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Cost-effective production process of scFv antibody fragments against Shiga toxin 2 via recombinant *E. coli*

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ABSTRACT

Shiga toxin (Stx)-producing *Escherichia coli* (STEC) and its subgroup enterohemorrhagic *E. coli* are significant pathogens responsible for diarrhea, which can progress to hemorrhagic colitis and hemolytic uremic syndrome (HUS), the leading cause of acute renal failure in children. Early diagnosis is crucial for effective clinical management, as antibiotic treatment is not recommended for STEC infections. The present study aimed to establish a cost-effective biotechnological platform for cultivating recombinant *E. coli* to produce scFv antibody fragments against Stx2 for diagnostic applications. The method was first evaluated through shake flask experiments and subsequently scaled up to bench-scale bioreactors operated in both batch and fed-batch modes using defined culture media. Optimal production conditions were achieved by inducing recombinant *E. coli* pLys at 18 °C for 18 h with 0.1 mM IPTG, resulting in a yield of 3.0 to 4.0 mg scFv/g cell biomass. A fed-batch, high-cell-density procedure with *E. coli* pLysS achieved a maximum production up to 150 mg scFv/L. A preliminary economic assessment demonstrated the production potential at a value of around $250/g scFv. Economic analysis also highlights that the relative cost of capital investment becomes important as production processes intensify. Therefore, technical parameters such as productivity (scFv mass/bioreactor volume * time) and scFv concentration (mass scFv mass/bioreactor volume) should be prioritized to maximize their values. Similarly, optimization of the recombinant *E. coli* microbial platform should be pursued to increase the Yp/x level.

Introduction

The increasing interest in antibody fragments can be attributed to their broad therapeutic applications (Sarker et al., 2019). Among the most widely utilized are Fab (fragment antigen-binding) and scFv (single-chain variable fragment) fragments. These fragments retain the variable heavy (VH) and light (VL) chains of antibodies, which are responsible for antigen specificity (Luz et al., 2018). With molecular masses ranging from approximately 28 kDa to 50 kDa, these fragments offer several advantages, including improved tissue penetration, reduced half-life in non-target tissues, and a lower incidence of immunogenic side effects compared to full-length antibodies, which are often mediated by the Fc (fragment crystallizable) region (Bates et al., 2019; Kholodenko et al., 2019). The simplified structure of these fragments also facilitates their production in microbial expression systems, such as recombinant *Escherichia coli*, making them suitable for large-scale production. From an industrial perspective, *E. coli* expression systems are advantageous due to their ability to utilize defined culture media, robust genetic constructs, rapid growth rates relative to animal cell cultures, and scalability to large bioreactor volumes, all of which contribute to reduced production costs (Spadiut et al., 2014).

Shiga toxin-producing *Escherichia coli* (STEC) is a zoonotic pathogen that infects humans through contaminated food or water. It is associated with severe conditions such as bloody diarrhea, hemorrhagic colitis (HC), hemolytic uremic syndrome (HUS), and neurological complications (Luz et al., 2018). The defining feature of STEC is its production of Shiga cytotoxins (Stx), which are ribosome-inactivating proteins. These toxins exist in two main forms: Stx1 and Stx2, with the latter being more strongly associated with HUS (Brusa et al., 2016). Stx exerts its cytotoxic effects via the pentameric B subunit, which binds to the

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globotriaosylceramide (Gb3) receptor on host cell membranes. This facilitates the translocation of the active A subunit into the cytoplasm, where it halts protein synthesis by targeting the 28S rRNA of 60S ribosomes, ultimately inducing apoptosis (Silva et al., 2019). The low infectious dose and ease of transmission make STEC a significant public health concern, often linked to outbreaks (Willshaw et al., 1994).

Brazil, the world's largest producer and exporter of beef, produced over 8.4 million tons of meat in 2019, valued at $7.59 billion (IBGE, 2022). To ensure the safety of meat products, Brazilian regulatory agencies, such as the National Health Surveillance Agency (ANVISA) and the Department of Inspection of Animal Origin Products (DIPOA), have established guidelines for monitoring pathogenic microorganisms, including verotoxigenic *E. coli*. These measures include testing processed meat products and monitoring major STEC serogroups in slaughterhouses and carcasses (Stromberg et al., 2018, de Assis et al., 2021).

The production of antibody fragments in recombinant *E. coli* has been well-documented (Huleani et al., 2022; Kim et al., 2018; Charoenpun et al., 2021; Bach et al., 2019; Sletta et al., 2004). While therapeutic antibodies are commercially produced using *E. coli* platforms (Walsh and Walsh, 2022; Slikboer and Walsh, 2021), identifying all STEC serogroups remains a challenge. Current diagnostic methods often rely on complementary bacterial tools, which are not universally accessible, particularly in resource-limited regions where cost and availability are significant barriers (Brusa et al., 2016; Silva et al., 2019).

Luz *et al.* developed two recombinant antibody fragments targeting Stx2: scFv fragments derived from mouse hybridomas and Fab fragments produced using phage display technology with a human synthetic antibody library (Luz et al., 2015). Both demonstrated high binding affinity to the GKIEF-SKYNEDDTF region of the Stx2 B subunit, neutralizing up to 80 % of the toxin's cytotoxicity *in vitro*. The scFv fragments also showed 79 % sensitivity and 100 % specificity in detecting STEC strains via ELISA (Stromberg et al., 2018). These findings underscore the potential of scFv fragments as an immunodiagnostic tool for early detection of STEC (Luz et al., 2018; Castro et al., 2019).

The present study aims to establish a cost-effective biotechnological platform for cultivating recombinant *E. coli* to produce scFv fragments against Stx2. Initial experiments were conducted in shake flasks, followed by scale-up to bench-scale bioreactors operated in batch and fed-batch modes using a defined culture medium. An economic analysis based on the experimental data was also performed.

The highlights of this study are as follows: a) demonstrating the bioreactor-scale production of a novel diagnostic tool for Shiga toxin detection using monoclonal antibody (mAb) fragments; b) establishing that *E. coli* pLysS outperforms *E. coli* Arctic Express in this process; and c) developing a high-cell-density recombinant *E. coli* system using a defined culture medium, enabling a cost-effective production of scFv anti-Stx2.

Materials and methods

Bacterial strain and plasmid expression

Recombinant *E. coli* strains BL21 (DE3), BL21(DE3) pLysS (Merck, USA), and Arctic Express (DE3) (Agilent, USA) were used to express the scFv fragment anti-Stx2 using the pscFv-Stx2 plasmid based on previous methodology (Luz et al., 2015).

The anti-Stx2 scFv gene was cloned into an expression vector (pscFv-Stx2) under control of the lac and pTac promoters. The construct includes an N-terminal PelB secretion signal leader sequence to direct expression to the periplasmic space, followed by a 6 × His tag and a Flag epitope for purification and detection. The plasmid also confers resistance to ampicillin.

The transformation of the plasmid pscFv-Stx2 into competent cells of *E. coli* strains BL21 (DE3) and BL21(DE3) pLysS. The protocol involves the following steps: a mixture consisting of 1 μL of DNA, 2 μL of a solution containing 0.5 M KCl, 0.15 M $CaCl_2$, and 0.25 M $MgCl_2$, along with 7 μL of distilled water, was incubated on ice for 5 min. Subsequently, 10 μL of competent cells were added, followed by another incubation on ice for 20 min and then at room temperature for 10 min. Afterwards, 200 μL of autoclaved 2xYT medium was added to the sample for cell growth, and the mixture was incubated at 37 °C for 1 h with agitation at 200 rpm. A volume of 15 μL of the transformed cell suspension was then plated onto LB-agar plates supplemented with 100 μg/mL ampicillin and incubated in a bacteriological oven at 37 °C for 18 h.

