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Optimization of CO fermentation by *Clostridium carboxidivorans* in batch reactors: Effects of the medium composition

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ABSTRACT

Objectives: The objective of this study was to investigate the effects of medium composition on CO fermentation by *Clostridium carboxidivorans*. The focus was to reduce the medium cost preserving acceptable levels of solvent production.
Methods: Yeast extract (YE) concentration was set in the range of 0–3 g/L. Different reducing agents were investigated, including cysteine-HCl 0.6 g/L, pure cysteine 0.6 g/L, sodium sulphide (Na2S) 0.6 g/L, cysteine-sodium sulphide 0.6 g/L and cysteine-sodium sulphide 0.72 g/L. The concentration of the metal solution was decreased down to 25 % of the standard value. Fermentation tests were also carried out with and without tungsten or selenium.
Results: The results demonstrated that under optimized conditions, namely yeast extract (YE) concentration set at 1 g/L, pure cysteine as the reducing agent and trace metal concentration reduced to 75 % of the standard value, reasonable solvent production was achieved in less than 150 h. Under these operating conditions, the production levels were found to be 1.39 g/L of ethanol and 0.27 g/L of butanol. Furthermore, the study revealed that selenium was not necessary for *C. carboxidivorans* fermentation, whereas the presence of tungsten played a crucial role in both cell growth and solvent production.
Conclusions: The optimization of the medium composition in CO fermentation by *Clostridium carboxidivorans* is crucial for cost-effective solvent production. Tuning the yeast extract (YE) concentration, using pure cysteine as the reducing agent and reducing trace metal concentration contribute to reasonable solvent production within a relatively short fermentation period. Tungsten is essential for cell growth and solvent production, while selenium is not required.

1. Introduction

Given the increasing global population and decreasing resources, as well as the environmental consequences of burning fossil fuels, it is imperative to discover alternative sources for fuels and chemicals. Our society's reliance on fossil fuels is evident, with the majority of everyday materials and primary energy originating from them. However, the fermentation of synthesis gas (syngas) by acetogenic bacteria offers a renewable and environmentally friendly option for producing low-carbon fuels and chemicals, and is gaining increasing attention [1].

Syngas is primarily composed of CO, H_2 and CO_2, and it is commonly produced from the gasification of biomass. This is advantageous when compared to the fermentation of sugar feedstocks derived from biomass, as it allows for access to the lignin fraction. Carboxydotrophic and homoacetogenic bacteria, such as *Clostridium carboxidivorans*, are capable of utilizing the carbon and electrons from syngas for growth, thanks to their unique Wood-Ljungdahl pathway for carbon fixation. The primary end-products of their metabolism are acetic acid and ethanol, although other products, such as butyrate or butanol, have also been observed [2]. With the increasing relevance of these microorganisms, understanding the fermentation process and their product formation profile is of significant interest [3].

Low cell density is a current issue in anaerobic bioprocesses, which can reduce productivity [4]. Media used for CO/CO_2 fermentation is typically similar to sugar-based media for *Clostridium* sp. growth and butanol production, with expensive components such as essential metals, vitamins, minerals, and nitrogen. This results in intensive labour

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Nomenclature

AA	acetic acid concentration (mg/L)
B	butanol concentration (mg/L)
BA	butyric acid concentration (mg/L)
CO	concentration of CO in the liquid phase (mg/L)
E	ethanol concentration (mg/L)
HA	hexanoic acid concentration (mg/L)
P_{CO}	CO partial pressure (atm)
Cys	cysteine (g/L)
Na_2S	sodium sulphide (g/L)
YE	yeast extract (g/L)
V_G	gas volume in serum bottles (mL)
V_L	liquid volume in serum bottles (mL)
X	cell concentration (g_{DM}/L)
$Y_{i/CO}$	CO-to-product "i" yield coefficient (g/g)
μ	specific cell growth rate (h^{-1})
μ_{max}	maximum specific cell growth rate (h^{-1})
ξ_{CO}	CO conversion degree (−)

and high costs. The challenge is to replace these compounds with cheaper and more complex sources. ATCC® medium 2713 and ATCC® medium 1754 (without fructose) are commonly used for *C. carboxidivorans* activation and growth in syngas fermentation. However, low specific growth rates ranging from 0.005 to 0.08 h^{-1} have been reported for these media using gaseous or soluble substrates [5–8]. Studies have been conducted to evaluate the effect of the carbon source on cell growth and solvent production using glucose-rich medium or only carbon monoxide as the carbon source, with maximum values for cell density and specific growth rate of 0.55 g dry weight of cells/L and 0.231 h^{-1}, respectively [5–7,9–11]. Additionally, the effects of trace metals on product formation have been evaluated, with maximum ethanol production reported at 3.5 g/L after 72 h of fermentation [12]; [13–16]. *C. carboxidivorans* strain P7 has been found to be greatly affected by trace metals in terms of cell growth and metabolite synthesis. These metals play a critical role as essential cofactors for metalloenzymes involved in both the WLP and alcohol synthesis pathways [17]. Additionally, they are known to regulate gene expression, leading to the reprogramming of the intracellular metabolic network in solvent-producing clostridia and acetogens [18]. However, there is a lack of research regarding the effects of combined trace metals on gene expression regulation during C1 gas fermentation [19].

