Model-based Optimization of Biopolymer Production from Glycerol

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The present study focuses on sustainable production of biodegradable polymers by *Cupriavidus necator* DSMZ 545 using glycerol as substrate. The batch growth and biopolymer production kinetics were established in a 7-L bioreactor, which resulted in a total biomass of 8.88 g L^{-1} and poly(3-hydroxybutyrate) (PHB) accumulation of 6.76 g L^{-1}. The batch kinetic and independently acquired substrate inhibition data were then used to develop a mathematical model for PHB production process. This was eventually used to design different nutrient feeding strategies under constant feed rate, decreasing feed rate, and pseudo steady state of substrate (glycerol) to optimize the PHB production during fed-batch cultivation. Among all the fed-batch cultivation strategies, the highest PHB accumulation and productivity of 13.12 g L^{-1} and 0.27 g L^{-1} h^{-1}, respectively, was achieved in fed-batch bioreactor cultivation where a pseudo steady state with respect to glycerol was maintained.

Keywords:
poly(3-hydroxybutyrate), *Cupriavidus necator*, glycerol, mathematical model, fed-batch cultivation strategies

Introduction

PHAs (polyhydroxyalkanoates) have received considerable attention as a substitute for synthetic polymers. Not only do they possess properties similar to conventional petrochemistry-derived plastics, but are also biodegradable in nature. However, the major abeyance in the large-scale production of biopolymers is the cost of PHA production of PHB, which is currently much higher than conventional plastic, thereby making them less popular than their counterpart[1]. This is primarily due to high substrate cost, low concentration of PHB in the growing cells, low rate of PHB accumulation, and expensive recovery protocols of the PHB. One way to reduce the overall cost of PHB production process is to use cheaper substrates (e.g., glycerol, which is a by-product of the biodiesel industry), which can be coupled with highly efficient isolation and purification protocols for PHB to economize the production cost further.

The major aim of the present study was, therefore, to investigate the use of glycerol as a renewable raw material for the fermentative production of PHB. With the increasing global interest in biofuel production, it was considered interesting to examine the availability of this industrial by-product (glycerol) of Jatropha biofuel industry for the microbial PHB production. Some reports[2–4] have indicated that *Cupriavidus necator* is able to consume glycerol and accumulate biopolymer (PHB) under specific cultivation conditions (excess availability of substrate and limiting nitrogen concentrations). Apart from this, optimization of process parameters (physical and chemical)[5–7] and bioprocess engineering strategies[8–10] are some other approaches generally employed to address this problem and achieve cost-effective PHB production. In the present studies, mutant strain of *C. necator* DSMZ 545 (of older wild type *C. necator* DSMZ 529) was used. Thus, the main aim was to optimize the medium recipe and develop a simple mathematical model, which not only satisfactorily describes the observed batch kinetics of cultivation, but also explicates substrate inhibition and limitation under different nutrient feed conditions of fed-batch cultivations.

Different nutrient feeding strategies have been implemented by different researchers during the fed-batch cultivation for process improvement with respect to PHB accumulation and/or productivity for other culture systems[11–14]. However, to date there are only very few reports on kinetic analysis of PHB production by *C. necator*[15–18] and particularly none on the use of mathematical model for the design of nutrient feeding of fed-batch cultivation strategies for growth associated PHB production us-

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ing *C. necator* DSMZ 545. Hence, the present work highly advocates that mathematical models could be an excellent tool to understand the system behaviour and help in the design of cultivation strategies for process optimization with minimum experimentation[19]. In addition, since none of the literature reports on *C. necator* has employed the use of mathematical model for the design of nutrient feeding strategy(ies) in the fed-batch cultivation, this can be considered as the steppingstone for employment of simple and logical approaches of fed-batch cultivations and their optimizations. This would significantly minimize the experimentation to increase the yield and productivity of PHB accumulation. The developed mathematical model could also serve as a useful tool to design appropriate reactor operation strategies to optimize the PHB concentration and/or productivity[19]. The present investigation thus focused on economical biopolymer production for societal applications using engineering optimization tools.

Material and methods

Microorganism and maintenance

The strain *Cupriavidus necator* DSMZ 545 was procured from German Collection of Microorganisms and Cell Cultures (DSMZ, Germany) and activated in Luria-Bertani Media (HI Media, India) by incubating at 30 °C for 48 h. Thereafter, the cells were grown and maintained on LB Agar plates/slants at 30 °C for 48 h, and then stored at 4 °C. These were then subcultured monthly to maintain the viability of the organism.

Culture media

No literature reports were available on optimized medium recipes for utilization of glycerol by *C. necator*. An attempt was, therefore, made to determine the highest concentrations of the key nutrients commonly used for cultivation of *C. necator* from existing literature studies[2,20–25] which formed the basis for statistical optimization in the present studies. The concentrations of these key nutrients were then varied by ±20 % to determine the range (low/high concentrations) to be used for identification of the statistically optimized media as described below: 40 g L^{-1} (pure) glycerol, 2 g L^{-1} $(NH_4)_2SO_4$, 1.5 g L^{-1} KH_2PO_4, 3.5 g L^{-1} Na_2HPO_4, 0.2 g L^{-1} $MgSO_4 \cdot 7H_2O$, 0.02 g L^{-1} $CaCl_2 \cdot 2H_2O$, 0.058 g L^{-1} Ammonium Fe(III) citrate, and 1 mL trace element solution (TES). TES consisted of 0.1 g L^{-1} $ZnSO_4 \cdot 7H_2O$, 0.03 g L^{-1} $MnCl_2 \cdot 4H_2O$, 0.30 g L^{-1} H_3BO_3, 0.2 g L^{-1} $CoCl_2 \cdot 6H_2O$, 0.02 g L^{-1} $NiCl_2 \cdot 6H_2O$, and 0.03 g L^{-1} $Na_2MoO_4 \cdot 2H_2O$. Sulphates and phosphates in the medium were autoclaved in separate flasks to prevent the precipitation of medium components during sterilization; ammonium Fe(III) citrate and TES were only filtered sterilized using 0.22 µm syringe filter (Millipore, Ireland). All the sterilized medium components were then mixed aseptically in the laminar hood chamber. All culture media throughout the experiments were adjusted to a pH value of 6.8 and incubated at 30 °C[26].

Inoculum development

Two loops of actively growing colonies on Luria Broth (LB) agar plates were transferred aseptically to 100-mL flask containing 20 mL sterile LB broth. These cultures were incubated overnight at 30 °C and later transferred to 50 mL of sterile medium in a 250-mL flask with 5 % v/v of the inoculum to ensure the viability of the cells.

The inoculum for shake flask and/or bioreactor cultivation was developed according to the aforementioned protocol to ensure reproducibility in repetitive cultivations. For chemically defined media, the concentration of major limiting nutrient, glycerol, was enhanced gradually from 10 g L^{-1} to 20 g L^{-1} in a stepwise manner (while the other medium components were kept the same), and then finally to statistically optimized concentration of 40 g L^{-1} in the final shake flask to let the cells slowly adapt to high glycerol concentration. An amount of 200 m L^{-1} of inoculum culture of *C. necator* (5 % of working volume) was then used to inoculate the bioreactors.

Selection of significant effectors- Plackett Burman Design

Plackett Burman (PB) experimental design was used as an initial screening tool for identifying significant process variables affecting the key responses, namely, biomass and PHB accumulation. Seven factors – glycerol, $(NH_4)_2SO_4$, KH_2PO_4, $MgSO_4 \cdot 7H_2O$, Na_2HPO_4, $CaCl_2 \cdot 2H_2O$, and TES were selected as the key effectors for growth and PHB production by *C. necator* (Table 1). Highest concentration ranges of above effectors were selected from the extensive literature studies available[13,14,22–25] for PHB fermentation process by *C. necator* (formerly known as *Waustersia eutropha* or *Ralstonia eutropha*). These nutrients were then varied by ±20 % to determine the range of statistical optimization protocol. Two concentration levels, high (+1) and low (–1) were thus identified, and their impact on the responses, biomass, and PHB formation was examined. A set of twelve experiments were formulated using Stat-Ease Design Expert Software (Design Expert 5.0.9, Stat Ease Inc., MN, USA). These trial experiments were then performed in the shake flask, and responses (biomass and PHB) were as-

sessed. Statistical analysis of the 12 trial experiments and their responses (biomass and PHB) by the Stat-Ease software yielded t-coefficient values for all seven effectors. Those medium components which yielded highest t-coefficient values were then selected for Response Surface Methodology (RSM) studies to obtain their optimum concentrations.

Response Surface Methodology for optimization of concentrations of effectors

Response Surface Method (RSM) was utilized to optimize the effect of varying concentration of the effectors in detail on output responses (biomass and PHB). The effect of changing the concentrations of each of the three critical effectors (screened by the experimental design of Plackett-Burman) on the overall responses (biomass and PHB) was studied in detail using Central Composite Design (CCD), and a suitably identified statistical model (describing linear squared and interactive effects of these effectors) was then developed. The 2^3- Factorial CCD was formulated using Design Expert (5.0.9) software (Stat-Ease Corporation, USA), which led to a total number of 20 trial experiments (Table 4). These were then performed in shake flask to optimize the concentration of those selected effectors having high 't'-values. The concentration of these nutrients and their responses were used for nonlinear regression analysis and identification of model parameters of responses (biomass and PHB) as described later. A special feature of the software, point-prediction, was used thereafter to identify the final optimized values of the effectors.

