J Ind Microbiol Biotechnol (2011) 38:761–767
DOI 10.1007/s10295-011-0951-6

REVIEW

Process challenges relating to hematopoietic stem cell cultivation in bioreactors

Marcin Kowalczyk · Kathryn Waldron · Penia Kresnowati · Michael K. Danquah

Received: 18 November 2010 / Accepted: 18 February 2011 / Published online: 9 March 2011

Abstract Hematopoietic stem cells (HSCs) are extremely useful in treating a wide range of diseases and have a variety of useful research applications. However, the routinely generated low in vitro concentrations of HSCs from current bioreactor manufacturing systems has been a hindrance to the full-scale application of these essential cellular materials. This has made the search for novel bioreactor systems for high-concentration HSC production a major research endeavour. This review addresses process challenges in relation to bioreactor development and optimisation for high-density HSC production under effective monitoring of essential culture parameters, such as pH, dissolved oxygen and nutrient uptake. It discusses different process strategies and bioreactor configurations for HSCs production from a commercial viability perspective, and also discusses recent advances in the field.

Keywords Hematopoietic stem cells · Cell growth · Stirred-tank bioreactor · Rotating-wall bioreactor · Continuous-perfusion bioreactor · Packed- and fluidised-bed bioreactor

Introduction

The use of living tissues as therapeutic agents has many important medical applications. Obvious examples include blood transfusion and organ donation, which have been established as sound medical techniques for many years. The use of living tissues for therapeutic applications in stem cell cultivation has gained a great deal of attention. Hematopoietic stem cells (HSCs) are, in general, responsible for the continual renewal of both red and white blood cells. HSCs derived from peripheral blood, bone marrow or umbilical cord blood have been used to successfully treat haematological diseases, hereditary disorders of metabolism, congenial immunodeficiency and central nervous system diseases [6]. Furthermore, HSCs are the progenitors of all blood cell types; hence, they could be used to produce blood cells for direct transfusion. Unfortunately, most of these cells are found in the bone marrow at very low concentrations and at even lower concentrations in the peripheral blood systems, umbilical cord blood and fetal liver [2]. The high demand for HSCs is not yet met by donors. Low in vivo concentration means that relatively large volumes of tissues are required to be donated. Also, the spread of infectious diseases has been a major safety concern. As a result, the search for methods to expand sources of HSCs is of immense research interest.

M. Kowalczyk · K. Waldron · P. Kresnowati · M. K. Danquah (✉)
Bio Engineering Laboratory,
Department of Chemical Engineering, Monash University,
Wellington Road, Clayton, VIC 3800, Australia
e-mail: michael.danquah@monash.edu

Background

Stem cells have the ability to either self-renew, differentiate into multiple cell lineages or both [9]. They remain dormant in an undifferentiated state awaiting process pathway signals to trigger proliferation and/or differentiation, depending on their function [2]. These capabilities make stem cells an ideal target for a huge variety of medical applications. Hematopoietic stem cells are self-renewing, with the capability to differentiate into more than eight cell lineages [12]. HSC differentiation (hematopoiesis) results in the continual production of mature red

and white blood cells. Three types of HSCs have been identified; long-term self-renewing, short-term self-renewing and multipotent progenitors [2, 13]. These are all grouped under pluripotent HSCs [16]. The HSCs eventually differentiate into the mature erythrocyte, granulocyte, macrophage, megakarocyte and lymphocyte cells—the components of blood, as shown in Fig. 1. The differentiation of HSCs is regulated by cytokines (proteins, peptides or glycoproteins used in cellular communication), which are in turn regulated and secreted by stromal cells [13]. Stromal cells are the adipocytes, endothelial cells, macrophages and fibroblasts that populate the extracellular matrix [2]. The role of bone marrow stromal cells, through their secreted cytokines and extracellular matrices, is to promote and regulate stem cell self-renewal, commitment, differentiation and proliferation [16]. The interactions between stromal cells, cytokines and HSCs in vivo give an indication of the complexities involved in the growth, proliferation and differentiation of HSCs. Ex vivo factors such as the use of serum [2], hydrodynamic shear stresses [9], oxygen concentration [2, 18] and pH [2, 13] are involved in the expansion and differentiation of HSCs. This complex interplay of cellular and chemical interactions in vivo has resulted in very little concrete knowledge about HSCs production. As a result, the convention of researchers has been to mimic the in vivo conditions as a baseline for further optimisation. As HSCs cultivated ex vivo are collected from either bone marrow, peripheral blood or umbilical cord blood and are only present in small concentrations, the sample is enriched via selection of surface antigen CD34 cells, a marker used to identify HSCs, as it is found on most of the primitive hematopoietic cells (stem cells and early progenitors) [13]. However, whereas the CD34 marker is useful in correlating relative amounts of HSCs, it is not an accurate indication of the number of HSCs collected, and further markers need to be developed to narrow the HSC type. A combination of the $CD34^{+}$ and $CD38^{-}$ (absence of that marker) is the most commonly used selection [2]. As a result of the inability to distinguish between stages of differentiation, it is impossible to isolate a specific stage; hence, studying the pathways of differentiation is difficult. This problem is overcome via the use of genetically modified cells or factor-dependent stem or progenitor cell lines that have been blocked at a range of

