International Journal of Natural and Engineering Sciences 3 (2): 53-58, 2009
ISSN: 1307-1149 www.nobel.gen.tr

Cultivation of Calcium-Alginate Encapsulated Myeloma Cells in a Bioreactor

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Received: December 11, 2008
Accepted: January 26, 2009

Abstract

Hybridization partners of hybridoma cells which produce monoclonal antibodies (MAb) are myeloma cells. Myeloma cells which are B-lymphoma cells can proliferate easily and be produced cheaply. This cell line shows many characteristic similarities with hybridoma cells. When calcium alginate encapsulated hybridoma cells are cultivated in bioreactors, the amount of MAb and viable cell number increase approximately 3-10 folds depending on the cell concentration used. In this study, concentration, time and other factors were optimized for MAb production with a high yield and quality. The toxicity of the Ca-alginate to the cells was determined. In order to optimize the conditions, different concentrations of sodium alginate and calcium chloride were used to keep myeloma cells alive for 15-20 days in the bioreactor. Under optimized conditions, Ag8 myeloma cells which were encapsulated in a 1 mm diameter beads were cultivated in a stirred bioreactor with a working volume of 700 ml for ten days. Encapsulation of the cells was carried out using 1.2% (w/v) Na-alginate (pH adjusted to 7.2) and 1.5% (w/v) $CaCl_2$ (pH adjusted to 7.4) which was used as a crosslinking agent. The pH of the medium, glucose consumption and lactic acid production were measured throughout the cultivation with daily intervals. The results indicated that after 2 days of logarithmic phase the Ca-alginate encapsulated myeloma cells stayed in stationary phase for about 6 days which was then followed by cell death.

Key words: Myeloma, hybridoma, calcium alginate, encapsulation

INTRODUCTION

Animal cell technologies involving the immobilization of cells within three dimensional gel microbeads provide several benefits [1]: Immobilized cells can be protected from mechanical stress; immobilized cell particles are about 50-1000 times larger than free cells that is why are easier to use in perfusion systems, the cells can be easily separated during the medium changes [2]; since any autocrine and/or paracrine factors produced and secreted by entrapped cells are not diluted by media immediately, immobilization of animal cells has indeed been reported to effectively enhance growth and cell viability [1, 3].

Because animal cells have a relatively low productivity, large amounts of culture supernatant are needed per clinical dose of final product. Extremely large volume cultures will be needed to produce kilogram quantities of a therapeutic monoclonal antibody [4]. That is why, immobilization seems a solution to achieve growing large amount of cells and cellular materials in small volume.

Monoclonal antibodies (MAb) are important reagents used in biomedical research, in diagnosis of diseases, and in treatment of such diseases as infections and cancer. These antibodies are produced by cell lines or clones obtained from animals that have been immunized with the material that is the focus of study. The cell lines are produced by fusing B cells from the immunized animal with myeloma cells [5]. The high amounts of MAb needed, particularly for their therapeutic applications. Since cell microencapsulation in biocompatible polymer matrices represents a powerful technology for biopharmaceutical manufacturing of protein therapeutics there is an increasing interest in using immobilized hybridoma cell systems for mAb production [6].

The polymer selection is very important issue. Polymer should have to be evaluated for some properties such as biocompatibility, degradability, biosafety, reproducibility, stability, durability etc. [7]. Alginate is a highly biocompatible, non-toxic hydrogel and it undergoes a mild gelation reaction. Encapsulation of cells in Ca-alginate matrices is a cheap, simple and rapid immobilization procedure so it is a favorable system [6]. Alginates are naturally derived polysaccharides and have been widely used as hydrogel synthetic extra cellular matrices. Alginates are composed of (1-4)-linked β-D-mannuronic acid (M units) and α-L-guluronic acid (G units) monomers which vary in amount and sequential allocation along the polymer chain depending on the source of the alginate. The alginate molecule is a block copolymer composed of regions of sequential M units (M-blocks), regions of sequential G units (G-blocks), and regions of a tactically organized M and G units (Figure 1). Upon interaction of divalent cations, like Ca^{+2}, ionic crosslinking occurs between the inter- and intra-chain G residues which cause gelling of aqueous alginate solutions [8].

