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FERMENTATION, CELL CULTURE AND BIOENGINEERING

Application of enzymatic apple pomace hydrolysate to production of 2,3-butanediol by alkaliphilic *Bacillus licheniformis* NCIMB 8059

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Abstract 2,3-Butanediol (2,3-BD) synthesis by a non-pathogenic bacterium *Bacillus licheniformis* NCIMB 8059 from enzymatic hydrolysate of depectinized apple pomace and its blend with glucose was studied. In shake flasks, the maximum diol concentration in fed-batch fermentations was 113 g/L (in 163 h, from the hydrolysate, feedings with glucose) while in batch processes it was around 27 g/L (in 32 h, from the hydrolysate and glucose blend). Fed-batch fermentations in the 0.75 and 30 L fermenters yielded 87.71 g/L 2,3-BD in 160 h, and 72.39 g/L 2,3-BD in 94 h, respectively (from the hydrolysate and glucose blend, feedings with glucose). The hydrolysate of apple pomace, which was for the first time used for microbial 2,3-BD production is not only a source of sugars but also essential minerals.

Keywords 2,3-Butanediol · Apple pomace hydrolysates · *Bacillus licheniformis* · Fermentation

Introduction

A wide range of industrial applications of 2,3-butanediol (2,3-BD) resulted in a growing interest in its biotechnological production. This alcohol is used in the manufacture of printing inks, perfumes, fumigants, moistening and softening agents, explosives, plasticizers, foods and pharmaceuticals [5, 9]. 2,3-BD is also a popular antifreeze agent (freezing point of −60 °C) and a valuable fuel additive with a heating value of 27.2 kJ/g, which compares favorably with other liquid fuels, such as methanol (22.1 kJ/g) and ethanol (29.1 kJ/g). Furthermore, it can be converted by dehydration to methyl-ethyl-ketone, an industrial solvent, or to 1,3-butadiene, an important monomer for synthetic rubber manufacturing while acetoin, the precursor of 2,3-BD, can be oxidized to diacetyl, which is a high-valued flavoring agent in food products, giving a buttery taste, and a bacteriostatic additive for food processing.

Industrial-scale microbial 2,3-BD production has been still in its early stage despite vast knowledge about biosynthesis pathway and effects of diverse factors on this process. A number of microbial species accumulate 2,3-BD but only a few do so in what might be considered significant quantities. Its efficient producers are risk group 2 (pathogenic) microorganisms such as *Klebsiella oxytoca*, *K. pneumoniae*, *Enterobacter aerogenes* and *Serratia marcescens*, which convert not only glucose but also other sugars occurring in hydrolysates of cereals and various lignocellulosic materials and glycerol to the diol. Also sugar beet [1] and sugarcane [7] molasses are suitable feedstocks for the diol synthesis by these bacteria. Until now, the highest 2,3-BD concentrations were achieved in fed-batch cultures of *S. marcescens* (152 g/L) [31, 32] and *K. pneumoniae* (150 g/L) [16], which cannot be used in industry because of the strict safety regulations. To facilitate the large-scale diol production by *K. pneumoniae*, its mutants devoid of pathogenicity factors like the capsule and outer core lipopolysaccharides were obtained by genetic modifications [24]. These nonpathogenic mutants are believed

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to be used in industry in the near future, however, none of them has hitherto reached the commercial stage.

Attractive alternative producers of 2,3-BD are *Bacillus* strains, which are nonpathogenic and exhibit satisfactory fermentative capabilities [5, 11]. Particularly promising *Bacillus* species are *B. licheniformis* DSM 8785, which produced 144.7 g/L diol from glucose in fed-batch fermentations in shake flasks [11] and *B. licheniformis* ATCC 14580, which accumulated 103 g/L diol during simultaneous saccharification and fermentation (SSF) of inulin [14].

Bacillus species produce the levorotatory (R,R)-2,3-BD and the optically inactive meso-2,3-BD. The wild-type strains accumulate the diol in the late-log or stationary phase of growth. To reduce fermentation time and increase 2,3-BD productivity, mutant strains which are able to express *bdhA* gene encoding acetoin reductase (converting acetoin to 2,3-BD) in the early log phase were obtained using genetic engineering methods [3].

For both aerobic bacteria of the genus *Bacillus* and facultative anaerobes belonging to the species *K. oxytoca*, *K. pneumoniae*, *E. aerogenes* and *S. marcescens*, 2,3-BD is a product of anaerobic metabolism, generated to increase NAD^+/NADH ratio. To find a balance between biomass growth and diol synthesis, usually intensive agitation and aeration are conducted in the first phase of fermentation and then microaerophilic conditions are established [3, 5]. However, cultivation of 2,3-BD producers under aerobic conditions also enables obtaining high diol levels [11].

One of advantages of *Bacillus* species is that they convert various sugars to 2,3-BD. As raw materials constitute a large part of microbial 2,3-BD production costs, the usage of lignocellulosic wastes may reduce the latter [5]. In past decades, the relatively high prices of conventional sugar substrates have been identified as a major factor affecting the economic viability of 2,3-BD fermentation. The replacement of food-grade glucose with sugar feedstocks derived by hydrolysis of inexpensive food processing residues is a promising approach to cutting costs of 2,3-BD biosynthesis. As the residues and their hydrolysates usually contain not only sugars but also other substances that are essential for microbial growth and metabolic activity, for instance vitamins and minerals, there is no need for additional supplementation of fermentation media. Minerals are also necessary for microbial 2,3-BD production. This was clearly demonstrated by the mentioned above high diol titers reported for *B. licheniformis* DSM 8785, which was cultivated in a rich medium, containing numerous micro- and macro-elements apart from glucose [11]. Also the study on acetoin reduction to 2,3-BD by acetoin reductase from *B. licheniformis* strains showed that this conversion is affected by minerals, including Mn^{2+}, Zn^{2+} and Ba^{2+} ions [30]. Thus, application of agro-industrial byproducts for 2,3-BD synthesis helps not only to solve disposal problems but also to replace expensive culture media supplements.

One of abundant processing wastes is apple pomace [27]. This produced worldwide, solid residue after juice pressing represents around 30 % of apple dry matter and has been mainly utilized as a fodder for ruminants despite low digestibility and nutritional value, resulting from the high lignin\cellulose ratio and low protein content. Apple pomace is also used as an abundant and inexpensive source of pectin for food manufacturing. The depectinization is beneficial because the material is partially pretreated to facilitate enzymatic degradation of cellulose and hemicelluloses. Currently, apple pomace has been increasingly used in biotechnology that helps to avoid environmental pollution related to direct disposal of this highly biodegradable waste. Despite numerous studies on apple pomace valorization, application of depectinized apple pomace for microbial 2,3-BD synthesis has not been proposed by other authors.

The objective of this study was to evaluate the efficiency of the nonpathogenic bacterial strain *B. licheniformis* NCIMB 8059 in 2,3-BD production from enzymatic hydrolysate of depectinized apple pomace via batch and fed-batch fermentations conducted in shake flasks and fermenters. The strain was selected based on literature data related to 2,3-BD synthesis by *B. licheniformis* bacteria.

