IOSR Journal Of Pharmacy And Biological Sciences (IOSR-JPBS)
e-ISSN:2278-3008, p-ISSN:2319-7676. Volume 14, Issue 3 Ser. II (May – June 2019), PP 54-59
www.Iosrjournals.Org

Isolation, Characterization and Identification ofBiocellulose Producing Strains of Bacteria Isolated from Selected Tropical Fruits

Peter KariukiNjenga
[1](*Department of botany, Jomo Kenyatta university of Agriculture and Technology, Kenya)*

Corresponding Author: Peter Kariuki Njenga

Abstract:*The principal objective of this research was to isolate and characterize cellulose producing bacteria from over ripe fruits through biochemical tests. The study majorly focused on the ability of the isolated strains of bacteria to synthesize cellulose and quantification of the synthesized cellulose so as to determine the modalities of scale up at the industrial level. The study involved culturing the over ripe fruits in Hestrin-Schram medium for 1 week then isolation of the bacterial strains for culturing in glucose yeast extract (GYE) agar from which the colony characteristics and further biochemical tests were carried out. In the project, 5 strains of bacteria were isolated from oranges, mangoes, water melon, lemon, grapes and pineapple. The isolates were 2 Gram positive rods and 3 Gram variables, catalase positive, positive for citrate utilization test and unable to ferment lactose, maltose and mannitol in the absence of peptone. All the strains were unable to liquefy gelatin. 2.023g of cellulose wasrecovered after analysis. The isolates were then identified as Bacillus strains, Gluconacetobacterxylinum, G. oboediensandSarcinaventriculi. The research concluded that tropical fruits are a rich source of BC producers and can therefore be used as a good nutrient source for production of good grade bacterial cellulose. The conditions in this research can be optimized at industrial level for production of cellulose so as to reduce the pressure exerted on the forests for timber to produce cellulose.*
Key words*: Bacterial cellulose, Cellulose, Culture media, Gluconacetobacter strains, Tropical fruits.*

Date of Submission: 27-01-2019 Date of acceptance: 11-06-2019

I. Introduction

Cellulose is a long chain of linked sugar molecules that gives wood its remarkable strength. It is the main component of plant cell walls and the basic building block for many textiles and paper. Cotton is the purest natural form of cellulose. Cellulose as a natural polymer consist of a long chain made by linking smaller molecules. The links in the cellulose chain are a type of sugar: beta- D glucose. The length of the chain varies greatly from few hundred sugar units in wood pulp to over 6000 for cotton [1]

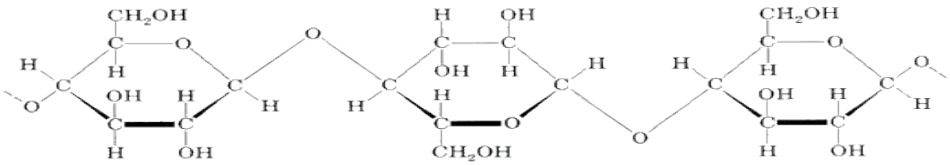

Fig. 1cellulose chain bristles with polar head groups [1].

Other than cellulose that comes from plant cell, there is certain strain of bacteria that can produce cellulose extracellular in the form of fibril that is attached to the bacterial cell. This cellulose is known as bacterial cellulose or bio cellulose. Bacterial cellulose is an extracellular polymer that is produced from monosaccharide or simple sugar such as glucose, xylose and galactose that act as a substrate or from other carbon source such as ethanol and glycerol [2].Bacterial cellulose is produced by acetic acid-producing bacteria, Gluconcetobacterxylinus. Bacteria from other species such as Aerobacter, Gluconacetobacter, Achromobacter, Agrobacterium, Alcaligenes, Azotobacter, Pseudomonas, Rhizobium and Sarcinaalso synthesize cellulose but only the Gluconacetobacter species produce enough cellulose to justify commercial interests [3]. All this bacteria produce bacterial cellulose in different forms, Gluconacetobacter produce cellulose in the form of pellicle composed of ribbon while Achromobacter, Aerobacter, Alcaligenes produce cellulose in fibrils form, Agrobacterium and Rhizobium produces cellulose in the form of short fibril, Pseudomonas produce bacterial

cellulose with no distinct fibril, Sarcina produce amorphous cellulose and Zooglea produce cellulose in not well defined form [4].

