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# Bioconversion of glycerol to 1,3-propanediol: A mathematical model-based nutrient feeding approach for high production using *Clostridium diolis*

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## HIGHLIGHTS

- Batch mathematical model for 1,3-PD production was developed.
- Developed model successfully simulated experimental batch data.
- Batch model was extrapolated to fed-batch for enhanced 1,3-PD production.
- 1,3-PD production up by 2.5-fold in pseudo-steady state fed-batch cultivation.

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## ABSTRACT

1,3-Propanediol (1,3-PD) is a bifunctional organic compound of particular importance in the polymer industry for the synthesis of polyesters, polyethers and polyurethanes. Its biotechnological production from glycerol features inherent problems of nutrient limitation and inhibition(s) by substrate and product. In the present study 1,3-PD batch mathematical model developed using average batch kinetics data and independently obtained inhibition data was used to identify fresh nutrient feeding strategies (*off-line* on the computer) for enhanced production of 1,3-PD. Experimental implementation of one such model-based fed-batch cultivation strategy involving pseudo-steady state of substrate featured a 1,3-PD concentration of 63.5 g/L with a 1,3-PD productivity of 1.35 g/L/h which were significantly higher than batch fermentation results. This demonstrated the potential application of developed model for the design of suitable nutrient feeding strategies for high production of 1,3-PD. The methodology can also be easily adopted for other cultivations.



## 1. Introduction

Bioenergy appears as a potential answer to the global problem of energy insecurity arising due to fast depleting petrochemical resources. It provides alternative biological production protocols for sustainable fuels and chemicals. There has been an increasing interest in the production and use of biofuels, including biodiesel mainly because of the national and international legislation which aim at greater non-dependence on the already disappearing fossil fuels (Papanikolaou et al., 2008). Biodiesel is considered as an alternative, renewable, non-polluting fuel to the conventional diesel fuel. It is produced by transesterification process wherein fatty acids from renewable feedstocks such as vegetable oil or animal fats are conjugated to an alcohol (Hoque et al., 2011; Sotoft et al., 2010). A predominant by-product of the biodiesel plant is glycerol which is a chemical commodity with major applications in cosmetic and food industry besides being the feedstock for many important metabolites (Da Silva et al., 2009; Kaur et al., 2012a). Surplus amounts of waste glycerol are generated by the biodiesel plant as 1 gallon of glycerol becomes available for every 10 gallons of biodiesel produced. With the global burgeoning interest in 'sustainable fuels' compounded by instability and historical increases in petroleum prices, the production of biodiesel is only expected to increase further (Kaur et al., 2012a). The latter would mean greater quantities of 'waste' glycerol than that can be supported by the current glycerol markets. Thus the biodiesel plant producing a clean, non-toxic fuel on one hand would leave behind large amounts of glycerol on the other hand, thereby raising serious concerns for the disposal of this waste. It is imperative to devise innovative solutions to convert this commercially important metabolite generated as 'waste' by the biodiesel plant into high value-added products (Posada et al., 2012). This would not only improve the sustainability and economic viability of the biodiesel plant, but

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**Nomenclature**

| | |
|---|---|
| $D$ | Dilution rate ($h^{-1}$) |
| $dC_i/dt$ | Rate of reaction for the component $C_i$ ($C_i$ = X, S, P) |
| $F$ | Feed flow rate (L/h) |
| $K_S$ | Saturation constant for substrate (g/L) |
| $S_m$ | Critical substrate concentration (g/L) |
| $P_m$ | Critical 1,3-PD concentration (g/L) |
| $K_1$ | Growth associated contribution for 1,3-PD production (g/g) |
| $K_2$ | Non-growth associated contribution for 1,3-PD production (g/g/h) |
| $m$ | Maintenance energy constant (g/g/h) |
| $P$ | 1,3-PD Concentration (g/L) |
| $q_p$ | Specific 1,3-PD production rate (g/g/h) |
| $q_s$ | Specific substrate (glycerol) consumption rate (g/g/h) |
| $S$ | Substrate concentration (g/L) |
| $S_O$ | Inlet substrate concentration (g/L) |
| SSWR | Sum of squares of weighed residues |
| $V$ | Volume (L) |
| $W_j$ | Weight of a process variable |
| $X$ | Biomass concentration (g/L) |
| $Y^{max}$ | Maximum yield (both biomass and 1,3-PD) with respect to substrate (g/g) |

also encourage the concept of biorefinery which aims to integrate the production of fuels, energy and chemicals.

