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Process Development for Enhanced 2,3-Butanediol Production by *Paenibacillus polymyxa* DSM 365

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Abstract: While chiral 2,3-Butanediol (2,3-BD) is currently receiving remarkable attention because of its numerous industrial applications in the synthetic rubber, bioplastics, cosmetics, and flavor industries, 2,3-BD-mediated feedback inhibition of *Paenibacillus polymyxa* DSM 365 limits the accumulation of higher concentrations of 2,3-BD in the bioreactor during fermentation. The Box-Behnken design, Plackett-Burman design (PBD), and response surface methodology were employed to evaluate the impacts of seven factors including tryptone, yeast extract, ammonium acetate, ammonium sulfate, glycerol concentrations, temperature, and inoculum size on 2,3-butanediol (2,3-BD) production by *Paenibacillus polymyxa* DSM 365. Results showed that three factors; tryptone, temperature, and inoculum size significantly influence 2,3-BD production ($p < 0.05$) by *P. polymyxa*. The optimal levels of tryptone, inoculum size, and temperature as determined by the Box-Behnken design and response surface methodology were 3.5 g/L, 9.5%, and 35 °C, respectively. The optimized process was validated in batch and fed-batch fermentations in a 5-L Bioflo 3000 Bioreactor, and 51.10 and 68.54 g/L 2,3-BD were obtained, respectively. Interestingly, the production of exopolysaccharides (EPS), an undesirable co-product, was reduced by 19% when compared to the control. These results underscore an interplay between medium components and fermentation conditions, leading to increased 2,3-BD production and decreased EPS production by *P. polymyxa*. Collectively, our findings demonstrate both increased 2,3-BD titer, a fundamental prerequisite to the potential commercialization of fermentative 2,3-BD production using renewable feedstocks, and reduced flux of carbons towards undesirable EPS production.

Keywords: *Paenibacillus polymyxa*; butanediol; acetoin; glycerol; optimization

1. Introduction

The compound 2,3-Butanediol (2,3-BD) is an industrial platform chemical with vast industrial applications, particularly for its potential use in the synthesis of 1,3-butadiene (1,3-BD), a monomer of synthetic rubber. Other applications of 2,3-BD include the synthesis of methyl ethyl ketone (MEK), a fuel additive with a higher heat of combustion than ethanol, and as solvents for lacquers and resins [1]. Furthermore, 2,3-BD finds applications as an antifreeze due to its low freezing temperature

of −60 °C [2], an ink additive, as a chemical feedstock for the production of acetoin and diacetyl, vital flavor enhancers in the food industry [3], and as an additive in aviation fuel. Due to the finite nature of petroleum and the need to reduce society's dependence on petroleum-derived feedstocks for industrial processes, it has become imperative to develop sustainable feedstocks such as 2,3-BD from renewable resources. At present, 2,3-BD is produced from hydrocarbons by the cracking of butane and 2-butene in which the resulting product is further hydrolyzed to 2,3-BD [4,5]. Recently, the often encountered instabilities in crude oil price have re-ignited interest in fermentative 2,3-BD production from cheap renewable feedstocks. To reach this goal, multifarious research efforts are currently underway to increase the yield, titer, and productivity of microbe-derived 2,3-BD. These include metabolic engineering of producer organisms to produce and tolerate higher 2,3-BD concentrations, and the optimization of fermentation media components and conditions for maximal 2,3-BD accumulation in the broth. In this study, we sought to optimize 2,3-BD production by *Paenibacillus polymyxa* DSM 365 (hereafter referred to as *P. polymyxa*) by assessing the impacts of both medium components and fermentation conditions on 2,3-BD accumulation.

P. polymyxa was chosen for this study due to its non-pathogenicity and capacity to produce 98% levo 2,3-BD; the industrially preferred 2,3-BD isomer due to its properties, which make it amenable to important chemical reactions that generate key industrially applicable products, such as dehydration to 1,3-BD (for synthetic rubber production), dehydrogenation to acetoin or diacetyl (flavor enhancers and essential components in fragrances), ketalization to Methyl tert-butyl ether (fuel additive), and esterification to 2,3-BD diester (used as a precursor in the synthesis and compounding of cosmetics, drugs, and thermoplastic polymers [1,6].

To assess the 2,3-BD production capacity of *P. polymyxa,* we first conducted batch fermentations in 100 mL Pyrex bottles, which resulted in a maximum 2,3-BD concentration of 24 g/L [7]. Batch fermentation in the bioreactor (6-L) produced 27 g/L 2,3-BD, whereas fed-batch fermentation (in the bioreactor) resulted in 47 g/L, despite excess glucose supply [7]. Therefore, we rationalized that in addition to other factors, 2,3-BD-mediated feedback inhibition might pose a significant roadblock to the accumulation of 2,3-BD during fermentation, and this assumption was confirmed by assaying 2,3-BD toxicity against *P. polymyxa* in fermentation cultures [7]. We observed that 2,3-BD exerts a concentration-dependent toxicity on *P. polymyxa* with ~50 g/L 2,3-BD as the toxic threshold above which cell growth stalls considerably and the accumulated 2,3-BD is converted backwards to acetoin, the precursor of 2,3-BD; most plausibly to alleviate 2,3-BD-mediated toxicity [7]. In addition, a significant portion of sugar substrates are diverted to exopolysaccharides (EPS) production during 2,3-BD fermentation, thereby lowering 2,3-BD yield and complicating its recovery from the fermentation broth [7,8]. Therefore, if *P. polymyxa* 2,3-BD fermentation is to reach an industrial-scale, it is critical to determine the optimal conditions and medium components necessary for marked 2,3-BD accumulation and tolerance during fermentation. Further, cheaply available substrates such as glycerol, which is currently accumulated in excess as a by-product of biodiesel production [9,10], holds significant promise towards improving the economics of 2,3-BD fermentation, either as a sole carbon source or as a supplement to glucose or other sugars. In fact, glycerol has been shown to support 2,3-BD production by *Klebsiella pneumoniae* as a sole carbon source [11,12]. Thus, we investigated the optimal conditions and medium components for high 2,3-BD production by *P. polymyxa* using a glycerol-supplemented medium. In addition to lowering the overall substrate cost, glycerol catabolism furnishes the cell with additional NADH [13,14], which supplies extra reducing power for 2,3-BD dehydrogenase, the final enzyme of the 2,3-BD pathway, which consumes NADH during the conversion of acetoin to 2,3-BD [15].

