Bulgarian Academy of Sciences

Institute of Chemical Engineering

Lidia Plamenova Tsigoriyna

PRODUCING 2,3-BUTANEDIOL FROM INULIN THROUGH A MODIFIED NON-PATHOGENIC PRODUCER

S U M M A R Y

of a dissertation for an educational and scientific degree "PhD"

Field of higher education: 4. Natural sciences, mathematics and informatics

Professional field: 4.2. Chemical Sciences

PhD program: Processes and apparatus in chemical and biochemical technology

Scientific consultant: prof. D.Sc. Kaloyan Petrov

Sofia, 2023

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Topic: Production of 2,3-butanediol from inulin by a modified non-pathogenic producer

Author: Lidia Plamenova Tsigoriyna

Materials are available in the office of the Institute of Engineering Chemistry - BAS.

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Used abbreviation

2,3-BD	2,3-Butanediol
BDH	Butanediol dehydrogenase
CCD	Central composite design
HPLC	High Performance Liquid Chromatography
MOPS	3-morpholino propane sulfonic acid
RI detector	Refractive index detector
rpm	revolutions per minute
UV detector	Ultraviolet detector
WT	Wild type
DO	Dissolved oxygen
OD	Optical density
DNS reagent	3,5-dinitorsalycilic acid

1. Introduction

Due to the depletion of fossil natural resources, the ever-increasing demand for energy as well as the increased concern about carbon emissions, attempts to change petroleum-based chemical production into environmentally friendly one is currently of strategic importance [1, 2, 3]. 2,3-butanediol | (2,3-BD) is a compound with versatile applications, which increasing demand in recent decades has led to a continuous increase in the volume of its production. Like many other valuable chemicals, 2,3-BD can be produced chemically and biologically, through the bacterial metabolism.

Compared to chemical techniques, the microbiological production of 2,3-BD has several advantages, including relatively high efficiency and the environmental friendliness of the method. Its effective production as a metabolite has been the subject of intensive scientific research in recent decades, but its chemical production from petroleum products remains more cost-effective. The main reasons are the high cost of the used substrates, the pathogenic nature of the best producers and energy-intensive and incomplete extraction from the fermentation medium. The cost of biotechnologically produced 2,3-BD should be under 1.2 $/kg for production to be commercialized. To achieve this result, main research is targeted at finding cheaper carbon sources, developing new extraction methods, as well as finding efficient producers of a non-pathogenic nature.

The aim of the present work is to develop a process for production of 2,3-BD that will help solve two of these problems. A non-pathogenic producer, *Bacillus licheniformis* 24, and a cheap carbon source – inulin from chicory flour were chosen.

2. Aim and Objectives

2.1. Aim

Development of biotechnological process for microbial production of 2,3-BD from inulin by genetically modified strain *Bacillus licheniformis* 24.

2.2. Objectives

1. Optimization of the nutrient medium for production of 2,3-BD;
2. Optimization of the process parameters for production of 2,3-BD;
3. Detection the ability of the wild strain *B. licheniformis 24* to convert inulin into 2,3-BD;
4. Heterologous expression of Inulinase Gene (EC 3.2.1.80) from *Lacticaseibacillus paracasei* B41 (DSM 23505) into *B. licheniformis* 24;
5. Determination of the maximum ability of the modified strain *B. licheniformis* 24 to produce 2,3-BD from inulin-containing chicory flour.

3. Material and methods

Bacterial Strain

The strain *Bacillus licheniformis* 24 was previously isolated from a soil sample, from the banks of the Yantra river (Bulgaria), and is stored in the microbiological collection of the Institute of Microbiology at the BAS. It is stored as 24-hour cultures frozen with added glycerol at a ratio of 1:1 in eppendorf tubes with a volume of 2 mL in a freezer at -80 °C.

The isolate was identified by 16S rDNA sequencing (NCBI GenBank accession no. MK461938).

Media Composition

The medium was originally developed for *P. polymyxa* by Okonkwo et al. [4], modified by Petrova et al. [5], with the following contents:

Glucose 20 g/L; Yeast extract 5 g/L; Tryptone 5 g/L; $(NH_4)_2SO_4$ 3 g/L; KH_2PO_4 3.5 g/L; K_2HPO_4 2.75 g/L; $MgSO_4$ 0.2 g/L; NH_4CH_3COO 1.5 g/L; $CoCl_2$ x 6 H_2O 0.09 g/L; salt solution* 3 mL/L

* Salt solution (g/L): $FeSO_4$ 0.4; H_3BO_3 0.8; $CuSO_4$*$4H_2O$ 0.04; $NaMoO_4$*$2H_2O$ 0.04; $MnCl_2$ 5; $ZnSO_4$ 0.1; $Co(NO_3)_2$*$6H_2O$ 0.8; $CaCl_2$*$2H_2O$ 1; Biotin 0.01.

The used inulin was from insoluble chicory flour Frutafit® HD (Sensus B.V., Roosendaal, The Netherlands) containing a minimum of 90% inulin with molecules with a degree of polymerization DP 8 – DP 13, and up to 10% sugars a mixture of sucrose, fructose and glucose.

Cultivation Conditions

Medium optimization experiments using Plackett–Burman and central composite (CCD) designs were performed in 500 mL Erlenmeyer flasks with 100 mL medium

containing 100 g/L glucose at 37 °C and 200 rpm on a rotary shaker (New Brunswick, San Diego, California, USA).

As an inoculum was used a overnight culture grown in 500 mL flasks with 50 mL medium containing 20 g/L glucose, at 37 °C, 200 rpm, on a rotary shaker. The inoculum was cultivated to OD = 2,400 (measured at wavelength $\lambda = 600$ nm), the amount of inoculum used was 2% for Plackett–Burman and 1% for the central composite design experiments.

Batch fermentations for the optimization of the process parameters, as well as those to validate the optimal values, were carried out on a 1 L fermenter (Biostat® A plus, Sartorius Stedim Biotech, Gottingen, Germany) additionally equipped with bumpers to ensure more aerobic conditions. An additional air pump and rotameter were also used to provide higher levels of airflow supply. The pH value was controlled by adding 6M NaOH or 5M HCl.

Batch cultivation was carried out using the already optimized culture medium and 10% inoculum (grown to OD_{600} = 2,400). In fed-batch fermentations, the additional amount of substrate was added as portions of filter-sterilized glucose stock.

Analytical Methods

Cell growth

Biomass concentration was determined by measuring the extinction of the sample on a spectrophotometer (VWR® UV-1600 PC) at a wavelength of $\lambda = 600$ nm, up to OD = 2,400, corresponding to the beginning of the stationary phase.

Determination of the concentration of metabolites

High performance liquid chromatography (HPLC) was used for qualitative and quantitative determination of substrate

and product concentrations. The amounts of glucose, lactic acid, glycerol, acetoin, 2,3-BD and ethanol were quantified using a YL Instrument 9300 HPLC system (YL Instrument Co., Ltd., An-yang, South Korea). After pre-filtering the fermentation samples with a bacterial filter, the separation of the sample components was carried out using an HPX-87H column (BioRad Laboratories, California, USA) at temperature 65 °C and mobile phase (eluent) 5 mmoL H_2SO_4 with flow rate 0.6 mL/min.

The detection of the components was performed based on their specific refractometric index on an RI detector (Perkin-Elmer, series 10, Waltham, Massachusetts, USA). The quantification of lactic acid and acetoin was further confirmed by a UV detector (YL9120UV/Vis detector) at a wavelength of 210 and 190 nm, respectively.

The statistical analyses

Statistical analysis of the experimental results was carried out by creating regressions models using the Minitab 17 software package (Minitab Inc., Pennsylvania, USA) (www.minitab.com).

Bacterial strains, media and culture conditions for gene cloning

Stain *Lc. paracasei* B41 e was isolated from boza [6] and has been deposited in the German Collection for Microorganisms and Cell Cultures GmbH (DSMZ) under accession number DSM 23505. The recombinant JET1.2/blunte construct used was previously obtained by Petrova et al. [7]. Both genes (*Inu* and *Inu-tr*) were cloned into pBE-S vector digested with *Xho*I and *Xba*I restriction enzymes.