Materials and methods

Materials

The commercial, depectinized and dried apple pomace used in this study was purchased from Mega-Sort Ltd. (Kraków, Poland). It was stored at 10 °C until enzymatic hydrolysis. The other byproducts from food processing that were also characterized in terms of chemical composition (all of them are presented in Table 1) were kindly donated by food manufacturers (Poland). All chemicals used in the study were analytical grade. They were purchased from Sigma-Aldrich (USA) unless otherwise stated.

Enzymatic preparations

Preparations used for enzymatic apple pomace saccharification were as follows: a multienzyme preparation from *Aspergillus niger* IBT 90 (Pectovin Ltd., Jasło, Poland) fabricated according to a technology developed in the Institute of Technical Biochemistry Lodz University of Technology [23]; Filtrase Premium (DSM, UK); Cell Conc L, Denimax 991, Cellustar XL, Cellusoft® CR, Cellusoft™ L, Cellusoft™ APL, Optimash™ VR, and Optimash™ BG (Novozymes A/S, Denmark).

Table 1 Composition of depectinized apple pomace and other tested byproducts from food processing

Material	Concentration (g/100 g d.w.)			
	Cellulose	Hemicelluloses and starch	Lignin	Reducing sugars (soluble)
Apple pomace	43.2 ± 0.50	24.3 ± 0.28	20.3 ± 0.23	5.0 ± 0.07
Sugar beet pulp	33.0 ± 0.40	32.0 ± 0.37	4.1 ± 0.06	2.1 ± 0.01
Sugar beet cossettes	33.0 ± 0.40	32.0 ± 0.37	4.1 ± 0.06	3.7 ± 0.06
Sugar beet leaves	27.2 ± 0.32	27.1 ± 0.30	38.2 ± 0.44	0.0
Potato pulp	16.0 ± 0.15	65.0 ± 0.64	2.1 ± 0.01	0.6 ± 0.05
Chokeberry pomace	33.1 ± 0.39	32.1 ± 0.39	23.0 ± 0.25	4.5 ± 0.03
Rapeseed cake	12.0 ± 0.11	28.0 ± 0.29	32.7 ± 0.32	0.4 ± 0.00
Soybean cake	6.1 ± 0.09	23.7 ± 0.27	36.3 ± 0.38	0.2 ± 0.00
Sunflower cake	22.0 ± 0.26	25.2 ± 0.28	17.0 ± 0.19	0.6 ± 0.00
Evening primrose cake	30.0 ± 0.35	21.1 ± 0.25	28.1 ± 0.31	0.4 ± 0.00

Pretreatment of dried apple pomace

Batches of dried apple pomace were suspended in deionized water (800 mL per 100 g dry weight) and thermally processed by autoclaving (121 °C, 20 min) prior to enzymatic digestion.

Enzymatic saccharification

Processes of enzymatic apple pomace saccharification were conducted in at least triplicate at 50 °C for 48 h (with mixing every 4–8 h) and at enzyme/substrate ratio of 1 mL/10 g dry weight in 5 L stainless steel reactors. Resulting hydrolysates were filtered through a nylon cloth, and monosaccharides and fermentation inhibitors contained in the filtrates were quantified. The filtrates were lyophilized and kept at +4 °C until use. Dry weight of enzymatic digestion insoluble residues was determined gravimetrically after drying to a constant weight at 105 °C.

Microorganism and culture media composition

The strain used for 2,3-BD synthesis was *Bacillus licheniformis* NCIMB 8059, purchased from The National Collection of Industrial, Food and Marine Bacteria (UK). This alkaliphilic strain grew over the pH range from 7.0 to 10.0. The strain was maintained at 4 °C on agar slants [15 g/L nutrient agar (BD, USA), 20 g/L glucose (POCh, Poland), 10 g/L yeast extract (BD, USA), and 20 g/L bactopeptone (BD, USA)]. The seed medium contained 10 g/L Nutrient broth (BD, USA). The fermentation medium for 2,3-BD biosynthesis contained 3.2 g/L yeast extract (BD, USA), 6.2 g/L NH_4Cl (POCh, Poland), 1.5 g/L Na_2HPO_4 (POCh, Poland) and a carbon source, being either a pure monosaccharide [glucose (POCh, Poland) or fructose (POCh, Poland)] or the enzymatic apple pomace hydrolysate. In the first case, initial concentrations of glucose and fructose ranged from 10 to 160 g/L. Their solutions were autoclaved separately and added to the sterile fermentation medium. Initial concentrations of the apple pomace hydrolysate, containing 20.1 % glucose on a dry weight, were equivalent to the initial glucose concentrations of 10, 30, 43 and 50 g/L. Because the highest 2,3-BD production in shake flasks was observed for the concentration equivalent to 43 g/L of glucose this variant was used in further fermentation experiments. Another variant of fermentation medium contained the blend of apple pomace hydrolysate (equivalent to 23 g/L glucose) and 20 g/L pure glucose. pH of all the media was adjusted before sterilization (121 °C, 15 min) to values ensuring the pH of 6.4–6.5 after the autoclaving. The strain was also cultivated in the rich culture medium described by Jurchescu et al. [11] which was supplemented with the apple pomace hydrolysate (in amounts equivalent to 10–30 g/L glucose) instead of pure glucose.

Fermentations in shake flasks and bioreactors

For seed cultures, a full loop of *B. licheniformis* NCIMB 8059 biomass from a fresh agar slant (after 18 h cultivation at 30 °C) was inoculated in a 100 mL flask containing 10 mL of the seed medium and the cultivation was conducted for 7 h at 30 °C in a rotary shaker at 130 rpm. Submerged cultures were conducted at 37 °C and 130 rpm in flasks containing the fermentation medium [the loading rate of 25 % (v/v)], which was inoculated with 2 % (v/v) of the seed culture. The pitching rate corresponded to an initial optical density (OD at 660 nm) of 0.7. Fermentations were also carried out in 0.75 L Sixfors (Infors, Switzerland) fermenters and a 30 L Techfors (Infors, Switzerland) tank, at loading rates of 53 and 33 %, respectively. These processes were conducted at 37 °C, aeration of 1.2 L/L × min and agitation rate of 250 rpm. Fermentation

media were inoculated with 2 % (v/v) seed culture. An antifoam agent used in these processes was AntiFoam 204. In fed-batch cultures, appropriate doses of glucose (POCh, Poland) solution were added periodically into the culture broth to avoid carbon source depletion (glucose concentration was adjusted to 50 g/L each time). Samples of culture broth were collected at fixed time intervals and biomass density was measured. Concentrations of 2,3-BD, glucose and other reducing sugars were assayed in supernatants from centrifuging (10,000 rpm, 15 min, 4 °C). All the fermentation processes and the assays were conducted in at least triplicate.

