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Production of monoclonal antibodies for breast cancer by HB8696 hybridoma cells using novel perfusion system

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ABSTRACT

Perfusion culture using spinfilters have been used for the production of health-care products using mammalian cells culture. However, available spinfilters are either highly prone to clog and/or are disposable and hence affects product formation. To address these problems, a novel non-woven *Bombyx mori* silk screen based spinfilter module for clog-free extended perfusion culture of hybridoma cells has been designed. The module is versatile in nature and reusable, after autoclaving and replacement of used polymeric membrane. Its application for clog-free extended perfusion culture was demonstrated by comparative perfusion experiments of HB8696 cells with stainless-steel spinfilter. HB8696 cells produce monoclonal antibodies (MAbs) 520C9 active against breast cancer oncoprotein. Silk spinfilter was found to be less prone to clog with cells and debris owing to its negatively charged hydrophobic screen compared to the positively charged hydrophilic stainless-steel spinfilter. Therefore, it provides extended cell growth phase and production phase of up to 56 h and 40 h respectively and 57.4% increase in MAb productivity compared to the stainless-steel spinfilter. The effect of different perfusion rates on MAb production was studied and an optimal MAb productivity of 1.6 g L^{-1} day^{-1} was achieved.

1. Introduction

Spinfilters have been primarily used for the perfusion culture of hybridoma cells due to their selective cell retention efficiency and low shear stress on cells [1–8]. But the primary drawbacks associated with existing spinfilter systems are either high filter clogging and/or disposability. For instance, positively charged hydrophilic stainless-steel spinfilters are highly prone to clog with negatively charged cells and cell debris [9–12] which lowers process productivity. To circumvent this problem, many strategies have been previously reported, such as the addition of deoxyribonuclease I to the culture medium [11], variation of perfusion rate, screen pore size and rotational speed of the spinfilter [13,14], application of neutral and hydrophobic filter screens [8,9,15] and use of ultrasonic vibrations [7], etc. Considering the fact that neutral or negatively charged hydrophobic polymeric filter screens are less prone to clog, polymeric spinfilters such as ethylene-tetrafluoroethylene (ETFE), polyamide and polytetrafluoroethylene (PTFE) screen based spinfilter [9,10], polyester screen based spinfilter P [16], etc. were also designed. However, most of these spinfilters are disposable in nature.

Therefore, for improving the process productivity during perfusion culture of hybridoma cells, a novel perfusion system has been designed. The biocompatibility of silk to mammalian cells [17] has been used as a selective advantage in designing a novel non-woven *Bombyx mori* silk membrane based reusable spinfilter module for clog-free extended perfusion operation of hybridoma cells. Since breast cancer is one of the most widespread invasive cancers worldwide and accounts for approximately 29% of all cancers in women [18], hybridoma cells produces MAb against breast cancer were used in this study as a model system. Comparative perfusion experiments of HB8696 hybridoma cells that produce MAb 520C9 active against breast cancer oncoprotein C-erbB2, were performed using stainless-steel spinfilter to demonstrate the applicability of silk spinfilter for clog-free extended perfusion operation. To enhance the MAb productivity, the perfusion rate for high MAb productivity by HB8696 hybridoma cells was also optimized while keeping the spinfilter rotation speed below the critical shear stress of cells. The

Abbreviations: MAb, monoclonal antibody; SS 316, stainless steel 316; DMEM, Dulbecco's modified Eagle's medium; FBS, foetal bovine serum; LDH, lactate dehydrogenase; IgG_1, immunoglobulin G subclass 1.

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variation in perfusion rate was chosen as the primary parameter for improving process productivity because increase in perfusion rate reduces nutrient depletion and waste product accumulation that maximizes cell growth and productivity [19,20]. Nevertheless, due to cell wash out and high shear stress on cells, at high perfusion rates [20], this parameter needs to be optimized.

