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Genomic and metabolic insights into solvent production by *Thermoanaerobacterium thermosaccharolyticum* GSU5

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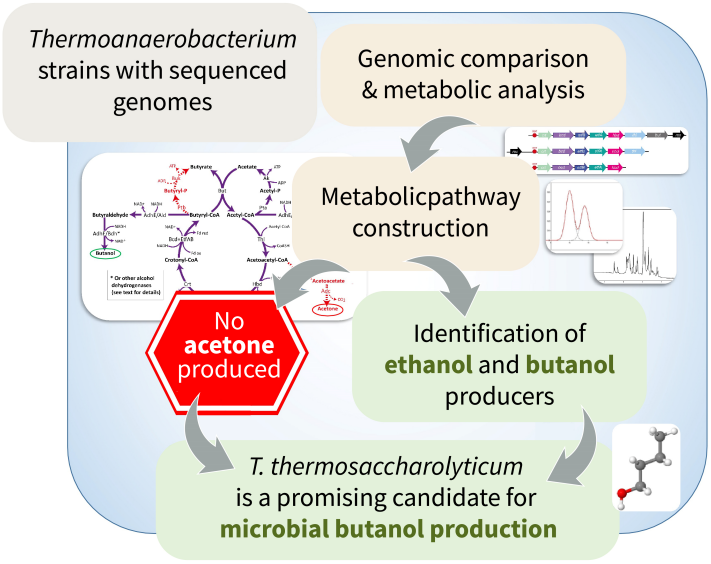
HIGHLIGHTS

- Comparative genome analysis of *Thermoanaerobacterium thermosaccharolyticum* GSU5 was performed.
- Ethanol and butanol production from different carbon sources without acetone was achieved.
- Solventogenic and acidogenic pathways in *Thermoanaerobacterium* were elucidated.

GRAPHICAL ABSTRACT



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ABSTRACT

Thermoanaerobacterium thermosaccharolyticum GSU5 was isolated from animal dung collected in a pasture plain in Buenos Aires, Argentina. This thermophilic and anaerobic microorganism was able to produce butanol and ethanol, but not acetone, using sugars such as xylose, arabinose, glucose, galactose, fructose, sucrose, and cellobiose. Key metabolic enzymes leading to solvent production were identified in its genome. A detailed analysis of the solvent and organic acid biosynthetic pathway genes of sequenced strains revealed new insights into the unique metabolic features of this species. Genes required for the synthesis of acetone are absent in the genomes of all sequenced *Thermoanaerobacterium*, suggesting that it is a general trait of the genus. Strains able to produce butanol synthesize butyrate through the one step pathway catalyzed by the butyryl-CoA:acetate-CoA transferase (But). The large range of fermentable substrates and the ability to produce both ethanol and butanol without acetone makes this species an interesting candidate for second generation biofuel production.

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1. Introduction

Climate change is a threat to various forms of life on the planet Earth. This phenomenon is partially due to the preferential use of fossil fuels to sustain human activities such as heating, agriculture, or transportation. The use of biofuels is one of the most promising solutions, as it would help mitigate the effects of climate change by avoiding the use of non-renewable resources as sources of energy such as petroleum or natural gas (Hill et al., 2006).

Among the most common biofuels are alcohols that can be obtained from microbial fermentation using different kinds of carbon sources. Ethanol is the best known and most studied alcohol that can be obtained by microbial fermentation using yeast or bacteria. While butanol is a less common but it is an increasingly attractive biofuel due to several characteristics, i.e., producing more energy if properly harnessed, having lower vapor pressure, and being less hygroscopic than ethanol (Dürre, 2007).

The production of both ethanol and butanol along with acetone, also known as acetone–butanol–ethanol (ABE) fermentation process, has been extensively studied in several *Clostridium* species, such as *C. acetobutylicum* (Jones and Woods, 1986). Although most butanol producers are strictly anaerobic, the halophile *Nesterenkonia* sp. strain F has also been reported to produce ABE from glucose under aerobic conditions (Amiri et al., 2016).

Many different processes have been developed to obtain alcohols from sugars or starch, but the use of these substrates to produce biofuels would trigger a competition over arable land and irrigation water; inputs upon which food security depends. To avoid this problem, biofuels should be obtained from non-food substrates, such as lignocellulosic biomass.

Several different approaches have been employed to exploit this abundant substrate, most of which start with the hydrolysis of the biomass to obtain sugars that can be subsequently fermented into the desired compounds. This hydrolysis step is normally accomplished through energy intensive processes, involving high temperatures and aggressive chemical conditions.

Consolidated bioprocessing is an alternative that integrates enzyme production, saccharification and fermentation (Yamada et al., 2013) and can be achieved under mild conditions. Consolidated bioprocesses that use anaerobic thermophilic organisms capable of degrading lignocellulosic biomass are expected to more effectively meet the sustainability standards, as they would enable production of biofuels from renewable resources by means of low energy demanding procedures (Lynd et al., 2002).

The use of thermophilic cellulolytic bacteria, such as *C. thermocellum*, has been extensively studied for the production of ethanol from cellulose (Lamed and Zeikus, 1980; Tian et al., 2016). However, most of these organisms are unable to produce butanol, and/or to ferment pentoses derived from hemicellulose degradation, thereby limiting the efficient use of lignocellulosic biomass (Demain et al., 2005).

In recent years, several species of the thermophilic genus *Thermoanaerobacterium* have received increased attention due to their capability to use different biomass substrates to produce solvents. The genus *Thermoanaerobacterium* comprises 8 validly described species: *T. aciditolerans*, *T. aotearoense*, *T. saccharolyticum*, *T. thermosaccharolyticum*, *T. thermostercoris* (formerly *T. thermostercus*), *T. thermosulfurigenes*, *T. xylanolyticum*, and *T. butyriciformans* (Onyenwoke and Wiegel, 2015). *T. saccharolyticum* and *T. thermostercoris* can produce ethanol (Shaw et al., 2008; Romano et al., 2010) while several *T. thermosaccharolyticum* isolates have been reported to produce hydrogen (Cao et al., 2009) and/or butanol (Li et al., 2018) from different biomass sources.

These results point out the potential of these microorganisms for the synthesis of diverse bioproducts, and particularly biofuels, from untreated or minimally treated biomass, and encourage further research in order to analyze their potential application in consolidated bioprocesses.

In light of the above, we have isolated and characterized a new strain, *T. thermosaccharolyticum* GSU5, an anaerobic thermophilic bacterium that is capable of producing ethanol and butanol from a variety of substrates. In this work, we present the genomic sequence of GSU5 and analyze its phenotypic traits, especially those pertaining to solvent production, in comparison with the type strain of the species. Additionally, genes related to solventogenesis of all sequenced *Thermoanaerobacterium* are compared to gain new insights into their unique metabolic properties.

2. Materials and Methods

2.1. Bacterial strains and growth conditions

Thermoanaerobacterium thermosaccharolyticum GSU5 was isolated from animal dung collected in a pasture plain in Buenos Aires, Argentina, in 1987. The strain was originally designated as *Clostridium thermopapyrolyticum* due to its phenotypic characteristics (Mendez et al., 1991). Stock cultures were kept at 4°C in Hungate tubes containing growth medium with a strip of filter paper for several decades. *Thermoanaerobacterium thermosaccharolyticum* DSM 571 was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Germany).

