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Research Article

A small-scale investigation process for the *Clostridium acetobutylicum* production of butanol using high-energy carbon heavy ion irradiation

Applied heavy ion irradiation technology and butanol industrial practices as a whole have been used as a strategy for the development of an attractive alternative to petroleum-based fuels. *Clostridium acetobutylicum* (*C. acetobutylicum*) strains are well documented as fermentation strains for the production of biobutanol. However, it has been reported that solvent production inhibits the growth of these strains, and the accumulation of acetate also inhibits biomass synthesis, rendering the production of butanol from acetone-butanol-ethanol (ABE) fermentation processes economically challenging. In this manuscript, we propose the use of high-energy carbon heavy ion irradiation from the Heavy Ion Research Facility in Lanzhou (HIRFL) to obtain a culture with an increased butanol yield. Our findings suggest that the use of a high-energy $^{12}\text{C}^{6+}$ heavy ion irradiation dose of 45 Gy with an energy of 135 AMeV and ion pulses/levels of 10^6 – 10^8 favours ABE solvent production in an irradiated strain compared with the non-irradiated strain. The strategy reported in this manuscript may contribute to the development of a cost-effective butanol fermentation process that is competitive with similar fermentation processes.

Keywords: ABE-fermentation / Applied HIRFL / Biobutanol / *Clostridium acetobutylicum* / $^{12}\text{C}^{6+}$ heavy ion

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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1 Introduction

The applications of n-butanol are not only limited to direct use but also include its utilization as a chemical raw material to generate other industrially important compounds [1, 2]. In recent years, the industrial process of oxo synthesis has been the predominant method used for the production of both n-butyraldehyde and the isomer iso-butyraldehyde [3]. Aldehyde mixtures are obtained in the first reaction step, which is followed by hydrogenation steps for the production of butanol [4, 5]. Depending on the reaction conditions, such as pressure, temperature, and type of catalyst (e.g., Rh, Pd, Pt, Ir, Au, Ni, Mo, Fe,

Co, Sn, Mn or Cu), different isomeric ratios of butanol can be obtained [6]. Because in the commercial synthesis of n-butanol, the compounds derived from these building blocks are dependent on the oxo reaction of propylene, the production of these chemicals is based on a non-sustainable source [7]. The increasing demand for the use of n-butanol to generate modern materials requires that alternative and sustainable processes for producing these chemicals be developed [8].

The cost of biobutanol production via conventional butanol fermentation by *C. acetobutylicum* is not economically competitive, hampering its industrial application. However, the productivity of metabolites can be improved by up to a factor of ten through suitable bacterium improvement techniques [9]. Mutagenesis is one of the most reliable and widely used approaches for strain improvement [10]. Chemical physics methods based on methyl methane sulfonate (MMS), hydroxyl amine

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Abbreviations: ECR, Electron Cyclotron Resonance; HIRFL, Heavy Ion Research Facility in Lanzhou; WDCM, World Data Centre for Microorganisms

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(HA), and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) have been adopted as important research methods for inducing mutations [11]. During organism breeding, mutations are induced using heavy-ion irradiation, ultraviolet (UV) rays, X-rays, γ -rays, lasers, neutrons, and thermophoresis [12]. These mutagenesis approaches tend to produce abundant mutant strains.

The Heavy Ion Research Facility in Lanzhou (HIRFL) is committed to finding solutions to real problems through basic research. Specifically, HIRFL comprises the superconducting ECR ion source, the 1.7-m Sector Focused Cyclotron (SFC, $K = 69$), the large Sector-Separated Cyclotron (SSC, $K = 450$), the Cooler-Storage Main Ring (CSRm), the Cooler Storage Experimental Ring (CSRe) of the newly built Cooler-Storage Ring (CSR), the radioactive ion beam lines (RIBLL1 and RIBLL2), and the experimental terminals. It is capable of providing ion beams from protons to uranium with energies of up to 2800 and 1000 MeV/u for protons and heavy ions, respectively [13, 14]. The studies on heavy ion physics and its related disciplines that are being conducted at HIRFL, include fundamental research on heavy ion nuclear physics, radioactive ion beam physics, nuclear astrophysics, high-energy density physics, highly charged atomic physics, hadronic physics, and experimental research in aerospace science and technology, materials science, biomedicine, and biological breeding.

HIRFL plays a central role in the commercial development of microbial fermentation processes. The HIRFL engineering of butanol-producing strains may allow even lower product inhibition and improved butanol yield and productivity. In addition to advanced biobutanol-producing mutant strains, the yield, productivity, and butyrate/acetate (B/A) ratio have also been improved by continuous fermentation processes with immobilized cells reactors [15], cell recycle membrane reactors [16], or packed bed reactors [17]. Continuous fermentation processes enable the use of highly concentrated sugar solutions, decrease product inhibition by integrated product removal, and lower the costs of wastewater treatment. *C. acetobutylicum* is one of the most important bacterial bioresources for high-efficiency butanol production. However, the economic feasibility of biobutanol production using ABE fermentation is suffering due to low product yield, product toxicity to the production strain, multiple end-products, production inhibitions, and inefficient product recovery from the produced alcohol mixtures [18–20].