Analytical methods

All assays were conducted in at least triplicate. Cellulose content in the apple pomace was determined gravimetrically after removal of lignin, hemicelluloses, proteins and other substances soluble in the mixture of acetic, nitric and trichloroacetic acids in 30 min at 100 °C [4]. Acid-insoluble (Klason) lignin was determined by the standard method [25]. The sum of hemicelluloses and starch was estimated based on concentrations of total reducing sugars and glucose in acid hydrolysates (25 % HCl, 3 h, 100 °C) of apple pomace [12]. Reducing sugars concentration was assayed using alkaline DNS solution, according to Miller [18]. Glucose was quantified enzymatically, using the commercial diagnostic kit (BioMaxima Ltd., Poland), employing glucose oxidase and peroxidase [2]. Activities of cellulases, xylanases, pectinases and invertase in the mentioned above multienzyme preparations were determined at 50 °C and pH 5.0 (0.1 M sodium acetate buffer solution, reaction time of 5 min) for 0.5 % carboxymethylcellulose (CMC), 0.5 % birch xylan, 0.5 % apple pectin and 1 % sucrose, respectively. Reducing sugars released from these substrates were determined according to Miller [18]. Enzyme activities were expressed as micromoles of reducing sugars released from the substrates in 1 min.

Bacterial biomass concentration in culture broths was determined based on measurements of absorbance at 660 nm.

2,3-BD was quantified in culture broth supernatants (30 µl samples) in at least triplicate by HPLC using a Merck-Hitachi liquid chromatograph system equipped with a Merck-Hitachi L-7250 autosampler, Merck-Hitachi L-7100 pump, a RI Merck Hitachi L-7490 detector and an Aminex HPX column (300 × 7.8 mm; Bio-Rad, USA). HPLC analyses were carried out at 65 °C using 0.005 M sulfuric acid as the mobile phase (flow rate of 0.5 mL/min). 2,3-BD was identified and quantified using the external standard method based on measurements of the surface area under peaks (measurements and computer integration of results were carried out using the Chromatography Data Station Software, Merck-Hitachi). 2,3-Butanediol has 3 isomers: *meso*-2,3-BD, D-(−)-2,3-BD, and L-(+)-2,3-BD. The D- and L-isomers were co-eluted in one peak (retention time, RT of 21.16 ± 0.05 min) visible in chromatograms while the *meso*-isomer (RT of 20.37 ± 0.06 min) was eluted separately. Quantification of 2,3-BD in culture broth supernatants was based on measurements of surface area under both these peaks. Before HPLC analyses, the supernatants were de-proteinized using 50 % (w/v) trichloroacetic acid solution and filtered through sterile microbiological filters (0.20 µm).

Monosaccharides contained in apple pomace hydrolysates were identified and quantified in at least triplicate by High-Performance Anion Exchange Chromatography–Pulsed Amperometric Detection (HPAEC–PAD) using a Dionex 600 system, governed by Chromeleon 6.40 software, and equipped with: a CarboPac PA1 analytical column (with a CarboPac PA1 Guard pre-column), an AS50 autosampler, a GP50 peristaltic pump for gradient formation and an ED50 detector. The column temperature and eluent flow rate were 20 °C and 1 mL/min, respectively. The following gradient of 3 eluents: A (deionized water), B (100 mM NaOH) and C (1 M sodium acetate in 100 mM NaOH) was used: 0–12 min: 3 % B and 97 % A, 12.1 min: 99 % B and 1 % C, 57 min: 60 % B and 40 % C, 57.1–82 min: 3 % B and 97 % A.

Concentrations of fermentation inhibitors such as furfural, 5-hydroxymethylfurfural (5-HMF), ferulic acid and 5-hydroxybenzoic acid in apple pomace hydrolysates were determined in triplicate using a HPLC system (Knauer, Germany) equipped with a C18 column (300 × 7.8 mm, Supelco), an autosampler, a pump for gradient formation and a Waters M2489 UV-detector. The inhibitors were eluted with a gradient of acetonitrile (0–100 %) in deionized water. Carbonyl chromatophores and double bonds were detected at 214 and 254 nm, respectively. The system was calibrated using standard solutions of furfural, 5-HMF, ferulic acid and 5-hydroxybenzoic acid (over the range of concentrations up to 0.5 µg/mL, 5 replicates for each of the standard solutions) to determine the correlation between the concentration of an inhibitor and the surface area under its peak.

To quantify metal ions in apple pomace hydrolysates, triplicate lyophilized samples (around 1 g d.w.) were mineralized using concentrated nitric (V) acid (Merck) and a Magnum 2 apparatus (Ertec, Poland). Concentrations of metal ions were determined using a GBC 932 Plus atomic absorption spectrometer (GBC, Australia) and relevant standard curves. Absorbance measurements were carried out at the following wavelengths: 766.5 nm (K), 589.0 nm (Na), 285.2 nm (Mg), 422.7 nm (Ca), 213.9 nm (Zn), 279.5 nm (Mn), 248.3 nm (Fe), 232 nm (Ni), 240.7 nm (Co), 217.0 (Pb), 279.5 nm (Mn), 357.9 nm (Cr), 324.7 nm

Table 2 Activities of tested enzymatic preparations and mean yields of reducing sugars released from thermally pretreated apple pomace by these preparations (24 h, 50 °C)

Multienzyme preparation	Activity (U/mL)				Reducing sugars released from apple pomace (μmol/g d.w.)
	Cellulases	Xylanases	Pectinases	Invertase	
1	36.2 ± 0.72	73.2 ± 0.98	123.2 ± 1.20	74.5 ± 0.87	2466.0 ± 45.0
2	33.5 ± 0.60	42.0 ± 0.71	34.0 ± 0.53	0.3 ± 0.00	1072.1 ± 28.0
3	36.7 ± 0.55	20.0 ± 0.33	0.0	0.0	724.1 ± 11.0
4	23.4 ± 0.42	18.0 ± 0.19	0.0	1.0 ± 0.01	651.3 ± 13.5
5	18.0 ± 0.27	39.4 ± 0.65	27.6 ± 0.18	0.0	1729.9 ± 26.4
6	39.2 ± 0.68	43.5 ± 0.73	20.5 ± 0.11	0.0	716.1 ± 6.6
7	32.0 ± 0.51	72.0 ± 0.74	20.8 ± 0.13	0.0	1692.5 ± 25.4
8	10.7 ± 0.30	0.0	16.2 ± 0.09	0.0	1574.1 ± 18.9
9	30.5 ± 0.41	57.9 ± 0.33	25.5 ± 0.11	0.0	1891.3 ± 13.8
10	16.7 ± 0.38	46.5 ± 0.45	18.5 ± 0.08	0.0	1023.4 ± 10.9

Means of triplicate assays (24 h enzymatic digestion of thermally pretreated (20 min at 121 °C) apple pomace, 1 mL of enzymatic preparation per 10 g d.w.)

