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Highly Efficient 2,3-Butanediol Production by *Bacillus licheniformis* via Complex Optimization of Nutritional and Technological Parameters

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Abstract: 2,3-Butanediol (2,3-BD) is a reagent with remarkable commercial use as a platform chemical in numerous industries. The present study aims to determine the capabilities of non-pathogenic and cellulolytic *Bacillus licheniformis* 24 as a 2,3-BD producer. By applying the Plackett–Burman design and response surface methodology through central composite design (CCD), a complex optimization of medium and process parameters was conducted. Thus, among ten studied factors of medium content, four components were evaluated with a significant positive effect on 2,3-BD formation. Their optimal values for 2,3-BD production (yeast extract, 13.38 g/L; tryptone, 6.41 g/L; K_2HPO_4, 4.2 g/L; $MgSO_4$, 0.32 g/L), as well as the optimal temperature (37.8 °C), pH (6.23) and aeration rate (3.68 vvm) were predicted by CCD experiments and validated in a series of batch processes. In optimized batch fermentation of 200 g/L of glucose 91.23 g/L of 2,3-BD was obtained, with the overall productivity of 1.94 g/L/h and yield of 0.488 g/g. To reveal the maximum 2,3-BD tolerance of *B. licheniformis* 24, fed-batch fermentation was carried out. The obtained 138.8 g/L of 2,3-BD with a yield of 0.479 g/g and productivity of 1.16 g/L/h ranks the strain among the best 2,3-BD producers.

Keywords: 2,3-butanediol; *Bacillus licheniformis*; Plackett–Burman design; response surface methodology

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1. Introduction

The possibility of microbial synthesis of the bivalent alcohol 2,3-butanediol (2,3-BD) was investigated for the first time more than a century ago by Harden and Walpole in *Klebsiella pneumoniae* and by Donker in *Paenibacillus polymyxa* [1,2]. Although its future use was only speculative at that time, today, the 2,3-BD market is notably fast-growing and is expected to reach USD 220 million in 2027 [3]. The most indispensable application of 2,3-BD is in the chemical, pharmaceutical and food industries, as a starting reagent in the production of rubber, solvents, varnishes, polyesters, polyurethanes and methacrylate. It is also a common constituent of liquid fuels, antifreeze, cosmetic products, drugs, antiperspirants and ointments [4–6]. A recent study reports 2,3-BD integration into biodegradable biofilms [7]. However, the emerging ecological problems, fossil fuel exhaustion and climate changes impose the development of biotechnologies for a bio-based alternative of 2,3-BD chemical synthesis despite cost competitiveness issues [8,9].

Current strategies for increased microbial production of 2,3-BD include (i) isolation of new bacterial producers, preferably non-pathogenic, that can utilize cheap, renewable and non-nutritional substrates, (ii) development of engineered strains that may produce optically pure isomers and (iii) selection of strains amenable to process optimization for increased 2,3-BD titer, yield and productivity. The "generally regarded as safe" (GRAS) 2,3-BD producers are the most desirable on an industrial scale; therefore, *Paenibacillus polymyxa* [10,11], *Bacillus subtilis* [12,13], *B. amyloliquefaciens* [14,15], *B. pumilus*, *B. siamensis* [16], *B. vallismortis* [17], *B. licheniformis*, *B. velezensis*, *B. toyonensis* and *B. safensis* have been evaluated as particularly promising [18–21].

However, *B. licheniformis* combines the greatest number of benefits. It has the potential to convert a variety of substrates into 2,3-BD, such as starch and corncob hydrolyzates [22,23], inulin [24] and most of the sugars included in plant biomass, such as glucose, cellobiose, galactose, mannose, xylose and arabinose. Although several attempts to optimize the medium content and culture conditions for *B. licheniformis* have been made, several unresolved issues with the species and the peculiar nature of the process remain to be studied in more detail. First, several process optimizations refer to thermophilic *B. licheniformis* strains [16,25], but information concerning the optimal conditions for high 2,3-BD production by mesophilic representatives is scarce. Second, it has to be noted that 2,3-BD is obtained via mixed acid fermentation yielding multiple products and the majority of them are undesirable and decrease the 2,3-BD yield. Beside 2,3-BD and its precursor, acetoin, some *B. licheniformis* strains produce mainly acetic acid and ethanol as by-products [26], while others secrete also lactate, formate and glycerol [27]. Therefore, different optimization approaches are required to decrease the specific spectrum of final metabolites produced by each industrial strain.

On the other hand, oxygen supply is a critical factor for acetoin and 2,3-BD interconversion. High levels of dissolved oxygen lead to acetoin synthesis, while lower levels favor 2,3-BD synthesis [21]. In addition, 2,3-BD can also serve as a carbon source for *B. licheniformis* at diminished glucose concentrations [28]. That is why a fed-batch process performance that requires additional improvement is frequently applied [16,20].

In our recent study, we revealed that Bulgarian isolate *B. licheniformis* 24 possesses particularly high 2,3-BD productivity using glucose, mannose and cellobiose, reaching 0.77, 0.64 and 0.46 g/L/h, respectively [18]. 2,3-BD yield from glucose was 83% of the theoretical maximum without any process optimization. Moreover, the strain was able to maintain low levels of acetoin even when the substrate was depleted and displayed significant natural extracellular cellulase activity [18]. Since *B. licheniformis* 24 is particularly promising as a producer of 2,3-BD from cellulose-containing substrates, we focused on detailed process optimization to obtain the highest production values from glucose. The purpose of the study was achieved by the use of Plackett–Burman and central composite design (CCD) for medium optimization and response surface design methodology for optimization of process parameters applied to both batch and fed-batch operation performance. The obtained record 2,3-BD amount also elucidates the maximal tolerance of the strain to this target metabolite.

2. Materials and Methods

2.1. Bacterial Strain and Basal Medium

B. licheniformis strain 24 was isolated from a soil sample collected near the Yantra river, Bulgaria, and is stored in the Microbial culture collection of the Institute of Microbiology, Bulgarian Academy of Sciences. It was identified by 16S rDNA sequencing (NCBI GenBank accession no. MK461938).

As a basic nutrient medium in the optimization experiments, we used the medium initially developed for *P. polymyxa* by Okonkwo et al. [29], modified by Petrova et al. [18], with the following content (g/L): glucose, 20–100; yeast extract, 5; tryptone, 5; $(NH_4)_2$ SO_4, 3; KH_2 PO_4, 3.5; K_2 HPO_4, 2.75; $MgSO_4$, 0.2; ammonium acetate, 1.5; $CoCl_2$ $6H_2O$, 0.09; 3-morpholino propane sulfonic acid (MOPS), 10; salt solution, 3 mL per liter. The salt solution contained (g/L): $FeSO_4$, 0.4; H_3BO_3, 0.8; $CuSO_4 \cdot 5H_2O$, 0.04; $NaMoO_4 \cdot 2H_2O$, 0.04; $MnCl_2 \cdot 4H_2O$, 5.0; $ZnSO_4 \cdot 7H_2O$, 0.1; $Co(NO_3)_2 \cdot 6H_2O$, 0.08; $CaCl_2 \cdot 2H_2O$, 1.0; Biotin, 0.01.

2.2. Cultivation Conditions

The experiments for media optimization (Plackett–Burman design and CCD) were performed in 500 mL Erlenmeyer flasks with 100 mL of media containing 100 g/L glucose at 37 °C and 200 rpm, on a rotary shaker (New Brunswick, San Diego, CA, USA). As an inoculum, we used overnight culture grown in 500 mL flasks with 50 mL of basal medium containing 20 g/L of glucose, at 37 °C, 200 rpm, on a rotary shaker. The inoculum was

grown to OD = 2.400 (measured at wavelength λ = 600 nm); its amount was 2% for the Plackett–Burman and 1% for the CCD experiments.

