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DOI:
10.1016/S0141-0229(03)00115-7

Document Version
Peer reviewed version

Citation for published version (Harvard):
Penfold, D, Forster, C & Macaskie, L 2003, 'Increased hydrogen production by Escherichia coli strain HD701 in comparison with the wild-type parent strain MC4100', *Enzyme and Microbial Technology*, vol. 33, no. 2-3, pp. 185-189. https://doi.org/10.1016/S0141-0229(03)00115-7

Penfold et al. (2003) Enzyme and Microbial Technology 33:185 - doi:10.1016/S0141-0229(03)00115-7

Increased hydrogen production by *Escherichia coli* strain HD701 in comparison with the wild-type parent strain MC4100

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Keywords: *E. coli*, hydrogen, sugar, glucose

Abstract *Escherichia coli* strain HD701 can evolve more hydrogen than its parental wild type, strain MC4100. Conditions were changed systematically to elucidate the best conditions for optimum H_2 production by cell suspensions of this strain. Increasing the temperature from room temperature (18-20 ºC) to 30 ºC increased the rate of H_2 production by ~ 4-fold, from 0.03 ± 0.01 ml/mg/l/h to 0.12 ± 0.01 ml/mg/l/h while decreasing the initial pH from 7.3 to 5.5 decreased the volume of evolved H_2 from 1.34 ± 0.07 ml/mg/l to 0.75 ± 0.08 ml/mg/l, attributable to more rapid attainment of an inhibitory pH. Increasing the cell concentration in resting cell suspensions gave in a higher rate of H_2 evolution. A 50% (v/v) inoculum was optimal, this producing 102.2 ± 5.1 ml/l of H_2 as compared to 22.0 ± 3.2 ml/l at a 10% inoculum. Increasing the cell concentration further gave no increased H_2 production. Stirring the medium doubled the rate of H_2 evolution. Combining these optimised conditions increased the rate of hydrogen production by ~ 6-fold as compared to the initial conditions, from 5.2 ± 0.8 ml/h/l to 29.8 ± 0.8 ml/h/l. Using these optimised conditions in a 6 l fermenter in evolution of *ca.* 3 l of hydrogen from 100 mM glucose. Hydrogen evolution was increased to 9 l using pH control at 5.5. This equates to a molar yield of 1 mol H_2/mol glucose.

Introduction

Hydrogen is regarded as the clean fuel of the future as its only combustion product is water. Hydrogen production is the focus of extensive research (Cammack *et al.* 2001). Conventional methods such as electrolysis of water are expensive (Das and Veziroglu 2001). Biological H_2 production can be coupled to the remediation of organic wastes and it is therefore seen as an economically viable approach. The majority of work has focused on *Citrobacter* sp. (Vos *et al.* 1983; Vatsala 1992), *Clostridium* sp. (Yokoi *et al.* 1998; 2001), *Enterobacter* sp. (Kumar and Das 2000; Palazzi *et al*. 2000), *Rhodobacter* sp. (Fascetti and Todini 1995; Eroglu *et al*. 1999) and mixed cultures (Mizuno *et al.* 2000a,b). Ideally, for industrial application of biohydrogen technology, an organism must be non-pathogenic, robust (with respect to its susceptibility to contaminant overgrowth) and should not produce methane, which is seen as a contaminant and may require removal. One candidate organism is *Escherichia coli.* Previous studies identified a H_2-overproducing strain, *E. coli* HD701, which evolves twice as much as hydrogen as its parental wildtype, strain MC4100 (Penfold *et al.* 2003). *E. coli* HD701 lacks the FHL repressor (Hyc A) of the formate hydrogenlyase (FHL) system, the enzymatic system responsible for converting formate into equimolar amounts of H_2 and CO_2 (Sauter *et al.* 1992; Penfold *et al*. 2003). Penfold *et al.* (2003) showed that *E. coli* HD701 produced H_2 from glucose solution using non-growing (resting cells) that did not require processing before use. Furthermore, the industrial potential of this strain was demonstrated by its ability to convert confectionery waste into hydrogen.