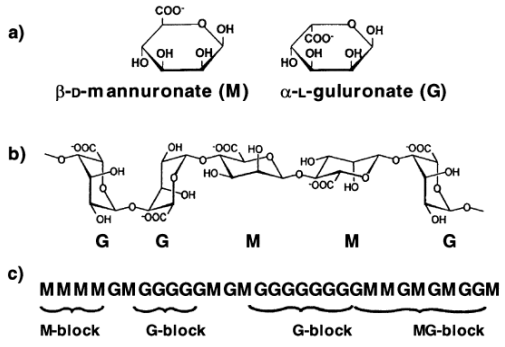

Figure 1: Structural characteristics of alginate: (a) alginate monomers, (b) chain confirmation, (c) block distribution [9].

The main objective of this study is to optimize the growth characteristics of the alginate encapsulated myelomas in a bioreactor. The data obtained will then be used as a tool in the next step for hybridoma encapsulation and MAb production.

MATERIALS AND METHODS

Cell Culture

Myeloma cells (X63AG8.653, BALB/c mouse myeloma, HUKUK, Ankara) were cultivated in DMEM (Sigma, USA) medium containing 10% FCS (Biochrom AG, Germany), 1% L-glutamine (Biochrom AG, Germany), 2% (v/v) gentamicin (Biochrom AG, A2712, Germany), 1% (v/v) Na-pyruvate (Biochrom AG, Germany) and 1% (v/v) HEPES Buffer (1M) (Biochrom AG, Germany) at 37°C with 5% CO_2 in humidified atmosphere before encapsulation. After immobilization, cells were transferred to the bioreactor containing the medium given above and then cultivated at 37ºC and 110 rpm. Half of the growth medium spent was replaced with fresh medium in every 48 hours.

Immob ilization

In this_study, in order to optimize the conditions different concentrations of sodium alginate and calcium chloride were used to keep myeloma cells alive for 15-20 days in the bioreactor. Encapsulation was carried out by gentle mixing of the cells (at the concentration of 2×10^6 cells/$ml_{alginate}$) with Na-alginate solution (Sigma A0682, ABD) [(%0.8-%1.5) (w/v) in %0.85 (w/v) NaCl] and cross linking with $CaCl_2$ (Merck, F1305779409, Germany) solution [1%-1.5% (w/v)] aseptically. Gel entrapped myeloma cells were allowed to stand for about 10 minutes in order to achieve proper gelation and the immobilized cells were then washed 3 times with washing solution [0.9 % (w/v) NaCl, 0.02 % HEPES (1M) (Biochrom AG, L1613, Germany), pH adjusted to 7.4]. Finally, gel beads with a 1 mm diameter were obtained and transferred into the bioreactor depicted in Figure 2.

Figure 2: Bioreactor system with Ca-alginate encapsulated X63AG8.653 myeloma cells.

Cell Viability Monitoring

Several methods were attempted for cell viability monitoring. Therefore, due to such problems depending on cell viability monitoring directly via MTT or trypan blue dye exclusion assay, the viability of the cells was observed by measuring the pH of the medium and monitoring the cells under the microscope throughout the cultivation. Moreover, some supernatant samples were drawn from the system right after medium replacement and used for glucose (GLU-12 TOADDK, Japan) and lactic acid (Roche, r-biopharm 14062300) measurements for indirect analysis in which metabolic activity of the cells were followed by glucose consumption and lactate production of the cells. Additionally, the pH of the growth medium was monitored throughout the cultivation of myeloma cells with two days intervals.

