Journal of Bioprocessing & Biotechniques
ISSN: 2155-9821
Tirado-Acevedo et al. J Bioproces Biotechniq 2011, S:2
DOI: 10.4172/2155-9821.S2-001
Research Article Open Access

# Influence of Carbon Source Pre-Adaptation on *Clostridium ljungdahlii* Growth and Product Formation

**Oscar Tirado-Acevedo[1], Jacqueline L. Cotter[2], Mari S. Chinn[2]* and Amy M. Grunden[1]**

[1]*Department of Microbiology, NC State University, 4548 Thomas Hall,Campus Box 7615, Raleigh, NC 27606,USA*
[2]*Department of Biological & Agricultural Engineering, NC State University, 277 Weaver Labs,Campus Box 7625,Raleigh, NC 27695-7625, USA*

## Abstract

Syngas fermentation is considered an alternate processing method for biofuel and biochemical production as part of thermochemical biomass conversion. Exposure of syngas fermenting microorganisms to sugars, either in the primary syngas fermentation or through pre-adaptation in the seed culture, has the potential to enhance overall fermentation performance and stress tolerance. In this rapid communication, *Clostridium ljungdahlii* was grown on different carbon sources including syngas only, syngas-fructose and fructose only to identify ideal pre-adaptation conditions for ethanol and acetate production from subsequent cultures grown in reactors containing syngas only or fructose-syngas substrates. In syngas only reactors, cultures pre-adapted to fructose had faster cell production rates (2X) and at least 83% higher ethanol and 16% higher acetate formation than cells pre-adapted on syngas or syngas-fructose. In syngas-fructose reactors, cultures did not show significant growth or acetate production differences under pre-adaptation treatments. Nevertheless, in these syngas-fructose reactors, cultures pre-adapted on syngas and syngas-fructose had nearly 20% higher ethanol production than those pre-adapted on fructose. Among pre-adaptation treatments, fructose had better results in syngas only reactors than syngas-fructose reactors. However, the presence of syngas in pre-adaptation cultures was better overall for ethanol production.

**Keywords:** *Clostridium ljungdahlii*; Synthesis gas fermentation; Pre-adaptation; Ethanol; Acetate; Fructose

## Introduction

Conversion of synthesis gas to liquid fuels by biological catalysts has been suggested as a promising technology to achieve oil independence [1]. In this method, cellulosic biomass and other carbon sources (coal, industry and/or municipal waste, etc.) are converted to synthesis gas (syngas) by a well known process called gasification [2]. Syngas typically consists of carbon monoxide, hydrogen, carbon dioxide, methane, trace amounts of $C_2$ hydrocarbons, water vapor, nitrogen and various contaminants including ash and tars [2]. This syngas is then fed to a microorganism which can utilize it as a carbon and energy source. The products of this fermentation could be alcohols (e.g. ethanol or butanol) and organic acids (e.g. acetate or butyrate) [3]. There have been a number of microorganisms isolated for use in this process, including *C. ljungdahlii*, *C. autoethanogenum*, *C. carboxidivorans* P7, *C. ragsdalei*, *Alkalibaculum bachi* and *Butyribacterium methylotrophicum* [4-7]. In addition, some companies are en route to commercializing their methods [8].

*C. ljungdahlii*, which has been studied in regard to synthesis gas fermentation [9,10], was one of the early microorganisms described to be able to ferment syngas components to ethanol and acetate via the Wood-Ljungdahl pathway. In addition to CO and $H_2$, *C. ljungdahlii* can use sugars as a carbon source, with fructose being its preferred sugar for growth and product formation [11]. Growth of *C. ljungdahlii* solely on fructose results in faster growth and product formation rates compared to cultures provided only syngas [10,12,13]. Fermentation on sugars provides cells with increased reductant and energy to support primary metabolism as well as synthesis of enzymes that help cells manage environmental stresses, thereby enhancing overall culture productivity. On the other hand, primary syngas fermentations present challenges to cell metabolism as CO fixation is a reductant intensive process, and the ability to overcome growth inhibition resulting from substrate utilization and product formation is compromised [14]. Exposure of *C. ljungdahlii* to sugars, either in the primary syngas fermentation or through pre-adaptation in the seed culture, may enhance overall fermentation performance and stress tolerance.

