
Environmental Technology
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/tent20

# Isolation and screening of carboxydotrophs isolated from composts and their potential for butanol synthesis

Sean Nguyen [a], Fatime Ala [a], Cisley Cardwell [a], Darlene Cai [a], Katelyn M. McKindles [a], Aaron Lotvola [a], Steven Hodges [a], Yiwei Deng [a] & Sonia M. Tiquia-Arashiro [a]
[a] Department of Natural Sciences, University of Michigan, Dearborn, MI, USA
Accepted author version posted online: 23 May 2013.Published online: 31 May 2013.

To cite this article: Sean Nguyen, Fatime Ala, Cisley Cardwell, Darlene Cai, Katelyn M. McKindles, Aaron Lotvola, Steven Hodges, Yiwei Deng & Sonia M. Tiquia-Arashiro (2013) Isolation and screening of carboxydotrophs isolated from composts and their potential for butanol synthesis, Environmental Technology, 34:13-14, 1995-2007, DOI: 10.1080/09593330.2013.795987

To link to this article: http://dx.doi.org/10.1080/09593330.2013.795987

*Environmental Technology*, 2013
Vol. 34, Nos. 13–14, 1995–2007, http://dx.doi.org/10.1080/09593330.2013.795987

Taylor & Francis
Taylor & Francis Group

# Isolation and screening of carboxydotrophs isolated from composts and their potential for butanol synthesis

Sean Nguyen, Fatime Ala, Cisley Cardwell, Darlene Cai, Katelyn M. McKindles, Aaron Lotvola, Steven Hodges, Yiwei Deng and Sonia M. Tiquia-Arashiro*

*Department of Natural Sciences, University of Michigan, Dearborn, MI, USA*

(*Received 3 January 2013; final version received 24 March 2013*)

Carboxydotrophs are known for their ability to convert carbon monoxide (CO) to butanol through fermentation. Such a platform offers a promising alternative approach to biofuel production from synthesis gas feedstocks. In this study, carboxydotrophs were isolated from various manure compost. Out of 500 isolates, only 11 carboxydotrophs (7 mesophiles and 4 thermophiles) were found to utilize CO as the sole source of carbon and energy. To assess the biochemical basis for their ability to produce biofuel (butanol), the level of activities of CO dehydrogenase (CODH), hydrogenase and butanol dehydrogenase (BDH) enzymes for these isolates against the known carboxydotroph, *Butyribacterium methylotrophicum* was assessed. All isolates showed evidence of enzyme activities (0.16–2.20 $\mu$mol min$^{-1}$), with the majority exhibiting higher activities compared with the known carboxydotroph, *B. methylotrophicum* (0.33–0.71 $\mu$mol min$^{-1}$). The level of activities for CODH and BDH ranged from 0.163–3.59 $\mu$mol min$^{-1}$ and 0.19–2.2 $\mu$mol min$^{-1}$, respectively. Three isolates (M7-1, T2-22, and T3-14) demonstrated enzymatic activity three to seven times higher than *B. methylotrophicum*. Of these, T2-22 exhibited the highest BDH activity and shows great promise in the conversion of toxic CO into butanol more so than other carboxytotrophs known thus far. This study revealed some biochemical basis for butanol production from CO by carboxydotrophs. However, more research is needed to discover a direct biological route for butanol production from CO to strengthen their potential for synthesis gas bioprocessing. Follow-up work will focus on whole-genome sequencing of the promising isolate T2-22 to provide system-level insights into how carboxydotrophs utilize and regulate their molecular machineries for butanol production.

**Keywords:** carboxydotrophs; carbon monoxide dehydrogenase; butanol dehydrogenase; biobutanol; syngas



## 1. Introduction

Most existing work on biofuel production from cellulosic biomass have been focused on the sugar platform, that is, decomposition of plant cell wall biopolymers (cellulose and hemicellulose) to small sugar monomers (mainly glucose and xylose) followed by sugar fermentation.[1–4] Some of the biggest challenges in this process are the sturdiness of the cellulosic material and the high cost associated with the saccharification step. In addition, some components in the cellulosic feedstock, such as lignin, are not degradable and thus cannot be converted to biofuels by sugar fermentation. An alternative might be to gasify organic biomass and to use the produced synthesis gas (or syngas) as a feedstock for the synthesis of ethanol and other valuable compounds. Syngas, formed by the gasification or reforming of coal, natural gas or biomass, is a key intermediate in the production of synthetic fuels.[5–8] As syngas can be produced from both fossil fuels and renewable resources, it also enables a gradual transition to more sustainable energy and chemical production. The carbon monoxide (CO) and $H_2$ present in syngas are substrates for microbial metabolism, which can be exploited for the synthesis of various interesting products.

Numerous studies indicate that CO participates in a broader range of processes than any other single molecule, ranging from subcellular to planetary scales.[9] Despite its toxicity to many organisms, a diverse group of bacteria that span multiple phylogenetic lineages metabolize CO.[9–11] These bacteria are globally distributed and include pathogens,[12] plant symbionts [13] and biogeochemically important lineages in soils and the oceans.[14,15] The term 'anaerobic carboxydotrophic bacteria' refers here to bacteria that can metabolize CO as a sole source of carbon, where the energy driving reaction is the oxidation of CO. Anaerobic carboxydotrophic bacteria can be further divided into three groups: acetogens, methanogens, sulphate reducers and elemental sulphur reducers.[16,17] Acetogens have attracted the most attention because they offer several promising routes to chemicals and fuels production.[17] They utilize CO as a sole source of carbon and energy source and produce a variety of products including acetate, ethanol, butyrate and butanol through syngas fermentation.[18] Carboxydotrophs are ubiquitously distributed in nature; however, locations of naturally enriched cultures are unknown.

*Corresponding author. Emails: smtiquia@umich.edu; smtiquia@yahoo.com

They have been isolated from composts, sewage, freshwater and marine sediments, hydrotherms, coal heap, rhizosphere, and volcanic soil.[19–21] Enrichment and culture of anaerobic carboxydotrophs from natural habitats are usually done in liquid batch cultures incubated anaerobically and supplied with CO as a sole source of carbon and energy.[22,23] Such conditions are highly specific because of the inability of the contaminating aerobic microflora to use CO for growth.

In the past two decades, new isolates and some known anaerobic microorganisms were shown capable of growth with CO and $H_2$ as substrates.[17,24–27] These anaerobic carboxydotrophic bacteria produce $H_2$, formate, acetate, ethanol, butyrate and/or butanol. Predominantly, mesophilic carboxydotrophs have been shown to form organic compounds from syngas. So far, few attempts have been made to isolate thermophilic carboxydotrophs that can produce organic compounds from syngas. Growth by the thermophiles at high temperatures could be advantageous, as less cooling of the syngas is required before it is introduced into the bioreactor. Additionally, higher temperatures can lead to higher conversion rates and benefit separation of the product by distillation. At present, suitable thermophiles for the production of organic compounds from syngas are not available. Their use could offer potential advantages over the use of mesophiles.

Interest in bio-butanol synthesis has increased due to rising oil prices and concerns of surrounding climate change. It has been increasingly regarded as a source of clean energy and alternative fuel.[28,29] Butanol, like ethanol, can be produced from sugars, synthesis gas, and glycerol. Butanol has a number of notable qualities that make it a suitable alternative fuel. Its energy content is 30% more than ethanol.[30] It can be mixed with gasoline in any proportion or be used as sole fuel component (100% butanol) in unmodified car engine.[31] It is less soluble to water and is less corrosive; therefore, it can be transported through existing gasoline pipelines.[32,33] Although several anaerobic carboxydotrophs have been isolated over the past 20 years, many of these strains did not produce butanol. A majority of the isolates produced acetate and a few (purple non-sulphur bacteria) were able to convert CO to $H_2$ to organic compounds.[17] Thus, it is necessary to isolate and characterize new anaerobic carboxydotrophs that will synthesize butanol from synthesis gas.

The main goals of this study were to isolate and characterize anaerobic carboxydotrophic bacteria from various composts and elucidate their potential for butanol production. Compost harbours a huge diversity of microorganisms [34–38] and can be an ideal medium to isolate new anaerobic carboxydotrophs. There are aerobic and anaerobic pockets within the compost,[39,40] which is ideal for isolating aerobic and anaerobic microorganisms. Composts also have a temperature gradient (20°C–75°C),[36,41–44] which is ideal for isolating mesophilic and thermophilic microorganisms. The enrichment and culturing techniques were employed to isolate anaerobic carboxydotrophs from five different composts: (1) dairy manure + straw + woodchips; (2) dairy manure + compost; (3) mature dairy manure; (4) mature leaf compost; and (5) dairy manure + sawdust. In addition to mesophilic carboxydotrophs, suitable carboxydotrophic thermophiles were isolated and characterize for their ability to utilize CO as a sole carbon source. A set of cellular, biochemical and molecular assays were performed to characterize the physiology and metabolic capabilities of the newly isolated strains.

## 2. Methods

### 2.1. Microorganism and culture conditions

The carboxydotrophic strain of *Butyribacterium methylotrophicum* was obtained from American Type Culture Collection, Manassas, Virginia, USA. A stock culture was maintained in 152 ml serum bottles (Wheaton Scientific, Millville, NJ) sealed with butyl rubber (Bellco Biotechnology, Vineland, NJ) and aluminium crimp seals (Bellco Biotechnology, Vineland, NJ). These serum bottles contained 50 ml of phosphate-buffered medium and an initial gas headspace of 100% CO gas at approximately 2.1. When applicable, 28 ml pressure tubes (Bellco Biotechnology, Vineland, NJ) were substituted for the bottles, containing 10 ml of liquid media. The stock culture was grown in the dark at 37°C on a shaking platform (Labnet 311DS Shaking Incubator, Labnet International, Edison, NJ) at 100 rpm. The culture was transferred to fresh bottles every two to three weeks. A complete set of 5 ml glycerol containing cell culture bottles was frozen at −80°C for extended storage. Growth studies were initiated by inoculation with 0.5%–2% (v/v) of actively growing *B. methylotrophicum* stock culture.

