A SCALABLE 30 ml BIOREACTOR FOR BIOPHARMACEUTICAL SCREENING

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Abstract.

Glycosylated Biopharmaceutcals are usually produced by suspended mammalian cells cultured in stirred tank bioreactors. However, screening protocols at the lab scale (i.e. to find optimum culture media or initial cell density) mainly rely on Petri dish, bottle or Erlenmeyer flask culture. There is an evident need for small scale fully scalable mammalian cell culture mini-bioreactors for screening processes. Here, we study the mixing performance, mass transfer capabilities, and culture adequacy of 30 ml eccentric stirred tanks. A detailed mixing characterization of the proposed bioreactor is presented. LIF experiments and CFD computations are used to identify operational conditions required for adequate mixing (globally chaotic conditions). Adult stem cells are cultured in this novel bioreactor system. Our results demonstrate good mixing performance, culture convenience, and potential scalability of the proposed screening bioreactor.

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INTRODUCTION

Nowadays, most biopharmaceuticals are recombinant glycoproteins secreted by mammalian cells under culture in large scale stirred tank bioreactors (Nienow, 2006), typically in the range from 50 to 20,000 L. Preceding that stage, the development path for a biopharmaceutical molecule implies extensive rounds of screening to select fast growth high producing clones adapted to suspension conditions. Commonly, the first portion of that selection process is done in very small volume systems, in vessel geometries that share nothing in common with industrial stirred tanks. Petri dishes and culture bottles of 20 to 50 ml continue to be the preferred containers to grow and select high producer cells at the lab scale. Afterwards, selected clones are adapted to suspension conditions in Erlenmeyer flasks ranging in volume from 200 to 1000 ml. However, the hydrodynamic conditions in Erlenmeyer flasks are very different with respect to the next scaling stage, typically a stirred tank bioreactor. Recently, new commercially available small scale bioreactors had appeared as options to the use of Erlenmeyer flasks. For example, DASGIP®, a medium size firm specialized in the manufacture of bioreactor systems for culture applications, offers platforms of fully instrumented bioreactors capable of controlling oxygen concentrations, pH, inlet flow rates, etc. to operate in the range of 300 to 1000 ml. Therefore, it is until scales of 300 and up are reached that stirred tank systems can be used to continue development. In consequence, there is an uncovered niche for screening systems, stirred tank-like, that can partially substitute the activities now done in culture bottles and Petri dishes. In this contribution, we examine the performance of a 30 ml stirred tank micro-reactor system potentially useful for mammalian cell culture operations.

Mixing and adequate oxygen mass transfer is not easily achievable in small scales. In larger systems, agitation with marine turbines causes turbulence as the main mixing mechanism. Under turbulent conditions, oxygen dosed through fine spargers is correctly distributed, and typical bubble residence times allow for adequate oxygen mass transfer. As scale reduces, adequate gas dispersion will demand higher agitation intensities. On the other hand, smaller scales will cause shorter residence time for air bubbles. More of them will achieve the surface of the tank, trapping cells at their surface. Both, high shear rates and bubble damage have been studied as possible factors of lethal and sub-lethal damage to mammalian cells, recognized as more fragile to forces than bacteria or yeast (Keane *et al.*, 2003; Amanullah *et al.*, 2004). This framework almost precludes the use of typical stirred tank or impeller designs for very small scales. Here we propose a geometry design, capable of adequately mix in the laminar and transitional regime, as we demonstrate by Laser Induced Fluorescence and CFD analysis. We culture human adult stem cells to demonstrate the potential of the proposed system for screening applications.

MATERIALS AND METHODS

Stirred tank system. The stirred tank geometry analyzed here is depicted in figure 1. A 30 ml glass round bottom vessel was agitated using a disc impeller eccentrically located. The impeller (0.6 cm radius) was positioned at an eccentricity of E=0.21, where eccentricity (E)

is defined as the distance between the shaft and the vertical centerline of the tank/tank radius. Agitation was set at 75 RPM. Temperature was maintained at 37°C by wrapping the tank with heated plastic tubing.