Both strains were grown at 60ºC in 5 mL screw cap tubes using the Hungate method (Hungate, 1950) in the Tryptose Sulphite Cycloserine (TSC) medium (Shaw et al., 2012) containing per liter: 2.0 g of sodium citrate tribasic dihydrate, 1.85 g of $(NH4)_2SO_4$, 0.1 g of $FeSO_4.7H_2O$, 2.0 g of $MgSO_4.7H_2O$, 1.0 g of KH_2PO_4, 0.1 g of $CaCl_2.2H_2O$, 2 mg of resazurin, 8.5 g of yeast extract, and 10 g of glucose. The pH was adjusted to 6.7 with NaOH 10 M.

For metabolite production analysis, the strains were grown in the TSC medium supplemented with 10 g/L of different carbon sources: monosaccharides xylose, arabinose, glucose, galactose, and fructose; and disaccharides sucrose and cellobiose. The pH was adjusted to 6.7 with NaOH 10 M. The strains were grown at 60ºC for 48 h.

Fermentations were performed in a 2.5 L BiostatA bioreactor with 1.5 L of the TSC supplemented with 10g/L of carbon sources (glucose or xylose). After 17 h, 5g/L of the corresponding sugar were added to avoid carbon source depletion. The agitation was kept at 100 rpm. The pH was adjusted initially at 7 and was regulated with NaOH 2M and H_2SO_4 1 M to keep it over 5. TSC pre-cultures were grown in serum bottles containing 50 mL of the medium at anaerobic conditions and 150 mL of inoculum was added to the bioreactor (DO= 0.3). The strains were grown at 60ºC for 35 h.

2.2. Sequence analysis

The sequence was obtained using a whole-genome shotgun strategy with a Roche 454 GS FLX Titanium pyrosequencer at INDEAR, Argentina, achieving ~37-fold coverage. Assembly was done using the Newbler version 2.6 and generated 73 contigs, the largest of which had 81,110 bases. The annotation was performed using the NCBI Prokaryotic Genomes Automatic Annotation Pipeline (PGAAP) and Rapid Annotations using Subsystems Technology (RAST) (Overbeek et al., 2014). The genome of the GSU5 was deposited in the Genbank with the accession number of MINB01000001-MINB01000073.

Genome to genome distance calculations were performed using the GGDC program (https://ggdc.dsmz.de/), that provides an *in silico* estimation of DNA-DNA hybridization (DDH) values (Auch et al., 2010) and Average Nucleotide Identity based on blast (ANIb) (http://jspecies.ribohost.com/jspeciesws) (Richter et al., 2016).

2.3. Analytical determinations

Cultures were centrifuged for 5 min at 6000 rpm, the supernatants were filtered through 0.22 µm nylon membranes (MSI, USA) and stored at -20ºC for analytical determinations.

Organic acids were determined by a high-performance liquid chromatography (HPLC) using an LC-20AT Prominance equipment (Shimadzu Corp., Kyoto, Japan) with an HPX-87-H Aminex column (Cat no. 125- 170 0140; Bio Rad Laboratories Inc., Hercules, CA) at 50ºC. The mobile phase consisted of 5 mM sulfuric acid at a flow rate of 0.6 mL/min. The metabolites were quantified with an SPD-20AV UV detector (Shimadzu 171 Corp.).

Ethanol and butanol were measured using an Agilent 7820A GC–FID with manual head space injection. The separation was conducted on a HP-INNO- WAX capillary column (30 m, 0.25 µm film thickness and 0.25 mm ID).

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The supernatants were diluted by adding 1 volume of K_2CO_3 1 g/mL, while 5 µl of isobutanol 5g/L were added as an internal standard. The samples were heated at 60°C for 1 h and 1 mL of the gas phase was injected manually in a GC-FID. The GC oven was initially heated at 35°C for 2 min, then to 45°C at a rate of 3°C/min and was held at this temperature for 1 min. Finally, the temperature was increased to 120ºC at a rate of 5ºC/min. The injector and FID temperatures were set at 150 and 300°C, respectively. Nitrogen was used as the carrier gas at a column flow of 2 mL/min with a 5:1 split ratio. The chromatographic method was validated for the experimental conditions used. The method was found to be precise and accurate on statistical evaluation with a linearity range of 0.01 to 1 g/L. The limit of quantification (LOQ= 0.004 g/L) and limit of detection (LOD= 0.0002 g/L) were also adequate.

3. Results and Discussion

3.1. Genomic relatedness of T. thermosaccharolyticum GSU5

The draft genome sequence of *T. thermosaccharolyticum* GSU5 was obtained in 73 contigs with a total length of 2.7 kb, with a mean G+C content of 33.9%. Analysis of the genome using the NCBI Prokaryotic Genomes Automatic Annotation Pipeline (PGAAP) revealed 2668 genes, comprising 2501 predicted coding regions, 102 pseudogenes, and 65 RNA genes, including one 16S rRNA gene. The RAST annotation covered 339 subsystems, including 44% of the coding sequences, and classified 897 open reading frames (ORFs) as hypothetical proteins.

Comparison of the 16S rRNA gene of GSU5 against sequences deposited in the Ribosomal Database Project revealed that GSU5 clusters together with other strains of *T. thermosaccharolyticum* and is closest to strain TG57 (**Fig. 1**). Strain SP-H2 could not be included in this analysis because the sequence of its rRNA gene is not available in spite of the fact that its genome has been sequenced.

The overall genomic phylogenetic relatedness of strain GSU5 with other strains of the genus *Thermoanaerobacterium* was analyzed through *in silico* genome comparisons. Values of *in silico* DDH between 87.30% and 71.6% were obtained when GSU5 was compared with *T. thermosaccharolyticum* strains TG57, DSM 571, M5, M0795, and SP-H2, indicating that it belongs to the *T. thermosaccharolyticum* species (species cutoff value: 70%), while the values obtained with all other strains ranged between 26.40 and 24.20% (**Table 1**). When ANIb was calculated, the values obtained (96.28 - 98.04%) when GSU5 was compared with other *T. thermosaccharolyticum* strains provided further evidence that it belongs to this species (species cutoff value: 95%) (**Table 1**).

Among the 12 *Thermoanaerobacterium* genomes available, the best represented species is *T. thermosaccharolyticum*, with 6 sequenced strains, followed by *T. saccharolyticum*, with two strains, and a single representative each for *T. xylanolyticum* and *T. aotearoense*. The other two genomes correspond to strains RBIITD and PSU2. When the genome of *Thermoanaerobacterium* sp. RBIITD was compared against all strains of the genus *Thermoanaerobacterium*, DDH values ranged between 21.3 and 24.70% and ANIb values ranged between 75.92 and 79.93% (**Table S1**). Similar results were obtained for *Thermoanaerobacterium* sp. PSU2, with DDH values ranging from 21.30 and 58.30% and ANIb values between 76.68 and 94.20% (**Table S2**). These results indicate that these strains do not belong to any of the species with sequenced representatives: *T. thermosaccharolyticum*, *T. saccharolyticum*, *T. xylanolyticum* or *T. aotearoense*.

When strain PSU-2 was first described, it was classified as *T. thermosaccharolyticum* (Sompong et al., 2008). However, when its genome was published, the authors reported that it was not a strain of *T. thermosaccharolyticum*, and although it had a high homology with *T. xylanolyticum*, it did not belong to this species either (Sompong et al., 2017).