Competent *E. coli* Arctic Express strain cells were transformed using the same pscFv-Stx2 plasmid. The cloning protocol consisted of adding 100 μL of competent cells into a microtube, followed by incubating the suspension on ice for 10 min, with shaking every 2 min. Subsequently, 1–50 ng of the plasmid was added and the sample was shaken again, followed by incubation on ice for 30 min. Afterwards, a heat pulse was applied at 42 °C for 20 s, followed by further incubation on ice for 2 min. After the incubation period, 900 μL of pre-warmed complex culture medium containing 20 g/L tryptone, 5 g/L yeast extract, 0.5 g/L NaCl, 10 mL/L 1 M $MgCl_2$, 10 mL/L 1 M $MgSO_4$, and 20 mL/L 20 % w/v glucose solution at 42 °C was added to the solution. It was then incubated at 37 °C for 1 h shaking. Following the incubation, the cell suspension was centrifuged at 5,000 x g for 3–5 min (Hettich Rotina 35R, Germany), after which part of the supernatant was discarded, leaving 200 μL for resuspension. The transformed cell suspension (15 μL) was then seeded onto LB-agar plates with 100 μg/mL ampicillin and incubated in a bacteriological oven at 37 °C for 18 h. All culture media were sterilized at 121 °C for 15 min.

Culture media

Inoculum preparation used 2xYT culture medium supplemented with 10 g/L glucose. The defined culture medium (HDF) adapted from Silva *et al.* (2013) used in batch mode experiments was composed of (amounts per liter): glucose (20 g), KH_2PO_4 (13.3 g), $(NH_4)_2HPO_4$ (4.0 g), $MgSO_4{\cdot}7H_2O$ (1.2 g), citric acid (1.7 g), EDTA (14.1 mg), $CoCl_2{\cdot}6H_2O$ (2.5 mg), $MnCl_2{\cdot}4H_2O$ (15.0 mg), $CuCl_2{\cdot}2H_2O$ (1.5 mg), H_3BO_3 (3.0 mg), $Na_2MoO_4{\cdot}2H_2O$ (2.1 mg), $Zn(CH_3COO)_2{\cdot}2H_2O$ (33.8 mg), Fe(III) citrate (100.8 mg), and thiamine HCl (4.5 mg). The culture media was supplemented with the antibiotics Amp 100 μg/mL (ampicillin) and Gen 20 μg/mL (gentamicin) for *E. coli* Arctic Express and Amp 100 μg/mL and Chl 34 μg/mL (chloramphenicol) for *E. coli* pLysS (Silva et al., 2013).

Experiments using fed-batch mode started as a batch mode as described above. After this phase a feed medium was used with the following per liter composition: glucose (500 g), KH_2PO_4 (13.3 g), $(NH_4).2HPO_4$ (4.0 g), $MgSO_4{\cdot}7H_2O$ (20 g), citric acid (1.7 g), EDTA (28.2 mg), Fe(III) citrate (201.6 mg), thiamine HCl (4.5 mg), and 2 mL of trace element solution containing per liter: $CoCl_2{\cdot}6H_2O$ (2.5 g), $MnCl_2{\cdot}4H_2O$ (15.0 g), $CuCl_2{\cdot}2H2O$ (1.5 g), H_3BO_3(3.0 g), $Na_2MoO_4{\cdot}2H_2O$ (2.1 g) and $Zn(CH_3COO)_2{\cdot}2H_2O$ (33.8 g).

Inoculum

The inoculum was prepared by transferring 50 μL of cryopreserved cell suspension to a 250 mL baffled or plain Erlenmeyer flask containing 50 mL of sterile modified 2xYT culture medium, with incubation overnight at 37 °C and 200 min^{-1} (Ecotron, Infors HT, Switzerland). A known volume of the resulting bacterial suspension was transferred to Erlenmeyer shake flasks or the bioreactor using a 2 % inoculum/volume of culture media.

Shaking flasks scFv production in HDF

Growth evaluation of recombinant *E. coli* was performed using HDF medium in 250 mL baffled Erlenmeyer flasks (50 mL working volume), inoculated with 1 mL of bacterial suspension prepared in modified 2 ×

YT culture medium as described above. The strains were evaluated for expressing the scFv fragment under induction using 0.1 mM IPTG or a lactose concentration of 10 g/L. IPTG is a non-metabolizable analog of allolactose, the natural inducer of the lac operon, and binds tightly to the LacI repressor, ensuring rapid and sustained induction of gene expression independent of cellular metabolism (Rosano and Ceccarelli, 2014). Lactose, on the other hand, is converted intracellularly by β-galactosidase into allolactose, which in turn binds LacI and promotes induction in a more gradual and physiological manner (Wheatley et al., 2013). Although the natural inducer of the system is allolactose, its direct use in expression assays is unfeasible due to its instability, low commercial availability, and short extracellular half-life (Huber et al., 1975).

Growth was analyzed every 0.5 h by measuring optical density of samples at OD600 nm up to approximately 0.6. Induction was then performed for 18 h, reducing the temperature to 18 °C.

Bioreactor scFv production in HDF

Table 1 summarizes the eight experimental runs (F1–F8) of growth and expression using the ArcticExpress and pLysS strains, performed in a 2.5 L bench-scale bioreactor (Bioflo 310, New Brunswick Scientific, USA) with 1.5 L of HDF culture medium, inoculated with 2 % (v/v) of a bacterial suspension prepared as described above.

Growth phases were conducted at temperatures ranging from 18 °C to 37 °C, depending on the experimental condition. Once cultures reached a predetermined optical density, induction was carried out for 18 h at 18 °C, using IPTG at a concentration of 0.5 mM for batch runs (F1–F4) and 0.1 mM for fed-batch runs (F5–F8).

The dissolved oxygen (DO) setpoint was maintained at 20 % for all fed-batch runs and ranged from 22 % to 52 % in batch runs. Agitation rates were dynamically adjusted between 200 and 550 min^{-1} to ensure adequate oxygenation throughout cultivation.

Recovery and purification procedures

The samples from experiments were first centrifuged at 4,500 × g for 60 min using a Hettich Rotina 35R centrifuge (Germany). The resulting pellets were then lysed for 60 min in 50.8 mL of a modified lysis buffer solution (PBS buffer pH 8.0 (50 mM NaH_2PO_4, 300 mM NaCl); 40 mM Imidazole; 0.5 % Triton 100; 10 mM $MgCl_2$; 1 % PIC 100 (100 mM PMSF and 100 mM benzamidine); 0.2 mg/mL lysozyme and 1 μL/sample benzonase) on a shaking ice bath. The total cell lysete material was submitted to another centrifugation at 4,500 × g for 60 min and the resulting supernatant containing the scFv fragments was collected for purification. Purification used an AKTA Prime Plus chromatographic system (Cytiva, USA) with a 5 mL His-Trap nickel resin column. The sample was washed with Buffer A (PBS buffer and 40 mM imidazole) and eluted with Buffer B (PBS buffer and 250 mM imidazole) to isolate the desired fragments.

Analytical procedures

Cell concentration was determined by measuring the dry weight of pellets obtained after centrifugation (10,000 × g, 10 min) and drying at 80 °C for 18 h. These values were used to construct a calibration curve correlating dry cell mass (g/L) with optical density readings at 600 nm (OD_{600}), enabling indirect estimation of biomass concentration during cultivation.

Proteins in the eluted fraction obtained after affinity chromatography were quantified according to Bradford method (Bradford, 1976) in a microplate reading (DMS/Synergy H1M, USA) at 595 nm using a BSA standard curve.

The eluted proteins were also analyzed by 12 % SDS-PAGE to assess the purity of the scFv. The electrophoretic assay was performed in eluted samples from the separation process, denatured for 5 min in sample buffer containing bromophenol blue at 95 °C, followed by application to a 12 % w/v SDS-PAGE gel. The run was performed for about 1.5 h at a current of 50 mA and voltage of 80 V to 110 V (Bio-Rad Vertical Electrophoresis System, USA). At the end of the run, the gel was stained using a Coomassie Brilliant Blue G-250 dye solution (Bio-Rad, USA) for 1 h, under gentle agitation. It was then decolored in ethanol and acetic acid solution until the bands were revealed. The gel was stored at 4 °C in a container with ultrapure water.

Yield coefficient Yp/x was calculated as the ratio between the mass of purified protein recovered (mg/L) and the corresponding biomass concentration (g/L) measured at the end of cultivation. This parameter reflects the efficiency of scFv production per unit of cell mass under each experimental condition. The protein concentration refers specifically to the eluted fraction obtained after affinity chromatography and was quantified by the Bradford assay. Maximum specific growth rate (μmax) was estimated during the exponential phase of cell growth by plotting the natural logarithm of biomass concentration over time [$Ln(X/X_0)$] and determining the slope of the resulting linear regression. Biomass concentration (X) was derived from a standard curve correlating optical density at 600 nm with dry cell weight (g/L), based on gravimetric analysis of centrifuged and dried culture samples.

