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Green Hydrogen exploiting Capnophilic Lactic Fermentation pathway in the anaerobic hyperthermophilic bacterium *Thermotoga neapolitana*

Experimental degree thesis

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Summary

1 Abstract

In recent years, both national and international politics switched their focus on researching and developing, sustainable fuels production processes in opposition to the fossil fuel-based ones.

Among them, special interest has been shown towards hydrogen, which, as the only fuel not chemically bound to carbon, has been considered an extremely promising source of energy.

In this field fall the research carried out on *Thermotoga neapolitana*, chosen as model species for biohydrogen production due to its versatility in various conditions and the ability to ferment different organic substrates.

The experiments performed aimed to assess the biological production of hydrogen, which can be performed by *T. neapolitana* through two different metabolic pathways: dark fermentation and Capnophilic (CO2-requiring) Lactic Fermentation (CLF), which involve the absorption of the insufflated CO_2 into the growth medium.

Both processes proceed through the formation of acetic acid and lactic acid as by-products, but, during the CLF, when the carbon dioxide gets absorbed into the liquid medium, a coupling with acetyl CoA occurs with consequent formation of lactic acid (possessing a way higher commercial value) instead of acetic acid, while maintaining unaltered the hydrogen production.

The analyses were carried out on two different sets of samples prepared by saturating the environment through insufflation of either CO_2 (CLF) or N_2 (dark), to properly compare the performance of the microorganism depending on which fermentation pathway was adopted.

Fundamental, and possibly inhibiting, parameters involved in the process, like pH, temperature, and agitation, were monitored at regular time intervals; particular attention was given to determine the variability of hydrogen and acids upon changes in temperature.

The entire experimental activity necessary to write this thesis was carried out in the laboratories of the Biomolecular Chemistry Institute of CNR (National Research Council) in Pozzuoli (Naples).

The results have shown the microorganism's intrinsic ability to successfully perform both capnophilic lactic and dark fermentation over a wide range of different temperatures.

The process led to promising production of hydrogen and lactic acid, both compounds with high commercial value.

2 Introduction

2.1 Global warming and Climate change

Over the past several decades, the impact of human life on our planet has continuously increased, in particular due to the exponential increase in global population.

Ever since the beginning of industrial revolution (around 1760), the combustion of fossil fuels to sustain humankind needs has only grown until present, ultimately resulting in the enhancement of greenhouse gases production (mainly CO_2).

Carbon dioxide, as every other greenhouse gas, is characterized by its transparency to UV radiation but, in turn, is able to trap IR radiation (which is a form of heat energy) emitted from the surface of the Earth; absorbing this kind of radiation, greenhouse gases are able to cause an increase in the average temperature of our planet, causing a phenomenon known as global warming.

Global warming is, in turn, responsible of drastic changes in the planet’s climate (climate change) that is accompanied by an increase in both frequence and intensity of dramatic events like droughts and heat waves, as well as abiotic stress like flooding, salinity and freezing (Zandalinas, Fritschi, and Mittler 2021).

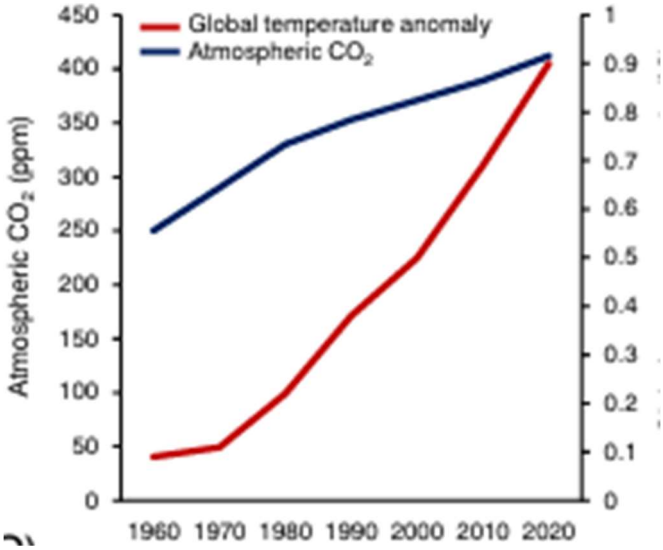

Figure 2.1: proportional increase in Atmospheric CO2 (ppm) and temperature from 1960 to 2020 (Zandalinas, Fritschi, and Mittler 2021).

2.2 Renewable sources of energy

The development of new technologies, especially in the industrial field, coupled with population expansion has caused an exponential increase in fossil fuels usage for energy production purposes, ultimately causing two extremely disruptive phenomena for the planet: global warming and climate change.

As a consequence, researchers all around the world started looking for various methods to restrain fossil fuels usage and contribution to climate change; three are the main strategies:

- enhancing the efficiency of conventional power conversion devices and systems through waste and heat recovery
- developing efficient energy conversion devices resulting friendly towards the environment, such as fuel cells
- shift toward renewable energies that comes from nature and have minimal negative effects on the environment

Among the renewable sources of energy most widely researched in recent years, the more successfully developed processes involve the use of solar radiation, wind and biomasses.

2.2.1 Solar energy

Solar energy is one of the sustainable alternatives developed in recent years, particularly promising mainly due to its worldwide availability, it has already been tested for various application such as residential, desalination, transportation, drying and irrigation.

In this regard, several research has been done to develop processes able to fill for some of these applications: among them, Concentrated Solar Power (CSP) technologies are one of the many promising technologies for generating both heat and electricity.

From a commercial point of view, consistent investments have been globally made and, since 2005, various systems have been installed for different purposes; the main issue is that, nowadays, the achieved development is still not sufficient to make CSP into a reliable and low-cos power source.

Other technologies developed to make use of solar radiation are called solar collectors: line focus, which focuses the solar energy along a collector's focal length (used for parabolic through collectors and linear Fresnel reflectors), and point focus, which focuses the sun on a specific point and is applied in thermal towers and parabolic dishes (Sayed et al. 2023).

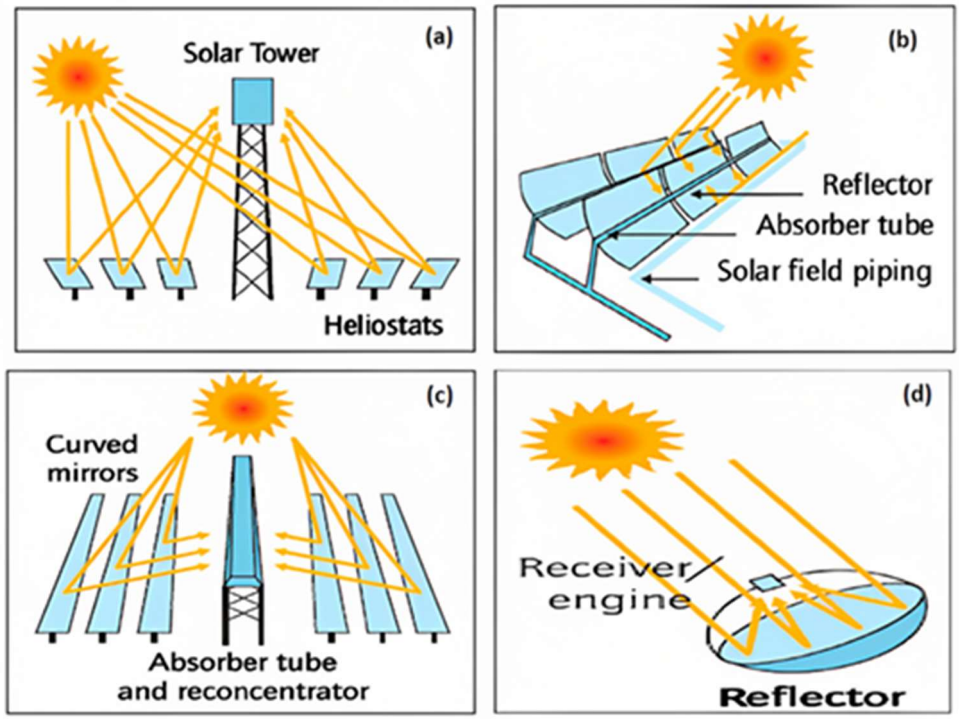

Figure 2.2: Recently available CSP technologies (a) solar thermal tower;(b) parabolic through collector; (c) linear Fresnel reflectors; (d) parabolic dish (Sayed et al. 2023).

2.2.2 Wind energy

Wind energy production is based on the conversion of wind's potential energy by a wind turbine either directly into mechanical energy or indirectly into electrical energy, which can then be used in various contexts.

At the beginning of the 20th century, the construction of the first wind turbine designed to generate electrical power began, and, since then, turbine technologies have been steadily advanced: refinements of its components coupled with advancement of the generators efficiency have allowed to achieve significant improvements in power production.

The main components of a wind turbine are its tower, blades, and the nacelle, which houses the generator, gears, and control system.

Wind turbines are divided into two different class: vertical axis wind turbines (VAWT) and horizontal axis wind turbines (HAWT), which dominate the majority of commercial field due to their major productions and efficiencies (Sayed et al. 2023).

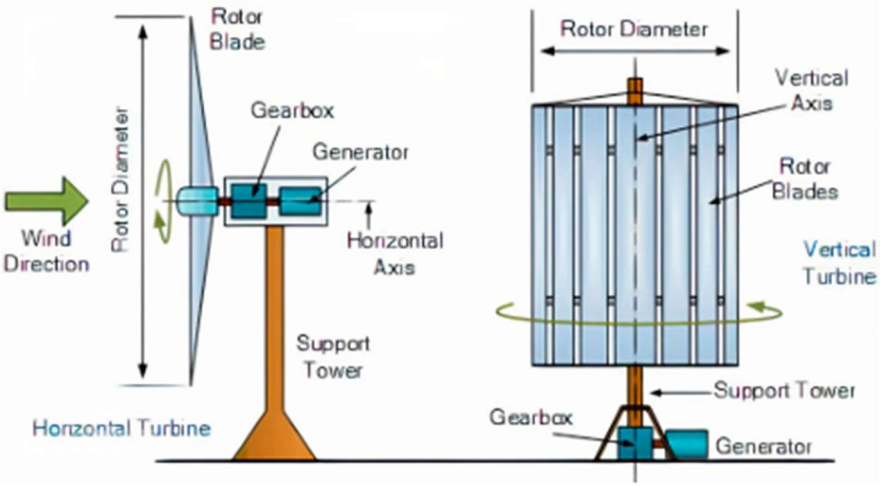

Figure 2.3: Wind turbine types VAWT ad HAWT (Sayed et al. 2023).

2.2.3 Biomasses

Biomasses are one of the most worldwide available energy forms: they are, in fact, abundantly produced in the form of waste in the agricultural and food industries.

The energy production from this raw material is involved in several applications, from the conventional burning, which doesn't allow to retrieve high value energy from the biomasses (thermal energy is, in fact, considered a low value energy in terms of conversion), to th direct use in fuel cells to generate electrical energy.

An especially promising application consists in their conversion into biodiesel and biochar, which opens the horizons for different new applications (Sayed et al. 2023).

2.3 Hydrogen

Among the different renewable energies recently developed, of outmost interest has been hydrogen firstly because, upon energy production, the only generated product would be water, which makes it an extremely clean source of energy.

Another attractive property of this gas is its energy density, of 140 MJ/kg, which is more than two times high than typical solid fuels (Shiva Kumar and Himabindu 2019).

The main production routes involve water electrolysis and recovery from fossil fuels treatments.

2.3.1 Water electrolysis

Electrolysis of water is one of the cleanest production processes for hydrogen as not only it uses renewable water as source, producing only pure oxygen as byproduct, but the energy needed to power up the system is provided by direct current power produced using other renewable energy sources like solar, wind or biomass.

In this process the reactant molecule, which is water, gets dissociated into pure hydrogen and oxygen under the effect of electric energy.

Four different types of water electrolysis can be classified based on their electrolyte, operating conditions, and ionic agents (OH^-, H^+ or O^{2-}) into:

- Alkaline water electrolysis (AWE)
- Solid oxide electrolysis (SOE)
- Microbial electrolysis cells (MEC)
- PEM water electrolysis, based on proton exchange membranes composed of polysulfonates

At present, unfortunately, only 4% of the global production of hydrogen is produced through this technology mainly due to its high costs (Shiva Kumar and Himabindu 2019).

2.3.2 Fossil fuels-based hydrogen

Hydrogen can be successfully produced from fossil fuels treatment processes; the most widely diffused are breakdown of the long-chained hydrocarbon through gasification, reforming, or pyrolysis, during these processes the main product consists of a mixture of hydrogen and carbon monoxide which has to be treated for a successful separation of the desire hydrogen.

Another well-established method to produce hydrogen consists in fossil fuels gasification, in particular coal gasification (Osman et al. 2022).

2.4 Biohydrogen production in *Thermotoga neapolitana*

Among the many processes developed to produce the cleaner possible hydrogen at industrial level, anaerobic digestion of organic material is regarded as a potential method for production from biomasses: hydrogen produced through this process is potentially competitive over both conventional processes and alternative biological methods (like photofermentation and photobiolysis).

Besides the use of monomeric carbohydrates, like glucose, *T.neapolitana* has been shown able to perform the fermentation also on more complex polymers like starch and cellulose, but, what is even more interesting lays in the ability of the microorganism to use also organic waste and agro-industrial matrices as carbonaceous substrates for the fermentation: this carries the possibility to produce clean energy, with good yields, while reduce wastes and greenhouse gases emissions(Pradhan et al. 2015).

2.4.1 Bacterial taxonomy

Originally isolated from shallow submarine hot spring near Lucrino, in the Bay of Naples, in 1986 *T. neapolitana* is a rod-shaped, not sporulating and Gram-negative bacterium belonging to the order *Thermotogales*.

It's a hyperthermophile bacterium, which is able to grow in a temperature range from 55°C to 90°C, with an optimal growth temperature of 80°C.

This bacterial order is characterized by a peculiar outer envelope called toga, that forms a large periplasmic space at the poles of each rod: although these regions might be involved in the formation of multicellular rods, the physiological role of this periplasmic space is still unknown.

Many studies agree on placing the order of *Thermotogales* among the deepest branches of bacteria, in terms of phylogenetic position, and so as main candidates for evolutionary studies.

An interesting characteristic is represented by their ability to synthesize many polysaccharide hydrolases, some of which are even exposed to the cell surface, that allow the adsorption of many matrices as source of carbon(Pradhan et al. 2015).

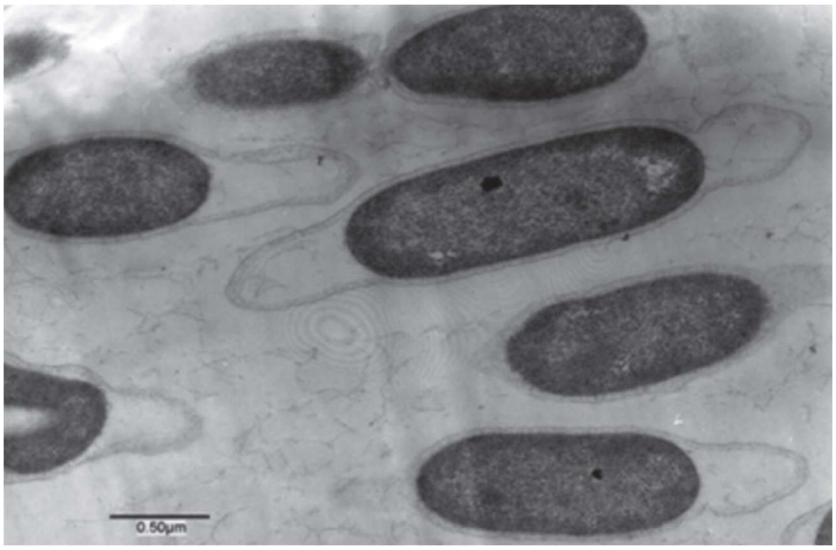

Figure 2.1: Scanning electron micrograph of *Thermotoga neapolitana* (Xu and d'Ippolito 2023).

2.4.2 Thermotoga sp. strain RQ7

Thermotoga sp. strain RQ7 was isolated from sediments of Ribeira Quente (Azores) and was gifted by Dr. Harald Huber at the University of Regensburg in Germany (Esercizio et al. 2022).

The strain is a member of the genus *Thermotoga* and belongs to the order *Thermotogales*: based on the 16S rRNA gene sequences, the closest relative of *T. sp. strain RQ7* is *T. neapolitana* (DSM 4359).

Equally to *T. neapolitana*, this bacterium is a hyperthermophile, strict anaerobe with optimal growth temperature at 80°C, possess rod-shaped cells, about 0.5 to 2 µm in length and 0.4-0.5 and possess a toga µm in diameter, and possess a toga, extension of their outer membrane, that pops out from both ends of the rod.

