

A New Strategy for Acetogenic Bacteriacell Growth and Metabolites Production Using Syngas in Lab Scale

Roberta R. Ribeiro, Fabiana Coelho, Tatiana F. Ferreira, Priscilla F. F. Amaral
(Escola de Química/Universidade Federal do Rio de Janeiro, Brazil)

Abstract: Syngas fermentation by acetogenic bacteria present a major bottleneck, which is the low solubility of the substrates (CO and CO₂) and, therefore, the low productivity caused by low cell density. In lab scale, many studies are conducted in serum bottles and agitated in incubation shakers with bottles kept vertically. In the present study, the serum bottles were kept horizontally in a new strategy to increase turbulence and, as a consequence, the mass transfer. Cell growth of *Clostridium carboxidivorans* (DSM 15243), *Clostridium ragsdalei* (DSM 15248) and *Clostridium ljungdahlii* (DSM 13528) was increased by the increase in the availability of CO and CO₂ in the liquid medium. Cell growth of *C. carboxidivorans* increased 3.7 times and the specific growth rate became 50% higher. Ethanol and acetic acid production were also favored by this strategy. *C. carboxidivorans* was the best specie for ethanol production.

Keywords: syngas, *Clostridium*, ethanol, hybrid process

I. Introduction

Currently there is a worldwide interest in using biomass as an environmentally friendly and renewable energy source. Many concerns point out the need to use renewable feedstock as part of the energy matrix and as a possible replacement for fossil fuels. An interesting pathway for biomass transformation is its thermochemical conversion through gasification, producing synthesis gas (syngas), a gas mixture composed mainly of CO, CO₂ and H₂. Subsequently, the syngas produced can be converted to a range of liquid fuels and hydrocarbons using transition metals as catalyst, which is known as Fischer-Tropsch process [1]. An alternative approach for biofuels production is the 'syngas fermentation', a biotechnological process which is currently under intensive research and development. This hybrid thermochemical/biochemical process can convert gas into ethanol and value-added liquid products using biocatalysts. This route is a promising technology and much more attractive due to several benefits over Fischer-Tropsch process and traditional ways of producing ethanol [2], [3].

Although carbon monoxide is highly toxic for most organisms, several microorganisms can tolerate and even live chemoautotrophically on this gas. Therefore, it offers an exciting opportunity to produce fuels and chemicals from non-dedicated crops, agriculture residues and forestry materials through fermentation. Some acetogenic bacteria have been described as capable of consuming CO and synthesizing acetate, ethanol and other products [4]. For syngas fermentation, the bacterial genus *Clostridium* is most used, which is categorized as carboxydotrophic homoacetogens. The metabolic pathway enabling CO utilization in *Clostridium* species is called reductive acetyl-CoA pathway, also known as the Wood-Ljungdahl pathway, in which syngas is converted to acetyl-CoA. Then, the acetyl-CoA can be: (i) incorporated into biomass; (ii) excreted as acetate, which can be further reduced to acetaldehyde and then ethanol; and (iii) directly reduced to acetaldehyde and then ethanol [4], [5]. During syngas fermentation, the acidogenesis occurs at favorable growth conditions with sufficient nutrient supply (vitamins and minerals), optimal pH and temperature, and no end-product inhibition but somewhat limited electron donor supply (CO or H₂). On the other hand, the solventogenesis is favored during slow growth in abundant presence of an electron donor, and disfavored at lower temperatures and pH and limited nutrient concentration. Another contributing factor for solventogenesis is a very likely end-product inhibition caused by high concentrations of undissociated acetic and butyric acids [6].

Syngas fermentation has many advantages such as its conduction at room temperature and pressure avoiding high energetic costs; feedstock flexibility, as in lignocellulosic and municipal solid wastes; and utilization of all components from the lignocellulosic biomass, including lignin. However, this process has a low productivity associated with low cell density, enzyme inhibition and mass transfer limitations [7]. The mass transfer limitation is a major challenge for syngas fermentation due to gases low solubility in water and media and reactors configuration [8], [9]. The gas-liquid mass transfer rates are only 77% (CO) and 65% (H₂) of that for oxygen. In lab scale, the serum bottles used for anaerobic bacteria growth are kept in vertical position, which compromises even more the mass transfer. Therefore, low concentrations of dissolved CO and H₂ result in an insufficient substrate supply for microorganisms and a major bottleneck for a full-scale application of syngas fermentation [7], [10]. The present work aims to study the kinetic growth of *Clostridium carboxidivorans* (DSM 15243), *Clostridium ragsdalei* (DSM 15248) and *Clostridium ljungdahlii* (DSM 13528) during syngas fermentation with serum bottles in vertical and horizontal position in order to investigate the influence of higher liquid turbulence in growth kinetic parameters.

