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Metabolic analysis of butanol production from acetate in *Clostridium saccharoperbutylacetonicum* N1-4 using ^{13}C tracer experiments

Ming Gao,[a] Yukihiro Tashiro,[b, c] Tsuyoshi Yoshida,[a] Jin Zheng,[a] Qunhui Wang,[d] Kenji Sakai,[b] Kenji Sonomoto* [a, e]

[a] *Laboratory of Microbial Technology, Division of Systems Bioengineering, Department of Bioscience and Biotechnology, Faculty of Agriculture, Graduate School, Kyushu University, 6-10-1 Hakozaki, Higashi-ku, Fukuoka 812-8581, Japan*

[b] *Laboratory of Soil Microbiology, Division of Systems Bioengineering, Department of Bioscience and Biotechnology, Faculty of Agriculture, Graduate School, Kyushu University, Japan, 6-10-1 Hakozaki, Higashi-ku, Fukuoka 812-8581, Japan*

[c] *Institute of Advanced Study, Kyushu University, 6-10-1 Hakozaki, Higashi-ku, Fukuoka 812-8581, Japan*

[d] *Department of Environmental Engineering, University of Science and Technology Beijing, Beijing 100083, China*

[e] *Laboratory of Functional Food Design, Department of Functional Metabolic Design, Bio-Architecture Center, Kyushu University, 6-10-1 Hakozaki, Higashi-ku, Fukuoka 812-8581, Japan*

***Corresponding author**

E-mail: sonomoto@agr.kyushu-u.ac.jp

Tel/Fax: +81-(0)92-642-3019

Abstract

During acetone-butanol-ethanol (ABE) fermentation by clostridia, acetate is reutilised for butanol production. In this study, we investigated the characteristics of ABE production from acetate and analysed the metabolism of exogenously added acetate by *Clostridium saccharoperbutylacetonicum* N1-4. Supplementation of 4 g/L exogenous acetate, to media containing glucose, increased not only concentrations of butanol (48.3%) and acetone (90.5%), but also the ratio of acetone to butanol (27.1%), which suggested that acetate addition altered the metabolic flux. Acetate could not be metabolized in the absence of glucose, thus glycolysis appeared to be necessary for acetate utilisation. In order to clarify the metabolism of exogenous acetate, ^{13}C tracer experiments were performed by supplementing [1, 2-$^{13}C_2$] acetate in culture broth. Based on the results of gas chromatography-mass spectroscopy analysis, we first confirmed both butanol and acetone formation from acetate. Further, the acetate-to-butanol efficiency will significantly decrease when more acetate than 2–4 g/L is added to the fermentation, while acetate-to-acetone efficiency may remain high (up to a ratio of 2 mol acetate per 1 mol glucose fed). Moreover, the culture supplemented with acetate exhibited an increase in conversion efficiency of glucose to butanol and acetone, from 0.196% to 19.5% and from 0 to 7.64%, respectively, even during acidogenesis. Thus, we first revealed quantitatively that acetate addition induced solvent production during early growth phase, and increased metabolic flux to acetone and butanol production from both acetate and glucose.

Key words: Butanol fermentation; acetate; ^{13}C-tracer experiment; metabolic analysis; *Clostridium saccharoperbutylacetonicum*

1. Introduction

Diminishing oil resources, and the increasing environmental concerns resulting from the impact of petroleum fuel emissions, has placed greater emphasis on the search for alternative renewable fuel sources.[1, 2] Butanol is an alternative liquid biofuel, which can be produced from renewable feedstock, such as agricultural and domestic wastes,[3-5] in a process referred to as the acetone-butanol-ethanol (ABE) fermentation. In contrast to the traditional alcohol-based biofuel ethanol, butanol is compatible with the existing fuel engines, and can be used in pure form or as a blend with gasoline. In addition, it has a higher energy density, lower vapour pressure, and is less corrosive.[6, 7] These attributes make butanol one of the most attractive liquid biofuels.

ABE-producing clostridia are gram-positive, spore forming, and obligate anaerobes.[8] The metabolism of ABE-producing clostridia is divided into the following two distinct phases: acidogenesis and solventogenesis.[9] During acidogenesis, organic acids such as lactic acid, acetic acid, and butyric acid are formed. Accumulation of these acids results in the induction of solventogenesis, during which these acids are reutilised to produce solvents (ABE). In consideration of the reutilisation pathway of ABE-producing clostridia, organic acids are of considerable value as substrates for butanol production. Compared with butyric acid and lactic acid, acetic acid is readily produced from hemicelluloses by extensive degradation during acid hydrolysis, which results in its accumulation.[10,11] Therefore, acetate is considered to be an available and feasible substrate for ABE fermentation.

The characteristics of ABE fermentation from acetate has been investigated using *C. acetobutylicum* and *C. beijerinckii*, with supplementation of glucose, xylose or hydrolysate derived from biomass.[12-14] Although additional acetate was shown to be consumed during ABE fermentation in all the previous studies, the effect of acetate on the ABE production depend on clostridia strains employed.[15-17] *Clostridium saccharoperbutylacetonicum* is one of the major species of ABE-producing clostridia, and we previously

reported on ABE production using *C. saccharoperbutylacetonicum* N1-4 from organic acids other than butyrate[18,19] and lactate.[20,21] However, to our knowledge, there are no reported studies on the investigation of the effect of exogenous acetate on ABE fermentation using *C. saccharoperbutylacetonicum*.

Considerable research has been conducted on the analysis of ABE metabolism in response to added acetate in *C. beijerinckii* or *C. acetobutylicum* using several approaches, including gene expression analysis,[22,23] assays of metabolic enzyme activities,[24] and metabolic flux analysis.[25] However, these approaches still have not demonstrated the direct conversion of additional acetate to butanol. In addition, there are no findings on whether or not ABE-producing clostridia generate acetate from sugar during the ABE fermentations with exogenous acetate addition. Because acetate has been found to be generated from glucose during acidogenesis through the routes of phosphotransacetylase (PTA) and acetate kinase (AK) reactions, and then reutilised via the reverse pathway of its formation, or the CoA transferase (CoAT) pathway during solventogenesis. Thus, it is impossible to distinguish the generation of ABE from added acetate via conventional measurements, such as gas chromatography (GC) and high performance liquid chromatography (HPLC). Previously, we reported on a powerful and efficient method, ^{13}C tracer experiments with GC-mass spectroscopy (MS), for the direct elucidation of butanol production from ^{13}C-labeled lactic acid.[20, 21] To the best of our knowledge, no studies have been reported on the metabolic analysis of ABE fermentation from acetate by ^{13}C tracer experiments with GC- MS and ^{13}C-labeled acetate.