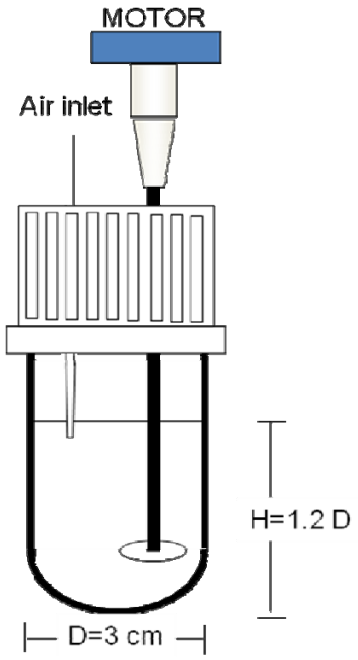

Figure 1. Schematic representation of the bioreactor system.

Flow visualization experiments. All visualization experiments reported here were done using glycerin (viscosity equal to 1200 cP/mPa s at 22°C) as fluid. Mixing 3D patterns within the bioreactor where observed through fluorescein injections (a fluorescent tracer) in a dark room where UV light was the only illumination source. To better resolve the evolution of mixing structure in time, laser induce fluorescence experiments (LIF) were conducted. In an otherwise dark room, a plane of laser light was projected towards the experimental system, to visually expose the mixing structure at a vertical 2D plane (slightly behind the rotation axes) of the bioreactor originated by the injection of a pulse of rhodamine dye. Photographic documentation of the visualization experiments was done using a professional digital camera model Rebel XSi from Canon®. In order to correct for optical aberration, the bioreactor was immersed in a transparent cubic chamber filled with glycerin to also match refractive index.

CFD simulations. Computational Fluid Dynamics (CFD) experiments were done using a xw8600 HP Workstation computer with 2 Intel Xeon Quad Core processors and a 512 Mb. Nvidia Quadro FX3700 graphic card. . Geometries were constructed using the software SolidEdge ® from UGS. Meshes were built using software from ACUSIM®. Velocity field was calculated by solution of the Navier Stokes Equations at 200,000 nodes using ACUSOLVE from ACUSIM® Sofware Inc., Mountview Ca. ACUSIM® is based on a special stabilized Galerkin/Least Squares (GLS) finite element technology formulation of the full transient 3D non-linear equations of fluid dynamics. The fluid was modeled primarily as a newtonian fluid with the nominal properties of water.

Stem cell culture. 25 ml of cell suspension at a concentration of $1.75X10^4$ cells/ml was seeded in the tank, such that the ratio of vessel width to liquid height was 1:1.2. DMEM F-12 supplemented culture media [10% FBS (lot), 1% penicillin-streptamycin solution, 10 mM HEPES,] was changed every 24 hours according to a feed batch protocol, where agitation was suspended for 5 minutes to allow cells to settle in the bottom of the tank and 80% of the culture media was removed from the upper portion of the tank using a sterile syringe and replaced by fresh media warmed to 37^{o}C. 1 ml samples of cell suspension were removed from the upper third portion of the tank for counting every 36 hours. Cells were stained with Trypan blue at a concentration of 10% and counted using a Neubauer camera. Here, we report results from daily replacement with pH-controlled culture media. RNA profiles were analyzed by PCR following addition of primers for CD133 and CD34 regions to verify if cells maintained un-differentiation during culture.

RESULTS AND DISCUSSION

The mixing visualization experiments reported here were conducted in glycerin. Glycerin retards the effect of diffusion, allowing for a cleaner observation of the structural effect of convective mixing at very low speed. Figure 2(a) and(b) shows the 3D mixing patterns arising from operation at laminar speed (Re~30, agitation=300 RPM in glycerin). In terms of Reynolds number values, and consequently mixing structure, these conditions will be approximately equivalent to those observed in water at 0.25 RPM.