To implement this technology commercially, the process must first be maximised with respect to the rate and extent of hydrogen production. The factors affecting hydrogen production by *E. coli* HD701 were investigated and hydrogen production was compared to previous studies.

Materials and methods

Bacterial strains and media

The strain used was *E. coli* HD701, a derivative of *E. coli* MC4100 that lacks the Hyc A regulator of the formate hydrogenlyase system (MC4100Δ*hycA*) and is up-regulated with respect to hydrogen evolution (Sauter *et al*. 1992; Penfold *et al*. 2003). Cultures were maintained on nutrient agar (NA) plates (Oxoid, UK) and grown in shake-flask cultures as described previously (Penfold *et al*. 2003) in nutrient broth no. 2 (Oxoid, UK). The OD_{600} of samples was measured (Ultraspec III Spectrophotometer, Pharmacia) and related to bacterial dry weight using a pre-determined calibration curve. The composition of phosphate buffered saline (PBS) was 0.8 g/l NaCl, 0.2 g/l KCl, 1.43 g/l Na_2HPO_4 and 0.2 g/l KH_2PO_4. The pH was adjusted to 7.3 with HCl. In scaled up experiments isotonic saline (8.5 g/l NaCl) was used.

Bacterial dry weight estimation

Bacteria were grown aerobically in nutrient broth (30^o C). Timed samples (50 ml) were transferred to pre-weighed 50 ml centrifuge tubes (Sarstedt) and centrifuged (10 min, 4 ºC, 6500 g), dried to constant weight (60 ºC), cooled (in a desiccator; 30 mins) and then weighed. Samples were not washed, to prevent loss of cells. To compensate for residual supernatant, the mass of residuum from a nutrient broth blank was subtracted. An optical density of 1.0 equated to ~0.5 g dry weight per litre of culture.

Hydrogen production tests

Hydrogen-production experiments used resting cells (320 ml reactors; working volume of 310 ml, at 30 ºC) as described by Penfold *et al* (2003). Unless otherwise specified experiments used 31 ml inocula pre-grown in nutrient broth overnight, 31 ml of 1 M glucose (final concentration 100 mM) and 248 ml of phosphate buffered saline (PBS) and were incubated statically (30 ºC) at pH 7.3. Suspensions were gassed with argon to initiate anaerobiosis as previously described (Penfold *et al*. 2003). Evolved H_2 was collected and its evolution was measured by displacement of 1 M NaOH, which was used to absorb CO_2 (Klibnov *et al.* 1982; Penfold *et al.* 2003).

Conditions were varied as specified in individual experiments with respect to the initial pH of the PBS, the temperature of the culture, the volume of *E. coli* inoculum and continuous stirring of the medium (magnetic stirrer and follower) throughout. Temperatures (19^o, 25^o and 30 ºC) were maintained as necessary using a heated water bath (Grant). Cells were inoculated to an initial OD_{600} of 0.17. The effect of pH was investigated by altering the ratio of the Na_2HPO_4 to KH_2PO_4 to pH of 5.5, 6.5 or 7.3 as required. Cells were inoculated at an OD of 0.19. To investigate the effect of cell concentration, *c*ells were inoculated to an initial OD_{600} of 0.08 (5%), 0.17 (10%), 0.33 (20%), 0.49 (30%), 0.56 (40%), 0.69 (50%), 0.94 (75%) and 1.10 (90%).

Scaled up experiments used a 6 l fermenter (Electrolab, UK), 1.6 l PBS or isoltonic saline (pH control experiments), 2 l of overnight *E. coli* culture and 400 ml of 1 M glucose (final concentration 100 mM). The culture was gassed with argon for 1 h. The culture was stirred continually at 600 rpm and temperature. The pH was maintained using an automated pH control system (Electrolab, UK) and 1 M HCL and 1 M NaOH.