This bacterial strand is able to grow in both rich and defined media, are free living and not pathogenic, and are able to ferment both simple and complex sugars to produce hydrogen (Xu et al. 2017).

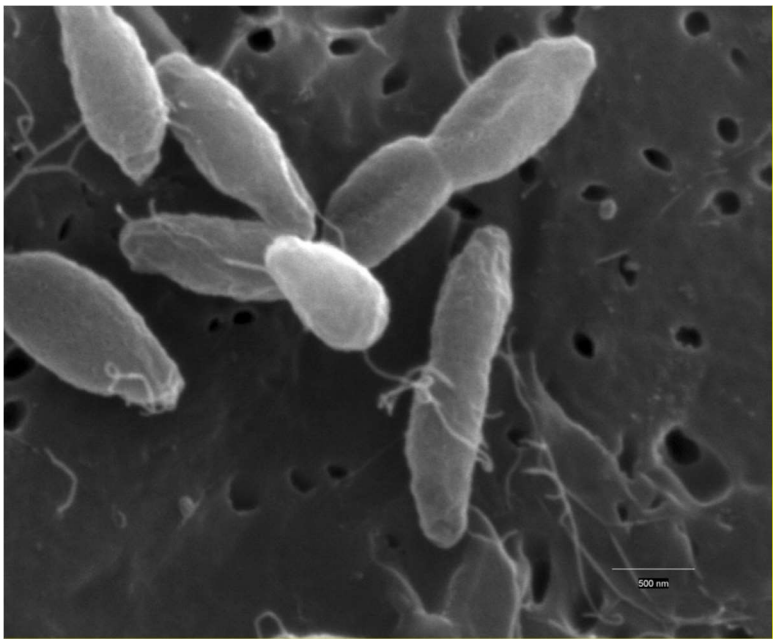

Figure 2.2: Scanning electron micrograph of *T. sp. strain RQ7* cells after 12 h of growth. Bar, 0.5 µm (Xu et al. 2017).

2.4.3 Dark fermentation pathway

The biological production of hydrogen in *Thermotoga neapolitana* occurs through a respiration process that uses protons (H^+) as electron acceptors, this process is coupled with the catabolism of carbohydrates, as *T. neapolitana* harvests energy mainly through glycolysis via the Embden-Meyerhoff pathway (EMP).

During EMP glucose or other hexoses are oxidized to supply energy (ATP), reducing equivalents (NADH) and pyruvate, which can either undergo a final oxidation to acetate or can be used for biosynthesis of cellular building blocks (for example of AcetylCoA).

The classical model proposed for the fermentation is called Dark Fermentation and a maximum of 4 moles can be theoretically produced per mole of glucose consumed, this limit is known with the name Thauer limit and represents the higher yield obtainable through a sugar-based fermentation performed by thermophilic bacteria.

Since hydrogen production represents a mean to dispose electrons, a direct relationship between the yield in terms of biogas and the type of organic products that will be released during the fermentation exists.

The yield can be optimized only when all reducing agent and electrons are fully consumed to produce the energy (when all the glucose is converted into acetate).

$$C_6H_{12}O_6 + 4ADP + 4Pi \longleftrightarrow 2\ CH_3CO_2H + 2CO_2 + 4H_2 + 4ATP + 2H_2O$$

On the contrary, in a redox neutral process, no synthesis of hydrogen occurs if the organic product released in the growth medium will be lactic acid:

$$C_6H_{12}O_6 + 2ADP + 2Pi \longleftrightarrow 2\ CH_3CH(OH)CO_2H + 2ATP + 2H_2O$$

The production of acetate is driven by the formation of additional ATP molecules, but, when hydrogen starts accumulating in the environment and the consumption of NADH stops, the pyruvate is directed towards the synthesis of other organic molecules.

In this way, mainly lactate production, catalysed by lactate dehydrogenase, takes place: lactate amounts that could rival the acetate yields are produced, alongside small quantities of alanine and ethanol.

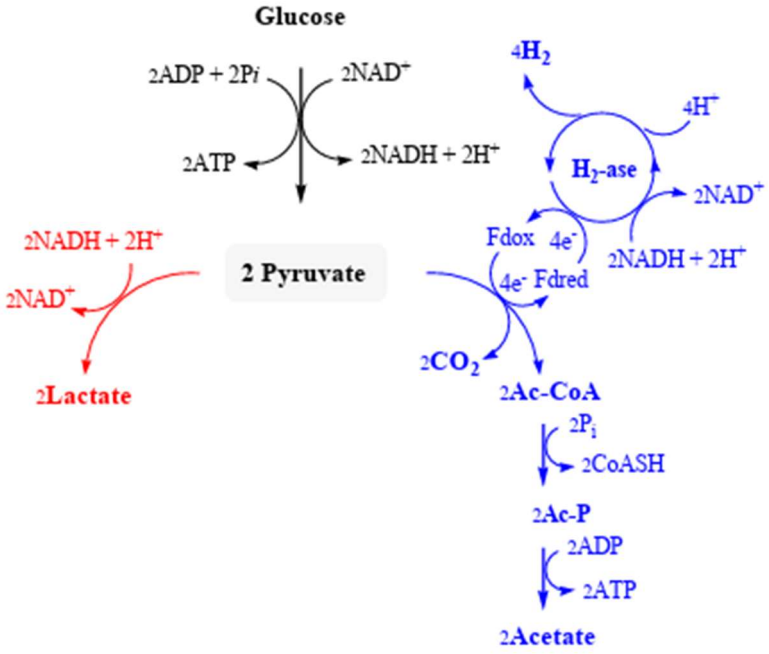

Figure 2.3: Streamlined biochemical pathway for H2 production by dark fermentation, adapted from Reference (Esercizio et al. 2022). Water is omitted for simplicity (Pradhan et al. 2015).

The high yields achieved by *T.neapolitana* are most likely due to the unique characteristic belonging to the heterotrimeric[FeFe]-hydrogenase that is responsible, as a hydrogenase, for the reduction of the protons to H_2.

Sequence analysis on the three proteins that form the hydrogenase (H2ase) , suggests that the β subunit is a flavoprotein that accepts electrons from NADH, and the γ subunit transfers electrons from the β subunit to the catalytic α subunit.

The catalytic site, also called H cluster, possesses the most complex Fe-S structure that was ever characterized and requires the assembling of three highly conserved proteins; despite the wide knowledge regarding the active site, how the endergonic production of hydrogen might be possible remains unknown for different reasons.

In fact, the reduction of the hydrogenase by the NADH is an energetically unfavourable reaction influenced by environmental conditions (e.g. pH), cellular growth and hydrogen partial pressure; for this reason, in many other thermophilic bacteria, the transfer of electrons to proton ion by the hydrogenase requires the presence of NADH-Ferredoxin oxidoreductase (NFOR).

On the other hand, for this reaction it's suggested that the NADH, formed during the carbon metabolism, performs the reduction of the oxidized ferredoxin (Fd), which, in turn, transfers the acquired electrons to the hydrogenase to form molecular hydrogen (Pradhan et al. 2015).

2.4.4 Capnophilic Lactic fermentation pathway

T. neapolitana, has been subjected to different studies to assess the biological production of hydrogen because of its ability to generate H_2 yields near the theoretical maximum corresponding to the Thauer limit (4 mol H_2/mol glucose).

As previously shown (Figure 2.3), this result should be achieved only if all the NADH from glucose oxidation is consumed to reduce protons to molecular hydrogen, but, in practice part of the reducing equivalents are also used to synthesize other fermentation products (specifically, lactic acid).

As a consequence, both the hydrogen yields and the low production of biomass during the fermentation process, suggested that the available pyruvate is only partially employed in other metabolic transformations under standard operating conditions.

The use of CO_2 as gas sparging (the inflow of gases is the most widely applied method to remove both oxygen and hydrogen form bacterial cultures in closed reactors) has produced a significant increase in the rate of both glucose consumption and hydrogen synthesis, but paradoxically also stimulated the production of lactic acid (Dipasquale, D'Ippolito, and Fontana 2014; Pradhan, Dipasquale, d'Ippolito, et al. 2016; Pradhan, Dipasquale, D'Ippolito, et al. 2016).

By performing experiments feeding labelled precursors, it was possible to prove that at least part of exogenous carbon dioxide is biologically coupled with acetyl-CoA to give lactic acid when the cultures are stripped by CO_2 gas or enriched in sodium bicarbonate: the process either recycles glycolysis-derived acetyl-CoA or employs exogenous acetate with ATP consumption.

In this latter case the overall outcome is a conversion of equimolar concentration of acetate and carbon dioxide into lactic acid, according to the equation:

$$CH_3CO_2H+CO_2+4H^++4e^- \longleftrightarrow CH_3CH(OH)CO_2H+H_2O$$

The fermentative CO_2-dependent synthesis of lactic acid and hydrogen was named Capnophilic Lactic Fermentation (CLF), and it pushed forward the possibility to fully convert sugar to lactic acid (or other reduced derivatives of pyruvate) without affecting hydrogen synthesis by means of an additional consumption of reducing equivalents deriving from other cellular processes (Pradhan et al. 2017).

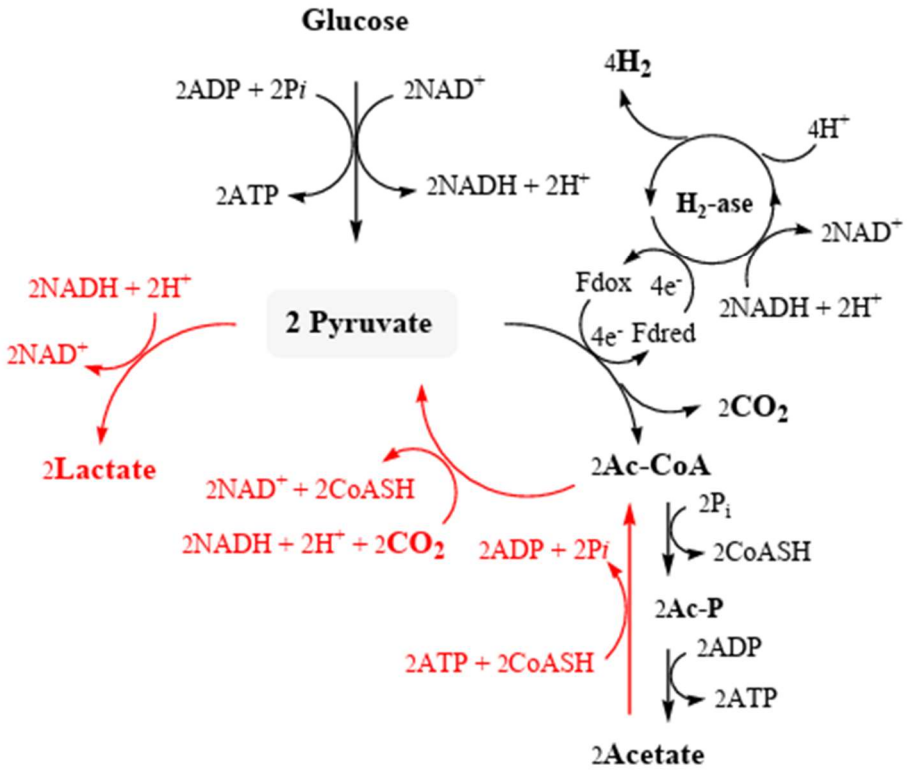

Figure 2.4: Proposed model of Capnophilic Lactic fermentation (CLF). Water is omitted for simplicity (Pradhan et al. 2015).

The key enzyme of the process is a Pyruvate Synthase (also named Pyruvate Oxido-Reductase) that utilizes reduced ferredoxin as source of electrons, as the reductive carboxylation of Acetyl-CoA most likely require the pool of Ferredoxin, also necessary for hydrogen production.

The Capnophilic Lactic Fermentation is ab example of CO_2 sequestration by coupling with an exogenous substrate, like glucose or acetate, but this mechanism isn't related to the autotrophic fixation of carbon dioxide (like other anaerobes perform); in fact, during this metabolic pathway CO_2 isn't converted into the final products but is, instead, completely released after the fixation in lactic acid (Pradhan et al. 2015).

3 Aim of the thesis

The aim of the following thesis was to assess the ability of *Thermotoga sp. strand RQ7* to perform the Capnophilic (CO2 requiring) Lactic Fermentation over a wide range of temperatures: 65°C, 70°C, 80°C and 85°C.

The experimental analysis was performed by setting the replicates under two different conditions through saturation of the system by insufflating sealed serum bottles with 120 ml of volume either with CO_2 or N_2.

The setting for the analyses was chosen in order to be able to compare the CLF yields in *Thermotoga neapolitana*, in terms of both hydrogen and organic acids production (mainly lactic acid was considered), to the classic Dark Fermentation pathway performed under nitrogen atmosphere.

The interest directed toward the Capnophilic Lactic Fermentation arises from the possibility to obtain, at the end of the process, together with the principal product (H_2) a higher amount of a second commercially valuable product, the lactic acid, in place of acetic acid, without hindering the production of hydrogen.

4 Material and methods

Anaerobic cultures of *Thermotoga sp. strain RQ7* were used as target organism for the production of pure hydrogen in laboratory trough fermentation of glucose performed in a batch system.

This microorganism was first isolated from marine sediments of Ribeira Quente (Azores) and was gifted by Dr. Harald Huber at the University of Regensburg in Germany.

4.1 Medium preparation and growth conditions

The protocol for the medium preparation was set up according to Hungate (1969) and appropriately modified to be applied to the use of flasks (Miller and Wolin, 1974; Van Ooteghem, Beer, and Yue 2002).

Thermotoga RQ7 was grown under anaerobic conditions at a temperature T=80°C with a pH=8 in a standard medium called TN (d'Ippolito et al. 2010).

The medium was prepared under aerobic conditions respecting the composition reported in Table 1.

Table 4.1: Growth medium composition

Compound	**Concentration (g/L)**
NaCl	10
KCl	0.1
MgCl2 6 H_2O	0.2
NH_4Cl	1
$CaCl_2$ 2 H_2O	0.1
K_2HPO4	0.3
KH_2PO4	0.3
Cisteine	0.5
Yeast extract	2
Glucose	5
Tryptone	2
Trace element solution	10 ml/L
Vitamin solution	10 ml/L
Resazurin	0.5 ml/L

The medium was supplemented with 10 ml/l of both filter sterilized trace element and vitamin solutions (Table 4.2 and 4.3).

Table 4.2: Trace element solution composition

Compound	**Concentration (g/l)**
Nitrilotriacetic acid	1.5
$MgSO_4$ 7H_2O	3
$MnSO_4$ 2H_2O	0.5
NaCl	1
$FeSO_4$ 7H_2O	0.1
$CoSO_4$ 7H_2O	0.18

$CaCl_2\,2H_2O$	0.1
$ZnSO_4\,7H_2O$	0.18
$CuSO_4\,5H_2O$	0.01
$KAl(SO_4)_2\,12H_2O$	0.02
H_3BO_3	0.01
$Na_2MoO_42H_2O$	0.01
$NiCl_26H_2O$	0.025
$Na2SeO_35H_2O$	0.3 mg

Table 4.3: Vitamin Solution composition

Compound	**Concentration (mg/L)**
Pyridoxine-HCl	10
Riboflavin	5
Nicotinic Acid	5
D-Ca-pantothenate	5
p-aminobenzoic acid	5
Thiamine-HCl $2H_2O$	5
Lipoic acid	5
Biotin	2
Folic acid	2
Vitamin B_{12}	0.1

To the medium were also added 0.5 ml per Liter of medium, of resazurin solution, a redox indicator to indicate the absence of oxygen in the medium, which turns the colour from a blue-purple shade to a pale yellow one under anaerobic conditions.

The pH was adjusted to 8 using a 2M aqueous solution of NaOH and the complete medium was then aliquoted in serum bottles with a volume of 120 ml (30 ml of solution in each of them): the ratio between headspace volume and culture volume was accurately calculated and set to 1:3, in order to narrow the inhibition due to the accumulation of the produced hydrogen in the headspace of the bottles.

Figure 4.1: aliquoted standard medium in serum bottles with 120 ml volume.

The oxygen is then removed by boiling the medium until the solution turn to a pale yellow colour: the generation of aqueous vapour, creates a pressure inside the bottle which pushes the air out of the system.

Figure 4.2: the medium solution turns to a pale yellow while boiling, going through a gradually lighter pink shade.

The bottles were immediately sealed using plugs made with butyl rubber and aluminium rings in order to maintain the anaerobic conditions and sterilized in autoclave at 110°C for 10 minutes.

4.2 Generation of inocula

During the inoculation phase, a bottle containing cells in exponential phase (usually after about 24 hours of growth at 80°C), stored in the fridge at 4°C, is retrieved.

After rising to room temperature, 5 ml for each flask are drawn from this bottle and inoculated in others (usually 4-5 in order to have it continuously available for other experiments).

Cells were grown in oven at 80°C for 24 hours and one of them will be the reactivated inoculum for the experiments.

