

Kinetic response of a *Drosophila melanogaster* cell line to different medium formulations and culture conditions

R. Bovo · A. L. L. Galesi · S. A. C. Jorge · R. A. M. Piccoli · A. M. Moraes ·
C. A. Pereira · E. F. P. Augusto

Received: 30 November 2007 / Accepted: 29 March 2008 / Published online: 17 April 2008
© Springer Science+Business Media B.V. 2008

Abstract In the past few years, *Drosophila melanogaster* cells have been employed for recombinant protein production purposes, and a comprehensive knowledge of their metabolism is essential for process optimization. In this work, the kinetic response of a Schneider S2 cell line, grown in shake flasks, in two different culture media, the serum-free SF900-II[®] and the serum-supplemented TC-100, was evaluated. Cell growth, amino acids and glucose uptake, and lactate synthesis were measured allowing the calculation of kinetic parameters. The results show that S2 cells metabolism was able to adjust to different environmental situations, as determined by medium formulation, as well as by the particular situation resulting from the culture conditions. Cells attained a 163% higher final cell concentration (1.4×10^7 cells mL⁻¹) in SF900 II[®] medium, when compared

to serum-supplemented TC-100 medium. Also, a maximum specific cell growth rate 52% higher in SF900 II[®] medium, when compared to serum-supplemented TC-100 one, was observed. Glutamine was the growth limiting factor in SF900 II[®] medium, while glucose, sometimes associated with glutamine, controlled growth in serum-supplemented TC-100 medium based formulation. The different pattern of lactate production is an example of the versatility of the metabolism of these cells. This by-product was produced only in glutamine limitation, but the amount synthesized depended not only on the excess glucose, but on other medium components. Therefore, in serum-supplemented TC-100 medium a much smaller lactate amount was generated. Besides, glucose was identified not only as a growth limiting factor, but also as a viability limiting factor, since its depletion accelerated cell death.

R. Bovo · S. A. C. Jorge · C. A. Pereira
Laboratório de Imunologia Viral, Instituto Butantan, Av.
Vital Brasil, 1500, 05503-900 São Paulo, SP, Brazil

A. L. L. Galesi · A. M. Moraes
Departamento de Processos Biotecnológicos, Faculdade
de Engenharia Química, Universidade Estadual de
Campinas, P.O. Box 6066, 13083-970 Campinas, SP,
Brazil

R. A. M. Piccoli · E. F. P. Augusto (✉)
Laboratório de Biotecnologia Industrial, Instituto de
Pesquisas Tecnológicas—IPT, Av. Prof. Almeida Prado,
532, Prédio 31, sala 107, 05508-901 São Paulo, SP, Brazil
e-mail: eaugusto@ipt.br

Keywords S2 cells · Metabolism · Glucose ·
Glutamine · Lactate · Alanine · Kinetic parameters ·
SF900 II[®] · TC-100

Introduction

Insect cells has been successfully employed for the expression of heterologous proteins of industrial interest showing advantages such as ease of culture, higher tolerance to osmolality, product concentration, and expression levels, when compared to mammalian systems (Ikonomu et al. 2003). The baculovirus

expression vector system (BEVS) is already widely used for large-scales applications. Specialized media, transfection reagents, and vectors have been developed in response to recent advances in insect cell culture and molecular biology methods. Nevertheless, in BEVS the infection step necessary for protein expression does not allow a continuous bioprocess system; also, proteolysis is a problem due to the BEVS lytic nature and can affect the quality and quantity of product; and finally, recombinant baculovirus may lose infectivity with increasing number of passages (Santos et al. 2007; McCarroll and King 1997).

Attempts to overcome some of these problems have led to the development of a stable, plasmid-based expression system capable of continuous protein production in *Spodoptera frugiperda* and *Drosophila melanogaster* cells (McCarroll and King 1997). Among them, the *Drosophila* expression system has been the most successful in achieving high level production of a number of recombinant proteins: antibodies (Kirkpatrick et al. 1995), receptors (Perret et al. 2003; Torfs et al. 2000; Millar et al. 1995; Tota et al. 1995; Vanden Broeck et al. 1995), enzymes (Banks et al. 2003; Gibson et al. 1993), inhibitors (Jeon et al. 2003; Valle et al. 2001), growth factor (Lee et al. 2000) and viral antigen (Yokomizo et al. 2007; Bachmann et al. 2004; Deml et al. 1999). This is largely due to the use of efficient promoters coupled to the ability to generate high copy numbers of the introduced plasmids.

Nevertheless, little information is available on propagation or production processes using *Drosophila* Schneider 2 (S2) cells (Galesi et al. 2007; Swiech et al. 2007; Valle et al. 2001; Park et al. 1999; Sondergaard 1996). The metabolism of S2 is scarcely discussed in those works and in most cases comparison is made with other insect cell lines, especially *S. frugiperda* 9 (Sf9) cells.

Nutrient utilization and by-product formation in cultured insect cells with potential industrial application have been investigated by several groups (Drews et al. 1995, 2000; Ferrance et al. 1993; Öhman et al. 1995; Rhiel et al. 1997). Some of these cell lines presented metabolic patterns very similar to those found in mammalian cell culture, synthesizing lactate, alanine and ammonia in response to excess of substrate (Rhiel et al. 1997). Sf9 cells are, on the other hand, distinguished by a lack of lactate and

ammonia production in conditions of glucose and glutamine excess. Nonetheless, Sf9 cell lines produce significant amount of ammonia under glucose limitation (Öhman et al. 1995), confirming that by-product formation pattern, apart from being cell-line specific, can also be influenced by the cultivation conditions. Alanine is a common by-product when there is an excess of glucose in the system (Öhman et al. 1995; Drews et al. 2000). The pathways that determine the transfer of amide-nitrogen and amine-nitrogen from glutamine to alanine was investigated by Drews et al. (2000). These authors also showed the formation of glycerol and ethanol by insect cells, an alternative sink to NADH not transported into the mitochondria by the NADH shuttle model.

The focus of our investigation was primarily to characterize the growth kinetic of a wild *D. melanogaster* S2 cells commonly employed for gene transfection purposes. These cells were cultured in two of the most common media formulation—the serum-free SF900 II[®] and the serum-supplemented TC-100. Besides the kinetic evaluation, the metabolic pattern was analysed to trace the influence of environmental conditions on cell growth and metabolism.

Materials and methods

Cell line and culture media

Drosophila melanogaster Schneider (S2) cell line (Invitrogen) was stored in liquid nitrogen and cultivated in a serum-supplemented TC-100 medium (Galesi et al. 2007), formulated with TC-100 medium (Gibco) and 10% (v/v) fetal bovine serum (FBS; Gibco), and a serum-free SF900 II[®] (Invitrogen) medium.

Inoculum preparation

Cells were adapted to both media in T-flasks (25 cm²) for 5–7 sub-cultures. Inoculum preparation, as well as the experiments performed, were carried out in 100 mL shake flasks (“Schott” bottles with working volume of 20 mL), incubated on a orbital shaker (NBS, USA), at 100 rpm, and 28 °C. Initial cell concentration was adjusted to 5×10^5 cells mL⁻¹.