Previous optimization studies focused largely on enhancing 2,3-BD production. These studies either targeted medium components only, or fermentation conditions without a holistic evaluation of both parameters (medium components and fermentation conditions; [8,16]). Medium components and fermentation conditions such as temperature, inoculum size, pH, and aeration rate most reasonably interact during fermentation to engender 2,3-BD production. Therefore, in this study, select medium

components and fermentation conditions were assessed collectively for their capacity to enhance 2,3-BD production by employing various optimization strategies. Plackett-Burman experimental design, path of steepest ascent method, Box-Behnken experimental design, and response surface methodology strategies were employed to optimize 2,3-BD production by *P. polymyxa*. The medium components tested in this study include yeast extract, tryptone, ammonium acetate, ammonium sulfate, and crude glycerol; whereas the fermentation conditions that were extensively investigated include temperature and inoculum size. These factors were shown to influence 2,3-BD production by *P. polymyxa* from our one-factor-at-a-time experiments.

2. Methodology

2.1. Experimental Methods

2.1.1. Microorganism and Culture Preparation

P. polymyxa was obtained from the German Collection of Microorganisms and Cell Culture, Braunschweig, Germany (DSMZ—Deutsche Sammlung von Mikroorganismen und Zellkulturen). Lyophilized cells were reactivated by inoculating into Luria bertani (LB) broth, grown overnight (12 h), and then stored as glycerol (50% sterile glycerol) stock at –80 °C until further use. Glucose, yeast extract, and tryptone were prepared and sterilized separately at 121 °C for 15 min followed by cooling to 50 °C prior to mixing with filter-sterilized components (buffer and trace element solution), and this mixture forms the final pre-culture medium. For inoculum preparation, 1 mL of *P. polymyxa* glycerol stock was inoculated into 30 mL of pre-culture medium. The pre-culture medium contained (g/L); 20.0 glucose, 5.0 yeast extract (YE; Sigma-Aldrich, St. Louis, MO, USA), 5.0 tryptone (Sigma-Aldrich, St. Louis, MO, USA), 0.2 $MgSO_4$, and 3.0 $(NH_4)_2SO_4$. The pre-culture was supplemented with 0.9 mL of phosphate buffer (pH 6.5) and 0.09 mL of trace element solution. The phosphate buffer (pH 6.5) contained (g/L); 3.5 KH_2PO_4, 2.75 K_2HPO_4, while the trace element solution was prepared by dissolving 0.4 g $FeSO_4$ in 3 mL 25% HCl, followed by the addition of 500 mL double-distilled H_2O and (g); 0.8 H_3BO_3, 0.04 $CuSO_4{\cdot}5H_2O$, 0.04 $NaMoO_4{\cdot}2H_2O$, 5.0 $MnCl_2{\cdot}4H_2O$, 0.1 $ZnSO_4{\cdot}7H_2O$, 0.08 Co $(NO_3)_2{\cdot}6H_2O$, 1.0 $CaCl_2{\cdot}2H_2O$, and 0.01 biotin. The trace element solution was made up to 1 L with double-distilled H_2O. The pre-culture was incubated aerobically at 37 °C and 200 rpm for 10–12 h in an incubator shaker (New Brunswick Scientific, Edison, NJ, USA). When the optical density (OD_{600nm}) of the pre-culture reached 1.0–1.2, 30 mL (10 mL each) of actively growing cells were distributed in three 250 mL flasks containing 90 mL sterile pre-culture medium each and incubated for another 2–3 h until the OD_{600nm} reached 1.0–1.2, and then the pre-culture was transferred to production medium. Phosphate buffer and trace element solution were prepared separately and filter-sterilized using a 0.22 μm PES filter (Corning Incorporated, Corning, NY, USA).

2.1.2. Batch and Fed-Batch Fermentations

Batch and fed-batch fermentations were conducted in a 5 L Bioflo 3000 Bioreactor (New Brunswick Scientific, Edison, NJ, USA) with a 2 L starting volume. The bioreactor was equipped with sensors for measuring pH and temperature and stirrers for medium agitation. The medium was continuously stirred by means of 2 Rushton impellers (3-plate). Fermentations were conducted aerobically by sparging sterile air into the medium at a flow rate of 150 mL/min through a 0.2 μm PTFE Acro®50 sterile filter (Pall Corporation, Ann Arbor, MI, USA) using a Masterflex L/S® Pump (Cole-Parmer Instrument Company, Vernon Hills, IL, USA) through the top of the bioreactor into the fermentation medium. In addition to the concentrations of the medium components studied, the production medium contained (g/L): 120 glucose, 3.5 KH_2PO_4, 2.75 K_2HPO_4, 0.2 $MgSO_4$, 0.05 $CoCl_2$, 10.0 3-(*N*-morpholino) propanesulfonic acid (MOPS), and 6 mL of trace element solution (described above: microorganism and culture preparation). All medium components were prepared separately and then mixed under aseptic conditions. Fermentation was started at an initial pH of 6.5 ± 0.1 and the pH was externally

controlled with 12.5% NH_4OH or 6.5 normal H_3PO_4 when the pH dropped below 6.0 ± 0.1 or increased above 6.5 ± 0.1. The fermentation medium was stirred at 300 rpm and the culture was fed when the broth glucose concentration fell below 15 g/L for fed-batch fermentations. Each round of sugar feeding was accompanied by the addition of half-strength of the other medium components (buffer and trace element solutions).

2.1.3. Analytical Methods

Cell growth was determined by measuring optical density (OD_{600}) in a DU® Spectrophotometer (Beckman Coulter Inc., Brea, CA, USA). Changes in pH were measured using an Acumen® Basic pH meter (Fischer Scientific, Pittsburgh, PA, USA). The concentrations of 2,3-BD, acetoin, ethanol, and acetic acid were determined using a 7890A Agilent gas chromatograph (Agilent Technologies Inc., Wilmington, DE, USA) equipped with a flame ionization detector (FID) and a J × W 19091 N-213 capillary column [30 m (length) × 320 µm (internal diameter) × 0.5 µm (HP-Innowax film thickness)]. The carrier gas was nitrogen, and the inlet and detector temperatures were maintained at 250 and 300 °C, respectively. The oven temperature was programmed to span from 60 to 200 °C with 20 °C min^{-1} increments, and a 5 min hold at 200 °C. Samples (1 µL) were injected with a split ratio of 10:1.

