Applied Biochemistry and Biotechnology (2023) 195:844–860
https://doi.org/10.1007/s12010-022-04174-2

ORIGINAL ARTICLE

L-Cys-Assisted Conversion of H_2/CO_2 to Biochemicals Using *Clostridium ljungdahlii*

Yuling Yang[1,2] · Weifeng Cao[2,3] · Fei Shen[2,3] · Zhiqian Liu[1] · Linli Qin[1] · Xinquan Liang[1] · Yinhua Wan[2,3]

Accepted: 28 September 2022 / Published online: 10 October 2022
© The Author(s), under exclusive licence to Springer Science+Business Media, LLC, part of Springer Nature 2022

Abstract

Carbon fixation and conversion based on *Clostridium ljungdahlii* have great potential for the sustainable production of biochemicals (i.e., 2,3-butanediol, acetic acid, and ethanol). Here, the effects of reducing agents on the production of biochemicals from H_2/CO_2 using *C. ljungdahlii* were studied. It was found that the element S and reducing power could significantly affect the production of biochemicals, and cysteine (Cys) was better than sodium sulfide for the production of biochemicals, especially for the production of 2,3-butanediol. Moreover, comparing to the control (i.e., without the addition of Cys), the gene expression profiles indicated that the *fdh* and a*dhE1* were significantly upregulated with the addition of Cys, which involved in pathways of the CO_2 fixation and ethanol production. Therefore, the irreplaceability of Cys on the production of biochemicals was both caused by its utilization as a reducing agent and its effect on the metabolic pathway. Finally, compared to the control, the production of 2,3-butanediol was increased by 2.17 times under the addition of 1.7 g/L Cys.

Keywords *Clostridium ljungdahlii* · Cysteine · Acetic Acid · 2, 3-Butanediol · Ethanol

Introduction

The utilization of syngas in biological processes is of interest, since they are often waste streams from large industries, such as the steel sector [1–3]. Usually, the syngas is composed of carbon monoxide (CO), carbon dioxide (CO_2), and hydrogen (H_2). As potential

✉ Weifeng Cao
wfcao@ipe.ac.cn

✉ Xinquan Liang
867862251@qq.com

1 College of Light Industry and Food Engineering, Guangxi University, Nanning 530004, China

2 State Key Laboratory of Biochemical Engineering, Institute of Process Engineering, Chinese Academy of Sciences, Beijing 100190, China

3 School of Chemical Engineering, University of Chinese Academy of Sciences, Beijing 100049, China

microbial catalysts for gas fermentation, several bacterial strains have been investigated in recent decades, such as *Clostridium ljungdahlii*, *C. autoethanogenum*, *C. carboxidivorans* P7, *C. ragsdalei*, *Butyribacterium methylotrophicum*, and *Eubacterium limosum* [3–10]. Both CO and H_2 can be utilized as energy sources during their growth, and they can be used to produce several products such as C2−C6 alcohols from syngas [11–13].

For the effective utilization of gases, many studies have been performed to enhance microbial growth and product formation by changing medium components and concentrations [8, 9, 14–18]. Meanwhile, a few studies have been conducted to reduce the cost of the medium by changing the buffer solution [19], replacing yeast extract with corn steep liquor, cotton seed extract, trypticase, and biochar [20–25]. In addition, agents, such as methyl viologen [17], neutral red [26], viologen dyes [27], and sodium sulfide [28, 29], were successfully used to improve the concentration of end-products. Previously, Panneerselvam et al. [17] studied the effects of various reducing agents on syngas fermentation by *C. ragsdalei*, and found that benzyl viologen caused cell death, and no ethanol production was detected; 0.1 mM methyl viologen promoted ethanol production compared to the control containing no reducing agent, while the addition of 0.2 or 0.3 mM methyl viologen delayed the production of ethanol compared with 0.1 mM methyl viologen; neutral red neither promoted nor detracted from ethanol production when compared to the control [17]. Oliveira et al. [10] found that a continuous sodium sulfide feed can increase ethanol production more than threefold by *C. ragsdalei*. Chandgude et al. [30] found that cysteine (Cys), ascorbic acid, and dithiothreitol had a different mechanism of action than conventional reducing agents such as viologens and neutral red. For example, Cys itself affects the distribution among the biochemicals, and thus affects the fermentation product distribution [31]. However, most of the initially added sulfur from Cys was stripped out within the first day of the batch process (first half of the exponential growth phase of *C. ragsdalei*) [10]. Meanwhile, it was also reported that cysteine was not a limiting factor for cell growth of *C. ljungdahlii* since its supplementation did not have a noticeable impact on product formation (i.e., acetic acid and ethanol) or overall gas consumption [32]. Bizarrely, in the culture medium for the production of alcohols from the syngas using *C. ljungdahlii*, a large amount of Cys or Cys-HCl was added [12, 20]. Thus, it need to further evaluate the effect of Cys on biochemical production using *C. ljungdahlii.*

C. ljungdahlii is capable of assimilating gaseous carbon sources such as pure CO or CO_2/H_2 [3, 12], and sugars such as fructose and sucrose [33, 34] for the production of biochemicals (i.e., ethanol, acetate, and 2,3-butanediol). Among the products, 2, 3-butanediol is a chemical platform used in several applications, such as polymers, cosmetics, fuels, and medicines [35, 36]. Less than 10% of current studies have focused on nutritional supplementation to improve 2, 3-butanediol production, and the redox balance achieved by reducing agents can affect the activity of key enzymes in 2, 3-butanediol metabolism [37]. However, the effect of Cys on the production of the biochemicals using *C. ljungdahlii* is still unclear. The addition of reducing agents has improved end-product formation in anaerobic fermentation processes [17, 30], where most enzymes preferentially utilize NADH as their cofactor [30, 38–40]. The reducing agents used can affect the parameters of redox potential (Orp), ATP, and cofactors, such as NADH. In turn, the affected parameters influence the expression of certain genes and activities of specific enzymes [41]. Recently, it was observed that the genome-wide transcriptional profile of gas-fermenting *C. ljungdahlii* differed significantly in the presence of sugars and C1 gases [42]. Therefore, we speculate first that the production of the biochemicals (i.e., ethanol, acetate, and 2,3-butanediol) may be improved with the addition of Cys. Furthermore, we conducted an in-depth investigation of the effect of L-Cys on the production of the biochemicals.