Conditions of runs

Table 1 summarizes the conditions tested in this work. Cells were cultivated in closed 100 mL shake flasks (working volume of 20 mL), incubated on a orbital shaker, at 100 rpm, and 28 °C. Initial cells concentration was adjusted to $5 - 10 \times 10^5$ cells mL⁻¹. Samples were taken from flasks every 12–24 h; an aliquot was used directly to determine cell concentration and viability and the remaining part was centrifuged at $200 \times g$ for 2 min to separate cells, allowing storage of the supernatants for later analysis.

During the fed batch runs (FB-1 and FB-2, Table 1), 0.7 g L⁻¹ of a glucose solution (Sigma Chemical Co.) was added to the flask when glucose concentration reached 0.5 g L⁻¹.

Analytical methods

Cell concentration (X) and viability were determined through a Neubauer hemocytometer and viability by the trypan blue dye exclusion method in an optical microscope (Freshney 2005).

Glucose (GLC), glutamine (GLN), and lactate (LAC) concentrations were determined enzymatically with an YSI Biochemical Analyser (model 2700) in culture supernatants after sample centrifugation at $200 \times g$ for 5 min.

Other amino acids were determined in culture supernatants, after sample centrifugation at $200 \times g$ for 5 min, by the Pico-tag system, using a reverse-phase HPLC column (Waters).

Table 1 Conditions employed in growth experiments with S2 cells carried out in SF900 II[®] and serum-supplemented TC-100 medium

Run	Meio	X_0 (cells mL ⁻¹)	GLC ₀ ^a (g L ⁻¹)	GLN ₀ ^a (mg L ⁻¹)	Operation mode	
SF-1	SF900 II [®]	10×10^5	10	1,900	Batch	
SF-2		10×10^5				
SF-3		5×10^5				
SF-4		5×10^5				
TC-1	Supplemented TC-100	5×10^5	1	600		
TC-2		5×10^5				
TC-3		10×10^5				
TC-4		5×10^5				
TC-5		5×10^5				
TC-6		5×10^5				4
TC-7		5×10^5				9
FB-1		5×10^5	1		Fed batch	
FB-2		5×10^5	1			

^a Concentrations for SF900 II[®] medium correspond to the values determined in this work. Values for serum-supplemented TC-100 medium correspond to the concentration stated by producer

Determination of kinetic variables and parameters

Maximum specific cell growth rate ($\mu_{X,MAX}$) was determined through the identification of a linear region in the curve $\ln(X_V) = f(t)$ followed by the linear regression of these data. X_V is the viable cell concentration, and t is time.

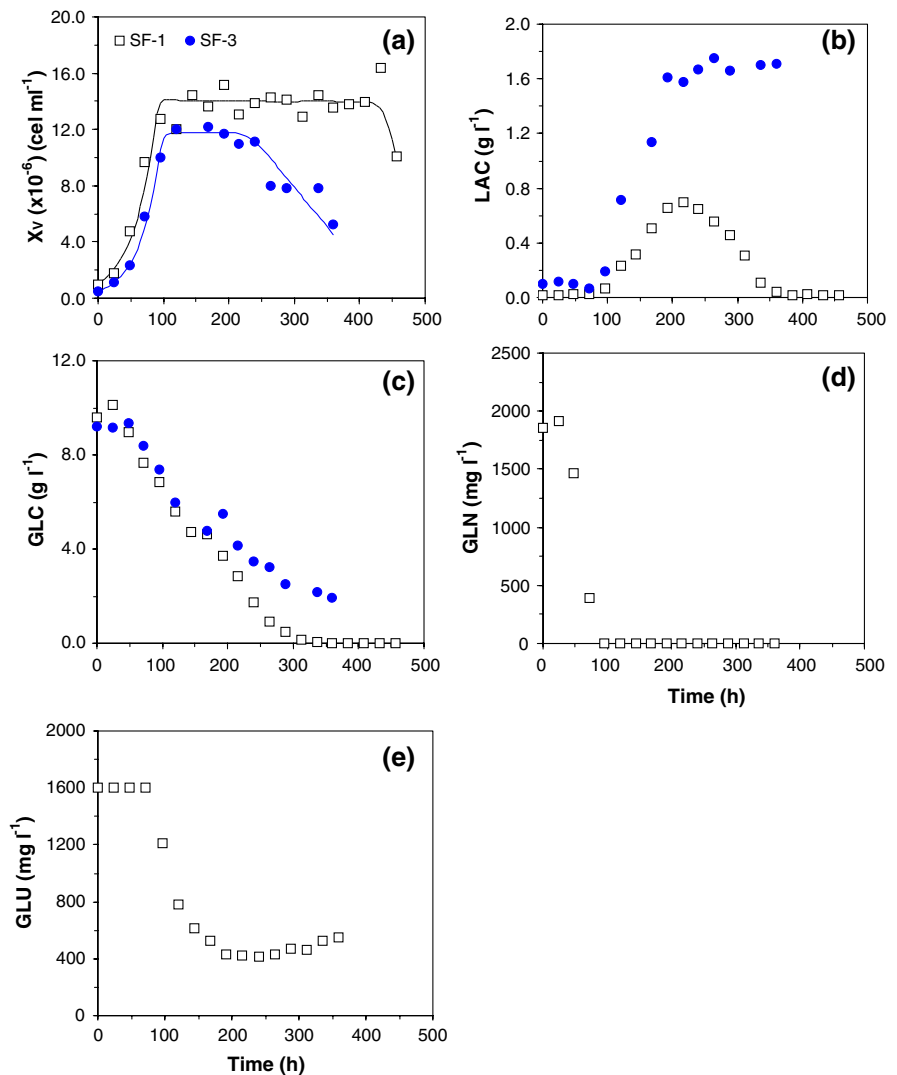
Glucose-to-cell yield coefficient ($Y_{GLC/X}$) was calculated from the linear regression of the curve $X_V = f(GLC)$, at the time interval characterized as the exponential growth phase. GLC indicates the glucose concentration. A similar methodology was applied for glutamine-to-cell yield facto ($Y_{GLN/X}$).

Results

Through the analysis of the following experiments, it was possible to establish the kinetic response of a Drosophila S2 cell line grown on two insect cells medium formulations: the serum-free SF900 II[®] medium and the serum-supplemented TC-100 medium. Distinct metabolic pattern and kinetic parameter values were observed as a function of the initial composition of those media, as well as a result of growth evolution. The study was based on data on growth, substrate consumption (glucose and amino acids), and by-product formation (lactate).

Figure 1 presents examples of typical runs carried out on SF900 II[®] medium, while Table 2 shows, for all experiments carried out on SF900 II[®] medium, the

Fig. 1 S2 cell growth, substrates uptake, and by-products synthesis for runs SF-1 and SF-3, carried out in shake flasks, in batch mode, in SF900 II[®] medium. (a) Cell growth; (b) Lactate production; (c) Glucose uptake; (d) Glutamine uptake; and (e) Glutamate uptake



variables and parameters calculated that will be taken in account to identify possible factors that can promote cell growth, or determine cell death. Thus, the kinetic behaviour of S2 cells was evaluated in three distinct instants of cultivation: at the end of exponential growth phase (indicated by “EXP”); when cells reached the maximum cell concentration (or beginning of stationary phase, indicated by “END”); and when cell concentration started to decrease (indicated by “DEATH”). The extent of the initial lag or adaptation phase (t_{LAG}) was also determined in all experiments shown in Table 1, but no correlation was established between this variable and the run conditions, so it will not be discussed.

