High cell density cultivation of *E. coli* in shake flasks for the production of recombinant proteins

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A B S T R A C T

Batch cultivation of recombinant bacteria in shake flasks typically results in low cell density due to nutrient depletion. Previous studies on high cell density cultivation in shake flasks have relied mainly on controlled release mechanisms. Here, we report a true fed-batch strategy to achieve high cell density of recombinant *E. coli* in shake flasks in 24 h by feeding a mixture of glycerol and yeast extract with a syringe pump. Feed composition and feed rate were obtained by cybernetic model-based, multi-objective optimization. Model parameters were estimated from time-course measurement of substrate, biomass, and dissolved oxygen levels. The optimized process yielded 20.7 g dry cell weight/L, in agreement with the model prediction. Volumetric protein productivity improved by 10–34-fold compared to batch cultivation with 2.8-fold further improvement when the fed-batch process was replicated in a 3 L bioreactor. The process has significance in the routine laboratory cultivations and in scaleup studies.

1. Introduction

Bacterial cultures are typically cultivated in shake flasks as they are easy to handle and do not require sophisticated instrumentation. Flask cultures are routinely used in the initial screening of culture conditions, media optimization, and for laboratory-scale production of recombinant proteins for biochemical and structural characterization. In addition, shaken cultures offer the advantage of parallelization and higher throughput with simple handling [1,2]. However, in contrast to bioreactors, the standard cultivation of recombinant *E. coli* in shake flasks is performed in batch mode. Batch growth in flasks is associated with multiple limitations, including low aeration, nutrient depletion, and lack of pH control, thus limiting biomass and protein yields [3].

The pioneering work by Weuster-Botz et al. has shown the potential of fed-batch strategy in improving biomass concentration in shake flask [4]. The authors have reported 4–6 g.dry cell weight/L (g.DCW/L) using an assembly for intermittent feeding of substrates. In more recent studies, improved cell densities have been obtained in shake flasks by simulating fed-batch processes using systems for the controlled release substrates. The Feedbeads and Feedplates (Kühner, Birsfelden,

Switzerland) systems are designed based on the slow release of solid substrates embedded in a silicone matrix [5]. The release rate of the substrate can be tweaked by altering the fill volume or selecting a polymer with the desired release rate. The commercially available EnBase® (BioSilta Oy, Oulu/Finland) [6] and EnPressoB systems [7] are based on controlled enzymatic degradation of starch to glucose. These processes can be combined with a high-aeration shake flask (Ultra Yield Flask™) [8] to achieve higher oxygen transfer rates and, in turn, higher biomass. A key limitation of the enzyme-based controlled-release systems is that they are sensitive to proteases and other enzyme inhibitors [9]. To address this, various membrane reservoirs have been developed for the diffusion of glucose and ammonium sulfate that can regulate the rate of carbon feeding while also facilitating pH stabilization [10–12].

The above platforms rely on specialized materials such as membranes or polymer tablets for controlled release of substrates and may need timely addition of feed booster, may require extended cultivation protocols, and handling efforts. Further, these platforms have been demonstrated predominantly for improved expression of soluble proteins; their application for protein production as inclusion bodies (IBs) in shaken cultures is relatively less explored. Protein expression in bacteria as IBs is now receiving increasing attention for various applications in

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Notations

t	Any time during the process
X	Biomass concentration at time t
S_1	Glycerol concentration at time t
S_2	Complex nitrogen-rich substrate concentration at time t
DO	Percentage of dissolved oxygen at time t
V	Volume of the mixture at time t
F	Feed flow rate
S_{10}	Glycerol concentration at time $t = 0$
S_{20}	Complex nitrogen-rich substrate concentration at time $t = 0$
$k_L a$	Volumetric mass transfer coefficient for oxygen
C^*	Maximum solubility of oxygen in media (0.0075 g. l^{-1})
MU	Mega units

medical and industrial Biotechnology [13,14]. Moreover, IBs can be produced at much higher levels than soluble proteins, thereby compensating for the higher downstream processing cost of the former [15,16]. Thus, there is a need to develop a simple yet automated fed-batch process for high cell density cultivation (HCD) in shake flasks.

Here, we report a cybernetic model-based optimization of a fed-batch method for HCD cultivation of *E. coli* in a shake flask. A cybernetic model for microbial growth assumes the organism as an optimal strategist with an inherent ability to regulate its cellular processes to utilize multiple substrates sequentially and parallelly. The model considers that a key enzyme is required to consume a given substrate in a multisubstrate environment [17,18]. The cybernetic model has been applied to optimize fed-batch recipes for the production of Rifamycin [19–21] and polyhydroxyalkanoates [22].

In the present work, optimized fed-batch process has been demonstrated for HCD cultivation of recombinant *E. coli* BL21(DE3) harboring (i) a reporter protein - Enhanced yellow fluorescence protein (eYFP), (ii) functional enzymes - formate dehydrogenase (FDH) and Alcohol dehydrogenase (ADH) and (iii) a therapeutic protein -Platelet-derived growth factor (PDGF). A syringe pump was used to feed a mixture of glycerol and yeast extract into the shake flask. Preliminary experiments were conducted with online monitoring of dissolved oxygen (DO) and offline measurements of biomass and glycerol to estimate the model parameters. The model was fitted using a hybrid approach comprising a genetic algorithm (GA) and a constrained nonlinear optimization. Our Multi-objective optimization strategy maximized biomass and minimized substrate feed rate. The fed-batch process showed significant improvement in both the biomass and production of all proteins tested.

2. Materials and methods

2.1. Bacterial strains

E. coli BL21 (DE3) (Novagen Inc, Madison, WI) was used as the host for heterologous protein expression. Transformation was carried out with the vector pET21a (Novagen Inc, Madison, WI) containing the codon-optimized genes for eYFP, *Lactobacillus kefir* ADH (*Lk*ADH), *Geotricum candidum* ADH (*Gc*ADH), *Candida boidinii* FDH (*Cb*FDH); or with the vector pET39b (Novagen Inc, Madison, WI) containing the gene for human PDGF (Table S1 and S2).

2.2. Batch fermentation

Batch cultivation was performed in Luria-Bertini broth (LB) (HiMedia Laboratories, Mumbai, India) containing, in g/L, tryptone 10, yeast extract 5, and NaCl 10 or in Super optimal broth (SOB) medium containing tryptone (HiMedia Laboratories, Mumbai, India) 20, yeast

extract (Sigma Aldrich, St. Louis, MO) 5, NaCl 0.5, and KCl 0.186, with an initial culture optical density (OD_{600}) of 0.05. The initial production medium was supplemented with 2.76 g/L glycerol, 10 mM $MgCl_2$/$MgSO_4$, and an appropriate antibiotic [23].

