UDC 579.222 https://doi.org/10.15407/biotech15.01.005

ACETONE-BUTYL FERMENTATION PECULIARITIES OF THE BUTANOL STRAINS-PRODUCER

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Received 18.12.2021
Revised 17.01.2022
Accepted 28.02.2022

The aim of this review was to generalize and analyze the features of acetone-butyl fermentation as a type of butyric acid fermentation in the process of obtaining butanol as an alternative biofuel.

Methods. The methods of analysis and generalization of analytical information and literature sources were used in the review. The results were obtained using the following methods such as microbiological (morphological properties of strains), chromatographic (determination of solvent concentration), spectrophotometric (determination of bacterial concentration), and molecular genetic (phylogenetic analysis of strains).

Results. The process of acetone-butyl fermentation was analyzed, the main producer strains were considered, the features of the relationship between alcohol formation and sporulation were described, the possibility of butanol obtaining from synthesis gas was shown, and the features of the industrial production of butanol were considered.

Conclusions. The features of the mechanism of acetone-butyl fermentation (the relationships between alcohol formation and sporulation, the duration of the acid-forming and alcohol-forming stages during batch fermentation depending on the change in the concentration of H_2, CO, partial pressure, organic acids and mineral additives) and obtaining an enrichment culture during the production of butanol as an alternative fuel were shown. The possibility of using synthesis gas as a substrate for reducing atmospheric emissions during the fermentation process was shown. The direction of increasing the productivity of butanol-producing strains to create a competitive industrial biofuel technology was proposed.

Key words: producer strains, biofuel, biobutanol, acetone-butyl fermentation.

The process of acetone-butyl fermentation is associated with the transformation (oxidation) of the organic molecules of the substrate; consequently, the part of the energy is released and accumulated in the molecules of adenosine triphosphate (ATP) due to substrate phosphorylation [1–3]. As a rule, during fermentation, the carbon skeleton of the substrate molecule is split [4], and the fermentation products are formed, such as various organic acids (lactic, butyric, acetic, formic), alcohols (ethanol, butanol, propanol, acetone), as well as gases (carbon dioxide and hydrogen) [5]. In the process of fermentation, two stages can be distinguished — oxidative and reductive stage [6]. The oxidation process is based on the electron detachment from certain metabolites with the help of enzymes (dehydrogenases) and its attachment to other molecules (anaerobic oxidation) [7, 8]. The energy released during this process is stored in the form of ATP. The second stage is the reduction, in which the formed intermediate compound is reduced due to the transfer of electrons and protons to it from a temporary carrier. Reduced organic compounds are released by microorganisms into the external environment.

The present paper considers the stages of acetone-butyl fermentation as a type of anaerobic butyric acid fermentation, features of alcohol formation and sporulation, preparing an enrichment culture, butanol obtaining from synthesis gas, and selected aspects of industrial butanol production.

Acetone-butyl fermentation and butanol production

Acetone-butyl fermentation is a biochemical process of carbohydrates decomposition carried out by selected bacteria, which passes anaerobically (without oxygen access) and results in the formation of acetone, butyl alcohol, as well as acetic, butyric acids and fermentation gases, hydrogen and carbon dioxide.

There are several types of bacteria that are able carry out the process of butyric acid fermentation, e.g., in one of its subtypes, an acetone-butyl fermentation [9–11]. In butyric acid fermentation, glucose is oxidized to pyruvate via the glycolytic pathway, wherein pyruvate is further converted to acetyl-CoA. Acetone-butyl fermentation is carried out by microorganisms that belong to the genera *Clostridium, Butyrvibrio, Butyribacterium, Sarcina, Eubacterium, Fusobacterium* and *Megasphera* [3, 12].

The *Clostridium* genus belongs to the *Bacillaceae* family, together with other members of this family (*Bacillus, Sporolactobacillus, Desulfotomaculum* and *Sporosarcina*). Clostridia are gram-positive, spore-forming bacteria, their dimensions vary from about 2–3 to 7–8 μm in length and 0.5–1 μm in width. Spore-forming anaerobes include giant vegetative cells, reaching 15–30 μm in length and 1.5–2.5 μm in width. They are highly mobile due to peritrichous flagella. Vegetative cells are rod-shaped. However, their shape may vary depending on environmental conditions. Presence of the oval or spherical endospores changes the shape of the rod-shaped mother cell, since their diameter is usually greater than the width of this cell [13].

Physiologically, clostridia are distinguished by a pronounced fermentative type of metabolism, as well as sensitivity to oxygen: their growth is possible only under anaerobic conditions. However, there are also transitional forms from strictly anaerobic species (*C. pasteurianum, C. kluyveri*) to almost aerotolerant ones (*C. histolyticum, C. acetobutylicum*). Clostridia, as a rule, do not contain hemoproteins (cytochrome and catalase). Some species are able to form cytochromes if their precursors are contained in the nutrient medium. Among the reserve substances, starch-like polysaccharides are widespread [14].

The temperature optimum for the growth of most known *Clostridium* sp. lies between 30 and 40 °C. Along with those mesophilic microorganisms, there are many thermophilic species with an optimum of 60–75 °C (*C. thermoaceticum* and *C. thermohydrosulfuricum*). They are able to grow, as a rule, in a neutral or alkaline medium, and their growth almost completely stops in acidic conditions [15, 16].

Clostridia vary in their ability to ferment different substrates [17]. Some types of those microorganisms can ferment a wide range of different substrates, while others are highly specialized and are able to ferment only one or several types of raw materials (Fig. 1). *Clostridia* are able to convert polysaccharides (starch, glycogen, cellulose, hemicelluloses, pectins), organic acids, proteins, amino acids, heterocyclic compounds [18]. Selected microorganisms use complex nutrient media and/or growth substances, while others use molecular nitrogen as the only nutrient (*C. pasteurianum*) [19].

According to the ability to ferment various substrates, microorganisms can be divided into saccharolytic and proteolytic. Saccharolytic clostridia break down mainly mono- or polysaccharides, while proteolytic clostridia break down proteins and amino acids [20].

Butyric acid fermentation is mainly carried out by anaerobic microorganisms *C. butyricum, C. tyrobutyricum, C. lactoacetophilum* [22]. Their main fermentation products are butyric and acetic acids. Acetic acid fermentation of carbohydrates is observed in *C. aceticum* and *C. thermoaceticum* [23]. Propionic acid fermentation is inherent to *C. propionicum*, resulting in the formation of propionic and acetic acid and carbon dioxide as main products [24].