II. Materials

2.1 Microorganism

Clostridium carboxidivorans (DSMZ 15243), *Clostridium ragsdalei* (DSMZ 15248) and *Clostridium ljungdahlii* (DSMZ 13528) were provided by the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany).

2.2 Synthesis Gas

The synthesis gas, or syngas, obtained from White Martins Gases Industriais Ltda. is composed of hydrogen (40%), carbon monoxide (25%), carbon dioxide (10%), nitrogen (10%) and methane (11%).

2.3 Media and Culture Preparation

The strains were cultivated separately in standard growth medium recommended by ATCC[®] 2713. The medium contains tryptone (10.0 g/L), gelatin peptone (10.0 g/L), yeast extract (5.0 g/L), glucose (1.0 g/L), sodium chloride (5.0 g/L), L-arginine (1.0 g/L), sodium pyruvate (1.0 g/L), menadione (0.0005 g/L) and haemin (0.005 g/L). This liquid media was degassed and dispensed anaerobically using syngas, and the 100 mL-glass serum bottles were sealed with impermeable butyl rubber septum-type stoppers and aluminum crimp seals before sterilization in autoclave at 121 °C and 0.5 atm for 15 minutes.

2.3.1 Inoculum

The inoculum was prepared in 100 mL serum bottles containing 50 mL of liquid media. The bottles were pressurized to 1.2 atm using syngas in order to create extremely anaerobic conditions. The pressure was maintained by impermeable butyl rubber septum-type stoppers and aluminum crimp seals. The cells were transferred from cultivated ATCC[®] 2713 media stored at refrigerator temperature (at 4°C) in one passage and were incubated in a shaker at 37 °C and 150 rpm for 24 h, with the bottles placed horizontally. The inoculum size used was 10% (v/v).

2.4 Bacterial growth kinetics

After 24 h inoculum cultivation, approximately 5.0 mL of cells were transferred to the sealed bottles containing ATCC[®] 2713 medium and syngas using sterilized syringe and needles. In order to study the bacterial growth, two strategies were implemented during cultivation in shaker. After inoculation, the bottles containing one bacteria species were incubated at 150 rpm and 37 °C for 120 h in a shaker and were placed horizontally or vertically during batch fermentation. The same procedure was adopted for three species: *C. carboxidivorans*, *C. ljungdahlii* and *C. ragsdalei*.

During sampling, syngas was added to the bottles to maintain the pressure in the bottle constant.

III. Analytical Methods

Cell growth was determined by measuring the optical density at 600 nm using a spectrophotometer Shimadzu UV-1201 (Japan). Samples of 2.0 mL were taken during fermentation using a syringe for product and by-product analysis. The quantitative analysis for fermentation products and by-products was conducted in a High Performance Liquid Chromatography (HPLC) equipped with a Refractive Index (RI) detector and an Aminex-HPX-87H ion exchange column (Biorad, Munich, Germany). The column was operated at 60 °C and sulfuric acid (0.005 M) was used as mobile phase. A 20 µL sample was injected at a constant flow of 0.8 mL min⁻¹. Peaks were identified by comparison with known concentrations of analytical standards of all fermentation products (Sigma Chemical Co., St. Louis, Missouri), which were diluted in Milli-Q water and injected in triplicate in order to prepare a standard curve relating the peak area to the products concentration.

IV. Results And Discussion

Cell growth, ethanol and acetate production were monitored for three species of *Clostridium* in ATCC medium with syngas. In order to investigate the influence of mass transfer in growth and metabolites production the bottles were kept positioned vertically or horizontally in the shaker, as shown in Fig. 1.

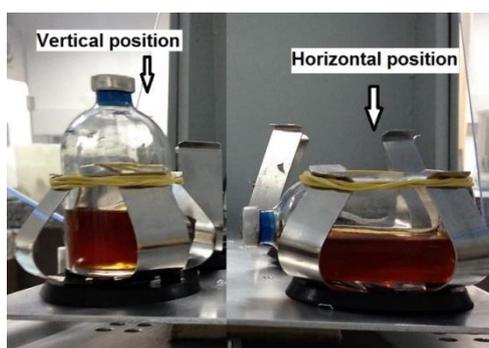


Figure 1. Serum bottles kept vertically and horizontally in the shaker.

The bottles kept horizontally present a higher turbulence in liquid and, therefore a higher mass transfer is possible. The results obtained are presented in Fig. 2 and Table 1. The growth kinetic profile of *C. rasgdalei* and *C. ljungdahlii* with bottles kept vertically are not shown because these were not significant.

