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Characterizing Novel Acetogens for Production of C2–C6 Alcohols from Syngas

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Abstract: Utilizing syngas components CO, CO_2, and H_2 to produce fatty acids and alcohols offers a sustainable approach for biofuels and chemicals, reducing the global carbon footprint. The development of robust strains, especially for higher alcohol titers in C4 and C6 compounds, and the creation of cost-effective media are crucial. This study compared syngas fermentation capabilities of three novel strains (*Clostridium carboxidivorans* P20, *C. ljungdahlii* P14, and *C. muellerianum* P21) with existing strains (*C. ragsdalei* P11 and *C. carboxidivorans* P7) in three medium formulations. Fermentations in 250-mL bottles were conducted at 37 °C using H_2:CO_2:CO (30:30:40) using P11, P7, and corn steep liquor (CSL) media. Results showed that P11 and CSL media facilitated higher cell mass, alcohol titer, and gas conversion compared to the P7 medium. Strains P7, P14, and P20 formed 1.4- to 4-fold more total alcohols in the CSL medium in comparison with the P7 medium. Further, strain P21 produced more butanol (0.9 g/L) and hexanol (0.7 g/L) in the medium with CSL, offering cost advantages over P7 and P11 media containing yeast extract. Enhancing strain activity and selectivity in converting syngas into C4 and C6 alcohols requires further development, medium formulation improvements, and characterization, particularly for the new strain P21.

Keywords: syngas; novel acetogens; ethanol; butanol; hexanol

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1. Introduction

Biofuel production gained significant attention due to its advantages in reducing dependence on fossil fuels and greenhouse gas emissions (GHG) [1–3]. The United States is the leading biofuel producer in the world, producing 17 billion gallons per year from corn ethanol in 275 biorefineries [4]. Second-generation biofuel production from lignocellulosic biomass is estimated to range from 30 to 60 billion gallons per year in the U.S.A. by 2050 [5]. In addition to ethanol, there has been a growing interest in making renewable butanol and hexanol [6,7]. Butanol is more compatible with the existing infrastructure and well-suited for the production of jet fuels [7–10]. Similarly, hexanol was used as a co-solvent in making biodiesel to improve the cold flow properties, making it more effective in cold temperatures [2].

The production of biofuels derived from crops like corn and sugarcane has raised concerns about potential impacts on food availability [11,12]. As an alternative, syngas fermentation is gaining attention for producing biofuels and biobased products. Syngas fermentation converts CO, CO_2, and H_2, which can be generated from non-edible feedstocks such as industrial waste gases and the gasification of agricultural residues and municipal solid wastes into biofuels [1,3,13]. Syngas fermentation can be integrated into existing industries like gasification, carbon capture, steel mills, and biogas production, which can help reduce GHG and generate revenues from waste streams [9,14–17]. Syngas fermentation has been used to produce two- to six-carbon (C2–C6) alcohols and acids, 2,3 butanediol,

and other products [7,13,18]. The balanced equations for making C2–C6 alcohols and acids from CO are listed below [3,13].

$$6CO + 3H_2O \rightarrow C_2H_5OH + 4CO_2 \quad \Delta G^\circ = -217 \text{ (kJ/mol)} \quad (1)$$

$$12CO + 5H_2O \rightarrow C_4H_9OH + 8CO_2 \quad \Delta G^\circ = -486 \text{ (kJ/mol)} \quad (2)$$

$$18CO + 7H_2O \rightarrow C_6H_{13}OH + 12CO_2 \quad \Delta G^\circ = -759 \text{ (kJ/mol)} \quad (3)$$

$$4CO + 2H_2O \rightarrow CH_3COOH + 2CO_2 \quad \Delta G^\circ = -154.6 \text{ (kJ/mol)} \quad (4)$$

$$10CO + 4H_2O \rightarrow C_3H_7COOH + 6CO_2 \quad \Delta G^\circ = -421 \text{ (kJ/mol)} \quad (5)$$

$$16CO + 6H_2O \rightarrow C_5H_{11}COOH + 10CO_2 \quad \Delta G^\circ = -663 \text{ (kJ/mol)} \quad (6)$$

Researchers investigated syngas fermentation, including the effect of minerals and trace metals [15,19], the use of defined and complex media [20–22], supplementation of media with biochar [8,9], genetic modification of strains [18,23], and the use of single or multistage bioreactors [24–26]. Furthermore, *C. ragsdalei*, *C. carboxidivorans*, *C. ljungdahlii and C. autoethanogenum* have been studied for syngas fermentation. *C. ljungdahlii*, *C. carboxidivorans,* and *C. autoethanogenum* have been genetically modified to produce higher alcohol titers or specific alcohol from syngas [2,13,18]. *C. ragsdalei* and *C. autoethanogenum* have already been studied at pilot and industrial scale to produce ethanol [27–29]. In addition, *C. carboxidivorans*, *C. ljungdahlii,* and *C. autoethanogenum* were reported to make butanol and hexanol from CO and CO_2 [2,8,18].

However, there are limiting factors to produce higher alcohols, especially its toxicity, which can negatively impact the strain's growth and fermentation abilities. Research findings have shown that hexanol, at around 1 g/L, reduced *C. ljungdahlii* activity, and a further increase to 5 g/L completely inhibited *C. ljungdahlii* while also inhibiting *C. carboxidivorans* at 1.2 g/L hexanol [30,31]. To tackle the toxicity issue, studies have employed extractive fermentation to increase hexanol production with *C. carboxidivorans*, achieving 1 g/L [32] and 5 g/L [2]. However, economic feasibility challenges remain to be addressed in syngas fermentation technology, including the need for more robust strains for higher alcohol titers, especially C_4 and C_6 compounds, and the development of low-cost media.

This study explores the potential of three new acetogens, namely *C. ljungdahlii* P14, *C. muellerianum* P21, and *C. carboxidivorans* P20, to make C4–C6 alcohols and fatty acids from syngas. It compares these new strains with two previously studied strains, *C. ragsdalei* P11 and *C. carboxidivorans* P7, for gas fermentation. Additionally, the study evaluates the activity of these acetogens in three syngas fermentation media. The characterization of new acetogens, particularly for C4–C6 products, makes this investigation important in advancing syngas fermentation.

2. Material and Methods

2.1. Microorganisms

The strains used in this study (*C. carboxidivorans* P7 and P20, *C. ragsdalei* P11, *C. ljungdahlii* P14, and *C. muellerianum* P21) were isolated and enriched by R.S. Tanner as previously described [33–36]. These microorganisms were preserved in the P11 medium, as previously reported [8].

2.2. Inoculum Preparation

The inoculum of the five *Clostridium* strains was prepared using P11 medium [8]. P11 medium contains (per L) yeast extract (0.5 g), mineral solution (25 mL), 4-morpholineethane sulfonic acid (MES, 10 g), trace metal solution (10 mL), cysteine sulfide reducing agent (10 mL), vitamin solution (10 mL), and 0.1% resazurin (1 mL) [37]. The medium's initial pH was modified to 6.0 with KOH (5N), falling within the optimum pH range for the growth of the five strains [33–35]. Table 1 provides the composition details of all stock solutions.

Table 1. Compositions of solutions for media (P7, P11, and CSL).