2.3. Fed-batch fermentation in shake flask

Two needles connected to silicone tubing were fitted in the mouth of the PreSens baffled flask of 500 mL size with a cotton plug. Shorter of the two needles delivers nutrient feed to the growing culture while the longer needle is used for drawing the sample intermittently. Each flask has integrated autoclavable sensor spots for pH and DO. These flasks were filled with 100 mL medium and inoculated with overnight grown preculture (initial OD_{600} 0.05). Unless specified otherwise, all the cultivations were carried out in a shaker incubator (Model: LT-X, Adolf Kuhner AG, Switzerland) of 5 cm pitch fitted with a PreSens shake flask reader (PreSens GmbH, Regensburg, Germany) shaken at 200 rpm and the chamber temperature maintained at 32 °C. Online measurements of pH and DO were recorded every 10 s by shake flask reader (SFR) software. The nutrient mixture containing glycerol and yeast extract (Sigma Aldrich, St. Louis, MO) at concentrations specified (in% w/v) and supplemented with 2 mM $MgCl_2$/$MgSO_4$ and 2X antibiotic was fed at a constant flow rate using an external 6 channel programmable syringe pump (NE-1600, New Era Pump Systems, Inc., Farmingdale, NY) (Fig. 1). Isopropyl β- D-1-thiogalactopyranoside (IPTG) was added at a final concentration of 0.5 mM to induce protein expression in the mid-log phase, and cultivation was continued till a decrease in OD_{600} or a sharp increase in the DO level was observed. To validate the optimized feed recipe without the online monitoring of DO and pH, the optimized fed-batch process was also carried out in an Eppendorf shaker that had a pitch of 2.5 cm and was shaken at 220 rpm.

### 2.4. Fed-batch fermentation in a 3 L bioreactor

Fed-batch cultivation for one of the representative proteins, eYFP was performed in 3 L capacity Applikon bioreactor (Model: Z310110011, Applikon Biotechnology, Delft, Netherlands) at 32 °C, with an agitation rate of 615 rpm and aeration rate of 1 vvm (volume per volume per minute) [24]. Briefly, the bioreactor was filled with 750 mL SOB medium and inoculated with preculture (initial OD_{600} 0.05). The initial medium was supplemented with 2.76 g/L glycerol, 10 mM $MgCl_2$/$MgSO_4$, and an appropriate antibiotic. The nutrient mixture containing 17.5% glycerol and 24.5% yeast extract was fed continuously at a flow rate of 5.4 mL/h from 6 h. The culture was induced with 0.5 mM IPTG at 12 h and the process was continued further for 12 h. Samples taken from bioreactor at different time points were analyzed as described below.

2.5. Offline measurements

Samples for protein analysis were drawn every 2 h through the sampling tube. Optical cell density at 600 nm was monitored using a UV–Vis spectrophotometer (UV-2600, Shimadzu, Singapore). Dry cell weight (DCW) was calculated at the end of cultivation in triplicates. Briefly, 1 mL sample was centrifuged in pre-weighed tubes. The pellet was washed with distilled water and dried at 95 °C to a constant weight. Residual glycerol concentration in the culture supernatant was estimated using a glycerol assay kit (Megazyme Co. Wicklow, Ireland).

2.6. Inclusion body extraction and solubilization

Bacterial cells were lysed by sonication and inclusion bodies were separated from cell lysate by centrifuging at 12,000 rpm for 30 min at 4 °C. Supernatant was used to perform glycerol assay. The pellet was washed sequentially once with 20 mM Tris containing 1% Triton X-100 (pH 7.8), and twice with 20 mM Tris (pH 7.8). The pellet resuspended in
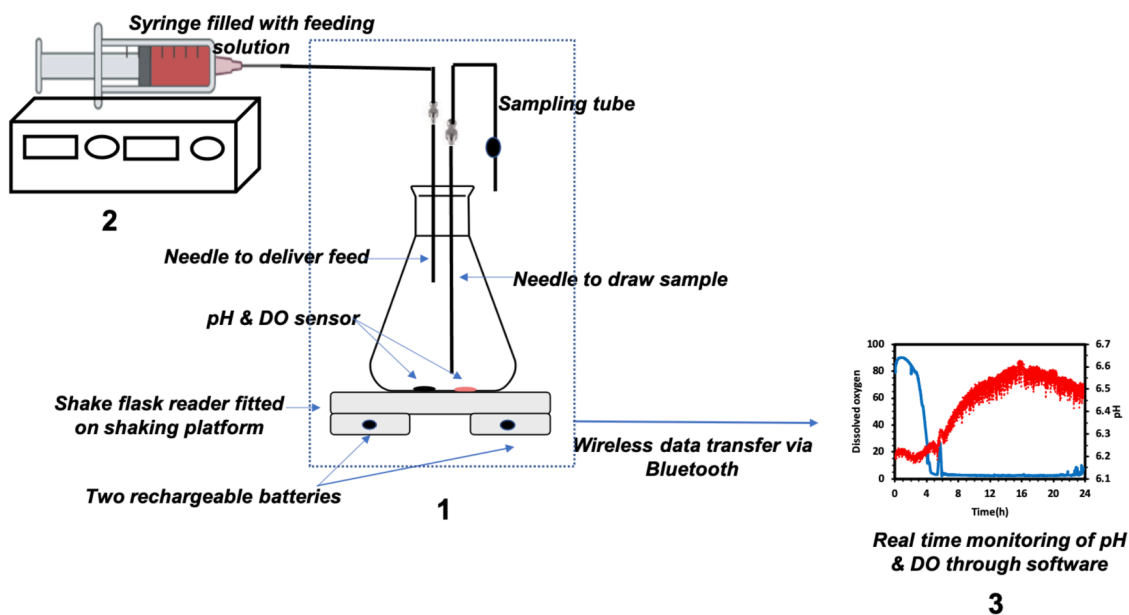
Fig. 1. Experimental setup of High cell density cultivation process in shake flask. The set up consist of 3 parts: 1. Shake flask placed on the shake flask reader (SFR) fitted on a shaking platform in a shaker incubator (shown with dotted lines), 2. External syringe pump placed outside of the incubator to deliver feeding solution to the growing culture, and 3. A module fitted inside SFR for the wireless transmission of the real-time data via Bluetooth to computer. Two needles are fitted within the cotton plug. One of them is utilized for drawing samples intermittently and another for delivering feeding solution.

respective wash buffers was incubated on a Wavex tube rotator (Abdos Labtech Pvt. Ltd, India) at 60 rpm for 15 min. IB solubilization was achieved by overnight incubation in 20 mM Tris containing 8 M Urea and 10 mM Dithiothreitol (pH 11.0) on a Wavex tube rotator at room temperature rotating at 60 rpm.

