EFFECT OF CARBOHYDRATES, AMINO ACIDS, AND PEPTONES ON SF-9 CELL CULTURE IN TUBESPIN BIOREACTORS

A DISSERTATION

Submitted in partial fulfillment of requirement to award of degree

MASTER OF TECHNOLOGY (ADVANCED CHEMICAL ANALYSIS)

by

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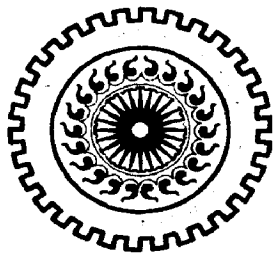

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CANDIDATE DECLARATION

I hereby declare that the work being presented in the dissertation entitled "EFFECT OF CARBOHYDRATES, AMINO ACIDS, AND PEPTONES ON SF-9 CELL CULTURE IN TUBESPIN BIOREACTORS" is an authentic record of work carried out jointly under the supervision of Prof.Florian Maria Wurm, and Dr. Kaushik Ghosh, during the period July 2011 - April 2012 at Ecole Polytechnique de Federale Lausanne, Switzerland and Indian Institute of Technology, Roorkee respectively. The dissertation is being submitted for the partial fulfillment for the award of Master of Technolgy (Advanced Chemical Analysis) degree at Department of Chemistry, Indian Institute of Technology Roorkee (Uttarakhand, India). I have not submitted the matter embodied in the dissertation report in any other institution for the award of any other degree.

Date: 15·06·2012

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CERTIFICATE

This is to certify that the above statement made by the candidate is correct to the best of my knowledge.

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A T T E S T A T I O N

ear Madam, Dear Sir,

'e confirm herewith that Mrs Archita Chaudhary did her Master Project in our

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est regards,

'of. Florian Wurm

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I. ABSTRACT

Lepidopteran insect cell culture technology has progressed to the point of becoming an essential part of one of the most successful eukaryotic expression systems and is increasingly used industrially on a large scale. Therefore, there is a constant need for convenient and low-cost culture media capable of supporting good insect cell growth and ensuring high yield of baculovirus as well as the strong expression of recombinant proteins. This study is performed to alter growth characteristics of Sf-9 cells with simple supplementation of basal IPL-41 with carbohydrates, amino acids and peptones. These compounds are an essential part of the insect cell metabolism. The process of this simple media development is highly cost effective and aids to obtain cell cultures with higher cell densities, superior viabilities and longer culture viabilities.

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1. INTRODUCTION

1.1 HISTORICAL BACKGROUND

Insect cell culture was first established in 1915 when the German entomologist Richard R. Goldschmidt worked with silk moth [1]. He accounted in vitro spermatogenesis and maintained cells from homologous species alive in hemolymph used as a culture medium. Stanley Gesser made efforts to develop insect tissue culture during 1917 and 1922. William Trager,1935 achieved the first long term cultivation of insect cells (a culture of silkworm ovary cells maintained for 3 weeks in medium consisting of hemolymph, inorganic salts, maltose and digested egg albumin).

Gerard R Wyatt in 1956 isolated cells from the silkworm *Bombyx Mori* and analysed the amino acids, organic acids, organic salts present in the hemolymph and attempted to maintain the ovarian cells alive for two weeks [2]. It was Thomas Grace in 1962 who isolated the first lepidopteran cell line namely *Spodoptera frugiperda Sf9*, and developed and media for their in vitro maintenance, which is widely used in cell culture even today with the name of Grace's media. Two years later Grace established the first mosquito cell line [2] and from here lepidopteran cell cultures took a spin.

In the same time frame exhaustive studies began on wild type baculoviruses infecting in vivo lepidopteran larvae came under exhaustive studies. These baculoviruses were originally propagated in larvae and were applied for the in vitro production of bio insecticides. In 1982 baculovirus were first applied in agriculture in 1892, the first extensive trials of baculovirus efficacy and safety were carried out in 1975 by the US Environmental Protection Agency (EPA) [3]. In the period of 70's and 80's more focused research was based on insect cell culture and the new arena of invertebrate cell cultivation developed independently of classical entomological studies. During this time Karl Maramorosch adapted insect cell cultivation to a large scale, and developed on universal insect cell medium both with (medium MM) [4] and without fetal calf serum (medium MM-SF) [5] [6].

The beginning of 80's observed the development of first serum free media [7], [8] supporting large scale cultivation by replacing serum by sterols and lipids to reduce costs and lot to lot variation, whereas today the use of serum free media is essentially dictated by safety considerations [9]. The first recombinant baculovirus was constructed by [10] to produce recombinant human interferon. The use of Baculovirus Expression Vector System (BEVS) with co-cultivated Insect Cells opened a new arena of activity in the field of protein expression, and with modifications in baculovirus genome a new line of bio pesticides can be produced. In 1988, the first recombinant baculovirus expressing an insect-specific neurotoxin was developed to increase its bio pesticide action [11]. In 1991, Hink et al. tested 3 different recombinant baculoviruses for protein expression in 23 insect cell lines [12].

With the whole genome sequencing of *Autographa californica* (AcMNPV), [13], the most widely used baculovirus, new fields of baculoviruses applications are opening up, such as Baculovirus Display [14] and the usage as vectors for gene delivery [15] [16] [17]. With recent ways of cultivating Sf-9 cells In TubeSpin Bioreactors 50 (Xie, Michel PO et al., 2011) transient gene expression using BEVS-insect cell culture (Shen, Michel PO, et al., 2011) is now an effective method for therapeutic protein production. Baculoviruses as bio insecticides are an efficient, cost-effective substitute to chemical pesticides [18]. Genetically modified baculoviruses constitute a powerful tool for the expression of glycosylated recombinant proteins (r-proteins), especially in the research and medical area [19]. They are used either as part of the IC-BEVS technology platform or with mammalian cells as transfer vectors [20]. The first therapeutic manufactured in IC-BEVS, a veterinary vaccine, was approved and commercialized in 2000 (Intervet International, The Netherlands) [21]. Interest in Insect-Cell technology shows a strong and consistent growth, as seen by the fact that today 3 future human vaccines produced by IC-BEVS are in clinical phase III trials (EMEA: European Medicines Agency). However, for all these aforesaid applications, large scale production of baculoviruses, or recombinant proteins after virus infection to requires batch process, it is required to have an effective and economical system [7] thus arises the need for suitable insect cell culture medium which can strongly support the cell growth and the high yield production of baculoviruses, as well as the expression of recombinant proteins, at a competitive price [22].

1.2 INSECT CELL PHYSIOLOGY

Since Thomas Grace established the first insect cell lines from *Antheraea eucalypti* in Australia in 1962, ca. 600 such lines have been isolated from more than 100 insect species encompassing more than 6 orders [23]. Some lines are used for entomological studies, while cell lines from the dipteran Drosophila melanogaster (whose genome is now sequenced) are mainly used for genetic studies and for constitutive gene expression [24]. Lepidopteran cell lines from *Bombyx mori* (B.mori: silkworm), *Mamestra brassicae*, *Spodoptera frugiperda* (*S. frugiperda*: "fall army worm") and *Trichoplusia ni* (*T.ni*: "cabbage looper") [25] are mainly used with the BEVS for the expression of r-proteins and for the production of baculovirus bio insecticides.

Among them, Sf-9, Sf-21, Tn-368 and BTI-TN-5B1-4 isolated from *S. frugiperda* and *T. ni* (nocturnal butterflies) are the cell lines most widely used in industrial applications. Sf-21, an ovarian cell line from *S. frugiperda* established by Vaughn and coworkers in 1977 was the first line to be intensively used in research and technological applications. The Sf-9 cell line was derived from Sf-21 [10] because of its, improved growth and baculovirus infection characteristics, it has progressively replaced Sf-21 in the research and production fields, and today it is the most widely used of all insect cell lines.

The Sf lines are adapted to suspension cultivation and are easily detached from cultivation surfaces by gentle agitation without trypsinization [25]. The use of Sf-21 has diminished to the benefit of Sf-9 cells which are less fragile than Sf-21, more tolerant to osmotic, pH and shear stress than Sf-21, and show higher growth rate [25]. Sf-9 is more resistant to thermal shock [26]. High-Five cells (15 μm) are bigger with higher protein content than Sf-9 cells (13 μm), and their cell size distribution is wider than Sf-9 cells .However, the cell size depends on medium osmolarity, shear stress, cell state (viable, apoptotic, etc.) [27] [28] [29] [30] [31] [32].

Table1.1 Characteristics of Sf-9 Cells

Physiology – Cell Culture		
Cell size		13um
Reached Cell Density (10e6cells/ml)		4-10
Growth rate		0.025h-1
Optimal growth temperature		27°C
Optimal Protein production temperature		25-27°C
Optimal pH		6.2-6.4
Metabolism		
Growth	Glucose consumption	+++
	Glutamine consumption	++
	Asparagine consumption	+
	Amino acid consumption	++
	Oxygen consumption	++++
	Lactate production	+
	Ammonia production	++
	Alanine production	++

-:almost null, +:weak, ++:moderate, +++:high, ++++:higher

1.3 INSECT CELL METABOLISM

The analysis of the chemical components in insect hemolymph the insect cell can provide ideas for medium design. Studies have shown that. Insects have no capacity for steroidogenesis [33], therfore the insect diet should be supplemented with chloestrol is used for the formation of cell membranes and for the synthesis of the important steroid hormone 20- hydroxy-ecdysone [33]. Through heamolypmh analysis from various species it has been demostrated of high osmotic pressure and total amino acid concentration varies from from 300 mg/l to 2100 mg ml/l. [33]. Most insect cell culture media, therefore contain high levels of amino acids [7] [33] [34]. Furthermore, insect blood contains an unusually high level of free organic acids such as citrate, alpha-ketoglutarate malate, fumarate, succinate, oxalate, and pyruvate; concentrations range from 0.1 to 25-35 mMoles per insect [33] [35] [36] [37].

A comparative analysis of vertebrate medium used for mammalian cell culture and insect cell culture medium points out striking differences [35] [36] [37]:

- The medium is more acidic, ranging in pH from 6.2-6.9, as observed in insect tissue fluids [33]. The media are buffered with sodium phosphate, no CO2 being required for insect cell culture. The media are clear to yellow due to the supplementation of protein hydrolysates. The presence of pH indicator is omitted.
- The osmotic pressure varies significantly from that in vertebrate blood, being more than twice as high [35] [36] [37].Commonly used insect cell culture media exhibit osmotic pressures of 340-390 mOsmol kg-1 compared to 290--330 mOsmol kg -l for vertebrate media. Variations of 4-50 mOsmol kg -1 do not have a significant effect on cell growth properties [38] [39] [40].
- The ratio of the Na+/K + ions differs in the base medium due to the different groups among the insects, although the general composition of the inorganic salt solutions resemble each other. Seawater (marine) invertebrates have a ratio of 1, the more specialized Lepidoptera exhibit a Na+/K + ratio of less than 1. In more primitive insects, on the other hand, the ratio is greater than 1 [35] [36] [37].

1.4 INSECT CELL CULTIVATION

Inspite of the similarities, the technique of insect cell cultivation has some notable differences from the mammalian cell cultivation (Table 1.2). However most insect cell lines are adapted to growth in suspension, but non-adherent Sf-9 and High-Five insect cells can be also immobilized with good results [41] [42] [43] [44] [45] [46] [47].

Table1.2 Comparison of mammalian and insect cell cultivation

	Mammalian cells	Insect cells
Culture Conditions		
Temperature of growth	35-37^{0}C	22-29^{0}C
Temperature of protein production	33-37^{0}C	25-29^{0}C
Optimum pH	7.0-7.3	6.0-6.3
Media osmolarity	280-320 mOsm/kg	330-380 mOsm/kg
Growth atmosphere	5% CO_2	0-5% CO_2

Media Requirements		
Glucose	Moderate (10-20mM)	High (10-40mM)
Amino acids	Moderate (1-2 mM)	High (2-10 mM)
Lipids	Often	yes
Buffer	CO_2/HCO_3^-	Phosphate
Surfactant	Yes	Yes (for suspension)
Serum	Not necessary	Not necessary
Undefined Extract	Less often	More often
Sensitivity		
Thermal shock	Weak	Moderate
Change in pH	High	Weak
Osmotic Shock	Weak	High
Change in DO	high	Moderate
Ammonia accumulation	High	Moderate
Lactate accumulation	weak	High
Adherent cell cultivation		
Attachment to cell surface	High	Weak
Detachment from cell surface	Trypsinization	Gentle Agitation
Contact Inhibition	High	Weak
Versatility of suspension/adherence	No	Yes
Suspension cultivation		
Growth rate	$0.017\text{-}0.025h^{-1}$	$0.018\text{-}0.027h^{-1}$
Dependence of growth on Inoculum size	yes	yes
Cell size in susupension	10-12μm	12-15μm

Source: Jean Christphope Drugmand

1.4.1 Effect of pH

pH values between 6.0 to 6.8 are essential for the growth of various insect cell lines [47]. The use of phosphate buffer is therefore fully adequate and there is no need to employ CO_2/HCO_3^- buffer system commonly used in mammalian cell culture. At lower or higher pH, a lengthening of the lag phase and a decrease of the growth rate and maximal cell density were observed. However, there was no difference in the numbers of apoptotic cells of Sf-9 or High-Five cultivated at 6.0 or 6.5. A pH value of 6.2 is generally used for Sf-9 and High-Five cells growth in controlled bioreactors. The same pH is generally used both for the growth and the infection phases.. Insect cell lines are less sensitive to variation and

increase of osmolality than mammalian cells [48]. They are able to stay alive in a medium whose osmolality varies from 250-450 mOsm/kg.