Components	P7 (g/L)	P11 and CSL (g/L)
Minerals solution		
NH_4Cl	100	100
KH_2PO_4	10	10
KCl	10	10
$CaCl_2 \cdot 2H_2O$	4	4
$MgSO_4 \cdot 7H_2O$	20	20
Vitamin solution		
Pyridoxine	-	0.010
Riboflavin	-	0.005
Thiamine	-	0.005
Thioctic acid	-	0.005
Nicotinic acid	-	0.005
Vitamin B_{12}	-	0.005
2-Mercaptoethanesulfonic acid sodium salt (MESNA)	-	0.010
Calcium pantothenate	0.005	0.005
p-(4)-Aminobenzoic Acid	0.005	0.005
Biotin	0.002	0.002
Trace Metal Solution		
Nitrilotriacetic acid	2.00	2.00
$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$	0.80	0.80
$ZnSO_4 \cdot 7H_2O$	0.20	1.00
$MnSO_4 \cdot H_2O$	1.00	1.00
$NiCl_2 \cdot 6H_2O$	0.02	0.20
Na_2SeO_4	0.02	0.10
$Na_2WO_4 \cdot 2H_2O$	0.02	0.20
$CoCl_2 \cdot 6H_2O$	0.20	0.20
$Na_2MoO_4 \cdot 2H_2O$	0.20	0.02

The inoculum of each strain (50 mL) was prepared in 250 mL bottles. P11 medium (40 mL) was transferred into 250 mL bottles and sterilized for 30 min at 121 °C. After sterilization, the P11 medium was cooled and then reduced with cysteine sulfide. Active cells (10 mL) of each strain were inoculated into the 40 mL of P11 medium. Then, the bottles were pressurized with H_2:CO_2:CO:N_2 (5:15:20:60) to 142.6 kPa and horizontally incubated at 37 °C for 68 h. The headspace in each bottle was replenished with H_2:CO_2:CO (30:30:40) to 101.3 kPa. Then, the same syngas was fed at 90 h to 142.6 kPa and at 114 h and 138 h to 170.2 kPa. The optical density (OD) of the inoculum was determined at 138 h. The OD for the P7, P14, P20, and P21 strains was measured at 600 nm, and for the P11 strain, it was measured at 660 nm. The culture pH was monitored, and if it dropped below 5, it was adjusted to 5.1 using 10% NH_4OH to minimize acid stress. Each strain inoculum was ready for syngas fermentation after 162 h with an OD of 0.6–0.7.

2.3. Syngas Fermentation Medium Preparation

For testing the five *Clostridium* strains, three fermentation media were used: corn steep liquor (CSL, Sigma-Aldrich, MO, USA), P7, and P11 media. Table 2 summarizes the composition of each medium used in the study. The P7 medium was previously formulated for alcohol production from syngas by strain P7 [7], while the CSL and P11 media were developed for the conversion of syngas to alcohol using strain P11 [9,20]. MES was added to the inoculum and fermentation media to prevent a rapid pH drop caused by acid production, which stresses cell activity. This choice was made due to the use of three new strains (P14, P20, and P21) and their performance in different medium formulations being unknown. In addition, CSL was selected as a cost-effective medium component, which

costs 2% of the industrial price of yeast extract [20]. For the CSL medium, the CSL was initially centrifuged at 13,000 rpm for 10 min to remove the solids, which was about 50% of CSL stock. The liquid portion was prepared to an initial concentration of 20 g/L CSL, which resulted in the best performance as previously reported [20,22]. After the addition of all components to deionized water (DI), the initial pH of the medium was modified to 6 using 5N KOH. The medium was then boiled for 2 min to remove dissolved O_2. Afterward, N_2 was purged through the medium to eliminate dissolved O_2. Further, 40 mL of the purged medium was placed into 250 mL bottles and autoclaved for 30 min at 121 °C. Following sterilization, each bottle containing medium was purged with H_2:CO_2:CO (30:30:40) for 2 min and reduced with cysteine sulfide. Syngas fermentation in triplicate was initiated with an inoculum of 20% (v/v). Bottles were fed with H_2:CO_2:CO (30:30:40) to 170.2 kPa and incubated at 125 rpm and 37 °C, with syngas replenished every 24 h for 360 h to ensure substate gases were not limiting. During fermentation, the culture pH was monitored and adjusted to 5.1 if it fell below 5, using 10% NH_4OH to lessen acid stress on cells.

Table 2. Media composition.

Media	P7	P11	CSL
Concentration		**mL/L**	
Mineral solution [a]	20	25	25
Trace metal solution [a]	10	10	10
Vitamin solution [a]	10	10	10
Resazurin	1	1	1
Cysteine-sulfide	5	10	10
Others		g/L	
Yeast extract	0.5	0.5	0.0
MES monohydrate [b]	10	10	10
CSL [b]	0	0	20

[a] Composition of solutions in Table 1. [b] CSL: corn steep liquor; MES: 4-morpholineethanesulfonic acid.

2.4. Analytical Procedures

2.4.1. Cell Mass

A 1.5 mL of culture sample was taken daily to determine the pH and cell mass concentration. The OD was measured at 660 nm and 600 nm, optimal for *Clostridium* bacteria due to clear cell visibility and compatibility with the redox indicator, oxidized resazurin, which doesn't absorb in this wavelength range. Although there's little practical difference between 600 and 660 nm, measuring at the reported literature wavelengths for strains P7 and P11 would facilitate comparison. To ensure accurate OD measurement, each sample was diluted with DI water so that the OD was below 0.4 (i.e., within the calibration curve liner range) [21]. Calibration equations were developed to estimate the cell mass (X_{cell}) in g/L: P7 ($X_{cell} = 0.337 \times OD_{600} - 0.004$), P11 ($X_{cell} = 0.377 \times OD_{660} - 0.003$), P14 ($X_{cell} = 0.359 \times OD_{600} - 0.001$), P20 ($X_{cell} = 0.364 \times OD_{600} - 0.002$) and P21 ($X_{cell} = 0.343 \times OD_{600} - 0.002$).

2.4.2. Solvent and Gas Analysis

After measuring the OD of the culture, the liquid samples were centrifuged (Microfuge 20R, Beckman Coulter, Brea, CA, USA) for 10 min at 13,000 rpm to remove the cells before product analysis. A gas-chromatograph (Agilent 6890N, Agilent Technologies, Wilmington, DE, USA) with an FID and DB-FFAP capillary column was used to determine C2 to C6 product titers, following the method described previously [7]. In addition, 100 µL gas samples every 24 h were analyzed on a Supelco PLOT 1010 column (Supelco, Bellefonte, PA, USA) using an Agilent 6890N GC a with a TCD as described previously [38].

2.4.3. Statistical Analysis and Product Yields

Tukey's multiple comparisons of means with a 95% confidence were conducted with JMP Pro 16.0 (SAS Institute Inc., Cary, NC, USA). The aim was to identify pairwise statistical variances in various parameters, such as cell mass concentration and yield (g/L and g/mol CO), utilization of H_2, CO, and CO_2 (%), total alcohol and total acid concentrations, and alcohol-to-acid ratios. These comparisons were made between each strain in the same medium and for the same strain among the three different media. The following equations were used to estimate the yields of cell mass, ethanol, butanol, and hexanol from CO and utilization of H_2 and CO. The yield of the specific C2–C6 alcohol was estimated based on the total experimentally measured CO consumed minus the estimated CO consumed to make other C2–C6 alcohols and acids measured in the culture over 360 h, divided by the theoretical yield according to equations 1 to 3. These equations evaluate the efficiency and selectivity of the production of a desired alcohol.

$$\text{Cell mass yield } \left(\frac{\text{g}}{\text{mol}}\right) = \frac{\text{Maximum cell mass} - \text{initial cell mass}}{\text{moles of CO consumed}} \quad (7)$$

$$\%\ \text{EtOH yield} = \frac{\frac{\text{Total moles of ethanol produced}}{\text{total moles of CO consumed} - \text{moles of CO consumed for other C2–C6 products}}}{\frac{\text{1 mol of ethanol produced}}{\text{6 mol of CO consumed}}} \times 100\% \quad (8)$$

$$\%\ \text{BuOH yield} = \frac{\frac{\text{Total moles of butanol produced}}{\text{total moles of CO consumed} - \text{moles of CO consumed for other C2–C6 products}}}{\frac{\text{1 mol of butanol produced}}{\text{12 mol of CO consumed}}} \times 100\% \quad (9)$$

$$\%\ \text{HeOH yield} = \frac{\frac{\text{Total moles of hexanol produced}}{\text{total moles of CO consumed} - \text{moles of CO consumed for other C2–C6 products}}}{\frac{\text{1 mol of hexanol produced}}{\text{18 mol of COconsumed}}} \times 100\% \quad (10)$$

$$\%\ \text{H}_2\ \text{utilization} = \frac{\text{Total moles of H}_2\ \text{consumed}}{\text{Total moles of H}_2\ \text{supplied}} \times 100\% \quad (11)$$

$$\%\ \text{CO utilization} = \frac{\text{Total moles of CO consumed}}{\text{Total moles of CO supplied}} \times 100\% \quad (12)$$

3. Results and Discussion

3.1. Syngas Fermentation in P7 Medium

Syngas fermentation profiles for the five *Clostridium* strains in the P7 medium are displayed in Figure 1. The culture's initial pH was about 5.7 for all strains (Figure 1A). Strains P7, P11, P14, and P20 exhibited similar pH drop trends, reaching a pH of 4.6 after 72 and 96 h. The pH remained above 5 after 120 h. However, the pH was adjusted to 5.1 using NH_4OH (10%) whenever it dropped below 5. All strains grew on syngas in the P7 medium, with similar growth patterns observed for the P7, P11, and P20 strains. However, strain P21 showed the highest cell mass production (0.5 g/L), while the lowest cell mass concentration was observed for strain P14 (Figure 1B).

