Improving biohydrogen production using *Clostridium beijerinckii* immobilized with magnetite nanoparticles

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Abstract In order to supplement the need for alternative energy resources within the near future, enhancing the production of biohydrogen with immobilized *Clostridium beijerinckii* NCIMB8052 was investigated. Magnetite nanoparticles were functionalized, with chitosan and alginic acid polyelectrolytes using a layer-by-layer method, to promote bacterial attachment. Cultivating *C. beijerinckii* with these nanoparticles resulted in a shorter lag growth phase and increased total biohydrogen production within 100-ml, 250-ml and 3.6-L reactors compared with freely suspended organisms. The greatest hydrogen yield was obtained in the 250-ml reactor with a value of 2.1 ± 0.7 mol H₂/mol glucose, corresponding to substrate conversion and energy conversion efficiencies of 52 ± 18 and 10 ± 3 %, respectively. The hydrogen yields obtained using the immobilized bacteria are comparable to values found in literature. However, to make this process viable, further improvements are required to increase the substrate and energy conversion efficiencies.

Keywords Biohydrogen · *Clostridium beijerinckii* · Magnetite · Chitosan · Alginic acid · Immobilization

Introduction

The global population's energy and carbon usage has increased significantly in recent years (Pielke et al. 2008), while current projections indicate energy consumption will increase another 35 % by 2035 compared to 2008. Of this future energy demand, countries outside the Organization for Economic Cooperation and Development (OECD) are expected to contribute an 85 % increase in their energy usage. The majority of this energy is derived from oil and coal resources (Conti and Holtberg 2011) and thus their use increases the atmospheric CO₂ concentrations, contributing to global climate change (Kothari et al. 2010). As a result, alternative energy resources with minimal atmospheric carbon contributions should be investigated to help offset future global energy needs. Finding such alternative resources would accomplish two significant objectives. First, atmospheric carbon emissions would be reduced, decreasing the overall impact on global climate change. Secondly, an alternative energy resource would help lower the differences in environmental footprints between nations having different levels of access to conventional sources of energy. Reaching these objectives would create a more sustainable global energy market (Cranston and Hammond 2010). Renewable energy resources typically produce lower carbon emissions and thus have a reduced environmental impact (Conti and Holtberg 2011; Cullen and Allwood 2010). Hydrogen is considered a strong alternative fuel source as it has the largest energy density per unit mass of all known fuels (142 kJ/g) (Ghosh and Hallenbeck 2014) and is considered a “clean fuel” as water is its primary byproduct (Brentner et al. 2010). At present, 95 % of hydrogen is produced through fossil fuel-dependent processes, primarily steam methane reforming of natural gas (Brentner et al. 2010). Other such processes include thermal cracking of natural gas, partial oxidation of hydrocarbons, and coal gasification (Hallenbeck and Ghosh 2009). Most other hydrogen is produced as a by-

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product of chemical processes with water electrolysis contributing a small amount (Brentner et al. 2010). However, not only do the majority of the above processes rely on non-renewable fossil fuel resources, but they also occur at high temperatures or pressures and are thus energy intensive (Hallenbeck and Ghosh 2009). Hydrogen production through biological processes (biohydrogen) is an alternative production scheme based solely on various renewable resources. Biohydrogen production that does not rely on fossil fuels is typically performed at moderate temperatures and is considered carbon neutral; therefore, it has a no net increase in carbon emissions. As a result, if biohydrogen can be produced in an economically feasible manner, it can be an important alternative to current fuel sources.

Biohydrogen is typically produced through one of four processes: biophotolysis of water, indirect biophotolysis, photofermentation, and dark fermentation. There are pros and cons to each method; however, biophotolysis and indirect biophotolysis have low photochemical efficiencies (Brentner et al. 2010; Hallenbeck and Ghosh 2009), while photofermentation systems do not currently produce hydrogen at a competitive rate (Brentner et al. 2010). Dark fermentation is performed by anaerobic organisms (strict and facultative) such as *Clostridium beijerinckii*. This process is often accompanied by low substrate conversion efficiency due to competing metabolic pathways as outlined in Fig. S1. However, dark fermentation produces the highest volumetric amount of hydrogen and offers numerous areas for improvement including immobilizing microorganisms, optimizing process conditions, and genetically modifying organisms.

