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Microbial Producers of Butanol

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Abstract—This review is written due to an increased interest in the production of energy carriers and basic substrates of the chemical industry from renewable natural resources. In this review, the microbiological aspects of biobutanol production are reflected and the microbial producers of butanol (both natural, i.e., members of the *Clostridium* genus, and recombinant), obtained by genetic modification of Clostridia and other microorganisms, are characterised.

Keywords: biofuel, butanol, renewable feedstock, clostridia, recombinant butanol producers, *Clostridium acetobutylicum*

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Production of energy from renewable natural resources is one of the most urgent tasks of our time. Limited resources of fossil fuels, unstable oil prices, and the necessity to reduce carbon dioxide emissions into the atmosphere stimulate the need to explore new technologies of liquid fuel production from plant-based materials. Four-carbon alcohol—butanol (1-butanol, *n*-butanol)—is of particular interest. Due to its high energy density, low vapor pressure, good miscibility with gasoline and ethanol, and high octane number (table 1), butanol is considered as a promising fuel for combustion engines.

It is suggested that in the future butanol may partially replace gasoline and diesel, and this will not require changes in existing engines and fuel supply and distribution systems [1, 2].

Butanol is currently used for dye, nitro enamel, plastificator, butyl acetate, phenol formaldehyde resin, and oil additive manufacturing, and it is also a solvent; butanol is an extracting agent for fats. On a commercial scale, butanol is produced by chemical synthesis from propylene or acetaldehyde; the most promising alternative to this is microbiological synthesis, which allows one to generate butanol from renewable carbon feedstock.

The ability of anaerobic bacteria to synthesise butanol was described by Pasteur in 1862 [3]. The first industrial process for manufacturing solvents by fermenting starch and other carbohydrates was developed by Fernbach and Strange in 1912 [4]. In 1912–1914, Weizmann isolated several bacterial strains that produced acetone and butanol. The most efficient of them (BY) was used to develop an industrial process for solvent production [5, 6]. Later, the BY strain was thoroughly characterised by McCoy et al. and was renamed as *Clostridium acetobutylicum* [7].

By the middle of the last century, the output of Weizmann's solvent manufacturing process from starch-containing feedstock was the second highest after the output of ethanol production by yeasts. Acetone- and butanol-producing plants were set up in the United States (the Terre Haute and Peoria plants), Japan, India, Taiwan, Australia, and South Africa (the Germiston plant). The history of acetone and butanol production in capitalist countries is described in detail elsewhere [8].

In the Soviet Union, industrial production of acetone and butanol was set up in 1929–1934; it was based on the technology developed by V.N. Shaposhnikov and colleagues and was developing independently from Western countries [9–11]. The growth stages of acetone and butanol producing bacteria were studied, the theory of a two–phase fermentation process was offered, and it was shown that the fermentation process is dependent on the environmental conditions: sterility, temperature, pH, nitrogen nutrition, fermentation products, redox potential, and other factors. In addition, methods were developed for active spore production and microbiological control of the production process, as well as a semi-continuous (battery) fermentation technique using *C. acetobutylicum* strains isolated by F.M. Chistyakov (1928–1929). The advantages of these strains were resistance to bacteriophage infections and high productivity at 37°. Eight acetone-butanol plants were built in the Soviet Union; the biggest among them were the Dokshukinskii and Efremovskii plants. Wheat and rye flour and potato starch were used as feedstock. The Dokshukinskii plant used mashes (fermentation media) consisting of

Table 1. Comparative characteristics of different automobile fuel types [1]

Fuel	Ethanol	*n*-Butanol	Isobutanol	Gasoline	Diesel	Biodiesel
heating value, mJ/l;	21	29	29	32	39	37
vapor pressure, kPa	7.58	0.53	1.17	0.7–207	<0.07	<0.07
vapor pressure of a mixture with gasoline, kPa	138*	44.1*	46.9*	53.8–103.4	–	–
average octane number	116	87	110	90	–	–
cetane number	–	–	–	–	45	49–58
freezing temperature, °C	–114.5	–89.5	–108	<–60	(–30)–(–9.9)	7.5–16
hygroscopicity	high	low	low	low	very low	very low
compatibility with modern infrastructure (see text)	–	+	+	+	+	-

* 10% alcohol + 90% gasoline; "–" – absent.

flour (30%) and a mixture of molasses, and hydrolyzates of corn stubs and sunflower shells (>70%) [9–11].

In Western countries, potato, corn, molasses, cassava (a type of manioc), and wheat starch (a side product of gluten production) were used as substrates for industrial fermentation. Using *Clostridium beijerinckii* NRRL B592 strain for the fermentation of partially hydrolyzed potato starch produced during potato growth and processing was economically beneficial [12–14]. Cheese whey [15–19], wood or other plant-based feedstock hydrolyzates [14, 20–24], agricultural wastes [25], palm oil and apple paste wastes [27], and soy molasses [28] can also be used as substrates.

The development of petro-chemical industry and the rise in prices on traditional fermentation substrates led to a decrease (and even total termination) in solvent production worldwide [29]. However, in the Soviet Union, acetone–butanol plants operated until the 1980s. In Egypt, solvent production based on the Soviet technology existed until 2008.

At present, biobutanol production is only successful in the People's Republic of China: there are 16 plants in the country that are either functioning or being developed. The estimated output of the largest plant (Ji-An Biochemical Co. Ltd) is 150000 tons per year. The second largest plant in Guangxi (Guinping Jinyuan Alcohol Industry Co. Ltd) with an estimated output of 100000 tons per year is using the bacterial fermentation process, the prototype of which was developed and introduced in the Soviet Union. Corn and cassava starch are used as substrates in Chinese plants [30]. Apart from China, a plant with an output of 10000 tons per year, using sugar cane juice as a fermentation substrate, is functioning in Brazil (David Jones, personal communication).

