Development and optimization of an *in vitro* process for the production of *Oryctes* nudivirus in insect cell cultures

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Dedicated to my loving parents,

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Abstract

The coconut rhinoceros beetle, an economically important pest of coconut and oil palms, is effectively managed by application of its natural pathogen, the *Oryctes* nudivirus (OrNV), which act as a bioinsecticide. While this approach offers an environment-friendly alternative to chemical pesticides, the current method of production in infected larvae suffers from inconsistencies in virus productivity and purity. While the anchorage-dependent DSIR-HA-1179 insect cell line has been identified as a susceptible and permissive host for OrNV and therefore would be suitable for the *in vitro* mass production of the virus, no attempts have been made toward the mass production of the virus, because of the technological challenges that working with DSIR-HA-1179 cells represent. Thus, the main objective of this research was to develop processes for the *in vitro* production of OrNV in the DSIR-HA-1179 cell line.

Knowledge of the growth kinetics and metabolic properties of the host cell line in a chosen culture medium, as well as the selection of an appropriate infection strategy, form the basis for the rational development of bioreactor-based virus production processes. However, characterization of these properties in the DSIR-HA-1179 cell line has been virtually precluded, due to its strongly adherent growth characteristics and the lack of a reliable method to accurately dissociate and count cells grown in monolayers. Using TrypLE™ Express enzyme, a technique allowing the precise counting of cells was developed.

The cell line was adapted to grow in four serum-supplemented culture media: TC-100, IPL-41, Sf-900 II and Sf-900 III, which were then individually screened for cell growth and virus production in 25 cm^2 attached T-flask cultures. TC-100 supplemented with 10% fetal bovine serum was chosen as a suitable culture medium, based on its capacity for achieving a high cell yield and OrNV production. The cell line metabolism was characterized with respect to nutrient consumption and metabolites production in this culture medium. Glucose, along with glutamine were found to be the nutrients that were consumed faster and to a greater extent, while other amino acids were not consumed to a significant degree. The production of metabolites was characterized by non-production of lactate and ammonia, and production of alanine, as a non-toxic alternative to ammonia.

The influence of cell density (CD) at time of infection (TOI) and multiplicity of infection (MOI) on OrNV production was evaluated in T-flask cultures that were infected at different CDs at the TOI and

a range of MOIs. The CD at TOI was found to significantly influence OrNV yields, while MOI influenced the dynamics of infection. The cell density effect was found to exist for the DSIR-HA-1179/OrNV system with the progressive decline in cell-specific yield beginning at low cell densities. It was found that in order to maximize OrNV volumetric yield, a combination of MOI and CD at TOI should be selected that allows to keep the maximum cell density reached by the infected culture within a range between 5.0 and 7.0 x 10^5 viable cells/ml.

The roller bottle system was evaluated for its potential to scale-up DSIR-HA-1179 cell growth and OrNV production, and culture parameters were optimized for the improvement of cell and virus yields. An inoculum density of 3.3 x 10^5 cells/ml and culture volume of 60 ml resulted in the highest cell yield of 1.5 x 10^6 cells/ml, in 490 cm^2 roller bottles. It was found that an optimal infection strategy for roller bottle cultures, which represented the most efficient use of viral inoculum, involved infecting cells at a density of 5.0 x 10^5 cells/ml and at a MOI of 1. The resulting OrNV volumetric yield of 2.5 x10^8 $TCID_{50}$/ml, improved significantly the viral yields obtained in attached T-flask cultures infected under similar conditions (6.8 x 10^7 $TCID_{50}$/ml).

The microcarrier system was also evaluated for culturing DSIR-HA-1179 cells and producing OrNV in spinner flask bioreactors. Three types of microcarriers (Cytodex-1, Cytodex-3 and Cultispher-G microcarriers) were screened for their ability to support DSIR-HA-1179 growth. Cells attached to Cytodex-1 and 3, but failed to attach to Cultispher-G microcarriers. The final cell density reached in microcarrier culture was dependent on bead type and concentration, and the cell to bead ratio. At an optimal bead concentration of 1 mg/ml and cell to bead ratio of 30, cells grew to a maximum density of 1.7 x 10^6 cells/ml on Cytodex-1, but only to 1.3 x 10^6 cells/ml on Cytodex-3 microcarriers. Since it supported higher cell yields, Cytodex-1 was chosen to study the kinetics of OrNV production in this system. Microcarrier cultures infected at a cell density of 5.0 x 10^5 cells/ml and a MOI of 1, produced OrNV at 1.4 x 10^8 $TCID_{50}$/ml, which was higher than the yield obtained in T-flask cultures infected under similar conditions.

A framework of knowledge on the physiology, metabolism and growth kinetics of the DSIR-HA-1179 insect cell line has been developed in this thesis. In addition, the feasibility of using roller bottles and microcarrier systems for the *in vitro* production of the virus has been ascertained. It is envisaged that these findings will contribute to the future development of a large-scale industrial process for the production of the OrNV biopesticide.

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List of Abbreviations used in the thesis

CD	Cell density
CHO	Chinese hamster ovary cell line
DEAE	Diethylaminoethanol
DIP	Defective interfering particles
DMSO	Di-methyl sulfoxide
D-PBS	Dulbecco's phosphate buffered saline
Dpi	Days post-infection
EBV gp105	Epstein-barr viral attachment protein
FBS	Fetal bovine serum
GS-GOGAT	Glutamine synthetase/glutamateoxoglutarate aminotransferase
Hpi	Hours post-infection
HPLC	High-performance liquid chromatography
IC-BEVS	Insect cell-baculovirus expression vector system
IPL-41	Commercially available basal medium for insect cell culture
LC-SFM	Low-cost, serum-free culture medium
MOI	Multiplicity of infection
MVCD	Maximum viable cell density
NPV	Nucleopolyhedrovirus
OrNV	*Oryctes* nudivirus
PDT	Population doubling time
PS-100	In-house prepared basal medium for culture of the DSIR-HA-1179 cell line
Sf-900 II	Commercial medium for insect cell culture
Sf-900 III	Commercial medium for insect cell culture
TC-100	Commercially available basal medium for insect cell culture
TCA	Tricarboxylic acid cycle
$TCID_{50}$	Tissue culture infectious dose
TOH	Time of harvest
TOI	Time of infection

Chapter 1

Literature Review

1.1 The coconut rhinoceros beetle (*Oryctes* Rhinoceros, L.) – A scarab pest

Coconut palms (*Cocos nucifera*) and Oil palms (*Elais guennis*) are economically important crops of the tropics. The oil extracted from the kernel is the most valuable product of the coconut palm, and is used in cooking, in the manufacture of soaps, cosmetics, margarine etc., while the residue is an important animal feed. Coir obtained from the fibrous husk of the coconut is used to make brushes, ropes and carpets. The inflorescences yield a sap with high sugar content, from which vinegar and alcoholic beverages like toddy are made. Village homes often have thatched roofs made from dried leaves of the coconut palm. Hence, the coconut palm has aptly been called 'the tree of life' (Harries, 2001). Oil palms are important sources for palm oil and palm kernel oil, which are used in the making of several processed foods as well as in the manufacture of oleochemicals such as fatty esters, fatty acids, fatty alcohols and glycerol compounds. In recent years, palm oil has also come to be used as a biofuel (Sayer *et al*., 2012).

Oryctes rhinoceros (L.) (Coleoptera: Scarabaeidae) (Fig. 1-1), or more commonly known as the coconut rhinoceros beetle, is a serious pest of coconut and oil palms. Economic losses associated with beetle damage to the palm run into millions of dollars annually (Jackson *et al*., 2005; Moore, 2009). Adult rhinoceros beetles cause damage by boring deep into the crown of the coconut palm or at the growing spear of oil palms, where they feed on the exuded sap (Young, 1986). This activity causes damage to the growing tissues within the apical meristem, causing V-shaped cuts in the fronds and the appearance of holes in the midribs of emerging leaves (Bedford, 2013). In light to intermediate attacks, a reduction in leaf photosynthetic area leads to a decrease in nut production. Heavy and repeated attacks, especially on young, growing palms, ultimately leads to the death of the palm (Wood, 1968).

Fig. 1-1 The coconut rhinoceros beetle - *Oryctes* rhinoceros (L.)

In 1909, the coconut rhinoceros beetle was accidently introduced into the Pacific Island of Upolo in Western Samoa, from where it spread to at least 42 of the neighbouring islands, including Tonga (1921), Wallis Island (1931), Papua New Guinea (1942), Palau Islands (1942), Fiji (1953), Tokelau Islands (1963) and Mauritius (1962) (Bedford, 1980; Monty, 1974). One of the reasons why the coconut rhinoceros beetle reached an unprecedented level of pestilence in the Pacific Islands could be attributed to it having very few natural enemies in these new habitats, in addition to having no natural pathogen present in the environment to regulate its population. Burgeoning beetle populations caused these island communities to sustain heavy losses due to widespread damage of plantations, across a period of fifty years. In the Palau Islands alone, the beetle destroyed 50% of the coconut palms within 10 years of its introduction (Gressit, 1953).

1.1.1 The search for a control agent against the coconut rhinoceros beetle

Initial efforts were made to control beetle outbreaks using chemical pesticides. For example, to discourage the breeding of beetle larvae in the trunks of mature palms, holes were drilled at one meter intervals, into which a mixture of dieldrin was injected. Another method involved placing a mixture of lindane granules and gamma benzene hexachloride powder mixed with damp sawdust, in the axils of the youngest fronds on the coconut palm (Wood BJ, 2002). Despite these measures,

it soon became apparent that the beetle could not be effectively controlled using chemicals, due to its unique biology and cryptic flying habits. While feeding during the day, adult beetles burrow deep into the crown of the palm and remain hidden, and fly only during the night time (Young, 1986). Larval stages of the beetle usually grow within breeding sites on the ground, which are piles of decomposing organic matter, accumulated saw dust and rotting tree stumps, where applied chemicals cannot easily penetrate (Huger, 2005).

The failure of chemical control led to an intensified search for a natural biocontrol agent against the coconut rhinoceros beetle, by the South Pacific Commission. In 1963, a diagnostic field survey of beetle populations in Malaysia led by Huger, led to the discovery of diseased larvae with distended, turgid midguts which appeared beige and waxen, with prolapsed rectums, and which displayed signs of lethargy, in comparison to healthy larvae. It was observed that healthy beetle larvae which orally imbibed a suspension of the diseased beetle macerate, developed the same symptoms of infection, and died within 1-4 weeks post-infection. Electron microscopy of thin sections of infected larval midgut epithelial and fat body cells revealed rod-shaped virions that reproduced in cell nuclei, satisfying Koch's postulates (Huger, 2005). Further investigations revealed that this was a DNA virus which was rod-shaped, enveloped and measured approximately 220 x 120 nm. The virus was found to differ from baculoviruses in that virions were not occluded with a proteinaceous crystalline occlusion body (Payne, 1974). Thus, the discovery of the *Oryctes* nudivirus was made.

1.1.2 Pilot release of OrNV and present scenario

The pilot release of the OrNV which was carried out in Samoa in 1964 by contaminating beetle breeding sites with titurated infected larvae, was a tremendous success in lowering coconut palm damage to acceptable levels, and has since been hailed as a 'landmark in classical biocontrol' (Caltagirone, 1981; Huger, 2005). The most interesting finding from the pilot release, was that infected beetles act as 'flying reservoirs of OrNV'. This highly efficient mechanism of transmission results from adult beetles spreading the virus among themselves during feeding, mating and breeding activities. Large amounts of virus are released from the gut and are expelled in droppings, which accumulate at communal sites. This leads to the effective spread of the viral disease in beetles via both peroral and parenteral routes. (Bedford, 1980; Huger, 2005).

Following its pilot release, OrNV was introduced into Western Samoa, Tonga, Papua New Guinea, Philippines in the coming years, where it controlled beetle outbreaks with success (Bedford, 2013). Since then, and until the present, the most effective control of the beetle has achieved through applying the *Oryctes* nudivirus as part of an integrated pest management (IPM) program. IPM is a comprehensive method of pest management that involves the use of multiple, common sense practices to help maintain productivity and restore the ecological balance in the most economical way. IPM programs adopt a sensitive approach to pest management utilizing knowledge of pests life cycle, its behavior, natural enemies and interaction with the environment with the watchwords being prevention, monitoring and control (Murhammer, 1996). The IPM toolkit for the coconut rhinoceros beetle includes myco-biocontrol using *M. anisopliae*, as well as field and cultural methods, to augment the action of OrNV in controlling the beetle. However though the fungus *Metarhizium anisopliae* is entomopathogenic to the beetle, its efficacy in initiating epizootics in less than that of OrNV. It has shown success when applied to single site beetle breeding grounds but is ineffective for large scale dispersion (Young, 1974). In addition, beetle populations can be reduced by trapping them. The male rhinoceros beetle produces an aggregation pheromone ethyl-4-methyloctanoate which is an attractant, effective in luring both males and females of the species. This is commercially sold as Oryctalure™. A simple yet effective pheromone trap can be made using a plastic bucket with a lid with two holes about 2.8cm wide made in the side of the bucket and two smaller holes are made on the center of the fitted lid. A sachet containing the pheromone lure is suspended securely from the lid and the trap is installed at a height of 5ft preferably away from direct sunlight. Because of its efficiency and economic value, the use of pheromones to lure and trap the rhinoceros beetle has gained popularity in developing nations in recent years (Hallett *et al*., 1995). In addition, cultural methods such as regular survey and detection of infested areas, sanitation of fields to remove breeding sites, and the use of cover crops have been implemented as part of the IPM strategy (Jackson et al., 2005; Wood, 1969). At present, while in some geographical locations, the beetle appears to be kept at low populations due to natural epizootics of OrNV persisting in the environment, in other regions the beetle has escaped control and continues to devastate coconut plantations. Examples of this include recent outbreaks of the beetle in Rarotonga since 2007, Guam since 2009 and Hawaii since 2013. Introduction of OrNV into these regions has been carried out by artificially infecting beetles and releasing them into the environment to spread the disease, and ongoing reduction in palm damage is being monitored (Jackson TA, 2009; Jackson *et al*., 2010).

1.2 The Oryctes nudivirus

1.2.1 Taxonomy and Biology

OrNV is a member of the newly assigned family 'Nudiviridae', which are a highly diverse group of rod-shaped, enveloped, and circular dsDNA viruses of arthropods, including insects and crustaceans. For many decades, the nudiviruses were classified under subgenus C of the family *Baculoviridae* and were known as 'non-occluded baculoviruses' alongside subgenus A (nucleopolyhedrosis viruses) and subgenus B (granuloviruses). They have also been referred to as 'intranuclear bacilliform viruses', indicating their similarity with baculoviruses in terms of their rod-shaped enveloped virions which replicate within the nucleus of infected host cells (Evans and Edgarton, 2002). Owing to a lack of convincing genetic data corroborating to their relatedness to baculoviruses, as well as their different morphological characteristics, they were eventually excluded from the *Baculoviridae* family and orphaned. Though the nudiviruses generally lack occlusion bodies, the facultative occurrence of occlusion bodies in midgut cells in infected beetles has been reported (Huger and Krieg, 1991). In 2013, a new family '*Nudiviridae*' was created, which included two genera and three species.

Table 1-1 The well-studied nudiviruses (modified from Wang *et al*., 2007).

Virus	Host	Host stage and/or tissue tropism	Virion size (nm)	Nucleocapsid size (nm)	Size (kb)	GC%	ORFs
HzNV-1	Heliothis zea	-	384-444 x 77-83	-	228,089	42	154
HzNV-2	Heliothis zea	Larvae and adults	415 x 80	500 x 25	231,621	42	113
OrNV	Oryctes rhinoceros	Larvae and adults	200-235 x 100-120	160 x 50	127,615	42	139
GbNV	Gryllus bimaculatus	Nymph and adults	145-240 x 80-100	162 x 66	96,944	28	98

The nudiviruses which have been well studied thus far are listed along with their properties in Table 1-1. These are the *Oryctes* nudivirus (OrNV) and the Gryllus bimaculatus nudivirus (GbNV) which belong to the genus alphanudivirus, and the Heliothis zea nudivirus 1 (HzNV-1) and the Heliothis zea nudivirus 2 which are betanudiviruses (HzNV-2). The *Oryctes* nudivirus consists of a rod-shaped, enveloped nucleocapsid containing a double-stranded super coiled DNA molecule of 92 - 106 daltons. Virions vary between 200-235 nm in length and 100-120 nm in width (Huger, 2005). Payne optimized a purification protocol for both virus and virus nucleocapsids using differential high and low speed centrifugation followed by two sucrose gradient steps. The presence of a unique tail like appendage within the nucleocapsid was reported (Payne 1974, Payne *et al.*, 1977). The protein composition of the virus is not very well analyzed, and proteomic data are missing, so far. OrNV have circularly closed ds DNA, which are 123-127 kb in length (Crawford *et al*., 1985; Mohan and Gopinathan, 1991). The 127, 615 base-pair genome has been sequenced for the isolate OrNV Ma07, and its G+C content determined to be 42% (Wang *et al*., 2011). Comparative gene and phylogeny analyses have been able to elucidate important relationships between nudiviruses and baculoviruses. Nudiviruses share 20 core baculovirus genes involved in RNA transcription, DNA replication, virion structural components and peroral infectivity. They also share a gene cluster of helicase, 19kda, 38K and lef-5 which is similar to the conserved core gene cluster of four helicase genes, 19kda, 38 K and lef-5, found in all baculoviruses (van Oers and Vlak, 2007; Wang *et al*., 2007). Based on comparisons of core gene content and gene clusters, it has been suggested that nudiviruses shared an ancient common ancestor with baculoviruses but evolved as a separate lineage of non-occluded double-stranded DNA viruses before the split between dipteran baculoviruses and the ancestors of hymenopteran and lepidopteran baculoviruses occurred.

1.2.2 *In vivo* pathogenesis of OrNV

Both larval and adult stages of the beetle can be infected by OrNV. This aspect is defining for nudiviruses, and is in contrast to baculoviruses, which are only able to infect larvae. OrNV infection begins in midgut epithelial cells and then spreads to the fat body cells, hemocytes and gonads in both larval and adult stages of male and female beetles (Majumder and Jacob, 1993). The virus replicates in these cells from within 7 hours post infection. After 48 hours of exposure, both infected larvae and adults stop feeding and display signs of lethargy. The midgut of diseased larvae fills up with white mucosal fluid, making the midgut line appear glassy and translucent when the

dorsal surface is held up to white light. Fat body cells disintegrate and the hemolymph content increases. Elevated levels of granular cells and plasmocytes, protein, amino acid and sugar concentrations, are present in infected larvae (Biju *et al*., 1993; Huger, 2005; Vincent *et al*., 1988). Increased turgor pressure within the abdomen often results in rectal prolapse in infected beetles. The virus' speed of kill in larvae was observed to occur faster at a temperature of 32ºC and the median periods of lethal infection for the 1st, 2nd and 3rd instar larvae were observed to be 8.5 ± 0.2, 12.6 ± 1.0 and 22.5 ± 2.8 days respectively (Zelazny, 1972) .

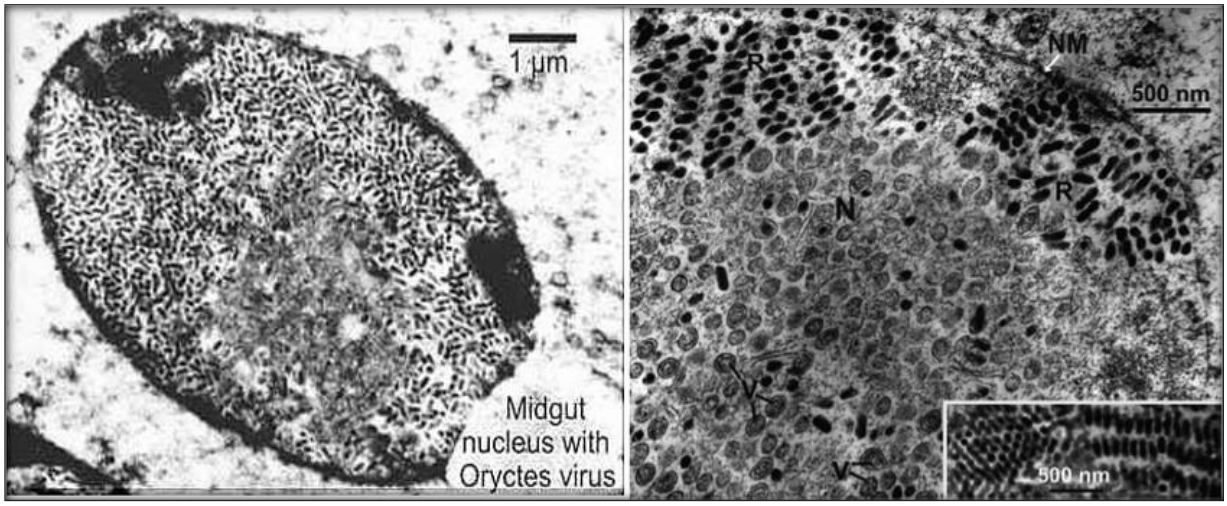

Fig. 1-2 Hypertrophied nucleus in infected fat body cell of a third instar coconut rhinoceros beetle, with virus rods (dark rods) accumulated at the nuclear periphery.

In adult beetles, the midgut epithelium swells up once infected and ruptures releasing infected nuclei into the gut lumen from where the virus can spread into other body tissues. In infected adults it can take up to 25 days for the infection to kill, during which period the beetle functions as a flying 'virus reservoir', auto disseminating the virus by excreting up to 0.3 mg of contaminated feces per day, contacting other beetles at feeding sites and mating at breeding sites (Monsarrat and Veyrunes, 1976) .

1.2.3 Detection and diagnosis of OrNV

While baculoviruses can be easily detected by using light microscopy to visualize their large polyhedral occlusion bodies, this is not possible for non-occluded viruses such as OrNV (Young,

1986). Instead, visual inspection of infected insects for signs of the disease, histological observation of tissue sections, electron microscopy, bioassays, biochemical tests such as ELISA, and molecular methods of detection such as PCR, can be used to diagnose OrNV disease in infected beetles and larvae.

Infected beetle midguts are usually swollen, white and fragile depending on the progression of the disease and are in contrast to healthy beetle midguts which are thin, brown and strong; thus providing a way to distinguish infection by visual inspection of insects (Gorick, 1980). The rapid smear technique involves making air-dried smears of dissected midgut fluid and epithelial cells, followed by alcohol fixation and staining of the smear with Giemsa (Zelazny, 1978). Classic indicators of infection at the cellular level are hypertrophied nuclei surrounded by a thin layer of cytoplasm, both staining mauve-pink (Huger, 2005). In many cases, a homogenously stained deep pink circular band can be observed at the periphery of the nucleus. In contrast, cells from uninfected beetles exhibit small, chromatin-dense nuclei which stain purple (Kinawy, 2004; Gopal *et al*., 2001, Gorick 1980). Additionally, electron microscopy of 1 mm thick, infected midgut slices, negatively stained with phosphotungstic acid or osmium tetroxide, can be used to detect the virus (Huger, 2005; Mohan *et al*., 1983; Mohan and Gopinathan, 1992). The virus can also be detected by a bioassay in which perorally infected healthy beetles or larval instars are infected with clarified homogenate of an infected beetle's midgut, and by observing the progression of the disease against an uninfected control group (Zelazny, 1971; Zelazny, 1978; Mohan *et al*., 1983). While the bioassay approach is a low-tech approach, it is also a tedious and laboursome process.

An indirect sandwich ELISA and using immune-osmophoresis, was developed in the early 1980's, which enabled the first semi-quantitative assessments of OrNV in infected beetle excreta and midgut macerate to be made (Mohan and Pillai, 1983; Longworth and Carey, 1981, Rajamannar and Indrawati, 2000). While the indirect sandwich ELISA could detect concentrations of virus as low as 20 ng/ml in buffer purified samples, the sensitivity of the test was reduced when titrating crude insect extract. This was attributed to host proteins in the insect extract interfering with the conjugation of virus particles to the primary and secondary antibodies during the ELISA (Mohan and Gopinathan, 1989b). More recently, the design of a 945 base pair PCR primer 15a/15b specific to OrNV has enabled the rapid and accurate detection of the virus using PCR technology. PCR amplification of viral gene fragments allows the detection of even very early stage infection in

larvae and adult beetles, which may not manifest visually as symptoms of disease yet. (Richards *et al*., 1999).

1.2.4 Production methods for OrNV: *in vivo* vs *in vitro* production

The production of *Oryctes* virus inoculum for application as a biopesticide was initially performed *in vivo* via infected beetle larvae. (Bedford, 1986). This was done by collecting already infected larvae from the field and triturating them to produce a crude virus suspension (Marschall KJ, 1970), or rearing them in insectaries and then infecting them (Prasad *et al*. 2008). This method requires the least skill or training, and virus extraction can be performed on any benchtop equipped with a blender, filter and a refridgerator to store the virus at 4 degrees C. Information about the cost of this process has not been reported, but presumably it is the most economical choice. A second method of preparation of inoculum was to collect infected beetles and dissect out the infected midguts which were then macerated to produce a virus solution. It requires, to start with, the use of techniques such as attractant/pheromone trapping, food bucket traps, detector dogs, acoustic detection etc. to trap adult beetle specimens from the field. Then, a lab worker with basic skill in dissecting out the infected guts is needed, which makes again for a cumbersome process (Marschall and Ioane, 1982; Ramle *et al*. 2005).

A third, slightly more sophisticated method of homogenising infected adult beetle midguts was developed, which involved purifying the virus-containing supernatant using glass permeation chromatography followed by formulating it with sucrose and tris-buffer. The infectivity and effectiveness of this chromatography-purified virus inoculum was tested against *in vitro* produced inoculum, and it was found that while both inocula showed comparable infectivity, the cell culture derived one had slightly higher stability (Zelazny *et al*., 1987). Finally, there is the *in vitro* method of producing OrNV, by its propagation in the susceptible and permissive host insect cell line DSIR-HA-1179 (Crawford, 1982). AgResearch Ltd, New Zealand, uses cell culture to produce low quantity stocks of the X2B strain of the *Oryctes* virus to provide farmers with viral inoculum. Their production capacity is limited to 20 ml (1×10^6 IU/ml viral titer) every 4-6 weeks (Jackson *et al.*, 2010).

The *in vivo* method is economical in terms of yield, activity and cost in the small scale. However there is a significant risk of contamination and poor process control given that the quantity and

quality of the wild type *Oryctes* virus produced in infected beetles are subject to a number of factors including environmental conditions, the quality of the viral inoculum used and type of diet of the grubs. The *in vivo* production of *Oryctes* virus is therefore an inconsistent process which has no commercial viability at a large scale. *In vitro* large-scale production of the *Oryctes* virus using cell culture technology would be advantageous over *in vivo* production due to the benefits such as a controlled and robust process that ensures consistent titer and purity of the viral product. However, despite these potential benefits, the use of the DSIR-HA-1179 cell line as a substrate for the mass production of *Oryctes* virus has not been extensively explored. The next section reviews the technological factors in insect cell culture which have an influence on *in vitro* virus production.

1.3 Insect Cell Culture

1.3.1 Insect cell lines for biopesticide production: Historical perspective & current state

The advent of invertebrate culture can be traced back to 1915 when Goldschmidt grew testicular cells of the silkmoth, *Samia cecropia* in hanging drops of insect hemolymph (Goldschmidt, 1915). Similar attempts were made by Glaser and Lazarenko who cultivated insect hemocytes in hanging drops in 1917 and 1925, respectively; and Frew who cultivated imaginal disks of blow-fly larvae in 1928 (Benz, 1986). While these studies were useful in studying *in vitro* gametogenesis, they were unsuccessful in demonstrating mitoses in *in vitro* cultures. In 1916, Lewis and Robertson grew testicular cells of the grasshopper *Chorthlppus curtlpennis* in a balanced salt solution containing glucose and peptones. Belar used a similar culture medium but without peptone, to grow spermatocytes of the grasshopper species *Chorthippus lineatus.* While mitosis was observed in both cases, the cultures failed to survive for more than three weeks (Lewis and Robertson, 1916; Belar 1929).

Trager demonstrated the first long term culture of insect cells by maintaining a culture of ovarian cells from the silkworm *Bombyx mori*. L for three weeks. He also demonstrated the production of silkworm grasserie virus by *in vitro* infection of these cultures (Trager, 1935). In 1956, Silver Wyatt reported an improvement in cell number, appearance and the rate of mitoses in *in vitro* cultures of *Bombyx mori* by making modifications to Trager's salt solution with the addition of tryptophan, cysteine, malate, fumarate, succinate and α-ketoglutarate and silkworm egg extract (Wyatt *et al*., 1956).

The first successful insect cell lines were established independently within a span of three years by Professor Shangyin Gao in Wuhan, China who established continuous cell lines from tissues of *Bombyx mori* in 1958; and by Dr. Thomas Grace in Canberra, Australia who established continuous cell lines from ovarian tissues of the emperor gum moth *Antherea Eucalypti* in 1961 (Gao, 1958; Grace, 1962). Of particular benefit was the formulation of Grace's culture medium which was tailored to the nutritional requirements of lepidopteran cell lines, which played an important role in ensuring the continuing success of establishment of over 500 continuous insect cell lines, derived from more than 100 insect species and encompassing more than 6 insect orders, over the next five decades (Fig. 1-3, Lynn, 2001; Lynn, 2007).

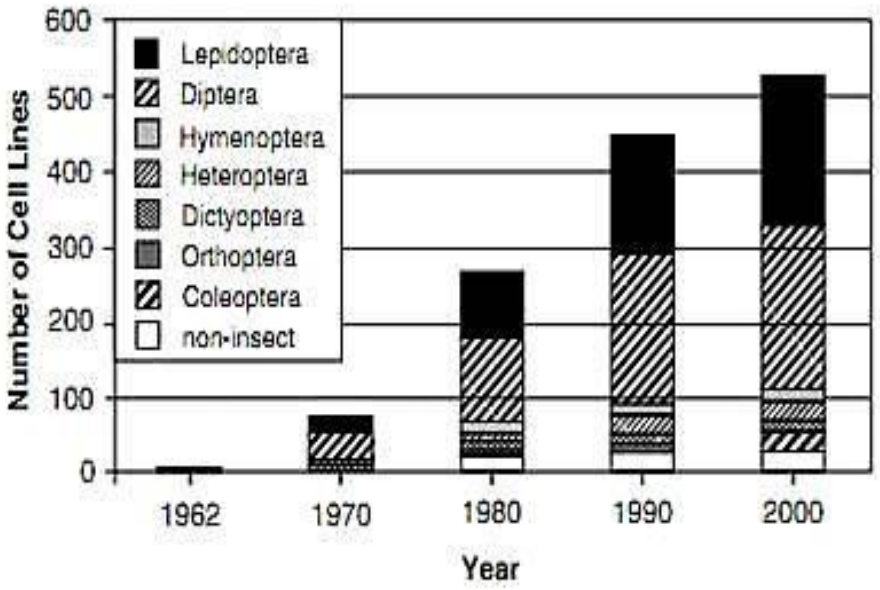

Fig. 1-3 Cell lines established within different insect species (Reproduced from Smagghe, 2007).

A general pre-requisite for the serious consideration of a host insect cell line for use in an *in vitro* biopesticide production process, is a population doubling time of around 24 h and a cell-specific capacity to produce at least 300 occlusion bodies per cell (Reid *et al*., 2013). At present, the lepidopteran cell lines derived from the fall armyworm *Spodoptera frugiperda* (Sf-9 and Sf-21), and the cabbage looper *Trichoplusia ni* (Tn-368 and BTI-TN-5B1-4 or High-Five), are regarded as the workhorses of the industry. These cell lines possess the aforementioned technological properties, are well-studied, and are widely use in the manufacture of heterologous gene products

using the baculovirus expression vector system. Their application in *in vitro* biopesticide production has also been well documented with respect to the AcMNPV bioinsecticide (Bonning *et al*., 1995; Vaughn, 1976; Drugmand *et al*., 2012). Sf-9, Sf-21 and High-Five cell lines have been adapted to grow in suspension culture in serum-free media, and culture conditions have been optimized for achieving high cell densities (Claus *et al*., 2012). In addition to AcMNPV, their susceptibility to infection with a range of wild-type and recombinant baculoviruses, as shown in Table 1-2, makes them suitable as hosts for the production of a spectrum of biopesticides. The use of these cell lines for biopesticide production has been demonstrated, but large-scale commercial production processes are yet to see the light of day, since technology costs still remain too high to ensure economic viability (Moscardi *et al*., 2011; Reid *et al*., 2013).

In addition, other insect cell lines have received attention for their suitability to be used as *in vitro* hosts for the production of specific narrow-spectrum viral biopesticides against damaging crop pests (Table 1-2). One of the most significant examples is the *H.zea* (HzAM1) cell line, used for the *in vitro* production of HearNPV (McIntosh and Ignoffo, 1981). HearNPV is entomopathogenic against the cotton bollworm (*Helicoverpa armigera*), the oriental tobacco budworm (*H. assulta*) and the corn earworm (*Heliothis zea*), which are polyphagous pests which attack economically important crops such as cotton, tobacco, vegetables, corn and sorghum. The *Anticarsia gemmatalis* (sa-UFL-Ag286) cell line, has also received a lot of attention for its *in vitro* capability for producing AgMNPV bioinsecticide (Sieburth and Maruniak, 1988). AgMNPV has been successfully applied for the biocontrol of the velvetbean caterpillar (*Anticarsia gemmatalis* Hübner), a devastating pest of soybean crop.

The HzAM1 and sa-UFL-Ag-286 insect cell lines have been well-characterized with respect to their growth and metabolic properties in culture, have been used to produce virus in small-scale bioreactor cultivations and are adapted to grow in low-cost serum-free culture media (Gioria *et al*., 2006; Lua *et al*., 2002; Lua *et al*., 2003; Micheloud *et al*., 2011; Nguyen *et al*., 2011). Other insect cell lines which show potential for *in vitro* biopesticide production include the Sf-9 cell line for producing SfMNPV for use against the fall armyworm pest (*Spodoptera frugiperda*) (Almeida *et al*., 2010), BM5 cell line for production of BmNPV for use against the silkworm (*Bombyx mori*) (Grace, 1967), and the IPLB-LdEIta cell line for LdMNPV production for control of the gypsy moth (*Lymantria dispar*) (Lynn *et al*., 1988). However, the technological properties of these cell

lines (adaptation to suspension culture, growth in serum-free culture media, in-depth knowledge of growth and metabolism and application to bioreactor cultivation), are not well-studied.

Table 1-2 Insect cell lines used frequently as hosts for the *in vitro* production of wild-type and recombinant virus biopesticides (adapted from Claus *et al*., 2012).

Cell Line	Insect	Tissue of Origin	*In vitro* baculovirus host range
Sf-9/ IPLB-Sf 21	*Spodoptera frugiperda*	Ovarioles	AcMNPV, AgMNPV, SfMNPV, SlNPV, TnSNPV
BTI-Tn-5B1-4	*Trichoplusia ni*	Embryos	AcMNPV, AgMNPV, TnSNPV, PxMNPV
Hz-AM1	*Heliothis zea*	Ovarioles	AcMNPV, HaNPV, HzSNPV
saUFL-Ag-286	*Anticarsia gemmatalis*	Embryos	AcMNPV, AgMNPV
Bm5	*Bombyx mori*	Ovarioles	AcMNPV, LdMNPV
IPLB-LdElta	*Lymantria dispar*	Embryos	AcMNPV, LdMNPV

It is interesting to note that the overwhelming majority of the available insect host cell lines for *in vitro* biopesticide production, were originally derived from lepidopteran insect tissue, and support baculovirus replication. This is because the majority of destructive field and forest insect pests are lepidopteran, and are susceptible to baculovirus infection. Despite Coleoptera accounting for a third of the world's insect species, the number of cell lines established from this order (22 cell lines) is scant, when compared to the large numbers of available lepidopteran cell lines. The need for development of more coleopteran cell lines has been emphasized, since these have great potential to be applied to the study of insect physiology, insecticide chemistries, and to be used as

host cell lines in *in vitro* biopesticide production against coleopteran pests (Zheng *et al*., 2014; Goodman *et al*., 2012; Long *et al*., 2002). Among the coleopteran cell lines currently available, the DSIR-HA-1179 cell line established in 1979 from surface-sterilized eggs of the black beetle *Heteronychus arator,* is an important one. In addition to having the distinction of being the first continuous coleopteran cell line established, the DSIR-HA-1179 cell line is a susceptible and permissive host for the *in vitro* replication of the *Oryctes* nudivirus. (Crawford, 1982; Crawford and Sheehan, 1985). The cell line therefore, has potential for use in an *in vitro* large-scale commercial process for the production of the OrNV biopesticide. However, despite these potential benefits, the use of the DSIR-HA-1179 cell line as a substrate for the mass production of *Oryctes* virus has not been extensively explored. At the time of its establishment in primary cultures, the cell line was grown in monolayers in 24 well tissue culture plates, and later in 25 cm^2 tissue-culture flasks (Crawford, 1982). Since then, no attempts have been made in the direction of understanding the cell line's physiology and metabolism, or its cultivation in large-volume attached culture for OrNV production. The challenge in developing a mass cultivation process for the DSIR-HA-1179 cell line is posed by intrinsic factors such as very slow cell growth (14 days to subculture), anchorage dependence, extreme susceptibility to shear stress and a tendency for cells to aggregate heavily in culture (Pushparajan *et al*., 2013). The very slow growth of the cell line is the key factor that has impeded the technical and commercial feasibility of a large scale cultivation process. No recent literature exists on the characterization of the DSIR-HA-1179 cell line. Thus, there is a need to first understand the DSIR-HA-1179 cell line in terms of its physiology, nutritional requirements and its growth in batch culture, which is one of the aims of this thesis. An account of the information on the DSIR-HA-1179 cell line, that is available in literature till date, is presented in the next sub-section.

1.3.2 The DSIR-HA-1179 insect cell line

In virology, susceptibility of a cell line to viral infection, implies that the virus is able to gain physical entry into cells through appropriate interactions with its cell surface receptors. Permissiveness of the cell line to the virus, is however, only confirmed if the virus is able to circumvent the host cell defenses, replicate within it and cause pathology (Roizman, 1996). Selection of a highly susceptible and permissive host cell line is a key requirement for *in vitro* production of insect viruses (Miltenburger *et al*., 1984). The first report of *in vitro* replication of OrNV was in primary cultures established from tissues of the coconut rhinoceros beetle. However,

these primary cultures failed to establish as a continuous cell line (Quiot, 1971; Quiot *et al*., 1973). The only continuous cell line that has proved susceptible and permissive to OrNV infection, is the DSIR-HA-1179 coleopteran cell line, established from primary cultures of the black beetle, *Heteronychus arator* (Crawford, 1981; Crawford, 1982). Further studies on the permissivity of the cell line to infection by other virus genera, showed that while the cell line was not susceptible to infection by AcMNPV (McIntosh *et al*., 2005), two scarab nodaviruses namely the black beetle virus and the flock house virus, were able to infect and replicate within its cells Crawford *et al*., 1984). Morphologically, DSIR-HA-1179 cells are spindle shaped, epithelial-like cells. Cells have been estimated to be, on average, 25-40 μm long and 15-20 μm wide. At the time of its establishment, the population doubling time of the cell line grown in Schneiders Drosophila medium in 24 well plates was estimated at 6 days, which was appreciably slower than population doubling times for lepidopteran and dipteran cell lines (Crawford, 1982; Hink, 1976). Analyses of the cell line's karyotype, at eighteen and thirty six months after its establishment, revealed that it was a highly unstable polyploid. Chromosome morphology was distinguished by a majority of metacentric chromosomes, as well as a few aberrations in the form of ring, double minute chromosomes, and di and tri-centric chromosomes (Crawford *et al*., 1983). The range of variation in chromosome numbers per cell increased greatly between the 18th and 36th month of the cell line in culture, as can be observed in Fig. 1-4. Therefore, it was concluded that karyotyping was not a suitable method for confirming cell line identity, and a recommendation was made for isozymes to be used for cell identification.

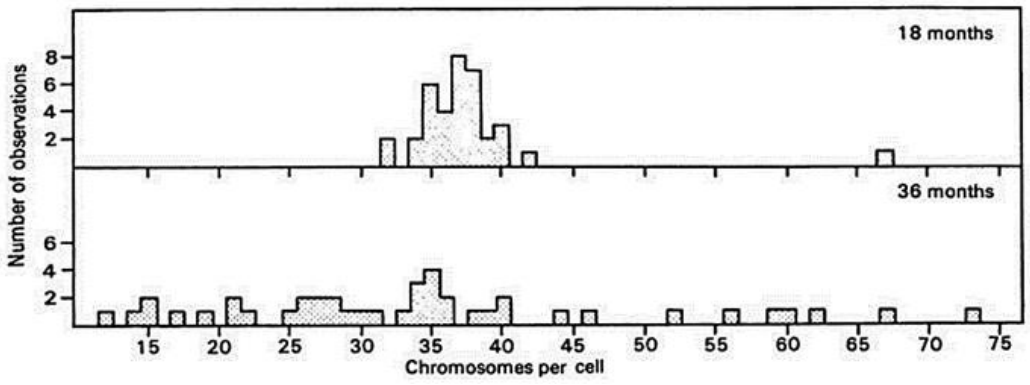

Fig 1-4 Histogram showing the variation in chromosome number in DSIR-HA-1179 cells at 18 and 36 months after the establishment of the primary cell culture (reproduced from Crawford *et al*., 1983).

Attempts at cultivating the cell line in suspension cultures have been unsuccessful till date, mainly due to the sensitivity of the cells to mechanical stress, as well as their tendency to form tight clumps when detached from a growth surface (Marshall *et al*., 2010). These reasons have precluded the development of a reliable method for dissociating cells from strongly adherent monolayers and the creation of single-cell suspensions which can be reliably enumerated using standard cell counting methods. An early study noted that considerable difficulties were faced in dissociating the attached cell monolayer, in so far as it took a combination of treatment with 0.05% trypsin in a 0.01 M EDTA/PBS mix for one hour at 37 ˚C, and sharp tapping of the flask surface, to dislodge between 80-90% of the attached cells. When subsequently inoculated in to new cultures, 50% of these cells failed to grow (Crawford, 1982). Collagenase treatment was found to be less effective than trypsin for dissociating cells (Crawford, 1982). The cell line was reported to have been cryopreserved in 10% dimethyl sulfoxide and revived successfully in an early report (Crawford, 1982), however, later reports have suggested the unreliability of cryopreserving these cells, and therefore, the cell line has been continually propagated in cell culture over the last three decades (Marshall *et al*., 2010).

DSIR-HA-1179 remains the sole available host cell line for the *in vitro* propagation of the OrNV biopesticide. Attempts to propagate the virus in other cell lines such as *Spodoptera frugiperda* and *Aedes albopictus* have been unsuccesful (Crawford, 1981). Since the virus does not produce plaques with DSIR-HA-1179 cells, end-point dilution assays were used to determine the infectious titer ($TCID_{50}$) of *in vitro* produced virus (Reed and Muench, 1938; Crawford and Granados, 1982). It was found that virus that was produced *in vitro*, at a titer of 10^7 $TCID_{50}$/ml and stored at 4˚C, showed no reduction in infectivity for up to a year. At 28˚C, which is likely to be the temperature in the field; the half-life of the virus was approximately two weeks (Crawford and Sheehan, 1984). The improved efficacy of *in vitro* produced OrNV inoculum over crude virus suspensions prepared from diseased insect larvae was largely due to the aseptic quality of the viral product, and its storage in sterile, lightproof containers until field application, thereby preventing degradation by microbial contamination or UV radiation. Furthermore, cell culture derived inocula could be easily formulated with sucrose, without affecting its shelf-life (Crawford and Sheehan, 1984); since beetles will more readily imbibe sugar-virus solution during peroral infection (Marschall and Ioane, 1982).

1.3.3 *In vitro* pathogenesis of OrNV in DSIR-HA-1179 cells

Fig. 1-5 is a schematic representation of the process by which OrNV infection occurs in DSIR-HA-1179 cells, according to Crawford and Sheehan (Crawford and Sheehan, 1985). Virus entry into the cells was found to occur via pinocytosis, a form of endocytosis in which virus particles are brought into the cell by forming narrow channels through its membrane that pinch off into vesicles, that subsequently fuse with lysosomes to subsequently hydrolyze or break down the particles (Wang and Jehle, 2009). These pinocytotic vesicles containing virus, were observed in the cytoplasm between 1 and 4 hours post-infection. The mechanism by which nucleocapsids are released from the viral envelope and into the cytoplasm, as well as how they are transported to the nucleus, is unknown. Quiot *et al*., have suggested that the nucleocapsids uncoat at the nuclear pore, followed by the release of the virus into the nucleoplasm; however, the hypothesis is yet to be confirmed (Quiot *et al*., 1973).

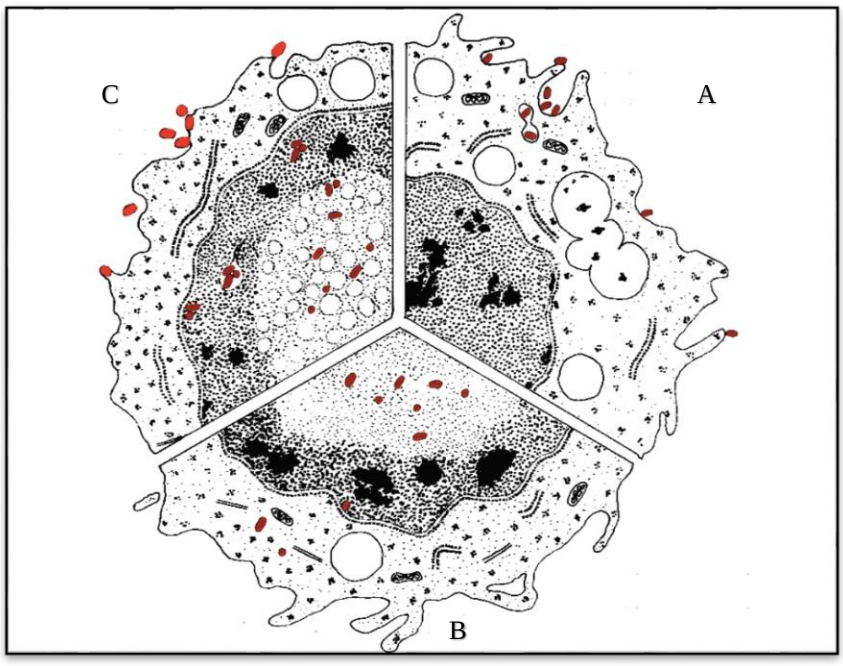

Fig. 1-5 Schematic representation of morphological changes in DSIR-HA-1179 cells post-infection with OrNV. (Quadrant A) 1 – 4 h post-infection: virions (shown in dark red) adsorb to the plasma membrane and are taken up in cytoplasmic vesicles (Quadrant B) 7 – 12 h post-infection: viral replication occurs in the clear area of the hypertrophied nucleus (Quadrant C) 16 h post-infection and beyond: newly synthesized progeny virus (shown in bright red), bud from the plasma membrane, along with the characteristic accumulation of viral envelope material in the nucleus. Adapted from Crawford and Sheehan, 1985.

The first sign of virus replication occurred 7 hours post-infection. At the nuclear level, signs of virus replication included nuclear hypertrophy, the migration of nuclear chromatin, and deposition of virus vesicular envelope material (around 160 nm in length) which formed virogenic stroma and filled up the chromatin-free clear nuclear spaces of the cell. The cells themselves increased in size upon infection and appeared spherical. Following virus replication in the nucleus, virions entered the cytoplasm, followed by budding from the cell plasma membrane. Virus budding from the cell plasma membrane began at 12 hours post-infection; peaked at 16 hours post-infection and continued beyond 36 hours post-infection. Three important dissimilarities between OrNV and baculoviruses were observed with regard to virus replication. Firstly, intracellular OrNV nucleocapsids were enveloped in a single membrane, unlike baculoviruses whose naked nucleocapsids acquire an envelope only upon budding from the cell. It was postulated that the OrNV envelope and nucleocapsid shell assemble first, followed by the insertion of double-stranded virus DNA into the core (Burand, 2008). Secondly, OrNV virions acquired a second unit membrane by budding from the cell plasma membrane. There was no difference in infectivity between extracellular double-enveloped budded OrNV particles and the single-enveloped particles found within the cell. Finally, there was the conspicuous absence of 'late' protein synthesis, as a consequence of lack of expression of the polh gene in OrNV infections of DSIR-HA-1179 cells. The non-expression of polyhedron coding genes, results in the non-production of occlusion bodies, and therefore, only one viral phenotype, i.e. the budded virus, is produced in OrNV infections. The lack of occlusion body formation is a key biological feature which distinguishes nudiviruses from baculoviruses, and which may also contribute to the increased susceptibility of OrNV to chemical and biochemical degradation in the environment (Crawford and Sheehan, 1985). Compared to baculoviruses, for which a wealth of information exists on their interaction with host cells at the molecular level; *in vitro pathogenesis* of nudiviruses are poorly studied, in general, and a better understanding of the molecular mechanism of OrNV trafficking and dynamics of infection, as well as elucidation of the gene pathways involved in the DSIR-HA-1179 host cell response to infection, is required.

1.3.4 Insect cell metabolism

Knowledge of nutrient requirements and metabolism of a cell line is necessary in order to optimize bioreactor cultures with respect to the design of low-cost culture media, nutrient feed, metabolite removal, pH and dissolved oxygen concentrations (Drugmand *et al*., 2011). This need becomes more pronounced when the end goal is virus production, since infected cells may have altered rates of nutrient consumption, metabolite production and oxygen uptake. Hence, for a particular virus-host cell system, there is a need to evaluate both uninfected and infected cell metabolism (Monteiro *et al*., 2012). The majority of studies have focused on the metabolism of the two most industrially important insect cell lines - Sf-9 and High-Five (Bedard *et al*., 1993; Benslimane *et al*., 2005; Drews *et al*., 1995; Drews *et al*. 2000; Donaldson and Shuler, 1998; Ferrance *et al*., 1993; Reuveny *et al*., 1993; Rhiel *et al*., 1997; Sugiura and Amann, 1996; Wang *et al*., 1993a). There are additional growing reports on the metabolism of other insect cell lines including UFL-Ag-286 (Gioria *et al*., 2006), Schneider S2 (Bovo *et al*., 2008), Bm-5 (Stavroulakis *et al*., 1991) and BCIRL-HZ-AM1 (Lua and Reid, 2003; Chakraborty *et al*., 1995). The metabolic nature of the DSIR-HA-1179 cell line has remained largely unstudied till date.

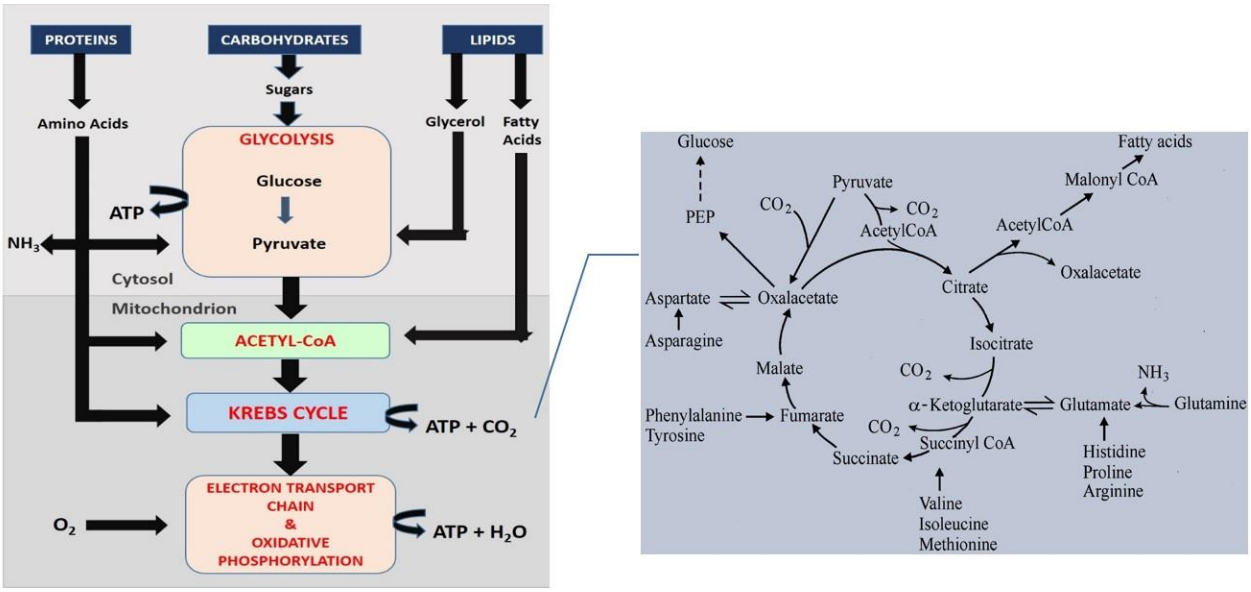

Fig. 1-6 Pathways for energy generation in animal cells, showing details of the cataplerotic and anaplerotic pathways in the Krebs cycle.

Insect cells require to be fuelled by energy supplied in the form of molecules of adenosine triphosphate (ATP), in order to grow and maintain viability in culture. ATP molecules are generated through the catabolism of substrates such as carbohydrates, proteins and lipids which are provided in the culture medium. Following cellular uptake, carbohydrates, proteins and lipids are broken down into their respective monomers namely six-carbon sugars, amino acids, glycerol and fatty acids (Fig 1-6). Glucose, the most common component of culture media, can be utilized as a primary energy source for most animal cells, including insect cells (Bhatia et al., 1997; Drews *et al.*, 1995). The generation of ATP from glucose molecules, occurs in the cytosol via a stepwise enzyme-catalyzed process called glycolysis. In this process, one six-carbon molecule of glucose is converted to two three-carbon molecules of pyruvate, with the net yield of 2 ATP molecules. Under fully aerobic conditions, two molecules of pyruvate are converted to two molecules of acetyl-coenzyme-A (acetyl-CoA) with the generation of CO_2 and the reducing equivalent of ATP - nicotinamide adenine dinucleotide (NADH). Within the mitochondrion, Acetyl-CoA feeds into the Krebs cycle (also known as the tricarboxylic acid cycle). The Krebs cycle consists of a series of oxidation reactions, converting citrate to oxaloacetate, in which molecules of CO_2, ATP, $FADH_2$ and NADH are produced. The net energy yield from the decomposition of two molecules of Acetyl-CoA is 2 ATPs. The Krebs cycle in insect cells is known to be fully functional (Bhatia *et al.*, 1997). $FADH_2$ and NADH molecules produced in the Krebs cycle, are further oxidized to release energy within the mitochondrial membrane, via the electron transport chain. An electric and pH potential is created by the shuttle of protons from NADH and $FADH_2$ to membrane bound electron-carrier molecules and onto other electron carriers, until final oxidation results in the production of water. The electrochemical gradient caused by the proton flux across the intermembrane space drives ATP synthetase to generate ATP molecules from ADP. This process, called oxidative phosphorylation, yields 34 ATP molecules per molecule of glucose (Stryer, 1995).

Studies across different insect cell lines have identified glucose as the preferred carbohydrate for insect cells. The lack of glucose retards growth of Sf-9 and High Five cells and triggers apoptosis in Sf-9 cells. The maximum specific growth rate in glucose deficient cultures is 25% lower than their glucose fed counterparts. In the absence of glucose, High Five cells back-consume the lactate they produce to remain viable (Drugmand *et al.*, 2012). On the other hand, excessive amounts of glucose can lead to an accumulation of lactate or alanine (Bedard *et al.*, 1993; Ohman *et al.*, 1995). In addition to glucose, Sf-9 and High Five cells can consume fructose and maltose, once these

sugars have been hydrolyzed outside the cell (Bedard *et al*., 1993; Palomares *et al*., 1996; Reuveny *et al*., 1992). Fructose is consumed only under conditions of glucose limitation and with reduced rates of uptake and by-product formation (Bedard *et al*., 1993; Mendonca *et al*., 1999). Sucrose, which is added to adjust medium osmolarity is consumed by Sf-9 cells at very low levels during the growth phase (Bernal *et al*., 2009), but at an enhanced rate upon baculovirus infection (Bernal *et al*., 2009, Benslimane *et al*., 2005; Sugiura and Amann, 1996). Patterns of carbohydrate consumption can vary between insect cell lines. Bm-5 cells actively consume sucrose during growth (Stavroulakis *et al*., 1991), while Aedes albopictus C6/36 cells consume sucrose only under conditions of glucose limitation (Clements and Grace, 1967; Stockdale and Gardiner, 1975). High Five and H.zea cells neither consume sucrose during growth nor infection (Lua and Reid, 2003; Rhiel *et al*., 1977). H.zea cells actively consume maltose even in the presence of high glucose concentrations, while its consumption by Bm-5 cells is less substantial (Lua and Reid, 2003; Stavroulakis *et al.*, 1991). With the exception of AA cells, the majority of insect cell lines will not consume trehalose when other carbon sources are present (Clements and Grace, 1967; Vaughn, 1973).

Amino acids fulfill the nitrogen requirements of cells as the building blocks for cell proteins. Additionally they are used to generate cellular energy and serve as precursors for lipids, signaling molecules and nucleic acids. Concerning amino acid metabolism, insect cells prefer to consume amino acids from the medium rather than synthesize their own (Bhatia *et al*., 1997; Doverskog *et al*., 1997; Ferrance *et al*., 1993). Glutamine is the most important amino acid for insect cells (Bedard *et al*., 1993; Ferrance *et al*., 1993; Reuveny *et al*., 1992). ATP is produced in a process initiated by the deamination of glutamine to glutamate, catalyzed by glutaminase; and the subsequent conversion of glutamate to α-ketoglutarate under the action of glutamate dehydrogenase. α-ketoglutarate feeds into the Krebs cycle and its oxidation can yield between 9 and 27 ATP molecules. The pyruvate formed can be converted to lactate or alanine, or be completely oxidized to CO_2 in an efficient TCA cycle. While most insect cell lines require glutamine to be provided in the culture medium, the Sf-9 and Sf-21 insect cell lines are able to grow in the absence of glutamine provided ammonium ions are provided in the medium, since they possess the glutamine synthetase gene, which allows them to synthesize endogenous glutamine (Drews *et al*., 2000; Ohman *et al*., 1995).

The main metabolites produced by insect cell lines include lactate, ammonia and alanine. Lactate can form as a result of anaerobic conditions in the cellular environment. Sf-9 cells do not produce lactate under sufficiently oxygenated conditions and grown in a medium with high glucose content of > 40 mM. On the other hand, High Five cells have been found to be high producers of lactate, accumulating up to 10-20 mM in culture (Sugiura and Amann, 1996; Yang *et al*., 1996). Ammonia and alanine, are produced from the catabolism of amino acids in insect cell lines, with the propensity to produce either depending on the specific metabolic pathway used by the cell line. Alanine and ammonia are not related to growth inhibition of Sf9 cell lines (Doverskog *et al*., 1997) and the reduced yield in insect cell cultures at high cell densities is most likely caused by nutrient depletion rather than inhibition by waste products (Drews *et al*., 1995).Ethanol, another by-product of glucose metabolism, is produced by certain insect cells to amounts ranging between 6 – 11.5 mM (Drews *et al*., 2000; Takahashi *et al*., 1995)

1.4 Physico-chemical factors affecting cell growth in insect cell cultures

1.4.1 Culture medium

The culture medium is an important component of insect cell culture based production processes for biopesticides or recombinant proteins. A complete growth medium consists of a chemically defined basal medium, which is supplemented with either vertebrate serum or in the case of serum free formulations, with protein hydrolysates and a lipid emulsion (Reid *et al*., 2013). In order to support proliferation, maintenance and viral replication in insect cells, the culture medium must be able to meet the nutritional demands of the cell line, both before and after infection (Gioria *et al*., 2006). Historically, the impetus for formulating new culture media came from the recognition of the fact that nutrition was central to long-term passaging of insect cell lines, as well as boosting their growth and productivity in culture. The first synthetic insect cell culture medium, Wyatt's medium, was developed based on the chemical composition of insect hemolymph. The basal medium contained sugars, inorganic salts, vitamins, amino acids, and organic acids, and was supplemented with heat treated insect hemolymph to provide essential growth-promoting substrates (Wyatt, 1956). The basal medium formulation was further improved in Grace's medium through the addition of B-complex vitamins (Grace, 1962). Replacement of insect hemolymph with a mixture of lactalbumin, yeast extract and fetal calf serum was first attempted in 1970, with the

development of the TNM-FH medium (Hink, 1970). Thereafter, fetal bovine serum which was cheaper and more easily available than hemolymph, became the preferred additive and was used in concentrations ranging between 5-20% depending on the cell line (Schlaeger, 1996). In 1975, the BML-TC/10 basal medium (now known as TC-100), was designed specifically to support AcMNPV production in *Spodoptera frugiperda* cells. This medium was formulated with glucose as a sole carbon source, the addition of tryptose phosphate broth and omission of organic acids (Gardiner and Stockdale, 1975). In 1981, a more complex basal medium called IPL-41 was developed which contained higher concentrations of amino acids, organic acids and vitamins than Grace's medium (Weiss *et al*., 1981). TNM-FH, TC-100 and IPL-41 media have all been used for the large-scale culture of cell lines from various insect orders. Basal media have also been specifically tailored for certain insect cell lines. For example, Schneider's drosophila medium and Shields and Sang's M3 medium were developed for *Drosophila melanogaster* cell lines (Schneider, 1964, Shields and Sang, 1977), while MM medium and Kitamura's medium were developed for culturing mosquito cell lines (Kitamura, 1965; Mitshuhashi and Maramarosch, 1964).

Supplementation of basal media with serum is vital for supplying cells with growth promoting elements including hormones, adhesion factors, transport proteins, fatty acids, lipids, sterols, minerals and protease inhibitors. Serum confers cells protection against toxic compounds and mechanical forces. However, the drawbacks of using serum include its high cost, lot-to-lot variability, inconsistent supply, the risk of contamination with adventitious agents such as viruses and prions, and its high protein content which could interfere with downstream processing (Barnes and Sato, 1980; Maiorella *et al*., 1988). The economic success of *in vitro* production of low margin products such as biopesticides at scales exceeding 1000 liters, hinges on the cost of culture medium being kept as low as $ 2-5 per liter (Godwin *et al*., 1991). The high cost of serum poses the biggest hindrance to achieving this goal. Therefore, research efforts have focused on substituting serum with a combination of low cost supplements such as protein hydrolysates, natural extracts and lipid microemulsion, which together confer the physiological benefits of serum. Hydrolysates are rich mixtures of free amino acids, vitamins, growth factors or oligopeptides derived from acidic or enzymatic digestion of substrates such as milk, meat or soy proteins. Examples of hydrolysates include lactalbumin and casein hydrolysate, whey ultrafiltrate, Primatone RL peptones, soy hydrolysate and tryptose. In general, plant or microbial based extracts are preferred over those of animal origin, which have the potential to be contaminated with animal viruses, mycoplasma or

prions (Kwon *et al*., 2005). Yeast autolysate (known as yeast extract or yeastolate) is the most widely used supplement which has proven efficacy in modifying the specific growth rate and maximum cell density reached in insect cell cultures (Donaldson and Shuler, 1988; Ikonomou *et al*., 2001; Marteijn *et al*., 2003). The role of the lipid micro-emulsion is to provide insect cells with hydrophobic nutrients such as lipids and sterols. Inclusion of Pluronic F-86 in the formulation of the lipid micro-emulsion is beneficial, because Pluronic F-86 mimics the shear-protective effects of serum. In early work, emulsions of egg yolk were used to provide lipids in serum-free culture. Later, microemulsions consisting of fatty acid methyl esters of cod liver oil, Tween-80, cholesterol, tocopherol acetate and Pluronic F-86 were developed. More recently, soybean oil, a cheaper and more abundant source of lipids, has been used to replace fish oil in the UNL-10 insect cell culture medium, which has been developed specifically for AgMNPV production in the saUFL-Ag-286 insect cell line (Micheloud *et al*., 2009).