Batch fermentations for process parameter optimization and the fed-batch with optimized parameters were conducted in a stirred 1 L fermenter (Biostat® A plus, Sartorius Stedim Biotech, Gottingen, Germany) additionally equipped with bumpers to provide more aerobic conditions. Likewise, an additional air pump and rotameter were used to ensure higher levels of airflow supply. The pH was controlled by the addition of 6M NaOH or 5M HCl. Batch processes were carried out using the medium with optimized content, supplemented with 200 g/L of glucose and 10% inoculum (grown to OD_{600} = 2.400), thus corresponding to an initial glucose concentration of 185–187 g/L. In fed-batch fermentation, the additional substrate amount was added as portions of filter-sterilized glucose stock with a concentration of 700 g/L.

2.3. Screening for Significant Factors in Media Composition

The Plackett–Burman design experiment was applied to estimate the significance of each compound of the nutrient medium [13,30]. An advantage of this design is the ability to study a large number of variables through a relatively small number of experiments. The influence of 10 components of the nutrient medium was studied, as corn steep liquor was additionally added to the 9 components of the basal medium to possibly replace the more expensive nitrogen sources yeast extract and tryptone (Table 1). A 15-run design including 3 central points and 12 cube points on two levels (+1, −1) was used. The influence of each factor on 2,3-BD production was described by the first-degree (linear) polynomial Equation (1):

$$Y = \beta_0 + \sum \beta_i X_i \quad (1)$$

where Y is the predicted response, β_0 is the intercept term, β_i is the linear coefficient and X_i is an independent variable.

Table 1. Media components selected for variation and their experimental range for 2,3-BD production using a ten-factor Plackett–Burman design.

Variables (Media Components)	Code	Experimental Levels		
		−1	**0**	**1**
Yeast extract (g/L)	X_1	0	5	10
Tryptone (g/L)	X_2	0	5	10
$(NH_4)_2\ SO_4$ (g/L)	X_3	1	3	5
$KH_2\ PO_4$ (g/L)	X_4	2	3.5	5
$K_2\ HPO_4$ (g/L)	X_5	2	2.75	3.5
$MgSO_4$ (g/L)	X_6	0.1	0.2	0.3
Ammonium acetate (g/L)	X_7	0.5	1.5	2.5
Corn steep liquor (g/L)	X_8	0	10	20
Salt solution (%)	X_9	1	3	5
MOPS [a] (g/L)	X_{10}	0	5	10

[a] MOPS, 3-morpholino propane sulfonic acid.

2.4. Media Optimization by Response Surface Design Methodology

The response surface methodology via CCD experiment was used to determine the optimal values of the components of the medium with significant influence on 2,3-BD production. According to the results obtained from the Plackett–Burman design, the variables were the following: yeast extract (X_1); tryptone (X_2); K_2HPO_4 (X_5); $MgSO_4$ (X_6). The four variables were tested at 5 levels (−2, −1, 0, +1, +2), in a matrix containing 31-run design with 7 central, 8 axial and 16 cube points. The variables levels and the corresponding values are shown in Table 2.

Table 2. Media components selected for variation and their experimental range for 2,3-BD production using a four-factor CCD.

Factors	Experimental Levels				
(Variable)	**$-\alpha$ ***	**−1**	**0**	**1**	**α ***
Yeast extract (g/L) (X_1)	5	7.5	10	12.5	15
Tryptone (g/L) (X_2)	5	7.5	10	12.5	15
K_2HPO_4 (g/L) (X_5)	2.5	3.0	3.5	4.0	4.5
$MgSO_4$ (g/L) (X_6)	0.2	0.25	0.3	0.35	0.4

* $\alpha = 2$.

An investigation of the effect of variables on 2,3-BD production was performed by a regression model, using the following second-degree polynomial Equation (2):

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j \quad (2)$$

where Y is the predicted response, β_0 is the intercept term, β_i is the linear coefficient, β_{ii} is the quadratic coefficient, β_{ij} is the coefficient of interaction and X_i and X_j are independent variables.

2.5. Process Parameter Optimization by Response Surface Design Methodology

The most important process parameters—temperature, pH and aeration—were subjected to optimization by applying the same response surface methodology used for the optimization of media components [31,32]. In this case, a three-factor central composite design experiment was applied, as the three variables were set at 5 levels (−1.682, −1, 0, +1, +1.682), in a matrix containing a total of 20 runs—6 central, 6 axial and 8 cube points. The relevant formula for the used regression model is the second-degree polynomial Equation (2). The coded levels of these 3 variables and their corresponding actual values are shown in Table 3.

Table 3. Process parameters selected for variation and their experimental range for 2,3-BD production using a three-factor central composite design.

Factors	Experimental Levels				
(Variable)	**$-\alpha$ ***	**−1**	**0**	**1**	**α ***
t (°C) (X_1)	28.9546	31	34	37	39.0454
pH (X_2)	5.1591	5.5	6.0	6.5	6.8409
Aeration rate (vvm) (X_3)	0.3182	1.0	2.0	3.0	3.6818

* $\alpha = 1.682$.

2.6. Analytical Methods

Cell growth was estimated by viable cells counts (CFU, colony-forming units, per mL) of decimal dilutions of samples, which were grown on Luria–Bertani (LB) agar plates. Glucose, lactic acid, glycerol, acetoin, 2,3-butanediol and ethanol amounts were quantified using the YL Instrument 9300 HPLC System (YL Instrument Co., Ltd., Anyang, Korea). The soluble components were analyzed by HPLC column Aminex HPX-87H (BioRad Laboratories, Hercules, CA, USA) at 65 °C. The mobile phase was 5 mmoL of H_2SO_4 at a flow rate of 0.6 mL/min. All compounds were detected by RI detector (YL 9170 RI Detector) as the quantification of lactic acid and acetoin was confirmed by a UV detector (YL9120 UV/Vis detector) at wavelengths of 210 and 190 nm, respectively. All standard substances were purchased from Merck KgaA, Darmstadt, Germany.

The statistical analyses were accomplished using the Minitab 17 software of Minitab Inc. (State College, PA, USA), www.minitab.com.

3. Results

3.1. Media Optimization

3.1.1. Screening for Significant Factors in Media Composition

Ten nutrient medium components were studied in order to assess their significance for 2,3-BD production (Table 1). As a response, according to the design of Plackett–Burman, the concentration of 2,3-BD after 24 h of fermentation was used. The statistical analysis of the experimental results showed that, within the defined ranges, the yeast extract, K_2HPO_4, $MgSO_4$ and the tryptone had a significant positive effect, the corn steep liquor had a significant negative effect and the other variables had no significant effect on the 2,3-BD formation (p value > 0.05). The results shown in Table 4 reveal that the most influencing factor appeared to be the yeast extract with a linear coefficient of 6.181 and p value of 0.001, followed by the corn steep liquor (−5.2680, p = 0.001), K_2HPO_4 (4.731, p = 0.002), $MgSO_4$ (2.758, p = 0.008) and the tryptone (1.421, p = 0.047).

Table 4. Estimated coded coefficients from the linear regression model for 2,3-BD production in a ten-factor Plackett–Burman design.