One of the most important biotechnological transformations of glycerol is its conversion to 1,3-propanediol (1,3-PD) (Papanikolaou et al., 2008). 1,3-PD is a valuable chemical compound with a wide range of applications. It has gained commercial attention for its use in the synthesis of a new polyester named polytrimethylene terephthalate (PTT) which has several end-uses such as fibre, clothing, carpeting, automobile lining etc. (Kaur et al., 2012a). 1,3-PD is also formulated into cosmetics, deodorants, solvents, adhesives, laminates, biocides and drugs (immunosuppressant and tranquilizer) (Maervoet et al., 2011). Conventional route to 1,3-PD is the chemical method which entails the use of high temperature and pressure conditions, expensive catalysts and generation of toxic intermediates. Biotechnological production, on the other hand, involves the metabolism of glycerol by an oxido-reduction process using microorganisms belonging to the genera *Klebsiella*, *Clostridium*, *Enterobacter* and *Citrobacter* under physiological cultivation conditions and no production of harmful by-products (Barbirato et al., 1995; Boenigk et al., 1993; Kaur et al., 2012b; Otte et al., 2009; Zhao et al., 2006).

One of the major barriers in the biological production route to 1,3-PD is the inhibition by substrate (glycerol) and major metabolite (1,3-PD) itself, especially when accumulated in high concentrations in the bioreactor, which reduces the overall growth and product formation rates (Colin et al., 2000; Horng et al., 2010). Fed-batch cultivation appears as an ideal means to achieve high product concentrations and/or productivities by ensuring adequate regulation of nutrient feed into the bioreactor. However success of such a controlled cultivation is rather limited due to the non-availability of *on-line* sterilizable substrate (glycerol) sensors which complicate the maintenance of right nutrient concentration inside the bioreactor. A mathematical model-based programmed feed forward nutrient feeding approach appears reliable, fast, intelligent and productive engineering solution for debottlenecking the limitation and/or inhibition problem(s) in bio-based 1,3-PD production. Application of mathematical models for 1,3-PD production has not been greatly explored and there are only few reports available in literature majorly describing the basic kinetic pattern only with practically no application thereof (Papanikolaou et al., 2004; Papanikolaou and Aggelis, 2003). Mathematical models are considered as important bioprocess engineering tools which help in detailed understanding of process behaviour and facilitate process optimization in minimum time without any trial and error. These are particularly useful for the elucidation of cultivation kinetics and design of experiments of the microorganism having intriguing and complex multiproduct fermentations. With the above objective in mind a batch 1,3-PD mathematical model was developed using the average batch kinetics and independently obtained inhibition data (Kaur et al., 2012c) which adequately reflected not only the substrate limitation/inhibitions but also product inhibitions during the dynamic cultivation process. The distinct utility of the model to design desirable nutrient feeding strategies in fed-batch cultivation which primarily addresses the severe problem of limitation and inhibition of substrate and yields high 1,3-PD production is demonstrated in this investigation.

## 2. Methods

### 2.1. Culture and maintenance

*Clostridium diolis* DSM15410 (previously *Clostridium butyricum* DSM5431) procured from DSMZ, Germany was used in the present study. It was maintained on Reinforced Clostridium Medium (RCM) (Himedia, India) and stored at 4 °C.

### 2.2. Growth medium and culture conditions

The medium used for cultivation of the microorganism consisted (per litre) glycerol 54.15 g, $K_2HPO_4$ 3.21 g; $KH_2PO_4$ 2.75 g; $(NH_4)_2SO_4$·2 g; $MgSO_4·7H_2O$ 0.2 g; $CaCl_2·2H_2O$ 0.02 g; $FeSO_4·7H_2O$ 5 mg; Yeast extract 2 g; Trace element solution (TES) 2 mL (Günzel et al., 1991). The trace element solution per liter contained: 70 mg $ZnCl_2$; 0.1 g $MnCl_2·4H_2O$; 60 mg $H_3BO_3$; 0.2 g $CoCl_2·2H_2O$; 20 mg $CuCl_2·2H_2O$; 25 mg $NiCl_2·6H_2O$; 0.9 mL HCl (37%). The medium pH was adjusted to 7.0 with 2 N NaOH/HCl. Inoculum development conditions were similar to those reported in our earlier studies (Kaur et al., 2012c).