The aims of this study were to investigate the effect of additional acetate and glucose on butanol production by *C. saccharoperbutylacetonicum* N1-4, and to analyse its metabolism of [1, 2-$^{13}C_2$] acetate to butanol by ^{13}C tracer experiments. We successfully verified butanol production from exogenously added acetate, and revealed its metabolic conversion, by distinguishing the acetate derived from different carbon sources.

2. Experimental

Microorganisms and media

Clostridium saccharoperbutylacetonicum N1-4 (ATCC 13564) was used for investigation of ABE fermentation.[26] Potato glucose (PG) medium was used for storage and germination of the strain spores with the following composition per litre of deionized water: 150 g mashed fresh potato, 0.5 g $(NH_4)_2SO_4$, 10 g glucose and 3 g $CaCO_3$. The mixture was incubated in boiling water for 60 min with interval mixing every 10 min, and then filtered through gauze, sterilized at 121ºC for 60 min. The strain was kept at 4ºC as spores in PG medium. One-millilitre of spore suspension was transferred aseptically to 9 ml of PG medium (10%, *v/v*), and heat-stocked in boiling water for 1 min, cultured at 30ºC for 24 h, and used as an inoculum.[27] Tryptone-yeast extract (TY) medium[28] was used for pre-culture and main culture with the following composition per litre of deionized water: 0–80 g glucose, 2 g yeast extract (Difco™; Becton Dickinson, Franklin Lakes, NJ, USA), 6 g tryptone (Difco™), 2.57 g $(NH_4)_2SO_4$, 0.3 g $MgSO_4 \cdot 7H_2O$, 0.5 g KH_2PO_4, and 10 mg $FeSO_4 \cdot 7H_2O$. Various amounts of potassium acetate (Sigma-Aldrich, St. Louis, MO, USA) were added to TY medium, separately, in different experiments, as indicated. The initial pH of the medium was adjusted to 6.5 using 3 M KOH. In all the experiments, glucose, and other components, were sterilized separately at 115ºC for 15 min, and then mixed aseptically.

Culture conditions

The pre-culture of the N1-4 strain was anaerobically grown, without agitation, at 30ºC for 15 h, in TY medium containing 20 g/L glucose. Main cultures were inoculated with 10% (*v/v*) of pre-culture, and grown at 30ºC, without pH control, under the anaerobic conditions by sparging with filtered (0.45 µm cellulose-acetate filter, ADVANTEC, Tokyo, Japan) oxygen-free nitrogen gas.

Batch culture was performed statically, in 500-ml Erlenmeyer flasks (300 ml working volume) with silicone rubber stoppers. The medium was sparged with filtered oxygen-free nitrogen for 15 min after

inoculation to ensure anaerobic conditions. To investigate the effect of additional acetate, 0, 2, 4, or 6 g/L acetate was added to TY medium containing 50 g/L glucose. Samples were withdrawn periodically, over a period of 72 h of cultivation, for analysis of products and substrates.

To investigate the effect of glucose concentration on the utilisation of acetate, batch cultures were carried out in 150-ml serum bottles containing 70-ml of TY medium supplemented with 4 g/L acetate, and different concentrations of glucose ranging from 0–80 g/L. In order to remove the residual glucose of the pre-culture broth, cells were washed prior to inoculation of the main culture. Pre-culture cells were washed as follows: the cells were harvested by centrifugation at 13,000 × *g* for 15 min, at 4°C, then washed twice and resuspended in the same volume of 0.85% sterile saline water. All operations were carried out under a N_2 atmosphere to ensure the anaerobic condition. Unless otherwise stated, all culture conditions were the same as described above.

To investigate the utilisation of adding exogenous acetate by ^{13}C tracer experiments, batch cultures were carried out in test tubes, with a 10-ml working volume of TY medium, at an initial pH of 6.5 (produced using 10 M KOH), containing 50 g/L $^{12}C_6$-glucose and 4 g/L [1,2-$^{13}C_2$] acetic acid (99% pure, Sigma-Aldrich, St. Louis, MO). After cultivation for 9 h (acidogenesis), and 24 h (solventogenesis), the culture broths were collected and analysed.

Analytical procedures

Cell growth was monitored by optical density at 562 nm (OD_{562}), with a spectrophotometer (V-530; JASCO, Tokyo, Japan), and the dry cell weight (DCW) was calculated using an OD_{562} of 1.0 equivalent to 0.301 g of DCW per litre.[21] Collected culture broths were centrifuged at 13,000 × g for 10 min to obtain the supernatant for quantification of ABE solvents, glucose, and organic acids. The concentrations of glucose and lactate were determined by HPLC (US HPLC-1210; JASCO, Tokyo, Japan), equipped with a refractive index detector, and a SH-1011 column (Shodex, Tokyo, Japan), at 50ºC. The mobile phase was 0.1% $HClO_4$,

at a flow rate of 1.0 ml/min. Acetate, butyrate and ABE solvents were determined using a gas chromatograph (6890A; Agilent Technologies, Palo Alto, CA, USA), equipped with a flame ionization detector, and a 15-m capillary column (INNOWAX; i.d. 0.53 mm; 19095N-121; Agilent Technologies), as described previously.[19] For the experiments using ^{13}C-labelled acetic acid, the supernatants were analysed by GC-MS (QP2010; Shimadzu, Kyoto, Japan). Acetone and butanol were separated on a 30-m capillary column (HP-INNOWAX; i.d. 0.25 mm; 19091 N-233; Agilent Technologies), with a split ratio of 25:1. The oven temperature program was 40ºC to 140ºC, at a rate of 16ºC/min, followed by 140ºC to 250ºC, at a rate of 45ºC/min. The injector temperature and ion source temperature were 250ºC and 200ºC, respectively. Acetic acid was separated on a DB-FFAP column (i.d. 0.25 mm; 122-3263; Agilent Technologies). The oven temperature program was 100ºC to 230ºC, at the rate of 10ºC/min. The injector temperature and ion source temperature were 250ºC and 230ºC, respectively. Helium was used as the carrier gas at a flow rate of 1.0 ml/min. The mass spectrometer was operated in the electron impact (EI) mode at 70 eV. Data were obtained by collecting the mass spectra within the scan range of 50-80 amu.