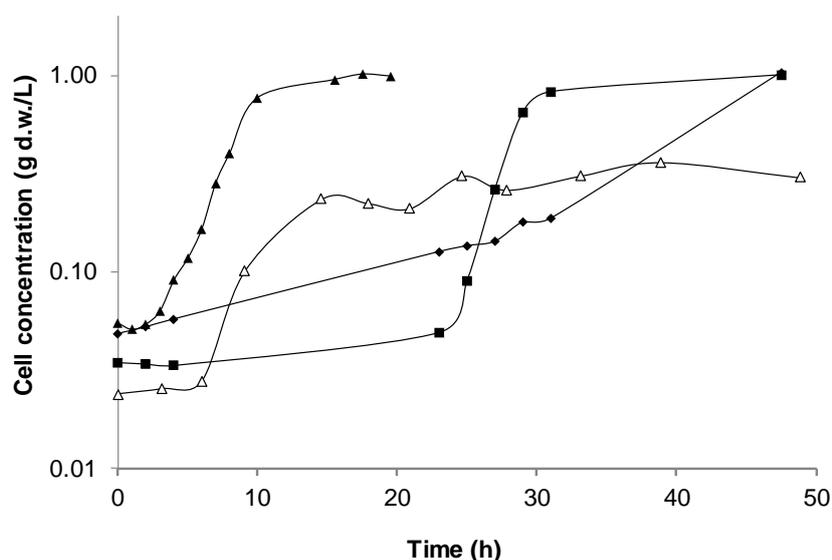


Figure 2. Kinetic of cell growth for *C.carboxidivorans* with bottles kept vertically (Δ), and *C.carboxidivorans* (\blacktriangle), *C.ljungdahlii* (\blacklozenge) and *C.rasgdalei*(\blacksquare) with bottles kept horizontally.

Table 1. Kinetic Parameters, ethanol and acetate concentration and productivity in fed-batch fermentation operating at 37 °C, 150 rpm and with serum bottles kept in different positions.

Organism	Serum bottle position	t_{lag} (h) ^a	\bar{X} (g/L) ^b	μ (h ⁻¹) ^c	P_{EtOH} (m g/L) ^d	Q_{EtOH} (mg/L.h) ^e	P_{Ac} (mg/L) ^f	Q_{Ac} (mg/L.h) ^g
<i>Clostridium carboxidivorans</i>	Vertically	6	0.337	0.24	ND	ND	1380.0	40.0
<i>Clostridium carboxidivorans</i>	Horizontally	3	1.245	0.37	378.7	15.8	1704.6	71.0
<i>Clostridium ljungdahlii</i>		27	0.987	0.10	144.2	6.3	930.8	192.0
<i>Clostridium rasgdalei</i>		23	0.968	0.38	150.5	5.2	802.9	32.1

a: lag phase time; b: Cell growth, calculated by the difference between final and initial cell concentration (in g dry weight of cells/L); c: Specific growth rate, calculated at exponential growth phase; d: Ethanol production (maximum concentration achieved); e: Ethanol productivity; f:acetateconcentration; g:acetate productivity.

Figure 2 depicts that the higher turbulence promoted by the horizontal position increased cell growth since no growth was detected for *C. rasgdalei* and *C. ljungdahlii* when a vertical position was used and a significant growth was observed for these species cultivated in the same conditions, but horizontally. For *C. carboxidivorans* it is also possible to observe that higher cell growth is achieved with higher mass transfer (bottles kept horizontally), with higher specific growth rate and cell production and inferior lag phase (Table 1). Comparing the growth kinetics of the tree species (Fig. 2, Table 1), similar profiles are observed for *C. carboxidivorans* and *C. rasgdalei*, with, approximately, the same specific growth rate (μ), except for the lag phase that was higher for *C. rasgdalei*. Allthree species presented a similar cell growth when conducting the fermentation with bottles horizontally positioned, reaching a cell concentration of approximately 1.0 g/L DW at the stationary phase. However, *C. carboxidivorans* had a smaller lag phase, which indicates a higher productivity in terms of biomass production.

Hurst and Lewis [12] conducted a study to evaluate the influence of pressure in cell growth during fermentation using *C. carboxidivorans* and obtained a lag phase of 17 hours and the beginning of the exponential phase at 22 hours with 1.35 atm. This study also shows a final cell concentration of 1.0 g/L at 2.0 atm after 70 hours of fermentation[12].In the present work,it was possible to achieve a similar cell concentration (1.245 g/L) after 15 hours of experiment, with a lower lag phase (3 hours) with lower pressure(1.2 atm). *C. ljungdahlii*in syngas fermentation presented a lag phase of approximately 10 hours and a final cell concentration of 1 g/L in 50 hours of fermentation at a pressure of 1.20 atm [13].Both studies ([12] and [13]) were conducted