Results and discussion

Choice of bacterial strain and optimisation of hydrogen production

E. coli strain HD701 produces more H_2 than its parental wild type MC4100, attributable to the lack of the Hyc A repressor of the formate hydrogenlyase (FHL) system, which renders the cells constantly up-regulated. Penfold *et al* (2003) showed that the optimum glucose concentration was 100 mM, and strain HD701 evolved approx. twice as much H_2 as the wild type. Increasing the glucose concentration further did not promote additional H_2 evolution. Industrial sugar waste supported H_2 evolution comparable to the glucose solution with a maximum rate of 31.63 ± 2.56 ml/h/OD unit/l. The present study showed that changing the needle gauge size on the gas outlet had no effect on hydrogen production

The rate of H_2 production increased with temperature (Fig 1), with evolution rates at room temperature, 25 ºC and 30 ºC of, respectively, 0.03 ± 0.01, 0.06 ± 0.07 and 0.12 ± 0.01 ml/mg/l/h. Increasing the temperature from room temperature to 30 ºC quadrupled the rate of H_2 evolution. It is

likely that further increasing the temperature would further increase the H_2 production but at industrial scale the energy required to maintain higher temperatures could offset the energy gained from use of the biohydrogen as a fuel. All subsequent tests were therefore done at 30 ºC.

The majority of work carried out to date has focused on biohydrogen production with little consideration for commercial application, e.g. it is probably not practical for cells to be centrifuged and washed and therefore this work used the direct transfer of organisms from pre-culture. The use of non-growing (resting) biomass allows a high cell density and exhaustion of residual substrates (e.g. nitrogen source) for growth would discourage contaminant organism overgrowth. Alternatively, conditions could be modified to further discourage contamination e.g. by decreasing the pH of the suspension. This was achieved with a predominantly *Clostridium* mixed culture with no adverse effect on H_2 production (R. Dinsdale, personal communication). Moreover, the *E. coli* FHL system is activated as a homeostatic response to low pH which results from the build up of organic acids, (in particular formate) during fermentation (Rossman *et al*. 1991). Therefore a low pH may also increase hydrogen production. The effect of decreased initial pH is shown in Fig 1b. Although the initial rate of hydrogen production was the same at all initial pH values, after 6 h the most H_2 (1.39 ± 0.07 ml/mg/l) was produced at initial pH of 7.3. This was significantly higher that obtained at pH 5.5 and pH 6.5 (0.94 ± 0.12 and 075 ± 0.08 ml/mg/l, respectively) (Fig 1b). The end point pH of all tests was *ca.* 4.2-4.5. The lower values of H_2 evolution at pH 5.5 and 6.5 can therefore probably be attributed to the shorter time taken to reach an inhibitory pH.

In initial tests (above) the cell suspensions were not stirred after the initial mixing. This can lead to concentration gradients with respect to pH and glucose. Lack of stirring can also lead to cell clumping. Together these factors may reduce the efficiency of hydrogen evolution. Indeed, Vatsala *et al* (1992) showed that stirring a culture of *Citrobacter freundii* increased hydrogen production. When cultures were continuously stirred in the present study the rate of H_2 evolution approximately doubled, from 0.15 ± 0.03 ml/mg/l/h to 0.28 ± 0.03 ml/mg/l/h.

Throughout this study resting cells (non-growing cell suspensions) were used and negligible further growth occurred on inoculation into the glucose-PBS solution. Where cell concentration was the focus it was no feasible to represent H_2 as per mg of biomass dry weight and instead the rate of H_2 evolution was calculated as ml/l/h. Increasing the inoculum from 5% to 50% (v/v) increased the rate of H_2 production (Fig 2); the rate for each concentration (ml/l/h) was 1.43 ± 0.03 (5%), 2.20 ± 0.32 (10%), 4.24 ± 0.28 (20%), 6.22 ± 0.34 (30%), 9.10 ± 0.15 (40%), 10.22 ± 0.51 (50%), 9.78 ± 0.05 (75%) and 10.60 ± 0.44 (90%). Thus, increasing the cell concentration from 10% to 50% increased the rate of hydrogen evolution by a factor of ~ 5 but increasing the inoculum above 50% further gave no further increase (Fig 2).