### 2.3. Batch cultivation of C. diolis in bioreactor

Batch cultivation of *C. diolis* for 1,3-PD production was carried out in a 3.7 L bioreactor (Bioengineering AG, Switzerland) (working volume 1.5 L) using statistically optimized medium. Bioreactor was inoculated with 10% (v/v) exponentially growing culture from the shake flasks. pH was controlled at 7.0 by automatic addition of 2 N NaOH/ HCl. Anaerobic conditions were maintained by sparging nitrogen gas at 0.5 l/l/m in the bioreactor. Agitation was achieved by a flat blade turbine impeller rotating at 150 rpm and temperature was maintained at 33 °C by circulation of chilled water using FP 50 chiller (Julabo, Germany). Analysis of biomass, substrate and 1,3-PD was done at regular intervals of 3 h.

### 2.4. Modelling glycerol to 1,3-PD bioconversion

A batch 1,3-PD mathematical model was developed by using the average batch kinetic data of three batch bioreactor experiments. Besides this data obtained from independent substrate

and product inhibition studies (as described in earlier studies) was also used for model development (Kaur et al., 2012c). The model consisted of a system of differential equations with respect to biomass formation, substrate consumption and 1,3-PD production. The optimal values of model parameters were obtained by non-linear regression technique by using the original algorithm of Rosenbrock (Rosenbrock, 1960) assisted by computer programs and methodologies described by (Volesky and Votruba, 1992; Votruba, 1982) which minimized the difference between original experimental data and model prediction. Model predictions were done by a numerical integration program based on Runge–Kutta method of 4th order using the system of differential equations which described the batch production kinetics of 1,3-PD. The optimization program for direct search of the minimum of a multivariable function was based on original algorithm of (Rosenbrock, 1960) wherein the minimization function SSWR was represented by:

$$\mathrm{SSWR} = \sum_{i=1}^{n}\sum_{j=1}^{m}\frac{\Delta_{ij}^2}{W_j^2}$$

where SSWR represents the Sum of the Square of Weighed Residues. '$n$' and '$m$' represent the total number of experimental data points and the process variables respectively, $W_j$ represents the weight of each variable (usually the maximum value of each process variable) and $\Delta ij$ accounts for the difference between the model and experimental value ($y_{\mathrm{model}}$–$y_{\mathrm{expt}}$).

### 2.5. Fed-batch cultivation of C. diolis involving pseudo-steady state with respect to substrate

Fed-batch cultivation was initiated as a batch with initial glycerol concentration of 54.45 g/L in the bioreactor. At 15 h when the culture was growing exponentially and the unconverted substrate glycerol concentration had fallen below 40 g/L, fresh glycerol feeding (along with other nutrients) was initiated. The feeding rate in this type of fed-batch cultivation was calculated by equating the rate of substrate consumption ($dS/dt$) of the model to zero and then back calculating the nutrient feed rate. The nutrient feeding was continued till 30 h after which the batch cultivation was resumed again for next 17 h in order to allow utilization of unconverted glycerol. Samples were withdrawn and analyzed at regular intervals of 3 h for biomass, substrate and 1,3-PD estimation.

### 2.6. Analytical methods

Biomass was measured turbidimetrically at 650 nm (optical density) and correlated with cell dry weight directly by a previously established correlation between optical density and Biomass concentration. The residual glycerol and 1,3-PD concentrations were determined by High Performance Liquid Chromatography (Agilent 1200 Series) using a Refractive-Index (RI) Detector. Separation was achieved on a Bio-Rad Aminex HPX-87H column using a solution of 0.5 mM $H_2SO_4$ as mobile phase at a flow rate of 0.5 mL/min and 30 °C temperature (Kaur et al., 2012d).

## 3. Results and discussion

### 3.1. Batch 1,3-PD fermentation

The kinetics of batch cultivation of *C. diolis* in the bioreactor is described in Fig. 1. The culture demonstrated an initial lag of 3 h after which it featured exponential growth with a final biomass concentration of 3.4 g/L and an accumulation of 1,3-PD concentration of 25.8 g/L at the end of 36 h. By-products of 1,3-PD fermentation namely butyric acid and acetic acid were accumulated in the bioreactor at reasonably low levels (i.e. 2.3 g/L and 1.38 g/L respectively) throughout the cultivation. Batch fermentation resulted in a 1,3-PD yield and productivity of 0.65 mol/mol and 0.72 g/L/h respectively which is the highest 1,3-PD yield achieved so far in the literature using *C. diolis* DSM 15410 (Kaur et al., 2012d).