Mean values, standard deviation, and coefficient of variance are also presented in Table 2, showing acceptable accuracy for most variables and parameters calculated, except for lactate measurement, what will be discussed later.

A similar set of results is shown in Fig. 2 and Table 3 for experiments carried out on serum-supplemented TC-100 medium.

In Fig. 1 and Table 2, maximum cell concentrations ($X_{V,\text{END}}$) of 1.2×10^7 to 1.61×10^7 cells mL^{-1} are reported after 120–144 h of cultivation. The mean value for $X_{V,\text{END}}$ observed on SF900 II[®] medium (Table 2) is statistically higher than the values listed in Table 3 for serum-supplemented TC100 medium (t -test for $\alpha = 0.05$). Besides this higher cell

Table 2 Kinetic variables and parameters for S2 cell growth runs in SF900 II® medium, considering three distinct instants of cultivation: the end of exponential phase (EXP); maximum cell concentration (END); cell concentration decreasing (DEATH)

Run	End of exponential phase						$\mu_{X,MAX}$ (h ⁻¹)	$Y_{X/GLC}$ (cells g ⁻¹)	$Y_{X/GLN}$ (cells mg ⁻¹)
	t_{LAG} (h)	t_{EXP}^a (h)	GLC _{EXP} (g L ⁻¹)	GLN _{EXP} (mg L ⁻¹)	LAC _{EXP} (g L ⁻¹)				
SF-1	0.0	96.0	6.84	0.0	0.070	0.0283	3.69E + 09	5.76E + 06	
SF-2	0.0	96.0	6.78	0.0	0.035	0.0298	4.37E + 09	5.30E + 06	
SF-3	0.0	120	6.21	–	0.263	0.0311	3.29E + 09	–	
SF-4	0.0	96.0	7.37	–	0.190	0.0318	4.63E + 09	–	
Mean	0.0	108	6.80	0.0	0.140	0.0303	3.99E + 09	5.53E + 06	
Standard deviation	0.0	17.0	0.474	0.0	0.106	0.00155	6.1E + 08	3.2E + 05	
CV (%)	–	15.7	6.98	–	75.8	5.1	15.4	5.86	

Run	Maximum cell growth						Beginning of death phase			
	t_{END}^a (h)	GLC _{END} (g L ⁻¹)	GLN _{END} (mg L ⁻¹)	LAC _{END} (g L ⁻¹)	$X_{V,END}$ (cells mL ⁻¹)	$P_{X,END}^a$ (cells mL ⁻¹ h ⁻¹)	t_{DEATH} (h)	GLC _{DEATH} (g L ⁻¹)	GLN _{DEATH} (mg L ⁻¹)	LAC _{DEATH} (g L ⁻¹)
SF-1	144	4.70	0.0	0.313	1.45E + 07	9.27E + 04	408	0.0	0.0	0.0
SF-2	144	4.11	0.0	0.194	1.61E + 07	1.04E + 05	336	0.0	0.0	0.0
SF-3	120	6.21	–	0.263	1.42E + 07	1.18E + 05	264	1.53	0.0	1.51
SF-4	120	5.97	–	0.710	1.20E + 07	1.00E + 05	216	4.10	0.0	1.58
Mean	120	5.25	0.0	0.370	1.42E + 07	1.09E + 05	240	–	0.0	–
Standard deviation	0.0	1.01	0.0	0.232	1.67E + 06	1.30E + 04	33.9	–	0.0	–
CV (%)	0.0	19.2	–	62.7	11.8	11.9	14.1	–	–	–

^a Mean and standard deviation consider only experiments carried out with initial cell concentration of 5.0×10^5 cells mL⁻¹

production, experiments carried out in SF900 II® medium also presents significant improvement in maximum specific growth rate ($\mu_{X,MAX}$) in relation to serum-supplemented TC-100 medium (Tables 2 and 3). The individual calculations for $\mu_{X,MAX}$ also exhibit good accuracy with exponential linear regression showing coefficients of correlation always superior to 0.94. According to this data, maximum specific growth rate is 52% higher in SF900 II® medium— 0.0303 h^{-1} in SF900 II® medium and 0.0199 h^{-1} in serum-supplemented TC-100 medium. These results corroborate the common sense idea that SF900 II® medium is one of the richest formulations for insect cell growth.

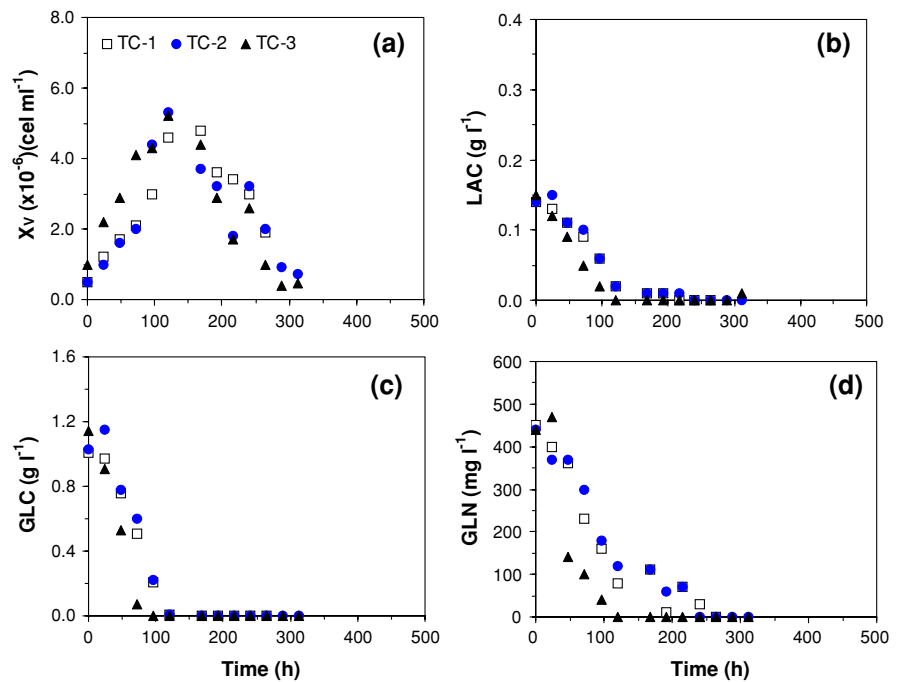
As previously shown, kinetic data of S2 cell line are not common in literature. Nevertheless, different authors present similar data for recombinant S2 cell lines. Swiech et al. (2007) and Galesi et al. (2007), both growing a recombinant S2 cell line transfected with rabies virus glycoprotein gene, achieved values of 0.027 and 0.037 h^{-1} for maximum specific growth rate

of cells cultivated on a serum-free medium based on TC-100 and on SF900 II® media, respectively.

Glutamine (GLN) and glucose (GLC) are the main substrates consumed to provide energy and carbon source for cell growth (Doverskog et al 1997). In the experiments reported here, both nutrients are consumed to promote cell growth, up to complete depletion in some cases (Figs. 1c, d and 2c, d). The initial substrates concentrations (GLC and GLN), as well as the ratio between the substrates are different in SF900 II® and serum-supplemented TC-100 media. Therefore, cell response varied not only within the same formula but also for the different media studied.

