ISSN 1996-3343

Asian Journal of Applied Sciences

http://knowledgiascientific.com

Knowledgia SCIENTIFIC
A Place to Publish Outstanding Research

Asian Journal of Applied Sciences 4 (3): 247-254, 2011
ISSN 1996-3343 / DOI: 10.3923/ajaps.2011.247.254

Production of Cellulose I Microfibrils from *Rhizobium* sp. and its Wound Healing Activity on Mice

[1]K. Parthiban, [2]S. Manikandan and [2]S. Ganesapandian
[1]Department of Microbiology, H.K.R.H College, Uthamapalayam, Theni (Dist), India
[2]Department of Marine and Coastal Studies, Madurai Kamaraj University, Madurai-21, India

Corresponding Author: K. Parthiban, Department of Microbiology, H.K.R.H College, Uthamapalayam, Theni (Dist), India

ABSTRACT

Bacterial cellulose essentially a high value special chemical with specific application and usage. Some is produced commercially as a source of highly pure polymer so called cellulose I. Current uses of bacterial cellulose are slowly gaining publicity with applications ranging from food additives and paper binding agents. The bacterial cellulose also used as temporary skin substitute. Modern medical biotechnology has accepted artificial skin as valid prospect. It has successfully applied by dermatologist and plastic surgeons. This can applied for human second and third degree skin burn, skin graft, face peeling, infectious dermolysis, trophic venous and chronic ulcers. The current study also aims to use the bacterial cellulose for the healing of wounds with slight alterations. Generally cellulose from *Acetobacter xylenum* was used for wound healing, but in this study cellulose from *Rhizobium* sp. was used. The bacterial cellulose is modified by mixing with alginate and examined their wound healing activity in experimental mice.

Key words: Bacterial cellulose, woundhealing material, cellulose I microfibril, modified bacterial cellulose

INTRODUCTION

Cellulose, the most wide spread biobased material on earth (Haigler and Benziman, 1982). Cellulose is synthesized by a variety of organisms ranging from multicellular and unicellular plants to bacteria. According to bacteria several general known to synthesize cellulose cite *Acetobacter* sp. and *Rhizobium* sp. so produce cellulose (Napoli *et al*., 1975). Cellulose synthesis is an intrinsic property of *Rhizobium* strains (Ross *et al*., 1991) cells produce cellulose fibrils as an extra cellulose product during growth (Cannon and Anderson, 1991) in the absence of plant cell (Delmer and Amor, 1995). Bacterial cellulose essentially a high value special chemical with specific application and usage (Brown, 1989). Bacterial cellulose essentially forms the basis for high quality diaphragm membranes in which the distribution of fibrils containing parallel joining of the glucan chains yield fibers possesing high tensile strength (Franz and Alban, 1995). Bacterial cellulose is essentially a high value special chemical with specific application and usage (Okiyama *et al*., 1993). Some is produced commercially as a source of highly pure polymer so called cellulose I. This Bacterial cellulose has a number of potential uses (Okiyama *et al*., 1992; White and Brown, 1989) and is manufactured in the form of dressing for patient with burns (Fontana *et al*., 1990), chronic skin ulcers, or other extensive less of tissue called Bio fill. The cellulose act as a temporary skin

substitute (Joris *et al*., 1994) with high mechanical strength in the wet state. The high water capacity of the oxygen permeable film appears to stimulate regrowth of skin tissue (Yoshinga *et al*., 1997; Hinman and Maibach, 1963). The cellulose act as a temporary skin substitute (Joris *et al*., 1994) with high mechanical strength in the wet state. Wound dressing based on alginic material is well known, in literature as well as from commercial point of view, in wound management (Paul and Sharma, 2004) alginate being a natural haemostat, alginate based dressings are indicated for bleeding wounds. The gel forming property of alginate helps in removing the dressing without much trauma and reduces the pain experienced by the patient during dressing changes. The combined use of alginate and a bio-occlusive membrane dressing in the management of split-thickness skin graft donor sites eliminated the pain and the problem of serum formation and leakage seen routinely with the use of a bio-occlusive dressing alone (Disa *et al*., 2001). The high water capacity of the oxygen permeable film appears to stimulate re-growth of skin tissue (Yoshinga *et al*., 1997; Hinman and Maibach, 1963).