Due to a revival of the interest in biobutanol production fuel and biotechnological companies, and scientific organizations combine their efforts to investigate the physiology, genetics, and metabolism of butanol-producing clostridia. Attempts to set up an efficient process of butanol biosynthesis, predominantly from renewable carbohydrate-based feedstock, are made by the BP and Dupont (the Butamax demonstration plant) companies, by the Chinese company Cathey Industrial Biothec (the estimated output of this plant, which is still being constructed, is 200000 tons per year) [30], and by the American company Cobalt Biofuels (a pilot plant with a productivity of 7.7 tons per year and a projected plant with an estimated output of 5000 tons per year), (personal communication, S.M. Burns-Guydish, 2010). Butyl Fuel LCC (United States) developed and patented a two-stage butanol production process, including the production of butyric acid; Green Biologics (Great Britain) developed strains that can tolerate a 4% concentration of butanol in media. The companies Tetra Vitae Bioscience (United States), Arbor Fuel Inc. (United States), Butalco (Switzerland), and Metabolic Explorer (France) are involved in developing an efficient process of biobutanol production from lignocelluloses. A technology of butanol production through the fermentation of wood hydrolyzate by immobilized *C. acetobutylicum* cells is being developed at the Aalto University (Finland) [31]. In Russia, a large-scale biobutanol production from wood hydrolyzates is planned at the Tulunskii hydrolysis factory based on the technology developed by ZAO Biosintezbelok.

The main purpose of this review was a comparative characterization of the currently existing butanol-pro-

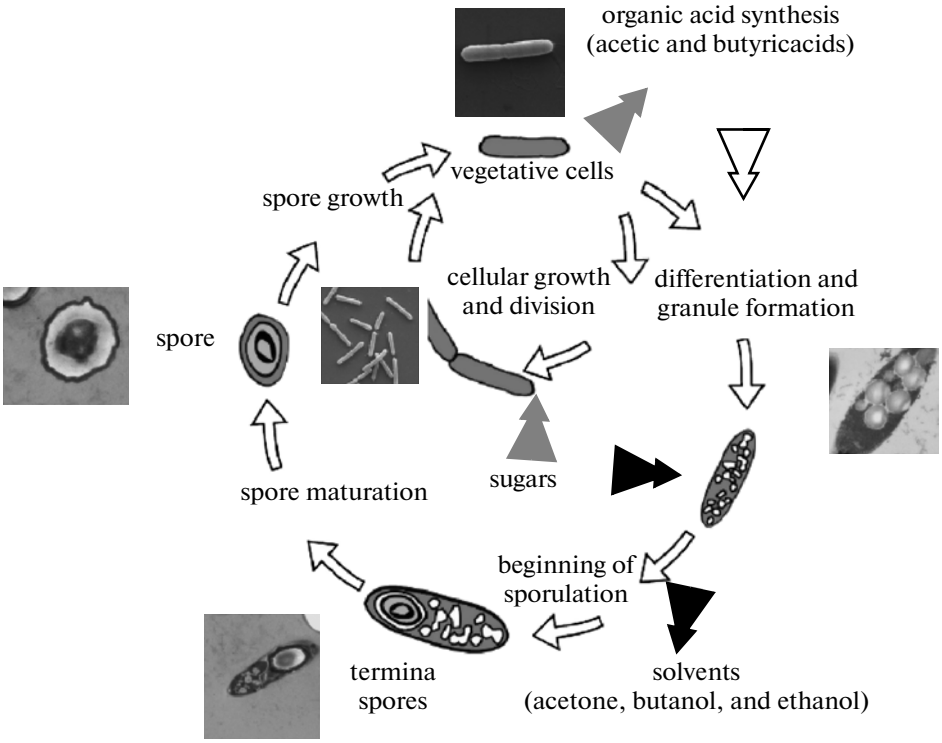

Fig. 1. Clostridia life cycle (from [40]) (schematic representation).

ducing microbial strains (both natural and obtained by selection and genetic modification).

BACTERIA OF THE GENUS *CLOSTRIDIUM* ARE NATURAL BUTANOL PRODUCERS

Traditionally, for industrial fermentative butanol production, anaerobic solventogenic (i.e., producing solvents—butanol, ethanol, and acetone/isopropanol) bacteria of the *Clostridium* genus were used, which are *C. acetobutylicum, C. beijerinckii, C. saccharobutylicum, C. saccharoperbutylacetonicum*, and *C. aurantibutyricum*. All industrial clostridia are mesophilic bacteria. The metabolic products of *C. acetobutylicum, C. beijerinckii, C. saccharobutylicum,* and *C. saccharoperbutylacetonicum* are CO_2, H_2, acetic and butyric acids, acetone, butanol and ethanol. *C. aurantibutyricum* and some strains of *C. beijerinckii* are able to convert acetone into isopropanol [32]. Unlike *C. botulinum* and *C. tetani*, solventogenic clostridia do not produce life-threatening toxins and are harmless for humans, animals, and plants [33].

Apart from the ones listed above, there are other species of solventogenic clostridia that have never been used in industry. The *C. pasteurianum* and *C. ljungdahli* produce insignificant amounts of butanol; the *C. puniceum* and *C. tetanomorphum* bacteria produce butanol in equimolar amounts with other compounds—acetone and ethanol, respectively. In addition, a small amount of butanol is produced by *Thermoanaerobacterium thermosaccharolyticum*, previously known as *Clostridium thermosaccharolyticum* [34–36]. The butanol- and isopropanol-producing strain *Clostridium toanum* Baba [37], used for large-scale production in Taiwan in 1942–1958, is not currently included in any international collection [14].

Morphology and Life Cycle of Solventogenic Clostridia

Like all other members of the genus *Clostridium*, solventogenic bacteria are obligate anaerobes and are able to form endospores [38]. Their vegetative cells have the shape of straight or slightly curved rods with rounded ends (either single or forming pairs and short chains). At the end of the exponential growth phase, cells start to accumulate granulose and form an extracellular capsule, which leads to a change from rod-shaped to cigar- or clostridium-shaped [39]. Morphological changes are usually associated with a metabolic switch from acid synthesis to neutral product synthesis—acetone and alcohols. Young vegetative forms of clostridia move using peritrichous flagella. In an old culture, cells lose their mobility and begin spore formation. The forming spores are oval or spherically shaped. The diameter of these spores is usually larger than that of a vegetative cell; therefore, if a forming spore is located in the middle, the cell becomes spindle-shaped, whereas, if spores are located terminally, the cell acquires the shape of a drumstick [32]. The clostridia life cycle is illustrated in Fig.1.

Difficulties in the Identification of Solventogenic Clostridia

An intraspecies classification of solventognenic clostridia based on phenotypical and biochemical characteristics can often be difficult due to the similarity of the bacteria with each other in this group. Some of the criteria, generally accepted for bacteria, are not applicable in the case of solventogenic bacteria [14]. For example, the membrane lipid content [14] and fermentation products of clostridia can change, depending on the cell stage and growth conditions. Therefore, molecular genetics methods, such as 16S rRNA analysis [42] and DNA hybridization [43], are necessary for a proper species identification. Since some clostridial species share more than 97% homology in 16S rDNA, a comparative analysis of the structure genes makes sense.