The most active pectinolytic species are *C. felsineum, C. laniganii, C. pectinolyticum, C. pectinovorum, C. virens,* and other pigmented and non-pigmented clostridia and plectridium [25]. Each species has its own specific details of metabolism, but their common property is the ability to decompose pectin substances with the formation of organic acids, alcohols and gases.

Some microorganisms have very stable pectinolytic properties and secrete pectinolytic enzymes into the media not containing

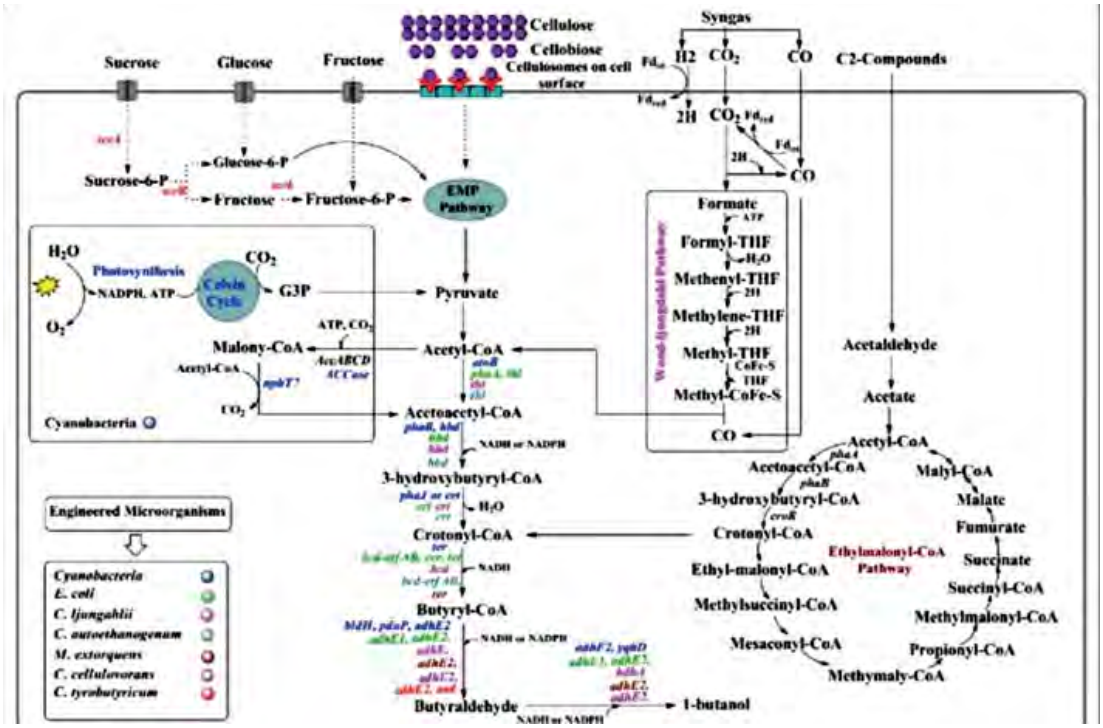

Fig. 1. **Schematic of 1-butanol production in heterologous hosts from various feedstocks. Different colors represent heterologous genes expressed in different hosts [21]**

pectins. In other anaerobes (for example, *C. multifermentans*), relevant enzymes are synthesized only when pectins are added to the media (induced enzyme synthesis) [26]. Pectinolytic anaerobes carry out the fermentation of sugars according to the butyric or acetone-butyl type. There is a group of highly specialized anaerobic spore-forming bacteria that obtain energy by fermenting cellulose with the fermentation end-products of acetic, propionic, butyric and lactic acids, ethyl alcohol, hydrogen and carbon dioxide, and intermediate products of glucose and cellobiose. For such bacteria, when glucose or sucrose is added to the nutrient medium, the fermentation process is practically absent (sugars are not assimilated), and when glucose and fiber are added simultaneously, mainly fiber is fermented. This indicates a high specialization of cellulose-decomposing microorganisms.

Cellulolytic bacteria differ not only in physiological but also in morphological features. Most cellulolytic spore-forming anaerobes have the appearance of thin long rods that form spores according to the plectridium type. Vegetative cells are usually present in an adsorbed state on cellulose fibers. Perhaps this is due to the fact that enzymes that hydrolyze cellulose (cellulases) are not released into the medium, but are attached to the cell surface. Spore-forming cells usually exist in solution; during spore formation, the nature of the cell connection with the environment changes, and spore formation occurs due to endogenous metabolism (due to intracellular nutrient reserves). Several specialized species of anaerobic bacteria have been identified that use organic acids and alcohols as a source of carbon and energy [27].

Microorganisms *C. kluyveri*, as a rule, obtain energy due to the conjugated oxidation-reduction system of ethyl alcohol–acetic acid, thus higher fatty acids are formed (mainly caproic and butyric acids). Not all *C. kluyveri* bacteria are capable of fermenting carbohydrates, amino acids, and purines. The accumulation of energy through ATP in such anaerobes occurs through the mechanism of oxidative phosphorylation [28].

There are three types of bacteria (*C. acidiurici, C. cylindrosporum* and *C. uracilicum*) that ferment heterocyclic compounds. They are able to destroy heterocycles with the formation of acetic acid, carbon dioxide and ammonia. The first two types of bacteria are not able to use carbohydrates and proteins (amino acids). These bacteria cleave xanthine, guanine, guanosine, 6,8-dioxipurine relatively quickly, and cleave hypoxanthine and inosine relatively slowly (even after adaptation) [29].

In spore-forming anaerobes, the specificity in relation to substrates is highly pronounced. The media containing a set of amino acids, carbohydrates, mineral salts, a complex of vitamins, and microbial growth activators may not be sufficient for selected proteolytic anaerobes (for example, *C. sporogens*) [30]. Such apparent heterotrophs grow only on the media containing proteins or products of their partial hydrolysis. However, there are anaerobes (sulfate-reducing bacteria) that ferment simple media, which include several mineral salts (including sulfates) and organic acid (atmospheric nitrogen can also be assimilated).