Glucose concentration was determined by HPLC using a Waters 2796 Bioseparations Module equipped with an Evaporative Light Scattering Detector (ELSD; Waters, Milford, MA, USA) and a 9 µm Aminex HPX-87P column; 300 mm (length) × 7.8 mm (internal diameter) connected in series to a 4.6 mm (internal diameter) × 3 cm (length) Aminex deashing guard column (Bio-Rad, Hercules, CA, USA). The column temperature was maintained at 65 °C. The mobile phase was HPLC-grade water maintained at a flow rate of 0.6 mL/min. The amounts of EPS produced were measured using a previously described method [7].

2.2. Experimental Design and Data Analysis

Other authors have previously reported that yeast extract, ammonium acetate, ammonium sulfate, glycerol, and tryptone, as well as temperature and inoculum size influence microbial 2,3-BD production [16–20]. Some of these studies concluded that using high concentrations of expensive yeast extract (up 60 g/L) was crucial for optimal 2,3-BD production. Therefore, we first conducted one-factor-at-a-time experiments to evaluate the degrees of effect exerted by these factors on the 2,3-BD production capacity of *P. polymyxa*. The one-factor-at-a-time experiments underlined the effects of yeast extract, ammonium acetate, ammonium sulfate, glycerol, and tryptone, as well as temperature and inoculum size on 2,3-BD production by *P. polymyxa*; hence, we employed the Plackett-Burman design, path of steepest ascent, Box-Behnken, and response surface methodology for further optimization studies.

2.2.1. Plackett-Burman Design

Placket-Burman design allowed for the evaluation of important factors that influence 2,3-BD production based on the assumption that the selected factors do not interact. In this design, each factor was defined at two levels; a high (+1) and a low (−1), which represent two different concentrations or condition set points. The actual experimental values were defined according to the equation;

$$X_i = x_i - x_0/\Delta x_i \ (I = 1, 2, 3, \ldots, k) \quad (1)$$

From the equation above, X_i is the defined value of an independent factor such as inoculum size, temperature, glycerol, tryptone, yeast extract, ammonium acetate, and ammonium sulfate, x_i is the real value of an independent factor, and x_0 represents the real value of an independent factor at a center point value. Furthermore, Δx_i is the difference between the real value at the center point (x_0) and the

real values at the lower or upper point of an independent factor. The data obtained from the design were fitted to a first-order model for 2,3-BD production as shown in the equation below;

$$Y = \beta_0 + \sum \beta_i X_i \quad (2)$$

Y is the concentration of 2,3-BD obtained from each experimental run, while β_0 is the intercept, and X_i represents the ith factor (X_1–X_7; see Table 1) and β_i is the regression coefficient of each factor (X_1–X_7; see Table 1) [21]. The resulting data were then analyzed and fitted to a linear regression using the Design Expert software package (version 10.0, Stat-Ease, Inc. Minneapolis, MN, USA). Analysis of variance (ANOVA) at 95% confidence interval ($p < 0.05$) was used to determine the significant factors. The significant factors were chosen for the path of steepest ascent experiment.

Table 1. Statistical analysis of Plackett-Burman design results showing the effect of medium components and fermentation conditions on 2,3-BD production by *P. polymyxa*.

Factor	Low Level (−1)	High Level (+1)	% Contribution	*t* Value	*p* Value
X_1: Inoculum size (%)	6	10	39.56	10.29	0.0260 *
X_2: Temperature (°C)	35	37	23.23	−7.88	0.0348 *
X_3: CH_3COONH_4 (g/L)	3	5	4.87	3.61	0.0753
X_4: $(NH_4)_2SO_4$ (g/L)	2	4	9.30	4.99	0.0595
X_5: Glycerol (g/L)	5	10	0.29	0.89	0.2112
X_6: Yeast extract (g/L)	5	7	4.51	3.47	0.1174
X_7: Tryptone (g/L)	5	7	18.23	−6.99	0.0377 *

$R^2 = 0.9993$, Adj $R^2 = 0.9951$ * Statistical significance.

2.2.2. Path of Steepest Ascent

The path of steepest ascent method enables the determination of three optimal levels at which to further optimize each of the significant factors obtained from the Plackett-Burman design. This is carried out by moving the center point value of each factor sequentially along the path of steepest ascent until no further increase in 2,3-BD is obtained; i.e., the center point value of each selected factor is either increased or decreased until the maximum 2,3-BD achieved begins to decline [22,23].

2.2.3. Box-Behnken Design and Response Surface Methodology

The optimal nutrient concentrations and fermentation conditions for maximum 2,3-BD production were determined by employing the Box-Behnken design and response surface methodology. The three significant factors—tryptone, temperature, and inoculum size—selected from the Plackett-Burman design were varied at three levels. These factors were shown to exert the most significant effects on 2,3-BD production. The Box-Behnken design, when integrated with response surface methodology quantifies the relationship between the independent input factors and the obtained response surfaces [24]. In this study, the association between the responses and the three important factors were determined according to the second order polynomial function:

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j \ (i,j = 1,2 \ldots ,k) \quad (3)$$

In the equation above, Y is the calculated 2,3-BD response function and β_0 is the estimated regression coefficient of the fitted response at the center point of the design, while X_i represents the corresponding actual value factors for inoculum size, temperature, and tryptone. The regression coefficient for the linear terms is represented by β_i, whereas β_{ij} is the interaction effects and β_{ii} is the quadratic effect. The Design Expert software package (Version 10.0, Stat-Ease Inc., Minneapolis, MN, USA) was used to calculate and analyze the second-order polynomial coefficients. Analysis of variance (ANOVA) was used to test the significance of independent factors (tryptone, inoculum size, temperature) and their interactions at an alpha (α) level of 0.05.

3. Results and Discussion

3.1. Plackett-Burman Design

To obtain an optimized medium and fermentation conditions for improved 2,3-BD production by *P. polymyxa*, medium components and fermentation conditions were evaluated. Nutrient components including tryptone, glycerol, ammonium sulfate, and other fermentation parameters (temperature and inoculum size) were studied to determine the extent to which these factors impact 2,3-BD production by *P. polymyxa*. All media contained crude glycerol. Crude glycerol served a dual purpose in the medium—a source of carbon and an additional source of NADH, as glycerol catabolism generates two additional molecules of NADH, relative to glucose on a molar basis [13,14]. The reducing equivalent furnished by NADH is critical for 2,3-BD biosynthesis, as NADH is required for the reduction of acetoin to 2,3-BD [25,26].

