Optimization of the Dark Fermentation Technique for Hydrogen production through Supplementation with Ascorbic Acid and/or L-cysteine by *Clostridium butyricum* CCDBC 11

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Abstract

This work focuses on engendering hydrogen production by adding of oxygen scavengers during the process of dark fermentation such as ascorbic acid and L-cysteine. Supplementation with them shortened the lag phase of bacteria and significantly accelerated the growth of the strain *Clostridium butyricum*, giving rise to the production of hydrogen. The amount of biogas formed in the fermenter was gauged by the water displacement method, with measurement of hydrogen content by gas chromatography. The highest cumulative hydrogen formation in the batch fermenter (2.20 mol H_2/mol glu) was achieved by adding 5 mg/L of ascorbic acid, utilizing saccharified corn scrap as substrate. This amount reduced the lag phase by 65.6% and increased the yield by 40.9% in comparison with the reference sample without such oxygen scavengers, and 11.4% compared to solely L-cysteine. A

modified Gompertz model was applied to perform kinetic analysis of hydrogen formation in connection with different amounts of ascorbic acid, employing glucose and saccharified corn scrap as energy sources. Results show that ascorbic acid acts as an affordable and effective bioactive agent for increasing the yield of hydrogen in the process of dark fermentation.

Keywords: Bio-hydrogen production; Dark fermentation; Ascorbic acid; L-cysteine; *Clostridium butyricum*

1. Introduction

The Czech Republic, as a member of the European Union, faces increasingly stringent climate targets, including a 55% reduction in carbon dioxide (CO_2) emissions by 2030 and the achievement of climate neutrality by 2050. Achieving these goals necessitates a significant expansion of renewable energy sources to replace carbon-intensive technologies. In response, the EU has intensified its efforts to decarbonize the energy sector through the updated „Fit for *55*“ program, now known as „RePowerEU“. This initiative envisions a renewable energy capacity of 1200 GW by 2030, with hydrogen playing a pivotal role [1,2]. Hydrogen is gaining global attention as a unique energy solution and a potential carbon-free fuel [3]. Various conventional methods have been employed to enhance hydrogen production, with biological hydrogen production playing a significant role [4]. This approach involves producing hydrogen through the cultivation of microorganisms [5], potentially via the biotechnological conversion of biomass.

Hydrogen production from organic waste biomass can generally be classified into two main processes: photosynthetic and dark fermentation. The latter offers distinct advantages, as it does not require light as an energy source, unlike photofermentation, and operates effectively with a simple reactor while achieving a higher hydrogen production rate. Moreover, a wide

range of renewable biomass and organic waste materials are applicable as the substrate, thereby lowering the cost involved [4–8].

The choice of substrate has demonstrable effect on the fermentation process, in accordance with the biodegradability of the material. Glucose, maltose and xylose are readily used, whereas others, e.g. starch, necessitate a preliminary transformation into glucose or maltose, either by acid or by enzymatic hydrolysis [7]. Several carbohydrate-rich substrates are also suitable, including first generation fuel crops such as sugarcane, wheat, corn, and sugar beet, as well as second generation (2G) biomass sources like agricultural residues, industrial waste, and wastewater, [9,10]. The use of 2G raw materials as a potential energy source for hydrogen production has gained significant interest due to its sustainability and its potential to enhance the comprehensive utilization of renewable energy resources [11].

Various microorganisms are capable of producing hydrogen, and notable amongst them are strict anaerobes, which cannot grow in the presence of oxygen. These microorganisms do not perform oxidative phosphorylation. Instead, generation of adenosine triphosphate (ATP) primarily transpires via substrate phosphorylation and a flavin-based electron bifurcation process during fermentation. Strict anaerobes with the ability to produce hydrogen include gram-positive bacteria of the genus *Clostridium* [5]. Although the maximum theoretical yield of hydrogen is 4 mol/mol glucose, the highest such values for genus *Clostridium* reported in the literature are below 3 mol H_2/mol glucose [5,12].

The efficiency of dark fermentation is associated with the activity of hydrogenase and nicotinamide-adenine dinucleotide (NAD+/NADH), whose activity is supported by an environment with a low oxidation-reduction potential (ORP). Some reducing amino acids contribute to inhibiting such an ORP [7,13], notably L-cysteine, a low-cost reducing agent.

The diminishing effect of L-cysteine on the oxidation-reduction potential of a fermentation system is exerted through the presence of a thiol group [14]. This particular amino acid has also been described as a mediator between the given fermentative bacteria and substrate, owing to its unique structure and affinity for certain bacterial proteins [15]. Additionally, the disulfide bond (-S-S-), which can be formed from the thiol group of L-cysteine, plays a crucial role in protein formation. Furthermore, the remaining thiol groups may contribute to maintaining protein structure, thereby regulating cellular metabolism [16]. This enables it to function as a bioactive agent, enhancing the growth of fermenting bacteria and supporting substrate utilization [17].

The influence of L-cysteine on hydrogen production has been investigated in several studies [6,14,18–21], wherein applying it shortened the lag phase and enhanced hydrogen production. In terms of the latter, Guo et al. (2013) reported a rise of 23.7% by adding 0.5 g/L of L-cysteine into an expanded granular sludge bed [22]. Zhang et al. (2016) recorded dark fermentative production as 1.6–2.0 times higher from cassava residues with the amino acid than a control group absent of it (0.5–2.0 g/L); [19]. Yuan et al. (2008) described how supplementation with 0.6 mM of L-cysteine increased the yield of hydrogen by 18.3% [15]. Another study by Qu et al. (2012) reported a reduction in reactor residence time to 21 days, which was 4 days shorter than the blank sample, while daily hydrogen production increased by 3.2% [20]. Additional examples are summarized by Yand & Wang (2018) in their review [17].

Although most studies emphasize the beneficial effect of L-cysteine on hydrogen production, a manuscript by Zhao et al. (2012) reported a decrease in such from in the *Clostridium beijerinckii* strain. Therein a drop of 1.73 to 1.46 mol/mol of sucrose (-15.6%) was observed upon the addition of 0.1 g/l cysteine [18]. For this reason it seemed advisable to determine the outcome of applying L-cysteine to each strain.