Residual growth from inoculum carry-over

Aerobic pre-cultures were inoculated directly into the anaerobic hydrogen-evolving vessel, and sparged with argon to ensure rapid onset of anaerobiosis. Residual growth on carried over medium could be problematic. To quantify this the initial and final OD_{600} were monitored. Changing the temperature and stirring the culture did not significantly increase the cell density. However, both altering the pH and cell concentration resulted in an increase in cell numbers over the duration of an experiment. This effect was smallest at in an initial pH of 5.5 (Table 1) and greatest (by ~ 2-fold) at pH 7.3. Increasing the inoculum size had a negative effect on the percentage increase in cell density over the duration of the experiment (Table 2). A final cell concentration of 90% (v/v) produced the smallest percentage increase in cell density whereas 5% produced the greatest increase. This can probably be attributed to a smaller amount of medium carried-over in the inoculum and also the dilution of inhibitory metabolic end-products.

Hydrogen production using the optimised system

The optimum conditions developed in this study were combined and hydrogen evolution was compared to that obtained in a previous study (Penfold *et al*. 2003). An initial pH of 7.3 was used,

with stirred cells. Separately, increasing the cell inoculum to 50% and stirring the culture both increased H_2 production (above). In comparison to the initial conditions the optimised system evolved ~ 6 times more hydrogen (Fig 3). This is a significant increase and is more than that is accountable as an increase in cell density alone (c.f. Table 1c).

The same conditions were applied to a scaled up 6 l fermenter. From 100 mM glucose, 2.86 ± 0.15 l of hydrogen was evolved over 10 h. After 10 hours the pH reached *ca*. 4.5 and hydrogen production would level off. This was not due to depletion of glucose as some still remained. To elucidate if this cessation was a result of end product inhibition from the organic acids or a low pH, the effect of pH control was examined. The PBS was replaced with isotonic saline as it was no longer needed. The pH control was tested at 5.5, 6, 6.5 and 7. A pH of less than 5.5 was not used as it had previously been noted that this pH resulted in a low rate of hydrogen production (data not shown). A pH of greater than 7 was not used as this is the maximum pH at which formate is transported into the cell during the fermentation process. Results of the experiment showed that 5.5 was the optimum for hydrogen evolution, with *ca.* 9 l of hydrogen being evolved over 48 h (Fig 4). This equates to 1 mol H_2/mol glucose. The rate of hydrogen production over the first 10 hours is slightly lower than with uncontrolled pH however this decreased rate is negated by the prolonged evolution and, moreover, full consumption of the glucose.

Application to industrial waste

The results obtained in this study indicate that the optimum conditions for H_2 production are a continually stirred 50% inoculum in PBS (pH 7.3) with 100 mM glucose at 30 ºC. Compared to the initial conditions increasing the cell concentration and stirring the culture were factors that increased hydrogen production, by ~ 5 and 2 times, respectively. Above a 50% inoculum, the rate of H_2 production did not increase; the cell concentration was probably not the rate-limiting step. Stirring the cells increased H_2 production by preventing the build up of concentration gradients in the culture medium. When the studied conditions were combined in an optimised system ~ 6 times more H_2 was produced in comparison to the conditions used in the initial studies. However, these results predict that the total increase should be ~ 9-fold. Reasons for this underperformance could be either that the maximum rate of H_2 evolution has been reached or that a buildup of H_2 in the headspace inhibited H_2 production. One way to overcome this is to remove the H_2 into a 'sink' as it is generated, using a process, which consumes hydrogen, for example a fuel cell. Preliminary tests using a scaled up 6 l reactor indicated that scaling up the process did not affect the H_2 production with industrial sugar waste producing rates of H_2 evolution similar to those observed with 100 mM glucose. This scaled up bioreactor (containing either synthetic sugar solution or industrial sugar waste) was coupled directly (without gas scrubbing or filtration) to a low temperature proton exchange membrane (PEM) fuel cell. This resulted in the generation of electricity to power a motorised fan. These tests further underline the potential for use of *E. coli* HD701 on an industrial scale and the fuel cell study will be reported in full at a later date.

