Biomass and Bioenergy 125 (2019) 17–22

Contents lists available at ScienceDirect

Biomass and Bioenergy

journal homepage: www.elsevier.com/locate/biombioe

Research paper

Simultaneous synthesis of lactic acid and hydrogen from sugars via capnophilic lactic fermentation by *Thermotoga neapolitana cf capnolactica*

Nirakar Pradhan[a,b,*,1], Giuliana d'Ippolito[a,**], Laura Dipasquale[a], Giovanni Esposito[b], Antonio Panico[c], Piet N.L. Lens[d], Angelo Fontana[a]

[a] National Research Council of Italy, Institute of Biomolecular Chemistry, Via Campi Flegrei 34, 80078, Pozzuoli, Napoli, Italy
[b] Department of Civil and Mechanical Engineering, University of Cassino and Southern Lazio, Via Di Biasio, 43, 03043, Cassino, FR, Italy
[c] Telematic University Pegaso, piazza Trieste e Trento, 48, 80132, Naples, Italy
[d] UNESCO-IHE Delft, Westvest 7, 2611AX, Delft, the Netherlands

ARTICLE INFO

Keywords:
Thermophilic bacteria
Hydrogen
Lactic acid
Fermentation
Salinity level
Buffering agent

ABSTRACT

This study investigated the effect of the salinity level, buffering agent and carbon source on the hydrogen (H_2) and lactic acid synthesis under capnophilic (CO_2-assisted) lactic fermentation (CLF) by *Thermotoga neapolitana cf capnolactica* (DSM 33003). Several series of batch fermentation experiments were performed either in 0.12 L serum bottles for selection of the best performing conditions or in a 3 L fermenter for the best possible combination of conditions. The serum bottle study revealed that change in the salinity level of the culture medium from 0 to 35 g L^{-1} NaCl increased lactic acid synthesis by 7.5 times without affecting the H_2 yield. Use of different buffers (MOPS, TRIS or HEPES) did not affect the average H_2 yield of 3.0 ± 0.24 mol H_2 mol^{-1} of glucose and lactic acid synthesis of 13.7 ± 1.03 mM when the cultures were sparged by CO_2. Among the carbon sources investigated, glucose was found to be the best performing carbon source for the CLF fermentation with 35 g L^{-1} of NaCl and 0.01 M of phosphate buffer. Hence, an up-scale experiment using a 3 L fermenter and the combination of the best performing conditions showed a 2.2 times more lactic acid synthesis compared to the 0.12 L serum bottle experiments. The study reveals the robustness and flexibility of the CLF-based technology using *T. neapolitana cf capnolactica* fermentation under various operating environmental conditions.

1. Introduction

Research efforts are currently focused on finding sustainable, clean and alternative energy sources, which are of urgent need for the increasing energy demand and growing concerns about greenhouse gas (GHG) emissions. Hydrogen (H_2) is a clean energy carrier, as it does not emit any GHGs upon its utilization. Currently, over 90% of the global H_2 is produced through physico-chemical routes, such as pyrolysis and gasification, thereby indirectly emitting GHGs (i.e. SO_x, NO_x, CO and CO_2) to the atmosphere [1,2]. On the other hand, biological methods to produce hydrogen (H_2) are free from net emission of GHGs and, for this reason, have raised interest in the scientific community. Among the biological routes, fermentation processes like dark fermentation (DF) are particularly promising because of additional advantages that include high H_2 production rates, low energy demand, low operational cost, cheap and easily available substrates and simple operational techniques with reliable process stability [3].

The introduction of the hyperthermophilic marine eubacterium *Thermotoga neapolitana* for H_2 production has had a significant impact in this research field, despite general pitfalls including the high risk of contamination due to use of a pure culture and the change of the fermentation products as a result of slight deviations of the operating conditions [3–7]. Recently, an unexpected high lactic acid production was achieved without compromising H_2 yield by using a stream of CO_2 gas to maintain the culture under anaerobic conditions [8].

After further investigation, a new metabolic pathway was discovered for this fermentation process, which was termed as capnophilic lactic fermentation (CLF) to underline the requirement of saturated CO_2 concentrations in the culture medium [8,9]. In the newly discovered pathway, exogenous CO_2 and acetic acid produced by the glycolytic

* Corresponding author. Department of Civil and Mechanical Engineering, University of Cassino and Southern Lazio, Via Di Biasio, 43, 03043, Cassino, FR, Italy.
** Corresponding author.
E-mail addresses: nirakar.pradhan@gmail.com, npradhan@ntu.edu.sg (N. Pradhan), gdippolito@icb.cnr.it (G. d'Ippolito), ldipasquale@icb.cnr.it (L. Dipasquale), giovanni.esposito@unicas.it (G. Esposito), antonio.panico@unipegaso.it (A. Panico), p.lens@un-ihe.org (P.N.L. Lens), afontana@icb.cnr.it (A. Fontana).
[1] Current address: Singapore Centre for Environmental Life Sciences Engineering, Nanyang Technological University, 60 Nanyang Dr, 637551, Singapore.

https://doi.org/10.1016/j.biombioe.2019.04.007
Received 4 May 2018; Received in revised form 28 March 2019; Accepted 8 April 2019
Available online 13 April 2019
0961-9534/

fermentation of sugars are recycled to synthesize pyruvate and lactic acid [3,8,10]. This new metabolic pathway is an absolute novelty in the field of microbial fermentation and it provides robust process stability and reproducibility [6,11,12].

Lactic acid is an important industrial chemical of wide commercial use in the food, pharmaceutical, cosmetic and chemical sectors [13]. However, these applications are currently limited by its modest synthesis and lack of cost effective extraction and purification techniques [13,14]. A CLF-based process has promising facets to be considered in the large-scale production that are necessary to address the increasing demand of lactic acid. In fact, various kinds of carbohydrate rich organic wastes/substrates can be fermented efficiently and effectively as well as the process has potential to sequestrate exogenous CO_2 to produce lactic acid [8].

The main aim of this study was to identify how the culture parameters in terms of salinity level, buffering agent and carbon source affect H_2 and lactic acid synthesis under CLF conditions. For the experiments, we used *Thermotoga neapolitana cf capnolactica* (DSM 33003) [11], a proprietary mutant strain derived from *T. neapolitana* DSMZ 4359^T. Additionally, the effects of the best performing parameters were assessed for the CLF-based process by up-scaling from a 0.12 L micro fermenter (serum bottles) to a laboratory scale 3.0 L fermenter.

