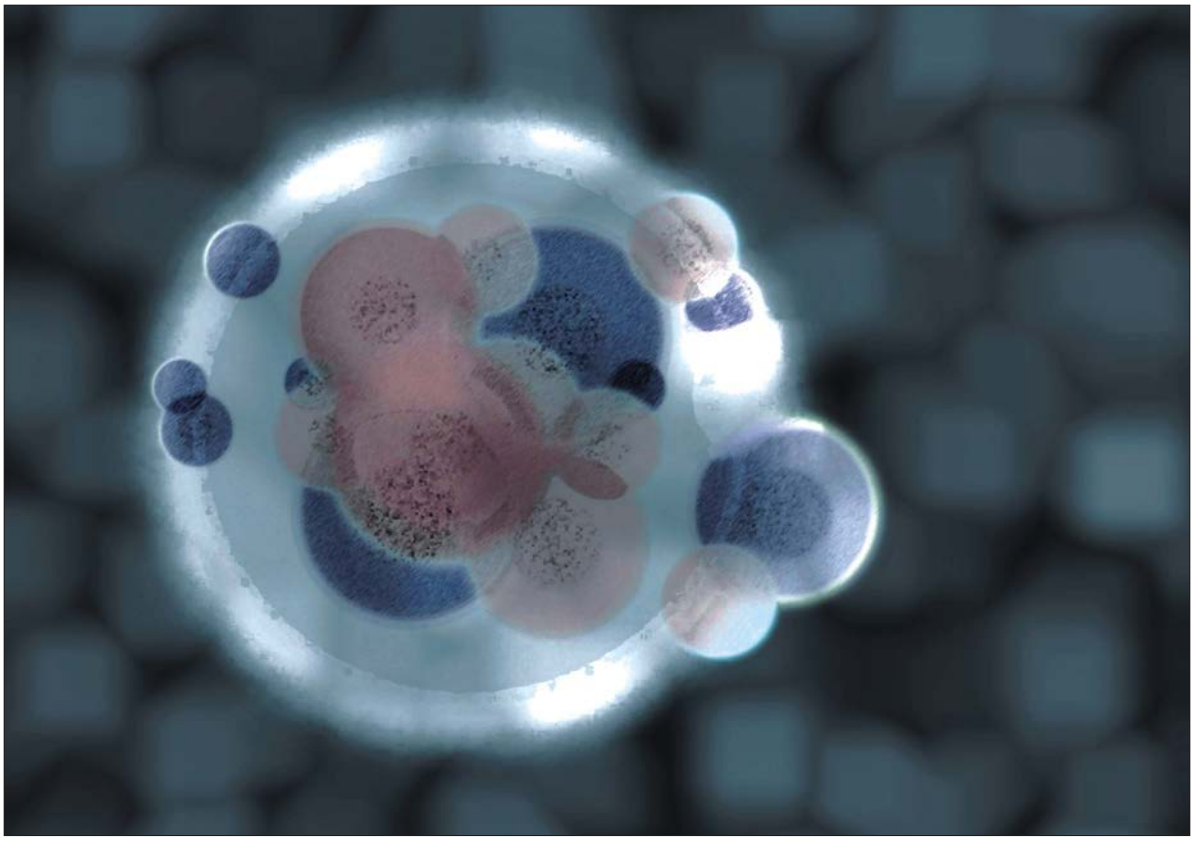

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HeLa-S3 Cell Growth Conditions in Serum-Free Medium and Adaptability for Proliferation in Suspension Culture

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Abstract: Serum-free cell culture methods are now routinely support mammalian cell growth, a practice adopted for ethical, scientific and safety concerns. The HeLa-S3 cell line is a subclone of the HeLa cell line that can grow in Serum-Free Medium (SFM) as well as suspension cultures. In order to optimize its culturing conditions in SFM, the present study investigated the efficacy of insulin and L-glutamine additives as biotic factors as well as osmotic stress as abiotic factor, all affecting growth kinetics and metabolism. Insulin was used with different concentrations ranging from 10 to 50 mg L^{-1}. It was found that cell growth is dependent on insulin up to a concentration of 25 mg L^{-1} at which maximum cell number as well as cell viability were achieved. Similarly, L-glutamine was used in the range of 3 to 8 mmol L^{-1} and was found optimum at 3 mmol L^{-1}. However, osmotic stress using saline solution addition showed that osmolality in the range of 314 to 350 mOsm kg^{-1} is preferable to cells. The study also showed the successful sequential cell adaptation from adherent culture mode to suspension culture in which cells were able to grow in small clumps of spherical-shaped cells. Based on this cultivation strategy, HeLa-S3 cells were completely adapted to proliferate suspended in serum-free medium with sustained growth kinetics and physiological properties.