### 3.2. Mathematical model development for 1,3-PD

Bioreactor batch kinetic data and independently acquired substrate and product inhibition data was utilized for the development of batch mathematical model for 1,3-PD production. The following assumptions were made for model development:

1. Glycerol is the only limiting nutrient.
2. There is no process limitation by nitrogen, phosphorous and other growth factors (e.g. yeast extract) as excess amount of these nutrients are available in the fermentation medium during the entire cultivation.
3. There is no change in pH and temperature throughout the cultivation.

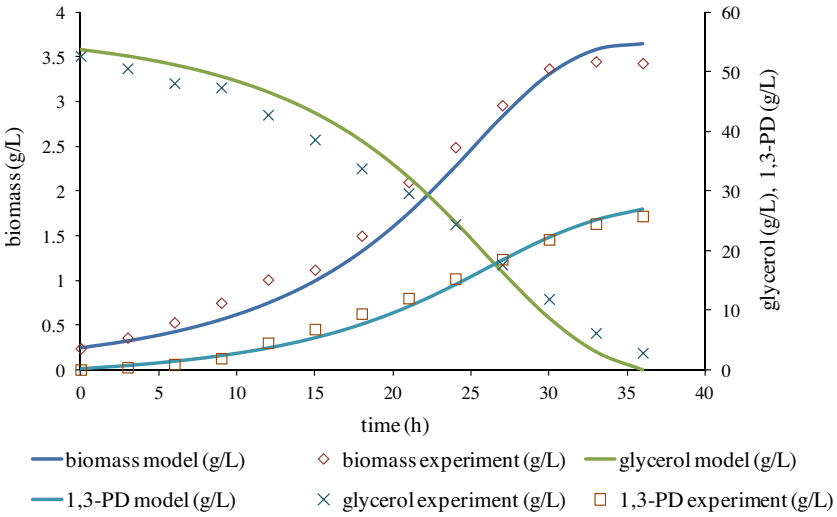


**Fig. 1.** Time course of Batch 1,3-PD fermentation using *C. diolis*. Data points represent experimental values. Smooth curve illustrates model predictions.

**Table 1**
Optimized values of model parameters of 1,3-PD fermentation.

| Parameter | Units | Value |
|---|---|---|
| $\mu_{max}$ | $h^{-1}$ | 0.65 |
| $K_s$ | g/L | 12.8 |
| $Y^{max}$ | g/g | 0.57 |
| $m$ | g/g/h | 0.23 |
| $K_1$ | g/g | 7.3 |
| $K_2$ | g/g/h | 0.15 |
| $S_m$ | g/L | 98.3 |
| $P_m$ | g/L | 65.2 |
| $a$ | Dimensionless | 1.12 |
| $b$ | Dimensionless | 1.0 |

Following model equations described the batch dynamics of 1,3-PD production:

$$\mu = \mu_{max}(S/S + K_S)[1 - (S/S_m)^a][1 - (P/P_m)^b] \quad (1)$$

$$dX/dt = \mu_{max}(S/S + K_S)[1 - (S/S_m)^a][1 - (P/P_m)^b]X \quad (2)$$

Biomass formation rate ($dX/dt$) featured limitation by substrate and inhibition by high levels of initial substrate and accumulated products (Eqs. (1) and (2)). The detailed kinetic and inhibitory data for derivation of the model has been adequately described by Kaur et al. (2012c).

The consumption of substrate was assumed to be for the biomass formation and cellular maintenance requirement as described below:

$$q_s = -(1/Y^{max})\mu + m \quad (3)$$

$$dS/dt = -[(1/Y^{max})\mu + m]X \quad (4)$$

here $Y^{max}$ represents a lumped parameter accounting for the cumulative maximum yield of both biomass and product based on substrate and '$m$' represents the maintenance energy requirement of the cell. Based on the batch kinetic data typically observed in this investigation and by Kaur et al. (2012c) 1,3-PD formation rate ($dP/dt$) was mathematically described by a combination of both growth and non-growth associated components as follows:

$$q_p = K_1\mu + K_2 \quad (5)$$

where $K_1$ and $K_2$ are the constants reflecting growth and non-growth associated product formations.

$$dP/dt = (K_1\mu + K_2)X \quad (6)$$

The optimized values of the model parameters are summarized in Table 1. The model Eqs. (1), (3), and (5) were numerically integrated on the computer using the optimized model parameter values to generate the batch kinetics. A comparison of model simulation and experimental kinetic data (Fig. 1) indicates a good correlation between the original experimental data of different process variables $X$, $S$ and $P$ (represented by points in Fig. 1) and model prediction (smooth curve).

