Clostridium acetobutylicum bacterial membrane responses to carbon ion beam irradiation perturbations

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Research

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Abstract

Background

Clostridium acetobutylicum is an important strain during acetone-butanol-ethanol (ABE) fermentation. However, butanol has toxic effects on cells, limiting the application of ABE fermentation. Accordingly, in this study, we aimed to elucidate the metabolic mechanisms through which *Clostridium* adapts to butanol stress to facilitate the industrial utilization of *Clostridium*.

Results

First, using cell morphology, cell membrane permeability and membrane potential, cell surface hydrophobicity, and cell membrane fatty acid composition analyses in wild-type (ATCC 824) and butanol-tolerant (Y217) strains under butanol stress, we explored the responses in the cell membrane to evaluate the damage caused by butanol poisoning. After 2.0% (v/v) butanol treatment, the extracellular conductivity of ATCC 824 increased, intracellular proteins and nucleotides were released in large quantities, the fluorescein diacetate staining rate decreased, the membrane potential decreased, and the cell membrane permeability increased. Under butanol shock, the cell surface of Y217 cells remained intact, and its butanol tolerance mechanism increased the integrity of cell membrane and reduced leakage of cell contents caused by changed in cell membrane permeability, thereby preventing butanol damage to the cell membrane. When stimulated with butanol, Y217 cells showed reduced surface hydrophobicity, thereby improving cellular tolerance to butanol. A comparison of differences in fatty acid compositions between ATCC 824 and Y217 cell membranes under butanol stress further demonstrated that maintenance of the normal physiological characteristics of cell membranes played important roles in resisting the impact of organic solvents.

Conclusions

Our findings clarified the changes in physiological and biochemical characteristics of the mutant Y217 cell membrane stimulated with butanol to enhance its tolerance. These results may provide important theoretical guidance for further accelerating the acquisition of bacteria with high butanol tolerance and promoting butanol production. Moreover, our study provided a scientific basis for improving the industrial and environmental adaptability of *Clostridium*.

Background

Clostridia spp. are gram-positive bacteria that have important applications in industrial processes [1]. Indeed, in industry, solventogenic *Clostridium* spp. utilize carbon sources, such as glucose, xylose, mannose, and arabinose, to produce acetone, butanol, and ethanol during anaerobic metabolism. This fermentation process is called acetone-butanol-ethanol (ABE) fermentation [2, 3]. The main component,

butanol, has the highest commercial value and is used as a multipurpose basic chemical raw material, high-quality renewable transportation fuel, and fuel additive [4]. *Clostridium acetobutylicum*, as the main strain used in ABE fermentation, expresses amylase and can be directly fermented with sugar and starchy raw materials without prior saccharification [1]. Owing to the unique sporulation and solvent production characteristics of *C. acetobutylicum* and its vital application value, many studies of *C. acetobutylicum* have been performed in the to evaluate the microbiological morphology, physiology, biochemistry, and molecular biology of the strain. Understanding the mechanisms through which *Clostridium* spp. adapt to environmental stress is essential for controlling these bacteria and taking advantages of its industrial applications [5].

The metabolic transition of *C. acetobutylicum* from the acidogenic period to the solventogenic period is an adaptive process resulting from the effects of acid toxicity on the bacterial cells [5]. During the solventogenic process, bacteria can metabolize the products accumulated during the acidogenic period through the reuse route, which causes the pH of the fermentation broth to rise, thereby partially detoxifying the acid [6]. Solvents produced during this process, particularly butanol, have major effects on the growth and metabolism of the bacteria and are therefore key factors limiting the fermentation yield [7, 8]. Therefore, butanol is considered the main toxic compound inhibiting the physiological activity of *C. acetobutylicum*.

Organic solvents first enter the cell membrane after coming in contact with the cells, and the phospholipid bilayer structure of the membrane is the main target of solvent toxicity [9]. The toxic effects of the solvent on the cell begin with binding of solvents to the phospholipid layer. After solvent accumulates on the cell membrane, membrane permeability to protons and ions increases [9], proton dynamics dissipate [10], energy conduction fails [11, 12], and intracellular pH control is suppressed, leading to penetration of intracellular macromolecules (such as RNA, phospholipids, and proteins) [11]. Moreover, solvent accumulation on the cell membrane also affects the activity of the mosaic protein by changing the physical and chemical properties of the membrane [13], such as alterations in the proton-K⁺ pump [11, 12], and may alter the membrane structure, resulting in increased membrane fluidity [13], reduced microbial metabolism, and decreased growth. Thus, after the solvent enters the membrane, it disrupts the order of the membrane, weakening the function of the membrane as a penetration barrier and protein embedding platform. The increased permeability, fluidity, and disorder of the cell membrane make it difficult for microorganisms to resist the toxic effects of solvents, resulting in cell death.

