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Experimental Design to Improve Cell Growth and Ethanol Production in Syngas Fermentation by *Clostridium carboxidivorans*

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Abstract: Fermentation of gases from biomass gasification, named syngas, is an important alternative process to obtain biofuels. Sequential experimental designs were used to increase cell growth and ethanol production during syngas fermentation by *Clostridium carboxidivorans*. Based on ATCC (American Type Culture Collection) 2713 medium composition, it was possible to propose a best medium composition for cell growth, herein called TYA (Tryptone-Yeast extract-Arginine) medium and another one for ethanol production herein called TPYGarg (Tryptone-Peptone-Yeast extract-Glucose-Arginine) medium. In comparison to ATCC® 2713 medium, TYA increased cell growth by 77%, reducing 47% in cost and TPYGarg increased ethanol production more than four-times, and the cost was reduced by 31%. In 72 h of syngas fermentation in TPYGarg medium, 1.75-g/L of cells, 2.28 g/L of ethanol, and 0.74 g/L of butanol were achieved, increasing productivity for syngas fermentation.

Keywords: syngas fermentation; *Clostridium carboxidivorans*; biomass production; ethanol; experimental design; anaerobic process

1. Introduction

The world growing energy demand has incited important discussions about the current energy system based on fossil fuels. Alternative energy sources from renewable resources are needed mainly due to ever-higher amounts of greenhouse gas emissions and oil price fluctuation [1].

In this context, biofuels, especially ethanol and butanol, represent potential substitutes of fossil fuels, such as gasoline. Currently, those alcohols are produced through fermentation processes, mostly using sugarcane and corn as the raw material. The major disadvantage of these processes is the competition between food and fuel production [2]. Lignocellulosic feedstocks from agricultural waste or directly from energy crops emerge as alternative raw materials for biofuel production as they are inexpensive, renewable, and do not affect food supply [3,4].

The biochemical conversion of lignocellulosic feedstocks into biofuels by anaerobic bacteria, such as *Clostridium* species, produces mainly acetone, butanol, and ethanol (ABE), through ABE fermentation [5]. However, a complex pre-treatment is required to obtain simple sugars from the polymeric structure of lignocellulose [6]. An alternative destination for lignocellulosic feedstocks involves biomass gasification, which eliminates the pre-treatment step. In this case, full use of lignocellulosic biomass, including lignin, is possible through thermochemical conversion. Besides,

urban solid residues can also be used as feedstock for gasification, with the advantage of reducing environmental impacts. The biomass gasification results in synthesis gas mainly composed of CO (carbon monoxide), CO_2 (carbon dioxide), and H_2 (hydrogen). Synthesis gas, also named syngas, obtained from gasification, can be converted into ethanol and higher alcohols as butanol and hexanol, through a hybrid thermo/biochemical process. This conversion can be performed by *Clostridum* sp., being *Clostridium ljungdahlii*, *C. carboxidivorans*, *C. autoethanogenum*, and *C. rasgdalei* the most studied species. These species use the acetyl-CoA metabolic pathway, also known as Wood–Ljungdahl pathway, for acetyl-CoA synthesis, energy conservation, and alcohol and acid production [7].

A current problem of anaerobic bioprocesses is low cell density, which reduces productivity [8]. Usually, media composition used in CO/CO_2 fermentation is similar to sugar-based media for *Clostridium* sp. growth and butanol production. Numerous and expensive components are mixed to provide essential metals, vitamins, minerals, and nitrogen needed for cell growth and metabolism [9]. As consequence, the preparation of those media requires intensive labor and is expensive. Replacing these compounds by cheaper and complex sources is an important challenge. ATCC® medium 2713 (Wilkins Chalgren Anaerobic Medium) is indicated by American Type Culture Collection (ATCC) for *Clostridium carboxidivorans* activation and growth and ATCC® medium 1754 (without fructose) is another common medium used for syngas fermentation by *Clostridium carboxidivorans*, reported by several authors [10–14]. Low specific growth rates, ranging from 0.005 to 0.08 h^{-1} were reported for those media with gaseous or soluble substrates [5,15–17]. Studies using glucose-rich medium or only carbon monoxide as the carbon source have been carried out to evaluate the effect of the carbon source on cell growth and solvents production [5,15,17–21] and the maximum values for cell density and specific growth rate were 0.55 g dry weight of cells/L and 0.231 h^{-1}, respectively. In addition, the effects of trace metals on product formation were assessed [1,22–26] and the maximum ethanol production reported was 3.5 g/L after 72 h of fermentation [22].

Therefore, the goal of this work was to obtain a low-cost culture medium, reduced also in number of components, by a sequence of experimental designs in order to improve cell growth and solvents production by *Clostridium carboxidivorans* using glucose and syngas as carbon sources.

2. Results

2.1. Nutrient Screening by Placket–Burman (PB) Design

Syngas fermentation by *C. carboxidivorans* must be supplemented with nutrient media containing nitrogen, vitamins, and ions sources. The American Type Culture Collection (ATCC) indicates ATCC® medium 2713 Wilkins Chalgren Anaerobic Medium for *C. carboxidivorans* growth, which is composed of tryptone, 10 g/L; gelatin peptone, 10 g/L; yeast extract, 5 g/L; glucose, 1 g/L; sodium chloride, 5 g/L; L-arginine, 1 g/L; sodium pyruvate, 1 g/L; menadione, 0.5 mg/L; and hemin, 5 mg/L. These components were screened by a PB experimental design using ATCC medium concentrations as the higher level (+1) and absence (zero) as lower level (−1).

For ethanol production, glucose was maintained at 1.0 g/L because of its importance as a simple sugar to induce microbial metabolism, and the results are present in Table 1.

Ethanol production ranged between 0.03 and 1.92 g/L (Table 2). Analysis of variance (ANOVA) and the significance of the results were verified using an *F*-test at 10% of significance. The components that influence ethanol production can be observed in the Pareto diagram with the bars that extend beyond the red vertical line (Figure 1). Tryptone, gelatin peptone, and L-arginine influence *C. carboxidivorans* ethanol production positively.

Table 1. Placket–Burman design matrix with real and coded values for *C. carboxidivorans* syngas fermentation with 1 g/L glucose. Response variables: ethanol production—P_{EtOH}(g/L).