Key words: HeLa cell line, serum-free medium, insulin, L-glutamine, osmolality

INTRODUCTION

HeLa cells were established by George Gey at the Johns Hopkins, Medical School, in 1951 from a human cervix adenocarcinoma of a 31 years old mother and called HeLa after the first two letters of Henrietta Lacks (Gold, 1986). These cells represent the first continuous immortalized human cell line that proliferate abnormally by rapidly applying telomeres during cell division. They can proliferate *in vitro* into unlimited number as long as basic cell requirements are provided (Masters, 2002). HeLa cells are routinely used for testing the efficacy of natural chemo-therapies (Tamayo *et al*., 2005; Abdul *et al*., 2008; Jing *et al*., 2011), drug quality control and as a good host for the expression of different recombinant proteins (Kretzmer, 2002; Bleckwenn *et al*., 2005; Hendrick *et al*., 2006). They are also widely used for viral propagation and anti-viral vaccine development for several viruses including; polio virus, human immunodeficiency virus, human papilloma virus, hepatitis C virus, adenovirus and herpes simplex virus (Kronenberger *et al*., 1998; Morizono and Harada, 1998; Kong *et al*., 2006; Soleimanjahi *et al*., 2007). Moreover, HeLa cell extracts have been used as a cell-free translation system for mRNAs and gene regulation (Witherell, 2000; Colli *et al*., 2006). Morphologically, HeLa cells are epithelial-like acquiring two unique cell morphologies one is similar to that of adherent cells and the other appeared with micro-carriers stirred under high shear condition changing from a sphere to the shape of fried egg (Masters, 2002).

HeLa cells are usually grown in Serum-Containing Medium (SM) such as Dulbacco's Modified Eagles Medium (DMEM) and Ham's F12 supplemented with 5-10% serum (Jun *et al*., 1996). However, supplementation of culture media with serum may have many disadvantages including; the threat to human health caused by contamination with viruses, mycoplasmas and prions, especially when the end product is human applicable such as vaccines (Wessman and Levings, 1999; Ozturk and Hu, 2006). Moreover, the limited availability of a good serum suppliers, the batch to batch variation causing inconsistency in cell growth patterns and product formation and the interference with the effect of hormones or growth factors upon studying their interaction with cells (Morris and Warburton, 1998). Taking in consideration, the cost of serum that can

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account for 70-85% of the overall cost of the medium and the difficulties of product purification from serum in large-scale cultivations (Latham and Chau, 1995; Even *et al*., 2006). Therefore, the need to switch to Serum-Free Medium (SFM) has become imperative. Indeed, many cell lines were successfully cultivated in SFM including non-tumor cells such as: human mucosal epithelial cells (Yahi *et al*., 1995), human endothelial cells (Labitzke and Friedl, 2001), Baby Hamster Kidney (BHK) cells (Perrin *et al*., 1995), vero cells (Butler *et al*., 2000), Chinese Hamster Ovary (CHO) cells (Haldankar *et al*., 1999) and hybridoma cells (Voigt and Zind, 1999). Moreover, SFM was used for manipulation of tumor cells such as: human prostatic carcinoma (Hedlund and Miller, 1994), lung carcinoma (Brower *et al*., 1986), human bladder carcinoma (Ruck *et al*., 1994), stem cells (Chicha *et al*., 2011; Su *et al*., 2011) and HeLa-S3 cells (El-Enshasy *et al*., 2009).

In general, *in vitro* culture of mammalian cells in SFM is affected by several factors including biotic such as insulin and L-glutamine additives and abiotic such as osmolality level. Insulin stimulates cell growth, cell cycle progression and regulation of glucose and lipid metabolism (Jayme and Smith, 2000; Saltiel and Khan, 2001). However, L-glutamine as a major source of energy can provide 30-65% of the energy required for cell growth (Butler and Christie, 1994). Furthermore, different cells might have somewhat different iso-osmotic points with optimum osmolality for most cells ranging from 260 to 320 mOsmol kg^{-1} (Xie and Wang, 1997; Freshney, 2005).

On the other hand, adaptation of cells for prolonged cultivation in suspension culture, without serum and at high cell densities develops novel cell phenotypes that may be useful for large-scale production of recombinant proteins (Sinacore *et al*., 2000; Griffiths, 2001). Suspension culture is preferable for scale-up due to the many advantages of ease, cheap, requirement of lesser space with no need for the use of proteolytic enzymes and the capability of culture in bioreactors with a total environmental control (Morris *et al*., 1997; Freshney, 2005). Moreover, suspended cell culture is characterized by homogeneity of nutrients and uniform cell population with good reproducibility from experiment to experiment. Suspension culture also provides cells in the logarithmic phase of growth with constant content of RNA, DNA and protein per cell (Mather and Roberts, 1998).

Taking all, it was therefore of interest to carry out experiments investigating the efficacy of insulin, L-glutamine and osmotic stress on HeLa-S3 cell growth and metabolism during cultivation in SFM as well as adaptability of cells to grow in suspension culture.

MATERIALS AND METHODS

Cell lines: HeLa-S3 (ACC161) cells derived from a human cervix adenocarcinoma (German Resource Center for Biological Material, DSMZ, Braunschweig, Germany). Cells grew in the form of adherent monolayer (anchoragenous-dependent). All cell culture procedures were carried out in Department of Bioprocess Development, Institute of Genetic Engineering and Biotechnology, Mubarak City for Scientific Research and Technology between 2009 and 2010.