Physiological studies of microorganisms have revealed that solvent toxicity is related to its logPo/w value [11]. The parameter logPo/w refers to the partition coefficient of a given solvent in equimolar octanol and water (indicating the hydrophobicity of the solvent) [14]. The greater the polarity, the smaller the logPo/w value and the greater the solvent toxicity [15]. The logPo/w values of common solvents range from 1 to 4.5, whereas that of butanol is 0.8 [16]. In addition, the solvent relies on its own hydrophobicity to enter and bind to different sites in the bilayer [14]. Binding of the solvent to different positions on the cell membrane can result in varying effects on membrane structure. For example, when a solvent is combined near the polar head, the effects of membrane disorder will be greater than that of the

solvent deeply bound to the center of the phospholipid fat chain [17]. The binding positions of alkanols (e.g., butanol, octanol, dodecanol, and myristyl alcohol) on the cell membrane have been examined by Westerman et al. [18] and Pope et al. [19] by X-ray and nuclear magnetic resonance technology. The results indicated that amphiphilic molecules, such as alkanol, position the hydrated part of the molecule close to the polar head of the phospholipid molecule, whereas the fat chain portion is inserted between the fat chains of the phospholipid.

Among the four alkanols described above, butanol causes the most serious cell membrane lipid damage in the glycerol skeleton [18]. Indeed, Ezeji et al. [20] found that butanol inhibited the activity of bacteria by destroying the cell membrane and altering its functions, i.e., reducing the substance transfer capacity of the membrane. A series of reliable experimental results showed that there are two systems in *C. acetobutylicum*, i.e., a specific self-induced peptide transport system and a signal transduction system; the signal transduction system involves membrane receptor histidine kinase function [21]. Butanol toxicity also results in destruction of the membrane transport system (i.e., the phosphoenolpyruvate-carbohydrate phosphotransferase system), thereby preventing the transport and phosphorylation of glucose and inhibiting the transmembrane transport and assimilation of sugars, amino acids, and other nutrients [22].

In response to butanol toxicity on the cell membrane, a series of studies have been conducted to improve the ABE fermentation and solvent tolerance of *C. acetobutylicum*. With improvements in molecular biology technology, researchers have attempted metabolic engineering of *C. acetobutylicum*. For example, Xu et al. [23] knocked out *CAC3319* (histidine kinase) in *C. acetobutylicum* and showed that the tolerance of the strain increased and that butanol production (18.2 g/L) and butanol yield (0.38 g/L/h) were both enhanced. Moreover, Borden et al. [24] screened the resistance genes of *C. acetobutylicum* by constructing a gene library and showed that recombinant strains carrying genes such as *CAC0977*, *CAC1463*, *CAC1869*, and *CAC2495* were enriched. *CAC1869* is a transcriptional regulator of the heterogeneous stress element, and its introduction into ATCC 824 increased the butanol tolerance of the recombinant strain by 90%. Mann et al. [25] overexpressed the genes *groESL*, *grpE*, and *htpG* in *C. acetobutylicum* and showed that 2.0% (v/v) butanol stress for 2 h improved butanol tolerance in engineered strains overexpressing these genes, with survival rates of 45% (*groESL*), 25% (*grpE*), and 56% (*htpG*). In addition to targeted genetic engineering methods, the genes and proteins related to butanol tolerance have been molecularly modified to enhance butanol tolerance and survival rates, thereby increasing butanol production. Furthermore, some induced mutations can also be used to obtain butanol-tolerant mutants. *C. beijerinichii* BA101, obtained by chemical mutagenesis in 1991, was used for this application [26]. *C. acetobutylicum* EA2018 strain was also chemical mutated to become an excellent butanol producer, showing a 10% increase in butanol production compared with the original strain; thus, this strain has been introduced in industrial applications in China [27]. Guo et al. [28] used *C. beijerinichii* NCIMB 8052 as the starting strain and utilized low-energy ion implantation (N⁺) for physical mutagenesis to obtain a mutant strain IB4 with high tolerance and high butanol production. Baer et al. obtained a solvent-tolerant mutant strain SA2 through mutagenesis screening and found that the saturation of cell membrane fatty acids was higher than that of the control strain *C. acetobutylicum* ATCC 824 under

various culture conditions between 22 and 37 °C. Moreover, the fluidity of the cell membrane remained unchanged under different concentrations of butanol (0–1.5%, v/v) [11].

