Hydrogen Production by *Desulfurococcus fermentans*

by

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Author's declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners. I understand that my thesis may be made electronically available to the public.

Abstract

Desulfurococcus fermentans is a hyperthermophilic archaeon growing optimally at 82°C. This microorganism is an obligate anaerobe with optimal growth pH of 6.0. It is capable of producing H_2 as an end metabolic product using cellulose as growth substrate. The major goal of this study was to optimize the growth conditions for the production of H_2 from various substrates such as cellulose, cellobiose, carboxymethyl cellulose (CMC), xylan, filter paper, avecil, starch and peptides. The highest cell density (2.83×10^8 cells/ml) was observed when yeast extract (0.2 g/L), starch (5 g/L) and xylan (4 g/L) were added to its growth media. The lowest generation time was shown to be 2.4 hours when yeast extract (0.2 g/L), starch (5 g/L) and cellobiose (4 g/L) were added to its growth medium. It was found that the growth of *D. fermentans* was obligately depended on the presence of yeast extract in the growth medium, and the H_2 production was positively correlated to its growth. Cells of *D. fermentans* were cocci with diameters varying from 1 to 3 μm. The largest cell size was observed using scanning electron microscopy when it grew in medium containing yeast extract (10 g/L) and starch (5 g/L). Maximum hydrogen production of 12% (v/v) was achieved when yeast extract (0.2 g/L), starch (5 g/L) and carboxymethyl cellulose (4 g/L) were added to the growth medium. Further studies are required to obtain the specific yield of H_2 from various substrates through the quantification of both the consumption of substrates and the production of H_2 by *D. fermentans*.

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overseas. I want to thank them for always being encouraging me to follow my heart and my dreams and always there for me when I stumbled along the way.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

CPD: critical point of drying

CMC: carboxymethyl cellulose

EDTA: Ethylenediaminetetraacetic acid

GC: gas chromatography

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

ND: not determined

Chapter 1

Introduction

1.1 Bio-hydrogen

Rapid consumption of fossil fuels causes negative impact on the environment, which makes scientists consider finding alternative energy sources that are environmentally friendly and renewable (Blumer et al., 2008). Biohydrogen is one of the clean and renewable energy sources. By gasification or fermentation processes, H_2 can be produced from cellulose. Cellulose is one of the low cost and available biomaterials. But one of the problems in the process of hydrogen production is very low digestibility of cellulose. In order to overcome this obstacle, pretreatment methods are essential (Chou et al., 2008). In fermentation processes, microorganisms such as yeast convert natural sugar content from a variety of raw materials into ethanol (Borneman et al, 1992). One of the other end products that may be produced by anaerobic fermentation is hydrogen under different conditions such as pH and temperature. H_2 can be produced from a wide range of waste materials. Microorganisms that are able to do this procedure do not suffer from inhibitory impact of oxygen and grow fast, due to anoxygenic process of fermentation (Nguyen et al., 2008). Microbial fermentation can potentially convert a variety of biomass - based substrates into H_2 at high rates. Hyperthermophiles show high potential to utilize raw materials with minimal pretreatment and to reach maximum bioconversion to renewable energy sources (Barnard et al., 2010).

Hydrogen also has an energy yield of 122 kJ/g, which is 2.75 times greater than that of hydrocarbon fuels. Glucose, sucrose and lactose as simple sugars are better substrates due to their degradability and untainted carbohydrate sources make them economically valuable. Starch and cellulose are rich in carbon contents. Starch can be hydrolyzed by acidic or enzymatic

treatments to glucose and maltose followed by hydrogen production. Cellulosic materials need further treatments such as chemical, mechanical and biological processes. Brewery wastewaters can be utilized as raw material for hydrogen production because of biodegradable carbohydrate contents and non-toxic industrial effluents. Wastewater treatments plants have carbohydrates and proteins that can be utilized for hydrogen production. The majority of bio-hydrogen is produced by the following procedures: dark and photo fermentative production of hydrogen, biophotolysis of water by algae and anaerobic digestion of organic matter during acidogenic phase of dark fermentation. Hydrogen can be produced by many anaerobic organisms which can utilize carbohydrate-containing organic wastes. Many *Clostridium* species such as *Clostridium paraputrificum* M-21, *Clostridium butyricum*, *Clostridium pasteurianum*, *Clostridium thermolacticum* and *Clostridium bifermentans* are obligate anaerobes which produce hydrogen during the exponential growth phase. *Enterobactriaceae* species including, *Enterobacter aerogenes* and *Enterobacter cloacae* can metabolize glucose by fermentation of butanediol or mixed acid and produce CO_2 and H_2. *Porphyromonos* spp. and *Actinomyces* spp. are found beside to *Clostridium* spp. in anaerobic sludge where the hydrogen is detected. Trace amounts of hydrogen can be produced by *Hafnia alvei* and *E. coli*. *Bacillus* sp., *Thermotogales* and other hyperthermophiles are able to produce hydrogen (Kapdan et al., 2006).