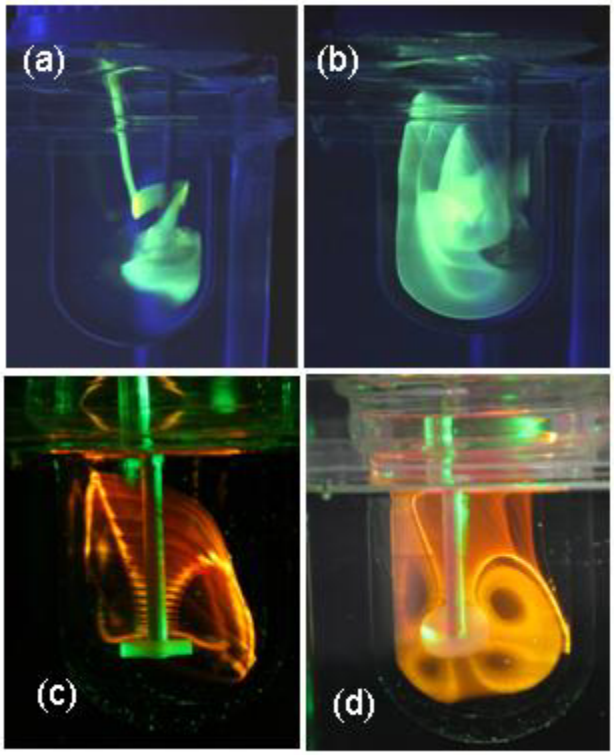

Figure 2. Mixing visualization experiments: (a) laminar flow patterns are revealed through a fluorescent tracer injection under UV light in a eccentric system (E=0.25) 10 seconds after injection, and (b) one

minute after injection; (c) LIF experiments reveal the mixing structure of eccentric stirred tank systems agitated by horizontal flat disc impellers and (d) 45 angle disc impellers at low Re number.

The mixing structure observed in the proposed bioreactor is highly complex. But even at this extremely low agitation values, liquid mixing is achieved in reasonable times (less than 10 minutes). Figure 2(c) and (d) show 2D planes of the mixing structure observed at such low Re values. Small structural features of the flow reveal the coexistence of chaos and order. Chaotic regions of adequate mixing coexist with areas of low mixing intensity and regular behavior, as previously reported (Lamberto *et al.*, 1999; Álvarez, 2002; Álvarez et al., 2002; Cervantes *et al.*, 2006).