The first serum-free medium IPL-SFM, was developed for lepidopteran cell lines (Inlow *et al*., 1989), and was followed by improved serum-free commercial media formulations including EX-CELL® 401 (Sigma Aldrich) and Sf-900™ II (Life Technologies). Further improvements were made to Sf-900™ II in developing the Sf-900™ III culture medium (Life Technologies) with reduced hydrolysate content. However, a limitation to the use of commercial serum-free culture media in large-scale biopesticide production processes is their high cost. An alternative strategy would be to formulate low-cost serum-free media (LC-SFM) in which most of the expensive purified amino acids are replaced by specific combinations of protein hydrolysates. Examples of LC-SFM which have been developed include ISYL SFM, YPR SFM and SF-1 for culture of Sf-9 and High Five cells (Donaldson and Shuler, 1988; Ikonomou *et al*., 2001; Schlaeger, 1996), VPM3 SFM for HearNPV production in H.zea cells (Huynh *et al*., 2012; Reid and Lua, 2005) and UNL-10 for AgMNPV production in saUFL-Ag286 cells (Micheloud *et al*., 2009). Since protein hydrolysates from different sources are likely to have divergent amino acid profiles, and coupled with the fact that nutritional requirements differ between insect cell lines, the rational formulation of an LC-SFM must be aided by a thorough understanding of the specific cell lines' nutrient requirements and metabolism, and involve the screening of various hydrolysate combinations for one which allows optimal growth and productivity. While LC-SFM's are cost-effective for large scale biopesticide production, its formulation with a large number of hydrolysates with undefined components introduces lot-to-lot variability within the culture medium. This could negatively

impact cell growth and infection and ultimately affect product quality between production runs. The problems associated with hydrolysates may be addressed by the development of an insect cell chemically defined medium (CDM), which are preferably animal component-free and protein-free, similar to the commercial CDM formulations which are widely available for mammalian cell lines (Reid *et al*., 2013).

With regard to culture medium development for the DSIR-HA-1179 cell line, research is at a very nascent stage. The cell line was cultured in Schneiders drosophila medium, supplemented with 10% fetal bovine serum, at the time of its establishment. Later, it was maintained in 10% serum-supplemented PS-100 culture medium for well over 25 years. PS-100 was a modified version of Grace's medium, made to mimic TC-100 (formulation described in Chapter 2, sub-section 2.2.1). Since the introduction of the cell line into our lab in 2010, the cell line was adapted to grow in TC-100, IPL-41, Sf-900 II™ and Sf-900 III™ culture media, respectively supplemented with 10% FBS. The screening of DSIR-HA-1179 cell growth and OrNV production in this range of culture media is presented in Chapter 2 of this thesis. While adaptation to these new media have improved cell yields at the lab scale, it is well recognized that the inclusion of serum renders the process unfeasible for large-scale production. The future outlook is that an LC-SFM must be tailored specific to the nutritional needs of the DSIR-HA-1179 cell line for OrNV production, in a similar vein to the work undertaken in formulating VPM3 culture medium for HearNPV production in H.zea cells, and UNL-10 for AgMNPV production in sa-UFLAg286 cells.

1.4.2 Temperature

Most insect cell lines are cultivated over a temperature range of 25-30 ºC, with the optimal range being 27-28 ºC (Agathos *et al*. 1990). The effects of temperature on cell growth and productivity have been most studied for the Sf-9 cell line. An incremental relationship between temperature and cell growth, glucose and oxygen consumption rates was observed when Sf-9 cells were cultivated between 22 and 30 ºC. At an optimal temperature of 27 ºC, the highest specific growth rate of 0.67/day was achieved, which was 30% higher than when the same cells were cultivated at 25 ºC. Increasing the temperature to 30 ºC led to an initial increase in the specific growth rate and a dramatic decline in cell viability upon the culture reaching its maximum density. A further temperature increase to 35 ºC led to decreased rates of cell growth, glucose and oxygen

consumption (Reuveny *et al*. 1993a). However, in a later study, the thermo-tolerance of Sf-9 cells to a temperature as high as 37 ºC has been demonstrated as a consequence of long-term passaging (Gerbal *et al*. 2000).

A number of studies have demonstrated the effects of temperature on the productivity of infected insect cells. It was found that increasing the temperature from 22 to 27 ºC in infected Sf-9 cell cultures expressing recombinant β-galactosidase led to earlier infection of cells and consequently, earlier product expression (Reuveny *et al*. 1993a, Reuveny *et al*. 1993b). Oscillating the temperature between 24 and 28 ºC led to prolonged uninfected and infected cell growth and an increase in virus production (Shao-Hua *et al*. 1998). In infected Bm-5 cells, increasing the temperature from 25 to 31 ºC led to earlier expression of recombinant chloramphenicol acetyltransferase, but total yield declined at temperatures above 28 ºC. Therefore an infection temperature of 25 ºC was deemed optimal for the Bm-5 cell line (Andersen *et al*. 1996). On the other hand it was found that lowering the temperature from 28 to 24 and even 20 ºC in infected High-Five cell cultures, improved the glycosylation of recombinant proteins produced (Donaldson *et al*. 1999). Thus, the temperature used for the cell growth and virus infection phases must be optimized for individual production systems. The DSIR-HA-1179 cell line has been cultivated at 27 ºC, during both uninfected and OrNV-infected growth (Crawford, 1982, Pushparajan *et al*. 2013), but no further study on the effect of varying temperatures on cell growth and OrNV production have been done to date.

1.4.3 pH and osmolarity

Insect cells are usually cultured at a pH range of 6.0 to 6.8 (Sohi 1980). Unlike mammalian cells, insect cells rely on a phosphate buffer system and do not require bicarbonate/CO_2 buffering to maintain an optimal pH. The optimal pH range for lepidopteran cell lines is 6.2 to 6.4 which is lower than those for mammalian cells (Agathos *et al*. 1996). A pH 6.2 to 6.4 is optimal for Sf-9 cells, 6.2 to 6.3 for High Five cells and 6.1 to 6.3 for Bm-5 cells (Drugmand *et al*. 2005, Hensler *et al*. 1994, Zhang *et al*. 1994a). Monitoring of pH is important especially in large-scale insect cell cultures since a shift towards a more acidic or basic pH may occur monotonically as a result of exhaustion of nutrients in culture medium and the excretion of metabolites by the cells. For example, pH drifts to 6.05 and 5.90 were observed when Sf9 cultures were grown in stirred tank

bioreactors in serum-containing TNM-FH medium and serum-free EXCELL®-401 medium, respectively; which occurred presumably due to lactate accumulation (Hensler *et al*. 1994). For Bm-5, an increase in pH from the optimal value of 6.3 to 6.4 led to a 50% reduction in recombinant CAT protein yield per 10^6 cells (Zhang *et al*. 1994a). Hence from an industrial perspective, maintaining an optimal pH is important to ensure productivity in both growth and infection phases. The effect of culture medium osmolality on insect cell growth is another parameter to be considered, though it has been found that insect cells are more tolerant to variations in osmotic pressure compared to mammalian cells. The osmolality of insect cell culture media range between 320-375 mOsm/kg (Agathos *et al*. 1990). In Bm-5 cells cultivated under well-controlled bioreactor conditions, the maximum specific growth rate was reached at 370 mOsm/kg, however it was found that varying the osmolality between 350-385 mOsm/kg did not alter the specific growth rate by more than 10% (Zhang *et al*. 1994a).

1.4.4 Dissolved Oxygen

Insect cells are strict aerobes with a high TCA activity. They require the adequate provision of oxygen in order to grow to high cell densities, and also to support their heightened metabolism during viral infection (Drugmand *et al*., 2012). Studies have shown that the percentage of dissolved oxygen in the culture medium can affect the cell specific growth rate in uninfected cultures, as well as recombinant protein production in infected cultures (Gotoh *et al*., 2004; Jain *et al*., 1991). Oxygen can be supplied to insect cells growing in bioreactors by surface aeration, membrane aeration or sparged aeration. With surface aeration, oxygen transfer occurs through its diffusion into the culture medium from the headspace of the culture. This form of oxygenation is only efficient in small scale systems such as T-flasks, roller bottles, shake flasks or spinner flasks. In membrane aeration, air or oxygen is introduced into the culture through permeable silicone tubes, and is also applicable only to small scale systems (Marks, 2003). Sparging is the most effective way to oxygenate large-scale bioreactors (Narang *et al*., 2013). Oxygen consumption is represented by the oxygen uptake rate, measured in mol/cell/s. For efficient growth, oxygen transfer rate in the bioreactor must match the oxygen uptake rate of the cells. The typical oxygen consumption rate for Sf-9 cells was found to vary between 4.3×10^{-17} mol/cell/s (Maoirella *et al*., 1988), 5.6×10^{-17} mol/cell/s (Kamen *et al*., 1991) and 1.2×10^{-16} mol/cell/s (Reuveny *et al*., 1993), in response to differing cultivation conditions. The type of culture medium, serum concentration in the culture medium, and baculovirus infection can all affect the oxygen uptake rate. Metabolic requirements

for growth and virus production differ between different insect cell lines, and consequentially, some cell lines have higher oxygen consumption rates than others. For example, typical specific consumption rates in High Five cells (5-16 x 10^{-17} mol/cell/s) are higher than for Sf-9 cells (2-5 x 10^{-17} mol/cell/s). Furthermore, it has been found that virally infected High Five cells consume oxygen at a specific rate that is 2 to 5 times higher than that of virally infected Sf-9 cells. High Five cells are larger than Sf-9 cells, and this size difference largely accounts for the higher oxygen uptake rate of High Five cells (Rhiel *et al*., 1997). A peak in the oxygen uptake rate was found to occur in the period immediately following viral infection, in both Sf-9 and High Five cell lines (Wong *et al*., 1994). Measurement of dissolved oxygen levels in infected Sf-21 roller bottle cultures also showed that dissolved oxygen levels increased by 25% in comparison with non-infected cultures, indicating an increased uptake rate during infection (Weiss *et al*., 1982). Dissolved oxygen values used in insect cell cultivation are generally between 10 and 30% of air saturation in airlift bioreactors, and between 40-60% in stirred tank reactors (Schmid, 1996). The flowrate of oxygen into the sparger is an important parameter to monitor and control in large-scale insect bioreactors, since the provision of too high levels can cause oxidative damage to cells, while on the other hand, too low levels may impair cell and virus yields.

1.4.5 Hydrodynamic shear

Insect cells are susceptible to damage resulting from hydrodynamic stresses (greater than 0.59 N/m^2) generated during the operation of bioreactors. Damage caused to insect cells during bioreactor cultivation is a function of the type, duration and magnitude of the hydrodynamic forces induced as a result of impeller agitation or sparging (Chalmers, 1996). Insect cells are susceptible to energy dissipation rates above 2.25 x 10^4 W/m^3, which is higher than that typically generated in stirred tank bioreactors (3 – 3.5 x 10^3) W/m^3 and bubble columns (0.2 -2 x 10^3 W/m^3) (Palomares *et al*., 2006). While exposure to too high levels of hydrodynamic stress can result in cellular necrosis, moderate levels of stress can induce physiological changes in cells, which lead them to apoptotic cell death (Chisti, 2000). With regard to the DSIR-HA-1179 cell line, no published information on its shear sensitivity is available, since until the time of this research, the cell line was only cultivated in static T-flasks. Though systematic investigations have not been performed, a general observation can be made on the susceptibility of the cell line to mechanical stresses based on the behavior of cells when subjected to strong pippeting forces. It has been observed that cells, when exposed to strong pipetting forces, have a tendency to aggregate in culture, and eventually

lose their viability if the strong mechanical force is continually applied. These observations highlight the relative sensitivity of the cell line to mechanical stresses, and therefore, it may be advantageous to operate spinner flask cultures of these cells at low to moderate stirring speeds. In larger scale bioreactors such as airlift bioreactors, interactions of entrained air bubbles at the gas-liquid interface, including bubble rupture at these interfaces, can cause cell damage. The addition of a shear protectant such as Pluronic F-68 or Methocel E-50 to the culture medium has been recommended in order to alleviate this problem in sparged bioreactors by reducing cell-bubble adhesion (Kunas and Papoutsakis, 1990; Michaels *et al*., 1995).

1.5 Bioreactors

The commercial exploitation of insect cell culture for the production of viral biopesticides requires the growth and infection of host insect cell lines in large-volume bioreactors, in a low cost process (Reid *et al*., 2013). Bioreactor technologies used for the *in vitro* propagation of insect cells are those originally developed for mammalian cell culture, which in turn were adapted from technologies based for microbial cell culture (Agathos, 1991). Bioreactors can be distinguished on the basis of culture mode (batch, fed-batch or continuous perfusion) and bioreactor configuration (suspension or attached culture systems); and on their configuration (suspension or attached culture systems).

1.5.1 Culture mode

Batch culture is the simplest mode of bioprocessing which involves the inoculation of cells into a fixed volume of culture medium in the reactor to achieve the desired seeding cell density. No further additions of culture medium or feeds are made to the reactor, hence, the volume of the reactor remains constant, except for periodic sampling in experiments where cell growth is monitored. Nutrients, temperature, pressure, aeration and other culture parameters are controlled at set levels. Cells can utilize nutrients in the culture medium to grow for a limited number of population doublings, and while nutrient concentrations gradually decrease in culture, the concentration of metabolites gradually increase (Butler, 1996). The potential toxicity of metabolites such as lactate and ammonia as they accumulate to high concentrations in culture, coupled with the depletion of key nutrients such as glucose and glutamine, contribute to a

progressively deteriorating environment for cell growth over time. Therefore, the maximum cell density which can be reached in batch culture is dictated by nutrient limitation, metabolites concentration, and in the case of anchorage-dependent cells which are contact inhibited, growth to confluence on the available growth surface area. Indiscriminate increase of nutrient levels in batch culture is not a viable strategy for surmounting yield limitations, since this leads to the formation of precipitates, as well as increase in osmolarity to high levels, which cells may not be able to tolerate.

In fed-batch culture, controlled nutrient feeding is performed over the culture period, either by partial medium changes at regular intervals, or the addition of specific nutrients at fixed time-points during the culture. Culture longevity, cell yield and overall volumetric productivity may be improved with a fed-batch approach. Fed-batch approaches have been applied to boost productivity of insect cell culture systems. For example, by using a single-pulse feed addition consisiting of glucose, tyrosine, vitamins, iron and trace metal solution, Bedard *et al*. (1994), found that Sf9 cell densities could be improved to 3 x 10^7 cells/ml. Further increase in cell density (52 x 10^6 cells/ml), was obtained by Elias *et al*., by using continuous feed addition (Elias *et al*., 2000). The continuous mode of culture is operated with continuously incoming feed or fresh medium into the bioreactor, and the removal of spent culture medium. This allows cell growth to proceed for longer periods than in batch and fed-batch culture. Continuous culture may be operated in chemostat or perfusion modes. Chemostat culture in unit bioreactors has not been widely used for the production of viruses in insect cells, since the continuous withdrawal of culture causes the dilution of cells and virus. Instead, a continuously operating cascade of reactors was used, with the first for cell growth and the second for infection (Zhang *et al*., 1993). The method was however, disadvantaged by the accumulation of defective interfering particles (van Lier *et al*., 1996). In perfusion mode, the culture is initiated as a batch culture, but nutrient feeding occurs continuously so that the inlet and outlet medium flow rates are equal, and the culture volume is kept constant. A cell retention device (such as centrifugation, internal and external spin filters, and hollow fiber modules) is present at the outlet stream in continuously perfused cultures, which allows high cell densities to build up in culture (Adams *et al*., 2011; Stiens *et al*., 2000). Insect cells have been cultivated to high cell densities in perfusion mode, for example, Sf-9 cells to a density of 3 x 10^7 cells/ml (Cavegn and Bernard, 1992), Sf-21 cells to 5.5 x 10^7 cells/ml (Deutschmann and Jäger, 1994) and High-Five cells to 15.5 x 10^6 cells/ml (Ikonomou *et al*., 2001).

1.5.2 Suspension growth systems

A major objective of the production process is to maximize volumetric productivity (i.e. the amount of product per unit volume per unit time of reactor), while assuring product quality. Volumetric productivity is influenced by the bioreactor configuration, its mode of operation and culture conditions. Improvements in volumetric yield (i.e. amount of product per unit time), may be achieved through increasing the cell density, increasing the cell-specific virus yield, or both (Rodrigues *et al*., 2010). Efficient scale up should aim for large volumes with high cell densities, with a high cell-specific productivity (Contreras-Gómez *et al*., 2013; Palomares *et al*., 2006). While, for recombinant protein production, the efficiency of downstream processing is crucial for obtaining high yields, this is not an issue in the production of viral biopesticides, since the virus recovered from the clarified culture broth is directly formulated without further purification (Moscardi *et al*., 2011).

One of the factors crucial to the commercialization of *in vitro* produced biopesticides, is the economies of scale achieved in large-scale suspension culture. Studies on the economic feasibility of *in vitro* production of baculovirus biopesticides have indicated the necessity of using large bioreactor scales, for example, of 10,000 l for HearNPV production, and 20,000 - 50,000 l for AfMNPV production, based on currently achieved volumetric yields (Jem *et al*., 1997; Reid *et al*., 2013). The only bioreactor configuration which is suited to hold such large volumes are stirred tanks, which currently have maximum capacities of 20,000L (De Jesus and Wurm, 2011). Stirred tank bioreactors are generally used to culture insect cells growing in single-cell suspensions, though immobilization systems such as microencapsulation or microcarrier culture can provide a method for anchorage-dependent cells to be cultivated in stirred tank bioreactors (Weiss and Vaughn, 1986; Warnock and Al-Rubeai, 2006). While stirred tank reactors are recognized for offering the highest volumetric capacities, suitability for multipurpose use and are well-studied systems, the level of shear generated by mixing in these vessels could be of detriment to the culture of shear sensitive cells. Airlift or wave bioreactors are alternative cultivation systems which can offer a low-shear culture environment. The airlift bioreactor has been ascertained as a scalable reactor choice for biopesticide production, and its use has been demonstrated for the mass production of AgMNPV biopesticide (Merchuk and Siegel, 1988; Visnovsky, 2003).

1.5.3 Attached cell culture systems

Historically, the large-scale propagation of anchorage dependent animal cells has relied on the linear expansion of the surface area available for cell growth. These were performed in roller tubes (Gey, 1933), roller bottles (Griffiths, 1995), prescription bottles (House and Wildy 1965), multi-plate propagators (Weiss and Schleicher, 1968), plastic films (Munder *et al*., 1971), spiral film propagators (House *et al.,* 1972), glass bead propagators (Wohler *et al*., 1972), artificial capillaries (Knazek *et al*., 1972), cell factories (Masters, 2000) and microcarriers (Van wezel, 1973). The two main criteria that govern the selection of a large-scale cultivation system are the potentially attainable volumetric cell productivity and the amenability of the system to scale up (Levine *et al*., 1977). Roller bottles and microcarriers are suitable cultivation systems for attachment-dependent insect cell lines. The application of roller bottles for insect cell culture has been demonstrated in studies which evaluated production of AcMNPV bioinsecticide in roller bottle cultures of *Spodoptera frugiperda* cell lines. Cell yields of 3 to 5 x 10^8 cells per bottle were obtained, along with a reduction in the population doubling time of the cell line compared to stationary cultures. The highest virus yields obtained were 8.5 x 10^5 polyhedra per ml or 6.3 x 10^7 polyhedra per bottle (Vaughn, 1976; Weiss *et al*., 1981a; Weiss *et al*., 1981b; Weiss et al., 1986). Production of recombinant Epstein-Barr viral attachment protein (EBV gp105) using the IC-BEVS system has also been demonstrated in roller bottle cultures of High-Five cells (Wickham and Nemerow, 1993).

Microcarriers are microscopic particles that are easily maintained in suspension in an agitated liquid medium, providing a pseudo-suspension environment for the culture of anchorage-dependent cells (Wurm FM, 2004). Cell growth and infection in microcarrier cultures of insect cells allows easier process scale-up, as well as improved volumetric production of *in vitro* propagated insect viruses. Microcarrier cultures of the AA mosquito cell line grown on DE-53 carriers were used to produce Sindbis and West Nile virus in a culture volume of 8 L (Lazar *et al*., 1987). Dengue virus production in the C6/36 mosquito cell line grown on Cytodex-1 microcarriers has also been demonstrated (Liu and Wu, 2004). The Tn-5 cell line has also been cultured on DEAE-based microcarriers and glass beads in roller bottles and in split-flow airlift bioreactors (Chung *et al*., 1993; Wickham and Nemerow, 1993). In the most recent report, several solid and macroporous microcarriers were screened for their ability to support cell growth and recombinant

β-galactosidase production in Sf-9 and High-five insect cell lines (Ikonomou *et al*., 2002). The widespread use of microcarriers for the cultivation of insect cells has been precluded mainly because most of the industrially relevant insect cell lines have been adapted to grow in free suspension (Archambault *et al*., 1994).

1.6 Factors affecting virus production in insect cell cultures

1.6.1 Multiplicity of infection

The multiplicity of infection (MOI) is defined as the ratio between the total number of infectious particles and cells of the infected culture. The MOI determines the percentage of the cell population initially infected as well as the rate of infection. The probability (*p*) of a cell being infected by (*w*) virus can be modelled by the Poisson distribution according to the following equation (O'Reilly *et al*., 1992).

$$p(w) = \left[\left(\frac{MOI^w}{w!}\right) * e^{-MOI}\right] \quad (1)$$

Infection at high MOI (greater than 3 $TCID_{50}$/cell) result in all cells in culture becoming infected near simultaneously, which is referred to as a synchronous infection. In synchronous infections, the viral replication process in all cells follows the same temporal pattern (assuming that all cells in culture are equally susceptible to the virus), and will result in a unique peak production of budded virus progeny. Infections at lower values of MOI (less than 1 $TCID_{50}$/cell), result in asynchronous infections, where only a small proportion of the overall cell population becomes infected initially. The dynamics of asynchronous infections are more complex than synchronous infections. In asynchronous infections, following the addition of virus, two different cell populations are observed – an infected cell population which has been infected by at least one virus (primarily infected cells), and an uninfected cell population. Infected cells will cease to proliferate, and their cellular machinery will be geared towards virus replication and production, while non-infected cells will continue to grow. Once primarily infected cells begin to produce virus progeny, these in

turn, go onto initiate a 'secondary' infection among the population of cells that were originally uninfected. Multiple 'secondary' infection cycles occur in the culture, until the entire uninfected cell population, is eventually infected (Aucoin *et al*., 2010).

A key difference between synchronously and asynchronously infected cultures, is that, in the former, viral replication occurs in cells that are all in a similar physiological state and culture environment; whereas in the latter, batches of cells become infected at different time-points post-infection, and are consequently infected under different physiological states and culture conditions. The significance of this difference is that the productive capacity of insect cells can vary depending on their 'age' in culture and the quality of the culture medium, at the time when they are actually infected (Claus *et al*., 2012). Therefore, cells becoming infected later in the growth phase as a consequence of the infection dynamics at low MOIs, can lead to reduce virus productivity in culture. Furthermore, the time of harvest will be indirectly influenced by the MOI used to infect a culture. The use of low MOIs can result in a longer time of harvest and therefore, a longer residence time for the viral product in the bioreactor. In the case of viral products susceptible to proteolytic degradation, cell lysis occurring in the later time-points of culture can lead to loss of infectivity (Maranga *et al*., 2003). Nevertheless, several groups have advocated using low MOIs for the infection of insect cell cultures, especially for the production of viral inoculum, since it minimizes the production of defective interfering particles in culture (Pijlman *et al*., 2002; Wickham *et al*., 1991; Wong *et al*., 1995; Zwart *et al*., 2008).

1.6.2 Cell density at the time of infection and the cell density effect

To ensure the economic viability of large scale viral biopesticide production, establishing high cell density culture has often been considered the cornerstone to improving virus yields (Bedard *et al*., 1994, Visnovsky and Claus 1994, Gioria *et al*., 2006, Lua and Reid 2003, Rodas *et al*., 2005, Micheloud *et al*., 2009). The highest cell densities are achieved later in the culture, so logically this would mean that infecting a culture during the late exponential or early stationary phase would lead to the highest virus yields. However, this has not been the case, and there are several reports of a reduction in cell specific yields of virus and recombinant proteins in cultures infected at high cell densities (Stockdale and Gardiner 1977, Wood *et al*., 1982, Chung and Shuler, 1993, Reuveny *et al*., 1993, Ljunggren *et al*., 2002). This phenomenon, termed the 'cell density effect', is caused by

a loss of the intrinsic ability of the cells to support viral replication as the culture ages (Claus *et al*., 2012). Strategies such as partial or total replacement of culture medium at the time of infection (Hensler and Agathos, 1994; Reuveny *et al*., 1993), glucose and/or glutamine supplementation (Taticek and Shuler, 1997; Wang *et al.*, 1993a), one step addition of a nutrient cocktail (Bedard *et al*., 1994) and fed-batch culture (Nguyen *et al*., 1993), have been used to restore cell-specific yields and to delay the cell density effect to higher cell densities, but were unable to totally overcome it. For example, in Sf cells grown in suspension cultures for the production of influenza vaccine, it was found that volumetric yields could not be improved above infection cell densities of 6 – 8 x 10^6 cells/ml, despite the use of a fed-batch approach (Meghrous *et al*., 2009). Several explanations for this phenomenon have been proposed; including nutrient limitation, the accumulation of toxic metabolites, the cell cycle distribution, oxygen deprivation due to culture mixing conditions, passage duration of host cells, inoculum age and length of lag phase (Claus *et al*., 2012, Hyunh, 2013). Most recently, Bernal *et al*. have noted, that at high cell densities, a down regulation of the metabolic fluxes associated with glycolysis and the tricarboxylic cycle, including anaplerotic pathways occurs, which leads to a drop in metabolic energy (Bernal *et al*., 2009). During the production of AgMNPV biopesticide in saUFL-Ag286 cells, cell specific virus yield declined upon cell densities reaching 4 - 8 x 10^5 cells/ml (Micheloud *et al*., 2009). For HearNPV infected HzAM1 suspension cultures, this threshold was reached at slightly higher cell densities of 0.5 – 1 x 10^6 cells/ml (Huynh, 2013). For the Sf-9 cell line infected with AcMNPV, the critical density above which specific recombinant protein productivity decreased was 3 - 4 x 10^6 cells/ml, despite 5 x 10^7 cells/ml being the highest uninfected cell density reached in suspension culture (Elias *et al*., 2000). When the same cell line was cultivated and infected in conditioned culture medium, productivity declined at cell densities as low as 1 - 2 x 10^6 cells/ml (Doverskog *et al*., 2000). On the other hand, in AcMNPV-infected High-Five cells, productivity only declined above an infection density of 5 - 6 x 10^6 cells/ml (Ikonomou, 2002, Drugmand, 2007). These examples tell us two important things – that, a) the critical density at which the cell density effect occurs varies depending on the cell line and virus combination and b) the critical density may vary for the same cell line - virus combination depending on the cultivation mode, culture media and mixing regime used.

Hence, the selection of an optimal time of infection in an *in vitro* virus production process is key in order to optimize yields. Infecting too early will lead to the early arrest of cell division thereby reducing the total number of infected cells. On the other hand, infecting too late will result in low

yields due to the aforementioned cell density effect. Furthermore, the interplay between the chosen multiplicity of infection and cell density at time of infection is of pertinence, especially when low MOI's (< 1) are used. In low MOI infections, cells are infected asynchronously and there is a percentage of the cell population which remains uninfected after the first round of infection, which continues to grow. These cells are then secondarily infected with virus progeny from the first round of infection (Zhang *et al*., 2005). The expectations of high virus yields from low MOI infections can only be fulfilled if the cell specific yields during the secondary infection phase are optimal. Thus the TOI must be carefully chosen, with due regard to the physiological state of the cells; to ensure that the density reached when cells are to be secondarily infected does not exceed the critical threshold at which the 'cell density' effect occurs.

1.6.3 Quality of viral inoculum

The quality of the virus inoculum directly impacts the level of virus replication in host cells, thereby impacting the yield and quality of the virus produced. In the case of baculoviruses, it is important to minimize the generation and propagation of genomic variants that result in low yields of budded virus as well as few-polyhedra (FP) mutants, however for nudiviruses only the former will apply. Viruses have their individual half-life and viral stocks are subject to inactivation over time (Palomares *et al*., 2006). Basing MOI calculations on outdated viral stock titers can run the risk of applying, in reality, an inactivated virus inoculum at a much lower MOI than desired. This can drastically affect overall process performance. While a remedy for this is to re-titer viral inoculum stocks periodically, caution must be paid to that fact that most methods for virus quantification such as end-point dilution and plaque assays have a coefficient of variation of 10-50% due to operator error (Nielsen *et al*., 1992). The use of virus inoculum with low viral concentrations, will require a larger volume of inoculum to be used than if a high titer stock was used to achieve the same MOI. Virus inoculum consist of infectious particles in spent culture medium. Spent culture medium may contain accumulated toxic metabolites, and its carry over could negatively impact the physiological state of the cells to be infected, thereby lowering virus yields. A way to limit the spent medium carryover is to concentrate viral stocks by centrifugation or ultrafiltration (Palomares *et al*., 2006).

Another factor affecting the quality of the virus inoculum is the presence of defective interfering particles (DIP's). DIP's are deletion mutants that arise during virus growth and replicate at the expense of the parent virus. They parasitize the normal virus replicative machinery which in turn parasitizes the infected cell, leading to a drop in productivity (Kirkwood and Bangham, 1994). It has been found that the accumulation of non-homologous repeated origin of replication regions of viral DNA leads to the formation of DIP's (Pijlman *et al*., 2003). At high MOI's, the likelihood of such an accumulation is increased as the distribution of viruses replicating per cell is high. For this reason, it is preferable to use low MOI's for the amplification of viral stocks, to ensure that cells are infected by not more than a single virus particle and the formation of DIP's are kept to the minimum (Wickham *et al.*, 1991).

1.7 Thesis Objectives

The main objective of this research work is:

- To develop processes for *in vitro* production of OrNV in the DSIR-HA-1179 insect cell line

The secondary objectives are:

- To develop a reliable method for the enumeration of DSIR-HA-1179 cells growing in attached monolayers.
- To select a suitable culture medium for cultivation and production of OrNV in the DSIR-HA-1179 cell line.
- To study the growth, metabolism and production of OrNV in attached DSIR-HA-1179 cultures in T-flasks.

- To study the growth, metabolism and production of OrNV in attached DSIR-HA-1179 cultures in roller bottles.

- To study the growth, metabolism and production of OrNV in microcarrier cultures of DSIR-HA-1179 cells in spinner flask bioreactors.

- To investigate the cell density effect in the DSIR-HA-1179/OrNV system.

- To characterize the DSIR-HA-1179 cell line from a cytogenetic perspective, through the evaluation of its karyotype.

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Chapter 2

Investigation of basic elements in the DSIR-HA-1179/OrNV system: karyotype, cell enumeration and culture medium selection

2.1 Introduction

The coconut rhinoceros beetle, *Oryctes rhinoceros,* L. (Coleoptera: Dynastinae) is a major pest of coconut and oil palms found throughout Southeast Asia, the Indian Subcontinent and the Pacific Islands (Bedford, 1980), that causes economic losses that reach millions of dollars annually (Jackson *et al*., 2005; Moore, 2009). Control of the beetle using chemical pesticides has had limited success owing to the life cycle of the beetle with damage to the tops of palms caused by feeding adults. The use of the *Oryctes* nudivirus (OrNV), a natural pathogen of the beetle, has been an effective method for controlling the coconut rhinoceros beetle (Young, 1986; Huger, 2005). The traditional *in vivo* method of production of the virus relies on its propagation in infected beetle larvae (Huger, 2005). However, this process has several disadvantages, including the requirement for a facility for growing and maintaining the insects, the need for numerous personnel and a final product that suffers from inconsistencies in virus concentration and purity. *In vitro* large-scale production of OrNV in insect cell cultures would be an important step towards the feasible and robust production of this virus, provided that engineering and economical constrains are solved (Visnovsky *et al.*, 2003; Gioria *et al.*, 2006).

The profitable *in vitro* production of viral biopesticides requires the efficient scale up of a host cell line, the use of low-cost chemically defined and preferably animal-component-free cell culture

media, and the maintenance of high viral specific productivity (Claus *et al.*, 2012). A fundamental process requirement is that the host cell line be well-characterized with respect to its cytogenetic, growth and metabolic properties. To date, the only cell line that has proved a susceptible and permissive host to infection with OrNV is the DSIR-HA-1179 cell line, established in 1979 by Crawford from sterilized eggs of the black beetle *Heteronychus arator* (Crawford, 1981; Crawford, 1982; Crawford and Sheehan, 1985). With the exceptions of an early estimate of population doubling time of 6 days and an observation that the cell line was a highly unstable polyploid (Crawford, 1982; Crawford *et al*., 1983), no current literature exists on the characterization of the DSIR-HA-1179 cell line from a technological perspective. The strong anchorage-dependent characteristics of the cell line, the fact that it grows forming clumped clusters that become even larger when the cells are dislodged from the flask surface, its particular fragility, and the lack of a method to dissociate and reliably count individual cells, are reasons that have precluded its study over the past thirty years. Additionally, of the 500 reported insect cell lines, only 22 are coleopteran derived and these, as a group, have not been fully understood in terms of their long term cultivation, manipulation, and the possibility of using them as hosts for mass production of viruses (Hoshino *et al.*, 2009; Goodman *et al.*, 2012).

The genotypic and phenotypic homogeneity of DSIR-HA-1179 cells in culture is highly desirable for achieving stable and reproducible cell growth and virus production. The cytogenetic characteristics of continuous cell lines are known to evolve over time (Sinacore *et al*., 2013). Such evolution maybe induced as a consequence of increasing passage number or selection pressures resulting from modifications made to the culture environment, for example, adaptation of the cell line to new culture medium, or a new mode of cultivation. Karyotyping is a technique which enables the visualization of the chromosomes of individual cells. Periodic evaluations of a cell line's karyotype will allow the identification of changes in chromosome number, structure, morphology and ploidy level which may have occurred over time (Wenger *et al*., 2004; Dzhambazov *et al*., 2001). Such chromosomal changes may have a direct effect on the performance of cells in culture. For example, chromosomal rearrangements in diploid and tetraploid clones of the Sf-9 cell line were correlated to changes in cell size and viability, glucose and oxygen uptake rates, intracellular pH and product yield (Jarman-Smith *et al*., 2004). Thus the need for routine cytogenetic characterization of industrially important insect cell lines is emphasized.

In animal cell cultures, rapid, accurate determination of cell concentration and viability are essential in monitoring cell growth and the effects that the culture environment has on the viability of cells, including their interaction with biological, chemical, and mechanical agents that are part of the same environment. Attachment-dependent cell lines can suffer a loss in their viability if they are not handled properly when sampled. In the case of DSIR-HA-1179 cells, the situation is even more complex, since the cells tend to aggregate when they are removed from the growth surface. Thus, there is an added requirement to dissociate cell clumps into a homogenous single cell suspension to allow individual cells to be scored for viability. The simplest methods for dissociating an adherent cell monolayer into a single cell suspension involve mechanical detachment, such as tapping the flask to dislodge cells, repeated aspiration through a pipette, and cell scraping. The addition of chemicals such as sulfated polyanions (e.g. heparin and dextran sulfate) to the culture medium may reduce aggregation in cell clumps, while the addition of chelators like EDTA, sequester divalent cations such as calcium and magnesium which play an important role in cell adhesion (Freshney, 1987). Alternatively, an adherent cell monolayer maybe dissociated using an enzymatic method which involves treating the monolayer with a proteolytic enzyme for a short duration (Freshney, 1987). Of these methods, enzymatic dissociation has been most widely used because of its ability to release a large number of cells whilst preserving cellular integrity and viability (Cunningham, 1999). Fragile cell lines that suffer damage upon treatment with strong enzymes such as trypsin and collagenase may instead be treated with TrypLE™ Express, a recombinant fungal trypsin-like protease that is gentler and less toxic to cells. TrypLE™ Express is a serine protease which cleaves peptides at the C-terminal of arginine and lysine amino acids much like trypsin. Its stability at room temperature and the fact that it does not require inactivation with serum or other protease inhibitors make it an attractive alternative to using trypsin (Nestler *et al.*, 2004).

Understanding cell growth kinetics will be very relevant to the management and prediction of the cell and virus production process, while an understanding of nutrient requirements and metabolite production are important in tailoring a specific culture medium that will support both high cell and virus yield. Nutritional limitations and by-products accumulation can affect cell multiplication and viability. This in turn, can have an effect on the synthesis of products produced by cells such as recombinant proteins or viruses. Among the metabolites involved in insect cell cultivation; glucose, lactate and ammonia emerge as important ones. Glucose has been indicated as one of the main

energy sources for insect cells (Drugmand *et al*., 2012), while lactate and ammonia have been demonstrated to adversely affect them because of the toxic effects they produce when they accumulate in the culture medium (Cruz *et al*., 2000). Metabolic profiles vary among insect cells lines. Glucose has been found to be the major carbohydrate source in a range of insect cell lines including Sf-9 (Reuveny *et al*., 1992), High-Five (Rhiel *et al*., 1997), saUFL-Ag-286 (Gioria *et al*., 2006) and BCIRL-HZ-AM1 (Lua and Reid, 2003). BTI-Tn-5B1-4 and BCIRl-HZ-AM1 cells display an inherent tendency to produce lactate and ammonia even when provided with sufficient glucose to grow in batch culture (Rhiel *et al.*, 1997; Lua and Reid, 2003), while Sf-9 cells will produce them only under glucose deprivation (Ohman *et al*., 1995; Drews *et al*., 2000). On the other hand, the metabolism of the saUFL-AG-286 cell line is characterized by the production of ammonia but not of lactate (Gioria *et al*., 2006). To date, no studies have been conducted on the cell growth kinetics or metabolism of DSIR-HA-1179 cells.

This chapter is based on the investigation of basic elements in the DSIR-HA-1179/OrNV system. In view of its potential for industrial application, the cell line's karyotype was re-evaluated. An original method was developed to allow the reliable enumeration of DSIR-HA-1179 cells in culture. The cell line was adapted to grow in a range of commercially available insect cell culture media and screened for their ability to support cell growth and OrNV production. The kinetic parameters of growth in the different culture media as well as glucose consumption, lactate and ammonia production were also evaluated, which culminated in the rational selection of a culture medium for use in the further development of the process.

2.2 Materials and Methods

2.2.1 Cell stock and culture media

DSIR-HA-1179 cells were obtained from the AgResearch (Lincoln, New Zealand) cell culture collection and were maintained as adherent cultures in 25 cm^2 tissue culture flasks (Corning®) at 27 ºC in 5 ml of 10% foetal bovine serum (FBS) (Life Technologies, New Zealand) supplemented PS-100 culture medium, which was prepared in-house. PS-100 is a modified preparation of Grace's insect medium made to mimic TC-100. Briefly, Grace's medium (Sigma) was supplemented with

2.95 g/l tryptose phosphate broth (Sigma) and 1 ml/l TC-100 vitamins. The pH of the culture medium was then adjusted to 6.2 using 10 M potassium hydroxide (Sigma). The TC-100 vitamins were originally made up as a 1000x stock solution in the following proportion: 200 mg/l thiamine hydrochloride, 110 mg/l calcium pantothenate, 200 mg/l pyridoxine hydrochloride, 200 mg/l para aminobenzoic acid, 200 mg/l folic acid, 200 mg/l nicotinic acid, 200 mg/l inositol (iso-, myo-, meso-), 100 mg/l cyanocobalmin, 200 mg/l riboflavin, 100 mg/l biotin (components sourced from Sigma).

2.2.2 Chromosome preparation and staining

DSIR-HA-1179 cell cultures for karyotyping were set up in 12.5 cm^2 tissue culture flasks by inoculating 0.5 ml of cell suspension in 2 ml of fresh PS-100 culture medium supplemented with 10% FBS. The cultures were incubated at 27 ºC for 8 days until they were 70-80% confluent (mid-exponential phase), at which point chromosome preparation and staining was performed (Crawford *et al*., 1983). Demecolchicine (Sigma) was added to experimental DSIR-HA-1179 cultures at a concentration of 0.1 µg per ml of culture medium and the cultures were incubated for 4 hours at 27 ºC to arrest cells in metaphase. At the end of the incubation period, cells were dislodged from the flask surface by tapping the side of the flask and the cell suspension was transferred to a 15 ml centrifuge tube and centrifuged at 117 x g for 5 minutes. The supernatant was decanted and 2.5 ml of hypotonic 0.075M KCl solution was added drop wise to the cell pellet retained in the centrifuge tube. This mixture was incubated at room temperature for 25 minutes and then centrifuged at 117 x g for 5 minutes. The supernatant was decanted and cells were fixed with three changes of cold Carnoy's solution (methanol: glacial acetic acid, 3:1 v/v) using volumes of 5 ml, 5 ml and 1 ml, in that order. Cell suspension was added drop-wise onto inclined glass slides to create even metaphase chromosome spreads. Slides were dried on a heating block and then stained with 4% Giemsa solution (Sigma) for 8 minutes. 100 metaphase chromosome spreads were examined under bright-field on an Olympus BX50 upright microscope. Representative spreads were photographed with an Olympus DP-12 digital camera. Images were subsequently processed using ImageJ™ software.

2.2.3 Adaptation of cells to new culture media

A five-months sequential process was used in adapting DSIR-HA-1179 cultures to grow from PS-100 to four commercial culture media: TC-100 (Sigma), IPL-41 (Life Technologies), Sf-900 II (Life Technologies) and Sf-900 III (Life Technologies), that were supplemented with 10% FBS. Once adapted, cells were passaged in their respective culture media every 10 days when the cell monolayer was approximately 80% confluent, using a dilution factor of 1:5 with fresh culture medium.

2.2.4 Cell dissociation and cell counting methods

For nuclei counting, a citric acid lysis buffer was made by dissolving 0.9 g of citric acid powder (Sigma) in 100 ml of distilled water to which 0.01 g crystal violet nuclear stain (Sigma) was added and homogenously dispersed. A 1 ml sample of cell suspension was centrifuged at 12,000 g for 5 minutes and the supernatant discarded. The cell pellet was re-suspended in 1 ml of the lysis buffer, vortexed in a whirl mixer for 1 minute, and then incubated for 1 hour at 37 °C. The individually stained nuclei were counted in duplicate using a Neubauer hemocytometer.

The role of heparin in eliminating cell aggregation was investigated by cultivating duplicate 25 cm^2 attached DSIR-HA-1179 cultures (seeded at approximately $2x10^5$ cells/ml) in 5 ml of 10% FBS-supplemented TC-100 containing heparin (Beparine®, 5000 I.U. /ml, Biological E, India) at concentrations of either 100, 250 or 667 µg/ml. Cultures were visually inspected over a 14 day growth period for dissociated single cells. Dissociated cells were collected and the trypan blue dye exclusion assay was used to assess culture viability. Briefly, cell suspensions were stained with 0.4% trypan blue (Sigma) and then both the total and viable cells were counted in duplicate using a Neubauer haemocytometer (Phillips, 1973).

TrypLE™ Express was used to dissociate cells. Prior to adding TrypLE™ Express (Life Technologies), 2 ml of Dulbecco's phosphate buffered saline (D-PBS) free of calcium and magnesium (Sigma) was used to wash the DSIR-HA-1179 cell monolayer. This washing step with buffer was essential in order to remove any calcium and magnesium ions present on the monolayer as well as residues of the spent cell culture medium. 1 ml of TrypLE™ Express, pre-warmed to 27 ºC was added per 25 cm^2 of a confluent cell monolayer in the tissue culture flask. The flask was

rocked gently to evenly coat the monolayer and then incubated at 27 ºC until cells had visibly detached. An appropriate volume of pre-warmed cell culture medium containing 10% FBS was added and the cell suspension was gently aspirated with a 10 ml pipette to break up any remaining cell aggregates. Cell counts were made on a Neubauer haemocytometer and trypan blue dye exclusion assay was used to assess cell counts and culture viability.

2.2.5 Cell growth kinetics

DSIR-HA-1179 cells growth kinetics were conducted on attached cultures in 25 cm^2 tissue culture flasks with a culture volume of 5 ml for each of the four commercial culture media. For each culture medium, TrypLE™ Express treatment (as described above) of a confluent cell monolayer in a 75 cm^2 tissue culture flask, provided sufficient number of cells to seed fourteen replicate 25 cm^2 flasks, each at an initial cell density of 2 x 10^5 viable cells/ml (Day 0) in a culture volume of 5 ml. Duplicate cultures were randomly selected and harvested every 48 hours and total and viable cell counts determined until day 14, when cultures in TC-100, Sf-900 II and Sf-900 III reached 100% confluence. At this stage, cultures in IPL-41 were 80% confluent.

2.2.6 Nutrient analyses

Glucose concentrations were determined using a YSI 2700 biochemistry analyzer (YSI, Yellow Springs, Ohio, USA). Lactate concentrations were determined using a reflectometric test kit (Merck, Germany). Ammonia concentration was determined using a commercial kit based on the Berthelot reaction (Wiener Labs, Rosario, Argentina) (Patton and Crouch, 1977).

2.2.7 Rates

The DSIR-HA-1179 cell specific growth rate (μ) was calculated by identifying the linear region from the semilog plot of viable cell density versus time, followed by linear regression of this data (Gioria *et al.*, 2006). Population doubling time (t_d) was calculated by equation 1:

$$td = \frac{Ln\,(2)}{\mu} \qquad (1)$$

Over the duration of the exponential growth phase, the mean specific consumption rate of glucose was calculated by determining cell yield (given by the slope of the plot of mean glucose concentration versus viable cell density) and then multiplying this value by the specific growth rate (Gioria *et al.*, 2006).

2.2.8 Virus and virus quantification

OrNV stock (strain X2B) at a concentration of 1 x 10^7 $TCID_{50}$/ml was obtained from AgResearch. X2B is a highly virulent strain originally isolated in 1983 from a field population of infected coconut rhinoceros beetles on Bugsuk Island, Palawan, Philippines (Crawford *et al.*, 1986; Zelazny *et al.*, 1990).

The virus stock used in these experiments was prepared by infection of attached cultures during the early exponential phase of growth (approximately 4 x 10^5 viable cells/ml) at a MOI of 0.1 $TCID_{50}$/cell. OrNV titer was quantified by end-point dilution analysis. Briefly, to determine the $TCID_{50}$, suspensions of DSIR-HA-1179 cells (2.5 x 10^5 cells/ml) were seeded onto 96 well plates (50 µl per well), and then an equal volume of each viral supernatant dilution (diluted in ten-fold series from 10^{-2} to 10^{-9}) was added with five replicates per supernatant. The plates were placed in a humidified, disinfected plastic container and incubated at 27 ºC for 11 to 14 days until the cytopathic effect was well developed, when the plates were scored for infection, and $TCID_{50}$ was calculated (Reed and Muench, 1938).

2.3 Results and Discussion

2.3.1 The DSIR-HA-1179 cell line karyotype

The DSIR-HA-1179 cell line karyotype was evaluated on the basis of chromosome number and morphology. Fig. 2-1 shows the histogram of the distribution of chromosome counts made on 100 giemsa-stained metaphase spreads. Chromosome number distribution varied from 42 to 145 chromosomes per cell with a modal number of 76. 85% of the metaphase spreads analyzed had between 70 and 80 chromosomes. In the remaining 15% of spreads that lay outside this range,

counts were predominantly clustered between 42 and 52 chromosomes per cell and between 142 and 145 chromosomes per cell.

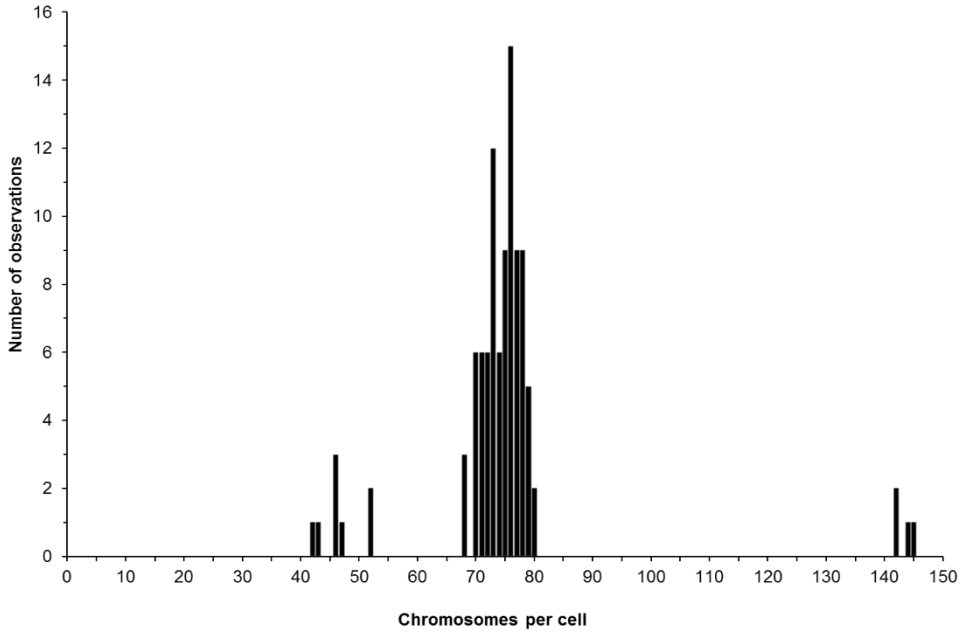

Fig. 2-1 Distribution of chromosome number in DSIR-HA-1179 cells (n=100)

Fig. 2-2 shows four representative metaphase spreads of the DSIR-HA-1179 cell line. For a representative metaphase spread bearing 76 chromosomes (Fig. 2-2 B), ImageJ™ software used in conjunction with the LEVAN plug-in was used to trace the arms of individual chromosomes and thereby assess arm ratio and centromeric position. 57 chromosomes (75% of the karyotype) were metacentric, 18 chromosomes (24% of the karyotype) were sub-metacentric and a single chromosome that appeared to be subtelocentric. In a few spreads, such as those represented in Fig. 2-2 A, C and D, chromosomal structural abnormalities such as di- and tri-centric chromosomes, ring chromosomes and double minutes were observed (identified by colored arrows in Fig. 2-2 A, C and D).

The only existing report of DSIR-HA-1179 cell karyotype was from eighteen and thirty six months after establishment of primary cultures (Crawford *et al*., 1983). It was found that eighteen months after the cell line was established, chromosome numbers ranged between 32 and 43 per cell with a modal number of 37. A single outlier with 67 chromosomes was also observed. Marked cytogenetic

changes occurred between the 18th and 36th month in culture, which was reflected in an unstable karyotype at 36 months in which the chromosome numbers were found to be highly variable between cells and ranged between 12 and 73 chromosomes per cell. In comparison, the present DSIR-HA-1179 cell karyotype shows a decrease in the variation of the spread of chromosomes per cell, despite the prevailing polyploid nature of the cell line (Fig. 2-1). Chromosomes appear to be unchanged with regard to their structure and morphology since the previous study. The reduction in the spread of chromosomes could be indicative of the cell line having tended to a relatively more stable state, which is a positive finding from an industrial perspective.

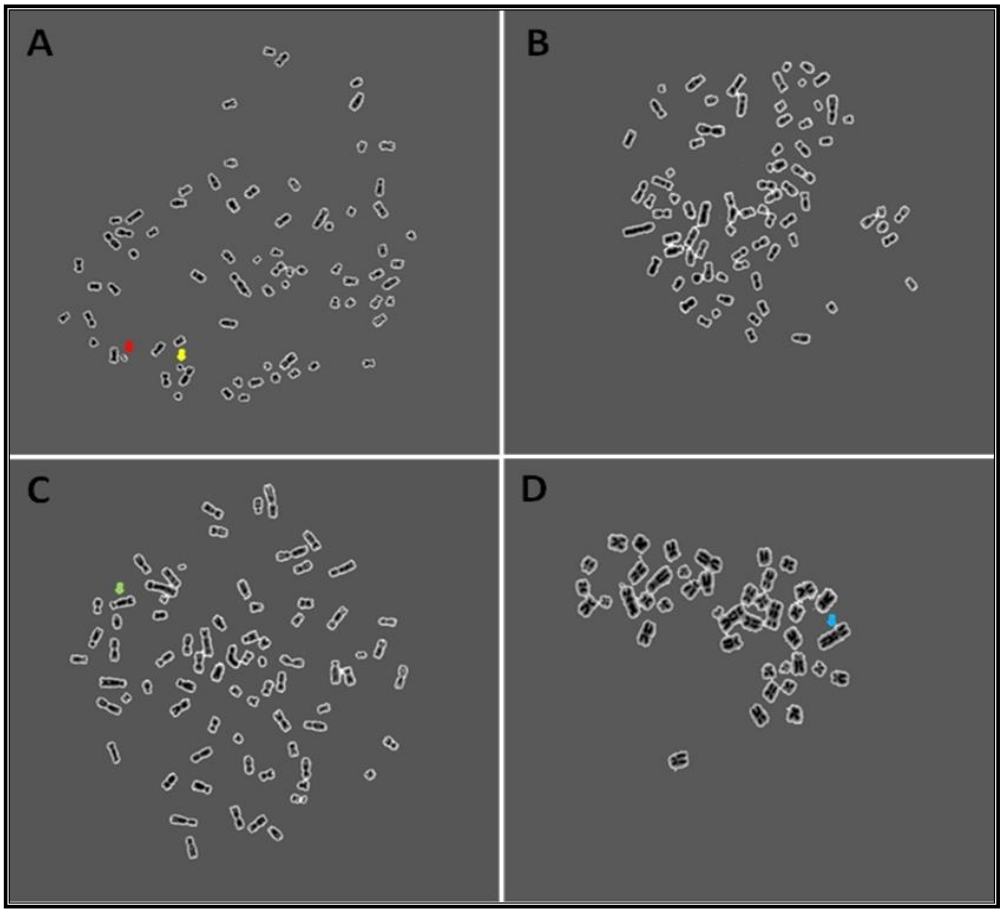

Fig. 2-2 Metaphase spreads from 4 representative DSIR-HA-1179 cells (A) Metaphase spread with 73 chromosomes (red and yellow arrows indicate double minute chromosome and ring chromosome respectively. (B) Metaphase spread with 76 chromosomes. (C) Metaphase spread with 77 chromosomes (green arrow indicates di-centric chromosome) (D) Metaphase spread with 42 chromosomes (blue arrow indicates tri-centric chromosome). Images were obtained using 1000 x magnification.

The chromosomal complement of *Heteronychus arator* cells from *in vivo* testicular tissue has diploid number of 20, and its stability *in vivo* contrasts with the chromosomal variability observed in the DSIR-HA-1179 cell line (Crawford *et al*., 1983). The accumulation of variation in chromosome number has been documented in insect cell lines, and particularly those derived from coleoptera (Fernon *et al*., 1995; Goodman *et al*., 2012; Iwabuchi, 1999; Zheng *et al*., 2014). The causes attributed to chromosomal changes in continuous cell lines are mainly associated with the cell culture methods used to keep them growing *in vitro*. These include long-term passaging, adaptation to new culture conditions, oxygen tension, exposure of cells to mechanical and enzymatic treatments for their detachment from monolayers and exposure to chemicals and radiation (Gaztelumendi and Nogu*é*s, *2014*).

Over the past thirty years, the DSIR-HA-1179 cell line was passaged more than 500 times and was adapted to grow in the PS-100 culture medium, which has a different chemical composition to Schneider's Drosophila medium in which the cell line was established and originally karyotyped. Therefore it is likely that evolution in the karyotype has been a result of long term culture and adaptation to a new culture medium. Given its industrial relevance as a host cell line in bio-pesticide production, greater emphasis must be placed on periodically re-evaluating the cell line's karyotype in future.

2.3.2 Adaptation of the DSIR-HA-1179 insect cell line to four culture media

Insect cell lines are typically cultured in a basal culture medium supplemented with 5-10% (v/v) of vertebrate serum (Marteijn *et al*., 2003). Basal culture media contain nutrients required for insect cell growth such as carbohydrates, amino acids, organic acids, organic salts, vitamins and trace elements (Schlaeger, 1996). The first step in developing a culture strategy for a new cell line would therefore be the selection of an appropriate basal medium, in accordance with the nutritional and metabolic requirements of the cell line. DSIR-HA-1179 cell cultures in the PS-100 culture medium were adapted to grow in four commercially available insect cell culture media (TC-100, IPL-41, Sf-900 II and Sf-900 III) in order to test those which might better support the nutritional requirements of the cell line and thereby improve cell yield and virus production, and to select a

culture medium that could be used as a model to conduct initial studies on this cell line. From the large spectrum of commercially available insect cell culture media, these were selected for this initial screening for the following reasons: TC-100 was chosen because of its similarity in composition to PS-100 (Gardiner and Stockdale, 1975) in which the cell line was originally maintained. The other three culture media were selected as they are formulated with additional carbohydrates and amino acids sources. For example, IPL-41 contains sucrose and maltose in addition to glucose as a carbohydrate source (Weiss *et al.*, 1981), while Sf-900 II and Sf-900 III culture media are very rich formulations with higher concentrations of asparagine and glutamine (Bovo *et al.*, 2008; Lua and Reid, 2003). All culture media were supplemented with 10% FBS to provide the cells a better chance to be adapted to the new culture media.

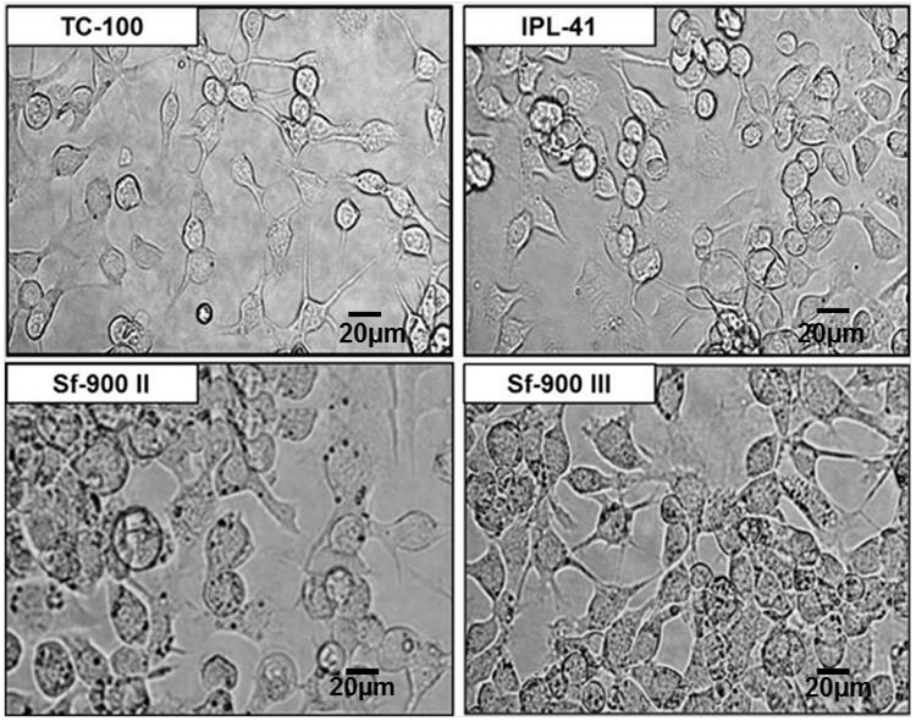

Fig. 2-3 Micrographs of healthy DSIR-HA-1179 cells grown in stationary culture in four culture media supplemented with 10% FBS. The images were obtained using 400 x magnification once cultures reached 70-80% confluence.

Distinct variations in cell morphology and growth patterns were observed between the four adapted cultures, as shown in Fig. 2-3. In the PS-100 culture medium, cells grew in a fibroblast-like shape with clear cytoplasm and the formation of cell 'towers' upon reaching confluence. Cells adapted to the TC-100 culture medium exhibited a similar morphology and growth pattern, which is to be expected if one considers that PS-100 was formulated to mimic the TC-100 culture medium. From visual observation, cells adapted to IPL-41 culture medium were smaller in size, appeared slightly rounded and had clear, non-vacuolar cytoplasm. On the other hand, cells adapted to Sf-900 II and Sf-900 III culture media were larger in size (Fig. 2-1). Cells in Sf-900 II appeared more round in shape and contained cytoplasmic vacuoles which could be observed as clear round spots at 400x magnification. Cells in PS-100, TC-100, IPL-41 and Sf-900 III adhered strongly to the flask surface, while cells grown in Sf-900 II showed a decreased degree of adherence to the flask. This tendency for reduced adherence and rounded morphology of cells in Sf-900 II indicates potential for this culture medium to be used in the future for adapting the cell line to grow as a free suspension culture. Cells that are round in shape have been found to be less susceptible to hydrodynamic stresses in the culture environment during cultivation in agitated suspension (Chisti, Y., 2001; Papoutsakis, E.T., 1991). Cultivation of DSIR-HA-1179 cells in suspension rather than attached cultures, would potentially improve the virus volumetric productivity (Micheloud *et al.*, 2011). At the beginning of this research, efforts were made to adapt the cell line to grow in suspension conditions for more than a year, using spinner flask bioreactors. However, these attempts were unsuccessful due to the damage caused to cells due to mechanical shear in the system. Though suspension adaptation of the DSIR-HA-1179 cell line is out of the scope of this thesis, it is recognized as a very important part of the process to work on in future studies.

2.3.3 Development of a technique to count DSIR-HA-1179 cells

To evaluate kinetic parameters of the DSIR-HA-1179 cell line, there was a need to develop a method for reliable cell counting; a task never achieved to date because of the particular growth characteristics of this cell line. It was first necessary to produce a homogenous suspension of single cells. Several methods, including mechanical dispersion, nuclei counting, heparin and enzymatic treatments were evaluated.