### 2.2. Culture media and gases

For the enrichments and cultures, a phosphate-buffered basal medium was adapted from Lynd et al.[23] The culture medium contained the following components (per 1 L of distilled water): 0.9 g NaCl; 0.2 g $MgCl_2H_2O$; 0.1 g $CaCl_2$ $2H_2O$; 1.0 g $NH_4Cl$; 10 ml trace metal II; and 1 ml of 0.2% resazurin. Trace element II solution is aqueous mineral salts containing 12.8 g $L^{-1}$ nitriloacetic acid; 0.10 g $L^{-1}$ $FeSO_4$ $7H_2O$; 0.10 g $L^{-1}$ $MnCl_2$ $4H_2O$; 0.17 g $L^{-1}$ $CoCl_2$ $6H_2O$; 0.10 g $L^{-1}$ $CaCl_2$ $2H_2O$; 0.10 g $L^{-1}$ $ZnCl_2$; 0.02 g $L^{-1}$ $CuCl_2$ $2H_2O$; 0.1 g $L^{-1}$ $H_3BO_3$; 0.01 g $L^{-1}$ sodium molybdate; 1.0 g $L^{-1}$NaCl; 0.017 g $L^{-1}$ $Na_2SeO_3$; and 0.026 g $L^{-1}$ $NiSO_4$ $6H_2O$. The nitriloacetic acid is not a carbon source, but serves to prevent precipitation of iron in the form of FeS. Resazurin is a colorimetric indicator of dissolved oxygen in the pH range of 5–8.

After autoclaving, 10 ml $L^{-1}$ of a 10% w/v yeast extract (Difco Laboratories, Franklin Lakes, NJ) solution and 25 ml $L^{-1}$ of an aqueous phosphate buffer containing

150 g $L^{-1}$ $KH_2PO_4$ and 290 g $L^{-1}$ $K_2HPO_4$ were added. Also added was 10 ml $L^{-1}$ aqueous vitamin solution containing 0.002 g $L^{-1}$ biotin; 0.002 g $L^{-1}$ folic acid; 0.010 g $L^{-1}$ $B_6$HCl (pyridoxine); 0.005 g $L^{-1}$ $B_1$HCl (thiamine); 0.005 g $L^{-1}$ $B_2$ (riboflavin); 0.005 g $L^{-1}$ nicotinic acid; 0.005 g $L^{-1}$ pantothenic acid; 0.0001 g $L^{-1}$ crystalline $B_{12}$ (cyanocobalamin); 0.005 g $L^{-1}$ PABA (para-aminobenzoic acid). The final addition was 25 ml $L^{-1}$ of a 2.5% w/v $Na_2S$ $9H_2O$ reducing aqueous solution. All post-autoclaving additions above were sterilized separately, either by autoclaving or filter sterilization. The medium thus consists mostly of inorganic salts, minerals, yeast extract, and vitamins and contains no carbon source except for small amount present in yeast extract.

The CO, $N_2$, $H_2$-$CO_2$ 80:20 (vol/vol) premixed, and $N_2$-$CO_2$ 95:5 (vol/vol) premixed gases used in this study were obtained from Cryogenic Gasses (Matheson Tri-Gas, Montgomeryville, PA). Gas purity averages were 99.0% for CO and 99.99% for $N_2$.

### 2.3. Enrichment and isolation of carboxydotrophs from composts

Five different composts were obtained from Ohio Agricultural Research and Development Center, The Ohio State University. These composts include: (1) dairy manure + straw + woodchips; (2) dairy manure + compost; (3) mature dairy manure; (4) mature leaf compost; and (5) dairy manure+sawdust. To obtain enrichments of CO-utilizing anaerobes, 158 ml serum vials (Wheaton Scientific, Millville, NJ) inoculated with composts (2 $cm^3$) were incubated at 37°C or 55°C. The enrichment was carried out in serum bottles containing phosphate-buffered medium containing glucose as the C source, and incubated at 37°C or 55°C. Growth was determined using light microscopy and formation of gaseous products. The enrichments underwent serial dilutions and were spread directly on the surface thioglycolate agar plates and incubated anaerobically at 37°C (to isolate mesophilic bacteria) or 55°C (to isolate thermophilic bacteria) for one week. Colonies arising on the plates were selected for isolation based on gross morphological features.

### 2.4. Characterization of isolates from composts

Fifty isolates were randomly selected from each of the five compost mesophilic and thermophilic enrichments. A total of 500 isolates were initially recovered, which included 250 mesophiles and 250 thermophiles. The isolates were characterized based on Gram reaction, $O_2$ requirements, motility, and physiological attributes. Morphological characteristics were determined by standard methods.[45] For the $O_2$ requirement assay, the stab inoculation technique was used to determine if the bacterial isolate is aerobic, anaerobic, facultative anaerobe or microaerophilic. The carbohydrate fermentation test was used to observe the carbohydrate fermentation patterns of the different isolates to screen for successful fermentation of an end product consisting of either an acid, gas ($CO_2$), alcohol or a combination thereof on glucose, lactose and sucrose. The methyl-red test was used to detect the production of varying acids in the end products to determine which of the isolates were mixed acid fermenters. The Vogues–Proskauer (VP) test was used to detect the presence of acetoin in the medium, which is a precursor in the synthesis of the compound 2,3-butanediol. The catalase test helped detect the presence of the catalase enzyme in the organisms, which plays an important role in destroying toxic compounds that can accumulate in the cell, such as $O_2$.

### 2.5. Growth on different carbon sources (glucose, CO and formate)

The cells were grown anaerobically in 10 ml phosphate-buffered medium containing either CO, formate (sodium formate), or glucose as the carbon source. Culture turbidities at 660 nm were determined by inserting anaerobic culture tubes into a Spectronic 20 spectrophotometer (Bausch & Lomb, Inc., Rochester, NY). When the turbidity exceeded an $OD_{660}$ of 0.4, the cells were diluted or a curve of diluted versus non-diluted $OD_{660}$ values was used. In serum bottle experiments, turbidities were measured by withdrawing 1 ml of the culture suspension, followed by dilution. Frequently, we used a curve relating $OD_{660}$ to dry weight. This relationship was linear with 344 mg of cells per litre at an $OD_{660}$ of 1.0 for CO-grown cells. Absorbance readings were taken at the following time intervals: 0, 30, 60, 90, 120, 180, 240 and 300 min. The growth of the isolates at different time intervals was compared with the growth of *B. methylotrophicum*, which served as a positive control.

### 2.6. Enzyme analysis

All assays were performed at 30°C spectrophotometrically, under strictly anaerobic conditions in plastic cuvettes (1.0 ml total volume) with 100% $N_2$ (unless otherwise specified) in the headspace as described by Rengpipat et al.[46] The cells were grown anaerobically until the late exponential phase (20 h) in 300 ml serum bottles (Bellco Biotechnology, Vineland, NJ) containing 55 ml of phosphate-buffered medium and CO as the carbon source. The phosphate-buffered medium was gassed with CO at 0.5 psi for 10 min. After 20 h of incubation at 37°C (thermophilic isolates) and 55°C (mesophilic isolates), the cells (~0.2 g) were harvested by centrifugation at 4000 *g* in a GT-2 table-top centrifuge (MSE, London, UK) and stored at −20°C prior to the enzyme assay. The assays were performed in 1.0 ml cuvettes that were made anaerobic by repeatedly evacuating and flushing with $N_2$. All enzyme activity was induced using 1.5 mM isopropyl-$\beta$-thiogalactoside in combination with phosphate-buffered medium, reducing agent,

and phosphate-buffered solution in a stoppered 300 ml serum bottle, then letting the cells grow overnight or until they reached a spectrophotometric reading of at least 0.2 at 660 nm. The cells were centrifuged at 4000 *g* for 10 min MSE GT-2 (London, UK) until 0.2 g of the cells were recovered and resuspended in lysis buffer (100 mM Tris pH 8.0; 150 mM NaCl; mg $ml^{-1}$ lysozyme; 1 mg $ml^{-1}$ antiprotease DNAse I; 2 mM $MgCl_2$).[47,48] The cells were then incubated 55°C for 10 min in a dry heating block (Accublock Digital Dry Bath, Labnet, Edison, NJ) and vortexed every 2 min. After 10 min of incubation, 0.1 mg of glass beads (Bio 101 systems) was added to the cells and vortexed for 10 min at maximum speed. Thereafter, the cells were then pelleted for 5 min at 16,000 *g* in a high-speed microcentrifuge (Eppendorf 5415D centrifuge, Happauge, NY). The crude cell extracts were used for the enzyme assays.

For the CO dehydrogenase (CODH) assay,[49] 0.25 ml of crude cell extracts were injected in cuvettes containing 0.75 ml of CODH standard reaction mixture (20 mM methyl viologen (MV) and 2 mM dithioerythritol in 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid–NaOH). The reaction was followed by monitoring the change in absorbance at 578 nm. Rates of MV reduction were calculated using an absorption coefficient of 9.7 $mM^{-1}$ $cm^{-1}$. One unit of CO or $H_2$ oxidation activity is defined as the reduction of 2 mmol of MV $min^{-1}$, which is equivalent to 1 mmol of CO or $H_2$ oxidized per $min^{-1}$.[49]

For the formate dehydrogenase (FDH) assay,[47,48] 0.25 ml of crude cell extracts were re-suspended in 0.75 ml of FDH standard reaction mixture (15 μmol 2-mercaptoethanol, 45 μmol potassium phosphate buffer (pH 7.5), 15 μmol MV and 13 mM sodium formate). The reaction was followed by monitoring the change in absorbance at 603 nm. Rates of MV reduction were calculated using an absorption coefficient of 11.3 $mM^{-1}$ $cm^{-1}$. A unit was the amount of enzyme that reduced 1 mmol of MV per minute.[48].

