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Review paper
Pregledni rad

A SHORT LOOK AT MICROBIAL PRODUCERS OF BIOBUTANOL – NEW TRENDS, POTENTIALITIES AND LIMITATIONS

KRATAK PREGLED MIKROBIOLOŠKIH PROIZVOĐAČA BIOBUTANOLA - NOVI TRENDOVI, POTENCIJALI I OGRANIČENJA

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ABSTRACT

Nowadays, the energy crisis, climate change and greenhouse effect have created a strong demand for the development of alternative energy sources. Researchers have made an attempt to produce biobutanol from various second-generation feedstocks with new microbial strains and new technologies. This paper presents the recent progress on efficient butanol production using various biomass sources and "designed" microorganisms. The advances in the butanol fermentation process, such as the use of numerous waste materials, improved strains and co-cultivation of mixed microbial populations are of particular interest in this review.

Keywords: *biobutanol; substrates ABE; clostridia, microorganisms.*

REZIME

Danas su energetska kriza, klimatske promene i efekat staklene bašte stvorili snažnu potražnju za razvojem alternativnih izvora energije. Istraživači su pokušali da proizvedu biobutanol iz različitih sirovina druge generacije novim sojevima mikroorganizama i novim tehnologijama. Ovaj rad pikazuje savremeni razvoj u efikasnoj proizvodnji butanola koristeći različite izvore biomase i „dizajnirane" mikroorganizme. Inovacije u procesu fermentacije butanola, kao što su upotreba brojnih otpadnih materijala, poboljšani sojevi i zajednička kultivacija mešovitih mikrobioloških populacija su od posebnog interesa u ovom pregledu.

Ključne reči: *biobutanol; podloge ABE; klostridije, mikroorganizmi.*

INTRODUCTION

The climate change and greenhouse effect, as well as limited availability and fluctuating price of fossil fuels, urge researchers and technologists to develop more efficient methods for the production of biofuels from safe and inexpensive sources. One of the European Union directives indicates that 10% of all fuels must be bio-synthesized by 2020 *(Ferreira et al., 2019)*.

Biofuels may be produced directly from available food resources, like sugar, starch, and oil, or from available crops, like sugar cane, corn, beets, wheat, sorghum, rapeseed, sunflower, soybean, palm, or coconut. They are then recognized as first-generation biofuels. The second-generation biofuels are produced from lignocellulosic materials: forest residue, woody biomass and nonfood crops *(Kolesinska et al., 2019)*. Second-generation production has some advantages over first-generation. For instance, second-generation substrates can grow on poor quality marginal land with less water and fertilizer and no direct competition with food crops. However, second-generation bioethanol production requires more capital cost due to the sophisticated processing equipment and lower energy density compared to first-generation biofuels. With the aim of addressing the issues associated with first- and second-generation biofuels, researchers have explored alternative feedstocks for biofuel production (Fig. 1). In turn, biofuel produced from algal biomass is considered third-generation.

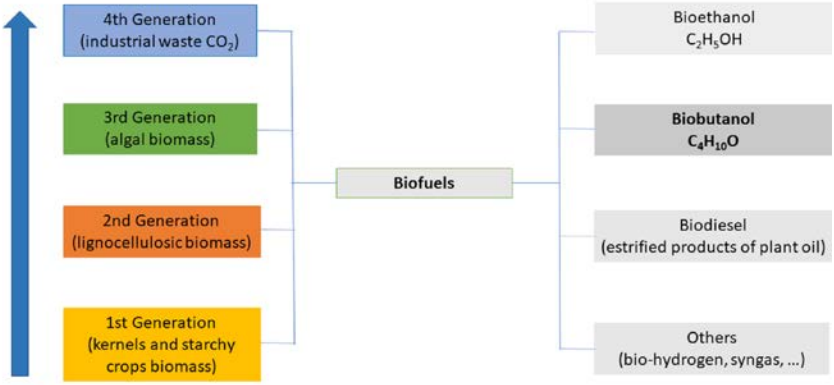

Fig. 1. Feedstock classification.

Third-generation materials represent a promising source due to the number of remarkable advantages over first- and second-generation feedstocks. For instance, microalgae can be cultivated on marginal land with a water environment, a low cost of cultivation, a high conversion efficiency, and a high energy density *(Halder et al., 2019)*.