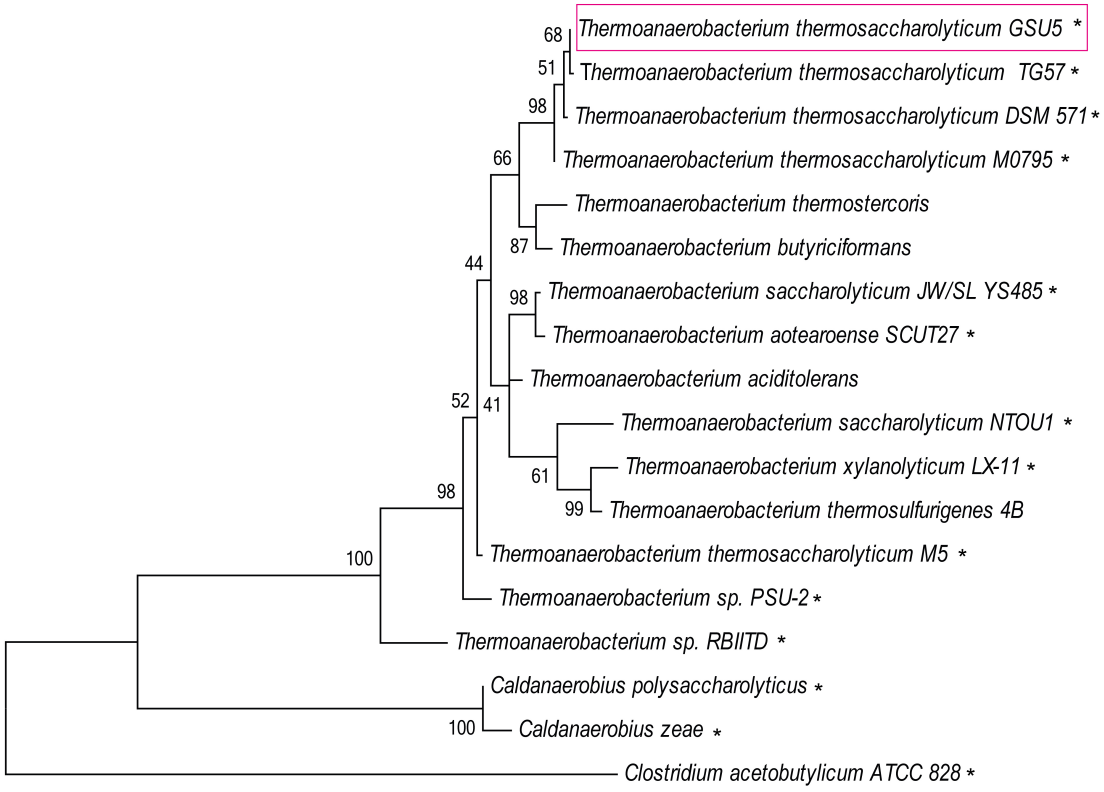

Fig. 1. Relatedness of GSU5 with other bacteria.
The 16s rRNA gene of GSU5 was compared against the sequences deposited in the Ribosomal Database Project. The phylogenetic tree was obtained using the Mega 6.0 program. At least one representative of each species of *Thermoanaerobacterium*, and all strains with sequenced genomes were included, along with the two species of *Caldanaerobium*, formerly classified within the *Thermoanaerobacterium* genus. *Clostridium acetobutylicum* was used as an outgroup.
*Strains with sequenced genomes

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Table 1.
Genome to genome comparisons of GSU5 with other species of *Thermoanaerobacterium*.

Strain	Accession number	DDH [1] estimate	ANIb [2]
T. thermosaccharolyticum GSU5	MINB01000001-MINB01000073	-	-
T. thermosaccharolyticum TG57	CP016893.1	87.30±2.50%	98.04%
T. thermosaccharolyticum DSM 571	CP002171	76.40 ± 2.88%	96.48%
T. thermosaccharolyticum M5	NKHD01000001-NKHD01000077	75.40±3.00%	96.93%
T. thermosaccharolyticum M0795	NC_019970	72.10 ± 2.92%	96.28%
T. thermosaccharolyticum SP-H2	VWOK00000000.1	71.6±3.9%	96.34%
T. saccharolyticum JW/SL-YS485	CP003184.1	24.30±2.39%	81.63%
T. saccharolyticum NTOU1	BBKT01000001-BBKT01000101	26.40±2,50%	83.52%
T. xylanolyticum LX-11	CP002739.1	25.00±2.40%	82.12%
T. aotearoense SCUT27	AYSN01000000	24.20±2.50%	81.76%
Thermoanaerobacterium sp. PSU-2	MSQD00000000.1	24.40±2.50%	81.82%
Thermoanaerobacterium sp. RBIITD	LT906662.1	24.30±2.50%	79.52%

[1] DNA-DNA hybridization (DDH) values were estimated using the Genome-to-Genome Distance Calculator 2.1 (Species cutoff value: 70%).
[2] ANIb: Average Nucleotide Identity based on BLAST (Species cutoff value =95%).

The results presented in this work indicate that neither PSU2 nor RBIITD belong to *T. saccharolyticum*, *T. thermosaccharolyticum*, *T. xylanolyticum* or *T. aotearoense*. It remains to be studied whether these strains belong to species with no sequenced representatives, or to previously undescribed *Thermoanaerobacterium* species.

3.2. Genetic analysis of solvent synthesis pathways

A search for genes coding for the key enzymes involved in the synthesis of butanol, ethanol, and acetone was performed in the genome of GSU5 and all other sequenced *Thermoanaerobacterium* strains using the RAST annotation Server, BLAST and the Biocyc database collection in order to analyze the corresponding pathways.

As shown in **Table 2**, all genes coding for key enzymes of butanol synthesis were detected in GSU5, in all sequenced *T. thermosaccharolyticum* strains, and in *Thermoanaerobacterium* sp. RBIITD (locus tags in **Table S3**). In contrast, these genes were absent in *T. xylanolyticum* LX-11, T. *saccharolyticum* JW/SL-YS485, *T. saccharolyticum* NTOU1, *T. aotearoense* SCUT27, and *Thermoanaerobacterium* sp. PSU-2, which suggests that they are not capable of producing butanol (**Table 2**).

In all strains of *T. thermosaccharolyticum*, genes coding for the crotonase (*crt*), butyryl-CoA dehydrogenase (*bcd*), electron transfer flavoprotein (*etfAB*), 3-hydroxybutyryl-CoA dehydrogenase (*hbd*), and acetyl-CoA C-acetyltransferase (*thl*), are located in a cluster. Next to these genes and in the same orientation are *but*, that codes for a butyryl-CoA: acetate-CoA transferase, and *rex*, corresponding to a redox dependent transcriptional regulator (**Fig. 2**).

In the well-known butanol producer *Clostridium acetobutylicum*, seven of the genes are also in a genomic cluster (Berezina et al., 2009) named *bcs* operon, albeit with a different organization: in this organism *thl* is in a monocistronic operon located in a different region, *rex* is situated upstream from *crt*, and *but* is absent (**Fig. 2**). In *C. acetobutylicum*, the transcriptional regulator Rex plays a fundamental role in the regulation of solvent synthesis (Panitz et al., 2014) and binds to specific sequences identified upstream from three genes: *thl*, *crt* (the first gene of the *bcs* operon), and *adhE* (Wietzke and Bahl, 2012). Putative binding sites for Rex (ROP: Rex operator site) were observed upstream from *crt* (**Fig. 2**) and *adhE* in the genome of GSU5, suggesting that Rex could regulate the expression of these genes in *Thermoanaerobacterium*.