In this rapid communication, the physiological advantage of growth on sugars is discussed in terms of *C. ljungdahlii's* ability to acclimate and perform in syngas cultures, which has not been previously addressed in the syngas fermentation literature. Specifically pre-adaptation of cultures was explored to establish its significance on end-product formation (ethanol and acetate). *C. ljungdahlii* was grown on different carbon sources (syngas and fructose combinations) in a continuous gas feed, batch liquid system. Growth and product formation were evaluated for the fermentations that were initiated using cells pre-adapted to fructose, syngas, and mixed fructose-syngas substrates.

## Materials and Methods

### Organism and medium preparation

*C. ljungdahlii* (ATCC 55383) was obtained from the American Type Culture Collection. Cells were grown at 37°C on a modified Reinforced Clostridial Basal medium with additional salts, vitamins, and trace elements adopted from the ATCC 1754 PETC medium (RCM.NA.SVE) under $N_2$ (pH 6.8) unless otherwise indicated [12]. Cultures provided with syngas (7.5 ml/min) were grown in a fermentation reactor presented in Figure 1A [13]. Bottled syngas used was an artificial mix of 50% $N_2$, 20% CO, 20% $CO_2$, and 10% $H_2$. When added to

***Corresponding author:** Mari S. Chinn, Department of Biological & Agricultural Engineering, North Carolina State University, 277 Weaver Labs, Raleigh, NC 27695-7625, Tel: 919-515-6744; Fax: 919-515-6719; E-mail: mschinn@ncsu.edu

**Received** September 08, 2011; **Accepted** October 22, 2011; **Published** October 24, 2011

**Citation:** Tirado-Acevedo O, Cotter JL, Chinn MS, Grunden AM (2011) Influence of Carbon Source Pre-Adaptation on *Clostridium ljungdahlii* Growth and Product Formation. J Bioprocess Biotechniq S2:001 doi:10.4172/2155-9821.S2-001

medium in combination with syngas, fructose was used at 2.5 g/L. All cultures were inoculated at 5% v/v.

### Inoculum preparation using different carbon sources

*C. ljungdahlii* was grown on RCM.NA.SVE (will now be referred to as medium). Cultures were initiated from a freezer stock (-80ºC) and incubated (37ºC, 72 h) before 3 serial transfers every 24 h to achieve steady growth in medium with fructose (5 g/L). Active cells (472 mg dry cells/L) were transferred to carbon sources [medium-syngas, medium-syngas-fructose (2.5 g/L) or medium-fructose (5g/L)] to start seed cultures for fermentation studies. These seed cultures had an adaptation period of 18-24 hours. Fermentation reactors containing medium-syngas and medium-syngas-fructose at a working volume of 250 ml were inoculated with the three different seed cultures (472 mg dry cells/L in inoculum; 5% v/v addition). Medium seed culture combinations were grown in triplicate at 37 ºC without mechanical agitation Figure1B. Cell growth was monitored and liquid and gas samples were taken every 6-8 h.

### Substrate and end product analysis

Headspace gases were analyzed by gas chromatography (GC) on a Carbosieve S-II, 100/120 mesh stainless steel column using a thermal conductivity detector (Shimadzu GC-17A). Ethanol and acetate concentrations were determined in acidified samples by GC (Shimadzu GC-17A). Analytes were quantified using a packed Supelco SP 1000 (1% H3 $PO_4$, 100/120 mesh) column and a flame ionization detector [12,13]. Fructose present in experimental cultures was determined by HPLC (Shimadzu LC20) as follows: liquid samples (600 µL) were centrifuged (10 min, 18,400 x g, 25ºC). Supernatants were filtered through a 0.22 µm syringe filter into a crimp vial and analyzed on a HPX-87H column (65°C) using a refractive index detector. The eluent (0.6 ml/min) was sulfuric acid (5 mM).