Cultivation media: Media included; serum-enriched medium (SM; Ham's F12, Cambrex Bioscience, Verviers, Belgium) supplemented with 10% (v/v) fetal bovine serum (FBS; Sigma, Lauis, USA) and Serum-Free Medium (SFM; Scharfenberg's modification 6 or SMIF-6, Biochrom, Berlin, Germany) which is a protein-free medium characterized by its high glucose concentration of 4 g L^{-1} with well-balanced medium constituents.

Medium preparation: All media were delivered in a powder form. They were dissolved in deionized distilled water (ddH_2O) obtained from a Milli Q reagent water deionization system (Millipore, Eschborn, Germany). For each of the SM, SFM and mixtures of them, pH was adjusted to 7 and medium osmolality was adjusted with saline solution to 320 mOsmol kg^{-1} using micro-osmometer (Model 3300, Norwood, Massachusetts, USA). The medium was then sterilized by membrane filtration in a 0.22 μm microbiological filter system (TPP-Europe, Switzerland). A sterility test was performed for 3 days at 37°C. Medium contamination was detected depending on the color change of the phenol red which gives a yellow-orange color through the change in pH to acidic value as a function of the microbial growth. A sample was also examined microscopically after passing the sterility test, medium was stored at 4°C until being used. For the serum-enriched medium preparation, 10% v/v FBS was added to the Ham's F12 medium. FBS was previously incubated for 30 min at 56°C in order to deactivate the complement cytolytic proteins.

Cell propagation and cultivation: All cell culture procedures were carried out under class II biological safety cabinets (Nuaire, Inc. Plymuth, MN 55447, USA). Cells were fed twice a week; cultivated and propagated in 25 or 75 mL T-flasks and 250 or 500 mL cell culture spinner flasks (Techne, Cambridge, UK). Confluent monolayer of HeLa-S3 cells was collected by trypsinization using trypsin/EDTA buffered solution (200 mg L^{-1} versene

EDTA, 500 mg L^{-1} trypsin; Cambrex Bioscience Verviers, Belgium). Adherent cells were washed with basal medium (Ham's F12 medium with L-glutamine) or Hank's Balanced Salts Solution (HBSS) without sodium bicarbonate (ICNS, biochemical Inc, Ohio, USA) and then incubated with 2 mL trypsin/EDTA buffered solution at 37°C for 2 min. Cell scrapers were used to help detaching cells and hence decreasing the time of exposure to trypsin, followed by addition of 5 mL of serum-enriched medium to stop trypsin action. Detached cells were then collected and washed twice with HBSS buffer, counted then inoculated in a fresh medium. Cultures were kept in 5% CO_2 incubator at 37°C and 80% air humidity (New Brunswick scientific Co., NJ, USA). Cell counting was carried out using heamocytometer (Improved Neubauer Type, Germany). Cell viability was determined using trypan blue exclusion method (Walford *et al*., 1964). The supernatant of a one ml sample of harvested cells was collected by centrifugation for 5 min at 112 g and was frozen immediately at -20°C for further analysis.

Adaptation of HeLa-S3 cells to grow suspended in SFM: HeLa-S3 cells were adapted to grow in SFM using sequential ratios between SM containing 10% FBS and SFM. This adaptation was carried out through a series of steps in which SM concentration was successively reduced as following: 1st passages were in 75% SM: 25% SFM, 2nd passages in 50% SM: 50% SFM, 3rd passages in 25% SM: 75% SFM, 4th passages in 10% SM: 90% SFM and 5th passages in 100% SFM (Sinacore *et al*., 1996). After establishment of stable growth rate and high viability, serum-free adapted cells were divided into two fractions, one preserved by freezing in serum-free freezing media and the other used for further adaptation to suspension culture in spinner flasks. This adaptation was carried out at stirring speed of 50 rpm which is sufficient to maintain the cells in suspension.

Efficacies of insulin, L-glutamine and osmotic stress on HeLa-S3 cells cultivated in SFM: Insulin was added in the form of proteomic zinc insulin (Fluka Chemie, Germany) dissolved in ddH_2O and sterilized by filtration. The hormone was used in different concentrations ranging from 10 to 50 mg L^{-1}. However, L-glutamine (Biochromkg, Leonorenstr, Germany) was used in different concentrations ranging from 3 to 8 mmol L^{-1} in SFM. Duplicated T-flasks were prepared for each concentration with an appropriate cell density of 1×10^5 cells mL^{-1}. After culturing time was elapsed, supernatants were collected for glucose and lactate analysis. Whereas, osmotic stress efficacy was estimated in the range of 314-425 mOsmol kg^{-1}, by adding saline solution to SFM.