2.7. Protein expression analysis

For recombinant protein expression analysis, cells were lysed by sonication (Q700, Qsonica L.L.C, Newtown, CT) in a buffer containing 50 mM Tris-Cl (pH 8.0), 0.5 mM EDTA, 1 mg/mL lysozyme, and 1 mM phenylmethylsulfonylfluoride. The crude cell lysate was clarified by centrifugation at 12,000 rpm for 30 min. Protein quantification was performed using the Bradford method [25]. The soluble and insoluble protein fractions were analyzed on 12% reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels stained with Coomassie Brilliant Blue.

2.8. Determination of enzyme activity

ADH activity was determined by monitoring NADH/NADPH absorption at 340 nm as described previously [26]. Briefly, the reaction mixture contained 10 mM acetophenone, 0.25 mM NADH in 100 mM potassium phosphate buffer (pH 7.0), and an appropriately diluted enzyme. One unit of activity (U) was defined as the amount of enzyme catalyzing the oxidation of 1 µmol NADH/NADPH per min under the predetermined assay conditions.

Similarly, FDH activity was measured by monitoring the increase in NADH absorbance at 340 nm for 5 min after adding the appropriate amount of diluted enzyme in a reaction mixture containing 0.25 mM NAD^+ and 10 mM sodium formate in 100 mM potassium phosphate buffer (pH 7.0). One unit of activity (U) was defined as the amount of enzyme catalyzing the reduction of 1 µmol NAD^+ per minute under the predetermined assay conditions [27].

2.9. eYFP fluorescence assay

E. coli culture was used for fluorescence estimation along with eYFP

standards in the range 0 − 100 µg/mL. Fluorescence was measured on a multimode fluorescent reader (TECAN, Infinite M200 PRO, Maríñedorf, Switzerland) using $\lambda_{excitation}$ of 500 nm and $\lambda_{emission}$ of 530 nm. Absorbance values at 600 nm obtained from the Tecan reader were multiplied by factor 2.91 to correlate with those obtained with the UV-Visible spectrophotometer (Figure S1). The purified eYFP used as a standard was produced in-house [28]. The standard curve of the purified eYFP was used to convert the relative fluorescence values to mg protein/g DCW.

3. Results

3.1. Initial media composition and operating parameters

Apart from substrate feed rates, the operating variables that affect the growth during fed-batch cultivations include shaker speed, temperature, pH, and the initial media composition. The cultures were shaken at 200 rpm, the highest speed that produced negligible foaming. The volumetric mass transfer coefficient (k_La) for oxygen was measured at different shaking speeds, culture volume, and pitch using the gassing-out, gassing-in technique as described previously [29]. The k_La was found to be 133 h^{-1} for the 500 ml PreSens baffled flask at 200 rpm shaking speed, 5 cm pitch and a fill ratio of 1:5 (Table S2). Preliminary experiments were performed to screen the initial media composition that support high biomass growth before the fed-batch phase. For this, we chose complex media such as LB and SOB with or without added glycerol (data not shown). As reported previously, an initial glycerol concentration of 2.76 g/L was included [23]. LB or SOB alone yielded OD_{600} of 3–4, in 6 h. However, the combination of SOB with glycerol yielded OD_{600} of 12–15 in the same period. A continuous decrease in DO was observed for the first 6 h, followed by a sharp rise concomitant with the depletion of glycerol. This landmark in the DO profile was considered as the starting point for feeding (Figure S2). Various feed compositions comprising different ratios of glycerol to yeast extract were delivered to the growing culture post 6 h using a multi-channel syringe pump (Table 1). Upon feeding, the DO levels decreased gradually and remained close to zero.

3.2. Model formulation and parameter estimation

It is well known that *E. coli* can grow aerobically on a complex, nitrogen-rich substrate (S_2), with or without added carbon substrate such as glycerol (S_1). The cybernetic model has been adapted from previous reports [30,31]. Briefly, the model assumes growth on two combinations of substrates: (1) S_1, S_2, and dissolved oxygen (DO), and (2) S_2 and DO, whose stoichiometries are defined in eq. (1) and (2), respectively. The substrate S_2 comprises several amino acids and peptides and can serve as both carbon and nitrogen sources. The growth kinetics is depicted by eq. (3)-(6). The net rates of synthesis of the key enzymes e_1 and e_2 are given by eq. (7). The material balances of the batch and fed-batch phase are described by eq. (8)-(11) and eq. (12)-(16), respectively. The model parameters have been defined in Table 2.

$$X - \frac{1}{Y_{11}}S_1 - \frac{1}{Y_{12}}S_2 - \frac{1}{Y_{o1}}DO = 0 \tag{1}$$

$$X - \frac{1}{Y_{22}}S_2 - \frac{1}{Y_{o2}}DO = 0 \tag{2}$$

$$r_1 = \left(\frac{S_1}{K_{11}+S_1}\right)\left(\frac{S_2}{K_{12}+S_2}\right)\left(\frac{DO}{K_{o,1}+DO}\right) \tag{3}$$

$$r_2 = \left(\frac{S_2}{K_{22}+S_2}\right)\left(\frac{DO}{K_{o,2}+DO}\right) \tag{4}$$

$$\mu_k = \mu_k^m\left(\frac{e_k}{e_k^m}\right)r_k \tag{5}$$

$$\mu = \sum_{k=1}^{2}\mu_k \tag{6}$$

$$\frac{d}{dt}\left(\frac{e_k}{e_k^m}\right) = \left(\mu_k^m + \beta_k\right)r_k - \left(\mu + \beta_k\right)\left(\frac{e_k}{e_k^m}\right) \tag{7}$$

Batch phase

$$\frac{dX}{dt} = \mu X \tag{8}$$

$$\frac{dS_1}{dt} = -\frac{\mu_1}{Y_{11}}X \tag{9}$$

$$\frac{dS_2}{dt} = -\left(\frac{\mu_1}{Y_{12}} + \frac{\mu_2}{Y_{22}}\right)X \tag{10}$$

$$\frac{d(DO)}{dt} = k_La(100 - DO) - \frac{100}{C^*}\left(\frac{\mu_1}{Y_{o1}} + \frac{\mu_2}{Y_{o2}}\right)X \tag{11}$$

