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Butanol tolerance of carboxydotrophic bacteria isolated from manure composts

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ABSTRACT

Carboxydotrophic bacteria (carboxydotrophs) have the ability to uptake carbon monoxide (CO) and synthesize butanol. The aims of this study were to determine the butanol tolerance and biological production of butanol carboxydotrophic strains. In this study, 11 carboxydotrophic strains were exposed to increasing *n*-butanol concentrations (1–3% vol/vol) to determine their effect on growth. Butanol production by the strains was quantified and the identity of the strains was elucidated using 16S rRNA sequencing. The carboxydotrophic strains possessed inherent tolerance to butanol and tolerated up to 3% *n*-butanol. Among the 11 strains, T1-16, M2-32 and M3-28 were the most tolerant to butanol. The 16S rRNA gene sequence of these strains was similar (99% nucleotide similarity) to the butanol-tolerant strains *Bacillus licheniformis* YP1A, *Pediococcus acidilacti* IMUA20068 and *Enterococcus faecium* IMAU60169, respectively. The carboxydotrophic strains screened in this study have two distinct features: (1) high tolerance to butanol and (2) natural production of low concentration of butanol from CO, which distinguish them from other screened butanol-tolerant strains. The butanol tolerance of these carboxydotrophic strains makes them ideal for genetic studies, particularly the molecular mechanisms that enable them to survive such hostile environmental conditions and the identification of genes that confer tolerance to butanol.

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1. Introduction

Butanol, like ethanol, can be produced from fermentable sugars, synthesis gas (a.k.a. syngas) and glycerol. Butanol has a number of notable qualities that make it a suitable alternative fuel: (1) its energy content is 30% more than ethanol;[1] (2) it can be mixed with gasoline in any proportion or be used as the sole fuel component (100% butanol) in unmodified car engines;[2] and (3) it carries less water and can therefore be transported through existing gasoline pipelines.[3] Even though the efficiency of butanol production from syngas is very low, syngas fermentation into butanol has several potential merits because the whole biomass, including lignin, can be utilized without the requirement of complex pretreatment and enzyme hydrolysis. Thus, it alleviates some of the problems associated with the utilization of lignocellulosic biomass. Hence, the development of syngas-based processes for butanol production can substantially improve the economics and viability of bio-based butanol production if the fermentation efficiency can be enhanced through metabolic engineering and process optimization.

Carboxydotrophs are micro-organisms that convert components of syngas (CO, CO_2, H_2) into liquid fermentation products, such as ethanol, butanol, butyric acid, and acetic acid.[4] These micro-organisms are able to (1) catalyze the oxidation of CO to CO_2; (2) use the electrons derived from this reaction for growth; (3) assimilate parts of the CO_2 ribulose biphosphate pathway; and (4) to withstand CO inhibition.[5] Carboxydotrophs are globally distributed and include pathogens, plant symbionts and biogeochemically important lineages in soils and the oceans.[4] Nguyen et al.[6] isolated 11 carboxydotrophic bacteria (7 mesophiles and 4 thermophiles). To assess the biochemical basis for their ability to produce butanol from CO, Nguyen et al.[6] assessed the CO dehydrogenase (CODH) and butanol dehydrogenase activities of each of the isolates. All 11 isolates showed evidence of CODH and BDH enzyme activities, with the majority exhibiting higher activities compared with the known carboxydotroph, *B. methylotrophicum* ATTC 33266. The level of activities for CODH and BDH ranged from 0.163 to 3.59 μmol min^{-1} and 0.19 to 2.2 μmol min^{-1}, respectively.[6]

One of the most critical problems in acetone–butanol–ethanol (ABE) fermentation is solvent toxicity. For instance, clostridial cellular metabolism ceases in the presence of 20 g L^{-1} or more solvents.[7] This limits the concentration of carbon substrate that can be used

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for fermentation resulting in low final solvent concentration and productivity. The lipophilic solvent butanol is more toxic than other solvents, as it disrupts the phospholipid components of the cell membrane causing an increase in membrane fluidity.[8] Moreira et al.[9] had attempted to elucidate the mechanism of butanol toxicity in *C. acetobutylicum* and they found that 0.1–0.15 M (about 0.74–1.1%) *n*-butanol caused 50% inhibition of both cell growth and sugar uptake rate by negatively affecting the ATPase activity. Increased membrane fluidity causes destabilization of the membrane and disruption of membrane-associated functions, such as various transport processes, glucose uptake and membrane-bound ATPase activity.[8] Also, *n*-butanol is the only solvent produced to the level that becomes toxic to the cells during the fermentation of clostridia.[10] It has been known that the addition of 7–13 g L^{-1} of butanol to culture medium results in a 50% inhibition of growth, while the addition of acetone and ethanol up to 40 g L^{-1} reduced growth by 50%.[10]

To date, very few butanol-tolerant bacterial species are capable of growing in greater than 2% (w/v) *n*-butanol.[11–14] Therefore, the need of strains capable of tolerating higher concentrations of butanol is economically desirable. Engineering butanol tolerance into known strains, such as *Clostridia* and *Escherichia coli*, requires well-developed genetic tools and sufficient information on tolerance mechanisms and pathways.[15–16] It is also time consuming to characterize the isolated mutants and elucidate the tolerance mechanism. Although a number of strains capable of tolerating up to 2.5–3% (w/v) *n*-butanol were screened previously,[11,13] anaerobic production of butanol with those isolates was not examined. Here we report the anaerobic production of butanol from CO by carboxydotrophic stains isolated from compost. Their ability to tolerate increasing concentrations was also examined. The carboxydotrophic strains screened in this study have two distinct features: (1) they have high tolerance to butanol and (2) they produce low concentration of butanol from CO. Three of the 11 carboxydotrophic strains grew best 3%t *n*-butanol, indicating their potential to be competitive for industrial use.

2. Materials and methods

2.1. Culture media and gases

For the cultures, a phosphate-buffered basal medium was adapted from Nguyen et al.[6] 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_6HCl (pyridoxine); 0.005 g L^{-1} B_1HCl (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 B12 (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_2 S $9H_2O$ 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.

2.2. Carboxydotrophic strains and culture conditions

The carboxydotrophic strain *Butyribacterium methylotrophicum* (ATTC 33266) was used as a reference organism in this study. The strain was obtained from American Type Culture Collection, Manassas, Virginia, USA. The 11 carboxydotrophic strains of which 7 mesophilic and 4 thermophilic, were isolated by Nguyen et al.[6] from various composts including dairy manure + straw + woodchips; dairy manure + compost; mature dairy manure; mature leaf compost and dairy manure + sawdust (Table 1).

Stock cultures were maintained in 152 ml serum bottles (Wheaton Scientific, Millville, NJ) sealed with butyl rubber (Bellco Biotechnology, Vineland, NJ) and aluminum 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 atm. When applicable,

Table 1. Carboxydotrophs were isolated from manure composts.

Isolate	Temperature class	Isolation source
T1-16	Thermophile	Dairy manure, straw, and woodchips
T2-22	Thermophile	Dairy manure, partially composted
T3-14	Thermophile	Mature dairy manure
T7-10	Thermophile	Dairy manure and sawdust
M3-28	Mesophile	Mature dairy manure
M3-29	Mesophile	Mature dairy manure
M6-36	Mesophile	Mature leaf compost
M3-16	Mesophile	Mature dairy manure
M2-32	Mesophile	Dairy manure, partially composted
M3-9	Mesophile	Mature dairy manure
M7-1	Mesophile	Dairy manure and sawdust

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 were frozen at −80°C for extended storage. Growth studies were initiated by inoculation with 0.5–2% (v/v) of actively growing *B. methylotrophicum* ATTC 33266 stock culture.

Prior to the butanol tolerance assay, the carboxydotrophic strains were characterized based on Gram reaction, O_2 requirements, motility and physiological attributes. Morphological characteristics were determined by standard methods.[17] 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 an acid, gas (CO_2), alcohol or a combination thereof on glucose, lactose and sucrose. The methyl-red (MR) 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.