Determination of glucose and lactate concentration: Glucose and lactate concentrations in the culture supernatants were determined using a biochemistry analyzer (YSI 2700 Select, Yellow Springs Instruments, Ohio, USA). The measurement is based on a coupled enzymatic-electrochemical process in which the enzyme (glucose oxidase or lactate oxidase) is immobilized between two membrane layers of polycarbonate and cellulose acetate. The substrate is oxidized at the enzyme layer producing hydrogen peroxide which passes through the acetate membrane to be oxidized on the platinum anode (Pt/AgCl/Ag) producing electrons. The electron flow is proportional to the peroxide concentration and hence the substrate concentration.

Morphological observation of cultured cells: Cultured cells were photographed using an inverted phase-contrast microscope (Olympus 1X70, Japan) equipped by digital camera (Cammedia C-4040 Zoom) and an image analysis system (Analysis 3.1 Soft Imaging System SIS, GmbH, Germany).

Statistical analysis: All statistical analyses were performed using SPSS 13 statistical software. Descriptive data of all criteria of cell growth kinetics were calculated as the mean of triplicate cultures in three independently repeated assays.

RESULTS

HeLa-S3 cell morphology during adaptation to SFM: The adaptation process from SM to SFM alters cell morphology. In SM, the cells grew as an adherent monolayer (anchorgenous-dependent). They initially appeared flattened triangular to irregular-shaped. Within 3 days, cultures were 70-80% confluent and within 4-6 days they were completely confluent. In SM confluent cultures, cells were tightly packed, polygonal with spiny projections and variable sizes. However, in SFM, cells were loosely attached especially in the initial passages of adaptation. They appeared smaller and spindle-shaped reaching the confluent monolayer delayed within 6-8 days. In SFM confluent cultures, small populations of cells tend to dislodge from the substratum (i.e., adapted to static suspension culture) and grew floated in colonies of spherical-shaped cells. These colonies were readily expanded and eventually become confluent (Fig. 1a-l).

Efficacy of insulin supplementation on HeLa-S3 cells cultivated in SFM: Insulin enhanced the cell growth rates up to 25 mg L^{-1}. It was found that increasing insulin