In 2001 a reclassification of more than 40 industrial strains, presented in the ATCC, DSM, NCP, and NCIMB collections, was performed using molecular genetic methods. As a result, the investigated strains were divided into four species: *C. acetobutylicum, C. beijerinckii, C. saccharobutylicum*, and *C. saccharoperbutylacetonicum* [44]. The names *C. kaneboi* (100% identical to *C. acetobutylicum* ATCC 824T), *C. butanologenum* (identical to *C. beijerinckii*), *C. madisonii*, *C. saccharoacetobutylicum*, *C. acetonigenum*, and several others are no longer used.

Due to the imperfection of the earlier classification, some of the data published prior to 2001 require a critical approach. For example, according to the first edition of Bergey's *Manual of Systematic Bacteriology*, *C. acetobutylicum* is unable to hydrolyze gelatine and to ferment amygdalin and raffinose [38]. This fact not only contradicts the formal description of *C. acetobutylicum* [7], but also the patent by Weizmann [5]. However, Keis et al., the founders of the molecular genetic classification of solventogenic clostridia, have established that all strains that were classified as the *C. acetobutylicum* species are able to hydrolyze gelatine and ferment amygdalin and raffinose, which also agrees with the data by McCoy and Weizmann [5, 7, 44].

The discrepancies in earlier descriptions of *C. acetobutylicum* are also due to the fact that some other solventogenic strains were mistakenly referred to this species. For example, the industrial strain *C. acetobutylicum* P262, used for solvent production until 1980, according to its molecular genetic and biochemical characteristics, was later separated into a different species—*Clostridium saccharobutylicum*. Among other strains that were referred to the *C. saccharobutylicum* species by Keis et al. [44], there were industrial strains, patented in 1937–1938 by the Commercial Solvent Corporation under the name of *Clostridium saccharobutyl-acetonicum-liquefaciens* [45–47]; they were considered to be members of the *C. acetobutylicum* species until 2001.

Characterization of the Main Species of Solventogenic Clostridia

The characteristics of the four main species of solventogenic clostridia are presented in Table 2.

All strains of the industrial clostridia, investigated by Keis et al., hydrolyzed aesculin and utilized arabinose, xylose, glucose, mannose, cellobiose, lactose, maltose, sucrose, methyl-glucopyranoside, raffinose, salicin, amygdalin, starch, and dextrin; they did not produce indole, urease, and catalase; they were also unable to efficiently hydrolyze microcrystalline cellulose [44].

Strains of the *C. acetobutylicum* species have been investigated most [7, 38, 44, 48]. These saccharolytic bacteria can be isolated from soil, sediments, intestines of some types of molluscs, and from cattle, as well as dog and human faeces [38]. *C. acetobutylicum* species are Gram-positive, although they can be Gram-negative in aging cultures. They are obligate anaerobes and their vegetative cells can survive in the presence of oxygen for several hours, whereas their endospores can survive for several decades [50]. *C. acetobutylicum* are able to fix nitrogen from the atmosphere [38], to reduce nitrites into ammonia; they do not form nitrites from nitrates; they form H_2S from thiosulfates and sulphites. *C. acetobutylicum* grow at a temperature of 20–47° (the optimum temperature is 37°) [48]. A theoretical mass balance of glucose fermentation by these bacteria is presented in Table 3.

Traditionally, the substrates used for *C. acetobutylicum* were sodden viscous mashes of wheat, and rye and corn flour or crushed grains, which did not require pH or anaerobiosis maintenance and already contained necessary vitamins and microelements. According to Weizmann [5], a periodic fermentation of 8.0–10.0% corn mash by a *C. acetobutylicum* strain was carried out at 34–39° for 40–60 h. The final concentration of solvents was 12–20 g/l with the ratio butanol : acetone : ethanol being equal to 6 : 3 : 1. In some cases, the ratio of solvents could vary, depending on the strain, substrate, and cultivation conditions, and could be, for example, 76.1 : 17.9 : 6.0, 75.6 : 22 .4 : 2.0, or 60 : 38 : 2 [6, 8, 51, 52]. The yield of solvents calculated as a mass equivalent of dry substrate was 25–26% [8]. The efficiency of the periodic fermentation process by solvents was 0.35–0.50 g/l per hour.

A battery fermentation technique with a *C. acetobutylicum* strain has been developed and used at the Dokshukinskii plant since 1961. In a 54-h cycle, 30–36 h were required to load the batteries; a mature culture fed to rectification moving from the tail fermenter to the head one (a new cycle was starting from the opposite end of the battery). The process could continue without infection of the mash for up to 25 days. The yield of solvents calculated for an average starch was ~37–38% when fermenting flour mashes and ~36% for mixtures of flour with hydrolyzates. The

Table 2. Phenotypic and biochemical characteristics of four industrial species of clostridia [44]

Species / Characteristics	*C. acetobutylicm*	*C. beijerinckii*	*C. saccharoperbutyl-acetonicum*	*C. saccharobutylicum*
type strain	ATCC 824, (=DSM 792, JCM 1419, LMG 5710, VKM B-1787)	DSM 791 (=NCIMB 8052)	N1-4 (=ATCC 13564)	NCP 262T (=DSM 13864 T, ATCC BAA-117T), NRRL B-643
Phenotypic characteristics				
number of characterized strains [44]	7	16	2	4
vegetative forms	straight rods, single, or forming pairs; do not form chains; size: 0.5–0.9 × 1.6–6.4 μm	straight rods, single, form pairs or short chains; size: 0.5–1.7 × 1.7–0.8 μm	short and long rods, single, sometimes form pairs; size: 0.4–0.8 × 3.1–6.2 μm	short and long rods; average size 1.4 × 6.3 μm . The cell length varies within a range of 3.8–10 μm
endospores	oval, subterminal	oval, eccentric or subterminal	oval, 0.8–1.5 × 1.6–2.2 μm	oval, 1.1 × 1.8 × 1.7–3.9 μm, terminal or subterminal, and up to 15% bipolar
colonies	colonies on blood agar 1–5 mm in diameter, flat or dome-shaped, granular, translucent, with irregular margins	colonies on blood agar 1–5 mm in diameter, round or irregular-shaped, may have irregular margins, translucent, grey, smooth and glistening	colonies on CBM* agar 2–3 mm in diameter, round domed, white, have smooth surfaces and undulated margins.	colonies on CBM agar 2–3 mm in diameter, domed, creamy yellow, with a smooth surface, round-shaped with irregular margins
Biochemical characteristics				
sensitivity to rifampicin, 10–100 ng/disk	+	(–)**	–	+
riboflavin synthesis	+	–	–	–
ability to hydrolyze gelatine	+	(–)	+	+
ability to utilize substrates:				
ribose	–	(+/–)	–	C
glycerol	C	C	–	–
D-arabitol	–	(+)	+	–/+
L-arabitol;	–	(+)	+	–/+
dulcitol	–	(+)	(+/–)	–
inositol	–	+	(+/–)	+
mannitol	+	+	+	–/+
sorbitol	(+/–)	+	(+/–)	–
melezitose	(–)	+	+	–
melibiose	(–)	(+)	+	+
ramnose	–	(C)	C	–
threhalose	(–)	+	+	+
turanose	(C)	+	+	+
glycogen	+	(+)	+	+
inulin	(+/–)	+	+	+/–
pectin	+	+	+	–