All strains exhibited growth-associated acetic acid production. Strain P11 had the highest acetic acid production of 4.7 g/L at 120 h, while strain P21 produced only 2.7 g/L at 196 h (Figure 1C). Ethanol production began after 48 h in P7 medium, with strain P21 showing significantly higher ($p < 0.05$) ethanol titers (8.9 g/L) compared to other strains: 2.6 g/L for strain P11, 3.5 g/L for strain P7, 1.4 g/L for strain P14 and 4.4 g/L for strain P20 (Figure 1D). Strain P7 produced the highest quantity of butyric acid of 0.24 g/L, while strain P11 did not produce C4 products (Figure 1E,F). The highest butanol titer (0.2 g/L) was produced by strain P21, while strains P11 and P14 did not produce butanol in the P7 medium. In comparison with the other strains, strain P21 also exhibited a significantly higher ($p < 0.05$) ethanol yield (91.6%), butanol yield (16.6%), ethanol to the acetic acid ratio (9.4 mmol/mmol), and butanol to butyric acid ratio (1.0 mmol/mmol) in P7 medium (Table 3). In addition, strain P21 produced 9.1 g/L total alcohol in the P7 medium, which

was 2- to 6-fold higher compared to other strains. On the other hand, total acid production in the P7 medium was highest with strain P7, at 3.5 g/L, which was 3 to 39% higher than for the other strains. The cumulative uptake of H_2 and CO for all strains in the P7 medium is illustrated in Figure 1G,H. Strain P21 in the P7 medium demonstrated the highest gas uptake (22 mmol H_2 and 34.5 mmol CO) of all strains. Moreover, strain P21 in the P7 medium converted significantly more ($p < 0.05$) H_2 (42%) and CO (44%) compared to the other strains (Table 3). However, none of the strains formed C6 products in the P7 medium, possibly due to nutrient limitation in this medium.

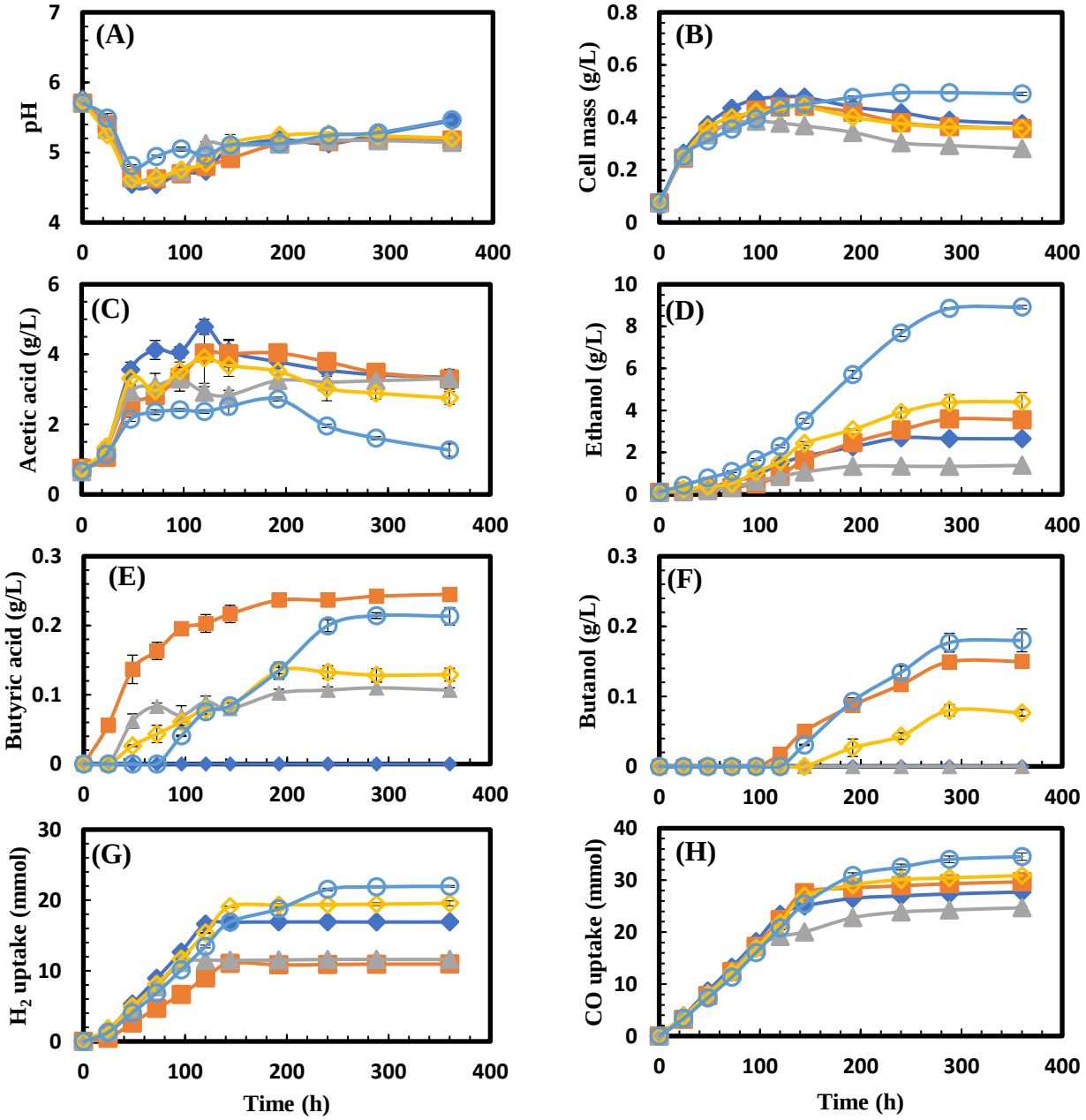

Figure 1. Profiles of syngas fermentation in P7 medium by strains P7 [■], P11 [◆], P14 [▲], P20 [◊], and P21 [○]. (**A**) pH; (**B**) cell mass; (**C**) acetic acid; (**D**) ethanol; (**E**) butyric acid; (**F**) butanol; (**G**) cumulative H_2 uptake; (**H**) cumulative CO uptake.

Table 3. Syngas fermentation parameters in P7, P11, and CSL media (n = 3).