2. Materials and methods

2.1. Cell line and cultivation medium

Two cell lines: the non-adherent mouse–mouse hybridoma cell line HB8696 (obtained from ATCC, LGC Promochem Ltd, Bangalore), which produces monoclonal antibody immunoglobulin G subclass 1 (IgG1) active against breast cancer onco-protein C-erbB2 and the adherent human fibrosarcoma kidney cell line HT1080, obtained from NCCS, Pune) were used in this study. HB8696 cells were grown in Hybricare medium (ATCC, 46-X) and HT1080 cells were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco, USA). Both the mediums were supplemented with Penicillin–Streptomycin solution (5%, v/v; 100 IU/mL and 0.1 mg/mL, respectively; Sigma–Aldrich Corp., MO) and heat inactivated foetal bovine serum (FBS; 20%, v/v; Biological Industries, Israel). Cells were thawed from frozen vials and grown in 25 cm^2 tissue culture flasks (Corning, USA) in a humidified 5% CO_2 incubator controlled at 37 °C.

2.2. Design of reusable spinfilter module

The reusable spinfilter module for perfusion culture of hybridoma cells was constructed using stainless-steel (SS) 316 and Teflon (Indian patent application number 2509/DEL/2012). Its cylindrical polymeric membrane supporting component was made up of Teflon with fine holes drilled in it that permits continuous media exchange during perfusion culture. It has two detachable silicone rubber O-rings and a SS-316 clamp to hold the polymeric filter membrane in place. The cylindrical component was attached to a SS-316 base, having an SS-316 Allen screw attached to it, which helps in its positioning at desired depth on the motor shaft of the bioreactor (Fig. 1a and b).

All the materials used in its construction were biocompatible to mammalian cells, impervious to cell culture media constituents, chemical and corrosion-resistant, and withstand high temperature up to 121 °C. Thus the designed spinfilter module is autoclavable and can be easily cleaned. The designed spinfilter module is reusable, as after completion of one perfusion run the same module can be used again after autoclaving and replacement of used polymeric membrane with the new one. The designed spinfilter module is versatile in operation, as any biocompatible polymeric membrane, selected according to the type and size of cells cultured, can be mounted over it as a filter screen. The module is presently designed for a 2-L bioreactor system, but it is amenable to scale-up to a higher volume (the data will be communicated shortly).

2.3. Characterization of silk membrane as filter screen

For selecting a non-woven *Bombyx mori* silk membrane as filter screen for retention of hybridoma cells, the following surface properties (wettability, surface charge density and pore size) of three different non-woven *Bombyx mori* silks: Seri-DSS, Seri-FSS and BF27PV (obtained from Sericare, Bangalore) and their effects on cell adhesion and proliferation were evaluated:

2.3.1. Wettability

Wettability of both surfaces of different silk membranes was evaluated by measuring their mean water contact angle value using contact angle goniometer (DSA100, Kruss GmbH, Germany). For each measurement, droplet of 3 μL of Milli-Q water was placed on 3 cm × 3 cm strip of each membrane and the contact angle value was measured. Three measurements were done for each side of the membrane and the mean value was calculated

2.3.2. Surface charge density

Surface charge density of each silk membrane was analyzed by measuring their zeta potential value using cylindrical cell of electrokinetic analyzer with Ag/AgCl disc electrodes (Anton Paar GmbH, Graz, Austria). For each measurement, an irregular plug of each silk membrane was placed between the two electrodes and the measuring fluid (0.001 mol/L KCl solution) was passed through them. For all the silk membranes, three zeta potential values were measured and the mean value was calculated. The pH was maintained by addition of 0.1 mol/L KOH or 0.1 mol/L HCl solution.

2.3.3. Pore size

Pore size of each silk membrane was measured in triplicates using their three scanning electron micrographs and IMAGE-J software (National Institutes of Health, USA, version 1.41). The images were captured using three strips of each silk membrane (5 mm × 5 mm each) by scanning electron microscope (Evo50, Zeiss, UK).