Notes: * CBM is clostridium basal medium [49].

** Extent of the characteristic: "+", – positive reaction; " – ", negative reaction; C, weak reaction; (+), the majority of the strains have a positive reaction; (–), the majority of the strains have a negative reaction; (C), the majority of the strains have a weak reaction; –/+, the reaction of the strains is sometimes positive; +/–, the reaction of a strain is sometime negative; (+/–), 40–60% of the strains show positive reactions

Table 3. Mass balance of glucose fermentation by *C. acetobutylicum*, mole of products per mole of fermenting glucose (from [8])

Products	Total fermentation	Acid production phase	Solvent production phase
H_2	1.35	2.5	1.4
CO_2	2.21	2.0	2.3
acetate	0.14	0.5	–*
butyrate	0.04	0.75	–
acetone	0.22	–	0.3
butanol	0.56	–	0.65
ethanol	0.07	–	0.1
solvent yield, %	32	–	36.7

* Products are not produced.

butanol yield in both cases was ~21– 22 or ~57% of the total amount of solvents [9].

C. saccharobutylicum strains (Table 2) were used for solvent production from corn substrates and various types of molasses, containing 6.0–7.5% of fermentable sugars, with the addition of ammonium salts or organic nitrogen. The most well-know strains are NRRL B-643 (supposedly, Commercial Solvents Co) and NCP 262 (National Chemical Products (NCP) Plant, Germiston, Republic of South Africa) [53]. The process was carried out at 29–33° for 29–33 h. The final pH in the culture was 5.2–6.4; the solvent yield was 27–33%, and the total concentration of solvents was 17–21 g/l, out of which 55–74% was butanol. Corn substrates usually required longer fermentation with a lower yield of solvents [54]. From all known clostridia strains, *C. saccharobutylicum* NCP 262 has the best characteristics for cheese whey fermentation. This strain not only utilized lactose, but also lactate (up to 7 g/l), from the fermentation medium, which is possibly the reason for its high productivity (0.31 and 0.7 g/L per hour for periodic and continuous fermentations, respectively) [19, 55].

Some of the *C. saccharobutylicum* and *C. acetobutylicum* strains are able to synthesise bacteriocins. Bacteriocin from the *C. saccharobutylicum* NCP 262T, produced at the end of the exponential growth phase, inhibits the growth of *C. saccharobutylicum* and *C. felsineum* and does not affect the growth of *Achromobacter, Escherichia coli, Serratia marcescens, Salmonella typhimurium*, and *Bacteroides fragilis* [56]. A similar bacteriocin from *C. acetobutylicum* ATCC 824 inhibits the growth of *C. butyricum* and Bacillaceae, and does not affect *Corynebacterium glutamicum, E. coli, Proteus mirabilis, Aerobacter aerogenes,* and *Zymomonas mobilis* [57].

C. beijerinckii strains (Table 2) were isolated from soil and fermented plants [38, 44, 48, 58, 59]. A distinguishing characteristic of *C. beijerinckii* is its ability to form a clot when cultivated on milk after 24 h of growth. *C. beijerinckii* fixes atmospheric nitrogen and does not form nitrites from nitrates. Its optimal growth temperature is 30°. When *C. beijerinckii* NCP265 and NCP270 were used for solvent production in South Africa [44, 60], the process was carried out in a fermenter with a volume of 90000 l and molasses was used as a substrate; initial pH was adjusted to 5.8–6.0 with ammonium and stabilized with calcium carbonate. The production cycle required 48 h, out of which 30–34 h was required for fermentation. 5850 kg of fermentable sugars of molasses were converted into, 1053 kg of butanol (18% of fermentable sugars or ~11.7 g/l), 526 kg of acetone (9%), 175 kg of ethanol (3%), 2900 kg of CO_2 (50%), and 117 kg of H_2. The total concentration of solvents was ~19.5 g/l [60].

C. beijerinckii cells were immobilized for the first time in order to organize an continuous solvent production process from whey permeate; this increased the productivity 16 times, compared to traditional fermentation by not immobilized cells [16, 17].

The *C. saccharoperbutylacetonicum* species (Table 2) was first described in 1960 [61]. The status of the species was confirmed in 1995 by the results of an analysis of 16S rDNA [53] and DNA-hybridization [62]. The strain *C. saccharoperbutylacetonicum* N1-4 and its derivatives N1-4 (HMT) and N1-504 are placed in the ATCC collection under the numbers 13564, 27021T, and 27022, respectively. Unlike *C. beijerinckii*, *C. saccharoperbutylacetonicum* cells hydrolyze gelatine and do not form a clot in milk during the first 48 h of growth. *C. saccharoperbutylacetonicum* does not produce riboflavin [62] and does not hydrolyze coagulated albumin (or hydrolyze in insignificant amounts). The N1–4 strain (unlike the N1–504 strain) ferments sorbitol, dulcite, and inositol. *C. saccharoperbutylacetonicum* does not reduce nitrates to nitrites, but it synthesise ammonium from nitrites. Hydrogen sulfide is mainly produced via reduction of thiosulfates and in small amounts via reduction of sulphites; however, it is not produced at all when *C. saccharoperbutylacetonicum* is cultivated on peptone-containing media [44]. The species poorly sporulates when cultivated on the majority of available laboratory media. The optimal temperature for the solvent biosynthesis is 25–35°; optimal pH is 5.6–6.7.