2.3. Butanol tolerance testing

Butanol tolerance testing [12] of the carboxydotrophic strains was performed by inoculating an overnight cell culture into fresh CO–phosphate medium supplemented with various concentrations (1%, 2%, and 3% vol/vol) of *n*-butanol (Sigma-Aldrich, St. Louis, MO). The mesophilic carboxydotrophic strains (M2-32, M3-9, M3-28, M3-16, M3-29 and M6-36) were cultivated at 37°C, while the thermophilic carboxydotrophic strains (T1-16, T2-22, T3-14, and T7-10) at 55°C. Optical reading at 660 nm (OD_{660}) was used as a parameter for cell viability and *n*-butanol tolerance. The growth of the strains was compared with the growth of *B. methylotrophicum* ATTC 33266, which served as a positive control, at different time intervals. Growth of the strains amended with *n*-butanol was also compared to isolates without butanol amendment.

Specific growth rates were calculated from the linear range of exponential growth. This typically occurred at OD values between 0.03 and 0.50 but varied for different treatments. The relative growth rate was also determined from the specific growth rate of the strains in the presence of butanol relative to that without butanol.

Solvent tolerance test by rhodamine 6G accumulation cells of overnight cultures (thioglycollate broth without and with 3% *n*-butanol) of the carboxydotrophic strains were spotted on an alternate thioglycollate (AT) agar with or without rhodamine 6G (100 µg ml^{-1}) and incubated at 37°C (for mesophilic carboxydotrophs) or 55°C (for thermophilic carboxydotrophs) in an anaerobic chamber for 24 h. Accumulation of rhodamine 6G was monitored under UV light.[18]

2.4. Analysis of fermentation products

For this assay, carboxydotrophic strains were kept anaerobically in CO–phosphate medium for 92 h. The mesophilic strains were incubated at 37°C and the thermophilic strains at 55°C. After 92 hours the cells were centrifuged at 4000 × *g* for 10 minutes and the supernatants were collected. The absorbance spectra of the supernatants collected from each carboxydotrophic strains were recorded to detect the fermentation products (acetic acid, butyrate, ethanol and butanol) expected for the carboxydotrophic bacterium, *B. methylotrophicum*.[19] For the ultraviolet and visible regions, measurements were made using a UV/Vis spectrophotometer (Beckman Coulter DU700 Series, Fullerton, California). The absorption peaks observed were then compared against the detection wavelengths of acetate (200–235 nm), butyrate (200 nm),ethanol (205–240 nm) and butanol (215–245 nm).[20–24]

Fermentation products were analyzed by High performance liquid chromatography (HPLC) (Shimadzu, Japan) equipped with Aminex HPX-87H Ion Exclusion Column (Bio-rad Laboratories, California, USA) and refractive index detector. The column was eluted isocratically with 5 mM sulfuric acid at a flow rate of 0.6 ml min^{-1} and 42°C. Confirmation of butanol in the fermentation

medium (CO–phosphate medium) was further performed by Gas chromatography mass spectrometry (GC-MS) analysis (Agilent Technologies, California, USA) with HP-5MS (5%-Phenyl)-methylpolysiloxane column (Agilent Technologies, California, USA). Samples were separated by mixing a 4 ml of ethyl acetate with 20 ml of the spent CO-phosphate medium. After centrifugation, the extracted butanol in ethyl acetate phase was subjected to GC-MS analysis.

2.5. Phylogenetic analysis based on 16S rrna gene sequences

The 16S rRNA gene sequences of each strain were determined as previously described.[25–27] All sequences were aligned with Clustal W [28] and the resulting alignments were used to construct phylogenetic trees. The maximum likelihood based on the Jukes–Cantor model was used to generate tree topologies. The evolutionary history was inferred by using the maximum likelihood method based on the Jukes–Cantor model.[29] The bootstrap consensus tree inferred from 10,000 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (10,000 replicates) is shown next to the branches. Phylogenetic trees and evolutionary distance calculations were generated using the distance Jukes–Cantor model (MEGA version 6.0).[30]

2.6. Nucleotide sequence accession numbers

The Genbank/EMBL/DDBJ accession numbers of the 16S rRNA gene sequences of the carboxydotrophic strains are KR858546 (strain M3-16); KR858547 (strain M3-29); KR858548 (strain M6-36); KR858549 (strain T1-16); KR858550 (strain T3-14); KR858551 (strain T7-10); KT026060 (strain M2-32), KT026061 (strain M3-9); KT026062 (strain M3-28); KT026063 (strain M7-1) and KT036434 (strain T2-32).

2.7. Culture collection accession numbers

The carboxydotrophic strains described in this study were deposited in USDA-ARC Culture Collection with the accession numbers NRRL B-65319 (strain M3-16); NRRL B-65317 (strain M3-29); NRRL B-65318 (strain M6-36); NRRL B-65315 (strain T1-16); NRRL B-65322 (strain T3-14); NRRL B-65322 (strain T7-10); NRRL B-65320 (strain M2-32), NRRL B-65324 (strain M3-9); NRRL B-65323 (strain M3-28); NRRL B-65325 (strain M7-1) and NRRL B-65316 (strain T2-32).

3. Results

3.1. Morphological, physiological and biochemical tests

All carboxydotrophic strains are Gram-positive facultative anaerobes, majority of which (10 out of 11 isolates) are coccus-shaped bacteria (Table 2). All tested positive for catalase test. Of the 11 strains, 2 (M3-16 and M7-1)

Table 2. Morphological, physiological and biochemical characteristics of the carboxydotrophic isolates.

	Carboxydotrophic strains										
Test	T1-16	T2-22	T3-14	T7-10	M3-28	M3-29	M6-36	M3-16	M2-32	M3-9	M7-1
O_2 requirement[a]	FA	FA	FA	FA	FA	FA	FA	FA	FA	FA	FA
Gram reaction[b]	+	+	+	+	+	+	+	+	+	+	+
Cell morphology[c]	R	C	C	C	C	C	C	C	C	C	C
Catalase test[d]	+	+	+	+	+	+	+	+	+	+	+
Methyl-red test[d]	–	–	–	–	–	–	–	+	–	–	+
Voges Proskauer[d]	–	–	–	–	–	–	–	–	+	+	–
Motility[d]	–	+	+	+	–	–	–	+	+	+	+
Fermentation capabilities											
Lactose fermentation[d]											
Gas	–	–	–	–	–	–	–	–	–	–	–
Acid	–	–	–	–	–	–	–	+	–	+	+
Alcohol	–	–	–	–	–	–	–	–	–	–	–
Sucrose fermentation[d]											
Gas	–	–	–	–	+	+	+	+	+	+	+
Acid	+	+	–	+	+	+	+	+	+	+	+
Alcohol	–	–	–	–	–	–	–	–	–	–	–
Glucose fermentation[d]											
Gas	–	–	–	–	+	+	+	+	–	–	–
Acid	+	+	–	+	+	–	+	+	+	+	+
Alcohol	–	–	–	–	–	–	–	–	–	–	–

[a]FA, Facultative anaerobe.
[b]G^-, Gram-negative; G^+, Gram-positive.
[c]R, rod-shaped; C, coccus-shaped.
[d]'–', negative result; '+', positive result.

tested positive for methyl-red test indicating that these strains are capable of performing mixed acid fermentation. Two strains (M2-32 and M3-9) showed positive results for Voges Proskauer test, suggesting that these strains produce 2,3-butanediol as a fermentation product from glucose. All strains were capable of fermenting glucose and sucrose with the exception of T3-14, which showed negative results for glucose, sucrose and lactose fermentation. Surprisingly, the strains that fermented glucose, sucrose and lactose showed positive results for acids and/or gas, but negative for alcohol (Table 2). These results suggest that these strains either did not produce alcohol as fermentation product in glucose, sucrose or lactose, or did produce alcohol but at concentration below the detection limit of the biochemical assay used in this study.