4.3 Experiments at different temperatures

For the batch growth the medium was divided into aliquots of 30 ml into flasks with 120 ml of volume.

The analysis was carried out using pure glucose, which was used by the cells as carbonaceous matrix substrate for the fermentation process during which the hydrogen (H_2) is produced.

The oxygen was removed by boiling the medium until the colour of the solution turn from the typical blue (due to the presence of resazurin) to a light yellow; the bottles where lastly sealed and sterilized in autoclave at 110°C for 5 minutes.

Serum bottles (120 ml total volume) containing 30 ml of complete medium were inoculated with 2 ml (6% v/v) of bacterial culture and, once they reached 80°C, insufflated for 3 minutes with carbon dioxide (CO_2) or nitrogen (N_2), to properly compare the behaviour of the microorganisms under the two different conditions.

The pH of the bottles, insufflated with CO2 get a value around 6.3; pH was adjusted to 7,5-8 using a 2M aqueous solution of NaOH and incubated in heater at 80°C for 24 hours.

The serum bottles were incubated in heater at different temperatures for 48 hours: 65°C, 70°C, 80°C, 85°C. All the experiments were done in 6 replicates for N2 insufflation and 6 replicates for CO2 insufflation, at each temperature.

At time 0h, 24h and 48 h, 2ml of culture and 500 µl of the headspace gas (only at 24 and 48 h) have been sampled for each bottle.

At time 24h, due to the production of acids, the values dropped to about 6.3 and 4.8 for the samples under CO_2 and N_2, respectively; the value was thus adjusted back to 7.5-8 using NaOH 2M. Before pH correction, new N_2 or CO_2 insufflation have been done to remove the remaining H_2 in the headspace of the bottle.

4.4 Measured parameters during the culture growth

For each experiment the following parameters were measured:

- Cellular growth by spectrophotometry: Abs540nm (aliquoted sample before centrifugation)
- pH
- Quantitative and qualitative measure of the produced gases in the flask's headspace
- Concentration of the fermentable sugar in the supernatant (after centrifugation)
- Concentration of the organic acids in the supernatant (after centrifugation)

4.4.1 pH measure and bacterial growth determination

The pH was measured at room temperature using a pH-meter (pHM210, meterlab) under not automatic conditions.

The cellular growth was determined through optical density (OP) using an UV/Vis spectrophotometer (V-650, Jasco) at wavelength λ=540 nm; the absorbance was measured both before and after centrifugation to evaluate the cells net growth (the supernatant was then retrieved in order to determine the remaining fermentation parameters).

The following procedure was followed:

- set to zero the spectrophotometer using distilled water

- read the absorbance of a 1ml aliquot of the culture
- centrifugate the sample at 13000 rpm (into a 2ml Eppendorf) at 4°C for 5 minutes
- retrieve the supernatant and read its absorbance

4.4.2 Quantitative and qualitative measure of produced gases

The main gases produced were measured by drawing 500 µL of gaseous sample from the headspace of serum bottles with a 500 µl sealed syringe (SGE syringe, Trajan).

Figure 4.3: sealed syringe for gas analysis.

The gas was injected into a gas chromatograph (Trace 1600, Thermo Scientific); the instrument is equipped with a thermal conductivity detector (TCD) at 200°C and fitted with a 3 m molecular sieve column (80/100 Hayesep Q) together with an injector at 50°C; nitrogen with 5,0 purity is used as inert gas carrier with a 20 ml/min flow.

The hydrogen, main product of interest, was assessed using a calibration line made by injecting different volumes of pure hydrogen into the gas chromatograph.

µl H_2 injected	**H_2 peak area**
0.050	2.0103
0.075	3.3017
0.100	4.545

0.150	6.6593
0.200	9.7136
0.250	11.8255
0.300	15.3474
0.350	17.7711
0.400	21.2806
0.450	23.8244
0.500	26.8178

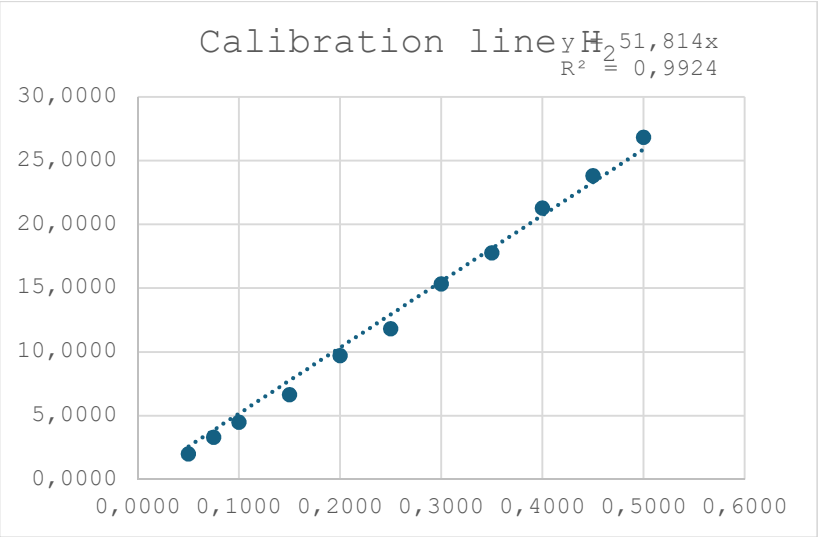

Figure 4.4: calibration line made injecting different volumes of pure hydrogen into the gas chromatography (3 measures for each volume, the line was obtained using the average).

4.4.3 Glucose concentration measurement

The supernatant was recovered by centrifugation at 13000 rpm at 4°C for 5 minutes is then analysed to determine the concentration of equivalent using Bernfeld colorimetric method also called dinitrosalicylic acid method.

The protocol followed for the assessment is:

- Reagent solution preparation

For a 100 ml volume of solution: 1 g of 3,5- dinitrosalicylic acid are dissolved into 20 ml of NaOH 2M and 50 ml of distilled water, 30 g o $KNaC_4H_4O_6$, which is called Rochelle Salt, are then added and the solution is brought at 100 ml of volume with distilled water.

The solution results photochemically degradable, thus, it has to be preserved by wrapping aluminium foil around the bottle.

- Sample preparation and calibration line: the samples are prepared as follows
  - White: 250 µl distilled H2O + 250 µl DNS solution
  - Samples containing glucose: x µl sample + y µl distilled H2O + 250 µl DNS solution (x + y= 250 µl): different samples volumes were used at different temperatures (Tabel 4)

Tabel 4.4: volumes of sample selected to perform DNS assay at 0h, 24h and 48h for different temperatures.

Temperaure	Insufflation	µl sample	µl distilled water
T0			
65°C	CO2	30	220
	N2	30	220
70°C	CO2	20	230
	N2	20	230
80°C	CO2	30	220
	N2	30	220
85°C	CO2	30	220
	N2	30	220
T24			
65°C	CO2	30	220
	N2	30	220
70°C	CO2	30	220
	N2	30	220
80°C	CO2	30	220
	N2	30	220
85°C	CO2	30	220
	N2	30	220
T48			
65°C	CO2	100	150
	N2	40	210
70°C	CO2	60	190
	N2	40	210
80°C	CO2	100	150
	N2	40	210
85°C	CO2	100	150
	N2	40	210

After this, all the samples are subjected to the same treatment: they are kept at 100°C for 5 minutes (hot bath containing boiling water), then transferred into ice to stop the thermically induced reaction (kept in for 2-3 minutes) and, lastly, 0.5 ml of water are added (making sure all the samples are well mixed to prevent uneven measures).

The calibration line is then determined by preparing the samples adding different volumes of a standard solution of glucose with concentration 1 mg/ml.

The measure is made using an UV/Vis spectrophotometer (V-650, Jasco) at wavelength λ=546nm.

µg glucose	Absorbance 546nm
50	0.1962
80	0.3348

100	0.4284
120	0.5422
150	0.7039
170	0.8198
200	0.9442

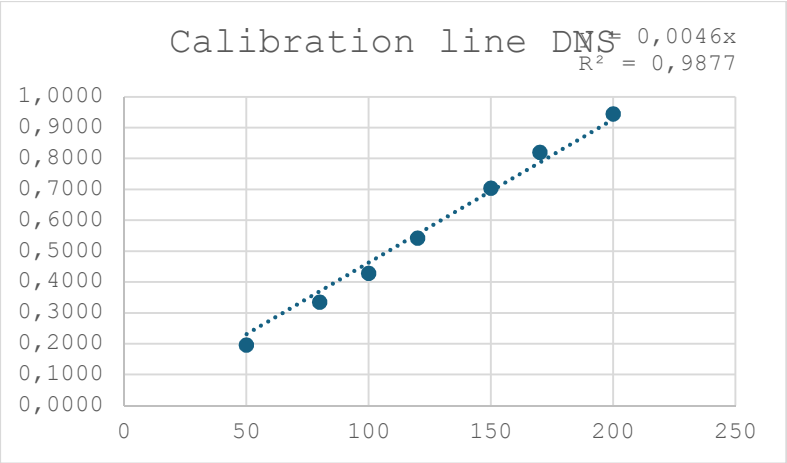

Figure 4.5: calibration line made selecting different volumes glucose solution 1 mg/ml (2 measures for each volume, the line was obtained using the average).

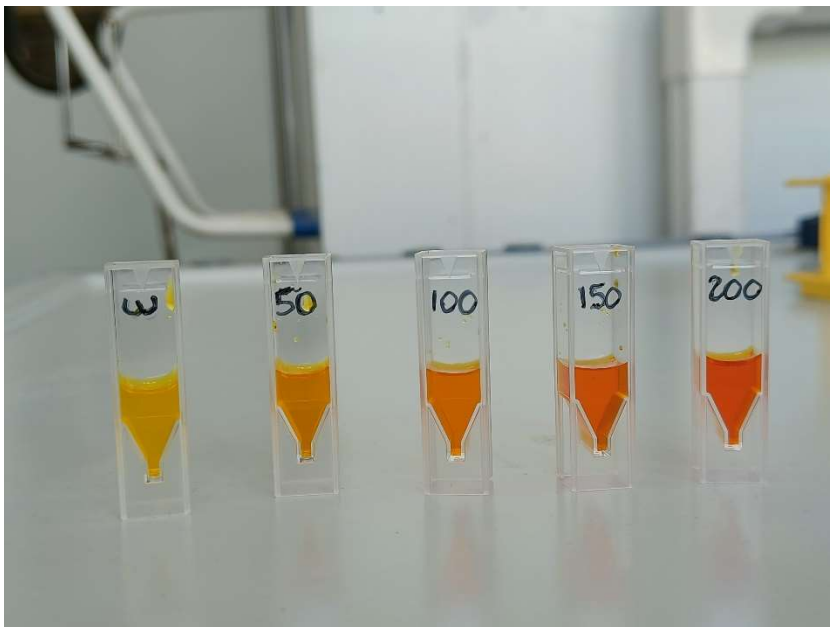

Figure 4.6: DNS assay for calibration line. Samples: 0 µl (white), 50 µl, 100 µl, 150 µl and 200 µl of glucose solution 1 mg/ml.

4.4.4 Determination concentration of organic acids

The quantitative and qualitative analysis of the fermentation products (acetic acid and lactic acid), was performed on culture supernatant, was performed by ^{1}H-NMR at 400 MHz of frequency (Bruker 400 MHz Prodigy, CryoProbe).

The samples are prepared by diluting 600 µl of medium with 100 µl of deuterium oxide (D_2O) solution containing 0.05% wt of 3-(trimethylsilyl) propionic-2,2,3,3-d_4 acid (TSP); this compound is used for the spectra calibration at 0 ppm.

NMR tubes treatment:

- Wash the tubes with water and soap and then with acetone, to remove all possible residues of soap, using a pipe cleaner
- Put all the clean tubes and caps under a vacuum bell to remove residues of acetone
- Write down identification codes in order to properly identify the samples (AF-xyz-1,2,…) on labels
- Prepare the samples into Eppendorf by diluting 600 µl of supernatant with 100 µl of deuterium oxide (D2O) solution
- Using a micropipette mix the samples, then transfer them into the tubes (previously remove from the bell) and seal the caps using parafilm preventing air input
- Tie the identification labels on each tube

Taking into consideration the relaxation times of the protons of the standards and those of the metabolites of interest the ^{1}H-NMR spectra are acquired using a single scan using the Eretic method.

To set a reference for the integration of the spectra for future experiments a 40 mM solution of both acetic and lactic acid was prepared, and the spectra was recorded; then, the spectra of progressive dilutions in water for both species separately were recorded.

The spectra belonging to the 20 mM dilution of lactic acid was set as reference for three main reasons, taking into consideration different concentrations it was found that:

- 20 mM is about the maximum concentration of lactic acid that can be produced through capnophilic lactic fermentation
- For a 10 mM concentration, even if the peak was well resolved for the aqueous solution, in the case of a fermentation sample it could easily be included into the background signals produced by other minor fermentation products

- A 40 mM concentration, on the other hand, is near saturation of the instrument and, thus, the signals aren't well resolved

To analyse the spectra and assess the concentration of organic acids inside the prepared samples, two signals were integrated: the singlet at 1.92 ppm, which correspond to the protons of methyl group of acetic acid and the doublet located at 1.33 ppm, which corresponds to the protons of methyl group of lactic acid.

4.5 ^{13}C labelled experiments

The aim of the analysis was to ascertain the amount of organic acids, and consequently of hydrogen, produced through the fermentation of organic matrices other than glucose, hypothetically contained into the yeast extract and/or tryptone added to the standard medium, and which presence is mandatory for the cellular growth (in fact, if the medium doesn't contain both components the cells can't grow and perform the fermentation).

The cultures were incubated at T=80 °C and the sample retrieving was performed as per usual right after gas sparging and after 24 and 48 hours of incubation; two replicates were prepared for both CO_2 and N_2.

The parameters and analysis carried out are:

- The bacterial growth was assessed measuring the absorbance at 540 nm
- The pH of the samples was measured using a pH-meter
- The concentration of fermentable reducing sugars was determined via colorimetric method using the dinitrosalicylic acid reactant solution
- The concentration of organic acids was assessed recording the spectra of the samples through ^{1}H-NMR

The main focus of the experiment lies in the replacement of the glucose normally used for all the other experiments (which contains only ^{12}C atoms) with a ^{13}C labelled glucose, in which all six carbon atoms are substituted.

The importance of this substrate is that, contrary to ^{12}C, ^{13}C can be detected during the analysis with Nuclear Magnetic Resonance: in fact, the signals recorded using not

substituted glucose are the result of the hydrogen atoms contained in the molecule of the two acids resonating exclusively with each other.

Using a ^{13}C labelled substrate, the hydrogen atoms will resonate but also with the carbon atoms to which they are bounded and, as a consequence, a change in the signals of the two atoms occurs: the singlet of the acetic acid at 1.92 ppm splits into a doublet, while the doublet of the lactic acid at 1.33 ppm splits into a quadruplet.

Operating with an organic substrate exclusively containing labelled carbon atoms means that every not splitted signal recorded during the analysis derived from the fermentation of organic matrices that weren't glucose.

5 Results and discussion

Thermotoga sp. strain RQ7 was used as model strain to study glucose consumption and conversion in fermentation products, such as hydrogen, acetic acid and lactic acid.

The fermentation process was studied at different temperatures (65°C, 70°C, 80°C, 85°C), under N_2 and CO_2 insufflation (Lanzilli et al. 2021).

5.1 Fermentation under static conditions

The aim of the analysis was to investigate the different production three fermentation products (hydrogen, acetic and lactic acids) deriving from glucose fermentation in Thermotoga sp. strain RQ7 after saturation of the culture headspace with carbon dioxide (CO_2) or nitrogen (N_2).