Although $^{12}\text{C}^{6+}$ heavy-ion irradiation plays a key role in the bioprocessing and the bio-fermentation design and scale-up, no studies have been performed on heavy-ion irradiation and its effect on the growth and butanol productivity of *C. acetobutylicum*. Hence, this work aimed to develop a high-energy carbon heavy ion irradiation pattern to explore this strain, and this process was used to track and evaluate the performance of the butanol fermentation system, as well as the traits of the production process.

2 Materials and methods

2.1 Cultures and medium

Clostridium acetobutylicum ATCC 4259 was used as the reference wild-type strain in an appropriate publicly accessible culture col-

lection belonging to the world data centre for microorganisms (WDCM). To test the production of butanol by the wild-type and mutant strains, a rich P2 medium containing 60 g/L glucose, 3.6 g/L yeast extract, 2.7 g/L peptone, 3.2 g/L K_2HPO_4 , 3.2 g/L KH_2PO_4 , 0.2 g/L MgSO_4 , 0.2 g/L MnSO_4 , 0.02 g/L FeSO_4 , 0.02 g/L NaCl, 1.5 g/L yeast extract (Difco, USA), 2.5 g/L ammonium acetate, 0.0005 g/L p-aminobenzoate, 0.0005 g/L thiamin, 0.00005 g/L biotin, and 35 $\mu\text{g}/\text{mL}$ thiamphenicol was used [21]. To test the production of butanol from different substrates, the same rich P2 medium with 30 g/L instead of 60 g/L glucose was used.

2.2 Serum bottles culture medium

A medium prepared under strictly anoxic conditions appears colourless with small black precipitates. Unless otherwise noted, the fermentation was conducted in serum bottles, each of which contained 40 mL of the medium and was inoculated with 1% (v/v) of an overnight culture in Reinforced Clostridial Medium (RCM; Difco, Detroit, MI, USA) at 37°C and with rocking at 250 rpm. The pH was maintained between 5.0 and 6.2 by adding a NaOH solution twice a day [22].

2.3 Experimental setup

The experiment was performed at the Cancer Therapy Terminal of the Heavy Ion Research Facility at HIRFL. The upgraded accelerator system of HIRFL consists of a Sector Focus Cyclotron (SFC), a Separated Sector Cyclotron (SSC), a main Cooling Storage Ring (CSRm), and an experimental Cooling Storage Ring (CSRe). High-energy $^{12}\text{C}^{6+}$ ions, with an energy of 234 AMeV, were extracted by CSRm. High-energy $^{12}\text{C}^{6+}$ ions, with an energy of 196 AMeV, were extracted by CSRm. Energies of 117 AMeV and 135 AMeV were obtained by adding the absorbers (water) and calibrating using the LISE programme, and the corresponding uncertainty of the energies is not higher than 0.27%. The extraction time of the carbon ions (approximately 10^6 – 10^8 ions/pulse) was approximately 3 s, and the priming dose was 15–100 Gy. The dose rates were up to 10 Gy/min.

2.4 Linear quadratic (LQ) model and MTT assay

The linear quadratic (LQ) model has been adopted as the model of choice to describe survival curves. The surviving fraction, $S(D)$, was calculated from the lineal energy spectrum after a single-dose irradiation, as follows [23]:

$$S(D) = e^{-\alpha D - \beta D^2} \quad (1)$$

where α is the component that represents non-repairable damage, and β is the component that represents damage that is repairable over time. $S(D)$ is the fraction of cells surviving a dose D . The constant α was used for the component proportional to the dose (αD), and the constant β was used for the component proportional to the square of the dose (βD^2).

In addition, for the irradiation experiments, the strain cells were grown in flasks (12 cm²) and allowed to reach 90%

confluence; the flasks were then completely filled with DMEM to avoid artefacts (air layers) due to irradiation. The protocol was adapted from methods described in the literature [24]. Briefly, the DMEM in each well was supplemented with 100 μ L of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reagent ($c = 0.5$ g/L), and the plate was incubated for 30 min at 37°C. The MTT solution was then removed. After the addition of 180 μ L of DMSO, the plates were incubated for 15 min at 37°C to dissolve the formazan crystals. The absorbances of the DMSO extracts were determined at 560 nm with a reference wavelength of 690 nm using a Tecan Infinite F 200 microplate reader (Crailsheim, Germany). The treated cells were harvested the next day by trypsinization, were counted, and a specific number of cells (900 and 450 strain cells) were plated in Petri dishes in triplicates for the clonogenic assay. Multiple MTT assays were performed using 96-well-plates with 3000 and 6000 cells per well. The survival fraction was calculated from the following equation:

$$Survival = 2^{-\frac{t_{delay}}{t_{doublingtime}}} \quad (2)$$

where the doubling time is the period of time required for a quantity of cells to double, and t_{delay} is the period of time required to reach a specific absorption value of the control cells versus the irradiated strains cells.