The ability to fix molecular nitrogen is widespread among spore-bearing bacteria. Such a process can be carried out by butyric, acetone-butyl and sulfate-reducing bacteria. The most active nitrogen fixers are saccharolytic anaerobes (clostridia). The relation to oxygen in different physiological groups of spore-forming anaerobes is not the same. Saccharolytic anaerobes are more resistant to oxygen. Some representatives of this group are aerotolerant forms of *C. carnis* and *C. histolyticum*, capable of weak growth on agar plates even under aerobic conditions. Sulfate-reducing bacteria are sensitive to oxygen and difficult to culture. Their growth is possible only under anaerobic conditions without oxygen in the cultivation medium [30].

Alcohol formation and sporulation

The mechanism of the regulation of alcohol formation has not been fully elucidated [31, 32]. This is especially relates to the switching phase of fermentation and the relationship between alcohol formation and sporulation (Fig. 2).

The spores represent specifically arranged resting germ cells that may withstand the action of high temperature, radiation, vacuum, various kinds of toxic substances and other unfavorable factors that lead to the death of vegetative cells. The formation of spores occurs at a certain stage of development at the moment when nutrient resources (sources of carbon and nitrogen) are exhausted in the environment, or toxic metabolic products accumulate [34]. The main purpose of spore formation is to transfer the culture to a resting (anabiotic) state, therefore, in mature spores, the metabolism occurs at extremely low level. This enables bacteria to survive in unfavorable environmental conditions, and when conditions change, they switch again to vegetative growth. For anaerobes (especially soil ones) it is also extremely important that the spores are not sensitive to oxygen. This allows them to survive under aerobic conditions that would have a detrimental effect on vegetative cells [35, 36].

Young, rapidly dividing anaerobic cells contain nucleoids in the form of dumbbells or V-shaped figures. Before sporulation, cell division stops, the cells sharply increase in size. At this time, an accumulation of a large amount of granulosa, a reserve nutrient, occurs, and it is being deposited in the form of granules, thus the cytoplasm becomes granular, and the cells swell, taking the form of a lemon (clostridium) or a drum stick (plectridium). In a minor part of proteolytic anaerobes cells do not change their original

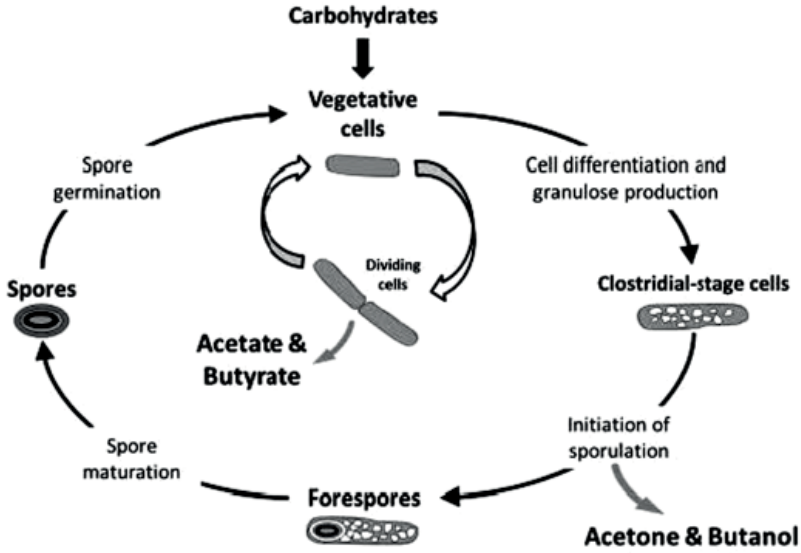

Fig. 2. **The general cell cycle of *Clostridium acetobutylicum* depicting different cell forms and major products during acidogenesis and solventogenesis [33]**

appearance, retaining the usual rod-shaped (bacillary) shape [37]. The first sign of the onset of spore formation is a change in morphology. Further, several nucleoids approach at one of the poles of the cell, merge and form a longitudinally located convoluted chromatin (nuclear) strand. The cytoplasm zone, where the nuclear cord is located, turns into a prospore [38]. Small cell bacteria usually have two separate nucleoids before sporulation, which fuse to form an axial chromatin strand. Subsequently, only part of this thread goes into a spore. The third type of nuclear behavior is found in many saccharolytic anaerobes. Their nuclear substance has the form of a chromatin mesh located throughout the cytoplasm. Part of this mesh is pulled together at one of the cell poles with the formation of a strand, which forms the center of the emerging prospore. Using a conventional microscope, three stages of spore formation can be observed [39]. At the first stage the sporogenous appears zone at one of the cell poles, in which the nuclear substance in the form of light rods is clearly visible. At the second stage, the sporogenic zone turns into a dark (optically dense) oval prospore with clearly defined contours [40]. In the prospores, the nuclear substance is no longer detected without the use of special methods (staining). At the third stage, the prospores gradually lighten, acquiring the ability to strongly refract light, and lose their ability to stain with dyes. Ripe spores look like light, sharply refracting light bodies with a strong shell. Prospore formation begins with invagination (ingrowth) of the cytoplasmic membrane closer to one of the cell poles [41]. In this case, the membrane moves to the center of the cell, and its poles merge to form a spore partition wall (septum). This process involves mesosomes, which help to stick together the converging sections of the invaginated membranes. The septum consists of two elementary membranes. At this moment, the second stage of spore formation is finished (if we take the formation of a chromatin strand as the first stage). The second stage can be considered as a modified cell division, which, as is known, also occurs due to the invagination of the cytoplasmic membrane and a septum formation. The next stage is an "absorption" process by the mother cell of the septate (cut-off) area of the cytoplasm with the nucleus [42]. This process is carried out by the growth and advancing of the peripheral sections of the membrane in the mother cell towards the cell pole. Then the converging sections of the membrane merge and a prospore is formed, which has two elementary (three-layer) membranes, internal and external. In some species, the prospore later remains at the cell pole (terminal location); in others, it moves inside the cytoplasm, occupying a central or subterminal position [43]. Thus, at the end of this stage, a kind of bicellular organism is formed: inside the cytoplasm of the mother cell, a new cell arises, a prospore, surrounded, unlike the mother, by two membranes. From this moment, a new irreversible phase of the development and metabolism begins, ending with the maturation of the spore and the death of the mother cell [44]. Unlike the fourth stage, the second and, in part, the third stages of sporulation are reversible. Thus, when, after the formation of a septum, the antibiotic chloramphenicol is added to the sporulating culture, protein synthesis will be suppressed. The movement of the peripheral sections of the membrane that absorbs the cut-off section of the protoplast will be stopped [45]. As a result, the process of spore formation that has begun will turn into a normal process of vegetative cell division, and cell wall material will accumulate between the two septa membranes. Such accumulation does not occur in the normal course of sporulation. At the fourth stage of sporulation, a cortical layer (cortex) is formed between the inner and outer membranes of the prospore [46]. First, the cortex appears as a thin dark layer, similar in structure and density to the cell wall of a vegetative cell. Then this layer sharply increases in thickness due to the formation of more electron transparent (light) layer. At the fifth stage, the spore shell is formed. At the beginning, the areas of a dark (electron-dense) substance in the form of scales appear around the prospore at some distance from the outer membrane of the prospore in the cytoplasm of the mother cell. At the sixth stage, the individual sheets of the shell elongate and, in the end, merge, forming a solid continuous dense layer. Between this layer and the outer membrane of the prospore, a cut-off layer of the cytoplasm of the vegetative cell remains [47]. On top of the first layer of the shell, one or two more layers can be deposited. In this case, they are divided into inner, middle and outer layers of the shell. These layers differ from each other in structure. In some species, the inner layer of the shell is lamellar, while the outer layer looks like a dense thick layer [48]. In other species, on the contrary, the lamellar layer may be external, and the denser layer may be internal. If the structure of the core is very similar in different species, then