The experimental procedure for the preparation of the 120 ml flasks was:

1. Starting from 30 ml of aliquoted standard medium, inoculate 2 ml of bacterial culture at exponential growth phase
2. Insufflation of N_2 or CO_2 for 3 minutes; three replicates were prepared for each condition

3. pH regulation between 7.5-8
4. Incubation in oven at 80°C
5. The fermentation was monitored at different incubation times: right after inoculation (0 hours) and after 24 and 48 hours

In order to assess the fermentation processes, different parameters were taken into consideration at 0h, 24 h and 48h:

- Bacterial growth was measured through optical density variation using an UV/Vis spectrophotometer at λ=540 nm
- pH was detected at room temperature using a pH-meter; pH decreases with the production of acids and can become an inhibiting parameter
- Glucose consumption was assessed using the DNS colorimetric method at 546 nm of wavelength
- Hydrogen production, using a gas chromatograph by sampling 500 µl of gas in the headspace of the serum bottles
- Organic acids production through 400 MHz ^{1}H-NMR

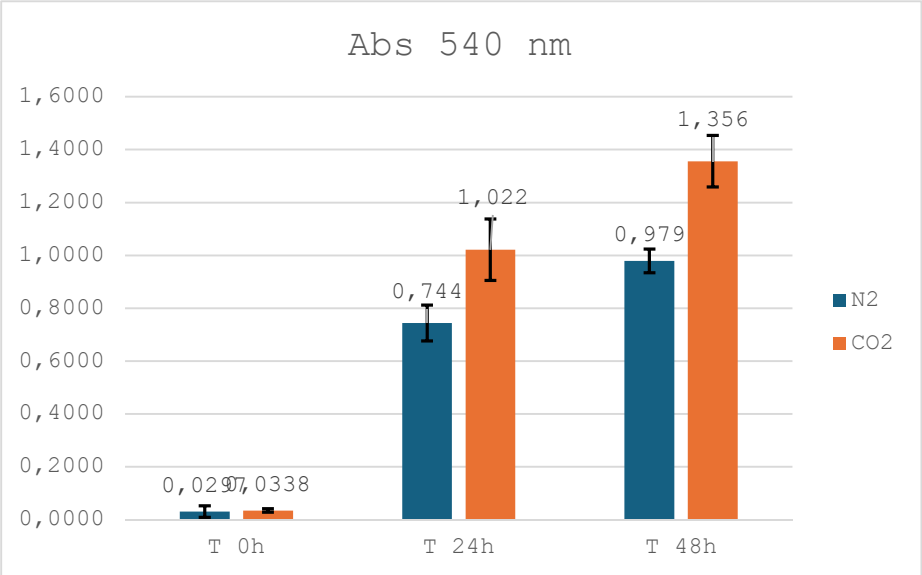

Figure 5.1: Measurements of optical density changes measured at 0h, 24h and 48 hours of fermentation.

At T_0 it's already possible to detected a slight change in absorbance at 540 nm due to the 2 ml inoculation.

From the cellular growth assessment, it's possible to appreciate the difference between the two conditions already after 24 hours of incubation, as the samples under carbon dioxide atmosphere exhibit a noticeably higher optical density.

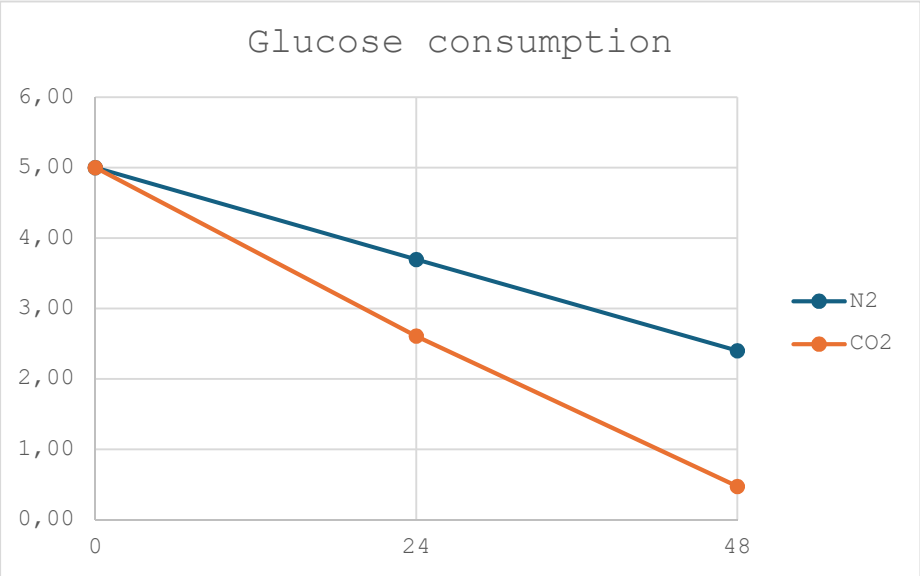

Figure 5.2: Glucose consumption (g/l) at 80°C.

This analysis shows a noticeably faster consumption of glucose in the samples under CO_2 atmosphere, which indicates a faster fermentation (Figure 2), as also supported by a major hydrogen production under CO_2 in comparison to N_2 (Figure 3).

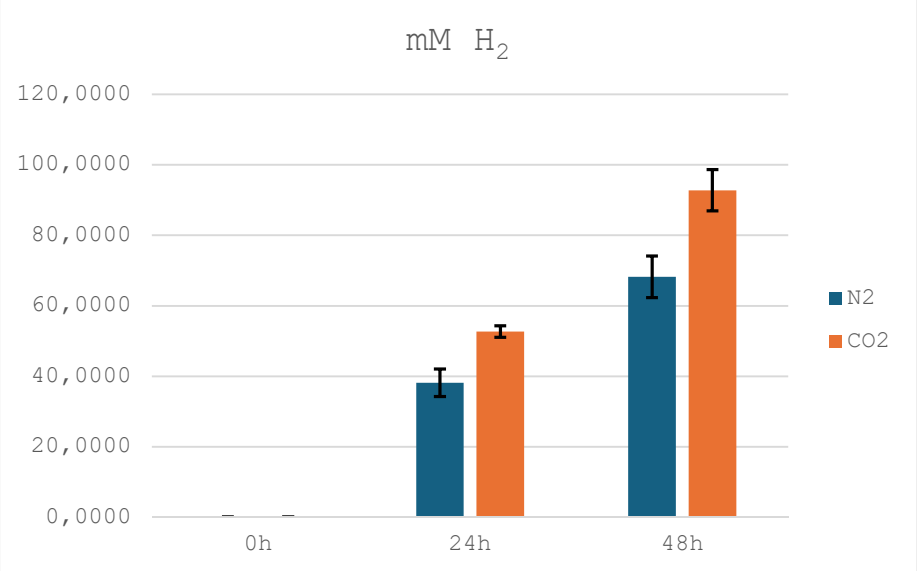

Figure 5.3: H_2 production (mmol/L culture) at 80°C. Data are expressed as mean ± SD, n=3 for N_2 and n=3 for CO_2.

Concentration of organic acids was assessed by ^{1}H-NMR on fermentation broth, by integrating diagnostic signals of the singlet at 1.92 ppm (methyl group of acetic acid) and the doublet at 1.33 ppm (methyl group of lactic acid) (Figure 6 and 7).

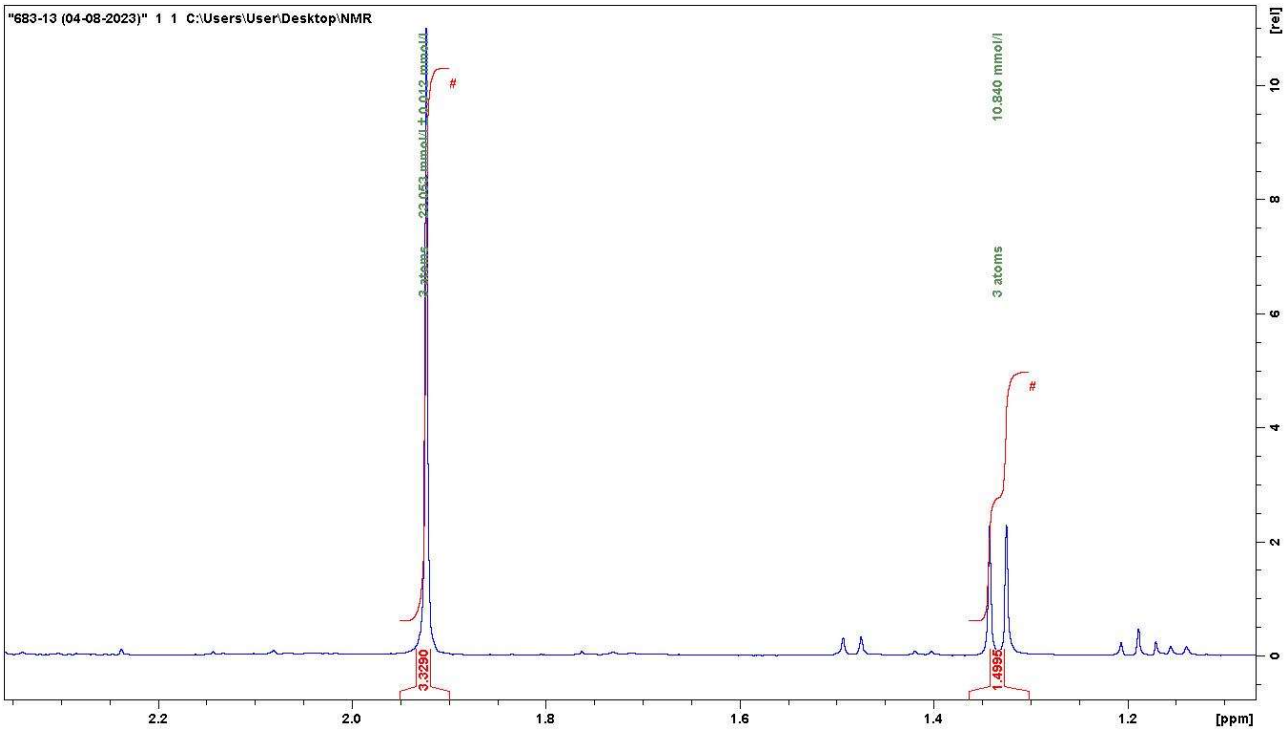

Figure 5.4: ^{1}H-NMR spectrum of medium of fermentation at 80°C under CO_2.

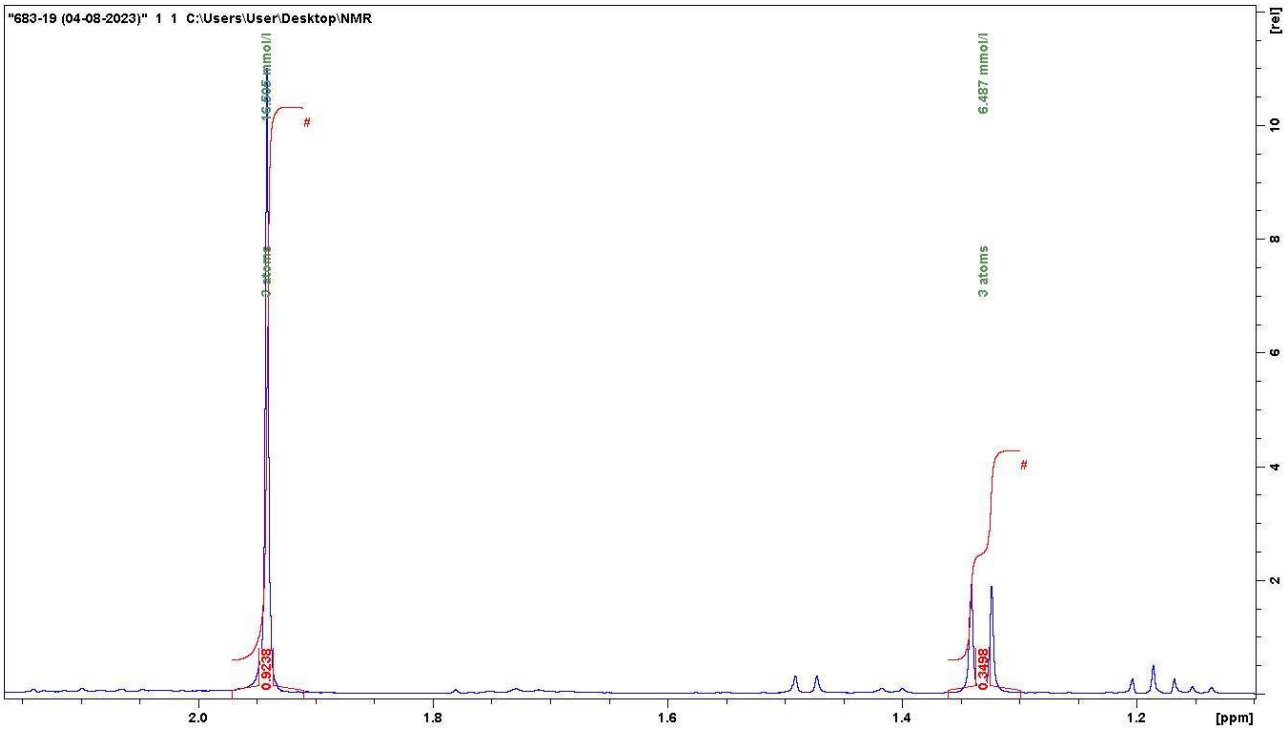

Figure 5.5: ^{1}H-NMR spectrum of medium of fermentation at 80°C under N_2.

From the integration of the two ^{1}H-NMR spectra (Figure 5.4 and 5.5) the mM concentrations in all the samples were determined, obtaining a considerably higher value for the samples under CO_2 than N_2.

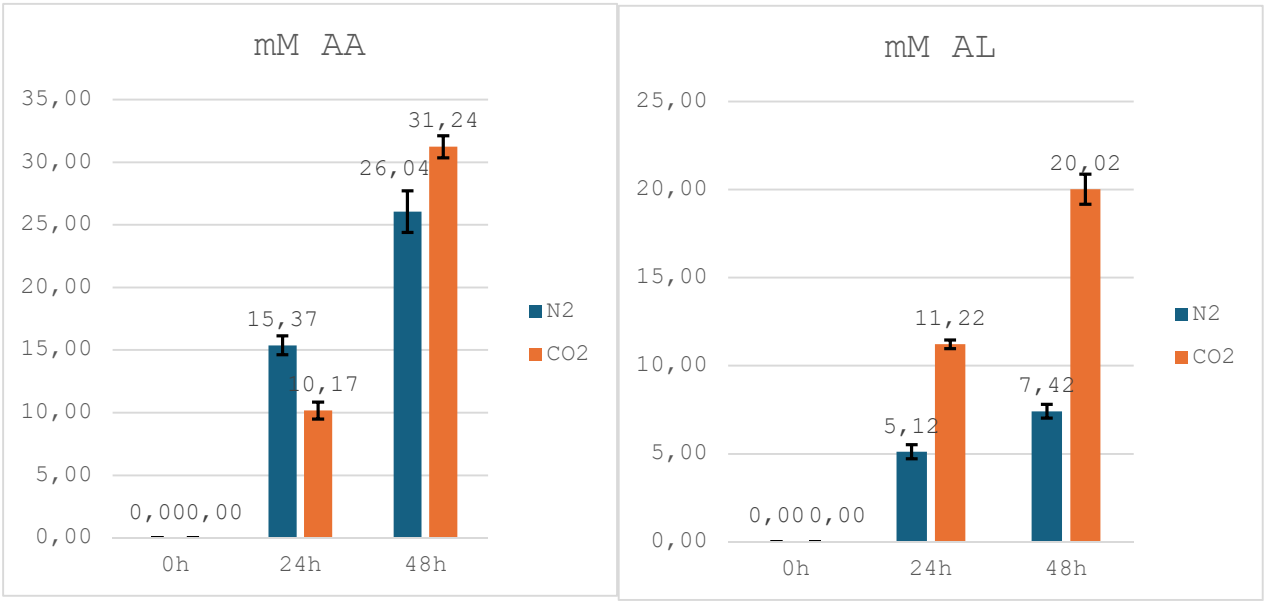

Figure 5.6: Production (mM) of acetic acid (AA) and lactic acid (LA) at 0h, 24h and 48h at 80°C. Data are expressed as mean ± SD, n=3 for N_2 and n=3 for CO_2.

From both the spectra and the average concentrations of the two acids, it's possible to deduce a higher efficiency of production under CO_2 conditions: regarding the lactic acid it was already noticeable at 24 hours, while the acetic acid production largely increased between 24 and 48 hours of incubation.

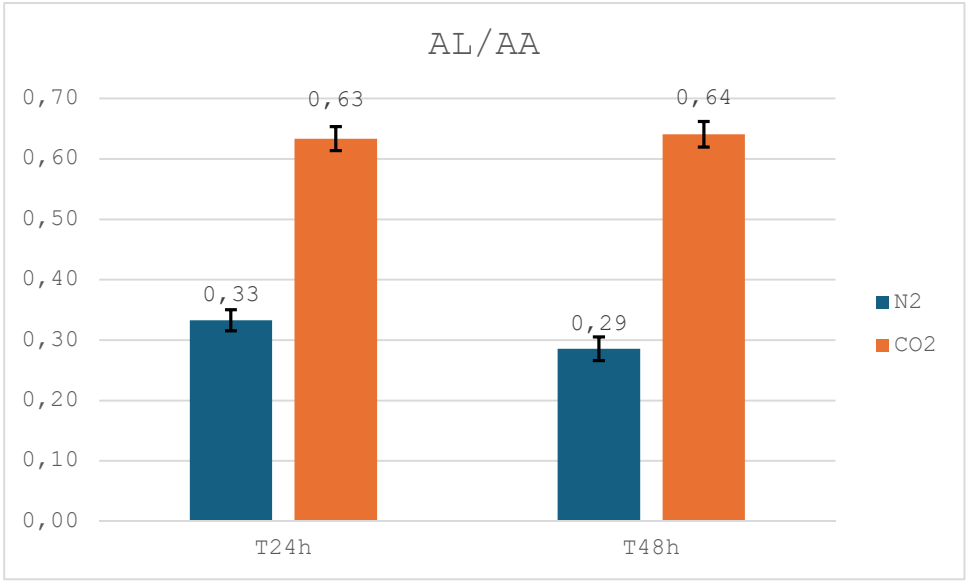

Figure 5.7: Ratios between lactic acid and acetic acid at 0h, 24h and 48h at 80°C. Data are expressed as mean ± SD, n=3 for N_2 and n=3 for CO_2.