In *Thermoanaerobacterium* sp. RBIITD, genes related to butanol formation have a different genetic organization, since *thl* is located in the *bcs* operon but *rex* is located upstream from *crt* (**Fig. 2**).

There are 5 genes encoding alcohol dehydrogenases distributed throughout the genome of GSU5 and one encoding an aldehyde dehydrogenase (*ald*). The gene coding for the bifunctional alcohol/aldehyde dehydrogenase (*adhE*) was detected in a monocistronic operon with a similar location in all genomes analyzed, while *ald*, corresponding to an aldehyde dehydrogenase, was only found in GSU5 and in some butanol producing and non-butanol producing strains (**Table 2**). *T. thermosaccharolyticum* DSM 571 and M5 produce both butanol and ethanol (Bhandiwad et al., 2013; Jiang et al., 2018; Li et al., 2018) but do not carry *ald*, suggesting that it is not essential for alcohol synthesis in *Thermoanaerobacterium.* This is in agreement with previous reports that

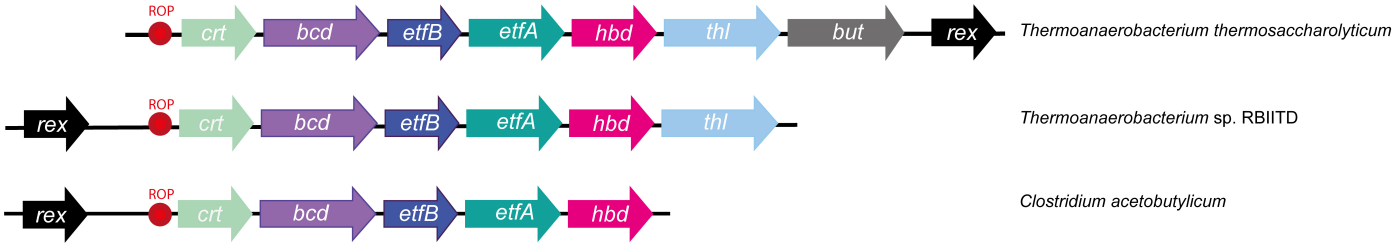

Fig. 2. Organization of the *bcs* operon in *Thermoanaerobacterium thermosaccharolyticum*, *Thermoanaerobacterium sp.* RBIITD and *Clostridium acetobutylicum*. Putative Rex operator sites (ROP) are indicated as red circles.

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Table 2.
Genes involved in ethanol, butanol, and butyrate synthesis in the sequenced *Thermoanaerobacterium* strains.

Strain	*crt*	*bcd*	*etf B*	*etf A*	*hbd*	*thl*	*but*	*ptb*	*buk*	*ald*	*adh1*	*adh2*	*adh3*	*bhd*	*adhE*	*pta*	*ak*	*ctfA*	*ctfB*	*adc*
T. thermosaccharolyticum GSU5	100*	100	100	100	100	100	100			100	100	100	100	100	100	100	100			
T. thermosaccharolyticum TG57	100	100	99	100	100	100	100			58	100	99	99	99	99	99	99			
T. thermosaccharolyticum DSM 571	91	98	99	99	100	100	100				99	99	100	99	99	99	99			
T. thermosaccharolyticum M5	99	99	99	99	100	100	100				99	100	99	99	99	99	99			
T. thermosaccharolyticum M0795	99	99	98	95	98	100	100			58		99	99	95	99	99	99			
T. saccharolyticum JW/SL-YS485								0	0	93		92	90	90	97	94	94			
T. saccharolyticum NTOU1								0	0		98	92	92	91	98	94	94			
T. xylanolyticuml X-11								0	0	92		92	92	91	97	94	94			
T. aotearoense SCUT27								0	0	93		92	90	90	97	94	94			
Thermoanaerobacterium sp. PSU-2								0	0			91	91	90	98	93	93			
Thermoanaerobacterium sp. RBIITD	77	96	91	96	88	90	82			58	67	89	89	84	55	87	87			
Clostridium acetobutylicum ATCC 824	58	72	66	58	69	71		0	0					45	53	63	72	0	0	0

* Numbers indicate percentage of protein sequence identity with the corresponding protein of strain GSU5.
- Red cells indicate that the gene is present in the corresponding strain and absent in GSU5.
- Blank cells indicate absence of the gene in the corresponding strain.
- Genes code for the proteins are indicated as follows, *crt:* crotonase; *bcd*: butyryl-CoA dehydrogenase; *etfAB:* electron transfer flavoprotein; *hbd*: 3-hydroxybutyryl-CoA dehydrogenase; *thl*: acetyl-CoA C-acetyltransferase; *but*: butyryl-CoA: acetate-CoA transferase; *ptb*: phosphotrans butyrylase; *buk*: butyrate kinase; *ald*: aldehyde dehydrogenase; *adh*: alcohol dehydrogenase; *bdh*: butanol dehydrogenase; *adhE*: bifunctional aldehyde/ alcohol dehydrogenase; *pta*: phosphotrans acetylase; *ak*: acetate kinase; *ctfAB*: acetoacetyl coenzyme A: acetate/butyrate:coenzyme A transferase; *adc:* acetoacetate decarboxylase. Accession numbers for each genome are indicated in **Table 1**.

indicated that AdhE was responsible for the synthesis of n-butanol from butyryl-CoA and ethanol from acetyl-CoA (Bhandiwad et al., 2014). All strains also carried genes coding for other dehydrogenases including Bhd that could also have a role in the synthesis of alcohols in *Thermoanaerobacterium*. Interestingly, one of the dehydrogenases, *adh3*, was highly conserved in all genomes, located upstream from genes coding for a NADH-dependent reduced ferredoxin: NADP oxidoreductase (*nfnAB*).

In recent years, analysis of the genome of several strains of *T. thermosaccharolyticum* revealed important differences in the biosynthesis of butanol and butyrate when compared to the ABE pathway known in the well characterized *C. acetobutylicum* (Jones and Woods, 1986). Previous reports indicated that the gene coding for the acetoacetate decarboxylase (*adc*) was absent in *T. thermosaccharolyticum* M5 (Jiang et al., 2018), and that both *adc* and *ctfAB* (that codes for the butyrate-acetoacetate CoA-transferase subunits A and B) were absent in *T. thermosaccharolyticum* TG57 (Li et al., 2018), indicating that these bacteria were unable to produce acetone. These genes were searched in the genomes of *T. thermosaccharolyticum* GSU5 and in all available *Thermoanaerobacterium* genomes using the genes from *C. acetobutylicum* as queries. None of the genomes analyzed carried *ctfAB* or *adc* (**Table 2**) indicating that none of the strains analyzed would be able to produce acetone during fermentation (**Fig. 3**). Since the analysis involved all available *Thermoanaerobacterium* genomes representing half of the known species, these results suggest that this is a common trait in this genus.