Calculations

The following equations were used to calculate the yield of butanol to carbon sources, and the yield of solvents to carbon sources, based on modifications of previously published equations.[20]

$$Y_{\text{butanol/carbon}} = (C_{\text{butanol}} \times 4) / (C_{\text{acetate}} \times 2 + C_{\text{glucose}} \times 6)$$

$$Y_{\text{solvents/carbon}} = (C_{\text{acetone}} \times 3 + C_{\text{butanol}} \times 4 + C_{\text{ethanol}} \times 2) / (C_{\text{acetate}} \times 2 + C_{\text{glucose}} \times 6)$$

Where $Y_{\text{butanol/carbon}}$ is the yield of butanol to carbon sources (C-mol/C-mol), $Y_{\text{solvents/carbon}}$ is the yield of solvents to carbon sources (C-mol/C-mol), C_{acetone} is the production of acetone (mM), C_{butanol} is the production of butanol (mM), C_{ethanol} is the production of ethanol (mM), and C_{acetate} and C_{glucose} are the utilisation of acetate (mM) and glucose (mM), respectively.

3. Results

Effect of added acetate on solvent production by *C. saccharoperbutylacetonicum* N1-4

To investigate the effect of acetate concentration on ABE fermentation by *C. saccharoperbutylacetonicum* N1-4, batch cultures (A0, A2, A4, and A6) were performed for 72 h in TY media, containing several concentrations of acetate (0, 2, 4, 6 g/L, respectively), and 50 g/L glucose. Although, in this experiment, GC analysis could not distinguish which source of acetate, exogenously added or intracellularly produced, was used for product formation, added acetate was apparently consumed, accompanying glucose consumption and butanol production (Fig. 1). As shown in Table 1, the apparent acetate consumptions increased from 0 (A0) to 1.66 g/L (A2), 3.36 g/L (A4), and 4.85 g/L (A6), with increasing added acetate concentrations (Table 1). On the other hand, A0 exhibited acetate production during acidogenesis (0–9 h), reaching a maximum concentration of 0.460 g/L, followed by complete reutilisation during solventogenesis. Butanol concentration, maximum butanol productivity, and total solvent concentration were increased in A2 (13.9 g/L, 0.749 g/L/h, 20.4 g/L), A4 (13.2 g/L, 0.755 g/L/h, 20.5 g/L) and A6 (12.7 g/L, 0.661 g/L/h, 22.1 g/L), compared with A0 (8.90 g/L, 0.443 g/L/h, 12.8 g/L). In addition, the acetone concentration was increased with increasing acetate utilisations; in particular, an acetone concentration of 8.38 g/L was produced in A6 (4.85 g/L consumed acetate), compared to 3.42 g/L in A0. However, while both butanol and solvent yields were greater in A2 and A4 than those in A0, A6 exhibited a lower butanol yield than A0, notwithstanding high acetate consumption. These data indicate that the consumption of exogenously added acetate resulted in significant improvements of, not only, the butanol concentration, production rate, and yield, but also the acetone concentration.

Effect of glucose concentration on butanol production and acetate utilisation

Regarding the metabolic pathway,[9] cofactors and energy carrying compounds, e.g., acetoacetyl-CoA, ATP

and NADH, supplied by glycolysis, appear to be essential for the reutilisation of acetate. To investigate the effect of glucose concentration on butanol production and acetate utilisation, batch cultures were performed for 72 h, in TY medium, containing various concentrations of glucose (0–80 g/L) and 4 g/L acetate. As shown in Table 2, acetate consumption and solvent production were not observed in the absence of glucose (G0), although the cell growth was slightly observed during the initial 3-h cultivation, maybe due to the intercellular nutrients (Fig. S1). However, in response to increasing the initial glucose concentration from G4 to G50, acetate consumption, glucose consumption and butanol levels gradually increased to 3.21 g/L, 45.4 g/L and 12.0 g/L in G50, compared with 1.06 g/L, 4.63 g/L, and 0.401 g/L with G4. However, these values were slightly decreased with further increases in glucose concentration, as shown for G60 (3.19 g/L, 44.7 g/L and 11.5 g/L) and G80 (3.14 g/L, 45.4 g/L and 10.8 g/L). Notably, there were no solvents produced within 36 h in G80 (Fig. S1), resulting from substrate inhibition of the high glucose concentration. These results demonstrated that glucose consumption should be necessary for conversion of acetate into butanol and acetone, in a similar manner as was previously described for conversion of butyric acid into butanol using either glucose[29] or syngas[30] to generate the necessary energy- (ATP) and reducing (NADH) equivalents. In consideration of these results, to investigate the metabolic flux of acetate conversion, sufficient cofactors from glycolysis need to be supplemented. Thus, 50 g/L glucose was considered to be appropriate for ABE fermentation in following experiments.

Verification of butanol and acetone production from added acetate using ^{13}C tracer experiments

To track the conversion of added acetate to butanol, and to distinguish the consumed acetate from acetate derived from glucose, batch cultures were carried out with supplementation of 4 g/L [1, 2-$^{13}C_2$] acetate and 50 g/L $^{12}C_6$-glucose as the substrates, and ^{13}C- labelled products were detected by GC-MS. Figure 2 shows

the mass spectra of differentially labelled butanol and acetone. When $^{12}C_4$-butanol was used as the standard, a peak corresponding to a molecular weight of 56.0 was observed, which indicated a mass shift of –18.0 because of the dehydration reaction of butanol (Fig. 2a). When [1, 2-$^{13}C_2$] acetate and $^{12}C_6$-glucose were used as substrates (Fig. 2b), GC-MS analysis of the culture broth showed three peaks for the butanol produced, with different relative intensities. Peaks 2 (m/z 58.0) and 3 (m/z 60.0) corresponded to dehydrated ^{13}C-butanol, in which 2 and all 4 carbons were substituted with ^{13}C-atoms derived from [1, 2-$^{13}C_2$] acetate, respectively. While peak 4 of $^{12}C_3$-acetone, corresponding to a molecular weight of 58.0 was observed in a standard solution (Fig. 2c), four peaks were observed for acetone produced using [1, 2-$^{13}C_2$] acetate and $^{12}C_6$-glucose as substrates (Fig. 2d). Peak 5 (m/z 59.0), peak 6 (m/z 60.0) and peak 7 (m/z 61.0) corresponded to^{13}C-acetone in which 1, 2 and all 3 carbons, were substituted with ^{13}C-atoms derived from [1, 2-$^{13}C_2$] acetate, respectively. Thus, we verified that added acetate could be converted to both butanol and acetone.