Source	Effect	Coefficient	*T* Value	*p* Value
Constant		10.477	24.09	0.000
Yeast extract (X_1)	12.362	6.181	14.25	0.001 [a]
Tryptone (X_2)	2.842	1.421	3.28	0.047 [a]
$(NH_4)_2$ SO_4 (X_3)	−0.465	−0.233	−0.54	0.629
KH_2 PO_4 (X_4)	−1.028	−0.514	−1.19	0.321
K_2 HPO_4 (X_5)	9.462	4.731	10.91	0.002 [a]
$MgSO_4$ (X_6)	5.515	2.758	6.36	0.008 [a]
Ammonium acetate (X_7)	−1.538	−0.769	−1.77	0.174
Corn steep liquor (X_8)	−10.535	−5.268	−12.15	0.001 [b]
Salt solution (X_9)	−2.395	−1.197	−2.76	0.070
MOPS (X_{10})	1.145	0.573	1.32	0.278

[a] Significant positive effect; [b] significant negative effect.

The variance analysis showed that the R^2 value (coefficient of determination) of the model is 99.53%, indicating that the model fits the experimental data almost completely. Likewise, the computed p value (0.003) and "Lack-of-Fit" value (0.741) of the model also suggest the statistical significance of the regression equation.

The four components (yeast extract, K_2HPO_4, $MgSO_4$ and tryptone) possessing a significant positive effect on 2,3-BD production were selected for further investigation of their optimal values. Corn steep liquor and MOPS were excluded from the media composition due to their significant negative effect in the range 0–20 g/L and lack of influence in the range 0–10 g/L.

3.1.2. Optimization of the Values of the Significant Factors in Media Composition

The significant factors were optimized for 2,3-BD production using the CCD experiment. The randomized design matrix is shown in Table 5.

Table 5. Real values of the four variables (X_1, X_2, X_5 and X_6) and the observed response (2,3-BD concentration after 24 h of fermentation) using CCD for media optimization. X_1, yeast extract (g/L); X_2, tryptone (g/L); X_5, K_2 HPO_4 (g/L); X_6, $MgSO_4$ (g/L).

Run Order	Experimental Values				2,3-BD *
	X_1	X_2	X_5	X_6	(g/L)
1	7.5	7.5	3.0	0.25	17.595
2	12.5	7.5	3.0	0.25	18.990
3	7.5	12.5	3.0	0.25	21.725
4	12.5	12.5	3.0	0.25	20.680
5	7.5	7.5	4.0	0.25	22.245
6	12.5	7.5	4.0	0.25	24.955
7	7.5	12.5	4.0	0.25	24.780
8	12.5	12.5	4.0	0.25	23.130
9	7.5	7.5	3.0	0.35	15.975
10	12.5	7.5	3.0	0.35	20.665
11	7.5	12.5	3.0	0.35	21.555
12	12.5	12.5	3.0	0.35	22.870
13	7.5	7.5	4.0	0.35	20.950
14	12.5	7.5	4.0	0.35	25.305
15	7.5	12.5	4.0	0.35	22.060
16	12.5	12.5	4.0	0.35	25.150
17	5.0	10.0	3.5	0.30	17.300
18	15.0	10.0	3.5	0.30	23.170
19	10.0	5.0	3.5	0.30	20.390
20	10.0	15.0	3.5	0.30	25.140
21	10.0	10.0	2.5	0.30	15.620
22	10.0	10.0	4.5	0.30	23.850
23	10.0	10.0	3.5	0.20	21.780
24	10.0	10.0	3.5	0.40	21.465
25	10.0	10.0	3.5	0.30	24.680
26	10.0	10.0	3.5	0.30	24.595
27	10.0	10.0	3.5	0.30	24.365
28	10.0	10.0	3.5	0.30	23.820
29	10.0	10.0	3.5	0.30	25.125
30	10.0	10.0	3.5	0.30	24.340
31	10.0	10.0	3.5	0.30	25.400

* Mean values of duplicates.

The central points (level 0) of the experimental ranges for each variable in the CCD scheme were determined to correspond to their higher level (+1) in the Plackett–Burman design. As a response, we used the obtained concentration of 2,3-BD after 24 h of fermentation. The analysis of the variance revealed that the response surface regression model can explain 96.59% of the variation in response (R^2 = 0.9659). According to Reddy et al. [31], values of $R^2 > 0.75$ indicate good model fitness. The p value of the model (<0.001) and "Lack-of-Fit" value (0.155) showed that the regression equation is suitable to describe the process of 2,3-BD production. The estimated coded coefficients of variables and their p values are shown in Table 6.

Table 6. Estimated coded coefficients from the regression model for 2,3-BD production in a four-factor CCD.

Source	Effect	Coefficient	*T* Value	*p* Value
Constant		24.618	91.59	<0.001
Yeast extract (X_1)	2.217	1.108	7.63	<0.001
Tryptone (X_2)	2.064	1.032	7.11	<0.001
K_2HPO_4 (X_5)	3.748	1.874	12.91	<0.001
$MgSO_4$ (X_6)	−0.017	−0.008	−0.06	0.955
${X_1}^2$	−1.958	−0.979	−7.36	<0.001
${X_2}^2$	−0.693	−0.347	−2.61	0.019
${X_5}^2$	−2.208	−1.104	−8.30	<0.001
${X_6}^2$	−1.264	−0.632	−4.75	<0.001
X_1X_2	−1.430	−0.715	−4.02	0.001
X_1X_5	0.269	0.134	0.76	0.461
X_1X_6	1.505	0.753	4.23	0.001
X_2X_5	−1.493	−0.746	−4.20	0.001
X_2X_6	0.276	0.138	0.78	0.449
X_5X_6	−0.465	−0.233	−1.31	0.209

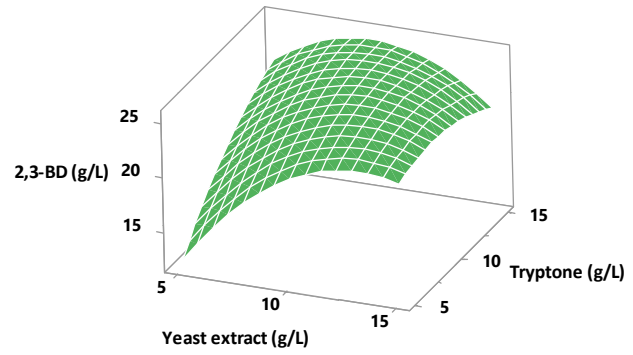
The presented data indicate that the model terms X_1, X_2, X_5, all quadratic terms, and the interaction terms X_1X_2, X_1X_6 and X_2X_5 have a significant effect on 2,3-BD production ($p < 0.05$). Thus, the second-degree polynomial Equation (2) takes the following form (3):

$$Y = 24.62 + 1.11\ X_1 + 1.03\ X_2 + 1.87\ X_5 - 0.98\ X_1^2 - 0.35\ X_2^2 - 1.10\ X_5^2 - 0.63\ X_6^2 - 0.72\ X_1X_2 + 0.75\ X_1X_6 - 0.75\ X_2X_5 \quad (3)$$

The influence of the varied media components on 2,3-BD formation can be seen in three-dimensional response surface graphs, presented in Figure 1. They suggest that yeast extract and K_2HPO_4 are the most influencing parameters. The response optimization procedure predicts a maximum 2,3-BD production (Y) of 25.825 g/L at the following variable settings: yeast extract (X_1), 13.38 g/L; tryptone (X_2), 6.41 g/L; K_2HPO_4 (X_5), 4.20 g/L; $MgSO_4$ (X_6), 0.32 g/L.