GLN was totally consumed after 96 h of cultivation on SF900 II® medium (Fig. 1d), determining the end of the exponential growth phase. In Table 2, it is observed that GLN was also a limiting factor on run SF-2 (GLN_{EXP} equal to zero). Although, data on GLN for runs SF-3 and SF-4 are not available, it is

Fig. 2 S2 cell growth, substrates uptake, and by-product synthesis for runs TC-1 to TC-3, carried out in, shake flasks, in batch mode, with serum-supplemented TC-100 medium. (a) Cell growth; (b) Lactate production; (c) Glucose uptake; and (d) Glutamine uptake



possible to infer that the same limiting condition applied also to these experiments.

Glutamate (GLU) is also consumed by S2 cells but only after GLN depletion ($t \sim 96$ h) and only partially as shown in Fig. 1e. Apparently, after a period of constant concentration (~ 260 h), GLU showed a slight increase, indicating a synthesis of these amino acid.

With a GLN to GLC relationship of 5:1 in SF900 II[®] medium, GLC is in excess at the end of the exponential phase and at the stationary phase (Fig. 1c; Table 2—GLC_{EXP} and GLC_{END}). A different ratio between GLN and GLC for TC-100 medium formulation (1:2) determined the complete depletion of glucose earlier or at the same time of glutamine (Fig. 2c, d). This can also be confirmed by values attributed to GLC and GLN in Table 3, at the end of exponential phase (“EXP”) or at the end of cell growth (stationary phase; “END”).

Significant lactate synthesis (LAC) was observed only in cultures with SF900 II[®] medium (Figs. 1b and 2b). Lactate is a common by-product of GLC anaerobic metabolism reported for many different cell lines and the amount produced is normally dependent on the excess of GLC (Doverskog et al. 1997). In our experiments, although GLC was in excess from the beginning of cultures, LAC synthesis only occurred after complete depletion of GLN, i.e., after the end of exponential growth phase (Fig. 1b, d).

At least in runs SF-1 (Fig. 1b) and SF-2 (not shown), lactate uptake was observed at the end of experiment, when glucose concentration was already low ($1\text{--}2\ g\ L^{-1}$). Nevertheless, in run SF-3 (Fig. 1b) and SF-4 (not shown) LAC accumulated up to approximately $1.5\ g\ L^{-1}$ and was not consumed by cells.

In contrast, no significant lactate production was observed in experiments carried out with serum-supplemented TC-100 medium. Nevertheless, LAC present at the initial phase of growth, probably derived from serum, was consumed during exponential phase (Mendonça et al. 1999). Since all GLC was also taken up during the exponential phase, no further by-product could be synthesised after that moment.

Finally, Figs. 1 and 2 also present a reduction in viable cells that can be attributed to different factors depending on culture media utilised and run performance. Glucose depletion is one of the hypotheses to explain cell death. This could be observed in run SF-1 (Fig. 1a, c) and SF-2 (not shown), with SF900 II[®] medium, as well in all experiments carried out with serum-supplemented TC-100 medium (Fig. 2a, c). Another possible explanation for the reduction of viable cells is an inhibitory effect caused by the high amount of LAC observed on runs SF-3 (Fig. 1a, c) and SF-4 (not shown).

In order to clarify the importance of GLC on S2 cells viability, a set of experiments with higher

Table 3 Kinetic variables and parameters for S2 cell growth runs in serum-supplemented TC-100 medium, in batch mode, considering three distinct instants of cultivation: the end of

exponential phase (EXP); maximum cell concentration (END); cell concentration decreasing (DEATH)

Run	End of exponential phase							
	t_{LAG} (h)	t_{EXP}^a (h)	GLC _{EXP} (g L ⁻¹)	GLN _{EXP} (mg L ⁻¹)	LAC _{EXP} (g L ⁻¹)	$\mu_{X,MAX}$ (h ⁻¹)	$Y_{X/GLC}$ (cells g ⁻¹)	$Y_{X/GLN}$ (cells mg ⁻¹)
TC-1	24.0	120	0.010	80.0	0.020	0.0167	3.26E + 09	9.41E + 06
TC-2	0.0	96.0	0.220	180	0.060	0.0205	30.87E + 09	1.07E + 07
TC-3	0.0	72.0	0.070	100	0.0	0.0188	2.99E + 09	8.67E + 06
TC-4	0.0	120	0.0	–	0.0	0.0192	3.19E + 09	–
TC-5	24.0	120	0.0	–	0.038	0.0244	4.75E + 09	–
Mean	10	114	0.060	120	0.024	0.0199	3.61E + 09	9.60E + 06
Standard deviation	13.1	12.0	0.094	52.9	0.026	0.0028	7.17E + 08	1.04E + 06
CV (%)	137	10.5	157	44.1	109	14.2	19.9	10.8

Run	Maximum cell growth						Beginning of death phase			
	t_{END}^a (h)	GLC _{END} (g L ⁻¹)	GLN _{END} (mg L ⁻¹)	LAC _{END} (g L ⁻¹)	$X_{V,END}$ (cells mL ⁻¹)	$P_{X,END}^a$ (cells mL ⁻¹ h ⁻¹)	t_{DEATH} (h)	GLC _{DEATH} (g L ⁻¹)	GLN _{DEATH} (mg L ⁻¹)	LAC _{DEATH} (g L ⁻¹)
TC-1	168	0.0	20.0	0.010	5.30E + 06	2.86E + 04	168	0.0	20.0	0.010
TC-2	120	0.010	120	0.020	5.30E + 06	4.42E + 04	120	0.01	120	0.020
TC-3	120	0.0	0.0	0.050	5.20E + 06	4.33E + 04	120	0.0	0.0	0.050
TC-4	144	0.0	–	0.0	4.70E + 06	2.92E + 04	144	0.0	–	0.0
TC-5	168	0.0	–	0.008	6.40E + 06	3.51E + 04	168	0.0	–	0.008
Mean	150	0.002	46.7	0.018	5.38E + 06	3.43E + 04	144	0.002	46.7	0.018
Standard deviation	23.0	0.004	64.3	0.019	6.22E + 05	7.24E + 03	24.0	0.004	64.3	0.019
CV (%)	15.3	224	138	111	11.6	21.1	16.7	224	138	111

^a Mean and standard deviation consider only experiments carried out with initial cell concentration of 5.0×10^5 cells mL⁻¹

amounts of initial glucose concentration (Fig. 3 and Table 4) or employing a feed strategy to ensure GLC concentration around 1 g L⁻¹ (see Fig. 4 and Table 5) were carried out.

