The Process Intensification of Biological Hydrogen Production by *Escherichia coli* HD701

By

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Abstract

Hydrogen is seen as a potential fuel for the future; its choice is driven by the increasing awareness of the necessity for clean fuel. Together with the simultaneous development of "green technologies" and sustainable development, a current goal is to convert waste to energy or to create energy from a renewable resource. Biological processing [of renewables] or bioremediation of waste to create hydrogen as a product fulfils this goal and, as such, is widely researched.

In this work, an already established process, using a hydrogenase up-regulated strain - was characterised and the important process parameters were established. This bacterial strain has the potential for industrial-scale hydrogen production from, for example, waste sugars. Previous work, repeated here, showed that hydrogen could be generated by *E. coli* HD701 using a two-phase process (growth in shake flasks, followed by hydrogen production within a bioreactor). Ideally a commercial process would need to be in a single vessel (bioreactor), which therefore resulted in this investigation of the scale-up of two-phase fermentations to 5 L stirred tank bioreactors.

Within the initial two-phase process, shake flask growth in 2 L shake flasks (employing a 50% working volume) achieved a dry cell weight of 1.33 ± 0.1 mg mL^{-1} which then, when transferred to a 5 L bioreactor (containing 2 L of culture and 2 L of hydrogen production substrate), achieved a maximum hydrogen production rate of (200 mL h^{-1}) 150 mL $g(dcw)^{-1}$ h^{-1}.

The first step in scale-up was to simply transfer the process to a bioreactor and see the effect it had on hydrogen production. This approach did not yield any hydrogen and therefore consequent experimentation sought to see if the hydrogen production was growth phase dependant. However all phases of growth evolved no hydrogen upon the addition of substrate. The next approach was to take the conclusion drawn from a literature survey that showed a need for microaerobiosis or anaerobiosis during growth (for mixed acid fermentation

to occur) along with a high formate concentration necessary for the transcription of the FHL complex (the hydrogen gas evolving enzyme). For this reason the K_La from the initial shake flask growth (calculated from literature correlations) was applied to the bioreactor. Experiments used to simulate the shake flask mass transfer coefficient (k_La) in a bioreactor did not generate hydrogen; the physical system within the shake flask used for growth in the initial process allows for this to occur, but the consequent process change to a bioreactor did not. This inability to produce hydrogen was concluded to be due to the lack of microaerobiosis/anaerobiosis required for mixed acid fermentation (the metabolic precursor to hydrogen production). The criterion of K_La was inappropriate for scale up in this case due to the physical differences between the shake flask and the bioreactor, as the oxygen transfer within the shake flask is not limited to transfer between the liquid and gas phase (the effect of transfer across the shake flask closure must be considered). This fact led to the novel use of gas blending for dissolved oxygen tension control. Gas blending was used in a bioreactor to track the changes observed during growth in the shake flask. This created a process that mirrored the shake flask in both growth and hydrogen production. The outcome was a dry cell weight of 1.34 ±0.02 mg mL^{-1} and a maximum hydrogen production rate of 200 mL h^{-1} i.e. 150 mL $g(dcw)^{-1}$ h^{-1}, exhibiting almost identical process results to the two-stage process.

This characterisation reinforced the necessity for microaerobiosis during growth to allow subsequent post-growth hydrogen production. Microaerobiosis in the latter stages of growth allows mixed acid fermentation to occur, which was found to be essential for hydrogen production.

Process intensification took place by increasing cell density. This was achieved by increasing the medium concentration, then by changing the medium (two differing fed batch media were chosen; each medium used was experimentally linked with multiple feeds) and finally by utilising the novel technique of combining gas blending with fed batch cultivation to ensure microaerobiosis during growth. This, along with the use of a low (μ=0.05 h^{-1}) growth rate for feed calculation, led to an eight-fold increase in cell density. The low growth rate was

employed to reduce inhibitory acetate formation while the multiple feeds were used to investigate nitrate depletion. The maximum increase in cell density led to a hydrogen evolution rate of 1800 mL h^{-1}, thus producing hydrogen that could be converted into energy at a rate eleven-fold greater than the rate at which it consumed energy for agitation.

Dedication

To my Mother and Sister for their endless support.

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Abbreviations

ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
AFC	Alkaline fuel cell
CSR	Corporate social responsibility
DMFC	Direct methanol fuel cell
DOT	Dissolved oxygen tension
EMP	Embden-Meyerhof-Parnas
FHL	formate hydrogen lyase
GHGs	Greenhouse gases
PPP/HMP	Hexose monophosphate pathway
HPLC	High performance liquid chromatography
LCA	Life cycle analysis
MMF	Mixed microbial flora
MCFC	Molten Carbonate Fuel Cell
Ni-Fe	Nickel-Iron
OD_{600}	Optical density
PAFC	Phosphoric Acid Fuel Cell
PHA	Polyhydroxyalkanoates
PEMFC	Proton Exchange Membrane [Polymer Electrolyte] Fuel Cell
PFOR	Pyruvate ferrodoxin oxidoreductase
PFL	Pyruvate formate lyase
RFC	Regenerative fuel cells
Re	Reynolds number
SOFC	Solid Oxide Fuel Cell
TCA	Tricarboxylic acid

1. Introduction – Biotechnology in the 21st century

1.1 A brief history of biotechnology

Fermentation processes are a fundamental to biotechnology and have been around for centuries within the food industry, with evidence of brewing found and dated back to 6000 B.C. in ancient Bablyonia (Hardwick, 1995). Apart from food uses (Wang *et al*., 1987) fermentation was actively researched throughout the late 19th century, with Pasteur being the first to grow bacteria in culture (Chain, 1971, Barnett, 2003) but the application of biotechnology to other industrial processes was advanced after the discovery of penicillin in 1928 by Alexander Fleming (Fleming, 1944). After the discovery of penicillin, Norman Heatley (Moberg, 1991) carried out its industrial production in conjunction with Howard Florey whilst Ernst Chain further researched into its pharmacological antibiotic effect. The discovery of methods to produce penicillin on a large scale occurred during the WW2, and while the production of penicillin had a dramatic effect on the war, its research was born out of scientific interest and not military endeavour (Chain, 1971).

Biotechnology changed in the 1980's with the introduction of recombinant DNA technology, first researched in the '70s by Herbet Boyer, Stanley Cohen and colleagues, (Nicholl, 2008), the protocols developed by these researchers led the way to technological advances such as the production of human insulin by *E. coli*, first marketed in the USA in the 1980s. With the ability to use bacteria to create non-native substances, the biological production of therapeutics became widely researched and industrially used, but the field of bioprocessing is further reaching than just therapeutics, it has extended from food, through medicine (therapeutics and preventative healthcare) and into agriculture**,** bioremediation, water treatment and polymers production.

1.2 The drivers for the increasing use of biotechnology

The increase in the use of biotechnology in differing industries can be attributed to many things, depending on the industry it is applied to, and the specific business model used by the specific companies. So-called "Green" processes, fulfil the role of being able to promote a company's corporate social responsibility (CSR), which is a measure of how a company affects its environment on all levels (Vogel, 2005). CSR is, of course, not the sole reason for the use of biological processing, its use and research must make good business sense as well as scientific sense. Initially enzymatic conversions were mostly employed for their regio-, stereo- and substrate specificity but the increasing environmental impact of chemical processes has led to biological processes being integrated into the field of clean chemical technology.

Clean chemical technology has been defined as " A means of providing a human benefit which, overall, uses less resources and causes less environmental damage than alternative means with which it is economically competitive"(Clift, 1995). Within the field of clean chemical technology is green chemistry, which has been defined by a set of 12 principles (Poliakoff, 2002) (see appendix 1). Bioprocessing is intrinsically linked to some of these principles shown below:

- The use of auxiliary substances (e.g., solvents, separation agents, and so forth) should be made unnecessary wherever possible and innocuous when used.
- Energy requirements should be recognized for their environmental and economic impacts and should be minimized. Synthetic methods should be conducted at ambient temperature and pressure.
- A raw material or feedstock should be renewable rather than depleting wherever technically and economically practicable.

- Unnecessary derivatization (blocking group, protection/de-protection, temporary modification of physical/chemical processes) should be avoided whenever possible.
- Catalytic reagents (as selective as possible) are superior to stoichiometric reagents.

It [bioprocessing] can also be applied to the remaining principles and because of this it is frequently used when replacing existing technologies and researching new technologies from a sustainable/green/ clean technology standpoint.

The overall development of clean technologies is said to be a mixture of paradigm shifting, one of which is considered to be the most essential, and states that it has been estimated that for human life on this planet to be sustainable, we must achieve an increase in efficiency of resource use by at least one order of magnitude (Clift, 1997). Alongside the use of "clean tech" another driver for research in this field is the creation of technologies that will replace the petrochemical industry, due to both its economic/environmental impact and dwindling resources.

1.3 Bioprocessing and sustainable development (waste minimization and renewable energy)

Currently the petrochemical industry creates a large proportion of energy used worldwide but also it supplies the world with the carbon building blocks necessary for the manufacture of many products. So along side biological energy production, research is carried out in the production of biopolymers, an example of this is the use of pyrolysed waste plastic as a substrate for bacteria to convert to polyhydroxyalkanoates (PHA), which is a biodegradable polymer (Nikodinovic *et al*., 2008).

In the field of biological energy production there are currently numerous strands of research, which (to simplify) is the production of liquids and gases. Bioprocessing as been employed to produce methane and hydrogen (the research into biological production of hydrogen is detailed in Section 2.1.2) and also liquid hydrocarbons such as ethanol, butanol and acetone. Industrial capability for some of the above processes has been demonstrated, with companies such as TMO renewables© (UK) and Biocaldol© (UK) employing thermophillic microorganisms for the production of bio-ethanol for petrol supplementation (thermophillic organisms are often employed for ethanol production due to it being produced in the vapour phase making it easier for downstream separation). While the liquid hydrocarbons are used to supplement fuel, hydrogen, via the proposed hydrogen economy (see appendix 2) is planned to be an all-encompassing, non-polluting energy source able to fuel the world of the future from renewable resources.

2. Literature Review

Alternative fuels are becoming increasingly sought after, due to dwindling petrochemical resources. Alongside this is the issue of climate change, a large proportion of which is said to be apportioned to the burning of fossil fuels. This has created the need for clean as well as renewable alternative energy sources which is why the production of hydrogen is currently being researched as a potential alternative fuel (Momirlan and Veziroglu, 1999). The use of hydrogen as a fuel forms an integral part of the so-called "Hydrogen Economy".

There are other potential energy technologies that are the subject of research or are currently used in industry. These include photovoltaic, wind, thermal, hydro and nuclear, all of which could be considered a renewable form of energy, which (with the exception of nuclear power) is also environmentally non-polluting. However; while these other energy production systems may be useful for portable applications, local (in house) power generation and to feed into the national grid, hydrogen and its use as a fuel has the advantage of being able to do this as well as be applicable for transportation.

The work in this study is devoted to the process intensification of hydrogen production by *Escherichia coli*. The literature reviewed in this chapter is used to set the scene and to further explain the decisions made during the body of work shown in the results and discussion section and is summarised here:

- The first section sets out by detailing other methods of hydrogen production and why this method was chosen.
- The next section in this chapter then explains the differing methods that could be used in scale-up, this is of importance, as process intensification will take place in a bioreactor whereas the current process utilises a shake flask for growth. These techniques can be used to transfer growth from a shake flask to a bioreactor. Also in this section is genetic engineering, which is the other technique that can be used during process intensification

- After describing ways of transferring from shake flask to bioreactor the next section uses the fact that growth is occurring in a bioreactor to expand on the different possible modes of growth, which is chosen and why, and also how some process parameters will affect the growth.
- The final section of the literature review details the bacterium species and strain, how and why it produces hydrogen and also includes the reason for strain choice and the aims for the study.

2.1 Hydrogen production

The methods used for the production of hydrogen can be split into three groups (Stoji *et al.*, 2003):

- Electrochemical methods
- Thermochemical methods
- Biochemical methods

All methods can be renewable, as they can utilise biomass or other renewable resources, however the use of non-biological methods is more likely to lead to environmentally harmful by-products and also tend to be more energy intensive (see Section 2.1). This Section (2.1) will detail the current literature in hydrogen production as a whole and then go on to summarise and compare the methods to show why biological hydrogen production is a viable research path.

Figure 2.1. Shows the potential routes from biomass to hydrogen, however as previously stated there is a further method, electrochemical production. !t will be considered here as electrochemical production is essentially renewable as it uses water which would be reproduced by the fuel cell. The level of "greenness" of this method however would be defined by the method used to obtain the electrical energy. This technique will be further discussed in Section 2.1.1.

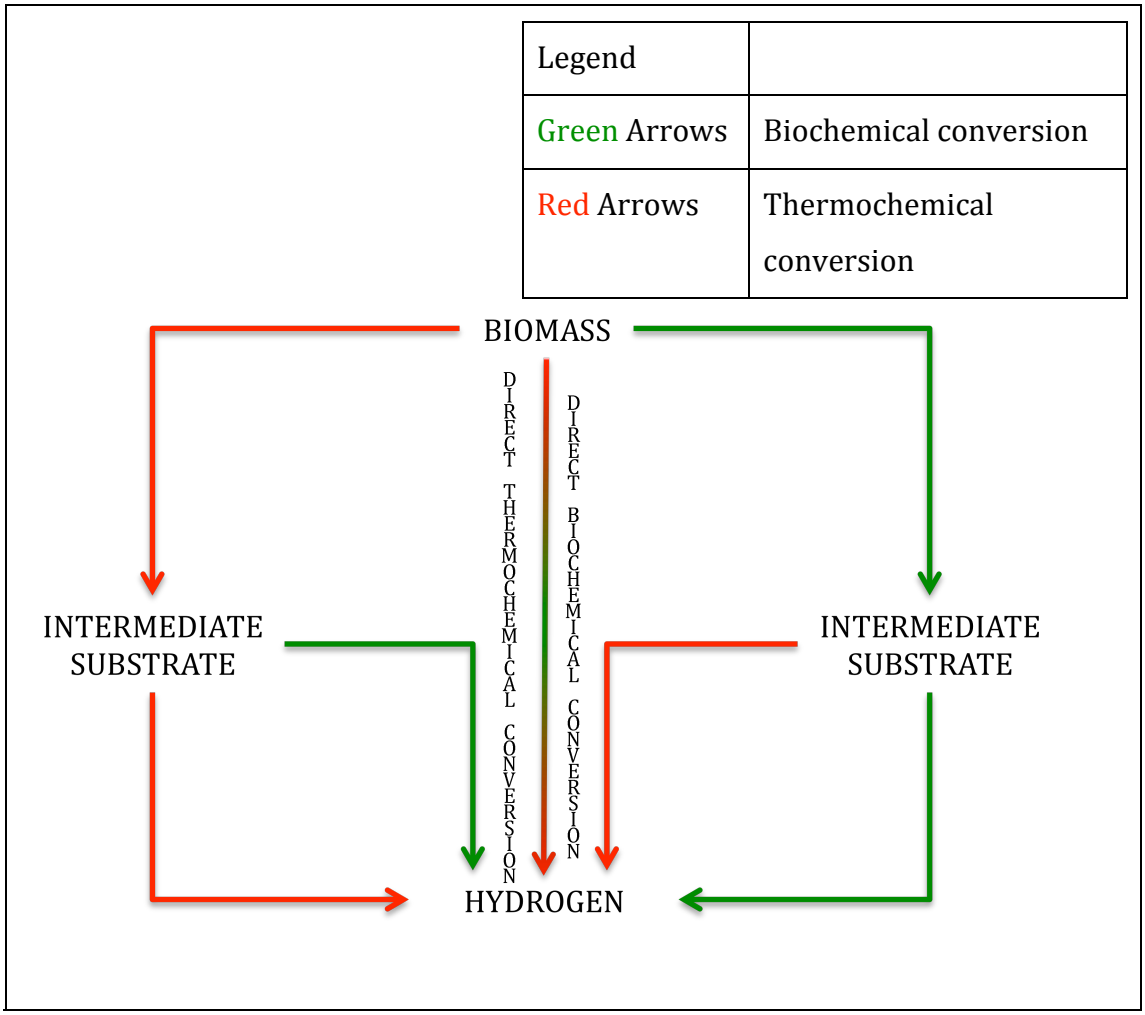


Figure 2.1 Potential routes from biomass to hydrogen.

2.1.1 Electrochemical methods of hydrogen production

The first method of hydrogen production is electrochemically. This is a well established technique with electrolytic hydrogen production being conducted since the early nineteenth century (Bockris *et al.*, 1985). Most of the research in this field is based on the electrolysis of water, which is simply the splitting of water into its constituent elements. The "greenness" of this method revolves around source of the electrical energy that splits the water. Potential sources of energy are, photovoltaic, conventional electricity (Turner *et al.*, 2008; Gardner,

2009). There are currently two commercial methods for the electrolytic production of hydrogen. These are using the alkaline electrolyser and the polymer electrolyte membrane (PEM) electrolyser (Marcelo and Dell'Era, 2008, Turner *et al.*, 2008), which have typical efficiencies of 56-73%.(Turner *et al.*, 2008)

The simplest methods suggested to improve electrolytic hydrogen production is simply an increase in scale and also moving from batch to continuous processes (Rogner, 1998). The current research in to methods used to improved the efficiency/decrease the energy input required when creating hydrogen electrochemically include:

- Integration of photovoltaics
- High temperature electrolysis
- Catalyst improvement
- Gas separation technologies
- Substrate variation

Each of these research themes will be detailed individually and then compared.

The electrochemical cells currently used to produced hydrogen have an analogue fuel cell which works in the opposite direction to produce electrical energy.

2.1.1.1 Integrating photovoltaic and electrochemical hydrogen production

The utilisation of solar energy to provide the electrical energy required for electrolysis is an attractive prospect for research (Momirlan and Veziroglu, 1999), processes involving enzymes that use solar energy are described in Section 2.1.1.4, however there are other ways of harnessing solar energy for electrochemical hydrogen production. This can occur by using TiO_2 as an electrode for oxygen evolution in an electrolytic cell, this does not reduce the energy requirement in a typical alkaline electrolysis cell but instead uses solar energy and a photovoltaic electrode to supply electrical energy (Fujishima *et al.*, 2000).

2.1.1.2 High temperature electrolysis

While on the face of it an increase in temperature would appear to be an unnecessary energy input to a hydrogen production process, when considering the electrochemical splitting of water vapour, the increase in temperature offers an increase in energy efficiency when compared to conventional alkaline water hydrolysis. This increase in efficiency arises from a reduced electrochemical cell potential and concomitantly reduced electrical energy requirements (Crosbie and Chapin, 2003, Utgikar and Thiesen, 2006, Eric, 2002).

Life cycle assessment of a HTE process has show that linking a process to an advanced nuclear reactor (to supply the heat and electrical energy) has a reduced environmental impact (both local and global) when compared to the current method for mass production (Reforming, shown in Section2.1.2.1(Utgikar and Thiesen, 2006)).

2.1.1.3 Catalyst improvement

Catalyst improvement spans the multitude of electrochemical production processes. This is due to the fact that, as with any chemical conversion, the addition or improvement of the catalyst employed will improve the process in some way. Due to the variety of research in catalyst use and improvement this section with briefly describe an example of each type of catalyst that has been employed/improved by firstly separating them into biological and non-biological catalysts.

2.1.1.3.a Biological catalysts

Biological catalysts (or enzymes and enzyme complexes) are employed in the electrochemical production of hydrogen. Only considering those that are removed from the whole cell, isolated and purified for use this group can be further divided into those that are linked to photolytic hydrogen production and those that are not. A study by Reisner *et al*. (2009), shows the use of a class of enzymes called hydrogenases (specifically the [NiFeSe] hydrogenase of *Desulfomicrobium baculatum*) linked to Ru dye sensitised TiO_2 nano-particles. This utilises the ruthenium based complex as a photo-sensitiser for the TiO_2

nano-particle that in turn supplies the electrons for the conversion of protons to gaseous hydrogen (Reisner *et al*., 2009). Along with this study other studies that utilise hydrogenases in conjunction with other enzymes as electron donors such as the [Fe] hydrogenase from *Desulfovibrio vulgaris* Hildenborough (Guiral-Brugna *et al.*, 2001) and the use of hydrogenases directly linked to he electrode within the electrolytic cell (Vincent *et al.*, 2007). An overall review on hydrogenase linked electrocatalyst design has been carried out (Artero and Fontecave, 2005).

2.1.1.3.b Other catalysts

Catalyst improvement involves many different technologies, discounting the use of enzymes and enzyme complexes previously discussed, the use of ionic activators is one such method. Attempting to improve the efficiency of the electrolytic cell using the addition of ionic activators had the effect of reducing the energy input to the electrolytic process by over 10% (Stoji *et al.*, 2003). de Souza *et al*. (2008) suggested the use of molybdenum and various other non-precious metal alloys in conjunction with ionic liquids to successfully improve the efficiency of electrolytic hydrogen production(Souza *et al.*, 2008).

2.1.1.4 Electrolytic cell improvement technologies

There are many potential ways of improving the design of an electrolytic cell. These include zero-gap cell geometry, development of new diaphragm/membrane materials, and cell assembly (Stoji *et al.*, 2003).

The reduction of the gap within the cell geometry has a positive effect on the reduction of energy usage by a standard alkaline electrolytic cell (Stoji *et al.*, 2003). The effect of the gap between electrodes has been investigated and the reason behind there being an optimum gap is due to the effect of bubble formation at the electrode diminishing the electrodes ability to create more gas (Nagai *et al.*, 2003). This phenomenon is not seen in all electrolytic cell assemblies as some have inbuilt gas separation systems which can negate this effect. The coproduction of oxygen during the electrolytic splitting of water is

one of the major kinetic obstacles encountered in this method of hydrogen production

Magnetolysis is a cost reduction technique for electrolytic hydrogen production processes. It was suggested by Bockris and Gutmann (1981) and removes the necessity for a transformation (accompanied by energy losses) of the electrical power (produced at a power station). The technique works by creating the necessary potential difference within the electrolyser using magnetic induction (an application of the homopolar generator concept conceived by Faraday (Bockris *et al.*, 1985)).

2.1.1.5 Substrate variation

While the bulk of electrochemical hydrogen production is performed using water as a substrate there are other potential substrates that can be used for hydrogen production. The two main substrates currently being researched is the use of ammonia (NH_4) and hydrogen sulphide (H_2S), but there is also the potential of halide electrolysis (Stoji *et al.*, 2003).

The use of aqueous NH_4 as a substrate is an advanced alkaline electrolysis cell, utilising the alkalinity of ammonia as well as its function as a hydrogen carrier (Vitse *et al.*, 2005). The advantage of ammonia as a hydrogen carrier its higher specific energy density (50% higher) when compared to hydrogen, its ease of transportation and the fact that it is already mass produced so global production could be easily scaled. These facts combined with the fact that the combination of ammonia oxidation with hydrogen production uses 95% less energy than conventional alkaline water hydrolysis (Vitse *et al.*, 2005) makes this an attractive research theme.

The use of hydrogen halides as an energy carrier is also researched, this can be combined to thermochemical cycles such as the use of HBr reported by Sivasubramanian et al., gained current densities of 2.0 A cm^{-2} (Sivasubramanian *et al.*, 2007).

2.1.1.6 Summary and comparison of Electrochemical hydrogen production

A recurring theme through all electrochemical production methods is the sustainability of the process. As such all productions methods are assumed to be supplied with energy from a renewable resource (Nuclear power/heat is considered sustainable). From this standpoint it is now possible to compare the electrochemical hydrogen production processes.

Based on thermodynamic considerations alone the cost of electrochemical hydrogen production will not decrease significantly through an increase in efficiency (Turner, 2004). Cost is a prohibitve factor in this technology also as it is thought to be the most expensive of the non-biological hydrogen production methods (Bockris *et al.*, 1985). Also, as is a recurring theme throughout energy production, efficiency is not the bottom line for choosing an energy production method. It is thought that the gain efficiency from high temperature electrolytic cells is only beneficial if the heat is "free" such as is the case where the electrolytic cell is coupled with an advanced nuclear reactor however one stumbling block that many methods of electrochemical hydrogen production have is the necessity for (at least relatively) pure water. While pure water could be supplied when an electrolyser is coupled with a fuel cell (perhaps in stationary applications). If this is not the case, the additional cost of water purification would have to be factored in to the decision making process. However photo-electro-chemical production may be able to circumvent the necessity for pure water (Turner, 2004).

While electrochemical hydrogen production may be the best option for near term, small scale, distributed hydrogen production (Eric, 2002) its overall use may be limited as is suggested by Vijh (2005) as some significant improvements are not thermodynamically feasible (this refers to one specific example, however the thermodynamic principles are valid) (Vijh and Bélanger, 2005).

2.1.2 Thermochemical methods of hydrogen production

As previously stated thermochemical processes form the bulk of current hydrogen production. It is favoured over electrochemical processes for large-scale production due to its greater efficiency. Thermochemical hydrogen production currently includes techniques, such as; Reforming, Thermal dissociation and thermal pre-treatment processes (Pyrolysis and gasification)(Freni *et al*., 2000; Funk, 2001).

2.1.2.1 Reforming

Reforming is the process that is currently used for the bulk worldwide production of hydrogen (Rostrup-Nielsen and Rostrup-Nielsen, 2002) and it is centred on the conversion of hydrocarbons to Synthesis Gas (commonly abbreviated to syn-gas, a mixture of CO and H_2). The reforming process either takes oil, diesel, coal, biomass or other hydrocarbons and partially oxidizes it (combustion with reduced oxygen input) or reacts methane (natural gas is the most efficient input (Haryanto *et al.*, 2005)), gasified coal and biomass (Ni *et al*., 2006) with steam over a catalyst (at between 700-1000°C) (The other hydrocarbons tend to produce hydrogen by catalytic reforming (Rostrup-Nielsen and Rostrup-Nielsen, 2002; Ni *et al*., 2007; Peña *et al*., 1996; Holladay *et al*., 2009)). The water-gas-shift reaction can also liberate more hydrogen converting CO into CO_2 (Peña *et al*., 1996; Freni *et al*., 2000; Holladay *et al*., 2009). While reforming is an established and efficient process, there is still research conducted into ways of potentially improving this technique. This research focuses on; the use of membranes for gas separation, energetic efficiency increases, the use of renewable starting materials (often requiring thermal pre-treatment).

2.1.2.1.a Membranes for gas separation

Membranes are not typically required for the actual reforming process however; reforming can produce carbon monoxide, which via the water-gas shift (WGS) reaction can be used to liberate more hydrogen. The WGS reaction combines carbon monoxide with water to create carbon dioxide and hydrogen. This reaction while advantages as it uses an abundant resource (water) to create more hydrogen, it does co-produce an equivalent molar amount of carbon dioxide, which along with not being an ideal waste stream will require separation (gas separation is always required from).

2.1.2.1.b Efficiency improvements

Moving the process to one that utilises filtration combustion, is a method employed to improve efficiency by Bingue (2002). A process was developed that used both rich and ultra-rich filtration combustion of methane and dihydrogen sulfide to produce syn gas and sulphur (Saveliev and Fridman, 2002). Catalytic reforming is also employed to increase energy efficiency (Pena *et al.*, 1996).

2.1.2.1.c Reforming of renewable starting materials

Some research is being carried out using renewable materials (biomass derived) for hydrogen production. This research is often linked to thermal pre-treatment technologies such as (pre- steam reforming) Pyrolysis (Navarro *et al.*, 2007). Research carried out by Bimbela *et al.* (2007) used acetic acid as a model compound for the product of a thermal pre-treatment process that underwent subsequent steam reforming (Bimbela *et al.*, 2007). This showed process shows the efficacy of steam reforming when linked to renewables, however the use of a model compound in place of the more complex bio-oil or bio-oil derivatives removes some of the main drawbacks of the steam-reforming of the complex oil quotient of biomass.

While the use of renewable materials often requires thermal pre-treatment, the use of simple biomass derived oils can circumvent the need for pre-treatment. Sunflower oil has be shown to be a viable potential starting point for hydrogen production by catalytic steam reforming (Marquevich *et al.*, 2000), as well as

steam reforming of potentially biologically derived; ethanol (Vasudeva *et al.*, 1996,;Haryanto *et al*., 2005) methanol (Palo *et al*., 2007), Glucose in supercritical water (Yu et al., 1993), fruit shells in supercritical water(Demirbas, 2004) and oil derived from biomass pyrolysis (Czernik *et al*., 2007).

2.1.2.2 Thermal dissociation

Thermal dissociation uses thermochemical processes to dissociate water into its constituent elements an example of this is the sulphur-iodine process (Simbolotti, 2006), but other techniques also exist and the research in this field tends towards the construction of new thermochemical cycles or the increase in efficiency of these cycles (also other starting materials other than petrochemicals, water and biomass are investigated such as hydrogen sulfide (Zaman and Chakma, 1995)).

A technique that does not involve these cycles is plasmolysis. Plasmolysis has been known as a hydrogen production method for sometime, a review "On the splitting of water" by Bockris *et al.* (1985) explains the method that basically uses extreme heat (in excess of 3000 °C) for dissociation (as plasma, water is substantially dissociated). As is common for both electrochemical techniques and thermochemical techniques, there is research that uses a combination of both to improve the efficiency and/or feasibility of a process. In the case of plasmolysis the research is designed to reduce the energy input by using electrically induced plasmas (other methods of direct thermal dissociation are also discussed) (Bockris *et al.*, 1985). Bockris (1985) also implies that catalytic thermal decomposition will not improve the Carnot efficiency of a process sufficiently without the use of thermochemical cycles. However even with this implication thermo-catalytic processes have been developed, such as the one described by Momirlan (1992), which described the use of zeolite catalysts doped with non-noble metals for hydrogen production (Momirlan and Veziroglu, 2002).

There are currently several (over 200 (Momirlan and Veziroglu, 2002)) thermochemical cycles utilised (and researched) for thermal dissociation, with

the only chemical/physical constraint being a thermodynamic one, where the sum of the enthalpies within the cycle must exceed the enthalpy of formation of water (if water is the species split to produce hydrogen (Bamberger and Richardson, 1976)). With such a large number this section will just outline a few examples and recent developments within the field of research. As with some forms electrochemical conversion, one proposed method for providing the heat energy required in this field or research is coupling the thermochemical production of hydrogen to a nuclear reactor, which would benefit both processes, reducing the need for cooling within the reactor and providing the heat energy for dissociation (Schultz, 2004). While nuclear reactors providing the energy for thermochemical reactions are attractive there are other potential options. One such potential energy source revolves around the design of reactors using concentrated solar energy for heat production, this could potentially be applied to or methods of thermochemical hydrogen production (Steinfeld and Palumbo, 2001; Steinfeld, 2005; Zedtwitz *et al.*, 2006).

2.1.2.2.a Metal oxide thermochemical cycles

A potential "new" thermo-cycle for hydrogen production has been proposed and investigated by Abanades (2005). It is one of the thermodynamically favourable two-step thermochemical cycles that utilise metal oxides (many cycles require four steps). It shows a cycle utilising the reduction of CeO_2 to Ce_2O_3 (at 1000 C and 100-200mbar) to produce hydrogen by the oxidation of Ce_2O_3 to CeO_2 with H_2O as the oxygen donor leaving gaseous hydrogen as the product. The high temperatures required in this process make it ideal for coupling to nuclear reactors as a source of heat energy, however some metal oxides are able to undergo the first (reduction) step using concentrated solar energy (Abanades and Flamant, 2006).

A study in to hydrogen production using a reverse Deacon cycle (the Deacon cycle is a 2 step thermochemical cycle that converts MgO in to $MgCl_2$ using HCl_{aq} and reverses the process using water and subsequently liberates hydrogen), has been carried out. This method uses both electrochemical and thermochemical reactions to improve efficiency and is also an improved version of the suggested Hallett Air Products cycle (Simpson *et al.*, 2006).

Further efficiency improvements of thermochemical cycles have been investigated using an adiabatic 4-step cycle containing CaO and Fe_3O_4 *(Sakurai et al.*, 1996) and ferrites (Han *et al.*, 2007) to improve thermodynamic efficiency

2.1.2.2.b Sulphur - iodine thermochemical cycle

The Sulphur-iodine process (also known as the iodine-sulphur process) is a long standing (established over 30 years ago (Kubo *et al.*, 2004)) thermochemical process and one of the more widely researched thermochemical cycles. Due to the amount of research in to this cycle it exists in more than one form with common starting materials.

Research into its use in liquid phase, has been carried out, detailing the separation technologies used to split the liquid phases (Sakurai *et al.*, 2000),

2.1.2.2.c Copper - chlorine thermochemical cycle

An example of this cycle has been assessed by Wang & Gabriel (2008); the specific research they conducted showed the use of the cycle (in the 5-, 3-, and 2-step variations) for hydrogen production and the reactor and reaction engineering that could be used for scale up of this process along with process inputs and energy requirements (Serban *et al.*, 2004; Wang *et al.*, 2008; Naterer *et al.,* 2008).

2.1.2.3 Thermal pre-treatment technologies

Pyrolysis and gasification are precursors to reforming and use thermal processing to convert substrates unsuitable for reforming into substrates that are (Turn *et al.*, 1998; Simbolotti, 2006; Ni *et al.*, 2006; Holladay *et al.*, 2009).

2.1.2.3.a Pyrolysis

Pyrolysis is a thermal pre-treatment technique used for splitting carbonaceous material into smaller constituents that can be used for hydrogen production. It differs from gasification, as its products are not all produced in the gaseous phase. Its use has been shown in conjunction with secondary decomposition (a

technique that in this case is analogous to catalytic steam reforming) to convert biomass (rice husks) into hydrogen (Zhao *et al.*, 2010).

As with many processes the addition of a catalyst has been researched. It has been shown that the addition of a catalyst improves the thermodynamic efficiency of the pyrolysis process and can also effect the initiation of the water-gas shift reaction within the process further increasing the hydrogen yield form the system (Chen *et al.*, 2003).

2.1.2.3.b Gasification

Gasification as a process is similar to reforming and uses steam or oxygen to break down carbonaceous material in to gaseous products. There are other methods of gasification however such as the use of supercritical water (Hao, 2003), which can be used for processes analogous to gasification or reforming. Along side other methods of gasification research is also conducted in to using various energy sources to produce the environment necessary for gasification such as solar energy (Z'Graggen *et al.*, 2006) and energy input reduction by catalytic gasification (Courson and Udron, 2002) as well as other renewable substrates (Midilli and Dogru, 2001).

2.1.2.3.c Other thermal pre-treatment techniques

While many thermal pre-treatment techniques are used to allow an input (substrate) stream to be converted in to a form that could be used by another thermochemical process, they are also used to convert material for use by non-thermochemical processes. An example of this is the use of steam explosion to convert the lingo-cellulosic biomass (corn stover) for subsequent fermentation (Datar *et al.*, 2007).

2.1.2.4 Summary of Thermochemical hydrogen production techniques

The use of thermal processes that utilise fossil fuels for hydrogen production is detrimental as it can include the co-production of relatively (when compared to

other fuel production methods) large amounts of carbon dioxide (Momirlan and Veziroglu, 2002). The advantages it has over electrochemical methods are mostly related to thermodynamic efficiency (but also includes a larger range of potential substrates), however its major drawback is the inability to cleanly use renewable materials (the use of biomass involves the formation of tar or other unwanted waste streams (Saxena *et al.*, 2008))or waste materials, it's high energy input (which may be offset by linking to other processes) and it's limited application in relation to substrate flexibility for hydrogen production.

2.1.3 Biological hydrogen production

Broadly speaking it is possible to split the species of microbe that can produce hydrogen in to two groups, photosynthetic and non-photosynthetic. This creates the light and dark fermentation routes for biohydrogen production. This section explains the main difference between the hydrogen producing bacteria and also why microorganisms produce hydrogen.

There are many different species employed in the biological production of hydrogen and in 2001, Das constructed a survey of literature (Das and Veziroğlu, 2001). In that survey, and other hydrogen production surveys (Lynd *et al.*, 2009; Ni *et al.*, 2006; Holladay *et al.*, 2009), Das stated that most processes are either photosynthetic or fermentative, in this section the processes will be further sub-divided and categorised as photosynthetic, obligate anaerobes and facultative anaerobes. The recurring theme in the area of hydrogen production is the use of either waste products or renewable substrates for the production of hydrogen. (, Taguchi *et al.*, 1996; Hussy *et al.*, 2005; Redwood and Macaskie, 2006; Kapdan and Kargi, 2006; Vijayaraghavan *et al.*, 2006; Wang and Zhao, 2009; Redwood *et al.*, 2009; Sung, 2009)

The reasons behind the research into biological production of hydrogen are many, however versatility and thermodynamic efficiency are key. Hydrogen as a fuel source is valued as it non-pollutant, but there are many methods of hydrogen production, every biological method of hydrogen production has its own merits and drawbacks (see Table 2.2) and each also present differing engineering and biotechnological problems to solve.

Table 2.1 A comparison of the hydrogen production technologies (reproduced from (Das and Veziroğlu, 2001))

Microorganism	Merits	Demerits
Green algae	Performs photolytic hydrolysis of water. Solar conversion energy increased by 10x when compared to trees and crops	O_2 sensitive, Light dependant
Cyanobacteria	Performs photolytic hydrolysis of water. Can fix N_2 from the atmosphere and nitrogenase enzyme produces H_2	Sun light dependant, uptake hydrogenases will remove H_2, produces O_2 which is inhibitory to nitrogenase
Photosynthetic bacteria	Able to utilise a variety of waste materials and a wide spectrum of light	Light dependant, supernatant may pollute water, produces CO_2
Fermentative bacteria	Not light dependant, can utilise a variety of substrates, by products can be reused, no O_2 limitation problems	Supernatant requires pre-treatment to not pollute water, produces CO_2

Dark fermentation is thought to be more valuable than photosynthetic, for hydrogen generation, due to the higher potential generation rates and the simpler engineering considerations (do not need to supply light at large scale), though a combination of both has also been assessed examples of which can be seen in Section 2.1.3.4.

2.1.3.1 Photosynthetic hydrogen production

The photosynthetic production of hydrogen has its own engineering considerations, which revolve around reactor engineering. Small-scale reactors within the biotechnology field are often constructed using glass and as such can be illuminated (the vessel height to diameter ratio can be altered at bench scale), however upon normal scale-up, vessel fabrication cannot be carried out in glass, so the delivery of light to the culture becomes a problem that requires is own unique engineering solutions via reactor design and vessel fabrication.

The research into photosynthetic production of bacteria is centred on production by *Rhodobacter sp*. and Cyanobacteria, but other bacteria are also used (*Thiocapsa roseopersicina*, (Kovacs *et al*., 2006) *Rhodopseudomonas* sp. (Barbosa *et al*., 2001)). The metabolic methods employed to produce hydrogen very between the species but centre on the removal of molecular nitrogen and ammonia from fermentation with the concomitant production of hydrogen by nitrogenases, as both nitrogen sources inhibit photosynthetic processes. (Zurrer and Bachofen, 1979).

*Rhodobacte*r Sp.

This species of hydrogen producing bacteria produces hydrogen from a variety of substrates many of which are organic acids such as; malic acid and glutamate (through anaerobic growth (Eroglu *et al*., 1999; Koku *et al*., 2003; Kars *et al*., 2008) and food waste including brewery and dairy wastes (Laniecki, 2007).

Rhodospirillum rubrum

Lactate and lactic acid containing wastes were converted to hydrogen by the bacterium. The waste lactate was sourced from the fermentation of *Streptococcus feacalis* in whey and yoghurt waste and hydrogen is produced in this species by a nitrogenase (as it is in cyanobacteria and other photosynthetic bacteria)(Zurrer and Bachofen, 1979).

Thiocapsa roseopersicina

This bacterium is from a class of purple sulphur phototrophic bacteria; it has two potential hydrogen evolving membrane associated NiFe hydrogenases. The two hydrogenases, while similar, one hydrogenase (HynSL) shows greater activity at 80°C, even though the bacterium only grows below 30°C. The other hydrogenase (HupSL) shows closer relationships to the other bacterial NiFe based enzymes and is sensitive to both heat and oxygen when removed from the photosynthetic membrane. In this strain nitrogenases are also able to produce hydrogen via a differing mechanism and under nitrogen fixing growth conditions hydrogen evolution occurs, but only in the pre-stationary phase of growth, while use of up-regulated hydrogenases can cause up to 10-fold slower gas evolution that can be sustained (Bagi *et al*., 2004, Kovacs *et al*., 2006).

Rhodopseudomonas palustris

The *Rhodopseudomonas* sp. like other photosynthetic bacteria can produce hydrogen using organic acids (in this case, lactate, malate, acetate and butyrate) as a carbon source. The use of organic acids is seen as advantageous as it is often found in organic waste streams.

Spirulina maxima

This species of cyanobacteria is capable of producing hydrogen, with hydrogen photoproduction being induced by illumination, while growth occurs in dark fermentation mode (Juantorena *et al*., 2007).

Gloeocapsa alpicola

Immobilisation of this bacterium on glass fibres is used to effect the conversion of glucose to hydrogen. *Gloeocapsa alpicola* is a nondiazotrophic cyanobacterium, so is not capable of fixing atmospheric nitrogen (Serebryakova

and Tsygankov, 2007) (Non immobilized cells were shown to effect the same conversion in this study (Antal and Lindblad, 2005)).

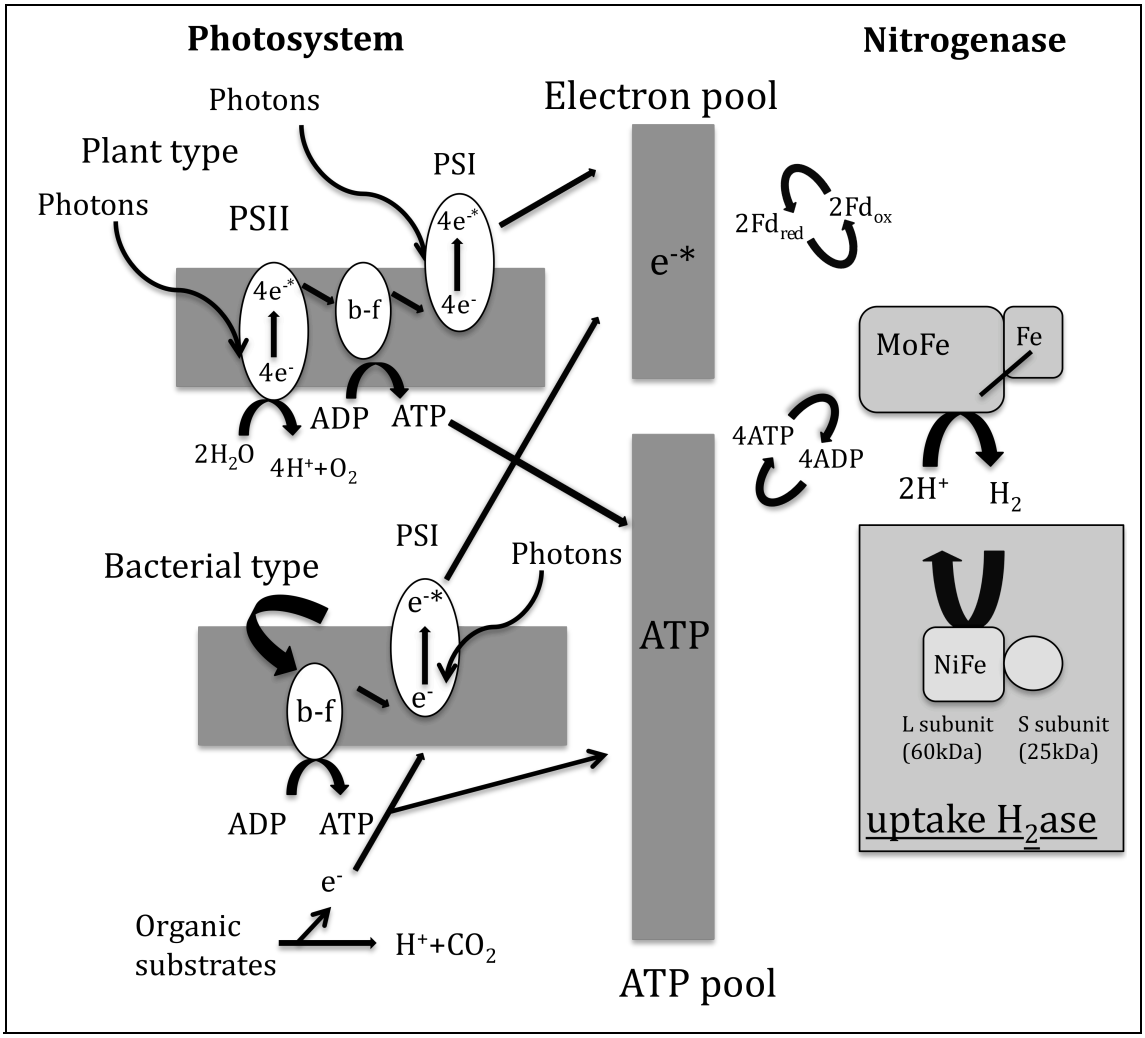


Figure 2.2 the hydrogen production mechanism in phototrophic organisms. Copied from(Miyake et al., 1999)

While there are many strains of photosynthetic organism capable of generating hydrogen, they all have a common method for doing as shown in Figure 2.2 for a more detailed look at the hydrogen production methods in cyanobacteria and phototrophic bacteria, it is beneficial to summarise the 2 systems separately.

2.1.3.1.a Photosynthetic bacteria

Photosynthesis in cyanobacteria and algae is similar to plant photosynthesis except that hydrogen can be produced instead of converting the substrate into more biomass. The photosynthetic process is a remnant from the earth's evolutionary past and is the way one way in which the planet derived oxygen (photosynthetic splitting of water) (Miyake *et al.*, 1999). With reference to Figure 2.2 Photosystem 1 (Labelled PSI) is used to supply the hydrogen producing nitrogenase with ATP. The ATP is converted to ADP by nitrogenase with the concomitant conversion of protons to hydrogen (Asada and Miyake, 1999).

2.1.3.1.b Algal production

Bacterial photosynthesis utilises organic acids whereas algal utilises water (Miyake *et al*., 1999). Algal production utilises a plant style photosynthetic mechanism and converts water to hydrogen, the nitrogenase reducing power is supplied by the oxidation of ferrodoxin, which is turn reduced by a supply of electrons from the electron pool. The electron pool is fed by a 2-photosystem mechanism (Figure 2.2) (Dutta *et al*., 2005).

2.1.3.2 Hydrogen production by obligate anaerobes

Obligate anaerobes form a large portion of the research into hydrogen production by 'Dark fermentation'. Obligate anaerobes appear to be less fastidious than facultative anaerobes, often employed for the conversion of a variety of substrates including mixed wastes. A variety of the employed strains will be described here and the generic methods they employ to produce hydrogen.

Clostridium sp.

The species of microbe has been widely researched in the field of biofuel production as they have the ability to perform 'ABE' (acetone, butanol and ethanol) fermentations from a variety of substrates, experimentation also reveals they can evolve hydrogen during growth. The species have been used to produce hydrogen from cellulose hydrolysate (Taguchi *et al*., 1996), from glucose, (Koskinen *et al*., 2008a, Koskinen *et al*., 2008b), from a mixture of acetate and lactate by *Clostridium diolis* (Matsumoto and Nishimura, 2007),

Thermotoga neapolitana

This bacterium is one of a class of thermophillic bacteria that are able to produce hydrogen, it is able to grow on defined media unlike many other strains in its class and it can produce hydrogen from cellulose, soluble starch, cellubiose, dextrose (Van Ooteghem *et al*., 2002) and glucose (Eriksen *et al*., 2007).

Caldicellulosiruptor saccharolyticus

This obligate anaerobe is another strain of bacterium capable of producing hydrogen from a variety of sources; it is a thermophile and produces hydrogen, carbon dioxide and acetate from cellulosic material and pectin containing biomass (Van De Werken *et al*., 2008).

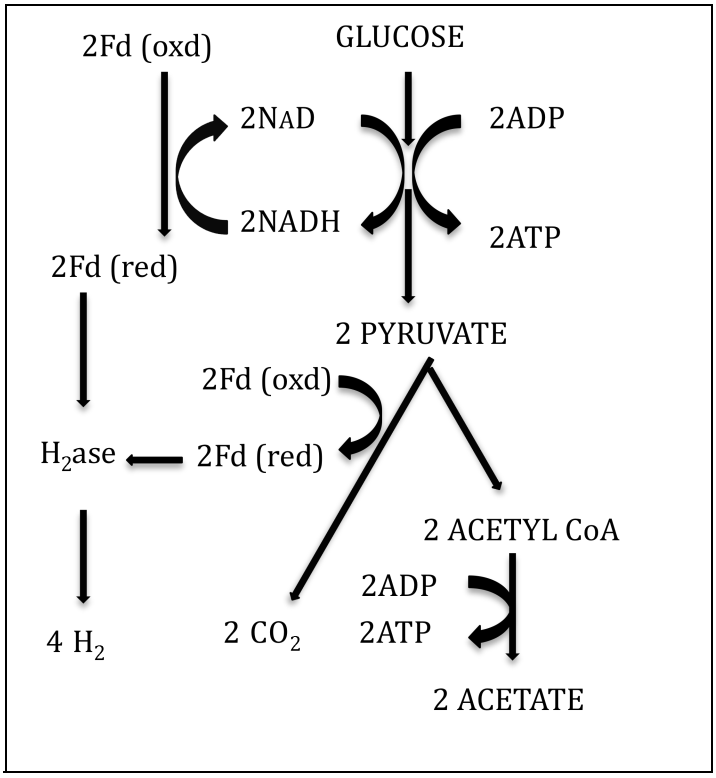


Figure 2.3 Hydrogen production from Clostridial type fermentations. Copied from (Hallenbeck, 2009)

As in conversion by facultative anaerobes (see Section 2.1.3.3) the branch point for the production of hydrogen occurs at pyruvate. The mechanism shown in Figure 2.3 is specific to clostridial type obligate anaerobes and involves the reduction of ferrodoxin. In the obligate anaerobes the fate of pyruvate (or phospho-enolpyruvate) is not mixed acids, but instead results in the production of carbon dioxide and acetate (via acetyl Coenzyme A), the enzyme pyruvate ferrodoxin oxidoreductase (PFOR) facilitates this initial conversion, and in doing so reduces the ferrodoxin. Ferrodoxin supplies electrons to the Fe-Fe hydrogenase that produces hydrogen. This reaction is effected by oxygen partial pressures; the lower the partial pressure the more NADH (generated in glycolysis) is re-oxidised for reuse.

2.1.3.3 Hydrogen production by facultative anaerobes

The current literature on the production of hydrogen by facultative anaerobes is focused on production by *Hafnia alvei, Enterobacter* sp*., Bacillus* sp*. and Escherichia coli*. The facultative anaerobes all generate hydrogen through the metabolism of glucose (or other carbohydrates sources that are able to be metabolised into glucose in the central metabolism) through the mixed acid fermentation (in anaerobic glycolysis) or the 2,3-butanediol route (Kapdan and Kargi, 2006). The mixed acid fermentation route is outlined in Section 2.4.4. There are many examples of facultative anaerobes producing hydrogen in literature, however the method they produce hydrogen differs, while the methods of metabolising carbohydrates are conserved the amount of hydrogen produced varies through strain or process alterations.

E. coli strain alterations tend to focus on the increase in efficacy of the H_2 generating hydrogenases and the formate hydrogen lyase (FHL) complex or the diversion of more substrate towards formate production (FHL over-expressing, (Yoshida *et al*., 2005); hydrogenase manipulation (up-regulation or deletion), (Nakashimada *et al*., 2002; Penfold *et al*., 2003; Maeda *et al.*, 2007b,)).

2.1.3.4 Other methods of biological hydrogen production

There are other methods of hydrogen production that do not fall neatly into the above categories. The first methods to be addressed are 2 stage methods of hydrogen gas production. One two-stage method of hydrogen production utilises the wastes from an *E. coli* hydrogen production process (organic acids) as a substrate for hydrogen production by *R. sphaeroides* (Redwood and Macaskie, 2006; Redwood *et al*., 2009).

There were also methods that employed recombinant DNA technology to either bestow a bacterium with the ability to convert more than one substrate (the

utilization of sucrose by *E. coli* (Penfold and Macaskie, 2004)) or to make it produce other non-native hydrogenases.

Aerobic cultures can also be used to produce hydrogen and recent research in to aerobic hydrogen production has shown that *Aeromonos sp*., *Pseudomonas sp*. and *Vibrio sp.* have this capacity (Kapdan and Kargi, 2006).

The last method of microbial hydrogen production to be assed is production using both anaerobic sludge and mixed microbial flora. Anaerobic sludge is typically sourced from anaerobic digesters within waste treatment plants whereas mixed microbial flora (MMF) may contain anaerobic sludge but can also consist of aerobic activated sludge, waste, faecal matter, soil and compost. Even though this is the case most MMFs in literature employ anaerobic sludge to produce hydrogen.

Anaerobic sludge

The anaerobic sludge in a study by Han, 2004, was isolated from an anaerobic digester in a sewage treatment plant, the substrate used for hydrogen production was food waste and the sludge was isolated by boiling to remove hydrogenotrophic organisms and leave spore-forming anaerobic bacteria (Han, 2004). Hyperthermophillic hydrogen production from glucose was assessed with the conversion taking place in an up-flow sludge blanket (Kotsopoulos *et al*., 2005). Anaerobic sludge can also be immobilised (in this case on ethylene-vinyl acetate copolymer discs) and utilise sucrose for hydrogen production (the sludge was isolated from a municipal sewage treatment plant)(Wu *et al.*, 2005). Lin *et al.* used anaerobic sludge obtained from a mesophillic sewage sludge digester in two studies. Both studies utilised the simple substrate glucose but in the latter study the anaerobic sludge was found to be dominated by *Clostridia sp*. (Lin and Chang, 1999; Lin, 2004). Lignocellulosics are a target renewable substrate for many biofuel production processes but can prove difficult to biodegrade, especially at mesophillic operating conditions, in a study by Sparling *et al.,* thermophillic sludge from anaerobic digesters (*Clostridium thermocellum* was

also employed) was used to convert lignocellulosic materials to hydrogen (Sparling *et al.*, 1997).