1.4.2 Effect of temperature

Insect cell lines can be cultivated over a temperature range of 25-30°C [49] [50] [51] [52]. But the optimal temperature in terms of specific growth rate and final cell density is 27°C for the Sf-9 cells [53] [54] [55] [56]. However, [26] have adapted Sf-9 cells to culture at 37°C over long-term passaging, thus showing that insect cells can become thermo-tolerant. At 25°C, the specific growth rate (μ) was found to be reduced while at 30°C, the cells viability and the maximal cell density decreased [53] [54] [55] [56]. The increase of the temperature of cultivation from 22 to 30°C is known to increase the growth rate and the consumption of oxygen and glucose by Sf-9 cells, their cultivation at 35°C decreases these parameters [53] [54] [55] [56].

1.4.4 Effect of shear stress on insect cells

Insect cells cultivated in bioreactor are subjected to relatively high shear stresses. The cell damages are a function of the type, duration and magnitude of the hydrodynamic forces caused by agitation and sparging. Insect cells that usually required high agitation speed in shake-flasks to sustain oxygenation (100-150 rpm) were found to be less sensitive to shear stress than mammalian cells. [57]. Cell exposure to high levels of hydrodynamic stress are damaged irreversibly and die principally by necrosis.

1.4.5 Effect of dissolved carbon dioxide

In order to improve the efficiency of oxygen transfer (Marks, 2003) small bubbles of air or, even, pure oxygen are often used for large scale mammalian and insect cultures,. Such small bubbles lead to almost complete dissolution of oxygen that, in turn, is transformed metabolically to CO_2 (1 mole CO_2 produced per mole O_2 consumed, [58]. Unfortunately, they cannot prevent the accumulation of CO_2 in the bioreactor, especially in high-density

cell cultures [59]. Mitchell-Logean and Murhammer showed that CO_2 can accumulate in Sf-9 cell cultures in a lab-scale bioreactor, to growth inhibitory levels (24 mM) unless the bioreactor headspace was purged.

1.4.3 Effect of dissolved oxygen

Insect cells are strict aerobes with a high TCA activity. They show a higher specific consumption rate of oxygen than mammalian cells. Although insect cells need more oxygen than mammalian cells, they can be cultivated in the same bioreactor types with similar gas process control.

In 1991, Jain et al. demonstrated that DO levels had a significant effect on the μ of Sf-9 cells: at DO values equal to 10 and 110 % of air saturation, the μ was ca. 25 % lower than at a DO of 65 %. [60]. Gotoh et al. (2004) demonstrated that the specific rate of consumption of oxygen (qo2) by Sf-9 cells varied with temperature and DO and was described by a Monod-type equation [61]. During the growth phase, insect cells are not very sensitive to variations in the concentration of dissolved oxygen (DO) and can grow optimally in a wide DO range from30 to 100% [27] [28] [29] [30] [31] [32] [62] [63] [64].

1.5 INSECT CELL MEDIA

Vertebrate sera or invertebrate hemolymph were essential supplements in first-generation insect cell media. These supplements, however, are cumbersome and expensive for routine large-scale culture; thus, their use is now circumvented by substituting the essential growth factors present in these supplements with serum-free substances. Such non-serum supplements are typically of non-animal origin and include protein hydrolysates, lipid emulsions, and specialized substances (e.g., surfactants and shear damage protecting chemicals). These supplements need to complement the defined, synthetic basal medium to ensure that the fundamental nutritional needs of the cells are satisfied. Although there is a significant number of proprietary serum-free and low-protein or protein-free media on the market, the lack of information concerning their detailed composition is a drawback in their

adoption for different applications, including their adaptation to the metabolic and kinetic analysis and monitoring of a given insect cell based bioprocess. Hence, there is wide appeal for formulating serum-free media based on a rational assessment of the metabolic requirements of the lepidopteran cells during both the growth and the production phases. Techniques such as statistical experimental design and genetic algorithms adapted to the cellular behavior and the bioreactor operation mode (batch, fed-batch, or perfusion) permit the formulation of versatile serum- and protein-free media. These techniques are illustrated with recent developments of serum-free media for the cultivation of commercially important Spodoptera frugiperda and Trichoplusia ni cell lines

Table 1.3 Insect cell media

A) MEDIA IN THE PUBLIC DOMAIN			
Medium	**Features**	**Cell lines**	**Ref.**
Grace's medium	Needs to be supplemented with 5-10% (FBS) serum	Invertebrates Cell lines	[33]
TNM-FH	Needs to be supplemented with 5-10% (FBS) serum	*Trichoplusia ni* cell lines	[12]
TC-100	Needs to be supplemented with 5-10% (FBS) serum	Insect cell lines	[34]
CDM	Chemically defined medium, Need Yeastolate to allow cell growth	*Spodoptera frugiperda* Cell lines	[7]
IPL-41	Chemically defined medium without serum, Need Yeastolate to allow cell growth	Insect cell lines	[8]
M-CSF	IPL-41 supplemented with Yeastolate and Pluronic F-68 Developed for large scale	Insect cell lines	[22]
SFM-LP	TNM-FH supplemented with Ex-Cyte VLE and Pluronic F-68	Sf-9 and TN-386 cells	[12]
EMIGG	Modified CDM supplemented with Yeastolate and Pluronic F-68	Sf-9 cells	[65]
SF-1(also named IP301)	Chemically defined medium supplemented with Primatone, Yeastolate, Lactalbumine, Glucose and Pluronic F-68 Developed for large scale	Sf-9, High-Five, SL-2 and SL-3 cells	[38] [39] [40]
KBM-10	Serum –free Developed for metabolic studies	Sf-9 cells	[67] [68] [69]
ISYL	Modified IPL-41 supplemented with soy hydrolysate	*Trichoplusia ni* and *Spodoptera frugiperda* cell lines	[69] [70]
YPR	Modified LPL-41 supplemented with Primatone, Yeastolate,Pluronic ,lipids, glucose and Gln Developed for large scale	High-Five and Sf-9 cells	[41] [42] [43] [44] [45] [46]

1.3 B Commerical Media

Medium	Features	Cell lines	Company
Drosophila SFM	Serum-free Proprietary composition	*Drosophila* cell lines	Invitrogen GIBCO (Carlsbad, CA)
Ex-cell 400	Serum –free, protein free medium Hydrolysate source yeast	*Spodoptera* cell lines	JRH Biosciences (Lenexa, KS)
Ex-cell 405	Serum-free Proprietary composition Developed in large scale	High-Five and Sf-9 cells	JRH Biosciences
Ex-cell 420	Serum-free Proprietary composition Developed in large scale	Sf-9, Sf-21 and SL-2 cells	JRH Biosciences
Express-Five	Serum-free Proprietary composition Developed in large scale	High-Five cells	Invitrogen GIBCO
HyQ CCM3	Serum-free Proprietary composition	Sf-9 cells	Hyclone (Logan, UT)
HyQ SFX	Serum-free Proprietary composition Developed in large scale	High-Five, Sf-9, Sf-21 and D.Mel(2) cells	Hyclone
Insectagro FIVE	Serum–free and protein free medium	High-Five cells	Mediatech Inc. (Hemdon, VA)
Insectagro Sf-9	Serum–free and protein free medium	Sf-9 cells	Mediatech Inc
Insect Express™ High Five	Serum–free and protein free medium Proprietary composition	High-Five cells	PAA Laboratories, GmbH (Pasching, Austria)
Insect Express™ Sf9-S2	Serum–free and protein free medium Proprietary composition	High-Five cells	PAA Laboratories, GmbH
Insect-Xpress	Protein free Proprietary composition Developed in large scale	Sf-9 and Sf-21 cells	Cambrex (Walkersville, MD)
IS-BAC	Serum–free, protein free medium Proprietary composition	Sf-9, Sf-21 and High-Five cells	Irvine Scientific (Santa Ana, CA)
SF900	Serum-free Proprietary composition	Sf-9 and Sf-21 cells	Invitrogen GIBCO
SF900-II	Serum-free Proprietary composition Developed in large scale	Sf-9 cells	Invitrogen GIBCO
Serum-free insect medium-I	Serum-free Proprietary composition	Insect cell lines	Sigma-Aldrich (Saint Louis, MO)
Serum-free insect medium-II	Serum-free Low protein content Proprietary composition	Insect cell lines	Sigma-Aldrich
Serum-free medium	Serum-free medium Custom-made media.	Insect cell lines	Chemicon International , CA,

Insect cell media are different from mammalian cell media. They contain the same basal elements (carbohydrates, amino acids, salt) but with concentrations adapted to insect cell metabolism. Contrary to mammalian media, they are supplemented with specific additives such as a lipid mixture, to supply to the cells some lipids that they are unable to produce [71]. Moreover, surfactants such as Pluronic F-68 are added at ca. 0.1-0.2% (w/v) to protect cells from shear stress occurring in an agitated reactor, and antifoam is also commonly added in sparged reactor cultivation [72]. Phenol Red as pH indicator is absent from insect media, hence they appear usually clear or yellow (due to the presence of serum or peptone hydrolysates). The addition of an antibiotic-antifungal solution that is typically used during the establishment of cell lines, must be avoided during cultivation for safety and purification reasons. [25].

First insect cells media were developed in the 60's and 70's based on the insect cell hemolymph composition. These basal media such as Grace's medium [33], TNM-FH [12] or TC-100 [34] are composed of carbohydrates, amino acids, organic acids, salts and basal mixtures of vitamins supplemented with 5% or 10% foetal bovine serum (FBS). Some media were originally msupplemented with hemolymph but serum became the preferred additive. Serum provides growth factors, proteins with detoxifying and antioxidant effects, carrier proteins and protease inhibitors and protects cells from shear stress. Unfortunately, it is expensive, has lot-to-lot variability, its supply is limited or unreliable and it may contain adventitious agents or contaminants. Unfortunately, insect cells are unable to grow in these basal media (Grace, TNM-FH, TC-100) if serum is removed: they can stay alive, but do not grow. Thus, these considerations, amongst many others, have motivated the development of serum-free media [49] [50] [51] [52].

Although serum-free medium development can be tedious and costly, in the last several years, substitutes of serum have been sought. Protein hydrolysates (also named peptones) are known to be promising supplements for the large-scale, serum-free culture of animal cells. They are complex mixtures of oligopeptides, polypeptides, vitamins and amino acids produced by enzymatic or chemical digestion of casein, albumin, plant or animal tissues or

yeast cells. Hydrolysates have similar protective effects as serum (antioxidant, shear stress, etc.), can supply growth factors and protease inhibitors, can have an antiapoptotic influence and may also have a nutritional role if basal media with lower amino acid content (or no amino acids) are used.

Moreover, integration of an ultrafiltration step in media preparation can eliminate hydrolysate lot-to-lot variability and ensure reproducibly good growth of insect cell lines [41] [42] [43] [44] [45] [46]. Efforts to eliminate serum from insect cell culture began in the early 80's. Wilkie et al. (1980) developed CDM that was the first serum-free insect cell medium containing [8]. Yeastolate hydrolysate for growth and infection of Sf cell lines. Yeastolate is a yeast extract ultrafiltrated from autolysed yeast biomass that can perform many serum functions. It contains vitamins of the B complex, that are important for the maintenance of insect cell lines [5] [6]. It is known to extend the growth phase of insect cells [73] [74] 75] [33] [34] [35]. Moreover, it was identified as essential for the production of some autocrine growth promoting factors by Sf-9 cells [76].

In 1981, Weiss et al. developed IPL-41, a serum-free basal medium adapted to large scale culture [7]. Supplemented with 4 g/l Yeastolate and a lipid mixture emulsified in Pluronic F-68, it supported the culture of Sf-9 cells. In the next few years, modifications of the IPL-41 and CDM supplemented with Yeastolate [22], egg yolk [77], lactalbumine [38] [39] [40] or Ex-Cyte [12] were developed to sustains the growth of Sf-9, Sf-21, Tn-386 and SL-2 cells (*Drosophila* Schneider S2 cell line). In 1995, Öhman et al. developed a serum-free media (KDM-10) specifically adapted to Sf-9 cells [66] [67].

