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Review

Current Advances in Microbial Production of Acetoin and 2,3-Butanediol by *Bacillus* spp.

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Abstract: The growing need for industrial production of bio-based acetoin and 2,3-butanediol (2,3-BD) is due to both environmental concerns, and their widespread use in the food, pharmaceutical, and chemical industries. Acetoin is a common spice added to many foods, but also a valuable reagent in many chemical syntheses. Similarly, 2,3-BD is an indispensable chemical on the platform in the production of synthetic rubber, printing inks, perfumes, antifreeze, and fuel additives. This state-of-the-art review focuses on representatives of the genus *Bacillus* as prospective producers of acetoin and 2,3-BD. They have the following important advantages: non-pathogenic nature, unpretentiousness to growing conditions, and the ability to utilize a huge number of substrates (glucose, sucrose, starch, cellulose, and inulin hydrolysates), sugars from the composition of lignocellulose (cellobiose, mannose, galactose, xylose, and arabinose), as well as waste glycerol. In addition, these strains can be improved by genetic engineering, and are amenable to process optimization. *Bacillus* spp. are among the best acetoin producers. They also synthesize 2,3-BD in titer and yield comparable to those of the pathogenic producers. However, *Bacillus* spp. show relatively lower productivity, which can be increased in the course of challenging future research.

Keywords: 2,3-butanediol; acetoin; *Bacillus*

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1. Introduction

The contemporary production of acetoin and 2,3-butanediol (2,3-BD) is primarily related to the use of oil and fossil fuels. However, the rising environmental problems such as global warming and greenhouse gas emissions require rapid replacement of chemical methods with environmentally friendly and sustainable biotechnologies for the synthesis of both platform chemicals [1]. More than fifteen years ago, the United States Department of Energy classified acetoin among the 30 chemical compounds, stating that its microbial synthesis needs priority scientific development [2–4]. Recent forecasts show that the global acetoin market will grow by 5.79% annually, and the market size of 2,3-BD will reach USD 300 million by 2030 [5,6].

Acetoin has a pleasant butter odor; therefore, it is widely applied as a taste and aroma enhancer in butter, milk, yogurt, and many other foods manufacturing [7,8]. In nature, acetoin is found in fruits (apple, blackcurrants, blackberries, and cantaloupes), wheat, vegetables (broccoli and brussels sprouts), maple syrup, or fermented products such as vinegar, butter, yogurt, and wine. This compound is responsible for the aroma of strawberries, raspberries, vanilla, butter, coconut, and coffee, and therefore, it is used as a flavoring in cosmetics and the liquids for electronic cigarettes [9]. In its optically pure form, acetoin is a precursor for the synthesis of heterocyclic compounds, as 2,3,5,6-tetramethylpyrazine, α-hydroxy ketone, liquid crystal composites, and pharmaceutical intermediates [10]. Currently, most of the industrial production of acetoin is carried out by chemical synthesis by partial reduction of diacetyl through deoxidization with zinc or other catalysts [11]. Other common approaches of acetoin production are the selective

oxidation of 2, 3-butanediol [12], and the oxidation of butan-2-one (MEK), followed by basic hydrolysis and hydrogenation of acetaldehyde [13].

Mainly used in the food, pharmaceutical, polymer, and cosmetic industries, 2,3-BD is used in the production of printing ink, perfumes, plasticizers (cellulose nitrates, polyacrylates, and polyvinyl chlorides, etc.), explosives, humidifiers, and emollients [14]. It is less toxic to microbial cells than other alcohols, but at the same time, its energy content is comparable to other liquid fuels obtained by microbial synthesis. The equimolar mixture of ethanol and 2,3-BD has an energy content of 27,700 kJ/kg, and comparatively pure ethanol, 2,3-BD, and methanol contain 29,100, 27,200, and 22,100 kJ/kg, respectively [15]. Dehydration of 2,3-BD results in the formation of methyl ethyl ketone (MEK), an important solvent and fuel additive [16,17]. In addition, 2,3-BD can be converted to 1,3-butadiene, which is the building block of synthetic rubber [18]. The dehydrogenation product of 2,3-BD, diacetyl, is a valuable flavoring in food products, such as original yogurt [19]. Esterification of 2,3-BD with maleic acid yields a polymeric material (polyurethane maleamide (PUMAs) used in medicine for the manufacturing of implants for cardiovascular disease [20]. Furthermore, 2,3-BD has antiseptic properties: 0.1% solution of the compound possesses a bactericide effect towards many pathogenic bacteria [21]. Due to its low freezing point of −60 °C, 2,3-BD can be used as an antifreeze [22,23]. It is also very promising as a cryoprotectant with properties similar to those of propylene glycol, DMSO, and glycerol [24] and creates fewer osmotic changes in the cell [25].

As a primer microbial metabolite, acetoin has been used in bacterial taxonomy as a classification marker of *Enterobacteriaceae* since 1898, when Voges and Proskauer, German bacteriologists at the Institute for Infectious Diseases in Berlin developed the legendary test that detects the oxidation of acetylmethylcarbinol to diacetyl in the presence of a strong base. Besides Gram-negative *Klebsiella pneumoniae*, *Salmonella*, and *Enterobacter aerogenes*, acetoin can be synthesized by many microorganisms, including Gram-positive wild-type strains of *Bacillus subtilis*, *Leuconostoc citrovorum*, *Leuc. mesenteroides*, *Lactococcus lactis*, *Lacticaseibacillus casei*, and yeasts *Saccharomyces carlsbergensis*, *Kloeckera apiculata*, and *Hanseniaspora guilliermondii* [26–34]. Most of them, however, produce less than 1 g/L acetoin and until 2011 the highest titer of 41.26 g/L was achieved by *B. licheniformis* [35].

Biotechnological production of 2,3-BD dates back to 1906, when Harden and Walpole studied its production by *Klebsiella pneumoniae* [36]. In 1926, Donker noticed it as a metabolic product of *Paenibacillus polymyxa* (*Bacillus polymixa*), but the idea for the industrial production of 2,3-BD was first suggested by Fulmer et al. in 1933 [37]. Later, during World War II, the interest in the production of 2,3-BD became more significant than ever. Due to the need for synthetic rubber, the first pilot plant for the production of 2,3-BD was built, as the process was carried out by microbial fermentation of strains of *K. oxytoca* and *P. polymyxa*. However, a large-scale industrial production never began, because it turns out that it is more cost-effective to produce 1,3-butadiene from oil. In the 1970s, the interest in 2,3-BD biotechnology returned, mainly due to rising oil prices. Pilot plants were built again in the United States, and later in China, this time with the idea of converting cheap lignocellulosic waste [38,39]. However, to date, despite great scientific progress, the industrial microbial production of 2,3-BD has not been established.

2,3-BD and acetoin are products of mixed-acid fermentation performed by many bacterial species with affiliation to genera *Klebsiella*, *Enterobacter*, *Serratia*, *Raoultella*, *Paenibacillus*, and *Bacillus* [40–45]. The first four of them belong to risk group 2 (pathogenic bacteria) and are therefore not suitable for industrial fermentation due to safety rules [15]. Conversely, bacilli are Generally Regarded as Safe (GRAS) producers of acetoin and 2,3-BD, suitable for large-scale industrial bioprocesses. These species are particularly promising due to their ability to grow well in both microaerophilic and aerobic conditions, their cultural adaptability, and in particular the capability to utilize a wide range of substrates. On the other hand, D(−), or (2R, 3R) stereoisomer of 2,3-BD is more desirable by the industry, because it could be used as an antifreeze. This isomer, as well as R-acetoin, are valuable reagents in chiral syntheses. In contrast to the strains of genus *Klebsiella* and *Enterobacter*,

which produce L(+) and *meso*-2,3-BD, many members of the genus *Bacillus* and *P. polymyxa* generate pure D(−) form [22]. However, current progress in microbial 2,3-BD production employing bacilli was mostly due to the development of bioprocess and strain engineering. That is why, in terms of 2,3-BD titer and yield, *Bacillus* spp. are already competitive to the "classical" pathogenic producers.

The members of genus *Bacillus* and *Paenibacillus polymyxa* (formerly *B. polymyxa*) are Gram-positive, motile, non-pathogenic bacteria ubiquitously spread in natural habitats. They are usually isolated from soil, water, and the rhizosphere [46–48], medicine plants [49], marine water [50], and even animals' guts [51,52]. Bacilli are widely used in the industrial manufacturing of enzymes, antibiotics, and exopolysaccharides with antimicrobial, antioxidant, and anticancer activities [53]. That is why the species is commonly used in horticulture and pharmacy, the biocontrol of plant diseases, and the development of probiotics [54–56].

This review summarizes the vast flow of experimental data involving the GRAS producers of 2,3-BD and acetoin, being the first focused on bacilli. Here we describe the significant advance in the employment of *B. subtilis* [57,58], *P. polymyxa* [59–62], *B. amyloliquefaciens* [63,64], and *B. licheniformis* [65–74]. In addition, the potential of the quite recently reported as 2,3-BD producers *B. atrophaeus*, *B. mojavensis*, *B. vallismortis*, *B. velesensis*, *B. tequilensis*, and *B. safensis* [69,75,76] for the valorization of plant biomass and agricultural wastes was assessed. The strains' limitations and the opportunities for their improvement by genetic engineering are also discussed.

