Journal Homepage: -www.journalijar.com
INTERNATIONAL JOURNAL OF ADVANCED RESEARCH (IJAR)
Article DOI:10.21474/IJAR01/6831
DOI URL: http://dx.doi.org/10.21474/IJAR01/6831

RESEARCH ARTICLE

BACTERIAL CELLULOSE: OPTIMIZED PRODUCTION FROM ACETOBACTER XYLINUM, RHIZOBIUM AND PSEUDOMONAS AERUGINOSA AND THEIR COMPARATIVE STUDY.

Aishwarya Bhat[1], Brinda V[1], Yash Malik[1] and Dr. Lingayya Hiremath[1].
Students of Department of Biotechnology R.V. College of Engineering Mysuru Road, R.V. Vidyanikethan Post, Bengaluru, Karnataka 560059 India.

Manuscript Info

Manuscript History

Received: 03 February 2018
Final Accepted: 05 March 2018
Published: April 2018

Keywords:-
Bacterial Cellulose, Waste water, Rhizobium, Pseudomonas, Acetobacter, purification

Abstract

Cellulose is an organic compound produced mainly in plants. Bacterial, or microbial, cellulose has different properties from plant cellulose and is characterized by high purity, strength, foldability and increased water holding ability. By controlling synthesis methods, the resulting microbial cellulose can be tailored to have specific desirable properties. With advances in the ability to synthesize and characterize bacterial cellulose, the material is being used for a wide variety of commercial applications including textiles, cosmetics, and food products, as well as medical applications. The objective of the paper is to successfully isolate cellulose from bacteria and study and analyze its characteristics for optimized growth and utilize it to purify waste water obtained from the sewage line, sugar industry effluents and paper industry white water in order to grow the cellulose in natural media and utilize the cellulose in fields like textiles or biomedicine.

Introduction:-

Cellulose is the most abundant natural polymer on Earth and it is found as a structural component, often bound to other polymers. Despite the fact that plant cellulose shares the same molecular formula $(C_6H_{10}O_5)_n$ with bacterial cellulose (BC), their physicochemical properties are different. BC is characterized by higher purity, due to the fact that it does not contain any hemicellulose or lignin, higher water holding capacity, hydrophilicity, degree of polymerization, mechanical strength, crystallinity, porosity, and a highly pure fiber network structure, compared to plant cellulose. The enhanced mechanical properties of BC occur due to the uniform, continuous and nano-scalar network of cellulosic fibers. These properties are affected by various factors, such as the culture conditions, the microorganism and the fermentation media employed. Bacterial cellulose (BC) is a versatile biopolymer with better material properties, such as purity, high degree of porosity, relative high permeability to liquid and gases, high water-uptake capacity, tensile strength and ultrafine network.

Acetobacter xylinum is nature's most prolific cellulose-producing bacterium. A typical single cell can convert up to 108 glucose molecules per hour into cellulose. A single cell of Acetobacter has a linear row of pores from which glucan chain polymer aggregates are spun. As many as one hundred of these pores can produce a composite cable of glucan polymers resulting in a ribbon. Time lapse analysis of individual Acetobacter cells assembling cellulose ribbons reveals a myriad of activities, each cell acting as a nano-spinneret, producing a bundle of sub-microscopic fibrils. Together, the entangled mesh of these fibrils produces a gelatinous membrane known as a pellicle.

Corresponding Author:- Aishwarya Bhat.
Address:- Students of Department of Biotechnology R.V. College of Engineering Mysuru Road, R.V. Vidyanikethan Post, Bengaluru, Karnataka 560059 India.

Pseudomonas aeruginosa thrives in many aqueous environments and is an opportunistic pathogen that can cause both acute and chronic infections. Environmental conditions and host defenses cause differing stresses on the bacteria, and to survive in vastly different environments, P. aeruginosa must be able to adapt to its surroundings. One strategy for bacterial adaptation is to self-encapsulate with matrix material, primarily composed of secreted extracellular polysaccharides. These polysaccharides differ in chemical structure and in their biosynthetic mechanisms.