In recent years, the use of ascorbic acid as an oxygen scavenger in the food industry has gained attention, both as a food additive and as a component of food packaging [23,24]. Although its beneficial effect on promoting the growth of anaerobic microorganisms, particularly lactic acid bacteria, has been proven [23], few studies have investigated its effect on hydrogen production. An example by Zhu et al. (2023) detailed this aspect in photofermentation experiments [25]; however, to our knowledge, no dark fermentation studies have been published.

Biological hydrogen production is regarded as a promising and efficient method due to its ability to utilize waste materials as substrates. However, despite its potential, challenges remain in optimizing the process to reduce production costs and enhance hydrogen yield. Ongoing research focuses on developing innovative strategies, including optimizing microbial consortia and enhancing metabolic pathways, to maximize efficiency and enable large-scale implementation [26]. Consequently, the authors aimed to evaluate the impact of ascorbic acid on hydrogen production. The study focused on determining the optimal concentration of ascorbic acid for hydrogen production by the strain *Clostridium butyricum* CCDBC 11 during dark fermentation, directly comparing its performance to L-cysteine – a comparison that has not been previously reported. Saccharified corn scrap (SCS), a second-generation (2G) raw material from ethanol production, were used as the substrate.

2. Materials and methods

2.1. Inoculum and medium

Clostridium butyricum CCDBC 11 was utilized herein, obtained from the Milcom a.s. collection (Tábor, Czech Republic; patent no. 305450) [27]. The bacteria were cultured in RCMB medium at the following concentration per 1000 mL: 10 g meat extract, 3 g yeast extract, 5 g peptone, 10 g glucose, 1 g soluble starch, 5 g sodium chloride, 3 g sodium acetate.

This formulation was previously identified as optimal for hydrogen production in an earlier study on the tested strain [28]. The resultant RCMB medium was employed in experiments with different quantities of ascorbic acid and L-cysteine. The pH was adjusted to 7.2, and the medium was autoclaved at 115 °C for 20 minutes and at 225 kPa [28].

2.2. Raw material

The corn scrap (2G raw material after ethanol production) was supplied by Ethanol Energy a.s. (Vrdy, Czech Republic) and saccharified by Novozyme enzymes sourced from the same company in accordance with the sated directions. In brief, 1 kg of the corns scrap was placed in 2.45 L of water; this was then heated to 82 °C and supplemented with 0.12 g of the EN1 A Alpha Amylase enzyme. Further heating transpired, up to the temperature of 105 °C, which was maintained for 5 minutes; the mixture was subsequent cooled to 85 °C. Afterwards 0.12 kg of EN1 B Alpha Amylase was added into it and stirring performed for 2.5 hours. The mixture was then topped up with water to the original volume and supplemented with the enzyme EN2 Gluco Amylase Spirizime ADV enzyme. Finally, it was stirred at 85 °C for 10 minutes, cooled to 30 °C and passed through KA 0 filter paper.

2.2.1 Raw material and SCS analysis

Moisture of raw material

The moisture content of corn scrap was measured using the conventional oven-drying method, which involved drying the samples at 105 °C for 6 hours.

Starch analysis

The starch content was determined according to the standard ČSN 467092-2. Corn meal was hydrolysed by boiling with HCl (1.124% HCl) for 15 minutes. Clarification was performed with phosphotungstic acid (4%, 10 ml). After filtration, polarimetric measurement was

conducted using an Inframatic 8600 instrument (Perten). Particle size was measured using a Analysette 3 PRO sieve shaker (Fritsch).

The Physiochemical composition of the corn scrap is presented in Table 1.

Table 1. Physiochemical composition of the corn scrap (2G raw material)

Starch (%)	Moistness (%)	Particle size >2 mm (%)	Particle size >1.4 mm (%)
69.40	12.49	5.83	21.26

Elemental analysis of SCS

The elemental composition of carbon, hydrogen, nitrogen, oxygen, and sulphur in the SCS samples was determined using a Flash 2000 CHNS/O+MAS200R analyser (Thermo Scientific) via the Dumas combustion method at 960 °C. The combustion products – CO_2, N_2, H_2O, and SO_2 – were transported by a helium carrier gas through a gas chromatography (GC) separation column and detected using a thermal conductivity detector (TCD). Quantification was performed using a calibration curve of standards, with identification facilitated by the Eager Experience software (Thermo Scientific).

The chemical composition of the SCS is presented in Table 2.

Table 2. Chemical composition of the SCS

Sample	Carbohydrates (g/L)		Elemental analysis results (%)				
	Glucose	Fructose	C	H	N	O	S
SCS	211.6	15.5	34.8±0.9	8.4±0.6	3.7±0.3	53.1±0.9	0±0

2.3. Experimental procedures

The experiments were conducted at 37 °C with a glucose concentration of 10 g/L and the pH was optimized at 7.2 for the growth of the *Clostridium butyricum* CCDBC 11 strain. During hydrogen production in the fermenter, the pH was maintained at 5.6 [28]. Initially, the optimal concentration of oxygen scavengers was determined using glucose as the substrate, after which glucose was replaced by SCS. Preliminary tests were first performed with small volumes of medium in glass syringes, and the results were subsequently validated in a fermenter.

2.3.1. Preliminary tests on optimal concentration of the ascorbic acid and L-cysteine

Experiments for this purpose involved adding 10 mL of the RCMB medium into 50 mL glass syringes and applying a magnetic stirrer. The plunger of the syringes had been lubricated with paraffin oil to create a seal and reduce friction, their tips having been sealed with a rubber septum to permit sampling for gas chromatography analysis with thermal conductivity detector (GC-TCD) during the tests. The concentrations of L-cysteine and ascorbic acid applied were 0, 0.63, 1.25, 2.5, 5, 10, 20, 40, 60 and 80 mg/L. The inoculation medium had an OD_{550} of 2.0 (a cell dry weight of 2.4 g/L) the final concentration equalling 3%. Fermentation was performed at 37 °C and 3 1/s. Tests were performed in triplicate, each one lasting 72 hours. Gas volume was measured by pushing out the plunger and reading the value on the syringe scale, this being monitored throughout the cultivation period. Samples were collected at 30 and 72 hours for GC-TCD.