Compared with traditional mutagenesis technology, as an advanced and efficient mutagenesis technology, heavy ion beam irradiation results in high mutagenesis rates and yields relatively stable mutants, leading to its broad applications as a tool for creating new species. In terms of microbial breeding, approximately 30 genera have been subjected to heavy ion beam irradiation to produce novel strains [29–31]. Cadmium resistance of *Arthrobacter* mutants selected by carbon ion beam irradiation was increased by 2-fold [32], and Lu et al. [33] increased the fermentation rate of *Saccharomyces cerevisiae* subjected to carbon ion beam irradiation by 25%. Zhou et al. [34] used carbon ion beam irradiation to significantly improve the resistance of *C. butyricum* to butyric acid and the ability to produce butyric. Accordingly, many excellent microbial mutants with applications in industry and research have been bred using heavy ion beam irradiation mutagenesis.

C. acetobutylicum Y217 is a stable and high-yield resistant mutant selected by *C. acetobutylicum* ATCC 824 as the starting strain through carbon ion beam irradiation. Based on previous studies, Y217 maintains better cell integrity under butanol stress than ATCC 824, potentially because of its high butanol tolerance. In this study, we systematically analyzed and compared the cell morphology, cell surface hydrophobicity, membrane potential, membrane permeability, cell fatty acid composition, and fermentation kinetics of high-butanol tolerant mutant and wild-type strains under butanol stress in order to clarify the membrane physiology and metabolic mechanisms of *C. acetobutylicum* under butanol stress.

Results

Effects of butanol stress on the growth and surface morphology of mutants

To explore the differences in the surface morphology of Y217 and ATCC 824 cells exposed to increased concentration of butanol, Y217 and ATCC 824 cells were plated on reinforced *Clostridium* medium (RCM) solid plates and cultured at 37 °C for 48 h. Single colonies were then selected and cultured in fresh medium for 24 h. The resulting seed suspension was inoculated into culture medium containing different concentrations of butanol and then subjected to dry cell weight (DCW) analysis and scanning electron microscopy (SEM). As shown in Fig. 1a, ATCC 824 grew well in medium without butanol, with a lag phase of approximately 4 h; in the presence of butanol, the lag phase increased significantly. In cultures of cells incubated with 1.0% (v/v) butanol, the DCW decreased by nearly 35%, and SEM (Fig. 1b) showed slight folds on the surface of some bacterial cells, which may be a specific response to butanol stimulation. In 2.0% (v/v) butanol, the cells maintained growth, the butanol concentration continued to increase, and bacterial growth entered a stagnation period. As shown in Fig. 1c, bacteria cultured with 3.0% (v/v) showed severe damage.

Figure 2d shows the growth of Y217 at different butanol concentrations. Notably, at butanol concentrations of up to 3% butanol, the growth lag phase of the cells did not differ in the presence or absence of butanol. In the presence of 1.0% or 2.0% (v/v) butanol, Y217 maintained excellent growth, although growth in the presence of 2.0% (v/v) butanol decreased slightly. The surface integrity of Y217 cells was intact in the presence of 1.0% (v/v) butanol. However, in the presence of 3.0% (v/v) butanol stimulation, SEM images showed that cell surface was slightly wrinkled and that integrity was slightly reduced; importantly, the growth of the mutant still met fermentation requirements.

Effects of butanol stress on cell surface hydrophobicity

To evaluate the relationships between changes in cell surface hydrophobicity and butanol tolerance, the microbial adhesion to hydrocarbons (MATH) value of the original strain ATCC 824 and mutant strain Y217 were measured during the logarithmic growth period without butanol and with 2.0% butanol RCM. As shown in Fig. 2, MATH values of ATCC 824 and Y217 were 42.3% and 35.6%, respectively, when butanol stress was absent in the culture environment, indicating moderate hydrophobicity on the cell surface for both strains. When both strains were exposed to butanol environmental stress (2.0% butanol), the hydrophobicity on the cell surface of both cells changed significantly, and MATH values increased to 63.5% and 52.7%, respectively. The MATH value of Y217 was lower than that of ATCC 824 under conditions of no butanol addition and 2.0% (v/v) butanol stress, suggesting that the surface hydrophobicity of Y217 cells was lower than that of ATCC 824 cells. Therefore, in a butanol stress environment, Y217 cells could reduce the amount of butanol entering the cell, thereby reducing butanol toxicity in cells. Overall, these findings demonstrated that reducing the cell surface hydrophobicity could improve the ability of the cells to resist butanol.