Recombinant constructs were transformed into *E. coli* STELLAR™ competent cells purchased from Clontech Laboratories Inc. (Takara Bio Company, Mountain View, CA,

USA). Plasmid DNA from *E. coli* was obtained with the Plasmid Miniprep DNA Purification Kit (EURx®, Gdansk, Poland).

E. coli and *B. licheniformis* 24 strains were cultured on LB medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl), with 1.5% agar and kanamycin (AppliChem GmbH, Darmstadt, Germany) at a concentration of 50 μg/mL in petri dishes. *Lc. paracasei* B41 was cultured in MRS medium (Merck, Darmstadt, Germany). The strains were stored in tubes on agar at 4 °C or frozen at −80 °C, with glycerol (20% v/v).

The batch cultivation of *B. licheniformis* 24 was carried out in 500 mL flasks containing 100 mL medium. As a substrate was used chicory flour containing 90% insoluble inulin and up to 10% sugars (Sensus B.V., Roosendaal, The Netherlands). Flasks were incubated on a rotary shaker at 37 °C and 140 rpm.

Cloning of the *Inu* gene into pBE-S *E coli* / *Bacillus* spp. shuttle vector

PCR amplification was performed in a QB-96 Satellite Gradient Thermal Cycler (LKB Vertriebs GmbH, Vienna, Austria). The primers used as well as the optimal annealing temperatures are listed in Table 1.

PCR reactions consisted of 15 ng DNA template, 0.4 μM primers, Premix Ex Taq Hot Start Version (Clontech Laboratories, Inc., A Takara Bio Company, Mountain View, CA, USA) and sterile water to a final volume of 25 μL.

Between the initial denaturation of 3 min and 30 sec at 98 °C and final extension for 5 min at 72 °C, the following temperature profile was used for 38 cycles: 10 sec denaturation at 98 °C, 45 sec heating at 57 °C or 60 °C, and 2.5 min extension at 72 °C.

Table 1. Primer for amplification of the inulinase gene from *Lc. paracasei* DSM 23505 with NCBI GenBank number KP663715. Inserted sites for endonuclease enzymes are underlined. Entered stop codons are shown in bold and italics.

Primer	Sequence (5′–3′)	Position in Gene	T (°C)	Purpose
InuF	atggatgaaaagaaacattac aagatg	1-27	60	PCR
InuR	ttagactcgcttcacccgcctc	3617-3645	60	PCR
InuF-tr_Xho	gtcaatctcgagatggatgaaa agaaacattacaagatgtat	1-30	60	Cloning
InuR-tr_*Xba*	ggtcattctaga***ctatta***gatag ttaagtcgctgatctttgtcgtg cc	2163-2193	60	Cloning
InuF_Nhe	gatcagctagcatggatgaaa agaaacattacaagat	1-26	57	Cloning
InuR_Nhe	cagtagctagcttagactcgct tcacccgcctctttaacc	3616-3645	57	Cloning

Plasmids and DNA fragments were visualized using gel electrophoresis on 1% agarose (AlfaAesar, Kandel, Germany) in TAE buffer (40 mM Tris-base, 20 mM acetic acid, 1 mM EDTA) stained with SimplySafeTM fluorescent dye (EURx®, Gdansk, Poland).

Transformation and clone selection

Transformation of *E. coli* STELLAR™ competent cells was performed following protocol PT5055-2 from the manufacturer's instructions.

Transformation of *B. licheniformis* 24 was performed by electroporation [8]. A overnight culture in standard LB medium was diluted 16 times in LB medium with 0.5 M sorbitol in a 500 mL Erlenmeyer flask and cultured to OD_{600} of 0.9. The flask was chilled on ice for 10 min and the bacteria were washed four times with ice-cold electroporation medium

(0.5 M sorbitol, 0.5 M mannitol, 10% glycerol). After a final centrifugation (3350 g/10 min/4 °C), competent cells were resuspended in 625 µL of electroporation medium. Aliquots of 60 µL were used for electroporation in ice-cold GenePulser cuvettes with 0.1 cm electrode spacing on a MicroPulser electroporator (BioRad Laboratories, Hercules, CA, USA). A 2.1 kV pulse was applied for 4–5 ms, after which 1 mL recovery medium (0.5 M sorbitol and 0.38 M mannitol in LB) was added as quickly as possible. The culture was transferred to glass tubes from 15 mL, incubated for 3 h, spread on petri dishes with LB-agar and kanamycin and left overnight at 37 °C. Competent cells were stored at –70 °C and reused several times with minimal loss of electroporation efficiency.

Clone selection was performed on agar medium with 1% inulin. Iodine staining and water destaining were used to visualize the areas of hydrolysis after 48 h of culture.

Inulinase activity

The inulinase activity of intact cells twice washed with water and cell-free culture supernatants was investigated. The samples were suitably diluted in phosphor citratebuffer (0.16 M Na_2HPO_4, 0.02 M citrate, pH 5.0, containing 1% inulin) and incubated at 50 ◦C for 60 min.

The amount of reducing sugars was estimated with DNS reagent (2.18% 3,5-dinitorsalycilic acid in 0.4 M NaOH c 30% (w/v) Rochelle salt). Absorbance at 540 nm was measured on a Helios Omega UV-VIS spectrophotometer (Thermo Scientific, Waltham, MA, USA) with a separate control for each reaction, and a fructose standard was used to estimate the increased amount of reducing sugars. One unit of enzyme activity was defined as the amount of enzyme that liberates 1 µmol of fructose per minute.

4. Results

4.1. Production of 2,3-BD from glucose

Optimization of nutrient medium components

To establish the components of the nutrient medium with a significant impact on the process of 2,3-BD formation, a first-order experimental design was applied, and the individual experiments were conducted according to the Plackett–Burman design scheme. The influence of 10 components of the nutrient medium was investigated, varying them at two levels with a central point. corn steep liquor was added into the design scheme to replace the more expensive nitrogen sources yeast extract and tryptone. The design included 15 run design, including 3 center points and 12 points at two levels (+1, -1). The influence of each factor on the production of 2,3-BD is described by a first-order linear equation:

$$Y = \beta_0 + \sum \beta_i X_i$$

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 2. Media components selected for variation and their experimental range for 2,3-BD productionusing in ten-factor Plackett–Burman design.

Variables (Media Components)		Experimental Levels		
		-1	0	+1
Yeast extract (g/L)	X_1	0	5	10
Tryptone (g/L)	X_2	0	5	10
$(NH4)_2SO_4$ (g/L)	X_3	1	3	5
KH_2PO_4 (g/L)	X_4	2	3.5	5
K_2HPO_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 (mL)	X_9	1	3	5
3-morpholino propane sulfonic acid. (MOPS) (g/L)	X_{10}	0	5	10

The concentration of 2,3-BD after 24 hours of fermentation was used as response. After conducting the experiments, the obtained results were subjected to statistical analysis to determine the influence of individual parameters. Statistical analysis (Table 3) showed that within the studied ranges, 4 of the studied parameters - the yeast extract, KH_2PO_4, $MgSO_4$ and tryptone had a significant positive effect, while one parameter, the corn hydrolyzate, had a significant negative effect on the formation of 2, 3-DB (P value < 0.05). The remaining variables had no significant effect on the formation of 2,3-BD in the respective ranges (P value > 0.05).

Table 3. Estimated coefficients in coded form from the linear regression equation for obtaining 2,3-BD in a ten-factor Plackett–Burman design.

Variables (Media Components)	Effect	Coefficient	*P* value
Constant		10.477	0.000
Yeast extract (g/L)	12.362	6.181	0.001 [a]
Tryptone (g/L)	2.842	1.421	0.047 [a]
$(NH_4)_2SO_4$ (g/L)	−0.465	−0.233	0.629
KH_2PO_4 (g/L)	−1.028	−0.514	0.321
K_2HPO_4 (g/L)	9.462	4.731	0.002 [a]
$MgSO_4$ (g/L)	5.515	2.758	0.008 [a]
Ammonium acetate (g/L)	−1.538	−0.769	0.174
Corn steep liquor (g/L)	−10.535	−5.268	0.001 [b]
Salt solution (mL)	−2.395	−1.197	0.070
3-morpholino propane sulfonic acid. (MOPS) (g/L)	1.145	0.573	0.278

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

After removing the terms with no significant effect on the yield of 2,3-BD (P > 0.05), the linear model took the following form:

$$Y = 10.48 + 6.18\,X_1 + 1.42\,X_2 + 4.73\,X_5 + 2.76\,X_6 - 5.27\,X_8$$

Analysis of variance of the thus derived regression model showed that the value of The R^2 (coefficient of determination) of the model is 0.9953. This shows that the model describes 99.53% of the experimental data. The

calculated P value for the model (0.003) and the "Lack-of-Fit" value (0.741) of the model also suggest statistical significance of the equation. The four components having a significant positive effect on 2,3-BD production were selected for further investigation to determine their optimum values. corn steep liquor and MOPS were excluded from the medium composition due to their significant negative effect and no influence in the 0–10 g/L range for MOPS.