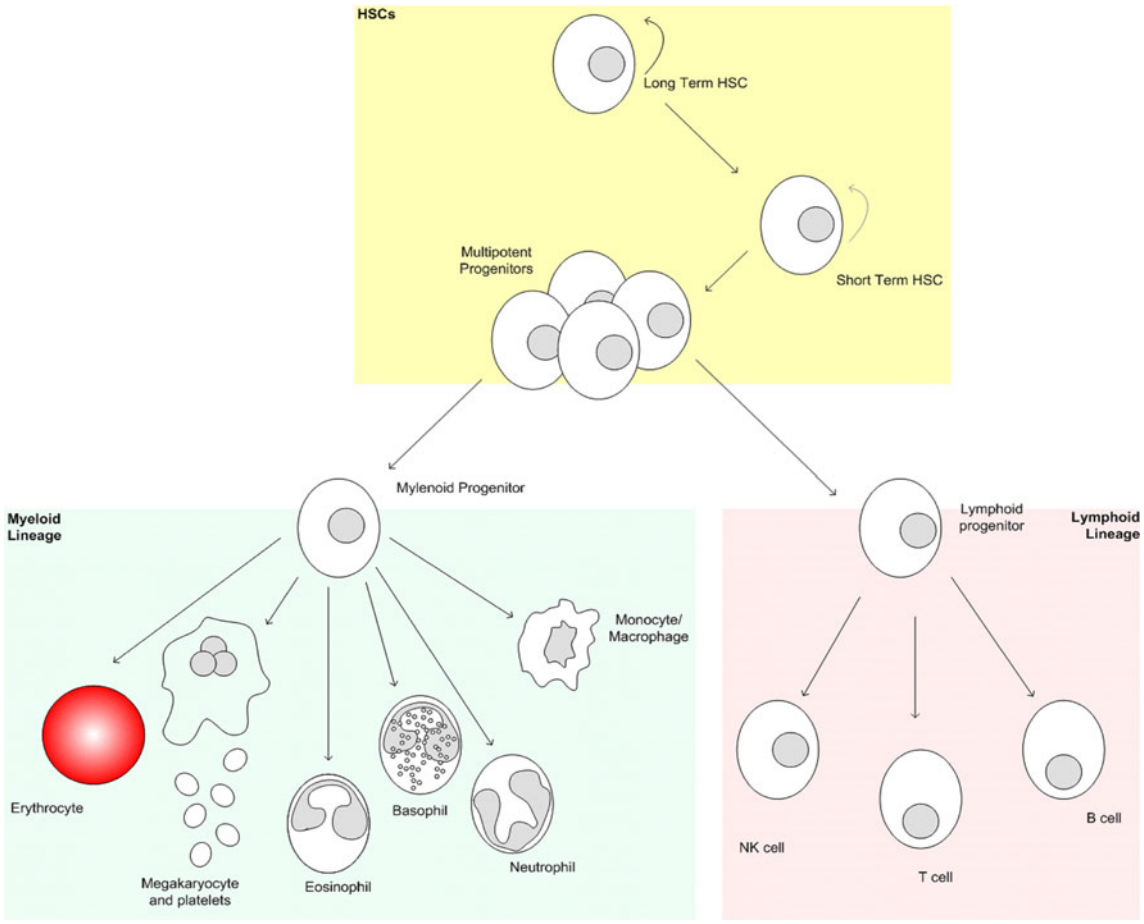

Fig. 1 Cell differentiation. *Straight arrows* indicate differentiation; *curved arrows* indicate self-renewal. Adapted from [2]

differentiation stages [5]. The $CD34^+$ acute myeloblastic leukaemia cell line KG1 is of the latter type. KG1 cells are useful to use as mock stem cells in developing bioreactor systems—they are easy to work with, possess known stages of differentiation, do not need cytokines to trigger growth/differentiation and there are no ethical issues associated with their use.