For the hydrogenase assay,[50,51] 0.25 ml of crude cell extracts was injected into the assay cuvette containing 0.75 ml of gas-free hydrogenase reaction mixture (50 mM Tris pH 8.5 and 1 mM MV. The reaction was followed by monitoring the absorbance at 578 nm. Rates of MV reduction were calculated using an absorption coefficient of 13.6 $mM^{-1}$ $cm^{-1}$. One unit (U) of hydrogenase activity was defined as the amount of enzyme, which catalyses the oxidation of 1 mmol $H_2$ per min.[50]

For the butanol dehydrogenase (BDH) assay,[32,52] 0.25 ml of crude cell extract was injected into an airtight cuvette flushed with CO at 0.5 psi for 1 min containing 0.75 ml of BDH standard reaction mixture (0.39 mM NAD+, 73.5 mM semicarbazide hydrochloride, 68.8 mM Tris-HCl pH 7.8). The reaction was monitored by observing the change in absorbance at $365_{nm}$. Rates of NAD+ reduction were calculated using an absorption coefficient of 3.4 $mM^{-1}$ $cm^{-1}$. A unit was the amount of enzyme that reduced 1 mmol NAD+ per minute.[32]

### 2.7. DNA isolation and 16S rRNA sequencing of isolates

The cells were centrifuged to obtain pellets for DNA extraction using DNeasy kit as suggested by manufacturer (Qiagen, Valencia, CA) to break up the cells and isolate the genomic DNA. Thereafter, nanodrop (Nanodrop 1000, Wilmington, DE) quantification was done to determine the DNA concentration values and quality (260:280 ratio) of the extracts. Identification of isolates was performed by amplifying the full-length 16S rRNA genes using bacteria-specific primers sequence FD1 (5′ AGA GTT TGA TCC TGG CTC AG 3′) and 1540r (5′ > AAG GAG GTG ATC CAG CC < 3′) with cycling conditions described previously.[53–55] Polymerase chain reaction (PCR) was performed in an iCycler Thermal cycler (Bio-Rad Laboratories, Inc., Hercules, CA) as 25 μl reactions consisting of 12.5 μl Choice *Taq* Blue DNA Polymerase Mastermix (Denville Scientific, Metuchen, NJ), 1.25 μl of each 10 μM primer, 0.15 μl BSA (bovine serum albumin), 0.75 μl DMSO (dimethyl sulphoxide), and 1–2 μl DNA template.[53–55] PCR products (~1500 bp) were verified on a 1% agarose gel and then purified using QIAquick PCR Purification Kit (Qiagen, Valencia, CA). The purified PCR products were sequenced and analysed directly without cloning. Chimeric sequences were checked by Check_Chimera program available at the Ribosomal Database Project (RDP II).[56] The similarity searches for sequences were carried out using the BLAST (N) program of the National Center for Biotechnology Information, MD, USA.

### 2.8. Absorbance spectra of the cell supernatant

The absorbance spectra of the cell supernatants (*B. methylotrophicum* and M3-29) were determined to detect the expected fermentation products (acetic acid, butyrate, ethanol and butanol). For the ultraviolet and visible regions, measurements were made using a UV/Vis spectrophotometer (Beckman Coulter DU 700 Series). The absorption peaks observed were then compared against the detection wavelengths of acetate (200–235 nm), butyrate (191–200 nm), ethanol (205–240 nm) and butanol (215–245 nm).[57–61]

## 3. Results

### 3.1. Characterization of isolates

Of the 250 mesophilic isolates, 93 (37%) were strict anaerobes; 145 (58%) were facultative anaerobes; and 3 (1%) were microaerobes (Table 1). Majority of the mesophilic isolates were from Gram-negative taxa. Fifty-nine per cent (147 isolates) and 41% (103 isolates) were Gram-positive. Cell morphologies tended to be more rods than cocci at 133 (53%) and 109 (44%), respectively, and others were filamentous or spiral. Most of the mesophilic isolates were motile and possess the enzyme catalase. Overall, 55 isolates tested positive for methyl-red test and 89 for VP test.

Table 1. Characterization of mesophilic and thermophilic isolates from composts.

| Test | Mesophilic isolates | Thermophilic isolates |
|---|---|---|
| Strictly anaerobes | 93 (37%) | 96 (38%) |
| Facultative anaerobes | 145 (58%) | 149 (60%) |
| Microaerophiles | 3 (1%) | 4 (2%) |
| Gram+ | 147 (59%) | 140 (56%) |
| Gram− | 103 (41%) | 110 (44%) |
| Cell morphologies | | |
| Cocci | 109 (44%) | 101 (40%) |
| Rods | 133 (53%) | 115 (46%) |
| Other morphologies | 5 (2%) | 19 (8%) |
| Catalase test | 162 (65%) | 65 (26%) |
| Methyl-red test | 55 (22%) | 38 (15%) |
| VP | 89 (36%) | 168 (67%) |
| Motility | | |
| Motile | 139 (56%) | 135 (54%) |
| Non-motile | 83 (33%) | 83 (33%) |
| Fermentation capabilities | | |
| Lactose fermentation | | |
| Acid | 165 (66%) | 25 (10%) |
| Gas | 65 (26%) | 1 (0%) |
| Alcohol | 3 (1%) | 0 |
| Sucrose fermentation | | |
| Acid | 247 (99%) | 162 (65%) |
| Gas | 207 (83%) | 1 (0%) |
| Alcohol | 0 | 0 |
| Glucose fermentation | | |
| Acid | 231 (92%) | 141 (56%) |
| Gas | 215 (86%) | 2 (1%) |
| Alcohol | 8 (3%) | 0 |

When lactose was provided as a carbon source, 165 isolates produced an acid, 65 produced a gas, and 3 produced an alcohol; when given sucrose as a carbon source, 247 isolates produced an acid, 207 produced a gas, but none produced an alcohol. On the other hand, when glucose was provided as a carbon source, 231 isolates produced acids, 215 produced gases, and 8 produced alcohols (Table 1).

Of the 250 thermophilic strains isolated, most were facultative anaerobes (60%) and the rest were either strict anaerobes (38%) or microaerobes (4%) (Table 1). Gram staining results showed that 44% of the thermophilic isolates were Gram-negative and the rest were Gram-positive. The majority of the isolates were motile rods, cocci and spirilli. The catalase test results included 65 positives (26%) and 181 negatives (72%). The methyl-red test showed 38 positive (15%) and 212 negative (85%) results, whereas the VP test generated 168 positives (67%) and 82 negatives (33%). Most of the isolates were capable of fermenting sucrose and glucose and very few were able to ferment lactose. None of these isolates produced alcohols as fermentation products (Table 1).

### 3.2. Growth and metabolism of the isolates on CO

Majority of the isolates (402 out of 500 isolates) did grow on CO, suggesting that they are not carboxydotrophs (Table 2).

Table 2. Growth of CO of the mesophilic and thermophilic isolates.

| Growth rate on CO[a] | Mesophilic isolates | Thermophilic isolates |
|---|---|---|
| No growth on | 189 | 213 |
| Low growth rate (10–50% growth change) | 54 | 33 |
| Medium growth rate (51–200% growth change) | 4 | 3 |
| High growth rate (>200% change) | 3 | 1 |
| Total number of isolates | 61(24%) | 38(15%) |

[a]The per cent change in growth was calculated as follows: (final absorbance−initial absorbance)/initial absorbance reading*100. The CO isolates were then grouped together based on low (10–50% growth change), medium (51–200% growth change), and high growth (>200% growth change).

The rest of the isolates (61 mesophiles and 38 thermophiles) either grew at low (10–50% growth change); medium (51–200% growth change), high rate (>200% growth change). Most of the isolates that grew on CO fell within the low growth rate category, with only 11 falling within the medium and high growth rate categories (Table 2). These strains include 7 mesophiles (M2-32, M3-9, M3-28, M3-29, M6-36, and M7-1) and 4 thermophiles (T1-16; T2-22, T3-14, and T7-10). The thermophilic isolate that grew best on CO was taken from the dairy manure + compost (T2) composts, while the mesophilic isolates that grew best on CO were retrieved from the dairy manure + compost (M2), mature dairy manure (M3) and mature leaf compost (M6). The mesophilic isolates retrieved from the dairy manure + compost (M2) showed the highest growth rates on CO.