2.3.2. Preliminary tests on the optimal concentration of SCS

Following on from those detailed above, such testing differed in that sample contained the RCMB medium in combination with the SCS (instead of glucose), at the concentrations (glucose present in SCS) of 0, 1.25, 2.5, 3.75, 5, 7.5, 10 and 12.5 g/L. Ascorbic acid or L-

cysteine was then added to give final amounts of 0, 2.5, 5, 10 and 20 mg/L. Additionally, a mixture of both scavengers was tested at concentrations of 1.25/1.25, 2.5/2.5 and 5/5 mg/L.

2.3.3. Batch fermentation in a fermenter for confirmation of results

To facilitate observation of the fermentation process in a larger medium volume (1.5 L), cultivation was conducted in a laboratory fermenter, specifically the Lambda Minifor “start-up kit” 3L (LAMBDA Instruments, Switzerland). The gas generated from the reaction in the fermenter was collected at the outlet using the water displacement method. The gas was directed into water-filled bottles connected in parallel, with an outlet leading to a measuring cylinder monitored overnight by a camera [14]. The water's pH was adjusted to 2 to prevent hydrogen from reacting with water, ensuring accurate measurement of hydrogen yield [29]. The composition of biogas was sampled periodically and analysed on GC-TCD. The amount of CO_2 was gauged continuously via a CO_2 probe fitted to the fermenter. The concentrations of ascorbic acid was tested ranged from 2.5 to 20 mg/L, and the total volume of the medium amounted to 1.5 L. The OD_{550} of the inoculation medium was 2.0 (a cell dry weight of 2.4 g/L), and the final concentration of the inoculation medium equalled 1%. The initial pH value was adjusted to 7.2, with subsequent monitoring of the fermentation medium enabled by a pH probe. During the lag phase, the pH dropped to 5.6, a level maintained by supplementation with 1M NaOH for the remaining period of fermentation. Prior to performing the experiment, the Lambda reactor was flushed with argon gas for 15 minutes to remove oxygen, upon which fermentation at 37 °C and 6 Hz. Liquid and gas samples were collected at frequent intervals throughout its duration.

Observation was made as to the growth of microorganisms within the fermentation process at an optical density of OD_{550}. The concentration of the biomass was determined by filtering a 5 mL sample through a 0.45 μm Millipore filter, followed by drying at 105 °C and determination of constant weight of the dried specimen [30]. The data obtained was

interpolated by applying a growth curve to a logistic model, thereby expressing the entirety of such a curve [31]; R^2 values constituted a measure of the goodness of fit, as reported in charts.

2.4. Analytical methods and data analyses

Gas chromatography was used to analyse the H_2 and CO_2 content. Such analyses were performed on a gas chromatograph (GC-TCD; Shimadzu GC-2010 Plus, Kyoto, Japan) equipped with a thermal conductivity detector. The subsequent data were processed in GC-Solution software. Injections were carried out manually by means of a Gastight side-hole syringe. A Carboxen 1010 PLOT column (internal diameter of 0.53 mm × 30 m long × 30 µm thick) was applied as the stationary phase. The carrier gas was argon at a flow rate 4.99 mL/min. The volume of sample injected was 100 µL and at a split ratio of 1:5. The temperature of the injector was maintained at 200 °C. A heating cycle was conducted in the oven that commenced at 35 °C for 4.2 minutes, with subsequent increase to 220 °C at 50 °C/min, which was held for 2 minutes; the total duration equalled 9.90 minutes. The temperature of the detector was set to 230 °C. Standard gases such as hydrogen, nitrogen, oxygen, carbon dioxide and methane were purchased from Siad (Italy).

The sugar content was analysed through high-performance liquid chromatography coupled with a refractive index detector (HPLC-RI) on a Waters Breeze QS HPLC unit (Waters, USA). Separation was achieved in a Luna NH_2 5 µm column (250 x 4.6 mm, Phenomenex USA) at 40 °C, the composition of mobile phase comprising acetonitrile (HPLC gradient grade, VWR International s.r.o., Czechia) and water (HPLC grade, VWR) in a ratio of 80:20 (v/v). All samples and standards were passed through PES 0.45 µm syringe filters (VWR,

Czech Republic) prior to injection. Each run was completed within 20 minutes. The data were recorded by and processed in EmPowerPro software (Waters, USA).

In order to determine the amount of volatile fatty acid (VFA) present, samples (10 mL) were centrifuged at 7500 g for 10 minutes, and the resulting supernatant then filtered via a 0.22 µm syringe filter. A 1:5 dilution of phosphoric acid to distilled water was constituted the inactivation acid for pretreatment of the samples, thereby eliminating the risk of peak tailing connected with high temperatures.

The concentrations of VFA were determined by gas chromatography (Shimadzu GC-2010 Plus, Kyoto, Japan), on a unit equipped with a flame ionisation detector and a Restek Stabilwax DA column (30 m x 0.22 mm, 0.25 µm). The temperatures for the injector and detector were set at 260 °C and 300 °C, respectively. Helium was applied as the carrier gas and delivered at flow rate of 1.2 mL/min. Each sample (0.5 µl) was injected with a split ratio of 1:40. The oven initially operated at 140 °C, a temperature maintained maintaining for 14 minutes. Quantification was performed using calibration data obtained from standard solutions of butyric acid, acetic acid, and propionic acid, diluted in distilled water at concentrations ranging from 0.1 to 10 g/L.

Data analysis

A modified Gompertz model (Eq. (1)) was applied for kinetic analysis of hydrogen production.

$$H_{H_2}(t) = P_{max} \cdot \exp\left\{-\exp\left[\frac{R_m \cdot e}{P_{max}}(\lambda - t) + 1\right]\right\} \quad (1)$$

where $H_{H2}(t)$ is cumulative hydrogen yield (mL), P_{max} represents the maximum potential of hydrogen production (mL), R_m stands for the maximum rate of hydrogen production (mL/h), e constitutes the Euler number, e = 2.71828…, λ symbolizes the lag time (h) and t refers to the duration of fermentation time (h); [32,33]. The measured biogas volume was standardized to 0 °C and 101.325 kPa pressure.