Preliminary substrate inhibition studies

To investigate the effect of limiting substrate(s) (glycerol and nitrogen) on the growth of *C. necator,* substrate inhibition studies were conducted separately in 1-L shake flasks with 250 mL optimized media by changing the concentrations of aforementioned key limiting nutrients, while keeping the concentrations of the rest of the medium components at their optimal value. The effect of the increase in the substrate (glycerol concentration) from 5 g L^{-1} to 100 g L^{-1} on *C. necator* growth was observed while keeping the rest of the medium components at optimum value during shake flask cultivation by monitoring the OD_{600nm} (Optical Density) during the initial growth phase. Samples were withdrawn at 2-h intervals and analyzed for biomass. Similarly, the effect of increase in nitrogen concentration from 0.5 g L^{-1} to 13 g L^{-1} on the growth of *C. necator* was monitored while keeping the glycerol concentration constant at 40 g L^{-1}. The maximum specific growth rate (μ_{max}) at different times for substrate inhibition studies was calculated as slope of a graph between ln$X$ vs time with respect to glycerol/nitrogen concentrations in the exponential growth phase.

Study of batch growth and PHB production kinetics of *C. necator* in 7-L bioreactor using statistically optimized media

Batch growth and PHB accumulation studies of *C. necator* were done in a 7-L stirred tank double-jacketed bioreactor equipped with two six-flat-blade impellers (Applikon Dependable Instruments, The Netherlands) and three baffles with pH, temperature, dissolved oxygen sensor, and ADI 1025 Controller containing 4-L optimized medium recipe as described in the section "Culture media". The dissolved oxygen (DO) concentration inside the bioreactor was measured by Applisens dissolved oxygen probe (Applikon Dependable Instruments, The Netherlands), and its concentration was maintained above 30 % by manually adjusting the speed of the agitator and/or flow rate of sterile air in the bioreactor. The temperature was maintained at 30 °C by circulating constant-temperature water in the jacket of the bioreactor through Chilled Water Circulator (Julabo FP50, Germany). Culture pH was maintained at 6.8 by automatic addition of 2N HCl/2N NaOH solution through ADI pH controller unit. Samples were withdrawn at intervals of 3 h, and analyzed for biomass, residual glycerol, nitrogen concentration, and PHB content. The batch bioreactor experiments were performed for 48 h (in triplicate) and average values of process variables (X, S, P) are reported.

Development of batch mathematical model

A mathematical model was developed using the batch growth and PHB accumulation kinetics obtained from bioreactor experiments. The independently acquired data obtained from substrate inhibition (glycerol and nitrogen) experiments were also used for the development of the mathematical model.

The following assumptions were made for the development of the mathematical model:

– Glycerol and nitrogen were the only limiting (substrates) affecting the growth and PHB production.

– The rest of the medium components were available in excess during the entire fermentation.

– The temperature (30 °C) and pH (6.8) of the culture broth was maintained constant throughout the course of cultivation.

Nonlinear regression technique as proposed by the original algorithm of Rosenbrock[27], and the computer programs and methodology described by

Volesky and Votruba[28] were then used to identify the optimized values of model parameters. This technique minimizes the differences between the experimental data points and corresponding model simulations for different process variables at different times to carefully define the objective function SSWR (explained in Equation 11).

Development of model-based nutrient(s) feeding strategy(ies) in fed-batch fermentation

From substrate inhibition studies it was established that high concentrations of the major nutrients (glycerol and nitrogen) inhibit the microbial growth and product accumulation, thereby indicating that only slow feeding of the nutrients in the bioreactor (fed-batch cultivation) would eliminate the substrate inhibition problem, and be a better choice for obtaining significantly high biomass with increased PHB accumulation and productivity in the bioreactor. Therefore, the mathematical model equations (Equations 1–10) were extrapolated to simulate fed-batch cultivations by taking overall mass balance around the bioreactor and incorporating the dilution terms in the model equations (Equations 12–17). This fed-batch mathematical model was then used to generate several offline computer simulations for different nutrient(s) feeding strategies as described further.

Fed-batch cultivation strategy

The bioreactor cultivation was initiated as a batch in a 7-L bioreactor (working volume 4-L) containing optimized media (initial glycerol concentration of 40 g L^{-1}). When the culture was in exponential growth phase, constant feeding of glycerol (175 g L^{-1}) and nitrogen (2.5 g L^{-1}) at 100 mL h^{-1} was initialized at 15 h (as identified by model simulations). The constant feeding of substrates glycerol and nitrogen were continued for 20 h (15 h –35 h) for maintenance of culture growth (as predicted by the model). After the nutrient feeding was stopped, the (secondary) batch cultivation was resumed further until 48 h for the consumption of the residual substrates (glycerol and nitrogen) in the bioreactor. For maintenance of pseudo steady state with respect to substrate (glycerol), variable feeding of glycerol at 200 g L^{-1} concentration (along with proportionately increased concentrations of other medium components) had started at 20 h and continued until 40 h of cultivation to ensure availability of a constant non-limiting and non-inhibitory concentration of (key) substrate glycerol concentration (as identified by model) during the fed-batch cultivation period. Secondary batch fermentation was then performed to consume the residual glycerol in the bioreactor. For decreasing feed rate, feeding of both glycerol (200 g L^{-1}) and nitrogen (3.5 g L^{-1}) was initiated at 16 h at a feed rate of 75 mL h^{-1}. The feeding rate thereafter was reduced to 55 mL h^{-1} after 24 h of cultivation. Feeding of substrate was then continued at this flow rate for another 8 h, and thereafter the flow rate was further reduced to 35 mL h^{-1}, which was then continued until the end of fermentation. The guidance for gradual decrease of nutrient feed rate emerged from several off-line model simulations on computer with the main aim that the need of secondary batch cultivation may be eliminated, and it should be possible to harvest the bioreactor immediately after the termination of the nutrient feed to reduce total cultivation time and eventually enhance the biomass/PHB accumulation.

Analytical methods

Optical density of appropriately diluted culture broth was measured by spectrophotometer (OPTIZEN model 3220UV, Mecasys, Korea) at 600 nm against the medium blank. An amount of 30 mL of the fermentation broth samples were withdrawn from the bioreactor every three hours. This was then centrifuged (Centrifuge 5810 R, Eppendorf India Limited, India) at 9,000 g for 15 min at a temperature of 4 °C, and the supernatant was used for the analysis of residual nutrients concentration. Glycerol was analyzed by high performance liquid chromatography (HPLC) (Waters 515), and separation was achieved using Phenomenex Rezex RCM-Monosaccharide Ca^{2+} (8 %) column (Column Dimensions: 300 x 7.8 mm ID; Elution Type: Isocratic; Eluent: Water; Flow Rate: 0.5 mL min^{-1}; Col. Temp.: 60 °C; Refractive Index (RI) detector). The Kjeldahl method was used for the analysis of residual ammonia nitrogen[29]. The cell pellet left in the centrifuge tube after centrifugation was dried at 90 °C in a hot air oven, and CDM (cell dry mass) was calculated. PHB concentration was quantified by gas chromatography (GC 2010 Shimadzu Co., Japan) using benzoic acid as an internal standard[30,31].

Results and discussion

Statistical optimization of nutrients for medium recipe

The initial screening of the nutrients (effectors) on the desired responses (biomass and PHB) was performed according to the Plackett-Burman protocol, which helped in prioritizing the effectors affecting the overall response. Each nutrient effector was evaluated at two concentration levels (as shown in Table 1), high (+1) and low (–1), as per the guidance available from the literature studies[12–14,22–25]. A nutrient recipe consisting of 12 experiments was designed by the software. Table 2 highlights the concentration distribution of these factors in the trial

experiment according to the Design Expert software and their responses in the study. From Table 2, it can be concluded that the high concentration of glycerol can affect biomass and PHB differently.