### 3.3. Growth comparison between CO-utilizing strains and B. methylotrophicum

The growth of the 11 CO-utilizing strains on different substrates (glucose, CO and formate) was compared with the known carboxydotroph, *Butyribacterium methylotrophicum*. *B. methylotrophicum* is a mesophilic, anaerobic, acid-producing bacterium that ferments one carbon compounds.[23] It has been known to grow on a wide range of substrates including glucose, CO, and formate as a sole source of carbon.[22,58] The growth curves of the 11 isolates and *B. methylotrophicum* in on glucose, CO and formate are shown in Figure 1. While all the isolates demonstrated growth in each medium, there were some differences noted in the growth characteristics of the isolates in the three culture media. The mesophilic isolates M2-32, M3-16, M6-36, and M7-1 grew better than *B. methylotrophicum* on glucose (Figure 1(a)). Growth of these isolates increased from 0.02 to 0.18. The mesophilic isolates grew better than the thermophilic isolates on glucose (Figure 1(a)

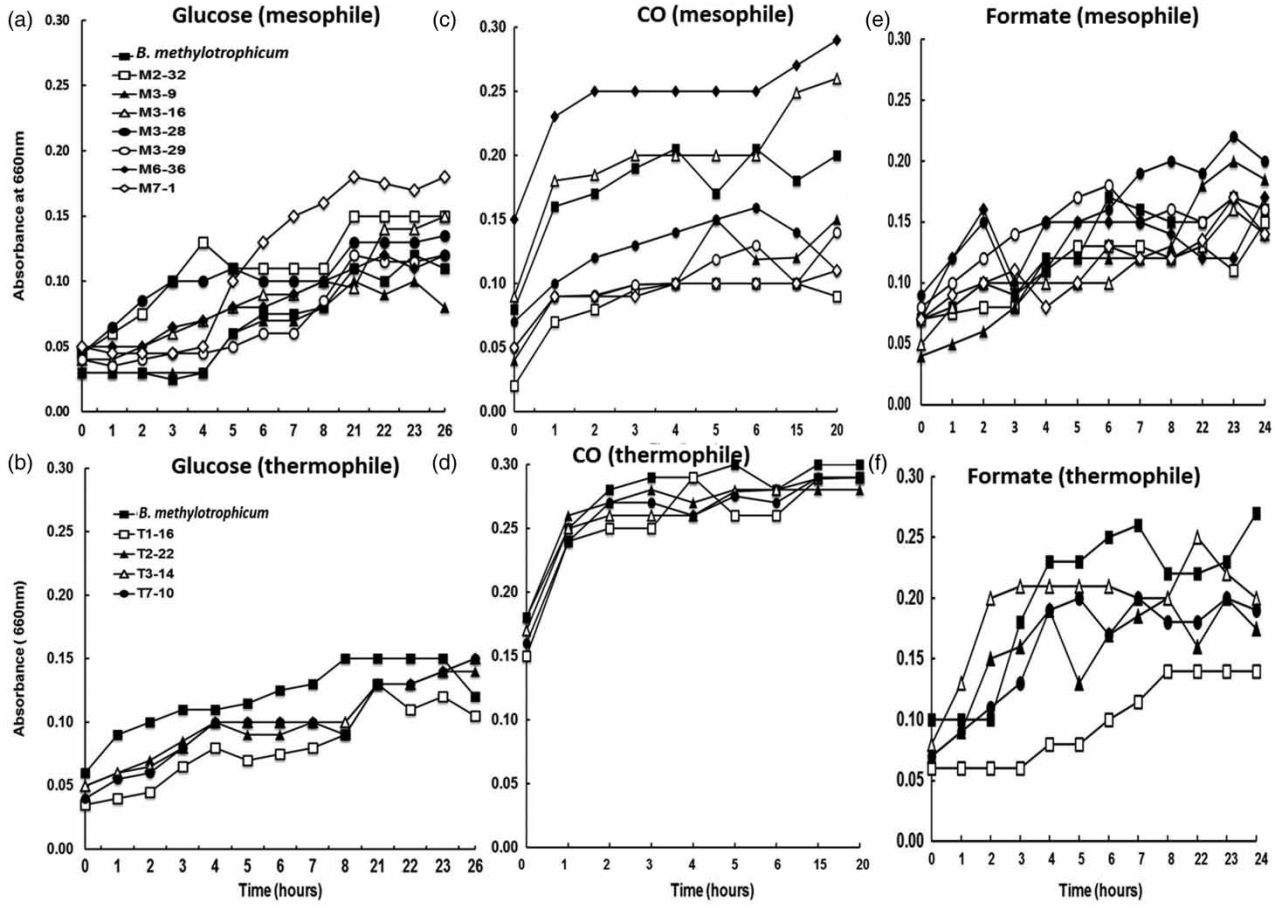


Figure 1. Growth of isolates on glucose (a = mesophilic; b = thermophilic), CO (c = mesophilic; d = thermophilic), and formate (e = mesophilic; f = thermophilic). *B. methylotrophicum* (control).



and 1(b)). The growth of the thermophilic isolates was lower than that of the mesophilic *B. methylotrophicum* (Figure 1(b)). When grown on CO, the thermophilic isolates showed better growth than the mesophilic isolates (Figure 1(c) and 1(d)), and grew at the same rate as *B. methylotrophicum* (Figure 1(d)). The growth pattern of the mesophilic isolates on formate was similar to that of *B. methylotrophicum* (Figure 1(e)), with growth rate ranging from 71 to 350%. Growth of the thermophilic isolates was comparable to the mesophilic isolates with the exception of T1-16, which demonstrated the lowest growth (Figure 1(e) and 1(f)).

The doubling time of the isolates and *B. methylotrophicum* varied depended on the substrate used (Table 3). While the isolates were able to grow both on organic (formate and glucose) and inorganic (CO) carbon, the doubling time was shorter when CO was used as the carbon source. The doubling times varied between 2 and 24 h on glucose; 1 and 6 h on CO and 1 and 11 h on formate. Thermophilic isolates had shorter doubling times than the mesophilic isolates for each provided carbon source. The doubling time of the known carboxydotroph *B. methylotrophicum* was the fastest on CO when grown at mesophilic temperature (37°C). On the other hand, it has the fastest (~2 h) doubling time on glucose when grown at thermophilic temperature (55°C) (Table 3). Among the 11 isolates, M6-36 and T2-22 had the fastest doubling time on CO. The period of time it takes for these isolates to double on CO was shorter than that of the *B. methylotrophicum* (Table 3).

### 3.4. Enzyme assay

The activities and concentration of the enzymes involved in CO-metabolism and butanol synthesis for the 11 isolates and *B. methylotrophicum* are summarized in Figures 3–6. The enzyme CODH is responsible for oxidizing CO to $CO_2$ and is also responsible for the formation of acetyl-CoA from bound methyl group (Figure 2). Results of the CODH assay indicated that the enzyme could be detected in all 11 isolates (Figure 3). The concentration of the enzyme varied between 0.04 and 0.50 mM $cm^{-1}$. Among the 11 isolates, M3-16 has the highest CODH concentration (0.50 mM $cm^{-1}$) (Figure 3). The CODH activities ranged between 0.16 and 2.01 μmol $min^{-1}$ per mg of protein. The mesophilic isolates M2-32, M3-16, M3-29 and M6-36, and thermophilic isolates T1-16 and T3-14

Table 3. Doubling time of the isolates and *B. methylotrophicum* (control) on glucose, CO, and formate as substrates.

| Isolate | Compost type | Doubling time (h)[a] | | |
|---|---|---|---|---|
| | | Glucose | CO | Formate |
| *Mesophiles* | | | | |
| M2-32 | Dairy manure and compost | 3.5 | 3.1 | 1.4 |
| M3-9 | Mature dairy manure | 5.7 | 2.9 | 9.5 |
| M3-16 | Mature dairy manure | 6.0 | 2.1 | 11.4 |
| M3-28 | Mature dairy manure | 14.7 | 1.6 | 3.5 |
| M3-29 | Mature dairy manure | 24.1 | 2.1 | 2.3 |
| M6-36 | Mature leaf compost | 6.0 | 1.4 | 6.8 |
| M7-1 | Dairy manure and sawdust | 11.0 | 5.7 | 2.7 |
| Control | *B. methylotrophicum* | 5.7 | 1.8 | 3.1 |
| *Thermophiles* | | | | |
| T1-16 | Dairy manure straw and woodchips | 6.0 | 3.7 | 6.1 |
| T2-22 | Dairy manure and compost | 2.2 | 3.2 | 2.5 |
| T3-14 | Mature dairy manure | 2.2 | 4.3 | 1.4 |
| T7-10 | Dairy manure and sawdust | 2.6 | 4.3 | 4.6 |
| Control | *B. methylotrophicum* | 1.8 | 3.7 | 6.9 |

[a]Doubling time was determined based on the absorbance values obtained during the initial and final exponential phase of growth. The following formula was used to determine the doubling time of the isolates: (final $absorbance_{log}$ - initial $absorbance_{log}$)/(0.301)* length of the log phase (h).

exhibited higher CODH activities (0.77–2.01 μmol $min^{-1}$) than *B. methylotrophicum* (0.63 μmol $min^{-1}$). The CODH activities of M3-16, M3-29 were higher than the thermophilic isolates (Figure 3).

FDH oxidizes formate to produce $CO_2$ and provide a carbon source for the pathway (Figure 2). FDH can be detected in all isolates (Figure 4). The activities of this enzyme were higher in the isolates than *B. methylotrophicum.* Highest FDH activities were observed on M3-29, T2-22 and T7-10 isolates. The lowest FDH activities were observed on M3-28 isolate and *B. methylotrophicum* (Figure 4).

Hydrogenase plays a vital role in anaerobic metabolism especially with bacterial fermentation. In CO-metabolism, hydrogenase is responsible for the oxidation of diatomic hydrogen (Figure 2). The concentration of hydrogenase enzyme in the isolates was lower (0.07–0.19 mM $cm^{-1}$) than the CODH enzyme (Figures 3 and 5). The thermophilic isolates T1-16 and T3-14 showed the highest levels of hydrogenase activity (0.54 and 0.76 μmol $min^{-1}$ per mg of protein, respectively) (Figure 5). Their activities were higher than *B. methylotrophicum* (0.29 μmol $min^{-1}$ per mg of protein). The hydrogenase activities of the mesophiles on the other hand were lower (0.16–0.38 76 μmol $min^{-1}$ per mg of protein) than *B. methylotrophicum* (0.52 μmol $min^{-1}$ per mg of protein) (Figure 5).