The cellulose synthesizing strains are obligate aerobe bacteria that are usually found in vinegar, alcoholic beverages, fruit juices, fruits and most likely in rotting ones as well [5]. The bacteria consume the sugar or carbohydrate from fruits as their main food. BC is formed on the air-liquid medium interface. Bacterial cellulose is widely used in the industry such as health, food industry, the making of audio component, wound care product and in production of paper product. It is the most abundant bio-polymer on earth with 180 billion tons per year produced in nature. The advantage of using bacterial system for production of cellulose is that the bacteria can grow rapidly under controlled conditions and produce cellulose from a variety of carbon sources including glucose, ethanol, sucrose and glycerol [6].

Bacterial cellulose has been considered as an alternative biomaterial since it possesses superior qualities to other cellulose. It exhibits many unique characteristics which are different from those of other plant cellulose such as high water holding capacity (over 100 times of its weight), high degree of crystallinity, great elasticity, high tensile strength, non-drying state, excellent biocompatibility and high purity, because its free from other contaminating components such as hemicelluloses, lignin or waxy aromatic substances [7]. These distinct physical and mechanical qualities have made bacterial cellulose more attractive than other materials well known as alternative materials in food, biomedical and other industries. Cellulose is of fundamental importance to human lives hence there is need for a constant supply.Currently the main source of cellulose is from plant resources, wood timber is cut from the forest and sent to the factories such as paper mills where wood is shredded into chips which are then processed into a thick watery pulp, this process requires a lot of energy and chemicals often harmful to the environment since unwanted lignin must be removed and the cellulose must be bleached. With our increased population explosion and the quest to continue using cellulose from wood and cotton, more land is required to meet the global demand. Bacterial cellulose is thus more favorable since it has high purity, biodegradable compared with the plant cellulose that is difficult to purify and limited in source which could decrease forest area [8].

II. Methodology

2.1 Preparation of fruit waste juice

100 g of pineapple, mango, grapes. Oranges, lemon and water melon were measured separately and weighed. The fruit residue were then crushed and blend with 200ml of water using a blender. After that, each fruit juice was filtered with a filter cloth to separate the fruit waste.

2.2 Isolation of cellulose synthesizing strains

10ml of each fruit was transferred into 90 ml of a modified Hestrin-Schramm (HS) [9] medium in a 250-mL flask containing 2.0% D-glucose (w/v), 0.5% peptone (w/v), 0.5% yeast extract (w/v), 0.27% Na_2HPO_4 (w/v), 0.12% citric acid (w/v), 0.2% acetic acid (v/v), 0.5% ethanol (v/v) and 0.01% cycloheximide (w/v) . The flasks with fruit and liquid medium were then incubated statically at 30C for 7 days. After incubation, the flasks with white pellicle covering the surface of the liquid medium were selected. The culture broth of the selected flasks were serially diluted with 0.85% NaCl (w/v) and 0.1 ml of each dilution spread on GYE agar, which comprised of 2.0% D-glucose, 1.0% yeast extract, 5% ethanol and 0.3% $CaCO_3$. The agar plates were incubated at 30 degrees Celcius until colonies are formed. Biochemical tests were then carried out to identify the isolated strains.

