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The effect of different insect cell culture media on the efficiency of protein production by *Spodoptera frugiperda* cells

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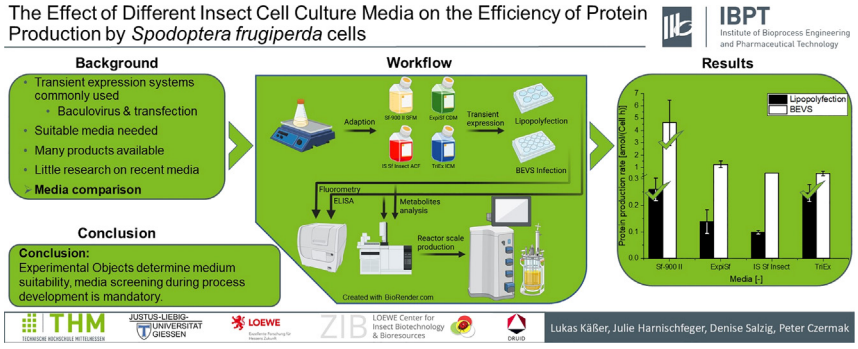
GRAPHICAL ABSTRACT



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ABSTRACT

Background: *Spodoptera frugiperda* (*Sf*-9) cells are often used to produce recombinant proteins by transient expression. The culture medium contributes to the efficiency of protein production, but the effect of modern insect cell culture media on *Sf*-9 cells has not been evaluated for different transient expression systems. Accordingly, we compared the baculovirus expression vector system (BEVS) and lipopolyfection (LP) for the production of schistosomal ALDH and the antimicrobial peptide BR033, respectively. We used *Sf*-9 cells adapted to four commercially available media: TriEx ICM, ExpiSf CDM, Sf-900 II SFM and IS Sf Insect ACF.

Results: During cell maintenance, the highest viable cell density was achieved using IS Sf Insect ACF, but the shortest doubling time was observed in TriEx ICM. All four media supported transient expression with standard protocols for LP and BEVS. We confirmed the typical switch in metabolic rates (glucose, lactate and amino acids) after transfection or infection, comparable to previous reports. The maximum protein production rates were 0.26 ± 0.04 amol/(cell h) for LP and 4.63 ± 1.85 amol/(cell h) for BEVS, both achieved using Sf-900 II SFM.

Conclusions: The experiments demonstrate that the most suitable medium for transient production in *Sf*-9 cells depends on the experimental objectives or production framework. We therefore recommend implementing the screening of different media during process development.

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1. Introduction

Insect cell lines are widely used for the production of gene therapy vectors, peptides, enzymes, and other recombinant proteins, typically by transient expression [1]. The *Spodoptera frugiperda* cell line *Sf*-9 is used routinely for this purpose [2] because it achieves high protein yields and has a greater passaging stability than other insect cell lines [3]. The major expression strategies used with *Sf*-9 cells are the baculovirus expression vector system (BEVS) and transient expression based on commercial transfection agents, although the latter is also used to generate stably transformed cells. Transient expression achieves yields of up to 300 mg/L in ~72 h (BEVS) or 100 mg/L in ~120 h (transfection) for various proteins and peptides [4]. The advantages of insect cells include a short doubling time, low cultivation temperature (27–28°C), high glucose and amino acid tolerance, growth in suspension, and the ability to carry out post-translational modifications [5]. They are suitable for large-scale manufacturing [6,7] and several approved biopharmaceuticals are produced commercially in insect cells, with others manufactured for clinical trials [1]. Two recent examples of recombinant proteins produced in *Sf*-9 cells are NanoFlu [8] and the SARS-CoV-2 vaccine candidate NVX-CoV2373, developed by Novavax.

The productivity of insect cells depends on an optimal production environment. Physical parameters such as temperature (25–29°C), pH (6.2–6.4) and dissolved oxygen (DO) concentration (30–100% air saturation) are important for all cells, but other more specific conditions must be provided by the culture medium [9]. The two major categories of insect cell culture medium are complex medium (CM), which contains various undefined components such as fetal bovine serum (FBS), and chemically defined medium (CDM), with only defined, low-molecular-weight components. Examples of serum-containing media used with *Sf*-9 cells include Grace's insect medium and FBS-supplemented IPL-41, but the biopharmaceutical industry increasingly favors serum-free medium (SFM) because FBS suffers from lot-to-lot variability [10] and carries a risk of contamination with viruses, mycoplasma and prions [11]. SFM still contains undefined components such as tissue extracts and hydrolysates, and is therefore defined as a type of CM [12]. Sf-900 II and Sf-900 III are the most common SFM types used for *Sf*-9 cells. The undefined components in SFM are often derived from animals, but are replaced with microbial, plant or synthetic counterparts in animal-component-free (ACF) media. The composition of CDM reduces the effort required during downstream processing. Examples include ExpiSf CDM for BEVS [13] and the recent TheraPEAK SfAAV medium for adeno-associated virus production in *Sf*-9 cells, both of which are ACF and yeast component free [14].

The culture medium provides essential nutrients (carbohydrates, lipids, amino acids, minerals and vitamins) as well as hormones and growth factors that promote cell growth and protein biosynthesis [15]. Glucose is the main energy source for uninfected *Sf*-9 cells and glucose depletion triggers apoptosis [9]. Glutamine (Gln) is the most important amino acid, under the formation of ammonia it is metabolized to glutamate (Glu), and further to α-ketoglutarate and fed into the tricarboxylic acid (TCA) cycle [16,17]. Similarly, asparagine (Asn) is converted to aspartate (Asp) via asparaginase under the formation of ammonia, and also fed into the TCA cycle [9]. Methionine (Met) and serine (Ser) are key components of insect cell energy metabolism [9]. Asn, Asp, Gln and Glu also influence the growth behavior of insect cells [16]. Growth limitation is also caused by a lack of isoleucine (Ile), leucine (Leu), histidine (His) or tyrosine (Tyr) [18].

The metabolism of insect cells can change during transient expression because resources are diverted toward recombinant protein synthesis. Lipopolyfection (LP) and similar methods [19] involve the direct introduction of plasmid DNA encoding the recombinant protein product [4,20]. In LP, the anionic DNA combines with the cationic lipid/polymer carrier to form complexes [19,21], which are taken up by endocytosis [22]. Protein expression is transient because the plasmid DNA is ultimately degraded [21]. LP is a simple transfection method that does not require viral components [23] and does not damage the cell, thus reducing the risk of product degradation by proteases [24]. The medium composition is important during LP because the DNA/carrier complexes are sensitive to pH, salt, and the presence of serum proteins [25,26,27]. BEVS is used more widely for large-scale expression (up to 2500 L), including products such as virus-like particles and subunits for human and veterinary vaccines [28,29,30]. BEVS is based on the well-characterized *Autographa californica* multiple nucleopolyhedrovirus (*Ac*MNPV), which is modified to carry the sequence of the target protein. Unlike standard plasmids, baculovirus vectors can tolerate the stable insertion of large transgenes encoding proteins of up to 600 kDa [31]. The infection of insect cells is followed by rapid transient protein expression with higher product titers than LP, and the excellent safety profile of the virus allows them to be handled in biosafety level 1 facilities. The host cells are lysed during infection, so careful process monitoring is necessary to optimize the time of harvest (TOH) for the target product [32], which can be predicted by dielectric spectroscopy [33]. The medium composition plays a key role in the product yield because the metabolism of insect cells shifts as soon as they are infected, including a spike in the consumption of glucose and Gln, and an increase in alanine (Ala) synthesis [34].

The optimal medium for recombinant protein production may also depend to some degree on the product, and we therefore evaluated two different candidates with distinct properties and applications: the antimicrobial peptide (AMP) BR033 from *Lucilia sericata* and an aldehyde dehydrogenase (SmALDH_312) from *Schistosoma mansoni*. BR033 is a promising drug candidate in the fight against multidrug-resistant pathogens. It is a member of the cecropin family (which shows broad-spectrum activity against bacteria and fungi) that inhibits the growth of *Escherichia coli*, with a minimal inhibitory concentration of 0.8 μM [35]. SmALDH_312 is a putative drug target, an essential enzyme in *S. mansoni*, the parasite responsible for the neglected tropical disease schistosomiasis [36]. SmALDH_312 is used as a screening target to identify interacting compounds as potential drug leads. The expression of these two recombinant proteins in insect cells provides the basis for characterization, drug discovery and scaled-up manufacturing, and it is therefore important to optimize the cell cultivation medium for each product and expression strategy, maximizing yields and product quality. We therefore compared four different commercially available insect cell culture media (three SFM and one

CDM) by characterizing the *Sf*-9 cells in terms of growth kinetics, nutrient consumption and metabolism. We determined the impact of the transient expression systems by comparing the infected/-transfected cells to controls. We tested the most promising media with BEVS (expressing SmALDH_312 in a bioreactor) and an LP-derived stable polyclonal *Sf*-9 cell line producing a BR033 fusion protein.