Optimizing the values of the significant components of the nutrient medium

To find the optimal values of the significant parameters, a central composite design (CCD) experiment planning was used, with the parameters varied at 5 levels (−α, −1, 0, +1, +α), in a matrix containing 31 experiments with 7 central, 8 axial and 16 cubic points. All 31 experiments were conducted twice. The levels of the variables and their corresponding real values are shown in Table 4.

Table 4. Coded levels and actual values of the significant components of the nutrient environment selected for optimization by four-factor CCD.

Factors (Variable)	Experimental Levels				
	-α	-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

* α = 2.

Since *B. licheniformis* 24, regardless of the used medium, it is able to fully assimilate 100 g/L of glucose. The maximum product concentration in all experiments would be approximately the same and could not be used as a response to

estimate the parameter value. For this reason, the obtained 2,3-BD concentration after 24 hours of fermentation was chosen as a function (response) of the model. The ranges of variation are chosen so that the center points (level 0) for each variable in the CCD plot correspond to their upper level (+1) of The Plackett–Burman design. The results obtained and the design of the randomized matrix are presented in Table 5.

Table 5. Experimental matrix according to the central composite design scheme. Actual values of the four variables and the observed response (concentration of 2,3-BD after 24 h fermentation).

Run Order	Experimental Levels				2,3-БД* (g/L)
	X_1	X_2	X_5	X_6	
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

* Average values of repetitions.

The study of the effect of the variables on the production of 2,3-BD was performed by a quadratic regression model using the following equation:

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

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

Table 6. Coded coefficients of the second-order regression model for 2,3-BD synthesis in a four-factor central composite design.

Source	Effect	Coefficient	P Value
Constant		24.618	<0.001
Yeast extract (X_1)	2.217	1.108	<0.001
Tryptone (X_2)	2.064	1.032	<0.001
K_2HPO_4 (X_5)	3.748	1.874	<0.001
$MgSO_4$ (X_6)	−0.017	−0.008	0.955
X_1^2	−1.958	−0.979	<0.001
X_2^2	−0.693	−0.347	0.019
X_5^2	−2.208	−1.104	<0.001
X_6^2	−1.264	−0.632	<0.001
X_1X_2	−1.430	−0.715	0.001
X_1X_5	0.269	0.134	0.461
X_1X_6	1.505	0.753	0.001
X_2X_5	−1.493	−0.746	0.001
X_2X_6	0.276	0.138	0.449
X_5X_6	−0.465	−0.233	0.209

The data presented (Table 6) show that X_1, X_2, X_5, all the quadratic terms and the interactions X_1X_2, X_1X_6 and X_2X_5 had a significant effect on the production of 2,3-BD ($P < 0.05$). Thus, the polynomial equation of the second degree takes the following form:

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

The model describes 96.59% of the experimental data ($R^2 = 0.9659$). According to Reddy et al. [9] values of $R^2 > 0.75$ indicate the adequacy of the model. The P value of the model (< 0.001) and the value "Lack-of-Fit" (0.155) showed that the model is adequate and the equation is suitable to describe the production process of 2,3-BD. The optimization procedure predicts the maximum amount of 2,3-BD obtained after 24 hours of fermentation (maximizing the regression equation) of 25.825 g/L, to be obtained at the following values of the variables: 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 (Table 7).

Table 7. Optimal values of the significant components of the nutrient medium.

Source	Value (g/L)
Yeast extract	13.38
Tryptone	6.41
K_2HPO_4	4.20
$MgSO_4$	0.32

Model validation

For experimental validation of the model, a nutrient culture process was carried out 13.38 g/L yeast extract, 6.41 g/L tryptone, 4.20 g/L K_2HPO_4 and 0.32 g/L $MgSO_4$.

After conducting three independent experiments, the average value obtained after 24 hours of cultivation was 25.74 ± 0.8 g/L 2,3-BD. This value is close to the predicted value 25.825 g/L 2,3-BD, which means that the model adequately describes the changes in the obtained amount of 2,3-BD. Thus, the optimal nutrient medium for obtaining 2,3-BD from *B. licheniformis* 24 have the following form: yeast extract 13.38 (g/L), tryptone 6.41 (g/L), K_2HPO_4 4.2 (g/L), $MgSO_4$ 0.32 (g/L), $(NH4)_2SO_4$ 1 (g/L), KH_2PO_4 3.5 (g/L),

ammonium acetate 2.5 (g/L), $CoCl_2$ x 6 H_2O 0.09 (g/L) and salt solution 3 ml/L.

Optimization of technological process parameters through central composite design

The most important process parameters - temperature, pH and aeration rate are optimized by applying the same methodology used to optimize the media components. A three-factor central composite design was applied, with the three variables set at 5 levels (−1.682, −1, 0, +1, +1.682), in a matrix containing a total of 20 experiments with 6 central, 6 axial, and 8 cubic points. Thus, the experiments will be described by a regression model a polynomial equation of the second degree. The coded levels of these 3 variables and their corresponding true values are shown in Table 8.

Table 8. Process parameters selected for variation with their corresponding experimental ranges.

Factors	Experimental Levels				
	−α *	−1	0	1	α*
T (°C) (X1)	28.9546	31	34	37	39.0454
pH (X2)	5.1591	5.5	6.0	6.5	6.8409
Aeration rate (vvm) (X3)	0.3182	1.0	2.0	3.0	3.6818

* α = 1.682.

The optimization of the process parameters was carried out in a series of batch fermentations carried out on a fermenter with the optimized nutrient medium containing 200 g/L glucose.

Table 9. Experimental matrix according to the CCD scheme.

Run Order	Experimental Levels			2,3-BD a (g/L)	2,3-BD a (g/L/h)	Y 2,3-BD a (g/g) b
	X_1	X_2	X_3			
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 Means of replicates; b Gram of 2,3-BD produced per gram of glucose consumed.

As response (Y) was used the maximum 2,3-BD concentration obtained after 48 hours of fermentation The statistical analysis of the obtained experimental data and the calculated coefficients of the varied parameters with their corresponding probability are presented in Table 10.

Table 10. Calculated coefficients of the regression model in coded form with their respective significance.

Factors	Effect	Coefficient	P Value
Constant		76.018	**<0.001**
t (°C) (X_1)	9.024	4.512	**<0.001**
pH (X_2)	−5.129	−2.565	**0.003**
Aeration rate (vvm) (X_3)	8.175	4.087	**<0.001**
X_1^2	−7.404	−3.702	**<0.001**
X_2^2	−8.416	−4.208	**0.001**
X_3^2	5.334	2.667	**0.002**
X_1X_2	12.585	6.292	**<0.001**
X_1X_3	2.400	1.200	0.186
X_2X_3	−1.515	−0.757	0.391

From the table it can be seen that the interactions X_1X_3 and X_2X_3 are not statistically significant ($P > 0.05$) and after their exclusion the polynomial equation of the second degree takes the following form:

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

The coefficient of determination of the model is $R^2 = 0.9659$, which means that this model describes the obtained experimental data extremely well.

After carrying out an optimization procedure (maximization of Y), the predicted maximum value for the amount of synthesized 2,3-BD is 93.77 g/L under the following

conditions: temperature (X_1) 37.82 °C; pH (X_2) 6.23; aeration rate (X_3) 3.68 vvm.