Metabolic analysis of added acetate conversion during ABE fermentation by ^{13}C tracer experiments

Based on the previously elucidated metabolic pathway of ABE-production by clostridia using GC-MS,[9] we illustrated the generation of intermediate metabolites resulting from [1,2-$^{13}C_2$] acetic acid input (Fig. 3). In addition, the percentages of fractional ^{13}C-labeled products (acetone and butanol) with different molecular weight, produced during the two phases of ABE fermentation are shown in Fig.3. During acidogenesis (at 9 h), four isotopes of acetone were formed, with 26.6% ($^{12}C_3$-acetone, m/z=58.0), 21.4% ($^{13}C_1$-acetone, m/z=59.0), 22.5% ($^{13}C_2$-acetone, m/z=60.0) and 29.4% ($^{13}C_3$-acetone, m/z=61). Moreover, three isotopes of butanol were generated with 30.0% ($^{12}C_4$-butanol, m/z=56.0), 43.8% ($^{13}C_2$-butanol, m/z=58.0) and 26.2% ($^{13}C_4$-butanol, m/z=60.0). In contrast, during solventogenesis (at 24 h), the percentages of ^{13}C-acetone and

185 ^{13}C-butanol molecules were all appreciably decreased, while $^{12}C_3$-acetone and $^{12}C_4$-butanol exhibited their
186 highest relative percentages, of 64.0% and 71.2% respectively. We also measured ^{12}C- and ^{13}C-acetate by
187 GC-MS in the culture broths after 9 h and 24 h of cultivation, and determined that 0.956 g/L and 3.04 g/L of
188 added ^{13}C-acetate were utilised, respectively, while 0.320 g/L and 0.175 g/L of glucose-derived ^{12}C-acetate
189 were observed, respectively (Fig. 3, Table 3). These results indicate that the N1-4 strain metabolised glucose
190 to acetate even during utilisation of added acetate, while there is no other enzymes related to acetate
191 formation in *C. saccharoperbutylacetonicum* besides PTA and AK in Fig. 3. In addition, after 9 h and 24 h of
192 cultivation, the actual ^{13}C-acetate consumption (0.956 g/L, 3.04 g/L, respectively) and butanol yield (0.230
193 C-mol/C-mol, 0.396 C-mol/C-mol, respectively), calculated from GC-MS data, exhibited similar values to
194 that of the apparent yields determined by GC alone, (0.636 g/L, 2.87 g/L, and 0.254 C-mol/C-mol, 0.403
195 C-mol/C-mol, respectively) (Table 3). Therefore, it was possible that the apparent butanol yield could be
196 used as an indicator of the efficiency of butanol production from acetate and glucose without using GC-MS.
197 Table 4 shows the carbon distribution of products, in terms of individual substrates or total consumed
198 substrates (^{12}C-glucose and ^{13}C-acetate), in batch cultures, with or without addition of ^{13}C-acetate. In the
199 presence of ^{13}C-acetate, high carbon distributions to butanol (61.6%) and acetone (36.6%), in terms of
200 ^{13}C-acetate, were obtained, even during acidogenesis (9 h), then slightly increased and decreased to 68.2%
201 and 27.9%, during solventogenesis (24 h), respectively, revealing that added acetate is mainly converted to
202 butanol and acetone by the N1-4 strain. In contrast, in terms of ^{12}C-glucose, at 9 h and 24 h, addition of
203 ^{13}C-acetate produced higher carbon distributions to butanol (19.5% and 37.3%) and acetone (7.64% and
204 11.6%), than observed without added acetate (butanol, 0.196% and 36.9%; acetone, 0 and 10.7%,
205 respectively). Moreover, similar results were observed in terms of the total substrates. These results
206 suggested that additional acetate induces the initiation of solvent production even during acidogenesis, and
207 may stimulate their productions from not only acetate but also glucose in both growth phases.

4. Discussion

As shown in Fig. 3, the metabolic pathway of ABE fermentation produces three organic acids, acetate, butyrate and lactate during acidogenesis, which are reutilised for solvent production during solventogenesis. In our previous studies using *C. saccharoperbutylacetonicum* N1-4, butanol concentration and yield were both found to be improved after supplementation with butyrate or lactate, and glucose as the primary substrate.[19,20] Further, we confirmed and established highly efficient butanol production systems from butyrate and lactate.[20,31] However, since there are no reports describing butanol production by *C. saccharoperbutylacetonicum* N1-4 using acetate as substrate, this study was aimed at investigating the effect of additional acetate and analysing the metabolism of the acetate using the N1-4 strain.

Previously, we proposed ^{13}C tracer experiments using [1, 2, 3-$^{13}C_3$] lactate and GC-MS analysis as a powerful approach to verify direct conversions of lactate to butanol, with supplementations of glucose[20] and arabinose.[21] This study, is the first to perform ^{13}C tracer experiments with exogenous [1, 2-$^{13}C_2$] acetate and $^{12}C_6$-glucose as substrates. As expected, three isotopes of butanol with different mass spectra ($^{12}C_4$-butanol, m/z=56.0; $^{13}C_2$-butanol, m/z=58.0; $^{13}C_4$-butanol, m/z=60.0) were detected in the culture broth (Figs. 2b and S2a). Similar to our previous studies on the addition of exogenous [1, 2, 3-$^{13}C_3$] lactate, [20,21] we suggest that three types of acetoacetyl-CoA ($^{12}C_4$-acetoacetyl-CoA, $^{13}C_2$-acetoacetyl-CoA, and $^{13}C_4$-acetoacetyl-CoA), are produced from $^{12}C_6$-glucose and [1, 2-$^{13}C_2$] acetate, which could be converted to butanol with different mass spectra (Fig. 3). Moreover, the conversion efficiencies of acetate to butanol were much higher in this study, 61.6% and 68.2% after 9-h and 24-h cultivation (Table 4), respectively, than those obtained by addition of lactic acid (52.6%[20]), possibly due to negligible carbon loss from the conversion of acetate to butanol (Fig. 3). Nevertheless, lactate was still considered as the more benefits co-substrate for butanol production than acetate, because conversion of 2 mol acetate to 1 mol butanol required 4 mol NADH from glycolysis, while

only 2 mol NADH was necessary for conversion of 2 mol lactate to 1 mol butanol (Fig. 3). Therefore, the proportion of required glucose per lactate should be significantly lower than per acetate. Correspondingly, the ratio of organic acids/glucose was higher for lactate/glucose (0.156 C-mol/C-mol[20]) than for acetate/glucose (0.0363–0.107 C-mol/C-mol as shown in Table S1). In addition, in this study we detected four isotopes of acetone with different mass spectra ($^{12}C_3$-acetone, m/z=58.0; $^{13}C_1$-acetone, m/z=59.0; $^{13}C_2$-acetone, m/z=60.0; $^{13}C_3$-acetone, m/z=61.0) by GC-MS analysis (Fig. 2d; Fig. S2b), consistent with the decarbonation of one molecule of ^{12}C- or ^{13}C-atom from three different isotopes of acetoacetate (Fig. 3). In addition, the apparent butanol yield to total carbon substrate, as determined by GC, showed similar values as those obtained using GC-MS (Table 3). Therefore, the butanol yield to total carbon substrate is suggested to be a useful parameter to evaluate the efficiency of the ABE fermentation process using exogenous acetate. To the best of our knowledge, this is the first report on the direct verification of the conversion of acetate to both butanol and acetone using ^{13}C tracer experiments.