2.5 Complete anoxic conditions

Serum tubes containing 7.0 mL of P2 medium were first purged with sterile 80% N₂, 10% CO₂, and 10% H₂ for 9 min. To prevent caramelization of the sugar, which is a browning reaction, a separate 60 g/L dextrose solution in distilled water was prepared in a 120 mL serum bottle and purged with nitrogen gas for 18 min to attain completely anaerobic conditions. Both vessels were tightly sealed with rubber stoppers and aluminium crimps to prevent the ingress of air and contamination with oxygen [25]. Both liquids were sterilized by autoclaving at 121°C and 15 psig for 25 min and were then cooled at room temperature. After 1.5 mL of dextrose solution was added to the first tube, the tube was inoculated with a 0.04 volume of each original stock culture. The anaerobic stock cultures of all of the strains were collected from the original serum tubes stored at 4°C. Prior to inoculation, the stock culture tubes were incubated at room temperature for 25 min to pre-activate the cells. The pre-culture was incubated at 37°C for 36 h to allow cell growth and was then inoculated to obtain the final fresh cell culture inoculant [26].

2.6 Fermentation kinetics study

One hundred and fifty millilitres of the cell suspension of each strain prepared in serum bottles was inoculated into the fermentor, and the cells were then allowed to grow for 65–72 h at 37°C under agitation at 170 rpm and a pH value in the range of 6.0 to 6.3, which was controlled by the addition of OH[−]. After approximately 48 h of continuous circulation, most of the strain cells were immobilized, and no change in the cell density in the medium could be identified. For some experiments,

the P2 medium was used instead of the RCM medium. The spent medium in the fermentor was then replaced with fresh medium, and the recirculation rate was increased to 200 mL/min for the new fermentation batch [27]. For the main fermentation, *Clostridium* growth medium (CGM), which contains 35–100 g of glucose, 0.56 g of K₂HPO₄, 0.63 g of MgSO₄·7H₂O, 0.015 g of MnSO₄·7H₂O, 0.015 g of FeSO₄·7H₂O, 1.7 g of NaCl, 2.1 g of asparagine, 6.5 g of yeast extract, and 1.35 g of (NH₄)₂SO₄ in 1.25 L of distilled water, was used. A modified reinforced *Clostridium* medium (RCM, Difco), which contains the following ingredients per litre of distilled water, was used for the pre-culture and solid culture: 15 g of glucose, 12 g of tryptose, 10 g of beef extract, 3.6 g of yeast extract, 4.5 g of NaCl, 1.3 g of soluble starch, 0.65 g of cysteine hydrochloride, and 2.5 g of sodium acetate in 15 g/L agar [28, 29]. After the strains were pre-cultured using RCM at 37°C and pH 6.0–6.2 under anaerobic conditions, the grown cells were inoculated (15%, v/v) into CGM containing glucose. The main fermentation was conducted in a 5 L stirred-tank fermentor (Marubishi MD-300) with a working volume of 1.5 L, and the pH was maintained at pH 6.0 using 2.5 M NaOH.

2.7 Analytical methods

A high-performance liquid chromatography (HPLC) system was used to analyse the carbohydrate compounds, including glucose, in the fermentation broth. The HPLC system consisted of an automatic injector (Agilent 1100, G1313A), a pump (Agilent 1100, G1311A), a Zorbax carbohydrate analysis column (250 mm × 4.6 mm, 5 μ m; Agilent, USA), a column oven maintained at 30°C (Agilent 1100, G1316A), and a refractive index detector (Agilent 1100, G1362A). The mobile phase was ethyl nitrile (ethyl nitrile/water = 75:25) at a flow rate of 1.5 mL/min. Butyric acid and acetic acid were analysed with a GC-2014 Shimadzu gas chromatograph (GC) (Shimadzu, Columbia, MD, USA) equipped with a flame ionization detector and a 30.0 m fused silica column (0.25 mm film thickness and 0.25 mm ID, Stabilwax-DA). The GC was operated at an injection temperature of 200°C, and 1 μ L of sample was injected using the AOC-20i Shimadzu auto injector. The column temperature was maintained at 80°C for 3 min, raised to 150°C at a rate of 30 K/min, and maintained at 150°C for 3.7 min. The cell density was analysed by measuring the optical density (OD) of the cell suspension at 600 nm using a spectrophotometer (Thermo Spectronics, Genesys 20, USA) with a conversion of 0.78 g/L of dry cell weight (DCW) per OD unit. The dry weight of the immobilized cell biomass was determined by centrifuging the fermentation broth at 10 000 × g for 10 min, washing the sediment with distilled water, and drying the sediment overnight at 180°C.