Mixed microbial flora (MMF)

In the first case of the use of MMF is the anaerobic digestion of jackfruit peel, which is a solid waste product from the fruit processing industry, the MMF in this case is isolated from cattle dung by pH adjustment and heat treatment, the MMF in this study is immobilized in an anaerobic upflow contact filter (Vijayaraghavan *et al.*, 2006). The next use of MMF, where the substrate used for hydrogen generation is palm oil mill effluent, the conversion is carried out at thermophillic temperatures 60°C. This is method is seen as beneficial as palm oil mill effluent is normally resistant to biodegradation (Othong *et al.*, 2007). MMFs are used in another study to produce hydrogen from non-sterile substrates of sucrose or sugarbeet, in this case the MMF was isolated from anaerobic sludge (Hussy *et al.*, 2005). Cattle manure is often used as a source of MMF, in this case the isolated MMF showed genetic similarities (99%) with three subspecies of *Caldanaerobacter subterraneus* after enrichment to a single species. In this study the substrate used for hydrogen generation was glucose and this is the simplest form of MMF, where the source is enriched to single or similar organisms (Yokoyama *et al*., 2009). A recent study involving the mixed microbial flora shows hydrogen production using sweet sorghum where the microbial flora is derived from the indigenous sorghum flora, (it also details production of hydrogen from mixed acidogenic cultures and pure cultures of *Ruminococcus albus* from the same substrate) (Antonopolou *et al*., 2007).

2.1.3.5 Summary of biological hydrogen production

While this shows a small section of the bacteria able to produce hydrogen in the literature there are many more. A survey in 2001 by Das *et al.,* displays a more extensive list. Each microbial method for hydrogen production has its advantages and however as previously mentioned those that require light suffer due to the novel engineering techniques required to supply light at large scale.

This leaves only dark fermentation and of the two groups involved in this the facultative anaerobes are of the most interest. This is due to the previous research that has been carried out on facultative anaerobes especially *Escherichia coli*, the wealth of knowledge about this bacterium, its use in bioprocesses, the scale-up of these processes and its genome makes it an ideal candidate for study, also the fact it is a facultative anaerobe makes bacterial growth simpler, so if a higher cell density is required from fermentation there are many techniques that have already been developed (see Section 2.3) to over come that hurdle. Also knowledge of the bacterial genome makes genetic manipulation a possibility in process intensification.

2.1.4 Summary and comparison of hydrogen production methods

There are differing takes on the future of hydrogen production, but they all agree on the necessity of the continuation of the use (and incremental improvement of) current/state of the art technology. This agreement however is only for the short-term status of hydrogen production (electrolysis and steam reforming are currently employed for hydrogen production and will be the methods of choice in the short term (Simbolotti, 2006)). The future of hydrogen production as suggested by Winter (2009) is shown in Table 2.2

Table 2.2 A potential future of hydrogen production technologies adapted from (Winter, 2009)

Timescale	Hydrogen production method
Current	Reformation of natural gas Coal gasification Partial oxidation of crude oil Electrolytic production
Mid Term (*c*.10 years)	Renewable electrolytic production Hydrogen from biomass
Long Term (*c*. 20 years)	Hydrogen from fossil fuels with carbon capture and storage (CCS) Nuclear heat facilitated hydrogen from coal High temp electrolysis Radiolysis, thermolysis and photocatalysis

While the future that Winter, (2009) suggests is technologically feasible it is unlikely. While some of the future hydrogen production needs may be met using high temperature/ nuclear heat combined electrolysis, the long-term production of hydrogen is likely to include the use of renewables and as such hydrogen production from fossil fuels is unsustainable.

Bringing together the conclusions derived from Sections 2.1.1.6, 2.1.2.4 and 2.1.3.5. The first point that should be made is that there will not be one solution for hydrogen production, this is due to a combination of contributory factors such as; differing natural resources available in regions and the fact that hydrogen production methods will probably change with time as technology progresses. As is the norm when considering current energy production methods, the scale, location and use of the energy there are differing methods of quantitative assessment, which will give potentially differing results, for this reason qualitative assessment is equally as important.

While it is possible to get greater energy and exergy efficiency from electrochemical processes when compared to thermal processes (Rosen, 1995)

but can produce far more CO_2 comparatively (Koroneos et al., 2004) this is not the whole story, a comparison is shown in Table 2.2 below.

Table 2.2 Comparison of hydrogen production technologies (Ramage and Agrawal, 2004)

Production Method	Temperature (°C)	Yield (%)	Pros	Cons
Electrolysis	>0	75-90	Eliminates CO_2 emissions	Low efficiency, Steam based process, requires high temp. reactors
Reforming	>700	70-80	40% reduction in CO_2 emissions	Linked to substrate price, Doesn't eliminate CO_2 emissions.
Thermochemical[1]	>600	>45	Eliminates CO_2 emissions	Aggressive chemistry, requires further development

Reforming and other thermochemical processes, require high temperatures, and more aggressive chemical degradation, while these techniques can be used with biomass as in Figure 2.1, the high temperatures and the possibility for the production of greenhouse gases (GHGs) (Lattin and Utgikar, 2007) means that

[1] Thermochemical conversion is the name applied to techniques such as Pyrolysis, Steam reforming, Water-Gas Shift, Gasification.

while research is being carried out into the use of these techniques, a more attractive solution lies in the use of biochemical conversion of substrates to hydrogen. While biochemical conversion could be applied to both in-vivo and ex-vivo techniques, there is limited research into in-vitro hydrogen production, but the research that exists shows that while its yield (on substrate) may be higher its rate of evolution is orders of magnitude lower (Woodward *et al*., 1996).

For this reason, and others including; its ability to use a variety or substrates, it's low energy input, and it's waste degradation potential, that the biological production of hydrogen is an attractive method currently researched to solve the energy gap.

2.2 Bioprocessing – Scale up and intensification

Within the field of bioprocessing, common techniques for improving the overall output of a process are scale up and genetic manipulation. There are many techniques used for both, the parameters used for scale up are process and strain dependant as well as being controlled by economic and engineering constraints. The techniques used for genetic manipulation tend to focus on the design of a strain whose metabolism is geared towards product formation rather than the evolutionary goal of reproduction. Techniques for both (scale up and genetic manipulation) are described and evaluated in this section.

2.2.1 Scale-up methodologies in bioprocessing

In the field of bioprocessing/ fermentation there is often a need for a change in scale. This can be done in more than one way; Scale-out (increasing the number but not the size of bioprocess units) and Scale-up (increasing the size but not number of bioprocess units) in some processes during Scale-up, the process may

have to change, as such Scale-in may occur (decreasing the number of bioprocess units to a single different unit), this process has occurred within this study changing from multiple shake flasks for growth to a single bioreactor.

There can be considered to be 3 differing scales of operation; lab scale, pilot scale and industrial scale. The definitive size of each scale is not fixed, but can be considered to increase by orders of magnitude (approximately $<10^1<10^2<10^3$L respectively). The main aim within scale-up is to ensure that the homogeneity remains within a culture (to the same degree) and to seek to ensure that each bacterium experiences the same physico-chemical environment at all scales. The typical parameters used for scale up in stirred, aerated vessels are (Ju and Chase, 1992, Amanullah, 1994):

- Maintaining geometric similarity
- Maintaining constant impeller tip speed
- Maintaining constant power input per liquid volume
- Maintaining constant impeller Reynolds number (Re)
- Maintaining constant oxygen uptake rate (OUR)
- Maintaining constant mass transfer coefficient (K_La)
- Maintaining dissolved oxygen tension (DOT) Profile
- Maintaining constant gas flow number (NA)
- Maintaining volumetric gas flow rate per unit volume of liquid (vvm)
- Maintaining superficial gas velocity (v_s)
- Maintaining constant mixing time

When embarking on theoretical scale up of a bioreactor and bioprocess, many factors may be used in conjunction to create an equivalent process at a larger scale, the choice of which parameters to focus on lies in the ability to define which parameters the process is most sensitive to (Schmidt, 2005). The number of parameters available to be maintained is limited by the degrees of freedom within the process (Ju and Chase, 1992). The above list is not exhaustive, there are other parameters that are used, ranges of dimensionless groups are also used, alone or in conjunction with each other (Schmidt, 2005).

2.2.1.1 Maintaining geometric similarity in scale up

This parameter is often the first consideration when scaling up a process (but also the first to be discarded), especially when fabrication is required. Maintenance of geometric similarity in a stirred vessel typically requires the conservation of a set of ratios. This parameter, while being the first to be considered, is also unlikely to be completely conserved. If the process involves moving from a shaken flask (as is the case in the process defined in section 3) to a bioreactor (stirred tank) geometric similarity cannot be conserved. Also if a large change in scale occurs the aforementioned ratios are likely to change (Table 2.6.1).

Examples of the ratios concerned in maintaining geometric similarity within reactors are (Junker, 2004):

- Vessel Height to Diameter (H_v/D_v)
- Impeller diameter to Vessel diameter (D_i/D_v)
- Vessel diameter to Vessel Volume (D_v/V)

The change in the above ratios, along with a possible change in the number, and design of the impellers within the vessel is designed to improve air utilisation and decrease energy input as bioreactors increase in scale.

2.2.1.2 Maintaining constant impeller tip speed during scale up

This parameter is often employed in the scale up of mycelial fermentations. This is because this parameter is directly related to shear, which mycelial fermentations can be more sensitive to (Ju and Chase, 1992). Bacterial fermentations however are not as sensitive to shear. Due to the small size of bacteria and their cell structure (section 2.4.2) they are less affected by hydrodynamic shear stress. The smallest scale at which turbulence and shear stress can occur is the is the Kolmogorov scale, this scale gives the minimum size of turbulent eddies which can occur within a bioreactor containing broth of a given viscosity. These eddies are too large to cause shear damage to bacterial cells(Joshi *et al.*, 1996). Scale up using this criterion results in a higher power input to a vessel, along with longer circulation times.

2.2.1.3 Scaling up by maintaining constant power input per liquid volume

This criterion is commonly used for scale up in the bioprocess field, especially in antibiotic fermentations and any process that contains shear sensitive operations or is an aerobic fermentation where gas dispersion is of importance (Ju and Chase, 1992). Constant power input per unit volume (P/V). The power input to the vessel can be expressed as both the gassed and ungassed power input. The volume is the current working volume (i.e. in a process where the volume changes such as a fed-batch process the value for P/V will change).
The power requirement for a gassed (gas sparged) fermentation is higher due to the formation of gas filled cavities behind the impeller blades, these cavities act to decrease the pumping power of the blades. The ratio of gassed power, P_g to ungassed power, P is linear (0-1) and dependent on the flow regime within the vessel.

Therefore $P \propto P_g$

And,

$$P = p_0 \rho D_i^5 N^3$$

where, P = Power (W)

p_0= power number (impeller)

D_i= Impeller diameter (m)

N= agitation speed (s^{-1})

One of the main difficulties in using the P_g/V as a scale up criterion is the calculation of the correlation that determines its value.

Another consideration of scaling up this criterion is the lack of complete characterisation of power input into shaken flasks. Research into this area tends to deal with empirical data and therefore gives system-specific correlations. A study into the power input into a shaken vessel was carried out by Kato *et al*. It correlated experimental observations and results to dimensionless groups (Reynolds number and Froude number), which could allow the relationship to be applied across a range of data sets, but the study was carried out using a cylindrical vessel which is geometrically dissimilar to a typical shake flask (Kato et al., 1996). The same type of study was carried out in shake flasks, using correlations with the dimensionless groups to calculate the power input in to the shaken vessel, focusing on the difference between different flow regimes (or phases) within the vessel (Büchs *et al*., 2000a; Büchs *et al*., 2000b; Büchs *et al*., 2007).

These studies gave the correlations;

$$Ne' = \frac{P}{\rho n^3 d^4 V_L^{1/3}}$$

$$\mathrm{Re} = \frac{\rho n d^2}{\eta}$$

$$Ne' = 70\mathrm{Re}^{-1} + 25\mathrm{Re}^{-0.6} + 1.5\mathrm{Re}^{-0.2}$$

where Ne, is the dimensionless power number; Re, is the dimensionless Reynolds number P, is power; ρ,is liquid density; n, is the shaking frequency; d, is the stirrer diameter/ maximum inner diameter of the flask ; V_L, is the flask filling volume; η,is the dynamic viscosity of the fluid.

Peter *et al.*, further improved this relationship with respect to relating the empirical correlations to this study by adding the effect of baffles on the volumetric power consumption in shake flasks. The un-quantified effect of phase of flow plays a larger role in power consumption in baffled shake flasks, but as the correlations found by Büchs *et al.*, are only inapplicable in systems with small shaking diameters and low liquid volumes, the difference is not applicable to this body of work, the only applicable conclusion is the increase in power consumption by baffled shake flasks (which is due to the hindrance to fluid flow afforded by the baffles) (Peter *et al.*, 2006).

2.2.1.4 Maintaining constant impeller Reynolds number (Re_i)

Reynolds number is a dimensionless number that give a numerical value to the amount of turbulence within the flow regime of a system. Turbulence in this case, is used as a measure of homogeneity within the bioreactor system.

$$\mathrm{Re}_i = \frac{\rho N D_i^2}{\eta}$$

This parameter is not generally used during scale up of bacterial fermentations as it does not account for the effect of aeration on the process and the deviation in geometric similarity during the process scale up also affects the Re_i (Junker, 2004; Ju and Chase, 1992).

Table 2.3 Examples of Reynolds number at different scales and agitation rates (Micheletti et al., 2006)

Bioreactor Geometry	N (rpm)	Re
Microwell (1000 μL)	500	700
	750	1060
	1000	1400
Shake flask (100 ml)	300	106800
Stirred tank (1.4 L)	700	24720
	1000	35320

The use of dimensionless groups in scale up can lead to infeasible operating conditions when used in conjunction with other parameters (Junker, 2004).

2.2.1.5 Maintaining constant volumetric mass transfer coefficient (K_La)

As with all mass transfer, K_La is affected by concentration gradients, the surface area available for mass transfer and the rate at which that transfer can occur. The use of K_La, as a scale up criterion therefore encompasses most of the hydrodynamic aspects of a bioreactor (Oldshue, 1985).

$$K_L a = A\left(\frac{P_g}{V}\right)^a v_s^b = \frac{OTR}{\left(C_L^* - C_L\right)}$$

with the variables A, a and b being defined by the operating conditions.

The quantification of the volumetric mass transfer coefficient within a shake flask is further complicated by the variation of both the flask closure and liquid volume within the flask (as well as the power input to the vessel). The material that is used as a closure device on a shake flask effects the K_La, as it mediates the replacement of air within the vessel, therefore the mass transfer across the closure will effect the mass transfer across the gas-liquid interface (all mass transfer is concentration gradient driven, therefore the gas phase must have a

higher oxygen concentration than the liquid phase)(McDaniel and Bailey, 1969, Nikakhtari and Hill, 2006).

One study into the effect that the vessel closures has on mass transfer, proposed that the main factors affecting it are the liquid volume (V_L), flask size (V) and rotating speed (N). As with the gas-liquid mass transfer the turbulence of the gas (T_G) has an effect (Nikakhtari and Hill, 2006).

$$T_G = \left(V / V_L\right) N^{\alpha}$$

With a foam closure as is used in this body of work this correlates to an across closure mass transfer coefficient (K_Ga) as below (where, α=1.31) ;

$$K_G a = 0.0013 T_G - 0.157$$

The study also shows that the type of closure used in the shake flask has the greatest effect on mass transfer into the vessel (Nikakhtari and Hill, 2006).

The actual volumetric mass transfer coefficient (at the gas-liquid interface, K_La), while being effected by the closure, has also been investigated separately, using both non-invasive techniques (Henzler and Schedel, 1991; Liu *et al.*, 2000; Gupta and Rao, 2003; Hermann *et al.*, 2003; Wittmann *et al.*, 2003,) and invasive probes (Van Suijdam *et al.*, 1978; Vasala *et al.*, 2006), with the non-invasive techniques including evaluation using non-invasive oxygen sensors and oxygen concentration measurement using sodium sulphite. There is general disparity between the between the findings of invasive and non-invasive measurement methods (this may be due to the additional "baffling effect" of the probe affecting the fluid flow regime, which would be more pronounced in an unbaffled vessel).

The correlation found in literature that would adequately model the experimental conditions used in the following study is:

$$K_L a = 3.18\left(h / D\right)^{-7.52}\left(V_G / V_L\right)^{0.75}\left(N^2\right)^{0.59}$$

where; h, is vessel height; D, is maximum vessel diameter; V_G, is volume of gas; V_L, is liquid volume; N, is the rotational speed (Liu *et al.*, 2000).

2.2.1.6 Maintaining dissolved oxygen tension (DOT) Profile

This criterion may be achieved by the control of other criteria, if however it is not, and the bioprocess is sensitive to the oxygen concentration within it at any given time then the DOT profile must be considered a standalone parameter and controlled as such. As suggested in section 2.6.3 the power input to a vessel is not the same power as that which is experienced by the aerated liquid within the vessel, for this reason, if this criterion is to be kept constant along with any other criterion that involves power input to the vessel the volumetric flow rate of air into the vessel must be kept constant (vvm). To allow the vvm to remain constant and still control the DOT of the fermentation, the constituent gases of air must be blended in non-atmospheric proportions to allow the DOT to be controlled (the proportion of N_2, O_2, within the inlet air varied). Gas blending can be used to either track a known DOT profile (as in this study) or to maintain the DOT at or above a certain level without changing the agitation or aeration rates (Korz *et al*., 1995; Onyeaka *et al*., 2003; Garciaarrazola et al., 2005; Antonio Rocha-Valadez *et al.*, 2006). The ability to perform experiments using this technique allows the separation of the compound effects of agitation and aeration of a bioreactor from the DOT within the reactor (Pollard *et al.*, 2002).
This scale-up method is used in processes where the process performance is linked to the DOT within the vessel.

2.2.1.7 Maintaining superficial gas velocity (v_s)

Superficial gas velocity is described as the ratio of the volumetric flow of the gas into the vessel and the vessels cross-sectional area. Maintaining the superficial gas velocity maintains gas holdup and prevents gas flooding within the vessel (Junker, 2004).

2.2.1.8 Maintaining volumetric gas flow rate per unit volume of liquid (vvm)

When scaling up using this criterion, the superficial gas velocity must also be addressed as increasing the vvm with increasing scale can lead to flooding, impeller overloading and excessive foaming in industrial size equipment (Ju and Chase, 1992; Junker, 2004). This parameter is effectively used to scale up processes that do not have mechanical agitation (Ju and Chase, 1992).

2.2.1.9 Maintaining constant mixing time

An increase in mixing time leads to increased temporal and spatial heterogeneity (Hewitt and Nienow, 2007), this would lead to the corollary that to keep the level of spatial and temporal homogeneity within a process constant upon scale up, mixing time should be held constant. The reality is somewhat different, maintaining constant mixing times during scale up, especially with geometrically similar vessels, leads to a large increase in power input (P/V)(Amanullah, 1994)

2.2.1.10 Evaluation of scale up methodologies

The description of the methods used for scale up in the preceding sections give an insight in to the difficulty that accompanies scale-up. In reality this complexity is reduced with process knowledge. In practice this means that any process, where the cell or the product is not sensitive to shear stress within the vessel, the parameters relating to power input are the lowest priority during scale up. A process that is sensitive to the level of oxygen within the vessel is often controlled by DOT, OUR or K_La. All methods and the methodology used to implement them are well established. The process used in this study, utilises *E.*

coli and as such, shear related parameters are unlikely to be useful in this case, with the bacterial size protecting it against sensitivity to shear forces, however sensitivity to parameters that affect the level of oxygen within the vessel is likely as the process is partly anaerobic.

2.2.2 Genetic engineering

This section briefly outlines the methods of manipulating the genetic information within bacteria. Due to the increase in the use of genetic manipulation over the last 35 years it has been possible to entirely characterize some specific genomes, and go on to isolate, and assess the functionality of specific sections of DNA within a genome (Nicholl, 2008). The techniques are used to enable bacteria to produce non-native compounds (or non-native amounts). This is done within microbial cells by either the cloning of all the genes for a biosynthetic pathway necessary, or to shut down/redirect substrates down certain native metabolic pathways.

2.2.2.1 Methods

The 2 methods employed for altering the genetic information of bacteria are mutagenesis and recombinant DNA technology. There are also various methods employed for screening the results of genetic manipulation, such as; Directed evolution (Arnold, 1998).

2.2.2.1.a Mutagenesis

This method of altering bacterial DNA can be either site specific or random. Site-specific mutagenesis in turn can be; oligonucleotide directed mutagenesis or

cassette mutagenesis, with the overall effect being a deletion, insertion or substitution of genetic material. Random mutagenesis is carried out by any medium that can mutate DNA, such as UV, mutator strains or error prone PCR (Berg *et al*., 2003).

2.2.2.1.b Recombinant DNA

This technology was developed by the pioneering work of Berg, Boyer and Cohen in the early 1970's, allowing novel conformations of genes to be cloned, amplified (by PCR) and introduced into a host cell. Genetic information is introduced in to a cell by suitable vectors, which include plasmid DNA (circular duplex DNA molecules) and bacteriophages (e.g. λ phage, M13 phage), which are viral vectors. Expression vectors stay as standalone genetic material, which can be used, whereas the other vectors can integrate themselves into the host genome for transcription (Berg *et al*., 2003).

2.2.2.1.c Metabolic engineering

This brief description of a section of genetic engineering may end up being the future of bioconversions. Essentially it revolves around the construction of a biosynthetic pathway (Bailey, 1991), but its advantage is the lack of necessity for the microbial cell, which in turn means no substrate is required for the stasis survival (Nyström and Gustavsson, 1998) of the cell during conversion giving higher yields (Woodward *et al*., 1996; Woodward *et al*., 2000).

2.2.2.3 Evaluation of genetic manipulation

In relation to this study, as the product can naturally be created by the bacterium, there is no need for recombinant DNA, however there is research that focuses on the use of recombinant DNA for the production of less fastidious non-native hydrogen producing enzymes within *E. coli*. This could be seen as

unnecessary as adequate process control can eliminate process under-performance by bacterial fastidiousness. When considering the production of hydrogen by *E. coli* the logical choice is mutagenesis, as the bacterial genome well known and researched, site specific mutagenesis to alter or remove parts of the genome that encode for metabolic processes that will compete for the substrate with the hydrogen production processes.

2.3 Bacterial growth and cultivation techniques

The kinetics of microbial growth are detailed in Appendix 3, this section shows how that background knowledge has been applied to develop methods of bacterial growth. There are three main modes used to cultivate bacteria; batch, fed-batch and continuous. Both batch and fed-batch modes will be considered and compared in detail as both were experimentally assessed during this study, whereas continuous culture will be described briefly.

2.3.1 Continuous culture

This method of bacterial cultivation was not used in this study; it involves the replenishment of nutrients by continuous addition. While nutrients are added, spent broth is removed to keep the culture volume constant, within that, nutrient poor, spent broth is biomass and potential products.

Control of continuous culture is carried out by feedback regulation of the medium flow rate (methods include the auxostat and turbidostat), but is often not used, and the process is operated in chemostat mode. Chemostat mode assumes culture homogeneity and that only the substrate required for growth is limiting and all others are present in excess. The culture growth rate is controlled by the rate of dilution and the cell death rate. This in turn means that in a chemostat the specific growth rate is always less than the maximum specific

growth rate (μ_{max}) and in practice the highest achievable growth rate is approximately 90% of the maximum (Pham, 1999).

2.3.2 Batch fermentation

In batch mode, the fermentation follows typical Monod growth kinetics as shown in Section 2.3.5 and Figure A.3.1, Appendix 3. The method is characterised by the addition of all the required nutrients at the beginning of growth, with the only additions being air, antifoam and pH control. Batch fermentations are typically carried out in both bioreactors and shake flasks

2.3.3 Fed-batch fermentation

The typical method used to achieve high cell density in both lab and industrial applications is fed-batch fermentation. In this method, high cell densities are achieved, by removing substrate related growth inhibition and controlling the growth rate of the bacterium. There are a variety of strategies utilised to feed the substrate to the culture:

- Linear feeding – there is a linear (constant) feed rate applied, the length of feeding is defined by this rate and the vessel volume, the specific growth rate will naturally decrease over time
- Step-wise feeding, this leads to higher cell densities than the linear method as more nutrients are fed in to the culture as the biomass increases and nutrient demand rises, cells can grow exponentially in this mode.
- Exponential feeding, the exponential increase in feeding rate, allows for control of the specific growth rate throughout the length of the fermentation (or until oxygen mass transfer becomes limiting), the ability to control specific growth rate is advantageous as it will avoid inhibitory acetate formation by over flow metabolism

Each method for feeding has its advantages, from simplicity, to the enhanced control and each can be further supplemented with feedback control (indirect and direct) to allow for any unforeseen process changes (Lee, 1996).

2.3.4 Comparison of techniques

While Sections 2.3.2 and 2.3.3 gave brief descriptions of batch and fed batch cultivation, this section details why they are chosen, compares the techniques and shows why they are used in differing circumstances. Continuous culture is the other option and during this study, it was not used. The decision not to use continuous culture for bacterial growth stems from its mode of operation. The growth is carried out as in Section 2.3.1, where there is constant replenishment of nutrients by the addition of growth substrate, and the removal of spent (lower substrate concentration) medium. This removal of medium is what is avoided when continuous culture is not used, reducing the increased potential for culture contamination. The precursor to hydrogen production (formate) is in the medium and constant removal of this would naturally limit the potential hydrogen production from the culture (low levels of extra-cellular formate imply low levels of intracellular formate which lead to a lack of transcription of FHL).

Batch growth is typically employed for shake flask and small-scale fermentation, due to ease of use. When shake flasks are used the difficulty of aseptic addition and the changing physical environment with increasing culture volume, in this mode all of the nutrients needed for cell mass are supplied which depending on the growth yield on the limiting substrate ($Y_{x/s}$) sets the maximum cell density achievable. While it would seem sensible to increase the concentration of all the components in the medium, the addition of such large quantities of substrate, especially simple molecules like glucose, can cause inhibition through a variety of processes, if growth is not inhibited by the substrate in excess it can cause growth to occur too quickly, which decouples energy utilisation from production

causing overflow metabolism, which is also detrimental to the cell, for this reason if an increase in cell density is required for process intensification fed-batch cultivation is used. As previously mentioned fed-batch growth works by utilising a specific feeding regime of a limiting substrate to control growth at a specific growth rate. There are a number of feeding regimes that could be employed; Linear Feeding, Step-wise Feeding, Exponential feeding and all can be subject to a form of feedback control. Exponential feeding is the most efficient feeding strategy as it allows for effective control of the specific growth of the bacteria while minimising the likelihood of substrate inhibition.

2.3.5 Microbial maintenance

Monod, while describing microbial growth kinetics, essentially assumed that the microbial energy requirement during growth for all non-growth associated processes was zero, but in 1965, Pirt suggested that microbial maintenance should be assessed to account for non-growth associated energy utilisation. This suggestion is another deviation from the (Pirt, 1965) assumptions postulated in Monod's growth kinetics stated previously. Another early reference to the necessity of maintenance energy that arose simultaneously as the work done by Pirt, was the work of Marr (Marr, 1991), the non-growth associated energy utilization was suggested due to the logical necessity for energy utilization by background metabolic processes that allow the bacterial cell to respond to environmental stress (It was found that β-Galactosidase production can be initiated and ceased very quickly in *E. coli* and this ability to respond to the cellular environment is an indication of maintenance energy) (Koch, 1997). Along with the ability to respond to environmental stress, other logical reasons for existence of a maintenance energy requirement include (Koch, 1997):

- The function and existence of mRNA
- The potential necessity for a cellular energy dump
- Endospore formation (in *Bacilli* etc.)

Microbial maintenance and its assessment/quantification have been studied for over 4 decades but it is still a concept that is debated. The debate centres around the fact that there is no fixed definition of the maintenance energy requirement, as the assumption that is the energy required by non-growth processes does not tell the entire story. The difficulties that arise when trying to quantify microbial maintenance are that this does not provide insight to underlying processes; non-growth components include (Bodegom, 2007):

- Metabolic pathway shifts
- Energy spilling reaction
- Microbial motility
- Changes in stored polymeric carbon
- Osmoregulation
- Extracellular losses of compounds not involved in osmoregulation
- Proofreading, synthesis and turnover of macromolecules
- O_2 stress defence

Quantification is difficult as it is unknown which factors are dominant and why, also, while all the above listed processes are non-growth associated they are not physiologically speaking all part of maintenance (when maintenance is defined as basic or endogenous metabolic processes)(Nyström and Gustavsson, 1998, Bodegom, 2007). The other issues surrounding the quantification of maintenance all arise out of ambiguity of definition, inference of a system constant from empirical data and the lack of inclusion of cell death in microbial cell dynamic models (Bodegom, 2007).

The issue of the effect of slow growth, on microbial maintenance has been addressed and as this study involves slowing growth by cultivation at reduced temperatures (see section 2.3.6), the effect that growth rate has on maintenance should be assessed.

The estimation of growth yield in respiratory metabolism shows the relationship between maintenance (m), growth yield (Y), maximum growth yield (Y^{max}) and growth rate (μ):

$$\frac{1}{Y} = m\left(\frac{1}{\mu}\right) + \left(\frac{1}{Y^{\max}}\right)$$

(Gadd, 2007).

This gives a linear plot as in Figure 2.4, but as maintenance is the slope of the curve it implies that maintenance is constant at all growth rates.

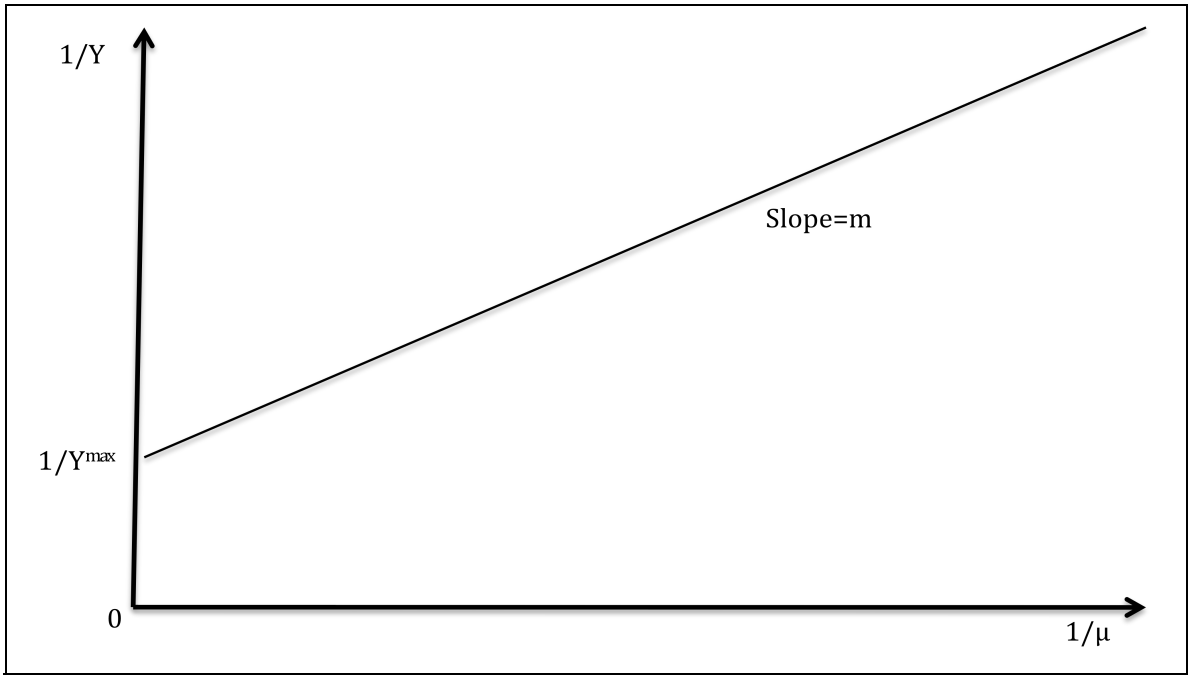


Figure 2.4 Relationship between growth rate, growth yield and maintenance energy. Reproduced from (Gadd, 2007)

Contrary to the graphical depiction Gadd *et al*., states the more intuitive relationship that at lower growth rates, the higher the maintenance energy, as maintenance is not constant for a given system or bacterium. It is postulated that the increase is due to the salient factors affecting maintenance changing when growth is slowed, and although the strictly non-growth associated parameters may have less of an effect, other factors such as energy spillage reactions and energy storage changes have an increased effect giving;

$$m_{tot} = d\frac{\mu_r}{\mu_{\max}}\left(\frac{\frac{1}{Y_G} + m_p}{1 - \left(\frac{d}{\mu_r}\right)}\right)$$

where; m_{tot}, is the total maintenance; d, is biomass excretion, leakage and cell death; μ_r, is the relative growth rate; μ_{max} is the maximum growth rate; Y_G, is the yield coefficient for growth purposes and mp, is the physiological maintenance.
This conceptual model shows that maintenance is not a constant, and that the fate of non-active biomass as well as the growth rate is of importance when defining the total maintenance of microbial cultures in any system (Bodegom, 2007). However this model does assume the relative growth rate cannot be zero.

Microbial maintenance has proved difficult to both adequately define and quantify, but it is of importance in all whole cell bioconversions. Its importance lies in the fact that if one can successfully cease bacterial reproduction and all competing metabolic reactions, one will still not achieve 100% yield on substrate, and this deviation from the maximum theoretical yield is due to the energy required for microbial maintenance. The greater this energy requirement the lower the yield will be.

2.3.6 Medium composition and design

E. coli (along with other bacteria) is able to grow on complex media, partially defined and fully defined media. The basic concept behind all media compositions is the necessity to provide the cell with all the nutrients required for growth. While it can be simplified to simply requiring the nutrients for growth in certain biotechnological situations, the medium requirement is more than that for growth (even with auxotrophs), but also includes specific components required for the synthesis of specific bacterium i.e. for hydrogen production from *E. coli*, molybdate, selenite and nickel are required for the production of hydrogenases and formate hydrogen lyase (Zinoni *et al*., 1984; Soini *et al*., 2008) and the improvement of phytase production by glucose addition to a complex medium (Sunitha *et al*., 1999). It can be shown that while bacterial growth is simple (with respect to nutrient supply), bacteria can be very fastidious if required to perform specific non-growth functions.

In medium design, defined medium is usually used to show the effect of the change in concentration or omission of a component (Zhang and Greasham, 1999). Strategies employed for medium design include mathematical models, neural networks, linear programming, statistical experimental design and stochastic global random search (Kennedy and Krouse, 1999; Kennedy *et al.*, 1994; Weuster-Botz, 2000).

The design of media for bacterial growth often forms stand-alone bodies of work. The necessity and relationship between different components evaluated, however a trend has been seen, that runs through much of the research that is linked to bacterial growth and that is that medium design tends to be focussed on growth in optimal conditions as has already been mentioned (and researched (Soini *et al.*, 2008)). Specific components are required for anaerobic metabolism/ mixed acid fermentation, and as such if growth is affected by oxygen limitation, other factors (such as temperature, discussed in Section 2.3.7) that affect the growth of bacteria should be researched or taken into consideration when media are chosen.

2.3.7 Effect of culture temperature on growth

It is known that temperature has an effect on bacterial growth; this is most easily seen at 'extremes', where sub-zero (°C) effectively inhibits bacterial growth, and temperatures in excess of 121°C are used for sterilisation. There is however a varying range of temperatures at which any microbe can grow, but a change in growth temperature can affect the microbial physiology during growth. Mesophiles such as *E. coli* usually grow in the temperature range of 30-45°C, the normal working temperature for *E. coli* being 37°C.

The general effect in a change of growth temperature on the rate of growth is analogous to a change in the reaction rate of any chemical reaction, for this reason Ratkowsky (Ratkowsky *et al.*, 1982) used an adapted form of the

Arrhenius equation to correlate the change in growth rate with the culture temperature.

$$k = Ae^{\left(-\frac{E}{RT}\right)}$$

The above equation is the typical Arrhenius eq. where; k, is the specific reaction rate constant; A, is the reaction frequency factor; E, is the activation energy; R, is the universal gas constant; T, is the absolute temperature. This can be converted into a microbial version (Ratkowsky *et al*., 1982);

$$r = Ae^{\left(-\frac{C_T}{RT}\right)}$$

where, the variable k has been changed for; r, the growth rate constant and E, for C_T the temperature characteristic.

The plots obtained from this relationship do not exhibit typical linear correlations between temperature and the growth rate constant, this shows that while E, experimental activation energy, is constant in chemical reactions, the analogy C_T, Temperature characteristic, is not a constant and is in fact a decreasing function of temperature (Ratkowsky *et al.*, 1982). This relationship put simply, shows that growth rate is not linearly effected by temperature and is also said to imply that unlike the chemical reaction it is analogous to, it is a complex multistep reaction (Ratkowsky *et al.*, 1982). Further to the work of Ratkowsky in 1982, his findings were extended to cover the entire biokinetic temperature range (Ratkowsky *et al.*, 1983), as in 1982, it was found that bacteria could not be simply related to the Arrhenius plot to estimate the effect of temperature on bacterial growth rate, but a relationship could be found empirically (Ratkowsky *et al.*, 1983).

$$\sqrt{r} = b\left(T - T_{\min}\right)\left(1 - e^{c(T - T_{\max})}\right)$$

where; r, is the growth rate constant; b, is a regression coefficient; T, is; Tmin, is the minimum temperature at which the growth rate is zero; Tmax, is the maximum temperature at which the growth rate is zero and c, is a model specific parameter for data fitting above optimal temperatures (Ratkowsky et al., 1983).

While this equation may effectively predict the effect of temperature on the growth of some bacteria, the change in physiology may have more of an effect on a process than the change in growth rate.

Graham Smith first reported the physiological effect of a change in temperature in 1920. Smith found that cell yields were greater at temperatures lower than those at which the maximum growth rate occurs. Many researchers (including Monod and Senez) have shown that more of the carbon source, which is utilised for both energy and as a cellular building block, is found as an integral part of the cell when growth occurs at a lower than optimum temperature (Ng, 1969). It has also been found however that if the temperature drops below that at which the Arrhenius equation is obeyed, cell yield will decrease (Ng, 1969). A process such as the one in this study, operating at 30°C can be assumed to not be deviating from the augmented Arrhenius equation and therefore cell yield will increase at this operating temperature, when compared to cell yield at the optimal growth temperature. Having established an increase in cell yield it is important to assess the effect of this change, i.e. how does this increase in yield effect the cell morphology or physiology? With respect to cell morphology, it has been shown that a decrease in growth temperature may affect the shape of the cell (Trueba and Woldringh, 1980), past the natural variation within the cell division cycle. There is also a case for the assumption that a decreased growth rate will increase the density and rigidity of the cell, as it has been shown that a decreased growth rate, leads to an increase in the cellular percentage of peptidoglycan, which forms part of the bacterial sacculus that defines the cell shape and rigidity (Driehuis and Wouters, 1987), this result is partly backed up by the models that try to explain the relationship between growth and cell size/shape during replication at differing growth rates (Koch, 1982). The research found showing the effect of temperature on cell physiology shows that growth at a low temperature (30°C) leads to larger cells (Shehata and Marr, 1975; Trueba *et al*., 1982). The assumption could be made that, as the Arrhenius equation is applicable to cell enumeration it may also be applicable to the biochemical processes within the cell, which would also be considered analogous to chemical reactions.

While there is research into the effect of slow growth (through both oxygen limitation and temperature reduction) on microbial physiology, it is not done in conjunction with consideration of process improvement, as such no literature has been found on the effect of slow growth on fed batch cultivation, and much is done to ensure sub-optimal growth conditions are not achieved. This is presumably due to fact that most processes that require the increase in cell density that can be found using fed batch cultivation, will not require slow growth and also it is counter intuitive (unless it is a physiological imperative) to concomitantly slow growth, while trying to improve process productivity.

2.4 *Escherichia coli*

The previous sections have shown initially why hydrogen is sought after as a potential fuel, why a facultative anaerobe, and specifically *E. coli* is the ideal candidate for research into biological hydrogen production, methods for process improvement and factors that may affect them. This section outlines the bacterium (*E. coli*) species, its structure and metabolism.

2.4.1 *Escherichia coli* Background

The *Escherichia coli* bacterium is widely researched in the bioscience, biotechnology and bioprocessing fields as well as also being used industrially. It was first identified by Theodor Escherich (a German paediatrician) in 1885. The reason the organism is widely researched is due to the level of genetic knowledge known about the wild type parent strains (Blattner *et al*., 1997) and its physiology. Genetic manipulation is widely studied and considered safe and

high cell density cultivation has been achieved and studied (Lee, 1996; Andersson *et al.*, 1996; Hewitt *et al.*, 2000).

E. coli is a typically robust enteric bacterium from the family Enterobacteriaceae, it is a mesophile and as such it is able to survive at temperature range from approximately 15-40°C, with an optimum growth conditions at 37°C and pH 6-8. It is a gram negative. Non-spore forming bacterium. *E. coli* is an aerobic organism able to function as a facultative anaerobe.

2.4.2 *Escherichia coli* Structure

The *E. coli* cell is a rod shaped organism, typically approximately 2μm in length and under 1μm in diameter (Figure 2.5, 2.6), Its structure allows for genetic information dissemination through the mechanism of conjugation, utilising the pili (Figure 2.6).

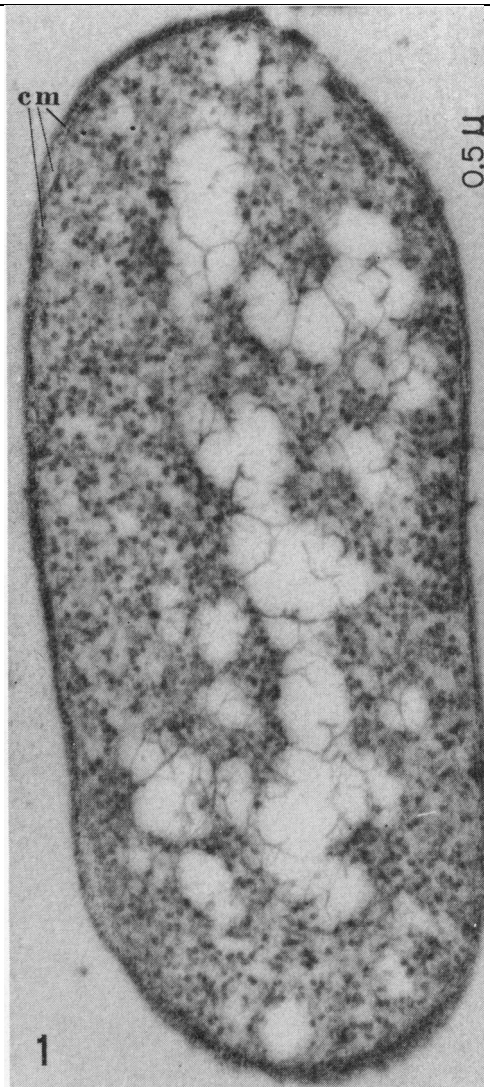


Figure 2.5 Electron micrograph of *E. coli*. Copied from(Conti and Gettner, 1962)

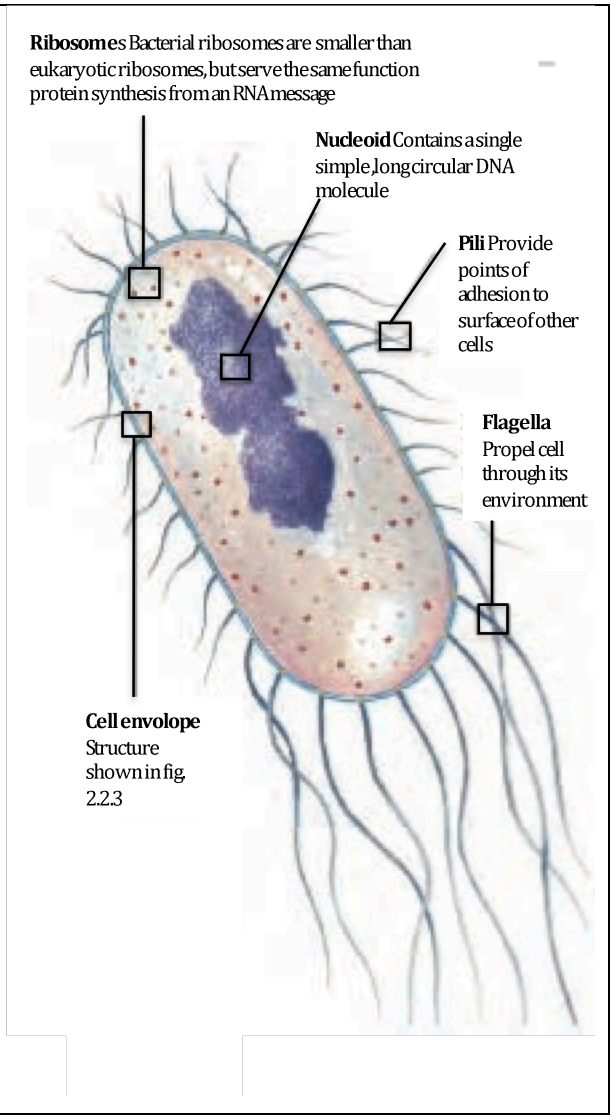


Figure 2.6 Structural representation of gram negative bacteria. Copied from (Cox, 2003)

The cell itself is made up mostly of water but exhibits a mechanically tough cell envelope. The cell envelope, as in other gram negative bacteria, consists of an outer membrane (structure is shown in Table 2.5), the peptidoglycan section forms the periplasmic space in E. coli with the cytoplasmic membrane forming the inner section of the cell wall (Kellenberger and Ryter, 1958). The peptidoglycan layer within the periplasmic space contains murein. The inner, cytoplasmic membrane acts as permeability barrier to a the transport of small cations within the cell, trans-cell envelope transport is carried out by a structure within the membrane that can span the entire cell wall called membrane vesicles

(Shechter *et al*., 1974), the membrane is also the site of manufacture for the cell wall constituents (Costerton *et al.*, 1974) also contains part of membrane bound hydrogenases (as well as other membrane bound enzymes and enzyme complexes), the enzymes used in hydrogen production and the redox safety valve to the microbial cell (Dubini *et al*., 2002; Gadd, 2007). The protein MreB is found attached to the cytoplasmic membrane, it, in conjunction with MreC and MreD helps define the shape of *E. coli* and MreB deficient mutants of the bacterium are spherical instead of rod shaped (Kruse *et al.,* 2005).

The periplasmic space contains the structural peptidoglycan (murein) polymer, which gives the cell its rigidity and protects against the osmotic pressure from within the cytoplasm. Mitchell originally defined this section of the cell envelope in 1961 (Costerton *et al.*, 1974) and apart from its structural feature, his concept was of a section of the cell envelope that contains enzymes and binding proteins (Costerton *et al*., 1974).

The outer membrane (components listed in Table 2.6) has an outer leaflet comprised of lipopolysaccharide, this confers the cell with more resistance to lysozyme, hydrolytic enzymes, surfactants, bile salts, hydrophobic antibiotics and other hydrophobic substances (Zimmermann and Rosselet, 1977; Gadd, 2007), the entire composition and function of the outer membrane is shown in Table 2.4

Table 2.4 The outer membrane (OM) components and corresponding functions in *E. coli* (Hong Kim and Michael Gadd, 2008)

Component	Function
Phospholipid	Inner leaflet
Lipopolysaccharide	Outer leaflet, hydrophilic in nature providing a barrier against hydrophobic compounds. Stabilises surface structure via bonding with metal ions (e.g. Mg^{2+})
Lipoprotein	Lipid section is embedded within the hydrophobic region of the OM, Sugar region is covalently bonded to murein stabilising the OM
Outer membrane protein A	Maintains OM stability, amino acid and peptide receptor, forms F-pilus in recipient cell during conjugation
Porin	3 different porins, OmpC, OmpF and PhoE, all consist of three peptides and act as channels for hydrophilic solutes
Receptor proteins	Act on aminoacids, vitamins and sugars etc.
Other proteins	Enzymes and extracellular protein export machinery

Another important structural feature in this bacterium is the pili shown in Figure 2.5, which are a form of fimbriae and enable adhesion to other cells and the transfer of genetic material (DNA) to other cells via the F-pilus (or sex pilus) (Gadd, 2007).

The final notable feature of *E. coli* physiology to be discussed is linked to its complex metabolism. It is the ability to respond to environmental stress (Stanier, 1951). While, like other mesophiles it cannot respond to extreme changes in temperature it can respond to other environmental stresses. The ability to respond and adapt is postulated to be one of the reasons for the requirement for microbial maintenance energy (see Section 2.3.5). An example of *E. col*i and its

ability to respond to environmental stress is the metabolism of arabinose. *E. coli* are not normally able to use this sugar efficiently, but if it becomes their sole carbon source, they are able to synthesise enzymes that will convert it into a useful carbon source, the ability to respond to environmental stress is advantageous due to the rapidly changing biochemical environment the cell inhabits. The cellular response can take the form of enzyme synthesis, metabolic changes, and membrane transport process changes (Berg *et al.*, 2003).

2.4.3 *Escherichia coli* Metabolism

In typical cell growth on a medium containing glucose, glucose is metabolised using the Embden-Meyerhof-Parnas (EMP) pathway (fig. 2.3.1) and the Hexose monophosphate pathway (HMP). All prokaryotes metabolise glucose using these pathways and combined with the TCA cycle they form the central metabolism (CM)(Gadd, 2007)

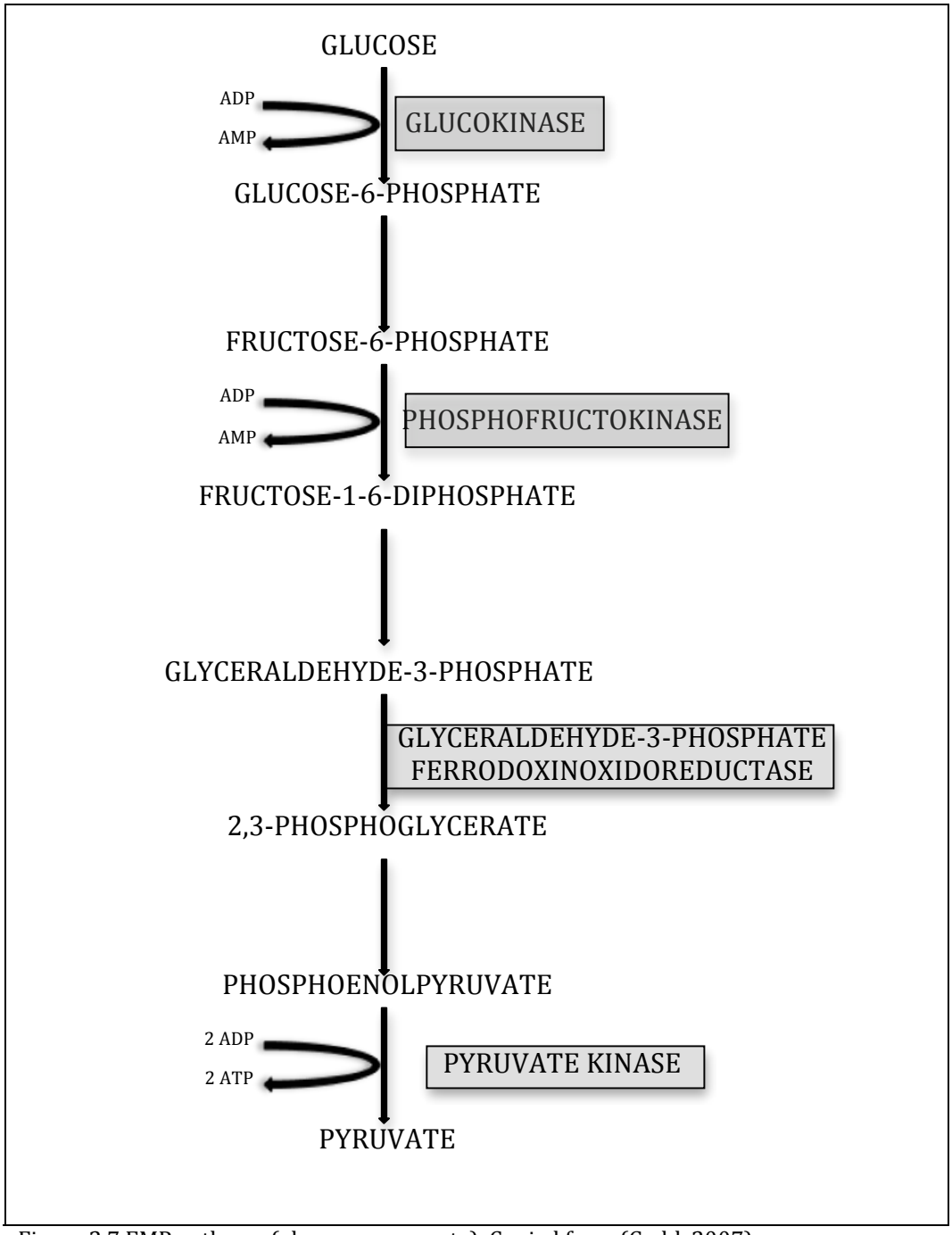


Figure 2.7 EMP pathway (glucose – pyruvate). Copied from (Gadd, 2007)

The central metabolic pathways of *E. coli* are used to generate energy, coenzymes, reducing agents and the building blocks for biosynthesis of new cellular material; it is split up in to 3 separate pathways (EMP, PPP and TCA cycle). These linked metabolic mechanisms show the pathways in aerobic fermentation and metabolic pathways change during anaerobic growth (see Section 2.4.4).

The EMP pathway is used to break down glucose and produce energy in the form of ATP. The pathway produces half of the intermediates used as carbon building blocks for biosynthesis, and while it is a major glycolitic route, other substrates can be converted to glucose, or other components within the pathway, for use in this pathway (Gadd, 2007; Durnin *et al*., 2009).

When glucose is the sole carbon source for *E. coli*, the EMP pathway can only metabolise about 72% of the substrate, so the remaining glucose enters the PPP pathway. The PPP (or hexose monophosphate pathway, HMP) pathway produces more precursors for biosynthesis and also more reducing agents that are used during biosynthesis (Gadd, 2007).

Under aerobic conditions the fate of pyruvate from the EMP pathway is the TCA cycle. Oxidation of pyruvate allows it to enter the TCA cycle as acetyl CoA, the compound is then oxidised within the cycle to produce reduced electron carriers (NAD^+, $NADP^+$ and FAD^+) which are then oxidised by the electron transport and oxidative phosphoryation processes. This reduction and oxidation of electron carriers allows the synthesis of ATP and the production of the proton motive force, which essentially leads to energy generation and transduction (Gadd *et al*, 2007).