2. Chemical Structure of Acetoin and 2,3-Butanediol and Their Stereoisomers

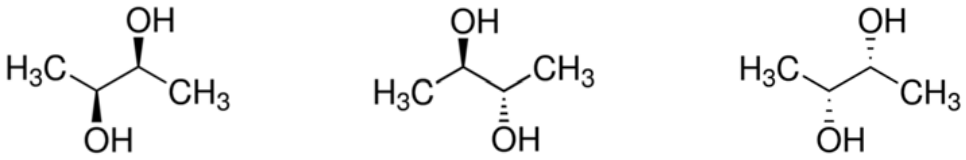
Acetoin (also named acetylmethylcarbinol), or 3-hydroxybutan-2-one, according to IUPAC, appears as slightly yellow liquid or crystals. It has a molecular weight of 88.11 g/mol and a boiling point of 143.0 °C. Acetoin has the molecular formula $C_4H_8O_2$ and exists in two chiral enantiomers: Levo-(R-), and Dextro-(S-), shown in Figure 1 [77].

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

Figure 1. Structural formulae of acetoin stereoisomers. (**a**) R-acetoin; (**b**) S-acetoin. Source: https://pubchem.ncbi.nlm.nih.gov (accessed on 3 November 2021).

Also known as 2,3-butylene glycol, dimethylene glycol, dimethyl ethylene glycol, or butane-2,3-diol (according to IUPAC), 2,3-Butanediol (2,3-BD) a is a compound that has the chemical formula $C_4H_{10}O_2$ and a molecular weight of 90.12 g/mol and density of 0.987 g/mL. It has three isomeric forms, two of which are optically active: L(+)-2,3-BD (2S, 3S) and D(−)-2,3-BD (2R, 3R), shown in Figure 2a,c, as well as an optically inactive form *meso*-2,3-BD (2R, 3S, or 2S, 3R), shown at Figure 2b [77]. In all three forms, 2,3-BD is found both as a colorless and odorless liquid and in crystalline form. It is soluble in water, alcohols, ethers, and ketones. In addition, 2,3-DB has a very high boiling point (depending on the stereoisomer, it varies between 177 °C and 182 °C) and a very low freezing point (−60 °C), which determines many of its characteristics and applications.

The key for the stereoisomer's formation is the enzyme specificity of butanediol dehydrogenase BDH, which is influenced not only by the nucleotide sequence of the responsible gene but also by factors such as carbon source, the mineral content of the medium (Ca, S, Mg), NADH/NAD^+ ratio, aeration, and the growth phase of the culture, etc. [67,78].



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

Figure 2. Stereoisomers of 2,3-bitanediol (2,3-BD). (**a**) L(+)-BD; (**b**) *meso*-2,3-BD; (**c**) D(−)-2,3-BD Source: https://pubchem.ncbi.nlm.nih.gov (accessed on 3 November 2021).

3. Metabolic Pathway for Acetoin and 2,3-Butanediol Synthesis by *Bacillus* spp.

Acetoin and 2,3-BD metabolic pathway can be determined both as anabolic and catabolic. The biological role of the synthesis of these compounds is to serve as an alternative to acid production in the course of mixed-acids fermentation and thus, to prevent excessive culture acidification. The second important function of the pathway is to support NADH/NAD^+ levels by the reversible transformation between acetoin and 2,3-BD, which is coupled with this redox balance. In addition, acetoin is an intermediate metabolite in amino acids biosynthesis. It, along with 2,3-BD, could be consumed by the microbial cells under starvation conditions.

In *Bacillus* spp. acetoin and 2,3-BD are produced from pyruvate, obtained from sugars and other carbohydrates metabolized either by the glycolytic pathway (hexoses) or via a combination with the pentose–phosphate metabolic pathway (pentoses). Both metabolites are obtained in the course of mixed-acid fermentation, which also generates other final metabolites such as lactic, acetic, succinic, and formic acids, ethanol, and glycerol. Depending on the type of producer and the cultivation conditions, these products are obtained in different proportions or do not accumulate at all. The same bacterial strain can produce predominantly 2,3-BD or acetoin as alternative final metabolites via the manipulation of process parameters.

The biosynthesis of 2,3-BD occurs through the intermediates α-acetolactate and acetoin, under the action of three key enzymes: (*i*) α-acetolactate synthase I/II/III (EC 2.2.1.6), which small and large subunits in bacilli are encoded by three genes: *ilvH*, *ilvB*, *alsS*; (*ii*) α-acetolactate decarboxylase (EC 4.1.1.5), encoded by *alsD* and catalyzing the conversion of α-acetolactate to acetoin; and (*iii*) 2,3-butanediol dehydrogenase (BDH), which displays multiple enzyme activities in bacilli, but its primary function is to reduce acetoin to 2,3-BD. Depending on the BDH type, R-acetoin can be reduced into *meso*-2,3-BD by R,S-BDH or D(−)2,3-BD by R,R-BDH, whereas S-acetoin could be converted into *meso*-2,3-BD by R,S-BDH, and L-2,3-BD by S,S-BDH.

In the presence of α-acetolactate and under aerobic conditions, acetoin is spontaneously decarboxylated to diacetyl (without enzymatic reaction), which under the action of diacetyl reductase (DAR) is irreversibly converted to acetoin. The secondary function of BDH in *Bacillus* spp. is to act as a stereospecific DAR.

According to the KEGG database [79], the conversion of pyruvate to 2,3-BD in *Bacillus* spp. can be presented by two reference pathways; the first, of *B. subtilis* subsp. *subtilis* 168 (Figure 3), and the second, of *B. licheniformis* ATCC 14580^T (Figures 3 and 4). The main difference between them is in *bdh* genes. *B. subtilis* genome contains *bdhA* (1041 base pairs) encoding (R,R)-butanediol dehydrogenase (346 amino acids), which produces D(−)2,3-BD from R-acetoin (EC 1.1.1.4). BdhA also displays DAR activity (EC 1.1.1.303) for diacetyl conversion to R-acetoin.

B. licheniformis ATCC 14580^T contains *meso*-BDH encoded by *budC* (EC 1.1.1-, 260 amino acids) with S-acetoin forming DAR stereo-specificity (EC 1.1.1.304). Figure 3 shows the conversion of the spontaneously formed diacetyl to S-acetoin, which, in turn, is reduced to (S,S)-2,3-BD by the same BDH BudC (EC 1.1.1.76). Although the gene encoding an enzyme with EC 1.1.1.4 has not been determined as active in the type strain ATCC 14580^T, it was identified in *B. licheniformis* strain MW3 by Ge et al. [45]. This enzyme, encoded by

gdh gene forms R-acetoin from diacetyl, and then D(−)2,3-BD stereoisomer (Figure 4), but also *meso*-2,3-BD from S-acetoin. Acetoin and 2,3-BD stereoisomers interconversion and the versatile catalytic activities of BDH are typical for *Bacillus* spp. However, this phenomenon is also the main obstacle to the successful production of 2,3-BD stereoisomers in pure form.

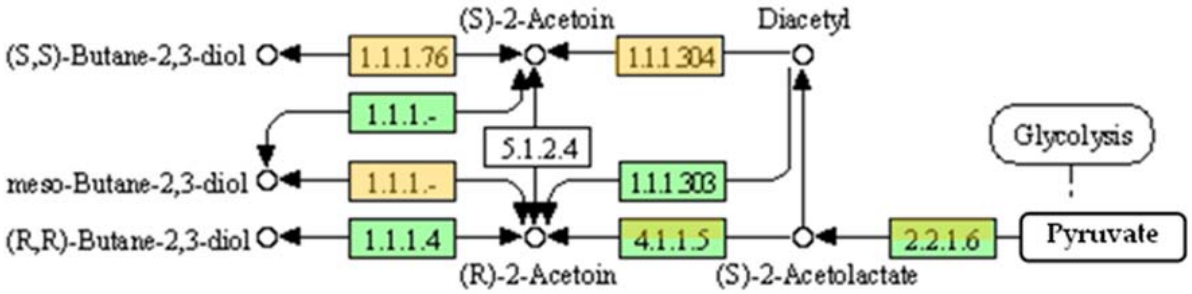


Figure 3. Scheme of enzymes responsible for 2,3-BD production by *B. subtilis* subsp. *subtilis* 168 (green) and *B. licheniformis* ATCC 14580^T (orange). Genes encoding the enzymes with EC 4.1.1.5 and EC 2.2.1.6 presented in both genomes, while the genes for the hypothetical enzyme with EC 5.1.2.4 were absent. Source: https://www.genome.jp/kegg-bin/show_pathway?bsu00650; https://www.genome.jp/pathway/bld00650, 4 December 2021.

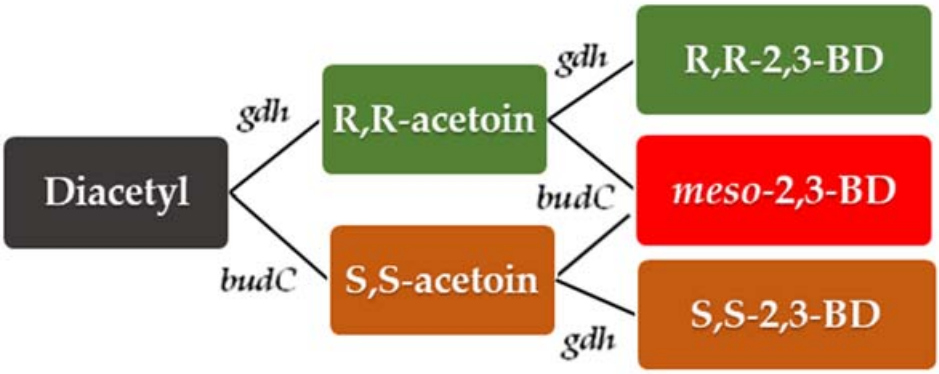


Figure 4. The genetic basis of alternative pathways of diacetyl conversion into 2,3-BD stereoisomers in *B. licheniformis.* The enzyme activities of BudC (EC 1.1.1.304 and EC 1.1.176), and Gdh (EC 1.1.1.4) were proven for *B. licheniformis* MW3 [45].