HSC cultivation: static versus dynamic

In vitro synthesis of HSCs has gained interest since the pioneering work of Dexter et al. [4]. As discussed earlier, due to the complex nature of the cellular system, it has become a convention to mimic conditions found in the body and use them as the basis for further optimisation. In their work, Dexter et al. used static cultures that, unfortunately, deviate from this convention, as cultivation is only performed at 33°C rather than the physiological temperature of 37°C. Further research into the limitations of the static culture system showed that it is not suitable for large-scale production of HSCs. Factors such as lack of mixing, which results in undesirable concentration gradients of key nutrients and oxygen, difficult online monitoring and control, low reproducibility and the need for repeated handling to obtain data result in dynamic cultures being the more desirable alternative when cultivating HSCs [13]. Dynamic cultures, however, suffer some disadvantages; mainly the build up of metabolic by-products and hydrodynamic shear stresses [9]. Large-scale production of HSCs will presumably have to occur at a steady state or at least on a batch processing basis, and therefore, dynamic cultures are better able to fulfil these process goals. Also, dynamic cultures are able to implement dedicated oxygenation systems, whereas a static culture relies of oxygen diffusion from the gas phase to the liquid phase and subsequently from the free surface to where the cells are growing. This results in an upper limit of cells able to grow in a static culture because oxygen transport is limited, and there is a minimum free surface area to media volume ratio. Also, due to constant volumetric flow and therefore better mixing, dynamic cultures remove problematic concentration gradients. The upper limit of cell growth within a dynamic culture is governed only by special considerations and cytokine availability. It should be noted, however, that both static and dynamic culturing methods are limited by a minimum cell-seeding density. Table 1 summarises the differences between the static and dynamic cultivation methods.

Growth media

The early work with ex vivo cultivation of HSCs involved serum-containing media [2]. The media is the nutritional source for the HSCs and a combination of human/animal serum with a cytokine cocktail [1]. To make allowances for the complexity of the microenvironment for hematopoiesis and the limitations in the knowledge of these complex interactions, serum is added to ensure that the ex vivo conditions mimic the in vivo conditions. The serum provides essential nutrients, hormones and growth factors and protects cells from potential damage in the culture environment [3]. However, despite these positive effects, the addition of serum has some disadvantages: unknown composition, which increases the uncontrolled variability of the experiment [2]; possible inhibitory factors [3], which could result in immunological rejection of cells when transplanted [1]. More recently a serum-free media has been used to overcome these disadvantages [2]. Serum-free media support the expansion of highly purified HSCs, whereas serum-containing media results in a more differentiated state of the HSCs [14]. The medium rheology is also an important factor to optimise cell growth, as it

Table 1 Comparison between static and dynamic cultivation methods

Parameters	Static culture	Dynamic culture
Nutrient transport	Diffusion-based	Convective transport to the region where cells are growing
Oxygen transport	Diffusion into the vessel followed by dissolving into the media and then diffusion to the cells	Dedicated oxygenation systems Convective transport to the region where cells are growing
pH	No online monitoring system	Online monitoring possible
Nutrient concentration monitoring	Must handle the culture	Sampling of the media possible without handling the culture directly
Handling	Culture must be handled to refresh media	Handling only occurs during seeding and harvesting
Size	Optimal density is based on limiting factors such as oxygen and nutrient transport	Density is limited by size of reactor vessel and cytokine availability

affects oxygen transfer and uptake rates, nutrient accessibility to cells and mixing requirement during cultivation under dynamic conditions.