Fermentation Parameters/Strains	P7	P11	P14	P20	P21
P7 Medium					
Cell mass yield (g/mol) [i]	$0.9 \pm 0.1^{A,b}$	$0.8 \pm 0.0^{B,c}$	$0.7 \pm 0.0^{E,c}$	$0.7 \pm 0.1^{C,c}$	$0.7 \pm 0.1^{D,c}$
Ethanol yield (%) [ii]	$54.8 \pm 2.3^{C,c}$	$43.6 \pm 0.8^{D,c}$	$25.6 \pm 0.8^{E,c}$	$61.2 \pm 6.4^{B,c}$	$91.6 \pm 7.2^{A,b}$
Butanol yield (%) [ii]	$16.7 \pm 2.6^{A,c}$	$0.0 \pm 0.0^{D,b}$	$0.0 \pm 0.0^{D,c}$	$14.5 \pm 3.1^{B,c}$	$10.5 \pm 1.2^{C,c}$
Hexanol yield (%) [ii]	$0.0 \pm 0.0^{A,c}$	$0.0 \pm 0.0^{A,b}$	$0.0 \pm 0.0^{A,c}$	$0.0 \pm 0.0^{A,c}$	$0.0 \pm 0.0^{A,c}$
EtOH/HAc (mol/mol) [iii]	$1.4 \pm 0.1^{C,c}$	$1.0 \pm 0.0^{D,c}$	$0.6 \pm 0.0^{E,c}$	$2.1 \pm 0.3^{B,a}$	$9.4 \pm 1.5^{A,a}$
BuOH/HBua (mol/mol) [iii]	$0.7 \pm 0.1^{B,c}$	$0.0 \pm 0.0^{D,b}$	$0.0 \pm 0.0^{D,c}$	$0.7 \pm 0.1^{C,c}$	$1.0 \pm 0.1^{A,b}$
HeOH/Hhex (mol/mol) [iii]	$0.0 \pm 0.0^{A,b}$	$0.0 \pm 0.0^{A,b}$	$0.0 \pm 0.0^{A,c}$	$0.0 \pm 0.0^{A,c}$	$0.0 \pm 0.0^{A,c}$
Total alcohols (g/L)	$3.7 \pm 0.0^{C,c}$	$2.7 \pm 0.1^{D,c}$	$1.4 \pm 0.0^{E,c}$	$4.5 \pm 0.4^{B,b}$	$9.1 \pm 0.1^{A,a}$
Total acids (g/L)	$3.5 \pm 0.3^{A,c}$	$3.3 \pm 0.0^{C,c}$	$3.4 \pm 0.1^{B,b}$	$2.9 \pm 0.2^{D,c}$	$1.5 \pm 0.2^{E,c}$
Sp. alcohol yield (g_{alcol}/g_x)	$10.4 \pm 0.4^{C,c}$	$7.0 \pm 0.1^{D,c}$	$4.9 \pm 0.1^{E,c}$	$12.5 \pm 1.3^{B,a}$	$18.6 \pm 0.1^{A,a}$
Sp. acid yield (g_{acid}/g_x)	$9.9 \pm 0.5^{B,c}$	$8.9 \pm 0.1^{C,b}$	$12.1 \pm 0.3^{A,b}$	$8.0 \pm 0.4^{D,c}$	$3.0 \pm 0.4^{E,c}$
CO consumption (%)	$37.6 \pm 1.3^{C,c}$	$35.1 \pm 0.9^{D,c}$	$31.3 \pm 0.7^{E,c}$	$39.0 \pm 0.7^{B,c}$	$44.2 \pm 1.0^{A,b}$
H_2 consumption (%)	$19.4 \pm 1.3^{E,c}$	$30.0 \pm 1.0^{C,c}$	$20.1 \pm 0.7^{D,c}$	$34.6 \pm 1.2^{B,a}$	$42.4 \pm 0.8^{A,a}$
P11 Medium					
Cell mass yield (g/mol) [i]	$0.9 \pm 0.0^{A,b}$	$0.9 \pm 0.0^{B,b}$	$0.8 \pm 0.0^{C,b}$	$0.9 \pm 0.0^{AB,b}$	$0.9 \pm 0.1^{A,b}$
Ethanol yield (%) [ii]	$92.2 \pm 1.8^{C,b}$	$97.4 \pm 0.8^{A,a}$	$90.5 \pm 1.8^{D,a}$	$63.4 \pm 1.7^{E,b}$	$93.7 \pm 1.6^{B,a}$
Butanol yield (%) [ii]	$23.1 \pm 0.6^{A,b}$	$0.0 \pm 0.0^{E,b}$	$16.5 \pm 0.2^{C,a}$	$15.1 \pm 1.4^{D,b}$	$17.2 \pm 0.5^{B,b}$
Hexanol yield (%) [ii]	$8.7 \pm 1.0^{B,b}$	$0.0 \pm 0.0^{E,b}$	$5.8 \pm 0.1^{C,b}$	$2.8 \pm 0.2^{D,b}$	$11.6 \pm 0.4^{A,b}$
EtOH/HAc (mol/mol) [iii]	$1.6 \pm 0.0^{D,a}$	$3.0 \pm 0.1^{B,a}$	$3.5 \pm 0.1^{A,a}$	$1.3 \pm 0.0^{E,b}$	$2.5 \pm 0.0^{C,b}$
BuOH/HBua (mol/mol) [iii]	$2.3 \pm 0.0^{B,a}$	$0.0 \pm 0.0^{E,b}$	$2.4 \pm 0.1^{A,a}$	$1.9 \pm 0.2^{C,a}$	$0.9 \pm 0.0^{D,c}$
HeOH/Hhex (mol/mol) [iii]	$1.1 \pm 0.1^{B,a}$	$0.0 \pm 0.0^{D,b}$	$1.7 \pm 0.1^{A,a}$	$1.1 \pm 0.1^{B,a}$	$0.8 \pm 0.0^{C,b}$
Total alcohols (g/L)	$6.1 \pm 0.0^{C,b}$	$8.1 \pm 0.0^{A,b}$	$6.8 \pm 0.1^{B,a}$	$4.4 \pm 0.1^{D,c}$	$8.1 \pm 0.1^{A,b}$
Total acids (g/L)	$4.7 \pm 0.0^{B,b}$	$3.5 \pm 0.2^{D,b}$	$2.7 \pm 0.1^{E,c}$	$4.5 \pm 0.1^{C,b}$	$5.0 \pm 0.1^{A,b}$
Sp. alcohol yield (g_{alcol}/g_x)	$17.0 \pm 0.6^{C,a}$	$21.4 \pm 0.1^{B,a}$	$24.3 \pm 0.5^{A,a}$	$12.3 \pm 0.3^{E,a}$	$16.5 \pm 0.1^{D,b}$
Sp. acid yield (g_{acid}/g_x)	$13.1 \pm 0.4^{A,a}$	$9.4 \pm 0.4^{D,b}$	$9.6 \pm 0.3^{D,c}$	$12.6 \pm 0.3^{B,a}$	$10.3 \pm 0.0^{C,b}$
CO consumption (%)	$42.5 \pm 0.9^{B,a}$	$47.6 \pm 0.9^{A,b}$	$41.8 \pm 0.6^{C,a}$	$40.6 \pm 0.9^{D,b}$	$47.4 \pm 0.8^{A,a}$
H_2 consumption (%)	$23.1 \pm 1.4^{E,b}$	$47.2 \pm 1.0^{A,a}$	$28.4 \pm 0.8^{C,a}$	$25.6 \pm 1.2^{D,b}$	$40.8 \pm 1.2^{B,b}$
CSL Medium					
Cell mass yield (g/mol) [i]	$1.3 \pm 0.0^{C,a}$	$1.5 \pm 0.1^{A,a}$	$1.2 \pm 0.0^{D,a}$	$1.4 \pm 0.1^{B,a}$	$1.1 \pm 0.1^{E,a}$
Ethanol yield (%) [ii]	$98.1 \pm 0.9^{A,a}$	$96.8 \pm 0.7^{B,b}$	$85.8 \pm 0.8^{E,b}$	$89.7 \pm 1.4^{C,a}$	$86.5 \pm 1.0^{D,c}$
Butanol yield (%) [ii]	$25.7 \pm 1.1^{B,a}$	$17.1 \pm 0.9^{D,a}$	$15.8 \pm 0.3^{E,b}$	$18.4 \pm 0.3^{C,a}$	$30.7 \pm 1.4^{A,a}$
Hexanol yield (%) [ii]	$12.3 \pm 1.7^{B,a}$	$3.7 \pm 0.2^{E,a}$	$8.2 \pm 0.4^{C,a}$	$3.9 \pm 0.2^{D,a}$	$25.6 \pm 0.9^{A,a}$
EtOH/HAc (mol/mol) [iii]	$1.5 \pm 0.0^{A,b}$	$1.3 \pm 0.0^{C,b}$	$1.0 \pm 0.0^{E,b}$	$1.3 \pm 0.0^{B,b}$	$1.20\pm 0.0^{D,c}$
BuOH/HBua (mol/mol) [iii]	$1.5 \pm 0.0^{B,b}$	$1.2 \pm 0.0^{C,a}$	$0.6 \pm 0.0^{E,b}$	$1.6 \pm 0.1^{A,b}$	$1.1 \pm 0.1^{D,a}$
HeOH/Hhex (mol/mol) [iii]	$1.2 \pm 0.1^{A,a}$	$0.8 \pm 0.0^{C,a}$	$0.5 \pm 0.0^{D,b}$	$0.5 \pm 0.0^{D,b}$	$1.1 \pm 0.0^{B,a}$
Total alcohols (g/L)	$7.9 \pm 0.0^{B,a}$	$8.7 \pm 0.1^{A,a}$	$5.0 \pm 0.0^{E,b}$	$6.5 \pm 0.0^{C,a}$	$6.2 \pm 0.1^{D,c}$
Total acids (g/L)	$7.0 \pm 0.1^{B,a}$	$9.0 \pm 0.2^{A,a}$	$6.8 \pm 0.0^{D,a}$	$6.7 \pm 0.1^{E,a}$	$6.8 \pm 0.1^{C,a}$
Sp. alcohol yield (g_{alcol}/g_x)	$14.0 \pm 0.3^{B,b}$	$14.4 \pm 0.2^{B,b}$	$13.2 \pm 0.1^{C,b}$	$11.0 \pm 0.1^{D,b}$	$16.3 \pm 0.8^{A,b}$
Sp. acid yield (g_{acid}/g_x)	$12.3 \pm 0.4^{C,b}$	$14.9 \pm 0.4^{B,a}$	$17.7 \pm 0.2^{A,a}$	$11.3 \pm 0.2^{D,b}$	$17.8 \pm 0.9^{A,a}$
CO consumption (%)	$41.2 \pm 1.0^{B,b}$	$48.0 \pm 0.9^{A,a}$	$36.3 \pm 0.6^{C,b}$	$42.8 \pm 0.8^{B,a}$	$35.2 \pm 0.8^{D,c}$
H_2 consumption (%)	$32.9 \pm 1.0^{C,a}$	$45.6 \pm 0.9^{A,b}$	$22.0 \pm 1.0^{E,b}$	$34.8 \pm 0.8^{B,a}$	$25.3 \pm 1.1^{D,c}$