Table 3 summarizes the statistical 't'-value coefficients for the different effectors (A-G) for the experimental study highlighting the significant effect of any effector on the responses (biomass and PHB). As may be seen from Table 3, the value of 't" for nutrients glycerol, $(NH_4)_2SO_4$, and TES were positive (in increasing order), and therefore these were the major nutrients (critical effectors) affecting the biomass and PHB formation by *C. necator*. This was an obvious conclusion also since carbon and nitrogen are the two most important factors that play a key role in PHB accumulation, which normally takes place under excess availability of carbon source and low (limiting) concentrations of nitrogen and/or oxygen[32]. Thus, by optimizing these two effectors were expected to yield larger values of concentration and productivity of PHB. Trace element solution (TES), which comprises a number of different micronutrients, is also essentially required for maintenance of protein structure, and functioning of key biosynthetic enzymes. Thus, these three factors, screened by the experimental design of Plackett-Burman, were then subjected to further analysis of Response Surface Methodology (RSM) to identify the appropriate concentration of selectively identified key nutrients. The analysis identifies the model parameters of equations (i) and (ii) by non-linear regression between concentrations of effectors of trial experiments with the corresponding concentrations of effectors by the solution of the model. Based on the Plackett–Burman design, three factors (glycerol, $(NH_4)_2SO_4$, and TES) that showed positive influence on growth and PHB were selected, and CCD was used to determine the optimum levels of these parameters. A total of 20 experimental runs with different combinations of glycerol (A), $(NH_4)_2SO_4$ (B), and TES (C) were performed (Table 4), and the responses with respect to biomass and PHB were established. The results were analyzed by Design Expert Software and the following quadratic regression equations were obtained in terms of the selected variables for growth and PHB production:

$$\text{Biomass} = 1.63222 + 0.377767A + 0.607749B + 0.233314C + 0.366739A^2 + 0.0308632B^2 + 0.331384C^2 - 0.05AB - 0.35AC + 0.425BC \quad \text{(i)}$$

$$\text{PHB} = 0.0163787 + 0.111404A + 0.00165526B - 0.0049446C + 0.0484388A^2 + 0.0430762B^2 + 0.0554507C^2 + 0.0343931AB + 0.0267856AC + 0.231374BC \quad \text{(ii)}$$

where A = Glycerol, B = Ammonium sulphate, C = TES (Trace Element Solution).

Table 1 – *Concentration ranges of effectors of the medium recipe for Plackett-Burman Optimization protocol*

S.no	Coded factors (g L^{-1})	Name of the effectors	Concentration Level	
			–1 (low) (g L^{-1})	+1 (high) (g L^{-1})
1	A	Glycerol	20	45
2	B	$(NH_4)_2SO_4$	0.5	2
3	C	KH_2PO_4	0.5	2
4	D	$MgSO_4 \cdot 7H_2O$	0.1	3
5	E	Na_2HPO_4	0.4	4
6	F	$CaCl_2 \cdot 2H_2O$	0.01	0.03
7	G	TES (Trace Element Solution)	0.5	1.5

Table 2 – *Experimental Design of Plackett-Burman Protocol along with their Responses (biomass and PHB)*

CODED EFFECTORS (A – G)								Response	
Exp No.	A	B	C	D	E	F	G	Biomass (g L^{-1})	PHB (g L^{-1})
1	20	0.5	0.5	3	4	0.03	0.5	1.78	0.49
2	20	2	2	0.1	4	0.01	0.5	2.59	0.24
3	20	2	2	3	0.4	0.03	1.5	2.75	0.26
4	45	0.5	2	0.1	0.4	0.01	1.5	2.15	0.52
5	45	2	0.5	3	0.4	0.01	0.5	1.78	0.49
6	20	2	0.5	0.1	0.4	0.03	1.5	1.99	1.06
7	45	0.5	2	3	0.4	0.03	0.5	3.04	0.59
8	45	2	0.5	3	4	0.01	1.5	4.52	0.32
9	45	0.5	0.5	0.1	4	0.03	1.5	2.29	0.11
10	20	0.5	0.5	0.1	0.4	0.01	0.5	1.63	0.59
11	45	2	2	0.1	4	0.03	0.5	2.89	0.19
12	20	0.5	2	3	4	0.01	1.5	1.99	0.67

Table 3 – *Statistical 't'-value coefficients for different effectors under study for growth and PHB accumulation by C. necator*

Coded Effectors[a]	Name	Biomass (g L^{-1})	PHB (g L^{-1})
A	Glycerol	1.90^1	0.12^2
B	$(NH_4)_2SO_4$	1.76^2	–0.11
C	KH_2PO_4	0.46	–0.54
D	$MgSO_4 \cdot 7H_2O$	1.20	–0.98
E	Na_2HPO_4	1.90	–1.34
F	$CaCl_2 \cdot 2H_2O$	0.024	–0.35
G	TES (Trace Element Solution)	1.29^3	0.30^1

The superscript numbers (1, 2, 3) describe the priority of significance on the respective responses[a]

Table 4 – *Experimental Design for Response Surface Methodology for three effectors along with their responses*

S.no	Effectors (A-C)			Responses	
	A (g L^{-1})	B (g L^{-1})	C (mL L^{-1})	Biomass (g L^{-1})	PHB (g L^{-1})
1	32.5	2.51	1	2.8	0.04
2	53.52	1.25	1	2.4	0.08
3	32.5	1.25	1	2.1	0.008
4	11.48	1.25	1	2.9	0.03
5	20	0.5	1.5	0.9	0.03
6	32.5	1.25	1	0.9	0.08
7	45	2	0.5	3.6	0.03
8	32.5	1.25	0.15	1.9	0.13
9	32.5	1.25	1.84	3.2	0.03
10	32.5	1.25	1	1.7	0.08
11	32.5	1.25	1	2.9	0.03
12	20	2	1.5	3.3	0.09
13	45	0.5	0.5	3.1	1.17
14	32.5	0	1	0.6	0.03
15	45	0.5	1.5	2.1	0.02
16	20	2	0.5	1.2	0.001
17	32.5	1.25	1	0.1	0.001
18	45	2	1.5	3.7	1.37
19	20	0.5	0.5	1.1	0.25
20	32.5	1.25	1	2.1	0.015

Table 5 – *Design matrix evaluation for Response Surface Quadratic Model*

Term	Std Err	VIF	Ri-Squared	0.5 Std Dec	1 Std. Dev	2 Std Dev
A	0.27	1.00	0.0000	13.3 %	38.6 %	91.4 %
B	0.27	1.00	0.0000	13.3 %	38.6 %	91.4 %
C	0.27	1.00	0.0000	13.3 %	38.6 %	91.4 %
AB	0.35	1.00	0.0000	9.8 %	24.9 %	72.2 %
AC	0.35	1.00	0.0000	9.8 %	24.9 %	72.2 %
BC	0.35	1.00	0.0000	9.8 %	24.9 %	72.2 %
A^2	0.26	1.02	0.0179	40.4 %	92.7 %	99.9 %
B^2	0.26	1.02	0.0179	40.4 %	92.7 %	99.9 %
C^2	0.26	1.02	0.0179	40.4 %	92.7 %	99.9 %

Ideal VIF (Variance Inflation factor) is 1.0. VIFs above 10 are cause for alarm, indicating coefficients are poorly estimated due to multicollinearity. Ideal Ri-squared is 0.0. High Ri-squared means terms are correlated with each other, possibly leading to poor models.

The equations (i) and (ii) in terms of coded factors can be used to make predictions of the responses for any given concentration levels of different effector(s). The model demonstrated an adequate precision of 4.65 with respect to biomass, and 4.70 with respect to PHB. Table 5 presents the design matrix evaluation for Response Surface Quadratic Model, which shows that the model will adequately predict the responses within the design space. Thus, it exhibited that the model is a good predictor of the responses. Optimized concentration of the medium components was established by examining point prediction feature of the Design Expert Software. The point prediction feature of the software allows the user to vary the concentration of different effectors at discrete levels with multiple combinations to probe their effect on the predicted responses in zero time. Point prediction uses the models fit during analysis on the factors to compute the point prediction and interval estimates. The predicted values are updated as the levels are changed. A maximum biomass and PHB concentration of 3.7 g L^{-1} and 1.36 g L^{-1}, respectively, were obtained by the statistical optimization protocol at the following optimized values of medium components: 40 g L^{-1} of glycerol, 2 g L^{-1} of $(NH_4)_2SO_4$ and 1 mL L^{-1} of TES.

Substrate (glycerol and nitrogen) inhibition studies

The effect of increasing glycerol concentration (from 5 to 100 g L^{-1}) on *C. necator* was assessed during shake flask cultivations, where the rest of the medium components were maintained at a constant level in the growth medium. The specific growth rate (μ) increased gradually as the concentration of glycerol was increased from 5 g L^{-1} to 25 g L^{-1} until a maximum value of specific growth rate (μ_{max} = 0.53 h^{-1}) was observed at 25 g L^{-1} (Fig. 1). This may be primarily due to substrate limitation; thereafter inhibition of culture growth and decrease in specific growth rate of *C. necator* was observed when the glycerol concentration was further increased beyond 25 g L^{-1}. The specific growth rate fell sharply to a value of 0.18 h^{-1} upon growth of *C. necator* at 60 g L^{-1} glycerol concentration. Specific growth rate continued to decrease until it reached 0.013 h^{-1} (almost zero) at a concentration of 100 g L^{-1}. Therefore, 100 g L^{-1} was considered as highest concentration at which complete growth inhibition sets in. This was considered as critical glycerol concentration (S_m) at which almost complete cessation of growth occurs.