3.2. Tolerance to butanol

The carboxydotrophic strains, including the known carboxydotroph, *B. methylotrophicum* ATTC 33266, grew in CO–phosphate medium without butanol (Figure 1(a)). The OD_{660} values increased from 0.03 to 0.50 by the end of the 92-hour incubation period, with a lag phase of 4 hours. CO–phosphate medium containing 1% (w/v) *n*-butanol showed no significant inhibition effect (Figure 1(b)) to carboxydotrophic strains. The overall trend of growth was similar to that of the untreated CO–phosphate medium (Figure 1(a)), although the butanol-treated strains had a longer lag phase (≥6 h) than the untreated strains. All strains were able to grow at 2% (w/v) *n*-butanol, although growths for M3-16, M3-29 and M7-1 were significantly lower ($P < .05$) than the rest of the strains (Figure 1(c)). At 3% (w/v) *n*-butanol, T1-16, T2-22, M6-36, M2-32 and *B. methylotrophicum* ATTC 33266 were able to sustain growth, while M3-16, M3-29 and M7-1 were significantly inhibited ($P < .05$) (Figure 1(d)).

Specific growth rates of carboxydotrophic strains in CO–phosphate medium without butanol ranged from 0.007 h^{-1} to 0.043 h^{-1} (Figure 2). Most of these strains showed higher specific growth rates than *B. methylotrophicum* ATTC 33266 (0.015 h^{-1}). The comparison of specific growth rates in 0% (no butanol)

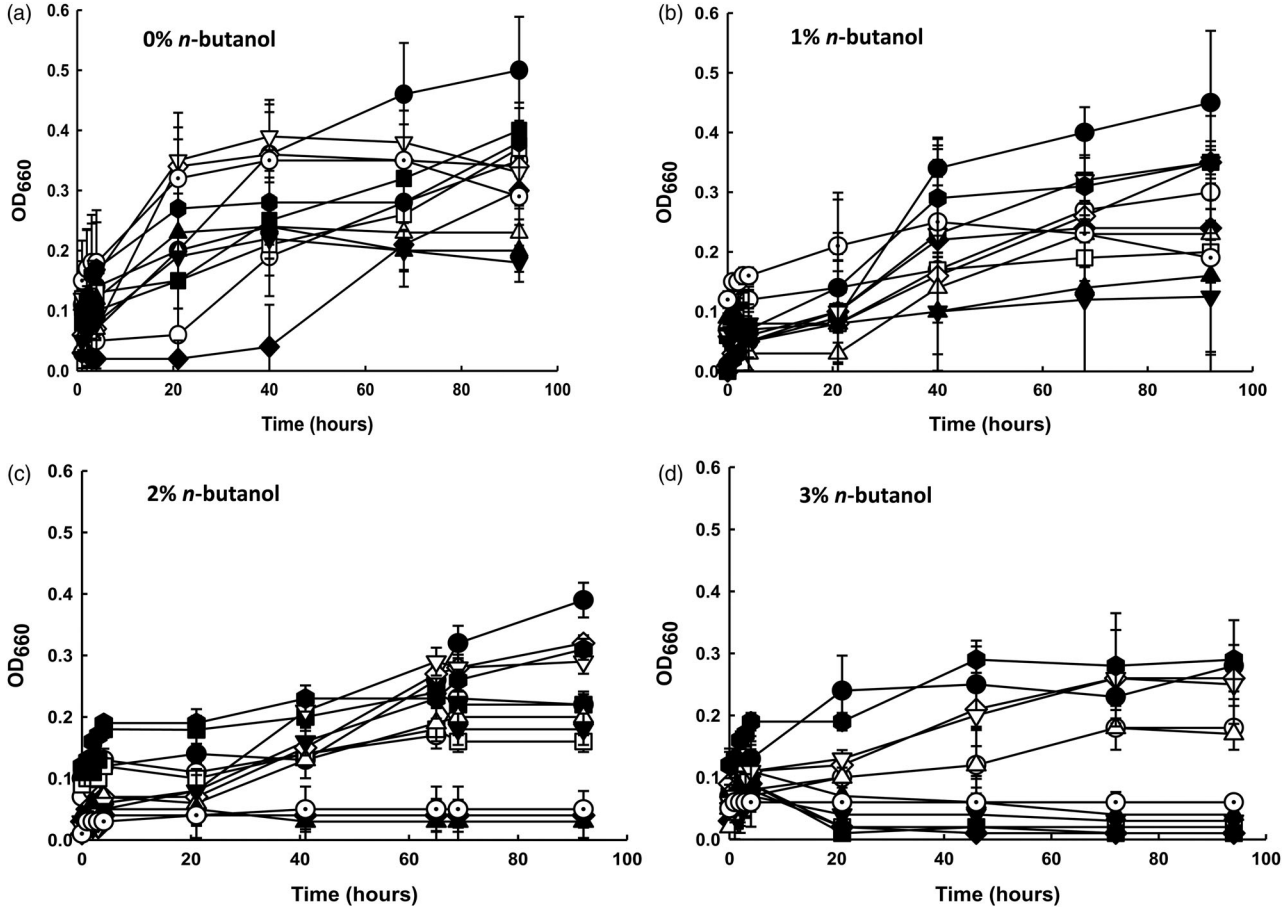

Figure 1. Growth curves of carboxydotrophic isolates at (a) 0%, (b) 1%, (c) 2% and (d) 3% butanol concentrations in the CO–phosphate medium. ○ (T-16); ● (T2-22); □ (T3-14); ■ (T7-10); ◊ (M3-28); ◆ (M3-29); △ (M6-36); ▲ (M3-9); ▽ (M2-32); ▼ (M3-9); ⊙ (M7-1); ⬢ (*B. methylotrophicum* ATTC 33266).

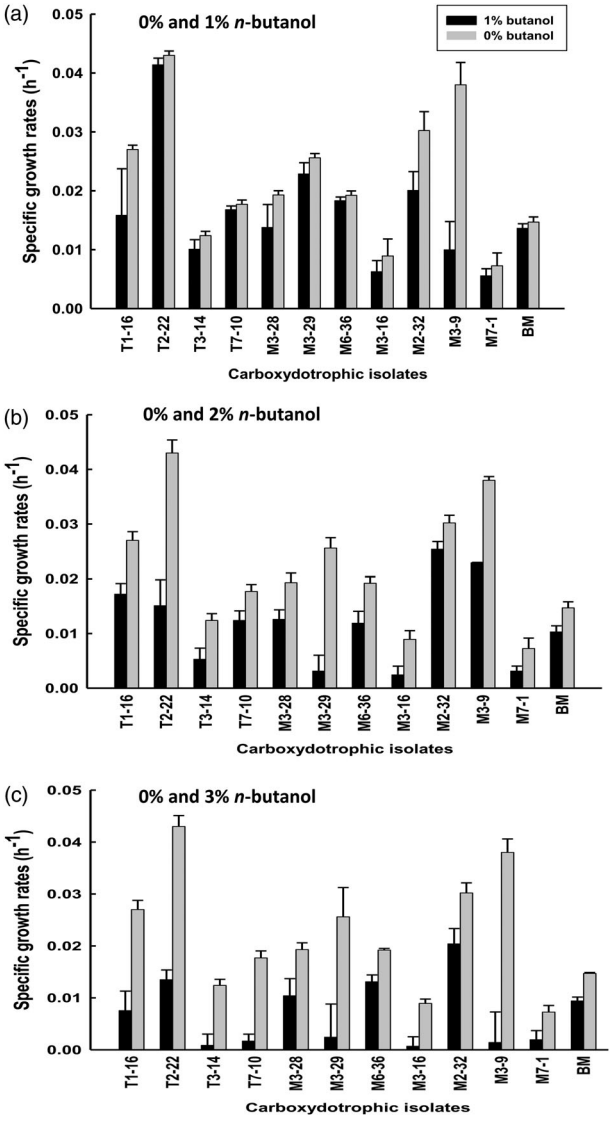

Figure 2. Specific growth rates between carboxydotrophic strains cultivated in the CO–phosphate medium without (0% *n*-butanol) and butanol: (a) 1% *n*-butanol; (b) 2% *n*-butanol and (c) 3% *n*-butanol.