3 Results and discussion

3.1 Mathematical and MTT assay describes the surviving fraction after high-energy carbon heavy ion irradiation

The HIRFL-CSR system was set to the full running mode as shown in Fig. 1A. In this full running mode, the ¹²C⁶⁺ ions

Figure 1. The survival data for *Clostridium acetobutylicum* obtained from mathematical, MTT assay plotted against high-energy carbon heavy ions irradiation from HIRFL. (A) The Heavy Ion Research Facility in Lanzhou comprises of the superconducting ECR ion source, the 1.7 m Sector Focused Cyclotron (SFC, $K = 69$), the large Sector-Separated Cyclotron (SSC, $K = 450$), the Cooler-Storage Main Ring (CSRm), the Cooler-Storage Experimental Ring (CSRe) of the newly built Cooler-Storage Ring (CSR), the radioactive ion beam lines (RIBLL1, RIBLL2), experimental terminals. It is capable of providing ion beams from protons to uranium with energies of up to 2800 MeV/u, 1000 MeV/u for protons, heavy ions, respectively. (B) The survival data for *C. acetobutylicum* obtained from mathematical description plotted against high-energy carbon heavy ions irradiation from HIRFL. (C) The survival data of *C. acetobutylicum* strains cell in natural logarithm by MTT assay.

produced by the ECR source were pre-accelerated, first to 4 AMeV by HIRFL-SFC and then to 26 AMeV by HIRFL-SSC. The 26 AMeV $^{12}\text{C}^{6+}$ were changed to $^{12}\text{C}^{6+}$ after passing through a stripper and were then injected into CSRm by multiple multi-turn injection (MMI) and accelerated to 234 AMeV by CSRm. The 234 AMeV $^{12}\text{C}^{6+}$ ions, extracted from CSRm, were transferred through RIBLL II and injected into CSRe for the calibration experiment. A total of 824 *C. acetobutylicum* strains were irradiated by two high energies generated by a $^{12}\text{C}^{6+}$ heavy ion accelerator: 117 AMeV and 135 AMeV. The irradiations were conducted at ordinary temperature and pressure with absorbed doses in the range of 15 to 100 Gy. For the high-energy carbon heavy ions, the dependence of the relative biological characteristics of microorganisms on the various physical and biological properties is greater and cannot be disregarded. The graph in Fig. 1B shows the survival curves of the strains with different

energies and doses. To explain linear quadratic theoretical models, a basic understanding of statistical derivatives is needed. The basic principle of calculating probability is derived from Poisson's statistics [30]. The probability P of event n occurring, $P(n)$, is given by [31]:

$$P(n) = \frac{(e^{-x}) \cdot (x^n)}{n!} \quad (3)$$

where $n!$ is the factorial of n , x is the average number of events, n is the number of occurrences of an event, the probability of which is given by the function, and e is Euler's number, which is expressed as:

$$e = \lim_{n \rightarrow \infty} \left(1 + \frac{1}{n}\right)^n \approx 2.71828182 \quad (4)$$

If the probability of being hit is assumed to be an inactivation, then the probability of not being hit, $P(0)$, is based on the following Poisson relationship:

$$P(0) = \frac{(e^{-1}) \cdot (1^0)}{0!} = e^{-1} = 0.36787944 \approx 36.79\% \quad (5)$$

where $x = 1$ and $n = 0$. The final slope of the survival curves obtained with 117 AMeV and 135 AMeV irradiation are similar, but the initial slopes are markedly different, which shows that the high-energy carbon heavy ion irradiation has a high α component, whereas the β component remains unchanged. Thus, irradiation of the strain cells with high-energy radiation levels produces linear survival curves. The survival of the strain cells as a function of the dose, is a common logarithm, and the survival curves are shown in Fig. 1B. The most prominent feature of most survival curves is the deviation from such a simple curve; namely, the energy and dose response curves typically show a shoulder. As already indicated in Fig. 1B, after reaching a maximum value at an irradiation dose of 50 Gy with an energy of 135 AMeV, the surviving fraction does not increase any further, but rather decreases towards higher dose values. For the high-energy $^{12}\text{C}^{6+}$ heavy ion irradiation (a dose of ≥ 40 Gy for 135 AMeV), surviving fraction values as low as 1% are observed. Interestingly, a dose of ≥ 70 Gy for 117 AMeV, the results in surviving fraction values as low as 1%. The *C. acetobutylicum* strain cells show survival curves that start with a moderate slope, but the slope increased with increasing energy and dose. Therefore, the efficiency per energy and dose increment also increases. This finding can be understood in terms of the effectiveness of the irradiation-induced mutations. At low energies and doses, only a few mutations with a large spatial separation are induced, and a considerable fraction of these mutations can be irradiated effectively. In contrast, at high energies and doses, the density of the mutations increases, leading to an interaction of mutations and thus a reduced surviving fraction. In addition, these cells gain more influence in the outcome of survival, as determined through the MTT assay. Cells from strains with low metabolic activity and slow proliferation, as well as cells from strains that ceased to proliferate, were excluded from the assay by washing and trypsinization during the plating step after irradiation. As expected, the survival curves of the assays show the same characteristics: the survival depends on the energy, dose, and ions/pulse of carbon ion irradiation, and an increase in the irradiation parameters leads to a decrease in the survival, which approaches zero for the strain cells plated after irradiation. The investigation of the growth and death of the irradiation-induced strain cells, which is defined as the time period required for the complete loss or exhaustion of the proliferative capacity, is one of the most commonly and reliably used methods for the study of the effects of irradiation on cells. The survival fraction, as obtained from Eq. (1), was compared with a representative set from the experimental data. The panels in Fig. 1C show the survival curve, plotted on a natural logarithmic scale, of the surviving fraction as a function of different physical parameters. The results of the cellular survival data from the MTT assay are plotted against the irradiation effects obtained with an energy of 117 AMeV and ion pulses/levels of 10^6 – 10^8 , which were $e^0 \rightarrow e^{-0.7}$ (100%–49.6%) for 30 Gy, $e^0 \rightarrow e^{-1.8}$ (100%–16.5%) for 60 Gy, $e^0 \rightarrow e^{-3.4}$ (100%–3.33%) for 75 Gy, and $e^0 \rightarrow e^{-6.9}$ (100%–0) for 105 Gy. The results were also