RESULTS

Encapsulated X63AG8.653 cells were maintained in a 700 ml working volume stirred bioreactor (Bellco, USA) for ten days. The optimization experiments revealed that encapsulation of the cells using 1.2% (w/v) Na-alginate (pH 7.2) and 1.5% $CaCl_2$ (w/v) (pH 7.4) was the best combination among the all optimization trials in terms of long lasting preservation of the cell viability and capsule stability. A slight decrease in pH of the medium was observed during the cultivation and it was considered as the indication of cell proliferation (Table 1). In the first 2 days, a sharp decrease in glucose concentration was detected while lactic acid concentration increased dramatically. However, after that period those values changed significantly. (Figure 3 and Figure 4).

Table 1: pH values of the culture medium in bioreactor.

Day	pH	
	Before media changing	After media changing
2	6.8	7.1
4	7.0	7.1
6	7.2	7.3
8	7.3	7.3
10	7.4	-

Glucose Consumption Rate and Lactate Accumulation

The major carbon and energy source, glucose, and the major byproduct, lactate, were measured with two days intervals during the cultivation period. Time course of glucose and lactate concentrations are shown in Figure 4, 5 and 6.

Glucose and lactate levels changed markedly within first four days of the culture. Although half of the growth medium was replaced with fresh medium in every two days of culture, glucose consumption rate (842,03 mg/L/day) was remained almost the same from 2nd to 6th day. Depending upon the medium replacement, oscillations were observed in glucose levels until the eighth day of culture. After that glucose concentration increased since most of the cells were dead.

Glucose utilization was accompanied by a corresponding accumulation of lactate. After the second day of culture the lactate concentration in the culture decreased, while the glucose concentration leveled off at a concentration of approximately between 2440-2600 mg/L. It was observed that the highest concentrations of lactate (230,7 mg/L) and glucose consumption (1900 mg/L) were overlapped indicating change in metabolite levels was growth-associated.