Many researchers have reported on the influence of exogenous acetate on solvent production using several species of ABE-producing clostridia, especially *C. acetobutylicum* and *C. beijerinckii*. As shown in Table 5, exogenously added acetate increased the concentrations of acetone (54.2–153%), and total solvents (9.86–28.4%) with an increased ratio of acetone to butanol (A/B ratio) (33.7–57.2%) by *C. acetobutylicum* strains, while mainly butanol production was stimulated (18.9‑2217%), with a decreased A/B ratio, by added acetate for *C. beijerinckii* strains. In contrast, in this study, we evaluated the effect of exogenous acetate on solvent production by *C. saccharoperbutylacetonicum*, with 4 g/L added acetate, demonstrating enhanced acetone production (90.5%) to a greater extent than butanol (48.3%), resulting in an increased A/B ratio (27.1%) (Table 5). Although, some researches seem to show inconsistent results, additional acetate is confirmed to improve solvents production, specifically acetone, and to some extent butanol. In addition, the characteristics of solvent production by addition of acetate can be slightly different, depending on the ABE-producing

Clostridium species used, and on the specific experimental conditions.

In addition to verifying the direct conversion of acetate to butanol and acetone, we distinguished the solvents derived from acetate from that derived from glucose, using ^{13}C tracer experiments, using supplementation with [1, 2-$^{13}C_2$] acetate and $^{12}C_6$-glucose (Table 4). We quantitatively reveal the changing metabolic flux using ^{13}C tracer experiments, particularly, that 4 g/L of acetate addition stimulated solventogenesis during the early acidogenic growth phase, and increased metabolic flux to acetone and butanol productions, derived from not only acetate but also glucose, using the N1-4 strain (Table 4). In comparison, previous studies have reported on the metabolic analysis of the stimulation of butanol and acetone productions by added acetate with the gene expression analysis [22,23] and assays of metabolic enzyme activities.[25] From these studies, the mechanisms of solvent production stimulation have been presumed to be due to the enhanced expression level of the *sol* operon, containing *ald* or *aad*, *ctf*AB, and *adc*, and the increase in CoAT activity, using *C. beijerinckii*,[23] and *C. acetobutylicum*. [12,22] The *sol* operon of *C. saccharoperbutylacetonicum* N1-4 exhibits a high degree of similarity with that of *C. beijerinckii* NCIMB 8052 strain,[33] which suggested that a similar mechanism could be responsible for the effect of acetate addition on the stimulation of butanol and acetone production by *C. saccharoperbutylacetonicum* N1-4. However, different solvent production and A/B ratios obtained with different strains after addition of acetate are caused by different enzyme expression patterns. To validate our hypothesis, future research needs to be performed, such as molecular analysis and enzyme activity assays.

Our previous studies have indicated that co-factors and energy compounds, such as NADH, acetyl-CoA, and ATP, need to be supplied by the metabolism of sugars, for the conversions of organic acids, such as butyrate[19] and lactate[20], to butanol. Similarly, acetate has been fermented to butanol with co-utilisation of various sugars using all the ABE-producing clostridia so far studied (Table 5). As shown in Fig. 3, the uptake of acetate to acetyl-CoA has been considered to proceed via two independent routes; one is the reverse

pathways of acetic acid formation by phosphotransacetylase and acetate kinase reactions with the dephosphorylation of ATP, and another is via the CoAT reaction with exchange of an acetyl- vs. an acetoacetyl moiety of acetoacetyl-CoA and resulting in acetone production via acetoacetate. In comparison, butanol production from acetoacetyl-CoA requires four molecules of NADH, supplied by glycolysis, as a cofactor in the reactions of β-hydroxybutyryl dehydrogenase, butyryl-CoA dehydrogenase, butyraldehyde dehydrogenase and butanol dehydrogenase (Fig. 3). In this study, additional acetate could not be converted to butanol in the absence of glucose (Table 2; Fig. S1a), which suggests that the cofactors, acetyl-CoA and NADH, and the energy compound ATP, primarily supplied by glycolysis from glucose, are necessary for acetate uptake and butanol production, as described above.

As shown in Fig. 3, the theoretical maximum butanol yield from glucose only is 1 mol butanol per 1 mol glucose (66.7 % carbon recovery, 0.41 g butanol/g glucose, 0.667 C-mol/C-mol), according to the conversion: 1 glucose → 2 CO_2 + 1 butanol. Practically achieved butanol yields are usually below 0.3 g/g, since other by-products (acetone, ethanol, butyrate, acetate, lactate) are always formed. On the other hand, based on the pathways of acetate uptake (Fig. 3), acetate can be converted to butanol without carbon loss, which would result in much higher theoretical maximum yields of butanol from acetate (1.0 C-mol/C-mol) than that from glucose (0.667 C-mol/C-mol). Correspondingly, we found consumptions of acetate (1.66–3.36 g/L) slightly increased yields of butanol to carbon sources, from 0.430 C-mol/C-mol in the absence of acetate, to 0.445–0.457 C-mol/C-mol in the presence of acetate. However, the improvement of the butanol yield by consuming more acetate seems unlikely (4.85 g/L), because redox-balance has to be maintained in metabolisms. Theoretically, 4 mol NADH from glucose could only reduce 2 mol glucose-derived acetyl-CoA into butanol. Reduction of 2 mol external acetate-derived acetyl-CoA into butanol would divert NADH from reduction of glucose-derived acetyl-CoA. Therefore, glucose-derived acetyl-CoA could no longer be reduced to butanol, and would follow the pathway to acetone. According to equation of 1 glucose + 2 acetate → 3

300 CO_2 + 1 butanol + 1 acetone, butanol yield would significantly decrease with high amount of acetate addition
301 (up to ratio of 2 mol acetate per 1 mol glucose fed), thus, only small amount of acetate addition could
302 slightly stimulate butanol yield.
303

