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RESEARCH ARTICLE

PREPARATION AND EVALUATION OF BACTERIAL CELLULOSE MEMBRANE AS AN ALTERNATIVE ADHERENT SURFACE MATERIAL FOR MAMMALIAN CELL CULTURE

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ABSTRACT

In mammalian cell culture, modified polystyrene (PS), polyethylene terephthalate (PET) discs are being used for adhesion of cells during packed bed fermentation. These discs are expensive, not easily available and non biodegradable. Hence, in the present study, bacterial cellulose (BC) membrane produced by *Gluconacetobacter intermedius* was tested as an alternative adherent surface material for anchorage dependent mammalian cell culture. The recombinant CHO cells were adhered poorly to native BC. However, charge modification of the BC carried out using 3-chloro-2-hydroxypropyl trimethyl ammonium chloride, supports CHO cell adherence, survival and proliferation. The production of recombinant erythropoietin from CHO cells adhered to modified BC was comparable to that of commercially available synthetic membranes. These results suggest potential of bacterial cellulose as an economical alternative to synthetic polymer substrate for adherent cell cultures.

INTRODUCTION

Mammalian cells are widely used for production of recombinant proteins for human use that include vaccines, hormones, antibodies and therapeutics. Majority of the animal cells used for the purpose are anchorage dependent and require attachment to a surface for their survival, growth and proliferation (Freshney 2005). Various substrates derived from natural and synthetic polymers have been used for cultivation of mammalian cell cultures. Commonly used synthetic polymers for adherent cell culture are of polystyrene (PS) and polyethylene terephthalate (PET). Although these synthetic polymers provide excellent anchorage dependent growth and proliferation of cells, the cost of using them limits their usage for large scale applications. Moreover, they are non-biodegradable thereby posing the problem for their disposal. Cellulose is considered as most abundant polymer in nature. It consists of polymer of glucose monomers joined together by β 1,4 glycosidic bond (Lubert Stryer, 2009). Cellulose has been widely used in textile, paper industry, food industry and pharmaceutical industry. It is found in cell wall of plant cell and synthesized by bacteria, algae, fungi and some animals. Recently, cellulose derived from bacterial source has garnered greater interests of researchers for its application in biomedical field (Zhijiang *et al.*, 2011 and Ahrenstedt *et al.*, 2010) due to

properties such as high mechanical strength, high crystallinity, hydrophilic nature and pure nanofibril network (Astley *et al.*, 2001). Bacterial cellulose (BC) has been used as scaffold for tissue engineering, wound healing and as a substrate for mammalian cell cultures. Studies have shown adherence of variety of cell types namely endothelial cells, smooth muscle cells, chondrocytes, neuronal cell, mesenchymal cells and stem cells to BC (Awad *et al.*, 1999 and Backdahl *et al.*, 2006).

Chinese Hamster Ovary (CHO) cells are the most preferred mammalian cell line for production of recombinant proteins in biopharmaceuticals. They are either grown as adherent cultures or as a suspension culture. The cost of recombinant bio-therapeutic product is mainly governed by cost of processes employed in the production and purification of protein. Keeping these views in mind present study was undertaken to evaluate the potential of BC to support the growth of recombinant CHO cells and subsequently to affect the production of recombinant proteins.

MATERIALS AND METHOD

Chemicals and materials

Recombinant Chinese Hamster Ovary (CHO) cells were obtained from Gennova Biopharmaceuticals Ltd, Pune (India).

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3-chloro-2-hydroxypropyl trimethyl ammonium chloride and Calcofluor white were procured from Sigma Aldrich (Germany). Sodium hydroxide and acetic acid was obtained from Merck specialities Private Ltd (India). Iscove's Modified Dulbecco's Medium (IMDM); Chinese hamster ovary cells - suspension culture – Serum Free medium (CHO-S-SMF-II); Dialyzed fetal bovine serum (DFBS) and Trypsin was procured from Invitrogen Corporation (India). High purified water was prepared by using Millipore (Milli-Q®) water purification system.

Isolation of cellulose producing bacteria from grape pulp.

The crushed Indian grape pulp was incubated in glass flask at room temperature for 15 days. At the end of the incubation, one ml of the grape pulp was inoculated in 50 ml of Hestrin and Schramm (HS) broth containing 40 μg/ml mycol as a fungicidal and incubated at 30°C for 6 days. At the end of the incubation, one ml of the broth was serially diluted and inoculated on HS agar media containing calcofluor white (50μg/ml) to identify the cellulose producing bacteria. The high yielding cellulose producing bacteria were screened and further identified based on 16s ribosomal sequencing.