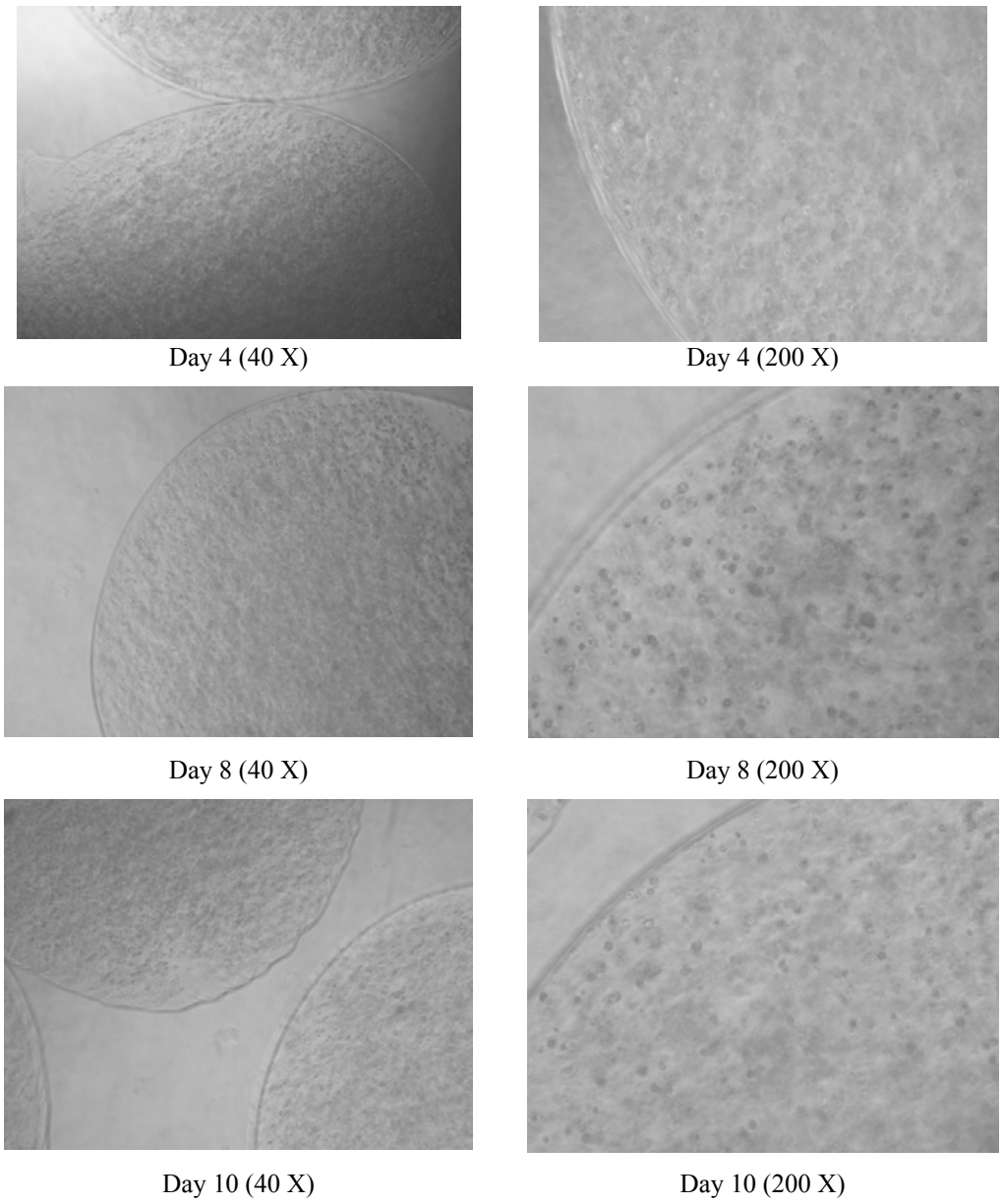

Figure 3: Cultivation of immobilized Ag8 myeloma cells in a bioreactor. Images of the Ca-alginate encapsulated cells on 4th, 6th, 8th and 10th days of the cultivation. The increasing number of black spots (cells) in the beads gives us a conclusion that the cell viability decreases gradually day by day (Olympus, Japan).

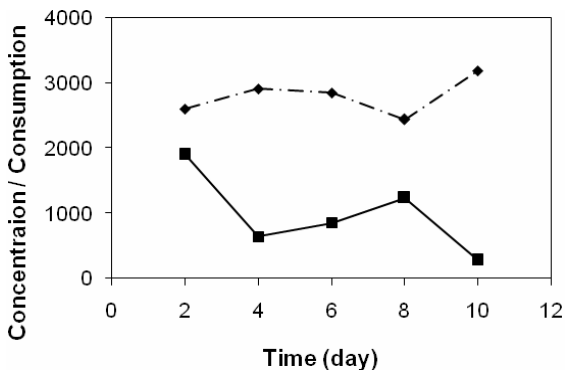

Figure 4: Variation of D-Glucose concentration and glucose consumption in bioreactor conditions. Half of the growth medium was replaced with fresh medium on 2nd, 4th, 6th and 8th days of the cultivation. --♦-- Glucose concentration in bioreactor medium (mg/L); —■— Glucose consumption rate in bioreactor medium (mg/L/day).

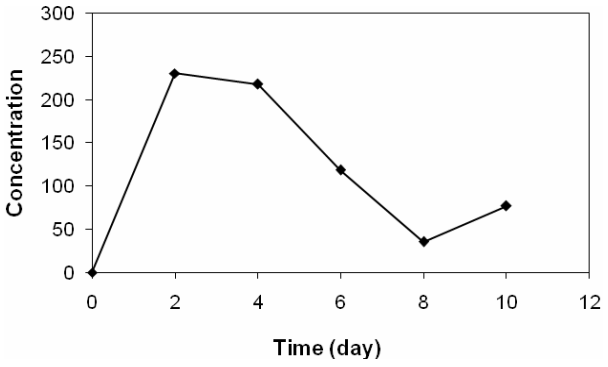

Figure 5: Variation of lactate concentration in the bioreactor medium (mg/L). Half of the growth medium was replaced with fresh medium on 2nd, 4th, 6th and 8th days of the cultivation