2. Materials and methods

2.1. Bacterial strain and culture medium

Unless otherwise specified, the experiments were carried out using *Thermotoga neapolitana cf capnolactica* (*Tn$_{cf}$*) (DSM 33003, a safe deposit with Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Germany), a recently described lab strain derived from *T. neapolitana* DSMZ 4359^T (Braunschweig, Germany) [11]. The bacterium was grown anaerobically in a standard culture medium (i.e. modified version of the ATCC 1977 culture medium) containing (g L^{-1}): NaCl 10.0; KCl 0.1; $MgCl_2.6H_2O$ 0.2; NH_4Cl 1.0; K_2HPO_4 0.3; KH_2PO_4 0.3; $CaCl_2.2H_2O$ 0.1; cysteine-HCl 1.0; yeast extract 2.0; tryptone 2.0; glucose 5.0; resazurin 0.001; 10.0 mL of filter-sterilized vitamins and trace element solutions (DSM medium 141) in 1.0 L distilled H_2O [4,9].

2.2. Experimental set-up

The effects of salinity level, buffering agent and carbon source on the H_2 yield and lactic acid synthesis by *Tn$_{cf}$* were investigated under CLF conditions [9] by varying one of these parameters while keeping the others constant. The culture medium was prepared according to the previously described methods [4]. All batch fermentation experiments were conducted in serum bottles with a working volume to headspace ratio maintained at 1:3. The culture medium was sparged with a stream of pure CO_2 gas for 3 min at 30 mL min^{-1} and then inoculated with the wet biomass (6%, *v* v^{-1}), previously washed twice in 10 g L^{-1} NaCl solution. The serum bottles were kept in the incubator at 80 °C without agitation for 72 h. The initial pH (t = 0 h) was corrected to 7.5 ± 0.1 by 1 M NaOH, except for the experiments assessing the effects of buffering agents and for the composite experiments when best performing parameters were combined. The successive pH corrections were carried out every 24 h unless stated otherwise. All batch fermentation experiments in serum bottles were triplicated.

2.2.1. Batch fermentation experiments

The effect of the salinity level was studied by varying NaCl concentrations from 0 to 35 g L^{-1} (i.e. 0, 5, 10, 20 and 35 g L^{-1}) in the standard culture medium with glucose as carbon source [4]. The effect of a buffering agent was investigated by using 0.01 M for each of the following chemicals: (a) diacid/monoacid phosphate; (b) 3-(*N*-morpholino) propanesulfonic acid (MOPS) (pH range = 6.4–7.8 with an optimal pH of 7.2 at 25 °C); (c) tris (hydroxymethyl) aminomethane (TRIS) (pH range = 7.0–9.0 with an optimal pH of 8.06 at 25 °C) and (d) 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH range = 6.8–8.2 with an optimal pH of 7.48 at 25 °C) in the standard culture medium without additionally supplementing the phosphate buffer. Additionally, two other sets of control experiments were conducted to understand the fermentability of glucose by *Tn$_{cf}$* in the absence of buffering agents. A set was sparged with only CO_2 and the other sparged with N_2 gas.

1. The effect of the carbon source was studied by using 5 g L^{-1} of arabinose, xylose, glucose, sucrose, laminarin and carboxymethyl cellulose (CMC) in the culture medium as done for the standard culture medium [4,15]. Two sets of composite experiments were conducted by combining the best performing culture parameters (i.e. salinity level, buffering agent and carbon source) in terms of H_2 and lactic acid synthesis in the serum bottles. The culture parameters selected for the composite experiments were: (a) 35 g L^{-1} NaCl, (b) 0.01 M phosphate buffer and (c) either 5 g L^{-1} (or 28 mM) of glucose or 5 g L^{-1} (or 33 mM) arabinose.

2.2.2. Scale-up experiment

The composite experiment with glucose was scaled up to a 3 L fermenter and the culture parameters were set as: (a) 35 g L^{-1} NaCl, (b) 0.01 M phosphate buffer and (c) 28 mM of glucose. The scale-up experiment was carried out in a jacketed 3 L reactor (Applikon Biotechnology, The Netherlands) containing 0.7 L culture medium and inoculated with the wet biomass (6%, *v* v^{-1}), previously washed twice in a 10 g L^{-1} NaCl solution. The mixture was sparged with a stream of pure CO_2 gas for 5 min at 30 mL min^{-1}. The initial pH of the fermentation mixture was adjusted to 7.5 by titrating with 1 M NaOH. The temperature was kept thermostatically constant at 80 ± 1 °C and the mixture was stirred at 50 rpm using an electro-magnetic stirring unit [10]. The fermentation was carried out for 24 h with samples taken at the beginning (t = 0 h) as well as at the end (t = 24 h) of the experiment. The experiment in the 3 L fermenter was conducted in triplicate.

2.3. Analytical methods

The gaseous metabolites (H_2 and CO_2) were measured by gas chromatography (Focus GC, Thermo Scientific) [9]. The biochemical analyses of water-soluble metabolites (i.e. acetic, lactic acid and alanine) were performed on the supernatants (previously centrifuged at 13,000 rpm for 5 min and stored at −20 °C) using a ^{1}H Nuclear Magnetic Resonance (NMR) 600 MHz spectrometer (Bruker Avance 600) without any processing of the samples [9]. Biomass growth was monitored through optical density measurements (λ = 540 nm) by a UV/Vis spectrophotometer (V-650, Jasco) [4,9]. Cell dry weight (CDW) was calculated according to the optical density and dry cell weight correlative equation for the *Tn$_{cf}$* bacterium [10].

The residual concentration of sugar was measured by the dinitrosalicylic acid (DNS) method calibrated on a standard solution of 1 g L^{-1} glucose for hexose and 1 g L^{-1} xylose for pentose [16]. The residual concentrations of di- and polysaccharides (i.e. sucrose, laminarin and CMC) were measured by the phenol/sulfuric acid method calibrated on a standard solution of 0.2 g L^{-1} of glucose [17]. The molar recovery of carbon and hydrogen after 72 h or 24 h fermentation was calculated as in agreement with previous methods [9]. The carbon recovery was calculated by considering initial and final concentrations of carbon contained in sugar, acetic acid, lactic acid, alanine and CO_2. Hydrogen recovery was calculated by considering initial and final concentrations of hydrogen contained in sugar, H_2, acetic acid, lactic acid and alanine.

Table 1
The effect of varying salinity (0–35 g L^{-1} NaCl) on CLF fermentation experiments supplemented with 28 mM glucose (72 h of incubation). The results are expressed as mean ± standard deviation (n = 3).