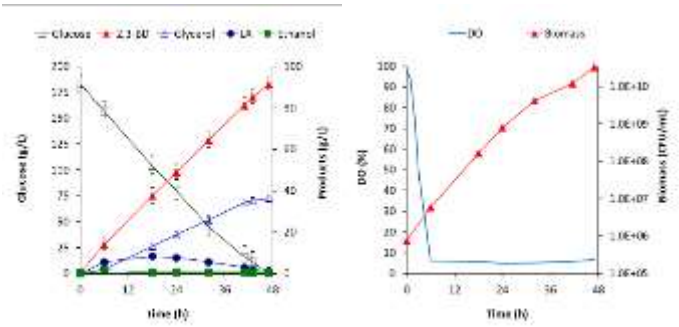
Table 11. Predicted optimal values of process parameters for maximum synthesis of 2,3-BD.

Factors	Value
t (°C)	37.82
pH	6.23
Aeration rate vvm	3.68

Experimental validation of the model

To validate the model, a batch fermentation with *B. licheniformis* 24 was performed under optimized media and process parameters. The results (mean values of three separate experiments) are presented in Figure 1. The maximum concentration of 2,3-BD obtained was 91.23 ± 2.9 g/L. This value is higher than all in the design matrix and is close to the estimated 93.77 g/L. This confirms the adequacy of the model. The experimentally obtained concentration was achieved with a productivity of 1.94 g/L/h 2,3-BD and a yield of 0.488 g/g (98% of the theoretical maximum 0.5 g/g), which shows that under the predicted optimal conditions the process for obtaining of 2,3-BD is extremely effective.

The main fermentation by-product is glycerol 35.20 g/L. The other soluble metabolites: lactic acid 1.2 g/L, acetoin and ethanol below 1 g/L. During fermentation, lactic acid reached the highest concentration at the 18th hour (8.37 g/L) and then slowly decreased. A similar profile was observed for ethanol up to 1.8 g/L at the 6th hour, then decreased to 0.5 g/L at the end of the process (Figure 1A). Acetoin is formed in the first few hours. Then, as dissolved oxygen in the medium decreases (Figure 1B), acetoin synthesis stops until the carbon source is completely exhausted. Glucose was completely consumed after 47 hours of fermentation (Figure 1A).

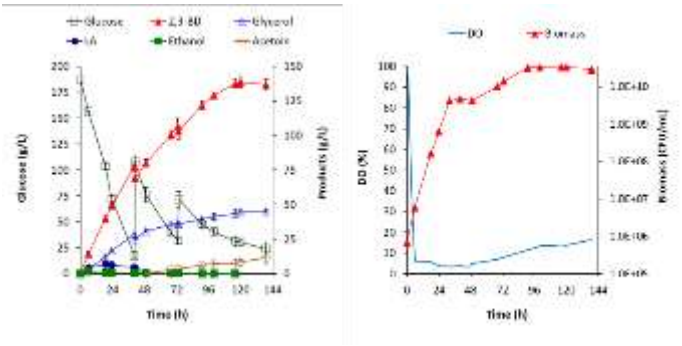


(A) (B)

Figure 1. Batch fermentation of 200 g/L glucose by *B. licheniformis* 24 in optimized media and process parameters: A) Glucose consumption and product accumulation (three replicates); B) Values of dissolved oxygen (DO) and biomass formed.

Fed-Batch process in optimized conditions from glucose

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 was carried out under the already established optimized conditions. The highest concentration of 2,3-BD achieved was 138.8 g/L, with a productivity of 1.16 g/L/h and a yield of 0.478 g/g. Strong inhibition by the product was observed at concentrations above 100 g/L, resulting in a decrease in the rate of glucose consumption. Glucose consumption stops completely after the 140th hour. The main fermentation by-product is glycerol, reaching a maximum of 45.38 g/L after 120 hours of fermentation. Acetoin accumulates after the 70th hour of fermentation, when the concentration of dissolved oxygen increases slightly from 6 to 14% at the end of the process (Figure 2B).



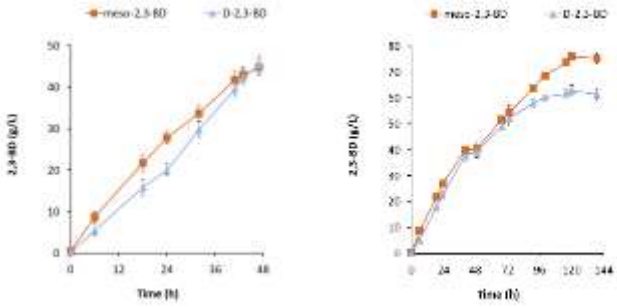
(A) (B)

Figure 2. Fed-batch process conducted in medium with optimized composition and at optimal process parameters: A) Glucose consumption and product accumulation (means of three replicates); B) Values of dissolved oxygen (DO) and biomass formed.

Isomeric ratios of the resulting 2,3-BD

B. licheniformis 24 produces 2,3-BD in two isomeric forms meso-2,3-BD and D-2,3-BD, in ratios of 1.6 : 1 to 1 : 1. The ratio was not observed to depend on the substrate used, but appeared to depend on the total concentration of 2,3-BD produced. For example, at the beginning of each fermentation the meso-form slightly predominated, while as the process progressed the ratio leveled off at a total 2,3-BD concentration of 80-90 g/L (Figure 2A). This phenomenon is observed in all batch fermentations. However, in fed-batch fermentations at optimized parameters, after the 70th hour the ratio changed again in favor of the meso-form and by the end of the process from 1.05:1 reached 1.23:1 (Figure 2B). A possible explanation is the production of acetoin during this period (Figure 3A), which was not observed in any of the other batch fermentations when the carbon source was available. On the other hand, upon complete depletion of the substrate, under the action of

butanediol dehydrogenase BDH, the D-form is rapidly converted to acetoin, while the meso-form decreases slowly and is only partially converted to acetoin.



(A) (B)

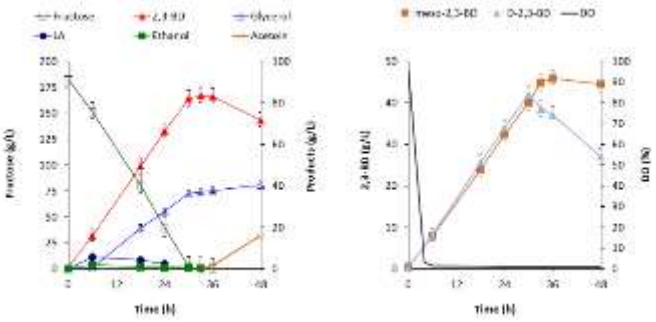
Figure 3. Kinetics of production of meso- and D-2,3-BD by *Bacillus licheniformis* 24 in: (A) Batch fermentation at 200 g/L glucose; (B) Fed-batch fermentation.

4.2. Production of 2,3-BD from fructose

Batch process with carbon source fructose under optimized conditions

The medium optimized for the production of 2,3-BD from glucose was used, replacing it with 200 g/L fructose. The process parameters pH, temperature and aeration are maintained at the already determined optimal values of 6.23, respectively, 37.8 °C and 3.68 vvm. A 10% inoculation was used at optical density (OD_{600} = 2,300) of the inoculums was reached.

When conducting a batch fermentation of 200 g/L fructose, the maximum 2,3-BD concentration was reached after 33 hours 83.5 ± 1.5 g/L, when fructose was completely exhausted (Figure 4A).



(A) (B)

Figure 4. Batch fermentation of 200 g/L fructose by *Bacillus licheniformis* 24: A) Time profiles of fructose consumption and product formation; B) Temporal profiles of meso- and D-2,3-BD accumulation and dissolved oxygen availability.

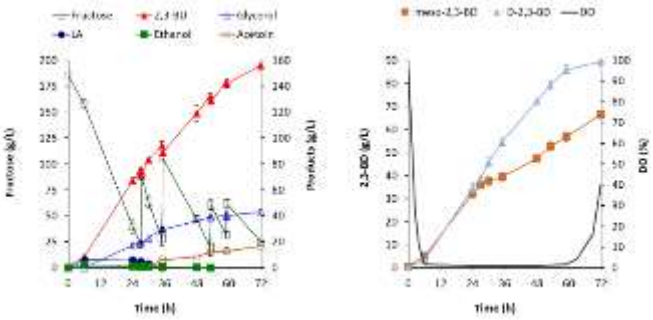
Fructose consumption rate is 5.52 g/L/h, and the main fermentation byproduct is glycerol. At the time when fructose was completely consumed, 37 g/L of glycerol was formed and no other soluble metabolites were detected, except for minor amounts of ethanol (about 0.1 g/L). Lactic acid is a temporary product of fermentation, reaching its maximum titer of 5.67 g/L at the 6th hour of fermentation. After that, the concentration decreases and lactic acid completely disappears after 24 hours. Ethanol reached its maximum of 2.05 g/L at the 6th hour and then slowly decreased (Figure 4A).