This modified bacterial cellulose with alginates act as temporary skin substitute or Biofill. The present study was aimed to extract bacterial cellulose from *Rhizobium* sp. and characterization of bacterial cellulose.

MATERIALS AND METHODS

Extraction and purification of bacterial cellulose: *Rhizobium* sp. were isolated from the root nodules of leguminous plant (*Sesbania sesban*) around Chinnamanur, Theni District on 7th July 2005 (De Gallardo *et al*., 1971; Gunasekaran, 1999). After that root nodules were taken to the laboratory and the root systems were carefully washed with water.

The collected nodules were washed with water. The nodules were then soaked in 4% H_2O_2 for 2 min and then washed with sterile water for 3 min. Finally nodule treatment included soaking in a 0.1% $HgCl_2$ soln for 2 min an pure culture of *Rhizobium* sp. (Fordan, 1984) was obtained by performing the streak plate technique. One loop full of the nodule juice (inoculum) was streaked on YEMA plate. The plates are incubated at 28°C for 4-5 days. After incubation the white colonies are formed on YEMA plates. The YEMA media consist of mannitol -10 g, K_2HPO_4 -0.5 g, yeast extract -0.5 g, Mg SO_4 -0.2 g, NaCl -0.1 g, $CaCO_3$ -3.0 g, Cangored -10 mL (1:400) (Somasegaran and Hoben, 1994; Thangaraju and Santhanakrishnan, 1999).

Isolated culture of *Rhizobium* was grown in YEM broth for 3 days at ±28°C. After 3 days the culture broth was homogenized aseptically Then the homogenate was filtered through filter paper and the filtrate was then analyzed (Okiyama *et al*., 1992; Oikawa *et al*., 1995). To obtain pure cellulose the culture broth was washed with distilled water to remove medium components and treated with 0.5 M NaOH at 90°C for 1 h to eliminate bacterial cells. Then, it is centrifuged and removes supernatant. The cellulose pellets were rinsed extensively with distilled water until the pH of the water become neutral (Masaoka *et al*., 1993). The purified cellulose was dried to constant weight at 105°C and weighed.

Characterization of bacterial cellulose

FTIR: The concentrated 0.05 g bacterial cellulose was mixed with 0.05 g of KBr and make pellet. The pellet was analyzed through IR spectroscopy for the detection of C=O bonds O-H bonds and linkages of bacterial cellulose (Napoli *et al*., 1975). For FTIR measurement cellulose was spotted on a KBr crystal. A spectrum one FTIR spectrophotometer was used to measure the absorbance in the range of 4,000-700 cm^{-1} with a resolution of 4 cm^{-1} and 128 9 integration.

SEM: Scanning electron microscopic analysis of Napoli *et al.* (1975) cellulose I micro fibrils were used to understand the surface morphology.

Temporary skin substitute (Biofill) production: According to moist wound therapy (Winter and Scales, 1963) the active wound dressing may be in the form of foams, gels, hydrogel and aerosol (Kannon and Garrett, 1995). The temporary skin substitute is made by modification of bacterial cellulose (Ciechanska, 2004) with alginate (O'Donoghue *et al.*,1997; Paul and Sharma, 2004). The alginate (Thomas, 2000; Suzuki, *et al.*, 1999) and bacterial cellulose mixed in the ratio of 1:3 and then boiled at 60-80°C for 5 min and then oven dried for 1 h.

Animal studies: Four Balb/c mice were selected for the animal studies and named as M1, M2, M3 and M4. Before performing animal studies weight of the mice is noted. And then, subjected to anesthesia (chloroxy ether or sulfoxy ether). After that the animals back of this were then, shaved throughout its length.

The animals had been submitted the wound of circular format, carried through with punch located in the medium line of the dorsal region. The size of the wound is noted. And then the temporary skin substitute is coated to the mice M1 and M2 and other two mices M3 and M4 were allowed for natural healing (without applying modified cellulose). And effect of curing, hypersensitivity and effect of contraction were measured (Cabral *et al.*, 1987).