NaCl (g/L)	Glucose consumed (mM)	Acetic acid (mM)	Lactic acid (mM)	Alanine (mM)	Lac/Ac[a]	C-recovery (%)[b]	H-recovery (%)[c]
0	25.62 ± 0.07	20.66 ± 0.27	2.80 ± 0.26	1.28 ± 0.09	0.14 ± 0.01	48.32 ± 2.31	74.58 ± 1 5.28
5	26.00 ± 0.14	24.59 ± 0.95	6.23 ± 3.26	1.61 ± 0.58	0.25 ± 0.12	62.30 ± 10.23	77.98 ± 25.31
10	26.12 ± 0.16	26.05 ± 4.69	11.61 ± 2.42	2.46 ± 0.24	0.45 ± 0.02	76.87 ± 16.00	96.96 ± 27.89
20	25.96 ± 0.11	25.58 ± 1.03	13.44 ± 0.94	2.41 ± 0.09	0.53 ± 0.04	79.70 ± 5.21	104.16 ± 9.56
35	25.68 ± 0.25	23.22 ± 0.81	21.63 ± 6.15	2.38 ± 0.10	0.93 ± 0.29	91.86 ± 14.47	109.56 ± 20.79

[a] Lac/Ac = Lactic acid/Acetic acid ratio.
[b] C-recovery = carbon recovery.
[c] H-recovery = hydrogen recovery.

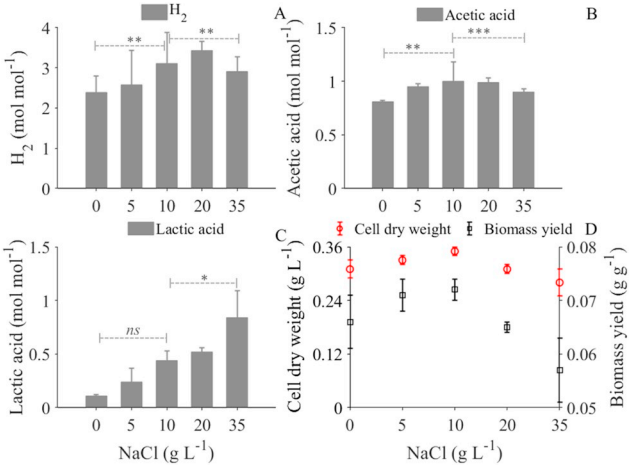

Fig. 1. The effect of salinity (0–35 g L^{-1} NaCl) on the yield (mol mol^{-1} of sugar) of (A) H_2, (B) acetic acid and (C) lactic acid and (D) cell dry weight and biomass yield (g g^{-1} of sugar) of *Tn*$_{cf}$ via the CLF pathway after 72 h of incubation. Error bar = standard deviation (n = 3). p-value was calculated and compared with experiments performed in standard culture medium with 10 g L^{-1} NaCl. *ns* = not significant; p value < 0.05 (*); p < 0.01 (**); p value < 0.001 (***).

3. Results

3.1. Effect of salinity

The effect of salinity on H_2 and organic acids (i.e. acetic and lactic acid) synthesis, amino acid synthesis (alanine), C and H recovery is reported in Table 1. Corresponding H_2, acetic acid, lactic acid and biomass yields are summarized in Fig. 1. The result showed that over 90% of the substrate was consumed within 72 h of incubation. H_2 synthesis increased by 43.5% when NaCl concentration increased from 0 to 20 g L^{-1} and decreased by 15% when NaCl concentration was further increased to 35 g L^{-1} (Fig. 1A). As evident from Fig. 1A and D, there was a clear link between the biomass yield and H_2 production. The highest biomass growth was observed at 10 g L^{-1} NaCl (i.e. standard culture medium) and the biomass growth decreased by 25% when subjected to 35 g L^{-1} NaCl in the culture medium.

The acetic acid synthesis showed a trend similar to that of H_2 synthesis and biomass growth with increase from 20.7 ± 0.3 mM with no NaCl to 26.1 ± 4.7 mM at 10 g L^{-1} NaCl (i.e. standard culture medium). In analogy with H_2 synthesis, acetic acid production decreased to 23.2 ± 0.8 mM at 35 g L^{-1} NaCl. However, this effect was associated to a remarkable boost (over 7.5 fold) in the lactic acid synthesis when NaCl concentration raised from 0 to 35 g L^{-1} NaCl, i.e. from 2.8 ± 0.3 mM to 21.6 ± 6.2 mM lactic acid (Fig. 1A).

These results are in good agreement with the production by *T. neapolitana* at 10 g L^{-1} NaCl [12]: 29.9 ± 1.3 mM acetic acid, 14.8 ± 0.8 mM lactic acid and 3.3 mol H_2 mol^{-1} of glucose. On the whole, the experiments with varying salinity 35 g L^{-1} NaCl suggested that the higher the salinity level, the better the fermentation performance with respect to H_2 and lactic acid synthesis, i.e. 2.91 ± 0.37 mol H_2 mol^{-1} of glucose and 21.6 ± 6.2 mM lactic acid. Interestingly, the experiments also revealed that a halophilic strain like *Tn*$_{cf}$ can degrade the organic substrate in the absence of NaCl in the culture medium.

3.2. Effect of buffering agent

The results of the effect of different buffering agents are presented in Table 2. On the whole, the H_2 yield ranged from 1.78 ± 0.29 to 3.27 ± 0.18 mol H_2 mol^{-1} of glucose. The H_2 synthesis was found to be the highest in MOPS buffer as shown in Fig. 2A. Biomass growth, substrate consumption and product formation were dependent on the buffering capacity of the culture medium. In the control experiment, the substrate was not completely consumed due to poor buffering capacity with an end point pH of 4.8. On the other hand the substrate was completely consumed in the well buffered experiments with a recorded end point pH of above 6.2 (Table 2).

The highest and lowest acetic acid synthesis were 26.8 ± 0.3 mM and 22.8 ± 0.4 mM in the TRIS buffer and control experiments, respectively. The highest value of lactic acid synthesis was 14.9 ± 0.3 mM with phosphate buffer and the lowest (11.3 ± 0.6 mM) for the control experiment (Table 2). The results from

Table 2
The effect of the buffering agent (0.01 M) on CLF fermentation experiments supplemented with 28 mM glucose (72 h of incubation). The results are expressed as mean ± standard deviation (n = 3).