and 1% *n*-butanol did not show a significant trend (Figure 2(a)). That is, addition of 1% *n*-butanol had little or no impact on specific growth rates except for M3-9 (Figure 2(a)). Specific growth rates decreased in 2% *n*-butanol for all strains (Figure 2(b)), with the mesophilic strains M3-32 and M3-9 displaying better growth (~0.02 h^{-1}) than the rest of the strains (<0.02 h^{-1}). The gap between the specific growth rates of strains in 0% (no butanol) and 3% *n*-butanol (Figure 2(c)) was more pronounced than 1% and 2% *n*-butanol (Figure 2(a) and 2(b)). Strong growth inhibition by 3% *n*-butanol was observed on most of the strains (T3-14, T7-10, M3-29, M3-16, M3-9 and M7-1), indicating that this concentration of butanol was toxic to these strains (Figure 2 (c)). These isolates showed little to no growth (Figure 1 (d)), indicating that they were not able to tolerate 3% *n*-butanol.

Relative growth rates were calculated for each strain in order to determine their growth in butanol relative to without butanol. At 1% and 2% *n*-butanol, strains reached up to an average of 96.2% and 73.3% of their relative growth rates, respectively (Figure 3). At 3% *n*-butanol, strains T3-14, T7-10, M3-29, M3-16, M3-9 and M7-1 showed no tolerance or growth. Only 5 (T-16, T2-22, M3-28, M3-36 and M2-32) out of the 11 strains grew in 3% n-butanol. Among these five strains, M3-28 (38.88%) and M2-32 (37.39%) showed the highest relative growth rates. These values were similar to the relative growth rate of *B. methylotrohicum* ATTC 33266 (43.99%) in 3% *n*-butanol (Figure 3).

3.3. Solvent tolerance test by rhodamine 6 G accumulation

A technique used to determine the role of multi-drug efflux pumps in conferring resistance to toxic compounds in bacteria is the rhodamine 6 G accumulation test.[18] Rhodamine 6 G is a P-glycoprotein substrate which mediates the energy-dependent efflux of toxic compounds from the bacterial cells.[31] Solvent-tolerant bacteria, which have the machinery to sustain the presence of toxic compounds, accumulate the dye leading to pink coloration of the colony. However, normal bacteria either get killed or expel the dye out when grown in the presence of rhodamine 6 G.[32] In the present study, the strains that tolerated 3% *n*-butanol (T-16, T2-22, M3-28, M3-36, M2-32 and *B. methylotrophicum* ATTC 33266 grew and accumulated increased amount of rhodamine 6 G, while the rest did not grow (T3-14, T7-10 and M3-16) or accumulated less amount of rhodamine 6 G (M3-29, M3-9 and M7-1).

3.4. Analysis of fermentation products

The detection wavelengths of the following fermentation products expected to be present in the reference micro-organism, *B. methylotrophicum*,[19,33] were: butanol (198 nm to 202 nm), butyric acid (200 nm), ethanol (196 nm), and acetic (222–224 nm), butyrate (222 nm), ethanol (205–240 nm) and butanol (215–245 nm). These absorption wavelengths were detected for *B. methylotrophicum* ATTC 33266 growing in the CO–phosphate medium after 92 h of incubation at 37°C (Table 3), implying that butanol, butyric acid, ethanol and acetic acid might be present in the cell supernatant. However, at 55°C, these absorption wavelengths were not detected for *B. methylotrophicum* ATTC 33266, suggesting that these fermentation products may not

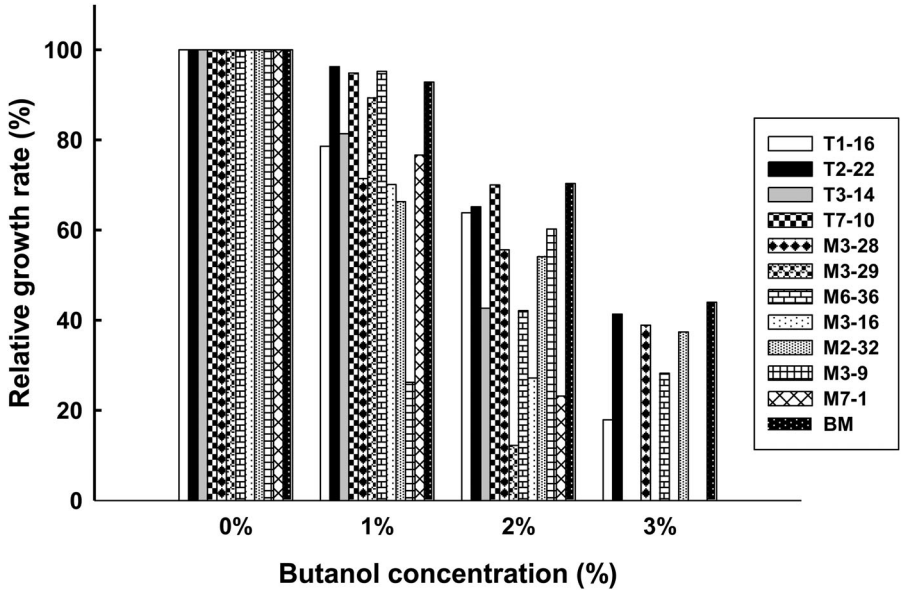

Figure 3. Relative growth rates of carboxydotrophic strains in the butanol-containing CO–phosphate medium relative to the medium without butanol.

be present in the cell supernatant and that fermentation is only possible at mesophilic temperatures (e.g. 37°C). Absorption wavelengths that correspond to ethanol and butanol were detected for all carboxydotrophic strains (Table 3). However, the true identity of these fermentation products should be confirmed by HPLC and GC-MS.

The fermentation products of representative strains that tolerated 3% *n*-butanol (*B. methylotrophicum* ATTC 33266, M2-32 and T1-16) were examined in the CO–phosphate medium (Figure 4). The representative strains produced increasing amounts of acetic acid (Figure 4(a)), ethanol (Figure 4(b)), butanol (Figure 4(c)) and butyric acid (Figure 4(d)). Butanol productivity is similar in all three strains (Figure 4(c)). Butanol reached up to about 6 g L^{-1} (for the mesophilic carboxydotrophic strain M2-32 and thermophilic strain T1-16) and 5.7 g L^{-1} (for *B. methylotrophicum* ATTC 33266) (Figure 4(c)). These butanol concentrations are lower than the tolerance concentrations (3% or 30 g L^{-1}) of these isolates to *n*-butanol (Figure 1). Butyric acid and two-phase fermentation were also observed. Butyric acid concentration peaked at 21 h to 6.0–8.0 g L^{-1} and then decreased dramatically to 1.8-2.6 g L^{-1} by day 40 (Figure 4(d)). The dramatic decrease in butyric acid concentration from 21 h to 40 h corresponded to rapid increase in butanol (Figure 4 (c) and 4(d)). The butanol concentration at this time period increased from 0.2–1.2 g L^{-1} to 3.4–4.0 g L^{-1} (Figure 4(c)).

Table 3. Presence or absence of the acetic acid, butanol, butrytic acid and ethanol in the cell supernatant.

