UDC 579.222 https://doi.org/10.15407/biotech10.01.034

DOMESTIC BUTANOL-PRODUCING STRAINS OF THE *Clostridium* GENUS

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Received 21.12.2016

The aim of the work was to summarize the results of own research concerning obtaining butanol producing strains of *Clostridium* genus, to identify them by physiological, morphological and genetic methods. Further study of characteristics and biological features of the strains, and various approaches in biotechnological process of butanol production are discussed. The work includes methods to increase butanol accumulation by producer strains. Perspectives of using chemical mutagenesis in *Clostridia* as a method of increasing butanol production are considered. The feasibility of using non-food raw material as a substrate for fermentation is discussed. Different methods of pretreatment and their impact on the accumulation of butanol in the liquid medium are compared. Butanol accumulation is shown to increase significantly if the synthesis precursors are added as components of enzymatic medium, and the "reverse bard" is used to reduce waste production without affecting the level of butanol synthesis. The problem of conservation of producing strains is given and protective medium for microorganisms during the freeze-drying is defined.

Key words: producing strains, mutagenesis, *Clostridium*.

Energy and environmental crises are forcing humanity to rational and efficient use of natural, especially renewable resources [1]. Microbiological conversion of renewable biosphere resources with the purpose to produce commercial products, including biofuels, is currently one of the challenges that biotechnology's facing. Anaerobic bacteria of *Clostridiaseae* family are known producers of one of the alternative biofuels, butanol. Microbiological synthesis of butanol during classical acetone-butanol-ethanol (ABE) fermentation is economically unprofitable nowadays. To create profitable ABE–fermentation highly solventogenic strains are required that would use affordable, renewable and cheap raw materials such as biomass or agricultural waste [2].

Industrial production of butanol by microbial synthesis emerged in the early twentieth century and was connected (as a side process) to the production of acetone [3]. The food materials were processed, corn flour as substrate by bacteria *Clostridium acetobutylicum* in production of acetone, butanol and ethanol in ratios of 3:6:1 [4]. In classic ABE fermentation, *C. acetobutylicum* bacteria produced on initial stages butyric, propionic, lactic and acetic acids (acid formation stage), then the pH value decreased and production stage of butanol, acetone, ethanol (alcohol phase formation) started. Increased demand for butanol and a sharp increase in chemical production have meant that biotechnological process for butanol became uneconomical and was replaced by more efficient chemical synthesis.

In recent years, the interest in microbial process for butanol production (Fig. 1) as an alternative fuel has resumed [5]. Today, biofuels account for only 2% of all fuels used. Experts predict that the volume of biofuels on the fuel market in the next ten years will reach 30% [7].

Recent studies are related to the search for new producing strains with increased butanol accumulation, and the use of non-food raw materials as the substrate [8, 9]. Thus, creation, search and identification of new strains of butanol producers, optimization of cultivation stages, and use of non-food raw materials as the substrate are an urgent problem.