plotted against the irradiation effects obtained with an energy of 135 AMeV and ion pulses/levels of 10^6 – 10^8 , which were $e^0 \rightarrow e^{-1.9}$ (100%–14.9%) for 45 Gy and $e^0 \rightarrow e^{-4.2}$ (100%–1.49%) for 60 Gy.

3.2 Effects of high-energy carbon heavy ion irradiation on cell growth

In a typical batch of ABE fermentation from carbohydrates, *C. acetobutylicum* has become the organism of choice because it converts both five- and six-carbon sugars to butyric, propionic, and acetic acids. First, the production by *C. acetobutylicum* (acidogenesis) during the exponential phase of cell growth undergoes a metabolic shift towards the formation of acetone, butanol, and ethanol as the main product solvents at an approximate ratio of 3:6:1 when the stationary phase is reached (solventogenesis). To further investigate the specific effect of high-energy carbon heavy ion irradiation on the cell growth profiles (based on OD₆₀₀ measurements), individual batch cultures were conducted in a chemically defined P2 medium (performed in serum bottles) containing 60 g/L glucose. As fully expected, all of the irradiated strains exhibited a biphasic metabolic pattern that was strongly influenced by the medium's pH. As a general trend, the cells initially consume glucose to grow, during which time they produce and excrete n-butanol, acetic acid, butyric acid, and acetone as primary metabolites; the accumulation of these metabolites and their increase to certain levels results in a decrease in the medium's pH. These differences in pH regulate the temporal switch associated with solvent formation for each strain. Usually, an external pH below 5.0 (optimal around pH 4.3), and an endogenous pH greater than 5.5, are required to induce solventogenesis. For the non-irradiated *C. acetobutylicum*, as described by the graphs shown in Fig. 2, the pH of the culture of *C. acetobutylicum* (Fig. 2) decreased to approximately 4.4 (ΔpH of 0.6 starting from pH 5.0) over 18 h; after periods of 27, 45, and 54 h, the pH values decreased to approximately 4.1 (ΔpH of 0.9 starting from pH 5.0), 3.9 (ΔpH of 1.1 starting from pH 5.0), and 3.8 (ΔpH of 1.2 starting from pH 5.0), respectively. When the culture was irradiated with 117 AMeV at a dose of 60 Gy (Fig. 2), the pH decreased to approximately 4.7 (ΔpH of 0.3 starting from 5.0) over a period of 18 h. While over periods of 27, 45, and 54 h, the pH values were approximately 4.8 (ΔpH of 0.2 starting from 5.0), 3.6 (ΔpH of 1.4 starting from 5.0), and 3.8 (ΔpH of 1.2 starting from 5.0), respectively. When the culture was irradiated with 135 AMeV at a dose of 45 Gy (Fig. 2), the pH dropped to approximately 4.8 (ΔpH of 0.2 starting from 5.0) over 18 h. After periods of 27, 45, and 54 h, the pH values decreased to approximately 4.5 (ΔpH of 0.5 starting from 5.0), 3.4 (ΔpH of 1.6 starting from 5.0), and 3.6 (ΔpH of 1.4 starting from 5.0), respectively. We ran 36 fermentation experiments at once using *C. acetobutylicum* wild-type strain (4259) at the pH value calculated during the growth process shown earlier in Fig. 2. We then weight-fitted straight line and quadratic equations, and by using this method, optimum straight-line equations and minimum non-linear errors can be calculated. The formula results in the following fitted straight-line equation model (yellow straight line) for the change in pH value during the process of growth in 54 h: $y = -0.016x + 4.6$. In addition,

Figure 2. Time-course of the wild type, irradiated strains cells for the change in pH value during the process of growth in 54 h of fermentation in serum bottles. Blue column represents the wild-type inherent the change in pH value for the effects observed. Orange column represents the irradiated strains cell the change in pH value for the effects observed, supported by available studies the $^{12}\text{C}^{6+}$ -ions irradiation dose (60 Gy) with 117 AMeV of energy, 10^6 – 10^8 ions/pulse levels. Yellow column represents the irradiated strains cell the change in pH value for the effects observed, supported by available studies the $^{12}\text{C}^{6+}$ -ions irradiation dose (45 Gy) with 135 AMeV of energy, 10^6 – 10^8 ions/pulse levels.

the formula results in the following fitted quadratic equation model (the purple curve) for the change in pH value during the process of growth in 54 h: $y = -0.00093x^2 - 0.083x + 5.6$, where y is the change in pH value and x is the time of growth of strain cells.