304 **5. Conclusion**

305 In conclusion, to our knowledge, this is the first report on the analysis of the conversion of exogenously
306 added acetate to butanol and acetone, using *C. saccharoperbutylacetonicum* N1-4 via ^{13}C-tracer experiments.
307 Furthermore, the acetate-to-butanol efficiency will significantly decrease when more acetate than 2–4 g/L is
308 added to the fermentation, while acetate-to-acetone efficiency may remain high (up to a ratio of 2 mol acetate
309 per 1 mol glucose fed). Butanol production can be improved via acetate addition by stimulating
310 solventogenesis during the early acidogenic growth phase, which only requires small amounts of acetate
311 (2–4 g/L). Moreover, acetate addition was found to shift the metabolic flux to acetone and butanol
312 production from both acetate and glucose. Thus, we successfully verified butanol production from acetate
313 and illustrated the metabolism of exogenous acetate in ABE fermentation.
314

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320

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Figure legends

Fig. 1 Time course of batch culture with and without added acetate (working volume, 300 ml). TY medium without added acetate (A0, a); 2 g/L acetate was supplied as co-carbon source (A2, b); 4 g/L acetate supplied as co-carbon source (A4, c); 6 g/L acetate supplied as co-carbon source (A6, d). Symbols for culture broth concentrations of glucose, *open squares*; acetate, *open triangles*; butanol, *closed circles*; acetone, *closed triangles;* pH, *dashed line*.

Fig. 2 Mass spectra of butanol and acetone by GC-MS analysis. (a) $^{12}C_4$-butanol solution standard. (b) Mass spectra of butanol in culture broth of the N1-4 strain with $^{12}C_6$-glucose and [1, 2-$^{13}C_2$] acetate for 24 h. Peak 1, 2 and 3, indicated by arrows, were derived from three isotopes of butanol: $^{12}C_4$-butanol, $^{13}C_2$-butanol in which two out of four carbons were replaced by ^{13}C-atom, and $^{13}C_4$-butanol, respectively. (c) $^{12}C_3$-acetone solution standard (d) Mass spectra of acetone in culture broth of the N1-4 strain with $^{12}C_6$-glucose and [1, 2-$^{13}C_2$] acetate for 24 h. Peaks 4, 5, 6 and 7, indicated by arrows, were derived from four isotopes of acetone: $^{12}C_3$-acetone, $^{13}C_1$-acetone in which one out of three carbons was replaced by ^{13}C-atom, $^{13}C_2$-acetone in which two out of three carbons were replaced by ^{13}C-atom and $^{13}C_3$-acetone, respectively.

Fig. 3 Metabolic pathways of fractional ^{13}C-labeled intermediate metabolites resulting from [1,2-$^{13}C_2$] acetate input using the N1-4 strain. Acetone and butanol molecules composed of different ^{12}C- and ^{13}C-atoms indicate the respective percentages (%) by GC-MS analysis. Each culture ($^{12}C_6$-glucose, 50 g/L; [1,2-$^{13}C_2$] acetate, 4 g/L; working volume, 10 ml) was performed three times, and the average is represented as the means ± standard deviation. Labeled ^{13}C-atoms, *closed circles*; ^{12}C-atoms, *open circles*; consumed carbon sources concentrations, *in boxes and red lettering*; product concentrations, *green lettering*. The left side

presents data at 9 h (acidogenesis); the right side presentes data at 24 h (solventogenesis). Enymes are abbreviated as follows: phosphotransacetylase (PTA); acetate kinase (AK); thiolase (THL); β-hydroxybutyryl dehydrogenase (BHBD); crotonase (CRO); butyryl-CoA dehydrogenase (BCD); CoA transferase (CoAT); acetoacetate decarboxylase (ADC); butyrate kinase (BK); phophotransbutyrylase (PTB); aldehyde/alcohol dehydrogenase (AAD); butanol dehydrogenase I (BDHA); butanol dehydrogenase II (BDHB). Putative mechanisms for acetate uptake: (a) reverse pathway of acetate production; (b) CoAT pathway.

Table 1 Kinetic parameters of batch cultures in TY medium containing exogenously added acetate

Additional acetate (g/L)	Maximum production (g/l)				Acetate consumption[a] (g/L)	$Y_{butanol}$[a] (C-mol/C-mol)	$Y_{solvents}$[a] (C-mol/-mol)	Maximum butanol production rate (g/L/h)
	Acetone	Ethanol	Butanol	Total solvents				
A0	3.42±0.09	0.552±0.108	8.90±0.02 (72 h)	12.8±0.1	0±0	0.430±0.004	0.604±0.002	0.443±0.029 (30 h)
A2	5.07±0.07	1.61±0.10	13.9±0.1 (48 h)	20.4±0.2	1.66±0.01	0.457±0.009	0.634±0.011	0.749±0.014 (18 h)
A4	6.67±0.16	1.17±0.05	13.2±0.1 (36 h)	20.5±0.2	3.36±0.02	0.445±0.005	0.638±0.013	0.755±0.063 (18 h)
A6	8.38±0.33	1.04±0.02	12.7±0.1 (36 h)	22.1±0.4	4.85±0.01	0.417±0.018	0.633±0.014	0.661±0.026 (18 h)

Each batch culture was performed three times, and the average was represented as means ± standard deviation. Batch cultures were performed by *C. saccharoperbutylacetonicum* N1-4 at 30°C for 72 h in TY medium containing 50 g/L glucose, without pH control (working volume, 300 ml).

[a] The parameters were calculated after a 72-h cultivation period.

Table 2 Effect of glucose concentration on butanol production and acetate utilization

Additional glucose (g/L)	Maximum production (g/l)				Acetate consumption[a] (g/L)	Glucose consumption[a] (g/L)	Y_{butaol}[a] (C-mol/C-mol)	$Y_{solvents}$[a] (C-mol/C-mol)	Maximum butanol production rate (g/L/h)
	Acetone	Ethanol	Butanol	Total solvents					
G0	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0
G4	0.444±0.035	0±0	0.401±0.060 (60 h)	0.845±0.056	1.06±0.06	4.63±0.42	0.114±0.027	0.235±0.030	0.0598±0.0022 (6 h)
G10	1.41±0.09	0±0	3.14±0.38 (60 h)	4.55±0.58	1.79±0.38	10.8±0.4	0.404±0.016	0.578±0.025	0.461±0.010 (12 h)
G20	2.87±0.18	0.305±0.108	5.87±0.66 (60 h)	9.04±1.03	2.73±0.66	20.3±1.9	0.413±0.009	0.600±0.021	0.620±0.024 (12 h)
G40	5.48±0.11	0.677±0.089	10.5±0.1 (60 h)	16.7±0.3	3.30±0.06	39.1±2.5	0.391±0.021	0.623±0.035	0.656±0.015 (18 h)
G50	5.80±0.05	0.896±0.274	12.0±0.2 (60 h)	18.7±0.6	3.21±0.04	45.4±0.2	0.400±0.003	0.609±0.003	0.592±0.007 (18 h)
G60	5.66±0.88	0.831±0.171	11.5± 0.5(60 h)	17.9±1.5	3.19±0.46	44.7±2.5	0.385±0.002	0.591±0.024	0.602±0.034 (24 h)
G80	5.80±0.03	0.774±0.08	10.8±0.1 (72 h)	17.4±0.2	3.14±0.12	45.4±0.1	0.361±0.003	0.567±0.005	0.373±0.013 (60 h)

Each batch culture was performed three times, and the average was represented as means ± standard deviation. Batch cultures were performed by *C. saccharoperbutylacetonicum* N1-4 at 30℃ for 72 h in TY medium containing 4 g/L acetate without pH control (working volume, 70 ml).