Simple mechanical means such as tapping the flask surface to dislodge cells and repeated gentle aspiration of the culture did not homogenously disperse the cell monolayer. Sections of the monolayer detached, and cells aggregated in clumps of differing sizes, preventing cell counting in a haemocytometer. More vigorous pipetting partially disaggregated the clumps but resulted in cell damage (visible cell debris), thus significantly reducing culture viability. Following these trials, it was confirmed that DSIR-HA-1179 is a strongly attachment dependent cell line, and its tendency to form dense clumps during growth, present a myriad of difficulties to the cell culturist. The presence of clumps introduces diffusion limitations in nutrients, including oxygen, in the culture media from reaching the cells. Furthermore, virus infection in such aggregated cultures will be less effective and therefore unproductive, because of the physical impediment of the virus to effectively reach every single cell. The DSIR-HA-1179 cell line is highly sensitive to shear forces so disruption of the clumps by mechanical means is not a viable option.

The possibility of using nuclei counting as a method to determine total cell number was investigated. The inability to monitor culture viability using nuclei counting is a disadvantage (Van Wenzel, 1973) because monitoring this parameter over batch growth is important and more so in large-scale cell cultivations. Moreover, the presence of bi-nucleated cells during the exponential growth phase introduces errors in the total cell count estimated by this method (Berry *et al*., 1996). Despite these disadvantages, the technique was still assayed on DSIR-HA-1179 cells since it was not known at that point of the research whether it would be possible to develop a more reliable method to determine total cell counts. Assessments of total cell counts of DSIR-HA-1179 cells by nuclei counting were later compared with those made using TrypLE™ Express (a more reliable technique for counting both viable and total cells that was developed in parallel). This confirmed that nuclei counting overestimated the total cell count by 14.8% (mean total cell count from DSIR-HA-1179 cultures on day 14 of growth that were treated with TrypLE™ Express was $17 \times 10^5 \pm 2.1$ cells/ml versus nuclei counts that estimated $19.5 \times 10^5 \pm 2.8$ cells/ml), corroborating the findings of Berry *et al.* (1996). In the light of this result and success achieved later with TrypLE™ Express, nuclei counting was deemed an unreliable method for total cell counting.

The use of heparin as an anti-aggregation agent to inhibit cell-to-cell adhesion has been reported for some cell lines. It has been successful in facilitating the suspension adaptation process in the HEK293 mammalian cell line (Tsao *et al*., 2001) and the TN 5B-1-4 insect cell line (Taticek *et al*.,

1997), both used as hosts in virus production applications. The optimal heparin concentrations that promoted the anti-aggregation effect in these two cell lines were 100 µg/ml and 667 µg/ml, respectively. On the other hand, for the suspension adaptation of recombinant CHO-TS28 cells, 250 µg/ml heparin was reported to effectively induce cell dispersion (Li *et al.*, 2011). Based on these previous findings, and aiming to reduce cell clumping, this range of heparin concentrations were tested on attached cultures of the DSIR-HA-1179 cells. Heparin treatment did not produce any disaggregation when assayed on the cells at the two lower concentrations, while the highest concentration of 667 µg/ml induced cell death possibly due to the dose of heparin being toxic to the cells. Further investigation contemplating the use of heparin in a concentration ranged between 250 and 667 µg/ml could be done to conclusively learn whether heparin can be effectively used to disaggregate this cell line.

It was then attempted to dissociate DSIR-HA-1179 cells grown in TC-100 supplemented with 10% FBS using the TrypLE™ Express enzyme treatment, which was preceded by a D-PBS wash step to ensure the removal of Ca^{2+} and Mg^{2+} ions from the cell monolayer, since these two ions have a role in increasing cell to substrate adhesion in animal cell cultures (Takeichi and Okada, 1972). In preliminary experiments, cultures that were treated with TrypLE™ Express were microscopically observed at 5 minute intervals to gauge the time it would take for the complete dissociation of the monolayer. It was found that the maximum number of cells released between 20-30 minutes post treatment. Due to a lack of literature on TrypLE™ Express incubation times and volumes to be used for the DSIR-HA-1179 cell line, an experiment was conducted to optimize these parameters according to the conditions shown in Table 2-1. Duplicate 25 cm^2 T-flasks containing 5 ml cultures of DSIR-HA-1179 cells labeled 1 to 4 were seeded at an estimated density of 2 x 10^5 viable cells/ml, and incubated at 27 ºC until day 10 of growth when 70% confluence was reached. TrypLE™ Express treatment was then used to dissociate cell monolayers (as described in the Materials and Methods section), using the treatment conditions listed in Table 2-1.

Table 2-1 Optimization of the conditions of TrypLE™ Express treatment for the DSIR-HA-1179 cell line.

Volume of TrypLE™ Express	Incubation Time and mixing conditions	Mean Culture Viability (%) ± SD
1 ml	30 minutes, gentle mixing	94.0 ± 0.9
1 ml	60 minutes, gentle mixing	91.8 ± 1.3
2 ml	30 minutes, gentle mixing	94.0 ± 1.0
2 ml	80 minutes, strong mixing	88.9 ± 0.8

All four treatments were equally effective in releasing cells from the attached monolayer and in dissociating cell clumps to enable the creation of a homogenous single cell suspension. From Table 2-1, cell viability in cultures 1 and 3 were comparable, appearing that the use of 2 ml of TrypLE™ Express instead 1 ml did not have a detrimental effect on culture viability (94.0%). However, since 1 ml of TrypLE™ Express was sufficient to coat the monolayer, this was deemed the optimal volume of TrypLE™ Express to use. Prolonged exposure of cells to TrypLE™ Express for up to 60 minutes followed by gentle mixing in culture 2 led to a slight decrease in culture viability (91.8%). Exposure for up to 80 minutes combined with stronger mixing in culture 4 led to a further decrease in viability (89.9%). Thus, the optimal duration of incubation of the DSIR-HA-1179 cells with TrypLE™ Express and handling approach were determined to be 30 minutes with gentle mixing of the cell suspension prior to counting. One explanation for the relatively long duration of TrypLE™ Express treatment could be because incubation was carried out at 27 ºC, which is lower than the enzyme's optimum operating temperature of 37 ºC. Nevertheless, this duration is similar to the mean time required for cell release of the highly adherent MDCK canine kidney cell line which is reported at 28.5 minutes (Nestler *et al.*, 2004).

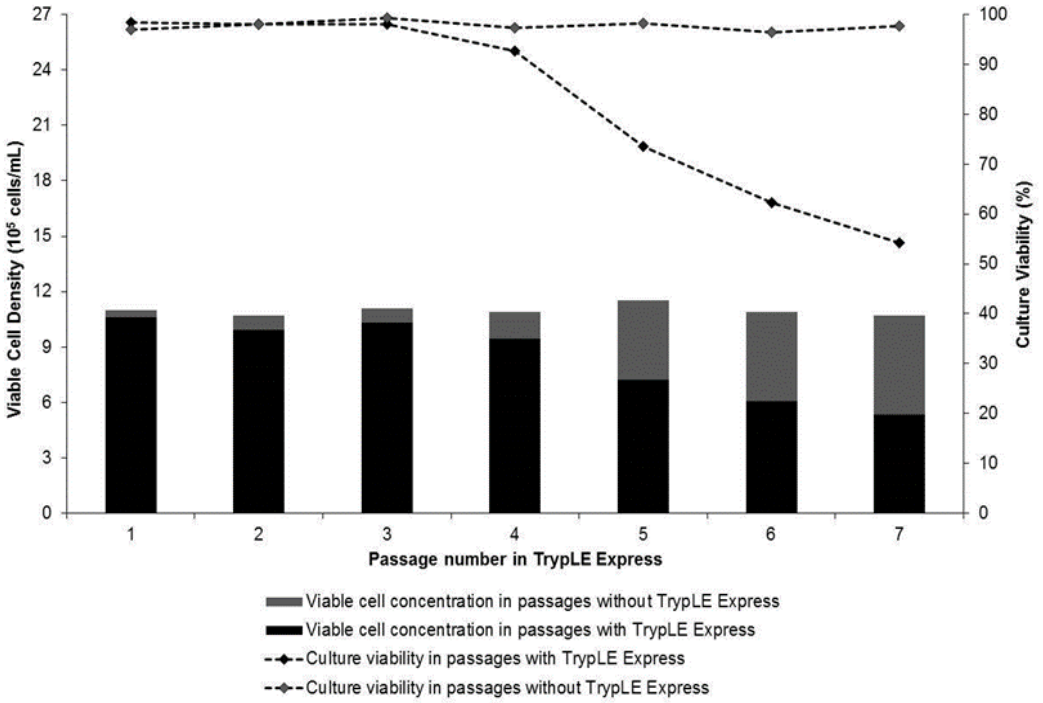

Fig. 2-4 Effect of prolonged use of TrypLE™ Express on viable cell concentration and viability of DSIR-HA-1179 cultures in TC-100 supplemented with 10% FBS.

To determine whether long term exposure to protease carryover would lead to a loss in cell growth and viability, the effect of passaging DSIR-HA-1179 cells with the inclusion of TrypLE™ Express at every passage was investigated (Fig. 2-4). At each passage, 1 ml of TrypLE™ Express was added to the parent flask to dissociate and count cells, which were then seeded into a new flask at a concentration of 2 x 10^5 viable cells/ml. The small percentage of TrypLE™ Express (4%) carried over at each passage did not affect cell growth and viability between the first and fourth passage. However, at the fifth passage, the culture viability suffered a significant drop (from 93% to 73%) and continued to decrease over the next two passages, suggesting that the use of TrypLE™ Express in routine passages after the fourth passage would not be recommended. The enzyme however proved to be an efficient tool in achieving the objective of creating a DSIR-HA-1179 single cell suspension culture, which was a prerequisite for both making accurate total and viable cell counts (i.e. to assess cell growth kinetics) and manipulating cells (i.e. for seeding a culture at a particular cell density).

2.3.4 Cell Growth Kinetics

To begin characterizing DSIR-HA-1179 cell growth kinetics in the four culture media selected for this study, cell density and culture viability assays were assessed. As shown in Fig. 2-5, cells went through an initial lag phase which lasted for approximately 48 hours after which the exponential phase of growth commenced. All cultures were allowed to grow until they reached confluence, as explained in the Material and Methods section. Of the four culture media, Sf-900 II gave the highest maximum viable cell density (17.9 x 10^5 viable cells/ml) and the mean highest specific growth rate over the batch culture (0.0074 h^{-1}, PDT: 3.9 days). Additionally, high culture viability was observed in Sf-900 II (99.2%), which is possibly because cells grew with a rounded shape in this culture medium and were therefore able to tolerate hydrodynamic forces of shear during cell dissociation and pipetting better. Kinetic parameters in Sf-900 III and TC-100 culture media were broadly comparable to Sf-900 II. Mean maximum viable cell densities of 16.9 x 10^5 and 16.8 x 10^5 viable cells/ml were reached respectively in Sf-900 III and TC-100 culture media, with mean specific growth rates of 0.0072 h^{-1} in Sf-900 III (PDT: 4.0 days) and 0.0069 h^{-1} in TC-100 culture media (PDT: 4.2 days). Mean culture viabilities in both of these culture media remained high, at 92.3% (Sf-900 III) and 92.9% (TC-100), over the batch growth period measured. In comparison, cultures in IPL-41 grew relatively slower with a mean specific growth rate of 0.0057 h^{-1} (PDT: 5.1 days). The mean maximum viable cell density reached in IPL-41 was 12.5 x 10^5 viable cells/ml, which was appreciably lower than the other three culture media though mean culture viability was 93%.

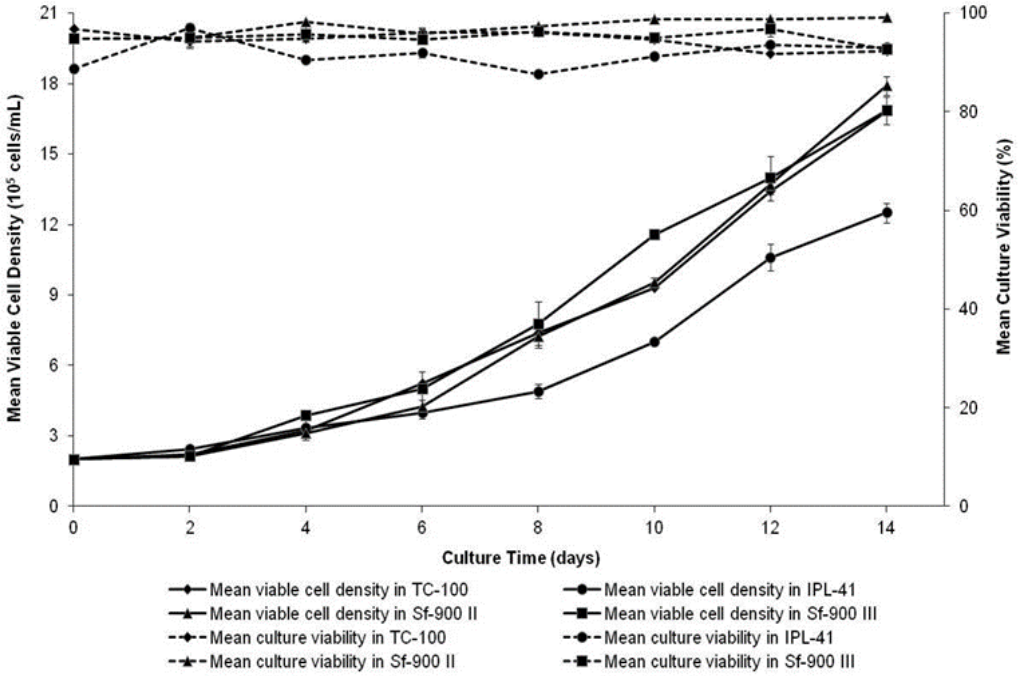

Fig. 2-5 Mean growth and culture viability profiles of the DSIR-HA-1179 cell line grown in four culture media (TC-100, IPL-41, Sf-900 II, Sf-900 III, supplemented with 10% FBS, respectively), in 25 cm^2 attached T-flask cultures. Error bars represent standard deviations from the mean of duplicate cell counts.

Typical specific growth rates for insect cell lines range between 0.01 h^{-1} and 0.03 h^{-1} (Schmid, 1996). In comparison, the specific growth rate of the DSIR-HA-1179 cell line is very slow. In nature, the development of the parent beetle, *Heteronychus arator* takes approximately 6-8 months. The very slow growth rate of the DSIR-HA-1179 cell line *in vitro* is likely an inherent characteristic of the embryonic tissue from which the cell line derives (Crawford, 1982). Across the four culture media tested, population doubling times varied between 3.9 – 5.1 days which is still an improvement from the population doubling time of 6 days estimated at the time of establishment of the cell line (Crawford, 1982). The growth characteristics of continuous cell lines are known to evolve over time along with putative changes at the genome level. Such evolution maybe induced by selection pressures resulting from modifications made to the culture environment. The improvement of growth rate could have been a result of adaptation of the cells to the new culture media – especially because the highest growth rate was observed in Sf-900 II, which is one of the richest existing insect cell culture media (Bovo *et al.*, 2008). Over the last thirty years the cell line

has been through over 400 passages. The growth rate improvement could also be attributed to an effect of this long-term passaging of the cell line (Donaldson and Shuler, 1998).

2.3.5 Metabolite Analyses

Glucose has been identified as a preferred energy and carbon source for insect cells and is considered the most important carbohydrate to monitor during batch culture (Drews *et al.*, 1995). As Fig. 2-6 shows, the glucose consumption profile of DSIR-HA-1179 cells in TC-100 indicates that this was the only culture medium in which glucose was exhausted at the end of the 14 days of batch growth. The mean overall specific rate of glucose consumption in TC-100 was determined to be 4.1 x 10^{-12} g $cell^{-1}$ h^{-1} while a higher value of 5.0 x 10^{-12} g $cell^{-1}$ h^{-1} was observed in IPL-41. Mean specific consumption rates of glucose in Sf-900 II and Sf-900 III were 4.3 x 10^{-12} and 3.1 x 10^{-12} g $cell^{-1}$ h^{-1} respectively.

Although specific rates of glucose consumption reported for insect cell lines vary under differing culture medium and cultivation conditions, one study has estimated the specific glucose consumption rates in suspension cultures of lepidopteran cell lines Sf-9, Sf-21 and BTI-Tn-5B1-4 at 3.7 x 10^{-12}, 5.1 x 10^{-12} and 14.5 x 10^{-12} g $cell^{-1}$ h^{-1} respectively (Kwon *et al.*, 2003). BTI-Tn-5B1-4 is one of the highest yielding cell lines (up to 8 x 10^{6} cells/ml in fed-batch culture) and correspondingly has a high specific rate of glucose consumption (Schmid, 1996). The inherent difference in the sizes of BTI-Tn-5B1-4 or High Five cells (15 μm) in comparison with Sf-9 cells (13 μm) could also explain the higher protein content and glucose consumption in the former (Drugmand, 2007; Drugmand *et al.*, 2011). The DSIR-HA-1179 cell line has a slower specific growth rate but not a slower specific rate of glucose consumption compared to Sf-9 and Sf-21 cell lines. The specific rate of glucose consumption ranged between 3-5 x 10^{-12} g $cell^{-1}$ h^{-1} across the four culture media and these differences could be attributed to the varying concentration of glucose present in the culture media (Micheloud *et al.*, 2011). However, the similarity in the specific rates of glucose consumption between cultures grown in TC-100 and Sf-900 II, despite the glucose concentration of Sf-900 II being almost 9 times higher than TC-100 (1 g/l), suggests that the cells are able to metabolize glucose very efficiently in the TC-100 culture medium.

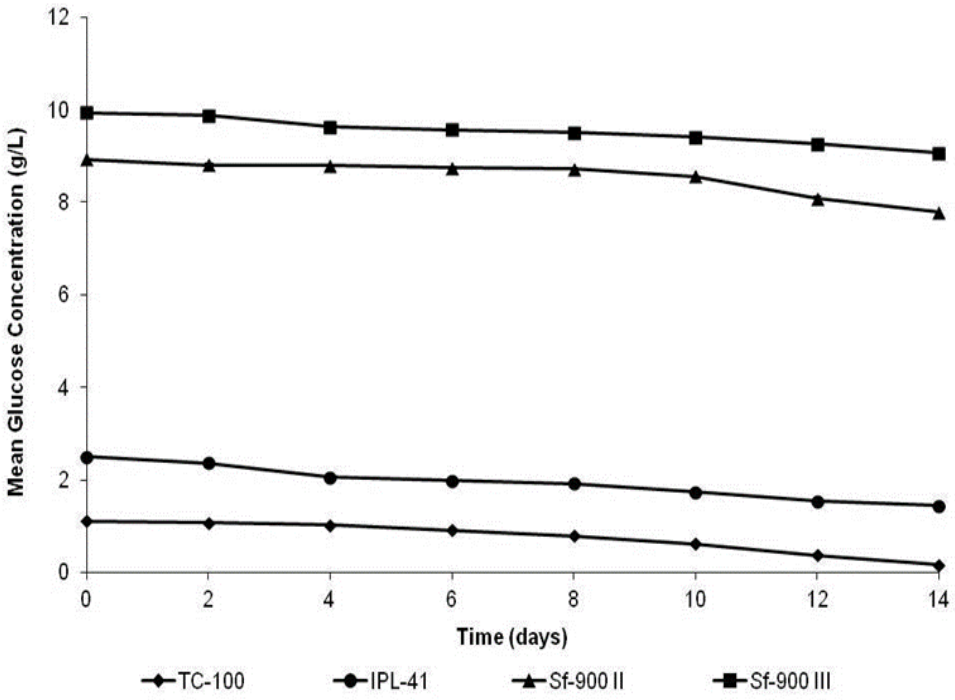

Fig. 2-6 Mean glucose consumption profiles for DSIR-HA-1179 cultures grown in four culture media (TC-100, IPL-41, Sf-900 II, Sf-900 III, supplemented with 10% FBS, respectively), in 25 cm^2 attached T-flasks. Error bars represent the standard deviations from the mean of duplicate samples. Error bars are difficult to see because standard deviations were very small.

The buildup of waste metabolites such as lactate and ammonia to high concentrations in batch culture could be potentially toxic to animal cells. Insect cell lines differ in their ability to produce lactate and ammonia. For example, Sf-9 cells under well oxygenated culture conditions do not produce lactate and ammonia, even in cultures supplemented with glucose concentrations higher than 40 mM (Rhiel *et al.*, 1997). In contrast, BTI-Tn-5B1-4 cells accumulate relatively high amounts of lactate, between 10-20 mM (Sugiura and Amann, 1996; Yang *et al.*, 1996). The saUFL-AG-286 cell line, for example, is characterized by the production of ammonia but not of lactate (Gioria *et al.*, 2006). This reinforces the idea that each specific cell line must be tested for production of these metabolites to shed light on its individual metabolic characteristics. It was found that lactate was produced to very low levels in DSIR-HA-1179 cells cultivated in TC-100 supplemented with 10% FBS with a mean value of 0.1 g/l, while the ammonia production was almost negligible at 0.02g/l. The very low level of lactate production indicates that the cell line is able to metabolize glucose from the culture medium efficiently. In this respect, DSIR-HA-1179

cells share a similarity with the metabolic nature of the Sf-9 cell line. It would be well worth investigating the production of ethanol and glycerol which have been identified as by-products of glucose metabolism in the Sf-9 cell line (Drews *et al.*, 2000). In addition, in order to gain a complete understanding into the metabolism of the cell line, detailed amino acid analyses must be performed. Nevertheless, this initial finding that DSIR-HA-1179 cells produce very low levels of lactate and ammonia is a promising prospect for the large-scale cultivation of the cell line to high densities for the production of OrNV in future.

2.3.6 Infection of DSIR-HA-1179 cell cultures with OrNV

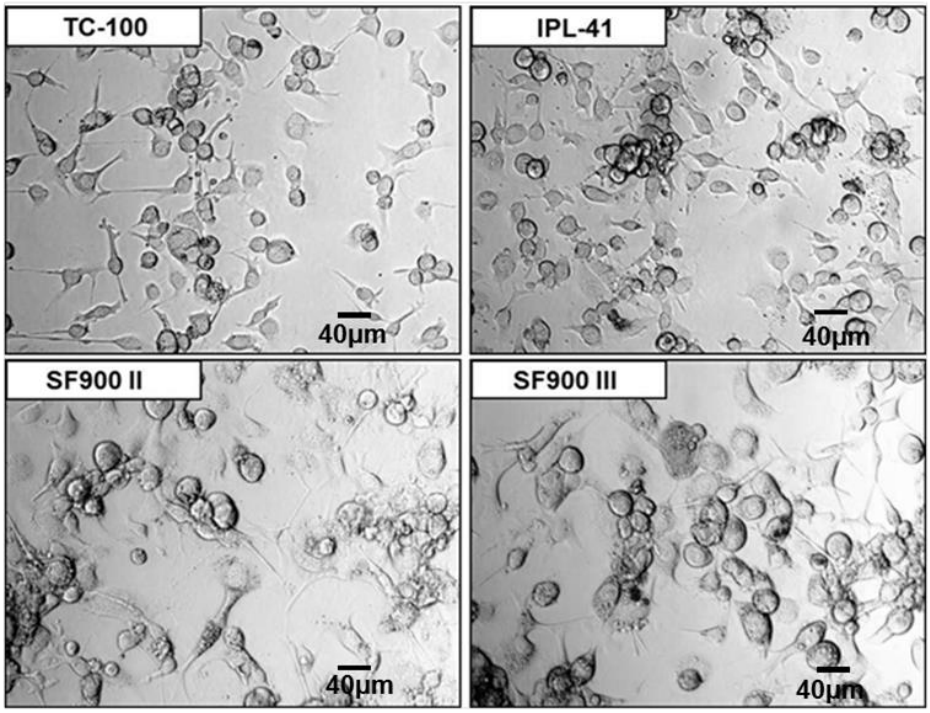

Fig. 2-7 Micrographs of DSIR-HA-1179 cells infected with *Oryctes* nudivirus in stationary cultures in four culture media. Cells were infected during the early-exponential growth phase at a MOI of 0.1. The images were obtained using 200 x magnification, at 14 days post infection.

Cultures of DSIR-HA-1179 cells adapted to the four culture media were infected with OrNV at a MOI of 0.1 $TCID_{50}$/cell at the fifth day post-seeding (early exponential growth phase). By visual observation, as the infection progressed the rate of growth slowed. The typical cytopathic effect in

OrNV infected cultures at the 14th day post-infection (d.p.i) is shown in Fig 2-7. (refer to Fig. 2-3 for comparison for morphology of uninfected cells). Infected cells round-up and small vesicles were observed. DSIR-HA-1179 cells infected in Sf-900 II and Sf-900 III were noticeably larger than cells infected in TC-100 and IPL-41 culture media.

Before the inclusion of TrypLE™ Express could be recommended in a process for producing OrNV in DSIR-HA-1179 cell cultures, it was necessary to ascertain that the enzyme would not affect replication of the virus. Infected cultures that were inoculated on cells either with or without TrypLE™ Express treatment, were harvested at the 14th day post infection, and the virus yields were determined (Fig. 2-8).

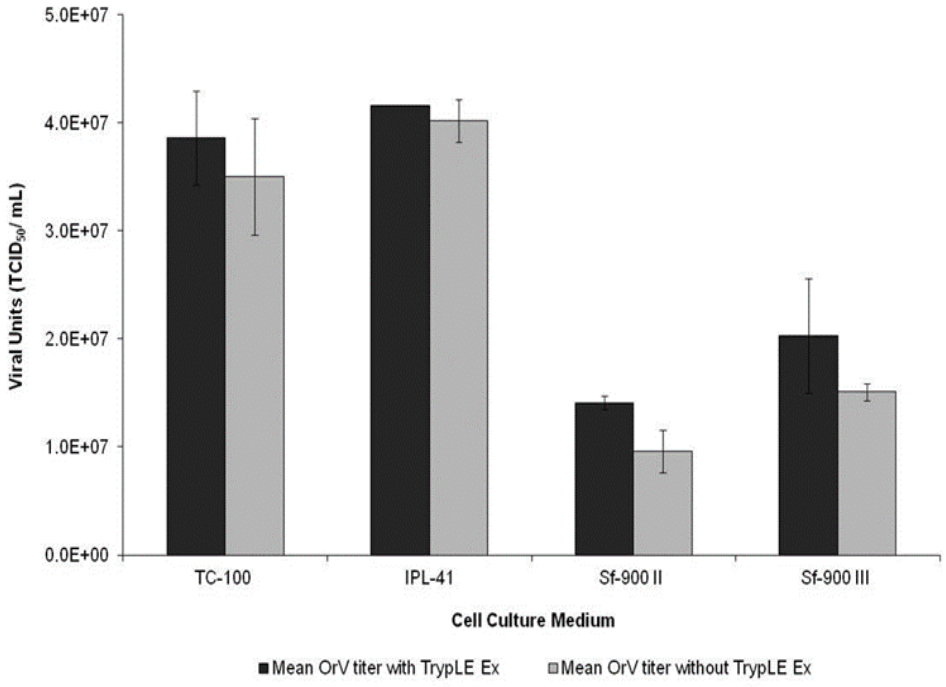

Fig. 2-8 Comparison of *Oryctes* nudivirus production with and without the use of TrypLE™ Express in four culture media. Error bars represent standard deviations from the mean of duplicate samples, except for IPL-41 with TrypLE™ Express where the same virus titer was recorded for both samples.

In all four culture media, OrNV production was consistently improved in the experimental condition where cells had been inoculated following treatment with TrypLE™ Express. A possible explanation for this could be that TrypLE™ Express effectively breaks down the extracellular

matrix of the treated culture and creates a homogenous single cell suspension free of cell aggregates. Such an evenly dispersed cell inoculum resulted in the formation of uniform, clump free cell monolayers that facilitated virus attachment and binding to a greater extent than in the absence of TrypLE™ Express, where cells growing in clumps caused a physical barrier to virus attachment and where mass transfer limitations of nutrients in the culture media could have occurred. From Fig. 2-8, it is interesting to note that despite Sf-900 II producing the highest cell yield, OrNV production in this culture medium was the lowest (1.4×10^7 $TCID_{50}$/ml). Virus production was only marginally improved in Sf-900 III (2.0×10^7 $TCID_{50}$/ml) despite the high specific rate of growth in this culture media. This could be due to some component of the culture media inhibiting virus binding. The formulations of Sf-900 II and III are proprietary and detailed testing of the culture media is required in order to elucidate this information.

It is interesting to note that despite having the lowest specific growth rate, the highest virus production was obtained in IPL-41 culture media (4.1×10^7 $TCID_{50}$/ml). TC-100 culture medium followed closely, yielding 3.8×10^7 $TCID_{50}$/ml. The complete exhaustion of glucose from the culture medium in TC-100 cultures on day 14 of batch growth (Fig. 2-4) could explain why the virus titer achieved in TC-100 was slightly lower than in IPL-41. For virus production, the infection of the cultures lasted for 14 days post time of infection (TOI), which was 5 days after the cells were seeded. Hence, the infection phase lasted through days 5-20 of batch culture. In asynchronous infections at low MOI, only a small proportion of cells become initially infected. Uninfected cells continue to grow and become targets during the subsequent round of 'secondary' infection. During this period, both infected and uninfected cells require glucose to support their basal metabolic needs, and the premature depletion of glucose might result in inhibition of viral replication in cells infected secondarily (Visnovsky and Claus, 1994). In IPL-41, an excess of glucose remaining in the culture medium on day 14 suggests the possibility that the cells were provided sufficient glucose for their metabolic activities during infection between days 14-20; which may not have been the case for TC-100 cultures. Nonetheless, the virus volumetric yields obtained in TC-100 were comparable with those obtained in IPL-41.

2.3.7 Selection of a culture medium

Based on the similarity in OrNV volumetric yields obtained in cultures grown in 10% serum-supplemented TC-100 (3.8×10^7 $TCID_{50}$/ml) and IPL-41 (4.1×10^7 $TCID_{50}$/ml), both culture media are recognized as potential candidates for use in an *in vitro* OrNV production process (Pushparajan *et al*., 2013). However, despite the similarity in OrNV yields in the two culture media, cells grew with a higher specific growth rate in TC-100 (0.0069 h^{-1}), than in IPL-41 (0.0057 h^{-1}). Furthermore, the mean maximum viable cell densities reached in TC-100 (16.8×10^5 cells/ml) were higher than in IPL-41 (12.5×10^5 cells/ml). Carbohydrates contained in basal culture medium are the main sources of energy for cell growth, and TC-100 and IPL-41 basal media differ in their qualitative and quantitative composition of carbohydrates. TC-100 contains only glucose at a concentration of 1 g/l, while IPL-41 is a more complex culture medium formulated with multiple carbohydrates such as glucose, maltose and sucrose at higher concentrations. It appeared that the multiple carbon sources in IPL-41 did not particularly benefit the growth or metabolism of DSIR-HA-1179 cells over TC-100. Furthermore, the use of TC-100, where glucose is the sole carbohydrate, allows the easiest monitoring of sugar exhaustion in the culture medium. Finally, at the operating scale, the cost of using TC-100 (NZD 79/l) is lower than IPL-41 (NZD 85/l), which makes it a more economical choice of culture medium at this stage of process development (Lecina *et al*., 2005). For these reasons, TC-100 was chosen as the basal culture medium to further develop the process.

2.4 Conclusions

This chapter describes for the first time, attempts to manipulate and characterize the unique DSIR-HA-1179 coleopteran cell line, the only cell line susceptible and permissive to infection by the *Oryctes* nudivirus known so far. The cell line in its present state, is polyploid, with a modal chromosome number of 76. The cell line's karyotype has evolved since it was last evaluated in the early 1980's, indicated by a reduction in the variation of chromosome numbers per cell and the range of chromosomal distribution. The cell line was successfully adapted to grow as attached cultures in a range of culture media supplemented with 10% FBS, including TC-100, IPL-41, Sf-900 II and Sf-900 III. It was found that the cells underwent morphological changes when adapted to new growth conditions. Simultaneously, a method for dissociating and accurately assessing

DSIR-HA-1179 cell numbers and viability using the recombinant serine protease TrypLE™ Express was developed and optimized. The use of 1 ml of pre-warmed TrypLE™ Express per 25 cm^2 of flask surface area and incubation for 30 minutes at 27 ºC enabled the creation of a homogenous single cell suspension of DSIR-HA-1179 cells while preserving cell viability (94%). TrypLE™ Express did not affect cell viability when it was used in routine cell passages until the fourth passage. In addition, it was verified that the inclusion of this enzyme in the cell culture process did not have any detrimental effect on the *in vitro* infection and production of OrNV.

Growth kinetics in TC-100, Sf-900 II and III culture media were comparable, with the highest mean specific growth rate of 0.0074 h^{-1} obtained in Sf-900 II. This value is an improvement over the specific growth rate estimated during establishment of the cell line, suggesting a possible benefit of adaptation of the cell line to more nutritionally suitable culture media. Glucose was determined to be a useful carbohydrate source in all four culture media, and it was totally depleted at the end of batch growth when its initial concentration was 1.0 g/l. Lactate and ammonia did not accumulate during batch growth of this cell line in TC-100, indicating an efficient use of nutrients in the culture medium. IPL-41 and TC-100 culture media supported the highest production of the virus (4.1 x 10^7 and 3.8 x 10^7 $TCID_{50}$/ml), compared to Sf-900 II and Sf-900 III (1.4 x 10^7 and 2.0 x 10^7 $TCID_{50}$/ml). TC-100 culture medium was selected for use in further development of the process since it assured both high cell and virus yields, in addition to offering a cost-benefit over IPL-41.

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Chapter 3

Growth, metabolism and *Oryctes* nudivirus production in attached T-flask cultures of the DSIR-HA-1179 insect cell line

3.1 Introduction

The *Oryctes* nudivirus (OrNV) is an entomopathogen which can be used as a biopesticide to control outbreak populations of the coconut rhinoceros beetle, a serious pest of coconut and oil palms (Huger, 2005).The current industrial method of OrNV production is based on infecting beetles larvae and adults with the virus and subsequently, harvesting the virus from macerated diseased insects (Gopal *et al*., 2001). Despite its economy, the *in vivo* production process is an artisanal method which suffers from inconsistencies in the concentration and purity of the final viral product, difficulties in process scale-up, control and validation of process parameters and maintenance of asepsis (Tramper and Vlak, 1986; Reid *et al*., 2013). *In vitro* production of OrNV which has been demonstrated in the susceptible and permissive anchorage-dependent DSIR-HA-1179 host insect cell line, is an attractive alternative production technology in which host cell growth and virus production can be closely controlled to assure a sterile product of known concentration and high purity.

The maximum cell density reached in *in vitro* attached cell cultures will depend on the surface area available for cell growth, whether cell growth is 'contact-inhibited' for the particular cell line, and the nutritional sufficiency of the culture medium to enable cells to grow to their maximal densities. The culture medium determines the nutritional and physicochemical environment of the culture, and influences the metabolic state of the cells; which in turn can impact cell growth and virus production (Hensler and Agathos, 1994; Matindoost *et al*., 2014). Insect culture media are supplied with nutrients such as carbohydrates, amino acids, vitamins, minerals, trace elements, lipids, organic salts and co-factors. Insect cells use carbohydrates supplied in the culture medium as their primary energy source to fuel their growth. While glucose is the preferred carbon source (Ferrance

et al., 1993), some cell lines have been shown to also actively consume other carbohydrates such as sucrose, maltose and trehalose reflecting a range of carbohydrate utilization profiles in insect cell lines (Clements and Grace, 1967; Lua and Reid, 2003; Stravoulakis *et al*., 1991). Amino acids are utilized by insect cells for the biosynthesis of proteins and nucleotides, but can also serve as energy sources by their incorporation into the tricarboxylic-acid (TCA) cycle as intermediates and via anaplerotic pathways. Glutamine, glutamate, asparagine and aspartate have been found to play an important role in energy generation in different insect cell lines. For Sf-9 cells, the most consumed amino acid is glutamine, which feeds into the TCA cycle as α-ketoglutarate via the glutamine/glutamate pathway (Drews *et al*., 2000). High Five cells on the other hand, consume asparagine extensively which is converted to aspartate and ultimately feeds into the tri-carboxylic acid cycle as oxaloacetate (Drugmand *et al*., 2012). Concomitant to the consumption of nutrients, the accumulation of potentially inhibitory by-products of metabolism such as lactate, ammonia, alanine, ethanol or urea may occur. H.zea cells accumulate high levels of alanine, lactate and ammonia when grown in asparagine and glutamine rich culture media, but not otherwise (Lua and Reid, 2003). When grown under non-limiting glucose and oxygen conditions, Sf-9 cells characteristically accumulate alanine but not lactate or ammonia; while High Five cells accumulate high levels of lactate and ammonia (Rhiel *et al*., 1997). On the other hand, saUFL-Ag-286 cells grown under similar conditions have a tendency to accumulate ammonia but no lactate or alanine (Gioria *et al*., 2006). These examples reveal the role of the culture medium in influencing cellular metabolism, and the diversity of metabolic profiles that exist between the different insect cell lines. Furthermore, virus infection induces major structural and functional changes in host insect cells. As a consequence, infected cells may display altered rates of nutrient consumption, metabolite production and oxygen uptake compared to uninfected cells (Kamen *et al*., 1996; Raghunand and Dale, 1999; Sugiura and Amman, 1996). Hence, for a particular virus-host cell system, there is a need to evaluate both uninfected and infected cell metabolism in order to anticipate and avoid nutrient limitations in order to obtain high virus yields (Gioria *et al*., 2006, Monteiro *et al*., 2012).

In Chapter 2 of this thesis, the preliminary characterization of DSIR-HA-1179 cell growth and metabolism in four different culture media was reported. Glucose was found to be a useful carbohydrate, while lactate and ammonia were produced at very low levels (Pushparajan *et al*., 2013). However, the amino acid metabolism of the cell line was not reported. Additionally, changes in the metabolic demands of the cells as a consequence of viral infection, if any, are yet to be

ascertained. The present chapter presents a detailed evaluation of the growth and metabolism of DSIR-HA-1179 cells grown in 10% serum supplemented TC-100 culture medium in attached batch cultures. Cell growth, nutrients consumption and metabolites production were quantified in uninfected cultures and in cultures that were infected with OrNV. The kinetics of OrNV production was also evaluated in the system. Analysis of these parameters over the entire culture period enabled a kinetic approach to allow a broader insight into nutrient utilization by the DSIR-HA-1179 cell line, which could potentially aid the design of low-cost culture media and/or the selection of appropriate feeding strategies for this cell line in future.

3.2 Materials and Methods

3.2.1 Cells and culture medium

The DSIR-HA-1179 cell line, originally established from surface sterilized eggs of the black beetle *Heteronychus arator* (Crawford, 1982), was obtained from the AgResearch (Lincoln, New Zealand) cell culture collection as adherent cultures in 25 cm^2 tissue culture flasks (Corning®). The cultures were maintained at 27 ºC, in 5 ml of 10% foetal bovine serum (FBS) (Life Technologies, New Zealand) supplemented PS-100 culture medium, which was prepared in-house. The cells were sequentially adapted to grow in 10% FBS supplemented TC-100 culture medium (Sigma) (Pushparajan *et al*., 2013). Cells were routinely maintained as attached working stock cultures at 27º C, in 25 cm^2 and 75 cm^2 tissue culture flasks with culture volumes of 5 and 15 ml respectively. Cultures were passaged every 10 days using a dilution factor of 1:5 with fresh culture medium. At the time of this study, the cells had been passaged 72 times in 10% serum supplemented TC-100 culture medium over a period of 24 months and displayed stable and reproducible growth.

In order to obtain a homogeneous cell suspension which could be counted on a Neubauer hemocytometer, it was necessary to dissociate confluent DSIR-HA-1179 cell monolayers in attached cultures by TrypLE™ Express treatment, as described by Pushparajan *et al*. (2013). Briefly, the spent culture medium was removed from the flask and 2 ml of Dulbecco's phosphate buffered saline (D-PBS) free of calcium and magnesium (Sigma) was used to wash the DSIR-HA-1179 cell monolayer. The spent D-PBS was removed, 1 ml of TrypLE™ Express (Life Technologies) pre-warmed to 27 ºC was added per 25 cm^2 of a confluent cell monolayer, and the

flask incubated at 27 ºC for 30 minutes. An appropriate volume of pre-warmed TC-100 culture medium supplemented with 10% FBS was added, and the cell suspension was gently aspirated with a 10 ml pipette to break up any remaining cell aggregates. In order to assess viable cell density and culture viability, a sample of the cell suspension was stained with 0.4% trypan blue (Sigma) and then both the total and viable cells were counted in duplicate using a Neubauer hemocytometer (Philips, 1973).

3.2.2 Cell growth kinetics in cultures supplemented with varying FBS concentrations

DSIR-HA-1179 cells growth kinetics were conducted on attached cultures grown in TC-100 culture medium supplemented with a range of FBS concentrations (0%, 1%, 2.5%, 5% and 10% FBS) in 25 cm^2 tissue culture flasks. Cultures in 1%, 2.5%, 5% and 10% FBS were passaged four times in their respective culture media before the experiment was set up. For each FBS concentration tested, TrypLE™ Express treatment (as described above) of a confluent cell monolayer in a 75 cm^2 tissue culture flask, provided sufficient number of cells to seed 14 replicate 25 cm^2 tissue culture flasks, each at an initial cell density of 2 x 10^5 viable cells/ml (day 0) in a culture volume of 5 ml. Duplicate cultures were randomly selected and harvested every 48 hours and total and viable cell counts determined until day 14 of batch growth. Culture supernatants were collected from cultures grown in 10% FBS over the growth phase. These were clarified by centrifugation at 10,000*g* for 5 min and stored at -20º C for analyses of nutrients and metabolites.

The specific growth rate (μ) was calculated by identifying the linear region from the semilog plot of viable cell density versus time, followed by linear regression of the data (Gioria *et al.,* 2006). Population doubling time (t_d) was calculated by equation 1:

$$\text{td} = \frac{Ln\,(2)}{\mu} \qquad (1)$$

3.2.3 Virus and virus quantification

OrNV stock (strain X2B) at a concentration of 1 x 10^7 $TCID_{50}$/ml was obtained from AgResearch. X2B is a highly virulent strain originally isolated in 1983 from a field population of infected coconut rhinoceros beetles on Bugsuk Island, Palawan, Philippines (Crawford *et al*., 1986, Zelazny

et al., 1990). The virus stock used in this study was prepared by infection of attached cultures during the early exponential phase of growth (approximately 5 x 10^5 viable cells/ml) at a MOI of 0.1 $TCID_{50}$/cell. OrNV infectious titer was quantified by end-point dilution analysis. Briefly, to determine the $TCID_{50}$, suspensions of DSIR-HA-1179 cells (2.5 x 10^5 cells/ml) were seeded onto 96 well plates (50 µl per well), and then an equal volume of each viral supernatant dilution (diluted in ten-fold series from 10^{-2} to 10^{-9}) was added with five replicates per supernatant. The plates were placed in a humidified, disinfected plastic container and incubated at 27 ºC for 11 to 14 days until the cytopathic effect was well developed, when the plates were scored for infection, and $TCID_{50}$ was calculated (Reed and Muench, 1938).

3.2.4 Virus production kinetics

OrNV production kinetics was assessed in attached cultures grown in TC-100 culture medium supplemented with 10% FBS in 25 cm^2 tissue culture flasks, each with a culture volume of 5 ml. TrypLE™ Express treatment (as described above) of a confluent cell monolayer in a 75 cm^2 tissue culture flask, provided sufficient number of cells to seed fourteen replicate 25 cm^2 flasks, each at an initial cell density of 2 x 10^5 viable cells/ml (Day 0) in a culture volume of 5 ml. The cultures were incubated at 27 ºC and were each infected with OrNV stock 144 h later (Day 6) when the cells had reached a density of 5 x 10^5 viable cells/ml at a MOI of 5 $TCID_{50}$/cell. Thereafter, duplicate cultures were randomly selected and harvested every 48 hours and viable cell counts determined until day 14 post-infection. Culture supernatants were collected from harvested cultures, clarified by centrifugation at 10,000*g* for 5 min and stored at -20º C for the quantification of extracellular OrNV infectious titers and for the analyses of nutrients and metabolites. The cell specific OrNV yield ($TCID_{50}$/cell) was calculated by dividing the maximum OrNV volumetric yield ($TCID_{50}$/ml) by the peak viable cell density reached post-infection (cells/ml) (Chakraborty *et al*., 1996; Micheloud *et al*., 2009). The time of harvest (TOH) was the post-infection time-point (day) when the maximum OrNV volumetric yield was reached. Volumetric productivity was calculated by dividing the maximum volumetric yield by the total culture time taken to reach this yield (Astray *et al*. 2012).

3.2.5 Nutrient and metabolite analyses

Glucose concentrations were determined using an enzymatic test kit (Sigma) based on the hexokinase-catalyzed oxidation of glucose (Slein 1963). Lactate concentrations were determined using a colorimetric test kit (Sigma) based on the oxidation of lactate by lactate dehydrogenase (Schweiger and Gunther, 1964). Ammonia concentration was determined using an enzymatic test kit (Sigma) based on the glutamate dehydrogenase-catalyzed oxidation of nicotinamide-adenine dinucleotide in the presence of ammonia (Mondzac *et al.,* 1965). Glutamine concentrations were determined using a YSI 2700 biochemistry analyzer (YSI, Yellow Springs, OH, USA). The total concentrations of 18 different amino acids were determined by reverse-phase high-performance liquid chromatography (HPLC) (AccQ.Tag, Waters Corporation, USA) using precolumn derivatization with 6-aminoquinolyl N-hydroxysuccinimidyl carbamate (Cohen, 2000). Prior to derivatization, sample proteins were hydrolyzed into their respective amino acids by treatment with 6N hydrochloric acid for 24 h at 110º C (AOAC official method 4.1.11: 994.12c, 1998). Cysteine and methionine were analyzed as cysteic acid and methionine sulfone after cold performic acid oxidation overnight prior to hydrolysis (AOAC official method 4.1.11: 994.12a, 1998). Tryptophan was determined after alkaline hydrolysis with sodium hydroxide for 22 h at 110º C (AOAC official method 988.15, 1995). For cells in the exponential growth phase, specific consumption rates of nutrients or production rates of metabolites were calculated by determining cell yield (given by the slope of the plot of concentration of the analyte of interest versus viable cell density), and then multiplying this value by the specific growth rate. For infected cells, the specific consumption rates of nutrients or production rates of metabolites were determined by evaluating the rate of change of the analyte of interest over a given time-period and then dividing by the average viable cell density during the respective time period (Gioria *et al.,* 2006).

3.3 Results

3.3.1 Cell growth

Supplementation of basal culture media with fetal bovine serum has been essential for the culture of DSIR-HA-1179 cells, with the cell line having been previously maintained in Schneider's Drosophila culture medium and in PS-100 culture medium, both supplemented with 10% FBS (Crawford, 1982; Scotti P.D., unpublished data). An experiment was conducted to determine the minimum concentration of FBS which permits the growth of DSIR-HA-1179 cells and the effect

of different FBS concentrations on the extent and kinetics of cell growth in TC-100 culture medium. Fig. 3-1 (A) shows the time course profiles of mean viable cell density and culture viability in attached DSIR-HA-1179 cultures grown in TC-100 supplemented with 0%, 1%, 2.5%, 5% and 10% FBS. Fig. 3-1 (B) is a plot of the maximum viable cell density and specific growth rate as functions of the FBS concentration.

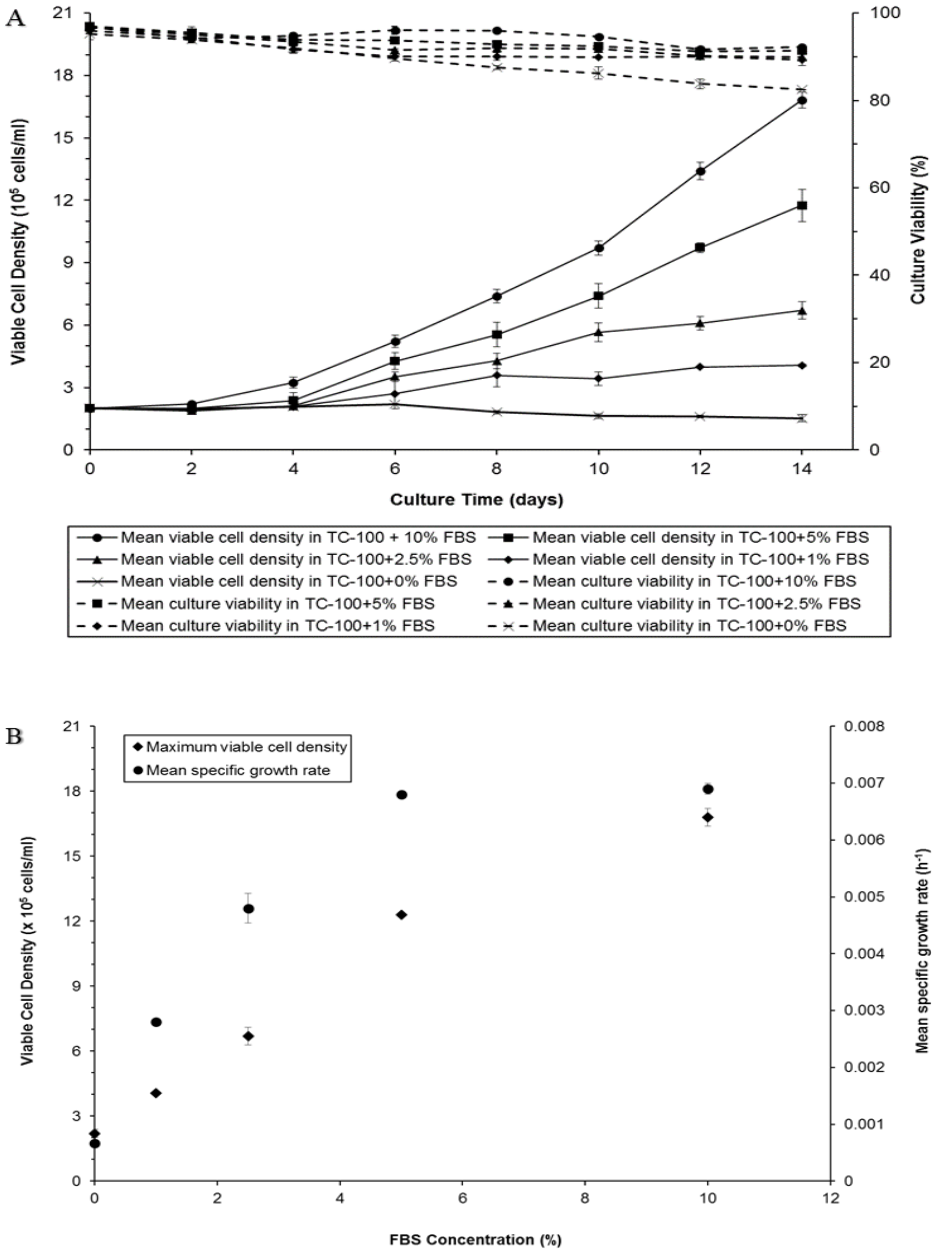

Fig. 3-1 (A) Cell growth (solid lines) and culture viability (dotted lines) in DSIR-HA-1179 cell cultures grown in TC-100 supplemented with varying amounts of fetal bovine serum (0%, 1%, 2.5%, 5% and 10% FBS) in 25 cm^2 T-flask cultures. (B) Mean maximum viable cell density and specific growth rate as

functions of the FBS concentration in DSIR-HA-1179 cell cultures grown in TC-100 in 25 cm^2 T-flasks. Error bars represent standard deviations from the mean of duplicate samples.

From Fig. 3-1 (A) it can be observed that in cultures grown without FBS, cells attached to the flask surface but failed to proliferate over the course of the culture. At the end of the batch growth period, mean viable cell density and culture viability in these cultures had declined to 1.53 x 10^5 cells/ml and 82.5%, respectively. In cultures supplemented with 1% FBS and 2.5% FBS, cells grew with mean specific growth rates of 0.0028 h^{-1} (t_d: 10.33 days) and 0.0048 h^{-1} (t_d: 6.01 days). The mean maximum viable cell density was reached on day 14 in cultures supplemented with 1% FBS (4.06 x 10^5 cells/ml) and with 2.5% FBS (6.7 x 10^5 cells/ml). In both conditions, mean culture viability remained at 90.3% and 91.5%, respectively. Cultures grown in TC-100 supplemented with 5% FBS and 10% FBS grew exponentially between days 4 and 14 of culture, with mean specific growth rates of 0.0068 h^{-1} (t_d: 4.23 days) and 0.0069 h^{-1} (t_d: 4.20 days). Mean culture viabilities in both of these conditions remained high at 92.3% and 92.9% respectively. However, the maximum viable cell density reached in cultures supplemented with 10% FBS (16.8 x 10^5 cells/ml) was higher than in cultures that were supplemented with 5% FBS (12.3 x 10^5 cells/ml). Consequently, whereas cultures grown with 10% FBS were 100% confluent on day 14 of culture, cultures grown with 5% FBS were only 80% confluent. From Fig. 3-1 (B), it appeared that supplementing DSIR-HA-1179 cultures with 10% FBS led to both the maximum cell yield and specific growth rate. Therefore, TC-100 supplemented with 10% FBS was chosen to further develop the process.

3.3.2 Nutrient consumption and metabolism in uninfected culture

To characterize the nutritional and metabolic profiles of DSIR-HA-1179 cells grown in TC-100 culture medium supplemented with 10% FBS, concentrations of glucose, glutamine, lactate and ammonia were assessed in supernatant samples obtained over the 14 day period of batch growth. The concentrations of eighteen amino acids in culture supernatants were also assessed at the beginning and at the end of the batch growth period.

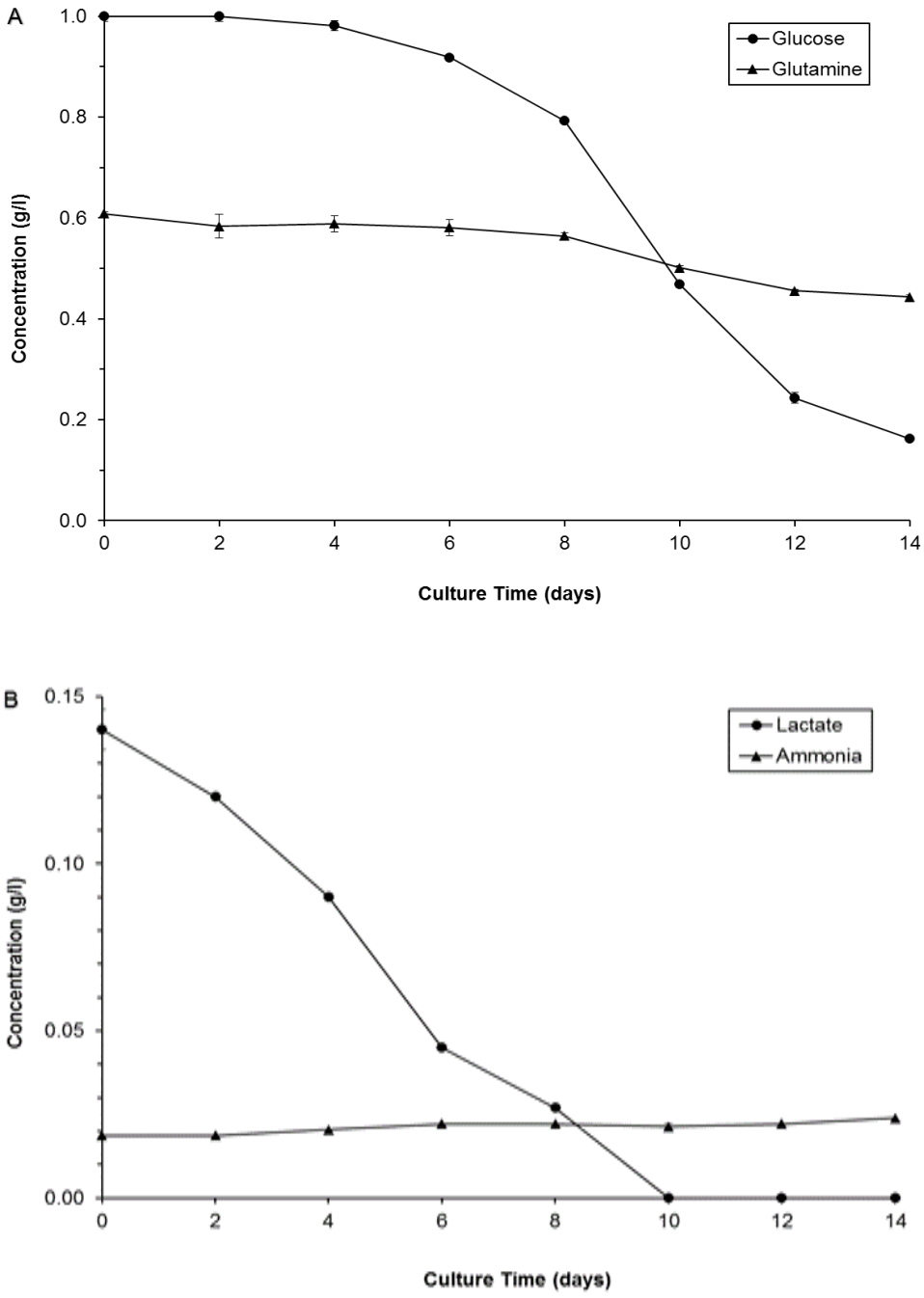

Fig. 3-2 (A) Glucose, glutamine and (B) lactate and ammonia profiles in uninfected cultures of the DSIR-HA-1179 cell line grown in in TC-100 supplemented with 10% FBS in 25 cm^2 T-flasks. Error bars represent the standard deviations from the mean of duplicate samples. Error bars are difficult to see because standard deviations were very small.

As shown in Fig. 3-2 (A), glucose, the sole carbohydrate in TC-100 culture medium, was consumed over the course of the culture. The consumption of glucose in the first 4 days of culture was minimal, which was expected as the cells were in the lag phase. Between days 4 and 14 of culture, correlating to the exponential growth phase, glucose was consumed at a mean specific rate of 4.1 x 10^{-12} g/c/h. At the end of batch growth, a small amount of glucose remained unconsumed which indicated that cells did not face glucose limitation over the course of the culture. In comparison with glucose, glutamine was consumed to a lesser extent (27.9% of its initial concentration), and at a slower rate (8.3 x 10^{-13} g/c/h) over the period of batch growth. From Fig. 3-2 (B), it can be seen that lactate which is a by-product of glucose and glutamine metabolism in many animal cell lines, was not produced by DSIR-HA-1179 cells. The small concentration of lactate (0.22 g/l) which was present at the start of the culture was rapidly consumed and was exhausted from the culture medium by day 10 of culture. Ammonia which generally accumulates as a result of amino acid catabolism, was detected at very low levels (0.02 g/l) and remained unchanged in its concentration over the course of batch growth.

Table 3-1 Amino acid concentration at the beginning and end of batch growth in uninfected cultures of the DSIR-HA-1179 cell line grown in TC-100 supplemented with 10% FBS in 25 cm^2 T-flasks. Concentrations of all amino acids are expressed in mg/g, except for glutamine which is expressed in g/l.

Amino Acid	Amino Acid Conc. 0 h	Amino Acid Conc. 336 h	% Consumption
Alanine	0.54	0.77	- 42.6
Arginine	0.83	0.83	0
Aspartic Acid	1.29	1.18	8.5
Cysteine	0.22	0.21	4.5
Glycine	0.79	0.80	- 1.3
Glutamic Acid	2.07	1.81	12.6
Histidine	2.41	2.54	- 5.4
Isoleucine	0.31	0.26	16.5
Leucine	0.74	0.64	13.5
Lysine	1.01	1.02	- 1.0
Methionine	0.18	0.25	- 18.9
Phenylalanine	0.51	0.48	5.9
Proline	0.75	0.65	13.3
Serine	0.70	0.65	7.1
Tyrosine	0.31	0.29	6.5
Threonine	0.48	0.44	8.3
Tryptophan	0.14	0.15	- 7.1
Valine	0.61	0.57	6.6
Glutamine	0.61	0.44	27.9

As shown in Table 3-1, none of the amino acids supplied were significantly consumed in DSIR-HA-1179 cell cultures. Only glutamine (27.9%), methionine (18.9%), and isoleucine (16.5%) were consumed in percentages exceeding 15% of its initial concentration. The percentage consumption of leucine, proline and glutamic acid were 13.5%, 13.3% and 12.6% respectively, while for the remaining amino acids, there was either minimal or no consumption noted. Alanine was the only amino acid that was significantly produced and accumulated in the culture medium to 42.6% of its original concentration by the end of batch growth.

3.3.3 OrNV infection

Attached DSIR-HA-1179 cultures were infected with OrNV at a cell density of 5.2 x 10^5 viable cells/ml and a multiplicity of infection of 5 $TCID_{50}$/cell. The kinetics of infection were evaluated based on the evolution of viral titers and the concomitant decrease in viable cell density post-infection (Fig. 3-3).

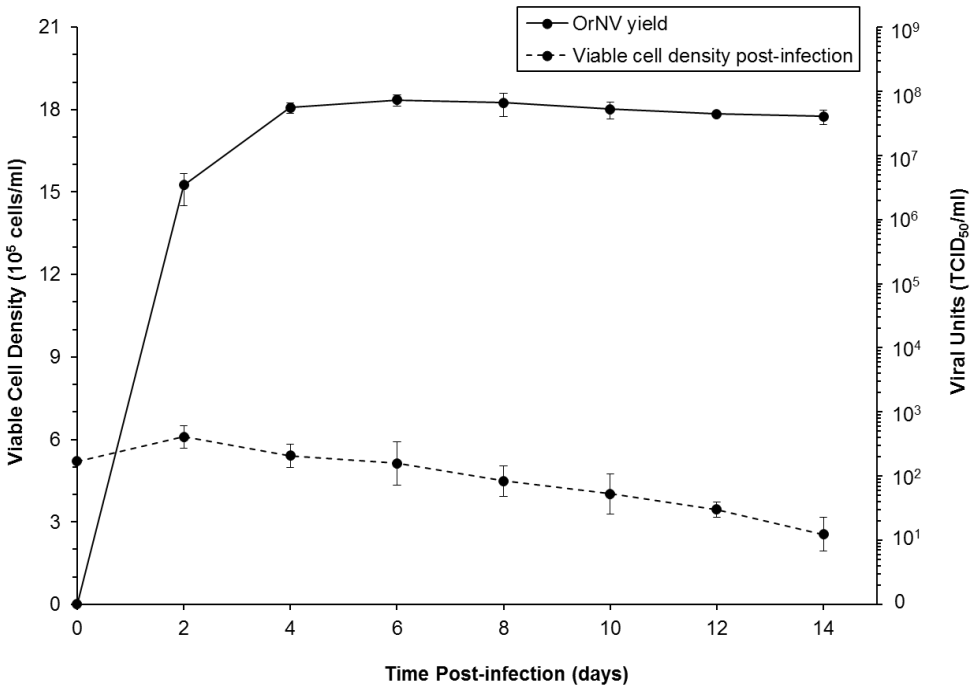

Fig. 3-3 Time course profiles of viable cell density post-infection (dashed line) and OrNV production (solid line) in infected cultures of the DSIR-HA-1179 cell line, grown in TC-100 supplemented with 10% FBS, in 25 cm^2 T-flasks. Cells were infected at a density of 5.2 x 10^5 viable cells/ml and multiplicity of infection of 5 $TCID_{50}$/cell. Error bars represent standard deviations from the mean of duplicate samples.

