l^{-1}. Finally at the end of the experiment the sugar content was only1.50 g l^{-1}.

4.24. Physical properties of bacterial cellulose from HS, MCW and CAJ medium

4.24.1. Colour analysis

As shown in Table 32, sju-1 yielded an opaque, white and a thick cellulose pellicle. The L* value of 51.42±0.11 shifted slightly toward the lightness side for the bacterial cellulose from coconut water medium. However, the values for L* (Lightness) for BC obtained from cashew apple juice detected higher values of positive b*(Yellow and blue), indicating yellowness, which contained a carotene colour. The a* (Red and green) values for all the three types of cellulose were almost same of 2.41±0.11, 2.45±0.02 and 2.17±0.01. The b* values for cellulose CAJ medium was 19.44±0.01 but it was 0.41±0.02 for BC from MCW medium. Values for the calculated Chroma (C*) for the celluloses obtained from three medium were 2.61±0.01, 2.65±0.04 and 18.77±0.01 respectively. The results clearly confirmed that cellulose from MCW medium was pure white in colour and was light yellowish from CAJ medium, but those produced from the HS medium (control) was light brown in colour.

4.24.2. Texture, water holding capacity, crude fibre and total dietary fibre of bacterial celluloses

The textural properties of bacterial cellulose obtained using texture analyzer based on the compression force exerted over the cellulose are presented in Table 33. The texture of bacterial cellulose produced from the HS medium was very higher than the other two types of bacterial cellulose obtained from MCW and CAJ medium. WHC of the celluloses from three medium were 84.44±0.04 per cent, 87.14±0.10 and 88.54±0.02 per cent. The crude fibre content was greater in HS medium of 11.25±0.01 g and MCW and CAJ medium recorded 10.74±0.01 g and 10.04±0.03 g respectively. The total dietary fibre constituted by soluble and insoluble fibre content recorded a higher value of 0.04±0.01 g and 8.50±0.35 g for BC from HS medium. There was a very little variation in the soluble dietary fibre content among the different medium. However the insoluble fibre content

showed a comparatively higher content in HS medium (8.50±0.35 g) followed by CAJ and MCW medium of 7.80±0.02 g and 7.20±0.04 g respectively.

4.25. Volatile acidity in mature coconut water vinegar produced using spent liquor of CAJ medium after cellulose production

The results of volatile acidity analyzed at 8 h interval in the coconut water vinegar produced using fermented liquor obtained after the extraction of bacterial cellulose from the CAJ medium is depicted in Table 34.

At 8.0 h after purification the volatile acidity was 1.2 ± 0.03 per cent and at 16 h there was only 1.0 per cent increase of 2.45 ± 0.06 per cent. Volatile acidity of 3.9 ± 0.09 and 4.8 ± 0.15 per cent was recorded at 24 and 32 h respectively. Thus when used in the preparation of coconut water vinegar, the resulted vinegar registered volatile acidity of 4.8 per cent.

4.26. Structural identification of bioactive compounds produced in fermented broth by Gas Chromatography Mass Spectrophotometer (GCMS)

The result of GCMS analysis of crude extract of fermented broth is presented in Table 35 and Fig. 19. The result revealed the presence of eight volatile compounds dominated by thiophene-2-carboxylic acid and santolin diacetylene (24.8 per cent), 1,5-dihydroxyl-3,3-dimethyl-7-acetylaminomethyl-tricyclododecane (10.75 per cent), 3-phenyl-3-m-tolylpropionamide and 1-(2-hydroxyethylthio)-1-phenyl-2-butanone (12.33 per cent), Nonane and 4-hepatanol (6.53 per cent) and 3-(4-nitrobenzoyl) cinnoline (5.8 per cent). Thiophene-2-carboxylic acid, santolin diacetylene, acetic acid, lavanduyl acetate, Benzaldehyde dioctyl acetal, Neopentyl-2-oxobutanoate 1-heptanol and 3-(4-nitrobenzoyl) cinnoline were the flavour compounds. Amide compounds like acetamide and diacetamide were produced in the crude extract. There was a relative abundance of alkane hydrocarbon like dodecane, heptadecane, octadecane, hexadecane, octacosane, heneicosane, tricosane and nonane. Other minor compounds present in the crude extract were piperazine, leucinol, crocusatin, sarracine, haliclorensin and quinoxaline.

4.27. Production of reinforced bacterial cellulose paper

The reinforced bacterial cellulose produced using calcium alginate incorporated HS medium, wet untreated cellulose pellicle, alkali treatment of pellicle and the BC pulp are depicted in Plates 15, 16, 17 and 18. When subjected for the production of BC paper, the physical and mechanical properties of the reinforced paper possessed significantly higher tensile strength and Young's modulus. The real image of dry BC sheets and BC reinforced paper are depicted in Plates 19 and 20.

The tensile strength and Young's modulus were recorded to be 120 ± 2.89 MPa and 4.9 ± 0.01 GPa respectively. The viscosity and degree of polymerisation are 127.4 ±0.29 cP and 2074±2.91 respectively (Table 36). Castor oil penetration test showed that it completely offered resistance to oil penetration and air could not penetrate into it as confirmed by the air penetration test. The physical and mechanical properties when compared to the reinforced paper obtained from wood cellulose, the tensile strength and Young's modulus, viscosity (cP) and degree of polymerization (DP) were comparatively lower for wood celluloses of 90±1.67 MPa, 4.2±0.10 GPa, 110.1±0.01 cP and 2001±1.20 DP respectively. With regard to air penetration test, it registered an air flow rate of 2.29±0.04 50 sec ml^{-1} and showed oil resistance permanently as that of BC reinforced paper.

4.28. Testing of laxative properties of bacterial cellulose by animal studies

The Effect of loperamide on feed, water intake and faecal properties of constipated Sprague-Dawley rats were studied and the results are presented in the Table 37. The constipated rats had more feed intake of 21.24±0.87 g compared to the normal control (21.24±0.87 g). Whereas water intake was significantly higher in the normal control (24.00±1.47 ml) than the constipated rats. Number of faecal pellets increased twice (70.77±3.00) in normal control producing a faecal weight of 7.12±0.63 g than the constipated rats which recorded 30.55±4.00 pellets with the faecal weight of 3.19±0.44 g. Similarly the moisture content of the faecal material was higher in the normal rats compared to the constipated ones.

The effect of colloidal bacterial cellulose laxative on feed intake, water intake and faecal properties of constipated rats were carried out and were presented in Tables 38.

Plate 21, shows the oral administration of colloidal bacterial cellulose laxative to a Sprague-Dawley rat. Among the four groups tested, Group I and III had higher feed intake (20.21±3.39 g and 20.21±1.21 g) compared to Group II (19.99±1.26 g) and IV (19.58±1.52 g). Water intake was found to be higher in Group II and Group IV. Significantly lower water uptake was recorded in Group I followed by Group III. Number of faecal pellets, a most important parameter dealing with the laxative property of the bacterial cellulose was higher in group IV, which excreted 46.34±2.55 number of faecal pellets followed by Group II (44.89±2.36). Similar results were obtained with respect to the moisture content and faecal weight. The animals that falls in group IV recorded a faecal pellet weight of 16.87±54 g with the moisture percent of 2.94±1.24 followed by group III rats which recorded 15.23±1.14 g with the moisture per cent of 2.15±1.11. Group II also recorded a very closer faecal weight of 15.21±0.65 g with a moisture per cent of 2.00±1.23. Group I recorded the least values for number of faecal pellet, moisture content of faecal pellet (%) and weight of faecal pellet of 29.66±1.28, 0.77±1.24 and 3.93±0.54 g of respectively. Plate 22, shows the faecal differences between the different treatment groups.

The results showed that loperamide administration induced severe experimental constipation in rats. The faeces content was decreased by loperamide. The water content in faeces as a constipation parameter was also decreased by loperamide. On the other hand, the dry weight of faeces was not significantly different among the groups. And also the feed intake was not significantly different between the control and constipated animals. This represents that the amount of food consumption might have been similar among the groups, and constipation was caused by delayed intestinal movement. Both the Isabgol and the colloidal laxative of BC treated rats for 7 days recovered from the loperamide induced constipation. This was evidenced by the faecal weight of the treated rats.

4.29. *Nata* production

Bacterial cellulose/ *nata* produced after processing in sugar syrup is depicted in Plate 23. In Plate 24, diced *nata* cubes coloured with beet root extract (2 per cent) and the uncoloured *nata* from CAJ medium is depicted. *Nata* in jelly base prepared from MCW and CAJ medium are shown in Plates 25 and 26. The prepared jelly was soft textured and showed very good acceptability over the control.

4.29.1. Sensory evaluation of *nata-de-coco* and *nata-de-cashew*

The results are presented in Table 39. The colour, appearance and flavour of *nata* derived from three medium did not show any significant difference, but in the case of texture a marked difference existed, where *nata* from HS medium recorded a lower acceptability of 8.4 and *nata-de-coco* and *nata-de-cashew* recorded a comparatively higher acceptability scores of 9.4 and 9.1. With respect to taste and tenderness *nata-de-coco* registered an high acceptability score of 9.5 followed by *nata-de-cashew* which recorded 9.2. In the case of *nata* from HS medium, very lower acceptability scores of only 8.7 was recorded. The overall acceptability scores of *nata* from HS medium, *nata-de-coco* and *nata-de-cashew* were 8.7, 9.2 and 9.02 respectively.

4.30. Cost of production of bacterial cellulose

The results of cost of production of bacterial cellulose are illustrated in Table 40. With regard to the feasibility of bacterial production, economic analysis was performed to analyze the Benefit Cost Ratio (BCR). Approximately 22 ml of cashew apple juice can be extracted from 100 g of cashew fruits and the mature coconut water in abundantly available in India. So with the use of these bio-wastes, media was formulated with minimal carbon cost and bacterial cellulose production was achieved. In the method standardized presently, fructose was used to develop bacterial cellulose. Due to easy availability and comparatively cheaper, cane sugar can be used instead of fructose and the same has been used to determine the cost of production. The raw material, cashew apple can be procured at cheaper cost due to larger availability as a bio-waste. Wet cellulosic mats have wide market avenues and are sold at the rate of Rs. 750/- per kg. When it is value added in the form of syrup base or jelly base it is sold at the rate of Rs. 1500/- per kg. From 1.0 litre of the fermentation media it is possible to produce 185 g of fresh cellulosic mat.

The BCR was worked out on the basis of income to expenditure incurred on non-recurring expenses, direct and indirect cost, interest on loan and depreciation was found to be 1.05 for the wet cellulose mat production and when the bacterial cellulose is value added into a syrup/jelly form the BC ratio hiked to 1.97. Hence the project seems to a viable one with a good techno-economic feasibility.

Table 4. Isolation and screening of cellulose producing bacteria from fruits

Sl.No	Sources	No. of morphologically distinct colonies producing zone of hydrolysis in GYC medium	No. of isolates producing cellulose pellicle in HS medium	Strain designation of cellulose pellicle producing isolates
1	Apple	8	5	app-1, app-2,app-3,app-4,app-5
2	Banana	5	1	ban-1
3	Cashew Apple	11	4	caap-1, caap-2,caap-3,caap-4
4	Citrus	7	1	cit-1
5	Cocoa Extract	7	4	coex-1,coex-2,coex-3,coex-4
6	Coconut Milk	8	4	com1,com2,com3,com4
7	Coconut water	7	4	cw-1,cw-2, cw-3, cw-4
8	Coffee Extract	9	4	cex-1, cex-2,cex-3, cex-4
9	Custard Apple	8	4	cuap-1, cuap-2, cuap-3, cuap-4
10	Dates	5	2	dat-1 , dat-2
11	Grapes	4	1	gra-1
12	Guava	6	1	gua-1
13	Hibiscus Flower Nectar	5	3	fln-1,fln-2,fln-3
14	Mango	7	2	man-1, man-2
15	Palm Fruit	6	4	paf-1, paf-2,paf-3,paf-4
16	Palm Vinegar (*Padaneer*)	9	3	pav-1,pav-2,pav-3
17	Papaya	7	1	papa-1
18	Pine Apple	6	3	pap-1, pap-2, pap-3
19	Pomegranate	2	1	pom-1
20	Sapota	4	1	sap-1
21	Tea Extract	5	3	tex-1, tex-2,tex-3
22	Wood Apple	5	3	wap-1, wap-2,wap-3
	Total	141	59	

Table 5. Isolation of cellulose producing bacteria from high sucrose containing sugarcane varieties

Sl.No	Fermented juice from high sucrose containing sugarcane varieties	No. of colonies producing zone of hydrolysis in GYC medium	No. of isolates producing cellulose pellicle in HS medium	Strain designation of cellulose pellicle producing isolates
1	Co1148	5	3	sju-1, sju-2,sju-3
2	Co 2000-12	5	2	sju-4, sju-5
3	Co 2001-13	4	3	sju-6, sju-7, sju-8
4	Co 2001-15	2	1	sju-9
5	Co 86032	4	2	sju-10, sju-11
6	Co 94008	4	1	sju-12
7	Co 94012	2	1	sju-13
9	Co 99004	3	2	sju-14, sju-15
10	Co 99008	4	2	sju-16, sju-17
11	Co 0218	5	2	sju-18, sju-19
12	Co 8371	3	2	sju-20, sju-21
13	CoC 671	5	2	sju-22, sju-23
14	Co 99008	4	2	sju-24, sju-25
15	Co 0218	5	2	sju-26, sju-27
16	Co 8371	3	2	sju-28, sju-29
	Total	58	29	

Table 6. Screening of the elite cellulose producing isolate based on Gallardo scale of thickness

Sl.No	Isolates from different sources	Cellulose thickness (mm)			
		Poor (1 mm)	Fair (2 mm)	Good (4 mm)	Excellent (5 mm)
1.	app-1	---	2	---	---
	app-2	---	2	---	---
	app-3	0.5	---	---	---
	app-4	0.5	---	---	---
	app-5	0.5	---	---	---
2.	ban-1	0.5	---	---	---
3.	caap-1	---	2	---	---
	caap-2	---	2	---	---
	caap-3	---	---	4	---
	caap-4	---	---	---	5
4.	cit-1	---	2	---	---
5.	coex-1	---	---	---	6
	coex-2	---	---	---	6
	coex-3	---	---	4	---
	coex-4	---	---	4	---
6.	com-1	---	2.5	---	---
	com-2	---	2.5	---	---
	com-3	---	3	---	---
	com-4	---	3	---	---
7.	cw -1	---	---	4	---
	cw-2	---	---	---	6
	cw-3	---	---	---	6
	cw-4	---	---	---	7
8.	cex-1	---	---	4	---
	cex-2	---	---	4	---
	cex-3	---	---	4	---
	cex-4	---	---	4	---
9.	cuap-1	---	2	---	---
	cuap-2	---	2	---	---
	cuap-3	---	---	4	---
	cuap-4	---	---	---	8
10.	dat-1	---	---	4	---
	dat 2	---	2	---	---
11.	gra-1	1.0	---	---	---
12.	gua-1	1.0	---	---	---
13.	fln 1	---	---	4	---
	fln 2	---	---	4	---
	fln 3	---	---	4	5
14.	man1	0.5	---	---	---
	man2	0.5	---	---	---

Sl.No	Isolates from different sources	Cellulose thickness (mm)			
		Poor (1 mm)	Fair (2 mm)	Good (4 mm)	Excellent (5 mm)
15.	paf 1	---	---	---	6
	paf 2	---	---	4	---
	paf 3	---	---	4	---
	paf 4	---	---	---	7
16.	pav 1	---	---	4	---
	pav 2	---	---	4	---
	pav 3	---	---	4	---
17.	papa 1	---	2	---	---
18.	pap 1	0.5	---	---	---
	pap 2	0.6	---	---	---
	pap 3	---	---	4	---
19.	pom1	---	2	---	---
20.	sap1	---	2	---	---
21.	sju-1	---	---	---	13
	sju-2	---	---	---	9
	sju-3	---	---	---	7
	sju-4	---	---	---	7
	sju-5	---	---	---	8
	sju- 6	---	---	---	10
	sju-7	---	---	---	10
	sju-8	---	---	---	11
	sju-9	---	---	---	8
	sju-10	---	---	---	7
	sju-11	---	---	---	9
	sju-12	---	---	---	10
	sju-13	---	---	---	9
	sju-14	---	---	---	9
	sju-15	---	---	---	9
	sju-16	---	---	---	10
	sju-17	---	---	---	10
	sju-18	---	---	---	10
	sju-19	---	---	---	10
	sju-20	---	---	---	9
	sju-21	---	---	---	9
	sju-22	---	---	---	8
	sju-23	---	---	---	8
	sju-24	---	---	---	8
	sju-25	---	---	---	10
	sju-26	---	---	---	8
	sju-27	---	---	---	8
	sju-28	---	---	---	7
	sju-29	---	---	---	8

Sl.No	Isolates from different sources	Cellulose thickness (mm)			
		Poor (1 mm)	Fair (2 mm)	Good (4 mm)	Excellent (5 mm)
22.	tex-1	---	---	---	8
	tex-2	---	---	4	---
	tex-3	---	---	4	---
23.	wap-1	---	---	4	---
	wap-2	---	---	4	---
	wap-3	---	---	4	---
24.	NCIM 2526	---	---	---	9

--- Indicates pellicle formation not detected

Table 7. Morphological characteristics of cellulose producing bacteria

Sl. No	Isolates	Cell and colony characters			
		Shape	Gram reaction	Morphology	Colour
1	caap-4	Short rods	-	Regular with serrated margin	Milky white
2	coex-1	Short rods	-	Regular with entire margin	Light cream
3	coex-2	Short rods	-	Regular with entire margin	Milky White
4	cw-2	Short rods	-	Irregular with entire margin	Milky White
5	cw-3	Short rods	-	Irregular with lobed margin	Pale yellow
6	cw-4	Short rods	-	Circular convex with entire margin	Yellow
7	cuap-4	Short rods	-	Flat with irregular margin	White
8	fln-3	Short rods	-	Very small colonies with regular margin	Yellow
9	paf-1	Short rods	-	Flat colonies with regular margin	Pale yellow
10	paf-4	Short rods	-	Raised colonies with irregular margin	Cream white
11	sju-1	Short rods	-	Regular with entire margin	Milky White
12	sju-2	Short rods	-	Regular with entire margin	Light brown
13	sju-3	Short rods	-	Slightly irregular with undulated margin	White
14	sju-4	Short rods	-	Slightly irregular with undulated margin	White
15	sju-5	Short rods	-	Circular convex with entire margin	Pale yellow
16	sju- 6	Short rods	-	Irregular with entire margin	Yellow
17	sju-7	Short rods	-	Circular convex with entire margin	White
18	sju-8	Short rods	-	Regular with entire margin	Cream white
19	sju-9	Short rods	-	Irregular with entire margin	Pale yellow
20	sju-10	Short rods	-	Circular entire margin	Cream white
21	sju-11	Short rods	-	Regular with wavy margin	White
22	sju-12	Short rods	-	Circular serrated margin margin	Cream white
23	sju-13	Short rods	-	Irregular with entire margin	Pale yellow
24	sju-14	Short rods	-	Circular wavy margin	Cream white
25	sju-15	Short rods	-	Irregular with entire margin	White
26	sju-16	Short rods	-	Circular convex with entire margin	Cream white
27	sju-17	Short rods	-	Regular with wavy margin	Pale yellow
28	sju-18	Short rods	-	Circular convex with entire margin	Cream white
29	sju-19	Short rods	-	Irregular with entire margin	Cream white
30	sju-20	Short rods	-	circular with irregular margin	Light yellow
31	sju-21	Short rods	-	Regular with wavy margin	Cream white
32	sju-22	Short rods	-	Circular convex with entire margin	Milky white
33	sju-23	Short rods	-	Irregular with entire margin	Cream white
34	sju-24	Short rods	-	Irregular with lobed margin	yellow
35	sju-25	Short rods	-	Regular with entire margin	Brown
36	sju-26	Short rods	-	Circular convex with entire margin	Light brown
37	sju-27	Short rods	-	Irregular with entire margin	Brown
38	sju-28	Short rods	-	Irregular with lobed margin	Dark brown
39	sju-29	Short rods	-	Irregular with lobed margin	Light yellow
40	NCIM 2526	Very short rods	-	Regular big sized colonies with serrated margin	Brownish yellow

Table 10. Effect of culture concentration on cellulose production by sju-1

S. No	Culture concentration (%)	Dry weight of cellulose (g l^{-1})
1.	5	1.14±0.02
2.	10	5.45±0.01
3.	15	5.50±0.03
4.	20	5.52±0.02
	SEd	0.027
	CD (0.05)	0.063

Data are mean ± SD of three replications

Table 11. Effect of incubation time on cellulose production by sju-1

S. No	Incubation time (days)	Dry weight of cellulose (g l^{-1})
1.	2	0.02±0.45
2.	4	0.07±0.01
3.	6	0.92±0.27
4.	8	1.97±0.54
5.	10	5.39±0.45
6.	12	9.87±0.16
7.	14	12.78±0.87
8.	16	14.11±0.28
9.	18	14.97±0.87
10.	20	14.02±0.29
	SEd	0.37
	CD (0.05)	0.78

Data are mean ± SD of three replications

Table 12. Effect of pH on cellulose production by sju-1

S. No	pH	Dry weight of cellulose (g l^{-1})
1	4.0	13.1±0.27
2	4.5	13.7±0.33
3	5.0	13.7±0.32
4	5.5	14.1±0.43
5	6.0	15.4±0.35
6	6.5	15.7±0.36
7	7.0	14.2±0.36
8	7.5	11.4±0.90
9	8.0	10.1±0.23
	SEd	1.66
	CD (0.05)	3.25

Table 13. Effect of temperature on cellulose production by sju-1

S. No	Temperature (°C)	Dry weight of cellulose (g l^{-1})
1	25	11.2±0.23
2	27	11.7±0.27
3	29	11.9±0.27
4	31	14.9±0.34
5	33	13.2±0.30
6	35	10.7±0.25
	SEd	0.50
	CD (0.05)	1.09

Table 14. Effect of culture conditions on the yield of cellulose by sju-1

S. No	Yield parameters	Culture conditions	
		Static culture	Shaken flask @ 120 rpm
1	Biomass (g l^{-1})	8.42±0.17	7.38±0.15
2	Viable cell count (cfu ml^{-1})	4.2 X 10^{11} (0.62±0.1)	3.7 X 10^{11} (0.56±0.1)
3	pH (final)	4.0±0.08	3.0±0.06
4	Wet weight (g l^{-1})	182.4±3.70	180.1±3.65
5	Dry weight (g l^{-1})	14.92±0.30	9.63±0.20
6	Moisture content (%)	91.8±1.86	94.6±1.92
7	Amount of soluble polysaccharides (g l^{-1})	0.48±0.01	1.65±0.03
8	Biosynthetic yield (%)	74.7±0.32	48.15±0.98

Log transformed values are indicated in the brackets

Data are mean ± SD of three replications

Table 15. Effect of different carbon sources on cellulose production by sju-1

S. No	Carbon sources	Dry weight of cellulose (g l^{-1})
1	Fructose	15.8±0.32
2	Glucose	14.1±0.35
3	Galactose	13.1±0.30
4	Glycerol	9.1±0.39
5	Inositol	7.5±0.23
6	Malic acid	11.2±0.46
7	Mannitol	12.5±0.29
8	Mannose	9.6±0.23
9	Rhamnose	10.1±0.23
10	Sorbitol	12.3±0.14
11	Starch	8.9±0.21
12	Sucrose	14.6±0.34
	SEd	0.38
	CD (0.05)	0.78

Table 16. Effect of different nitrogen sources on cellulose production by sju-1

S. No	Nitrogen sources	Dry weight of cellulose (g l^{-1})
1	Ammonium carbonate	11.2±0.55
2	Ammonium chloride	12.0±0.23
3	Ammonium nitrate	10.4±0.35
4	Ammonium sulphate	10.9±0.30
5	Beef extract	11.9±0.45
6	Calcium nitrate	11.5±0.35
7	Casein hydrolysate	12.9±0.31
8	Malt extract	13.4±0.38
9	Peptone	14.1±0.74
10	Potassium nitrate	13.1±0.33
11	Sodium nitrate	9.81±0.35
12	Yeast extract	15.1±0.37
	SEd	0.517
	CD (0.05)	1.06

Data are mean ± SD of three replications

Table 17. Effect of growth hormones on cellulose production by sju-1

S. No	Growth hormones (ppm)	Dry weight of cellulose (g l^{-1})	
		Kinetin	Indole Acetic Acid
1	200	14.1±0.03	15.1±0.03
2	400	15.2±0.21	15.9±0.10
3	600	15.7±0.05	16.7±0.04
4	800	15.4±0.07	16.9±0.02
5	1000	15.8±0.01	16.4±0.12
SEd		0.249	0.26
CD (0.05)		0.555	0.58

Table 18. Effect of different additives @ 0.1 (%) on cellulose production by sju-1

S. No	Additives	Dry weight of cellulose (g l^{-1})
1	Agar	14.7±0.30
2	Agarose	14.5±0.29
3	Acetan	15.4±0.92
4	Calcium alginate	17.8±0.38
5	Chitin	15.9±0.56
6	Chitosan	15.5±0.32
7	Cellulose	11.1±0.23
8	Carboxy methyl cellulose	14.7±0.30
9	Dextran	14.1±0.49
10	Gelatin	15.7±0.32
11	Gellan	16.1±0.33
12	Levan	15.2±0.20
13	Lignosulfonate	17.8±0.41
14	Poly ethylene glycol	17.0±0.39
15	Sodium alginate	14.2±0.73
SEd		0.25
CD (0.05)		0.51

Data are mean ± SD of three replications

Table 19. Physicochemical properties of Mature Coconut Water (MCW) and Cashew Apple Juice (CAJ)

S.No.	Particulars	Mature coconut water (MCW)	Cashew apple juice (CAJ)
1	pH	5.2±0.01	4.29±0.12
2	Total Soluble Solids °Brix	5.4±0.11	12.9±0.37
3	Total Titrable Acidity (%)	0.1±1.22	0.27±0.01
4	Reducing sugars (g/ 100g)	0.2±0.01	11.3±0.33
5	Protein (g/ 100g)	0.1±0.01	3.32±0.10
6	Fat (g/ 100g)	0.60±0.01	0.50±0.01
7	Phosphorous (mg/ 100g)	6.3±0.92	211.2±6.14
8	Potassium (mg/ 100g)	247.0±0.02	69.5±0.61
9	Sodium (mg/ 100g)	48.0±0.11	39.1±0.47
10	Calcium (mg/ 100g)	40.0±0.12	46.1±0.57
11	Magnesium (mg/ 100g)	15.0±0.97	178.2±0.14
12	Copper (µg/ 100g)	26.0±0.15	0.01±6.10
13	Iron (µg/ 100g)	79.0±0.35	0.04±1.33

Data are mean ± SD of three replications

Table 20. Pre-optimisation of mature coconut water (MCW) media for bacterial cellulose production

S.No.	Treatment combinations	Dry weight of bacterial cellulose ($g\ l^{-1}$)
1.	Mature coconut water alone (1000 ml)	0.20±0.03
2.	Mature coconut water (1000 ml)+ 10 g fructose	6.50±0.13
3.	Mature coconut water (1000 ml) + 15 g fructose	7.50±0.15
4.	Mature coconut water (1000 ml)+ 20 g fructose	6.90±0.30
5.	Mature coconut water (1000 ml) + 15 g fructose + 5.0 g yeast extract	11.70±0.24
6.	Mature coconut water (1000 ml) + 15 g fructose +7.5 g yeast extract	11.24±0.47
7.	Mature coconut water (1000 ml) + 15 g fructose + 10.0 g yeast extract	12.74±0.23
8.	Mature coconut water (1000 ml)+ 15 g fructose + 10.0 g yeast extract +1.5 g citric acid	14.11±0.29
9.	Mature coconut water (1000 ml)+ 15 g fructose + 10.0 g yeast extract +2.0 g citric acid	14.77±0.30
10.	Mature coconut water (1000 ml)+ 15 g fructose + 10.0 g yeast extract +2.5 g citric acid	13.80±0.20
12.	Mature coconut water (1000 ml) + 15 g fructose + 10.0 g yeast extract +2.0 g citric acid+ 1.10 g disodium hydrogen phosphate	15.95±0.32
13.	Mature coconut water (1000 ml) + 15 g fructose + 10.0 g yeast extract +2.0 g citric acid+ 1.15 g disodium hydrogen phosphate	16.12±0.33
14.	Mature coconut water (1000 ml) + 15 g fructose + 10.0 g yeast extract +2.0 g citric acid+ 1.20 g disodium hydrogen phosphate	16.00±0.48
	SEd	0.42
	CD (0.05)	0.87

Table 21. Pre-optimisation of Cashew Apple Juice (CAJ) media for bacterial cellulose production

S.No.	Treatment combinations	Dry weight of bacterial cellulose (g l^{-1})
1.	Cashew apple juice alone (250 ml)	0.75±0.04
2.	Cashew apple juice alone (500 ml)	1.25±0.03
3.	Cashew apple juice alone (750 ml)	1.95±0.04
4.	Cashew apple juice alone (1000 ml)	2.50±0.05
5.	Cashew apple juice (1000 ml) + 2.0 g fructose	3.14±0.31
6.	Cashew apple juice (1000 ml)+ 4.0 g fructose	9.27±0.19
7.	Cashew apple juice (1000 ml) + 6.0 g fructose	8.56±0.17
8.	Cashew apple juice (1000 ml) + 8.0 g fructose	8.17±0.41
9.	Cashew apple juice (1000 ml) + 4.0 g fructose + 2.0 g yeast extract	11.21±0.23
10.	Cashew apple juice (1000 ml) + 4.0 g fructose + 4.0 g yeast extract	12.11±0.25
11.	Cashew apple juice (1000 ml) + 4.0 g fructose + 6.0 g yeast extract	14.57±0.30
12.	Cashew apple juice (1000 ml)+ 4.0 g fructose + 8.0 g yeast extract	13.47±0.27
13.	Cashew apple juice (1000 ml) + 4.0 g fructose + 6.0 g yeast extract +0.5 g citric acid	12.31±0.28
14.	Cashew apple juice (1000 ml)+ 4.0 g fructose + 6.0 g yeast extract +1.0 g citric acid	15.12±0.35
15.	Cashew apple juice (1000 ml) + 4.0 g fructose + 6.0 g yeast extract +1.5 g citric acid	15.84±0.37
16.	Cashew apple juice (1000 ml) + 4.0 g fructose + 6.0 g yeast extract +2.0 g citric acid	14.71±0.33
17.	Cashew apple juice (1000 ml)+ 4.0 g fructose + 6.0 g yeast extract +1.5 g citric acid+ 0.5 g disodium hydrogen phosphate	16.11±0.37
18.	Cashew apple juice (1000 ml)+ 15 g fructose + 6.0 g yeast extract +1.5 g citric acid+ 0.75 g disodium hydrogen phosphate	16.00±0.44
19.	Cashew apple juice (1000 ml) + 15 g fructose + 6.0 g yeast extract +1.5 g citric acid+1.0 g disodium hydrogen phosphate	16.00±0.37
	SEd	0.33
	CD (0.05)	0.65