Run	Real Values (Corresponding Coded Levels)								P_{EtOH}
	TRYP [1]	PEP [2]	YE [3]	NaCl [4]	L-ARG [5]	PYR [6]	MEN [7]A	HEM [8]	
1	10(1)	0(−1)	5(1)	0(−1)	0(−1)	0(−1)	0.5(1)	5(1)	0.79
2	10(1)	10(1)	0(−1)	5(1)	0(−1)	0(−1)	0(−1)	5(1)	0.90
3	0(−1)	10(1)	5(1)	0(−1)	1(1)	0(−1)	0(−1)	0(−1)	0.69
4	10(1)	0(−1)	5(1)	5(1)	0(−1)	1(1)	0(−1)	0(−1)	0.78
5	10(1)	10(1)	0(−1)	5(1)	1(1)	0(−1)	0.5(1)	0(−1)	1.21
6	10(1)	10(1)	5(1)	0(−1)	1(1)	1(1)	0(−1)	5(1)	1.87
7	0(−1)	10(1)	5(1)	5(1)	0(−1)	1(1)	0.5(1)	0(−1)	0.51
8	0(−1)	0(−1)	5(1)	5(1)	1(1)	0(−1)	0.5(1)	5(1)	0.25
9	0(−1)	0(−1)	0(−1)	5(1)	1(1)	1(1)	0(−1)	5(1)	0.03
10	10(1)	0(−1)	0(−1)	0(−1)	1(1)	1(1)	0.5(1)	0(−1)	0.99
11	0(−1)	10(1)	0(−1)	0(−1)	0(−1)	1(1)	0.5(1)	5(1)	0.51
12	0(−1)	0(−1)	0(−1)	0(−1)	0(−1)	0(−1)	0(−1)	0(−1)	0.04
13 (C)	5(0)	5(0)	2.5(0)	2.5(0)	0.5(0)	0.5(0)	0.25(0)	2.5(0)	1.65
14 (C)	5(0)	5(0)	2.5(0)	2.5(0)	0.5(0)	0.5(0)	0.25(0)	2.5(0)	1.92
15 (C)	5(0)	5(0)	2.5(0)	2.5(0)	0.5(0)	0.5(0)	0.25(0)	2.5(0)	1.79

[1] TRYP: tryptone concentration (g/L); [2] PEP: peptone concentration (g/L); [3] YE: yeast extract concentration (g/L); [4] NaCl: sodium chloride concentration (g/L); [5] L-ARG: L-arginine concentration (g/L); [6] PYR: sodium pyruvate concentration (g/L0; [7] MEN: menadione concentration (mg/L); [8] HEM: hemin concentration (mg/L); (C) central point.

Table 2. Placket–Burman design matrix with real and coded values for *C. carboxidivorans* syngas fermentation with 1 g/L glucose. Response variables: biomass concentration—[X_{24}] (g d.w.cells/L)—and maximum ethanol production—P_{EtOH} (g/L).

Run	Real Values (Corresponding Coded Levels)									[X_{24}]
	TRYP [1]	PEP [2]	YE [3]	NaCl [4]	GLU [5]	ARG [6]	PYR [7]	MEN [8]	HEM [9]	
1	0(−1)	0(−1)	0(−1)	0(−1)	0(−1)	0(−1)	0(−1)	0(−1)	5(+1)	0.044
2	10(+1)	0(−1)	0(−1)	0(−1)	1(+1)	0(−1)	1(+1)	0.5(+1)	0(−1)	0.400
3	0(−1)	10(+1)	0(−1)	0(−1)	1(+1)	1(+1)	0(−1)	0.5 (+1)	0(−1)	0.423
4	10(+1)	10(+1)	0(−1)	0(−1)	0(−1)	1(+1)	1(+1)	0(−1)	5(+1)	0.735
5	0(−1)	0(−1)	5(+1)	0(−1)	1(+1)	1(+1)	1(+1)	0(−1)	0(−1)	0.407
6	10(+1)	0(−1)	5(+1)	0(−1)	0(−1)	1(+1)	0(−1)	0.5(+1)	5(+1)	0.714
7	0(−1)	10(+1)	5(+1)	0(−1)	0(−1)	0(−1)	1(+1)	0.5(+1)	5(+1)	0.606
8	10(+1)	10(+1)	5(+1)	0(−1)	1(+1)	0(−1)	0(−1)	0(−1)	0(−1)	0.868
9	0(−1)	0(−1)	0(−1)	5(+1)	0(−1)	1(+1)	1(+1)	0.5(+1)	0(−1)	0.202
10	10(+1)	0(−1)	0(−1)	5(+1)	1(+1)	1(+1)	0(−1)	0(−1)	5(+1)	0.481
11	0(−1)	10(+1)	0(−1)	5(+1)	1(+1)	0(−1)	1(+1)	0(−1)	5(+1)	0.286
12	10(+1)	10(+1)	0(−1)	5(+1)	0(−1)	0(−1)	0(−1)	0.5(+1)	0(−1)	0.805
13	0(−1)	0(−1)	5(+1)	5(+1)	1(+1)	0(−1)	0(−1)	0.5(+1)	5(+1)	0.301
14	10(+1)	0(−1)	5(+1)	5(+1)	0(−1)	0(−1)	1(+1)	0(−1)	0(−1)	0.591
15	0(−1)	10(+1)	5(+1)	5(+1)	0(−1)	1(+1)	0(−1)	0(−1)	0(−1)	0.796
16	10(+1)	10(+1)	5(+1)	5(+1)	1(+1)	1(+1)	1(+1)	0.5(+1)	5(+1)	0.984
17 (C)	5(0)	5(0)	2.5(0)	2.5(0)	0.5(0)	0.5(0)	0.5(0)	0.25(0)	2.5(0)	0.701
18 (C)	5(0)	5(0)	2.5(0)	2.5(0)	0.5(0)	0.5(0)	0.5(0)	0.25(0)	2.5(0)	0.692
19 (C)	5(0)	5(0)	2.5(0)	2.5(0)	0.5(0)	0.5(0)	0.5(0)	0.25(0	2.5(0)	0.582

[1] TRYP: tryptone concentration (g/L); [2] PEP: peptone concentration (g/L); [3] YE: yeast extract concentration (g/L); [4] NaCl: sodium chloride concentration (g/L); [5] GLU: glucose (g/L); [6] ARG: L-arginine concentration (g/L); [7] PYR: sodium pyruvate concentration (g/L); [8] MEN: menadione concentration (mg/L); [9] HEM: hemin concentration (mg/L); (C) central point.

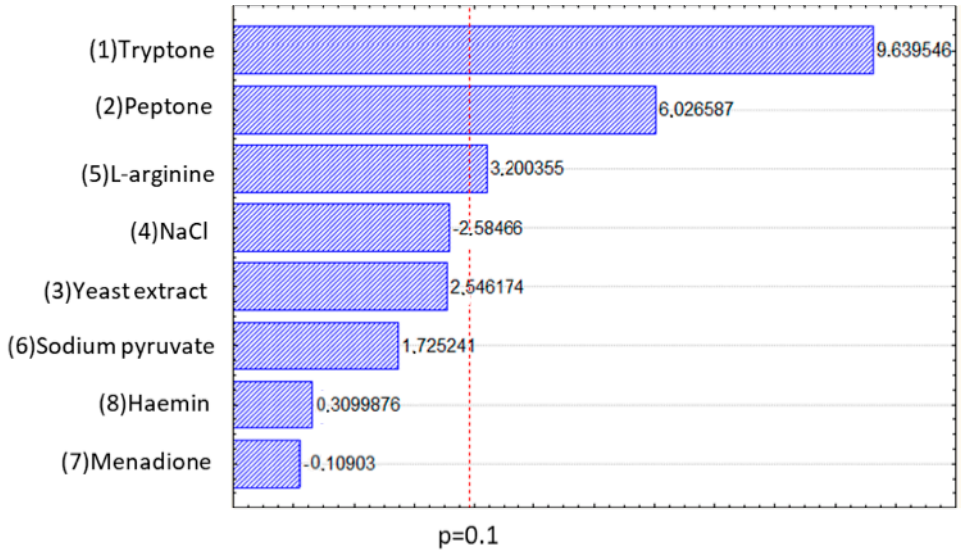


Figure 1. Pareto diagram for the estimated effect of each variable of the Placket–Burman design for *C. carboxidivorans* syngas fermentation with 1 g/L glucose: maximum ethanol production. The point at which the effects estimates were statistically significant (at $p = 0.1$) is indicated by the broken vertical line.