After the 33rd hour, when the carbon source is no longer available, 2,3-BD is rapidly converted to acetoin. Quantification of the different 2,3-BD isomer concentrations showed that the D-2,3-BD form was mainly converted to acetoin, while the meso form remained at almost the same concentration for at least 15 h after complete substrate depletion (Figure 4B). During this period, the concentration of acetoin in the medium increased from 0 to 16.1 g/L, and the

concentration of dissolved oxygen remained at its lowest level (1-2%), indicating that there were still living cells (Figure 4B).

Batch process by feeding the carbon source fructose under optimized conditions

In the fed-batch process, additional amounts of fructose were added as a sterile aqueous solution when the fructose concentration in the culture medium dropped below 50 g/L. Thus, after 72 h of fermentation, a total of 370 g/L fructose was consumed, reaching a maximum concentration of 156.1 ± 3.6 g/L 2,3-BD (Figure 5A).



(A) (B)

Figure 5. Periodic process with feeding carried out in an environment with optimized composition and optimal process parameters: A) Time profiles of fructose consumption and product formation; B) Temporal profiles of meso- and D-2,3-BD accumulation and dissolved oxygen availability.

Main byproducts are glycerol (43.1 g/L) and acetoin (16.7 g/L). Lactic acid and ethanol were not detected after 48 h, and acetoin began to accumulate in the medium after 30 h (Figure 5 A). At the 72nd hour, when the maximum concentration of 2,3-BD was reached, the ratio of the stereoisomers was: meso-2,3-BD:D-2,3-BD=1.00:1.35. By the

24th hour, both isomers are produced in approximately equal amounts, after which the amount of D-2,3-BD increases significantly, with the ratio reaching 1.00:1.51 (meso : D-shape) at the 60th hour. Between the 60th and 72 h, when acetoin accumulation begins, although the concentrations of both isomers continue to increase, their ratio returns to 1.00:1.35 (Figure 5 B). This indicates that the production of acetoin is mainly at the expense of D-2,3-BD. The level of dissolved oxygen in the environment drops sharply - from 95.8% to 1.7% in the first 6 hours of fermentation and remained almost constant until the 60th hour, then gradually increased from 1.7% to 40.9% at the 72nd hour, most likely due to the decrease in the number of viable cells (Figure 5 B).

In conclusion, it can be said that the obtained concentration of 156.1 g/L 2,3-BD in fermentation with the carbon source fructose is the highest amount reached by a non-pathogenic microorganism to date. The productivity of 2.17 g/L/h of *B. licheniformis* 24 is relatively high for a member of the genus *Bacillus*.

4.3. Production of 2,3-BD from inulin

Batch process with carbon source inulin under optimized conditions

The results obtained in the cultivation of *B. licheniformis* 24 in fructose as a carbon source suggests that the strain is highly suitable for the conversion of fructose-containing substrates to 2,3-BD. To establish the ability of *B. licheniformis* 24 to convert an inulin-containing substrate in the already optimized medium, the strain was cultivated with a carbon source chicory flour (Frutafit® HD (Sensus B.V., Roosendaal, The Netherlands)) with an initial concentration of 100 g/L. The process was carried out at the already optimized process parameters: pH 6.23, temperature 37.8 °C and aeration of 3.68 vvm. Although *B. licheniformis* 24 possessed some

natural inulinase activity, the strain was unable to hydrolyze inulin molecules to fermentable sugars, and the total amount of acetoin+2,3-BD produced (about 6 g/L) during fermentation was almost entirely from free sugars found in chicory flour (~10 g/L). Therefore, it is necessary, to additionally introduce a gene encoding an enzyme with strong inulinase activity in strain 24.

Cloning of the *Inu* gene from *Lacticaseibacillus paracasei* DSM 23505

For this purpose, the *Inu* gene of *Lacticaseibacillus paracasei* DSM 23505 (GenBank KP663715.1), encoding a cell-bound fructan-β-fructosidase (EC 3.2.1.80) containing 3645bp, was chosen (Figure 6).

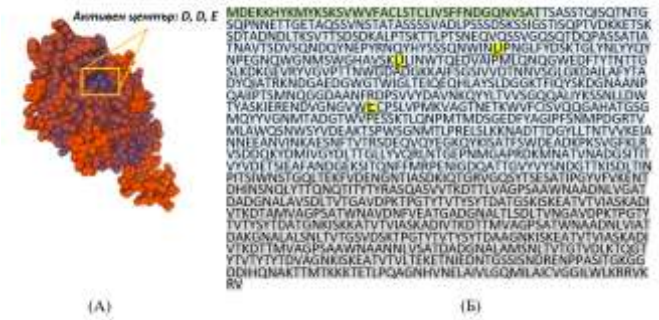


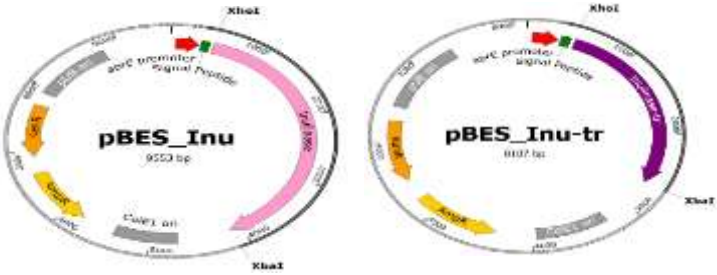
Figure 6. (A) 3D model of the enzyme (EC 3.2.1.80) of *Lc. paracasei* DSM 23505, the amino acids of the catalytic center: two aspartates (D) and glutamate (E), are circled and shown in purple. The model was made by homology in the SWISS-MODEL Workspace [10] (B) Schematic representation of the amino acid sequence of the enzyme. The signal peptide is shown in green; the 731 amino acid polypeptide belonging to the GH32 family is shown in blue; the Big3 domains responsible for cell wall anchoring are represented in gray; amino acids of the catalytic center are highlighted in yellow and conserved regions are underlined.

The enzyme contains a theoretical signal peptide from 38 amino acids, a β-fructosidase catalytic domain, a C-terminal domain and four Big3 domains responsible for attachment to the cell wall [7].

Cloning of the *Inu* gene into pBE-S *E. coli*/*Bacillus* spp. shuttle vector

The *Inu* gene was cloned into two different shuttle vectors in two different length variants with (pBES_Inu) and without (pBES_Inu-tr) the Big3 cell wall attachment domains. The constructs were introduced into competent *E. coli* cells, then propagated by culturing the transformants.

Maps of the constructs used to transform *E. coli* and *B. licheniformis* 24 are shown in Figure 7.



(A) (B)

Figure 7. Graphic representation of the recombinant constructs containing the *Inu* gene. (A) pBES_Inu; (B) pBES_Inu-tr; Maps of plasmid constructs were made with SnapGene software (GSL Biotech LLC).

After isolation of the plasmid content from the developed culture of *E. coli*, the resulting recombinant constructs were restricted with the appropriate enzymes, and

those with the correct *Inu* gene sequence were isolated after confirmation by PCR and sequencing (Figure 8).