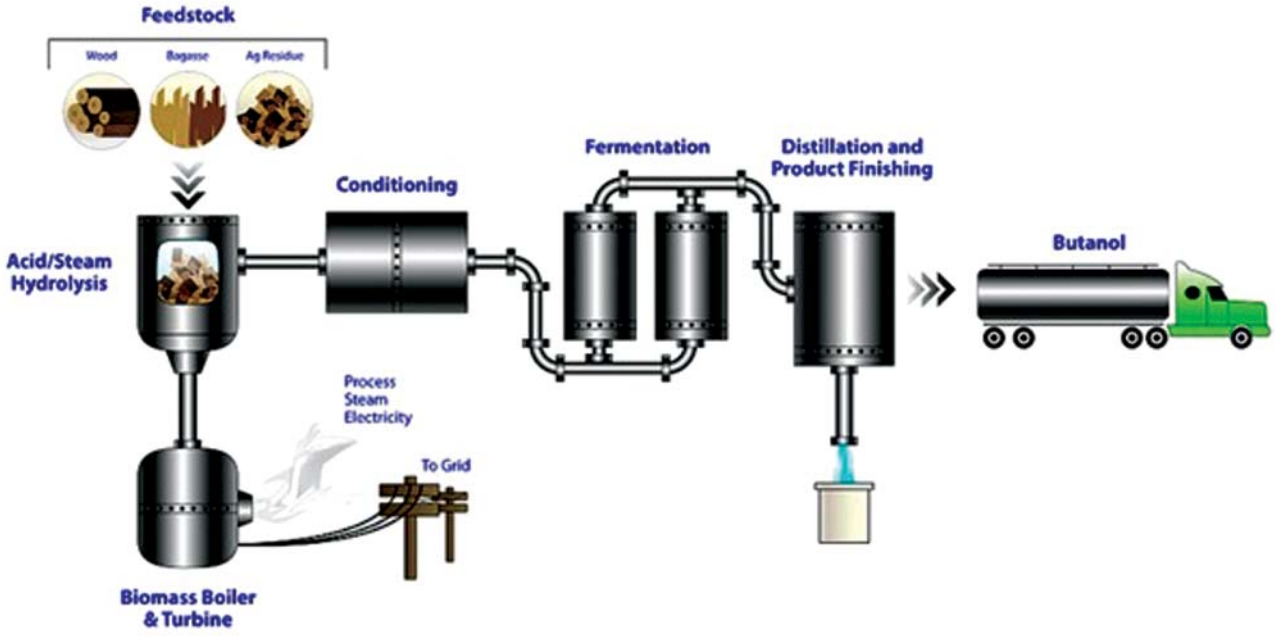

Fig. 1. **Technology of butanol production [6]**

Most existing butanol producing strains were isolated from soil [1, 10–15], so we collected 9 samples of soil and silts of Kyiv ponds. Samples of silt and soil have been previously thermally processed for 10 min to kill non-spore-forming microorganisms [10–13].

For proper dilution of soil suspension, the number of microorganisms was determined on bean-peptone agar (BPA). The numbers of microorganisms in samples were used to find the best dilution of soil and sludge suspensions for further research of the selection of butyric and acetonic acid bacteria.

Each clostridia species has different growth needs [1, 4, 8–15], so several media were selected for culturing samples of silt and soil. Integrated medium BPB (bean-peptone broth) [16] with sucrose as a carbon source was used for the accumulation of butyric bacteria cultures. Winogradsky medium with glucose as the carbon source was chosen as other medium for selection [17]. This choice was made because most clostridia are anaerobic nitrogen fixers [10–13]. Ashby's medium was used as selective medium for clostridial forms of bacteria [17]. All media were liquid and contributed to the accumulation of bacterial mass during cultivation process. Growth of *Clostridium* was marked by medium opacity, indicating accumulation of bacterial biomass, and gas bubbles on the surface of the medium (emission of fermentation gas) and a noticeable odor of butyric acid, by-product of butyric-acid bacteria activity. The opacity levels of the medium and increase of gas production varied depending on silt, soil samples and medium used. The data showed that butyric bacteria grew in all media after culturing the samples of silt and soil under nitrogen atmosphere at 30 ± 1 °C.

To confirm the presence of *Clostridium*, liquid media samples were studied using light microscopy [17]. Thecells of *Clostridium* bacteria include granulosa, amyloid substance, which when exposed to iodine is stained blue, so to differentiate these bacteria the iodine solution was used. The cytological studies showed that in all samples one or more rod-shaped bacterial cultures were present, mobile and containing granulose. In many samples, mixtures of cultures were found which were further separated by method of limiting dilutions [17].

To confirm the presence of butyric and acetic-acid bacteria all samples were transferred to potato slices rubbed with chalk. In this medium clostridia colonies were convex shaped, yellow in color with bubbles of gas within [17]. Typical colonies of different appearance on the surface of potato slices were obtained in the study. The data showed the existence of butyric bacteria in all collected samples. According to the analysis of the shape, size, edge, color, structure, texture and surface of colonies, different strains of butyric bacteria were found in all samples.

To support bacterial growth of *Clostridia* considering the complexity of the cultivation process [10–13], modified Winogradsky's medium with agar was created. In this medium, single characteristic colonies were obtained and pure cultures were isolated [18]. Genus and purity of the colony were confirmed by conventional methods [17].