Butyrate production has been reported for several *T. thermosaccharolyticum* strains (Freier-Schröder et al., 1989; Li et al., 2018), and is the main metabolite produced from most carbon sources by both strains tested in this work. Among the known butyrate producing pathways, the most common is the acetyl-CoA pathway, that has two variants: i) the two-step conversion catalyzed by the phosphotransbutyrylase (Ptb) and butyrate kinase (Buk) with a phosphorylated intermediate that allows the formation of ATP, commonly found in bacteria that have the ABE pathway, and ii) the one step conversion of butyryl-CoA to butyrate catalyzed by a butyryl-CoA:acetate-CoA transferase (But) (Vital et al., 2014). Only the one step pathway seems to be performed by the strain GSU5 and all other *T. thermosaccharolyticum* strains, as they carry *but* (denominated *ach* in *T. thermosaccharolyticum* TG57), and not *ptb* or *buk* (Bhandiwad et al., 2013; Li et al., 2018) (**Fig. 3**). The two-step pathway has been proposed to exist in the strain M5 (Jiang et al., 2018) but its genome does not carry genes coding for Ptb or Buk.

The one step conversion of butyryl-CoA to butyrate has been extensively studied in *C. kluyveri*, that seems to compensate for the lack of the ATP producing step by means of an electron bifurcating mechanism involving the crotonyl-CoA reductase that couples the reduction of crotonyl-CoA to the reduction of ferredoxin using NADH as the electron donor for both reactions. The reduction of crotonyl-CoA to butyryl-CoA is catalyzed by the cytoplasmic butyryl-CoA dehydrogenase complex, coded by *bcd* and *etfAB*, while the reduction of the ferredoxin is catalyzed by NfnAB, a NADH-dependent reduced ferredoxin:NADP oxidoreductase (Wang et al., 2010). These genes are present in all strains of *T. thermosaccharolyticum* and in *Thermoanaerobacterium* sp. RBIITD that have the capability to produce butanol and butyrate (**Table 2**). Furthermore, the ferredoxin-dependent activity of the butyryl-CoA dehydrogenase was experimentally demonstrated in *T. thermoanaerobacterium* DSM 571 (Bhandiwad et al., 2014). Based on this information, it can be hypothesized that all butanol producing *Thermoanaerobacterium* are able to obtain energy during the one step butyrate synthesis through the ferredoxin mediated electron bifurcation mechanism (**Fig. 3**). In contrast, genes coding for Ptb and Buk were identified in *T. xylanolyticum*, *T. saccharolyticum*, *T. aotearoense*, and *Thermoanaerobacterium* sp. PSU-2. These strains lack genes needed for butanol synthesis (*bcs* operon), and are also devoid of genes coding for the cytoplasmic butyryl-CoA dehydrogenase complex (*bcd* and *etfAB*), suggesting that they are unable to synthesize either butanol or butyrate. In these microorganisms, *pbt* and *buk* are clustered together with a gene that codes for a leucine/valine/phenylalanine dehydrogenase. This genetic organization has been previously observed in *Bacillus megaterium*. In this organism, Ptb expression was induced in the presence of valine and isoleucine, and the enzyme could use butyryl-CoA and 2-methyl-propionyl CoA as substrates (Vazquez et al., 2001). In *B. subtilis*, these genes are part of the *bkd* operon, involved in the degradation of branched chain amino-

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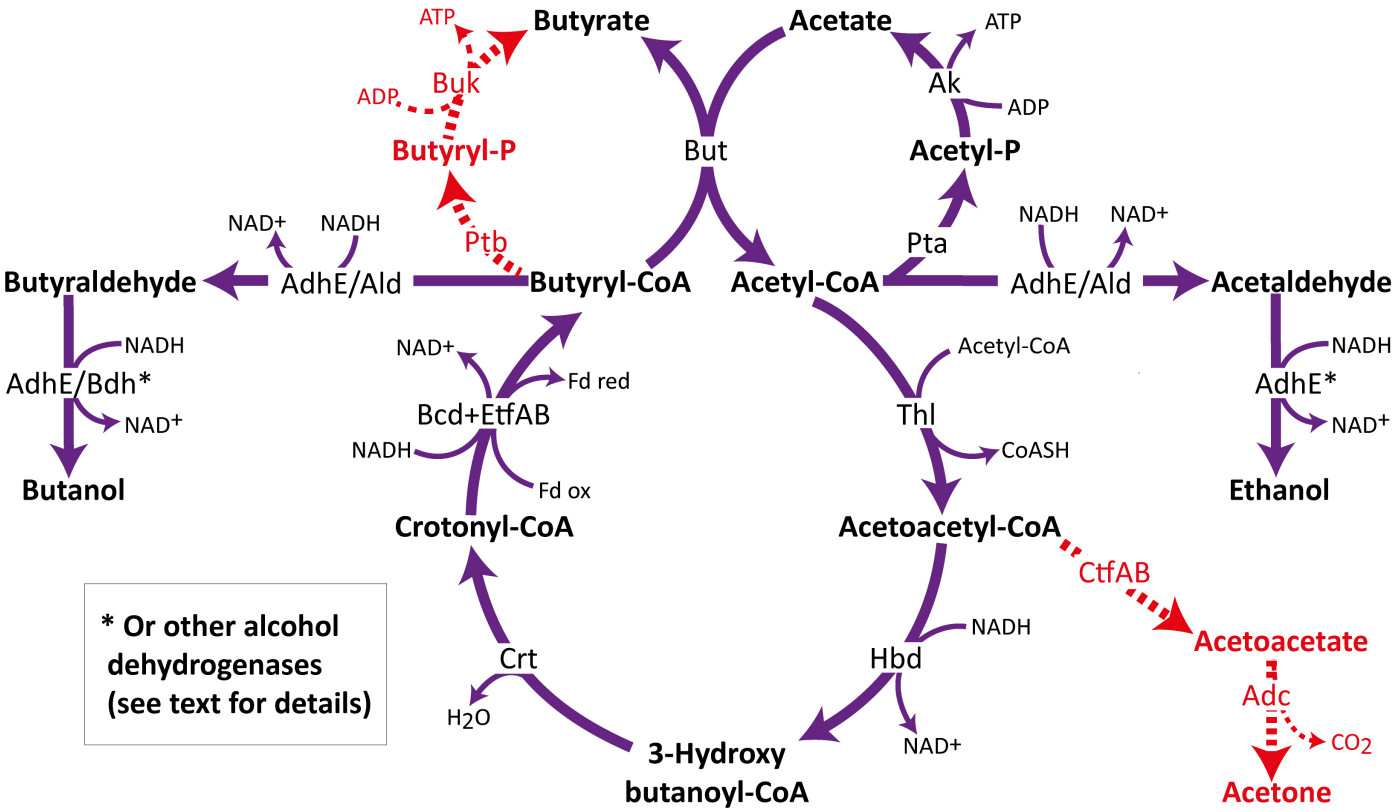

Fig. 3. Proposed main metabolic pathways in *T. thermosaccharolyticum*.
Purple solid arrows indicate reactions catalyzed by genes present in the genome. Red dotted arrows indicate reactions catalyzed by genes absent in the genome. Enzymes separated by a slash and/or with asterisks indicate reactions that could be potentially catalyzed by more than one enzyme. *Abbreviations:* Crt: crotonase; Bcd: butyryl-CoA dehydrogenase; EtfAB: electron transfer flavoprotein; Hbd: 3-hydroxybutyryl-CoA dehydrogenase; Thl: acetyl-CoA C-acetyltransferase; But: butyryl-CoA: acetate-CoA transferase; Ptb: phosphotrans butyrylase; Buk: butyrate kinase; Ald: aldehyde dehydrogenase; Bdh: butanol dehydrogenase; AdhE: bifunctional aldehyde/ alcohol dehydrogenase; Pta: phosphotrans acetylase; Ak: acetate kinase; CtfAB: acetoacetyl coenzyme A:acetate/butyrate:coenzyme A transferase; Adc: acetoacetate decarboxylase; Fd red: reduced ferredoxin; Fd ox: oxidized ferredoxin.

acids (Debarbouille et al., 1999). It is possible that in *T. xylanolyticum*, *T. saccharolyticum*, *Thermoanaerobacterium* sp. PSU-2, and *T. aotearoense*, Ptb and Buk could be involved in branched chain amino-acid degradation as in *Bacillus*.