Taking into consideration that the Capnophilic Lactic fermentation proceed through the production of lactic acid in replacement of acetic acid, without affecting neither hydrogen production nor glucose consumption, the ratio between the two acids is the only indicator that can be used to assess the contribution of the CLF metabolic pathway to the overall H_2 production.

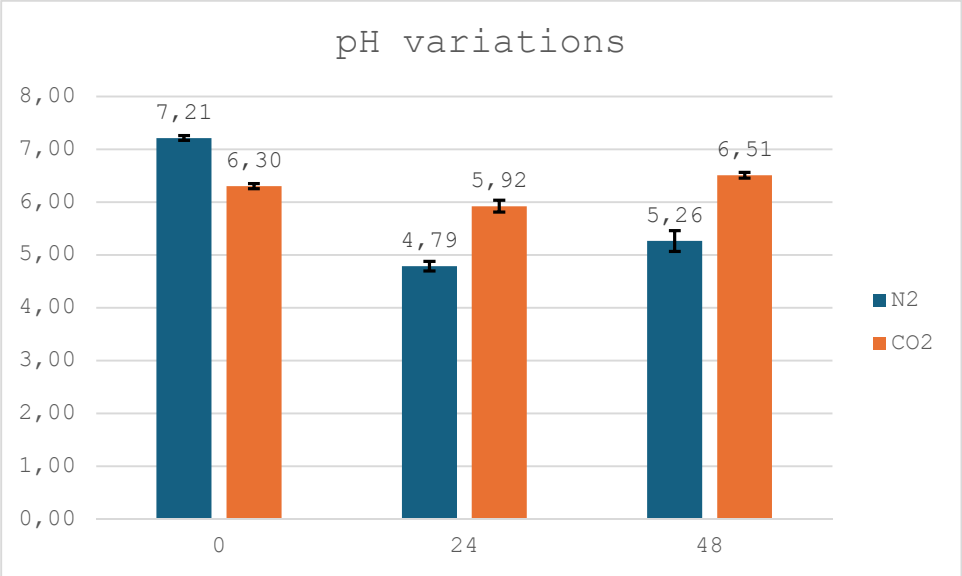

Figure 5.8: pH measurement at 0h, 24h and 48h at 80°C. Data are expressed as mean ± SD, n=3 for N_2 and n=3 for CO_2.

From the pH measurement it was possible to appreciate the mild buffer effect of carbon dioxide preventing an excessive drop in pH value, as it could become inhibiting for the fermentation process: at 24 and 48 hours of incubation, in spite of the higher productions of acids, the pH of the samples under CO_2 conditions was found to be around one order of magnitude higher (N_2 is an inert gas).

In aqueous environment, in fact, the insufflated CO2 gets absorbed in the liquid medium and develop the following interaction with water:

$$CO_2 + H_2O \longleftrightarrow H_2CO_3 \longleftrightarrow HCO3^- + H^+$$

This is called Bicarbonate system: as soon as the CO_2 is absorbed in water, contained in the culture medium, it tends to form carbonic acid which, at the pH the analyzes were conducted (7.5-8), dissociates to produce hydrogen carbonate anions and protons in equal quantities; this set of reactions represent the fundament of the buffer activity performed by carbon dioxide in water.

5.2 Temperature variation analysis

The aim of this analysis was to compare the glucose-based fermentation process in *Thermotoga sp. strain RQ7* at different temperatures (65°C, 70°C, 80°C, 85°C), under N_2 and CO_2 insufflation.

In order to thoroughly assess the variability of the parameters, the number of replicates was increased to six for both carbon dioxide and nitrogen; the measurements were done at 65, 70, 80 and 85°C.

The parameters taken into consideration were, as per usual:

- bacterial growth measuring the absorbance at 540 nm
- pH
- concentration of glucose via DNS assay
- concentration of organic acids by ^{1}H-NMR

5.2.1 T= 65°C

The twelve serum bottles, inoculated using a preculture at the exponential phase (maintained at 80°C for about 24 h), were incubated in heater at a temperature of 65°C for a total of 48 hours.

The samples were drawn at the standard times: 0h (right after gas insufflation) and after 24 and 8 hours of incubation.

Table 5.1: Concentration of glucose and fermentation products at 65°C, at T_{24} and T_{48}. Data are expressed as mean ± SD, n = 6 for N_2 and n=6 for CO_2.

24h				
Insufflation gas	***Residual Glucose (g/l)***	***mM Hydrogen***	***mM Acetic acid***	***Mm lactic acid***
CO_2	3.690 (±0.201)	27.93(±1.82)	10.17(±0.58)	20.75 (±3.65)
N_2	4.197 (±0.269)	24.25(±0.71)	9.07(±0.23)	2.66(±1.12)

48h				
Insufflation gas	***Residual Glucose (g/l)***	***mM Hydrogen***	***mM Acetic acid***	***mM lactic acid***
CO_2	1.291(±0.116)	44.35(±2.69)	13.83(±0.60)	10.85 (±1.09)
N_2	2.886(±0.122)	40.88(±0.98)	16.20(±0.61)	3.96 (±0.14)

As previously determined during the experiment into static conditions, at T=80°C, the samples under CO_2 atmosphere tend to consume glucose way faster than the samples under N_2 even at a temperature at which the product formation results slowed down, compared the performance observed at 80°C which is considered the optimal operating temperature.

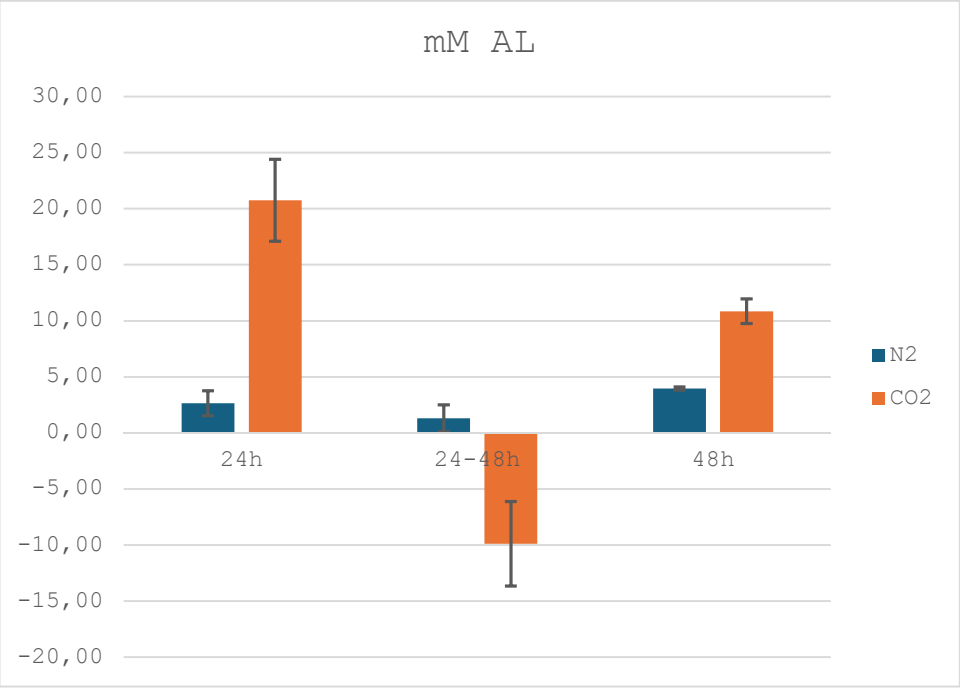

Figure 5.6: Production (mM) of lactic acid (LA) at 24h, interval 24h-48h and at 48h at 80°C. Data are expressed as mean ± SD, n=3 for N2 and n=3 for CO2.

A peculiar behaviour was observed in all samples under CO_2 conditions during the first 24 hours of incubation: in fact, a very high concentration of lactic acid was produced (in average 20.75 mM), to then decrease to an average concentration of 10.85 mM at 48 hours of incubation.

The reasons for these results are unknown, as *Thermotoga sp. strain RQ7* wasn't thoroughly tested to evaluate its performances towards capnophilic lactic fermentation at different temperatures, but further investigations might be carried out in the future.

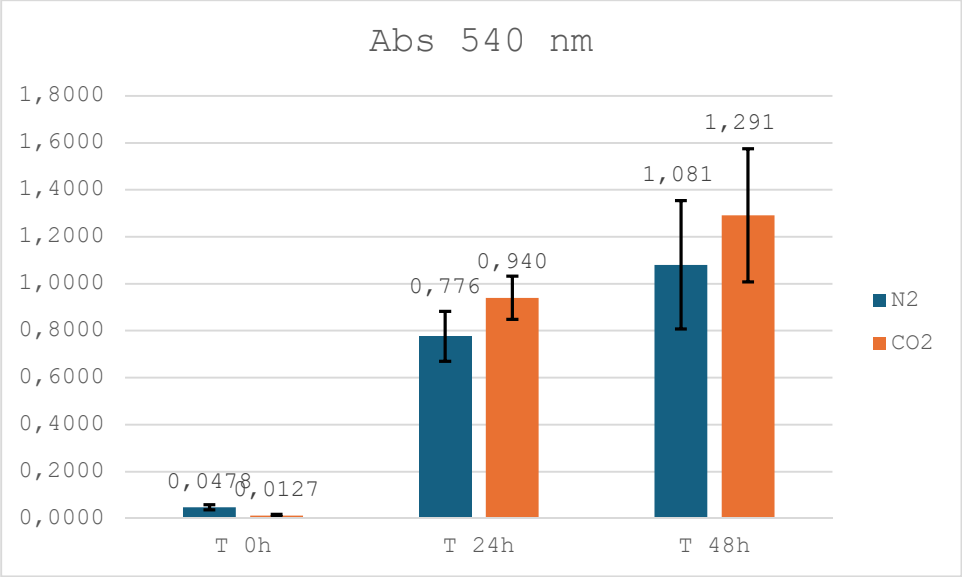

Figure 5.7: Measurements of optical density at 0h, 24h and 48 hours of fermentation at 65°C. Data are expressed as mean ± SD, n = 6 for N_2 and n=6 for CO_2.

This measure allows to determine the culture net growth at 0, 24 and 48 hours of fermentation and, a slight variation, due to the 2 ml starting inoculation, is already detected at T_0.

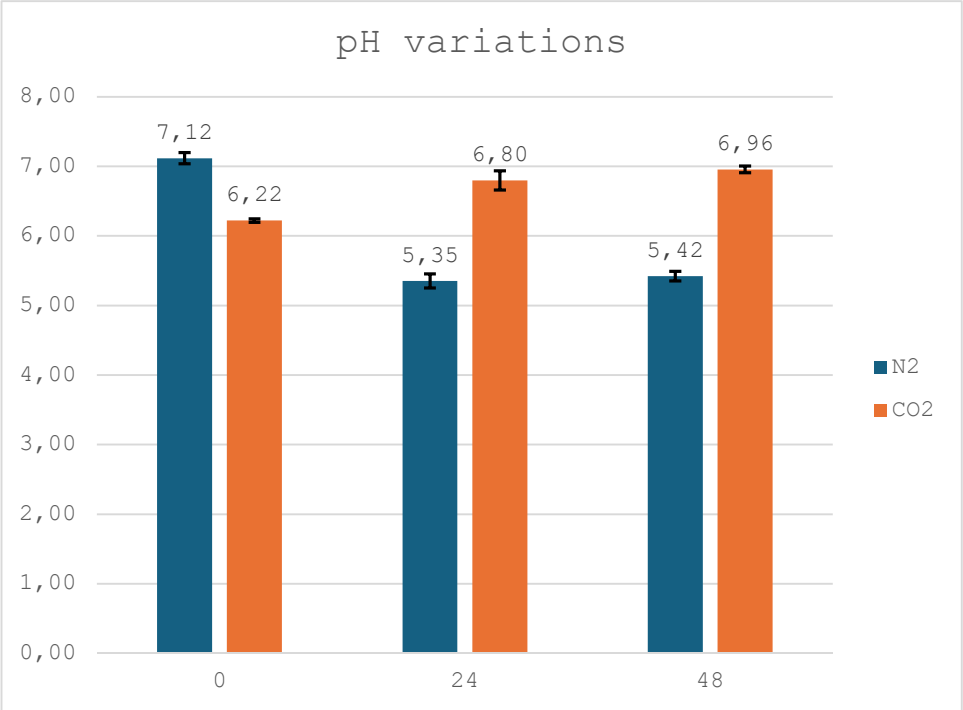

Figure 5.8: pH measurement at 0h, 24h and 48h at 80°C. Data are expressed as mean ± SD, n = 6 for N_2 and n=6 for CO_2.

At 24 hours, pH was adjusted at 7.5-8 using a NaOH 2M solution (NaOH 0.5 M for N_2 T_0 samples because the starting pH is very near to the desired range.

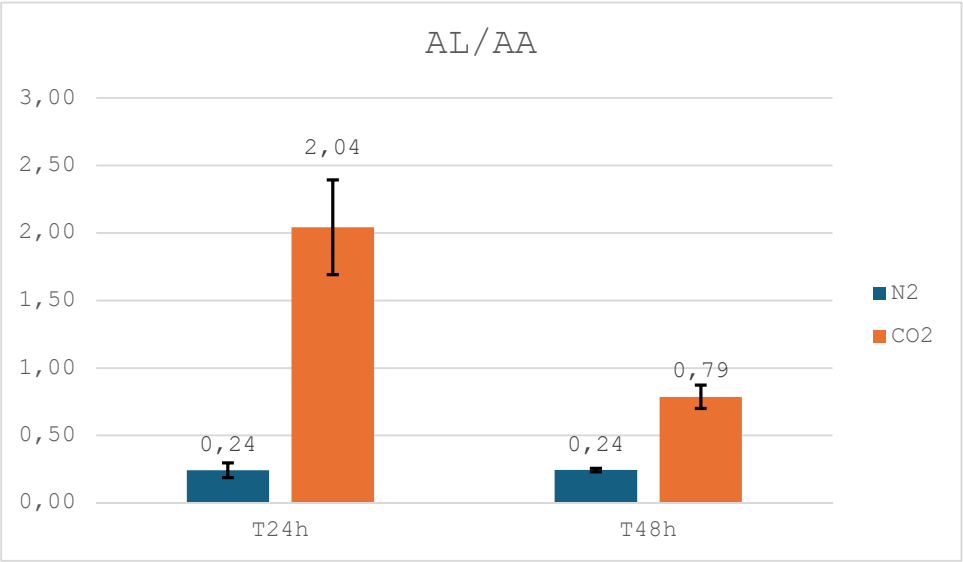

Figure 5.9:Ratios between lactic acid and acetic acid at 0h, 24h and 48h at 80°C. Data are expressed as mean ± SD, n = 6 for N_2 and n=6 for CO_2.

Through the determination of the ratio between the two acids it's possible to estimate the contribution of the capnophilic lactic fermentation to the overall process, as this pathway proceed through the formation of hydrogen with production of lactic acid in place of the acetic acid.

High ratio between lactic and acetic ratio was found at 24 hours through the fermentation (with lactic acid double more abundant compared to the acetic in the samples under CO_2 conditions).

5.2.2 T=70°C

Six replicates for each insufflated gas were prepared and, after adjustment of the pH to 7.5-8 using two different NaOH aqueous solutions: 2M for CO2 samples (gas bubbling tend to decrease the pH) and 0.5 M for N2 samples as the starting value is very near to the desired range (less than 130 µl of solution were enough to reach a pH of about 7.7).

Table 5.2: Concentration of glucose and fermentation products at 70°C, at 24h and 48h. Data are expressed as mean ± SD, n = 6 for N_2 and n=6 for CO_2.