1.2 Hyperthermophiles

Hyperthermophilic microorganisms are both archaea and bacteria and have an optimal temperature of 80°C and above. They are a subset of extremophiles and they are also able to withstand other environmental extremes such as high acidity or radiation levels. Coleman et al (2010) divided these microorganisms into two groups. The first group is ancient hyperthermophiles that have been hyperthermophiles for their entire evolutionary history and the second group is the recent ones, such as *Thermotoga maritima* (Chou et al., 2008). *Methanopyrus kandleri* "isolate 116" strain is able to grow at temperatures up to 122°C and high pressure of 200 bar (Koki et al., 2011). *Thermococcus kadakaraensis*, a hyperthermophilic archaeon, with optimum growth temperature of 85ºC is a hydrogen producer (Kapdan et al., 2006). *T. maritima* is an extremophilic organism which is capable of fermenting both simple and complex sugars. *Pyrococcus horikoshkii* is an anaerobic heterotrophic microorganism that utilizes peptides as its main carbon source (Blumer et al., 2008). *Caldicellulosiruptor bescii* is a thermophilic, anaerobic, cellulolytic bacterium which utilizes a variety of polymeric carbohydrates as well as monosaccharaides. It can grow on carboxy methyl cellulose (CMC), starch, crystalline cellulose, and glycogen to produce CO_2, H_2, lactate, acetate and a trace amount of ethanol (Dam et al., 2011).

There are diverse enzymes that have been extracted from hyperthermophiles. Their thermostable cellulases including endoglucanases, cellobiohydrolases and β-glucosidases which are responsible for cleavage of β-1,4-glycosidic bonds of cellulose to glucose, which catalyze the hydrolysis of cellulose (Park et al., 2011).

Another enzyme that plays a vital role in anaerobic metabolism is hydrogenase. Hydrogenases are oxygen-sensitive enzymes and can be found in all three domains of life and have cofactors and multiple subunits. These enzymes can catalyze the reverse reduction of protons to molecular hydrogen (Jenney et al., 2008).

1.2.1 *Desulfurococcus fermentans*

D. fermentans was isolated by Perevalova et al (2005) from a freshwater hot spring of the Uzon caldera. It is an obligately anaerobic, hyperthermophilic archaeon and has a growth temperature range from 63 to 89° C (T_{opt} = 82° C). It can grow from pH 4.8 to 6.8 (pH_{opt} = 6.0). Its cells are cocci, 1-4 μm in diameter, and with one polar flagellum (Perevalova et al., 2005). It is an obligate chemoorganoheterotroph and obtains energy by fermentation of arabinose, agarose, amygdalin, arbutin, casein hydrolysate, cellulose, dextran, dulcitol, fructose, laminarin, lactose, α-keratin, maltose, lichenan, pectin, ribose, peptone, starch and sucrose. No growth is observed on glucose, gelatin, albumin, casein, mannitol, sorbitol or xylose (Perevalova et al., 2005; Kublanov et al., 2009).

D. fermentans belongs to *Desulfurococcus* genus (Table 1.1). Elemental sulfur, thiosulfate, sulfate, hydrogen and nitrate do not have any effect on its growth (Zillig et al., 1983). *Desulfurococcales* are able to utilize peptides (Perevalova et al., 2005). Table 1.2 shows different characteristics of different species of the genus *Desulfurococcus. D. fermentans* is the only microorganism in this genus that is able to grow on cellulose. However, it was not known about

optimal growth conditions for its production of H_2 using various substrates including cellulose (Kublanov et al., 2009).