Biomass concentration after 24 h ([X_{24}]) (g d.w. cells/L) of fermentation was included as dependent variable in another PB design, in this case, including glucose as independent variable. The results are shown in Table 2.

Cell concentration ranged from 0.04 to 0.98 g d.w. cells/L, while deviation of center point was inferior to 10%, indicating a good reproducibility. Analysis of variance (ANOVA) and the significance of the results were verified using Fisher's statistical test (*F*-test) at 10% of significance. The Pareto diagram (Figure 2) shows tryptone, peptone, yeast extract, and L-arginine concentrations as the variables that influences cell growth, indicating a positive effect. Considering yeast extract is not only a vitamin source, but also a compound that contains carbon, nitrogen, and other elements, it is possible that it can replace other compounds for cell growth, as the results show.

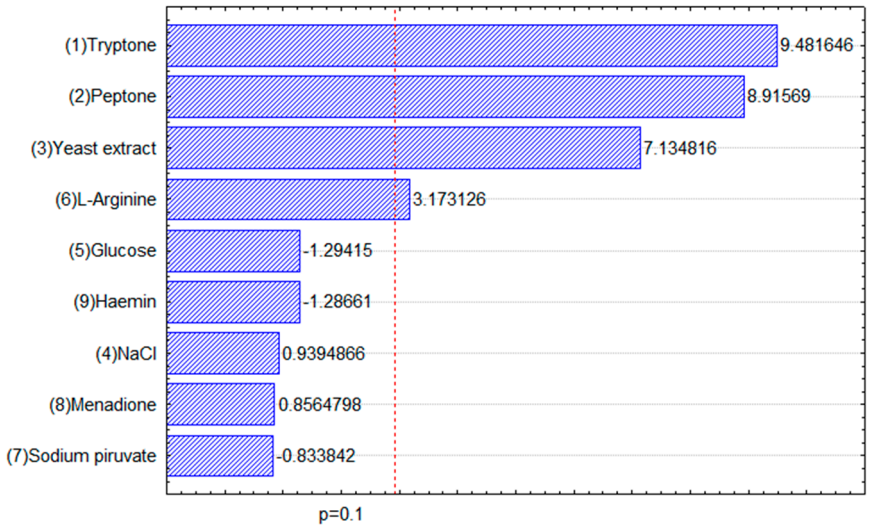


Figure 2. Pareto diagram for the estimated effect of each variable of the Placket–Burman design for *C. carboxidivorans* syngas fermentation on cell concentration ([X_{24}]). The point at which the effects estimates were statistically significant (at $p = 0.1$) is indicated by the broken vertical line.

Therefore, tryptone, peptone, yeast extract, and L-arginine were relevant to biomass production and they were all considered to optimize cell growth medium. For ethanol, tryptone, gelatin peptone, and L-arginine were investigated in the next steps, but yeast extract was also included since cell growth is also important during ethanol production.

2.2. Increasing Biomass Production by Central Composite Rotational Design (CCRD)

A central composite rotational design (CCRD) was considered in order to increase biomass production during syngas fermentation, excluding the components that were not relevant to cell growth according to PB design ($p > 0.1$). For this design, the concentrations of the selected variables were adjusted, as indicated by the effect of each one of them. Cell concentrations after 24 h of syngas fermentation by *C. carboxidivorans* at different conditions of CCRD are shown in Table 3.

Table 3. Central composite rotational design (CCRD) matrix with real and coded values and cell concentration after 24 h of syngas fermentation by *C. carboxidivorans*—[X_{24}] (g d.w. cells/L).

Run	Real Values (Coded Levels)				[X_{24}]
	TRY [1]	PEP [2]	YE [3]	ARG [4]	
1	8(−1)	8(−1)	3(−1)	0.8(−1)	0.99
2	8(−1)	8(−1)	3(−1)	1.2(+1)	0.85
3	8(−1)	8(−1)	7(+1)	0.8(−1)	1.05
4	8(−1)	8(−1)	7(+1)	1.2(+1)	1.05
5	8(−1)	12(+1)	3(−1)	0.8(−1)	1.24
6	8(−1)	12(+1)	3(−1)	1.2(+1)	1.03
7	8(−1)	12(+1)	7(+1)	0.8(−1)	0.88
8	8(−1)	12(+1)	7(+1)	1.2(+1)	1.29
9	12(+1)	8(−1)	3(−1)	0.8(−1)	0.99
10	12(+1)	8(−1)	3(−1)	1.2(+1)	1.22
11	12(+1)	8(−1)	7(+1)	0.8(−1)	1.17
12	12(+1)	8(−1)	7(+1)	1.2(+1)	1.36
13	12(+1)	12(+1)	3(−1)	0.8(−1)	1.02
14	12(+1)	12(+1)	3(−1)	1.2(+1)	1.02
15	12(+1)	12(+1)	7(+1)	0.8(−1)	1.29
16	12(+1)	12(+1)	7(+1)	1.2(+1)	1.26
17	6(−2)	10(0)	5(0)	1(0)	0.83
18	14(+2)	10(0)	5(0)	1(0)	1.23
19	10(0)	6(−2)	5(0)	1(0)	1.12
20	10(0)	14(+2)	5(0)	1(0)	1.20
21	10(0)	10(0)	1(−2)	1(0)	1.02
22	10(0)	10(0)	9(+2)	1(0)	1.35
23	10(0)	10(0)	5(0)	0.6(−2)	1.06
24	10(0)	10(0)	5(0)	1.4(+2)	1.07
25 (C)	10(0)	10(0)	5(0)	1(0)	1.06
26 (C)	10(0)	10(0)	5(0)	1(0)	1.16
27 (C)	10(0)	10(0)	5(0)	1(0)	1.06

[1] TRY: tryptone concentration (g/L), [2] PEP: gelatin peptone (g/L); [3] YE: yeast extract concentration (g/L); [4] ARG: L-arginine concentration (g/L). (C) Central point.

Table 3 shows that the cell concentration ranged from 0.83 to 1.35 g d.w. cells/L, both values higher than those found in the Placket–Burman design (Table 2), showing an improvement in biomass production by the statistical design. Analysis of variance (ANOVA) and the significance of the results were verified using Fisher's statistical test (*F*-test) at 10% of significance. The ANOVA (Table 4) showed that tryptone and yeast extract were relevant ($p < 0.1$) to cell growth. The complete predicted model was not significant (data not shown) and therefore it was improved by eliminating non-significant terms ($p > 0.1$). The ANOVA for the reduced model is presented in Table 4.