Immobilizing microorganisms on solid substrates retains biomass within the reactor, which has been shown to increase volumetric hydrogen production (Bartacek et al. 2007). For example, *Pseudomonas delafieldii*, used in an immobilized process to desulfurize a model oil (Li et al. 2009), showed that immobilized and free cultures had similar desulfurizing activity during the first batch cycle. However, immobilized cells could be reused for up to seven batch cycles while retaining 80 % of their specific desulfurizing activity compared to a 15 % retained activity for the suspended cells (Li et al. 2009). Further, the production of biohydrogen from different carbon sources has been studied extensively in *Clostridium* sp. (Ghosh and Hallenbeck 2014), but limited immobilization studies with the objective of producing hydrogen have been carried out with this strain. Some work has been done using immobilizing materials such as polyethylene glycol (Singh et al. 2013), calcium alginate beads (Zhao et al. 2012), polyurethane-activated carbon matrix (Mitchell et al. 2009), ethylene-vinyl acetate copolymer (Lin et al. 2009), polyurethane foam (Jo et al. 2008), silicone gel (Lin et al. 2006), and acetyl cellulose filter (Karube et al. 1982). Although in these works hydrogen yield and productivity were improved using immobilized bacteria

compared to suspended cells, major issues still remain about the reusability, stability, and regeneration of these immobilizing materials.

In this study, a novel immobilization technique was investigated, utilizing super paramagnetic nanoparticles (NPs) as the solid substrate for attachment. Using a substrate of this nature would allow organisms to be retained in the bioreactor from batch to batch by applying a magnetic field. This magnetic field separates biomass from the liquid media, allowing fresh liquid to be added to begin new growth and biohydrogen production phases. Therefore, it was hypothesized that successful immobilization of the anaerobic dark fermentation organism *C. beijerinckii* would reduce the lag growth phase between dark fermentation batches; resulting in higher volume of hydrogen produced over a given time period compared to a freely suspended culture. Therefore, overall volumetric hydrogen production would increase compared to a non-immobilized system over repeated batch cycles, defined as the time from fresh media addition to spent liquid removal. Such a process based on magnetic separation of immobilized bacteria has not yet been designed for hydrogen production, and thus this novel research may help to reduce one of the economical barriers to scale up of an industrial biohydrogen production process.

Materials and methods

Chitosan (low molecular weight, 75–85 % deacetylated), NaCl, and magnetite nanoparticles (nano powder, <50 nm) were purchased from Sigma-Aldrich (Oakville, ON, Canada). Glacial acetic acid and NaOH were purchased from Fisher Scientific (Ottawa, ON, Canada). Alginic acid (low viscosity) was purchased from MP Biomedicals (Solon, OH, USA).

Bare magnetite NPs were functionalized prior to immobilization using a layer-by-layer (LbL) technique in order to enhance growth of the bacteria on the surface of the particles. The outermost layer must possess traits to promote bacterial attachment such as biocompatibility, electrostatic, or hydrophobic characteristics or induce nano-size effects when interacting with the bacterial cell wall (Li et al. 2009).

This LbL method relies on electrostatic interactions between the core nanoparticles and each subsequent polyelectrolyte layer of opposite charge. Thus, negatively charged core nanoparticles are first coated with a cationic polyelectrolyte, inverting the overall surface charge. An anionic polyelectrolyte can then be deposited as a second layer, again inverting the overall surface charge. This process is repeated for any number of layers whereby the final surface characteristics of the NP–polyelectrolyte hybrid are determined by the final layer (Decher 1997; Masereel et al. 2011). Appropriate polyelectrolytes must be chosen when working with biological

samples. Primarily, the layer materials must be biocompatible and non-toxic; two common characteristics of many biopolymers. Thus, the procedure used here consisted in depositing chitosan (cationic polyelectrolyte) onto the magnetite NP surface followed by alginic acid (anionic polyelectrolyte) as depicted in Fig. S2. Chitosan is a polyaminosaccharide composed of repeating glucosamine units. Thus, it contains amino groups providing a cationic charge (Pillai et al. 2009). Conversely, alginic acid is composed of α -L-guluronate and β -D-mannuronate units with carboxyl functional groups providing a net anionic charge (Ye et al. 2005). This alginic acid layer is expected to provide the desired conditions discussed above for bacterial attachment to the particle surface. A 2-g/L chitosan solution was prepared in 2 % acetic acid containing 0.01 M NaCl (prepared in reverse osmosis (RO) water). This solution was filtered using P8 filter paper and degassed for 20 min in a Branson 2510 sonicating bath. Dry magnetite NPs were added to the chitosan solution under sonicating to a final concentration of 0.1 g/L magnetite. This solution was sonicated for 1 h, shaking by hand every 15 min. NPs were washed twice with RO water (pH 3 adjusted using acetic acid) by centrifugation at 10,000 rpm for 20 min followed by re-suspension in RO water (pH 3). After two wash cycles, the particles were again centrifuged at 10,000 rpm for 20 min before being re-suspended in RO water (pH 3). Chitosan-coated NPs (C-NPs) were then coated with alginic acid (A-C-NPs) according to the same procedure, except all aqueous solutions were set to pH 7 with NaOH.