In industrial production, *C. saccharoperbutylacetonicum* was used for solvent production from various sugar- and starch-containing substrates (including molasses with the addition of ammonium salts or organic nitrogen). Fermentation on molasses (4–6% of utilizable sugars) was carried out for 48–72 h at 25–35°. At the end of the fermentation process, pH reached 5.5–8.0; the solvent yield was 27–34%, and

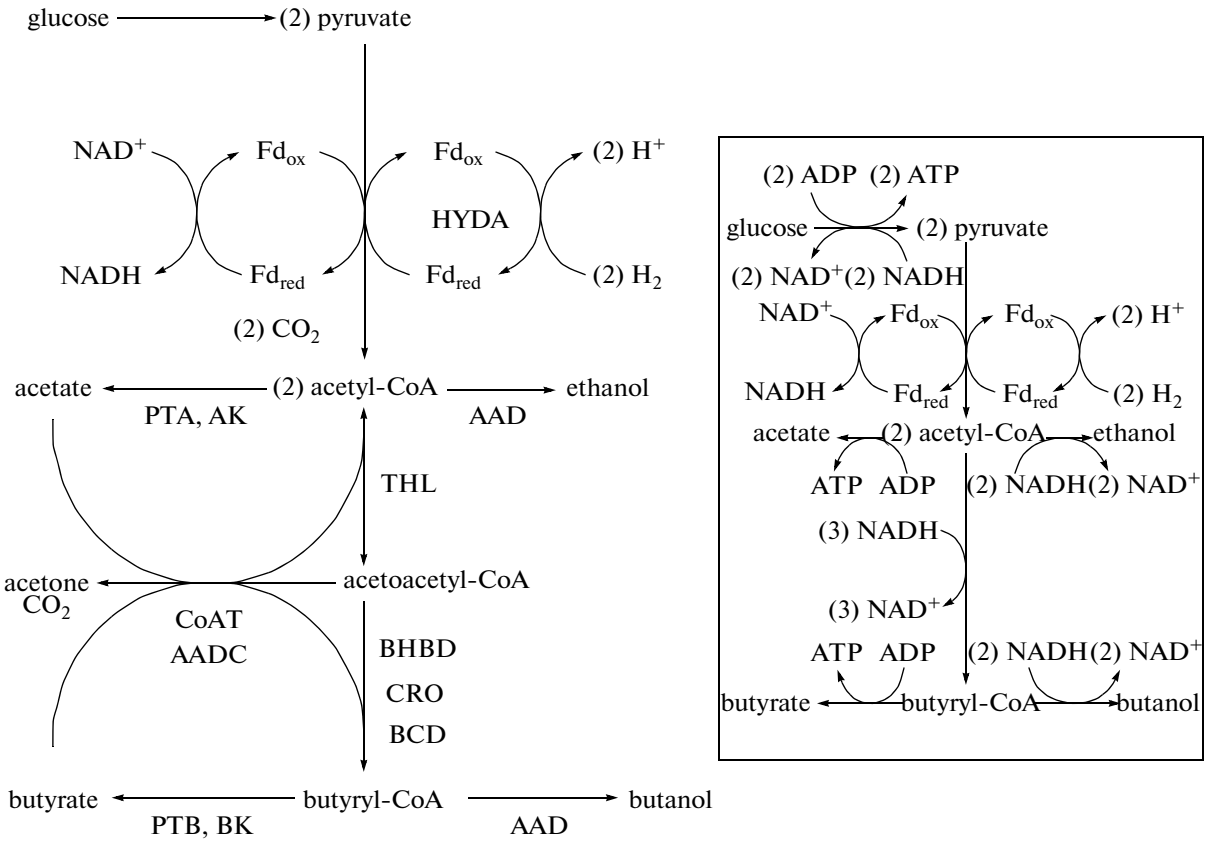

Fig. 2. Metabolic pathways of butanol and organic acid production in *C. acetobutylicum*: HYDA, hydrogenase; PTA, phosphotransacetylase; AK, acetate kinase; THL, thiolase; BHBD, 3-hydroxybutyryl-CoA-dehydrogenase; CRO, crotonase; BCD, butyryl-CoA-dehydrogenase; CoAT, CoA-transferase; AADC, acetoacetate decarboxylase; BK, butyrate kinase; PTB, phosphotransbutyrylase; AAD, aldehyde/alcohol dehydrogenase; Fd_{ox}, oxidised ferredoxin; and Fd_{red}, reduced ferredoxin. The boxed pathway shows the ATP generation and NADH production occurring during metabolism (from [79]).

the final concentration was 15–19 g/l (the amount of butanol was 73–85%). More time was required for the fermentation of flour compared to molasses, whereas the yield and concentration of solvents were significantly lower [54].

During batch fermentation of 7.2% (m/v) glucose [36], *C. puniceum* bacteria produce butanol and acetone at a ratio of 10 : 1 (179 and 16.8 mM, respectively); they are also able to ferment starch. *C. puniceum* are pectinolytic bacteria, producing a pink-coloured pigment [63], which differs from the pink–lavender pigment of *C. beijerinckii* NRRL B592. According to the data of a 16S rDNA analysis, *C. puniceum* is phylogenetically close to *C. beijerinckii*.

C. pasteurianum bacteria produce acetone (90 mM), butanol (135 mM), and small amounts of ethanol on synthetic mineral media, containing 12.5% (m/v) of glucose [64]. On media containing 3.5% (m/v) of glucose or less, they only produce small amounts of butanol and acetone [64–66]. One of the *C. pasteurianum* isolates produces acetone and butanol when cultivated on 3% inulin [67]. When cultivated on glycerol, *C. pasteurianum* produces butanol, 1,3-propanediol, and ethanol [66, 68, 69]. *C. aurantibutyricum* produces butanol, isopropanol, and acetone from glucose [65]. Differences between *C. aurantibutyricum*, *C. beijerinckii* and *C. butyricum* were identified by DNA hybridization [70].