The analyses of *Bacillus* spp. draft genomes by the metabolic model of Rapid Annotations using Subsystems Technology (RAST) by ModelSEED v2.3 [80,81] allow for building a complex 2,3-BD synthesis model (Figure 5).

The enzyme acetoin racemase (EC 5.1.2.4) was postulated previously by Volloch [82], or the responsible gene was not found in *Bacillus* spp. genomes. The conversion of R-acetoin in acetaldehyde is performed by the acetoin dehydrogenase enzyme system ADH ES (EC 2.3.1.190) [83]. The step from pyruvate to Acetyl-CoA is carried out by the pyruvate dehydrogenase multienzyme complex (PDH) encoded by the *pdhABCD* operon [84]. Acetoin biosynthesis pathway genes *alsD* and *alsS* are organized in an operon, and the expression is regulated by AlsR, and encoded by *alsR* gene. This is a LysR-type transcriptional activator that switches "on" the operon in conditions of high medium acidification (especially in the presence of acetate). The genomes of both *B. subtilis* and *B. licheniformis* contain *acoABCL* operon, which is responsible for acetoin and 2,3-BD degradation. This operon is regulated by *acoR* gene with sigmaL-dependent promoter. Its transcription is repressed by glucose and activated by acetoin [85].

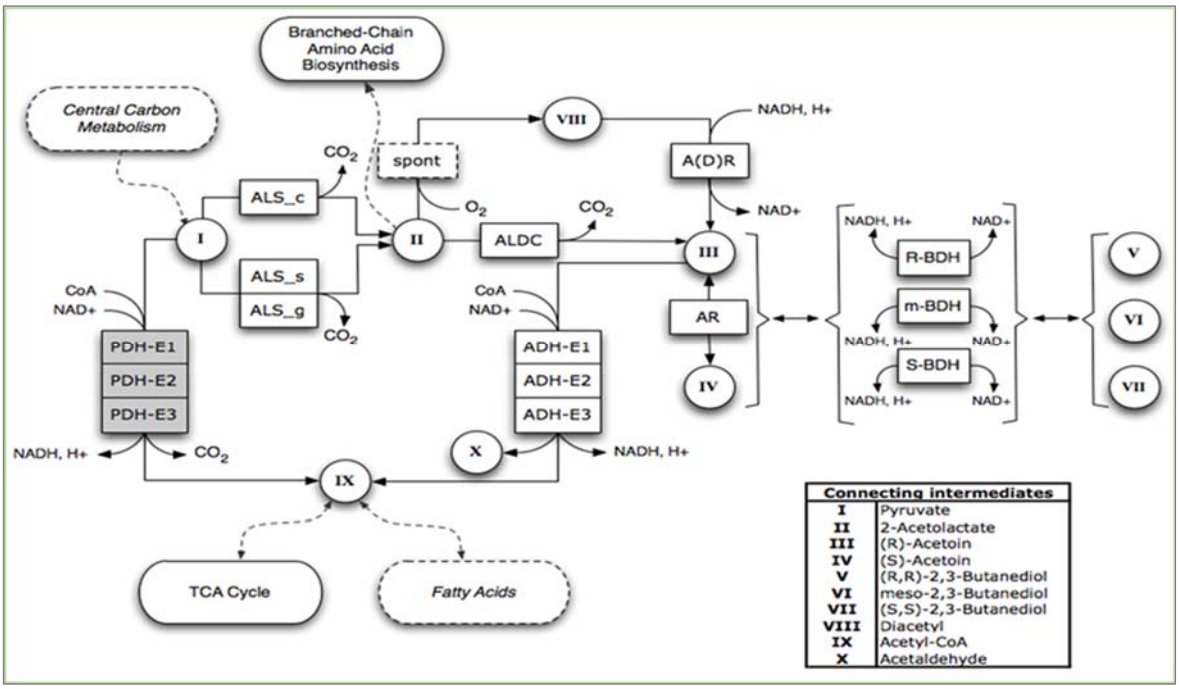


Figure 5. RAST model of the metabolic network for acetoin and 2,3-BD synthesis in *Bacillus* spp. Designations: PDH-E1, PDH-E2, PDH-E3, pyruvate dehydrogenase multienzyme complex, [85]; ALS_c, ALS_s, ALS_g, acetoin dehydrogenase enzyme system; spont, spontaneous reaction; ADLC, α-acetolactate decarboxylase, A(D)R, acetoin dehydrogenase, AR, acetoin racemase; BDH, 2,3-butanediol dehydrogenase.

4. Natural Producers of Acetoin and 2,3-BD

4.1. B. licheniformis

Numerous attempts have been made over time to optimize the content of the medium and the cultivation conditions of *B. licheniformis*. The strains and bioprocesses that reached high titer, yields, and productivities of 2,3-BD are presented in Table 1; those of acetoin are shown in Table 2.

As top acetoin and 2,3-BD producers among bacteria, native bacilli are selected just by ordinary enriching and screening procedure. However, as it was mentioned above, the main problem of mixed-acid fermentation is the synthesis of undesirable by-products, which reduces the carbon flux to acetoin and 2,3-BD. In addition to the target metabolites, the strains of *B. licheniformis* also produce acetic acid and ethanol [65], lactate, formic acid, and glycerol [86,87]. In 1993, Romano et al. [33] managed to improve the yield of acetoin produced by *B. licheniformis* MEL09, varying one factor at a time and using orthogonal array tests. In this way, after medium composition and culture conditions' improvement, the maximum acetoin concentration achieved 41.26 g/L, and had a 41.26% glucose conversion efficiency (84.39% of theoretical). Then, a series of experiments engaging thermophilic *B. licheniformis* strains were performed by Li et al. in 2013 and 2014 [70,73,88]. At optimal temperature 50 °C and pH 7.0, with a two-stage agitation speed control strategy, 115.7 g/L of 2,3-BD was obtained from glucose by fed-batch with productivity 2.4 g/L/h and yield 94% of the theoretical value. The same authors used *B. licheniformis* strain ATCC 14,580 for efficient 2,3-BD synthesis from fructose (at 50 °C), and for simultaneous saccharification and fermentation of inulin, receiving 103.0 g/L 2,3-BD in 30 h with a productivity of 3.4 g/L/h [88]. Recent trials of Song et al. [75] showed that among 4000 *Bacillus* candidates isolated by pre-treatment and enrichment, the thermophilic *B. licheniformis* GSC3102 produced the highest 2,3-BD titer of 92.0 g/L. However, it was accompanied by accumulation of the toxic formate; the amount was dependent on the nitrogen source and reached up to 29.1 g/L [75].

Table 1. Natural producers of 2,3-butanediol belonging to genera *Bacillus* and *Paenibacillus*.

Species	Strain	Substrate	2,3-BD (g/L)	Yield (g/g)	Productivity (g/L/h)	Process Mode	Reference
B. licheniformis							
	NCIMB 8059	Corn starch hydrolysate	6.44	-	0.126	Batch	[68]
	Isolate	Glucose	8.70	-	-	Batch	[66]
	10-1-A	Glucose	115.7	0.47	2.4	Fed-batch	[70]
	ATCC 14580	Inulin	103.0	-	3.4	Fed-batch	[88]
	DSM 8785	Glucose	76.2	0.42	0.86	Batch	[71]
	DSM 8785	Glucose	144.7	0.4	1.14	Fed-batch	[71]
	NCIMB 8059	Apple pomace hydrolysate/glucose	87.71	0.36	0.55	Fed-batch	[89]
	24	Glucose	91.23	0.49	1.94	Batch	[67]
	24	Glucose	138.8	0.48	1.16	Fed-batch	[67]
	X10	Corn stover hydrolysate	74	0.47	2.1	Fed-batch	[73]
	GSC3102	Glucose	95.9	0.431	1.50	Fed-batch	[75]
B. subtilis							
	AJ1992	Glucose	2.5	0.33	0.38	Batch	[90]
	35	Glycerol	11.10	0.27	0.12	Batch in flasks	[69]
	CS13	Sucrose	132.4	0.45	2.45	Fed-batch	[58]
B. amyloliquefaciens							
	B10-127	Glucose	92.3	-	0.96	Fed-batch	[63]
	18025	Molasses	48.7	0.4	0.83	Fed-batch	[78]
	Wild type	Bakery waste hydrolysate	55.2	0.42	1.19	Batch	[5]
	B10-127	Glycerol+molasses	83.3	0.42	0.87	Fed-batch	[91]
Other *Bacillus* spp.							
B. velezensis	5RB	Glucose	18.1	-	-	Batch in flasks	[69]
B. safensis	14A	Glucose	13.9	-	-	Batch in flasks	[69]
B. toyonensis	11RA	Glucose	12.0	-	-	Batch in flasks	[69]
B. atrophaeus	NRS-213	Glucose	29.9	0.33	1.0	Batch	[76]
B. mojavensis	B-14698	Glucose	37.8	0.26	1.1	Batch	[76]
B. vallismortis	B-14891	Glucose	59.1	0.37	1.18	Batch	[76]
P. polymyxa							
	ZJ-9	Inulin	36.92	0.51	0.89	Batch	[92]
	CJX518	Glucose	71.71	-	1.33	Fed-batch	[93]
	DSM 365	Glucose	68.5	0.34	0.70	Fed-batch	[61]
	DSM 365	Glucose	111.0	-	-	Batch	[62]
	ICGEB2008	Cellulose hydrolysates	16.50	0.33	2.01	Batch	[94]
	ATCC 12321	Glucose/xylose	18.8	0.31	1.13	CRCF *	[95]
P. brasiliensis							
	PB24	Glucose	27.0	0.43	0.68	Batch in flasks	[96]

* CRCF, cell-recycling continuous fermentation.