In 2001, Ikonomou et al. developed a serum-free media (YPR media) based on IPL-41 and containing lipids, Primatone and Yeastolate. YPR media is specifically adapted to High-Five cells and allows perfusion cultures with high cell densities and productivities. A number of commercial media have been specifically developed for Sf and Tni cells and have appeared on the market in the last decade. The media Sf-900 II and Express-Five SFM from Invitrogen Gibco (Carlsbad, CA), Insect-XPRESS from Cambrex (Walkersville, MD), EX-Cell 400, 405 and 420 from JRH Biosciences (Lenexa, KS) and HyQ SFX-Insect and

HYQ CCM3 from Hyclone (Logan, UT) are now available and used routinely for the cultivation of insect cells. Most contemporary, insect cell growth media are serum-free and contain yeast, meat or soy hydrolysates, some of them are protein or animal-free. These media, although able to support high cell densities and protein titers, are expensive, cell-line specific and of proprietary composition. Altough culture conditions are not the same for all lepidopteran cell lines, substantial similarities exist [50] [51] [52]. Insect cell media are usually developed for a unique cell line, or a narrow spectrum of cell lines (e.g. Sf cell lines). Even if lepidopteran cells can stay alive in a non-specific medium, they usually need a specific medium well adapted to their physiology and metabolism to sustain cultivation leading to high densities and to high production levels. Today almost all serum-free media in the public domain are developed for Sf-9 cells.

1.6. INSECT CELL TECHNOLOGY: APPLICATIONS, MARKET AND PRODUCTS

The initial field of application of insect cell technology was the production of baculoviruses as insecticides, well developed since the 70's. The production of r-proteins in insect cells is more recent. Lately, the use of baculoviruses as gene delivery vehicles opens exciting new applications for gene therapy. Today, insect cells in culture are mainly used to produce r-proteins in the areas of research and biomedicine and continue to be used for the production of biopesticides.

1.6.1. Insecticides

The cost of damages inflicted by pest insects and their control is worth US$ 1.25 billon each year in the United Stated alone (FAO: Food and Agriculture Organization of the United Nation). The total world market of all insecticides is estimated at ca. US$ 8 billon (in 2005) and at US$ 150 million for bioinsecticides, of which baculoviruses account for US$ 10 million of sales. The first use of baculovirus in agriculture was in 1892, although they had been intensively studied as wild-type viruses since 1975 [78] [79] [80]. Today,

more than 40 wild-type baculoviruses are used as biopesticides against damaging lepidopteran insects (moths, butterflies, larvae, etc.) that damage cotton, corn, tobacco, vegetables, grapes and many more crops [81] [28] [29] [30]. Since 1988, many recombinant baculoviruses have also been used. They represent today the majority of the market.

It was estimated that ca. 7 million of hectares of crops were treated with baculoviruses (FAO). These baculoviruses sold as biopesticides could be conditioned in form of liquids, powders and sprays. Treating 1 ha requires between 1012 to 1013 viruses[28] [29] [30]. The cost of treatment, which was very expensive in the 80's, is today around US$ 0.5/hectare [28] [29] [30] [82]. At this cost, viral products are competitive with chemical insecticides. Although public safety concerns about genetically engineered products must be addressed whenever this issue is raised, these baculoviruses are, objectively, more health-care friendly and environmentally benign than chemical insecticides [79] [80] [3]. The latter can accumulate in environment, can be dangerous for human and animal health and higher quantities of them are required per hectare, while baculoviruses are safe and lower amounts are needed to spread per hectare. They have no known adverse effects on non-target organisms [3]. However, although no deleterious effects of baculoviruses as biopesticides on human health have been observed, no strict demonstration of complete safety has been offered so far [83] [27] [28] [29] [30] [31] [32]. They are specific against a narrow spectrum of pest insects (Maeda, 1995). Until now, insects show a lower tendency to develop resistance against baculovirus than against chemical insecticides [84]. Nevertheless, this environmentally friendly advantage could become harmful when crops are infested by various several different pest insect species.

The baculovirus pesticides need to correctly identify the target pest due to their high specificities of and usually these biopesticides are not preventive. Contrary to chemicals, the insecticidal activity of baculoviruses is more susceptible to adverse environmental conditions (rain, sun, temperature, dryness, etc.) and to fertilizers. Wild-type baculoviruses do not kill pest insects as rapidly as chemical insecticides. Their lethality can be delayed between 3 days and 3 weeks. During this time, damages to crop can continue [3].

Usually, wild-type baculoviruses intended as pesticides are produced in vivo in larvae while recombinant baculoviruses are generally produced in vitro. In vivo production requires breeding of caterpillars from eggs in humidified containers at 25-27°C containing ca. 15 larvae where their diet is controlled [25]. Infections are done by spraying occluded viruses onto the larvae or by addition them into the diet. Viruses are harvested after the second virus cycle from larval hemolymph and their yield is ca. 10^9 viruses/caterpillar [85] [86]. Non-occluded virus (*polh* gene deleted) cannot be produced in vivo [3] Lawrence, 1996). In vitro production of baculoviruses is more expensive than in vitro production and requires qualified staff, but it is more practical for quality control, especially in the production of recombinant viruses, as long as the market size does not exceed 1 million hectares a year [87][27] [28] [29] [30] [31] [32].

1.6.2. Recombinant proteins

Today, in addition to the production of pesticides, the IC-BEVS technology is widely used for the production of r -proteins for various commercial, biomedical and medical research applications and for production of diagnostic reagents. More than 500 genes have been expressed in IC-BEVS and this system is chosen in almost 50 % of the scientific papers reporting a higher eukaryotic protein expression [27] [28] [29] [30] [31] [32]. These sectors of applications need great amounts of r-proteins. Biological activity and similarity to native proteins represent great potential for the production in insect cells.

Although the majority of r-proteins is produced in vitro using the IC-BEVS, these proteins may be also produced in vivo in larvae, or in stable (constitutively engineered) insect cells lines [25] [88].

The yield of production of r-protein in larvae could represent 50 % of biomass protein of the larvae [89]. However, in vivo production needs experience in growing and maintaining larvae. It also needs efficient purification steps of the r-protein and is only used when the amount produced is more important than the purity required (in diagnostic applications or in the research area). This technology uses mainly silkworm larvae (*Bombyx mori*) infected with BmNPV virus but can also use larvae of the cabbage looper *Trichoplusia ni* infected

with AcMNPV. This approach is more widespread in Asia where silkworm cultures are well developed [16] [17].

Concerning stable cell lines (constitutively engineered), insect cells are able to produce in continuous mode up to several mg/l, sometimes with higher secreted protein yield than the BEVS and occasionally without the risk of protein degradation due to lysis in the BEVS [90] [91] [92] [93] .Despite these advantages, the establishment of stable insect cell lines expressing r-proteins in a non-lytic system does not seem to have much of a future in industrial application, because cells generally produce less protein, the time for establishment of a stable cell line is longer and more expensive than BEVS and, overall, this approach is not really less expensive than the production in mammalian cells. The latter are preferred by industry because they are more under control, there is more known on their bioprocess behavior, and they possess the capacity for posttranslational modifications nearest to human proteins [94].

1.6.3. Therapeutics

The efficacy, safety and cost issues appear to indicate that for some products the of recombinant protein or viral drugs using IC-BEVS will be useful. The BEVS constitutes a good system the production of vaccine subunits by expressing antigen proteins or by production of virus-like particles (VLP) as vaccines [63] [64]. A VLP is a virus identical to the native virus expressing surface protein from one or more different pathogen viruses, but without their genetic material. This approach is particularly attractive for producing vaccines against viruses whose viral replication is impossible with a cell culture-based technology, such as human papilloma virus and hepatitis virus. Today, the presence of glycosylation a bit different from mammalian cells is still considered as a disadvantage for insect cell based production of human therapeutics. However, for vaccine production, the lack of human-like glycosylation of the antigen is less important, and it can even enhance the immune response to the vaccine [63] [64].

The first therapeutic produced using IC BEVS was a veterinary vaccine against the swine fever virus, approved in 2000 (Intervet International, Boxmeer, The Netherlands).Today, four commercialized veterinary drugs are made using IC-BEVS (Table 1-10) [21]. The market of IC-BEVS is worth ca. 1 % of the biopharmaceutical market (US$ 478 billion in 2004) but will probably increase in the next years [95].

Table1.4 Recombinant veterinary drug produced using the Insect cell-Baculovirus Expression System approved in European Union.

Product	Type	Company	Therapeutic Indication	Approval
Porsilis pesti	Vaccine	Intervet International Boxmeer The Netherlands	Immunization of pigs against classic swine fever virus.	2000
Bayovac	Vaccine	Bayer Leverkusen,Germany	Immunization of pigs against classic swine fever virus.	2001
Virbagen Omega	Interferon	Virbac Carros, France	Immunization of dogs against canine parvovirus and cats against feline retrovirus.	2001
Adavsure	Vaccine	Pfizer NY,USA	Immunization of pigs against classic swine fever virus.	2004

Concerning human drugs, the fear of new technology and the cost of drug development has been one of the brakes on their development using insect cells (between US$500 and 800 million for the development of a human vaccine). However, the benefits of the BEVS and the recent approval of veterinary products by the American Food and Drug Administration (FDA), the European Medicines Agency (EMEA), the Australian Therapeutic Goods Administration (TGA), The Japanese Ministry of Health, Labour and Welfare (MHCW) and the Public Health Agency of Canada have allowed pharmaceutical manufacturers to develop new human therapeutic products using insect cells. [96] [97] [98] [99] [100].

Nowadays, many therapeutic vaccines and drugs using IC-BEVS technology are under development ("in the pipeline"). Amongst vaccines, Protein Sciences (Meriden, CT) has

begun the phase III clinical trials of an Influenza type A vaccine, the most common type of virus causing human flu. They have also a vaccine program against HIV-AIDS based on similar approach. They have developed an avian vaccine against bird flu (Influenza H5) in vivo with caterpillars. The company has, in phase II clinical trials, an epidemiologic human vaccine produced in vitro by IC-BEVS, which is usable in case of a pandemic of avian flu virus and transmission to humans. Concerning cancer vaccines, Dendreon Coorporation (Seattle, WA) and GlaxoSmithKline (Rixensart, Belgium) have, respectively, a prostate cancer vaccine candidate and a human papilloma virus vaccine candidate in phase III clinical trials. MedImmune Inc (Gaithersburg, MD) has just licenced a human papilloma virus vaccine and will commercialize it in 2006 or 2007. Concerning hepatitis, GlaxoSmithKline has an hepatitis E vaccine candidate in development using insect cells. Wyeth (Madison, NJ) has development programs including production from insect cells of a HIV, a malaria and a needle-free flu vaccine.

As far as therapeutic drugs are concerned, Protein Sciences will soon bring to human testing an erythropoietin produced with IC-BEVS. Amgen (Thousand Oaks, CA) and Sanofi-Pasteur (Paris, France) have programs of vaccine and therapeutic drug development using insect cells that are in preclinical phase. Human Genome Sciences (Rockville, MD) has under development some therapeutics using insect cells that are in the pre-clinical and clinical phases. Bayer (Leverkusen, Germany), Intervet International, Pfizer (New York, NY) and Vibrac (Carros, France) are supposed to continue to develop veterinary products using IC-BEVS. Pfizer and Bayer are not disclosing any information regarding the development of human therapeutic using IC-BEVS. However their choices to produce veterinary vaccines using this technology indicate an interest in the IC-BEVS platform and perhaps a future development of human therapeutics. Nowadays, most other human and animal health companies involved in the production of r-proteins as therapeutics are using mammalian cells: Baxter (Deerfield, IL, U.S.A), Eli Lilly (Indianapolis, IN), Genentech (South San Francisco, CA, U.S.A.), Genzyme (Cambridge, MA), Hoffmann-La Roche (Basel, Switzerland), Merial (Saint- Priest, France) do not officially communicate an interest in BEVS technology.

Other vaccine projects using IC-BEVS are under development by several companies and institutes: veterinary vaccines in preclinical phase against the foot-and mouth-disease and the canine leishmaniasis; the development of a human vaccine candidate against HIVAIDS, malaria, hepatitis A, B, C and E, human hookworm disease, human parvovirus and human polyomavirus [100]. Various therapeutic drugs are currently under investigation, e.g. antistasin, erythropoietin, interleukins, tPA, glucocerebrosidase, etc. [27] [28] [29] [30] [31].

Despite the growing interest in insect cell culture and technology, it seems clear that several large pharmaceutical companies have opted, at least for the time being, not to develop the production of r-proteins using the BEVS, due to key patents controlling insect cell technology (over than 5000) such as patents on media, cell lines, applications, vector constructions, etc.

The moment the major such patents will expire and the first product for human therapy approved, a lot of people will probably start using the IC-BEVS for commercial applications, including human health. As already shown, the use of baculovirus and insect cell technology offers many opportunities for the\ production of vaccines. However, some companies prefer to usemammalian cells that have better glycosylation than insect cells [101] [102] [91] [92].

The recent studies on insect cells expressing "human like" glycosylation patterns by eliminating "insect-like" glycosylation and replacing the insect genes with genes encoding for enzymes that mimic mammalian/human glycoform synthesis, could change this [91] [92] [103]. Insect cells stably transformed with mammalian glycosyl transferases [104] [105] [106] [91] [92] have given rise to SfSWT-3 cell line (a transgenic derivative of Sf9 cell line) [107] which can form monosialylated biantennary complex N-glycans in serum-free medium supplemented with N acetylmannosamine. Its parental cell line, SfSWT-1 is commercially available by Invitrogen (Carlsbad, CA) under the name "Mimic cells" and can produce biantennary N-glycans like SFSWT-3, but requires a serum supplemented medium. The establishment of insect cell line expressing proteins with "human-like" glycosylation is a challenge to improve the quality of vaccines and therapeutics produced in insect cells [91] [92] [16] [17].

