ARC Final Report – MicroH_2

SUMMARY

Introduction

In this project, hydrogen production has been studied in parallel in two different biological processes, involving different types of microorganisms: the photoproduction by the green microalga *Chlamydomonas reinhardtii*, and the fermentative production by bacteria of the *Clostridia* genera. In the photoproduction process, the photosynthetic apparatus in the chloroplast is involved in the reduction of protons under anoxia whereas, in the fermentative process, only dark-fermentation pathways from sugars are involved. At the start of the project, the state of the art in the two processes was quite different.

For the microalgal photoproduction process, it was clear that basic aspects related to the interactions between the photosynthetic apparatus in the chloroplast and the whole cellular energetic metabolism had first to be better understood in order to define ways of improving the photoproduction rate. This research was essentially conducted through a collaboration between the Laboratory of Plant Biochemistry and Photobiology (LPBP) and the Laboratory of Genetics of Microorganisms (LAG).

For the bacterial dark-fermentative process, basic knowledge on the cellular biochemical pathways was already well-advanced and it was possible to concentrate research on the design of the reactors for scaling up the process, on bacterial strain comparisons and on the problems of process stability in relation with changing bacterial populations in the reactors. This research was performed by the Centre Wallon de Biologie Industrielle (CWBI) and the Centre d'Ingénierie des Protéines (CIP).

In spite of this operational work division, frequent collaborations and exchanges of ideas between the two sub-consortia took place, with a very fruitful synergy that was natural because the two biological processes share common aspects and the analytical approaches involved were partly the same. This collaboration led to several common publications.

We summarize below the progress achieved during the four-year project in the two fields *i.e.*

A. Hydrogen photoproduction by the green microalga *Chlamydomonas reinhardtii*
B. Bacterial fermentative hydrogen production

A. Hydrogen photoproduction by the green microalga *Chlamydomonas reinhardtii*

Partners : Laboratory of Plant Biochemistry and Photobiology (LPBP) and Laboratory of Genetics of Microorganisms (LAG)

For sustained hydrogen photoproduction by Chlamydomonas, a two stage approach must be applied. In the first stage, algae are grown mixotrophically in complete medium. In the second stage, algae are transferred to a sulphur-deprived medium. Sulphur deprivation causes a down-regulation of photosynthetic activity in such way that dissolved oxygen is consumed in the light through respiration, allowing anoxia to be established and maintained. When anoxia is reached, hydrogenases are expressed and hydrogen photoproduction starts. Previous works had identified two different pathways for the hydrogen photoproduction by Chlamydomonas cells in such conditions. The first pathway, noted '**PSII-dependent pathway**, involves water photolysis by photosystem II (PSII) followed by electron transport from reduced plastoquinones through photosystem I (PSI) to the hydrogenase, which catalyses proton reduction to molecular hydrogen. The second pathway, noted '**PSII-independent pathway**', starts with plastoquinone reduction in a light-independent manner, followed (as in the first pathway) by electron transport through PSI to the hydrogenase.

When this project was written, the identity of the chloroplastic dehydrogenase (Nda) that catalyses plastoquinone reduction for the PSII-independent pathway, was unknown. Preliminary data from our labs suggested that this dehydrogenase belongs to a family of nucleus-encoded type-II dehydrogenases. The type-II family comprises six genes encoding different proteins that can be potentially targeted either to the mitochondria or to the chloroplast. Our bioinformatics and proteomic analysis had suggested that one of these genes, Nda-5, could be the chloroplastic hydrogenase. However, subsequent work countered this suggestion. Shortly before the beginning of the project, we demonstrated that it was not Nda-5, but Nda-2, that was the chloroplastic dehydrogenase involved in non-photochemical plastoquinone reduction. RNA-interference (RNAi) mutants, that were deficient for Nda2, were obtained and their analysis was continued during the project in the frame of WP1. These mutants were extensively used as unique opportunity to better understand the relationship between Nda2 function and hydrogen photoproduction in sulphur-deprived Chlamydomonas. On the other hand, we focused rather on under-expression than on over-expression effects, because overexpressing genes in a stable way in Chlamydomonas proved unrealistic at the present stage of technical advances, whereas knock-down mutants are efficiently produced through RNA-interference.