The first 48 hours of infection were characterized by a slight increase in the viable cell density to 6.09 x 10^5 viable cells/ml. Subsequently, the number of viable cells decreased steadily to a concentration of 2.5 x 10^5 viable cells/ml on the 14^{th} day post-infection. The cytopathic effects of OrNV infection characterized by the rounding up and hypertrophy of infected cells, could be observed microscopically from 24 hours post-infection. From Fig. 3-3, it can be seen that the rate of OrNV production was highest during the first 48 hours post-infection, followed by a period of slower incremental production between 48 and 144 hours post-infection. The maximum volumetric yield of 7.38 x 10^7 $TCID_{50}$/ml (cell specific yield of 121 $TCID_{50}$/cell) was reached at 144 hours post-infection. The volumetric productivity of the culture was 6.15 x 10^6 $TCID_{50}$/ml/day. Subsequent to the attainment of the maximum OrNV yield, it was found that prolonging the infection period between days 8 and 14 post-infection led to a slight decay in the OrNV volumetric yield to 4.04 x 10^7 $TCID_{50}$/ml on day 14 post-infection.

3.3.4 Nutrient consumption and metabolism in OrNV-infected cultures

The consumption of glucose, glutamine (Fig 3-4A), lactate and ammonia (Fig 3-4B) were evaluated in OrNV-infected DSIR-HA-1179 cultures grown in TC-100 culture medium supplemented with 10% FBS over the 14 day period of infection. Similar to uninfected cultures, glucose and glutamine were the nutrients most consumed by infected cultures. Glucose was completely exhausted from the culture by day 12 post-infection. In comparison, glutamine was only consumed to 29.3% of its concentration at the time of infection. The specific rates of consumption of these nutrients were evaluated between the time of infection and the post-infection time when the maximum OrNV volumetric yield was reached (144 h.p.i). In the first 48 hours of infection, the specific rate of glucose consumption increased from 2.2 x 10^{-12} g/c/h to 9.17 x 10^{-12} g/c/h. Between 48-96 hours post-infection, this value decreased to 2.54 x 10^{-12} g/c/h and again increased to 7.34 x 10^{-12} g/c/h between 96-144 hours post-infection. The specific rate of glutamine consumption increased from 2.68 x 10^{-13} g/c/h to 1.52 x 10^{-12} g/c/h during the first 48 hours post-infection. Between 48-96 hours post-infection, its value decreased to 6.27 x 10^{-13} g/c/h and again increased to 1.1 x 10^{-12} g/c/h between 96-144 hours post-infection. From Fig 3-4 (B), it can be observed that a very low concentration of lactate (0.03 g/l) was present in the culture medium at the time of infection, which

was rapidly consumed by day 4 post-infection. Ammonia, which was detected at very low levels of approximately 0.02 g/l, was neither consumed nor produced in infected cultures.

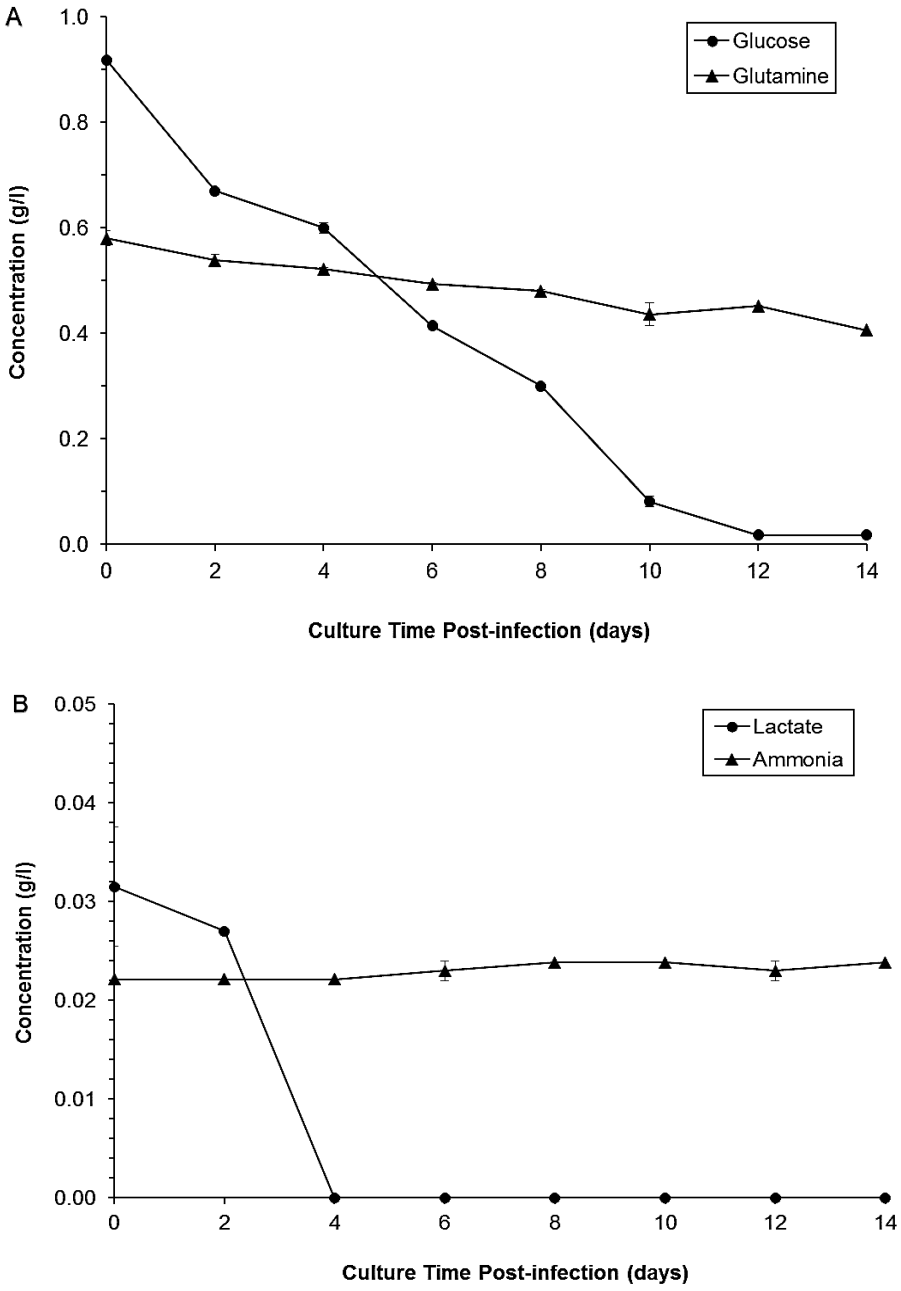

Fig. 3-4 (A) Glucose, glutamine and (B) lactate and ammonia profiles in infected cultures of DSIR-HA-1179 cell line grown in TC-100 supplemented with 10% FBS in 25 cm^2 T-flasks. Cultures were infected at a density of 5.2 x 10^5 viable cells/ml and multiplicity of infection of 5 $TCID_{50}$/cell. Error bars represent the standard deviations from the mean of duplicate samples. Error bars are difficult to see because standard deviations were very small.

Table 3-2 Amino acid concentration at the beginning and end of batch growth in infected cultures of the DSIR-HA-1179 cell line grown in TC-100 supplemented with 10% FBS in 25 cm^2 T-flasks. Cultures were infected at a density of 5.2 x 10^5 viable cells/ml and multiplicity of infection of 5 $TCID_{50}$/cell. Concentrations of all amino acids are expressed in mg/g, except for glutamine which is expressed in g/l.

Amino Acid	Amino Acid Conc. 0 hpi	Amino Acid Conc. 336 hpi	% Consumption
Alanine	0.53	0.70	- 32.1
Arginine	0.79	0.80	- 1.3
Aspartic Acid	1.16	1.14	1.7
Cysteine	0.19	0.17	10.5
Glycine	0.76	0.80	- 5.3
Glutamic Acid	1.89	1.73	8.5
Histidine	2.48	2.59	- 4.4
Isoleucine	0.27	0.26	3.7
Leucine	0.61	0.61	0
Lysine	0.97	0.96	1.0
Methionine	0.25	0.24	4
Phenylalanine	0.46	0.44	4.4
Proline	0.65	0.59	9.2
Serine	0.66	0.65	1.5
Tyrosine	0.30	0.24	20
Threonine	0.41	0.43	- 4.9
Tryptophan	0.15	0.16	- 6.7
Valine	0.51	0.52	- 1.9
Glutamine	0.58	0.41	29.3

The concentrations of eighteen amino acids in culture supernatants at the beginning and at the end of infection, were assessed in order to evaluate the amino acid requirements of infected DSIR-HA-1179 cells (Table 3-2). The highest percentage consumption was noted for glutamine (29.3%), followed by tyrosine (20%). All other amino acids were consumed minimally, to less than 10% of their original concentrations. The only amino acid which was significantly produced was alanine (32.1%), while other amino acids such as arginine, glycine, histidine, valine, threonine and tryptophan were produced at very low levels.

3.4 Discussion

Effects of FBS concentration on DSIR-HA-1179 cell growth

Fetal bovine serum is a complex mixture of growth factors, vitamins, carrier proteins, protease inhibitors, detoxifying proteins, mineral oligoelements, cholesterol and lipids, all of which are vital for insect cell growth (Barnes and Sato, 1980). Supplementation of basal culture medium with FBS has been necessary for the continued propagation of a variety of insect cell lines. However, the concentration of FBS which results in maximal growth differs between cell lines. For example, for *A. aegypti* cells, maximum growth was obtained with 10% FBS, while maximum growth of *A. eucalypti* and *B. mori* cells was obtained in 5% FBS (Sohi and Smith, 1970). The results of our study showed that FBS supplementation of TC-100 culture medium was essential for the growth of DSIR-HA-1179 cells. The failure of DSIR-HA-1179 cells to proliferate in the absence of FBS can be explained by deficiencies of specific molecules contained only in serum, such as fibronectin and cholesterol, which are essential for cell adhesion and growth (Lery and Fediere, 1990). Cell growth was observed in cultures grown in 1% and 2.5% FBS, however, the specific growth rate was impaired and cells grew to very low maximum cell densities, indicating that at these concentrations, one or more crucial components of FBS required for cell growth was limiting. Between 5% and 10% FBS, serum concentration did not affect the specific growth rate, but affected the maximum cell density obtained, in a dose-dependent manner. Consequently, in the concentration range tested, the highest maximum viable cell density was reached in cultures supplemented with 10% FBS. Therefore, at this stage of process development, supplementation of TC-100 culture medium with 10% FBS was found to be necessary. Despite its growth promoting effects, the use of FBS entails a number of disadvantages including high cost, undefined composition, batch to batch variability, risk of adventitious agents and difficulties in downstream processing. The need to replace FBS in culture medium becomes especially pertinent for the *in vitro* production of low-margin products such as biopesticides, since commercial production can become economically viable only through the use of a low-cost and serum-free culture medium (Claus *et al*., 2012). Combinations of supplements such as protein hydrolysates, microbial extracts and lipid and sterol microemulsions have been used as serum substitutes without reductions in cell and virus productivity (Batista *et al.,* 2005; Donaldson and Shuler, 1998, Ikonomou *et al*., 2001; Maiorella *et al.,* 1988; Roder, 1982). The finding that FBS at the concentration of 10% v/v is

essential for optimal DSIR-HA-1179 cell growth, is an indicator that an important aspect of process optimization will be the formulation of a specific nutrient mixture to replace serum in these cultures; despite this goal being outside the scope of this thesis.

Metabolism of uninfected DSIR-HA-1179 cells

Similar to other insect cell lines, glucose and glutamine are the main sources for carbon and energy in DSIR-HA-1179 cell cultures. Over the batch growth period, neither glucose nor glutamine were consumed to the extent where they became limiting for cell growth. The mean specific rate of glucose consumption in uninfected DSIR-HA-1179 cells (4.1×10^{-12} g/c/h) was broadly comparable to values published for some insect cell lines, such as Sf-9 (1.98×10^{-12} g/c/h), Sf-21 (5.1×10^{-12} g/c/h), sa-UFL-Ag286 (5.77×10^{-12} g/c/h) and Hz-AM1 (18×10^{-12} g/c/h) (Gioria *et al.,* 2006; Kamen *et al.,* 1991; Kwon *et al.,* 2003;; Lua and Reid, 2003). However it was much lower compared to the glucose consumption rates of High Five cells (45.4×10^{-12} g/c/h) grown in high density culture (Rhiel *et al.,* 1997). The moderate rate of glucose consumption in DSIR-HA-1179 cells may be due to the low concentration of glucose in TC-100, compared to other insect cell culture media (Bedard *et al*., 1993). It may also be explained by the slow growing nature of the DSIR-HA-1179 cell line whose specific growth rate is much lower than other insect cell lines (Pushparajan *et al*., 2013). Glutamine was consumed at a lower specific rate compared to other insect cell lines. The peak in cell-specific consumption rates of both glucose and glutamine occurred between day 8 and 10 of uninfected growth, which was the mid-exponential phase of growth in which cells are most actively dividing, and hence most metabolically active. A similar trend of peak consumption of these nutrients during the exponential phase, has been described for IPLB-Sf-21AE and Sf-9 cells cultivated in serum-supplemented EXCELL401 and TNM-FH culture media respectively (Mendonca *et al.* 1999; Neerman and Wagner, 1996).

The metabolism of the DSIR-HA-1179 cell line is characterized by a lack of production of lactate and ammonia, but an accumulation of alanine. In these respects, DSIR-HA-1179 cell line metabolism may be likened to that of cell lines derived from *Spodoptera frugiperda* ovarioles (Sf-9 and Sf-21). Lactate is a by-product of the anaerobic metabolism of glucose and the amount accumulated by insect cells is generally dependent on the amount of excess glucose present in the culture medium. The small amount of lactate that was present at the beginning of the culture, which was probably derived from FBS, was consumed by the cells during the exponential growth phase.

The consumption of this residual lactate and the lack of lactate production over batch growth, indicated aerobic respiration i.e. that the oxygen transfer in 25cm^2 attached T-flask cultures was sufficient to meet cellular demand (Bovo *et al.,* 2008; Maranga *et al.,* 2002a). Sf-9 cells are characterized by low yields of lactate on glucose (0.02), high glycolytic flux and conversion of approximately 60% of glucose consumed into TCA intermediates (Neerman and Wagner, 1996). The active TCA cycle in these cells may account for their high specific growth rate (0.025 h^{-1}). While the possibility exists that the non-accumulation of lactate in DSIR-HA-1179 cell cultures might be due to a high glycolytic flux and efficient TCA cycle in these cells, this has not manifested in a high cell specific growth rate of the cell line. A more likely explanation could be that, rather than being completely oxidized, the carbon from glucose is derived to other non-measured metabolites such as ethanol or glycerol. A thorough investigation of the cell lines' metabolism through radio-labelling studies is required before either hypothesis can be confirmed (Bhatia *et al.,* 1997; Doverskog *et al.,* 1997). Nevertheless, the fact that the cell line is a non-producer of lactate is a promising aspect for its high-density culture, from an industrial perspective. In future work, it would be interesting to study the lactate consumption behavior of DSIR-HA-1179 cells during uninfected and infected growth, by growing and infecting the cell line in culture medium containing higher concentrations of lactate.

Most of the amino acids supplied in the TC-100 culture medium were not consumed by DSIR-HA-1179 cells to a significant extent. Although the percentage consumption of glutamine (27.9%) was the highest compared to the other amino acids, more than half of the glutamine supplied in the culture medium remained unconsumed at the end of batch growth. While glutamine is essential as an energy source and for biomass and nucleic acid synthesis, the hydrolysis of glutamine is also a major source of ammonia production. The significant production of alanine by DSIR-HA-1179 cells can be explained in context of the non-accumulation of ammonia in these cultures. Alanine is produced as a means of detoxifying the culture of ammonia produced; a salient metabolic feature of insect cell lines such as Sf-9 and *H.zea* when grown under non-limiting glucose conditions (Bedard *et al*., 1993, Lua and Reid, 2003). The percentage of alanine produced (31.1%) was comparable to that produced in Sf-9 cells (38.7%), under similar culture conditions and was not toxic to cells at this concentration (Drews *et al.,* 1995). However, the conversion of ammonia to alanine requires the transamination of pyruvate, and is an energetically wasteful reaction (Ferrance *et al.,* 1993; Ohman *et al,.* 1995). Therefore, in the light of the fact that most of the amino acids

supplied in TC-100 culture media were not utilized by the cells, a recommendation can be made for lower amino acid concentrations including glutamine in a low-cost culture medium formulated specifically for the DSIR-HA-1179 cell line. This can be done by reducing concentrations of individual amino acids or replacing them with hydrolysates which are cheaper sources of their supply. The reduction of amino acid concentrations may result in enhanced cell metabolism, and ensure process economy since amino acids are the most expensive components of basal culture media.

Infected DSIR-HA-1179 cell metabolism

The efficient realization of virus replication in host insect cell lines may trigger changes in the physiology and metabolism of the host cells. Since the supply of nutrients in the culture medium is central to the physiology of cells, it must therefore be expected that viral infection will induce changes in the nutrient requirements by the cells. If the nutritional demands of the cells, exceed the capacity of the culture medium to provide them with the required nutrients, then viral production could be limited. DSIR-HA-1179 cultures that were infected during the early-exponential phase, did not face any nutritional limitation at the time of infection, as the culture medium contained almost 90% and 95% of the original concentrations of glucose and glutamine, and lactate and ammonia concentrations were negligible.

In the immediate 48 hour period following infection with OrNV, there was a marked increase in the specific uptake rates of both glucose and glutamine. Similar behavior have been observed in other virally infected insect cell lines (Radford *et al*., 1997; Raghunand and Dale, 1999; Rhiel *et al.,* 1997; Rodas *et al.,* 2005; Sugiura and Amman, 1996) and mammalian cell lines (Gray *et al.,* 1986; Warren *et al*., 1986). Previous studies on OrNV pathogenesis in DSIR-HA-1179 cells observed that in a single infection cycle, virus replication lasted between 7-12 hours post-infection, followed by virus assembly and budding which continued for more than 36 hours post-infection (Crawford and Sheehan, 1985). Therefore, increased glucose uptake during the first 48 hours post-infection can be correlated to the active virus replication occurring in the cells. One possible explanation for this would be that the nutritional and metabolic requirements to support OrNV replication in DSIR-HA-1179 cells are higher than that required for cellular multiplication. An alternative hypothesis could be that, the stress of viral infection causes glucose transport into cells

to become up-regulated, rather than increased metabolism (Raghunand and Dale, 1999). OrNV production was not affected by nutrient deprivation, since both glucose and glutamine were in plentiful supply from the time of infection and until day 6 post-infection when the maximum OrNV volumetric yields were reached.

Infected cells were deprived of glucose but not glutamine in the last 4 days of the infection phase. Glucose limitation may explain the 37% drop in viable cell density over this period, but glutamine was still being consumed and its conversion to α-ketoglutarate could still fuel the survival of the remaining cells in culture. Glucose limitation would result in a diminished production of pyruvate, reducing both its flux into the TCA cycle for energy production as well as alanine synthesis via transamination (Mendonca *et al*., 1999). This would explain the lower percentage of alanine accumulation during the infection, as compared to uninfected growth. There is also the possibility that alanine was back-consumed by cells and converted to pyruvate using alanine transaminase. Rapid consumption of lactate in infected cultures could also indicate lactate dehydrogenase-mediated conversion to pyruvate to sequester energy.

Under conditions of glucose limitation but glutamine excess, up-regulation of the glutaminase/glutamate pathway could lead to an accumulation of ammonia in the culture, with no means for it to be non-toxically eliminated as alanine (Ohman *et al*., 1995). Such ammonia accumulation under glucose limitation, due to glutamine catabolism through the successive activity of glutaminase and glutamate dehydrogenase, has been documented for the Sf-9 cell line (Benslimane *et al.,* 2005; Drews *et al.,* 2000). However, ammonia was not produced in infected DSIR-HA-1179 cultures under glucose limitation. An alternative mechanism for the detoxification of ammonia from the culture medium for example, the synthesis of uric acid, urates, allantoin or allantoic acid via the uricotelic pathway, could be a possibility. Uricotelism is the main method of nitrogen excretion in insects. Production of uric acid has been demonstrated as a mechanism for ammonia removal in the Bm-5 insect cell line (Stavroulakis *et al*., 1991). Further tests for the presence of uric acid or its intermediate metabolites in glucose-deprived DSIR-HA-1179 cultures must be undertaken before this hypothesis can be accepted. Another possibility for the metabolic disposal of ammonia, could be the presence of an active glutamine synthetase-glutamate synthase (GS-GOGAT) pathway in DSIR-HA-1179 cells, similar to that described for Sf-9 cells (Drews *et al.,* 2000). Glutamine synthetase catalyzes the production of endogenous glutamine in cells, via a

condensation reaction incorporating ammonia and glutamate. Further tests are required to confirm the presence of this enzyme in DSIR-HA-1179 cells.

OrNV production in attached DSIR-HA-1179 cell cultures

The maximum OrNV volumetric yield obtained through the infection of DSIR-HA-1179 attached cell cultures in this study (7.38 x 10^7 $TCID_{50}$/ml), was higher than the maximum yield obtained from infected DSIR-HA-1179 attached cultures in a previous work (5.79 x 10^7 $TCID_{50}$/ml) (Crawford and Sheehan, 1984). The differences in the virus yields can be ascribed to the use of a different culture medium (Schneider's tissue culture medium) and indeterminate infection parameters such as the multiplicity and time of infection, in the earlier work. Nevertheless the improvement in virus yield in our study confirms that the cells we have adapted to grow in TC-100 culture medium are highly susceptible and permissive to OrNV infection. OrNV is the only nudivirus that has been propagated in *in vitro* insect cell cultures till date, and therefore there is no antecedent to compare yields within the same viral group. The volumetric and cell specific virus yields of OrNV produced in this study (7.38 x 10^7 $TCID_{50}$/ml, 121 $TCID_{50}$/cell) are slightly lower in comparison with yields obtained in other industrially important *in vitro* production systems for baculovirus; for example, AcMNPV production in Sf-9 cells (1 x 10^9 pfu/ml, 1300 pfu/cell), AgMNPV in saUFL-Ag-286 cells (6.3 x 10^8 TCID50/ml, 692 $TCID_{50}$/cell) and HearNPV in HzAM1 cells (1 x 10^8 pfu/ml, 50-100 pfu/cell) (Gioria *et al.,* 2006; Kloppinger *et al.,* 1990; Reid *et al.,* 2013). The lower volumetric yield is a consequence of a relatively low cell-specific yield, combined with the reduced cell density in T-flask cultures (Pollard and Khosrovi, 1978). The lower cell-specific OrNV yield may be due to individual cell line and culture medium characteristics, which can influence virus binding kinetics and replication (Pedrini *et al.,* 2011). Nutritional deficiencies or accumulation of toxic metabolites in the culture medium could be potentially inhibitory to virus replication, however, this was confirmed not to have occurred in this study since glucose and glutamine were not limiting during viral replication, nor was there accumulation of lactate or ammonia. Another factor which may have affected the OrNV yield is the possibility of the onset of the cell-density effect in DSIR-HA-1179 cultures at the time of infection. The cell-density effect is characterized by an intrinsic reduction in the capacity of insect cells to support viral replication as the infection is delayed in time. The critical cell density at which cell-specific virus productivities drop varies depending on the specific cell line-virus combination and

cultivation regime. This phenomenon has been attributed to several causes including contact inhibition, nutrient deficiencies, accumulation of by-products, autocrine factors, changes in the cell cycle and drop in central metabolic energy (Bedard *et al.,* 1994; Bernal *et al.,* 2009; Calles *et al.,* 2006; Taticek and Shuler, 1997; Wood *et al.,* 1982). In particular, for cells growing as attached cultures, cell-to-cell contact was found to inhibit viral replication (Stockdale and Gardiner, 1977; Wood *et al.,* 1982). In attached cultures of the BTI-Tn-MG-1, BTI-EA-88 and MB0503 insect cell lines, the optimal cell densities for virus infection were found to be low as 2.5 x 10^4, 5 x 10^4 and 7.5 x 10^4 cells/cm^2, respectively (Davis *et al.,* 1993). The general strategy to avoid the cell-density effect is to infect cultures synchronously and during the early exponential phase of growth (Visnovsky and Claus, 1994). Despite this infection strategy having been followed in this study, the possibility of a reduced OrNV yield due to the cell-density effect cannot be ruled out, since no studies on the susceptibility of the DSIR-HA-1179 cell line to the cell-density effect, have been performed to date. Future work will focus on understanding the cell-density effect and its impact on OrNV production in DSIR-HA-1179 cell cultures. This knowledge will aid in the selection of optimal infection parameters to infect DSIR-HA-1179 cells such as the multiplicity and time of infection, which can potentially improve OrNV specific yields.

For the routine production of OrNV, infected cell cultures are generally incubated for at least 10 days post-infection, before the harvest of the virus-containing cell culture supernatant (Scotti P.D., unpublished observations). The rationale for this was not based on a systematic study of OrNV infection kinetics, but on empirical observation of a full-blown cytopathic effect in infected cultures after 10 days post-infection. Under the conditions of infection employed in this study, it was found that the highest OrNV volumetric yield was reached on day 6 post-infection. The gradual reduction in OrNV infectious titer between day 6 and 14 post-infection, highlights the need to optimize the 'time of product harvest' in infected DSIR-HA-1179 cultures. Reductions in infectious virus titers in infections continued past the point of peak productivity have been observed in insect cell-baculovirus systems as well (Maranga *et al.,* 2002, Pedrini *et al.,* 2011). The loss of viral infectivity could result from cell lysis occurring late in the infection phase which would release proteases into the culture medium, which could potentially affect the virus (Cruz *et al.,* 1999; Hu and Bentley, 1999). Loss of viral infectivity could also be a consequence of thermal degradation as the infectivity half-life of OrNV at 28ºC, is only 2 weeks (Crawford and Sheehan, 1984). This finding highlights the need to harvest OrNV as soon as maximum volumetric yield is reached in infected DSIR-HA-

1179 cell cultures, regardless of the observable cytopathic effects of infection, in the interest of preserving product quality and for maintaining process economy.

3.5 Conclusions

The aim of this study was to investigate elements relating to DSIR-HA-1179 cell growth, metabolism and OrNV production in attached batch cultures in 25 cm^2 T-flasks, in greater detail. The necessity of supplementing FBS, an expensive yet beneficial additive, to DSIR-HA-1179 cell cultures grown in TC-100 basal medium, was ascertained. Though cells could grow in TC-100 supplemented with FBS at concentrations as low as 1%, the highest specific growth rate ($0.0069h^{-1}$) and viable cell concentration (16.8×10^6 cells/ml) were obtained only when cultures were supplemented with 10% FBS. Therefore, the supplementation of TC-100 with 10% FBS was recommended for DSIR-HA-1179 cell culture, at this stage of process development. Carbohydrate consumption, amino acid metabolism and the production of key metabolites were studied during cell growth in this culture medium. Glucose, and to a lesser extent glutamine were the major nutrients consumed to fuel cell growth. With the exception of glutamine, the consumption of all other amino acids to less than 20% of their original concentrations implied the over-supply of amino acids in the TC-100 culture medium, which may be reduced in the formulation of a low-cost culture medium specific to the needs of the DSIR-HA-1179 cell line, in future. The lack of lactate and ammonia production are promising from an industrial perspective, indicating the potential for high-density culture of these cells without the build-up of toxic metabolites. Alanine was produced as a non-toxic alternative to ammonia, indicative of a metabolism similar to Sf-9 cells. OrNV infection of DSIR-HA-1179 cells induced physiological and metabolic changes which included an increased rate of glucose and glutamine uptake immediately after infection. Despite the increased uptake rates, the TC-100 culture medium was found to be nutritionally sufficient over the main infection period and did not limit the maximum OrNV volumetric yield obtained (7.38×10^7 $TCID_{50}$/ml) on the 6^{th} day post-infection. The time of harvest was identified as a factor influencing virus yields, and harvesting the viral product immediately upon attainment of the peak yield was recommended in order to avoid loss in virus infectivity. The improvement of OrNV yields through the selection of an optimal culture strategy (multiplicity of infection, time of infection and time of product harvest), will be investigated in future work.

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Chapter 4

The Influence of Cell Density at Time of Infection and Multiplicity of Infection on the DSIR-HA-1179/OrNV system

4.1 Introduction

The *Oryctes* nudivirus (OrNV) is a natural pathogen of the coconut rhinoceros beetle which has been applied as a biopesticide to effectively manage beetle outbreaks with great success (Caltagirone 1981, Huger 2005). OrNV biopesticide is currently produced *in vivo* by propagating the virus in infected beetle larvae. While *in vivo* production is economical at the small scale, it has several disadvantages which include a non-sterile process environment with the risk of contamination from other microorganisms, lack of quality control of the final viral product produced in terms of its titer and purity, and difficulties in scaling-up production (van Beek and Davis, 2007).

In vitro production of biopesticides in insect cell cultures, overcomes the disadvantages associated with *in vivo* production, and has been recognized as a strong requirement for commercial production in the future (Claus *et al*., 2012; Szewczyk, 2009). Efforts toward *in vitro* mass production of viral biopesticides have thus far, focused only on baculoviruses, and OrNV is the first nudivirus to have options for exploring its *in vitro* production. *In vitro* production of OrNV has been demonstrated in the susceptible and permissive DSIR-HA-1179 insect cell line, however several technical issues remain to be solved before OrNV production at the industrial scale can become feasible (Crawford, 1982; Pushparajan *et al.,* 2013). The success of large-scale OrNV production in the DSIR-HA-1179 host cell line will ultimately rest on the maintenance of high cell-specific virus yields in cultures infected at high cell densities. In this regard, the infection strategy used is a major determinant of the efficiency of viral propagation and consequently the virus yield, and is an important aspect of the process to optimize.

The virus infection strategy is based primarily on the selection of two interrelated infection parameters - the multiplicity of infection (MOI) and the cell density at the time of infection (CD at TOI) (Carinhas *et al*., 2009; Micheloud *et al*., 2009). MOI is defined as the number of infectious viruses per cell added to the culture at the time of infection. The fraction of the cell population initially infected depends on the MOI, and consequently, MOI influences the dynamics of the infection process. The 'CD at TOI' parameter describes the physiological state of the cells at the time of infection, taking into account both the viable cell density as well as the composition of the culture medium at that point in the growth curve (Chico and Jäger, 2000). The combination of MOI and CD at TOI used to infect a culture can influence the maximum yield of virus, the time taken to reach this yield, and consequently the time of harvest (TOH). The selection of MOI and CD at TOI must be optimized for each individual insect cell/ virus system since the modalities of infection are influenced by the characteristics of the specific host cell line and culture medium used in the production process (Wong *et al*., 1996; Yang *et al*., 1996).

One of the major limitations affecting yields of viruses and recombinant proteins in insect cell cultures is the reduction of cell specific virus yields as infections are delayed in time; a phenomenon which has been termed the 'cell density effect'(Aucoin *et al*., 2010; Wood *et al*., 1982). Thus, choosing to infect a culture late in the growth phase when cells have reached their highest densities, might not necessarily result in the highest virus volumetric yields, due to the reduced specific-capacity of individual cells to produce virus. In light of the yield limitations posed by the 'cell density effect', the selection of an optimal cell density to infect cultures becomes critical. The cell density effect has been observed for a range of insect cell lines used as hosts for the *in vitro* production of baculoviruses, including Sf-9, Sf-21, High Five, UFL-Ag-286 and HzAM 1 cell lines (Chakraborty *et al.,* 1996; Kloppinger *et al.,* 1990; Micheloud *et al.,* 2009; Stockdale and Gardiner, 1977; Visnovsky and Claus, 1994). However, whether this phenomenon occurs for the DSIR-HA-1179 cell line infected with OrNV is not known. The present work sought to address the following questions: Does the cell density effect occur in the DSIR-HA-1179/ OrNV system; and if so, at what threshold cell density do cell specific yields begin to decline? Furthermore, when infecting at a particular cell density, what influence does MOI exert on the virus yield and the time of harvest? For this purpose, DSIR-HA-1179 cell cultures were infected at three different cell densities corresponding to the early, mid and late exponential phase of culture, and at a range of high and low MOI's, in each cell density condition tested. The time course profiles for viable cell density

and OrNV yields in infected cultures were evaluated for each MOI and CD at TOI combination, in order to elucidate relationships between the two parameters and to select an optimal infection strategy for the production of OrNV in DSIR-HA-1179 cell cultures.

4.2 Materials and Methods

4.2.1 Cells and culture medium

The DSIR-HA-1179 cell line, originally established from surface sterilized eggs of the black beetle *Heteronychus arator* (Crawford, 1981), was obtained from the AgResearch (Lincoln, New Zealand) cell culture collection as adherent cultures in 25 cm^2 tissue culture flasks (Corning®). The cultures were maintained at 27 ºC, in 5 ml of 10% foetal bovine serum (FBS) (Life Technologies, New Zealand) supplemented PS-100 culture medium, which was prepared in-house. The cells were sequentially adapted to grow in TC-100 culture medium (Sigma) supplemented with 10% FBS (Pushparajan *et al*., 2013), and maintained at 27º C as attached cultures in 25 cm^2 and 75 cm^2 tissue culture flasks (Corning®) with culture volume of 5 and 15 ml, respectively. Cultures were passaged every 10 days using a dilution factor of 1:5 with fresh culture medium. At the time of this study, cultures had been maintained in 10% serum supplemented TC-100 culture medium for over a year and displayed stable and reproducible growth.

In order to obtain a homogeneous cell suspension which could be counted on a Neubauer hemocytometer, it was necessary to dissociate confluent DSIR-HA-1179 cell monolayers in attached cultures by TrypLE™ Express treatment, as described by Pushparajan *et al*. (Pushparajan *et al*., 2013). Briefly, the spent culture medium was removed from the flask and 2 ml of Dulbecco's phosphate buffered saline (D-PBS) free of calcium and magnesium (Sigma) was used to wash the DSIR-HA-1179 cell monolayer. The spent D-PBS was removed, 1 ml of TrypLE™ Express (Life Technologies) pre-warmed to 27 ºC was added per 25 cm^2 of a confluent cell monolayer, and the flask incubated at 27 ºC for 30 minutes. An appropriate volume of pre-warmed TC-100 culture medium supplemented with 10% FBS was added, and the cell suspension was gently aspirated with a 10 ml pipette to break up any remaining cell aggregates. In order to assess viable cell density and culture viability, a sample of the cell suspension was stained with 0.4% Trypan blue (Sigma) and

then both the total and viable cells were counted in duplicate using a Neubauer hemocytometer (Phillips 1973).

4.2.2 Virus and virus quantification

OrNV stock (strain X2B) at a concentration of 1 x 10^7 $TCID_{50}$/ml was obtained from AgResearch. X2B is a highly virulent strain originally isolated in 1983 from a field population of infected coconut rhinoceros beetles on Bugsuk Island, Palawan, Philippines (Crawford *et al*., 1986; Zelazny *et al*., 1990).

The virus stock used in this study was prepared by infection of attached cultures during the early exponential phase of growth (approximately 5 x 10^5 viable cells/ml) at a MOI of 0.1 $TCID_{50}$/cell. OrNV infectious titer was quantified by end-point dilution analysis. Briefly, to determine the $TCID_{50}$, suspensions of DSIR-HA-1179 cells (2.5 x 10^5 cells/ml) were seeded onto 96 well plates (50 µl per well), and then an equal volume of each viral supernatant dilution (diluted in ten-fold series from 10^{-2} to 10^{-9}) was added with five replicates per supernatant. The plates were placed in a humidified, disinfected plastic container and incubated at 27 ºC for 11 to 14 days until the cytopathic effect was well developed, when the plates were scored for infection, and $TCID_{50}$ was calculated (Reed and Muench, 1938).

4.2.3 Virus production kinetics at various MOI and CD at TOI

OrNV production kinetics were assessed in attached DSIR-HA-1179 cell cultures grown in TC-100 culture medium supplemented with 10% FBS in 25 cm^2 tissue culture flasks. A total of 15 independent infection experiments were performed which tested various combinations of MOI and CD at TOI. The CD at TOI was varied to three levels (5.2 x 10^5 viable cells/ml, 9.3 x 10^5 viable cells/ml and 1.2 x 10^6 viable cells/ml) which corresponded to cell densities at the early, mid and late exponential growth phase respectively; and at each level, cultures were infected at each of five different MOI's (0.01, 0.1, 1, 5 and 10 $TCID_{50}$/cell). The following method was followed to set up each individual infection experiment: TrypLE™ Express treatment (as described above) of an uninfected confluent DSIR-HA-1179 cell monolayer in a 75 cm^2 tissue culture flask, provided sufficient number of cells to seed fourteen replicate 25 cm^2 flasks, each at an initial cell density of 2 x 10^5 viable cells/ml in a culture volume of 5 ml. The cultures were incubated at 27 ºC and were later, individually infected at a specific time-point in culture (to attain the desired cell density at

infection) and with an appropriate volume of OrNV stock, according to the particular CD at TOI and MOI combination tested in the experiment. Thereafter, duplicate infected cultures were randomly selected and harvested every 48 hours and viable cell counts determined until day 14 post-infection.

For each infection condition, the cell specific yield ($TCID_{50}$/cell) was calculated by dividing the maximum volumetric yield obtained ($TCID_{50}$/ml) by the maximum viable cell density post-infection (cells/ml) (Chakraborty *et al*., 1996; Micheloud *et al*., 2009). The time of harvest was determined to be the post-infection time-point (day) when the maximum OrNV volumetric yield was reached. Volumetric productivity was calculated by dividing the maximum volumetric yield by the total culture time taken to reach this yield (Astray *et al*. 2012). The amplification factor (ŋ) was calculated as the ratio between the maximum volumetric yield at the time of harvest and the number of viral units at the time of infection, given by the product of the initial cell density at the time of infection and the multiplicity of infection used in that particular infection condition (Carinhas *et al*., 2009). Statistical analysis of data was performed using OriginPro 8.1 software.

4.3 Results

4.3.1 Infections at high MOIs 5 and 10 (synchronous infections)

In the range of conditions tested in this experiment, cultures were infected at cell densities of 5.2 x 10^5, 9.3 x 10^5 and 1.2 x 10^6 viable cells/ml. These cell densities were reached on days 5, 9 and 11 of growth, and corresponded to the early, mid and late exponential phase of growth, respectively. MOIs of 5 and 10 were chosen in order to reach a probability level that upon infection, at least 99% of the cells would be infected with at least 1 virus particle, resulting in the entire cellular population being infected near simultaneously i.e. synchronous infection (Claus *et al*,. 2012). The temporal evolution of viable cell density and volumetric OrNV yield post-infection in cultures infected at MOIs of 5 and 10 are shown in Figs. 4-1 A and B. A minimal amount of cell growth was observed from the time of infection until day 2 post-infection in all cultures, indicating that even at the highest MOI used in this study, a proportion of the cells did not become infected, or remained refractory to the consequences of the infection. On the other hand, the rapid increase in OrNV titer from the time of infection to day 2 post-infection indicates the productive infection of DSIR-HA-1179 cells when infected at MOI's 5 and 10.

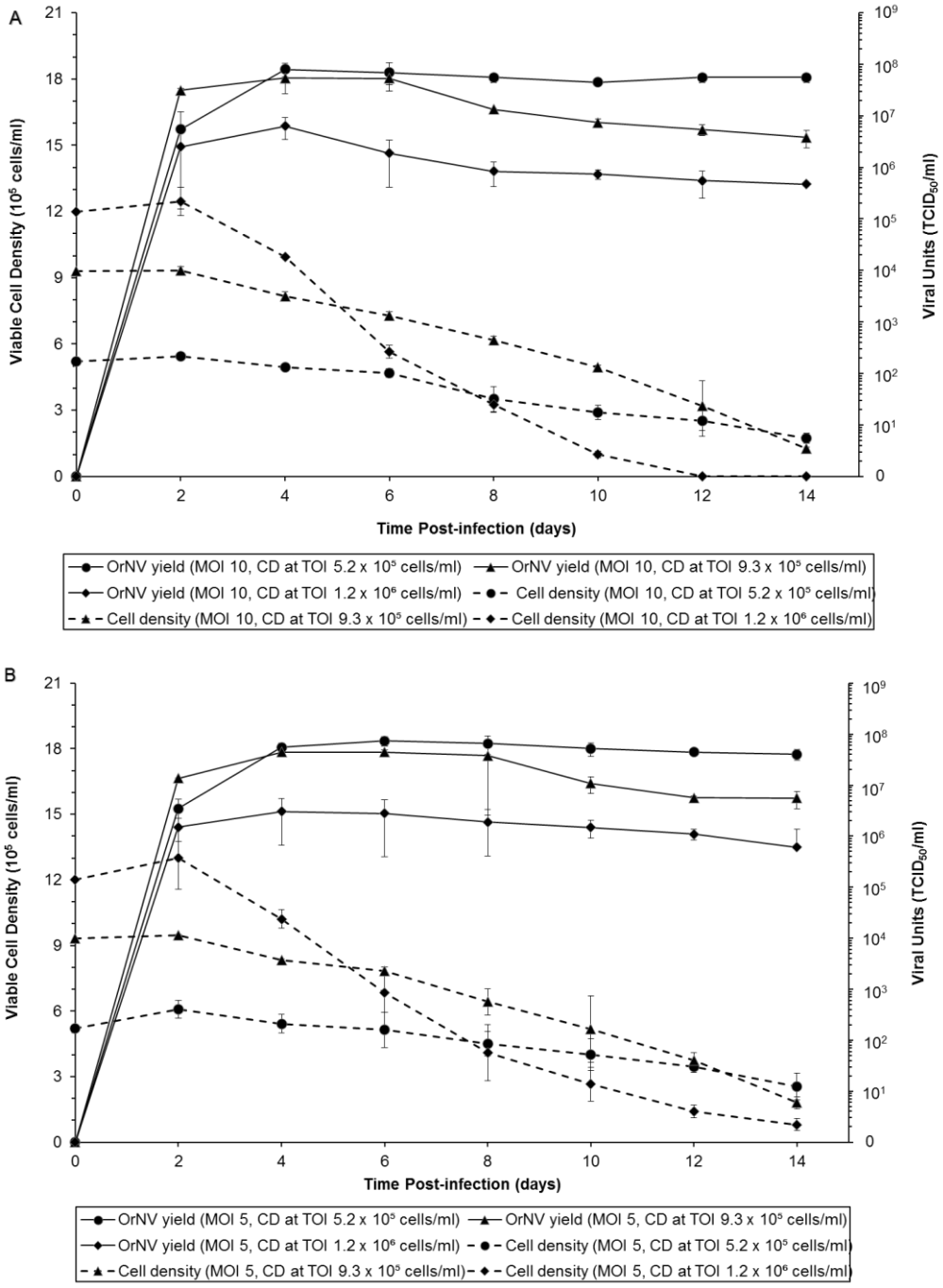


Fig. 4-1 Time course profiles of viable cell density post-infection (dashed lines) and OrNV production (solid lines) in cultures of the DSIR-HA-1179 cell line grown in TC-100 supplemented with 10% FBS in 25 cm^2 T-flasks and infected at high MOI's and at three different cell densities. (A) Cells were infected at MOI of 10 when cultures reached densities of 5.2 x 10^5, 9.3 x 10^5 and 1.2 x 10^6 viable cells/ml respectively. (B) Cells were infected at MOI of 5 when cultures reached densities of 5.2 x 10^5, 9.3 x 10^5 and 1.2 x 10^6 viable cells/ml respectively. Error bars represent standard deviations from the mean of duplicate samples.

The behavior of cell growth post-infection and OrNV production was influenced by differences in the combinations of infection parameters used. The main parameters that have highlighted the existence of these differences were the maximum viable cell density (MVCD) reached post-infection, the maximum OrNV volumetric yield, the maximum cell specific OrNV yield and the time of harvest (Table 4-1). A positive correlation was found between CD at TOI and MVCD. On the other hand, for each CD at TOI, a negative correlation was found between increasing values of MOI and the MVCD. Thus the highest MVCD was reached in cultures infected at 1.2×10^6 viable cells/ml and MOI 5, while the lowest MVCD was reached in cultures infected at 5.2×10^5 viable cells/ml and MOI 10 (Table 4-1). A low average percentage of post-infection cell growth (6.1%) was observed in infections performed at high MOIs. The time taken to reach the MVCD was two days post-infection in all cultures. From that point on, viable cell density in infected cultures declined at variable rates, which were dependent on the MOI and CD at TOI used. It was observed that the higher the CD at TOI and the MOI, the greater was the rate of decline in the viable cell density that occurred following the MVCD peak. Therefore, the greatest decline (100%) was observed in cultures infected at 1.2×10^6 viable cells/ml and MOI 10, wherein the cell monolayer completely detached and lysed by the end of the infection period (Fig. 4-1 A), while the least extent of decline in viable cell density (58%), occurred in cultures infected at 5.2×10^5 viable cells/ml and MOI 5 (Fig 4-1 B).

From Figs. 4-1 A and B, a rapid increase in OrNV titer was observed in the first two days of infection in all cultures. The initial rate of OrNV accumulation was positively correlated with increasing CD at TOI up to the infection cell density of 9.3×10^5 cells/ml and with increasing MOI. Subsequently, the rate of virus production decreased but production continued until maximum yields were reached between day 4 and day 6 post-infection. The maximum OrNV volumetric and cell-specific yield (7.96×10^7 $TCID_{50}$/ml, 146 $TCID_{50}$/cell) was obtained in cultures infected during the early exponential growth phase at a cell density of 5.2×10^5 viable cells/ml and at the highest MOI of 10. The lowest OrNV volumetric and cell-specific yield (3.05×10^6 $TCID_{50}$/ml, 2 $TCID_{50}$/cell) was obtained in cultures infected during the late exponential growth phase at a cell density of 1.2×10^6 viable cells/ml and MOI of 5 (Table 4-1). For synchronous infections, statistical analysis showed that both OrNV volumetric and cell-specific yield were significantly affected by

the CD at the TOI, but not the MOI for MOI's of 5 and 10, and nor was the interaction between the two factors statistically significant (Table 4-2 A and B).

In order to study the importance of the time of harvest on OrNV yield, the duration of the infection phase in experimental cultures was prolonged until day 14 post-infection. In all cultures, infections prolonged past the point when the maximal OrNV volumetric yields were reached led to a decrease in virus yield. From Figs. 4-1 A and B, it can be observed that this effect was most pronounced in cultures that were infected at 1.2×10^6 and 9.3×10^5 viable cells/ml (late and mid-exponential phase), than in cultures infected at 5.2×10^5 viable cells/ml in the early exponential phase. The decrease in infectious virus titers in the former conditions can be linked to the rapid reduction in viable cell density which occurred in these cultures between day 2 and 14 of infection, with many cells lysing at the same time. Cell lysis may have led to the release of cellular proteases into the culture medium, which could have affected the integrity of the produced virus, thereby lowering its infectivity. Thus it is important that the harvest of infected cultures be timed for when peak OrNV yields are reached so as to maximize OrNV yields.

Table 4-1 Summary of cell growth post-infection and OrNV yields from DSIR-HA-1179 attached cell cultures grown in TC-100 supplemented with 10% FBS in 25 cm^2 T-flasks, and infected at various CDs at TOI (5.2×10^5, 9.3×10^5, 1.2×10^6 viable cells/ml) and at high MOIs (10 and 5).

MOI	CD at TOI (cells/ml)	MVCD (cells/ml)	OrNV Yield ($TCID_{50}$/ml)	Cell Specific Yield ($TCID_{50}$/cell)	Time of Harvest (d.p.i)	Amplification factor (ŋ)	Volumetric Productivity ($TCID_{50}$/ml/d)
10	5.2×10^5	$5.45 \times 10^5 \pm 5 \times 10^3$	$7.96 \times 10^7 \pm 2.34 \times 10^7$	146 ± 43	4	16	$8.84 \times 10^6 \pm 2.60 \times 10^6$
10	9.3×10^5	$9.33 \times 10^5 \pm 1.8 \times 10^4$	$5.40 \times 10^7 \pm 2.69 \times 10^7$	58 ± 29	4	6	$4.15 \times 10^6 \pm 2.07 \times 10^6$
10	1.2×10^6	$1.25 \times 10^6 \pm 6.4 \times 10^4$	$6.32 \times 10^6 \pm 2.83 \times 10^6$	5 ± 2	4	0.5	$4.21 \times 10^5 \pm 1.89 \times 10^5$
5	5.2×10^5	$6.09 \times 10^5 \pm 4.1 \times 10^4$	$7.38 \times 10^7 \pm 1.49 \times 10^7$	121 ± 26	6	30	$6.71 \times 10^6 \pm 1.35 \times 10^6$
5	9.3×10^5	$9.48 \times 10^5 \pm 4 \times 10^3$	$4.45 \times 10^7 \pm 4.11 \times 10^6$	47 ± 4	4	10	$3.42 \times 10^6 \pm 3.16 \times 10^6$
5	1.2×10^6	$1.30 \times 10^6 \pm 1.4 \times 10^5$	$3.05 \times 10^6 \pm 1.2 \times 10^6$	2 ± 1	4	0.5	$2.03 \times 10^5 \pm 8.0 \times 10^4$

Table 4-2 Analysis of the main effects and interaction effects of the factors: CD at TOI (5.2×10^5, 9.3×10^5, 1.2×10^6 viable cells/ml) and MOI (5, 10) on: (A) OrNV volumetric yield and (B) cell-specific yield, in DSIR-HA-1179 batch attached cultures, grown and infected in 10% serum-supplemented TC-100 in 25 cm^2 T-flasks.

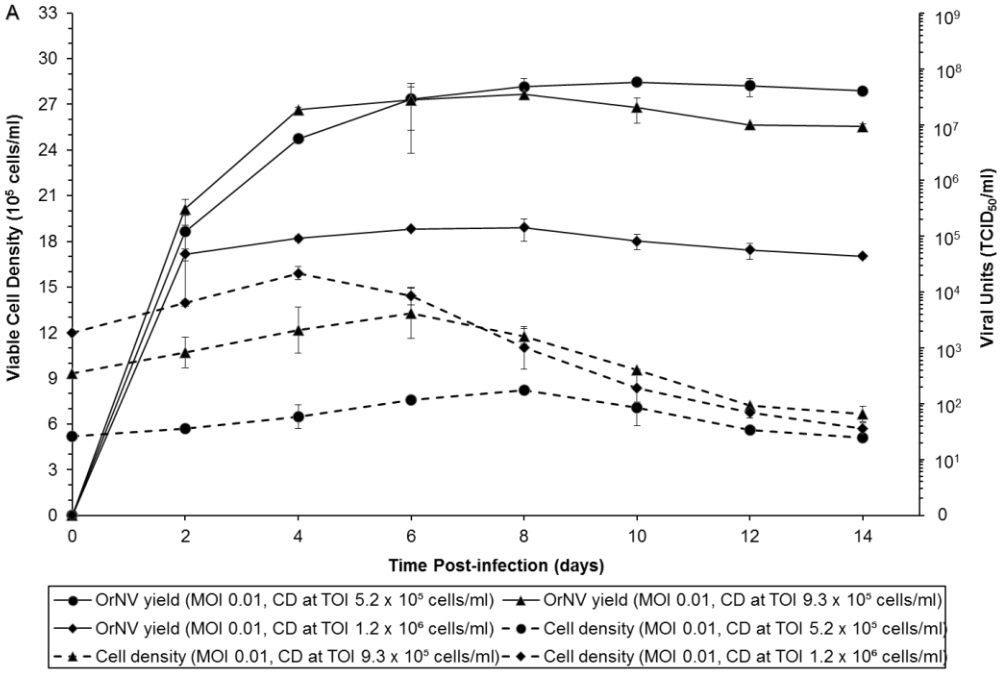
Factor	Degrees of freedom	Sum of squares	Mean square	F value	P value
(A) Volumetric yield					
CD at TOI	2	3.845	1.917	42.456	2.875E-04*
MOI	1	0.066	0.066	1.462	0.272
Interaction	2	0.074	0.037	0.819	0.485
Model	5	3.975	0.795	17.603	0.002
Error	6	0.271	0.045	-	-
Corrected total	11	4.245	-	-	-
(B) Cell-specific yield					
CD at TOI	2	34482.667	17241.333	32.747	5.911E-04*
MOI	1	533.333	533.333	1.013	0.353
Interaction	2	272.667	136.333	0.259	0.780
Model	5	35288.67	7057.733	13.405	0.003
Error	6	3159	526.5	-	-
Corrected total	11	38447.67	-	-	-

*Significant factors ($p < 0.05$)

4.3.2 Infections at low MOI (asynchronous infections)

The strategy of infecting at a high MOI is almost routinely used for virus production at the lab-scale, since synchronous infections are easier to monitor and high yields may be obtained in shorter process times. However, at the industrial production scale, the use of high MOIs is discouraged,

mainly due to the need for large viral volumes to be used as inoculum. Creation of large volumes of viral inoculum will require a scale-up train in parallel with cell inoculum, an expensive process which requires large volumes of culture media and monitored bioreactor operation (Wong *et al.*, 1996; Zhang, 2005). Additionally, infecting at a high MOI will result in the transfer of a high percentage of spent supernatant into the production bioreactor, running the risk of metabolites or cell debris in the spent supernatant affecting process performance (Maranga *et al.*, 2003). Furthermore, the use of high MOIs increases the risk of defective interfering particles (DIP's) being propagated in infected cultures which severely undermines the production of stable, infectious viruses (Bangham and Kirkwood, 1990; Casal, 1996). For these reasons, infecting cultures asynchronously using low MOIs could be a better option. In this set of experiments, the influence of low MOIs (0.01, 0.1 and 1) on post-infection cell growth and virus production, was investigated in DSIR-HA-1179 cell cultures infected at cell densities of 5.2 x 10^5 viable cells/ml, 9.3 x 10^5 viable cells/ml and 1.2 x 10^6 viable cells/ml, respectively.



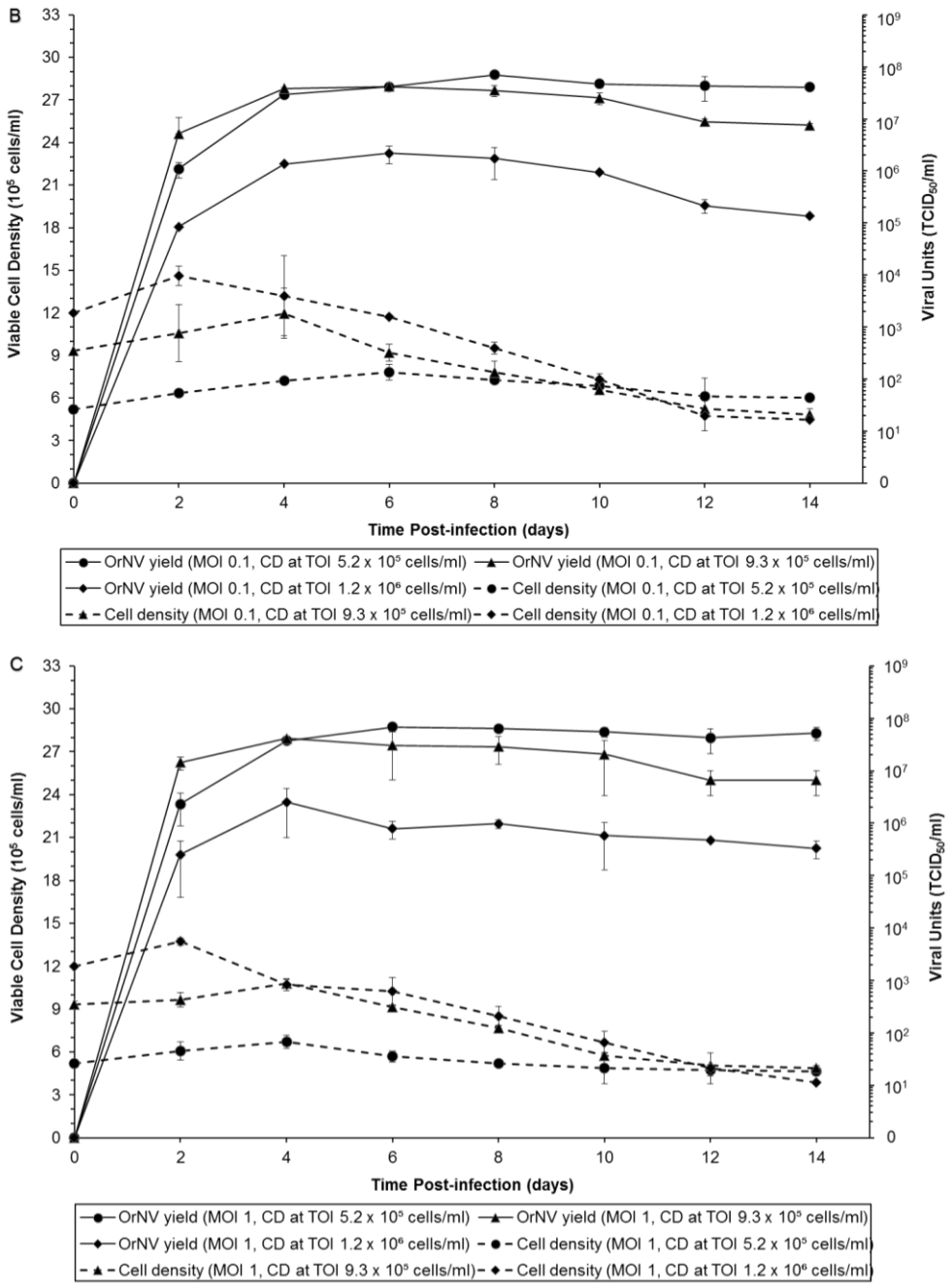


Fig. 4-2 Time course profiles of viable cell density post-infection (dashed lines) and OrNV production (solid lines) in cultures of the DSIR-HA-1179 cell line grown in TC-100 supplemented with 10% FBS in 25 cm^2 T-flasks, and infected at low MOIs and at three different cell densities. (A) Cells were infected at MOI of 0.01 when cultures reached densities of 5.2 x 10^5, 9.3 x 10^5 and 1.2 x 10^6 viable cells/ml respectively. (B) Cells were infected at MOI of 0.1 when cultures reached densities of 5.2 x 10^5, 9.3 x 10^5 and 1.2 x 10^6 viable cells/ml respectively. (C) Cells were infected at MOI of 1 when cultures reached densities of 5.2 x 10^5, 9.3 x 10^5 and 1.2 x 10^6 viable cells/ml respectively. Error bars represent standard deviations from the mean of duplicate samples.

From Figs. 4-1 A, B and C, it can be observed that MVCD values in infections performed at low MOIs followed a trend that was similar to that observed in high MOI infections. Increasing the CD at TOI led to an increase in the MVCD, while increasing the MOI had the opposite effect. Thus the highest MVCD was reached in cultures infected at the highest cell density and at the lowest MOI (1.2×10^6 viable cells/ml and MOI 0.01), while the lowest MVCD was reached in cultures infected at the lowest cell density and at the highest MOI (5.2×10^5 viable cells/ml and MOI 1) (Table 4-2). Differences between low and high MOI infections, manifested themselves in the percentage of cell growth post-infection and time taken for cultures to reach their MVCD. Across all infections carried out at low MOIs, the average percentage of post-infection cell growth was 51.8%, which was higher than that observed in the previous experiment (6.1%). This shows that, at low MOIs, a lower fraction of cells are initially infected by virus, which allows for a greater percentage of cell growth to occur post-infection and ultimately for higher MVCDs to be reached. With regard to the time taken to reach the MVCD, cultures infected earlier at a cell density of 5.2×10^5 viable cells/ml and MOI 1 reached its MVCD on day 2 post-infection, while cultures infected in the late growth phase and very low MOI of 0.01, took up to 8 days to reach the MVCD (Figs. 4-2 A and C). This was in contrast to high MOI infections, where the MVCD was reached on day 2 post-infection, regardless of the CD at TOI and MOI.

The initial rate of OrNV accumulation was the highest in cultures infected at 9.3×10^5 viable cells/ml, followed by cultures infected at 5.2×10^5 viable cells/ml and finally those infected at 1.2×10^6 viable cells/ml. At each CD at TOI, a higher MOI ensured higher accumulation of virus in the initial 48 hours of infection. Infected cultures continued to produce OrNV until peak volumetric yields were reached. The maximum volumetric yield (7.09×10^7 $TCID_{50}$/ml) was obtained in cultures infected at the cell density of 5.2×10^5 viable cells/ml and a MOI of 0.1. This was followed by cultures infected at the same cell density at a MOI of 1 (6.84×10^7 $TCID_{50}$/ml) and MOI 0.01 (5.80×10^7 $TCID_{50}$/ml). Increasing the CD at TOI to 9.3×10^5 and 1.2×10^6 viable cells/ml, in that order, resulted in a severe inhibition of virus yields (Table 4-3). Therefore, at low MOIs within the range of 0.1 to 1, similar maximum OrNV volumetric yields can be achieved, so long as cultures are infected at a cell density of 5.2×10^5 viable cells/ml (i.e. during the early exponential growth phase). The cell-specific OrNV yield in these experiments, also decreased with increasing CD at TOI. Within each CD at TOI tested, increasing the MOI led to a slight increase in the cell-specific yield. Therefore, as can be seen in Table 4-3, the maximum cell specific yield (102 $TCID_{50}$/cell)

was obtained in cultures infected at cell density of 5.2 x 10^5 viable cells/ml and MOI 1, while the lowest cell-specific yield (0.1 $TCID_{50}$/cell) occurred in cultures infected at cell density 1.2 x 10^6 viable cells/ml and MOI 0.01. Statistical analysis for asynchronous infections (Table 4-4 A and B) showed that OrNV volumetric and cell-specific yield were both significantly affected by CD at TOI and MOI, and that the interaction between the two factors was statistically significant (Table 4-4 A and B). An interesting result from this study is that the use of the very low MOI of 0.01 in early-infected cultures resulted in an appreciable volumetric yield of OrNV (5.80 x 10^7 $TCID_{50}$/ml). Consequently, the highest amplification factor of 11,154 was achieved in this condition. This amplification factor was one and two orders of magnitude higher than the amplification factor achieved with MOIs 0.1 and 1 respectively; and three orders of magnitude higher than that of high MOI infections. However, the drawbacks in using very low MOIs are a longer time of harvest and lower volumetric productivity. In this study, the time to harvest was highest in early-infected cultures infected at the lowest MOI of 0.01 (10 days post-infection), compared to infections carried out at a MOI of 1 (6 days post-infection) and a MOI of 10 (4 days post-infection). Furthermore, volumetric productivity in cultures infected at MOI of 0.01 at 5.2 x 10^5 viable cells/ml was 37.8% and 56.2% lower than in infections carried out at the same infection cell density at MOIs of 1 and 10, respectively.