Statistical significance was evaluated by applying one-way ANOVA ($P < 0.05$); (OriginPro 2024 SR1, OriginLab Corporation, Massachusetts, USA); experimental data are reported as the mean values of three replicates ± standard deviation.

3. Results and discussion

Preliminary experiments were conducted on a smaller scale, using glass syringes for this purpose. Sealed perfectly, these were placed under optimal conditions to encourage growth and hydrogen production. Tests informed by the initial findings were then carried out in a fermenter containing 1.5 L of fermentation medium, with the aim of verifying such results.

3.1. Effect of oxygen scavengers and SCS on hydrogen production in preliminary tests

Optimal concentration of ascorbic acid and L-cysteine for hydrogen production

The optimal medium and glucose concentration of 10 g/L for carbon production by this strain was determined by the authors Havlíková et al. (2011), who also discerned the optimum pH for producing hydrogen equalled 5.6 [28]. Initial tests conducted herein verified that the same values applied under the given conditions (data not shown), hence the optimal concentration of ascorbic acid for hydrogen production was investigated with a concentration of 10 g/L glucose and an initial pH of 7.2 to promote bacterial growth.

Although L-cysteine has been documented in the literature as an oxygen scavenger [13,14], its effects on the *Clostridium butyricum* CCDBC 11 strain had not been previously explored. Therefore, experiments were conducted using varying concentrations of both tested oxygen scavengers. Preliminary tests were performed in small volumes of medium within syringes, and the total volume of biogas produced was measured after 30 and 72 hours of cultivation. The biogas was simultaneously analysed using GC-TCD.

Figure 1 shows a steep increase transpired in hydrogen yield after adding 0.63 mg/L of such oxygen scavengers. Preliminary tests revealed a maximum of 2.24 mol H_2/mol Glu achieved by the addition of 5 mg/L ascorbic acid after only 30 hours of cultivation (Fig. 1). In contrast, the control group without oxygen scavengers achieved its maximum yield only after 72 hours, reaching 1.25 mol H_2/mol Glu − 44.2% lower than the maximum yield obtained with the addition of ascorbic acid. Nevertheless, results did not indicate a statistically significant difference ($P > 0.05$) in hydrogen yield brought about by applying ascorbic acid at concentrations of 2.5-20 mg/L. The experiments with L-cysteine revealed contrasting findings, since the greatest effect was observed for the amount of 5 mg/L (1.98 mol H_2/mol Glu). Furthermore, supplementation with both oxygen scavengers reduced the lag phase. Samples with ascorbic acid reached maximum yield within 30 hours of incubation, while those with L-cysteine achieved 90% of the maximum yield in the same period. In contrast, samples without oxygen scavengers reached only 33% of the maximum yield during this time. These findings align with the results reported in previous studies by Bao et al. (2013), Yang and Wang (2018), and others [14,15,17,18], wherein a shortening of the lag phase is reported in connection with the presence of L-cysteine. Such accelerated production and a heightened yield of hydrogen, as instigated by the oxygen scavengers, could have been caused by L-cysteine and ascorbic acid reducing the value for oxidation-reduction potential (ORP) in the fermentation system, initiating cell growth as a consequence [15]. Preliminary tests

indicated that supplementing oxygen scavengers led to an earlier onset of hydrogen production. Moreover, the maximum yield with ascorbic acid supplementation was approximately 17.5% higher than with L-cysteine alone, representing a significant difference ($P < 0.05$).