Appropriate devices for reliable experimentation on hydrogen photoproduction had to be implemented. For this purpose, a set of four photobioreactors were constructed and tested for reproducibility.

H_2 photoproduction experiments were carried out 1 liter semi-closed tubular photobioreactors. Constant atmospheric pressure could be maintained during the process, which was at variance with most devices used in previous works, which generally used closed bottles in which gas pressure progressively increased. The volume of evolved gas was measured from an outlet of the reactor using a home-made gas-trap. A home-made Clark-type electrode was used to monitor alternatively the O_2 and H_2 concentrations in the gas phase. Gas samples could be collected and analysed by gas chromatography at the CWBI.

Investigating the relative contributions of the PSII-dependent and the PSII-independent pathways of hydrogen photoproduction using genetically modified strains.

As the outcome of our experimentations on the relative contributions of the two pathways, the following conclusions can be drawn:

- In Nda2-deficient strains, the addition of the PSII inhibitor DCMU abolishes the hydrogen photoproduction almost completely, which demonstrates that the PSII-independent pathway is totally dependent of the Nda2 dehydrogenase.
- When comparing H_2 photoproduction kinetics of different algal strains, or of wild-type strains in the presence or absence of PSII inhibitor, care must be taken to distinguish variations in maximal photoproduction rates (measured at the beginning of the process) from variations in

total amounts of H_2 produced at the end of the photoproduction. Variations in maximal rates are more appropriate to appreciate direct effects, whereas total amounts also depend on metabolic interactions and adaptations on the long term.

- As far as maximal rates are concerned, the PSII-dependent pathway contributes for as high as 70 % of hydrogen production capacity, whereas the remaining 30 % can be attributed to the Nda2-based (PSII-independent) pathway.
- In the long term, the impairment of the PSII-independent pathway by Nda2 silencing causes a decrease of the total amount of produced H_2 by 40 to 50 %, which indicated indirect effects of Nda2 deficiency, or an increasing contribution of the PSII-independent pathway with time.

These functional analysis were completed by a proteomic analysis using a 2D-DIGE approach in order to quantitatively compare the cellular proteomes of the Nda2-deficient and wild type strains. This analysis confirmed the large implications of Nda2 deficiency on the whole cellular metabolism. Evidence for interactions with fermentative pathways and with mitochondrial oxidative phosphorylation was found.

Moreover, observations of variations in the kinetics of H_2 photoproduction, both in wild type and in Nda2-deficient strains, led us to consider the possibility that the H_2 photoproduction process could be inhibited by its product, H_2, which accumulated with time in dissolved form in a way that depended on the volume of the gas phase in the photobioreactor and on the mixing velocity. The possibility of product inhibition was also pointed out during exchanges of ideas with the CWBI group working on bacterial hydrogen production, the latter process being known for its sensitivity to H_2 accumulation. Product inhibition of hydrogen photoproduction in *Chlamydomonas* was then confirmed by different approaches: variations in the gas phase volume (to modify H_2 partial pressure build-up in the gas phase), nitrogen flushing (to strip out H_2) and H_2 flushing. In conditions where H_2 was repeatedly removed by nitrogen flushing, the H_2 production could be prolonged during several days and the total H_2 amount was more than three-fold compared to conditions where H_2 was allowed to accumulate. The H_2 inhibition was found more pronounced in the Nda2-deficient strain, which suggested that Nda2 activity favours H_2 photoproduction by modifying local redox conditions.

Identifying a mitochondrial NAD(P)H dehydrogenase

In the frame of WP2, aimed at modifying the expression levels of mitochondrial alternative NAD(P)H dehydrogenases belonging to type-II dehydrogenases, a functional characterization of the Nda1 dehydrogenase was carried out. Type-II NAD(P)H dehydrogenases form a multigene family that comprise six members in the green microalga *Chlamydomonas*. At the beginning of the project, only one enzyme (Nda2) located in the chloroplast had been characterized in this alga and demonstrated to participate in the reduction of the plastoquinone pool. Another NAD(P)H dehydrogenase, Nda1, was characterized at the functional level during this project.