Bioreactor types for HSC cultivation

It is somewhat difficult to compare different bioreactors for HSC cultivation based on expansion rates and/or achievable cell densities. This is because a wide variety of different bioreactor systems are employed under different growth conditions and parameters, such as medium and serum composition and concentration, type of cells, passage number, presence of growth factors/inhibitors and other physicochemical conditions such as media exchange rates and shear considerations. Also, different bioreactors could be useful at different stages of cell-culture development, and this further complicates the issue. The exact nature of the effect of each of these parameters on HSC cultivation is not well understood. An example of this is the use of serum, the presence of which does not conclusively affect the expansion rate of the cell culture [6]. The reported data on hematopoietic cell expansion in different types of bioreactors is an excellent example of the complex interplay of factors [2]. Packed-bed reactors have shown excellent expansion rates in some experiments ($\sim$100-fold), yet average expansion in others are $\sim$7-fold [8, 11]. Similarly, stirred-tank bioreactors have shown similar expansion rates to perfusion bioreactors ($\sim$10-fold) [2]. The aforementioned factors are very important in determining overall cell expansion, and the advantages and disadvantages of each bioreactor type must be considered before a selection is made. Achievable cell density in a bioreactor is a strong function of bioreactor type. Bioreactor type affects essential growth-driven parameters, such as culture-flow dynamics, temperature distribution, aeration profile, agitation and nutrient distribution. For example, stirred-tank bioreactors achieve relatively higher cell densities (5×10^5 cells/ml) [1] compared with rotating-wall bioreactors (2×10^5 cells/ml) [10], and this could be attributed to inferior mixing in the case of rotating-wall vessels. Culture methods also affect achievable cell densities, with spinner flasks and static 3D matrices being able to achieve 1×10^6 cells/ml and 2.5×10^5 cells/ml, respectively [1].

Stirred-tank bioreactor

The stirred-tank bioreactor overcomes the many shortfalls of the static culture system, such as concentration gradient, low reproducibility, frequent handling that can result in contamination and the inability to closely monitor, sample and control culture conditions [2]. This homogenous environment promotes HSC growth and differentiation. However, the process of stirring the culture produces a shear stress that, as the HSCs are shear sensitive [15], can damage the HSC's surface-marker expression [2] and hence have adverse effects on growth and differentiation. These adverse effects result in stirred-tank bioreactors producing a lesser-fold cell expansion when compared with other types of bioreactors [1].

Rotating-wall bioreactor

Rotating-wall vessels enable the reduction of concentration gradients necessary for cultivating HSCs without the complications of shear stress [1, 10]. This is done by inducing a free-fall state in the bioreactor that has significantly less impact on the cells [1]. A schematic representation of a rotating-wall bioreactor is shown in Fig. 2. A rotating-wall vessel is vastly more suited to the expansion of HSCs than a T-flask (static culture), and a greater HSC expansion is achieved in a shorter time period than other types of bioreactors.

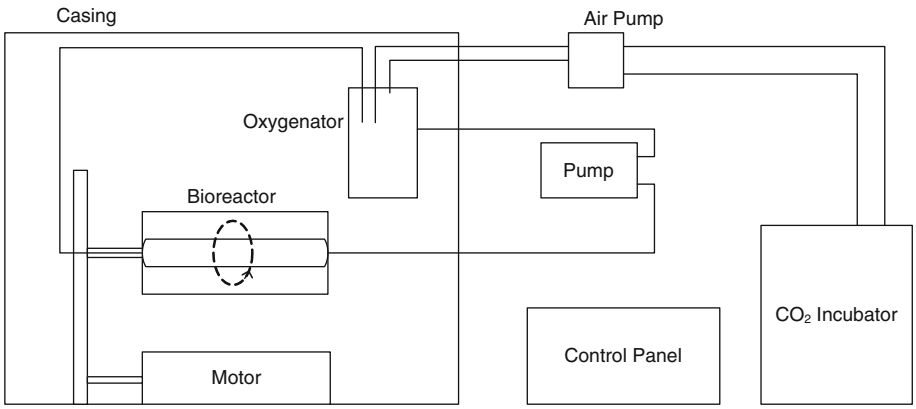

Fig. 2 Rotating-wall bioreactor. Adapted from [1]