The composition of the medium can affect the biomass growth, gas utilization, and product formation of anaerobic bioprocesses [20]; [21]. In a proteome analysis, Richter et al. [22] discovered that the sulphur assimilation pathway in *C. ljungdahlii* lacked the genes for sulfate reduction. Therefore, they suggested substituting sulfate with sulfide or cysteine in the syngas fermentation medium. Indeed, a medium without sulfate but with cysteine as sulphur source has been demonstrated to enhance the growth of *C. autoethanogenum, C. ljungdahlii*, and *C, ragsdalei* [23]. The yeast extract (YE) is an essential component of the fermentation medium too. Indeed, syngas-fermenting microorganisms do not growth without YE [24]. Nevertheless, it has been shown that decreasing YE concentration can result in a transition towards ethanol in some cases [24,25]. As a matter of fact, a depth investigation of the role of the medium components in syngas fermentation is worth to note [21].

This paper focuses on investigating the impact of different medium components (yeast extract, reducing agents and trace metals) and their concentrations on both cell growth and production of acids/solvents by *C. carboxidivorans*. Although there are several studies that have explored the effects of medium composition on solvent production, many of them focus solely on optimizing the production of solvents while neglecting the impact on cell growth (or vice versa). This study, on the other hand, takes into account both aspects and provides a comprehensive analysis of the effects of various components and their concentrations on both cell growth and solvent production. In particular, the study focuses on the impact of yeast extract, reducing agents, and trace metals on the fermentation process, since they are crucial factors that influence the growth and metabolism of microorganisms. Therefore, this study contributes to the existing literature by providing a more complete understanding of the relationship between medium composition, cell growth, and solvent production, which can lead to more effective strategies for optimizing the CO fermentation process by *C. carboxidivorans*.

2. Materials and methods

2.1. Microorganism and culture media

The microorganism used for this work is *Clostridium carboxidivorans* DSM 15243, it was supplied by Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) in the form of dried pellets. Reactivation and storage of the microorganism were carried out following the protocol reported in Lanzillo et al. [1].

Tests were carried out in biological triplicate to support reproducibility. The reported results are the mean values. The composition of the standard fermentation medium was [9]: yeast extract 1 g/L; mineral solution 25 mL/L, trace metal solution 10 mL/L, vitamin solution 10 mL/L, resazurin1 mL/L. A spectrum of reducing agents was investigated at several concentrations.

The composition of the mineral stock solution was: NaCl 80 g/L, NH_4Cl 100 g/L, KCl 10 g/L, KH_2PO_4 10 g/L, $MgSO_4$ 20 g/L, $CaCl_2$ 4 g/L.

The composition of the trace metal solution was: $C_6H_9NO_6$ 2 g/L, MnO_4S H2O 1 g/L, $H_8FeN_2O_8S_2$ H_2O 0.8 g/L, Cl_2Cu $2H_2O$ 0.2 g/L, O_4SZn $7H_2O$ 0.02 g/L, Cl_2Co $6H_2O$ 0.02 g/L, Cl_2Ni $6H_2O$ 0.02 g/L, $MoNa_2O_4$ $2H_2O$ 0.02 g/L, Na_2O_3Se 0.02 g/L, Na_2O_4W 0.02 g/L.

The composition of the vitamin solution was: pyridoxine 0.1 g/L, thiamine 0.05 g/L, riboflavin 0.05 g/L, calcium pantothenate 0.05 g/L, lipoic acid 0.05 g/L, para-amino-benzoic acid 0.05 g/L, nicotinic acid 0.05 g/L, vitamin B12 0.05 g/L, biotin D 0.02 g/L, folic acid 0.05 g/L, 2 mercaptoethane-sulphonic acid 0.05 g/L.

The investigated yeast extract concentrations were: 0 g/L; 0.5 g/L; 1 g/L; 3 g/L. Trace metal (TM) composition was tuned by reducing the fraction of trace metal stock solution supplemented to the medium: the amount was reduced down to 25 %. Some tests were carried out using the 100 % TM stock solution without selenium (Se) or tungsten (W). The spectrum of reducing agents and concentrations was: cysteine-HCl 0.6 g/L (Cys-HCl), pure cysteine 0.6 g/L (Cys), sodium sulphide 0.6 g/L (Na_2S), cysteine-sodium sulphide 0.6 g/L and cysteine-sodium sulphide 0.72 g/L (Cys-Na_2S).

2.2. Batch tests

Fermentation tests were carried out in 250 mL serum bottles. A preset volume of medium was loaded in bottles and boiled. N_2 stream was sparged into the bottles during the cooling down of the medium. The reducing agent was supplemented to the bottles at medium temperature lower than 40 °C. The pH was adjusted at 5.75 with NaOH or HCl. The bottles were sealed with Viton stoppers, capped with aluminium crimps, and autoclaved for 20 min at 121 °C. Bottles were: kept under anaerobic conditions; pressurized with 100 % CO. As inoculated, the bottles were kept under agitation on an orbital shaker operated at 130 rpm and housed in an incubation chamber at 35 °C. The initial culture medium was set at 55 mL (gas-liquid volume ratio: 0.28). The CO pressure in the headspace was set at 1.7 atm.