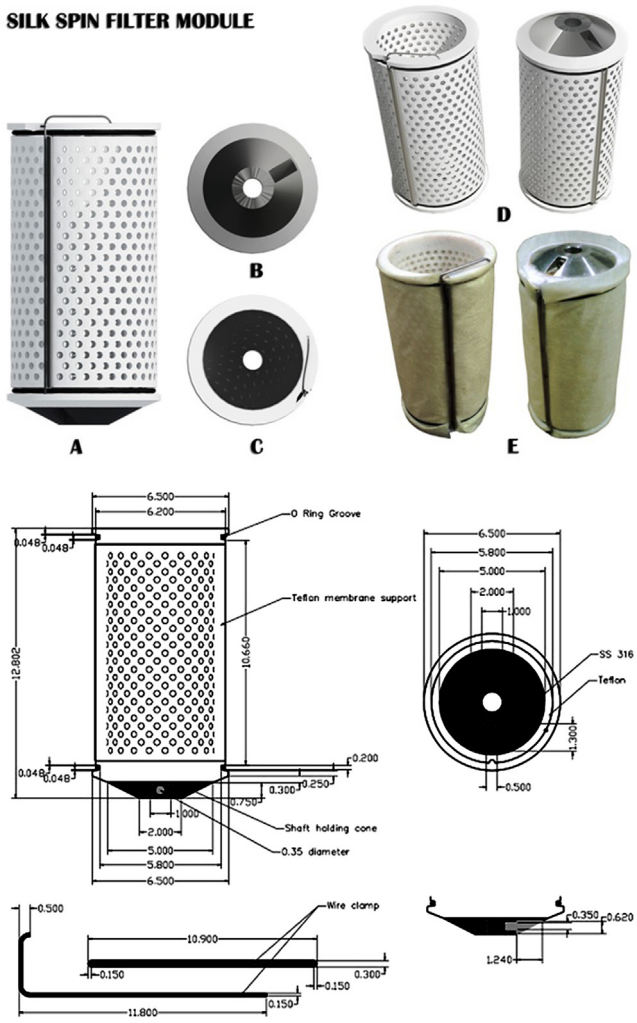

Fig. 1. (a) 3D image of silk spinfilter generated by Autodesk 3ds max software. (A) Porous teflon membrane support, (B) shaft holding cone of stainless steel with Allen screw, (C) metallic clamp for fixing the membrane, (D) top and bottom view of spinfilter module, (E) top and bottom view of spinfilter module with silk. **(b)** Dimensions of different components of silk spinfilter. The components clockwise from the top left panel are porous teflon membrane support, stainless steel shaft holding cone, Allen screw for fixing the module and stainless steel clamp for holding the membrane. All the dimensions are in centimetres.

2.3.4. Cell attachment supporting nature of silk membranes

The mammalian cell attachment supporting nature of each silk membrane (Seri-DSS, Seri-FSS and BF27PV taken separately) was tested by culturing adherent HT1080 cells on them in 24-well plates (Corning, USA). Growth medium used was DMEM supplemented with 20% heat inactivated FBS and 5% penicillin–streptomycin solution. For each of the three membranes, two 24-well plates were used. The two other 24-well plates without membrane were used as control. Together, these eight 24-well plates constituted a set for one experiment. Three sets of these experiments were performed. In each of the wells, 1 cm diameter circular piece of membrane was taken. The seeding density of 1.4×10^5 cells/mL was used for each well and incubated in a humidified 5% CO_2 incubator at 37 °C. After every 8 h, samples were drawn from three wells of each membrane-type and control plates by trypsinization using 0.25% trypsin (Gibco, USA). At each sampling instant viable cell densities were measured in triplicates.

Along with this, for visualization of attached adherent HT1080 cells on different silk membranes, their scanning electron micrographs were also captured after 72 h using the Evo50 scanning electron microscope (Zeiss, UK).

2.3.5. Cell proliferation supporting nature of silk membranes

Proliferation of mammalian cells in the presence of three different silk membranes (Seri-DSS, Seri-FSS and BF27PV taken separately) was tested by culturing non-adherent HB8696 hybridoma cells on them in 24-well plates in exactly the same manner as described in Section 2.3.4. Growth medium used was Hybricare 46-X medium supplemented with 20% heat inactivated FBS and 5% penicillin–streptomycin solution. The seeding density of 1.4×10^5 cells/mL was used