Confirming coatings and preparing for immobilization

The presence of chitosan and alginic acid coatings were confirmed through zeta potential measurements and TEM images. The zeta potential of bare magnetite nanoparticles, C-NPs and A-C-NPs were determined using a Malvern Zetasizer Nano ZS90. TEM images were obtained using a Philips CM200 200 kV TEM. Even though C-NPs would in theory have a greater electrostatic interaction with a negatively charged bacterial cell wall, C-NPs poor solubility in neutral solutions resulted in agglomeration, reducing the overall surface area available for immobilization. Therefore, only A-C-NPs were used for immobilization studies. Prior to use in the biohydrogen production experiments, A-C-NPs were transferred to 100 ml serum bottles sparged with argon gas to remove all dissolved oxygen, sealed with butyl rubber stoppers and capped with aluminum crimps before being autoclaved at 121 °C for 30 min and cooled down for later use in test bottles.

Immobilizing *C. beijerinckii* (NCIMB8052) and hydrogen production experiments at the 100-ml scale

C. beijerinckii (NCIMB 8052) were inoculated into 100-ml culture bottles containing 50 ml of oxygen free 2xTY media

(5 g/L glucose as a carbon source) and grown to mid-log phase at 35 °C and 100 rpm. 2xTY media was chosen because of its main constituents: tryptone providing nitrogen sources and amino acids required for bacterial growth and yeast extract acting as an adequate vitamin source. Glucose was added to the media considering that our preliminary work with *C. beijerinckii* in 2xTY media without glucose (results not shown) resulted in very poor growth (O.D around 0.2–0.3 after 18 h). Autoclaved A-C-NPs prepared previously were then added to the growing 100-ml culture bottle to a final concentration of 0.25 g/L and incubated for 6 h. This initial incubation, known as the pre-culture preparation, was meant to allow exponentially growing bacteria to interact with the A-C-NPs and complete the immobilization process. After this time period, a permanent magnet was applied to the culture bottle to separate the nanoparticles and immobilized biomass from the liquid media. The liquid phase was removed and 4 ml of anaerobic phosphate buffered saline (PBS) buffer were added as a washing step. A permanent magnet was again applied to the culture bottle, separating the nanoparticles and immobilized biomass from the PBS buffer. The buffer was then removed and 30 ml of fresh 2xTY media with 5 g/L glucose was added in order to start the biohydrogen production experiment. All the transfers mentioned above were performed using the Hungate technique in order to maintain anaerobic conditions in the test bottles.

Total gas production was measured every 2 h over the sampling period of up to 24 h and analyzed for hydrogen concentration. Suspended biomass growth was measured at specific time intervals via optical density measurements. After approximately 24 h of growth, a permanent magnet was again applied to the culture bottle, separating the immobilized biomass from the liquid media and non-immobilized bacteria. This spent liquid and non-immobilized bacteria was then removed and 30 ml of fresh 2xTY media with 5 g/L glucose was added to begin the second fermentation cycle. After approximately 17 h of growth, this cycling process was repeated for a third fermentation cycle of approximately 12.5 h in length. Cycling time periods were ended when the stationary growth phase was reached. All trials were performed in duplicate. Controls consisted of test bottles without nanoparticles.

Scale-up of biohydrogen production

In order to further evaluate the effects of immobilizing *C. beijerinckii* on the prepared NPs, scale-up trials involving 100-ml, 250-ml, and 3.6-L batch reactors were performed. The fermentation conditions and cycling procedures were identical to the previously described method for the immobilization trials with slight differences: 100-ml trials were performed without the PBS washing step; the 250-ml reactors consisted of 250-ml Wheaton bottles with screw caps

containing gray butyl rubber septa and the 3.6-L batch trials were performed in a 3.6-L Infors HT Labfors 4 bioreactor with X-DDC operating panel. The 3.6-L reactor was also supplied with 10 % (w/v) NaOH to maintain reactor pH at 7.0 and a 1 % (v/v) solution of Antifoam A to prevent foaming. A water-circulating jacket was also used to maintain temperature at 35 °C within the reactor and agitation was applied via a dual impeller at 500 rpm. The 3.6-L reactor trials represent preliminary scale-up as only one trial was performed.