No significant differences ($p > 0.05$) between strains in the same medium share the same capital letter in each row, while no significant differences ($p > 0.05$) for the same strain between three media are indicated by the same small letter in each row. [i] Estimated at highest cell mass concentration. For P7 medium → strains P11 and P7 at 120 h, strains P14, P20 at 144 h, strain P21 at 196 h. For P11 medium → all strains at 120 h. For CSL medium → strains P11, P7, P20 and P21 at 96 h, strain P14 at 120 h. [ii] CO consumed and calculated over 360 h. [iii] EtOH/HAc (ethanol/acetic acid); BuOH/HBua (butanol/butyric acid); HeOH/Hhex (hexanol/hexanoic acid).

3.2. Syngas Fermentation in P11 Medium

All strains grew on syngas in the P11 medium and produced C2–C6 products except strain P11, which produced only C2 products (Figure 2). P11 medium contains higher levels of vitamins, Zn, Ni, Se, and W in comparison with P7 medium (Table 1). The initial pH of the cultures was 5.8 (Figure 2A). The pH changes in the P11 medium for all strains

were nearly identical. When the pH in the P11 medium with all strains was below 5, it was adjusted back to 5.1 using NH_4OH (10%). The growth patterns observed in the P7 medium (Figure 1B) and the P11 medium (Figure 2B) were similar, with more growth observed in the P11 medium (Table 3). The highest cell mass in the P11 medium was achieved by strains P11 and P21.

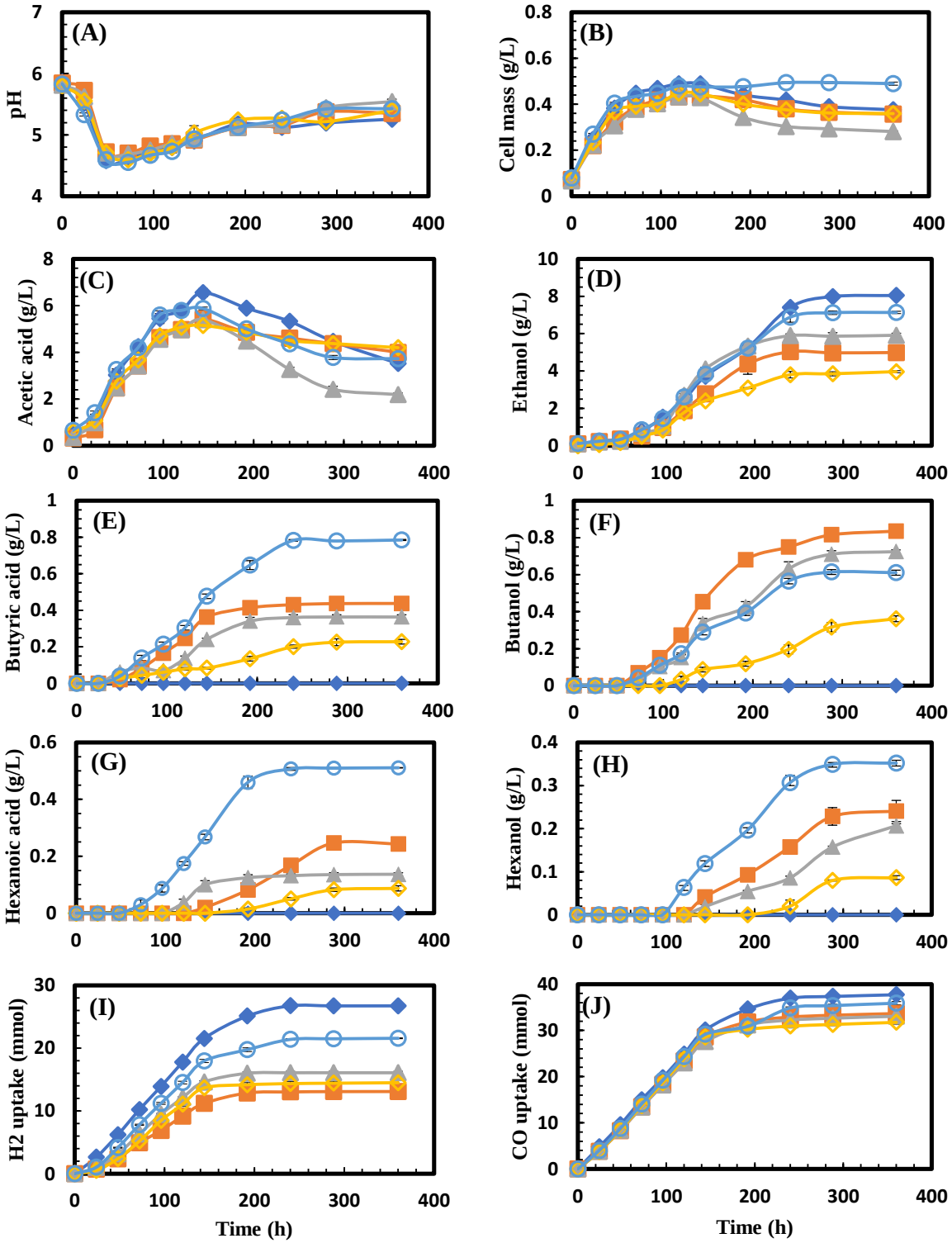

Figure 2. Profiles of syngas fermentation in P11 medium by strains P7 [■], P11 [◆], P14 [▲], P20 [◊], and P21 [○]. (**A**) pH; (**B**) cell mass; (**C**) acetic acid; (**D**) ethanol; (**E**) butyric acid; (**F**) butanol; (**G**) hexanoic acid; (**H**) hexanol; (**I**) cumulative H_2 uptake; (**J**) cumulative CO uptake.

Strain P11 demonstrated a peak acetic acid concentration (6.6 g/L) at 144 h in the P11 medium, significantly ($p < 0.05$) more than with other strains (Figure 2C). After 144 h, all strains exhibited a gradual decrease in acetic acid concentration, likely due to its conversion into ethanol. Strain P11 also exhibited the highest ethanol production (8.0 g/L) in the P11 medium, which was 1.2- to 2-fold more ($p < 0.05$) than that of the other strains (Figure 2D). Table 3 further shows that strain P11 had a higher ($p < 0.05$) ethanol yield at about 97%, with an ethanol to acetic acid ratio of 3.0 mmol/mmol compared to the other strains. Strain P11 only produced C2 compounds in the P11 medium, whereas the other four strains produced C2–C6 products. Among these strains, strain P7 exhibited the highest butanol production (Figure 2F). However, strain P21 had the highest concentrations of butyric acid (0.8 g/L), hexanoic acid (0.5 g/L), and hexanol (0.4 g/L) in the P11 medium. Strain P20 produced the lowest amounts of C4 and C6 compounds (Figure 2E–H).