Buffering agents	Glucose consumed (mM)	End point pH	Acetic acid (mM)	Lactic acid (mM)	Lac/Ac	C-recovery (%)	H-recovery (%)
Control[a]	18.54 ± 0.15	4.82 ± 0.19	22.76 ± 0.40	11.35 ± 0.62	0.50 ± 0.09	74.75 ± 15.11	83.50 ± 7.88
CO_2/HCO_3^-[b]	25.62 ± 0.10	6.20 ± 0.11	22.82 ± 0.84	14.63 ± 3.23	0.55 ± 0.12	70.33 ± 10.57	80.75 ± 20.68
Phosphate	26.17 ± 0.26	6.22 ± 0.08	24.70 ± 0.59	14.92 ± 0.25	0.60 ± 0.02	75.71 ± 3.82	90.84 ± 12.05
MOPS	26.42 ± 0.05	6.22 ± 0.06	26.65 ± 0.87	14.23 ± 0.22	0.53 ± 0.02	77.36 ± 4.65	98.38 ± 6.72
TRIS	25.55 ± 0.06	6.30 ± 0.04	26.77 ± 0.29	12.08 ± 0.89	0.45 ± 0.04	76.05 ± 6.56	94.19 ± 13.14
HEPES	25.99 ± 0.03	6.28 ± 0.05	25.56 ± 0.49	13.58 ± 0.88	0.53 ± 0.03	75.22 ± 3.63	89.92 ± 11.62

[a] Culture medium without buffering agent and sparged with N_2 instead of CO_2.
[b] Culture medium without buffering agent but sparged with CO_2.

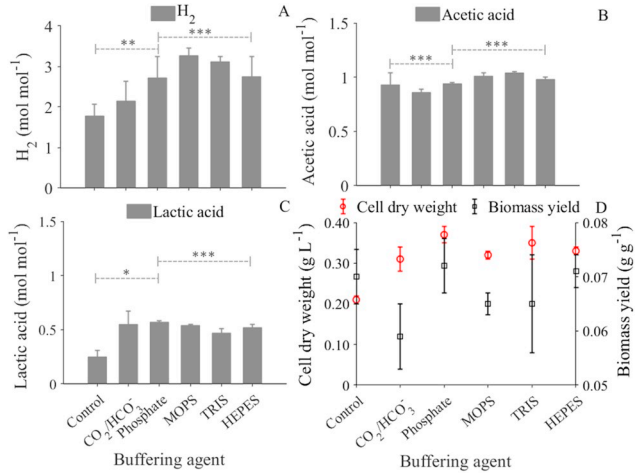

Fig. 2. Effect of buffering agent (0.01 M) on the yield (mol mol^{-1} of sugar) of (A) H_2, (B) acetic acid and (C) lactic acid and (D) cell dry weight and biomass yield (g g^{-1} of sugar) of *Tn*$_{cf}$ via the CLF pathway after 72 h of incubation. Error bar = standard deviation (n = 3). p-value was calculated and compared with experiments performed in standard culture medium with phosphate as a buffering agent. p value < 0.05 (*); p < 0.01 (**); p value < 0.001 (***).

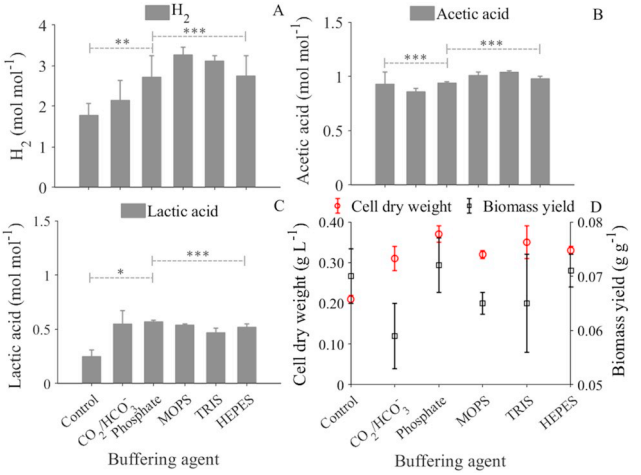

Fig. 3. Effect of carbon source (5 g L^{-1}) on the yield (mol mol^{-1} of sugar) of (A) H_2, (B) acetic acid and (C) lactic acid and (D) cell dry weight and biomass yield (g g^{-1} of sugar) of *Tn*$_{cf}$ via the CLF pathway after 72 h of incubation. Error bar = standard deviation (n = 3). CMC = Carboxymethyl cellulose. p-value was calculated and compared with experiments performed in standard culture medium with glucose as carbon source. *ns* = not significant; p value < 0.05 (*); p < 0.01 (**); p value < 0.001 (***).

the buffering agent experiments indicate that buffering agents along with CO_2 (or HCO_3^-) played a crucial role in maintaining the pH, thereby facilitating better substrate degradation.

3.3. Effect of carbon source

Tn$_{cf}$ can metabolize both simple and complex organic substrates to produce H_2 and organic acids [3]. The results of H_2 and organic acid synthesis from various types of carbon sources are presented in Table 3. In addition, H_2, acetic acid, lactic acid and biomass yield are reported in Fig. 3. H_2 yield under CLF conditions from pentose sugars like xylose and arabinose was 3.2 ± 0.1 and 2.8 ± 0.3 mol H_2 *per* mole of sugar, respectively. Similarly, the H_2 yield from glucose, sucrose and laminarin was, respectively, 3.34 ± 0.02 mol H_2 mol^{-1} of glucose, 2.56 ± 0.1 mol H_2 *per* mole glucose equivalent and 3.70 ± 0.17 mol H_2 *per* mole glucose equivalent. From CMC, the H_2 yield was 2.05 ± 0.13 mol H_2 mol^{-1} of glucose equivalent with only 10% of the substrate being consumed after 72 h of fermentation. The CMC fermentation with *Tn*$_{cf}$ is rather slow and not comparable with other simple sugars, indicating that it requires pretreatment of CMC in order to improve its fermentability [18].

The lactic acid synthesis was found to be significantly higher with glucose and sucrose as the carbon source, i.e. 14.8 ± 0.3 and 17.0 ± 1.3 mM, respectively, compared to the other sugars (Table 3). The lactic acid to acetic acid ratio (Lac/Ac) for both arabinose and glucose amounted to > 0.47 ± 0.01, whereas the highest Lac/Ac ratio (0.7 ± 0.1) was observed for sucrose as the carbon source. From the experiments with various carbon sources, it is evident that both glucose and arabinose performed best with respect to H_2 yield and lactic acid synthesis. Hence, both glucose and arabinose were selected for further investigation by combining the best performing parameters with respect to salinity, buffer and carbon source.