Butanol production by microorganisms was first reported by Louis Pasteur in 1861. However, it was not until 60 years later that Chaim Weizmann used anaerobic bacteria of the genera *Clostridium* for butanol production on a large scale. Interest in acetone-butanol-ethanol fermentation was developed in the 1980s, due to the escalating price of petroleum.

Although biotechnological production of biobutanol is more expensive and complicated than bioethanol production, its numerous advantages over bioethanol might outweigh the

balance in biobutanol favour. Biobutanol shows better characteristics over bioethanol, such as: (i) miscibility with gasoline at any concentration, (ii) availability for use with current gasoline infrastructure due to its lower corrosiveness, (iii) lower vapor pressure and water compatibility, (iv) higher energy density *(Mařík et al., 2014; Kuroda and Ueda, 2016; Xin et al., 2018; Halder et al., 2019; Li et al., 2019; Kolesinska et al., 2019)* (Tab. 1).

Table 1. Comparison of the basic properties of ethanol, butanol and gasoline.

Fuel	Ethanol	Butanol	Gasoline
Formula	C_2H_5OH	C_4H_9OH	$C_4 \div C_{12}$
Molecular weight	46.07	74.12	100-105
Density at 15 °C (kg m^{-3})	795	810	750
Viscosity at 20 °C (mm^2 s^{-1})	1.52	3.64	0.4–0.8
Calorific value (MJ kg^{-1})	26.4	32.5	43.3
Octane number VM	108	96	95
Boiling point (°C)	78	118	30–190
Vapor pressure at 20-25°C (kPa)	~12,4	~0,9	~75
Oxygen content (% vol)	34.7	21.6	< 2.7

In addition, butanol has been regarded as an important chemical of various industrial applications. For example, it can be used as the solvent for the production of hormones, drugs, antibiotics, cosmetics, and even vitamins *(Lee et al., 2008)*.

MATERIAL AND DICUSSION

Microorganisms

Many strains being capable of butanol formation are recognized and can be categorized as the genus *Clostridium* (e.g. *Clostridium acetobutylicum, C. beijerinckii, C. saccharoperbutylacetonicum, C. saccharobutylicum*) and non-*Clostridium* strains (e.g. *Escherichia coli, Lactobacillus brevis, Saccharomyces cerevisiae*) *(Nimbalkar et al. 2018; Li et al., 2019b).*

The examples of butanol production using clostridia and various carbon sources are presented in Table 2.

Fermentation by *C. acetobutylicum* and *C. beijerinckii* is known as acetone - butanol - ethanol (ABE) fermentation, where acetone, 1-butanol, and ethanol are produced at a ratio of 3:6:1. *C. acetobutylicum* was originally isolated and grown on starch, while *C. beijerinckii* was performed better on molasses-based feedstocks *(Niglio et al., 2019)*.

Table 2. The productivity of butanol by Clostridium spp. using different carbon sources (Kolesinska et al., 2019)

Strain	Carbon source	Productivity (g L^{-1})
C. beijerinckii	soy molasses	8
C. beijerinckii BA101	peanuts	18.9
C. acetobutylicum	sago starch	16
C. acetobutylicum	fresh domestic wastes	3
C. acetobutylicum DSM 792	wasted vegetables	9.96–10.65
C. acetobutylicum YM1	deoiled rice bran	6.48

Uncertainty for investments in the ABE industry is especially created by acetone. Acetone, in contrast to biobutanol, is corrosive to rubber engine parts and has poor fuel properties *(Li et al., 2019a)*. The acetone issue may be addressed by metabolic engineering to decrease acetone production by ABE producers. This solvent can be converted into isopropanol by specific solventogenic *Clostridium* sp. strains in the isopropanol – butanol - ethanol (IBE) fermentation *(Dos Santos Vieira et al., 2019)*.

Classic batch butanol fermentation is divided into two stages, (i) acidogenesis (sugar conversion into organic acids) and (ii) solventogenesis (solvent production) (Fig. 2). In the beginning, bacterial cells grow exponentially producing acids, mostly acetate and butyrate. This stage leads to a decrease in pH to around 4.5. Then, acid production rate falls as the bacterial cells shift their metabolic activity from acidogenesis to solventogenesis, in response to the low pH. The organic acids re-enter the cells and act as co-substrates for solventogenesis. The major product of this second phase is butanol, with an admixture of acetone and ethanol *(Kolesinska et al., 2019)*.