RESULTS

Pure culture isolation: The leguminous plants (*Sesbania sesban*) were collected from the various area arround chinnamanur, the number of nodules and their weight were measured and named (Table 1). Among these the weight of nodule R4 is high when compare with others. The bacteria *Rhizobium* were isolated from all the nodules. White, translucent, glistering elevated and comparatively small colonies were obtained on YEMA medium. Moreover, it don't take cango red dye, but some strains of *Rhizobium* sp. absorb cangored strongly (Subba-Rao, 1977). The symbiont isolated from the healthy root nodules was identified to be a *Rhizobium* species but the species was not ascertained.

Extraction of cellulose I fibrils: The cellulose I Fibrils were extracted from the *Rhizobium species* from the root nodules of all plant. The amount of cellulose I Fibrils produced was determined by dry weight basis. Table 2 shows the dry weights of the cellulose I fibrils. All *Rhizobial* strains produced cellulose I fibrils in fairly detectable amounts. But, the amount produced is variable. The high yield strain R4 was selected for further analysis and test. The nodule weight of R4 is high at the mean time the strain from R4 produced high amount of cellulose.

Table 1: Weight of nodules

Plant name	Place (Chinnamanur)	Soil type	No. of nodules/plant	Nodule weight (g)
R1	Site 1	Red soil	36	0.46
R2	Site 2	Red soil	28	0.38
R3	Site 3	Red soil	34	0.45
R4	Site 4	Red soil	38	0.48
R5	Site 5	Red soil	28	0.17

Table 2: Dry weight of cellulose I fibrils

S. No.	Nodule sample	Dry weight (g)/100 mL
1	R1	0.96
2	R2	0.83
3	R3	0.93
4	R4	0.97
5	R5	0.78

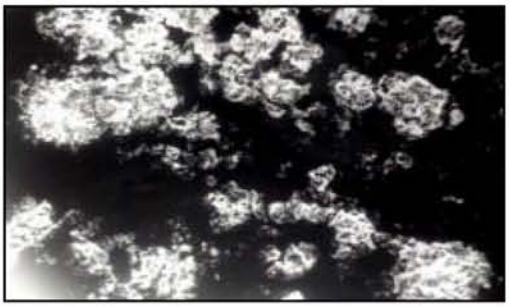
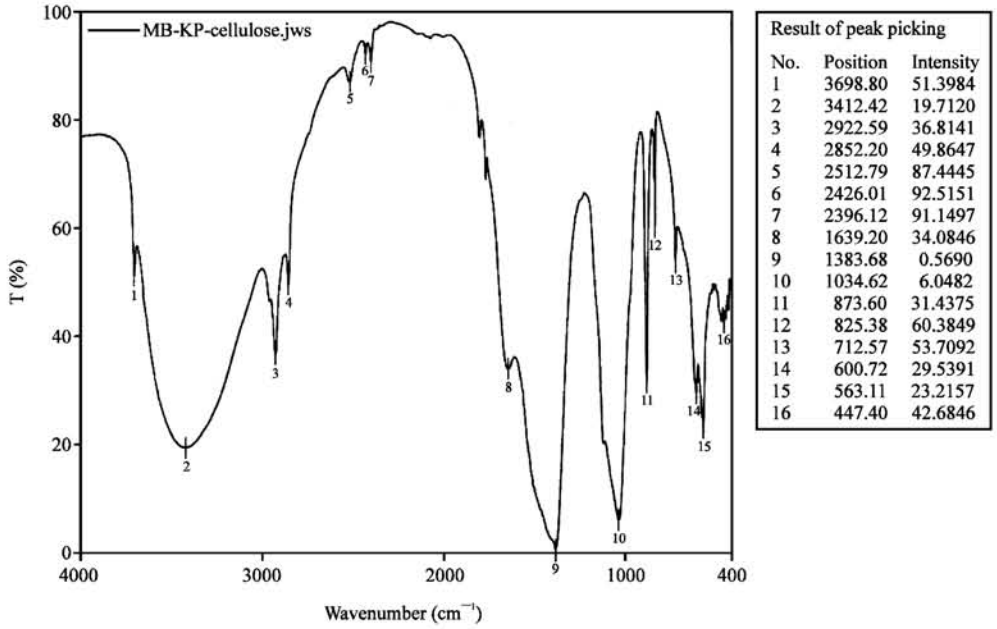