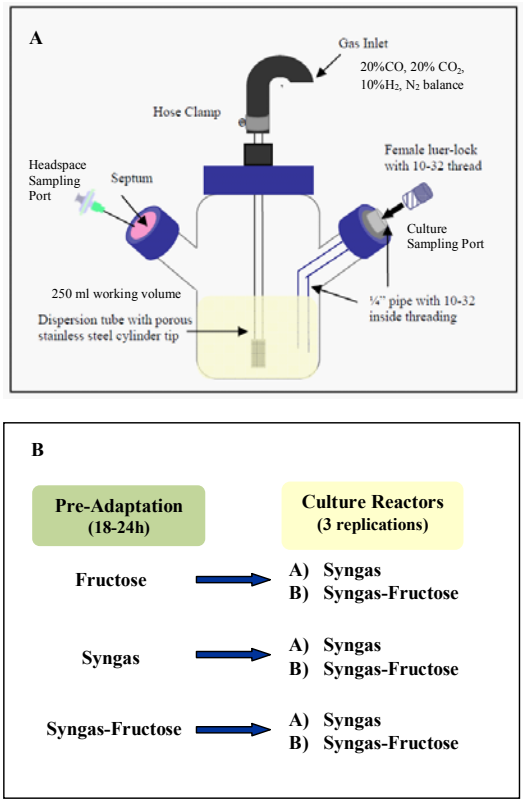


**Figure 1:** (A) Schematic of continuous syngas, batch liquid medium reactor and (B) Summary diagram of *C. ljungdahlii* culture treatment combinations.

### Statistical analysis

Cell growth and liquid product analysis from different pre-adaptation sources were evaluated using the General Linear Model (GLM) in SAS® Version 9.1 (SAS Inc., Cary, NC, USA). Assessment of statistical significance for pre-adaptation on different carbon sources and different production growth substrates was set at $P < 0.05$.

## Results and Discussion

### Growth of *C. ljungdahlii* and product formation in syngas reactors

*C. ljungdahlii* cultures were pre-adapted on three different carbon source combinations (syngas, syngas-fructose or fructose). Subsequently, these cells were transferred to fermentation reactors with either syngas or syngas-fructose medium. In syngas only reactors, cells pre-adapted on fructose produced the highest cell densities reaching maximum growth after 24 h Figure 2A. This was unexpected given that these cells would have to switch from fructose to syngas fermentation. Syngas and syngas-fructose pre-adapted cells reached statistically significant maximum densities after 40 and 56 h, respectively. Cells pre-adapted on syngas-fructose showed the longest lag-time, contributing to increased time to reach peak growth, where fructose only cultures had cell production rates of 10.4 mg/L/h, while syngas and syngas-fructose cultures both had cell production rates of 5 mg/L/h (calculated during exponential growth phase). Interestingly, after 56 h cell density was statistically similar independent of pre-adaptation medium, possibly due to product inhibition or depletion of an essential nutrient in the cultures pre-adapted to fructose.

Ethanol production in syngas reactors was significantly higher ($P < 0.05$) when cells were pre-adapted on fructose, reaching a maximum of 2.2 mM at 64 h of growth Figure 3A and seemed to be non-growth associated since these cells reached stationary phase after 24 h. Cells pre-adapted on syngas also reached maximum ethanol production after 64 h, while cells pre-adapted on syngas-fructose reached a maximum after 48 h. Although acetate is considered to be a growth associated product, it was continuously accumulated during the growth phase as well as after the stationary phase was reached Figure 3C. Cells pre-adapted on fructose produced significantly higher acetate concentrations than cells pre-adapted on syngas and syngas-fructose after 56 h ($P < 0.05$) with a maximum of 34.3 mM. Again, in syngas reactors, cells pre-adapted to syngas-fructose show a lag time in acetate production when compared to fructose or syngas pre-adapted cells but reached comparable concentrations by the end of the experiment. Despite final cell concentrations reached, cells pre-adapted on fructose demonstrated increased productivity and rate of cell, ethanol and acetate production. If culture conditions were modified to limit growth inhibition, cells pre-adapted on fructose would provide even greater conversion efficiency in syngas fermentations, especially those cultured beyond 24 h.