Table 22. Central composite design matrix of different parameters of independent variables and their corresponding experimental and predicted values of dry weight of cellulose using MCW medium

Standard order	Independent variables				Dry weight of cellulose (g l^{-1})	
	Fructose concentration (g l^{-1})	Yeast extract concentration (g l^{-1})	Citric acid Concentration (g l^{-1})	Disodium Hydrogen Phosphate Concentration (g l^{-1})	Observed	Predicted
1	10	5	1.5	1.1	14.3	14.49
2	**20**	**10**	**1.5**	**1.1**	**16.11**	**16.74**
3	10	10	1.5	1.1	9.23	9.27
4	20	10	1.5	1.1	14.49	14.62
5	10	5	2.5	1.1	14.22	14.30
6	20	5	2.5	1.1	14.45	14.62
7	10	10	2.5	1.1	10.21	10.65
8	20	10	2.5	1.1	14.42	14.07
9	10	5	1.5	1.2	13.25	13.82
10	20	5	1.5	1.2	13.87	13.68
11	10	10	1.5	1.2	13.25	13.33
12	20	10	1.5	1.2	16.14	16.29
13	10	5	2.5	1.2	13.22	13.34
14	20	5	2.5	1.2	11.09	11.27
15	10	10	2.5	1.2	14.82	14.42
16	20	10	2.5	1.2	15.38	15.45
17	5	7.5	2	1.15	12.35	12.13
18	25	7.5	2	1.15	15.47	15.41
19	15	2.5	2	1.15	15.42	14.29
20	15	12.5	2	1.15	13.59	13.25
21	15	7.5	1	1.15	15.11	14.29
22	15	7.5	3	1.15	13.44	13.26
23	15	7.5	2	1.05	13.88	13.42
24	15	7.5	2	1.25	14.21	14.13
25	15	7.5	2	1.15	13.59	13.77
26	15	7.5	2	1.15	13.41	13.77
27	15	7.5	2	1.15	13.88	13.77
28	15	7.5	2	1.15	13.36	13.77
29	15	7.5	2	1.15	13.65	13.77
30	15	7.5	2	1.15	13.38	13.77

Table 23. Analysis of variance for optimization of different parameters for maximum cellulose production for MCW medium

Source	Sum of squares	Degree of freedom	Mean square	*F* value	Significance of *F*
Model	64.79	14	4.63	19.92	< 0.0001
Residual	3.48	15	0.23	-	-
Total Model	68.27	29	-	-	-

R^2 = 0.9490, adjusted R^2 = 0.9013

Table 24. Regression coefficient for optimization of different parameters for maximum cellulose production using MCW medium

Factor	Regression co efficient	Standard error	P value
Intercept	13.77	0.09	< 0.0001
A-Fructose concentration	0.82	0.10	< 0.0001
B-Yeast extract concentration	-0.26	0.10	0.0149
C-Citric acid concentration	-0.26	0.10	0.0157
D-Disodium hydrogen phosphate	0.18	0.10	0.0835
AB	0.77	0.12	< 0.0001
AC	-0.48	0.12	0.0007
AD	-0.60	0.12	< 0.0001
BC	0.39	0.12	0.0038
BD	1.18	0.12	< 0.0001
CD	-0.07	0.12	0.5453
A^2	0.00	0.09	0.9760
B^2	0.15	0.09	0.1204
C^2	0.09	0.09	0.3230
D^2	0.04	0.09	0.6968

Table 25. Experiment employing different parameters for maximum cellulose production as predicted by the model of dry weight of cellulose from MCW medium

Dependent variable	Independent variables				Yield ($g\ l^{-1}$)	
	Fructose	Yeast extract	Citric acid	Disodium hydrogen phosphate	Observed	Predicted
Dry weight of cellulose ($g\ l^{-1}$)	20	10	1.5	1.1	16.11	16.74

Table 26. Central composite design matrix of different parameters of independent variables and their corresponding experimental and predicted values of dry weight of cellulose using CAJ medium

Standard order	Independent variables				Dry weight of cellulose (g l^{-1})	
	Fructose concentration (g l^{-1})	Yeast extract concentration (g l^{-1})	Citric acid Concentration (g l^{-1})	Disodium Hydrogen Phosphate Concentration (g l^{-1})	Observed	Predicted
1	2	4	0.5	0.5	9.64	10.84
2	6	4	0.5	0.5	14.22	14.28
3	2	8	0.5	0.5	10.84	11.38
4	6	8	0.5	0.5	14.66	15.01
5	2	4	2.5	0.5	14.35	13.07
6	6	4	2.5	0.5	14.11	14.88
7	2	8	2.5	0.5	11.87	12.51
8	6	8	2.5	0.5	14.35	14.50
9	2	4	0.5	1.0	9.25	9.23
10	6	4	0.5	1.0	14.64	14.67
11	2	8	0.5	1.0	11	10.90
12	**6**	**8**	**0.5**	**1.0**	**15.12**	**16.53**
13	2	4	2.5	1.0	10.17	10.49
14	6	4	2.5	1.0	14.7	14.29
15	2	8	2.5	1.0	10.98	11.05
16	6	8	2.5	1.0	15.58	15.05
17	0	6	1.5	0.75	7.44	7.16
18	8	6	1.5	0.75	15.11	14.60
19	4	2	1.5	0.75	10.74	10.81
20	4	10	1.5	0.75	12.97	12.11
21	4	6	-0.5	0.75	16.12	14.79
22	4	6	3.5	0.75	15	15.54
23	4	6	1.5	0.25	16	15.19
24	4	6	1.5	1.25	14.11	14.13
25	**4**	**6**	**1.5**	**0.75**	**16.18**	**16.24**
26	**4**	**6**	**1.5**	**0.75**	**16.14**	**16.24**
27	**4**	**6**	**1.5**	**0.75**	**16.2**	**16.24**
28	**4**	**6**	**1.5**	**0.75**	**16.34**	**16.24**
29	**4**	**6**	**1.5**	**0.75**	**16.22**	**16.24**
30	**4**	**6**	**1.5**	**0.75**	**16.35**	**16.24**

Table 27. Analysis of variance for optimization of different parameters for maximum cellulose production for CAJ medium

Source	Sum of squares	Degree of freedom	Mean square	*F* value	Significance of *F*
Model	175.87	14	12.56	17.30	< 0.0001
Residual	10.89	15	0.73	-	-
Total Model	186.76	29	-	-	-

$R^2 = 0.9417$, adjusted $R^2 = 0.9872$

Table 28. Regression coefficient for optimization of different parameters for maximum cellulose production using CAJ medium

Factor	Regression co efficient	Standard error	P value
Intercept	16.24	0.35	<0.0001
A-Fructose concentration	1.86	0.17	<0.0001
B-Yeast extract concentration	0.32	0.17	0.0821
C-Citric acid concentration	0.19	0.17	0.2781
D-Disodium hydrogen phosphate	-0.27	0.17	0.1473
AB	0.048	0.21	0.8216
AC	-0.41	0.21	0.0743
AD	0.50	0.21	0.0331
BC	-0.28	0.21	0.2144
BD	0.28	0.21	0.2047
CD	-0.24	0.21	0.2705
A^2	-1.34	0.16	<0.0001
B^2	-1.19	0.16	<0.0001
C^2	-0.27	0.16	0.1197
D^2	-0.39	0.16	0.0283

Table 29. Experiment employing different parameters for maximum cellulose production as predicted by the model of dry weight of cellulose from CAJ medium

Dependent variable	Independent variables				Yield ($g\ l^{-1}$)	
	Fructose	Yeast extract	Citric acid	Disodium hydrogen phosphate	Observed	Predicted
Dry weight of cellulose ($g\ l^{-1}$)	4.0	6.0	1.5	0.75	16.22	16.24

Table 30. Fermentation kinetics of bacterial cellulose production from static model fermentor using CAJ medium

S. No	Fermentation kinetic parameters	Observed values
1	Growth rate (K) ($g\ l^{-1}d^{-1}$)	0.0079 ±0.11
2	Specific growth rate (μ) (h^{-1})	0.00316 ±0.04
3	Maximum BC concentration (C max) ($g\ l^{-1}$)	16.22 ±0.17
4	BC production rate (Qp) ($g\ l^{-1}\ h^{-1}$)	0.27 ±0.10
5	BC produced per 100 gram of the substrate (Yp/s) ($g\ 100g^{-1}$)	1.62 ±0.01
6	BC produced per 100 g of cells (Yp/x) ($g\ 100g^{-1}$)	1.30 ±1.11

Table 31. Changes in viable cell count, pH and total sugars of CAJ medium in model fermentor during the course of fermentation

S. No	Incubation time (days)	Viable cell count number (cfu ml^{-1})	pH	Total sugars ($g\ l^{-1}$)
1	0	0.002x 10^{11} (8.30±0.21)	6.5±0.01	18.00±0.45
2	2	0.011 x 10^{11} (9.04±0.11)	6.3±0.21	16.14±0.10
3	4	0.777 x10^{11} (10.89±0.72)	6.0±0.27	12.12±0.34
4	6	04.21 x10^{11} (11.62±0.41)	6.0±0.11	10.88±0.31
5	8	13.24 x10^{11} (12.12±0.49)	5.8±0.17	9.14±0.22
6	10	25.21x10^{11} (12.40±0.21)	5.4±0.21	7.45±0.23
7	12	26.10x10^{11} (12.42±0.09)	5.0±0.22	5.28±0.24
8	14	31.24x10^{11} (12.49±0.97)	5.0±0.37	3.97±0.71
9	16	34.12x10^{11} (12.53±0.71)	4.5±0.31	2.18±0.88
10	18	31.22x10^{11} (12.49±0.33)	4.0±0.32	2.00±0.48
11	20	33.14x10^{11} (12.52±0.51)	3.5±0.15	1.50±0.36
	SEd	0.33	0.22	0.27
	CD (0.05)	0.69	0.47	0.57

Log transformed values are indicated in the brackets

Data are mean ± SD of three replications

Table 32. Colour analysis of bacterial cellulose from three different media

S. No.	Media used	CIELab Values			
		L*	a*	b*	C*
1.	HS medium (Control)	47.45±0.13	2.41±0.11	1.18±0.01	2.61±0.01
2.	Coconut water medium	51.42±0.11	2.45±0.02	0.41±0.02	2.65±0.04
3.	Cashew apple juice medium	54.41± 0.21	2.17±0.01	19.44±0.01	18.77±0.01
	SEd	0.41	0.02	3.24	2.11
	CD (0.05)	0.87	0.04	6.32	4.46

L* - Lightness, a* - Red and Green, b* - Yellow and Blue, C* - Chroma

Table 33. Determination of texture, water holding capacity and fibre content of bacterial cellulose from three different media

Sl. No.	Media used	Characteristics				
		Texture (N)	Water holding capacity (%)	Crude fibre content (g/ 100g)	Soluble dietary fibre content (g/100g)	Insoluble dietary fibre content (g/100g)
1	HS medium (control)	45.00±2.21	84.44±0.04	11.25±0.01	0.04±0.01	8.50±0.35
2	Coconut water medium	07.00±1.37	87.14±0.10	10.74±0.01	0.03±0.01	7.20±0.04
3	Cashew apple juice medium	11.00±1.24	88.54±0.02	10.04±0.03	0.03±0.01	7.80±0.02
	SEd	0.54	0.036	0.140	NS	0.136
	CD (0.05)	1.32	0.089	0.342	NS	0.330

Table 34. Measurement of volatile acidity in mature coconut water vinegar produced using spent liquor of CAJ medium after cellulose production

Sl. No	Incubation (h)			
	8	16	24	32
Volatile Acidity (%)	1.2 ± 0.03	2.45 ± 0.06	3.9 ± 0.09	4.8 ± 0.15

Table 35. GCMS Analysis of bioactive compounds in the crude extract of fermented broth of cashew apple juice medium

Sl. No.	Retention Time (min)	Bioactive compounds	Area (%)
1	3.04	Acetic acid	1.25
2	4.31	Buyladamantan-1-ol	1.68
3	6.18	Illimaquinone-epoxide	0.19
4	6.77	2-(5-chloromehoxyphenyl) pyrrole	0.77
5	8.98	3-ethylaminosulpholane, 2-propenoic acid	0.59
6	9.44	Dodecane, heptadecane, octadecane, hexadecane, octacosane, tricosane, nonane	1.86
7	9.72	Heneicosane, 1-pentanol	1.02
8	11.29	2,2,6-trimethyl-5-hepten-3-one	6.68
9	13.13	Benzaldehyde dioctyl acetal	0.45
10	16.07	1-piperazine	0.37
11	17.76	Nonane	0.17
12	18.54	Crocusatin B	0.30
13	22.56	Thiophene-2-carboxylic acid, santolin diacetylene	24.80
14	25.59	1,5-dihydroxyl-3,3-dimethyl-7acetylaminomethyl-tricyclododecane	10.75
15	30.22	Lavandyl acetate, 1-heptanol	0.13
16	31.17	3-phenyl-3-m-tolylpropionamide	12.33
17	34.85	Nonane, 4-hepatanol	6.53
18	37.48	3-(4-nitrobenzoyl) cinnoline	5.80
19	41.71	Methylsilylpropyne	0.78

Table 36. Physical property of bacterial cellulose fibre incorporated paper and plant cellulosic paper

S. No	Physical properties	BC incorporated paper	plant cellulosic paper
1	Tensile strength (MPa)	120 ± 2.89	90±1.67
2	Young's Modulus (GPa)	4.9 ±0.01	4.2±0.10
3	Viscosity (cP)	127.4 ±0.29	110.1±0.01
4	Degree of polymerisation (DP)	2074±2.91	2001±1.20
5	Air penetration test (sec $50ml^{-1}$)	Air impenetrate	2.29±0.04
6	Castor oil penetration (min $0.05ml^{-1}$)	oil resistant permanently	oil resistant permanently

Table 37. Effect of loperamide on feed intake, water intake and faecal properties of constipated rats

S. No	Parameters	Normal Control	Constipated rats
1.	Feed intake (g)	20.21± 1.23	21.24±0.87
2.	Water intake (ml)	24.00±1.47	13.21±1.21
3.	Number of faecal pellet	70.77±3.00	30.55±4.00
4.	Moisture content of faecal pellet (%)	1.99±0.45	0.68±0.15
5.	Weight of faecal pellet (g)	7.12±0.63	3.19±0.44
	SEd	0.56	0.35
	CD (0.05)	1.33	0.72

Data are mean ± SD of three measurements

Table 38. Effect of colloidal bacterial cellulose laxative on feed and water intake and faecal properties of constipated rats

S. No	Parameters	Group I	Group II	Group III	Group IV
1.	Feed intake (g)	20.21±3.39	19.99±1.26	20.21±1.21	19.58±1.52
2.	Water intake (ml)	12.38±1.11	19.99±2.65	17.57±1.77	18.54±2.34
3.	Number of faecal pellet	29.66±1.28	44.89±2.36	40.63±2.00	46.34±2.55
4.	Moisture content of faecal pellet (%)	0.77±1.24	2.00±1.23	2.15±1.11	2.94±1.24
5.	Weight of faecal pellet (g)	3.93±0.54	15.21±0.65	15.23±1.14	16.87±54
	SEd	0.27	0.39	0.37	0.41
	CD (0.05)	0.61	0.88	0.82	0.91

Data are mean ± SD of three measurements

Table 39. Sensory evaluation of *nata* – a value added product from bacterial cellulose

S. No	Characters	Product scoring*		
		***Nata* (HS medium)**	***Nata-de-coco***	***Nata-de-cashew***
1.	Colour and appearance	8.8	8.9	8.8
2.	Flavour	9.0	9.0	9.0
3.	Texture/ juiciness	8.4	9.4	9.1
4.	Taste/ tenderness	8.6	9.5	9.2
5.	Overall acceptability	8.7	9.2	9.02
	SEd	0.14	0.15	0.14
	CD (0.05)	0.31	0.33	0.32

*****Scoring is the mean of fifteen member judge panel

Table 40. Cost of production of bacterial cellulose

I. Total Expenditure (tonnes per year)

Sl. No	Particulars	Value (Rs.)
A.	**Expenditure details**	
a.	Fixed investments	4,28,500.00
b.	Recurring expenses i. Direct cost ii. Indirect cost	 1,58,000.00 26,000.00
c.	Interest/ Depreciation	96,412.00
	Sub Total A (a+b+c)	**7,08,912.00**

A. Expenditure details

Sl. No	Particulars	Quantity (Nos.)	Value (Rs.)
a.	**Fixed Investments**		
1	Building	1	2,00,000.00
2	Autoclave	1	60,000.00
3	Incubator	1	25,000.00
4	pH meter	1	10,000.00
5	Weighing balance	2	2,000.00
6	Refractometer	2	3,000.00
7	Refrigerator	1	15,000.00
8	U-V laminar airflow chamber	1	60,000.00
9	Working table	1	10,000.00
10	Thermometer	5	500.00
11	Squeezer	3	1000.00
12	Mixer grinder	1	5000.00
13	Sealing machine	1	5000.00
14	5 litre autoclavable jar fermentors with lid	20	20,000.00
15	Stainless steel containers	10	2000.00
16	Glass containers (storage bottles with lid)	25	1000.00
	Total		**4,28,500.00**

Sl. No	Particulars	Quantity (Nos.)	Value (Rs.)
b.	**Recurring expenses**		
I.	**Direct cost**		
	i. Raw materials (chemicals)	20 kg	20000.00
	ii. Packaging materials	10 kg	20000.00
	iii. Starter cultures	10 l	10000.00
	iv. Labour cost @5 Mandays/ week @Rs. 150/ person/day	3	1,08,000.00
	Total		**1,58,000.00**
II.	**Indirect cost**		
	i. Water		6000.00
	ii. Fuel (LPG)		10,000.00
	iii. Electricity		10,000.00
	Total		**26,000.00**
c.	**Interest/ Depreciation on fixed investments**		
	i. Interest @10 % per year		42,850.00
	ii. Depreciation @12.5% per year		53,562.50
	Total		**96,412.00**
	Total expenditure (a+b+c)		**7,08,912.00**

II. Product Pricing

	Amount in Rs.
Cost of 1.0 Kg of wet BC pellicle	750.00
Cost of 1.0 Kg of BC as *nata* in syups/ jelly base	1500.00

If production efficiency = 185 g l^{-1}
For 5 litres = 925 g
Production rate for 100 litres per harvest= 18500 g or 18.5 kg
Production rate @ two harvests per month = 37 kg per month
Production rate for one year = 888 kg per year

III. Income

	Amount in Rs.
For 1000 kg of wet BC pellicle @Rs.750 per kg	7,50,000/-
For 1000 kg of BC in syrup/ jelly base	1,50,0000/-

IV. BC ratio: Income ÷ Expenditure

	BC ratio
Then BC ratio (wet cellulose) (7,50,000.00 ÷ 7,08,912.00)	1.05
If an additional cost of Rs. 50,000/- is added to expenditure	
Then BC ratio (BC in syrup/ jelly base) (15,00,000.00 ÷ 7,58,912.00)	1.97

Table 8. Biochemical characterization of elite cellulose producing isolate (sju-1)

Sl. No.	Isolates	Biochemical Tests									
		Cellulose production	Oxidase	Catalase	Indole production	Urea utilisation	Citrate utilisation	Gelatin lique-faction	Oxidation of ethanol	Overoxidation of acetic acid	Ketogenesis of glycerol
1.	sju-1	+	-	+	-	-	-	-	+	+	+
2.	NCIM 2526	+	-	+	-	-	-	-	+	+	+

+ presence of growth ; - absence of growth

Table 9. Similarity analysis of elite cellulose producing isolate sju-1 with the related taxa

Strains	Accession Number	Similarity (per cent)	nt differences /compared
Gluconacetobacter intermedius	JF718417	99.02	6/1456
Gluconacetobacter oboediens	JF794003, FJ794002	99.00	6/1456
Gluconacetobacter xylinus	AB680481	99.00	3/1456

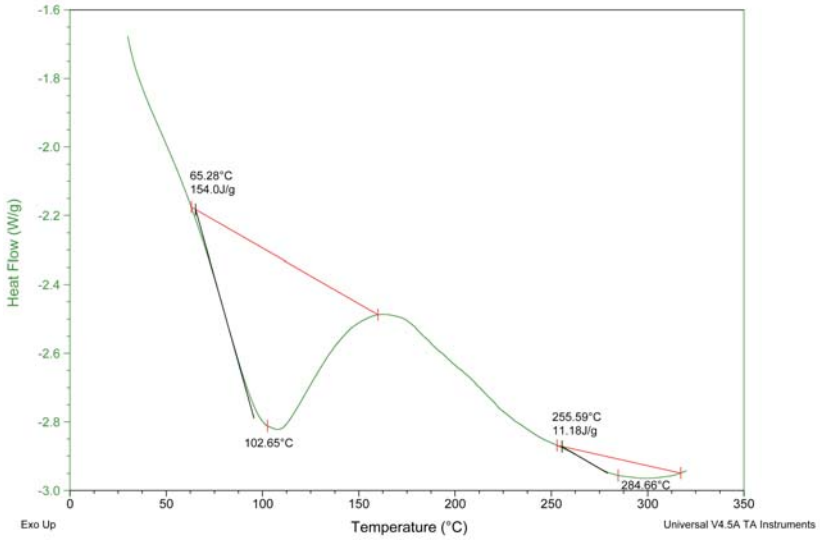

Fig. 10a. Differential Scanning Calorimetric Thermograms of Micro Crystalline Cellulose

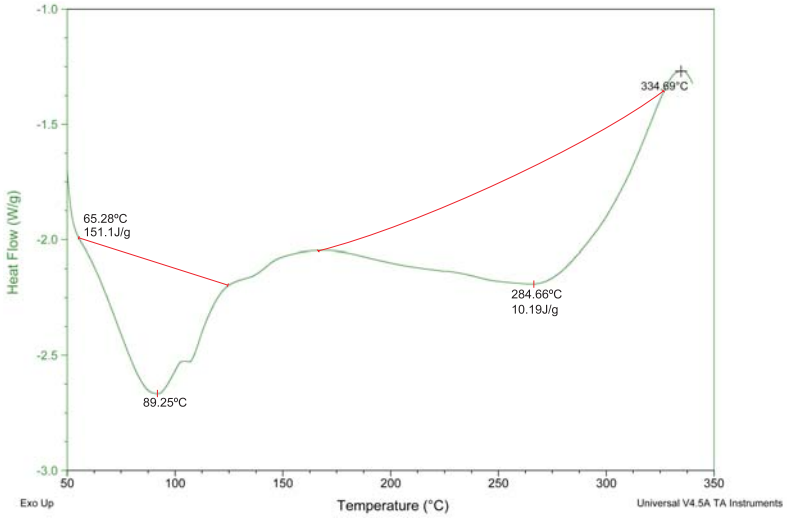

Fig. 10b. Differential Scanning Calorimetric Thermograms of Bacterial Celluloses from *Acetobacter xylinum* NCIM 2526

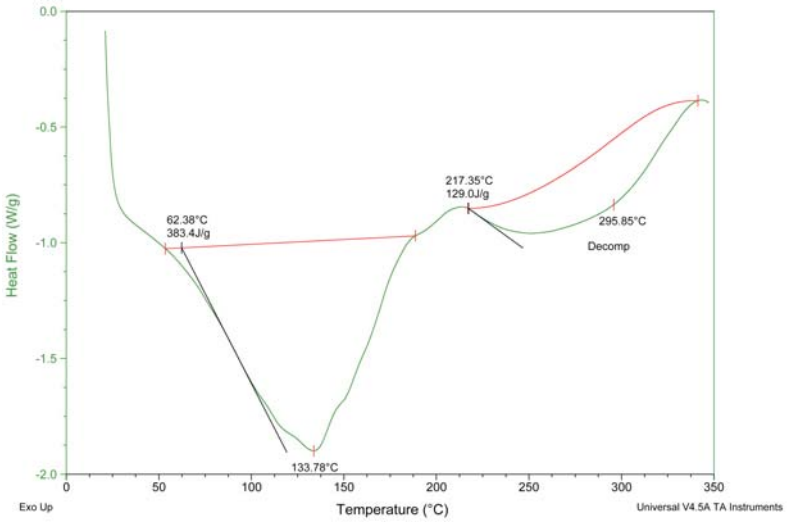

Fig. 10c. Differential Scanning Calorimetric Thermograms of Bacterial Celluloses from *Gluconacetobacter* sp. sju-1

Fig. 10. Differential Scanning Calorimetric Thermograms of Bacterial Celluloses

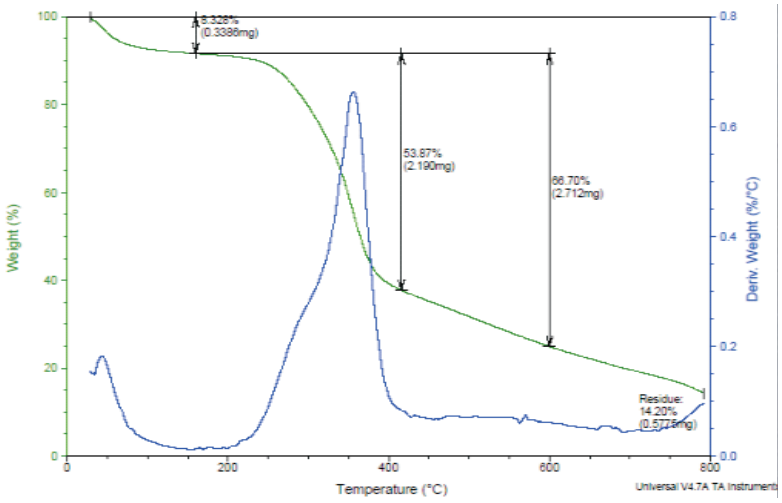

Fig. 9a. Thermo Gravimetric Analysis of Micro Crystalline Cellulose

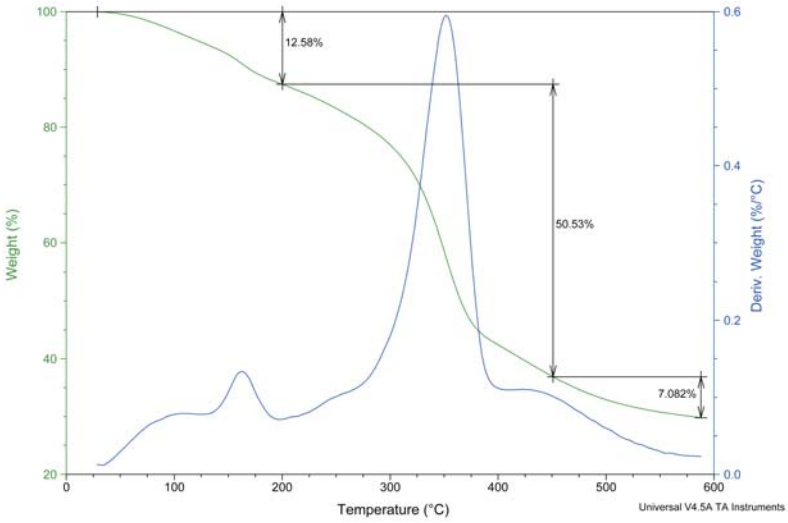

Fig. 9b. Thermo Gravimetric Analysis of bacterial celluloses from *Acetobacter xylinum* NCIM 2526

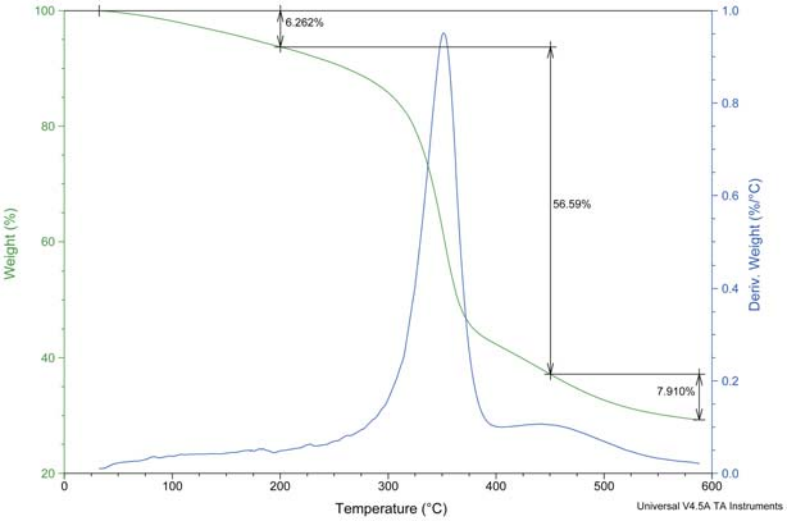

Fig. 9c.Thermo Gravimetric Analysis of bacterial celluloses from *Gluconacetobacter* sp. sju-1