3.4. Composite experiments

A salinity level of 35 g L^{-1} NaCl in 0.01 M of phosphate buffer and either 5 g L^{-1} of glucose (28 mM) or arabinose (33 mM) as carbon source were selected for the composite experiments. The results of the composite experiments are presented in Table 4. About 80% of the substrate was consumed after 72 h of incubation. The H_2 yields from arabinose and glucose were 2.99 ± 0.10 mol H_2 *per* mole of arabinose and 3.08 ± 0.27 mol H_2 mol^{-1} of glucose, respectively. The lactic acid synthesis from arabinose and glucose fermentation were 0.22 ± 0.10 and 0.54 ± 0.02 mol *per* mole of sugar, respectively. The composite experiments showed that a higher quantity of lactic acid was synthesized with glucose as the carbon source compared to arabinose under similar operating conditions.

3.5. Process scale-up

To validate the performance of the composite experiment, the process was scaled up from 0.12 L serum bottles to a 3 L fermenter containing 35 g L^{-1} NaCl, 0.01 M phosphate buffer and 5 g L^{-1} (or 28 mM) glucose in the culture medium (Table 4). The performance of the composite experiment in the 3 L fermenter was further improved with over 90% glucose consumed in 24 h of fermentation compared to about 80% in the 0.12 L serum bottles after 72 h of incubation. The H_2 yield

Table 3
The effect of the different carbon source on CLF fermentation experiments supplemented with 5 g L^{-1} of carbon source. The results are expressed as mean ± standard deviation (n = 3).

Carbon sources	Sugar consumed (mM)	Acetic acid (mM)	Lactic acid (mM)	Alanine (mM)	Lac/Ac	C-recovery (%)	H-recovery (%)
Xylose	29.57 ± 0.13	26.28 ± 0.32	3.79 ± 0.23	2.48 ± 0.13	0.14 ± 0.01	66.02 ± 4.62	88.91 ± 10.05
Arabinose	30.51 ± 0.11	23.08 ± 0.33	10.94 ± 0.43	2.55 ± 0.07	0.47 ± 0.03	71.91 ± 2.91	91.53 ± 8.78
Glucose	26.30 ± 0.01	30.34 ± 0.09	14.79 ± 0.26	2.64 ± 0.12	0.49 ± 0.01	90.81 ± 1.99	107.47 ± 4.14
Sucrose	23.30 ± 0.69	25.12 ± 1.43	16.95 ± 1.34	3.15 ± 0.34	0.68 ± 0.09	97.10 ± 7.76	136.52 ± 11.19
Laminarin	24.73 ± 0.40	28.75 ± 0.81	7.60 ± 0.27	2.11 ± 0.14	0.26 ± 0.01	77.79 ± 4.15	101.07 ± 6.40
CMC[a]	2.75 ± 0.25	3.40 ± 0.30	1.18 ± 0.05	1.27 ± 0.04	0.35 ± 0.04	106.48 ± 16.48	102.55 ± 17.31

[a] CMC = Carboxymethyl cellulose.

Table 4
Substrate consumption and major fermentation products via the CLF pathway by *Tn*$_{cf}$ with 35 g L^{-1} NaCl and 0.01 M phosphate buffer after 72 h of fermentation in serum bottles and 24 h of fermentation in the fermenter. The results are expressed as mean ± standard deviation (n = 3).

Carbon sources	Sugar consumed (mM)	H_2 (mol mol^{-1} sugar)	Acetic acid (mol mol^{-1} sugar)	Lactic acid (mol mol^{-1} sugar)	Lac/Ac	C-recovery (%)	H-recovery (%)
Arabinose[a]	23.38 ± 0.18	2.99 ± 0.10	0.68 ± 0.03	0.22 ± 0.10	0.32 ± 0.04	87.40 ± 15.97	97.18 ± 3.65
Glucose[a]	23.62 ± 0.36	3.08 ± 0.27	0.80 ± 0.02	0.54 ± 0.02	0.67 ± 0.01	79.21 ± 4.06	106.67 ± 9.53
Glucose[c]	25.09 ± 2.79	3.07 ± 0.23	0.90 ± 0.06	1.17 ± 0.21	1.32 ± 0.31	107.31 ± 31.01	135.08 ± 46.22

[a] Best performing culture parameter experiments in 0.12 L serum bottles; [b]Process scale-up experiments in a 3 L fermenter.

was 3.07 ± 0.23 mol H_2 mol^{-1} of glucose, which was comparable to that of the composite experiment conducted in the serum bottles (Table 4). The lactic acid synthesis was 29.4 ± 6.9 mM with a Lac/Ac ratio of 1.32 ± 0.31 compared to the Lac/Ac ratio of 0.67 ± 0.01 in the composite experiment with serum bottles. The scale-up experiment not only improved the overall fermentation efficiency, but also enhanced the lactic acid synthesis by 2.2 fold compared to the composite batch experiments in serum bottles under similar culture parameters.

4. Discussion

4.1. Best performing culture parameter for CLF by *Tn*$_{cf}$

4.1.1. Effect of salinity

This study showed that *Tn*$_{cf}$ has a great adaptability to a wide range of salinity levels (0–35 g L^{-1} NaCl) (Table 1) for simultaneous synthesis of H_2 and lactic acid under CLF conditions. The serial experiments showed that lactic acid synthesis increased by 7.5 fold when the NaCl concentration in the culture medium was increased from 0 to 35 g L^{-1}. Similarly, a recent study on H_2 producing *Vibrionaceae* showed that increasing salinity levels from 9 to 75 g L^{-1} of NaCl increased the lactic acid synthesis significantly [19]. We also showed that there is a direct correlation between lactic acid synthesis and salinity level (Fig. 1A), thus suggesting that increase of salinity level can induce an additional enhancement of the recycling of acetic acid to lactic acid under CLF conditions. It is possible that a sodium ion gradient potentially fuels ATP synthesis and transport process, thus driving the coupling of exergonic and endergonic reactions in the cell. High salt concentrations are also related to the bioenergetic balance within the cells and supports availability of reducing equivalents necessary for the lactic acid synthesis. An incomplete carbon and hydrogen recovery were observed as presented in Table 1, which could be attributed due to the exclusion of *Tn*$_{cf}$ biomass component, residual concentrations of inorganic carbon (HCO_3^- and CO_3^{2-}), the contributions from components present in the fermentation media and other minor byproducts of the process.