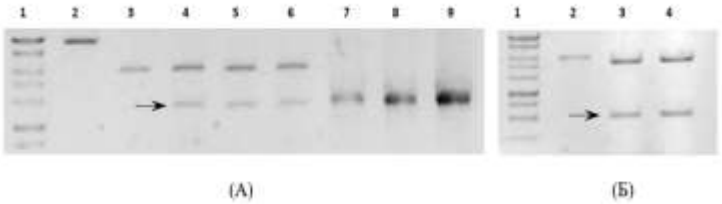


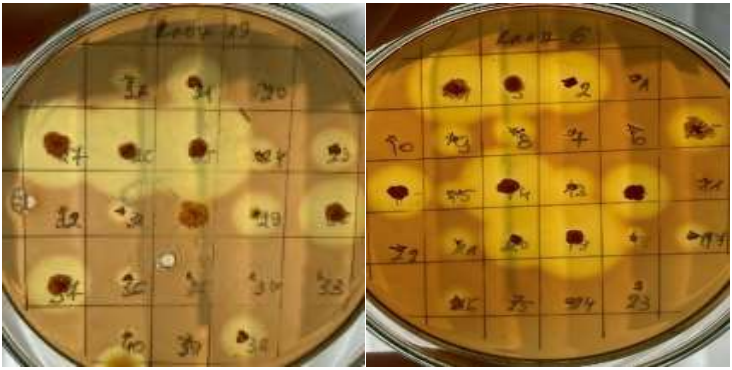
Figure 8. Analysis of recombinant constructs isolated from *E. coli* STELLAR^{TM}(A) Row 2: linearized construct pBES_Inu; Lane 3: linear pBE-S vector, Lanes 4, 5 and 6: plasmid DNA of pBES_*Inu* clones cut with *Xba*I and *Xho*I; Rows 7, 8 and 9: PCR amplification of the 3.6 kb gene. (B) Lane 2: linearized pBE-S vector; Lanes 3 and 4: plasmid DNA of pBES_Inu-tr clones 2.2 kb. cut with *Xba*I and *Xho*I. A Perfect Plus 1 kb DNA marker (EURx, Gdansk, Poland) with the following sizes was used to determine the size of the fragments:10.0, 8.0, 6.0, 5.0, 4.0, 3.0, 2.5, 2.0, 1.5 kb.

All recombinant constructs were introduced into *B. licheniformis* 24 by electroporation. A summary of the results obtained from the transformant analysis is presented in Table 12. *B. licheniformis* 24 clones resistant to the antibiotic kanamycin were selected based on their hydrolase activity (Figure 9), which was assayed by culturing the clones on petri dishes with nutrient medium LB-agar containing 1% insoluble inulin and 50 μg/mL kanamycin. Colonies forming zones larger than those of the wild strain were scored as positive. Thus, 7 positive clones carrying the pBES_*Inu* construct were reported, 7 pBES_Inu-tr.

Table 12. Total number of transformants, number of clones analyzed and number of positive clones containing the three types of constructs.

Construct	Number ofTransformants	Clones Analyzed	Positive Clones*
pBES_Inu	2638	89	7
pBES_Inu-tr	2415	62	7

*Hydrolysis zones analyzed in petri dishes containing LB medium with 1% insoluble inulin. Clones forming zones larger than those of the wild strain *B. licheniformis* 24 containing the "empty" vector were considered positive.



(A) (B)

Figure 9. Clonal selection of *B. licheniformis* transformed with pBES_*Inu* (Figure 9 A) and pBES_Inu-tr (Figure 9 B) cultured on petri dishes with 1% insoluble inulin.

The largest zones (2.5–2.7 cm) were formed by *B. licheniformis* clones containing pBES_*Inu* (Figure 9 A). Of these, clone T26 (Figure 9A) was selected for further work. Of the clones carrying the pBES_Inu-tr (Figure 9 B) construct, the largest area (2.2–2.3 cm) was clone T14 (Figure 9B), which was also selected for 2,3-BD production.

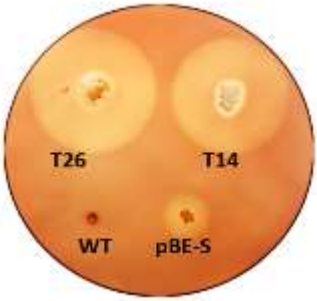


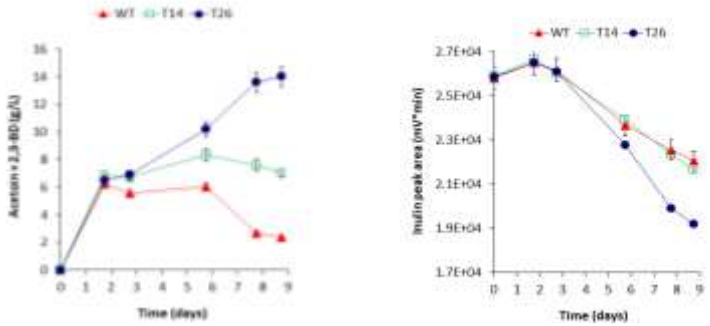
Figure 10. Comparison of inulin hydrolysis zones obtained from selected *B. licheniformis* transformants in petri dishes Clone T26 *B. licheniformis* containing the pBES_*Inu* construct; Clone T14 *B. licheniformis* with pBES_Inu-tr; pBE-S, *B. licheniformis* transformed with the "empty" vector; WT – wild type *B. licheniformis* 24 (does not grow in kanamycin medium).

Batch fermentation with *B. licheniformis* clones T14 and T26 in flasks

As a substrate was used chicory flour with an initial concentration of 100 g/L, containing 90% insoluble inulin DP8-DP13 and 10% mixture of fructose, sucrose and glucose. Inulin was hydrolyzed most efficiently by clone T26, T14 and wild type showing close profiles. By the 40th hour, T26, T14, and wild type had completely consumed the free sugars, synthesizing about 6 g/L acetoin+2,3-BD. After that, the wild type and the T14 clone formed no more product. Clone T26 synthesized approximately 8 g/L more 2,3-BD and acetoin to14 g/L (Figure 11).

Evaluation of the inulinase activity of the recombinant clones showed a significant increase over the wild type. The activity of T26 was more than eight times higher (average 8.7 U/mL) compared to the wild type

(ranging from 0.5 to 1.5 U/mL) and more than three times higher than that of clone T14 (2.7 U/ mL). Establishing the localization of the heterologous enzyme by examining cells and cell-free supernatants showed predominantly cell-bound inulinase activity of T26 and predominantly extracellular of T14 and wild type.



(A) (B)

Figure 11. Batch fermentation by recombinant clones T26, T14 and wild-type *B. licheniformis* 24 (A) Synthesized acetoin + 2,3-BD. (B) Hydrolyzed inulin (peak area of oligosaccharides DP3–DP9)

Batch fermentation by *B. licheniformis* T26 under optimized conditions

Batch cultivation was carried out with an initial substrate concentration of 200 g/L chicory flour. In this process, free sugars were completely consumed within 16 h, yielding 7.22 g/L 2,3-BD and 3.96 g/L acetoin. The biomass increased exponentially until the 72nd hour, up to 8.8×10^7 CFU/mL, and reached the stationary phase after the 96th hour (1.2×10^8 CFU/mL). No degradation of inulin was observed during the first 48 hours.

As a result, between the 16th and the 48th hour, a certain amount of 2,3-BD is converted to acetoin. When the degradation of inulin begins, the concentration of 2,3-BD rises again. However, a sharp accumulation of sucrose in the culture medium was observed after the 66th hour. After 160 h of fermentation, although approximately 140 g/L inulin was degraded, 53 g/L sucrose and 18.5 g/L 2,3-BD accumulated. (Figure 12)

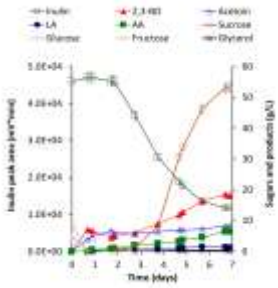


Figure 12. Batch fermentation of 200 g/L chicory flour by *B. licheniformis* T26.

5. Discussion of the results

5.1. Optimizing the culture medium for the production of 2,3-BD

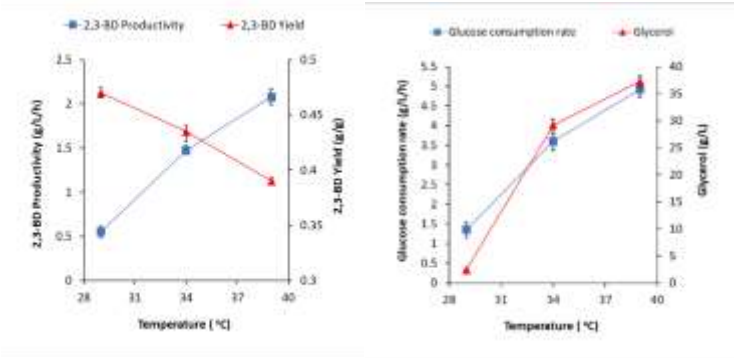
To optimize the composition of the nutrient medium for the production of 2,3-BD, a screening design (Plackett-Burman) was successively applied to establish the significant components of the nutrient medium and a central composite design to establish the optimal concentrations of the components that showed a significant effect on the production of 2,3-BD. The results of conducting the Plackett-Burman design showed that 4 of the 10 studied components had a significant positive influence on the production of 2,3-BD. Four of them: yeast extract, K_2HPO_4, $MgSO_4$ and tryptone. This observation is in line with the suggestion made by Song et al [11]. On the other hand, the addition of corn hydrolyzate has a significant negative effect. For this reason, corn hydrolyzate was excluded from the medium. MOPS was also excluded from the medium due to its negligible influence in the range of 0 to 10 g/L. This indicates that unlike *P. polymyxa* [4], *B. licheniformis* does not require MOPS, making the culture medium cheaper. The four components showing a significant positive effect on the 2,3-BD production process were varied in a central composite design scheme to establish their optimal concentrations. After conducting an optimization procedure to maximize the regression equation obtained from the statistical analysis, it was predicted that the maximum amount of 2,3-BD would be obtained at the following values of the variables: yeast extract 13.38 g/L; K_2HPO_4 4.20 g/L; tryptone 6.41 g/L; $MgSO_4$ 0.32 g/L. These values were confirmed as optimal by triplicate experimental validation of the model.