Fig. 1: Scanning electron microscopic view of cellulose I microfibrils at 600X



Result of peak picking

No.	Position	Intensity
1	3698.80	51.3984
2	3412.42	19.7120
3	2922.59	36.8141
4	2852.20	49.8647
5	2512.79	87.4445
6	2426.01	92.5151
7	2396.12	91.1497
8	1639.20	34.0846
9	1383.68	0.5690
10	1034.62	6.0482
11	873.60	31.4375
12	825.38	60.3849
13	712.57	53.7092
14	600.72	29.5391
15	563.11	23.2157
16	447.40	42.6846

Fig. 2: IR spectrum of bacterial cellulose

Scanning electron microscopic observation: Analyzing the cellulose-I fibrils on SEM Fig. 1 reveals that the fibrils form a unique ribbon 3-8 nm thick and approximately 100 nm wide (Reynolds, 1963).

Spectroscopy

FTIR spectroscopy: The extracted bacterial cellulose I fibrils were analyzed in IR spectroscopy. They exhibited absorption bands characteristic of the chemical groups and bonds in the cellulose

molecule. The band at 3650-3584 cm^{-1} indicates the presence of OH group (Napoli *et al.*, 1975) (Fig. 2). The band at 835-805 cm^{-1} indicates the presence of substitution. The band at 1385-1380 cm^{-1} indicates the presence of C-H vibrations. The band at 1750-1782 cm^{-1} indicated the C=O. Among the above results, it finally confirms the cellulose fibrils are produced by the bacteria (spectro metric identification of organic compounds-silverstein bassler).

Animal studies

Effect of biofill on wound recovery: The temporary skin substitute (modified bacterial cellulose with alginate) is applied to the experimental wound created in mice. The analysis of morphometric and morphological parameters (Table 3) were carried. The (Biofill) temporary skin has effective in curing wounds. Bio fill is a natural product that is derived from cellulose and alginate. This material is known in the wound management field for its haemostatic properties. Further, it also possesses other biological activities and affects macrophage function that helps in faster wound healing (Balassa and Purdden, 1984). It also has an aptitude to stimulate cell proliferation and histo architectural tissue organisation (Muzzarelli, 1989). The biological properties including bacteriostatic and fungistatic properties are particularly useful for wound treatment. On compairing the cellulose treated mice M2 heal faster than M1.

Like alginate material, there is also number of references on cellulose in wound treatment (Prudden *et al.*, 1970). Flexible, thin, transparent, novel cellulose alginate complex membranes caused an accelerated healing of incision wounds in a rat model compared with others application of the bio fill on full-thickness skin incisions made on the backs of mice significantly induced wound contraction and accelerated wound closure and healing compared with the untreated controls (Table 4).

Early returns to normal skin color at biofill areas were demonstrated. Treatment with cellulose and alginate demonstrated a substantial decrease in treatment time with minimum scar formation

Table 3: Wound recovery of Balb/c mice (M2) on applying with biofill

Day	Weight of wounded mice (g)	Regrowth or healing of skin	Hyper sensitivity reactions Yes/No
1	34.694	-	No
3	34.786	-	No
5	34.899	√	No
9	35.032	√	No
14	35.032	√	No
21	35.092	√	No

-: Absence of growth; √: Regrowth or healing of skin

Table 4: Wound recovery of Balb/c mice(M3) on without applying with Biofill

Day	Weight of wounded mice (g)	Regrowth or healing of skin	Hyper sensitivity reactions Yes/No
1	35.424	-	No
3	35.424	-	No
5	35.429	-	No
9	35.429	√	No
14	35.434	√	No
21	35.434	√	No
26	35.441	√	No
31	35.445	√	No

-: Absence of growth; √: Regrowth or healing of skin

on animals (Muzzarelli, 1989). An enzyme lysozyme is excreted from the wounds. These enzymes act on the bacterial cellulose and break them into bio active mono saccharides and bio active oligosaccharides which stimulates angiogenesis and tissues regeneration.