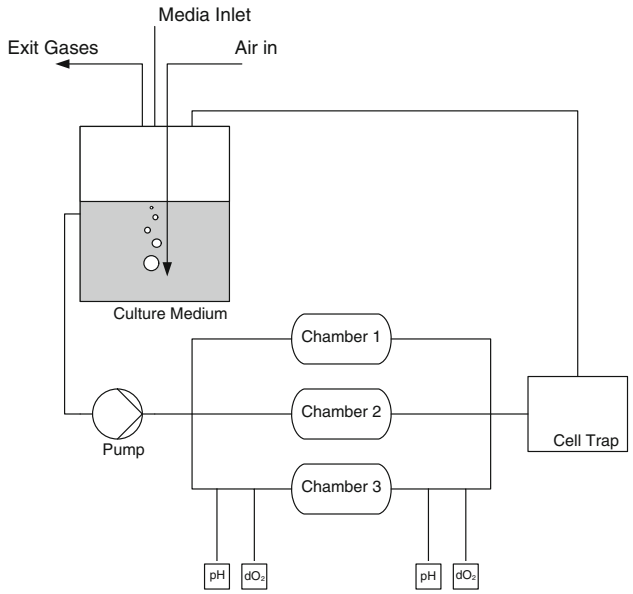

Fig. 3 Continuous-perfusion bioreactor. Adapted from [3]

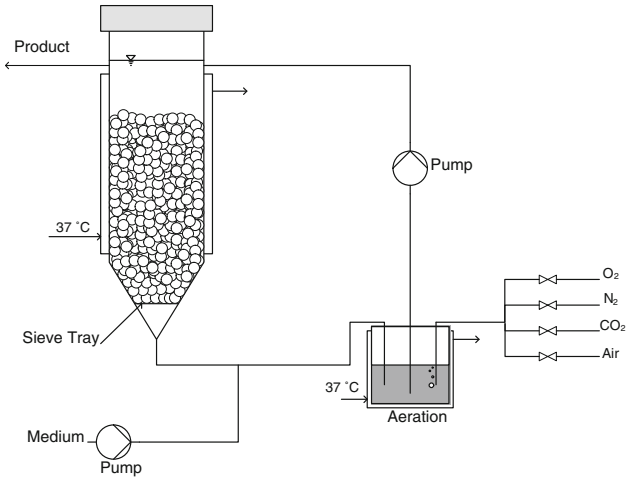

Fig. 4 Packed-bed bioreactor system. Adapted from [11]

Continuous-perfusion bioreactor

It has been found that continuous perfusion of fresh or recycled medium results in an increase in oxygen transport to cells and thus increases cell growth and proliferation [9]. This is the basis for the design of a continuous-perfusion or perfusion bioreactor [2, 3]. Another advantage of this type of bioreactor is that no stromal-cell layer is required [15], thus reducing the complexity of the system in vitro. A continuous-perfusion bioreactor containing highly porous collagen microspheres could provide an increased surface area and scaffolding for cell growth and could also allow for the control of physical and biochemical parameters. A schematic diagram of the continuous-perfusion bioreactor is presented in Fig. 3.

Packed- and fluidised-bed bioreactor

As the in vivo conditions for the growth and proliferation of HSCs involve a 3D extracellular matrix, packed-bed reactors attempt to mimic this by incorporating a 3D scaffold for the attachment of cells [2]. These systems typically function by embedding stromal cells on porous glass carriers. During cultivation, the HSCs attach to the stromal cells and experience a microenvironment that mimics in vivo conditions more closely [11]. This system has had some success but needs further investigation [11]. Fluidised-bed bioreactors operate on a similar principle while overcoming the issue that high immobilization densities have on the concentration gradient [17]. Packed-bed bioreactors are a better alternative for the early stages of HSC cultivation, whilst fluidised-bed bioreactors may have a role in the later stages of cultivation. A schematic diagram of a packed-bed bioreactor is shown in Fig. 4.

Table 2 summarises the advantages and disadvantages of each bioreactor type.

Essential online monitoring parameters

pH

As with many variables that can be controlled in the bioreactor cultivation system, there remain some uncertainties about pH. Studies suggest that a bioreactor can experience pH variations of up to 0.5 pH units if the pH is not controlled [1, 2]. There are also studies that report that the type of cell differentiation is tuned by pH. Myeloid differentiation occurs optimally in the pH range 7.2–7.4 [13], whereas in the erythroid lineage, differentiation occurs at a greater rate in the pH range 7.1–7.6 [1, 2]. Also reported is the optimum pH for granulocyte–macrophage differentiation (7.2–7.4), where higher values in this range (7.35–7.4) promote differentiation, maturation and apoptosis of these kinds of cells. Furthermore, there is some evidence that the method by which pH is adjusted can have a significant effect on the behaviour of the culture. Cell cultures with pH adjusted by sodium hydroxide behave differently to those adjusted with ammonium nitrate [2]. This could be due to the foreign species affecting the equilibrium position of the constituents of the media. It has also been reported that pH affects the resistance of cells to damage as a result of hydrodynamic shear [15].