2.3. Analytical methods

Fermentation analysis was carried out using the protocol reported in Lanzillo et al. [1]. Small (compared to the gas and liquid volume in the

reactor) samples of gas and liquid were regularly collected from the reactor for analysis using the diagnostic methods described below.

pH was measured off-line by a pH-meter (Hanna Instruments). The optical density (ODλ) was measured at 600 nm by using a UV–visible spectrophotometer (SPECORD 50 UV-VIS, Analytik Jena). The concentration of water-soluble metabolites - acetic acid, butyric acid, hexanoic acid, ethanol, butanol, hexanol – was measured by using an HPLC (HP1100, Agilent Co.) equipped with Rezex ™ ROA-Organic Acid H+ column (8 %), 150 × 7.8 mm and a UV detector at a wavelength of 284 nm, at room temperature. The mobile phase was 7 mM H_2SO_4 solution fed at 0.8 mL/min flow rate. Gas-phase concentration of CO and CO_2 were measured by a gas chromatograph (GC, HP 6890 Agilent Technologies) equipped with a thermal conductivity detector (TCD). The GC was fitted with a 15-mHP-PLOT Molecular Sieve 5A column (ID, 0.53 mm; film thickness, 50 μm). The pressure in the fermentation bottles was measured using a pressure manometer (Keller) before and after each gas sampling.

2.4. Data processing

Data measured during the fermentation tests were processed to assess a pool of fermentation indicators.

The CO to ethanol yield ($Y_{E/CO}$), the CO to butanol yield ($Y_{B/CO}$), and the CO conversion degree (ξ_{CO}) were calculated according to the Eq. s (1)–(3).

$$Y_{E/CO} = \frac{V_L * E_f}{V_G * (CO_i - CO_f)} \quad (1)$$

$$Y_{B/CO} = \frac{V_L * B_f}{V_G (CO_i - CO_f)} \quad (2)$$

$$\xi_{CO} = \frac{CO_i - CO_f}{CO_i} \quad (3)$$

where E_f, B_f are the are the final concentrations of ethanol and butanol in the liquid phase, resepctively, CO_i and CO_f the initial and final concentrations of carbon monoxide in the gas phase, V_L and V_G the liquid and gas volumes.

The specific cell growth rate (μ) was estimated at the beginning of the exponential phase as the slope of the biomass concentration (X) vs. time curve, on a semilog scale.

3. Results & discussion

The objective of this study was to evaluate the impact of three factors on cell growth and metabolite production, namely: i) the concentration of yeast extract, ii) the type and concentration of reducing agent, and iii) the composition and concentration of the metal solution. Fermentation tests were characterized in terms of maximum concentration of cells (X), ethanol (E), butanol (B), acetic acid (AA), butyric acid (BA), and hexanoic acid (HA). CO to ethanol yield (YE/CO), CO conversion degree (ξ_{CO}), and specific cell growth rate (μ) were assessed according to the procedure reported in section 2.4 and detailed in Lanzillo et al. [1]. No data were reported on hexanol because its production was negligible. Data reported in tables are the mean values among repeated (biological triplicate) tests.

3.1. Effect of yeast extract

This section presents the evaluation of how the growth of *C. carboxidivorans* and the production of metabolites (concentration and production rate) are affected by YE. The aim of the test campaign was to determine the minimum concentration of YE needed for satisfactory fermentation performance. Table 1 shows the key results of the tests conducted, while Table 2 presents a comprehensive summary of studies from the scientific literature investigating the impact of varying concentrations of YE on the fermentation performance of *Clostridia* strains; in particular, the ratio of the final concentration of solvents (ethanol + butanol) to the initial concentration of YE ($Y_{totS/YE}$) was chosen as a critical indicator of the incidence of the YE on the overall process costs. However, the complete evaluation of the cost incidence must also consider the recovery and concentration of the solvents, which is a function of their final concentration (out of the scope of this work).