2. Materials and methods

2.1. Cultivation of Sf-9 cells

Sf-9 TriEx cells (Merck, Darmstadt, Germany) were thawed as recommended and cultivated in baffled 100-mL Erlenmeyer flasks in TriEx ICM (Merck). The cells were counted using the trypan blue exclusion method and passaged to $0.5*10^6$ cells/mL twice weekly.

2.2. Determination of cell concentration by trypan blue staining and flow cytometry

For trypan blue staining, a sample of *Sf*-9 cells was diluted and 125 μL of the diluted cells was mixed with 75 μL trypan blue. The solution was then pipetted into the Neubauer-improved counting chamber (Marienfeld, Königshofen, Germany) the cells were counted, and the viable cell concentration was calculated according to the manufacturer's instructions. Cell concentration and viability were also assessed by flow cytometry and propidium iodide (PI) staining using the Guava easyCyte 6HT-2L flow cell cytometer (Luminex, Austin, TX, USA). We mixed 20 μL of the diluted cells with 180 μL 0.005 g/L PI solution immediately before analysis.

2.3. Media adaption

Sf-9 TriEx cells were adapted from TriEx Insect Cell Medium (ICM) to ExpiSf CDM, Sf-900 II SFM (both from Thermo Fisher Scientific, Waltham, MA, USA) and IS Sf Insect ACF (FUJIFILM Irvine Scientific, Santa Ana, CA, USA) by passaging them through a mixture of 75% TriEx ICM and 25% of the new medium. The proportion of TriEx ICM was reduced by 25% every time the culture reached >90% viability in the new mixture. After complete adaption, the cells were passaged four times before transfer to freezing medium: 46.25% conditioned medium, 46.25% fresh medium, 7.5% DMSO (Sigma-Aldrich, St. Louis, MO, USA). Cells were stored in the gas phase of a liquid nitrogen freezer.

2.4. Transfection of Sf-9 cells and harvesting

The plasmid DNA used for LP encoded a 60-kDa fusion protein consisting of the fluorescent reporter protein tdTomato with a His_6 tag and the AMP BR033 (peptide sequence: GWLKKIGKKIERVGQHTRDATIQTIGVAQQAANVAATLKG). We seeded 2 mL of exponentially growing *Sf*-9 cells ($1*10^6$ cells/mL) in a flat-bottom 12-well plate approximately 24 h before transfection. The plate was incubated at 28°C and 80 rpm on an Infors Celltron shaker (25-mm throw). Transfection was carried out using Mirus TransIT Insect Transfection Reagent (MITR; Mirus Bio, Madison, WI, USA) according to the manufacturer's instructions. We transferred 2.4 mL sterile Grace's insect SFM to a 5-mL tube and sequentially added 24 μg plasmid DNA (1 μg/μL) and 48 μL MITR with gentle mixing. After incubation for 30 min, we added 206 μL of the mixture dropwise to each well and rocked the plate gently to distribute the lipopolyplexes. The cells were incubated in a Cytation 3 plate reader (BioTek, Winooski, VT, USA) at 28°C with linear continuous shaking. The fusion protein was secreted into the culture medium and protein expression was monitored by fluorimetry every 30 min against a dilution series of tdTomato standards (0–22 mg/L). The fusion protein was harvested by transferring the suspension in each well to a 2-mL Eppendorf tube and centrifuging at 500 × *g* for 5 min at room temperature. The supernatant was transferred to a fresh tube, mixed with 1.5 μM aprotinin and 10 μg/mL trypsin inhibitor, and stored at −80°C.

2.5. Infection of Sf-9 cells and enzyme harvesting

For BEVS production, $TCID_{50}$ was determined by titration using the *Sf*-9 Easy Titer cell line (Kerafast, Boston, Massachusetts, USA) according to the manufacturer's protocol. We subsequently seeded 2 mL of exponentially growing *Sf*-9 cells ($1*10^6$ cells/mL) in a flat-bottom suspension six-well plate and infected them at a multiplicity of infection (MOI) of 0.01 with recombinant baculoviruses encoding the 56 kDa protein SmALDH_312 (GeneID: Smp_050390 and Smp_312440). After taking a zero sample to measure cell viability and density, the plate was wrapped in aluminum foil and incubated at 28°C, shaking at 80 rpm. To control the infection, cell viability was monitored by regular sampling and analysis by flow cytometry following 1:10 sample dilution in 0.005 g/L PI. SmALDH312 was secreted into the supernatant and recovered at a cell viability of ∼80%. The suspensions were removed from the wells, transferred to sterile 2-mL centrifuge tubes, and centrifuged at 250 × *g* for 10 min at room temperature. The supernatants were transferred to fresh tubes and centrifuged at 3000 × *g* for 10 min at room temperature. The final supernatants were transferred to light-proof 2-mL tubes, mixed with 1.5 μM aprotinin and 10 μg/mL trypsin inhibitor, and stored at −80°C.

2.6. Amino acid analysis

We analyzed 100-μL samples 24 h post-transfection/infection and at the end of the experiments, with samples of the four different media as controls. Samples were prepared using the EZ:faast gas chromatography kit (Phenomenex, Torrance, CA, USA) and analyzed on a 7890B gas chromatograph system (Agilent Technologies, Santa Clara, CA, USA) using OpenLab ChemStation C.01.08 [244] software. L-alanyl-L-glutamine was analyzed in a Cedex Bio Analyzer with the *Ala-Gln Assay* in accordance with the manufacturer's protocol (Roche Diagnostics, Mannheim, Germany).

2.7. Protein analytics

2.7.1. Fluorimetry

BR033 was tagged with tdTomato, and was detected by placing the 12-well plate in a Cytation 3 plate reader at 28°C and reading the relative fluorescence (excitation 554 nm, emission 581 nm) every 30 min. Expression levels were plotted against standard curves of tdTomato in all four media to calculate the fusion protein content from the fluorescence signal.

2.7.2. His-ELISA

BR033 and SmALDH_312 carry an N-terminal His_6 tag, which was detected by ELISA to determine the protein concentration. We used 0.4% (w/v) BSA in PBS as a blank and for the preparation of 10-fold serial dilutions of BR029 (0.01–1.0 mg/L). The detection antibody was *Penta·His HRP Conjugate* (Qiagen, Venlo, Netherlands) used at a dilution of 1:10,000. His-Tag antibody plates (GenScript, Piscataway, NJ, USA) were brought to room temperature before adding 100 μL of each sample, standard and blank. After incubation for 2 h at room temperature, shaking at 300 rpm, the plate was washed four times with 0.05% Tween-20 in PBS (PBST). We then added 100 μL of the prepared antibody solution, followed by incubation and washing as above. Finally, we added Ultra TMB substrate (Thermo Fisher Scientific), incubated the plates for 20 min

as above, and stopped the reaction by adding 50 μL 0.5 M sulfuric acid per well. The absorbance at 450 nm was determined in the Cytation 3 plate reader.

2.7.3. Bioreactor preparation

The bioreactor setup for *Sf*-9 cells was similar to the setup we used for the cultivation of Schneider 2 (S2) cells [33]. However, the *Sf*-9 cells were cultivated at 28°C and at pH 6.3. For BEVS the bioreactor was inoculated with $1*10^6$ cells/mL, whereas BR033 production was initiated with $0.5*10^6$ cells/mL. Infection was started immediately after inoculation with a MOI of 1. We used a constitutive promoter so the stable cell line did not require induction for the production phase.

3. Results

3.1. Biochemical profiling of the four insect cell media

We compared the concentrations of amino acids, glucose and lactate in the four insect cell media as the basis for subsequent comparisons during transient expression experiments (Table 1). The glucose content ranged from ~8 g/L (IS Sf Insect ACF) to ~15 g/L (ExpiSf CDM) whereas the Gln content was more diverse, ranging from undetectable (TriEx ICM) to 12.17 ± 1.62 μmol/mL (Sf-900 II SFM). We considered it unlikely that TriEx ICM would contain no Gln at all, so we also tested for L-alanyl-L-glutamine and detected a concentration of 10 ± 0.02 μmol/mL. Notably, IS Sf Insect ACF did not contain any Asn, and the Asn content of TriEx ICM was 1.4-fold lower than Sf-900 II SFM and 2.2-fold lower than ExpiSf CDM. We detected no striking differences in the content of other key amino acids, such as Asp, Ile, Leu, His and Tyr (Table 1).

3.2. Growth of Sf-9 cells in the four different insect cell media

Following the adaptation of *Sf*-9 TriEx cells from TriEx ICM to Sf-900 II SFM, ExpiSf CDM and IS Sf Insect ACF, we analyzed the cell concentration, cell viability, and the glucose and lactate concentration in the medium of *Sf*-9 TriEx cells cultivated in shake flasks. The growth phases were identified for all four media, starting with a lag phase of 24–48 h after inoculation (Fig. 1).