5.2. Optimization of process parameters for obtaining 2,3-BD

Cultivation temperature (t °C), medium acidity (pH), and aeration (sterile air flow rate) were optimized by a central composite design scheme, with all experiments performed at agitation speed 500 rpm. As optimal values for the process parameters were determined: temperature 37.82 °C; pH 6.23; aeration rate 3.68 vvm. These values were validated experimentally.

Effect of temperature

Temperature is the parameter with the greatest impact, but the expected optimum values are quite different for maximum 2.3-BD concentration, 2.3-BD productivity and 2.3-BD yield. Comparing the central and axial points of the CCD design shows that higher temperatures accelerate glucose consumption, leading to higher productivity in the production of 2,3-BD. (Figure 13 B).



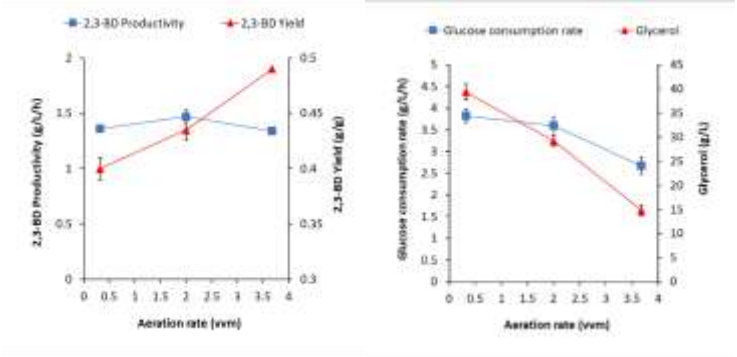
(A) (B)

Figure 13. Effect of temperature on fermentation by *B. licheniformis* 24 on: (A) productivity and yield of 2,3-BD;(B) rate of glucose consumption and glycerol formation.

For example, at a constant pH of 6 and an aeration rate of 2 vvm, increasing the temperature from 29 °C to 39 °C increases productivity from 0.55 to 2.08 g/L/h. However, due to increased glycerol formation, the yield of 2,3-BD decreased from 0.47 to 0.39 g/g (Figure 13 A). As the rate of glucose consumption increased (from 1.35 to 4.92 g/L/h), glycerol accumulation increased from 2.42 to 37.25 g/L

Effect of aeration

Higher aeration rates favored an increase in 2,3-BD concentration and yield and had no significant effect on yield (Figure 14 A). In terms of glucose consumption and glycerol accumulation, increasing aeration had the opposite effect to increasing temperature (Figure 14 B).



(A) (B)

Figure 14. Effect of aeration rate on fermentation by *B. licheniformis* 24 on: (A) productivity and yield of 2,3-BD; (B) rate of glucose consumption and glycerol formation.

Under fully aerobic conditions, oxygen is the electron acceptor for NAD+ regeneration and reduction of acetoin to 2,3-BD does not occur. The observation that acetoin production is greater under highly aerobic conditions determines the

development of complex two-stage aeration regimes in the processes for the production of 2,3-BD by *B. licheniformis* [11, 12, 13, 14].

Fermentations were carried out at pH 6 and temperature 34 °C. Mean values from at least two individual experiments are presented. *B. licheniformis* 24 is an extremely aerobic producer of 2,3-BD and is able to consume significant amounts of oxygen. As shown in Figure 14 B, the amount of dissolved oxygen in the medium remained consistently low from the 6th hour until the end of fermentation. This high oxygen consumption prevents acetoin formation even at the highest aeration levels tested. However, glycerol synthesis cannot be avoided, even at high levels of aeration.

Effect of pH

A specific characteristic of *B. licheniformis* 24 is the production of glycerol as a major by-product. Due to the formation of glycerol, acidity of the medium changes little during fermentation. Changes in pH affected the production of 2,3-BD to a lesser extent (Figure 15).

At lower pH (5.16), acetoin appeared as the major byproduct, reaching concentrations of 28.64 g/L. It accumulates at low glucose consumption, in this case under conditions combining lower pH (≤ 5.5) and lower temperature (≤ 31 °C). This effect leads to a strong accumulation of acetoin in the culture liquid at a low level of sugar consumption is characteristic of *B. licheniformis* 24 [5].

The other significant byproduct, glycerol, is not formed at lower pH (5.16) but increases at pH 6.0. Logically, lactic acid is a major fermentation co-product only at higher pH values, reaching 14.11 g/L at pH 6.84.

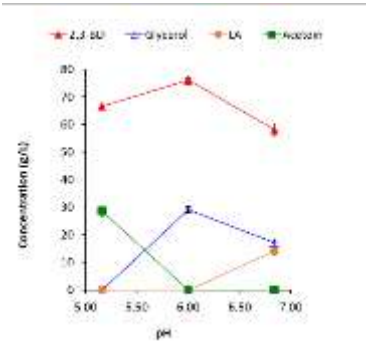


Figure 15. Effect of pH on product formation in the fermentation of 200 g/L glucose by *B. licheniformis* 24. Fermentations are carried out at T 34 °C and aeration rate 2 vvm. Mean values from at least two individual experiments are presented.

In conclusion, it can be said that the effect of the presented complex optimization of the composition of the medium and process parameters is most obvious when the results of batch fermentation from glucose before and after optimization are compared. At the absorption of 200 g/L of glucose, the application of the optimized composition of the nutrient medium and process parameters increases the maximum concentration of 2,3-BD by 28.9% from 70.8 g/L to 91.23 g/L. The yield increased by 6.8%, from 0.457 g/g to 0.488 g/g substrate. The increase in process productivity is particularly significant 0.38 to 1.94 g/L/h (more than 5 times).

5.3. Production of 2,3-BD from glucose

To establish the maximum amount of 2,3-BD that B. licheniformis 24 could produce from glucose, a fed-batch process was carried out. The process was carried out with the already optimized nutrient medium and process parameters, and from a total of 290 g/L, 138.8±4.3 g/L 2,3-BD were

obtained with a productivity of 1.16 g/L/h and a yield close to the theoretical (0.479 g/g). This result ranks *B. licheniformis* 24 among the top nonpathogenic producers. To date, the highest concentration of 2,3-BD obtained from a non-pathogenic producer (*B. licheniformis* DSM 8785) was 144.7 g/L, obtained with a productivity of 1.14 g/L/h [15] This indicates that the limits of the species as a producer have probably been reached. Comparing the achieved results with those of pathogenic producers, it can be said that *B. licheniformis* 24 produced 2,3-BD in almost the same concentration, but with significantly lower productivity. For example, representatives of pathogenic species *K. pneumoniae* or *K. oxytoca* produce 2,3-BD in a concentration of up to 150 g/L, but with a productivity of up to 4 g/L/h [16].