Fig. 9. Thermo Gravimetric Analysis of bacterial celluloses

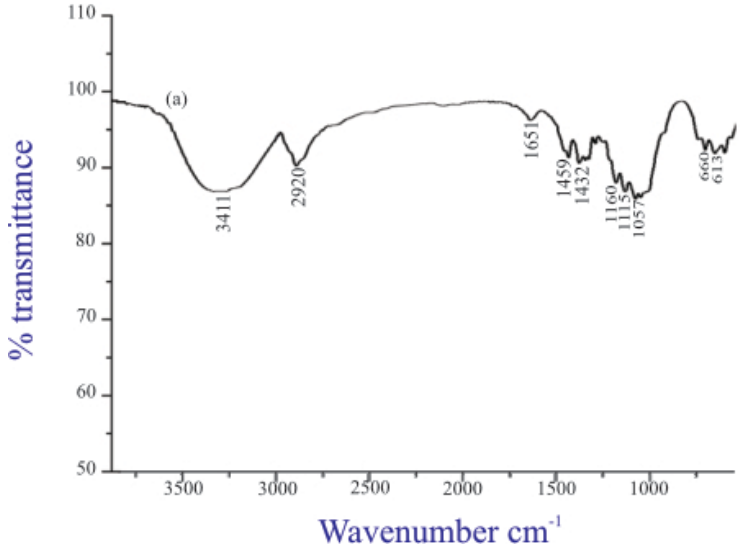

Fig. 8a. FTIR Spectrum of Micro Crystalline Cellulose

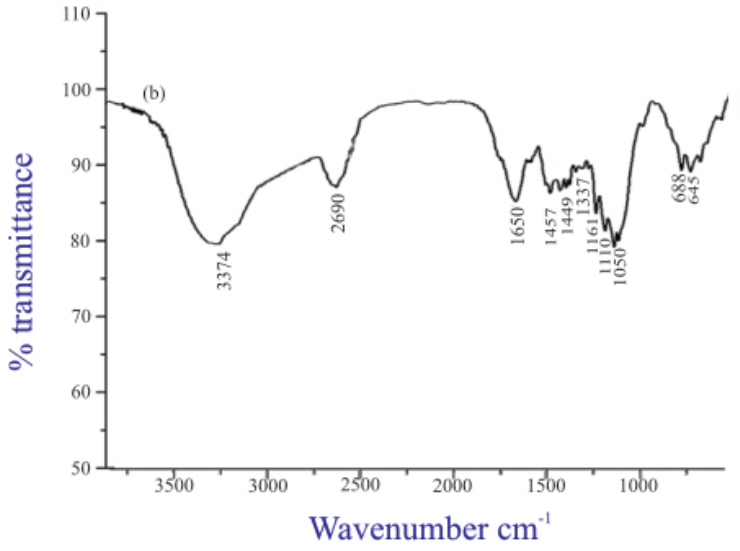

Fig. 8b. FTIR Spectrum of bacterial cellulose from *Acetobacter xylinum* NCIM 2526

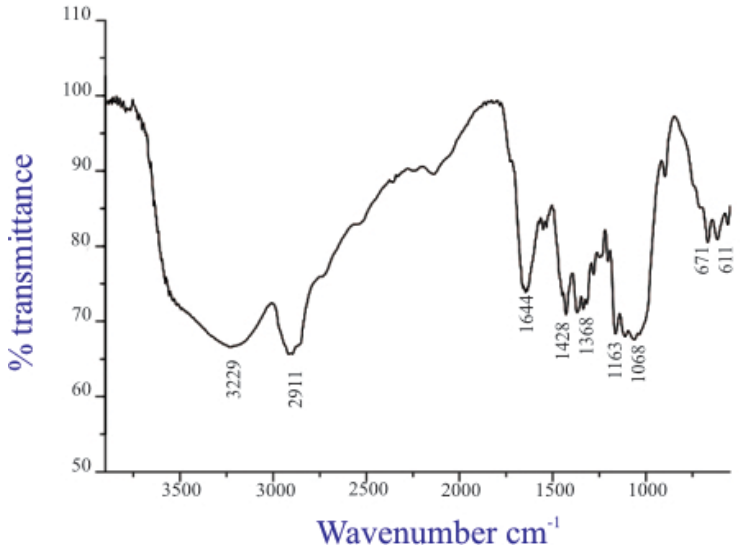

Fig. 8c. FTIR Spectrum of Bacterial Cellulose from *Gluconacetobacter* sp. sju-1

Fig. 8. FTIR Spectrum of bacterial celluloses

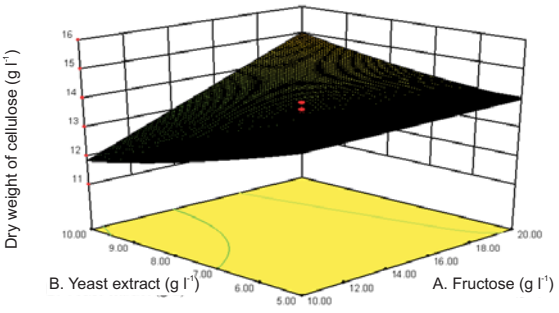

Fig. 15a.

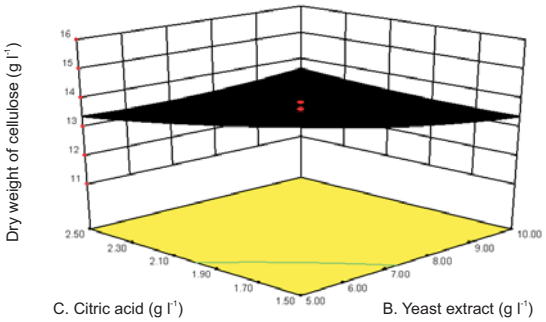

Fig. 15d.

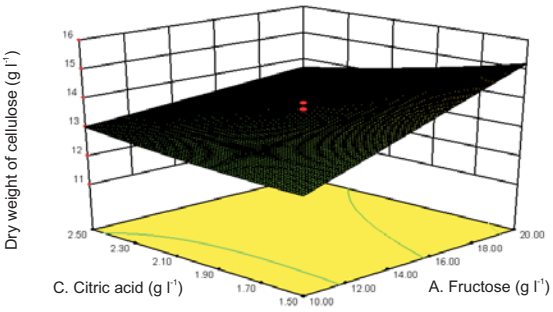

Fig. 15b.

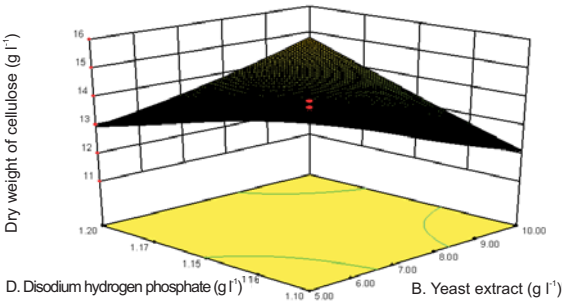

Fig. 15e.

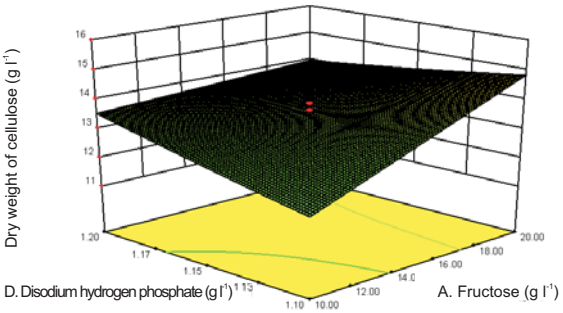

Fig. 15c.

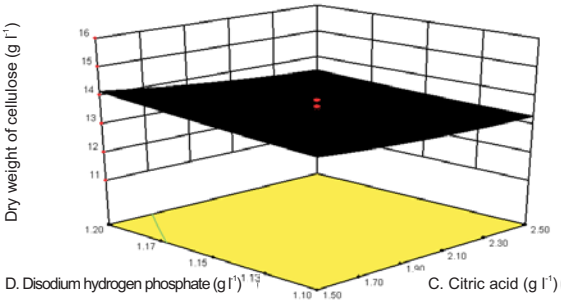

Fig. 15f.

Fig. 15. Response surface curve showing the effect of MCW medium on dry weight of bacterial cellulose

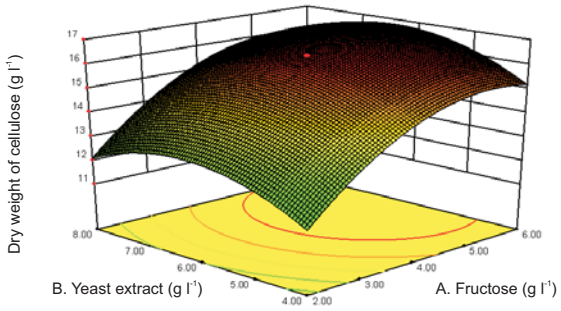

Fig. 16a.

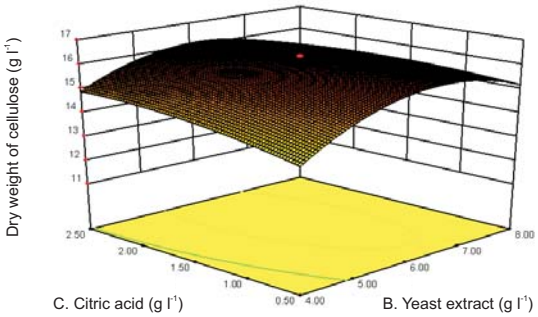

Fig. 16d.

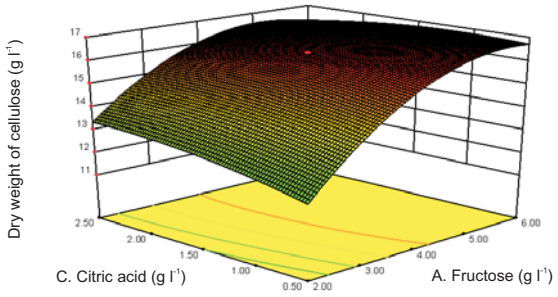

Fig. 16b.

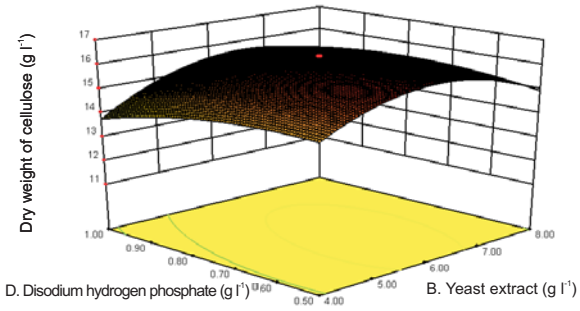

Fig. 16e.

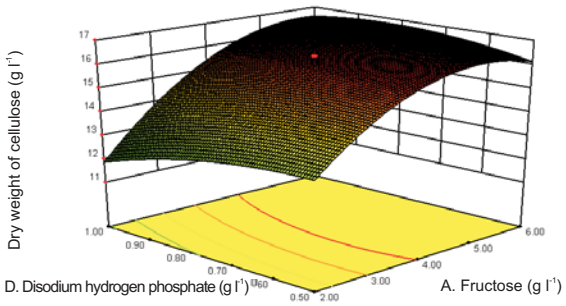

Fig. 16c.

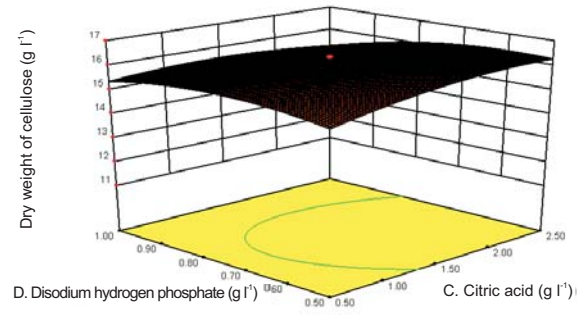

Fig. 16f.

Fig. 16. Response surface curve showing the effect of CAJ medium on dry weight of bacterial cellulose

Plate 1. Purified colonies of *Gluconacetobacter* sp. sju-1 in Hestrin Schramn medium

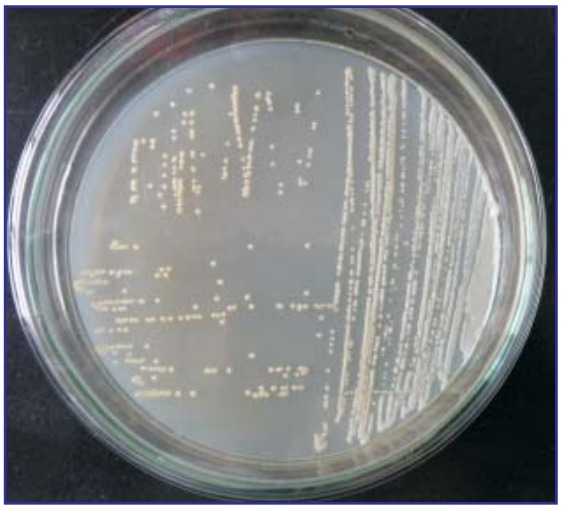

Plate 2. Purified colonies of *Gluconacetobacter* sp. sju-1 in Glucose Yeast extract Calcium carbonate medium



Plate 3. Short rods of *Gluconacetobacter* sp. sju-1 under phase contrast microscope at 40X

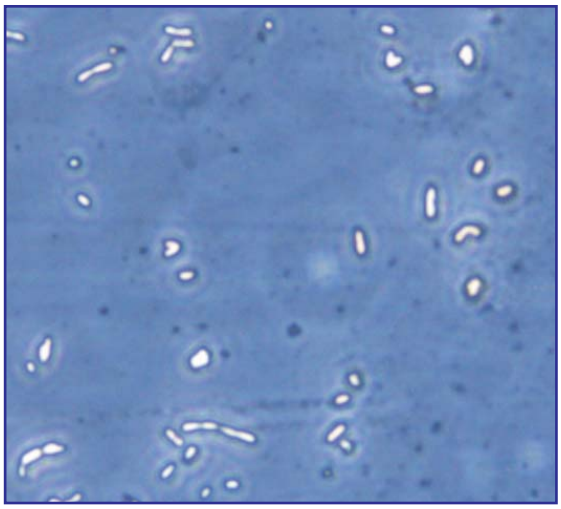

Plate 4. Scanning Electron Micrographs (SEM) of *Gluconacetobacter* sp. sju-1

Plate 4a. SEM view of short rods of *Gluconacetobacter* sp. sju-1 embedded in the cellulose pellicle at 6000X

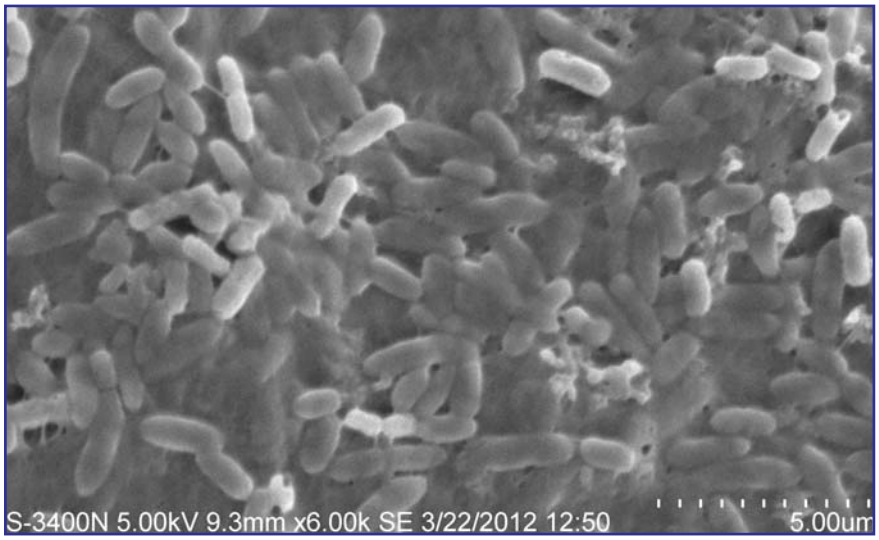

Plate 4b. SEM view of cellulose fibrils of *Gluconacetobacter* sp. sju-1 at 8000X

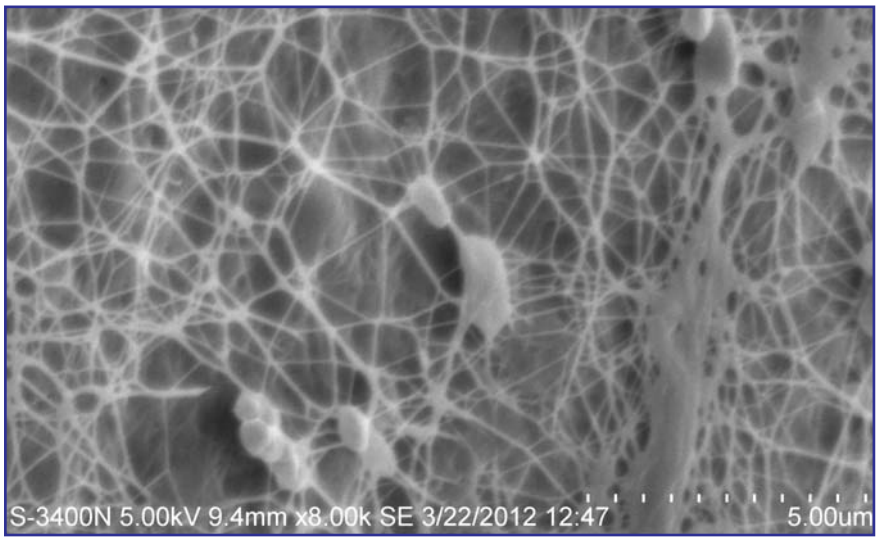

Plate 4c. SEM view of single cell of *Gluconacetobacter* sp. sju-1 producing cellulose fibrils from the cell membrane at 37000X

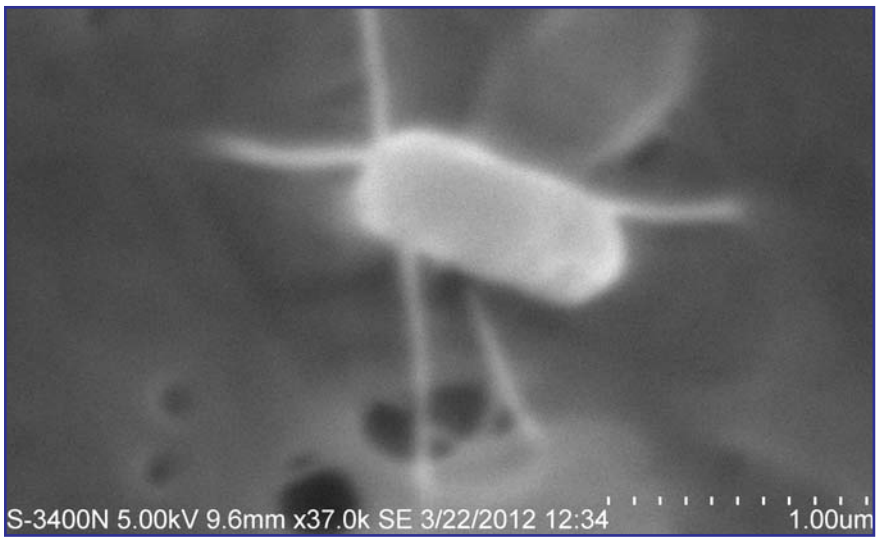

Plate 5. Formation of cellulose pellicle by *Gluconacetobacter* sp. sju-1 in HS medium

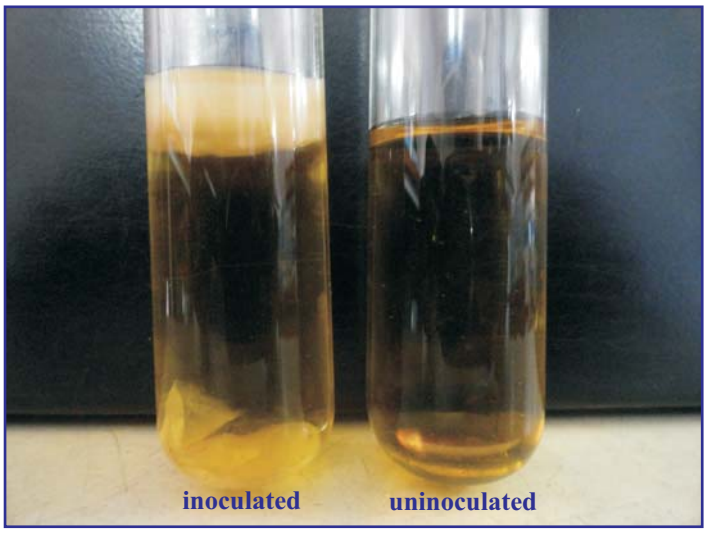

Plate 6. Real image of cellulose pellicle (a) *Acetobacter xylinum* NCIM 2526 and (b) *Gluconacetobacter* sp. sju-1

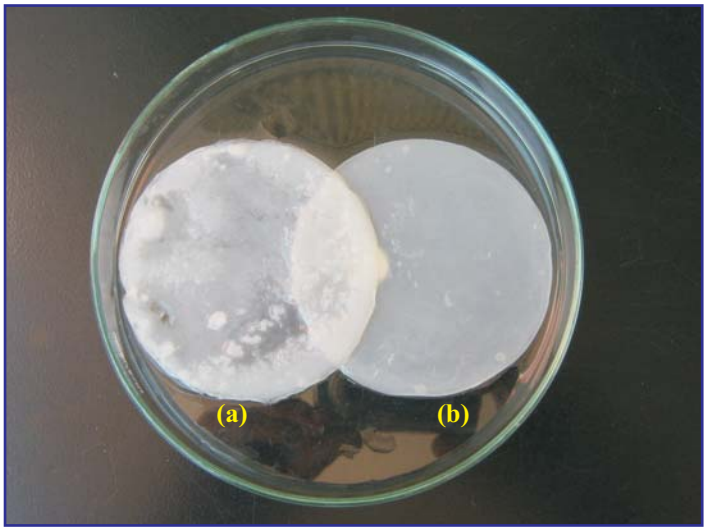

Plate 7. Purified colonies of *Gluconacetobacter* sp. sju-1 in Congo red medium

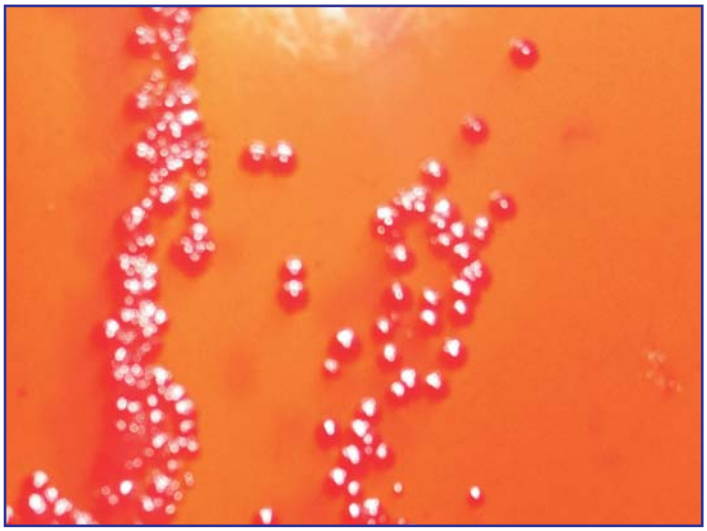

Plate 8. Comparison of cellulose fibrils viewed under SEM at 8000X

Plate 8a. Cellulose fibrils of Micro Crystalline Cellulose (MCC)

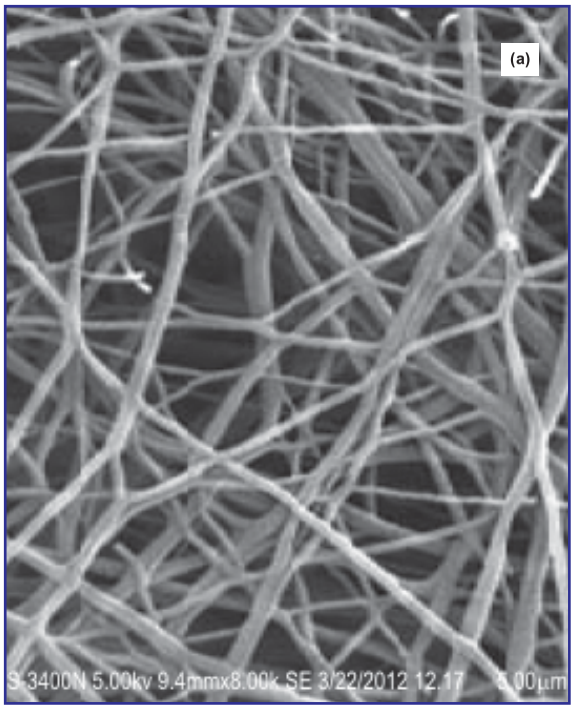

Plate 8b. Cellulose fibrils of *Acetobacter xylinum* NCIM 2526

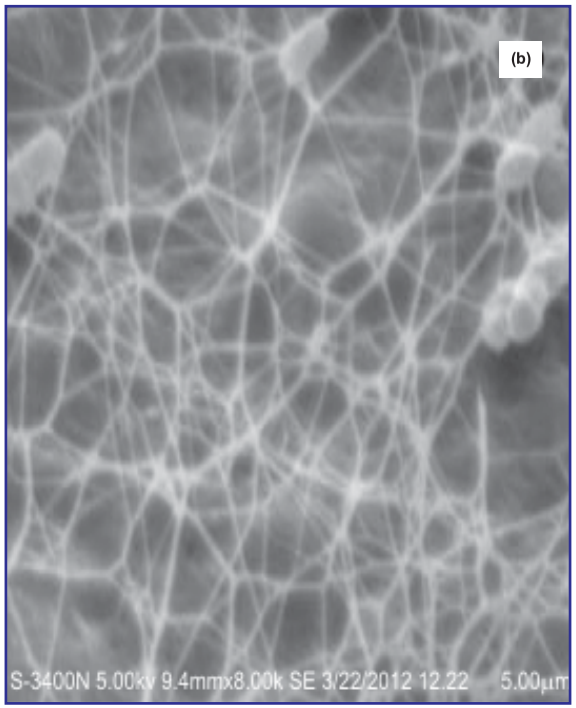

Plate 8c. Cellulose fibrils of *Gluconacetobacter* sp. sju-1

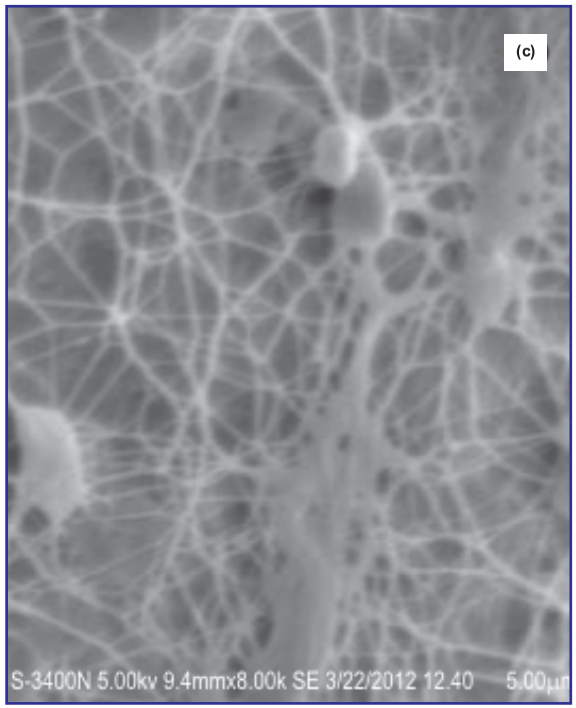

Plate 9. Cellulose formation by *Gluconacetobacter* sp. sju-1 in static and shaken culture condition



Plate 10. Effect of different additives on cellulose production by *Gluconacetobacter* sp. sju-1

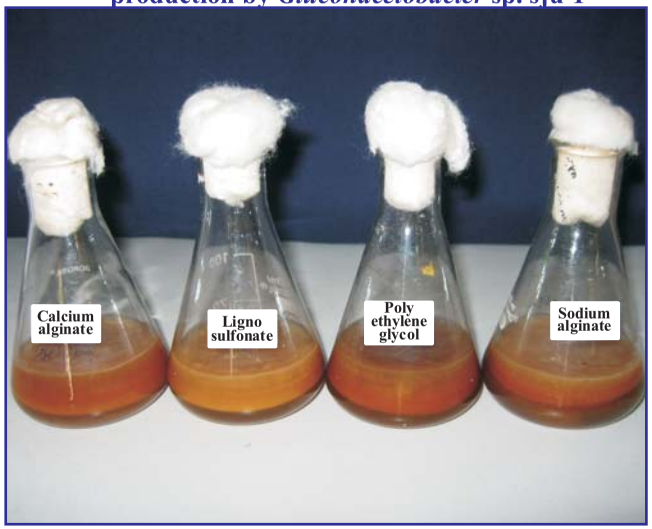

Plate 11. Effect of cashew apple juice on cellulose production by *Gluconacetobacter* sp. sju-1