24h				
Insufflation gas	***Residual Glucose (g/l)***	***mM Hydrogen***	***mM Acetic acid***	***Mm lactic acid***
CO_2	3.95 (±0.092)	26.23 (±5.28)	10.11 (±0.21)	5.70 (±0.26)
N_2	4.34 (±0.093)	27.55 (±3.31)	11.11 (±1.26)	4.77(±0.58)
48h				
Insufflation gas	***Residual Glucose (g/l)***	***mM Hydrogen***	***mM Acetic acid***	***mM lactic acid***
CO_2	1.725 (±0.198)	54.52 (±7.14)	18.74 (±1.14)	15.75 (±2.30)
N_2	2.46 (±0.247)	52.21(±14.54)	20.56 (±0.72)	11.05 (±1.15)

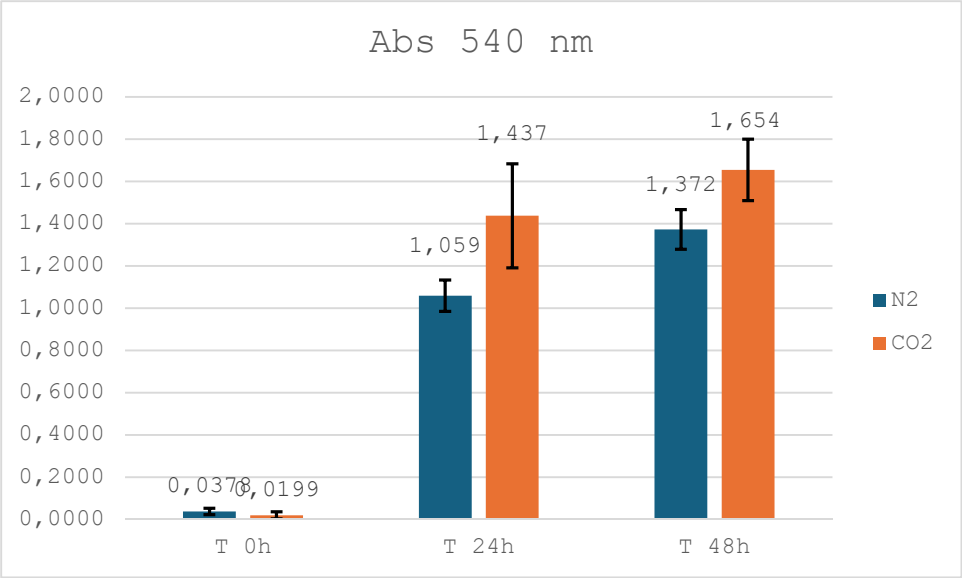

Figure 5.13: Measurements of Optical Density (Abs=540 nm) at 0h, 24h and 48 hours of fermentation.

Comparing these measures to the optical density measured at 65°C and 80°C (Figure 5.7 and 5.17) it results easily noticeable that the samples under N_2 experienced a better cellular growth at temperatures under 80°C with the maximum growth reached at 70°C.

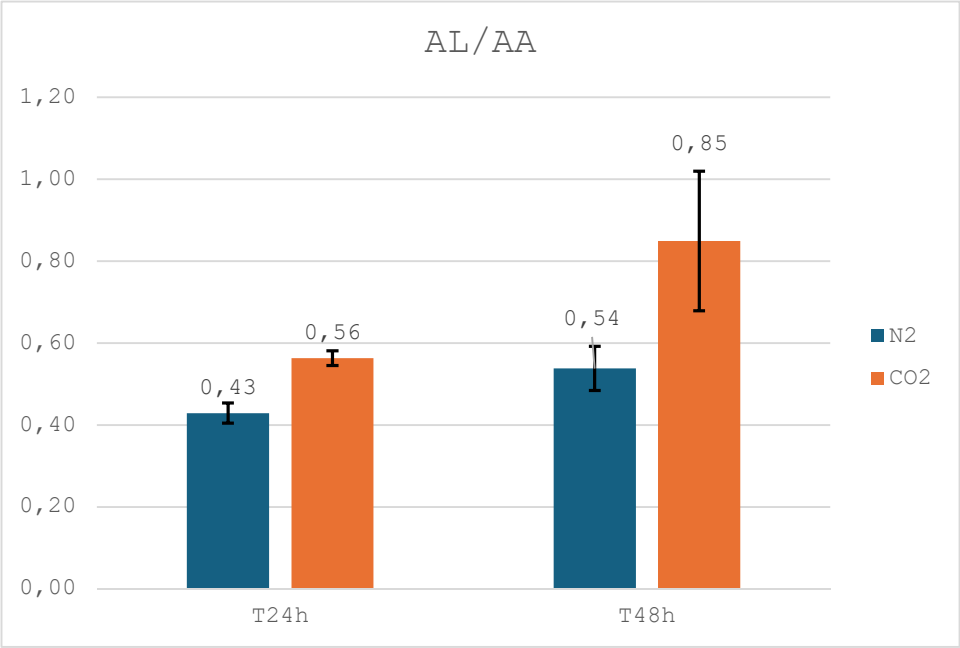

Figure 5.14: Ratios between lactic acid and acetic acid at 0h, 24h and 48h at 70°C. Data are expressed as mean ± SD, n=6 for N_2 and n=6 for CO_2.

Compared to the previous temperature (Figure 5.9) an inversion of tendency of this parameter can be appreciated: at 70°C, the ratio of the acids tends to be higher at 48h than at 24h (similarly to T 80°C, Figure 5.16).

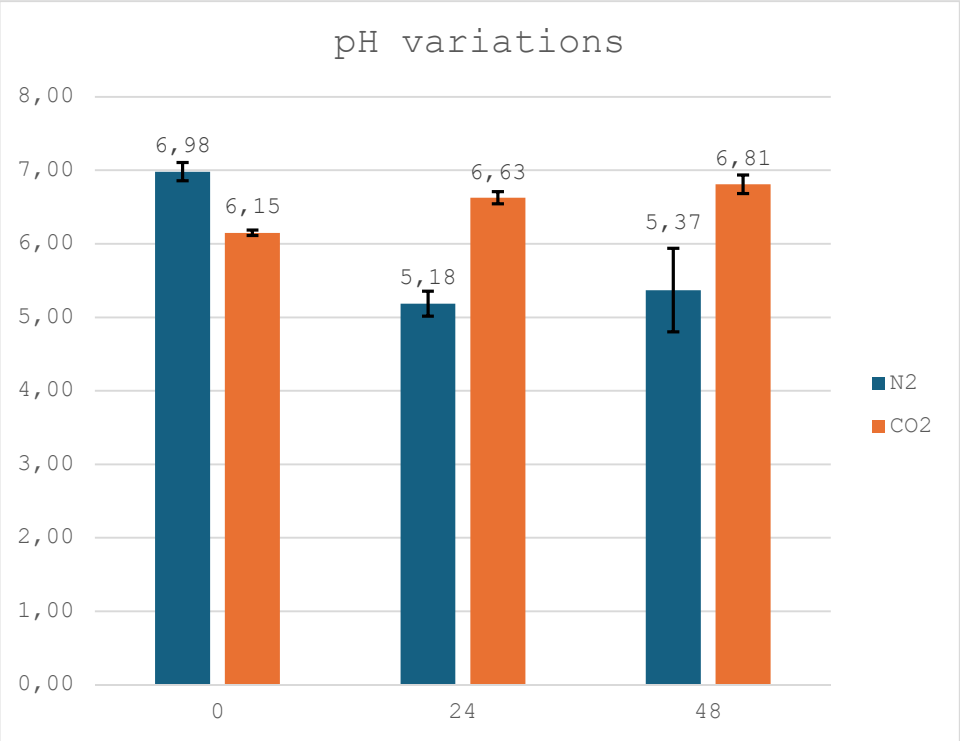

Figure 5.15: pH measurement at 0h, 24h and 48h at 80°C. Data are expressed as mean ± SD, n=6 for N_2 and n=6 for CO_2

It's possible to notice that the drop in pH under N_2 atmosphere is reduced the lower the acids production results, while the samples with CO_2 have shown an overall constant drop in pH at all the temperatures taken into consideration.

5.2.3 T=80°C

Table 5.3: : Concentration of glucose and fermentation products at 80°C, at T_{24} and T_{48}. Data are expressed as mean ± SD, n = 6 for N_2 and n=6 for CO_2.

24h				
Insufflation gas	***Residual Glucose***	***mM Hydrogen***	***mM Acetic acid***	***Mm lactic acid***

	(g/l)			
CO_2	2.76 (±0.165)	49.83 (±3.57)	14.53 (±0.61)	8.71 (±0.25)
N_2	3.78 (±0.173)	37.08 (±2.10)	11.69 (±1.10)	4.81 (±0.48)
48h				
Insufflation gas	***Residual Glucose (g/l)***	***mM Hydrogen***	***mM Acetic acid***	***mM lactic acid***
CO_2	0.71 (±0.087)	85.77 (±3.91)	25.04 (±0.97)	16.59 (±0.82)
N_2	2.48 (±0.106)	59.86 (±2.84)	11.69 (±1.10)	7.46 (±0.85)

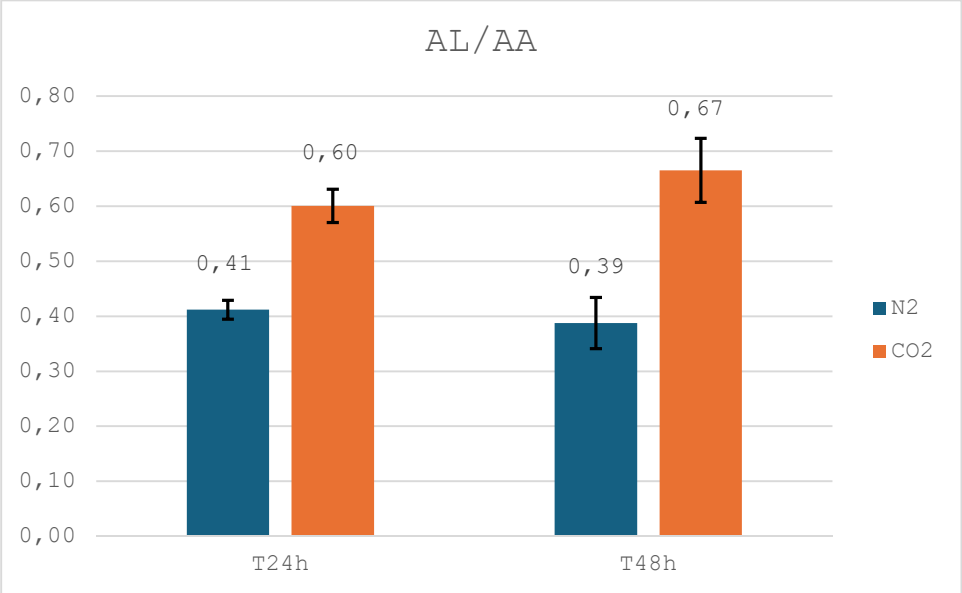

Figure 5.16: Ratios between lactic acid and acetic acid at 0h, 24h and 48h at 80°C. Data are expressed as mean ± SD, n=6 for N_2 and n=6 for CO_2.

From the evaluation of the overall results, 80°C was found to be the best temperature to ascertain the difference in contribution of the capnophilic fermentation when compared to the dark fermentation performed under nitrogen atmosphere.

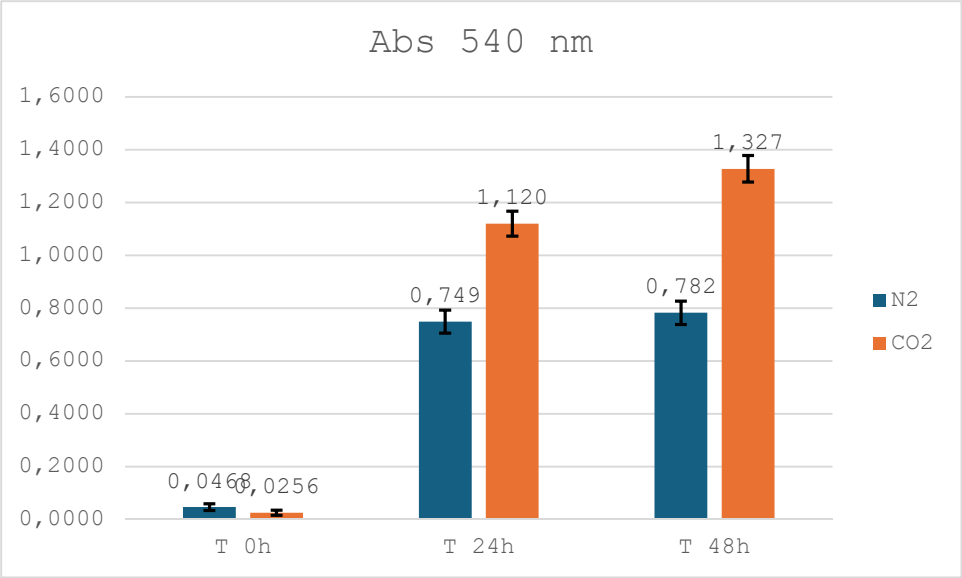

Figure 5.17: Measurements of Optical Density (Abs=540 nm) at 0h, 24h and 48 hours of fermentation.

In spite being the best yielding temperature, the cellular growth for the samples under both conditions (N_2 and CO_2) results slightly lower compared to the measures obtained at 70°C.

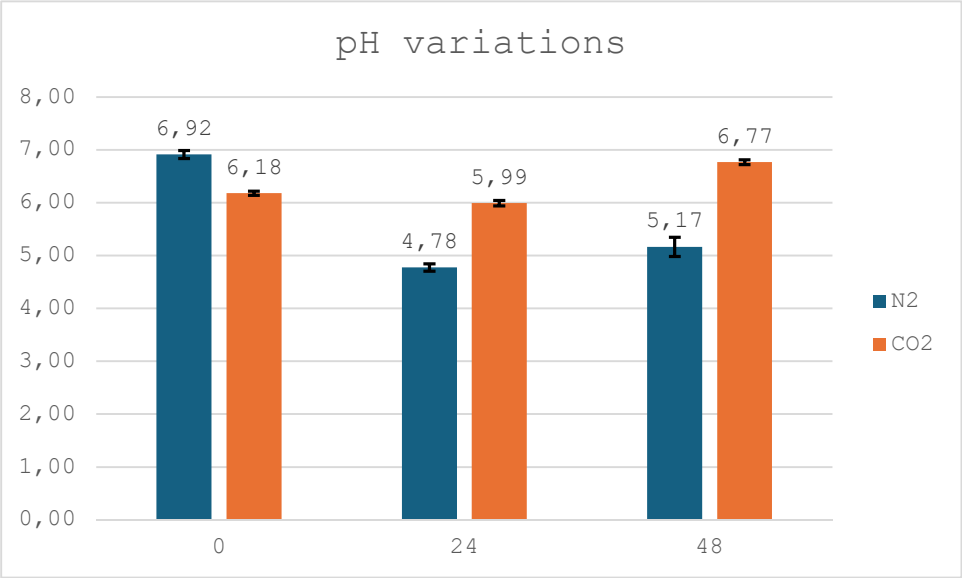

Figure 5.18: : pH measurement at 0h, 24h and 48h at 80°C. Data are expressed as mean ± SD, n=3 for N_2 and n=3 for CO_2.

Due to the major production of organic acids, the pH drop measured in the samples under nitrogen atmosphere is the largest observed, as N_2 is an inert gas.

5.2.4 T=85 °C

Table 5.4: : Concentration of glucose and fermentation products at 85°C, at T_{24} and T_{48}. Data are expressed as mean ± SD, n = 6 for N_2 and n=6 for CO_2.

24h				
Insufflation gas	***Residual Glucose (g/l)***	***mM Hydrogen***	***mM Acetic acid***	***Mm lactic acid***
CO_2	2.516 (±0.194)	80.11 (±3.19)	20.67 (±1.24)	5.89 (±0.53)
N_2	4.658 (±0.122)	38.48 (±2.6)	12.98 (±0.63)	3.11 (±0.44)
48h				
Insufflation gas	***Residual Glucose (g/l)***	***mM Hydrogen***	***mM Acetic acid***	***mM lactic acid***
CO_2	0.794 (±0.067)	104.46 (±6.55)	31.65 (±0.54)	8.09 (±0.68)
N_2	3.352 (±0.191)	65.57 (±5.04)	21.32 (±1.79)	4.54 (±0.28)

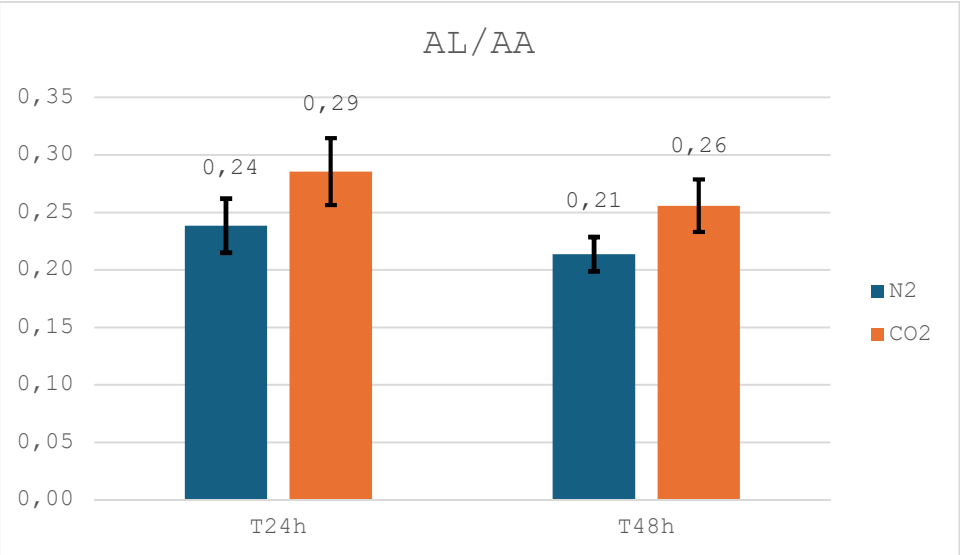

Figure 5.19: Ratios between lactic acid and acetic acid at 0h, 24h and 48h at 80°C. Data are expressed as mean ± SD, n=6 for N_2 and n=6 for CO_2.