Table 4-3 Summary of cell growth post-infection and OrNV yields from DSIR-HA-1179 attached cell cultures grown in TC-100 supplemented with 10% FBS in 25 cm^2 T-flasks, and infected at various CD's at TOI (5.2 x 10^5, 9.3 x 10^5, 1.2 x 10^6 viable cells/ml) and at low MOIs (0.01, 0.1 and 1).

MOI	CD at TOI (cells/ml)	MVCD (cells/ml)	OrNV Yield ($TCID_{50}$/ml)	Cell SpecificYield ($TCID_{50}$/cell)	Time of Harvest (d.p.i)	Amplification factor (ŋ)	Volumetric Productivity ($TCID_{50}$/ml/d)
0.01	5.2 x 10^5	8.20 x 10^5 ± 7 x 10^3	5.80 x 10^7 ± 8.13 x 10^6	71 ± 10	10	11154	3.87 x 10^6 ± 5.42 x 10^5
0.01	9.3 x 10^5	1.33 x 10^6 ± 1.66 x 10^5	3.48 x 10^7 ± 7.50 x 10^6	26 ± 6	8	3742	2.05 x 10^6 ± 4.41 x 10^5
0.01	1.2 x 10^6	1.59 x 10^6 ± 4.21 x 10^4	1.43 x 10^5 ± 6.08 x 10^4	0.1 ± 0.04	8	12	7.53 x 10^3 ± 3.20 x 10^3
0.1	5.2 x 10^5	7.80 x 10^5 ± 5.70 x 10^4	7.09 x 10^7 ± 7.07 x 10^5	91 ± 7	8	1363	5.45 x 10^6 ± 5.40 x 10^4
0.1	9.3 x 10^5	1.20 x 10^6 ± 1.77 x 10^5	4.20 x 10^7 ± 1.41 x 10^6	35 ± 5	6	452	2.80 x 10^6 ± 9.40 x 10^4
0.1	1.2 x 10^6	1.46 x 10^6 ± 7.1 x 10^4	2.18 x 10^6 ± 8.13 x 10^5	1 ± 0.6	6	18	1.28 x 10^5 ± 4.78 x 10^4
1	5.2 x 10^5	6.73 x 10^5 ± 4.6 x 10^4	6.84 x 10^7 ± 1.20 x 10^6	102 ± 7	6	131	6.22 x 10^6 ± 1.09 x 10^5
1	9.3 x 10^5	1.08 x 10^6 ± 2.8 x 10^4	4.16 x 10^7 ± 0	39 ± 1	4	45	3.20 x 10^6 ± 0
1	1.2 x 10^6	1.37 x 10^6 ± 2.5 x 10^4	2.54 x 10^6 ± 1.45 x 10^6	2 ± 1	4	2	1.69 x 10^5 ± 9.67 x 10^4

Table 4-4 Analysis of the main effects and interaction effects of the factors: CD at TOI (5.2×10^5, 9.3×10^5, 1.2×10^6 viable cells/ml) and MOI (0.01, 0.1, 1) on: (A) OrNV volumetric yield and (B) cell-specific yield, in DSIR-HA-1179 batch attached cultures, grown and infected in 10% serum-supplemented TC-100 in 25 cm^2 T-flasks.

Factor	Degrees of freedom	Sum of squares	Mean square	F value	P value
(A) Volumetric yield					
CD at TOI	2	12.776	6.388	251.713	1.261E-08*
MOI	2	0.816	0.408	16.078	0.001*
Interaction	4	1.083	0.271	10.669	0.002*
Model	8	14.675	1.834	72.283	2.938E-07
Error	9	0.228	0.025	-	-
Corrected total	17	14.903	-	-	-
(B) Cell-specific yield					
CD at TOI	2	23043.204	11521.602	450.912	9.476E-10*
MOI	1	730.542	365.271	14.295	0.002*
Interaction	2	465.604	116.401	4.555	0.028*
Model	5	24239.35	3029.919	118.579	3.302E-08
Error	6	229.966	25.552	-	-
Corrected total	11	24469.317	-	-	-

*Significant factors ($p < 0.05$)

4.3.3 OrNV volumetric and cell-specific yield as functions of the MVCD

To clarify the cell density effect, the maximum volumetric and cell-specific OrNV yields obtained in the various infection conditions were plotted against their associated MVCD values, in Figs. 4-3 and 4-4 respectively. MVCD, rather than CD at TOI, was chosen to represent the variation in

volumetric and cell-specific OrNV yield. This was because the MVCD reached in an infection condition is determined by the specific combination of MOI and CD at TOI used to infect cells in that condition, and is therefore, more likely to relate to the final virus yield than the CD at TOI.

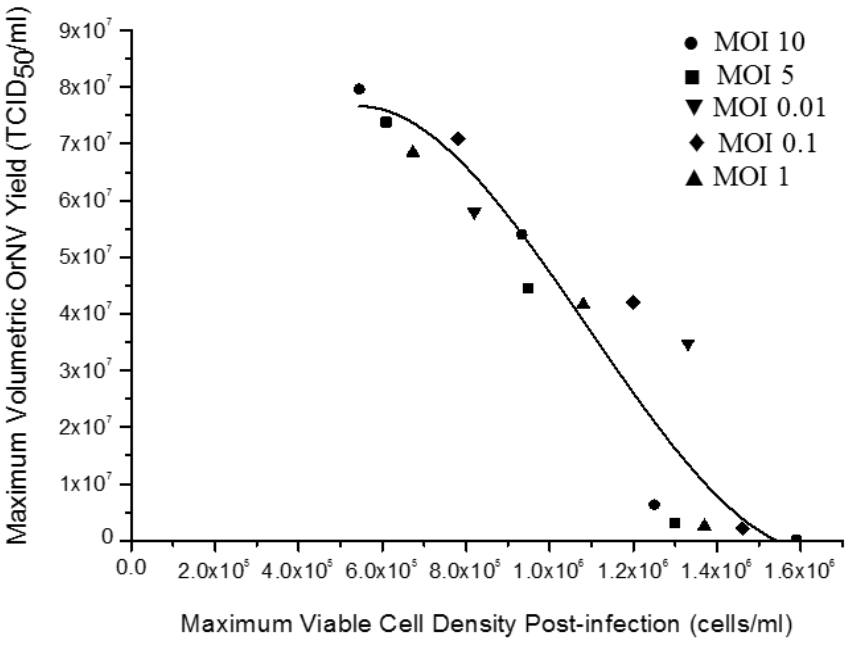


Fig. 4-3 Plot of the maximum OrNV volumetric yield achieved versus maximum viable cell density reached post-infection, in cultures of the DSIR-HA-1179 cell line grown in TC-100 supplemented with 10% FBS in 25 cm^2 T-flasks, and infected at a range of MOIs (10, 5, 1, 0.1, 0.01) at various cell densities at time of infection (5.2 x 10^5, 9.3 x 10^5 and 1.2 x 10^6 viable cells/ml). Solid line represents data fitted to a 3rd order polynomial equation: $y = -1.9 + 323.12 x - 3.94 \times 10^{-4} x^2 + 1.20 \times 10^{-10} x^3$, adj. $r^2 = 0.87$.

From Fig. 4-3 it can be observed that OrNV volumetric yields between 7 – 8 x 10^7 $TCID_{50}$/ ml could be obtained as long as the MVCD was kept within the range of 5 – 7 x 10^5 viable cells/ml. An increase in the MVCD above 7 x 10^5 viable cells/ml, led to a decline in OrNV volumetric yields.

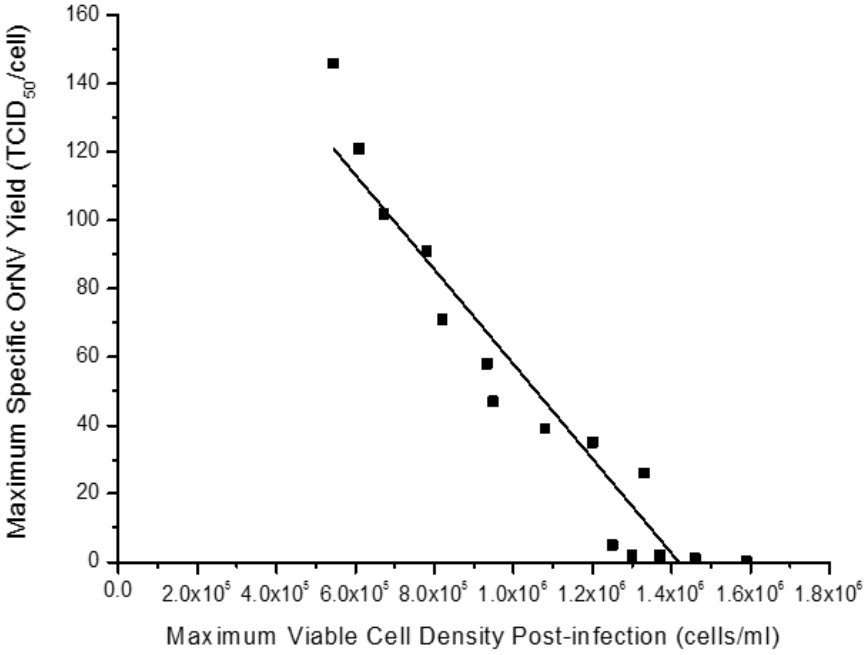


Fig. 4-4 Plot of the maximum cell specific OrNV yield achieved versus maximum viable cell density reached post-infection in cultures of the DSIR-HA-1179 cell line grown in TC-100 supplemented with 10% FBS in 25 cm^2 T-flasks, and infected at a range of MOIs (10, 5, 1, 0.1, 0.01) at various cell densities at time of infection (5.2×10^5, 9.3×10^5 and 1.2×10^6 viable cells/ml). Solid line represents data fitted to the equation $y = -1.4x + 196.4$, $r^2 = 0.91$.

On the other hand, from the plot of the maximum OrNV cell-specific yield against the MVCD (Fig. 4-4), it can be observed that the cell-specific OrNV yield declined continuously with increasing MVCD from the lowest MVCD (5.45×10^5 viable cells/ml). An MVCD increase between 5×10^5 viable cells/ml and 7×10^5 viable cells/ml, caused a reduction in the cell-specific virus yield in the cells by almost 50%.

4.4 Discussion

OrNV infection of DSIR-HA-1179 cells begins with the virion attaching to the cell surface receptor followed by its entry into the cell by pinocytosis. The virus then moves to the nucleus where it

sheds its nucleocapsid and viral DNA takes over the cellular machinery, causing cell division to halt (Crawford and Sheehan, 1985). Across the different infection conditions tested in this study, the eventual cessation of cell growth that was observed, was therefore, a direct consequence of OrNV infection. Despite the halting of cell division, in order for productive infections to occur, host cells must continue to respire and metabolize in order to support viral replication. Cellular energy will be directed toward facilitating the synthesis, packaging and budding of virions. For example, in baculovirus infections there is evidence that shortly after virus is internalized, the cellular machinery must synthesize G-actin cables to transport virus nucleocapsids to the nucleus (Monteiro *et al*., 2012). Furthermore, upon virus multiplication, localization of the newly synthesized virions within the cell depends upon the reorganization of the cells' cytoskeleton proteins (Cudmore *et al*., 1997). From the morphological changes in OrNV-infected DSIR-HA-1179 cells, it appeared that the effects of OrNV infection are similar to those observed for other virus-infected insect cells in terms of changes in cell size (hypertrophy) and cell shape (rounding up of cells), which indicate that the cellular machinery of the host cell is directed towards virus production. The finding of a strong negative correlation between CD at TOI and OrNV volumetric yield, further suggests that the DSIR-HA-1179 host cell machinery is more efficient at supporting the requirements of OrNV production when infections occur earlier, rather than later in the growth phase. The choice of CD's at TOI (5.2×10^5, 9.3×10^5 and 1.2×10^6 viable cells/ml) which corresponded to the early, mid and late exponential growth phases, reflected not only increases in cell density, but also represented cells of differing 'age' in culture and growing in a culture medium which was 'spent' to varying degrees at the time of infection. The differing 'age' of cells would reflect the physiological effects of auto regulatory events, cell cycle changes and changes in energy metabolism at different points in culture, which could in turn affect an individual cells' efficiency in replicating virus. On the other hand, the degree to which the culture medium is 'spent' at the time of infection, would indicate nutrients depletion and/ or accumulation of metabolites which could also undermine viral replication. The lower volumetric OrNV yields in cultures infected at higher cell densities during mid to late exponential growth, must therefore result from the reduced capacity of the cellular machinery of individual older cells to support viral replication. This is linked to the concept of the cell density effect which has been widely observed in a number of baculovirus/insect cell systems and been proposed as the major bottleneck to the improvement of *in vitro* virus yields. The cell density effect is characterized by a progressive reduction in cell-specific yields of the products of viral replication (budded virus, occlusion bodies, virus-like

particles and recombinant proteins), as the infection is delayed to higher cell densities (Caron *et al*., 1990; Kloppinger *et al.,* 1990; Vincente *et al*., 2011; Visnovsky and Claus, 1994). This means that for a particular insect cell line/ virus system there is a critical threshold cell density above which any further cell growth will come at the expense of viral product formation. In order to clarify the cell density effect, when maximum cell-specific OrNV yields were plotted against the MVCD from the different infection conditions (Fig 4-4), a drop in cell-specific OrNV yield was observed from the lowest MVCD value, which continued to decline continuously with increasing MVCD. Similar declines in cell-specific yield with increasing MVCD, with no stable region, have been previously reported for saUFL-Ag-286 cells infected with AgMNPV (Micheloud *et al.,* 2009) and for HzAM1 cells infected with HearNPV (Huynh, 2014). These results demonstrate that a cell density effect exists for the DSIR-HA-1179 cell/OrNV system which is influenced strongly by the cell density at the time of infection. While the cell density effect has been reported for a number of baculovirus/ insect cell systems, this is the first report of this phenomenon occurring for an insect cell line infected with a nudivirus.

The variation of the maximum OrNV volumetric yield with the MVCD showed a short stable region between the MVCD values of approximately 5 - 7 x 10^5 viable cells/ml which corresponded to the highest OrNV volumetric yields of 7 - 8 x 10^7 $TCID_{50}$/ml. Beyond this point, OrNV volumetric yields decreased with increasing MVCD (Fig 4-3). It may be reasoned that, within the small window of MVCD increase up to 7 x 10^5 viable cells/ml, despite the continuous reduction in the cell-specific yield, the increase in the number of cells in the culture which were actively producing virus, counterbalanced the reduced specific capacity of each cell to replicate the virus. Based on this finding, it would appear that any combination of CD at TOI and MOI which results in a MVCD of or below 7 x 10^5 viable cells/ml, would be successful in achieving similarly high OrNV volumetric yields. Within the range of infection conditions tested, this could only be achieved in cultures infected in the early exponential phase (CD at TOI 5.2 x 10^5 viable cells/ml). Infections carried out at higher CDs at TOI during the mid and late-exponential growth phase led to reduced yields since their starting infection cell densities (9.3 x 10^5 and 1.2 x 10^6 viable cells/ml), were themselves higher than the threshold MVCD value of 7 x 10^5 viable cells/ml after which point, the cell density effect affected volumetric yield.

Several reasons have been proposed to explain the cell-density effect in baculovirus-infected insect cells. The earliest reports of the phenomenon were in attached cultures, where cell-to-cell contact in cultures seeded at high cell densities was thought to inhibit viral synthesis (Stockdale and Gardiner, 1977; Wickham *et al*., 1992; Wood *et al*., 1982). Several others have considered nutrient depletion in high density cultures, including trace element and vitamin deficiencies to be a main cause for the cell density effect. Partial or total replacement of culture medium at the time of infection (Hensler and Agathos, 1994; Reuveny *et al*., 1993), glucose and/or glutamine supplementation (Taticek and Shuler, 1997; Wang *et al.*, 1993a), one step addition of a nutrient cocktail (Bedard *et al*., 1994) and fed-batch culture (Nguyen *et al*., 1993), have proven efficient strategies to restore cell-specific yields and to delay the cell density effect to higher cell densities, but not totally overcome it. From another perspective, the build-up of toxic metabolites (such as ammonia) which are inhibitory to cell and virus replication have also been attributed to the cell density effect (Micheloud *et al*., 2009; Taticek and Shuler, 1997). Other studies on the cell density-dependent decrease in productivity have implicated oxygen deprivation (Palomares *et al.*, 2004), auto-regulatory events including the accumulation of autocrine factors, and the decreasing degree of cell cycle synchronization in cultures as they grow to high densities (Braunagel *et al*., 1998; Calles *et al*., 2006; Doverskog *et al*., 2000). A more conclusive cause for the cell density effect has been revealed through metabolic flux analysis: the progressive inhibition of central metabolism in cells grown to high densities which affects virus replication (Bernal *et al.*, 2009). More recently, the cell density effect has been linked to reduction in the cell specific yield of intracellular viral DNA replication, indicating that the overall decline in cell specific and volumetric yields with increasing CD at TOI are a result of impaired early upstream events such as virus gene transcription. (Huynh *et al*., 2013).

Since DSIR-HA-1179 cells were grown as attached cultures in this study, the theory of contact inhibition cannot be ruled out as a reason for the cell density effect. Nutrient depletion could also explain the loss in productivities, especially for cultures infected during the mid and late exponential phase. In Chapter 3 of this thesis, nutrient consumption and metabolism was studied in attached DSIR-HA-1179 cultures grown in 10% FBS-supplemented TC-100 culture medium and infected on day 5 of the culture (CD at TOI 5.2 x 10^5 viable cells/ml, MOI 5). The infection conditions of that experiment was the same as the early-exponential phase infection experiment at a MOI of 5 performed in the present study. The infection phase lasted 14 days post-infection which

corresponded to days 5-20 of batch culture. It was found that glucose, the main energy source for cells, was exhausted from the culture medium only on day 12 post-infection in early-infected cultures (or day 18 of batch culture) (Chapter 3, section 3.3.4, this thesis). This occurred much later after the MVCD and peak volumetric OrNV yield were reached on days 2 and 6 post-infection, respectively in this condition. These facts confirm that glucose was not limiting when cultures were infected synchronously and in the early exponential growth phase. The decline of cell-specific productivity that was observed in early infected cultures, might then be alternatively explained by possible deficiencies in trace elements, vitamins or hormones in the culture medium. DSIR-HA-1179 cells did not accumulate lactate or ammonia during growth and infection, ruling out at least known toxic metabolites as a cause for the cell density effect. For cultures infected at higher cell densities of 9.3×10^5 and 1.2×10^6 viable cells/ml, the 14 day infection period corresponded to days 9-24 and days 11-26 of culture, respectively. During this time, glucose might have become completely exhausted from cultures, which might have caused the observed rapid decline in both cell-specific and volumetric OrNV yields. The rapid cell lysis that occurred toward the end of the infection period in these infection conditions, in addition to being virally induced, may be partly due to nutrient-deprived cell death. If this possibility is true, then partial or total replacement of the culture medium at the time of infection or the application of a nutrient feed, could be suitable strategies to reverse the cell density effect. This has not been evaluated in the present work but must be considered in the future as a means to improve virus yield in late infected cultures. However, within the range of infection conditions tested in this study and without nutrient replacement or supplementation at the time of infection, infecting DSIR-HA-1179 cultures during the early exponential growth phase was necessary in order to maximize OrNV yields.

While final OrNV yields were found to be influenced mainly by the CD at TOI, the MOI played a role in influencing the dynamics of the process. At infections carried out at high MOIs, cell growth arrest was more rapid than in infections at low MOIs. In cultures infected at low MOIs, only a fraction of the cell population is initially infected and uninfected cells continue to grow. Progeny viruses' budded from the primarily infected cells go on to infect another fraction of growing cells in a 'secondary' infection. Successive infection cycles may occur, until either all cells become infected or lose their propensity for virus attachment (Zhang *et al.,* 2005). This explains the higher MVCD's reached and the longer duration taken for complete growth arrest to occur in asynchronous infections at low MOIs. On the other hand, synchronous infections carried out at

MOIs above 5, should result in the entire cell population being infected at the same time and therefore, a minimal increase in post-infection cell density (O'Reilly *et al.*, 1994). However, it was observed that even at the highest MOI, DSIR-HA-1179 cell growth was not completely arrested immediately post-infection. The low percentage of cell growth in high MOI infections could have resulted from either a lower than desired MOI having been incorrectly applied, or more likely, due to slow virus binding kinetics of OrNV to DSIR-HA-1179 cells; a rate limiting step during which time cell growth could have occurred. While in some synchronously infected baculovirus/ insect cell systems, complete cell growth arrest was observed upon infection (Radford *et al.*, 1997; Visnovsky and Claus, 1994), in other studies a minimal amount of post-infection cell growth occurred (Licari and Bailey, 1992; Micheloud *et al.*, 2009). This may be explained by the differences in the cell lines, virus, culture media and bioreactor configurations used in the studies. Differences in virus binding kinetics have been observed between Group I and II nuclear polyhedoviruses (NPV's), with Group I NPV's displaying faster binding rates than Group II NPV's due to the gp64 envelope fusion protein in the former being more fusogenic than the F protein envelope in the latter (Pedrini *et al.*, 2011). The nature of the virus envelope of OrNV and its binding affinity to DSIR-HA-1179 cell receptors has not been studied so far. Characteristics of the culture medium can also affect virus binding, for example the elimination of FBS from the culture medium during infection has been shown to enhance virus binding (Petricevich *et al.*, 2001). Additionally, the mode of cultivation induces spatial limitations in the availability of cell-surface receptors for virus binding to cells. Virus binding in attached cultures has been found to be 10 fold lower than in suspension cultures (Dee and Shuler, 1997). Thus, despite the significant influence of MOI on the level of post-infection cell growth, other influences such as the ones just described may also affect the kinetics of infection.

The kinetics of OrNV production was influenced by the MOI, with cultures infected at high MOIs reaching their maximum OrNV volumetric yield at a faster rate than those infected at low MOIs. In infections performed at high MOI's, this phenomenon can be explained by the synchronal infection and budding of virus from a large fraction of simultaneously infected cells during the primary infection cycle. On the other hand, at low MOIs the longer time taken for viral titers to accumulate is indicative of multiple rounds/ asynchronies of infection taking place. An interesting finding from early-infected cultures in this study, was that the use of low and high MOIs of 0.1, 1, 5 and 10; all produced similar OrNV volumetric yields (approximately 7 - 8 x 10^7 $TCID_{50}$/ml).

Generally, cultures infected at the highest MOI produce higher yields because a large proportion of cells are effectively infected early when they are in their active multiplication phase; while at low MOI, a large proportion of cells are infected later in the culture, when they are in a non-proliferating state and where cell cycle changes, drop in central energy metabolism or nutrient depletion affect their productivity. However, in this case it appeared that until the MVCD of 7.8 x 10^5 viable cells/ml - reached in early-infected cultures at MOI 0.1, secondarily infected DSIR-HA-1179 cells did not suffer a drop in virus productivity.

The possibility to infect DSIR-HA-1179 cells with OrNV at a low MOI and still achieve high virus yields is a positive finding from the perspective of process economy. This is mainly because it allows for reduction in virus inoculum volumes which is an expensive part of the process. The use of low MOIs also alleviates the risk of defective interfering particles forming, which can compromise product yield (Wong *et al.*, 1995; Bangham and Kirkwood, 1990). However, a disadvantage of using low MOIs is that longer infection durations are required before the time of harvest. Consequently, while the use of MOI of 0.1 would take 8 days post-infection until the time of harvest, choosing to infect cultures at MOI 10, would reduce this timeframe by half. Similar to reducing the amount of virus inoculum, choosing a process with a shorter production timeframe is also in the interest of process economy. Furthermore, as observed by Radford, the use of MOIs below 1 creates the risk of obtaining suboptimal yields. This is due to the extremely limited predictability of cell growth at very low MOI's because inherent inaccuracies in virus titration techniques could easily lead to considerable differences between the actual and theoretical MOIs applied (Radford *et al*., 1997). The choice of the MOI must involve judicious consideration of the maximum obtainable virus yield and the amount of virus inoculum required, as well as the time of harvest in the process. Based on these considerations it would seem that a suitable infection strategy for attached DSIR-HA-1179 cell cultures with OrNV, would involve infecting cultures early in the exponential phase and at an intermediate MOI of 1. In this way, optimal volumetric OrNV yields can be achieved, reducing the volume of viral inoculum to $1/10^{th}$ of that required in high MOI infections without loss in yield, and a time of harvest of 6 days which is an acceptable compromise.

4.5 Conclusions

In conclusion, the current study has investigated the influence of the CD at TOI and MOI on the production of OrNV in batch attached cultures of the DSIR-HA-1179 cell line grown in 10% serum-supplemented TC-100 culture medium, in 25 cm^2 T-flasks. It was found that CD at TOI was the main infection parameter which affected OrNV yield, while MOI influenced the dynamics of the infection. Of the range of MOI-TOI combinations tested in the study, the combination of a low infection cell density of 5.2 x 10^5 viable cells/ml in the early-exponential growth phase and an intermediate MOI of 1 was deemed optimal. With this infection strategy, an OrNV volumetric yield of up to 7 x 10^7 $TCID_{50}$/ml could be produced, with a time of harvest of 6 days. The cell density effect was found to exist for the DSIR-HA-1179/OrNV system with the progressive decline in cell-specific yield beginning at low cell densities. Through the correlation of CD at TOI and the MVCD on the one hand, and the correlation of MVCD and maximum cell-specific and volumetric OrNV yields on the other; it was found that in order to maximize OrNV volumetric yield, any combination of CD at TOI and MOI could be used, as long as the MVCD was kept between 5 – 7 x 10^5 viable cells/ml. There is potential for the cell density effect to be delayed to higher cell densities in this system, through the application of strategies such as partial culture medium replacement at TOI, fed-batch culture or the use of alternative culture systems such as perfusion.

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Chapter 5

Roller bottle culture of the DSIR-HA-1179 insect cell line for the production of *Oryctes* nudivirus

5.1 Introduction

The use of entomopathogenic viruses for the control of insect pests is an environment-friendly alternative to the use of toxic chemical pesticides. At present, the global market for biopesticides is estimated to be between 400 and 1600 million US dollars, of which viral biopesticides enjoy a market share of 5 to 10%, with a projected annual growth rate of 14% (Glare *et al*., 2012; Ravensberg, 2011a). In order to meet this growing demand and to ensure their success in the marketplace, there is a need to develop robust, high-yielding production systems for viral biopesticides, which can achieve economies of scale.

The *Oryctes* rhinoceros nudivirus (OrNV) is a biopesticide which finds its application in the management of the coconut rhinoceros beetle; a serious pest of coconut and oil palms found throughout the Southeast-Asia and Pacific region (Huger, 2005). In the current global scenario of burgeoning beetle outbreaks in non-endemic regions such as Guam and Hawaii, it is possible that large volumes of OrNV will be required for the control of this pest in future (Moore and Marshall, 2014). The current method of producing the virus is based on its *in vivo* propagation in diseased larvae and in adult beetles (Marschall and Ioane, 1982; Prasad *et al*., 2008; Ramle *et al*., 2005; Zelazny *et al*., 1987). While this method is economical in the small scale, it presents a myriad of disadvantages including the lack of process validation, inconsistencies in purity and titer of the

virus produced and difficulties in process scale-up (Shuler *et al*., 1995). *In vitro* production of OrNV is an attractive alternative which overcomes the disadvantages posed by *in vivo* production. *In vitro* production of OrNV in the susceptible and permissive DSIR-HA-1179 insect cell line has been demonstrated, however, several technical challenges remain to be solved before such a process can become commercially feasible (Crawford, 1982; Pushparajan *et al*., 2013).

One of the key challenges to the ease of process scale-up is posed by the strongly attachment-dependent and fragile nature of the DSIR-HA-1179 cell line (Pushparajan *et al*., 2013). Attempts to adapt the cell line to grow in suspension culture have been unsuccessful, till date. The choice of the culture system exerts a significant impact on virus production and process scalability (Weiss *et al*., 2012). Production of OrNV has been restricted to attached DSIR-HA-1179 cell cultures grown in 25 and 75 cm^2 T-flasks with culture volumes of 5-15 ml. While T-flasks are suitable for small scale research applications, they are impractical as a bioreactor choice for OrNV production at the industrial scale due to the considerable time and labour that would be involved in handling multiple unit flasks. Therefore, there is a need to evaluate alternative bioreactor configurations for scaling-up attached DSIR-HA-1179 cell cultures in order to produce larger volumes of the virus.

Roller bottles are suitable for scaling-up virus production in attachment-dependent cells as they provide greater surface areas for cell growth (490 – 1750 cm^2) and hold larger culture volumes than tissue culture flasks. The operational features of the roller bottle system include gentle mechanized rolling with only 15 – 20% of the cell monolayer surface covered by a thin film of culture medium at any point of time, creating a dynamic culture environment which is different from stationary T-flask cultures. Improved mass transfer due to dynamics of the system, in turn can improve cell and virus yields (Weiss *et al*., 1980; Weiss *et al*., 1982). Additionally, the low shear environment within roller bottles could be particularly beneficial for the culture of a fragile cell line such as DSIR-HA-1179. A further advantage of large-scale cell and virus production in roller bottles is that contamination can be confined to a single unit, if it occurs. Currently, the most widespread use of roller bottle technology is in the vaccine industry for the manufacture of human and veterinary vaccines (Aunins, 2000). The application of roller bottles for insect cell culture has been demonstrated in studies which evaluated production of an AcMNPV bioinsecticide in roller bottle cultures of *Spodoptera frugiperda* cell lines (Vaughn, 1976; Weiss *et al*., 1981a; Weiss *et al*., 1981b; Weiss et al., 1986). Production of recombinant Epstein-Barr viral attachment protein

(EBV gp105) using the IC-BEVS system has also been demonstrated in roller bottle cultures of High-Five cells (Wickham and Nemerow, 1993).

The aim of this study was to assess roller bottle technology as a means to scale up OrNV production at the laboratory scale, and to assess its suitability for large-scale manufacturing. A procedure for growing DSIR-HA-1179 cell monolayers in roller bottles was established. Efficient operation of the roller bottle system required the assessment of factors that were expected to have a significant influence on DSIR-HA-1179 cell growth and productivity, such as the inoculum cell density, culture volume and the culture medium. Under optimal conditions of culture, DSIR-HA-1179 cells growth kinetics, nutrient consumption and metabolite production were quantified, both in uninfected cultures and in cultures infected with OrNV. The influence of infection parameters i.e. multiplicity of infection (MOI) and cell density at the time of infection (CD at TOI), on OrNV production, were also studied in roller bottle cultures. The kinetics of OrNV production were evaluated in the system and partial replacement of culture medium at the time of infection was investigated as a strategy for yield improvement. Overall, this work represents a comprehensive first attempt at optimizing a process for OrNV mass production using the roller bottle system.

5.2 Materials and Methods

5.2.1 Cells and culture media

DSIR-HA-1179 cells, originally established from surface sterilized eggs of the black beetle *Heteronychus arator* (Crawford, 1982), were obtained from the AgResearch (Lincoln, New Zealand) cell culture collection as adherent cultures in 25 cm^2 tissue culture flasks (Corning®). The cultures were maintained at 27 ºC, in 5 ml of 10% foetal bovine serum (FBS) (Life Technologies, New Zealand) supplemented PS-100 culture medium, which was prepared in-house. The cell line was sequentially adapted to grow in four different culture media: TC-100 (Sigma), IPL-41 (Life Technologies), Sf-900 II (Life Technologies) and Sf-900 III (Life Technologies), each supplemented with 10% FBS (Life Technologies, New Zealand). In each culture medium, cells were maintained at 27 ºC as attached working stock cultures in 25 cm^2 and 75 cm^2 tissue culture flasks (Corning®), with culture volumes of 5 and 15 ml respectively. Cultures were passaged every

10 days when the cell monolayer was approximately 80% confluent, using a dilution factor of 1:5 with fresh culture medium. At the time of this study, the cells had been passaged for more than 2 years in the respective culture media and displayed stable and reproducible growth.

5.2.2 Roller bottle system

The CELLROLL (Integra Biosciences) was the roller bottle system used in this study. It consisted of two roller racks mounted with a drive unit (0.1 to 2 rpm) and connected to a control unit and power supply. Roller bottles (Corning®, Cat. 430195) had surface areas of 490 cm^2 and were made of optically clear, tissue culture treated polystyrene with plug seal caps. Roller bottle cultures were initiated by aseptically dispensing an appropriate volume of the DSIR-HA-1179 cell suspension into culture medium and were incubated at 27 ºC. A rolling speed of 0.1 rpm was used over the duration of the culture.

5.2.3 Inoculum preparation, cell harvest and cell counting procedure

In order to prepare a homogenous cell suspension to inoculate roller bottle cultures, confluent cell monolayers in 75 cm^2 tissue culture flasks were dissociated using TrypLE™ Express (Life Technologies). Briefly, the spent culture medium was removed from the flask and 2 ml of Dulbecco's phosphate buffered saline (D-PBS) free of calcium and magnesium (Sigma) was used to wash the DSIR-HA-1179 cell monolayer. The spent D-PBS was removed, 1 ml of TrypLE™ Express (Life Technologies) pre-warmed to 27 ºC was added per 25 cm^2 of a confluent cell monolayer, and the flask incubated at 27 ºC for 30 minutes. An appropriate volume of pre-warmed TC-100 culture medium supplemented with 10% FBS was added, and the cell suspension was gently aspirated with a 10 ml pipette to break up any remaining cell aggregates, thereby creating a single cell suspension of DSIR-HA-1179 cells.

In order to harvest cells from roller bottle cultures, a slight modification was made to the procedure described above. The spent culture medium was removed from the roller bottle and 20 ml of Dulbecco's phosphate buffered saline (D-PBS) free of calcium and magnesium (Sigma) was added. The roller bottle was returned to the incubator and rolled for 10 minutes at 0.1 rpm in order for the PBS to wash the cell monolayer. The spent D-PBS was removed and 20 ml of TrypLE™ Express pre-warmed to 27 ºC was added to the roller bottle. The roller bottle was returned to the incubator

and rolled at 27 ºC for 30 minutes until cells detached from the monolayer. An appropriate volume of pre-warmed culture medium supplemented with 10% FBS was added, and the cell suspension was gently aspirated with a 25 ml pipette to break up any remaining cell aggregates. 1 ml samples of cell suspension were taken in order to assess viable cell density and culture viability and for the analysis of nutrients and metabolite concentrations. In order to assess viable cell density and culture viability, a sample of the cell suspension was stained with 0.4% Trypan blue (Sigma) and then both the total and viable cells were counted in duplicate using a Neubauer hemocytometer (Philips, 1973). Culture supernatants harvested for nutrient and metabolite analyses, were clarified by centrifugation at 10,000g for 5 min and stored at -20 ºC until they were analyzed.

5.2.4 Virus and virus quantification

OrNV stock (strain X2B) at a concentration of 1 x 10^7 $TCID_{50}$/ml was originally obtained from AgResearch Ltd. (Lincoln, New Zealand). X2B is a highly virulent strain originally isolated in 1983 from a field population of infected coconut rhinoceros beetles on Bugsuk Island, Palawan, Philippines (Crawford *et al*., 1986; Zelazny *et al*., 1990).

The virus stock used in this study was prepared by infection of attached DSIR-HA-1179 cultures grown in 10% serum-supplemented TC-100 culture medium in tissue culture flasks, during the early exponential phase of growth (approximately 5 x 10^5 viable cells/ml) and at a MOI of 0.1 $TCID_{50}$/cell. OrNV infectious titer was quantified by end-point dilution analysis. Briefly, to determine the $TCID_{50}$, suspensions of DSIR-HA-1179 cells (2.5 x 10^5 cells/ml) were seeded onto 96 well plates (50 µl per well), and then an equal volume of each viral supernatant dilution (diluted in ten-fold series from 10^{-2} to 10^{-9}) was added with five replicates per supernatant. The plates were placed in a humidified, disinfected plastic container and incubated at 27 ºC for 11 to 14 days until the cytopathic effect was well developed, when the plates were scored for infection, and $TCID_{50}$ was calculated (Reed and Muench, 1938).

5.2.5 Nutrient and metabolite analyses

Glucose concentrations were determined using an enzymatic test kit (Sigma) based on the hexokinase-catalyzed oxidation of glucose (Slein, 1963). Lactate concentrations were determined using a colorimetric test kit (Sigma) based on the oxidation of lactate by lactate dehydrogenase (Schweiger and Gunther, 1964). Ammonia concentration was determined using an enzymatic test

kit (Sigma) based on the glutamate dehydrogenase-catalyzed oxidation of nicotinamide-adenine dinucleotide in the presence of ammonia (Mondzac *et al.,* 1965). Glutamine concentrations were determined using a YSI 2700 biochemistry analyzer (YSI, Yellow Springs, OH, USA). The total concentrations of 17 other amino acids were determined by reverse-phase high-performance liquid chromatography (HPLC) (AccQ.Tag, Waters Corporation, USA) using precolumn derivatization with 6-aminoquinolyl N-hydroxysuccinimidyl carbamate (Cohen, 2000). Prior to derivatization, sample proteins were hydrolyzed into their respective amino acids by treatment with 6N hydrochloric acid for 24 h at 110º C (AOAC official method 4.1.11: 994.12c, 1998). Cysteine and methionine were analyzed as cysteic acid and methionine sulfone after cold performic acid oxidation overnight prior to hydrolysis (AOAC official method 4.1.11: 994.12a, 1998).

5.2.6 Rates

Cell specific growth rate (μ) was calculated by identifying the linear region from the semilog plot of viable cell density versus time, followed by linear regression of this data (Gioria *et al*., 2006). Population doubling time (t_d) was calculated by equation 1:

$$td = \frac{Ln\,(2)}{\mu} \qquad (1)$$

For cells in the exponential growth phase, specific consumption rates of nutrients or production rates of metabolites were calculated by determining cell yield (given by the slope of the plot of concentration of the analyte of interest versus viable cell density), and then multiplying this value by the specific growth rate. For infected cells, the specific consumption rates of nutrients or production rates of metabolites were determined by evaluating the rate of change of the analyte of interest over a given time-period and then dividing by the average viable cell density during the respective time period (Gioria *et al.*, 2006).

OrNV cell specific yield ($TCID_{50}$/cell) was calculated by dividing the maximum volumetric yield obtained ($TCID_{50}$/ml) by the maximum viable cell density post-infection (cells/ml) (Chakraborty *et al*., 1996; Micheloud *et al*., 2009). The time of harvest (TOH) was the post-infection time-point (day) when the maximum OrNV volumetric yield was reached. Volumetric productivity was

calculated by dividing the maximum volumetric yield by the total culture time taken to reach this yield (Astray *et al*. 2012). The amplification factor (ŋ) was calculated as the ratio between the maximum volumetric yield at the time of harvest and the number of viral units at the time of infection, given by the product of the initial cell density at the time of infection and the multiplicity of infection used in that particular infection condition (Carinhas *et al*., 2009).

5.3 Results

5.3.1 Procedure for establishing roller bottle cultures

Preliminary experiments were performed in which DSIR-HA-1179 cells (1 x 10^7 cells per roller bottle) were inoculated directly into 60 ml of 10% serum-supplemented TC-100 culture medium and the bottle rolled at 0.1 rpm. Microscopic examination of cultures revealed that this inoculation procedure led to poor initial cell attachment, the formation of unattached cell clumps, and sparse and uneven monolayers, as the culture progressed. In order to reduce these problems and to obtain even DSIR-HA-1179 cell monolayers, the following protocol was adopted. The first step involved conditioning the roller bottle surface with culture medium prior to cell inoculation. This was done by adding 25 ml of fresh culture medium to the roller bottle and rolling it at 0.1 rpm for 24 hours. DSIR-HA-1179 cells (1 x 10^7 cells per roller bottle) was then inoculated into the reduced culture volume of 25 ml, and the bottle was rolled at 0.1 rpm for a further 24 hours. During this time, more than 90% of the cells attached and began spreading on the roller bottle surface. At the end of the 24 hour cell attachment period, the culture was adjusted to its final volume (60 ml) by the addition of fresh culture medium, and the roller bottle was rolled at 0.1 rpm for the rest of the batch growth period. With this procedure, cells grew as an even monolayer on the roller bottle surface. The first roller bottle cultures established using this procedure yielded 5.4 x 10^7 cells/ roller bottle (9 x 10^5 cells/ml) on day 14 of the culture. Since this cell yield was low compared to T-flask cultures, efforts were made to improve cell yields through the optimization of basic culture parameters such as the inoculum cell density, the final culture medium volume and the culture medium used.

5.3.2 Evaluation of basic culture parameters in roller bottles

5.3.2.1 Inoculum level

The inoculum cell density used to initiate a culture is a critical parameter which has been found to influence cell growth kinetics as well as the maximum cell yield (Taticek *et al*., 2001). In experiments performed in 25 cm^2 T-flasks in Chapters 2-4, DSIR-HA-1179 cells were inoculated at a density of 4 x 10^4 cells/cm^2. For the purpose of OrNV production, the final virus yield was presumed to be related to the total cell yield in roller bottles (Bouillant and Vineer, 1988; Vaughn *et al*., 1976). Therefore, the effect of varying the inoculum level between 1, 2 and 3 x 10^7 cells/ roller bottle (i.e. 2 x 10^4, 4 x 10^4 and 6 x 10^4 cells/ cm^2), was evaluated on final cell yields from roller bottle cultures grown in 60 ml of 10% serum-supplemented TC-100 culture medium and harvested on day 14 of culture (Fig. 5-1).

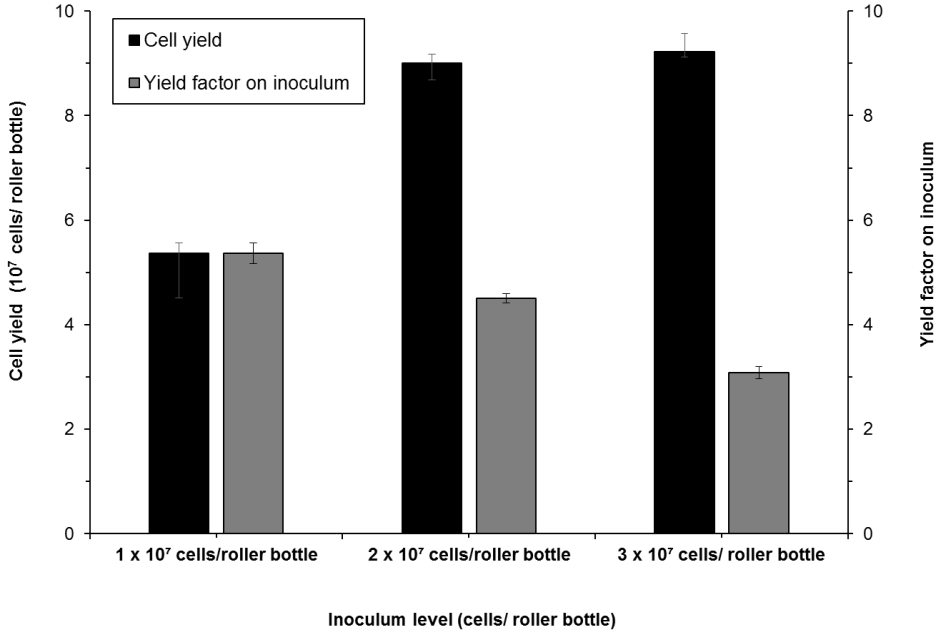


Fig. 5-1 Effect of inoculum cell density (1 x 10^7, 2 x 10^7 or 3 x 10^7 cells/ roller bottle) on cell yield at harvest and yield on inoculum, in roller bottle cultures of DSIR-HA-1179 cells grown in 60 ml of TC-100 culture medium supplemented with 10% FBS. A rolling speed of 0.1 rpm was used. Error bars represent standard deviations from the mean of duplicate samples.

From Fig. 5-1 it can be observed that while the DSIR-HA-1179 cell yield in roller bottles increased as the magnitude of the inoculum increased, the yield factor on inoculum followed the opposite trend. Therefore while an inoculum of 1 x 10^7 cells/ roller bottle gave the highest yield factor on inoculum (5.36), and represented the most efficient use of culture medium, the cell yield (5.36 x 10^7 cells/ roller bottle) in this condition was the lowest. Increasing the inoculum level to 2 x 10^7 cells/ roller bottle led to a 68% increase in cell yield to 9.01 x 10^7 cells/ roller bottle and a yield factor on inoculum of 4.5. Further increasing the inoculum level to 3 x 10^7 cells/ roller bottle, led to a 2.4% increase in the cell yield to 9.23 x 10^7 cells/ roller bottle with a yield factor on inoculum of 3.08. Considering that the yield improvement with an inoculum level of 3 x 10^7 cells/ roller bottle was very minimal and also considering that the yield factor on inoculum obtained in this condition was lower than that obtained with 2 x 10^7 cells/ roller bottle, a judicious choice was made for 2 x 10^7 cells/ roller bottle to be the inoculum level used in the process.

5.3.2.2 Culture volume

The culture medium plays a critical role in providing nutrients to fuel cellular energetic processes during cell growth and infection. It is therefore conceivable that the amount of culture medium used would affect DSIR-HA-1179 cell and OrNV yields in infected roller bottle cultures. The first experiment aimed to evaluate the influence of the final culture volume on DSIR-HA-1179 cell yield in uninfected roller bottle cultures. Experimental cultures were inoculated with 2 x 10^7 cells/ roller bottle in 25 ml of 10% serum-supplemented TC-100 culture medium, according to the procedure described in subsection 5.3.1. Once cells had attached to the roller bottle surface, the final culture volume was adjusted to different levels (45-100 ml). Total and volumetric cell yields were assessed on day 14 of culture (Fig. 5-2). From Fig. 5-2, it can be observed that total cell yield increased with increasing final culture medium volume. A culture medium volume of 100 ml gave the highest cell yield (1.1 x 10^8 cells/ roller bottle), followed by 80 ml (9.13 x 10^7 cells/ roller bottle), 60 ml (9.03 x 10^7 cells/ roller bottle) and finally 45 ml (5.68 x 10^7 cells/ roller bottle). On the other hand, the highest volumetric cell yield (1.51 x 10^6 cells/ml) was obtained in a culture medium volume of 60 ml, representing the most efficient use of culture medium in this condition.

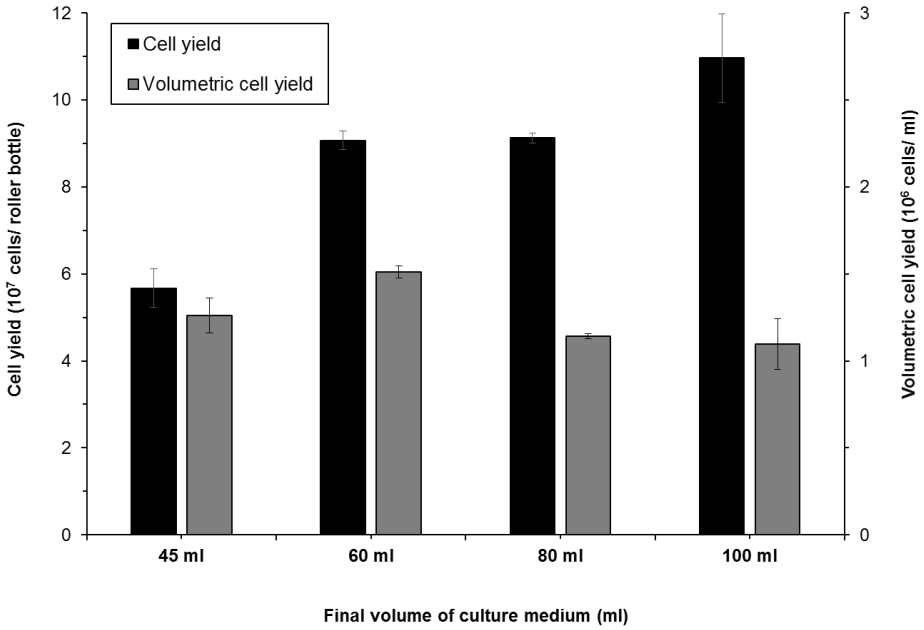


Fig. 5-2 Effect of final culture medium volume (45, 60, 80 or 100 ml) on total and volumetric cell yields in roller bottle cultures of DSIR-HA-1179 cells grown in TC-100 culture medium supplemented with 10% FBS. Roller bottles were inoculated at a cell density of 2 x 10^7 cells/ bottle. A rolling speed of 0.1 rpm was used. Error bars represent standard deviations from the mean of duplicate samples.

A second experiment was performed to evaluate the influence of the final culture medium volume on OrNV production in roller bottle cultures. Experimental cultures were inoculated with 2 x 10^7 cells/ roller bottle in 25 ml of 10% serum-supplemented TC-100 culture medium, according to the procedure described in subsection 5.3.1. Once cells had attached, the final culture volume was adjusted to different levels (45-100 ml) by adding fresh culture medium. Cultures were infected with 1 ml of OrNV containing 1 x 10^7 $TCID_{50}$ on day 4 of culture when the cell monolayer was ~30% confluent (a MOI of approximately 0.3). OrNV yields were assessed in the culture supernatant harvested from roller bottles on day 14 post-infection. The OrNV yields obtained in this experiment are graphically represented in Fig. 5-3.

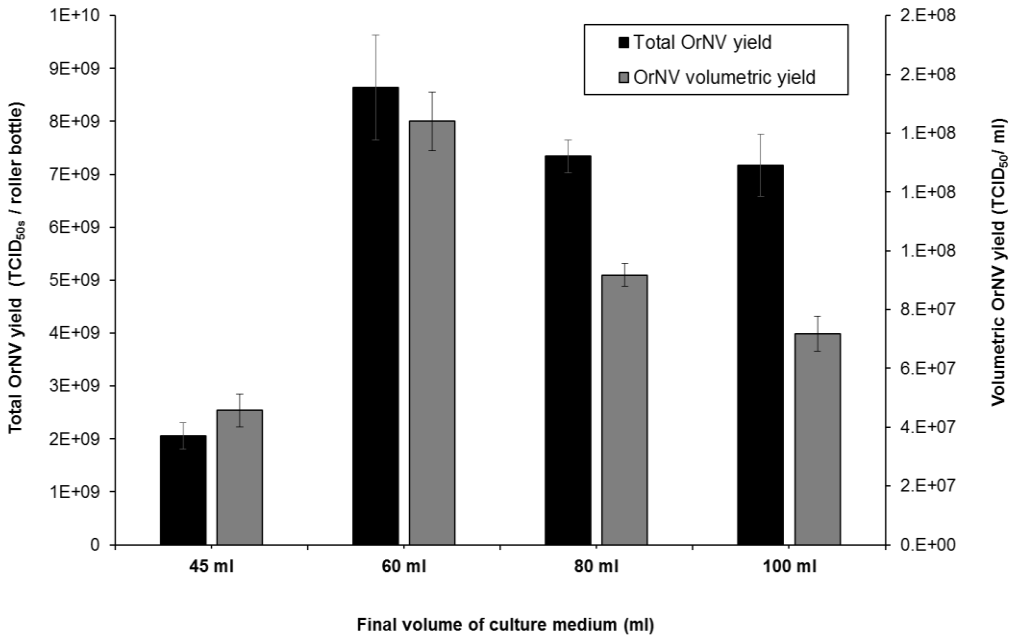


Fig. 5-3 Effect of final culture medium volume (45, 60, 80 or 100 ml), on total and volumetric OrNV yields, in roller bottle cultures of DSIR-HA-1179 cells grown in TC-100 culture medium supplemented with 10% FBS. Roller bottles were inoculated at a cell density of 2 x 10^7 cells/ bottle and were rolled at 0.1 rpm. Cultures were infected with 1 x 10^7 $TCID_{50}$ of virus on day 4 of culture. OrNV was harvested and titered on day 14 post-infection. Error bars represent standard deviations from the mean of duplicate samples.

From Fig. 5-3, it can be observed that the maximum total and volumetric OrNV yields of 8.64 x 10^9 $TCID_{50}$/ roller bottle and 1.44 x 10^8 $TCID_{50}$/ml, were obtained in roller bottle cultures with a final culture medium volume of 60 ml. In comparison, progressively lower OrNV volumetric yields were produced in culture medium volumes of 80 ml (9.18 x 10^7 $TCID_{50}$/ml), 100 ml (7.18 x 10^7 $TCID_{50}$/ml) and 45 ml (4.58 x 10^7 $TCID_{50}$/ml). Therefore, a final culture medium volume of 60 ml was deemed the best choice among those tested, for further use in the process.

5.3.2.3 Type of culture medium

The chosen culture medium must be nutritionally sufficient to support cellular metabolic demands both during growth and viral infection (Gioria *et al*., 2006). In Chapter 1 of this thesis, four culture media: TC-100, IPL-41, Sf-900 II and Sf-900 III, each supplemented with 10% FBS, were screened

for their ability to support DSIR-HA-1179 cell and OrNV yields in stationary T-flask cultures. TC-100 and IPL-41 were recognized as potential candidates as they supported the highest OrNV yields up to 4.1 x 10^7 $TCID_{50}$/ml (Pushparajan *et al*., 2013). However, it is known that media used in stationary cultivation systems, may not necessarily produce the best results when used with dynamic cultivation systems such as roller bottles (Johnson, 2001; Silva *et al*., 2008). For this reason, the same range of culture media were screened again for DSIR-HA-1179 cell yield and OrNV production in the roller bottle system.

Fig. 5-4 shows the cell yields obtained at the end of the 14 day batch growth period in experimental cultures that were inoculated with 2 x 10^7 cells/ roller bottle with 60 ml culture volumes, in each of the four culture media. Comparable cell yields were obtained in Sf-900 II (9.09 x 10^7 cells/ roller bottle), TC-100 (9.01 x 10^7 cells/ roller bottle) and Sf-900 III culture media (8.1 x 10^7 cells/ roller bottle). On the other hand, a lower cell yield of 4.29 x 10^7 cells/ roller bottle was obtained in the IPL-41 culture medium. The general pattern of DSIR-HA-1179 cell growth was similar in roller bottles and 25 cm^2 T-flasks, where cells grew at a lower specific growth rate and to a lower maximum cell density in IPL-41 than in Sf-900 II, Sf-900 III and TC-100 culture media (Chapter 2 of this thesis; Pushparajan *et al*., 2013). However, the yield factor on inoculum obtained in roller bottle cultures in IPL-41 (2.15), was much lower than in T-flask cultures (6.25). From visual observation of cultures, it appeared that the major difference between T-flask and roller bottle cultures grown in IPL-41, was in the initial attachment of cells to the growth surface. While in T-flasks, DSIR-HA-1179 cells that were inoculated in IPL-41 completely attached to the flask surface within 12 hours of culture; attachment to the roller bottle surface was, in comparison, considerably impaired with a large number of cells remaining unattached even four days post-seeding. These floaters eventually lysed, evidenced by the progressive accumulation of cell debris in the culture medium. It appeared that the density of cells attached to the roller bottle surface was too low to initiate the minimum rate of growth that was necessary to form a cell monolayer. The need for a minimum inoculum level maybe explained by the carry-over of growth promoting metabolites in the inoculum, which mediate cell-to-cell interactions in attachment-dependent cell lines, including the secretion of hormones and autocrine factors which drive their proliferation in attached cultures (Lauffenburger and Cozens, 1989).

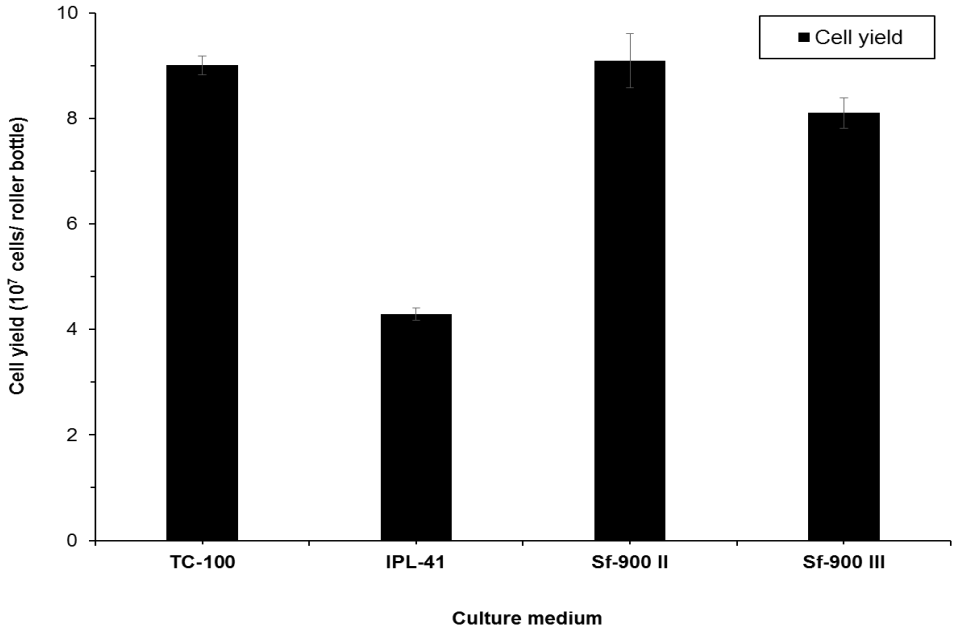


Fig. 5-4 Effect of the culture medium used (TC-100, IPL-41, Sf-900 II or Sf-900 III, each supplemented with 10% FBS), on DSIR-HA-1179 cell yield in roller bottle cultures. Roller bottles were inoculated at a cell density of 2 x 10^7 cells/ bottle. A final culture volume of 60 ml was used for each culture medium evaluated. Bottles were rolled at 0.1 rpm. Error bars represent standard deviations from the mean of duplicate samples.

In order to select an optimal culture medium, OrNV volumetric yields were screened in DSIR-HA-1179 roller bottle cultures that were grown and infected in each of the four culture media. Roller bottle cultures inoculated at 2 x 10^7 cells/ roller bottle and grown in a culture volume of 60 ml in each culture medium, were infected with 1 ml of OrNV containing 1 x 10^7 $TCID_{50}$ on day 4 of the culture when the monolayer appeared ~30% confluent. OrNV volumetric yields were assessed in the culture supernatant harvested from roller bottles on day 14 post-infection. From Fig. 5-5 it can be observed that the highest virus production was obtained in TC-100 culture medium (1.44 x 10^8 $TCID_{50}$/ml). A much lower yield was obtained in IPL-41 (2.87 x 10^7 TCID50/ml) which can be attributed to poor cell yield in those cultures. Finally, it was interesting to note that despite the high cell yields obtained in Sf-900 II and Sf-900 III culture media, OrNV yields in these media were very low at 1.71 x 10^7 and 2.46 x 10^7 $TCID_{50}$/ml respectively. Based on these results, at this stage

of process development, 10% serum-supplemented TC-100 was chosen as a suitable culture medium for roller-bottle based production of OrNV.

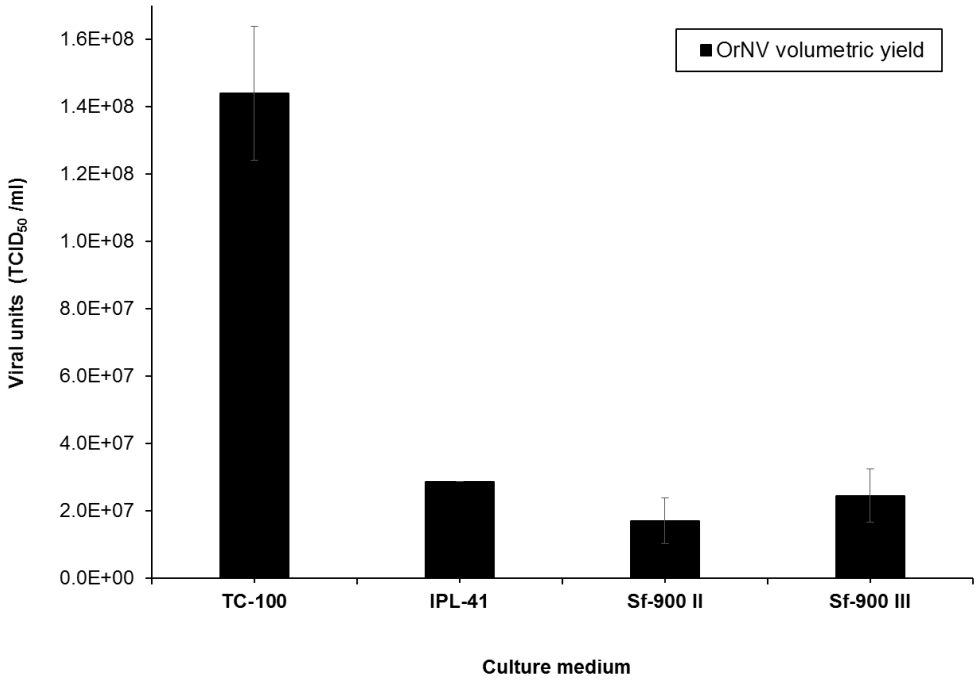


Fig. 5-5 Comparison of OrNV production in four culture media (TC-100, IPL-41, Sf-900 II or Sf-900 III, each supplemented with 10% FBS). Roller bottles were inoculated at a cell density of 2 x 10^7 cells/ bottle. Bottles were rolled at 0.1 rpm and a final culture volume of 60 ml was used for each culture medium. Cultures were infected with 1 x 10^7 $TCID_{50}$ of virus on day 4 of culture. OrNV was harvested and titered on day 14 post-infection. Error bars represent standard deviations from the mean of duplicate samples.

5.3.3 Cell growth kinetics in roller bottle cultures

DSIR-HA-1179 cell growth kinetics were conducted on attached DSIR-HA-1179 cultures in 490 cm^2 roller bottles grown in 10% serum-supplemented TC-100. Ten replicate roller bottle cultures were each inoculated with 2 x 10^7 viable cells. The final culture volume in each roller bottle was 60 ml. Duplicate cultures were randomly selected and harvested on days 4, 8, 10 and 14 of culture.

Viable cell concentration and culture viability were assessed in each harvested culture, and supernatant samples stored for nutrient and metabolites analyses. From the cell growth and viability profiles over 14 days of batch growth presented in Fig. 5-6, it can be observed that cells went through a lag phase which lasted for approximately 72 hours, after which the exponential phase of growth commenced. The mean specific growth rate over the exponential growth phase was 0.0052 h^{-1} (PDT: 5.58 days). The maximum viable cell density (14.5 x 10^5 viable cells/ml) was reached on day 14 of culture. At this point the cell monolayer appeared to be ~ 80% confluent. Prolonging the culture past this point resulted in the detachment of some parts of the cell monolayer, which made cell estimation difficult. Hence, day 14 was chosen as the end of batch growth. Cell viability remained greater than 90% over the entire culture period.