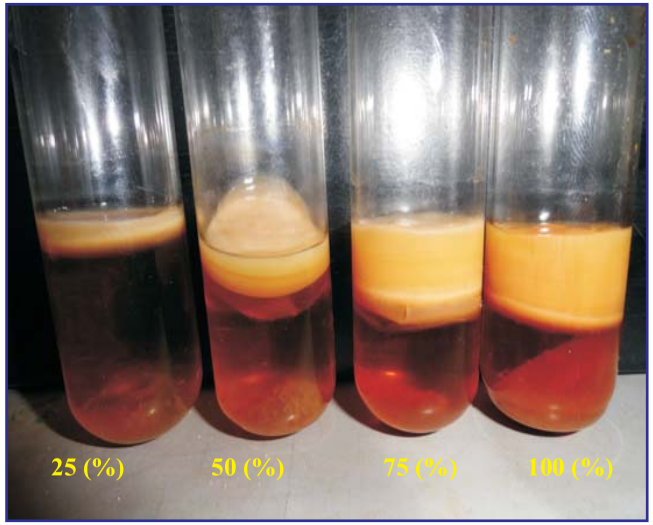

Plate 12. Comparison of cellulose production in three different medium

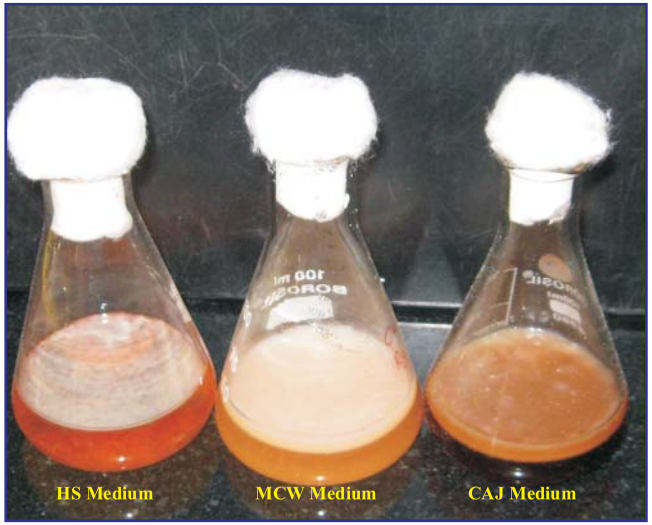

Plate 13. Optimisation of fermentation condition by Response Surface Methodology (RSM)



Plate 14. Model fermentor for bacterial cellulose production (5 l capacity)



Plate 15. Growth of *Gluconacetobacter* sp. sju-1 in calcium alginate incorporated HS medium



Plate 16. Fresh untreated cellulose pellicle



Plate 17. Alkali treatment of cellulose pellicle



Plate 18. Reinforced bacterial cellulose pulp



Plate 19. Dried sheets of bacterial cellulose



Plate 20. Reinforced paper sheet produced from bacterial cellulose



Plate 21. Oral dosing of colloidal bacterial cellulose laxative to Sprague-Dawley rats



Plate 22. Fecal pellets of Sprague-Dawley rats fed with colloidal bacterial cellulose laxative

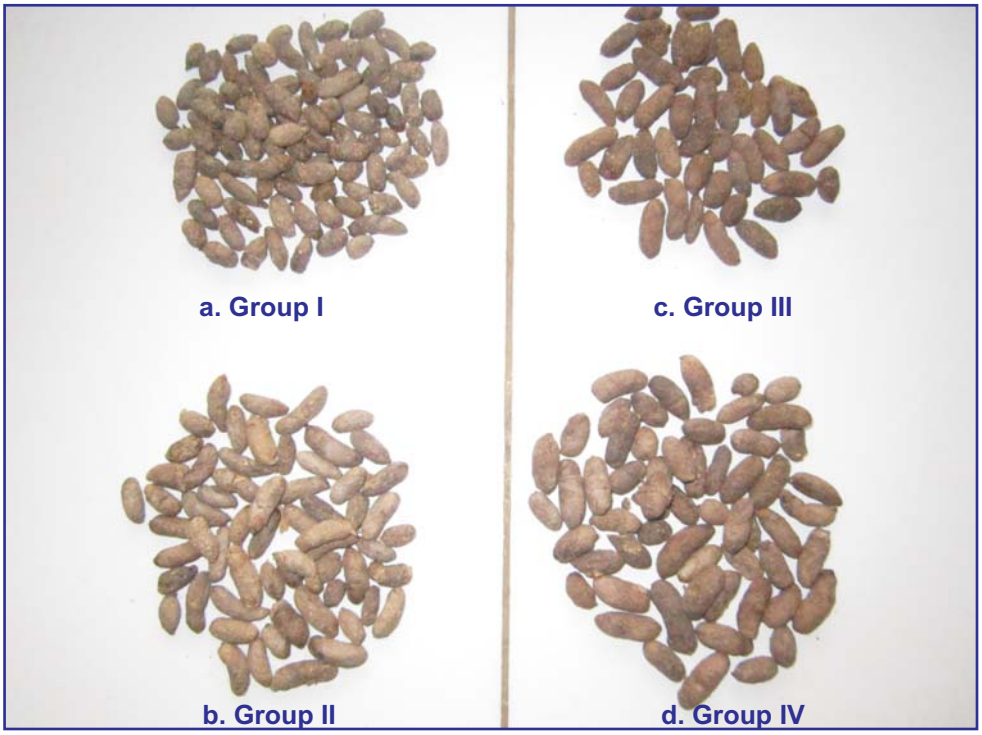

Plate 23. Fresh and wet bacterial cellulose from cashew apple juice medium (*nata-de-cashew*)

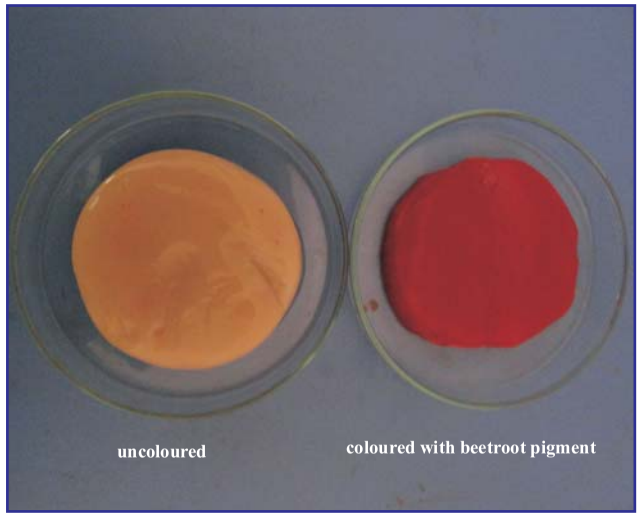

Plate 24. Diced cubes of *nata-de-cashew*



Plate 25. *Nata-de-coco* in jelly base from mature coconut water medium



Plate 26. *Nata-de-cashew* in jelly base from cashew apple juice medium



CHAPTER V

DISCUSSION

Microbial cellulose has gained an economic importance because of its high purity, light weight, fine network structure and fast biodegradation. In addition to it, exceptional mechanical strength especially in wet state, enormous water retention value, low roughness of the inner surface and high hydrophilicity makes the microbial cellulose suitable for numerous applications. These type of new functional materials obtained from renewable resources have gained much attention in the past decades due to the increasing global demand for alternatives to fossil resource. For example, composites based on cellulose polymers and natural fibres are very attractive materials because of their good mechanical properties, sustainability and environmental-friendly connotation. These materials have been widely used in the automotive and packaging industries and their applications in other areas are being actively sought (Kamel *et al*., 2008).

Bacterial cellulose produced by some selected species of *Gluconacetobacter* sp. is a new type of biopolymer, and far superior to plant based one because of its exceptional purity, ultra fine network structure, high biodegradability and unique mechanical strength. The use of natural fibres as reinforcing element in composite materials presents important advantages, such as eco-friendly, biodegradable, high availability, low cost, low energy consumption, low density, and high specific strength and modulus. Bacterial cellulose is expected to be used for many industrial applications as a high-strength construction material, a component of biodegradable products such as paper and food additives. As a food it is called as *nata* which is solid, sturdy, strong, white, transparent, flexible and widely used as an in mixing ice cream mix, fruit cocktail, syrup and other snacks (Lucia and Yudianti, 2008).

However, there are some limitation in production of bacterial cellulose due to high cost of pure carbon sources used for bacterial cellulose production besides low yield. India being an agricultural country, is having enormous quantity of bio-wastes which can be used as a potential alternative to carbon source for the production of bacterial cellulose. Among them the fruit and fruit processing wastes and their conversion into useable materials is high. Many fruit wastes have both food and feed value specific constituents like proteins and carbohydrates. Hence, considerable potential exists for the conversion of these wastes into higher value added products through microbial fermentation (Kumalaningsih *et al*., 2011).

5.1. Isolation, screening and selection of cellulose producing acetic acid bacteria bacteria

In general, Acetic Acid Bacteria (AAB) especially *Acetobacter* and *Gluconacetobacter* tolerate high concentration of acetic acid. All the acetic acid bacteria are

able to produce organic acid specifically acetic acid and some species of *Gluconacetobacter* could produce gluconic acid, lactic acid and acetic acid as end product of fermentation. Cells of *Gluconacetobacter* were capable of producing both organic acids and water insoluble cellulose pellicle. *Acetobacter* and *Gluconacetobacter* are usually selected based on the formation of clearing zone in ethanol containing glucose yeast extract calcium carbonate medium (Sokollek *et al*., 1998).

Bacterial cellulose is formed by species of acetic acid bacteria on the surface of a liquid containing sugar, juice, or other plant extracts. *Acetobacter* are found in rotten fruits, flowers, alcoholic drinks and in human intestine.

Bernardo *et al*. (1998) identified and characterized two wild type cellulose producing *Acetobacter* sp. (ITDI 2.1 and PA 2.2) from thirty-eight colonies that produced clearing zone in GYC medium and production of cellulose pellicle of 10 mm and 11 mm thickness respectively in HS medium. Tahir *et al*. (1999) have also isolated clearing zone producing strains in GYC medium from sugarcane juice, fruit juice, crushed apples, alcohol, vinegar, flower nectars. They were found to be excellent producers of vinegar rather than cellulose.

In this study, an attempt was made to isolate, screen and select a potent cellulose producing acetic acid bacteria. Isolation and screening of cellulose producing acetic acid bacteria is a typical and unique method where the elite cellulose producer was screened based on thickness of the surface pellicle formed in the Hestrin Schramn medium using a grading prescribed by Gallardo scale of thickness. A total of 88 bacterial isolates (59 from rotten fruits, juices and 29 from different high TSS containing sugarcane varieties) were isolated, screened and selected for cellulose production using HS medium. In the present investigation, many colonies produced zone of hydrolysis, but only few were cellulose former. According to Gallardo scale of thickness, isolates from many of the fruits produced cellilose pellicle thickness ranging from 0.5 mm to 2.0 mm. Cashew apple and pine apple produced 7.0 mm thickness of cellulose pellicle. Amongst all the sources used for selection, fermented sugarcane juice yielded a cellulose pellicle with maximum thickness of 13.00 mm and the isolate was designated as sju-1.

Andelib and Nuran (2009) have also demonstrated the ability of *Gluconacetobacter* sp. to produce acetic acid that dissolve calcium carbonate of GYC medium and the formation of clear zone around colonies. Hwan *et al*. (2004) have also isolated *G. persimmonis* sp. nov., from Korean traditional persimmon vinegar using HS medium. Ashley (2006) have indicated that *Acetobacter xylinum* 1FO 13693 was the best cellulose-producing bacterium among 41 strains belonging to the genus *Acetobacter* and *Agrobacterium*. Cleenwerck *et al*. (2007) isolated 23 strains of *Acetobacter ghanensis* sp. nov

from cocoa fermentation heap of Ghana. They were able to oxidize ethanol to acetic acid, a non-cellulose producer and were unable to produce 2-ketogluconic acid, 5-ketogluconic acid and also 2,5-diketogluconic acid from glucose, which is a striking difference between the *Gluconacetobacter* sp. that produced cellulose. This clearly indicated that, in the present work, acetic acid bacteria capable of producing cellulose have produced both acid and pellicle belonged to *Gluconacetobacter* sp. and those that produced only acid might be *Acetobacter* sp. or a non-cellulose producing *Gluconacetobacter* sp. Hence the results of present study are in accordance with above findings.

5.1.1. Phenotypic, biochemical and molecular characterization of cellulose producing bacteria

Acetic acid bacteria isolated from viticultural soil was found to be small, round shaped, white to light brown in colour and those isolated from red wine was dark brown in colour (Carmen *et al*., 2000). Yukphan *et al*. (2004) characterized *Gluconacetobacter* sp. biochemically and indicated that they produced both acetic acid, cellulose pellicle and were tested catalase positive. Sievers and Swings (2005) have observed that the colonies of *Gluconacetobacter xylinum* are typically white to brownish white, small and convex in appearance, eventually developing uneven edges where the bacterial cells break through the cellulose. Kanchanarh *et al*. (2010) isolated strains such as *Acetobacter pasteurianus* IFO3283, SKU1108, and MSU10 from acetic acid fermentation conditions. The isolates were short rods forming white coloured colonies in HS medium and showed high resistance for acetic acid by the aggregated cells covered by amorphous cellulosic layer over the colonies.

Poblet *et al*. (2000) identified acetic acid bacteria by RFLP analysis of PCR amplified fragment of 16SrRNA and the amplified rDNA were approximately 870 bp long for all the *Acetobacter* sp. Iyer *et al.* (2010) have performed the genome sequence of a cellulose-producing bacterium, *Gluconacetobacter hansenii* ATCC 23769. Ogino *et al*. (2011) have also found the cellulose non-producing strains of *Gluconacetobacter xylinus*. Whole genome sequence of *G. xylinus* NBRC 3288, have revealed the cellulose non-producing strains of *Gluconacetobacter* sp. producing organic acids only.

The results of colony morphology obtained from the present study showed that the selected isolate sju-1 developed milky white colonies and were short rods. When grown in Congo red incorporated LB medium, the colonies obtained the colour of the dye due to presence of cellulose aggregates over the colonies. Further in ethanol containing GYC medium, the colonies were milky white in colour and produced organic acid around the colonies. Further some of the isolates such as sju-25, sju-27 and sju-28 were brown in colour. Based on the biochemical tests the selected isolate sju-1 belonged to *Gluconacetobacter* sp.

sju-1 which was oxidase, urease, gelatinase, citrate and indole negative, and positive for catalase and glycerol utilization. The isolate was able to oxidize ethanol to acetic acid and overoxidation of acetic acid to CO_2 and H_2O.

In the present investigation, the 16S rRNA gene sequence of 1456 nucleotides were determined and the strain sju-1 was a member of the genus *Gluconacetobacter* and most closely related to *G.intermedius* JF18417 and *G. oboediens* JF94003. When BLAST was performed using NCBI, BLAST+ the strain showed 99 per cent similarity to three species of *Gluconacetobacter* namely *G. intermedius, G. obediens* and *G. xylinum*. When the sequence analysis of sju-1 was compared with the type strains using EXTAXON SERVER 2.1, the strain showed closest match with 99.02 per cent similarity to *Gluconacetobacter intermedius*. Hence the exact species of the isolate sju-1 could not be ascertained and was identified as *Gluconacetobacter* sp.

These results of biochemical tests obtained in the present study were in accordance to those described by Bergey's Manual of Systematic Bacteriology (Jozef *et al*., 1986). *G. intermedius* was isolated from Indian grapes and was found to be milky white convex shaped colonies (Pokalwar *et al*., 2011). Karahan *et al*. (2011) isolated acetic acid bacteria from vinegar, fruit, vegetable, and soil from different regions of Turkey and the cellulose production ability of 153 isolates was determined by 16S rRNA sequence analysis. The selected elite strain (A06O2) when compared with type strain *Gluconacetobacter xylinus* NRRL B-759, it had 99 per cent similarity with *Gluconacetobacter* sp. and strain A06O2 was named at the genus level as *Gluconacetobacter* sp. The results are in accordance to the above and the acetic acid tolerance may be rendered to the cells by cellulose formation mechanism. A novel and potent cellulose-producing bacterium was newly isolated from a rotten fruit and identified as *Gluconacetobacter* sp. F6 through morphological, cultural, and biochemical characteristics and by 16S rDNA sequencing (Jahan *et al*., 2012). *Gluconacetobacter* and *Acetobacter* from traditional *kombucha* fermentation of tea showed higher acetic acid production but cellulose thickness was very lower in *Gluconacetobacter* sp. than the reference strains recorded earlier (Soheir, 2012). But the selected isolate obtained through the present study produced more thickness of cellulose pellicle rather than acetic acid.

5.1.1.1. Structural, physical and thermal properties of bacterial cellulose

Chemically, the fibre contained in bacterial cellulose is highly pure without lignin, pectin, and hemicelluloses that are commonly found in plant cellulose (Rahayu *et al*., 1994). Yang *et al*. (2007) showed that cellulose decomposition started at 315ºC and continued until 400ºC and the maximum weight loss rate reached at 355ºC. The

thermal degradation temperature for BC obtained from *A. xylinum* was found to be 300ºC (Slusarska *et al*., 2008).

In the present study, confirmation of the structural groups present in the bacterial cellulose using infra red spectroscopy revealed that it was a pure form of cellulose with more of cellulose type Iα. It can be evidentially proved that cellulose is the only polysaccharide present in the BC formed by *Gluconacetobacter* sp.sju-1 and no other polysaccharides like pullulan, dextran and levan were produced. The results are in line with the findings of Hesse and Kondo (2005) who illustrated that the bacterial cellulose shows the presence of carbonyl, ketone, carboxylic acid and hydroxyl group when investigated using infra red spectral analysis. Johari *et al*. (2012) have developed hydrogels by treating BC with acrylic acid and the IR spectrum of these hydrogels showed C-O stretching in the region between 985 to 1000 cm^{-1} and at 1163 cm^{-1} and 2926 cm^{-1} it showed the presence of C-O-C of ether linkage and C-H stretching respectively. The prominent peak at 3440 cm^{-1} corresponding to O-H stretching of intermolecular hydrogen bonding was the main characteristic feature of pure celluloses. The results of IR spectrum for the bacterial cellulose obtained for the standard culture and *Gluconacetobacter* sp. sju-1 was in closer proximity to the above findings.

In the present study, the BC obtained from *Gluconacetobacter* sp. sju-1 registered a degradation temperature of 350ºC. This was in conformity with the findings of Costa *et al*. (2011) who have found that the thermal degradation temperature of 400ºC in cellulose nanocomposites. Nuruddin *et al*. (2011) have indicated the degradation temperatures for the fibre obtained from rice straw, wheat straw, corn stalks and *Dhaincha*. The degradation temperature for the microfibrils extracted from rice straw started at 332ºC and continued up to 370ºC. The degradation peaks for microfibrils from *Dhaincha* and corn stalks were of 368 and 353ºC respectively. The microfibrils of cellulose obtained for *Acetobacter xylinum* NCIM 2526 showed very nearer degradation temperature of 375ºC and the results are closer to the above findings of degradation temperature of plant celluloses.

5.2. Effect of culture conditions, process parameters and media composition on the production of bacterial cellulose

5.2.1. Effect of fermentation condition on BC production

Oxygen is required for the aerobic growth and cellulose formation by *G. xylinum.* The formation of cellulose occurs only at the upper film/air interface and mature cellulose is pushed gradually down, while the new cellulose layers are constantly being built on the top of the mature cellulose due to the need of oxygen (Fiedler *et al.*, 1989).

Toda *et al*. (1997) have established that the rate of cellulose production by *Acetobacter pasteurianus* AP-1SK was about five times higher in static culture than stirred or shaken cultures. Yoshinaga *et al*. (1997) have indicated that the biotechnological production of cellulose is possible in submerged fermentors as well as in surface cultures. Although the chemical structure of cellulose from surface culture is identical with the submerged material, the phenotype of both of these forms were totally different. In agitated or aerated submerged culture, the cellulose accumulates in the form of huge pellets. The final product of the static surface culture is a 2 to 4 cm thick cellulose layer situated on the broth surface.

Lee (1999) have indicated that microbial cells produced under shaking conditions were aggregated and were different from those under static culture conditions. The aggregation caused by shaking was accelerated by the fibrils of microbial cellulose due to their sticky characteristics and this might be the one of the reasons why shaking culture generates much less or no cellulose than static cultures.

In this investigation, there occurred a visible and textural difference in both the top and bottom layer of cellulose pellicle formed by *Gluconacetobacter* sp. sju-1. The bottom layers were highly mature and harder than those formed at the top most layer and this phenomenon must have happened due to the high affinity to oxygen for the growing cells. The results of the present study were very similar to the previous reports, where sju-1 yielded more dry weight of cellulose in static rather than shaken flask culture condition. Further the moisture content of the pellicle was comparatively higher (94.6 per cent) in shaken condition. In addition to it, the amount of soluble polysaccharides at the end of 20 days of incubation was 1.65 g l^{-1} in shaken culture whereas it was only 0.48 g l^{-1} in static culture. Hence it is concluded that due to high moisture content and formation of other water soluble polysaccharides which may be levan or dextran or due the presence of unutilized sugars in the medium, the cellulose production might have reduced in shaken culture condition.

Budhiono *et al*. (1999) also established that glucose conversion to cellulose in the coconut water during bacterial cellulose production using *Acetobacter xylinum* was greatly influenced by static culture condition and oxygen permeability into the medium.

Hungund and Gupta (2010b) has explained that UV and EMS mutated strains of *A. xylinum* GHEM4 gave cellulose yield of 5.96 g l^{-1} which was 50 per cent more than the parent strain and *A. xylinum* GHUV4 yielded 98 per cent more than the wild strain *A. xylinum* NCIM 2526 under static fermentation condition.

A clear cut confirmation on the decrease in the yield in shaken or stirred condition has been established by Krystynowicz *et al*. (2005). That is, the frequency of Cel^{+} to Cel^{-}

conversion depends on culture conditions and their changes. Cellulose producing cells dominate in stationary cultures and produce on the surface of culture medium, a thick cellulose mat, called as pellicle, in which the embedded bacterial cells have contact with the oxygen rich liquid/air interface. Sufficient and uniform aeration of liquid culture media under agitated culture conditions favours spontaneous appearance of Cel^{-} cells, which become dominating in the population. Further molecular identification by 2D gel electrophoresis revealed that Cel^{-} cells lacked two enzymes: phosphoglucomutase and glucose-1-phosphate uridylyltransferase, which generates UDP-glucose which are the main substrate for cellulose synthase. The reasons for low yield in shaken conditions obtained from the present study might be similar as that of the findings above cited.

Hesse-Ertelt *et al*. (2010) have indicated that *G. xylinus* NQ-5 and AY-201 preferred static culture condition. During the first and second day of incubation, no significant differences in the cultivation behaviour of both bacterial strains occurred. The bacteria always produced gelatinous and transparent substances on the surface of the HS medium. After three incubation days the maximum cellulose production could be observed in both the strains under static condition rather than shaken method. After 6 days of cultivation period BC pellicles were able to bind high amount of water and gained thickness and the medium become turbid. The maximum cellulose production could be observed after 6 days of cultivation period. The results of present study were in accordance to the above findings.

5.2.2. Effect of process parameters on BC production

The process parameters such as, pH, temperature, oxygen availability and the way in which the cellulose producer was cultivated and the composition of the media such as carbon, nitrogen, buffers, additives and growth hormones highly influenced the cellulose production potential of the selected strain.

Forng *et al*. (1989) have established that cellulose formation greatly decreased over the course of time and beyond 12th day the formation rates reduced. The average dry weight of cellulose produced by *A. xylinum* ATCC 23769 in HS medium was 9.76 g l^{-1} and the viable cell count taken after 240 hours of fermentation in the HS medium was $4.2x10^{8}$ cfu ml^{-1}. Verschuren *et al*. (2000) has indicated that the pellicle formation rate was greatly influenced by pH and the most favourable pH was 5.0 which decreased to 4.0 during fermentation.

The present study on viable cell count gradually increased from 2nd day of fermentation and dry weight of cellulose increased till 16th day and thereafter it remained constant till the 20th day. The isolate sju-1 preferred acidic range rather than the alkaline pH range. The maximum dry weight of cellulose was in temperature range between 30°C to 32°C. The higher biomass content directly correlated with the increase in cellulose dry weight.

Teoh *et al*. (2004) have established that *Acetobacter xylinum* culture needs a warm and static condition with temperature not below 20°C and not above 30°C. The ideal temperature is about 23-30°C. Fermentation was conducted in a temperature (20-22°C) for up to 14 days. Black tea, sweetened with sucrose incubated at the temperatures of 30°C, for 21days gave yield of 250 g l^{-1} (wet weight). In the present study , also a significant decrease in cellulose yield have been noticed in shaken culture of 9.63 g l^{-1} as compared to static culture which yielded 14.92 g l^{-1}.

According to Pourramezan *et al*. (2009), the optimum temperature was obtained at 30°C and the lowest yield was obtained at 45°C. Maal and Shafie (2010) have suggested that *Acetobacter* could tolerate 5 to 9 percent of ethanol and temperature upto 34°C and produces vinegar. The results obtained from the present research were in concurrent with the above findings.

Maal and Shafiee (2009) have isolated *Acetobacter* strain from Iranian white red cherry that tolerates 40°C and ethanol stress. Further, the strain showed 9.5 per cent acetic acid tolerance after one week incubation which is a very good characteristic in vinegar production in a short period of time. The strain isolated in the present study showed vinegar production of 4.8 per cent in 32 h and the acid tolerance characteristics of *Gluconacetobacter* sp. sju-1 were comparable with the above findings.

5.2.3. Effect of carbon, nitrogen and growth hormones on BC production

With regard to carbon utilization, *Acetobacter* are capable of using different carbon substrates such as ethanol glucose, lactate, acetate, and mannitol. It was very obvious from the present investigation that fructose was the highly utilized carbon source rather than glucose established in the earlier studies conducted by Navarro and Komagata (1999). Ramana *et al*. (2000) established that *G. liquefaciens* produce reddish brown water soluble pigments whereas *G. xylinum* produced water insoluble pellicle made of cellulose and 5-ketogluconic acid by using glucose as a carbon source. Sucrose, glucose and mannitol were found to be suitable for optimum levels of cellulose production.

As per Cvetkovic and Markov (2002) presence of sugars such as glucose and fructose, in the medium contributes to faster fermentation and maximum yield of cellulose in a shorter time. The presence of these sugars along with malt extract yielded high bacterial cellulose. It was found in the present study, that fructose was the most preferred carbon source followed by sucrose and glucose and the results are in accordance to the above findings.

Heo and Son (2002) have optimized a complex medium to increase cell growth and cellulose production in static cultures and established that *G. xylinum* requires a complex

medium containing sucrose plus yeast extract as base nutrients for optimal cellulose production. In the present findings, the most optimum carbon source was fructose rather than sucrose, because its utilization as electron donor and carbon source may not require previous hydrolysis as in case for sucrose.

Park *et al*. (2003b) have established that *G. hansenii* PJK (KCTC 10505 BP) produced 1.72 g l^{-1} of cellulose when glucose was provided as the carbon source. With regard to *Gluconacetobacter* sp. sju-1 used in the present study yielded more dry weight in fructose rather than glucose. Tamura and Tokura (2004) have established the use of tri-carbon sugar increases the production of bacterial cellulose. In the present findings, no such results were obtained when the tri-carbon sugar, glycerol was used to test the effect on dry weight of bacterial cellulose.

Chavez-Pacheco *et al*. (2005) have also formulated a new media (BEGG- medium) composition to increase the cell biomass and extraction of cells from the pellicle. They have established that cell growth and cellulose production were increased about 2 fold when the basal media was supplemented with 1 to 4 per cent ethanol, 1 per cent sodium glutamate and the original sucrose replaced by 5 per cent glucose. But the results obtained from the present investigation on preparing the media with fructose instead of glucose or sucrose gave a comparatively higher dry weight of cellulose.

Cellulose production by a strain of *Acetobacter xylinum* was affected by the type and concentration of sugar, nitrogen source and pH. Fructose, fructose + sucrose, fructose + lactose and sucrose gave the highest yields (5.25 to 7.38 g l^{-1}). Glucose gave low yields when used alone or in combination with other sugars. The pH of all glucose-containing media decreased (Embuscado *et al*., 2009). Panesar *et al*. (2009) indicated that, glucose and sodium nitrate were the best carbon and nitrogen source for the production of cellulose using *Acetobacter aceti* MTCC 2623. In the present research also similar trend was observed. The carbon utilization pattern and reduction in pH were in conformity with the above findings.

Earlier investigation on the process and media optimisation by Pourramezan *et al*. (2009) confirmed that cellulose was found to be produced at the liquid surface in static cultures and the optimum pH for cellulose production was 4.0 to 6.0. Glucose, fructose and glycerol were preferred carbon sources for cellulose production. The yield of cellulose, relative to the glucose consumed, decreased with an increase in initial glucose concentration, and gluconic acid accumulated at a high initial of glucose concentration. Similar results were obtained from the present study and a comparative decrease in cellulose yield in HS medium might be due to conversion of glucose to gluconic acid.

Coban and Biyik (2011) have suggested that the cellulose producing output by *A. lovaniensis* HBB5 strain for the different carbon and nitrogen sources changed from medium to medium. In the medium that contained glucose or ammonium sulphate, cellulose production by *A. lovaniensis* HBB5 was about 3.6 fold lower than that of glucose or yeast extract medium. But in the present study, the carbon utilisation pattern was completely different where fructose in combination with yeast extract contributed to The high production rate of cellulose and was probably because, fructose could be directly used in the metabolic pathway of BC production and the organic source yeast extract contains various amino acids renders for the survival of the bacterium.

Goh *et al*. (2012) indicated that tea broth with sucrose concentration of 90 g l^{-1} produced highest yield of bacterial cellulose (66.9 per cent). The thickness and yield of bacterial cellulose increased with fermentation time of 21 days. The bacterial cellulose yield increased progressively over the whole course of fermentation, with a maximum production of 199.3 g l^{-1} with a thickness of 20 mm after 18 days. The present findings were in close proximity with the above results where in the present research the fermentation time of 20 days increased the yield of cellulose to about 182.5 g l^{-1} (wet weight) under static condition.

One of the important element in nutrient media for *Acetobacter* is phosphorus. Phosphorus is crucial in the synthesis of substances such as phosphoproteins, phospholipids, nucleoproteins, nucleic acids, phophorylated polysaccharides and is also present in cells as inorganic orthophosphate, pyrophosphate, metaphosphate and polyphosphate. Phosphate is added to nutrient media in the form of potassium, ammonium or sometimes sodium phosphates. Ammonium salts (sulphate, phosphate, nitrate) are common inexpensive nitrogen supplements used in the fermentation. Ammonium salts of organic acids are better utilized than salts from inorganic acids as the decomposition produces weak acids that can serve as an additional carbon source. Strong organic acids however, change the pH levels and have an inhibitory effect on cells. An exception is ammonium phosphate as phosphorus is a principal biogenic element and phosphoric acid acts a good buffer system (Halim, 2000).
In the present study, among the inorganic sources of nitrogen, ammonium nitrate and ammonium chloride have favoured high dry weight of BC because, apart as a nitrogen source their acidic nature must have highly contributed to an improved yield.