Materials and Methods

Microorganism, Media, and Cultivation Conditions

C. ljungdahlii DSM 13528 was purchased from the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) and conserved by freezing mid-exponential phase cultures at −80 °C with 20% glycerol for long-term storage. The modified DSMZ 879 medium with the following composition was used (per liter) [12, 33]: 1.0 g NH_4Cl, 0.1 g KCl, 0.2 g $MgSO_4·7 H_2O$, 0.8 g NaCl, 0.02 g $CaCl_2·2 H_2O$, 0.1 g KH_2PO_4, 2.5 mg $Na_2WO_4·2 H_2O$, 1.0 g $NaHCO_3$, 1.0 g Cys-HCl·H_2O, 1 g yeast extract, 5.0 g fructose, 0.5 g Cys, 0.5 mg resazurin, 10 mL trace element solution, and 10 mL vitamin solution. Trace element solution contains 2.0 g nitrilotriacetic acid, 1.3 g $MnCl_2·H_2O$, 0.4 g $FeSO_4·7 H_2O$, 0.2 g $CoCl_2·7 H_2O$, 0.2 g $ZnSO_4·7 H_2O$, 0.2 g $Na_2MoO_4·2 H_2O$, 0.02 g $NiCl_2·6 H_2O$, and 0.1 g $Na_2SeO_3·5 H_2O$ in 1 L distilled water. Vitamin solution per liter involves 2 mg biotin, 2 mg folic acid, 10 mg pyridoxine-HCl, 25 mg thiamine-HCl·2 H_2O, 5 mg riboflavin, 5 mg nicotinic acid, 5 mg D-Ca-pantothenate, 0.1 mg vitamin B_{12}, 5 mg ρ-aminobenzoic acid, and 5 mg lipoic acid in 1 L distilled water. The modified DSMZ 879 medium was used in all the fermentation experiments, and the medium was assembled in anaerobic chamber. With a constant pressure of 0.8 bar, the headspace of gas mixture was H_2: CO_2, 60:40 which was the same as reported in the literatures [12]. Analytical grade chemicals used in the medium were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). The medium was assembled in anaerobic chamber (Ruskinn technology Ltd., Sony Technology center, Pencoed, Bridgend Mid Glamorgan, UK). After autoclaving, $FeSO_4$, vitamins, Cys-HCl, and $NaHCO_3$ were added using syringe with a 0.2-μm filter. In all fermentation experiments, the seed culture of the strain DSM 13528 was inoculated by the 7.5 mL freezing mid-exponential phase cultures. Then, to obtain the seed culture, a 250-mL screw-cap bottle with a 75 mL working volume of modified DSMZ 879 medium was cultured at 37°C for 2 days in a rotary shaker (HYG-A, Taicang Experimental Equipment Factory, China) at 150 rpm. Batch fermentations were performed in 250-mL screw-cap bottles under the addition of 4 g/L $CaCO_3$ with a working volume of 75 mL. The gas in the headspace was substituted by the syngas as required with a pressure of 0.8 Bar. Then, for bioreactor culture, the seed culture broth (150 mL) was transferred to a 2.7 L bioreactor (BioFio®110, New Brunswick Scientific, San Francisco, USA) with a 1500 mL working volume. The temperature and stirring speed in the bioreactor were kept at 37°C and 200 rpm, respectively. Fermentation was carried out under the completely closed exhaust pipe case (i.e., no syngas was escaped from the bioreactor), and the syngas in the headspace of the bioreactor was kept at 0.8 Bar with the syngas that entered the bioreactor through a microflowmeter. Meanwhile, no matter the experiments were carried out in bioreactor or screw cap flasks, the syngas in the headspace was replaced every 1 day.

Analytical Methods

A total of 5 mL samples were withdrawn from the culture for cell density monitoring and products analysis. The concentrations of ethanol, acetic acid, and 2,3-butanediol were measured by a HPLC apparatus (LC-20AT, Shimadzu, Kyoto, Japan) equipped with an Aminex HPX-87H ion exclusion column and refractive index detector. The process was performed at a temperature of 50 °C, and a flow rate of 0.6 mL/min with 5 mmol/L

H_2SO_4 as the moving phase. The growth of *C. ljungdahlii* was monitored by using a UH5300 spectrophotometer (Hitachi high-tech science corporation, Tokyo, Japan) to measure the optical densities at 600 nm. In the screw-cap bottle conditions, the samples were analyzed after culturing for 7 days, and the excess $CaCO_3$ was removed by the addition of 1 M HCl before the measurement of biomass. In the bioreactor conditions, the samples were analyzed every 12 h. According to the genome analysis [33], genes involved in the ethanol production (*adhE1 and aor1*), acetic acid production (*ack*), and carbon fixation (*metF, fold* and *fdh*) were analyzed by Majorbio (Beijing, China). Analysis of genome-wide differential message RNA (mRNA) expression provides us with greater insights into biological pathways and molecular mechanisms that regulate cell fate and development. Cell pellets from cultures in the bioreactor were collected by centrifugation at 10,000×g under 4 °C for 10 min at 72 h, frozen in liquid nitrogen immediately, and stored at −80 °C. The mRNA isolation and high-throughput mRNA sequencing (RNA-Seq) were performed by Majorbio (Beijing, China). Total RNA was extracted using the TruSeqTM Stranded Total RNA Library Prep Kit (Ambion, Santa Clara, CA, USA) following the manufacturer's protocol. RNA integrity was evaluated using the Agilent 2100 Bio-analyzer (Agilent Technologies, Santa Clara, CA, USA). The samples with RNA Integrity Number (RIN) ≥ 7 were subjected to subsequent analysis. The libraries were constructed using the TruSeq Stranded mRNA LTSample Prep Kit (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. Then, these libraries were sequenced on the Illumina sequencing platform (HiSeqTM 2500) and 150-bp/125-bp paired-end reads were generated. Based on reads per kilobase of transcript per million mapped reads (RPKM) normalization, the gene expression profiles were analyzed. The differential genes were analyzed using Bioconductor edgeR (V3.4.6); information was from Clusters of Orthologous Groups (COG, https://www.ncbi.nlm.nih.gov/research/cog/api/cog/accessed on 5 January 2022) and Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.genome.jp/kegg/ accessed on 5 January 2022). Meanwhile, KEGG annotation results were derived from the KAAS (KEGG Automatic Annotation Server). Statistical analysis of the different experimental groups was conducted by subjecting the experimental data to one-way analysis of variance (ANOVA) using OriginPro 2018 software (Origin Lab Corporation, Northampton, MA, USA) at a 95% confidence level. The data presented in the figures are the average values with error bars.

Results and Discussion

The Effect of Cys and Sodium Sulfide on the Production of Biochemicals

In the modified DSMZ 879 medium, 1.0 g/L Cys-HCl×H_2O (i.e., 0.69 g/L Cys) and 0.5 g/L Cys were added [12, 33]. However, the effect of Cys on the production of biochemicals (i.e., 2,3-butanediol, ethanol, and acetic acid) is still unclear. Thus, to evaluate the effect of Cys on the production of the biochemicals, different concentrations of Cys were first supplemented at the beginning of fermentation in screw cap flasks. It should be indicated here that the concentration of Cys used in the modified DSMZ 879 medium was the sum of Cys from Cys-HCl×H_2O and Cys where the ratio of Cys-HCl×H_2O to Cys was 2:1 (w/w). As shown in Fig. 1a, without the addition of Cys, the final 2,3-butanediol production was 0.69 g/L with a yield of $Y_{P/X}$ (i.e., product (g)/dry cell weight (g)) =258.4. When the Cys concentration was below 0.24 g/L,