Indirect ELISA for scFv functional evaluation

ELISA assays were performed to evaluate the functional activity of the produced scFv fragments and are described below.

MaxiSorp 96-well plates (Sigma-Aldrich) were coated with purified Stx2 toxin in twofold serial dilutions starting at 100 μg/mL in PBS and incubated overnight at 4 °C. After blocking with PBS + 1 % BSA for 1 h under agitation at room temperature (RT), purified scFv (40 μg/mL in PBS-T) was added and incubated for 45 min at RT.

Table 1
Summary of the experimental conditions for runs F1 to F8 performed in bioreactor assays. Parameters include *E. coli* strain, cultivation mode (batch or fed-batch), dissolved oxygen, temperatures during the growth and induction phases, temperature during the fed-batch stage (when applicable), agitation rates, IPTG concentration used for induction, and optical density at the time of induction.

Run	Strain	Mode	Dissolved O2 (%)	Temperature during growth/ induction (°C)	Temperature during fed-batch mode (°C)	Induction (mM IPTG)	OD_{600} at induction phase
F1	ArcticExpress	Batch	52.3	37/18	–	0.5	0.8 – 1.0
F2	ArcticExpress	Batch	32.0	37/18	–	0.5	0.8 – 1.0
F3	pLysS	Batch	22.0	37/18/18	–	0.5	0.8 – 1.0
F4	pLysS	Batch	20.0	37/18	–	0.5	0.8 – 1.0
F5	pLysS	Fed-batch	20.0	37/18	37	0.1	25 – 30
F6	pLysS	Fed-batch	20.0	37/18	37	0.1	25 – 30
F7	pLysS	Fed-batch	20.0	37/18	18	0.1	25 – 30
F8	pLysS	Fed-batch	20.0	37/18	37	0.1	25 – 30

Wells were then incubated with anti-Flag-HRP antibody (1:5000 in PBS) for 30 min at RT. Detection was performed with OPD (1 mg in 2 mL citrate/phosphate buffer + 1 μL H_2O_2) for 15 min in the dark at RT. The reaction was stopped with 1 N HCl, and absorbance was measured at 492 nm. All assays were performed in duplicate.

Economic assessment based on obtained data

The hypotheses used for the economic evaluation were as follows: a) 500,000 doses/year, b) 500 mg active agent/dose, and c) internal rate of return of 15 % per year over 10 years of amortization. The scenarios for this preliminary economic analysis used the data from experiments, considering the cell concentration (X), the anti-Stx2 scFv concentration in the culture broth (Pb), the fermentation time plus the downtime (t), and the mass yield of protein/mass of cell (Yp/x). The costs of the equipment used at different production scales were calculated using Equation (1):

$$Ct = Co\ (Bt/Bo)^{\delta} \quad (1)$$

where Ct and Co are the equipment costs at different scales, Bt and Bo, and δ is a scale factor with a value of 0.6. The scenarios in the economic analysis used data obtained in the experiments performed previously. The equipment, raw materials, and utility costs were obtained from the literature (Lebozec et al., 2018) and local suppliers. The analysis was performed using Excel spreadsheets (Microsoft, USA).

Results and Discussion

Expression of scFv

The production of monoclonal antibodies using less demanding microorganisms, such as various strains of *E. coli* has evolved in recent years as a promising alternative to animal cells, particularly to produce antibody fragments. This approach shows great potential for the development of diagnostic kits and medicines (Spadiut et al., 2014).

Several factors influence the biosynthesis of recombinant products, including the choice of recombinant *E. coli* strain, the composition of the culture medium, and the type of inducer used for protein expression. Preliminary tests were conducted to evaluate these factors, using IPTG and lactose as inducers in three different recombinant *E. coli* platforms: BL21 (DE3), BL21 (DE3) pLys, and Arctic Express. The strains were cultivated in a defined culture medium, adapted from Silva *et al.* (2013) (Silva et al., 2013), widely used for recombinant protein synthesis in *E. coli* and referred to here as HDF.

The recombinant *E. coli* strains were grown to an optical density (O. D.) of approximately 0.6, followed by an 18-hour induction period at 18 °C, using either IPTG (0.1 mM) or lactose (10 g/L) as inducers.

Table 2
Obtained results of recombinant *E coli* BL21 (DE3) pLysS and *E coli* (DE3) Artic scFv production in baffle shaking flasks using HDF-defined culture media induced with 0.1 mM IPTG. Pb: protein concentration in the eluted fraction (mg/L) relative to the original culture volume determined by the Bradford assay at 595 nm. The values of Yp/x, the mass of protein produced/mass of cells obtained, were calculated with the data obtained on protein concentration in the fermented medium (Pb)/cell concentration (X), inform the efficiency of protein production for each condition, and tested construction. The μmax values, maximum specific growth rate pre-induction, were accessed by graphing Ln (X/Xo) against time.

Strain/Induction	μmax (h^{-1})	Pb (mg/L)	X (g/L)	Yp/x (mg/g)
BL21/IPTG	0.57	0.226	4.39	0.052
BL21/lac	0.60	0.066	4.18	0.016
pLysS/IPTG	0.54	0.280	4.08	0.069
pLysS/lac	0.57	0.066	4.12	0.016
Arctic/IPTG	0.55	0.440	4.02	0.110
Arctic/lac	0.46	0.269	4.52	0.060

Table 2 summarizes the results of these experiments.

The final cell concentrations achieved across all strains were comparable, ranging between 4.0 and 4.5 g/L. Also, the maximum specific growth rate calculated during the exponential phase were about the order of magnitude with mean value of 0.55 h^{-1}. On the other hand, a significant difference in expression levels was observed between the samples, depending on the *E coli* and induction used.

The highest production levels (Pb) were achieved with IPTG (0.1 mM) induction in *E. coli* Arctic Express, yielding up to 0.44 mg/L of protein, followed by *E. coli* pLysS and BL21, which produced approximately 0.2 mg/L. The calculated product yields per unit biomass (Yp/x) were 0.110, 0.069, and 0.052 mg protein/g cell for *E. coli* Arctic Express, *E. coli* pLysS, and *E. coli* BL21, respectively. In contrast, lactose induction at 10 g/L was less effective than IPTG induction at 0.1 mM. Although lactose induction resulted in slightly higher final cell concentrations compared to IPTG, the product yield (Yp/x) was substantially lower across all tested *E. coli* strains.

Fig. 1 shows the results of SDS-PAGE electrophoresis analyses performed after the lysis and purification steps. The lysate supernatant SDS-PAGE analysis was conducted on recombinant *E. coli* synthesizing the scFv-stx2 fragment in HDF-defined culture medium for 18 h at 18 °C, using either IPTG (0.1 mM) or lactose (10 g/L) as inducers. A prominent band at 25 kDa was observed in lane 7, corresponding to *E. coli* Arctic Express induced with IPTG (0.1 mM). This result corroborates the higher protein concentration (Pb = 0.440 mg/L) reported in Table 2 for this condition. Lane 6, corresponding to *E. coli* pLysS induced with 0.1 mM IPTG, also displayed a less intense band, warranting further attention.

Based on these findings (Table 2, Fig. 1), the recombinant strains *E. coli* Arctic Express and *E. coli* pLysS were selected for experiments in a bench-scale bioreactor. The HDF-defined culture medium was used in both batch and fed-batch modes, with induction carried out using IPTG (0.1 mM). The characteristics and results of each experiment are presented and discussed below.

Functional evaluation of scFv binding to Stx2 by indirect ELISA

The indirect ELISA results demonstrated that the anti-Stx2 scFv was able to specifically recognize the target toxin. A concentration-dependent binding response was observed, with mean absorbance values decreasing progressively as the Stx2 concentration was reduced: 0.580 for 100 μg/mL, 0.313 for 50 μg/mL, and 0.175 for 25 μg/mL. The lowest concentration tested (0.78125 μg/mL) yielded a mean absorbance of 0.048. The data showed good reproducibility between duplicates, with standard deviations below 0.02 for all points (Fig. 2).

The binding profile exhibited a typical saturation curve, with a gradual decrease in signal as the toxin concentration declined. These results indicate that the recombinant scFv displays specific affinity for Stx2, confirming its functionality after expression and purification.

Bioreactor batch experiments

Bioreactor batch experiments were conducted using the two best-performing strains from the previous shaking flask experiments. The results are summarized in Fig. 3 and Table 3, as described below.