We determined that the enzyme is located on the inner face of the inner mitochondrial membrane. A knockdown mutant was obtained, which was only slightly impaired in mitochondrial function, indicating the Nda1 was not essential for respiration. A more pronounced phenotype was obtained when the reduction of Nda1 was combined in a double mutant with the lack of complex I, the type I dehydrogenase of mitochondria. Respiration and growth rates in heterotrophic conditions were significantly altered in the double mutants investigated. The conclusion of this work was that Nda1 plays a role in the oxidation of NADH from the mitochondrial matrix in the absence of complex I. However, no effect of Nda1 on the hydrogen photoproduction was found, which indicated that this enzyme was not essential in the process.

Defining the functions of Chlamydomonas hydrogenases

Although the role of Fe-hydrogenase enzymes in catalyzing proton reduction for H2 photoproduction is well established, less is known on their importance for the adaptation of the cell to anaerobic conditions. This important physiological aspect was investigated during this project by making use of a mutant devoided of the HydEF factor, essential for the assembly of functional

hydrogenase. It was shown that this mutant has a decreased ability for photosynthetic activation after a shift from darkness to light. This established the hydrogen photoproduction process as a transient device for allowing sufficient ATP production by photophosphorylation, which is needed for activating photosynthetic carbon fixation. Under oxic conditions, oxygen photoreduction plays a similar transient function.

For the study of photosynthetic activation under anaerobiosis, a protocol for the fluorimetric evaluation of the time-course of the activation had been established. The principle of this protocol was further used in the context of WP3 in order to obtain a rapid screening method, which could be used for the isolation of new mutants affected in hydrogen photoproduction. Based on the specific chlorophyll fluorescence induction kinetics typical of hydrogenase-deficient mutants, we set up an *in vivo* fluorescence imaging screening protocol allowing to isolate mutants impaired in hydrogenase expression or activity, as well as mutants altered in related metabolic pathways required for energy production in anaerobiosis. Compared to previously described screens for mutants impaired in H_2 production, our screening method is remarkably fast, sensitive and non-invasive. Out of 3000 clones from a small-sized insertional mutant library, five mutants were isolated and the most affected one was analyzed and shown to be defective for the hydrogenase HydG assembly factor. The new screening protocol will be helpful for future investigations on the factors that control hydrogenase activity in Chlamydomonas.

The effect of the nitrogen source (nitrate or ammonium) on the photosynthetic response of *Chlamydomonas* to sulphur deprivation.

In order to optimize hydrogen photoproduction by *Chlamydomonas*, it is important to thoroughly characterize its reponse to sulphur deprivation as a function of time. On the other hand, all investigations have up to now been conducted by using ammonium as nitrogen source in the medium, although *Chlamydomonas* can use also nitrate (and generally does so in natural conditions). The effect of the nitrogen source on the behavior of the algae during sulphur deprivation was never studied. We have therefore carried out a complete study of the kinetics of important photosynthetic parameters during sulphur starvation, using either nitrate or ammonium as nitrogen source. Abundance of proteins of the photosynthetic apparatus, electron transport activities and starch accumulation were monitored. The analysis of the consequences of the nitrogen source has revealed unexpected effects. In a general way, the decline of photosynthetic electron transport and of protein abundances (Rubisco, D1 protein of PSII) was found to be largely delayed on nitrate compared to ammonium. The persistence of photosynthetic activity on nitrate leads to a massive starch accumulation when cells stop to divide due to sulphur starvation. Since starch is the substrate for the PSII-independent hydrogen photoproduction, this effect is quite interesting in the perspective of hydrogen photoproduction improvement in *Chlamydomonas*.