Effects of butanol stress on membrane permeability

In order to study the relationships between membrane permeability and butanol tolerance in Y217 and ATCC 824 cells under butanol stress, Y217 and ATCC 824 cells were inoculated into RCM liquid medium and cultured for 24 h. Butanol was then added (final concentration: 2.0%), and changes in membrane permeability were determined every 2 h. As shown in Fig. 3a, at 2 h after the addition of butanol, the fluorescence intensity of ATCC 824 decreased to 51.7%, indicating that the cell membrane permeability increased by 48.3%. In contrast, for Y217, the fluorescence intensity only decreased to 94.6%, and the cell membrane permeability increased by 5.4%. When the cell encounters an adverse environment, stress responses are produced to facilitate adaptation, which may be why fluorescence intensity decreased rapidly and then stabilized when butanol was added. After 10 h, the fluorescence intensity of ATCC 824 and Y217 cells decreased to 30.5% and 91.2%, respectively, and the permeability of ATCC 824 and Y217 cells increased by 69.5% and 8.8%, respectively. As shown in the Fig. 3b, the conductivity of Y217 remained stable throughout the cultivation cycle, whereas that of ATCC 824 increased with time. The conductivity of ATCC 824 reached a maximum of 1080 $\mu\text{S}/\text{cm}$ at 8 h of treatment, and there was a significant difference in the conductivity of 623 $\mu\text{S}/\text{cm}$ when no butanol was added ($p < 0.05$). At 6 h,

Y217 showed a slight increase in conductivity, although this increase was not significant, indicating that butanol did not affect the permeability of this strain.

Nucleic acid molecules show a maximum absorption peak at a wavelength of 260 nm, whereas that of proteins is 280 nm. Thus, we next evaluated the effects of butanol on the absorbance of intracellular biological macromolecules of *C. acetobutylicum* at 260 and 280 nm (Fig. 3c). Without butanol stimulation, the absorbance values of ATCC 824 bacterial supernatants at 260 and 280 nm were 0.180 ± 0.015 and 0.150 ± 0.02 , respectively; those of the Y217 bacterial supernatants were 0.175 ± 0.01 and 0.142 ± 0.02 , respectively. After stimulation with butanol, the absorbance of the ATCC 824 supernatant increased as the treatment time increased. After 10 h of butanol treatment, the absorbance values at 260 and 280 nm reached 0.390 ± 0.02 and 0.290 ± 0.015 , respectively, indicating that as the butanol treatment time was prolonged, the permeability of the cell membrane of *C. acetobutylicum* gradually increased, resulting in leakage of intracellular nucleic acids, proteins, and other substances and leading to cell inactivation. However, for Y217, compared with ATCC 824, the amount of macromolecular spillage was small, indicating that relatively stable permeability was maintained in the presence of butanol.

Effects of butanol stress on transmembrane potential

Decreased membrane potential is a characteristic change in the cell membrane. In order to detect the effects of butanol stress on membrane potential, we examined the fluorescence intensity of rhodamine 123 (Rh123), a lipophilic cationic fluorescent dye that is permeable to membranes and very sensitive to membrane potential, in the two strains. Y217 and ATCC 824 were inoculated into RCM liquid medium and cultured for 24 h. Butanol (final concentration: 2.0%) was then added to the fermentation broth, and samples were evaluated every 4 h to determine changes in membrane potential. Flow cytometry was used to detect the intensity of the bound Rh123 to determine the level of membrane potential. Butanol stress in ATCC 824 cells reduced the membrane potential of the cells to a certain extent (Fig. 4), and this difference was statistically significant ($P < 0.01$). In contrast, after stimulation with butanol, Y217 showed no significant difference in cell membrane potential (Fig. 4). Thus, these findings demonstrated that the mutant strain Y217 maintained a relatively stable membrane potential under butanol stress, thereby ensuring cell membrane integrity and cell activity.

Effects of butanol stress on membrane fatty acids (FAs)

FAs are important components of cell membranes. Changes in the content and ratio of saturated and unsaturated FAs (SFAs and UFAs, respectively) in FAs can affect the fluidity of cell membranes. Accordingly, Y217 and ATCC 824 cells were inoculated into liquid culture medium and cultured for 24 h. Butanol was then added to the fermentation broth (final concentration: 2.0%). After 4 h, samples were evaluated for changes in membrane FAs. As shown in Fig. 5, FAs of Y217 and ATCC 824 cells mainly included C12–C22 FAs, with C16 and C18 FAs being the main components. After the addition of butanol, the FA composition of the cell membranes of Y217 and ATCC 824 cells was significantly different. In the Y217 cell membrane, SFAs accounted for 62.32% of total FAs; in the ATCC 824 cell membrane, SFAs accounted for 49.39% of total FAs, indicating that there was a 12.93% increase in SFAs in the Y217 cell

membrane compared with that of the ATCC 824 cell membrane. The SFA/UFA ratio in the Y217 cell membrane was 1.65, whereas that in the ATCC 824 cell membrane was 0.97. In the membranes of Y217 and ATCC 824, mono-unsaturated FAs (MUFAs) accounted for 35.17% and 48.71% of total FAs, respectively; the percentage of MUFAs was significantly lower in Y217 cells than in ATCC 824 cells, consistent with the changes in total UFAs.