Materials and Methods:-

Isolation of Acetobacter Xylinum:-

*Acetobacter Xylinu*m bacteria was isolated from rotten pears obtained from a local vendor present in Vijayanagar. The experiment proceeded with isolation through serial dilution method where the 6 test tubes with 9ml ml distilled water and one test tube with 10ml double distilled water were taken and autoclaved in order to sterilize the test tube and the water against present bacteria in order to prevent any contamination. The rotten pear was DE fleshed and 1gm of the rotten region of the fruit was weighed and vortexed in with the 10ml the test tube. In the case of serial dilution, 1ml from the 10ml test tube was pipetted out and injected into the next 9ml test tube in the laminar airflow and marked as 10^{-1}, and mixed thoroughly. The similar procedure done till we obtained a test tube numbered 10^{-5}, and mixed well. The agar media was prepared for 250ml as yeast extract 1.25g/ml, peptone 1.25g/ml, Disodium phosphate 0.675g/ml, citric acid 0.288 g/ml, agar 3.75g/ml and autoclaved. The agar media was poured into Petri plates and allowed to solidify in the laminar air flow. The petri plates were later labelled after solidification.

The first petri plate, was cultured via spread plate methodology where 1ml of the 10^{-5} test tube was pipetted out and poured onto the solidified agar which was placed on a rotating stand. Using a sterilized glass rod, the inoculum was spread on the plate till there the liquid had been absorbed by the agar The second petri plate was cultures using streaking method where a ethanol sterilized loop was dipped into the 10^{-5}testube and streaked across the plate. The plates were then sealed with film and incubated at 32 degrees for 3 days.

Fig 1:- Isolation of Acetobacter Xylinum from rotten pear

Isolation of Rhizobium from Pea Plant:-

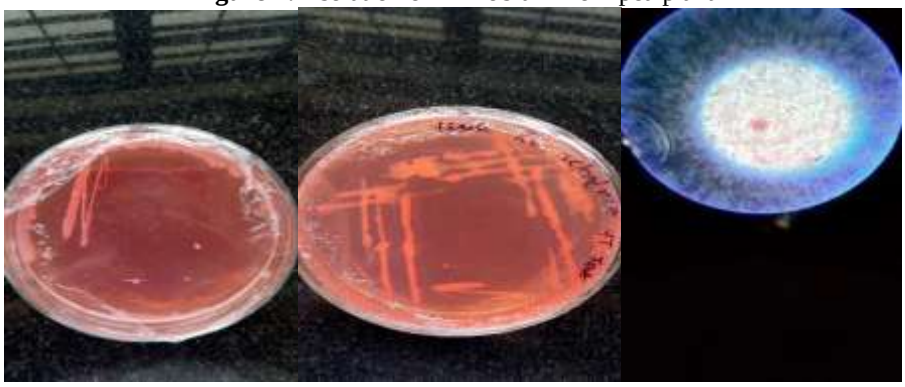
The isolation of Rhizobium was executed by collected nodules from CAMSON BIOTECHNOLOGY PVT LTD from a pea plant grown in the fields maintained by the industry. The isolation of rhizobium was done in two different methodologies; serial dilution method and bleaching method.

In case of the serial dilution the nodules were cut open with a sterile blade and the red part of the nodule was removed and the vortexed into 9 ml of autoclaved water. It was further diluted till the power -7 and streaked onto the YMA agar in a spatial pattern. The experiment proceeded with isolation through serial dilution method where the 6 test tubes with 9ml ml distilled water and one test tube with 10ml double distilled water were taken and autoclaved in-order to sterilize the test tube and the water against present bacteria in-order to prevent any contamination. vortexed in with the 10ml the test tube. In the case of serial dilution, 1ml from the 10ml test tube was pipetted out and injected into the next 9ml test tube in the laminar airflow and marked as 10^{-1}, and mixed thoroughly.