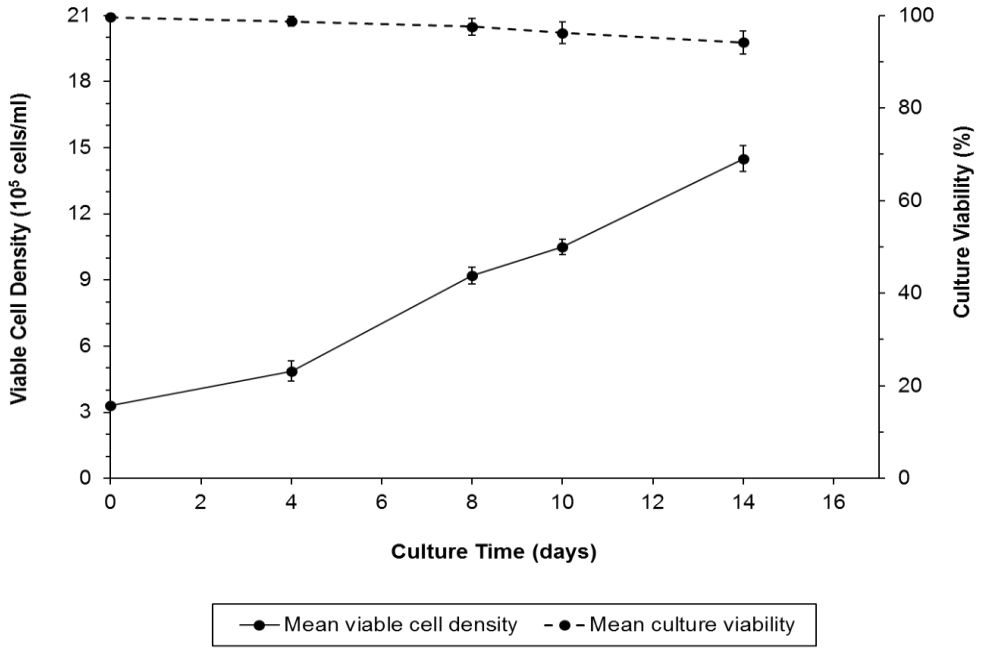


Fig. 5-6 Cell growth (solid line) and culture viability (dotted line) profile of the DSIR-HA-1179 cell line grown in TC-100 supplemented with 10% FBS in 490 cm^2 roller bottles. Error bars represent standard deviations from the mean of duplicate samples.

5.3.4 Nutrient consumption and metabolism in uninfected roller bottle cultures

To characterize the nutritional and metabolic profiles of DSIR-HA-1179 cells grown in TC-100 culture medium supplemented with 10% FBS in roller bottles, concentrations of glucose,

glutamine, lactate and ammonia were assessed in supernatant samples obtained over the 14 day period of batch growth. The concentrations of eighteen amino acids in culture supernatants were also assessed at the beginning and at the end of the batch growth period.

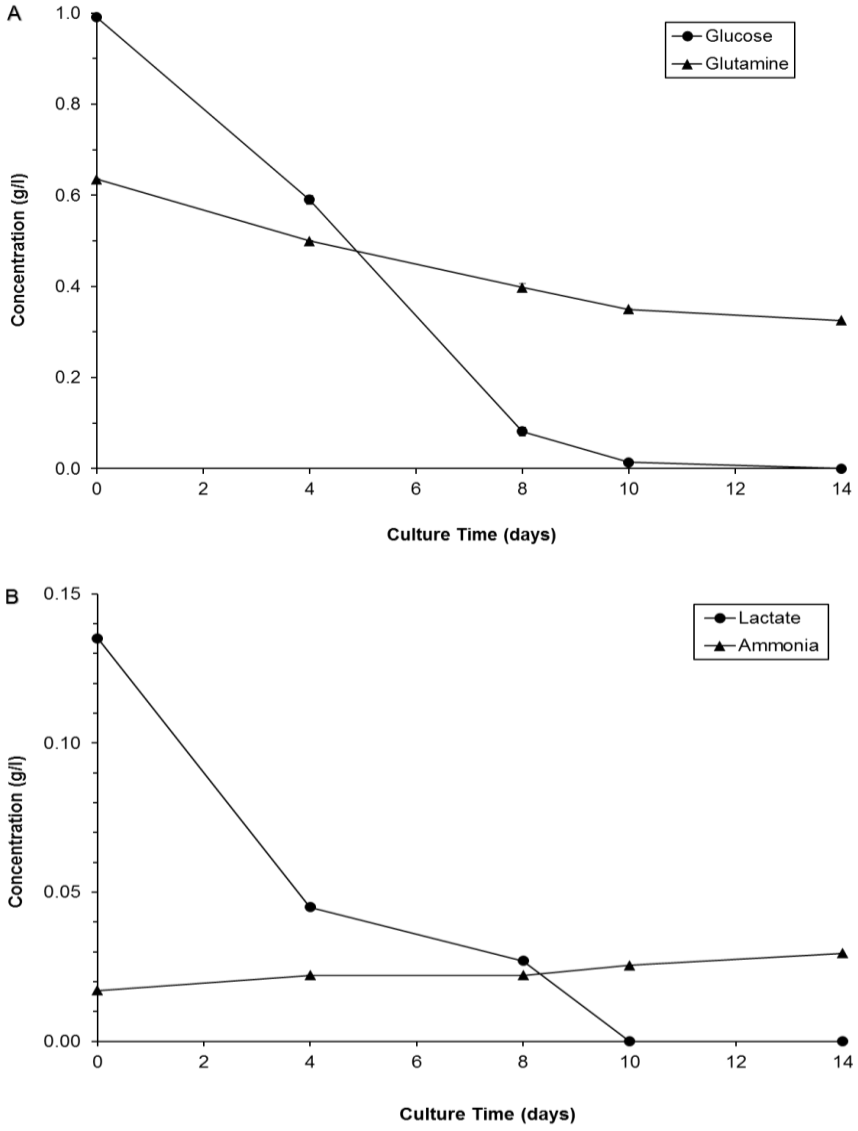


Fig. 5-7 (A) Glucose, glutamine and (B) lactate and ammonia profiles in uninfected cultures of the DSIR-HA-1179 cell line, grown in TC-100 supplemented with 10% FBS, in 490 cm^2 roller bottles. Error bars represent the standard deviations from the mean of duplicate samples. Error bars are difficult to see because standard deviations were very small.

As shown in Fig. 5-7 (A), glucose, the sole carbohydrate in TC-100 culture medium, was the most consumed nutrient over the course of the culture. Glucose was consumed rapidly from the start of

the culture, even during the lag phase. Glucose was completely exhausted from the culture medium by day 10 of the culture. Cells faced glucose limitation between days 10 and 14 of growth. Between days 4 and 10 of culture, correlating to the exponential growth phase, glucose was consumed at a mean specific rate of 5.2×10^{-12} g/c/h. In comparison with glucose, glutamine was consumed to a lesser extent (40.6% of its initial concentration), and at a slower rate (9.5×10^{-13} g/c/h) over the period of batch growth.

From Fig. 5-7 (B) it can be observed that lactate and ammonia which are by-products of carbohydrate and nitrogen metabolism in animal cell lines, were not produced by DSIR-HA-1179 cells. A very small concentration of lactate (0.14 g/l) which was present at the start of the culture was rapidly consumed and was exhausted from the culture medium by day 10 of culture. Negligible concentrations of ammonia of approximately 0.02 g/l were detected over the course of batch growth in culture, confirming the fact that DSIR-HA-1179 cells are non-producers of ammonia.

Table 5-1 shows that none of the amino acids were consumed to more than half of their original concentrations during DSIR-HA-1179 batch growth in roller bottle cultures. The highest consumed amino acids were proline (41.2%), glutamine (40.6%), methionine (30%), glutamic acid (29.1%) and aspartic acid (25.2%). Isoleucine, leucine, arginine, serine, tyrosine and valine were consumed between 10-20% of their original concentrations, while minimal consumption (< 10%) was noted for the remaining amino acids. Alanine was the only amino acid that significantly accumulated in the culture medium to 44.2% of its original concentration by the end of batch growth.

Table 5-1 Amino acid concentration at the beginning and end of batch growth in uninfected cultures of the DSIR-HA-1179 cell line grown in TC-100 supplemented with 10% FBS in 490 cm^2 roller bottles. Concentrations of all amino acids are expressed in mg/g, except for glutamine which is expressed in g/l.

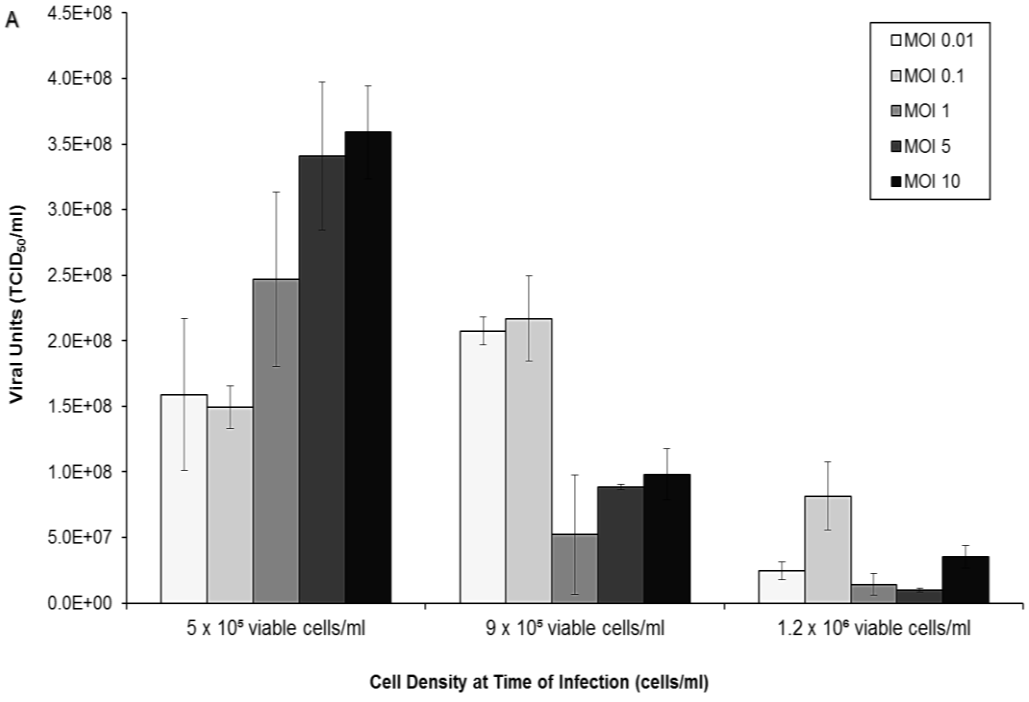
Amino Acid	Amino Acid Conc. 0 h	Amino Acid Conc. 336 h	% Consumption (negative sign indicates production)
Alanine	0.52	0.75	- 44.2
Arginine	0.80	0.72	10
Aspartic Acid	1.23	0.92	25.2
Cysteine	0.19	0.18	5.3
Glycine	0.74	0.70	5.4
Glutamic Acid	1.99	1.41	29.1
Histidine	2.35	2.21	5.9
Isoleucine	0.28	0.24	14.3
Leucine	0.66	0.58	12.1
Lysine	0.97	0.88	9.3
Methionine	0.10	0.07	30
Phenylalanine	0.46	0.42	8.7
Proline	0.68	0.40	41.2
Serine	0.65	0.55	15.4
Tyrosine	0.28	0.25	10.7
Threonine	0.45	0.41	8.9
Valine	0.54	0.48	11.1
Glutamine	0.64	0.38	40.6

5.3.5 OrNV infection in roller bottle cultures

5.3.5.1 Selection of infection parameters: MOI and CD at TOI

The infection strategy is largely determined by the combination of the multiplicity of infection (MOI) and the cell density at the time of infection (CD at TOI) used to infect a culture, and is a major determinant of the efficiency of viral propagation and consequently, the virus yield. The influence of various combinations of CD at TOI and MOI on OrNV production in 25 cm^2 T-flask cultures was studied in Chapter 4 of this thesis. It was found that infecting cells during the early exponential growth phase was necessary in order to maximize virus yields, and to avoid the cell density effect. Given that the dynamics of cell growth and virus infection are likely to be different in T-flasks and roller bottles, it is arguable that the optimal values of the parameters of infection in the two systems can be similar. Therefore, the influence of various combinations of CD at TOI and MOI on OrNV yield were evaluated in roller bottle cultures. A total of 15 independent infection

experiments were performed in which CD at TOI was varied to three levels (5 x 10^5 viable cells/ml, 9 x 10^5 viable cells/ml and 1.2 x 10^6 viable cells/ml), which corresponded to cell densities in the early, mid and late exponential growth phase, respectively; and at each level, cultures were infected at each of five different MOI's (0.01, 0.1, 1, 5 and 10 $TCID_{50}$/cell). Duplicate roller bottle cultures were set up for each infection experiment. Each roller bottle culture was inoculated with 2 x 10^7 cells, and had a final culture volume of 60 ml. Cultures were incubated at 27 ºC and were later, individually infected at a specific time-point in culture (to attain the desired cell density at infection), and with an appropriate volume of OrNV stock, according to the particular CD at TOI and MOI combination tested in the experiment. Infected cultures were harvested on day 14 post-infection and virus yields were determined. Infected cell density was not determined because of the high level of cell detachment from the roller bottle surface that was observed on day 14 post-infection, especially in cultures that were infected in the mid and late exponential growth phase, which made it difficult to accurately perform cell counts.



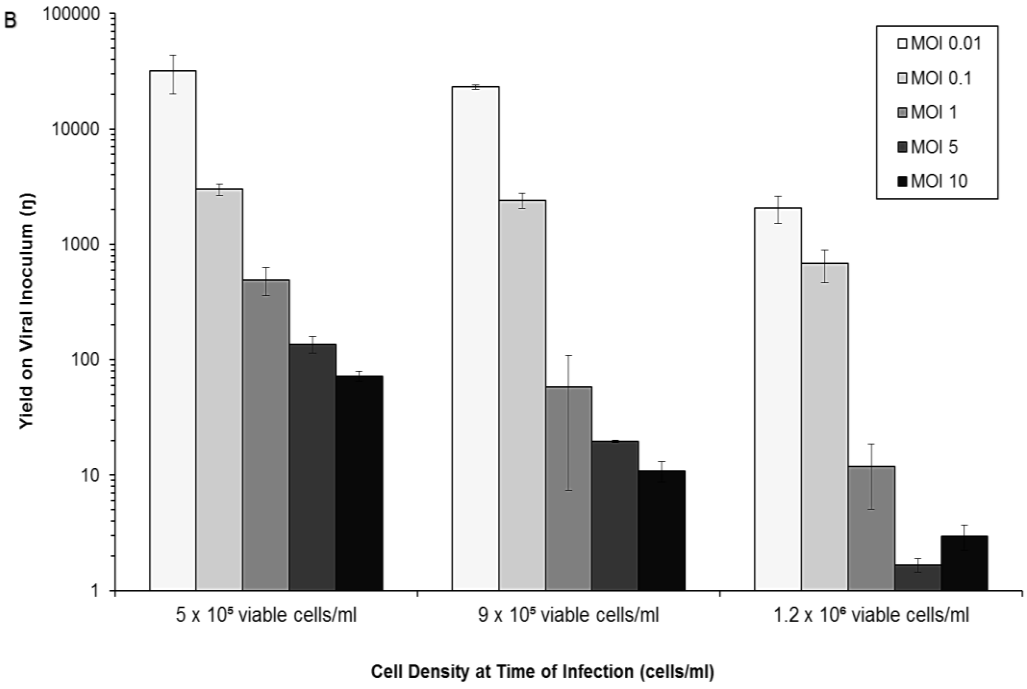


Fig. 5-8 (A) OrNV volumetric yield (B) Yield on viral inoculum (ŋ) achieved 14 days post-infection in DSIR-HA-1179 cell cultures grown in TC-100 supplemented with 10% FBS in 490 cm^2 roller bottles and infected at various CD's at TOI (5 x 10^5, 9 x 10^5 and 1.2 x 10^6 viable cells/ml) and MOI's (0.01, 0.1, 1, 5, 10). Error bars represent standard deviation from the mean of duplicate samples.

Across the range of MOI's evaluated, it was found that cultures infected at a cell density of 5 x 10^5 viable cells/ml produced on average, the highest OrNV volumetric yield (2.51 x 10^8 $TCID_{50}$/ml), compared to those infected at 9 x 10^5 (1.33 x 10^8 $TCID_{50}$/ml) and 1.2 x 10^6 viable cells/ml (3.32 x 10^7 $TCID_{50}$/ml). Statistical analysis showed that OrNV volumetric yield was significantly affected by CD at TOI and not by the MOI, but the interaction between the two factors was statistically significant (Table 5-2). From Figs. 5-8 A and B, it can be observed that the lowest volumetric yields and yield on viral inoculums were obtained with an infection cell density of 1.2 x 10^6 viable cells/ml. Therefore, this CD at TOI was deemed unsuitable for OrNV production in roller bottles. In cultures infected at 9 x 10^5 viable cells/ml and asynchronously, using MOI's of 0.01 and 1, the respective OrNV yields obtained (2.08 x 10^8 and 2.17 x 10^8 $TCID_{50}$/ml), were similar to those obtained in cultures infected at a lower cell density of 5 x 10^5 viable cells/ml and at the same MOI's (1.50 x 10^8 and 1.69 x 10^8 $TCID_{50}$/ml respectively). Despite the similarities in yield, it appeared

advantageous to infect cultures at a CD at TOI of 5 x 10^5 viable cells/ml since the yield on virus inoculum (ŋ) obtained at MOI's 0.01 and 1, were respectively 28% and 19% higher than in cultures infected at 9 x 10^5 viable cells/ml at the same MOI's. In synchronous infections performed at higher MOI's of 1, 5 and 10, both OrNV volumetric yield and ŋ in cultures infected at 9 x 10^5 viable cells/ml were considerably lower than those infected at 5 x 10^5 viable cells/ml. Furthermore, process times are likely to be reduced in earlier-infected cultures, than those infected later in the growth curve. For cultures infected at 5 x 10^5 viable cells/ml, OrNV volumetric yield was positively correlated with increasing MOI, while ŋ showed the opposite effect. Based on comparison of the respective yields in early-infected cultures between MOI 1 (2.5 x 10^8 $TCID_{50}$/ml, ŋ = 494), MOI 5 (3.41 x 10^8 $TCID_{50}$/ml, ŋ = 136) and MOI 10 (3.59 x 10^8 $TCID_{50}$/ml, ŋ = 72); a MOI of 1 was selected for use at this stage of process development since it offered a suitable trade-off between the quantity/cost of viral inoculum required to infect DSIR-HA-1179 roller bottle cultures and the volumetric yield of OrNV that could be obtained.

Table 5-2 Analysis of the main effects and interaction effects of the factors: CD at TOI (5 x 10^5, 9 x 10^5, 1.2 x 10^6 viable cells/ml) and MOI (0.01, 0.1, 1, 5, 10) on OrNV volumetric yield in DSIR-HA-1179 roller bottle cultures, grown and infected in 10% serum-supplemented TC-100.

Factor	Degrees of freedom	Sum of squares	Mean square	F value	P value
CD at TOI	2	2379.83	1189.9	104.9	1.52E-09*
MOI	4	124.52	31.13	2.745	0.068
Interaction	8	1158.84	144.85	12.77	1.99E-05*
Model	14	3663.19	261.66	23.07	1.28E-07
Error	15	170.13	11.34	-	-
Corrected total	29	3833.32	-	-	-

*Significant factors ($p < 0.05$)

5.3.5.2 OrNV production kinetics: Influence of partial culture medium replacement at TOI

OrNV production was assessed over a 14 day infection period in roller bottle cultures that were infected at various cell densities, either with or without partial replacement of culture medium at the time of infection. Partial replacement of culture medium involved removal of 75% of the culture medium and its replacement with an equal volume of fresh medium immediately prior to infection of the culture. A total of 4 independent infection experiments were performed at a MOI of 1 $TCID_{50}$/cell. In the first experiment (a control experiment), cultures were infected at a cell density of 5 x 10^5 viable cells/ml without culture medium replacement at TOI. In the other three experiments, partial culture medium replacement was carried out immediately prior to infection of cultures at the cell densities of 5 x 10^5, 7.3 x 10^5 and 8.5 x 10^5 viable cells/ml, respectively. The following procedure was followed to set up each experiment: Eight replicate roller bottle cultures were each inoculated with 2 x 10^7 cells/ bottle and with final culture volume of 60 ml. The cultures were incubated at 27 ºC, and were each infected with OrNV stock at a MOI of 1 $TCID_{50}$/ml, according to the experimental conditions described above. Duplicate cultures were randomly selected and harvested on day 4, 8, 10 and 14 post-infection and viable cell counts were determined. Culture supernatants from harvested cultures were clarified by centrifugation at 12,000g for 5 min and stored at 4 ºC, for the quantification of OrNV infectious titers. Another set of culture supernatants were similarly harvested, and stored at -20 ºC for analyses of nutrients and metabolites.

Table 5-3 Summary of OrNV production parameters in infection experiments in DSIR-HA-1179 roller bottle cultures at CD's at TOI (5 x 10^5, 6.5 x 10^5 and 8.5 x 10^5 viable cells/ml), performed with or without partial culture medium replacement at TOI.

CD at TOI (viable cells/ml)	75% culture medium replacement at TOI	OrNV volumetric yield ($TCID_{50}$/ml)	Cell-specific OrNV yield ($TCID_{50}$/cell)	Volumetric Productivity ($TCID_{50}$/ml/d)	TOH (d)
5 x 10^5 (Control)	No	2.13 x 10^8	250	1.78 x 10^7	8
5 x 10^5	Yes	2.81 x 10^8	320	2.01 x 10^7	4
6.5 x 10^5	Yes	1.89 x 10^9	1897	7.71 x 10^7	8
8.5 x 10^5	Yes	7.98 x 10^8	763	2.28 x 10^7	8

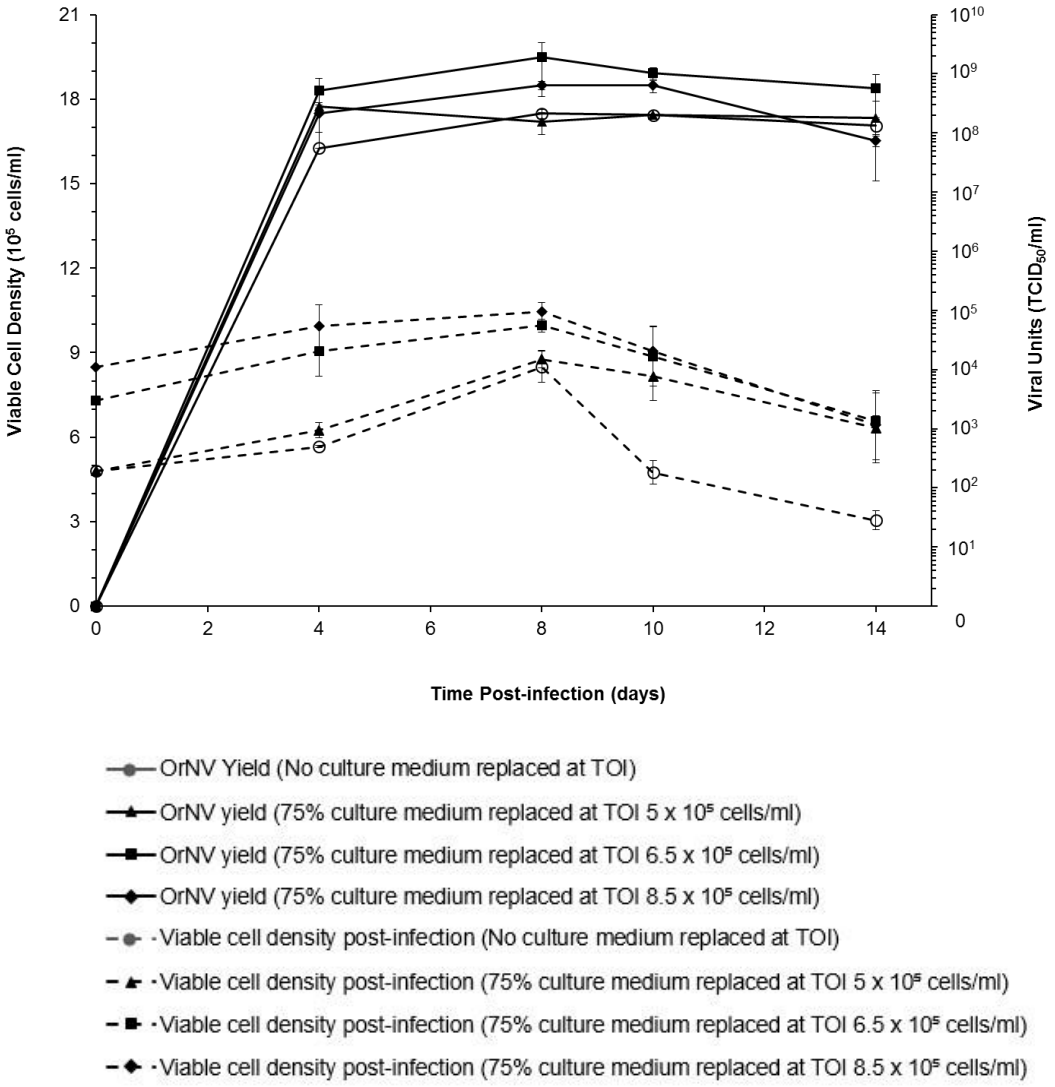


Fig. 5-9 Time course profiles of viable cell density post-infection (dashed line) and OrNV production (solid line) in infected DSIR-HA-1179 cell cultures grown in TC-100 supplemented with 10% FBS in 490 cm^2 roller bottles. Infections were carried out at the following CD's at TOI: 5 x 10^5, 6.5 x 10^5 and 8.5 x 10^5 viable cells/ml, with 75% of the culture medium replaced immediately prior to infection. A control infection experiment (open circles) was carried out at CD at TOI 5 x 10^5 viable cells/ml without culture medium replacement at TOI. A MOI of 1 $TCID_{50}$/cell was used in all experiments. Error bars represent standard deviations from the mean of duplicate experiments.

Two main trends were observed with regard to the evolution of viable cell density and maximum OrNV yield in the different infection conditions. Firstly, that increasing CD at TOI led to an increase in the MVCD; and secondly, that partial replacement of culture medium at TOI led to improved OrNV production over control cultures that were infected without medium replacement. In early-infected control cultures (CD at TOI: 5 x 10^5 viable cells/ml), a 41.1% increase in cell growth occurred over 8 days of infection to reach an MVCD of 8.5 x 10^5 viable cells/ml. By the end of the infection phase, viable cell density had declined by 64.2% to 3.04 x 10^5 viable cells/ml. The maximum OrNV volumetric yield (2.13 x 10^8 $TCID_{50}$/ml) was reached on day 8 post-infection. This corresponded to a cell specific yield of 250 $TCID_{50}$/cell and volumetric productivity of 1.78 x 10^7 $TCID_{50}$/ml/day (Table 5-3).

Partial culture medium replacement at TOI at the same infection cell density (5 x 10^5 viable cells/ml), led to a slight increase in MVCD to 8.77 x 10^5 viable cells/ml and in OrNV volumetric and cell-specific yield to 2.81 x 10^8 $TCID_{50}$/ml and 320 $TCID_{50}$/cell, respectively. Increasing the CD at TOI to 6.5 x 10^5 viable cells/ml and 8.5 x 10^5 viable cells/ml under the same partial culture medium replacement regime, led to respective increases in MVCD to 9.96 x 10^5 and 10.45 x 10^5 viable cells/ml. Between the MVCD and end of infection, viable cell densities declined by 42.9%, 34% and 38.5%, respectively. Compared to control cultures, the lower percentage decline in viable cell densities in cultures with medium replacement at TOI, was presumably due to the nutrient supplementation having improved the cells' physiological state post-infection which might have offset cell death and cell lysis to a degree.

Partial culture medium replacement led to an overall improvement in volumetric and cell-specific OrNV yields (Table 5-3). Interestingly, increasing the CD at TOI to 6.5 x 10^5 viable cells/ml, improved OrNV yields by an order of magnitude (1.89 x 10^9 $TCID_{50}$/ml, 1897 $TCID_{50}$/cell). The highest volumetric productivity (7.71 x 10^7 $TCID_{50}$/ml/day) occurred in this condition. It was found that further increasing the infection cell density to 8.5 x 10^5 viable cells/ml, however, led to lower volumetric and cell-specific yields of 7.98 x 10^8 $TCID_{50}$/ml and 763 $TCID_{50}$/cell; which may be possibly explained as a consequence of the cell-density effect.

5.3.6 Nutrient consumption and metabolism in infected roller bottle cultures

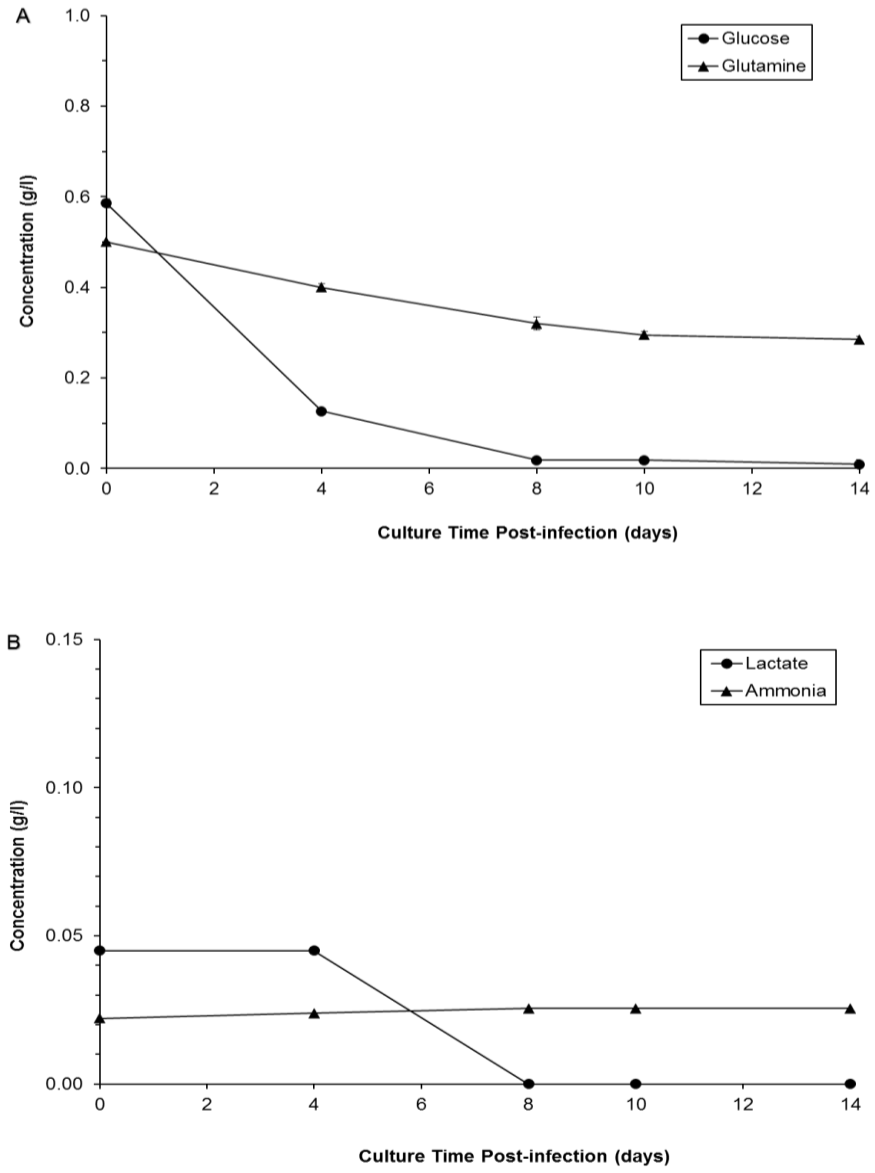


Fig. 5-10 (A) Glucose, glutamine and (B) lactate and ammonia profiles in infected cultures of the DSIR-HA-1179 cell line grown in TC-100 supplemented with 10% FBS in 490 cm^2 roller bottles. Cultures were infected at a density of 5 x 10^5 viable cells/ml and multiplicity of infection of 1 $TCID_{50}$/cell. Error bars represent the standard deviations from the mean of duplicate samples. Error bars are difficult to see because standard deviations were very small.

Table 5-4 Amino acid concentration at the beginning and end of batch growth in infected cultures of the DSIR-HA-1179 cell line grown in TC-100 supplemented with 10% FBS in 490 cm^2 roller bottles. Cultures were infected at a density of 5 x 10^5 viable cells/ml and multiplicity of infection of 1 $TCID_{50}$/cell. Concentrations of all amino acids are expressed in mg/g except for glutamine which is expressed in g/l.

Amino Acid	Amino Acid Conc. 0 hpi	Amino Acid Conc. 336 hpi	% Consumption (negative sign indicates production)
Alanine	0.69	0.70	- 1.5
Arginine	0.71	0.72	- 1.4
Aspartic Acid	1.05	0.99	5.7
Cysteine	0.17	0.18	- 5.9
Glycine	0.67	0.69	- 3.0
Glutamic Acid	1.57	1.40	10.8
Histidine	2.15	2.17	- 0.9
Isoleucine	0.24	0.25	- 4.2
Leucine	0.57	0.59	- 3.5
Lysine	0.87	0.88	- 1.2
Methionine	0.08	0.08	0
Phenylalanine	0.41	0.41	0
Proline	0.58	0.50	13.8
Serine	0.57	0.56	1.7
Tyrosine	0.24	0.25	- 4.2
Threonine	0.40	0.41	- 2.5
Valine	0.47	0.49	- 4.3
Glutamine	0.50	0.29	42

Glucose, glutamine (Fig 5-10 A), lactate and ammonia (Fig 5-10 B), were evaluated in DSIR-HA-1179 roller bottle cultures grown in 10% serum-supplemented TC-100, that were infected at a CD at TOI of 5 x 10^5 viable cells/ml, and MOI of 1 $TCID_{50}$/cell, over a 14 day infection period. Similar to uninfected cultures, glucose and glutamine were the nutrients most consumed by infected cultures. Glucose was entirely consumed between day 0 and 8 post-infection and was limiting between day 8 and 14 post-infection. Glutamine was consumed to 42% of its concentration at the time of infection. The specific rates of consumption of these nutrients were evaluated between the time of infection and day 4 post-infection and the post-infection time point when the maximum OrNV volumetric yield was reached, i.e. day 8 post-infection. In the first 4 days post-infection, the specific rate of glucose consumption increased from 6.96 x 10^{-12} g/c/h to 9.15 x 10^{-12} g/c/h. Between day 4 and 8 post-infection, this value decreased to 1.65 x 10^{-12} g/c/h. The specific rate of glutamine consumption followed a similar trend and increased from 1.39 x 10^{-12} g/c/h to 1.99 x 10^{-12} g/c/h during the first 4 days of infection. Between days 4 and 8 post-infection, its value decreased to 1.18 x 10^{-13} g/c/h. From Fig 5-10 B it can be observed the very low concentration of lactate (0.05

g/l) present in the culture medium at the time of infection was rapidly consumed and exhausted from the culture medium between day 4 and 8 post-infection. Ammonia, which was detected at very low levels of approximately 0.02 g/l, was neither consumed not produced in infected cultures.

Concentrations of amino acids in the culture supernatant from infected roller bottle cultures was assessed at the beginning and at the end of the infection phase (Table 5-4). With respect to amino acids, the overall trend during infection was accumulation over consumption. A range of amino acids including alanine, arginine, cysteine, glycine, histidine, isoleucine, leucine, lysine, tyrosine, threonine and valine accumulated to between 0 and 10% of their concentration at the time of infection. Significant consumption during the infection phase was observed only with glutamine (42%), while other amino acids such as proline, serine, glutamic acid, aspartic acid were consumed to a lesser extent between 0 – 15%. The concentrations of methionine and phenyalanine remained unchanged over the infection period with no consumption nor production noted.

5.4 Discussion

Roller bottles were chosen for scaling-up OrNV production in DSIR-HA-1179 cell cultures, based on the strong attachment-dependent nature of the cell line and the prevailing inability to adapt it to grow in suspension conditions (Pushparajan *et al*., 2013). Scale-up of virus production from T-flasks to roller bottles with the aim of maximizing volumetric productivity, should ideally target an increase in the overall number of cells available for virus infection and enhanced cell-specific virus productivity (Maranga *et al*., 2002). The results presented in this chapter, establish on both counts that the roller bottle system is an excellent platform for scaling-up OrNV production from tissue culture flasks. While improvements between T-flasks and roller bottles relate to the increase in surface area available for cell growth, a more profound role is played by the operational dynamics of the roller bottle system which is characterized by constant but gentle agitation and improved gas exchange over stationary T-flasks (Fenge and Lüllau, 2006; Isayeva *et al*., 2003). With regard to the DSIR-HA-1179 cell – OrNV system, differences between T-flasks and roller bottles manifested themselves in several facets of the process including cell attachment and growth, metabolism and virus production, which are discussed in this section.

Cell attachment and growth in roller bottle cultures

Cell attachment involves a dynamic interplay between characteristics of the cell line, of the growth surface and any surface modifications made to it, and the culture medium (Whitford, 2013). Though cell attachment kinetics have not been specifically quantified for DSIR-HA-1179 cells; it was observed that it took up to 12 h from the time of inoculation until complete cell attachment to occur in stationary T-flasks. Compared to other animal cell lines, DSIR-HA-1179 cell attachment appeared to be an intrinsically slow process (Grinnell and Feld, 1979; Volger and Bussian, 1987). Cell adhesion is a step-wise process initiated by adsorption of serum proteins on the growth surface followed by cell contact with the surface, attachment and finally, spreading of attached cells (Grinnell, 1978). Fibronectin and vitronectin (attachment glycoproteins found in serum), as well as divalent cations (in the basal culture medium), promote the adsorption, attachment and spreading of cells. In contrast to cell attachment directly onto growth surfaces which is a non-specific process mediated by electrostatic forces, adsorption to serum proteins occurs through specific interactions with cell surface integrin receptors. The serum proteins are subsequently incorporated into the extracellular matrix of the spread cells (Carter *et al*., 1981; Hayman and Rouslahti, 1979; Hughes *et al*., 1979; Xu and Mosher, 2011). This could explain why cell attachment and subsequent monolayer formation was improved when the roller bottle surface was conditioned with 25 ml of serum-containing culture medium prior to inoculation. Inoculation of cells in a reduced culture volume of 25 ml also improved their attachment efficiency. A lower culture medium volume might have resulted in lower drag force on cells which facilitated better adhesion to the roller bottle surface (de Villiers, 2012). It may also be reasoned that the resulting high inoculum cell density in the reduced culture volume led to a higher concentration of growth promoting fractions which promoted attachment of DSIR-HA-1179 cells to the roller bottle surface (Taticek *et al*., 2001).

Improvements to insect cell attachment and growth in roller bottles as a consequence of surface-modifications and modified initial culture procedures have been demonstrated in previous studies. In establishing the first roller bottle cultures of IPLB-21 and IPLB-1254 cell lines in glass roller bottles, Vaughn recommended pre-treating the bottle's surface with 25 ml serum-containing culture medium prior to cell inoculation (Vaughn, 1976). Weiss *et al*. reported improvements in cell growth when the IPL-21 AEIII cell line was cultured in plastic roller bottles (Weiss *et al*., 1981a). Later, Weiss *et al*. demonstrated growth improvements in a perfused system (the Dyna Cell

Propagator) where a film of Melinex was coated on the plastic growth surface (Weiss and Vaughn, 1986). Improvements in the growth of certain insect cell lines on positively charged Primaria surfaces have also been reported (Goodwin, 1985). In another study, problems with Tn-5 cell attachment to the surface of standard polystyrene roller bottles were offset when roller bottles were pre-coated with DEAE-based microcarriers (Wickham and Nemerow, 1993). The Corning® roller bottles used in the present study, were made of negatively charged, hydrophilic polystyrene and were found to be suitable for DSIR-HA-1179 attachment and growth using the modified initial culture procedure. Other surface-treated roller bottles which have been developed specifically for use with hard-to-attach cell lines; for example, Corning® CellBIND or Primaria products, are available in the market, and could be potentially tested for DSIR-HA-1179 cell culture in future.

In attached cultures, the magnitude of the cell inoculum determines the initial cell density per cm^2 of growth surface area. An inoculum of 2 x 10^7 cells/ roller bottle (4 x 10^4 cells/ cm^2) led to considerable improvement in cell yield over an inoculum of 1 x 10^7 cells/ roller bottle (2 x 10^4 cells/cm^2), and represented the most efficient use of inoculum. Interestingly, further increasing the inoculum level to 3 x 10^7 cells/ roller bottle (6 x 10^4 cells/cm^2) did not result in a proportional increase in cell yield. This confirms the existence of a critical inoculum size necessary for optimal cell growth, which for attached DSIR-HA-1179 cultures was 4 x 10^4 cells/cm^2. Similar observations on a critical initial cell density have been made for Sf-9, Sf-21, S2 and High Five lepidopteran cell lines, grown in attached and suspension culture systems (Calles *et al*., 2006; Doverskog *et al.*, 1997; Kioukia *et al*., 1995; Moraes *et al*., 2012; Vaughn, 1976). The lag phase of growth was prolonged in cultures initiated at low initial cell densities compared to those initiated at high initial cell densities (Calles *et al*., 2006). This can be attributed to the accumulation of growth-promoting autocrine factors to critical concentrations earlier in culture which in turn, caused DNA replication to commence earlier and with greater synchronicity (Calles *et al*., 2006). Doverskog *et al*. found endogenous expression of insulin-like growth factor I as well as an insulin-binding soluble protein with a molecular weight of 27 kDa by Sf-9 cells grown in serum-free culture medium. At high inoculum cell densities, the proliferative effect of these growth factors was directly correlated to improvements in the specific growth rate and maximum cell yield (Doverskog *et al*., 1997; Doverskog *et al*., 1999). On the other hand, negative effects of exceeding the optimal initial cell density include the rapid decline in pH and dissolved oxygen in the culture medium, higher degree of cell detachment and formation of cell debris, have been reported in roller

bottle cultures (Tsao *et al*., 1992). These effects may be explained by conditioned medium consisting of carryover metabolites with the likelihood to cause cellular toxicity, in addition to the presence of stimulatory autocrine factors. While the identification of autocrine factors secreted by DSIR-HA-1179 cells was not within the scope of this thesis, the modulation of cell growth observed in response to varying inoculum levels in roller bottle experiments, hints at the possibility of autocrine regulation of cell proliferation occurring in this cell line.

Cell metabolism in roller bottle cultures

For purposes of industrial scale-up, it is desirable to obtain high cell and virus yields with minimum nutrient consumption, and consequently the use of lower volumes of culture medium. Despite roller bottle culture volumes of 80 ml and 100 ml resulting in higher cell yields over a culture volume of 60 ml, the latter was initially selected because it produced the best percentage of improvement in virus titer. However, in a later experiment it was found that in a culture volume of 60 ml, glucose - the main carbon and energy source of the cells - was rapidly consumed from the start of culture and exhausted by day 8 of culture. Glucose was consumed at a higher specific rate in uninfected roller bottle cultures (5.2×10^{-12} g/c/h) than in stationary T-flask cultures (4.1×10^{-12} g/c/h) (Chapter 3 of this thesis). Glucose was a limiting factor in relation to the maximum viable cell density achievable in the culture medium since the maximum viable cell density was achieved on day 14 of batch growth, 6 days after glucose exhaustion from the culture medium. The lower specific growth rate in roller bottle cultures ($0.0052\ h^{-1}$) compared to T-flask cultures ($0.0069\ h^{-1}$), could have been due to glucose limitation as well as an increased 'cell maintenance energy' requirement in roller bottle cultures (Raghunand and Dale, 1996; Xiang-ming and Yuan-xing, 2001). Supplementing the culture medium with glucose or increasing the culture volume would avoid glucose limitation and could potentially boost cell productivity in roller bottle cultures. Glutamine was consumed to a higher extent (40.6%) and at a higher specific rate (9.5×10^{-13} g/c/h) in roller bottles cultures, than in stationary T-flask cultures (27.9% and 8.3×10^{-13} g/c/h, respectively). The continuance of cell growth and maintenance of high cell viabilities in roller bottle cultures under glucose limitation, could be due to the use of glutamine as a secondary energy source and because glutamine did not deplete to limiting concentrations during the culture. It is also possible that alanine was back-consumed to form pyruvate, to meet the energy demands for cell growth. Two reasons may account for the increased nutrient uptake rates in roller bottle

cultures, (a) the constant, gentle mixing prevents concentration gradients forming in the culture medium enabling better nutrient mass transfer to cells (b) improved oxygen transfer in the roller bottle system (Jensen, 1981; Madihally, 2010; Spier and Griffiths, 1984). In roller bottles, as with T-flasks, oxygen transfer occurs by diffusion. However in roller bottles, an increased amount of dissolved oxygen in the medium can be obtained because a thin film of the medium which facilitates gas exchange between gas and liquid phases is formed on the unsubmerged inner surface of the bottle during rotation. Weiss *et al*. found that the surface area to volume ratio in a 490 cm^2 roller bottle, permitted sufficient diffusion of oxygen to supply the amount of dissolved oxygen (1.81×10^5 µl) required by a 100 ml culture of IPL-Sf-21AE cells (Weiss *et al*., 1982).

Compared to T-flask cultures, amino acid consumption was, in general, up-regulated in roller bottle cultures. In addition to glutamine, some of the highest consumed amino acids, proline and glutamic acid, could both be converted to glutamate which upon deamination would form α-ketoglutarate. Methione and aspartic acid could be converted into oxaloacetate and succinyl CoA, repectively. In the absence of glucose, the anaplerotic sequestering of carbon intermediates of the TCA cycle would be a means to generate cellular energy (Ferrance *et al*., 1993). Other amino acids that were minimally consumed (including isoleucine, leucine, histidine, valine, tyrosine etc.), may have been incorporated in cellular protein but not significantly used for cellular energy generation (Drews *et al*., 1995). None of the amino acids became limiting in roller bottle cultures as none were consumed to more than half their original concentration. The oversupply of amino acids in insect cell culture media with respect to cellular requirements, has been reported for *Spodoptera frugiperda, Anticarsia gemmatalis* and *Drosophila melanogaster* insect cell lines and appears to be the case for the DSIR-HA-1179 cell line as well (Bedard *et al*., 1993; Bovo *et al*., 2008; Chapter 3 of this thesis; Gioria *et al*., 2006). As previously observed in Chapter 3, DSIR-HA-1179 metabolism is characterized by a lack of production of lactate and ammonia, and the production of alanine. Lactate is a by-product of anaerobic glycolysis and its non-production in roller bottle cultures is a further testament to the adequacy of oxygenation of cells in this system. Despite the increased uptake of amino acids in roller bottle cultures, the complete lack of ammonia production can be explained by the high level of alanine production. In line with increased amino acid consumption, cells in roller bottle cultures accumulated alanine to a greater extent (44.2%) than in T-flask cultures (31.1%). The preferential accumulation of alanine over ammonia points to an active transamination pathway mediated by alanine aminotransferase, where ammonia derived from glutamate catabolism and

pyruvate are converted to alanine and α-ketoglutarate (Bedard *et al*., 1993). This means of culture detoxification may have contributed to the longevity and viability of DSIR-HA-1179 cells over the extended batch culture period in roller bottles.

OrNV production

Careful adjustment of the multiplicity of infection (MOI) and time of infection (TOI) are crucial for maximizing virus yields in roller bottle cultures (Weber *et al*., 2001). Batch culture is a dynamic process in which the physiological state of the cells, as well as the composition of the culture medium, are modified over time. These changes can affect the susceptibility and permissivity of cells to viral replication. Consequently, maximal virus production occurs when cells are infected at a density when they are most permissive to viral replication. On the other hand, at a given TOI, the MOI may be manipulated in order to determine the fraction of the cell population that is initially infected (Aucoin *et al*., 2010). The synchronicity of infection will ultimately have a bearing on the rate of virus production, time of harvest and product quality. In Chapter 4 of this thesis, the influence of the CD at TOI was described for DSIR-HA-1179 cultures growing in 25 cm^2 T-flasks. Decline in OrNV volumetric yields was found to occur at infection cell densities of 9.3 x 10^5 viable cells/ml or higher, as a consequence of the cell-density effect. In the present study, from screening a range of CD at TOI's and MOI's in roller bottle cultures, it was found that CD at TOI influenced OrNV volumetric yield to a greater extent than MOI. Regardless of MOI, infections carried out during the late exponential phase of growth (CD at TOI 1.2 x 10^6 cells/ml) resulted in very poor OrNV yields. This can be explained as a result of the cell density effect, for which several explanations have been proposed including cell-to-cell contact inhibition, nutrient deficiencies, accumulation of by-products, autocrine factors, cell cycle changes and drop in central metabolic energy (Claus *et al*., 2012). Of these, contact inhibition in the near-confluent monolayer and nutrient deficiency (in particular, glucose limitation) seem to be the most plausible explanations for the cell-density effect in infected roller bottle cultures. For an infection cell density of 9 x 10^5 viable cells/ml (corresponding to the mid-exponential phase of batch growth), higher OrNV volumetric yields were observed in infections performed at MOI's of 0.01 and 0.1; than at higher MOI's of 1, 5 and 10. The long time to harvest (day 14 post-infection) was a drawback in the experiment, and it possible that the low virus yields obtained in the latter MOI conditions, were a result of proteolytic decay in infectious virus due to exacerbated cell lysis by the time of harvest

(Chapter 3 of this thesis; Maranga *et al*., 2003). Infecting roller bottle cultures during the early-exponential phase (5 x 10^5 viable cells/ml) led to the highest OrNV volumetric yields. MOI of 1 was chosen in order to be able to obtain comparable yields (2.47 x 10^8 $TCID_{50}$/ml) to those reached at MOI's 5 and 10 (3.4 - 3.6 x 10^8 $TCID_{50}$/ml), whilst reducing the viral inoculum requirement. Radford *et al*. have cautioned against use of MOI's of less than unity in large-scale bioreactors due to the fluctuations of cell growth and risk of producing sub-optimal yields associated with these conditions (Radford *et al*., 1997). While a MOI of 0.01 was not used for production runs in roller bottles, it was identified as a suitable MOI to infect cells in early-exponential growth phase for the production of virus inoculum for the infection of multiple roller bottles. The motivations for using MOI's of less than 1 in the viral inoculum train were: (a) the cost-efficiency of using lower viral inoculum volumes; (b) ability to obtain OrNV volumetric yields as high as 1.6 x 10^8 $TCID_{50}$/ml at a MOI as low as 0.01, albeit with a longer time of harvest, which was not a prohibitive factor in the case of inoculum expansion; (c) reduced risk of accumulating defective interfering particles in low MOI infections (Krell, 1996; Rhodes, 1996).

One of the effects that rolling has on cells is to mechanically 'stress' them. Cells stressed by different forms of motion may have increased cell proliferation, decreased population doubling times, activated genes, altered cell metabolism, increased longevity and increased productivity. Specifically, it has been proposed that rolling and rocking of cells activates or stimulates them to produce more virus or antigens (Hughes, 1993). Secondly, high oxygen transfer rates in roller bottles can support the increased oxygen uptake rates of infected cells that result from the increased metabolic demands which occur as a consequence of virus replication and virus-induced macromolecule biosynthesis (Schmid, 1996). Thirdly, the dynamics within the roller bottle system improve both virus mass transfer as well as nutrient mass transfer to infected cells. While the rate of virus attachment to cells, governed by Brownian motion, would not be affected by hydrodynamics *per se*; it has been suggested that the rolling motion leads to the creation of multiple infectious foci on the roller bottle monolayer from which infection can spread, thereby enhancing the efficiency of virus production (Dee and Shuler, 1997; Unger *et al*., 2002). These factors may provide explanations for the improvement in OrNV volumetric and cell-specific yield in roller bottles (2.13 x 10^8 $TCID_{50}$/ml, 250 $TCID_{50}$/ml) over T-flask cultures (6.84 x 10^7 $TCID_{50}$/ml, 102 $TCID_{50}$/cell) at the same infection conditions. Improvement in virus volumetric yields in roller bottle cultures have been reported for a number of animal cell-virus systems (Hughes, 1993), with

some studies specifically finding these improvements in baculovirus-infected insect cell cultures (Vaughn, 1976; Weiss *et al*., 1981b). Viral infection also conferred an increased metabolic burden on cells, evidenced by increased specific consumption rates of glucose and to a lesser extent, glutamine; which occurred in the immediate 96 h period following infection. However, after this initial period, glucose and glutamine uptake rates were down-regulated. OrNV production in DSIR-HA-1179 cells may be viewed as consisting of two phases, the first involving viral entry and a switch in cellular metabolism towards viral needs; and the second, characterized by virus accumulation, release and cell damage (Genzel *et al*., 2004). Increased nutrient uptake occurred in the first phase, concomitant with higher cellular oxygen demand and respiratory activity; and is supported by studies in which the maximum specific oxygen uptake rate was observed between 10-20 hours post-infection in Sf-9 cultures (Hensler and Agathos, 1994; Schopf *et al*., 1990; Wong *et al*., 1994) On the other hand, the slight accumulation of amino acids observed at the end of infection was related to the second phase of infection, where cell lysis could have led to the release of amino acids in cells into the intracellular metabolite pool. There was a 42.7% reduction in the percentage of alanine produced between the growth and infection phases of culture, which may be explained by the transamination of alanine to pyruvate in order to meet the cells' energetic needs (Ohman *et al*., 1995). The consequence of this reaction would have been the accumulation of ammonia. However, the fact that the concentration of ammonia was as low as 0.03 g/l at the end of the infection phase, points to an alternate metabolic route for its disposal, for example uricoltely, which must be investigated in future work (Stavroulakis *et al*., 1991).

Improvement of OrNV yield with partial culture medium replacement at TOI

Nutrient limitation is the most commonly ascribed cause for reductions in cell-specific virus yields in baculovirus-infected cultures at high cell densities. Several studies have demonstrated that through the maintenance of a sufficient nutrient supply and aeration, specific productivity could be restored to that of a low-density infection or even enhanced i.e. the cell-density effect delayed to higher infection cell densities (Hensler and Agathos, 1994; Lindsay and Betenbaugh, 1992). Maintaining high cell-specific yields at higher cell densities ultimately results in higher volumetric yield in the process. Strategies that have been used to improve nutrient supply include replacement of spent culture medium with fresh medium at TOI (Doverskog *et al*., 2000; Hensler and Agathos, 1994; Jesionowski and Ataai, 1997; Reuveny *et al*., 1993), addition of fresh culture medium after

viral infection (Lazarte *et al*., 1992), supplementation of fresh culture medium with selective nutrients followed by medium replacement at TOI (Reuveny *et al*., 1993) and application of nutrient feeds in fed-batch cultures (Chan *et al*., 1998; Elias *et al*., 2000; Jardin *et al*., 2007). However despite best efforts, nutritional improvements have been able to sustain yield improvements only up to certain threshold cell densities, above which the cell-density effect sets in. This is one of the main reasons why *in vitro* production of baculoviruses are unable to achieve economic viability yet (Reid *et al*., 2013).

In the culture medium replacement strategy used in OrNV infected-DSIR-HA-1179 roller bottle cultures, only 75% of the culture medium was replaced at TOI in order to preserve at least a small percentage of autocrine factors in the conditioned medium which might have stimulatory effects on cell growth and virus infection. Partial culture medium replacement led to improved cell-specific and volumetric OrNV yields at all infection cell densities tested, when compared to cultures without medium replacement. While the nutritional sufficiency of the culture medium was a clear reason for the yield improvements, another cause which could be implicated in this occurrence was the cell-cycle state. The eukaryotic cell cycle progresses through four phases, namely, the Gap 1 (G 1), DNA synthesis (S), Gap 2 (G2) and Mitosis (M) phase. It has been found that infection of cells in the G1/S phase of the cell cycle is more efficient that infection in the G2/M phase (Saito *et al*., 2002). The synchronicity with which individual cells in culture enter the G1 and S phases are progressively reduced after the initial lag phase of growth. As the culture progresses it consists of populations of cells at different points in the cell cycle. The effect of culture medium replenishment is to re-synchronize all cells in G2/M phase, after which they enter the following G1 and S phases with greater synchronicity. Consequently, with culture medium replacement immediately prior to the TOI, a greater population of cells would be infected in their most productive phase, leading to improved cell-specific yields (Lindskog, 2006). While the remarkable order-of-magnitude increase in cell-specific yield in infections carried out at 6.5×10^5 viable cells/ml after medium replacement (1897 $TCID_{50}$/cell), compared to infections carried out at 5×10^5 viable cells/ml (320 $TCID_{50}$/cell), could have possibly occurred due to a better physiological cell state and cell cycle synchronization in the former condition, it is not a conclusive explanation given that the two infection cell densities were not that different. It is possible that the

results are erroneous due to experimental errors and it is therefore recommended that the experiment be replicated in future to re-confirm these findings.

At a higher infection cell density of 8.5 x 10^5 viable cells/ml, despite the higher maximum number of viable cells available for virus replication, it appeared that the specific capacity of individual cells to replicate virus was reduced to 763 $TCID_{50}$/cell, due to the onset of the cell density effect. While it was unlikely that nutritional deficiency, toxic metabolite accumulation or cell cycle status caused the cell-density effect, explanations such as cell-to-cell contact inhibition and/or drop in central metabolic energy are more plausible in this case. Nevertheless, while culture medium replacement at TOI is an effective strategy for improving OrNV volumetric yields in roller bottle cultures, it is not a viable strategy to use in large-scale industrial production, due to the prohibitive costs of using large volumes of serum-supplemented TC-100 culture medium. Therefore, the lines of inquiry in future research must be on the formulation of a low-cost culture medium, free of animal sera and specifically tailored to the DSIR-HA-1179 cell line's nutritional needs, as well as the development of low-cost nutrient feeds with the potential to improve peak cell density and cell-specific productivity in the system.

5.5 References

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Chapter 6

Microcarrier-based process for the production of *Oryctes* nudivirus in DSIR-HA-1179 insect cell cultures in spinner-flask bioreactors

6.1 Introduction

The advent of *in vitro* insect cell culture has enabled the production of insect viruses for use as biopesticides within the sterile, controlled environment of bioreactors. While *in vitro* production offers several advantages over the traditional *in vivo* method of propagating viruses in live insect colonies, this technology has not seen commercial success till date as the cost of production remains too high for a low margin product such as a biopesticide. Some of the challenges to be surmounted in this regard relate to the development of low cost culture media, the ability to cultivate cells to high densities in scalable bioreactors, and the maintenance of high cell-specific virus yields in infections carried out at high cell densities (Claus *et al*., 2012; Reid *et al*., 2013). Bioreactor selection for the large-scale production of an insecticidal virus must be made with due consideration of the characteristics of the specific cell line and virus combination. For example, the bioreactor configuration will depend on whether the cell line is attachment-dependent or can grow in free suspension (Fenge and Lüllau, 2006). The aeration and agitation mechanisms used within the bioreactor will depend on the cell line's shear sensitivity to hydrodynamic forces (Cherry and Papoutsakis, 1986; Chisti, 2000). The choice of bioreactor mode of operation between batch, fed batch or continuous perfusion will largely depend on the cells' growth characteristics and metabolism, such as whether nutrient feeds are required to boost productivity or whether toxic metabolite build-up could be alleviated through continuous medium perfusion (Butler, 1996; Chico and Jäger, 2000; Ozturk, 1996). Furthermore, the nature of the virus (lytic or non-lytic) will dictate

the method used for product harvest and the subsequent downstream processing steps (Wu and Goosen, 1996).

The DSIR-HA-1179 insect cell line is uniquely distinguished as being the only available cell line which is susceptible and permissive to replication of the *Oryctes* nudivirus (OrNV), a useful biopesticide (Crawford and Sheehan, 1985). Since the 1960's, OrNV has been applied for biocontrol of the coconut rhinoceros beetle, which is a major defoliator of coconut and oil palms throughout the Asia-Pacific region (Huger, 2005; Bedford, 2013). Large-scale production of OrNV in the DSIR-HA-1179 cell line cultivated in large-volume and scalable suspension bioreactors such as stirred tanks and airlift bioreactors would be desirable. However, a major challenge in accomplishing this is posed by the strong anchorage-dependent nature of the cell line and the inability to adapt it to grow in free suspension (Pushparajan *et al*., 2013). Since the maximum cell density reached by anchorage-dependent cells is directly proportional to the surface area available for their growth, scale-up is based on the amplification of the growth surface area. In this vein, successful production of OrNV in DSIR-HA-1179 cells using a roller bottle system has been demonstrated (Chapter 5 of this thesis); however drawbacks of this system include a large footprint, labor-intensiveness, poor monitoring and control of process parameters and the likelihood of product variation between individual roller bottle cultures. The use of microcarriers for the culture of DSIR-HA-1179 cells is an attractive alternative to roller bottle culture.

A key advantage in using microcarriers is their extremely high surface area to volume ratio which allows high cell densities and in turn, high virus volumetric productivities to be reached in culture (Hirtenstein *et al*., 1980). With microcarrier culture, DSIR-HA-1179 cells would reap the benefits of growing in a dynamic suspension-like environment and be easily integrated with a wide range of scalable unit bioreactors used in industry. While microcarrier technology is an industry standard for the production of a range of biologicals such as vaccines, interferons, monoclonal antibodies and erythropoetin, there are few reports on its application to biopesticide or recombinant protein production in insect cell cultures (Chu and Robinson, 2001; Ikonomou *et al*., 2002). The earliest report of microcarrier culture of insect cells was for the production of Sindbis and West Nile arboviruses in the AA mosquito cell line grown on DE-53 carriers (Lazar *et al*., 1987). Dengue virus production in the C6/36 mosquito cell line grown on Cytodex-1 microcarriers has also been demonstrated (Liu and Wu, 2004). The Tn-5 cell line has also been cultured on DEAE-based

microcarriers and glass beads in roller bottles and in split-flow airlift bioreactors (Chung *et al*., 1993; Wickham and Nemerow, 1993). In the most recent report, several solid and macroporous microcarriers were screened for their ability to support cell growth and recombinant β-galactosidase production in Sf-9 and High-five insect cell lines (Ikonomou *et al*., 2002). The widespread use of microcarriers for the cultivation of insect cells has been precluded mainly because most of the industrially relevant insect cell lines have been adapted to grow in free suspension (Archambault *et al*., 1994). In this respect, the DSIR-HA-1179 cell line is an anomaly and its cultivation on microcarriers represents an ideal use for the technology. This chapter explores the feasibility of a microcarrier-based process for DSIR-HA-1179 cell culture and OrNV production in spinner flask bioreactors.