This was followed by medium-dependent exponential growth, with high cell viabilities (>80%). The cells cultivated in ExpiSf CDM showed a prolonged viability of ≥80% even during the stationary phase, whereas viability dropped significantly in the other three media. In IS Sf Insect ACF but not the other media, the end of the exponential growth phase coincided with the depletion of glucose. For comparison, initial glucose concentrations are shown in Table 1 and no lactate was present in the fresh media. IS Sf Insect ACF featured the lowest glucose content, minimal Gln and no Asn, suggesting that no carbon source other than glucose was available. In the other three media, the end of exponential growth occurred before glucose was depleted, suggesting glucose was not the growth-limiting substrate. Lactate accumulation was not observed during these experiments.

We calculated the growth rate μ [1/h], doubling time t_D [h], and yield coefficient $Y_{X/S}$ [cells/$g_{Glucose}$], as well as the glucose and lactate metabolic rates $q_{Glucose}$ and $q_{Lactose}$ [fmol/cell h] (Table 2). Based on the yield coefficient, cells grew most efficiently in TriEx ICM ($2.7*10^9$ cells/$g_{Glucose}$) with lower yield coefficients of 1.2–$1.7*10^9$ cells/$g_{Glucose}$ in the other three media.

The growth rates of *Sf*-9 cells in all four media fell within the range 0.024–0.038 1/h. TriEx ICM promoted the highest growth rate, also associated with the highest yield coefficient and the fastest consumption of glucose (79.9 fmol/(cell h)). ExpiSf CDM supported the lowest growth rate, but this was not far below the growth rates in Sf-900 II SFM and IS Sf Insect ACF.

3.3. Metabolic profiling of transfected/infected Sf-9 cells

Next we compared the transient expression systems for their impact on *Sf*-9 cell metabolism in the four different media. We measured glucose consumption, lactate production, and amino acid metabolism.

We found that glucose consumption increased significantly in three of the media following transfection, only falling in the IS Sf Insect ACF cultures (Fig. 2). Glucose consumption increased by 1.4-fold in Sf-900 II SFM, 1.6-fold in ExpiSf CDM, and 1.3-fold in TriEx ICM, but decreased by 20% in IS Sf Insect ACF. Infection with BEVS caused glucose consumption to increase in all four media: 2.5-fold in Sf-900 II SFM, 3.0-fold in ExpiSf CDM, 2.4-fold in IS Sf Insect ACF, and 2.2-fold in TriEx ICM. Lactate production declined in most of the transfected/infected cells, but not in the BEVS-infected cultures in ExpiSf CDM (2.3-fold increase) or in IS Sf Insect ACF (1.6-fold increase).

Gln consumption decreased 60% after transfection in Sf-900 II SFM whereas BEVS infection triggered Gln production (Fig. 3). Gln consumption in ExpiSf CDM increased 24-fold after transfection and 38.4-fold after infection. Cells growing in IS Sf Insect ACF produced Gln before treatment and the production rate increased by 5.3-fold following BEVS infection, but transfection had the opposite effect, triggering the consumption of Gln. The L-alanyl-L-glutamine content of TriEx ICM could not be quantified, but it was converted to Gln after BEVS infection.

Glu consumption also decreased 60% after transfection in Sf-900 II SFM but it increased by 2.3-fold after BEVS infection. In ExpiSf CDM, there was no significant change in Glu consumption during either transient expression process. In IS Sf Insect ACF, Glu production declined by 80% after transfection but increased 15.2-fold after BEVS infection. The rate of Glu consumption did not significantly differ in TriEx ICM after transfection but increased 1.3-fold after BEVS infection.

The rate of Asp consumption in Sf-900 II SFM was reduced 80% by transfection but was only minimally affected (10% increase) by BEVS infection. In ExpiSf CDM, a small amount of Asp was produced before treatment, and this increased 3.2-fold after transfection and 22-fold after infection. In IS Sf Insect ACF, Asp consumption fell by 80% following transfection but increased 2.3-fold after infection. There was no significant difference between untreated and transfected cells in TriEx ICM, but the rate of Asp consumption fell by 50% after BEVS infection.

Among the other amino acids Ala production increased significantly in all four media after transfection and BEVS infection (data not shown), whereas the consumption of Leu, threonine (Thr), Ser, Tyr and cystine (C–C) increased significantly after both treatments (Table 3). The highest increase was observed for Thr in ExpiSf CDM, with a 10.5-fold increase following transfection and a 38.6-fold increase following BEVS infection. Ser was the only amino acid that switched from production to consumption following transfection or infection. The consumption of these amino acids was generally higher in the BEVS-infected cells than transfected cells. We did not detect significant changes for Gly, Val, Ile, Pro, Asn, Met, Phe, Lys, His or Trp, and we were unable to detect Arg using our analytic method.

3.4. Production kinetics and recombinant protein yields

BR033 was expressed as a secreted fusion protein with the fluorescent reporter tdTomato, so we characterized the production kinetics by fluorimetry (Fig. 4). In contrast, no fluorescent marker

Table 1
Concentrations of glucose, lactate and amino acids in Sf-900 II SFM, ExpiSf CDM, IS Sf Insect ACF and TriEx ICM (n = 3).

Component	Unit	Sf-900 II SFM	ExpiSf CDM	IS Sf Insect ACF	TriEx ICM
Glucose	g/L	10.43 ± 0.04	14.93 ± 0.10	8.08 ± 0.06	9.77 ± 0.07
Lactate	g/L	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Ala	µmol/mL	1.97 ± 0.25	0.046 ± 0.00	1.62 ± 0.03	3.20 ± 0.04
Gly	µmol/mL	2.35 ± 0.33	1.95 ± 0.12	2.04 ± 0.01	1.63 ± 0.02
Val	µmol/mL	3.65 ± 0.40	4.47 ± 0.24	3.41 ± 0.05	4.44 ± 0.08
Leu	µmol/mL	1.53 ± 0.17	2.60 ± 0.12	1.86 ± 0.02	3.36 ± 0.02
Ile	µmol/mL	4.05 ± 0.01	5.45 ± 0.36	3.06 ± 0.05	4.62 ± 0.03
Thr	µmol/mL	1.81 ± 0.01	2.31 ± 0.21	1.59 ± 0.04	2.20 ± 0.08
Ser	µmol/mL	3.02 ± 0.02	4.19 ± 0.29	4.78 ± 0.23	5.96 ± 0.89
Pro	µmol/mL	3.29 ± 0.02	5.15 ± 0.72	2.87 ± 0.13	2.57 ± 0.14
Asn	µmol/mL	6.32 ± 0.01	9.60 ± 0.53	0.00 ± 0.00	4.44 ± 0.15
Asp	µmol/mL	7.20 ± 0.47	7.11 ± 0.17	5.47 ± 0.56	4.00 ± 0.18
Met	µmol/mL	3.72 ± 0.47	5.12 ± 0.32	2.47 ± 0.04	3.96 ± 0.02
Glu	µmol/mL	8.15 ± 0.26	8.47 ± 0.43	0.00 ± 0.00	9.66 ± 0.33
Phe	µmol/mL	3.55 ± 0.044	4.33 ± 0.26	2.66 ± 0.06	3.09 ± 0.03
Gln	µmol/mL	12.17 ± 1.62	10.49 ± 0.68	3.91 ± 0.01	0.00 ± 0.00
Lys	µmol/mL	2.67 ± 0.08	3.31 ± 0.24	1.94 ± 0.01	3.16 ± 0.20
His	µmol/mL	0.93 ± 0.10	0.91 ± 0.11	0.72 ± 0.13	0.82 ± 0.06
Tyr	µmol/mL	0.82 ± 0.10	1.07 ± 0.63	0.82 ± 0.0	1.09 ± 0.01

was attached to SmALDH_312, so we characterized the production kinetics only for the transfection experiments.

During the first 12 h post-transfection, no increase in RFU was observed indicating no protein production (negative control groups showed no increase in RFU over the whole experiment). After 12 h, the RFU increased in all media. Protein production was least efficient in ExpiSf CDM and most efficient in TriEx ICM based on the final RFU. The production kinetics and protein yield in Sf-900 II SFM and IS Sf Insect ACF were comparable. No further increase in RFU was observed ∼88 h post-transfection. The measured total protein yields confirmed the RFU data (Table 4). We also compared the endpoint values for total protein yields in the transfection experiment to the yields achieved by BEVS (Table 4). Overall, the yields in TriEx ICM and IS Sf Insect ACF were comparable, whereas BEVS achieved much higher yields than LP in Sf-900 II SFM and ExpiSf CDM.