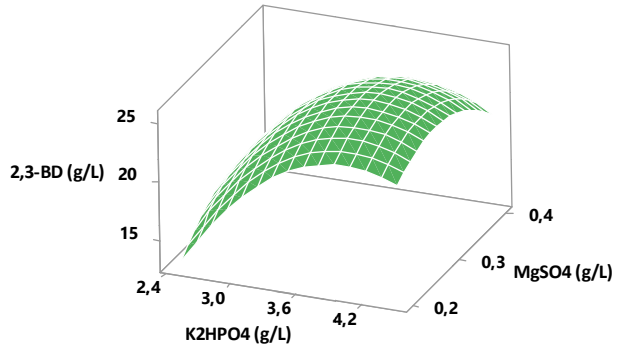
3.1.3. Experimental Verification of the Model

In order to verify the reliability of the model, a set of experiments with predicted optimal parameter values were performed. In these conditions, the observed concentration of 2,3-BD after 24 h of fermentation was 25.74 ± 0.8 g/L (in triplicate trials). This experimentally obtained value is very close to the predicted one of 25.825 g/L. Therefore, the regression equation quite accurately describes the changes in 2,3-BD production as a function of media content. The completely optimized medium had the following composition (g/L): yeast extract, 13.38; tryptone, 6.41; K_2HPO_4, 4.2; $MgSO_4$, 0.32; $(NH_4)_2SO_4$, 1; KH_2PO_4, 3.5; ammonium acetate, 2.5; $CoCl_2 \times 6H_2O$, 0.09; salt solution, 3 mL/L.



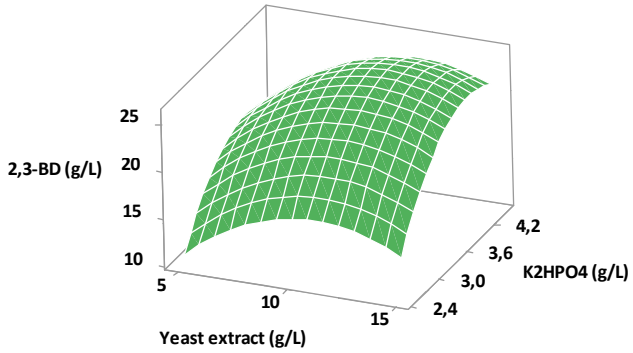
Hold values: K_2HPO_4, 3.5 g/L; $MgSO_4$, 0.3 g/L.

(**a**)



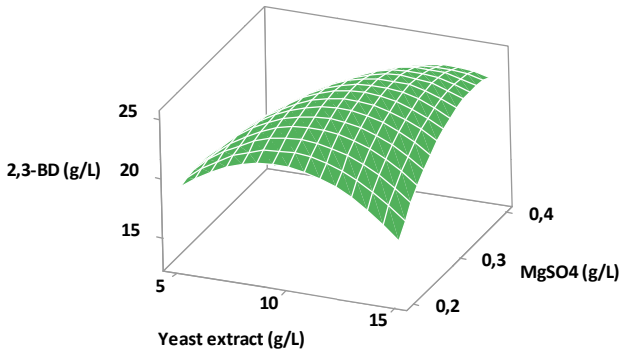
Hold values: yeast extract, 10 g/L; tryptone, 10 g/L.

(**b**)



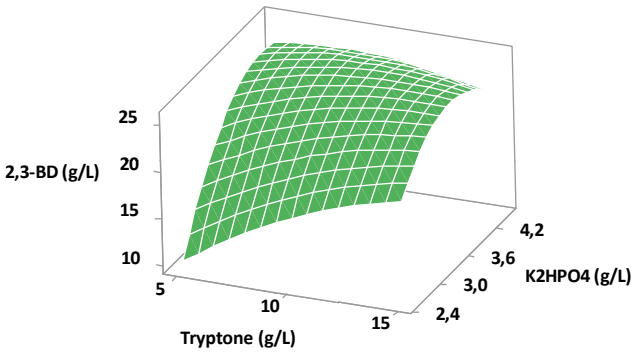
Hold values: tryptone, 10 g/L; $MgSO_4$, 0.3 g/L.

(**c**)



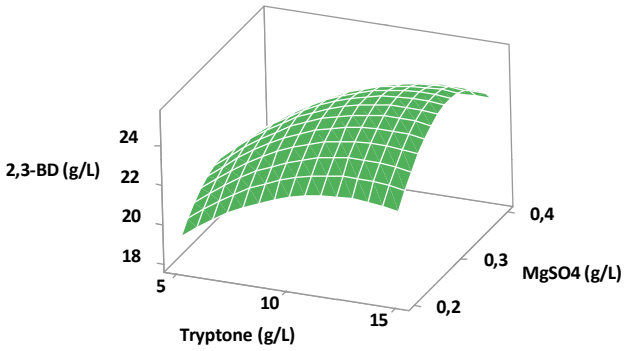
Hold values: tryptone, 10 g/L; K_2HPO_4, 3.5 g/L.

(**d**)



Hold values: yeast extract, 10 g/L; $MgSO_4$, 0.3 g/L.

(**e**)



Hold values: yeast extract, 10 g/L; K_2HPO_4, 3.5 g/L.

(**f**)

Figure 1. Response surface graphs of 2,3-BD production after 24 h of fermentation: (**a**) influence of yeast extract and tryptone; (**b**) influence of K_2HPO_4 and $MgSO_4$; (**c**) influence of yeast extract and K_2HPO_4; (**d**) influence of yeast extract and $MgSO_4$; (**e**) influence of tryptone and K_2HPO_4; (**f**) influence of tryptone and $MgSO_4$.

3.2. Process Parameter Optimization

3.2.1. Optimization of the Process Parameters

The optimization of the process parameters was carried out in series of batch experiments performed with an optimized medium containing 200 g/L of glucose. Three parameters were subjected to optimization: temperature, pH and aeration flow. The response surface methodology was applied, following the scheme of the CCD experiment. The maximum concentration of 2,3-BD obtained during the process was chosen for the response. The design matrix and the observed responses are presented in Table 7.

Table 7. Real values of the three variables (X_1, X_2 and X_3) and the observed response (2,3-BD concentration) using CCD for process parameters optimization. X_1, temperature (°C); X_2, pH; X_3, aeration rate (airflow supply in vvm).

Run Order	Factor Levels in Real Values			2,3-BD [a]	2,3-BD [a]	$Y_{2,3\text{-BD}}$ [a]
(Randomized)	X_1	X_2	X_3	(g/L)	(g/L/h)	(g/g) [b]
1	37	5.50	3.0	76.90	1.71	0.41
2	31	6.50	1.0	58.50	0.56	0.34
3	31	5.50	3.0	80.20	1.64	0.48
4	37	5.50	1.0	68.42	1.51	0.38
5	39.0454	6.0	2.0	71.81	2.08	0.39
6	34	6.0	3.6818	90.86	1.34	0.49
7	34	6.8409	2.0	58.10	1.05	0.32
8	34	6.0	2.0	75.45	1.45	0.43
9	34	6.0	2.0	76.79	1.48	0.44
10	31	5.50	1.0	73.01	0.71	0.41
11	34	6.0	0.3182	72.70	1.36	0.40
12	34	5.1591	2.0	66.57	0.67	0.32
13	37	6.50	1.0	75.57	1.23	0.43
14	37	6.50	3.0	84.53	1.61	0.48
15	28.9546	6.0	2.0	55.72	0.55	0.47
16	31	6.50	3.0	59.15	0.78	0.39
17	34	6.0	2.0	76.08	1.46	0.43
18	34	6.0	2.0	76.81	1.49	0.44
19	34	6.0	2.0	76.10	1.46	0.43
20	34	6.0	2.0	75.49	1.45	0.43

[a] Mean values of duplicates; [b] gram produced 2,3-BD per gram consumed glucose.

