Chapter 2
Pretreatment to Increase Hydrogen Producing Bacteria (HPB)

This Chapter focuses on the investigation of an easy and efficacious method of obtaining a hydrogen-producing bacteria (HPB) culture to the detriment of hydrogen-consuming bacteria (HCB), such as methanogens and homoacetogenic bacteria. Although the use of mixed microflora is more viable from both the practical and biological points of view, important limitations arise from the co-activity of HPB and HCB. In this respect, pretreatment is one of the most important issues in anaerobic hydrogen production, in order to produce suitable inocula of HPB. In particular, we investigated the effectiveness of acid pretreatment applied to mixed microflora in order to stop methanogen activity. We evaluated the content of *Clostridium* bacteria, which are the main ones responsible for H_2 fermentation in two of the most widely used inoculum sources: anaerobic sludge from wastewater treatment plants and rumen microorganisms from cow stomachs.

2.1 Physiological Differences Between HPB and HCB

Bacteria belonging to the genus *Clostridium* are the main ones responsible for H_2 production. They are obligate anaerobes, Gram-positive and rod-shaped. *Clostridium* spp. have a substantial characteristic that distinguishes them from other bacteria allowing the production of bioH$_2$ in anaerobic processes instead of bioCH$_4$: they are capable of producing protective end spores by undergoing a process called sporulation. This occurs when bacteria are exposed to harsh environmental conditions for bacterial growth, such as high temperature, ultraviolet radiation, presence of oxygen, extreme acidity and alkalinity, harmful chemicals like antibiotics and disinfectants, drying out, freezing, and many others that would easily kill a normal vegetative cell. To be precise, endospores are metabolically inactive dormant bodies, like seeds, which wait until the environment again becomes favorable to life. These endospore-forming bacteria, mainly Bacilli and Clostridia, have essentially two phases during their life cycle: vegetative cells and endospores. Once environmental conditions change, the endospores germinate back into living

vegetative cells that can grow and thrive. In extremely restrictive conditions, the spores might be very resistant and not easily destroyed, as opposed to HCB that are methanogens without such an ability to resist [1]. It happens in our specific case, in fact, that when the *Clostridium* spores are placed in favorable conditions, with nutrients and anaerobic conditions, the germination and metabolism processes can restart [2], and consequently hydrogen and other metabolic products can be produced. *Enterobacter* spp. are also H_2-producing microorganisms with the advantage that they are facultative bacteria able to grow in the presence of oxygen. Based on phylogenetic analysis of the rDNA sequences, Fang et al. [3] found that 64.6 % of all the clones were affiliated with three *Clostridium* species, 18.8 % with Enterobacteriaceae and 3.1 % with *Streptococcus bovis* (Streptococcaceae). The remaining 13.5 % belonged to eight operational taxonomic units whose affiliations were not identified. Methanogens play a vital ecological role in anaerobic environments by removing excess hydrogen and fermentation products yielded by acetogenic bacteria, producing methane. Methanogens are usually coccoid rods or rod-shaped bacteria. There are over 50 described species of methanogens, which do not form a monophyletic group, although all of them belong to the Archaea. Methanogens are strict anaerobes and when they are exposed to an aerobic environment, the oxygen lowers their adenylate charge and causes their death [4]. The physiological differences between HPB (also called *acidogenic* bacteria) and HCB (methanogens, Archaea and homoacetogenic bacteria) are the basis of the scientific rationale behind the development of the various methods proposed to prepare hydrogen-producing seeds [5]. The following list summarizes the main differences between HPB and HCB:

- Most methanogens are limited to a relatively narrow pH range (about 7–8) [4], while most HPB can grow over a broader pH range (4.5–7) [5].
- HPB have much faster growth kinetics than HCB.
- HPB are able to resist harsh environmental conditions due to protective spore formation, while HCB are very sensitive and do not have this capacity.

2.2 Methods of Obtaining HPB

Various authors have described several pretreatment methods applied to sewage sludge in order to select HPB and to inhibit HCB. All of these methods are based on the physiological differences between HPB and HCB described in the previous paragraph. In particular, most of them are based on the ability of HPB to form endospores in unfavorable growth conditions:

- *Thermal treatment*: heat shock (80–110 °C) for a short time (20–60 min), boiling (several hours), sterilization and freezing/thawing (−20/25 °C for 6 h in two cycles) [6].