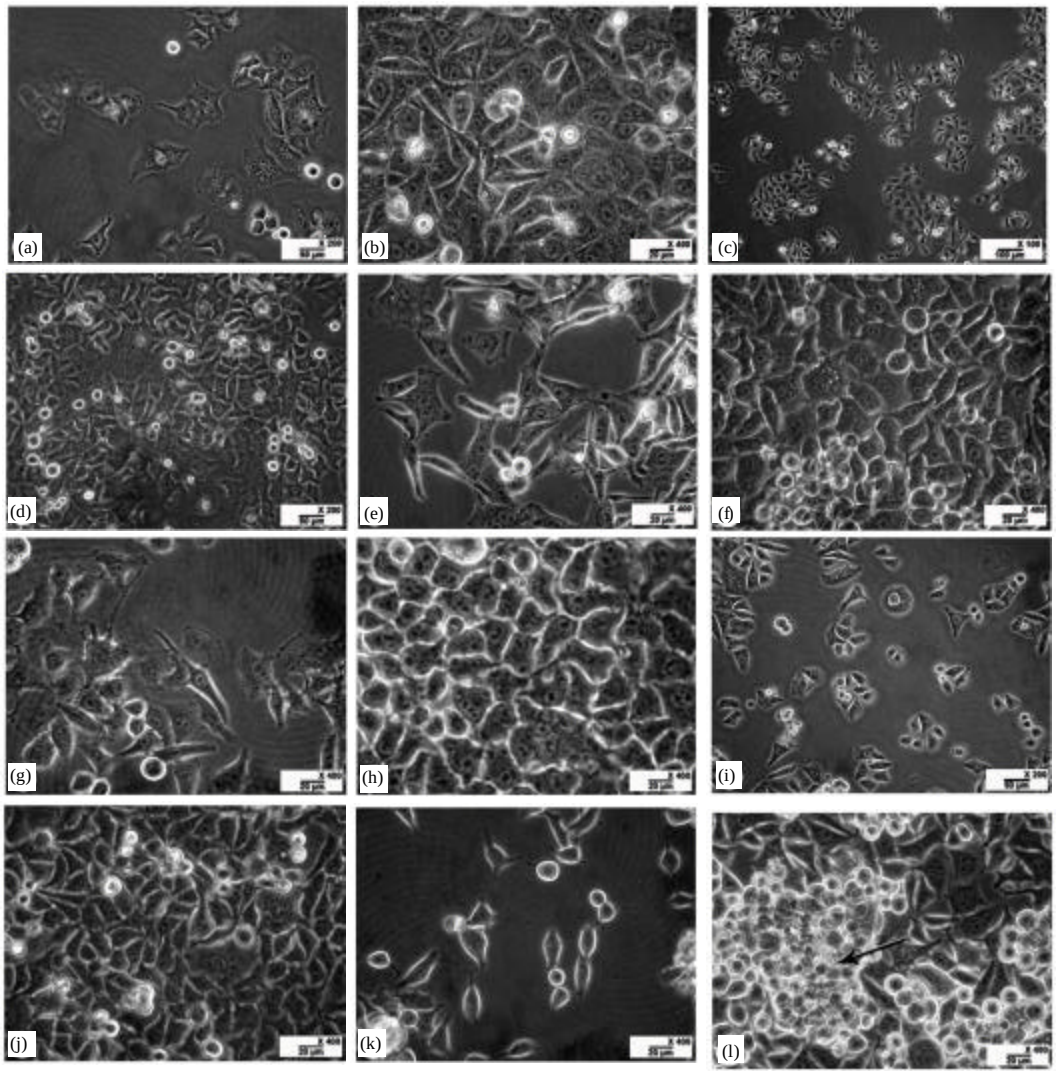

Fig. 1 (a-l): Photomicrographs of HeLa-S3 cells during adaptation to SFM from SM. Steps of adaptation included: (a, b) cells cultured in SM at low and high cell densities, respectively, (c, d) cells in 75% SM: 25% SFM, (e, f) cells in 50% SM: 50% SFM. Cells showed no significant change during these steps of adaptation. They appeared triangular to irregular-shaped, (g, h) cells in 25% SM: 75% SFM, (i, j) cells in 10% SM: 90% SFM and (k, l) cells in 100% SFM. During these passages, the cells acquire a spindle-shaped appearance. Moreover, some populations of cells (pointed by an arrow in L) have dislodged from the substratum and grew as colonies of spherically-shaped cells upon the confluent monolayer (adapted to static suspension culture)

concentration increased both total cell number and cell viability from 9.83×10^5 cells mL^{-1} with viability 81.82% in SFM without insulin to 11.20×10^5 cells mL^{-1} with viability 95.54% in SFM containing 10 mg L^{-1} insulin. Whereas, further increase of insulin to 25 mg L^{-1} increased cell viability to 98.21% but with sustained total cell number of 11.20×10^5 cells mL^{-1}. Moreover, cells required 120 h to reach their maximum cell number in all cultures except for the 50 mg L^{-1} culture where cells needed only 72 h to reach their maximum cell number. On the other hand, higher metabolic activities (as indicated by higher glucose consumption and lactate production rates) were demonstrated in cultures with insulin at 50 and 25 mg L^{-1} followed by 10 mg L^{-1} and cultures without insulin,

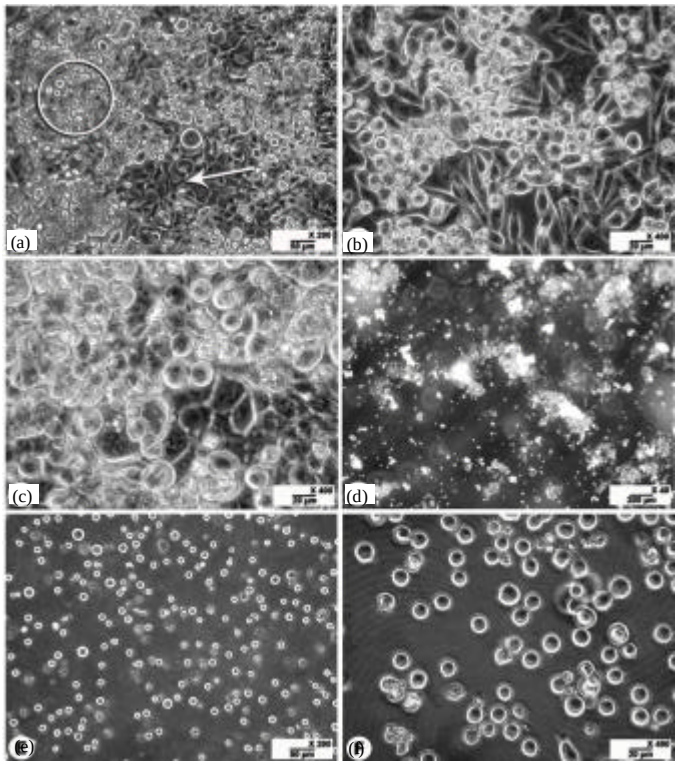

Fig. 2 (a-f): Photomicrographs of HeLa-S3 cells during adaptation to suspension culture in SFM. Adaptation steps included: (a, b) the initiation of cell dislodging, (c) cells grew floated upon the confluent monolayer, (d) cells escape from anchoragenous-dependent inhibition and grew floated in clumps and (e, f) cell dispersion with agitation. Cells acquire two phenotypes in a, b and c; adherent spindle-shaped (arrow) and spherical-shaped (circle) adapted to static suspension

respectively. From all of these records, insulin was found optimum at a concentration of 25 mg L^{-1} (Table 1).