Fig. 3 presents four plots illustrating the evolution of dissolved O_2 concentration and the natural logarithm of relative biomass concentration ($Ln(X/X_0)$) over time for the bioreactor batch production of scFv with recombinant *E. coli* in HDF-defined culture medium: *E. coli* Arctic Express in experiments (a) F1 and (b) F2, and *E. coli* pLysS in experiments (c) F3 and (d) F4.

Experiment F1 was conducted in batch mode with Arctic Express. Cultivation was maintained at 37 °C until induction, with pH controlled at 7.0 using 4 N H_2SO_4 and 4 N NaOH. Dissolved oxygen concentration was maintained at 50 % air saturation by adjusting agitation rates between 200 and 550 min^{-1}. Exponential growth persisted for approximately 7 h, reaching a biomass concentration of 1.9 g/L, with a

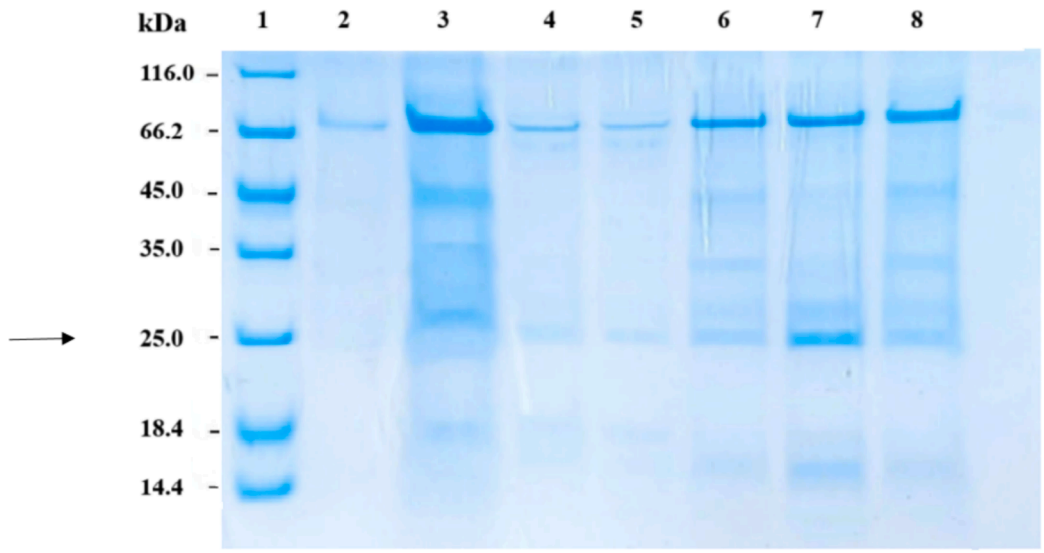

Fig. 1. Lysate supernatant SDS-PAGE electrophoresis of the recombinant *E. coli* synthesizing the scFv-stx2 fragment in HDF-defined culture medium for 18 h, at 18 °C; IPTG 01 mM or lactose 10 g/L. Lanes: 1 – marker, 2 – empty, 3 – BL21/IPTG, 4) BL21/lactose, 5 – pLyss/ lactose, 6 – pLyss/IPTG, 7 – Arctic/IPTG, 8 – Arctic/ lactose. The marker axis indicated the molar mass of 25 kDa corresponding to the scFv.

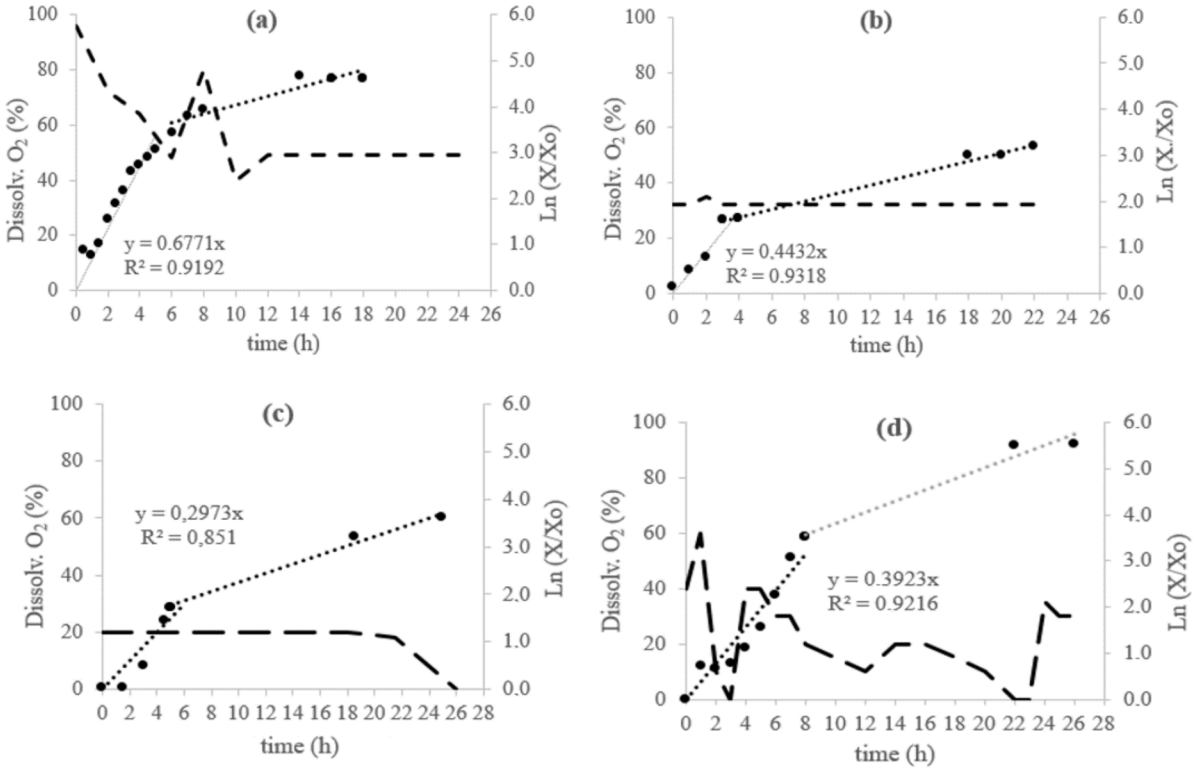

Fig. 2. Indirect ELISA assessing the binding capacity of recombinant anti-Stx2 scFv to Shiga toxin 2. After incubation with purified scFv (40 μg/mL) and detection with anti-Flag-HRP, absorbance was measured at 492 nm. Data represent mean values ± standard deviation of duplicates. The curve shows a concentration-dependent binding profile, confirming the specific interaction between the scFv and its target antigen.

maximum specific growth rate (μmax) of 0.677 h^{-1} (Fig. 3a). Induction with 0.1 mM IPTG occurred during the exponential phase at cell concentrations between 1.0 and 1.5 g/L. After induction, the temperature was reduced to 18 °C, slowing cell growth over the subsequent 18 h (Fig. 3a). At the end of the experiment, the scFv fragment concentration reached 4.03 mg/L, with a final cell concentration of 3.88 g/L and a yield coefficient (Yp/x) of 1.03 mg protein/g cells (Table 3).