Generally the cellulose from *Acetobacter* origin is used in wound healing. But, this study is attempted to use cellulose from Rhizobacterial origin. The physio chmical properties of cellulose from *Rhizobium* sp. is similar to *Acetobacter* sp. In order to reduce the pain of wound dressing the bacterial cellulose is mixed with alginate. This alginate cellulose dressing maintains the wound in wet condition and helps to reduce the function of macrophages. As a result the animal is free of allergic reaction and the wound healing is faster than natural healing.

ACKNOWLEDGMENT

We thank to Tamilnadu State Council for Science and Technology an autonomous body run by Govt of Tamilnadu for providing financial assistance to this study.

REFERENCES

Balassa, L.L. and J.F. Purdden, 1984. Applications of Chitin and Chitosan in Wound Healing Acceleration. In: Chitin, Chitosan and Related Enzymes, Zikakis, J.P. (Ed.). Academic Press, San Diego, pp: 296-305.

Brown, R.M. Jr., 1989. Bacterial Cellulose. In: Cellulose: Structural and Functional Aspects, Kennedy, J.F., G.O. Phillips and P.A. Williams (Eds.). Ellis Horwood Ltd., England, pp: 145-151.

Cabral, L.M., M.D. Gattaz, L.A.P. Factore, J.A. Matter, D. Diament and A.M. Ollveira, 1987. Curativo biologico no tratamento do grandequeimado. Rev. Bras Cir., 77: 383-389.

Cannon, R.E. and S.M. Anderson, 1991. Biogenesis of bacterial cellulose. Crit. Rev. Microbiol., 17: 435-447.

Ciechanska, D., 2004. Biological dressing materials from modified cellulose. Research Project Report No 4T09B04222, Institute of Chemical Fibres, Lodz.

De Gallardo, D.E.J., R.M. Andres and E.T. Magno, 1971. A study on the isolation and screening of microorganism for production of diversed textured Nata. Phillipine J. Sci., 100: 41-51.

Delmer, D.P. and Y. Amor, 1995. Cellulose biosynthesis. Plant Cell, 7: 987-1000.

Disa, J.J., K. Alizadeh, J.W. Smith, Q.Y. Hu and P.G. Cordeiro, 2001. Evaluation of a combined calcium sodium alginate and bio-occlusive membrane dressing in the management of split-thickness skin graft donor sites. Ann. Plast. Surg., 46: 405-408.

Fontana, J.D., A.M. de Souza, C.K. Fontana, I.L. Torriani and J.C. Moreschi *et al.*, 1990. Acetobacter cellulose pellicle as a temporary skin substitute. Appl. Biochem. Biotechnol., 24-25: 253-264.

Fordan, D.C., 1984. Family lll *Rhizobiacea*. In: Bergey's Manual of Systematic Bacteriology. Krieg, N.R. and J.G. Holt (Eds.). Vol. I., The Williams and Willkins Co., Baltimore, pp: 233-234.

Franz, G. and S. Alban, 1995. Structure-activity relationship of antithrombotic polysaccharide derivatives. Int. J. Biol. Macromol., 17: 311-314.

Gunasekaran, S., 1999. Practical manual on microbial inoculants. Isolation and Characterization of *Rhizobium*. TN. Av. Coimbatore, pp: 3-8.

Haigler, C.H. and M. Benziman, 1982. Cellulose and other Natural Polymer Systems. Brown Plenum Press, New York, pp: 273-295.

Hinman, C.D. and H. Maibach, 1963. Effect of air exposure and occlusion on experimental human skin wounds. Nature, 200: 377-378.

Joris, K., F. Billiet, P. De Wulf and E.J. Vandamme, 1994. Enhanced Bacterial Cellulose Yield in Aerated *Acetobacter xylinum* Cultures by Adding Micro-Particles. In: Cellulosics: Materials for Selective Separations and Other Technologies (Polymer Science and Technology), Kennedy, J.F., G.O. Phillips and P.A. Williams (Eds.). Prentice Hall Publ., UK., pp: 239-245.