Tryptone was used as an organic nitrogen source in addition to yeast extract, whereas ammonium acetate and ammonium sulfate served as inorganic sources of nitrogen. For fermentation conditions, temperature and inoculum size were selected for investigation. Like most microbial processes, 2,3-BD biosynthesis is enzyme-controlled, therefore, fermentation temperature impacts substrate consumption and 2,3-BD production, because enzyme activity is temperature-dependent [19,20]. Inoculum size has been reported to increase substrate utilization with improved 2,3-BD production and yield [19,27]. To our knowledge, this is the first report of combined optimization of fermentation nutrients and conditions for enhanced 2,3-BD production by *P. polymyxa*. To determine the factors with significant influence on 2,3-BD production, the Plackett-Burman design was employed to test each of the factors at two levels. Our preliminary experiments involving each factor (one-factor-at-a-time experiment) showed that the concentrations of yeast extract, tryptone, ammonium acetate, ammonium sulfate, and glycerol that had significant influence on 2,3-BD production were approximately 5.0, 5.0, 4.0, 3.0, and 7.0 g/L, respectively (Figures S1–S5). Furthermore, the approximate temperature and inoculum size that exerted marked effects on 2,3-BD accumulation from the one-factor-at-a-time experiments were 36 °C and 8%, respectively (Figures S6 and S7).

The results obtained from our one-factor-at-a-time experiments informed the selection of two levels for each factor, which were then tested using the Plackett-Burman design. The two levels for each of the factors were determined by employing Equation (1) above; designated as the low and high levels, respectively, as shown in Table 1. The ANOVA generated from the experimental runs using the Plackett-Burman design is shown in Table 1. The linear regression coefficient of the model, R^2, was 0.9993 and the adjusted determination coefficient, *Adj* R^2, was 0.9951, which are both significantly close to unity, indicating the robustness of the model for further studies. R^2 measures variations in 2,3-BD response that are explained by the tested factors for a linear regression model, whereas *Adj* R^2 is the measure of goodness-of-fit for the model. As shown in Table 1, the *p*-values for tryptone, temperature, and inoculum size were 0.0377, 0.0348, and 0.0260, respectively, indicating that tryptone, temperature, and inoculum size were the most important factors that influenced 2,3-BD production by *P. polymyxa* among the factors studied, at a 95% confidence interval.

Among the medium components tested, tryptone exerted the most significant effect on 2,3-BD production by *P. polymyxa*. However, it was observed that further increase in the concentration of tryptone in the fermentation medium increased biomass formation without increasing 2,3-BD production as shown by the t-value of tryptone (Table 1). The pattern observed with tryptone was not unusual considering that tryptone supplies amino acids for protein (including enzymes) biosynthesis, as well as serves as a source of nitrogen for the biosynthesis of nucleic acids. In light of this, we speculated that a concentration threshold may exist for tryptone, within which both biomass and 2,3-BD accumulations by *P. polymyxa* occur optimally. On the other hand, increasing the temperature of the fermentation medium negatively impacted 2,3-BD production by *P. polymxa*, as revealed by the t-value of temperature in Table 1. Temperature regulation is essential for cells to function optimally, and when temperature falls below, or exceeds the optimum range for an organism, cellular metabolism

is impeded, which in this case, adversely affects 2,3-BD production [6]. Increasing inoculum size in the fermentation medium was found to positively influence 2,3-BD production by *P. polymyxa* as shown by the t-value of inoculum (Table 1). Inoculum size determines the population of viable cells in the fermentation medium at time zero, and the greater the number of cells in the medium at time zero, the shorter the lag phase of growth, which ultimately translates to a faster conversion of substrates to 2,3-BD. For instance, the use of high inoculum size has been shown to decrease the duration of bacterial acclimation to culture medium with increased productivity as opposed to when a low inoculum volume is used [28]. A study conducted using *Bacillus licheniformis* showed that increasing inoculum size from 0.5 to 10 g/L increased volumetric 2,3-BD productivity and 2,3-BD yield from ~0.04 to 0.35 g/L/h and 0.11 to 0.35 g/g, respectively [19]. Furthermore, Jyothi et al. [29] obtained higher glutamic acid concentrations when the inoculum size of *Brevibacterium divaricatum* was increased from 3% to 7% with concomitant higher glutamic acid yields.

3.2. Path of Steepest Ascent Design

The path of steepest ascent method was used to determine the optimum levels for each of the three significant factors obtained by the Plackett-Burman design. The optimum level for each of the factors is critical for the Box-Behnken design and response surface methodology. For the path of steepest ascent study, the center point (value) between the low and high levels in the Plackett-Burman design was employed. The center point value for each factor was moved along a path that ensures an increase in 2,3-BD production. The direction for which the center point of each factor is moved was informed by the t-values shown in Table 1. The effects of tryptone and temperature were negative (−6.99 and −7.88, respectively), whereas that of inoculum size was positive (+10.29). These imply that to increase 2,3-BD production by *P. polymyxa*, the center point of each factor with a positive effect needs to be increased sequentially while that with a negative effect is to be decreased until no further increase in 2,3-BD production is observed. The center points for tryptone, temperature, and inoculum size were 5 g/L, 36 °C and 8%, respectively. Consequently, tryptone and temperature were decreased sequentially from 5 g/L and 36 °C to 2.5 g/L and 32 °C, respectively, while inoculum size was sequentially increased from 8% to 10.5% until no further increase in 2,3-BD production was observed (Table 2). From Table 2, the best three experiments in terms of 2,3-BD concentration were experiments 2, 3, and 4. The levels of each factor corresponding to experiments 2, 3, and 4 in the path of steepest ascent were selected for further optimization using the Box-Behnken design and response surface methodology. Based on these, the low, center, and high values, respectively, selected for temperature were 33, 34, and 35 °C, and those of inoculum size were 8.5%, 9.0%, and 9.5%, whereas the selected concentrations for tryptone were 3.5, 4.0, and 4.5 g/L.

Table 2. The path of steepest ascent experimental design and 2,3-BD production by *P. polymyxa*.

Run	Factors			2,3-BD (g/L)
	Inoculum Size (%)	Temperature (°C)	Tryptone (g/L)	
1	8.0	36	5.0	46.56
2	8.5	35	4.5	47.87
3	9.0	34	4.0	51.96
4	9.5	33	3.5	48.26
5	10	32	3.0	45.50

3.3. Box-Behnken Design and Response Surface Methodology

Based on the results obtained from the Plackett-Burman design and the path of steepest ascent method, the Box-Behnken design was used to conduct 15 experimental runs to further optimize the levels of temperature, tryptone, and inoculum size as shown in Table 3. Data obtained from the design

matrix were analyzed using multiple regression and a second order polynomial equation model was obtained as shown below:

$$Y = 51.97 + 0.64X_1 - 0.16X_2 + 1.17X_7 + 2.36X_1X_2 - 0.79X_1X_7 + 0.64X_2X_7 - 2.31{X_1}^2 - 0.71{X_2}^2 - 0.059{X_7}^2 \quad (4)$$

where Y was the predicted response, and X_1, X_2, and X_7 were the defined values of inoculum size, temperature, and tryptone, respectively.