Fed-batch phase

$$\frac{dX}{dt} = \left(\mu - \frac{F}{V}\right)X \tag{12}$$

$$\frac{dS_1}{dt} = \frac{(S_{10} - S_1)F}{V} - \frac{\mu_1}{Y_{11}}X \tag{13}$$

$$\frac{dS_2}{dt} = \frac{(S_{20} - S_2)F}{V} - \left(\frac{\mu_1}{Y_{12}} + \frac{\mu_2}{Y_{22}}\right)X \tag{14}$$

$$\frac{d(DO)}{dt} = k_La(100 - DO) - \frac{(DO)F}{V} - \frac{100}{C^*}\left(\frac{\mu_1}{Y_{o1}} + \frac{\mu_2}{Y_{o2}}\right)X \tag{15}$$

$$\frac{dV}{dt} = F \tag{16}$$

To fit the model parameters, eight fed-batch experiments were carried out using different feeding rates of glycerol and yeast extract (Table 1). It is well known that microbial growth is dependent on C: N ratio apart from the substrate feeding rates [23,32]. Therefore, the ratio

Table 2

Model parameters for growth of *E. coli* in complex media for batch and fed-batch process.

Parameters	Notation	Units	Value
Maximum specific growth rate for combination 1	μ_{m1}	h^{-1}	1.497
Maximum specific growth rate for combination 2	μ_{m2}	h^{-1}	0.834
Half-saturation constant for substrate S_1 for combination 1	K_{11}	g/L	0.131
Half-saturation constant for substrate S_2 for combination 1	K_{12}	g/L	36.154
Half-saturation constant for substrate S_2 for combination 2	K_{22}	g/L	1.001
Half-saturation constant for *DO* in combination 1	$K_{o,1}$	%	0.326
Half-saturation constant for *DO* in combination 2	$K_{o,2}$	%	8.944
Yield coefficient for substrate S_1 for combination 1	Y_{11}	g of biomass/g of S_1	0.440
Yield coefficient for substrate S_2 for combination 1	Y_{12}	g of biomass/g of S_2	0.881
Yield coefficient for substrate S_2 for combination 2	Y_{22}	g of biomass/g of S_2	0.254
Yield coefficient for *DO* in combination 1	Y_{o1}	g of biomass/g of oxygen	0.925
Yield coefficient for *DO* in combination 2	Y_{o2}	g of biomass/g of oxygen	1.219
Ratio of initial key Enzyme concentration to its maximum concentration for combination 1	$\frac{e_{10}}{e_1^m}$	–	0.082
Ratio of initial key Enzyme concentration to its maximum concentration for combination 2	$\frac{e_{20}}{e_1^m}$	–	0.874
Enzyme Degradation constant for combination 1	β_1	h^{-1}	0.001
Enzyme Degradation constant for combination 2	β_2	h^{-1}	0.028
Minimum value of Objective function	OF	–	2.277

Table 1

Set of fed-batch experiments consisting of different combinations of feed composition and flow rate used for model fitting and validation.

Protein	Glycerol Conc. (% w/v)	Yeast extract Conc. (% w/v)	Yeast extract/Glycerol	Volumetric flow rate (mL/h)	Applied in Fitting/Validation	Reference to figure[#]
eYFP	25	50	2	0.6	Fitting	S3
PDGF	25	35	1.4	0.5		S4
PDGF	30	40	1.33	0.52		2
PDGF	20	32	1.6	0.78		S7
PDGF	20	32		0.72		S5
PDGF	20	32		0.75		S6
eYFP	25	50	2	0.5	Validation	S8
PDGF	20	30	1.5	0.78		3

[#] S: Supplementary figure and only numbers: main text figure.

of yeast extract to carbon source glycerol was varied between 1.33 and 2. The model parameters were estimated by fitting the model to six fed-batch experiments (Table 1). The nonlinear constraints were set to have DO, glycerol, and complex nitrogen-rich substrate greater than zero. The lower and upper bounds for model parameters were selected based on the literature [33–37]. Model fitting was achieved by minimizing the objective function using the Hybrid Scheme in GA available in MATLAB R2020b (The MathWorks, Natick, MA). This involves two optimization solvers working in tandem, (1) 'Genetic Algorithm', to find the local area of the optimum and (2) 'fmincon' function, to find the exact value of the optimum once GA finds the local area. The dimensionless objective function (OF) for a single fermentation experiment was defined as the deviation of the estimated variables from the experimental values and normalized with the maximum achievable value of the given variable eq. (17). The overall objective function ($OF_{overall}$) is the sum of all the OFs of 6 individual fermentation experiments, as shown in eq. (18).

remaining experiments (Figures S3-S7). The model was validated on two different experimental combinations (Table 1, Fig. 3, and Figure S8). The goodness of fit of the model was evaluated using the χ^2 test. The null hypothesis was accepted for all the experimental runs within a 99.5% confidence interval. This confirms that the model satisfactorily predicts the time course measurements of the process.