Acetate is often a fermentation product during high cell culture of *E. coli*, this accumulation of this fermentation by product is said to be due to the inability of the oxidative metabolism (perhaps the TCA cycle) to keep up with the anabolic and catabolic requirements of the cell. Acetate production is thought to be chosen as it is energetically favourable as a secondary route, producing the second largest amount of ATP (Han *et al.*, 1992). It is thought that a reduction in

glucose concentration will decrease the glucose uptake rate and stop acetate production (Akesson *et al.*, 2001). Acetate can be re-assimilated into the cell (Diaz-Ricci *et al.*, 1991), and it is beneficial to do so as the presence of acetate within the culture can have a detrimental effect on the cell physiology (Kleman and Strohl, 1994; Farmer and Liao, 1997).

2.4.4 Anaerobic glycolysis

The strain used within this project is a facultative anaerobe, as with other microbes of its species it produces hydrogen through anaerobic glycolysis. Figure 2.7 shows the EMP pathway, which as stated before shows the breakdown of glucose to pyruvate, during anaerobic glycolysis the EMP leads to mixed acid production, with a branch point at Phosphoenolpyruvate to form succinate and all other products (lactate, ethanol, acetate and formate) being formed from pyruvate (Ataai and Shuler, 1985).

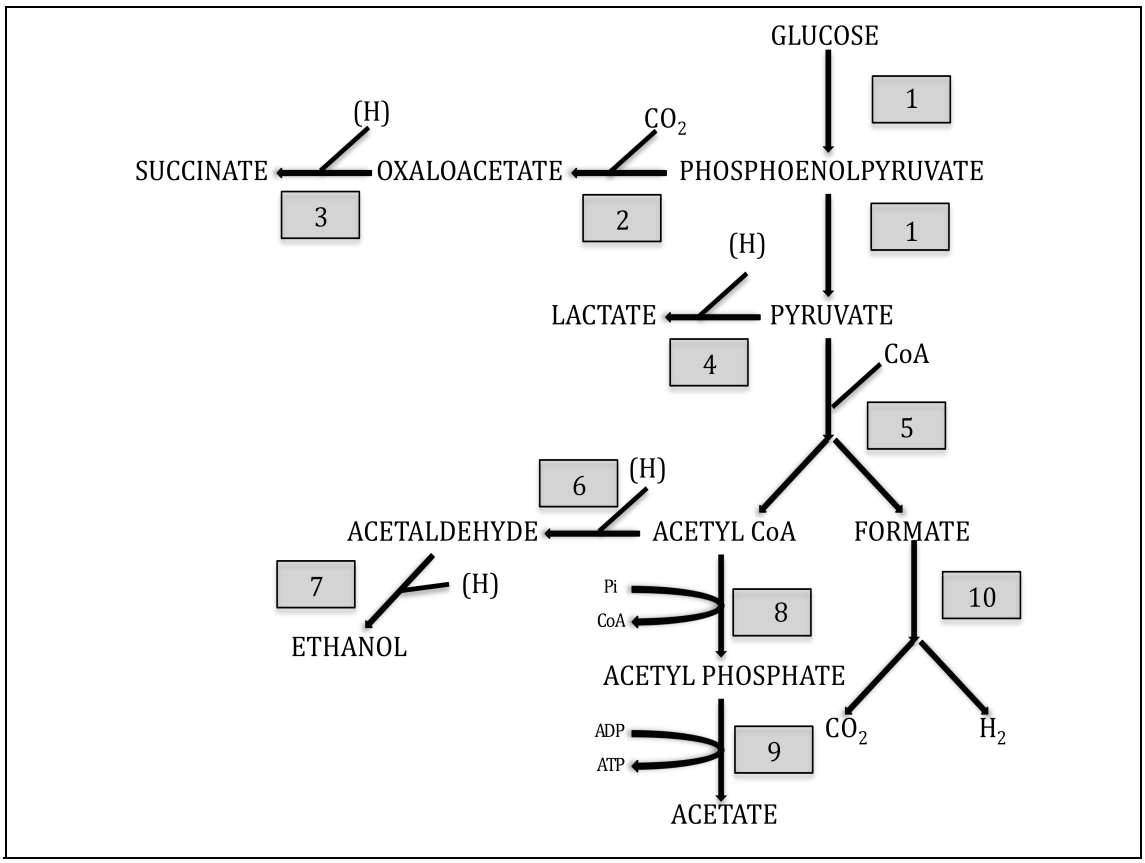


Figure 2.8 Anaerobic glycolysis. Reconstructed from (Ataai and Shuler, 1985; Gadd, 2007)

Table 2.5 Enzymes and pathways used in anaerobic glycolysis

Number (Figure 2.7)	Enzyme/pathway
1	EMP pathway
2	Phosphoenolpyruvate carboxylase
3	TCA cycle
4	Lactate dehydrogenase
5	Pyruvate formate lyase
6	Acetaldehyde dehydrogenase
7	Alcohol dehydrogenase
8	Phosphotransacetylase
9	Acetate kinase
10	Formate hydrogen lyase

The anaerobic production of these mixed acids are facilitated by specific enzymes, but with respect to the biological production of hydrogen, only two are of importance; pyruvate formate lyase (PFL) and formate hydrogen lyase (FHL) (Gadd, 2007).

2.4.4.1 Pyruvate formate lyase

Pyruvate and phosphoenolpyruvate are the branch points in anaerobic metabolism, and it from these two compounds all the organic acids are made. In the route from pyruvate to hydrogen production the first enzyme is PFL. The necessity of this enzyme for hydrogen production enforces some culture condition restrictions on the fermentation. PFL is found to be present in aerobically grown cultures, but the level can be increased by up to ten fold by switching the culture to anaerobiosis. The expression of PFL is repressed by nitrate, and this repression is controlled by NarL (Sawers and Bock, 1988), which is part of a two-component system, that represses the transcription of many operons encoding anaerobic respiratory enzymes, while it activates

transcription of operons encoding nitrate respiratory enzymes (Goh *et al*., 2005), NarL is an oxygen and nitrate dependant regulation system (Stewart, 1982). This leads to the conclusion that for effective PFL activity, there should be a low oxygen and nitrate level within the fermentation.

2.4.4.2 Formate hydrogen lyase

FHL is the final enzyme involved in the production of hydrogen during anaerobic glycolysis. It is a membrane bound enzyme complex consisting of the hydrogenase 3 (Hyd-3) and formate dehydrogenase-H enzymes (FDH-H) as in Figure 2.8 (Yoshida *et al*., 2005; Maeda *et al.*, 2007a). The enzyme complex requires the elements selenium, molybdenum (FDH-H) and nickel (Hyd-3) (Yoshida *et al*., 2005), which leads to the necessity for these elements to be in the medium. Transcription of the FHL complex is induced by intracellular formate concentration (Sawers, 1994a; Sawers, 2005) and repressed by oxygen and nitrate (Sawers, 1994a; Richard *et al.*, 1999; Self and Shanmugam, 2000) in the same way as the PFL.

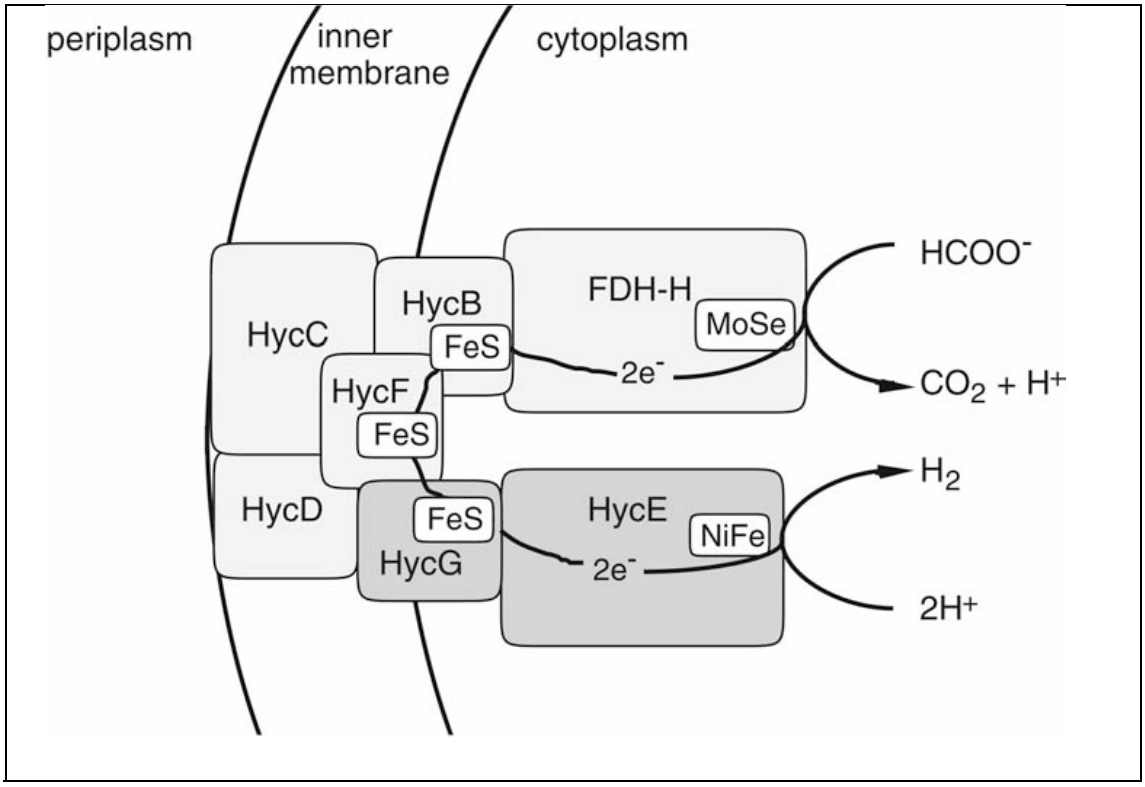


Figure 2.9 Structural model of the FHL complex. Copied from (Leonhartsberger *et al.*, 2002; Bagramyan and Trchounian, 2003).

2.4.4.3 Overview of anaerobic glycolysis and hydrogen production

While aerobic metabolism is an important consideration for bacterial growth, it is only the anaerobic metabolism that will produce hydrogen within *Escherichia coli*.

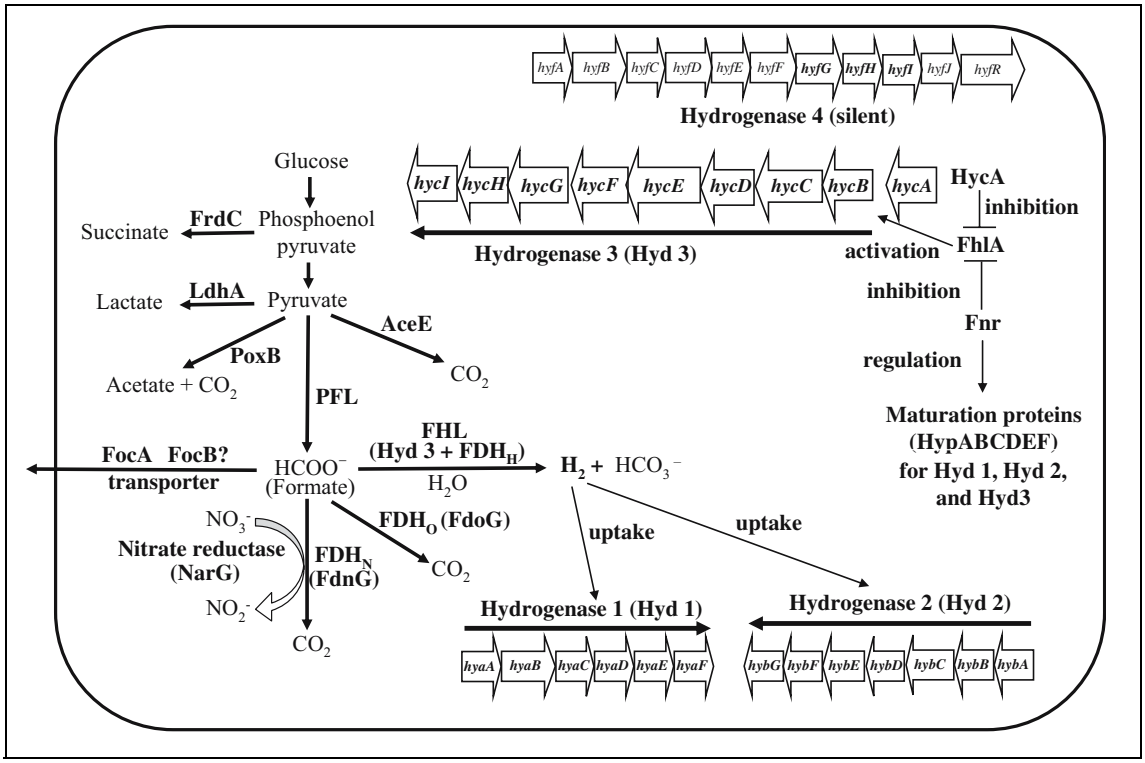


Figure 2.10 Schematic of fermentative hydrogen production in *E. coli.* Copied from (Maeda *et al.*, 2007a)

Figure 2.10 shows the potential routes of formate and how hydrogen is produced and taken up, and the genes that encode the synthesis of each hydrogenase.

The route which formate takes depends on many factors, diverted from hydrogen production by nitrate and oxygen, as well as the accompanying pH drop from formate production diverting the metabolism towards lactate production (Hallenbeck, 2009).

2.4.4.4 Evolutionary reason for hydrogen production

While there are many chemical elements, bacteria only contain 12 in significant quantities one of which is hydrogen. Hydrogen can be considered a major constituent of the bacterial cell but its presence, as a building block does not explain the reason for its production. Bacteria are also noted for their ability to adapt to environmental stress to survive. As can be seen from Section 2.1., the production of hydrogen is coupled with low oxygen levels in fermentation (being

carried out by obligate and facultative anaerobes). This phenomenon occurs during catabolism, hydrogen is produced by "branched fermentative catabolic" pathways, which leads to the coproduction of oxidized molecules (Hong Kim and Michael Gadd, 2008). Hydrogen is also used as an electron donor by some denitrifying prokaryotes (facultative anaerobic chemoorganotrophs and chemolithotrophs) e.g. *Ralstonia eutropha, Paracoccus denitrificans, Hydrogenobacter thermophilus* and *Thiolalkalivibrio sp.* and also as an oxygen donor in methanogenisis, in this capacity it is able to release energy through the reduction of other compounds.

Hydrogen is produced as necessary (for utilisation) within the cell as an electron donor and as part of catabolism, but it is also produced as a response to an extracellular stress. In anaerobic glycolysis its production acts as a cellular defence to the drop in extracellular and intracellular pH caused by the production of formic acid. The defence mechanism works by formate export from within the cell, and subsequent extracellular formate breakdown by the cell membrane bound formate hydrogen lyase (FHL) complex.

A brief summary of the necessity for hydrogen production by bacteria can be made by considering the action of hydrogenases. The hydrogenases act to generate hydrogen gas or take hydrogen back in to the cell and as thus they act as a redox safety valve and effectively control the amount of reducing power within the cell (Kovacs *et al.*, 2006).

2.4.5 *Escherichia Coli* HD701 as a bacterium for biohydrogen production

The strain used in this study has been upregulated with respect to hydrogen production. This upregulation has occurred by deletion of *hycA*, part of the hyc operon located in the 58/59- min of the *E. coli* chromosome (Rossmann *et al.*, 1994). The genetic mutation that this strain has undergone, removes the inhibition of FHL by the hyc operons (as shown in Figure 2.9), this makes it a

more effective producer of hydrogen when compared to the parent strain (Penfold *et al.,* 2003). There are many other mutations that could have been made to the strain, such as the removal of hydrogenase 1 and 2, which are both uptake hydrogenases, but as hydrogen production is seen as a redox valve for the cell, the utilisation of hydrogen uptake for reducing power may be essential for the stasis survival of the cell, as such could make the cell more fastidious to process conditions. The only other potential way to improve the strain would be to over-express FHL in conjunction with the removal of inhibition.

The strain itself was [supplied by August Böck], was not the only candidate for use in this study, while the main aim of the study was to intensify a process that already existed, the first step could have been a strain change or augmentation. The choice of Strain occurred due to the reasons already stated.

2.6 Literature conclusions

From the literature just reviewed, the strain used in this study (*Escherichia coli* HD701) was a good choice due to its simple genetic mutation that allows improved hydrogen production. The wealth of literature makes *E. coli* a good bacterial candidate among the other facultative anaerobes, and this division of bacterium is chosen due to the simpler engineering considerations (when compared to photosynthetic organisms) and simpler process control (when compared to strict anaerobes). Process intensification should be carried out by sensible (preliminary) medium choice/design and by switching cultivation from

batch to fed batch mode. When scale up is attempted, geometric similarity cannot be conserved as the process is currently carried out in a shake flask, consequently due to the use of *E. coli* and its lack of sensitivity to shear, the change should be made initially considering oxygen mass transfer.

2.7 Aims of the study

The process in this study consists of bacterial growth in a shake flask, followed by subsequent hydrogen production in a bioreactor. The growth medium initially used is nutrient broth[#2] and the substrate for hydrogen production is glucose (further detailed in Chapter 3). The principal aim of the study was process intensification, the increase of volumetric production of hydrogen from the process so that the energy that can be produced surmounts the energy used by the process, making it a net energy producer. This leads to further aims:

- Fully characterise and replicate the initial shake flask-growth orientated batch process
- Define conditions under which the process can occur in one vessel (bioreactor)
- Identify a suitable medium for an increase in cell density through batch growth
- Show that the increase in cell density, increases the volumetric production of hydrogen
- Identify a suitable medium for fed batch cultivation
- Identify process conditions where fed-batch cultivation can lead to hydrogen production

The work presented in this thesis set out to fulfil these goals.

Chapter 4 forms the Results and Discussion section of this thesis; the aims set out above are addressed in this section. Section 4.1 outlines the initial process

replication and the process characterisation. Section 4.2 shows the conditions necessary for growth and hydrogen production to occur in one vessel, shows the effect of an increase in cell density on the process and identifies a medium to do so. Section 4.3 further investigate a suitable medium for increasing cell density and moves the mode of growth to fed-batch growth.

3. Materials and methods

3.1 Materials

All cultivation and fermentation media were supplied by the Biocentre, School of Chemical Engineering, University of Birmingham (Birmingham. UK) (purchased from Sigma-Aldrich, UK).
Gases were either supplied by BOC Gases (Guilford, UK) or compressed in house using a Fluidair 67 Rotastar Rotopak Compressor (FluidAir international Ltd., Bolton, UK) and an Atlas Copco NG10 nitrogen generator (Atlas Copco Ltd, Hemel Hempstead, UK).

3.2 Strain

Escherichia coli HD701 was provided by Professor A. Böck (Lehrstühl fur Mikrobiologie der Universitat, Munich, Germany)

3.3 Culture maintenance.

Master and Working Cell Banks (MCB and WCB respectively) were created using the same method.

Single bacterial colonies were grown on Nutrient agar at 37 °C, having being taken from a Nutrient agar slope provided. The single colonies were then selected and used to inoculate a 500 ml baffled Erlenmeyer shake flask containing 50 ml of sterile Nutrient broth (NB), which was subsequently incubated for 8 h at 30 °C and 200 r.p.m. in an orbital shaker (New Brunswick, USA). 1ml aliquots were then taken and combined with 20% glycerol (v/v), which acts as a cryo-preservant and stored at – 80 °C in cryogenic vials (Nalgene, NY, USA). This formed the MCB. The WCB was created using the same method, inoculating with a 1mL aliquot from the MCB.

3.4 Overall Process

The process as a whole can be thought of as 2 distinct phases; the cell growth phase and the hydrogen production phase as shown in fig. 3.1. During the Cell growth phase the methods employed are as in section 3.5 and during the hydrogen production phase the methods are as in section 3.6.

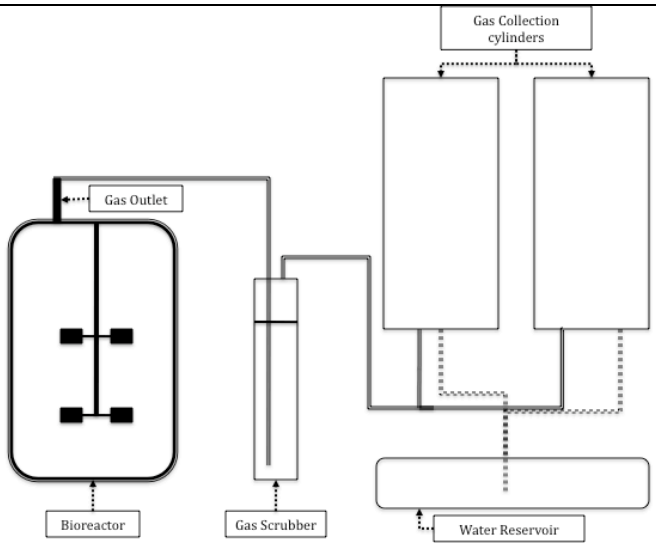
Cell Growth	Hydrogen Production
Shake Flask	
Bioreactor	

Figure 3.1 Process Description Diagram

3.5 Experimental protocols

While carrying out this body of research there was some experimental commonality that carried throughout all the experiments. All experiments were carried out in duplicate and for each data point 2 samples were taken and an average of these values is shown as the upper and lower limit of error bars on the graphical plots.

3.5.1 Cell Growth

Bacterial colonies were picked from an agar slope, which was prepared weekly from the WCB. Nutrient agar plates were spread with these colonies and incubated overnight at 37°C

All starter cultures were grown at 30°C in 100mL Erlenmeyer shake flasks containing 10mL of culture medium. The length of cultivation was 8 hours in all experiments and two starter cultures were grown for use as an inoculum (in case one culture did not grow). Inoculum size was experiment dependant, but the removal and introduction of the inoculum to the next phase of growth took place aseptically using a 200 μL pipette (Biohit Ltd., Devon, U.K.) from the inoculum flask (50 mL shake flask) and inserted aseptically to the medium by removal of the foam bung in the case of shake flask growth and inserted through an addition port in the case of bioreactor growth. When the inoculum size was increased to 5%, the addition occurred through the utilisation of a 500 mL shake flask (with a side-arm) employing a 20% working volume. The flask in this case was then attached to an addition port using a sterilised length of silicone tubing.

Further growth was carried out at 30°C in either 2L shake flasks or 5L bioreactors (experiment dependant). In all cases of batch growth the total culture volume was 2L (achieved by using two 2L shake flasks with 50% working volume or by using a single bioreactor with 40% working volume), the bioreactor based experiments were agitated at 300 rpm and aerated at 0.75 vvm (unless experimental conditions required this to change).

During all modes of growth, samples were taken hourly to determine OD_{600} and organic acid levels (off-line sampling methods shown in section 3.9). Culture samples were removed aseptically by pipette in the case of shake flask cultures and in the case of bioreactor sampling, all samples were taken aseptically using the reactor sampling port. Reactor Geometry and medium composition are shown in Section 3.6 (along with gas blending apparatus protocols and feeding strategies). For all experiments cell growth took place four times, the primary duplicate to determine a growth curve, then the secondary duplicate to attempt hydrogen production. During the second set of duplicates sampling did not occur in batch phase, but did continue in fed-batch phase. During cell growth the off gas from the fermentation (when using a bioreactor) was vented so as not to affect the hydrogen collection equipment. Sample preparation took place as in Section 3.9

During fed-batch growth feeding took place using the exponential strategy outlined in section 3.6.2.3. The feeding point was chosen to be the point at which the DOT level reached a minimum and began to rise. This time point within the fermentation was found by performing batch cultivation (in duplicate) with the fed-batch medium (not including the feed) prior to fed-batch growth. This preparatory experimentation gave not only the time at which to begin feeding but also the optical density (and therefore cell mass) that would be found allowing the approximate level of feeding to be calculated before fed-batch fermentation began.

3.5.2 Hydrogen production

Experimental procedure during the hydrogen production phase had some commonalities with cell growth. All hydrogen production occurred in a bioreactor. The bioreactor typically contained (some experiments called for a change in this ratio, see Section 4.3) 2 L of fermentation culture (derived from either; 2 x 2 L shake flasks employing a 50% working volume or 2 L of culture from a bioreactor) and 2 L of substrate. During hydrogen production the temperature was maintained at 30 using the bioreactor based temperature control (see Section 3.8.3) with constant agitation at 300 rpm and no aeration (in between the growth and hydrogen production phase the system is agitated and sparged with nitrogen to remove any residual oxygen).

3.6 Fermentation

There were two modes of fermentation used, batch fermentation (in shaken flasks and bioreactors) and fed-batch fermentation (in bioreactors).

3.6.1 Batch

During batch mode fermentation, 2 different vessels were used.
Shake flasks of varying sizes but with geometric similarities and stirred tank bioreactors. When shake flasks were employed for cultivation they were incubated at 30°C in an orbital shaker (Newbrunswick, USA) at 200 r.p.m.

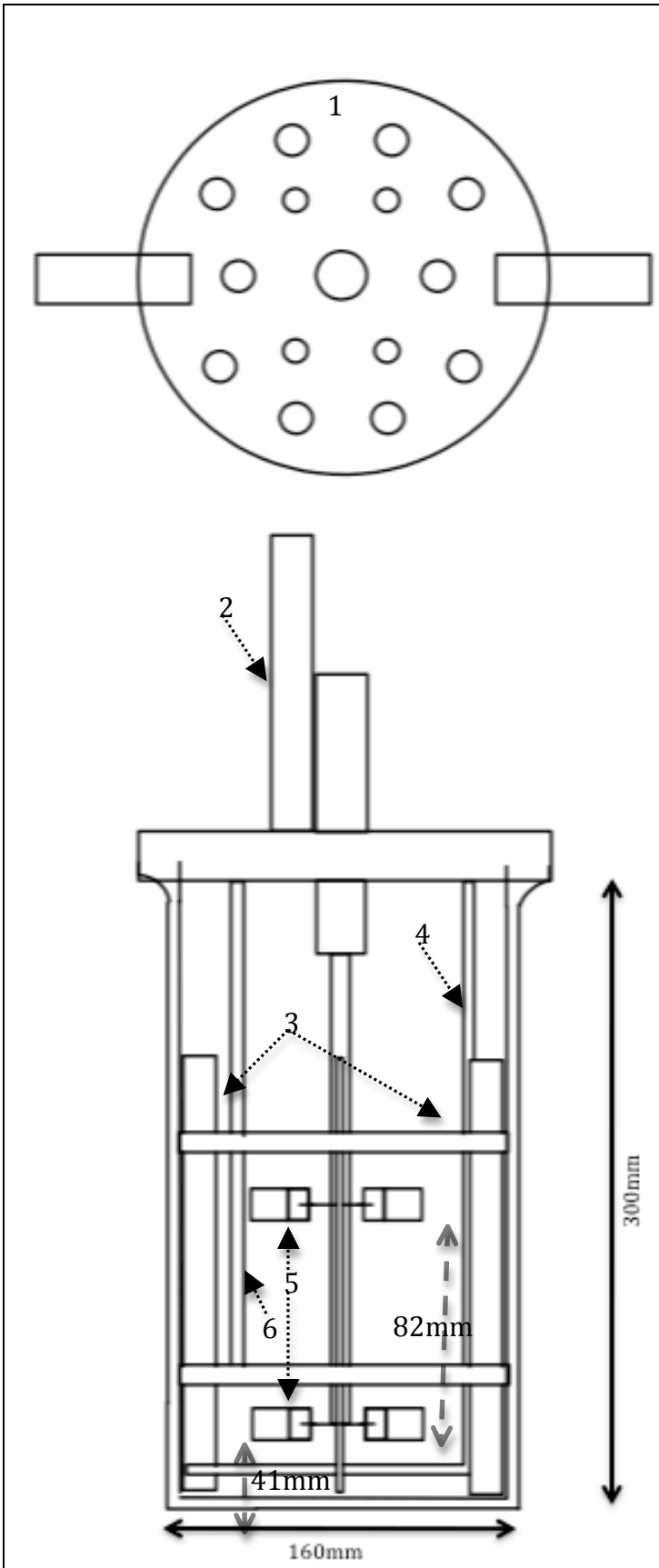
3.6.1.1 Vessel geometry

The shake flasks used were standard Erlenmeyer flasks, baffled and varying between 250ml and 2000ml in size. The working volume of the flasks varied from 10 - 50%. The opening was sealed with a porous foam bung.

The Bioreactor used (in both batch and fed-batch mode) was a 5L Electrolab bioreactor FerMac 300 series (Electrolab, Tewkesbury, UK). The bioreactor had a 4L working volume and is equipped with two 6-bladed paddle type impellers 0.082m in diameter and 4 equally spaced baffles for mixing, the variety of ports in its top plate were sealed apart from those used for:

- pH probe
- DOT probe
- Feed addition (during fed-batch)
- Gas outlet
- Air inlet
- Acid/base additions

As shown in Figure 3.2.



Label	Apparatus	Dimensions (mM)
1	Top plate	200 (diameter)
2	Gas outlet	220x20
3	Baffles	20x200
4	Gas sparger	n/a
5	Impellers	82 (diameter)
6	Temp. probe holder	6x230

Figure 3.2 Bioreactor diagram (1:3 scale)

3.6.1.2 Medium preparation

All sterilisable medium components were sterilised using a Boxer autoclave (Wolf Labs, York, UK) at 121°C and a pressure of 1atm. Non-sterilisable components were filter sterilised using a 0.2μm filter and added aseptically before the addition of cells to the medium.

Table 3.1 Nutrient Broth#2 Composition

Component (chemical Formula)	Concentration (g/L)
Tryptone	10
Lab – lemco powder	10
NaCl	5

Table 3.2 Batch Medium Composition

Batch:

Component (chemical formula)	Concentration (g/L)
KH_2PO_4	3
Na_2HPO_4	6
NaCl	0.5
Casein	2
$(NH_4)_2S0_4$	10
Glycerol	35
Yeast extract	20

Post sterilisation additions:

Component (chemical formula)	Concentration (g/L unless stated)
$MgSO_4.7H_2O$	0.5
$CaCL_2.2H_2O$	0.029
Thiamine	0.008
$FeSO_4$	0.04
Citric acid	0.02
Trace elements (see table below)	0.5 ml/l

Trace elements:

Component (chemical formula)	Concentration (g/L unless stated)
$AlCl_3.6H_2O$	0.2
$CoCl_2.6H_2O$	0.08
$CrK(SO_4).12H_2O$	0.02
$CuCl_2.2H_2O$	0.02
Boric acid	0.01
KI	0.2
$MnSO_4.7H_2O$	0.26
$NiSO_4.7H_2O$	0.009
$Na_2MoO_4.2H_2O$	0.004
$ZnSO_4.7H_2O$	0.004

Table 3.3 TYGEP Medium Composition

Component (chemical formula)	Concentration (g/L unless stated)
Tryptone	10
Glucose	4
Yeast extract	5
Potassium phosphate buffer (pH 6.5)	0.1 M
Sodium molybdate	1 μM
Sodium selenite	1 μM
Nickel chloride	5 μM

Table 3.4 Modified Super Broth Composition

Component (chemical formula)	Concentration (g/L unless stated)
Soytone	12.5
Glycerol	6.3
Yeast extract	24
Potassium phosphate buffer (pH 6.5)	0.1 M
Sodium molybdate	1 μM
Sodium selenite	1 μM
Nickel chloride	5 μM

3.6.1.3 Inoculum preparation

The inoculum for each experiment was prepared in the same way, an appropriately (experiment and vessel dependant) sized aliquot of the batch medium in use was transferred aseptically to a shake flask (sized so that the medium was 10% of its volume)

3.6.1.4 Gas Blending

The gas blending equipment used during a series of batch and fed-batch experiments was designed and supplied by LSL Biolafitte (Luton, England). The gas blender allows the proportion of nitrogen, air and Oxygen entering the vessel to be mixed (blended) so as to alter the dissolved oxygen tension (DOT) within the bioreactor without changing the volumetric flowrate of gas to the vessel.

Calibration of the gas blender was carried out using air, nitrogen and oxygen. The gas blender is equipped with a mass flow controller this is set to equal the

maximum volumetric flowrate of gas into the reactor (1vvm). The airflow potentiometer was zeroed using nitrogen at 1vvm without the presence of air or oxygen. Air at 50% of the air valve opening (and at 1 vvm) was used to calibrate the 50% point. The value for the mass flow was set and maintained, while the set point for the DOT in the fermentation vessel was fixed initially then monitored and changed through out each experiment.

3.6.2 Fed-batch Fermentation

During fed-batch fermentation the scale was altered slightly (through feeding and sampling), however the approximate culture volume remained at 2 L, the vessels used for batch cultivation were re-used.

3.6.2.1 Media preparation

Table 3.5 TGYEP medium Composition (Fed-Batch)

Batch:

Component (chemical formula)	Concentration (g/L unless stated)
Tryptone	10
Glucose	4
Yeast extract	5
Potassium phosphate buffer (pH 6.5)	0.1 M
Sodium molybdate	1 μM
Sodium selenite	1 μM
Nickel chloride	5 μM

Feed A:

Component (chemical formula)	Concentration (g/L unless stated)
Tryptone	20
Glucose	250
Yeast extract	10
Potassium phosphate buffer (pH 6.5)	0.2 M
Sodium molybdate	2 μM
Sodium selenite	2 μM
Nickel chloride	10 μM

Feed B:

Component (chemical formula)	Concentration (g/L unless stated)
Tryptone	40
Glucose	250
Yeast extract	20
Potassium phosphate buffer (pH 6.5)	0.4 M
Sodium molybdate	4 μM
Sodium selenite	4 μM
Nickel chloride	20 μM

Table 3.6 Modified super broth medium Composition (Fed-Batch)

Batch:

Component (chemical formula)	Concentration (g/L unless stated)
Soytone	12.5
Glycerol	6.3
Yeast extract	24
Potassium phosphate buffer (pH 6.5)	0.1 M
Sodium molybdate	1 μM
Sodium selenite	1 μM
Nickel chloride	5 μM

Feed a:

Component (chemical formula)	Concentration (g/L unless stated)
Glucose	250

3.6.2.2 Inoculum preparation

For fed-batch cultures the inoculum was prepared in a manner identical to that of batch cultures (section 3.6.1.3)

3.6.2.3 Feeding strategy

In the fed-batch fermentations an exponential feeding rate was used to maximise cell density and minimise growth substrate inhibition. This was calculated using the following equation (Strandberg *et al*, 1994):

$$F = \left(\frac{1}{s}\right) \times \left(\frac{\mu}{Y_{xs}} + M\right) \times X_0 \times e^{\mu t}$$

Where, F, is the substrate feed rate; s, is substrate concentration; Y_{xs}, is bacterial yield on the limiting substrate; μ,is the specific growth rate; M, is the maintenance coefficient; X_0, is the initial cell mass; t, is time, see Appendix 5 for further details.

3.7 Hydrogen evolution

3.7.1 Volumetric measurement of Gas evolution

The volumetric amount of hydrogen produced during the experiments was quantified using water displacement in 2L measuring cylinders (Figure 3.1). It was assumed that during the anaerobic respiration of *Escherichia coli*, only 2 gases were produced H_2 and CO_2 and as such to more accurately measure the volume of hydrogen evolved during experiments, the CO_2 was removed by passing the fermentation oulet gas through a solution of 3M NaOH, which acts as a gas-scrubber and removes the CO_2 using the following reaction $CO_2 + NaOH = NaHCO_3$

This allows only the volume of H_2 to be measured.

3.8 On line analysis and control

3.8.1 Dissolved Oxygen Tension (DOT)

The dissolved oxygen tension within the fermentation cultures and during hydrogen production was measured using a polargraphic steam sterilisable oxygen electrode (Ingold). The aeration rate within all fermentations (unless otherwise stated) was kept at 0.75 vvm (volume of gas/ volume of culture/ minute) and this typically kept the DOT level high enough that there was no oxygen limitation exhibited. When DOT control was necessary, gas blending was used (section 3.5.1.4) so that the aeration rate could be kept constant.

3.8.2 pH

pH Measurements of the fermentation medium within the bioreactor were made online using a steam sterilisable pH electrode. (pH Fermprobe, Broadly-James, UK)

3.8.3 Temperature

Fermentation temperature was measured using an external temperature probe, and controlled using a heating pad and cooling circuit ("Cold finger").

3.8.4 Exit Gas analysis - Mass Spectroscopy

Initial experiments only sought to compare volumetrically the amount of gas evolved during the anaerobic hydrogen production phase (section 3.6) but during the scale-up and optimisation of the process mass spectroscopy was employed to analyse the outlet gas during the anaerobic hydrogen production phase. As the minimum working gas flowrate of the Prima δB Process MS (Thermo Scientific, UK) was above that of the expected gas evolution, nitrogen was used as a carrier gas, and mass ratios of the evolved gas re-calculated to remove the carrier gas.

3.9 Off line analysis

3.9.1 High Performance Liquid Chromatography (HPLC)

Chromatographic analysis of the experimental samples was performed using a Cecil CE4000 series HPLC (Cecil Instruments, Cambridge, UK), utilising a Bio-Rad Aminex HPX-87H column (Bio-Rad laboratories Ltd., Herts., UK) to effect the separation of compounds within each sample. Samples were run at a pump flowrate of 0.6 mL/min and a column temperature of 50°C. The use of both a UV detector and a refractive index detector was employed for detecting organic acids and sugars respectively. The UV detector was set at 210 nm. A Basic Marathon Auto-sampler (Spark Holland B.V., Netherlands) was used to run a sequence of samples.

3.9.1.1 Calibration

The calibration standards were chosen using the end products from anaerobic glycolysis. Each standard was prepared using volumetric flasks, and the differing concentrations were obtained using serial dilutions.
The calibrations standards are shown in Appendix 4.

3.9.1.2 Sample preparation

All samples were pre-filtered using a Millipore Millex 0.2 μm filter (Millipore UK Ltd., Watford, UK) prior to analysis.

3.9.1.3 Column regeneration

Column regeneration took place periodically; the column was regenerated following the procedure provided by the manufacturer, which is shown below.

1. Cleaning

a. 5% acetonitrile in 0.005M H_2SO_4, 0.2 mL/min, 65°c for 4 hours in reversible flow mode.

b. 30% acetonitrile in 0.005M H_2SO_4, 0.2 mL/min, 65°c for 12 hours in reversible flow mode.

c. 0.005M H_2SO_4, 0.2 mL/min, 65°c until steady baseline

2. Regeneration

a. 0.025M H_2SO_4, 0.2 mL/Min, 65°c for 4-16 hours

b. 0.005M H_2SO_4, 0.2 mL/min, 65°c until steady baseline

3.9.2 Optical density measurements

Optical density measurements were taken using a Uvikon 922 Spectrophotometer (Kontron Instruments, Bletchley, UK). The measurements were taken at 600 nm and all samples were removed asceptically from the culture and diluted so that the sample was within the working range of the spectrophotometer (0.2-1 Optical density units).

4. Results and discussion

Processes can be extremely sensitive to changes when the key parameters are not understood. This may lead to problems when relocating (as in this study) or outsourcing which can in turn impact the economic viability of the product. The solution of these potential problems often lies in process characterisation to enhance process understanding. The work presented in this Chapter shows the effect that differing changes had on the process as a whole. In detail, the effect of differing physico-chemical parameters and environments had on the process, during intensification. The overall objective is process intensification, which is to increase process productivity without increasing overall process size, this was carried out by:

Initial process characterisation (Section 4.1)

Process translation from shake flask growth to bioreactor (Section 4.2)

Process intensification, batch to fed batch growth (Section 4.3)

4.1 Process characterisation

The initial process was carried out as in Section 3.4 using a 2 L shake flask for cell cultivation and the medium described in Table 3.1. A series of experiments (in duplicate) were carried out to both ensure that the process as described by Penfold et al (Penfold, 2004), had been successfully transferred to a different laboratory facility. During growth OD_{600}, pH and HPLC (off line) analysis were carried out and during hydrogen evolution, OD_{600}, pH and HPLC (off line) and volumetric hydrogen evolution was assessed.

Table 4.1 Process parameters for shake flask growth (experiment in Fig. 4.1)

Parameter	
Shake flask size	2 L
Working volume	1 L
Shaking speed	200 rpm
Inoculation size	0.1 mL
Medium	Table 3.1 - Nutrient Broth #2
Growth temperature	30°C

Table 4.2 Process parameters for hydrogen production (experiment in fig. 4.2)

Parameter	
Bioreactor size	5 L
Working volume	4 L
Impeller speed	300 rpm
Substrate volume	2 L
Substrate composition	Glucose (100 mM), NaCl (17 gL^{-1})
Culture size	2 L
Process temperature	30°C

Penfold *et al*., 2004 (Penfold, 2004), initially described the operating parameters for growth and hydrogen production in this study; the parameters differ from "normal" operating parameters for growth in a shake flask (lower filling [*ca.* 10%] volumes are commonly used to enable sufficient oxygen supply to the culture (Henzler and Schedel, 1991)).

The process (both the described in this study and described by Penfold, (2004)) employed a high working volume during the growth phase as well as a sub-optimal growth temperature. The high working volume of the flask had an effect on the mass transfer within the vessel as well as the volumetric power input to the vessel.

The sub-optimal growth temperature, may prove to be an energy saving device, but the decrease in temperature has an affect on the cell growth kinetics (Ratkowsky *et al.*, 1982; Ratkowsky *et al.*, 1983), microbial metabolism and

physiology (Ng, 1969), the necessity for the decrease in growth temperature has not been previously investigated and its effect will not be, but it can be said that all research cited uses a method for slowing growth (mostly through fermentative growth,(Penfold *et al.*, 2003; Yoshida *et al.*, 2005; Redwood and Macaskie, 2006; Hallenbeck, 2009)). In the hydrogen production phase of the process, Penfold, found that the optimal impeller speed for gas evolution was 300 rpm., this will not be assessed in this study, however as this impeller speed correlates to a Reynolds number (Re) at the lower end of the turbulent flow regime (when calculated assuming the medium is similar to water). It may be the case that in this specific study the medium components and the addition of the sugar solution may affect the medium viscosity enough to make the flow regime transition, but also, that transition phase flow (if it occurs) is sufficient to create a system that while it may not be fully homogenous, the degree of heterogeneity is low.

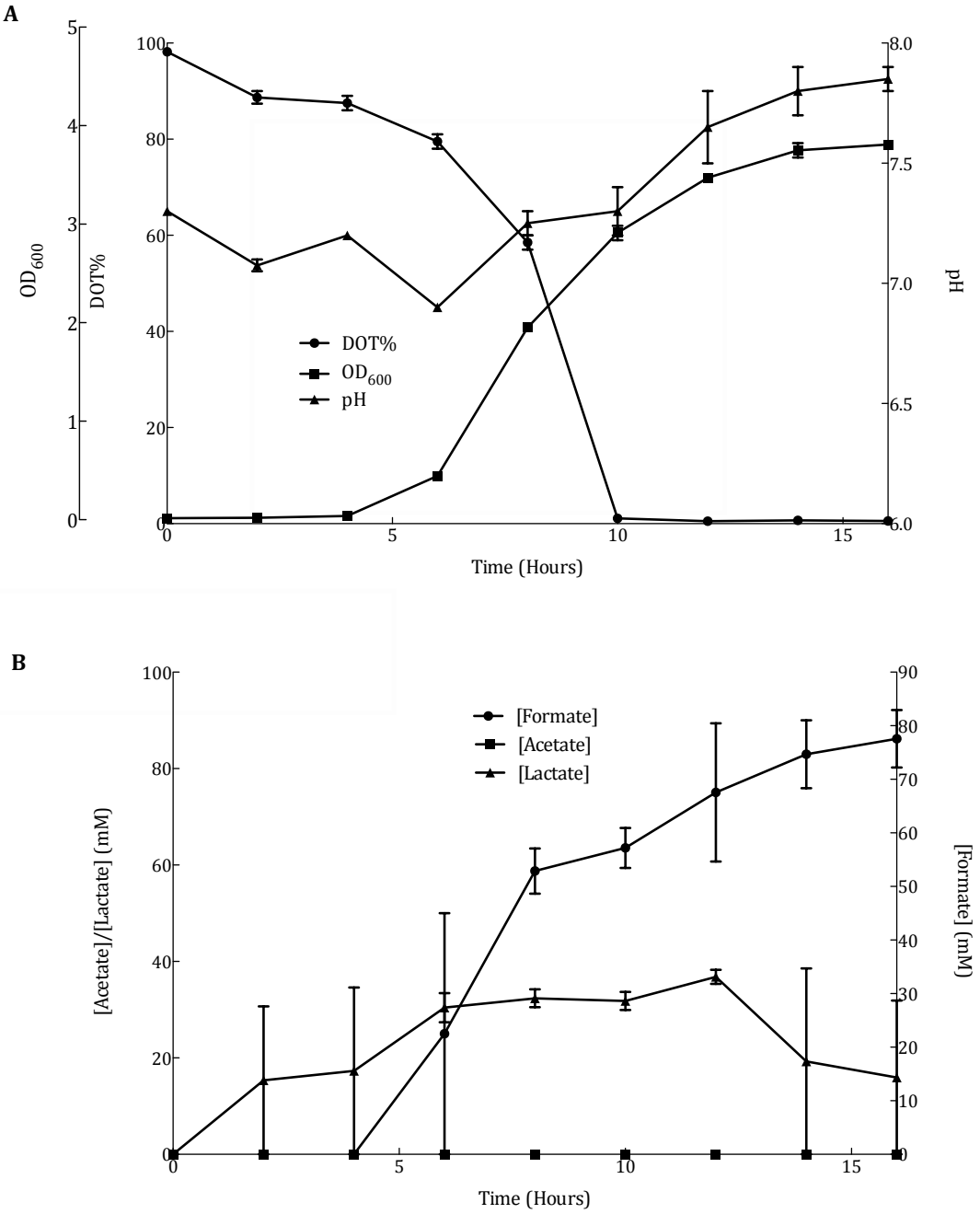


Figure 4.1 Graph showing the initial process growth of *E. coli* HD701 in a shake flask. Process parameters detailed in Table 4.1. Graph A shows the variation of OD_{600}, pH and DOT with time, μ_{max} = 0.7 h^{-1}. Graph B shows the fermentation organic acid profile, where acetate was not accumulated, but both formate and lactate were, increasing through the fermentation as oxygen limitation increased.

Figure 4.1 shows the variation of the fermentation parameters over time, the bacterial cell density (as characterised by the OD_{600} values) increased in a typical sigmoidal curve for batch growth, with stationary phase attained in approximately 16 hours. The maximum optical density achieved was 3.94. The culture temperate was below the optimal growth temperature (as in all experiments carried out). This will limit the overall bacterial number as lowering the culture temperature leads to a higher level of assimilation of the carbon source into each bacterial cell (Ng, 1969). The fermentation profile also showed the typical drop in DOT over the period of the fermentation, however in typical shake flask fermentations the working volume of the culture is lower, therefore the headspace is larger and the corresponding amount of oxygen within that space is greater, also the area available for mass transfer is affected (conical geometry implies an inverse proportionality between decreasing surface area for mass transfer and increasing liquid volume), so the DOT level dropping to 0% during the course of the fermentation was a function of the process parameters, with the factors that effect the oxygen transfer within the vessel being; agitation, liquid filling volume, shake flask closure and the use of baffles (McDaniel and Bailey, 1969; Van Suijdam *et al*., 1978; Nikakhtari and Hill, 2006; Micheletti *et al*., 2006).

The organic acid profile gives more information into the process. The production of formate and lactate during the early stages of the fermentation implies a lack of homogeneity within the reactor; this is due to mixed acid fermentation only being employed during anaerobiosis (Smith and Neidhardt, 1983; Riesenberg *et al*., 1991; Hewitt *et al*., 2000; Alexeeva *et al*., 2003). The level of formate produced through the fermentation rose slowly, which implies an increase in the proportion of anaerobiosis (due to culture heterogeneity), microaerobic conditions, or a simple increase in oxygen limitation as is confirmed by the DOT level in the last 7 hours of the fermentation, the extracellular level of formate is intrinsically linked to the intracellular level of formate through the FocA and FocB, transporter mechanisms (Maeda *et al.*, 2007a). While the production of formate and its concentration within the medium does not give an insight into the activity and or transcription of the FHL complex, it does show that in these conditions the PFL enzyme is functioning, as formate is being produced, and this

further shows that there were no inhibitory substances within the vessel (PFL is inhibited by oxygen and nitrate) and that the PFL system, while it is inhibited by oxygen and nitrate, was able to overcome that inhibition (Sauter and Sawers, 1990; Sawers, 2005).

During the hydrogen production phase of the process (Fig. 4.2) the DOT stays at a low enough level to assume anaerobiosis, the culture was sparged with pure nitrogen prior to the beginning of the phase, also acetate remains absent from the culture (or at a level below detection). At the beginning, prior to any hydrogen evolution lactate is re-assimilated into the cell, which is known to occur rapidly (Hewitt *et al*., 2000), while the formate level remains relatively constant at 60 mM. The glucose concentration profile varies in relation to the hydrogen production showing typical michaelis-menten enzyme kinetics (Madison, 1967; Fiechter *et al*., 1972; Berg *et al*., 2003). The maximum evolution rate of hydrogen was found to be approximately 200 mL/hour.

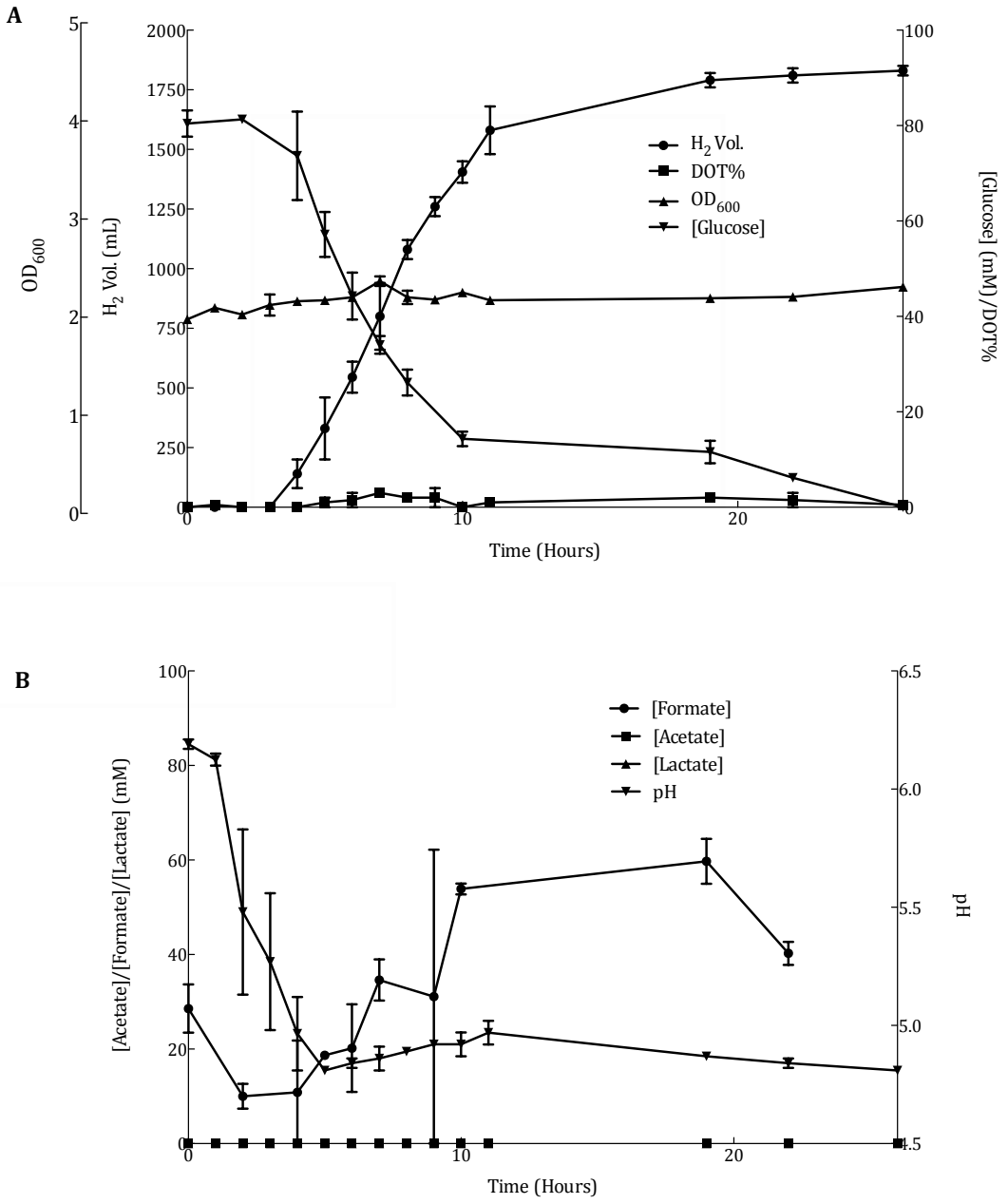


Figure 4.2 Graph showing the initial process hydrogen production *E. coli* HD701 in a bioreactor subsequent to shake flask growth. Process parameters detailed in Table 4.2. Graph A shows the variation of [glucose], hydrogen evolution, DOT and OD_{600} with time, where the utilisation of glucose mirrors the evolution rate of hydrogen, OD_{600} remains approximately static and the process is anoxic. Graph B shows the fermentation organic acid profile, where acetate and lactate were not present (both plots run along the base line), but formate was present, the pH dropped through the initial hydrogen evolution phase but levelled out at approximately 4.7

The process as a whole was transferred from and generally mirrors the work done in a previous study (Penfold, 2004). When the data is analysed specifically there are some surprising differences;

- Increase in cell density
- Higher hydrogen evolution rate
- Change in organic acid profile

While the slightly higher hydrogen evolution rate can be explained by the increase in cell density (this increase shown is possibly due to the use of baffled shake flasks for growth which improve mass transfer within the culture that in turn increases the growth rate) a further study (Redwood and Macaskie, 2006), which details the use of the end-products of this process, states that the end-products are as shown in Table 4.3.

Table 4.3 Properties of fermentation liquor post *E. coli* HD701 hydrogen production (diluted 1:1)(Redwood and Macaskie, 2006) [Bracketed results from this study].