Table 2 Summary of the advantages and disadvantages of different bioreactors

Types	Advantages	Disadvantages
Stirred tank	Relatively inexpensive and simple to operate	Cells are exposed to high shear conditions due to stirrer blades and bursting bubbles at the surface of the tank
	Online monitoring possible depending on setup	Results in lower overall expansion
		Sampling is impossible without handling the culture directly
Rotating wall	Induces a free-fall state that has significantly less impact on the cells	Complex system (potentially high cost and difficult to scale up)
	Greater HSC expansion in a shorter time period compared with other types of bioreactors	
Continuous perfusion	Good oxygen and nutrient transport capabilities resulting in good expansion	Potentially difficult harvesting
	Good mimic of in vivo conditions	Further optimisation is required to explore full-scale application
	No stromal-cell layer needed	
	Easy scale up	
Packed/ fluidised bed	Provides 3D scaffold for the attachment of cells	Further optimisation is required to explore full-scale application
	Mimics in vivo conditions	
	One system capable of handling both early and late stages of cell growth	

Dissolved oxygen

As to the optimum value of dissolved oxygen concentration in the bioreactor system, there are some discrepancies in reported literature values even for the same cell line, further highlighting the complexity and variability of the system. It is clear that cells experience growth inhibition and even toxicity at high oxygen concentrations and anoxia at low oxygen concentrations [2, 9]. There is some evidence to suggest that low oxygen concentrations result in greater differentiation and proliferation of the population [9], whereas moderate concentrations enhance expansion rates [2]. Also noteworthy is the fact that a limited oxygen environment significantly reduces nitric oxide, hydrogen peroxide and oxygen radical concentration, which is beneficial, as these species are all known to be cell-proliferation inhibitors.

Nutrients and wastes

Monitoring nutrient and waste concentration during cultivation is possible through the use of commercially available assays. As the culture must be sampled for analysis in this manner, especially in the case of dynamic systems, it is desirable to use bioreactor types that have the capacity to separate the cell culture from the bulk of the media. This allows sampling of the media to occur while maintaining culture sterility. For this reason, the continuous-perfusion and packed-/fluidised-bed bioreactor systems have an advantage in this capacity.

Flow rate and shear considerations

The flow rate of the bioreactor system is an important consideration, as it must take into account the shear stress exerted on the cells due to hydrodynamic forces. Cell death due to shear stress is a function of both the magnitude of that stress as well as exposure time [15]. The flow rate must therefore be selected to mitigate the shear stress while ensuring sufficient nutrient and oxygen transport to sustain cell growth. Cell damage due to shear stress comes from two main sources; eddies in the turbulent flow that have a size of the same order of magnitude as the diameter of a single cell, aggregate or microcarrier [9]; and the fast draining of liquid films [15]. Eddies in the flow are generated primarily at the impeller blade of a centrifugal pump, and these can therefore be mitigated by using a peristaltic pump. A larger problem is presented in the fast draining of liquid films. This occurs primarily when persistent bubbles form in the system and presents a major problem if the oxygenation system bubbles gas through cell-containing media. Also, it is conceivable that the fittings of the bioreactor system may develop leaks that could introduce bubbles into the bioreactor. Papoutsakis [15] shows that bubbles bursting at the free surface is the main cause of cell death when dealing with systems such as these—nonbursting bubbles simply travelling through the fluid do not induce the high shear conditions necessary to cause cell death. Also, foaming causes unwanted conditions in which cells can be subjected to undesirably high shear stresses. To overcome this issue, care must be taken