At this temperature the samples under CO_2 atmosphere show the highest hydrogen production while consuming glucose nearly as fast as it happens at T=80°C but, comparing the difference in organic acid ratios (Figure 5.16), it can be deduced that the contribution of the CLF to the production of the gaseous product isn't nearly as important as the previous temperature.

This experiment, as a consequence, is able to highlight a greater efficiency of the samples under CO_2 conditions in performing mostly a dark fermentation pathway.

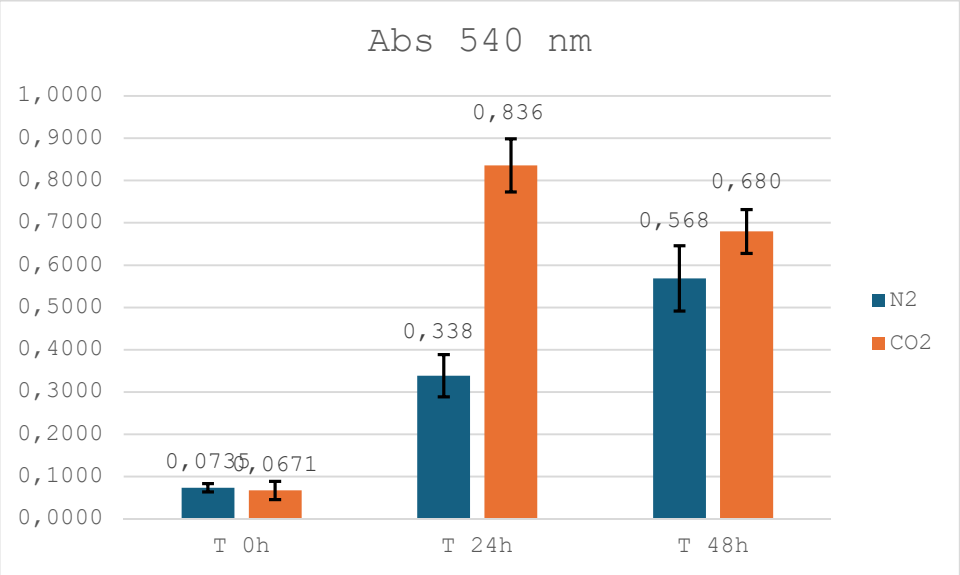

Figure 5.20: Optical density variations measured at 540 nm highlighted a reduction in cellular density between 24 and 48 hours.

The drop in cellular density measured for the drawn samples is the first indication that the highest limit in temperature was reached during the research: in fact, at 90°C it was measured only a mild production of hydrogen with limeted cellular growth during the first 24 hours of incubation.

5.2.5 Temperature comparison

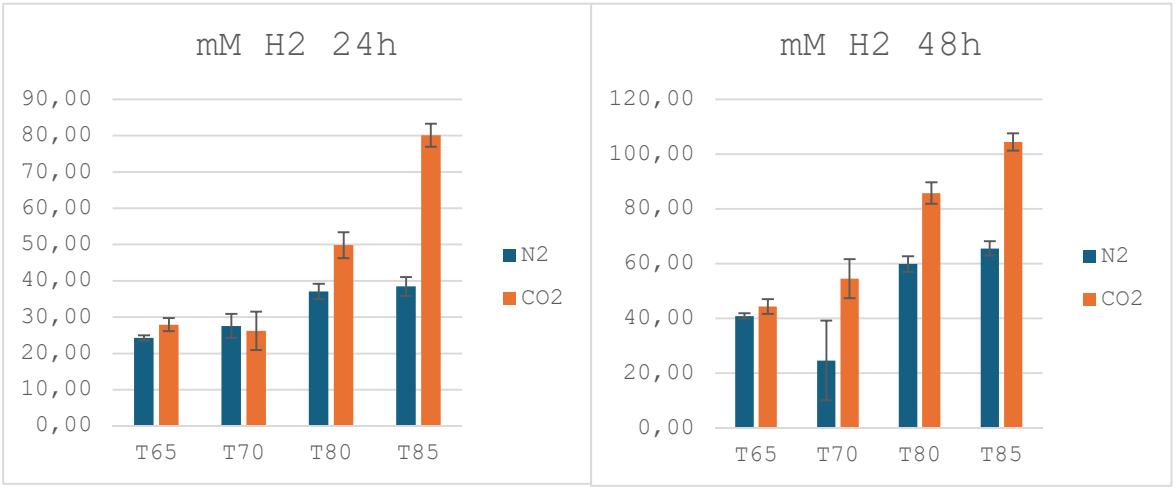

Figure 5.21: Comparison of hydrogen production (mM) at different temperatures at 24h and 48 h.

At 85 °C the highest hydrogen and acetic acid productions (Figure 5.21 and 5.22), coupled with the major glucose consumption, were determined through gaschromatpography and ^{1}H-NMR, respectively; taking into consideration the molar ratio between lactic acid and acetic acid, it was extremely similar to the samples that peformed under nitrogen conditions.

Thus, it's safe to assume that at this tempeature the fermentation proceeds through a dark fermentation pathway, rather than a capnophilic lactic one.

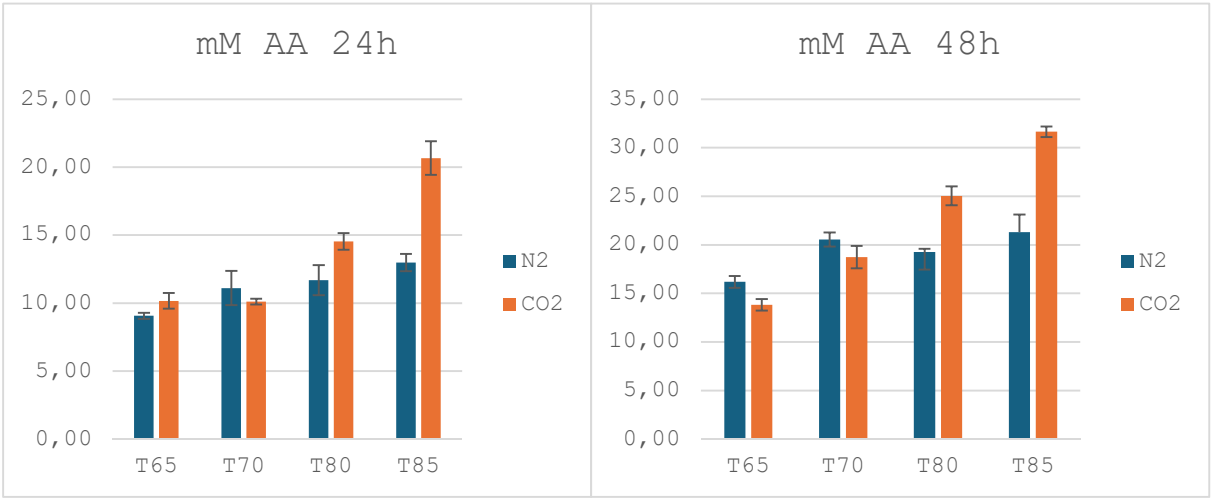

Figure 5.22: Comparison of acetic acid production (mM) at different temperatures at at 24h and 48h.

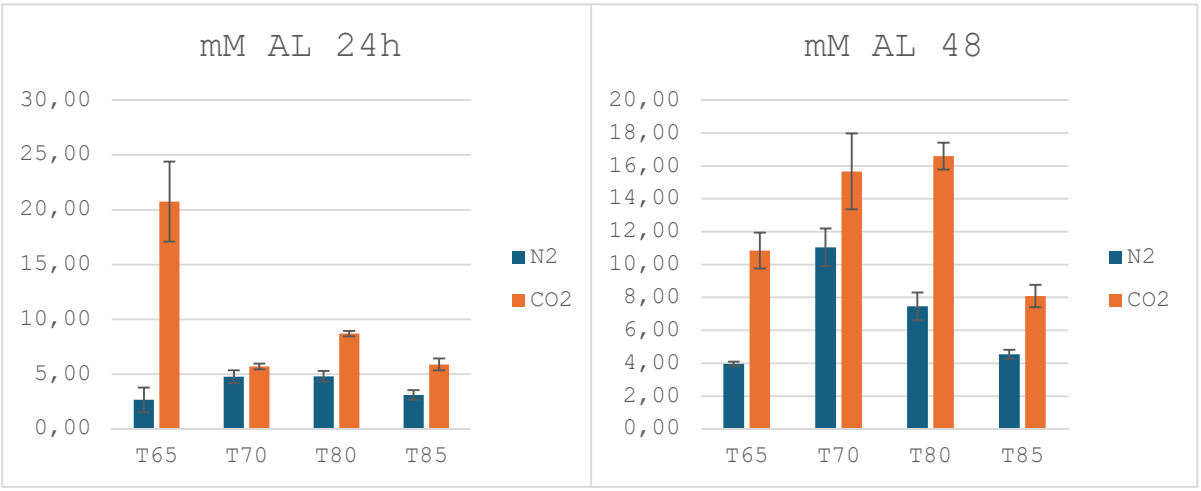

Figure 5.23: Comparison of lactic acid production (mM) at different temperatures at at 24h and 48h.

The lactic acid concentration at 48 hours of the experiment at 70 and 80°C resulted quite similar, with the first having higher lactic/acetic acid ratios, but, the highest measured concentration in terms of lactic acod wasascertain at 24 hours during the test at 65°C.

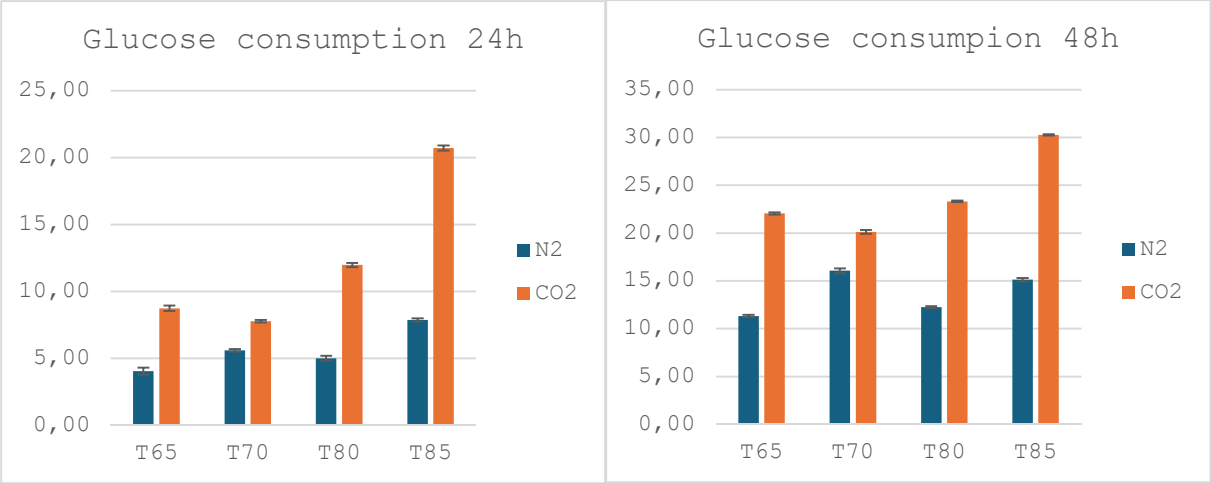

Figure 5.24: Comparison of glucose consumption (mM) at different temperatures at at 24h and 48h.

Taking into consideration the combined results of all the performed tests, this analysis confirmed the temperature T=80°C as the optimal one to assess and compare the differences in performance of the microorganism, through the two different fermentation pathways adopted .

Tabel 5.5: Conparison of glucose (g/l) and yiels (mmol/mmol glucose) of hydrogen, acetic acid and lactic acid at all the tested temperatures.

T 48 h				
Insufflation gas	***Residual Glucose (g/l)***	***Hydrogen yield***	***Acetic acid yield***	***Lactic acid yield***
T 65 °C				
CO2	1.291 (±0.11)	2.08 (±0.11)	0.63 (±0.03)	0.49 (±0.05)
N2	2.886 (±0.122)	3.79 (±0.51)	1.45 (±0.17)	0.30 (±0.04)
T 70 °C				
CO2	1.275 (±0.198)	2.62 (±0.40)	0.92 (±0.08)	0.78 (±0.08)
N2	2.46 (±0.247)	3.15 (±0.33)	1.26 (±0.13)	0.58 (±0.06)
T 80°C				
CO2	0.71 (±0.087)	3.69 (±0.25)	1.09 (±0.07)	0.68 (±0.07)
N2	2.48 (±0.106)	4.89 (±0.54)	1.58 (±0.22)	0.13 (±0.02)

T 85 °C				
CO2	0.794 (±0.067)	3.45 (±0.21)	1.05 (±0.02)	0.27 (±0.02)
N2	3.352 (±0.191)	4.49 (±0.26)	1.46 (±0.18)	0.27 (±0.03)

5.3 Incorporation of ^{13}C-glucose

In the determination of fermentation product yields, the calculation correlates formation of H_2 and organic acids to glucose consumption. The contribution of yeast and tryptone as fermentable matrix is generally considerable negligible. In *Thermotoga neapolitana*, throughout experiments with 1-^{13}C-glucose and 6-^{13}C-glucose, it has been estimated that the contribution of yeast extract (2 g/l) and tryptone (2 g/l) on acetate production accounted for about 12% on the basis of the differences between labelled and unlabelled products (d'Ippolito et al. 2010).

In order to assess the contribution of yeast extract and tryptone in *Thermotoga* sp. strain RQ7, a new labelling experimental set has been developed. The experiments involve the feeding of the cultures with $^{13}C_6$-glucose and the analysis of labelled and unlabelled organic acids by 1H-NMR. In this way, it's possible to discriminate the contribution of oganic sources to the fermentation products, since unlabelled organic acids can derive only by unlabelled organic source in the medium (yeast extract and tryptone), whereas labelled products can only derive form labelled glucose ($^{13}C_6$-glucose).

ERETIC ^{1}H-NMR spectra of fermentation broth showed the splitting of the singlet of methyl protons of acetate at 1.9 ppm into a doublet of doublets doublets with $^1J_{CH}$ = 127.5 Hz and $^2J_{CH}$ = 5.8 Hz, corresponding to 1,2,$^{13}C_2$-acetate (Figure 5.24).

Doublet at 1.33 pm corresponding to the methyl group of lactate was splitted into a doublet of complex sextets, with each sextet half of the doublet separated by $^1J_{HC}$=128 Hz. To explain the sextet structures, we considered the ^{1}H-^{13}C interactions: $^2J_{CH}$ = 4.2 Hz, $^3J_{CH}$ = 4.2 Hz and $^3J_{HH}$ = 7.0 Hz (Figure 5.25)

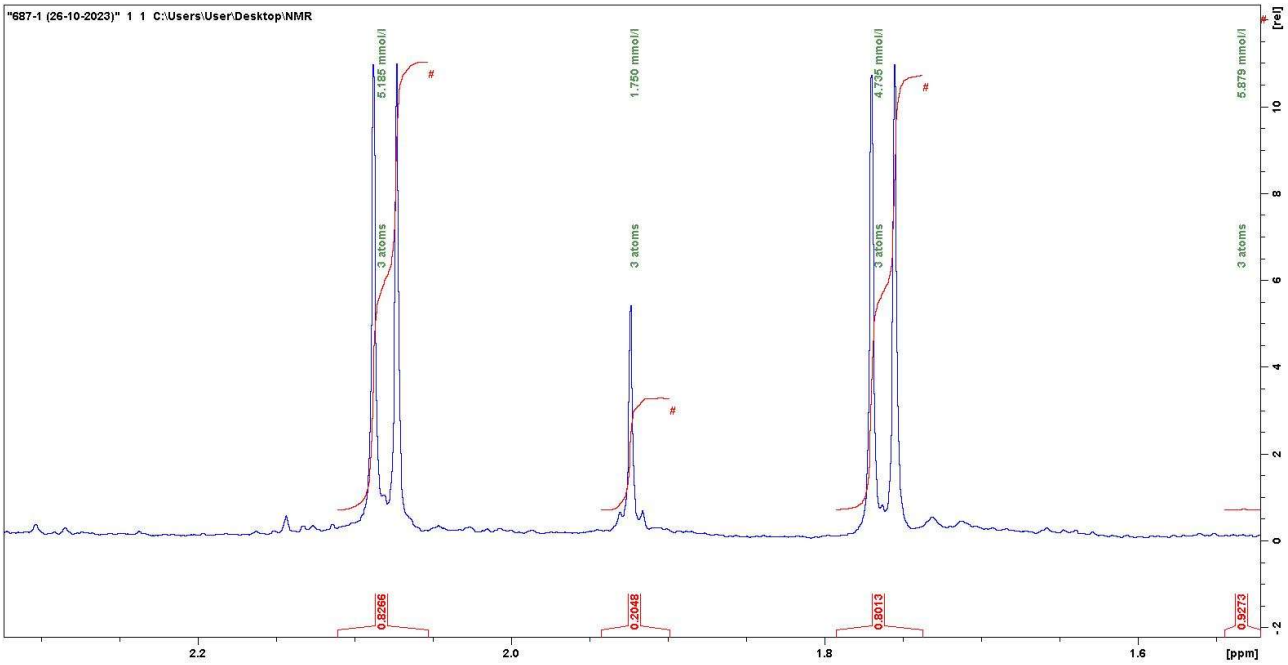

Figure 5.24 ^{1}H-NMR of fermentation broth of *Thermotoga* cultures fed with $^{13}C_6$-glucose, sparged with CO_2 (T=24h). Doublets at 2.09 ppm and 1.8 ppm correspond to methyl protons of $^{13}C_2$-acetate, singlet at 1.92 ppm corresponds to methyl protons of ^{12}C-acetate.