[a] The parameters were calculated after 72-h cultivation period.

Table 3 Comparison of apparent and actual parameters by distinguishing ^{12}C-acetate and ^{13}C-acetate

Fermentation time (h)	^{12}C-acetate generation (g/L)	^{13}C-acetate consumption (g/L)		^{12}C-glucose consumption (g/L)	$Y_{butanol}$ (C-mol/C-mol)	
		apparent (GC)[a]	actual (GC-MS)[b]		apparent (GC)[a]	actual (GC-MS)[b]
9	0.320	0.636	0.956	4.30	0.254	0.230
24	0.175	2.87	3.04	27.8	0.403	0.396

Batch cultures were performed by *C. saccharoperbutylacetonicum* N1-4 at 30°C for 9-h or 24-h cultivation in TY medium containing 50 g/L $^{12}C_6$-glucose and 4 g/L [1,2-$^{13}C_2$] acetic acid without pH control (working volume, 10 ml).

[a] Parameters were calculated using the detected value of acetate from GC-FID analysis, the apparent acetate concentration in broth. [b] parameters were calculated by using ^{13}C-acetate detected value from GC-MS, the actual additional exogenous acetate consumption, avoid the influence of ^{12}C-acetate generated from ^{12}C-glucose.

Table 4 Carbon distributions of products in terms of consumed ^{12}C-glucose and ^{13}C-acetate by N1-4 strain

Added ^{13}C-acetate	Time (h)	Substrate	Product (%)							Recovery (%)
			Acetone	Butanol	Ethanol	Lactate	^{12}C-acetate	Butyrate	CO_2	
Without	9	^{12}C-glucose	0	0.196	0	0	16.7	13.4	33.4	63.6
	24	^{12}C-glucose	10.7	36.9	0	0	2.45	0	36.9	86.9
With	9	^{13}C-acetate	36.6	61.6	0	0	0	N.D.[a]	12.2[b]	110
		^{12}C-glucose	7.64	19.5	0	10.8	7.44	N.D.	32.3[c]	77.7
		Total	12.9	23.0	0	8.88	6.12	17.3[d]	28.9	97.1
	24	^{13}C-acetate	27.9	68.2	N.D.	0	0	N.D.	9.88[b]	106
		^{12}C-glucose	11.6	37.3	N.D.	6.04	0.63	N.D.	35.1[c]	90.8
		Total	13.4	39.6	3.72[d]	5.46	0.57	0.38	32.8	96.0

Batch cultures were performed using *C. saccharoperbutylacetonicum* N1-4, at 30℃, for 9-h or 24-h cultivation, in TY medium containing 50 g/L $^{12}C_6$-glucose and 4 g/L [1,2-$^{13}C_2$] acetic acid, without pH control (working volume, 10 ml).

[a] Not detected by GC-MS. [b] $^{13}CO_2$ was formed by acetoacetate decarboxylation reaction, $M_{13CO2} = M(^{13}C_1\text{-acetone}) + M(^{13}C_3\text{-acetone})$. [c] $^{12}CO_2$ was calculated according to pathway of pyruvate decarboxylation and $^{12}C_3$-acetone, $^{13}C_2$-acetone formation, $M_{12CO2} = (2\times M_{glucose} - M_{lactate}) + M(^{12}C_3\text{-acetone}) + M(^{13}C_2\text{-acetone})$. [d] ^{12}C- and ^{13}C- in ethanol and butyrate were not distinguished by GC-MS, it was calculated as molecular weights were 46 g/mol and 88 g/mol, respectively.

Table 5. The data of solvents production with supplementing acetate in previous researches

Strains	Acetate addition	Co-substrates	Acetone (g/L)	Butanol (g/L)	Total solvents (g/L)	Acetone/Butanol (g/g)	Reference
C. acetobutylicum ATCC 824	None	65.8 g/L Glucose	4.80	15.0	21.3	0.320	Martin et al., 1983[12]
	2.0 g/L	66.6 g/L Glucose	7.40	14.7	23.4	0.503	
			(+54.2%)[a]	(–2.00%)[a]	(+9.86%)[a]	(+57.2%)[a]	
C. acetobutylicum 77	None	70 g/L Glucose	4.00	14.8	21.8	0.270	Matta et al., 1985[16]
	3.5 g/L[b]	67 g/L Glucose	10.1	15.1	28.0	0.361	
			(+153%)[a]	(+2.03%)[a]	(+28.4%)[a]	(+33.7%)[a]	
C. acetobutylicum EA 2018	None	60 g/L Cassava starch	2.60	9.90	15.4	0.263	Gu et al., 2009[22]
	30 mM[c]	60 g/L Cassava starch	5.10	13.0	19.4	0.392	
			(+96.2%)[a]	(+31.3%)[a]	(+26.0%)[a]	(+49.0%)[a]	
C. beijerinckii NCIMB 8052	None	60 g/L Glucose	0[d]	0.6	——[e]	0	Chen and Blaschek, 1999[23]
	60 mM	60 g/L Glucose	4.4[d]	13.9	——[e]	0.317	
				(+2217%)[a]			
C. beijerinckii BA 101	None	55 g/L Glucose	3.9[d]	13.2[d]	17.8	0.295	Ezeji, 2007[32]
	8.9 g/L	55 g/L Glucose	4.1	15.7	20.3	0.261	

			(+5.13%)[a]	(+18.9%)[a]	(+13.4%)[a]	(–11.5%)[a]	
C. beijerinckii TISTR 1461	None	60 g/L Glucose	4.84	7.98	13.07	0.607	Boonsombuti et al., 2013[14]
	80 mM[c]	60 g/L Glucose	5.21[d]	12.01	17.63	0.434	
			(+7.64%)[a]	(+50.5%)[a]	(+34.9%)[a]	(–28.5%)[a]	
C. saccharoperbutylacetonicum N1-4	None	50 g/L Glucose	3.42	8.90	12.8	0.384	This study
	4.0 g/L	50 g/L Glucose	6.67	13.2	20.5	0.488	
			(+90.5%)[a]	(+48.3%)[a]	(+60.2%)[a]	(+27.1%)[a]	

[a] The variation in percentage obtained by comparison with the value without acetate addition. [b] The maximum acetate concentration during cultivation. [c] Supplement with ammonium acetate. [d] The data were cited from the approximate values of figures in published papers. [e] Not mentioned.