Table 3. Box-Behnken design and response results for 2,3-BD production.

Run	Coded Values			Actual Values			2,3-BD (g/L)
	X_1	X_2	X_7	X_1 (%)	X_2 (°C)	X_7 (g/L)	
1	−1	0	+1	8.5	34	4.5	50.65
2	+1	0	+1	9.5	34	4.5	50.99
3	−1	+1	0	8.5	35	4.0	46.20
4	0	0	0	9.0	34	4.0	52.56
5	0	−1	−1	9.0	33	3.5	50.98
6	−1	−1	0	8.5	33	4.0	51.04
7	0	0	0	9.0	34	4.0	51.98
8	+1	−1	0	9.5	33	4.0	46.99
9	0	+1	+1	9.0	35	4.5	52.70
10	+1	0	−1	9.5	34	3.5	50.13
11	+1	+1	0	9.5	35	4.0	51.58
12	0	+1	−1	9.0	35	3.5	49.18
13	0	0	0	9.0	34	4.0	51.37
14	0	−1	+1	9.0	33	4.5	51.94
15	−1	0	−1	8.5	34	3.5	46.65

X_1, inoculum size; X_2, temperature; X_7, tryptone. Error bars show standard deviations of the means (n = 3).

Analysis of variance (ANOVA) was used to test the statistical significance of the model as shown in Table 4. The model had a p-value of 0.0017 which is far less than 0.05 (indicative of significance). The regression coefficient, R^2, of the model was 0.9750 and the adjusted determination coefficient, *Adj* R^2, of the model was 0.9300, implying that 93% of variation in the response can be explained by the model. The lack of fit p-value was 0.5904, which indicates that the lack of fit was not significant, which confirms that the model was adequate for predicting 2,3-BD production. The lack of fit test is used to compare residual errors to the pure errors and gives an F-value for the model [30]. The F-value of the model was 21.65, which is low, thereby confirming that the model is significantly robust.

Table 4. ANOVA for 2,3-BD production by *P. polymyxa* according to the response surface quadratic model (lack of fit is not significant).

Factors	Sum of Squares	Degree of Freedom	Mean of Squares	*F*-Value	*p*-Value
Model	61.55	9	6.84	21.65	0.0017 *
X_1	3.32	1	3.32	10.50	0.0230 *
X_2	0.21	1	0.21	0.66	0.4539
X_7	10.90	1	10.90	34.52	0.0020 *
X_1 X_2	22.23	1	22.23	70.38	0.0004 *
X_1 X_7	2.46	1	2.46	7.80	0.0383 *
X_2 X_7	1.64	1	1.64	5.19	0.0718
${X_1}^2$	19.64	1	19.64	62.18	0.0005 *
${X_2}^2$	1.87	1	1.87	5.91	0.0592
${X_7}^2$	0.013	1	0.013	0.040	0.8487
Residual	1.58	5	0.32		
Lack of fit	0.87	3	0.29	0.82	0.5904
Pure error	0.71	2	0.35		
Cor. Total	63.13	14			

R^2 = 0.9750, Adj R^2 = 0.9300; *Cor.* = Corrected; * Statistical significance.

To further evaluate the optimal levels of the individual factors, the significance of each factor and their interaction terms were tested using *F*-test, and the corresponding *p*-values for each of the model terms are shown in Table 4. Model terms with a *p*-value less than 0.05 shows that the terms are significant. The model terms for inoculum size, tryptone, inoculum size and temperature (inoculum size *x* temperature), inoculum size and tryptone (inoculum size *x* tryptone), and the quadratic term, inoculum size and inoculum size (inoculum size)^2 were all found to be significant. As shown in Table 4, tryptone was found to exert the largest effect on 2,3-BD production by *P. polymyxa* amongst the individual terms studied, whereas inoculum size and temperature (inoculum size *x* temperature) exhibited the largest effect when the individual and interaction terms were compared.

The interaction effects of factors were also evaluated by response surface methodology. The response surface is a three-dimensional plot that graphically represents the regression equation and shows relationship between the response and the independent factors [31,32]. Concave or convex response surfaces show that the maximum or minimum response is located within the experimental region, whereas a saddled surface shows a relative maximum and a relative minimum response, respectively [16,33]. Furthermore, the contour plots are two-dimensional representations of the response surface, which enhance the visual interpretation of the response surface [32,33]. Plots showing elliptical contours indicate significant interactions between the independent factors and the center of the smallest ellipse refers to a point of maximum or minimum response [31]. Also, plots with circular contours show that the interactions between the independent factors are negligible [32].

In the present study, the interaction between temperature and inoculum size when tryptone is maintained at the center value (4.0 g/L) shows a concave surface (Figure 1a), suggesting the presence of an apparent optimum condition. The corresponding elliptical contour plot shows that the interaction between temperature and inoculum size has significant effect on 2,3-BD production by *P. polymyxa* (Figure 1b). Additionally, the interaction between inoculum size and tryptone when the temperature is kept at the center point value also shows a concave surface (Figure 1c) with an elliptical contour (Figure 1d), thereby indicating a significant interaction. Conversely, the interaction between temperature and tryptone shows a contour that is not elliptical, therefore, not significant (Figure 2b). The optimum levels for the factors where maximum 2,3-BD production by *P. polymyxa* is predicted was obtained from the elliptical contour plot of Figure 1b, where strong interactions was observed. The maximum levels of inoculum size and temperature were indicated at the point of intersection between the major and minor axes confined by the smallest ellipse in Figure 1b [16,34,35]. The optimum conditions for maximum predicted 2,3-BD production was calculated when the coordinates of the important points (from Figure 1b) were inserted into Equation (3), and the partial derivatives were set to zero. The maximum predicted 2,3-BD was 51.52 g/L, which corresponds to a temperature of 34.98 °C and an inoculum size of 9.45%. The concentration of tryptone at this maximum predicted 2,3-BD was 3.5 g/L. The contour plots showing interactions between inoculum size and tryptone and between temperature and tryptone were not fully elliptical. The lack of a perfect elliptical contour is an indication that little or minimal interaction exists between the factors under evaluation. Thus, the optimized fermentation medium and conditions obtained in this study were 9.5% inoculum size, 120 g/L glucose, 3.5 g/L tryptone, and a temperature of 35 °C with the addition of yeast extract, 5 g/L; ammonium acetate, 4 g/L; NH_4SO_4, 3 g/L, crude glycerol, 7 g/L; KH_2PO_4, 3.5 g/L; K_2HPO_4, 2.75 g/L; $CoCl_2$, 0.05 g/L, $MgSO_4$, 0.2 g/L; MOPS, 10 g/L; and trace element solution.