3.3. Optimized feeding strategy

A multi-objective optimization strategy was implemented to obtain the optimum feeding recipe. Constraints of operating conditions used in optimization are described in Table S3. The three objectives for multi-objective optimization were to maximize biomass concentration and minimize the glycerol and yeast extract mass flow rates described by eq. (19) with the decision variables shown in eq. (20)-(22) and their bounds in eq. (24).
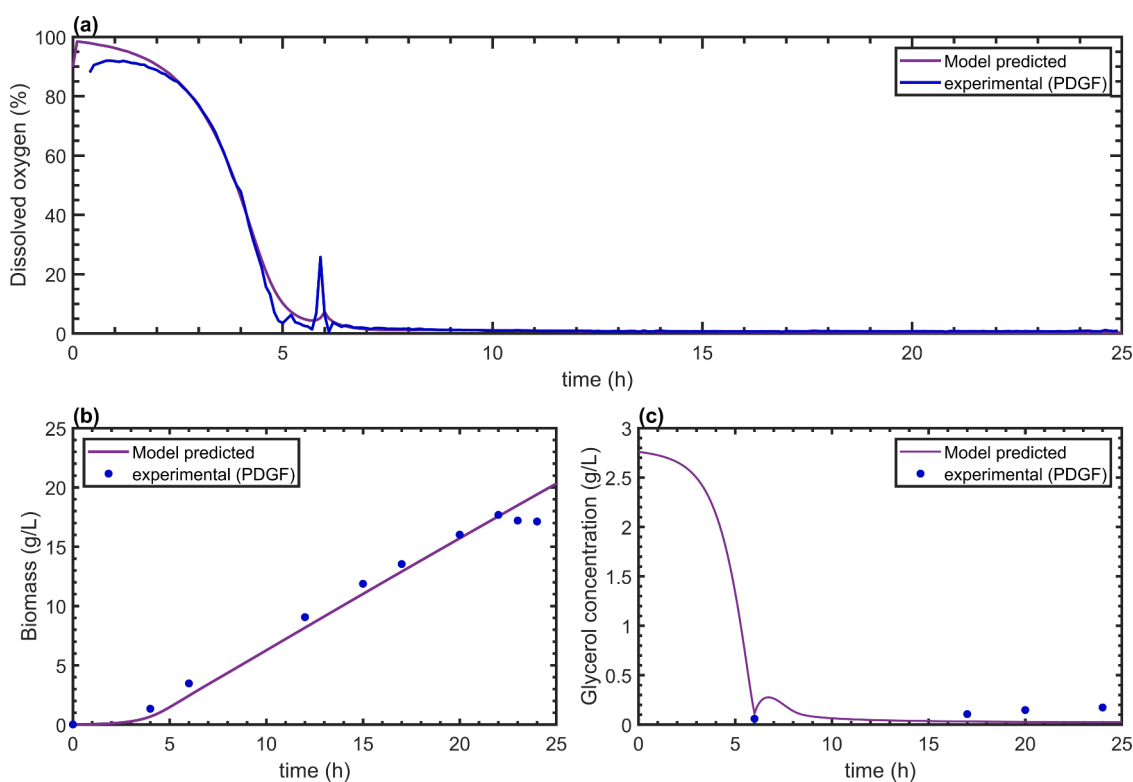
$$Objective = \min(f_1(\mathbf{V}), f_2(\mathbf{V}), f_3(\mathbf{V})) \tag{19}$$

$$OF_i = \frac{1}{100 n_{DO}} \sum_{i=1}^{n_{DO}} \left(DO_{exp} - DO_{pre}\right)^2 + \frac{1}{2.76 n_S} \sum_{i=1}^{n_S} \left(S_{exp} - S_{pre}\right)^2 + \frac{1}{n_X \cdot max(X_{exp})} \sum_{i=1}^{n_X} \left(X_{exp} - X_{pre}\right)^2 \text{for } i^{th} \text{ experiment} \tag{17}$$

$$f_1(\mathbf{V}) = -\max[X(\mathbf{V}, t)] \tag{20}$$

$$OF_{overall} = \sum^{O} F_i \tag{18}$$

$$f_2(\mathbf{V}) = S_{10}F \tag{21}$$

$$f_3(\mathbf{V}) = S_{20}F \tag{22}$$

The parameter values obtained by fitting the experimental data are shown in Table 2. Fig. 2 shows a representative model fitting with the experimental data. Refer to Supplementary Information for the fitting of

$$\mathbf{V} = \begin{bmatrix} S_{10} & S_{20} & F \end{bmatrix} \tag{23}$$

Fig. 2. Model fitting: Comparison of the model predicted values with the experimental data set with feeding composition of 30% glycerol and 40% yeast extract fed at 0.52 mL/h flow rate a) dissolved oxygen b) Biomass c) glycerol concentration (● – experimental values, – modelled values).
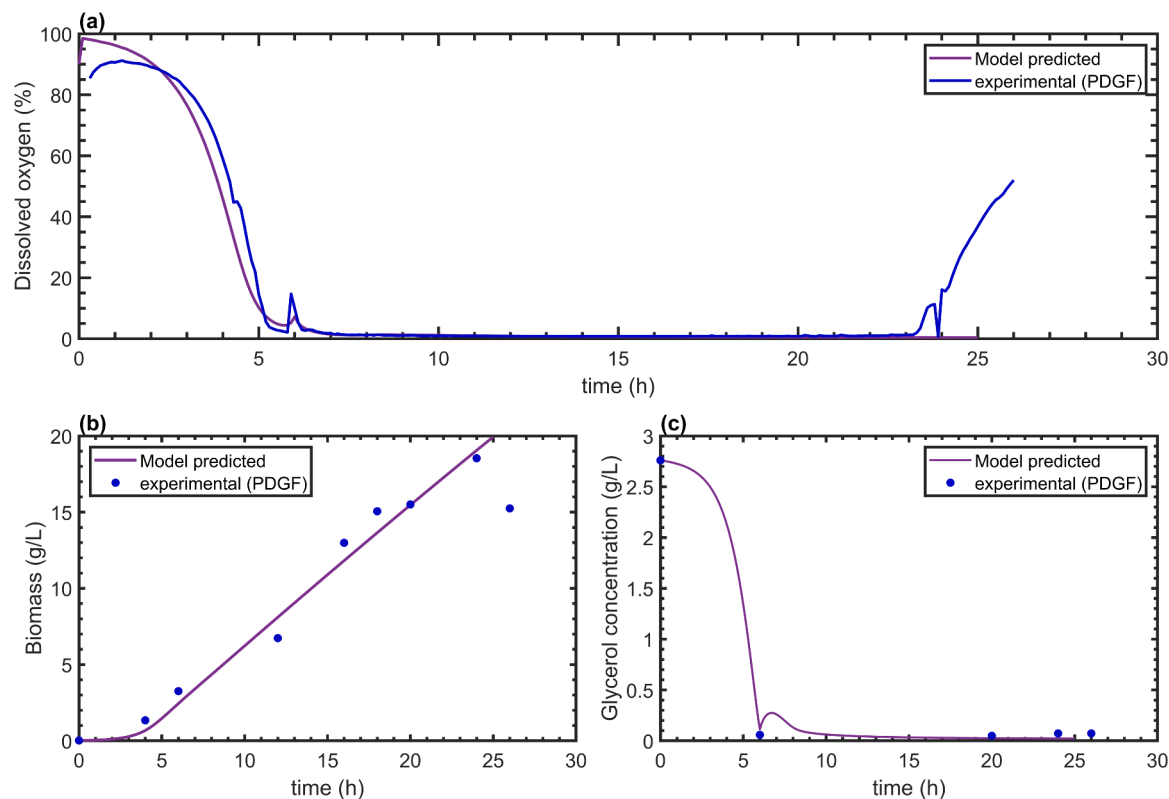
Fig. 3. Model validation: Goodness of fit of the model with the experimental data that was not used for estimation of model parameters. The figure represents data with feeding composition of 20% glycerol, 30% yeast extract fed at 0.78 mL/h flow rate for a) dissolved oxygen b) Biomass c) glycerol concentration (●– experimental values, - – modelled values).
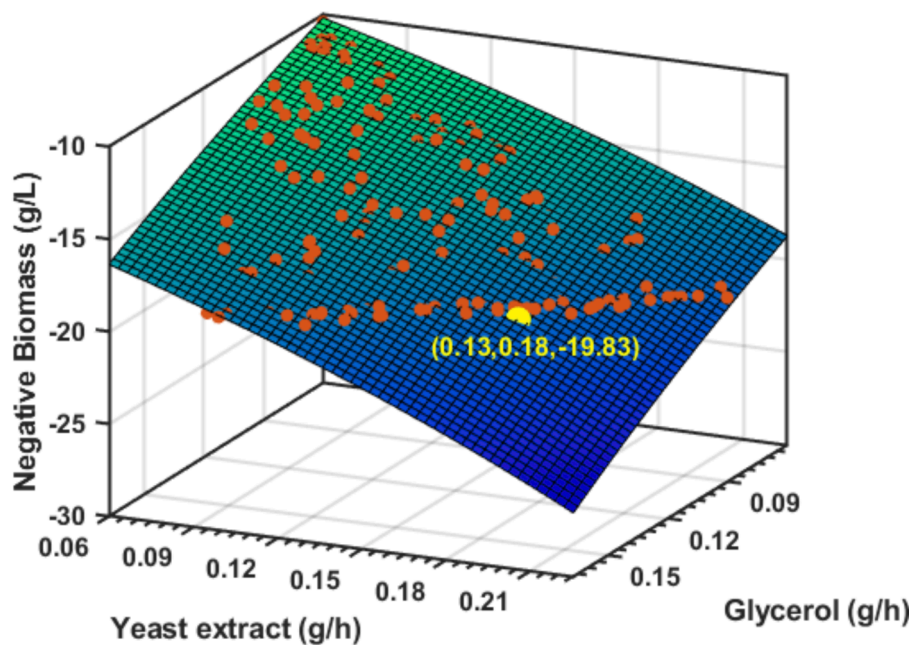
Fig. 4. Pareto front obtained from multi-objective optimization algorithm. The points represent optimum combinations of mass flow rate of glycerol, yeast extract and biomass concentration. The points are fitted with a 3D rectangular hyperboloid as the expected pattern of the pareto front surface. Point marked in yellow color represents optimal feeding recipe of 17.5% glycerol, 24.5% yeast extract and at a flow rate of 0.72 mL/h.