The A–C–NP concentration was also increased in an effort to achieve a greater amount of immobilized biomass and in accordance with NP concentrations used in literature (Ghosh and Pramanik 2010; Ghosh et al. 2010, 2011). Cycling time periods were decreased to a maximum of 13 h to prevent stationary phase cell death from influencing the activity of immobilized bacteria. The only exception to this rule came if biomass growth in all samples had not reached late exponential phase by 13 h. Finally, a greater volume of fresh media was used to initiate each cycle. Differences in reactor operational parameters are outlined in Table 1. Initial and cycled media volumes refer to the amount of fresh 2xTY media used for initial immobilization (prior to cycle 1) and 2xTY media added following each cycling period, respectively.

Analytical methods

Total gas production was measured using a frictionless syringe, every 2 h during the immobilization studies and every 3–10 h during the scale up study. Hydrogen concentration in the biogas produced was analyzed using a Hewlett Packard GC model 5890 gas chromatograph equipped with a 6A molecular sieve column and a thermal conductivity detector (TCD). The column temperature was maintained at 80 °C, injection temperature at 100 °C and TCD at 100 °C with 2.0 ml/min Argon as a carrier gas. Two-milliliter gas samples were collected from the reactor using a gas-tight Hamilton 2-ml syringe for injection into the GC. Biomass production was also measured every 3–10 h via optical density measurements. The permanent magnet was first placed against the side of the reactor to separate immobilized biomass from the bulk

solution. From this bulk solution, 1-ml samples were extracted and measured at 600 nm with Ocean Optics CHEMUSB4-UV–VIS spectrophotometer, (200–850 nm). Substrate consumption during initial immobilization trials was analyzed using the Glucose GO Assay kit from Sigma-Aldrich (Oakville, ON, Canada), following the manufacturer's experimental procedure.

During scale-up trials, glucose and fermentation metabolite concentrations were also analyzed by high-performance liquid chromatography (HPLC). An HP model 1050 HPLC equipped with a Resex ROA Organic Acid H+ 8 % 150×7.8 mm column was used to measure concentrations of pyruvate, lactate, formate, acetate, butyrate, ethanol, and glucose. The mobile phase used was 0.035 M H₂SO₄ at a flow rate of 0.8 ml/min and run time of 12 min. The column temperature was maintained at 35 °C. UV and refractive index (RI) detectors were used to quantify organic acids and alcohols, respectively. The RI detector was used to measure glucose concentration in each sample. The UV detector was operated at 195 nm while the RI detector was maintained at 35 °C.

Results

Nanoparticles coating confirmation

The zeta potential (ζ) of bare NPs, C–NPs, and A–C–NPs were determined to verify the presence of each coating. Chitosan carries a characteristic positive charge, while alginate acid induces an anionic surface charge. Each value reported in Table 2 represents an average of six zeta potential readings from different batches and errors represent standard deviations between repeated readings. The zeta potentials of coated particles were also measured following autoclaving to determine the effect of sterilization on the coatings. Comparison of the zeta potential before and after autoclaving indicated some coating loss; as indicated by a change of 12–23 % of zeta potential.

Table 2 Zeta potential of bare magnetite nanoparticles, C–NPs and A–C–NPs under varying conditions

Sample	ζ , pH 3 (mV)	ζ , pH 7 (mV)	ζ , post-autoclave, pH 7 (mV)
Bare NPs	−5.3 (±0.9)	−28.2 (±1.2)	N/A ^d
C–NPs	47.1 (±2.1)	11.4 (±1.7)	7.2 (±1.2)
A–C–NPs	N/A ^a	−45.9 (±3.8)	−35.3 (±2.0)

NPs nanoparticles, C–NPs chitosan nanoparticles, A–C–NPs alginate–chitosan–nanoparticles

^a N/A represents unachievable conditions due to the polyelectrolyte properties (A–C–NPs, pH 3) or irrelevant conditions to immobilization (autoclaved bare nanoparticles, pH 7)

Table 1 Operational parameter differences between the three reactor scales

Reactor	A–C–NP Conc. (g/L)	Initial media volume (L)	Cycled media volume (L)
100 ml ^a	0.25	0.040	0.030
100 ml	0.30	0.050	0.030
250 ml	0.30	0.125	0.125
3.6 L	0.31	1.125	1.000

^a Immobilization

TEM images were also analyzed to visually confirm the presence of the layers and ensure that coated particles remained below 100 nm in diameter. Figure 1 displays images confirming that the nanoparticles size remained below 100 nm and the white arrows in Fig. 1b, c indicate the presence of chitosan and chitosan–alginate layers (lighter color surrounding the particles), respectively. Therefore, the layer-by-layer method successfully functionalized the magnetite NPs, preparing them for bacterial immobilization.