YE is an indispensable component of the medium for supporting the growth of syngas-fermenting microorganisms and the effect of its concentration is strain dependent. The outcomes of fermentation tests provide clear evidence that YE is indispensable for the growth of *Clostridium* species, as no observable growth occurred without YE [24]. The cell concentration and the maximum specific cell growth rate did not significantly change with the YE concentration when tuned between 0.5 and 3.0 g/L. They were about 0.50 g_{DM}/L and 0.11 h^{-1}, respectively (Table 1). The analysis of Tables 1 and 2 pointed out the effects of the concentrations of YE on the fermentation process. An increase in the YE concentration led to an improvement in acid production. The literature suggests that YE is required during the initial CO fermentation phase of *C. carboxidivorans* to promote rapid cell growth [24]. San-Valero et al. [28] - an investigation of the effect of YE concentration on the growth of *C. kluyveri* and the production of acids and solvents - found that the supplement of YE to the medium plays a key role in the fermentation process, enhancing biomass growth and chain elongation of secreted metabolites. Results reported in the literature and in the present study support the idea that increasing the concentration of YE could promote rapid cell growth at the beginning of the CO fermentation process, improve acid production (related to the cell growth stage). As a consequence, the CO fermentation time reduces and gas pumping costs reduce too. This is because YE provides the necessary nutrients for the bacteria to grow rapidly and produce more acids, while the reduction in CO fermentation phase saves gas pumping costs. As a further effect of the YE, solvent production was characterized by a non-monotonic trend with the concentration of YE. Indeed, results reported in the present study points out that: i) the increase in YE concentration from 0.5 to 1 g/L caused a slight increase in solvent production and yield; ii) the increase in YE concentration from 1 to 3 g/L caused a decrease in solvent production and yield, which is consistent with previous observations of CO-bioconversion investigations [24]. One plausible explanation is that the bacteria utilized YE as a source of both nutrients and energy for cell growth, potentially leading to the inhibition of ethanol production. It is worth to remember that *C. ljungdahlii* is characterized by an opposite behaviour: the reduction of YE concentration resulted in an enhancement of ethanol production in *C. ljungdahlii* [24]. Thi et al. [27] and Abubackar et al. [25] carried out studies on the effect of YE on the fermentation performances of *C. autoethanogenum* and *C. carboxidivorans*, respectively. They pointed out that the maximum cell

Table 1
Data from the fermentation tests carried out at different YE concentration. Batch fermenters: 250 mL bottles. Gas-liquid volume ratio: 0.28. $P_{CO} = 1.7$ atm. TM 100 %. Cell concentration at inoculation 0.02 g_{DM}/L (g_{DM} indicates grams of biomass referred to dry mass).

YE g/L	X g_{DM}/L	AA mg/L	BA mg/L	HA mg/L	E mg/L	B mg/L	$Y_{E/CO}$ g/g	$Y_{B/CO}$ g/g	ξ_{CO} %	μ h^{-1}
0.5	0.50 ± 0.01	1170 ± 6	158 ± 5	16 ± 1	825 ± 7	158 ± 2	$0.14 \pm 9 \times 10^{-4}$	$0.027 \pm 7 \times 10^{-4}$	78.7 ± 0.5	$0.114 \pm 5.5 \times 10^{-4}$
1.0	0.51 ± 0.01	1450 ± 20	140 ± 3	35 ± 2	898 ± 5	168 ± 1	$0.20 \pm 1.5 \times 10^{-3}$	$0.034 \pm 6 \times 10^{-4}$	78.6 ± 0.5	$0.117 \pm 1.6 \times 10^{-3}$
3.0	$0.52 \pm 1.4 \times 10^{-2}$	1720 ± 30	285 ± 4	65 ± 1	322 ± 7	145 ± 1	$0.067 \pm 1.5 \times 10^{-3}$	$0.031 \pm 1.3 \times 10^{-4}$	65.0 ± 0.6	$0.102 \pm 1.8 \times 10^{-3}$

Table 2
Effects of YE on the performances of *Clostridia* strains.

Strain	Reactor	Operating conditions	Yeast extract	Growth	Alcohols	Acids	$Y_{totS/YE}$ (g_S/g_{YE})	Reference
Clostridium ljungdahlii	Batch Reactor volume: 2.5 L, Working volume: 1.5 L	Temperature: 37 °C pH: 5.9 Carbon source: CO:CO_2:H_2:N_2 32.5:16:32.5:19 (% vol)	0.5 g/L; 1.0 g/L	Reduction of biomass with 1.0 g/of YE	Reduction of ethanol concentration with 1.0 g/L of YE	Acetate concentration not influenced by YE concentration	4.0 @ * YE = 0.5 g/L 1.3 @ YE = 1.0 g/L	Infantes et al. [26]
Clostridium autoethanogenum	Batch Reactor volume: 150 mL, Working volume: 25 mL	Temperature: 37 °C pH: Carbon source: CO:CO_2:H_2:N_2 20:20:10:50 (% vol)	0.5 g/L; 5.0 g/L	Maximum growth: 5.0 g/L	Maximum ethanol concentration: 0.5 g/L	Maximum acetic acid concentration: 5.0 g/L	4.32 @ YE = 0.5 g/L 0.34 @ YE = 5.0 g/L	Thi et al. [27]
Clostridium carboxidivorans	Batch reactor Reactor volume: 200 mL, Working volume: 75 mL	Temperature: 30 °C pH: 4.75–5.75 Carbon source: CO 0.8–1.6 bar	0.6 g/L; 1.6 g/L	Maximum growth: 1.6 g/L	Maximum ethanol concentration: 0.6 g/L	Maximum acetic acid concentration: 1.6 g/L	1.08 @ YE = 0.6 g/L 0.12 @ YE = 1.6 g/L	[25]
Clostridium carboxidivorans	Batch reactor Reactor volume: 250 mL, Working volume: 55 mL	Temperature: 35 °C pH: no control Carbon source: CO 1.7 bar	0.5 g/L; 1.0 g/L; 3.0 g/L	Maximum growth: 3.0 g/L	Maximum ethanol concentration: 1.0 g/L	Maximum acetic acid concentration: 3.0 g/L	1.95 @ YE = 0.5 g/L 1.07 @ YE = 1.0 g/L 0.16 @ YE = 1.0 g/L	This paper

(*) estimated from data reported on figures.

concentration and acid production were observed at the highest YE concentration (5 and 1.6 g/L, respectively), while the maximum solvent production was observed at the lowest YE concentration (0.5 and 0.6 g/L, respectively). Infantes et al. [26] reported that high YE concentrations inhibited *C. ljungdahlii* growth and ethanol production. However, it should be noted that a minimum YE concentration of 0.01 g/L is required for *C. carboxidivorans* to maintain structural integrity [25].