It is worth noting that the presence of solvents affects the cell membrane fluidity and metabolic functions. Therefore, ABE fermentation using wild-type clostridia is limited by weak solvent tolerance, sluggish growth and low cell density during the solventogenic phase.

Compared to the genus *Clostridium*, non-*Clostridium* strains can reduce or eliminate the major byproducts of acetone and ethanol *(Kuroda and Ureda, 2016)*. Recent research has been performed which investigates the combination of modifications to conventional fermentation processes and/or strain development using metabolic engineering in order to obtain the levels of butanol yield. Other interesting alternatives develop acetone-free butanol production by yeast *Saccharomyces cerevisiae (Swidah et al., 2018, Zhao et al., 2018)*. Several rational strategies were also tested in *E. coli* to increase butanol productivity *(Ferreira et al. 2019)*.

The first report of butanol production by a wild microorganism that does not belong to clostridia was published by *Amiri et al. (2016).* Recently, researchers have focused on halophiles and thermophiles for biofuel production *(Amoozegar et al., 2019; Xin et al., 2018)*. The production of butanol was observed in halophile strain *Nesterenkonia* sp. F (family

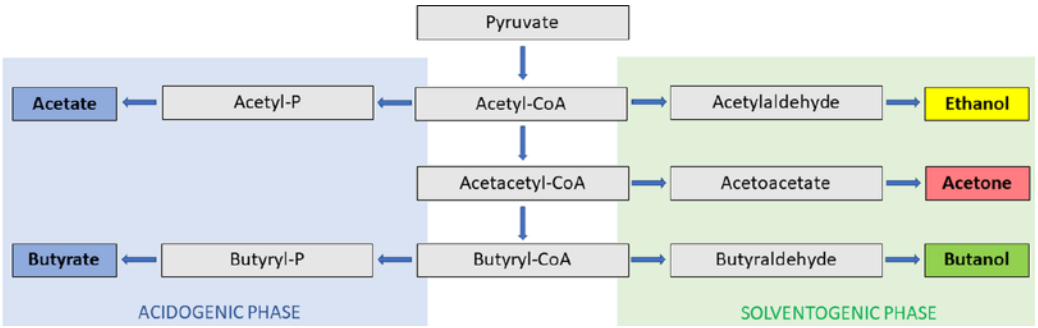

Fig. 2. ABE fermentation.

Micrococcaceae of the order *Actinomycetales*) under aerobic or anaerobic conditions. It was reported that this bacterium, isolated from a hypersaline lake in Iran, shows the ability to ABE fermentation under aerobic and anaerobic conditions. Cultivation of the isolate under anaerobic conditions with 50 g L^{-1} of glucose for 72 hours resulted in the production of butanol equal to 0.105 g L^{-1}. It was the first report of butanol and ethanol production by a wild microorganism that does not belong to *Clostridium* sp. *Jiang et al. (2018)* and *Xin et al. (2018)* also isolated and characterized a new thermophilic strain *Thermoanaerobacterium* sp. M5, which could directly produce butanol from hemicellulose at 55 °C. The isolate possessed a unique pathway with acetone elimination.

Strain improvement

The key factors determining the metabolic response of *Clostridium* spp. to butanol stress are thought to be changes in the lipid and fatty acid compositions of bacterial cells, intracellular metabolic changes and the osmoregulator concentrations. The outer membrane is the first defense for bacteria against unfavorable environmental conditions. It was documented that under butanol stress *C. acetobutylicum* cells might change their levels of long acyl chain saturated fatty acids and branched-chain amino acids to adjust the fluidity and maintain the integrity of their cell membranes *(Kolesinska et al., 2019).*

Liu et al. (2014) developed a novel strategy called "1-butanoleglycerol storage" to enhance butanol tolerance in *C. acetobutylicum* during long-term preservation. Under optimal storage conditions, in a solution containing 16 g L^{-1} butanol mixed with 200 g^{-1} glycerol at temp. 37°C, after 12 months the cell survival rate was very high and equaled 80%. In addition, the bacterial cells showed enhanced butanol tolerance of 32 g L^{-1} that was 2-fold higher in comparison to the control strain. These results show that the preservation conditions for butanol producers are very important for enhancing butanol tolerance and preventing loss of productivity.