4.1.2. Effect of buffering agent

Experiments with 0.01 M of different buffering agents along with CO_2 sparging showed that H_2 and lactic acid synthesis were comparable and had no significant difference (Table 2). A past study on *Thermotoga* strains showed the effect of varying concentrations of different buffering agents and found that 0.1 M of HEPES was the best performing buffering agent under N_2 sparging atmosphere, yielding 1.6 ± 0.1 mol H_2 mol^{-1} of glucose and 1.1 ± 0.1 mol acetic acid mol^{-1} of glucose [20]. In another study, 0.05 M HEPES was found to be sufficient in maintaining the buffering capacity of the culture medium and produced 2.7 ± 0.1 mol H_2 mol^{-1} of glycerol consumed under N_2 sparging atmosphere [21]. In a sharp contrast, Table 2 showed that only 0.01 M of buffers (either of phosphate or MOPS or TRIS or HEPES) under CLF conditions provided better results in terms of both H_2 and lactic acid synthesis by *Tn*$_{cf}$.

The buffering agent along with dissolved CO_2 in the form of HCO_3^- played a major role in maintaining the pH of the culture medium (pH ~ 6.5), which ensured complete substrate degradation and desired byproduct formation. However, the application of carbon based buffering agents like MOPS, TRIS and HEPES are expensive and not recommended for large-scale applications. Therefore, the buffering capacity of the culture medium can be maintained by using a non-carbon based buffer like phosphate along with bicarbonate (due to dissolved CO_2). The phosphate buffer along with dissolved CO_2 was found to be a suitable combination in maintaining the desired buffering capacity of the CLF fermentation process [10,22].

4.1.3. Effect of carbon source

2. Ability of *T. neapolitana* DSMZ 4359^T (wild-type strain) to ferment different carbon sources under N_2 sparging atmosphere has been previously investigated [3]. The reported H_2 yields from xylose, arabinose, glucose, sucrose, laminarin and CMC under N_2 sparging atmosphere were quite similar to the results obtained under CO_2 sparging atmosphere by *Tn*$_{cf}$ fermentation in the present study (Table 3) [5,10,18,23]. The ability of *Tn*$_{cf}$ to utilize pentose sugar as the carbon source opens a new prospective for handling agriculture-based waste (e.g. cellulosic feedstocks) where xylose is one of the key sugars found upon hydrolysis [18]. The present study with CO_2 sparged culture also showed that Lac/Ac ratio for glucose as carbon source exceeds 0.49 (Table 3). This represents a significant change in organic acid production since N_2 sparged cultures under similar experimental conditions gave Lac/Ac ratios of only 0.2 [4] and 0.3 [10].

4.2. Process scale-up with best performing culture parameters

According to the conventional dark fermentation model, the lactic acid synthesis is always associated with a metabolic shift due to changes in the optimal operating environmental conditions (e.g. pH, partial pressure of gases, organic loading rate and hydraulic retention time), indicating that the culture was not adapted to the new environmental conditions [24]. It has been reported that accumulation of 5–10 mM of H_2 in the gas phase of the culture medium initiated a metabolic shift towards lactic acid synthesis for the extreme thermophile *Caldicellulosiruptor saccharolyticus* [25]. Another study reported that the dissolved H_2 in the culture medium and the osmotic pressure determined a metabolic shift towards lactic acid synthesis [26]. Although the energy yield and the redox potential range of *Tn*$_{cf}$ cells under CLF conditions are not fully understood, the results of the present study clearly showed that both CO_2 in the form of HCO_3^- and Na^+ ions in the culture medium were responsible to trigger unconventional lactic acid synthesis without affecting the H_2 yield.

The process scale-up experiments in the 3 L fermenter improved both lactic acid production and enhanced the overall fermentation efficiency possibly due to better mixing, buffering and mass transfer compared to the experiments in 0.12 L serum bottles [10,12]. In addition, higher hydrogen recovery as fermentation end product suggests that CO_2 sparging also stimulated another metabolic process or utilization of non-sugar substrates in the fermentation medium [4,9]. However, further studies are required to understand why acetic acid is not fully recycled by the CLF mechanism.

5. Conclusions

This study was designed to evaluate the performance of the CLF

process using Tn_{cf} strain by varying salinity, buffering agents and bioenergy relevant carbon sources as a function of H_2 and lactic acid synthesis. The experiments were conducted by varying NaCl concentrations in the range of 0–35 g L^{-1} and with mono-, di- and polysaccharides as a carbon source as well as with different buffering agents. The results showed that increasing the NaCl concentration (0–35 g L^{-1}) has a positive impact on the CLF process where the lactic acid synthesis increased by 7.5 fold without significantly affecting the overall H_2 yield. Nonetheless, we found that the CLF process is particularly suitable for simple sugars as carbon source. The process scale-up experiment with the best performing conditions showed a further improvement in lactic acid synthesis in comparison with results in serum bottles. This study showed a novel approach for handling and treating carbohydrate rich organic substrates, such as high salinity wastewaters and organic solid wastes for faster recovery of fermentation products (i.e. H_2 and lactic acid) compared to the conventional anaerobic digestion process.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgements

This work was funded by the Italian Ministry of Education, University and Research (MIUR) through the projects: “Sfruttamento Integrato di Biomasse Algali in Filiera Energetica di Qualità (SIBAFEQ)” (PON01_02740); the European Union’s Horizon 2020 Research and Innovation Programme Project BioRECO2VER under grant agreement No. 760431 and the Erasmus Mundus Joint Doctorate program ETeCoS^3 (Environmental Technologies for Contaminated Solids, Soils and Sediments) under EU grant agreement FPA No. 2010-0009. The authors are thankful to Miss Nunzia Esercizio for her contribution in processing and depositing the strain *Thermotoga neapolitana cf capnolactica* (DSM 33003) to Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Germany.