The similar procedure done till we obtained a test tube numbered 10^{-5}, and mixed well. The agar media from Table 4 was prepared and autoclaved. The agar media was poured into petri plates and allowed to solidify in the laminar air flow. The petri plates were later labelled after solidification. The whole nodules were first incubated in 100%

ethanol so as to surface sterilize the nodule, it was then immediately drenched with bleach for 3 minutes. It was then inserted into a Petri plate of autoclaved distilled water to remove the bleach from the surface and was further washed with subsequent dipping's into distilled water. It was then put on a clean plate, cut open and crushed so that the liquid is released. the liquid was then taken with a sterile loop and streaked.

Figure 2:- Isolation of Rhizobium from pea plant



Isolation of Pseudomonas Aeruginosa:-

Pseudomonas sp. bacteria was isolated from culture obtained from Bangalore University. The experiment proceeded with isolation through serial dilution method where the 6 test tubes with 9ml ml distilled water and one test tube with 10ml double distilled water were taken and autoclaved in-order to sterilize the test tube and the water against present bacteria in-order to prevent any contamination.

1ml of the culture was added and vortexed in with the 10ml the test tube. In the case of serial dilution, 1ml from the 10ml test tube was pipetted out and injected into the next 9ml test tube in the laminar airflow and marked as 10^{-1}, and mixed thoroughly.

The similar procedure done till we obtained a test tube numbered 10^{-5}, and mixed well. The agar media from Table 4 was prepared and autoclaved. The agar media was poured into petri plates and allowed to solidify in the laminar air flow. The petri plates were later labelled after solidification. The first petri plate, was cultured via spread plate methodology where 1ml of the 10^{-5} test tube was pipetted out and poured onto the solidified agar which was placed on a rotating stand.

Using a sterilized glass rod, the inoculum was spread on the plate till there the liquid had been absorbed by the agar. The second Petri plate was cultures using streaking method where a ethanol sterilized loop was dipped into the 10^{-5} test tube and streaked across the plate. The plates were then sealed with film and incubated at 32 degrees for 3 days.

Figure 3:- Isolation of Pseudomonas Aeruginosa

Extraction and harvesting of cellulose:-

With respect to A.Xylinum and pseudomonas, the bacterial growth was taken as a loopful and incubated into the stock media which HS media of two flasks each containing 100ml of media.

The autoclaved media is inoculated with one loopful of the grown bacterial culture and mixed well. It is then incubated statically without any movement at 32 degrees in direct sunlight for 3 days, till a mucous layer is formed.

The Rhizobium petriplates were taken and 4 different flasks were prepared with 100ml of stock from Table 3, and with a loopful of inoculum was scooped and mixed into the stock each. Two flasks from different isolation methods were kept static at a degree of 32 Celsius and the other two in a rotary shaker at 37 degree for one week without discontinuity. The mucous layer was filtered through a filter paper and the media obtained was into a sterilized flask and the strands of the cellulose on the filter paper is scraped out on to a petri plate which is preweighed. The rhizobium was plucked with the help of forceps and splayed on a petri plate which was also preweighed. A snipping of the strands was isolated with autoclaved forceps and splayed on a microscope glass and focused at 40X.

Figure 4:- Harvsting of Bacterial Cellulose from A.Xylinum, Rhizobium and Pseudomonas sp.

Results:-

Cellulose extraction and Assay:-

The produced cellulose layers on the interface of the media and air were then harvested by pouring through a filter media in the case of *Acetobacter Xylinum* and *Pseudomonas sp*. The filtered strands were then noticed under the microscope. The experiment resulted in the sheet produced from *Acetobacter Xylinum* dissociate into strands which can be seen in the figure 5. It was also observed that no sheet was formed by Pseudomonas sp. And thus, there was neither strand nor sheet when filtration was performed. The sheet produced by Rhizobium was extracted and harvested using forceps and analysed under microscope to find both cellulose as well as contaminants in a link.

Figure 5:-Harvested Bacterial Cellulose

Media Constituent Analysis:-

In- order to understand the constituents used by the bacteria to produce bacterial cellulose- Lipid test, carbohydrate test, protein estimation, acetic acid test which includes both odour and sodium carbonate test and citric acid test.