2.3 Biochemical and physiological tests

Morphology of the cells was examined under light microscope where Gram stain, capsule stain and spore stain was performed. The motility of cells was observed by use of SIM medium. Purified cultures were streaked onto $CaCO_3$, agar plates to confirm acid production and investigate overoxidation ofacetic acid. $CaCO_3$ agar medium comprised of 0.05% D-glucose, 0.3% peptone, 0.5% yeast extract, 1.5% $CaCO_3$, 1.2% agar and 1.5%(v/v) ethanol. Microbial growth was examined after incubation at 30ºC for 2-7 days. Acid forming colonies were subjected to further biochemical tests including: Catalase, gelatin liquefaction, oxygen requirement, citrate utilization, nitrate reduction, starch hydrolysis, coagulase and hydrogen sulphide production using BD reagent droppers. Sugar fermentation was considered in glucose, maltose, lactose, glycerol by using 1% of tested sugar solution as the only source of carbon. Citrate utilization was tested using citrate broth (0.1% $NH_4H_2PO_4$, 0.1% KH_2PO_4, 0.5% NaCl, 0.02% $MgSO_4$, 0.2% sodium citrate, 0.008% bromthymol blue and, 1.3% agar). Growth on blood agar was considered using basic blood agar consisting of proteose peptone 15g , liver digest 2.2g, yeast extract 5g, NaCl 5g and 12g of agar per litre. Acid production from mannitol was tested using mannitol salt agar. Hydrolysis of gelatin was investigated by inoculation of nutrient gelatin tubes (0.5% peptone, 0.3% beef extract and 12% gelatin) incubated at 37ºC.

2.4 Synthesis and analysis bacterial cellulose

Subsequently, the colonies with a clear zone around $CaCO_3$ were selected and transferred into flask containing 1000 ml of HS medium and then incubated at 30C for 3-7 days. Subsequently, only the flasks with white pellicle on the surface were collected for further purification. The resulting pellicle was harvested and washed three times with distilled water and its appearance observed by the unaided eye. The pellicles were confirmed by boiling with 0.5N NaOH for 15 min. to remove bacterial contaminants. Finally, the purified cellulose was further confirmed by drying at 80 degrees Celcius in a hot air oven to a constant weight since they may not be cellulose.

III. Results

From the six fruits collected, 5 bacterial isolates were obtained and named as BCST1, BCST2, BCST3, BCST4 and BCST5 depending on their morphological characteristics as shown below