OBJECTIVES

The studies on limiting components in basal media IPL-41 for insect cell culture the following goals are targeted:

- To obtain higher cell growth in terms of cell densities (10e6 cells/ml),
- To obtain higher viability (%).
- Increase culture longevity.
- Development of cost effective media.

2. MATERIALS AND METHODS

2.1 MEDIA PREPARATION

2.1.1 BASAL MEDIA IPL-41

Basal media IPl-41 was prepared from IPL-41 Insektenmedium powder (with l-glutamine, without $NaHCO_3$) (Genaxxon Biosciences, Germany) by dissolving 25.63gm/l in Ultra filtered water (Sartorius Arium 611 UF, Vitaris AG, Switzerland). 20ml/l of 10% Pluronic F-68 (AppliChem, Darmstadt, Germany) was added externally. The pH (340, Mettler Toledo, USA) is adjusted to 6.3 and solution is sterlized by using 250ml PES 0.22um filter (TPP, Trasadingen, Switzerland) coupled with vaccum pump (Vacuskan, Skan AG, Switzerland). Table2.1 mentions the composition of IPL-41.

2.1.2 SUPPLEMENTATION OF IPL-41

2.1.2.1 Carbohydrates

The basal media IPL-41 contains 2.5gm/l of D-Glucose, 1.0gm/l of D-Maltose and 1.6gm/l of D-Sucrose. In order to supplement the metabolic needs of insect cells, it was necessary to supplement the basal media with optimal carbohydrate concentration. Weighed quantities of D-Glucose, D-Maltose and D-Sucrose were added externally to prepare different IPl-41-Carbohydrate stocks with final concentrations of each type of sugar twice (2X), thrice (3X), five times (5X) and eight times (8X) of the initial concentration present in IPL-41. Table2.2 lists the final concentration of sugars present in IPl-41 after supplementation. The pHs of the stocks were adjusted to 6.3 and are sterilized by using 250ml PES 0.22um filter. Suppliers of sugars are mentioned in Appendix 1(a). To further optimize the key sugar, five-fold concentrated mix of individual and combination of sugars were prepared as follows:

- Glucose
- Maltose
- Sucrose

- Glucose & Maltose
- Maltose & Sucrose
- Glucose & Sucrose.
- Glucose, Maltose & Sucrose

Table2.2 Final Concentration of sugars in different IPL-41 stock.

Sugars gm/l	IPL 41	2x	3x	5x	8x
Glucose	2.5	5	7.5	12.5	20
Maltose	1.02	2.04	3.06	5.1	8.16
Sucrose	1.6	3.2	4.8	8	12.8

Table2.1 Composition of IPL-41 *(Source:Genaaxon Biosciences)*

Supplements		mg/L
Inorganic salts	Ammonium heptamolybate X $4H_2O$	0.04
	$CaCl_2.6H_2O$	662.31
	$Co(II)Cl_2.H_2O$	0.05
	$Cucl_2.H2O$	0.20
	KCl	1200.00
	$Fe(II)$-sulfate.$7H_2O$	0.55
	$MgCl_2.6H_2O$	0.00
	$MgSO_4$ wfr.	1193.40
	$MnCl_2.4H_2O$	0.02
	NaCl	2850.00
	$NaHPO_4.H_2O$	1160.00
	NaH_2PO_4	0.00
	$SnCl_2$	0.04
Other components	DL-Malic acid	53.60
	Succinic acid	4.80
	Fumaric acid	4.40
	D(-)- Fructose	0.00
	D(+)-Glucose wfr.	2500.00
	Yeast extract	0.00
	α-Ketoglutaric acid sodium salt	34.05
	D(+)-Maltose X H2O	1052.58
	Lactalbumin hydrolysat	0.00
	D(+)-Saccharose	1650.00
Amino acids	β-Alanine	300.00
	L- Alanine	0.00
	L- Arginine xHCl	800.00
	L- Asparagine x H2O	1477.14
	L-Aspartic acid	1300.00
	L-Cysteine	100.00
	L-Glutamine	1000.00
	L-Glutamic acid	1500.00
	Glycine	200.00
	L-Histamine base	200.00
	L-Hydroxyproline	800.00
	L-Isoleucine	750.00
	L-Leucine	250.00
	L-Lysine X HCl	700.00
	L-Methionine	1000.00
	L-Phenylalanine	1000.00
	L-proline	500.00
	L-Serine	200.00
	L-Threonine	200.00
	L-Tryptophan	100.00
	L-Tyrosine	250.02
	L-Valine	500.00
Vitamins	p-Aminobenzoic acid	0.32
	D(+)-Biotin	0.16
	D-Ca-Pantothenate	0.008
	Choline chloride	20.00
	Folic acid	0.08
	Myo-Inositol	0.40
	Nicotinic acid	0.16
	Nicotinamide	0.16
	Pyridoxine X HCl	0.40
	Riboflavin	0.08
	Thiamine X HCl	0.08
	Vitamin B12	0.24
	Total Weight (g/L)	25.49

2.1.2.2 Amino Acids

The concentration of different amino acids in IPL-41 is tabulated in Table 2.1. Weighed quantities of all amino acids were added to IPL-41 to increase the amino acid concentration two fold (2X) and four fold (4X) of the initial concentration in IPL-41. These IPL-41-Amino Acid stocks are free of additional carbohydrates. The mix is sterilized using 250ml PES 0.22um filter set after adjusting the pH to 6.3. Table 2.4 Table2.2 lists the original and final concentrations of amino acids present in IPl-41 after supplementation. Suppliers of amino acids are mentioned in Appendix 1(b).

Table2.3 Final Concentration of amino acids in different IPL-41 stock.

Amino Acids gm/l	IPL 41	2x	4x
Alanine	0.3	0.6	1.2
Arginine	0.8	1.6	3.2
Asparagine	1.4	2.8	5.6
Aspartic	1.3	2.6	5.2
Cystine	0.1	0.2	0.4
Glutamic	1.5	3	6
Glutamine	1	2	4
Glycine	0.2	0.4	0.8
Histidine	0.2	0.4	0.8
Hydroxy proline	0.8	1.6	3.2
Isoleucine	0.7	1.4	2.8
Leucine	0.2	0.4	0.8
Lysine	0.7	1.4	2.8
Methionine	0.1	0.2	0.4
Phenylalanine	0.1	0.2	0.4
Proline	0.5	1	2
Serine	0.2	0.4	0.8
Threonine	0.2	0.4	0.8
Tryptophan	0.1	0.2	0.4
Tyrosine	0.2	0.4	0.8
Valine	0.5	1	2

A separate stock solution of IPL-41 with five fold (5X) carbohydrates containing all the three sugars and two fold amino acids (2X) was prepared using the concentrations tabulated in Table2.2 and 2.3. The stock is sterilized using 250ml PES 0.22um filter set after adjusting the pH to 6.3.

2.1.2.3 Peptones

A 50 ml 200gm/l stock solutions were prepared for following peptones:

- Wheat peptone.
- Tryptone N1.
- Caesin pepto.ne plus
- Soya peptone.
- HyPep 5603.
- Ultrafiltrate soy RXD AM41.
- Hydrolysate Caesin.

Refer appendix 1(c) for list of suppliers. 20gm/l of each peptone was added individually to IPL-41 supplemented with five fold sugars and two fold amino acids during passaging of Sf-9 cells from the seed culture. A 1:1 ratio ie 10gm/l each of Ultrafiltrate soy RXD AM41 and HyPep 5603 was also added to test the mutual effect of both peptones on cell growth if present together in the media.

All stock solutions, media, and trypan blue were stored at 4^0C in refrigerator (Profiline, Liebherr, Germany).

2.2 CELL CULTURE

Suspension adapted Sf-9 cells [10] were grown in Sf-900 II SFM (Invitrogen, Basel, Switzerland). The cells were supplied with 10 ml media and cultivated in TubeSpin Bioreactor 50 (TPP, Trasadingen, Switzerland). To maintain the seed, the cells were passaged twice per week by dilution and seeded into pre-warmed media at cell density of 1 X 10e6 cells/ml.

For experimental procedure, the seed culture was passaged via dilution in 10 ml pre-warmed (29^0C) basal IPL-41, and supplemented IPL-41 stocks ie IPL-Carbohydrate, IPL-

Amino acid and IPL-Carbohydrate-Amino acid stock at cell density of 1 X 10e6 cells/ml and cultivated in TubeSpin Bioreator 50.

The cultures were maintained at 28^0C in shaking incubator ClimoShaker FSF1-X (Kuhner AG, Switzerland) with a shaking diam.of 2.5 cm and shaking speed of 180 rpm (Xie Q, Michel PO., 2011). The cell density and viabily were determined by measurement of Packed Cell Volume (PCV) (Stettler et al., 2006) and manually by Trypan Blue exclusion method using a Neubauer hemocytometer. PCV value of 0.3 corresponds to cell density of 1 X 10e6cells/ml. The cell's metabolic behavior during growth phase was determined by measurement of pH, and concentration of glucose, lactic acid, glutamine, and ammonia by Nova analyzer (Nova Biomedical, Waltham, MA).

$$\text{Viability (\%)} = \frac{\text{Total number of living cells} \quad — \quad \text{Total number of dead cells X 100}}{\text{Total Number of cells}}$$

3. RESULTS AND DISCUSSION

3.1 GROWTH CURVE AND VIABILITY OF SF-9 CELLS IN SF-900 II SFM AND IPL-41.

Sf-9 cells were cultivated in TubeSpin Bioreator 50 vessels in Sf-900 II SFM and basal IPL-41. A stark difference in growth profiles of cells in the two media. With doubling time of approximately 20 hours, in Sf-900 II SFM the maximum cell density reached was 12X 10e6 cells/ml with a viability of 97% at day four, while in IPL-41 cell density was 3.8X 10e6cells/ml with 93% viability **(Figure 3.1)**. The reason for poor growth can be accounted due to absence of important metabolites as in carbohydrates, amino acids, inorganic salts, vitamins and growth factors. These metabolites are essential to synthesize new materials during the lag phase of cells and show exponential growth. It can be thus thought that on supplementation of additional carbohydrates, amino acids, peptones cells can behave similarly in IPL-41.

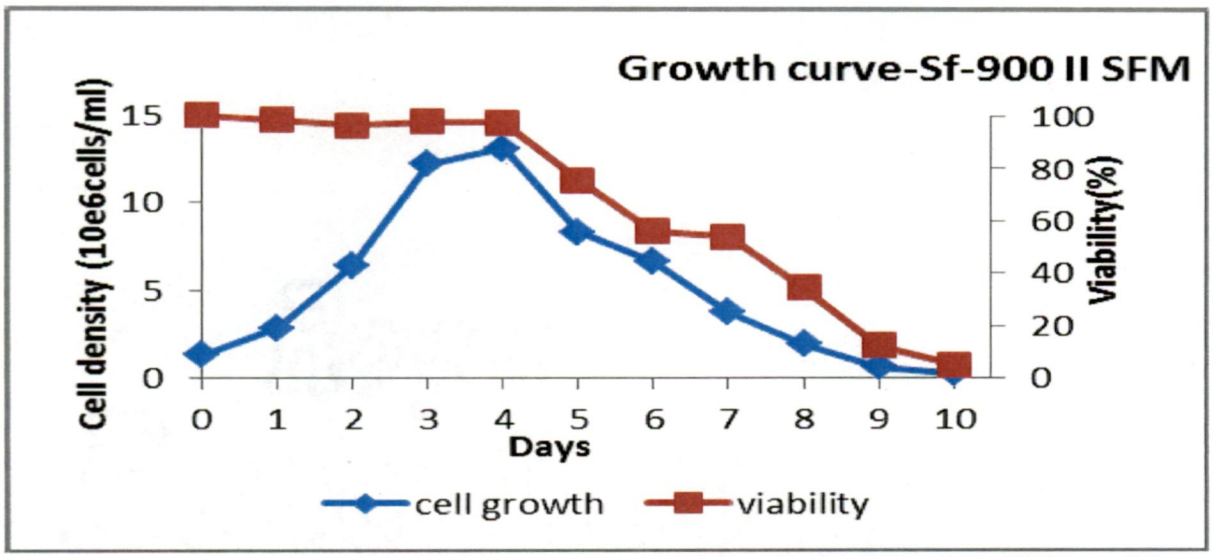


Figure 3.1 (a) Growth and Viability curves of Sf-9 cells in Sf-900 II SFM and basal IPL-41