3.3. Production of metabolites from different carbon sources

The synthesis of solvents and acids by *T. thermosaccharolyticum* GSU5 was analyzed in cultures grown using different substrates in 5 mL tubes, and compared with the type strain of the species, DSM 571. Both strains grew well on several monosaccharides: glucose, xylose, arabinose, and galactose, and also on the disaccharides, sucrose and cellobiose. They grew slightly more when using fructose that on pentoses and other hexoses, and also showed a modest preference for cellobiose compared to sucrose (despite their specific epithet).

Ethanol was the most abundant alcohol, and butanol was also observed in all cultures in lower amounts. Acetone was not produced, as previously reported for the butanol producing strains RBIITD and TG57 (Biswas et al., 2018; Li et al., 2018). Among the acids, butyrate was the most abundant, followed by acetate and lower amounts of lactate (**Fig. 4**). The relative production of the metabolites depended on the substrate used, and some differences were observed between the strains. The main product of GSU5 was butyrate in all the conditions tested, ranging from 0.93 to 2.07 g/L, while DSM 571 synthesized butyrate as a main product (0.99 to 1.35 g/L) in all cultures except those using glucose and xylose, in which similar amounts of ethanol and butyrate were observed (**Fig. 4**). Both strains produced much more ethanol than butanol. GSU5 produced between 0.12 and 0.73 g/L ethanol and 0.005 to 0.048 g/L butanol, with the highest amount of butanol produced in sucrose. DSM 571 produced between 0.25 and 1.25 g/L ethanol and 0.014 to 0.034 g/L butanol with the highest amount of butanol obtained in glucose.

Strain GSU5 produced 3.2 times more butanol than DSM 571 in sucrose (0.048 *vs.* 0.015 g/L; $p<0.05$), and also accumulated more butanol than DSM 571 in arabinose, while the opposite was observed in fructose and cellobiose, in which strain DSM 571 produced more butanol than GSU5. When ethanol production was compared, strain DSM 571 produced a greater concentration of ethanol than GSU5 in all hexoses and in xylose, in which the highest amount was observed (0.98 g/L), while GSU5 accumulated more ethanol in arabinose and with the disaccharides sucrose and cellobiose, with the maximum production in this last carbon source (0.73 g/L).

These results are similar to those obtained in previous studies performed using *T. thermosaccharolyticum* DSM 571 grown on cellobiose (Bhandiwad et al., 2013), and also using strain M5 grown on xylan (Jiang et al., 2018). In contrast, Li et al. (2018) report that *T. thermosaccharolyticum* TG57 produced butyrate, acetate, and butanol, but no ethanol, when grown using glucose, cellobiose, cellulose or xylan, in spite of the fact that it has all enzymes required for the synthesis of both alcohols.

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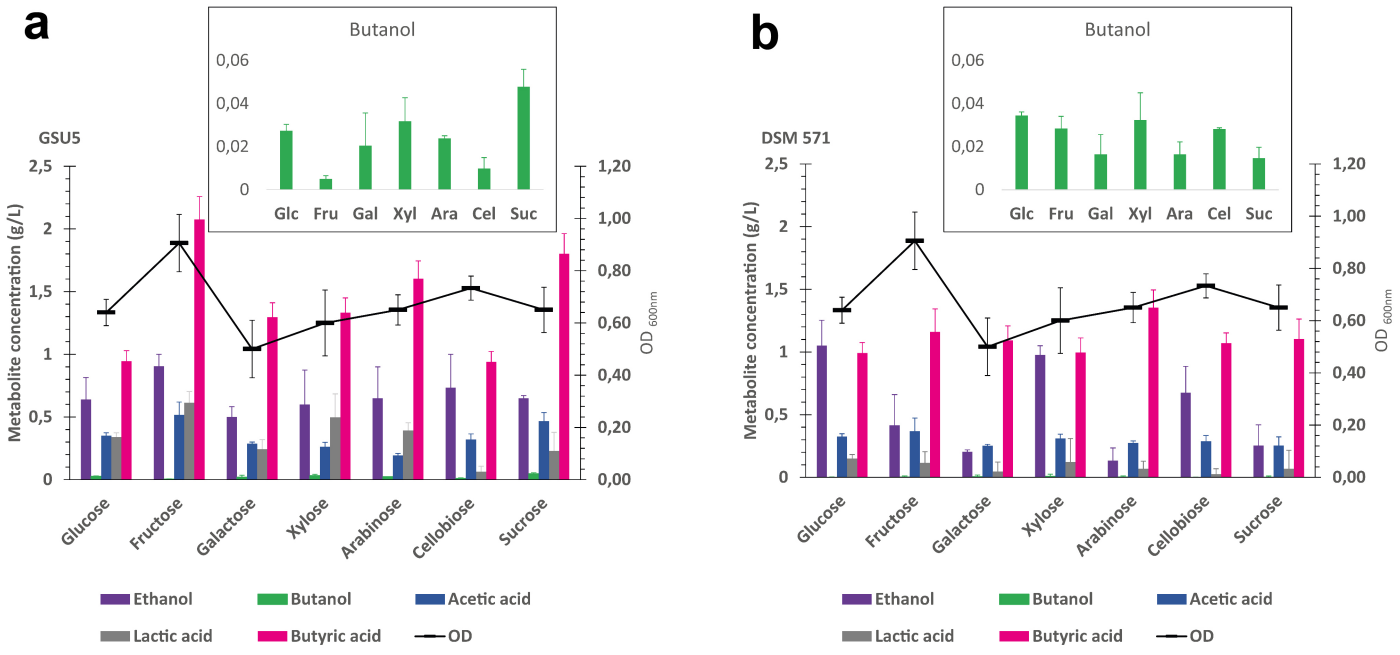

Fig. 4. Production of metabolites in *T. thermosaccharolyticum* GSU5 (A) and *T. thermosaccharolyticum* DSM 571 (B). Strains were grown in TCS supplemented with different carbon sources in 5 mL Hungate tubes under anaerobic conditions for 48 h. Results represent the mean value ± standard deviation of three independent replicates.

3.4. Bioreactor cultures

Strain GSU5 was grown in bioreactor batch cultures using glucose or xylose as carbon sources in order to analyze solvent production in a larger scale. Several tests were performed to determine the best pH conditions for growth and solvent production, since previous studies had indicated that solvent production was enhanced by high pH in other *T. thermosaccharolyticum* strains (Jiang et al., 2018). Preliminary tests in glucose cultures with initial starting pH of 6.7 or 7.5 did not allow adequate growth, so all cultures were performed with an initial pH of 7, and pH was controlled to prevent values lower than 5. Sugars were added at an initial concentration of 10 g/L, and replenished after 17 h by adding half the initial amount to avoid sugar depletion.