Heat shock has been widely used [7–12]. It is advantageous because it can assist in sludge solubilization [7, 12]. Kotay and Das [6] found that, among many pretreatments (acid, alkaline, heat, freeze/thaw, microwave, ultrasonication, chloroform), heat shock best augments H_2 production. Mu et al. [13] comparing three pretreatment methods (acid, base and heat shock) also found that heat shock was the best one. Wang and Wan [14] reached the same conclusion: among acid, base, heat shock, aeration and chloroform, they found that heat shock is the best pretreatment, achieving the highest H_2 yield and substrate degradation efficiency. On the other hand, thermal treatment has the disadvantage of a lower net energy yield of the bioH$_2$ process due to high energy demand.

- *Wave and radiation stress*: ultrasonication, microwave and ultraviolet radiation. Some authors have tried to use ultrasonication, microwave and ultraviolet radiation pretreatments, among several others [6], but these do not stand out significantly against the others.
- *pH stress*: acid or alkaline pretreatment.
  Both acid and alkaline pretreatments are generally carried out by adding a strong acid or base, respectively, until a set pH value is maintained for 24 h in anaerobic conditions. Acid chloride (1 or 2 N) and sodium hydroxide (1 or 2 N) are generally used to reach the desired pH. In particular, Chen et al. [15] conducted pretreatments at different pH values: 3–5 for acid pretreatment, and 10–12 for alkaline pretreatment. They found that HPB enrichment at pH 3 (acid) and pH 10 (alkaline) were the most efficacious. However, treatment at pH 3 gave the best HPB enrichment of all the pH values.
- *Use of chemicals*: chloroform, sodium 2-bromoethansulfonate (BESA) and iodopropane.
  These pretreatment methods selectively inhibited methanogenic activity without influencing H_2 production [5, 6]. Zhu and Béland [5] compared six pretreatment methods (acid, base, heat-shock, aeration, 2-bromoethanesulfonic acid and iodopropane) for enriching HPB from digested wastewater sludge. They concluded that the iodopropane pretreatment was the best of the six studied methods. Hu and Chen [16] compared three pretreatment methods (acid, heat-shock and chloroform) and concluded that chloroform was the best. However, the use of these strong chemicals mitigates against the sustainability of the bioH$_2$ process.
- *Aerobic stress*: Giordano et al. [17] evaluated the use of *aerobic stress* to develop HPB: the result was that 3–4 days are a sufficient time to obtain HPB from anaerobic granular sludge. Furthermore, Zhu and Béland [5] found that the aeration method of completely flushing the sludge with air for 30 min was unsuccessful. In fact the methanogenic activity was not totally suppressed, although the seeds prepared by this method should have a more complex bacterial community than those obtained by heat-shock, acid and base pretreatments [5].

In addition, it is possible to continuously inhibit HCB during dark fermentation metabolism by controlling various parameters, such as the pH and the solid retention time, during acetogenesis, based on so-called *kinetic selection*:

- *Kinetic selection* is based on different growth kinetics of microorganisms by an appropriate loading rate or solid retention time. Kinetic selection needs to work in continuous mode with a hydraulic retention time (HRT) shorter than that utilized in CH_4 production in order to have continuous methanogen washout. Biokinetics studies have showed that the specific growth rate μ is greater for HPB than HCB; it is in fact approximately 4–5 times higher than HCB [18]:

$$\mu_{\text{max HPB}} = 0.215 \text{ h}^{-1} \tag{2.1}$$

$$\mu_{\text{max HCB}} = 0.055 \text{ h}^{-1} \tag{2.2}$$

  This means that methanogens need 4–5 times longer residence time in a bioreactor to maintain their vital activity. The fast rate of growth of HPB, higher than that of methanogens, has two consequences: either on the gas production rate or on the HRT as the main process parameter. In terms of productivity, hydrogen production can potentially be obtained much faster than methane, bearing in mind that hydrogen and methane are the metabolites of different populations which grow at different rates. The very marked difference between μ_{HPB} and μ_{HCB} can also be exploited through the management of HRT to slow the process. Methanogenesis needs a much higher HRT than does hydrogen production. The choice of a HRT similar to the characteristic time of the hydrogen-producing reaction means that HCB, on average, are not in contact with the substrate for the necessary time and consequently they are not able to carry out their metabolic functions, with strong inhibition of their activity [19, 20]. Many reviews in the literature report values of the maximum specific growth rate μ_{max} of HPB in the range of 0.08–0.125 h^{-1} [13, 21]. Yang and Shen [22] selected the hydrogen producers by holding the HRT at 12 h ($\mu = 0.083$ h^{-1}) in a continuous fermentation, so HCB were probably washed out at low HRTs since their growth rates were lower than HPB. Because of the differences in the literature about the $\mu_{\text{max HPB}}$ values, this topic needs a deeper analysis from an experimental point of view because of the presence in both HPB or HCB of many microorganisms with different μ_{max} values.
- *Working at a pH outside the optimal range of HCB*: working at a pH outside the optimal range of methanogens is a good way of avoiding continuous HCB activity during acidogenesis: at pH values lower than 6.3 or higher than 7.8 the methanogenesis rate decreases or shuts down [13].

In some cases, a combination of the aforementioned methods may be more effective. Argun and Kargi [23] found in practice that heat pretreatment (boiled sludge), followed by exposure to chloroform, renders more effective the elimination of HCB present in such anaerobic sludge. Venkata Mohan et al. [24] also showed that integration of pH 3 and chemical pretreatment with BESA gives a higher H_2 production. Among the various parameters, pH is considered to be the most useful one, thanks to its easy application and its low energy cost.

After this short review, it will be noted that there have been several studies comparing various pretreatment methods for enriching HPB bacteria from seed

sludge, but with some conflicting conclusions [14]. Since disagreement on the best pretreatment methods exists, a cost-effective method is required, avoiding some technical and economical difficulties which could be present working at the industrial scale. For this reason, acid pretreatment was chosen for our tests, with a view to scaling up pretreatment for H_2-anaerobic technology.

2.3 Experimental Evaluation of Acid Pretreatment of Anaerobic Microflora to Produce Bio-H_2

This study aimed to test the effectiveness and the reproducibility of acid pretreatment of sewage sludge and bovine manure to suppress methanogen activity and to increase HPB activity. Acid pretreatment has several advantages: it could be adapted well in a full-scale plant in which the bioreactor is initially filled with an acid solution and it does not need energy consumption, as occurs with heat treatment, hence no additional devices to generate and transfer heat are necessary. Several experimental tests were performed showing the efficiency of acid conditioning of sewage sludge by using the treated bacteria consortium as inoculum to produce hydrogen by dark fermentation. Both anaerobic wastewater sewage sludge and bovine manure were used, previously treated with 1 N HCl, as inoculum in tests on H_2 production conducted in batch mode by a glucose medium with micro- and macro-nutrients in order to test the effectiveness of acid treatment of both consortia.

2.3.1 Applied Methodology

2.3.1.1 HPB Sewage Sludge Enrichment

The anaerobic microflora that we used was obtained from a digester of municipal waste water treatment plant and from a cow-breeding farm. The pH, density, volatile suspended solid (VSS) and total solid concentration (TSS) of the sewage sludges used were 7.1–7.4, 1,010–1,200 g/L, 10,875–1,325 mg/L and 14,500–18,500 mg/L, respectively. The sludges, before beginning tests on H_2 production, were pretreated with 1 N HCl until pH 3 for 24 h at 35 °C in anaerobic conditions [13, 15]; the pretreatment test was repeated five times with different portions of sewage sludge for the two sludges.