the structure of the spore shells in them varies greatly both in fine structure and in the number and thickness of layers [49]. After the final maturation of the spore, the lysis of the parent vegetative cell occurs: the cell wall is destroyed, and the spore enters the external medium (seventh stage). The shape of mature spores can be different in different types of anaerobes: spherical, oval, ovoid, cylindrical [50].

In many anaerobes, another structure is found on top of the spore membrane, i.e., the exosporium. The exosporium has the appearance of a multilayer sheath, which the spore is located in. Such structure is observed in *C. pasteurianum, C. bifermentans, C. tyrobutyricum* [51]. In the exosporium of many anaerobic species, the layers contain subunits which are placed in specific order. The spherical subunits in the lamellar layer of the exosporium have a hexagonal packing. Adjacent subunits may sometimes fuse, forming ring-shaped structures with pores in the center. Such exosporium layers comprise perforated membrane films. Exosporium occurs at an early stage of spore formation in the form of a small bubble on the outer membrane of the prospore. This vesicle grows, turning into a sheath covering the spore from all sides [52].

The core of the spore, surrounded by a layer of cortex, is a protoplast with its own membrane, nucleus, and cytoplasm. The core of a mature spore is a resting vegetative cell. It is characterized by a very low metabolic rate and although it contains all the necessary enzymes, their activity is somehow suppressed [53].

The cortex is composed of mucopeptides that are very similar to cell wall mucopeptides. The cortex also contains diaminopimelic acid. In spores, dipicolinic acid ($C_7H_5O_4N$) is found in fairly large quantities. It is an active chelating agent, forming the claw-like complexes with metals. This substance is absent in vegetative cells. Dipicolinic acid is released from spores in the form of calcium and magnesium salts, which play a major role in the thermal stability of spores. Dipicolinic acid is also involved in the process of transferring the spore protoplast to a dormant state [54]. The mechanism of these processes has not been elucidated yet. Possibly, dipicolinic acid is localized in the cortex, since there is a certain correlation between cortex formation and the accumulation of dipicolinic acid and calcium in the spore [55]. The cortex of mature spores plays a protective role. It protects the core from lytic enzymes that destroy cells. This assumption was confirmed for mutants that have lost the ability to form a cortex. At the final stage of spore formation, there is a sharp increase in the activity of lytic enzymes, which completely destroy the parent vegetative cell. Spores without cortex are also lysed [56].

The shell (or cover) is a unique structure of bacterial spore that is not found in other microorganisms. It mainly consists of protein substances enriched with cystine. The volume of the shell reaches 50% of the total spore volume. The substance of the spore shell is not sensitive to the action of various lytic enzymes. The shape of the spores, specific for each type of bacteria, is maintained due to the structural rigidity of the membranes. The shell also plays the role of a protective structure that protects spores from premature germination. Spores of mutant strains lacking the shell usually germinate immediately after emerging from sporangia in an environment unfavorable for growth (even in distilled water), which leads to the death of germinated cells. However, the role of the spore membranes, as well as the cortex, remains largely enigmatic [57].

The exosporium is a membranous structure; it often has a multilayer composition. The exosporium probably plays the role of a barrier that regulates the penetration of various substances into the spore. In many anaerobic bacteria, the exosporium is not a confined system, as its polar part, immersed in the cytoplasm of the mother cell, contains very large pores up to 0.5 μm in diameter. After mechanical removal of the exosporium, the spores remain normal, their germination process is not disturbed. A feature of spores in anaerobes is the formation of special outgrowths of various structure. Each type of anaerobic bacteria tends to have its own type of outgrowth structure. This feature is strictly specific, hereditarily fixed and very stable. Even in defective spores that have lost the ability to form a shell, the outgrowths are preserved and do not change their specific structure [58].

On *C. taeniosporum* spores, the outgrowths have a ribbon-like shape. A bundle of such outgrowths is attached to the spore with the help of a special organ — the pad. The outgrowths appear at an early stage of prospore formation, before the initiation of the cortex and shell; then they grow, lengthen, and penetrate the cytoplasm until they reach the opposite pole of the cell. The cytoplasm around the outgrowths gradually lyses. The mother cell is destroyed. On the free mature spores emerging from the sporangium, the outgrowths bloom in the form of an umbrella [59].

The spores of *C. sporogenes* have a single large and complex outgrowth. It has an

appearance of a long thick bundle or trunk, forming a ring at the end, from which antennae extend, a tubular rod-shaped outgrowths. The trunk has a coarse-grained structure and transverse striation, fine-grained antennae have a capsular layer. The formation of outgrowths in this species can be traced on intact cells [60]. At first, the processes are poorly visible, since they are surrounded by dense areas of the cytoplasm, then the cytoplasm becomes lighter and the outgrowths become clearly visible. A ring-shaped structure and antennae are clearly visible at one of the poles of the cell [61]. The function of outgrowths on spores has not yet been finally elucidated. Some researchers suggest that the outgrowths on spores are specific sensitive (chemosensory) organelles that give the spore a "command" for germination (under favorable conditions). Others believe that outgrowths play an important role in the process of spore maturation, participating in the formation of spore covers and the cortex. Some studies postulate that outgrowths on spores are the result of some disturbances in normal metabolism. The question of the enzymatic activity of outgrowths is very important [62].