Strains P11 and P21 produced similar amounts of total alcohol ($p > 0.05$) in P11 medium (Table 3). However, strain P11 produced only ethanol, while strain P21 produced ethanol, butanol and hexanol. Furthermore, strain P21 in the P11 medium exhibited significantly more ($p < 0.05$) total acid formation than with other strains. In terms of gas uptake, strain P11 had higher ($p < 0.05$) cumulative H_2 uptake (26.7 mmol) than other strains in the P11 medium, while strain P7 had the lowest H_2 uptake (13.1 mmol), as shown in Figure 2I. Similarly, strain P11 had the highest CO uptake (37.7 mmol), while strain P20 had the lowest CO uptake (31.7 mmol) (Figure 2J). Additionally, strain P11 in the P11 medium showed higher ($p < 0.05$) CO and H_2 conversion efficiencies in comparison to other strains (Table 3).

3.3. Syngas Fermentation in CSL Medium

Figure 3 shows the fermentation profiles during syngas consumption in a CSL medium. The initial pH in the CSL medium for all strains was 5.8 (Figure 3A). The pH remained relatively stable until 24 h, after which strains P11, P20, and P7 exhibited a rapid decrease in pH from 5.7 to 4.6 between 48 and 96 h. Except for strain P21, the pH of the cultures with the other strains dropped below pH 5 between 48 and 144 h and was subsequently adjusted to 5.1 using 10% NH_4OH. The profiles of cell mass concentration for strains P11, P7, and P20 in the CSL medium were similar (Figure 3B), with higher cell mass measured compared to strains P14 and P21 (Table 3).

Unlike P7 or P11 media (Figures 1 and 2), all strains formed C2–C6 acids and alcohols in the CSL medium (Figure 3C–H). Strain P11 in the CSL medium produced more ($p < 0.05$) acetic acid (8.6 g/L) than other strains (Figure 3C). Furthermore, strain P11 produced significantly more ($p < 0.05$) ethanol (8.1 g/L) than strain P7 (7.0 g/L), strain P14, P21 (4.6 g/L), and strain P20 (6.1 g/L). Ethanol yields (>95%) were the highest for strains P7 and P11 in the CSL medium (Table 3).

Among the tested strains, P21 produced more ($p < 0.05$) butanol (0.9 g/L), butyric acid (1.0 g/L), hexanol (0.7 g/L), and hexanoic acid (0.7 g/L), indicating its superior production ability of C4–C6 products. Conversely, strain P11, known for its ethanol production, produced butanol (0.5 g/L) and hexanol (0.1 g/L) in the CSL medium. Previous reports have highlighted strain P11's potential to produce C4 and C6 alcohols using CSL as a medium [20]. Additionally, strain P11 exhibited higher H_2 and CO uptakes (25.8 mmol and 38.15 mmol, respectively) ($p < 0.05$) in the CSL medium (Figure 3I,J), along with H_2 and CO conversion efficiencies of 46% and 48%, respectively, surpassing the other strains (Table 3). The 20 g/L CSL medium initially contained 4.3 g/L of sugar. In the first 72 h, 85% of the sugars were consumed by all strains, with minimal consumption observed in subsequent measurements (data not presented). Assuming 85% sugar utilization for ethanol production, the strains can yield a maximum of 1.8 g/L, representing about 12% of the total products generated. This emphasizes that syngas resulted in most product formation. Furthermore, distinguishing the extent to which growth and products originate from the consumption of sugars or syngas is challenging. The availability of amino acids

and other essential nutrients in CSL, including some sugars, further enhanced *Clostridium* strains' ability to form higher alcohols [19,20,24].

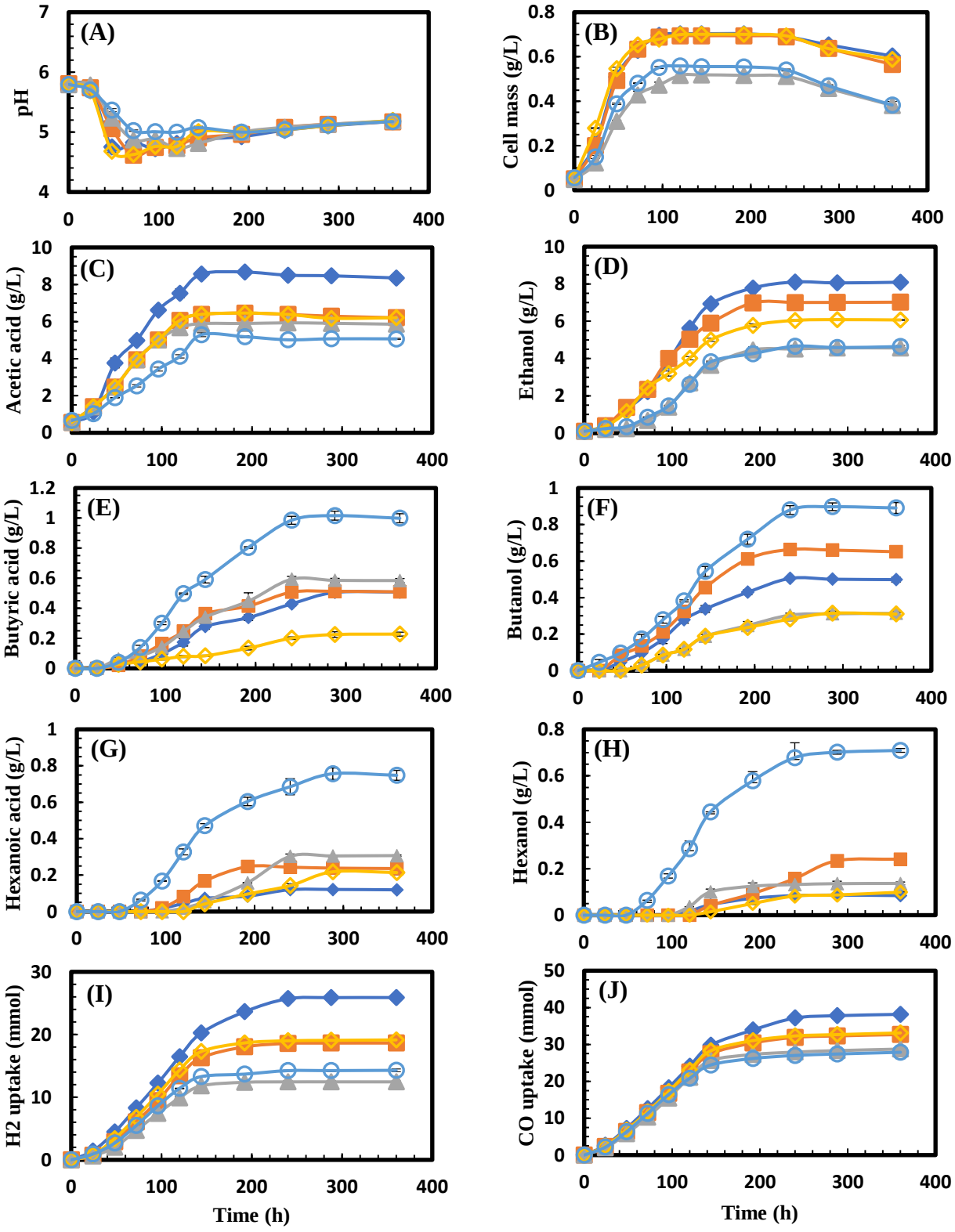

Figure 3. Profiles of syngas fermentation in CSL medium by strains P7 [■], P11 [◆], P14 [▲], P20 [◊], and P21 [○]. (**A**) pH; (**B**) cell mass; (**C**) acetic acid; (**D**) ethanol; (**E**) butyric acid; (**F**) butanol; (**G**) hexanoic acid; (**H**) hexanol; (**I**) cumulative H_2 uptake; (**J**) cumulative CO uptake.

All strains successfully converted syngas into C2, C4, and C6 alcohols and acids using mainly P11 and CSL media. However, the concentrations of these compounds varied among the strains and type of medium. CSL medium provided the highest growth potential for all strains (Table 3). P11 medium exhibited the second highest growth, while P7 medium supported the least growth among the tested strains. Strain P11 exhibited robust ethanol production, especially in P11 and CSL media, with ethanol yield above 95% (Table 3). However, ethanol concentration with strain P11 in the P7 medium was significantly lower ($p < 0.05$), about 3-fold lower than in P11 or CSL media, likely due to the lack of certain vitamins and other nutrients.