TriEx ICM outperformed all other media in terms of protein yields, but the transfection efficiencies were not comparable (∼21% in Sf-900 II SFM, ∼24% in ExpiSf CDM, ∼49% in IS Sf Insect ACF and ∼38% in TriEx ICM). For better comparability, the final fusion protein content was normalized to the amount of protein (amol) per transfected cell and hour (Fig. 5). This revealed that Sf-900 II SFM achieved the highest overall productivity of 0.26 ± 0.04 amol/(cell h), followed by TriEx ICM (5% lower, difference not significant), then ExpiSf CDM (47% lower) and IS Sf Insect ACF (62% lower). Sf-900 II SFM achieved by far the highest protein yield in BEVS infection experiments (Table 4). The yield was ∼10-fold lower in ExpiSf CDM and IS Sf Insect ACF, and ∼4-fold lower in TriEx ICM. In terms of production rates, BEVS performed better than LP by a factor of 3.8–17.8 in all media. The highest protein production rate of 4.63 ± 1.85 amol/(cell h) was achieved in Sf-900 II SFM, whereas the production rates in Expi Sf CDM (1.25 ± 0.27 amol/(cell h)), IS Sf Insect ACF (0.54 ± 0.01 amol/(cell h)) and TriEx ICM (0.51 ± 0.17 amol/(cell h)) were significantly lower.

3.5. Laboratory-scale production

Before transfer to the 1-L bioreactor, optimized infection parameters were established for BEVS and a stable cell line was generated for the production of BR033. We selected the most promising media based on the earlier experiments, and therefore used Sf-900 II SFM for BEVS and both Sf-900 II SFM and ExpiSf CDM for the stable cell line (Fig. 6). For the BEVS process, no remarkable increase in product formation was observed before ∼30 h post-infection. Given that the cells stop growing after infection, it was not surprising to see the cell concentration drop over the process time. Cell viability declined immediately after infection, and with the optimized MOI a high product concentration of 54.22 ± 27.21 mg/L was achieved, which is comparable to the product concentration achieved in the six-well format. The high variability on the product yield is most likely due to high absolute error limits based on the inherent variance of the $TCID_{50}$ titration method [37], leading to a variance in MOI.

Growth of the stable cell line was comparable to the kinetics described in Section 3.1 and the maximum cell concentration achieved in Sf-900 II SFM was twice that achieved in ExpiSf CDM. Cell viability in both media remained >90% throughout the process. The product concentration increased from the beginning of cultivation, and the final product concentrations were 1.25 mg/L in Sf-900 II SFM and 0.98 mg/L in ExpiSf CDM. Surprisingly, the product concentration continued to increase in a linear manner in ExpiSf CDM during the stationary phase (after 144 h) whereas the rate of product accumulation declined in Sf-900 II SFM. This may reflect the availability of amino acids, given that 14 of the 19 amino acids we quantified were more concentrated in ExpiSf CDM than Sf-900 II SFM. The product concentration was lower than that achieved by transient transfection, but cell-specific productivity can be increased by developing a monoclonal high-producer cell line [38,39]. Our overall findings in terms of protein expression levels in different media are consistent with those in the LP experiments.

4. Discussion

Glucose is the main carbon and energy source for both mammalian and insect cells, but the glucose concentrations in the four insect media were higher than those typically found in mammalian cell media because insect cells tolerate higher glucose levels [40]. Gln is the second most important metabolite for the cells, but the four media contained widely different levels. TriEx ICM contained no Gln at all, but L-alanyl-L-glutamine was present instead. The growth rate of *Sf*-9 cells in Sf-900 II SFM was comparable to earlier reports [16,41]. Furthermore, the observed doubling times in Sf-900 II SFM and TriEx ICM were comparable to those claimed by the manufacturers [42,43]. Interestingly, the

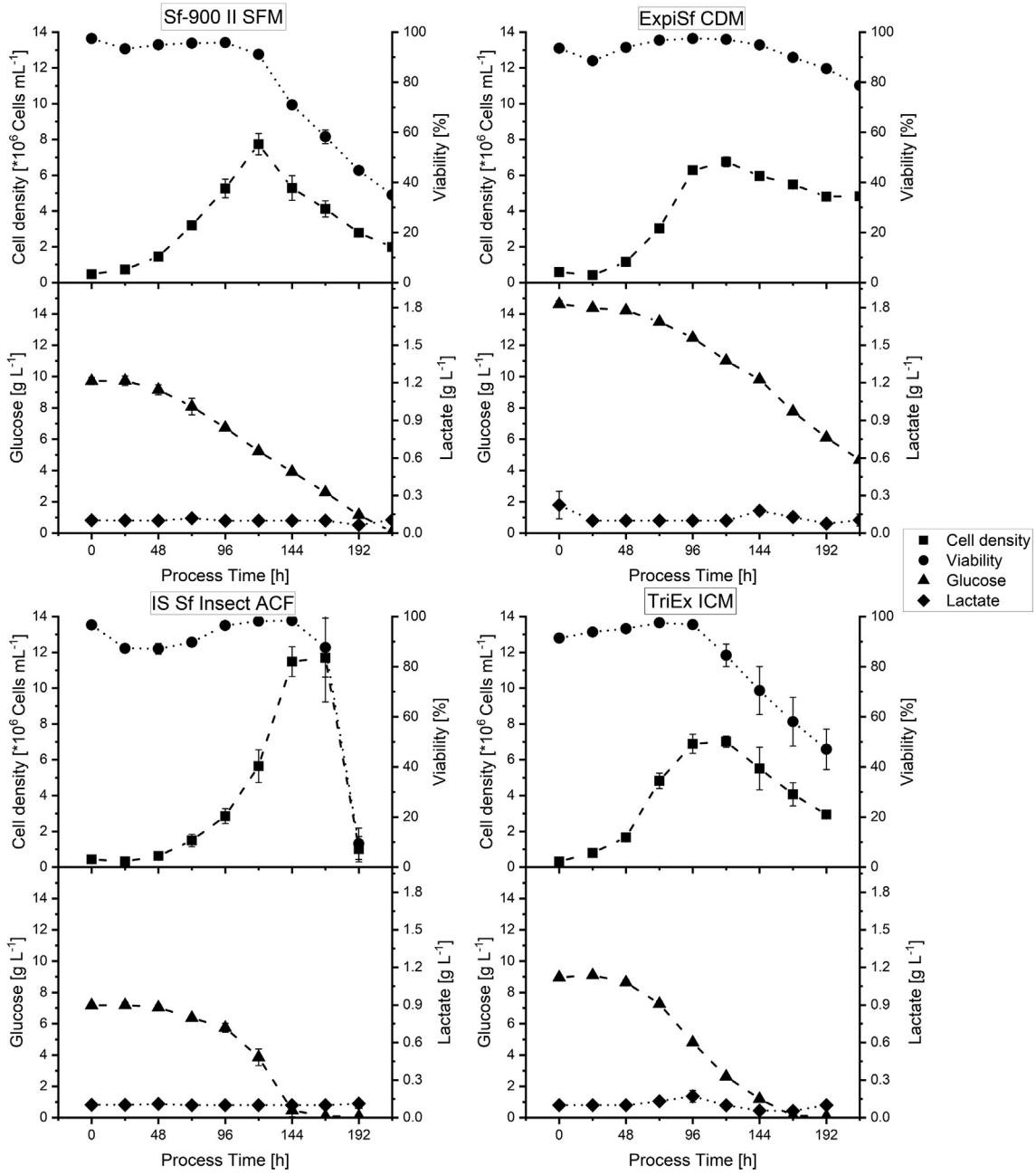

Fig. 1. Growth kinetics of adapted Sf-9 cells and the offline parameters of cell density, cell viability, glucose concentration and lactate concentration (n = 3).

Table 2
Parameters and growth kinetics of TriEx Sf-9 cells in the four different media (n = 3).