Experiment F2 replicated the parameters of F1, except for the dissolved oxygen profile, which was adjusted to maintain a minimum of 30 % air saturation during induction. Agitation rates were adapted to keep dissolved oxygen levels around 20–30 % air saturation throughout cultivation (Fig. 3b). In F2, the cell concentration reached 2.4 g/L, approximately half that of F1, and μmax during exponential growth decreased to 0.443 h^{-1} (Fig. 3b). Despite the lower growth rate, a higher scFv concentration of 3.4 mg/L was achieved post-induction, with a yield coefficient (Yp/x) of 1.57 mg protein/g cells over the 14-hour

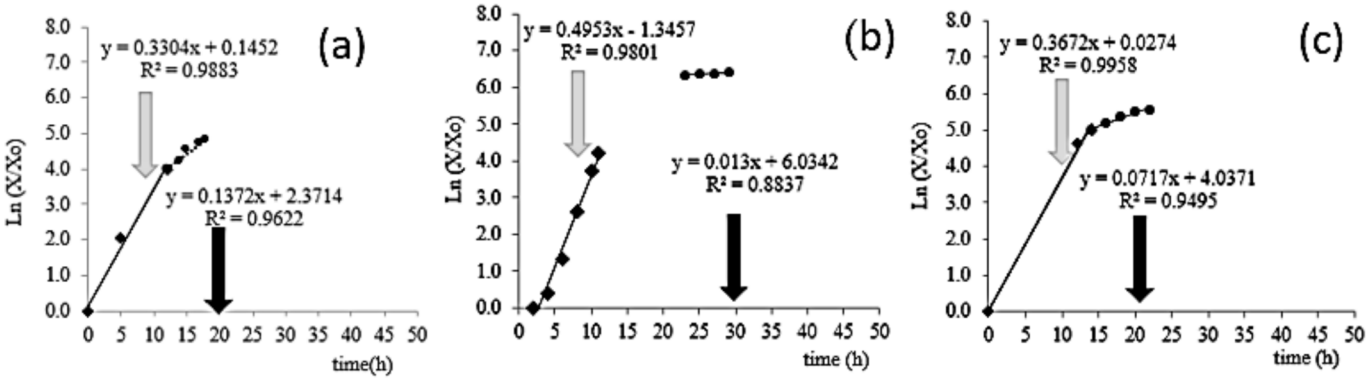

Fig. 3. Evolution of dissolved O_2 concentration (dashed lines) and cell growth described by $Ln(X/X_0)$ (dotted lines) with time for bioreactor batch production of scFv with recombinant *E. coli* in HDF defined culture media: (a) F1, *E. coli* Arctic Express; (b) F2, *E. coli* Arctic Express; (c) F3, *E. coli* pLysS; and (d) F4, *E. coli* pLysS.

Table 3
Results of scFv-stx2 production assays carried out in batch mode stirred tank bioreactor using recombinants *E. coli* Arctic Express and *E. coli* pLysS; HDF medium, induction by 0.1 mM IPTG for 18 h at 18 °C. Pb: protein concentration in the fermented medium (mg/L). The values of Yp/x, the mass of protein produced/mass of cells obtained, were calculated with the data obtained on protein concentration in the fermented medium (Pb)/cell concentration (X) and inform the efficiency of protein production for each condition and tested construction. The μmax values, maximum specific growth rate pre-induction, were accessed by graphing Ln (X/Xo) against time.

Run	Strain	Induction/growth time (h)	Dissolved O_2 (%)	Temperature growth/induction (°C)	Pb (mg/L)	Xf (g/L)	μmax pre-induction (h^{-1})	$Y_{P/X}$ (mg/g)
F1	ArcticExpress	6/24	52.3	37/18	4.03	3.88	0.68	1.03
F2	ArcticExpress	6/24	32.0	37/18	3.38	2.15	0.44	1.57
F3	pLysS	8/26	20.0	18/18	16.7	1.18	0.29	5.29
F4	pLysS	8/26	N.C.	37/18	6.25	5.75	0.39	3.00

N.C. Dissolved O_2 variable due to instability in automatic control (Fig. 3.d).

induction period (Table 3). These results suggest that reducing dissolved oxygen concentration, while lowering μmax, can enhance scFv biosynthesis — highlighting the critical role of oxygen availability in optimizing recombinant protein production in *E. coli*.

The primary difference between experiments F3 and F4 was the temperature applied during the growth phase: F3 was conducted at 18 °C, while F4 was performed at 37 °C. As expected, the increased temperature in F4 led to an enhanced *E. coli* growth rate, with calculated μmax values of 0.29 h^{-1} in F3 and 0.39 h^{-1} in F4 (Fig. 2c and 2d).

On the other hand, F4 exhibited dissolved oxygen concentration (DO %) instability, likely caused by oxygen transfer limitations and a delayed automatic oxygen control response, attained DO% values below 20 % during the induction phase, as shown in Fig. 3.d. It is possible that transient oxygen limitation redirected cellular metabolism impairing recombinant protein production (Hajihassan et al., 2018; Araújo et al., 2019).

Interestingly, the experiments using the *E. coli* pLysS demonstrated that the decrease in μmax observed in growth phase of experiments F3 (Fig. 3c) and F4 (Fig. 3d) from approximately 0.39 h^{-1} to 0. 0.29 h^{-1} corresponded to an increase in the Yp/x value from 3.00 mg/g to 5.29 mg/g (Table 3).

The best results in terms of Yp/x achieved with the pLysS strain (Table 3) led us to used it in the fed batch high cell density experiments.

Bioreactor Fed-Batch experiments

Process intensification through high cell-density bioreactor cultivation is widely recognized as a powerful strategy for reducing bioproduct costs. In this context, fed-batch experiments were conducted with the *E. coli* pLysS strain, which demonstrated promising Yp/x values in low-cell-density batch experiments (Table 2).

Assays F5 to F8 were performed using the HDF culture medium, optimized for high cell-density cultivation, with a target biomass concentration of up to 50 g/L in fed-batch mode. The process began with batch cultivation in HDF medium, as described in Section 2.1.2, followed by a fed-batch phase with a constant feed flow rate. The feed rate during the pre-induction phase was adjusted based on the experiment, aiming to achieve a cell density of approximately 25–30 g/L before induction. At this point, the feed flow rate remained constant, the temperature was reduced to 18 °C, and IPTG was added to a final concentration of 0.1 mM. The process continued for 18 h to induce recombinant protein expression, following the approach established in previous experiments. Dissolved oxygen level was maintained at 20 %. The results are summarized in Fig. 4 and Table 4.

In assay F5, exponential growth occurred for approximately 14–15 h post-inoculation, reaching a cell concentration of ~ 15 g/L with a calculated maximum specific growth rate (μmax) of 0.33 h^{-1} (Fig. 4a). During the fed-batch phase, μmax decreased to 0.13 h^{-1} between 15 and 22 h. IPTG induction was initiated at this point, and cultivation continued until a final cell concentration of 40 g/L was achieved. The scFv protein concentration in the fermentation broth reached 120 mg/L, with a calculated yield coefficient (Yp/x) of 3 mg/g of cells (Table 4).

In contrast, assay F6 exhibited markedly different behavior due to a protocol modification. In this experiment, the exponential growth phase was shortened, and fed-batch mode began when the cell concentration reached ~ 8 g/L (Fig. 4b). IPTG induction occurred only after 29 h of cultivation, once the biomass concentration had reached ~ 40 g/L. Notably, both the protein concentration and Yp/x values were 40–50 times lower than those observed in assay F5 (Table 4). This outcome appears to be related to the significantly lower μmax observed during the pre-induction phase (0.013 h^{-1}), which was approximately 10 times lower than in F5. Assay F7 followed a modified fed-batch protocol, starting with a pre-induction phase at a reduced temperature of 18 °C. The initial growth phase lasted 14 h, producing a biomass concentration of ~ 7 g/L. The fed-batch phase continued at 18 °C, with μmax decreasing to ~ 0.062 h^{-1} between 16 and 22 h (Fig. 4c). IPTG induction