Table 1.1 classifications of *Desulfurococcus* (Zillig et al., 1983)

Domain	**Archaea**
Kingdom	*Crenarchaeota*
Phylum	*Crenarchaeota*
Class	*Thermoprotei*
Order	*Desulfurococcales*
Family	*Desulfurococcaceae*
Genus	*Desulfurococcus*

Table 1.2 Characteristics of species in the genus *Desulfurococcus* (Kublanov et al., 2009)

Characteristic	*D. mucosus*	*D. mobilis*	*D. amylolyticus*	*D. fermentans* Z-1312^T	*D. kamchatkensis*
Shape and size of cells	Cocci	Cocci	Irregular cocci	Regular cocci, 1–4 μm	Cocci, 0.6-1 μm
Flagellation	No flagella	One flagellum	No flagella	One flagellum	No flagellum
Growth temperature (°C) (min./opt./max.)	ND/85/ND	ND/85/ND	68/90–92/97	63/80–82/89	65/85/87
Growth pH (min./opt./max.)	4·5/6·0/7·0	4·5/6·0/7·0	5·7/6·4/7·5	4·8/6·0/6·8	5.5/6.5/7.5
Growth substrates	Peptides	Peptides	Peptides, starch	Peptides, monosaccharaides, polysaccharides including cellulose	Peptides, monosaccharaides
Influence of sulfur on growth	Stimulating	Stimulating	Stimulating	No effect	Stimulating
Influence of hydrogen on growth	Inhibiting*	No data	Inhibiting*	No effect	Inhibiting

*Data from Slobodkin & Bonch-Osmolovskaya (1994)

1.3 Objectives

The specific goals of this research project were the following:

1. To determine optimal pH for the growth of *D. fermentans.*
2. To investigate the effect of various substrates including CMC, cellobiose, avecil, cellulose, arabinose, filter paper, xylan and peptides on the growth of *D. fermentans* and the production of H_2 from each substrate.
3. To determine the cell size variation of *D. fermentans* grown on yeast extract using a scanning electron microscope.

Chapter 2

Methods and Materials

2.1 Microorganism and Chemicals

D. fermentans was obtained from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH) Braunschweig in Germany. The chemicals used were of the highest purity commercially available.

2.1.1 Growth of *D. fermentans*

D. fermentans was grown in 160 mL serum bottles sealed with a butyl rubber stopper, which contained 50 mL of a medium modified from Perevalova et al. (2005) at 82^0C and pH 6. The modified medium contained the following (per liter): KCl, 0.33 g; NH_4Cl, 0.33g; KH_2PO_4, 0.33 g; $MgCl_2$•$6H_2O$, 0.33 g; $CaCl_2$•$2H_2O$, 0.33 g; Na_2S•$9H_2O$, 0.5 g; starch, 5 g; yeast extract, 0.2 g; resazurin, 0.2 ml (of a 0.5 g/L stock solution); trace element solution, 0.1 mL, vitamin solution, 0.1 mL.

Trace element solution contained (per liter): EDTA (TRIPLEX IV, from Sigma-Aldrich Canada -Oakville, ON, Canada), 500 mg; $FeSO_4$, 200 mg; $ZnSO_4$•$7H_2O$, 10 mg; $MnCl_2$, 3 mg; H_3BO_3, 30 mg; $CoCl_2$•$6\ H_2O$, 20 mg; $CuCl_2$•$2H_2O$, 1 mg; $NiCl_2$•$6\ H_2O$, 2 mg; Na_2MoO_4•$2H_2O$, 3 mg. Vitamin solution was composed of (per liter): biotin, 2 mg; folic acid, 2 mg; pyridoxine – HCl, 10 mg; thiamine–HCl•$2H_2O$, 5 mg; riboflavin, 5 mg; nicotinic acid, 5 mg; D-Ca – pantothenate, 5 mg; vitamin B_{12}, 0.1 mg; p-aminobenzoic acid, 5 mg; lipoic acid, 5 mg (Perevalova et al., 2005).

The medium was sterilized by autoclaving (American Sterilizer Autoclave Cyclomatic control, USA) at 121^0C (Nguyen et al., 2008). Sterile filtered (0.2 µm pore size) vitamin solution was added to the medium after autoclaving, and then the medium containing bottles were degassed in a manifold. The strictly anaerobic conditions for growth were ensured by adding 830 µl of a 3% (w/v) $Na_2S•9H_2O$ solution (which was degassed and kept under N_2). The pH of the medium was measured using two methods: a pH meter (Fisher Scientific, Corning AB accumet, PC – 353 stirrer, Canada) was used if the medium was at room temperature and pH paper (Baker-pHIX, Mallinckrodt Baker, Inc. Canada) was used if the medium was hot. To obtain the desired pH, 2M NaOH or 2M HCl was added drop by drop. Adding $Na_2S•9H_2O$ and doing several gas-degassing resulted in pH fluctuations. Either phosphate buffer (0-500 mM) (Table 2.1) or HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] (10 mM) was added to culture medium to maintain a stable pH for the growth of *D. fermentans* (Ngo et al., 2011). The final concentration of HEPES in the media was 0.01 M.