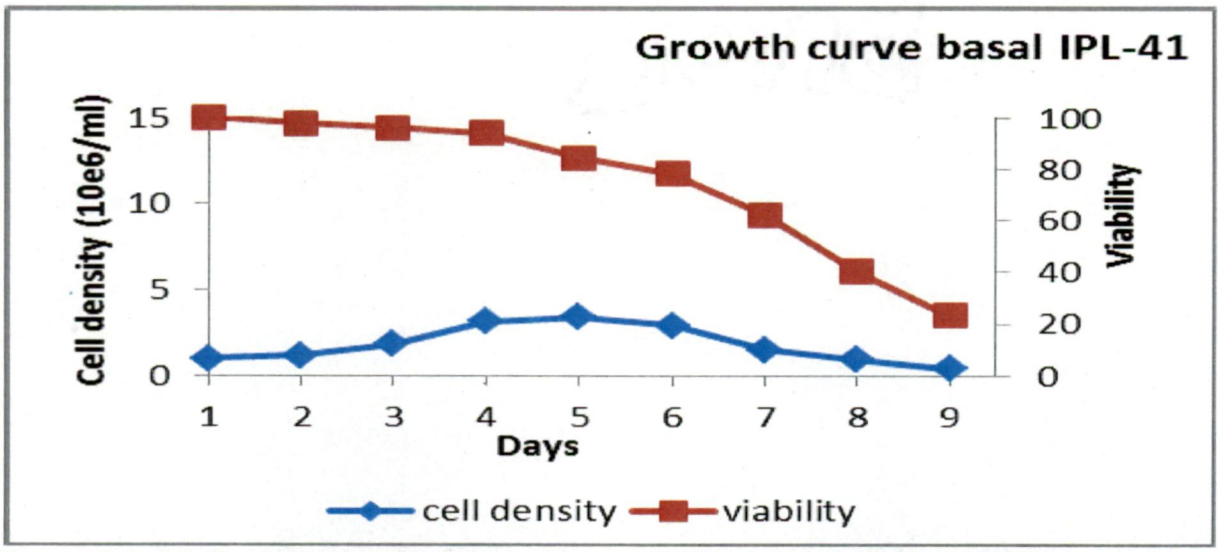


Figure 3.1 (b) Growth and Viability curves of Sf-9 cells in Sf-900 II SFM and basal IPL-41

3.2 OPTIMIZING CARBOHYDRATE CONCENTRATION

Sf-9 cells are cultivated in IPL-41 supplemented with carbohydrates ie: glucose, maltose and sucrose with twice, thrice, fivefold and eight fold of their initial concentration IPL-41. The growth profiles in this excessive amount of sugars were compared with the growth profile of cells in basal IPL-41 (control). From the **(figure 3.2.1),** a trend in the growth of insect cells is observed; as the amount of sugar is increased, the cell density also increases. Cell density reached to a maximum of 2.8X10e6 cells/ml with viability of 93 % on day two in control. Whereas in twice, thrice, fivefold and eight fold IPL-carbohydrate media maximum cell densities were 2.6 X 10e6, 3.4 X10e6, 4.1 X 10e6 and 4.6 X 10e6 cells/ml with viabilities of 90%, 95%, 90%, 65% on day four respectively. It is evident from here that fivefold sugar concentrations can aid in reach higher cell densities with good viability.

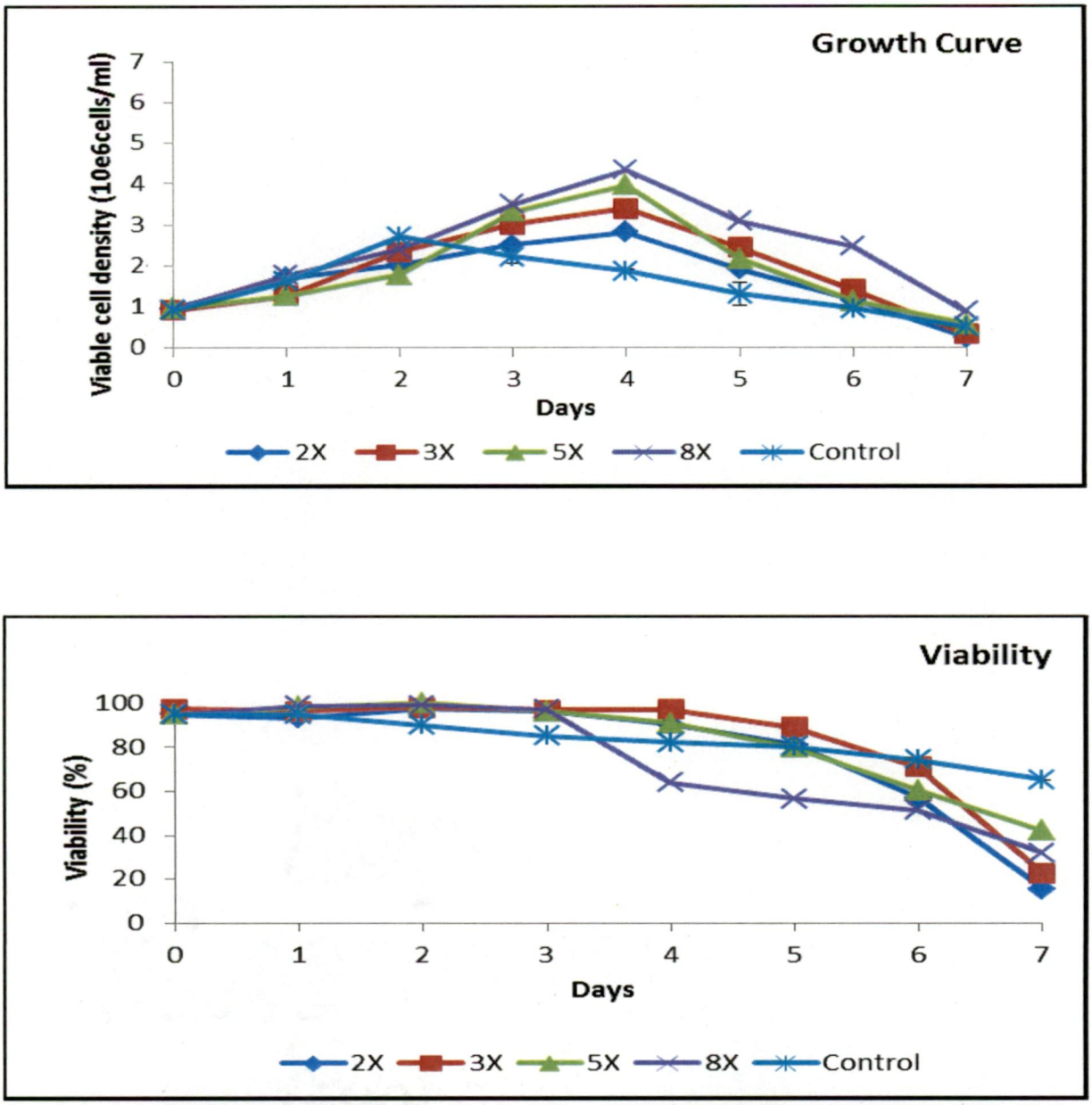


Figure 3.2.1 Growth and Viability curves for Sf-9 cells in IPL-41 with twice, thrice, fivefold and eight fold concentration of carbohydrates.

To understand the effect of different carbohydrates better, experiments were conducted by growing Sf-9 cells in IPl-41 with fivefold concentration of differernt sugars, individually and in combination with other sugars **(Figure3.2.2).** Basal IPl-41 was setup as a control for comparison of growth curves and viabilites. All sugars present individually and in combination performed better than the control, but display a longer lag phase. For better understanding of growth of cells integral viable cell densities are calculated for day-zero to

day-seven **(Figure 3.2.3).** IVCD represents sustainable growth of cells on media supplementation with different sugars. The maximum value was observed for maltose ie 37x10e6 cells/ml followed by combination of glucose + maltose with 33x10e6 cells/ml. On five fold supplementation of all sugars, glucose+maltose+sucrose the observed IVCD value was 18x10e6 cells/ml, which is although better than the control but dosen't meet expectations of fair preformance. On a close observation at growth and viability profiles of five fold glucose, glucose+maltose and all three sugars togther, it is observed that glucose, a combination of glucose+maltose fair well than control with cell densities of 7.8x10e6, 6.4x10e6 and 2.6x10e6 with viabilities of 78%, 72%, and 76% on day-four respectively **(Figure3.2.4).** Growth in a combination of glucose+maltose+sucrose is also positive with cell density of 5.0x10e6 and viability of 55% on day four. This may be either due to adverse effects of high sugar concentrations eg :changes in osmolarity of the medium or dependent metabolic uptake of one sugar in the presence of other.

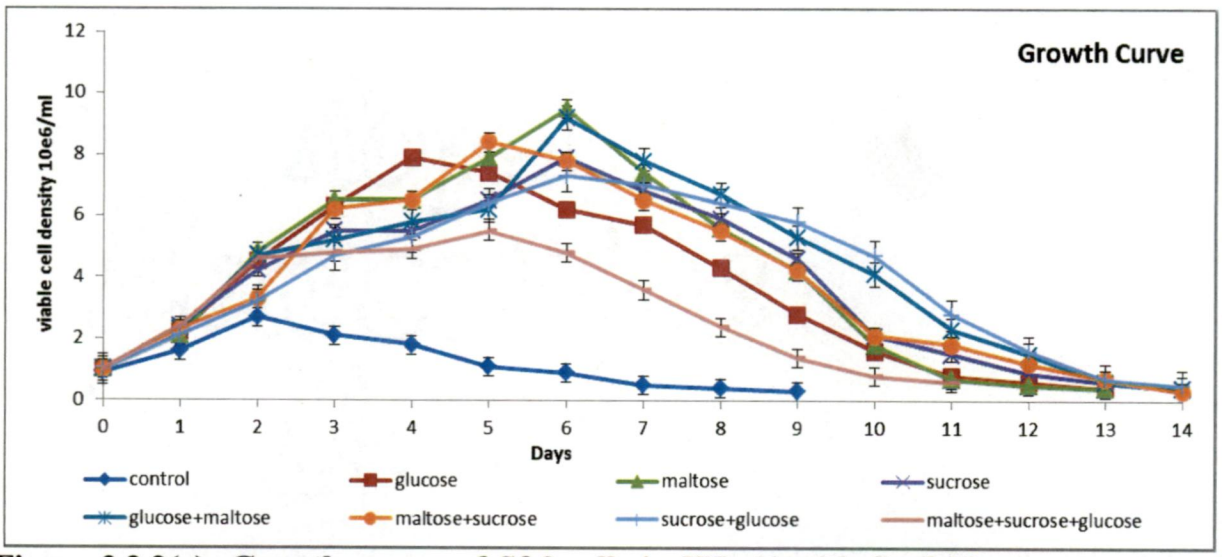


Figure 3.2.2(a): Growth curves of Sf-9 cells in IPL-41 with fivefold concentration of different carbohydrates.

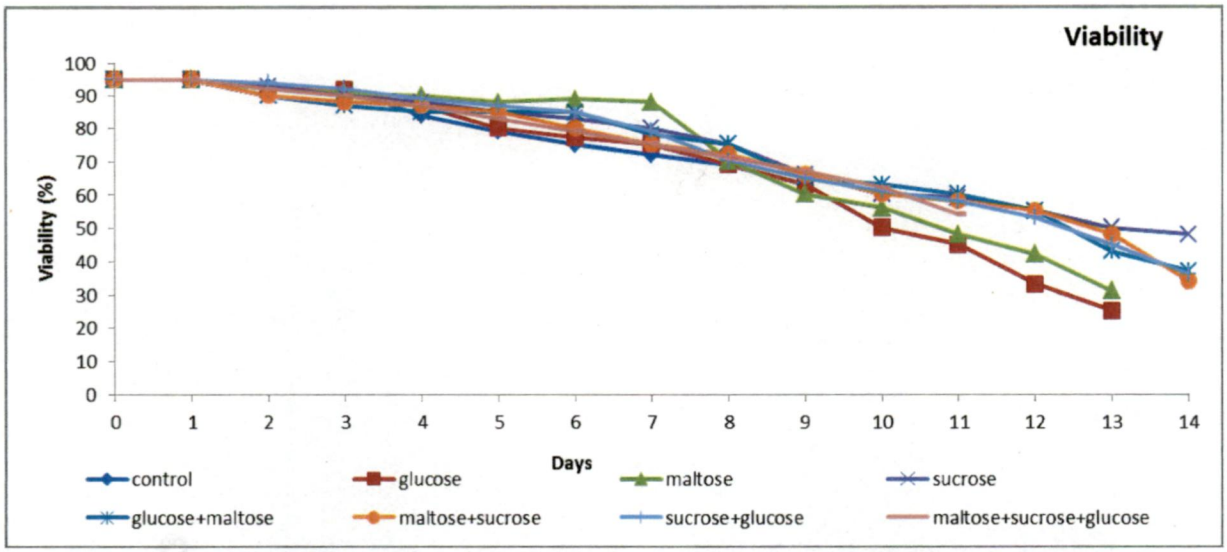


Figure 3.2.2(b): Viability curves of Sf-9 cells in IPL-41 with fivefold concentration of different carbohydrates.