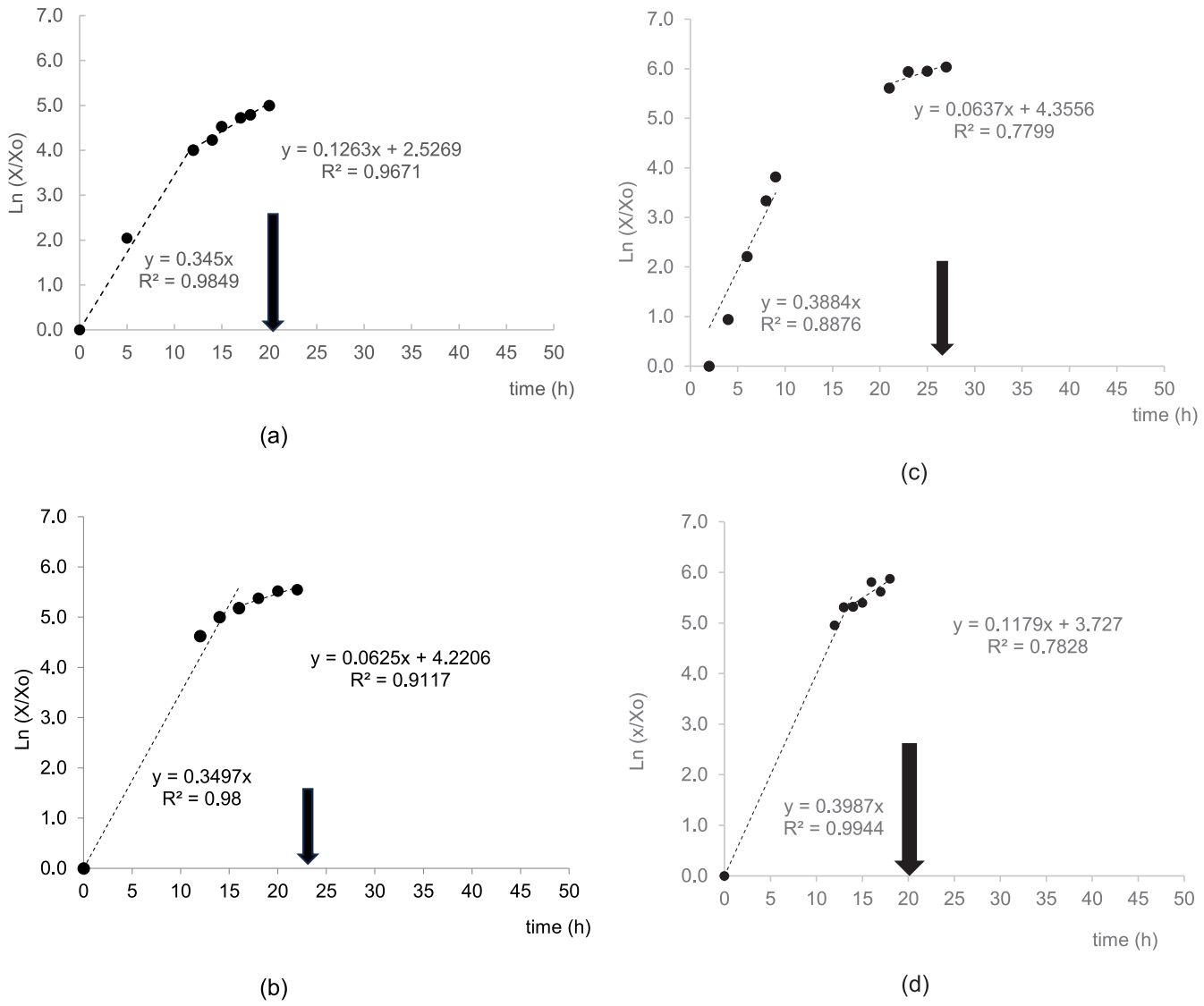

Fig. 4. Ln (X/Xo) with time course during the growth phase in fed-batch mode scFv production with recombinant *E. coli* pLysS in HDF-defined culture media: (a) run F5; (b) run F6, (c) run F7 and (d) run F8 (replicate of F5). The arrows indicate the time of induction.

Table 4
Results of scFv-stx2 production fed-batch mode assays carried out in a stirred tank bioreactor using *E. coli* pLysS; HDF medium, induction by 0.1 mM IPTG for 18 h, at 18 °C. Pb: protein concentration in the fermented medium (mg/L). The values of Yp/x, the mass of protein produced/mass of cells obtained, were calculated with the data obtained on protein concentration in the fermented medium (Pb)/cell concentration (Xf) and inform the efficiency of protein production for each condition and tested construction. The μmax values, maximum specific growth rate pre-induction, were accessed by graphing Ln (X/Xo) against time. Temperature profile indicates values at cell growth phase during exponential and fed-batch mode, followed by induction phase.

Run	Induction time/Final time (h)	Temperature profile (°C)	Pb (mg/L)	Xf (g/L)	μmax pre-induction (h^{-1})	$Y_{P/X}$ (mg/g)
F5	20/38	37/37/18	138.0	39.6	0.137	3.49
F6	29/47	37/37/18	46.0	44.8	0.013	1.02
F7	24/42	37/18/18	12.0	11.1	0.072	1.08
F8	18/26	37/37/18	148.0	36.4	0.118	4.06

(0.1 mM) was initiated during this period, and cultivation proceeded for a total of 42 h. The final protein concentration was 11.5 mg/L, with a biomass concentration of 11 g/L and a Yp/x value of 1.04 mg/g (Table 4).

Experiment F8 was ran as a replicate of F5 assay, and was conducted under condition same to those of run F5, particularly with respect to the pre-induction specific growth rate, as well as identical temperature, dissolved oxygen, and inducer concentrations.

The close similarity observed in the final biomass concentration (X_f), maximum specific growth rate prior to induction (μmax pre-induction), and yield coefficient ($Y_{p/x}$) (Table 4), as well the time profile between the two runs (Fig. 3.a and 3.d) confirms the reproducibility of the developed process.

It is important to highlight the obtained values of Pe equal 138 and 148 mg/L, as well $Y_{p/x}$ of 3.49 and 4.06 mg/g, respectively for F5 and F8 experiments, validated the proposed high-cell-density protocol.

Moreover, present results highlight the impact of growth conditions, induction timing, and oxygen availability on recombinant protein production in high-cell-density fed-batch processes. Notably, assay F5 and F8 demonstrated the best performance, suggesting that balancing growth rate and biomass accumulation before induction is critical for optimizing protein yields.

Comparison with literature data

Table 5 summarizes typical results found in the literature to produce scFv against various targets using recombinant *E. coli* expression systems, compared with the best results obtained in this study.

Jeong *et al.* (Jeong and Rani, 2011) reported high-cell-density cultivations of *E. coli* XL1-Blue for preparative-scale production of the M18 scFv antibody fragment against anthrax toxin PAM18, using a fed-batch pH-stat feeding strategy in a bench-scale fermenter (Araújo et al., 2019). The culture was grown in a defined medium with 2 % (w/v) glucose as the carbon source at 37 °C during the growth phase, followed by induction at 25 °C with 1.0 mM IPTG and 100 g/L yeast extract. This strategy resulted in high post-induction cell growth and scFv production of approximately 665 mg/L.

Petrus *et al.* used an *E. coli* BL21 strain with a rhamnose-inducible expression system for periplasmic production of an anti-HIV scFv (Petrus et al., 2019). Batch cultivation in TB medium (containing yeast extract, peptone, and phosphate salts) at 37 °C was followed by induction with 10 mM L-rhamnose at OD600 of 0.6–0.8, reducing the temperature to 25 °C. This process yielded 762 mg/L of scFv, with a Yp/x of 33 mg/g cell dry weight at a cell concentration of ~ 20 g/L.

Bu *et al.* evaluated scFv production against human brain natriuretic peptide (BNP) using *E. coli* Rosetta (DE3) pLysS cells grown in LB medium (Bu et al., 2013). After IPTG induction (1 mM) at OD600 0.4–0.6, they achieved a scFv concentration of 10.2 mg/L, with Yp/x = 3 mg/g cell. For anti-HER2 scFv production, *E. coli* BL21 (DE3) cells were cultivated in TB medium at 37 °C, induced at OD600 ~ 0.6 with 0.2 mM IPTG at 15 °C, and supplemented with 0.5 % Triton X-100 and 0.05 % Tween-20 to promote extracellular secretion. Despite the low yield (2.5 mg/L), this method demonstrated the potential for extracellular scFv production (Hyun et al., 2021).

Aghdam *et al.* explored the periplasmic expression of an anti-G17-Gly scFv using *E. coli* HB2151 in 2xYT medium, with IPTG induction ranging from 0.05 to 1 mM with a DoE approach, evaluating the impact of temperature, OD_{600} at induction, induction time, and IPTG concentration. scFv production ranged from 0.4 to 15 mg/L depending on the tested conditions. Additional optimization revealed that the highest levels of functional scFv expression were achieved either when using TB medium or by supplementing the culture medium with 0.5 M sorbitol or 100 mM glycine betaine (Aghdam et al., 2021).

Kasli *et al.* used *E. coli* BL21 as the host strain to express an scFv antibody fragment targeting 13R4 β-galactosidase. Expression was induced with 0.05–2 % arabinose in TB medium, resulting in scFv concentrations ranging from approximately 2 to 20 mg/L. A design of experiments (DoE) approach was applied to optimize variables such as temperature, inducer concentration, and induction point. The study also compared periplasmic targeting efficiency using PelB and DsbA signal peptides, with PelB showing superior performance in terms of scFv solubility. The highest scFv production was observed under conditions of low temperature and low inducer concentration (Kasli et al., 2019).