P7 medium contained lower concentrations of Se (5X), W (10X), Ni (10X), and Zn (5X) compared to P11 medium [19]. For example, the increase in Se, W, Ni, and Zn concentration in the P11 medium was reported to increase ethanol production by strain P11 by 2- to 5-fold compared to a base medium [19]. Details on the effect of these elements on the growth and solvent production of acetogens, like strains P7 and P11, were reported previously [7,20]. As shown in Table 3, all strains except strain P21 exhibited higher total alcohol formation in the CSL medium than in either the P7 or P11 medium. However, ethanol remained the dominant alcohol produced by all strains in all three media. P11 and CSL media, with more nutrients, facilitated higher butanol and hexanol production compared to the P7 medium [19,20]. Strain P21 demonstrated specific total alcohol yields from 16 to 19 g/g dry cell weight, surpassing other strains, especially in P7 and CSL media (Table 3). Strain P21 demonstrated the highest specific total acid yield (17.8 g/g dry cell) in the CSL medium. Strain P14 in the P11 medium showed the highest specific total alcohol yield (24.3 g/g dry cells), followed by strain P11. Strain P20 exhibited a specific total alcohol yield of 11–13 g/g dry cells, performing similarly in all media. All strains except P14 and P21 produced total alcohols in the CSL medium, with generally lower alcohol production in the P7 medium. In both CSL and P11 media, all strains achieved 2- to 4.5-fold greater specific total alcohol production than previously reported for strain P11 [20]. Additionally, the specific total alcohol yields of the five strains in the present study were 1- to 4-fold higher than reported previously in the P7 medium [8]. Total acids made by all strains were consistently higher in the CSL medium (Table 3). While strain P11 in the CSL medium yielded the highest total acid concentration, the maximum specific total acid production (17.8 g acid/g dry mass) was measured in the CSL medium with strains P14 and P21 (Table 3).

There was a net CO_2 production during syngas fermentation by all strains in the three media (Figure 4). CO_2 was formed from CO and H_2 utilization, where CO is used as a carbon and energy source. Acetogens prefer utilizing CO and H_2 over CO_2 and H_2 due to thermodynamic favorability [3]. Their gas preference is influenced by their metabolic capabilities, environmental conditions such as gas partial pressure and pH, and the presence of specific enzymes for gas utilization [3,39]. The lower H_2 consumption compared to CO (Figure 1G,H, Figure 2I,J and Figure 3I,J) can be due to hydrogenase inhibition by CO and the thermodynamic disadvantage of H_2 utilization with the presence of CO [39]. The CO_2 production profiles in Figure 4 align with the observed CO uptake profiles for all strains in the three media.

Figure 5 shows the maximum product titers and cumulative gas uptake. Strain P11 in P11 and CSL media demonstrated the highest ethanol production, which was 3-fold more than in the P7 medium. The top butanol producers were strains P7 and P21, each producing about 0.9 g/L butanol. In the P11 medium, strain P7 produced 6-fold more butanol than in the P7 medium and 1.3-fold more than in the CSL medium. The highest butanol titers were formed by strain P21 in the CSL medium. Moreover, strain P21 produced the highest amounts of butyric acid, hexanol, and hexanoic acid in the CSL medium (Figure 5). None of the strains produced C6 products in the P7 medium. Strains P14 and P20 produced C2–C6 alcohols in P11 and CSL media. Strain P14 produced 1.3-fold more ethanol, 2-fold more butanol, and 1.5-fold more hexanol in the P11 medium than in the CSL medium (Figure 5). Strain P20 in the CSL medium produced 1.4-fold more ethanol than in the P11 medium. However, butanol and hexanol production by strain P20 were almost identical in P11 and

CSL media. Strain P11 uptake of CO and H_2 was higher ($p < 0.05$) in P11 and CSL media than in P7 medium (Figure 5). Strain P21 demonstrated similar gas uptake in P7 and P11 media, which were slightly higher than in the CSL medium.

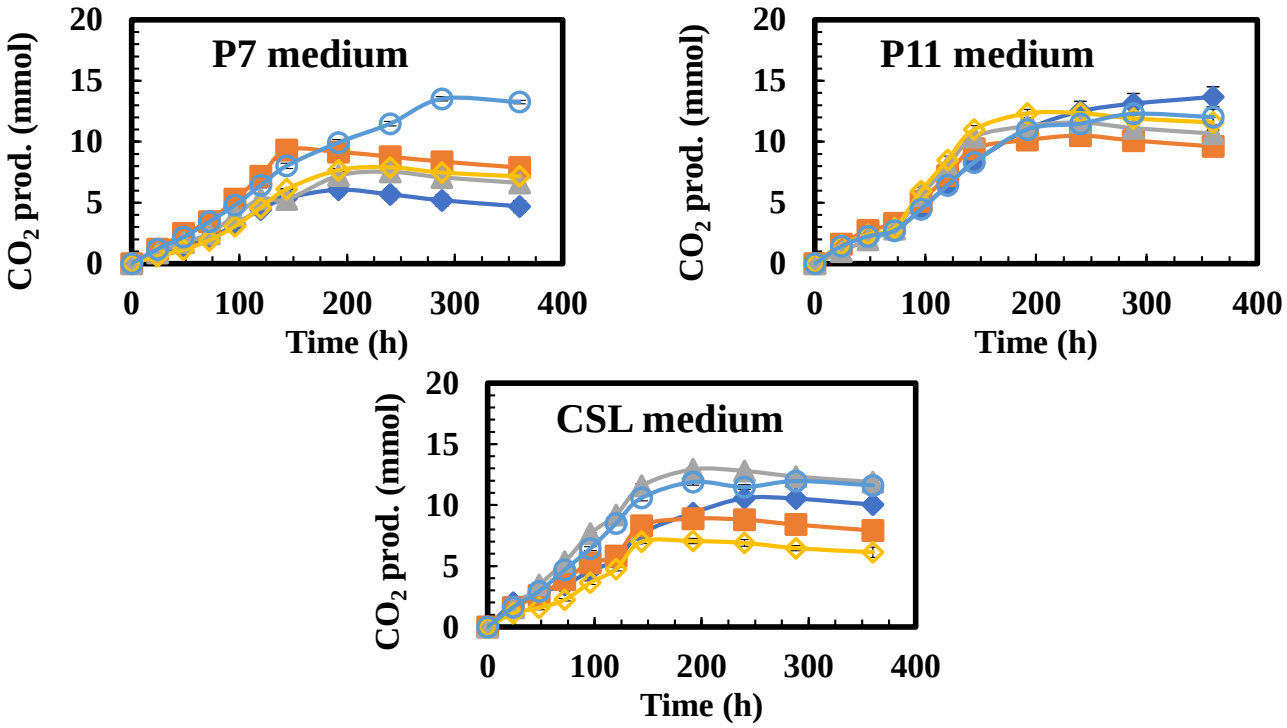

Figure 4. Profiles of cumulative CO_2 formation during syngas fermentation in P7, P11, and CSL media by strains P7 [■], P11 [◆], P14 [▲], P20 [◊], and P21 [○].

Strain P11 is known as one of the best ethanol producers in gas fermentations, achieving high yield in various media: 9.6 g/L ethanol in CSL medium in a 3-L CSTR [20], 13 g/L ethanol in P11 medium with biochar [26] and 20 g/L ethanol in P11 medium supplemented with activated carbon [6]. Ethanol production in the bottles by strain P11 (8.1 g/L) in the present study was 1.7- to 4-fold higher than previously reported [9,20]. In addition, ethanol production by strain P11 in the bottles in the present study was 6-fold and 3.5-fold higher than produced by *Alkalibaculum bacchi* strain CP15 in yeast extract and CSL media [40] and 1.5-fold higher than by strain P11 in a trickle bed reactor with yeast extract medium [41]. Similar to the findings in the present study, the CSL medium enabled strain P11 to produce 0.5 g/L butanol and 0.1 g/L hexanol from syngas [20].

Similarly, strain P7 is known for its ability to produce butanol from syngas, in addition to ethanol. In P11 and CSL media, strain P7 achieved higher ethanol titers (5 g/L and 7 g/L, respectively) than in P7 medium. In this study, ethanol formed by strain P7 in P11 and CSL media was 1.5- to 2-fold higher than the previous reports in the P7 medium [7] and in the P7 medium supplemented with biochar [8,9]. Additionally, strain P7 showed a remarkable 9-fold increase in butanol production compared to *A. bacchi* CP15 in CSL medium [40] and a 1.4-fold increase compared to P7 medium supplemented with biochar [9]. The butanol titer (0.9 g/L) formed by strain P7 in the P7 medium in the present study was consistent with a previous report [7].