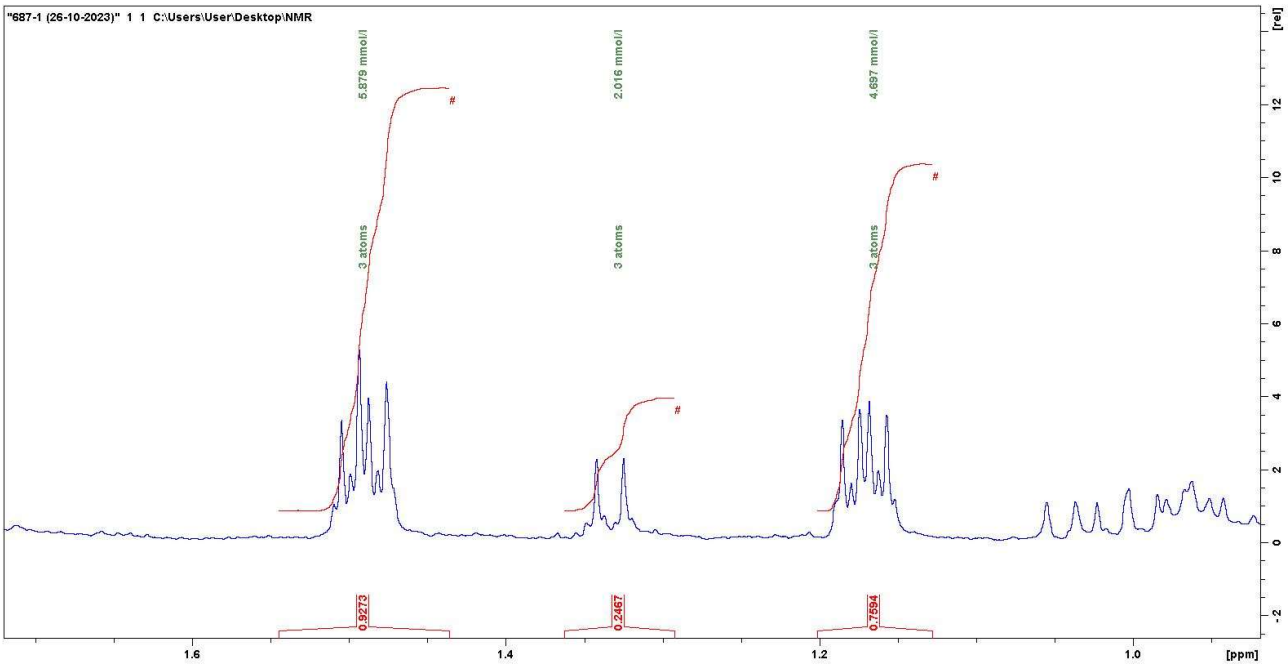

Figure 5.25^{1}H-NMR of fermentation broth of *Thermotoga* cultures fed with $^{13}C_6$-glucose, sparged with CO_2 (T=24h). Sextets at 1.49 ppm and 1.17 ppm correspond to methyl protons of $^{13}C_3$-lactate, doublet at 1.33 ppm corresponds to methyl protons of ^{12}C-lactate. Doublets at 2.09 ppm and 1.8 ppm correspond to methyl protons of $^{13}C_2$-acetate, singlet at 1.92 ppm correspond to methyl protons of ^{12}C-acetate.

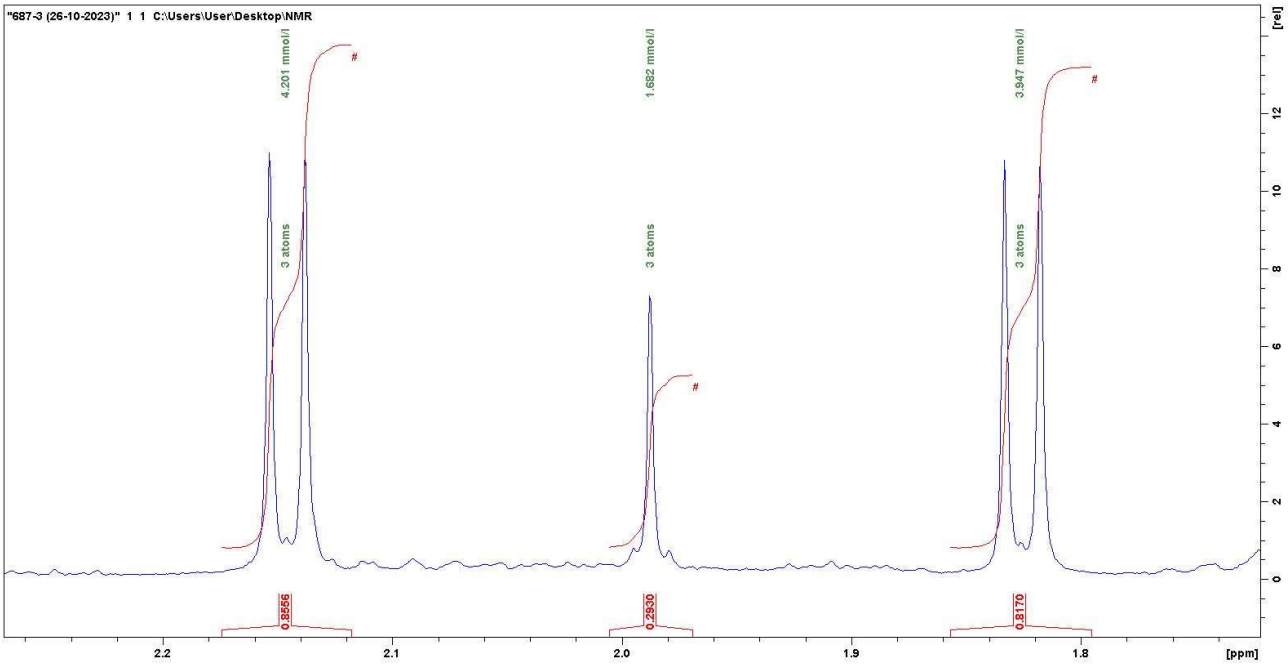

Figure 5.26: ^{1}H-NMR of fermentation broth of *Thermotoga* cultures fed with $^{13}C_6$-glucose, sparged with N_2 (T=24h). Doublets at 2.09 ppm and 1.8 ppm correspond to methyl protons of $^{13}C_2$-acetate, singlet at 1.92 ppm corresponds to methyl protons of ^{12}C-acetate.

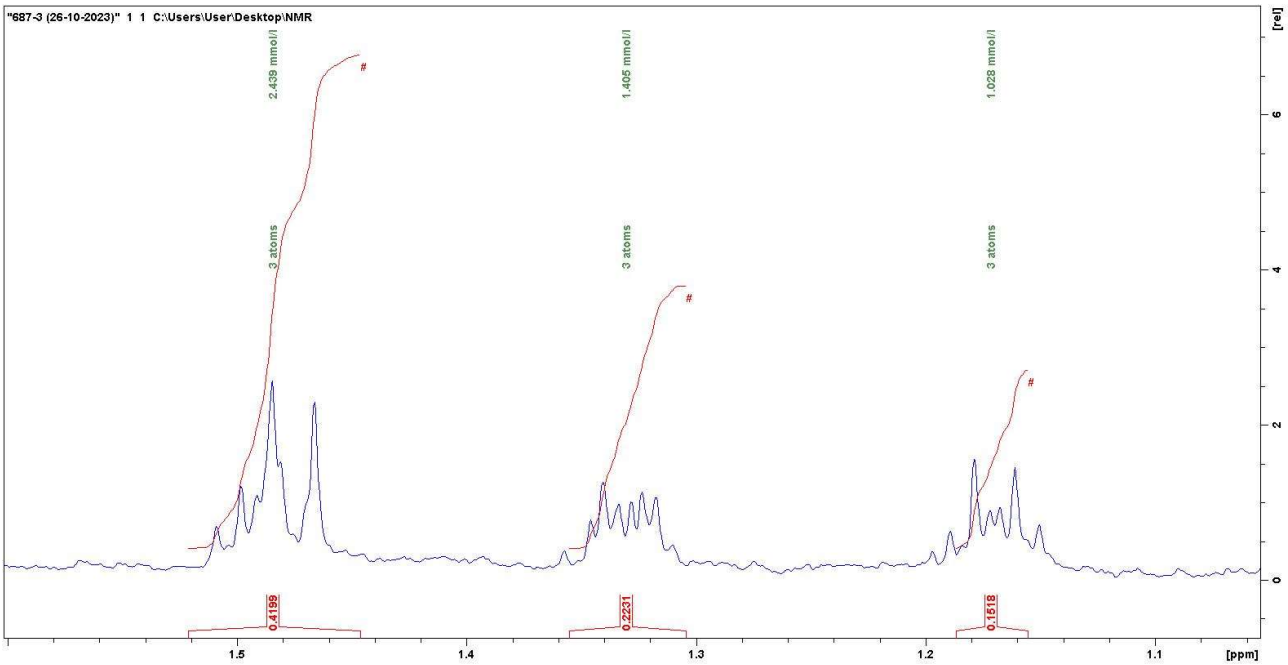

Figure 5.27: : ^{1}H-NMR of fermentation broth of Thermotoga cultures fed with 13C6-glucose, sparged with N_2 (T=24h). Multiplets at 1.49 ppm and 1.17 ppm correspond to methyl protons of $^{13}C_3$-lactate, doublet at 1.33 ppm corresponds to methyl protons of ^{12}C-lactate. Doublets at 2.09 ppm and 1.8 ppm correspond to methyl protons of $^{13}C_2$-acetate, singlet at 1.92 ppm correspond to methyl protons of ^{12}C-acetate.

Besides the complexity in the interpretation of NMR data, we assumed that the integration of labelled and unlabelled diagnostic signals led to the quantitative assessment of different products.

In particular, doublets at 2.09 ppm and 1.8 ppm accounted for $^{13}C_2$-acetate, singlet at 1.92 ppm accounted for ^{12}C-acetate, sextets at 1.49 ppm and 1.17 ppm accounted for 1,2,3 $^{13}C_3$-lactate, doublet at 1.33 ppm accounted for the ^{12}C-lactate.

On the basis of the differences between labelled and unlabelled products, we estimated that the contribution of yeast extract (2 g/l) and tryptone (2 g/l) on acetate production accounted for about 10.7-13.9 % under CO_2 and 11.6-18.2 % under N_2 (Tabel 5.6).

About the contribtuion of protein hydrolizates on lactate production, we estimated 17.7-22 % under CO_2 and 27-36.5% under N_2 (Table 5.7).

Tabel 5.6: average percentuals of ^{13}C-glucose derived and protein-derived acetic acid for samples sparged with CO_2 and N_2 at 24h and 48h of fermentation.

	% Glucose-derived acetic acid	**% Protein-derived acetic acid**
24 h		
CO_2	86.13 (±1.73)	13.87 (±1.73)
N_2	81.75 (±0.69)	18.25 (±0.69)
48h		
CO_2	89.31 (±0.05)	10.69 (±0.05)
N_2	88.36 (±0.76)	11.64 (±0.76)

Tabel 5.7: average percentuals of ^{13}C-glucose derived and protein-derived lactic acid for samples sparged with CO_2 and N_2 at 24h and 48h of fermentation.

	% Glucose- derived lactic acid	**% Protein-derived lactic acid**
24 h		
CO_2	77.97 (±1.90)	22.03 (±1.90)
N_2	63.54 (±8.79)	36.46 (±8.79)
48h		
CO_2	82.29 (±2.13)	17.71 (±2.13)
N_2	72.91 (±0.79)	27.09 (±0.79)

6 Conclusions

During the experimental evaluation, *Thermotoga sp. strain RQ7*, was found able to successfully perform both CLF and dark fermentation in a wide range of different temperatures, ascertaining its versatility also in conditions that resulted quite far from the optimal operating temperature.

At every temperature, the culture was found to be more productive in terms of hydrogen production and, of course lactic acid synthesis, in the systems saturated with carbon dioxide, in spite showing, rather clearly, a reduction of the concentrations the lower the operating temperature was.

Special attention was put towards the identification of the best operating parameters, especially temperature, to optimise the Capnophilic Lactic Fermentation since, both principal products (hydrogen and lactic acid) are considered to possess a high commercial value.

During the experiments performed at different temperatures, the competition for reducing equivalents (NADH) between hydrogen and lactic acid production was highlighted; in fact, two temperatures were found to be especially promising in terms of possible applications in the future for completely different reasons:

- T=85°C was found to be the condition at which the best production of hydrogen occurred, but at the expence of lactic acid production, as lowest ratio with acetic acid was calculated
- At 80°C a slightly lower production of hydrogen was measured but a way higher ratio between acids was determined

In terms of a possible industrial application, it will be essential to define the desired outcome: to obtain the highest possible synthesis of hydrogen it will be more convenient to operate at 85°C, but mainly obtaining a low commercial value byproduct (acetic acid).

On the other hand, the best compromise to have good productions of both hydrogen and lactic acid, products possessing a high commercial value, should be to perform the fermentation at 80°C.

During the analyses, the pH reduction arising from the synthesis of the acids was found out as especially inhibiting toward the fermentation process, in fact, an excessive drop in value (mainly occurred in the samples under N_2 atmosphere) would cause the complete interruption of the fermentation.

As a consequence, a future perspective for further development, could consist in the scale up of the process using a fermenter: the possibility of automatically adjust the pH to a set value, would allow to establish a continuous regime of production, ultimately leading to a more efficient fermentation.

Great interest is also related to the ability of *Strand RQ7* to perform the fermentation using organic matrices other than glucose as substrates (as ascertained during the ^{13}C labelled analysis); the principal target for future research could be glycerol.

Crude glycerol is the main byproduct deriving from the base catalysed transesterification for the production of biodiesel; it's a green, biodegradable and abundant feedstock which is commercially used in the pharmaceutical, cosmetic and paint industries.

In recent years, mainly due to the wide development of biodiesel production its commercial value experienced a sudden drop; in fact, 1kg of crude glycerol is obtained every 10 kg of produced biodiesel.

For this reason, research aiming at the development of sustainable process that could make use of crude glycerol, without increasing the refining costs, are currently taking place; among them fatty acid production, animal feed and biological conversions were studied.

Particularly interesting was reported to be the anaerobic digestion to biogas, mainly to hydrogen and methane, from fermentative microorganisms due to advantages like low nutrients requirements, energy savings and generation of a stabilized digestate (Esercizio et al. 2021).

In this field would fall the anaerobic fermentation to produce green hydrogen performed by *Thermotoga sp. strand RQ7*: this bacterium, in fact, possess the incredible ability to internalize and hydrolyse complex molecules to simpler monomers, which allows it to use a wide range of organic molecules as substrates for the fermentation.

In this way, the perspective of utilizing crude glycerol as fermentable molecule would be particularly promising; especially considering that previous experiments carried out on *Thermotoga neapolitana*, which shares with strand RQ7 an average 98.48% of nucleotide identity (Xu et al. 2017), which have already proven the bacteria able to perform the biosynthesis on such material.

As a consequence, the possibility to replace glucose with glycerol opens the horizons for the production of two high value products starting from a cheap and abundant waste material with high chances of success.

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