There has been no substantial evidence established so far about the incorporation of growth hormones into the media of BC production. But there has been many reports that waste coconut water is good enough for the manufacture of *nata-de-coco*. Coconut water contains a variety of nutrients that can be utilized by cellulose producing bacteria. The presence of a growth promoting factor in the media is capable of enhancing the growth of *Acetobacter xylinum* producing *nata*. The

sucrose in coconut water is utilized by *Acetobacter xylinum* as an energy source, or sources of carbon to form the metabolites. Compounds like growth promoting factor will increase the growth of microbes, while the presence of minerals in the substrate will help to improve the activity of kinase enzymes in cell metabolism of *Acetobacter xylinum* to produce cellulose (Shantz and Steward, 1952).

With this view two growth hormones were incorporated into the HS medium. Both kinetin and Indole acetic acid increased the dry weight of the cellulose substantially. The increase in dry weight by IAA was comparatively higher which might be due to the utilization of acetic acid both as a carbon source and growth promoter. Embuscado (1997) have isolated *Acetobacter xylinum* DA from a continuous surface culture for acetic acid production using *Acetobacter aceti*. The strain *A. xylinum* DA produced a gelatinous cellulosic pellicle in the presence of more than 2 per cent acetic acid. The production of cellulose in static culture increased about 4-fold as a result of adding 2 g acetic acid per litre of the glucose medium. The cellulose pellicle produced was characterized by a higher water holding capacity, about 2-fold that of the standard culture *Acetobacter pasteurianus*. Similar results were obtained in the present study where acetic acid concentration of 0.8 to 1.0 g per litre favoured for the production of bacterial cellulose.

5.2.4. Effect of additives on BC production

Tokoh *et al*. (1998) cultured *Acetobacter xylinum* in Hestrin-Schramm medium incorporated with acetyl glucomannan. Cellulose microfibrils formed at the end of incubation were loose bundles in contrast to the normal ribbons produced in the control containing HS medium. The presence of acetyl glucomannan in the medium prevents the assembly of cellulose microfibrils and changes the crystal structure of cellulose. In the results of present study, none of the cellulose fibrils formed by incorporation of polysaccharides were of loose textured but were very strong and thicker, and the results were contradictory to the above findings.

Iwata *et al*. (1998) have proved the affinity of hemicelluloses for the cellulose production by *A. xylinum* ATCC 53524. Cellulose produced under shaking and agitation conditions in the presence of 2 per cent lignin-carbohydrate complexes (LCCs) and six different types of hemicellulosic polysaccharides including glucuronoxylan, glucomannan, O-acetyl-glucuronoxylan, arabinoglucuronoxylan, arabinogalactan and xyloglucan were resistant to alkali. In the present study, also similar results were obtained. Ishida *et al*. (2003) have established on the use of negatively charged water-soluble cellulose derivative, carboxymethylcellulose (CMC), agar and sodium alginate to enhance BC production in static culture. The improvement of BC production with the presence CMC was proportionately

higher (8.2 g l^{-1}) compared to those of agar and sodium alginate. The results of the present investigation recorded a maximum yield when agar (14.7 g l^{-1}), CMC (14.7 g l^{-1}) and sodium alginate (14.2 g l^{-1}) were amended into the HS medium.

Seifert *et al*. (2003) have investigated that polymeric additives such as xanthan, agar, acetan hinder formation of large clumps of high moisture content bacterial cellulose and enhance production in either jar fermentor or airlift reactor. But there were remarkable differences in BC production extent according to strains, type of additives and its concentration used. Basically 0.05 to 0.1 per cent incorporation of additives gives enhanced yield in static cultures. The findings of the present research are in accordance with the above.

Bae *et al*. (2004) established that the maximum BC production of *A. xylinum* BPR2001 at 0.4 per cent (w/v) agar was 12.8 g l^{-1} compared with 8 g l^{-1} without agar and the mutant *A. xylinum* EP1, generated from *A. xylinum* BPR2001 produced 11.6 g l^{-1} of BC at 0.6 per cent agar, while only 5.5 g l^{-1} was produced in the control in a 10 litre stirred-tank reactor. The yield or the dry weight of the cellulose obtained from *Gluconacetobacter* sp. sju-1 of our study clearly envisages that with the addition of lignosulfonate and calcium alginate at the rate of 0.1 per cent increases the yield at static condition itself. Hence an extra input incurred for mutant selection and energy required for stirred tank condition can be overruled.

Zhou *et al*. (2007) have established on the bacterial cellulose production using *Acetobacter xylinum* NUST4.1 in shake flask and in a stirred-tank reactor by means of adding sodium alginate (NaAlg) 0.04 per cent into the HS medium. The production recorded a maximum of 6.0 g l^{-1} and the control recorded only 3.7 g l^{-1}. The results obtained from the present study on the addition of calcium alginate into the HS medium recorded an increased yield of 17.8 g l^{-1} and the yield was 66 per cent higher over the above findings. Cheng *et al.* (2009) have also established that the addition of additives, namely CMC yielded highest BC production (8.2 g l^{-1}) compared to the control (1.3 g l^{-1}). The findings of the present study were in line with the above research.

5.3. Production of bacterial cellulose from low cost substrates

Sucrose in the coconut water acts as an energy source and also as a cellulose precursor. The monosaccharide is also converted by membrane-bound dehydrogenase into (keto) gluconic acid (Masaoka *et al*. 1993). Embuscado *et al*. (1994) have explained that excess sugar utilization resulted in a decreased cellulose production. Therefore, an appropriate level of sugar is necessary for optimum bacterial cellulose production. The pre-optimisation performed to find out the low and high levels of carbon and nitrogen sources required for optimum production of bacterial celluloses in the present study also proved the above findings, the reason for the decrease in yield might be due to the carbon

catabolite repression or might be due to the accumulation of more organic acids in the medium.

Keshk and Sameshima (2005) have illustrated that, when fructose was used as a sole carbon source, the BC production was not significantly higher. But fructose along with molasses, resulted in higher quantities of bacterial cellulose. Similar results were obtained when fructose was used in combination with cashew apple juice. Therefore it can be concluded that with the presence of carbohydrate sources (sucrose and reducing sugar), nitrogen sources and mineral salts, together with low cost gives cashew apple juice an edge over using glucose or fructose to produce the BC.

Keshk and Sameshima (2006) found that BC production increases in molasses medium compared to the HS medium. Further, the pH does not decrease to suboptimal levels in the molasses medium and the average pH values of the HS and MO media after the incubation were 2.7 and 4.2 respectively. Faridah *et al.* (2010) have established that *Arenga pinnata* juice and coconut water can be used as a basal medium to produce bacterial cellulose by using *Acetobacter xylinum*. Edible film or the bacterial cellulose yielded from the media containing *Arenga pinnata* juice and coconut water (25:75 per cent) recorded a maximum production of 300 g l^{-1} on wet weight basis.

It is noticed from the present study that yield of bacterial cellulose was comparatively very higher in CAJ than the MCW and HS medium. The reason may be due to the presence of more readily available carbon sources such as glucose and fructose and acidic pH of the cashew apple juice is more suited for the growth and survival of *Gluconacetobacter* sp. sju-1. It was confirmed from the present findings that in the CAJ, after 20 days of fermentation yield of BC has maximised linearly and the pH reduction was only 3.5 from the initial 6.5. But the use of MCW involves an additional carbon cost in the form of glucose, sucrose or fructose. But in the case of CAJ when used alone without any sugar amendment, dry weight of BC was 2.5 g l^{-1}. The primary goal of this research is to cut down the production cost by using sugary rich substrates and it has been achieved with the use of MCW and CAJ. Further to improve the yield of BC, HS medium was amended with various polysaccharides and calcium alginate addition highly influenced for higher dry weight of bacterial cellulose. The textural properties of calcium alginate incorporated HS medium was found to be very high and was more suitable for use in paper making rather than for edible purpose.

In the present investigation, the production of bacterial cellulose was relatively high with similar properties to that produced in HS medium. These results suggest that it is possible to produce bacterial cellulose from low-cost resources such as cashew apple juice in order to increase BC production to a larger scale.

While using cashew apple juice alone, the yield was 3.14 g l^{-1} and upon addition of 0.4 per cent fructose into the cashew apple juice, the yield increased to 9.27 g l^{-1} (dry weight basis). Earlier reports by Castro *et al*. (2011) on the production of bacterial cellulose from pineapple peel juice (2.8 g l^{-1}) was higher than that produced from HS medium (2.1 g l^{-1}). The carbon and nitrogen resources in pineapple peel and sugar cane juice were sufficient for the microorganism development. The findings of the present research were in conformity to the above.

The presence of sugar in coconut water is utilized by *Acetobacter xylinum* as an energy source, or sources of carbon to form the metabolites. Compounds such as growth promoting factor will increase the growth of microbes, while the presence of minerals in the substrate will help to improve the activity of kinase enzymes in cell metabolism of *Acetobacter xylinum* to produce cellulose (Kus and Sri Retno, 2012).

Supakod and Wongwicharn (2012) have identified a cheap media for the preparation of seed culture of *Acetobacter xylinum.* Coconut water, banana juice and a mixture of coconut water and banana juice (ratio 1:1) were used as inoculation media and compared with HS and glucose ethanol yeast extract medium, the commonly used media for acetic acid bacteria. *Acetobacter aceti* TISTR102, *Acetobacter xylinum* TISTR975, *Acetobacter xylinum* AGR60 and the isolated *Acetobacter xylinum* Coc5 were used as the test strains. The results found that all strains grew well in each medium and viable cells was 10^6 cfu ml^{-1} when cultured for 12 hours at 30°C. The results of present study are in line with the above findings. Kamarudin *et al*. (2013) have found that **c**oconut water in HS medium is the most suitable medium for BC production with a productivity of upto 0.044 g l^{-1} day^{-1}. Carbon sources like mannitol and fructose showed relatively low yield than glucose. But in the present study *Gluconacetobacter* sp. sju-1 used almost all the carbon sources.

5.4. Physicochemical properties of MCW and CAJ

Vandamme *et al*. (1998) have suggested that coconut water, due to the presence of high glucose and sucrose content, helps in the growth and polysaccharide production of wide variety of bacteria especially *A. xylinum.* Richter *et al*. (2005) have established the content of MCW as phosphorous (9.2 mg), potassium (273 mg), sodium (45.4 mg), calcium (47.7 mg), magnesium (11.7 mg), copper (112 µg) and iron (28.7 µg). With regard to the proximate mineral composition of CAJ obtained from Northern Savannah forests of Ghana by Lowor and Agyento-Badu (2009), 100 ml of juice contained phosphorous (0.79 mg), potassium (76.0 mg), sodium (0.41 mg), calcium (43.0 mg), magnesium (10.92 mg), copper (0.05 µg) and iron (0.08 µg).

From the experimental results, it was explored that the proximate composition of the juices namely mature coconut water and cashew apple juice was found to contain TSS of 5.4 and 12.9 °Brix respectively. It was evident from the TSS content that CAJ is sugar rich than MCW, and can very well be used as a substrate for microbial fermentation with little or no added carbon source. Further the pH of MCW and CAJ were found to be in the acidic range of 5.2 and 4.29 respectively.

Rocha *et al*. (2009) have evidenced that CAJ possess high sucrose, glucose and fructose content which supports the growth of yeasts and can be converted into wine or ethanol of high purity. The results of TSS and pH correlated with the findings of Sivagurunathan *et al*. (2010) who have established that total sugars and pH of cashew apple juice collected from Ariyalur district were 13.3 g and 4.8 g respectively. Further the protein content was established to be 6.21 g. But the protein content obtained from the CAJ used in this study was only 3.32 g. The reducing sugars content of CAJ were 11.3 g and the results were in accordance to the findings of Vergera *et al*. (2010) who have estimated a very closer reducing sugar content of 11.8 g. When the results of TSS, reducing sugar and pH of mature coconut water obtained from Malayan Tall variety of coconut estimated by Prades *et al*. (2012) were compared, they were in close proximity with the results of present study and the mineral composition of MCW obtained from the present study .

5.4.1. Media optimisation using Response surface Methodology

Dobre *et al*. (2008) have illustrated and optimised the BC using 2^4 factorial experiment RSM. In their study, quantitative correlations between the two independent variables such as fructose concentration and temperature against the dependent variable of BC yield were accomplished. The analysis emphasized that the most important factors influencing the BC yield were operating temperature and fructose concentration. Hutchens *et al*. (2007) used RSM for optimising sucrose concentration, ethanol addition and temperature against the production of cellulose by *Gluconacetobacter hansenii*, and found that the addition of ethanol serves as a carbon substrate for the acetic acid bacteria, and favours for the oxidation of ethanol to acetic acid.

In the present study, MCW and CAJ was taken as a basal medium, four independent variable such as concentration of fructose, yeast extract, and two buffer sources namely citric acid and disodium hydrogen phosphate were taken as independent variables and correlated with the dependent variable namely, dry weight of cellulose.
It was interesting to note that fructose concentration highly correlated with the dry weight of bacterial cellulose rather than the yeast extract concentration. But the interaction of fructose

with yeast extract showed positive correlation in the case of MCW medium, whereas no such correlation was found to exist in the case of CAJ medium.

In the case of buffer sources, no prior standardization have been explained earlier using the central composite design of RSM. Hence in this attempt, the concentration of the buffer sources were also taken as independent variables and disodium hydrogen phosphate buffer found to significantly influence the dry weight of cellulose by interaction with fructose and yeast extract ($p < 0.0001$) in the MCW medium. In the case of optimization of buffer sources in CAJ medium, no such interaction effect was found to significantly influence the dry weight of bacterial cellulose.

Response surface curves were illustrated by Jagannath *et al*. (2008), indicating that sucrose, pH and ammonium sulphate concentration were the independent variables and highly correlated with the wet weight, texture and water holding capacity of bacterial cellulose. The study showed that *A. xylinum* could effectively use sucrose as the sole carbon source present in coconut water medium and that cellulose production was more dependent on pH than sucrose or ammonium sulphate concentrations. Maximum thickness of *nata* was obtained at pH 4.0 with 10 per cent sucrose and 0.5 per cent ammonium sulphate concentrations. Lin *et al*. (2011) have illustrated that by using Taguchi method of RSM, yield of bacterial cellulose could be optimized. At the glucose concentration of 5 per cent and at pH 4.5, the wet film of cellulose could be increased to 37.5 per cent.

5.5. Value added products from bacterial cellulose

Three different types of products such as reinforced paper, *nata* and coconut water vinegar were developed from the present study. Firstly bacterial cellulose was produced from the HS medium and was subjected to produce paper at Sheshasayee Paper Boards, Erode, Tamil Nadu, but due to very high moisture content (91.8) and very finer fibril (207 nm) thickness of BC, paper could not be formed, hence, amendment of media with polymer additives were tried so as to reinforce the texture and structure of BC. Incorporation of calcium alginate into HS medium at the rate of 0.1 per cent favoured for more fibril thickness and improved the tensile strength. The BC pulp obtained from calcium alginate incorporation could be easily made into reinforced paper sheets of high tensile strength (120 MPa). Secondly *nata, a* soft textured dietary fibre rich food product of aesthetic value was developed from MCW and CAJ medium, as the BC developed from these sources was very softer showing the hardness of 7 and 11 N respectively. Coconut water vinegar was prepared using the spent liquor left after the extraction of bacterial cellulose from cashew apple juice medium as inoculum source for vinegar making. The resulted vinegar registered 4.8 per cent of acetic acid.

5.5.1. Production of special paper from bacterial cellulose

Sheets obtained from bacterial cellulose possessed greater mechanical strength compared to the sheets prepared from wood celluloses. Young's modulus of unprocessed BC was around 15 GPa and after alkaline and oxidation solution treatment it reached to 30 GPa (Nishi *et al*. 1990). Ochaikul *et al*. (2000) have established that the use of chitosan in the range 0.1 to 1.0 per cent to coconut water medium gave the highest yield of bacterial cellulose. The paper from bacterial cellulose prepared from culturing *Acetobacter xylinum* TISTR 976 in coconut water medium had the highest tensile strength of 112.2 MPa when 0.2 per cent chitosan solution was added. In addition to it, the presence of chitosan helped to lower water absorption in the paper. Tabuchi *et al*. (2005) have also established that polysaccharide incorporated bacterial cellulose can be used for reinforced paper production and various other cellulose based products of high value. Radiman and Yuliani (2008) have found that BC from coconut water has a potential to be used in the fabrication of membranes by converting it to cellulose diacetate membranes which gives an added value to its original nature. Acetylated bacterial cellulose increased the degree of crystallinity of pulp and results in higher membrane permeability.

Based on the results of the present study, it can be concluded that use of polysaccharide in general improved the quality attributes of bacterial cellulose and also the yield to certain extent. The reinforced paper prepared from the calcium alginate incorporated HS medium was comparatively stronger and even air cannot penetrate into it and was resistant to castor oil permanently which might be due to calcium alginate present in the bacterial cellulose would have increased the degree of crystallinity and resulted in thicker fibrils of cellulose.

Basta and El-Saied (2009) have confirmed that the use of glucose phosphate along with glucose significantly increases the production of phosphate containing bacterial cellulose. Incorporating 5 per cent of this phosphate containing bacterial cellulose with wood pulp during paper sheet formation was found to improve retention strength and fire resistance properties as compared to paper sheets produced from incorporating bacterial cellulose alone. In the present research, no phosphate was blended into the medium, but the buffers used in HS medium contains disodium hydrogen phosphate and in addition to it calcium alginate additive incorporation must have improved the strength of the paper and favoured for high tensile strength of 120 MPa and Young's modulus of 90 GPa.
Due to polymer addition in the HS medium, the number of hydrogen bonds might have increased and conferred high tensile strength and Young's modulus.

Ciechanska (2004) have established that modifying bacterial cellulose with chitosan during its biosynthesis results in a composite material with glucosamine and N-acetylglucosamine units incorporated into the cellulose chain and yielded cellulose with good mechanical properties in wet state. The results obtained from the present study were in conformity with the above findings. Hsieh *et al*. (2008) studied on a single filament of bacterial cellulose and Young's modulus reached 114 GPa. Gindl and Keckes (2004) established that the mechanical properties of cellulose composite such as cellulose-pectin or xyloglucan, and CAB (Cellulose Acetate Butyrate) were comparatively superior in quality than the usual cellulose membrane.

Bacterial cellulose behaves as a viscoelastic material, with brittle failure reached at approximately 20 per cent strain and 1.5 MPa stress under uniaxial tension. Treatment with 0.1 M NaOH resulted in minimal effects on the mechanical properties of bacterial cellulose. Fermentation time had a large effect on both bacterial numbers and cellulose yield but only minor effects on mechanical properties (Mc Kenna *et al.,* 2009). The results of the mechanical properties of the reinforced paper obtained from the present study were in conformity to the above findings. The enhancement of the mechanical properties of reinforced bacterial cellulose paper might be due to the improvement caused by the calcium alginate incorporation that have made the cross links very closer and made of the fibre-matrix interface very stronger.

Saputra and Darmansyah (2010) prepared bacterial cellulose using urea, acetic acid and sucrose and reinforced with resin filled with SiO_2 and Al_2O_3. The properties of cellulose obtained possessed tensile strength of 390.2 MPa and 226.90 MPa respectively. The mechanical properties of the reinforced paper obtained from the present research were only 120 GPa. Hence, it is vivid that addition of metallic compounds may still improve the strength of the BC.

Radiman and Rifathin (2013) prepared an environmental-friendly and cheap material as the polymer electrolyte membrane from bacterial cellulose obtained from coconut water fermented with *Acetobacter xylinum.* The membrane was phosphorylated using phosphoric acid into the HS medium and the resultant membrane showed high tensile strength. The Young's modulus and tensile strength obtained from the present study were in agreement with the above findings.

5.5.2. Production of *nata* - a value added organic dietary food

Mina and Penalosa (1994) used additives like CMC, carrageenan, sodium alginate to improve the textural properties of *nata-de-coco*. Carrageenan produced thickest and softer cellulose pellicle, but CMC and sodium alginate made the texture very harder and rendered unfit as an additive of *nata-de-coco*. Booranasawettathum *et al*. (1998) have developed *nata-de-coco* product from fruit juices. Sensory evaluation studies of *nata,* from apple juice has high acceptability scores than from cherry juice and grape juices. There was no significant difference in odor and texture evaluation among the samples. Sheu *et al*. (2001) produced colourful *nata* by culturing *Monascus purpureus* in the media containing bacterial cellulose derived from *Acetobacter aceti* sub sp*. xylinum*, and rice powder and monosodium glutamate. The scanning electron microscopic observations showed that the *Monascus* mycelium could grow through the cellulose network of *nata* and proved that the *Monascus-nata* complex has the potential to be a new vegetarian foodstuff. Lynd *et al*. (2002) have also established that the micro-fibril cellulose is more delicate, softer and superior than other types of fibre found in fruits and vegetables. Further it was indicated that *nata* is a suitable health food for weight control and solves excretory problem.

The gel-like properties of microbial cellulose combined with its complete indigestibility in the human intestinal tract, make this an attractive food base. In addition to it, an unique property of high water retention capacity gives a texture to prevent water being released from 3-dimensional structure (Dubey *et al*., 2002).The crude fibre content of *nata* prepared from pine apple solid waste was found to be 15.53 g (Kumalaningsih *et al*. 2011). Setyawaty *et al*. (2011) has explored the possibilities of using cassava starch into *nata* with higher fibre content. Production was comparatively lower in cassava starch but was found to be superior in quality than the control.

In the present research the hardness of bacterial cellulose from HS medium was comparatively higher than MCW and CAJ medium, which might be due to use of multiple type of sugars present in the MCW and CAJ by *Gluconacetobacter* sp. sju-1. The *nata-de-cashew* prepared using cashew apple juice was on par in quality attributes to *nata-de-coco* and taste wise it was found to be more acceptable than *nata* from HS medium. In the present study, involving the organoleptic evaluation of *nata* in jelly base from MCW and CAJ showed no significant difference and the crude and soluble fibre content also did not show any differences. Texture analysis a measure of product firmness, hardness, tactile properties, smoothness, fracture ability and resilience was higher of 45 N in the BC produced from HS medium, whereas biowaste substrates such as MCW and CAJ produced more soft textured bacterial cellulose. The softness in texture might be due to high moisture

content of BC from MCW and CAJ and also the fibrils were looser. The insoluble and soluble fibre content of bacterial cellulose from HS medium were significantly higher of 8.50 g and both bacterial celluloses from MCW and CAJ medium contained lower dietary fibres compared to HS medium. Invariably the fibre value of three cellulose was comparable with fibre rich products.

The studies on laxative effect of *nata-de-cashew* from the present investigation also proved the above findings. Crude fibre might have acted as base of various elements like Fe, Mn, Mg that may support the activity of enzyme. Apparently the crude fibre presence in the media has the potential role to distribute the nutrition during fermentation.

Sutanto (2012) have established on the production of pine apple juice *nata* which had superior quality with fibre content of 9.25 per cent and the thickness of the cellulose was 12.4 mm with a wet weight of 272.4 g l^{-1}. *Nata-de-cashew* prepared in the present study have also evidenced similar characteristics.

Ochaikul *et al*. (2006) produced bacterial cellulose using *Acetobacter xylinum* TISTR 967 and fermented it with *Monascus purpureus* TISTR 3090 in a culture medium on a rotary shaker at 150 rpm at 30°C for 12 days. Good and appealing colour was developed at the end of incubation and the product demonstrated a good resistance to washing, heat, freezing, acidification and irradiation. Similarly, in the present research the *nata* obtained from CAJ was coloured using pigments derived from beet root extract. The appearance of the developed *nata* possessed a meaty texture and possessed a high overall acceptability. There were only few studies established on colouring of bacterial cellulose. The colouring of bacterial cellulose can improve its appearance and provide more variety for application as food stuff.

Dumadaug (2012) have evaluated texture of *nata* from Eucheuma pulp extract and established that the texture of *nata* was 1.79 and 0.29 N. The results obtained from the present findings were much higher than the above findings.

The fibre value of *nata* was also explained by Santosa *et al*. (2012) who have established that fibre rich instant drinks can be developed from bacterial cellulose which is processed and value added into *nata* in gel base by appropriate incorporation of CMC and dextrin as binding and filler agents respectively. Further the crude fibre value increased even to 38.2 per cent by such type of preparations and proved to be very beneficial for health, especially the digestive health for which it enhances the absorption of excess cholesterol in the body. The results of the present experiment on the laxative properties of bacterial cellulose were proved to be in line with the above findings.

5.5.3. Production of coconut water vinegar using spent liquor of BC culture media

The spent liquor of the fermentation broth is rich in variety of important compounds. According to Kocher *et al.* (2006) vinegar can be produced using fermented sugarcane juice using acetic acid bacteria. Pinto *et al*. (2008) have explained that immersion of complete denture in 10 per cent vinegar solution reduced *Candida* sp. and proved that vinegar acts as an antimicrobial agent. Santos *et al*. (2009) have indicated that the crude extract of fermented broth shows a good antibiotic spectrum against *Staphylococcus aureus* and *Salmonella typhimurium.* Hiroyuki *et al*. (2009) suggested that sphingolipids are usually present in acetic acid bacteria (AAB) and it improves the cognitive function when administered as AAB capsule containing *Acetobacter malorum* to the elderly persons.The medicinal importance of *nata* and its fermented liquor was studied by Bhattacharya *et al*. (2011). Consumption of *nata* along with the liquor as *Kombucha* tea has been shown to have beneficial effects on gastric, intestinal, and glandular activities and to overcome arteriosclerosis, toxin excretion, diabetes, nervousness, and aging problems. Additionally, it can also act as a laxative and are known to relieve joint rheumatism, gout and haemorrhoids.

Haigh (1973) has illustrated that induction of bifringence (orientation of microfibrils) were due to the liberation of 1:1 mixture of saturated and monounsaturated tetra hydroxy terpene. Previous publications established that the compounds such as 1-octadecene, 1-heptadecene found in algae, bacteria and plants show anticancer, antioxidant and antimicrobial activity (Jayabalan *et al*., 2008).

In the present study, organic acids were one of the important compound present in the fermented broth which was primarily made of acetic acid. The pH of the fermented broth was more in the acidic range from the 6th day of incubation, even in the low pH range, multiplication of bacteria was in a linear range and this shows the acid tolerance of the bacterium, the reason may be attributed due to the formation of cellulosic layer around the cell membrane that protects the cell from diffusion of acid from the medium. In such a low pH condition, acetate from the metabolic pathway might have been protonated into acetic acid which can penetrate into the cells through passive diffusion and decrease the intracellular pH by releasing the protons. This was evidenced from the studies of Kanchanarh *et al*. (2010) who have proved that acetic acid fermentation and pellicle formation capacity of *Acetobacter pasteurianus* IFO3283 and *A. pasteurianus* SKU1108 provides multiple resistance to stress conditions.

Further, results of GCMS showed the presence of terpenoids and this may have favoured for the orientation of microfibrils of bacterial cellulose. Most of antioxidants were present in the fermented broth of *Gluconacetobacter* sp. sju-1. There was a relative abundance of alkane hydrocarbon or fatty acid molecules like dodecane, heptadecane, octadecane, hexadecane, octacosane, heneicosane, tricosane and nonane which are found to possess

antibacterial activity. Some other medically important and flavanoid compounds were also present in the broth.

5.6. Static fermentor and fermentation kinetics of BC production

Bae and Shoda (2005) studied the production of bacterial cellulose by *A. xylinum* BPR2001 using molasses medium in a jar fermentor. They have developed an efficient cultivation method for bacterial cellulose production using molasses at concentration of 20 g l^{-1}. Hornung *et al*. (2006a) have established that bacterial cellulose production is greatly enhanced by wide mouthed containers in static condition rather than cultivation in conical flasks or other conical structures due to the availability of large surface area. Further extraction or removal of the cellulosic layer is also rendered convenient in wide mouthed vessels. In the present research, the wide mouthed model fermentor gave a good and an economical yield. *Gluconacetobacter* sp. sju-1 was cultivated in a similar jar type model fermentor. It was user friendly and gave economically high yield. With respect to fermentation kinetics, the population reached the maximum of 34.12 x 10^{11} cfu ml^{-1} with a decrease in pH of 4.5. Stationary phase was attained on the 18th and 20th day respectively. Hornung *et al*. (2006b) have explained the mass transfer reactions during the growth of *A. xylinum.* The cellulose growth rate ceases after about 15 days, but remaining glucose diffuses through the layer and continues to be consumed even after the stagnation of product formation is used for cell maintenance. After a brief lag phase, the number of cells which produce BC increases exponentially, reaches the maximum value of 8.3 x 10^9 cells and remains constant on the maximum value between days eight to ten of incubation. From day eleven, the number of BC producing cells seems to decrease due to the cessation of cellulose production. The results obtained from the present study paralleled with the above findings.

5.7. Animal experiment to test the laxative effect of bacterial cellulose

The use of various hygroscopic celluloses and hemicelluloses has gained a wide use in the treatment of constipation. Normally celluloses have stool bulking and laxative effect. Cellulose based laxative act by release of lower volatile fatty acids and primarily based on a chemical and not by mechanical stimulation (Brummer *et al*., 1954). Dietary fibre increases the faecal weight associated with the improvement of undesirable constipation, which can be mediated through the water-holding capacity of unfermented fibre (Kayashita *et al*., 1995). Laxative effect of cellulose is due to the hygroscopic nature, which enables to imbibe and hold water in the intestinal tract and increases the quality and quantity of stools. In addition to the mechanical stimulus due to distention, the biochemical end product which arises from the decomposition of cellulose by intestinal microorganisms stimulates the evacuation of bowel.

More the number of pentose sugar in the laxative, the effect of bowel evacuation is more (Furda, 2001).