ABE fermentation process is biphasic, divided into stages of acid and alcohol formation. The acids, including butyric acid [12, 19], accumulated at the first stage, so the determination of butyric acid in the culture liquid using qualitative reaction with iron chloride ($FeCl_3$) with forming of butyric iron was a rapid method for the detection of ABE bacteria. Solutions of butyric iron were characterized by "chestnut brown" color of reflected light (Fig. 2), and "blood-red" color in the transmitted light [17]. The results confirmed the presence of butyric clostridia in the samples.

To identify butanol producers, acetone rapid test was utilized for all cultures of nine samples of soil and silt. The results showed the presence of acetone in the samples IFBG C6H, IFBG C7P, IFBG C2M. To determine the alcohol concentration in the culture liquid of these samples, gas chromatography was performed. Conducted studies have helped to select two butanol producing strains — IFBG C6H and IFBG C7P, and one butyric acid producing strain IFBG C4B. The results for the IFBG C6H butanol producing strain are given in Fig. 3.

Identification of new cultures of biofuels producing strains is the first step towards further development of butanol biotechnology. Species identification of strains of *Clostridium* is rather complex [20–23], so initial identification of obtained strains was performed by classical methods [17]. It is shown that according to Bergey's bacteria identification manual, the cultures belong to the *Clostridium* genus.

One of more precise species identification methods for strains is comparing gene sequences of 16S rRNA [24]. To determine family relationships, phylogenetic analysis was conducted and phylogenetic tree was developed. According to molecular phylogenetic analysis of 16S rRNA gene sequence of strain IFBG C6H that isolated strain belongs to *C. pasteurianum* [25].

To estimate the performance of producing strains IFBG C7P and IFBG C6H the butanol accumulation in the culture liquid was examined and compared with that of other known strains. Isolated producing strains IFBG C7P and IFBG C6H produced butanol on the glucose-containing medium as well as the classic strain *C. acetobutyricum* ATCC 824 [26–29]. In the glucose-containing medium, the strain IFBG C6H accumulated more butanol compared with the strains of *C. beijerinckii* NCIMB 8052 and *C. saccharoperbutylacetonicum* N1–4 [30]. In the glucose-containing medium the strain IFBG C6H accumulated amount of butanol less than *C. pasteurianum* strain

Fig. 2. **Qualitative reaction with $FeCl_3$, where K — negative control; IFBG C4B, IFBG C6H, IFBG C7P, IFBG C2M — samples**