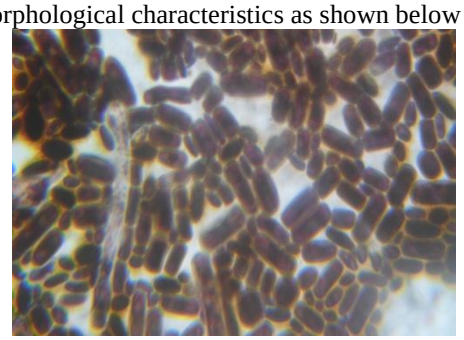

Fig. 2 light microscope slide of BCST1

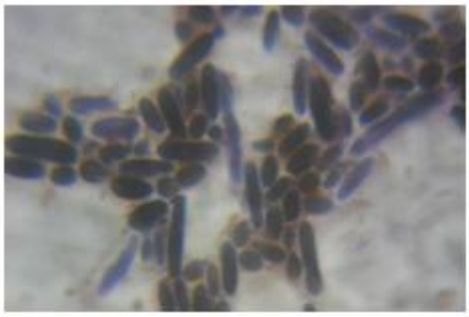

Fig. 3 light microscope slide of BCST2

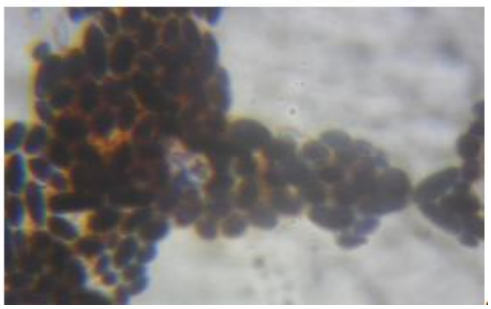

Fig. 4 light microscope slide of BCST3

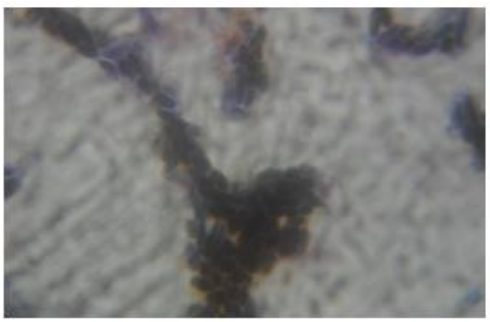

Fig.5 light microscope slide of BCST4

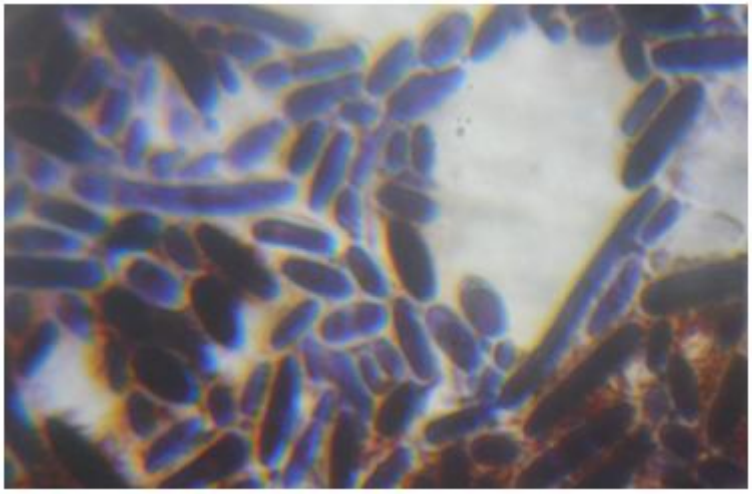

Fig. 6 light microscope slide of BCST5

They were then examined for BC-production where 3 isolates were recorded as BC producing strains. Accordingly, all the isolated strains were gram positive with BCST 1, BCST 3 and BCST 4 being Gram variables since they stained lightly in the classical Gram stain. The cells were ellipsoidal to rod shape. All the isolates were able to grow on basic blood agar where BCST 1 had brown muccoid colonies, BCST 2, BCST 4 and BCST 5 had limited growth with creamy colonies, and BCST 3 had an intense growth on basic blood agar having thick white spreading colonies.

All the strains tested positive for capsule stain characterized by green spots on the cells against the red background of safranin. All the strains except BCST 1 tested positive for spore stain. All the isolated strains were aerobic except BCST 1 and BCST 2 that were microaerophilic. Isolates BCST 1 and BCST 5 were immotile on SIM media while the rest were motile. On further analysis, all the strains showed positive results for: citrate utilization, catalase and glycerol fermentation. They also showed negative results for: lactose, maltose, xylose fermentation in the sugar fermentation test, TSI and nitrate reduction. They however exhibited a mixed reaction for: glucose fermentation where only BCST 2 and BCST 5 could ferment glucose and only BCST 3 could ferment mannitol. In starch hydrolysis, BCST 1 and BCST 4 could hydrolyze starch while the rest could not. Finally, only BCST 2 and BCST 3 tested positive for coagulase test.