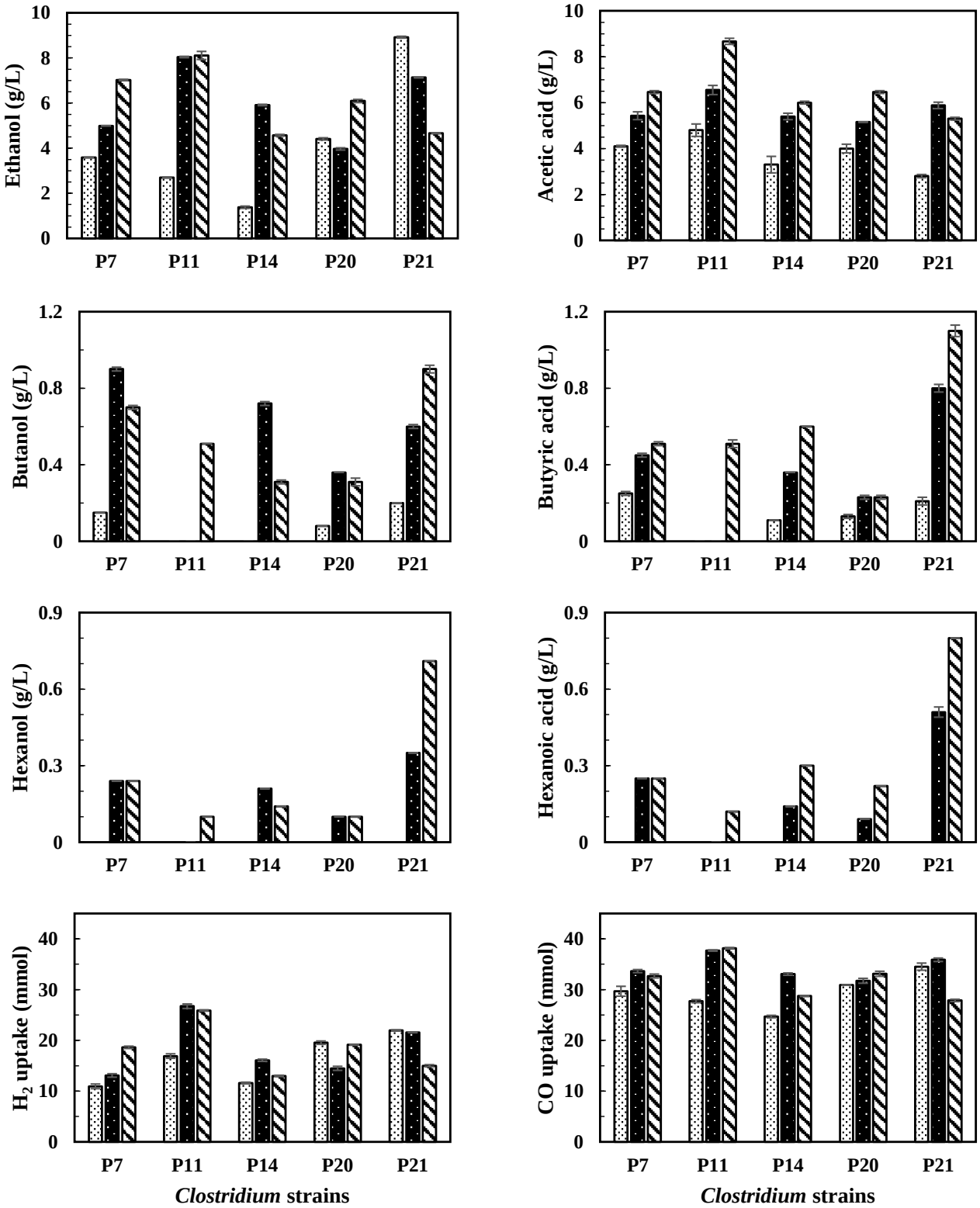

Figure 5. Maximum product concentrations and cumulative gas uptake during syngas fermentation in P7 medium ( ), P11 medium ( ), and CSL medium ( ).

The new *C. ljungdahlii* P14 and *C. carboxidivorans* P20 strains showed abilities to make C2–C6 products (Figure 5). While both strains produced similar amounts of C2 products in each medium, strain P14 produced more C4 and C6 products than strain P20. The maximum ethanol synthesized by strain P14 (6 g/L) in the P11 medium in this study was

comparable to strain P11 reported by [41], 3-fold more than strain P7 in the P7 medium [23] and 6-fold higher than *C. autoethanogenum* in the modified mineral medium reported by [42]. Further, strain P14's highest butanol titer (0.7 g/L) in the P11 medium was 1.1-fold more than strain P7 in a P7 medium supplemented by biochar [9], 1.4-fold higher than genetically modified strain P7 [23] and 4- to 6-fold higher than genetically modified *C. ljungdahlii* in complex yeast extract, tryptone, fructose medium [43]. With further development, the new *C. ljungdahlii* strain P14 can potentially compete with strains P7, P11, and genetically modified *C. ljungdahlii* strains in producing C2 and C4 products from syngas. This is achieved through medium modification, biochar supplementation, and genetic engineering to increase selectivity for specific alcohols and improve titer.

C. carboxidivorans P20 produced 2- to 3-fold more ethanol from syngas than previous studies with strain P11 in CSL medium [20] and cotton seed extract medium [44]. However, strain P20 butanol and hexanol production abilities were 2- to 3-fold and 2.4- to 7-fold lower, respectively, compared to strains P7, P14 and P21 (Figure 5). Additional improvements, such as adding sugars and biochar to the medium or increasing headspace pressure, could enhance strain P20's ability to produce C4 and C6 alcohols. *C. muellerianum* P21 showed great promise for the production of C4 and C6 alcohols. In the CSL medium, it achieved the highest butanol (0.9 g/L) and hexanol (0.7 g/L) titers from syngas. Moreover, strain P21 produced 1.4-fold more butanol from syngas than strain P7 in a P7 medium supplemented with biochar [9]. Strain P21 also produced similar amounts of hexanol from syngas reported in a previous study using strain P7 with temperature variance [45]. However, strain P21 showed slightly lower hexanol production from syngas compared to strain P7 in previous studies, particularly those utilizing extractive syngas fermentation [32,46–48].

In contrast to CO_2 fermentation in P7, P11, and CSL media using a gas mixture (H_2:CO_2:N_2 60:20:20) [36], the five strains in the present study produced 2-fold more ethanol and comparable amounts of butanol and hexanol from syngas. Furthermore, the CSL medium yielded the highest total alcohol and acid titers with syngas in the current study and previously reported CO_2 fermentation [36].

The results highlighted the influence of medium composition on *Clostridium* strains' growth and syngas fermentation capabilities, especially for making higher-chain fatty acids and alcohols via the acetyl-CoA pathway. The new P14, P20, and P21 strains demonstrated the potential to produce C4 and C6 alcohols. Differences in vitamin and nutrient content in the P7 medium might have limited C6 product formation (Table 1). P11 medium, with more vitamins and specific trace metals, enhanced C_2, C_4, and C_6 product titers, particularly with the new strains. Utilizing CSL as a nutrient-rich source instead of yeast extract improved cell mass and alcohol titers while reducing cost. Further development, medium formulation improvements, and characterization of the new strains, especially P21, are needed to enhance the strain's activity and selectivity in converting syngas into C4 and C6 alcohols.

4. Conclusions

Clostridium muellerianum P21 was the best butanol and hexanol producer from syngas, particularly in CSL medium, while *C. ragsdalei* P11 showed the highest ethanol production. *C. carboxidivorans* P7, *C. ljungdahlii* P14, *C. carboxidivorans* P20, and *C. muellerianum* P21 demonstrated potential in generating C4 and C6 products in P11 and CSL media. CSL medium supported higher cell mass, alcohol titers, and gas conversion compared to the P7 medium. The highest ethanol (8.0 g/L) was produced by strain P11 in P11 and CSL media, which was 3-fold more than in P7 medium. Strain P21 achieved ethanol, butanol, and hexanol yields of 87%, 31%, and 26%, respectively, in the CSL medium. These results confirm the viability of the novel strains, particularly strain P21, and the efficacy of CSL medium for C4 and C6 alcohol synthesis from syngas.

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