Effects of exogenous butanol on the fermentation kinetics of the mutant

Based on the above research on the physiological characteristics of the cell membrane of the mutant strain, we compared the fermentation abilities of the starting strain and the mutant following the addition of 2.0% (v/v) butanol (Fig. 6). The net butanol output of Y217 cells was 7.62 g/L. Additionally, acetone production was 1.8 g/L higher than the control, and ethanol production remained similar in the two strains. During the acid production stage, the amount of acid in ATCC 824 cells was low, and butanol stimulation decreased cell activity during the acid production stage, thereby affecting the progression of the solvent stage. Notably, the final concentration of butanol in the fermentation broth reached 21.27 g/L for Y217 cells, which was 6.04 g/L higher than that in ATCC 824 cells, indicating that the mutant could still grow normally and participate in the fermentation process to yield butanol when stimulated with 2.0% butanol. The cells showed ultimate butanol tolerance when the butanol concentration exceeded 20 g/L.

Discussion

Heavy ion beam irradiation, which can induce genetic variations in the genome with high mutation rates, is commonly used for mutation breeding. This approach has several advantages in the improvement of biological varieties and has yielded important advancements in the breeding of microorganisms, plants, and other biological varieties. In a previous study, *C. acetobutylicum* Y217, obtained from $^{12}\text{C}^{6+}$ ion beam irradiation generated by the Heavy Ion Research Facility of Lanzhou (HIRFL), showed resistance to butanol and membrane integrity disruption. Accordingly, in this study, we aimed to explore the mechanisms through which the mutant responded to butanol stress in terms of changes to the membrane.

The relationships between cell morphology and cell tolerance to organic solvents have been studied based on changes in cell size, specific surface area, and capsules. Capsules have protective effects on cells and can also act as a permeability barrier [35, 36]. Therefore, the presence of capsules can increase the ability of cells to resist adverse environments. In this study, our SEM results showed that under butanol stress, Y217 cells still exhibited a typical rod-shaped appearance; the cells were also full and intact, without damage. However, after stimulation with butanol, ATCC 824 cells showed folds and depressions on the cell surface. Owing to the uneven extracellular morphology, the cell walls and cell membranes of bacterial cells may have been damaged under butanol pressure. Thus, these findings indicated that the cell surface barrier played important roles in resisting the impact of organic solvents. According to a

previous work [37], when the MATH value is higher than 50%, the cell surface hydrophobicity is strong. In contrast, when the MATH value is between 20% and 50%, the cell surface hydrophobicity is moderate, and when the MATH value is less than 20%, the cell surface becomes hydrophilic. Accordingly, in the context of butanol stress, lower cell surface hydrophobicity was associated with reduced entry of organic solvent in the cells. The mutant strain Y217 could tolerate butanol to a certain extent, possibly because this strain reduced the penetration of butanol into the cell membrane and thereby suppressed the toxic effects of organic solvent.

The cell membrane is a protective barrier for microorganisms. Biological mechanisms actively regulate the dynamic and structural characteristics of the membrane through subtle changes in chemical components and the interactions between components [38]. When microorganisms are in an unfavorable environment or are poisoned by drugs, the protective barrier of the bacteria will be broken, resulting in changes in the fluidity and permeability of the cell membrane; large amounts of electrolytes and biomacromolecules can then be released from the cells [39]. Electrolyte leakage can cause changes in the conductivity of the culture fluid, and such changes in conductivity and nucleic acid and protein leakage can reflect changes in cell membrane permeability [40]. The conductivity of the solution mainly depends on the number of ions and the speed of movement of the ions [41]. According to the results of our conductivity experiment, after treatment with butanol, intracellular electrolyte leakage occurred, and ion concentration in the supernatant began to increase, resulting in increased conductivity. However, when the ion concentration increases to a certain level, the interaction between ions is enhanced, resulting in reduced speed of ion movement and stable or decreased electrical conductivity. Fluorescein diacetate (FDA) can penetrate the cell membrane to enter the inside of the cell. The esterase produced by living cells can decompose FDA into fluorescein to generate fluorescence and change its polarity to prevent it from penetrating the cell, allowing the compound to be retained in living cells. When the cell membrane is damaged (but the cell has not died), the permeability of the cell membrane increases, and fluorescein flows out of the cytoplasm, resulting in decreased fluorescence intensity [42, 43]. Therefore, after staining the cells with FDA, the difference in the integrity and permeability of the cell membrane of the original strain ATCC 824 and the mutant Y217 can be determined according to the change in fluorescence intensity. In our study, we compared the UV absorbance and conductivity values of the supernatants of the two strains at 260 and 280 nm and showed that as the processing time increased, leakage of intracellular substances, such as nucleic acids and proteins, was lower in Y217 than in ATCC 824. The conductivity of Y217 was relatively stable, whereas the conductivity of ATCC 824 tended to increase first and then stabilize. Our findings also showed that butanol could increase the cell membrane permeability of *C. acetobutylicum*, which may cause cell death, and that Y217, as a butanol-tolerant strain, could resist the damage to cell membrane permeability caused by butanol [40]. When the cells are exposed to butanol stress, butanol can penetrate the cell membrane and enter the interior of the cells, thereby causing changes in the cell membrane. Changes in cell membrane permeability and membrane potential can accurately reflect changes in cell membrane structure [44]. Therefore, FDA and Rh123 can be used to stain the cells and judge the integrity of the cell membrane according to changes in fluorescence intensity [45, 46]. Our results showed that after addition of butanol, the membrane potential and permeability of