	Unit	Sf-900 II SFM	ExpiSf CDM	IS Sf Insect ACF	TriEx ICM
μ	1/h	0.027	0.024	0.030	0.038
t_D	h	25.36	28.51	23.30	18.34
$Y_{X/S}$	cells/$g_{Glucose}$	$1.5*10^9$	$1.2*10^9$	$1.7*10^9$	$2.7*10^9$
$q_{Glucose}$	fmol/(cell h)	40.5	54.3	38.6	79.9
$q_{Lactate}$	fmol/(cell h)	0.2	− 1.9	-	-
C_{MAX}	Cells/mL	$7.74*10^6$	$5.96*10^6$	$1.17*10^7$	$7.01*10^6$

doubling time in ExpiSf CDM exceeded the manufacturer's specifications [44], probably reflecting the specific, adapted *Sf*-9 cell line recommended by the manufacturer, differing from the most commonly used *Sf*-9 cells. We found no comparative reports for the IS Sf Insect ACF, which is surprising because we achieved the highest viable cell concentration in this medium. Notably, the depletion of glucose in this medium coincided with the end of the exponential growth phase, indicating that the nutrients

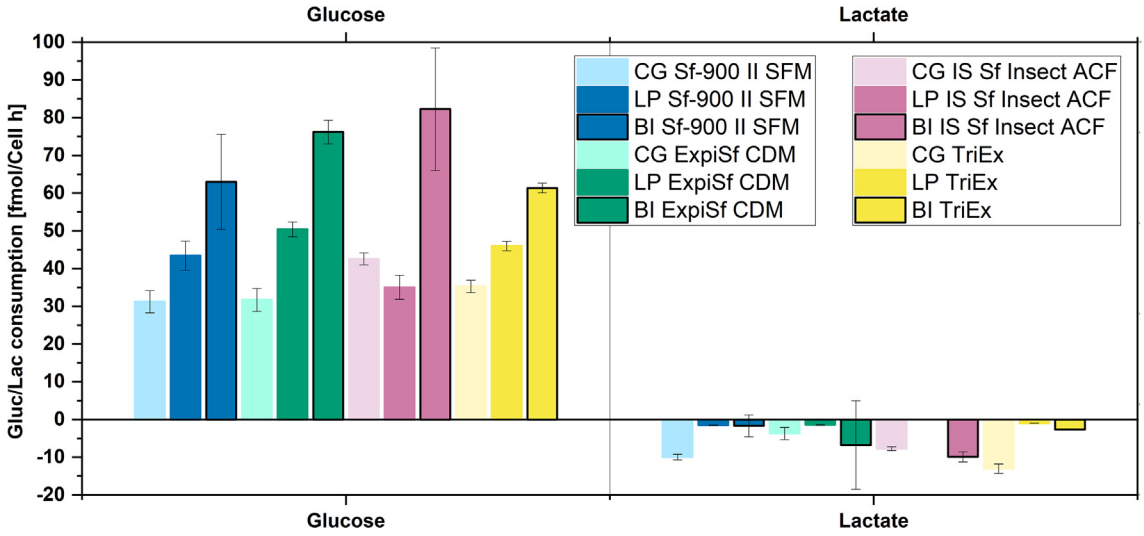

Fig. 2. Glucose consumption and lactate production in the four different media (n = 3). Lighter colors represent untreated control cultures, dark unboxed bars represent the transfected cells, and dark boxed bars represent the infected cells. Data were normalized per cell and the process time of 114 h. CG = control group; LP = lipopolyfection; BI = BEVS-infection.

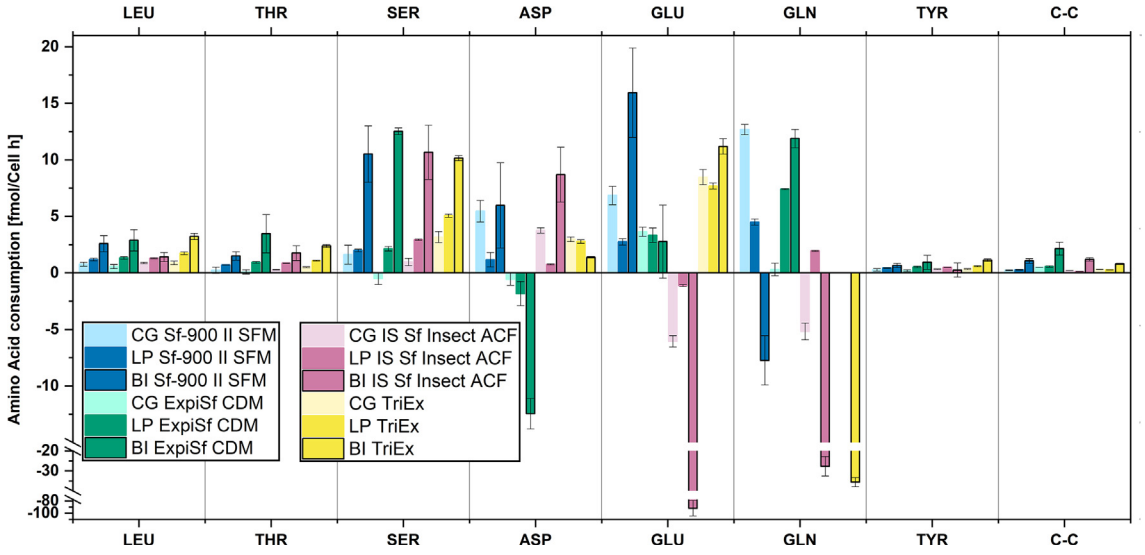

Fig. 3. Amino acid consumption and production in the four different media (n = 3). Lighter colors represent untreated control cultures, dark unboxed bars represent the transfected cells, and dark boxed bars represent the infected cells. Data were normalized per cell and the process time of 114 h. CG = control group; LP = lipopolyfection; BI = BEVS-infection; C–C = cystine.

Table 3
Relative increase (fold change) in amino acid consumption for leucine, threonine, serine, tyrosine and cystine after transfection or BEVS infection in four different media. LP = lipopolyfection; BI = BEVS-infection; C–C = cystine.

AA/Medium	Sf-900 II SFM		ExpiSf CDM		IS Sf Insect ACF		TriEx ICM	
	LP	BI	LP	BI	LP	BI	LP	BI
LEU	1.5	3.4	2.3	5.1	1.5	1.6	2	3.6
THR	3	6.4	10.5	38.6	3	6.0	2.1	4.7
SER	1.2	6.4	−4.2	−24.6	3	11.0	1.6	3.2
TYR	1.6	2.4	2.6	4.6	1.5	0.8	1.8	3.3
C–C	1.1	4.4	1.1	4.3	0.7	5.9	0.9	2.5

were well balanced. Although the glucose concentration was not the highest in TriEx ICM, it nevertheless was the most promising medium for protein production based on the calculated growth rate and yield (cells/$g_{Glucose}$). This may be linked to the replacement of Gln with L-alanyl-L-glutamine, thus increasing the availability of the stabilized amino acid. Furthermore we assume TriEX ICM to comprise an additional carbohydrate source as such as maltose or fructose, which can also be utilized by *Sf*-9 cells

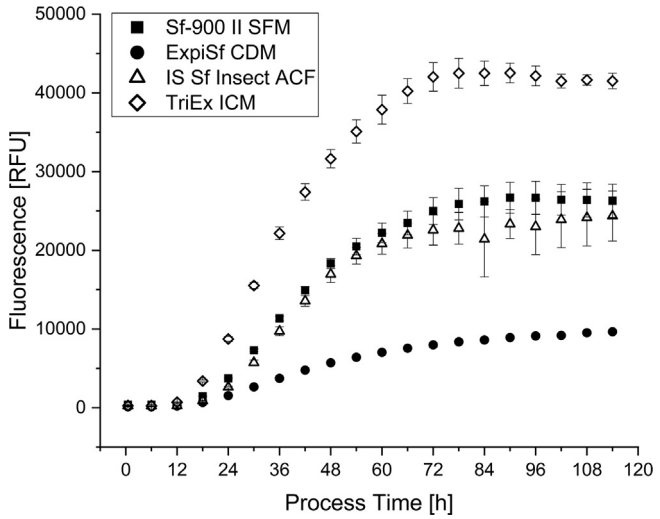

Fig. 4. Fluorescence signal in relative fluorescence units (RFU) showing the production kinetics for BR033 fused to tdTomato (n = 3). Data points are shown every 6 h rather than every 0.5 h for clarity.

Table 4
Protein yields achieved by transfection and BEVS infection in the four different media (n = 3).

	Lipopolyfection Protein Yield [mg/L]	BEVS Protein Yield [mg/L]
Sf-900 II SFM	4.95 ± 0.46	47.78 ± 10.71
ExpiSf CDM	2.09 ± 0.66	5.95 ± 0.88
IS Sf Insect ACF	4.72 ± 0.41	4.57 ± 0.62
TriEx ICM	8.53 ± 1.02	11.37 ± 3.25

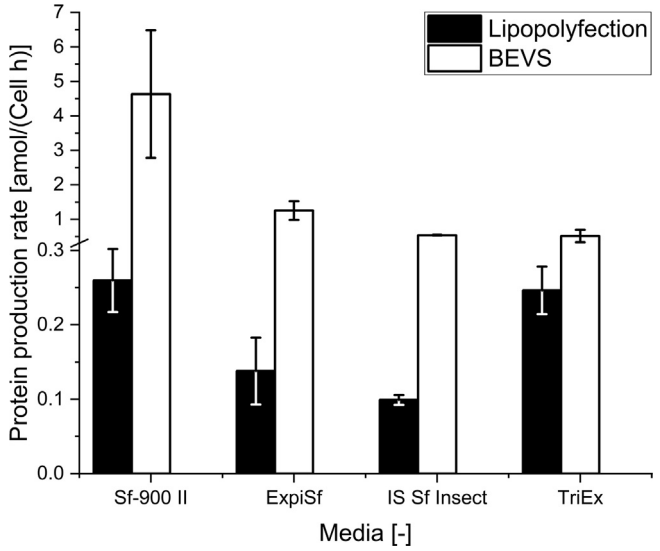

Fig. 5. Cell specific protein production rates after lipopolyfection/BEVS infection (n = 3). The fusion protein was harvested from the four media 114 h post-transfection. BEVS-related post-infection harvest times were specific for each medium: 70 h for Sf-900 II SFM, 77 h for ExpiSf CDM, 87 h for IS Sf Insect ACF and 80 h for TriEx ICM. The y-axis scale changes from 0.1 to 1 per major tick after the break at y = 0.3.