Cellulose membrane preparation

Cellulose membrane was prepared using the method described by Hestrin and Schramn (1954). Briefly, the identified high yielding cellulose producing bacteria was inoculated in 50 ml HS broth (pH 5.0) in a 250 ml conical flask and incubated at 30°C for 6 days as a static culture. At the end of the incubation, the pellicle produced on the surface of the broth was harvested and washed twice with distilled water and further treated with 0.5 M NaOH solution at 90°C for 30 minutes. The treated cellulose was washed with distilled water until pH of the washed solution became neutral. The washed BC was dried in an oven at 105°C to obtain bacterial cellulose sheet.

Preparation of cationic cellulose membrane

The cationic membrane was prepared using the method described by Peter *et al* (2004). Briefly, the obtained bacterial cellulose sheet (5 g) was cut into small discs (6mm) and soaked overnight with constant stirring at room temperature in 69% w/v solution of 3-chloro-2-hydroxypropyl trimethyl ammonium chloride. The pH of the solution was adjusted to 10.5 using NaOH solution (50% w/v). At the end of the soaking, the temperature of the solution was raised to 95°C and maintained for 4 hours in water bath. After attaining the room temperature the pH of the solution was adjusted to 7.0 using acetic acid solution. The treated bacterial cellulose discs were collected and dried in an oven at 105°C for 20 minutes for use in cell culture.
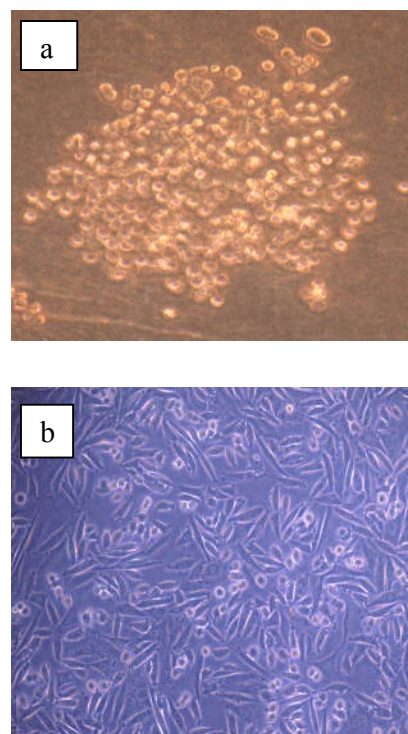
CHO cell culture using Bacterial cellulose membrane

The recombinant CHO cells were cultured in tissue culture flask (TCF-175) and grown to 90% confluence in Iscove's Modified Dulbecco's Medium (IMDM). The cells were harvested by trypsinization and further suspended in 120 ml of IMDM. Thirty milliliter of cell suspension were transferred separately in three untreated tissue culture flasks (T-175)

containing 150 mg of previously sterilized bacterial cellulose membrane discs, Fibra discs and BioNOC-II discs. Remaining 30 ml of the suspension was transferred to untreated T-175 flask as control. The flasks were incubated at 37°C for 6 hrs in a CO_2 incubator (Heracel 150). At the end of the incubation, the medium was decanted and replenished with 30ml of IMDM containing 10% dialyzed fetal bovine serum (Growth Medium) and flasks were incubated at 37°C for 48 hrs in a CO_2 incubator . After incubation, growth of the cells on supporting materials and control flask was observed by microscopy. Medium was replaced from IMDM containing 10% DFBS to CHO-S-SMF-II containing 10% dialyzed FBS as a production medium. All flasks were incubated at 37°C in CO_2 incubator. After every 48 hours interval the spent medium was collected and replaced with fresh medium and the cultures were maintained for one month. The collected medium was estimated for the production of recombinant erythropoietin by ELISA method using quantkine EPO ELISA kit from R&D system (DEP00).At the end of experiment, the cells were trypsinised in 30 ml Chinese hamster ovary suspension serum free medium (CHO-S-SFM-II medium) and cell count and viability was estimated using trypan blue.

RESULTS AND DISCUSSION

Substrates for growing mammalian cell culture incur substantial cost to production of vaccines and biotherapeutics. In the present study, bacterial cellulose derived from *G. intermedius* was tested for ability to support adherence and growth of recombinant CHO cells.