Similarly, experiments were conducted by varying initial concentrations of nitrogen (as ammonium sulphate) to establish its effect on the growth of *C. necator*. Varying initial nitrogen concentrations (0.5–13 g L^{-1}) were taken in the medium, which

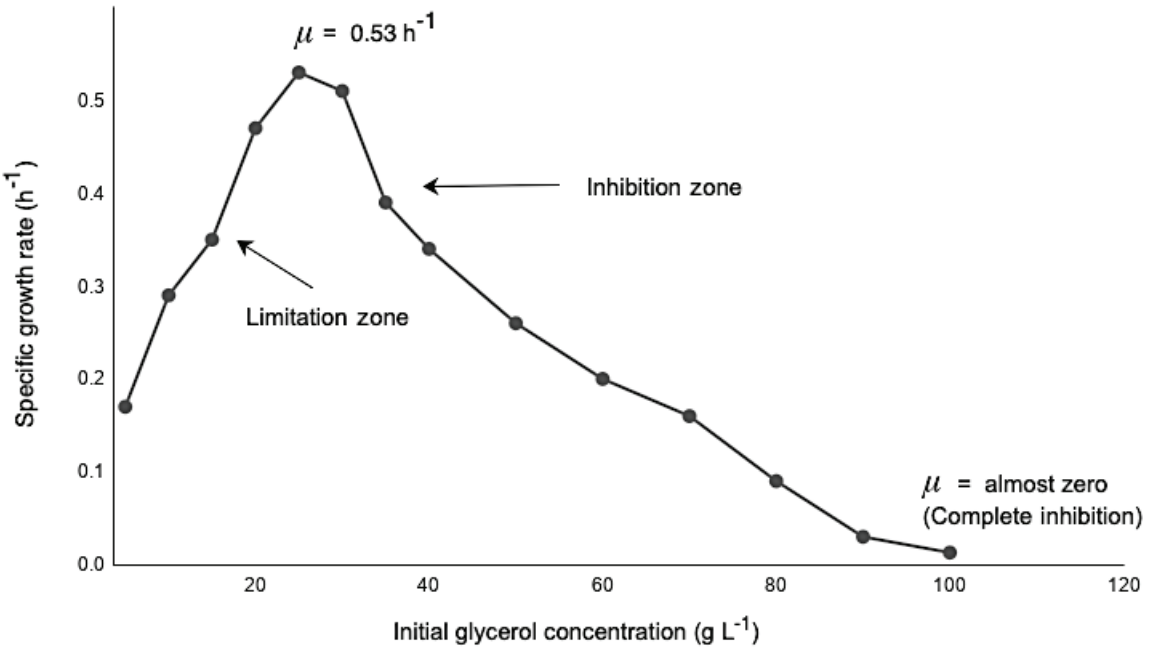

Fig. 1 – *Effect of increasing initial glycerol concentration on growth of C. necator*

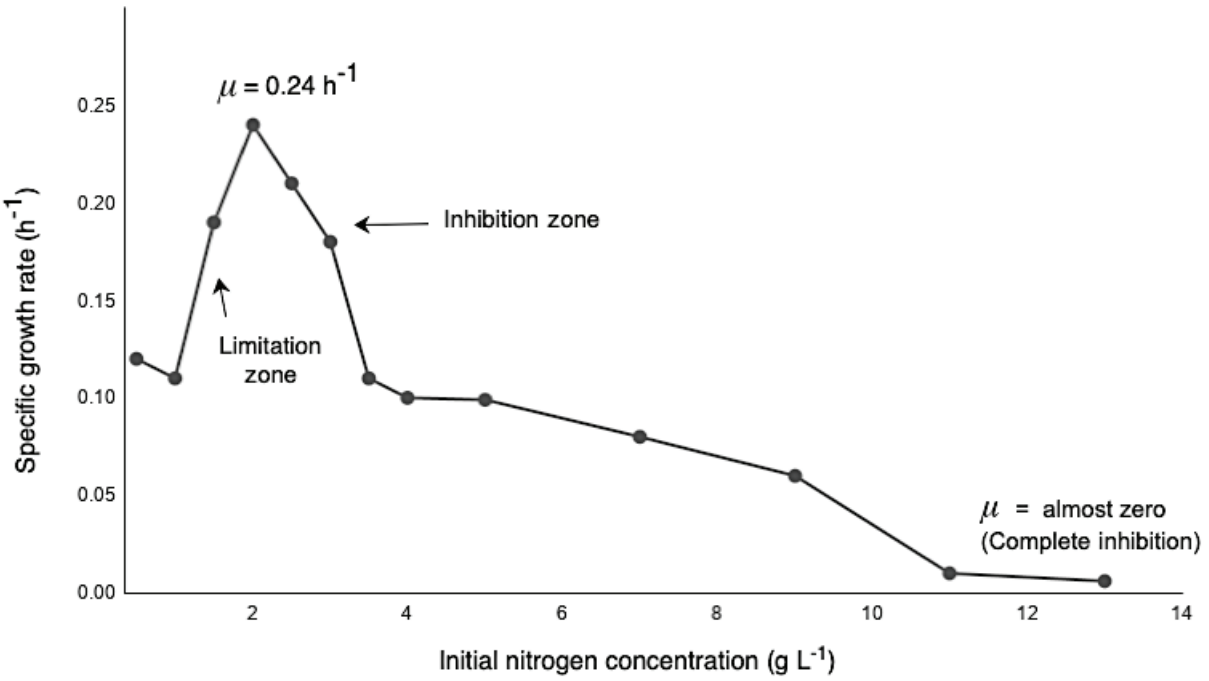

Fig. 2 – *Effect of increasing initial nitrogen concentration on growth of C. necator*

contained 40 g L^{-1} glycerol (constant) as the carbon source. A maximum specific growth rate (μ_{max}) of 0.24 h^{-1} was obtained at nitrogen concentration of 2 g L^{-1} ammonium sulphate, while complete inhibition of culture growth was observed at nitrogen concentration of 13 g L^{-1}, as shown in Fig. 2.

These effects of initial glycerol and/or nitrogen concentration with respect to specific growth rate helped in the identification of appropriate substrate inhibition constants of the model equations for the batch mathematical model for growth and PHB production as described later.

Batch kinetics on *C. necator* using statistically optimized media

Growth kinetics of the *C. necator* was then studied in a lab-scale bioreactor (7 L) under controlled pH and temperature of 6.8, and 30 °C, respectively. Fig. 3 demonstrates the time course of

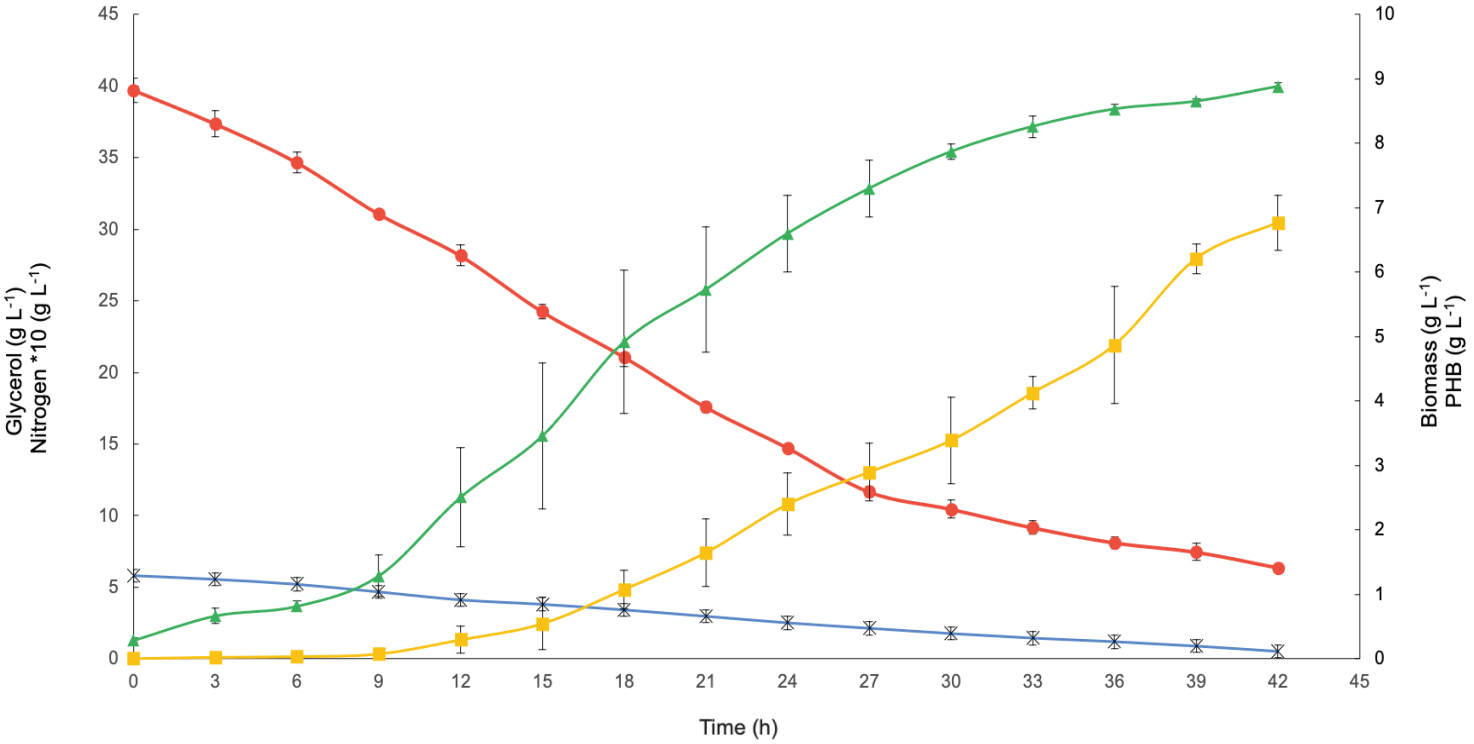

Fig. 3 – *Batch kinetic data of total biomass, nutrient consumption, and PHB production for C. necator in a 7-L bioreactor (glycerol concentration – circle, biomass concentration – triangle, PHB concentration – square, nitrogen concentration – cross)*

batch cultivation of *C. necator* for PHB production. The culture exhibited an initial lag phase of around 9 h, after which it featured exponential growth. Batch fermentation featured overall accumulation of 8.88 g L^{-1} biomass, and 6.76 g L^{-1} PHB concentration in 42 h of cultivation period, thereby resulting in a maximum PHB productivity of 0.16 g L^{-1}.