[45]. We also observed only low levels of lactate production by *Sf*-9 cells in all media. In contrast to mammalian cells, *Sf*-9 cells can metabolize lactate [46] despite their high glucose consumption [9,47].

About 80% of the carbon consumed by the *Sf*-9 cells enters the TCA cycle and is used to provide energy and metabolic intermediates [48,49]. The transfected and infected *Sf*-9 cells consumed more glucose than untreated controls, which is unsurprising due to the additional metabolic burden of recombinant protein synthesis. Compared to untreated cells, lactate production declined after transfection in all media, and no lactate at all was detected in IS Sf Insect ACF, which may reflect the increased flux through the TCA cycle fueled by pyruvate [49]. In contrast, BEVS-infected cells produced small amounts of lactate in ExpiSf CDM and TriEx ICM, which may be associated with oxygen limitation. However, a decline in lactate production after BEVS-infection has already been reported in Sf-900 II SFM, reflecting the metabolic flux from glucose redirected into the TCA cycle [9,48]. The enhanced conversion of glucose to pyruvate inhibits lactate dehydrogenase, thus reducing lactate production [48]. The amino acids Leu, Thr and C–C were consumed more quickly after infection, as previously suggested [50]. However, Glu production in IS Sf Insect ACF was conspicuous after infection, possibly reflecting glucose limitation. Under these conditions, *Sf*-9 cells can use glutaminase to convert Gln into glutamate, with ammonia as a by-product. *Sf*-9 cells can then use glutamine synthetase to recycle ammonia to Gln [17]. Glucose limitation in IS Sf Insect ACF after infection was ruled out because considerable amounts of glucose and only a slight accumulation of ammonia were detected.

The consumption rates we observed are close to the values available in the literature [49,51] as summarized in Table 5. However, these articles differentiate between growth phases and low or high MOI, whereas we calculated the overall rates following transfection/infection until the end of cultivation. The transfection experiments reported herein agreed with the published values for BEVS infection [49,51], but our BEVS-infected cells produced more Asp, Gln and Glu than claimed in the previous studies.

As well as investigating the metabolic shift in *Sf*-9 cells after transfection/infection, we screened the media to determine which was most suitable for recombinant protein production. We compared BR033 expression by transfection and SmALDH_312 expression following BEVS infection. Using the standard LP protocol, the highest final protein concentration was achieved using TriEx ICM and was 1.7-fold higher than Sf-900 II SFM, 1.8-fold higher than IS Sf Insect ACF and 4-fold higher than ExpiSf CDM. The normalized protein production rate in TriEx ICM was similar to Sf-900 II SFM, but about 50% lower in ExpiSf CDM and IS Sf Insect ACF. This is comparable to the lower range of values achieved for the PEI-mediated transfection of *Sf*-9 cells with a TNFR-Fc construct [4] although we used a non-optimized transfection protocol. Our total protein yields (2.1–8.3 mg/L) were also in agreement with the lower range of values reported in the literature (1–100 mg/L) [4,20]. In contrast, we achieved 3.8–17.8 times higher protein production rates in all four media when using BEVS, which is commensurate with literature values ranging from 1–600 mg/L [52], and much higher than virus-free transient expression methods [53]. The maximum yield we achieved using BEVS was 47.78 mg/L in Sf-900 II SFM, which is comparable to other BEVS-based production processes [54,55]. Sf-900 II SFM is the most frequently mentioned insect cell culture medium in the literature in reports spanning 2017–2021, suggesting it is the most popular commercial product for *Sf*-9 cells. Comparing the four modern insect cell culture media for transient expression systems, we achieved the highest overall protein yields in Sf-900 II SFM, but TriEx ICM is a suitable alternative for LP-based transfection. Laboratory-scale production of recombinant proteins in Sf-900 II SFM confirmed its suitability for BEVS and transfection. If a CDM is required, ExpiSf CDM is also suitable for the stably transformed cell line.

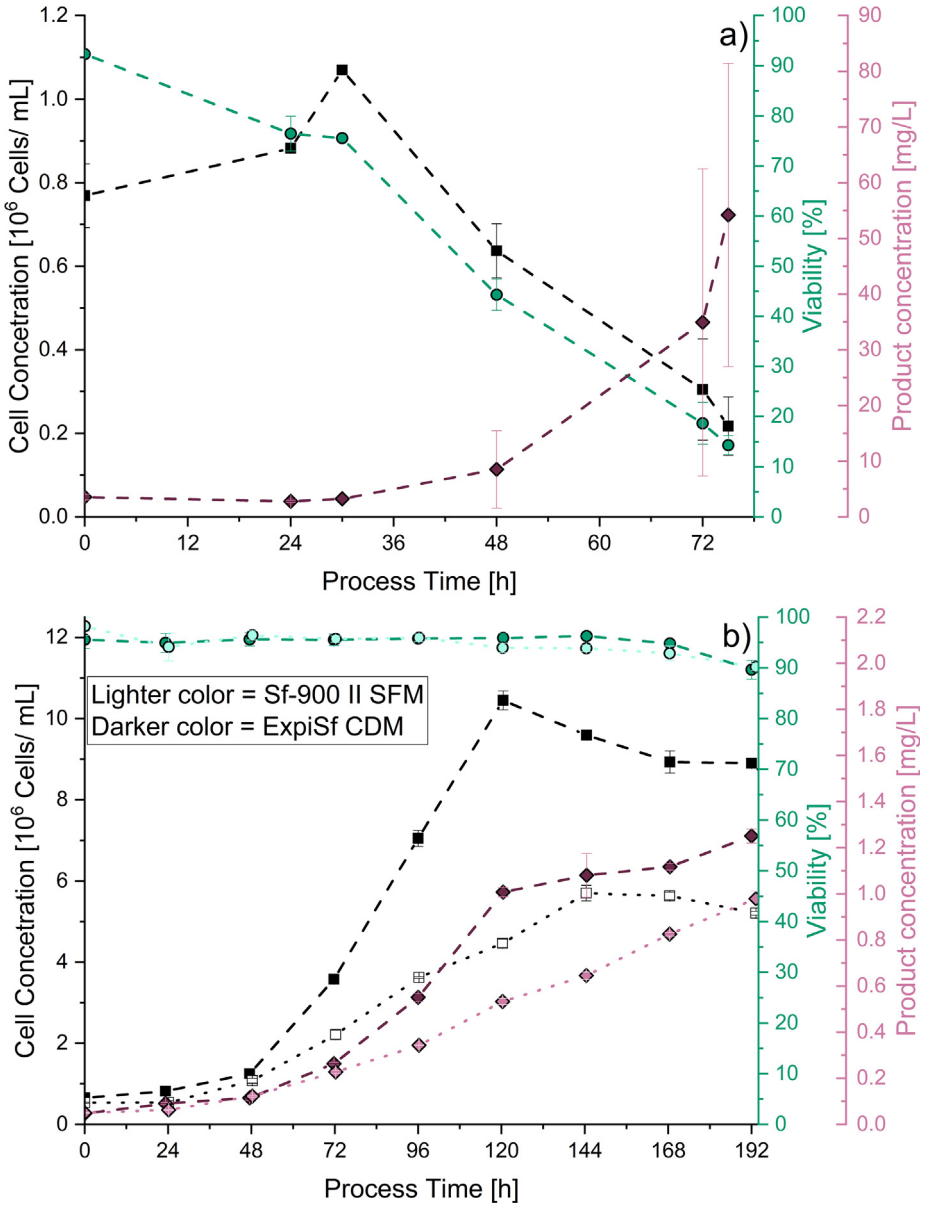

Fig. 6. Laboratory-scale production of ALDH with BEVS (a, biological triplicate) and the BR033 fusion protein in the stable Sf-9 cell line (b, technical triplicates).

Table 5
Comparison of published consumption/production rates for glucose, lactate and amino acids [49,51] and data obtained in this study (n = 3). Consumption rates in fmol/(cell h), negative values indicate production rates. CG = control group; BI = BEVS-infection; LP = lipopolyfection.