Growth was more vigorous in glucose cultures, achieving 52% more biomass than when using xylose (1.9 *vs.* 1.0 g/L). Solvent production was also higher in glucose, while xylose cultures produced more acids than solvents (Fig. 5). Butyric acid remained the main product in xylose cultures, but glucose cultures produced slightly more ethanol than butyrate. The final butanol concentration obtained was 0.33 ± 0.01 g/L in glucose and 0.26 ±

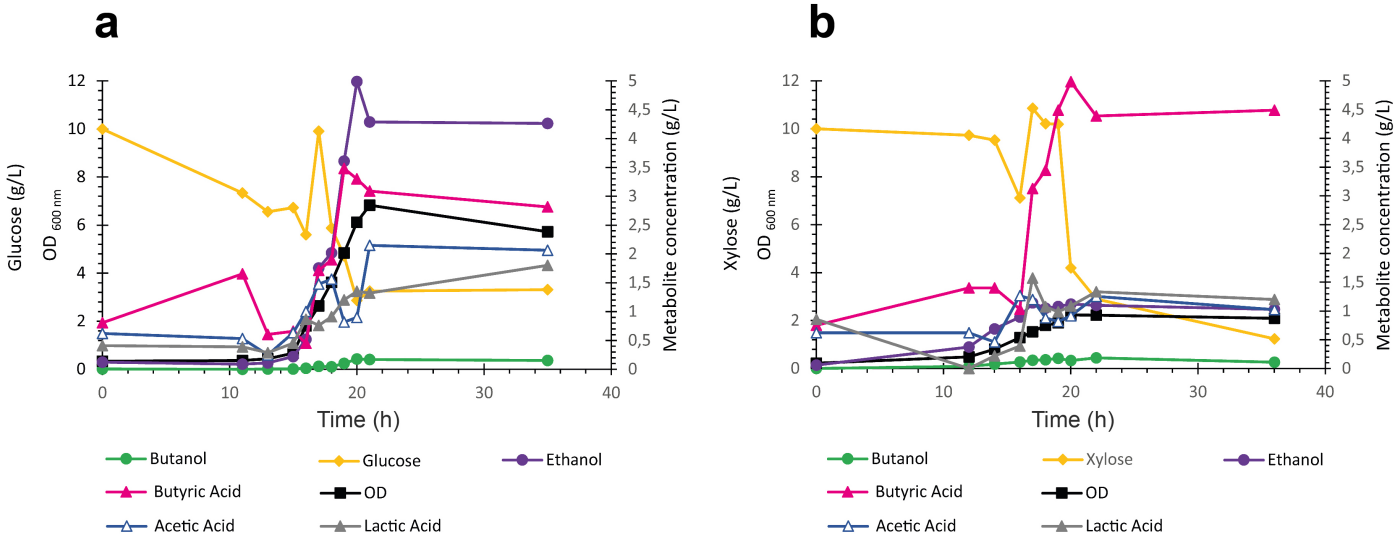

Fig. 5. Production of metabolites in *T. thermosaccharolyticum* GSU5 grown in TCS supplemented with glucose **(A)** or xylose **(B)** at 10 g/L in a bioreactor under anaerobic conditions for 36 h. Data from a representative fermentation of three independent replicates.

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Table 3.
Fermentation products of *T. thermosaccharolyticum* GSU5.*

Fermentation Product	Product concentration (g/L)		Yield[a] (g/g)		Yield[b] (mol /mol)		Volumetric productivity[c] g/(L.h)	
	Glu	Xyl	Glu	Xyl	Glu	Xyl	Glu	Xyl
Butanol	0.33±0.10**	0.26±0.15	0.034±0.008	0.022±0.007	0.09±0.02	0.05±0.01	0.015±0.004	0.013±0.006
Ethanol	4.35±0.06	1.06±0.05	0.46±0.07	0.08±0.01	1.8±0.1	0.31±0.05	0.19±0.01	0.050±0.002
Butyrate	3±1	6±3	0.39±0.24	0.33±0.19	0.8±0.3	0.6±0.3	0.12±0.08	0.28±0.09
Acetate	2.3±0.9	0.96±0.2	0.13±0.08	0.10±0.07	0.5±0.2	0.15±0.07	0.11±0.04	0.05±0.01
Lactate	1.1±0.3	1.2±0.8	0.15±0.07	0.09±0.07	0.22±0.06	0.2±0.1	0.05±0.01	0.06±0.04
Biomass	1.9±0.7	1.0±0.5	0.19±0.09	0.09±0.05	-	-	-	-

* Cultures were grown in TSC medium supplemented with glucose or xylose at 10/L in a bioreactor under anaerobic conditions for 36 h.
** Results represent means ± the standard deviations corresponding to three independent cultures (Glu: Glucose and Xyl: xylose).
[a] Yield: g of product/g of substrate
[b] Yield: mol of product/mol of substrate
[c] Volumetric productivity: g of product/(time . volume)

0.02 g/L in xylose, while the final ethanol concentration was 4.35 ± 0.06 g/L in glucose and 1.06 ± 0.05 g/L in xylose (Table 3).

As expected, the bioreactor cultures corresponding to both sugars had a much higher biomass and solvent production that the 5 mL tube cultures. Although the main products were the same as observed in tube cultures, the relative amounts of acids and alcohols varied. A higher relative amount of butanol was observed with both substrates when all major metabolites were considered, showing a slight increase in carbon fluxes towards butanol in bioreactor cultures (Fig. 6). When cultures grown using glucose were compared to tube cultures, solvent production was observed to increase more than biomass, with the most important increase measured in butanol production. While an 8-fold change was recorded in biomass (1.78 *vs.* 0.22 g/L), final concentrations of ethanol and butanol increased by 10 and 14 times, respectively, so that the amount of alcohols produced in the bioreactor was similar to that of acids (Fig. 6). Results obtained with xylose were different, as a 6-fold increase in biomass (1.04 *vs.* 0.20 g/L) was accompanied by a similar increase in butanol concentration, while ethanol concentration only increased by 1.5 folds when the bioreactor cultures were compared with the 5 mL cultures (Fig. 6).