Table 4. Analysis of variance (ANOVA) for central composite rotational design (CCRD) for biomass production of syngas fermentation by *Clostridium carboxidivorans* after 24 h.

Factor	SS [1]	Df [2]	MS [3]	*F*-Value [4]	*p*-Value [5]
(1)Tryptone(L)	0.126702	1	0.126702	37.56348	0.025604
(3)Yeast extract(L)	0.117852	1	0.117852	34.93986	0.027448
(4)L-arginine(L)	0.008221	1	0.008221	2.43742	0.258861
1L by 3L	0.029087	1	0.029087	8.62357	0.099034
3L by 4L	0.029224	1	0.029224	8.66407	0.098638
Lack of Fit	0.225563	19	0.011872	3.51964	0.244182
Pure Error	0.006746	2	0.003373		
Total SS	0.543396	26			

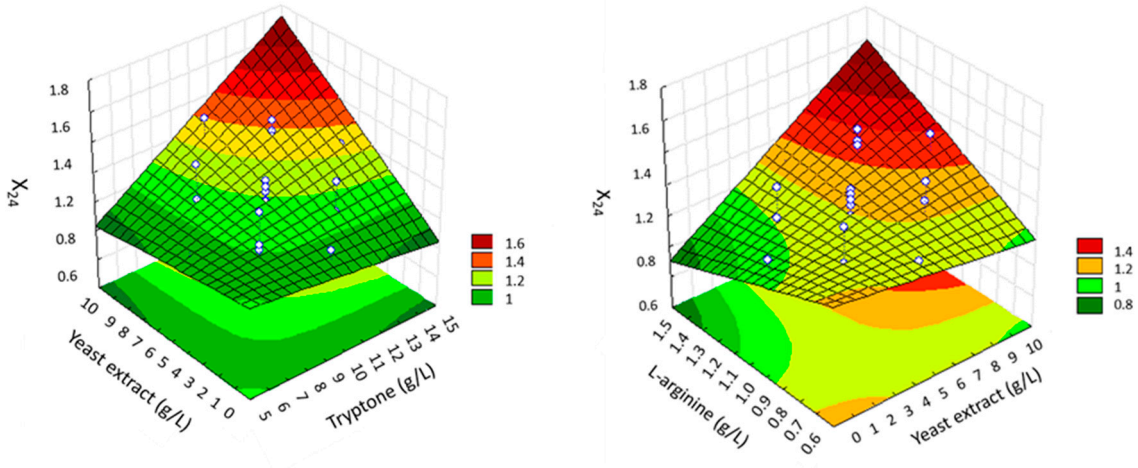
[1] SS: sum of squares; [2] Df: degree of freedom; [3] MS: mean square; [4] *F*-value: Test comparing model variance with residual (error) variance; [5] *p*-value: significant <0.1; (L) linear term; (R^2 = 0.58).

Table 4 shows that there was no lack of adjustment ($p > 0.1$) for the predicted model, which indicates that it is adequate to describe the process. Despite the non-significance of L-arginine, it was considered in the model as the linear interaction between this component and yeast extract was significant (Table 4). The first-order polynomial model proposed for cell growth including all the linear, quadratic, and linear interaction coefficients that were significant to this variable is presented in Equation (1).

$$[X_{24}] = 1.54158 - 0.01697 \times T - 0.17840 \times YE - 0.44168 \times A + 0.01066 \times T \times YE + 0.10684 \times YE \times A \quad (1)$$

where T, YE, and A represent real values of tryptone, yeast extract and L-arginine concentrations (g/L), respectively.

From the presented model, the response surface plots were obtained (Figure 3).



(**a**) (**b**)

Figure 3. Response surface plots for *C. carboxidivorans* syngas fermentation with cell concentration ([X_{24}]) as function of: (**a**) tryptone and yeast extract and (**b**) yeast extract and L-arginine.

Due to the non-significance of the quadratic terms of the model, it was not possible to obtain the conditions that would result in maximum value for cell growth. However, new experiments were performed in order to validate the obtained model by setting tryptone, yeast extract at higher levels (14 and 9 g/L, respectively), peptone at a lower level (6 g/L) or absence and L-arginine at a higher (1.4 g/L), lower (0.6 g/L) levels, or absence, as shown in Table 5. Indeed, absence or presence of peptone (comparing medium A with C and D with E) did not influence cell growth as predicted by the model. Furthermore, the absence of L-arginine reduced cell growth (medium B), also as predicted by the model.

The best condition was obtained with L-arginine at higher level (medium E), similar to the predicted by the model under these same conditions (1.77 g d.w. cells/L). As a result, we propose a new medium for *C. carboxidivorans* cell growth during syngas fermentation - TYA medium: Tryptone 14 g/L; yeast extract 9 g/L; and L-arginine 1.4 g/L.

Table 5. Biomass production in syngas fermentation by *Clostridium carboxidivorans* after 24 h.

Component	Medium				
	A	**B**	**C**	**D**	**E**
Tryptone	14	14	14	14	14
Yeast extract	9	9	9	9	9
L-arginine	0.6	0	0.6	1.4	1.4
Peptone from gelatin	6	6	0	6	0
Model prediction [X_{24}]	1.35	1.04	1.35	1.77	1.77
[X_{24}] (g d.w. cells/L) [1]	1.18 ± 0.02	1.09 ± 0.05	1.19 ± 0.04	1.69 ± 0.02	1.73 ± 0.06

[1] Experimental value.

2.3. Improving Ethanol Production by Fractional Factorial Design (FFD)

Considering the results of the PB design for ethanol production, a fractional factorial design (FFD) was proposed to further investigate the influence of the significant variables on ethanol production during syngas fermentation (tryptone, gelatin peptone, and L-arginine). Yeast extract was also included in this design because of its effect on cell growth. Glucose was maintained constant (1 g/L) as in the PB design. Results of ethanol production after 144 h of syngas fermentation by *C. carboxidivorans* at different conditions of FFD are present in Table 6.

Table 6. Fractional factorial design matrix with real and coded values and ethanol production after 144 h of syngas fermentation by *Clostridium carboxidivorans*—P_{EtOH} (g/L).

Run	Real Values (Corresponding Coded Levels)				P_{EtOH}
	TRY [1]	**PEP** [2]	**YE** [3]	**L-ARG** [4]	
1	2(−1)	2(−1)	1(−1)	0.2(−1)	0.287
2	12(1)	12(1)	1(−1)	1.2(1)	0.350
3	2(−1)	2(−1)	1(−1)	1.2(1)	0.340
4	12(1)	12(1)	1(−1)	0.2(−1)	0.257
5	2(−1)	2(−1)	7(1)	1.2(1)	0.216
6	12(1)	12(1)	7(1)	0.2(−1)	0.671
7	2(−1)	2(−1)	7(1)	0.2(−1)	0.677
8	12(1)	12(1)	7(1)	1.2(1)	1.614
9 (C)	7(0)	7(0)	4(0)	0.7(0)	0.985
10 (C)	7(0)	7(0)	4(0)	0.7(0)	0.967

[1] TRYP: tryptone concentration (g/L); [2] PEP: peptone concentration (g/L); [3] YE: yeast extract concentration (g/L); [4] L-ARG: L-arginine concentration (g/L); (C) central point.