The highest yield and productivity of *B. licheniformis* to date was reported by Jurchescu et al. [71]. Fed-batch cultivation of the strain DSM 8785 with substrate glucose resulted in 144.7 g/L 2,3-BD, yielding 0.4 g/g, with a productivity of 1.14 g/L/h. A very similar titer was recently obtained by Tsigoriyna et al. [67]. By applying the Plackett–Burman design and RSM through CCD, a complex optimization of medium and process parameters for 2,3-BD production by *B. licheniformis* strain 24 was performed. The highest concentration of 2,3-BD was 138.8 g/L, the productivity-1.16 g/L/h, and the yield—close to the theoretical—0.479 g/g. The most important factors affecting the process were the amounts of yeast extract, tryptone, K_2HPO_4, and $MgSO_4$, as well as the aeration rate. Comparison with the results of Jurchescu et al. [71] reveals that the values of 2,3-BD around 140 g/L are probably the highest that can be obtained using the species *B. licheniformis* and indicate maximal natural capabilities of the species as a 2,3-BD producer.

Table 2. Natural producers of acetoin belonging to genera *Bacillus* and *Paenibacillus*.

Species	Strain	Substrate	Acetoin (g/L)	Yield (g/g)	Productivity (g/L/h)	Process Mode	Reference
B. licheniformis							
	MEL09	Glucose	41.3	0.42	-	Batch	[35]
B. subtilis							
	JNA 3-10	Glucose	42.2	0.32	-	Batch in flasks	[7]
	CICC10025	Molasses	35.4	0.41	0.63	Batch	[97]
	SF4-3	Glucose	48.9	0.39	0.56	Batch	[2]
	DL01	Molasses	61.2	0.34	0.807	Batch	[98]
B. pumilus							
	DSM 16187	Glucose	63.0	-	-	Batch in flasks	[99]
B. amyloliquefaciens							
	FMME044	Glucose	51.2	0.43	1.42	Batch	[64]
	Wild type	Bakery waste hydrolysate	65.9	0.31	1.57	Fed-batch	[5]
P. polymyxa							
	CS107	Glucose	55.3	-	1.32	Fed-batch	[100]

4.2. *B. subtilis*

The natural strains of *B. subtilis*, which produce acetoin, have been isolated from soil, distiller's grains, and agricultural controlled residues, spirits or vinegar factories, and even from food as natto. Zhang et al. [7], after optimization of several process parameters, efficiently produced acetoin by B. subtilis strain JNA 3-10 (42.2 g/L), but the targeted product was mixed with 2,3-BD (15.8 g/L). A similar titer, but from a cheaper substrate, was obtained by *B. subtilis* CICC 10,025 [97]. RSM was applied to determine the optimal levels of acid-treated molasses and soybean meal hydrolysate for acetoin production, thus obtaining 35.4 g/L acetoin in 56 h (Table 2). Tian et al. [2] also showed that the carbon and nitrogen source and agitation play a crucial role in acetoin production. By the use of *B. subtilis* strain SF4-3, from 150 g/L glucose, the authors obtained 48.9 g/L acetoin (with a yield corresponding to 79.90% of the theoretical conversion). High acetoin production was achieved by Dai et al. [98]. New marine *B. subtilis* strain DL01 demonstrated high sugar tolerance and relatively low oxygen requirement. From 210 g/L glucose, 76 g/L acetoin (60.9 g/L R-form) was received, with a productivity of 1 g/L/h and a yield of 0.421 g/g. From the cheaper molasses (200 g/L sugar), the strain produced a smaller amount of acetoin (61.15 g/L) [98].

4.3. *B. amyloliquefaciens*

As a close relative to *B. subtilis*, *B. amyloliquefaciens* is able to produce an enantiomer pure form of acetoin and 2,3-BD. Noticing that the lower agitation speeds favored 2,3-BD accumulation, while the higher agitation speeds favored reverse transformation of 2,3-BD to acetoin, Zhang et al. (2013) proposed a two-stage agitation speed control (350 rpm for 24 h, then-500 rpm) for acetoin production [64]. Following this strategy, through the use of *B. amyloliquefaciens* FMME044, the authors achieved yield of 51.2 g/L acetoin [64].

A record-high concentration of acetoin obtained from this species was achieved by Maina et al. [5]. The new approach of variation of the volumetric oxygen transfer for either acetoin or 2,3-BD production was applied. Thus, when the volumetric oxygen transfer coefficient kL a value was 64 h^{-1}, 55.2 g/L D(−)2,3-BD and 47.4 g/L acetoin were received; when kL a value was 200 h^{-1}, the acetoin reached 65.9 g/L with a productivity of 1.57 g/L/h. The same effect was observed when cane sugar with very high polarity (VHP) was used as a substrate. Increasing the value of k_L a to 104 h^{-1}, led to acetoin titer of 25.6 g/L, while the k_L a at 49 h^{-1}, led to an increase in 2,3-BD yield. High titer of 2,3-BD, 92.3 g/L has been achieved by Yang et al. [45] by process parameters optimization (agitation and pH) [63].

4.4. Other 2,3-BD and Acetoin Producers

4.4.1. *Bacillus* spp.

The interest in the selection of other GRAS microorganisms for the synthesis of 2,3-BD and acetoin is due to the need to reduce the cost of bioprocesses and in the hope that hitherto unknown bacilli will be effective producers. Thus, the species *B. atrophaeus*, *B. mojavensis*, *B. vallismortis*, *B. pumilus*, *B. velezensis*, *B. toyonensis*, and *B. safensis* are considered as particularly promising, because they can use the sugars falling into the lignocellulose content and may be applied in biomass valorization. For the development of economical production of 2,3-BD, the screening procedures included tests of the utilization of xylose, fructose, sucrose, or corn steep liquor [75]. For instance, *B. vallismortis* B-14891 converts 14 different substrates that can be obtained from residual biomass sources to 2,3-BD [76]. Recently, Petrova et al. [69] added three new species (*B. velezensis*, *B. toyonensis*, and *B. safensis*) to the group of 2,3-BD producers. They all displayed cellulolytic activity but also degraded arabinoxylan, arabinan, galactomannan, xyloglucan, xylan, and galactan (included in hemicellulose), as well as starch and fructans. *B. velezensis* 5RB was able to convert nine explored substrates to acetoin and D(−)2,3-BD, as relatively high 2,3-BD titer was obtained from glucose (18.1 g/L), cellobiose (14.8 g/L), mannose (13.8 g/L), and inulin (11.7 g/L), but low from xylose (2.6 g/L) and galactose (0.6 g/L), differing from *B. toyonensis* 11RA that yielded 4.48 g/L 2,3-BD from galactose [69]. However, the sugar that is most difficult to convert to acetoin and 2,3-DB is xylose. Of the bacilli studied, only two strains convert xylose to significant amounts of the target products-*B. vallismortis* B-14891 [76], and *B. velezensis* 5RB [69].

4.4.2. *Paenibacillus* spp.

In addition to glucose and cellulosic hydrolysates, *P. polymyxa* is able to ferment crude inulin to almost pure D(−)-stereoisomer of 2,3-BD [61,62,93–96]. A good natural producer of acetoin was the strain CS107 isolated by Zhang et al. [100], yielding 55.3 g/L by precise oxygen supply control. Dai et al. [93] observed the whole cellular redox status and oxygen availability as a tool to enhance 2,3-BD production by *P. polymyxa*. The neccessarry high NADH/NAD+ ratio was reached by decreasing agitation speed and adding ascorbic acid (vitamin C), which elevated the 2,3-BD titer 71.71 g/L. Okonkwo et al. [60] determined 50 g/L of 2,3-BD as a toxic threshold on *P. polymyxa* DSM 365, but after optimization of the medium content by Box–Behnken design received 68.5 g/L 2,3-BD [61]. These authors stated as problematic the synthesis of exopolysaccharide (EPS) by the used strain. Indeed, *P. polymyxa*, as well as *B. licheniformis*, produce high amounts of EPS along with 2,3-BD fermentation, especially when high substrate concentration or fed-batch fermentation mode is performed. EPS can reach 4.97 g/L for *P. polymyxa* [61] and 12.6 g/L for *B. licheniformis* [53].

P. brasilensis PB24 produces D(−), and *meso*-2,3-BD through activity of S-2,3-butanediol dehydrogenase encoded by *butA*, presented in its genome [96]. The obtained 2,3-BD by *P. brasilensis* was 27 g/L without any optimization. Regarding the great similarity of the genomes (including 2,3-BD operons) of *P. brasilensis*, *P. terrae*, and *P. peoriae*, we could expect the emergence of new producers belonging to the little-studied species of the genus *Paenibacillus*.