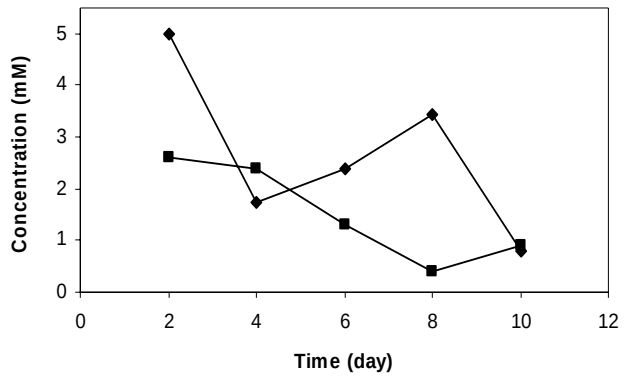

Figure 6: Glucose consumption rate versus lactate production. Half of the growth medium was replaced with fresh medium on 2nd, 4th, 6th and 8th days of the cultivation. --♦-- Glucose consumption rate in bioreactor medium (mM/day); —■— Lactate concentration in bioreactor medium (mM).

DISCUSSION

In the face of monoclonal antibody (MAb) production using hybridoma cells entrapped in gel beads has attracted considerable interest due to a number of advantages over free-suspended cell culture; there are also many drawbacks of encapsulation in alginate beads such as, diffusional and core limitations, product inhibition and mechanical stability of the beads [7,10].

Several methods were attempted for cell viability monitoring. Cells under the alginate polymers could not been contacted with MTT or trypan blue dye to be counted accurately. Therefore, due to such problems depending on cell viability monitoring directly via MTT or trypan blue dye exclusion assay, the viability of the cells was observed by measuring the pH of the medium and monitoring the cells under the microscope throughout the cultivation.

Chemical and mechanical stability

The gelation of calcium alginate is an almost instantaneous and irreversible process, which is governed by the relative rate of diffusion of calcium ions and polymer molecules into the gelling zone. The gelling properties of an alginate depend strongly upon its monomeric composition, block structure, molecular size and concentration of polymer and calcium ions [11].

As it is very well-known that the dissolution of beads may easily be affected by some parameters such as $CaCl_2$ and Na-alginate concentrations, retention time in $CaCl_2$ solution, alginate purity and quality, metabolic activity of the cells and medium components [12, 13]. In our study, the time period for the proper cultivation of the cells encapsulated within the beads was 10 days, in terms of mechanical and chemical stability. Thereafter, cell leakage and deterioration in bead structure were observed visually as well as under microscope.

Optimization of sodium alginate and $CaCl_2$ concentrations and their effects on cell viability

Blandino et al. (1999) has reported that increasing sodium alginate concentration, the thickness of the membrane decreases at a given gelation time. This effect is presumably due to the fact that on increasing the number of biopolymer molecules per unit solution volume in the locality of the core capsule, the number of binding sites for Ca^{2+} ions also increases. As a result, a more compactly cross-linked gel structure will probably form and, consequently, it will have a smaller thickness. In correlation with this phenomenon, the observed qualitatively that the capsules obtained from 0.75% (w/v) sodium alginate solutions were more resistant, from a mechanical point of view, than those obtained from less concentrated solutions. The effect of $CaCl_2$ concentration on capsule formation kinetics was studied by fixing the anionic solution at 0.5% (w/v) sodium alginate. On increasing calcium chloride concentration, the thickness of the membrane increases at a given gelation time [11]. When all these parameters are taken into consideration membrane thickness can cause diffusional limitations.