	Bernal et al. [49] and Monteiro et al. [51]		This work		
	CG	BI	CG	LP	BI
Glucose	43.51–82.74	20.6–68.89	24.74–33.69	27.77–39.92	61.38–82.28
Lactate	(−3.36) – (−17.05)	1.41 – (−21.4)	(−2.91) – (−10.29)	0 – (−1.15)	(−1.67) – (−9.88)
LEU	1.06–8.5	0.02–8.3	0.57–0.89	1.19–1.74	1.41–3.22
THR	0.93–6.4	0.2–8	0.09–0.51	0.71–1.08	1.5–4.56
SER	3.32–12.4	0.82–9.9	3.17 – (−0.51)	1.99–5.06	1.53–10.15
ASP	0.93–13.6	24.1 – (−2.96)	5.46 – (−0.56)	2.78 – (−1.83)	8.68 – (−12.45)
GLU	4.25–19.64	41.1 – (−0.33)	8.46 – (−6.05)	7.68 – (−1.11)	15.94 – (−97.56)
GLN	5.7–36.1	1.64–25.6	12.68 – (−5.17)	0–7.41	11.89 – (−35.62)
TYR	0.32–5.5	0.1–4.2	0.2–0.34	0.44–0.6	0.26–1.13
C–C	N.A.	N.A.	0.2–0.5	0.13–0.57	0.81–2.15

5. Conclusions

BEVS is a reliable platform for the large-scale production of recombinant proteins, and is currently used for the production of multiple pharmaceutical products, including the NVX-CoV2373 COVID-19 vaccine candidate [47]. Although BEVS achieved the highest overall yields, LP was a simpler approach with fewer process steps, which is suitable for the production of recombinant

peptides [56,57]. Among the four media we tested, Sf-900 II SFM was most suitable for the production of SmALDH_312 (BEVS) and BR033 (LP). However, IS Sf Insect ACF supported the highest final cell concentration and TriEx ICM achieved the shortest doubling time. If a CDM such as ExpiSf CDM is required, the LP protocol can be optimized, which was previously shown to increase protein yields by ~2.8-fold [4,24]. Furthermore, the pre-adapted ExpiSf9 cell line can increase the maximum cell concentration and yield with ExpiSf CDM for LP, but especially BEVS, according to the manufacturer [58]. Our data show that the medium has a profound impact on protein production and final yield, and we therefore recommend the inclusion of media screening as a process development option whenever possible.

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Conflict of interest

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analysis, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

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Data availability

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

References

[1] Kumar N, Pandey D, Halder A. Diagnostic and therapeutic applications of baculovirus expression vector system. In: Kumar D, Gong C, editors. Trends in insect molecular biology and biotechnology. Cham: Springer International Publishing; 2018. p. 163–91. https://doi.org/10.1007/978-3-319-61343-7_9.
[2] Scholz J, Suppmann S. A new single-step protocol for rapid baculovirus-driven protein production in insect cells. BMC Biotechnol 2017;17:83. https://doi.org/10.1186/s12896-017-0400-3. PMid: 29145860.
[3] Druzinec D, Salzig D, Brix A, et al. Optimization of insect cell based protein production processes – online monitoring, expression systems, scale up. Adv Biochem Eng Biotechnol 2013;136:65–100. https://doi.org/10.1007/10_2013_205. PMid: 23995041.
[4] Shen X, Hacker DL, Baldi L, et al. Virus-free transient protein production in Sf9 cells. J Biotechnol 2014;171:61–70. https://doi.org/10.1016/j.jbiotec.2013.11.018. PMid: 24333123.
[5] Ghosh A, Dhall H, Dietzgen RG, et al. Insect cell culture as a tool in plant virus research: a historical overview. Phytoparasitica 2020;48:287–303. https://doi.org/10.1007/s12600-020-00795-7.
[6] Cox MMJ. Recombinant protein vaccines produced in insect cells. Vaccine 2012;30:1759–66.
[7] Vicente T, Roldão A, Peixoto C, et al. Large-scale production and purification of VLP-based vaccines. J Invertebr Pathol 2011;107:S42–8. https://doi.org/10.1016/j.jip.2011.05.004. PMid: 21784230.
[8] Sia ZR, Miller MS, Lovell JF. Engineered nanoparticle applications for recombinant influenza vaccines. Mol Pharm 2021;18:576–92. https://doi.org/10.1021/acs.molpharmaceut.0c00383. PMid: 32787280.
[9] Drugmand J-C, Schneider Y-J, Agathos SN. Insect cells as factories for biomanufacturing. Biotechnol Adv 2012;30:1140–57. https://doi.org/10.1016/j.biotechadv.2011.09.014. PMid: 21983546.
[10] Chan LCL, Reid S. Development of serum-free media for lepidopteran insect cell lines. Methods Mol Biol 2016;1350:161–96. https://doi.org/10.1007/978-1-4939-3043-2_8. PMid: 26820858.
[11] Gstraunthaler G, Lindl T, van der Valk J. A plea to reduce or replace fetal bovine serum in cell culture media. Cytotechnology 2013;65:791–3. https://doi.org/10.1007/s10616-013-9633-8. PMid: 23975256.
[12] Yao T, Asayama Y. Animal-cell culture media: History, characteristics, and current issues. Reprod Med Biol 2017;16:99–117. https://doi.org/10.1002/rmb2.12024. PMid: 29259457.
[13] Mi Y, Xie T, Zhu B, et al. Production of SARS-CoV-2 virus-like particles in insect cells. Vaccines 2021;9(6). 10.3390/vaccines9060554. PMid: 34073159.
[14] Joshi PRH, Venereo-Sanchez A, Chahal PS, et al. Advancements in molecular design and bioprocessing of recombinant adeno-associated virus gene delivery vectors using the insect-cell baculovirus expression platform. Biotechnol J 2021;16:2000021. https://doi.org/10.1002/biot.202000021. PMid: 33277815.
[15] Gstraunthaler G, Lindl T. Zell- und Gewebekultur: Allgemeine Grundlagen und spezielle Anwendungen. 7th ed. Berlin, Heidelberg, s.l.: Springer, Berlin Heidelberg; 2013.
[16] Rhiel M, Mitchell-Logean CM, Murhammer DW. Comparison of *Trichoplusia ni* BTI-Tn-5B1-4 (high five™) and *Spodoptera frugiperda* Sf-9 insect cell line metabolism in suspension cultures. Biotechnol Bioeng 1997;55:909–20. https://doi.org/10.1002/(SICI)1097-0290(19970920)55:6<909:AID-BIT8>3.0.CO;2-K.
[17] Drews M, Doverskog M, Öhman L, et al. Pathways of glutamine metabolism in *Spodoptera frugiperda* (Sf9) insect cells: evidence for the presence of the nitrogen assimilation system, and a metabolic switch by $^{1}H/^{15}N$ NMR. J Biotechnol 2000;78:23–37. https://doi.org/10.1016/S0168-1656(99)00231-X.
[18] Radford KM, Reid S, Greenfield PF. Substrate limitation in the baculovirus expression vector system. Biotechnol Bioeng 1997;56:32–44. https://doi.org/10.1002/(SICI)1097-0290(19971005)56:1<32:AID-BIT4>3.0.CO;2-W.
[19] Kim TK, Eberwine JH. Mammalian cell transfection: the present and the future. Anal Bioanal Chem 2010;397:3173–8. https://doi.org/10.1007/s00216-010-3821-6. PMid: 20549496.
[20] Li E, Brown SL, Dolman CS, et al. Production of functional antibodies generated in a nonlytic insect cell expression system. Protein Expr Purif 2001;21:121–8. https://doi.org/10.1006/prep.2000.1329. PMid: 11162396.
[21] Bus T, Traeger A, Schubert US. The great escape: how cationic polyplexes overcome the endosomal barrier. J Mater Chem B 2018;6:6904–18. https://doi.org/10.1039/c8tb00967h. PMid: 32254575.
[22] Longo PA, Kavran JM, Kim M-S, et al. Transient mammalian cell transfection with polyethylenimine (PEI). Meth Enzymol 2013;529:227–40. https://doi.org/10.1016/B978-0-12-418687-3.00018-5. PMid: 24011049.
[23] Lin S-Y, Chung Y-C, Hu Y-C. Update on baculovirus as an expression and/or delivery vehicle for vaccine antigens. Expert Rev Vaccines 2014;13:1501–21. https://doi.org/10.1586/14760584.2014.951637. PMid: 25155825.
[24] Puente-Massaguer E, Gòdia F, Lecina M. Development of a non-viral platform for rapid virus-like particle production in Sf9 cells. J Biotechnol 2020;322:43–53. https://doi.org/10.1016/j.jbiotec.2020.07.009. PMid: 32673687.
[25] Syga M-I, Nicolì E, Kohler E, et al. Albumin incorporation in polyethylenimine-DNA polyplexes influences transfection efficiency. Biomacromolecules 2016;17:200–7. https://doi.org/10.1021/acs.biomac.5b01308. PMid: 26652656.
[26] Rezvani Amin Z, Rahimizadeh M, Eshghi H, et al. The effect of cationic charge density change on transfection efficiency of polyethylenimine. Iran J Basic Med Sci 2013;16:150–6.
[27] Sang Y, Xie K, Mu Y, Lei Y, Zhang B, Xiong S, et al. Salt ions and related parameters affect PEI-DNA particle size and transfection efficiency in Chinese hamster ovary cells. Cytotechnology 2015;67:67–74.
[28] van Oers MM, Pijlman GP, Vlak JM. Thirty years of baculovirus-insect cell protein expression: from dark horse to mainstream technology. J Gen Virol 2015;96:6–23. https://doi.org/10.1099/vir.0.067108-0. PMid: 25246703.
[29] Felberbaum RS. The baculovirus expression vector system: A commercial manufacturing platform for viral vaccines and gene therapy vectors. Biotechnol J 2015;10:702–14.
[30] Buckland B, Boulanger R, Fino M, et al. Technology transfer and scale-up of the Flublok® recombinant hemagglutinin (HA) influenza vaccine manufacturing process. Vaccine 2014;32:5496–502. https://doi.org/10.1016/j.vaccine.2014.07.074. PMid: 25131727.
[31] Zhai Y, Zhang D, Yu L, et al. SmartBac, a new baculovirus system for large protein complex production. J Struct Biol: X 2019;1:100003. https://doi.org/10.1016/j.yjsbx.2019.100003. PMid: 32337507.
[32] Harnischfeger J, Käßer L, Zitzmann J, et al. Bioreactor-based antigen production process using the baculovirus expression vector system. Methods Mol Biol 2021;2183:95–118. https://doi.org/10.1007/978-1-0716-0795-4_8. PMid: 32959243.
[33] Käßer L, Zitzmann J, Grein T, et al. Turbidimetry and dielectric spectroscopy as process analytical technologies for mammalian and insect cell cultures. Methods Mol Biol 2020;2095:335–64. https://doi.org/10.1007/978-1-0716-0191-4_20. PMid: 31858478.
[34] Palomares LA, López S, Ramírez OT. Utilization of oxygen uptake rate to assess the role of glucose and glutamine in the metabolism of infected insect cell cultures. Biochem Eng J 2004;19:87–93. https://doi.org/10.1016/j.bej.2003.12.002.
[35] Pöppel A-K, Vogel H, Wiesner J, et al. Antimicrobial peptides expressed in medicinal maggots of the blow fly *Lucilia sericata* show combinatorial activity