Ethanol production ranged between 0.216 and 1.614 g/L (Table 6), increasing the lower value of the PB design (Table 1), but maintaining the higher value, indicating that the concentrations studied in the PB design were already close to maximum response. All variables of the FFD were relevant to ethanol production during syngas fermentation by *C. carboxidivorans*, including yeast extract (Figure 4). Different concentrations of yeast extract were used in FFD in comparison with PB design, which might explain this result. All variables affected the response positively, which means that higher concentrations stimulate ethanol production. Therefore, a culture medium composed of all those components at the higher levels (+1) of concentration is proposed for syngas fermentation, herein called TPYGarg, containing: 12 g/L of tryptone, 12 g/L of peptone from gelatin, 7 g/L of yeast extract, 1.2 g/L of L-arginine, and 1 g/L of glucose.

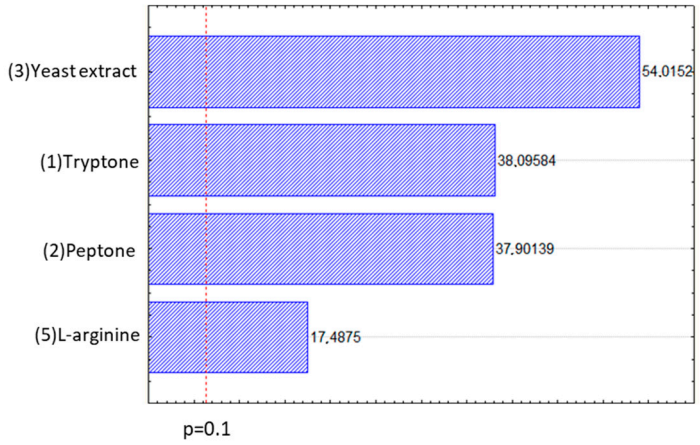


Figure 4. Pareto diagram for the estimated effect of each variable of the Fractional Factorial Design on ethanol production—P_{EtOH}—in the syngas fermentation by *Clostridium carboxidivorans*. The point at which the effects estimates were statistically significant (at p = 0.10) is indicated by the broken vertical line.

2.4. Tryptone-Peptone-Yeast Extract-Glucose-Arginine Medium (TPYGarg) for Ethanol Production

2.4.1. Cell Growth

During ethanol production, cell growth is important since more cells produce more ethanol. Therefore, *C. carboxidivorans* growth in TPYGarg and ATCC® 2713 media was monitored for comparison. A short lag phase of approximately 2 h for TPYGarg and 4 h for ATCC® 2713, is depicted in Figure 5a. Even though 40% more biomass is achieved for TPYGarg after 8 h of growth, in relation to ATCC® 2713 medium, both growth profiles meet after 24 h (Figure 5a). Specific growth rate obtained for TPYGarg (0.82 h^{-1}) was higher than that obtained for ATCC® 2713 (0.64 h^{-1}). These values are much higher than those recently reported for ABE fermentation (0.080 h^{-1}) and HBE fermentation (0.086 h^{-1}) by Fernández-Naveira et al. [18].

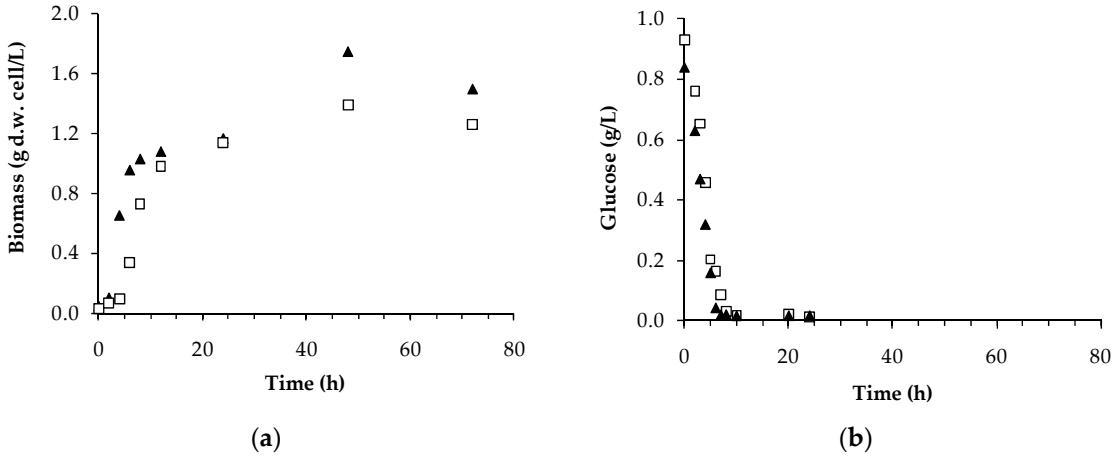


Figure 5. *C. carboxidivorans* growth (**a**) and glucose consumption (**b**) on Tryptone-Peptone-Yeast extract-Glucose-Arginine medium (TPYGarg) (▲) and ATCC® 2713 (□).

Glucose consumption was also monitored during syngas fermentation. Figure 5b shows that after 7 h of fermentation glucose was completely depleted from TPYGarg medium while total glucose consumption occurred after 8 h of fermentation on ATCC® 2713 medium. It is possible to observe that cells are still growing (Figure 5) even after glucose is completely consumed, mainly on TPYGarg medium.

2.4.2. Metabolites Production

Two phases are observed during *C. carboxidivorans* growth on TPYGarg and ATCC® 2713 with no pH control (Figure 6). At the beginning of fermentation (4 to 5 h), a decrease in pH values before glucose depletion is detected, and this is followed by an increase in pH levels on both media. These phases are described in literature as acidogenesis and solventogenesis [27].

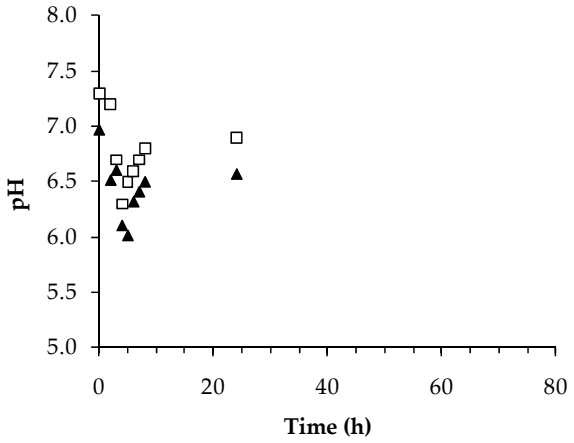
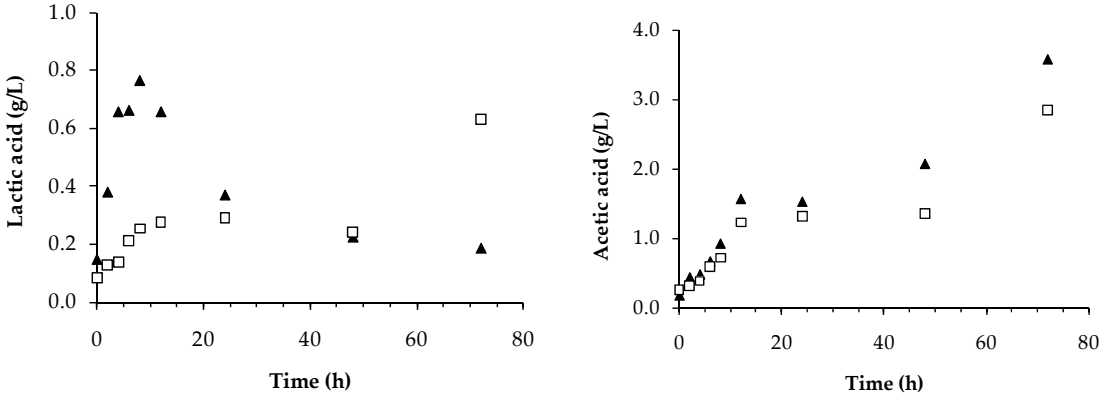