Fig. 1. Adherence of CHO cells to a) Modified bacterial cellulose b) Tissue culture flask

The comparison was done with commercially available Fibra discs and Bionoc-II substrates for cell adherence as viewed by phase contrast microscopy and production of recombinant
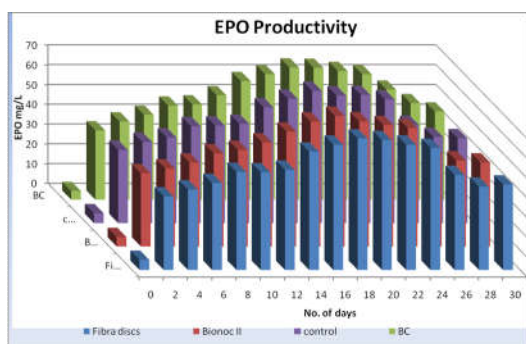
Fig. 2. Productivity of recombinant protein using different discs for period of one month

erythropoietin. Initially we tried to grow recombinant CHO cells on native BC derived from static culture of *G. intermedius* albeit less success. The cells adhered poorly to the native BC while Fibra disc and Bionoc-II supported adherence and growth of recombinant CHO cells. However, charge modification of native BC using 3-chloro-2-hydroxypropyl trimethyl ammonium chloride to cationic charge exhibited greater cell viability and adherence compared to native BC (Figure 1). The modified BC was further used to assess the efficacy of erythropoietin production. The production of erythropoietin gradually increased in culture supernatant of cells seeded on modified BC. Peak concentration was found on day 18 of culturing after which the levels decreased gradually till the end of observation period. Similar results were observed when the cells were seeded on Fibra discs and Bionoc-II membranes. The productivity of recombinant erythropoietin in culture supernatant ranged from 35.01 to 66.77 mg/liter, 37.15 to 66.43 mg/liter, 37.01 to 66.04 mg/liter and 37.00 to 66.80 mg/liter for bacterial celluloses membrane discs, Fibra discs, BioNOC-II and control TC flask, respectively (Figure 2).

Table 1. CHO cell counts

Sr. No.	Description	CHO Cell count per flask
1	Control flask	1.7×10^7
2	Bacterial cellulose	1.8×10^7
3	Fibra Discs	1.8×10^7
4	BioNoc II	1.85×10^7

The cell counts after one month was also near about same in all supporting materials (Table 1). These results suggest that productivity of recombinant protein using modified BC membrane is comparable to control and other commercially available discs used in the study. Different bacterial genus have been reported to produce cellulose viz *Aerobacter, Gluconacetobacter, Achromobacter, Agrobacterium, Alacaligenes, Azotobacter, Pseudomonas, Rhizobium and Sarcina* (Deinema and Zevenhuizen 1971 and Ross *et al.* 1991). The genus *Gluconoacetobacter* is the most efficient producer of cellulose which is preferred for applied studies on cellulose (Cannon and Anderson 1991). In a suitable culture media, it produces an extra-cellular gel-like material or pellicle, which comprises of a random assembly of cellulose ribbons; composed of a number of micro-fibrils (Lynd *et al.* 2002) have the properties of high tensile strength, water absorbing and dynamic fiber forming capacity (Ring *et al.* 1986 and Hoenich 2006). Studies have shown that BC when used as a substrate for cell culture is not harmful to cells (Kim

et al. 2010 and Anderson *et al.* 2010). In addition, BC implants were well tolerated by mice generating only a mild inflammatory response suggesting reduced risk of leachable molecules from BC substrates (Mendes *et al.* 2009). Wantanabe *et al.* (1993) investigated BC to support adherence of mammalian cell culture. They found that untreated BC membrane did not support the adherence and growth of various cell lines. However, when the membranes were charge modified the cell line was able to adhere, grow and proliferate on the BC indicating adherence of cells to BC is charge dependent. A limitation of BC is that nanofibrils from a strong interwoven network that prevent cell infiltration. To improve the cell infiltration into the nanofibrils porous membranes are being developed by using porogens. Better infiltration of smooth muscle cells into the cellulose pores have been demonstrated (Backdahl *et al.* 2006). Chemical engineering of cost effective, biodegradable BC has potential to replace synthetic polymer for cell culture fermentation in future.

Conclusion

During this investigation, cellulose producing bacteria was isolated from Indian grape pulp and identified as *Gluconacetobacter intermedius* on basis of 16s ribosomal sequencing (Gen Bank accession no. Y14694). The bacterial cellulose membrane was developed successfully as an adherent surface material for mammalian cell culture. The adhesive efficiency an d productivity were found to be comparable to that of commercially available discs which are very costly. Hence, the bacterial cellulose membranes can be used as an alternative cost effective and environment friendly adherent surface material for cell culture in biopharmaceutical industries for commercial production of health care products.

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