Table 1: Summary of the tests carried on the bacterial isolates

Type of test	**Parameter recorded**	**Bcst1**	**Bcst2**	**Bcst3**	**Bcst4**	**Bcst5**
Macroscopic	Colony characteristic and morphology in:					
	Glucose yeast agar	White circular	Cream-clustered	Brown circular	Brown circular	Brown circular
	Blood agar	Brown muccoid	Cream	White spreading	Cream	Cream
	Gram reaction	Variable	Positive	Variable	Variable	Positive
Microscopic	Morphology	Short rods	Short rods	Coccus and short rods	Short rods	Long rods
	Cell arrangement	Mono	Mono	Cluster	Cluster	Mono
Biochemical	Spore stain	-	+	+	+	+
	Capsule	+	+	+	+	-
	Oxygen requirement	Micro	Micro	Aero	Aero	Aero
	Motility	-	+	+	+	-
	Gelatin	-	-	-	-	-
	Citrate	+	+	+	+	+
	Coagulase	-	-	+	+	-
	TSI	-	-	-	-	-
	Starch	+	-	-	+	-

Nitrate	-	-	-	-	-
Catalase	+	+	+	+	+
Sugar fermentation from:					
Lactose	-	-	-	-	-
Glucose	-	+	+	-	-
Maltose	-	-	-	-	-
Manitol	-	-	+	-	-
Glycerol	+	+	+	+	+
Xylose	-	-	-	-	-

+ = Positive - = Negative

Treatment of the sieved pellicle with boiling NaOH resulted in hydrolysis of the cytoplasmic material. On storage in a hot air oven at 80 degrees celcious for 2 days, a white powder was formed. The powder weighed 2.023g.

Fig. 7: On the fourth day the flasks were covered with a thick pellicle to signify for presence of cellulose synthesizing bacteria.

Fig 8: Purified cellulose obtained from the study

IV. Discussion

The results of the present study indicate that the over ripe fruits present ideal conditions for the survival and growth of many types of microorganisms. The internal tissues are nutrient rich. Their main structure is mainly composed of polysaccharide, cellulose, hemicelluloses and pectin. The principal storage polymer is starch. The microorganisms exploit the fruits using extracellular lytic enzymes that degrade those polymers and other intracellular constituents for use as nutrients [10].

From a total of 6 fruit samples, 5 bacterial isolates were obtained 3 belonged to Gluconacetobactersppbecause they gave clear zones on glucose yeast extract agar medium after 72 hours of incubation because of their ability to produce acetic acid that dissolves $CaCO_3$ of glucose yeast extract medium and formation of zones around colonies of those isolates . According to Bergey's Manual of Determinative Bacteriology [11], Gluconacetobacter strains are individual cells that are ellipsoidal to rod shape, occurring singly or in pairs or in short or long chains. Young cells are Gram negative while old cells are Gram variable. Another research done by [12], the isolated strains of Gluconacetobacter were found to be either Gram negative or Gram variable. It is in the same vein and after analysis of the results of identification BCST 3 and BCST 5 were confirmed to be strains of Gluconacetobacterxylinum and BCST 2 was confirmed to be Gluconacetobacteroboediens. According to the morphological, cultural and biochemical analysis, they gave negative results for: lactose, maltose, xylose fermentation, Gelatin liquefaction, nitrate reduction and TSI. They also gave positive results for cataalase and glycerol fermentation. These results coincides with those described in Bergey's manual of determinative bacteriology [13].

The morphological and physical characteristics together with the fermentation of products establish that the bacteria labeled BCST 1 and BCST 4 are Bacillus substilis and sarcinaventriculi respectively. Sarcinaventriculi had a negative test for: lactose, glucose, maltose, mannitol, xylose, gelatin liquefaction and nitrate reduction. They showed positive results for catalase and starch hydrolysis. These characteristics coincide with those in bergey's manual. It is indicated that growth of Sarcinae only occurs in sugar media containing peptone. Bacillus substilis gave negative results for fermentation of lactose, glucose, maltose and xylose. They also gave negative results for coagulase, TSI and nitrate reduction. The bacteria gave positive results for citrate reduction, starch hydrolysis and catalase.