Bacterial immobilization confirmation

Initial trials in the 100-ml reactor indicated increased gas production and a shorter lag growth phase with A–C–NPs compared to controls (no nanoparticles) but only for the second immobilization cycle, as depicted in Fig. 2a (gas production) and Fig. 2b (growth). There were no large differences in hydrogen production between the A–C–NP samples and control during the first and third cycles. However, during the second cycle, A–C–NP samples produced a 3-fold increase in hydrogen compared to the control. The differences observed in the various cycles were not explained but hypothesis are provided in the discussion section. The results obtained, particularly the shorter lag growth phase, indicated that more biomass was retained within the A–C–NP reactors than in the controls. To confirm this retention was due to the attachment of NPs to the bacterial cell wall, samples incubated with NPs during the pre-culture step were imaged. Results shown in Fig. 3a confirm that immobilization occurred in the first cycle. A second sample, consisting of bacteria that had been cycled once, is shown in Fig. 3b and confirms that the cycling process did not reverse the immobilization procedure or strip NPs from the cell wall. Although Fig. 3a suggests a possible agglomeration of NPs on the surface of the bacteria, these images confirm that the immobilization procedure was successful in attaching A–C–NPs to bacterial cell walls.

Fermentation results

Over the course of three fermentation cycles, the 100-ml reactors containing NPs produced 85.1 ml of hydrogen as depicted in Fig. 2a. This equates to a volumetric production of $14.2 (\pm 1.5)$ ml- H_2 /L/h and molar production of $0.6 (\pm 0.1)$

mmol/L/h. Liquid samples collected from the reactors containing NPs were analyzed using the HPLC method described in the analytical methods section. The only liquid metabolites produced in measureable quantities were ethanol, acetate, and formate, as shown in Fig. 4. The patterns of production were similar from one cycle to another, with glucose conversion in the 16–32 % range, ethanol and acetate produced in comparable amounts and a lower production of formate (about three times less than the other metabolites).

The resulting total hydrogen, ethanol, acetate, and formate production across all cycles of each reactor are presented in Fig. 5 to facilitate comparison of results at the different scales tested. The 250-ml reactors experienced a significant loss of immobilized biomass during the cycling process, which resulted in large errors in hydrogen production and biomass growth during cycle two. This loss was due to limited ability to retain the NPs using the magnets available at that time. Due to such a large loss of A–C–NPs, the 250-ml reactor was only cycled twice (as opposed to three times). Bigger magnets were used for the 3.6-L reactor and it was possible to achieve three cycles in this larger reactor. The 250-ml reactor resulted in a volumetric hydrogen production of $77.6 (\pm 33.4)$ ml/L/h (comparable to the value of 85.1 ml/L/h obtained in the immobilization study) and molar production of $3.2 (\pm 1.4)$ mmol/L/h (see Fig. 5). The 3.6-L dataset are preliminary in nature (no replicate) but results indicated a volumetric hydrogen production of 37.6 ml/L/h and molar production of 1.6 mmol/L/h, values lying between the other two scales tested. The relative production profiles of all metabolites were similar across all scales (ethanol and acetate representing the primary and secondary metabolites respectively), however, formate was not detected at the largest scale. The 3.6-L reactor dataset suggest a shift in metabolic processes at this larger scale as no formate was produced in measureable quantities.

Process efficiencies

Substrate conversion efficiency (SCE), energy conversion efficiency (ECE) and specific hydrogen productivity (SHP) were determined to evaluate the overall performance of the

Fig. 1 TEM images of bare magnetite (a), C–NPs (b) and A–C–NPs (c). The presence of coating can be seen as a light color layer surrounding the particles, as indicated with white arrows

Fig. 2 Cumulative hydrogen production (a) and biomass profile (b) observed in 100-mL reactors containing bacteria immobilized on A–C–NPs (A–C–NP) or suspended bacteria (Control). Error bars represent the maximum and minimum values of the experimental runs. Suspended biomass growth was measured by optical density at 600 nm

hydrogen production process. SCE was calculated based on the complete theoretical conversion of glucose to hydrogen, yielding 4 mol of hydrogen for every 1 mol of glucose consumed (Levin et al. 2004). Therefore, the SCE was calculated according to Eq. 1:

$$\text{SCE} = \frac{\text{mol}_{\text{H}_2 \text{ produced}}}{\text{mol}_{\text{glucose added}}} \cdot \frac{1 \text{ mol glucose}}{4 \text{ mol}_{\text{H}_2}} \cdot 100\% \quad (1)$$