Regarding the solvent yield on YE ($Y_{totS/YE}$), as detailed in Table 2, $Y_{totS/YE}$ reduces with the YE concentration, and the specific value is strain dependent. The decrease in $Y_{totS/YE}$ is in agreement with the anticipated increase in biomass growth observed during the acidogenic stage. As a matter of fact, the operating conditions addressing the best cost-effectiveness (highest $Y_{totS/YE}$ value) are those characterized by a low initial YE concentration.

3.2. Effect of reducing agents

The present section concerns the investigation of the impact of various reducing agent compositions on the growth of *C. carboxidivorans* and the production of metabolites in terms of concentration and rate. The selected reducing agents and their respective concentrations for the fermentation tests were as follows: cysteine-HCl at 0.6 g/L (Cys-HCl), pure cysteine at 0.6 g/L (Cys), sodium sulphide (Na_2S) at 0.6 g/L, cysteine-sodium sulphide at 0.6 g/L (Cys-Na_2S), and cysteine-sodium sulphide at 0.72 g/L (A fermentation test without any reducing agent was carried out; no growth was observed in these conditions). The reason for focusing on sulphur-bearing agents is that sulphur is an essential component of metal clusters in various enzymes involved in the Wood-Ljungdahl pathway [22]. Thus, sulphur plays a crucial role in the autotrophic carbon fixation process by acetogens.

Main results of tests are reported in Table 3, while Table 4 presents a comprehensive summary of studies from the scientific literature investigating the impact of different reducing agents on the fermentation performance of *Clostridia* strains. Analysis of Table 3 points out that the tests conducted with Na_2S at a concentration of 0.6 g/L yielded the highest concentration of both butanol and ethanol. However, this

Table 3
Data measured/assessed during fermentation tests carried out with different reducing agents. Batch fermenters 250 mL bottles. Gas-liquid volume ratio: 0.28. P_{CO} = 1.7 atm. YE = 1 g/L. TM 100 %. Cell concentration at inoculation 0.02 g_{DM}/L.

Reducing agent	X g_{DM}/L	AA mg/L	BA mg/L	HA mg/L	E mg/L	B mg/L	$Y_{E/CO}$ g/g	$Y_{B/CO}$ g/g	ξ_{CO} %	μ h^{-1}
Cys-HCl 0.6 g/L	$0.51 \pm 1.4 \times 10^{-2}$	1445 ± 20	140 ± 3	35 ± 2	898 ± 5	168 ± 1	$0.20 \pm 1.5 \times 10^{-3}$	$0.034 \pm 6 \times 10^{-4}$	78.6 ± 0.5	$0.117 \pm 1.6 \times 10^{-3}$
Pure Cys 0.6 g/L	$0.60 \pm 9.5 \times 10^{-3}$	1630 ± 94	248 ± 30	98 ± 16	1208 ± 45	139 ± 2.5	$0.22 \pm 1.0 \times 10^{-3}$	$0.022 \pm 5.6 \times 10^{-4}$	78.8 ± 0.2	$0.114 \pm 1.5 \times 10^{-3}$
Na_2S 0.6 g/L	$0.33 \pm 3.5 \times 10^{-2}$	830 ± 55	105 ± 33	20 ± 2	1430 ± 186	206 ± 64	$0.22 \pm 3.3 \times 10^{-2}$	$0.033 \pm 6.1 \times 10^{-4}$	86.15 ± 1.4	$0.074 \pm 7.5 \times 10^{-3}$
Cys-Na_2S 0.6 g/L	$0.42 \pm 3.7 \times 10^{-2}$	1119 ± 42	221 ± 16	103 ± 3	460 ± 80	148 ± 9	$0.11 \pm 1.0 \times 10^{-2}$	$0.032 \pm 4.7 \times 10^{-4}$	70.6 ± 1.5	$0.092 \pm 1.5 \times 10^{-2}$
Cys-Na_2S 0.72 g/L	$0.25 \pm 3.9 \times 10^{-3}$	510 ± 0.5	97 ± 4.5	27 ± 3	207 ± 1	30 ± 2	$0.081 \pm 3.2 \times 10^{-2}$	$0.011 \pm 3.9 \times 10^{-4}$	34.04 ± 0.6	$0.079 \pm 1.9 \times 10^{-3}$

Table 4
Effects of reducing agents on different *Clostridia* strains.