Fermentation properties	General properties
Glucose 40mM [0mM]	pH 4.5 [4.8]
Ethanol 20mM	Turbidity Low
Total organic acids 38mM [Formate 40mM]	Ammonia 4.4mM
Acetate 20mM [0mM]	Protein 2.30g/L
Lactate 15mM [0mM]	Chloride 46mM
Succinate 3mM [0mM]	Phosphate 5.2mM

This fermentation liquor shows notable differences from the liquor that was left at the end of fermentation that was carried out in Figures 4.1 and 4.2. It is assumed that the low level of succinate could have been too low to detect utilising the HPLC method of organic acid quantification, but the main difference was the presence of acetate, and ethanol. When conducting the analysis of the fermentation liquor from the previous experiments, it was difficult to determine a method to quantify ethanol concentration within the medium; however, it is co-produced in equal amounts with acetate (following the metabolic pathway for

anaerobic glycolysis) (Gadd, 2007). However the combined effect of the ability of the cell to take up acetate but not ethanol and the shift of metabolism from acetate production to lactate production at a pH below 7 should mean there was a different organic acid profile at the end point of the fermentation (Kirkpatrick *et al.*, 2001). The other differing factor was the lack of formate in the spent liquor, the bioconversion had not gone to completion as can be demonstrated by the high level of glucose still present in the fermentation liquor, yet no formate the sole precursor of hydrogen production was present, these factors lead to the conclusion that the organic acid profile found at the end of the fermentations shown in Figure 4.1 and 4.2 was closer to reality than shown in the stated literature[2].

Throughout the entirety of this Section, the level of individual organic acids within the medium is assumed to be analogous to its level within the bacterial cell and while the values will not be equivalent, it is a reasonable assumption that an increase in the level of any organic acid produced through metabolic processes in the medium occurs concomitantly with the increase of the intracellular level of that acid.

4.2 Process translation from shake flask growth to bioreactor

The next step in the intensification process is to perform the whole process in a bioreactor. The transfer from growth in a shake flask to growth in a bioreactor is due to the later use of fed-batch culture to increase cell density (Section 4.3). The current method used to grow bacteria for hydrogen production involves the addition of formate to the medium (Nandi *et al.*, 2001; Yoshida *et al.*, 2005; Redwood *et al.*, 2008; Maeda and Wood, 2008) and in some cases the centrifugation, washing and re-suspension of the bacterial culture (Maeda *et al.*,

[2] Ideally all quantification methods would be cross verified with another method to further clarify the data

2007a). For this study that was deemed superfluous, as it was unnecessary to do so in the shake flask orientated process, it was postulated that there must be a process that could be defined within a bioreactor that would have the same physico-chemical characteristics as the process where growth occurred in the shake flask and therefore hydrogen production could occur without the necessity for a vessel change subsequent to growth.

Other reasons for forgoing the addition of formate and the cell treatment stem from the research area that the work falls in to. Life cycle analysis (LCA) is a green process metric used to assess products and processes throughout their entire lifetime and as such any unnecessary input is detrimental to the process from a LCA standpoint. Formate[3] addition at an industrial scale could prove costly, especially as it could be seen as unnecessary, and the use of centrifugation would create an unnecessarily high energy input to the process, that would need to be further offset by the hydrogen production.

Prior to transferring the process to a bioreactor, all the reasons for the switch must be considered. The main reason being the proposed hypothesis:

'An increase in cell density will increase the volumetric rate of hydrogen production'

Therefore process optimisation and intensification through an increase in cell density is the initial goal and as such the first step in this process was to first evaluate the effect of an increase in cell density on the production of hydrogen by increasing it within the shake flask. The initial cell density increase was achieved by simply doubling the concentration of the medium components (Table 3.1) in the shake flask. The other physical process parameters used in these experiments were unchanged from those stated in Table 4.1 and 4.2.

[3] Formate is technically a renewable resource as it can be produced from fermentation of other sustainable sources and is also found in plants.

As can be expected the increase in the concentration of the medium components increased the overall cell number, but not in a linear fashion. The concentration of the medium was doubled, and as such, if the bacterial cell yield with respect to the substrate were constant, the overall bacterial concentration would have doubled which would have correlated to a doubling of the optical density value in stationary phase. Another notable change was the length of the fermentation, part of the increase in fermentation time was due to the increase of the lag phase (shown in Figure 4.3), with the culture inoculum being grown in the original strength medium the change in the bacterial environment upon addition to the increased concentration medium would have affected the lag phase of bacterial growth. Substrate inhibition could lead to the increased lag time, or be a component effect in this change in the fermentation profile. These conclusions are drawn from the suggested reason for the appearance of lag in bacterial cultures suggested by Monod (1949), which is it being the time taken for the bacterial inoculum to adapt to its new environment. Also the effect of microaerobiosis would be different at high cell density, this assumption occurs due to the effect of microaerobiosis occurring in cultures that are supplied with oxygen at a rate at which it is immediately used and not accumulated, at higher cell densities, utilising the same physical system it is increasingly less likely that microaerobiosis will occur and anaerobiosis will take place within the vessel.

Figure 4.3 also shows that the fermentation stage of the process has many similarities to the process shown in Figures 4.1 and 4.2, showing that microaerobiosis (or anaerobiosis) is seen in the final 6 hours of the fermentation allowing mixed acid fermentation to occur. It draws parallels with the same fermentation profile as it has a similar organic acid profile, but the increase in cell density has caused an increase in the formate and lactate concentrations within the fermentation broth, again in contrast to that shown by (Redwood and Macaskie, 2006), no acetate is produced (or not at a detectable level) and the differences in the fermentation and hydrogen production profile can be attributed to the same reasons given in Section 4.1.

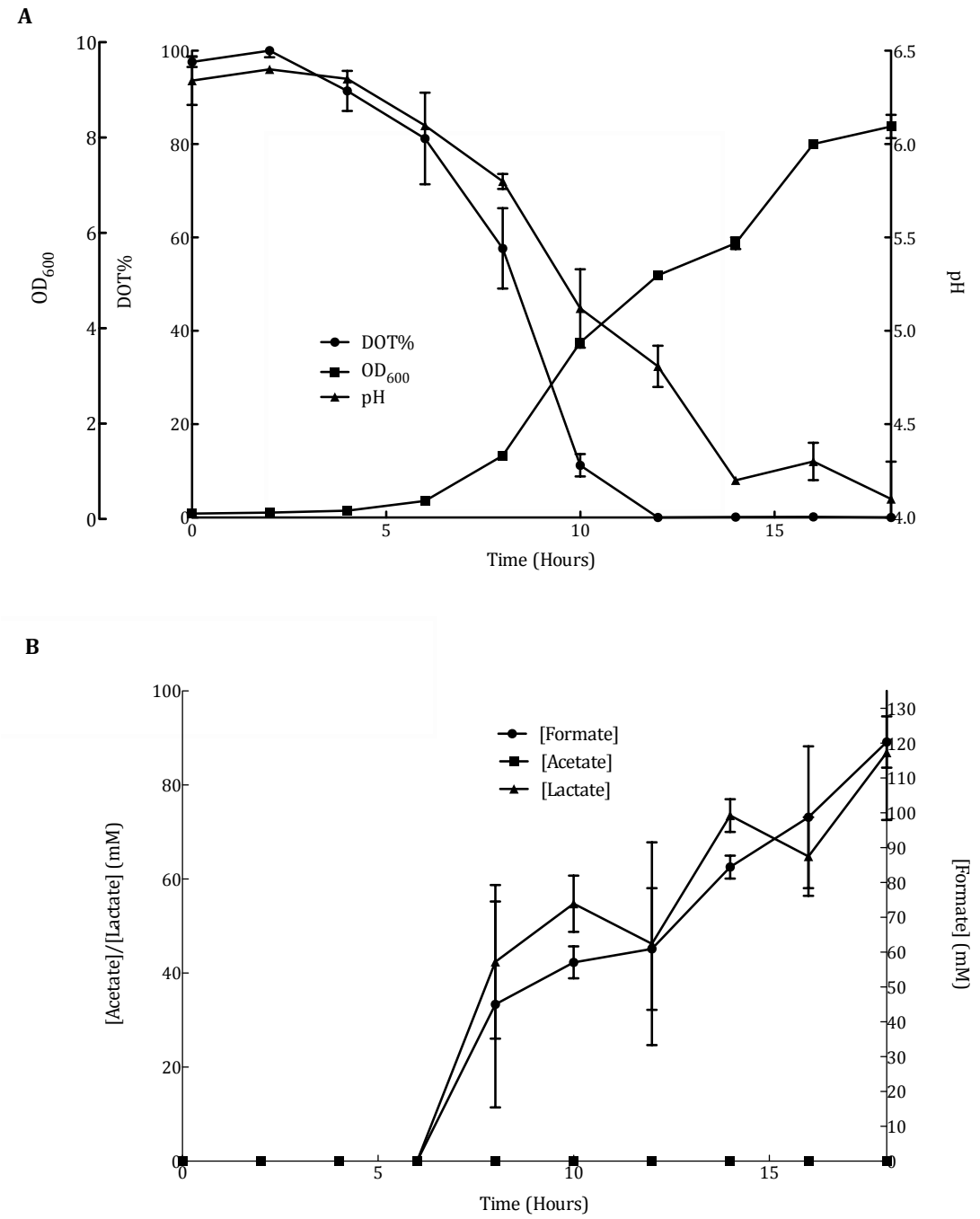


Figure 4.3 Graph showing the growth of *E. coli* HD701 in double strength NB #2medium in a shake flask. Process parameters detailed in Table 4.1. Graph A shows the variation of OD_{600}, pH and DOT with time, μ_{max} = 0.52 h^{-1}. Graph B shows the fermentation organic acid profile, where acetate was not accumulated, but both formate and lactate were, increasing through the fermentation as oxygen limitation increased.

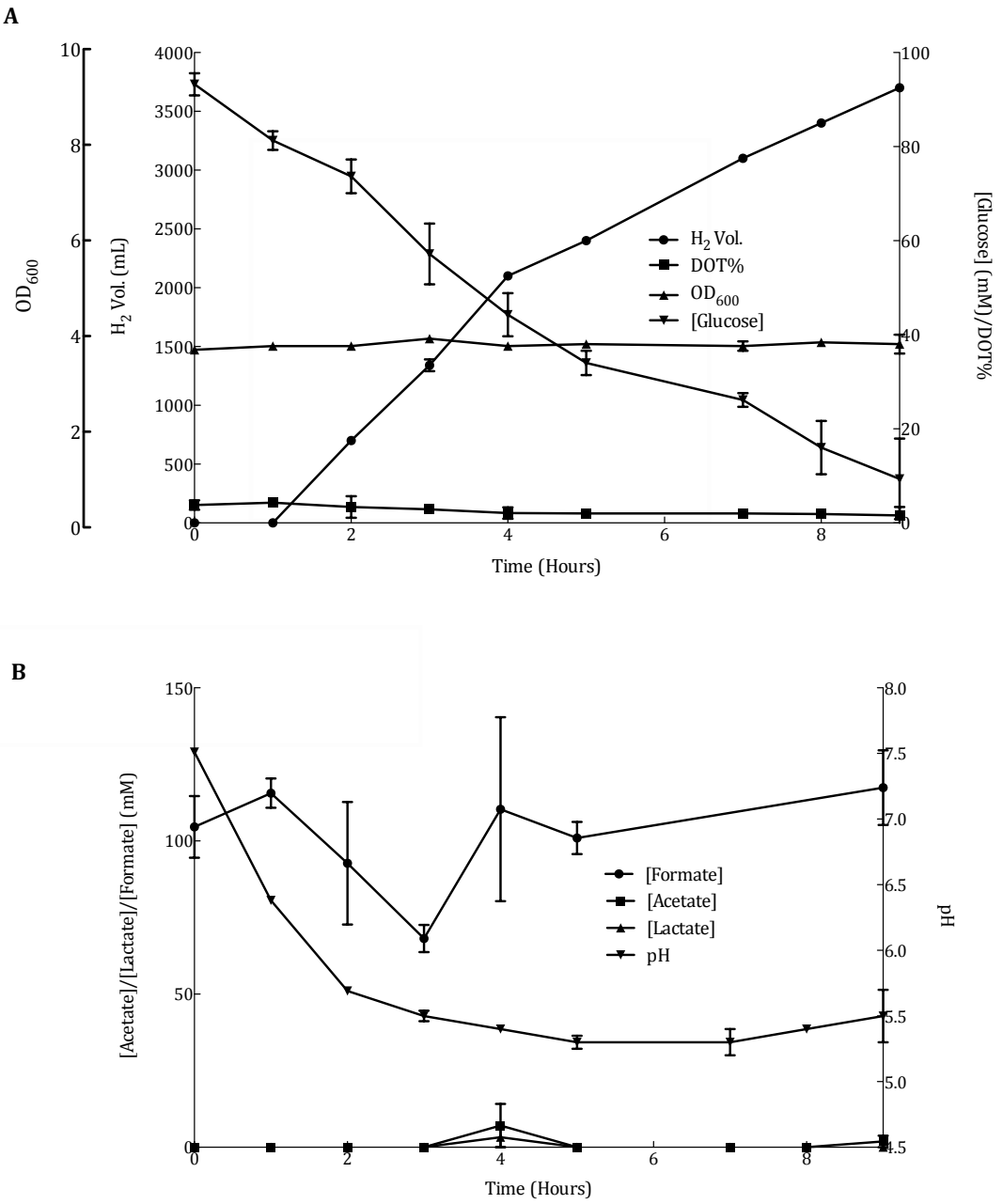


Figure 4.4 Graph showing the hydrogen production in a bioreactor subsequent to shake flask growth by *E. coli* HD701 in double strength NB[2] medium. Process parameters detailed in Table 4.2. Graph A shows the variation of [glucose], hydrogen evolution, DOT and OD_{600} with time, where the utilisation of glucose mirrors the evolution rate of hydrogen, OD_{600} remains approximately static and the process is anoxic. Graph B shows the fermentation organic acid profile, where acetate and lactate were not present (both plots run along the base line), but formate was present, the pH dropped through the initial hydrogen evolution phase but levelled out at approximately 4.9

The increase in cell density had the desired effect of increasing hydrogen production rate, further increase in cell density may require a change in the method for bacterial enumeration, but before this change can take place, as the final process will require the use of a bioreactor for growth, both growth and hydrogen production must be carried out in one vessel. Consequently the next step in process translation was to simply see the effect on growth in the bioreactor (Figure 4.5)

The growth parameters shown in Table 4.3 describe those that were used for the preliminary growth in a bioreactor. The culture volume was below the maximum working volume due to the necessity of the addition of hydrogen production substrate (glucose), post growth. The impeller speed was chosen for ease of use (no change of impeller speed through the differing phases of operation), the previously mentioned, potential lack of turbulence may have led to increased heterogeneity within the culture. Aeration rate is chosen as a standard rate to ensure the culture does not become oxygen limited. The other parameters chosen are the same as used in the shake flask, with the same percentage volume used for the inoculation culture and the same growth temperature employed.

Table 4.4 Process parameters for bioreactor growth (experiment in Figure 4.5)

Parameter	
Bioreactor size	5L
Culture volume	2 L
Impeller speed	300 rpm
Aeration rate	0.75 vvm
Inoculation size	0.2 mL
Medium	Nutrient Broth #2
Growth temperature	30°C

Table 4.5 Process parameters for hydrogen production (experiment in fig. 4.6)

Parameter	
Bioreactor size	5 L
Working volume	4 L
Impeller speed	300 rpm
Substrate volume	2 L
Substrate composition	Glucose (100 mM), NaCl (17gL^{-1})
Culture size	2 L
Process temperature	30°C

The bioreactor growth experiments, data shown in Figure 4.5 and 4.6, did not yield any hydrogen. Analysis of the growth phase and hydrogen production phase gives an insight to the reasons why this occurred; only low levels (< 15mM) of the organic acids produced during anaerobic metabolism (formate, acetate) were produced, this leads to the assumption that there is only a low level of intracellular formate, which in turn leads to the possibility that as intracellular formate concentration is an inducer for the transcription of FHL, this may not have occurred. There was no glucose in the medium so this implies a reduced possibility of the acetate being produced by overflow metabolism (Han *et al*., 1992; Kleman and Strohl, 1994), meaning that the glycolytic route goes through pyruvate to acetyl co-a and straight in to the TCA. Another potential source of acetate is the de-coupling of the metabolic needs of the cell and the energy production by oxidative metabolism however at slow growth rates this is unlikely (Han *et al*., 1992), in this study the growth rate is sub-optimal due to the process temperature. Consideration of both the organic acid profile and the DOT profile throughout the fermentation shows that, unlike the shake flask experiment, the bioreactor stayed well aerated throughout the entirety of the process. It is also concluded that the low level of formate and acetate shown at the end of the fermentation was due to the heterogeneous nature of fermentations. Mixed acid fermentation only occurs at low (or anoxic) oxygen levels (Gadd, 2007) and it is these compounds that lead to the production of hydrogen (Ataai and Shuler, 1985). Furthermore it was shown that simply by

removing oxygen during the stationary phase of growth and feeding the hydrogen production substrate, did not promote the production of hydrogen. This fact implies that either the glucose was not used or PFL was not formed, so the formate endpoint of anaerobic glycolysis could not be reached. There was however a decrease in glucose concentration, that could be attributed to microbial maintenance if it were not for the corresponding decrease in pH. The decrease in pH implies the production of protons most likely in the form of an acid that has not been quantified. A lack of the PFL enzyme complex means that the acid which decreases the pH could be pyruvate, but it equally could be succinate from phosphoenolpyruvate (Gadd, 2007). The overall result could have been predicted through the lack of use of this method for growth in literature.

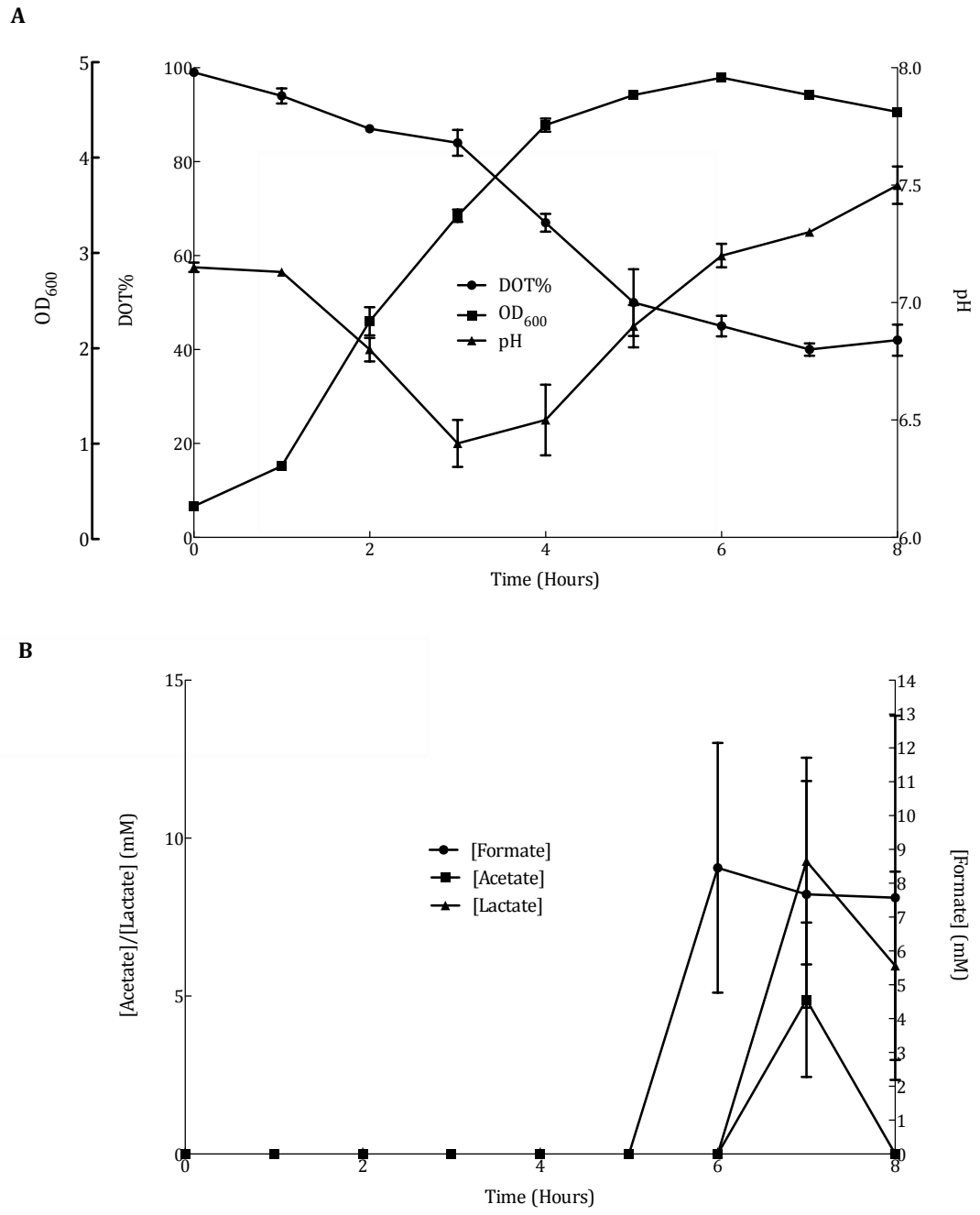


Figure 4.5 Graph showing the growth of *E. coli* HD701 in a bioreactor. Process parameters detailed in Table 4.4. Graph A shows the variation of OD_{600}, pH and DOT with time, where the DOT never falls below 40%, μ_{max} = 0.64 h^{-1}. Graph B shows the fermentation organic acid profile, where acetate, formate and lactate were accumulated in comparatively low concentrations (to initial shake flask process) at the very end of the fermentation.

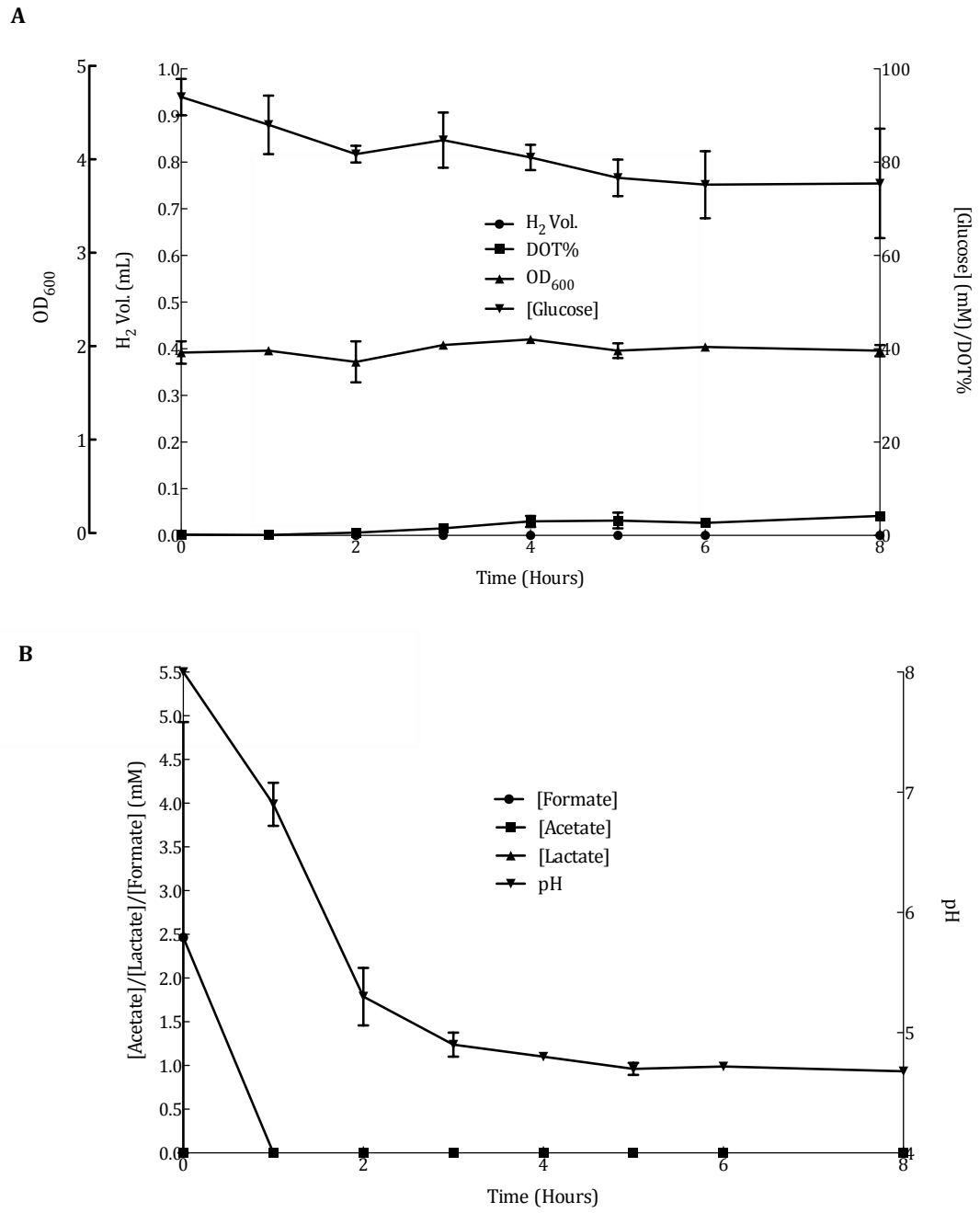


Figure 4.6 Graph showing the hydrogen production in a bioreactor subsequent to bioreactor growth by *E. coli* HD701. Process parameters detailed in Table 4.5. Graph A shows the variation of [glucose], hydrogen evolution, DOT and OD_{600} with time, where no hydrogen is evolved, the [glucose] decreases, OD_{600} remains approximately static and the process is anoxic. Graph B shows the fermentation organic acid profile, where acetate formate and lactate were not present for the majority of the phase (all plots run along the base line), the pH dropped through the initial hydrogen evolution phase but levelled out at approximately 4.7

As it has been shown that with this strain, aerobic growth, cannot be the precursor to hydrogen production (under the conditions in this study), the next step was to check if hydrogen could be produced during the differing phases of growth in a bioreactor, to elucidate if anaerobiosis had to occur at a specific part of the growth curve (Figure 4.5 and 4.7) and that the reason for the lack of hydrogen production was not the necessity of PFL transcription (and/or FHL) through a certain phase of growth, to assess this growth was stopped during each phase of growth (after 2 hours, early exponential; after 4 hours, late exponential; after 6 hours, stationary) and anaerobiosis was induced by sparging with nitrogen, and subsequent hydrogen production was attempted by the addition of the glucose substrate.

While it was apparent that there was no hydrogen production, it was not clear why. The oxygen limited growth conditions in the shake flask however, do lead to hydrogen production, but performing the growth aerobically then removing the oxygen does not. This implies that there was a necessity for oxygen limitation during growth.

Figure 4.7 show the points along the growth curve at which growth was stopped and hydrogen production was attempted.

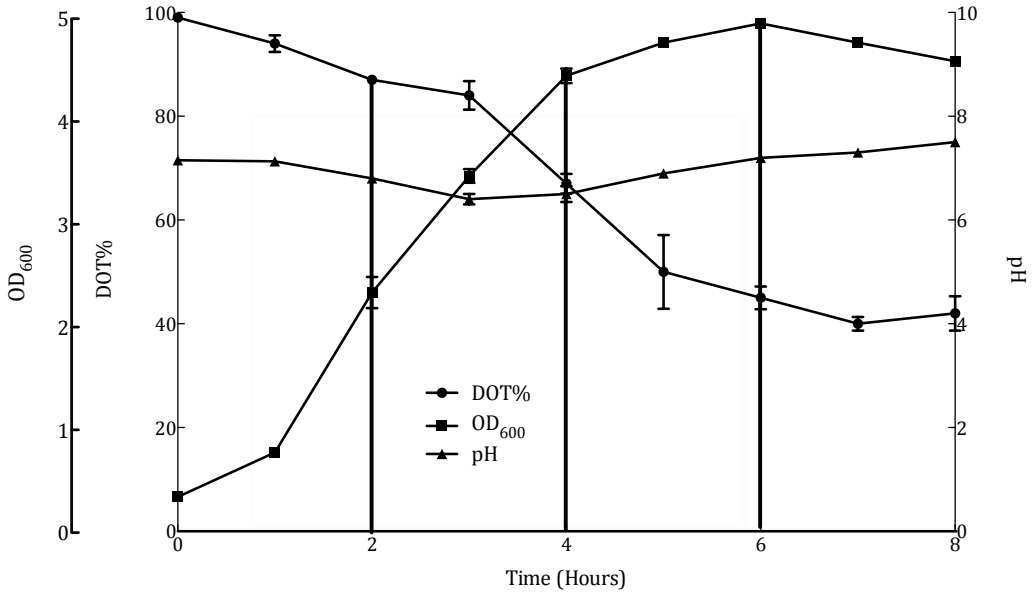


Figure 4.7 Growth of *E. coli* HD701 showing the points at which growth was stopped and hydrogen production was attempted

Figures 4.8-4.10 show combined with Figures 4.3 and 4.4 that growth in a normally aerated bioreactor will not yield a culture capable of producing hydrogen. There are many possible reasons for the lack of hydrogen production and the precise reason for the lack of hydrogen production cannot be deduced. If the process for producing hydrogen is considered metabolically, the route of anaerobic glycolysis must be considered. Anaerobic glycolysis branches at pyruvate, where PFL is used to produce formate (Gadd, 2007), which is then in turn converted into hydrogen and carbon dioxide by FHL. PFL is an enzyme complex that is inhibited by both nitrate and oxygen (Sawers and Bock, 1988), so it can be assumed that for the early exponential and late exponential phase growth, the reason that hydrogen cannot be produced is the inability to produce a precursor (formate) from pyruvate in the metabolic pathway and when oxygen is removed PFL is not synthesised due to the presence of nitrate in the fermentation medium.

The lack of hydrogen production in the two later stages of fermentation leads to the conclusion that the process is sensitive, probably to multiple parameters, and the bacterium is fastidious with respect to hydrogen production. As with the earlier stages the lack of hydrogen production must be explained metabolically, the relatively small amount of formate and acetate (compared to the shake flask process in Figure 4.2) present at the end of the fermentation indicates that again PFL was not present, as the fermentation was nearing its end, there is a lower probability that this is due to nitrate repression and as such the repression in the later stages of the fermentation may stem from the transcriptional regulators FNR, ArcA and ArcB. These regulators act to detect the change in redox potential as a culture moves from aerobic growth to anaerobiosis (Sawers and Suppmann, 1992) and as such the lack of hydrogen production from the latter stages of fermentation can be attributed to the level of oxygen within the culture and the action of these transcriptional regulators when growth is limited.

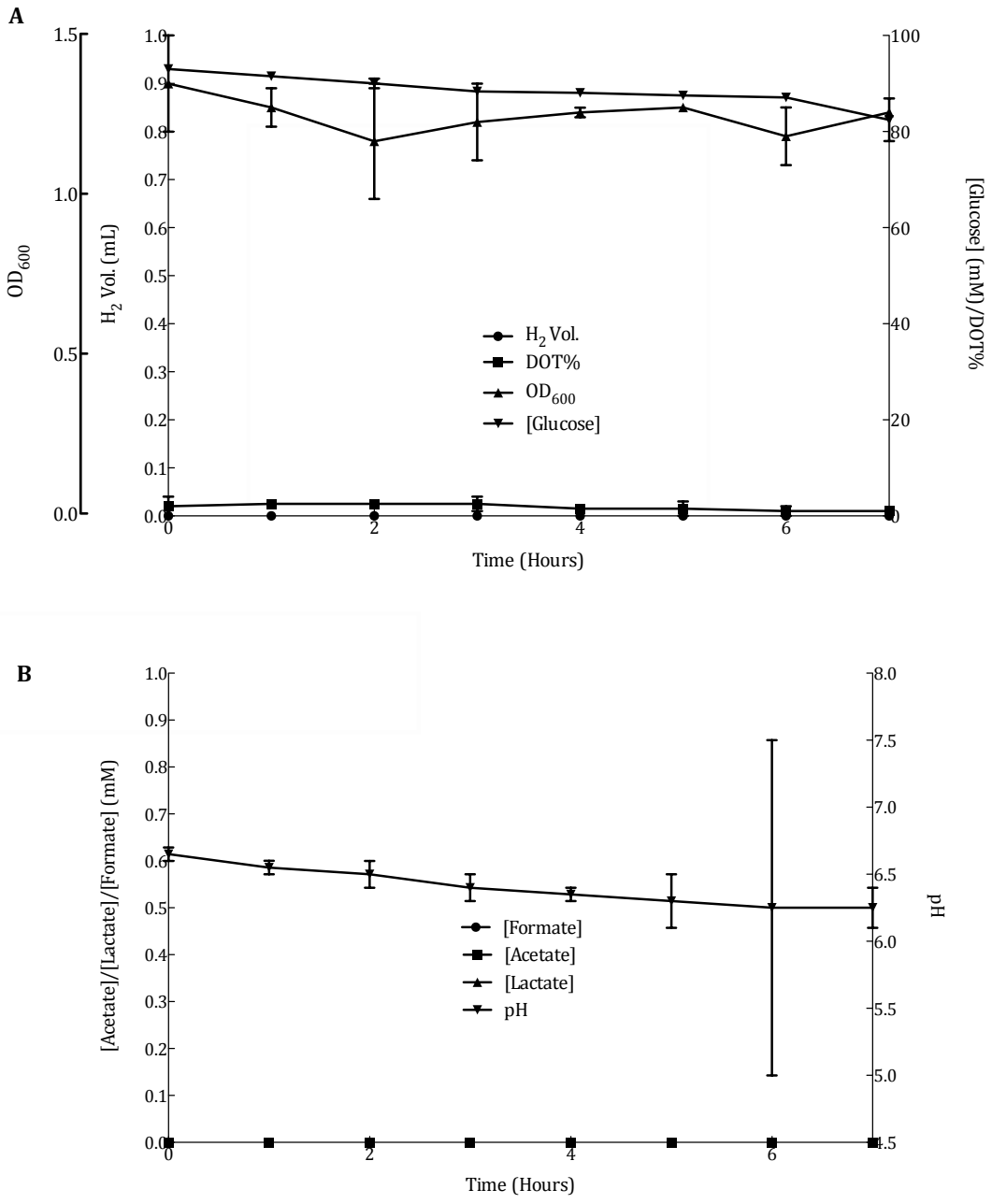


Figure 4.8 Graph showing the hydrogen production by *E. coli* HD701 production in a bioreactor subsequent to 2 hours of bioreactor growth by *E. coli* HD701. Process parameters detailed in Table 4.5. Graph A shows the variation of [glucose], hydrogen evolution, DOT and OD_{600} with time, where no hydrogen is evolved, OD_{600} remains approximately static and the process is anoxic. Graph B shows the fermentation organic acid profile, where acetate formate and lactate were not present (all plots run along the base line), the pH remained at approximately 6.3

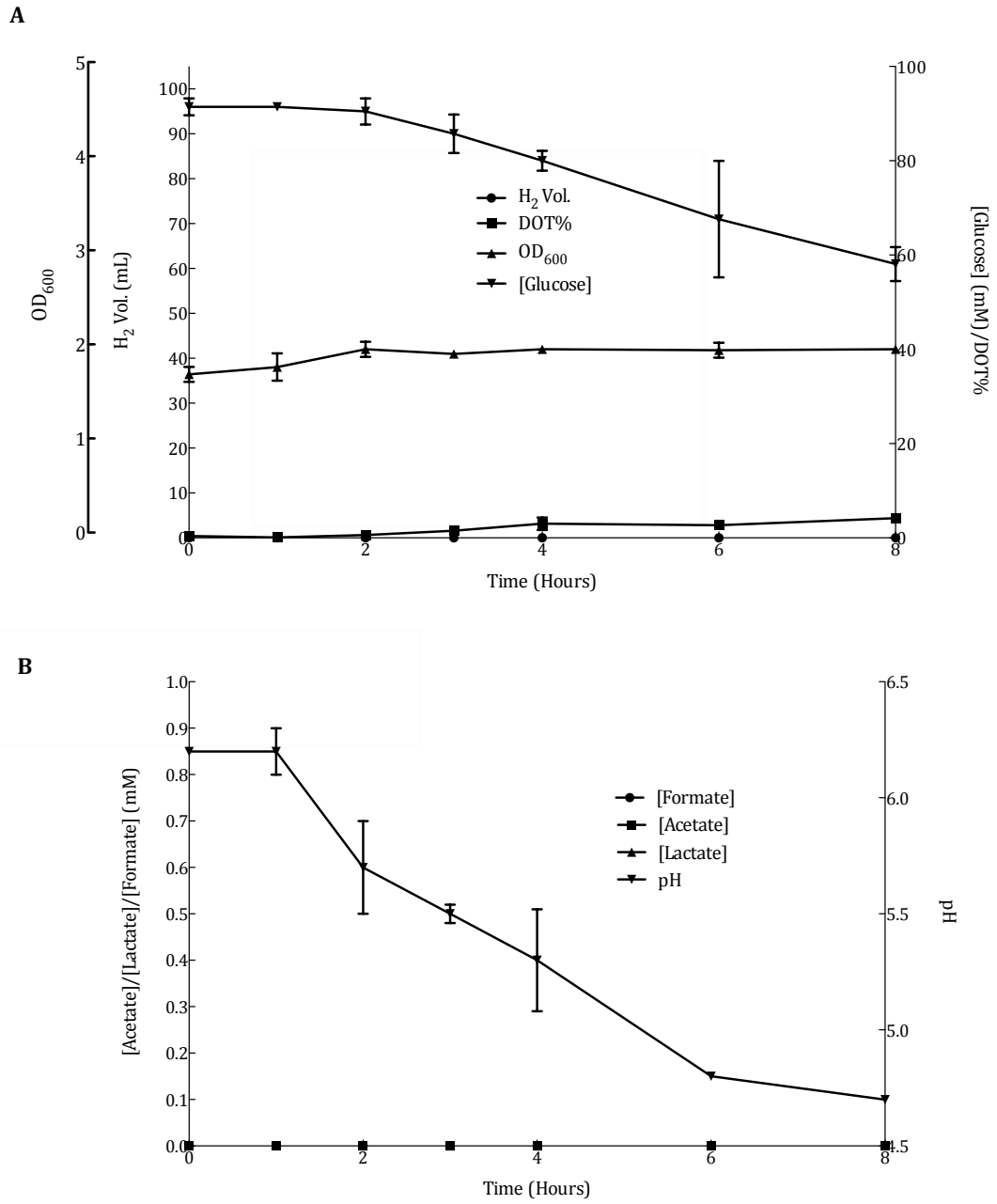


Figure 4.9 Graph showing the hydrogen production by *E. coli* HD701 production in a bioreactor subsequent to 4 hours of bioreactor growth by *E. coli* HD701. Process parameters detailed in Table 4.5. Graph A shows the variation of [glucose], hydrogen evolution, DOT and OD_{600} with time, where no hydrogen is evolved, OD_{600} remains approximately static, [glucose] decreased and the process is anoxic. Graph B shows the fermentation organic acid profile, where acetate formate and lactate were not present (all plots run along the base line), the pH decreased through the phase.

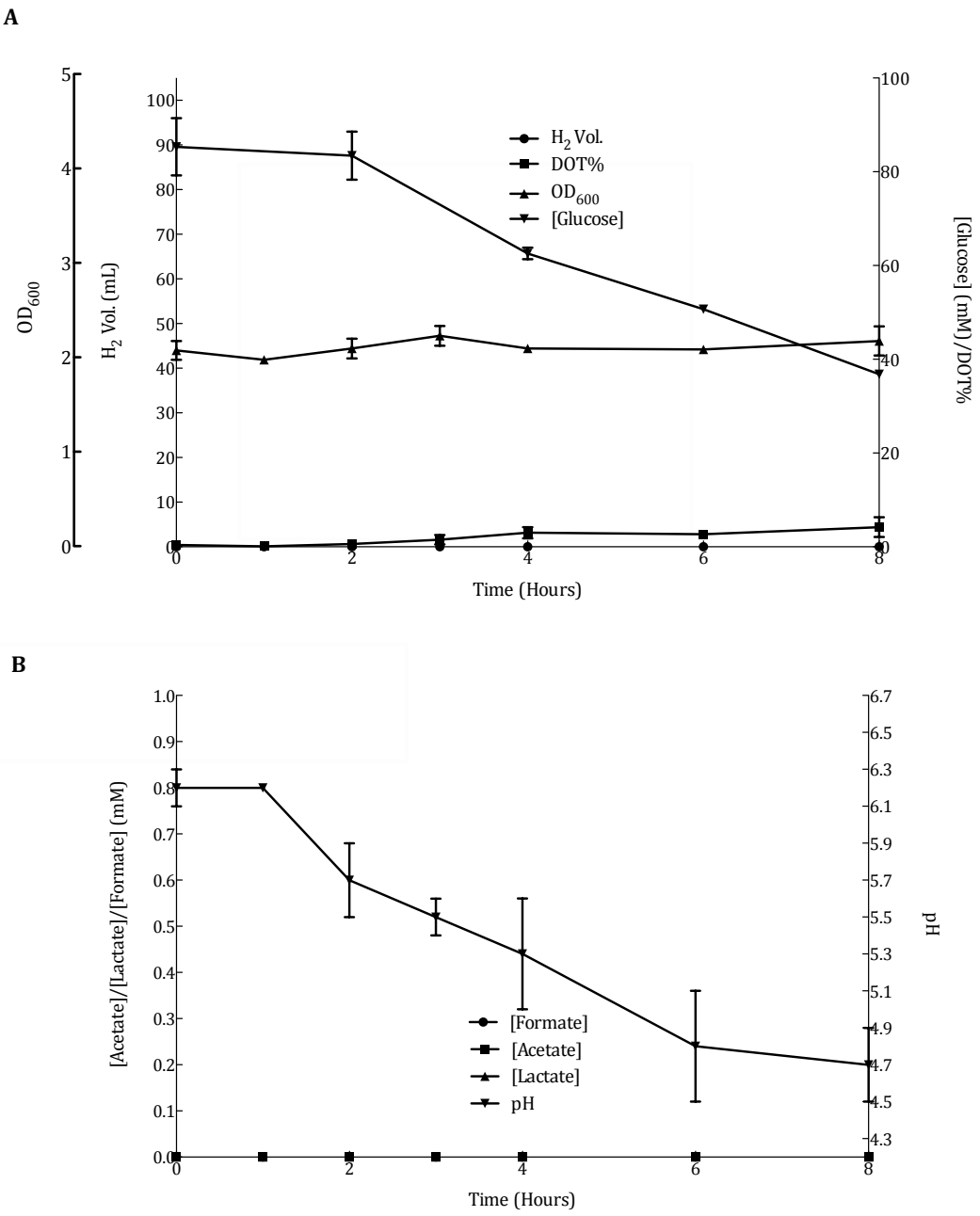


Figure 4.10 Graph showing the hydrogen production by *E. coli* HD701 production in a bioreactor subsequent to 6 hours of bioreactor growth by *E. coli* HD701. Process parameters detailed in Table 4.5. Graph A shows the variation of [glucose], hydrogen evolution, DOT and OD_{600} with time, where no hydrogen is evolved, OD_{600} remains approximately static, [glucose] decreased and the process is anoxic. Graph B shows the fermentation organic acid profile, where acetate formate and lactate were not present (all plots run along the base line), the pH decreased through the phase.

This set of results and the conclusions drawn from them further highlight the need for microaerobic conditions within the culture, during the later stages of the fermentation to allow cells to grow to a relatively high cell density and utilise nitrates and also provide oxygen limitation during growth to allow the transcription level regulation of the metabolic pathways involved in mixed acid fermentation and hydrogen production. A similar effect was found in a study of the succinic acid production of *E. coli* (Vemuri *et al.*, 2002). This study is of importance as succinic acid is another potential endpoint of anaerobic glycolysis. It details the use of dual phases for growth and succinate production, which could be considered analogous to the system employed in this study, the study concluded that there was an optimum time for transition to anaerobiosis. The transition point in this case is dependant on a set of genes, some of which are not applicable to this study, but the overall development method is of use, especially as it utilises fed-batch growth, which is an eventual aim.

It is clear that the physical and chemical environment is very different within the shake flask, when compared to the bioreactor. As such they should be assessed separately. The physical parameters that were assessed within the shake flask include:

K_La (volumetric oxygen transfer coefficient)

DOT

Volumetric power input

Shown in Table 4.8.

There are many possible parameters that could have been assessed (as detailed in Section 2.2.1) but when transferring a process from shake flask to bioreactor there is a natural limitation due to the comparative physical simplicity of a shake flask, also there was consideration of the previous study that used K_La as a parameter for assessment (Vemuri *et al*., 2002).

Figures 4.11 and 4.12 show that pseudo-fermentative[4] growth of *Escherichia coli* HD701 was possible and led to subsequent hydrogen production, pseudo-fermentative growth of the bacterium did however lead to lower cell densities. This result was typical of fermentative/anaerobic growth due to the increasing microbial maintenance and energy requirements of bacterial cultures (Pirt, 1965; Ataai and Shuler, 1985). Along with these lower cell densities the organic acid profile of the fermentation was similar to that shown in Figures 4.1 and 4.2, but only formate is being produced and at a lower level. It is not known if there is no acetate and lactate produced as these acids could be being produced at an undetectably low level and continuously re-assimilated into the carbon metabolism, the rate of acetate and lactate re-assimilation is relatively high (in relation to formate) (Hewitt *et al.*, 2000). The lower level of organic acids produced is in agreement with the higher level produced at higher cell density in Section 4.1. In reality the no aeration experiments in Figures 4.11 and 4.12 do not depict a process that was true fermentative growth, instead to allow the biomass to accumulate rapidly in the early stages of the fermentation (and potentially decrease lag time) air was sparged into the bioreactor until the DOT level within the vessel was 100% and then the vessel was sealed. The bioreactor was agitated and the process parameters for the experiments are shown in Table 4.6 and 4.7. This mode of operation was essentially the first step to mimicking the shake flask and it served to show the effect of the shake flask closure on oxygen transfer as if there was no mass transfer across that boundary the shake flask would also be a sealed vessel (other factors are involved such as surface aeration)(McDaniel and Bailey, 1969; Nikakhtari and Hill, 2006).

[4] Fermentative growth implies the absence of any exogenous electron acceptors, in this study oxygen is present at the beginning but not supplied.

Table 4.6 Process parameters for bioreactor growth (No aeration experiment in Fig. 4.11)

Parameter	
Bioreactor size	5L
Culture volume	2 L
Impeller speed	300 rpm
Aeration rate	0 vvm
Inoculation size	0.2 mL
Medium	Nutrient Broth #2
Growth temperature	30°C

Table 4.7 Process parameters for hydrogen production (No aeration experiment in fig. 4.12)

Parameter	
Bioreactor size	5 L
Working volume	4 L
Impeller speed	300 rpm
Substrate volume	2 L
Substrate composition	Glucose (100 mM), NaCl ($17gL^{-1}$)
Culture size	2 L

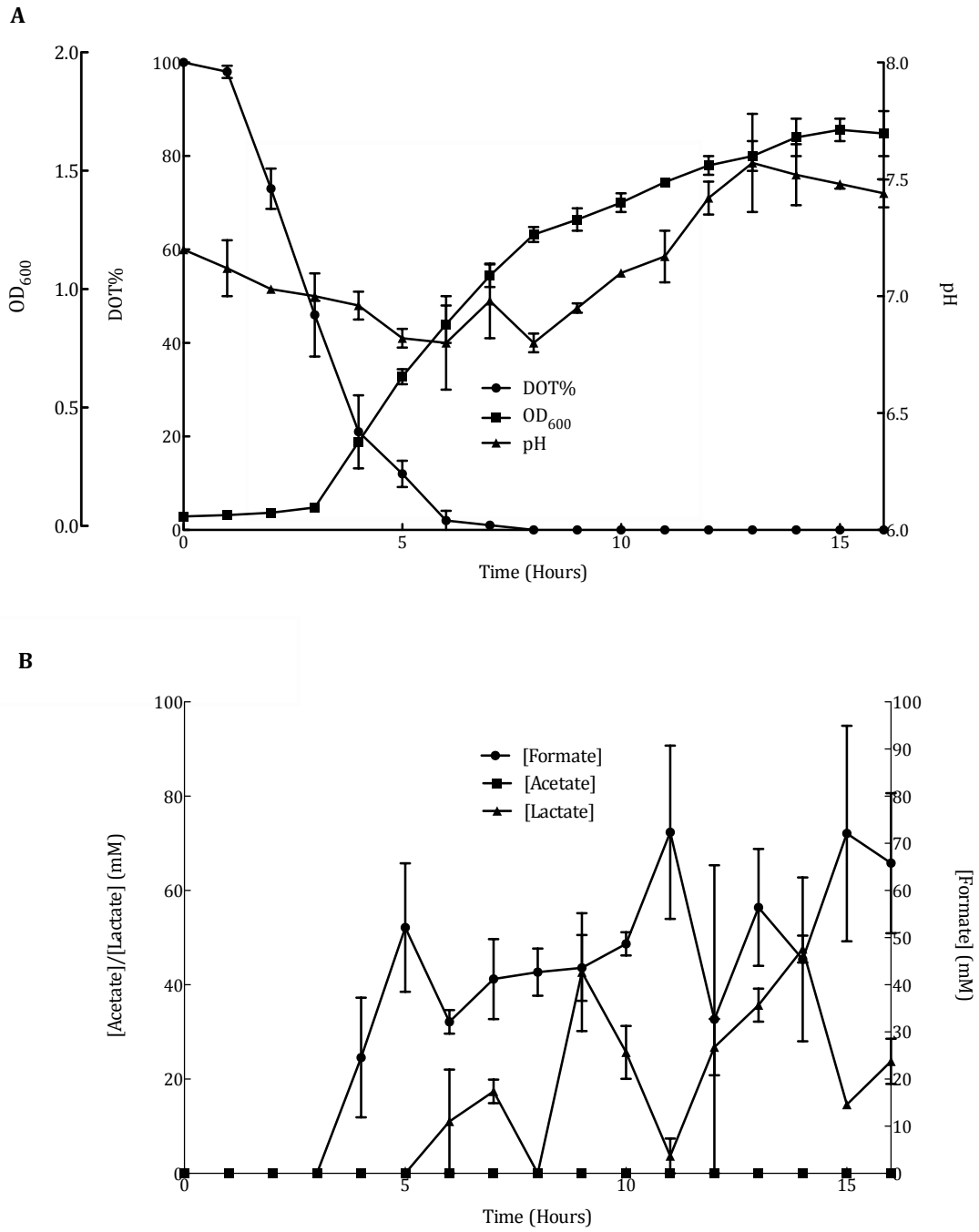


Figure 4.11 Graph showing the growth of *E. coli* HD701 in a bioreactor without aeration. Process parameters detailed in Table 4.6. Graph A shows the variation of OD_{600}, pH and DOT with time, where the DOT has an extended period of 0% DOT when compared to the original shake flask process, μ_{max} = 0.43 h^{-1}. Graph B shows the fermentation organic acid profile, where acetate was not accumulated, but both formate and lactate were, increasing through the fermentation as oxygen limitation increased.

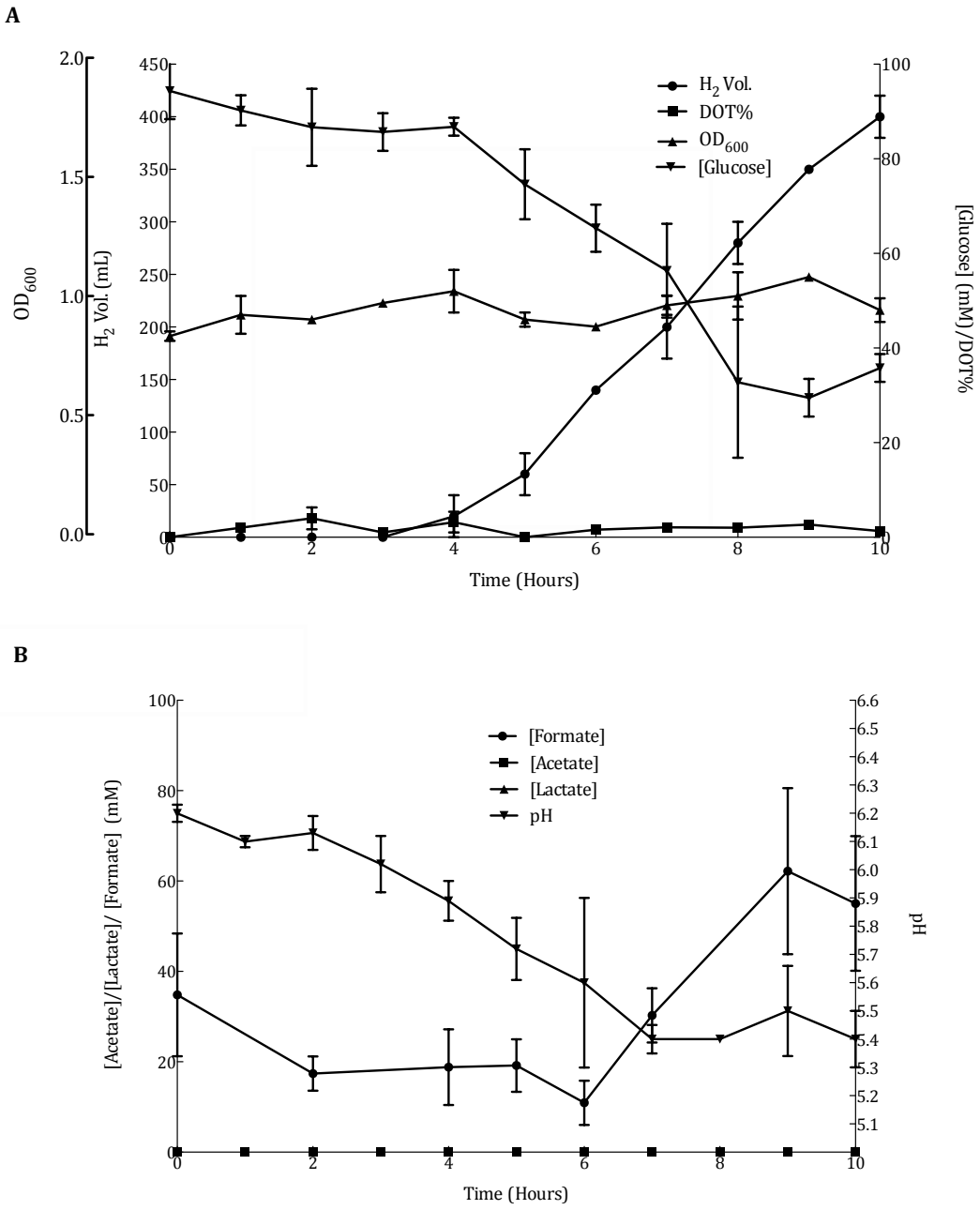


Figure 4.12 Graph showing the hydrogen production in a bioreactor subsequent to growth by *E. coli* HD701 in a bioreactor without aeration. Process parameters detailed in Table 4.7. Graph A shows the variation of [glucose], hydrogen evolution, DOT and OD_{600} with time, where the utilisation of almost glucose mirrors the evolution rate of hydrogen, OD_{600} remains approximately static and the process is anoxic. Graph B shows the fermentation organic acid profile, where acetate and lactate were not present (both plots run along the base line), but formate was present, the pH dropped through the initial hydrogen evolution phase but levelled out at approximately 5.4

A comparative hydrogen production study has been performed using a sealed cylindrical flask that was mechanically agitated (Maeda *et al*., 2007b). The mode of cell growth employed is similar to this study as there is partial (pseudo-) fermentative growth, allowing the biomass to initially increase using the oxygen available in the closed system. The strain employed in the study was similar to the one employed in this study and it also produced hydrogen under these conditions.