5.4. Production of 2,3-BD from fructose

To establish the ability of *B. licheniformis* 24 to produce 2,3-BD from fructose, a batch process and a fed-batch process were conducted under the already optimized conditions. Comparing the batch processes for obtaining 2,3-BD from glucose and fructose, it was observed that the consumption rate of fructose uptake (5.52 g/L/h) significantly exceeded the consumption rate of glucose uptake (3.98 g/L/h) under similar conditions . Although the obtained 2,3-BD titer from fructose was lower than that from glucose (83.5 vs. 91.2 g/L), it was obtained with significantly higher productivity 2.53 g/L/h instead of 1.94 g/L/h of glucose. The yield of 2,3-BD from fructose was 0.46 g/g, lower than that from glucose 0.488 g/g. The same dependence was observed when fed batch process was condunted. The consumption rate of fructose (5.14 g/L/h) is more than twice that of glucose (2.41 g/L/h), correspondingly the productivity with which 2,3-BD is produced from fructose is 2.17 g/L/h versus 1.16 g/L/h of glucose (87.1% increase). In contrast to the comparison made for batch processes, here also the maximum concentration of 2,3-BD is higher by 12.5%

156.1 g/L against 138.8 g/L. These results indicate that fructose is a more suitable carbon source for the production of 2,3-BD by *B. licheniformis* 24. Since glucose is considered the preferred carbon source, one possible explanation for this phenomenon lies in the ability of *B. licheniformis* 24 to produce exopolysaccharides (EPSs) [17]. With a glucose substrate, the exopolysaccharide produced is at an extremely high concentration (12.6 g/L), which makes the medium extremely viscous and difficult to control. Another feature that is noticed during fructose fermentation is the increased production of D-2,3-BD, with fructose being the only substrate where *B. licheniformis* produced more D-2,3-BD than meso-2,3-BD.

5.5. Production of 2,3-BD from inulin

In a process to determine the ability of *B. licheniformis* 24 to produce 2,3-BD from inulin, it was found that the strain produced 2,3-BD solely from sugars in chicory flour, but could not hydrolyze inulin molecules to fermentable sugars. To increase the weak native inulinase activity of the strain,a cell-bound inulinase gene from *Lacticaseibacillus paracasei* DSM 23505 was successfully introduced via a plasmid into strain 24. Two types of gene transfer constructs were tested. The pBES_Inu construct containing the domains of the cell wall anchoring gene proved to be the most successful. The *B. licheniformis* T26 strain modified with this construct showed 8.7 times higher inulinase activity than the wild type. However, the obtained 18.5 g/L 2,3-BD with a yield of 0.1 g/g is not sufficient to consider the process efficient. On the other hand, transformant T26 hydrolyzed 140 g/L inulin, but most of the carbon accumulated in the culture fluid as sucrose, which was not metabolized. Therefore, recombinants need further "improvement" to make the process industrially applicable.

6. Conclusions

1. By planning the first-order experiment, it was found that to obtain 2,3-BD via *B. licheniformis* 24, the most important components of the nutrient medium are the organic nitrogen sources yeast extract and tryptone, as well as the salts K_2HPO_4 and $MgSO_4$.
2. Through a second-order experimental design, it was found that the optimal concentrations for obtaining 2,3-BD of these components were as follows: 13.38 g/L yeast extract, 6.41 g/L tryptone, 4.20 g/L K_2HPO_4, and 0.32 g /L $MgSO_4$.
3. By planning the second-order experiment, it was found that the optimal values of the process parameters for obtaining 2,3-BD by *B. licheniformis* 24 are the following: temperature 37.82 °C; pH 6.23; aeration rate 3.68 vvm.
4. In the fermentation of glucose by *B. licheniformis* 24, as the temperature increases: (i) the rate of glucose uptake increases; (ii) the rate of 2,3-BD formation (productivity); (iii) the production of the by-product glycerol, while the yield of 2,3-BD decreases.
5. In the fermentation of glucose from *B. licheniformis* 24, with increasing aeration, the yield of 2,3-BD increases, its productivity does not change, but the rate of glucose absorption and glycerol formation decrease.
6. In the fermentation of glucose by *B. licheniformis* 24, 2,3-BD is the main fermentation product at pH 5.00 to 7.00. Depending on the acidity of the medium, however, the main by-products of fermentation are different: at pH < 5.50 acetoin, at pH from 5.50 to 7.00 glycerol, at pH > 7.00 lactic acid.
7. In a fed-batch process, the maximum amount of 2,3-BD that strain *B. licheniformis* 24 could produce under optimal

conditions from glucose was 138.8 g/L, with a yield of 0.478 g/g and a productivity of 1.16 g/L/ h.

8. In a fed-batch process, the maximum amount of 2,3-BD that strain *B. licheniformis* 24 could produce under optimal conditions from fructose was 156.1 g/L, with a yield of 0.46 g/g and a productivity of 2.17 g/L/h .

9. A reason for the more efficient production of 2,3-BD from the substrate fructose than from glucose is that *B. licheniformis* 24 produces from glucose more and a different polysaccharide with which it forms a polysaccharide capsule. This makes the fermentation medium viscous and difficult to control with a glucose substrate.

10. *B. licheniformis* strain 24 has native inulinase activity that is not sufficient to hydrolyze Frutafit® HD chicory flour inulin to fermentable sugars.

11. Introduction of the *Inu* gene from *Lacticaseibacillus paracasei* DSM 23505 (GenBank KP663715.1) encoding a cell-bound fructan-β-fructosidase (EC 3.2.1.80) into *B. licheniformis* 24 resulted in an 8.7-fold increase in enzyme activity (transformant T26).

12. Introduction of the *Inu* gene from *Lacticaseibacillus paracasei* DSM 23505 into *B. licheniformis* 24 without its cell wall binding domains to render the enzyme extracellular resulted in a 4.7-fold increase in enzyme activity (transformant T14).

13. The modified strain *B. licheniformis* 24 T26 was able to hydrolyze 140 g/L inulin, but the maximum amount of 2,3-BD obtained was only 18.5 g/L, due to the accumulation of sucrose in the culture liquid, which was not metabolized to product.

7. Contributions

1. After complex optimization of culture medium and process parameters, strain *B. licheniformis* 24 produced from glucose 138.8 g/L 2,3-BD, the second best result for a non-pathogenic producer, and 156.1 g/L 2,3-BD from fructose the highest score in the world to date.

2. The inu gene from *Lacticaseibacillus paracasei* DSM 23505 was successfully introduced into *B. licheniformis* 24 via the pBE-S vector in two variants as a pBES_Inu construct containing the entire gene and as a pBES_Inu-tr construct containing the gene without its cell wall binding domains of the host.

3. By introducing the construct pBES_Inu, the thus modified strain *B. licheniformis* 24 T26 was given the quality to produce 2,3-BD from inulin in a process of simultaneous saccharification and fermentation.

Dissertation Publications

1. **Tsigoriyna L**., Ganchev D., Petrova P., Petrov K. (2021) Highly efficient 2,3-butanediol production by Bacillus licheniformis via complex optimization of nutritional and technological parameters., Fermentation, 7, 3, 118.(IF 5.11-2023) Q1
2. **Tsigoriyna L**., Petrova P., Petrov K. (2021) Production of 2,3-butanediol from fructose by Bacillus licheniformis 24., Acta Microbiologica Bulgarica,37,4,183-187 (IF 0.14-2021) Q4
3. **Tsigoriyna L**., Arsov A., Petrova P., Gergov E., Petrov K. (2023) Heterologous Expression of Inulinase Gene in Bacillus licheniformis 24 for 2,3-Butanediol Production from Inulin., Catalysts, 13, 5, (IF 4.501-2022) Q2

Citations:6

Participation in scientific forums

1. **Tsigoriyna L**., Ganchev D., Petrova P., Petrov P. Optimization of the microbial production of 2,3-butanediol from glucose by Bacillus licheniformis 24 Conference "Modern Trends in Science" – FMNS-2021, 15 - 19.09.2021, Blagoevgrad, South-West University "Neofit Rilski" – Blagoevgrad.
2. **Tsigoriyna L**., Arsov A., Petrova P., Gergov E., Petrov K. Heterologous expression of inulinase gene in Bacillus licheniformis 24 for 2,3-butanediol production from inulin - 2nd Symposium on Challenges in Chemical and Biochemical Technologies and Environmental Protection & 18th Workshop on Transport Phenomena in Two-Phase Flow, September 10 - 13, 2023, Sandanski, Bulgaria

Awards

1. Award for the best work of a young bulgarian microbiologist in 2021 of the Akd. Prof. Dr. Stefan Angelov"
2. BAS "Ivan Evstatiev Geshov" award for the youngest scientists under 30 years for 2023

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