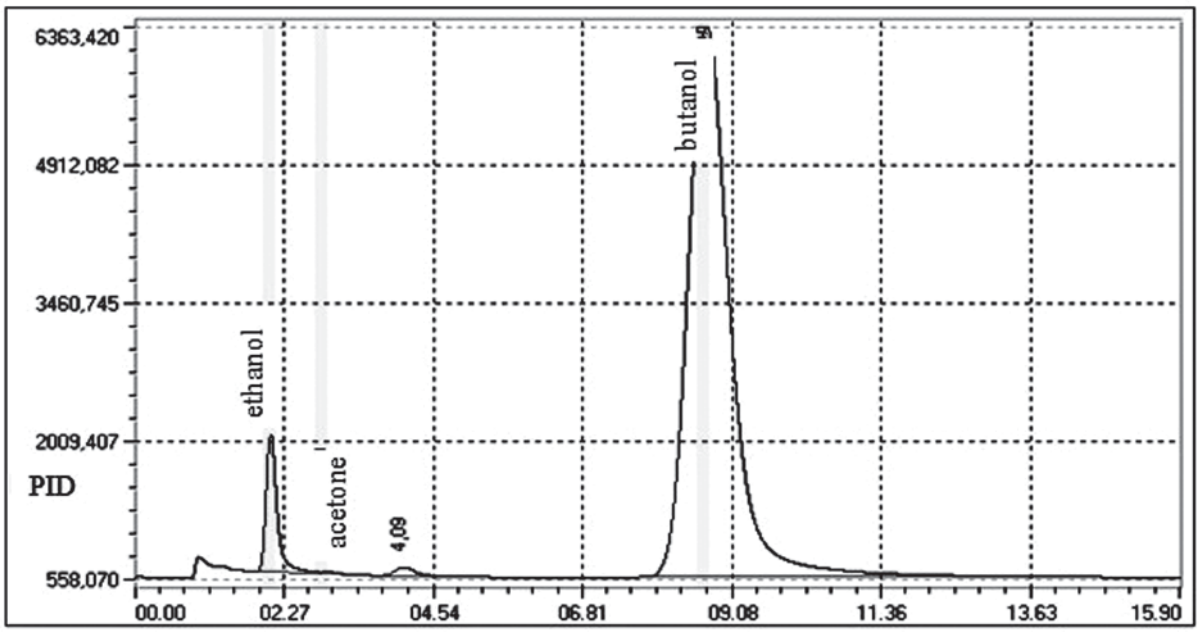

Fig. 3. **Chromatogram of alcohols of culture liquid of IFBG C6H sample**

ATCC 6013 [31]. Strains IFBG C7P and the IFBG C6H fermented xylose, arabinose and cellulose unlike *C. pasteurianum* ATCC 6013 and fermented glycerol unlike *C. beijerinckii* NCIMB 8052 [32–34]. These data suggest that the experimental producing strains in comparison with those already known can ferment a wide range of raw materials, producing butanol. Hence, strains IFBG C7P and IFBG C6H can be the basis for further research for commercial development of butanol biotechnology [35].

Further studies were aimed at increasing butanol accumulation and obtaining productive strain by chemical mutagenesis [36]. The strains were selected for mutagenesis among the new producing strains IFBG C6H and IFBG C7P, using the method for determining the strain performance described in [1]. Strain IFBG C6H was selected for mutagenesis. The clones obtained using mutagenesis accumulated butanol differently; several clones have completely lost the ability to produce alcohols. Among the obtained clones one was selected for further research of butanol accumulation on non-food raw materials. To confirm the stability of the mutant strain, the study was conducted on the accumulation of butanol during a year of storage. The unchanging butanol concentration in the culture liquid showed stability of the mutant strain [17]. Further studies were performed with the mutant strain comparing it with the original.

Storage on artificial nutritious media can lead to rapid drying, dying of microorganisms and reduction or loss of physiological and enzymatic properties. For the "conservation" of materials of biological origin, freeze-drying method is widely used [37]. Through the selection and use of cryoprotectants — protective media of different composition — negative consequences of eutectic concentrations during freezing and freeze-drying on the microorganisms can be prevented [38]. The effect of the protective medium (glucose and sucrose) on hydrophilic bacterial suspensions after freeze-drying was studied depending on their concentration [39]. It is shown that the use of glucose and sucrose in an amount of 10% resulted in the lowest residual moisture in the lyophilized suspension. For freeze-drying of new butanol producing strains, the composition of protective medium was optimized (%: sucrose — 10.0; gelatose — 10.0; agar — 0.02). The biological activity of the cells did not decrease in samples of lyophilized bacteria after one month storage at 4 °C. If storage temperature was higher, the cell activity gradually lowered and a temperature of 30 °C it was reduced by 40% [39]. Storage of samples of lyophilized bacteria for six months with that cryoprotectant at 4 °C had virtually no effect on the productivity of strains.

In addition to strain productivity, the cost of the end product largely depends on the enzymatic medium, the substrate [40]. It is desirable to determine both the quantitative composition and individual components of the substrate [41–49]. Analysis of biomass makes it possible to determine substances that compose it [50–52] with sufficient accuracy for future use of plant biomass as a substrate. Chemical composition of biomass of *Panicum virgatum L.* (switchgrass) and definition of its components (29% hemicellulose, 26% lignin, 36% cellulose) were studied.

The butanol producing strains were tested for their ability to ferment the minor components that make up the biomass of switchgrass. The study showed that the new butanol producing strains can ferment cellulose and monosaccharides, which are parts of hemicellulose. Similar results were obtained by authors [26, 31, 32] on the fermentation process of model mixtures with the corresponding sugars.

The substrate composition can significantly affect the synthesis of butanol [40], so comparative cultivation was conducted on classical (maize and potato mashes) and alternative substrates (mash with sawdust, savings, switchgrass and crude glycerol). Fermentation of wide range of raw materials by the producing strains and the use of "hungry" substrates to produce butanol (without additional costs) showed the possibility of using alternative substrates in butanol biotechnology [35].

The cost of the target product is affected not only by the strain nature and substrate composition and cost; pretreatment and concentration of substrate are also important. The influence of the concentration, milling and autoclaving of switchgrass biomass for production of butanol were studied [40].