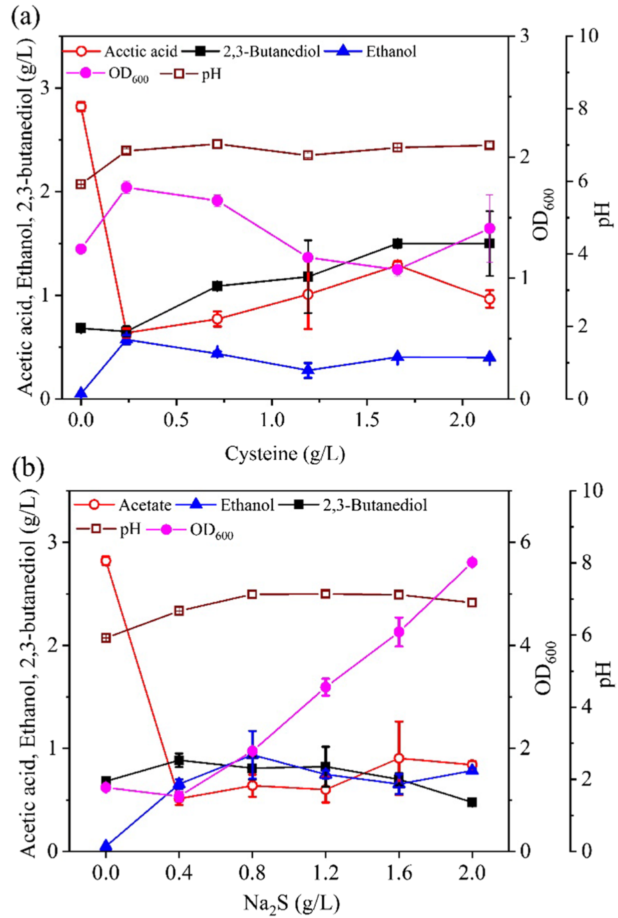

Fig. 1 The effect of cysteine (**a**) and sodium sulfide (**b**) on the production of biochemicals. The fermentations were carried out in 250-mL screw-cap bottles under 40% CO_2 plus 60% H_2. In (a), different concentrations of Cys were first supplemented at the beginning of fermentation. In (**b**), without the addition of Cys, different concentrations of Na_2S were first supplemented at the beginning of fermentation. Data are given as mean ± SD, $n = 3$

2,3-butanediol production showed little difference; when Cys concentration was beyond 0.24 g/L, 2,3-butanediol production increased with the enhanced concentration of Cys, and the maximal 2,3-butanediol concentration (1.5 g/L) with a yield of $Y_{P/X}$=694.4 was obtained under the addition of 1.7 g/L Cys which was increased 2.17 times compared to the no-addition of Cys case. For acetic acid production, when Cys concentration was below 0.24 g/L, acetic acid concentration decreased sharply from 2.82 to 0.64 g/L with the enhanced concentration of Cys; when Cys concentration was beyond 0.24 g/L, acetic acid production increased and the maximal acetic acid only reached 1.29 g/L under the addition of 1.7 g/L Cys, which was decreased by 54.3% compared to the no-addition of Cys case. For ethanol production, when Cys concentration was below 0.24 g/L, ethanol concentration increased sharply from 0.051 to 0.57 g/L with the enhanced concentration of Cys; when Cys concentration was between 0.24 and 1.2 g/L, ethanol production decreased; when Cys concentration was beyond 1.2 g/L, ethanol production remained almost constant. For cell growth, when the Cys concentration was below 0.24 g/L, cell growth increased with the enhanced concentration of Cys; then, after the Cys concentration was above 0.24 g/L, the cell concentration decreased. These results suggested that the Cys showed significant effects on the production of biochemicals from the syngas

H_2/CO_2. Similarly, it was reported that Cys itself affected the distribution among the biochemicals [31].

In addition, it was assumed that Cys was used as a reducing agent, and the production of the biochemicals responded to the different reducing values. To verify this assumption, the effect of another reducing agent, sodium sulfide (Na_2S) on the production of 2,3-butanediol was investigated. As shown in Fig. 1b, when the Na_2S concentration was below 0.4 g/L, 2,3-butanediol production increased slightly; when the Na_2S concentration was beyond 0.4 g/L, 2,3-butanediol production decreased with the enhanced concentration of Na_2S, and the maximal 2,3-butanediol concentration (0.89 g/L) was obtained under the addition of 0.4 g/L Na_2S. For the production of acetic acid, when the Na_2S concentration was below 0.4 g/L, acetic acid concentration decreased sharply from 2.82 g/L to 0.51 g/L with the enhanced concentration of Na_2S; when the Na_2S concentration was beyond 0.4 g/L, acetic acid production increased and the maximal acetic acid only reached 0.91 g/L under the addition of 1.6 g/L Na_2S. For ethanol production, when the Na_2S concentration was below 0.8 g/L, the ethanol concentration increased sharply from 0.051 to 0.94 g/L with the enhanced concentration of Cys; when the Na_2S concentration was beyond 0.8 g/L, ethanol production decreased. In addition, it should indicated that the excess $CaCO_3$ was removed by the addition of 1 M HCl before the measurement of biomass in screw-cap bottle culture, which resulted that the values of OD in Fig. 1b cannot really reflect the cell growth, since some black precipitated materials, such as FeS, were formed during the acidization. These results also suggested that Na_2S had significant effects on the production of the biochemicals from H_2/CO_2 syngas. In detail, the reducing power or element S can slightly promote the production of 2,3-butanediol and ethanol, while it decreased the production of acetic acid. Although Cys and Na_2S could both be used as reducing agents, their effects on the production of the biochemicals were significantly different, especially Cys was better than Na_2S for the production of 2,3-butanediol. Thus, besides Cys was used as a reducing agent, it may be used as a metabolic regulator or an extra carbon source. If Cys was used as an extra carbon source, the amino acids having the equal concentration of C may be used to replace the Cys. If the Cys was used as a S donor, the amino acids having the element S may be used to replace the Cys. If Cys was used as a metabolic regulator, the cysteine may be used to replace the Cys. However, we still cannot clearly identify these factors. Therefore, in the following sections, the effect of Cys on the production of the biochemicals will be further studied.

The Effect of the Elements in Cys on the Production of Biochemicals

To further verify the elements in Cys on the production of biochemicals, the effect of different amino acids on the production of biochemicals was investigated (Fig. 2a). The amino acids used included two categories: sulfur-containing amino acids (i.e., methionine, cystine, and Cys) and sulfur-free amino acids (i.e., glycine, phenylalanine and arginine). There was 29.46 mmol/L C from the amino acids used, which was the same concentration of C from 1.0 g/L Cys-HCl×H_2O and 0.5 g/L Cys in the modified DSMZ 879 medium. It should be indicated here that the Cys-HCl×H_2O and Cys were removed from the DSMZ 879 medium in this section (Fig. 2a). Under equal C and S from Cys and cystine (Cys-Cys), the concentration of 2,3-butanediol was 1.54 g/L and 1.51 g/L with a $Y_{P/X}$ of 523.8 and 474.8, respectively. Meanwhile, the 2,3-butanediol production was higher using Cys than that using other amino acids. However, compared to those using Cys-Cys, the concentration of acetic acid (i.e., 0.289 g/L with a $Y_{P/X}$ of 98.3) using Cys was decreased, while the

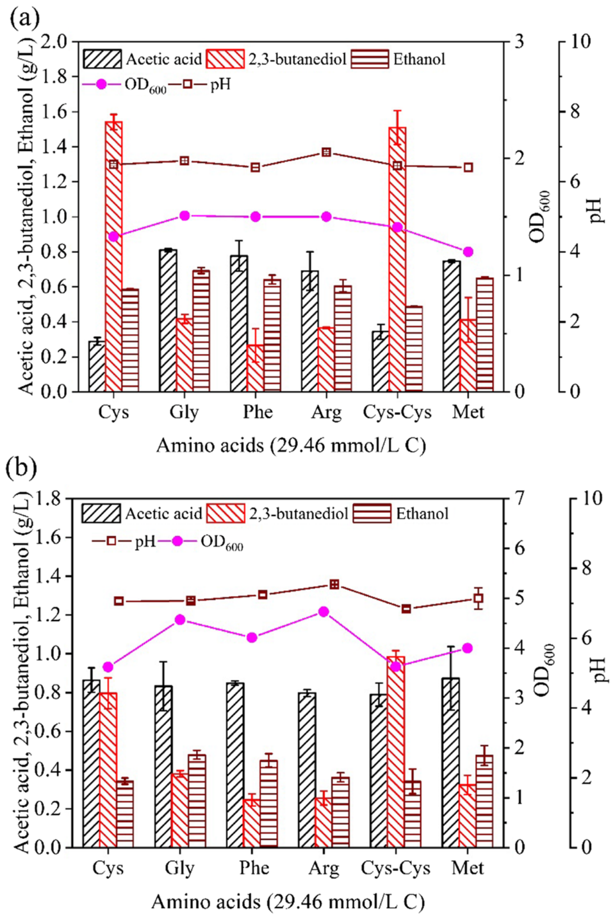

Fig. 2 The effect of the elements in cysteine on the biochemicals production. In (**a**), different amino acids were added without extra reducing agents. In (**b**), different amino acids were added under the equal Na_2S of 1.6 g/L. There was equal 29.46 mmol/L C of the different amino acids in (**a**) and (**b**). The fermentations were carried out in 250-mL screw-cap bottles under 40% CO_2 plus 60% H_2. Data are given as mean ± SD, $n = 3$