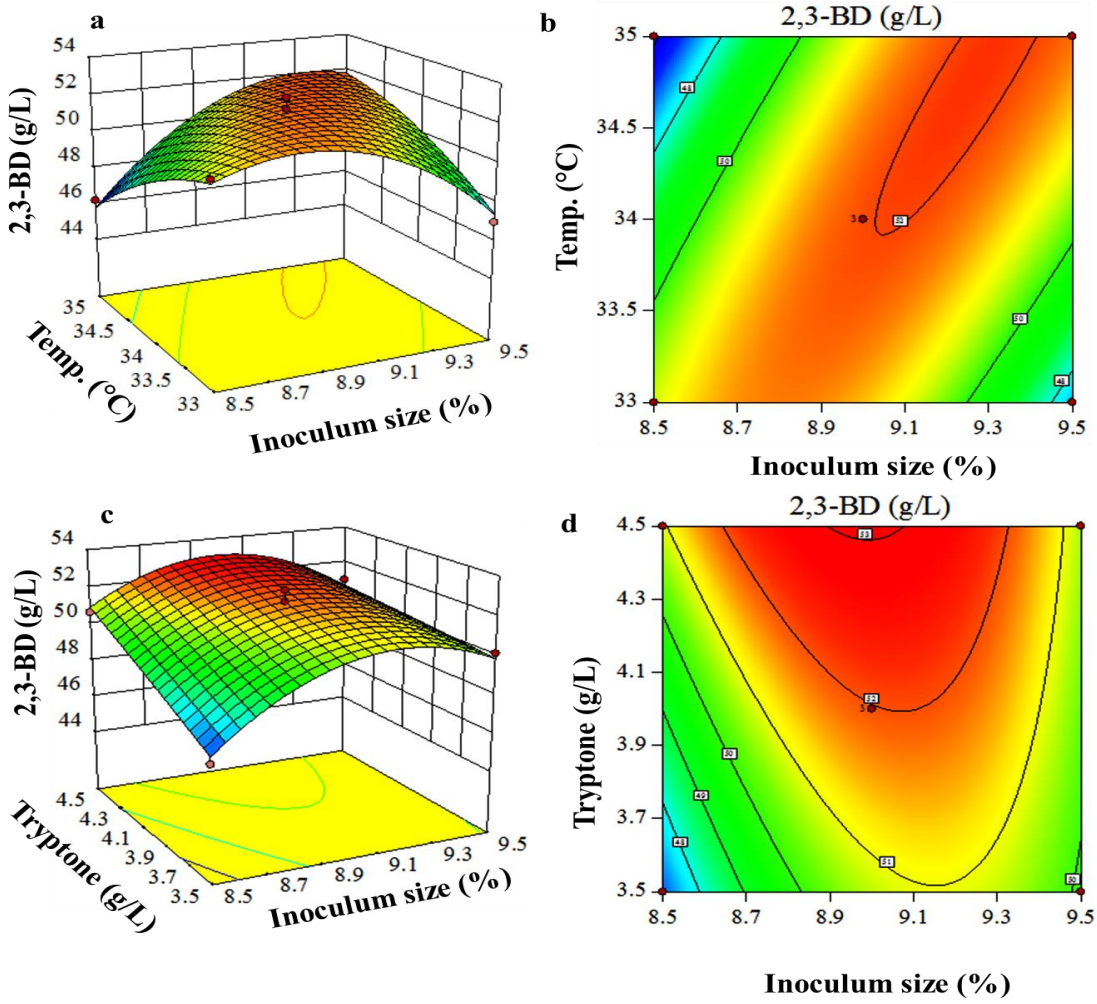

Figure 1. Contour and response surface plots. (**a**) The response surface plot; (**b**) the resultant contour plot showing the effects of temperature and inoculum size on 2,3-BD production by *P. polymyxa* with tryptone fixed at 4 g/L; (**c**) response surface plot and (**d**) the resultant contour plot depicting the effects of tryptone and inoculum size on 2,3-BD production by *P. polymyxa* with the temperature fixed at 34 °C.

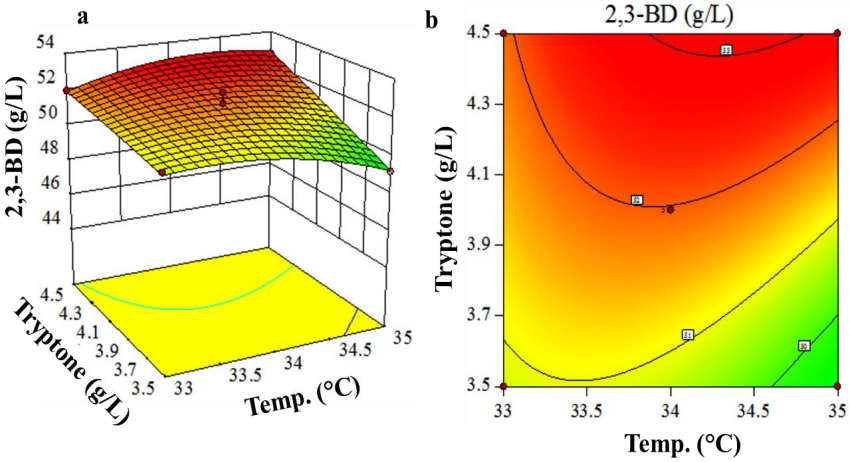

Figure 2. Contour and response surface plots. (**a**) The response surface plot and (**b**) the resultant contour plot (**b**) showing the effects of tryptone and temperature on 2,3-BD production by *P. polymyxa* with the inoculum size fixed at 9%.