Metabolism of Acetone–Butanol Fermentation

All known solventogenic clostridia share similar metabolism. During the fermentation, solvents (acetone, ethanol, and butanol) and gases (H_2 and CO_2) are produced. The fermentation process is two-staged. During the first stage, acids are produced. Accumulation of butyric acid in the medium and decrease of pH are signals leading to a switch in intracellular metabolic pathways, starting active solvent production [32, 66, 71–73]; this is induced at the gene transcription level via the genes coding the enzymes of corresponding metabolic pathways [74]. However, the molecular mechanism of transcription regulation is not completely clear and is currently under investigation [75–78]. Metabolic pathways of solventogenesis by *C. acetobutylicum* are presented in Fig. 2.

At present, the genomes of *C. acetobutylicum* ATCC824 [80] and *C. beijerinckii* NCIMB 8052 are

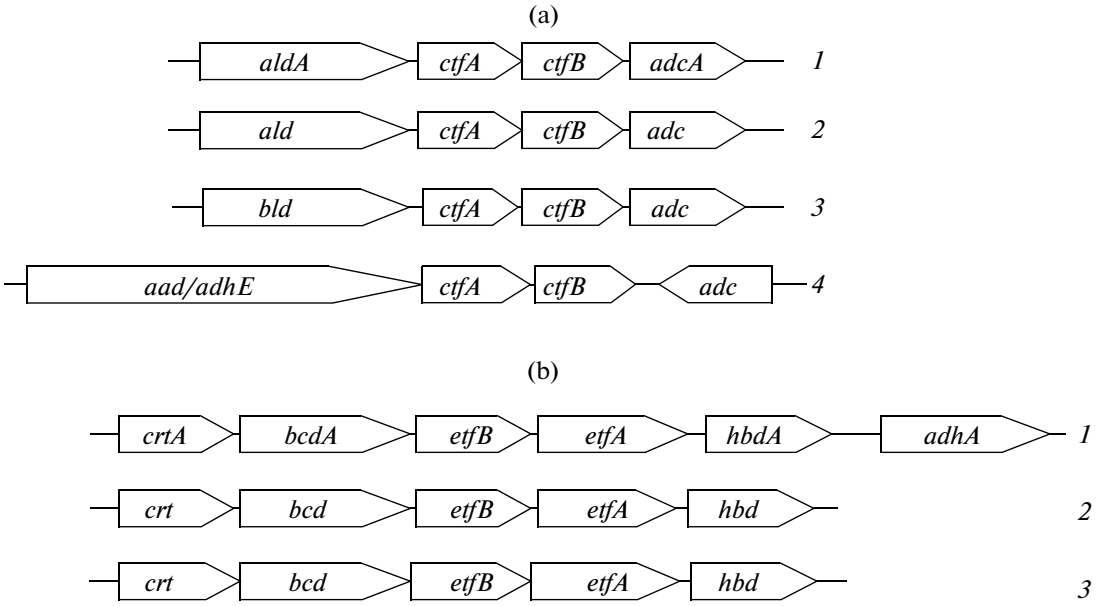

Fig. 3. Organization of the genes responsible for solventogenesis in four clostridia species (from [82]): (a) *sol*- and *adc*-operons (*1*, *C. saccharobutylicum* Ox29; *2*, *C. beijerinckii* NCIMB 8052; *3*, *C. saccharoperbutylacetonicum* N 1–4; and *4*, *C. acetobutylicum* ATCC 824); (b) *bcs*- and *adhA*-operons (*1*, *C. saccharobutylicum* Ox29; *2*, *C. beijerinckii* NCIMB 8052; and *3*, *C. acetobutylicum* ATCC 824). See the text for the names of the enzyme-encoding genes.

sequenced which made the identification and localization of solventogenesis-responsible genes significantly easier. The genome of *C. acetobutylicum* consists of a circular 3941-kb (kilobase) chromosome (AE001437 GenBank) and a 192-kb megaplasmid (AE001438 GenBank). The chromosome includes the genes of thiolase *thl* GenBank CAC 2873, butanol dehydrogenases *bdhA* and *bdhB* (CAC 3299 and CAC 3298), phosphotransacetylase *eutD* (CAC 1742), acetate kinase *askA* (CAC 1743), butyrate kinase *buk* (CAC 3075), phosphotransbutyrylase *ptb* (CAC 3076), and *bcs*-operone: *hbd*, *etfA*, *etfB*, *bcd*, and *crt* (CAC 2708, CAC 2709, CAC 2710, CAC 2711, and CAC 2712). A megaplasmid contains *sol*-operon genes, including the bifunctional aldehyde/alcohol dehydrogenase genes *aad*/*adhE*(*adhel*) (CAP 0162) and subunits of the CoA-transferase *ctfA* and *ctfB* genes (CAP 0163) and *ctfB* (CAP 1064). It also contains the genes of acetoacetate decarboxylase *adc* (CAP 0165), bifunctional aldehyde/alcohol dehydrogenase *adhe* (CAC 2873), and thiolase *thil* (CAP 0078) and sporulation genes. The plasmid is unstable and only maintains in cells during the sporulation stage [80].

The genome of *Clostridium beijerinckii* NCIMB 8052 consists of a circular chromosome of approximately 6700 kb in length (CP000721, GenBank).

The genome of *C. saccharobutylicum* NCP 262 consists of a circular chromosome of approximately 5300 kb in length. Although the complete genome sequence of *C. saccharobutylicum* has not been published yet, the genes for certain groups of enzymes responsible for the synthesis of acids and solvents have been identified and cloned for the NCP 262 and Ox29 strains. Some of the solventogenesis genes have also been identified for *C. saccharoperbutylacetonicum* N1–4 [81, 82]. The genomic organization of several genes responsible for solventogenesis in four clostridia species is presented in Fig. 3.

Strain Degeneration Phenomenon in Solventogenic Clostridia

Solventogenic clostridia lose their productivity over time if the cells are maintained in the vegetative state for a prolonged period of time [83, 84]. Strain degeneration is a result of genetic changes and is not the same as the loss of ability to produce solvents under unfavourable cultivation conditions [85]. Degeneration is also different from the loss of the solvent production ability due to a megaplasmid loss—a phenomenon only common for *C. acetobutylicum*. Asporogenous mutations are susceptible to degeneration during continuous cultivation [86]. Thus the maintenance of cells in the vegetative state via repassaging or continuous cultivation leads to an accumulation of degenerated cells within the culture, while the density of the culture and the rate of metabolism decrease [87]. Compared to normal cells, degenerated *C. beijerinckii* and *C. saccharobutylicum* cells are longer and thinner, and they form larger colonies with less regular shapes [86, 88, 89]. The development of mutants with a reduced level of degeneration along with an identification of the Spo0A factor as a main regulator of the solvent production initiation should promote the

elucidating mechanisms of degeneration and a metabolic shift. Occasionally, some of the degenerated cells can spontaneously return to their original condition. Degeneration is more prominent in *C. beijerinckii* strains compared to *C. acetobutylicum* [86].