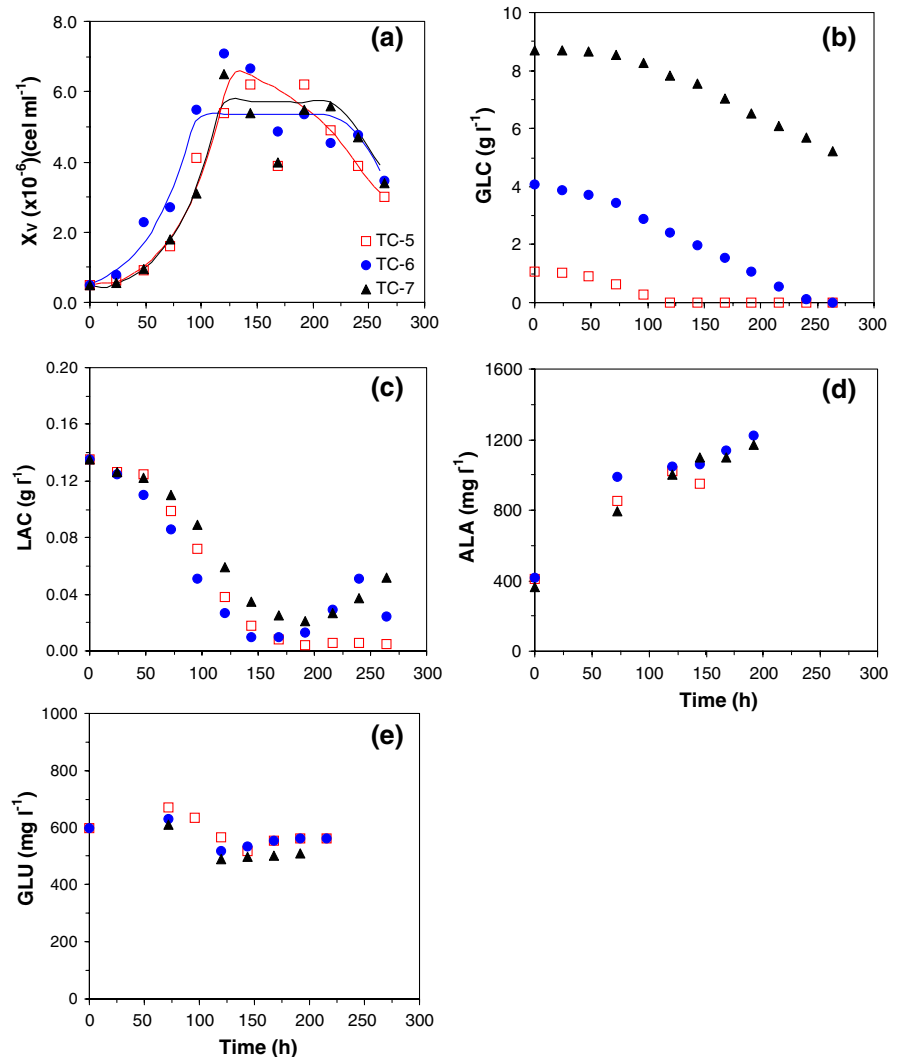
The surplus of GLC at the beginning of runs TC-6 and TC-7 (Fig. 3b) resulted in long-lasting stationary growth phases, 70–100 h (Fig. 3a), when compared to the data obtained with supplemented TC-100 medium formulation (Fig. 2a). Data on GLN was not available for those experiments, but as the initial GLN concentration in these runs was the same as in other experiments with supplemented TC-100 medium (Table 1), a GLN limitation was expected at the end of exponential phase, with maximum cell concentration around 5.4×10^6 cells mL⁻¹ (Table 3). Also confirming our previous observations, GLU

was consumed only after the limitation on GLN (Fig. 3e).

No other amino acid was completely consumed during these experiments (Fig. 5), barely constituting further limiting factors of the process. Alanine (ALA) was produced during the whole experiment, even after cell culture reached the stationary phase, i.e., after GLN depletion, usually considered as the main amino acid for alanine synthesis (Drews et al. 2000). It indicates that alanine can be the main destination of the exceeding pyruvic acid generated from GLC uptake through the glycolytic pathway (Ikonou et al. 2003).

In GLC-supplemented experiments, lactate was again produced and consumed (Fig. 3c, run TC-6 at ~250 h), a pattern very similar to the one previously

Fig. 3 Influence of glucose concentration on S2 cell growth and death, substrates uptake, and by-product synthesis for runs TC-5 to TC-7 carried out in serum-supplemented TC-100 medium. (a) Cell growth; (b) Glucose uptake; (c) Lactate production; (d) Alanine production; and (e) Glutamate uptake



observed with SF900 II[®] medium (Fig. 1b). The fact that GLC was in excess in media was essential for the synthesis of LAC, nevertheless the amount of LAC produced was much lower than in cultures with SF900 II[®] medium, where similar initial GLC concentration was present.

A comparison between parameters obtained in experiments with and without GLC supplementation indicates no significant difference (t -test, $\alpha = 0.05$) for maximum cell concentration (X_{END}), cell productivity ($P_{X,END}$), and glucose-to-cell yield coefficient ($Y_{X/GLC}$). The only exception is the maximum specific cell growth rate ($\mu_{X,MAX}$) that shows an increase of approximately 28%.

In runs operated in fed batch mode, GLC was maintained at an average of $1 g L^{-1}$ (Fig. 4c), and a

stationary phase of 100–120 h was demonstrated (Fig. 4a). When compared to supplemented batch experiments (TC-5, TC-6, and TC-7), this fed batch runs showed lower lactate and alanine concentrations, probably as a consequence of lower glucose availability (Figs. 3b and 4c).

Discussion

The data presented above demonstrate that the *D. melanogaster* S2 cells used in this study could adjust its metabolism according to their environment, this variability being determined either by the specific medium formulation or through the culture condition of a given experiment.

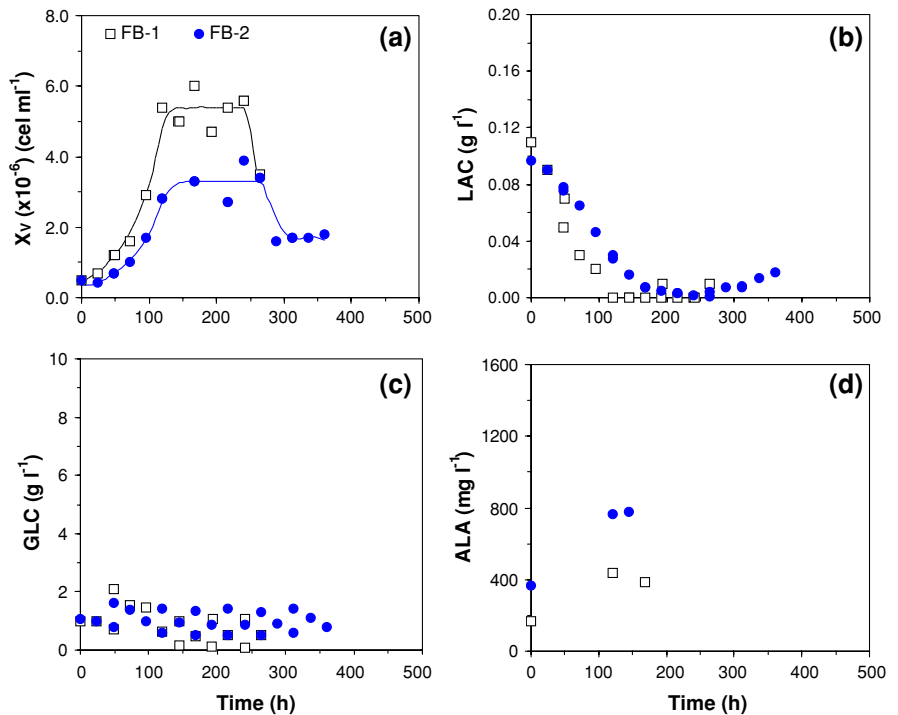
Table 4 Kinetic variables and parameters for S2 cell growth runs in serum-supplemented TC-100 medium, in batch mode, with different initial GLC, considering three distinct instants of

cultivation: the end of exponential phase (EXP); maximum cell concentration (END); cell concentration decreasing (DEATH)