Efficacy of L-glutamine supplementation on HeLa-S3 cells cultivated in SFM: L-glutamine showed an inhibitory effect on HeLa-S3 cell viability in the range of 3 to 8 mmol L^{-1}. It was found that 3 mmol L^{-1} L-glutamine is the most suitable for cultivation. At this concentration, the cells grew exponentially reaching maximum viability of 88.04% after 120 h compared to lower percentages of viability obtained with higher concentrations of L-glutamine. On the contrary, L-glutamine addition showed an enhancing effect on cellular metabolism in the range of 3 to 6 mmol L^{-1} as judged by glucose consumption and lactate production rates that recorded their maximum values at a concentration of 6 mmol L^{-1} (Table 2).

Efficacy of medium osmolality on HeLa-S3 cells cultivated in SFM: The efficacy of medium osmolality was studied in the range of 314 to 425 mOsm kg^{-1}. It was found that 314 mOsm kg^{-1} is favorable to cells that recorded maximum viability of 84.4% and minimum dead cells of 1.5×10^5 cells mL^{-1} after 120 h of cultivation. Although there was an increase in both cell growth (13.6×10^5 cells mL^{-1} total cells) and metabolic activity (0.0203 g/L/h glucose consumption and 0.0210 g/L/h lactate production) at 350 mOsm kg^{-1}, this was associated with increased dead cells that reached about 2.8×10^5 cells mL^{-1} and lowered viability of about 78.8%. Moreover, further increase in osmolality in the range of 375 to 425 mOsm kg^{-1} inhibits cell growth, viability and metabolism (Table 3).

Adaptation of HeLa-S3 cell to suspension culture from adherent culture: Transfer of the colonies of static suspension cells that grew floated upon a confluent monolayer in SFM to agitated cultivation system resulted in dispersion of cells (Fig. 2a-f). They initially appeared solitary and with time, they grew in small clumps of cells (Fig. 3a-c). However, kinetics of cell growth and metabolism demonstrated improved levels through ten

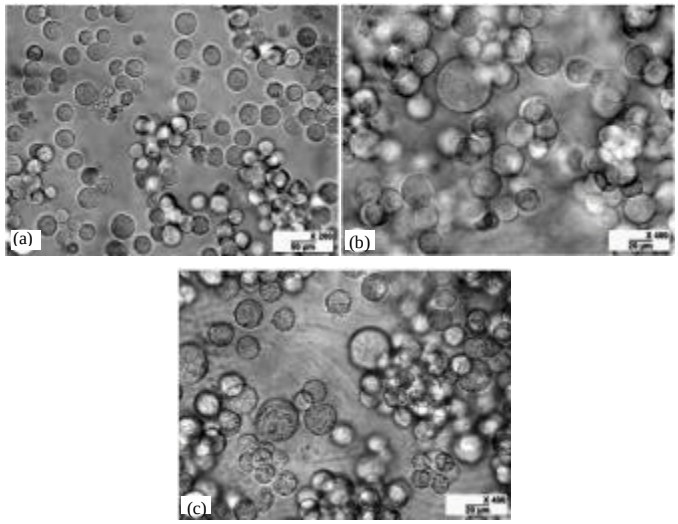

Fig. 3 (a-c): Photomicrographs of suspension adapted HeLa-S3 cells. (a-c) are representatives of the cells in day 1, 2 and 3 after adaptation, respectively, (a) cell clumps dispersed, (b) 2-4 cells attached to each other as a result of cell proliferation and (c) 6-12 cells aggregate in clumps with continuing cell proliferation. These clumps of cells start again to disperse upon agitation once cells have finished division

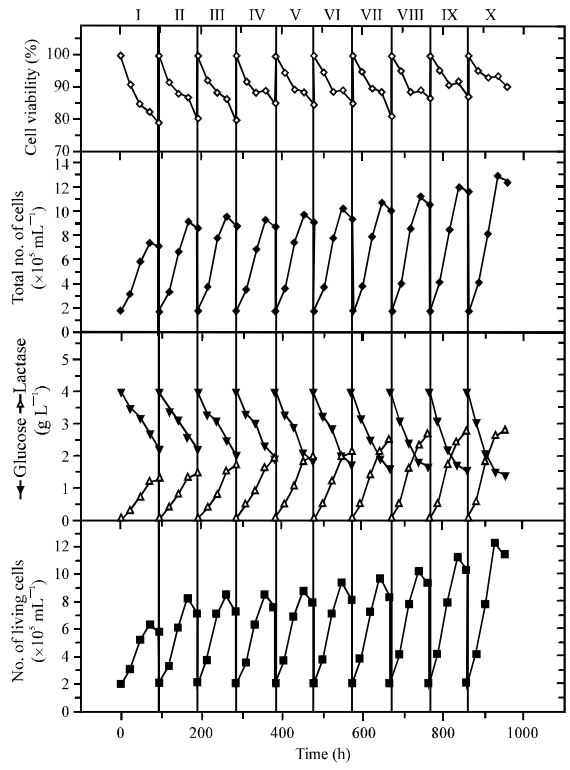

Fig. 4: Cell growth, viability, glucose consumption and lactate production during adaptation to suspension culture

passages of 96 h each. Adapted suspension cells recorded maximum total cell number of about 13.11×10^5 cells mL^{-1} at the final passage comparing with 7.6×10^5 cells mL^{-1} during the initial adaptation passage. In addition, cell viability recorded more that 90% in the last two passages compared with lower percentages from 82.8 to 86.7% during the initial passages of adaptation. Furthermore, the metabolic activity of the cells showed an increase in both glucose consumption and lactate production rates in the last two passages as a response to the elevation in the number of viable cells (Fig. 4).