Table 4.8 Process parameters at different operating conditions

Parameter	Low agitation	Low aeration	Shake flask
K_La (s^{-1})	0.0027	0.0027	0.003 s^{-1} (adapted from (Henzler and Schedel, 1991; F. Veglio, 1998; Veglio, 1998; Maier and Büchs, 2001,)
N (rpm)	200 rpm	300 rpm	200 rpm
P/V (KWm^{-3})	0.465	1.565	≈0.4 (adapted from (Kato *et al*., 1995; Büchs *et al*., 2000a,))
Aeration rate (vvm)	1.25	0.48	N/A

Table 4.8 shows that there were two operating conditions (low aeration and low agitation) that were initially used to effect the process change from shake flask to bioreactor, conserving K_La in both cases but alternating the conservation of impeller speed and volumetric power input. It has led to overall process parameters shown in Tables 4.9-4.12.

Table 4.9 Process parameters for bioreactor growth (Low aeration experiment in Fig. 4.13)

Parameter	
Bioreactor size	5L
Culture volume	2 L
Impeller speed	300 rpm
Aeration rate	0.48 vvm
Inoculation size	0.2 mL
Medium	Nutrient Broth #2
Growth temperature	30°C

Table 4.10 Process parameters for hydrogen production (Low aeration experiment in fig. 4.14)

Parameter	
Bioreactor size	5 L
Working volume	4 L
Impeller speed	300 rpm
Substrate volume	2 L
Substrate composition	Glucose (100 mM), NaCl ($17gL^{-1}$)
Culture size	2 L

Table 4.11 Process parameters for bioreactor growth (Low agitation experiment in Fig. 4.15)

Parameter	
Bioreactor size	5L
Culture volume	2 L
Impeller speed	200 rpm
Aeration rate	1.25 vvm
Inoculation size	0.2 mL
Medium	Nutrient Broth #2
Growth temperature	30°C

Table 4.12 Process parameters for hydrogen production (Low agitation experiment in fig. 4.16)

Parameter	
Bioreactor size	5 L
Working volume	4 L
Impeller speed	300 rpm
Substrate volume	2 L
Substrate composition	Glucose (100 mM), NaCl (17 gL^{-1})
Culture size	2 L

While the pseudo-fermentative growth in Figures 4.11 and 4.12 proved positive for hydrogen production, it was effectively a step back due to the lower cell density and lower hydrogen production rates, as such further process scale up techniques were applied. The parameters shown in Table 4.8 were used to try and create a process that would allow a higher cell density to be achieved along with a complimentary higher rate of hydrogen evolution. Figures 4.13 - 4.16 show the effect of using K_La as the overall parameter for scale up and subsequent hydrogen production.

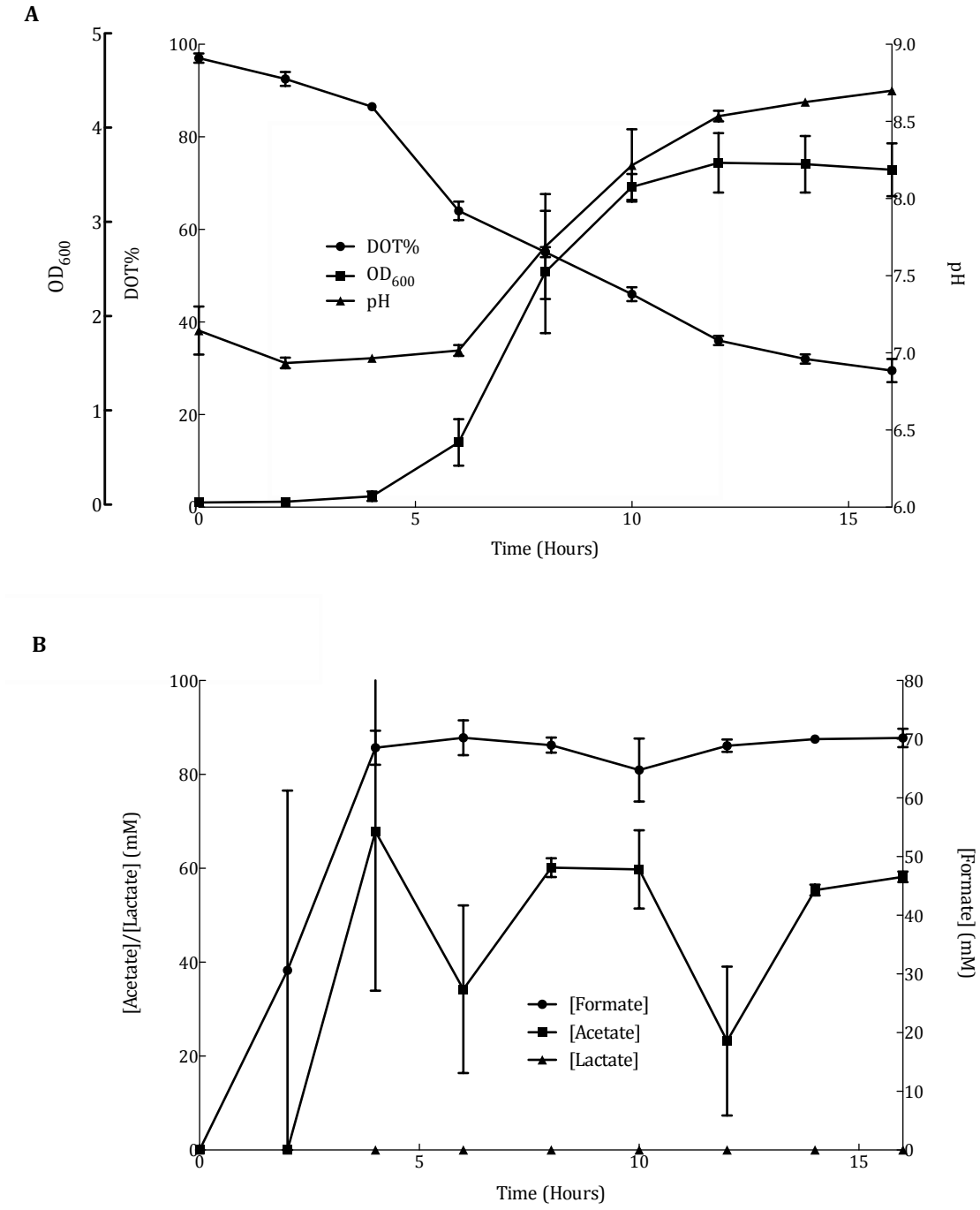


Figure 4.13 Graph showing the growth of *E. coli* HD701 in a bioreactor with low aeration. Process parameters detailed in Table 4.9. Graph A shows the variation of OD_{600}, pH and DOT with time, where the DOT never reaches 0%, μ_{max} = 0.71 h^{-1}. Graph B shows the fermentation organic acid profile, where lactate was not accumulated, but both formate and acetate were.

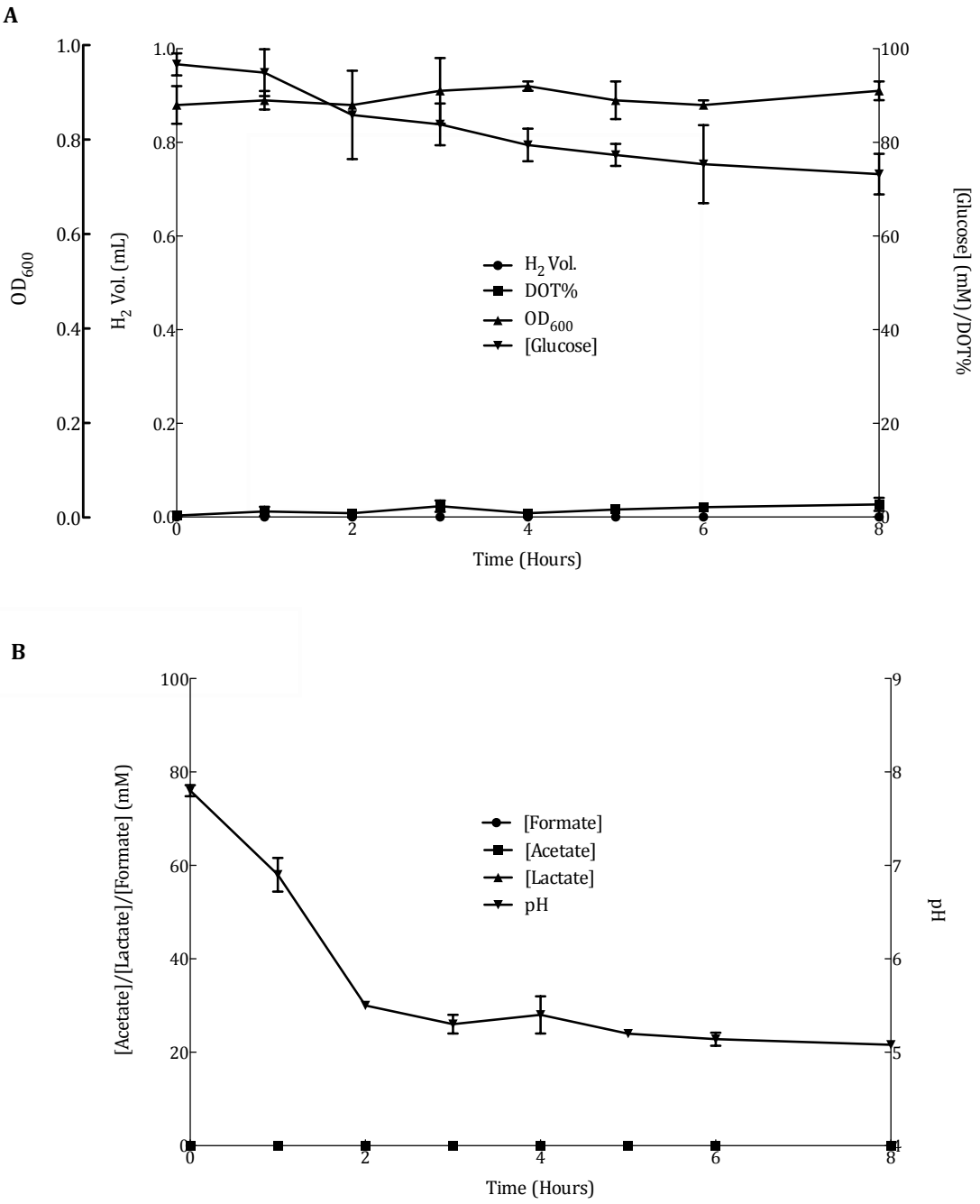


Figure 4.14 Graph showing the hydrogen production in a bioreactor subsequent to growth by *E. coli* HD701 in a bioreactor with low aeration. Process parameters detailed in Table 4.10. Graph A shows the variation of [glucose], hydrogen evolution, DOT and OD_{600} with time, where there is no hydrogen evolution, the [glucose] decreases OD_{600} remains approximately static and the process is anoxic. Graph B shows the fermentation organic acid profile, where formate, acetate and lactate were not present (all plots run along the base line), the pH dropped through the initial hydrogen evolution phase but levelled out at approximately 5.1

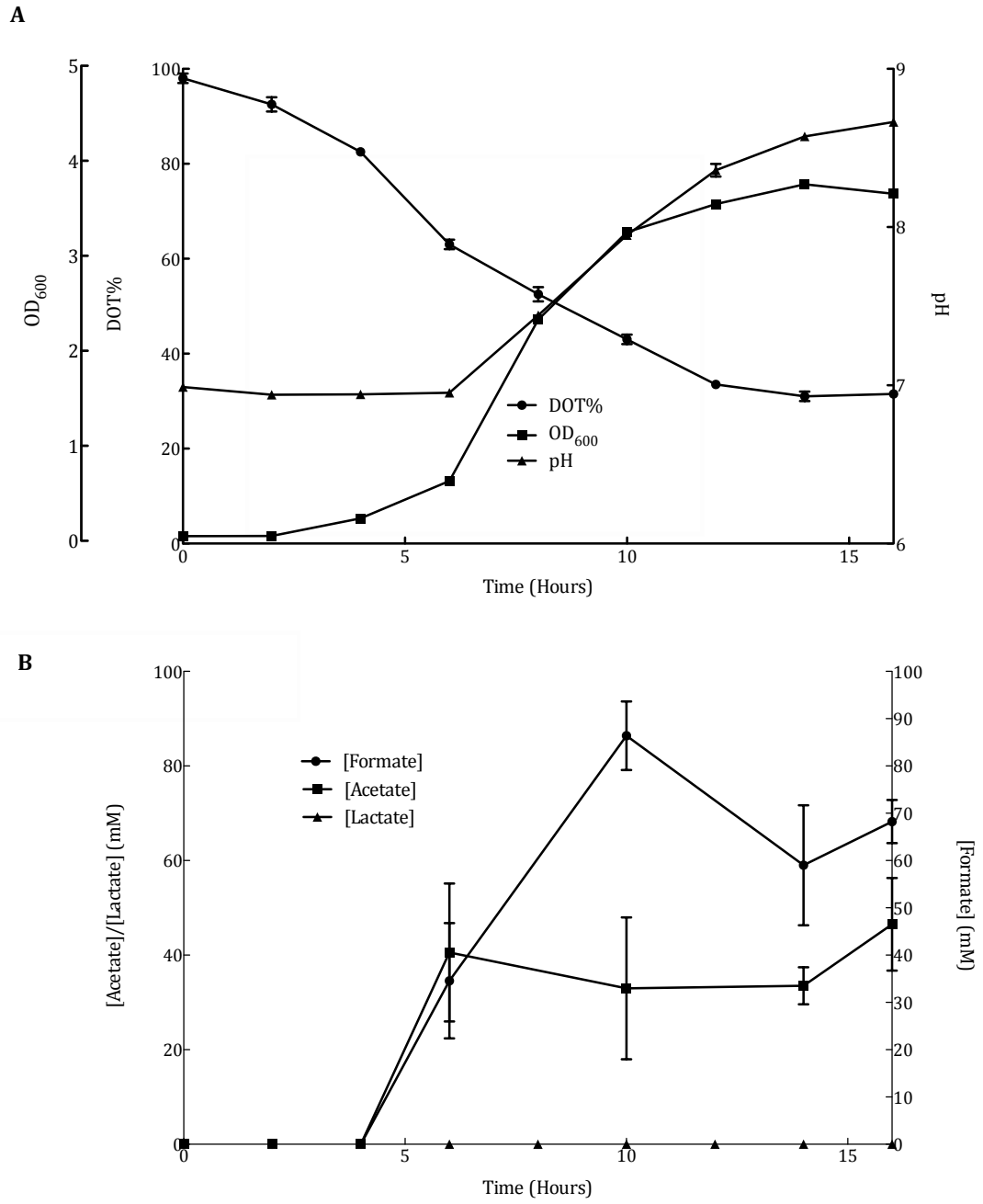


Figure 4.15 Graph showing the growth of *E. coli* HD701 in a bioreactor with low agitation. Process parameters detailed in Table 4.11. Graph A shows the variation of OD_{600}, pH and DOT with time, where the DOT never reaches 0%, μ_{max} = 0.65 h^{-1}. Graph B shows the fermentation organic acid profile, where lactate was not accumulated, but both formate and were.

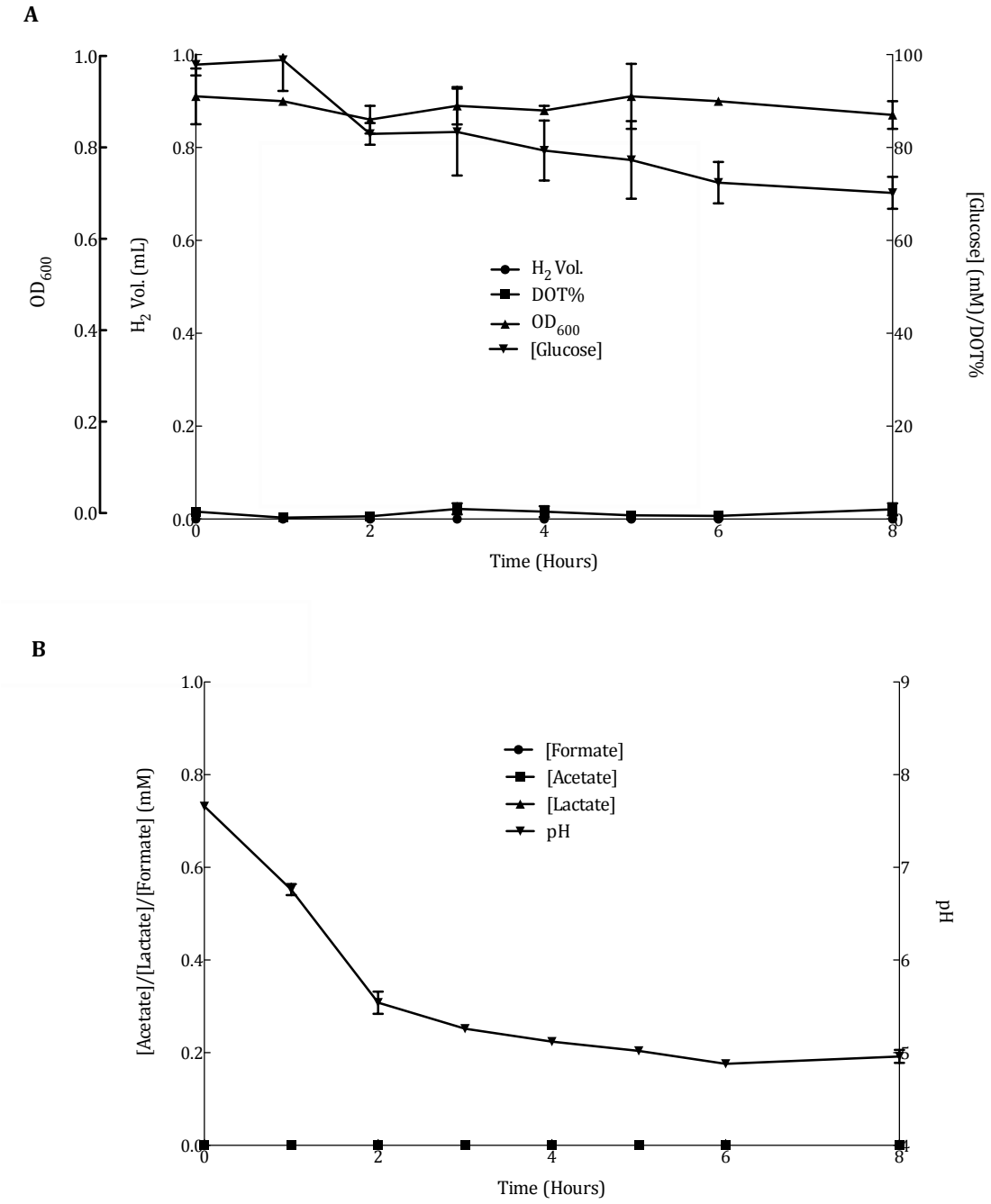


Figure 4.16 Graph showing the hydrogen production in a bioreactor subsequent to growth by *E. coli* HD701 in a bioreactor with low agitation. Process parameters detailed in Table 4.12. Graph A shows the variation of [glucose], hydrogen evolution, DOT and OD_{600} with time, where there is no hydrogen evolution, the [glucose] decreases OD_{600} remains approximately static and the process is anoxic. Graph B shows the fermentation organic acid profile, where formate, acetate and lactate were not present (all plots run along the base line), the pH dropped through the initial hydrogen evolution phase but levelled out at approximately 5.0

The first conclusion that can be drawn from the results is that the conservation of a constant K_La proved to be a good technique for process control, as both processes with differing parameters (shown in Table 4.9) but constant K_La had similar fermentation profiles.

In both experimental fermentation profiles the change in K_La had slowed growth, this can be shown by the increase in length the fermentation time, but this slowing of growth, in both cases was accompanied by a decrease in bacterial density in comparison to experimental profile shown in Figure 4.1 This decrease in bacterial enumeration, typically accompanies a decrease in growth rate as a larger proportion of the growth substrate is assimilated into the cell and not used as energy for growth (Ng, 1969). Consideration of the organic acid profile and the pH profile during the fermentation shows that alongside a steady amount of formate being present acetate is produced. The production of acetate is in contrast to the previous experiments in which hydrogen production occurred, however the increase in pH (above pH 7) is said to shift mixed acid fermentation from the production of lactate to the production of acetate (Hallenbeck, 2009). The final similarity between the two fermentation profiles was during the hydrogen production phase of the process in both cases the OD_{600} remained approximately static, which implies there was no bacterial growth or death. The drop in pH over the time course means acidification of the culture was occurring, it could only be said that the acidification was not due to the production of formate, acetate and lactate. The lack of formate correlates with the lack of hydrogen production and also implies that during the entirety of both processes no functional FHL was produced, which can be attributed to either the lack of significant oxygen depletion during growth to allow for the transcription of the FHL complex (the FHL complex is oxygen sensitive, so while it requires a specific level of intracellular formate to be attained, it also requires an anoxic environment) or inactivation/inhibition of the enzyme complex (Birkmann *et al.*, 1987; Abaibou *et al.*, 1997).

As it has been established that the change in scale and geometry of the bioreactor (with respect to the shake flask) cannot be overcome by maintaining

k_La and (to some extent) power input in the vessel. However hydrogen evolution can be achieved by the conditions in the no aeration/anaerobic growth (shown in Figure 4.11). As previously stated there was a lower hydrogen evolution rate in the "No aeration/anaerobic growth" experiment, but this was correlated with a lower optical density and therefore a decreased bacterial number in the hydrogen evolution phase of the experiment. This fact leads to two conclusions:

- It reinforces the hypothesis/result that an increase in cell density increases the hydrogen evolution rate.
- It implies that the DOT within the culture during growth is of importance and later growth phases require microaerobiosis.

The second conclusion was reached by considering the results gained in this study alongside the study by Vemuri *et al.*, (Vemuri *et al.*, 2002). These conclusions led to the assessment of the DOT within the shake flask (DOT curves shown in Figure 4.17), and the employment of gas blending (Figures 4.18 and 4.19) as a technique to allow control of the DOT within the bioreactor. The other scale up techniques shown in Section 2.2.1 were not assessed as the similarity between two hydrogen producing experiments were the oxygen limitation during the later stages of growth. While further experimentation could have been carried out to find out if there were physical parameters that could be used to model the shake flask process it was deemed unnecessary as gas blending could perform the function of allowing the transition from aerobic – anaerobic/microaerobic growth to occur at a point at which hydrogen production was known to occur.

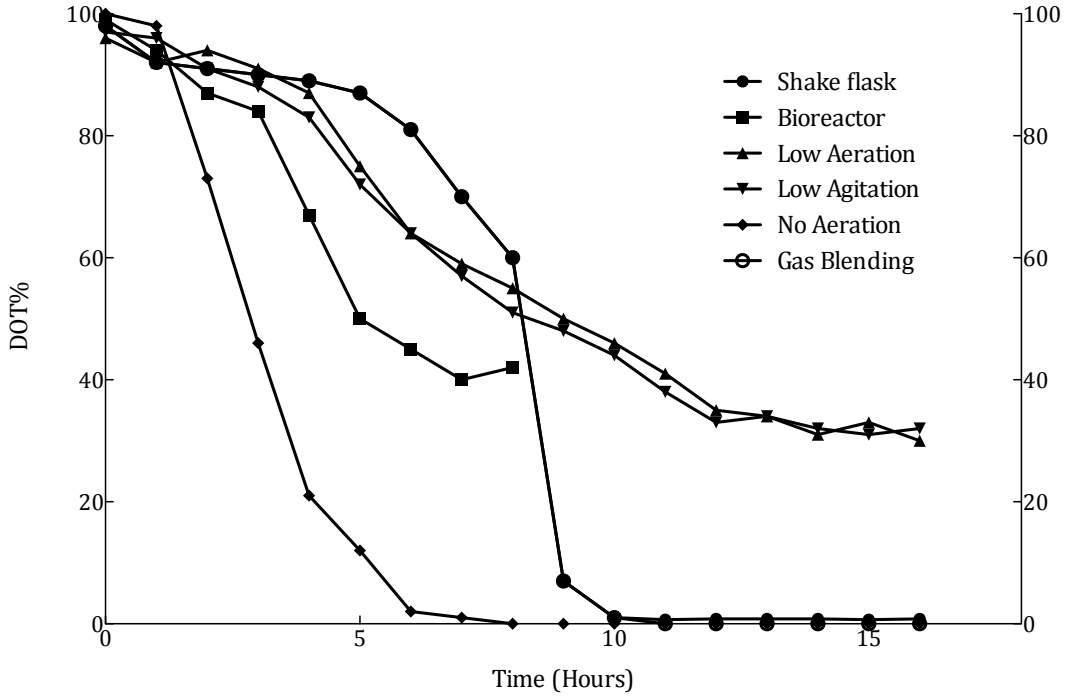


Figure 4.17 DOT Curves - differing modes of growth of *E. Coli* HD701, The gas blending experiment sets out to mirror the shake flask and as such the plot runs underneath the shake flask plot.

Figure 4.17 Shows the DOT curves of all the differing experiments used to try and mirror the physical characteristics of the shake flask, it shows the ability of gas blending to create the necessary DOT within the bioreactor so that it mirrors the process within the shake flask using the parameters listed in Table 4.13 with subsequent hydrogen production carried out using the parameters in Table 4.14. The curve for gas blending within Figure 4.17 cannot be seen as it runs underneath the shake flask DOT curve.

Table 4.13 Process parameters for bioreactor growth (Gas Blending experiment in Fig. 4.18)

Parameter	
Bioreactor size	5L
Culture volume	2 L
Impeller speed	300 rpm
Aeration rate	0.75 vvm
Inoculation size	0.2 mL
Medium	Nutrient Broth #2
Growth temperature	30°C

Table 4.14 Process parameters for hydrogen production (Gas Blending experiment in fig. 4.19)

Parameter	
Bioreactor size	5 L
Working volume	4 L
Impeller speed	300 rpm
Substrate volume	2 L
Substrate composition	Glucose (100 mM), NaCl ($17gL^{-1}$)
Culture size	2 L

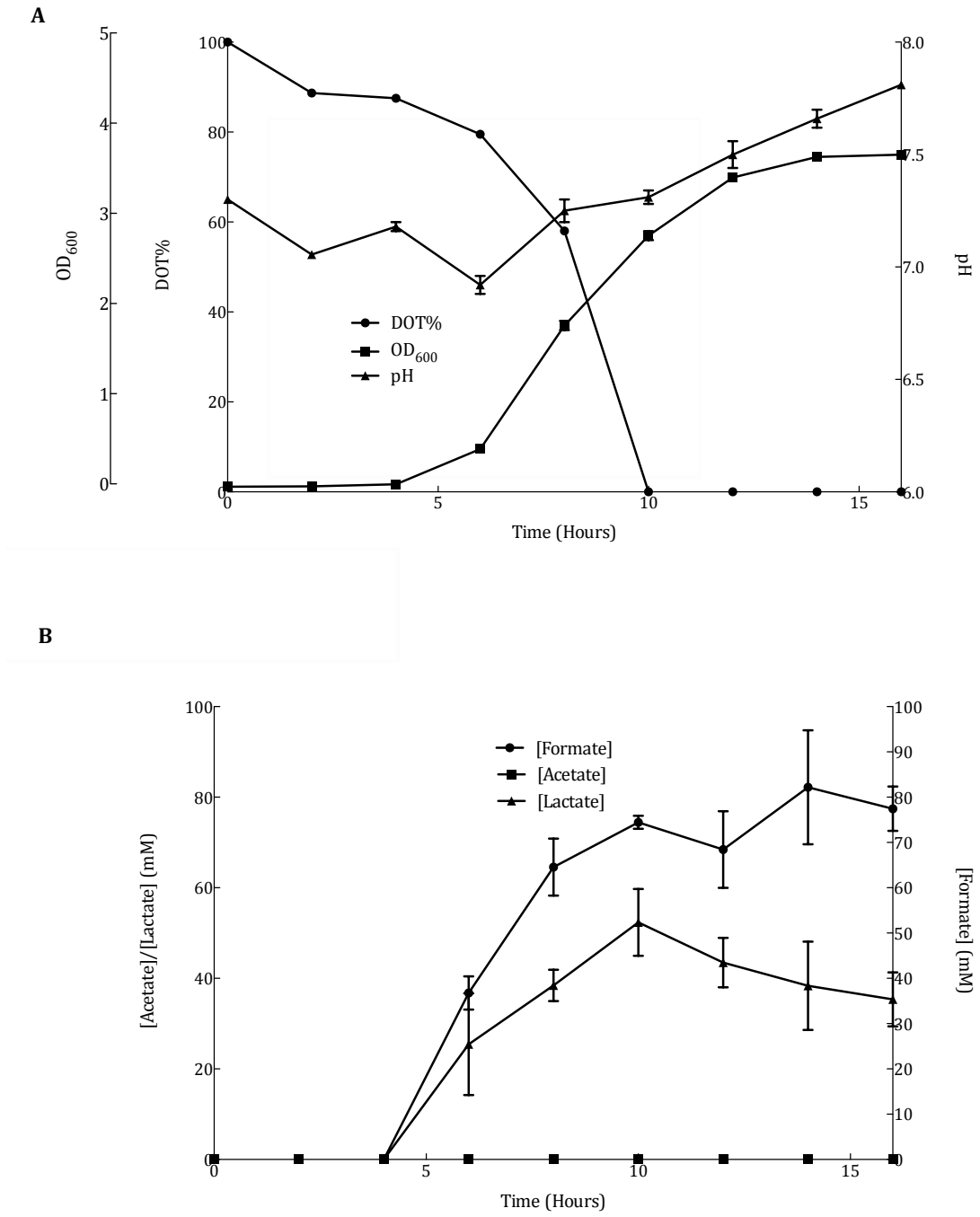


Figure 4.18 Graph showing the growth of *E. coli* HD701 in a bioreactor with gas blending for DOT control. Process parameters detailed in Table 4.13. Graph A shows the variation of OD_{600}, pH and DOT with time, μ_{max} = 0.68 h^{-1}. Graph B shows the fermentation organic acid profile, where acetate was not accumulated, but both formate and lactate were, increasing through the fermentation as oxygen limitation increased.

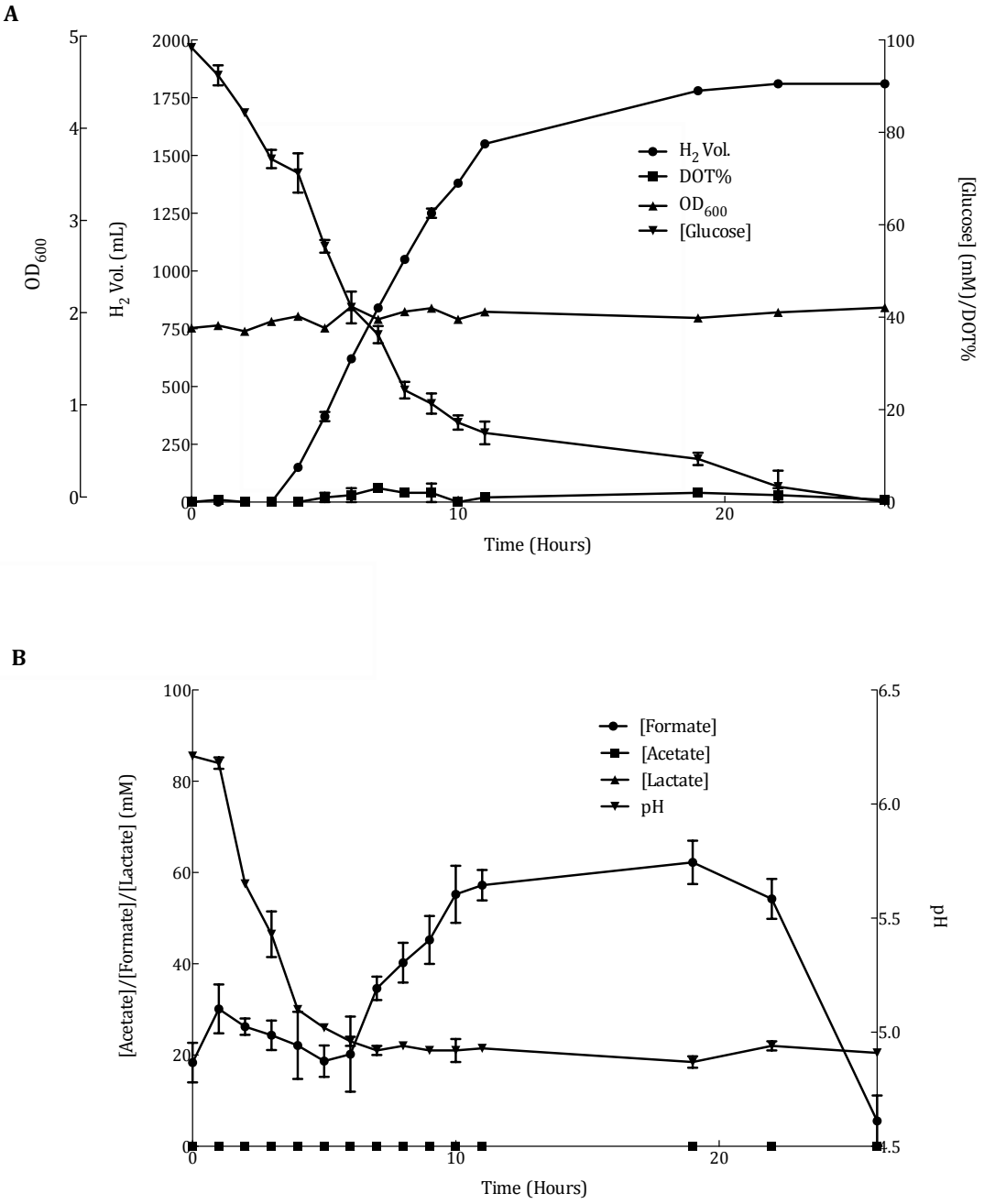


Figure 4.19 Graph showing the hydrogen production by *E. coli* HD701 in a bioreactor subsequent to bioreactor growth with gas blending for DOT control. Process parameters detailed in Table 4.14. Graph A shows the variation of [glucose], hydrogen evolution, DOT and OD_{600} with time, where the utilisation of glucose mirrors the evolution rate of hydrogen, OD_{600} remains approximately static and the process is anoxic. Graph B shows the fermentation organic acid profile, where acetate and lactate were not present (both plots run along the base line), but formate was present, the pH dropped through the initial hydrogen evolution phase but levelled out at approximately 4.7

In this study gas blending was used to control the DOT within the bioreactor, without affecting the power input to the vessel. When comparing the results of these experiments to those shown in Figures 4.1 and 4.2 the process proceeds in a similar fashion but with less batch-to-batch variation in results implying greater process control achieved through the use of gas blending to control DOT.

It has been shown in Figures 4.18 and 4.19 that the physical and geometric differences exhibited in the bioreactor when compared to a shake flask can be overcome by using gas blending. The main difference between the fermentation and hydrogen production profiles of the shake flask and bioreactor (with gas blending) experiments was the slightly lower biomass attained, and while this does not have an appreciable effect on hydrogen production, it was counter intuitive. The DOT profile shown in the gas blending experiment, implies a smooth transition between defined points on the DOT curve, where as in fact the transition from point to point was carried out in a stepwise fashion at set intervals throughout the fermentation, this would logically lead to there being a higher DOT level within the bioreactor, than the shake flask for the transition period between any two set interval points. This higher DOT level should serve to increase the growth rate, which should not lead to the lower biomass concentration, which is implied by the lower OD_{600} value at the end of the fermentation. An explanation for this is there was an increase in growth rate, that increase in growth rate has the effect of countering the decrease in growth rate that was created by the below optimum growth temperature, and this has a knock on effect on the cell physiology, which would in turn effect the bacterial cell density. Another potential reason for this difference could be the change in flow regime from shake flask to bioreactor. While the Reynolds number (Re) necessary for turbulent flow has not been evaluated it can be shown that:

$$\mathrm{Re}_{Shakeflask} = 15.86\mathrm{Re}_{Bioreactor}$$

An increase in the turbulence within the reactor would increase the homogeneity of the culture and improve bacterial growth, by the removal or minimisation of nutrient gradients removing inhibition of growth by both starvation and excess substrate.

After it had been shown that gas blending was an effective way to mirror the process within the shake flask, within a bioreactor and achieve similar results. The same technique was applied to the shake flask process in which the medium concentration had been doubled. The repetition of the process shows the efficacy of using gas blending at increasing cell densities. Table 4.15 and 4.16 and Figures 4.20 and 4.21 show the process parameters used during the experiments in which gas blending was used to replicate the shake flask process that utilised the double strength medium.

Table 4.15 Process parameters for bioreactor growth (Gas Blending experiment in Figure 4.20)

Parameter	
Bioreactor size	5L
Culture volume	2 L
Impeller speed	300 rpm
Aeration rate	0.75 vvm
Inoculation size	0.2 mL
Medium	Double strength Nutrient Broth #2
Growth temperature	30°C

Table 4.16 Process parameters for hydrogen production (Gas Blending experiment in Figure. 4.21)

Parameter	
Bioreactor size	5 L
Working volume	4 L
Impeller speed	300 rpm
Substrate volume	2 L
Substrate composition	Glucose (100 mM), NaCl ($17gL^{-1}$)
Culture size	2 L

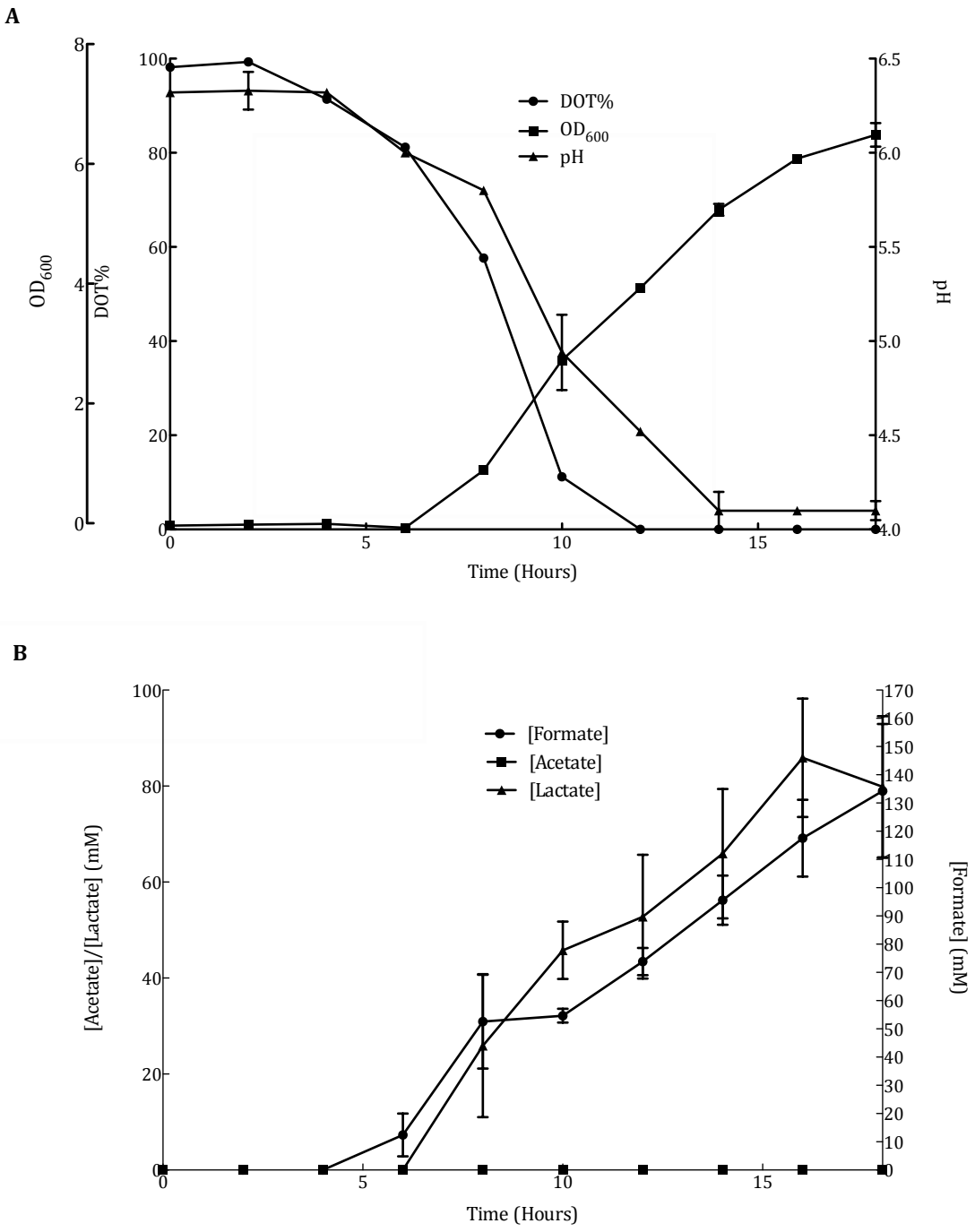


Figure 4.20 Graph showing the growth of *E. coli* HD701 in a bioreactor with double strength nutrient broth and gas blending for DOT control. Process parameters detailed in Table 4.15. Graph A shows the variation of OD_{600}, pH and DOT with time, μ_{max} = 0.53 h^{-1}. Graph B shows the fermentation organic acid profile, where acetate was not accumulated, but both formate and lactate were, increasing through the fermentation as oxygen limitation increased.

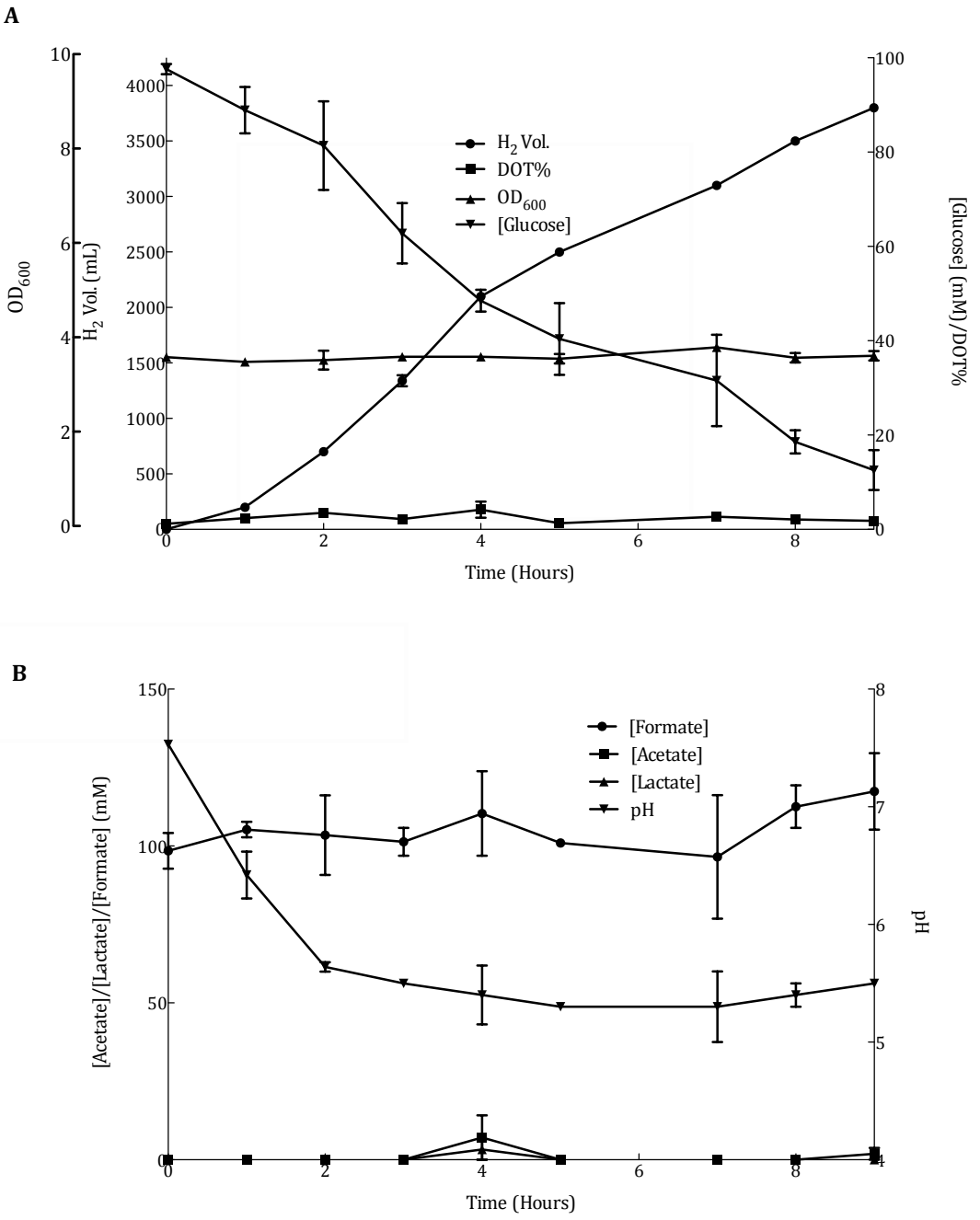


Figure 4.21 Graph showing the hydrogen production by *E. coli* HD701 in a bioreactor subsequent to bioreactor growth in double strength nutrient broth with gas blending for DOT control. Process parameters detailed in Table 4.16. Graph A shows the variation of [glucose], hydrogen evolution, DOT and OD_{600} with time, where the utilisation of glucose mirrors the evolution rate of hydrogen, OD remains approximately static and the process is anoxic. Graph B shows the fermentation organic acid profile, where acetate and lactate were not present for the majority of the phase (both plots run along the base line), but formate was present, the pH dropped through the initial hydrogen evolution phase but levelled out at approximately 5.7

The use of gas blending had once again, provided the correct environment for growth and subsequent hydrogen production. The results gained were again similar to the shake flask process, with the feature of a slight decrease in cell density. The potential reasons for this decrease have already been discussed with the previous experiment that utilised gas blending. Gas blending as a technique is not commonly used in bacterial fermentation processes, but it allows for control of the DOT within a bioreactor and as such has relevance to research in all processes where productivity is affected by the DOT, whether it be, as in this study, to allow for microaerobiosis to occur in a process where it may not naturally occur, or as in other processes where there is the need to maintain good aeration within the vessel (Flores *et al.,* 1994; García-Arrazola *et al.,* 2005). An additional novel factor of this process is the use of gas blending to track a DOT profile, whereas it normally functions to maintain DOT at (or above) a certain level.

This set of experiments (Figures 4.17 – 4.21) showed that there were important physical parameters that must be conserved within the bioreactor to allow hydrogen evolution to occur. The system that has been created within the shake flask was far more complex than at first assumed. It has been shown that for hydrogen evolution to occur, subsequent to growth in a bioreactor, the later stages of the bacterial growth curve, from mid-late exponential must occur with oxygen limitation. If growth is stopped by oxygen limitation at any phase of the growth in a bioreactor, this does not have the same effect as growth in microaerobic/anaerobic/ oxygen limited conditions (it is assumed that the actual conditions are microaerobic as the switch to anaerobiosis may have a more marked effect on the growth rate).

After physical assessment and characterisation of the process has allowed for a process change, and the parameters for that change have been identified, it was possible to see the effect of a change of the chemical environment within the shake flask by changing the fermentation growth medium. When conducting this change in batch mode operation, cell growth media were chosen with the view of

firstly optimising batch culture by increasing the cell density and secondly with the view that it should be able to be adapted for fed-batch growth.

4.3 Process intensification

As there has been an increase in process efficiency (shown by the higher hydrogen evolution rate), at higher cell density, the next step was to further increase the cell density by changing the mode of growth from batch to fed-batch. The method chosen to do this was to firstly identify a semi-defined medium that would produce hydrogen in batch mode growth and then utilise the medium for fed-batch growth. When carrying out this process, the technique developed in Section 4.2 was used, i.e. the medium is first assessed for growth in a shake flask and hydrogen production in a bioreactor, then gas blending is used to create the conditions necessary for batch growth and hydrogen production within the bioreactor, prior to fed-batch growth being attempted. Previous sections within this Chapter have also demonstrated (alongside the use of gas blending) the necessity for microaerobiosis during the later stages of fermentation to produce the necessary environment during growth to allow for hydrogen production to occur. The experiments in this chapter were conducted with this knowledge and as such hydrogen evolution was assessed but not analysed (experiments were assessed to see if they would evolve hydrogen in contradiction to earlier findings, but full analysis was not conducted) if microaerobiosis or anaerobiosis did not occur.

Before a new medium was assessed, the first process change to take place was the effect of the inoculum size. The initial inoculum was 0.01% of the overall fermentation volume; this is far lower than is typical in the bioprocess industry so the inoculum size was increased to 5%. This change in inoculum size affects the overall process parameters as shown in Tables 4.17 and 4.18

Table 4.17 Process parameters for Shake flask growth (Inoculum size change experiment in Figure 4.22)

Parameter	
Bioreactor size	5L
Culture volume	2 L
Agitation speed	200 rpm
Aeration rate	N/A
Inoculation size	100 mL
Medium	Double strength Nutrient Broth #2
Growth temperature	30°C

Table 4.18 Process parameters for hydrogen production (Inoculum size change experiment in Figure 4.23)

Parameter	
Bioreactor size	5 L
Working volume	4 L
Impeller speed	300 rpm
Substrate volume	2 L
Substrate composition	Glucose (100 mM), NaCl ($17gL^{-1}$)
Culture size	2 L

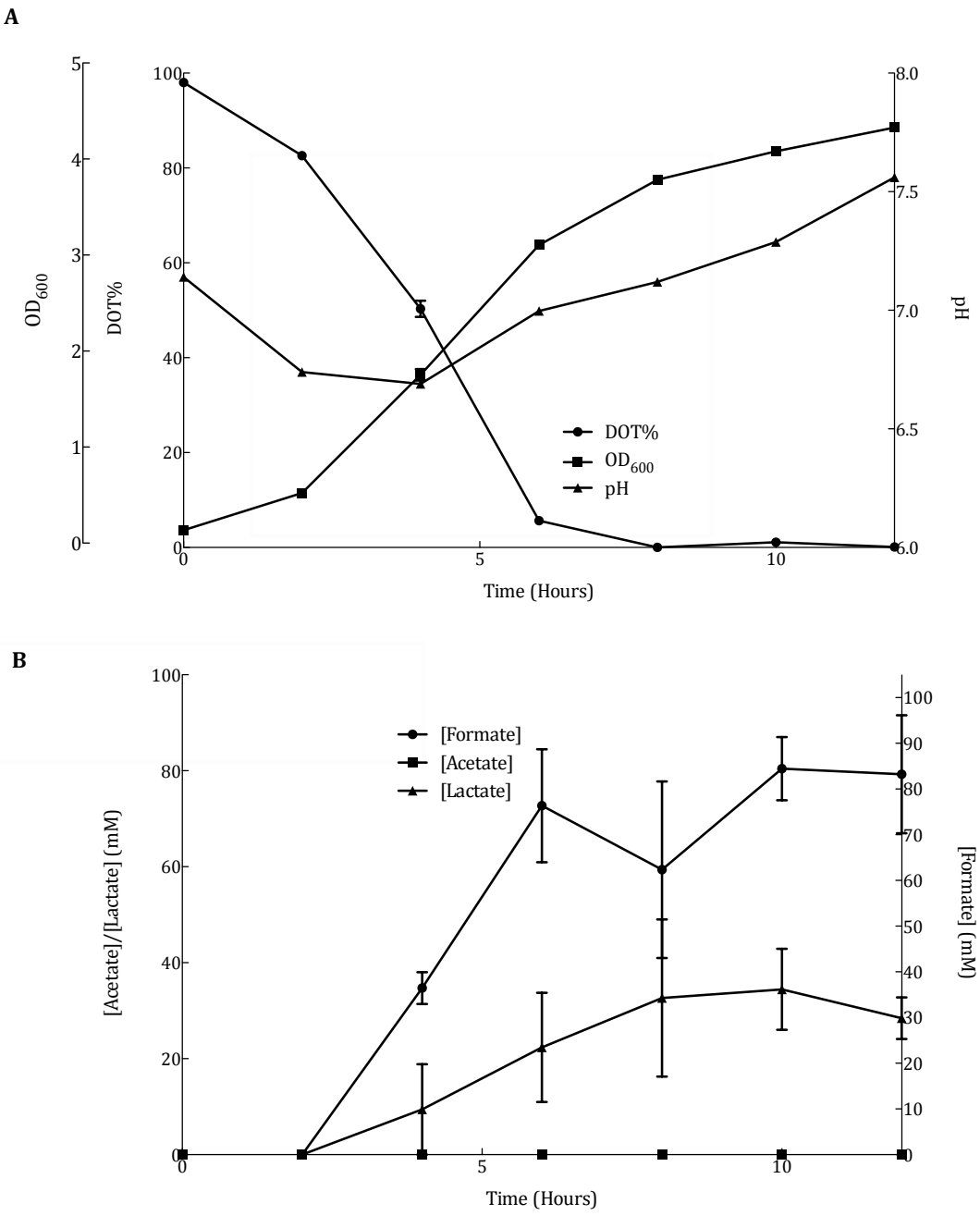


Figure 4.22 Graph showing the growth of *E. coli* HD701 in a shake flask with an increased in inoculum size. Process parameters detailed in Table 4.17. Graph A shows the variation of OD_{600}, pH and DOT with time, μ_{max} = 0.43 h^{-1}. Graph B shows the fermentation organic acid profile, where acetate was not accumulated, but both formate and lactate were, increasing through the fermentation as oxygen limitation increased.