Run	t_{LAG} (h)	End of exponential phase				$\mu_{X,MAX}$ (h ⁻¹)	$Y_{X/GLC}$ (cells g ⁻¹)
		t_{EXP} (h)	GLC _{EXP} (g L ⁻¹)	LAC _{EXP} (g L ⁻¹)			
TC-6	24.0	120	7.84	0.059	0.0257	5.35E + 09	
TC-7	0.0	96.0	2.88	0.051	0.0251	4.14E + 09	
Mean	12.0	108	5.36	0.055	0.0254	4.74E + 09	
Standard deviation	17.0	17.0	3.51	5.66E-03	4.26E-04	8.59E + 08	
CV (%)	141	15.7	65.4	10.3	1.68	18.1	

Run	Maximum cell growth					Beginning of death phase		
	t_{END} (h)	GLC _{END} (g L ⁻¹)	LAC _{END} (g L ⁻¹)	$X_{V,END}$ (cells mL ⁻¹)	$P_{X,END}$ (cells mL ⁻¹ h ⁻¹)	t_{DEATH} (h)	GLC _{DEATH} (g L ⁻¹)	LAC _{DEATH} (g L ⁻¹)
TC-6	144	7.53	0.035	5.50E + 06	3.40E + 04	240	0	0.05
TC-7	96.0	2.88	0.051	5.40E + 06	5.21E + 04	216	6.10	0.03
Mean	120	5.21	0.043	5.45E + 06	4.31E + 04	228	3.05	0.04
Standard deviation	33.9	3.29	0.011	7.07E + 04	1.28E + 04	17.0	4.31	0.01
CV (%)	28.3	63.2	26.3	1.30	29.7	7.44	141	35.4

Fig. 4 S2 cell growth and death, substrates uptake, and by-product synthesis for runs FB-1 and FB-2, in shake flasks, in fed batch mode, with serum-supplemented TC-100. (a) Cell growth; (b) Lactate production; (c) Glucose uptake; and (d) Alanine production



The fact that S2 cells presented different values for maximum specific growth rate ($\mu_{X,MAX}$) in SF900 II[®] and in serum-supplemented TC-100 media (Tables 2

and 3) is indicative of use of different metabolic pathways rather than a surplus of nutrients. A richer medium formulation, as that of SF900 II[®] medium,

Table 5 Kinetic variables and parameters for S2 cell growth runs in serum-supplemented TC-100 medium, in fed batch mode, considering three distinct instants of cultivation: the end

of exponential phase (EXP); maximum cell concentration (END); cell concentration decreasing (DEATH)

Run	End of exponential phase					
	t_{LAG} (h)	t_{EXP} (h)	GLC_{EXP} (g L ⁻¹)	LAC_{EXP} (g L ⁻¹)	$\mu_{X,MAX}$ (h ⁻¹)	$Y_{X/GLC}$ (cells g ⁻¹)
FB-1	0.0	120	0.654	0.000	0.0196	2.46E + 09
FB-2	24.0	120	0.900	0.028	0.0192	2.72E + 09
Mean	12.0	120	0.777	0.014	0.0194	2.59E + 09
Standard deviation	17.0	0.0	0.174	0.020	0.0003	1.83E + 08
CV (%)	141	0.0	22.4	141	1.31	7.07

Run	Maximum cell growth					Beginning of death phase		
	t_{END} (h)	GLC_{END} (g L ⁻¹)	LAC_{END} (g L ⁻¹)	$X_{V,END}$ (cells mL ⁻¹)	$P_{X,END}$ (cells mL ⁻¹ h ⁻¹)	t_{DEATH} (h)	GLC_{DEATH} (g L ⁻¹)	LAC_{DEATH} (g L ⁻¹)
FB-1	145	0.65	0.000	5.20E + 06	3.24E + 04	241	1.00	0.0
FB-2	144	0.90	0.016	3.30E + 06	1.94E + 04	264	0.900	0.0
Mean	145	0.78	0.008	4.25E + 06	2.59E + 04	253	0.950	0.00
Standard deviation	0.85	0.18	0.011	1.34E + 06	9.14E + 03	16.3	0.071	0.00
CV (%)	0.59	22.8	141	31.6	35.3	6.4	7.4	–

would lead to a long-lasting exponential phase, but Tables 2 and 3 showed no significant difference in the extent of exponential phase (t_{EXP}) for the media studied.

The analysis of the exponential phase, particularly at the moment the specific growth rate started to decrease, indicated that the GLN depletion in SF900 II[®] medium (Fig. 1d), and the GLC total consumption, eventually associated with the GLN depletion, in the case of serum-supplemented TC-100 medium formulation (Fig. 2c) determined the growth limitation. Different limiting factors found in those media were a consequence of the balance between initial concentrations of GLN/GLC in the formulations: 1:5 in SF900 II[®] medium and 1:2.5 in serum-supplemented TC-100 medium formulation.

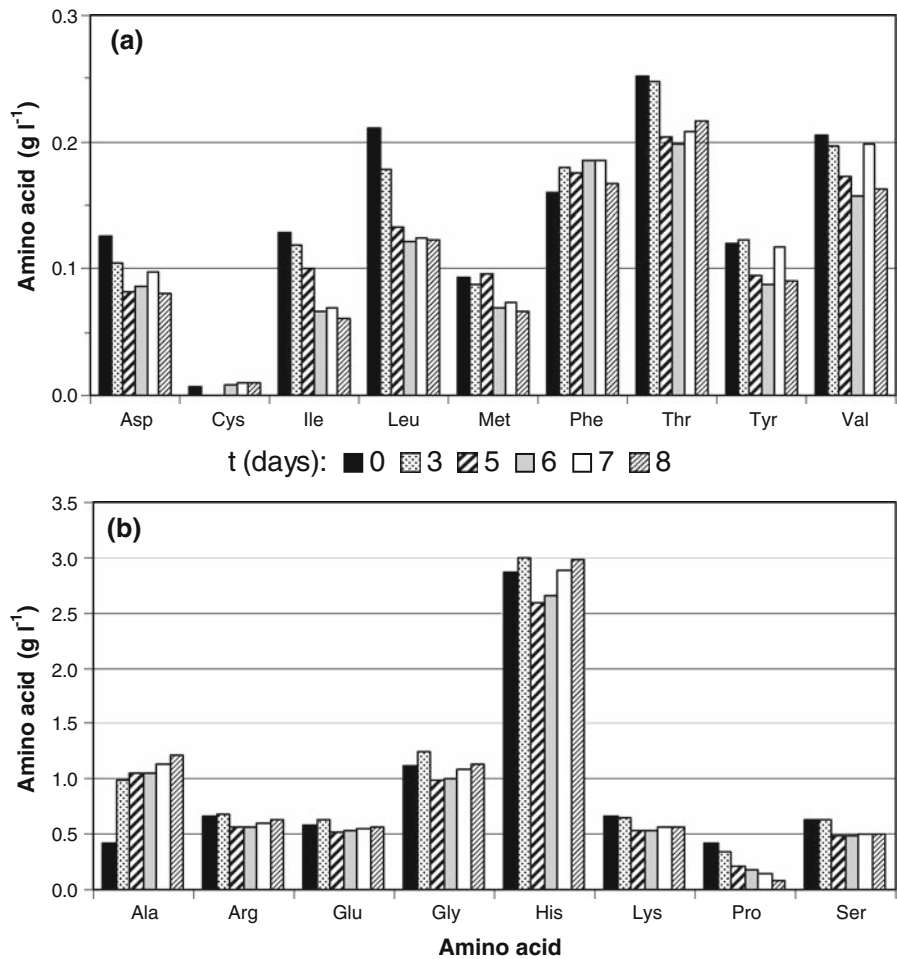
Although there is a correlation between the end of exponential growth phase and the depletion of GLN and/or GLC, the possibility of a double limiting condition must be considered. Swiech et al. (2007), studying a recombinant S2 in SF900 II[®] medium, indicated that proline was the main limiting substrate. Even though cells are not exactly the same in our work and in Swiech et al (2007), the metabolic machinery is likely to be similar, so a double limiting substrate is