### 3.3. Fed-batch model and model-based fed-batch cultivation involving pseudo-steady state

The developed batch mathematical model was extrapolated to fed-batch cultivation by taking the mass balance around the bioreactor and incorporating the dilution terms in the model equations primarily to account for feeding fresh nutrient feed into the bioreactor. The fed-batch model (consisting of the following equations) was then used to design fresh nutrient feeding strategies to facilitate improvement in the concentration and/or productivity of 1,3-PD.

$$dV/dt = F \quad (7)$$

$$D = F/V$$

$$dX/dt = \mu_{max}(S/(S + K_S))[1 - (S/S_m)^a][1 - (P/P_m)^b]X - DX \quad (8)$$

$$dS/dt = -[(1/Y^{max})\mu + m]X + D(S_0 - S) = 0 \quad (9)$$

$$dP/dt = [K_1\mu + K_2]X - DP \quad (10)$$

It was necessary to introduce yet another model equation (Eq. (7)) primarily to account for changing volume in the bioreactor in fed-batch cultivation condition, where '$F$' is the glycerol feed rate, '$V$' is the volume of the bioreactor and '$D$' is the dilution rate.

Low product concentrations (yield) and productivities are invariably observed in the highly dynamic batch fermentation conditions primarily due to disappearing nutrient availability during the entire cultivation period. Therefore it is highly desirable to have non-limiting and non-inhibitory concentration of limiting nutrient(s) for entire cultivation period in fed-batch fermentation in order to attain high growth and product formation rates. This scenario of nutrient availability is rather unique and would yield high product accumulation as opposed to batch fermentation. With this concept in mind, fed-batch cultivation which ensured constant substrate availability i.e. pseudo-steady state of substrate was attempted in the present study. However it is important to understand that implementation of pseudo-steady state of substrate right from the beginning of cultivation is rather difficult as there is a risk of creating a chaotic cultivation scenario arising out of either too high or too low fresh nutrient additions. Therefore attempt was made to create pseudo-steady state of substrate for a major period of cultivation during the exponential phase of biomass growth. This peculiar cultivation strategy was designed and simulated off-line on computer using the model and then experimentally implemented at time when reasonable biomass concentration, in vigorously growing phase, was available in the bioreactor.

Fed-batch cultivation was initiated as batch and the feeding of glycerol at 180 g/L (along with proportional concentration of other nutrients) was initiated at 15 h of cultivation when the culture was vigorously growing in the exponential phase and unconverted glycerol concentration in the bioreactor had dropped below 40 g/L. Nutrient feeding was continued till 30 h of fed-batch cultivation. At 30 h the model was utilized to simulate yet another batch cultivation condition until the residual glycerol in the broth was totally consumed. By the adoption of above fed-batch cultivation strategy it was possible to achieve 1,3-PD concentration of 64.32 g/L in 47 h. Experimental implementation of this nutrient feeding strategy during the fed-batch cultivation resulted in 63.5 g/L 1,3-PD at the end of 47 h (Fig. 2). It also featured an accumulation of 12.4 g/L butyric acid and 7.2 g/L acetic acid as by-products of 1,3-PD fermentation along with a reasonably high biomass production of 4.24 g/L. It was demonstrated that an improvement not only in concentration (63.5 g/L vs 25.8 g/L in batch) but also 1,3-PD productivity of 1.35 g/L/h (as compared to 0.72 g/L/h in batch cultivation) was feasible in the present model-based fed-batch cultivation strategy. A comparison between observed data (points) and model simulation (smooth curve) exhibited excellent correlation as shown in Fig. 2.

From above experimentation it can be concluded that application of developed model resulted in a high 1,3-PD production which was significantly better than the batch fermentation results in this study. The productivity achieved in the present study was even higher than the model-based fed-batch cultivation with constant feeding of nutrients (Kaur et al., 2012c). Different fed-batch strategies have been adopted in the literature for enhanced