1, preparation from *A. niger* IBT-90; 2, Filtrase Premium (DSM); 3, Cell Conc L (Novozymes); 4, Denimax 991 (Novozymes); 5, Optimash™ VR (Novozymes); 6, Optimash™ BG (Novozymes); 7, Cellustar XL (Novozymes); 8, Cellusoft® CR (Novozymes); 9, Cellusoft™ L (Novozymes); 10, Cellusoft™ APL (Novozymes). The activities were assayed at 50 °C and pH 5.0 as described in "Materials and methods"

(Cu), 228.8 nm (Cd) and 253.7 nm (Hg). The results were expresses as mean ± SD in mg of metal ions per 1 g d.w. of the hydrolysate.

Results

Composition of apple pomace and its hydrolysates

The depectinized apple pomace was selected as a source of glucose and other fermentable sugars for 2,3-BD biosynthesis because of the relatively high content of cellulose (43.2 % d.w.), hemicelluloses (24.3 % d.w.) and soluble reducing sugars (5 % d.w.) compared to the other characterized byproducts from food processing that are presented in Table 1, including sugar beet pulp, potato pulp and cakes from oil pressing. The drawback of apple pomace used in this study was a high lignin level (20.3 % d.w. Klason lignin). However, certain residues like sugar beet leaves, soybean, rapeseed and evening primrose cakes, and chokeberry pomace contained even more lignin. This polymer protects chains of cellulose and hemicelluloses from the attack of cellulases and hemicellulases that reduces the extent of their enzymatic saccharification. The composition of apple pomace shown in Table 1 is consistent with data presented by Nawirska and Kwaśniewska [19] but in general, it is difficult to compare our results with chemical parameters of apple pomace, reported in literature, because they depend on the apple variety, technology of processing and analytical protocols [27]. Despite around 20 % d.w. lignin content, its extraction with bases or organic solvents was omitted not to generate additional costs and environmentally harmful wastes and apple pomace was subjected only to relatively mild thermal pretreatment (autoclaving of its dense suspensions in water for 20 min at 121 °C) before enzymatic digestion. Autoclaving increased the dynamics of saccharification and suppressed microbial contamination, appearing when it was omitted (unpublished data). Then, triplicate portions of thermally pretreated material were treated with enzymatic preparations listed in "Materials and methods" to select the most efficient of them. Results shown in Table 2 demonstrate that the highest yield of reducing sugars was achieved using the multienzyme preparation from *A. niger* IBT 90, containing cellulases, xylanases, pectinases and invertase. Therefore, this preparation was used to produce hydrolysates of apple pomace for 2,3-BD production. The insoluble residue after digestion with this preparation accounted for approximately 35 % apple pomace dry weight and pH of the digests was around 3.1. Their filtrates contained 23.8–30.5 mg/mL glucose while the total reducing sugars concentration was 82.6–105.9 mg/mL. HPAEC-PAD analysis revealed that the hydrolysates contained tenfold less xylose than glucose while the level of fructose was higher than that of glucose (Table 3). Apart from the sugars, the apple pomace hydrolysate contained minerals, including sodium (around 4 mg/g d.w.), magnesium (around 3 mg/g d.w.), potassium (around 0.6 mg/g d.w.), calcium (around 0.4 mg/g d.w.), iron (around 0.03 mg/g d.w.), manganese (around 0.01 mg/g d.w.) and zinc (around 0.003 mg/g

Table 3 Monosaccharides released from apple pomace by the multienzyme preparation from *A. niger* IBT 90

Monosaccharide	Percentage of the hydrolysate dry weight	Percentage of total reducing sugars
Glucose	20.1 ± 0.50	28.8 ± 0.59
Fructose	39.9 ± 0.68	57.1 ± 0.81
Galacturonic acid	4.8 ± 0.16	6.9 ± 0.23
Xylose	1.7 ± 0.18	2.4 ± 0.16
Arabinose	1.6 ± 0.15	2.3 ± 0.19
Galactose	1.0 ± 0.08	1.4 ± 0.13
Mannose	0.5 ± 0.01	0.7 ± 0.02
Rhamnose	0.1 ± 0.00	0.1 ± 0.00

Thermally pretreated apple pomace (20 min, 121 °C) was digested with *A. niger* IBT 90 multienzyme preparation (1 mL/10 g d.w., 48 h, 50 °C) and the hydrolysates were filtered. The filtrates were lyophilized to dryness and analyzed by HPAEC-PAD as described in "Materials and methods"

Table 4 Concentrations of metal ions in the lyophilized apple pomace hydrolysate

Element	Concentration (mg/g)
Na	4.048 ± 0.048
Mg	3.011 ± 0.006
K	0.640 ± 0.010
Ca	0.405 ± 0.002
Fe	0.030 ± 0.001
Mn	0.010 ± 0.000
Zn	0.003 ± 0.001

Concentrations of Ni, Co, Pb, Cr, Cu, Cd and Hg were below detection levels

d.w.) (Table 4). Advantageously, concentrations of toxic metal ions like Ni, Co, Pb, Cr, Cu, Cd and Hg were below their detection levels. Also concentrations of fermentation inhibitors were relatively low (approximately 1000-fold lower compared with those of reducing sugars), of 0.03, 7.6×10^{-3}, 20.3×10^{-3}, and 1.1×10^{-3} mg/mL, for ferulic acid, 5-hydroxybenzoic acid, furfural and 5-HMF, respectively. This was ascribed to the mild conditions of apple pomace pretreatment (20 min, 121 °C) before enzymatic digestion because when it was subjected to more drastic thermal pretreatment (2 h, 100 °C), its enzymatic hydrolysates contained more fermentation inhibitors, and neither the growth of *B. licheniformis* NCIMB 8059 nor 2,3-BD biosynthesis was observed in fermentation media containing the latter hydrolysates (unpublished data).

2,3-Butanediol production in batch cultures

Batch cultures of *B. licheniformis* NCIMB 8059 were conducted as described in "Materials and methods" in shake flasks and 0.75 L fermenters, in the simple medium containing yeast extract, NH_4Cl, Na_2HPO_4 and either pure sugars or the enzymatic hydrolysate of apple pomace, which was obtained using the multienzyme preparation from *A. niger* IBT 90 (Table 2), or its blend with glucose. Because of the simple composition, supplementation of this medium with the complex carbon source such as the apple pomace hydrolysate, which contained not only the sugars but also minerals (Tables 3, 4), was expected to give discernible effects. The results shown in Table 5 demonstrate that when either pure glucose or fructose (10–160 g/L) was the sole carbon sources in the culture medium, the *Bacillus* strain synthesized 2,3-BD in neither shake flasks nor 0.75 L fermenters. These results suggest that the simple medium did not contain all nutrients that are essential for 2,3-BD biosynthesis by *B. licheniformis* NCIMB 8059. To provide *B. licheniformis* NCIMB 8059 not only with sugars but also essential minerals, the simple culture medium was supplemented with enzymatic apple pomace hydrolysate instead of pure glucose or fructose. Amounts of 2,3-BD produced by the strain in shake flasks increased (up to 23.76 g/L in 36 h) with the initial concentration of the hydrolysate but only in the range equivalent to glucose levels from 10 to 43 g/L (Table 5). Supplementation of the culture medium with a mixture of pure glucose (20 g/L) and the hydrolysate (their sum was equivalent to glucose concentration of 43 g/L) increased not only 2,3-BD level to 27.14 g/L (in 32 h) but also its yield (from 0.36 to 0.44 $g/g_{red\ sug}$). When *B. licheniformis* NCIMB 8059 was cultivated in the rich medium, described by Jurchescu et al. [11], in which glucose was replaced with the apple pomace hydrolysate (equivalent to glucose concentration of 10 g/L) only 10.05 g/L 2,3-BD was observed after 12 h. When larger amounts of the hydrolysate were added to the rich medium (equivalent to glucose level of 30 g/L) the strain did not produce 2,3-BD. Apparently, too high concentration of nutrients inhibited 2,3-BD biosynthesis by *B. licheniformis* NCIMB 8059.