3.4. Experimental Validation of the Optimized Medium and Conditions in Batch and Fed-Batch Fermentations

The optimized fermentation medium and conditions obtained from the analyses above were then used to conduct batch and fed-batch fermentations in a 5 L bioreactor to validate the fermentation medium and conditions. In each case, fermentation was conducted with a starting volume of 2 L. As shown above, apart from inoculum size, temperature, and tryptone, all the other factors that were tested by the Plackett-Burman experimental design were kept at their center point values. Batch and fed-batch fermentations were conducted in triplicate. The concentration of 2,3-BD obtained from the mean of three biological replicates using the optimized medium and conditions was 51.10 g/L, which was 99% of the predicted maximum 2,3-BD concentration of 51.52 g/L by response surface methodology. The yield and productivity of 2,3-BD obtained in the batch fermentation were 0.42 and 1.70, respectively (Table 5). The batch fermentation profile in Figure 3a shows complete glucose utilization, which is an indication of efficient glucose conversion to 2,3-BD with minimal formation of competing products. Thus, in addition to increased 2,3-BD production, the concentrations of ethanol, acetic acid, EPS, and acetoin were considerably diminished (Table 5 and Figure S8), when compared to non-optimized fermentations [7]. Due to reduced diversion of carbon to the EPS and ethanol biosynthesis pathways, and most likely, increased carbon flux from acetoin and acetic acid to 2,3-BD production during batch fermentation by *P. polymyxa*, the 2,3-BD yield increased from 0.32 g/g glucose under non-optimized fermentation conditions [7] to 0.42 g/g glucose under optimized conditions, accounting for a 31% increase in 2,3-BD yield.

To further determine the maximum 2,3-BD that *P. polymyxa* can accumulate using the optimized fermentation medium and conditions, a fed-batch fermentation was conducted. During the fed-batch process, glucose was intermittently replenished in the culture, accompanied by the addition of half-strength of the other nutrient components until no further increase in 2,3-BD production or glucose consumption was observed. The maximum 2,3-BD obtained from the mean of three independent fed-batch fermentations was 68.54 g/L with a yield and productivity of 0.34 and 0.70, respectively (Table 5; Figure 3b). Similarly, the concentrations of competing products, namely acetoin, ethanol, and acetic acid were considerably reduced, indicating that the optimized process enabled the efficient conversion of substrate carbon to the desired product, 2,3-BD. Nonetheless, acetoin was observed to increase towards the end of the fermentation, at which point, a corresponding decline in 2,3-BD was observed; a mechanism that *P. polymyxa* is thought to adopt for reducing the toxicity of 2,3-BD at an elevated concentration [7]. Notably, the yield and productivity of 2,3-BD reduced as the fermentation mode was switched from batch to fed-batch (Table 5). This is not unusual considering that greater amounts of glucose are consumed in the fed-batch cultures, some of which is funneled to cell maintenance and growth. In fact, the cell dilution effect resulting from intermittent feeding of glucose and other nutrients into the broth engenders a lag phase; albeit transient, thereby triggering transient cell growth following glucose supplementation, which diverts glucose away from 2,3-BD biosynthesis, momentarily. On the other hand, the concentration of acetoin in the fed-batch fermentation increased 3-fold whereas those of ethanol and acetic acid exhibited 1.2-fold increases, when compared to the batch fermentation (Table 5). A portion of the additional glucose fed into the fed-batch cultures were further converted to ethanol and acetic acid, which were observed to increase relative to the batch cultures that were not fed additional glucose. Interestingly, the optimized culture medium and conditions resulted in a 19% reduction in EPS production in both the batch and fed-batch fermentations (Table 5; Figure S8).

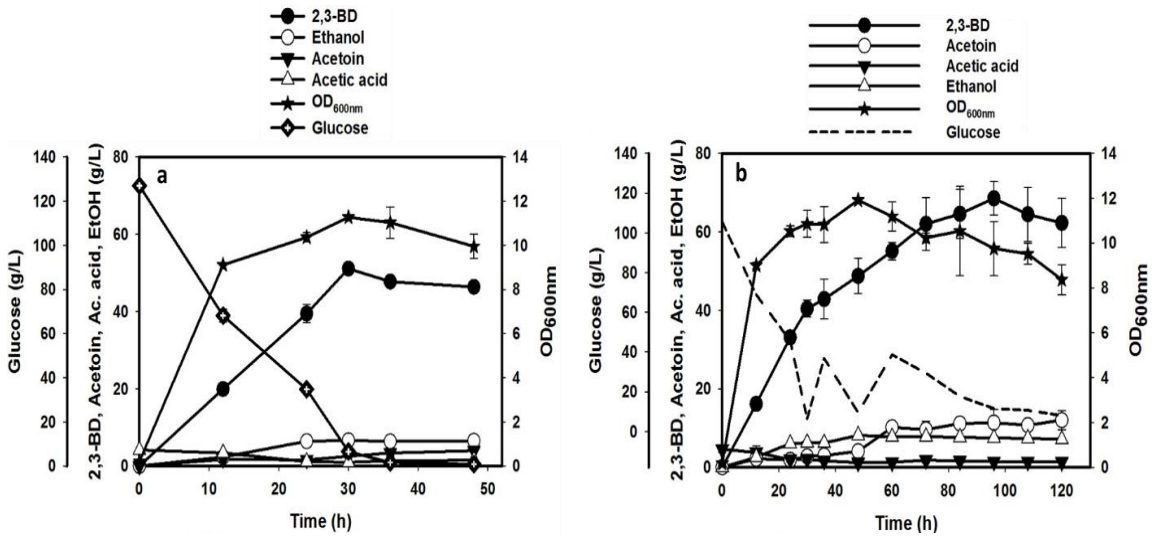

Figure 3. The fermentation profiles of *P. polymyxa* using optimized culture medium and conditions. (**a**) Batch and fed-batch (**b**) fermentations.

Table 5. The product profiles of *P. polymyxa* grown in batch and fed-batch fermentations under optimized conditions.

Product Profile	Batch Fermentation			Fed-Batch Fermentation		
	Max. conc. (g/L)	Yield (g/g)	Productivity (g/L/h)	Max. conc. (g/L)	Yield (g/g)	Productivity (g/L/h)
2,3-BD	51.10 ± 0.61	0.42 ± 0.01	1.70 ± 0.02	68.54 ± 4.25	0.34 ± 0.03	0.70 ± 0.04
Ethanol	6.64 ± 0.19	0.06 ± 0.00	0.22 ± 0.01	8.15 ± 0.51	0.06 ± 0.00	0.17 ± 0.01
Acetoin	3.96 ± 0.08	0.03 ± 0.00	0.08 ± 0.00	12.01 ± 2.43	0.05 ± 0.01	0.10 ± 0.02
Acetic acid	1.51 ± 0.07	0.01 ± 0.00	0.03 ± 0.00	1.82 ± 0.71	0.01 ± 0.00	0.03 ± 0.01
EPS	4.97 ± 0.15	0.04 ± 0.00	0.41 ± 0.01	4.69 ± 0.02	0.02 ± 0.00	0.39 ± 0.00
Glucose consumed	120.54 ± 1.55	N/A	N/A	199.97 ± 8.53	N/A	N/A

N/A: Not applicable. Error bars show standard deviations of the means (n = 3).