In the present study, faecal content and moisture percent was found to be greater compared to the constipated rats. Basically, BC laxative increased the weight of the stools and also the moisture content. The present findings on the effect of colloidal laxative of bacterial origin correlates with the studies of Kim *et al*. (2009) who have established that probiotic cultures at 1×10^6 cfu ml^{-1} administered for 5 days partially recovered or prevented constipation in the loperamide induced constipated rats.
The laxative studies of bacterial cellulose conducted in the present study highly correlated with the findings of Kakino *et al*. (2010) who have demonstrated that ethanol extract of *Aquilaria sinensis* (EEA) increases both the intestinal tension of the small intestine, stool frequency and weight of stools, and the rate of gastrointestinal transit.
The present study was in agreement with Hong-Geun *et al*. (2011) that constipation can be relieved by the intake of fibre rich foods and natural laxatives like fig which contains more amount of cellulose that relieves chronic constipation.

Mikkelsen *et al*. (2011) have evaluated the use of methyl bacterial cellulose as a colloid laxative and evidenced that at the dose of 10 g day^{-1}, methyl cellulose doubled the volume of the stools. Likewise in the present study, laxative formulation prepared using bacterial cellulose obtained from *nata-de-cashew* was found to have stimulatory effect on bowel evacuation and increased the stool weight in the experimental rats.It is evident for the present research that bacterial cellulose can be derived from mature coconut water and cashew apple juice. 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 mature coconut water which is discharged as waste from coconut processing industries. As cheap source of carbon both MCW and CAJ 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. *Nata* contains dietary fibre that is needed in the body's physiological processes. In terms of appearance, *nata* have a high aesthetic value. From an economic point of production *nata* is a promising value-added product. *Nata-de-cashew* production using cashew apple juice produced *nata* with good quality and showed higher overall acceptability. *Nata-de-cashew* production can gain benefit cost ratio of 1 to 2.
The production has got both economical and ecological benefits for the coconut and fruit

processing industries. Further the spent liquor is rich in monosaccharides, various types of organic acids, free radical scavenging molecules and antioxidant substances and can be utilised for the production of vinegar. 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 hydrogel in dryland agriculture also.

CHAPTER VI

SUMMARY

The present study was taken up with the main objective to isolate an efficient cellulose producing bacteria from various fruits and sugary juices for the production of bacterial cellulose using bio-wastes such as mature coconut water and cashew apple juice. The bacterial cellulose (BC) obtained was value added into reinforced paper, *nata* and vinegar.

Cellulose producing bacteria was isolated, screened and selected from various fruits and different sugarcane varieties. Since the isolate sju-1 produced comparatively thicker cellulose pellicle (13 mm), it was selected for the analysis of biochemical characteristics and compared with reference culture *Acetobacter xylinum* NCIM 2526. Based on the cellulose pellicle thickness, an elite cellulose producing bacteria was isolated from fermented sugarcane juice and was named as sju-1. Based on the morphological, biochemical and molecular characterization, the best performing isolate was identified as *Gluconacetobacter* sp. sju-1. The 16S rRNA gene sequence of *Gluconacetobacter* sp. (sju-1) with 1456 bases possessed 99 per cent similarity to *G. obediens*, *G. intermedius* and *G. xylinum* and was deposited in the NCBI GenBank database under the accession number KF164613.

Scanning Electron Microscopy (SEM) revealed the structural properties of cellulose fibrils. The size of the fibrils ranged between 128 and 207 nm. Fourier Transforming Infra Red Spectroscopy (FTIR) of cellulose microfibrils registered the transmittance peak at 3229 cm^{-1} and 2911 cm^{-1}, showing the presence of OH and CH_2 groups indicating a pure form of cellulose type Iα in the BC. The thermograms obtained from Thermo Gravimetric Analyser (TGA) and Differential Scanning Colorimeter (DSC) showed the thermal resistance of BC from *Gluconacetobacter* sp. sju-1, by registering degradation or the decomposition temperature more than 300°C.

Two sugar rich, edible bio-wastes namely Mature Coconut Water (MCW) and Cashew Apple Juice (CAJ) were used for bacterial cellulose production. From the bacterial cellulose value added products namely reinforced paper, *nata* and vinegar were developed. The effect of various process parameters and media components were optimized in the Hestrin Scramn (HS) medium. The optimum incubation time, pH, temperature, carbon, nitrogen and growth hormones for maximum cellulose production were found to be 20 days (480 h), 6.5, 31°C, fructose, yeast extract and Indole Acetic Acid respectively. With regard to aeration or fermentation condition, dry weight of BC was 14.92±0.30 g l^{-1} with a biosynthetic yield of 74.7±0.32 per cent under static culture condition. But shaken culture recorded only 9.63±0.20 g l^{-1} dry weight of BC and the biosynthetic yield was 48.15±0.98. In general, the moisture

content of BC was above 90 per cent and for making paper, BC has to be reinforced with polysaccharide additives to achieve high tensile strength paper. In this study, the HS medium was optimized by incorporating various polysaccharide additives, among which calcium alginate and lignosulfonate improved the dry weight of BC to 17.8±0.38 and 17.8±0.41 g l^{-1} respectively.

From the results of texture analysis, the hardness of calcium alginate incorporated BC was 45 N which makes it highly amenable for high quality paper production rather than for edible purpose. So, with the pulp of BC obtained from calcium alginate incorporated HS medium, reinforced paper was produced with modular paper making machine at Sheshasayee Paper Boards, Erode, Tamilnadu, India. The viscosity and degree of polymerization of BC pulp were127.4 ±0.29 cP and 2074±2.91 respectively. The tensile strength and Young's modulus of BC reinforced paper was recorded to be 120 ± 2.89 MPa and 4.9 ± 0.01 GPa respectively. The viscosity and degree of polymerization of BC pulp 127.4 ±0.29 cP and 2074±2.91 respectively.

The use of MCW and CAJ as a basal medium for bacterial cellulose production for development of an edible dietary fibre rich product *nata*, was pretested to optimize the low and high levels of C, N and buffer sources. Media optimization was carried out to find the interaction effect of each of the individual components using a statistical based Response Surface Methodology (RSM). The model was found to be highly significant with a predicted R^2 values for the dry weight of cellulose of 0.9490 for MCW medium and 0.9417 of CAJ medium respectively. With MCW as the basal medium, the concentration of fructose (20 g l^{-1}), yeast extract (10 g l^{-1}), citric acid (1.5 g l^{-1}) and disodium hydrogen phosphate (1.1 g l^{-1}) at the end of 20th day of fermentation recorded the maximum predicted dry weight of BC with 16.74 g l^{-1} which was slightly higher than the actual experiment which recorded 16.11 g l^{-1}. With CAJ as the basal medium, concentration of fructose (4.0 g l^{-1}), yeast extract (6.0 g l^{-1}), citric acid (1.5 g l^{-1}) and disodium hydrogen phosphate (0.75 g l^{-1}) had the maximum predicted dry weight of BC with 16.24 g l^{-1} than the actual experiment which resulted in 16.22 g l^{-1}. From the results, it was confirmed that, CAJ medium recorded a significantly higher dry weight of BC, with a low input of carbon. Hence, CAJ medium was selected for further studies pertaining to mass production in a model fermentor and testing of laxative properties of BC using animal experiment.

Animal experiment was conducted at KMCH College of Pharmacy, Coimbatore to test the laxative properties of BC. The Effect of loperamide on feed, water intake and faecal properties of constipated Sprague-Dawley rats were studied. The constipated rats had more feed intake of 21.24±0.87 g compared to the normal control (21.24±0.87 g). Number of faecal

pellets increased twice (70.77±3.00) in normal control producing a faecal weight of 7.12±0.63 g than the constipated rats which recorded 30.55±4.00 pellets with the faecal weight of 3.19±0.44 g. Similarly the moisture content of the faecal material was higher in the normal rats compared to the constipated ones.

Among the four groups tested, Group I (loperamide treated rats) and Group III (Loperemide induced constipated rats fed with colloidal bacterial cellulose laxative at the rate of 2.5 ml twice daily) had higher feed intake (20.21±3.39 g and 20.21±1.21 g) compared to Group II (Loperemide induced constipated rats fed with Isabgol laxative at the rate of 5.0 ml twice daily) (19.99±1.26 g) and Group IV (Loperemide induced constipated rats fed with bacterial colloidal laxative at the rate of 5.0 ml twice daily) (19.58±1.52 g). Water intake was found to be higher in Group II and Group IV. Number of faecal pellets, a most important parameter dealing with the laxative property of the bacterial cellulose was higher in group IV, which excreted 46.34±2.55 number of faecal pellets followed by Group II (44.89±2.36). Group IV animals recorded a faecal pellet weight of 16.87±54 g with the moisture percent of 2.94±1.24 followed by group III rats which recorded 15.23±1.14 g with the moisture per cent of 2.15±1.11.

From the results of the ethical studies, it was evident that the effect of colloidal bacterial cellulose laxative on feed intake, water intake and faecal properties of loperamide induced constipated Sprague-Dawley (3 weeks old) rats were comparable with those of the control laxative, Isabgol (*Psyllium* sp.). An increase in the number and weight of faecal pellets was comparatively higher in colloidal bacterial cellulose laxative administered rats (5.0 ml dose twice daily) of 46.34±2.55 and 16.87±54 g respectively. The loperamide induced constipated rats administered with colloidal bacterial cellulose laxative twice a day at the rate of 5.0 ml per dose significantly increased the faecal weight, which is a good indicator of laxative property of BC.

A wide mouthed, autoclavable jar type model fermentor of 5 litre capacity was designed for mass production using CAJ medium. The studies on fermentation kinetics revealed that Specific growth rate (μ) of *Gluconacetobacter* sp. sju-1 was 0.00316 ±0.04 h^{-1} with the BC production of 1.62 ± 0.01 g $100g^{-1}$ of the substrate and 1.30 ± 1.11 g $100g^{-1}$ of cells respectively.

Colour analysis (L*, a*, b*, C*) performed using Hunter colorimeter, clearly confirmed that cellulose from MCW medium was pure white in colour and was light yellowish from CAJ medium, but those produced from the HS medium (control) was light brown in colour. The texture of bacterial cellulose produced from the HS medium was very higher than the other two types of bacterial cellulose obtained from MCW and CAJ medium.

WHC of the celluloses from three medium were 84.44±0.04 per cent, 87.14±0.10 and 88.54±0.02 per cent respectively. The crude fibre content was greater in HS medium of 11.25±0.01 g and MCW and CAJ, medium recorded 10.74±0.01 g and 10.04±0.03 g respectively. Bacterial cellulose from MCW and CAJ medium due to higher water holding capacity and desirable texture was processed into a jelly form called as *nata-de-coco* and *nata-de-cashew*. The sensory evaluation of the developed products showed the overall acceptability score of 9.2 and 9.02 respectively.

GCMS analysis of fermented broth of CAJ medium after extraction of bacterial cellulose revealed the presence of eight volatile compounds dominated by thiophene-2-carboxylic acid and santolin diacetylene (24.8 per cent), 1,5-dihydroxyl-3,3-dimethyl-7-acetylaminomethyl-tricyclododecane (10.75 per cent), 3-phenyl-3-m-tolylpropionamide and 1-(2-hydroxyethylthio)-1-phenyl-2-butanone (12.33 per cent), Nonane and 4-hepatanol (6.53 per cent) and 3-(4-nitrobenzoyl) cinnoline (5.8 per cent).

Vinegar, a value added product was developed from coconut water using the spent liquor of CAJ medium after BC extraction. The spent liquor at the rate of 10 per cent was used as a mother culture for coconut water vinegar production. The resulted vinegar contained 4.8 per cent of acetic acid.

The cost benefit ratio for the production of *nata* was worked out and it was found to be 1.05 and 1.97 for unprocessed *nata* and *nata* in jelly base respectively.

REFERENCES

Alaban, C.A. 1962. Studies on the optimum conditions of *nata-de-coco* bacterium on *nata* formation in coconut water. **Phillip. Agri.,** 45(45): 490-515.

Andelib, Y.A. and D.A. Nuran, 2009. Isolation of cellulose producing bacteria from wastes of vinegar fermentation. *In:* **Proc. of the World Congress on Engineering and Computer Science,** San Francisco, USA. 1st January. pp. 20-22.

*Anonymous, 1993. Annual book of ASTM Standards. section 8, plastics, (ed.) ASTM, Pennsylvania. vol. 8. p. 78.

AOAC. 1990. Official methods of analysis of the Association of Official Analytical Chemists. (14th ed.), Washington, D.C. p. 1094.

Arahal, D. R., Sanchez, E., Marcian, M. C. and E. Garay. 2008. Value of recN sequences for species identification and as a phylogenetic marker within the family Leuconostocaceae". **International Microbiology: The Official Journal of the Spanish Soc. Microbiol.,** 11(1): 33-39.

Arindam, K., Anshu, S., Mainak, M. and B. Rintu. 2011. Process optimization for aqueous extraction of reducing sugar from cashew apple bagasse: A potential, low cost substrate. **Food Sci. Technol.,** 44: 62-66.

Ashley, O. M. 2006. Bacteria may allow recreation of bones. **The Daily Beacon**, Student Newspaper, September 19, University of Tennessee. p. 3.

Bae, S. O. and M. Shoda. 2005. Production of bacterial cellulose by *Acetobacter xylinum* BPR2001 using molasses medium in a jar fermentor. **Appl. Microbiol. Biotechnol.,** 67: 45-51.

Bae, S., Sugano, Y. and M. Shoda. 2004. Improvement of bacterial cellulose production by addition of agar in a jar fermentor. **J. Biosci. Bioeng.,** 91(1): 33-38.

Bar, R., Gainer, J.L. and D. J. Kirwan. 1986. An unusual pattern of product inhibition: batch acetic acid fermentation. **Biotechnol. Bioeng.,** 119: 796-798.

Bas, D. and I. H. Boyaci. 2007. Modelling and optimization I: Usability of response surface methodology. **J. Food Eng.,** 78: 836-845.

Basta, A.H. and H. El-Saied. 2009. Performance of improved bacterial cellulose application in the production of functional paper. **J. Appl. Microbiol.,** 107 (6): 2098-2107.

Bernardo, E.B., Neilan, B.A. and I. Couperwhite. 1998. Characterization, differentiation and identification of wild-type cellulose-synthesizing *Acetobacter* strains involved in *nata-de-coco* Production. **Syst. Appl. Microbiol.,** 21(4): 599-608.

Bhattacharya, S., Gachhui, R. and P. C. Sil. 2011. Changes in thickness and yield of bacterial cellulose during prolonged fermentation of tea broths for a period of 21 days. **Pathophysiol.,** 18: 221-234.

Bielecki, S., Krystynowicz, A., Turkiewicz, M. and H. Kalinowska. 2005. *In:* **Bacterial Cellulose: Polysaccharides and Polyamides in the Food Industry** (eds.) A. Steinbuchel and S.K. Rhee, Wiley-VCH Verlag, Weinheim, Germany. pp. 31-85.

Booranasawettathum, S., Punsri, P. and M. Kitchaicharoen. 1998. Optimum pH of *nata-de-coco* product in grape, apple and cherry juice. *In:* **Proc. of the 15th Rajamangala Institute of Technology Annual Conference: V.3 Agro-Industry and Home Economics,** Rajamangala Institute of Technology, Research and Development Inst., Bangkok, Thailand. pp. 227-232.

*Bourne, M.C. 2002. Food texture and viscosity: concepts and measurements. Academic Press, UK. p. 540.

Brenner, D.J., Krieg, N. R. and J.T. Stanley. 2005. Bergey's Manual of Systematic Bacteriology, Volume 2(c), USA: Springer. pp. 41-99.

Brown, R.M., Willison, J.H.M. and C.L. Richardson. 1976. Cellulose biosynthesis in *Acetobacter xylinum*: Visualization of the site of synthesis and direct measurement of the *in vivo* process. **PNAS.,** 73:4565-4569.

Brummer, P. 1954. The significance of intestinal decomposition products of cellulose on the effect of cellulose laxatives. **Gastroenterologia,** 82:10-14.

Budhiono, A., Rosidi, B., Taher, H. and M. Iguchi. 1999. Kinetic aspects of bacterial cellulose formation in *nata-de-coco* culture system. **Carbohydr. Polym.,** 40: 137-143.

Bungay, H. R., Serafica, G. and R. Mormino. 1997. Environmental implications of microbial cellulose. **Stud. Environ. Sci.,** 66: 691-700.

Carmen, V., Mihail Drogos, S., Luminita, O. and P. Aurel. 2000. The resistance of acetic acid bacteria at the acetic acid. **Natura Montenegrina Podgorica,** 7(3): 109-116.

Carr, J.G. and S.N. Passmore. 1979. Methods for identifying acetic acid bacteria. Identification methods for microbiologists. **Soc. Appl. Bacteriol. Tech. Ser.,** 14: 33.

Carvalho, J.M., Maia, G.A., Figueiredo, R.W., Brito, E.S. and S. Rordrigues. 2007. Development of a blended beverage consisting of coconut water and cashew apple juice containing caffeine. **Int. J. Food Sci. Technol.,** 42: 1195-1200.

Castro, C., Zuluag, R., Putauxc, J. L., Caro, G., Mondragon, I. and P. Ganan. 2011. Structural characterization of bacterial cellulose produced by *Gluconacetobacter swingsii* sp. from Colombian agroindustrial wastes. **Carbohydr. Polym.,** 84: 96-102.

Cavalcante, A.A.M., Rubensam, G., Picada, J.N., Silva, E.G., Moreira, J.C.F. and J.A.P. Henriques. 2003. Mutagenicity, antioxidant potential, and antimutagenic activity against hydrogen peroxide of cashew (*Anacardium occidentale*) apple juice and *cajuina*. **Environ. Mol. Mutagenesis.,** 41: 360-369.

Chao, Y.P., Mitarai, M., Sugano, Y. and M. Shoda. 2001. Effect of addition of water-soluble polysaccharides on bacterial cellulose production in a 50-L airlift reactor. **Biotechnol. Prog.,** 17: 781-785.

Chavez-Pacheco, J.L., Martınez-Yee, S., Contreras, M.L., Gomez-Manzo, S., Membrillo-Hernandez, J. and J.E. Escamilla. 2005. Partial bio-energetic characterization of *Gluconacetobacter xylinum* cells released from cellulose pellicles by a novel methodology**. J. Appl. Microbiol.,** 9: 1130-1140.

Chawla, P., Bajaj, I.B., Survase, S. and R. Singhal. 2009. Fermentative production of microbial cellulose. **Food Technol. Biotechnol.,** 47: 107-124.

Cheng, K. C., Catchmark, J. M. and D. Ali. 2009. Effect of different additives on bacterial cellulose production by *Acetobacter xylinum* and analysis of material property. **Cellul.,** 16: 1033-1045.

Cheng, K. C., Catchmark, J. M. and D. Ali. 2009. Enhanced production of bacterial cellulose by using a biofilm reactor and its material property analysis. **J. Biol. Eng.,** 3: 12.

Chiciudean, T.G. 2011. Production Methods and Characteristics of Bacterial Cellulose Composites. Ph d., Thesis, "POLITEHNICA" University of Bucharest, Faculty of Applied Chemistry and Materials Science, Chemical Engineering Department, Romania. p.154.

Chopra, S.L. and J.S. Kanwar. 1976. Analytical Agricultural Chemistry. (3rd ed.) 12, pp. 332-341.

Christensen, W.B. 1946. Urea decomposition as a means of differentiating proteins and paracolon culture from each other and from *Salmonella and Shigella* types. **J. Bacteriol.**, 52: 461.

Chung, Y. and Y. Shyu. 1999. The effects of pH, salt, heating and freezing on the physical properties of bacterial cellulose-*nata.* **Int. J. Food Sci. Technol.,** 34: 23-26.

Ciechanska, D. 2004. Multifunctional Bacterial Cellulose/Chitosan Composite Materials for Medical Applications. **Fibres. Tex. Eastern. Eur.,** 12 (4(48)): 69-74.

Cleenwerck, I., Camu, N., Engelbeen, K., De Winter, T., Vandemeulebroeke, K., De Vos, P. and L. De Vuyst. 2007. *Acetobacter ghanensis* sp. nov., a novel acetic acid bacterium isolated from traditional heap fermentations of Ghanaian cocoa beans. **Int. J. Sys. Evol. Microbiol.,** 57: 1647-1652.

Clydesdale, F.M. 1978. Calorimetry methodology and applications. **CRC Crit. Rev. Food Sci. Nutri.,** 10: 243 - 302.

Coban, E. P. and H. Biyik. 2011. Effect of various carbon and nitrogen sources on cellulose synthesis by *Acetobacter lovaniensis* HBB5. **Afri. J. Biotechnol.,** 10(27): 5346-5354.

Collins, C.H. and P.M. Lyne. 1970. **Microbiological Methods**. Co-published by Butterworths, London and University park press, Baltimore. p. 314.

Costa, J.M.C., Felipe, F.M.F., Maia, G.J., Hernandez, F.F.F. and I.M. Brasil. 2009. Production and characterization of the cashew apple (*Anacardium occidentale* L.) and guava (*Psidium guajava*) fruit powders. **J. Food Process. Preserv.,** 33: 299-312.

Costa, L. M.M., De Olyveira, G.M., Basmaji, P. and L. X. Filho. 2011. Bacterial Cellulose towards functional green composites materials. **J. Bio. Nano. Sci.,** 5: 167-172.

Costerton, J.W. and K.J. Cheng. 1982. Microbe - microbe interactions at surfaces. *In:* **Experimental Microbial Ecology** (eds.) R.G. Burns and J. H. Slaters, London, Blackwell Scientific Publications, pp. 275-290.

Cummings, J.H., Bingham, S.A., Heaton, K.W. and M.A. Eastwood. 1992. Fecal weight, colon cancer risk and dietary intake of non-starch polysaccharides (dietary fiber). **Gastroenterology,** 103: 1783-1789.

Cvetkovic, D. D. and S. L. Markov. 2002. Cultivation of tea fungus on malt extract medium. **APTEFF,** 33: 1-174.

Dobre, T., Stoica, A., Parvulescu, C., Stroescu, M. and G. Iavorschi. 2008. Factors influence on bacterial cellulose growth in static reactors. **Rev. Chem.,** 59(5): 591-594.

Dubey, V., Saxena, C., Singh, L., Ramana, K.V. and R.S. Chauhan. 2002. Pervaporation of binary water-ethanol mixtures through bacterial cellulose membrane. **Sep. Purif. Technol.,** 27: 163-171.

Dubois, M., Gilles, D.A, Hamilton, J.K., Rebers, P.A. and F. Smith. 1956. Colorimetric method for the determination of sugars and related substances. **Anal. Chem.,** 28**:** 350-356.

Dumadaug, N. T. 2012. Eucheuma pulp extract: Source of *nata* and *nata*-producing microorganisms. **Int. J. Sci. Clin. Lab.,** 2: 82-103.

Dudman, W. F. 1959. Cellulose production by *Acetobacter acetigenum* in defined medium. **J. Gen. Microbiol.,** 21: 327-337.

*Edward, C.A. 1995. The physiological effects of dietary fibre. *In:* **Dietary fibre in health and disease** (eds) D. Kritcheveski and C. Bonfield, Minnesota, Eagen press, pp. 58-72.

Embuscado, M. E., Marks, J. S. and J. N. Be Miller. 1994. Bacterial cellulose. I. Factors affecting the production of cellulose by *Acetobacter xylinum*. **Food Hydrocoll.,** 8: 407-418.

Embuscado, M. E., Marks, J. S. and J. N. Be Miller. 2009. Production of bacterial cellulose by agitated and static culture system. **Carbohydr. Polym.,** 76(2): 333-335.

Embuscasdo, M. E. 1997. Acetic acid production by *Acetobacter xylinum* strain DA. **J. Ferment. Bioeng.,** 84: 228-231.

Faparusi, S.I. 1973. Origin of initial microflora of palm wine from oil palm trees (*Elaeis guineensis*). **J. Appl. Bacteriol.,** 36: 559-565.

Faparusi, S.I. 1974. Microorganisms from oil palm trees (*Elaeis guineensis*) tape holes. **J. Food Sci.,** 39: 755-757.

Faridah, Y. M., Zahra, F. and S. Rahmayani. 2010. The Effect of culture medium on bacterial cellulose production as edible film material. **Sci. Tech.,** 4: 20 -25.

Fiedler, S. Fussel, M. and K. Sattler. 1989. Production and application of bacterial cellulose: I. A survey on state of research and investigations concerning fermentation kinetics. **Zentralbl. Mikrobiol.,** 144: 473-484.

Forng, E. R., Anderson, S. M. and R. E. Cannon. 1989. Synthetic Medium for *Acetobacter xylinum* that can be used for isolation of auxotrophic mutants and study of cellulose biosynthesis. **Appl. Environ. Microbiol.,** 55(3): 1317-1319.

Freeman, H.J, Spiller, G.A. and Kim, Y.S. 1978. A double-blind study on the effect of purified cellulose dietary fiber on 1,2-dimethylhyrazine-induced rat colonic neoplasia. **Cancer Res.,** 38: 2912-2917.

Furda, I. 2001. Chitin and chitosan - a special class of dietary fibre. *In:* Hand book on dietary fibre in human nutrition. (3rd edn.) ed. G. A. Spiller. CRC Press, USA. p. 683.

Gallardo De Jesus, R.M.A. and T.M. Elvira. 1971. A study on the isolation and screening of microorganisms for production of diverse texture *nata*. **Philipp. J. Sci.,** 100(1): 41.

Gao, W. H., Chen, K. F., Yang, R. D., Yang, F. and W. J. Han. 2011. Bacterial cellulose for paper. **Bioresour.,** 6(1): 144-153.

George, J., Ramana, K. V., Sababathy, S. N. and A.S. Bawa. 2005. Physic mechanical properties of chemically treated bacterial (*Acetobacter xylinum*) cellulose membrane. **World J. Microbiol. Biotechnol.,** 21: 323-327.

Gerhardt, P., Murray, R. G., Costelow, R. N., Nexter, E. W., Wood, W. A., Kreig N. P., and G. B. Phelleps. 1981. Manual methods for general bacteriology. **Amer. Soc. Microbiol.,** 23: 400-450.

Gillus, R.P. 1956. An evaluation of two composite media for preliminary identification of *Shigella* and *Salmonella*. **J. Clin. Pathol.,** 9: 368.

Gindl, W. and J. Keckes. 2004. Tensile properties of cellulose acetate butyrate composites reinforced with bacterial cellulose. **Compos. Sci. Technol.,** 64: 2407-2413.

Goh, W.N., Rosma, A., Kaur, B., Fazilah, A., Karim A.A. and R. Bhat. 2012. Fermentation of black tea broth (*Kombucha*): I. Effects of sucrose concentration and fermentation time on the yield of microbial cellulose. **Int. Food Res. J.,** 19(1): 109-117.

Hagiwara, A., Imai, N., Sano, M., Kawabe, M., Tamano, S., Kitamura, S., Omoto, T., Asai, I., Yasuhara, K and S. Hayashi. 2010. A 28-day oral toxicity study of fermentation-derived cellulose, produced by *Acetobacter aceti* sub sp. *xylinum* in F344 rats. **J. Toxicological Sci.,** 35(3): 317-325.

Haigh, W. G., 1973. Induction of orientation of bacterial cellulose microfibrils by a novel terpenoid from *A. xylinum*. **Biochem. J.,** 135: 145-149.

Halib, N., Mohd Amin, M. C. I. and I. Ahmad. 2012. Physicochemical properties and characterization of *nata-de-coco* from local food industries as a source of cellulose. **Sains Malaysiana,** 41(2): 205-211.

*Halim, N. F. 2000. Synthesis of bacterial cellulose by *Acetobacter xylinum* sp. using pineapple pith for biocomposite application. M.Tech., Thesis, Chemical Engineering and Natural Resources, University of Malaysia, Pahang, Malaysia.

Hameed, N. D., Majed H. Al-Gelawi. and H.M. Jasim. 2012. The Role of plasmid in cellulose producing *Gluconacetobacter xylinum.* **J. Plant Mol. Biol. Biotechnol.,** 3(2): 16-20.

Helenius, G., Backdah, H, Bodin, A, Nannmark, U, Gatenholm, P. and B. Risberg. 2006. *In-vivo* biocompatibility of bacterial cellulose. **J. Biomed. Mater. Res.,** 76: 431-438.

Heo, M.S. and H.J. Son. 2002. Development of an optimized, simple chemically defined medium for bacterial cellulose production by *Acetobacter* sp. A9 in shaking culture. **Biotechnol. Appl. Biochem.,** 36: 41-45.

Hermansson, A.M. 1986. Water and fat holding. *In:* **Functional properties of food macromolecules.** (eds.) J.R Mitchell and D.A. Ledward. Elviser Applied Science publications, London, Englandand New York. pp. 101-135.

Hesse, S. and T. Kondo. 2005. Behaviour of cellulose production of *Acetobacter xylinum* in ^{13}C-enriched cultivation media including movements on nematic ordered cellulose templates. **Carbohydr. Polym.,** 60(4):457-465.

Hesse-Ertelt, S., Heinze, T., Togawa, E. and T. Kondo. 2010. Structure elucidation of uniformly ^{13}C-labeled bacterial celluloses from different *Gluconacetobacter xylinus* strains. **Cellul.,** 17:139-151.

Hestrin, S. and M. Schramn. 1954. Synthesis of cellulose by *Acetobacter xylinum*: preparation of freeze dried cells capable of polymerizing glucose to cellulose. **Biochem. J.,** 58: 345-352.

Hillman, L., Peters, S., Fisher, A. and Pomare, E.W. 1983. Differing effects of pectin, cellulose and lignin on stool pH, transit time and weight. **Br. J. Nutr.,** 50: 189-195.

Hiroyuki, F., Hideki, T., Mikiya, K., Takayuki, K., Yasukazu, T., Yoshihiko, K. and S. Takuji. 2009. Continuous ingestion of acetic acid bacteria: Effect on cognitive function in healthy middle - aged and elderly persons. **J. Anti. Aging Med.,** 6(7): 60-65.

Holt, J. G., Krieg, N. R., Sneath, P. H. A., Staley, J. T. and S. T. Williams. 1994. "Bergey's Manual of Determinative Bacteriology, New York, Williams and Wilkins. pp. 267-279.