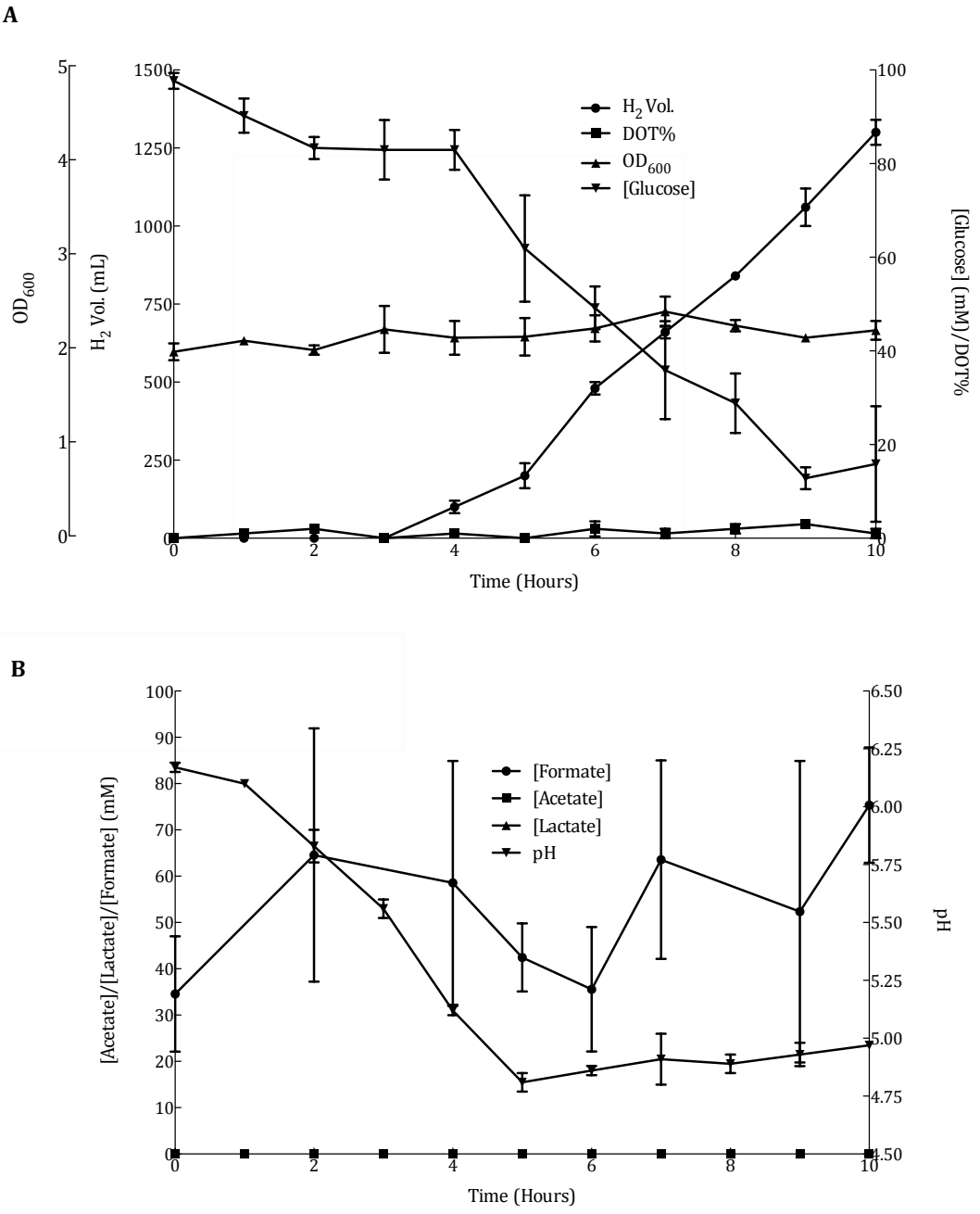


Figure 4.23 Graph showing the hydrogen production by *E. coli* HD701 in a bioreactor subsequent to shake flask growth with an increased inoculum size. Process parameters detailed in Table 4.18. Graph A shows the variation of [glucose], hydrogen evolution, DOT and OD_{600} with time, where the utilisation of glucose mirrors the evolution rate of hydrogen, OD_{600} remains approximately static and the process is anoxic. Graph B shows the fermentation organic acid profile, where acetate and lactate were not present (both plots run along the base line), but formate was present, the pH dropped through the initial hydrogen evolution phase but levelled out at approximately 4.9

Figures 4.22 and 4.23 show the effect of increasing the inoculum size was primarily to decrease the lag phase of the fermentation profile, shortening the overall fermentation time. This is a probable conclusion to this process change, as an increase in inoculum size should decrease the length of the lag phase of growth due to the decrease in viable population variance and single cell lag time variance (Davis *et al*., 2009; Malakar and Barker, 2009).

This decrease in fermentation time may have little effect on the overall process length but a decrease in process length with the same output/ yield leads to an overall decrease in the energy input to the process over its duration.

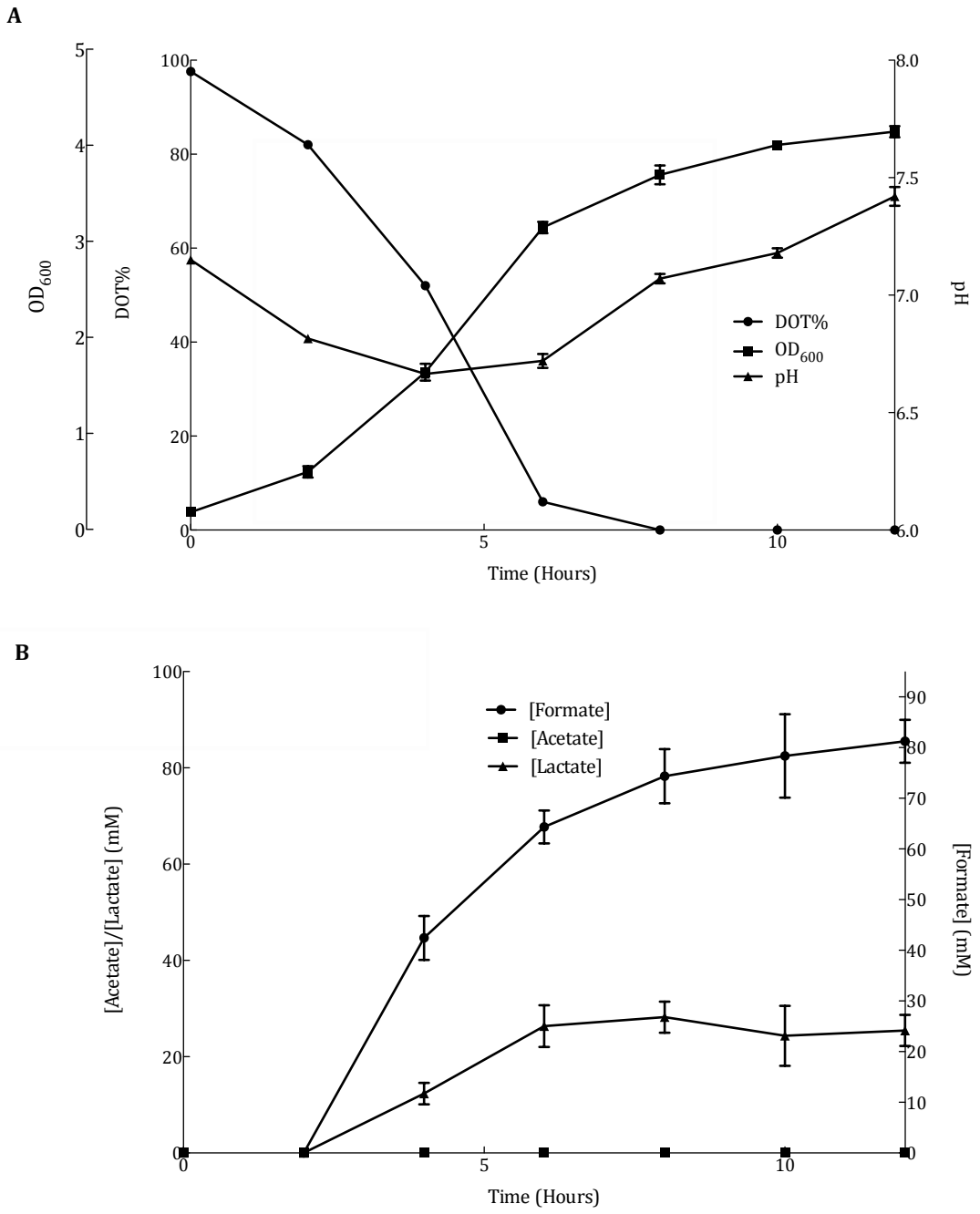


Figure 4.24 Graph showing the growth of *E. coli* HD701 in a bioreactor with an increased in inoculum size and gas blending used to control DOT. Process parameters detailed in Table 4.17. Graph A shows the variation of OD_{600}, pH and DOT with time, μ_{max} = 0.33 h^{-1}. Graph B shows the fermentation organic acid profile, where acetate was not accumulated, but both formate and lactate were, increasing through the fermentation as oxygen limitation increased.

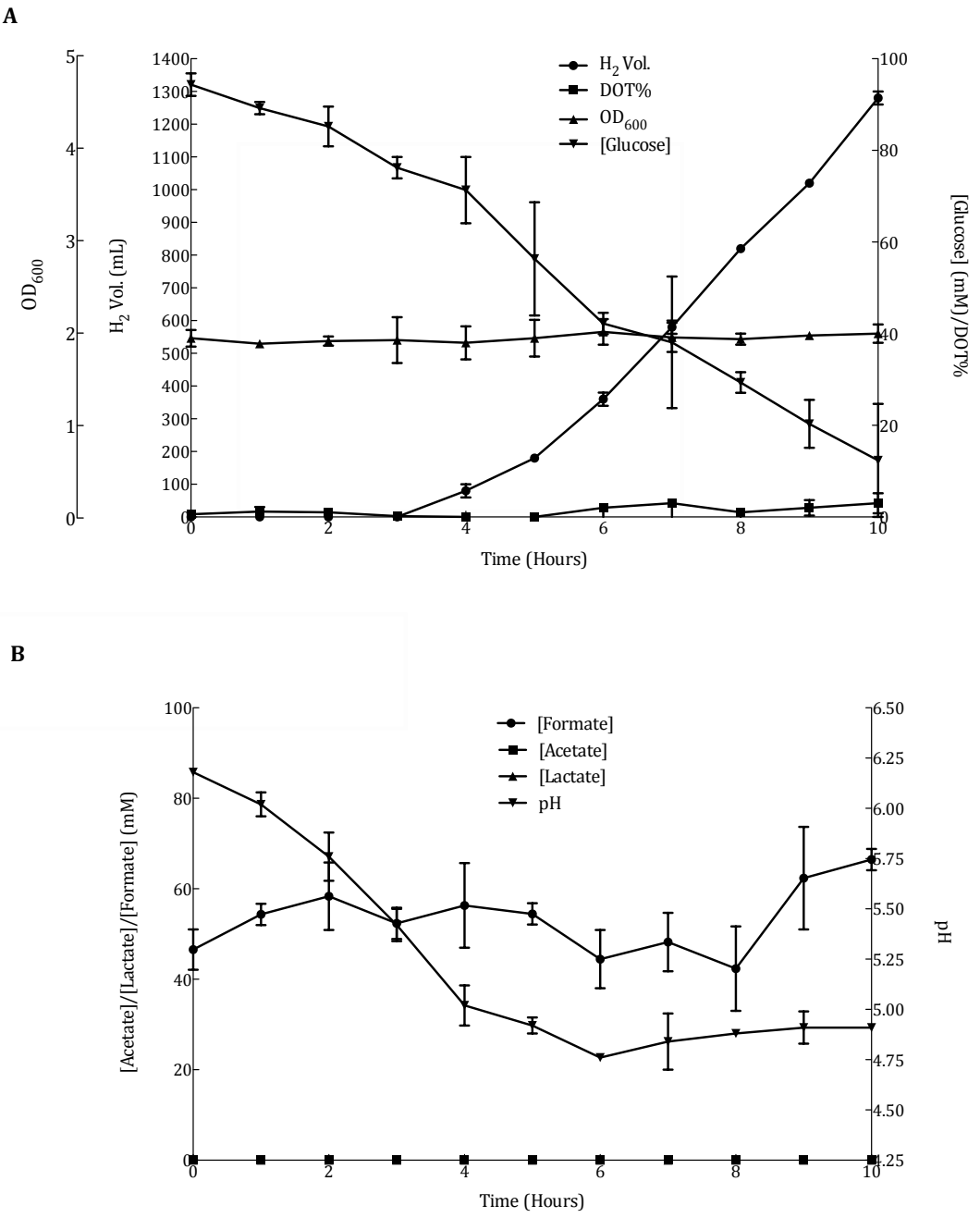


Figure 4.25 Graph showing the hydrogen production by *E. coli* HD701 in a bioreactor subsequent to growth with an increased inoculum size and gas blending for DOT control. Process parameters detailed in Table 4.18. Graph A shows the variation of [Glucose], hydrogen evolution, DOT and OD_{600} with time, where the utilisation of glucose mirrors the evolution rate of hydrogen, OD_{600} remains approximately static and the process is anoxic. Graph B shows the fermentation organic acid profile, where acetate and lactate were not present (both plots run along the base line), but formate was present, the pH dropped through the initial hydrogen evolution phase but levelled out at approximately 4.9

Figures 4.24 and 4.25 show the data for the experiments carried out using the parameters in Tables 4.17 and 4.18, with gas blending used so that the process could proceed in a bioreactor (instead of the shake flask used in Figures 4.22 and 4.23).

The change in inoculum size brings the process parameters closer to the norm for bacterial cultivation and having done so, the next step was to screen different media for suitability. The criteria the medium needed to fulfil were;

- Must contain a separate defined carbon source to allow for feeding in later experiments.
- Must be able to evolve hydrogen after the typically successful shake flask – batch growth.

This led to the selection of a batch medium that had been used to grow *E. coli* to high cell density previously (Lewis *et al.*, 2004).

Table 4.19 Process parameters for Shake flask growth (Medium change to Batch Medium in Figure 4.22)

Parameter	
Bioreactor size	5L
Culture volume	2 L
Agitation rate	200 rpm
Aeration rate	N/A
Inoculation size	100 mL
Medium	Batch Medium
Growth temperature	30°C

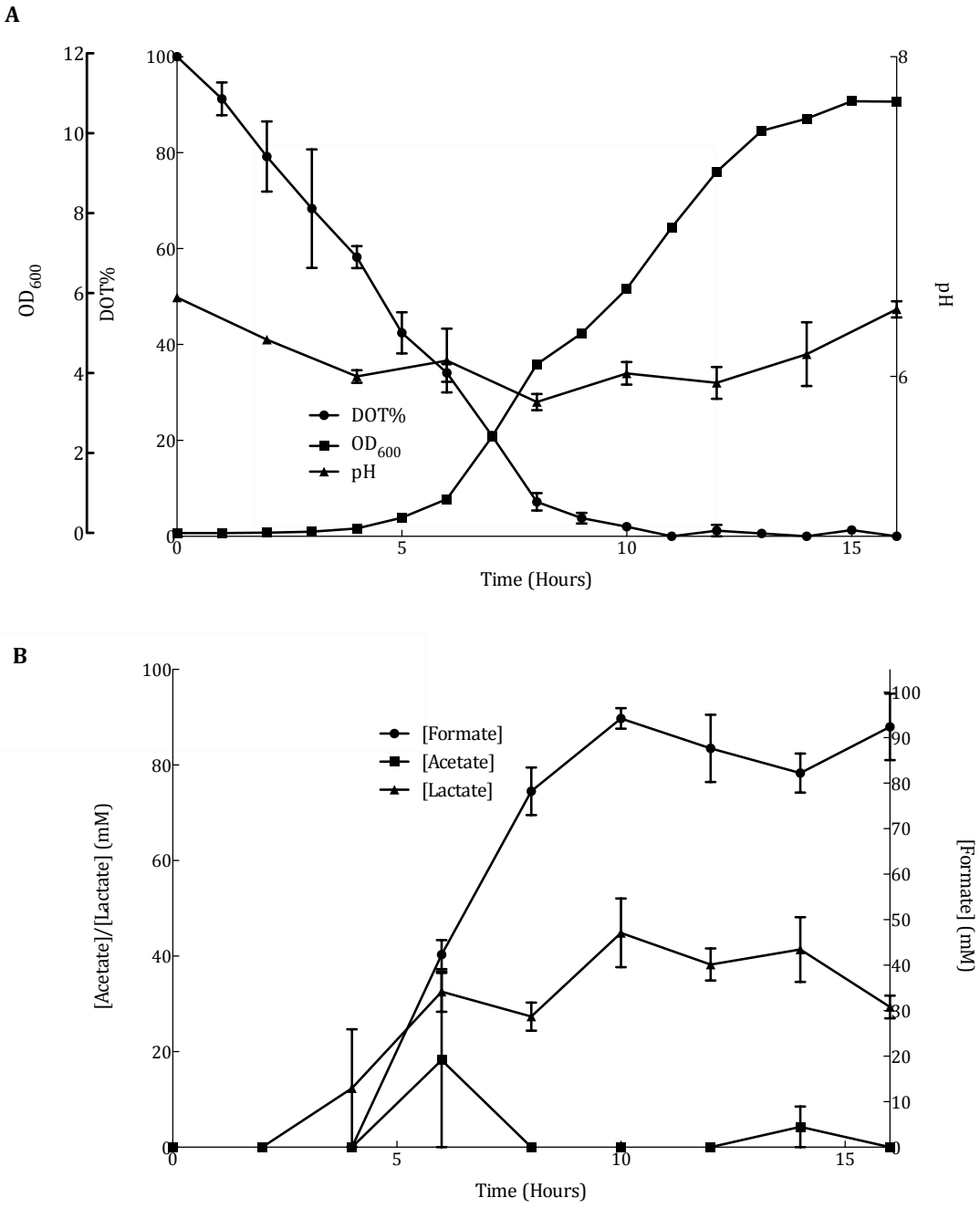


Figure 4.26 Graph showing the initial process growth of E. coli HD701 in a shake flask with a medium change to the Batch medium. Process parameters detailed in Table 4.19. Graph A Shows the variation of OD_{600}, pH and DOT with time. μ_{max} = 0.50 h^{-1}. Graph B shows the fermentation organic acid profile, where acetate was not accumulated, but produced in small amounts, but both formate and lactate were, increasing through the fermentation as oxygen limitation increased.

Figure 4.26 above, shows the fermentation profile of growth in the changed batch medium, from the profile hydrogen production would be predicted and the further increase in cell density (previous maximum; OD600≈6.5), should further increase the hydrogen evolution. However hydrogen production was not achieved from this process and prior to the potential assessment of the hydrogen production phase, the reason for the lack of production was given in literature. The batch medium did not contain a source of selenium within its composition, selenium is an integral part of the formate dehydrogenase (FDH-H), section of the FHL enzyme complex (Boonstra *et al*., 1975; Axley *et al*., 1990; Lutz *et al*., 1991; Bagramyan and Trchounian, 2003) FDH-H is a selenocysteine and molybdenum containing peripheral membrane protein whose function is to deprotonate formate in the hydrogen production process. Further evaluation of the necessity for these medium supplements could be made and have been suggested in Chapter 6 – Further Work.

As the batch medium did not achieve hydrogen production (for the reasons stated), the decision was made to utilise a semi-defined medium that has already been shown to produce hydrogen from a similar strain of bacteria. Figures 4.27 and 4.28 show the fermentation and hydrogen production profiles from that process carried out using the parameters in Table 4.20. Section 4.2 showed the use of gas blending and how it can be adopted to transfer a hydrogen production process from a shake flask to a bioreactor, this technique was then applied to create processes within the bioreactor. As previously stated, the first step is always to characterise the shake flask, then use gas blending to mimic the conditions within the shake flask in the bioreactor as shown in Figures 4.28 and 4.29. Tables 4.20 – 4.22 show the process parameters for all the following batch mode experiments.

Table 4.20 Process parameters for Shake flask growth (Medium change)

Parameter	
Bioreactor size	5L
Culture volume	2 L
Agitation rate	200 rpm
Aeration rate	N/A
Inoculation size	100 mL
Medium	TGYEP / MSB
Growth temperature	30°C

Table 4.21 Process parameters for Bioreactor growth (Medium change)

Parameter	
Bioreactor size	5L
Culture volume	2 L
Agitation rate	300 rpm
Aeration rate	0.75 vvm
Inoculation size	100 mL
Medium	TGYEP / MSB
Growth temperature	30°C

Table 4.22 Process parameters for hydrogen production (Medium change)

Parameter	
Bioreactor size	5 L
Working volume	4 L
Impeller speed	300 rpm
Substrate volume	2 L
Substrate composition	Glucose (100 mM), NaCl ($17gL^{-1}$)
Culture size	2 L

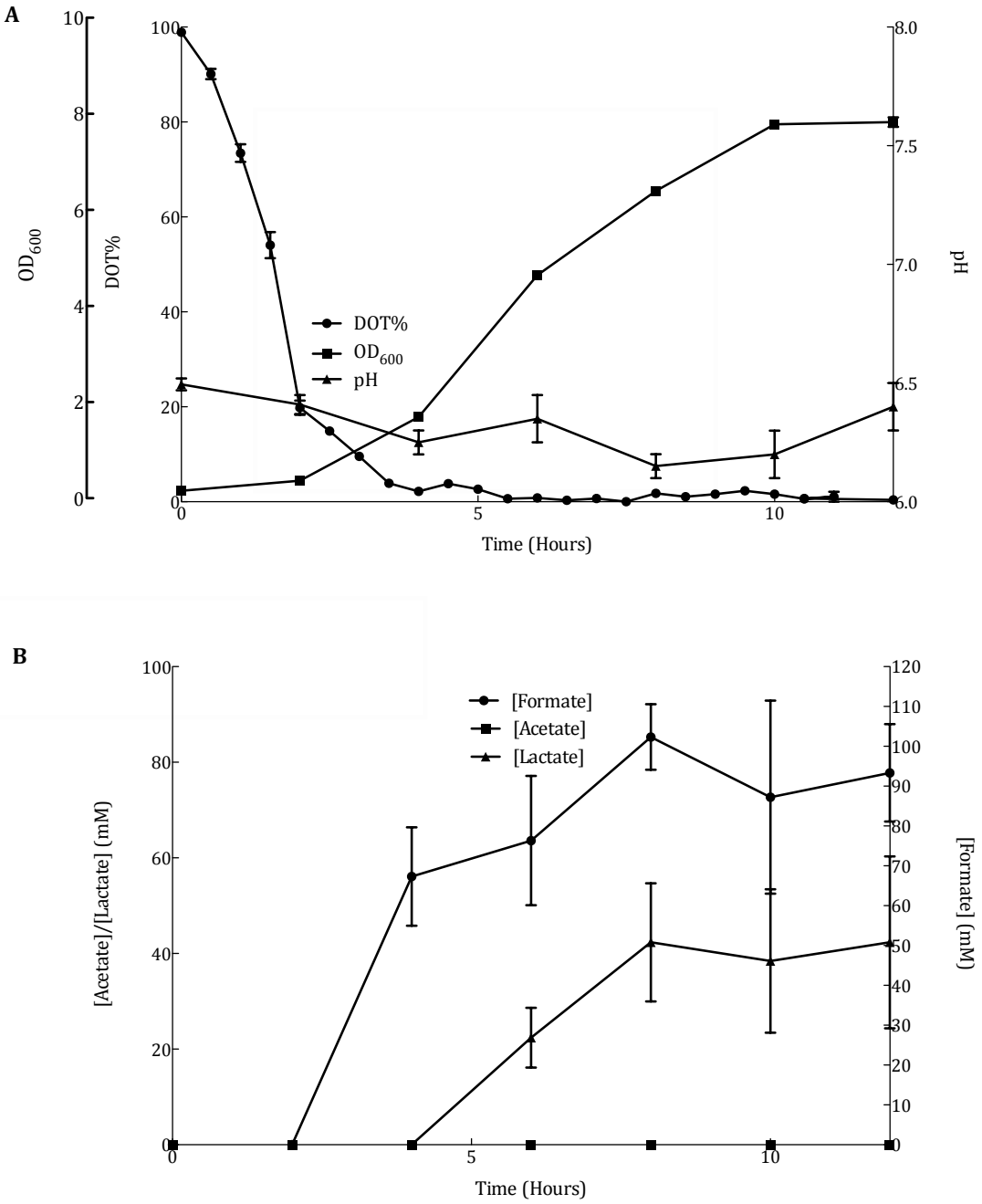


Figure 4.27 Graph showing the initial process growth of *E. coli* HD701 in a shake flask with a medium change to TGYEP medium. Process parameters detailed in Table 4.20. Graph A Shows the variation of OD_{600}, pH and DOT with time, μ_{max} = 0.49 h^{-1}. Graph B shows the fermentation organic acid profile, where acetate was not accumulated, but produced in small amounts, but both formate and lactate were, increasing through the fermentation as oxygen limitation increased.

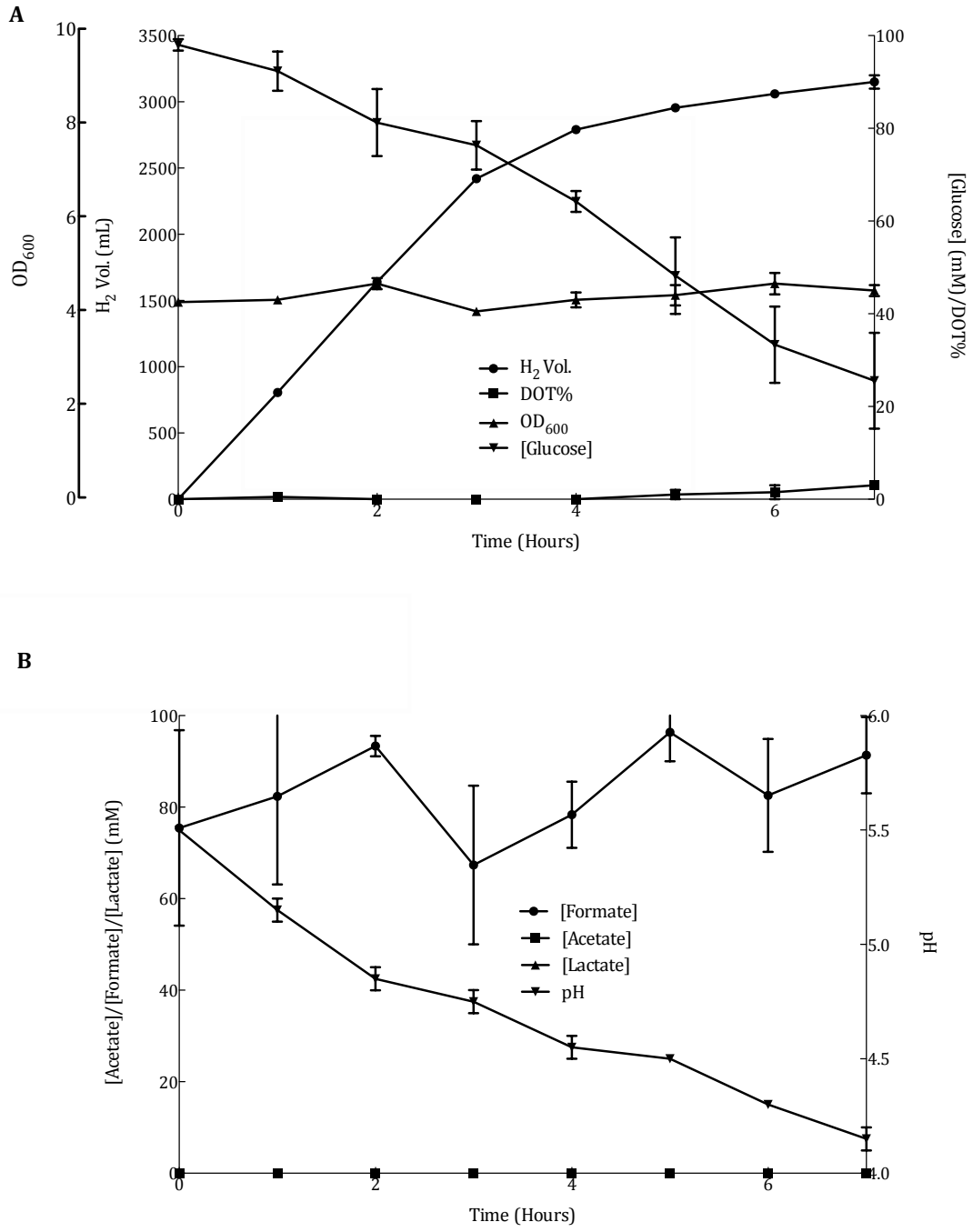


Figure 4.28 Graph showing the initial process Hydrogen production *E. coli* HD701 in a bioreactor subsequent to growth in a shake flask with TGYEP. Process parameters detailed in Table 4.22. Graph A Shows the variation of [Glucose], Hydrogen evolution, DOT and OD_{600} with time, where the utilisation of glucose mirrors the evolution rate of hydrogen, OD_{600} remains approximately static and the process is anoxic. Graph B shows the fermentation organic acid profile, where acetate and lactate were not present (both plots run along the base line), but formate was present, the pH dropped through the hydrogen evolution phase.

After correcting and simplifying the medium choice and ensuring the requisite supplementation was made to the medium, a new intensified process has been created. As with previous batch mode growth experiments the pH remained below 7 (there was little pH fluctuation, most likely due to the phosphate buffer in the medium) and only Formate and lactate were produced. The initial increase in cell density shown in Figure 4.3 had the effect of increasing the organic acid concentration in the medium during growth where as this set of experiments did not yield a further increase in the concentration of organic acids, indicating an upper limit for the concentration. The other effect of the increase in cell density that may not be clear from graphical representation was the increase in variability of the process, the larger error bars do not show the true level of the fluctuating trend within the organic acid profile, this may be due to the increasing effect of heterogeneity and potentially sub-turbulent flow regimes at higher concentrations. The increase in hydrogen evolution has not been linear from previous experiments with the medium change doubling the evolution rate to 800mL/hr, where as the OD_{600} value has only increased to approximately 8, medium supplementation could be the reason for the increase in specific productivity (on a per gram of cells basis).

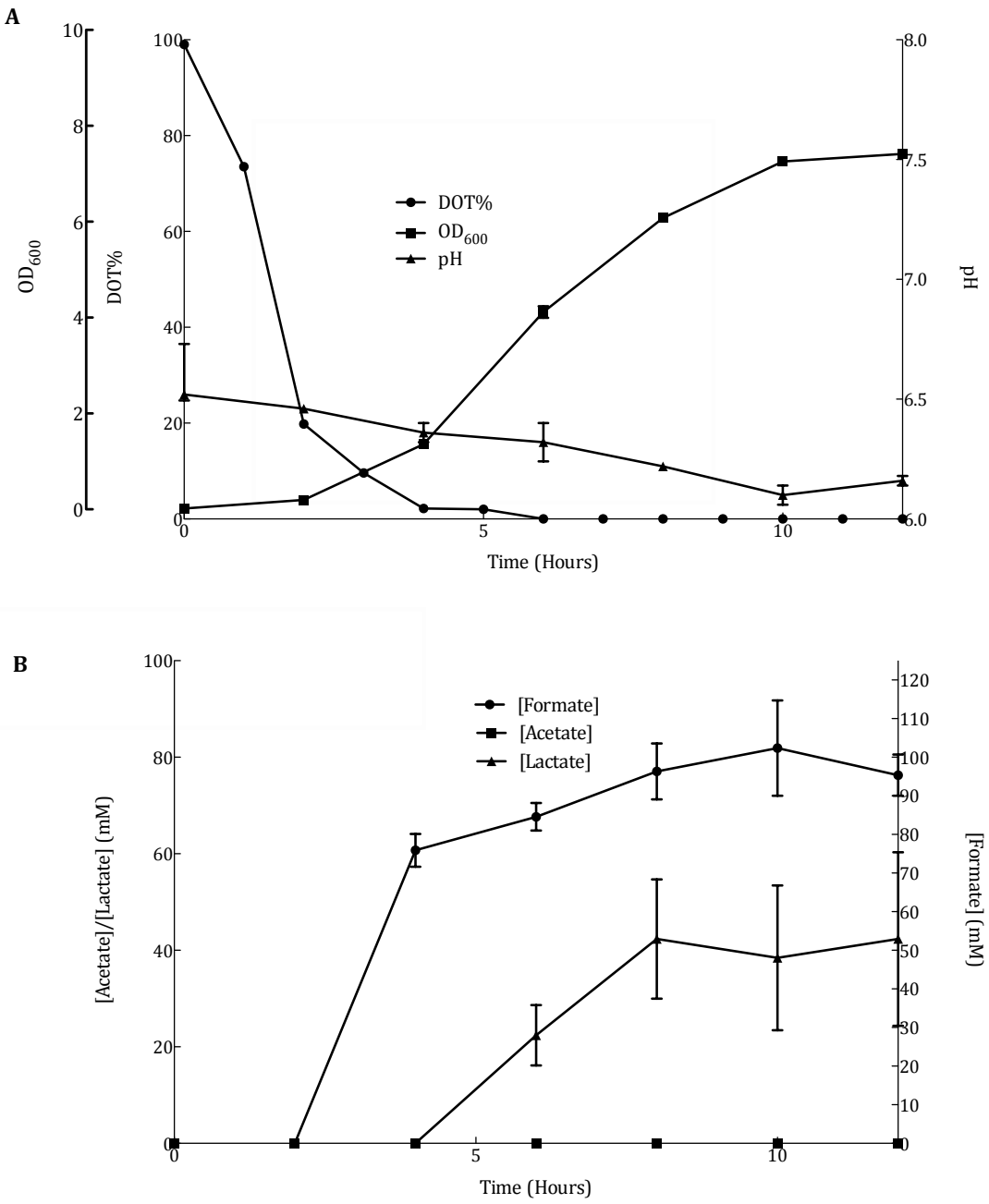


Figure 4.29 Graph showing the growth of *E. coli* HD701 in a bioreactor with a medium change to TGYEP medium and DOT control by gas blending Process parameters detailed in Table 4.21. Graph A Shows the variation of OD_{600}, pH and DOT with time, μ_{max} = 0.50 h^{-1}. Graph B shows the fermentation organic acid profile, where acetate was not accumulated, but produced in small amounts, but both formate and lactate were, increasing through the fermentation as oxygen limitation increased.

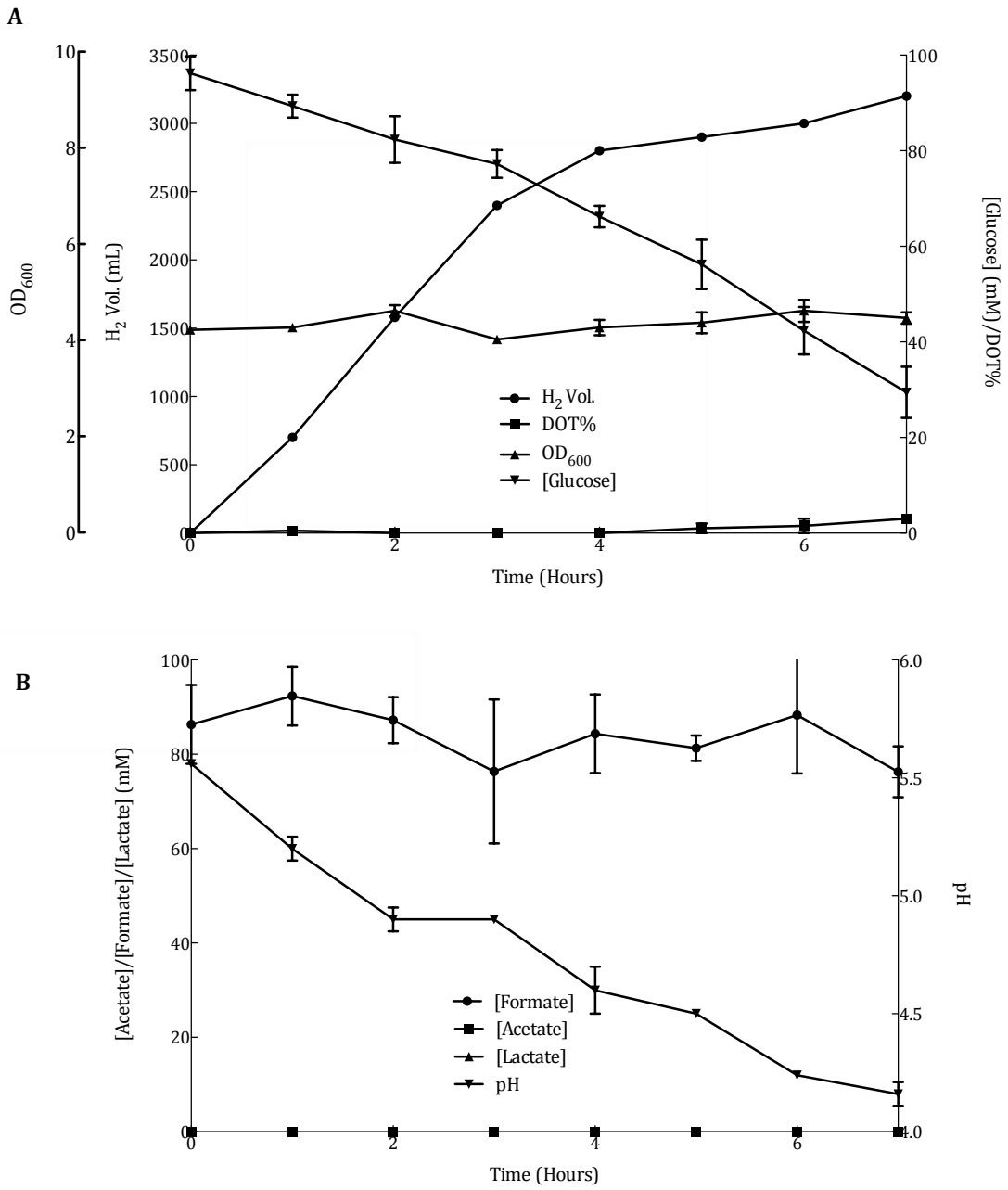


Figure 4.30 Graph showing the hydrogen production by *E. coli* HD701 in a bioreactor subsequent to growth in a bioreactor containing TGYEP medium with gas blending for DOT control. Process parameters detailed in Table 4.22. Graph A Shows the variation of [Glucose], Hydrogen evolution, DOT and OD_{600} with time, where the utilisation of glucose mirrors the evolution rate of hydrogen, OD_{600} remains approximately static and the process is anoxic. Graph B shows the fermentation organic acid profile, where acetate and lactate were not present (both plots run along the base line), but formate was present, the pH dropped through the hydrogen evolution phase.

Typical of the gas blending experiments, Figures 4.29 and 4.30 shows the effect of tracking the DOT level throughout the fermentation, similar levels of organic acids are produced but a typically lower level of biomass was produced. The lower level of biomass produced during the gas-blending experiment, is counter-intuitive as previously explained but may not be statistically significant. The use of the semi defined medium led to the discovery of the essential medium additions, which reinforced the conclusions drawn from the initial batch medium (Figure 4.26), with this data it was possible to repeat the previous experiment with a new medium (Modified Super Broth, MSB) known to produce high (batch growth) cell density. To ensure that hydrogen evolution occurred MSB was further modified by the addition of the essential medium additions shown in Figures 4.31 -4.34, the parameters for the growth phase are shown in Table 4.20 and the parameters for Hydrogen evolution are as shown in Table 4.22.

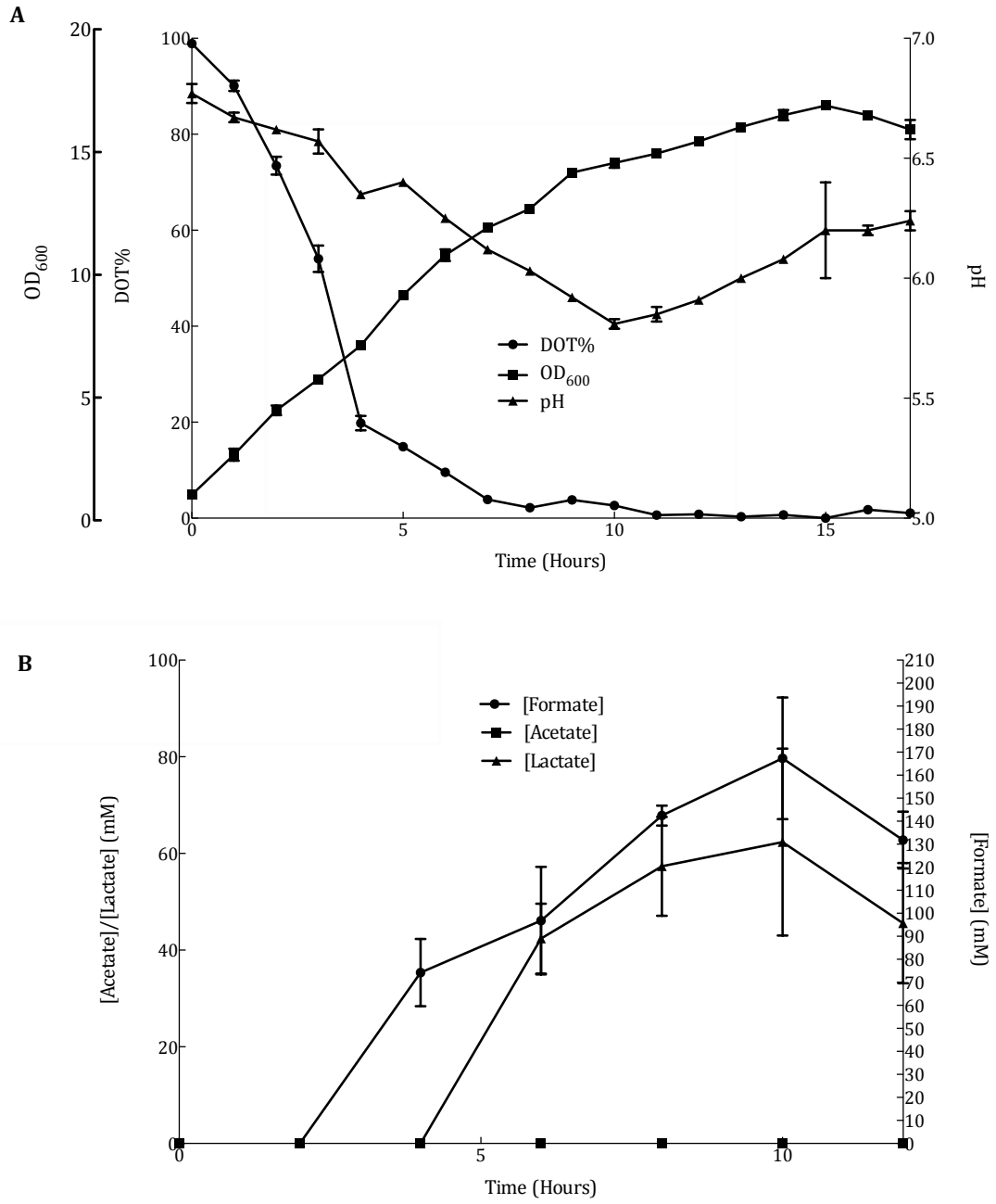


Figure 4.31 Graph showing the growth of *E. coli* HD701 in a shake flask with a medium change to MSB medium. Process parameters detailed in Table 4.20. Graph A Shows the variation of OD_{600}, pH and DOT with time, μ_{max} = 0.21 h^{-1}. Graph B shows the fermentation organic acid profile, where acetate was not accumulated, but produced in small amounts, but both formate and lactate were, increasing through the fermentation as oxygen limitation increased.

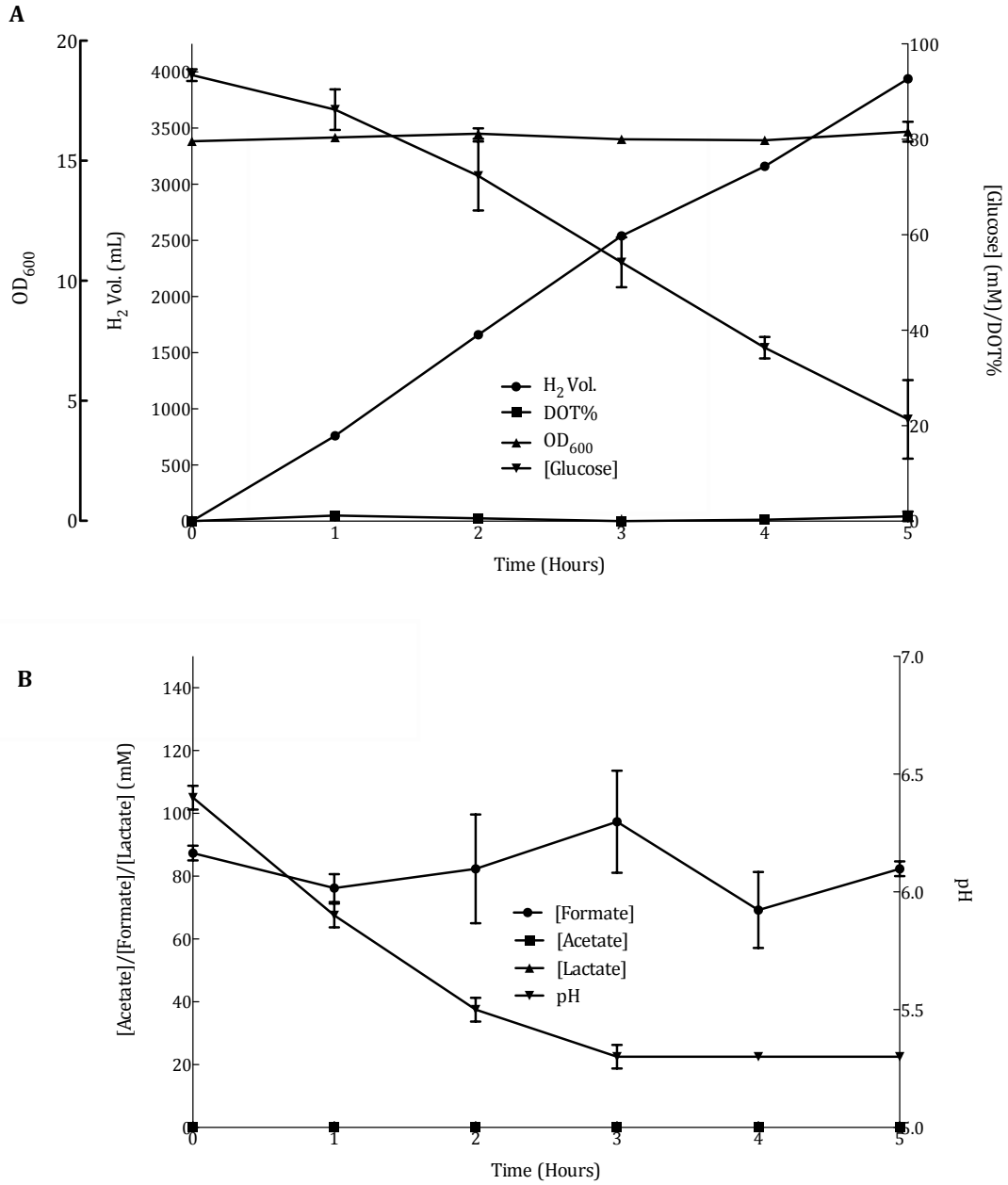


Figure 4.32 Graph showing the hydrogen production *E. coli* HD701 in a bioreactor subsequent to growth in a shake flask with MSB medium. Process parameters detailed in Table 4.22. Graph A Shows the variation of [Glucose], Hydrogen evolution, DOT and OD_{600} with time, where the utilisation of glucose mirrors the evolution rate of hydrogen, OD_{600} remains approximately static and the process is anoxic. Graph B shows the fermentation organic acid profile, where acetate and lactate were not present (both plots run along the base line), but formate was present, the pH dropped slightly through the hydrogen evolution phase but was controlled by the phosphate buffer in the medium.

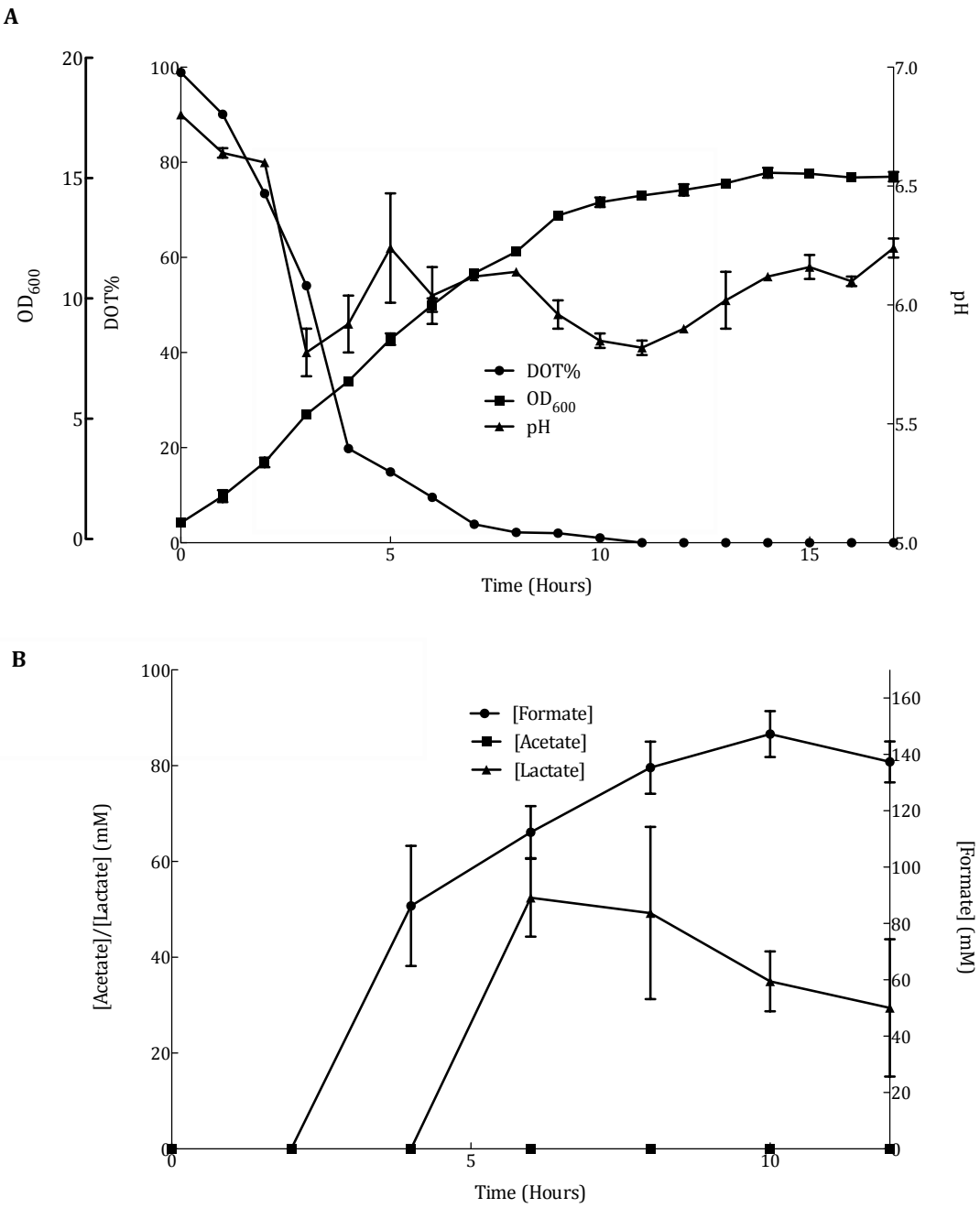


Figure 4.33 Graph showing the growth of *E. coli* HD701 in a bioreactor with a medium change to MSB medium and DOT control by gas blending Process parameters detailed in Table 4.21. Graph A Shows the variation of OD_{600}, pH and DOT with time, μ_{max} = 0.27 h^{-1}. Graph B shows the fermentation organic acid profile, where acetate was not accumulated, but produced in small amounts, but both formate and lactate were, increasing through the fermentation as oxygen limitation increased.

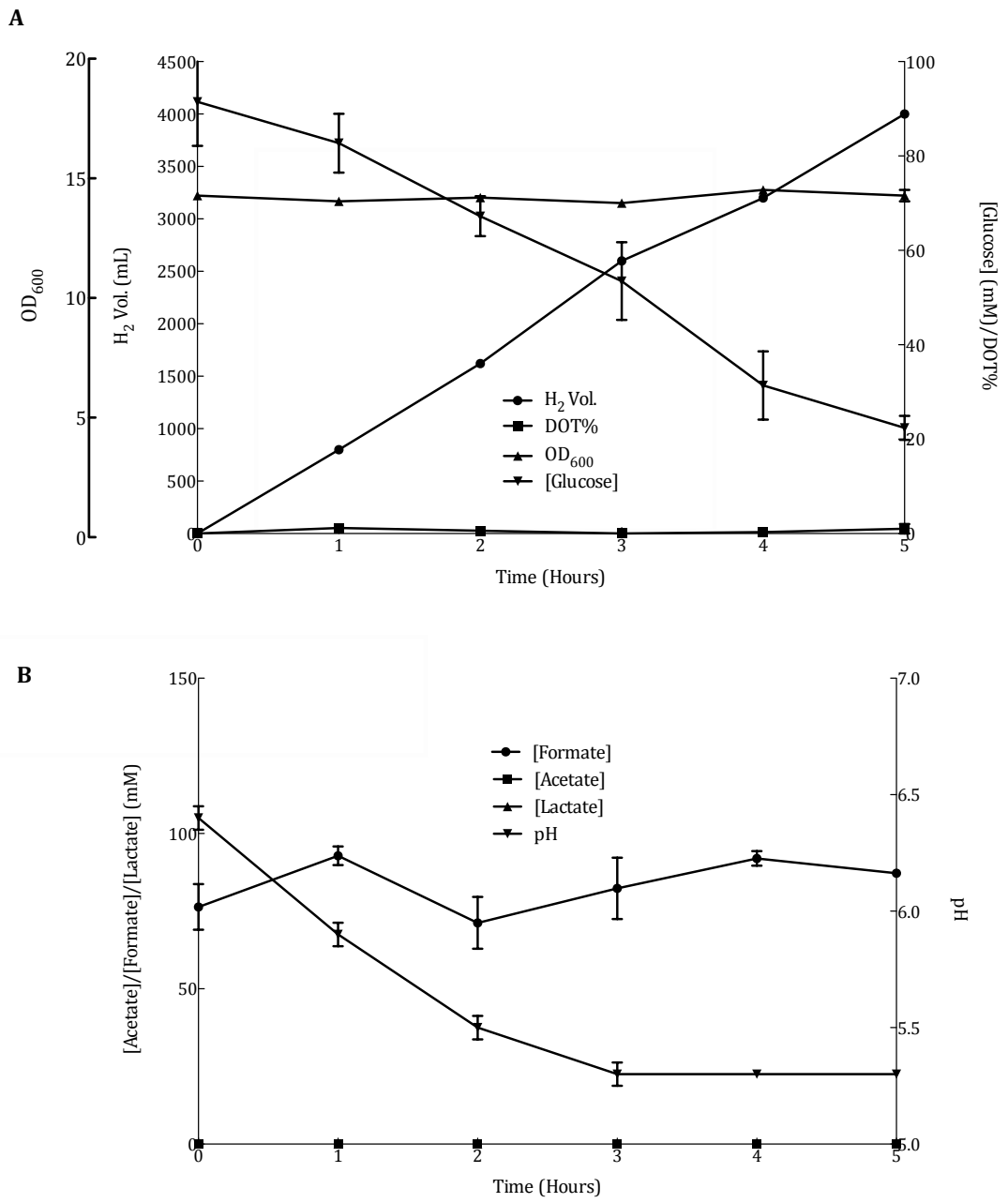


Figure 4.34 Graph showing the hydrogen production by *E. coli* HD701 in a bioreactor subsequent to growth in a bioreactor containing MSB medium with gas blending for DOT control. Process parameters detailed in Table 4.22. Graph A Shows the variation of [Glucose], Hydrogen evolution, DOT and OD_{600} with time, where the utilisation of glucose mirrors the evolution rate of hydrogen, OD_{600} remains approximately static and the process is anoxic. Graph B shows the fermentation organic acid profile, where acetate and lactate were not present (both plots run along the base line), but formate was present, the pH dropped slightly through the hydrogen evolution phase but was controlled by the phosphate buffer in the medium.