Acknowledgements This work was funded by the BBSRC studentship number 00/B1/E/0698 (to DWP) The authors wish to thank Professor A. Böck (Lehrstuhl fur Mikrobiologie der Universitat, Munich, Germany) for providing the strains of *E. coli.*

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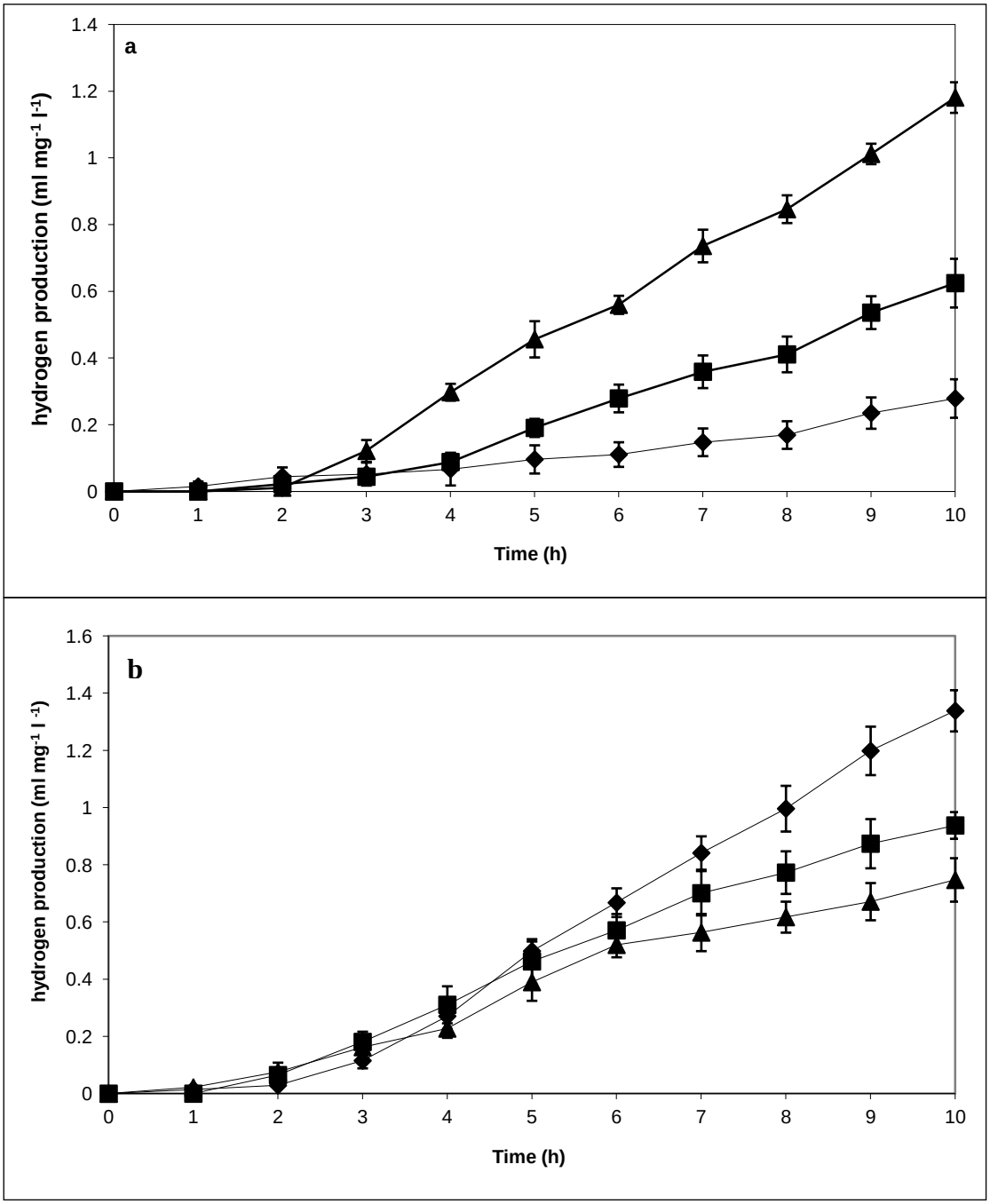