indeed possible. Oxygen is another possible limiting substrate, since volumetric oxygen transfer coefficient ($K_L a$) in shake flasks is low particularly with the procedure adopted in our experiments that allowed gas exchange only during sampling, once or twice a day. As Swiech et al. (2007) discussed, S2 cells are remarkably well adapted to hypoxia, maintaining viability for long periods, but this characteristic does not avoid a growth limitation by oxygen. Such a limiting condition is much likely to happen in SF900 II[®] medium because cell concentration is higher, and consequently oxygen uptake should be also higher, leading to a lower profile of dissolved oxygen values.

Lactate production in many animal cell lines is a way of regenerate NADH when complete oxidation through the electron transport chain is not possible, as is the case when there is a limitation in oxygen supply or a an excess of GLC that generates a high glycolytic flux (Drews et al. 2000; Doverskog et al. 1997). Considering insect cell lines, the metabolic responses to such situations seems to be characteristically cell-dependent.

In non-limiting oxygen conditions, Sf9 cells are distinguished by the lack of accumulation of lactate even at high substrate concentrations (Drews et al. 2000), showing that these cells possess a fully

Fig. 5 Amino acids uptake for run TC-6, carried out in serum-supplemented TC-100 medium, supplemented with glucose (4 g L^{-1}), operated in batch mode. Different columns indicate the sampling time (days)



functional TCA cycle (Bhatia et al. 1997). On the other hand, *Trichoplusia ni* and BTI-Tn-5B1-4 cells display a mammalian cell-like metabolism, characterized by the accumulation of alanine, lactate, and ammonium ions (Sugiura and Amann 1996; Rhiel et al. 1997). Finally, Galesi et al. (2007) and Swiech et al. (2007) showed that a recombinant S2 cell line did not produce expressive LAC ($0.05\text{--}0.2 \text{ g/L}$) concentration even under oxygen limiting conditions.

This variability of responses is also verified within the experiments shown in this article. Different concentrations of LAC were observed in the media studied (Figs. 1b, 2b, and 3c), as well as within a specific formulation (Fig. 1b), under GLC excess conditions and possibly under oxygen limitation. It seems that a clear correlation between the depletion of GLN and the beginning of lactate production exists. Thus, the LAC appearance in culture media could be the result of S2 cell incapacity to synthesise

ALA, after depletion of GLN, a conditions that that applies more to SF900 II[®] medium cultivation.

Glutamine is the major source of energy, carbon, and nitrogen for animal cells (Ikonomou et al. 2003; Mendonça et al. 1999). It plays two important functions: acts as an energy donor and plays a critical role in nitrogen metabolism, acting as a collection point for amino groups. According to Drews et al. (1995) the majority of other amino acids (histidine, lysine, threonine, glycine, valine, leucine, isoleucine, phenylalanine, tyrosine, tryptophane) are incorporated in cellular protein, but are not significant for energy production purposes. The low consumption of those amino acids by S2 cells as shown in this study (Fig. 5), as well as in Swiech et al. (2008), is in accordance to the previous observation.

Concerning ALA production, Drews et al. (2000) demonstrated a lower production of this amino acid in glutamine-free medium, glutamate replacing

glutamine as the major nitrogen-donor for ALA synthesis. The results shown in Fig. 3d indicated that ALA synthesis decreased after GLN depletion (possibly around 80 h), probably using other amino acids as ammonia donor. For S2 cell cultures in our study, it is not clear if GLU played this role, since it started to be consumed when GLN was limited, but did not have the same profile of ALA synthesis (Fig. 3d, e). Also, an increase in GLU is observed at the end of cultivation, while ALA is still being produced. Thus, another amino acid must be the responsible for ALA generation. As shown in Fig. 5, proline is the amino acid most consumed after GLN depletion and could play this role.

The apparently lower capacity of S2 cells to synthesise ALA after GLN depletion, led to a higher LAC accumulation in SF900 II[®] medium, up to deleterious concentrations (Fig. 1b). It occurred only in runs SF-3 and SF-4 (not shown), as a consequence of cultivation conditions. In comparison to experiments SF-1 and SF-2, runs SF-3 and SF-4 showed a high GLC at the end of exponential phase, when cells started to synthesise lactate. As presented before, cells produced lactate until GLC became limiting ($1\text{--}2\text{ g L}^{-1}$) in cultivation, then LAC is consumed. As runs SF-3 and SF-4 had a relatively higher GLC availability, higher amounts of LAC were produced up to 1.5 g L^{-1} . As a consequence, viable cells started to decrease and LAC synthesis was interrupted and no consumption of this by-product was observed in this condition.

Although different amounts of LAC were observed during our experiments, it is important to indicate that the S2 cell line did not alter its metabolism in GLC excess. The values for glucose-to-cells coefficient ($Y_{X/GLC}$) obtained for both media are similar (Tables 2–4). On the other hand, it was observed that the excess of GLN induced a less efficient metabolism, measured by the parameter $Y_{X/GLN}$.

Ammonia (NH_3) was not determined in our experiments, but data on a derived recombinant S2 cell line indicate that the amount of NH_3 synthesise, and its deleterious effects also depend on the culture medium employed (Swiech et al. 2008; Galesi et al. 2008). For SF900 II[®] (Swiech et al. 2008), it could be observed concentrations up to 340 mg L^{-1} at the end of exponential phase, without any indication of cell growth inhibition. Nevertheless, for the TC-100-based formulation (Galesi et al. 2008), a cell growth

inhibition was attributed to the $50\text{--}60\text{ mg L}^{-1}$ of NH_4^+ accumulated in culture. As the initial GLN in our experiment was six times smaller than in Galesi et al. (2008), a much lower NH_3 is expected, and possibly no growth inhibition could be attributed to this by-product.

Finally, this study demonstrated that GLN is an essential substrate for S2 cell growth, since its depletion limited the cell growth in all the experiments, while the GLC level, besides interrupting cell growth, is also responsible for maintenance of cell viability. Lactate synthesis is determined by GLN depletion and excess of GLC, but the amount release in culture depends on medium formulation and on experiment conditions. Its synthesis is lower in serum-supplemented TC-100 medium, possibly because in this case, alanine can be produced from other amino acids rather than GLN. Also, an excess of LAC accumulated in culture can lead to cell death.