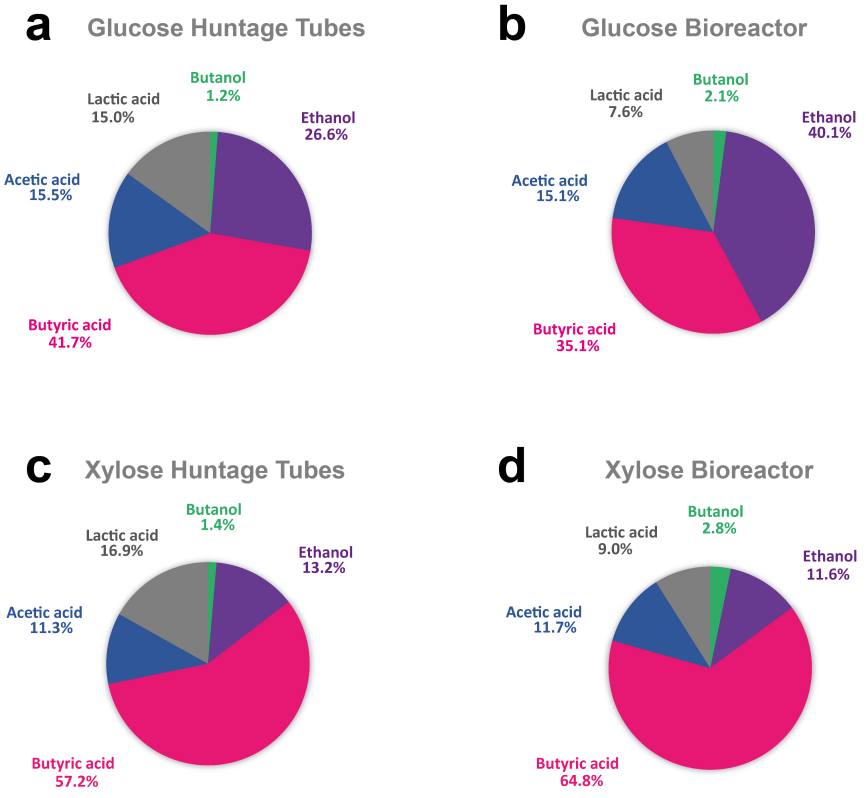

Fig. 6. Relative distribution (%) of metabolites produced by *T. thermosaccharolyticum* GSU5 in Hungate tubes with glucose **(A)**, in a bioreactor with glucose **(B)**, in Hungate tubes with xylose **(C)**, and in a bioreactor with xylose **(D)**.

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These results could suggest that in higher density cultures, carbon flow towards the synthesis of alcohols increases contributing to the re-oxidation of excess NADH or NADPH, thus favoring the production of alcohols over acids.

Solventogenic anaerobes like the broadly studied model bacterium *C. acetobutylicum* have a fermentation behavior characterized by two distinct phases: formation of acids during the first phase followed by a solventogenic phase in which growth slows down, and solvents are produced (Amador-Noguez et al., 2011). When the dynamics of acids and alcohols production was analyzed in *T. thermosaccharolyticum* GSU5, metabolite synthesis was observed to accompany growth, and no clear solventogenic phase could be distinguished. This had been reported for *T. thermosaccharolyticum* M5 (Jiang et al., 2018) and careful observation of metabolite curves displayed in studies carried out with different solventogenic *Thermoanaerobacterium* strains show that in all cases alcohols and acids are synthesized throughout growth. This can be clearly perceived in an early work performed using strain DSM 571 (Freier-Schröder et al., 1989) and in the metabolite curves shown in studies that analyze the production of hydrogen in different strains of *T. thermosaccharolyticum* (Cao et al., 2009 and 2014; Khamtib and Reungsang, 2012), using both sugars or lignocellulosic biomass for growth. These results suggest that solventogenesis in *Thermoanaerobacterium* is not subject to the same regulatory mechanisms described for the ABE metabolism in *Clostridium*.

4. Conclusions

Several species of *Thermoanaerobacterium* have been studied for the production of different bioproducts. Among them, *T. thermosaccharolyticum* seems to be the most promising, and different strains have been analyzed as candidates for the production of hydrogen (Cao et al., 2009 and 2014), ethanol (Bhandiwad et al., 2013 and 2014; Jiang et al., 2018) and 1,2 propanediol (Cameron and Cooney, 1986; Altaras et al., 2001) from several carbon sources, including lignocellulosic biomass. *T. thermosaccharolyticum* GSU5 is able to produce ethanol and butanol from a wide variety of substrates, including hexoses, pentoses and the discacharides sucrose and cellobiose. Analysis of the genome of *T. thermosaccharolyticum* GSU5 and comparison with other members of the genus enabled new insights into the particular metabolic characteristics of these bacteria, leading to a detailed description of the metabolic routes present in *Thermoanaerobacterium*, with clear differences between butanol producing and non-butanol producing strains. The metabolism of the butanol producing *Thermoanaerobacterium* differs from the metabolism of the well-known butanol producer *C. acetobutylicum*. Among the most important differences are the lack of acetone synthesis, a different butyrate production pathway, and a different dynamic of solvent and acid synthesis. Knowledge on their physiology and unique metabolic properties will enable the use of these microorganisms to obtain biotechnologically relevant bioproducts in sustainable and environmentally friendly processes.

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Supplementary Data

Table S1.
Genome to genome comparisons of *Thermoanaerobacterium* sp. RBIITD with other species of *Thermoanaerobacterium*.

Strain	Accession	DDH [1] estimate	ANIb [2]
Thermoanaerobacterium sp. RBIITD	LT906662.1	-	-
T. thermosaccharolyticum GSU5	MINB01000001-MINB01000073	24.30±2.50%	79.53%
T. thermosaccharolyticum TG57	CP016893.1	24.30±2.50%	79.03%
T. thermosaccharolyticum DSM 571	CP002171	24.70±2,70%	79.18%
T. thermosaccharolyticum M5	NKHD01000001-NKHD01000077	23.50±2.50%	78.94%
T. thermosaccharolyticum M0795	NC_019970	24.50±2.50%	79.19%
T. saccharolyticum JW/SL-YS485	CP003184.1	21.50±2,40%	75.92%
T. saccharolyticum NTOU1	BBKT01000001-BBKT01000101	23.30±2.50%	77.39%
T. xylanolyticum LX-11	CP002739.1	21.30±2.40%	76.16%
T. aotearoense SCUT27	AYSN01000000	21.40±2.40%	76.19%
T. sp. PSU-2	MSQD00000000.1	21.30±2.70%	76.16%

[1] DNA-DNA hybridization (DDH) values were estimated using the Genome-to-Genome Distance Calculator 2.1 (Species cutoff value: 70%).
[2] ANIb: Average Nucleotide Identity based on BLAST (Species cutoff value =95%).

Table S2.
Genome to genome comparisons of *Thermoanaerobacterium* sp. PSU-2 with other species of *Thermoanaerobacterium*.

Strain	Accession	DDH [1] estimate	ANIb [2]
Thermoanaerobacterium sp. PSU-2	MSQD00000000.1	-	-
T. thermosaccharolyticum GSU5	MINB01000001-MINB01000073	24.40±2,50%	82.30%
T. thermosaccharolyticum TG57	CP016893.1	24.60±2,50%	81.96%
T. thermosaccharolyticum DSM 571	CP002171	24.80±2.40%	82.15%
T. thermosaccharolyticum M5	NKHD01000001-NKHD01000077	24.10±2.5%	81.56%
T. thermosaccharolyticum M0795	NC_019970	58.30±2.80%	81.93%
T. saccharolyticum JW/SL-YS485	CP003184.1	48.90±2.60%	92.35%
T. saccharolyticum NTOU1	BBKT01000001-BBKT01000101	48.20 ±2.60%	91.96%
T. xylanolyticum LX-11	CP002739.1	58.30±2,80%	94.20%
T. aotearoense SCUT27	AYSN01000000	48.70±2,60%	92.24%
Thermoanaerobacterium sp. RBIITD	LT906662.1	21.30±2.70%	76.68%

[1] DNA-DNA hybridization (DDH) values were estimated using the Genome-to-Genome Distance Calculator 2.1 (Species cutoff value: 70%).
[2] ANIb: Average Nucleotide Identity based on BLAST (Species cutoff value =95%).

Table S3.
Locus tags of genes analyzed in this study.
https://www.biofueljournal.com/jufile?ar_sfile=1007034

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