concentration of ethanol (i.e., 0.587 g/L with a $Y_{P/X}$ of 199.7) was enhanced. In addition, under the same C and the lower S in methionine (Met) compared to Cys or Cys-Cys, the concentration of 2,3-butanediol was 0.41 g/L with a $Y_{P/X}$ of 160.8 which was significantly decreased, while the productions of acetic acid and ethanol were enhanced. Moreover, compared to the addition of Cys, 2,3-butanediol production was significantly decreased, and the productions of acetic acid and ethanol were enhanced under the addition of sulfur-free amino acids (i.e., glycine, phenylalanine, and arginine). Furthermore, compared to methionine (Met) with less S, the productions of 2,3-butanediol, acetic acid, and ethanol showed no similar trends to those under Cys. For the cell growth, the concentrations of cell were a little higher under the addition of sulfur-free amino acids compared to those under the addition of sulfur-containing amino acids. In detail, the concentrations of cell were similar among the sulfur-containing amino acids, and between Cys-cys and Cys, while the Met gave the lowest concentration of cell. Thus, we can conclude that the different amino acids showed different effects on the production of biochemicals, and the Cys-Cys or Cys was appropriate to high 2,3-butanediol production; besides the reducing power introduced by Cys, the element S also participated in regulating metabolic processes for the production of biochemicals, which was similar to the results in Fig. 1a.

To further verify the element S in Cys on the production of the biochemicals, different amino acids were added in the initial fermentation process under the addition of 1.6 g/L Na_2S. As showed in Fig. 2b, the 2,3-butanediol production was higher using Cys-Cys or Cys than that using other amino acids, which was similar to that in Fig. 2a. Meanwhile, the 2,3-butanediol concentration was slightly higher using Cys-Cys than using Cys, which was different from that in Fig. 2a. However, for the addition of sulfur-free amino acids (i.e., glycine, phenylalanine, and arginine), the 2,3-butanediol concentration was not enhanced, as predicted, compared to those in Fig. 2a, although the S and reducing power were both introduced in the broth. This phenomenon was consistent with that in Fig. 1, which suggested that the element S from Na_2S could not be used to replace the element S from Cys for high 2,3-butanediol production, and the element S from Cys was more suitable for the production of 2,3-butanediol. Compared with the concentrations of ethanol in Fig. 2a, ethanol production decreased regardless of the addition of sulfur-free amino acids or sulfur-containing amino acids when Na_2S was introduced in the broth (Fig. 2b). However, acetic acid production showed the reverse trend to ethanol production under the same conditions (Fig. 2b). These results indicated that the metabolic process for the production of the biochemicals could be regulated by the element S regardless of the reducing power which was similar to those reported by Oliveira et al. [10]. Meanwhile, these results were different from those reported in Hu et al. [28], who found that an increased concentration of sulfide would lead to a more negative redox potential and consequently resulted in improved ethanol production. In view of the results in Figs. 1 and 2, it can be concluded that the Cys itself served as a metabolic regulator in the production of the biochemicals, which offered the element S, reducing power and extra C/H donor. In addition, the extra H in Cys may serve as an extra energy source in the production of the biochemicals since H_2 can be utilized as an energy source during the autotrophic growth of *C. ljungdahlii* [12]. Thus, the gas composition under the addition of Cys may affect the production of biochemicals, which will be evaluated in the following section.

The Effect of Gas Composition on the Production of Biochemicals

To investigate the gas composition on the production of the biochemicals, batch cultures were further performed in a 2.7-L bioreactor with a working volume of 1.5 L under the addition of 1.2 g/L Cys. When the syngas only contained CO_2 (Fig. 3a), the maximal concentrations of 2,3-butanediol, ethanol, and acetic acid were 3.16, 0.65, and 0.42 g/L with a $Y_{p/x}$ of 3427.0, 705.0, and 455.5, respectively. The cell grew to its maximal concentration at 60 h with an OD_{600} of 1.21, and then it began to decrease until the end of fermentation at 168 h with an OD_{600} of 0.66. When the syngas contained 40% CO_2 and 60% H_2 (Fig. 3b), the maximal concentrations of 2,3-butanediol, ethanol, and acetic acid were 2.57, 0.61, and 0.49 g/L with a $Y_{p/x}$ of 1404.3, 333.3, and 267.8, respectively. The cells grew to their maximal concentration at 60 h with an OD_{600} of 1.07, and then it remained approximately constant until the end of fermentation at 168 h. Compared with those in Fig. 3a, although the maximal concentrations of ethanol and acetic acid showed little difference between the two cases, the final concentrations of cell and acetic acid in Fig. 3b were increased by 53.1% and 45.5%, respectively. Compared with those in Fig. 3a, although the maximal concentrations of cells in Fig. 3b was decreased by 11.6%, the finial concentration of 2,3-butanediol was similar, while the final concentration of ethanol was decreased by 41.3%. Moreover, the variation trends in the whole fermentation process for the production of 2,3-butanediol, ethanol, and acetic acid were the same, that is, the productions of 2,3-butanediol and

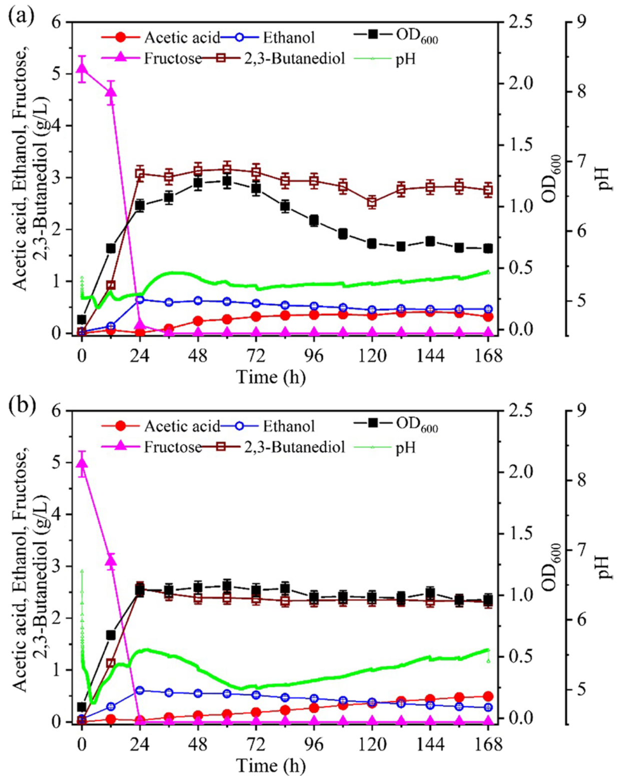

Fig. 3 The effect of gas composition on the biochemicals production. The time courses refer to 100% CO_2 (**a**) and 40% CO_2 plus 60% H_2 (**b**). The fermentations were carried out in a 2.7-L bioreactor. Data are given as mean ± SD, $n = 2$

ethanol decreased until the end of fermentation after reaching their maximal concentrations, while the production of acetic acid increased until the end of fermentation. Compared with the results in Fig. 3a, it can be concluded that the introduced H_2 in the syngas mainly decreased 2,3-butanediol production. In addition, it also indicated that the 2,3-butanediol was only produced during growth on fructose and not during growth on gaseous carbon sources. However, the concentrations of C and H were different between Fig. 3a and b. Thus, it needed to further study the effect of Cys on the production of 2,3-butanediol.