Overall, literature results typically report Yp/x values of 3–5 mg/g cell dry weight under IPTG induction (up to 1 mM) (Araújo et al., 2019; Hyun et al., 2021), which aligns with our findings (Table 5). However, Petrus *et al.* achieved exceptionally high productivity (Yp/x = 33 mg/g) and scFv titers (760 mg/L) with the rhamnose-inducible system, and Jeong *et al.* reported 665 mg/L with IPTG induction supplemented with 100 g/L yeast extract (Petrus et al., 2019; Jeong and Rani, 2011).

These discrepancies likely stem from differences in plasmid constructs, induction types, scFv sequences, *E. coli* strains, media compositions, and cultivation conditions. Notably, using *E. coli* BL21 with rhamnose induction or *E. coli* XL1-Blue with yeast extract supplementation enhanced Yp/x values (Petrus et al., 2019; Jeong and Rani, 2011).

Economic assessment based on obtained data

An economic scFv-stx2 cost production assessment was performed using the obtained data. It is important to emphasize that this a preliminary evaluation. Nevertheless, this preliminary result may be served as a guide to the process development. As indicated in the literature selecting a production process that ensures quality and safety is essential, but equally critical is evaluating manufacturing costs early, as process changes can re-strict market access (Walsh, 2018; Rathore and Winkle, 2009; Papathanasiou and Kontoravdi, 2020). Subsequently, the process would advance to scale-up, with optimal conditions being established in larger-capacity fermenters, followed by a cost analysis, which could ultimately alter the production cost.

Table 5
Comparative results of scFv production in recombinant *E. coli.*

scFv target	*E. coli* strain	Plasmid vector	Expression site	Culture mode/ Equipment	Culture media/ Induction	scFv (mg/ L)	Yp/x (*) (mg/ g)	Ref.
BNP	Rosetta (DE3) pLysS	pET22b(+)	Cytoplasm	Batch/Shaking flask	LB/IPTG 1.0 mM	10.2	3.0	(Bu et al., 2013)
HER2	BL21 (DE3)	pCold-1-Mv	Extracellular	Batch/Shaking flask	TB/IPTG 0.2 mM	2.5	––	(Hyun et al., 2021)
HIV	BL21	pD881-SR	Periplasm	Batch/ Bioreactor	TB/L-rhamnose 10 mM	760	33.0	(Petrus et al., 2019)
Anthrax toxin	XL1-Blue	pMoPac16	Periplasm	Fed-batch/ Bioreactor	R2/IPTG 1.0 mM + 100 YE g/L	665	30.0	(Jeong and Rani, 2011)
G17-Gly	HB2151	pIT2	Periplasm	Batch/Shaking flask	2xYT/IPTG 0.05 – 1 mM	0.4 – 15	––	(Aghdam et al., 2021)
13R4 (β-gal)	BL21	pLBAD2-DsbA-scFv13R4 + A and pLBAD2-PelB-scFv13R4 + A	Periplasm	Batch/Shaking flask	TB/Arabinose 0.05 – 2 %	2 – 20	––	(Kasli et al., 2019)
Stx2	pLysS	pscFv-Stx2	Periplasm	Batch/ Bioreactor	HDF/IPTG 0.1 mM	17.0	2.9	This work
Stx2	pLysS	pscFv-Stx2	Periplasm	Fed-batch /Bioreactor	HDF/IPTG 0.1 mM	120.0	3.0	This work

(*) 1.0 OD600nm = 0.3 g/L.
(https://bionumbers.hms.harvard.edu/bionumber.aspx?s = n&v = 2&id = 109837).
TB: Terrific Broth culture media.
LB: Luria-Bertani culture media.
R2: Defined culture media (Jeong *et al.*, 2011).
HDF: Defined culture media, as described in 2.2.
β-gal: β-galactosidase.

Fig. 5 presents a simplified flowchart outlining the primary steps proposed for the biosynthesis, separation, and purification of the scFv-stx2 fragment using genetically modified *E. coli*. The culture media were prepared and supplied to the bioreactors (a). The inoculum grown in the Seed bioreactor (b) was transferred to the Production bioreactor (c), where cultivation occurred at 37 °C in a defined culture medium (HDF defined culture me-dia), followed by induction with 0.1 mM IPTG at 18 °C. The cells were centrifuged (d) and sent to the Holding tank (e). The recovered cells were suspended in a lysis buffer, and the cell suspension was introduced into a high-pressure cell homogenizer system (f) to promote lysis of the biomass. The suspension was then pumped into a microfiltration system device, which separates cellular debris and produces a clarified effluent (g) sent to an IMAC column with Ni Sepharose 6 Fast Flow (Cytiva, USA) as the stationary phase (h). Elution of the column with buffer generates effluent rich in the scFv-stx2 fragment. If necessary, a lipopolysaccharide (LPS) removal chromatography column (Pierce™, Thermo Fisher, USA) eliminates residual endotoxin, and purification with a dialysis membrane removes residues from the culture medium (not depicted in the diagram).

The Scenarios F1 to F5 are based on the results of experiments F1 to F5 (Tables 3 and 4). The technical parameters and the cost of leading equipment of flowchart (Fig. 5) for each of the analyzed scenarios are depicted, respectively, in Table S1 and Table S2 in the Supplementary Material.

Table 6 details each of the items that make up the cost of scFv production for each of the different scenarios. The ROI (Return on Investment) was calculated using the PMT function in Excel, considering the equipment cost for each scenario (Table S2, Supplementary Material) and an internal rate of return of 15 % per year over a period of 10 years.

Scenarios F1, F2, and F3 were increase in the Yp/x increased about 10 times from *c.a* 0.5 to 5.0 mg/g and scFv productivity from 0.05 to 0.13 mg/g (Table S1, Suppl. Material). In consequence these optimizations reduced the production cost to US$ 2,320/g in scenario F3 down to US$ 1,430/g in scenario 4.

Scenario F4 is particularly noteworthy, as it demonstrates the pronounced impact of increasing cell concentration on production costs. Using the defined HDF medium and maintaining Yp/x at approximately 5 mg/g, a fivefold increase in cell concentration (Table 3) led to a substantial cost reduction, bringing the production expense down to US$ 660/g of scFv (Table 6). Notably, the relative costs of ampicillin, the IMAC phase, and IPTG were markedly reduced in this scenario.

Even greater cost efficiencies were achieved in scenario F5, which operated in fed-batch mode with an HDF culture medium designed for high-cell density cultivation (cell mass concentration around 40 g/L), while maintaining Yp/x at 3.5 to 4.0 mg/g (Table 4). This approach further reduced the production cost to about 270 US$ /g (Table 6).

It is expected that, during process scale-up, the results achieved in laboratory-scale bioreactors may not be fully reproducible due to system heterogeneity, which can lead to concentration gradients in the fermentation medium and negatively affect culture performance. For instance, Sandoval-Basurto et al. (2005) (Sandoval-Basurto et al., 2005) reported that dissolved oxygen concentration gradients had a detrimental effect on recombinant *E. coli* producing insulin, with significant losses in product yield when the circulation time — defined as the time required for a fluid element to complete a full loop through the mixing pattern generated by the agitation system — exceeded 20 s. In the present study, the bioreactor volume, based on the results of experiment F5, is estimated to be approximately 1.5 m^3 working volume (Table S1, Supplementary Material).

It is assumed that a bioreactor of this scale would be less prone to this phenomenon, provided it is properly engineered with suitable design parameters, such as the type, size, and number of impellers; an appropriate height-to-diameter ratio; sufficient agitation speed; an optimal power-to-volume ratio; and an adequate aeration rate.

Nonetheless, the present data underscore the significant impact of bioprocess parameters—particularly cell density and product yield—on production costs, highlighting the crucial role of optimizing upstream cultivation strategies to enable commercially viable scFv production.

It is also possible that the yields of the unit operations for separation and purification of the target product decrease during scale-up compared to bench scale. Although this parameter is challenging to estimate, assuming, for instance, an overall scFv recovery yield of approximately 50 % (equivalent to about 87 % yield per purification step), the production cost could rise to around US$ 550 per gram of scFv. Despite this increase, the cost would remain within the same order of magnitude as the reported production cost for antibody fragments in recombinant *E. coli*, estimated at US$ 296 per gram of protein (Rosano and Ceccarelli, 2014).