BDH is the final enzyme in the butanol synthesis, which catalyses the reduction of butyraldehyde to butanol (Figure 2). The BDH concentration for the mesophilic and thermophilic isolates ranged from 0.03–0.59 mM $cm^{-1}$ and 0.001–0.89 mM $cm^{-1}$, respectively (Figure 6). Isolates

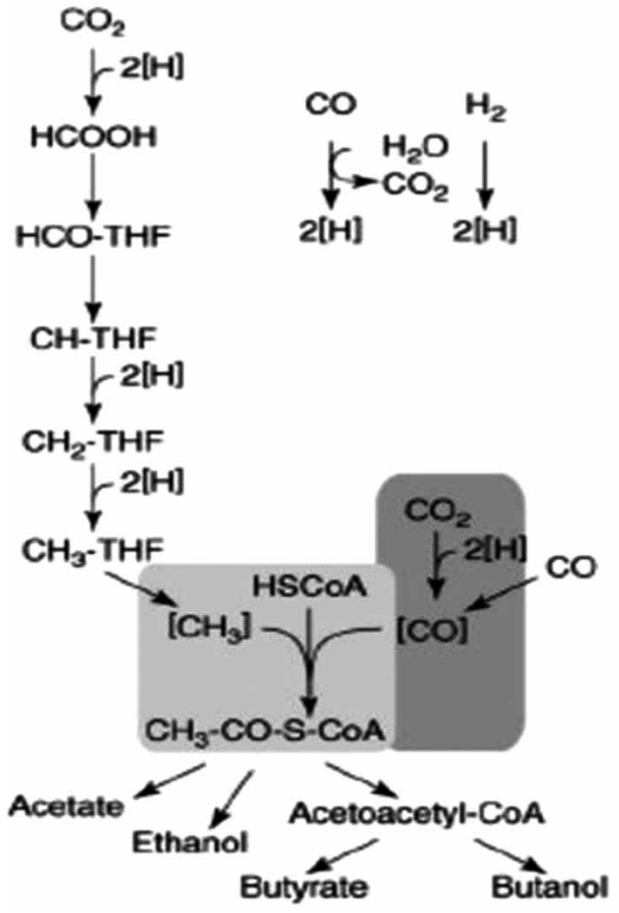


Figure 2. Proposed carbon and electron flow scheme to explain the mechanism of CO tolerance and the production of butanol via metabolism of CO and formate by carboxydotrophs.
*Source*: Jones and Woods [64] and Henstra et al.[17]

M3-29, T2-22 and T3-14 exhibited higher BDH activities (0.99–2.21 μmol $min^{-1}$) compared to *B. methylotrophicum* (0.71 μmol $min^{-1}$). The thermophilic isolates performed better than mesophilic isolates. Their activities are higher than that of the mesophilic isolates. Among the 11 isolates, the thermophilic isolates T2-22 (2.2 μmol $min^{-1}$ per mg of protein) and T3-15 (1.21 μmol $min^{-1}$ per mg of protein) showed the highest BDH activities (Figure 6).

### 3.5. *Identification of the CO-utilizing isolates*

The 11 isolates were identified using by 16S rRNA gene sequencing. All of the isolates belong to the phylum *Firmicutes* with the exception of T1-16, which belongs to the phylum *Actinobacteria* (Table 4). The mesophilic isolates were associated with the families *Enterococcaceae*, *Carnobacteriaceae*, and *Lactobacillaceae*, while the thermophilic isolates were associated with the families *Brevibacteriaceae* and *Bacillaceae.* The isolates that exhibited higher CODH activities than *B. methylotrophicum* were related to the 16S rRNA sequences of *Enterococcus devriesei* (M2-32; 95% sequence similarity); *Isobaculum melis* (M3-16; 98%

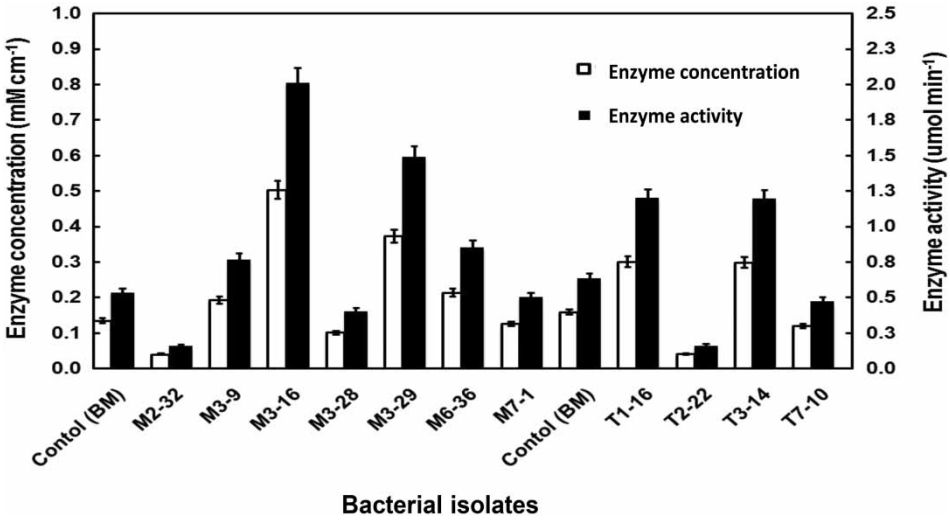


Figure 3. CODH concentration and activities of the isolates and the known carboxyudotroph, *B. methylotrophicum* (control).

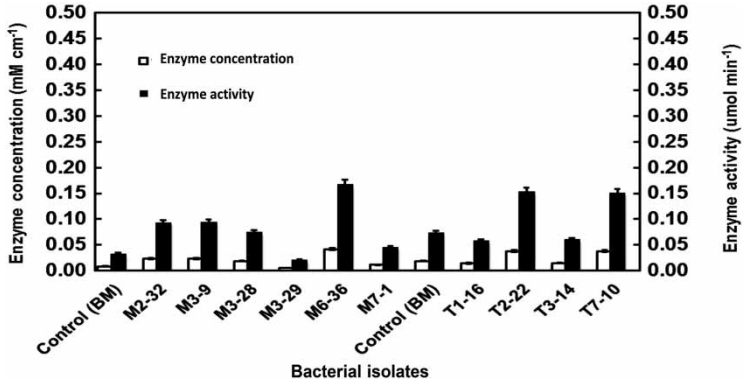


Figure 4. FDH concentration and activities of the isolates and the known carboxyudotroph, *B. methylotrophicum* (control).

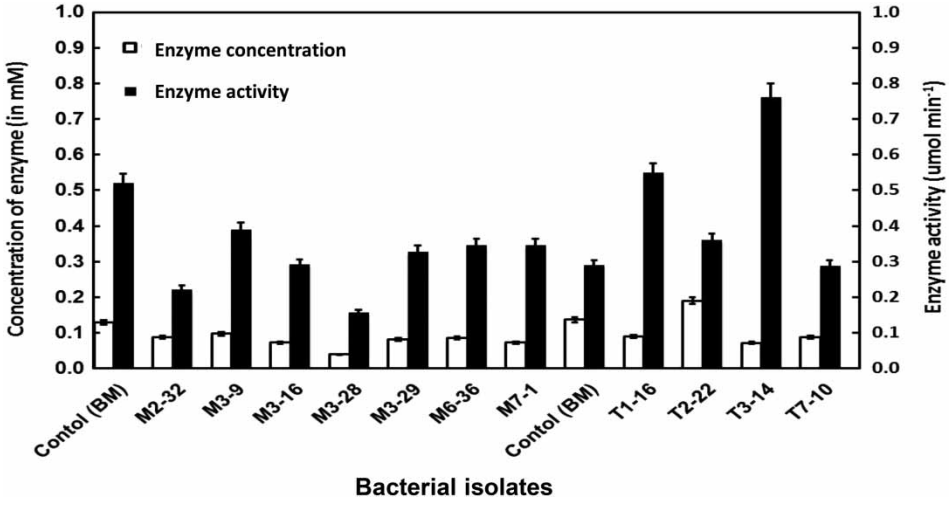


Figure 5. Hydrogenase concentration and activities of the isolates and the known carboxyudotroph, *B. methylotrophicum* (control).

sequence similarity; *Carnobacterium funditum* (M3-29; 95% sequence similarity); *Enterococcus ratti* (M6-36; 94% sequence similarity); *Brevibacterium halotolerans* (T1-16; 93% sequence similarity); and *Bacillus sonorensis* (T3-14; 99% sequence similarity). The isolate that displayed the highest BDH activities was related to the 16S rRNA sequences of *Bacillus carboniphilus* (T2-22; 92% sequence similarity) (Table 4).

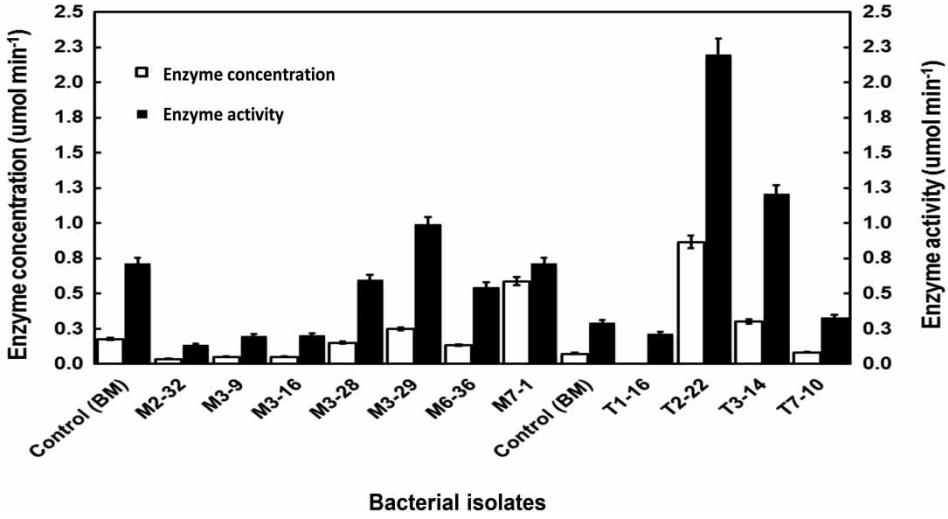


Figure 6. BDH concentration and activities of the isolates and the known carboxyudotroph, *B. methylotrophicum* (control).