with bottles kept in a vertical position, and, therefore, with lower gas transfer rate. With bottles kept horizontally it was possible to increase CO and CO₂ transfer to the liquid phase, which might have reduced lag phase because of the availability these two substrates promptly. Higher gas transfer caused by the turbulence of the bottles in the horizontal position increased also ethanol and acetic acid production as Table 1 shows. No production was detected for *C. ljungdahlii* and *C. rasgdalei* with bottles kept vertically and inferior values were observed for *C. carboxidivorans* in relation to the bottles kept horizontally. Productivity of ethanol and acetic acid were also higher when CO and CO₂ were rapidly available. Table 1 also shows that *C. carboxidivorans* was a better ethanol producer between the three clostridia species studied.

V. Conclusions

The increase in the availability of substrates caused by the higher turbulence when serum bottles were kept in a horizontal position reduced lag phase in 3 hours and cell growth was 3.7 times higher with a 50% increase in the specific growth rate in relation to the bottles kept vertically, for *C. carboxidivorans*. It also increased acetic acid and ethanol production. For *C. ljungdahlii* and *C. reasgdalei*, this strategy made possible cell growth and metabolites production, which was not observed when syngas was slightly dissolved in the medium (bottles kept vertically). *C. carboxidivorans* was the best acetogenic specie for acetic acid and ethanol production.

References

- [1]. B. S. Rahardjo, The assessment of syngas utilization by fichtertropsch synthesis in the slurry-bed reactor using Co/SiO₂ catalyst, *International Journal of Engineering and Applied Sciences*, 4, 2013, 20-39.
- [2]. M. Mohammadi, G. D. Najafpour, H. Younesi, P. Lahijani, M. H. Uzir, and A. R. Mohamed, Bioconversion of synthesis gas to second generation biofuels: a review, *Renewable and Sustainable Energy Reviews*, 15, 2011, 4255-4273.
- [3]. J Vandecasteele, *Experimental and modelling study of pure-culture syngas fermentation for biofuels production*, master diss., Faculty of Bioscience Engineering from Ghent University, Ghent, Belgium, 2016.
- [4]. M. Köpke, C. Mihalcea, J. C. Bromley, and S. D. Simpson, Fermentative production of ethanol from carbon monoxide, *Current Opinion in Biotechnology*, 22, 2011, 320-325.
- [5]. M. Köpke, C. Held, S. Hujer, H. Liesegang, A. Wiezer, A. Wollherr, A. Ehrenreich, W. Liebi, G. Gottschalk, and P. Dürre, *Clostridium ljungdahlii* represents a microbial production platform based on syngas, *Proceedings of the National Academy of Sciences*, 107, 2010, 13087-13092.
- [6]. H. Richter, M. Martin, and L. Angenent, A two-stage continuous fermentation system for conversion of syngas into ethanol, *Energies*, 6, 2013, 3987-4000.
- [7]. M. D. Bredwell, P. Srivastava, and R. M. Worden, Reactor design issues for synthesis gas fermentations, *Biotechnology Progress*, 15, 1999, 834-844.
- [8]. M. Devarapalli, H. K. Atiyeh, J. R. Phillips, R. S. Lewis, and R. L. Huhnke, Ethanol production during semi-continuous syngas fermentation in a trickle bed reactor using *Clostridium ragsdalei*, *Bioresource Technology*, 209, 2016, 56-65.
- [9]. Y. Shen, R. Brown, and Z. Wen, Syngas fermentation of *Clostridium carboxidivorans* P7 in a hollow fiber membrane biofilm reactor: Evaluating the mass transfer coefficient and ethanol production performance, *Biochemical Engineering Journal*, 85, 2014, 21-29.
- [10]. P-H Lee. *Syngas fermentation to ethanol using innovative hollow fiber membrane*, doctoral diss., Iowa State University, Iowa, 2010.
- [11]. P. Maddipati, H. K. Atiyek, D. D., Bellmer, R. L. Huhnke, Ethanol production from syngas by *Clostridium* strain P11 using corn steep liquor as a nutrient replacement to yeast extract, *Bioresource Technology*, 102, 2011, 6494-6501.
- [12]. K.M. Hurst, and R.S. Lewis, Carbon monoxide partial pressure effects on the metabolic process of syngas fermentation, *Biochemical Engineering Journal*, 48, 2010, 159-165.
- [13]. A. Dadak, M. Aghbashlo, M. Tabatabaei, H. Younesi, and G. Najafpour, Using exergy to analyze the sustainability of fermentative ethanol and acetate production from syngas via anaerobic bacteria (*Clostridium ljungdahlii*), *Sustainable Energy Technologies and Assessments*, 15, 2016, 11-19.