V. Conclusion

The research has confirmed that tropical fruits are a rich source of BC producers. Many fruit juices are a rich in carbohydrates, proteins and trace elements and can therefore be used as good nutrients for the production of good grade cellulose. To reduce production costs, optimization of the culture conditions and use of alternative cheaper carbon sources such as agricultural wastes are desirable. Discrepancies were noted in citrate utilization, and glycerol fermentation but the available data could be used to expeditiously identify the strains. Since BC producing strains are prone to inactivation under harsh environmental conditions and their source of isolation is majorly limited to fruits and other food products, future work needs to be done so as to genetically engineer the most abundant strains of bacteria like E. coli, Bacillus spp. that can survive harsh environmental conditions and enhance BC production capacity.

Reference

[1]. **CDoree (1947)**, The Methods Of Cellulose Chemistry,London, Chapman and Hall

[2]. **Brown, R.M., Jr., J.H.M. Willison, and C.L. Richardson (1976)**, Cellulose biosynthesis in Acetobacterxylinum: 1. Visualization of the site of synthesis and direct measurement of the in vivo process. Proc. Nat. Acad. Sci. U.S.A. **73**(12):4565-4569.

[3]. **AmornratSuwanposri, Pattaraporn Yukphan, Yuzo Yamada and DaungjaiOchaikul (2013),** Identification and biocellulose production of Gluconacetobacter strains isolated from tropical fruits in Thailand, Maejo Int. J. Sci. Technol. 7(01), 70-82.

[4]. **Andelib Y. A and Nuran D. A(2009),** Isolation Of Cellulose Producing Bacteria From Wastes of Vinegar Fermentation, Vol. 1, October 20-22, 2009,Proceedings of World Congress On Engineering and Computer Science. San Francisco, USA.

[5]. **Klemm, D., Schumann, D., Udhart, U., &Marsch, S. (2001) ,** Bacterial Synthesis Cellulose - Artificial Blood Vessels for Microsurgery. Progress in Polymer Science 26, 1561-1603.

[6]. **Junaidizacharia and MuhamadAzlanNazeri (2012),** Optimization of Bacterial Cellulose Production from Pineapple Waste: Effect of Temperature, pH and Concentration, Engineering Conference, Kuching sarawak.

[7]. **Son HJ, Kim HG, Kim KK, Kim HS, Kim YG, Lee SJ (2003),** Increased production of bacterial cellulose by Acetobacter sp. V6 in synthetic media under shaking culture conditions.Bioresour. Technol86: 215-219.

[8]. **Barbara Surma-Slusarska, Sebastian Presler and DariuszDanielewicz (2008),** Characteristics of bacterial cellulose obtained from Acetobaccterxylinum culture for application in paper making, institute of paper making and printing, 350-370

[9]. **Hestrin S (1963)** , Bacterial Cellulose, in Methods in Carbohydrate Chemistry, (R. L. Whisler, editor), New York, Academic Press, Inc 3-4.

[10]. **Moosavi-Nasab M, Yousefi AR (2010),** Investigation of physicochemical properties of the bacterial cellulose produced by Gluconacetobacterxylinus from date syrup. Oral presented in ICBN , Paris, France, World Academy of Science. Eng Technol. 68: 893-899.

[11]. **Andelib Y. A and Nuran D. A(2009),** Isolation Of Cellulose Producing Bacteria From Wastes of Vinegar Fermentation, Vol. 1, October 20-22, 2009,Proceedings of World Congress On Engineering and Computer Science. San Francisco, USA.

[12]. **Beveridge T. J (1990),** Mechanisms of Gram variability in select bacteria, J.Bacterial 172(3):1609-1620.

[13]. **Jozef D L, Monique G and Jeans S (1986) ,** Bergey's Manual Of Systematic Bacteriology, PP 267-274, USA, Williams and Wilkins publishing.

Peter Kariuki." Isolation, Characterization and Identification of Biocellulose Producing Strains of Bacteria Isolated from Selected Tropical Fruits" IOSR Journal of Pharmacy and Biological Sciences (IOSR-JPBS) 14.3 (2019): 54-59.