$$V_{min} < V < V_{max} \tag{24}$$

Multi-objective optimization was performed on MATLAB R2020b using the 'gamultiobj' function to obtain the Pareto front surface. The

Pareto front represents a set of optima that are trade-offs between potentially conflicting objective functions [38]. The end-user can select any of the optimal solutions based on additional criteria or practical considerations [39]. Fig. 4 shows the distribution of the Pareto front

fitted with hyperboloid. For experimental validation, we chose an optimal solution corresponding to a feed composition of 17.5% glycerol, 24.5% yeast extract, and a feed rate of 0.72 mL/h.

The selected optimized recipe was tested for a range of proteins that show different extent of soluble expression in *E. coli*. These included eYFP and *Lk*ADH, primarily expressed in the soluble fraction, *Cb*FDH and *Gc*ADH, which are partially soluble, and human PDGF, which forms inclusion bodies. These fed-batch results were compared with the conventional batch cultivation of these proteins (shake flask without fed-batch). The biomass concentrations achieved in 24 h were 19.9–21.5 g DCW/L, in agreement with the model predicted value of 19.44 g DCW/L. For all the fed-batch trials, experimental data fitted well with the model predictions as determined by the χ^2 test (Fig. 5). SDS-PAGE was performed to analyze the time-course expression of the tested proteins. An increase in protein content was observed over time, as shown in the representative Fig. 6. Next, it was of interest to quantify protein titer in the optimized fed-batch process. All the tested proteins which are predominantly expressed in soluble form showed significant improvement in the titer, volumetric productivity, and protein yield (Table 3 and 4) compared to shake flask without fed-batch. PDGF IBs titer was 3.14 ± 0.11 g/L in the fed-batch compared to 0.12 ± 0.02 g/L in the conventional batch cultivation in SOB. The Fed-batch process resulted in about a 26-fold increase in the yield of solubilized PDGF IBs. *Lk*ADH and *Gc*ADH showed 40–50 fold improvement in titer, whereas the *Cb*FDH titer was enhanced by 133 fold.

Next, it was of interest to ascertain if the high protein productivities can be obtained without the online monitoring of DO and pH. Therefore, we investigated the reproducibility of the optimized process on a different shaker using an ordinary baffled shake flask and without online monitoring. To match the k_La, we used a shaking speed of 220 rpm on the Eppendorf shaker that has a pitch of 2.5 cm (Table S4). All other experimental conditions were kept unchanged. Feeding was started at 6 h, culture induced at 12 h, and cultivation was continued for 10–12 h

post-induction. HCD cultivation of all the recombinant proteins and enzymes tested were reproduced with comparable outcomes in terms of biomass concentration, protein yield, and enzyme activities (Table 3 and 4). This shows that the optimized protocol can be carried out in a routine laboratory shaker by connecting a feeding tube and a syringe pump.

### 3.4. Validation of the fed-batch recipe in a 3 L bioreactor

We replicated the proposed fed-batch process in a 3 L Bioreactor for a representative protein, eYFP. The agitation and aeration rates were chosen to closely match the k_La of the shake flask. The trends in DO observed in the 3 L bioreactor were comparable to that of the optimized shake flask process with fed-batch. While the biomass concentration was comparable, the specific activity of the eYFP in the bioreactor was 2.8-fold higher than that observed for the shake flask with fed-batch (Table 4). Therefore, the process developed for shake flask is portable across scales of fermentation.