Moreover, without the addition of Cys and sodium sulfide (Fig. 4a), the maximal productions of 2,3-butanediol, ethanol, and acetic acid were 0.42, 0.72, and 1.17 g/L with a $Y_{p/x}$ of 276.3, 473.63, and 769.7, respectively. With the addition of sodium sulfide (Fig. 4b), the maximal productions of 2,3-butanediol, ethanol, and acetic acid were 0.36, 0.86, and 1.29 g/L with a $Y_{p/x}$ of 112.1, 267.9, and 401.9, respectively. With the addition of Cys (Fig. 4c), the maximal productions of 2,3-butanediol, ethanol, and acetic acid were 1.56, 0.64, and 0.81 g/L with a $Y_{p/x}$ of 559.1, 229.3, and 290.3, respectively. These results indicated again that the addition of Cys could enhance the production of 2,3-butanediol, which was similar to the results in Figs. 1 and 2. Moreover, the exogenous Cys slightly decreased the acetic acid production (Fig. 4b), which were similar to the results in Fig. 1a. However, the exogenous S or reducing power from the addition of Na_2S slightly increased the acetic acid production (Fig. 4c), which were different to the results in Fig. 1b. Under the addition of Cys, the above results in the bioreactor (Figs. 3 and 4) showed that the concentration of 2,3-butanediol and ethanol decreased after reaching their maximum

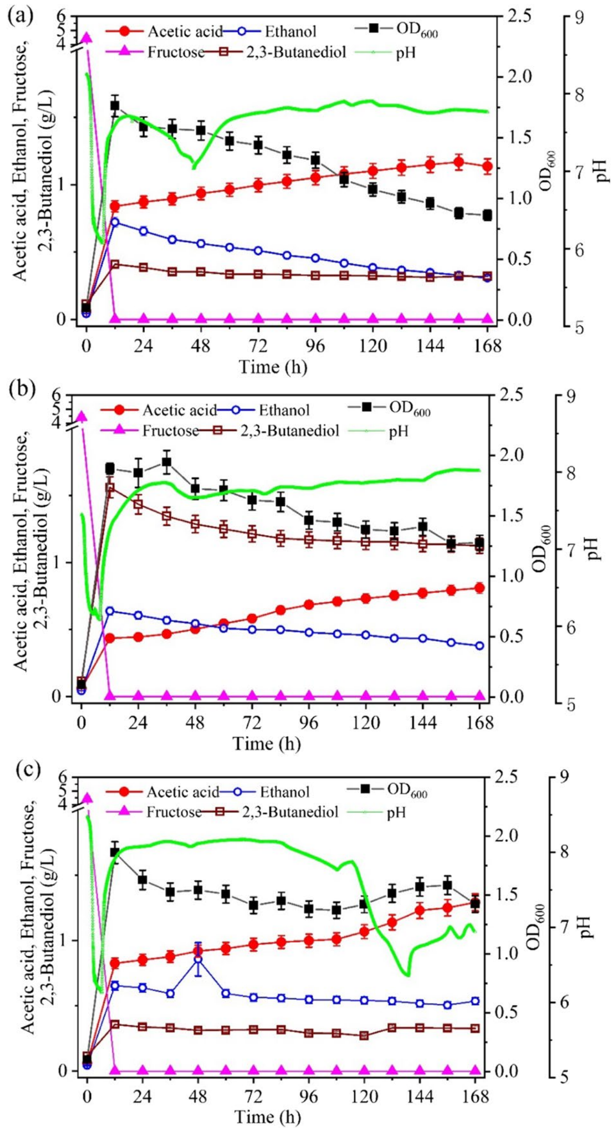

Fig. 4 The effect of cysteine and sodium sulfide on the biochemicals production in a 2.7-L bioreactor. The time courses refer to **a** without the addition of cysteine and sodium sulfide (i.e., Fig. 4a = control), **b** with the addition of 1.2 g/L cysteine (i.e., Fig. 4b =cys), and **c** with the addition of 1.6 g/L Na_2S (i.e., Fig. 4c = Na_2S). The fermentations were carried out in a 2.7 L bioreactor under 40% CO_2 plus 60% H_2. Data are given as mean ± SD, $n = 2$

concentration. However, as showed in Fig. 4, when the 2,3-butanediol concentration was higher, the produced 2,3-butanediol was much more easily assimilated by the strain DSM 13528 after the fructose was depleted. Thus, it was speculated that the three products themselves can be mutual transformation, which should be studied. Firstly, different concentrations of exogenous acetic acid were added in the initial fermentation at the same concentration of Cys (Fig. 5a) since acetate formation can produce ATP [12]. It was found that 2,3-butanediol production increased with the increasing acetic acid concentration,