2.3.1.2 Experimental H_2 Production

Tests were carried out using 500 ml Erlenmeyer flasks without agitation, flushed at the beginning with nitrogen gas for 5 min in order to reach strictly anaerobic

Fig. 2.1 Schematic view of batch test conducted in Erlenmeyer flask Gas production was measured by the water-replacement method

conditions. The treated sludges were used as inocula in a ratio of 10 % v/v in five batch anaerobic tests using 50 g/L glucose as carbon source and macro- and micro-nutrient composition as follows (units mg/L): $NaHCO_3$ 1,250, NH_4Cl 2,500; KH_2PO_4 250; K_2HPO_4 250; $CaCl_2$ 500; $NiSO_4$ 32; $MgSO_4 \cdot 7H_2O$ 320; $FeCl_3$ 20; $Na_2BO_4 \cdot H_2O$ 7.2; $Na_2MoO_4 \cdot 2H_2O$ 14.4; $CoCl_2 \cdot 6H_2O$ 21; $MnCl_2 \cdot 4H_2O$ 30; yeast extract 50. The initial pH of the media was set in the range 7–7.5. The experiments were conducted at 35 ± 1 °C in a thermostatically-controlled room. A picture of the simple and effective configuration for the tests is shown in Fig. 2.1.

2.3.1.3 Analytical Methods

Before and after acid treatment, *Clostridium* bacteria and total bacteria content were evaluated by the "*Clostridium* spp. plate count protocol" and "total bacteria count method", respectively [25]. *Clostridium* bacteria were grown in anaerobic conditions for 3 days at 37 °C on "Reinforced Clostridial Agar", adding Polymyxin B sulfate. Total bacteria were grown on "Plate Count Agar" in aerobic conditions for 48 h at 37 °C. The gas evolution during the fermentation tests was monitored using a water-replacement method. The gas composition was evaluated at the end of each test as average composition. Measurements were performed by gas chromatography (Varian CP, 4900) equipped with a thermal conductivity detector (TCD) and two columns of 10 m, using argon as a carrier gas; pH and redox potential were measured with a pH meter (Infors, AG Switzerland) and Pt4805-DXK-S8/120 electrode (Mettler Toledo, Switzerland), respectively; glucose and ammonia concentrations were evaluated via enzymatic bio-analysis (Biopharm-Roche). Total cell growth was monitored by measuring the optical density (OD) at 620 nm (HP 8452A Diode Array Spectrophotometer).
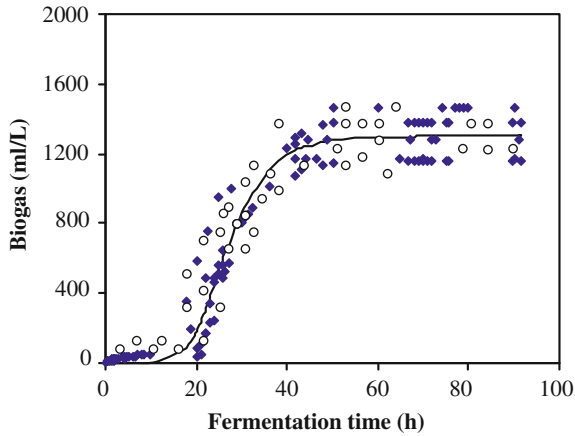
Fig. 2.2 Best fit of total biogas production (H_2 and CO_2) with logistic equation at initial pH (7–7.5), temperature ≈35 °C and initial glucose 50 g/L. ♦ Test with anaerobic wastewater sewage sludge and ○ test with bovine manure, both treated at pH 3 for 24 h at 31 °C

2.3.1.4 Development of Kinetic Model of Biogas (H_2 and CO_2) Production

In this study, cumulative biogas production curves versus time were obtained from the hydrogen production experiments (Fig. 2.2). The experimental data were elaborated by a best-fit procedure, following the modified logistic equation (2.3) known as the Gompertz equation [13] to model the biogas production:

$$V_{gas} = V_{max} \exp\left\{ -\exp\left[\frac{R_{max} * e}{V_{max}}(\lambda - t) + 1 \right] \right\} \qquad (2.3)$$

V_{gas} (mL L^{-1} broth) is the cumulative amount of biogas product (CO_2 and H_2) at reaction time t(h). Equation (2.3) permits evaluation using the potential maximal amount of biogas product $V_{gas, max}$, the maximum biogas production rate R_{max} (mL L^{-1} h^{-1}) and the lag time λ(h).