4. Discussion

Previously reported protocols for HCD in shake flask have been based primarily on the controlled release of one or more substrates to the growing culture. The present study demonstrates a true fed-batch process for HCD cultivation in shake flasks. The optimized process resulted in cell densities of 19.9–21.5 g DCW/L and a significant reduction in process time. This process compares favorably with the previously reported HCD protocols in terms of the cell densities. For instance, the EnBase cultivation system yielded cell densities of 6–9 g DCW/L [6]. Higher cell densities of 10–15 g DCW/L were reported with the EnBase® Flo cultivation system [40]. Likewise, HCD cultivation using a dialysis shake flask resulted in cell densities of 8.5 g DCW/L [11]. Philip et al. reported ∼ 25 g DCW/L by feeding glucose and ammonium carbonate parallelly by using control release mechanism [12].
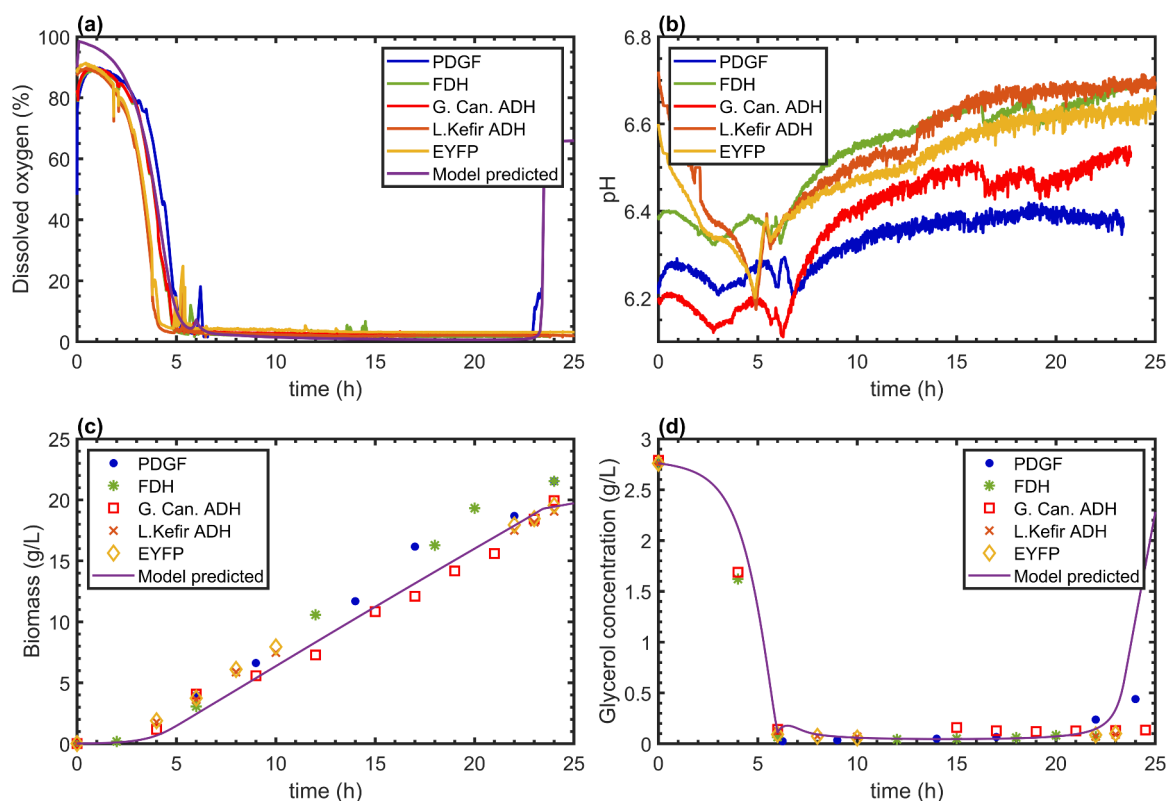
Fig. 5. Model prediction for the experimental data from the selected optimum. The figure represents data with feeding composition of 17.5% glycerol, 24.5% yeast extract fed at 0.72 mL/h of flow rate for a) dissolved oxygen b) pH c) Biomass d) glycerol concentration
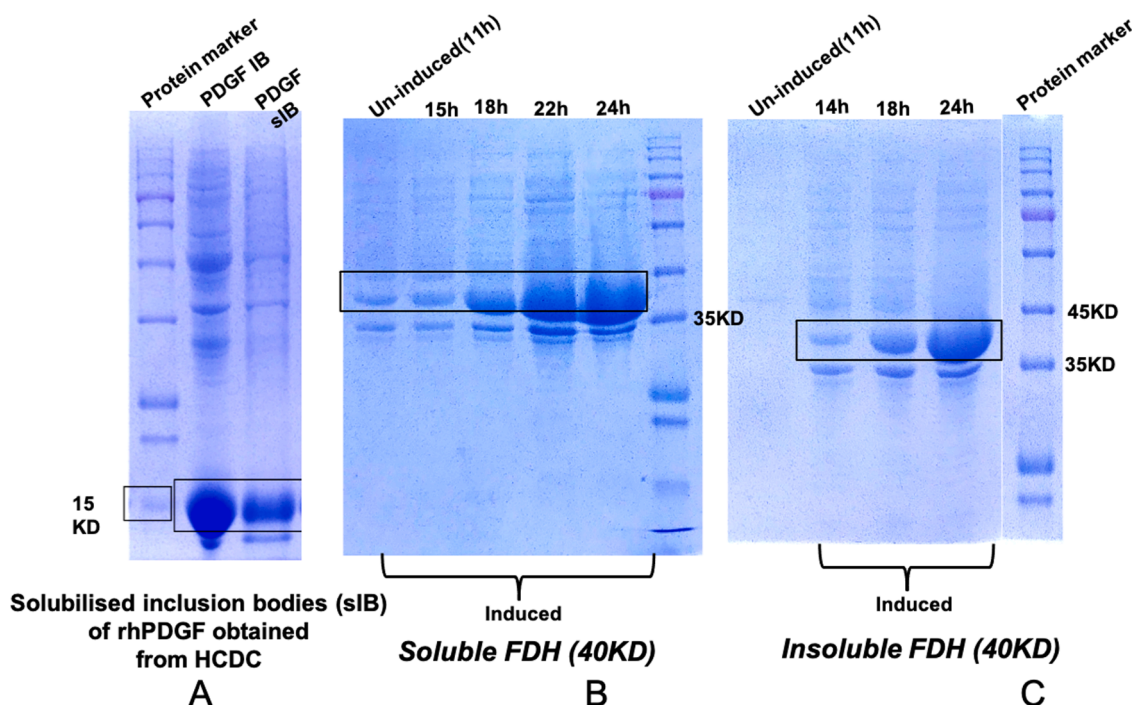
Fig. 6. PDGF and FDH expression analysis and solubilization. (a) Time-course expression analysis of PDGF; (b) and (c) solubilized PDGF inclusion bodies; (d) and (e)Time course expression analysis of soluble FDH and insoluble FDH, respectively; performed on 12% SDS-PAGE.