References

[1] B. Johnston, M.C. Mayo, A. Khare, Hydrogen: the energy source for the 21st century, Technovation 25 (2005) 569–585.
[2] K. Nath, D. Das, Improvement of fermentative hydrogen production: various approaches, Appl. Microbiol. Biotechnol. 65 (2004) 520–529.
[3] N. Pradhan, L. Dipasquale, G. d'Ippolito, A. Panico, P.N. Lens, G. Esposito, A. Fontana, Hydrogen production by the thermophilic bacterium *Thermotoga neapolitana*, Int. J. Mol. Sci. 16 (2015) 12578–12600.
[4] G. d'Ippolito, L. Dipasquale, F.M. Vella, I. Romano, A. Gambacorta, A. Cutignano, A. Fontana, Hydrogen metabolism in the extreme thermophile *Thermotoga neapolitana*, Int. J. Hydrogen Energy 35 (2010) 2290–2295.
[5] M.A. Basile, C. Carfagna, P. Cerruti, G. Gomez d'Ayala, A. Fontana, A. Gambacorta, M. Malinconico, L. Dipasquale, Continuous hydrogen production by immobilized cultures of *Thermotoga neapolitana* on an acrylic hydrogel with pH-buffering properties, RSC Adv. 2 (2012) 3611–3614.
[6] L. Dipasquale, N. Pradhan, G. d'Ippolito, A. Fontana, Potential of hydrogen fermentative pathways in marine thermophilic bacteria: dark fermentation and capnophilic lactic fermentation in *Thermotoga* and *Pseudothermotoga* Species, in: P.H. Rampelotto, A. Trincone (Eds.), Grand Challenges in Marine Biotechnology, Springer, Cham, Switzerland, 2018.
[7] M.E. Nissilä, C.H. Lay, J.A. Puhakka, Dark fermentative hydrogen production from lignocellulosic hydrolyzates - a review, Biomass Bioenergy 67 (2014) 145–159.
[8] G. d'Ippolito, L. Dipasquale, A. Fontana, Recycling of carbon dioxide and acetate as lactic acid by the hydrogen-producing bacterium *Thermotoga neapolitana*, ChemSusChem 7 (2014) 2678–2683.
[9] L. Dipasquale, G. d'Ippolito, A. Fontana, Capnophilic lactic fermentation and hydrogen synthesis by *Thermotoga neapolitana*: an unexpected deviation from the dark fermentation model, Int. J. Hydrogen Energy 39 (2014) 4857–4862.
[10] N. Pradhan, L. Dipasquale, G. d'Ippolito, A. Fontana, A. Panico, P.N.L. Lens, F. Pirozzi, G. Esposito, Kinetic modeling of fermentative hydrogen production by *Thermotoga neapolitana*, Int. J. Hydrogen Energy 41 (2016) 4931–4940.
[11] N. Pradhan, L. Dipasquale, G. d'Ippolito, A. Panico, P.N.L. Lens, G. Esposito, A. Fontana, Hydrogen and lactic acid synthesis by the wild-type and a laboratory strain of the hyperthermophilic bacterium *Thermotoga neapolitana* DSMZ 4359 T under capnophilic lactic fermentation conditions, Int. J. Hydrogen Energy 42 (2017) 16023–16030.
[12] N. Pradhan, L. Dipasquale, G. d'Ippolito, A. Fontana, A. Panico, F. Pirozzi, P.N.L. Lens, G. Esposito, Model development and experimental validation of capnophilic lactic fermentation and hydrogen synthesis by *Thermotoga neapolitana*, Water Res. 99 (2016) 225–234.
[13] H.G. Joglekar, I. Rahman, S. Babu, B.D. Kulkarni, A. Joshi, Comparative assessment of downstream processing options for lactic acid, Separ. Purif. Technol. 52 (2006) 1–17.
[14] N. Pradhan, E. Rene, P. Lens, L. Dipasquale, G. D'Ippolito, A. Fontana, A. Panico, G. Esposito, Adsorption behaviour of lactic acid on granular activated carbon and anionic resins: thermodynamics, isotherms and kinetic studies, Energies 10 (2017) 665.
[15] L. Dipasquale, G. d'Ippolito, C. Gallo, F.M. Vella, A. Gambacorta, G. Picariello, A. Fontana, Hydrogen production by the thermophilic eubacterium *Thermotoga neapolitana* from storage polysaccharides of the carbon dioxide fixing diatom *Thalassiosira weissflogii*, Int. J. Hydrogen Energy 37 (2012) 12250–12257.
[16] P. Bernfeld, Alpha and beta-amylases, Methods Enzymol. 1 (1955) 149–158.
[17] M. Dubois, K.A. Gilles, J.K. Hamilton, P.A. Rebers, F. Smith, Colorimetric method for determination of sugars and related substances, Anal. Chem. 28 (1956) 350–356.
[18] T.A.D. Nguyen, J.P. Kim, M.S. Kim, Y.K. Oh, S.J. Sim, Optimization of hydrogen production by hyperthermophilic eubacteria, *Thermotoga maritima* and *Thermotoga neapolitana* in batch fermentation, Int. J. Hydrogen Energy 33 (2008) 1483–1488.
[19] M. Pierra, E. Trably, J.J. Godon, N. Bernet, Fermentative hydrogen production under moderate halophilic conditions, Int. J. Hydrogen Energy 39 (2014) 7508–7517.
[20] M. Cappelletti, G. Bucchi, J.D.S. Mendes, A. Alberini, S. Fedi, L. Bertin, D. Frascari, Biohydrogen production from glucose, molasses and cheese whey by suspended and attached cells of four hyperthermophilic *Thermotoga* strains, J. Chem. Technol. Biotechnol. 87 (2012) 1291–1301.
[21] T.A. Ngo, M.S. Kim, S.J. Sim, High-yield biohydrogen production from biodiesel manufacturing waste by *Thermotoga neapolitana*, Int. J. Hydrogen Energy 36 (2011) 5836–5842.
[22] L. Dipasquale, A. Adessi, G. d'Ippolito, F. Rossi, A. Fontana, R. De Philippis, Introducing capnophilic lactic fermentation in a combined dark-photo fermentation process: a route to unparalleled hydrogen yields, Appl. Microbiol. Biotechnol. 99 (2015) 1001–1010.
[23] N.T. Eriksen, M.L. Riis, N.K. Holm, N. Iversen, Hydrogen synthesis from pentoses and biomass in *Thermotoga* spp, Biotechnol. Lett. 33 (2011) 293–300.
[24] M.F. Temudo, R. Kleerebezem, M. van Loosdrecht, Influence of the pH on (open) mixed culture fermentation of glucose: a chemostat study, Biotechnol. Bioeng 98 (2007) 69–79.
[25] E.W.J. van Niel, P.A.M. Claassen, A.J.M. Stams, Substrate and product inhibition of hydrogen production by the extreme thermophile, *Caldicellulosiruptor saccharolyticus*, Biotechnol. Bioeng. 81 (2003) 255–262.
[26] K. Willquist, S.S. Pawar, E.W.J. van Niel, Reassessment of hydrogen tolerance in *Caldicellulosiruptor saccharolyticus*, Microb. Cell Factories 10 (2011) 111–121.