Isolate	Acetic acid	Butanol	Butyric acid	Ethanol
Mesophiles				
M2-32	–	+	–	+
M3-9	–	+	+	+
M3-16	+	+	+	+
M3-28	–	+	+	+
M3-29	–	+	–	–
M6-36	–	+	–	+
M-7	–	+	–	+
B. methylotrophicum	+	+	+	+
Thermophiles				
T1-16	–	+	–	+
T2-22	+	+	+	+
T3-14	–	+	–	+
T7-10	+	+	+	+

3.5. Phylogenetic analysis of carboxydotrophic strains based on 16S rRNA gene sequences

The phylogenetic analysis based on 16S rRNA gene sequencing indicated that the carboxydotrophic isolates were affiliated with the genera *Bacillus*, *Enterococcus* and *Pediococcus* (Figure 5) within phylum *Firmicutes*. The nucleotide sequence of 16S rRNA of the carboxydotrophic strains were closely related to the 16S rRNA gene sequences of known butanol-tolerant bacteria. For instance, strain T1-16 demonstrated close relationship (level of 16S rRNA gene sequence similarity, 99%) with the butanol-tolerant strain *Bacillus lichineformis* YP1A. Strain M2-32 exhibited the closest affiliation (level of 16S rRNA gene sequence similarity, 99%) with the known butanol-tolerant strain *Pediococcus acidilacti* IMUA20068. Similarly, strains T2-32, T3-14, T7-10, M3-16, M3-29 and M6-36 were closely related to the 16S rRNA gene

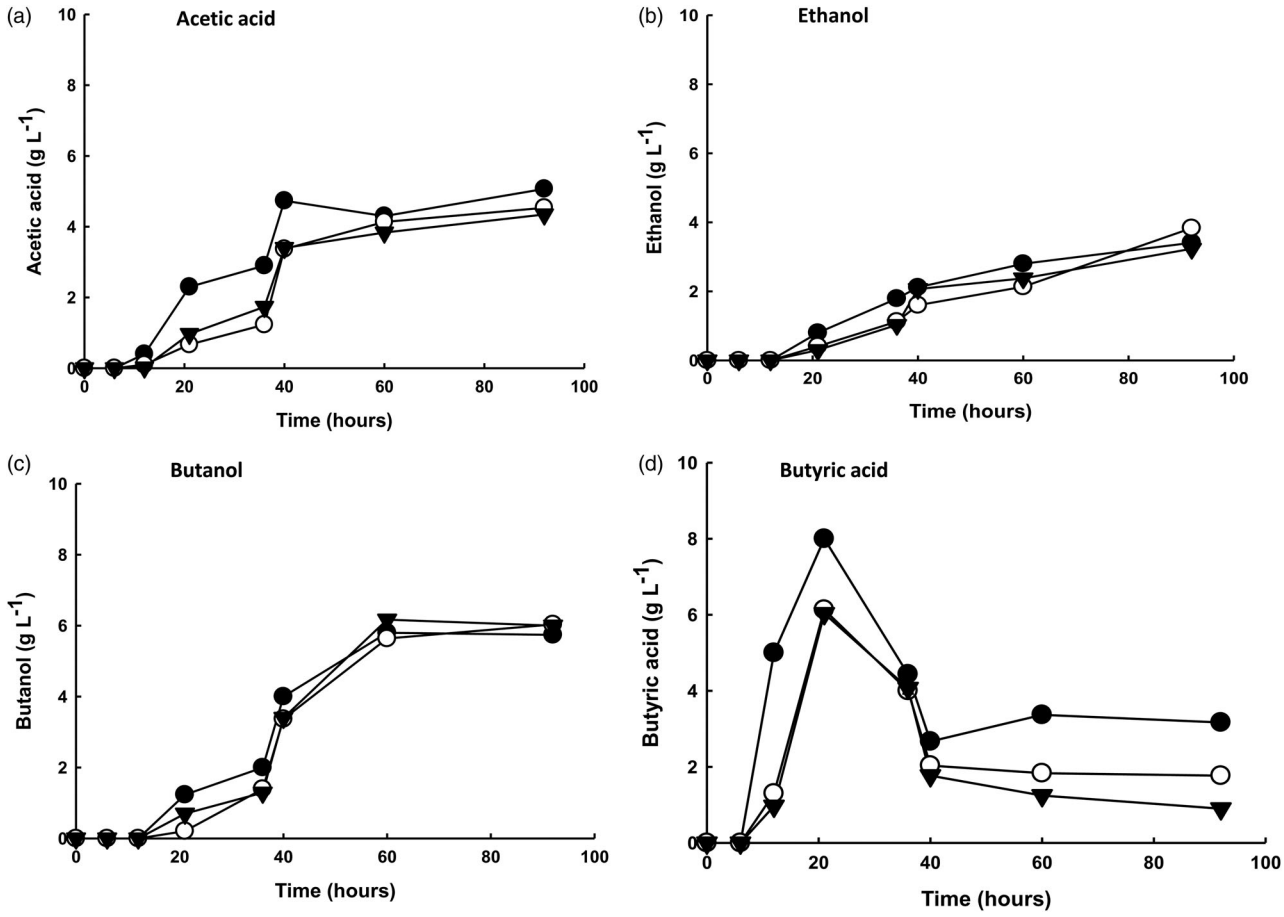

Figure 4. Fermentation profiles of carboxydotrophic strains. • (*B. butyribacterium*); ○ (M2-32) and ▼ (T1-16).

sequences of known butanol-tolerant strains *Enterococcus faecium* CM4A (98%); *Enterococcus faecium* IMAU60169 (96–99%), *Enterococcus casseliflavus* IMAU10148 (95–99%) *and Enterococcus italicus* IMAU50096 (98–99%) (Figure 5). The phylogenetic analysis revealed that the 16S rRNA gene sequence of the carboxydotrophic strains was distantly related to that of the known carboxydotrophic strain *B. methylotrophicum* ATTC 33266 (Figure 5). The carbxydotrophic strains exhibited less than 80% nucleotide identity to 16S rRNA gene sequences of *B. methylotrophicum* ATTC 33266.

4. Discussion

The need to improve the solvent resistance of solvent-producing strains is crucial for a sustainable butanol production. This problem can be overcome by either engineering known butanol-producing strains [15–16] or screening of new butanol-resistant bacteria.[11,14] Although there are research efforts on genetic engineering for the known butanol-producing strains, such as *Clostridia* and recombinant *E. coli*,[15,16,34] engineering of these strains for butanol-tolerant phenotype requires sufficient information on butanol tolerance mechanisms and pathways. Screening of butanol-tolerant strains on the other hand, is relatively simple and the isolated strains may have higher tolerance than existing known strains.

In this study, we adopted the screening strategy for isolation of new carboxydotrophic butanol-tolerant strains. Isolated from manure composts,[6] these strains convert components of synthesis gas (CO, CO_2, H_2) into liquid fermentation products, such as ethanol, butanol, butyric acid and acetic acid.[4,19,35]. The production of fuels and chemicals through syngas fermentation offers several advantages over metal catalytic conversion, which include higher specificity of the biocatalyst, lower energy costs and greater resistance to catalyst poisoning.[36–37] In the past two decades, new isolates and some known anaerobic micro-organisms were shown capable of growth with CO and H_2 as substrates, [4,19,35] however, none of these strains were screened for butanol toxicity.