ATCC 824 change greatly, indicating that under high butanol stress, ATCC 824 cell membrane damage was greater. In contrast, the membrane potential of Y217 remained stable, indicating that under butanol stress, the Y217 cell membrane showed better integrity. These results are consistent with the results of SEM, indicating that the mutant strain had better ability to resist the impact of butanol. Because the integrity of the cell membrane is essential for maintaining cellular activity, cell death can result when membrane damage is severe. Overall, Y217 showed much better butanol tolerance than ATCC 824.

The fluidity of the cell membrane depends on many factors and is a result of interactions of various membrane components. However, its regulation is mainly achieved through changes in fatty acids [47]. Environmental stress causes changes in various characteristics, including increased density of the membrane lipid bilayer. The cell membrane can alter its own composition, called the “compensation effect” [48], to modulate membrane fluidity. FAs, as important components of bacterial cell membranes, are highly sensitive to changes in the external environment and are often used as indicators to assess the survival status of microorganisms [49, 50]. The membrane lipid structure of the cell membrane is also critical for maintaining the function of the cell membrane and for the growth and reproduction of the cell. The membrane lipid structure is closely related to the FA composition, and changes in the FA composition inevitably affect the relevant membrane protein activity and membrane function [51–53]. Generally, the UFA component in the cell membrane is related to the fluidity of the cell membrane [54]. If the SFA content is high, the fluidity of the cell membrane will be reduced. Changes in cell membrane fluidity can alter the functional characteristics of the cell and the induction of apoptosis channels [55]. In our study, the proportion of SFAs in the cell membrane of Y217 was 13% higher than that of the wild-type strain ATCC 824. Compared with UFAs, the chains of SFA are straight and hard, which can increase the rigidity of the cell membrane [56]. When the rigidity of the cell membrane increases, the fluidity of the cell membrane will inevitably decrease, thereby reduce the entry of organic solvents into the cell, weakening the adverse effects of solvents, and enhancing the tolerance of the cell to organic solvents [47]. However, these changes in membrane composition in response to stress are rapid responses. The long-term solution is to enhance isomerization, such as altering the composition of the polar head group to enhance phospholipidity agglomeration. Further studies are required to fully elucidate these mechanisms.

Conclusions

In this study, we analyzed and compared the cell morphology, cell surface hydrophobicity, cell membrane permeability, membrane potential, and FA contents of a butanol-tolerant mutant strain and wild-type strain of *C. acetobutylicum* under butanol stress. Our goals were to elucidate the physiological and metabolic mechanisms related to the adaptation of the *C. acetobutylicum* cell membrane to a high concentration of butanol and provide a theoretical basis for further improving the ability of *C. acetobutylicum* to synthesize butanol and tolerate organic solvents. The tolerance of cells to organic solvents is the result of interactions of multiple factors, and it is difficult to explain changes in tolerance based on analysis of only a single factor. Therefore, in the future, systems biology research methods should be used to further explore the solvent resistance of cell and provide a scientific exploration for the

relationships among solvent tolerance, metabolic disturbance law, and butanol production of *C. acetobutylicum*.

Methods

Strains and cultivation

C. acetobutylicum ATCC 824 and Y217 were stored with 40% glycerol at -80 °C in the Biophysics Laboratory of the Institute of Modern Physics at the Chinese Academy of Science, Lanzhou, China. In order to study the relationships of changes in cell membrane permeability, membrane potential, and cell membrane FA contents with butanol tolerance under butanol stress conditions, strains were inoculated into RCM to logarithmic phase (~ 20 h), and butanol was then added to the fermentation broth (final concentration: 2.0%). The composition of RCM in distilled water was as follows: beef extract, 10.00 g/L; peptone, 10.00 g/L; yeast extract, 3.00 g/L; glucose, 5.00 g/L; starch, 1.00 g/L; NaCl, 5.00 g/L; sodium acetate, 3.00 g/L; cysteine hydrochloride, 0.50 g/L.

SEM sample preparation

Cells used for SEM were collected from the late logarithmic growth period (~ 24 h), centrifuged at 5000 rpm for 20 min, resuspended, and immobilized with 2.5% glutaraldehyde at 4 °C for 3 h. The cells were centrifuged and washed twice with phosphate buffer, and a series of ethanol concentrations (10%, 30%, 50%, 70%, 90%, and 100%) was used for gradient dehydration (15 min each; 100% dehydration was performed two times). Finally, specimens were dried at room temperature and coated with gold. Samples were observed with an FEI Nova NanoSem 450 (working voltage: 15 kV, amplification factor: 80,000).