### Growth and product formation in syngas-fructose reactors

Significant growth differences were not observed among the three pre-adaptation sources in syngas-fructose reactors Figure 2B. These cultures did not show a lag-time and reached maximum cell density after 24 h. Cell production rates were approximately 43 mg/L/h for syngas and fructose pre-adapted cultures and 37 mg/L/h for syngas-fructose

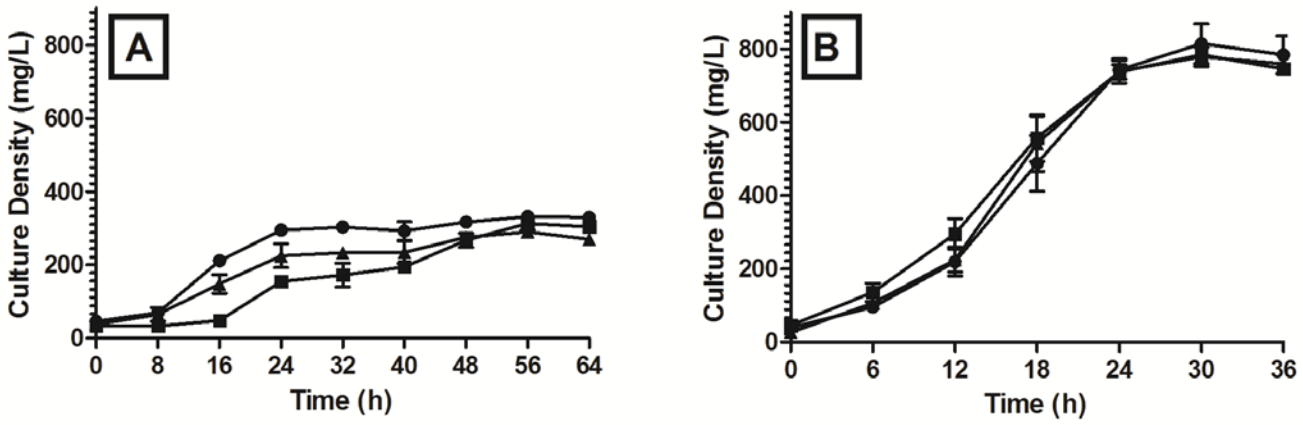


**Figure 2:** *C. ljungdahlii* culture density in (A) syngas reactors and (B) syngas-fructose reactors inoculated with cells pre-adapted on fructose (circles), syngas-fructose (squares), and syngas (triangles).