Investigating the H_2-production-related metabolism using mitochondrial mutants

It was the aim of WP4 to decipher the possible interactions between H_2 photoproduction in the chloroplasts and the mitochondrial oxidative phosphorylation. Despite the anoxic conditions (that are established through the S-deficiency protocol), mitochondrial respiration is active in the light due to the intracellular recycling of the oxygen produced by residual photosystem II activity. It was therefore of interest of investigating the H_2-producing properties of mitochondrial mutants which were altered at different degrees in their abilities to produce ATP through oxidative phosphorylation. Mitochondrial mutants of *Chlamydomonas reinhardtii* defective for respiratory complex I (NADH:ubiquinone oxidoreductase), complex III (ubiquinol cytochrome c oxidoreductase) and both complexes I and III were therefore analyzed for H_2 photoproduction. Several parameters were followed during the S-deficiency stage and the anaerobic stage leading to H_2 photoproduction. At the early aerobic S-deficiency stage, starch and neutral lipids accumulated in all strains but their amount was significantly decreased in mutants compared to wild type. During the H_2 photoproduction process, whereas starch content strongly decreased in all strains, neutral lipid amount remained nearly unchanged, suggesting that starch degraded by glycolysis is the preferential substrate for energy production during anaerobiosis. The mutants displayed a decrease in H_2 photoproduction correlating to the number of active mitochondrial proton-pumping sites lost in the strains.

The results of this study thus highlighted the critical role of oxidative phosphorylation during the first (aerobic) stage of S-starvation when carbon resources are accumulated.

The research performed on H_2 photoproduction by Chlamydomonas during this project led to seven publications in peer reviewed journal. Three PhD thesis were carried out in LAG and LPBP on this subject.

B. Bacterial fermentative hydrogen production

CWBI and CIP partners

The micro-H_2 ARC project aimed to study and exploit the microbial production of hydrogen (H_2). In addition to building a competence centre in University of Liege around the H_2 production by microorganisms and the molecular monitoring of the processes, this project answers two main socio-economic issues.

- First, transport and many economic activities will be based on *hydrogen energy* in the near future. Indeed, hydrogen is being considered as an ideal and clean energy carrier since the utilization of H_2, either via combustion or via fuel cells, results in pure water.
- Secondly, many research and technology developments deal with *renewable resources*. Therefore, a new integrated technology for a sustainable development should be promoted. Photofermentation and dark-fermentation are the most promising ways to produce biohydrogen.

The bacterial H_2-production can be performed by the dark-fermentation of different sugars or starch. Carbohydrate-rich wastewaters (such as crop residues, livestock, and food waste) may be used as substrates, thereby valorising waste and reducing waste treatment and disposal costs. Previous research at the CWBI had shown promising results but also the need of molecular monitoring of the bacterial diversity and activities. Therefore, the collaboration maintained through the ARC project between the CWBI, the CIP and the LPBP allowed us to apply and compare different molecular ecology methodologies currently used or used for the first time in environmental biotechnology.

Five PhD theses have been carried out on the topic in CWBI and CIP labs. About 15 papers have been published in international journals with peer-reviewing or will be submitted soon. In addition, two international post-doctoral workers spent one year period to investigate specific topics in both labs.

In the frame of this research project, the CWBI isolated and selected interesting hydrogen-producing strains from the most H_2-producing bacteria groups i.e. *Clostridium* genius and enterobacteriaceae. Their performances were compared on different substrates (glucose, starch, etc.) and their operational culture parameters (pH, culture medium composition, substrate concentration, mixing state, …) were optimised for H_2 production. Molecular tools designed by the CIP enabled the interdisciplinary study of hydrogen production and its biochemistry in order to increase knowledge, stability potentialities and investigation skills about pure strains and consortia composed of different H_2-producing bacteria. These tools such as RT-qPCR and 2D-DIGE were used to unveil some characteristics of the