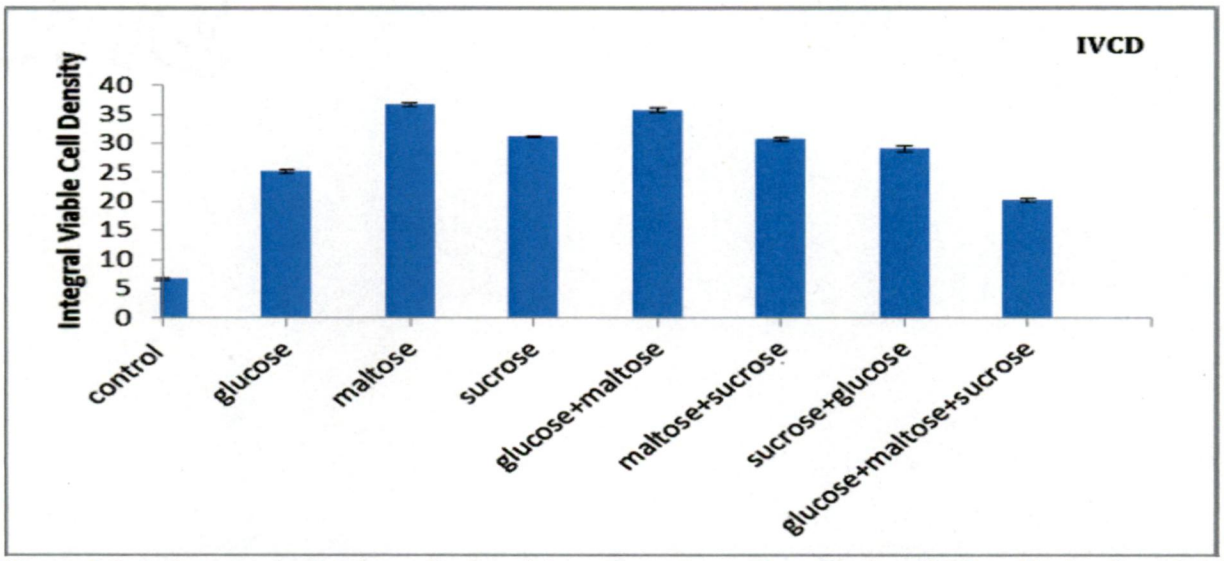
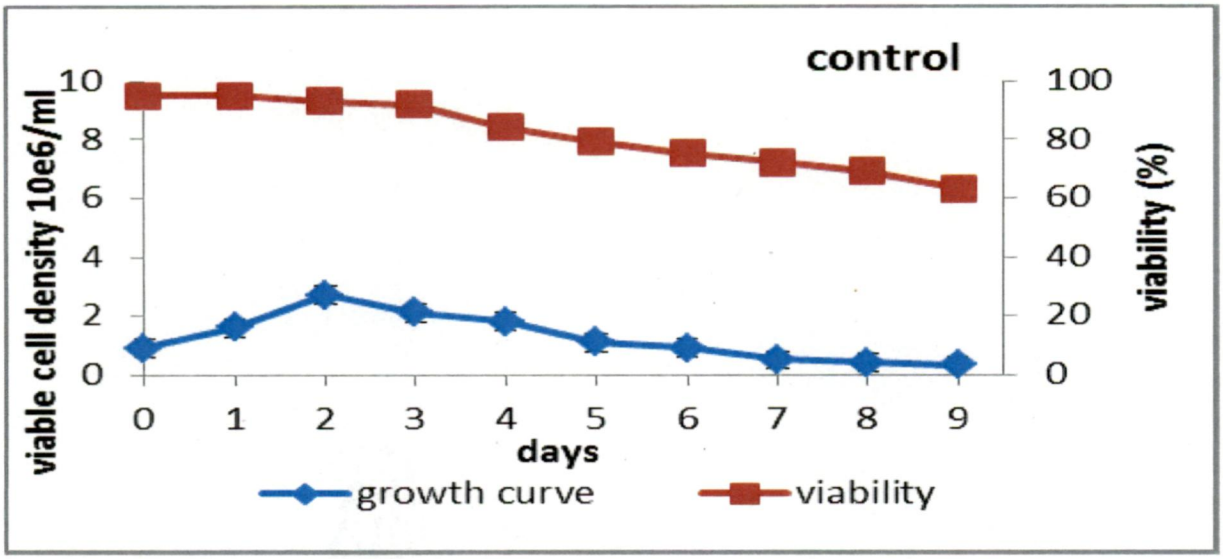


Figure3.2.3 Integral viable cell densities (Day-0 to Day-7) for Sf-9 cells in five fold sugar concentrations



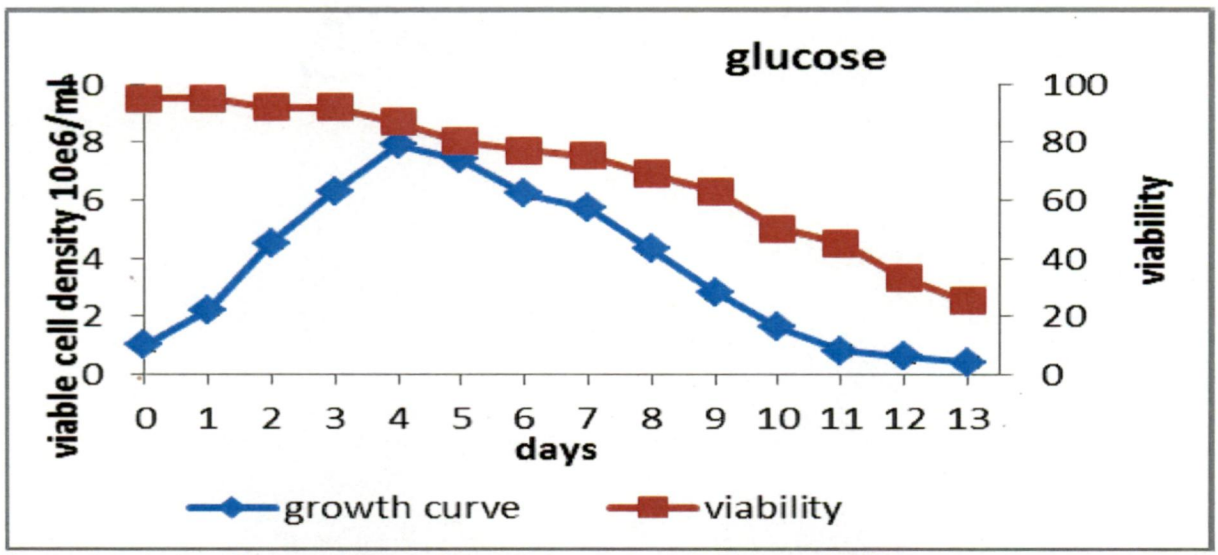
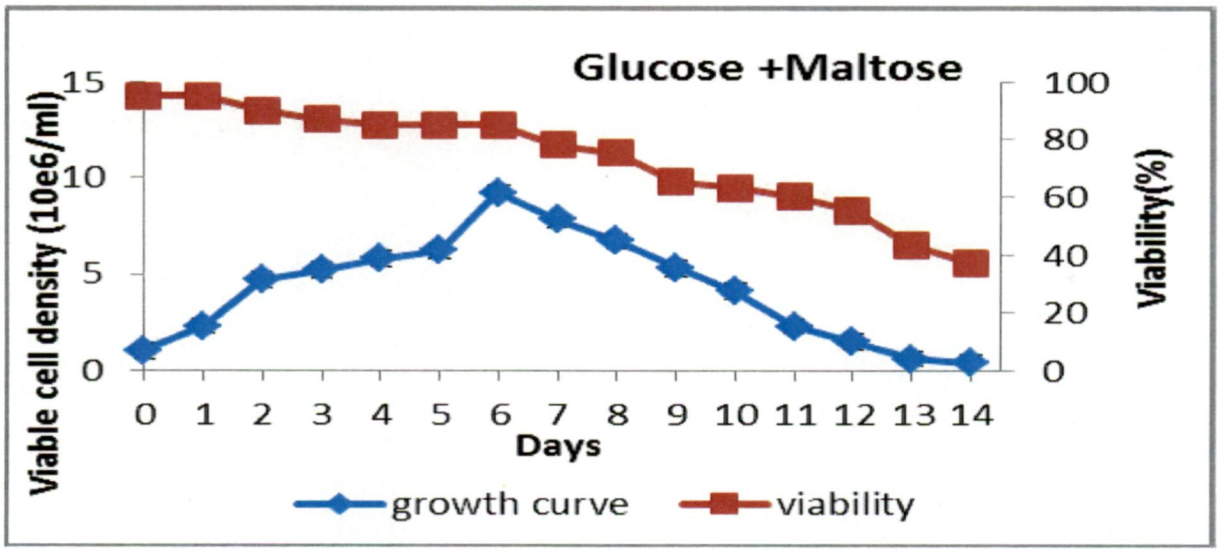


Figure3.2.4 (a) Comparison of growth and viabilities of Sf-9 cells in basal IPl-41 (control) and IPL-41 with fivefold sugar concentration of glucose.



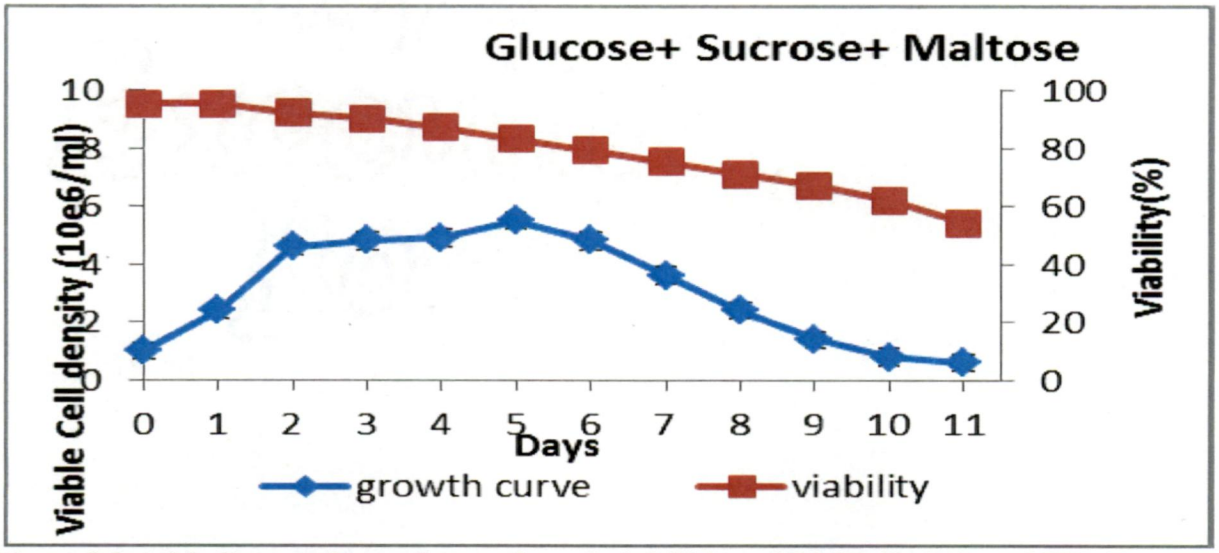


Figure3.2.4 (b) Comparison of growth and viabilities of Sf-9 cells in basal IPl-41 (control) and IPL-41 with fivefold sugar concentration of glucose + maltose and glucose + sucrose + maltose.

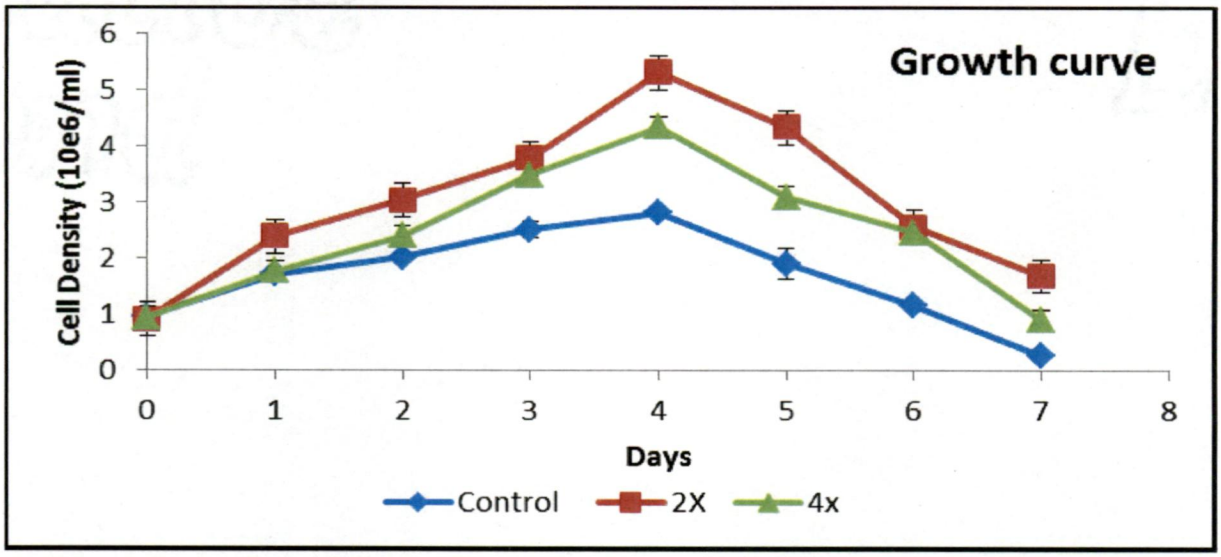
From these results, glucose appears to be an essential sugar for cell growth which is in accordance with metabolic studies of Sf-9 cells performed by Bedard and Bhatia and is considered as the most important carbohydrate for insect cell growth (Bedard et al. 1993; Bhatia et al. 1997; Drews et al. 1995; Mendonca et al. 1999; Reuveny et al. 1992).