2.3.2 Results and Discussion

2.3.2.1 Clostridium and Total Bacteria Growth

Total bacteria count and *Clostridium* spp. count were performed on the sludge before and after acid pretreatment and during fermentation. Table 2.1 shows that only 25 % of the total bacteria survived the treatment whereas 72 % of *Clostridium* spp. survived the treatment. This implies that the acid treatment is highly selective

Table 2.1 Effect of acid treatment on *Clostridium* spp. and total bacteria growth

	Before acid treatment (CFU/mL)	After acid treatment (CFU/mL)	Surviving (%)	Fermentation 10 h (CFU/mL)	Fermentation end (CFU/mL)
Clostridium spp.	56,000 ± 1,000	40,400 ± 1,200	72.5 ± 3.5	491,000 ± 1,800	350 × 10^6 ± 9 × 10^6
Total bacteria	371,000 ± 1,500	93,000 ± 950	24.9 ± 0.15	n.d.	n.d.

n.d. not determined

for *Clostridium* with respect to the other bacteria. The protocol utilized for the determination of *Clostridium* spp. is probably not able to detect *Clostridium* spores but only the vegetative form, hence the spores present after acid treatment can germinate when they are placed in favorable conditions. In fact, during the fermentation process, *Clostridium* spp. grew from 4.91×10^5 CFU/mL at 10 h from the beginning of the fermentation to 3.50×10^8 CFU/mL at the end of the test (mean values), increasing more than 700-fold. Total cell mass, monitored over time by the optical density OD, shows a rapid increase during the exponential phase of hydrogen production, as shown by Fig. 2.3a.

2.3.2.2 H$_2$ Production

In Table 2.2 the results of the whole series of experiments are reported. The biogas produced was composed of hydrogen (range 50–70 %) and carbon dioxide (range 50–38 %). The biogas was free of methane and hydrogen sulfide, indicating the lack of methanogenic activities in the sludge after acid treatment.

In order to obtain a regression curve of the 10 tests (Fig. 2.2), (2.3) (and subsequently) was used to best fit the experimental data. The application of equation (2.3) permits estimation of the potential maximal amount of biogas, maximum biogas production rate and lag time, which results in 1,300 ml/L reactor, 75 mL L^{-1} h^{-1} and 18 h, respectively. Figure 2.2 shows that biogas developed after 18–20 h (lag phase), the time in which the microorganisms probably reorganized their molecular constituents to adapt to the new environmental conditions when they were transferred from an extremely unfavorable condition to a favorable one. During the exponential phase (20–48 h) there are the major quantities of biogas evolution, while after 48 h biogas evolution was very low and at approximately 60 h biogas shut-down occurred.

2.3.2.3 Effect of pH, Redox Potential and Substrate Utilization

In Fig. 2.3, a plot of equation (2.1) is shown in relation to cell growth (a), pH and redox (b) and substrate utilization (c) for test No. 4 of Table 2.2 only.
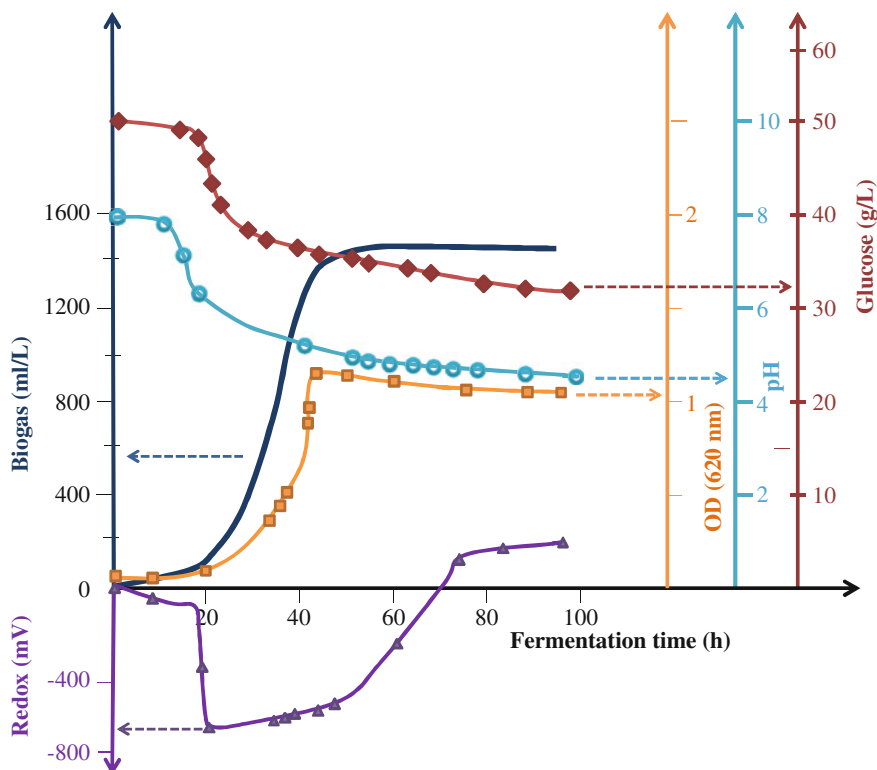
Fig. 2.3 Cumulative biogas (H_2, CO_2) production at 35 °C in relation to cell growth, pH, redox and substrate utilization during fermentation time