A comparison of the 2,3-BD concentration obtained in this study to those from other studies is summarized in Table 6. Different strains of *P. polymyxa* have been shown to possess the enzymatic repertoire for metabolizing several carbon sources to 2,3-BD, with yields ranging from 0.33 to 0.51 g/g glucose (Table 6). Notably, Häßler et al. [8] reported the production of 111 g/L 2,3-BD using a complex fermentation medium containing 60 g/L yeast extract. The use of 60 g/L yeast extract in large-scale 2,3-BD fermentation would increase the cost of production astronomically. Hence, an important achievement of this study is the development of an inexpensive fermentation medium for 2,3-BD fermentation. To assess the degree of impact exerted on 2,3-BD production by tryptone and yeast extract in this study, each of the nitrogen sources (yeast extract and tryptone) were incorporated in the growth medium up to 7 g/L (0, 3, 5, 7 g/L) prior to optimization studies (one-factor-at-a-time experiments). As depicted in Figures S4 and S5, the addition of yeast extract and tryptone in the fermentation medium resulted in increased 2,3-BD production in a concentration dependent manner. However, the increase in 2,3-BD accumulation in the fermentation broth diminished at tryptone and yeast extract concentrations above 5 g/L (that is, at 7 g/L), suggesting that increasing tryptone and yeast extract concentrations above 5 g/L may not engender a further increase in 2,3-BD production. In light of this, we rationalized that lower tryptone and yeast extract concentrations may be ideal for 2,3-BD production by *P. polymyxa*; an economic benefit for large-scale fermentation. Indeed, optimization studies demonstrated that 5 and 3.5 g/L yeast extract and tryptone, respectively, resulted in increased 2,3-BD production from ~27 and ~47 (in un-optimized) to 51 and 68.5 g/L 2,3-BD (in optimized) batch and fed-batch fermentations, respectively.

It is worth mentioning that Häßler et al. [8] used varying fermentation parameters, namely 500 rpm, 0.2 vvm, 37 °C, and sucrose as a carbon source whereas 300 rpm, 0.075 vvm, 35 °C, and glucose (a more readily available sugar from different biomass materials) were used in the present study. However, it is unlikely that interactions between medium components and fermentation conditions contributed to such high levels of 2,3-BD production (111 g/L). Indeed, Okonkwo et al. [7] demonstrated recently that 2,3-BD-mediated toxicity on *P. polymyxa* increased remarkably when the total 2,3-BD concentration in the fermentation broth exceeded the toxic threshold (48 g/L), thereby necessitating backward conversion to acetoin (acetoin ↔ 2,3-butanediol), possibly to alleviate 2,3-BD toxicity. Overall, the 2,3-BD yield of 0.42 and productivity of 1.70 obtained in the batch fermentation in this study compares favorably to those reported by other authors. It is worth noting that the yield and productivity obtained in this study were achieved using lower amounts of organic nitrogen sources in the forms of tryptone and yeast extract when compared to the other studies; a critical economic consideration for large-scale operations.

Table 6. Comparison of 2,3-BD concentrations obtained in this study to those of other studies using *P. polymyxa*.

Carbon Source	2,3-BD (g/L)	2,3-BD Yield (g/g)	2,3-BD Prod. (g/L/h)	GO	ONS (g/L)	PP	FM	Ref.
Glucose	51.10 ± 0.61	0.42 ± 0.01	1.70 ± 0.02	11.27 ± 0.06	YE, 5; tryp., 3.5	PP DSM 365	B	This work
Glucose	68.54 ± 4.25	0.34 ± 0.03	0.70 ± 0.04	11.92 ± 0.11	YE, 5; tryp., 3.5	PP DSM 365	FB	This work
Raw inulin extract from Jerusalem artichoke tubers	37.57 ± 0.32	0.51	0.89	26.79 ± 0.35 *	YE, 3; pep, 2	PP ZJ-9	B	[16]
Glucose	71.71	ND	1.33	13	YE, 10	PP CJX518	FB	[36]
Sucrose	111	ND	2.06	23 †	YE, 60	PP DSM 365	FB	[8]
Glucose	16.50	0.33	2.01	9.5	YE, 15	PP ICGEB2008	B	[37]
Inulin	51.3	ND	ND	NS	YE, 6; Pep., 3	PP ZJ-9 (XG-1)	FB	[38]
Inulin	36.8	ND	ND	11 †	YE, 6; Pep., 3	PP ZJ-9 (XG-1)	B	[38]

* Unit in g/L; † Determined at OD_{660nm}; NS—Not shown; ND—not detected; GO—Growth (OD_{600nm}); ONS—Organic nitrogen source; PP—*Paenibacillus polymyxa*; FM—Fermentation mode; B—batch; FB—Fed-batch; YE—yeast extract; Pep.—peptone; tryp.—tryptone; Ref.—references. The data shown are maximum product concentrations and cell growth achieved during fermentation.

4. Conclusions

Based on the results from the Box-Behnken design and response surface methodology, an optimized medium (7 g/L crude glycerol included) and culture conditions for enhanced 2,3-BD production by *P. polymyxa* were developed. The optimized conditions were validated in batch and fed-batch fermentations, leading to the production of 51.10 and 68.54 g/L, respectively, of 2,3-BD. These account for 47% and 31% increases in 2,3-BD production in batch and fed-batch cultures, respectively, with attendant diminished generation of competing co-products, especially EPS, relative to the non-optimized fermentations. The results presented here underline the interplay between medium components, culture conditions, and product-mediated toxicity (feedback inhibition), as the earlier determined toxic threshold of 2,3-BD (50 g/L) on *P. polymyxa* in a non-optimized medium [7] was significantly exceeded in this work (68.54 g/L). However, it is worth mentioning that glycerol was incorporated in the fermentation medium used in this study, which may contribute to 2,3-BD biosynthesis via improved NADH regeneration, especially in the optimized medium, relative to the un-optimized control medium. Collectively, we demonstrate that lower amounts of the expensive organic nitrogen sources, tryptone and yeast extract, can be used for optimal 2,3-BD production. This represents a significant reduction in operating costs in the efforts to commercialize biological production of 2,3-BD.

Supplementary Materials: The following are available online at http://www.mdpi.com/2311-5637/3/2/18/s1.

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Conflicts of Interest: The authors declare no conflict of interest.

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