Conclusion

This study successfully established a scalable and cost-effective bioprocess to produce scFv-Stx2 using recombinant *E. coli* cultivated in simple, chemically defined media. The highest yield coefficients (Yp/x) were achieved with *E. coli* pLysS strains under both batch and fed-batch conditions.

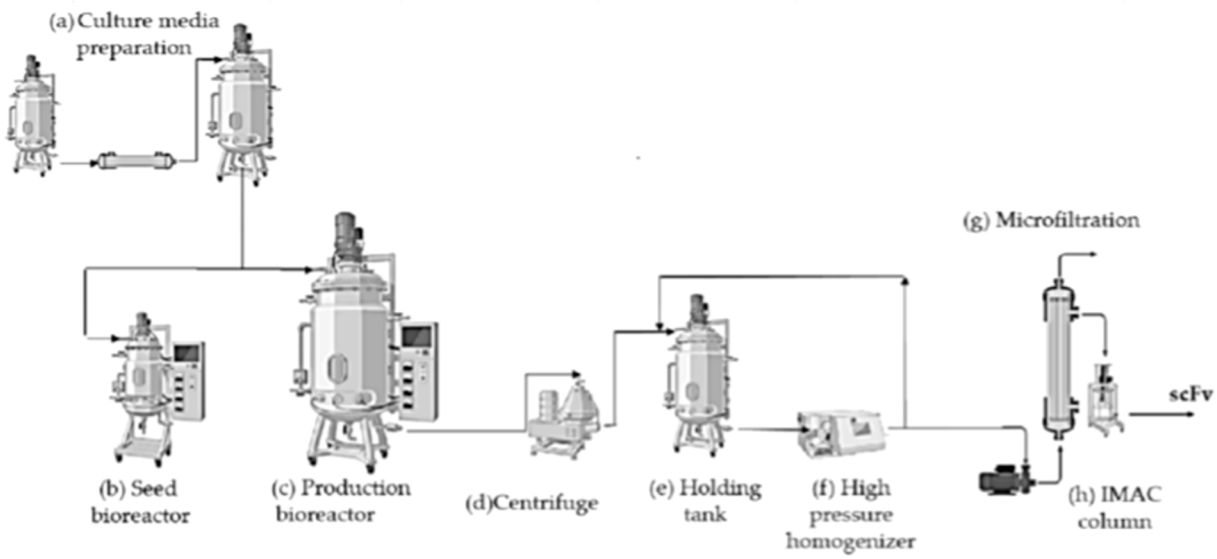

Fig. 5. Flowchart for production of scFv-stx2 using recombinant *E. coli*.

Table 6
Cost of scFv production using recombinant *E. coli* for scenarios F1 to F5.

		Scenario F1		Scenario F2		Scenario F3		Scenario F4		Scenario F5	
Item	**Unit**	**amount**	**Total**	**amount**	**Total**	**amount**	**Total**	**amount**	**Total**	**amount**	**Total**
ROI	US$		23,09,097.9		14,38,515.4		10,56,006.6		9,79,778.2		9,46,937.0
Managment	US$/y	1.0	1,44,000.0	1.0	1,44,000.0	1.0	1,44,000.0	1.0	1,44,000.0	1.0	1,44,000.0
Supervision	US$/y	4.0	3,45,600.0	4.0	3,45,600.0	4.0	3,45,600.0	4.0	3,45,600.0	4.0	3,45,600.0
Technician plant	US$/y	4.0	2,88,000.0	4.0	2,88,000.0	4.0	2,88,000.0	4.0	2,88,000.0	4.0	2,88,000.0
Technician lab	US$/y	2.0	1,44,000.0	2.0	1,44,000.0	2.0	1,44,000.0	2.0	1,44,000.0	2.0	1,44,000.0
Maintenance	10 % CAPEX/y	0.1	11,58,882.8	0.1	7,21,957.6	0.1	5,29,985.3	0.1	4,91,728.0	0.1	4,75,245.8
Glucose	kg	64,184.9	7,38,125.8	40,355.1	4,64,083.9	13,451.7	1,54,694.6	24,715.8	2,84,231.3	23,809.5	2,73,809.5
YE + Tryptone	kg	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Salts	kg	6,418.5	32,092.4	4,035.5	2,01,775.6	1,345.2	67,258.5	2,471.6	1,23,578.8	2,381.0	1,19,047.6
Ampicillin	kg	641.8	12,836,970.5	401.6	80,32,773.7	241.4	48,28,818.4	90.3	18,05,325.7	12.5	2,50,000.0
IPTG	kg	152.8	64,15,917.8	95.6	40,14,780.3	57.5	24,13,443.4	21.5	9,02,301.8	3.0	1,24,950.0
Lysis buffer	m3	855.8	17,11,596.1	535.5	10,71,036.5	321.9	6,43,842.5	120.4	2,40,710.1	16.7	33,333.3
Washing buffer	m3	2,567.4	51,34,788.2	1,606.6	32,13,109.5	965.8	19,31,527.4	361.1	7,22,130.3	50.0	1,00,000.0
Elution buffer	m3	171.2	3,42,319.2	107.1	2,14,207.3	64.4	1,28,768.5	24.1	48,142.0	3.3	6,666.7
Process water	m3	19,255.5	23,106.5	12,049.2	14,459.0	7,243.2	8,691.9	2,708.0	3,249.6	375.0	450.0
IMAC His-Tag	mL	5,70,532.0	22,821,280.8	357,012.2	14,280,486.6	214,614.2	85,84,566.0	80,236.7	32,09,467.9	16,666.7	6,66,666.7
LPS extraction	mL	5,70,532.0	0.0	357,012.2	0.0	214,614.2	0.0	80,236.7	0.0	16,666.7	0.0
MF membranes	m2	5.0	25,000.0	5.0	25,000.0	5.0	25,000.0	5.0	25,000.0	5.0	25,000.0
Electricity	kWh	11,30,783.1	2,17,458.3	977,048.8	1,87,894.0	777,770.4	1,49,571.2	732,000.0	1,40,769.2	732,000.0	1,40,769.2
Total			54,688,236		34,801,679		21,443,774		98,98,013.0		40,84,475.7
ScFv production cost (US$/g)			3,646		2,320		1,430		660		272

Process optimization, particularly the control of dissolved oxygen at 20 % air saturation and the fine-tuning of specific growth rates pre-induction, led to substantial improvements in protein yield and overall productivity. These strategies not only enhanced process efficiency but also contributed directly to reducing production costs, underscoring the critical role of precise bioprocess control.

The economic assessment identified key cost drivers, particularly the relationship between the IMAC His-tag purification step and protein output, which significantly influences overall production costs. Addressing this by improving product concentration upstream is essential. Furthermore, the data suggest that exploring higher IPTG concentrations is economically viable, as its contribution to production costs at high cell densities remains modest (~3%), offering room to evaluate potential gains in productivity.

As the process intensifies, capital costs (CAPEX) become increasingly relevant. Consequently, maximizing critical performance metrics such as volumetric productivity (mass of scFv per bioreactor volume per unit time) and product concentration (mass of scFv per bioreactor volume) should be prioritized. This can be achieved through further optimization of operational parameters—including feeding profiles, temperature, and dissolved oxygen levels during both growth and induction phases—as well as through genetic improvements to enhance Yp/x.

Overall, the findings demonstrate that targeted bioprocess and strain optimization can significantly improve the economic feasibility of scFv-Stx2 production. Continued efforts in these areas will be pivotal for enabling large-scale, cost-effective manufacturing of recombinant antibody fragments.

CRediT authorship contribution statement

Marcela Guimarães: Methodology, Writing – review & editing. **Daniela Luz:** Conceptualization, Writing – original draft. **Elisabeth de Fátima Pires Augusto:** Conceptualization, Writing – original draft. **Lucia Vieira:** Writing – review & editing. **Maricilia Silva Costa:** Writing – review & editing. **Roxane Maria Fontes Piazza:** Conceptualization, Writing – original draft, Formal analysis. **José Geraldo da Cruz Pradella:** Conceptualization, Writing – original draft, Formal analysis.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: This work was supported by FAPESP (Grant number 2019/25623-7). The authors declare they have no financial or competing interests. All authors contributed to the study conception and design. All authors read and approved the final manuscript. All data generated or analyzed during this study are included in this published article and its supplementary information files.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crbiot.2025.100310.

Data availability

Data will be made available on request.

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