Figure 3 represents the cell growth types based on optical density (OD600) measurements. A similar lag phase of 9–10 h was found in the chemically defined P2 medium (performed in serum bottles) containing 60 g/L glucose. As shown in Fig. 3A, we observed a reduction in cell counts at approximately 54 h for the wild-type strain. This can be explained by the fact that as glucose in consumed, consumption which requires the bacterial strain is forced to enter a transition zone before it can start utilizing the other substances in the P2 medium. By the end of cell growth experiment, the wild-type strain's OD value in a medium containing 60 g/L glucose was on average 1.61 (OD600). Furthermore, we found that the wild-type strain's cell growth exhibited a downward trend after 20 h, as well as after 54 h. The linearized equation of descending trend is $y = 0.02x + 1.57$, shown as a yellow straight line in Fig. 3A. This result shows that the non-radiated *C. acetobutylicum* culture inoculated into glucose-minimal media begins to consume sugar almost immediately. Interestingly, as shown in Fig. 3B, the radiated *C. acetobutylicum* (with 135 AMeV at a dose of 45 Gy) culture, inoculated into glucose-minimal media, began to consume sugar almost 9 h after inoculation. We found that the cellular growth of irradiated strains exhibits an increasing trend after 12 h. The linearized equation of the increasing trend is $y = 0.011x + 0.58$, shown as the yellow straight line in Fig. 3B. This finding

Figure 3. Time-course of the physiologic properties of the wild type, irradiated strains cells growth types based on optical density (OD600) measurements during the process of growth in 54 h of fermentation in serum bottles. (A) The wild-type inherent physiologic properties for the effects observed. (B) The irradiated strains cell physiologic properties for the effects observed, supported by available studies the $^{12}\text{C}^{6+}$ -ions irradiation dose (45 Gy) with 135 AMeV of energy, 10^6 – 10^8 ions/pulse levels.

suggests that the wild-type and irradiated strains exhibit a biphasic metabolic pattern that is strongly influenced by the pH of the medium. As a general trend, the cells initially consume glucose to support growth and produce and excrete organic acids as primary metabolites (acidogenesis), which cause a decrease in the medium's pH when they accumulate to certain levels. This increase in the broth acidity shifts the formation of acids towards the production of solvents when the culture reaches the stationary phase of cell growth (solventogenesis). At high pH, organic acids are mainly formed, whereas at low pH, solvent production is stimulated. The reutilization of organic acids for the formation of solvents results in an increase in the medium's pH. As expected, the nature of the metabolic shift and the kinetic pattern of solvent formation are strain-dependent because the irradiated

strains exhibit their own intrinsic genetic and metabolic characteristics. It was not the purpose of this work to provide an extensive explanation based on the inherent biochemical properties of the irradiated strains, but to assign possible causes for the effects observed, supported by studies documented in the literature.

3.3 The impact of supplemented butyric acid on growth rates and biomass concentration

Improvements in productivity were made by employing continuous cultures with partial cell recycling. The main challenges that need to be approached in order to make fermentation economically viable are the following: (i) butyric acid has previously been reported to inhibit cell growth, (ii) the production of solvents, especially n-butanol, is manifestly influenced by butyric acid, and (iii) rapid cell growth has a higher energy demand and preferentially produces acetic acid. In addition to shifting the metabolic stage of the culture due to a decrease in the medium's pH, butyric acid is also utilized by the cells as a co-substrate in the formation of butanol. As such, the regulation of butyrate amounts in the culture media is of great industrial importance. To examine in more detail the effect of added butyrate, the cell growth profiles (based on optical density measurements) for the wild-type and irradiated strains (different physical parameters) were compared. Supporting Information Fig. 1 shows the impact of butyric acid supplementation and 117 AMeV irradiation levels on optical density (OD_{600}) and biomass concentration (dry cell weight) during different times of fermentation. For the non-irradiated *C. acetobutylicum* (control), individual batch cultures were incubated in chemically defined P2 media (performed in serum bottles) containing 75 g/L glucose and supplemented with 2.2, 4.4, 8.8, 11, or 13.2 g/L butyric acid. The results show that, irrespective of the wild-type strain used, there was a gradual inhibition of cell growth, and no growth was observed at butyric acid concentrations higher than 11 g/L. The extended period of minimal metabolism is due to the butyrate causing an inhibition in the log phase of cellular growth. However, irrespective of the irradiated strains used, no gradual inhibition of cellular growth was observed, and no growth was observed for butyric acid concentrations higher than 13.2 g/L. Additionally, the panels in Fig. 4 show the impact of butyric acid supplementation and 135 AMeV irradiation levels on optical density (OD_{600}) and biomass concentration (dry cell weight), calculated using a conversion rate of 0.78 g/L of dry cell weight (DCW) per OD unit, during different times of fermentation. The DCW was used to determine the specific growth rate, as described by Shuler and Kargi [32]. The DCW data points from the logarithmic growth phase were plotted on a semi-log graph to identify the period during the phase in which the culture experienced the fastest growth. These points were then used in the following equation: $\mu(1/h) = [(DCW_2 - DCW_1)] / \Delta Time$. The results show that the specific growth rate of the irradiated strains (117 AMeV and a dose of 60 Gy) in the non-butyric acid supplemented culture was $0.21\ h^{-1}$ and was reduced by 11.8% compared to that of the wild-type strain. The specific growth rate of the irradiated strains (135 AMeV and a dose of 45 Gy) in the non-supplemented culture was $0.18\ h^{-1}$ and was reduced by 17.3% compared with