Lee et al. (1992) investigated the effect of different concentrations of calcium chloride on cell viability. They observed that the cells in 1.3% (w/v) calcium chloride solution appeared to lose their viability faster than the cells in 1.5% (w/v) calcium chloride solution [14]. Similarly, as long as during the optimization stage of this study, we also treated the cells with different concentrations of $CaCl_2$ solutions in a changing range of 1% to 1.5% (w/v). The results indicated that the cell treated with 1.3% (w/v) calcium chloride solution showed a longer lag phase than the cells treated with 1.5% (w/v) calcium chloride solution, confirming the previous results that the cells in 1.3% (w/v) $CaCl_2$ solution lost their viability faster than the cells in 1.5% (w/v) $CaCl_2$ solution during calcium chloride treatment. Accordingly, in order to minimize cell damage during calcium alginate entrapment, it is desirable to use a 1.5% (w/v) calcium chloride solution rather than a 1.3% (w/v). In addition, the exposure time of the cells to a calcium chloride solution should be minimized. In this study, the use of 1.5% (w/v) $CaCl_2$ resulted in better bead stability and cell viability.

Cell number, bead size, diffusion and mass transfer

In order to facilitate diffusion of materials from the calcium alginate beads, the beads should be as porous as possible. One way to get this goal is to lower the concentration of alginate. However, the calcium alginate gels at concentrations less than 0.8% (w/v) were found to be too soft to remain stable in spinner culture. The use of alginate with a high molecular weight and a low mannuronic acid/guluronic acid ratio can also increase the porosity of calcium alginate beads. Conversely, the increase in porosity of calcium alginate beads augments the cell concentration in the beads, which may increase the diffusion limitation [5].

Sinacore et al. (1989), used 1.0-1,5 mm diameters of sodium alginate (0.8% (w/v)) beads with 1×10^6 cell/ml alginate. The results of this study showed that, few microcolonies within the 1.0-1.5 mm diameter alginate beads would exceed the threshold diameter where mass transfer would become limiting. The high cell viabilities (> 90%) observed in cultures of entrapped cells is reliable with this hypothesis. This literature also indicates that using Na-alginate concentration higher than 0.8% is limiting the mass transfer and inhibits cell proliferation and growth [2]. However, in our case, this amount of Na-alginate caused dissolution of beads in shorter time comparing to that of 1.2% (w/v) Na-alginate beads.

Arus et al. (2005), found out that the highest cell seeding density ($1x10^7$ cell/ml) can be obtained with the beads >300μm diameter where the worst was $1\text{-}5x10^6$ [15]. However, in the present study, the diameters of the beads were approximately 1 mm. Therefore, unlike the previous work, in our study, the mass transfer limitation might have been a restrictive factor for cell viability when higher cell densities were used, that is why 2×10^6 cell/ml cell density was used in this study.

Glucose and lactic acid metabolism

Since glucose utilization may vary with growth and culture conditions and 80% of glucose is theoretically metabolized into lactate, it is impossible to derive an exact relation between cell yield and glucose consumption [16]. Various metabolic changes have been observed in tumors [17]. Mammalian cells in culture, especially continuous cell lines, have an inefficient glucose metabolism where most of the glucose consumed is converted into lactate, while very little is oxidized in the tricarboxylic acid cycle (TCA). Due to the low energy gain, the cell specific glucose uptake rates are in excess to what is normally required for cellular metabolism [18]. Apoptosis can be accounted for up to 80% of the cell death in bioreactors as a result of such stresses as nutrient or growth factor depletion, hypoxia and hyperoxia, hydrodynamic stress, elevated osmolality, and toxic byproduct accumulation with associated changes in the culture pH [19]. With HLM cells, Paul (1965) showed that a change in medium pH from 7.2 to 6.8 caused 10-fold decrease in lactate formed per mole of glucose utilized. Such a pH change could readily occur over several hours with a high concentration of a rapidly metabolizing cell such as mouse lymphoma [20]. Correspondingly, due to cell line properties of myeloma cells, encapsulation of them in Ca-alginate microbeads and some pH changes could cause lower lactate production per mole of glucose.