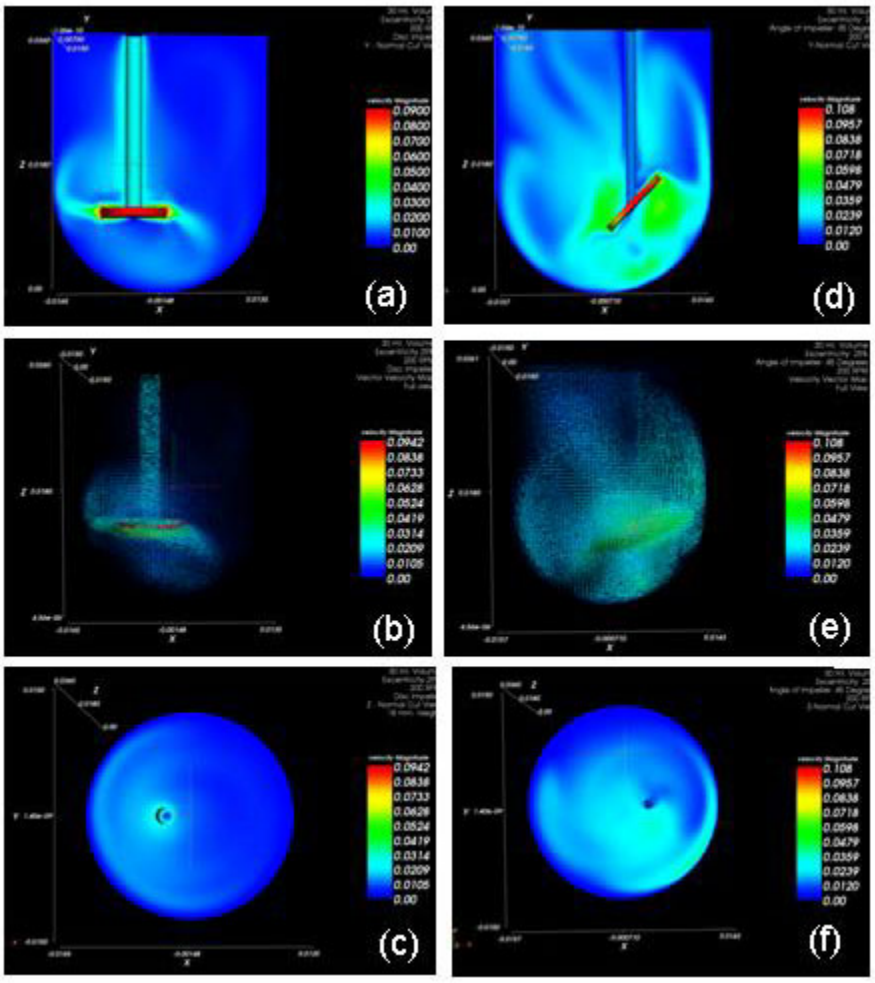

Figure 3. Velocity fields (obtained by CFD calculations) within eccentric stirred tanks (a) agitated by a horizontal disc impeller(vertical velocity contour at the axes level); (b) (vertical velocity field), and (c) (horizontal velocity contour at the impeller level); and (d) agitated by a 45° disc impeller (vertical velocity contour at the axes level); (b) (vertical velocity field), and (c) (horizontal velocity contour at the impeller level).

Figure 3 shows CFD results from simulations done with two different disc impeller configurations (varying in angle) rotating at 150 RPM in a water like liquid (matching average culture media properties) at an eccentric position (E=0.25) in a round bottom flask. A flat,

horizontal disc, impeller geometry previously analyzed by Alvarez *et al.* (2002) and Cervantes *et al.* (2006), is compared versus a disc impeller, were the angle of the impeller with respect to its axes is different to zero (for the case reported here, 45 degrees). The effect of the modification in the impeller angle is significant. In general, circulation patterns were dramatically modified, as observed in the velocity field presented in Figure 3. Particularly, axial circulation was greatly improved.

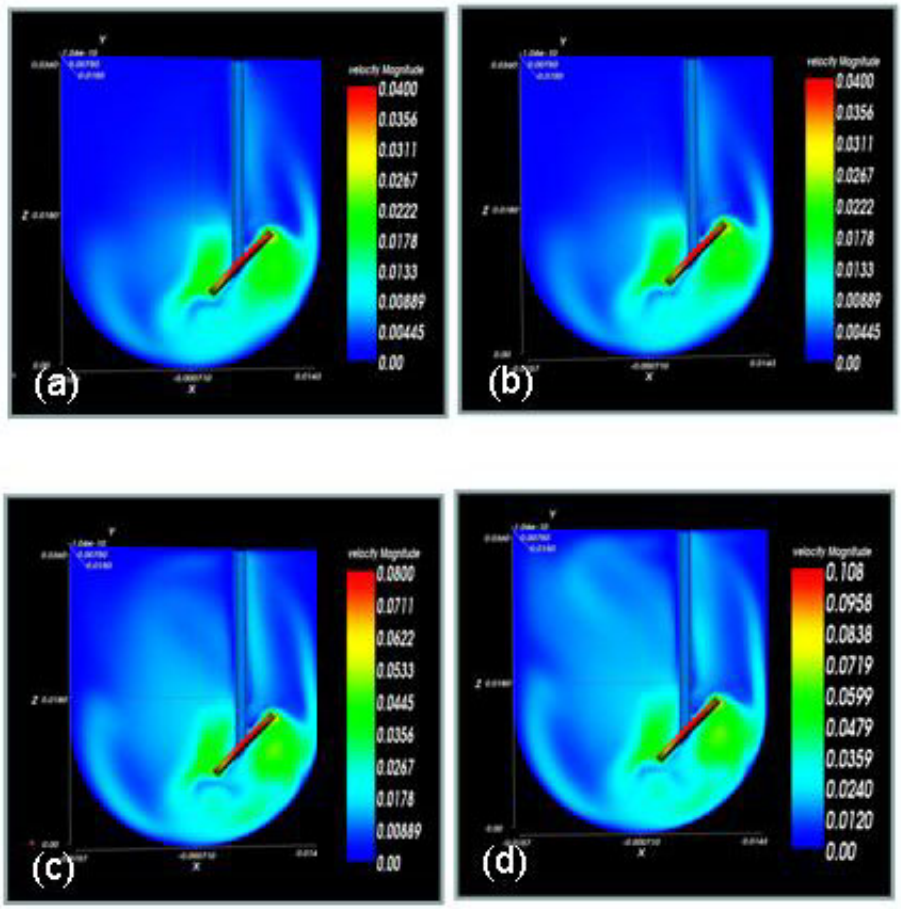

Figure 4. Velocity fields (obtained by CFD calculations) within eccentric stirred tanks agitated by a 45° disc impeller agitated at (a) 75 RPM; (b) 100 RPM; (c) 150 RPM; and (d) 200 RPM.