Given the diversity in physical and chemical properties of commercially available microcarriers and the fact that different cell lines have specific requirements for their attachment and growth on substrates, the starting point of this work was the preliminary screening of DSIR-HA-1179 cell growth on three types of solid and macroporous microcarriers in stationary cultures. This culminated in the selection of a suitable microcarrier for the cultivation of DSIR-HA-1179 cells. It has been previously recognized that the initial attachment of cells to microcarriers is critical to the success of the culture. Furthermore, the conditions which ensure the best initial attachment of cells to microcarriers may be different from those which promote cell growth later in the culture (Forestell *et al*., 1992; Kong *et al*., 1999). Based on these considerations, culture parameters (cell attachment procedure, inoculum cell concentration and microcarrier concentration) which maximized cell attachment and yield on microcarriers in spinner flask cultures were selected. Cell growth and metabolism was evaluated in uninfected microcarrier cultures, and those that were infected with OrNV. Finally, the kinetics of OrNV production was evaluated in the system.

6.2 Materials and Methods

6.2.1 Cells and culture medium

DSIR-HA-1179 cells, originally established from surface sterilized eggs of the black beetle *Heteronychus arator* (Crawford, 1982), were obtained from the AgResearch (Lincoln, New

Zealand) cell culture collection as adherent cultures in 25 cm^2 tissue culture flasks (Corning®). The cultures were maintained at 27 ºC, in 5 ml of 10% foetal bovine serum (FBS) (Life Technologies, New Zealand) supplemented PS-100 culture medium, which was prepared in-house. The cells were sequentially adapted to grow in TC-100 culture medium (Sigma) supplemented with 10% FBS (Pushparajan *et al*., 2013), and maintained at 27º C as attached cultures in 25 cm^2 and 75 cm^2 tissue culture flasks (Corning®) with culture volume of 5 and 15 ml, respectively. Cultures were passaged every 10 days using a dilution factor of 1:5 with fresh culture medium. At the time of this study, cells had been passaged in 10% serum supplemented TC-100 culture medium for more than 2 years and displayed stable and reproducible growth. All experiments in this study were carried out using 10% serum-supplemented TC-100 medium.

In order to prepare a single cell suspension for use as inoculum in microcarrier cultures, confluent cell monolayers in 75 cm^2 tissue culture flasks were dissociated using TrypLE™ Express (Life Technologies). Briefly, the spent culture medium was removed from the flask and 2 ml of Dulbecco's phosphate buffered saline (D-PBS) free of calcium and magnesium (Sigma) was used to wash the DSIR-HA-1179 cell monolayer. The spent D-PBS was removed, 1 ml of TrypLE™ Express (Life Technologies) pre-warmed to 27 ºC was added per 25 cm^2 of confluent cell monolayer, and the flask incubated at 27 ºC for 30 minutes. An appropriate volume of pre-warmed TC-100 culture medium supplemented with 10% FBS was added, and the cell suspension was gently aspirated with a 10 ml pipette to break up any remaining cell aggregates, thereby creating a single cell suspension of DSIR-HA-1179 cells (Pushparajan *et al*. 2013). In order to assess viable cell density and culture viability, a sample of the cell suspension was stained with 0.4% Trypan blue (Sigma) and then both the total and viable cells were counted in duplicate using a Neubauer hemocytometer (Philips, 1973).

6.2.2 Preparation of microcarriers

Cytodex-1, Cytodex-3 and Cultispher-G microcarriers were purchased from Sigma (St Louis, MO, USA). Table 6-1 lists the properties of the microcarriers used in this study. For each microcarrier, stock suspensions of 10 g/l were prepared in Dulbecco's phosphate-buffered saline free of Ca^{2+} and Mg^{2+} (D-PBS) (Sigma). Briefly, 1 g of weighed dry microcarriers was added to 100 ml D-PBS in a Schott-Duran glass bottle. The bottle was gently swirled for 5 minutes to evenly disperse the

microcarriers and was incubated at 27 ºC for 5 hours for the microcarriers to hydrate. Upon hydration, the supernatant was decanted and the microcarriers were washed twice in 50 ml of fresh D-PBS. The microcarriers were sterilized by autoclaving at 121 ºC and 15 psi for 20 minutes and stored at 4 ºC until use.

At the time of use and prior to inoculation of the culture, it was necessary to equilibrate microcarriers in culture medium. To do this, microcarriers were allowed to settle and the D-PBS supernatant was decanted. The microcarriers were washed twice with two exchanges of pre-warmed serum-free TC-100 culture medium (50 ml culture medium added per gram of microcarrier). The washing steps were done in order to reduce the dilution of D-PBS trapped between and within the microcarriers. Microcarriers were then re-suspended in 10% serum-supplemented TC-100 culture media and equilibrated at 27 ºC for 10 minutes. Microcarrier cultures were established by inoculating DSIR-HA-1179 cells into an appropriate volume containing the desired microcarrier concentration in tissue culture flasks or in spinner flasks.

Table 6-1 Properties of microcarriers tested in this study (GE Healthcare, 2005; Ikonomou *et al*., 2002)

Property	**Cytodex-1**	**Cytodex-3**	**Cultispher-G**
Manufacturer	Sigma	Sigma	Sigma
Type	Microporous	Microporous	Macroporous
Matrix composition	Cross-linked dextran	Cross-linked dextran	Gelatin
Surface composition	DEAE	DEAE- denatured collagen	Gelatin
Microcarrier charge	+	Nearly neutral	Uncharged
Avg. microcarrier size (µm)	60 - 87	60 - 87	130 - 380
Avg. porosity (µm)	N/A	N/A	10 - 20
Surface area (cm^2/g)	4400	2700	Unknown
Microcarriers per gram	6.8×10^6	4×10^6	Unknown

6.2.3 Culture Vessels

25 cm^2 tissue culture flasks with culture volume of 5 ml were used for the preliminary screening of cell growth on microcarriers. 75 cm^2 tissue culture flasks with culture volume of 20 ml were used for cell-attachment to microcarriers under static conditions in the first 12 h of culture prior to transfer into spinner flasks. Microcarrier culture was performed in 125 ml spinner flasks (Techne, Cambridge, UK) with culture volume of 60 ml and agitation speed of 40 rpm. All cultures were maintained at a temperature of 27 ºC.

6.2.4 Microcarrier Culture

6.2.4.1 Preliminary screening of microcarriers in stationary cultures

The preliminary screening of DSIR-HA-1179 cell attachment and growth on microcarriers was performed in stationary 25 cm^2 tissue culture flasks (Corning®). Cytodex-1 and Cytodex-3 were used at the respective concentrations of 3 g/l and 5 g/l, giving equivalent growth surface areas of 66 cm^2. Cultispher-G was used at a concentration of 1 g/l. For each microcarrier tested, duplicate T-flasks were inoculated at an initial cell density of 2 x 10^5 viable cells/ml (day 0) in a culture volume of 5 ml. At the end of batch growth (day 14), the culture supernatant was harvested in each culture and cell concentration was assessed by nuclei counting according to the procedure described in section 6.2.6.1.

6.2.4.2 Cell growth studies on microcarriers: Influence of cell attachment procedure, cell to bead ratio, microcarrier concentration and growth kinetics

Cell attachment procedure

Cell attachment efficiency to Cytodex-1 microcarriers was evaluated in three different attachment methods during the first 12 h of culture. Each method was evaluated in duplicate cultures set up with a microcarrier concentration of 0.5 g/l and initial cell density of 2 x 10^5 viable cells/ml (with respect to the final culture volume of 60 ml). In the first method, cells and microcarriers were inoculated directly into 60 ml of culture medium in spinner flasks which were stirred continuously at 40 rpm for 12 h. In the second method, cells were inoculated on microcarriers in a reduced volume of culture medium (20 ml) in spinner flasks, and stirred intermittently (3 min at 40 rpm

every 30 min) for 12 h, after which the culture volume was adjusted to 60 ml with fresh culture medium. In the third method, cells were inoculated on microcarriers in a reduced volume of culture medium (20 ml), in 75 cm^2 T-flasks, and allowed to attach for 12 h under static conditions. Cell-laden microcarriers and culture medium were then aseptically transferred into spinner flasks, the culture volume was adjusted to 60 ml with fresh culture medium and the culture stirred continuously at 40 rpm. Cell attachment efficiency was calculated by equation 1 (Ikonomou *et al*., 2002).

$$\text{Cell attachment efficiency (\%)} = \frac{\mathrm{XoVo} - \mathrm{XfVf}}{\mathrm{XoVo}} * 100 \qquad (1)$$

Where X_o and X_f are viable cell concentration in the liquid phase at t = 0 h and t = 12 h, and V_o and V_f are liquid volumes (ml) at t = 0 h and t = 12 h, respectively.

Cell-to-bead ratio

Duplicate 60 ml spinner flask cultures were inoculated at each of the four cell densities (5 x 10^4 cells/ml, 1 x 10^5 cells/ml, 2 x 10^5 cells/ml, 4 x 10^5 cells/ml) with a constant concentration of Cytodex-1 microcarriers (0.5 g/l). According to the manufacturer, 1 g of Cytodex-1 contained 6.8 x 10^6 beads, therefore cell-to-bead ratios (λ) of 15, 30, 60 and 120 were tested. Between day 0 and 14 of culture, 1 ml samples of microcarrier-cell suspension were taken from spinner flasks at 48 h intervals, and total cell density was assessed by nuclei counting as described in section 6.2.6.1.

The multiplication ratio of cells on microcarriers was calculated according to equation 2 (Forestell *et al*., 1992).

$$\text{Multiplication Ratio} = \frac{\mathrm{Ln\ (Xf)} - \mathrm{Ln(Xi)}}{\mathrm{Ln\ (2)}} \qquad (2)$$

Where X_f and X_i are maximum and initial total cell densities in culture, respectively.

Microcarrier concentration

Duplicate 60 ml spinner flask cultures were set up at each of four Cytodex-1 microcarrier concentrations (0.5 g/l, 1 g/l, 2 g/l and 3 g/l) with a constant cell: bead inoculation density of 30 cells per bead. Between day 0 and 14 of culture, 1 ml samples of microcarrier-cell suspension were taken from spinner flasks at 48 h intervals, and total cell density was assessed by nuclei counting as described in section 6.2.6.1.

Cell growth kinetics

The combination of culture parameters optimized with respect to the initial cell attachment procedure, cell-to-bead ratio and microcarrier concentration, were applied to compare DSIR-HA-1179 cell growth kinetics on Cytodex-1 and Cytodex-3 microcarriers over 14 days of batch culture, in duplicate experiments. The following procedure was followed: Cytodex-1 and Cytodex-3 microcarriers were used at the respective concentrations of 1 g/l and 1.63 g/l (with respect to a final spinner flask culture volume of 60 ml), to give equivalent growth surface areas of 264 cm^2. For the initial cell attachment step, microcarriers were transferred to a 75 cm^2 T-flask with a culture volume of 20 ml. An appropriate volume of DSIR-HA-1179 cell suspension containing 1.2 x 10^7 viable cells to give an inoculum cell density of 2 x 10^5 viable cells/ml with respect to the final culture volume (i.e. a cell-to-bead ratio of 30) was added, and allowed to attach to microcarriers for 12 h. At 12 h, the cell-laden microcarriers and culture medium were aseptically transferred into spinner flasks, the culture volume was adjusted to 60 ml with fresh culture medium and continuous agitation at 40 rpm was started. 1 ml culture samples were withdrawn from spinner flasks at 48 h intervals in order to assess cell density and nutrients and metabolite concentrations, until day 14 of culture. Culture supernatants were clarified by centrifugation at 10,000g for 5 min and stored at -20 ºC until they were analyzed for nutrient and metabolites.

The specific growth rate (μ) was calculated by identifying the linear region from the semilog plot of viable cell density versus time, followed by linear regression of the data (Gioria *et al.*, 2006). Population doubling time (t_d) was calculated by equation 3:

$$td = \frac{Ln\,(2)}{\mu} \qquad (3)$$

6.2.5 Virus production studies in microcarrier culture

Duplicate spinner flask cultures (1 g/l Cytodex-1, 60 ml culture volume) were set up with an inoculum cell concentration of 2 x 10^5 viable cells/ml. Cultures were infected with OrNV stock at a multiplicity of infection of 1 $TCID_{50}$/cell when they reached a cell density of 5 x 10^5 cells/ml, and agitated continuously at 40 rpm. 1 ml culture samples were withdrawn from spinner flasks at 48 h intervals in order to assess cell density, nutrients and metabolite concentrations and extracellular virus titer, until day 14 post-infection. Culture supernatant samples, clarified by centrifugation at 10,000g for 5 min, were stored at -20 ºC and 4 ºC, until the quantification of nutrients and metabolites, and virus titer, respectively.

In order to evaluate the effects of time of infection (TOI) on virus production, duplicate spinner flask cultures, set up as described above, were infected with OrNV stock at a multiplicity of infection of 1 $TCID_{50}$/cell when they reached the respective cell densities of 5 x 10^5 cells/ml and 7 x 10^5 cells/ml, and agitated continuously at 40 rpm. 1 ml samples were withdrawn at 48 h intervals and assessed for cell density and extracellular virus titer, until day 14 post-infection.

In order to evaluate the effects of partial culture medium replacement on virus production, duplicate spinner flask cultures, set up as described above, were grown until they reached the respective infection cell densities of 5 x 10^5 cells/ml and 7 x 10^5 cells/ml. Immediately prior to infection, 75% of the spent culture medium was removed and replaced with an equal volume of fresh culture medium. The cultures were infected with OrNV stock at a multiplicity of infection of 1 $TCID_{50}$/cell and agitated continuously at 40 rpm. 1 ml samples were withdrawn at 48 h intervals and assessed for cell density and extracellular virus titer, until day 14 post-infection.

6.2.6 Analytical Procedures

6.2.6.1 Nuclei counting

To measure total cell concentration, nuclei staining with crystal violet was carried out according to Sandford *et al*., 1951. Briefly, a citric acid lysis buffer was made by dissolving 0.9 g of citric acid powder (Sigma) in 100 ml of distilled water to which 0.01 g crystal violet nuclear stain (Sigma)

was added and homogenously dispersed. Microcarrier culture samples, held in 1.5 ml Eppendorf microcentrifuge tubes, were centrifuged at 12,000g for 5 min to separate cell-bound microcarriers from the culture supernatant. The culture supernatant was removed and an equal volume of citric acid lysis buffer containing crystal violet was added to the cell-microcarrier pellet. The mixture was vortexed in a whirl mixer for 1 min, and then incubated overnight at 27 ºC. Citric acid did not affect the microcarriers, and only lysed the cells' plasma and nuclear membranes to release their nuclei. The individually stained nuclei were counted in duplicate using a Neubauer hemocytometer in order to assess total cell density.

6.2.6.2 Virus and virus quantification

OrNV stock (strain X2B) at a concentration of 1 x 10^7 $TCID_{50}$/ml was obtained from AgResearch. X2B is a highly virulent strain originally isolated in 1983 from a field population of infected coconut rhinoceros beetles on Bugsuk Island, Palawan, Philippines (Crawford *et al*., 1986; Zelazny *et al*., 1990).

The virus stock used in this study was prepared by infection of attached cultures during the early exponential phase of growth (approximately 5 x 10^5 viable cells/ml) at a low multiplicity of infection (MOI) of 0.1 $TCID_{50}$/cell. OrNV infectious titer was quantified by end-point dilution analysis. Briefly, to determine the $TCID_{50}$, suspensions of DSIR-HA-1179 cells (2.5 x 10^5 cells/ml) were seeded onto 96 well plates (50 µl per well), and then an equal volume of each viral supernatant dilution (diluted in ten-fold series from 10^{-2} to 10^{-9}) was added with five replicates per supernatant. The plates were placed in a humidified, disinfected plastic container and incubated at 27 ºC for 11 to 14 days until the cytopathic effect was well developed, when the plates were scored for infection, and $TCID_{50}$ was calculated (Reed and Muench, 1938).

6.2.6.3 Nutrients and metabolite analyses

Glucose concentrations were determined using an enzymatic test kit (Sigma) based on the hexokinase-catalyzed oxidation of glucose (Slein, 1963). Lactate concentrations were determined using a colorimetric test kit (Sigma) based on the oxidation of lactate by lactate dehydrogenase (Schweiger and Gunther, 1964). Ammonia concentration was determined using an enzymatic test

kit (Sigma) based on the glutamate dehydrogenase-catalyzed oxidation of nicotinamide-adenine dinucleotide in the presence of ammonia (Mondzac *et al.,* 1965). Glutamine concentrations were determined using a YSI 2700 biochemistry analyzer (YSI, Yellow Springs, OH, USA). The total concentrations of 17 other amino acids were determined by reverse-phase high-performance liquid chromatography (HPLC) (AccQ.Tag, Waters Corporation, USA) using precolumn derivatization with 6-aminoquinolyl N-hydroxysuccinimidyl carbamate (Cohen, 2000). Prior to derivatization, sample proteins were hydrolyzed into their respective amino acids by treatment with 6N hydrochloric acid for 24 h at 110º C (AOAC official method 4.1.11: 994.12c, 1998). Cysteine and methionine were analyzed as cysteic acid and methionine sulfone after cold performic acid oxidation overnight prior to hydrolysis (AOAC official method 4.1.11: 994.12a, 1998).

6.2.6.4 Imaging of cells and microcarriers

Immobilized cells on microcarriers were examined under an inverted microscope (Leica DM IL) attached to a digital camera (Leica Digital DFC499). In order to differentiate between viable and non-viable cells during cell growth and infection, microcarrier-cell suspensions were stained with 0.2% Trypan blue (Ikonomou *et al.*, 2002). This resulted in the preferential staining of microcarriers and non-viable cells, allowing the easy differentiation of viable cells which remained unstained and appeared as bright, clear spots.

6.3 Results

6.3.1 Preliminary screening of DSIR-HA-1179 cell growth on microcarriers

A preliminary experiment was conducted in stationary T-flasks to identify whether DSIR-HA-1179 cells would attach and proliferate on the three types of microcarriers tested. It took up to 12 hours for cells to attach on Cytodex-1 and Cytodex-3. On the other hand, cells failed to attach to Cultispher-G microcarriers. From images taken of the respective microcarriers on day 2 of culture (Fig. 6-1 A, B and C), cell proliferation was observed on Cytodex-1 and Cytodex-3, but not on Cultispher-G microcarriers. By day 4 of culture, cells inoculated with Cultispher-G microcarriers had preferentially attached and spread on the surface of the (tissue-culture treated) flask, instead of on microcarriers (Fig. 6-1 D).

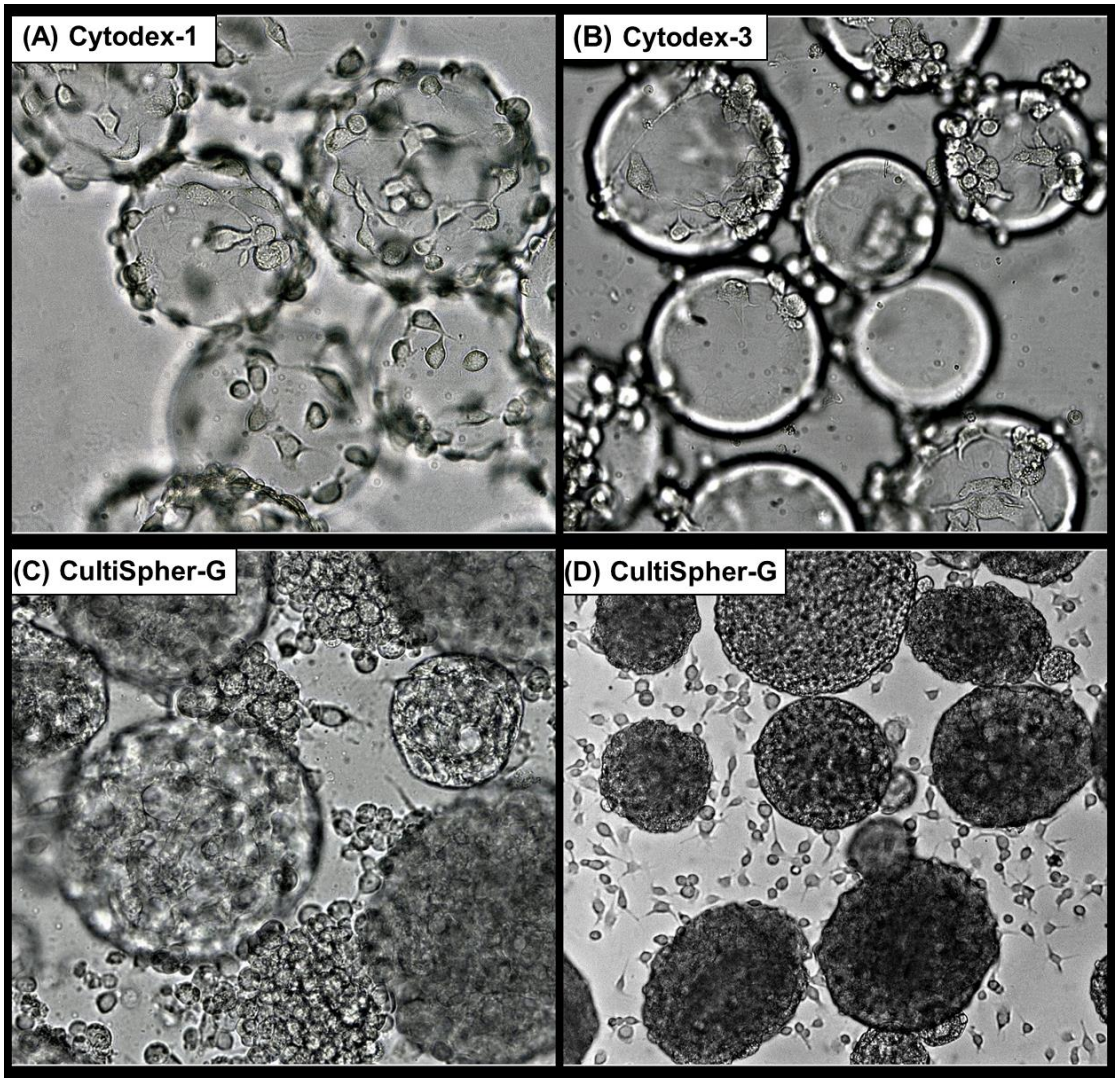

Fig. 6-1 Micrographs of DSIR-HA-1179 cells attachment and spreading on various microcarriers in stationary cultures (A) Cytodex-1 (200x magnification), (B) Cytodex-3 (200x magnification), (C) CultiSpher-G (200x magnification) and (D) CultiSpher-G (100x magnification) – where cells preferentially attached and grew on the T-flask surface, and no cell growth on microcarriers occurred.

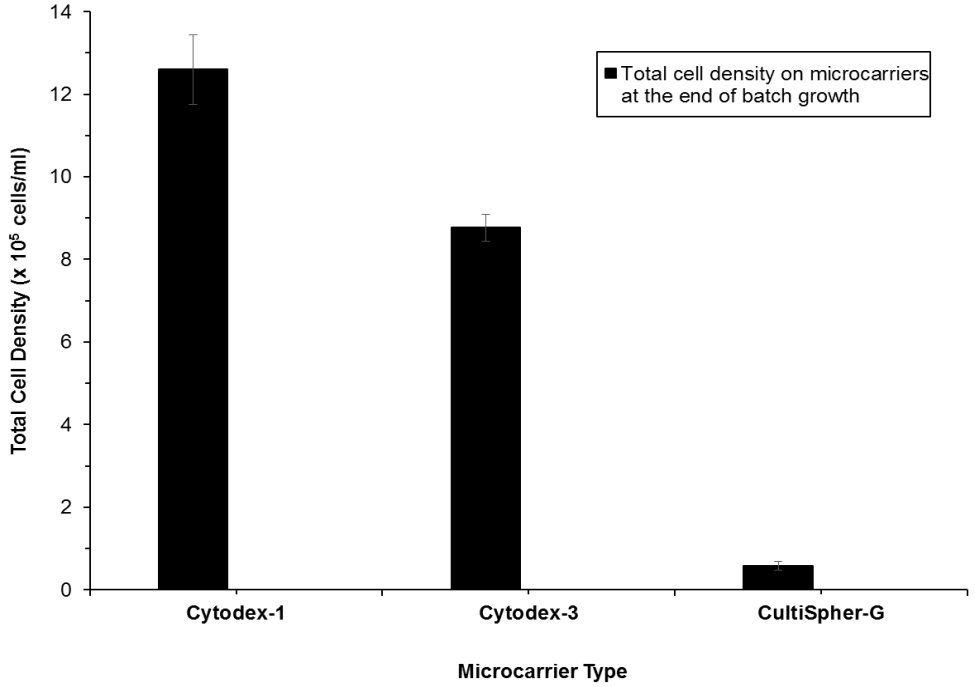

Fig. 6-2 Mean total cell density at the end of batch growth of DSIR-HA-1179 cells on Cytodex-1, Cytodex-3 and CultiSpher-G microcarriers in TC-100 supplemented with 10% FBS in stationary T-flask cultures. Error bars represent standard deviations from the mean of duplicate samples.

From Fig. 6-2, it can be observed that at the end of the 14 day batch growth period in stationary flasks, the highest mean cell density was reached on Cytodex-1 microcarriers (12.6 x 10^5 cells/ml), followed by Cytodex-3 (8.77 x 10^5 cells/ml) and finally Cultispher-G microcarriers (0.5 x 10^5 cells/ml). Because it supported the highest cell yield, Cytodex-1 microcarriers were chosen to further develop the process.

6.3.2 Influence of culture parameters on cell attachment and growth on microcarriers

The initial phase of microcarrier culture is regarded as the most critical stage in the culture. The key to achieving maximum cell yields in microcarrier culture is to ensure that all microcarriers are inoculated with cells right from the beginning of the culture (Hirtenstein and Clark, 1980). In addition to ensuring that all cells have attached to microcarriers, the even distribution of a minimum number of cells on individual microcarriers is highly desirable in order to avoid uncolonized beads or bare spots developing on microcarriers later in culture. Furthermore, for cells with an intrinsically slow attachment rate, initial culture conditions must allow cells to attach as fast as

possible. Several culture parameters affect the efficiency of the initial phase of microcarrier culture; including how well microcarriers have equilibrated to the culture medium prior to cell inoculation, the quality of the cell inoculum, the cell attachment/ initial stirring regime, microcarrier concentration and inoculum cell concentration. The choice of culture parameters depends on the cell line's attachment and growth characteristics, and must be optimized for each cell line and microcarrier. In this study, equilibration of microcarriers was carried out according to the manufacturer's instructions, by allowing microcarriers to soak in pre-warmed culture medium for 10 minutes at 27 ºC, prior to addition of cells. Cell inoculum consisted of healthy cells taken from cultures in their exponential growth phase. In this section, results are presented on the optimization of the latter three culture parameters – cell attachment procedure, inoculum cell density and microcarrier concentration, in DSIR-HA-1179 cells cultivated on Cytodex-1 microcarriers in spinner flask bioreactors.

6.3.2.1 Cell attachment procedure

The purpose of agitation in the initial phase of microcarrier culture is to increase the rate of collision between cells and microcarriers kept in suspension so that productive attachment of cells to microcarriers can occur. However, detrimental effects of using either too high or low a stirring speed during the initial attachment phase can be that either cells are killed due to excessive shear or that they are distributed unevenly on microcarriers. The attachment efficiency of DSIR-HA-1179 cells to Cytodex-1 microcarriers in the first 12 h of culture was investigated in three different procedures (Table 6-2).

Cell attachment efficiency to microcarriers was lowest under the continuous stirring regime, with 28.1% of the cells remaining unattached at the end of the 12 h period. Despite the high cell attachment efficiency (> 90%) achieved under intermittent stirring in a reduced culture volume, microscopic observation showed that cells were unevenly distributed on microcarriers. Some microcarriers were heavily colonized with cell clumps, while others remained completely bare. The highest attachment efficiency (99.4%) occurred when cells were inoculated on microcarriers in stationary T-flasks and allowed to attach under static conditions for the first 12 h of culture. This procedure also resulted in a more even initial cell distribution on microcarriers (Fig. 6-6 A), and was therefore, followed for the rest of the experiments in this work. However, despite the initial

even distribution of cells on microcarriers, it was found that microcarrier-cell aggregates formed by day 6 of batch growth in spinner flasks (Fig. 6-6 C, D).

Table 6-2 Attachment efficiency of DSIR-HA-1179 cells to Cytodex-1 microcarriers in various cell attachment procedures during the initial 12 hours of culture.

Cell attachment procedure	Cell attachment efficiency ± SD (%)
Continuous stirring at 40 rpm in final culture volume (60 ml) for 12 h	71.9 ± 0.8
Intermittent stirring (3 min stirring every 30 min) at 40 rpm in 1/3rd culture volume (20 ml) for 12 h	90.7 ± 2.1
No stirring in 1/3rd culture volume (20 ml) in stationary 75 cm^2 T-flask for 12 h	99.4 ± 0.9

6.3.2.2 Cell–to-bead ratio (λ)

The cell-to-bead ratio is a critical parameter which affects cell yield in microcarrier culture (Hu *et al*., 1985). For attachment-dependent cell lines, a minimum inoculum cell density (cells/cm^2) is necessary to initiate cell growth on a growth surface. In the case of microcarriers, the growth surface comprises of multiple beads forming discrete units of growth surface area, which individually must be provided with a critical number of cells per carrier for optimal cell growth to occur. The cell-to-bead ratio has been found to affect both the proportion of microcarriers bearing cells at the end of culture, as well as cell yield (Mered *et al*., 1981; Ng *et al.*, 1996). The effects of varying the cell-to-bead ratio of DSIR-HA-1179 cells on Cytodex-1 microcarriers were evaluated for the values of λ = 15, 30, 60 and 120 cells per bead.

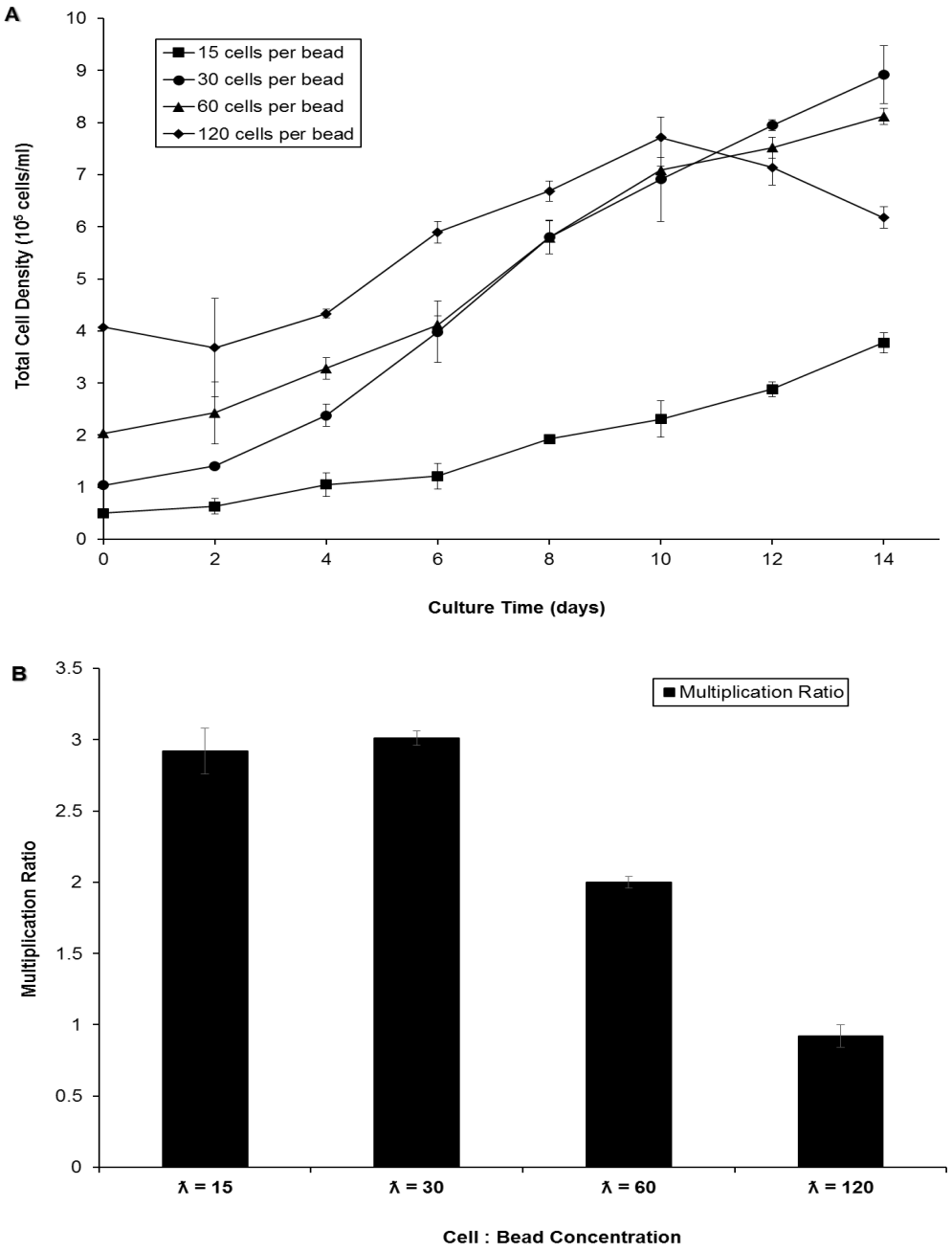

Fig. 6-3 (A) Mean cell growth profiles and (B) Mean multiplication ratios of DSIR-HA-1179 cells grown on Cytodex-1 microcarriers in TC-100 supplemented with 10% FBS in 60 ml spinner flask bioreactors at different cell to bead concentrations (λ) of 15, 30, 60 and 120. Error bars represent standard deviations from the mean of duplicate samples.

The selection of an optimal cell-to-bead ratio was based on a comparison of mean maximum cell density and the cell multiplication ratio obtained on a fixed microcarrier surface area of 132 cm^2 in spinner flask cultures. As shown in Fig. 6-3 (A), a ratio of 30 cells per bead resulted in the highest cell density (8.92 x 10^5 cells/ml), followed by 60 cells per bead (8.12 x 10^5 cells/ml), 120 cells per bead (7.72 x 10^5 cells/ml) and finally, 15 cells per bead (3.38 x 10^5 cells/ml). Furthermore, since the highest multiplication ratio (3.01) for DSIR-HA-1179 cells on Cytodex-1 microcarriers was obtained when cells were inoculated at $\lambda = 30$ (Fig. 6-3 (B)), a cell-to-bead ratio of 30 was selected.

6.3.2.3 Microcarrier concentration

One of the factors influencing cell yield in microcarrier culture is the surface area available for cell growth (i.e. the concentration of microcarriers) (Nilsson, 1989). Studies have shown that both increasing and decreasing microcarrier concentration indiscriminately, whilst maintaining a constant cell-to-bead ratio can have adverse effects on cell growth (Clark and Hirtenstein, 1981; Forestell *et al*., 1992; Levine *et al*., 1979). A microcarrier concentration of 0.5-5 mg/ml has been typically recommended for Cytodex-1 microcarriers. However, the maximum cell yield obtained at a particular microcarrier concentration depends on the characteristic saturation density of the cell type and on the nutritional capacity of the volume of culture medium used (GE Healthcare, 2013). A range of Cytodex-1 microcarrier concentrations (0.5 g/l, 1 g/l, 2 g/l and 3 g/l) were screened for DSIR-HA-1179 cell growth at a constant cell-to-bead ratio of 30 in a culture volume of 60 ml in spinner flasks.

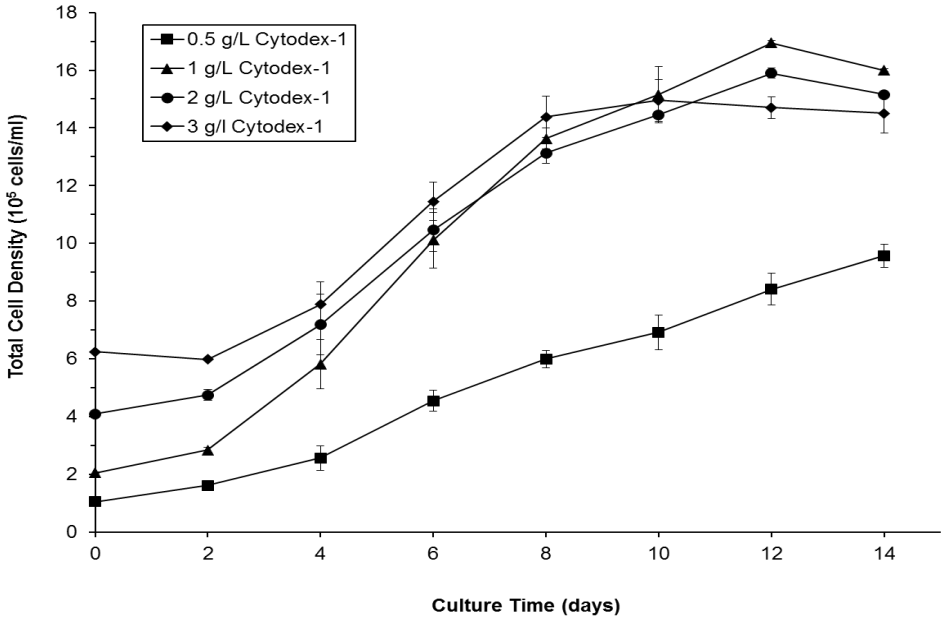

Fig. 6-4 Mean cell growth profiles of DSIR-HA-1179 cells grown on Cytodex-1 microcarriers in TC-100 supplemented with 10% FBS in 60 ml spinner flask bioreactors at different microcarrier bead concentrations of 0.5 g/l, 1 g/l, 2 g/l and 3 g/l. Error bars represent standard deviations from the mean of duplicate samples.

From the evolution of cell growth profiles at the different microcarrier concentrations in Fig. 6-4, it can be observed that using the lowest microcarrier concentration of 0.5 g/l resulted in poor cell growth with the mean maximum cell density in this condition (9.57 x 10^5 cells/ml) reached on day 14 of culture. Doubling the microcarrier concentration to 1 g/l led to an approximate doubling in the mean maximum cell density (16.95 x 10^5 cells/ml), reached on day 12 of culture. However, further doubling the microcarrier concentration to 2 g/l, despite the increase in growth surface area, led to a decrease in the mean maximum cell density reached on day 12 of culture (15.9 x 10^5 cells/ml), compared to 1 g/l. A still higher microcarrier concentration of 3 g/l resulted in further impairment of the maximum volumetric cell yield to 14.95 x 10^5 cells/ml. It was therefore concluded that a 60 ml volume of 10% serum-supplemented TC-100, could support maximum cell yields at a microcarrier concentration of 1 g/l Cytodex-1 beads and inoculum of 30 cells per bead, in spinner flask culture.

6.3.3 DSIR-HA-1179 cell growth kinetics on microcarriers

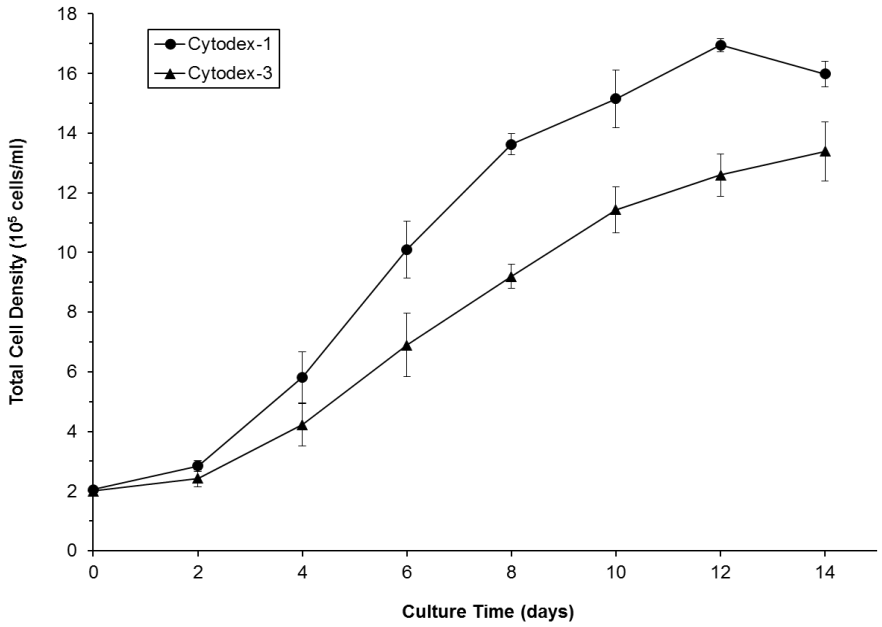

Fig. 6-5 Mean cell growth profiles of DSIR-HA-1179 cells on Cytodex-1 and Cytodex-3 microcarriers in TC-100 supplemented with 10% FBS in 60 ml spinner flask bioreactors. Error bars represent standard deviations from the mean of duplicate samples.

The combination of culture parameters selected in preceding sub-sections were applied to compare DSIR-HA-1179 cell growth kinetics on Cytodex-1 and Cytodex-3 microcarriers in spinner flask cultures. The cell growth profiles of DSIR-HA-1179 cells on the respective microcarriers over a 14 day batch growth period are shown in Fig. 6-5. In both Cytodex-1 and Cytodex-3 microcarrier cultures, cells went through an initial lag phase which lasted for approximately 48 hours, after which the exponential growth commenced and continued until the maximum cell density was reached. Cells growing on Cytodex-1 reached the highest maximum cell density on day 12 of batch growth (16.95 x 10^5 cells/ml) and the mean highest specific growth rate (0.0074 h^{-1}, PDT: 3.9 days). A 5.7% reduction in cell density occurred in the last 48 hours of culture. On the other hand, while cell density increased until day 14 in Cytodex-3 microcarrier cultures, the maximum cell

density reached (12.6 x 10^5 cells/ml) and mean specific growth rate (0.0069 h^{-1}, PDT: 4.2 days) were lower compared to Cytodex-1 microcarrier cultures.

Since nuclei counting could only be used to estimate total cell density, cell viability was not evaluated in Cytodex-1 microcarrier cultures. However, microcarrier cell suspensions were sampled at 3 day intervals over the course of batch growth and stained with Trypan blue in order to better visualize cell growth and to differentiate between live and dead cells on microcarriers. This allowed a rough assessment of culture viability to be made (Fig. 6-6). Two observations could be drawn from Fig. 6-6 (A-D), the first being the presence of only a very few dark stained cells on microcarriers (i.e. non-viable cells), indicating high culture viability during batch growth. The second observation made was, that despite the use of a 12 hour initial cell attachment procedure in static T-flasks which ensured high seeding efficiency and even cell distribution on microcarriers at the start of the culture (sub-section 6.3.2.1) (Fig 6-6 A: 12 h post-seeding), progressive aggregation of cells and microcarriers occurred, ultimately leading to inhomogeneous cell distribution on microcarriers. It appeared that clumping of microcarriers early in the culture was mediated by the formation of cell-bridges between individual beads and attachment of small free floating cell clumps onto attached microcarriers (Fig. 6-6 B). As the culture progressed, macro-aggregates of cells and microcarriers increased in size and density (Fig. 6-6 C and D). It was interesting to note a lack of necrotic cores in the macro-aggregates on day 12 of the culture, despite being very densely populated with cells (Fig. 6-6 D). In an attempt to reduce aggregation of cells and microcarriers, the experiment was repeated with a higher stirring speed of 60 rpm, but this failed to produce any significant reduction in aggregate size, confirmed by microscopic observation of microcarriers.

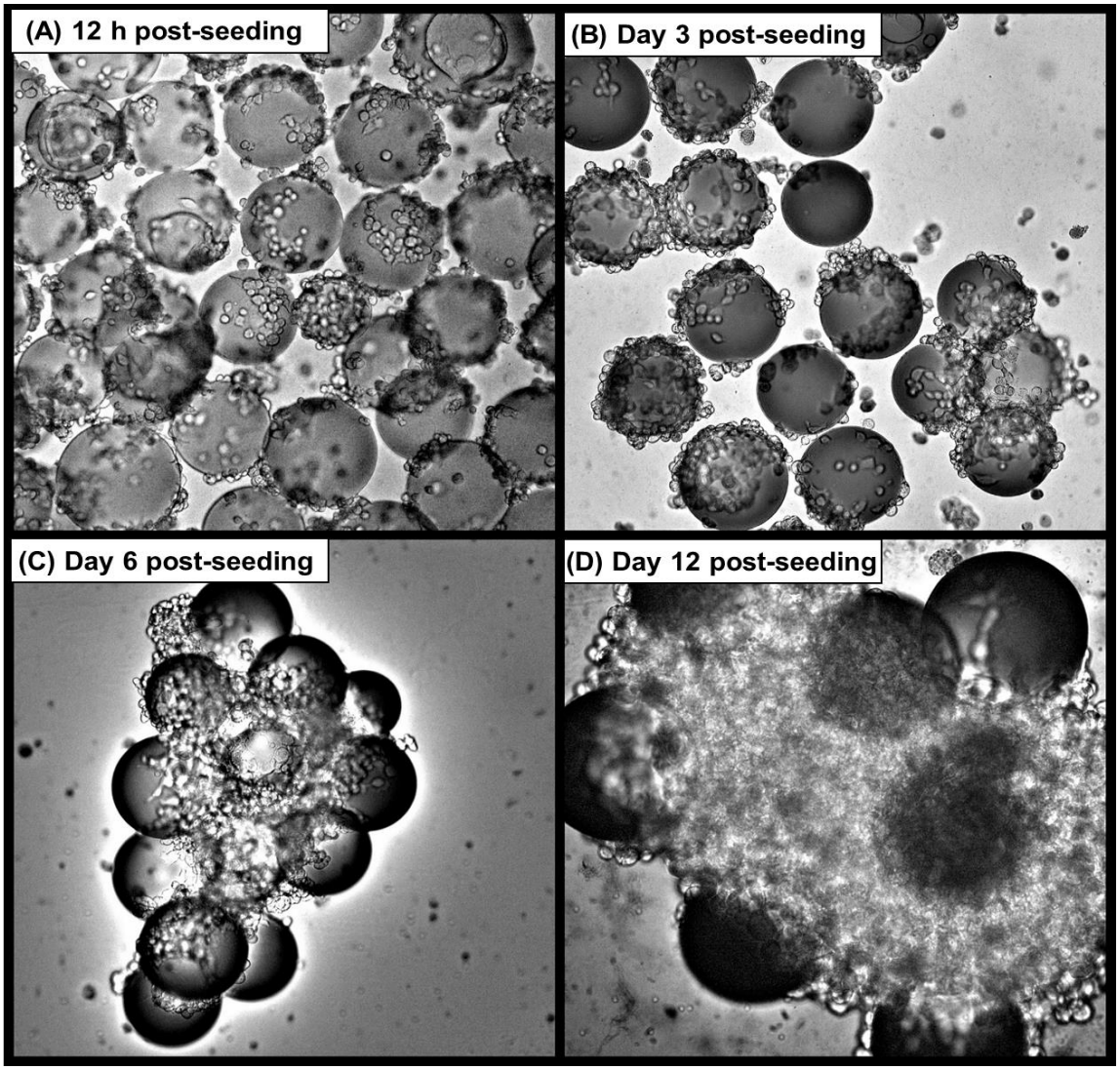

Fig. 6-6 Micrographs of DSIR-HA-1179 cell growth on Cytodex-1 microcarriers during batch culture under optimized culture conditions. (A) Cell attachment and spreading at 12 h post-inoculation (i.e. time of starting spinner flask culture) (B) Day 3 of culture (early-exponential growth phase) (C) Day 6 of culture (mid-exponential growth phase) (D) Day 12 of culture (late exponential growth phase). Samples were stained with trypan blue and photographed at 100x magnification. Non-viable cells and microcarriers are preferentially stained with trypan blue and appear dark in the images.

6.3.4 Nutrient consumption and metabolism in uninfected microcarrier cultures

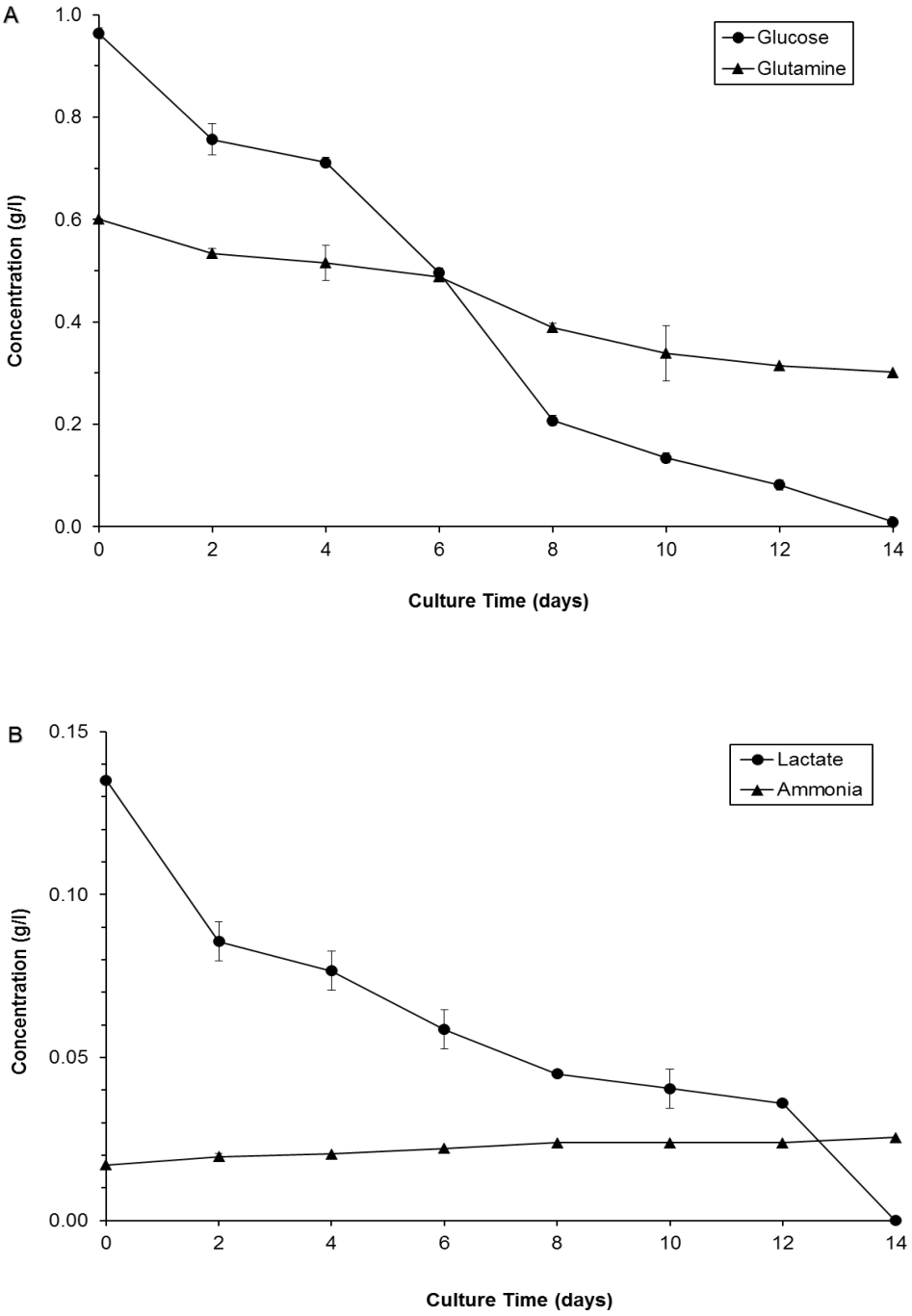

Fig. 6-7 (A) Glucose, glutamine and (B) lactate and ammonia profiles in uninfected cultures of the DSIR-HA-1179 cell line grown in TC-100 supplemented with 10% FBS on Cytodex-1 microcarriers in 60 ml spinner flasks. Error bars represent the standard deviations from the mean of duplicate samples.

Fig. 6-7 (A) shows the time course of change in glucose and glutamine concentrations in microcarrier cultures. Glucose was consumed rapidly by 20% of its initial concentration in the first 48 h of culture, despite the culture being in its lag phase. Between days 4 and 12 of culture, correlating to the exponential growth phase, glucose was consumed at a mean specific rate of 3.9 x 10^{-12} g/c/h. Glucose was exhausted from the culture medium only by day 14, which indicated that cells did not face glucose limitation during their active growth phase. In comparison with glucose, glutamine concentration decreased gradually and was consumed to a lesser extent (50% of its initial concentration) and at a lower specific rate (1.2 x 10^{-12} g/c/h) than glucose. From Fig. 6-7 (B) it can be observed that the metabolites lactate and ammonia did not accumulate in microcarrier cultures. Lactate which was present at a very small concentration (0.14 g/l) at the start of the culture, was gradually consumed over the course of culture. Ammonia was detected at very low levels (0.02 g/l) and remained unchanged in its concentration throughout the batch growth period.

Table 6-3 Amino acid concentrations at the beginning and end of batch growth in uninfected cultures of the DSIR-HA-1179 cell line grown on Cytodex-1 microcarriers in TC-100 supplemented with 10% FBS in 60 ml spinner flasks. Concentrations of all amino acids are expressed in mg/g except for glutamine which is expressed in g/l.

Amino Acid	Amino Acid Conc. 0 h	Amino Acid Conc. 336 h	% Consumption (negative sign indicates production)
Alanine	0.51	0.80	- 56.9
Arginine	0.77	0.71	7.8
Aspartic Acid	1.20	0.99	17.5
Cysteine	0.20	0.17	15
Glycine	0.71	0.66	7
Glutamic Acid	1.94	1.50	22.7
Histidine	2.29	2.15	6.1
Isoleucine	0.27	0.23	14.8
Leucine	0.64	0.57	10.9
Lysine	0.95	0.85	10.5
Methionine	0.09	0.07	22.2
Phenylalanine	0.44	0.40	9.1
Proline	0.66	0.50	24.2
Serine	0.64	0.56	12.5
Tyrosine	0.27	0.24	11.1
Threonine	0.43	0.39	9.3
Valine	0.52	0.47	9.6
Glutamine	0.60	0.30	50

All amino acids with the exception of alanine, were consumed during DSIR-HA-1179 cell growth in microcarrier culture, however none were depleted to the extent that they were limiting for culture growth. Glutamine which was a secondary energy source for cells, was consumed to 50% of its initial concentration. Only proline, glutamic acid and methionine were consumed in percentages exceeding 20% of their initial concentrations, while aspartic acid, tyrosine, serine, lysine, leucine, isoleucine and glycine were consumed to between 10 – 20% of their initial concentrations. Other amino acids were consumed in very minimal percentages < 10%. Alanine accumulated in culture medium to 56.9% of its original concentration by the end of batch growth.

6.3.5 OrNV infection in microcarrier cultures

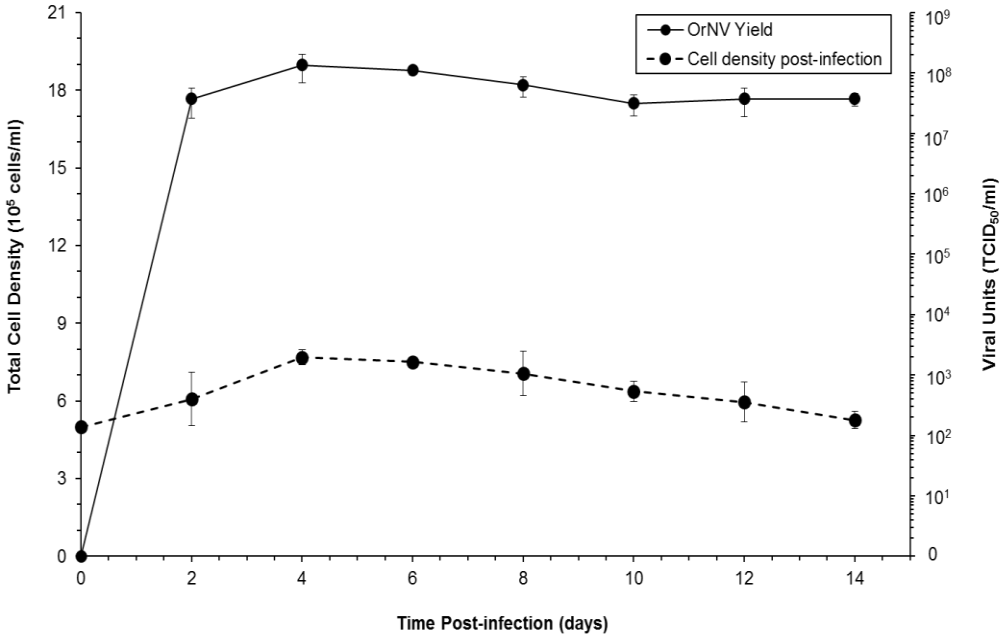

Fig. 6-8 Time course profile of total cell density (dashed line) and OrNV production (solid line) in infected Cytodex-1 microcarrier cultures of the DSIR-HA-1179 cell line, grown in TC-100 supplemented with 10% FBS, in spinner flasks. Cells were infected at a density of $5x10^5$ cells/ml and multiplicity of infection of 1 $TCID_{50}$/cell. Error bars represent standard deviations from the mean of duplicate experiments.

DSIR-HA-1179 cells growing on Cytodex-1 microcarriers in 60 ml spinner flask cultures were infected with OrNV, at an approximate cell density of 5 x 10^5 cells/ml (day 4 of culture), and

multiplicity of infection of 1 $TCID_{50}$/cell. The kinetics of infection were evaluated based on the evolution of viral titers and cell density over a 14 day period of infection (Fig. 6-8). The first 96 hours of infection were characterized by an increase in infected cell density to 7.68 x 10^5 cells/ml. Subsequently, infected cell density declined steadily to a final concentration of 5.26 x 10^5 cells/ml on the 14^{th} day post-infection. The rate of OrNV production was highest during the first 48 hours post-infection, followed by a slower increase between 48 and 96 hours post-infection, to reach the maximum volumetric yield of 1.36 x 10^8 $TCID_{50}$/ml at 96 hours post-infection. The maximum cell specific OrNV yield and volumetric productivity of the culture were calculated as 177 $TCID_{50}$/cell and 1.70 x 10^7 $TCID_{50}$/ml/day, respectively. Subsequent to the attainment of the maximum OrNV yield, it was found that prolonging the infection period until day 14 post-infection led to a decay in the OrNV volumetric yield to 3.72 x 10^7 $TCID_{50}$/ml on day 14 post-infection.

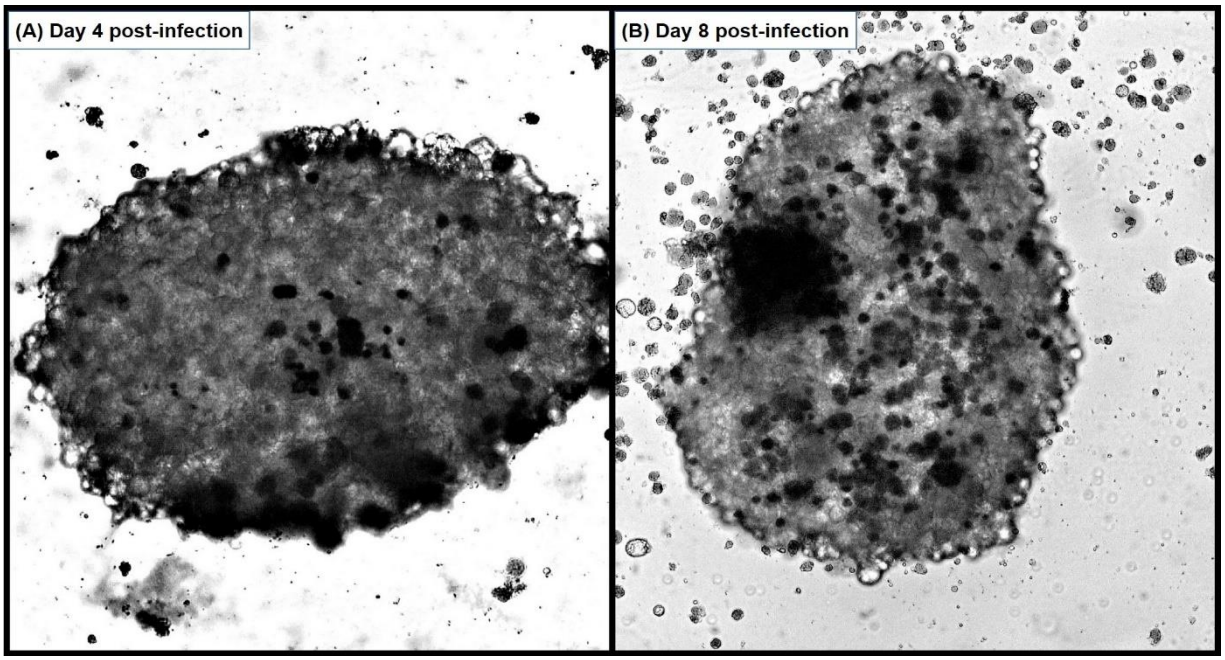

Fig. 6-9 Micrographs of OrNV-infected macro-aggregates of DSIR-HA-1179 cells and Cytodex-1 microcarriers on (A) Day 4 post-infection (B) Day 8 post-infection. Macroaggregates were stained with trypan blue on the respective time-points and photographed at 100x magnification. Dark spots indicate non-viable cells stained with trypan blue.

Fig. 6-9 shows the cytopathic effects of OrNV infection in trypan-blue stained microcarrier-cell aggregates, on the days 4 and 8 post-infection. Typical cytopathic effects of OrNV infection such as the rounding up and hypertrophy of infected cells, were observed particularly on cells on the

outer surfaces of microcarrier-cell aggregates. Progressive loss of cell viability can also be observed, as the infection progressed.