Figure 2.3a shows that the H_2 production is coupled with microorganism growth. Figure 2.3b shows that, at the begin of the exponential phase, redox suddenly reached a minimum value of –518 mV, but increased thereafter to reach positive values at the end of fermentation, confirming that the substrate was not oxidized in the oxidative environmental conditions, and hence H_2 was not produced [26].

As shown in Table 2.2, the conversion efficiencies (right-hand column) of H_2 from glucose ranged from about 10–15 %, based on the theoretical stoichiometry of 3 mol H_2 from 1 mol glucose, taking into account the average values between the maximum theoretical production of H_2 from acetic acid (4 mol H_2/mol glucose) and butyric pathways (2 mol H_2/mol glucose), considering, as a rough approximation, that glucose is equally converted into the two volatile fatty acids. These conversion efficiencies are rather less than that obtained by Mu et al. [13] of 43 % in batch tests with acid pretreatment of sewage sludge (based on the assumption of a maximum 3 mol H_2/mol glucose). Our low production efficiency of H_2 from glucose probably

Table 2.2 Summary of total biogas, hydrogen content, glucose utilization, H_2 yield and H_2 conversion efficiency

Run	Gas evolved (ml/L culture)	H_2 content (%)	H_2 content (mmol)	$C_6H_{12}O_6$ consumed (mmol)	H_2 yield $\frac{molH_2}{molC_6H_{12}O_6}$	Y_{H2}/Yth[a] (%)
Anaerobic wastewater sewage sludge						
1	1463.64	60.40	39.44	93.88	0.42	14.00
2	1373.82	61.82	37.89	101.94	0.37	12.33
3	1154.53	62.85	32.37	105.06	0.31	10.33
4	1274.58	52.18	29.67	102.28	0.29	9.67
5	1176.44	55.53	29.14	99.33	0.29	9.67
Mean	1289 ± 131	59 ± 5	34 ± 5	100 ± 4	0.34 ± 0.06	11.20 ± 1.91
Bovine manure						
1	1560.31	61.32	42.71	91.81	0.46	15.33
2	1480.14	71.84	47.47	100.10	0.47	15.66
3	1250.36	56.70	31.65	95.06	0.33	11.00
4	1110.42	65.72	32.58	103.15	0.32	10.66
5	1325.10	58.32	34.50	98.75	0.35	11.66
Mean	1347 ± 181	63 ± 6	38 ± 7	98 ± 4	0.39 ± 0.07	12.86 ± 2.4

[a] Y yield; Yth (theoretical yield) = 3 $molH_2/molC_6H_{12}O_6$ taking into account the average values between the maximum theoretical H_2 production from the acetic and butyric pathways of 4 and 2 $molH_2/mol$, respectively

depends on the absence of agitation, leading to a high dissolved H_2 concentration that inhibits H_2 and produces enzymes. It is important to point out that acid treatment has the same effect on the different sources of anaerobic microflora (anaerobic wastewater sewage sludge and bovine manure), as confirmed by the conversion efficiency.