Strain	Reactor	Operating conditions	Reducing agent	Growth	Alcohols	Acids	Reference
Clostridium radsdalei	Fed-batch Reactor volume: 2.0 L, Working volume: 1.0 L	Temperature: 37; 32 °C pH: 5.5; 6.0 Carbon source: CO:CO_2:H_2 60:20:20	Na_2S 0.05 and 0.1 mmol S L^{-1} h^{-1}	Maximum concentration: Na_2S 0.1 mmol S L^{-1} h^{-1}	Maximum ethanol concentration: 7.67 g/L Na_2S 0.05 mmol S L^{-1} h^{-1}	Maximum acetate concentration: Na_2S 0.05 mmol S L^{-1} h^{-1}	[34]
Clostridium ljungdahlii	Gas-fed stirred tank reactor Reactor volume: 200 mL, Working volume: 75 mL	Temperature: 37 °C pH: Carbon source: CO_2:H_2 40:60 (%vol)	Cys-HCl H_2O Na_2S	Maximum OD (value not reported) Na_2S (2 g/L)	Maximum ethanol concentration: 0.94 g/L Na_2S (0.8 g/L) Maximum 2,3- butanediol concentration: 1.5 g/L Cys-HCl (1.7 g/L)	Maximum acetic acid concentration: 2.82 g/L Without reducing agent addiction	[35]
Clostridium acetobutylicum	Fed-batch/ batch Reactor volume: 125 mL, Working volume: 95 mL	Temperature: 37 °C pH: 6.5 not controlled Carbon source: Glucose	Ascorbic acid, l- cysteine, Dithiothreitol (DTT)	Not reported	Maximum butanol concentration: 9.19 g/L DTT 0.3 mM (batch)	Faster ri-assimilation with L-Cys Solvents to acids ratio of 27.97 (Fed-batch)	[36]
Clostridium carboxidivorans	Batch reactor Reactor volume: 200 mL, Working volume: 75 mL	Temperature: 30 °C pH: 4.75–5.75 Carbon source: CO 0.8–1.6 bar	Cys-HCL H_2O 0.5–1.2 g/L	Maximum concentration: 326 mg/L Cys-HCl 0.5 g/L	Maximum ethanol concentration: 0.649 g/L Cys-HCl 1.2 g/L	Maximum concentration of acetic acid: 2.522 g/L Cys-HCl 1.2 g/L	[25]

operating condition led to relatively low biomass production. Assuming the enhancement of solvent production as the key performance indicator, the most effective reducing agent among the tested ones was Na_2S at 0.6 g/L. The yield of both solvents did not significantly change with the reducing agent (specie and concentration), except for the Cys-Na_2S at 0.72 g/L. Indeed, a poor solvent production was measured at Na_2S = 0.72 g/L. The mechanism underlying the effect of the reducing agent is not fully understood, but the decrease in cell concentration may be due to the toxic effects of Na_2S on cells, as suggested by Wang et al. [29]. The analysis of Table 3 also suggests that the presence of cysteine in the medium positively affect biomass growth throughout the fermentation process. However, when cysteine was used as the reducing agent instead of Na_2S, the production of solvents was considerably lower. This indicates that while Cys had a beneficial effect on the growth of *C. carboxidivorans*, Na_2S had a greater impact on solvent production. The analyis of the results of tests carried out with Cys-HCl and pure Cys points out that the use of pure Cys as a reducing agent led to improved fermentation performance in terms of cell, acid and solvent production, except for butanol (which experienced a slight decrease). The addition of Cl− ions with Cys-HCL may be responsible for the detrimental effect observed. Further research is needed to clarify this issue. The results obtained distinctly indicate that the presence of the reducing agent modifies the microorganism metabolism, causing alterations in the distribution of end products. This evidence is corroborated by results reported in the literature. Investigation on *Thermoanaerobacter ethanolicus* has demonstrated that high concentration of reducing agents is characterized by increased ethanol production. However, it was noted that high concentration of reducing agents led to the inhibition of cell growth [30]. Some studies have pointed out that the supplementation of artificial electron carriers - such as methyl viologen [31] and neutral red [32] - to *C. acetobutylicum* cultures results in an augmented production of alcohols. The rationale behind this phenomenon is believed to be the alteration of the NAD/NADH + ratio upon the addition of these electron carriers, triggering an increase in solventogenesis to maintain the natural balance of the NADH/NAD + ratio [33].

The results of other studies focusing on the importance and beneficial effect of reducing agents in *Clostridia* fermentation are reported in Table 4. Oliveira et al. [34] investigated the impact of sulphur availability in the fermentation broth of *C. ragsdalei* using a fully controlled, continuously gassed stirred-tank bioreactor. They monitored cysteine concentration in the medium and H_2S fraction in the exhaust gas and found that almost all the initially added sulphur was stripped out within the first day of the batch process. To support fermentation performance, they continuously fed sodium sulphide (Na_2S), which significantly increased ethanol concentration. Yang et al. [35] studied the effects of reducing agents on the production of acids/alcohols from H_2/CO_2 using *C. ljungdahlii*. They reported that sulphur and reducing power significantly affected metabolite production. They found that cysteine (Cys) was superior to Na_2S in terms of biochemical production, especially for 2,3-butanediol production. Chandgude et al. [36] conducted experiments to investigate the effect of a range of reducing agents, including ascorbic acid, cysteine, and dithiothreitol, on *C. acetobutylicum* fermentation. They demonstrated that these agents improved the uptake of acids to produce solvents. Abubackar et al. [25] tested the same strain used in the present study (*C. carboxidivorans*) using cysteine-HCl at two concentrations. They found that increasing the Cys-HCl concentration increased acid and solvent production but decreased biomass growth, with a minimum biomass growth observed at 0.5 g/L Cys-HCl.