The amount of hydrogen produced was converted from volume to moles assuming hydrogen acted as an ideal gas. The pressure was assumed to be atmospheric (1 atm) and at room temperature in the lab (21 °C). The ECE was calculated comparing the total specific energy of glucose added to the system to the total specific energy of hydrogen produced. The specific energy (or energy density) of hydrogen is 142 kJ/g (Ghosh and Hallenbeck 2014), while that of glucose is 16 kJ/g (Kraisid 2003). Therefore, the ECE is given by Eq. 2:

$$\text{ECE} = \frac{m_{\text{H}_2} \cdot E_{\text{H}_2}}{m_{\text{glucose}} \cdot E_{\text{glucose}}} \cdot 100\% \quad (2)$$

Fig. 3 TEM images of pre-cultured bacteria (a) and cycled bacteria (b). Black arrows represent visual confirmation of NPs attached to the bacterial cell wall

m_x represents the mass of hydrogen produced or glucose added in grams, while E_x represents the specific energy of the corresponding component.

The SHP based on biomass growth was determined from the ratio of hydrogen produced to maximum biomass reading according to Eq. 3:

$$\text{SHP} = \frac{\text{mmol}_{\text{H}_2 \text{ produced}}}{\text{OD}_{600}} \quad (3)$$

Figure 6 depicts the efficiencies and specific productivity calculated for each reactor size. This data indicates an initial increase in efficiency when scaling up to the 250-ml reactors, followed by a large decrease when scaled up further to the 3.6-L reactor. The exception to this trend was the specific hydrogen productivity normalized against biomass optical density reading, which significantly increased at the highest reactor scale. Large changes in optical density measurements were not expected from scale to scale; however, the amount of hydrogen produced was expected to vary directly with reactor size, which might explain this result.

Fig. 4 Profiles of the concentration of glucose and three metabolites over 3 cycles of production in 100-mL reactors containing bacteria immobilized on A–C–NPs. Metabolites include ethanol, acetate, and formate. Error bars represent the maximum and minimum values of the experimental runs

Discussion

Cycling and scale up of the reactors

Based on the results presented above, the addition of A–C–NPs can provide greater biomass retention between cycles and shorten each lag phase (Fig. 2, data not shown for the 250-mL and 3.6-L reactors). The greater hydrogen production obtained in the second fermentation cycle of the 100-mL reactors containing A–C–NP (3-fold increase) was likely due to an acclimation period during the first cycle. An increase in total hydrogen production was also observed in the larger-scale reactors containing A–C–NP when compared to their respective controls operated with suspended cells (23 % increase for the 250-mL reactor and 98 % increase in the 3.6-L reactor, data not shown). However, acclimation over the cycle was not significant for the 250-mL and 3.6-L reactors. The variability observed between the cycles performed in the 100-mL reactor

Fig. 5 Total hydrogen and metabolite production for the three reactor scales using bacteria immobilized on A–C–NPs. Error bars represent the maximum and minimum values of the experimental runs

Fig. 6 Substrate conversion efficiency (SCE), energy conversion efficiency (ECE) and specific hydrogen productivity (SHP) for the three reactor scales. Error bars represent the maximum and minimum values of the experimental runs

but not in the larger scale reactors might be explained by the small total initial population inoculated in the 100-mL reactors.

The overall SCE increased during scale-up to 250 ml but decreased significantly when scaled up to 3.6 L. This may be due to changes in metabolic pathways as the large decrease in SCE was not accompanied by a corresponding increase in the ethanol detected. In evaluating the specific trends in metabolite production (Fig. 5), ethanol concentrations remained relatively constant between scales while acetate and formate concentrations exhibited a pattern following the changes in SCE and were at lower concentrations in the 3.6-L reactor. One hypothesis is that the differences in SCE are associated with the formation of numerous other metabolites that were produced in quantities below the limit of quantification of our method and instrument. Such potential metabolites include lactate, propionate, acetaldehyde, and butyrate. The formation of butyrate would have a compounding effect, as its production would also result in higher CO_2 production (Levin et al. 2004). Looking at the hydrogen concentration measured in the reactor head spaces, which ranged from 27 % to 45 % in the 100-mL reactors, 27–74 % in the 250-mL reactors, and 20–37 % in the 3.6-L reactor, no clear trend was observed between the various scales suggesting that butyrate was not produced in greater quantities. This is likely due to the primary production of ethanol competing with the butyric acid pathway (Lee et al. 2008). The production of acetate during fermentation is an unavoidable by-product of hydrogen production and it is only produced under the regeneration of ATP (cellular energy), which can be used to further produce biomass (and thus indirectly produce hydrogen) as shown in Fig. S1. Therefore the presence of acetate is not intolerable. Ethanol, however, is a by-product of a metabolic pathway that not only terminates without additional hydrogen production, but produces metabolites which compete with hydrogen producing reactions. This pathway utilizes 1 mol of NADH to