Hong, F. and K. Qiu. 2007. An alternative carbon source from *konjac* powder for enhancing production of bacterial cellulose in static cultures by a model strain *Acetobacter aceti* subsp. *xylinus* ATCC 23770. **Carbohydr. Polym.,** 72(3): 545 - 549.

Hong-Geun, O.H., Lee, H. K., Seo, M. Y. Kang, Y. R., Kim, J. H., Park, J. W., Kim, O. K., Back, H. I., Kim, S. Y. and S. W. Chae. 2011. Effects of *Ficus carica* paste on constipation induced by a high-protein feed and movement restriction in beagles. **Lab Anim. Res.,** 27(4): 275-281.

Honorato, T.L., Rabelo, M.N., Gonçalves, L.R.B., Pinto, G.A.S. and S. Rodrigues. 2007. Fermentation of cashew apple juice to produce high added value products. **World J. Microbiol. Biotechnol.,** 23: 1409-1415.

Hornung, M., Ludwig, M., Gerrard, A. M. and H.P. Schmauder. 2006a. Optimizing the production of bacterial cellulose in surface culture: Evaluation of substrate mass transfer influences on the bioreaction (Part 1). **Eng. Life Sci.,** 6(6): 537- 545.

Hornung, M., Ludwig, M., Gerrard, A. M. and H.P. Schmauder. 2006b. Optimizing the production of bacterial cellulose in surface culture: Evaluation of product Movement influences on the bioreaction (Part 2). **Eng. Life Sci.,** 6(6): 546-551.

Hsieh, Y.C., Yano, H. and M. Nogi. 2008. An estimation of the Young's modulus of bacterial cellulose filaments. **Cellul.,** 15: 507-513.

Hungund, B. S. and S. G. Gupta. 2010a. Factors affecting production of cellulose from *Gluconacetobacter xylinus.* **Asian J. Microbiol. Biotech. Env. Sci.,** 12 (3): 517-522.

Hungund, B. S. and S. G. Gupta. 2010b. Strain improvement of *Gluconacetobacter xylinus* NCIM 2526 for bacterial cellulose production. **Afri. J. Biotechnol.,** 9(32): 5170-5172.

Hutchens, S.A., Leon, R.V., O'Neill, H.M. and B.R. Evans. 2007. Statistical analysis of optimal culture conditions for *Gluconacetobacter hansenii* cellulose production. **Lett. Appl. Microbiol.,** 44:175-180.

Hwan, Y.S., Lee, O.S., Lee, I.S., Kim, H.S., Yu, T.S. and Y.J. Jeong. 2004. *Gluconacetobacter persimmonis* sp. nov., isolated from Korean traditional persimmon vinegar. **J. Microbiol. Biotechnol.,** 14: 276-283.

Ibrahim, A.D., Musa, K., Sani, A., Aliero, A.A. and B.S. Yusuf. 2011. Microorganisms associated with the production of volatile compounds in spoilt tomatoes. **Biotechnol. Res.,** 2(2): 82-89.

Iguchi, M., Yamanaka, S. and A. Budhiono. 2000. Bacterial cellulose – a masterpiece of nature's arts. **J. Mater. Sci.,** 35: 261-270.

International Committee on Systematic Bacteriology. 1998. Validation number. 65. **Int. J. Syst. Evol. Microbiol.,** 48: 365-368.

Ishida, T., Mitarai, M. and Y. Sugano. 2003. Role of water soluble polysaccharides in bacterial cellulose production. **Biotechnol. Bioeng.,** 83(4): 474-478.

Ishihara, M., Matsunaga, M., Hayashi, N. and V. Tiller. 2002. Utilization of D-xylose as carbon source for production of bacterial cellulose. **Enzyme Microbiol. Technol.,** 31: 986-991.

Ishikawa, A., Matsuoka, M., Tsuchida, T. and F. Yoshinaga. 1995. Increase in cellulose production by sulfaguanidine-resistant mutants derived from *Acetobacter xylinum* subsp. *sucrofermentans*. **Biosci. Biotechnol. Biochem.,** 59(12): 2259-2262.

Iwata, T., Indrarti, L. and J. Azuma. 1998. Affinity of hemicelluloses for cellulose produced by *Acetobacter xylinum*. **Cellul.,** 5: 215-228.

Iyer, P. R., Geib, S. M., Catchmark, J., Kao, T. H. and M. Tien. 2010. Genome sequence of a cellulose-producing bacterium, *Gluconacetobacter hansenii* ATCC 23769. **J. Bacteriol.,** 192: 4256-4257.

Jagannath, A., Kalaiselvan, A., Manjunatha, S.S., Raju, P.S. and A.S. Bawa. 2008. The effect of pH, sucrose and ammonium sulphate concentrations on the production of bacterial cellulose (*nata-de-coco*) by *Acetobacter xylinum*. **World J. Microbiol. Biotechnol.,** 24: 2593-2599.

Jahan, F., Kumar, V., Rawat, G. and R. K. Saxena. 2012. Production of microbial cellulose by a bacterium isolated from fruit. **Appl Biochem. Biotechnol.,** 167(5): 1157-1171.

Jayabalan, R., Subathradevi, P., Marimuthu, S., Satiskumar, M. and K. Swaminathan. 2008. Changes in free radical scavenging ability of *kombucha* tea during fermentation. **Food Chem.,** 109: 227-234.

Johansson, N.G., Hallmer, H. and M. J. Siljestrom. 1983. Dietary fibre agriculture. Food Chem., 3: 476.

Johari, N. S., Ahmad, I. and N. Halib. 2012. Comparison study of hydrogels properties synthesized with micro and nano size bacterial cellulose particles extracted from *nata-de-coco.* **Chem. Biochem. Eng.,** 26 (4): 399-404.

Johnson, D. C. and A. R. Winslow. 1990. Bacterial cellulose has potential application as new paper coating. **Pulp and Paper,** 25: 105-107.

Jonas, R. and L. F. Farah. 1997. Production and application of microbial cellulose. **Polym. Degrad. Stab.,** 59: 101-106.

Joyeux, A., Lafon-Lafourcade, S. and P. Ribereau-Gayon. 1984. "Evolution of acetic acid bacteria during fermentation and storage of wine," **Appl. Environ. Microbiol.,** 48: 153-156.

Jozef, D.L., Monique, G. and S. Jean. 1986. Bergey's Manual of Systematic Bacteriology, Williams and Wilkins, USA. pp.267-274.

Jung, H. O., Lee, O.M, Jeong, J. H, Jeon, Y. D, Park, K.H, Kim, H. S., An, W. G. and H. J. Son. 2010. Production and characterization of cellulose by *Acetobacter sp*. V6 using a cost-effective molasses-corn steep liquor medium. **Appl. Biochem. Biotechnol.,** 162: 486-497.

Junsangsree, P., Srikamsukh, P. and W. Pankhaw. 2002. Substitution of coconut juice by pineapple juice in *nata-de-pina* production. **J. Agric.,** 18(1): 46-55.

Kakino, M., Tazawa, S., Maruyama, H., Tsuruma, K., Araki, Y., Shimazawa, M., Hara, H. and Kakino. 2010. Laxative effects of agarwood on low-fiber diet-induced constipation in rats. **BMC Comp. Alt. Med.,** 10: 68.

Kamarudin, S., Sahaid, M., Sobri, K. M., Mohtar, T. W., Radiah, Y. D. and H. Norhasliza. 2013. Different media formulation on biocellulose production by *Acetobacter xylinum* (0416). **Pertanika. J. Sci. Technol.,** 21(1): 29-36.

Kamel, S., Ali, N., Jahangir, K., Shah, S. M. and A. A. El-Gendy. 2008. Pharmaceutical significance of cellulose: A review. **Polym. Lett.,** 2(11): 758-778.

Kanchanarh, W., Theera gool, G., Inque, T., Yakushi, T., Adachi, O. and K. Matsushita. 2010. Acetic acid fermentation of *Acetbacter pasteurianus*: Relationship between acetic acid resistance and pellicle polysaccharide formation. **Biosci. Biotechnol. Biochem.,** 74(8): 1591-1597.

Karahan, A.G., Akoglu, A, Cakir, I., Kart, A., Cakmakci, M. L., Uygun, A. and F. Goktepe. 2011. Some properties of bacterial cellulose produced by new native strain *Gluconacetobacter sp*. A06O2 obtained from Turkish vinegar. **J. Appl. Polym. Sci.,** 121(3): 1823-1831.

Katikaneni, S. P. R. and M. Cheryan. 2002. Purification of fermentation-derived acetic acid by liquid-liquid extraction and esterification. **Ind. Eng. Chem. Res.,** 41: 2745-2752.

Kayashita, J., Shimaoka, I., Yamazaki, M. and N.Kato. 1995. Buckwheat protein extract ameliorates atropine-induced constipation in rats. **Curr. Adv. Buckwheat Res.,** 19: 941 - 946.

Kersters, K., Lisdiyanti, P., Komagata, K. and J. Swings. 2006. The family Acetobacteraceae: the genera *Acetobacter*, *Acidomonas*, *Asaia*, *Gluconacetobacter*, *Gluconobacter* and *Kozakia*. *In:* **The Prokaryotes**, Springer, New York, pp. 163-200.

Keshk, S. and K. Sameshima. 2006. The utilization of sugar cane molasses with/without the presence of lignosulfonate for the production of bacterial cellulose. **Appl. Microbiol. Biotechnol.,** 72(2): 291-296.

Keshk, S. and K. Sameshima. 2005. Evaluation of different carbon sources for bacterial cellulose production. **Afri. J. Biotechnol.,** 4:478-482.

Keshk, S., Taha, M., Razek, A. and K. Sameshima. 2006. Bacterial cellulose production from beet molasses. **Afri J. Biotechnol.,** 5(17): 1519-1523.

Kim, J. H., Park, S. C., Kim, J. Y. and Y. K. Park. 2009. The effect of probiotics on constipation in rats. **Yeungnam Univ. J. Med.,** 26 (2): 102-107.

Kim, J. Y., Kim, J.N., Wee, Y.J., Park, D.H. and H.W. Ryu. 2007. Bacterial cellulose production by *Gluconacetobacter* sp. RKY5 in a rotary biofilm contactor. **Appl. Biochem. Biotechnol.,** 137: 529-537.

Klemm, D., Shumann, D., Udhardt, U. and S. Marsch. 2001. Bacterial synthesized cellulose-Artificial blood vessels for microsurgery. **Prog. Polym. Sci.,** 26(9): 1561-1603.

Kocher, G. S., Kalra, K. L. and R. P. Phutela. 2006. Comparative production of sugarcane vinegar by different immobilization techniques. **J. Inst. Brew.,** 112: 264-266.

Kojima, Y., Tonouchi, N., Tsuchida, T., Yoshinaga, F. and Y. Yamada. 1998. The characterization of acetic acid bacteria efficiently producing bacterial cellulose from sucrose: the proposal of *Acetobacter xylinum* subsp. *nonacetoxidans* subsp. nov. **Biosci. Biotechnol Biochem.,** 62(1): 185-187.

Kongruang, S. 2008. Bacterial Cellulose Production by *Acetobacter xylinum* strains from agricultural waste Products. **Appl. Biochem. Biotechnol.,** 148: 245-256.

Kouda, T., Yano, H. and F. Yoshinaga. 1997. Effect of agitator configuration on bacterial cellulose productivity in aerated and agitated culture. **J. Ferment. Bioeng.,** 83: 371-376.

Krystynowicz, A., Koziolkiewicz, M., Jezierska, A. J., Bielecki, S., Klemenska, E., Masny, A. and A. Płucienniczak. 2005. Molecular basis of cellulose biosynthesis disappearance in submerged culture of *Acetobacter xylinum*. **Acta Biochem. Polonica.,** 52(3): 691- 698.

Kubo, I., Masuoka, N., Ha, T.J. and K. Tsujimoto. 2006. Antioxidant activity of anacardic acids. **Food Chem.,** 99: 555-562.

Kulkarni, P.K., Anil Dixit, S. and U.B. Singh. 2012. Evaluation of bacterial cellulose produced from *Acetobacter xylinum* as pharmaceutical expient. **Amer. J. Drug. Dis. Dev.,** 2(2): 72-86.

Kumalaningsih, S., Wignyanto, H.Y.S. and A.D. Wardanu. 2011. An innovative natural dietary fibre (*nata*) product made from solid waste of pineapple concentrate industry using *Acetobacter xylinum In:* **Proc. of 12th ASEAN food conference**, 16 -18 June, BITEC Bangna, Bangkok, Thailand.

Kurosumi, A., Sasaki, C., Yamashita, Y. and Y. Nakamura. 2009. Utilization of various fruit juices as carbon source for production of bacterial cellulose by *Acetobacter xylinum* NBRC 13693. **Carbohydr. Polym.,** 76(2): 333-335.

Kus, M. and D.A. Sri Retno. 2012. *Kimia Sehari-hari.* **Surakarta**: UNS Press. p.970.

Lam, D. M., Nguyen, T. T. V. and D.T.K. Nhung. 2013. Isolation, selection and identification of bacterial strain BHN2 producing bacterial cellulose. **J. Biol.,** 35(1): 85-89.

Lapuz, M.M., Gallardo, E.G. and M. A. Palo. 1967. The *nata* organism cultural requirements. characteristics and identity. **Philipp. J. Sci.,** 96(2): 91-108.

Lee, H.C. 1999. Reduced production of microbial cellulose caused by aggregation of *Acetobacter xylinum* under shaking culture conditions – observation by scanning electron microscope. **Appl. Chem.,** 3(2): 92- 95.

Lee, H.J. and E.H. Hwang. 1997. Effects of alginic acid, cellulose and pectin level on bowel function in rats. **J. Nutr.,** 30(5): 465-477.

Lee, K.J. and P.L. Rogers. 1983. The fermentation kinetics of ethanol production by *Zymomonas mobilis*. **J. Chem. Eng.,** 27: 31-38.

Lee, S.I. 2002. Treatment of constipation. **Kor. J. Gastroenterol.,** 40: 37-47.

Lin, J.H., Kuo, J. C., Lin, Y.J., Chen, T. Y. and W. P. Sung. 2011. Production of bacterial cellulose by *Gluconacetobacter xylinus* using Taguchi methods. **Appl. Mech. Mater.,** 44-47: 605-609.

Lowor, S. T. and C. K. Agyente-Badu. 2009. Mineral and proximate composition of cashew apple (*Anacardium occidentale*) juice from Northern Savannah, forest and coastal Savannah regions in Ghana. **Amer. J. Food. Technol.,** 4(4): 154-161.

Lucia, I. and R. Yudianti. 2008. Effect of water soluble polymer on structure and mechanical properties of bacterial cellulose composites. **J. Appl. Sci.,** 8: 177-180.

Lynd, l. R., Weimer, P. J., Willem, H. and V. Zyl. 2002. Microbial cellulose utilisation. Fundamentals and biotechnology. **Microbiol. Mol. Biol.,** 66: 506-577.

Lysenko, O. 1961. *Pseudomonas* -an attempt of general classification. **J. Gen. Microbiol.,** 25: 379-383.

Maal, B.K. and R. Shafiee. 2009. Isolation and identification of an *Acetobacter* strain from Iranian white-red cherry with high acetic acid productivity as a potential strain for cherry vinegar production in food and agriculture biotechnology. **World. Acad. Sci. Eng. Technol.,** 30: 201-204.

Maal, B. K. and R. Shafie. 2010. Characterization of an *Acetobacter* strain isolated from Iranian peach that tolerates high temperatures and ethanol concentrations. **World. Acad. Sci. Eng. Technol.,** 62: 141 -145.

Makoto, S. and S. Yasushi. 2005. Recent advances in bacterial cellulose production. **Biotech. Bioprocess Eng.,** 10:1-8.

Marci, G., Mele, G., Palmisano, L., Pulito, P. and A. Sannino. 2006. Environmentally sustainable production of cellulose-based superabsorbent hydrogels. **Green Chem.,** 8 (5): 439-444.

Marzieh, M. N., Yousefi, A. R., Askari, H. and M. Bakhtiyari. 2010. Fermentative production and characterization of carboxy methyl bacterial cellulose sing Date Syrup. **World. Acad. Sci. Eng.Technol.,** 68: 1467-1470.

Masaoka, S., Ohe, T. and N. Sakota. 1993. Production of cellulose from glucose by *Acetobacter xylinum*. **J. Ferment. Bioeng.,** 75: 18-22.

Matsui, K.N., Granado, L.M., Oliveira, P., De, P. and C. C. Tadini. 2005. Peroxidase and polyphenol oxidase thermal inactivation by microwaves. **Food Sci. Tech.,** 40: 852-859.

Matsuoka, M., Tsuchida, T. Matsushita, K., Adachi, O., and F. Yoshinaga. 1996. A synthetic medium for bacterial cellulose production by *Acetobacter xylinum* subsp. *sucrofermentans*. **Biosci. Biotechnol. Biochem.,** 60: 575-579.

Matsushita, K., Inoue, T., Theeragool, G., Trcek, J., Toyama, H. and O. Adachi. 2005. Survival and death of bacteria. Research sign post, Kerala, pp. 169-181.

McKenna, B. A.., Mikkelsen, D., Wehr, J. B., Gidley, M. J. and N.W. Menzies. 2009. Mechanical and structural properties of native and alkali treated bacterial cellulose produced by *Gluconacetobacter xylinum* strain ATCC 53524. **Cellul.,** 16: 1047-1055.

Melody, S.C. 1997. Plant Molecular Biology - A laboratory manual. Springer-Verlag, New-York. p. 340.

Mesomya, W., Cuptapun, Y., Hengsawadi, D, Tangkanakul, P., Boonvisut, S. and S. Poosimuang. 2002. Protein bioavailability - lowering in rats fed high dietary Fibre from cereal and *nata-de-coco*. **Kasetsart J. Nat. Sci.,** 35: 66 - 73.

Mesomya, W., Pakpeankitvatana, V., Komindr, S., Leelahakul, P., Cuptapun, Y., Hengsawadi, D., Tammarate, P. and P. Tangkanakul. 2006. Effects of health food from cereal and *nata de coco* on serum lipids in human. **J. Sci. Technol.,** 28(1): 24-28.

Mikkelsen, D., Gidley, M. J. and B.A. Williams. 2011. *In-vitro* fermentation of bacterial cellulose composites as model dietary fibers. **J. Agric. Food Chem.,** 59(8): 4025-4032.

*Mina, L.A. and C. A. Penalosa. 1994. Effects of different additives on the texture of *nata de coco*. *In:* **YRAP [Youth Research Apprenticeship Program]'94 research reports**, gamma sigma delta honour society of agriculture, UPLB Chapter, Laguna, Philippines, pp. 399-425.

Mini, C, Mathew, J. and A. Augustin. 2005. Technologies for cashew apple processing. Directorate of Extension, Kerala Agricultural University, Mannuthy, Trissur, India. p. 55.

*Montgomery, R.R. 1982. Polymers. *In:* **Toxicology**. Vol. II, Part C (eds.) G.D. Clayton and F.E. Clayton. 3rd edn. John Wiley and Sons, Inc., New York, USA.pp.37-54.

Moon, S. H, Park, J. M, Chun, H.Y. and S. J. Kim. 2006. Comparisons of physical properties of bacterial celluloses produced in different culture conditions using saccharified food wastes. **Biotechnol. Bioprocess Eng.,** 11: 26-31.

Moonmangmee, S., Kawabata, K., Tanaka, S., Toyama, H., Adachi, O. and K. Matsushita. 2002. A novel polysaccharide involved in the pellicle formation of *Acetobacter aceti*. **J. Biosci. Bioeng.** 93: 192-200.

Moonmangmee, S., Toyama, H., Adachi, O., Theeragool, G., Lotong, N. and K. Matsushita. 2002. Purification and characterization of a novel polysaccharide involved in the pellicle produced by thermotolerant *Acetobacter* strains. **Biosci. Biotechnol. Biochem.,** 66: 777-783.

Nadia, H., Amin, M.C.I.M. and I. Ahmad. 2012. Physicochemical properties and characterization of *nata-de-coco* from local food industries as a source of cellulose. **Sains Malaysiana,** 41(2): 205-211.

Naritomi, T., Kouda, T., Yano, H. and F. Yoshinaga. 2002. Influence of broth exchange ratio on bacterial cellulose production by repeated-batch culture. **Process Biochem.,** 38: 41-47.

Navarro, R. R. and K. Komagata. 1999. Differentiation of *Gluconacetobacter liquefaciens* and *Gluconacetobacter xylinus* on the basis of DNA base composition, DNA relatedness and oxidation products from glucose. **J. Gen. Appl. Microbiol.,** 45: 7-15.

Nguyen, V.T., Flanagan, B., Michael J. G. and G. A. Dykes. 2008. Characterization of cellulose production by a *Gluconacetobacter xylinus* strain from *Kombucha*. **Curr. Microbiol.,** 57: 449-453.

Nishi, Y., Uryu, M. and S. Yamanaka. 1990. The structure and mechanical-properties of sheets prepared from bacterial cellulose and Improvement of the mechanical-properties of sheets and their applicability to diaphragms of electro-acoustic transducers. **J. Mater. Sci.,** 25: 2997-3001.

Nuruddin, M., Chowdhury, A., Haque, S. A., Rahman, M. S., Farhad, F., Jahan, M. S. and A. Quaiyyum. 2011. Extraction and characterization of cellulose microfibrils from agricultural wastes in an integrated biorefinery initiative. **Cellul. Chem. Technol.,** 45 (5-6): 347-354.

Ochaikul, D., Rukchonlatee, S., Naranong, N. and P. Pongsakul. 2000. Effect of chitosan on paper production from bacterial cellulose and its properties. **Process Chem.,** 24(7): 42-58.

Ochaikul, D., Chotirittikrai, K.C. and S. Wutigornsombatkul. 2006. **S**tudies on the fermentation of *Monascus purpureus* TISTR 3090 with bacterial cellulose from **KMITL Sci. Tech. J.,** 6 (1): 113-119.

Ogino, H., Azuma, Y., Hosoyama, A., Nakazawa, H., Matsutani, M., Hasegawa, A., Otsuyama, K., Matsushita, K., Fujita, N. and M. Shirai. 2011. Complete Genome Sequence of NBRC 3288, a unique cellulose-nonproducing Strain of *Gluconacetobacter xylinus* isolated from vinegar. **J. Bacteriol.,** 193(24): 6997-6998.

Omojola, A.B. and A.O.K. Adesehinwa. 2007. Performance and carcass characteristics of broiler chickens fed diets supplemented with graded levels of Roxazyme G®. **Int. J. Poult. Sci.,** 6: 335-339.

Panesar, P.S. Chavan, Y.V. Bera, M.B. Chand, O. and H. Kumar. 2009. Evaluation of *Acetobacter* Strain for the Production of Microbial Cellulose. **Asian J. Chem.,** 21 (10): 99-102.

Panse, V. G. and P.V. Sukhatme. 1976. **Statistical methods for agricultural workers.** Indian Council of Agricultural Research, New Delhi, p.180.

Park, J. K., Park, Y. H. and J. Y. Jung. 2003a. Production of bacterial cellulose by *Gluconacetobacter hansenii* PJK isolated from rotten apple. **Biotechnol. Bioprocess Eng.,** 8(2): 83-88.

Park, J.K., Jung, J.Y. and Y.H. Park. 2003b. Cellulose production by *Gluconacetobacter hansenii* in a medium containing ethanol. **Biotechnol. Lett.,** 25: 2055-2059.

Pinto, T. M.S., Neves, A. N. C., Leao, M. V.P. and A.O.C. Jorge. 2008. Vinegar as an anti microbial agent for the control of Candida spp. In complete dental wearers. **J. Appl. Oral Sci.,** 16(6): 385-390.

Poblet, M., Rozes, N., Guillamon, J. M. and A. Mas. 2000. Identification of acetic acid bacteria by restriction fragment length polymorphism analysis of the PCR amplified fragment of the gene coding for 16SrRNA. **Lett. Appl. Microbiol.,** 31: 63- 67.

Pokalwar, S. U., Mishra, M. K. and A. V. Manwar. 2010. Production of cellulose by *Gluconacetobacter* sp. **Rec. Res. Sci. Technol**., 2(7): 14-19.

Pokalwar, S. U., Mishra, M. K. and A. V. Manwar. 2011. Production and characterisation of bacterial cellulose from *Gluconacetobacter intermedius* isolated from Indian grapes. **Bionano. Front.,** 4(1): 23-27.

Pourramezan, G.Z, Roayaei, A.M. and Q.R. Qezelbash. 2009. Optimization of culture conditions for bacterial cellulose production by *Acetobacter* sp. 4B-2. **Biotechnol.,** 8: 150-154.

Prades, A., Dornier, M., Diop, M. and J. P. Pajn. 2012. Coconut water uses, composition and properties: a review. **Fruits,** 67: 87- 107.

Premjet, S., Ohtani, Y. and K. Sameshima. 1994. The contribution of high molecular lignosulfonate to the powerful bacterial cellulose. **Seni Gakkaishi,** 50: 458-463.

Premjet, S., Premjet, D. and Y. Ohtani. 2007. The effect of ingredients of sugar cane molasses on bacterial cellulose production by *Acetobacter xylinum* ATCC 10245. **Seni Gakkaishi,** 63: 193-199.

Premjet, S., Srisawat, C. and D. Premjet. 2012. Enhanced cellulose production by ultraviolet (UV) irradiation and N-methyl-N'-nitro-N-nitrosoguanidine (NTG) mutagenesis of an *Acetobacter* species isolate. **Afri. J. Biotechnol.,** 11(6): 1433-1442.

Radiman, C. and G. Yuliani. 2008. Coconut water as a potential resource for cellulose acetate membrane preparation. **Polymr. Int.,** 53(3): 502-508.

Radiman, C. L. and A. Rifathin, 2013. Preparation of phosphorylated *nata-de-coco* for polymer electrolyte membrane applications. **J. Appl. Polym. Sci.,** 10: 180-190.

Rahayu, S. E., Basrah, A. E. and A. Budhiono. 1994. Bacterial cellulose: prospects of its industries in developing countries. *In:* **Proc. of International Workshop on Green Polymers.** Institute for Research and Development of Agro-Based Industry (IRDABI), 4 - 6 November, Bandilllg-Bogor, Indonesia.

Ramana, K., Tomar, A. and L. Singh. 2000. Effect of various carbon and nitrogen sources on cellulose synthesis by *Acetobacter xylinum*. **World. J. Microbiol. Biotechnol.,** 16(3): 245-248.

Richter, E. M., De Jesus, D. P., Munoz, R. A. A., Lago, C.L.D. and L. Angnes. 2005. Determination of anions, cations and sugars in coconut water by capillary electrophoresis. **J. Braz. Chem.,** 16: 1134 - 1139.

Rocha, M. V. P., Rodrigues, T. H. S., Macedo, G. R. D. and L. R. B. Gonçalves. 2009. Enzymatic hydrolysis and fermentation of pre-treated cashew apple bagasse with alkali and diluted sulfuric acid for bioethanol production. **Appl. Biochem. Biotechnol.,** 155: 104-114.

Ross, P., Mayer, R. and M. Benziman. 1991. Cellulose Biosynthesis and Function in Bacteria, **Microbiol. Rev., 55(1):** 35 - 58.

Sambrook, J., Fritsch, E.F. and J. Maniatis. 1989. Molecular cloning - A laboratory manual. (2nd ed.), Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, USA.

Santos, R.J., Batista, R.A., Filho, L.X. and A.S. Lima. 2009. Antimicrobial activity of broth fermented with *Kombucha* colonies. **J. Microbial. Biochem. Technol.,** 1: 72-78.

Santosa, B., Ahmadi, K. G. S. and D. Taeque. 2012. Dextrin concentration and Carboxy Methyl Cellulose (CMC) in making of fiber-rich instant beverage from *nata-de-coco.* **Int. J. Sci.Technol.,** 1(1): 6-11.

Saputra, A. H. and Darmansyah. 2010. Evaluation of physical and mechanical properties composite of *nata-de-coco* fibers/resin filled SiO_2, and Al_2O_3. *In:* **Proc. of the 1st International seminar on fundamental and application of chemical engineering**. Nov' 3-4, Bali-Indonesia.

Schmitt, D.F., Frankos, V.H and J. Westland. 1991. Toxicologic evaluation of Cellulon™ fiber: genotoxicity, pyrogenicity, acute and sub-chronic toxicity. **J. Amer. Toxicol.,** 10: 541-542.

Seifert, M., Hesse, S., Kabrelian, V. and D. Klemm. 2003. Controlling the water content of never dried and reswollen bacterial cellulose by the addition of water-soluble polymers to the culture medium. **J. Polym. Sci: Part A: Polym Chem.,** 42: 463-470.

Setyawaty, R., Hirayama, K. K., Kaneko, H. and Hirayama, K. 2011. Current tapioca starch waste water management in Indonesia. **World Appl. Sci. J.,** 14(5): 658-665.

Shah, J. and R.M. Brown. 2005. Towards electronic paper displays made form microbial cellulose. **Appl. Microbiol. Biotechnol.,** 66: 352-355.

Shalini, Y. 2010. Economics of cashew in India. National Bank for Agriculture and Rural Development, Department of Economic analysis and Research, Bandra (East) Mumbai. pp. 1-97.

Shanshan, G., Wang, J. and J. Zhengwei. 2012. Preparation of cellulose films from solution of bacterial cellulose in NMMO. **Carbohyd. Polym.,** 87: 1020-1025.

Shantz, E. M. and F.C. Steward. 1952. Coconut milk factor: the growth promoting substances in coconut milk. **J. Amer. Chem. Soc.,** 74: 6133-6135.

Sheu, F., Wang, C.L. and Y.T. Shyu. 2001. Fermentation of *Monascus purpureus* on bacterial cellulose-*nata* and the color stability of *Monascus-nata* complex. **J. Food Sci.,** 65(2): 342-345.

Shivakumar, S. and S. V. N. Vijayendra. 2006. Production of exopolysaccharides by *Agrobacterium* sp. CFR-24 using coconut water - a byproduct of food industry. **Lett. Appl. Microbiol.,** 42(5): 477-482.

Shoda, M. and Y. Sugano. 2005. Recent advances in bacterial cellulose production. **Biotechnol. Bioprocess. Eng.,** 10: 1-8.