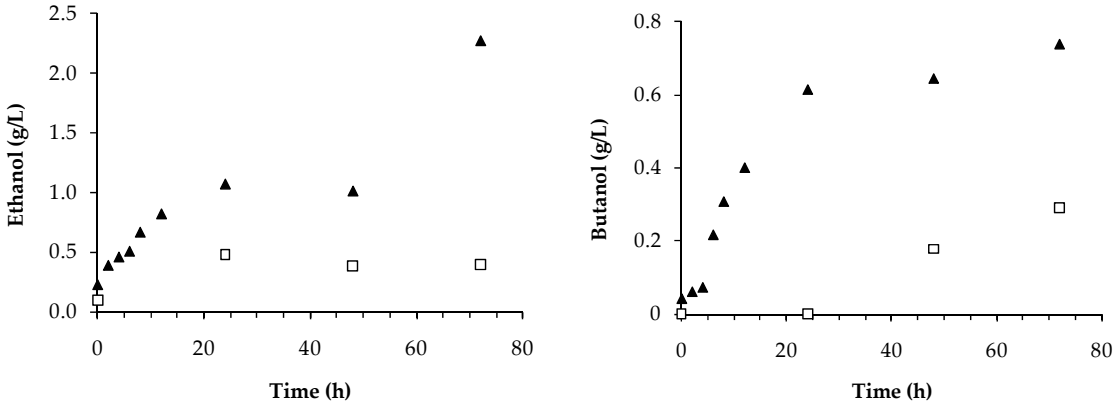
Figure 6. pH during syngas fermentation by *C. carboxidivorans* on Tryptone-Peptone-Yeast extract-Glucose-Arginine medium (TPYGarg) (▲) and ATCC® 2713 (□).

In the present study, with glucose and CO/CO_2 as carbon sources, acid production starts immediately after seed culture inoculation and along with bacterial biomass increase, as Figure 7 depicts. Most abundant acids obtained were acetic and lactic acids and their concentrations still increase even after glucose depletion, suggesting that those acids can be produced from carbon sources other than glucose, as CO and CO_2 from syngas, or from tryptone and peptone. Maximum concentrations of acetic acid were 3.57 g/L and 2.85 g/L after 72 h of fermentation on TPYGarg and ATCC® 2713 media, respectively. For lactic acid, 3-times more acid is produced after 8 h on TPYGarg in relation to ATCC® 2713, but at the end (70 h) a similar value is obtained on ATCC® 2713.



(**a**) (**b**)

Figure 7. *Cont.*



(**c**) (**d**)

Figure 7. Lactic acid (**a**), acetic acid (**b**), ethanol (**c**), and butanol (**d**) production during syngas fermentation by *C. carboxidivorans* on Tryptone-Peptone-Yeast extract-Glucose-Arginine medium (TPYGarg) (▲) and ATCC® 2713 (□).

Maximum ethanol production on TPYGarg was 5.7-times higher than on ATCC® 2713 after 72 h of fermentation (Figure 7c). Ethanol productivity (considering 24 h) was also improved on TPYGarg (0.045 g/L·h) in comparison to ATCC ® 2713 (0.020 g/L·h). Fernández-Naveira et al. [5] reported an inferior ethanol productivity (0.03 g/L·h) using the same microorganism and a medium containing 30 g/L of glucose, 1 g/L of yeast extract, minerals, trace metal, and vitamin solutions, with no syngas addition. This ethanol productivity was higher than those reported on syngas fermentation [14,19,24], which might be related to medium composition. Regarding butanol production, TPYGarg performance was also better than ATCC® 2713, with a butanol production almost 2.5-times higher. For butanol productivity, Fernández-Naveira et al. [5] reported 0.007 g/L·h, while 0.04 g/L·h was obtained in this study.

Maximum biomass and solvents production on ATCC® 2713 and TPYGarg over 72 h of syngas fermentation with *C. carboxidivorans* are presented in Table 7. Biomass concentration on ATCC® 2713 was 26% inferior in comparison to TPYGarg. Ethanol and butanol production were 4.75- and 2.55-times higher, respectively, on TPYGarg medium in comparison to ATCC® 2713.

Table 7. Cell concentration ([X_{24}] (g d.w. cells/L)), ethanol and butanol production (P_{EtOH} and P_{But}, respectively, in g/L) and productivities (Q_{EtOH} and Q_{But}, respectively, in g/L•h) after 72 h of syngas fermentation by *Clostridium carboxidivorans*.

Medium	Maximum Values in 72 h				
	$[X_{máx}]$	P_{EtOH}	P_{But}	Q_{EtOH}	Q_{But}
ATCC® 2713 [1]	1.38	0.47	0.29	0.020	0.004
TPYGarg [2]	1.75	2.28	0.74	0.045	0.039

[1] 10 g/L tryptone, 10 g/L peptone from gelatin, 5 g/L yeast extract; 1 g/L glucose, 5 g/L NaCl, 1 g/L L-arginine, 1 g/L sodium pyruvate, 0.0005 g/L menadione and 0.005 g/L hemin; [2] 12 g/L tryptone, 12 g/L peptone from gelatin, 7 g/L yeast extract, 1 g/L glucose, and 1.2 g/L L-arginine.

2.4.3. Cost Comparison

The competitiveness of new technologies compared to those already consolidated is very important to enable its implementation on industrial scale. As important as metabolite production and growth analysis, a cost-effectiveness study represents a fundamental step. A cost prospect was calculated for ATCC® 2713, TYA, and TPYGarg media. As shown in Table 8, TYA and TPYGarg are 47% and 31% cheaper than ATCC®2713 and promote higher cell growth and ethanol production, respectively.

Table 8. Medium composition and cost of ATCC® 2713, Tryptone, yeast extract, and L-arginine (TYA) and Tryptone-Peptone-Yeast extract-Glucose-Arginine (TPYGarg).