When spores are transferred to a fresh nutrient medium they begin to germinate. Firstly, they swell, darken, then, through the hole formed in the spore shell, the young cell exits into the outer medium. In this case, the cortex layer is destroyed, and the spore shell, together with outgrowths (if any), is shed. In anaerobes, it is rarely possible to study the germination by observing the same single spore. At the last stage of the exit, the vegetative cell is blasted off. The hole in the spore shell is formed not strictly at the pole of the spore, but somewhat on the side, and the young vegetative cell, when exiting, is located at an angle to the long axis of the spore. In other anaerobes, the germination process may look different [63].

Germination characteristics of *C. pasteurianum* are used to differentiate this species from other spore-forming anaerobes [64]. Finally, three species of Clostridium, *C. pectinovorum, C. butyricum*, and *C. tetani*, differ in that their spores germinate inside the sporangium [65]. The cell wall (or part of it) in these species is not lysed, but remains on mature spores, covering them in the form of a sheath. But this sheath is not identical in origin and structure with the exosporium described above. *C. acidiurici* and *C. cylindrosporum* are physiologically very close, but clearly differ by morphological features. In the case of *C. acidiurici*, the spores are oval, located terminally, and the cells swell during sporulation [66]. In the case of *C. cylindrosporum*, the spores are cylindrical, located centrally or subterminally, and the sporangia do not swell [67].

Obtaining an enrichment culture

To obtain enrichment cultures of *Clostridium*, some of their features could be used. Their main feature is the thermal resistance of spores, which facilitates the isolation of microorganisms by the method of preliminary pasteurization of the inoculum [68]. To maintain the ability for intensive fermentation, pasteurized inoculums are used when working with isolated strains. Another feature is their anaerobicity or aerotolerance [69]. By creating strictly anaerobic conditions, the growth of all aerobic bacteria is excluded in advance. Strains can be isolated from soil, sewage, animal wastes, potatoes, roots of nitrogen-fixing legumes, milk, and cheese. When using a substance containing large particles as an inoculum, for example, starch grains or cellulose particles from the rumen of ruminants, those particles are firstly washed and then used as material for inoculation. Industrial strains isolated from natural sources are contained in different microorganism collections , such as ATCC (American Collection of Culture Types), DSM (German Collection of Microorganisms), NCIMB (National Collection of Industrial and Marine Bacteria, United Kingdom), NRRL (Northern Regional Research Laboratory — Agricultural Research Service Culture Collection, US Department of Agriculture) [70].

Most acetone-butyl bacteria share a similar phenotype, metabolic pathways, and end products. The taxonomy of these bacteria is quite complicated and time-consuming. Acetone- and butanol-producing strains are now divided into four species (Fig. 3), according to genetic features, namely *C. acetobutylicum, C. beijerinckii, C. saccharoperbutylacetonicum* and *C. saccharobutylicum* [71–73].

C. beijerinckii synthesizes solvents at approximately the same rate as *C. acetobutylicum*, but it synthesizes isopropanol instead of acetone [74, 75]. *C. aurantibutyricum* synthesizes isopropanol in addition to butanol and acetone [76]. The main source of carbon for the growth of *C. tyrobutyricum* bacteria is lactose, and the fermentation products are butyric acid, hydrogen, and carbon dioxide [77].

C. tetanomorphum is a relatively new producer that synthesizes almost equimolar amounts of butanol and ethanol and does not synthesize other alcohols [78].

Clostridium producer strains have different productivity and the end-product accumulation rates (Table 1). The accumulation of the corresponding products of microbial synthesis depends both on the strain itself and the cultivation medium, as well as on growth factors, pH, and temperature [79].

Production of butanol from synthesis gas

In addition to the classical scheme of ABE fermentation, it is necessary to note an alternative way of butanol production from synthesis gas using the *C. carboxidivorans* bacteria [81]. *C. carboxidivorans* binds CO, fixes CO_2 and convert them into acetyl-CoA according to the Wood-Ljungdahl scheme (Fig. 4).

In this scheme, two CO_2 molecules are used, but for completely different purposes: one molecule is used as a carbon source, and the second molecule is used as an electron acceptor. At the first stage, carbon dioxide is fixed with the help of tetrahydrofolate using the energy of ATP, and at the second stage, acetyl-coenzyme A (acetyl-CoA) is synthesized from CH_3-H_4-folate. The transfer of the methyl group to coenzyme A is carried out by a special methylase, a cobalt-containing iron-sulfur protein.

Two processes take place in parallel:

The first process. CO-dehydrogenase (CODH) reduces the CO_2 molecule to CO, and the electrons for reduction are usually taken from H_2:

$$CO_2 + 2\,e^- + 2H^+ = CO + H_2O.$$

The reduction of CO_2 to CO occurs at the so-called Fe_4NiS_5-active center of the "C-cluster"protein.

The second process. Acetyl-CoA synthetase condenses a carbonyl group with a methyl group at the so-called "A-cluster", giving a metal-bound acetyl group, which is released from the enzyme via thiolysis by acetyl-CoA.

$$CO + H_2O = CO_2 + 2e^- + 2H^+ \quad (1)$$

$$CH_3\text{-}Co^{3+}FeSP + CO + CoASH = CH_3C(O)SCoA + Co^{1+}FeSP + H^+ \quad (2)$$

$$H^+ + Co^{1+}FeSP + CH_3\text{-}THF = CH_3\text{-}Co^{3+}FeSP + THF \quad (3)$$

$$CH_3C(O)SCoA + H_4SPT + H_2O = CH_3.H_4SPT + CO_2 + 2e^- + 2H^+ + CoASH, \quad (4)$$

where H_4SPT is tetrahydrosarcinapterin, the archaeal analogue of tetrahydrofolate (THF). CoFeSP in reaction (2) is a corrinoid-[FeS]-protein, a heterodimer containing the nucleotide cofactor cobalamin in one subunit and the Fe_4S_4 cluster in the other. Reduced Co^{1+}cobalamin accepts the methyl group from CH_3-THF in the reaction (3).