Table 3 Hydrogen yield and molar productivity comparison under different reactor scales and operations

Responses	Volume of the reactor			Rittmann Herwig index (Rittmann and Herwig 2012)		
	100 ml	250 ml	3.6 L	Min.	Med.	Max.
$Y_{(H_2/S)}^a$	1.0 (± 0.1)	2.1 (± 0.7)	0.6	0.51	0.51	2.8
$P_{H_2}^b$	0.6 (± 0.1)	3.2 (± 1.4)	1.6	2.09	17.9	27.2

^a Molar hydrogen yield (in mole per mole of glucose)^b Molar hydrogen productivity (in millimole per liter per hour)

produce acetaldehyde and 1 mol of NAD^+ . Acetaldehyde is then further metabolized using 1 mol of NADH to produce ethanol and 1 mol of NAD^+ . Therefore, every time ethanol is produced, at least 2 mol of NAD^+ are released. NAD^+ can then be recycled back to NADH in a reaction competing with hydrogen production (Lee et al. 2008). It is thus clear that the high presence of ethanol indicates the organisms are not efficiently producing hydrogen. The fact ethanol is the primary metabolite produced over all three reactor scales also indicates

there is significant room for improving process efficiency on a cellular level.

ECE remained relatively unchanged between the 100-ml and 250-ml reactors; however it decreased when scaling up to the 3.6-L reactor. Again, this trend was likely due to the changes in metabolic pathways. The SHP steadily increased with each scale-up as expected. The maximum optical density between different-sized reactors did not change significantly, however, the total amount of hydrogen produced changed in proportion to reactor size. Perhaps the most intriguing difference was the changes in molar hydrogen yield between scales. Hydrogen yield increased from the 100-ml reactors to the 250-ml reactors, but then decreased when scaling up was done to the 3.6-L reactor. This indicates unwanted side reactions or metabolic pathways taking place within the 3.6-L reactor. This discrepancy may be due to large changes in environmental conditions such as type of agitation (stirred incubator vs. impeller), pH (decreasing vs. constant), antifoaming agent (not-present vs. present), oxygen partial pressure (pO_2) control (small volume vs. large volume) (Bartacek et al. 2007), and overall changes in process techniques as described in the scale-up of biohydrogen production section.

Table 4 Overview of hydrogen yield obtained using different immobilization approaches and free-cell systems

Microorganism	Parameters	Immobilizing material	$Y_{(H_2/S)}$ (mol/mol glucose)	Reference
<i>C. beijerinckii</i> NCIMB8052	250-ml batch 37 °C, pH 7.0, 5 g/L glucose	Alginate–chitosan–NPs	2.1	Present study
<i>Clostridium</i> sp. LS2	5-L continuous UASB 37 °C, pH 5.5 HRT 12 h with 5 g COD/L/h	Polyethylene glycol gel	0.35 L H_2 /g COD removed (PEG)	Singh et al. (2013)
<i>Clostridium</i> sp. T2	1-L CSTR 37 °C, pH 7.0, 10 g/L xylose	Sodium alginate/mycelia pellets	Not available	Zhao et al. (2012)
<i>C. beijerinckii</i> RZF-1108	100-ml batch 35 °C, pH 7.0, HRT 8–12 h, 9 g/L glucose	Free suspension	1.97	Zhao et al. (2011)
<i>C. tyrobutyricum</i> ATCC 25755	Fed batch 2-L working volume 37 °C, pH 5.7, HRT 8 h, 30 g glucose/L	Polyurethane matrix	2.0	Mitchell et al. (2009)
<i>C. tyrobutyricum</i> JM1	Continuous Fixed bed reactor 2.5-L working volume 37 °C, pH 6.7, HRT 2 h, 5 g glucose/L	polyurethane foam	223 ml /g hexose	Jo et al. (2008)
<i>C. beijerinckii</i> Fanp3	140-ml batch 36 °C, pH 6.5, 200 g/L glucose	Free suspension	1.86	Pan et al. (2008)
<i>Clostridium</i> sp. AK15	300-ml batch 60 °C, pH 6.0	Free suspension	0.8	Koskinen et al. (2008)
<i>Clostridium</i> sp. W1	800-ml batch 35 °C, potato wastewater	Free suspension	0.51	Wang et al. (2007)
<i>Clostridium</i> sp.	1-L batch 36 °C, pH 6.0, 10 g/L glucose	Free suspension	1.8	Taguchi et al. (1994)
<i>C. butyricum</i>	Batch (shrenk flask) 37 °C, pH 7.0, 55 μ moles glucose	Acetylcellulose filter	2.0	Karube et al. (1982)
Anaerobic sludge	2-L fluidized bed 40 °C, 6 h HRT 20 gCOD/L sucrose	Ethylene–vinyl acetate copolymer	4.26	Lin et al. (2009)
Anaerobic sludge	8-L draft tube Fluidized bed 40 °C, HRT 8.9 h, 40 gCOD/L sucrose	Silicone gel	4.98	Lin et al. (2006)

Overall, the proposed immobilizing and cycling procedure was successful when applied across all reactor sizes but variations were observed from one cycle to another. There is still room for improvement in cycling conditions and techniques, which might further enhance the effect of A–C–NPs on the sustainable production of hydrogen.