that of the wild-type strain. When supplemented with 4.4 g/L butyric acid, the specific growth rates of the strains irradiated with 117 AMeV and a dose of 60 Gy and 135 AMeV and a dose of 45 Gy were 0.15 and $0.13\ h^{-1}$, respectively. This observation is not surprising because the use of high-energy carbon heavy ion irradiation results in a low specific cell growth rate or an increase in cell mass over time. Additionally, the observation represents a shift in selectivity at different growth rates, which has a significant impact on the ABE fermentation process. At high growth rates, the preferential production of acetic acid has a higher energy demand. At low growth rates, the production of butyric acid is favoured over that of acetic acid. It should be noted that the short-term results of high-energy carbon heavy ion irradiation are associated with higher selectivity in the late exponential or early stationary phases compared with those obtained in the lag phase. Thus, we theorized that an increase in the cell mass in a continuous system at low dilution rates and/or a high cell recycle ratio may also increase the selectivity of butyric acid.

3.4 Comparison of solvent production at different irradiation levels

One experiment was conducted in the fermentation mode using CGM media containing 60 g/L glucose as the primary carbon source and without butyrate supplementation to determine the solvent production capability of the wild-type and irradiated strains (135 AMeV and a dose of 45 Gy). Figure 5A shows that the minimum butanol concentration detected was 1.132 g/L at 20 h in *C. acetobutylicum* strain 4259 fermentation using CGM media and a starting pH at 6.5. This concentration increased to 6.137 g/L by the end of 60 h of fermentation. The low butanol production is thought to be a result of the poor uptake of sugars and acids by the *C. acetobutylicum* strain 4259. These strain characteristics can be further improved by generating mutant versions of *C. acetobutylicum* strain 4259 with a higher capability for sugar uptake. As Fig. 5B shown, in the case of irradiated strains (135 AMeV and a dose of 45 Gy), the production concentration increased from 2.132 g/L at 20 h of fermentation to 8.096 g/L at the end of 60 h of fermentation. This value is more than 1.32 times the concentration produced from the *C. acetobutylicum* wild-type strain. Concentrations and selectivity results of the 60 h fermentation experiment were summarized in Fig. 5 for both the *C. acetobutylicum* 4259 and irradiated strains (135 AMeV and a dose of 45 Gy). At the current stages of biofuel research, total solvent production is of more interest, especially that butanol yield is fairly similar in wild-type strains (0.29). However, the cost of the separation of acid by-products that may result in serious corrosion risk to pipelines and storage reservoirs, is high. Therefore, we decided to use the irradiated strains to determine the optimum pH in feedstocks for the maximum production of butanol, until the uptake issues are resolved with the *C. acetobutylicum* 4259 wild-type strain.

Another experiment was conducted in the fermentation mode using CGM media containing 90 g/L glucose as the primary carbon source to determine the capacity of *C. acetobutylicum*, after high-energy carbon heavy ion irradiation, to produce solvents. The experiment required that the cells be acclimatized for 130 h,

Figure 4. Impact of supplemented butyric acid (BA), 135 AMeV irradiation levels on Optical Density (OD₆₀₀), biomass concentration (dry cell weight) during different times of fermentation.

even though the irradiated strain fermentations were tested under the same conditions and were supplemented with 4.0 g/L butyric acid. It is well known that high amounts of organic acids accumulate in the culture broth, inhibit cellular growth, and consequently cause cell death. Previous studies demonstrated that the cellular growth is mainly dependent on the dissociation constants of organic acids (pK_a) and the pH of the culture broth. Based on the fact that the butyric acids used in this study have similar pK_a values of 4.82, the pH of the fermentations in this study was controlled at 6.5. The graphs in Supporting Information Figs. 1 and 2, Figs. 4 and 6 show the individual kinetic profiles for the radiated strains, which were unaffected by the addition of butyrate. In contrast, the wild-type strains supplemented with butyrate exhibit very low rates of alcohol and acetone formation compared with the formation of acids. Clearly, as shown in Figs. 5B and 6, the growth of the strains is inhibited by high-energy carbon heavy ion irradiation, which appears to enhance the substrate consumption parameters, even