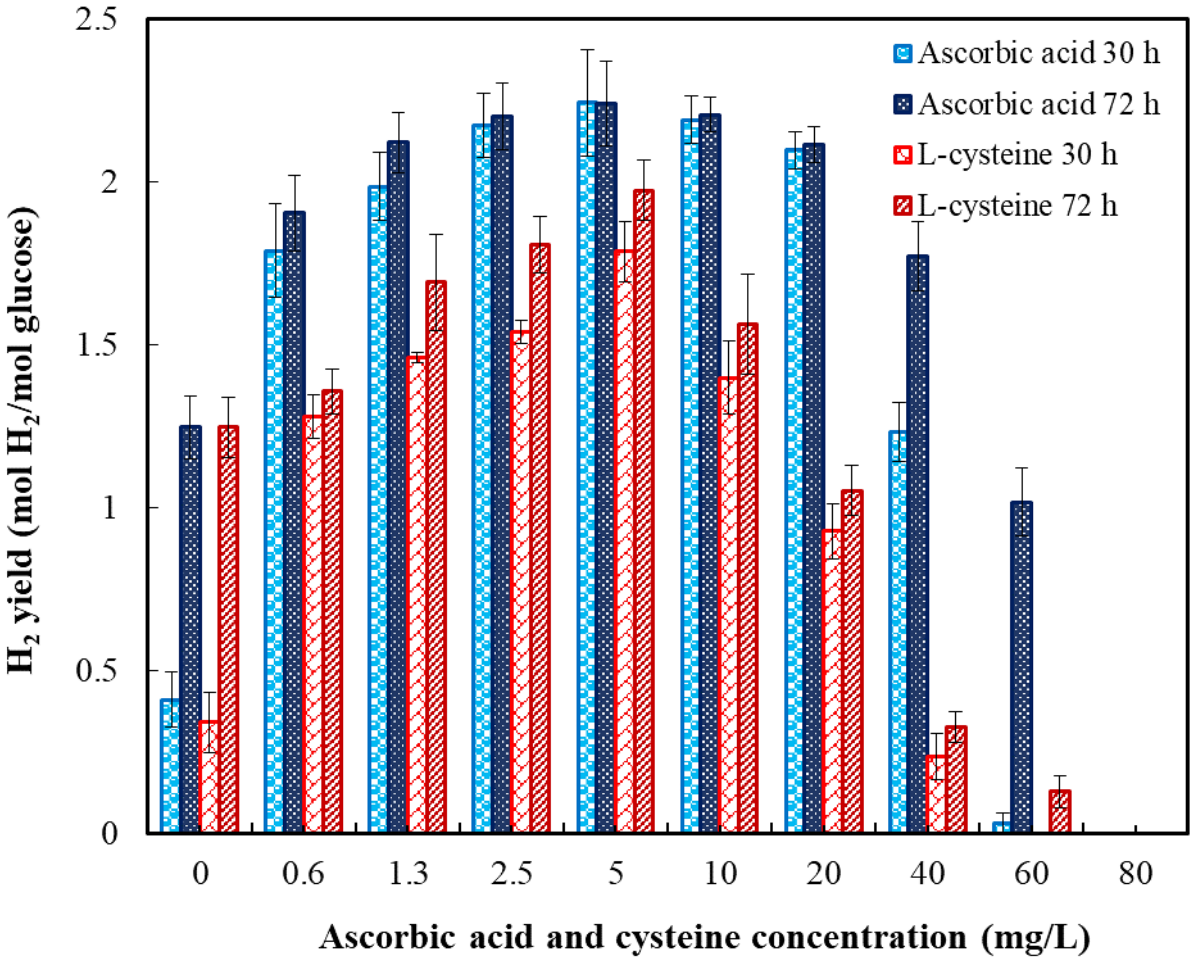


Fig.1. Dependence of H_2 yield on the presence of ascorbic acid and L-cysteine across a range of concentrations: ascorbic acid (30 h cultivation), ascorbic acid (72 h cultivation), L-cysteine (30 h cultivation) and L-cysteine (72 h cultivation).

GC-TCD analysis revealed that the gas formed during fermentation contained hydrogen and carbon dioxide. The proportion of hydrogen varied between 66% and 72%, the amount of it decreasing slightly in parallel with a rise in the biogas produced. The data indicate that neither the addition nor the type of oxygen scavenger influenced the hydrogen content in the

biogas. (The corresponding values are provided in the supplementary materials (Table S4). No significant difference was observed in the levels of hydrogen generated by the two oxygen scavengers.

Optimal concentration of SCS for hydrogen production

Preliminary tests were conducted to discern the optimal concentration of the substrate and oxygen scavengers in 50 mL syringes for maximum hydrogen yield. HPLC-RI analysis showed that a concentrated solution of SCS contained 211.6 g/L of glucose and 15.5 g/L of fructose.

Samples were prepared for testing with glucose at concentrations of 0, 1.25, 2.5, 3.75, 5, 7.5, 10 and 12.5 g/L. The previous experiment informed which combinations and concentrations of the oxygen scavengers were selected, i.e. those demonstrating the greatest yield of hydrogen.

The highest value for hydrogen production of 2.82 mol H_2/mol Glu was recorded after 72 hours of cultivation for the sample containing 7.5 g/l of glucose. This represents a significant ($P < 0.05$) increase of up to 20% compared to using glucose as the sole energy source. The maximum in this regard was observed for the sample with 5 g/L of ascorbic acid, 11% higher than that with the L-cysteine (see Tables 3 and 4). Combinations of the oxygen scavengers also underwent testing, though the absolute values for hydrogen yield were below the achieved maximum. These findings indicated that mixing the two oxygen scavengers led their effects being combined. Thus, the total optimal concentration discerned was 5 mg/L.

The results in Tables 3 and 4 further confirm that the addition of oxygen scavengers reduces the lag phase, particularly under optimal conditions. When 5 mg/L ascorbic acid and 7.5 g/L glucose were added, 96% of the maximum yield of 2.82 mol H_2/mol glucose was achieved

within 30 hours of cultivation. Whereas the control samples without oxygen scavengers reached only 35% of their final maximum yield (2.21 mol H_2/mol Glu) in the same period.

The literature reports that raising the level of glucose increases osmotic pressure and reduces water activity, causing stress which inhibits bacterial growth and diminishes hydrogen production [34]. These findings confirm a gradual decrease in hydrogen yield at higher glucose content in the medium above 7.5 g/L.

Table 3. Hydrogen yield engendered by concentrations of the substrate and oxygen scavengers upon 30 hours of cultivation.

Oxygen scavengers	**Scavengers concentration (mg/mL)**	**Hydrogen yield (mol H_2/mol Glu)[a] at 30 hours**							
Glucose concentration in SCS (g/L)		**0**	**1.25**	**2.5**	**3.75**	**5**	**7.5**	**10**	**12.5**
Ascorbic acid	**0**	0	0	0	0	0.54	0.78	0	0
	2.5	0	1.37	1.75	1.86	2.29	2.57	1.13	0.56
	5	0	1.40	2.00	2.49	2.67	2.72	1.83	1.79
	10	0	1.33	2.03	2.13	2.27	2.30	0	0
L-cysteine	**5**	0	1.44	1.95	2.02	2.05	2.24	0.87	0
Ascorbic acid/ L-cysteine	**1.25/1.25**	0.52	1.52	1.98	2.01	2.15	2.26	0.14	0
	2.5/2.5	0.11	1.38	2.02	2.33	2.57	2.66	0.87	0
	5/5	0.58	1.31	1.67	1.87	2.02	2.33	1.30	0

[a]Relative Standard Deviations (RSD) for hydrogen yield ranged from 1% to 17%, based on three or more measurements. The RSD values are provided in the supplementary documents (Table S5).

Table 4. Hydrogen yield engendered by concentrations of the substrate and oxygen scavengers upon 72 hours of cultivation

Oxygen scavengers	**Scavengers concentration (mg/mL)**	**Hydrogen yield (mol H_2/mol glu)[a] at 72 hours**							
Glucose concentration in SCS (g/L)		**0**	**1.25**	**2.5**	**3.75**	**5**	**7.5**	**10**	**12.5**

	0	0	0.95	1.47	1.89	2.20	2.21	0	0
Ascorbic	**2.5**	0.53	1.43	1.77	1.94	2.48	2.60	2.44	0.67
acid	**5**	0.53	1.49	2.02	2.50	2.72	2.82	2.74	1.85
	10	0	1.34	2.03	2.29	2.42	2.47	2.37	0
L-cysteine	**5**	0.46	1.50	1.98	2.28	2.42	2.51	2.08	0
Ascorbic	**1.25/1.25**	0.60	1.56	2.01	2.30	2.33	2.48	2.13	0
acid/ L-	**2.5/2.5**	0.22	1.45	2.04	2.41	2.72	2.76	1.90	0
cysteine	**5/5**	0.60	1.35	1.85	2.08	2.15	2.36	1.70	0

[a]RSD for hydrogen yield ranged from 1% to 17%, based on three or more measurements. The RSD values are provided in the supplementary documents (Table S6).