5. Engineered Producers of 2,3-BD and Acetoin

5.1. Engineered Producers of 2,3-BD

The genetic manipulations of bacilli for 2,3-BD and acetoin production have two main purposes: (i) to obtain enantiopure stereoisomers; and (ii) to enhance the fermentation process for higher titer, yield, and productivity of the target metabolite.

The first strategy is usually performed by eliminating specific *bdh* genes (Table 3).

Table 3. Engineered producers of 2,3-butanediol belonging to genera *Bacillus* and *Paenibacillus*.

Species	Strain	Substrate	2,3-BD (g/L)	Yield (g/g)	Productivity (g/L/h)	Modification	Reference
B. licheniformis							
	MW3	Glucose	123.7 (D(−))	0.508	2.95	Δ*budC*	[45]
	MW3	Glucose	90.1 (*meso*)	0.424	2.82	Δ*gdh*	[45]
	WX-02	Glucose	30.7 (D(−))	-	1.28	Δ*budC*	[101]
	WX-02	Glucose	98.0 (*meso*)	-	0.94	Δ*gdh*Δ*acoR*	[102]
B. subtilis							
	LOCK 1086	Sugarbeet molasses, glucose	75.73	-	0.66	*bdhA* overexpression, *vhb* cloning	[103]
B. amyloliquefaciens							
	B10-127	Glucose					[64]
	B10-127	Crude glycerol+molasses	102.3	0.44	1.16	$NADH/NAD^+$ regeneration system introduction, *alsR* under *pbdhA* promoter control	[104]
	B 10-127	Glucose	132.9	-	2.95	GAPDH, BDH overexpression	[105]
P. polymyxa							
	ZJ-9	Glucose	25.88	-	-	Δ*dudA*	[106]
	XG-1	Glucose	51.3	-	-	NAD^+-dependent *fdh* gene cloning	[107]

Knowing that *B. licheniformis* MW3 produces a mix of *meso*- and D(−)2,3-BD, Ge et al. [45] consecutively determined and eliminated the genes encoding the stereospecific 2,3-BD dehydrogenases. The engineered MW3Δ*budC* strain produced 123.7 g/L D(−)2,3-BD with 99.4% purity, while MW3Δ*gdh* produced 90.1 g/L meso-2,3-BD, which was 99.2% pure. The same approach was applied to *B. licheniformis* strain WX-02. Qi et al. [101] deleted *budC*, while Qui et al. [102] eliminated *gdh* gene, but also *acoR* gene (which encodes the activator of *acoABCL* operon). The recombinant WX-02Δ*gdh*Δ*acoR* produced 99% pure *meso*-2,3-BD in a higher amount than WX-02Δ*budC*.

In *P. polymyxa* ZJ-9, by elimination of *dudA* gene (encoding DAR) by homologous recombination [106], D(−)2,3-BD stereoisomer was produced to purity of 99%.

The main approach to enhance 2,3-BD production is the redistribution of the carbon flux towards 2,3-BD branch by $NADH/NAD^+$ ratio increase. This goal was successively accomplished by Yang et al. [104] using *B. amyloliquefaciens* B10-127 with crude glycerol as a substrate. The manipulation of carbon flux was conducted as an extra copy of an $NADH/NAD^+$ regeneration system was introduced into the strain by overproduction of two enzymes: glycerol dehydrogenase and acetoin reductase. This co-overexpression accelerated the $NADH/NAD^+$ regeneration rate and, finally, caused a decrease in by-products acetoin and acids. In addition, the transcriptional regulator *alsR* was cloned under P_{bdhA} promoter control, which also increased the carbon flux towards the 2,3-BD(−)branch. These genetic manipulations, accompanied by three-stage dissolved oxygen control and two-stage pH control, led to a significant increase in 2,3-BD titer (102.3 g/L).

Employing the same strain *B. amyloliquefaciens* B10-127 for glucose fermentation, Yang et al. [105] increased NADH/NAD+ ratio using another approach, by the complex effect of the over-production of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and NADH-dependent BDH. NAD^+-dependent GAPDH-catalyzed reaction is coupled with reduction of NAD^+ to NADH, while the conversion of acetoin to 2,3-BD by BDH is concomitant with oxidation of NADH to NAD^+. That is why GAPDH excess increased 2,3-BD by 12.7%, and decreased acetoin yield by 44.3%. By its side, BDH excess increased 2,3-BD yield by 16.6%. Moreover, the fermentation was accelerated, and the overall process time was reduced. Thus, the final fed-batch fermentation yielded 132.9 g/L 2,3-BD in 45 h.

The desired increased $NADH/NAD^+$ ratio for higher 2,3-BD titer was achieved by the cloning of NAD^+-dependent formate dehydrogenase (*fdh*) gene from *Candida boi-*

dinii in *P. polymyxa* strain ZJ-9 [107]. The recombinant *P. polymyxa* strain XG-1 reached 51.3 g/L 2,3-BD.

Białkowska et al. [103] tried to intensify the production of 2,3-BD *B. subtilis* by homologous overexpression of *bdhA* and heterologous expression of *vhb* gene encoding bacterial hemoglobin in *Vitreoscilla stercoraria*. The recombinant LOCK 1086 did not display higher productivity in flasks, however, the fed-batch processes were successful and reached 75.73 g/L 2,3-BD concentration.

5.2. Engineered Producers of Acetoin

When the goal is to produce the maximum amount of acetoin, the redox balance of the cell should be shifted exactly the opposite i.e., to NADH/NAD+ ratio reduction. In this way, Bao et al. [108] developed *B. subtilis* 168 derivate for high acetoin production with NAD^+ regeneration system through homologous co-expression of 2,3-BDH and NADH oxidase. This decreased intracellular NADH concentration and NADH/NAD^+ ratio and in the batch process, the recombinant produced 91.8 g/L acetoin. Another attempt of manipulation of the NADH levels in *B. subtilis* for high acetoin yield was performed by Zhang et al. [109] by deletion of *bdhA* and moderate overexpression of *yodC* gene encoding NADH oxidase. Thus, 2,3-BD decreased by 92.3%, while acetoin productivity reached 0.639 g/L/h (Table 4).

Table 4. Engineered producers of acetoin belonging to genera *Bacillus* and *Paenibacillus*.

Species	Strain	Substrate	Acetoin (g/L)	Yield (g/g)	Productivity (g/L/h)	Modification	Reference
B. licheniformis							
	WX-02	Glucose	78.79	0.31	0.58	Δ*budC*Δ*acoR*	[111]
B. subtilis							
	ZB02	Xylose/glucose/arabinose	62.2	0.29	0.86	*ARSRCP*Δ*acoA*Δ*bdhA*, cloned *araE*, *xylA*, *xylB*	[8]
	ZB02	Lignocellulose hydrolysate	11.2	-	-	*ARSRCP*Δ*acoA*Δ*bdhA*, *cloned araE*, *xylA*, *xylB*	[8]
	168	2,3-BD	91.8	0.765	2.30	NADH oxidase and BDH coexpression	[108]
	BMN	Glucose	56.7	0.378	0.639	Δ*bdhA*, *yodC* overexpression	[109]
	168	Glucose	24.6	-	-	Δ*bdhA*	[110]
	PAR	Glucose	41.5	0.35	0.43	*alsR* promoter control	[112]
	BSUW06	Glucose	20	-	0.273	Δ*bdhA*, Δ*acoA*, Δ*pta*, overexpression of *alsSD*	[113]
	TH-49	Glucose	56.9	-	-	Mutagenesis	[114]
	JNA-UD(−)6	Glucose	53.9	-	-	Mutagenesis	[115]
	IPE5-4-UD(−)4	Lignocellulose hydrolysate	22.76	0.46	0.38	Mutagenesis	[116]
B. amyloliquefaciens							
	E-11	Glucose	71.5	0.41	1.63	Adaptive evolution	[117]

The deletion of (R,R)-2,3-BD dehydrogenase gene *bdhA* of *B. subtilis* 168 enhanced four-fold the acetoin synthesis by the recombinant variant reaching 24.6 g/L [110]. The amount of 2.4 g/L of *meso*-2,3-BD from 100 g/L glucose indicated that the *bdhA* gene was the only gene responsible for (2R,3R)-2,3-BD synthesis. A similar approach was applied by Liu et al. [111] for high acetoin production by engineered *B. licheniformis* WX-02. Further, two- and three-genes deletion mutants were developed, as the highest acetoin was obtained by the recombinant WX-02Δ*budC*Δ*acoR*. In optimized fed-batch fermentation, by the use of the latter, 110.04 g/L 2,3-BD was accumulated and then converted to 78.79 g/L acetoin.

For enhanced acetoin production from lignocellulosic sugars, Zhang et al. [8] used recombinant *B. subtilis* 168 *ARSRCP*Δ*acoA*Δ*bdhA* strain, which was able to use xylose. This strain was engineered to produce acetoin from glucose, xylose, and arabinose mixtures by cloning of *araE*, *xylA*, and *xylB* encoding sugar transport protein, xylose isomerase, and

xylulokinase in *E. coli*. The recombinant strain simultaneously utilized glucose, xylose, and arabinose, and produced 62.2 g/L acetoin from mixed sugars (fed-batch process), and 11.2 g/L acetoin from lignocellulosic hydrolysate fermentation in flasks. Zhang et al. [112] reported that moderate enhancement of *alsR* expression under P_{bdhA} promoter control significantly improves the acetoin production in *B. subtilis*. This was due to the two-fold increase in the activities of α-acetolactate synthase (ALS) and α-acetolactate decarboxylase and yielded 41.5 g/L of acetoin.