Table 4. Identity of isolates that is based on 16S gene sequence comparison from known bacteria from GenBank.

| Isolate | Closest matched sequence from GenBank | Taxonomic classification | % nucleotide sequence identity | Accession number |
|---|---|---|---|---|
| *Mesophiles* | | | | |
| M2-32 | *Enterococcus devriesei* | *Firmicutes/Enterococcaceae* | 95 | NR-042389 |
| M3-9 | *Enterococcus saccholyticus* | *Firmicutes/Enterococcaceae* | 99 | NR-041707 |
| M3-16 | *Isobaculum melis* | *Firmicutes/Carnobacteriaceae* | 98 | NR-025445 |
| M3-28 | *Enterococcus casseliflavus* | *Firmicutes/Enterococcaceae* | 96 | NR-041704 |
| M3-29 | *Carnobacterium funditum* | *Firmicutes/Carnobacteriaceae* | 95 | NR-041703 |
| M6-36 | *Enterococcus ratti* | *Firmicutes/Enterococcaceae* | 94 | NR-041933 |
| M7-1 | *Lactobacillus animalis* | *Firmicutes/Lactobacillaceae* | 94 | NR-041610 |
| *Thermophiles* | | | | |
| T1-16 | *Brevibacterium halotolerans* | *Actinobacteria/Brevibacteriaceae* | 93 | NR-042638 |
| T2-22 | *Bacillus carboniphilus* | *Firmicutes/Bacillaceae* | 92 | NR-0.24690 |
| T3-14 | *Bacillus sonorensis* | *Firmicutes/Bacillaceae* | 99 | NR-025130 |
| T7-10 | *Bacillus aerius* | *Firmicutes/Bacillaceae* | 94 | NR-0423381 |



### *3.6. Detection of fermentation products*

The absorption wavelengths that correspond to butyrate, acetate, ethanol, and butanol were detected in supernatant (Figure 7); however, the true identity of these fermentation products needs to be confirmed by gas chromatography-mass spectrometry or high performance liquid chromatography-mass spectrometry analysis.

## 4. Discussion

Even though several anaerobic carboxydotrophs have been isolated with the ability to convert synthesis gas to biofuels,[17] the list is still small and extensive physiological and metabolic research is deficient for these isolates. We have demonstrated in this study that carboxydotrophic bacteria can be isolated from various manure composts. Most of the CO metabolizing strains were isolated from the mature dairy manure composts, suggesting that mature manure compost served as a better natural environment for carboxydotrophs than immature composts (partially composted manure). Attempted enrichment and isolation of carboxydotrophs from composts, however, yielded growth of a minority of isolates. While the CO in the culture medium made the isolation procedure highly selective, many of the isolates were inhibited by and unable to grow on high CO (100% in the headspace). Out of 500 isolates, only 11 (7 mesophiles and 4 thermophiles) proved to utilize CO as the sole source of carbon and energy. These isolates grew well on high CO and were considered true carboxydotrophs.

All 11 carboxydotrophs isolated in this study were Gram-positive facultative anaerobes (Table 5) and were able to grow in various organic (glucose and formate) and inorganic (CO) carbon sources. The 16S rRNA gene sequences of these isolates were very similar (92–99%) to the 16S rRNA gene sequences of *Bacillus* (*Bacillus carboniphilus*, *Bacillus sonorensis* and *Bacillus aerius*), *Enterococci* (*Enterococcus devriesei*, *Enterococcus saccholyticus*, *Enterococcus casseliflavus* and *Enterococcus ratti*), *Isobaculum* (*Isobaculum melis*), *Lactobacillus*

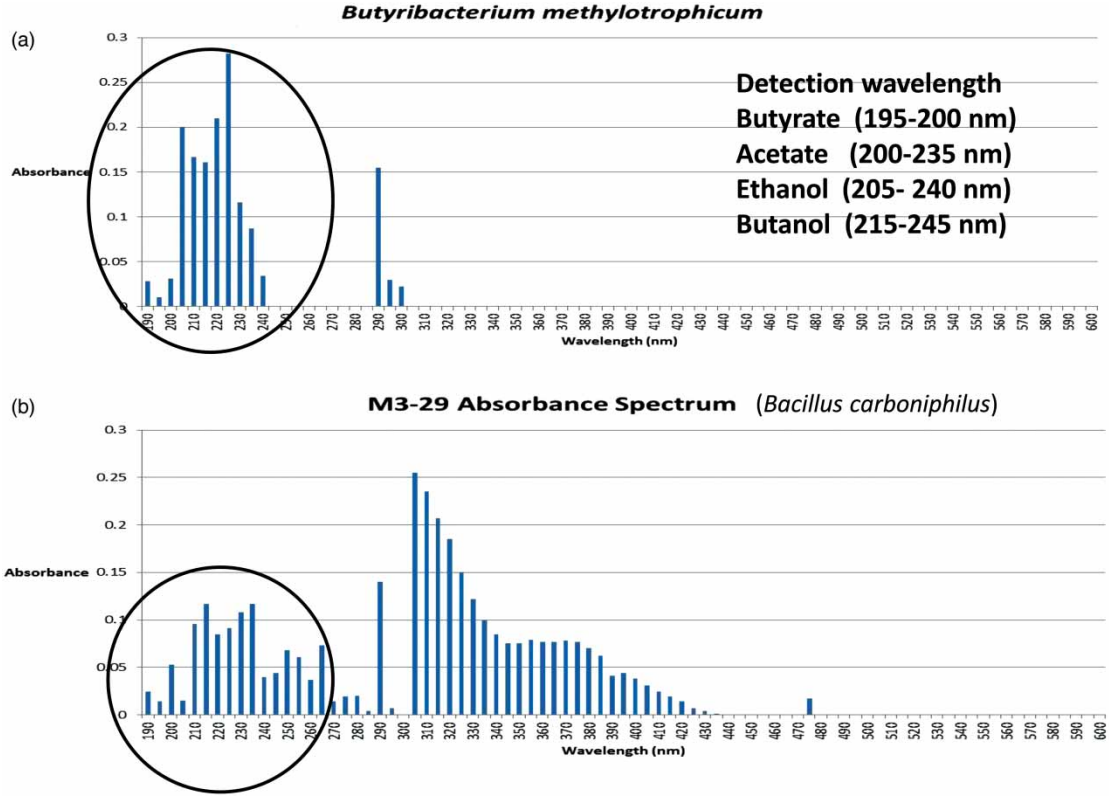


Figure 7. Absorption spectra of (a) *B. methylotrophicum* and (b) M3-29 cell suspension.

Table 5. Characteristics of carboxytrophic isolates.

| | M2-32 | M3-9 | M3-16 | M3-28 | M3-29 | M6-36 | M7-1 | T1-16 | T2-22 | T3-14 | T7-10 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| $O_2$ requirement | FA | FA | FA | FA | FA | FA | FA | FA | FA | FA | FA |
| Gram reaction | + | + | + | + | + | + | + | + | + | + | + |
| Motility | + | + | + | − | − | − | + | − | + | + | + |
| Shape | C | C | R | C | C | C | R | R | R | R | R |
| Catalase test | + | + | + | + | + | + | + | + | + | + | + |

Note: '−', negative result; '+', positive result; FA, facultative anaerobe; R, rod-shaped; C, coccus-shaped.

(*Lactobacillus animalis*), *Carnobacterium* (*Carnobacterium funditum*) and *Brevibactrium* (*Brevibacterium halotolerans*) (Table 4). Currently known microorganisms capable of fermentating syngas into ethanol, butanol and other bioproducts are mesophilic,[17,62] which grow favourably at temperatures between 37 and 40°C. Mesophilic microorganisms that have been widely used in butanol synthesis from CO fermentation belong to the genera *Clostridium*, *Acetobacterium*, and *Butytibacterium*.[62, 63] None of the mesophiles isolated in the present study belong to these microbial groups, nor have been identified as carboxydotrophs in the past. Thus, these carboxydotrophic mesophiles may represent new microbial groups that can be used to explore butanol synthesis from CO.

The use of thermophiles in syngas fermentation is still in infant stage. Thermophiles grow favourably at temperature between 55°C and 80°C although some thermophilic microbes, however, can operate at higher temperature.[17] In the past few years, there has been an increase in the number of carboxydotrophic thermophilic bacteria being isolated and characterized. In 2008, four moderate carboxydotrophic thermophiles were isolated including *Methanothermobacter thermautotrophicus*, *Moorella thermoautotrophica*, *Thermoanaerobacter kivui*, and *Moorella perchloratireducens*.[18] Since 1991, when the first hydrogenogenic carboxydotrophic thermophilic bacterium was isolated from the marine hydrothermal vents of Kuril Islands, 15 species of such organisms have been isolated from hot environments relating to *Firmicutes*, *Dictyoglomi*, *Euryarchaeota*, and *Crenarchaeota*.[18] These thermophilic strains are predominantly carboxydotrophs that grow chemolithotrophically through the conversion of CO and $H_2O$ to $H_2$ and $CO_2$. Some of these strains can grow organotrophically or can reduce various electron acceptors using CO or $H_2$. However, none can produce ethanol or butanol from CO.[17,18,49] In the present study,

four thermophiles belonging to the genera *Bacillus* (T2-22, T3-14, and T7-10) and *Actinobacteria* (T1-16) isolated from composts grew on CO at rates higher than the known carboxydotroph *B. methylotrophicum.* These thermophilic isolates possess the enzymes important to CO metabolism (CODH) and butanol synthesis (BDH). This study demonstrated that if conditions are chosen properly, it is possible to isolate thermophiles that will grow well on CO and produce organic compounds of great interest (i.e. butanol).