Proposal of mathematical model and assessment of model parameters

Differential mass balance equations were used for the description of observed batch fermentation kinetics of microbial growth, substrate consumption, and PHB accumulation, as described below:

$$\mu = \mu_{\max}\left[\frac{S_1}{S_1 + K_{S_1}}\right]\left[\frac{S_2^{n_1}}{S_2^{n_1} + K_{S_2}^{n_1}}\right] \tag{1}$$

$$\frac{\mathrm{d}X}{\mathrm{d}t} = \mu_{\max}\left[\frac{S_1}{S_1 + K_{S_1}}\right]\left[\frac{S_2^{n_1}}{S_2^{n_1} + K_{S_2}^{n_1}}\right]X \tag{2}$$

Equation (2) describes the biomass formation rate ($\mathrm{d}X/\mathrm{d}t$), which featured limitation by two major nutrients, glycerol (S_1) and nitrogen (S_2) featuring applicability of Monod kinetics with respect to key substrate glycerol and Sigmoidal kinetics with respect to minor nutrient nitrogen, respectively.

From the preliminary experiments on the growth inhibition by increasing initial substrate (glycerol) concentration (Fig. 1), it was indicated that the specific growth rate started to decrease after a particular concentration of glycerol (above 25 g L^{-1}). However, almost complete culture growth inhibition was recorded only at a glycerol concentration (S_m) of 100 g L^{-1}. This indicated that an empirical correlation proposed by Luong[33] describing the inhibition kinetics of culture growth substrate (glycerol) was more appropriate to describe the observed experimental inhibition pattern by the glycerol, as given below:

$$\mu = \mu_{\max}\left(1 - \left(\frac{S_1}{S_{m_1}}\right)^{a_1}\right) \tag{3}$$

Similarly, culture growth inhibition studies with respect to nitrogen exhibited a slow decrease in specific growth rate, followed by its decrease to zero with increasing initial nitrogen concentration, as shown in Fig. 2. The inhibition of culture growth by increasing concentrations of nitrogen was also described by an empirical correlation proposed by Luong[33].

$$\mu = \mu_{\max}\left(1 - \left(\frac{S_2}{S_{m_2}}\right)^{a_2}\right) \tag{4}$$

The differential mass balance equation for culture growth by incorporating the substrate inhibition terms was described as follows:

$$\frac{dX}{dt} = \mu_{max}\left[\frac{S_1}{S_1 + K_{S_1}}\right]\left[\frac{S_2^{n_1}}{S_2^{n_1} + K_{S_2}^{n_1}}\right]\left[1-\left(\frac{S_1}{S_{m_1}}\right)^{a_1}\right]\left[1-\left(\frac{S_2}{S_{m_2}}\right)^{a_2}\right]X \quad (5)$$

$$\mu = \mu_{max}\left[\frac{S_1}{S_1 + K_{S_1}}\right]\left[\frac{S_2^{n_1}}{S_2^{n_1} + K_{S_2}^{n_1}}\right]\left[1-\left(\frac{S_1}{S_{m_1}}\right)^{a_1}\right]\left[1-\left(\frac{S_2}{S_{m_2}}\right)^{a_2}\right] \quad (6)$$

Specific rate of glycerol consumption (q_{S_1}) was described by Eq. 7, as follows:

$$\frac{dS_1}{dt} = q_{S_1} X = -\left[\frac{1}{Y_{\frac{X}{S_1}}}\mu + m_{S_1}\right]X \quad (7)$$

The following equation represents the specific nitrogen consumption rate (q_{S_2}):

$$\frac{dS_2}{dt} = q_{S_2} X = -\left[\frac{1}{Y_{\frac{X}{S_2}}}\mu + m_{S_2}\right]X \quad (8)$$

where $Y_{\frac{X}{S_2}}$ represents the yield of biomass with respect to nitrogen, and m_{S_2} is the maintenance energy requirement of the cell on nitrogen. The product formation was observed during growth phase as well for non-growth phase, therefore specific rate of product formation (q_P) was adequately described by the growth-associated component and non-growth-associated component as follows:

$$q_P = k_1\mu + k_2 \quad (9)$$

$$\frac{dP}{dt} = (k_1\mu + k_2)X \quad (10)$$

where k_1 and k_2 represent the growth-associated and non-growth-associated product formation constants, respectively. Hence, Eqs. (5, 7, 8, and 10) represent the batch mathematical model equations for growth, substrate consumption, and PHB accumulation by *C. necator*.

Estimation of model parameters

The optimized values of the model parameters (Table 6) were determined by minimizing the difference between the experimental data points and corresponding model simulations using a non-linear regression technique[27,34] developed by computer program[28]. For the estimation of model parameters, a system of differential equations, Eqs. (5, 7, 8, and 10), was solved using a numerical integration program based on Runge-Kutta method of 4th order. Thereafter, the search for the minimum of the multivariable objective function (SSWR) was performed by the original algorithm of Rosenbrock[27], as described further, and which was extensively used before by several researchers:

$$SSWR = \sum_{i=1}^{n}\sum_{j=1}^{m}\frac{\Delta_{ij}^2}{W_j^2} \quad (11)$$

where,

– SSWR describes the sum of the square of the weighted residues
– i reflects the data point and has the limit of 1 to n, while j describes the process variable and has the limit of 1 to m
– W_j = the weight of each variable (normally taken as the maximum value of each process variable) to normalize the error between experimental data points and model simulation.
– Δ_{ij} = difference between the model simulated process variable at a particular data point and corresponding experimental data point ($y_{model} - y_{expt}$).

Fig. 4 shows the comparison of the model simulation and experimental data points wherein a good agreement between the two is clearly reflected. The developed model was able to describe successfully the experimental batch kinetics of *C. necator*.

Table 6 – *Optimized value of the model parameters for PHB fermentation process by C. necator*

Parameters	Units	Values
μ_{max}	h^{-1}	0.16
K_{S_1}	g L^{-1}	3.14
K_{S_2}	g L^{-1}	2.6
n_1	Dimensionless	3.49
$1/Y_{(X+P)/S_1)}$	g g^{-1}	4.11
m_{S_1}	g g^{-1} h^{-1}	0.002
$1/Y_{(X/S_2)}$	g g^{-1}	0.61
m_{S_2}	g g^{-1} h^{-1}	0.00
k_1	g L^{-1}	0.14
k_2	g L^{-1}	0.02
S_{m_1}	g L^{-1}	95.58
a_1	Dimensionless	3.07
S_{m_2}	g L^{-1}	13.0
a_2	Dimensionless	2.85