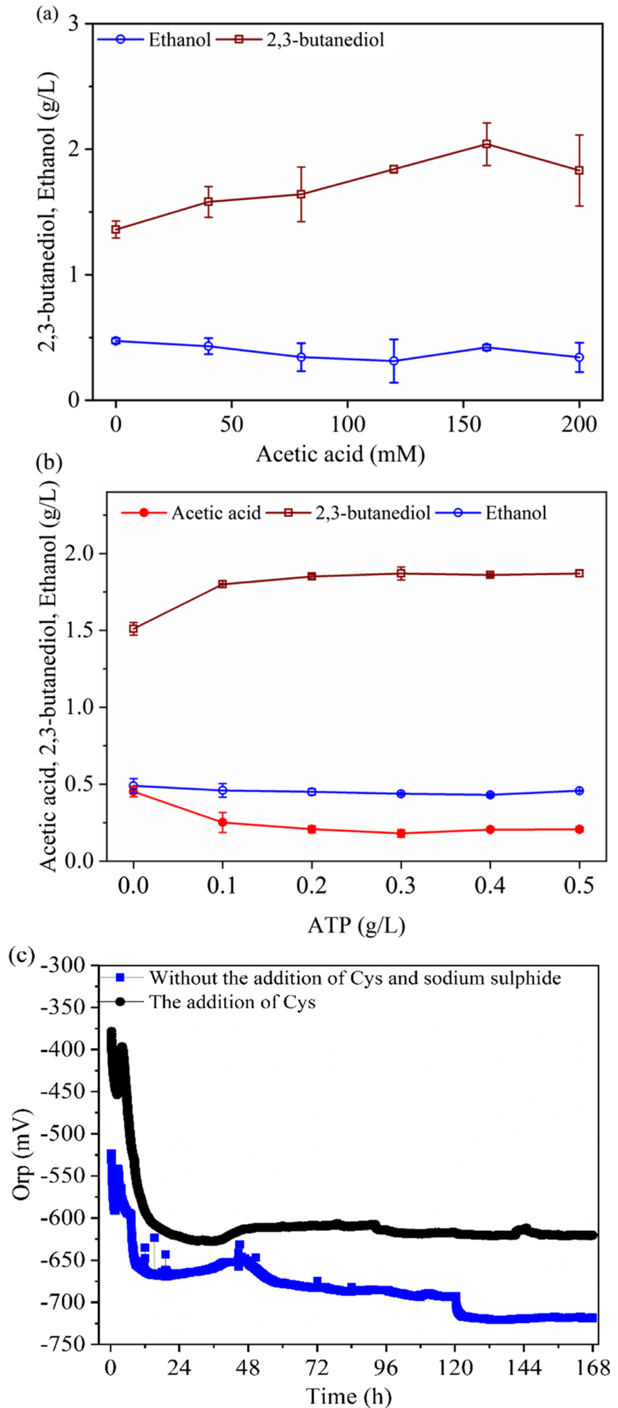

Fig. 5 The effect of acetic acid (**a**), ATP (**b**), and the Orp (**c**) on the biochemicals production. Batch fermentations for (**c**) were the same as those in Fig. 4a and b. In (**a**) or (**b**), the fermentations were carried out in 250-mL screw-cap bottles under 40% CO_2 plus 60% H_2. Data are given as mean ± SD, $n = 3$ in (**a**) or (**b**), and n=2 in (**c**)

while when the acetic acid concentration was beyond 160 mM, 2,3-butanediol production decreased. However, ethanol production showed the reverse trend to 2,3-butanediol production. In the whole fermentation process, a small amount of the added exogenous acetic acid was assimilated by strain DSM 13528. Thus, 2,3-butanediol can be synthesized from the added acetic acid (Fig. 4), which may be used as an additional carbon source or to produce ATP [12]. Secondly, the effect of exogenous ATP on the production of biochemicals was further evaluated (Fig. 5b). It was found that 2,3-butanediol production increased with increasing ATP concentration below 0.3 g/L ATP, while acetic acid production showed the reverse trend. When the ATP concentration was beyond 0.3 g/L, 2,3-butanediol production was not further increased, and the acetic acid concentration was also not further decreased. In addition, when 0.3 g/L ATP was supplied, the intracellular concentration of ATP showed no much difference compared to the case without the addition of ATP. Thus, a higher concentration of ATP can inhibit the acetic acid production (Fig. 5b), which will increase the 2,3-butanediol production at the same concentration of carbon source (i.e., CO_2). Moreover, 2,3-butanediol formation requires NADPH as a cofactor during gas fermentation of *C. ljungdahlii* [12, 33]. Thus, it indicated that the reducing power and S donator were only two factors of Cys on 2,3-butanediol production, which were also verified by the results in the bioreactor (Figs. 3 and 4). Moreover, the genes of *C. ljungdahlii* response to the addition of Cys should be further evaluated.

Unraveling the Effect of Cys on Production of Biochemicals

A high concentration of 2,3-butanediol can be produced under the addition of Cys (Fig. 4b) compared with the case without the addition of Cys (Fig. 4a). Then, the Orp between the two cases was further investigated (Fig. 5c). It was found that Orp was higher under the addition of Cys, since the biosynthesis process of 2,3-butanediol can consume NADPH. Thus, we proposed that the reducing power introduced in the broth by the added Cys was not the reason for the high production of 2,3-butanediol. To verify this hypothesis, sodium sulfide was used to replace some Cys (Fig. 6). It was found that 2,3-butanediol production decreased when Cys was replaced by sodium sulfide. At the same concentration of Cys, the 2,3-butanediol production decreased when the concentration of sodium sulfide was increased. Previously, the addition of Cys into the hydrogen production system accelerated the formation of ethanol-type fermentation and enhanced the hydrogen production by creating an optimal low Orp environment, and by increasing the biomass growth simultaneously [43]. The plausible provision of more electrons into the culture in the presence of Cys-HCl enhanced ethanol production (48%) and the ethanol to acetate production ratio (24%) compared to the cells cultivated in standard growth medium recommended by ATCC [44]. When sodium sulfide was added to the broth during syngas fermentation using *Clostridial* bacteria denoted as P11, the increased concentration of sulfide led to a more negative redox potential and consequently resulted in improved ethanol production [28]. However, 2,3-butanediol production showed different responses to Cys and sodium sulfide, and Cys could not be replaced by some sodium sulfide (Fig. 6). Thus, the reducing power introduced in the broth by the added Cys was not the sole reason for the high production of 2,3-butanediol, and we proposed that the Cys shifted the metabolic pathways toward the production of 2,3-butanediol was another main reason.

Furthermore, according to the genome analysis [33, 45], genes involved in the ethanol production (*adhE1 and aor1*), acetic acid production (ack), carbon fixation (*metF*, *fold*,

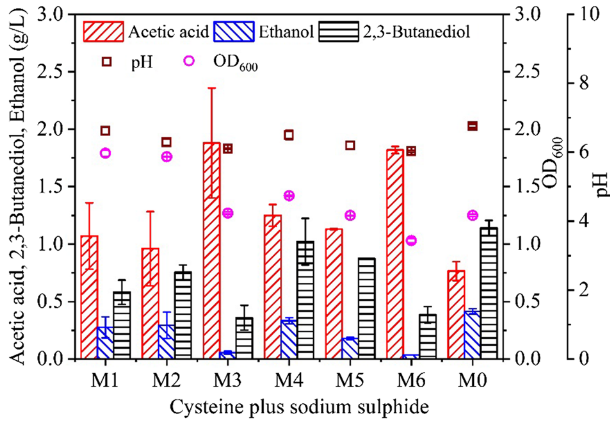

Fig. 6 The effect of cysteine plus sodium sulfide on the biochemical production. The M0 means that 1.2 g/L cysteine (i.e., 1.0 g/L cysteine-HCl ×H_2O and 0.5 g/L cysteine) was added in the fermentation medium. The M1, M2, and M3 means that 0.75, 0.5, and 0.25 g/L cysteine were added in the fermentation medium under the addition of 1.6 g/L sodium sulfide, respectively. The M4, M5, and M6 means that 0.75, 0.5, and 0.25 g/L cysteine were added in the fermentation medium under the addition of 0.4 g/L sodium sulfide, respectively. The fermentations were carried out in 250-mL screw-cap bottles under 40% CO_2 plus 60% H_2. Data are given as mean ± SD, n=3

and *fdh*), and acetoin reductase were further analyzed at 72 h (Table 1), since the fermentation processes were more similar between the control (i.e., no-addition of Cys and sodium sulfide) and the case with the addition of Cys at 72 h (Fig. 4). The gene expression of *fdh* that is involved in the methyl sub-unit synthesis from CO_2 had significant alternation during the fermentation of H_2/CO_2 after the fructose was depleted. As major enzymes involved in the same gene cluster for the acetate synthesis pathway, *ack* was only slightly down-regulated. However, the ethanol production-related gene *dhE1* were significantly upregulated, and the acetoin reductase-related gene showed no much difference. Therefore, the irreplaceability of Cys on the production of 2,3-butanediol was both caused by its utilization as a reducing agent and its effect on the metabolic pathway flux.