Hydrogen yield obtained

In order to facilitate comparison of the hydrogen production obtained to previously published data, Table 3 presents the equivalent molar yield of hydrogen ($Y_{H_2/S}$; in mole of H_2 per mole of glucose) and molar hydrogen productivity (in millimole per liter per hour) obtained at each scale. The hydrogen yields ($Y_{H_2/S}$) obtained in the present study lie within the range of data reported in a review of dark fermentation published by Rittmann and Herwig (2012). However, the hydrogen productivity obtained for all three reactor sizes were relatively low (0.6 to 3.2 mmol/L/h) when compared to the range reported in the same review (2.09 to 27.1 mmol/L/h).

Considering that the major objective of this study was to improve hydrogen production using *C. beijerinckii* immobilized on chitosan–alginate acid polyelectrolyte functionalized magnetite nanoparticles, results obtained are compared to molar hydrogen yields (in moles of hydrogen per mole of glucose) reported in literature. Table 4 summarizes the key parameters and results of the most relevant studies. All the previous studies performed using immobilized *Clostridium butyricum*, either using PEG (Singh et al. 2013), alginate bead (Zhao et al. 2012), polyurethane foam (Jo et al. 2008), polyurethane-activated carbon matrix (Mitchell et al. 2009), or acetyl cellulose fiber (Karube et al. 1982) demonstrated similar or increased molar hydrogen yield when compared to free-cell systems. There are also indications in literature that *Clostridium* sp. CFPA-20 is thermodynamically favored for immobilization (Nomura et al. 2014). The molar hydrogen yield obtained in this study is in the range of the values reported in literature for immobilized cells. However, for all the studies reported, major issues still remain about the reusability, stability, and regeneration of immobilizing materials that had been previously used. The approach used here would facilitate such recovery of the immobilized cells.

Based on the results obtained in this study, magnetite nanoparticles were successfully coated with chitosan and alginate acid layers and for the first time, attachment of *C. beijerinckii* cultures to this immobilization material was confirmed. This immobilization technique enabled the retention of biomass within bioreactors of varying sizes and use in successive batch fermentations for hydrogen production. This process has the advantages of reducing downtime between batches and increasing the volumetric production of hydrogen over a given time period. Although efficiency and processing issues presented themselves, it was shown that the use of a strong

enough magnetic field can retain the immobilized biomass within the reactor, leading to greater amounts of hydrogen being produced than in a control reactor operated with a free suspension over the same time period. However, the low substrate and energy conversion efficiencies compared to previously published data indicate that there is room for improvement. Considering the decrease in some process efficiencies during scale up from 250-ml to 3.6-L reactors, operating factors such as agitation, pH, antifoaming, and pO_2 must be optimized in order to maximize hydrogen yields. Optimization of media components or reactor conditions could also be considered in order to promote greater hydrogen production from a metabolic standpoint while suppressing ethanol formation. A promising avenue for improvement of the hydrogen production is based on the metabolic carbon flow and reduced equivalent regeneration and utilization (e.g., NADH, NAD[P]H). NADH regeneration and utilization is predominantly dominated by ethanol, lactate and butanol metabolic networks, which are indirectly competing with hydrogen generation involving PFOR and NFOR pathways, as shown in Fig. S1. Previous studies showed that regeneration of reduced equivalents by immobilized *C. butyricum* is feasible but that the bottleneck is the hydrogen partial pressure (Matsunaga et al. 1985). In the current study, ethanol and acetate were the most predominant end metabolites and ethanol was produced over all three reactor scales, indicating space for improving the hydrogen yield by knocking out those competing metabolic pathways. The immobilization on chitosan–alginate acid polyelectrolyte functionalized magnetite nanoparticles of metabolically engineered *C. beijerinckii* may be a promising future option for sustainable production of biohydrogen.

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Conflict of interest The authors do not have potential conflict of interest to disclose.

Ethical statement The authors confirm that principles of ethical and professional conduct have been followed in this research and in the preparation of this manuscript.

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