In fact, as discussed in Chap. 1, the pool of reduced Fd is generated from two sources: (1) pyruvate oxidation by ferredoxin oxidoreductase (PFOR) and (2) NADH oxidation by NADH, Fd oxidoreductase (NFOR). These enzyme systems can be thermodynamically regulated by the H_2 concentration dissolved in the liquid phase; PFOR can function at the H_2 concentrations observed in fermentative systems so there will always be some H_2 produced, whereas NADH oxidation by NFOR is inhibited at a concentration of H_2 measured as partial pressure in the off-gas $P_{H2} > 60$–100 Pa (~ 0.5–0.8 μM).

It is emphasized that the purpose of the present experimental work was not the kinetics set-up of H_2 production from glucose, but only the effectiveness and reproducibility of the acid pretreatment of such anaerobic microorganism consortia, in order to use them as enriched HPB as inoculum in a bioreactor plant to produce hydrogen.

2.4 Conclusion

The experimental results obtained in this chapter clearly reveal that acid pretreatment is an effective method of increasing hydrogen-forming bacteria in an anaerobic microorganism consortium and is able to avoid methanogenesis during fermentation. The results of the tests performed on the activity of the treated sludge, repeated five times for each consortium, as bio-hydrogen-producing inoculum in anaerobic fermentation are more than acceptable, indicating that acid pretreatment is a suitable process for preparing the inoculum for a bioreactor producing hydrogen in a full-scale application. The high H_2 gas concentration (50–70 %) and the absence of methane confirm the feasibility of acid treatment. Thus, acid treatment is highly selective for *Clostridium* spp. with respect to other bacteria, including facultative bacteria. Facultative anaerobic bacteria are less sensitive to oxygen than strict anaerobes, i.e. Clostridia, and they are sometimes able to recover hydrogen production activity after accidental oxygen introduction by rapidly depleting oxygen which might be present in the digester. Therefore, it could be feasible to use in a full-scale plant in which the bioreactor is filled with an acid solution in the first phase; this approach does not need high energy expenditure, as occurs in the case of heat treatment. The H_2 production monitored during the evolution of fermentation shows that H_2 is mainly produced by a cellular growth phase in which glucose (except that utilized for cellular reproduction) is converted to volatile fatty acids (VFA), as shown by the decrease in pH.

References

 1. T.D. Brock, M.T. Madigan, J.M. Martino, J. Parker, *Biology of microrganisms* (Prentice-Hall, New york, 1994)
 2. R.Y. Stanier, L. John Ingraham, L. Mark, P.D. Wheelis, P.R. Painter, *The Microbial World* (Prentice Hall, Englewood Cliffs, 1986)
 3. H.H.P. Fang, T. Zhang, H. Liu, Microbial diversity of a mesophilic hydrogen-producing sludge. Appl. Microbiol. Biotechnol. **58**, 112–118 (2002)
 4. R.S. Oremland, in *Biology of Anaerobic Microorganisms*, ed. by A.J.B. Zehnder. Biogeochemistry of Methanogenic Bacteria (Wiley Press, New York, 1988), pp. 641–705
 5. H. Zhu, M. Béland, Evaluation of alternative methods of preparing hydrogen producing seeds from digested wastewater sludge. Int. J. Hydrogen Energy **31**, 1980–1988 (2006)
 6. S.M. Kotay, D. Das, Novel dark fermentation involving bioaugmentation with constructed bacterial consortium for enhanced biohydrogen production from pretreated sewage sludge. Int. J. Hydrogen Energy **34**, 7489–7496 (2009)
 7. M. Yang, H.Q. Yu, G. Wang, Evaluation of three methods for enriching H_2-producing cultures from anaerobic sludge. Enzyme Microb. Technol. **40**, 947–953 (2007)
 8. J.J. Lay, Y.J. Lee, T. Noike, Feasibility of biological hydrogen production from organic fraction of municipal solid waste. Water Resour. **33**(11), 2579–2786 (1999)
 9. S.S. Cheng, M.D. Bai, S.M. Chang, K.L. Wu, W.C. Chen and W.C. Chen, *Studies on the Fasibility of Hydrogen Production Hydrolyzed Sludge by Anaerobic Microorganism*, in The 25th Wastewater Technology Conference, Yunlin, Taiwan, 2000
10. S.M. Kotay, D. Das, *Studies on Maximization of Microbial Production of Hydrogen from Pretreated Sewage Sludge, IIChE- CHEMCON 2006* (Ankleshwar, India, 2006)