Lignocellulosic biomass (savings, switchgrass) was fractionated into its constituent components (lignin, arabinogalactan, cellulose, water-soluble substances) after thermobaric treatment (explosive autohydrolysis). The strains were cultured on different components of switchgrass after explosive autohydrolysis. The possibility of using the lignocellulose components after explosive autohydrolysis processing as a substrate for microbial synthesis of butanol was described [53]. It was revealed that before cultivation the products of switchgrass autohydrolysis have to be additionally cleaned of furfurol, which is formed by thermobaric treatment and inhibits microbial growth. The results showed the possibility of culturing strains and obtaining butanol using all derived components (cellulose, arabinogalactan and lignin). It was established that the use of explosive autohydrolysis for pretreatment of waste has twice increased butanol accumulation [53].

Optimization of technological parameters such as temperature, pH and duration of cultivation and are important in microorganism culturing and can increase the accumulation of the target product [40]. Optimization of main cultivation parameters of new producing strains was conducted and the optimum process parameters (T 35 °C, pH 7, cultivation duration 120 hours) were discovered [35]. Optimization of cultivation process parameters taking into account the needs of cultures according to [34] allowed to increase the accumulation of the target product by 20% for the original and by 50% for the mutant strains. The accumulation of acetone, butanol and ethanol by different producing strains on different carbon sources are showed in the Table.

ABE fermentation is one of the varieties of butyric fermentation [29], thus intensification of the butanol accumulation using precursor synthesis, such as lactic, acetic and butyric acid was studied [54]. The results show that using precursor synthesis increased butanol accumulation for 1.7 times. The results are caused by the biphasic nature of ABE process and the required synthesis of lactic, acetic and butyric acids for further alcohol accumulation. The aforementioned precursors, added to the enzyme medium, trigger alcohol synthesis [8, 34, 54].

An important issue of butanol technology is waste recycling. To solve this problem, it was suggested to return the leftovers of acetone-butanol fermentation (so-called "bards") to the process of cultivation. The possibility of successful use of "reverse" bards to reduce waste production was shown [54].

This work paper analyzes the main stages of butanol biotechnology (Fig. 4) using new producing strains of the *Clostridium* genus.

Determined characteristics and biological features of the strains, the methods of increasing accumulation of butanol are suggested, the possibility of using non-food raw materials as the substrate is showed, cryoprotectants for protection and stability of microorganisms during freeze-drying are identified.

Synthesis of ABE products by different producing strains of *Clostridium* genus on different substrates [35]

Producing strains	Accumulation of acetone, butanol, ethanol on different carbon sources, g/l				
	Glucose	Xylose	Arabinose	Cellulose	Glycerin
C. acetobutyricum ATCC 824	Acetone, butanol and ethanol:5.7:10.0:1.1	0.2:1.9:0.2	0.2:1.9:0.2	0.1:2.4:0.2	0.1:3.0:0.1
C. beijerinckii NCIMB 8052	1.7:5.1:4.6	0.9:2.7:2.4	0.9:2.6:2.4	1.4:4.2:3.8	–
C. saccharoperbutylacetonicum N 1–4 (Al -Shorgani, 2011)	0.6:4.8:0.8	0.8:1.2:0.2	0.8:1.2:0.2	0.1:0.3:0.1	–
IFCG C6H	0.7:8.0:0.05	0.02:1.9:–	0.02:1.5:0.01	0.01:1.1:0.01	0.5:6.0:0.5
IFBG C6H 5M	0.2:4.0:0.03	0.02:2.0:0.02	0.02:2.3:–	0.02:2.8:–	0.1:3.4:0.1
C. pasteurianum ATCC 6013	5.0:10.0:2.0	–	–	–	1:5:2