Table 3 Summary of some cell-expansion results and conditions in the literature

Bioreactor type	Cell type	Media	Cultivation conditions	Expansion	Reference
Perfusion bioreactor	Human bone marrow	IMDM with 10% calf serum and 10% horse serum	37°C and 20% O_2	~25-fold expansion (2 weeks)	[14]
Fluidised bed	Peripheral-blood stem cells/ mononucleated cord blood	McCoy's 5a medium with 10% calf serum	37°C	No expansion	[11]
Packed bed	Peripheral-blood stem cells/ mononucleated cord blood	McCoy's 5a medium with 10% calf serum	37°C	7-fold expansion	[11]
Stirred tank	Human *t* cells/KG1 cells	IMDM containing 10% fetal calf serum	37°C 5% CO_2	8-fold expansion	[7]
Rotating wall	Cells from full-term umbilical cords	Iscove's modified Dulbecco's medium (IMDM) with 10% fetal bovine serum and 10% horse serum	95% air with 5% CO_2 37°C	~430-fold expansion (~8 days)	[10]

to ensure that if bubbling does occur, then the bubbles must be small and nonbursting to minimise the resulting shear stresses. Furthermore, filtration systems may be required to separate cell-containing media from the oxygenation systems. Table 3 reports a summary of some of the work carried out in HSC production.

References

1. Andrade-Zaldivar H, Santos L, Rodriguez AL (2008) Expansion of human hematopoietic stem cells for transplantation: trends and perspectives. Cytotechnology 56::151–160
2. Cabrita GJ, Ferreira BS, da Silva CL, Goncalves R, Almeida-Porada G, Cabral JM (2003) Hematopoietic stem cells: from the bone to the bioreactor. Trends Biotechnol 21(5):233–240
3. Colllins PC, Papoutsakis ET, Miller WM (1996) Ex vivo culture system for hematopoietic cells. Curr Opin in Biotechnol 7: 223–230
4. Dexter TM, Allen TD, Lajtha GL (1977) Conditions controlling the proliferation of haemopoietic stem cells in vitro. J Cell Physiol 91:335–344
5. Gudmundsson KO, Thorsteinssona L, Sigurjonsson OE, Keller JR, Olafsson K, Egeland T, Gudmundsson S, Rafnar T (2007) Gene expression analysis of hematopoietic progenitor cells identifies Dlg7 as potential stem cell gene. Stem Cells 25: 1498–1506
6. Hai-Jiang W, Xin-Na D, Hui-Jun D (2008) Expansion of hematopoietic stem/progenitor cells. Am J Hematol 83:922–926
7. Jelinek N, Schmidt S, Hilbert U, Thoma S, Biselli M, Wandrey C (2002) Novel bioreactors for the ex vivo cultivation of hematopoietic cells. Eng Life Sci 2:15–18
8. Kawada H, Ando K, Tsuji T, Shimakura Y, Nakamura Y, Chargui J, Hagihara M, Itagaki H, Shimizu T, Inokuchi S, Kato S, Hotta T (1999) Rapid ex vivo expansion of human umbilical cord hematopoietic progenitors using a novel culture system. Exp Hematol 27:904–915
9. King JA, Miller WM (2007) Bioreactor development for stem cell expansion and controlled differentiation. Curr Opin Chem Biol 11:394–398
10. Liu Y, Liu T, Fan X, Ma X, Cui Z (2006) Ex vivo expansion of hematopoietic stem cells derived from umbilical cord blood in rotating wall vessel. J Biotechno 124:592–601
11. Meissner P, Schroder B, Herfurth C, Biselli M (1999) Development of a fixed bed bioreactor for the expansion of human hematopoietic progenitor cells. Cytotechnology 30:227–234
12. Nakano T (2003) Hematopoietic stem cells: generation and manipulation. Trends in Immunol 24(11):589–594
13. Nielsen LK (1999) Bioreactors for hematopoietic cell culture. Annu Rev Biomed Eng 1:129–152
14. Palsson BO, Paek S-H, Schartz RM, Palsson M, Lee G-M, Silver S, Emerson SG (1993) Expansion of human bone marrow progenitor cells in a high cell density continuous perfusion system. Nat Biotechnol 11:368–372
15. Papoutsakis ET (1991) Fluid-mechanical damage of animal cells in bioreactors. Trends Biotechnol 9(12):427–437
16. Takagi M (2005) Cell processing engineering for ex vivo expansion of hematopoietic cells. J Biosci Bioeng 99(3):189–196
17. Thommes J, Gatgens J, Biselli M, Rundstadler PW, Wandrey C (1993) The influence of dissolved oxygen tension on the metabolic activity of an immobilized hybridoma population. Cytotechnology 13:29–39
18. Wang T-Y, Wu JHD (1992) A continuous perfusion bioreactor for long-term bone marrow culture. An NY Acad Sci 665:274–284