against bacteria. Antimicrob Agents Chemother 2015;59:2508–14. https://doi.org/10.1128/AAC.05180-14. PMid:25666157.
[36] Ludolf F, Patrocínio PR, Corrêa-Oliveira R, et al. Serological screening of the *Schistosoma mansoni* adult worm proteome. PLoS Negl Trop Dis 2014;8:e2745. https://doi.org/10.1371/journal.pntd.0002745. PMid: 24651847.
[37] Keiser PT, Anantpadma M, Staples H, et al. Automation of infectious focus assay for determination of filovirus titers and direct comparison to plaque and $TCID_{50}$ assays. Microorganisms 2021;9(1). 10.3390/microorganisms9010156. PMid: 33445537
[38] Fernandes F, Vidigal J, Dias MM, et al. Flipase-mediated cassette exchange in Sf9 insect cells for stable gene expression. Biotechnol Bioeng 2012;109:2836–44. https://doi.org/10.1002/bit.24542. PMid: 22565981.
[39] Zitzmann J, Weidner T, Czermak P. Optimized expression of the antimicrobial protein Gloverin from *Galleria mellonella* using stably transformed *Drosophila melanogaster* S2 cells. Cytotechnology 2017;69:371–89. https://doi.org/10.1007/s10616-017-0068-5. PMid: 28132128.
[40] Schlaeger EJ. Medium design for insect cell culture. Cytotechnology 1996;20:57–70. https://doi.org/10.1007/BF00350389. PMid: 22358474.
[41] Bernal V, Monteiro F, Carinhas N, et al. An integrated analysis of enzyme activities, cofactor pools and metabolic fluxes in baculovirus-infected *Spodoptera frugiperda* Sf9 cells. J Biotechnol 2010;150:332–42. https://doi.org/10.1016/j.jbiotec.2010.09.958. PMid: 20933551.
[42] Novagen. User Protocol TB314 Rev. C 0108: Tri Ex Sf9 Cells 2008.
[43] Gibco invitrogen cell culture. Sf-900 II SFM, Insert No. 3408 2005.
[44] Gibco Thermo Fisher Scientific. ExpiSf CD Medium USER GUIDE: Catalog Numbers: A3767801, A3767803, A3767804, A3767805 2019.
[45] Bédard C, Tom R, Kamen A. Growth, nutrient consumption, and end-product accumulation in Sf-9 and BTI-EAA insect cell cultures: insights into growth limitation and metabolism. Biotechnol Prog 1993;9:615–24. https://doi.org/10.1021/bp00024a008. PMid: 7764350.
[46] Palomares LA, Realpe M, Ramírez OT. An Overview of Cell Culture Engineering for the Insect Cell-Baculovirus Expression Vector System (BEVS). In: Al-Rubeai M, editor. Animal Cell Culture. Cham: Springer International Publishing; 2015 (9):501–19.
[47] Tian J-H, Patel N, Haupt R, et al. SARS-CoV-2 spike glycoprotein vaccine candidate NVX-CoV2373 immunogenicity in baboons and protection in mice. Nat Commun 2021;12:372. https://doi.org/10.1038/s41467-020-20653-8. PMid: 33446655.
[48] Monteiro F, Bernal V, Alves PM. The role of host cell physiology in the productivity of the baculovirus-insect cell system: Fluxome analysis of *Trichoplusia ni* and *Spodoptera frugiperda* cell lines. Biotechnol Bioeng 2017;114:674–84. https://doi.org/10.1002/bit.26089. PMid: 27568545.
[49] Bernal V, Carinhas N, Yokomizo AY, et al. Cell density effect in the baculovirus-insect cells system: a quantitative analysis of energetic metabolism. Biotechnol Bioeng 2009;104:162–80. https://doi.org/10.1002/bit.22364. PMid: 19459142.
[50] Wong TK, Nielsen LK, Greenfield PF, et al. Relationship between oxygen uptake rate and time of infection of Sf9 insect cells infected with a recombinant baculovirus. Cytotechnology 1994;15:157–67. https://doi.org/10.1007/BF00762390. PMid: 7765927.
[51] Monteiro F, Bernal V, Saelens X, et al. Metabolic profiling of insect cell lines: Unveiling cell line determinants behind system's productivity. Biotechnol Bioeng 2014;111:816–28. https://doi.org/10.1002/bit.25142. PMid: 24258249.
[52] Stolt-Bergner P, Benda C, Bergbrede T, et al. Baculovirus-driven protein expression in insect cells: A benchmarking study. J Struct Biol 2018;203:71–80. https://doi.org/10.1016/j.jsb.2018.03.004. PMid: 29545204.
[53] Mirzaei M, Xu Y, Elias CB, et al. Nonviral production of human interleukin-7 in spodoptera frugiperda insect cells as a soluble recombinant protein. J Biomed Biotechnol 2009;637942. 10.1155/2009/637942. PMid: 18949045
[54] Tao L-N, Liu Z-H, Xu H-L, et al. *Lv*YY1 activates WSSV *ie1* promoter for enhanced vaccine production and efficacy. Vaccines 2020;8:510. https://doi.org/10.3390/vaccines8030510. PMid: 32911686.
[55] Cao W, Cao H, Yi X, et al. Development of a simple and high-yielding fed-batch process for the production of porcine circovirus type 2 virus-like particle subunit vaccine. AMB Express 2019;9:164. https://doi.org/10.1186/s13568-019-0880-8. PMid: 31605297.
[56] Jarvis DL. Recombinant protein expression in baculovirus-infected insect cells. Meth Enzymol 2014;536:149–63. https://doi.org/10.1016/B978-0-12-420070-8.00013-1. PMid: 24423274.
[57] Bleckmann M, Schmelz S, Schinkowski C, et al. Fast plasmid based protein expression analysis in insect cells using an automated SplitGFP screen. Biotechnol Bioeng 2016;113:1975–83. https://doi.org/10.1002/bit.25956. PMid: 26913471.
[58] Yovcheva M, Thompson K, Barnes S, et al. High-titer recombinant protein production. Genet Eng Biotechnol News 2018;38:20–1. https://doi.org/10.1089/gen.38.13.08.