The synthesis of butanol from CO begins with the anaerobic CO oxidation via CODH (Figure 2). The oxidation of CO by CODH yields $CO_2$, which is then reduced to formic acid and complexed with tetrahydrofolate (THF), a methylating molecule. Utilizing the enzyme FDH, formate can also be oxidized to produce $CO_2$ and provide a carbon source for the pathway. After the formic acid is complexed with THF, this carbon group is reduced several times to produce a methylated THF ready to transfer its methyl group to another molecule. The reducing power used to synthesize the methyl group comes from the initial oxidation of CO, the oxidation of diatomic hydrogen by hydrogenase, as well as the oxidation of formate by FDH. Acetyl-CoA synthase can then utilize the methyl group from the methylated THF, as well as another CO molecule and Coenzyme A to synthesize Acetyl-CoA. Acetyl-CoA becomes the first organic molecule in the pathway to be reduced. After a series of reactions involving the reduction of these organic intermediates, butyraldehyde can be reduced to butanol by the enzyme BDH. During these fermentative reactions, NADH, H ($^+$) is oxidized to NAD ($^+$), regenerating the electron acceptors necessary for the oxidation of carbon molecules earlier in the pathway.[17,65] All of the isolates used in this study possessed the four enzymes (CODH, FDH, hydrogenase and BDH) involved in the CO to butanol pathway. Key isolates that showed higher levels of enzyme CODH activities than *B. methylotrophicum* (a known carboxydotroph) were the thermophilic isolates T1-16 and T3-14, and the mesophilic isolates M3-16, M3-29 and M6-36. These isolates exhibited similar growth rates to the control (*B. methylotrophicum*) when grown on CO, except for the thermophiles which had lower growth and consequently slower doubling time than *B. methylotrophicum*. Thermophilic isolates were shown to express higher levels of CODH activity compared to mesophilic isolates which may help catalyse enzymatic activity. However, in this study, the mesophilic isolates seemed to express higher levels of CODH activity than the thermophilic isolates (Figure 3). Doubling times for the mesophilic isolates also were much greater when grown on CO (Table 3). The thermophilic isolates, on the other hand, expressed higher levels of BDH activities than the mesophilic isolates (Figure 6), suggesting that these thermophilic isolates may be the ideal microorganisms for syngas conversion to butanol. The key isolate that stood out in this study was T2-22. It exhibited BDH activity seven times greater than *B. methylotrophicum*. Such elevated levels of the final enzyme in the butanol pathway (BDH) may make this isolate an ideal organism for further biochemical analysis. Hydrogenase activities were evident in all isolates indicating their ability to metabolize $H_2$. Hydrogenase is very important in anaerobic metabolism. Various types of hydrogen-consuming anaerobes obtain energy from hydrogen to produce methane (methanogens) or acetate (acetogens) from carbon dioxide; sulphide from sulphate (sulphate reducers); ferrous from ferric form (iron-reducers) and nitrite from nitrate (nitrate-reducers).[66] In this study, the hydrogen gas was not used as an electron donor. When CO and $H_2$ are present in the culture medium, anaerobic caboxydotrophs preferentially use CO before consumption of $H_2$ started,[67] which is consistent with the results obtained by Kerby and Zeikus.[62] Carbon dioxide is thermodynamically more favourable electron donor for the reduction of $CO_2$ than $H_2$ because the $CO_2$/CO pair has lower standard redox potential than the $H^+/H_2$ pair in all organisms that contain CODH. The $H_2$ in the present study is oxidized by hydrogenases to $2H^+$ to provide the reducing equivalents for the reduction of $CO_2$ to formate ($HCO_2H$) and the energy (protons) necessary in the reductive steps to acetyl-CoA synthesis (Figure 2). For the synthesis of butyrate and butanol, anaerobic carboxydotrophs depend on acetyl-CoA.[17]

Solvent production by microorganisms, in particular, the production of butanol, holds great promise as a means for generating sustainable transportation fuel and commodity chemicals. This study demonstrated that anaerobic carboxydotrophs can be isolated from composts. The mesophilic and thermophilic carboxydotrophs isolated in this study represent new microbial groups that can be used to explore butanol synthesis from CO. In future work, we will focus on quantifying the fermentative butanol outputs of the isolates, as well as CO uptake to determine a ratio for CO input to biofuel output. We may also seek to sequence the entire genome of this isolate to better understand the regulation of enzymes pertaining to butanol synthesis. Bioreactors with enhanced efficiencies for this specific type of fermentation and tailored for the isolated anaerobic carboxydotrophic strains will also be developed.

**Acknowledgements**

We thank the University of Michigan Energy Institute for financial support. We also thank Kurt Cafini, Linda Grimm, Bob Quattro, Kathy Leach and Michael Skorupta of the Department of Natural Sciences for their assistance with configuring the gas system setup for the experiments.

**References**

[1] Himmel ME, Ding S-Y, Johnson DK, Adney WS, Nimlos MR, Brady JW, Foust TD. Biomass recalcitrance: engineering plants and enzymes for biofuels production. Science. 2007;315:804–807.

[2] Huber GW, Iborra S, Corma A. Synthesis of transportation fuels from biomass: chemistry, catalysts, and engineering. Chem Rev. 2006;106:4044–4098.