metabolism of *Clostridium* and its hydrogenases and further to monitor experimentations in bioreactors.
Different bioreactor-types have been investigated in order to improve the hydrogen production yields and rates : mixed systems with suspended microorganisms, fixed-bed and biodisc-like systems with immobilized cells. Discontinuous, sequenced-batch, pseudo-continuous and continuous modes were tested in order to study the effect of organic loading rate, hydraulic retention time, etc. Anaerobic bioreactors up to 20-litres total volume were operated with pH and temperature regulation and with data recording for biogas flowrate and composition. Gas and liquid chromatography techniques were developed to monitor substrate consumption and the production of different major metabolites such as H_2, CO_2, ethanol, lactate, formate, volatile fatty acids (acetic, propionic, butyric, …).
A chemical engineering approach was also developed to investigate and quantify the crucial impact of soluble H_2 concentration (often related to H_2 partial pressure) on the bacterial performances for hydrogen production. Moreover, metallic nanoparticles were used to significantly improve these performances.
Finally, and because more than 60 % of the organic content remains in the form of by-products in the bioreactor at the end of dark fermentation, it was necessary to test the coupling of this process with another one that has the ability to lower the carbon load of effluents, such as anaerobic digestion (*i.e.* methane fermentation).

Methods and techniques used

- HPLC-RID was used to analyse carbohydrates substrates (mono- and di-saccharides directly or polysaccharides after acidic hydrolysis) and soluble metabolites (ethanol, lactate, formate, volatile fatty acids) in culture medium
- HPLC-UV/Vis was used to analyse VFA in low concentrations and to analyse the 24 aminated acids after derivatisation (carried out automatically by HPLC equipment)
- Ritter gasmeters and replacement equipments were used to monitor the biogas production in bioreactors
- GC-TCD and ABB catharometric gas analyser (EL1020) were used to analyse biogas composition; a simple method using a replacement equipment filled with KOH 9 N was also validated for that purpose
- DGGE analysis followed by 16S rRNA sequencing was performed to identify bacteria strains
- FISH (Fluorescence *in situ* hybridisation) and qPCR (quantitative real-time PCR) were used to assess the evolution of the constructed consortiums involving different *Clostridium* strains in bioreactors
- a PCR detection tool was designed to characterize the hydrogenase gene content of *Clostridium* strains
- RT-qPCR (Reverse Transcription qPCR) and RNAseq were used to study the transcriptome and 2D-DIGE to study the proteome of the strain *Clostridium butyricum* CWBI1009.

The experimental results related to WP6 compared different strains and consortia of bacteria producing hydrogen from different carbohydrate substrates. The main results achieved in anaerobic serum bottles and 2 to 20 L bioreactors are listed below.

- A **comparison of the hydrogen production yields** was carried out with 19 different pure strains and sludges : facultative and strict anaerobic H_2-producing strains along with anaerobic digester sludges thermally pre-treated (to enrich the microflora in high H_2-producers) or not. Significant yields variations were recorded even between different strains of the same species (up to 20% of variation). The **pure *Clostridium butyricum*** (*C. but.*) strains achieved the highest yields i.e. up to 172 L H_2 produced per kilogram of glucose consumed (1.38 mol H_2 /

mol glucose). They are also able to use other carbon substrates : mono-, di- and polysaccharides such as starch.