When 2,3-BD concentration (Y) was used as a response, the response surface regression equation has a coefficient of determination $R^2 = 0.9659$, which means that this model fits the experimental data extremely well. The obtained results from the statistical analysis are presented in Table 8.

Table 8. Estimated coded coefficients from the regression model for 2,3-BD production in a three-factor CCD.

Source	Effect	Coefficient	*T* Value	*p* Value
Constant		76.018	78.01	<0.001
t °C (X_1)	9.024	4.512	6.98	<0.001
pH (X_2)	−5.129	−2.565	−3.97	0.003
Aeration rate (vvm) (X_3)	8.175	4.087	6.32	<0.001
X_1^2	−7.404	−3.702	−5.88	<0.001
X_2^2	−8.416	−4.208	−6.69	0.001
X_3^2	5.334	2.667	4.24	0.002
X_1X_2	12.585	6.292	7.45	<0.001
X_1X_3	2.400	1.200	1.42	0.186
X_2X_3	−1.515	−0.757	−0.90	0.391

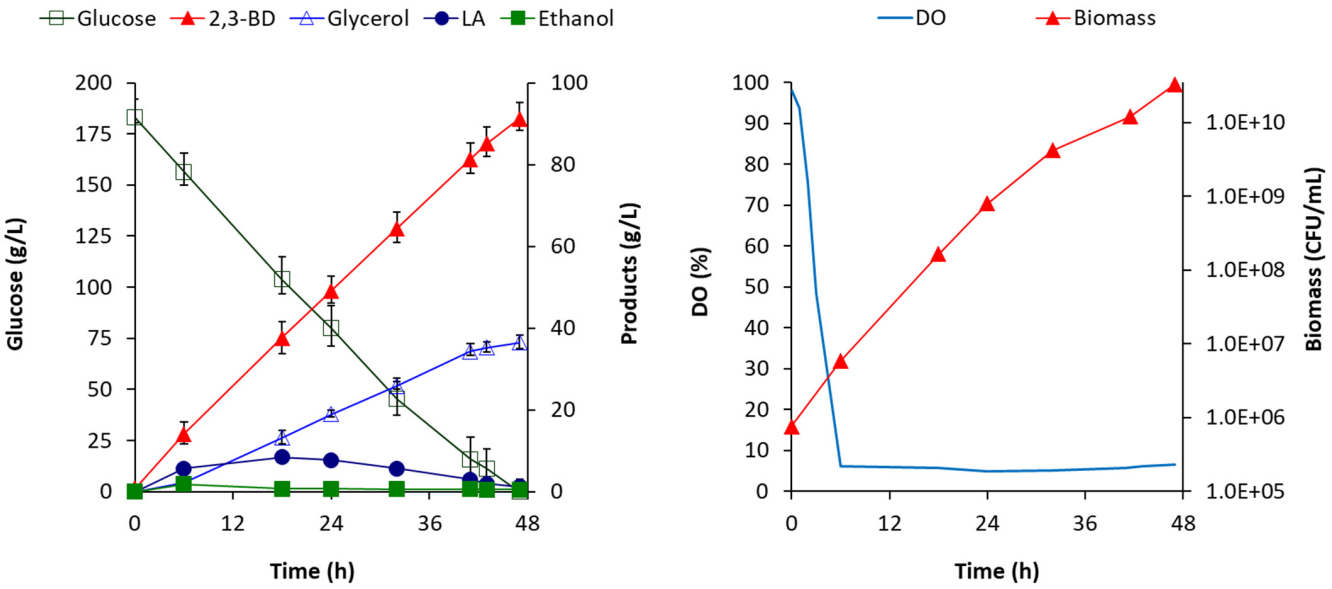
Interaction terms X_1X_3 and X_2X_3 are not statistically significant ($p > 0.05$) and, after their exclusion, the second-degree polynomial equation takes the following form:

$$Y = 76.02 + 4.51\ X_1 - 2.57\ X_2 + 4.09\ X_3 - 3.70\ X_1^2 - 4.21\ X_2^2 + 2.67\ X_3^2 + 6.30\ X_1X_2 \quad (4)$$

Response optimization predicts the maximum value of 93.77 (g/L) for Y (2,3-BD maximum concentration) at the following conditions: temperature (X_1), 37.82 °C; pH (X_2), 6.23; aeration flow (X_3), 3.68 vvm.

3.2.2. Experimental Verification of the Model

For the validation of the model (regression Equation (4)), we performed batch fermentation by *B. licheniformis* 24 in optimized media and process parameters. The results (mean values of three separate experiments) are presented in Figure 2. The highest obtained concentration of 2,3-BD was 91.23 ± 2.9 g/L. This value is higher than all observed responses in the design matrix (Table 7) and is close to the predicted 93.77 g/L. Thus, the verification of the model was successful. Similarly, the overall achieved productivity of 1.94 g/L/h 2.3-BD and 2,3-BD yield of 0.488 g/g (98% of the theoretical maximum of 0.5), also shows that the whole process is almost completely optimized for 2,3-BD production.



(**a**) (**b**)

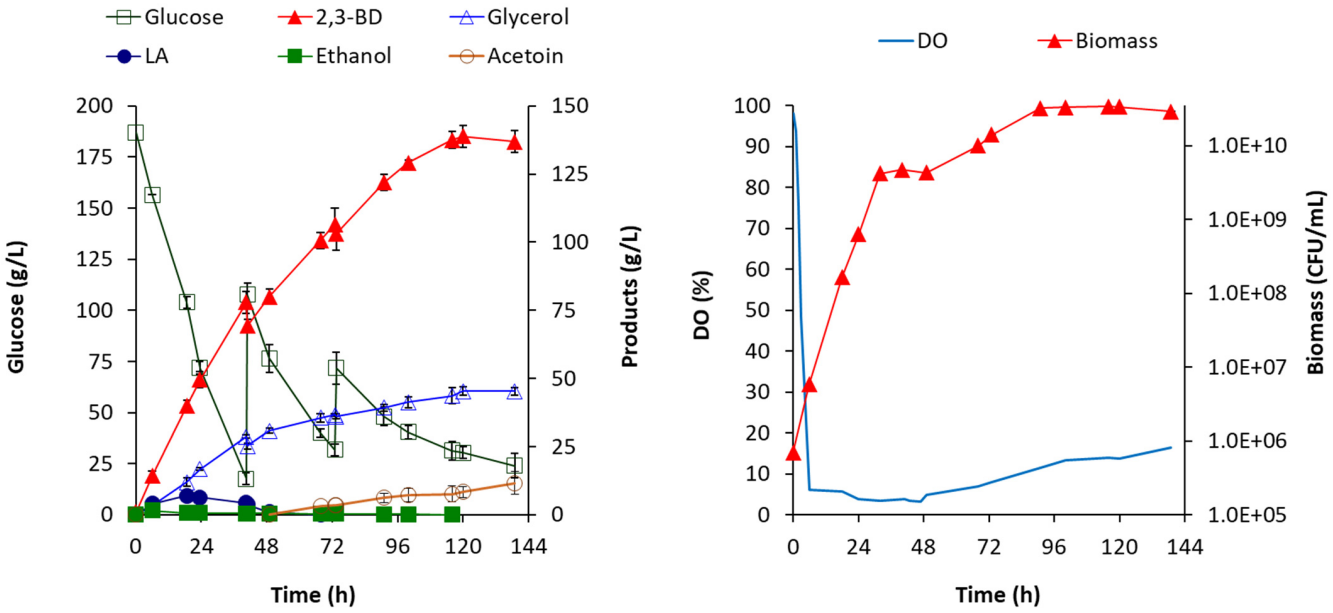
Figure 2. Batch fermentation of 200 g/L of glucose by *B. licheniformis* 24 in optimized medium and process parameters. (**a**) Glucose consumption and products accumulation (triplicates). (**b**) Time course of the dissolved oxygen (DO) and biomass formation.