Supplementation of glucose to basal media to improve growth characteristics is thus a simple option and some media containing glucose as the sole carbohydrate have been reported by Landureau and Jolles 1969; Reuveny et al. 1992; Wilkie et al. 1980). Also from the growth and viability curves of fivefold glucose in Figure 3.2.4(a) a linear exponential phase can be observed till day four with maximum cell densities reached are 7.8x10e6 cell/ml, this can be accounted due to consumption of glucose as the prime carbon source for furnishing energy needs and when glucose starts depleting from the medium, the cell do not replicate further and we observe decreasing viabilities. The observation is very well in sync with data from literature. Bedard also found that Sf-9 cells in serum-supplemented medium consumed glucose, fructose and maltose, but that glucose appeared to be the key carbohydrate substrate for the cells. Similarly, Reuveny et al. (1992) concluded that only glucose, fructose and maltose were used as carbon sources. When using a fivefold combination of glucose and maltose, maximum cell densities 9.7x10e6 cells/ml on day six, thus cell survive and replicate for longer duration as glucose was identified as the preferred energy and carbon source (Drews et al. 1995); and other carbohydrates (here maltose) were consumed after glucose depletion. As mentioned by Bedard et al. 1993; Drews et al. 1995; Reuveny et al. 1995 sucrose is not consumed by Sf-9, but the supplementation of media with fivefold glucose + maltose + sucrose combination helps in reaching to cell densities of $5.8x10e^6$ cells/ml with 57% viability on day six. The curve for this experiment shows a plateau of stationary phase where the cells are sustaining growth with good viabilities as they are consuming carbohydrates for energy requirements. It can be thus assumed that even if there is no sucrose uptake, the presence of sucrose in the culture media help in uptake of other carbohydrate sources.

3.3 OPTIMIZING AMINO ACID CONCENTRATION

For the growth of a living system the presence of a nitrogen source is critical for biosynthesis of proteins essential for building up cellular structure. For this purpose Sf-9 cells cultivated in IPL-41 supplemented with a twofold and four fold amino acid mix of their initial concentration IPL-41 in basal IPl-41 which was set up as control. The growth profiles in this excess amount of amino acids were compared with the growth profile of

cells in basal IPL-41 (control). From the figure a linear increase in cell growth was observed. On day-four maximum cell densities were 5.6X10e6, 4.6X10e6 and 2.7X10e6 of twofold, four fold and control respectively with viabilities of 64%, 75% and 95%. **(Figure 3.3.1).** It can be observed that with twice and four fold supplementation to IPL-41 changes the growth characteristics and cell reach the exponential peak faster. Two fold supplementation performs better than four fold amino acid mix as a more stable viability curve is obtained. A hypothesis can be proposed that high amino acid concentration is toxic to insect cells.



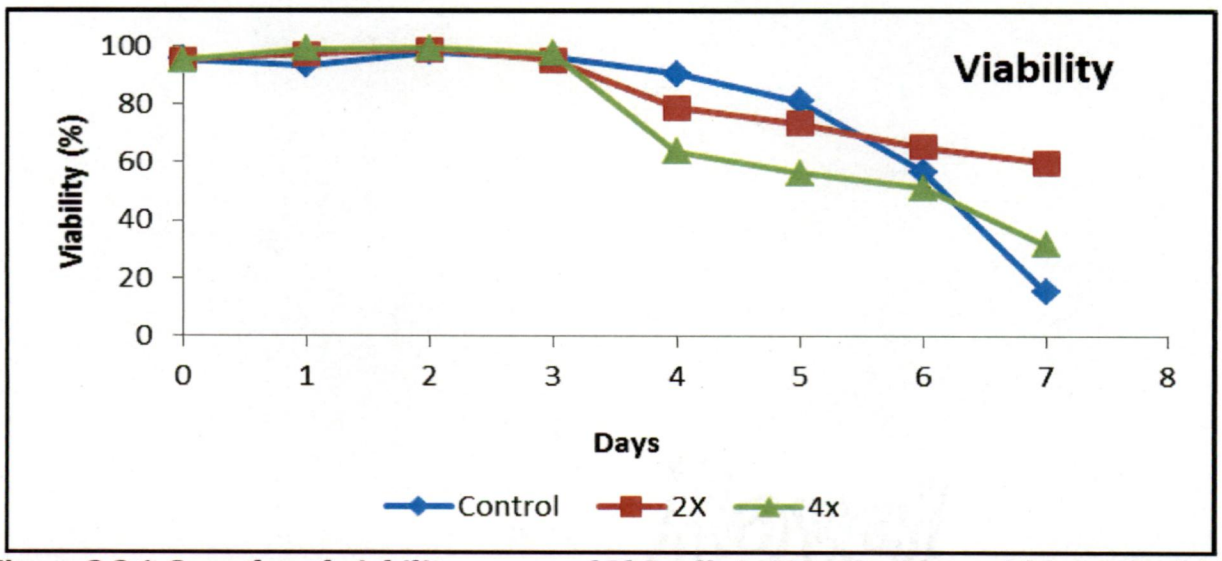


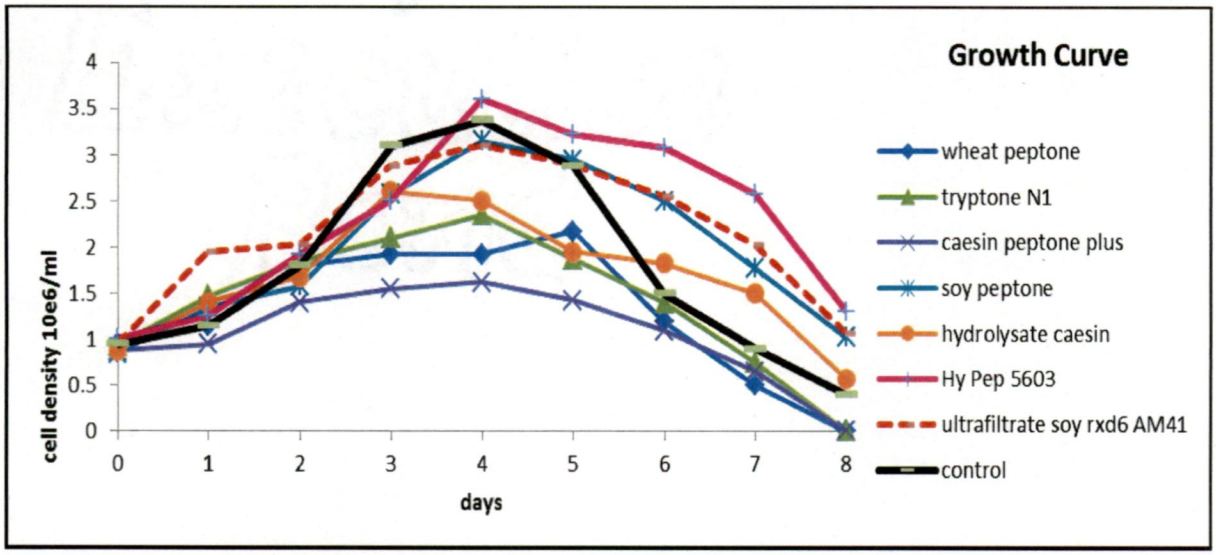
Figure 3.3.1 Growth and viability curves of Sf-9 cells in IPL-41 with twofold and fivefold amino acid concentration.

Literature survey was very useful in an attempt to understand the importance of different amino acids for insect cell culture. As mentioned by Drews et al. 1995 amino acids such as glutamine, glutamate, aspartate, serine, arginine, asparagine and methionine are used for energy production. It has been an assumption that the majority of amino acids are not synthesized by insect cells (Bhatia et al. 1997). Supplementation of methionine and tyrosine was found to retard cell death in Sf-9 culture (Mendonca et al. 1999). Addition of methionine and cysteine was critical for the growth of two insect cell lines in the IBL10 serum-free medium (Vaughn and Fan 1997) and cystine was the only amino acid to be depleted in high density culture of Sf-9 cells. This cell line was believed to be auxotrophic on glycine and cysteine (Tremblay et al. 1992). Nevertheless, more recent work (Doverskog et al. 1998) has shown that Sf-9 cells can grow in cystine-free medium, provided that cell inoculum is taken early on (47–53 h) from the previous culture. In this case, more methionine was consumed by the cells, used in the biosynthesis of cysteine. The same research group showed that Sf-9 and Sf-21 cell growth was also possible in a glutamine-, glutamate- and aspartate-free medium, provided that ammonium ions are present (Ohman et al. 1996). However, Mendonca et al. (1999) noted that glutamine deprivation could affect cell growth rate and thus its synthesis by insect cells was not as efficient as its supply from the medium. As the basal media IPL-41 was supplemented with all amino acids in the form of a mixture, the consequences on growth are positive. This depicts a mutualistic effect on uptake and utilization of one amino acid in presence of other. Amino acids are thus crucial to take the insect cells from lag phase to log phase and reach the exponential peak with higher viabilities.

3.4 OPTIMIZING PEPTONES

Building on the previous results of fivefold sugars and two fold amino acids supporting good cell densities and viabilities, 20gm/lit of each listed (Annexure I) peptones were supplemented to IPL-Carbohydrate-Amino acids. Out of seven peptones tested three of them, namely Ultrafiltrate Soy RXD6 AM41, HyPep5603 and soy peptone performed better than control **(Figure3.4.1)**. The maximum cell densities and viabilities were 2.8X

10e6, 3.9X10e6, 3.1X10e6 for control, HyPep5603, and Ultrafiltrate Soy RXD6 AM41, respectively with viabilities as 92%, 83%, 81% on day four **(Figure 3.4.2)**. The reason why some peptones work to increase cell densities and other don't can be accounted due to difference in membrane permeability, integration to metabolic pathways, or toxicological effects on insect cell culture.



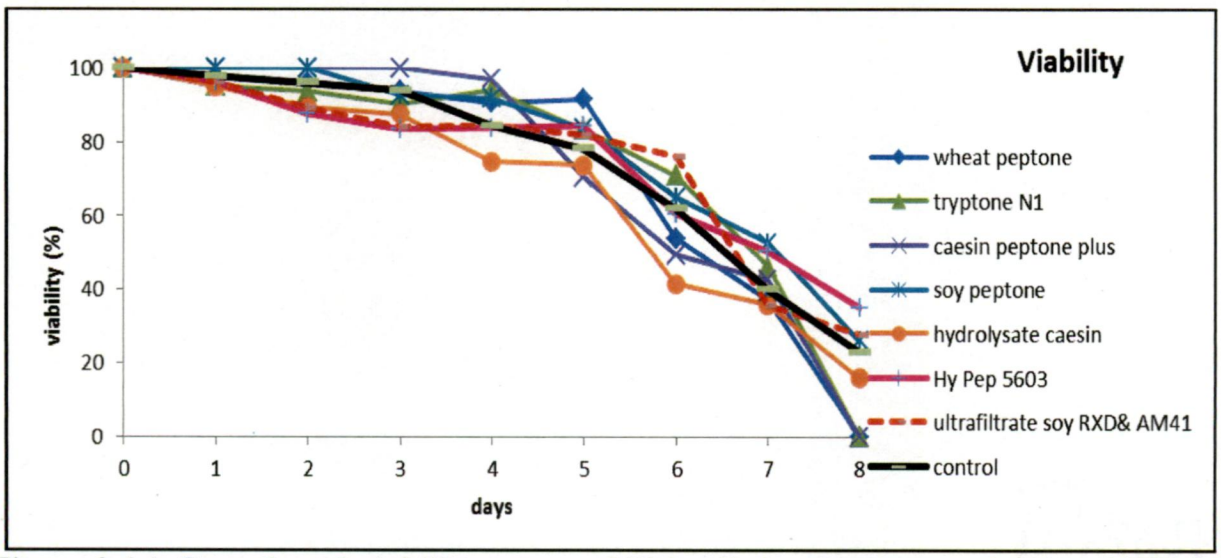


Figure 3.4.1 Growth and viability curves of Sf-9 cells in peptone supplemented IPL-41 with fivefold carbohydrates and twofold amino acid concentration.