Process up-scaling, it means the replacement of shake flasks by 0.75 L fermenters only slightly affected the efficiency of 2,3-BD production by *B. licheniformis* NCIMB

Table 5 The effect of carbon source on the maximum 2,3-BD concentration, yield and productivity in batch fermentations conducted in shake flasks and 0.75 L fermenters

Carbon source	Time (h)	2,3-BD concentration (g/L)	Yield ($g/g_{red\ sug}$)	Productivity (g/L × h)
Glucose (10–160 g/L)	0–120	0.00/0.00[a]	0.00/0.00[a]	0.00/0.00[a]
Fructose (10–160 g/L)	0–120	0.00/0.00[a]	0.00/0.00[a]	0.00/0.00[a]
Apple pomace hydrolysate[b]				
10 (g/L)	12	11.60 ± 0.10	0.47 ± 0.02	0.97 ± 0.02
30 (g/L)	36	16.41 ± 0.21	0.30 ± 0.01	0.46 ± 0.01
43 (g/L)	36/32[a]	23.76 ± 0.18/27.55 ± 0.14[a]	0.36 ± 0.01/0.39 ± 0.01[a]	0.66 ± 0.01/0.86 ± 0.02[a]
50 (g/L)	0–60	0.00	0.00	0.00
Apple pomace hydrolysate and pure glucose (23[b] g/L + 20 g/L, respectively)	32/28[a]	27.14 ± 0.22/31.04 ± 0.26[a]	0.44 ± 0.01/0.48 ± 0.01[a]	0.85 ± 0.01/1.11 ± 0.02[a]

[a] Maximum 2,3-BD production in the 0.75 L fermenter

[b] Shown as the concentration of glucose contained in the hydrolysate

Table 6 2,3-BD production by *B. licheniformis* NCIMB 8059 in fed-batch processes conducted in shake flasks

Process	Initial glucose concentration (g/L)	Feeding with glucose (g/L)	Process duration[d] (h)	Max 2,3-BD conc. (g/L)	Yield ($g_{2,3\text{-BD}}/g_{red\ sug}$)	Productivity (g/L × h)
1	43	3 × 50	138	102.6 ± 1.05	0.48 ± 0.01	0.74 ± 0.02
2	43	4 × 50	162	112.2 ± 1.30	0.47 ± 0.01	0.69 ± 0.03
3[a]	43	3 × 50	163	106.1 ± 1.10	0.48 ± 0.01	0.65 ± 0.02
4[b]	43	3 × 50	163	113.0 ± 1.20	0.49 ± 0.01	0.69 ± 0.04
5	23 + 20[e]	3 × 50	84	87.8 ± 0.90	0.49 ± 0.01	1.04 ± 0.07
6	23 + 20[e]	4 × 50	94	97.6 ± 0.75	0.47 ± 0.01	1.04 ± 0.08
7[c]	23 + 20[e]	4 × 50	168	110.5 ± 0.95	0.49 ± 0.01	0.66 ± 0.03

[a] After 72 h the agitation rate was reduced from 130 to 60 rpm

[b] After 72 h the fermentation was conducted under stationary conditions at 37 °C

[c] After 48 h the fermentation was conducted under stationary conditions at 37 °C

[d] Time corresponding to the maximum 2,3-BD concentration

[e] Pure glucose

8059 (Table 5). In the hydrolysate-based medium (at the initial glucose concentration of 43 g/L), the maximum 2,3-BD level reached 27.55 g/L after 32 h (the yield of 0.39 $g/g_{red\ sug}$) while in the medium containing the hydrolysate-glucose blend it was increased to 31.04 g/L after 28 h (the yield increased to 0.48 $g/g_{red\ sug}$). In both these media, the 2,3-BD productivity was increased by approximately 30 %, which is thought to result from more friendly conditions for bacterial growth in the fermenter compared with those in the shake flasks. Even small bioreactors ensure more uniform and stable fermentation conditions than shake flasks. The higher 2,3-BD concentrations (in shake flasks and fermenters) in the media supplemented with the blend of glucose and apple pomace hydrolysate compared to those supplemented with the hydrolysate are thought to be a consequence of lower content of fermentation inhibitors (like ferulic acid, furfural, 5-HMF) in the first media.

Fed-batch fermentations

2,3-BD production by *B. licheniformis* NCIMB 8059 was enhanced via fed-batch fermentations in shake flasks and fermenters. Fed-batch cultures were carried out to avoid too high initial concentration of carbon sources and maintain glucose concentration at most appropriate level for 2,3-BD biosynthesis [13]. To avoid accumulation of fermentation inhibitors contained in the hydrolysate of apple pomace, only glucose solution was added periodically to the culture broth during fed-batch processes.

Fed-batch fermentations in shake flasks

Fed-batch fermentations in shake flasks were conducted using 2 variants of culture media (Table 6). One of them contained the enzymatic hydrolysate of apple pomace in the concentration equivalent to 43 g/L glucose, while

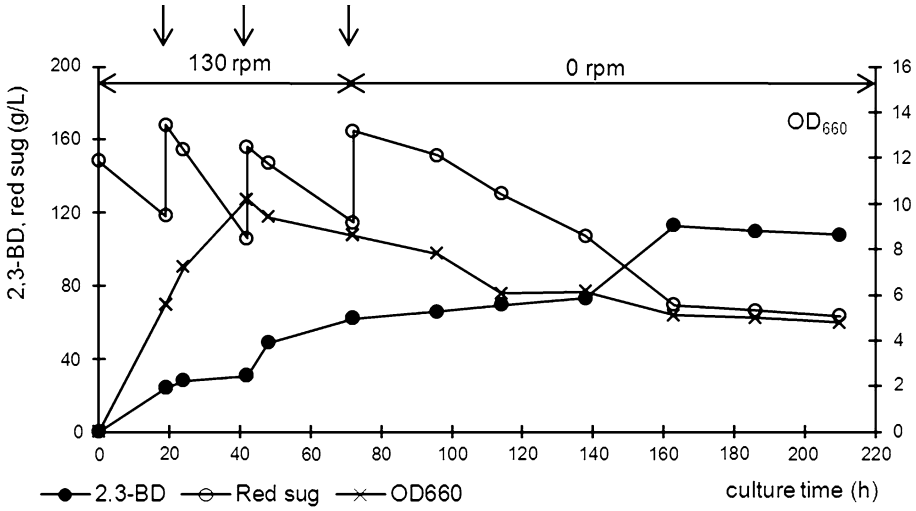

Fig. 1 Time course of fed-batch fermentation of *B. licheniformis* NCIMB 8059 in shake flasks with loading rate of 25 % (v/v), medium with 43 g/L glucose (contained in the apple pomace hydrolysate), initial pH 6.5 (pH-uncontrolled process), 37 °C, 130 rpm for 72 h, followed by the stationary conditions. *Arrows* indicate the feeding points