Fig. 1. Effect of temperature (a) and initial pH (b) on hydrogen production by unstirred cultures. a: ♦, room temperature; ■, 25 ºC; ▲,30ºC. Experiments were done three times, in triplicate. Error bars (means ± SEM) that are not shown are within the dimensions of the symbols. b: ♦ pH 7.3; ■, 6.5 and ▲, 5.5 The final pH in each case was ~ 4.2-4.5.

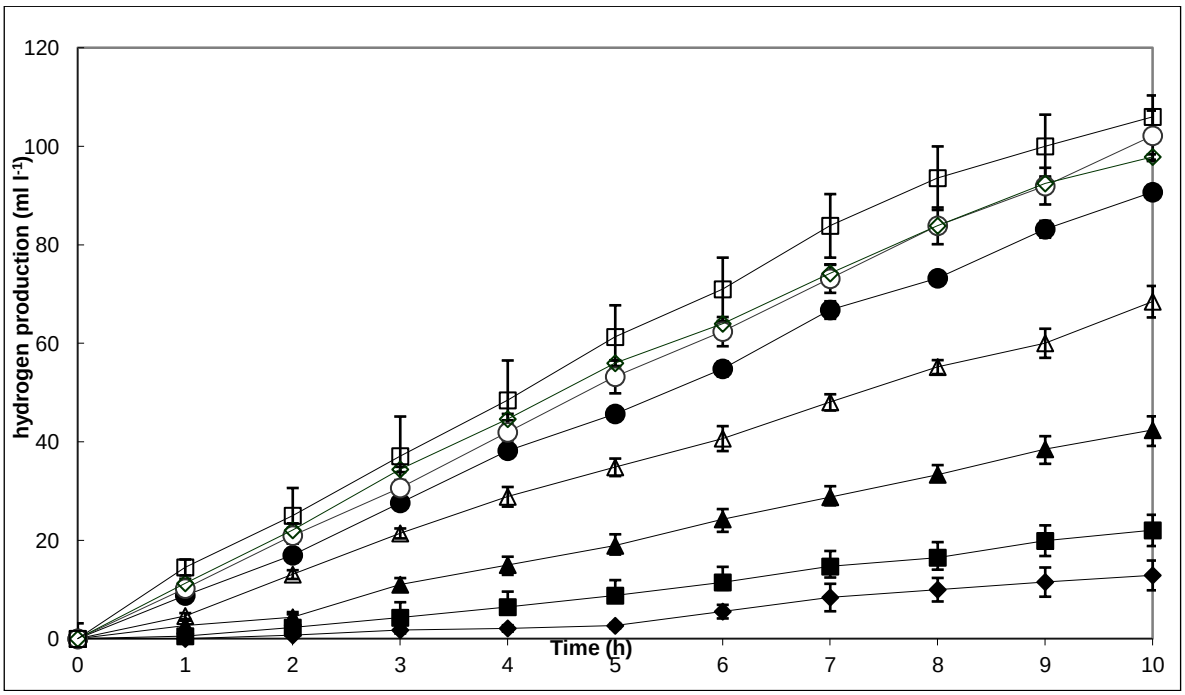

Fig 2. Effect of cell concentration on hydrogen production in unstirred cultures. The cell inoculum was varied (v/v) as follows: ♦, 5%; ■,10%; ▲, 20%; Δ, 30%; ●, 40%; ○, 50%; ◊, 75%; □, 90%. Experiments were done three times, in triplicate. Error bars (means ±SEM) that are not shown are within the dimensions of the symbols.