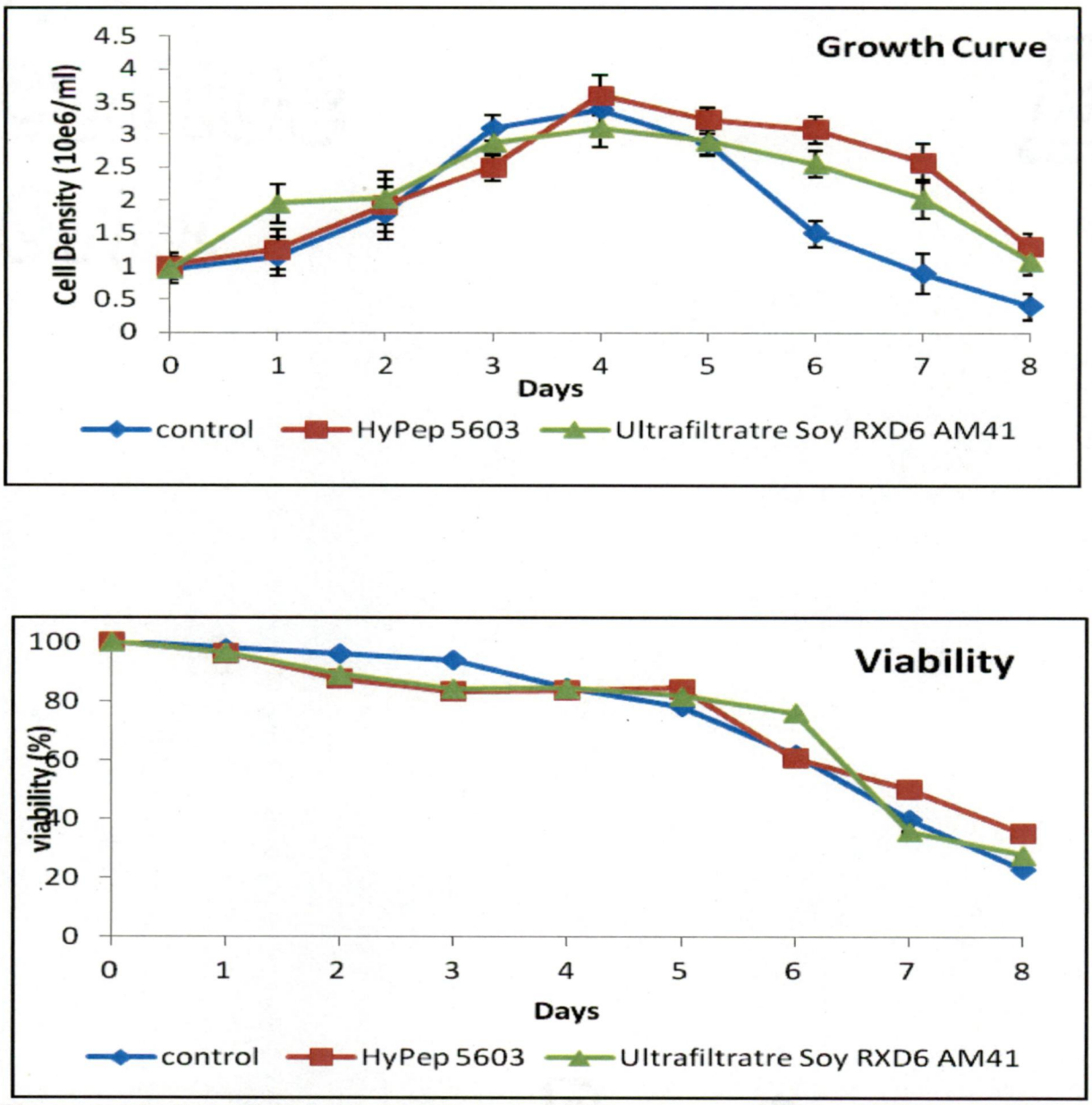


Figure 3.4.2 Comparison of growth and viability curves of Sf-9 cells in basal IPL-41 and peptones supplemented IPL-41 with fivefold carbohydrates and twofold amino acid concentration.

Peptones acted as additional source of energy and accomplishment to amino acid content. The benefits of peptone supplementation in cell culture applications have been well documented for many years. Due to the complex composition of peptones, they provide a wide range of benefits to the cells. Peptones (protein hydrolysates) are an important media

supplement for cell culture processes that do not exhibit optimal performance in chemically defined media. A strong tendency is currently emerging to remove not only serum but also any product of animal origin from animal cell culture media during production of recombinant proteins. This should facilitate downstream processing and improve bio safety (Masuro Shiratori, Genentech Inc). In many cell culture processes, peptones have been shown to enhance product titers as well as overall viable cell densities and culture longevity.

Unfortunately, the composition of peptones is undefined and that contributes to possible disadvantages including: 1) process variability, 2) possible introduction of adventitious agents, and 3) the use of single-sourced raw materials by definition. One way consists in the fortification of protein-free nutritive media with plant protein hydrolysates. In some cases, peptides of various lengths have resulted in increased cell performance. Others have benefited from free amino acids and other low molecular weight nutrients. With the number of peptones that are available, it is critical to evaluate a wide variety of products. Since every peptone is different, multiple peptones produced using the same base material should be included. In order to run the most effective peptone screen, the list of potential peptones should be narrowed to include only the best candidates. This can be accomplished with an understanding of the specific regulatory and process requirements.

3.5 CELL GROWTH AND VIABILITY OF SF-9 CELLS IN SUPPLEMENTED IPL-41.

After understanding the effect of sugars, amino acids and peptones, a choice was made to cultivate cells in two different media *a)* IPL-41-sugar (5X) and *b)* IPL-41+ Sugars (5X) + Amino Acids (2X) under the same conditions as the seed **(Figure3.5.1).** The seed was taken from Sf-9 cells growing in basal IPl-41 for passaging at 10e6 cells/ml.

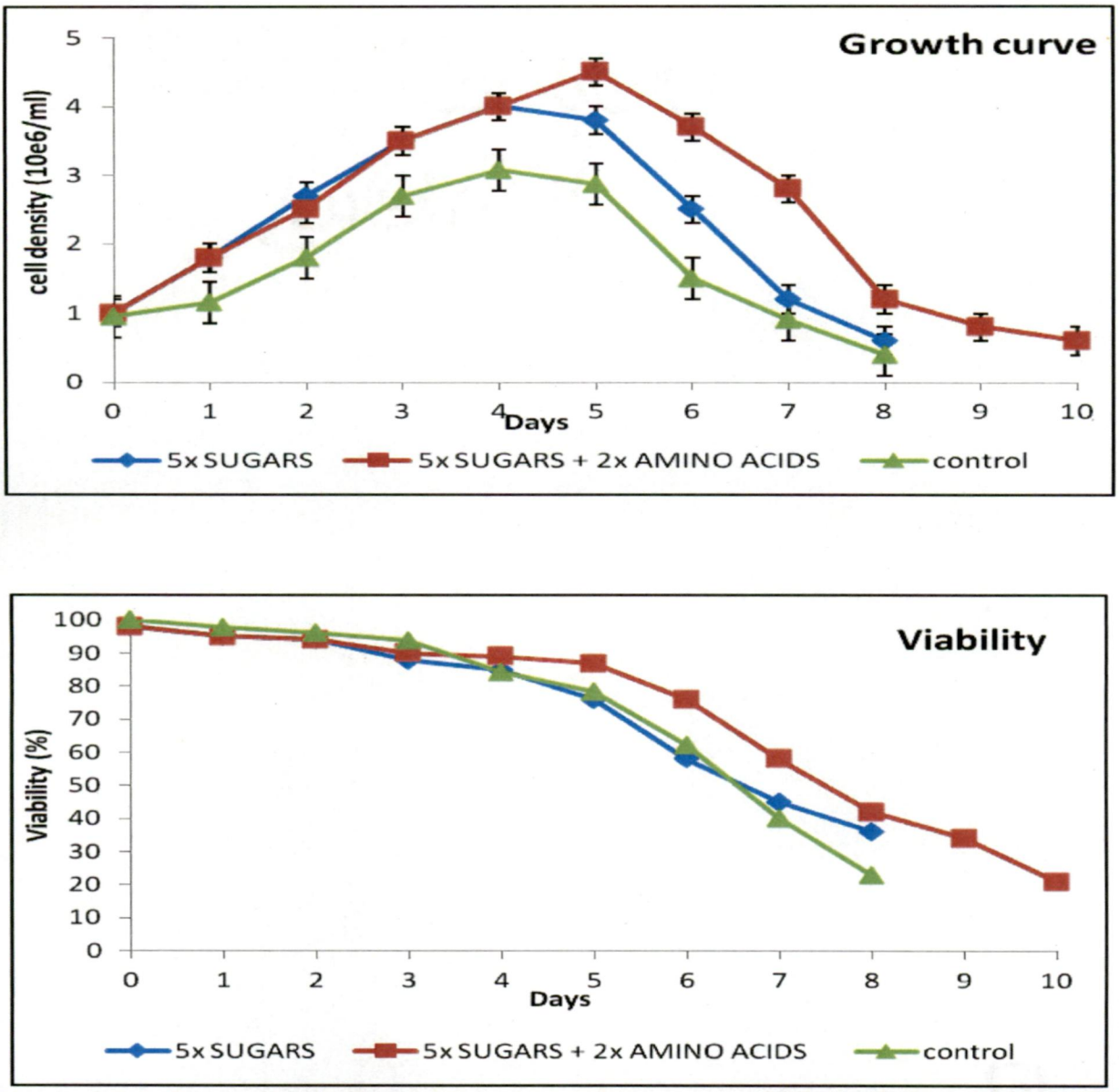


Figure 3.5.1 Comparison of growth and viability curves of Sf-9 cells in supplemented IPL-41 with 5X sugars (blue) and 5Xsugars+2X amino acid (red).

As compared to the control (basal IPL-41), both supplemented media with performed better than the control. Maximum cell densities (cells/ml) and viabilities attained were 3.6 X10e6, 75% in IPL-41-sugar (5X); 4.7 X10e6, 88% in IPL-41+ Sugars (5X) + Amino Acids (2X); and 2.7X10e6, 74% in the control on day four. With such supplementation it is possible to keep viable cells for longer duration with higher viabilities. The doubling time however was not constant to 20 hours and a longer lag phase was observed in all the three cases which might be due to lack of growth factors.

4. CONCLUSIONS

The studies on limiting components in basal media IPL-41 for insect cell culture the following objectives were targeted:

- Higher cell growth in terms of cell densities ($10e^6$cells/ml),
- Higher viability (%).
- Increased culture longevity.
- Cost effective.

The above aims can be achieved through supplementation of basal IPL-41 with

- Fivefold sugars than originally present in IPL-41 to fulfill the prime energy requirements
- Twice amino acids (all)
- Peptones: 20g/l each of either HyPep 5603 or Ultrafiltrate Soy RXD6 AM41.

Supplementation of basal IPL-41 , with carbohydrates, amino acids increases the cost of media to 15€/l whereas SF900-II SFM media supplied by GIBCO (Invitrogen NY,USA) costs 75€/l. Thus, supplementation of media and preparation of stocks for future cell cultures is cheaper and very cost effective.

FUTURE PROSPECTS

After these studies, there is more left to explore for development of an efficient and cost effective media. Other components of IPL-41 which demand exhaustive studies are

- Individual role of each amino acid.
- Inorganic Salts.
- Lipids.
- Vitamins.
- Growth Factors.
- pH indicator.
- Buffering systems other than phosphate.

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ANNEXURE

I. List of Chemical Suppliers

CHEMICALS	SUPPLIERS
I. Carbohydrates	
Glucose	Applichem, Darmsdadt , Germany
Maltose	Sigma Aldrich, Germany
Sucrose	Bethesda Research Labs, NY, USA
II. Amino Acids	
Alanine	Sigma Aldrich ,Steinheim, Germany
Arginine	Sigma Aldrich ,Steinheim, Germany
Asparagine	Sigma Aldrich ,Steinheim, Germany
Aspartic	Sigma Aldrich ,Steinheim, Germany
Cystine	Sigma Aldrich ,Steinheim, Germany
Glutamic	Sigma Alrich, France
Glutamine	Fluka Chemie, CH, AG
Glycine	Sigma Aldrich ,Steinheim, Germany
Histidine	Sigma Aldrich ,Steinheim, Germany
Hydroxy proline	Biochemica
Isoleucine	Sigma Aldrich ,Steinheim, Germany
Leucine	Sigma Aldrich ,France
Lysine	Sigma Aldrich ,Steinheim, Germany
Methionine	Applichem, Darmsdadt , Germany
Pheny alanine	Sigma Aldrich ,Steinheim, Germany
Proline	Fluka Chemie, CH, AG
Threonine	Applichem, Darmsdadt , Germany
Tryptophan	Sigma Aldrich ,Steinheim, Germany
Tyrosine	Sigma Aldrich ,Steinheim, Germany
Valine	Applichem, Darmsdadt , Germany

III.Peptones	
Wheat Peptone	Sheffield Pharma Ingredients, United Kingdom
Tryptone N1	Sheffield Pharma Ingredients, United Kingdom
Casein Peptone Plus	Sheffield Pharma Ingredients, United Kingdom
Soya peptone	Sheffield Pharma Ingredients, United Kingdom
HyPep 5603	Sheffield Pharma Ingredients, United Kingdom
Ultrafiltrate Soy RXD AM41	Sheffield Pharma Ingredients, United Kingdom
Hydrolysate Casein	Organotechi e SAS, France