The 11 carboxydotrophic strains examined in this study showed similar butanol tolerance at concentrations comparable with isolates reported by Kanno et al.[38] In their study, the isolates grew at maximum concentrations of 2–3.5% *n*-butanol. Most of our strains

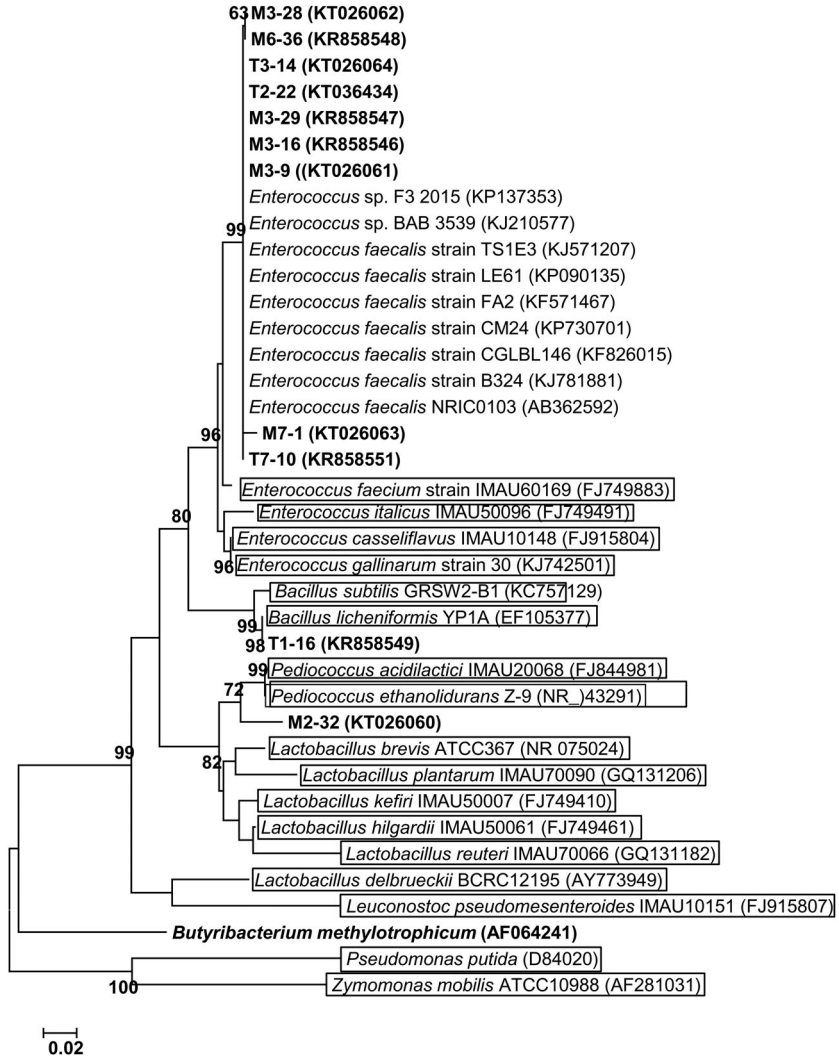

Figure 5. Maximum likelihood tree showing the relationship 16S rRNA gene sequences of carboxydotrophic strains (boldface type), their relatives and other butanol-tolerant bacteria (boxed clusters). The bootstrap values that were above 50% are shown at the nodes. Bar, 0.02 substitution per nucleotide position.

showed growth at a similar range (2–3% *n*-butanol), including the reference carboxydotrophic bacterium, *B. methylotrophicum* ATTC 33266 (Figure 3). Butanol toxicity was observed in our experiment, as growth was inhibited as the concentration of butanol in the CO-phosphate medium was increased. Significant reduction in growth was observed when the isolates were grown in the CO–phosphate-buffered medium containing >2% *n*-butanol. Butanol has been found to primarily accumulate in the cytoplasmic membrane, where it leads to disruption of the phospholipid bilayer. This phenomenon produces an increase in membrane fluidity, which results in the loss of intracellular molecules (including proteins, RNA and ATP), as well as an ability to maintain transmembrane ion gradients.[8]

The carboxydotrophic strains screened in this study have two distinct features: (1) high tolerance to butanol and (2) natural production of low concentration of butanol from CO, which distinguish them from other screened butanol-tolerant strains.[11,14] A sought threshold of tolerance in bacteria is 2% *n*-butanol.[38] Five of the isolates (T-16, T2-22, M3-28, M3-36 and M2-32) grew at that concentration up to 3% *n*-butanol (higher than the 2% threshold), indicating their inherent tolerance to butanol and their potential to be competitive for industrial use. Among these five strains, M3-28 (38.88%) and M2-32 (37.39%) showed the highest relative growth rates, which were similar to the relative growth rate of *B. methylotrophicum* ATTC 33266 (43.99%) in 3% *n*-butanol. Both strains exhibited superior tolerance, even though they were not grown in their

optimum medium. Besides high tolerance to butanol and butanol production from CO, the carboxydotrophic isolates assayed in this study are facultative anaerobes, which could help maximize the growth rate and butanol productivity at low dissolved oxygen concentration. Ting et al.[14] reported that fermentation at high cell density under the microaerophilic condition may potentially lead to higher butanol productivity.

Known butanol-tolerant micro-organisms were screened either from culture collections [39] or from environmental samples.[11] In the present study, the carboxydotrophic strains were screened from environmental samples (manure composts). The strains, which belong to the genera *Bacillus*, *Enterococcus* or *Pediococcus* (Table 1) were comparable to the butanol-tolerant strains from Li et al.[11] For instance, the 16S rRNA gene sequence of strain T1-16 and M2-32 was similar to the butanol-tolerant strains *Bacillus subtilis* GRSW2-B1 [12] and *Pediococcus acidilacti* IMUA20068, respectively. Similarly, strains T2-32, T3-14, T7-10, M3-16, M3, 29, M3-29 and M6-36 were closely related to the 16S rRNA gene sequences of known butanol-tolerant strains *Enterococcus faecium* IMAU60169 (96-99%), *Enterococcus casseliflavus* IMAU10148 (95-99%) and *Enterococcus italicus* IMAU50096 (98–99%).[11,14] Taken together, these results suggest that the species from the genera *Bacillus*, *Enterococcus* and *Pediococcus* inherently tolerate a higher concentration of butanol.

The carboxydotrophic strains in this study produced acetic acid, ethanol, butyrate and butanol under anaerobic conditions, which implied a major fermentation pathway similar to syngas fermenter, *B. methylotrophicum*. [40–41] Based on those products, we proposed the following pathway for the carboxydotrophic strains (Figure 6). The oxidation of CO with H_2O to CO_2 and $2H^+$ provides reducing equivalents for the reduction of CO_2 to formate (HCOOH), of methylene-tetrahydrofolate (CH-THF) to methenyl-tetrahydrofolate (CH_2-THF), of CH_2-THF to methyl-tetrahydrofolate (CH_3-THF), and of CO_2 to CO (Figure 6(a)). The electron flow from reduced ferredoxin to NAD is coupled by this oxidoreductase and the ferredoxin-NAD reductase is not inhibited by high NADH levels (Figure 6(a)). Consequently, the carboxydotrophic strains can tolerate high CO levels and forms butyrate from CO (Figure 6(b)) because CO oxidation reduces ferredoxin, which is then oxidized to form NADH by the ferredoxin-NAD oxidoreductase. The NADH can then be used to reduce acetyl-CoA to butyrate (Figure 6(b)). It is likely