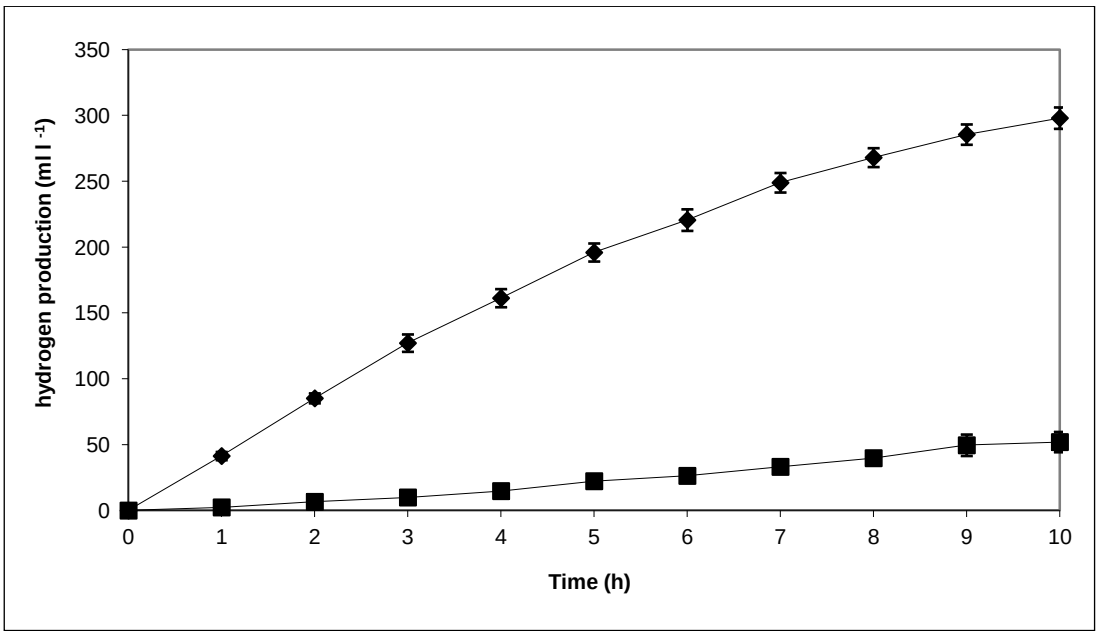

Fig 3. Comparison of hydrogen production by *E. coli* HD 701 using the ■ initial (unstirred 10% inoculum in PBS: pH 7.3; 100 mM glucose, 30 ºC) and ♦ optimised (continually stirred 50% inoculum in PBS: pH 7.3 with 100 mM glucose at 30 ºC) conditions Experiments were done three times, in triplicate. Error bars that are not shown (means ±SEM) are within the dimensions of the symbols.