In conclusion, the results obtained in the present study (reported in Table 3) are consistent with - and support - observations reported in the literature. On one hand, the addition of reducing agents to *C. carboxivorans* cultures increased solvent production. On the other hand, high concentrations of reducing agents caused cell growth inhibition.

3.3. Effect of trace metals

The present section provides an analysis of the influence of trace metals (TMs) and their concentration on the growth of *C. carboxidivorans* and the production of metabolites, including their concentration and

Table 5
Data measured/assessed during fermentation tests carried out under a wide spectrum of Trace Metal (TM) composition. Batch fermenters 250 mL bottles. Gas-liquid volume ratio: 0.28. P_{CO} = 1.7 atm. YE = 1 g/L. Reducing agent Pure Cysteine.

Trace Metal	X g_{DM}/L	AA mg/L	BA mg/L	HA mg/L	E mg/L	B mg/L	$Y_{E/CO}$ g/g	$Y_{B/CO}$ g/g	ξ_{CO} %	μ h^{-1}
TM 25 %	0.29 ± 0.03	633 ± 50	110 ± 18	28 ± 4	830 ± 90	210 ± 2	0.27 ± 1.2 × 10^{-3}	0.0675.4 × 10^{-4}	42.5 ± 0.5	0.090 ± 1.3 × 10^{-4}
TM 40 %	0.33± 0.04	930 ± 40	120 ± 30	22 ± 5	1000 ± 25	188 ± 7.5	0.28 ± 2.5 × 10^{-3}	0.0436.1 × 10^{-4}	49.6 ± 0.2	0.096 ± 1.1 × 10^{-4}
TM 50 %	0.43 ± 0.02	1450 ± 24	280 ± 26	88 ± 15	750 ± 27	193 ± 1	0.15 ± 5.5 × 10^{-3}	0.0384.5 × 10^{-4}	68.8 ± 0.1	0.114 ± 2.0 × 10^{-3}
TM 75 %	0.59 ± 0.02	1960 ± 130	260 ± 17	82 ± 12	1400 ± 28	270 ± 7	0.24 ± 1.0 × 10^{-2}	0.0475.1 × 10^{-4}	78.4 ± 1.5	0.114 ± 1.0 × 10^{-2}
TM 100 %	0.60 ± 0.01	1630 ± 94	250 ± 30	98 ± 16	1210 ± 45	139 ± 2.5	0.22 ± 2.0 × 10^{-3}	0.022 ± 5.6 × 10^{-4}	78.8 ± 0.2	0.114 ± 1.5 × 10^{-3}
TM 100 % NO Se	0.56 ± 0.03	1350 ± 20	266 ± 6	60± 8	1030 ± 60	260 ± 11	0.36 ± 1.5 × 10^{-3}	0.073 ± 6.0 × 10^{-4}	42.4 ± 0.9	0.100 ± 1.6 × 10^{-3}
TM 100%NO W	0.26 ± 0.01	1070 ± 43	42 ± 3	0	440 ± 15	18 ± 2.5	0.26 ± 4.0 × 10^{-3}	0.012 ± 5.2 × 10^{-4}	34.2 ± 0.2	0.097 ± 1.4 × 10^{-3}

rate. The TM composition was manipulated by adjusting the fraction of the stock solution added to the medium, which ranged from 25 % to 100 %. In addition, some experiments were conducted using a 100 % TM stock solution lacking selenium (Se) or tungsten (W). The major outcomes of these investigations are summarized in Table 5, while Table 6 presents a comprehensive summary of studies from the scientific literature investigating the impact of different trace metals on the fermentation performance of *Clostridia* strains.

The results outlined in Table 5 underscore the pivotal role of trace metals (TMs) in the fermentation process. The addition of TMs is essential for supporting microorganism growth as well as the production of acids and solvents. At low concentrations of TMs, bacteria face challenges in the growth and in the synthesis of acids and solvents. Specifically, when the TM concentration was less than 50 % of the stock solution, both cell and acid/solvent concentrations were approximately half of those measured when the TM stock solution was utilized. However, with a TM concentration of 75 % of the stock solution, fermentation performance matched or exceeded that observed with the full TM stock solution. Consequently, a 25 % reduction in TM concentration may offer cost savings without compromising process performance. Fermentation tests with a 100 % TM solution, excluding selenium, displayed a slight decline in performance compared to tests utilizing the complete TM stock solution. Conversely, tests conducted with a 100 % TM solution devoid of tungsten exhibited significantly poorer performance. Specifically, the production of butyric acid and solvents was notably lower by an order of magnitude without tungsten. Tungsten serves as a crucial cofactor for certain WLP enzymes, including formate dehydrogenase, a pivotal enzyme in the upstream segment of the metabolic pathway [39]. Therefore, the absence of tungsten is anticipated to lead to a reduction in several end-metabolites. Similar outcomes were reported by Abubackar et al. [40] and by Chakraborty et al. [39].