- Two efficient H_2-producing strains (*C. but.* CWBI1009 and *Citrobacter freundii* CWBI952) were further studied in order to determine the **optimum culture conditions** for the production of hydrogen. A 2.3 L bioreactor was operated at 30 °C in batch and sequenced-batch mode using glucose and starch as substrates. For glucose the maximum yield (211 L H_2 / kg or 1.7 mol H_2 / mol glucose) was measured with the *C. but.* strain when the pH was maintained at 5.2. In **sequenced-batch reactor (SBR)** a 35% increase in H_2 yield was obtained with removal–addition of 40% of the culture medium at the beginning of each sequence. By this way, we showed that it was feasible to **direct the carbon metabolism** to acetate and butyrate production *i.e.* the only pathways leading to hydrogen production.
- In the same conditions, a **mixed culture enriched** in spore-forming microorganisms (thermal treatment as reported by many authors working on biohydrogen production) achieved interesting performances but rather unstable on the long term.
- By contrast, a **constructed consortium involving *C. but.* and *Cit. fr.* strains** reached stable, comparable or higher performances than the scientific literature : 1.2 to 1.7 mol H_2 / mol glucose. These should be compared to the maximum performances reached by the sole *Cit. fr.* (0.83 mol H_2 / mol glucose and 33.2 mL H_2/L.h) obtained at the optimal pH 5.9 in a semicontinuous culture with a dilution rate of 0.012 h^{-1}. In addition, this strain does not use starch substrate. The results achieved in association of both pure strains confirm the interest of our approach in order to install rapidly the strong anaerobic conditions suitable for growth and activity of the strict anaerobe *C. but.* without addition of often expensive reagents *e.g.* L-cysteine. It is however to notice that the experiments performed in SBR with the pure *Clostridium* strain needed **no special care** for addition of 40% of culture medium even containing dissolved oxygen.
- Different **artificial co-cultures of pure *Clostridium* strains** were also tested **in 20 L SBR using glucose and starch as carbon sources**. The aim was to achieve robust microbial consortia with complementarities against environmental changes since for instance their respective optimal pH are different. According to the monitoring by qPCR a **stable coexistence** was obtained between *C. pasteurianum* and *C. butyricum* during 13 days of glucose fermentation. In addition, the gene expression pattern of **seven [FeFe] hydrogenases**, analysed by RT-qPCR (Reverse Transcription-qPCR) suggested a major contribution of *hydB2* and *hydA2* from *C. pasteurianum* to the overall H2 production when cultured with *C. but.* On the contrary, co-cultures of these strains with C. felsineum. DSM794 lead to outcompetition of this strain.
- The experimentations in batch and SBR mode unveiled (i) the **ability of *C. but.* CWBI1009 to form biofilms** and (ii) the **crucial impact of the dissolved hydrogen concentration in the liquid media**. Its quantification showed that in exponential growth phase and H_2 production the medium can reach a 7-fold supersaturation in H_2 compared to equilibrium conditions. This hugely affect the H_2 production yields and rates since yields 55 to 100% higher (up to 3.1 mol H_2/mol glucose) were recorded by improving the mass transfer conditions. Therefore, for operation in continuous mode, original bioreactors such as an **anaerobic biodisc reactor** (AnBDR) were designed to both fix biomass and enable rapid liquid to gas transfer of hydrogen produced.
- Other improvement parameters for H_2 production were also tested such as iron concentration or nitrogen source and concentration. A significant increase of the hydrogen production rate by about 40 to 100% was recorded after the addition in the culture medium of small quantities of **metallic nanoparticles**, suggesting a rather **innovative catalytic-like effect** related to this biological process.

In the frame of the WP7 the dark fermentation process was improved in order to be economically affordable for future industrial biohydrogen production *i.e.* with stable performances, operated under non-sterile conditions, using readily available complex feedstocks such as agro-industrial or

municipal wastes and wastewaters. For this reason and taking into account the former results, **robust strains and microbial consortia have been tested in semi- and continuous bioreactors, with a mixture of carbohydrates and with high biomass retention**.