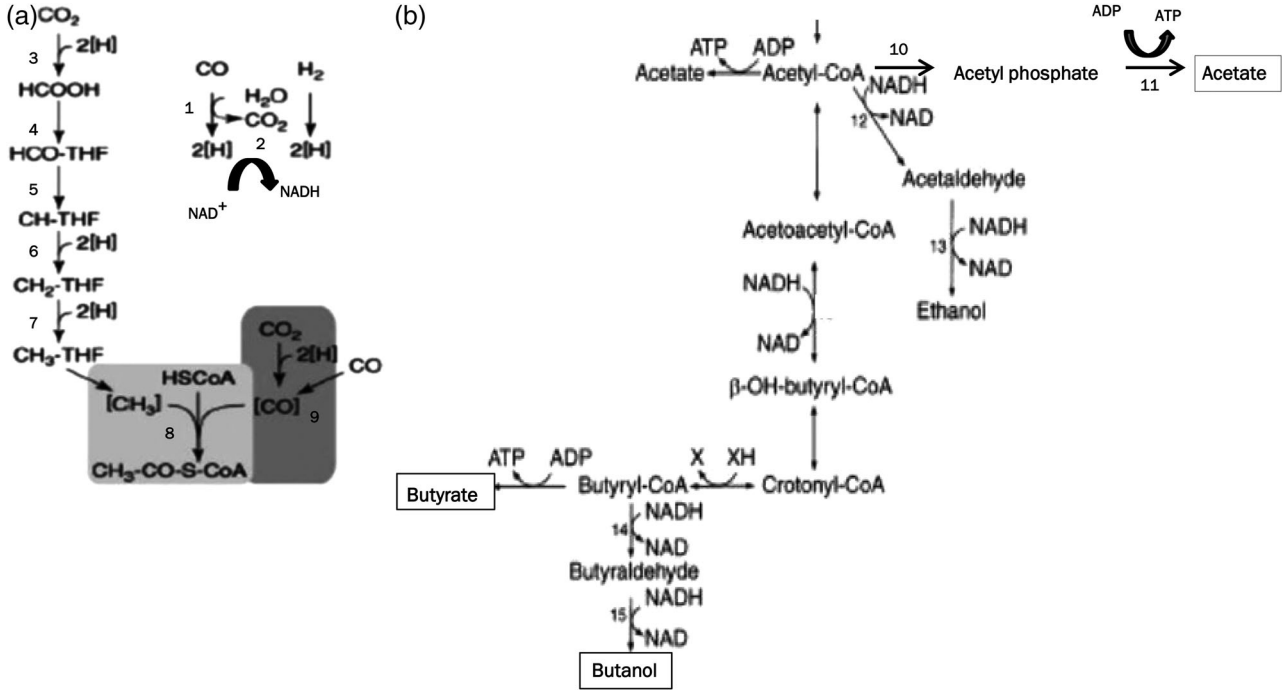

Figure 6. Proposed butanol fermentation pathways for the carboxydotrophic strains. (a) Synthesis of acetyl-CoA from CO. (b) Production of butanol from acetyl-CoA. Boxed are fermentation products. The numbers refer to the following enzymes: (1) hydrogenase; (2) ferredoxin-NAD reductase; (3) CO dehydrogenase; (4) formate reductase; (5) methylene-tetrafolate reductase; (6) methynyl-tetrahydrofolate reductase; (7) methyl-tetrahydrofolate reductase; (8) acetyl-CoA synthase; (9) CO dehydrogenase; (10) phosphotransacetylase, (11) acetate kinase; (12) acetaldehyde dehydrogenase; (13) alcohol dehydrogenase; (14) butyraldehyde dehydrogenase; (15) butanol dehydrogenase.

that the acetyl-CoA pathway is restricted to anaerobes. In the non-cyclic pathway coenzyme A (CoA), a carbonyl and a methyl group are joined by an acetyl-CoA synthase/carbon monoxide dehydrogenase.[42–43] The methyl group is obtained by the reduction of CO_2 in several successive steps with formyl, methenyl, methylene and methyl intermediates bound to a pterin cofactor. The CO_2 is first reduced to formate which is then activated at the expense of ATP to form a formyl bound to the pterin tetrahydrofolate complex (ACS/CODH) to form acetyl-CoA (Figure 6(a)). The formation of acetyl-CoA from H_2/CO_2 has a negative energy balance. Acetate is formed from acetyl-CoA to recover metabolic energy that is invested earlier in the acetyl-CoA pathway. Further reduction of acetate yields ethanol. The production of butyrate or butanol proceeds via acetoacetyl-CoA that is formed from two acetyl-CoA molecules (Figure 6(b)). Energy conservation in carboxydotrophic strains is dependent on the acetyl-CoA pathway (Figure 6(b)). In other carboxydotrophs such as carboxydotrophic hydrogenogens,[19] energy is conserved through the formation of H_2. In these micro-organisms CO is oxidized by a monofunctional CO dehydrogenase. Electrons released by the oxidation are transferred to an energy-converting hydrogenase (ECH) that reduces protons to molecular hydrogen.[19] In addition, ECH couples the formation of H_2 to the membrane translocation of protons or sodium ions, generating a chemiosmotic ion gradient that can drive ATP synthesis through an ATP-synthase.[44–45] Thus, energy conservation in carboxydotrophic hydrogenogens is independent of the acetyl-CoA pathway. However, it is expected that most thermophilic carboxydotrophic hydrogenogens contain the acetyl-CoA pathway for carbon fixation, while the currently known mesophilic carboxydotrophic hydrogenogen strains employ a different route.[19]

The biochemical basis for butanol production by carboxydotrophic strains in this study is associated with both the regulation of the ferredoxin ± NAD reductase activity and the presence of NAD-linked butyraldehyde dehydrogenase and butanol dehydrogenase activities. The alcohol production was regulated by activity and the presence of NAD-linked butyraldehyde dehydrogenase and butanol dehydrogenase activities of NAD-linked acetaldehyde dehydrogenase and ethanol dehydrogenase. These activities complete the pathway for solvent production from CO. In the present study, the carboxydotrophic strains tolerated *n*-butanol concentrations of 3% (30 g L^{-1}). The butanol production (Figure 4(c)) of the carboxydotrophic strains in this study was lower (5.7–6.0 g L^{-1}) than their tolerance concentration to *n*-butanol (30 g L^{-1}).

Tolerance mechanisms to butanol include adaptations to the cell-wall composition and the activity of stress response proteins, such as the synthesis of solvent efflux pumps.[14,46] In the present study, the existence of solvent efflux pumps in carboxydotrophic strains was confirmed by rhodamine 6G accumulations (100 µg ml^{-1}) in the cells. Accumulation of rhodamine 6G in the cells was observed by fluorescence of rhodamine 6G under UV light. According to the literature,[18] rhodamine 6G is P-glycoprotein substrates, which mediate energy-dependent efflux of certain toxic compounds, such as butanol, from bacterial cells. The rhodamine G assays showed significant difference between cells incubated without butanol (control) and those in the presence of 3% *n*-butanol. Strains that tolerated 3% *n*-butanol (strains T1-16, T2-22, M3-28, M3-36, M2-32 and *B. methylotrophicum* ATTC 33266) grew and accumulated increased amount of rhodamine 6G, while the rest did not grow (strains T3-14, T7-10 and M3-16) or accumulated less amount of rhodamine 6G (strains M3-29, M3-9 and M7-1) compared to the control.

For application, the thermophilic isolates are most useful in syngas fermentation as less cooling of the syngas is required before it is introduced in the bioreactor. Additionally, higher temperatures can lead to higher conversion rates and benefit separation of the product by distillation. However, higher temperatures may have a negative impact on the solubility of CO and H_2 [19] and butanol tolerance.[11,39] In the present study, butanol tolerance of two (strains T1-16 and T22) of the four thermophilic strains (Table 1) was not affected by high temperature (55°C). These strains tolerated butanol concentrations of ≥2.5% *n*-butanol.