the second—its mixture (equivalent to 23 g/L glucose) with pure glucose (20 g/L). In the first case, the feedings were carried out either 3 times (after 18, 42 and 48 h) or 4 times (after 18, 24, 42 and 48 h). Each time the concentration of glucose in the culture broth was adjusted to 50 g/L. When the feedings were conducted 3 times, the concentration of 2,3-BD was increased more than fourfold (to 102.6 g/L after 138 h), compared with the batch fermentation (23.76 g/L in 36 h). In case of the process with 4 feedings, the concentration of 2,3-BD was increased to 112.2 g/L. Because this occurred 24 h later (after 162 h), compared to the fermentation with 3 feedings, the overall fermentation productivity was lower (around 0.69 versus 0.74 g/L × h) (Table 6). The same frequency of feedings with pure glucose (either 3 times, after 18, 24 and 48 h or 4 times, after 18, 24, 42 and 48 h, to rise glucose level to 50 g/L) was used when *B. licheniformis* NCIMB 8059 was cultivated in the culture medium supplemented with the mixture of apple pomace hydrolysate and pure glucose. In this medium, it synthesized less 2,3-BD since the highest concentrations of the diol were only 87.8 g/L in 84 h (for 3 feedings) and 97.6 g/L in 94 h (for 4 feedings). However, these 2,3-BD concentrations were above threefold higher than in the batch fermentations in shake flasks (27.14 g/L after 32 h).

Also, the effect of agitation and aeration conditions on 2,3-BD biosynthesis in fed-batch fermentations carried out in shake flasks was investigated. The aeration was the highest at the beginning of these processes to ensure optimum oxygen concentration for the growth of *B. licheniformis* NCIMB 8059 and then it was reduced to intensify metabolic processes that operate under conditions of limited oxygen availability, like biosynthesis of 2,3-BD. Therefore, the agitation rate was kept at 130 rpm during the phase of intensive growth of the *Bacillus* strain and was reduced to either 60 or 0 rpm (the stationary culture) during the phase of 2,3-BD biosynthesis. When the feedings with glucose were carried out in the first phase of fermentation in the apple pomace hydrolysate-based medium (3 times after 24, 48 and 72 h, at 130 rpm) to adjust glucose concentration to 50 g/L and then (after 72 h) the agitation rate was reduced to 60 rpm, the maximum 2,3-BD concentration reached 106.1 g/L after 163 h. When the second phase of fermentation was performed under stationary conditions, the 2,3-BD level was increased to 113 g/L after 163 h. Also, fermentations in the medium supplemented with the blend of the apple pomace hydrolysate and pure glucose were conducted with 4 feedings with glucose (after 18, 24, 42 and 48 h) and at variable agitation rate (at 130 rpm for the first 48 h followed by the stationary culture at 37 °C). In this case, the maximum concentration of 2,3-BD was 110.5 g/L after 168 h. The results of all fed-batch processes in shake flasks are presented in Table 6 and the time course of fermentation in which the agitation rate was reduced from 130 to 0 rpm after 72 h and which yielded the highest 2,3-BD concentration (113 g/L) is shown in Fig. 1.

The changes in the agitation rate during the fermentations carried out in shake flasks undoubtedly caused changes in the dissolved oxygen level but the expected increase in 2,3-BD production by *B. licheniformis* NCIMB 8059 was not observed. Because both under constant and variable agitation and aeration conditions the maximum concentration and productivity of 2,3-BD were almost the same (113.0 and 112.2 g/L, 0.69 g/L × h), the cultures in the fermenters were conducted at the constant agitation and aeration rate.

Fed-batch processes in 0.75 and 30 L fermenters

To up-scale 2,3-BD production, fed-batch fermentations were carried out in the 0.75 L Sixfors fermenters and the 30 L Techfors fermenter, in the culture medium supplemented

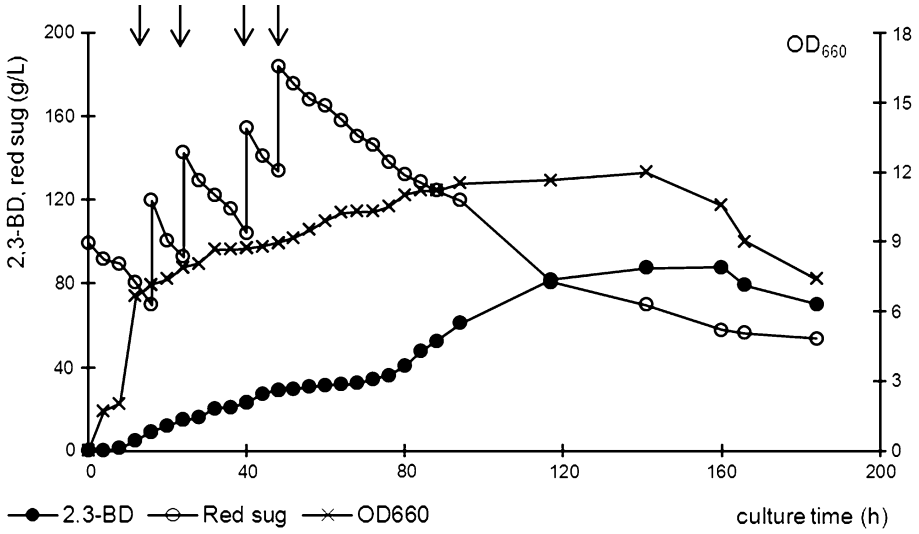

Fig. 2 Time course of fed-batch fermentation of *B. licheniformis* NCIMB 8059 in 0.75 L bioreactor, loading rate of 53 % (v/v), medium with the blend of apple pomace hydrolysate (equivalent to 23 g/L glucose) and 20 g/L pure glucose, initial pH 6.5 (pH-uncontrolled process), 37 °C, 250 rpm, aeration of 1.2 L/L × min. *Arrows* indicate the feeding points

Table 7 2,3-BD production by *B. licheniformis* NCIMB 8059 in fed-batch fermentations carried out in fermenters

Process duration[a] (h)	2,3-BD concentration (g/L)	Yield ($g_{2,3\text{-BD}}/g_{\text{red sug}}$)	Productivity (g/L × h)
0.75 L fermenter			
Apple pomace hydrolysate (43 g/L glucose) + 4 feedings with pure glucose			
184	77.58 ± 0.85	0.32 ± 0.01	0.42 ± 0.01
0.75 L fermenter			
Pure glucose (20 g/L) and apple pomace hydrolysate (23 g/L glucose) + 4 feedings with pure glucose			
160	87.71 ± 0.90	0.36 ± 0.01	0.55 ± 0.01
30 L fermenter			
Pure glucose (20 g/L) and apple pomace hydrolysate (23 g/L glucose) + 4 feedings with pure glucose			
94	72.39 ± 0.65	0.38 ± 0.01	0.77 ± 0.01