In these conditions, the main by-product of the fermentation was glycerol—35.20 g/L—as both glycerol and 2,3-BD increased their titers continuously during the process. The other soluble metabolites were accumulated in small quantities at the end of fermentation, including lactic acid, 1.2 g/L, and acetoin and ethanol in amounts less than 1 g/L. In the course of the fermentation, lactic acid achieved the highest concentration at the 18th h (8.37 g/L) and then slowly decreased. A similar profile was observed for ethanol; its accumulation reached a maximum of 1.8 g/L at the 6th hour, then decreased to 0.5 g/L at the end of the process (Figure 2a). Acetoin was formed only in the first few hours. Then, with the decrease in dissolved oxygen in the broth (Figure 2b), acetoin synthesis ceased until complete depletion of the carbon source. Glucose was entirely consumed after 47 h of fermentation (Figure 2a), whereas the D-isomer of 2,3-BD was sharply converted to acetoin (data not shown).

3.3. Fed-Batch Process in Optimized Conditions

To reveal the maximum tolerance of *B. licheniformis* 24 to 2,3-BD, which determines its maximum capabilities as a producer, a fed-batch process under the described optimized conditions was performed. The highest achieved concentration of 2,3-BD was 138.8 g/L, with 2,3-BD productivity of 1.16 g/L/h and yield of 0.478 g/g. High product inhibition was observed at concentrations above 100 g/L, leading to a decrease in glucose consumption

rate. Glucose fermentation ceases completely after the 140th hour. The main fermentation by-product was glycerol, reaching its maximum of 45.38 g/L after 120 h of fermentation. Lactic acid and ethanol were accumulated temporarily in the broth and were in insignificant concentrations at the end of the process (Figure 3a). Acetoin was accumulated after the 70th hour of fermentation, when the DO concentration slightly increased from 6 to 14% at the end of the process (Figure 3b).



(**a**) (**b**)

Figure 3. Fed-batch fermentation of glucose by *B. licheniformis* 24 in optimized medium and process parameters. (**a**) Glucose consumption and products accumulation (mean values of duplicates). (**b**) Time course of the dissolved oxygen (DO) and biomass formation.

4. Discussion

For more than a decade, the fermentation of 2,3-BD has been the subject of continuous laboratory research, including the use of different types of natural and modified producers, a variety of substrates, innovative methods to control the process and new, promising ways of product extraction [5,33]. At present, however, this process has never been successfully commercialized. The reasons lie in the high price of the substrates, the pathogenic nature of the best producers and the expensive extraction of the product from the fermentation mixture (further discouraged by significant losses).

B. licheniformis 24 has several advantages as a producer of 2,3-BD; it is non-pathogenic, has a broad substrate spectrum, possesses a high consumption rate and high yield, regardless of the substrate [18]. The present study establishes the strain as a super-producer of 2,3-BD with a future industrial application by employing complex optimization via series of Plackett–Burman design and CCD experiments.

The application of the Plackett–Burman design showed that among the components of the nutrient medium with significant influence, the yeast extract, K_2HPO_4, $MgSO_4$ and tryptone had the most pronounced positive effect on 2,3-BD production. The addition of corn steep liquor and MOPS had a negative impact on 2,3-BD production and these two compounds were excluded from the media. This observation is in agreement with Song et al. [16], who revealed that corn steep liquor is less suitable than yeast extract as a nitrogen source for *B. licheniformis*. Unlike *P. polymyxa* [32], *B. licheniformis* does not need MOPS, which makes the nutrient medium cheaper. The establishment of the optimal values of the components with the greatest impact on 2,3-BD production was achieved by

CCD experiments. The computed optimal values were: yeast extract, 13.38 g/L; K_2HPO_4, 4.20 g/L; tryptone, 6.41 g/L; $MgSO_4$, 0.32 g/L. In this experiment, as revealed by the screening design, yeast extract and K_2HPO_4 had the highest impact on 2,3-BD production. However, $MgSO_4$ did not affect the response in the second experimental range. The response surface graph revealed that the yeast extract is the preferred nitrogen source, but a total concentration of yeast extract and tryptone above 20 g/L led to a decrease in 2,3-BD formation (Figure 1a).

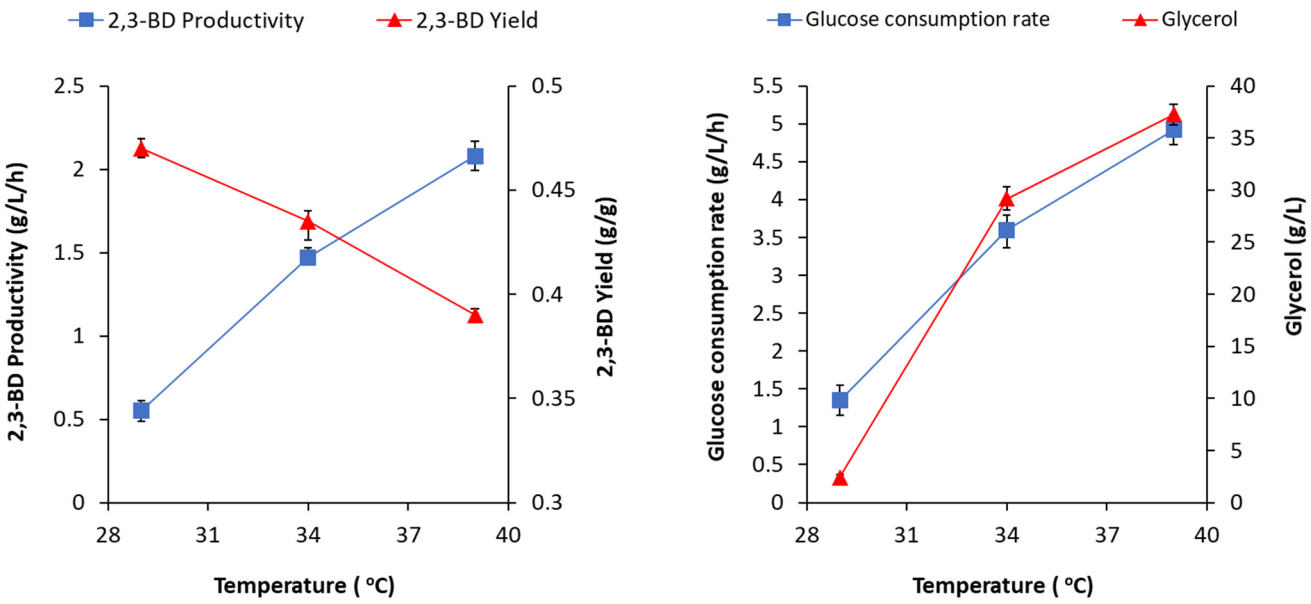
A specific characteristic feature of *B. licheniformis* 24 is the production of glycerol as the main by-product of glucose fermentation. This finding distinguishes *B. licheniformis* 24 from most of the 2,3-BD-producing bacilli. Importantly, due to glycerol formation, the acidity of the medium changes slightly during the process and the fermentation could be performed without any pH control. Unlike other strains of the species, *B. licheniformis* 24 does not produce formic acid at all. The accumulation of the toxic formic acid [33] remains a serious problem against the industrial application of *B. licheniformis*, as its amount could reach between 29.1 g/L and 42 g/L at the end of the process [16,19].