Detection of cell surface hydrophobicity

MATH [37, 57] was evaluated as follows. First, cells were cultured at 37 °C for 18–20 h (logarithmic phase) and then transferred to fresh medium (2.0% [v/v] butanol RCM medium and original RCM medium). After 3 h of incubation, cells were collected by centrifugation (4 °C, 5000 rpm), washed with phosphate buffer (pH 7.2), and suspended in the buffer to achieve an OD₆₀₀ of 0.6. Next, a 1-mL suspension was removed and added to 300 µL butanol. The mixture was shaken for 2 min and then left to rest for 15 min at room temperature. The butanol phase was removed, and the OD₆₀₀ of the water phase was determined. The MATH value was calculated as follows: $MATH = ([OD_{600}]_{\text{pretreatment}} - [OD_{600}]_{\text{post-treatment}}) / ([OD_{600}]_{\text{pretreatment}})$.

Evaluation of membrane permeability and membrane potential

FDA (maximum excitation light wavelength: 490 nm, emission light wavelength: 526 nm; Sigma-Aldrich, St. Louis, MO, USA) was prepared at 5 mg/mL as a stock solution. Rh123 (maximum excitation light wavelength: 507 nm, emission wavelength: 529 nm; Sigma-Aldrich) was prepared at 1 mg/mL as a stock solution. Cells (10^6 – 10^7) were washed with phosphate buffer and resuspended. The dye was added to

the sample solution for appropriate incubation. The fluorescence intensity was immediately detected using a multimode reader and flow cytometer. Changes in cell membrane permeability and cell membrane potential in the wild-type and mutant strains in the presence of butanol stress were evaluated by measuring changes in fluorescence intensity.

Determination of intracellular biological macromolecular spillage and electrical conductivity

The bacterial suspension of strains stimulated with butanol was centrifuged at 8000 rpm for 15 min. Supernatants were collected, and the ultraviolet absorbance of nucleic acids and proteins was measured at 260 and 280 nm, respectively. Extracellular conductivity was detected with a conductivity meter. Saline was evaluated as a blank. The experiment was repeated three times.

Determination of FA contents

For FA extraction, strains were grown to the logarithmic growth phase and centrifuged at 3000 rpm for 5 min. The supernatants were discarded, and an inoculating ring was used to pick up 40 mg (wet weight) cells into a clean, dry screw-cap test tube. Saponification was then carried out by adding 1.0 mL saponification reagent (45 g sodium hydroxide + 150 mL methanol + 150 mL water). The samples were shaken for 5–10 s, placed in boiling water at 95–100 °C for 5 min, cooled at room temperature, incubated in a water bath for 25 min, and then cooled again at room temperature. Methylation was performed by adding 2.0 mL methylation reagent (325 mL of 6 M hydrochloric acid + 275 mL methanol), shaking for 5–10 s, incubating in a water bath at 80 °C for 10 min, and cooling to room temperature with running tap water. For extraction, 1.25 mL of extraction reagent (200 mL n-hexane + 200 mL methyl tert-butyl ether) was added, and samples were gently mixed by rotation for 10 min. The upper organic phase was used for gas phase detection.

Cellular butanol tests and fermentation experiment

For butanol-challenge growth experiments, the seed culture was grown to an OD₆₀₀ of 1.0 and inoculated into 10 mL fresh RCM at an inoculation amount of 8%. Cells were then grown anaerobically at 37 °C for 24 h, and samples are collected every 2 h. Butanol was added at different concentrations in RCM medium (0%, 1%, 2.0%, 2.5%, and 3.0% [v/v]) at an initial pH of 6.5.

The batch fermentation study was carried out in a 250-mL screw capped bottle with 50 mL *Clostridium* growth medium (CGM). The seed suspension of *C. acetobutylicum* ATCC 824 or the mutant strain Y217 was anaerobically cultured to the stationary phase at 37 °C in RCM. Screw capped bottles were inoculated with 8% seed culture of the total fermentation culture volume and stationary culture at 37 °C. The composition of CGM in distilled water was as follows: 50 g/L glucose, 2.0 g/L (NH₄)₂SO₄, 3.0 g/L CaCO₃, 0.75 g/L K₂HPO₄·3H₂O, 1.25 g/L KH₂PO₄, 0.71 g/L MgSO₄·7H₂O, 0.01 g/L MnSO₄·H₂O, 0.01 g/L FeSO₄·7H₂O, 1 g/L NaCl, 2 g/L asparagine, and 5 g/L yeast extract. Glucose, FeSO₄, and asparagine were dissolved independently and added to sterile medium with a 0.22-μm filter. All media were purged with N₂ to remove O₂ to maintain complete anoxic conditions.