To ensure a higher possibility of success for the subsequent fed-batch experiments the MSB and TGYEP batch growth experiments were carried out in parallel. The MSB medium had a higher concentration of necessary nutrients for growth and as such the further increase in OD_{600} is expected. The increase takes the OD_{600} value to 17, which again doubled the optical density and therefore the bacterial number, there is a further non-linear increase in the rate of hydrogen evolution, (decreasing the specific hydrogen production) with a maximum hydrogen evolution rate now approximately 900 mL/hr. Once again the increase in cell density has also increased the variability of the process with respect to organic acid concentration. As is expected the gas blending experiment performs in the same way as the shake flask, with the typical deviations (slightly lower cell density).

There were now two viable candidates as a medium choice for fed-batch mode fermentation. Both media (MSB and TYGEP) had the ability to evolve hydrogen post bioreactor growth (utilising gas blending to create a similar environment to the shake flask). The hypothesis employed when making the switch to fed-batch growth was that this process change will increase the cell density within the bioreactor; a corollary of this was an assumed increase in the rate of hydrogen evolution. The physical characteristics (with respect to the DOT level within the vessel) cannot be evaluated in fed-batch culture, as there was no current working process with which to compare this process to. The second reason for making this process change was the assumption that the increase in cell density that should accompany fed-batch growth should create sufficient oxygen limitation within the bioreactor to simulate the microaerobic/anaerobic conditions during growth that were needed to produce hydrogen.

All fed batch growth was carried out using the parameters in Table 4.23. With the differences between each experiment shown in Table 4.24. During the fed-batch experiments the growth was commenced in batch mode and feeding began

when the DOT had reached its minimum (level observed in prior batch cultivations).

Table 4.23 Process parameters for Bioreactor - Fed-batch growth

Parameter	
Bioreactor size	5L
Culture volume	2 L
Agitation rate	300 rpm
Aeration rate	0.75 vvm
Inoculation size	100 mL
Medium	TGYEP / MSB
Growth temperature	30°C

Table 4.24 Feeding parameters for Bioreactor - Fed-batch growth

Experiment	Medium	Feed	Growth rate, μ
TGYEP 1	TGYEP	A	0.2
TGYEP 2	TGYEP	B	0.2
TGYEP 3	TGYEP	B	0.1
MSB 1	MSB	N/A	0.2
MSB 2	MSB	N/A	0.05

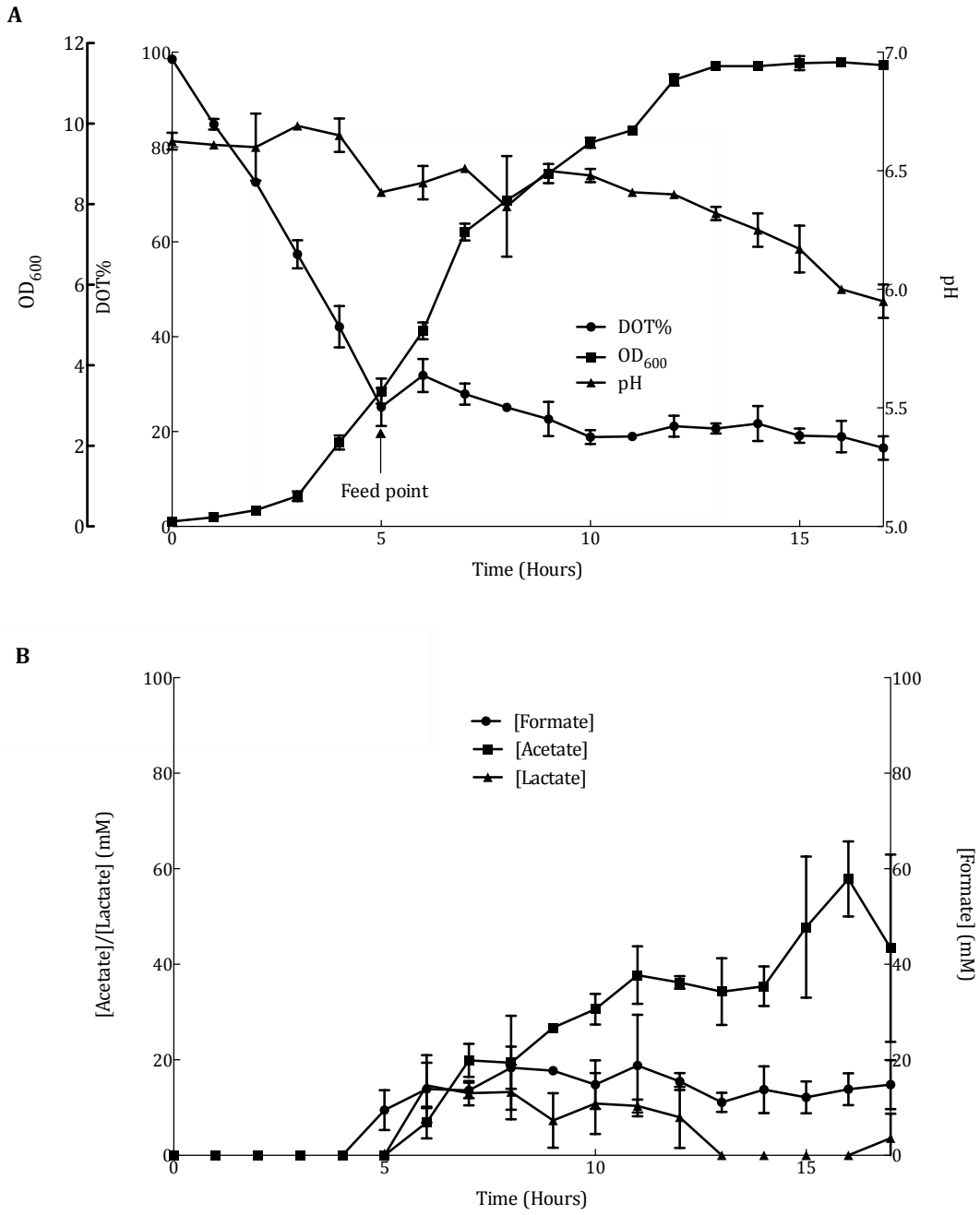


Figure 4.35 Graph showing the initial fed batch growth of *E. coli* HD701 in a bioreactor with TGYEP medium. Process parameters detailed in Table 4.23. Feeding parameters detailed in Table 4.24 (TGYEP1). Graph A Shows the variation of OD_{600}, pH and DOT with time. Graph B shows the fermentation organic acid profile, where acetate was accumulated to a comparatively high amount, but lactate and formate were produced in small amounts. The feed point is indicated with an arrow.

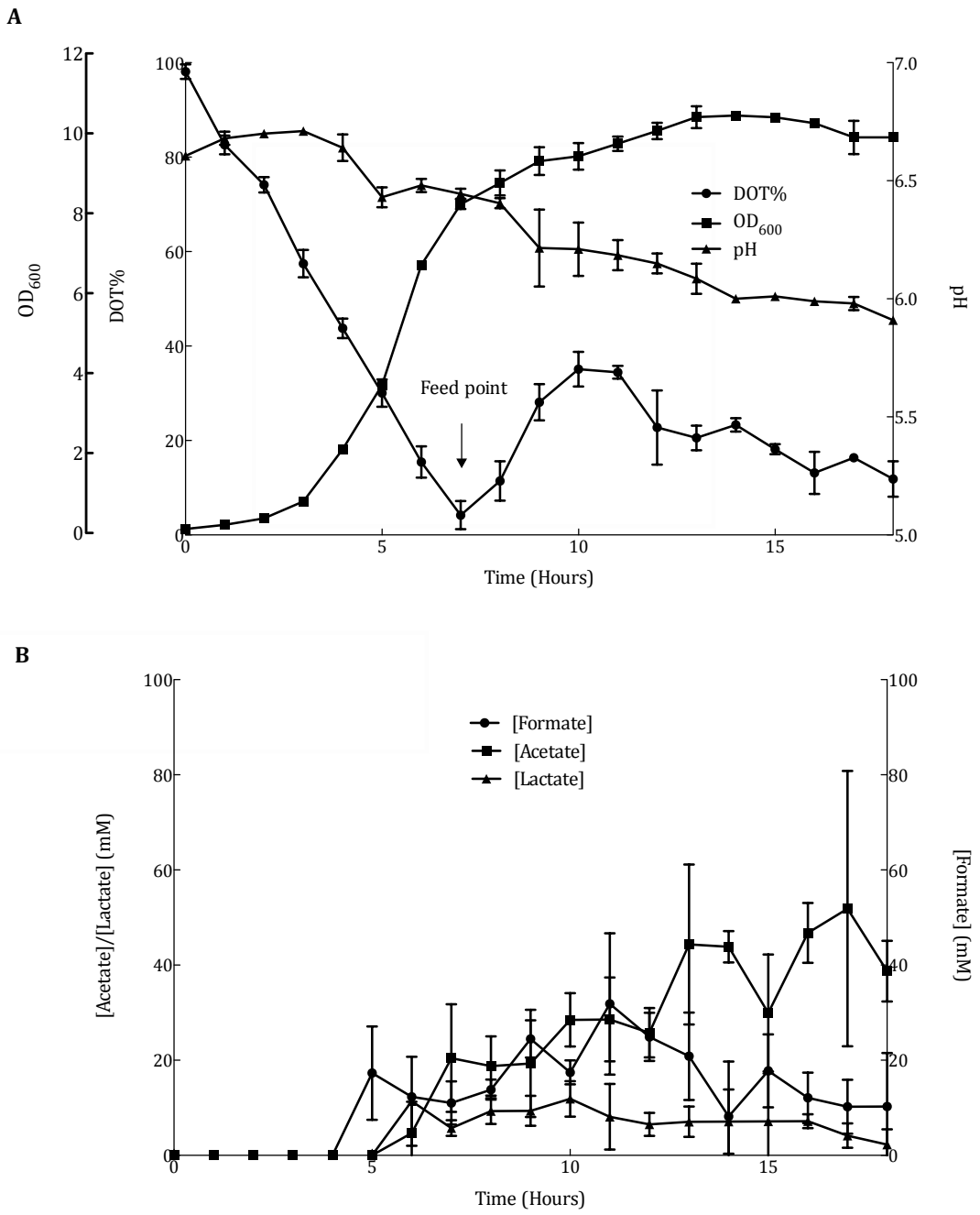


Figure 4.36 Graph showing the fed batch growth of *E. coli* HD701 in a bioreactor with TGYEP medium. Process parameters detailed in Table 4.23. Feeding parameters detailed in Table 4.24 (TGYEP2). Graph A Shows the variation of OD_{600}, pH and DOT with time, microaerobiosis does not occur. Graph B shows the fermentation organic acid profile, where acetate was accumulated to a comparatively high amount, but lactate and formate were produced in small amounts. The feed point is indicated with an arrow.

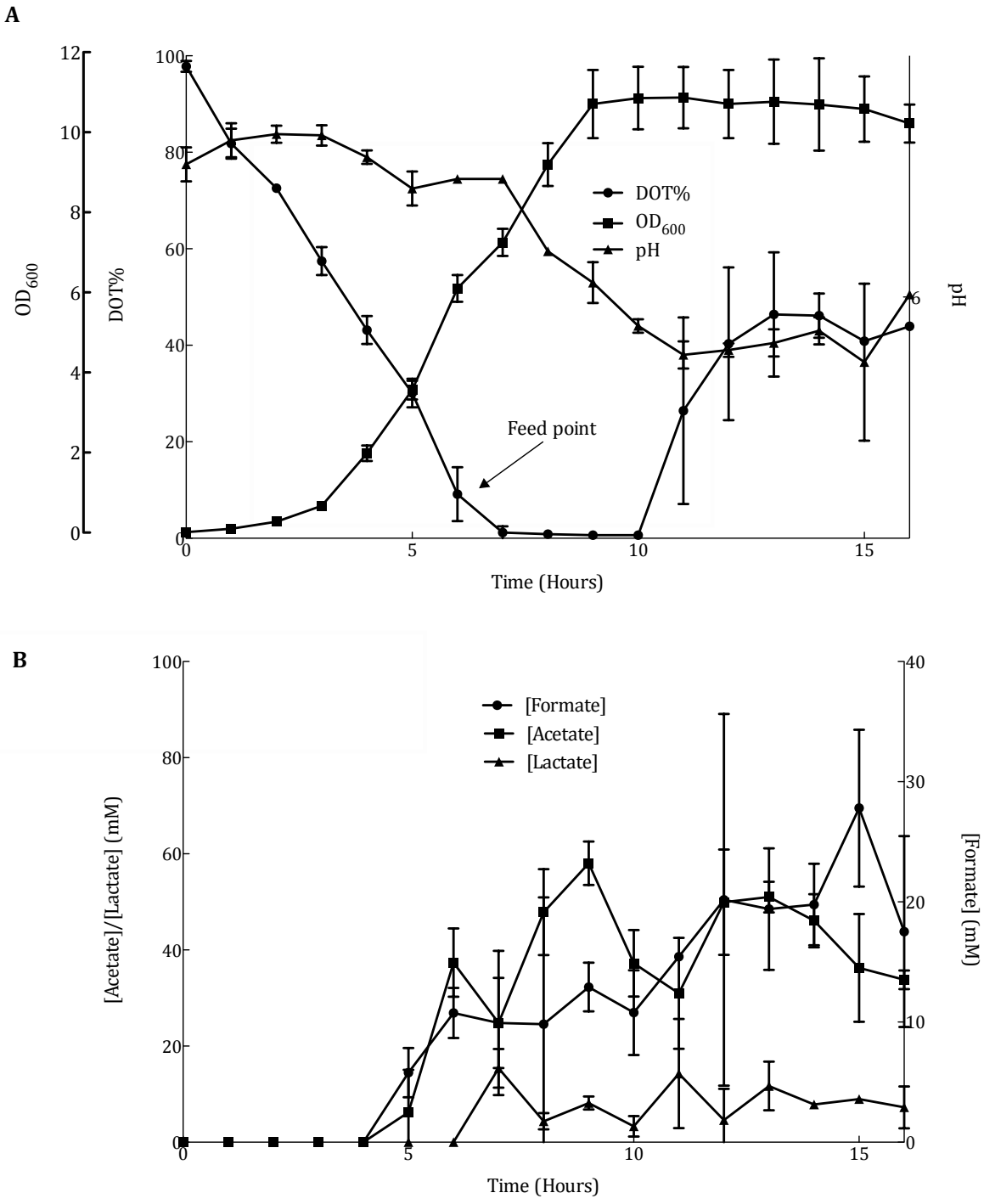


Figure 4.37 Graph showing the fed batch growth of *E. coli* HD701 in a bioreactor with TGYEP medium. Process parameters detailed in Table 4.23. Feeding parameters detailed in Table 4.24 (TGYEP3). Graph A Shows the variation of OD_{600}, pH and DOT with time, microaerobiosis does not occur. Graph B shows the fermentation organic acid profile, where acetate was accumulated to a comparatively high amount, but lactate and formate were produced in small amounts. The feed point is indicated with an arrow.

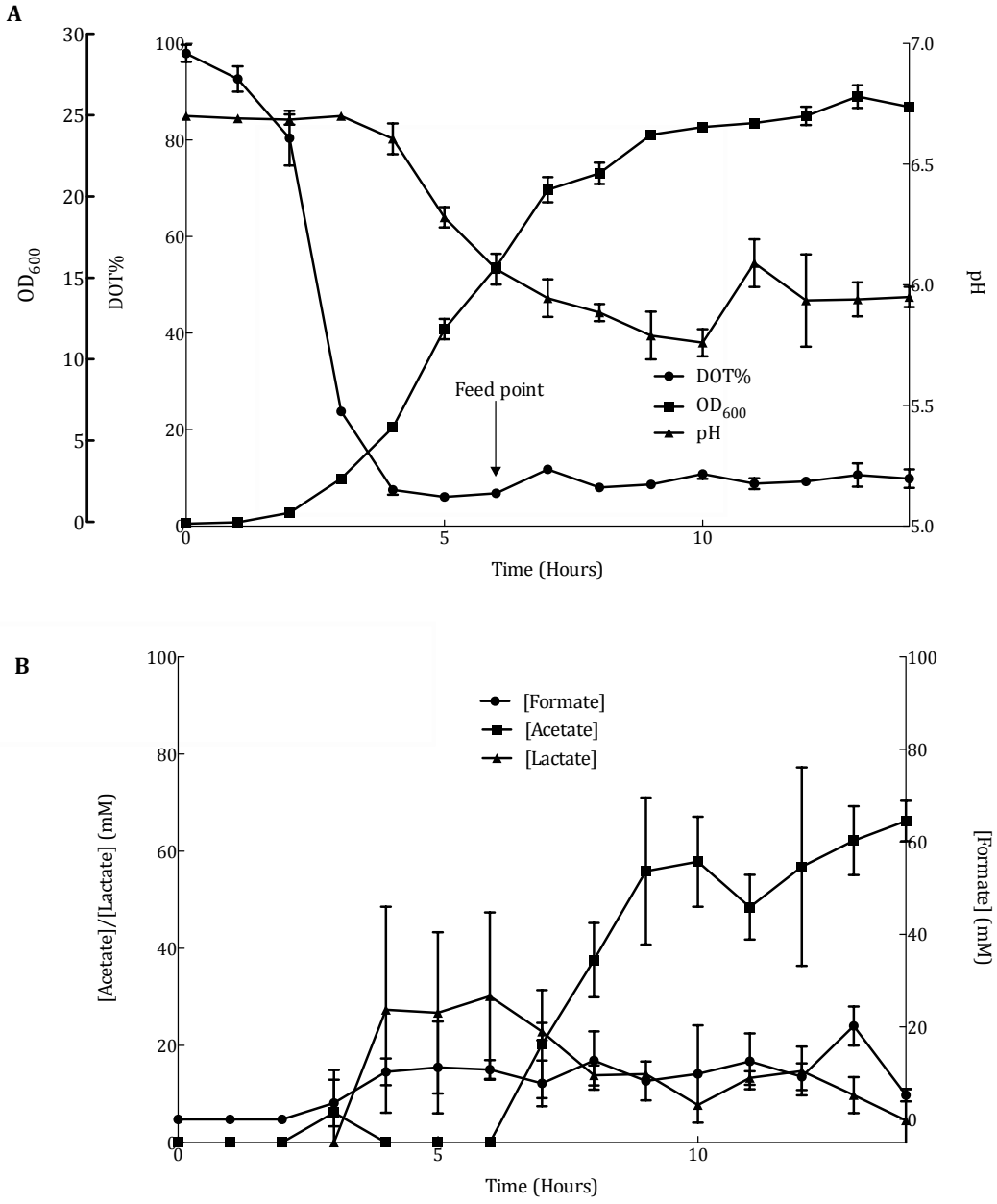


Figure 4.38 Graph showing the fed batch growth of *E. coli* HD701 in a bioreactor with MSB medium. Process parameters detailed in Table 4.23. Feeding parameters detailed in Table 4.24 (MSB1). Graph A Shows the variation of OD_{600}, pH and DOT with time, microaerobiosis does not occur. Graph B shows the fermentation organic acid profile, where acetate was accumulated to a comparatively high amount, but lactate and formate were produced in small amounts. The feed point is indicated with an arrow.

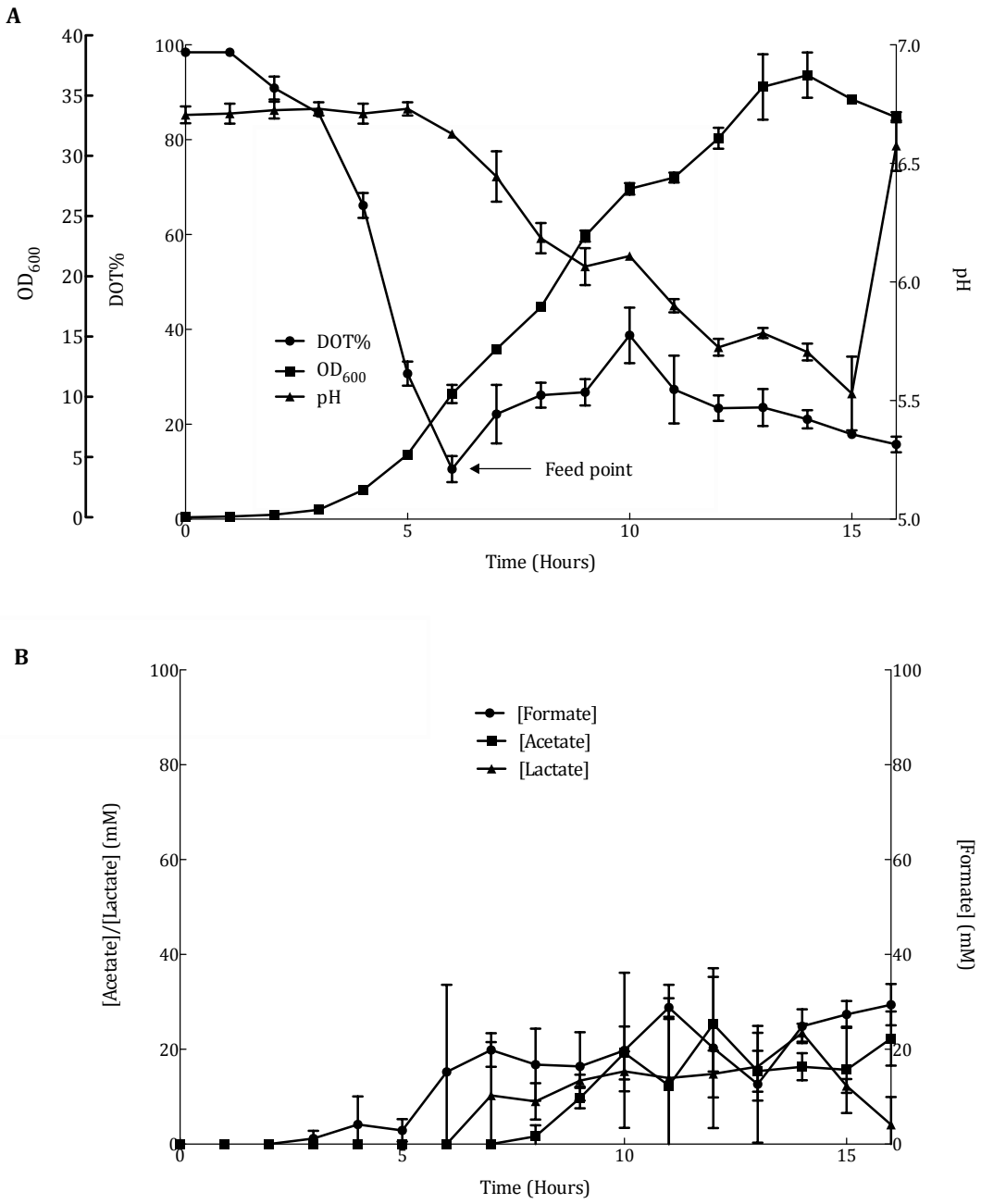


Figure 4.39 Graph showing the fed batch growth of *E. coli* HD701 in a bioreactor with MSB medium. Process parameters detailed in Table 4.23. Feeding parameters detailed in Table 4.24 (MSB2). Graph A Shows the variation of OD_{600}, pH and DOT with time, microaerobiosis does not occur. Graph B shows the fermentation organic acid profile, where acetate was accumulated to a comparatively high amount, but lactate and formate were produced in small amounts. The feed point is indicated with an arrow.

The Fed batch experiments had some commonality, the feeding regime was an exponential strategy, designed to maximise cell growth, via controlling the growth rate by limiting the carbon source, which is fed to the system at an exponentially increasing rate as discussed in Section 2.3.3. Another common theme through the results gained from these fed-batch experiments is the production of acetate, which will be discussed after the reasoning for the progression of experiments, has been explained.

As previously stated there were two different mediums assessed for the fed-batch growth experiments. Initially considering the fed-batch experiments conducted using the TGYEP medium. The initial experiment (TGYEP 1) using a growth rate of 0.2 h^{-1} as a growth rate of between 0.1 and 0.3 was recommended for effective growth without acetate production (Lee, 1996). After growth stalled in experiment "TGYEP 1", the next experiment (TGYEP 2) used the same feeding strategy as the first experiment, but the feed was changed to include a larger proportion of the component that contains a nitrogen source (yeast extract). In the experiment "TGYEP 2" growth again stalled, so the decision was taken to decrease the growth rate to 0.1 h^{-1}.
The MSB experiments were based on the assumption that the limiting factor for the previous experiments were the accumulation of acetate within the fermentation, so experiment "MSB 1" utilised a growth rate of 0.2h^{-1} whereas experiment "MSB 2" utilised a growth rate of 0.05h^{-1} with the reduction of growth rate designed to limit the production of acetate.

The set of fed-batch experiments that utilised TGYEP as a medium with a variety of feeds and controlled growth rates, were not used for further investigation, along with the experiment "MSB 1" the growth in each case was far below that which is expected from shake flask growth. The reason behind the lack of effective growth, was at first assumed to be due to a low level of the nitrogen source within the fermentation, but experimentation showed this was not the case, as increasing the nitrogen containing component of the TGYEP fed-batch medium had little effect on growth. The next assumption was that the most likely

problem with a fed-batch process was acetate accumulation. Acetate is produced during aerobic fermentation when the carbon, which enters the central metabolic pathway exceeds the cells biosynthetic and energetic needs (overflow metabolism)(Lee, 1996; Akesson *et al*., 2001). The reason that acetate production is avoided during cell growth, is that acetate can inhibit the growth rate of the bacterium (Kleman and Strohl, 1994). It is known that the cells under certain conditions can alter their metabolism to reuse the acetate, but this phenomenon was not seen here, perhaps due to experiments ending before this could take place, with acetate accumulation high enough to inhibit growth but not to trigger the "acetate switch" which would trigger the uptake and utilisation of acetate during growth (Wolfe, 2005). It is postulated that the reason for the severe acetate accumulation in this study, even at low specific growth rates, is the assumption that the specific growth rate range that should control the acetate production within normal fed-batch cultivation is not applicable for growth at sub-optimal temperatures, where the cellular biosynthetic pathways and energetic needs are very different. Due to this change in energetic/ metabolic needs fed batch cultivation of the strain used in this study at sub-optimal growth temperatures was difficult, the specific growth rate required to stop significant acetate accumulation was far lower than literature suggests (Han *et al*., 1992; Lee, 1996). Experiment "MSB 2" exhibits the optimum conditions assessed for growth, however the bacterial growth failed to reach a density at which microaerobiosis occurred. If microaerobiosis had occurred in this experiment it would have been expected to produce hydrogen, as it produced a relatively high amount of formate, and while it also produced acetate during growth it may have been produced through a different mechanism when compared to other experiments that failed to produce hydrogen and had acetate present, this is presumed due to the pH level within the fermentation being acidic which tends to divert mixed acid fermentation away from acetate being produced, so the acetate production must be a product of the over-flow metabolism associated with growth as in the previous fed-batch experiments, however in this experiment the level of acetate production was considerably lower than the previous experiments, so the level may be below that which was inhibitory to growth in this system.

The previous fed-batch experiments did not evolve hydrogen, the hydrogen phase was not fully assessed once more, as hydrogen evolution did not occur, the reason why hydrogen did not occur can not be fully concluded, but the lack of microaerobiosis in the late stages of growth imply an in ability to produce hydrogen for the reasons previously stated

It became clear that fed-batch growth would be difficult under normal parameters and as such the next step taken prior to attempting more fed-batch growth was to increase the bacterial population within the reactor by increasing the volume of cell culture. To increase the cell culture volume the NaCl was removed from the process and the volume of glucose reduced to 1L, this created a process as shown in Figure 4.40 and Table 4.25

Table 4.25 Process parameters for hydrogen production (3L Batch culture)

Parameter	
Bioreactor size	5 L
Working volume	4 L
Impeller speed	300 rpm
Substrate volume	1 L
Substrate composition	Glucose (100 mM)
Culture size	3 L

Growth was carried out in shake flasks as in the previous MSB medium shake flask based experiment in Figure 4.31.

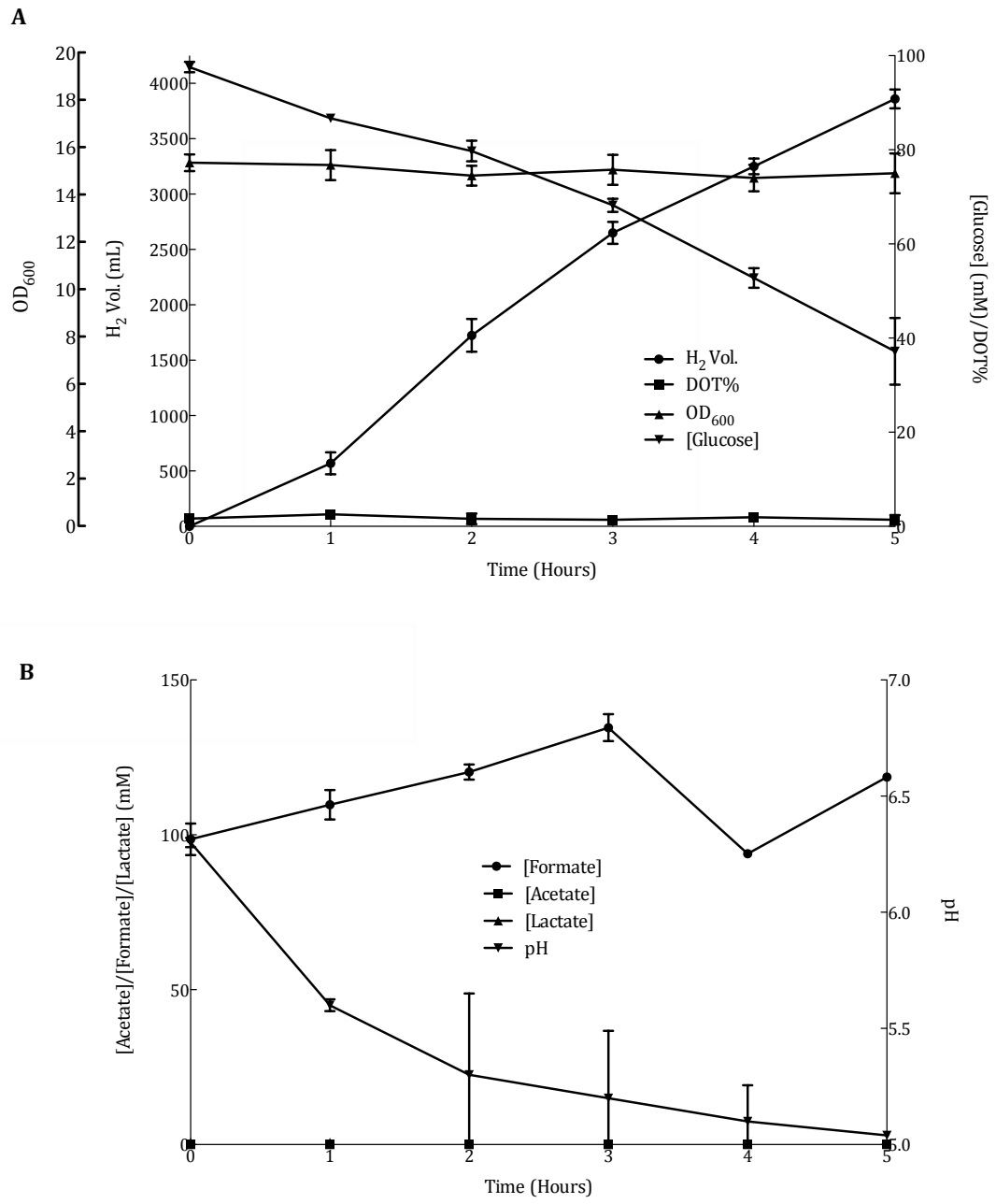


Figure 4.40 Graph showing the hydrogen production by *E. coli* HD701 in a bioreactor with an increased culture volume subsequent to growth in a shake flask with MSB medium. Process parameters detailed in Table 4.25. Graph A Shows the variation of [Glucose], Hydrogen evolution, DOT and OD_{600} with time, where the utilisation of glucose mirrors the evolution rate of hydrogen, OD_{600} remains approximately static and the process is anoxic. Graph B shows the fermentation organic acid profile, where acetate and lactate were not present (both plots run along the base line), but formate was present, the pH dropped through the hydrogen evolution phase and was not controlled by the phosphate buffer in the medium.

Figure 4.40 shows that in the process that utilises the MSB medium, there is no need for the NaCl solution to be present during hydrogen production, the reason for its use was not give in the initial study by Penfold (2004), and as such a viable method of increasing the bacterial number is to change the ratio of culture to substrate. This fact may prove useful for further experimentation, as the removal of unnecessary components is always advantageous. NaCl is known to be inhibitory to hydrogen production in anaerobes (MMFs) (Zheng *et al*., 2005), but it does have a role in bacterial metabolism, with a special function in hydrogen metabolism, Na^+ ions are involved in the transport of H^+ ions from the bacterial cell (West and Mitchell, 1974).

The final set of experiments utilised the unorthodox experimental method of gas blending for only part of the growth phase. It was postulated that if the growth was controlled by both the limiting substrate and the DOT% within the bioreactor then the later stages of the fermentation may proceed with oxygen limitation during growth, this would extend the fermentation time and allow for a potentially higher cell density to be achieved. The difficulty was in deciding how to control the DOT within the fermentation as the feeding strategy had already been chosen. The approach taken was to use gas blending to mimic the latter stages of the shake flask process, during feeding; creating an artificial DOT profile for the fermentation as shown in Figure 4.41 was the starting point for this methodology.

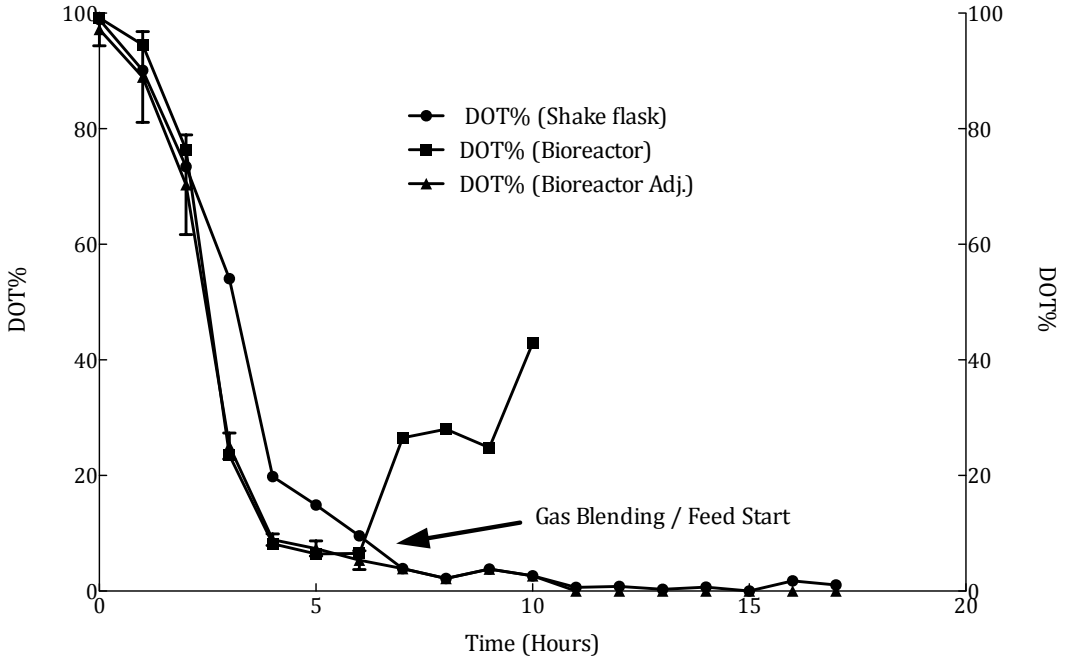


Figure 4.41 Graph showing the MSB medium DOT curves along with the augmented new DOT profile

Figure shows the Adjusted DOT% profile, as indicated on the graph feeding and gas blending were started at the same point during growth, the time point where this occurred was chosen as before (the low point in the DOT profile for normal batch growth). The DOT profile during the feeding stage was selected by matching a similar level of DOT from the shake flask growth and then using gas blending to simulate the DOT in the bioreactor.

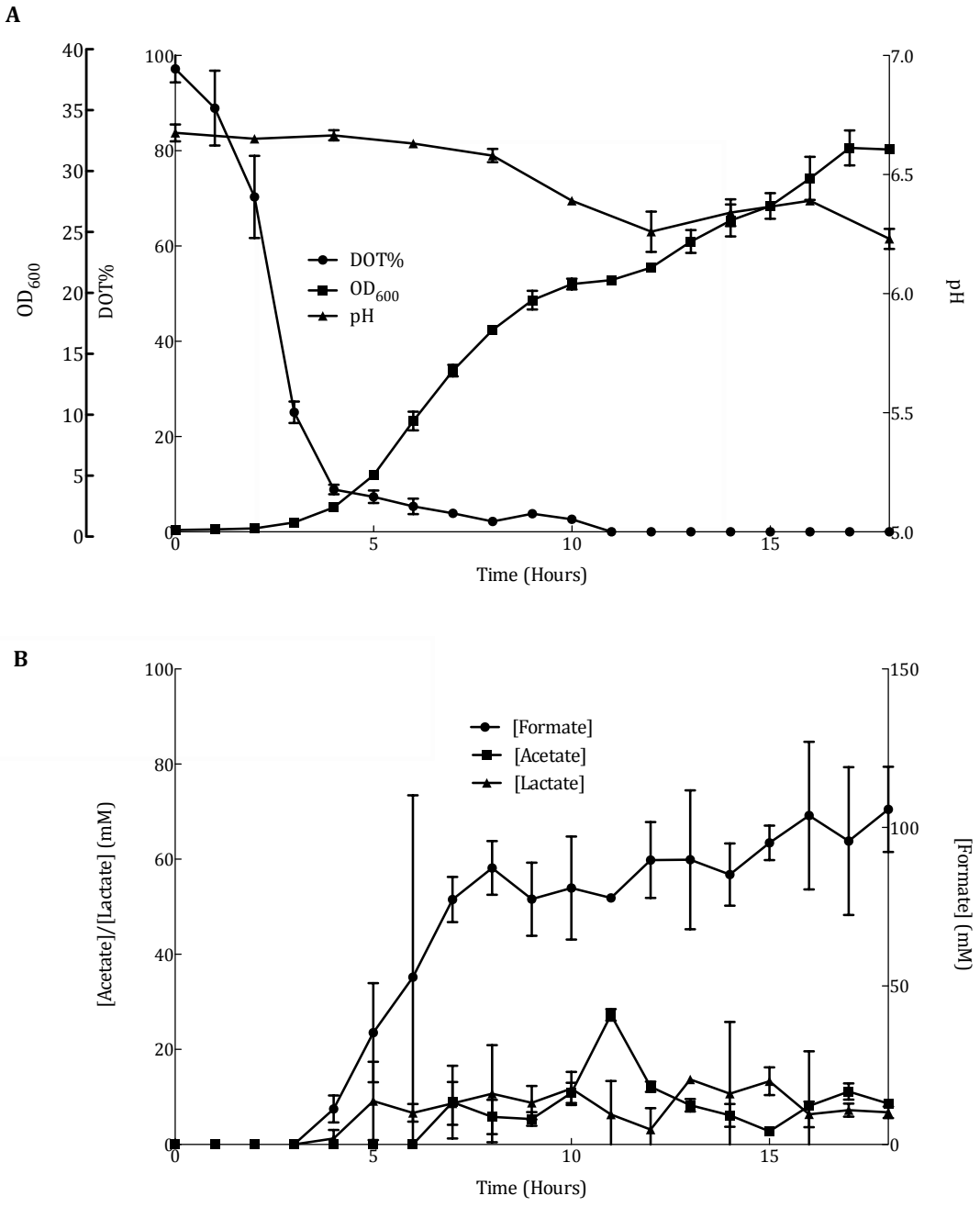


Figure 4.42 Graph showing the fed batch growth of *E. coli* HD701 in a bioreactor with MSB medium, using gas blending for DOT control. Process parameters detailed in Table 4.23. Feeding parameters detailed in Table 4.24 (MSB2). Graph A Shows the variation of OD, pH and DOT with time, microaerobiosis occurred in the latter stages of the process. Graph B shows the fermentation organic acid profile, where acetate accumulation was minimal, but lactate and formate were produced in increased amounts. The feed point is indicated with an arrow in Figure 4.41.

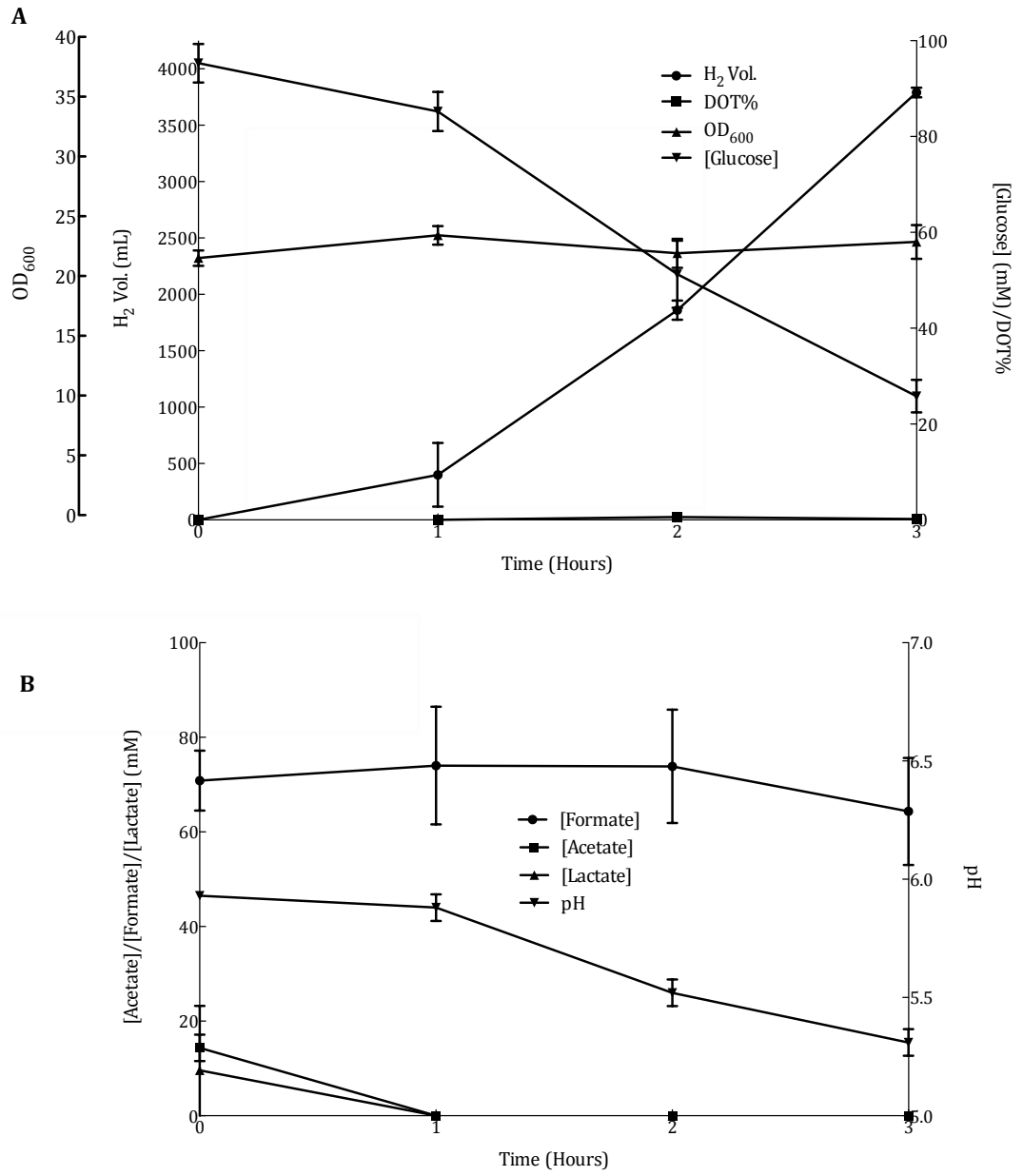


Figure 4.43 Graph showing the hydrogen production by *E. coli* HD701 in a bioreactor subsequent to fed batch growth in a bioreactor with MSB medium and DOT control by gas blending. Process parameters detailed in Table 4.22. Graph A Shows the variation of [Glucose], Hydrogen evolution, DOT and OD_{600} with time, where the utilisation of glucose mirrors the evolution rate of hydrogen, OD_{600} remains approximately static and the process is anoxic. Graph B shows the fermentation organic acid profile, where acetate and lactate were not present for the majority of the phase (both plots run along the base line), but formate was present, the pH dropped through the hydrogen evolution phase and was not controlled by the phosphate buffer in the medium.

The final experiment utilised a mixture of gas-blending and fed-batch cultivation to achieve an OD_{600}= 33 and this final increase in cell density created process that evolved 1800ml/hr, which represented an nine fold increase when compared to the original shake flask process.

4.4 Summary

To summarise the results found in this study, non-linear regression of the results gained was carried out, utilising the Michaelis-Menten model for enzyme kinetics. This was carried out using cell number as an analog for enzyme concentration and hydrogen production rate an analog for reaction rate, an ability to do this would be useful as it would help predict the maximum reaction rate and suitable enzyme concentrations to operate at, however the correlation did not show any useful data (Shown in Appendix 5). The inability to use this method to predict information for potential future work is presumably due to the ways in which the reaction does not fit the Michaelis-Menten model (not able to use initial reaction rate, the enzyme-substrate complex concentration unlikely and enzyme concentration being constant) and the assumptions made such as cell number being an analog for enzyme concentration not being valid. Without this technique it is still possible to summarise the results found in this study and compare them as below in Table 4.26

Table 4.26 Summary of hydrogen production experiments

Experiment	OD_{600}	Max. Hydrogen evolution rate (mlh^{-1})	Specific Hydrogen production rate ($mLg^{-1}h^{-1}$)	Yield coefficient (mol/mol)	Process conversion efficiency (%)
Initial process	2.1	200	120	0.9	120
Nutrient broth[2] gas blending	1.9	200	130	0.8	120
Double strength nutrient broth[2]	3.9	700	220	1.8	430
No aeration	0.9	90	120	0.2	55
TGYEP medium	4.2	800	240	1.8	490
MSB Medium	15.2	900	70	1.9	550
MSB Fed batch	21	1800	100	1.9	1100

This shows that the process intensification had both improved the hydrogen yield to 95% of its theoretical maximum (yield=2) as well it evolving eleven times the hydrogen needed to run the process (calculation shown in Appendix A.2.1.2) at the maximum hydrogen evolution rate (gas composition verified using mass spectroscopy).

The disparity between the increase in the cell density and the increase in hydrogen evolution could be down to the medium supplementation. The level of supplementation was consistent however the media used varied in the cell density they produced, further work (see Chapter 6) could be carried out to see if the hydrogen evolution rate can be further improved.

5.Conclusion

The first conclusion drawn from this study, is the inability for any whole cell process to achieve 100% yields (with respect to substrate) if the bioconversion taking place is one which utilises the cells normal metabolic processes or if the substrate is able to be utilised by the cell for these processes. This is due to the inherent energy required by the bacterium for stasis survival and metabolic processes known as the maintenance energy. While this yield [product yield with respect to substrate] is of importance, a potentially more important factor with respect to an energy producing process, is the overall process efficiency, which relates the amount of energy used by the process to the amount of energy that is produced by the process. The summary of the main results show that process intensification has occurred, increasing the yield to 95% of its theoretical maximum, increasing the hydrogen evolution rate 9-fold and producing 1100% of the energy required to run the process.

While these general conclusions show the overall picture that can be drawn from this study, more specific conclusions can be made, by relating the findings from this study to the initial aims

5.1 Fully characterised and replicated the initial shake flask-growth orientated batch process

The initial process was fully characterised, the organic acid profile along with the pH profile gained from this process gave an insight into the process. For hydrogen evolution to occur, formate had to be accumulated in the medium, also acidification of the medium was necessary, so as not to preferentially produce acetate. The later stages of the growth phase was found to proceed with

microaerobiosis, which in turn was also found to be integral to hydrogen production.

5.2 Defined conditions under which the process can occur in one vessel (bioreactor).

Experimental procedure led to the discovery of the importance of the DOT profile within the process. This in turn led to the application of Gas blending to the process. Gas blending was used to allow the microaerobiosis (assumed, as anaerobiosis may have an increased effect on the cell growth) that is required within the reactor for a high level of mixed acid fermentation to occur during cell growth, a necessary precursor to hydrogen production. This study used the novel technique of utilising gas blending to track a DOT profile, where it is usually used to ensure the DOT does not fall below a set point. Gas blending gave the ability to bypass the potentially difficult recreation of the physical parameters found within the shake flask in the bioreactor.

5.3 Identified a suitable medium for an increase in cell density through batch growth

Experimental results showed that the medium used for growth was important with respect to hydrogen production. The use of a complex medium in the initial process made it difficult to determine what the necessary medium components were, however literature showed that the medium supplementation must take place. The necessity for nickel, molybdenum and selenium within the medium is documented in the literature and was essential for hydrogen production (it is assumed that the Nutrient broth[#2] medium contained it in one of its

components). The metals added form the metallic centres of some of the essential enzymes utilised in hydrogen production.

5.4 Shown that the increase in cell density increases the volumetric production of hydrogen

The study showed that an effective way to intensify the process was to increase the cell density. When considering the original process, the results showed a non-linear proportionality between increasing volumetric hydrogen production and increasing cell density. The increase in cell density also had the effect of increasing yield.

5.5 Identified a suitable medium for fed-batch cultivation

The medium supplementation that was deemed necessary in batch cultivation was kept in this case. The media chosen [TGYEP and MSB] were suitable as they contained separate carbon sources that could be fed to the culture during growth. The importance of supplementation was noted and found to be more important than overall medium choice.

5.6 Identified process conditions where fed-batch cultivation can lead to hydrogen production

Achieving an appreciable increase in cell density using fed-batch culture proved difficult. Controlling the specific rate at a level at which acetate would not accumulate was the main hurdle to overcome. A specific growth rate of $0.05h^{-1}$

proved to be the most effective, but this had to be linked to gas blending to induce microaerobiosis in the fermentation as is found in the batch process. The decision was made to utilise gas blending within this mode of growth. This novel step was used in conjunction with feeding to allow the decrease of the DOT alongside continued cell growth, till microaerobiosis occurred. The use of gas blending for only par of the growth phase further shows that it is the transition point from aerobic to microaerobic growth that is important for effective hydrogen production, as this point was unknown for fed batch growth, a pseudo-DOT profile was constructed by the effective mapping of a batch DOT profile to the end of the most successful fed batch DOT profile.

6. Future work

As with many studies of this nature possible future work is aimed at both understanding the process further and its improvement.

6.1 Growth temperature

One of the main hurdles to overcome whilst intensifying this process was the difficulty in performing fed-batch cultivation (as shown in Section 4.3). That difficulty was postulated to be due to the low operating temperature (30 °C) of the process slowing the growth to a point that the addition of a glucose feed (at a rate suggested by literature) caused acetate production through overflow metabolism. The process, initially developed by Penfold (2004), may or may not require this temperature in order to be productive (the effects of temperature change on cell physiology and morphology are shown in Section 2.3.7). However, the reason for the choice was not stated by Penfold (2004). The use of an optimal temperature for growth (possibly a value closer to the optimal value for *E. coli*, 37 °C) could potentially alleviate the problem of slow growth and could also lead to a system/process that does not require gas blending during fed batch growth. This possible effect would be due to an increase in cell density increasing the oxygen demand leading to the required microaerobiosis. However, it is likely that some control of dissolved oxygen tension would still be essential, to create the correct balance between oxygen supply and utilisation.

Due to the potential process simplifications that could be afforded through growth at optimal temperatures, evaluating the necessity for growth at suboptimal temperatures should be the next study performed in any future work.

6.2 Strain

There are many other viable strains that could have been employed. A strain developed in a study on "Metabolic engineering to improve hydrogen production" by Maeda *et al*., (2007b), was shown to improve hydrogen evolution by 141 times. These workers took the route of fully engineering the metabolism, mutating uptake hydrogenases, directing metabolism to formate, by further deletions and deleting the inhibitory control mechanisms for FHL (Maeda *et al*., 2007b).