Therefore, an important achievement of the present study is the clarification of the process parameters that are suitable for maximum production of 2,3-BD by a strain, producing glycerol as a main by-product. To obtain the highest 2,3-BD concentration, according to maximized regression equation (4), the following process parameters were determined: temperature, 37.82 °C; pH, 6.23; airflow rate, 3.68 vvm. The temperature was found as the parameter with the highest impact, but the estimated optimal values were quite different for 2,3-BD concentration, 2,3-BD productivity and 2,3-BD yield. Indeed, the higher temperatures accelerate glucose consumption, which results in higher 2,3-BD productivity. For example, at a constant pH of 6.0 and aeration rate of 2 vvm, an increase in temperature from 29 °C to 39 °C raised 2,3-BD productivity from 0.55 to 2.08 g/L/h. However, due to higher glycerol formation, 2,3-BD yield decreased from 0.47 to 0.39 g/g (Figure 4a). With a glucose consumption rate increase (from 1.35 to 4.92 g/L/h), glycerol accumulation rose from 2.42 to 37.25 g/L (Figure 4b); therefore, at higher temperatures and low aeration, glycerol production appears to be favored.

Conversely, the higher aeration levels favored 2,3-BD concentration increase and 2,3-BD yield and did not possess any significant effect on 2,3-BD productivity (Figure 5a). Regarding glucose consumption and glycerol accumulation, the increase of aeration definitely affected the process in the opposite way with respect to the temperature rise (Figure 5b).

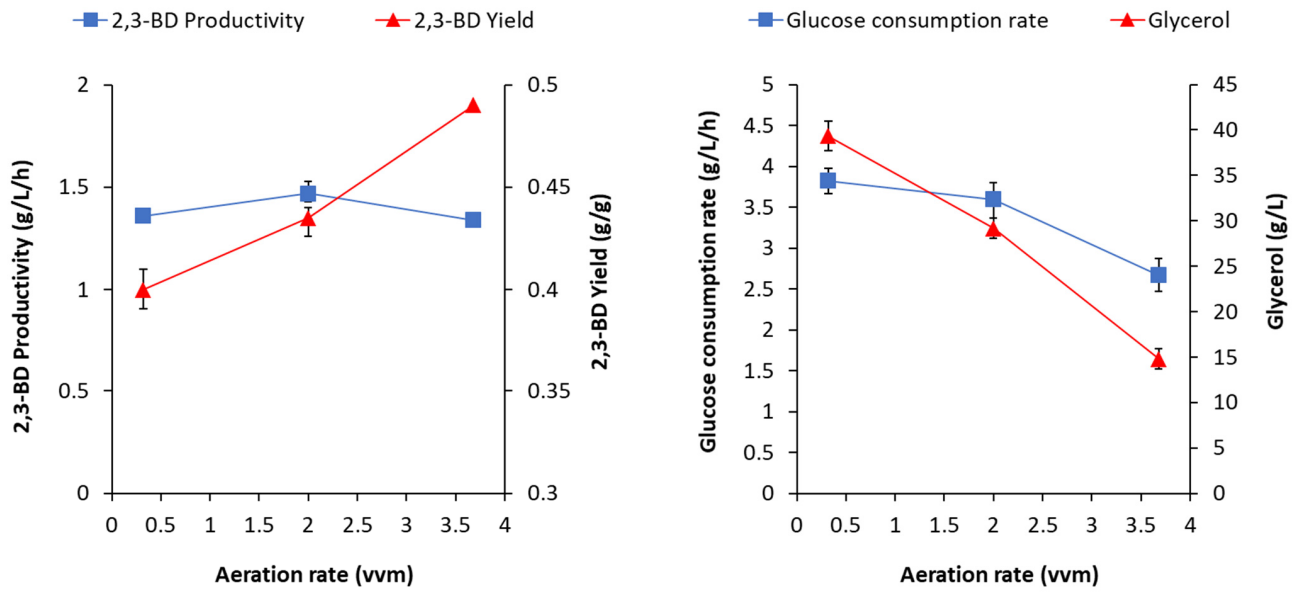
According to Li et al. [19] and Rebecchi et al. [34], acetoin synthesis is strongly influenced by oxygen availability. Under fully aerobic conditions, the oxygen is the electron acceptor for NAD+ regeneration and acetoin reduction to 2,3-BD does not occur. The observation that acetoin production is greater at highly aerobic conditions determined the development of complex two-stage aeration regimes in the processes for obtaining 2,3-BD by *B. licheniformis* [16,19,34,35]. On the contrary, when the oxygen supply is insufficient, the appropriate regeneration of co-factors such as NAD+ cannot be achieved by electron transfer onto oxygen, but, alternatively, NAD+ regeneration could be achieved by the reduction of dihydroxyacetone phosphate to glycerol-3-phosphate, which is then dephosphorylated yielding glycerol.

It turns out that *B. licheniformis* strain 24 is an extremely aerobic 2,3-BD producer and is capable to consume huge oxygen amounts. As it was shown in Figure 2b, the retention of dissolved oxygen in the medium remained constantly low, after the 6th hour, till the end of fermentation. This high oxygen consumption prevented acetoin formation even at the highest tested aeration levels. However, glycerol synthesis cannot be avoided even at high aeration levels.



(**a**) (**b**)

Figure 4. Influence of temperature on the fermentation of 200 g/L glucose by *B. licheniformis* 24 on (**a**) 2,3-BD productivity and 2,3-BD yield and (**b**) glucose consumption rate and glycerol formation. Fermentations were carried out at a pH of 6.00 and an aeration rate of 2 vvm. Mean values of at least two separate experiments are presented.



(**a**) (**b**)

Figure 5. Influence of aeration rate on the fermentation of 200 g/L glucose by *B. licheniformis* 24 on (**a**) 2,3-BD productivity and 2,3-BD yield and (**b**) glucose consumption rate and glycerol formation. Fermentations were carried out at a pH of 6.00 and temperature of 34 °C. Presented are mean values of at least two separate experiments.

pH changes affect 2,3-BD production to a lesser extent. At lower pH (5.16), acetoin appeared as the main by-product, reaching concentrations of 28.64 g/L. Acetoin is accumulated at a low glucose consumption rate, in this case, at conditions combining lower pH (≤5.5) and lower temperature (≤31 °C). This positive effect of the high consumption rates

of sugars (glucose, cellobiose and mannose) on 2,3-BD production at the expense of acetoin was always observed in *B. licheniformis* 24 [18].

The other significant by-product, glycerol, was not formed at lower pH (5.16), but increased at a pH of 6.0. However, Raspoet et al. [27] showed that the effect of pH on glycerol produced is strain specific. Expectedly, lactic acid is accumulated in the highest amounts, 14.11 g/L, in the process with a pH of 6.84 (Figure 6).