Sievers, M. and J. Swings. 2005. Genus VIII *Gluconacetobacter*. *In:* **Bergeys manual of Systematic Bacteriology (2nd edn, Vol. 2 Part C)** (eds.) D. J. Brenner.,

N.R. Krieg. and J. T. Stanley, Springer Science and Business Media Inc., New York, pp.72-77.

Simmons, J.S. 1976. A culture method for differentiating organisms of typhoid colour aerogenes group and for isolation of certain fungi. **J. Infect. Dis.,** 39-209.

Sivagurunathan, P., Sivasankari, S. and S. M. Muthukkaruppan. 2010. Characterisation of cashew apple (*Anacardium occidentale* L.) fruits collected from Ariyalur District. **J. Biosci. Res.,** 1(2): 101-107.

Slusarska, B.S., Presler, S. and D. Danielewicz. 2008. Characteristics of bacterial ellulose obtained from *Acetobacter xylinum* culture for application in papermaking. **Fibres Tex. Eastern Eur.,** 16(4): 108-111.

Smibert, R.M. and N.R. Kreig. 1981. General characterization. *In:* **Methodology for General Bacteriology.** (ed.) P. Gerardt, Academic Publishers, New York. pp. 400-450.

Smith, B. 1996. Fundamentals of Fourier Transform Infrared Spectroscopy. CRC Press, Washington, D.C.p.550

Soheir, S. A. S. 2012. 16S rRNA Gene sequence detection of acetic acid bacteria isolated from tea *Kombucha*. **New York Sci. J.,** 5(3): 55-61.

Sokollek, S. J., Hertel, C. and W. P. Hammes. 1998. "Description of *Acetobacter oboediens* sp. nov., and *Acetobacter pomorum* sp. nov., two new species isolated from industrial vinegar fermentations", **Int. J. Syst. Bacteriol.,** 48: 935-940.

Sokolnicki, A. M., Fisher, R. J., Harrah, T. P. and D. L. Kaplan. 2006. Permeability of bacterial cellulose membranes. **J. Membr. Sci.,** 272: 15-27.

Son, C., Chung, S., Lee, J. and S. Kim. 2002. Isolation and cultivation characteristics of *Acetobacter xylinum* KJ-1 producing bacterial cellulose in shaking cultures. **J. Microbiol. Biotechnol.,** 12: 722-728.

Son, H.J., Heo, M.S., Kim, Y.G. and S.J. Lee. 2001. Optimization of fermentation conditions for the production of bacterial cellulose by a newly isolated *Acetobacter* sp. A9 in shaking cultures. **Biotechnol. Appl. Biochem.,** 33: 1-5.

Sousa, R. A., Borges, N., Poppi, W., Baccan, N. and S. Cadore. 2006. Classificaco de agua de coco processada e natural por meio de HCA, PCA eteores de íons metalicos determinados por ICP OES. **Quimica Nova.,** 29: 654-656.

Sowden, L.C. and J.R. Colvin. 1978. Morphology, microstructure and development of colonies of *Acetobacter xylinum*. **Can. J. Microbiol**., 24: 772-779.

Stasiak, L. and S. Blazejak. 2009. Acetic acid bacteria – perspectives of application in biotechnology – a review. **Pol. J. Food Nutr. Sci.,** 59(1): 17-23.

Supakod, S. and A. Wongwicharn. 2012. Cheap media for inoculum preparation of acetic acid bacteria. **Adv. Mater. Res.,** 506: 575-580.

Sutanto, A. 2012. Pineapple liquid waste as *nata de pina* raw material. **Makara, Teknologi.,** 16(1): 63-67.

Suwannapinunt, N., Burakorn, J. and S. Thaenthanee. 2007. Effect of culture conditions on bacterial cellulose production from *Acetobacter xylinum* TISTR 976 and physical properties of BC parchment paper. **J. Sci. Technol.,** 14(4): 357- 365.

Suwanposri, A., Yukphan, P., Yamada, Y. and D. Ochaikul. 2013. Identification and biocellulose production of *Gluconacetobacter* strains isolated from tropical fruits in Thailand. **Maejo Int. J. Sci. Technol.,** 7(1): 70-82.

Swings, J. 1992. The genera *Acetobacter and Gluconobacter. In:* **The Prokaryotes**, 2nd edn., Vol. III (eds.) T. Balows., H. Dworkin and S. Schleifer, Springer-Verlag, New York, pp. 2268-2286.

Tabuchi, M. and Y. Baba. 2005. Design for DNA separation medium using bacterial cellulose fibrils. **Anal. Chem.,** 77: 7090-7093.

Tabuchi, M., Kobayashi, K. and Fugimoto. 2005. Biosensing on a chip with compact discs and nanofibres with compact discs and nanofibres. **Lab Chip.,** 5: 1412-1415.

Tahir, Z., Siddique, F. and U. Farooq. 1999. Isolation and charecterisation of vinegar culture (*Acetobacter aceti*) from indegenious sources. **J. Biosci. Bioeng.,** 88(2): 183-188.

Tamura H. and S. Tokura. 2004. Participation of tri-carbon compounds on the biosynthesis of bacterial cellulose by *Acetobacter xylinus*. **Book of Abstracts, 227th ACS National Meeting,** March 28 - April 1, Anaheim, USA.

Tamura, K., Dudley, J., Nei, M. and S. Kumar. 2007. "MEGA5: Molecular evolutionary genetics analysis (MEGA) software version 5.0". **Mol. Biol. Evol.,** 24: 1596-1599.

TAPPI, 1993. TAPPI test method. T460 om-96, T462 om-93. Atlanta.

TAPPI, 2005. T230 om-89, viscosity of pulp (capillary viscometer method). TAPPI, Atlanta.

Tayama, K., Minakami, H., Entani, E., Fujiyama, S. and H. Masai. 1985. Structure of an acidic polysaccharide from *Acetobacter* sp. NBI 102. **Agric. Biol. Chem.,** 49: 959-966.

Teoh, A. L., Heard, G. and J. Cox. 2004. " Yeast ecology of *kombucha* fermentation." **Int. J. Food Microbiol.,** 95(2): 119-126.

Thompson, D. N. and M.A. Hamilton, 2001. Production of bacteria from alternate feedstocks. **Appl. Biochem. Biotechnol.,** 91: 503-513.

Tigressa, H. S. R., Gustavo, A. S. P. and R. B. G. Luciana. 2008. Effects of inoculum concentration, temperature and carbon sources on tannase production during solid state fermentation of cashew apple bagasse. **Biotechnol. Bioprocess Eng.,** 13: 571-576.

Toda, K., Asakura, T., Fukaya, M., Entani, E. and Y. Kawamura.1997. Cellulose Production by acetic acid-resistant *Acetobacter xylinum.* **J. Ferment. Bioeng.,** 84(3): 228-231.

Tokoh, C., Takabe, E., Fujita, M. and H. Saiki. 1998. Cellulose synthesized by *Acetobacter xylinum* in the presence of acetyl glucomannan. **Cellul., 5:** 249-261

Ton, N.M.N. and V.V.M. Le. 2011. Application of immobilized yeast in bacterial cellulose to the repeated batch fermentation in wine-making. **Int. Food Res. J.,** 18(3): 983-987.

Trcek, J. 2005. Quick identification of acetic acid bacteria based on nucleotide sequences of the 16S-23S rDNA internal transcribed spacer region and of the PQQ-dependent alcohol dehydrogenase gene. **Sys. Appl. Microbiol.,** 28(8): 735-745.

Vandamme, E.J., De Baets, S., Vanbaelen, A., Joris, K. and P. De Wulf. 1998. Improved production of bacterial cellulose and its application potential**. Polym. Degrad. Stab.,** 59: 93-99.

Vergera, C.M.A.C., Honorato, T.L., Maia, G.A. and S. Rodrigues. 2010. Prebiotic effect of fermented cashew apple (*Anacardium occidentale*) juice. **LWT - Food Sci. Technol.,** 43: 141-145.

Verschuren, P. G., Cardona, T. D., Nout, Z M. J. R., De Gooijer, K. D. and J. C. V. Heuvel. 2000. Location and limitation of cellulose production by *Acetobacter xylinum* established from oxygen Profiles. **J. Biosci. bioeng**., 89(5): 414-419.

Vincent, J. F. V. 1999. From cellulose to cell. **J. Exp. Biol.,** 202: 3263-3268.

Wee, Y.J., Kim, S. Y., Yoon, S. D. and R.W. Ryu. 2011. Isolation and characterization of a bacterial cellulose producing bacterium derived from the persimmon vinegar. **Afri. J. Biotechnol.,** 10(72): 16267-16276.

Weisberg,W.G., Barns, S.M., Pelletier, D.A. and D.J. Lane. 1991. 16S ribosomal DNA amplification for phylogenetic study. **J. Bacteriol.,** 173(2): 697-703.

Williams, W. S. and R. E. Cannon. 1989. Alternative environmental roles for cellulose produced by *Acetobacter xylinum*. **Appl. Env. Microbiol.,** 55(10): 2448-2452.

Wintola, O.A., Sunmonu, T.O. and A.J. Afolayan. 2010. The effect of *Aloe ferox* mill in the treatment of loperamide-induced constipation in Wistar rats. **BMC Gastroenterol.,** 10: 95.

Yamada, Y. 2000. Transfer of *Acetobacter oboediens* Sokollek *et al*. 1998 and *Acetobacter intermedius* Boesch *et al*. 1998 to the genus *Gluconacetobacter* as *Gluconacetobacter oboediens* comb. nov., and *Gluconacetobacter intermedius* comb. nov., **Int. J. Sys. Evol. Microbiol.,** 50: 2225-2227.

Yamada, Y. and K. Kondo. 1984. *Gluconoacetobacter*, a new subgenus comprising the acetate-oxidizing acetic acid bacteria with ubiquinone-10 in the genus *Acetobacter*. **J. Gen. Appl. Microbiol.,** 30: 297-303.

Yamada, Y. and P. Yukphan. 2008. Genera and species in acetic acid bacteria. **Int. J. Food. Microbiol.,** 125 :15-24.

Yang, H. Yan, R., Chen, H., Dong, H. L. and C. Zheng. 2007. Thermal properties of cellulose. **Fuel,** 86: 17-81.

Yang, Y.K., Park, S.H., Hwang, J.W., Pyun, Y.R. and Y.S. Kim. 1998. Cellulose production by *Acetobacter xylinum* BRC5 under agitated condition. **J. Ferment. Bioeng.,** 85: 312-317.

Yoshinaga, F., Tonouchi, N. and K. Watanabe. 1997. Research progress in production of bacterial cellulose by aeration and agitation culture and its application as a new industrial material. **Biosci. Biotech. Biochem.,** 61: 219-224.

Yukphan, P., Malimas, T., Takahashi, M., Potacharoen, W., Busabun, T., Tanasupawat, S., Nakagawa, Y., Tanticharoen, M. and Y. Yamada. 2004. "Re-identification of *Gluconacetobacter* strains based on the restriction analysis of 16S-23S rDNA Internal Transcribed Spacer regions", **J. Gen. Appl. Microbiol.,** 50: 189-195.

Zhou, L.L., Sun, D.P., Hu, L.Y., Li, Y.W. and J.Z. Yang. 2007. Effect of addition of sodium alginate on bacterial cellulose production by *Acetobacter xylinum*. **J. Int. Microbiol. Biotech.,** 34: 483-489.

Zogaj, X., Nimtz, M., Rohde, M., Bokranz, W. and U. Romling. 2001. The multicellular morphotypes of *Salmonella typhimurium* and *Escherichia coli* produce cellulose as the

second component of the extracellular matrix. **Mol. Microbiol.,** 39: 1452-1463.

*Originals not seen.

ANNEXURE I

GROWTH MEDIA COMPOSITIONS

HS medium

Glucose	20.0 g
Yeast extract	5.0 g
Peptone	5.0 g
Na_2HP0_4	2.7 g
Citric acid	1.15 g
Distilled water	1000 ml
pH	6.0 – 6.2
Agar	15.0 g

GYC medium (Isolation medium)

Glucose	20.0 g
Yeast extract	5.0 g
$CaCO_3$	2.0 g
Cycloheximide	0.01 g
Absolute ethanol	2.0 ml
Distilled water	1000 ml
pH	5.0

Luria Bertani medium

Tryptone	10.0 g
Sodium chloride	10.0 g
Yeast extract	5.0 g
Distilled water	1000 ml
pH	7.0
Agar	15.0 g

Gram stain solutions

a) Crystal violet solution

Crystal violet 10.0 g	
Ammonium oxalate	4.0 g
Ethanol	100 ml
Distilled water	1000 ml

b) Iodine solution

Iodine	1.0 g
Potassium iodide	2.0 g
Ethanol	25.0 ml
Distilled water	100 ml

c) Counter stain

2.5% safranin in ethanol	10.0 ml
Distilled water	100 ml

d) Ethyl alcohol

Ethanol	95 ml
Distilled water	5 ml

ANNEXURE II

Components of central composite rotatable design of experiments

No of factors	Number of points			Total	Scale value for α $[(2^k)^{1/4}]$
	Factorial (2^k)	Star (2k)	Base level n_0		
2	4	4	5	13	1.414
3	8	6	6	20	1.682
4	16	8	7	32	2.000
5	32	10	10	52	2.378

$\alpha = [number\ of\ factorial\ runs]^{1/4}$ ….. (3.1)

If the factorial portion is a full factorial, then

$\alpha = [(2^k)^{1/4}]$ ….. (3.2)

The number of base level points 'n_0' to be carried out depend on the no. of factors 'k'.

Total number of experiments in the first order design $(n_0) = 2^k + n_0$ ….. (3.3)

Total number of experiments in the second order design $(n_s) = 2^k + 2k + n_0$ ….. (3.4)

Natural levels, codes and intervals of variation of the independent variables in the design of cellulose production experiments using MCW

Factors	Codes	Levels					Interval of variation
		-α	-1	0	+1	+ α	
Fructose concentration (FC)	FC	5	10	15	20	25	5
Yeast extract concentration (YC)	YC	2.5	5.0	7.5	10	12.5	2.5
Citric acid (CC)	CC	1	1.5	2.0	2.5	3	0.5
Disodium hydrogen phosphate (DC)	DC	1.05	1.1	1.15	1.2	1.25	0.05

where,

$$FC = \left(\frac{FC - 15}{10}\right) \quad \text{..... (3.5)}$$

$$YC = \left(\frac{YC - 7.5}{5.0}\right) \quad \text{..... (3.6)}$$

$$CC = \left(\frac{CC - 2.0}{1.5}\right) \quad \text{..... (3.7)}$$

$$DC = \left(\frac{DC - 1.15}{1.1}\right) \quad \text{..... (3.8)}$$

Fifteen experiments starting from A to O were formulated for the optimization of cellulose production process. The base level treatment I was replicated 6 times to calculate the reproducibility of the method and the other treatments were replicated thrice. The maximum and minimum variable levels were selected on the basis of preliminary studies. The experiments were randomized in order to minimize the effects of unexplained variability in the observed responses due to extraneous factors. The experiments were conducted with second order design so that both the first and second order models can be postulated according to the adequacy of fit. For each combination of the independent variables in the experimental design, the dependent parameters were found out.

Experimental design used for optimization of cellulose production using MCW medium

Experiment No.	Independent Variable Levels								
	Natural Values				Coded Values				
	FC	Y	CC	DC	FC	Y	CC	DC	
1 (A)	10	5	1.5	1.1	-1	-1	1	1	2^k type factorial experiments
2 (B)	20	5	1.5	1.1	1	-1	1	-1	
3 (C)	10	10	1.5	1.1	1	1	-1	-1	
4 (D)	20	10	1.5	1.1	-1	1	-1	1	
5 (E)	10	5	2.5	1.1	-1	-1	1	1	
6 (F)	20	5	2.5	1.1	1	-1	1	-1	
7 (G)	10	10	2.5	1.1	1	1	-1	-1	
8 (H)	20	10	2.5	1.1	1	1	1	1	
9 (J)	10	5	1.5	1.2	0	0	-2	0	2k extra experiments with star points
10 (K)	20	5	1.5	1.2	0	0	2	0	
11 (L)	10	10	1.5	1.2	-2	0	0	0	
12 (M)	20	10	1.5	1.2	2	0	0	0	
13 (N)	10	5	2.5	1.2	0	-2	0	0	
14 (O)	20	5	2.5	1.2	0	2	0	-2	
15 (P)	10	10	2.5	1.2	0	0	0	2	
16 (Q)	20	10	2.5	1.2	0	0	-2	0	
17 (R)	5	7.5	2	1.15	0	0	2	0	
18 (S)	25	7.5	2	1.15	-2	0	0	0	
19 (T)	15	2.5	2	1.15	2	0	0	0	
20 (U)	15	12.5	2	1.15	0	-2	0	0	
21(V)	15	7.5	1	1.15	0	2	0	-2	
22(W)	15	7.5	3	1.15	0	0	0	2	
23(X)	15	7.5	2	1.05	0	2	0	-2	
24(Y)	15	7.5	2	1.25	0	0	0	2	
25(I_1)	15	7.5	2	1.15	0	0	0	0	n0 base level experiments
26(I_2)	15	7.5	2	1.15	0	0	0	0	
27(I_3)	15	7.5	2	1.15	0	0	0	0	
28(I_4)	15	7.5	2	1.15	0	0	0	0	
29(I_5)	15	7.5	2	1.15	0	0	0	0	
30(I_6)	15	7.5	2	1.15	0	0	0	0	

Statistical modeling of responses

Empirical statistical modeling was used to develop an appropriate approximating model between the response (y) and independent variables $(X_1, X_2, X_3, \ldots\ldots X_k)$.

In general the relationship is

$$Y = f(X_1, X_2, X_3, \ldots\ldots X_k) + \varepsilon \qquad \ldots\ldots (3.9)$$

The variables $X_1, X_2, X_3, \ldots\ldots X_k$ in the Eqn. 3.9 were usually called the natural variables in RSM . It is convenient to transform the natural variables to coded variables $x_1, x_2, x_3, \ldots\ldots x_k$, which are dimensionless having mean zero and variance equal to 1. In terms of the coded variables, the response function (Eqn. 3.10) is

$$y = f(x_1, x_2, x_3, \ldots\ldots x_k) + \varepsilon \qquad \ldots\ldots (3.10)$$

The function 'f' is called the response surface. The form of the function 'f' is unknown. The term 'ε' represents other sources of variability not accounted for in 'f' and usually it is treated as statistical error. As the form of the response function 'f' is unknown, it must be approximated. Polynomials are often chosen because they usually offer an adequate approximation of the true response surface. In many cases, either a first order or a second order model as shown below is used.

$$y = b_0 x_0 + \sum_{i=1}^{k} b_i x_i + \varepsilon \qquad \ldots\ldots (3.11)$$

$$y = b_0 x_0 + \sum_{i=1}^{k} b_i x_i + \sum_{i=1}^{k} b_{ii} x_i^2 + \sum_{i=1}^{k} \sum_{j=1}^{k} b_{ij} x_i x_j + \varepsilon \qquad \ldots\ldots (3.12)$$

The first model (Eqn. 3.11) is likely to be appropriated when the experimenter is interested in approximating the true response surface over a relatively small region of the independent variables space in a location where there is no curvature in 'f'. If there is a curvature in the system, then a polynomial of higher degree such as second order model (Eqn. 3.12) must be used.

To find out the effect of the independent variables on the dependent variables, the first order linear equation (Eqn. 3.13) was fitted between 'x' and 'y'. For optimization of the independent variables and to check the sufficiency of the experimental design, the

second order non-linear regression equation (Eqn. 3.14) was fitted between dependent and independent variables.

$$y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_4x_4 \quad \text{….. (3.13)}$$

$$y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_4x_4 + b_{11}x_1^2 + b_{22}x_2^2 + b_{33}x_3^2 + b_{44}x_4^2 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3 \quad \text{…… (3.14)}$$

where,

y is the response variable

b_0, b_1, b_2, b_3 and b_4 are regression coefficients of linear terms

b_{11}, b_{22}, b_{33} and b_{44} are regression coefficients of quadratic terms

b_{12}, b_{13} and b_{23} are regression coefficients of cross-product terms

x_1, x_2, x_3 and x_4 are the coded values of the independent variables X, *viz.*, fructose (X_1), yeast extract concentration (X_2), citric acid concentration (X_3) and disodium hydrogen phosphate concentration (X_4) respectively. The quality of fit of the second order equation was expressed by the coefficient of determination R^2, and its statistical significance was determined by F-test. The significance of the regression coefficient was determined by p-value. The coefficients of the equation were determined by employing design expert software version 8.0.7.1. Analysis of variance (ANOVA) for the final predictive equation was done using design expert software.

Natural levels, codes and intervals of variation of the independent variables in the design of cellulose production experiments using CAJ

Factors	Codes	Levels					Interval of variation
		-α	-1	0	+1	+ α	
Fructose concentration (FC)	FC	0.0	2.0	4.0	6.0	8.0	2.0
Yeast extract concentration (YC)	YC	2.0	4.0	6.0	8.0	10.0	2.0
Citric acid (CC)	CC	-0.5	0.5	1.5	2.5	3.5	0.5
Disodium hydrogen phosphate (DC)	DC	0.25	0.5	0.75	1.0	1.25	0.25

α = *[number of factorial runs]*$^{1/4}$ ….. (3.1a)

If the factorial portion is a full factorial, then

$\alpha = [(2^k)^{1/4}]$ ….. (3.2a)

The number of base level points 'n_0' to be carried out depend on the no. of factors 'k'.

Total number of experiments in the first order design $(n_0) = 2^k + n_0$ ….. (3.3a)

Total number of experiments in the second order design $(n_s) = 2^k + 2k + n_0$ ….. (3.4a)

where,

$$FC = \left(\frac{FC - 4.0}{2.0}\right) \quad \text{….. (3.5a)}$$

$$YC = \left(\frac{YC - 6.0}{4.0}\right) \quad \text{….. (3.6a)}$$

$$CC = \left(\frac{CC - 1.5}{0.5}\right) \quad \text{….. (3.7a)}$$

$$DC = \left(\frac{DC - 0.75}{0.5}\right) \quad \text{….. (3.8a)}$$

Fifteen experiments starting from A to Y were formulated for the optimization of cellulose production process. The base level treatment II was replicated 6 times to calculate the reproducibility of the method and the other treatments were replicated thrice. The maximum and minimum variable levels were selected on the basis of preliminary studies. The experiments were randomized in order to minimize the effects of unexplained variability in the observed responses due to extraneous factors. The experiments were conducted with second order design so that both the first and second order models can be postulated according to the adequacy of fit. For each combination of the independent variables in the experimental design, the dependent parameters were found out.

Experimental design used for optimization of cellulose production using CAJ medium

Experiment No.	Independent Variable Levels								
	Natural Values				Coded Values				
	FC	Y	CC	DC	FC	Y	CC	DC	
1 (A)	2	4	0.5	0.5	-1	-1	1	1	2^k type factorial experiments
2 (B)	6	4	0.5	0.5	1	-1	1	-1	
3 (C)	2	8	0.5	0.5	1	1	-1	-1	
4 (D)	6	8	0.5	0.5	-1	1	-1	1	
5 (E)	2	4	2.5	0.5	-1	-1	1	1	
6 (F)	6	4	2.5	0.5	1	-1	1	-1	
7 (G)	2	8	2.5	0.5	1	1	-1	-1	
8 (H)	6	8	2.5	0.5	1	1	1	1	
9 (J)	2	4	0.5	1.0	0	0	-2	0	2k extra experiments with star points
10 (K)	6	4	0.5	1.0	0	0	2	0	
11 (L)	2	8	0.5	1.0	-2	0	0	0	
12 (M)	6	8	0.5	1.0	2	0	0	0	
13 (N)	2	4	2.5	1.0	0	-2	0	0	
14 (O)	6	4	2.5	1.0	0	2	0	-2	
15 (P)	2	8	2.5	1.0	0	0	0	2	
16 (Q)	6	8	2.5	1	0	0	-2	0	
17 (R)	0	6	1.5	0.75	0	0	2	0	
18 (S)	8	6	1.5	0.75	-2	0	0	0	
19 (T)	4	2	1.5	0.75	2	0	0	0	
20 (U)	4	10	1.5	0.75	0	-2	0	0	
21(V)	4	6	-0.5	0.75	0	2	0	-2	
22(W)	4	6	3.5	0.75	0	0	0	2	
23(X)	4	6	1.5	0.25	0	2	0	-2	
24(Y)	4	6	1.5	1.25	0	0	0	2	
25(I_1)	4	6	1.5	0.75	0	0	0	0	n0 base level experiments
26(I_2)	4	6	1.5	0.75	0	0	0	0	
27(I_3)	4	6	1.5	0.75	0	0	0	0	
28(I_4)	4	6	1.5	0.75	0	0	0	0	
29(I_5)	4	6	1.5	0.75	0	0	0	0	
30(I_6)	4	6	1.5	0.75	0	0	0	0	

Statistical modeling of responses

Empirical statistical modeling was used to develop an appropriate approximating model between the response (*y*) and independent variables *(X_1, X_2, X_3, …..X_k).*

In general the relationship is

$$\boldsymbol{Y = f\,(X_1, X_2, X_3, \ldots\ldots X_k) + \varepsilon} \qquad \ldots\ldots (3.9a)$$

The variables $X_1, X_2, X_3, \ldots\ldots X_k$ in the Eqn. 3.9a were usually called the natural variables in RSM . It is convenient to transform the natural variables to coded variables $x_1, x_2, x_3, \ldots\ldots x_k$, which are dimensionless having mean zero and variance equal to 1. In terms of the coded variables, the response function (Eqn. 3.10a) is

$$\boldsymbol{y = f\,(x_1, x_2, x_3, \ldots\ldots x_k) + \varepsilon} \qquad \ldots\ldots (3.10a)$$

The function 'f' is called the response surface. The form of the function 'f' is unknown. The term 'ε' represents other sources of variability not accounted for in 'f' and usually it is treated as statistical error. As the form of the response function 'f' is unknown, it must be approximated. Polynomials are often chosen because they usually offer an adequate approximation of the true response surface. In many cases, either a first order or a second order model as shown below is used.

$$\boldsymbol{y = b_0 x_0 + \sum_{i=1}^{k} b_i x_i + \varepsilon} \qquad \ldots\ldots (3.11a)$$

$$\boldsymbol{y = b_0 x_0 + \sum_{i=1}^{k} b_i x_i + \sum_{i=1}^{k} b_{ii} x_i^2 + \sum_{i=1}^{k} \sum_{j=1}^{k} b_{ij} x_i x_j + \varepsilon} \qquad \ldots\ldots (3.12a)$$

The first model (Eqn. 3.11a) is likely to be appropriated when the experimenter is interested in approximating the true response surface over a relatively small region of the independent variables space in a location where there is no curvature in 'f'. If there is a curvature in the system, then a polynomial of higher degree such as second order model (Eqn. 3.12a) must be used.

To find out the effect of the independent variables on the dependent variables, the first order linear equation (Eqn. 3.13a) was fitted between 'x' and 'y'. For optimization of the independent variables and to check the sufficiency of the experimental design, the second order non-linear regression equation (Eqn. 3.14a) was fitted between dependent and independent variables.

$$\boldsymbol{y = b_0 + b_1 x_1 + b_2 x_2 + b_3 x_3 + b_4 x_4} \qquad \ldots\ldots (3.13a)$$

$$y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_4x_4 + b_{11}x_1^2 + b_{22}x_2^2 + b_{33}x_3^2 + b_{44}x_4^2 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3 \quad \ldots\ldots (3.14a)$$

where,

y is the response variable

b_0, b_1, b_2, b_3 and b_4 are regression coefficients of linear terms

b_{11}, b_{22}, b_{33} and b_{44} are regression coefficients of quadratic terms

b_{12}, b_{13} and b_{23} are regression coefficients of cross-product terms

x_1, x_2, x_3 and x_4 are the coded values of the independent variables X, *viz.*, fructose (X_1), yeast extract concentration (X_2), citric acid concentration (X_3) and disodium hydrogen phosphate concentration (X_4) respectively. The quality of fit of the second order equation was expressed by the coefficient of determination R^2, and its statistical significance was determined by F-test. The significance of the regression coefficient was determined by p-value. The coefficients of the equation were determined by employing design expert software version 8.0.7.1. Analysis of variance (ANOVA) for the final predictive equation was done using design expert software.

ANNEXURE-IV

Score card for sensory evaluation of the *nata-de-coco* and *nata- de-cashew*

Characteristics	***Nata***	
	Nata-de-coco	***Nata-de-cashew***
Colour and appearance 9----------5----------1 **Flavour** 9----------5----------1 **Texture/juiciness** 9----------5----------1 **Taste/tenderness** 9----------5----------1 **Over all acceptability** 9----------5----------1		

Comments:

Scores:

1. Dislike Extremely
2. Dislike Very much
3. Dislike Moderately
4. Dislike Slightly
5. Neither like or Dislike
6. Like Slightly
7. Like Moderately
8. Like Very much
9. Like Extremely

LIST OF PUBLICATIONS

Sl. No.	ARTICLES	Journal Sl. No.	Journal In. No.	NAAS Rating
1.	**Gayathry, G. and G. Gopalaswamy.** 2013. Studies on cellulose producing G*luconacetobacter sp.* sju-1 isolated from fermented sugarcane juice. **J. Pure Appl. Microbiol.,** 7(3): 2389-2395	**873**	**I073**	**3.1**
2.	**Gayathry, G. and G. Gopalaswamy.** 2013. Production of bacterial cellulose from coconut liquid endosperm using *Acetobacter xylinum* sju-1. **Ind. J. Sugarcane Technol.,** 27(2): (In Press)	**1312**	**J333**	**6.4**
3.	**Gayathry, G. and G. Gopalaswamy.** 2013. **Isolation of cellulose producing** G*luconacetobacter xylinum* from fermented sugarcane juice. **Adv. Appl. Res., 5(1):** 1-4	**37**	**A037**	**3.0**

Dr. G. GOPALASWAMY

Chairman of advisory Committee