[3] Williamson C. The energy sector: a hidden Goliath. In: Walker JCF, editor. Primary wood processing. Netherlands: Springer; 2006. p. 535–556.
[4] Van Kasteren JMN. Co-gasification of wood and polyethylene with the aim of CO and $H_2$ production. J Mater Cycles Waste Manage. 2006;8:95–98.
[5] Demirbas A. Progress and recent trends in biofuels. Prog Energy Combust Sci. 2007;33:1–18.
[6] Young S, Do S, Smeenk J, Broer KM, Kisting CJ, Brown R, Heindel TJ, Bobik TA, DiSpirito AA. Growth of *Rhodospirillum rubrum* on synthesis gas: conversion of CO to $H_2$ and poly-$\beta$-hydroxyalkanoate. Biotechnol Bioengin. 2007;97:279–286.
[7] Kopke M, Mihalcea C, Bromley JC, Simpson SD. Fermentative production of ethanol from carbon monoxide. Curr Opin Biotechnol. 2011;22:320–325.
[8] Mohammadi M, Najafpour GD, Younesi H, Lahijani P, Uzir MH, Mohamed AR. Bioconversion of synthesis gas to second generation biofuels: a review. Renew Sust Energy Rev. 2011;15:4255–4273.
[9] Meyer O, Schlegel HG. Biology of aerobic carbon monoxide-oxidizing bacteria. Annu Rev Microbiol. 1983;37:277–310.
[10] Krueger B, Meyer O. Thermophilic bacilli growing with carbon monoxide. Arch Microbiol. 1984;139:402–408.
[11] Gadkari D, Schricker K, Acker G, Kroppensetdt RM, Meyer O. *Streptomyces thermoautotrophicus* sp. nov., a thermophilic CO- and H2-oxidizing obligate chemolithotroph. Appl Environ Microbiol. 1990;56:3727–3734.
[12] Park SW, Hwang EH, Park H, Kim JA, Heo J, Lee KH, Song T, Kim E, Ro YT, Kim SW, Kim YM. Growth of mycobacteria on carbon monoxide and methanol. J Bacteriol. 2003;185:142–147.
[13] Lorite MJ, Tachil J, Sanjuan J, Meyer O, Bedmar EJ. Carbon monoxide dehydrogenase activity in *Bradyrhizobium japonicum*. Appl Environ Microbiol. 2000;68:1871–1876.
[14] Dunfield KE, King GM. Molecular analysis of carbon monoxide-oxidizing bacteria associated with recent Hawaiian volcanic deposits. Appl Environ Microbiol. 2004;70:4242–4248.
[15] Tolli JD, Taylor CD. Biological CO oxidation in the Sargasso Sea and in Vineyard Sound, Massachusetts. Limnol Oceanogr. 2005;50:1205–1212.
[16] Sipma J, Henstra AM, Parshina SM, Lens PN, Lettinga G, Stams AJ. Microbial CO conversions with applications in synthesis gas purification and bio-desulfurization. Crit Rev Biotechnol. 2006;26:41–65.
[17] Henstra AM, Sipma J, Rinzema A, Stams AJM. Microbiology of synthesis gas fermentation for biofuel production. Curr Opin Biotechnol. 2007;18:200–206.
[18] Choi DW, DiSpirito A, Chipman D, Brown R. Hybrid processing. In: Brown RC, editor. Thermoprocessing of biomass: conversion into fuels, chemicals and power. New York: Wiley; 2011. p. 280–306.
[19] Lyons CM, Justin P, Colby J, Williams E. Isolation, characterization and autotrophic metabolism of a moderately thermophilic carboxydobacterium, *Pseudomonas thermocarboxydovorans* sp. nov. J Gen Microbiol. 1984;130: 1097–1105.
[20] Svetlitchnyi V, Sokolova TG, Gerdhardt M, Kostrikina NA, Zavarzin GA. Anaerobic extremely thermophilic bacteria in hydrotherms of Kuril Islands. Microb Ecol. 1991;21: 1–10.
[21] King G, Weber C. Distribution, diversity and ecology of aerobic CO-oxidizing bacteria. Nature Rev Microbiol. 2007;5:107–118.
[22] Lynd L, Zeikus JG. Metabolism of $H_2$-$CO_2$, methanol, and glucose by *Butyribacterium methylotrophicum*. J Bacteriol. 1983;153:1415–1423.
[23] Lynd L, Kerby R, Zeikus JG. Carbon monoxide metabolism of the methylotrophic acidogen *Butyribacterium methylotrophicum*. J Bacteriol. 1982;149:255–263.
[24] Grethlein AJ, Worden RM, Jain MK, Datta R. Evidence for production of n-butanol from carbon monoxide by *Butyribacterium methylotrophicum*. J Ferment Bioeng. 1991;72:58–60.
[25] Tanner RS, Miller LM, Yang D. *Clostridium ljungdahlii* sp. nov., an acetogenic species in clostridial ribosomal-RNA homology group-I. Int J Syst Bacteriol. 1993;43: 232–236.
[26] Abrini J, Naveau H, Nyns EJ. *Clostridium autoethanogenum*, sp. nov., an anaerobic bacterium that produces ethanol from carbon monoxide. Arch Microbiol. 1994;161:345–351.
[27] Shen GJ, Shieh JS, Grethlein AJ, Jain MK, Zeikus JG. Biochemical basis for carbon monoxide tolerance and butanol production by *Butyribacterium methylotrophicum*. Appl Microbiol Biotechnol. 1999;51:827–832.
[28] Duerre P. Fermentative production of butanol – the academic perspective. Curr Opin Biotechnol. 2011;22:331–336.
[29] Green EM. Fermentative production of butanol – the industrial perspective. Curr Opin Biotechnol. 2011;22:337–343.
[30] Qureshi N, Ezeji TC. Butanol 'a superior biofuel', production from agricultural residues (renewable biomass): recent progress in technology. Biofuels Bioprod Biorefining. 2008;2:319–330.
[31] Ramey DE. Butanol: the other alternative fuel. In: Eaglesham A, Hardy RWF, editors. Agricultural biofuels: technology, sustainability and profitability. Vol. NABC, Report 19; 2007. p. 264.
[32] Duerre P, Kuhn A, Gottwald M, Gottschalk G. Enzymatic investigations on butanol dehydrogenase and butyraldehyde dehydrogenase in extracts of *Clostridium acetobutylicum*. Appl Microbiol Biotechnol. 1987;26: 268–272.
[33] Lee SY, Park JH, Jang SH, Nielsen LK, Kim J, Jung KS. Fermentative butanol production by Clostridia. Biotechnol Bioeng. 2008;101:209–228.
[34] Tiquia SM. Evaluation of organic matter and nutrient composition of partially decomposed and composted spent pig-litter. Environ Technol. 2003;24:97–108.
[35] Krause MS, De Ceuster TJJ, Tiquia SM, Michel FC Jr, Madden LV, Hoitink HAJ. Isolation and characterization of Rhizobacteria from composts that suppress the severity of bacterial leaf spot of radish. Phytopathology. 2003;93: 1292–1300.
[36] Tiquia SM. Microbiological parameters as indicators of compost maturity. J Appl Microbiol. 2005;99:816–828.
[37] Tiquia SM. Microbial community dynamics in manure composts based on 16S and 18S rDNA T-RFLP profiles. Environ Technol. 2005;26:1104–1114.
[38] Tiquia SM, Ichida JM, Keener HM, Elwell D, Burt E Jr, Michel FC Jr. Bacterial community structure on feathers during composting as determined by terminal restriction fragment length polymorphism analysis of 16S rDNA genes. Appl Microbiol Biotechnol. 2005;67:412–419.
[39] Tiquia SM, Tam NFY. Co-composting of spent pig litter and sludge with forced-aeration. Bioresour Technol. 2000;72: 1–7.
[40] Tiquia SM, Tam NFY, Hodgkiss IJ. Microbial activities during composting of spent pig-manure sawdust litter at different moisture contents. Bioresour Technol. 1996;55: 201–206.

[41] Tiquia SM, Tam NFY, Hodgkiss IJ. Effects of bacterial inoculum and moisture adjustment on further composting of pig manure. Environ Pollut. 1997;96:161–171.
[42] Tiquia SM, Tam NFY. Composting of spent pig litter in turned and forced aerated piles. Environ Pollut. 1998;99:329–337.
[43] Tiquia SM, Richard TL, Honeyman MS. Effects of windrow turning and seasonal temperatures on composting of hog manure from hoop structures. Environ Technol. 2000;21:1037–1046.
[44] Tiquia SM, Wan JHC, Tam NFY. Microbial population dynamics and enzyme activities during composting. Compost Sci Util. 2002;10:150–161.
[45] Gerhardt P, Murray RGE, Costilow RN, Nester EW, Wood WA, Kreig NR, Phillips GB. Manual of methods for general bacteriology. Washington (DC): American Society for Microbiology; 1994.
[46] Rengpipat S, Lowe SE, Zeikus JG. Effect of extreme salt concentration on the physiology and biochemistry of *Halobacteriodes acetoethylicus*. J Bacteriol. 1988;170:3065–3071.
[47] Schauer NL, Ferry JG. Properties of formate dehydrogenase in *Methanobacterium formicicum*. J Bacteriol. 1982;1:1–7.
[48] Schauer LN, Ferry JG. Metabolism of formate in *Methanobacterium formicicum*. J Bacteriol. 1982;142:800–807.
[49] Svetlitchnyi V, Peschel C, Acker G, Meyer O. Two membrane-associated NiFeS-carbon monoxide dehydrogenases from the anaerobic carbon-monoxide-utilizing *Eubacterium Carboxydothermus hydrogenoformans*. J Bacteriol. 2001;183:5134–5144.
[50] Laffly E, Garzoni F, Fontecilla-Camps JC, Cavazza G. Maturation and processing of the recombinant [FeFe] hydrogenase from *Desulfovibrio vulgaris* Hildenborough (DvH) in *Escherichia coli*. Int J Hydrog Energy. 2010;35:10761–10769.
[51] Kuchenreuther J, Grady-Smith C, Bingham A, George S, Cramer S, Swartz J. High-yield expression of heterologous [FeFe] hydrogenases in *Escherichia coli*. PLoS One. 2010;5:1–7.
[52] Andersch W, Bahl H, Gottschalk G. Level of enzymes involved in acetate, butyrate, acetone and butanol formation by *Clostridium acetobutylicum*. Eur J Appl Microbiol Biotechnol. 1983;18:327–332.
[53] Cho K, Zholi A, Frabutt D, Flood M, Floyd D, Tiquia SM. Linking bacterial diversity and geochemistry of uranium-contaminated groundwater. Environ Technol. 2012;33:1629–1640.
[54] Tiquia SM, Schleibak M, Schlaff J, Floyd CJ, Benipal B, Zakhem E, Murray KS. Microbial community profiling and characterization of some heterotrophic bacterial isolates from river waters and shallow groundwater wells along the Rouge River, Southeast Michigan. Environ Technol. 2008;29:651–663.
[55] Tiquia SM, Davis D, Hadid H, Kasparian S, Ismail M, Murray KS. Halotolerant bacteria from river waters and shallow groundwater along the Rouge River of Southeastern Michigan. Environ Technol. 2007;28:297–307.
[56] Hugenholtz P, Huber T. Chimeric 16S rDNA sequences of diverse origin are accumulating in public databases. Int J Syst Evol Microbiol. 2003;53:289–293.
[57] Krieger PA. High purity solvent guide. MacGraw Park (IL): Burdick and Jackson Laboratories; 1984.
[58] Sommer L. Analytical absorption spectrophotometry in the visible and ultraviolet. Longson: Elsevier; 1989.
[59] Stackler L, Christensen EN. Quantitative determination of ethanol in wine by gas chromatography. Am J Enol Vitic. 1974;25:202–207.
[60] Wasinski FAH, Andersson JT. Qualitative analysis of phenols and alcohols in complex samples after derivitization to esters to ferrocene carboxylic acid by gas chromatography with mass spectrometric detection. J Chromatogr A. 2007;1157:376–385.
[61] Langer S, Ljungstrom E, Sehested J, Nielsen OJ. UV absorption spectra, kinetics and mechanism for alkyl and alkyl peroxy radicals originating from t-butyl alcohol. Chem Phys Lett. 1994;226:165–170.
[62] Kerby R, Zeikus JG. Catabolic enzymes of the acetogen *Butyribacterium methylotrophicum* grown on single-carbon substrates. J Bacteriol. 1987;169:5605–5609.
[63] Munasinghe PC, Khanal SK. Biomass-derived syngas fermentation into biofuels: opportunities and challenges. Bioresour Technol. 2011;101:5013–5022.
[64] Sokolova TG, Henstra A, Sipma J, Parshina SN, Stams AJM, Lebedinsky AV. Diversity and ecophysiological features of thermophilic carboxydotrophic anaerobes. FEMS Microbiol Ecol. 2009;68:131–141.
[65] Jones D, Woods D. Acetone-butanol fermentation revisited. Microbiol Rev. 1986;50:484–524.
[66] Adams MW, Stiefel EI. Biological hydrogen production: not so elementary. Science. 1998;282:1842–1843.
[67] Heiskanen H, Virkajarvi I, Viikari L. The effect of syngas composition on the growth and product formation of *Butyricum methylotrophicum*. Enzyme Microb Technol. 2007;41:362–367.