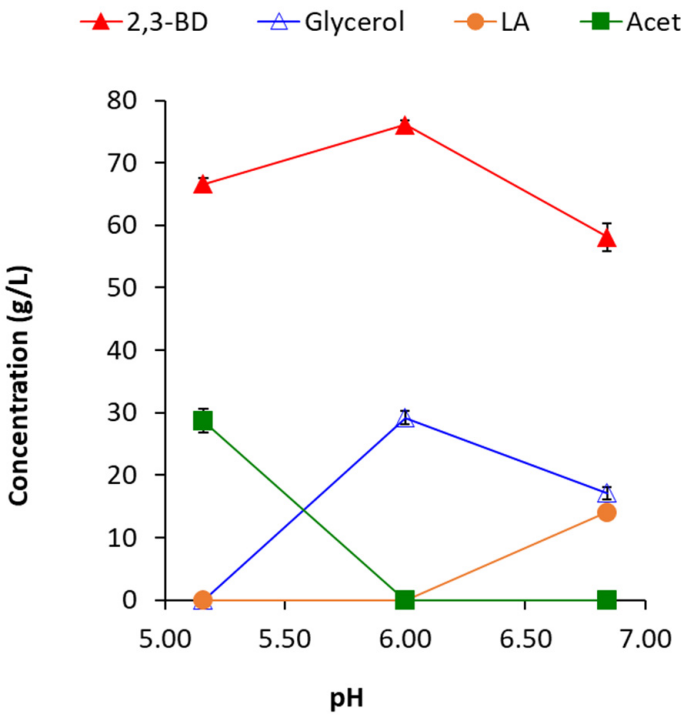
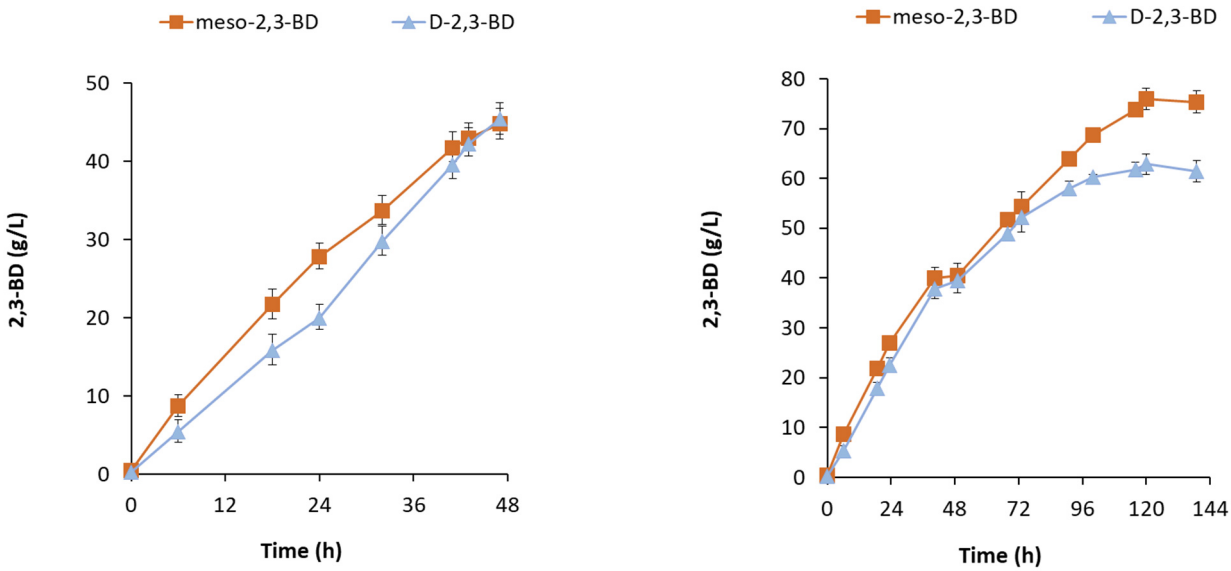


Figure 6. Influence of pH on product formation in the fermentation of 200 g/L of glucose by *B. licheniformis* 24. Fermentations were carried out at t = 34 °C and aeration rate of 2 vvm. Mean values of at least two separate experiments are presented.

B. licheniformis 24 produces 2,3-BD in two isomeric forms, meso-2,3-BD and D-2,3-BD, in a ratio of 1.6:1–1:1. It has not been observed that the ratio depends on the substrate used, but it seems to depend on the total concentration of 2,3-BD produced. For example, at the beginning of each fermentation, the meso-form slightly prevailed, followed by equalization of the ratio at a total concentration of 80–90 g/L of 2,3-BD (Figure 7a). This occurrence was observed in all batch processes. However, in the fed-batch process with optimized parameters, after the 70th h, the ratio changed again in favor of the meso-form and, by the end of the process, from 1.05:1, it became 1.23:1 (Figure 7b). A possible explanation is the production of acetoin during this period (Figure 3a), which was not observed in any of the batch processes when the carbon source was available. On the other hand, upon the complete depletion of the substrate, under the action of butanediol dehydrogenase BDH, the D-form was rapidly converted to acetoin, while the meso-form decreased slowly and was converted to acetoin only partially.

The effect of the presented complex optimization of medium composition and process parameters is the most obvious when the batch processes are compared before and after optimizations. The application of optimized parameters increased the maximal concentration of 2,3-BD by 28.9%, from 70.8 g/L, obtained without optimization [18], to 91.23 g/L. The yield increased by 6.8%, from 0.457 g/g to 0.488 g/g substrate. Notably, the productivity increased more than 5-fold, from 0.38 g/L/h to 1.94 g/L/h.



(**a**) (**b**)

Figure 7. Production of meso- and D-2,3-BD by *Bacillus licheniformis* 24: (**a**) in batch fermentation of 200 g/L of glucose and (**b**) in fed-batch glucose fermentation.

After performing all the steps of optimization and validation, we conducted a fed-batch process, which aimed to reveal the full potential, or the upper limit, of the strain as a producer of 2,3-BD. The obtained 2,3-BD concentration of 138.8 ± 4.3 g/L, the productivity of 1.16 g/L/h and the yield, close to the theoretical (0.479 g/g), ranks *B. licheniformis* 24 among the best non-pathogenic producers. Our results are very similar to the results presented by Jurchescu et al. [20] and obtained by the use of *B. licheniformis* DSM 8785: 2,3-BD concentration of 144.7 g/L and productivity of 1.14 g/L/h. A comparison of these results reveals that these values are probably the highest that can be obtained using the species *B. licheniformis* and comprise its maximum capabilities as a 2,3-BD producer. Compared to the best pathogenic producers as *Klebsiella pneumoniae* or *K. oxytoca*, it is obvious that they have comparable tolerance to 2,3-BD and produce relatively equal quantities of 2,3-BD, reaching 150 g/L. However, *Klebsiella* strains achieve this concentration up to four times faster [36–38]. Therefore, the low productivity continues to be an obstacle for industrial application of even the most successful non-pathogenic 2,3-BD producers of the species *B. licheniformis*.

5. Conclusions

Non-pathogenic producers of 2,3-BD are preferred for industrial applications. Here we present a complex optimization of the nutrient medium and process parameters for the production of 2,3-BD with the purpose to disclose and validate the suitability of the strain *B. licheniformis* 24 for commercialization. All optimization steps were carried out in course of batch processes, since this performance enables the optimization of many more factors, compared to fed-batch mode. The high final titer of 2,3-BD and the high yield from glucose allow the evaluation of the strain as one of the best producers of 2,3-BD. Although very promising, the focus of future research with this strain should be on improving its 2,3-BD productivity, which can be significantly increased, but only at the expense of reducing the final 2,3-BD concentration. Therefore, much effort is still needed to address this issue, which is a common problem for all non-pathogenic producers of 2,3-BD.

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