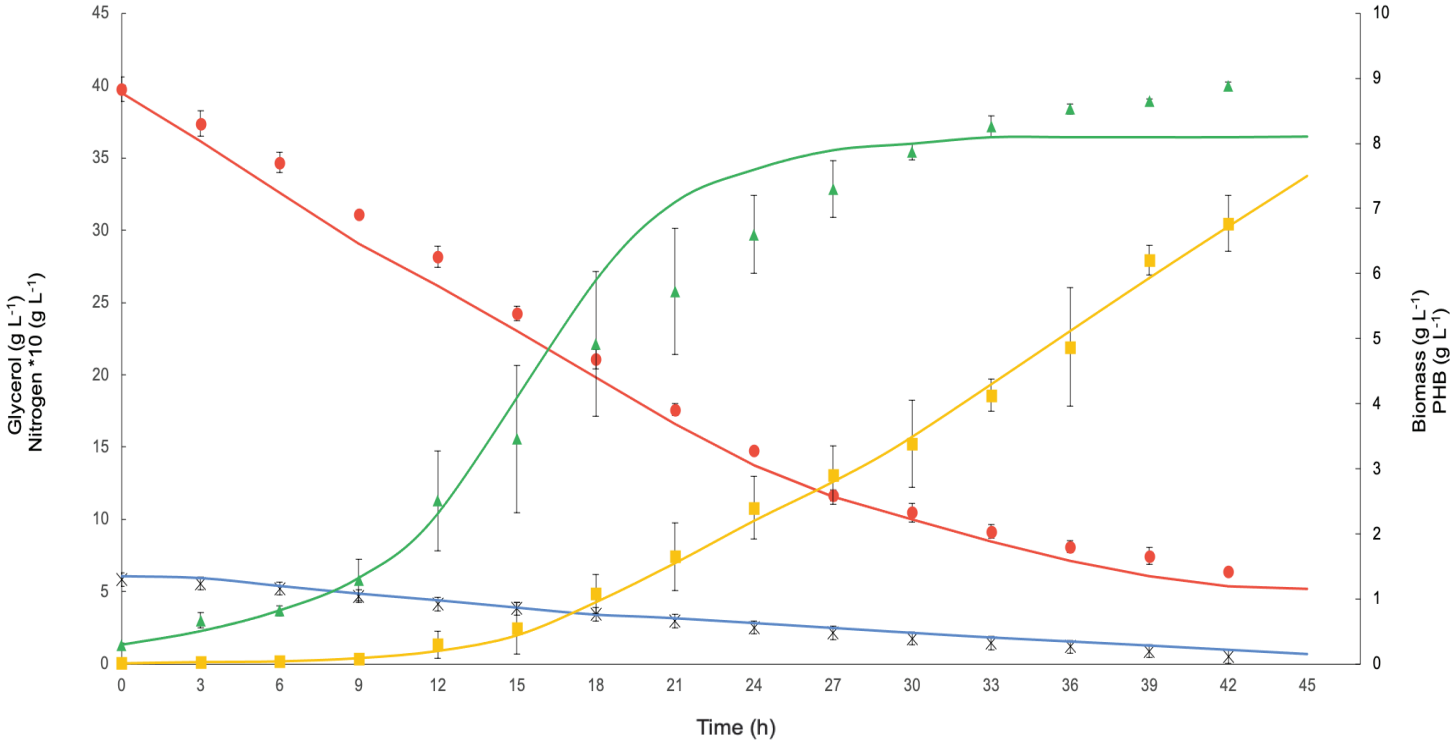

Fig. 4 – *Comparison of model simulations (smooth lines) and experimental values (data points) of batch fermentation kinetics of C. necator. Data points (● Glycerol, × Nitrogen, ■ PHB, ▲ Biomass) represent the average values of the samples (triplicate).*

Development of model-based nutrient(s) feeding strategy(ies) during fed-batch cultivation in bioreactor

In the present investigation, the nutrient feedings were designed to ensure non-growth phase of cultivation, characterized by excess availability of major substrate (glycerol) and limiting concentration of nitrogen to facilitate enhanced PHB accumulation. Several factors, including limitation of the fresh key (major) nutrients feed, its optimal concentration, on/off time, and its rate of addition play an important role in successful design of fed-batch cultivations. Thus, the developed batch mathematical model can be utilized as a tool to simulate nutrient feeding strategies of key limiting nutrients (carbon and nitrogen) to yield highest product concentration with minimal unconverted substrate at the end of fermentation. The batch model equations were extrapolated to describe the fed-batch model, as follows:

$$\frac{\mathrm{d}V}{\mathrm{d}t} = F_1 + F_2 = F \tag{12}$$

$$D = \frac{F}{V} \tag{13}$$

$$\frac{\mathrm{d}X}{\mathrm{d}t} = \mu_{\max}\left[\frac{S_1}{S_1 + K_{S_1}}\right]\left[\frac{S_2^{n_1}}{S_2^{n_1} + K_{S_2}^{n_1}}\right]\left[1 - \left(\frac{S_1}{S_{\mathrm{m}_1}}\right)^{a_1}\right]\left[1 - \left(\frac{S_2}{S_{\mathrm{m}_2}}\right)^{a_2}\right] - \left[\frac{F_1 + F_2}{V}\right]X \tag{14}$$

$$\frac{\mathrm{d}S_1}{\mathrm{d}t} = -\left[\frac{1}{Y_{\frac{X}{S_1}}}\mu + m_{S_1}\right]X + \frac{F_1}{V}(S_{01}) - \frac{(S_1)F}{V} \tag{15}$$

$$\frac{\mathrm{d}S_2}{\mathrm{d}t} = -\left[\frac{1}{Y_{\frac{X}{S_2}}}\mu + m_{S_2}\right]X + \frac{F_2}{V}(S_{02}) - \frac{(S_2)F}{V} \tag{16}$$

$$\frac{dP}{dt} = [k_1\mu + k_2]X - \left[\frac{F_1 + F_2}{V}\right]P \quad (17)$$

where "D" represents the dilution rate= F/V.
F = total flow rate
F_1 = flow rate for glycerol
F_2 = flow rate for nitrogen
V = working volume of bioreactor
S_{01} and S_{02} are inlet concentrations of substrate(s) glycerol and nitrogen, respectively, in the feed reservoir.

The different fed-batch cultivation strategies used in the present investigation are described further.

Fed-batch cultivation with constant feed rate

With an aim to improve the PHB concentration and productivity over the batch cultivation, the developed model was used to simulate the fed-batch strategy with constant feed rate offline and was later experimentally implemented. Initially, cultivation of *C. necator* was conducted in batch mode in a 7-L bioreactor (4-L working volume) with statistically optimized medium recipe. When the culture was in an active growing stage, constant feeding of glycerol (175 g L^{-1}) and nitrogen (2.5 g L^{-1}) at 100 mL h^{-1} was initiated, as shown in Fig. 5, keeping other medium nutrients at their optimized value. The feeding was continued for 20 h in order to sustain the exponential growth of the culture. At 35 h, the reactor was again operated in batch mode (secondary batch) for the complete consumption of residual substrates. The strategy proved extremely advantageous because the limiting (disappearing) nutrient availability at the time when the biomass concentration was extremely high (at hour 15) was overcome by addition of constant feed of glycerol and nitrogen, which eventually featured increased rates of biomass and product accumulation, and better glycerol consumption. This model-designed nutrient feeding strategy ensured a reasonably high glycerol and limiting nitrogen availability, which ensured PHB accumulation in the latter phase of the cultivation. A maximum biomass of 18.79 g L^{-1} and PHB accumulation of 11.37 g L^{-1} (60 % of CDM) was obtained experimentally in 48 h in the fed-batch cultivation, which was in close proximity to the model-predicted values. Fig. 5 describes the different experimental data points and the corresponding model simulations (smooth lines) for the fed-batch cultivation under constant feed rate, as identified above. This fed-batch cultivation strategy demonstrated a significant

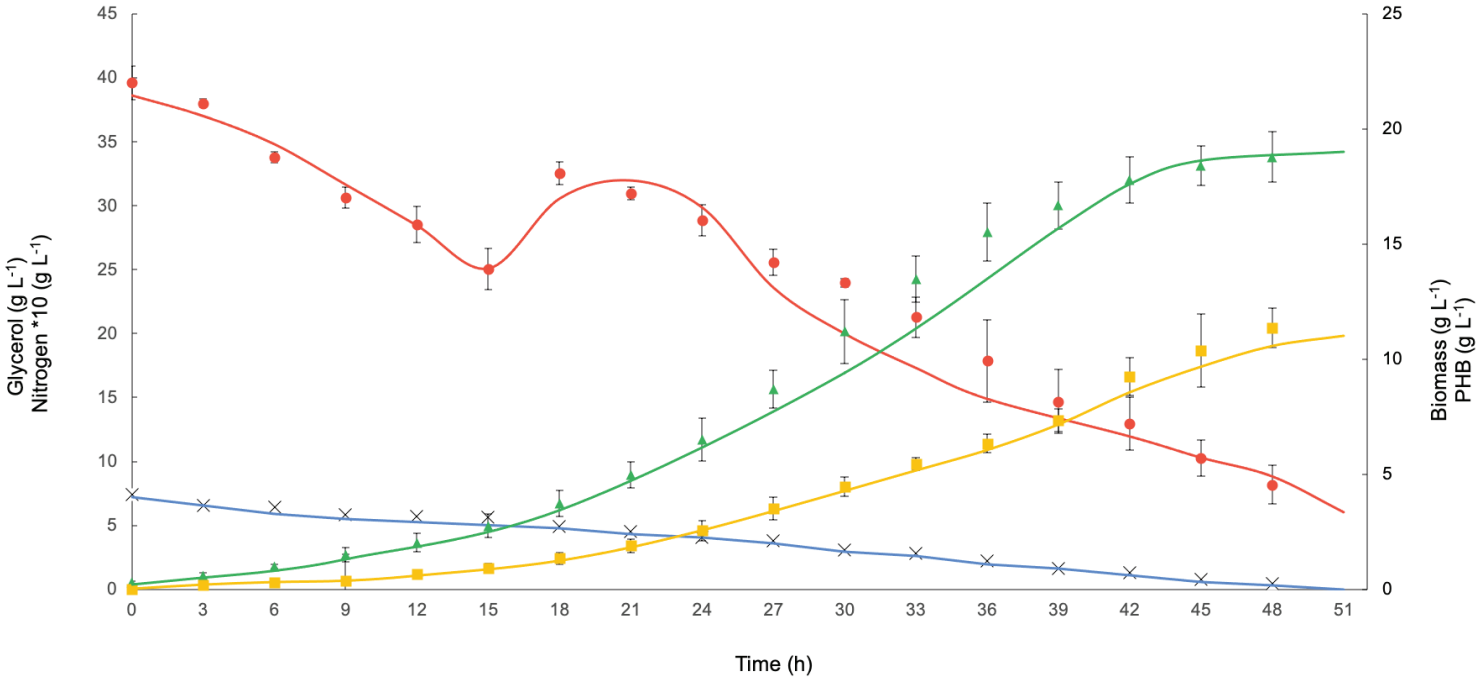

Fig. 5 – *Model-based fed-batch cultivation at constant feed rate. Comparison between model predictions (smooth lines) and experimental values (data points) (0–15 h-batch; 15–35 h fed-batch at constant feed rate of 100 mL h^{-1}; 35–48 h-batch fermentation). Data points (● Glycerol, × Nitrogen, ■ PHB, ▲ Biomass) represent the average values of the samples (triplicate).*

improvement in PHB productivity (0.23 g L^{-1} h^{-1}) as opposed to 0.16 g L^{-1} h^{-1}observed during batch cultivation.