This process contains fewer steps, does not require organic seeds such as citric acid to initiate reactions, and carbon fixation in it occurs in only one reaction. In addition, the Wood-Ljungdahl pathway is the only way to fix carbon without the use of ATP or other triphosphates. It should be noted that all genes encoding enzymes in the classical scheme of ABE fermentation are present in the genome of *C. Carboxidivorans*, and the genome also has the *sol* operon and alcohol formation genes, which, for some reasons, are not included in fermentation. [83–85]. It is possible that the same genetic process had occurred here, as in the degenerated (DGN) strains which lost the ability to produce solvents after repeated cultivation. For *C. acetobutylicum* ATCC 824, the complete loss of the pSOL1 plasmid, which

Table 1. **Productivity of butanol-producing strains [80]**

Strain	Substrate	Type of fermentation	Solvent accumulation, g/l
Clostridium sp. BOH 3	Xylose	ABE*	5.32; 14.94; 1.25
C. tetanomorphum DSM 665	Glucose	Butanol-ethanol (BE)	9.8; 1.01
C. pasteurianum DSM 525	Glycerol	Butanol-propanediol	7.13; 6.79
*Clostridium sp.*BT 10-6	Glucose	Isopropanol	5.26
*Clostridium sp.*NJP 7	Glucose	Isopropanol-methanol	12.21; 1.92
C. pasteurianum GL11	Glycerol	Butanol-ethanol	14.7; 0.01

Note: * — The name of the type of fermentation directly characterizes the products of fermentation, for example, ABE — acetone-butanol-ethanol.