though the patterns for the sugar utilization rate are somewhat different for the different irradiation parameters. Thus, the irradiated strains were unaffected by butyric acid addition and consume glucose mainly for growth, producing butyrate simultaneously as a primary metabolite. As butyrate builds up in the system, the medium's pH decreases; at this stage of the fermentation, the irradiated strains shift their metabolic state from acidogenesis to solventogenesis. The irradiated (117 AMeV, 60 Gy) *C. acetobutylicum* culture inoculated into CGM media containing 90 g/L glucose began to consume sugar almost immediately and began to produce butanol 20 h after inoculation (Supporting Information Fig. 2). However, the irradiated (135 AMeV, 45 Gy) *C. acetobutylicum* culture inoculated into CGM media containing 90 g/L glucose began to consume sugar almost immediately and began to produce butanol 25 h after inoculation (Fig. 6). The same culture inoculated into media containing 4 g/L butyrate equivalents needed to be acclimatized for more than 15 h to the butyrate added to the medium, even though

Figure 5. Concentrations, selectivity results of the 60 h fermentation experiment were summarized in the fermentation mode using CGM media containing 60 g/L glucose as the primary carbon source, without butyrate supplementation to determine the solvent production capability of strains. (A) The *C. acetobutylicum* 4259 wild-type strain. (B) Irradiated strains (135 AMeV, a dose of 45 Gy).

both fermentations were performed under the same conditions. The extended period of minimal metabolism and productivity is due to the high-energy carbon heavy ion irradiation causing a delay in the log phase of cellular growth. After the irradiated (135 AMeV, 45 Gy) *C. acetobutylicum* culture had adapted to the 4 g/L-butyratesupplemented media, the culture behaved similarly to the control, resulting in complete glucose utilization, and in the production of more than 7.2 g/L butanol, and similar levels of cell density (Fig. 6). This result appears to show that a major fraction of the glucose is channelled towards the generation of energy associated with the metabolic shift towards solventogenesis, which requires new enzymes and other components, whereas the cellular growth of the irradiated strains uses a smaller portion. In addition, due to the accumulation of butyrate in the system, acetoacetyl-CoA is converted into butyryl-CoA, thereby raising the levels of this metabolite, rather into acetoacetate, which results in lower acetone production. In addition,

enhanced butanol production requires the efficient transition from acidogenesis to solventogenesis and, therefore, it is very important to show the impacts of the applied light source on the biochemistry underlying the transition. Although the interaction details may be unclear, a general principle has been identified that the acidogenesis-solventogenesis transition is a stress response which can be caused by various sources of toxicity acting alone or together [33,34]. The high-energy irradiation can be regarded as a stressor which may enhance the transition and butanol production accordingly. If *C. acetobutylicum* (4259 strain) were to be utilized in anaerobic batch fermentation, it would be required to develop a mutant version having a high capability to uptake various agriculture sugars, leading to butanol production since the overall competitiveness of the bioprocess highly depends on the strain's successful metabolic performance.

4 Concluding remarks

The enthusiastic attention for biobutanol promotes the development of butanol production from renewable feedstocks. In this study, an applied high-energy carbon heavy ion irradiation for the ABE fermentation of *C. acetobutylicum* (strain 4259) was proposed. Due to the limitation of current biotechnologies, such as homologous recombination, antisense RNA and Mobile group II intron, the advent of the CRISPR-Cas-based technology makes large-scale genome editing of *Clostridium* a reality [35]. Therefore, our future work will use CRISPR-Cas-based editing toolkits to research well-characterized genetic and metabolic backgrounds in the mutant. Overall, high-energy carbon heavy ion irradiation mutagenesis and targeted manipulation technologies integration, are promising tools to use for the efficient and targeted mutant cell engineering in order to enhance biobutanol production.

Practical application

The cost of biobutanol production via conventional butanol fermentation by *C. acetobutylicum* is not economically competitive, which has hampered its industrial application. Thus, the genetic manipulation and process engineering strategies developed based on *C. acetobutylicum* can also be implemented in other species of *Clostridium* and heavy-ion radiation producing strains. The rheology of the bioprocess and the downstream processing of the product are heavily dependent on the production ability of *C. acetobutylicum* mutants and require carefully designed $^{12}\text{C}^{6+}$ heavy-ion radiation parameters. In this work, a $^{12}\text{C}^{6+}$ heavy-ion beam plays a key role in the bioprocessing and the bio-fermentation design and scale-up. Related empirical models for the bio-fermentation of mutants are proposed to describe the optimal operation conditions for mutant cell growth, butanol formation, and glucose consumption. Improving the butanol yield is critical for the future applications of this process in the fermentation industry.

Figure 6. Time-course studies of various activities for irradiated *C. acetobutylicum* strains fermentation as a function of added 4 g/L butyric acid concentrations, supported by available studies the 135 AMeV (at a dose rate of 45 Gy) high-energy carbon heavy ion irradiation from HIRFL. (A) Graph: pH, cell density (OD₆₀₀), glucose conc. (B) Graph: acetic acid, ethanol, butyric acid, acetone, butanol.

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