Fed-batch cultivation at pseudo steady state

Another fed-batch cultivation strategy of pseudo steady state was simulated and experimentally implemented. A large number of off-line simulations was carried out to identify the glycerol concentration such that its constant concentration (pseudo steady state) was maintained inside the reactor, so that neither limitation nor inhibition of *C. necator* would occur. The model-based cultivation was initiated as a batch, and when the culture was actively growing and the residual glycerol concentration was reduced to 19.13 g L^{-1}, variable feeding of glycerol at 200 g L^{-1} (along with proportionately increased concentrations of other medium components) was started at 20 h and fed until 40 h of cultivation. At 40 h, the mathematical model was again utilized to simulate secondary batch fermentation so that the higher concentration of accumulated residual glycerol is consumed completely before the termination of the experiment. For this fed-batch cultivation, the model predicted an overall biomass of 29.94 g L^{-1} and PHB accumulation of 13.84 g L^{-1}. Fig. 6 shows the comparison of experimental (data points) along with the corresponding model simulations (smooth lines) for the fed-batch cultivation featuring the pseudo steady state with respect to glycerol for 20–40 hours. Reasonably high biomass concentration of 24.44 g L^{-1} and PHB accumulation of 13.12 g L^{-1} (53 % of CDM) was obtained experimentally in 48 h of cultivation of *C. necator*. This cultivation strategy exhibited major improvement in PHB productivity (0.27 g L^{-1} h^{-1}) as opposed to 0.16 g L^{-1} h^{-1} obtained during batch cultivation.

Fed-batch cultivation with decreasing feed rate

In the fed-batch cultivation strategies, it was observed that there was a need of secondary batch cultivations to consume the unconverted glycerol when the nutrients feed was completed at full reactor volume condition. Therefore, it was considered necessary to design the nutrient feeding strategy in such a way that eliminates the need of secondary batch cultivations, where termination of feeding of substrate (glycerol) coincides with the end of fermentation. Out of several off-line computer simulations of the model, one such model-simulated fed-batch strategy was experimentally implemented where the batch cultivation lasted for 16 h, and thereafter feeding of substrate (200 g L^{-1} glycerol and 3.5 g L^{-1} of nitrogen) was continued until 24 h at a feed rate of 75 mL h^{-1}. The feeding rate was then reduced to 55 mL h^{-1} after 24 h of cultivation, and was then continued for another 8 h; thereafter, the flow rate was further reduced to 35 mL h^{-1} until the end of fermentation (48 hours). Design of such a strategy predicted 20.57 g L^{-1} of biomass and an

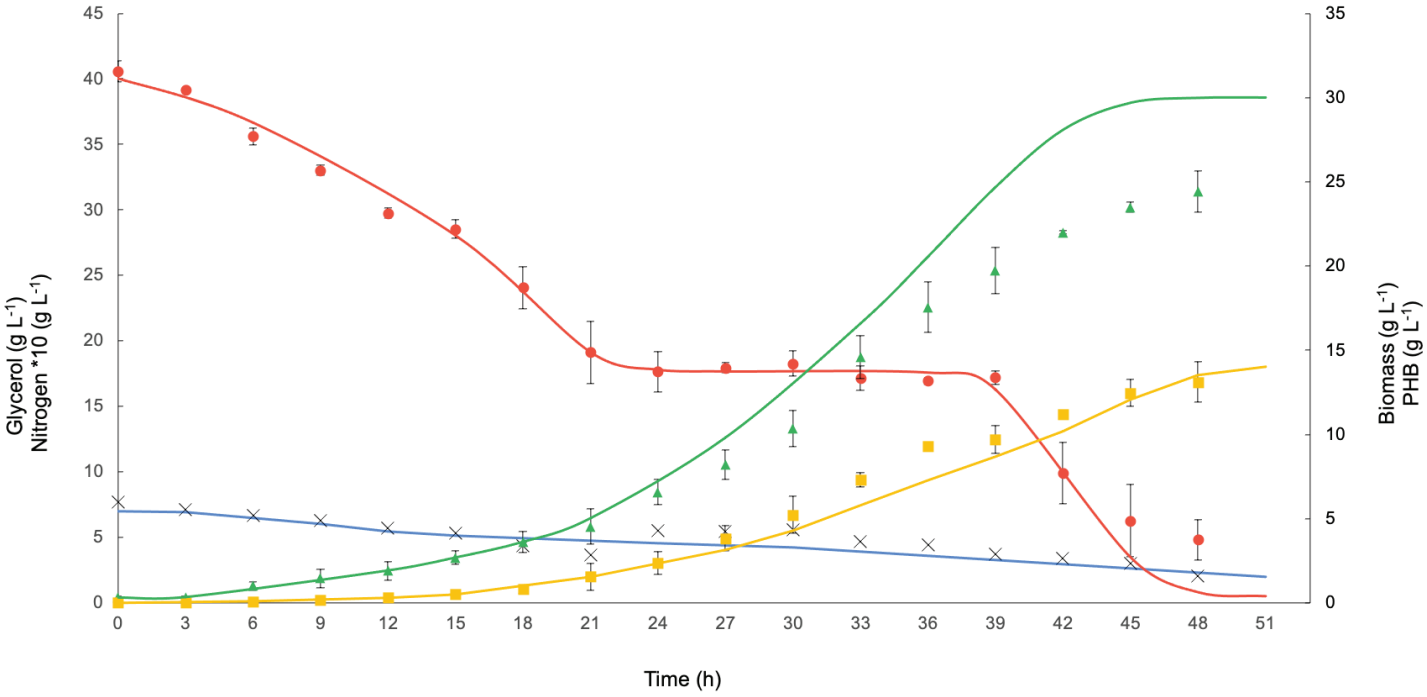

Fig. 6 – *Model-based fed-batch cultivation involving constant availability of substrate. Data points represent experimental values, and smooth lines indicate model predictions. 0–20 h batch cultivation, 20–40 h – fed-batch cultivation, 40–48 h – secondary batch cultivation. Data points (● Glycerol, × Nitrogen, ■ PHB, ▲ Biomass) represent the average values of the samples (triplicate).*