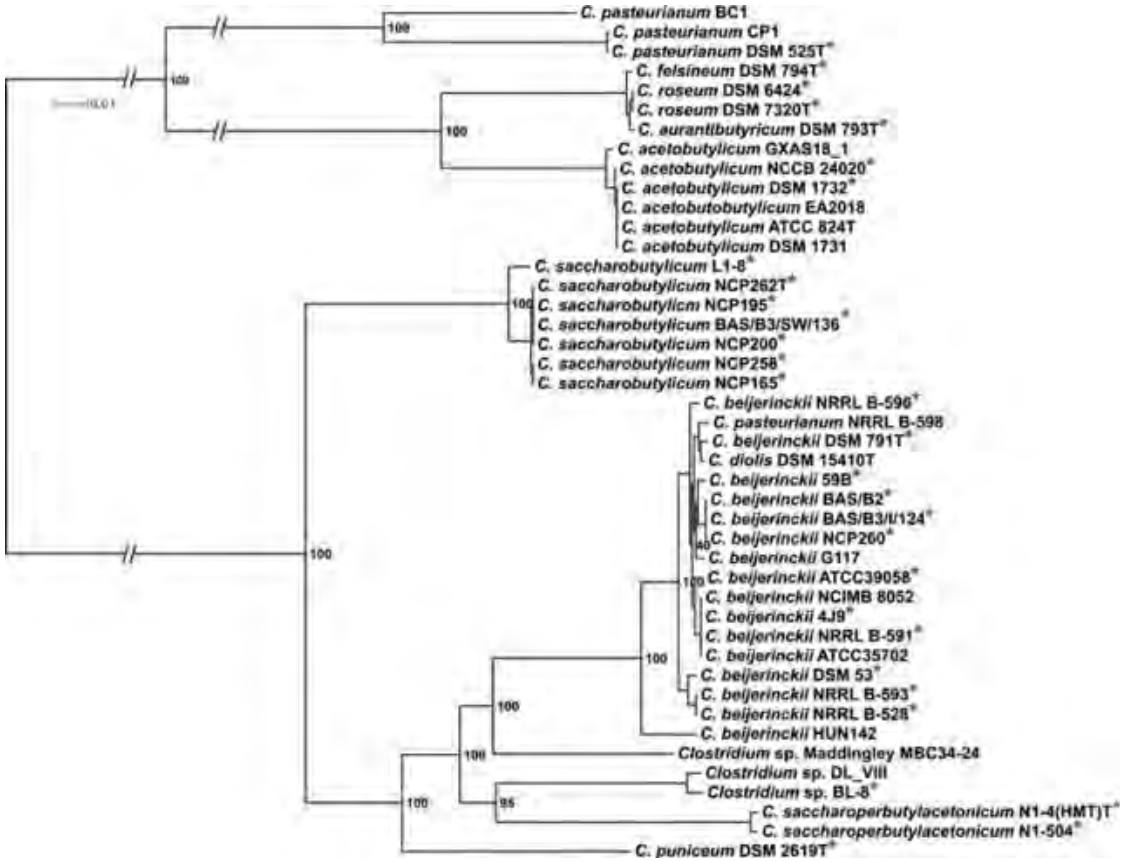

Fig. 3. **Phylogenetic tree of 44 sequenced solventogenic clostridia [10]**

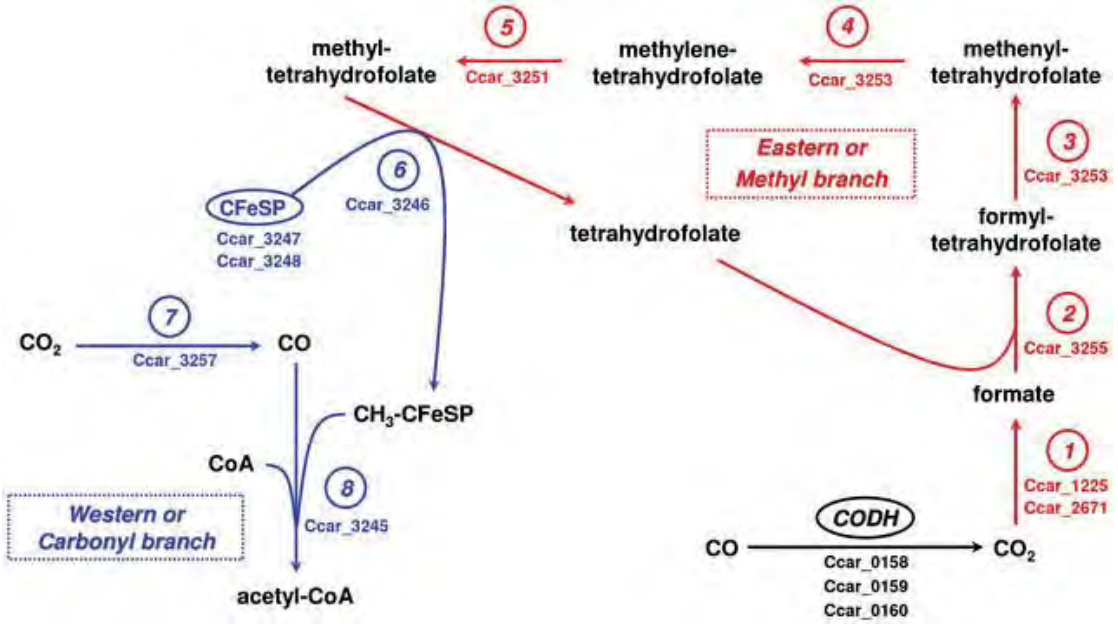

Fig. 4. **Scheme of the Wood-Ljungdahl pathway of *C. carboxidivorans* P7T** [82]:
where 1 is formate dehydrogenase; 2 — formate-tetrohydrofolate ligase; 3 and 4, bifunctional methenyltetrahydrofolate cyclohydrolase/methylenetetrahydrofolate dehydrogenase ($NADP^+$); 5 — 5,10-methylenetetrahydrofolate reductase; 6–5-methyltetrahydrofolate:corrinoid/iron-sulfur protein Co-methyltransferase; 7 — carbon monoxide dehydrogenase; 8 acetyl-CoA synthetase; CoFeSP is a cobalt-iron-sulfur protein that catalyzes the transfer of a methyl group from tetrahydrofolate to coenzyme A and carbon reduced to (+2), CODH is an additional carbon monoxide dehydrogenase complex. The corresponding genes of the strain are shown below the enzyme

contains the *sol* operon, consisting of the alcohol-producing genes *aad, ctfA, ctfB,* and *adc*, was shown during the degeneration [86, 87].

One species of Clostridia, *C. cylindrosporum*, is able to generate formyl tetrahydrofolic acid formate and tetrahydrofolate in a reaction that is accompanied by ADP phosphorylation. In this species, this reaction is the main way to obtain ATP. All reactions of substrate phosphorylation are localized in the cell cytosol, which suggests the simplicity of the chemical mechanisms underlying substrate phosphorylation. The degree of oxidation and the amount of free energy, as well as the nature of the products formed, are related to the nature of the final electron acceptors. In the process of fermentation, the final electron acceptors are mainly organic compounds: metabolites obtained from the original substrates (pyruvic acid, acetaldehyde), or the substances present in the cultivation medium. The main function of hydrogenases in Clostridia is to remove excess catabolic reactions of reducing equivalents (electrons) that have been produced and are removed from the cell in the form of molecular hydrogen. Other ways to obtain hydrogen are also possible. For example, $NADH_2$, which is produced in the glycolytic pathway, can reduce ferredoxin with the help of $NADH_2$: ferredoxin oxidoreductase, and H_2 is released from the reduced ferridoxin by hydrogenase [88].

Features of industrial butanol production

It is not only the genetic features of the producer strain are a key factor in the microbiological synthesis of butanol; substrate (raw materials) and, in fact, the technology also make a major contribution into the cost of the final product.

By selecting a producer strain and an appropriate substrate, pretreatment of the substrate, optimization of technological parameters (pH, temperature, aeration, and nutrient supply), one can change the productivity of the producer organism and the accumulation of the final product [89]. The following microorganisms of the genus Clostridium are used in the industrial production of butanol: *C. acetobutylicum, C. beijerinckii, C. saccharobutylicum* and *C. saccharoperbutylacetonicum*.

Some species of Clostridia are characterized by an altered metabolic pathway of synthesis and, and consequently, the yield of solvents differs significantly from that obtained in classical ABE fermentation. In this regard, the selected producer strain must be checked using metabolomic analysis. The first stage of such an analysis is the performing of classic "direct" fermentation with subsequent study of all intermediates and final products. For fermentation, pure cultures of bacteria, or preliminarily prepared spores for inoculation are used [90].

Spore preparation is an essential and necessary step for cultivation in industrial production [91]. The accumulation of spores is carried out on a 6% mash of corn or rye flour. For the formation of spores, tubes with fresh sterile mash are inoculated with a culture of bacteria and the entire fermentation cycle is carried out at 37 °C. Spores could be prepared by pouring as well. For each new portion, spores obtained from cultures that have shown the maximum results of product accumulation during fermentation under industrial conditions are used [92]. The first stage of fermentation is carried out in a vessel, from which the mash is poured into sterile test tubes where the process ends with the formation of spores. The tubes with spores are sealed and placed in a thermostat. After 18–60 hours, the fermentation gases are released, the test tubes are sealed again and stored at room temperature. After two months of storage, the spores are tested by test-tube fermentation. The spores satisfying the technological conditions are recognized as suitable and the passports are issued for them [93].

After the accumulation of spores, ABE fermentation is carried out directly. It can be proceeded both in batch, semi-continuous and continuous mode. Batch fermentation is a relatively simple process [94].

Large amounts of pure culture are needed to carry out a batch process under industrial conditions. The preparation of the required amount of pure bacterial culture for production begins with the inoculation of spores in a pure culture apparatus (PCA) [95]. After 28 hours, the fermentation contents of the PCA are sterilely transferred to a large inoculator (LIN). Sterile mash for LIN is taken when it is hot. It is cooled in a LIN or refrigerator. From the inoculator, the bacterial culture is transferred under sterile conditions to the fermenter-activator of the production battery. In batch fermentation, in the first hours after inoculation of the mash with active culture, fermentation is observed, noticeable by gas release from the surface. Gas release peaked after 24–26 hours and subsided towards the end of fermentation.

During the period of maximum gas release, a characteristic stratification of the substrate occurred: a "loose" mucous layer moved upward to the surface, a cloudy opalescent liquid remained in the lower layer, and the entire medium acquired a yellowish color. This phenomenon in production is referred to as the "rise" of the mash and is one of the signs of normal fermentation. By the end of fermentation, the solid part of the substrate precipitated to the bottom.