Other studies have investigated the effects of trace metals on the growth and metabolite production of other microorganisms (Table 6).

Table 6
Effects of Trace Metals on different *Clostridia* strain.

Strain	Reactor	Operating conditions	Trace Metal (*)	Cell growth	Alcohols	Acids	Reference
Clostridium carboxidivorans	Batch Reactor volume: 120 mL, Working volume: 20 mL	Temperature: 37 °C pH: 6.0 Carbon source: CO:CO_2:H_2 50:35:15 ($\%_{vol}$)	Ni Fe Se Co	++ ++ 0 0	++ ++ 0 0	++ ++ 0 0	[37]
Clostridium radsdalei	Batch Reactor volume: 500 mL, Working volume: 50 mL	Temperature: 36 °C pH: Carbon source: CO	Ni Zn Se W Cu Fe Co Mo Mn	++ + 0 + 0 0 + 0 0	++ + + ++ – + + + 0	++ 0 0 0 0 0 0 0 0	[14]
Clostridium ljungdahlii	Batch Reactor volume: 100 mL Working volume: 50 mL	Temperature: 37 °C pH: 4.5–5.0 Carbon source: CO:CO_2:H_2: CH_4:N_2 60:10:10:10:10 ($\%_{vol}$)	Mg Mn Cu W Zn	+ + + + 0	/ / + + 0	/ / / / 0	[19]
Clostridium. autoethanogenum	Batch Reactor volume: 250 mL, Working volume: 75 mL	Temperature: 37 °C pH: Carbon source: CO:CO_2:H_2 20:20:10 ($\%_{vol}$)	1 mL/L 100 mL/L (10 mL/L basal medium condition)	+ –	/ /	/ /	[38]

(*) TM concentration set at 1%$_V$ of the stock solution, if no further information is reported.
++ indispensable.
+ positive effect.
0 no effect.
- detrimental effect.
/not reported.

Han et al. [37] reported that nickel and ferrous ions were essential for the growth of *C. carboxidivorans*, molybdate was vital for triggering CO utilization and alcohol production, while selenium and cobalt had no significant effects on cell growth and metabolite production. Park et al. [38] found that trace metals inhibited the growth of *C. autoethanogenum* at high concentrations, while Saxena and Tunner (2011) demonstrated that nickel was necessary for cell growth and ethanol production, while cobalt, iron, molybdate and tungsten were necessary for ethanol production in *C. ragsdalei*. Sertkaya et al. [19] reported that tungsten and copper had the most significant effects on ethanol and cell production, respectively, in *C. ljungdahlii*.

In summary, the findings presented in Tables 5 and 6 demonstrate that the effects of trace metals on fermentation performance are strain dependent. Evaluating the influence of trace metals on the chosen biocatalyst is crucial, given their substantial impact on the economics of the process. As a general guideline, tungsten supplementation is deemed necessary when seeking to augment solvent production.

4. Conclusions

Fermentation runs were carried out to evaluate the impact of different components and concentrations in the medium - YE, reducing agents and trace metals included - on the cell growth kinetics and fermentation performances of *Clostridium carboxidivorans*.

The increase of the concentration of YE did not affect cell growth, but it led to enhanced acid production and reduced ethanol production. The optimal initial concentration of YE for the fermentative process was determined to be 1 g/L. The optimal concentration as regard the balance between economy and efficiency must take into account these results.

The fermentation process was proved to depend on the type and concentration of reducing agents. The comparison between tests utilizing Cys-HCl and pure Cys pointed out that the use of pure Cys as a reducing agent enhanced overall fermentation outcomes, encompassing cell, acid and solvent production. However, butanol production experienced a slight decrease. While cysteine presence positively affected biomass growth, its use instead of Na_2S resulted in a reduced solvent production, emphasizing the superior impact of Na_2S in this regard. It is worth to note that Na_2S at 0.6 g/L was the most effective reducing agent when solvent production was the priority.

The supplement of trace metals to the medium was found to be essential for supporting microorganism growth and the production of acids and solvents. Insufficient trace metal concentrations hindered bacteria growth and reduced acid/solvent production. At TM concentration smaller than 50 % of the stock solution, acid/solvent concentrations dropped at about half the value measured by supplementing the stock solution. However, at 75 % of the stock solution, fermentation performance matched or exceeded those assessed by supplementing the TM stock solution. As a consequence, it is possible to reduce the incidence of the TM cost of the 25 % on the overall process cost without compromising process performance. Moreover, while selenium was not strictly necessary for *C. carboxidivorans* fermentation, the presence of tungsten was crucial for cell growth and solvent production.

In conclusion, the optimization of medium components, including their species and concentrations, plays a crucial role in ensuring the sustainability of solvent production through *C. carboxidivorans* syngas fermentation. Reducing the amount of components has a dual significance: it reduces the mass supplement required for each unit of solvent produced and lowers the production cost of solvents. Therefore, there is potential for increasing the sustainability of solvent production by *C. carboxidivorans* through syngas fermentation.

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CRediT authorship contribution statement

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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