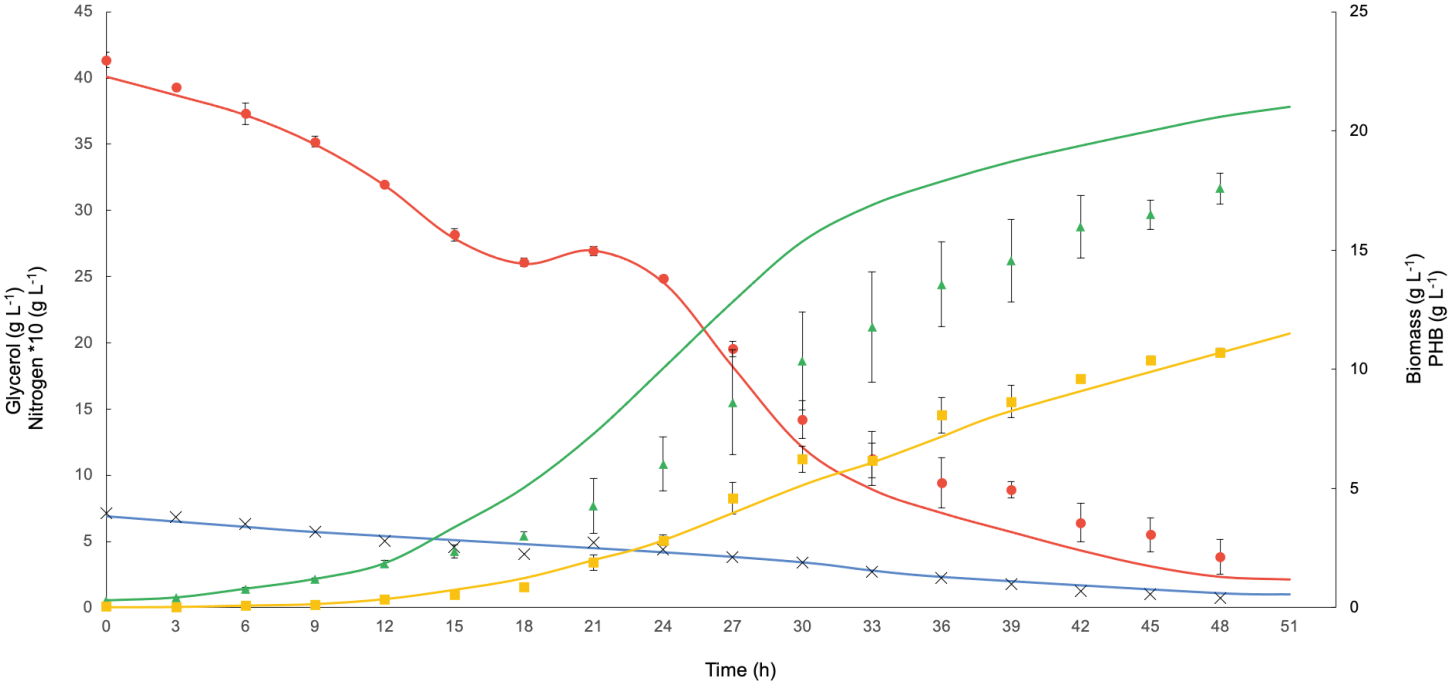

Fig. 7 – *Model-based fed-batch cultivation with decreasing feed rate. Comparison between model predictions (smooth lines) and experimental values (data points). 0–16 h Batch, 16–48 h fed-batch; 16–24 h – feeding rate of 75 mL h^{-1}, 24–32 h – feeding rate of 55 mL h^{-1}, 32–48 h feeding rate of 35 mL h^{-1}. End of feeding coincides with end of fermentation (48 h) Data points (● Glycerol, × Nitrogen, ■ PHB, ▲ Biomass) represent the average values of the samples (triplicate).*

accumulation of 11.54 g L^{-1} of PHB. Experimental implementation of aforementioned fed-batch cultivation strategy resulted in accumulation of 17.59 g L^{-1} biomass and 10.71 g L^{-1} PHB concentration in 48 h (as compared to corresponding biomass and PHB values of batch cultivation (8.88 g L^{-1} and 6.76 g L^{-1}, respectively). Fig. 7 describes a comparison of model simulation and experimental observation of fed-batch cultivation, when a decreasing feed rate strategy was implemented in the bioreactor. This fed-batch cultivation strategy also resulted in an increase in overall productivity of PHB to 0.23 g L^{-1} h^{-1} as opposed to 0.16 g L^{-1} h^{-1} obtained during batch cultivation.

Figs. 5, 6, and 7 clearly suggest that the experimental process variables were matching with the model simulations for almost the entire cultivation period. This, along with Table 8, demonstrated the validity of the mathematical model particularly during highly dynamic fed-batch cultivation conditions, as well as established its use for enhancing the productivity for PHB accumulation.

Discussion

To date, there are very few reports on PHB production by *C. necator*[15,17,18,20,21,36,37] and none on the use of mathematical models for the design of nutrient feeding strategies for growth-associated PHB production using *C. necator* DSMZ 545 on glycerol.

It has been invariably observed that during batch cultivation, limitation of essential nutrients (carbon) occurs during a major part of cultivation of microbial cells, which significantly hinders growth of *C. necator* and accumulation of PHB. For keeping high growth and product accumulation rates, a fresh supply of nutrient(s) is necessary at appropriate times. Therefore, a set of model equations was simulated by a computer program, and the different fed-batch cultivation strategies were designed by varying the carbon and nitrogen concentrations in the feed at different time intervals. Table 7 shows some of the best possible strategies demonstrating high biomass and PHB accumulation in the present study, and Table 8 shows predicted and experimental data of fed-batch cultivation of *C. necator*. The main objective of all fed-batch simulations was to ensure high biomass with maximum PHB accumulation at the end of the fermentation. Among all experimented strategies, the best results were obtained with pseudo steady state of glycerol wherein maximum PHB accumulation of 13.12 g L^{-1} in 7-L bioreactor was observed. The developed model predicted high PHB accumulation, which was experimentally verified with minimum experimental efforts by cultivation of *C. necator* on glycerol, thus demonstrating the high predictive power of the mathematical model for enhanced PHB production.

Different researchers have also implemented different nutrient feeding strategies during the fed-

Table 7 – *Comparison of batch and fed-batch cultivation strategies adopted in present study*

	Batch cultivation	Fed-batch at constant feed rate	Fed-batch at Pseudo steady state	Fed-batch at decreasing feed rate
Time (h)	42	48	48	48
Biomass (g L^{-1})	8.88	18.79	24.44	17.59
PHB (g L^{-1})	6.76	11.37	13.12	10.71
Productivity (g L^{-1} h^{-1})	0.16	0.24	0.27	0.23

Table 8 – *Model predictions and experimental data of fed-batch cultivation of C. necator*

Cultivation strategy	Feeding mode	Maximum biomass (g L^{-1})		Maximum PHB (g L^{-1})	
		Mod[a]	Exp[b]	Mod[a]	Exp[b]
Fed-batch	Constant feed rate	18.65	18.79	10.6	11.37
Fed-batch	Pseudo steady state	29.94	24.44	13.84	13.12
Fed-batch	Decreasing feed rate	20.57	17.59	11.54	10.71

Mod[a] – *model predicted values,* Exp[b] – *results of experimental observations*

batch cultivation for process improvement with respect to PHB accumulation and/or productivity. Fed-batch cultivation of *A. latus* ATCC 29713 was used for PHB accumulation wherein the effect of constant rate feeding, exponentially increasing feeding rate, and pH stat fed batch cultures were examined on the maximum PHB accumulation. It was possible to accumulate 18.2 g L^{-1} PHB under pH-stat fed batch cultivation. In addition, the distinct capability of the mathematical model to successfully predict highly dynamic fed-batch cultivation strategies was demonstrated by their experimental implementation[14]. A significantly high PHB concentration of 22.65 g L^{-1} and an overall PHB content of 76 % was achieved during constant feed rate fed-batch cultivation by using the model-based cultivation. This was the highest PHB content reported so far using *Azohydromonas australica.* Hence, the present work further demonstrated that mathematical models are excellent tools for understanding the culture behaviour without extensive trial experiments, as well as help greatly in the design of bioreactor cultivation strategies for process optimization with minimum experiments. The scope of the present mathematical model can be further enhanced by making it pH- and temperature-sensitive, and used for the design of more complex fed-batch/continuous cultivation strategies for over production of PHB by *C. necator*.

Conclusions

In the present study, glycerol, an inexpensive carbon source, was used for the production of PHB. The methodology featured statistical media optimization for the cost-effective production of PHB by *C. necator*. Thereafter, mathematical model for PHB production was developed using batch kinetic data and culture growth inhibition data of *C. necator*. The developed batch kinetic model was extrapolated to fed-batch cultivations, and used for the design of different nutrient feeding strategies for high PHB accumulation. Different model-based fed-batch cultivation strategies were then experimentally implemented. Among all cultivation strategies, maximum PHB accumulation and productivity of 13.12 g L^{-1} and 0.27 g L^{-1} h^{-1}, respectively, were obtained when the fed-batch was carried out under maintenance of pseudo steady state with respect to substrate (glycerol) for a major period of cultivation. This strategy featured maintenance of high substrate availability and limiting concentrations of nitrogen, which led to high intracellular PHB accumulation. The manuscript summarizes a comprehensive engineering optimization strategy for improvement of productivity of PHB accumulation, which involves maintenance of specific nutrient availabilities (high or low) during the cultivation. The methodology adopted in this investigation is system-independent, and can be applied to other cultivation systems for process optimization in minimum trial and error experiments.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

List of symbols

K_{S1} – saturation constant for glycerol consumption, g L^{-1}

K_{S2} – saturation constant for nitrogen consumption, g L^{-1}

a_1 – exponent indicating type of relationship between S_1 (glycerol) and μ

a_2 – exponent indicating type of relationship between S_2 (nitrogen) and μ

S_{m1} – critical glycerol concentration at which complete inhibition occurs

S_{m2} – critical nitrogen concentration at which complete inhibition occurs

$Y_{\frac{X}{S_1}}$ – yield with respect to glycerol, g g^{-1}

$Y_{\frac{X}{S_2}}$ – yield with respect to nitrogen, g g^{-1}

m_{S_1} – maintenance energy requirement of the cell on glycerol

m_{S_2} – maintenance energy requirement of the cell on nitrogen

q_{S_1} – specific rate of glycerol consumption, h^{-1}

q_{S_2} – specific rate of nitrogen consumption, h^{-1}

q_P – specific rate of product formation, h^{-1}

K_1 – growth-associated product formation constant, g g^{-1}

K_2 – non-growth-associated product formation constant, h^{-1}

S_1 – glycerol concentration, g L^{-1}

S_2 – nitrogen concentration, g L^{-1}

X – biomass concentration, g L^{-1}

Greek symbols

μ_{max} – maximum specific growth rate, h^{-1}

μ – specific growth rate, h^{-1}

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