DISCUSSION

HeLa-S3 cell line is commonly used in the biotechnology industry to produce recombinant proteins. The current study shows the optimization of its culturing conditions in order to maintain cell viability and reduce cell death in the absence of serum as well as adaptability of cells to grow in suspension culture as a step towards the large-scale cultivation.

Comparison of growth kinetics in insulin-free to insulin-supplemented media, in the range of 10 to 50 mg L^{-1}, revealed that insulin supplementation increased the rate of cell growth and the entire cellular metabolism up to 25 mg L^{-1}. At which the maximum number of living cells was obtained with lowered number of dead cells (Table 1). These results agree with previous

Table 1: Kinetics of cell growth and metabolism during cultivation of HeLa-S3 cells in SFM supplemented with different insulin concentrations

	Insulin supplemented to SFM (mg L^{-1})			
Parameters	Without	10	25	50
Maximum no.of living cells ($\times 10^5$ mL^{-1})	9.00	10.70	11.00	9.9
Maximum no. of dead cells ($\times 10^5$ mL^{-1})	1.80	0.50	0.20	0.16
Maximum no. of total cells ($\times 10^5$ mL^{-1})	9.83	11.20	11.20	10.5
Cell viability (%)*	81.82	95.54	98.21	98.39
$-Q_{glucose}$ (g/L/h)	0.0197	0.0225	0.0233	0.0232
$Q_{lactate}$ (g/L/h)	0.0167	0.0197	0.0208	0.0217

*Viability calculated after 120 h of cultivation

studies reported that insulin induces the growth of HeLa cells (Blaker *et al*., 1971), human appendix cells (Lieberman and Ove, 1959), human stem cells of exfoliated deciduous teeth (Chadipiralla *et al*., 2010) and together with the basic fibroblast growth factor maintain viability and enhance the proliferation of CHO cells (Liu and Wu, 2009). However, at 50 mg L^{-1} insulin, the number of viable cells slightly declined as a sign for stopping cell division, a consequence probably due to the inability of cells to take sufficient nutrients at confluency (Griffiths, 1970b). Although insulin induces pinocytosis and glucose uptake and metabolism to help the cell to take up and utilize nutrients more efficiently in confluent cultures, it might not prevent but rather delay halting cell growth (Griffiths, 1970a; Vinci and Parekh, 2003).

In support of the finding that higher cell growth in insulin-supplemented cultures was accompanied by higher glucose consumption and lactate production, it has been reported that insulin stimulated glycolysis and lactic acid formation (Paul *et al*., 1966; Gould and Holman, 1993). In general, cancer cells preferentially utilize glucose in order to satisfy their increased energetic and biosynthetic requirements (Yalcin *et al*., 2009). Insulin also increases the biosynthesis of fatty acids and nucleic acids when the supply of energy-rich compounds, such as glucose and glutamine are limited (Paul and Pearson, 1960; Komolov and Fedotov, 1978). In addition, the decreased viability of mouse L cell line in insulin deficient cultures confirmed the role of insulin for supporting cell growth (Waymouth and Reed, 1965).

Concerning the efficacy of L-glutamine supplementation, in the range of 3 to 8 mmol L^{-1}, the highest cell viability was achieved with 3 mmol L^{-1} and further increase of L-glutamine resulted in decreased cell viability. However, both glucose consumption and lactate production rates increased with L-glutamine addition in the range of 3 to 6 mmol L^{-1} which was not reflected on the cell division but rather resulted in higher death rate. Further increase of L-glutamine beyond 6 mmol L^{-1} resulted in a reduction in all cellular activity; cell division and cell viability as well (Table 2). In agreement with the present study, it has been suggested that glutamine influenced the consumption of glucose (Jeong and Wang, 1995). In HeLa cell culture, the main products of glutamine are CO_2 and lactate (Reitzer *et al*., 1979; Windmueller, 1982). Glutamine also induces 2 to 3 fold stimulation in the synthesis of glycogen (Mojena *et al*., 1985) and consumed similar to glucose but CO_2 yield from glutamine is at least 4 times greater than that of glucose (Reitzer *et al*., 1979). Although, glutamine is a major source of nitrogen for the synthesis of biomass (Lanks and Li, 1988; Street *et al*., 1993), its consumption is associated with ammonia production. Hence, the maximum viability of cells obtained at lower L-glutamine concentration may be due to the induction of lower ammonia and subsequently lower ammonia toxicity. Theoretically, consumption of one mole of glutamine produces two moles of ammonium. Indeed, the production of ammonia from glutamine is not only dependent on glutamine concentration but is also influenced by the concentration of other nutrients (Meijer and van Dijken, 1995). Therefore, L-glutamine was found optimum at 3 mmol L^{-1} that was confirmed by previous reports suggesting lower levels of glutamine (Blaker *et al*., 1971; Vriezen *et al*., 1997).