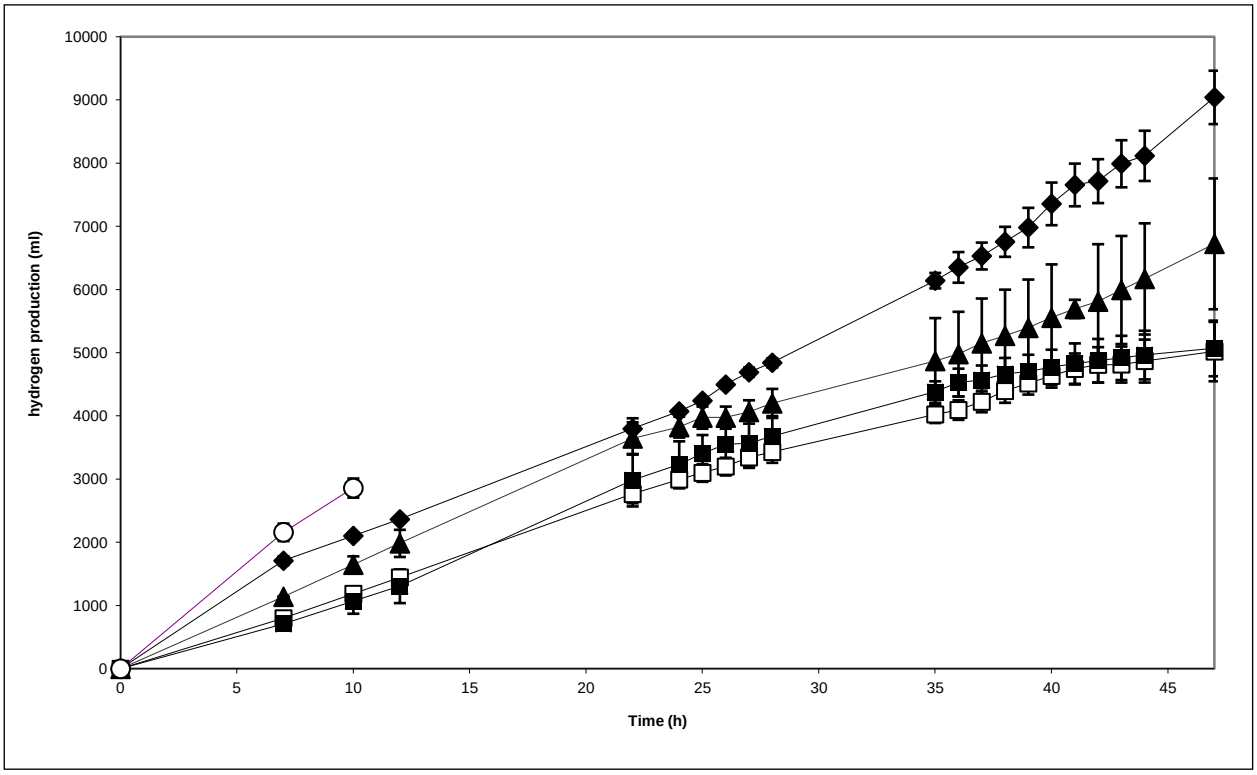

Fig 4. Effect of pH control on hydrogen production in a 6 l fermenter. The cell inoculum was varied (v/v) as follows: ♦, pH 5.5; □, pH 6; ▲, pH 6.5; ■, pH 7; ○, uncontrolled pH. Experiments were done in duplicate. Error bars (means ±SEM) that are not shown are within the dimensions of the symbols.

Table 1. a. Changes in OD_{600} at different initial pH values after 10 h

Initial pH	Initial OD_{600}	Final OD_{600}
5.5	0.19 ± 0.00	0.22 ± 0.01
6.5	0.20 ± 0.00	0.24 ± 0.01
7.3	0.19 ± 0.00	0.37 ± 0.01

b. Changes in OD_{600} at different cell concentrations in 10 h

	5%	10%	20%	30%
Initial OD_{600}	0.08 ± 0.00	0.17 ± 0.00	0.33 ± 0.01	0.49 ± 0.00
Final OD_{600}	0.18 ± 0.00	0.30 ± 0.00	0.49 ± 0.01	0.67 ± 0.00
	40%	50%	75%	90%
Initial OD_{600}	0.56 ± 0.00	0.69 ± 0.00	0.94 ± 0.00	1.11 ± 0.00
Final OD_{600}	0.75 ± 0.01	0.91 ± 0.02	1.14 ± 0.01	1.30 ± 0.01

c. Changes in OD_{600} of the optimised and initial cultures after 10 h

System	Initial OD_{600}	Final OD_{600}
Optimised	0.96 ± 0.01	1.1 ± 0.02
Initial	0.26 ± 0.01	0.47 ± 0.01