Figure 4 presents CFD results for 45 degree angle configurations at slightly higher eccentricity values (E=0.33). Different velocity field contours (from 75 to 200 RPM) are compared. Although the flow structure is similar among them, there are significant differences in the axial outreach of the flow field. This type of analysis, complementing experimental information, is useful to take decision like where to inject reagents for fast dispersion, or how low to locate the impeller to achieve adequate suspension. In our experiments with stem cells and CHO cells, agitation was set at 75 RPM and 200 RPM (in water like media) to allow for adequate mixing while minimizing mechanical stress. Although adequate liquid-liquid mixing is achievable at much lower speeds, visualization experiments with neutrally buoyant 20 microns polystyrene particles (results not presented here) demonstrate that higher speeds are needed

to maintain cells in suspension. The “just suspension velocity” for polystyrene particles in water was estimated in 200 RPM.

Cells cultured in the eccentric stirred tank bioreactor at an initial concentration of $1.75X10^4$ cells/ml exhibited the growth curve shown in Figure 5(a), with characteristic latency, exponential and stationary phases. Cell doubling time, calculated using a linear regression analysis on the exponential growth phase, was 31.46 hours, 19.18% increase from doubling time in stationary culture in Petri dishes (standard culture protocol for this type of cells). Cell viability in time is presented in Figure 5(b).Cell viability is maintained high (in the range of 80% to 100%). Remarkably, in exponential phase, cell viability is sustained in the range of 95 to 100% for several hours, evidencing that the flow field conditions are acceptable to favor survival and growth of extremely shear sensitive cells. Cells remained non-differentiated (CD 34^+ CD 133^+) during culture, as demonstrated by expression analysis using PCR protocols.

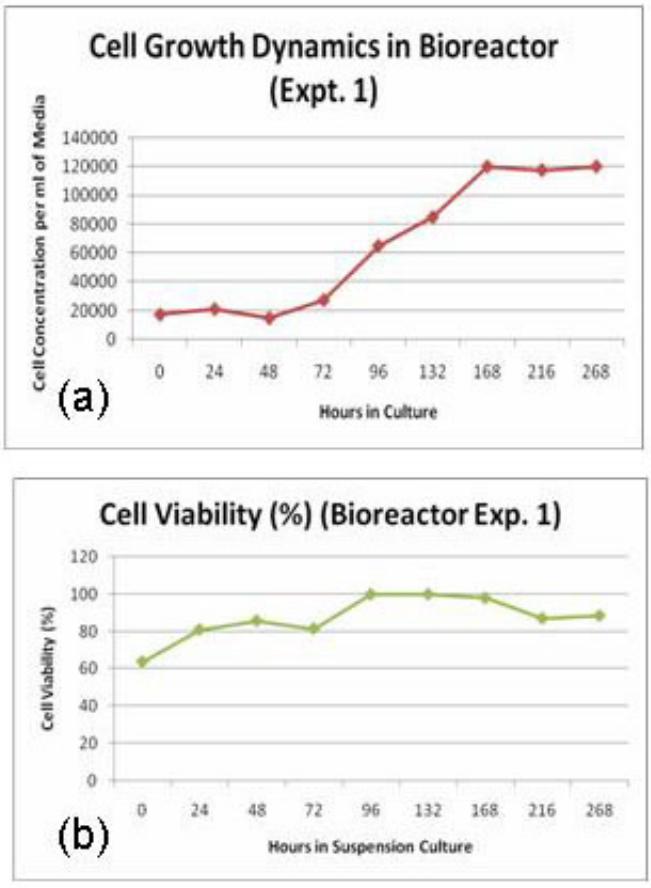

Figure 5. Non-embryonic stem human hematopoietic stem cells growth in an eccentric stirred tank system: (a) cell growth profile in time; (b) culture cell viability in time.

CONCLUSIONS

A 30 ml round bottom vessel stirred by a disc impeller eccentrically located at relative low speed has been proposed for biopharmaceutical screening applications. A mixing analysis was conducted using fluorescent tracer experiments under UV light, LIF visualizations and CFD simulations. Based on this analysis, operational conditions were decided for the culture of human non-embryonic stem cells. The stem cell culture was monitored over a period of 10 days, showing adequate performance in terms of growth rates, cell densities and viability.

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