[a] Time corresponding to the maximum 2,3-BD concentration in the culture broth

with either the apple pomace hydrolysate (equivalent to 43 g/L glucose) or its mixture with pure glucose (23 g/L glucose and 20 g/L, respectively). The feedings with glucose were carried out 4 times (after 16, 24, 40 and 48 h) to adjust its concentration to 50 g/L. The fed-batch fermentations in the 0.75 L stirred tanks (Fig. 2) caused an approximately threefold increase in the maximum 2,3-BD concentration compared with the batch processes in the same scale (for both the carbon sources) (Tables 5, 7). Fermentation in the 30 L fermenter in the medium supplemented with the mixture of glucose and apple pomace hydrolysate yielded 72.39 g/L 2,3-BD (Fig. 3). Although this concentration was 15 g/L lower compared with that observed in the 0.75 L fermenters (Table 7), it was achieved in 94 h while in the smaller fermenters—in 160 h. Therefore, the productivity and yield of 2,3-BD were higher in the 30 L vessel than in the smaller ones (0.77 and 0.55 g/L × h, and 0.38 and 0.36 $g_{2,3BD}/g_{\text{red sug}}$, respectively). The maximum 2,3-BD concentration achieved in the fed-batch fermentations in the medium containing the mixture of the enzymatic apple pomace hydrolysate and pure glucose (with 4 feedings with glucose) was the highest in shake flasks (97.6 versus 87.7 g/L in the 0.75 L fermenter and 72.39 g/L in the 30 L vessel). In case of fed-batch fermentations in the medium containing the apple pomace hydrolysate and supplemented 4 times with pure glucose, the difference between the maximum 2,3-BD concentrations in shake flasks (112.2 g/L) and the 0.75 L fermenter (77.58 g/L) was even higher. The lower 2,3-BD concentrations achieved in the fermenters indicate that fermentation conditions need further optimization.

Discussion

The results of batch and fed-batch fermentations performed in this study showed that enzymatic hydrolysate of apple pomace, which has not been used as a sugar feedstock for 2,3-BD production by other authors, is a suitable source of not only sugars but also minerals that are essential for this process. In the absence of the complex mixture of minerals, *B. licheniformis* NCIMB

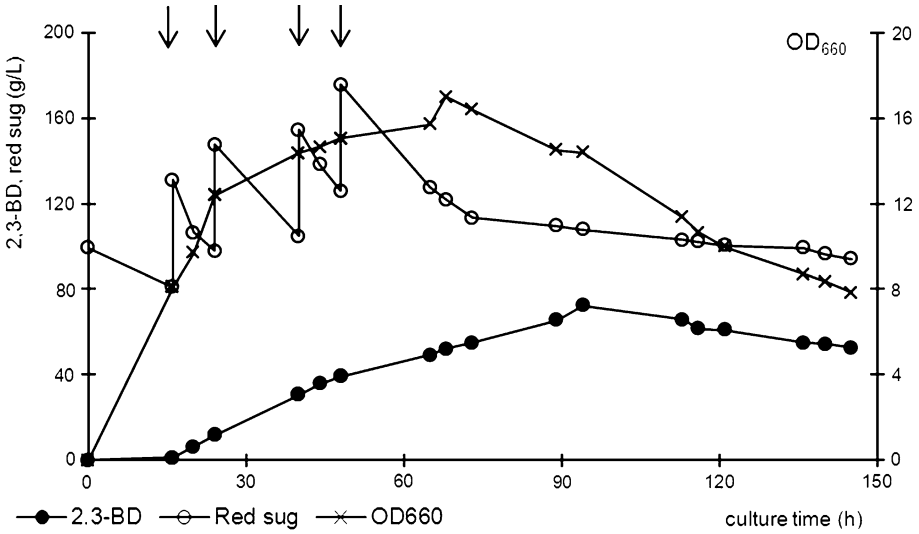

Fig. 3 Time course of fed-batch fermentation of *B. licheniformis* NCIMB 8059 in 30 L bioreactor, loading rate of 33 % (v/v), medium with the blend of apple pomace hydrolysate (equivalent to 23 g/L glucose) and 20 g/L pure glucose, initial pH 6.5 (pH-uncontrolled process), 37 °C, 250 rpm, aeration of 1.2 L/L × min. *Arrows* indicate the feeding points

8059 synthesized 2,3-BD neither from glucose nor from fructose. The replacement of pure sugars with the enzymatic apple pomace hydrolysate triggered the diol biosynthesis. This result is in compliance with findings of other authors, including Wang et al. [30] who observed the dependence of acetoin conversion to 2,3-BD on the presence of minerals in the fermentation medium. Also higher diol yields from natural substrates than from pure sugars seem to result among others from the occurrence of minerals in the first carbon sources. For instance, the replacement of glucose with inulin extract from Jerusalem artichoke tubers in the fermentation medium of *Paenibacillus polymyxa* ZJ-9 caused fivefold increase in 2,3-BD concentration (to around 38 g/L) [8]. Rich in minerals are also various sorts of molasses, which are excellent feedstocks for diverse fermentations, including 2,3-BD biosynthesis. One of examples is a high concentration of 2,3-BD (118 g/L) produced from sugar beet molasses by *K. oxytoca* [1].

The maximum 2,3-BD concentrations achieved in batch cultures of *B. licheniformis* NCIMB 8059 in the culture medium based on the apple pomace hydrolysate were around 23.8 and 27 g/L in shake flasks and 0.75 L fermenters, respectively. Partial replacement of the hydrolysate with glucose enabled to increase these concentrations to around 27 and 31 g/L, respectively. The latter diol concentration corresponded to the highest values of the yield (0.48 $g/g_{red\ sug}$) and productivity (1.11 g/L × h) reached in the batch fermentations. These concentrations are considerably higher than those reported by Perego et al. [21] for the same *B. licheniformis* strain (NCIMB 8059), which produced in batch fermentations only 6.44, 5.16 and 3.93 g/L 2,3-BD from cornstarch hydrolysate, glucose and sucrose (at low productivity of 0.126, 0.085 and 0.056 g/L × h), respectively. For comparison, Nilegaonkar et al. [20] achieved 8.70 and 6.65 g/L diol from glucose and sucrose, respectively, using a *B. licheniformis* strain.