Conclusion

In this work, the element S and reducing power all could significantly affect the production of the biochemicals, and Cys was better than sodium sulfide for the production of 2,3-butanediol. Compared to the no-addition of Cys case, the 2,3-butanediol production was enhanced 2.17 times under the addition of 1.7 g/L Cys. Meanwhile, the gene expression profiles indicated that the *fdh* and *dhE1* were significantly upregulated under the addition of Cys case, which involved in pathways of the CO_2 fixation and ethanol production. Therefore, the irreplaceability of Cys on the production of biochemicals was both caused by its utilization as a reducing agent and its effect on the metabolic pathway.

Table 1 Gene expression profiles during the fermentation with H_2 and CO_2

Gene ID	Log2FC	*p* value	Regulation[a]	Description[b]	Significant
CLJU_c12780	−0.636642127167	3.61×10^{-248}	Down	Acetate kinase (ack)	No
CLJU_c07030	2.15562681877	2.01×10^{-168}	Up	NADH dehydrogenase subunit E	Yes
CLJU_c16520	0	1	no change	Alcohol dehydrogenase (a*dhE2*)	No
CLJU_c16510	2.87975420399	0	Up	Aldehyde dehydrogenase (*adhE1*)	Yes
CLJU_c20110	−1.03042222618	1.15×10^{-25}	Down	Aldehyde oxidoreductase (*aor1*)	Yes
CLJU_c06990	4.63204964177	2.16×10^{-53}	Up	Formate dehydrogenase (fdh)	Yes
CLJU_c37630	−0.98695225997	9.79×10^{-66}	Down	Methylene-tetrahydrofolate dehydrogenase (*folD*)	No
CLJU_c37610	0.0641955632423	0.65969396072	Up	Methylene-tetrahydrofolate reductase (*metF*)	No
CLJU_c23220	−0.736408317	0.000120581	Down	Acetoin reductase	No

[a]The genes from no-addition of cysteine and sodium sulfide were used as the control. The differential genes were between no-addition of cysteine and sodium sulfide and the addition of 1.2 g/L cysteine

[b]The fermentations were carried out in a 7.5-L bioreactor under 40% CO_2 plus 60% H_2

Author Contribution YY carried out the experiment and analyzed data. WC (Weifeng Cao) conceived and designed research. FS contributed new reagents or analytical tools. QL and YW conducted experiments. WC and YY wrote the manuscript. All authors read and approved the manuscript.

Funding The authors received financial support from the Beijing Natural Science Foundation, China (No. 5182025); the Fundamental Research Funds for the Public Research Institutes of Chinese Academy of Inspection and Quarantine (No. 2020JK004); the National Natural Science Foundation of China, China (No. 21406240); and the National High Technology Research and Development Program of China (Nos. 2015AA021002 and 2014AA021005) .

Data Availability The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics Approval Not applicable.

Consent to Participate Not applicable.

Consent for Publication All authors consent to publish the manuscript.

Conflict of Interest The authors declare no competing interests.

References

1. Allwood, J. M., Cullen, R. M., & Milford, R. L. (2010). Options for achieving a 50% cut in industrial carbon emissions by 2050. *Environmental Science & Technology, 44*, 1888–1894.
2. Salehizadeh, H., Yan, N., & Farnood, R. (2020). Recent advances in microbial CO_2 fixation and conversion to value-added products. *Chemical Engineering Journal, 390*, 124584.
3. Zhang, L., Zhao, R., Jia, D., Jiang, W., & Gu, Y. (2020). Engineering *Clostridium ljungdahlii* as the gas-fermenting cell factory for the production of biofuels and biochemicals. *Current Opinion in Chemical Biology, 59*, 54–61.
4. Abrini, J., Naveau, H., & Nyns, E. J. (1994). *Clostridium autoethanogenum* , sp. nov., an anaerobic bacterium that produces ethanol from carbon monoxide. *Archives of Microbiology, 161*, 345–351.
5. Chang, I. S., Kim, B. H., Lovitt, R. W., & Bang, J. S. (2001). Effect of CO partial pressure on cell-recycled continuous CO fermentation by *Eubacterium limosum* KIST612. *Process Biochemistry, 37*, 411–421.
6. Henstra, A. M., Sipma, J., & Rinzema, A. J. M. (2007). Microbiology of synthesis gas fermentation for biofuel production. *Current Opinion in Biotechnology, 18*, 200–206.
7. Kundiyana, D. K., Wilkins, M. R., Maddipati, P., & Huhnke, R. L. (2011). Effect of temperature, pH and buffer presence on ethanol production from synthesis gas by *Clostridium ragsdalei*. *Bioresource Technology, 102*, 5794–5799.
8. Shen, Y., Brown, R. C., & Wen, Z. (2017). Syngas fermentation by *Clostridium carboxidivorans* P7 in a horizontal rotating packed bed biofilm reactor with enhanced ethanol production. *Applied Energy, 187*, 585–594.
9. Li, D., Meng, C., Wu, G., Xie, B., Han, Y., Guo, Y., Song, C., Gao, Z., & Huang, Z. (2018). Effects of zinc on the production of alcohol by *Clostridium carboxidivorans* P7 using model syngas. *Journal of Industrial Microbiology & Biotechnology, 45*, 61–69.
10. Oliveira, L., Rhrenbach, S., Holzmüller, V., & Weuster-Botz, D. (2022). Continuous sulfide supply enhanced autotrophic production of alcohols with *Clostridium ragsdalei*. *Bioresources and Bioprocessing, 9*, 1–13.
11. Phillips, J. R., Atiyeh, H. K., Tanner, R. S., Torres, J. R., Saxena, J., Wilkins, M. R., & Huhnke, R. L. (2015). Butanol and hexanol production in *Clostridium carboxidivorans* syngas fermentation: Medium development and culture techniques. *Bioresource Technology, 190*, 114–121.