Along with the gas release, the shape of the titratable acidity curve is also a characteristic feature of ABE fermentation. The growth of bacteria was characterized by the increase in titratable acidity, reaching its maximum (4.0–4.6 mL of 0.1 NaOH per 10 mL of mash) by 12–16 hours of fermentation, and then sharply decreased by 24–25 hours, after which there was a slight increase in acidity towards the end of fermentation. In the process of increasing acidity, the pH of the medium decreased from 6.0 to 4.1 and virtually remained at this level. The formation of alcohols began starting from approximately the 6th hour of fermentation, but become the most intensive after the "break" in the acidity curve. Up to 35% of total carbohydrates were converted into alcohols, and the final mash contained about 2% of solvents. The cost of the substrate in the cost of butanol obtained by the classical method is 60%, which makes the process of obtaining butanol economically unprofitable [96–100].

To increase the accumulation of butanol in the process of "direct" batch fermentation, the duration of the acid-forming and alcohol-forming stages can be changed by changing the concentration of H_2, CO, partial pressure, organic acids and mineral additives. The addition of CO during the batch fermentation using *C. acetobutylicum* tends to inhibit hydrogenase activity [101]. During batch cultivation of *C. saccharoperbutylacetonicum*, it was demonstrated that the removal of hydrogen from the bioreactor leads to the accumulation of only H_2, while alcohols did not accumulate [102].

Increasing the culture productivity is possible at the stage of metabolites formation. Artificial electron carriers such as methyl viologen and neutral red drastically change the production of metabolites. Microelements of the environment can also influence the electron transfer. The conversion of pyruvate to acetyl-CoA involves the use of iron-sulfur proteins (ferredoxin oxidoreductases), and iron is also an important mineral supplement. A change in the iron concentration significantly affected the process of butanol synthesis [103–107].

A semi-continuous fermentation process was used to avoid fermentation inhibition by high substrate concentrations. However, due to the inhibitory properties of butanol, fed-batch culture is ineffective. In industrial production, a semi-continuous fermentation process is known as a battery fermentation. The battery consists of 6–8 bioreactors serially connected into an integral device. The main reactor (the activator) is inoculated with a culture from the inoculator and after the "break" of the acidity curve (after about 12 hours) they are loaded with flour mash. The entire battery is filled through the activator. The battery is unloaded (the worth is transferred for rectification) from the last "tail" bioreactor. After sterilization, the "tail" bioreactor becomes the activator of the next battery, consisting of the same bioreactors, but loaded in the opposite direction. To optimize the semi-continuous fermentation process, the technological system for a continuous fermentation process was created.

Continuous fermentation made it possible to reduce (up to 70%) the flour usage and replace it with cheaper raw materials: sugar beet molasses (syrup) and hydrolysates of vegetable waste [108].

In this scheme, the use of flour mash for the first phase is preferred. This ensures the rapid growth of bacteria and the formation of enzymes for the synthesis of solvents. Molasses and hydrolysates are introduced during the transferring of the fermentation to the second phase. The scheme of continuous two-phase fermentation has been introduced into industrial production. According to this scheme, for the process of continuous ABE fermentation, an additional stage was proposed, a pure production culture apparatus (PPCA), which was sown from LIN. After 10–12 hours of fermentation, the entire culture from it was transferred to the first bioreactor of the production battery, and its loading with flour mash had immediately began. The flour mash was transferred to the first (head) bioreactor of the battery, as well as for the further breeding of a pure culture in the inoculator and PCA. The molasses mash was sent to the second fermenter, where the transition to the second phase of fermentation took place.

This technology allowed the fermentation to continue for a long time and with a high productivity of the producer strain. The rate of butanol synthesis intensification in a

continuous process is similar to that for a batch process. An increase in butanol accumulation can also be obtained by adding precursors to the cultivation medium [109–113].

Conclusions

The features of the acetone-butyl fermentation mechanism (the relationships between the alcohol formation and sporulation, the duration of the acid-forming and alcohol-forming stages during batch fermentation depending on the change in the concentration of H_2, CO, partial pressure, organic acids and mineral additives) and obtaining an enrichment culture were shown. The possibility of synthesis gas use as a substrate for reducing emissions into the atmosphere during the fermentation process was demonstrated. The direction of increasing the productivity of butanol-producing strains to create a competitive industrial biofuel technology has been proposed.

This study was carried out within the project "Development of the ultrasonic disintegration technology for plant biomass of non-grain parts from agricultural crops" of the Ministry of Education and Science of Ukraine (State registration No. 0120U102083), and the project "Intensification of butanol accumulation using various plant raw materials as a substrate and the domestic producer strains" of the National Academy of Sciences of Ukraine (State registration No. 0120U101706).

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ОСОБЛИВОСТІ АЦЕТОНОБУТИЛОВОГО БРОДІННЯ ШТАМІВ–ПРОДУЦЕНТІВ БУТАНОЛУ

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Метою даного огляду узагальнення та аналіз особливостей ацетонобутилового бродіння як процесу отримання альтернативного біопалива — бутанолу.

Методи. Застосовано методи аналізу й узагальнення аналітичної інформації та літературних джерел. Результати отримано з використанням мікробіологічних (морфологічні властивості штамів), хроматографічних (визначення концентрації розчинників), спектрофотометричних (визначення концентрації бактерій) та молекулярно-генетичних (філогенетичний аналіз штамів) методів.

Результати. Досліджено процес ацетонобутанолового бродіння, розглянуто основні штами-продуценти, описано особливості взаємозв'язку спиртоутворення та споруляції, показано можливість отримання бутанолу із синтез-газу, розглянуто особливості промислового виробництва.

Висновки. Показано особливості механізму ацетонобутилового бродіння (взаємозв'язок спиртоутворення та споруляції, тривалість кислотоутворючого та спиртоутворючих етапів за переодичної ферментації в залежності від зміни концентрації H_2, CO, парціального тиску, органічних кислот та мінеральних добавок) та отримання накопичувальної культури за отримання бутанолу, як альтернативного палива. Показано можливість використання синтез-газа як субстрата для зменшення викидів в атмосферу в процесі ферментації. Запропоновано напрямок збільшення продуктивності штамів-продуцентів бутанолу для створення конкурентноспроможної промислової технології бутанолу.

Ключові слова: штами-продуценти, біопаливо біобутанол, ацетонобутилове бродіння.