2,3-BD concentrations which were achieved in fed-batch fermentations of *B. licheniformis* NCIMB 8059 were several fold higher compared to those from batch processes, which is consistent with results published, among others, by Jurchescu et al. [11]. The highest diol level, of 113 g/L (in 163 h), was observed in shake flasks in the medium based on the apple pomace hydrolysate with 3 feedings with glucose. Although the corresponding diol yield (0.49 $g/g_{red\ sug}$) was also high, the productivity (0.69 g/L × h) was relatively low. The latter parameter was increased to around 1.04 g/L × h, despite lower 2,3-BD levels (87.8 and 97.6 g/L) due to significantly shorter fermentation time (84 and 94 h, respectively) in fed-batch cultures in the medium based on the blend of hydrolysate and glucose. The maximum diol concentrations, yields and productivity achieved in fed-batch cultures conducted in either 0.75 or 30 L fermenters were lower compared to those in shake flasks (up to 87.7 g/L, 0.36 $g/g_{red\ sug}$, and 0.55 g/L × h, and 72.4 g/L, 0.38 $g/g_{red\ sug}$, and 0.77 g/L × h, respectively). This difference was thought to be caused by different agitation and aeration conditions in the shake flasks and fermenters. The agitation and aeration rates during successive phases of *B. licheniformis* NCIMB 8059 growth and 2,3-BD accumulation in the fermenters need optimization to achieve at least the same diol concentration, yield and productivity as were observed in the fed-batch fermentations in shake flasks.

The comparison of literature data related to the diol biosynthesis from various natural substrates by fed-batch fermentation, which are collected in Table 8, with the results of fed-batch processes performed in this study (Tables 6, 7), allows to conclude that *B. licheniformis* NCIMB 8059 is a promising 2,3-BD producer and may be applied in large-scale processes. Also, some other *B. licheniformis* strains

Table 8 Microbial 2,3-BD production based on natural substrates and fed-batch processes

Microorganism	Carbon source	Fermentation type	Max. 2,3-BD conc. (g/L)	Fermentation time (h)	References
Klebsiella pneumoniae CICC10011	Jerusalem artichoke tubers powder	Fed-batch SSF	84.00	40	[26]
Klebsiella pneumoniae CICC10011	Jerusalem artichoke tubers hydrolysates	Fed-batch SHF	60.95	56	[26]
Klebsiella pneumoniae SDM	Corncob molasses	Fed-batch	78.90	61	[28]
Klebsiella pneumoniae G31	Glycerol	Fed-batch	70.00	150	[22]
Klebsiella oxytoca ACCC 10370	Corncob acid hydrolysate	Fed-batch	35.70	60	[6]
Klebsiella pneumoniae CICC 10011	Jerusalem artichoke stalk and tuber hydrolysates	Fed-batch SSF	67.40	68	[13]
Enterobacter aerogenes	Sugarcane molasses	Fed-batch	98.69	36	[10]
Enterobacter cloacae subsp. *dissolvens* SDM	Cassava powder	Fed-batch SSF	93.90	47	[29]
Enterobacter cloacae CGMCC 605	Sugarcane molasses	Fed-batch	99.50	60	[7]
E. coli K19 MG1655	Seaweed hydrolysate	Fed-batch	14.10	45	[17]
Bacillus licheniformis X-10	Corn stover hydrolysate	Fed-batch	74.00	36	[15]
B. licheniformis ATCC 14580	Inulin hydrolysate	Fed-batch SSF	103.00	30	[14]
Bacillus licheniformis NCIMB 8059	Apple pomace hydrolysate	Fed-batch	113.00	163	This study

are not worse 2,3-BD producers from natural substrates than *Klebsiella* or *Enterobacter* species. For instance, *B. licheniformis* ATCC 14580 accumulated as much as 103 g/L 2,3-BD in the simultaneous saccharification and fermentation (SSF) in a medium based on inulin with 3 feedings with a mixture of inulin and specific glycoside hydrolase. The considerably shorter time of fermentation (30 h) gave rise to a high productivity of 3.4 g/L × h of this process [14]. The lower diol concentration, of 74.0 g/L (in 36 h), was achieved in fed-batch fermentations using a thermophilic *B. licheniformis* X-10 and corn stover hydrolysates [15]. Because this strain simultaneously assimilated both glucose and xylose, which were contained in the hydrolysates, the high 2,3-BD productivity (2.1 g/L × h) and yield (94.6 %) were observed. This productivity was higher than in case of the diol production from Jerusalem artichoke tubers by *K. pneumoniae* CICC10011. In the latter case, the separated hydrolysis and fed-batch fermentation (SHF) with 4 feedings of the culture medium with the hydrolysate, yielded 60.95 g/L 2,3-BD in 56 h at the productivity of 1.25 g/L × h [26]. Both these parameters were increased (to 84 g/L 2,3-BD in 40 h and 2.29 g/L × h, respectively) through the SSF carried out with 3 feedings with the powdered Jerusalem artichoke tubers mixed with hydrolytic enzymes. The high 2,3-BD concentrations of 99.5 g/L (in 60 h) and 93.9 g/L (in 47 h) were obtained from sugarcane molasses using *E. cloacae* CGMCC 605 [7] and from cassava powder using *E. cloacae* subsp. *dissolvens* SDM [29], respectively. Less diol was produced from corncob molasses by *K. pneumoniae* SDM (78.9 g/L in 61 h) in fed-batch fermentations [28]. Another *K. pneumoniae* strain (G31) efficiently converted glycerol to 2,3-BD and accumulated around 70 g/L diol in 150 h in fed-batch fermentations with forced pH fluctuations [22]. The diol concentrations that were achieved from corncob acid hydrolysate using *K. oxytoca* ACCC10370 (35.7 g/L 2,3-BD in 60 h) [6] and seaweed hydrolysate using *E. coli* K19 MG1655 (14.10 g/L in 45 h) [17] were significantly lower compared to those obtained using *B. licheniformis* NCIMB 8059.

Conclusions

The results of this work demonstrate that apple pomace being an abundant, under-utilized, renewable agro-industrial residue may be used as a feedstock for biosynthesis of 2,3-BD using the wild-type nonpathogenic strain, *B. licheniformis* NCIMB 8059. Production of the diol is one of potential options of valorization of this easily available residue.

Mild pretreatment of depectinized apple pomace before digestion by the multienzyme preparation from *A. niger* IBT-90 resulted in low levels of fermentation inhibitors in the hydrolysate, which contained not only simple sugars (mainly glucose and fructose) but also the minerals essential for the diol accumulation. The high 2,3-BD concentration of 113 g/L (in 163 h, the yield and productivity of 0.49 $g/g_{red\ sug}$ and 0.69 g/L × h, respectively) achieved in fed-batch fermentations (in shake flasks) in the simple medium based on this hydrolysate (with 3 feedings with glucose) was around fourfold higher compared to batch

fermentations (around 27 g/L in 32 h and 31 g/L in 28 h, in shake flasks and 0.75 L fermenters, respectively). Up-scaling of fed-batch fermentation and partial replacement of the enzymatic apple pomace hydrolysate with pure glucose enabled to produce 87.71 g/L 2,3-BD in 160 h in the 0.75 L fermenters, and 72.39 g/L 2,3-BD in 94 h in the 30 L stirred tank. Optimization of agitation and aeration conditions in fermenters is necessary to increase 2,3-BD titers.

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