Glycosyl Hydrolases of Solventogenic Clostridia

Almost all clostridia that are able to efficiently hydrolyse crystalline cellulose form cellulosomes for this purpose—multienzyme extracellular protein complexes, consisting of endo- and exoglucanases and cellulose-binding proteins, joined by scaffolding molecules [90]. Genes encoding at least 11 cellulosome proteins were identified in the genome of *C. acetobutylicum* ATCC 824 [80]. However, functional cellulosomes, as well as an ability to efficiently hydrolyze microcrystalline cellulose, have not been found in solventogenic clostridia. Inducible activity of endoglucanase and cellobiase has been reported in the strains *C. acetobutylicum* NRRL B527 and ATCC 824 [91] and *C. beijerinckii* NCP270 [92], and high hemicellulase activity has been detected in several *C. acetobutylicum* and *C. saccharobutylicum* strains [82, 93]. The genes encoding endoglucanase and xylanase were cloned from *C. saccharobutylicum* NCP 262 [94, 95]. Solventogenic clostridia can grow on xylan and pentoses [38, 44, 96], producing moderate amounts of solvents [21]. Arabinose is an excellent substrate for solvent production by the *C. beijerinckii* NRRL B592 strain; however, it is not very suitable for *C. acetobutylicum* NRRL B527 (ATCC 824) or *C. beijerinckii* NRRL B593 [21]. The genes supposedly encoding the enzymes of xylan degradation form an operone located on the megaplasmid of *C. acetobutylicum* ATCC 824 [80]. Although the main substrate for industrial solvent production is starch, the amylolytic system of clostridia is not fully investigated [96, 97]. It is known that *C. acetobutylicum* ATCC 824 produces several amylolytic enzymes [98, 99]; the genes of some amylases are identified [80].

Most Active Butanol Producers

The choice of a strain for industrial production depends on a number of factors: feedstock, fermentation method (periodic, continuous, or battery), technology of solvent separation, required composition and ratio of the end metabolic products, requirement for additional minerals and vitamins, resistance to bacteriophage and contamination with lactic acid bacteria [100, 101]. In most cases, strains with the required characteristics were isolated from the nature; however, recently several attempts have been made to create new butanol-synthesizing bacterial strains via mutagenesis or genetic engineering. A classical approach to improving the properties of solventogenic clostridia is still mutagenesis (irradiation with UV light, exposure to alkylating agents, and insertion of transposons) with consequent selection [14]. At present, the best producers of butanol, obtained by mutagenesis (selection), are *C. beijerinckii* BA 101 and *C. acetobutylicum* VKPM B10290. The first one has an increased amylolytic activity and produces 18.6 g/l of butanol (251 mM), 8.6 g/l of acetone (148 mM), and 0.3 g/l of ethanol (6.5 mM) in 48.5 h on 6% glucose. Under the same conditions, the wild strain *C. beijerinckii* NCIMB 8052 produces 9.2 g/l of butanol (124 mM), 4.4 g/l of acetone (75 mM), and 0.9 g/l of ethanol (19 mM). The weight fraction of butanol in the BA 101 strain is 18% from the total amount of solvents; the mass yield of butanol to a mole of glucose is 33%. The productivity of the BA 101 strain during continuous fermentation is 1.74 g/l/h compared to 1.17 g/l/h for the parent NCIMB 8052 strain [102].

C. acetobutylicum VKPM B10290 cultivated on starch produces up to 20 g/l of butanol, which is 53.5% of the total amount of solvents. The mass yield of butanol to a mole of glucose equivalent from starch is 32.3% [103].

Metabolic Engineering of Solventogenic Clostridia

In the last few years, the development of methods and approaches to the genetic modification of solventogenic clostridia has received a lot of attention [96, 104]. As a result, a large set of tools for targeted genetic modification of clostridia has been developed for creating strains with higher levels of solvent synthesis, improved selectivity, and higher resistance to butanol [105]. Cloning, expression, and gene inactivation methods are widely used to change the metabolic pathways of solventogenesis. In particular, a ClosTron system was developed for the rapid and specific disruption of clostridial genes by insertion of group II introns from *Lactococcus lactis* [106]. An inactivation of the *pta* gene (phosphotransacetylase) in *C. acetobutylicum* led to a decrease in the phosphotransacetylase and acetate kinase activity, which significantly reduced the production of acetate. An inactivation of the *buk* gene led to a decrease in the butyrate kinase activity, a reduction in butyrate production, and an increase in butanol, acetone, and ethanol production up to 16.7 g/l (225 mM), 4.4 g/l (76 mM), and 2.6 g/l (57 mM), respectively [107, 108].

A superexpression of the gene cluster *adc*, *ctfA*, and *ctfB*, responsible for acetone synthesis, in *C. acetobutylicum* led to an increase in butanol and acetone production up to 13.2 and 8.6 g/l, respectively, which is 37 and 90% higher than in the wild strain [109].

One of the best genetic modifications of *C. acetobutylicum* ATCC 824 was an inactivation of the *sol*R gene combined with a superexpression of the *aad* gene (aldehyde/alcohol dehydrogenase). The obtained strain synthesised 17.6 g/l of butanol (238 mM), 8.2 g/l of acetone (141 mM), and 2.2 g/l of ethanol (48 mM), which is 51, 66, and 194% higher, respectively, than in the type strain [110, 111].

An increase in the level of selectivity for butanol biosynthesis can make the process of purification more efficient and economical. The combined use of ctfB-asRNA (antisense RNA to the *ctfB* gene, encoding a subunit of CoA-transferase) and superexpression of the *aad* gene in *C. acetobutylicum* allowed for an increase in the ratio of butanol to acetone from 1.83 ± 0.05 to 4.89 ± 0.29, although the synthesis of butanol decreased from 10.2 to 9.8 g/l [112].