12. Zhu, H. F., Liu, Z. Y., Zho, X., Yi, J. H., Lun, Z. M., Wang, S. N., & Tang, W. Z. (2020). Energy conservation and carbon flux distribution during fermentation of CO or H_2/CO_2 by *Clostridium ljungdahlii*. *Frontiers in Microbiology, 11*, 416.
13. Diender, M., Stams, A., & Sousa, D. Z. (2016). Production of medium-chain fatty acids and higher alcohols by a synthetic co-culture grown on carbon monoxide or syngas. *Biotechnology for Biofuels, 9*, 82.
14. Guo, Y., Xu, J., Zhang, Y., Xu, H., Yuan, Z., & Li, D. (2010). Medium optimization for ethanol production with Clostridium autoethanogenum with carbon monoxide as sole carbon source. *Bioresource Technology, 101*, 8784–8789.
15. Saxena, J., & Tanner, R. S. (2011). Effect of trace metals on ethanol production from synthesis gas by the ethanologenic acetogen, *Clostridium ragsdalei*. *Journal of Industrial Microbiology & Biotechnology, 38*, 513–521.
16. Saxena, J., & Tanner, R. S. (2012). Optimization of a corn steep medium for production of ethanol from synthesis gas fermentation by *Clostridium ragsdalei*. *World Journal of Microbiology & Biotechnology, 28*, 1553–1561.
17. Panneerselvam, A., Wilkins, M. R., Delorme, M. J. M., Atiyeh, H. K., & Huhnke, R. L. (2010). Effects of various reducing agents on syngas fermentation by *Clostridium ragsdalei*. *Biological Engineering, 2*, 135–144.
18. Mann, M., Munch, G., Regestein, L., & Rehmann, L. (2020). Cultivation strategies of *Clostridium autoethanogenum* on xylose and carbon monoxide combination. *ACS Sustainable Chemistry & Engineering, 8*, 2632–2639.
19. Jie, G., Atiyeh, H. K., Phillips, J. R., Wilkins, M. R., & Huhnke, R. L. (2013). Development of low cost medium for ethanol production from syngas by *Clostridium ragsdalei*. *Bioresource Technology, 147*, 508–515.
20. Thi, H. N., Park, S., Li, H., & Kim, Y. K. (2020). Medium compositions for the improvement of productivity in syngas fermentation with *Clostridium autoethanogenum*. *Biotechnology and Bioprocess Engineering, 25*, 493–501.
21. Sun, X., Atiyeh, H. K., Kumar, A., & Zhang, H. (2018). Enhanced ethanol production by *Clostridium ragsdalei* from syngas by incorporating biochar in the fermentation medium. *Bioresource Technology, 247*, 291–301.
22. Cotter, J. L., Chinn, M. S., & Grun, A. M. (2009). Ethanol and acetate production by *Clostridium ljungdahlii* and *Clostridium autoethanogenum* using resting cells. *Bioprocess & Biosystems Engineering, 32*, 369–380.
23. Kundiyana, D. K., Huhnke, R. L., Maddipati, P., Atiyeh, H. K., & Wilkins, M. R. (2010). Feasibility of incorporating cotton seed extract in *Clostridium* strain P11 fermentation medium during synthesis gas fermentation. *Bioresource Technology, 101*, 9673–9680.
24. Maddipati, P., Atiyeh, H. K., Bellmer, D. D., & Huhnke, R. L. (2011). Ethanol production from syngas by *Clostridium* strain P11 using corn steep liquor as a nutrient replacement to yeast extract. *Bioresource technology, 102*, 6494–6501.
25. Sun, X., Atiyeh, H. K., Kumar, A., Zhang, H., & Tanner, R. S. (2018). Biochar enhanced ethanol and butanol production by *Clostridium carboxidivorans* from syngas. *Bioresource Technology, 265*, 128–138.
26. He, A. Y., Yin, C. Y., Xu, H., Kong, X. P., Xue, J. W., Zhu, J., Jiang, M., & Wu, H. (2016). Enhanced butanol production in a microbial electrolysis cell by *Clostridium beijerinckii* IB4. *Bioprocess and Biosystems Engineering, 39*, 245–254.
27. Du, Y., Jiang, W., Yu, M., Tang, I. C., & Yang, S. T. (2015). Metabolic process engineering of *Clostridium tyrobutyricum* Δack–adhE2 for enhanced n-butanol production from glucose: Effects of methyl viologen on NADH availability, flux distribution, and fermentation kinetics. *Biotechnology & Bioengineering, 112*, 705–715.
28. Hu, P., Jacobsen, L. T., Horton, J. G., & Lewis, R. S. (2010). Sulfide assessment in bioreactors with gas replacement. *Biochemical Engineering Journal, 49*, 429–434.
29. Cao, W. F., Luo, J. Q., Zhao, J., Qiao, C. S., Ding, L. H., Qi, B. K., Su, Y., & Wan, Y. H. (2012). Intensification of β-poly(L-malic acid) production by *Aureobasidium pullulans* ipe-1 in the late exponential growth phase. *Journal of Industrial Microbiology & Biotechnology, 39*, 1073–1080.
30. Chandgude, V., Välisalmi, T., Linnekoski, J., Granström, T., Pratto, B., Eerikäinen, T., Jurgens, G., & Bankar, S. (2021). Reducing agents assisted fed-batch fermentation to enhance ABE yields. *Energy Conversion and Management, 227*, 113627.
31. Trachootham, D., Lu, W., Ogasawara, M., Nilsa, R. D., & Huang, P. (2008). Redox regulation of cell survival. *Antioxidants & redox signaling, 10*, 1343–1374.

32. Infantes, A., Kugel, M., & Neumann, A. (2020). Evaluation of media components and process parameters in a sensitive and robust fed-batch syngas fermentation system with *Clostridium ljungdahlii*. *Fermentation, 6*, 61.
33. Xie, B. T., Liu, Z. Y., Tian, L., Li, F. L., & Chen, X. H. (2015). Physiological response of *Clostridium ljungdahlii* DSM 13528 of ethanol production under different fermentation conditions. *Bioresource technology, 177*, 302–307.
34. Yang, Y., Deng, T., Cao, W., Shen, F., Liu, S., Zhang, J., Liang, X., & Wan, Y. (2022). Effectively converting cane molasses into 2,3-butanediol using Clostridium ljungdahlii by an integrated fermentation and membrane separation process. *Molecules, 27*, 954.
35. Kim, D., Yoo, S., Kim, M., Ko, J., Um, Y., & Oh, M. (2020). Improved 2,3-butanediol yield and productivity from lignocellulose biomass hydrolysate in metabolically engineered *Enterobacter aerogenes*. *Bioresource Technology, 309*, 123386.
36. Ji, X. J., He, H., & Ouyang, P. K. (2011). Microbial 2,3-butanediol production: a state-of-the-art review. *Biotechnology Advances, 29*, 351–364.
37. Tinco, D., Borschiver, S., Coutinho, P. L., & Freire, D. (2020). Technological development of the bio-based 2,3-butanediol process. *Biofuels, Bioproducts and Biorefining, 2*, 1–20.
38. Park, J., & Choi, Y. (2017). Cofactor engineering in cyanobacteria to overcome imbalance between NADPH and NADH: A mini review. *Frontiers of Chemical Science and Engineering, 11*, 66–71.
39. Meng, H., Liu, P., Sun, H., Cai, Z., Zhou, J., Lin, J., & Li, Y. (2016). Engineering a d-lactate dehydrogenase that can super-efficiently utilize NADPH and NADH as cofactors. *Scientific Reports, 6*, 24887.
40. Han, S., Gao, X. Y., Ying, H. J., & Zhou, C. (2016). NADH gene manipulation for advancing bioelectricity in *Clostridium ljungdahlii* microbial fuel cells. *Green Chemistry, 18*, 2473.
41. Liu, C. G., Qin, J. C., & Lin, Y. H. (2017). Fermentation and redox potential. *Fermentation Processes, License In tech, Chapter, 2*, 23–41.
42. Aklujkar, M., Leang, C., Shrestha, P. M., Shrestha, M., & Lovley, D. R. (2017). Transcriptomic profiles of *Clostridium ljungdahlii* during lithotrophic growth with syngas or H_2 and CO_2 compared to organotrophic growth with fructose. *Scientific Reports, 7, 13135*.
43. Qu, Y. Y., Guo, W. Q., Ding, J., & Ren, N. Q. (2012). Effect of l-cysteine on continuous fermentative hydrogen production. *Applied Mechanics & Materials, 178-181*, 406–410.
44. Mohammadi, M., Mohamed, A., Najafpour, G., Younesi, H., & Uzir, M. (2016). *Clostridium ljungdahlii* for production of biofuel from synthesis gas. *Energy Sources, Part A: recovery, utilization, and environmental Effects, 38*, 1–8.
45. Tan, Y., Liu, Z. Y., Liu, Z., & Li, F. L. (2015). Characterization of an acetoin reductase/2,3-butanediol dehydrogenase from *Clostridium ljungdahlii* DSM 13528. *Enzyme and Microbial Technology, 79-80*, 1–7.

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Springer Nature or its licensor holds exclusive rights to this article under a publishing agreement with the author(s) or other rightsholder(s); author self-archiving of the accepted manuscript version of this article is solely governed by the terms of such publishing agreement and applicable law.