Some *B. subtilis* acetoin overproducers were selected after chemical or UV mutagenesis. The mutants fulfilled the requirements, although the genetic basis of these properties was not elucidated. Such a mutant is TH-49, which reached a yield of 56.9 g/L acetoin in 100 L fermentation of glucose [114]. Other such examples are *B. subtilis* JNA-UD(−)6 that derives from UV-light and diethyl sulfate-treated JNA 3-10, which was selected by a deficiency in BDH [115], and IPE5-4-UD(−)4, which acquired the ability to ferment simultaneously glucose and xylose to high acetoin concentrations, converting lignocellulose hydrolysates [116].

B. amyloliquefaciens E-11 mutant was selected after adaptive evolution by acetoin stress of parental strain FMME044. In comparison with the parent, the accumulated mutations resulted in a higher unsaturated fatty acid content and a lower unsaturated fatty acid content. E-11 was able to resist 80 g/L acetoin and possessed a 2,5-fold higher growth rate compared to its parent [117].

6. Substrates

An important advantage of bacilli is the wide range of substrates they can convert. Because substrate costs account for the largest share of production costs, bioprocesses based on renewable, non-food, and cheaper sources are being sought. A decade ago, Ji et al. [22] classified the cheap alternative substrates for 2,3-BD production as two types: non-cellulosic and lignocellulosic. In the first category fall the raw material or hydrolysates of plants containing sucrose (sugar cane and beet), starch, and inulin (chicory and Jerusalem artichoke).

The first alternative substrates employed for 2,3-BD production were starch and inulin. *B. licheniformis* NCIMB 8059 produced 2,3-BD and acetoin with a productivity of 0.58 g/L/h from cornstarch hydrolysate [68]. The one-step fermentation of raw inulin extract from Jerusalem artichoke tubers by *P. polymyxa* ZJ-9 was developed to produce R,R-2,3-BD [92], while Li et al. [88] performed SSF process for 2,3-BD synthesis with thermophilic *B. licheniformis* ATCC 14,580 (with productivity 3.4 g/L/h).

A total of two recent reports revealed the high potential of sucrose for 2,3-BD production by *B. subtilis*. Wang et al. [58] fermented sucrose using *B. subtilis* strain SC13 and reached 132.4 g/L 2,3-BD, with a productivity of 2.45 g/L/h, and yield of 0.45 g/g in fed-batch process. Feng et al. [118] developed four combinations of heterologous sucrose utilization pathways and introduced them into two different *B. subtilis* strains for successful conversion of sucrose and sugar cane juice into 2,3-BD.

Both raw and decolored molasses are widely employed in 2,3-BD fermentations by all bacilli. *B. subtilis* strains CICC10025 [97], engineered 1A1 [119], and DL01 [98] yielded between 35 and 65 g/L 2,3-BD from molasses. *B. amyloliquefaciens* TUL 322 also fermented molasses to 25.3 g/L 2,3-BD but this strain needed some glucose feeding for better results [120]. A higher concentration (75 g/L) was obtained by *B. subtilis* strain TUL 322 [121], in a medium containing sugar beet molasses in a course of a fed-batch process with four glucose additions.

A series of experiments devoted to apple pomace as a substrate were performed, but for effective 2,3-BD production, glucose addition was needed. *B. subtilis* TUL 322, with substrate apple pomace hydrolysate, needed three glucose feedings to produce 51 g/L 2,3-BD [121]. The more potent *B. licheniformis* NCIMB 8059 yielded 113 g/L 2,3-BD at the same conditions [89]. In a medium based on the apple pomace hydrolysate with three feedings with sucrose, the recombinant *B. subtilis* LOCK 1086 produced 51.53 g/L 2,3-BD (with a productivity of 0.43 g/L/h) [103].

The techno-economical evaluation of 2,3-BD production by Koutinas et al. [122] showed that among the three most promising substrates for the industrial production of 2,3-BD, glycerol is the most profitable, followed by molasses and sucrose. Although the highest yields are obtained from sucrose and molasses, the minimum selling price of 2,3-BD produced from glycerol is lower, because of the low glycerol price.

A substrate combination of molasses (15%) with biodiesel-derived glycerol (80%) appeared to be very promising for 2,3-BD production by *B. amyloliquefaciens*. Beet molasses as a co-substrate stimulated 2,3-BD production and reduced the duration of fermentation to reach the productivity of 0.87 g/L/h. From this substrate, a very high titer of 2,3-BD (102.3 g/L) was obtained by an engineered *B. amyloliquefaciens* strain [104]. Hence, other new-isolated strains that can convert glycerol as sole carbon source into 2,3-BD, such as *B. subtilis* 35 [69], may be also promising for 2,3-BD synthesis in future experiments.

Cellulose and lignocellulose containing plant biomass is a renewable, abundant, environmentally friendly, sustainable carbon feedstock for microbial biorefinery. Agro-industrial residues typically contain a mix of both, for example, corncob, cereals, and potato wastes contain cellulose and residual starch; sugar beet, sugar cane, fruits, and vegetables are rich in sucrose and fructans. The wheat straw, barley hull, rice husks, oat, ray, and sorghum straw, corn stover, sugarcane bagasse, and wood are composed of 30–50% cellulose, 16–28% hemicellulose, and 6–12% lignin [69]. Several processes for 2,3-BD and acetoin production using hydrolysates have been reported. The thermophilic strain *B. licheniformis* X10 effectively converted lignocellulose hydrolysates to 2,3-BD [73]. Batch fermentation with corn stover hydrolyzate as a substrate reached a titer of 74.0 g/L 2,3-BD, with a productivity of 2.1 g/L/h. Furthermore, X10 had a high tolerance to the toxic compounds of lignocellulose hydrolysis such as furfural and vanillin, and the fermentation by-products such as formic and acetic acids.

B. licheniformis mutants, WX-02Δ*budC* and WX-02Δ*gldA*, were engaged in optically pure 2,3-BD production from *Miscanthus floridulus* (silver grass) hydrolysate [123]. This application of the recombinants is promising because in the course of fed-batch fermentation, 32.2 g/L D(−)2,3-BD and 48.5 g/L *meso*-2,3-BD were obtained, respectively.

From alkali-pretreated corncob, the thermotolerant (52 °C) acetoin producer *B. subtilis* IPE5-4 produced 22.76 g/L acetoin, with a yield of 0.46 g/g and productivity 0.38 g/L/h [116]. This is by far the highest acetoin yield from lignocellulosic biomass in a process of simultaneous (enzymatic) saccharification and fermentation.

Another species that could utilize the lignocellulose sugars is *P. polymyxa*. Adlakha et al. [94] and Ma et al. [95] employed natural *P. polymyxa* strains and lignocellulose hydrolysate as a substrate, but applied different process performances. However, both teams obtained a similar yield of 2,3-BD (0.3 g/g sugar). Some *Bacillus* strains can directly degrade cellulose, hemicelluloses, and lignin owing to the action of their glycoside-hydrolase enzymes [124,125]. Among them, the plant-associated rhizobacteria (*B. amyloliquefaciens*, *B. velezensis*, *B. siamensis*), belonging to "*B. amyloliquefaciens* operational group" [126,127] contain the richest genetic pool of cellulases and xylanases [69]. *B. subtilis* BY-4 isolated from Tibetan's pig gut [128], *B. subtilis* UMC7 from termites [129], or *B. paralicheniformis* strains from the Red Sea [130], are a source of powerful endoglucanases.

Hence, the attempts to develop bioprocesses for direct microbial conversion of plant biomass into 2,3-BD and acetoin by genetic improvement of strains are worthy and prospective. For example, Liu et al. [131] cloned and expressed several clostridial genes encoding cellobiohydrolases in *B. subtilis* strains. Later, the genes encoding cellulases *cel8A* and *cel48S* of *Acetivibrio thermocellus* were cloned and expressed in 2,3-BD producers *B. licheniformis* 24 and *B. velesensis* 5RB [132]. However, despite successful expression and high cellulase activity, the accumulation of fermentable sugars was not observed in these studies.

7. Media and Process Parameters for 2,3-BD and Acetoin Production by Bacilli

7.1. Medium Content

In addition to the carbon source, the nitrogen source is crucially important for the microbial synthesis of high amounts of 2,3-BD and acetoin. Tryptone, peptone, yeast extract, corn steep liquor (or powder), casamino acids, urea, and ammonium salts are known to highly improve the yield [9,22,60,61]. That was why Häßler et al. [62] added up to 60 g/L yeast extract in the medium for 2,3-BD synthesis by *P. polymyxa*. Later, Okonkwo et al. [61] assessed the impact of tryptone and yeast extract and showed that concentrations above 5 g/L may not engender a further increase in 2,3-BD production by this species. However, concerning *B. licheniformis*, the estimated optimal values were 13.38 g/L yeast extract and 6.41 g/L tryptone [67]. Corn steep liquor was frequently applied as an inexpensive organic nitrogen source [104], however, its addition is less suitable than yeast extract for 2,3-BD yield by *B. licheniformis* [67,75]. Taking into account the high price of yeast extract, it should be replaced by raw materials in industrial-scale fermentation [11,28]. Xiao et al. [97] estimated that molasses and soybean meal hydrolysate are more productive than pure sucrose and yeast extract plus peptone in acetoin fermentation by *B. subtilis*.