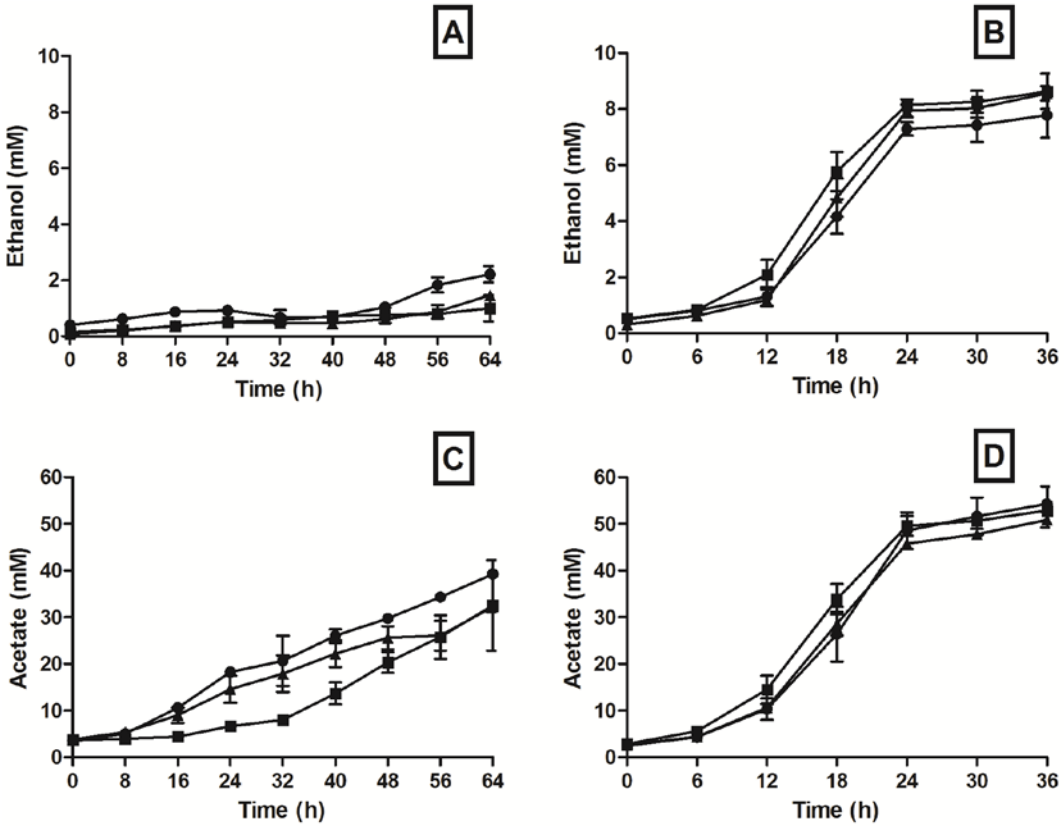


**Figure 3:** *C. ljungdahlii* production of (A) ethanol and (C) acetate in syngas reactors and (B) ethanol and (D) acetate in syngas-fructose reactors inoculated with cells pre-adapted on fructose (circles), syngas-fructose (squares), and syngas (triangles).

pre-adapted cultures (calculated during exponential growth phase). Cells growing in syngas-fructose reactors had higher cell densities ($P < 0.05$) than cells growing in syngas reactors (787 mg/L vs. 332 mg/L), independent of pre-adaptation source.

Ethanol concentration in syngas-fructose reactors was statistically higher in cultures pre-adapted to syngas only and syngas-fructose than those pre-adapted on fructose Figure 3B. Pre-adaptation had no effect on acetate production as all cultures produced statistically the same amount of acetate Figure 3D. Independent of pre-adaptation, both ethanol and acetate production seem to be growth associated as production decreases when cells reach stationary phase. It has been previously suggested that ethanol production occurs in non-growth conditions [10]. We observe this trend in syngas only medium but not when fruc-

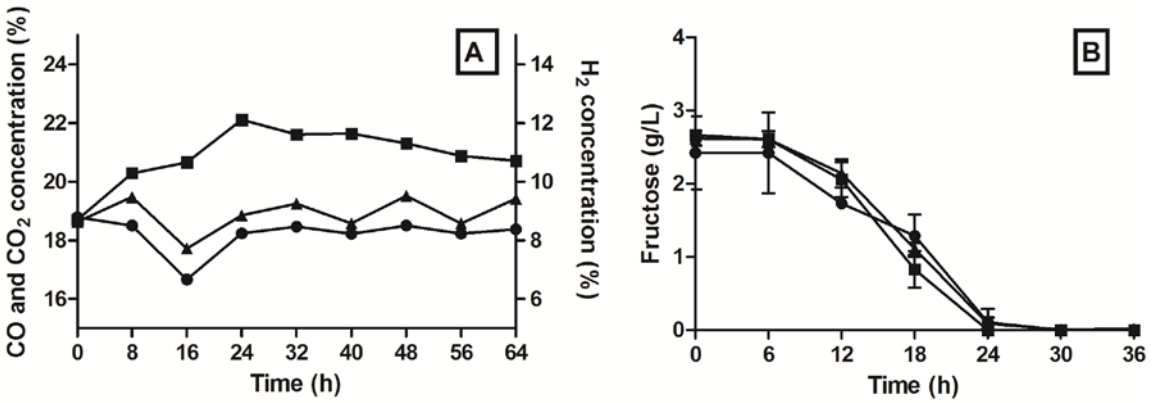


**Figure 4:** *C. ljungdahlii* (A) syngas use in syngas reactors inoculated with cells pre-adapted on syngas. Squares denote $CO_2$ concentrations, circles represent CO concentrations, and the triangles indicate $H_2$ concentrations. (B) fructose use in syngas-fructose reactors inoculated with cells pre-adapted on fructose (circles), syngas-fructose (squares), and syngas (triangles).

tose is present Figure 3A, B. Independent of pre-adaptation source, cells grown in syngas-fructose reactors produced significantly more ethanol and acetate than cells grown in syngas reactors Figure 3, with as much as 8- and 1.5-fold more ethanol and acetate respectively. This difference exceeds the theoretical amount of product that can be produced from the fructose alone and indicates that the fixation of syngas carbon is improved. This difference was also observed with addition of 5 g/L of fructose to syngas-grown cultures, where increases up to 25% and 35% for ethanol and acetate concentration, respectively, were seen compared to syngas alone (unpublished data). This demonstrates that addition of 0.25% w/v of sugar in the medium not only improves total product concentration, but promotes shift increases towards ethanol for *C. ljungdahlii*. A number of low-cost sugar sources have been identified that could act as a carbon supplement for this process without interfering with food supply, including corn steep liquor, cane molasses and fruit processing residues [15-18]. Use of low sugar concentrations may be worth examining to improve commodity chemical production from *C. ljungdahlii*.