Glucose in the medium enters the cell via the glucose transport protein (GLUT-1), located in the outer cell membrane. Glucose enters the glycolysis pathway where hexokinase catalyzes the phosphorylation of glucose to glucose-6-phosphate. Subsequent glycolytic steps reduce the sugar to pyruvate and lactate. Pyruvate enters the mitochondrial matrix and the TCA cycle, which generates NADH and $FADH_2$, substrates for oxidative phosphorylation. ATP generated via oxidative phosphorylation exits the mitochondria via ANT in the iner mitochondrial membrane and VDAC in the outer mitochondrial membrane; ADP returns to the mitochondria via VDAC and ANT for regeneration of ATP. IMM, inner mitochondrial membrane; IMS, intermembrane space; OMM, outer mitochondrial membrane; VDAC, voltage-dependent anion channel; ANT, adenine nucleotide translocator; GLUT-1, glucose transport protein; TCA, tricarboxylic acid; G-6-P, glucose-6-phosphate [19].

The sugar can be metabolized to generate ATP and metabolic intermediates through glycolysis, the citric acid cycle, and oxidative phosphorylation. Alternatively, glucose may be oxidized to provide precursors for nucleic acid and protein synthesis and NADPH for reducing power in biosynthetic reactions. The glucose metabolism pathway relies upon transport proteins (e.g., glucose transporter and voltage-dependent anion channel) and metabolic enzymes (e.g., hexokinase, aldolase) to ensure glucose levels can meet the energy demands of a proliferating cell. Glucose is phosphorylated to pyruvate. Pyruvate, the main product of glycolysis (the others being ATP and lactate), enters the mitochondria matrix for utilization in the citric acid cycle. The citric acid cycle provides precursors for the production of ATP via oxidative phosphorylation, and ATP exits the mitochondria through specific transporters for use within the cell (Figure 7) [19]. Therefore, in this study, despite the decrease in lactate formation, increase in glucose utilization from the fourth to eighth days could be a result of either converting glucose to ATP or glucose usage in TCA cycle.

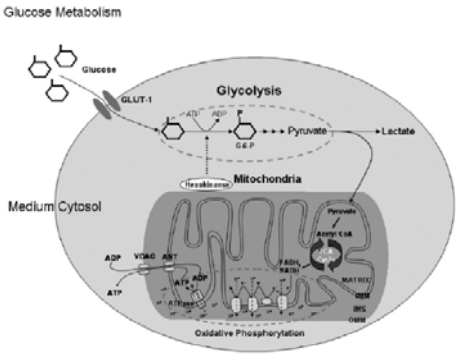

Figure 7: The glucose metabolic pathway in mammalian cells [19].

The overall results show that the cell line used for the encapsulation is very important for the cell viability as well as production ability. To prove this approach we also encapsulated L929 Mouse fibroblast cells in Ca-alginate, and it was seen that fibroblast cells could live longer than myeloma cells (data was not shown).

CONCLUSION

In this study, we immobilized the myeloma cells in Ca-aligante beads in succes, albeit, relatively short time cell viability was observed. However, this study is the important source for the next step in which Ca-alginate encapsulated hybridoma cells will be cultivated also in bioreactors.

REFERENCES

[1] S. Sinacore M., Creswick, BC and Buehler, R. (1989) Entrapment and growth of murine hybridoma cells in calcium alginate gel microbeads. *Nature Bio/Technology* 7:1275-1279.

[2] Lee M. G., Varma A., Palsson B. O. (1991) Production of monoclonal antibody using free-suspended and immobilized hybridoma cells: Effect of serum. *Biotechnology an d Bioengineering* 38: 821-830.