Other medium supplements affecting 2,3-BD production are phosphate and acetate [9,67]. Fed-batch processes by feeding glucose and acetate at a ratio of 0.35 mol acetate per mol glucose at constant pH 6.8 for *P. polymyxa* were found to the enhancement of 2,3-BD [133]. Fe^{2+}, Mn^{2+}, K^{+}, and Mg^{2+} affect the enzymes activity and their stereospecificity [134]. The highly productive media also contain vitamins (biotin), and trace elements, supplied by the following salts: $MgSO_4$, KH_2PO_4, K_2HPO_4, $FeSO_4$, $CuSO_4$, $NaMoO_4$, $MnCl_2$, 0.1 $ZnSO_4$, $Co(NO_3)_2$, and $CaCl_2$ [61].

7.2. Process Parameters

A total of three modes of process performance are applied for bacilli: flask-batch, batch, and fed-batch, which can overcome the inhibition of the process by the high substrate concentrations. The most influencing process parameters are temperature, pH of the media, aeration rate, and agitation speed, as the last two are crucial for acetoin/2,3-BD production ratio and are an object of optimization most frequently (Table 5).

7.2.1. Temperature

The optimal temperature for acetoin/2,3-BD production is strain-dependent, although the species-specific characteristics should also be taken into account. For most producers of *B. subtilis* and *B. amyloliquefaciens*, the optimal temperature is usually around 37 °C. In most cases, using producers of these two species, this temperature is accepted to be optimal without being an object of optimization. In one of the few optimizations performed, Yang et al. [63] reported that the optimal temperature of *B. amyloliquefaciens* strain B10-127 for 2,3-BD production is 37 °C, using an experimental range from 25 to 40 °C. At 40 °C, the obtained titer 2,3-BD is slightly lower than the maximum (57.4 vs. 59.1 g/L at 37 °C). Similarly, in the studied range 25–40 °C, the strain *B. subtilis* JNA 3-10 produced acetoin with maximum titer and yield at 37 °C [7]. However, detailed monitoring of the effect of the temperature revealed that some strains of *B. subtilis* and *B. amyloliquefaciens* possess a higher temperature optimum for 2,3-BD and acetoin production. For example, the strain *B. amyloliquefaciens* 18025 produced 2,3-BD with a maximum titer, yield, and productivity at 40 °C, when the range between 27 and 44 °C was tested [78]. Likewise, when the fermentations were performed in the range between 20 °C and 50 °C, *B. subtilis* 168 displayed the highest conversion rate of 2,3-BD to acetoin at 40 °C [108]. When *B. licheniformis* is employed, the optimal temperature for 2,3-BD or acetoin production varies in a wide range from 30 to 50 °C, depending on the specificity of the strain used [70], (Table 6).

Table 5. Process parameters used for 2,3-BD production by strains belonging to genera *Bacillus* and *Paenibacillus*.

Species	Strain	Temperature (°C)	pH	Agitation (rpm)	Aeration (vvm)	Reference
B. licheniformis						
	10-1-A	50 *	7.0 *	400/200 *	1.0	[70]
	NCIMB 8059	37	6.5 †	250	1.2	[89]
	24	37.8 *	6.23 *	500	3.68 *	[67]
	X10	50	7.0	400/200	1.0	[73]
	GSC3102	50	7.0	500/400	1.0	[75]
	DSM 13^T	37	6.0 *		Anaerobic	[86]
	DSM 394	37	6.0–6.5 *		Anaerobic	[86]
	NCIB 8069	37	6.0 *		Anaerobic	[86]
	NCIB 8062	37	6.0 *		Anaerobic	[86]
	NCIB 8549	37	6.0 *		Anaerobic	[86]
	DSM 392	37	6.0–6.5 *		Anaerobic	[86]
	DSM 603	37	6.0 *		Anaerobic	[86]
B. subtilis						
	CS13	37	6.5	500 *	2.0 *	[58]
B. amyloliquefaciens						
	B10-127	37 *	6.5 † *	100 f *		[63]
	B10-127	37	6.5 ‡	350/400/350 *	0.66	[104]
	pBG	37	6.5 †	350	0.33	[105]
	18025	40	6.0	300 *	1.0	[5]
	18025	40 *	6.0 *	k_L a = 49 h^{-1} *	1.0	[78]
Other *Bacillus* spp.						
B. velezensis	5RB	37	6.5 †	200 f	-	[69]
B. safensis	14A	37	6.5 †	200 f	-	[69]
B. toyonensis	11RA	37	6.5 †	200 f	-	[69]
B. atropaeus	NRS-213	39	6.7 †	700 mp	-	[76]
B. mojavensis	B-14698	39	6.7 †	700 mp	-	[76]
B. vallismortis	B-14891	39	6.7 †	700 mp	-	[76]
P. polymixa						
	DSM 365	35 *	6.5 †	300	0.075	[61]
	DSM 365	37	6.0	500 *	0.2 *	[62]
	ATCC 12321	37	6.5	500	0.2	[95]
	CJX518	37 *	5.2	400 *	0.1 *	[93]
P. brasiliensis						
	PB24	32		200 f		[96]

*—Optimized values; †—Initial pH of pH uncontrolled process; ‡—Two stage pH control; f—Experiments in flasks (rpm of shaker); mp—Experiments in microplate wells (rpm of shaker).

When glucose to acetoin is converted by the strain *B. licheniformis* MEL09, it was shown that temperature has no significant effect on the process between 34 °C and 40 °C [35]. However, the temperature appeared to be a significant factor in starch hydrolysate conversion into 2,3-BD by *B. licheniformis* NCIMB 8059. In this case, a maximum 2,3-BD concentration was obtained at 37 °C [68].

The temperature was also the object of optimization of glucose fermentation to 2,3-BD by *B. licheniformis* 24 [67]. The statistical analysis of the experimental results showed that a maximum 2,3-BD titer should be obtained at 37.8 °C. However, higher temperatures accelerate glucose consumption rate and 2,3-BD productivity, respectively. Thus, maximum productivity can be obtained at temperatures $\geq$ 39 °C [67]. On the other hand, there are many thermophilic *B. licheniformis* strains among the best 2,3-BD producers. Strains such as *B. licheniformis* ATCC 14,580 [88], 10-1-A [70], X10 [73], and BL1 [135], all have a temperature optimum of 50 °C for 2,3-BD production. Curiously, the highest 2,3-BD

concentration obtained to date by *Bacillus* spp. (144.7 g/L from glucose) is received by *B. licheniformis* DSM 8785 at 30 °C [12].

Table 6. Process parameters used for acetoin production by strains belonging to genera *Bacillus* and *Paenibacillus*.

Species	Strain	Temperature (°C)	pH	Agitation (rpm)	Aeration (vvm)	Reference
B. licheniformis						
	10-1-A	50 *	7.0 *	400 *	1.0	[70]
B. subtilis						
	CS13	37	6.5	500 *	2.0 *	[58]
	CICC10025	37	7.0 †	700	1.0	[97]
	SF4-3	37	7.0 †	300 *	0.5	[2]
	DL1	37	6.5 †	200/300/400 *	0.4 *	[98]
	JNA-310	37 *	4.0 † *	160 f		[7]
	ZB02	37	7.0	400 *	1.0	[8]
B. amyloliquefaciens						
	B10-127	25 *	4.5 † *	200 f *		[63]
	18025	40	6.0	600 *	1.0	[5]
	18025	40	6.0 *	k_L a = 104 h^{-1} *	1.0	[78]
	FMME044	37	-	350/500 *	1.0	[64]
B. pumilus						
	DSM 16187	37	-	180 f	-	[99]
P. polymixa						
	DSM 365	37	6.0	800 *	0.2 *	[62]
	CS107	37	6.0	500 *	0.5	[100]

*—Optimized values; †—Initial pH of pH uncontrolled process; f—Experiments in flasks (rpm of shaker).

Using *P. polymyxa* DSM 365, Okonkwo et al. [61] optimized the temperature as a factor in the Box–Behnken design during the process of glucose conversion into 2,3-BD. The optimal value appeared to be 35 °C. Significant quantities of acetoin from glucose [100] and 2,3-BD from corn stover hydrolysate [95] were received by the cultivation of *P. polymyxa* strains at 37 °C. Dias et al. [96] cultivated *P. brasilensis* at 32 °C for 2,3-BD production from glucose.

7.2.2. pH

When bacilli are employed for 2,3-BD production, a proper pH control taking into account their distinguishing characteristics should comply. The opposite of *Enterobacteriaceae* producers, in acetoin/2,3-BD fermentation, *Bacillus* spp. form organic acids in relatively low quantities. Thus, when pH is not controlled, its changes are limited and follow subsequent periods of decrease and increase. Hereof, depending on the specificity of the strain and its by-products formation, in many fermentations, the pH is not necessary to be maintained during the process, which is an important advantage of the *Bacillus* producers. In these cases, only initial pH is determined, as its value is the object of optimization and maintenance during the process. In such optimization for acetoin production from glucose by *B. licheniformis* MEL09, in the tested range of initial pH from 6.0 to 8.5, the optimal initial pH was determined to be 6.5 [35]. The same was the optimal initial pH for 2,3-BD production from glucose by *B. amyloliquefaciens* B 10-127 [63]. In other studies, when fermentations without pH control have been conducted, the most frequently used initial pH was 6.5 [98], or 7.0 [2,97] for acetoin production, and 6.5 [103,105] for 2,3-BD production, with no species specificity.