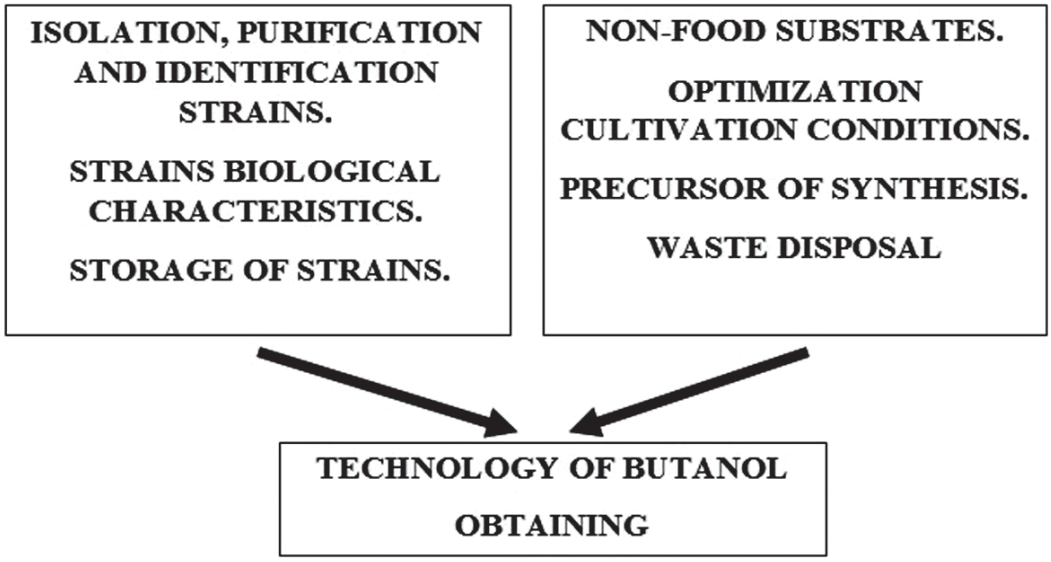

Fig. 4. **Stages of obtaining strains of butanol-producers and increasing their productivity**

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ВІТЧИЗНЯНІ ШТАМИ-ПРОДУЦЕНТИ БУТАНОЛУ РОДУ *Clostridium*

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Метою роботи було підсумувати результати власних досліджень отримання нових штамів-продуцентів бутанолу роду *Clostridium*, провести їх ідентифікацію фізіолого-морфологічними і генетичними методами. Обговорюється подальше вивчення характеристик та біологічних особливостей штамів, різні шляхи біотехнологічного процесу одержання бутанолу. Розглянуто методи підвищення накопичення бутанолу штамами-продуцентами, окреслено перспективу використання хімічного мутагенезу клостридій як методу збільшення продукції бутанолу. Показано можливість використання нехарчової сировини як субстрату для культивування, проведено порівняння різних методів попередньої підготовки сировини та впливу їх на накопичення бутанолу в культуральній рідині. Доведено суттєве збільшення накопичення бутанолу за рахунок використання попередників синтезу як додаткових компонентів до ензиматичного середовища та застосування зворотної барди для зменшення відходів виробництва без впливу на рівень синтезу бутанолу. Подано характеристику проблеми зберігання штамів-продуцентів та визначено середовище для захисту мікроорганізмів під час ліофільного висушування.

Ключові слова: штами-продуценти, мутагенез, *Clostridium.*

ОТЕЧЕСТВЕННЫЕ ШТАММЫ-ПРОДУЦЕНТЫ БУТАНОЛА РОДА *Clostridium*

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Целью работы было обобщение результатов собственных исследований получения новых штаммов-продуцентов бутанола рода *Clostridium*, осуществление их идентификации физиолого-морфологическими и генетическими методами. Обсуждаются дальнейшее изучение характеристик и биологических особенностей штаммов, разные пути биотехнологического процесса получения бутанола. Рассмотрены методы повышения накопления бутанола штаммами-продуцентами, очерчена перспектива использования химического мутагенеза клостридий как метода повышения продукции бутанола. Показана возможность использования непищевого сырья в качестве субстрата для культивирования, проведено сравнение разных методов предварительной обработки сырья и их влияния на накопление бутанола в культуральной жидкости. Доказано существенное увеличение накопления бутанола за счёт использования предшественников синтеза как добавочных компонентов к энзиматической среде и применение обратной барды для уменьшения отходов производства без влияния на уровень синтеза. Дана характеристика проблемы сохранения штаммов-продуцентов и определена среда для защиты микроорганизмов во время лиофильного высушивания.

Ключевые слова: штаммы-продуценты, мутагенез, *Clostridium.*