Acknowledgments This work was supported in part by grants from FAPESP, CNPq, and Fundação Butantan. A.M. Moraes and C. A. Pereira are the recipients of a CNPq research fellowship.

References

- Bachmann AS, Corpuz G, Hareld WP et al (2004) A simple method for the rapid purification of copia virus-like particles from *Drosophila* Schneider 2 cells. *J Virol Methods* 115:159–165
- Banks DJ, Hua G, Adang M (2003) Cloning of a *Heliothis virescens* 110 kda aminopeptidase N and expression in *Drosophila* S2 cells. *Insect Biochem Mol Biol* 33:499–508
- Bhatia R, Jesionowski G, Ferrance J et al (1997) Insect cell physiology. *Cytotechnology* 24:1–9
- Deml L, Wolf H, Wagner R (1999) High level expression of hepatitis B virus surface antigen in stably transfected *Drosophila* Schneider-2 cells. *J Virol Methods* 79:191–203
- Doverskog M, Ljunggren J, Öhman L et al (1997) Physiology of cultured animal cells. *J Biotechnol* 59:103–115
- Drews M, Paalme T, Vilu R (1995) The growth and nutrient utilization of insect cell line *Spodoptera frugiperda* Sf9 in batch and continuous culture. *J Biotechnol* 40:187–198
- Drews M, Doverskog M, Öhman L et al (2000) Pathways of glutamine metabolism in *Spodoptera frugiperda* (Sf9) insect cells: evidence for the presence of the nitrogen assimilation system, and a metabolic switch by $^1\text{H}/^{15}\text{N}$ NMR. *J Biotechnol* 78:23–37
- Ferrance JP, Goel A, Ataai MM (1993) Utilization of glucose and amino acids in insect cell cultures: quantifying the metabolic flows within the primary pathways and medium development. *Biotechnol Bioeng* 42:697–707

- Freshney RI (2005) Culture of animal cells: a manual of basic technique, 5th edn. Wiley-Liss, New York, 642 p
- Galesi ALL, Pereira CA, Moraes AM (2007) Culture of transgenic *Drosophila melanogaster* Schneider S2 cells in serum free media based on TC100 basal media. *Biotechnol J* 2(11):1399–1407
- Galesi ALL, Aguiar MA, Astray RM et al (2008) Growth of recombinant *Drosophila melanogaster* Schneider 2 cells producing rabies virus glycoprotein in bioreactor employing serum-free medium. *Cytotechnology*. doi: [10.1007/s10616-008-9139-y](https://doi.org/10.1007/s10616-008-9139-y)
- Gibson KR, Vanek PG, Kaloss WD et al (1993) Expression of dopamine-beta-hydroxylase in *Drosophila* Schneider 2 cells—Evidence for a mechanism of membrane-binding other than uncleaved signal peptide. *J Biol Chem* 268:9490–9495
- Ikonomou L, Schneider YJ, Agathos SN (2003) Insect cell culture for industrial production of recombinant proteins. *Appl Microbiol Biotechnol* 62:1–20
- Jeon HK, Chang KH, Kim KI et al (2003) Functional expression of recombinant tumstatin in stably transformed *Drosophila melanogaster* S2 cells. *Biotechnol Lett* 25:185–189
- Kirkpatrick RB, Ganguly S, Angelichio M et al (1995) Heavy chain dimers as well as complete antibodies are efficiently formed and secreted from *Drosophila* via a BiP-mediated pathway. *J Biol Chem* 270(34):19800–19805
- Lee JM, Park JH, Park JO et al (2000) Expression of recombinant erythropoietin in stably transformed *Drosophila melanogaster* S2 cells. *In Vitro Cell Dev Biol Anim* 36:348–350
- McCarroll L, King LA (1997) Stable insect cell cultures for recombinant protein production. *Curr Opin Biotechnol* 8:590–594
- Mendonça RZ, Palomares LA, Ramírez OT (1999) An insight into insect cell metabolism through selective nutrient manipulation. *J Biotechnol* 72:61–75
- Millar NS, Baylis HA, Reaper C et al (1995) Functional expression of a cloned *Drosophila* muscarinic acetylcholine receptor in a stable *Drosophila* cell line. *J Exp Biol* 198:1843–1850
- Öhman L, Ljunggren J, Häggström L (1995) Induction of a metabolic switch in insect cells by substrate-limited fed-batch. *Appl Microbiol Biotechnol* 43:1006–1013
- Park JH, Lee JM, Chung IS (1999) Production of recombinant endostatin from stably transformed *Drosophila melanogaster* S2 cells. *Biotechnol Lett* 21:729–733
- Perret BG, Wagner R, Lecat S et al (2003) Expression of EGFP-amino-tagged human mu opioid receptor in *Drosophila* Schneider 2 cells: a potential expression system for large-scale production of g-protein coupled receptors. *Protein Expr Purif* 31:123–132
- Rhiel M, Mitchell-Logean CM, Murhammer DW (1997) Comparison of *Trichoplusia ni* BTI-Tn-5B1-4 (High Five TM) and *Spodoptera frugiperda* Sf-9 insect cell line metabolism in suspension cultures. *Biotechnol Bioeng* 55:909–920
- Santos MG, Jorge SAC, Brillet K et al (2007) Improving heterologous protein expression in transfected *Drosophila* S2 cells as assessed by EGFP expression. *Cytotechnology* 54:15–24
- Sondergaard L (1996) *Drosophila* cells can be grown to high cell densities in bioreactor. *Biotechnol Tech* 10:161–166
- Sugiura T, Amann E (1996) Properties of two insect cell lines useful for the baculovirus expression system in serum-free culture. *Biotechnol Bioeng* 51:494–499
- Swiech K, Silva CL, Arantes MK et al (2007) Characterization of growth and metabolism of *Drosophila melanogaster* cells transfected with the rabies virus glycoprotein gene. *Biotechnol Appl Biochem* 49:41–49
- Swiech K, Rossi N, Silva BG et al (2008) Bioreactor culture of recombinant *Drosophila melanogaster* S2 cells: characterization of metabolic features related to cell growth and production of the rabies virus glycoprotein. *Cytotechnology*. doi:[10.1007/s10616-008-9130-7](https://doi.org/10.1007/s10616-008-9130-7)
- Torfs H, Shariatmadari R, Guerrero F et al (2000) Characterization of a receptor for insect tachykinin-like peptide agonists by functional expression in a stable *Drosophila* Schneider 2 cell line. *J Neurochem* 74:2182–2189
- Tota MR, Xu L, Sirotina A et al (1995) Interaction of [fluorescein-Trp25] glucagon with the human glucagon receptor expressed in *Drosophila* Schneider-2 cells. *J Biol Chem* 270:26466–26472
- Valle MA, Kester MB, Burns AL et al (2001) Production and purification of human menin from *Drosophila melanogaster* S2 cells using stirred tank reactor. *Cytotechnology* 35:127–135
- Vanden Broeck J, Vulsteke V, Huybrechts R et al (1995) Characterization of a cloned locust tyramine receptor cDNA by functional expression in permanently transformed *Drosophila* S2 cells. *J Neurochem* 64:2387–2395
- Yokomizo AY, Jorge SAC, Astray RM et al (2007) Rabies virus glycoprotein expression in *Drosophila* S2 cells. I. Functional recombinant protein in stable co-transfected cell line. *Biotechnol J* 2:1–8