There are also strains that are derived from the strain [*E. coli* HD701] used in the present study. The further alteration that they have undergone is the mutation of the gene that encodes for the uptake hydrogenases (Hyd1 and Hyd2). These hydrogenases have different actions; it has been shown (Lukey *et al*., 2009) that Hyd1 functions in oxidative environments, cannot function in both directions and is also tolerant to oxygen, whereas Hyd2 functions in more reducing environments. The series of mutants that have been created are listed in Table 6.1

Table 6.1 The Other potential biohydrogen candidates and their mutations

Strain	Mutation
E. coli FTD 89	Hyd1 and Hyd2 deficient
E. coli FTD 67	Hyd2 deficient
E. coli FTD 22	Hyd1 deficient

The effect that these mutations have should be evaluated in the bioreactor before being assessed for process intensification potential. A previous study has shown the effect that the mutations have on hydrogen production (Redwood *et al*., 2008) and the fact that the hydrogen production process is conducted in an anaerobic environment suggests the mutation on Hyd1 will have a limited effect, whereas the Hyd2 deficient strain will increase the production of hydrogen. The use of the further engineered strains could further improve the process.

6.3 Growth medium/feeding strategy

While the medium choice and feeding strategy were successful, further improvements need to be made. If it is found that a decreased growth temperature is essential (evaluated following the work suggested in Section 6.1), then a study into the effect of this temperature on the specific growth rate needed to avoid acetate accumulation in fed-batch cultivation would be both beneficial to this process and to any process which operates at suboptimal growth temperatures (and require fed batch cultivation). When considering the growth medium, the level and timing of supplementation (the supplementation of the medium are discussed in Section 2.3.6) need to be considered, as these could have played a part in the non-linearity of the relationship between cell density and hydrogen evolution rate. There may also be an economic evaluation of a move from complex to a more defined medium or the substitution of potentially expensive components with cheaper ones. The semi-defined "batch medium" should also be assessed to see if hydrogen production will occur when the medium has had the necessary supplementation.

As previously mentioned the level of supplementation should also be investigated. The level used in the study was suggested for the TGYEP medium, presumably in excess but when the medium was changed in batch mode the supplementation amount remained unchanged. This could have been the reason for the non-linear increase in hydrogen production with respect to cell number. This hypothesis is based on the necessity of the metals found in the medium supplementation for the formation of the enzymes and complexes that are used in anaerobic metabolism. Adding these substances in limiting amounts will have a detrimental effect on the production of active enzyme.

The transition point from aerobic to anaerobic growth may have an effect on hydrogen production; also if the transition point can occur later in growth,

higher bacterial cell numbers may be achieved and as such this should be investigated.

The necessity of gas blending can also be evaluated. While gas blending was effective in this study, its use may not be essential to hydrogen production within the bioreactor. If gas blending proves to be too difficult to achieve at large scale, then the need for the knowledge of physical parameters that allow hydrogen evolution to occur would be enhanced.

6.4 Substrate concentration and feeding/Bioconversion optimisation

Although the process did not follow Michaelis-Menten type enzyme kinetics (see Appendix 5), it is still essentially an enzymatic conversion and as such there will be an optimum substrate concentration for any given enzyme [in this case whole cell] concentration. Knowledge of this parameter would potentially further improve the process. Another potential process improvement would be to move from batch conversion to a continuous process. This would enhance the process, decreasing the portion of the process that is dedicated to cell growth. It would have its own engineering problems associated with it however as there is a finite space within the reactor so feeding could not be infinite unless there is constant removal of broth, this could be partially resolved with the use of a highly concentrated feed.

6.5 Substrate variation

Glucose is an ideal substrate for the biological production of hydrogen, but more complex substrates could be utilised. It is known that glucose containing

confectionary (nougat) waste was suitable for the conversion. With waste utilisation being the ideal another possibility would be the use of glycerol which can also be metabolised to pyruvate and then enter the mixed acid fermentation route under the correct physical conditions, glycerol would be a good candidate (Ito *et al*., 2005) due to both its place in the *E. coli* metabolism (Durnin *et al*., 2009) and the fact that it is a by-product of biodiesel production (Ma *et al*., 1999). Although glycerol would constitute another waste source, it may require pre-treatment as it often contains salts that are residual from the base catalysed biodiesel production process (Ito *et al*., 2005). Other renewable resources (apart from those which would supply unrefined sugar to the process) would potentially require further genetic modification to enable the cell to convert other substrates to hydrogen.

6.6 Analytical methods, experimental techniques and data manipulation

Further experimental verification could have been carried out and should be done in any future work. The dynamic gassing out method used for determining K_La should be employed to further characterise the physical parameters within the process and to see if other forms of dissolved oxygen control than gas blending would be possible.

The use of more than one technique for data verification is recommended along with the use of techniques based on their sensitivity to the substance/metabolite of interest.

Firstly, linked to Section 6.4, the use of enzyme kinetic plots to predict process performance at differing conditions would be beneficial. As previously mentioned (Section 6.4) the use of cell density as an analogue enzyme concentration is not acceptable (due to the potential variability in enzyme concentration per cell) and the fact that the system employs a whole cell bioconversion. Therefore protein quantification and activity measurements

should be used to fully explain the enzyme kinetics of the system. This would also be beneficial in relation to section 6.5 allowing the relationship between the level of medium supplementation and the amount of active enzyme to be found.

The evaluation of the enzyme kinetics will only be useful for the final metabolic step in the process (using FHL) and further to this metabolic flux analysis should be carried out between pyruvate and the end points of anaerobic glycolysis. This information would identify bottlenecks with in the metabolic pathway and give an insight in to the rate and amount of any other potential product formation

Appendix 1

A.1 The 12 principles of green chemistry

- It is better to prevent waste than to treat or clean up waste after it is formed.
- Synthetic methods should be designed to maximize the incorporation of all materials used in the process into the final product.
- Wherever practicable, synthetic methodologies should be designed to use and generate substances that possess little or no toxicity to human health and the environment.
- Chemical products should be designed to preserve efficacy of function while reducing toxicity.
- The use of auxiliary substances (e.g., solvents, separation agents, and so forth) should be made unnecessary wherever possible and innocuous when used.
- Energy requirements should be recognized for their environmental and economic impacts and should be minimized. Synthetic methods should be conducted at ambient temperature and pressure.
- A raw material or feedstock should be renewable rather than depleting wherever technically and economically practicable.
- Unnecessary derivatization (blocking group, protection/de-protection, temporary modification of physical/chemical processes) should be avoided whenever possible.
- Catalytic reagents (as selective as possible) are superior to stoichiometric reagents.
- Chemical products should be designed so that at the end of their function they do not persist in the environment and break down into innocuous degradation products.

- Analytical methodologies need to be developed further to allow for real-time in-process monitoring and control before the formation of hazardous substances.
- Substances and the form of a substance used in a chemical process should be chosen so as to minimize the potential for chemical accidents, including releases, explosions, and fires.

Appendix 2

A.2 The hydrogen economy

There are many facets to the hydrogen economy apart from hydrogen production (discussed in Section 2.1). The following section discusses the use of fuel cells, the different types of fuel cell and how their efficiency effects the results of this study.

In 1997 the Kyoto protocol was created under the UNFCCC (United Nations Framework Convention on Climate Change), the protocol came into force in 2005 and essentially commits all countries that ratify the protocol to reducing the emission of gaseous substances that contribute to climate change (out of over 170 countries the US of A and Kazakhstan are the only 2 protocol signatories that have not ratified the protocol)(Dhir, 2008). The increasing public and consequentially political awareness of the problems of climate change is the social driver for the research into clean and renewable energy the a potential loss of petrochemical resources is an economical driver, the combination of these factors have led to research into potential energy resources (Wind, Solar, Tidal/Wave/Hydro, Biomass, Geothermal, Fuel cells and Waste to energy technology) (Dhir, 2008, Gehl *et al*., 2005).

The Hydrogen economy is fundamentally linked to fuel cells as they are predominantly powered by hydrogen (some are also able to utilise hydrocarbons). The "Hydrogen Economy" is centred on the transportation, production, delivery and storage of hydrogen. The research into producing, transporting, delivering and storing the lightweight, explosive gas is outlined in the following sections.

A.2.1 Fuel cells

A fuel cell is essentially an electrochemical cell, where the reactants can continually be replenished as they are consumed and the electrodes are unaffected by the reaction (Larminie *et al*., 2000). Research into Fuel cell technology has been in the public eye recently but the technology has been around for many decades, being the electrical cell of choice for the American space race in the late 1950s. However, William Grove first demonstrated Fuel cells in 1839(Larminie *et al*., 2000). Fuel cells fall into the category of clean power generation as they only produce energy and water. They Utilise electrochemical reactions involving either methane or hydrogen to generate power, the specific reaction or fuel is dependant on the fuel cell utilised.

There are primarily 5 different types of Fuel cell:

Alkaline Fuel Cell (AFC)

Proton Exchange Membrane [Polymer Electrolyte] Fuel Cell (PEMFC)

Phosphoric Acid Fuel Cell (PAFC)

Molten Carbonate Fuel Cell (MCFC)

Solid Oxide Fuel Cell (SOFC)

All fuel cells work in approximately the same way as illustrated by Figure A.2.1, they derive their names from the electrolyte employed and their specific differences and applications are also outlined (JHHirschenhofer *et al*., 2006, Dhir, 2008).

Table A.1 Fuel cells and their applications (Larminie *et al*., 2000)

Fuel Cell	Application
AFC	Transportation, Domestic CHP 1-100KW
PEMFC	Portable electronics, Transportation, Domestic CHP 1W-100KW
PAFC	Transportation, Domestic CHP , National grid supply 10KW-1MW
MCFC	Transportation, National grid supply 100KW- 10MW
SOFC	Transportation, National grid supply, Domestic CHP 1KW – 10MW

Table A.2.2 Fuel cell operating temperatures and electrolyte (Larminie *et al.*, 2000)

Fuel Cell	Mobile Ion	Electrolyte	Operating Temperature (°c)
AFC	OH^-	Alkali (aq)	50-200
PEMFC	H^+	Polymer mebrane	30-100
PAFC	H^+	Phosphoric acid (aq)	220
MCFC	CO_3^{2-}	Molten Carbonate (Li, Na & K)	650
SOFC	O^{2-}	Ceramic	500-1000

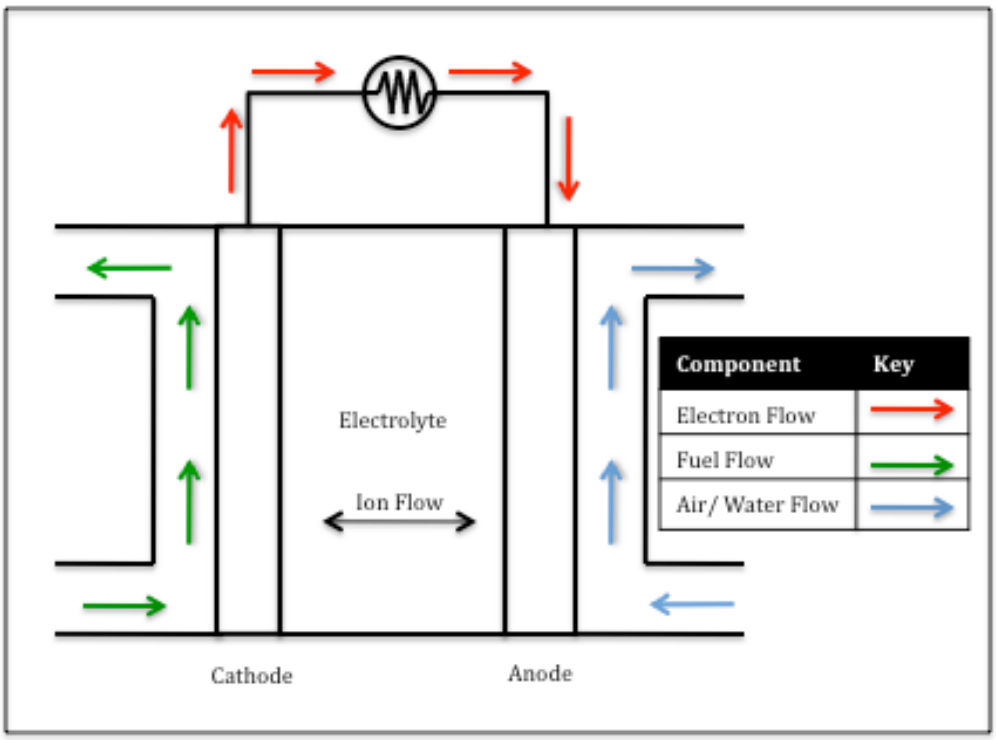


Figure A.2.1 General Fuel Cell Schematic (JHHirschenhofer et al., 2006, Dhir, 2008)

A.2.1.1 Proton Exchange Membrane Fuel Cell (PEMFC)

Due to the nature of the process involved in this study, on the action of the PEMFC will be assessed due to its ambient operating conditions lending it self

well to being applied to electrical energy production in conjunction with the process investigated. PEMFCs were used on the first manned spacecraft, and address the problem of slow reaction rates found in the AFCs (Larminie *et al.*, 2000). As the name suggests, the electrolyte employed is a proton exchange membrane, solid polymers are used for this function e.g. Nafion®, which also give rise to there alternative name – Polymer electrolyte fuel cells. The cell reactions are as follows:

Anode: $2H_2 \rightarrow 4H^+ + 4e^-$

Cathode: $O_2 + 4H^+ + 4e^- \rightarrow 2H_2O$

Overall: $2H_2 + O_2 \rightarrow 2H_2O$

So at the anode diatomic hydrogen is split to Hydrogen ions and electrons are released to the external circuit and at the cathode, oxygen is combined with hydrogen ions to form water. PEMFC's have carbon supported platinum electrodes and also require pure hydrogen and oxygen. Another form of the PEMFC is the Direct methanol fuel cell (DMFC)(Larminie *et al*., 2000), which uses methanol as a fuel, which changes the cell reaction to:

Anode: $2CH_3OH + 2H_2O \rightarrow 12H^+ + 2CO_2 + 4e^-$

Cathode: $3O_2 + 12H^+ + 12e^- \rightarrow 6H_2O$

Overall: $2CH_3OH + 3O_2 \rightarrow 2CO_2 + 4H_2O$

So the difference between the two is at the anode methanol and water combine to make hydrogen ions, carbon dioxide with the electrons being released to the external circuit (Larminie *et al.*, 2000).

A.2.1.2 Energy efficiency of biohydrogen production process combined with a PEMFC

As with any technology, the method and standpoint at which you evaluate them can give differing results. It is for this reason, when considering process metrics there are many different choices that could be made. As the process in this study is essentially a energy production process

The way in processes are measured form a body of research in itself. As with anything that can be measured in more than one way, differing methods of process assessment will give differing conclusions. It can be said that differing process metrics have differing value depending on the process, however it is probable that to fully compare differing processes more than one method will be needed.

With the potential techniques for measuring the process shown above, it is clear that these are more beneficial for chemical production processes, and with the potential for the process to be a localised energy producer and the utilisation of waste as a substrate, LCA may be skewed if conducted. However if the process is simplified it is an energy production system and it therefore should produce more energy than it uses. To be able to quantify these amounts further simplifications were made.

As has been shown in previous sections, Fuel cells essentially work by utilizing electrochemical reactions, at opposing electrodes. The overall cell reaction is the formation of water from hydrogen and oxygen gas. Although the half-cell reactions are electrochemical conversions they still undergo the same kinetic constraints as other reactions, this is why catalysts are employed (to speed up the reaction and reduce the activation energy of the reaction).
With any power generation technology, the issue of the efficiency of the technology is questioned. Unlike mechanical power generation or other methods such as combustion where the energy input is clear and therefore efficiency is easily calculated by comparing it with energy output, the efficiency of a fuel cell is more complicated, it is for this reason that the efficiency of the fuel cell is defined using the 'Gibbs free energy' of the system. Gibbs free energy is defined as the energy available to do external work in a system and it neglects any work done by pressure and/or volume changes. In a fuel cell the external work is the work-done in moving electrons around the circuit. The use of the Gibbs free energy, for efficiency calculations also allows us to set a more convenient base point, this is because the important factor is the change in the Gibbs free energy

ΔG, from the Gibbs free energy for the formation of the reactants to the Gibbs free energy for the formation of the product (Larminie *et al*., 2000)

$$\Delta G_{formation} = G_{formation-prods} - G_{formation-reacts}$$

And if we define the point of zero energy to be the Gibbs free energy associated with the formation of reactants at standard temperature and pressure (25c, 0.1Mpa)(Larminie *et al*., 2000).

$$\Delta G_{formation} = G_{formation-prods}$$

While the Gibbs free energy allows us to calculate the electromotive force (EMF) of the cell

$$E_{/Mol} = \frac{-\Delta G_{formation}}{2F}$$

It alone cannot give us an efficiency value (Larminie *et al*., 2000).

In standard heat engines the limit is the Carnot efficiency, this of course is not applicable here. As the ΔG varies with temperature and pressure, we cannot simply relate it to the electrical energy produced as taking all factors into account it would frequently arrive at an over-inflated efficiency (≈100%). In most cases the efficiency stated is used to relate the efficiency to other engines and employs the Enthalpy of formation of the products ($\Delta h_{formation}$). This gives rise to an equation for the maximum efficiency of a fuel cell (or thermodynamic efficiency, TE) that compares the energy left to do external work (Gibbs free energy) after product formation, with the energy released by product formation (calorific value/enthalpy of formation) (Larminie et al., 2000) through combustion.

$$TE_{/mol} = \frac{\Delta G_{formation}}{\Delta h_{formation}} \times 100\%$$

This gives rise to a thermodynamic efficiency of ≈83% at process operating temperature (in a PEMFC)(Larminie *et al*., 2000). This gives the maximum "working" efficiency of the fuel cell by relating the amount of energy it produces to the theoretical maximum amount of energy that could be produced carrying out the same reaction.

The Actual fuel cell efficiency (E_{fc}) can be evaluated by comparing the open circuit voltage (V_c) with the maximum theoretical circuit voltage (V_{max}), taking in to account the amount of unused fuel (μ_f),

$$E_{fc} = \mu_f \frac{V_c}{V_{\max}}$$

The E_{fc} gives the specific fuel cell efficiency, which allows you to show how well an individual fuel cell converts the energy it produces. (Larminie *et al*., 2000) To allow us to calculate the electrical power potentially obtainable from a hydrogen producing process, Fuel cell efficiency must be considered.

The overall process efficiency can be assessed in many ways, the ratio of products and the theoretical maximum amount of products (reaction yield) can be used along with other process metrics (i.e. atom efficiency and reaction selectivity) but more importantly, as the end product of the process is electrical energy, is the ratio of energy input to energy output. The input of energy to the system is almost entirely through mixing, if process control equipment and the variable heating load is not considered. Therefore the energy used by the process is;

$$P = p_0 \rho D i^5 N^3$$

The Power produced by the system is related to the volume of hydrogen evolved by the system as follows;

$$P = IV = I^2R = \frac{V^2}{R}$$

Where, P= Power (W)

I = Current (A)

V = Potential difference (V)

R = Resistance (Ω)

Also,

$$P = \frac{\Delta W}{\Delta t}$$

Where,

W= Work done (J)

t= time (s)

If we consider a process, is a reversible system with no losses then we can also go on to say;

$$W = QV = -2FV = \Delta G_{formation}$$

Where, Q= charge (C)

F= Farad (the charge on a mole of electrons, C)

Which shows that the work done is equivalent to the Gibbs free energy of formation of the product, so finally taking into consideration the thermodynamic efficiency of the fuel cell;

$$P = TE\left(\frac{\Delta G_{Formation}}{\Delta t}\right)$$

Giving an overall process energetic efficiency (E_{EP});

$$E_{EP} = \frac{P_{input}}{P_{output}} \times 100 = \frac{\left(p_o \rho D_i^5 N^3\right)}{\mu_f \left(TE\left(\frac{\Delta G_{formation}}{\Delta t}\right)\right)} \times 100$$

This shows the relationship between the rate of power input and output from the process and the efficiency of the process, including the fuel utilisation coefficient, μ_f (= 0.95) (Levin, 2004) (negating heating requirements and mechanical losses within the bioreactor).

Appendix 3

This section outlines the background of bacterial growth kinetics

A.3.1 Bacterial growth

Bacterial growth or enumeration occurs simply by providing the cultures with the necessary substances for growth, these substances vary between microbial species, with auxotrophic species occurring (Haddock and Jones, 1977). Bacterial species typically require a carbon, nitrogen and oxygen source, long with various other elements (Major microbial cell constituents Table A.3.1), which must be provided for growth (growth medium components discussed in Section 2.3.6), Methods for bacterial growth are discussed in Section 2.3.

Table A.3.1 12 Major microbial cell constituents with corresponding functions (Gadd, 2007)

Element	Chemical form used by microbes	Function
C	Organic compounds, CO, CO_2	Major constituent of cell materials; proteins, nucleic acids, lipids, carbohydrates etc.
O	Organic compounds, CO, CO_2, O_2, H_2O	
H	Organic compounds, H_2, H_2O	
N	Organic compounds, NH_4^+, NO_3^-, N_2	
S	Organic sulphur compounds, SO_4^{2-}, HS^-, $S_2O_3^{2-}$	Proteins, coenzymes
P	HPO_4^{2-}	Nucleic acids, phospholipids, coenzymes, teichoic acid
K	K^+	Major inorganic cation. Enzyme cofactor
Mg	Mg^{2+}	Enzyme cofactor, bound to cell wall, membrane and phosphate esters including nucleic acids and ATP
Ca	Ca^{2+}	Enzyme cofactor, bound to cell wall
Fe	Fe^{2+}, Fe^{3+}	Cytochromes, ferredoxin, Fe-S proteins, enzyme cofactor
Na	Na^+	Involved in transport and energy transduction
Cl	Cl^-	Major inorganic anion

Along with providing the necessary food for bacterial growth in the form of growth media, other factors must be conserved for effective bacterial enumeration. Using the parameters of pH and temperature each bacterial species will have an optimal and working range for growth, furthermore some specific

species have strict growth consideration, such as anaerobes, where as some merely change there behaviour and growth characteristics in response to environmental changes (i.e. Facultative anaerobes and the response of Fungi to mixing/ power input to a culture). Most bacteria that are studied are chemoorganotrophs (requiring an organic carbon and energy source).

In 1949 J. Monod postulated the kinetics of growth in bacterial cultures (Monod, 1949). Monod suggested that bacterial growth could be expressed in terms of phases of growth:

Lag phase
Acceleration phase
Exponential phase
Retardation phase
Stationary phase
Death phase

With the view being, the differences in the growth phases are attributed to a change in bacterial growth rate. As well as describing the phases of growth, Monod also attempted to model them algebraically, which gave rise to the Monod equation.

$$\mu = \frac{\mu_{\max} s}{K_s + s}$$

Where; s, is the limiting substrate concentration, μ, is the specific growth rate, μ_{max}, is the maximum specific growth rate and K_s is the value of limiting substrate concentration at which $\mu=0.5\mu_{max}$.

Monod's equation, while being frequently used in bacterial cell culture is an oversimplification, it assumes (Koch, 1997, Monod, 1949):

All bacteria in the population are able to produce a clone
$s>>K_s$
Single limiting substrate
Substrate uptake is hyperbolically dependant on substrate concentration
A single enzymatic step controlled cell growth
Substrate uptake is carried out by a single enzyme

These assumptions do not always hold true. As it has already been stated Monod's equation forms the basis of most growth kinetics but there are other methods used to model bacterial growth kinetics, some of which are based on Monod's equation.

A.3.2. Lag phase

Monod, assumed that the length of the lag phase (and the acceleration phase) merely constituted the length of time the bacterial culture took to reach steady-state growth (steady state growth occurs in the exponential phase). Due to the lack of knowledge about microbial physiology, many cellular processes were unknown and as such assumptions had to be made (these assumptions also lead to the creation of the Monod equation and are stated above and are based on Michaelis-Menten type enzyme kinetics). The length of the Lag phase can be attributed to many factors but all are part of the cellular response to the adaptation of a new environment (Monod, 1949), and throughout the phase the cell number remains constant (Buchanan, 1918).

A.3.3 Acceleration phase

The acceleration phase can often be overlooked, its length can be short enough to be considered part of the lag phase or even negligible, it accounts for the length of time require to go from no quantifiable growth in the lag phase to the maximal growth rate of the exponential phase (Buchanan, 1918, Monod, 1949).

A.3.4 Exponential phase

This phase of growth is the growth rate is constant and at it's highest. It is called the exponential phase because the cell number during this phase increases exponentially (Buchanan, 1918, Monod, 1949).

A.3.5 Retardation phase

During this phase the growth rate, rate of cell division decreases or cell generation time increases (Buchanan, 1918)

A.3.6 Stationary phase

During Stationary phase the growth rate is zero, however unlike in lag phase there is still cell division (Kolter *et al*., 1993). The growth rate is unknown and effectively zero, as the cell death rate is equivalent to the growth rate. During this phase the average generation time is infinity (Buchanan, 1918). Another feature of the stationary phase is stringent response, this is part of a survival strategy and involves the synthesis of many proteins, a change in cell shape and size and an increased resistance to environmental stresses (Gadd, 2007).

A.3.7 Death phase

The death phase contains an acceleration of the death rate where the rate is effected either by virtue of still dividing and reproducing cells, or just the time taken for the rate of cell death to reach a maximum (or a combination of both processes) (Buchanan, 1918).

Appendix 4

This section outlines the background calculations used in the results and discussion.

A.4.1 Operating pressures

The process as a whole has an intrinsic hydrostatic pressure requirement, this is of importance as its quantification will give and insight in to the amount of hydrogen that is lost to the atmosphere[5]. The expression used to derive the approximate operating pressure is a partial differential, as it changes according the volume of gas collected. The process diagram given in Section 3.4, Figure 3.1 and replicated here in Figure A.4.1 is not a scale description but gives an insight in to the layout of the process that is to be evaluated.

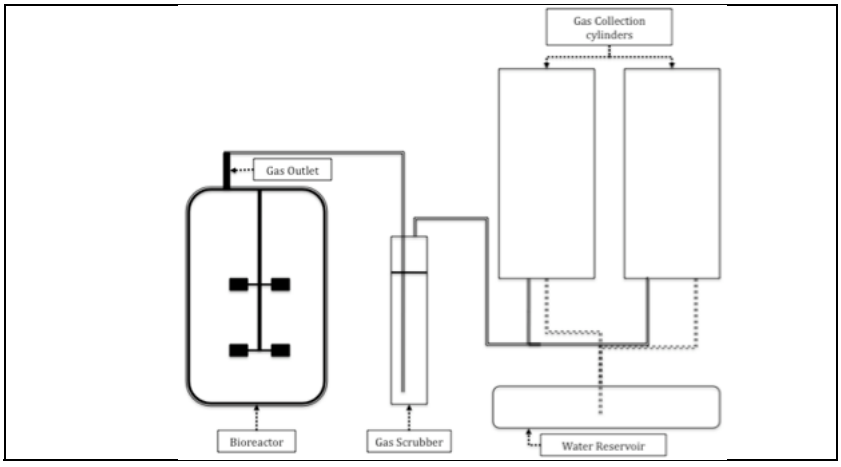


Figure A.4.1 Biohydrogen production process schematic

The equation that determines the hydrostatic pressure exerted by water (assumed an incompressible fluid) is given by;

$$P = \rho g h$$

[5] The silicone tubing used in the experiment is porous to hydrogen, its porosity is dependant on operating pressure

Where P, is the hydrostatic pressure (Pa); ρ is the liquid density (kg/m^3); g is the body force per unit volume acting on the fluid (N/m^3) and force exerted on the fluid in this case is gravity, g and h is the height above the liquid level denoted the baseline.

In this case the pressure, p has hydrostatic pressure exerted on it by the liquid it has to displace in the gas scrubber and the volumetric hydrogen collection cylinders, giving;

$$P = \rho_{NaOH} g h_{NaOH} + \rho_{water} g h_{water}$$

Where the subscripts NaOH and water denote the gas scrubber and the hydrogen collection cylinders respectively.

This give an overall process pressure of 1.54 atms (15,923.974 KgM^{-2}, 117.1303 cmHg).

The quantification of process pressure allows the calculation of the approximate amount of hydrogen lost to the atmosphere. This is done by using Darcy's Law, which relates the permeability of a substance to the rate of fluid diffusion through the substance.

Darcy's Law;

$$Q = \frac{\kappa A}{\mu_{viscocity}} \cdot \frac{\Delta P}{L}$$

Where the volumetric rate of gas loss equals Q; permeability, κ ; cross-sectional area, A; the pressure drop, ΔP; the dynamic viscosity, μ; and the length, L the pressure drop is taking place over.

Giving the maximum rate of loss of gas approximately equal to 60 mLh^{-1}

The process pressure changes throughout the hydrogen collection time period however the initial process pressure is known. The different methods used to collect the gas within literature (both physically and or theoretically) means there is a difficulty in accurately comparing results between different studies, however it is suitable for internal comparison within this study as the mode of

gas collection was constant. The use of Boyle's Law allows the calculation of the volume of gas at s.t.p. (Boyle's Law states the product of pressure and volume of a gas is a constant), which gives an evolution rate of 300 ml h^{-1} (again an approximation as the pressure would reduce as more gas is collected). This goes on to give a mass evolution rate of 0.013g h^{-1} (using the ideal gas laws). Mass evolution rates are an appropriate method for reporting the production of a gas, due to the variability of gas volumes with temperature and pressure.

A.4.2 Process parameters

Within the results and discussion section, certain process parameters are mentioned but not quantified. These are evaluated in this section.

A.4.2.1 Reynolds number/flow regimes

The dimensionless Reynolds number (Re) gives an insight into the flow regime within any object with a fluid flowing in or through it.
In an stirred tank the flow regimes correlate to Re as shown in Table A.4.1 below

Table A.4.1 Flow regimes

Flow Regime	Laminar flow	Transition flow	Turbulent flow
Re (approximate)	1-1x10^2	1x10^2-1x10^4	>1x10^4

In a stirred tank the Re, is given by the following equation;

$$\mathrm{Re} = \frac{\rho N D_i^2}{\mu}$$

This gives an Re= 33,620 (assuming the medium is similar to water flowing within the vessel) which is turbulent flow, however if the fluid viscosity increases enough (more than three fold) as may happen with fermentation utilising complex media this value could decrease to the transition flow regime. The addition of the 0.2M glucose solution during the hydrogen production phase will also further increase the viscosity taking the flow regime closer to (or in to) the transition flow regime.

A.4.3 Fed batch growth

Feeding strategies were calculated using the relationship developed by (Strandberg *et al*, 1994). An exponential feeding rate was used to maximise cell density and minimise growth substrate inhibition. This was calculated using the following equation

$$F = \left(\frac{1}{s}\right) \times \left(\frac{\mu}{Y_{xs}} + M\right) \times X_0 \times e^{\mu t}$$

Where, F, is the substrate feed rate; s, is substrate concentration; Y_{xs}, is bacterial yield on the limiting substrate; μ, is the specific growth rate; M, is the maintenance coefficient; X_0, is the initial cell mass; t, is time. The variables bacterial yield, and specific growth rate were calculated by performing batch experiments prior to fed batch cultivations. Maintenance however was assumed using a literature correlation (Bodegom, 2007).

An example of this is shown in Figure A.4.2

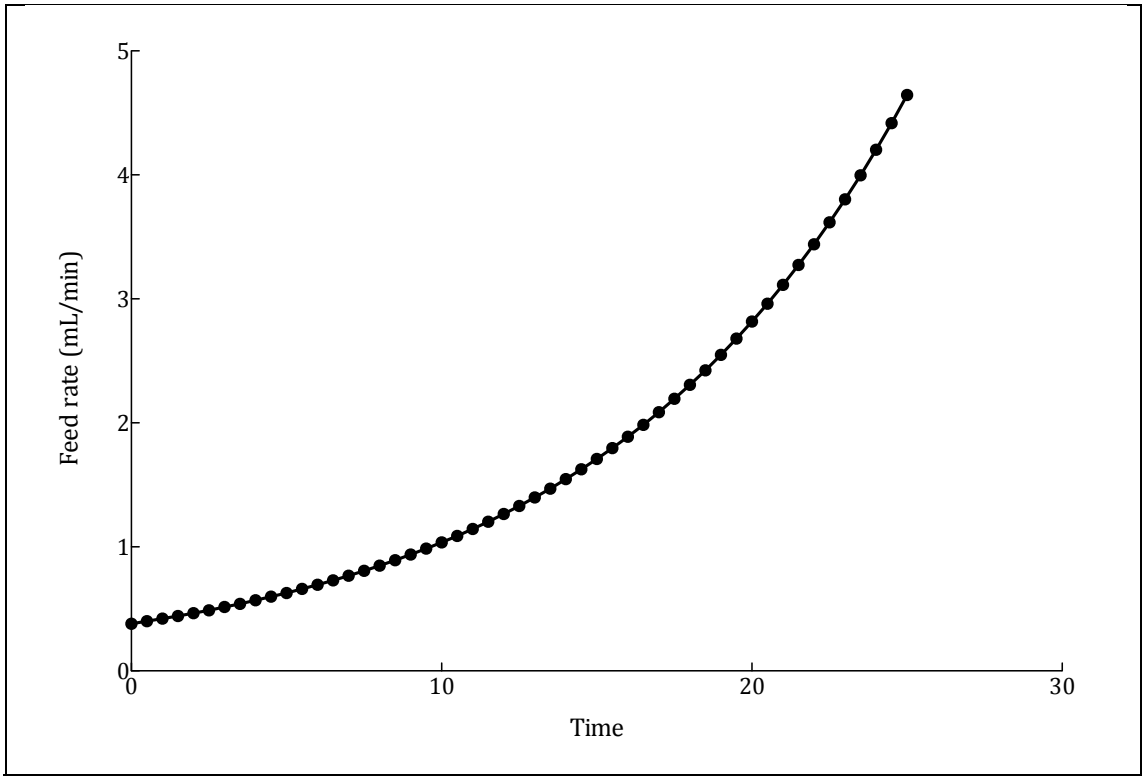


Figure A.4.2 An example feeding strategy for a TGYEP fed batch process (X_0=15, μ=0.1

A. 4.4 Calibration curves

A.4.4.1 HPLC calibration curves

Table A.4.2 Calibration equations and retention times for HPLC analysis

Substance	RI Calibration equation	UV Calibration equation	Retention time (Minutes)
Formate	[formate]=245.295(Peak area)+5.894	[formate]=16.3313(Peak area)+5.756	13:29
Lactate	[lactate]=120.2611(Peak area)+0.766	[lactate]=18.5066(Peak area)-0.847	12:30
Acetate	[acetate]=275.154(Peak area)+5.894	[acetate]=22.924(Peak area)+1.541	14:43
Glucose	[formate]=40.6445(Peak area)-4.461	N/A	4:28

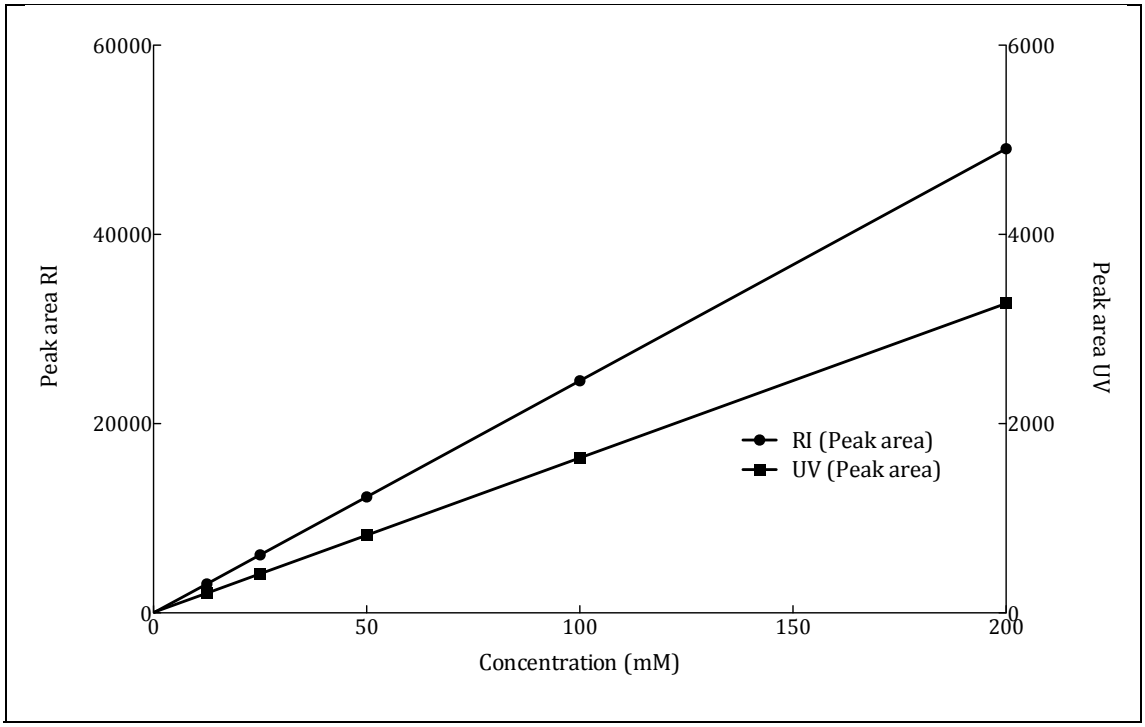


Figure A.4.3 Formate Calibration curve

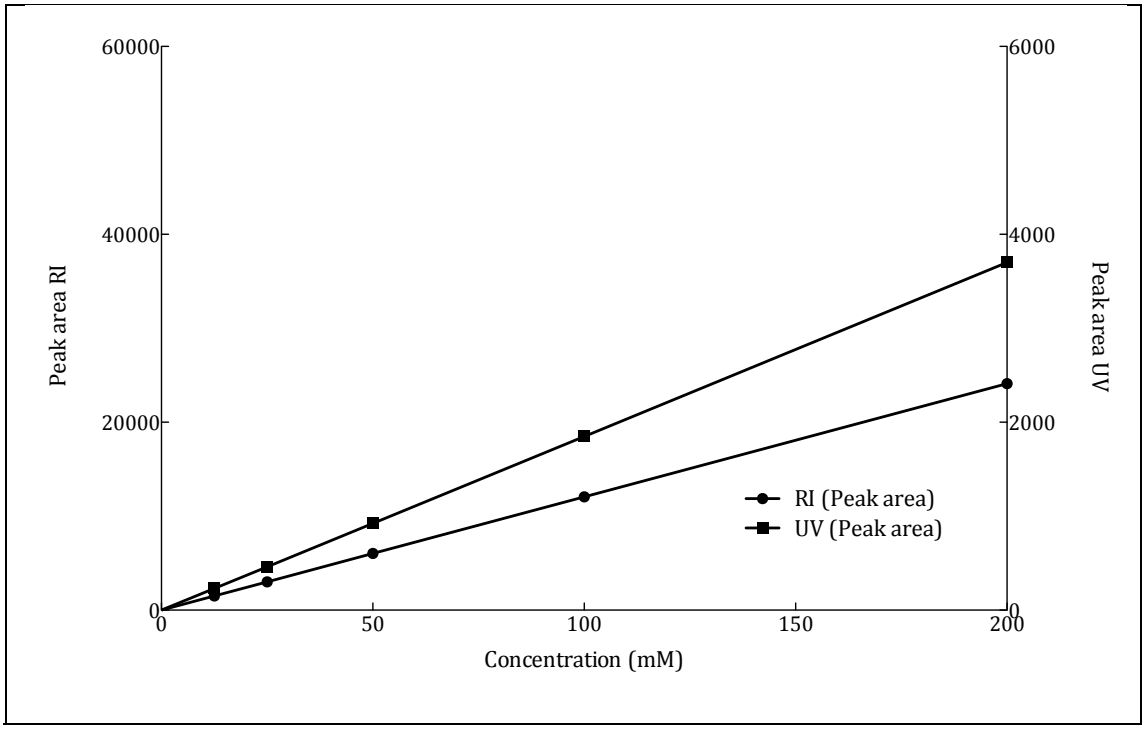


Figure A.4.4 Lactate Calibration curve

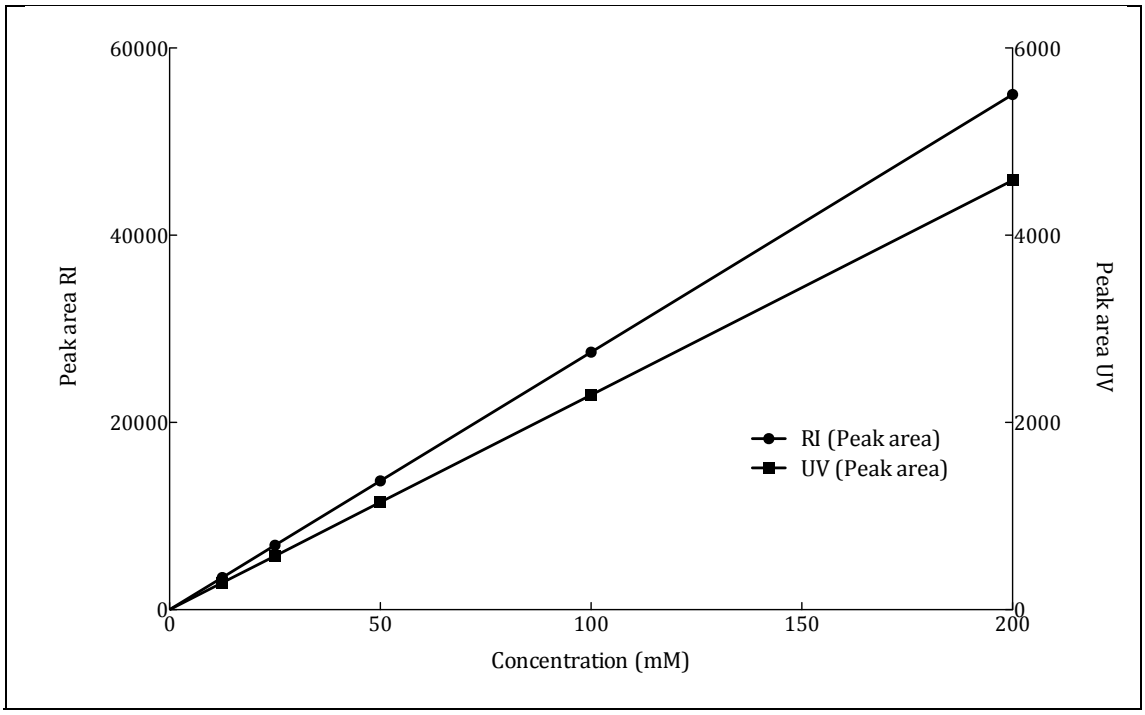


Figure A.4.5 Acetate Calibration curve

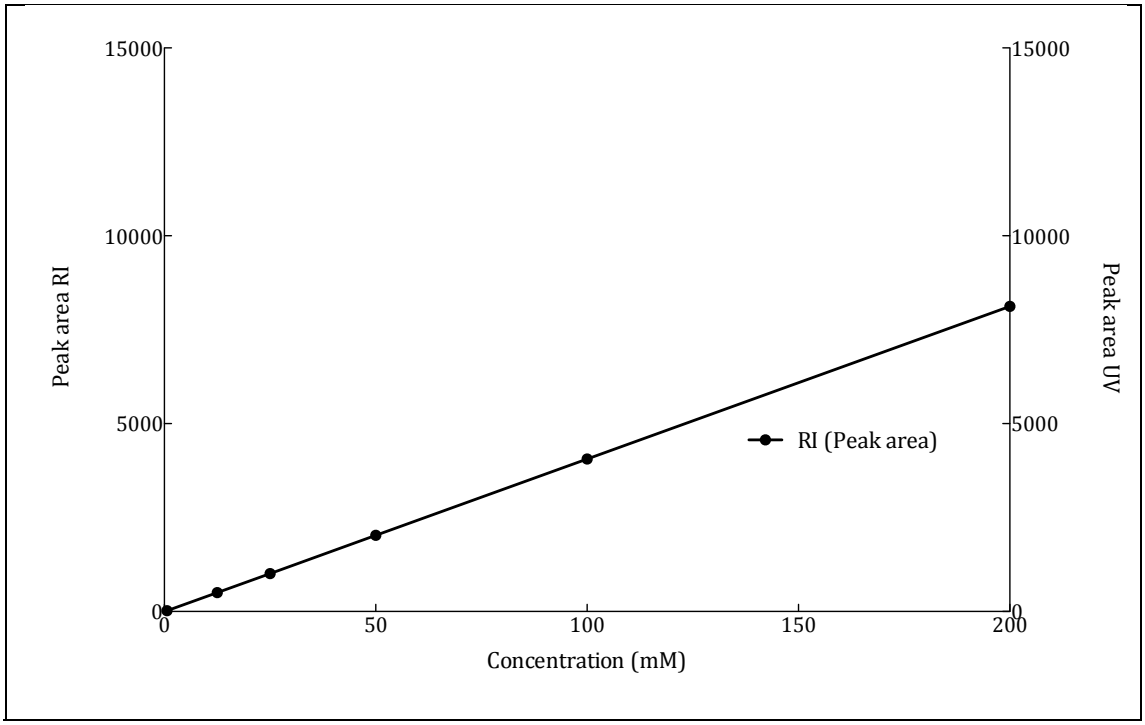


Figure A.4.6 Glucose Calibration curve

Appendix 5

This section of the appendices outlines some of the further calculations that are referred within the results and discussion section of the thesis.

A.5.1 Growth calculations

As well as the calculations used to determine the feeding strategy, the maximum specific growth rate was also calculated. It was calculated assuming Monod growth kinetics (Monod, 1949). The equation used gives the maximum specific growth rate when the cell concentrations are taken during the exponential growth phase and is as below.

$$X_t = X_0\left(e^{\mu_{max} t}\right)$$

Where X is the cell concentration (at time, 0 and time, t) and μ_{max} is the maximum specific growth rate.

A.5.2 Enzyme kinetics

Table A.5.1 Summary of the kinetic data established from a range of experiments

	Enzyme Activity Shake flask	Enzyme Activity DS Medium	Enzyme Activity No Aeration	Enzyme Activity TGYEP Medium	Enzyme Activity MSB Medium	Enzyme Activity MSB Fed-Batch
Michaelis-Menten						
Best-fit values						
Vmax	387.6	961.8	90.72	941.6	872.0	2335
Km	24.78	28.97	14.49	14.41	5.629	21.13
Std. Error						
Vmax	143.4	254.2	19.51	162.6	98.10	773.6
Km	23.33	19.17	12.58	17.35	6.309	20.11
95% Confidence Intervals						
Vmax	36.75-738.4	407.9-1516	45.72-135.7	490.1-1393	645.7-1098	187.7-4483
Km	0.0-81.86	0.0-70.75	0.0-43.50	0.0-62.57	0.0-20.18	0.0-76.97
Goodness of Fit						
Degrees of Freedom	6	12	8	4	8	4
R square	0.3804	0.4905	0.2435	0.1955	0.1082	0.6834
Absolute Sum of Squares	12392	286857	1029	2440	89321	705612
Sy.x	45.45	154.6	11.34	24.70	105.7	420.0
Constraints						
Km	Km > 0.0	Km > 0.0	Km > 0.0	Km > 0.0	Km > 0.0	Km > 0.0
Number of points Analyzed	8	14	10	6	10	6

Steady state reaction kinetics were evaluated by nonlinear regression analysis using Prism (GraphPad Software Inc, La Jolla, CA), using the Michaelis-Menten equation [$v = (V_{max}[S])/K_m[S])$], where K_m represents the substrate concentration at half saturation, Vmax represents the maximum substrate conversion rate and [S] is the substrate concentration. Figure A.5.1 is a graphical representation of this data.

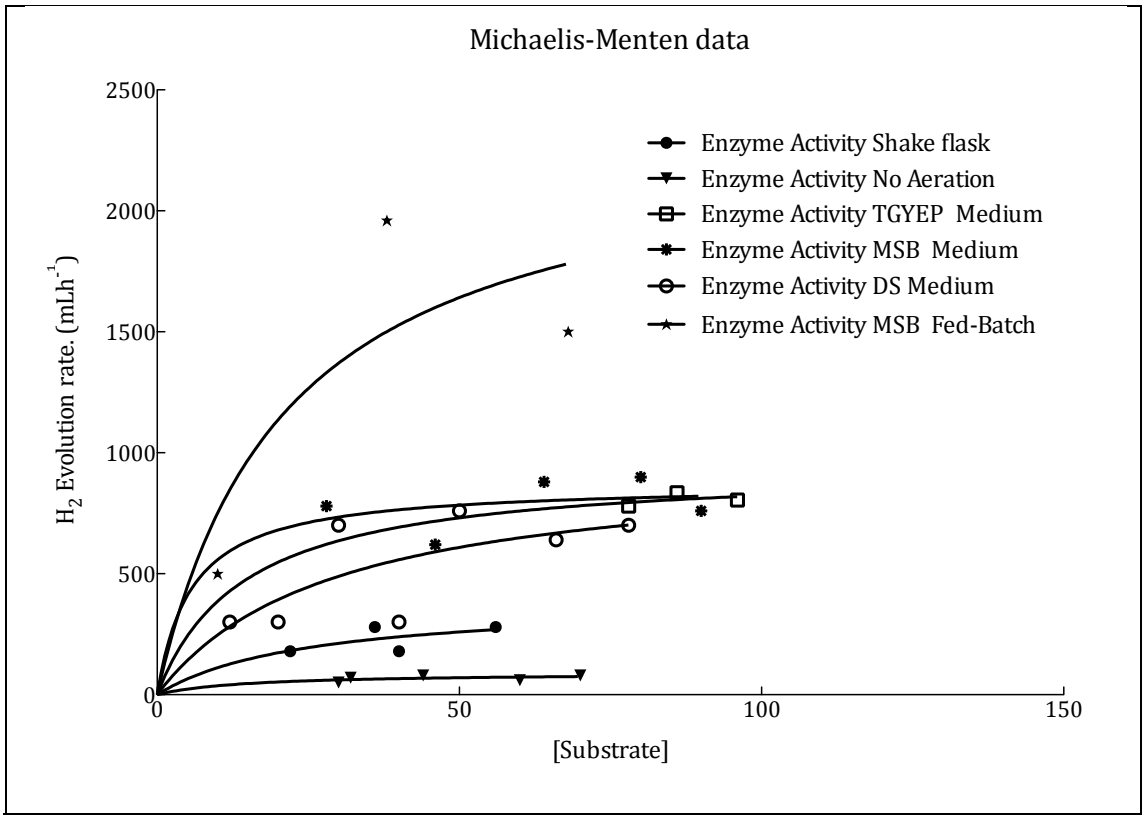


Figure A.5.1 Summary of the kinetic data established from a range of experiments

Appendix 6

This section of the appendices shows example chromatograms.

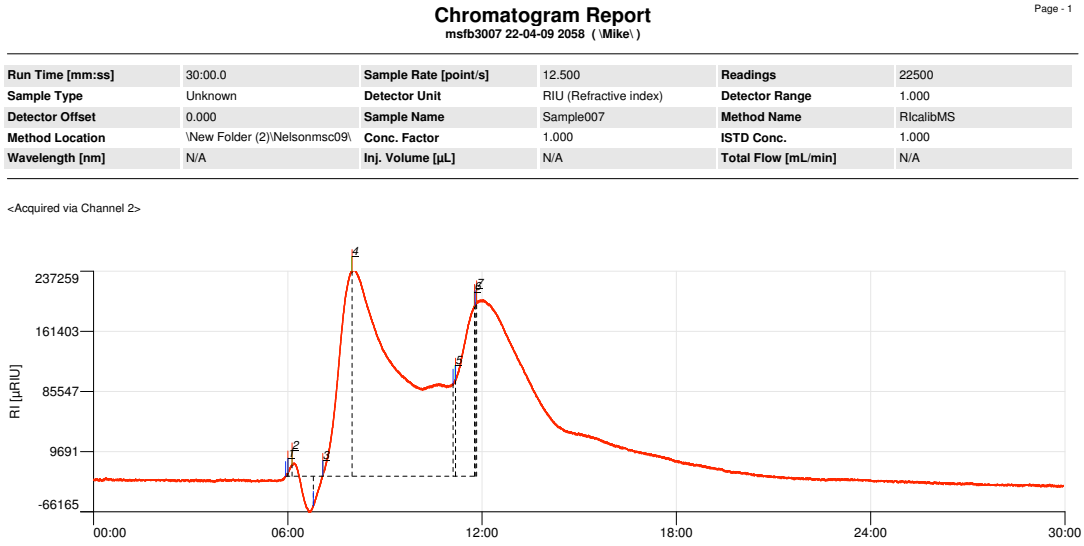


Figure A.6.1 An example chromatogram showing peaks corresponding to glucose and formate

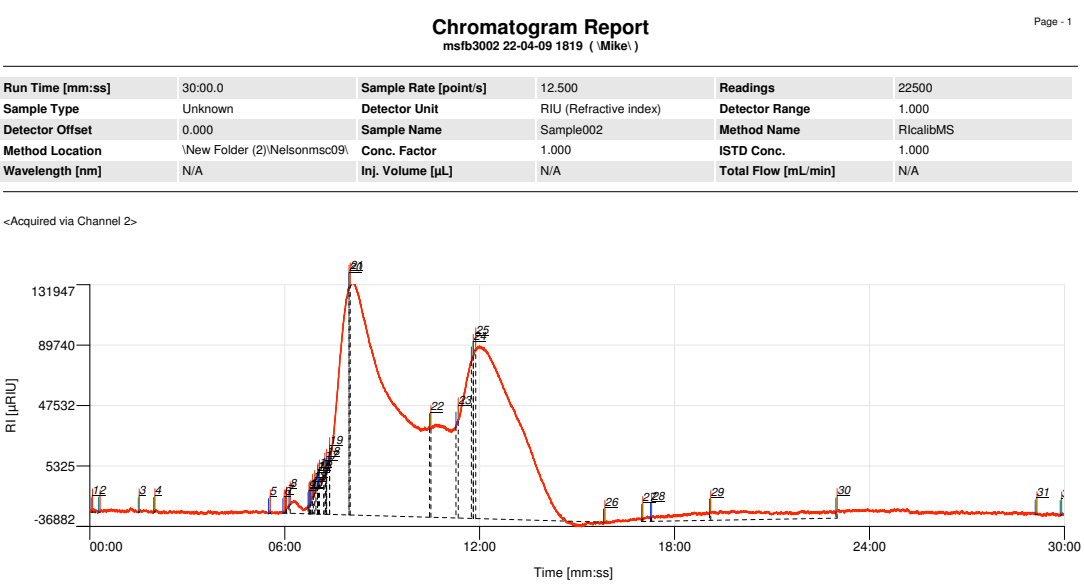


Figure A.6.2 An example chromatogram showing peaks corresponding to glucose and formate

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