The modification of membrane structure could enhance cell viability not only in *Clostridium* spp. producers. *Guo et al (2019; 2020)* found that butanol accumulates at the phospholipid headgroups, therefore it causes only partial interdigitation of cells at physiological temperatures. Then they determined experimentally that reducing the lipopolysaccharide (LPS) core length and charge increased *E. coli* sensitivity to butanol. The resistance to ingress of butanol correlates with both core length and charge, where a lower charge density is more conducive to butanol assimilation. In contrast to systems with short-length LPS cores, butanol intercalation into membranes with longer LPS cores increases membrane order and rigidity, which might be due to their more porous internal structure. These findings will assist the development of more butanol-tolerant bacteria, where thicker, more compact and less polar LPS-core surfaces reinforce the integrity of membranes and further improve resilience in extreme environments.

Strategies to prevent the destruction of *Clostridium* spp. cells by the butanol synthesized in fermentation processes include the genetic engineering methods (Fig. 3). The first attempts to improve clostridia for butanol production were taken by *Lin et al. (1983).* They obtained the mutant *C. acetobutylicum* from the native strain ATCC 824 that was developed by serial enrichment of diluted n-butanol. The new strain showed significantly higher butanol tolerance (121%) than the native strain. Another novel mutant was developed from *C. acetobutylicum* by a combination of N-methyl-N'-nitro-N-nitrosoguanidine (MMNG), ethyl methane sulphonate and UV exposure *(Syed et al., 2008).* This strain showed greater potency (20%) in molasses and gave higher butanol yields in comparison to the native strain.

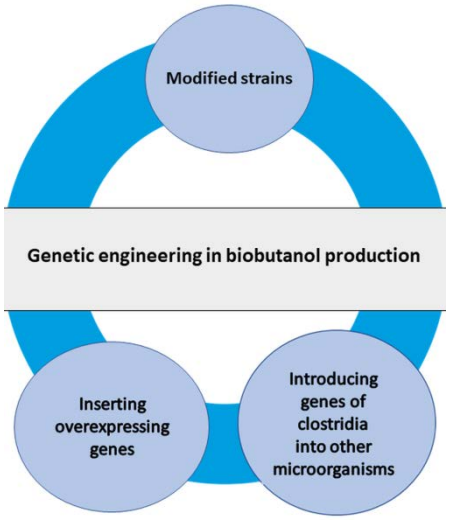

Fig. 3. Genetic engineering strategies in biobutanol production.

Identifying and modelling the key enzymatic reactions for butanol ratio in *C. acetobutylicum* is an important first step towards the construction of metabolically-engineered strains. Systems-level metabolic engineering of clostridia could lead to the discovery of entirely new biosynthetic pathways for butanol, and to the development of new strains that might overcome the current limitations of butanol fermentation.

It was found that the strain *C. beijerinckii* NJP7 IBE was able to secrete extracellular xylanases and convert hemicellulose of xylan directly to butanol and isopropanol *(Jiang et al., 2018).* This strain was able to partially convert 60 g L^{-1} birchwood xylan into 0.5 g L^{-1} isopropanol, 2.1 g L^{-1} butanol, and 3.2 g L^{-1} ethanol in 120 h (Xin et al., 2017).

In the first decade of the twenty-first century, the genomes of two butanol producing clostridia, namely *C. acetobutylicum* ATCC 824 and *C. beijerinckii* NCIMB 8052 were sequenced by *Garcia et al. (2011).* Therefore, sequencing the genomes for more hyper-butanol producing bacteria gives the possibility to enhance the fermentation process. Once the butanol and acetone producing genes had been identified, genetic modifications were attempted, to decrease or eliminate the acetone production. Lately, a special technology called TargeTron was used to disrupt the acetoacetate decarboxylase gene (*adc*), which is responsible for acetone production *(Máté de Gérando et al., 2018).* As a result, butanol production was increased from 70% to 80% and acetone production was reduced to 0.21 g L^{-1}.