- The artificial consortium involving *C. butyricum* CWBI1009 and *C. pasteurianum* DSM525 was tested on a mix of carbohydrates (glucose, sucrose, starch) in a fixed-bed SBR. Progressively, other microorganisms developed in the bioreactor : *Enterobacter sp, Citrobacter sp.,* other *Clostridium* strains, etc. As shown by qPCR and FISH, the dominance of the different species was subjected to significant changes throughout the experimentation, with the contaminant species constituting more than 70 % of the whole population. Surprisingly, the hydrogen production performances were not significantly affected with a mean hydrogen yield of **2.59 mol H_2/ mol glucose**. Moreover the two **clostridia remained the most metabolically active** species whereas they became the minority species at some stage of the fermentation.
- Since the design of the fixed-bed reactor was not quite suitable for control of environmental conditions and channeling, an **anaerobic biodisc reactor (AnBDR) was designed to enable homogeneous sampling, fix biomass and enable rapid liquid to gas transfer**. The highest and stable H_2 production rate (**703 L H_2 per hour and per m³** of liquid volume inside the bioreactor) and yield (302 L / kg glucose consumed *i.e.* 2.4 mol/mol) with the pure culture of *C. but.* CWBI1009 were recorded in the AnBDR with 300 mL culture medium (total volume 2.3 L) at pH 5.2 and a glucose loading rate of 2.87 kg / m³.h. In mixed culture after contaminations, only 15% lower H_2 production rate was reported. These results are relevant compared to the highest H_2 yields and rates reported in the literature with mixed cultures and achieved in reactors, such as trickle bed bioreactors, with high gas transfer performances.
- *Clostridium butyricum* CWBI1009 was also cultured in a trickling bed SBR (TBSBR) packed with a Lantec **HD Q-Pac® packing material** as projected in WP7. The reactor was operated for 62 days under sequenced batch reactor mode by removal and addition of 40% of the mixed liquid and medium respectively. Controlled parameters were pH, temperature, and recirculation flow rate and inlet glucose concentration at 10 g/l. The maximum specific hydrogen production rate and hydrogen yield found from this study were respectively 3.3 m³ H_2 per day and per m³ of liquid volume and 1.67 mol H_2/mol glucose consumed. The maximum hydrogen composition in the biogas found from this study was 83%. For all the sequences, soluble metabolites were dominated by the presence of butyric acid and acetic acid compared in other volatile fatty acids. After contaminations, **thermal treatment** improved both hydrogen yield and hydrogen production rate without adding fresh inoculum in the subsequent feeding. This bioreactor operating conditions should be improved to increase H_2 production rate and to be operated in continuous mode. However, efficient **biofilm formation** was evidenced **without clogging risks** even taking into account the high specific area of this packing. This confirms the results achieved in 60 mL rolling serum bottles with different packing material.

The objectives of the WP8 was to increase the knowledge on the fermentative hydrogen production using bacteria of the genus *Clostridium*.

- By analysing sequenced clostridial **genomes,** we described the **genetic diversity** of **potential [FeFe] hydrogenases**, thus pointing out to the existence of novel pathways leading to H_2 production in this genus..
- By designing and applying **[FeFe] hydrogenase cluster-specific primers**, we could amplify and sequence **new hydrogenase genes** from several species selected to produce H_2 in bioreactors but for which no genetic information was known, *e.g. C. sartagoforme, C. felsineum, C. pasteurianum.*
- The application of different **molecular tools**, including RT-qPCR, and high throughput RNA-seq and 2D-DIGE, proved to be very useful in starting to decipher a complex H_2 metabolism in clostridia.

- Based on different experiments performed in this study, we hypothesized for the first time, that depending on the environmental conditions clostridia can produce H_2 using alternatively **hydrogenases or/and nitrogenases**.
- Based on the differential regulation of different pathways and processes in different conditions, respectively with low and high H_2 yields, we put forward an hypothesis that clostridia can efficiently grow and ferment glucose without any H_2 production. Instead, activation of the sporulation process at lowering pH, where the production of H_2 is increased in comparison to neutral pH, led us to the conclusion, that clostridia activate different H_2 production pathways in response to **stress conditions**.
- **Molecular monitoring** of the bacterial communities used to produce H_2 in different bioreactors, showed that in artificial co-cultures usually one of the species is becoming dominant. However the other strain is never completely overgrown and washed out from the bioreactor. Moreover, we have shown that in some conditions, the minority species can be the most metabolically active one.
- The main conclusion of this work is that **we still do not know much about H_2 metabolism in clostridia**, and additional studies are necessary to decipher the physiological functions of different [FeFe] and [NiFe] hydrogenases and the role of nitrogenase in the overall H_2 production.

Valorisation

Different opportunities for results valorization have been identified in some project carried out by CWBI since they involve hydrogen production or mediation :

- FRFC project (Coord D. Toye, LGC-ULg; n° 14730278) on the study of liquid/gas transfer in hydrogen-producing bioreactors
- Greenwin project “Minerve” (Coord Shanks SA) and 2 PhD thesis on anaerobic digestion improvement in landfills and biodigestors
- Greenwin project “Nanomicro” (Coord Sanifox SA) on hydrocarbon biodegradation in soils

Starters of Clostridia are also involved in new products on the market to improve anaerobic digestion from agriculture and agro-industries wastes.