Analytical methods

The optical density of the appropriate dilution at 600 nm was detected with an epoch spectrophotometer (Times Legend Bio-Scientific Co., Ltd., Shanxi, China) to determine bacterial growth. Fluorescence intensity was determined by a multimode reader (Infinite 200 PRO; Tecan). The blank was sterile RCM liquid medium. Before analysis of the product concentration, the fermentation broth was analyzed by centrifugation at 8000 rpm for 5 min, and the supernatant was filtered using a 0.22- μ m syringe filter. Solvents (acetone, ethanol, and butanol), acids (acetic acid and butyric acid), and FAs were determined using 456-GC gas chromatography (Bruker, Germany) on a HP-INNOWAX column (30 m $\times$ 0.320 mm $\times$ 0.50 μ m; Agilent J&W, USA) equipped with a flame ionization detector. FA gas chromatographic detection was performed using the heating program of the column oven, as follows: initial temperature, 50 °C; maintained at 50 °C for 1 min; increased at 25 °C/min to 175 °C; increase at 40 °C/min to 230 °C; maintained at 230 °C for 15 min. The injection port temperature was set at 250 °C, the split ratio was 50:1, the detector temperature was 280 °C, the carrier gas was hydrogen (40 mL/min), the air was 400 mL/min (nitrogen, 25 mL/min), the precolumn pressure was 14.00 psi, and the injection volume was 1 μ L. Conductivity was detected using a conductivity meter (DDBJ-350; Electronic Scientific Instrument Co., Ltd., Shanghai, China). Cell membrane potential was measured using a FlowSight flow cytometer (Merck Millipore, Burlington, MA, USA) and analyzed by IDEAS Application software v6.0. For flow cytometry analyses, 20,000 events were recorded per sample.

Statistical analysis

SPSS 11.5 software was used for statistical analysis. The experimental data were expressed as means of at least three independent experiments; error bars indicate standard deviations. Means of multiple groups of samples were compared by one-way variance analysis. Pairwise comparisons were performed using least significant difference *t*-tests. Results with *P* values of less than 0.05 were considered statistically significant.

Abbreviations

ABE: acetone-butanol-ethanol fermentation; DCW:dry cell weight; OD:optical density; RCM:reinforced *Clostridium* medium; CGM:*Clostridium* growth medium; SEM:scanning electron microscopy; MATH:microbial adhesion to hydrocarbon; FDA:fluorescein diacetate; Rh123:rhodamine 123; FA:fatty acid; SFA:saturated fatty acid; UFA:unsaturated fatty acids; MUFA:monounsaturated fatty acid.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

Not applicable

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

DL and XZ coordinated and supervised the project. YG designed the experiments and wrote the manuscript. YG and MZ analyzed the data. YG, MZ, and XG performed the experiments. MZ, DL, and WL corrected the manuscript. All authors read and approved the final manuscript.

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Figures

Figure 1

SEM images of strains cultivated in butanol medium: ATCC 824 with 1.0% (B), ATCC 824 with 3.0% (C), Y217 with 1.0% (E), Y217 with 3.0% (F). Effects of butanol stress on dry cell weight (DCW) in ATCC 824 (A) and Y217 (D).

Figure 2

Ability of ATCC 824 and Y217 to tolerate butanol at a concentration of 2.0% (v/v). MATH values were determined as described by Zhang et al. [37], as follows: $\text{MATH value} = ([\text{OD600}] \text{ pretreatment} - [\text{OD600}] \text{ post-treatment}) / ([\text{OD600}] \text{ pretreatment})$ (300 μL butanol treatment). * $P < 0.05$, ** $P < 0.01$.

Figure 3

Comparison of the membrane permeability of ATCC 824 and Y217 cells. Intensity of FDA staining (A), conductivity (B), and macromolecular spillage (C).

Figure 4

Comparative analysis of membrane potential in the wild-type strain (ATCC 824) and mutant strain (Y217). Histograms (A, B) and significance analysis (C, D) are shown. * $P < 0.05$, ** $P < 0.01$.

Figure 5

Effects of butanol stress on membrane fatty acid composition in ATCC 824 and Y217 strains. Relative content composition of fatty acids (A), ratio of SFAs and UFAs to total FAs (B), ratio of SFAs to UFAs (C), and ratio of MUFAs (D) and polyunsaturated FAs (PUFA) (E) to TFAs. Cn1: n2, where n1 represents the number of carbon atoms, and n2 represents the number of double bonds. *P < 0.05, **P < 0.01.

Figure 6

Comparison of the fermentation kinetics of ATCC 824 (A) and Y217 (B) with addition of exogenous butanol.