Compounds	Price	ATCC® 2713		TYA		TPYGarg	
	USD/kg [1]	g/L [2]	US$/L [3]	g/L [2]	USD/L [3]	g/L [2]	USD/L [3]
Tryptone	94.2	10	0.94	14	1.32	12	1.13
Peptone	70	10	0.70	-	-	12	0.84
Yeast extract	48.2	5	0.24	9	0.43	7	0.34
D (+) Glucose	9.96	1	0.01	-	-	1	0.01
NaCl	10.56	5	0.05	-	-	-	-
Sodium pyruvate	1430.00	1	1.43	-	-	-	-
Menadione	1630.00	0.0005	≈0	-	-	-	-
Hemin	5960.00	0.005	0.03	-	-	-	-
L-arginine	46	1	0.48	1.4	0.06	1.2	0.06
Total (USD/L) [4]		3.52		1.82		2.37	

[1] Cost of each component was calculated based on Sigma–Aldrich prices (Accessed on October 2019); [2] amount of component (g) per liter of medium; [3] Cost of component per liter of medium; and [4] Total cost of medium per liter.

3. Discussion

In the exploratory study to evaluate medium components effect on cell growth, tryptone, peptone, yeast extract, and L-arginine concentrations were considered significant for biomass production. Zhang et al. [14] reported that trace metals are required for *C. carboxidivorans* growth and these elements might be present in yeast extract. Peptone and tryptone are both sources of amino acids. However, as they are obtained from different protein sources (peptone is pancreatic digested gelatin and tryptone is the pancreatic digested casein), they might have complementary functions for cell growth. Another important observation is that glucose was not significant for cell growth, which might be related to the presence of CO and CO_2, indicating that syngas was used as the carbon source. Although the best result was obtained for the composition of the ATCC® medium 2713 (Table 1, run 16), the statistical evaluation shows that some components are not significant to cell growth, which means that its addition to medium composition just adds costs.

Considering the positive effect of these components on cell growth, the experimental design to optimize this medium was planned with higher concentrations. In that case, peptone was not significant to cell growth, which shows that it was only significant in PB design because tryptone concentration was not enough. The medium proposed for *C. carboxidivorans* cell growth after optimization, is reduced to three components, besides syngas. ATCC® medium 2713 is composed of nine components and many other media reported in literature have more than that, for example BDM—base defined medium (22 components) and P7Mt medium (20 components) [17]. In those laborious media, cell growth for *C. carboxidivorans* ranges from 0.4 to 0.6 g/L [5,17,18]. Therefore, it is evident that the medium proposed in the present work not only has the advantage of being easy to prepare but also yields a higher biomass.

For ethanol, experimental design revealed that a culture medium composed of 12 g/L of tryptone, 12 g/L of peptone from gelatin, 7 g/L of yeast extract, 1.2 g/L of L-arginine, and 1 g/L of glucose was the best for syngas fermentation, herein called TPYGarg. The development of a culture medium with no yeast extract, but with vitamins (biotin, pantothenic, and p-aminobenzoic acid), ammonia, trace metals, minerals, cysteine, sodium sulfide, and resazurin was reported by Phillips et al. [17]. After 200 h, around 3.0 g/L of ethanol (approximately, 0.015 g/L·h) was obtained in a medium 27-times cheaper than the standard one [14]. Despite cheaper, this medium still requires 20 components and demands time to prepare it. A ATCC® 1754 modified medium was proposed by Ramachandryia [28], using cotton seed extract (CSE) and morpholinoethanesulfonic acid (MES), with no yeast extract, resulting in 2.78 g/L of ethanol after 350 h of fermentation (approximately, 0.008 g/L·h). A modification in the ATCC® 1754 medium with MES as buffer was also reported, leading to 6.1 g/L of ethanol in a two

stirred-tank bioreactor (in series) [21], without considering the high cost of MES. Enhancement of alcohol synthesis was reported using CO-rich off-gas fermentation by *C. carboxidivorans* by adding molybdenum/iron/cupper, aiming at the enzymes of the microorganism metabolism [22]. A final ethanol concentration of 3.5 g/L was obtained after 72 h of fermentation (approximately, 0.049 g/L·h) [19], the higher productivity found for this process in literature so far, but without considering the culture medium cost.

Cell growth, glucose consumption, and medium pH were monitored during syngas fermentation in TPYGarg and high biomass was obtained as well as the detection of two phases: pH reduction in the beginning followed by pH increase. In classical ABE fermentation using glucose as sole carbon source or in HBE fermentation using gaseous substrate, the bioconversion by Clostridia occurs in two steps. First, the exponential bacterial growth and organic acids production can be observed, known as the acidogenesis phase. Then, those acids are converted into solvents as ethanol, butanol, and acetone in the ABE process, and hexanol, butanol, and ethanol in the HBE process, known as the solventogenic phase. The acidogenic phase usually shows greater efficiency at neutral or slightly high pH values, leading to the medium acidification due to acid production. Concerning ABE fermentation, studies have reported that the solventogenic phase is stimulated during medium acidification [5]. On the other hand, HBE fermentation studies have demonstrated that alcohol production usually occurs near pH 6.0 [29]. Below this pH level, low alcohol production and a decreasing growth capacity are usually attributed to the "acid crash" phenomenon [30].

In the present study, solvent production started while glucose was being consumed and cells were growing. Although, it is expected that solventogenic phase only starts after medium acidification as result of acid production, the production of organic acids and solvents occurred simultaneously in the present study. Formation of organic acids occurs during exponential growth, decreasing the culture pH level, making it unfavorable for the cell population and resulting in a metabolic change at the end of the exponential phase. Therefore, those organic acids are partly re-assimilated and rebuilt into neutral products as the solvents. However, not all species and strains of solventogenic clostridia have the same behavior. Lipovsky et al. [31] and Branska et al. [32] verified butanol production associated with cell growth by clostridia via the acetone–butanol–ethanol (ABE) pathway.

When using syngas as the only carbon source for *C. carboxidivorans* fermentation, a mixture of organic acids as acetic, butyric, and hexanoic acids was expected, and further partial conversion of those acids into respective alcohols, following HBE fermentation process. Using glucose as the only carbon source, ABE fermentation was expected, producing lactic, acetic, formic, and propionic acids, which then could be converted into acetone, ethanol, and butanol. However, it was observed that *C. carboxidivorans* does not follow classical ABE or HBE fermentations when both carbon sources were used (glucose and CO/CO_2), since acids and alcohols production were detected at the same time on TPYGarg and ATCC® 2713 media.

Higher growth rates and alcohol productivities obtained in the mixotrophic fermentation (glucose and CO/CO_2) when compared to heterotrophic (glucose) and autotrophic (CO/CO_2) processes reported in the literature [33], is probably the result of the two metabolic pathways synergy, glycolysis and Wood–Ljungdahl. The glycolysis converts glucose into pyruvate, producing 2 mol of pyruvate, two ATP, and four electrons. The pyruvate is converted to acetyl-CoA, an important intermediate for biofuel production, which results in the release of 2 mol of CO_2 and 4 additional electrons [33]. The autotrophic metabolism, known as Wood–Ljungdahl (WL) pathway, consists of two branches. In the methyl branch, CO_2 is reduced to a methyl group consuming six electrons and one ATP. While in the carbonyl branch, CO_2 is reduced to CO consuming two electrons. The CO and the methyl group formed in this first step of WL pathway are converted into 1 mol of acetyl-CoA [33–35]. Thereby, the glycolysis and WL are complementary pathways as CO_2 and electrons produced in the glycolysis are fully utilized by WL to produce one additional mol of acetyl-CoA increasing its yield by 50% in relation to glucose metabolism. Besides, the syngas feed contributes to enhance the metabolic flow of the WL pathway, as CO, CO_2, and H_2 are introduced [33].