11. L. Guo, X.M. Li, X. Bo, Q. Yang, G.M. Zeng, D.X. Liao, J.J. Liu, Impacts of sterilization, microwave and ultrasonication pretreatment on hydrogen producing using waste sludge. Bioresour. Technol. **99**(9), 3651–3658 (2008)
12. J. Kim, C. Park, T.H. Kim, M. Lee, S. Kim, S.W. Kim, J. Lee, Effects of various pretreatments for enhanced anaerobic digestion with waste activated sludge. J. Biosci. Bioeng. **95**(3), 271–275 (2003)
13. Y. Mu, H.Q. Yu, G. Wang, Evaluation of three methods for enriching H_2-producing cultures from anaerobic sludge. Enzyme Microb. Technol. **40**(4), 947–953 (2006)
14. J. Wang, W. Wan, Comparison of different pretreatment methods for enriching hydrogen-producing bacteria from digested sludge. Int. J. Hydrogen Energy **33**, 2934–2941 (2008)
15. C.C. Chen, C.Y. Lin, M.C. Lin, Acid-base enrichment enhances anaerobic hydrogen production process. Appl. Microbiol. Biotechnol. **58**, 224–228 (2002)
16. B. Hu, S. Chen, Pretretment of methanogenic granules for immobilized hydrogen fermentation. Int. J. Hydrogen Energy **32**(15), 3266–3273 (2007)
17. A. Giordano, R. Farina, M.C. Lavagnolo, A. Spagni, Biological Hydrogen production from mixed cultures using aeration pretreatment, in *Proceedings Second International Symposium on Energy from Biomass and Waste* in Venice, 2008
18. B.E. Ritmann, P.L. McCarty, *Environment Biotechnology*: *Principle and Application* (McGraw Hill Press, New York, 2001)
19. I. Valdez-Vazquez, H.M. Poggi-Varaldo, Alkalinity and high total solids affecting H2 production from organic solid waste by anaerobic consortia. Int. J. Hydrogen Energy **34**, 3639–3646 (2009)
20. I.C. Clark, R.H. Zhang, S.K. Upadhyaya, The effect of low pressure and mixing on biological hydrogen production via anaerobic fermentation. Int. J. Hydrogen Energy **37**, 11504–11513 (2012)
21. W.M. Chen, Z.-J. Tseng, K.-S. Lee, J.-S. Chang, Fermentative hydrogen production with Clostridium Butyricum CGS5 isolated from anaerobic sewage sludge. Int. J. Hydrogen Energy **30**(10), 1063–1070 (2005)
22. H. Yang, J. Shen, Effect of ferrous iron concentration on anaerobic bio-hydrogen production from soluble starch. Int. J. Hydrogen Energy **31**(15), 2137–2146 (2006)
23. H. Argun, F. Kargi, Effect of acid pretreatment method on bio-hydrogen production by dark fermentation of waste ground wheat. Int. J. Hydrogen Energy **34**, 8543–8548 (2009)
24. S. Venkata Mohan, V. Lalit Babu, P.N. Sarma, Effect of various pretreatment methods on anaerobic mixed microfolora to enhance biohydrogen production utilizing dairy wastewater as substrate, Bioresour. Technol. **99**(1), 59–67 (2008)
25. A. Galli, L. Franzetti, *Metodi per l'analisi Microbiologica Degli Alimenti* (DISTAM Università degli Studi di Milano Press ,Milano, 2003)
26. A.C. Van Handel, G. Lettinga, *Anaerobic Sewage Treatment- A Practical Guide for Regions with a Hot Climate* (Wiley Press, New York, 1994)