Lipid test analysis:-

0.5gm powdered potassium bi-sulphate (KHSO4) was taken in a clean dry test tube, and 1ml of sample was added to each. It was mixed thoroughly and heated on a flame for 3 minutes. The sample taken was the filtered media in which the cellulose was produced. The result of the lipid test showed that there was a pungent and fruity smell when

the samples were heated in the test tubes containing the *A. Xylinum* and *Pseudomonas sp.* whereas the sample containing the Rhizobium Sample was devoid of any kind of smell.

Figure 6:- Lipid test result table

SAMPLE NAME	ODOUR	RESULT
A.XYLNIUM	Presence of pungent smell	Positive presence of lipids
PSEUDOMONAS SP.	Presence of pungent smell	Positive presence of lipids
RHIZOBIUM	Devoid of any smell	Negative presence of lipids

Carbohydrate analysis:-

The method used to check the if any carbohydrates are present especially sucrose was the usage of Benedicts test. The procedure used included the addition to 1.8ml of each media and control (distilled water), 10 drops of freshly prepared Benedict's reagent was added and mixed. It was then incubated in 45-degree water bath for 5 minutes. The benedict's reagent was prepared by 100g of anhydrous sodium carbonate, 173g of sodium citrate and 17.3g of copper sulphate pentahydrate to 1000ml of distilled water and mixed thoroughly and diluted. The result included a colour change present in the testubes containing *A. Xylinum and Pseudomonas sp.* , whereas the there was no colour change in the testube containing Rhizobium and the control which was added as distilled water.

SAMPLE	COLOR BEFORE HEATING	COLOUR AFTER HEATING	RESULT
A. XYLINUM	Dark green	Brick red	Carbohydrates present
PSEUDOMONAS SP.	Dark green	Brick red	Carbohydrates present
RHIZOBIUM	Light green	No change in color	Carbohydrates absent
CONTROL (D.W)	Light blue	No change in color	Carbohydrates absent

Figure 7:-Carbohydrate test analysis result

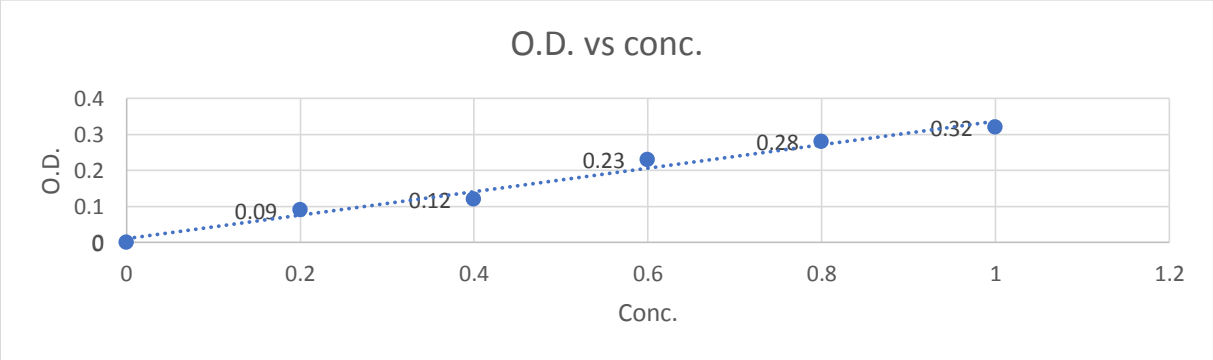
Citric Acid test:-

The citric acid test was done with the filtered media, where 10 test tubes was taken and labelled from 0 to 6 with 0, .2, .4, .6., .8, 1.0 and three test samples with 1.0ml each from the media in which the cellulose was produced. The testubes were made upto 1ml with distilled water and mixed well. 1ml of 9N sulphuric acid was added to the testtubes and then .3ml of 40% metaphosphoric acid and mixed well. It was then incubated in icebath for 10 min. 0.5ml of 2M potassium bromide was added along with 1.5 ml of 6% $KMNO^{4.}$ This was then vortexed and incubated in ice bath for 10 min.