Update

Biomass and Bioenergy
Volume 150, Issue , July 2021, Page

DOI: https://doi.org/10.1016/j.biombioe.2021.105999

Biomass and Bioenergy 150 (2021) 105999

Contents lists available at ScienceDirect

Biomass and Bioenergy

journal homepage: http://www.elsevier.com/locate/biombioe

Corrigendum to "Simultaneous synthesis of lactic acid and hydrogen from sugars via capnophilic lactic fermentation by *Thermotoga neapolitana cf capnolactica*" [Biomass and Bioenergy 125 (2019) 17–22]

Nirakar Pradhan [a,b,*,1], Giuliana d'Ippolito [a,**], Laura Dipasquale [a], Giovanni Esposito [b], Antonio Panico [c], Piet N.L. Lens [d], Angelo Fontana [a]

[a] National Research Council of Italy, Institute of Biomolecular Chemistry, Via Campi Flegrei 34, 80078, Pozzuoli, Napoli, Italy
[b] Department of Civil and Mechanical Engineering, University of Cassino and Southern Lazio, Via Di Biasio, 43, 03043, Cassino, FR, Italy
[c] Telematic University Pegaso, Piazza Trieste e Trento, 48, 80132, Naples, Italy
[d] UNESCO-IHE Delft, Westvest 7, 2611AX, Delft, the Netherlands

The authors regret Fig. 3, the actual figure was inadvertently repeated as that of Fig. 2. The correct figure has been printed below.

The authors would like to apologise for any inconvenience caused.

DOI of original article: https://doi.org/10.1016/j.biombioe.2019.04.007.

* Corresponding author. Department of Civil and Mechanical Engineering, University of Cassino and Southern Lazio, Via Di Biasio, 43, 03043, Cassino, FR, Italy. ,
** Corresponding author.
E-mail addresses: nirakar.pradhan@gmail.com, npradhan@ntu.edu.sg (N. Pradhan), gdippolito@icb.cnr.it (Giuliana d'Ippolito), ldipasquale@icb.cnr.it (L. Dipasquale), giovanni.esposito@unicas.it (G. Esposito), antonio.panico@unipegaso.it (A. Panico), p.lens@un-ihe.org (P.N.L. Lens), afontana@icb.cnr.it (A. Fontana).
[1] Current address: Singapore Centre for Environmental Life Sciences Engineering, Nanyang Technological University, 60 Nanyang Dr, 637551, Singapore.

https://doi.org/10.1016/j.biombioe.2021.105999

Available online 15 February 2021

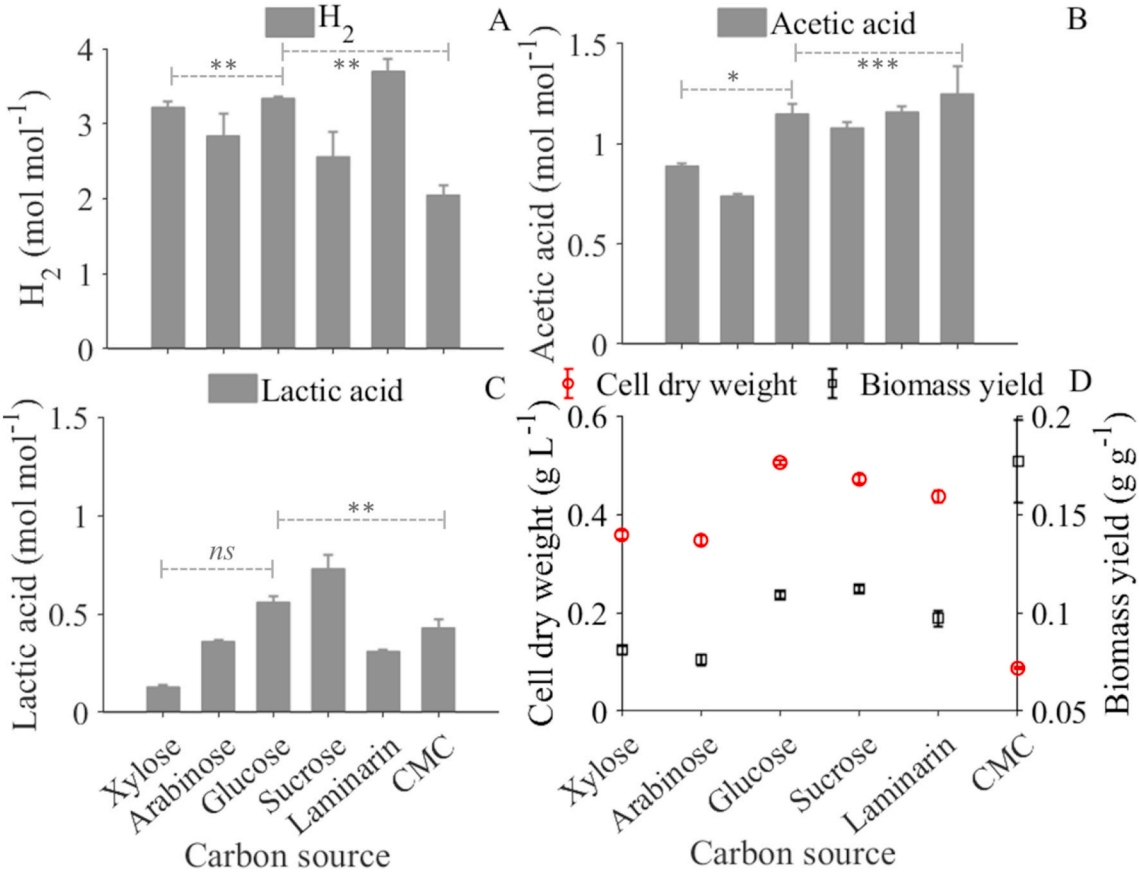

Fig. 3. Effect of carbon source (5 g L^{-1}) on the yield (mol mol^{-1} of sugar) of (A) H_2, (B) acetic acid and (C) lactic acid and (D) cell dry weight and biomass yield (g g^{-1} of sugar) of *Tn*$_{cf}$ via the CLF pathway after 72 h of incubation. Error bar = standard deviation ($n = 3$). CMC = Carboxymethyl cellulose. *p*-value was calculated and compared with experiments performed in standard culture medium with glucose as carbon source. *ns* = not significant; p value < 0.05 (*); $p < 0.01$ (**); p value < 0.001 (***).