6.3.6 Nutrient consumption and metabolism in infected microcarrier cultures

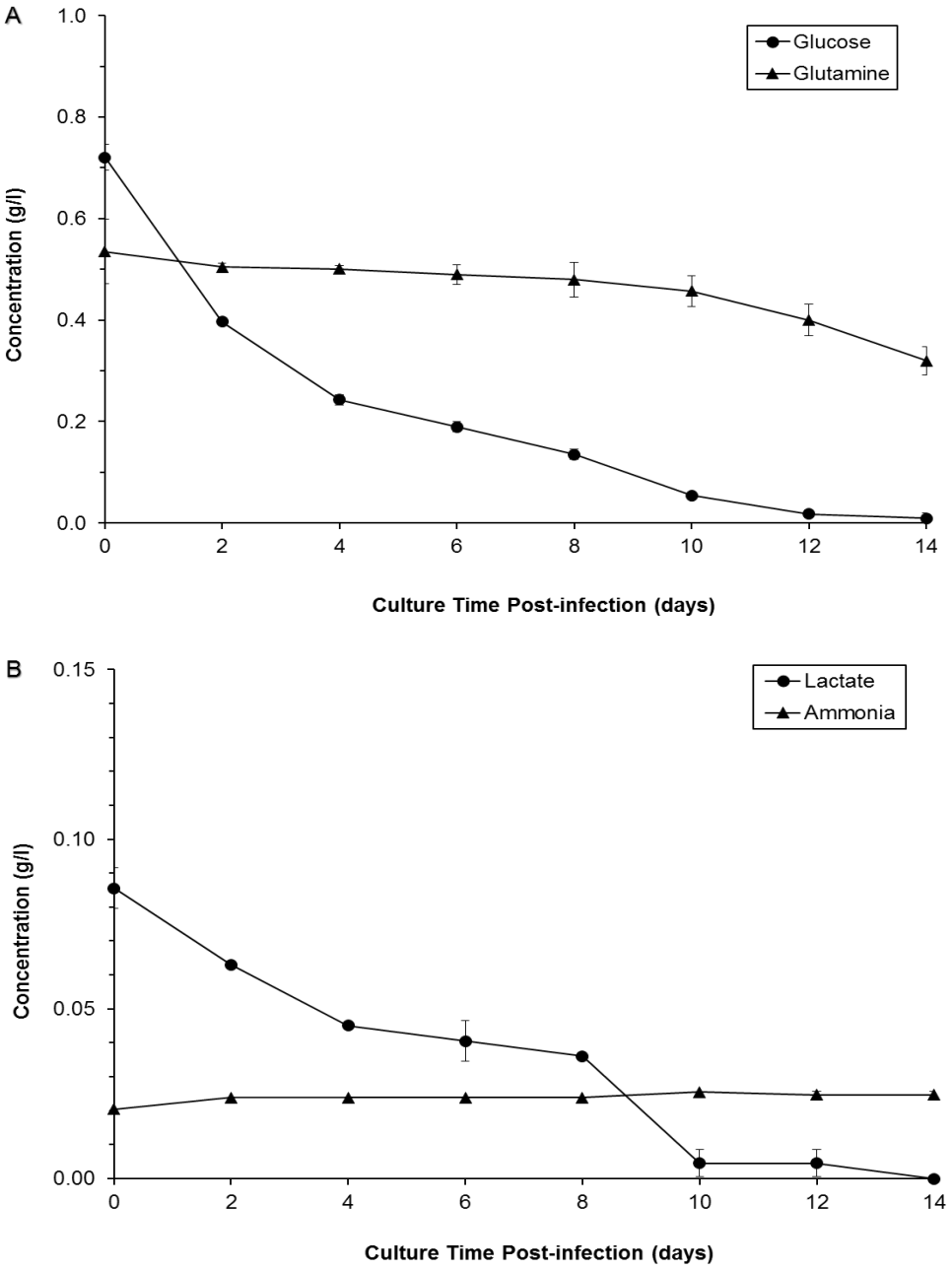

Fig. 6-10 (A) Glucose, glutamine and (B) lactate and ammonia profiles in infected cultures of the DSIR-HA-1179 cell line grown in TC-100 supplemented with 10% FBS on Cytodex-1 microcarriers in 60 ml spinner flasks. Error bars represent the standard deviations from the mean of duplicate samples.

The consumption of glucose, glutamine (Fig 6-10 A), lactate and ammonia (Fig 6-10 B) were evaluated in OrNV-infected microcarrier cultures over the 14 day period of infection. Similar to uninfected cultures, glucose and glutamine were the nutrients most consumed during infection. Glucose reached limiting concentrations by day 12 post-infection and was completely exhausted from the culture medium at day 14 post-infection. Glutamine was consumed to a lesser extent (40.7%) with respect to its concentration at the time of infection. The specific rates of consumption of these nutrients were evaluated between the time of infection and 96 hours post-infection, when the maximum OrNV volumetric yield was reached. In the first 48 hours of infection, the specific rate of glucose consumption increased from 1.12×10^{-12} g/c/h to 12.2×10^{-12} g/c/h; and then decreased to 4.76×10^{-12} g/c/h between 48 and 96 hours post-infection. A similar incremental trend was observed in the specific rate of glutamine consumption from 0.45×10^{-12} g/c/h to 1.13×10^{-12} g/c/h in the first 48 hours following infection, after which it decreased to 1.28×10^{-13} g/c/h between 48 and 96 hours post-infection. Lactate and ammonia were not produced in infected microcarrier cultures. The low concentration of lactate (0.09 g/l) present in the culture medium at the time of infection, was rapidly consumed and exhausted by the end of the infection phase. Ammonia, which was detected at very low levels of approximately 0.02 g/l, was neither consumed nor produced in infected cultures.

The concentrations of eighteen amino acids in culture supernatants were assessed at the beginning and at the end of infection, in order to evaluate amino acid requirements in infected microcarrier cultures (Table 6-4). The only amino acid consumed to any significant extent was glutamine (40.7%). Proline, methionine and glutamic acid were consumed between 10 – 20% of their initial concentrations at the time of infection. In addition, serine and aspartic acid were consumed minimally (< 5%); while valine, threonine, tyrosine, leucine, isoleucine and arginine were neither consumed not produced. The accumulation of alanine (38.6%) over the infection phase, was in contrast to amino acids such as phenylalanine, lysine, cysteine, glycine and histidine which accumulated to less than 6%.

Table 6-4 Amino acid concentrations at the beginning and end of batch growth in infected cultures of the DSIR-HA-1179 cell line grown on Cytodex-1 microcarriers in TC-100 supplemented with 10% FBS in 60 ml spinner flasks. Concentrations of all amino acids are expressed in mg/g except for glutamine which is expressed in g/l.

Amino Acid	Amino Acid Conc. 0 h	Amino Acid Conc. 336 h	% Consumption (negative sign indicates production)
Alanine	0.57	0.79	- 38.6
Arginine	0.70	0.70	0
Aspartic Acid	1.06	1.01	4.7
Cysteine	0.17	0.18	- 5.9
Glycine	0.65	0.66	- 1.5
Glutamic Acid	1.67	1.50	10.2
Histidine	2.07	2.12	- 2.4
Isoleucine	0.24	0.24	0
Leucine	0.58	0.58	0
Lysine	0.85	0.87	- 2.4
Methionine	0.08	0.07	12.5
Phenylalanine	0.40	0.41	- 2.5
Proline	0.58	0.52	10.3
Serine	0.58	0.56	3.4
Tyrosine	0.24	0.24	0
Threonine	0.40	0.40	0
Valine	0.47	0.47	0
Glutamine	0.54	0.32	40.7

6.3.7 Yield improvement in infected microcarrier cultures

6.3.7.1 Influence of TOI on OrNV production

Having ascertained the nutritional sufficiency of the culture medium for infections performed at a cell density of 5 x 10^5 cells/ml, it was further investigated whether delaying the infection to a higher cell density of 7 x 10^5 cells/ml when cells were still in the exponential growth phase, would lead to improved OrNV production. Fig. 6-11 shows the temporal progression of infected cell density and OrNV volumetric yield in infections carried out at the two respective infection cell densities.

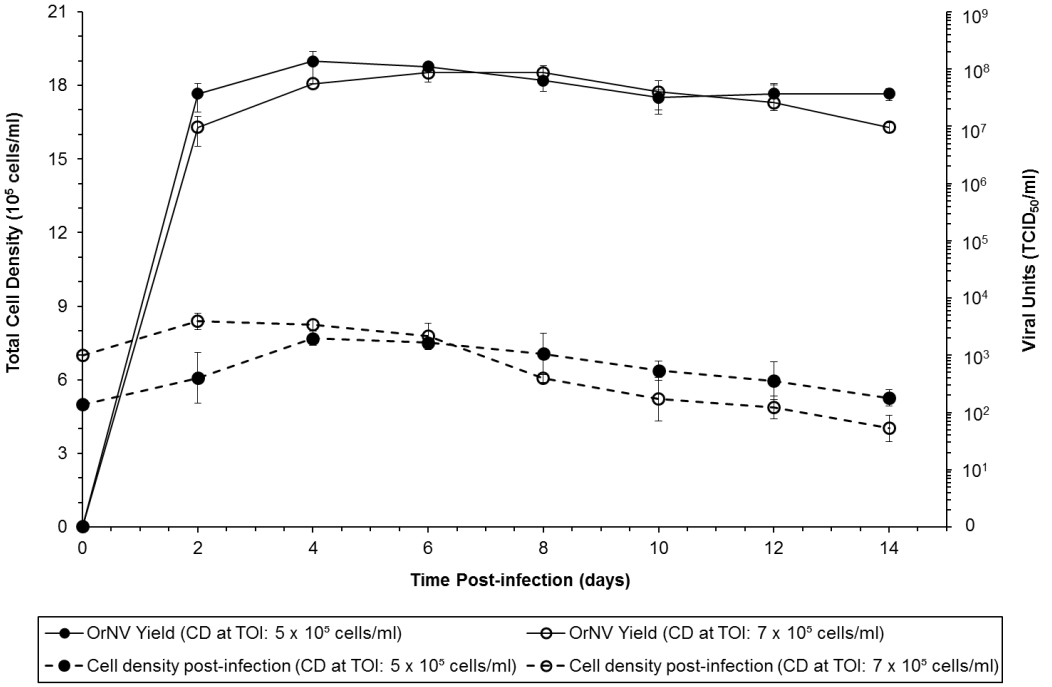

Fig. 6-11 Time course profile of total cell density (dashed lines) and OrNV production (solid lines) in infected Cytodex-1 microcarrier cultures of the DSIR-HA-1179 cell line, grown in TC-100 supplemented with 10% FBS, in spinner flasks, and infected at cell densities of $5x10^5$ cells/ml and $7x10^5$ at a multiplicity of infection of 1 $TCID_{50}$/cell. Error bars represent standard deviations from the mean of duplicate experiments.

The results demonstrate that while cultures infected at a higher cell density of 7 x 10^5 cells/ml reached a higher maximum post-infection cell density (8.4 x 10^5 cells/ml) than those infected at 5 x 10^5 cells/ml (7.68 x 10^5 cells/ml), the improved infected cell growth in the former condition did not translate into improved virus yields. The maximum volumetric and cell-specific yield of virus in cultures infected at 7 x 10^5 cells/ml (8.6 x 10^7 TCID50/ml, 102 $TCID_{50}$/cell) was lower than that achieved in cultures that were infected at 5 x 10^5 cells/ml (1.36 x 10^8 $TCID_{50}$/ml, 177 $TCID_{50}$/cell). Another factor to be taken into account with respect to the calculation of volumetric productivity is the longer culture time required to reach a higher initial cell concentration, in this case the extra time it takes to reach a density of 7 x 10^5 cells/ml instead of 5 x 10^5 cells/ml.

6.3.7.2 Partial culture medium replacement at TOI on OrNV production

Partial (75%) culture medium replacement at TOI was attempted as a means to boost cell-specific OrNV yields in microcarrier cultures infected at 5 x 10^5 and 7 x 10^5 cells/ml. Fig. 6-12 shows the temporal evolution of infected cell density and OrNV titer in each condition.

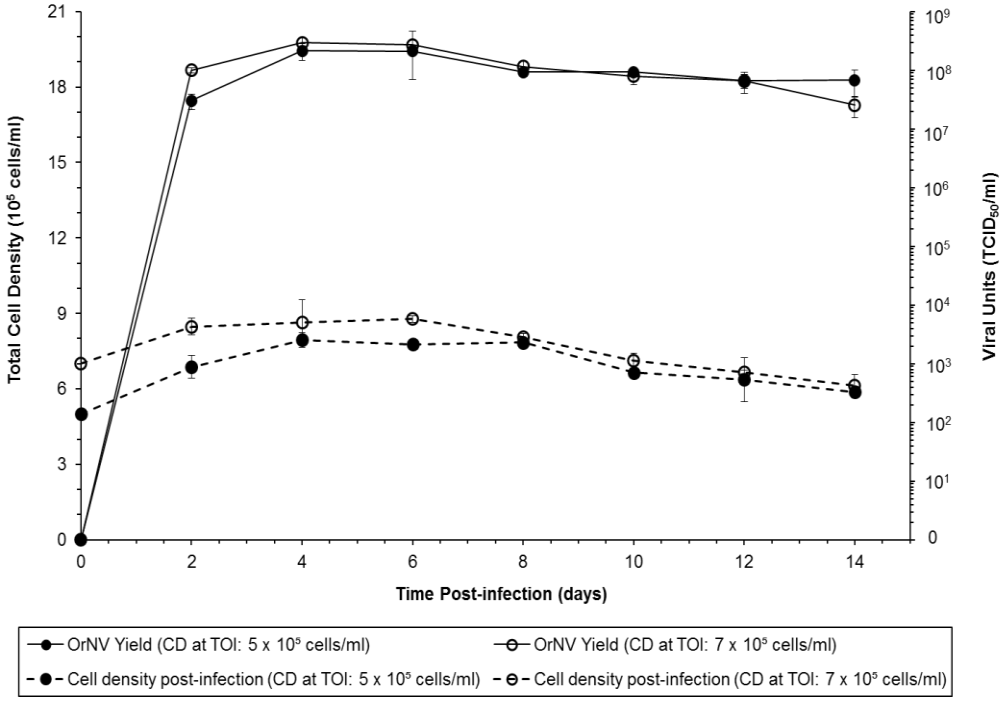

Fig. 6-12 Time course profile of total cell density (dashed lines) and OrNV production (solid lines) in infected Cytodex-1 microcarrier cultures of the DSIR-HA-1179 cell line, grown in TC-100 supplemented with 10% FBS, in spinner flasks, and infected at the respective cell densities of 5x10^5 cells/ml and 7x10^5 with 75% of the culture medium replaced immediately prior to infection. All cultures were infected at a multiplicity of infection of 1 $TCID_{50}$/cell. Error bars represent standard deviations from the mean of duplicate experiments.

In infections carried out at the cell densities of 5 x 10^5 cells/ml and 7 x 10^5 cells/ml, partial culture medium replacement at TOI led to slight improvements in post-infection cell density by 3.4% and 4.6% respectively, compared to cultures without medium replacement at TOI. Therefore,

maximum post-infection cell densities of 7.95 x 10^5 cells/ml and 8.78 x 10^5 cells/ml were reached at 96 and 48 hours post-infection, in infections performed at 5 x 10^5 cells/ml and 7 x 10^5 cells/ml, respectively.

The effects of partial culture medium replacement also manifested in improved OrNV volumetric and cell-specific yields in the two conditions, with infections at 7 x 10^5 cells/ml showing slightly enhanced productivity over those infected at 5 x 10^5 cells/ml. Similar maximum OrNV volumetric yields (2.15 x 10^8 $TCID_{50}$/ml and 2.94 x 10^8 $TCID_{50}$/ml) were achieved 96 hours post-infection, in infections carried out at 5 x 10^5 cells/ml and 7 x 10^5 cells/ml, respectively. The maximum cell-specific yield and volumetric productivity in infections carried out at 7 x 10^5 cells/ml were 334 $TCID_{50}$/cell and 1.87 x 10^7 $TCID_{50}$/ml/day, and for the lower cell density of 5 x 10^5 cells/ml, were, 270 $TCID_{50}$/cell, 1.54 x 10^7 $TCID_{50}$/ml/day, respectively.

6.4 Discussion

Cell attachment to microcarriers

The DSIR-HA-1179 cell line has an absolute requirement for attachment to a substrate in order to proliferate in culture. The interaction of cells with the substrate is thought to affect the arrangement of cellular structures involved in the control of growth and other essential functions (Varani *et al*., 1986). That cell behavior may be directly influenced by the properties of the growth substrate, was the rationale for evaluating three types of microcarriers with different conformational and surface properties in this study. Two of the microcarriers chosen, Cytodex-1 and Cytodex-3, were uniformly spherical and microporous; while the third, Cultispher-G was macroporous and of variable sphericity. Microporous carriers allow cells to create a micro-environment inside the beads, with short diffusion paths in spherical microporous microcarriers improving nutrient supply in general. On the other hand, macroporous microcarriers have a large internal matrix providing a greater, three-dimensional surface area per bead for cell attachment and growth (GE Healthcare, 2013). While cellular growth within macroporous carriers may offer protection against shear forces generated from bead collisions or culture agitation, oxygen or virus mass transfer could be less efficient in these carriers, due to diffusional limitations (Hu and Peshwa, 1991; Ng *et al*., 1996).

DSIR-HA-1179 cell attachment to microcarriers was observed to occur in four phases – the first, where inoculated cells were still spheroidal and adhered very slightly to the microcarrier surface. By 1 h post-inoculation, cells had adhered more firmly to microcarriers but were still round in shape. By 6 h post-inoculation, cells had flattened and adhered more firmly on the microcarrier surface, but had not begun spreading yet. By 12 h post-inoculation, cells had just begun spreading in their characteristic fibroblast-like manner and were reliably immobilized on carriers. The relatively long duration for complete cell attachment, was despite the provision of cultural factors which promote attachment, such as, the equilibration of carriers in culture medium containing serum and divalent cations, and the inoculum consisting of round, single cells after TrypLE™ Express treatment with disrupted cytoskeletons and newly produced cell surface receptors (GE healthcare, 2013; Ng *et al*., 1996; Sayer *et al*., 1987). The time taken for cell attachment to a particular microcarrier type can vary between cell lines. For example, it was found that CHO cells took up to 24 h to attach to the same Cytodex-1 microcarriers, which Vero cells could attach to within 1 h (Kong *et al*., 1999; Ng *et al*., 1996). With regard to DSIR-HA-1179 cell attachment to Cytodex-1 microcarriers, since cells take approximately the same amount of time to attach to T-flask surfaces, the underlying cause for slow attachment is probably related to an intrinsic slow cell cycle which is an inherent characteristic of the *in vivo* tissue from which the cell line derives, rather than inadequacies of the microcarrier surface (Crawford, 1982; Pushparajan *et al*., 2013).

While cells attached and grew on both Cytodex-1 and Cytodex-3 microcarriers, Cytodex-1 performed better in batch culture, resulting in a maximum cell density and specific growth rate that were 26% and 6.7% higher than Cytodex-3. On the other hand, cells failed to attach to Cultispher-G microcarriers. Previous studies indicate that the attachment and growth characteristics of insect cells on microcarriers appear to be cell-line specific. While the AA mosquito cell line could attach and grow on Cytodex-1 and DE-53 microcarriers (Lazar *et al*., 1987), and Tn-5 on Cytodex-3 microcarriers (Wickham and Nemerow, 1993); minimal cell growth was observed for Sf-9 and High-Five cell lines on Cytodex-1 and glass bead microcarriers (Ikonomou *et al*., 2002). Cultispher-S macroporous microcarriers failed to support the growth of the Sf-9 cell line, but supported the growth of the High-Five cell line to high densities. On the other hand, Cytopore 1 and Cytopore 2 carriers did not support growth of either cell line (Ikonomou *et al*., 2002). Cell attachment and spreading on a microcarrier may be determined by one or more factors, including;

the charge density and to a lesser extent, charge-polarity of the microcarrier, the material of its construction, any surface coatings/proteins that may be present, cell concentration, the spherical geometry of the carrier, and carrier pore size (Gebb *et al*., 1982; Jacobson and Ryan, 1982; Kong *et al*., 1999). Cytodex-1 and Cytodex-3 are both dextran based microcarriers, but Cytodex-1 has a high positive charge density (1.1-1.4 meq/g), while Cytodex-3 is nearly neutrally charged as a consequence of having a thin layer of denatured collagen chemically coupled to its surface. Cultispher-G is composed of gelatin and is uncharged. The high charge density of Cytodex-1 microcarriers would have resulted in enhanced electrostatic interactions of the cell surface with charged DEAE groups on the carrier, leading to better attachment. In addition to microcarrier surface charge, the type of serum-proteins adsorbed onto the microcarrier can also mediate cell adhesion. It has been found that collagen coated microcarriers have a lower capacity to adsorb serum proteins on their surface than uncoated microcarriers (Gebb *et al*., 1982). The first-order rate constant for attachment of the FS-4 human fibroblast cell line was found to be two-fold lower on Cytodex-3 microcarriers, than on Cytodex-1 microcarrier (Tao *et al*., 1987) As observed in Chapter 5 of this thesis, serum protein adsorption onto the growth surface is crucial for DSIR-HA-1179 cell attachment; and its lack thereof on Cytodex-3 microcarriers, may have been a reason why it underperformed Cytodex-1 in culture. The failure of cells to attach and spread on Cultispher-G microcarriers, may be related to the porosity of the carrier (10-20 µm), being too small to accommodate DSIR-HA-1179 cells which are on average 25-40 µm long and 15-20 µm wide (Crawford, 1982). Even had cells migrated into pores, it is likely that their tendency to grow as aggregates would have been precluded by the narrow pore channels, as similarly observed in the aggregative cell lines KM-12 and HT-29 cultivated on Cultispher-G (Rasey *et al*., 1996). It may also relate to interaction of cells with the gelatin surface of Cultispher-G, occurring at a lower rate than with Cytodex microcarriers (Ng *et al*., 1996). In future work, it would be interesting to evaluate macroporous microcarriers with a larger average pore size and/or different material composition, for example, Cultispher-S, and Cytopore 1 and 2 microcarriers, for their ability to support DSIR-HA-1179 cell growth.

A key element in establishing efficient microcarrier culture is ensuring that the available bead surface area is homogenously colonized by cells, right from the beginning of culture. The stirring regime, culture volume during initial cell attachment and the level of agitation can influence this process (Blüml, 2007; Butler, 1996; Voight and Zintl, 1999). The relatively lower attachment

efficiency in continuously stirred cultures (71.9%), compared to those that were intermittently stirred in a reduced culture volume (90.7%) and those attached under static conditions (99.4%), could be due to the contact time between cells and microcarriers being below the optimal value which allows sufficiently strong interactions between the cell-surface receptors and the surface to be formed, in the former case. Furthermore, continuous agitation in the form of stirring, induces flow stresses which are capable of affecting cells as well as microcarriers (Croughan *et al*., 1987). Shear stress from impeller agitation could have inhibited cell attachment, detached cells that were weakly bound to microcarriers, and/or affected cell viability, particularly since the DSIR-HA-1179 cell line is known for its fragility and sensitivity to mechanical stress (Chapter 2 of this thesis; Croughan and Wang, 1999; Forestell *et al*., 1992, Sinskey *et al*., 1981). Intermittent stirring in a reduced culture volume ensured an increased contact time between cells and microcarriers, as well as diminishing shear stress on cells. The reduced culture volume simulated a higher carrier concentration; which might have also improved cell attachment efficiency. By entirely doing away with agitation and attaching cells in a reduced culture volume in stationary T-flasks, cell attachment was further enhanced, as well as a more homogenous cell distribution on microcarriers achieved, due to improved spatial distribution of cells on microcarriers on the flat, horizontal surface. While static attachment in T-flasks is practicable in small-scale laboratory experiments and was therefore used in this work, it is recognized that it is not a suitable strategy for microcarrier culture at a larger-scale, either in spinner flask or stirred tank bioreactors, due to the labour and costs involved in the two-step process. Therefore, it is recommended that in future scale-up of the process, intermittent agitation in a reduced culture volume be used during the initial 12 hour cell attachment period of DSIR-HA-1179 cells to microcarriers.

Uninfected cell growth and metabolism in microcarrier culture

Despite ensuring the homogenous distribution of DSIR-HA-1179 cells on microcarriers at the start of a continuously agitated culture in spinner flasks, the progressive aggregation of cells on microcarriers was found to occur over the course of the culture. Cells grew in multiple layers, forming masses of dense cellular overgrowth on the bead surface. It appeared that the formation of large macro-aggregates (> 500 µm in size), consisting of cells and microcarriers (Fig. 6-6 D), was initially mediated by the formation of ‘cell-bridges’ between microcarriers. The process of macro-aggregate formation is represented schematically in Fig. 6-13.

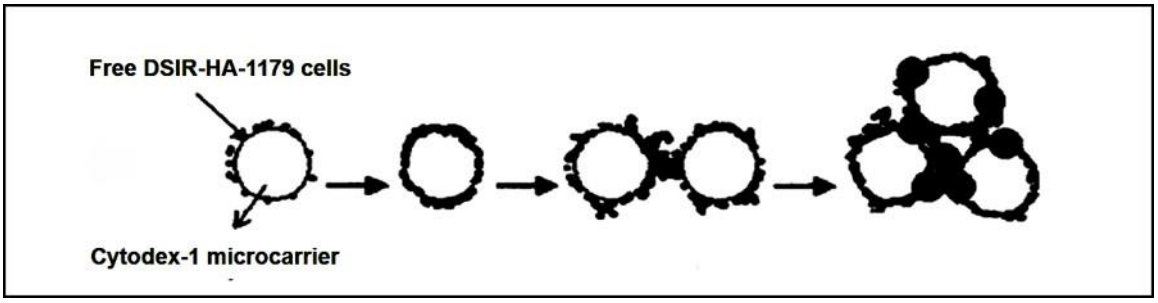

Fig. 6-13 Presumed mechanism of the formation of large DSIR-HA-1179 cell-microcarrier macro-aggregates during batch culture based on microscopic observation. Picture adapted from Drugmand *et al.*, 2001.

In addition to cell-bridging between microcarriers, actively growing cells on the outer surfaces of macro-aggregates, formed clumps, which in turn contacted and spread onto other uncolonized or partially colonized microcarriers that they came in contact with. Similar aggregation of cells on microcarriers have been reported for a variety of mammalian cell lines cultivated on solid and macroporous microcarriers (Clark and Hirtenstein, 1981; Goetghebeur and Hu, 1991; Schulz *et al*., 1987, Varani *et al*., 1983), as well as for the High-Five insect cell line cultivated on Cultispher-S microcarriers (Ikonomou *et al*., 2002). Cellular aggregation on microcarriers is widely considered a disadvantage due to the mass transfer limitations it entails, except in cases where cellular overgrowth is the only means of achieving high cell densities and the colonization of new beads in culture, and where cell-specific productivity is not affected by clumping (Avgerinos *et al*. 1990; Cherry and Papoutsakis, 1988; Goetghebeur and Hu, 1991). For cell cultures applied to the production of virus, mass transfer rates of oxygen, nutrients, waste metabolites and the virus may become limiting in areas of dense cellular overgrowth. One study has estimated that glucose limitations may be faced when the size of cell clumps exceed 900 μm; while another study has estimated that nutrient limitations typically occur at a distance of 100 – 220 μm from the periphery of porous microcarriers (Bliem and Katinger, 1988; Cahn, 1990). Oxygen transfer rates may become growth limiting when clumps exceed 300 μm in size (Bliem and Katinger, 1988). While mass limitations have been linked to cellular necrosis occurring at the cores of large microcarrier-cell aggregates (Preissmann *et al*., 1997), it was interesting that this did not occur in DSIR-HA-1179 macro-aggregates, even at the later stages of culture (Fig. 6-6 D). An explanation for this

could be a relatively loose connection of microcarriers and cells, which creates channels for the diffusion of nutrients to cells at the core of the macro-aggregate – a mechanism which has been previously proposed to explain the lack of necrotic cores in High-Five cell macro-aggregates on Cultispher-S microcarriers (Drugmand *et al*., 2001).

The specific growth rate of cells (0.0074 h^{-1}) and the maximum cell density (16.95 x 10^5 cells/ml) reached in microcarrier culture, were both comparable to the values obtained in monolayer culture in 25 cm^2 T-flasks (0.0069 h^{-1} and 16.8 x 10^5 cells/ml). The specific rates of glucose and glutamine consumption in microcarrier cultures (3.9 x 10^{-12} g/c/h and 1.2 x 10^{-12} g/c/h, respectively), were also similar to the respective consumption rates for glucose (4.1 x 10^{-12} g/c/h) and glutamine (8.3 x 10^{-13} g/c/h) in T-flask cultures (Chapter 3 of this thesis). Neither of these key nutrients became limiting during the batch growth period. Furthermore, lactate was not produced, while alanine was produced as a non-toxic alternative to ammonia, to similar levels observed in T-flask cultures (Bedard *et al*., 1993; Chapters 2, 3 and 5 of this thesis). The complete lack of production of toxic metabolites in microcarrier cultures, rules out the possibility of cell growth having been inhibited on that account. Taken together, these findings give credence to the hypothesis that the high extent of cellular overgrowth in macro-aggregates in microcarrier cultures, did not pose a significant impediment to cell growth *per se*, within the culture conditions evaluated in this work. Nevertheless, a main line of enquiry in future work must be the reduction of aggregation in DSIR-HA-1179 microcarrier cultures and evaluation of its effect on cell growth and metabolism kinetics. Strategies to reduce aggregation in microcarrier culture include increasing the agitation intensity in culture (Borys and Papoutsakis, 1992; Clark and Hirtenstein, 1981; Cherry and Papoutsakis, 1988), as well as the use of polar solvents such as DMSO, which have demonstrated their potential to inhibit cellular overgrowth on microcarriers (Borys and Papoutsakis, 1992).

OrNV production in microcarrier culture

The OrNV volumetric yield in microcarrier cultures infected in the early exponential growth phase at a cell density of 5 x 10^5 cells/ml and MOI of 1 $TCID_{50}$/cell, was higher than yields obtained in T-flask cultures infected under the same conditions (Chapter 4 of this thesis). The yields from microcarrier cultures were however, approximately two fold lower than those obtained in roller bottle cultures (Chapter 5 of this thesis). The superior yield obtained in roller bottle cultures may

be due to better oxygen and/or nutrients and/or virus mass transfer in the roller bottle system, compared to microcarrier cultures. This can be a consequence of the differing spatial distribution of cell growth surface in the two systems, which could have an influence on the virus binding kinetics (Borys and Papoutsakis, 1992). While cells in roller bottles grew as a single cell monolayer that was free of clumps, cells grew on microcarriers as large macro-aggregates. It has been recognized cell clumps can cause physical barriers to the efficient attachment of virus to individual cells. The inability for the virus to penetrate and infect cells at the core of the macro-aggregate, could have determined that the actual MOI is less than the theoretical MOI of 1. On the other hand, the exposure of the entire cell monolayer surface to efficient virus infection in roller bottle cultures, would have led to the creation of a number of infectious foci right at the start of the infection, when cells were in a better physiological state, and therefore, more permissive to virus infection. The cell density effect has been observed to occur in the DSIR-HA-1179/OrNV system, starting from infection cell densities as low as 5×10^5 cells/ml. In heavily aggregated microcarriers with regions of dense cellular overgrowth on them formed by multi-layering of cells, the localized infection cell density, may have been higher than intended. Therefore, reductions in cell-specific yield due to the cell density effect occurring in localized high-cell density regions on macro-aggregates, are a possibility (Berry *et al*., 1998).

Oxygen deprivation of cells growing in the inner regions of the cell-microcarrier macro-aggregates may also have occurred, since the size of many macro-aggregates exceeded 300 µm, which has been reported as the clump size above which oxygen limitations can occur (Bleim and Katinger, 1988). Indeed, as was observed in infected macro-aggregates photographed on day 4 post-infection (Fig. 6-9 A), the dark-stained non-viable cells are found at the core of the aggregate. On day 4 post-infection, cells were actively replicating virus as volumetric yields peaked on day 4 post-infection. In this scenario, while cell death could have occurred as a consequence of infection, the fact that only cells in the center of the aggregate became non-viable, points to an alternate explanation such as nutrient or oxygen deprivation leading to a necrotic core. While the concentration of the main nutrients, glucose and glutamine, did not become limiting over the course of infection in microcarrier cultures, the concentration of unmonitored species such as micronutrients in the culture medium might have been depleted during infection. The cell density effect has also been linked to the depletion of micronutrients in culture (Claus et *al*., 2012). Therefore it is possible that such micronutrient deficiencies, in tandem with the other mentioned limitations in oxygen and

virus mass transfer, could have also been a cause for the lower productivity in microcarrier macroaggregates, compared to roller bottle cultures. This could also be the reason why partial replacement of culture medium at the time of infection, improved OrNV volumetric yields by two fold, since it would have replenished any limiting nutrients that caused the cell-density effect. This may also explain the delay in the drop in cell-specific yield of the higher infection cell density of 7 x 10^5 cells/ml, which occurred when culture medium was partially replenished at the time of infection.

It is suggested that the reduction of cellular overgrowth in macro-aggregates or the complete inhibition of aggregation, especially during the infection phase, may be beneficial for improving OrNV volumetric yields in microcarrier cultures. Strategies for reducing clumping of microcarriers and cellular overgrowth, must be further investigated in future work. Nevertheless, and despite macroaggregate formation, the improved OrNV volumetric yields obtained in microcarrier cultures over T-flask cultures, have demonstrated that it is a feasible cultivation system for DSIR-HA-1179 cells and for production of OrNV in these cultures. The great advantage of microcarrier culture is that, unlike roller bottles, novel and scalable options for its cultivation can be explored. Bioreactor configurations that will be suitable for the scale-up of microcarrier culture include stirred tanks and particularly, airlift bioreactors which may provide the benefits of a low shear environment for DSIR-HA-1179 cell growth on microcarriers.

6.5 Conclusions

The emergence of microcarrier technology has brought about a fresh wave of interest in the culture of anchorage-dependent cell lines. In addition to the wide application of microcarrier technology for the production of vaccines, the system has potential for application to other virus-production systems, specifically for the *in vitro* production of viral biopesticides. The results of this study demonstrate that the OrNV biopesticide can be produced in microcarrier cultures of DSIR-HA-1179 cell line in spinner flask bioreactors. Three types of microcarriers were investigated in this study – Cytodex-1, Cytodex-3 and Cultispher-G – of which, only the former two were found to be conducive for cell attachment and proliferation of DSIR-HA-1179 cells. Cytodex-1 was selected as it produced the highest cell yields in culture (16.95 x 10^5 cells/ml), and achieved the highest

specific growth rate (0.0074 h^{-1}), when used with optimized culture conditions of a microcarrier concentration of 1 g/l and cell to bead ratio of 30, in a 60 ml culture volume in spinner flasks. A cell attachment strategy whereby cells attached to microcarriers in static culture in the initial 12 h of culture, ensured high cell attachment efficiencies and an even distribution of cells on microcarriers at the start of the culture. However, as the culture progressed, progressive cell clumping occurred, which resulted in the formation of large macro-aggregates. The maximum OrNV volumetric yield achieved in cultures infected in the early exponential phase (CD at TOI: 5 x 10^5 cells/ml), was 1.36 x 10^8 $TCID_{50}$/ml. OrNV volumetric yields could be improved to 2.94 x 10^8 $TCID_{50}$/ml by using an infection strategy wherein, the CD at TOI was increased to 7 x 10^5 cells/ml, and 75% of the culture medium was replaced at TOI. Further process improvements may be sought by finding ways to reduce cell-microcarrier aggregation, development of low-cost nutrient feeds in lieu of medium replacement at TOI, as well as attempting the scale-up of OrNV production in a suitable large-scale bio-reactor such as an airlift bioreactor.

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Chapter 7

Conclusions

The purpose of this chapter is to re-state the aims and expectations set out at the beginning of this thesis, and to present a compendium of conclusions drawn from results obtained in the different investigations carried out in this work. The global goal of the research carried out in this thesis, was to apply the tools and techniques of 'insect cell biotechnology' to the *in vitro* production of the OrNV biopesticide, in the unique DSIR-HA-1179 coleopteran host insect cell line. The justification for a shift from the current *in vivo* method of OrNV production in infected beetle larvae, to *in vitro* production in the DSIR-HA-1179 cell line, is two-fold. Firstly, *in vivo* production technology, while it is advantaged by process economy, it remains an artisanal method which cannot guarantee a sterile, quality-controlled biopesticide product. The need for large insectaries for rearing and infecting beetle larvae, coupled with the significant time and labour that this entails, can be easily offset by *in vitro* production technologies, where the virus is propagated in cell cultures grown in a sterile and controlled bioreactor environment. Secondly, in the present scenario of burgeoning beetle outbreaks in new territories such as Guam in 2009, and in Hawaii as recently as 2014, there is likely to be an increased demand for large volumes of sterile and highly virulent OrNV inoculum in the near future. A major advantage of production in insect cell bioreactors, is that they can reach economies of scale, and can achieve cost-efficiency when operated at higher scales and with large volumes.

I*n vitro* production is clearly advantageous over *in vivo* production of biopesticides, and insect cell biotechnology has now reached a stage, where cell culture and virus quantification methods are well developed for monitoring *in vitro* production processes. However, technical problems related to the formulation of low-cost culture media, achieving adequate nutrient and oxygen mass transfer in bioreactors for high cell yields, and the improvement of virus yields in cultures infected at high

cell densities; continue to impede the profitable production and commercialization of *in vitro* produced biopesticides – issues which are well recognized by several research groups working on the *in vitro* production of baculovirus biopesticides. Baculoviruses have been the largest studied group for *in vitro* production as biopesticides in insect cell cultures. On the other hand, the nudivirus family to which OrNV belongs, has been poorly studied in comparison, and OrNV is the first nudivirus to have options explored for its *in vitro* production. Nevertheless, at the beginning of this research work, it was recognized that the challenges for OrNV production were likely to be similar to those faced for baculovirus production in *in vitro* production systems. Therefore, the optimization of OrNV production were conducted along similar lines.

The following requirements were identified for successful *in vitro* production of OrNV – (1) a susceptible, permissive and fast-growing host cell line (2) a virulent strain of virus for infecting cell cultures (3) the use of a low-cost and serum-free culture medium (4) the ability to cultivate cells to high densities and in scalable bioreactors (5) the maintenance of high cell-specific virus yields when infecting cells at high densities.

The DSIR-HA-1179 cell line has the distinction of being the only available cell line, which is known to be permissive and susceptible to the replication of OrNV. However, its strongly adherent nature, its slow growth rate, and the difficulties faced in manipulating the cell line faced by early researchers, discouraged further studies of the cell line since the 1980's. Until the cell line was received in our hands at the start of this research, it had only been cultured in 25 cm^2 T-flasks with typical culture volumes/OrNV production of 5 ml. There was no prior literature on the characterization of the basic technological properties of the cell line, its mass production in scaled-up bioreactor configurations, or on infection strategies for the efficient production of OrNV. Therefore, the main goals of the project were to investigate each of the aforementioned aspects i.e. cell growth kinetics, uninfected and infected cell metabolism and OrNV production in DSIR-HA-1179 cultures grown in T-flasks, roller bottles and microcarrier cultures. Table 7-1 provides a comparison of cell growth, metabolism and OrNV production parameters in the three bioreactors evaluated in this thesis. The table is followed by a list of conclusions that can be drawn from the results of this research work.

Table 7-1: Comparison of cell growth, metabolism and OrNV production (with and without partial culture medium replacement at TOI) in the DSIR-HA-1179 cell line grown in three alternative culture systems (T-flasks, roller bottles and microcarriers).

	Culture Vessel		
	T-flask	Roller Bottle	Microcarrier culture in spinner flasks
Culture Parameters			
Growth surface area (cm^2)	25	490	264
Final culture volume (ml)	5	60	60
Culture mode	Batch	Batch	Batch
Agitation	None	Continuous rolling at 0.1 rpm	Continuous stirring at 40 rpm
Culture medium	TC-100 +10% FBS	TC-100 + 10% FBS	TC-100 + 10% FBS
Cell Growth			
X_{vmax} (cells/ml)	16.8×10^5	14.5×10^5	16.95×10^5
μ (h^{-1})	0.0069	0.0052	0.0074
PDT (days)	4.2	5.5	3.9
Cell yield on inoculum	8.4	4.4	8.5
Cell Metabolism			
q_{GLUC} (g/c/h)	4.1×10^{-12}	5.2×10^{-12}	3.9×10^{-12}
q_{GLN} (g/c/h)	8.3×10^{-13}	9.5×10^{-13}	1.2×10^{-12}
Lactate Production	No	No	No
Ammonia Production	Negligible	Negligible	Negligible
Alanine Production	Yes	Yes	Yes
OrNV Production			
Cell density at TOI (cells/ml)	5.2×10^5	5×10^5	5×10^5
MOI ($TCID_{50}$/cell)	1	1	1
Volumetric yield ($TCID_{50}$/ml)	6.84×10^7	2.13×10^8	1.36×10^8
Cell-specific yield ($TCID_{50}$/cell)	102	250	177
Volumetric productivity ($TCID_{50}$/ml/day)	6.22×10^6	1.78×10^7	1.7×10^7
Overall yield/culture vessel ($TCID_{50}$)	3.42×10^8	1.28×10^{10}	8.16×10^9
Time of harvest (day post-infection)	6	8	4
Viral amplification factor	131	426	272
OrNV Yield Improvement with Partial (75%) Culture Medium Replacement at TOI			
Cell density at TOI (cells/ml)	Not evaluated	6.5×10^5	7×10^5
MOI ($TCID_{50}$/cell)		1	1
Volumetric yield ($TCID_{50}$/ml)		1.89×10^9	2.94×10^8
Cell-specific yield ($TCID_{50}$/cell)		1897	334
Volumetric productivity ($TCID_{50}$/ml/day)		7.71×10^7	1.87×10^7
Overall yield/culture vessel ($TCID_{50}$)		1.13×10^{11}	1.76×10^{10}
Time of harvest (day post-infection)		8	4
Viral amplification factor		3780	588

7.1 The DSIR-HA-1179 cell line was particularly difficult to manipulate in culture, due to its strong adherent nature, fragility, and tendency to form cell clumps when mechanically stressed. The lack of a reliable methodology for creating homogenous single-cell suspensions from attached cultures of the DSIR-HA-1179 cell line, was a fundamental problem that had to be surmounted. Various methods of cell detachment/dissociation of DSIR-HA-1179 cell monolayers by mechanical, chemical and enzymatic means, were evaluated. Simple mechanical means such as tapping the flask surface to dislodge cells and repeated gentle aspiration of the culture did not homogenously disperse the cell monolayer, but instead, exacerbated cell clumping and mortality. Treatment of cultures with the chemical disaggregation agent, Heparin, in doses of 100 µg/ml and 667 µg/ml, also proved ineffective in dispersing cell monolayers. The cell line could be reliably dissociated using the TrypLE™ Express enzyme. The use of 1 ml of pre-warmed TrypLE™ Express per 25 cm^2 of flask surface area and incubation for 30 minutes at 27 ºC, enabled the creation of a homogenous single cell suspension of DSIR-HA-1179 cells while preserving cell viability (94%).

7.2 The effect of passaging DSIR-HA-1179 cells with the inclusion of TrypLE™ Express at every passage was investigated in order to determine whether long term exposure to protease carryover would lead to a loss in cell growth and viability. It was found that culture viability remained high (> 90%) until the 4^{th} continuous passage with TrypLE™ Express, but significantly declined to 73% at the 5^{th} passage and continued to decline in subsequent passages. Therefore, use of TrypLE™ Express in routine passages after the fourth passage was not recommended.

7.3 Before the inclusion of TrypLE™ Express could be recommended in a process for producing OrNV in DSIR-HA-1179 cell cultures, it was necessary to ascertain that the enzyme would not affect *in vitro* replication of the virus. It was found that inclusion of TrypLE™ Express in the process, in fact, led to slight improvements in OrNV volumetric yields, compared to cultures which had not been treated with the enzyme. It was postulated that this occurred as a consequence of TrypLE™ Express treatment, having effectively creating an evenly dispersed cell inoculum, which in turn resulted in the formation of uniform, clump-free cell monolayers. This facilitated virus attachment and binding to a greater extent than in the absence of TrypLE™ Express, where cells

growing in clumps caused a physical barrier to virus attachment, and where mass transfer limitations of nutrients in the culture media could have occurred.

7.4 The cell line was successfully adapted to grow in four different culture media (TC-100, IPL-41, Sf-900 II and Sf-900 III, each supplemented with 10% FBS), in batch attached T-flask cultures. This was done to test those which might better support the nutritional requirements of the cell line and thereby improve cell yield and virus production, and to select a culture medium that could be used as a model to conduct initial studies on this cell line. TC-100 supplemented with 10% FBS was selected as an optimal growth medium for the DSIR-HA-1179 cell line grown in attached T-flask cultures, based on the maximum cell density (16.8×10^5 viable cells/ml) and OrNV volumetric yields (3.8×10^7 $TCID_{50}$/ ml) reached in this culture medium.

7.5 The use of serum in culture media contributes to the high cost of *in vitro* production of biopesticides, in addition to introducing undefined animal-derived components into the culture environment. It is therefore desirable to reduce the percentage of serum in the culture medium or to adapt the cell line to grow in a serum-free medium. The effects of varying FBS concentration (0%, 1.0%, 2.5%, 5.0% and 10.0% v/v) in the TC-100 culture medium on DSIR-HA-1179 cell growth in 25 cm^2 T-flask cultures, was evaluated. Cells failed to grow in the complete absence of serum, while maximum viable cell densities and specific growth rates were impaired at FBS concentrations below 5.0%. The specific growth rate was comparable in cultures supplemented with 5 and 10% FBS (0.0069 h^{-1}), however the highest viable cell density (16.8×10^5 viable cells/ml) was obtained only when cultures were supplemented with 10% FBS. Therefore, the supplementation of TC-100 with 10% FBS was recommended for DSIR-HA-1179 cell culture, at this stage of process development.

7.6 The DSIR-HA-1179 cell line's karyotype was re-evaluated after a period of thirty years in continuous culture. A decrease in the variation of the spread of chromosomes per cell was observed, despite the prevailing polyploid nature of the cell line. The reduction in chromosomal instability is

indicative of the cell line having tended to a relatively more stable genetic state, which is a positive finding from an industrial perspective.

7.7 A range of culture media (TC-100, IPL-41, Sf-900 II and Sf-900 III, each supplemented with 10% FBS) were screened for cell growth and virus production in roller bottle cultures, and TC-100 + 10% FBS was chosen based on the high cell (9.0×10^7 cells/ roller bottle) and virus yields (1.4×10^8 $TCID_{50}$/ml) achieved in this culture medium.

7.8 Culture parameters were optimized for DSIR-HA-1179 cell growth in roller bottle cultures, including the roller bottle pre-conditioning procedure, inoculum cell density and culture volume to be used.Under these conditions, cells grew with a specific growth rate of 0.0052 $h^{-1,}$ and reached a maximum cell density of 14.5×10^5 viable cells/ml on day 14 of culture.

7.9 Three types of microcarriers: Cytodex-1, Cytodex-3 and Cultispher-G, were evaluated for their ability to support DSIR-HA-1179 cell attachment and proliferation in 10% serum-supplemented TC-100 medium. It was found that cells attached and grew on Cytodex-1 and Cytodex-3 microcarriers, but failed to attach to Cultispher-G microcarriers. Between Cytodex-1 and Cytodex-3, cells grew to the highest maximum cell density (16.95×10^5 cells/ml) and specific growth rate (0.0074 h^{-1}) on Cytodex-1 microcarriers, compared to a lower maximum cell density (12.6×10^5 cells/ml) and specific growth rate (0.0069 h^{-1}) on Cytodex-3 microcarriers in 60 ml spinner flask bioreactors that were continuously stirred at 40 rpm. Based on these findings, Cytodex-1 was chosen as a suitable microcarrier for DSIR-HA-1179 cell culture.

7.10 In order to maximize cell attachment efficiency, as well as the distribution of cells on Cytodex-1 microcarriers, three different attachment procedures were investigated in the first 12 h of culture (i.e. the attachment phase). Cells attached to microcarriers in a reduced culture volume of 20 ml, in 75 cm^2 T-flasks under static conditions during the first 12 h of culture, produced the highest cell attachment efficiency (99.4%), and a more even distribution of cells on microcarriers.

Therefore, these conditions were adopted to perform the rest of the microcarrier experiments done in this work.

7.11 DSIR-HA-1179 cell growth on Cytodex-1 microcarriers was evaluated at a range of cell to bead ratio's (15, 30, 60 and 120 cells per bead). A cell to bead ratio of 30 cells per bead was found to be optimal, as it led to the highest multiplication ratio (3.01) of DSIR-HA-1179 cells on Cytodex-1 microcarriers. Cell growth in a range of microcarrier concentrations (0.5 g/l, 1 g/l, 2 g/l, 3 g/l), were also evaluated with a constant cell to bead ratio of 30. It was found that highest maximum cell density (16.95 x 10^5 cells/ml) and specific growth rate (0.0074 h^{-1}), was reached when a microcarrier concentration of 1 g/l was used in a 60 ml spinner flask culture and inoculated at a density of 30 cells per bead (or 2 x 10^5 cells/ml) (Table 7-1).

7.12 Despite ensuring that cells were evenly distributed on microcarriers during the initial phase of cell attachment, large macro-aggregates consisting of dense cellular overgrowth on several clumped microcarriers, occurred between the mid to late-exponential growth phase in microcarrier cultures. The lack of necrotic cores in these macro-aggregates suggests that despite their large formatted structures, cells did not suffer from mass transfer limitations during batch growth, possibly due to loose connection of cells on carriers which allowed nutrient and oxygen diffusion into the center of the macro-aggregates.

7.13 Nutrient consumption in uninfected DSIR-HA-1179 cultures growing in T-flasks, roller bottles and in microcarrier cultures was broadly similar with respect to the active consumption of glucose, and to a lower extent, of glutamine, during batch growth (Table 7-1). While neither nutrient became limiting in T-flask and microcarrier cultures, roller bottle cultures suffered glucose limitation between days 8 – 14 in culture. Supplementation of glucose to the culture medium, or an increase in the culture volume above 60 ml in roller bottles is recommended to avoid cell growth limitations occurring as a consequence of glucose deprivation.

7.14 Metabolites production in uninfected batch DSIR-HA-1179 cultures growing in T-flasks, roller bottles and in microcarrier cultures were also similar, and were characterized by the lack of production of lactate and ammonia, and the production of alanine as a non-toxic alternative to ammonia, during the batch growth (Table 7-1). From an industrial perspective, the non-production of potentially toxic metabolites in culture is promising for high-density culture of the cell line in future.

7.15 Nutrient consumption in infected DSIR-HA-1179 cultures growing in T-flasks, roller bottles and in microcarrier cultures were characterized by an increased specific rate of glucose and glutamine consumption during the first 48 hours of infection, in response to the increased metabolic energy required to sustain virus replication in cells (Table 7-1). No nutritional limitations of glucose and glutamine were faced during virus production in T-flask and microcarrier cultures, but occurred in infected roller bottle cultures.

7.16 Metabolites production in infected DSIR-HA-1179 batch cultures growing in T-flasks, roller bottles and in microcarrier cultures, were all characterized by the lack of production of lactate and ammonia, and by the production of alanine (Table 7-1).

7.17 Volumetric yields of OrNV were influenced significantly by the cell density at the time of infection, and to a lesser extent by the MOI. This factor, however, played a role in influencing the dynamics of infections and the time of harvest. Infecting cells at an approximate cell density of 5.0 x 10^5 viable cells/ml during the early-mid exponential growth phase, was critical for maximizing OrNV volumetric and cell-specific yields in batch infections carried out in T-flasks, roller bottle and microcarrier cultures.

7.18 The cell density effect was found to exist for the DSIR-HA-1179/OrNV system with the progressive decline in cell-specific yields beginning at cell densities as low as 5.0 x 10^5 viable cells/ml. Through the correlation of CD at TOI and the maximum viable cell density (MVCD) on

the one hand, and the correlation of MVCD and maximum cell-specific and volumetric OrNV yields on the other; it was found that in order to maximize OrNV volumetric yield, any combination of CD at TOI and MOI could be used, as long as the MVCD was kept between 5.0 – 7.0 x 10^5 viable cells/ml.

7.19 In OrNV infections performed at an optimal infection cell density of 5.0 x 10^5 viable cells/ml using a MOI of 1 $TCID_{50}$/cell, and without culture medium replacement at the TOI, the highest volumetric and cell-specific OrNV yields were obtained in roller bottle cultures, followed by microcarrier cultures and finally, T-flask cultures (Table 7-1).

7.20 Partial (75%) culture medium replacement at the time of infection, was shown to be a useful strategy for improving OrNV volumetric and cell-specific yields in both roller bottle and microcarrier cultures (Table 7-1). It effectively delayed the onset of the cell density effect to higher infection cell densities, in both roller bottle and microcarrier systems. This strategy led to a remarkable improvement in volumetric yield in infected roller bottle cultures by an order of magnitude. The same magnitude of yield improvement was not observed in microcarrier cultures, possibly due to nutrient, oxygen or virus mass transfer limitations having occurred in cell-microcarrier macro-aggregates. Despite the yield improvements obtained with partial culture medium replacement at TOI, it is unlikely that this strategy will be industrially applicable, due to the prohibitive costs of replacing culture medium. However, the facts of the improvements reached in delaying the onset of the cell density effect and increasing of virus yield, open an important gate to the future in the terrain of culture medium optimization for this particular insect cell line/virus system.

Chapter 8

Future Directions

Having achieved the main objectives proposed in of this thesis, it would be useful to explore the avenues for future work in the area of *in-vitro* production of the *Oryctes* nudivirus (OrNV) biopesticide in DSIR-HA-1179 insect cells.

Despite the clear benefits that *in-vitro* biopesticide production offer over *in-vivo* production from a technological and quality assurance perspective, the global biopesticide market is currently dominated by industries producing viral biopesticides using the latter method solely because of the process economy (Ravensberg, 2011a), particularly in underdeveloped countries where the manpower is inexpensive. Taking into consideration the narrow profit margins for biopesticides, there is consensus on the fact that the future of *in-vitro* biopesticides rests on making their commercial production economically feasible by developing cheaper and better-yielding processes in scalable bioreactors (Reid *et al*., 2013). An assessment of the suitability of the *in-vitro* production methods for OrNV that have been developed in this thesis and the scale at which they must be operated in industry, is best informed by estimating the global demand for OrNV biopesticide.

8.1 Global demand for OrNV biopesticide

The coconut rhinoceros beetle is a pest of coconut palms (*Cocos nucifera* L.) and oil palms (*Elaeis guineensis*) which are cultivated over 12 and 15 million hectares worldwide, respectively (FAO, 2007; Fitzherbert *et al*., 2008). Therefore, a total of 27 million hectares of palms could potentially require treatment with OrNV to combat the beetle pest. While the baculoviruses of several lepidopteran pests are applied as biopesticides by spraying them on fields, this is not the case with OrNV due to the unique ecology of the beetle. Instead, nudivirus infection relies on the potential for the beetle to auto-disseminate the virus in the environment (Huger, 2005). The current approach involves using healthy adults periodically trapped on plantations, orally dosed with virus, and

released into the environment to spread the infection (Jackson, 2010). With an estimated 15 beetles that are required to be released per hectare (Gopal *et al*., 2001), a global total of 400 million beetles would require inoculation with OrNV. At an infectious dose of 2 x 10^5 $TCID_{50}$ administered per beetle (Jackson, 2010), this would mean that 8 x 10^{13} $TCID_{50}$ will potentially be required to infect 400 million beetles. Using the roller bottle production method developed in this thesis, yields of approximately 6 x 10^9 $TCID_{50}$ can be obtained per 490 cm^2 roller bottle each with a production volume of 60 ml. Therefore, in order to produce 8 x 10^{13} $TCID_{50}$ to meet the global demand using the current process, 15,000 roller bottles should be operated, which is industrially feasible. It is important to note that if the OrNV yield could be improved two folds only - a reasonable assumption given that the maximum potential of this system is yet to be explored, the number of roller bottles of similar volume to be used will be reduced to half, making the industrial process using this production system even more attractive.

An alternative option would be microcarrier-based production of OrNV in large-scale unit bioreactors. Assuming successful scale-up which maintains the current yield of 10^8 $TCID_{50}$/ml, only 800 L would be required to potentially meet the global demand. Both stirred tank and airlift reactors could be used to reach this target. Comparatively low volume production of this nature may become industrially feasible, particularly when for humanitarian reasons, in order to treat affected regions in a timely manner, government subsidies are obtained for OrNV production.

Therefore, and subject to successful process scale-up, both production systems evaluated in this thesis (roller bottles and microcarriers) are suitable for the industrial production of OrNV.

8.2 Future work

- Development of a low-cost, serum-free and preferably animal component free culture medium for the cultivation DSIR-HA-1179 cell line

The culture medium used is the most expensive consumable in an *in-vitro* biopesticide production process. Insect cell culture media in general are partially defined in terms of their chemical composition - consisting of a defined basal medium supplemented with undefined elements such

as fetal bovine serum, microbial extracts, vitamins and protein hydrolysates. In the experiments performed in this thesis, the basal medium used to grow DSIR-HA-1179 cells was the TC-100 insect cell culture medium, which was supplemented with 10% fetal bovine serum. From experiments done in this thesis, we now have an understanding of the main nutritional requirements and metabolism of the DSIR-HA-1179 cell line. While TC-100 was found to be nutritionally sufficient for the cell line, we know that it provides the cells an oversupply of amino acids. Amino acids are an expensive part of the basal medium and their concentrations could be lowered specific to the cell line's requirements. At present, however, the inclusion of serum in the culture medium still presents the main cost barrier to achieving commercial feasibility of producing OrNV using an *in vitro* production process. It is in the interest of the process economy to lower the cost of the culture medium by developing a low-cost culture medium specifically designed according to the nutritional requirements of the DSIR-HA-1179 cell line in future, which both support the growth of these cells to high densities and the production of OrNV to high titers. This will require development of a basal medium, as well as the replacement of serum with cheaper sources of growth factors, amino acids and hydrophobic nutrients which it supplies. These replacements may be provided by a combination of yeast extract, tryptose broth, plant or animal derived hydrolysates and lipid micro-emulsions (Claus *et al*., 2012). The development of a low-cost serum-free culture medium (LC-SFM) for the DSIR-HA-1179 cell line could be aided by a design of experiment (DoE) approach (fractional factorial assays and response surface methodology) for screening a large number of medium components, in a similar approach to the works that were done in tailoring the VPM3 LC-SFM for the production of HearNPV biopesticide in H.zea cells (Reid *et al*., 2013), and UNL-10 for AgMNPV production in saUFL-AG-286 cells (Micheloud *et al*., 2009). The development of a tailored serum-free culture medium for DSIR-HA-1179 cell line will play without any doubts an important role in its adaptation to grow in suspension.

- Suspension adaptation of the DSIR-HA-1179 cell line

Suspension adaptation of the DSIR-HA-1179 cell line could aid its high-density culture in large-scale unit bioreactors and improve volumetric productivity of OrNV – again improving the commercial feasibility of producing OrNV in an *in vitro* process. Compared to suspension cultures, the scale-up of adherent cultures is expensive and time consuming. In suspension cultures, OrNV

production could potentially be scaled up in stirred tank bioreactors up to 20,000 L and airlift bioreactors up to 5,000 L (De Jesus and Wurm, 2011; Pörtner, 2014). In the research done in this thesis, the suspension adaptation of DSIR-HA-1179 cells in spinner flask bioreactors was unsuccessful due to the tendency of the cells to aggregate as well as their sensitivity to shear. Suspension adaptation using shaker flasks could be evaluated in future work. The selection and propagation of populations of single cell clones from the mixed population of cell aggregates can be attempted in future. If successful, further experiments to characterize DSIR-HA-1179 cell growth, metabolism and OrNV production in suspension adapted cultures must be undertaken.

- Optimization of cell growth and scale-up of OrNV production in roller bottle and microcarrier-based culture systems

There is potential for several lines of research to be pursued relating to the engineering aspects of large-scale OrNV production in bioreactors. The experiments performed on DSIR-HA-1179 cell growth and OrNV production in roller bottle cultures as part of this thesis, have established this system as a robust production platform for OrNV. However, only roller bottles of 490 cm^2 surface area were evaluated for cell growth and virus production in the experiments performed in this thesis. Roller bottles providing larger surface areas for cell growth, i.e. 850 cm^2 and 1,750 cm^2, are commercially available, and it would be interesting to evaluate OrNV production using them. Additionally, it would be worth evaluating the effect of different rolling speeds (other than 0.1 rpm which was used in our experiments) on cell growth and OrNV production in roller bottles.

Microcarrier-based production of OrNV was demonstrated in spinner flask cultures in this thesis, however, the formation of large macro-aggregates of DSIR-HA-1179 cells on Cytodex-1 microcarriers in this system was recognized as an aspect that might induce mass transfer limitations which, in turn, could affect virus production in the system. Therefore, future work should evaluate strategies to reduce the formation of DSIR-HA-1179 cell aggregates on microcarriers. A potential strategy could involve the incorporation of 1% DMSO in the culture medium – such an approach was used to inhibit cellular overgrowth on CHO cell cultures growing on Cytodex-3 microcarriers (Borys and Papoutsakis, 1992). The next step of process development would be scaling-up microcarrier cultures of DSIR-HA-1179 cells to large-scale bioreactors such as stirred tank and

airlift bioreactors and the optimization of the engineering parameters related to each of these systems. Additionally, the routine testing of the in-vivo activity of OrNV produced in the different culture systems must be undertaken, as well as the identity of the virus produced routinely confirmed using PCR. With respect to virus production, improvement of cell-specific OrNV yield may be sought via strategies to delay the onset of the cell density effect to higher infection cell densities. This could be attempted either through partial culture medium replacement with fresh medium at the time of infection, and the development and application of nutrient feeds and feeding strategies (i.e. feedback, perfusion). Finally, it would be interesting to quantify the total number of viral genomes of OrNV produced via qPCR and compare these results with the infectious titer obtained via $TCID_{50}$ assay in the different culture systems, which would give an indication of the infectivity of *in vitro* produced OrNV.

- Cryopreservation of the DSIR-HA-1179 insect cell line

The cryopreservation of cell lines is an important part of animal cell culture, and the creation of a master cell bank ensures that a back-up of cells of a lower passage number exist in the event of contamination or a loss of growing cell cultures. DSIR-HA-1179 is a unique and important cell line, being the only currently available cell line which can support the *in vitro* replication of OrNV. Cryopreservation of this cell line is an important aspect of its maintenance, however, its failure to reliably cryopreserve in previous attempts led to it being maintained through continuous passaging over the past thirty years (Marshall *et al*., 2010). The failure of earlier attempts to cryopreserve the cells could have been a result of the tendency of the cells to form clumps when dispersed in suspension. The work done in this thesis now allows the cell line to be dissociated using the TrypLE™ Express enzyme and enabled the creation of a single-cell suspension, aspects which might now permit the cells to be reliably cryopreserved. It is imperative therefore, that cryopreservation of DSIR-HA-1179 cells and establishment of a master cell bank be investigated as part of future work.

- Improvement of OrNV formulation to ensure longevity in the field

The current method of formulating OrNV for oral delivery to the coconut rhinoceros beetle is simple and involves constituting the infectious dose of the virus in 10% sucrose solution (Moore and Marshall, 2014). Since nudiviruses lack a protective crystalline polyhedral body and therefore are inactivated easily by solar ultraviolet (UV) radiation, the current formulation method does nothing to improve the persistence in the virus in the field. Optical brightener compounds and adjuvants have been included in the formulation of other virus-based biopesticides to confer them UV protection. For example: Tinopal C1101 (an ethenedyl benzenesulfonic derivative) has been used to improve the UV resistance of SfMNPV (Mondragon *et al*., 2007). Such an approach could be of benefit in improving the persistence of OrNV in the field, and improving the formulation of the final OrNV product must be undertaken in future research.

8.3 References

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