A strategy for the use of antisense RNA to the *buk* and *ptb* genes was used to decrease the butyrate titer and to increase the butanol titer in *C. acetobutylicum* [113]. However, despite the expectations, the strain transformed with buk-asRNA synthesised a third more butyrate (111 mM), as well as butanol (154 mM or 11.4 g/l), compared to the wild strain (81 mM butyrate and 113 mM or 8.4 g/l of butanol). The strain transformed with ptb-asRNA synthesised 2.1 g/l of butanol, and no significant uptake of butyrate was observed during the stationary growth phase [73].

Superexpression of the *aad* gene in the mutant *C. acetobutylicum* M5, not showing butyraldehyde dehydrogenase, acetoacetate decarboxylase, and CoA-transferase activity and not synthesising acetone and butanol, led to the restoration of butanol (to 11.1 g/l at pH 5.75), but not acetone production [79, 114]. An additional superexpression of the thiolase gene (*thl*) led to a decrease in acetate and ethanol titers, although the butanol level also decreased (to 0.8 and 5.7 g/l, respectively, at pH 5.5 and 6.0) [79]. Apparently, the level of solvent synthesis is strongly related to the intracellular level of NADH, an excess of which is necessary for an increase in butanol titers and a decrease in acetone titers during fermentation.

Superexpression of the *groESL* gene in *C. acetobutylicum*, which encodes the class I heat shock protein, led to an increase in the *C. acetobutylicum* resistance to butanol [115].

In attempts to increase butanol production, an emphasis was placed on increasing the amount of enzymes involved in butanol biosynthesis. However, this approach does not give a significant increase in productivity [73]. It might be due to the fact that the anaerobic metabolism of *C. acetobutylicum* is accompanied by only a small output of energy, whereas protein biosynthesis is an energy-consuming process and can lead to serious disruptions of total metabolism [116]. An alternative solution for constructing butanol-producing strains with certain characteristics could be an improvement of enzyme qualities via genetic engineering methods, using rational design and targeted evolution (laboratory evolution) as approaches. An example of successful laboratory evolution is the construction of highly active alkane hydrolase, the turnover rate of which was increased 20 times compared to the wild type enzyme [117].

Construction of Butanol-Producing Strains Based on Other Microorganisms

Despite the numerous attempts to improve solventogenic clostridia, so far it has not been possible to overcome the difficulties related to the slow growth speed, production of solvent mixture, and the two-phased nature of the fermentation process. These are the main restrictions in creating a highly efficient strain, ensuring the profitability of *n*-butanol production in the current economic conditions. At present, the world's leading research centers and biotechnological companies are exploring ways to develop butanol producers from other microorganisms via cloning the required complex of genes encoding the metabolic pathways of butanol biosynthesis. The main criteria in the choice of a recipient microorganism are sufficient understanding of the metabolism and genome of this strain, an availability of genetic engineering tools, increased butanol resistance of the recipient, a wide range of utilising substrates, and metabolism intensity. The first attempts of a butanol synthesis gene transfer into microorganisms, such as *E. coli* (butanol production 0.55–1.2 g/l) [118, 119], *Saccharomyces cerevisiae* (2.5 mg/l) [120], *Lactobacillus brevis* (300 mg/l) [121], *Pseudomonas putida* (580 mg/l), and *Bacillus subtilis* (120 mg/l) [122], were not particularly successful. The small yield of butanol during heterologous synthesis demonstrates the low efficiency of the clostridia metabolic pathway functioning within other microorganisms. Recombinant strains with high levels of ethanol (50 g/l) [123, 124], isobutanol (20–50 g/l) [125, 126], and isopropanol (40–140 g/l) [127] production were created when using appropriate metabolic pathways. In all these cases, the driving force, directing the metabolic flux to the end product, was the presence of an irreversible reaction of decarboxylation in a synthetic chain. However, when cloning a clostridium CoA-dependent pathway of n-butanol synthesis into a heterologous organism, a significant driving force directing the carbon flow to butanol does not develop. Another possible reason for the low production level of butanol in heterologous organisms is the low functioning efficiency of the Bcd/EtfAB clostridium enzyme complex, which performs the conversion of crotonyl-CoA to butyryl-CoA.

A significant achievement on the way to the creation of recombinant butanol-producing microorganisms was the development of an *E. coli* strain, containing a chimerical pathway of butanol synthesis, combined form reactions, encoded by the genes of three different organisms. For this procedure, the following genes were used: *phaA* and *phaB* from *Ralstonia eutrophus*, encoding thiolase and hydroxybutyryl-CoA dehydrogenase; *crt* from *C. acetobutylicum*, encoding crotonase; and *ccr* from *Streptomyces collinus*, encoding crotonyl-CoA reductase, which, unlike the butyryl-CoA dehydrase enzyme complex Bcd/EtfAB from *C. acetobutylicum*, reveals a high activity when expressed in *E.coli*. For converting butyryl-CoA to

butanol, the butyraldehyde/butanol dehydrogenase AdhE2 from *C. acetobutylicum* possessing high affinity to C_4-substrates was selected. The *phaA*, *phaB*, and *crt* genes were localized under the control of a relatively weak arabinose promoter to avoid their superexpression and to compensate for the high activity of the encoded proteins. The *ccr* and *adhE2* genes were placed under the control of a strong promoter—T7*lac*—aiming to increase the expression of key enzymes, determining the driving force of the carbon flow towards butanol synthesis. To produce an excess of acetyl-CoA and NADH, the level of expression of the *aceEF-lpd* operone was increased, which led to a threefold increase in the pyruvate dehydrogenase activity. The *E. coli* strain constructed with all of the above modifications produced 4.65 ± 0.72 g/l of butanol with 28% yield calculated to glucose [128].

However, the best result was achieved by expressing the thiolase (*ato*B) and formate dehydrogenase (*fdh*) genes from *E. coli*; *hbd*, *crt*, and *adhE2* from *C. acetobutylicum*; and enoyl-Coa reductase (*ter*) from *Treponema denticola* in an *E. coli* strain, containing deletions in the aldehyde/alcohol dehydrogenase (*adh*E), lactate dehydrogenase (*ldh*A), fumarate reductase (*frd*), and phosphotransacetylase (*pta*) genes. Under anaerobic conditions, the butanol synthesis level by this strain reached 15 g/l in flasks and 30 g/l in a fermenter, and butanol reached 88 and 70%, respectively. The productivity of the strain by butanol was 0.2 g/l/hour, which is comparable with and even surpasses the performance of some natural butanol producers [129].

Thus, recent achievements in the developments of recombinant butanol producers on the platform of *Clostridia* or other microorganisms open the way for efficient production of the new generation biofuels.

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