Kannon, G.A. and A.B. Garrett, 1995. Moist wound healing with occlusive dressings. Dermatol. Surg., 21: 583-590.

Masaoka, S., T. Ohe and N. Sakota, 1993. Production of cellulose from glucose by *Acetobacter xylinum*. J. Ferment. Bioeng., 75: 18-22.

Muzzarelli, R.A.A., 1989. Amphoteric Derivatives of Chitosan and their Biological Significance. In: Chitin and Chitosan, Sjak-Braek, G., T. Anthonsen and P. Sandford (Eds.). Elsevier, New York, pp: 87-89.

Napoli, C., F. Dazzo and D. Hubbell, 1975. Production of cellulose microfibrils by *Rhizobium*. Applied Microbiol., 30: 123-131.

O'Donoghue, J.M., S.T. O'Sullivan, E.S. Beausang, J.I. Panchal, M. O'Shaughnessy and T.P. O'Connor, 1997. Calcium alginate dressings promote healing of split skin graft donor sites. Acta Chir. Plast., 39: 53-55.

Okiyama, A., H. Shirae, H. Kano and S. Yamanaka, 1992. Bacterial cellulose I. Two-stage fermentation process for cellulose production by *Acetobacter aceti*. Food Hydrocolloids, 6: 471-477.

Okiyama, A., M. Motoki and S. Yamanaka, 1993. Bacterial cellulose III. Development of a new form of cellulose. Food Hydrocolloids, 6: 493-501.

Oikawa, T., T. Morino and M. Ameyama, 1995. Production of cellulose from D-Arabitol by *Acetobacter xylinum* KU-1. Biosci. Biotechnol. Biochem., 59: 1564-1565.

Paul, W. and C.P. Sharma, 2004. Chitosan and alginate wound dressings: A short review. Trends Biomater Artif. Organs, 18: 18-23.

Prudden, J.F., P. Migel, P. Hanson, L. Friedrich and L. Balassa, 1970. The discovery of a potent pure chemical wound-healing accelerator. Am. J. Surg., 119: 560-564.

Reynolds, E.S., 1963. The use of lead citrate as an electron opaque stain in electron microscopy. J.Cell. Biol., 17: 208-213.

Ross, P., R. Mayer and M. Benziman, 1991. Cellulose biosynthesis and function in bacteria. Microbiol. Rev., 55: 35-58.

Somasegaran, P. and H.J. Hoben, 1994. Hand Book for Rhizobia: Methods in Legume-Rhizobium Technology. 1st Edn., Springer, Verlag, New York, ISBN-10: 0387941347, pp: 450.

Subba-Rao, N.S., 1977. Soil Microorganisms and Plant Growth. Oxford and IBH Publishing Co. Pvt. Ltd., New Delhi, India, pp: 250.

Suzuki, Y., M. Tanihara, Y. Nishimura, K. Suzuki and Y. Yamawaki *et al.*, 1999. *In vivo* evaluation of a novel alginate dressing. J. Biomed. Mat. Res. Applied Biomat., 48: 522-527.

Thangaraju, M. and P. Santhanakrishnan, 1999. Practical Manual on Microbial Inoculants. TNAU, Coimbatore, pp: 3-8.

Thomas, S., 2000. Alginate wound dressing in surgery and wound management--Part 1. J. Wound Care, 9: 56-60.

White, D.G. and R.M. Brown Jr., 1989. Prospects for the Commercialization of the Biosynthesis of Microbial Cellulose. In: Cellulose and Wood - Chemistry and Technology, Schuerch, C. (Eds.). John Wiley and Sons Inc., New York, NY., pp: 573-590.

Winter, G.D. and J.T. Scales, 1963. Effect of air drying and dressings on the surface of a wound. Nature, 197: 91-92.

Yoshinga, F., N. Tonouchi and K. Watanabe, 1997. A novel polysaccharide involved in the pellicle formation of *Acetobacter aceti*. Biosci. Biotechnol. Biochem., 61: 214-224.