## Gas and fructose analysis

Headspace gas composition was monitored in both syngas and syngas-fructose fermentations. Figure 4A shows a representative headspace gas profile. CO and $H_2$ consumption at 8 to 24 h coincides with a $CO_2$ increase. This suggests *C. ljungdahlii* is most likely using the Wood-Ljungdahl pathway for metabolism of CO and $H_2$, thereby providing cells with carbon for biomass and energy production as well as reducing equivalents [19]. However, by the end of culture growth, head-space gases return to near starting concentrations, which may be related to several conditions, including cells that no longer consume gases for growth or that gas flow rates exceed the cells' consumption rates. Scaled processes with a continuous gas flow system like the one presented here could achieve more complete utilization of syngas with gas recirculation [20].

Fructose use was analyzed over time for syngas-fructose reactors. Fructose is known to be transported into *C. ljungdahlii* using either the fructose- or mannitol-specific phosphotransferase system, wherein it is metabolized via the Embden-Meyerhof-Parnas pathway, resulting in the net production of two ATP and four reducing equivalents per fructose [21]. In our experimental cultures, fructose was consumed at equal rates independent of pre-adaptation source and exhausted by 24 h Figure 4B. Fructose use and liquid product synthesis appear to be tightly associated as disappearance of fructose correlates with lower ethanol and acetate production. However, after fructose is all consumed, the ethanol production rate is still higher in syngas-fructose reactors compared to syngas. In this case, fructose is likely providing cells with excess reducing equivalents, which in turn could be used to reduce acetate to ethanol [21].

In syngas reactors, pre-adaptation source was found to have an effect on cell production rate, final cell concentration and ethanol formation. In these reactors, the highest cell concentration and ethanol production was found in reactors with cells pre-adapted with fructose. These reactors produced around 2.5 and 1.5 times more ethanol than reactors inoculated with syngas and syngas-fructose pre-adapted cells, respectively. Fructose pre-adaptation likely provides higher levels of energy equivalents (ATP/GTP) and reducing power (NADH/NADPH) to cells, which enables cells to efficiently use a carbon source (syngas) that initially requires an energy and reductant input [21].

In syngas-fructose reactors, pre-adaptation source did not appear to have an impact on cell density, liquid product yield, or fructose utilization. This suggests that independent of pre-adaptation, in syngas-fructose reactors, cells have the capacity to readily use fructose as a carbon and electron source. Overall, syngas-fructose reactors had about 2.5 times higher cell densities and 8 and 1.5 times higher ethanol and acetate, respectively than syngas reactors. This indicates that use of fructose (0.25%w/v) in cultures may be a reasonable approach to increase ethanol production in syngas fermentation of *C. ljungdahlii* as excess reducing equivalents translate to better production.

Based on this study, it was concluded that for syngas-fructose reactors, pre-adaptation is not necessary as the overall fermentation was not affected, but in syngas only reactors a fructose pre-adaptation will be beneficial as it increases cell numbers and liquid products. The growth history of *C. ljungdahlii,* and likely other autotrophic bacteria, as it relates to metabolic capacity (i.e. bioenergetics, pathway induction) is important to cell performance in syngas reactor systems.

### Acknowledgments

This work was partially funded by the NC State University Faculty Research and Professional Development grant. In addition, this material is based upon work supported in part by the North Carolina Biotechnology Center.