Although butanol is toxic to bacteria because they accumulate in and disrupt cell membranes, more and more bacterial strains have been obtained that can adapt to and survive higher butanol concentration. The butanol tolerance of the carboxydotrophic strains assayed in this study makes them ideal for genetic studies, particularly the molecular mechanisms enabling them to survive such hostile environmental conditions, which will provide new insights into the general stress response of bacteria. Future studies will focus on identifying the genes that confer tolerance to butanol and enhancing their function – either by selection, mutagenesis or over-expression.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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References

[1] 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.
[2] Ramey DE. Butanol: The other alternative fuel. In: Eaglesham A, Hardy RWF, editor. Agricultural biofuels: technology, sustainability and profitability. NABC Report; New York: National Agricultural Biotechnology Council Ithaca. 19; 2007. p. 264.
[3] Dűrre P. Biobutanol: an attractive biofuel. Biotechnol J. 2007;2:1525–1534.
[4] Tiquia-Arashiro SM. Thermophilic carboxydotrophs and their biotechnological applications. Springerbriefs in microbiology: extremophilic microorganisms. New York: Springer International; 2014a. p. 131.
[5] Meyer O, Schlegel HG. Biology of aerobic carbon monoxide-oxidizing bacteria. Annu Rev Microbiol. 1983;37:277–310.
[6] Nguyen, S, Ala, F, Cardwell, C, et al. Isolation and screening of carboxydotrophs isolated from composts and their potential for butanol synthesis. Environ Technol. 2013; 34:1995–2007. doi:10.1080/09593330.2013.795987.
[7] Woods DR. The genetic engineering of microbial solvent production. Trends Biotechnol. 1995;13:259–264.
[8] Bowles LK, Ellefson WL. Effect of butanol on *Clostridium acetobutylicum*. Appl Environ Microbiol. 1985;50:1165–1170.
[9] Moreira, AR, Ulmer, DC, Linden, JC. Butanol toxicity in the butylic fermentation. Biotechnol Bioeng Symp. 1981; 11:567–579.
[10] Jones DT, Woods DR. Acetone-butanol fermentation revisited. Microbiolog Rev. 1986;50:484–524.
[11] Li, J, Zhao, JB, Zhao, M, Yang, YL, Jiang, WH, Yang, S. Screening and characterization of butanol-tolerant microorganisms. Lett Appl Microbiol. 2010;50:373–379. doi:10.1111/j.1472-765X.2010.02808.x.
[12] Kataoka N, Kato J, Rachadech W, Vangnai A. Development of butanol-tolerant *Bacillus subtillis* strain GRSW2-B1 as a potential bioproduction host. AMB Exp. 2011;1:1–10.
[13] Ruhl J, Schmid A, Blank LM. Selected *Pseudomonas putida* strains can grow in the presence of high butanol concentrations. Appl Environ Microbiol. 2009;75:4653–4656.
[14] Ting CNW, Wu J, Takahashi K, Endo A, Zhao H. Screened butanol-tolerant *Enterococcus faecium* capable of butanol production. Appl Biochem Biotechol. 2012;168:1672–1680.
[15] Atsumi S, Wu TY, Eckl EM, Hawkins SD, Buelter T, Liao JC. Engineering the isobutanol biosynthetic pathway in *Escherichia coli* by comparison of three aldehyde reductase/alcohol dehydrogenase genes. Appl Microbiol Biotechnol. 2010;85:651–657.
[16] Rutherford BJ, Robert H, Dahl RH, et al. Functional genomic study of exogenous *n*-butanol stress in *Escherichia coli*. Appl Environ Microbiol. 2010;76:1935–1945.
[17] Gerhardt P, Murray RGE, Costilow RN, et al. (1994) Manual of methods for general bacteriology. Washington DC: American Society for Microbiology; 1994. p. 524.
[18] Nashino K, Yamaguchi A. Role of histone-like protein H-NS in multidrug resistance of *Escherichia coli*. J Bacteriol. 2004;186:1223–1429.
[19] Henstra AM, Sipma J, Rinzema A, Stams AJM. Microbiology of synthesis gas fermentation for biofuel production. Curr Opin Biotechnol. 2007;18:200–206.
[20] Krieger PA. High purity solvent guide. MacGraw Park, IL: Burdick and Jackson Laboratories; 1984. p. 153.
[21] Sommer L. Analytical absorption spectrophotometry in the visible and ultraviolet. London: Elsevier;1989. p. 313.
[22] Stackler, L, Christensen, EN. Quantitative determination of ethanol in wine by gas chromatography. Am J Enol Vitic. 1974; 25:202–207.
[23] 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.
[24] 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.
[25] Tiquia, SM, Davis, D, Hadid, H, et al. Halophilic and halotolerant bacteria from river waters and shallow groundwater along the Rouge River of Southeastern Michigan. Environ Technol. 2007; 28:297–307. doi:10.1080/09593332808618789.
[26] Tiquia, SM, Schleibak, M, Schlaff, J, Floyd, C, Hadid, H, 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.
[27] Tiquia, SM. Salt-adapted bacteria isolated from the Rouge River and potential for degradation of contaminants and biotechnological applications. Environ Technol. 2010; 31:967–978. doi:10.1080/09593331003706226.
[28] Thompson, JP, Wedin, DG, Gibson, TJ. ClustalW: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acid Res. 1994; 22:4673–4680. doi:10.1093/nar/22.22.4673.
[29] Jukes TH, Cantor CR. Evolution of protein molecules. In: Munro HN, editor. Mammalian protein metabolism. New York, NY: Academic Press; 1969. p. 21–132.
[30] Tamura K, Stecher G, Peterson D, Filipski A, and Kumar S. MEGA6: Molecular evolutionary genetics analysis Version 6.0. Mol Biol Evol. 2013;30:2725–2729.
[31] Baer SH, Blaschek HP, Smith TL. Effect of butanol challenge and temperature on lipid composition and membrane fluidity of butanol-tolerant *Clostrodium acetobutylicum*. Appl Environ Microbiol. 1987;53:2854–2861.
[32] Lăzăroaie, MM. Mechanisms involved in organic solvent resistance in gram-negative bacteria. World Acad Sci Eng Technol. 2009; 30:643–653.
[33] Grethlein A, Worden M, Jain M, Datta R. Evidence for production of n-butanol from carbon monoxide by *Butyribacterium methylotrophicum*. J Ferment Bioeng. 1991;72:58–60.
[34] Janssen H, Grimmler G, Ehrenreich A, Bahl H, Fischer R. A transcriptional study of acidogenesis in *Clostridium acetobutylicum* in a chemostat culture. J Biotechnol. 2012;161:354–365.

[35] Tiquia-Arashiro SM. Biotechnological applications of thermophilic carboxydotrophs. In: Thermophilic carboxydotrophs and their applications in biotechnology. New York: Springer International; 2014b. p. 29–101.
[36] Bredwell MD, Srivastava P, Worden RM. Reactor design issues for synthesis-gas fermentations. Biotechnol Prog. 1999;15:834–844.
[37] Klasson KT, Ackerson CMD, Clausen EC, Gaddy JL. Biological conversion of synthesis gas into fuels. Int J Hydrogen Energy. 1992;17:281–288.
[38] Kanno, M, Katayama, T, Tamaki, H, et al. Isolation of butanol- and isobutanol-tolerant bacteria and physiological characterization of their butanol tolerance. Appl Environ Microbiol. 2013; 79:6998–7005. doi:10.1128/AEM.02900-13.
[39] Knoshaug, EP1; Zhang, M. Butanol tolerance in a selection of microorganisms. Appl Biochem Biotechnol. 2009; 153:13–20. doi:10.1007/s12010-008-8460-4.
[40] Tiquia-Arashiro SM. Microbial CO metabolism. In: Thermophilic carboxydotrophs and their applications in biotechnology. New York: Springer International; 2014c. p. 5–9.
[41] Tiquia-Arashiro SM. CO-oxidizing microorganisms. In: Thermophilic carboxydotrophs and their applications in biotechnology. New York: Springer International; 2014d. p. 11–28.
[42] Lindahl PA. The Ni-containing carbon monoxide dehydrogenase family: light at the end of the tunnel? Biochemistry. 2002;41:2097–2105.
[43] Ragsdale, SW. Life with carbon monoxide. Crit Rev Biochem Mol Biol. 2004; 39:165–195. doi:10.1080/10409230490496577.
[44] Hedderich R. Energy-converting [NiFe] hydrogenases from archaea and extremophiles: ancestors of complex I. J Bioenerg Biomembr. 2004;36:65–75.
[45] Singer SW, Hirst MB, Ludden PW. CO-dependent H_2 evolution by *Rhodospirillum rubrum*: role of CODH:CooF complex. Biochim Biophys Acta – Bioenergy 2006;1757:1582–1591.
[46] Sardessai, YN, Bhosle, S. Organic solvent-tolerant bacteria in mangrove ecosystems. Curr Sci. 2002; 82:622–623.