[3] Breguet V., Gugerli R., Stockar U., Marison I. W. (2007) CHO immobilization in alginate/poly-L-lysine microcapsules: an understanding of potentials and limitations. *Cytotechnology* 53: 81-93.

[4] Microcarrier Cell Culture Handbook, Principles and Methods; Edition AA 18: 1140-62.

[5] *A Report of the* Committee on Methods of Producing Monoclonal Antibodies Institute for Laboratory Animal Research National Research

Council: Monoclonal Antibody Production. *National Academy Press* Washington, DC; 1999.

[6] Pajic-Lijakovic´ I., Bugarski D., Plavsic´ M., Bugarski B. (2007) Influence of microenvironmental conditions on hybridoma cell growth inside the alginate-poly-L-lysine microcapsule. *Process Biochemistry* 42:167–174.

[7] Orive G.,. Herna´ndez R. M, Gasco´ n A. R., Calafiore R., Chang T. M. S., Vos P., Hortelano G., Hunkeler D., Laci´k I., and Pedraz J. L. (2004) History, challenges and perspectives of cell microencapsulation. *Biotechnology* Vol.22 No.2.

[8] Rowley Jon A., Madlambayan G., Money D. J. 1999) Alginate hydrogels as synthetic extracellular matrix materials. *Biomaterials* 20: 45–53.

[9] Draget I. K., Smidsrød O., Skjåk-Bræk G. (2005). Alginates from algae: Polysacchrides and polyamids in the food industry; properties, production, and patents. *Wiley-Vch.*

[10] S. J. Kim, B. K. Han, G. M. Lee. (1995) Feasibility study on the hybridoma cells for IgM use of calcium alginate-entrapped production. *Bioprocess Engineering* 12: 65-70.

[11] Blandino A., Macias M., Cantero D. (1999). Formation of Ca-alginate gel capsules: Influence of sodium alginate and $CaCl_2$ concentration on gelation kinetics. *Journal of Bioscience and Bioengineering* Vol. 88, 686-689.

[12] Smidsrød O., Skjak-Braek G. (1990) Alginate as immobilization matrix for cells. *Tibtech* Vol.8.

[13] Lee G. M. and Palson B. O. (1990). Simplified method of making alginate-polylysine microcapsules for hybridoma cell culture using RPMI-1640 medium. *Biotechnology Te chniques* Vol. 4, 341-344.

[14] Lee G. M., Han B. K., Kim J. H., and Palsson B. O. (1992) Effect of calcium chloride treatment on hybridoma cell viability and growth. *Biotechnology Letters* Vol. 14, 891-896.

[15] Arús L., Orıve G., Hernández R., Rodriguez A., Rojas A. and Pedraz J. L. (2005) The influence of cellular seeding density in the microencapsulation of hybridoma cells. *J. Bi omater. Sci . P olymer Ed n.* Vol. 16, 521–529.

[16] Aslankaraoğlu E., Gümüşderioğlu M., Gürhan İ.S. (2003) Hybridoma Cells Immobilized on Nonwoven Polyester Fabric Discs: Proliferation and Monoclonal Antibody Production in Stationary Culture *Journal Of B ıomaterıals Ap plıcatıons* Vol. 18, 137–148.

[17] Chi V. D. and Gregg L. S. (1999) Oncogenic alterations of metabolism. *Elsevier Scienc e* TIBS 24.

[18] Hinterkörner G., Brugger G., Müller D., Hesse F., Kunert R., Katinger H., Borth N. (2007). Improvement of the energy metabolism of recombinant CHO cells by cell sorting for reduced mitochondrial membrane potential. *Journal of Biotechnology* 129, 651–657.

[19] Majorsa B. S., Betenbaugha M. J., Chiang G. G. (2007). Links between metabolism and apoptosis in mammalian cells: Applications for anti-apoptosis engineering. *Metabolic Engineering* 9, 317–326.

[20] Rothblat H. G. and Cristofalo J. V. Growth, nutrition, and metabolism of cells in culture. Vol 1, 85.