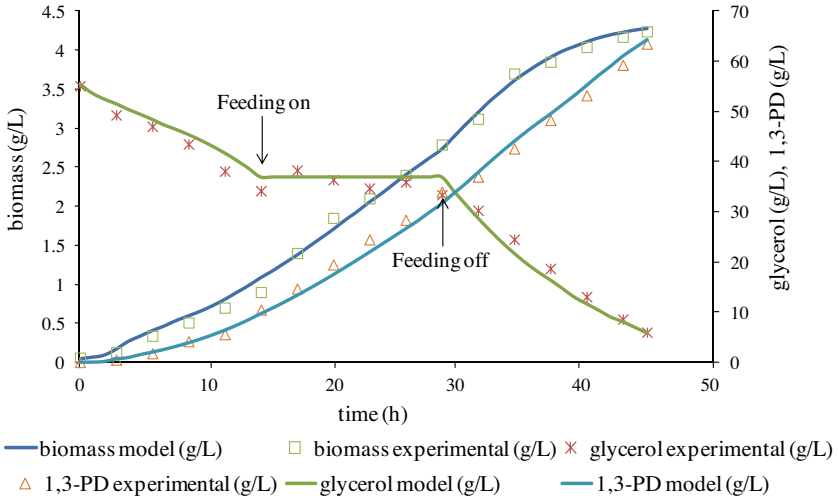


**Fig. 2.** Model-based Fed-batch 1,3-PD fermentation involving pseudo-steady state of substrate. 0–15 h – Batch, 15–30 h – Fed-batch, 30–47 h – Batch. Data points represent experimental values. Smooth curve illustrates model predictions.

**Table 2**
1,3-PD batch and fed-batch fermentation literature results using different *C. butyricum* strains.

| Fermentation | *C. butyricum* strain | Glycerol | 1,3-PD[a] | $Q_{1,3\text{-PD}}$[b] | Reference |
|---|---|---|---|---|---|
| Batch | CNCM1211 | Raw | 63.4 | 1.85 | Barbirato et al. (1998) |
| | DSM5431 | Pure | 29.5 | 2.3 | Biebl et al. (1992) |
| | F2b | Raw | 47.1 | 1.12 | Papanikolaou et al. (2008) |
| | CNCM1211 | Raw | 65.4 | 1.67 | Himmi et al. (1999) |
| | VPI3266 | Pure | 7.35 | 0.31 | González-Pajuelo et al. (2004) |
| | DSM15410 | Pure | 25.6 | 0.72 | Present study |
| Fed-batch | DSM5431 | Pure | 56 | 2.3–2.9 | Günzel et al. (1991) |
| | DSM5431 | Pure | 47.5 | 0.66 | Abbad-Andaloussi et al. (1995) |
| | DSM5431 | Pure | 47.5 | 2.4 | Reimann and Biebl (1996) |
| | VPI3266 | Pure | 65 | 1.21 | Saint-Amans et al. (1994) |
| | IK124 | Raw | 77.5 | 1.2 | Hirschmann et al. (2005) |
| | VPI1718 | Raw | 67.9 | 0.78 | Chatzifragkou et al. (2011a) |
| | VPI1718 | Raw | 70.8 | 0.70 | Chatzifragkou et al. (2011b) |
| | AKR102a | Raw | 76.2 | 2.3 | Wilkens et al. (2011) |
| | DSM15410 | Pure | 61.2 | 1.02 | Kaur et al. (2012c) |
| | DSM15410 | Pure | 63.5 | 1.35 | Present study |

[a] 1,3-PD concentration (g/L).
[b] 1,3-PD productivity (g/L/h).

1,3-PD concentration and/or productivity. The results of batch and fed-batch fermentation studies using different strains of *C. butyricum* have been summarized in Table 2. Based on which it is clearly observed that a 1,3-PD concentration of 63.5 g/L obtained in the present investigation is the highest 1,3-PD concentration ever reported in the literature using the native strain of *C. diolis* DSM15410 by following a model-based nutrient feed design approach. The results reported in this investigation clearly demonstrate that the present model was not only adequately robust in nature but could possibly be used as a 'guide' to improve 1,3-PD concentration and /or productivity by designing different feeding/cultivation strategies in fed-batch and/or continuous 1,3-PD fermentation. Development and testing of such newer feeding strategies shall be the topic of our future investigation.

## 4. Conclusion

A simple batch 1,3-PD mathematical model was developed which could successfully simulate the observed batch kinetics. Potential application of the developed mathematical model for simulation of different strategies for enhanced 1,3-PD production was demonstrated. A fed-batch cultivation strategy involving pseudo-steady state with respect to substrate was identified by model and experimentally verified. A 1,3-PD concentration of 63.5 g/L with a 1,3-PD productivity of 1.35 g/L/h could be obtained which was very close to the model predictions. The model based design of nutrient feeding strategy (ies) adopted in this investigation for the enhancement of 1,3-PD production is a rather logical and unique approach and can be easily adopted for other microbial cultivations to design fed-batch/continuous cultivation strategies in order to enhance product concentration and productivities with minimum experimentation.

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