## References

1. Zeikus JG (1980) Chemical and fuel production by anaerobic bacteria. Annu Rev Microbiol 34: 423-464.
2. Bridgwater AV (1995) The technical and economic feasibility of biomass gasification for power generation. Fuel 74: 631-653.
3. Worden RM, Grethlein AJ, Jain MK, Datta R (1991) Production of butanol and ethanol from synthesis gas via fermentation. Fuel 70: 615-619.
4. Hurst KM, Lewis RS (2010) Carbon monoxide partial pressure effects on the metabolic process of syngas fermentation. Biochem Eng J 48: 159-165.
5. Kundiyana DK, Wilkins MR, Maddipati P, Huhnke RL (2011) Effect of temperature, pH and buffer presence on ethanol production from synthesis gas by "*Clostridium ragsdalei*". Bioresource Technol 102:5794-5799.
6. Allen TD, Caldwell ME, Lawson PA, Huhnke RL, Tanner RS (2011) *Alkalibaculum bacchi* gen. nov., sp. nov., a CO-oxidizing, ethanol-producing acetogen isolated from livestock-impacted soil. Int J Syst Evol Microbiol 60:2483-2489. http://www.ncbi.nlm.nih.gov/pubmed/19965999
7. Slivka RM, Chinn MS, Grunden AM (2011) Gasification and synthesis gas fermentation: an alternative route to biofuel production. Biofuels 2: 405-419.
8. Tirado-Acevedo O, Chinn MS, Grunden AM (2010) Production of Biofuels from Synthesis Gas Using Microbial Catalysts. Adv Appl Microbiol 70: 57-92.
9. Phillips J, Klasson K, Clausen E, Gaddy J (1993) Biological production of ethanol from coal synthesis gas. Appl. Biochem Biotechnol 39-40: 559-571.
10. Klasson KT, Ackerson MD, Clausen EC, Gaddy JL (1992) Bioconversion of synthesis gas into liquid or gaseous fuels. Enzyme Microb Technol 14: 602-608.
11. Tanner RS, Miller LM, Yang D (1993) *Clostridium ljungdahlii* sp. nov., an Acetogenic Species in Clostridial rRNA Homology Group I. Int J Syst Bacteriol 43: 232-236.
12. Cotter JL, Chinn MS, Grunden AM (2009) Ethanol and acetate production by *Clostridium ljungdahlii* and *Clostridium autoethanogenum* using resting cells. Bioprocess Biosyst Eng 32: 369-380.
13. Cotter JL, Chinn MS, Grunden AM (2009) Influence of process parameters on growth of *Clostridium ljungdahlii* and *Clostridium autoethanogenum* on synthesis gas. Enzyme Microb Technol 44: 281-288.
14. Drake HL, Gossner AS, Daniel SL (2008) Old acetogens, new light. Ann NY Acad Sci 1125: 100-128.
15. Lawford H, Rousseau J (1997) Corn steep liquor as a cost-effective nutrition adjunct in high-performance *Zymomonas* ethanol fermentations. Appl Biochem Biotechnol 63-65: 287-304.
16. Jiang L, Wang J, Liang S, Wang X, Cen P, Xu Z (2009) Butyric acid fermentation in a fibrous bed bioreactor with immobilized *Clostridium tyrobutyricum* from cane molasses. Bioresour Technol 100: 3403-3409.
17. Gullón B, Yáñez R, Alonso JL, Parajó JC (2008) l-Lactic acid production from apple pomace by sequential hydrolysis and fermentation. Bioresour Technol 99: 308-319.
18. Maddipati P, Atiyeh HK, Bellmer DD, Huhnke RL (2011) Ethanol production from syngas by *Clostridium* strain P11 using corn steep liquor as a nutrient replacement to yeast extract. Bioresource Technol 102: 6494-6501.
19. Ragsdale SW, Wood HG (1991) Enzymology of the Acetyl-CoA Pathway of CO2 Fixation. Crit Rev Biochem Mol Biol 26: 261-300.
20. Nie Y, Liu H, Du G, Chen J (2008) Acetate yield increased by gas circulation and fed-batch fermentation in a novel syntrophic acetogenesis and homoacetogenesis coupling system. Bioresour Technol 99: 2989-2995.
21. Kopke M, Held C, Hujer S, Liesegang H, Wiezer A, et al. (2010) *Clostridium ljungdahlii* represents a microbial production platform based on syngas. PNAS 107: 13087-13092.

This article was originally published in a special issue, **Bioprocessing** handled by Editor(s). Dr. Mark R. Wilkins, Oklahoma State University, USA; Stefano Curcio, University of Calabria, Italy