It should be noted that genetic manipulations, particularly in native butanol producer *Clostridium* sp., are extremely difficult by both the problems in performing genetic manipulations and the formation of spores during the solventogenic phase. The modified strain after serial sub-culturing was unable to produce butanol and acetone, probably due to the destruction of solvent producing genes (*ctfA, ctfB, adc, aad*). Then plasmid pSOLI containing proper genes was inserted in bacterial mutants. Unfortunately, the engineered strains were still unable to produce butanol and acetone, due to the destruction of the inserted plasmid. In addition, non-clostridial strains have been attracted as alternative hosts for butanol production. Butanol-producing genes were introduced into bacterial cells belonging to genera: *Escherichia*, *Lactobacillus*, *Bacillus* as well as yeast *Saccharomyces cerevisiae (Kolesinska et al., 2019*; *Russmayer et al., 2019).* The expression of the clostridia butanol pathway in

E. coli was firstly implemented by *Atsumi et al. (2008)*. In a study conducted by *Inui et al. (2008)*, the genes *thiL, hbd, crt, bcd-etfB-etfA, adhE1* and *adhE2* from *C. acetobutylicum* ATCC 824, coding acetyl-CoA acetyltransferase, β-hydroxybutyryl-CoA dehydrogenase, 3-hydroxybutyryl-CoA dehydratase, butyryl-CoA dehydrogenase, butyraldehyde dehydrogenase and butanol dehydrogenase were introduced into *E. coli*. In another study, the genes of *C. saccharobutylicum* encoding crotonase, butyryl-CoA dehydrogenase, electron-transport protein subunits A and B, 3-hydroxybutyryl-CoA, dehydrogenase, alcohol dehydrogenase, CoA-transferase, acetoacetate decarboxylase and aldehyde dehydrogenase were inserted into this microorganism *(Berezina et al., 2009)*.

The low butanol production obtained show that many challenges remain in turning *E. coli* into a viable butanol production *(Ferreira et al., 2019)*. Until now, besides the clostridial route, a few alternative pathways for producing butanol have been tested in *E. coli*. *Dellomonaco et al. (2011)* and *Gulevich et al. (2012)* engineered *E. coli* to activate the reverse β-oxidation cycle in the absence of the inducing substrate (fatty acids) or by expressing enzymes to convert butyryl-CoA into butanol. Also, *Abdelaal et al. (2019)* engineered a synthetic pathway in the genome of *E. coli* MG1655 to produce n-butanol. Deletion of competing pathway followed by fed-batch cultivation of the engineered strain in a bioreactor with glucose-containing complex medium yielded 5.4 g L^{-1} n-butanol. In turn, Ferreira and co-workers designed novel strains of *E. coli* capable of producing butanol through the algorithm pathway using 2-oxoglutarate as the precursor *(Ferreira et al., 2019)*. The greatest titer (75 ± 4 mg L^{-1}) was obtained by strain cultivation in a high-density medium and induction by 0.5 mM of isopropyl β-1-thiogalactopyranoside. Unfortunately, the maximum titers obtained for this novel pathway were still far below those required for industrial purposes.

The genes from clostridia were also expressed in the host bacteria *Lactobacillus brevis (Berezina et al., 2010)* and *S. cerevisiae (Steen et al., 2008)*, but without significant improvement in butanol production. Recently, butanol production by *S. cerevis*iae was first attempted by transferring the 1-butanol-producing metabolic pathway for isobutanol synthesis. Utilizing alternative enzymes, eliminating competitive pathways, and maintaining cofactor balance achieved significant improvements in butanol production *(Kuroda and Ueda, 2016). Tan et al. (2016)* developed dynamic redirection of glucose from glycolysis to the isobutanol pathway.

Renewed interests in biobutanol as biofuel and rapid development in genetic tools stimulate technological advances to strain modifications. However, there is still a lack of sufficient tools to significantly improve the final butanol titer, and butanol production is still maintained at a low level below 20 g L^{-1}. On the other hand, more wild type solventogenic strains as butanol producers with unique properties were also isolated and characterized. Integration of novel strain isolation and metabolic evolution seems to be the best strategy to obtain more promising butanol producers *(Xin et al., 2018)*.

CONCLUSIONS

Butanol is a promising, renewable fuel that may be obtained from various agricultural residues. These materials have great potential as feedstock in biobutanol production processes. As they are co-produced with various food raw materials, their production does not compete with food production and will not result in land-use changes. Substantial research towards converting lignocellulosic feedstocks to biobutanol and other biofuels has been conducted during the last years. Establishing technologies to produce butanol from waste materials will create opportunities best adapted to the local conditions and market demands. However, it should be emphasized that commercial production by clostridial strains is hindered due to their poor tolerance to butanol and inhibitors. Metabolic engineering of *Clostridium* sp. producers and other 'complementary strains' seems to be essential to solving the technological problems in biobutanol production.

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