To these test tubes 1X hydrogen peroxide was added dropwise till the colour became colourless or white. 5ml of petroleum ether was then added to the testtubes and mixed till two layers were formed. 2ml from the upper layers were pipetted out and added to new clean testtubes which were already marked. To these testtubes 6ml of 6% thiourea borax was added and mixed well till a golden colour was observed. It was then checked for O.D. at 440 nm.

SAMPLE	O.D.nm	Conc. Ug/ml
A.Xylinum	0.15	.44
Pseudomonas sp.	0.29	.90
Rhizobium	.03	.08

Figure 8:- citric acid test value and chart



Protien Estimation:-

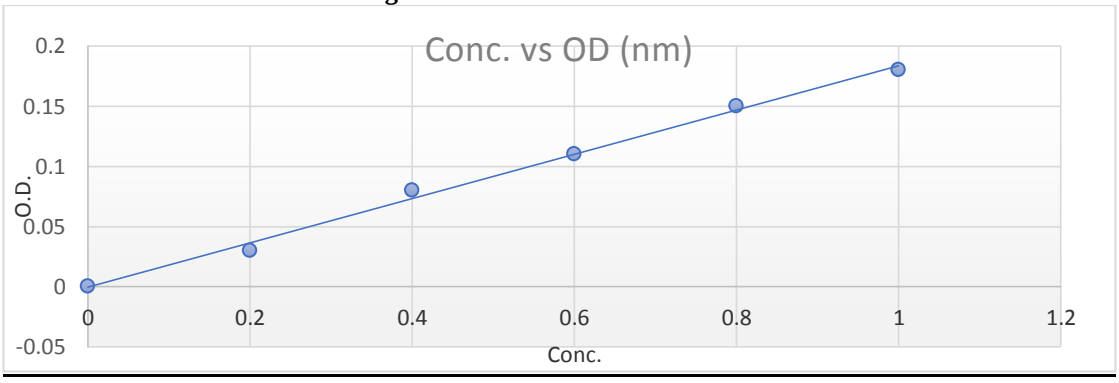
For the estimation of protiens, the usage of biuret method was utilized. This included tow different steps included preparation of standard and preparation of the sample.

Preparation of standard - Standard solution of bovine serum albumin was taken with conc. of 10mg/ml along with 6 test tubes each having 0,.2,.4….1ml of BSA respectively. It was then made up to 1ml volume with distilled water. To this, 9ml of biuret reagent was added and incubated at 37 degrees Celsius for 20 minutes. The OD of which was taken and noted at 540 nm. Preparation of Sample- The sample from the three media were taken and from each media 0.5 and 1 ml was taken and made up to volume. 9ml of biuret reagent was added to the test tubes and mixed well. They were incubated at room temperature for 2 minutes.

From these OD values a graph was drawn of conc. Vs OD and the conc. of protein in each of the samples was verified.

Sample amount Taken		O.D. (nm)
A. XYLINUM	*1.0 ml*	0.12
A. XYLINUM	*0.5ml*	0.04
PSEUDOMONAS	*1.0ml*	0.16
PSEUDOMONAS	*0.5ml*	0.08
RHIZOBIUM	*1.0ml*	0.07
RHIZOBIUM	*0.5ml*	0.02

Figure 9:- Protien estimation values and table



Discussion:-

The tests that were done proved that each media contained different amounts of various components and the amount of base feed was utilized in order to optimize the given situation. The experiment proved that the there was still sugar present in the first two bacteria and that rhizobium has completely converted the amount of sugar present in the media which shows that the amount of dextrose present in the media must be increased. It also proved that the

amount of citric acid present in the media attributed to having the lowest concentration present in the rhizobium media showing that the citric acid has been completely utilized as compared to pseudomonas while *Acetobacter xylinum* has a better utilization of citric acid and that that amount of citric acid present in the HS media must be reduced.

After the entire optimization of the media, the future step in this experiment includes the optimized production of the bacterial cellulose which will then be incubated into tanks with sewage water, paper effluent, and sugar industry effluent which will then help in the concept of waste water treatment and can also use this produced cellulose in cosmetics or in clothing industry.

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