3.2. Effects of the oxygen scavengers and SCS on hydrogen production in the fermenter

Throughout the tests involving the fermenter, observation was made of the cumulative volume of total biogas and proportion of carbon dioxide. Samples were taken at selected intervals and analysed accordingly, with GC-TCD being employed to determine the exact content of hydrogen in the gas generated, HPLC-RI evaluating the extent of carbohydrate loss and finally cell growth was determined.

The findings were in agreement with those of the preliminary tests, having been verified by discerning the effect of oxygen scavengers on hydrogen production in the fermenter, utilizing glucose (10 g/L) as the only energy source. The highest yield of hydrogen (1.80 mol H_2/mol Glu) was achieved with 5 mg/L of ascorbic acid. Details results are given in the supplementary materials file (Table S1, Fig. S1 and Fig. S2).

Effect of SCS on hydrogen production in the fermenter

On the basis of preliminary research, the optimal concentrations of glucose in the saccharified corn scrap (7.5 g glu/L) and oxygen scavengers (5 g/L) were determined and tested in a larger volume of medium (1.5 L) in the fermenter.

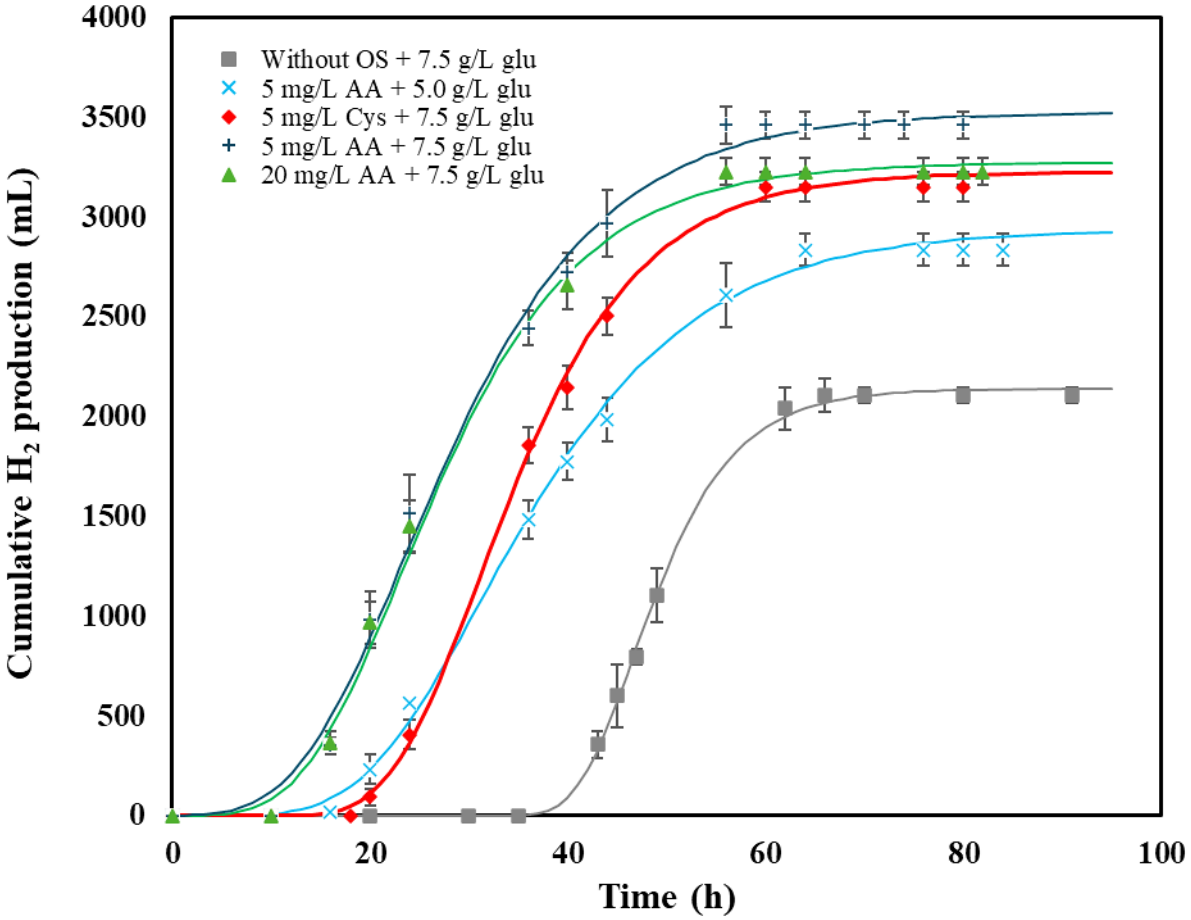
Peak figures for hydrogen yield and cumulative volume were recorded for 7.5 g/L glucose in the SCS with 5 mg/L of ascorbic acid (Fig. 2). Cumulative hydrogen volume reached 3526 mL, corresponding to a hydrogen yield of 2.20 mol H_2/mol Glu; 11.4% greater than in the presence of 5 mg/L of L-cysteine and 40.9% higher than without the scavengers. The lag phase was significantly ($P < 0.05$) shortened by up to 65.6% with the addition of 5 mg/L ascorbic acid and by up to 38,9% with the addition of L-cysteine.

The Gompertz equation coefficients for oxygen scavengers, SCS concentration, and hydrogen yield are summarized in Table 5.