Table 3
Process summary at different fermentation scale. Data represented as Mean±Standard Error of Mean.

Recombinant Enzyme	*Cb*FDH			*Gc*ADH		
Fermentation scale	Shake flask without fed-batch[a]	Shake flask with Fed-batch		Shake flask without fed-batch[a]	Shake flask with Fed-batch	
		With online monitoring[b]	Without online monitoring[a]		With online monitoring[a]	Without online monitoring[a]
Activity titer (U/L)	179±6	23,883 ± 1493	24,499 ± 637	2269 ± 150	98,149 ± 736	88,852 ± 366
Specific activity (U/g DCW)	137±10	1150 ± 76	1229 ± 24	1047 ± 43	4722 ± 103	4343 ± 73
Volumetric Productivity (U/L/h)	30 ± 1	995 ± 62	1021 ± 27	378 ± 25	4090 ± 31	3702 ± 15
Protein yield (U/g complex substrate)	7 ± 0.24	420 ± 26	431 ± 11	91 ± 6	1726 ± 13	1563 ± 4

[a] =average of two biological replicates,.
[b] = average of three biological replicates.

Table 4
Comparison of the fed-batch process at different fermentation scales for *Lk*ADH and eYFP.

Recombinant Protein	*Lk*ADH[e]		eYFP[e]		
Fermentation scale	Shake flask without fed-batch	Shake flask with Fed-batch	Shake flask without fed-batch	Shake flask with Fed-batch	3 L fermenter with fed batch
Titer[a]	35,852 ± 714	17,06,627 ± 17,111	24 ± 0.6	1167 ± 8	3093 ± 409
Specific activity[b]	19,395 ± 774	88,885 ± 1576	16 ± 1	61 ± 1	170 ± 9
Volumetric Productivity[c]	5975 ± 119	71,109 ± 713	4 ± 0.1	48 ± 1	130 ± 17
Protein yield[d]	1434 ± 29	30,020 ± 301	1 ± 0	21 ± 0.1	54 ± 7

[a] Units of enzyme/L for *Lk*ADH and mg of protein/L for eYFP.
[b] U/g DCW for *Lk*ADH and mg protein/g DCW for eYFP.
[c] U/L/h for *Lk*ADH and mg of protein l^{-1} for eYFP mg of protein/L/h.
[d] U/g complex substrate for *Lk*ADH and mg of protein/ g complex substrate for eYFP

Here, we use cybernetic model to optimize the feed composition and feeding rate. The model considers S_1 as the carbon source and S_2 as the nitrogen source for combination 1 while S_2 is considered as both carbon or nitrogen source for combination 2. The yield coefficient value for S_2 is higher for combination 1 compared to combination 2. Further, the specific growth rate on substrate combination 1 is higher than that of combination 2 suggesting that the former is preferred for biomass growth. The model can simulate a sharp rise in DO at approximately 6 h, thus validating the shift of phase from combination 1 to combination 2 upon glycerol exhaustion. It can be concluded that a feed composition containing glycerol and yeast extract would allow better growth of biomass and the presence of yeast extract will not hamper the growth in the glycerol limiting conditions. The multi-objective optimization approach implemented in this study would be beneficial for scaling up of

the process to an industrial scale. Further, the proposed optimization strategy could be used to predict the induction and harvest time while working with smaller culture volumes.

With the optimized protocol, the final biomass concentrations were 15–20 times higher than the conventional batch cultivation. The higher protein yield obtained in the fed-batch process could be attributed to the prolonged period of protein synthesis under optimized growth conditions. The high cell density cultivation process was reproduced in a standard baffled, Erlenmeyer flask without the online monitoring of DO and pH, demonstrating the economic feasibility of the process with a minimum 'hardware' setup.

It was of interest to compare the titer of the proteins studied here with the best-reported titers regardless of the process. For instance, for *CbFDH*, the maximum specific activity obtained in this study was about 3.5-times higher than that obtained by using a combination of parallelly operated milliliter-scale stirred tank bioreactors and EnBase media [41]. Similarly, for *Lk*ADH, Weckbecker and Hummel obtained 1.0 MU/L of *Lk*ADH in HCD fermentation compared to 1.72 MU/L of *Lk*ADH obtained in this study [42]. Further, majority of the previous protocols have been demonstrated mainly for soluble protein production. We demonstrate the suitability of the fed-batch process for the production of both IBs and soluble proteins. Thus, the protocol can be applied to a variety of recombinant proteins that can be expressed in *E. coli*.

The proposed process opens a wide range of applications. For high throughput screening, the process can be readily parallelized by using a multi-channel syringe pump. We tested a 6-channel syringe pump without any loss of productivity. Further, commercially available automated liquid delivery systems such as those developed by INFORS HT (Infors AG, Bottmingen, Switzerland) may provide better process control and minimize manual intervention. Based on the results of the DO profile, it is seen that the growth is limited by oxygen availability beyond 6 h. Therefore, strategy of constant feed rates was employed. As the feed flow rate has been optimized for a specific value of k_La, additional process improvements may be feasible by designing a specialized shake flask that affords higher oxygen transfer rates (OTR) (Figure S10). Likewise, if a different shaker speed or shake flask geometry is chosen, the feed flow rates may need to be fine-tuned to be in sync with the k_La. Finally, the optimized cultivation strategy was validated in a 3 L bioreactor. Note that much higher OTR can be achieved in a bioreactor thus providing scope for further improvement in biomass and protein productivities. Our results show that shake flask with fed-batch could act as the bridge between the initial shake flask screening trials and the subsequent scale-up studies in a bioreactor.

5. Conclusion

The fed-batch recipe provided higher cell densities of 19.9–21.5 g DCW/L and improved protein production in the shake flask. With the readily available optimization tools like 'Hybrid Genetic Algorithm' and 'Multi-objective Optimization' along with monitoring of DO and pH, a fed-batch process was developed without the need for extensively controlled systems like bioreactors. The process enabled protein expression and production over a prolonged period. With a simple setup, the proposed process will have important applications for the cultivation of *E. coli* in routine lab-scale experiments and subsequent scale-up in a bioreactor.

CRediT authorship contribution statement

Snehal D Ganjave: Conceptualization, Formal analysis, Writing – original draft. **Hardik Dodia:** Data curation, Writing – original draft. **Avinash Vellore Sunder:** Conceptualization, Formal analysis, Writing – review & editing. **Swati Madhu:** Formal analysis. **Pramod P Wangikar:** Conceptualization, Writing – review & editing, Funding acquisition, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.btre.2021.e00694.

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