In pH-controlled fermentation, the optimal control values of pH are strain-dependent and if the maximum concentration of the product is intended, they varied from pH 6.0 to

7.0. At pH $\geq$ 7.0 the metabolic pathway shifts to acids production and the yields of both acetoin and 2,3-BD sharply decreased, while at pH $\leq$ 6.0 the substrate conversion was slow, reflecting on reduced productivity [9,67,86]. Raspoet et al. investigated the effect of pH on ten *B. licheniformis* strains and found out that eight of them produced a maximum 2,3-BD from glucose at pH 6.0, and the other two produced equal amounts at pH 6.0 and 6.5 [86]. The maximum 2,3-BD titer, yield, and productivity obtained by *B. licheniformis* 10-1-A from glucose were at pH 7.0. The highest acetoin titer also was achieved at pH 7.0 [70].

It remains unclear whether pH has an impact on the 2,3-BD/acetoin production ratio or not. Bao et al. [108] investigated the process of 2,3-BD conversion to acetoin using an engineered strain of *B. subtilis*. The authors found out that BDH (acetoin reductase/butanediol dehydrogenase), possessing reversible activities, has different pH optima for oxidation (pH 8.5) and reduction (pH 6.5). Thus, the strain showed the highest conversion rate of 2,3-BD to acetoin at pH 8.5 [108]. This effect of pH on the acetoin reductase activity was confirmed later for *B. amyloliquefaciens* B10-127 [104]. However, in the process of sugar conversion to acetoin and 2,3-BD, the highest amounts of both products were obtained at equal [70], or close [78] values of pH. Using *B. amyloliquefaciens* 18,025 in the process of bioconversion of VHP cane sugar, Maina et al. [78] noticed that at a certain aeration mode (k_L a = 49 h^{-1}) maximum D(−)2,3-BD was formed at pH 6.0 (32.3 g/L), but the highest acetoin titer was reached at pH 6.4 (8.5 g/L). However, at higher levels of aeration, when acetoin is the main product, the highest titer, yield, and productivity of acetoin was observed at pH 6.0. This indicates that pH and aeration are most likely interdependent parameters.

7.2.3. Aeration

Aeration is the crucial parameter determining the acetoin/2,3-BD production ratio. In the same manner as all producers of 2,3-BD, *Bacillus* spp. can obtain energy by two different pathways, fermentation and respiration [22], and metabolites formation depends on the relative activity of both pathways [78]. The high oxygen supply favors respiration, and in conditions with an excess of oxygen, the carbon flux is mainly channeled toward cell mass formation [136], resulting in a low yield of fermented products. On the opposite, in conditions with a lack of oxygen, cell mass is insufficient, which results in slow carbon consumption and low productivity.

Thus, for effective production of acetoin and 2,3-BD, both pathways should work simultaneously, in a proportion determined by the product specificity. Because the reduction of acetoin to 2,3-BD by BDH requires simultaneous oxidation of NADH to NAD+, NADH availability is essential for 2,3-BD formation. Under conditions of oxygen excess, through the oxidative phosphorylation more NADH is converted to NAD+, which hampers 2,3-BD production at the expense of acetoin accumulation [78]. Thus, acetoin appears to be a more aerobic product than 2,3-BD. Using *B. subtilis* culture, Moes et al. [90] found that, at a level of dissolved oxygen (DO) more than 100 ppb, acetoin is mainly accumulated, and vice versa, when DO is below 100 ppb–2,3-BD production is predominant. The required DO level can be obtained by manipulation of aeration flow rate or agitation speed. On the other hand, oxygen demand depends on the specificity of the producer, hence, aeration is generally an object of optimization to ensure effective production.

To provide optimal aeration, the most usual optimized process parameters are aeration flow rate and agitation speed (Tables 5 and 6). Wang et al. [58] optimized both aeration flow rate and agitation speed in batch processes of sucrose utilization by *B. subtilis* CS13. They found that maximum acetoin and 2,3-BD are produced at aeration rates of 2 vvm and 1 vvm, respectively. For both products, the optimal was agitation speed of 500 rpm [58]. In the process of glucose conversion by *B. amyloliquefaciens* FMME044, using an aeration rate of 1 vvm at different agitation speeds was found that 300 rpm is optimal for 2,3-BD, and 400 rpm–for acetoin production [64]. Similarly, in glucose conversion by *P. polymyxa* CS107 at 0.5 vvm, 400 rpm is the optimal agitation for 2,3-BD, and 500 rpm–for acetoin production [100].

It is clear from all indicated optimizations so far, that acetoin production is maximized at higher aerations level than 2,3-BD for any organism used. However, in the course of fermentation, DO level is not constant. As a rule, DO sharply drops in the first few hours and slowly increases when the culture is exhausted [67]. This also affects the production of acetoin and 2,3-BD. On the other hand, when maximum 2,3-BD production is desired, at the beginning of the process more oxygen is needed for biomass formation, followed by a period with less oxygen for product formation.

Based on these presumptions, for an additional increase in product formation, two-stage or three-stage aeration strategies are applied. For 2,3-BD production from glucose by strain *B. licheniformis* 10-1-A, using aeration of 1 vvm, agitation rate was kept at 400 rpm for the first 10 h, and 200 rpm from then to the end of the process. Thus, 115.7 g/L 2,3-BD was obtained [70]. Yang et al. [104] used a three-stage aeration strategy for 2,3-BD production from a mix of glycerol and molasses by *B. amyloliquefaciens* B10-127. Agitation was kept consistently at 350 rpm (0–10 h), 400 rpm (5–22 h), and 350 rpm (22–88 h). Song et al. [75] applied two-stage agitation control for 2,3-BD production from glucose by *B. licheniformis* GSC3102. At this aeration strategy, the agitation rate was changed from 500 to 400 rpm when acetoin concentration passes over 10 g/L. To obtain maximum acetoin concentration from sugarcane molasses by *B. subtilis* DL01, Dai et al. [98] used three-stage aeration–agitation rate of 200 rpm (0–6 h), 300 rpm (6–24 h), and 350 rpm (after 24 h). The maximum obtained acetoin was 61.2 g/L.

Optimizations of aeration and agitation rates have the disadvantage that they apply only to the specific apparatus. Therefore, to obtain more information about the process, control of DO levels (%) or k_L a (volumetric oxygen transfer coefficient, h^{-1}) can be applied. As it was mentioned, during acetoin and 2,3-BD production from bakery waste hydrolysate, Maina et al. [5] found that for optimal acetoin formation, k_L a = 203 h^{-1} (corresponding to 1 vvm and 600 rpm), while the optimal for 2,3-BD production is a far lower value of k_L a-64 h^{-1} (corresponding to 1 vvm and 300 rpm). Similarly, in the process of sugarcane molasses conversion, for optimal acetoin production, k_L a = 104 h^{-1}, and for 2,3-BD production, k_L a = 30 h^{-1} [78].

7.3. By-Products Reduction Strategies

The techno-economic evaluation of the production of acetoin and 2,3-BD reveals that efficiency of fermentation is the most important factor in the industry, followed by the process of separation and purification [137,138]. Industrial feasibility depends on the high titer of the target metabolite and the reduced concentration of the by-products. Because acetoin is a precursor to 2,3-BD, both metabolites present in all processes performed with bacilli. As already noted above, the directing of the production towards 2,3-BD or acetoin can be conducted by fine-tuning the process parameters, with aeration and agitation being of the utmost importance. EPS synthesis was successively diminished by optimization of the medium content [61]. As for strategies to reduce other by-products such as acetate, lactate, and succinate, this is often performed through genetic engineering improvement of *Bacillus* spp. producers. For example, Yang et al. achieved significant reduction of by-products formed by *B. amyloliquefaciens* B10-127 after over-expression of glyceraldehyde-3-phosphate dehydrogenase and NADH-dependent 2,3-BDH. The simultaneous action of these enzymes redistributed glucose flux, decreasing molar yields of unwanted by-products acetoin, lactate, and succinate by 80.8%, 33.3% and 39.5%, compared to the parent strain [105]. To decrease the accumulation of acetate and L-lactate by *B. subtilis* 168, Fu et al. disrupted both *pta* (coding phosphate acetyltransferase) and *ldh* (coding L-lactate dehydrogenase) genes in the genome of the strain [139]. The absence of *pta* gene resulted in less acetate and succinate accumulation. The deletion of *ldh* gene did not affect the metabolic flux in 2,3-BD pathway directly, but increased the NADH/NAD+ ratio, which is the key factor for 2,3-BD production. The deletion of *ldh* gene in *B. licheniformis* BL1 fully eliminated the lactate production [135]. The deletion of *acoA* (encoding acetoin

dehydrogenase E1 component) in *B. subtilis* reduced acetate synthesis and increased acetoin concentration [11,139].

8. Conclusions

The two most important factors influencing the successful application of the microbial process on an industrial scale are: the cost of the carbon source in the composition of the fermentation medium, and the stability and safety of the microbial producer. In both respects, the GRAS producers of acetoin and 2,3-BD belonging to *Bacillus* and *Penibacillus* genera are promising. In addition to their non-pathogenic nature, another important property of bacilli is that they can synthesize pure stereoisomers of 2,3-BD and acetoin that can be used in chiral chemical syntheses. Another advantage of bacilli is their ability to convert raw, cheap, and waste substrates, including plant biomass, which makes the processes cost-effective and a good basis for the development of biotechnology. Finally, *Bacillus* spp. are highly tolerant to 2,3BD and acetoin, which allows greater production. Advances in mathematical and statistical methods for modeling and analyzing processes have led to the development of highly efficient fermentations, almost reaching the limits of process optimization. Therefore, a further impetus to increase the production of 2,3-BD and acetoin will be devoted to the genetic improvement of the strains through genetic and metabolic engineering.

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