Fig. 1

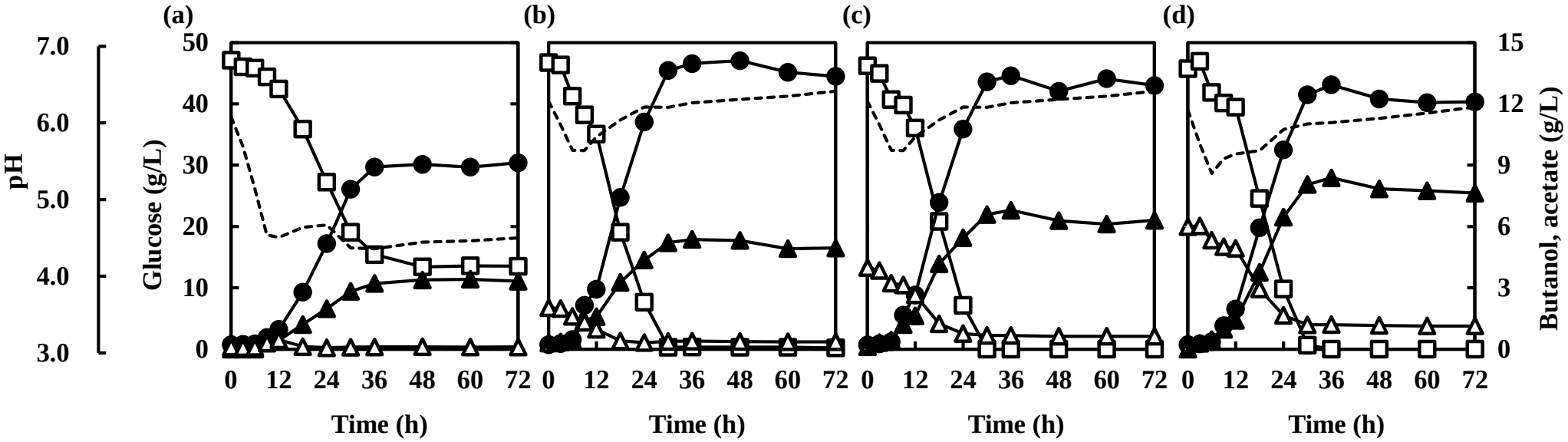

Fig. 2

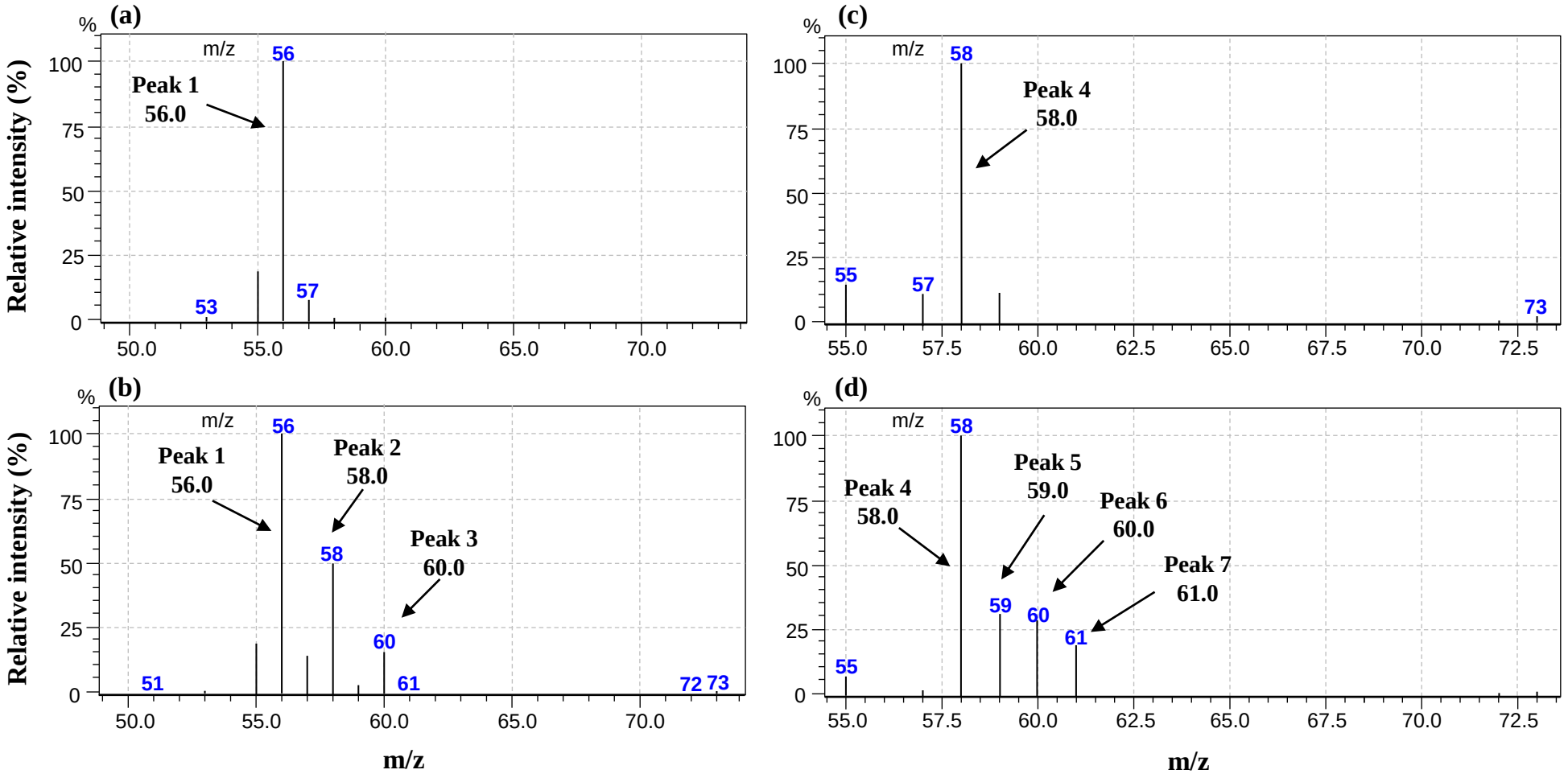

Fig. 3