In the current study, HeLa-S3 cells showed an increase of cell growth with the increase of osmotic pressure in the range of 314 to 350 mOsm kg^{-1}. The maximum total cell number as well as the maximum number of viable cells was obtained with osmolality of 350 mOsm kg^{-1} (Table 3). Optimized osmolality may enhance the transport of nutrients, particularly amino acids into the cells (Oh *et al*., 1993). Albeit, other cell lines such as murine hybridoma cells and human bone marrow-derived stem cells require higher osmotic pressures to reach their maximum growth (Oh *et al*., 1995; El-Enshasy *et al*., 2007). In case of HeLa-S3 cells, further increase in medium osmolality beyond 350 mOsm kg^{-1} declines cell growth and metabolism probably due to cell shrinkage (Reusch *et al*., 1995).

Furthermore, adaptation of HeLa-S3 cells to agitated suspension culture revealed that cell viability increases with continued sub-culturing (passages). Cells recorded the lowest viability of about 79.5% during the first passage while, forward adaptation through several subsequent passages brings an improved viability in the

Table 2: Kinetics of cell growth and cellular metabolism during cultivation of HeLa-S3 cells in SFM supplemented with different L-glutamine concentrations

	L-glutamine concentration in SFM media (mmol L^{-1})					
Parameters	3	4	5	6	7	8
Maximum no. of living cells (× 10^5 mL^{-1})	9.20	9.40	9.80	9.20	6.80	5.80
Maximum no. of dead cells (× 10^5 mL^{-1})	1.10	1.80	3.20	3.90	2.70	2.80
Maximum no. of total cell (×10^5 mL^{-1})	9.82	10.15	11.00	11.20	8.60	7.40
Cell viability (%)*	88.04	81.25	70.09	65.18	64.00	60.00
$-Q_{glucose}$ (g/L/h)	0.0190	0.0204	0.0225	0.0242	0.0208	0.0192
$Q_{lactate}$ (g/L/h)	0.0167	0.0192	0.0220	0.0229	0.0192	0.0160

*Viability calculated after 120 h of cultivation

Table 3: Kinetics of cell growth and cellular metabolism during cultivation of HeLa S3 cells in SFM of different osmotic pressures

	Medium osmolarity (mOsmol kg^{-1})					
Parameters	314	325	350	375	400	425
Maximum no. of living cells (×10^5 mL^{-1})	9.0	9.50	11.10	8.70	7.10	5.90
Maximum no. of dead cells (×10^5 mL^{-1})	1.5	2.00	2.80	4.501	5.10	5.10
Maximum no. of total cells (×10^5 mL^{-1})	9.83	10.50	13.60	11.20	10.60	9.70
Cell viability (%)*	84.4	80.90	78.80	59.10	48.50	37.80
$-Q_{glucose}$ (g/L/h)	0.0192	0.0196	0.0203	0.0182	0.0158	0.0108
$Q_{lactate}$ (g/L/h)	0.0166	0.0183	0.0210	0.0167	0.0144	0.0102

*Viability calculated after 120 h of cultivation

range of 80-90% (Fig. 4). The decrease in cell viability is usually associated with the substrate depletion or accumulation of acid or other metabolic products (Maclimans *et al*., 1957; Freshney, 2010). This effect was avoided with harvesting cells after 96 h. Hence, the poor adaptability of cells especially during the initial passages improved exponentially with time and reflected on the increased viability and metabolic activity. After 960 h, the cells lose their adherent dependency and become adapted to suspension culture with a viability of more than 90%.

During adaptation to SFM from SM, HeLa-S3 cells transform from an epithelial-shaped (polygonal or irregular-shape) to a spindle-shaped appearance and tend to dislodge from the substratum. The newly detached cells take a spherical shape with smooth membrane (Fig. 1). In SFM, the cells require longer time to reach confluency more than in SM and tend to dislodge from the substratum, result of serum deprivation since serum is an attachment factor that help spreading the cells (Freshney, 1987; Butler, 1996). Moreover, in confluent cultures which become crowded, the cells tend to reduce their sizes, rounded up and escape from contact inhibition (Linge, 2005). Contrarily to Freshney (2005), not all detached cells from substratum are dead and many tumor cell lines do not produce attachment factors and remain in suspension (Morris *et al*., 1997). HeLa-S3 cells were able to grow first as dispersed single rounded cells and continued to grow and proliferate giving finally small clusters of cells.

CONCLUSION

The optimization of HeLa S3 cell growth in SFM and the adaptation to suspension culture described herein is useful for the development of large-scale cell culture processes for the manufacture of recombinant protein biotherapeutics in the absence of animal-derived medium components. This may reduce the potential for introduction of adventitious contamination and greatly simplifies the purification of the produced protein.

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