Table 5. Gompertz equation coefficients for the various concentrations of the oxygen scavengers

Oxygen scavengers	**Scavengers concentration (mg/mL)**	**SCS (g/L)**	**Pmax (mL)**	**Rm (mL/h)**	**λ (h)**	**R^2**	**HY (mol H_2/mol glu)**
Ascorbic acid	0	7.5	2106	278	36.0	0.999	1.30
	5	5.0	2941	89	19.1	0.999	1.75
	5	7.5	3526	117	12.4	0.997	2.20
	20	7.5	3272	120	13.0	0.998	2.00
L-cysteine	5	7.5	3226	131	22.0	0.999	1.95

Where HY is hydrogen yield (mol H_2/mol glu) and λ (h) the lag phase.



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374 *Fig. 2. Plots for cumulative H_2 per time for various concentrations of ascorbic acid, L-*
375 *cysteine and SCS concentrations:* ■ *without the oxygen scavengers (7.5 g/L glucose);*
376 ♦ *5 mg/L of L-cysteine (7.5 g/L glucose);* x *5 mg/L of ascorbic acid (5 g/L glucose);* + *5 mg/L*
377 *of Ascorbic Acid (7.5 g/L glucose); and* ▲ *20 mg/L of ascorbic acid (7.5 g/L glucose).*

378

379 GC-TCD analysis showed that the proportion of hydrogen during fermentation ranged from
380 61% to 72%. Although the presence of hydrogen increased during the fermentation, no
381 difference was observed for the various concentrations of the oxygen scavengers or SCS. The
382 highest total volume of biogas (5191 mL) was achieved with the addition of 5 mg/L
383 of supplemented ascorbic acid. Hourly variations in cumulative H_2 and CO_2 volume

37
38

for the tested concentrations of oxygen scavengers and SCS are given in the supplementary file (Fig. S3).

Bao et al. (2013) state the significance of L-cysteine as an important nutrient in the hydrogen production process. It acts as a bioactive agent during fermentation, facilitating interactions between bacteria and the substrate. Moreover, similar to ascorbic acid, L-cysteine acts as a reducing agent, effectively lowering the ORP in the fermentation system. This reduction in ORP enhances the growth of certain hydrogen-producing bacteria [14]. The findings herein indicated that ascorbic acid, like L-cysteine, increased hydrogen production and substantially shortened the lag phase. Ascorbic acid heightened the hydrogen yield of hydrogen by more than 11% and reduced the lag phase by nearly 44% in comparison with L-cysteine.

It is not possible to contrast the peak yield recorded of 2.20 mol H_2/mol Glu in the fermenter with results published by Havlíková et al. (2011), since they only gave proportions as percentages and did not list hydrogen yields. Several authors utilized bacteria from the genus *Clostridium* due to their high hydrogen production rates [26,35,36]. The authors Davila-Vazquez et al. (2009) reported a maximum hydrogen yield of 2.8 mol H_2/mol Glu with the strain *Clostridium butyricum* CM-C86 [37]. Liu et al. (2012) and Plangklang et al. (2012) achieved values of 1.15 and 1.34 mol H_2/mol Glu respectively, in experiments with the *Clostridium butyricum* strain CGS5 and TISTR 1032 [38,39]. Herein, the *Clostridium butyricum* strain demonstrated yield of 2.20 mol H_2/mol Glu, hence it would appear to have potential in industrial hydrogen production.

Bacterial growth in the fermenter

The growth of microorganisms within the fermentation process was monitored by the cell weighing technique. The data obtained was interpolated through a logistic growth curve model (Fig. 3). The R^2 values for all fits for the logistic model were above 0.98, i.e. an

excellent match with the experimental data. Each concentration of the oxygen scavengers brought about bacterial growth, the course of which being similar in character, differing merely in the length of the lag phase. Results showed that adding between 2.5-20 mg/L of ascorbic acid shortened the lag phase by more than half and increased the number of cells. This corresponds with findings by Bao et al. (2013), who state that L-cysteine is an important nutrient for encouraging the growth of anaerobic bacteria [14]. The maximum biomass concentration (12.4 g/L) was obtained with the supplementation of 5 mg/L of ascorbic acid. At this concentration, the highest hydrogen yield of 2.20 mol H_2/mol glucose was also recorded. However, further increases in ascorbic acid levels resulted in declines in both cell growth and hydrogen yield. These findings suggest that the addition of oxygen scavengers promotes cell proliferation in the *Clostridium butyricum* strain CCDBC 11. The experiments demonstrated that exponential hydrogen production began when amount of cells reached the level of 4.7 to 5.0 g/L.

Applying SCS as a substrate meant that the lag phase was shortened slightly, although the number of cells did not increase (data not shown).