4. Material and Methods

4.1. Microorganism and Culture Medium

Clostridium carboxidivorans DSM15243 was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany). The strain was anaerobically activated in ATCC® 2713 medium. ATCC® 2713 contains the following components (per liter): tryptone, 10 g; peptone from gelatin, 10 g; yeast extract, 5 g; L-arginine, 1 g; sodium pyruvate, 1 g; menadione, 0.5 mg; hemin, 5 mg; and glucose, 1 g. Next, the cells were grown in 100 mL serum glass bottles (Wheaton, NJ, USA) containing 50 mL of Brain Heart Infusion (BHI) medium from Sigma-Aldrich and syngas. Syngas was provided by White Martins Praxair Inc. (Joinville–SC, Brazil) and it is composed of 25% CO, 43.9% H_2, 10.02% CO_2, 10.05% N_2, and 11.01% methane.

4.2. Syngas Fermentation

All fermentations were performed in 50 mL serum glass bottles containing 30 mL of each culture medium studied. Media composition is detailed in Tables 1–3, Table 5, and Table 6. After media preparation, syngas was flushed in the liquid phase for 5 min. The glass bottles were sealed with gas impermeable butyl rubber septum stopper and aluminum seal and sterilized in autoclave at 0.5 atm for 20 min. After sterilization, seed culture was aseptically inoculated in all glass bottles to achieve 0.05 g d.w. cells/L. Syngas was added in the headspace at 1.22 atm and the bottles were incubated horizontally [36] at 37 °C and 150 rpm in TECNAL TE-420 shaker. Fermented culture mediums were sampled for optical density (OD) measurement and high-performance liquid chromatography (HPLC) analysis.

4.3. Experimental Designs to Increase Cell Growth and Ethanol Production

4.3.1. Screening Medium Components by Plackett-Burman Design

Plackett–Burman (PB) design was applied to screen culture medium components that influence biomass and ethanol production during syngas fermentation. Eight components and their concentrations were based on ATCC® 2713 medium (tryptone, 10 g/L; gelatin peptone, 10 g/L; yeast extract, 5 g/L; glucose, 1 g/L; sodium chloride, 5 g/L; L-arginine, 1 g/L; sodium pyruvate, 1 g/L; menadione, 0.5 mg/L; and hemin, 5 mg/L). The concentration of each nutrient at different levels (minimum, −1; central, 0 and maximum, +1) can be found in Tables 1 and 2. A 12-run PB design plus 3 central level (0) was performed for ethanol production, setting glucose was at 1.0 g/L (Table 1) and a 16-run PB design with 3 central level (0) was performed for biomass production. All experiments were conducted in random order and duplicates.

Biomass concentration after 24 h of fermentation—[X_{24}] and ethanol concentration after 144 h of fermentation—[P_{EtOH}] were used as response variables.

4.3.2. Central Composite Rotational Design (CCRD) to Increase Cell Growth

A central composite rotational design (CCRD) was proposed after the PB screening in order to maximize biomass production during syngas fermentation. Cell concentration after 24 h of fermentation—[X_{24}] was the response variable. The parameters set and used as independent variables were: tryptone, peptone, yeast extract, and L-arginine concentrations as shown in Table 3, with high (+1) and low levels (−1), as well as axial (−2, +2), and central (0) levels. A CCRD with 24 runs plus 3 central level assays were performed, totalling 27 tests (Table 3). All experiments were conducted in random order and duplicates. The results obtained were evaluated by Analysis of Variance (ANOVA) and the effects were considered significant when $p < 0.10$.

4.3.3. Fractional Factorial Design (FFD) to Improve Ethanol Production

A fractional factorial design (FFD) was used to evaluate the relevant components influencing ethanol production during syngas fermentation. Four parameters (Tryptone, Peptone from gelatin, yeast extract, and L-arginine concentrations) were screened in this study to determine the most significant input factors for ethanol production by a two-level fraction factorial design (2^{4-1}) with three central points. The response variable was ethanol production after 144 h of fermentation—P_{EtOH}. Table 6 shows the values representing the levels for each parameter studied. All experiments were conducted in random order and duplicates.

4.4. Analytical Methods

4.4.1. Cell Growth

Optical density (OD) was measured at 600 nm using a UV-VIS spectrophotometer (Bell SP 2000 UV). Cell concentration (g dry weight cells/L) was estimated using a standard curve.

4.4.2. Product and by-Products Quantification

Metabolites were analyzed by HPLC (High performance Liquid Chromatography) from Shimadzu equipped with Aminex® HPX-87H, 300 × 7.8 mm (Bio-Rad Laboratories Ltd., São Paulo–SP, Brazil) column and RI (refractive index) detector (Shimadzu®, Kyoto, Japan). The mobile phase was H_2SO_4 5 mM, 0.6 mL/min flow rate, and 20 µL injection volume. The column temperature was set at 55 °C.

4.5. Statistical Analysis

The statistical analysis was performed using STATISTICA 7.1 software (StatSoft, Inc., Tulsa, OK, USA). Analysis of variance (ANOVA) and the significance of the results were verified using Fisher's statistical test (*F*-test) at 10% of significance.

5. Conclusions

Experimental design is a powerful tool for reducing culture medium components. In the present study, a PB design followed by CCRD indicated that tryptone, yeast extract, and L-arginine were the most important culture medium components for *C. carboxidivorans* growth from syngas. The proposed medium for *C. carboxidivorans* growth, herein named TYA (14 g/L tryptone, 9 g/L yeast extract, and 1.4 g/L L-arginine) is cheaper (47%) than ATCC® 2713 and reduced in components. For ethanol production, peptone and glucose were also relevant. After applying an experimental design sequence, a culture medium for syngas fermentation by *C. carboxidivorans* was proposed and named TPYGarg (12 g/L tryptone, 12 g/L peptone from gelatin, 7 g/L yeast extract, 1.2 g/L L-arginine, and 1 g/L glucose). Almost five-times higher ethanol concentration and more than two-times higher butanol concentration were produced on TPYGarg in comparison to ATCC® 2713. TPYGarg represents a 31% reduction in cost of culture medium for syngas fermentation by *C. carboxidivorans* compared to ATCC® 2713. The increase in ethanol productivity with this new medium in comparison to literature reports is outstanding.

Author Contributions: C.B., A.B., T.F., and P.A. conceived and planned the experiments. C.B., A.B., M.B., and R.R. carried out the experiments. A.P. and A.C.V. were responsible for analyses and A.P. conducted the statistical analysis. C.B. wrote the manuscript with support from T.F. and P.A. T.F. and P.A. supervised the project. All authors provided critical feedback and helped shape the research, analysis, and manuscript. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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