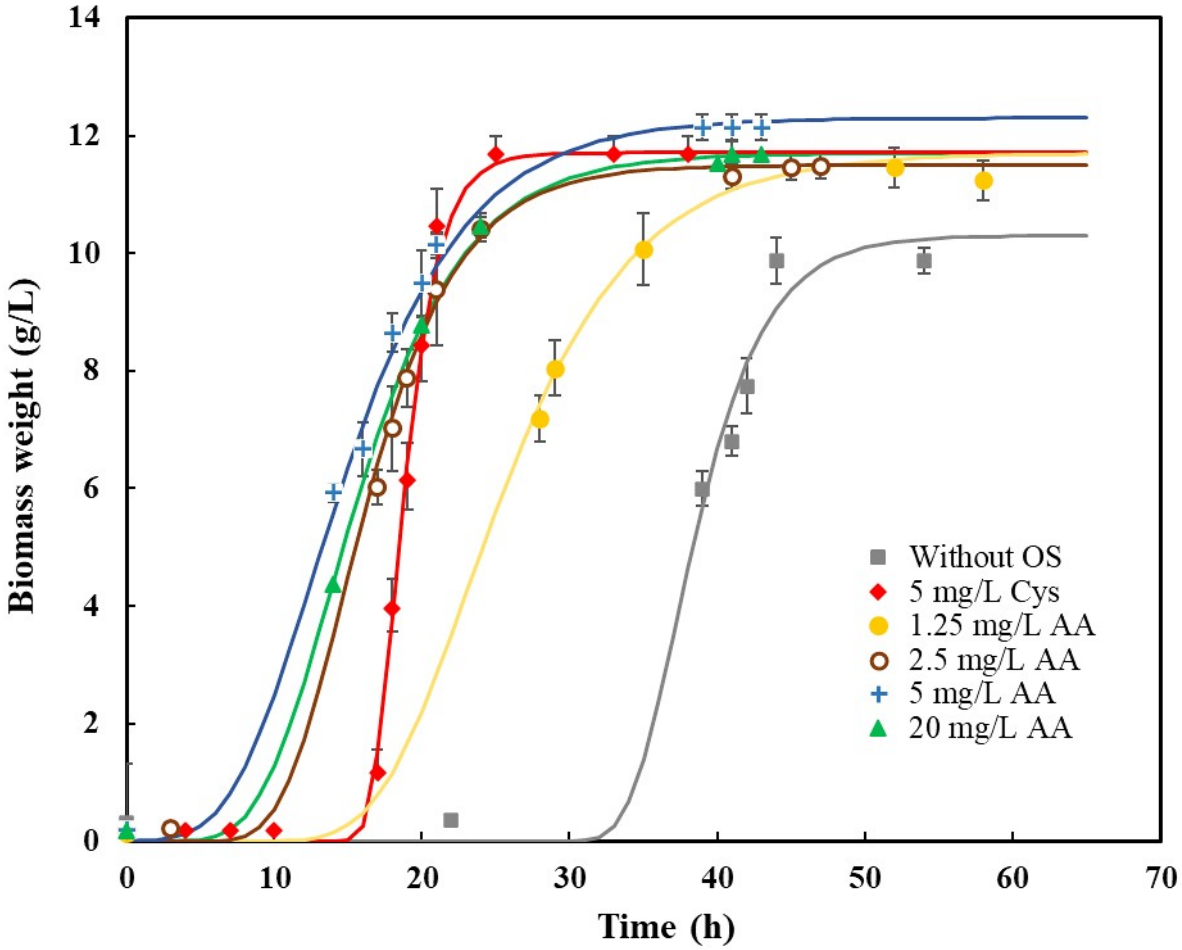

Fig. 3. Growth curves for various concentrations of ascorbic acid and L-cysteine; ■ without the oxygen scavengers; ♦ 5 mg/L L-cysteine; ● 1.25 mg/L ascorbic acid; ○2.5 mg/L ascorbic acid; + 5 mg/L ascorbic acid, ▲ 20 mg/L ascorbic acid.

Although numerous studies have demonstrated the stimulatory effect of L-cysteine on hydrogen production, the underlying mechanisms of L-cysteine and ascorbic acid remain under investigation. To our knowledge, only Zhao et al. (2012) have examined the impact of L-cysteine supplementation on hydrogenase activity in *Clostridium beijerinckii* RZF-1108. Their findings indicated that the effect of L-cysteine on hydrogen production in *C. beijerinckii* RZF-1108 is complex. While L-cysteine slightly enhanced *hydA* gene expression, hydrogen production was highly dependent on the interplay between cell growth and *hydA* gene

435 expression [18]. The influence of the environmental factor L-cysteine on enzymatic function
436 and activity requires further investigation.

437 HPLC-RI analysis revealed that 0.8 g of glucose per hour was lost in the exponential phase
438 of hydrogen production. No significant difference existed between the individual tests. When
439 using SCS as a substrate, however, only glucose was consumed, not fructose (data not
440 shown).

441 **Fermentation metabolites**

442 Volatile fatty acids (VFAs) are important by-products that stem from the metabolic activity
443 of microorganisms within the production of hydrogen via fermentation. Their type
444 and concentration directly depend on the substrate and the species of microorganism present
445 [32].

446 Table 6 shows the metabolites of the *Clostridium butyricum* strain CCDBC 11 formed
447 by fermentation during hydrogen production. The results herein agree with those
448 in the literature, indicating that the metabolic activity of hydrogen-producing acidogenic
449 bacteria primarily gives rise to acetate and butyrate [6,28]. The presence of butyric acid
450 increased by adding the oxygen scavengers, a finding consistent with heightened hydrogen
451 yield and cell growth. Discerning that a greater concentration of butyric acid is brought about
452 through supplementation with the oxygen scavengers is in agreement with other studies,
453 wherein their positive effect on bacterial cell growth and hydrogen production is reported
454 [6,17]. The cause of this phenomenon is reduction in ORP.

Table 6. Final values for metabolites upon applications of oxygen scavengers at various concentrations (37 °C)

Oxygen	Concentration of oxygen scavengers	HY (mol	Final ORP (mV)	Acetic acid (g/L)	Butyric acid (g/L)

scavenger	(mg/L)	H_2/mol glu)			
Ascorbic acid	0	0.70	-165	4.82	1.30
	1.25	1.21	-185	4.28	3.48
	2.5	1.62	-199	5.12	3.05
	5	1.80	-214	4.08	3.18
	20	1.65	-278	4.74	2.50
L-cysteine	5	1.52	-206	4.05	3.22

Where HY is hydrogen yield (mol H_2/mol glu) and ORP oxidation-reduction potential

Conclusion

Under the optimal conditions, a highly positive effect on hydrogen production and cell growth was exerted by adding of ascorbic acid at the extent of 2.5-20 mg/L. Results showed that the adding of ascorbic acid reduced the lag phase and further boosted hydrogen yield, surpassing even L-cysteine. The optimal concentration of ascorbic acid and L-cysteine for hydrogen production in a fermenter containing 1.5 L of medium was determined to be 5 mg/L. The peak yield of hydrogen determined was 2.20 mol H_2/mol Glu; 11.4% more than with supplemented L-cysteine and 40.9% higher than without the oxygen scavengers. Moreover, the lag phase was shortened by 65.6% with the addition of ascorbic acid and by 38,9% through supplementation with L-cysteine, compared to the control sample without the oxygen scavengers. The findings revealed that using ascorbic acid instead of L-cysteine increased the yield and reduced the cultivation time for the tested strain, suggesting potential cost savings in an industrial environment. These results also suggest that the tested strain of *Clostridium butyricum* CCDBC 11 shows a good potential in hydrogen production at scale.

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.

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