Removing of O_2 from culture bottles and media was carried out by using a manifold. The manifold had different parts including oil vacuum pump, moisture trap, O_2-scavenging catalyst column, gas supply and glass manifold. Inside the trap should be cleaned before use each time. Then vacuum pump was turned on and the valve of the gas cylinder was opened (the nitrogen gas cylinder for this purpose). The catalyst column should be checked to make sure it is hot enough. The manifold should be gassed and degassed three times before use. A sterile filter (0.2 µm pore size) was connected to a needle to each connector port of the manifold. Then a sterile needle was connected to the filter. The needle will penetrate the thick part of the stopper sealing the culture bottle.

Table 2.1 Preparation of phosphate at different concentrations in 50 ml media

mM	KH_2PO_4	K_2HPO_4
0 mM	0	0
10 mM	0.068 g	0.087 g
50 mM	0.34 g	0.43 g
100 mM	0.68 g	0.87 g
200 mM	1.36 g	1.74 g
500 mM	3.40 g	4.35 g

The following substrates were used as carbon and energy sources for the growth of *D. fermentans.*

1) L-(+)-arabinose from Sigma-Aldrich Canada (Oakville, ON, Canada).

2) D-(+)-cellobiose from Sigma-Aldrich Canada (Oakville, ON, Canada): It is a disaccharide derived from the condensation of two glucose molecules linked in a β(1→4) bond or the hydrolysis of cellulose. It can be hydrolyzed by bacteria and converted to glucose (Shi et al., 2011 and Zhang et al., 2011).

3) Carboxymethyl cellulose (CMC) from Sigma-Aldrich Canada (Oakville, ON, Canada) contains carboxymethyl groups (-CH_2-COOH) which are bound to some of the hydroxyl groups of the glucopyranose monomers. It is manufactured by the alkali-catalyzed reaction of cellulose with chloroacetic acid (Chang et al., 2009).

4) Xylan from beechwood purchased Sigma-Aldrich Canada (Oakville, ON, Canada) is composed of complex polysaccharides. That contains xylose units (a pentose sugar). Xylan is a kind of hemicellulose with heteropolymers such as arabinoxylans, and it is composed of different sugar monomers, for example, xylose, mannose, galactose and arabinose. Normally, it has the D-pentose sugars and sometimes L-sugars (Biely et al., 1985). It has random amorphous structure with little strength and can be easily hydrolyzed by dilute acid or base and many hemicellulase enzymes.

5) Cellulose (Sigma-Aldrich Canada, Oakville, ON, Canada). It is present primarily in cell walls of green plants and it can also be found in algae. It contains only anhydrous glucose. Cellulose is crystalline, strong and resistant to hydrolysis.

6) Whatman filter paper- GF/A- 70 mm contains polymers containing of β1→4 bonds of glucose to glucose (From Whatman®, Whatman International Ltd Maidstone England, England).

7) Avicel (microcrystalline cellulose) type PH 105 NF- Lot 50901C from FMC BioPolymer, Newark, ON, Canada.

2.1.2 Monitoring the growth of *D. fermentans*

The growth of *D. fermentans* was monitored by direct cell count using a counting chamber (Hausser Scientific Partnership- Horsham, PA, USA). A growth curve was obtained using a semi-log plot. From the growth curve, generation time was calculated using the following equation (White 1995) where g is the generation time, x is the cell count (cells/mL) at the point after a period of t within the log phase, x_0 is the cell count at the start point within the log phase, and t is the time between points x_o and x:

$$g = \frac{0.301t}{\log x - \log x_0}$$

2.2 Determination of H_2

2.2.1 Gas chromatography method

Hydrogen in the headspace of the culture bottle was sampled with gas tight syringe (100 µL). The amount of H_2 in the sample was determined by using a gas chromatograph (GC-2014, SHIMADZU GAS CHROMOTOGRAPH) equipped with a thermal conductivity detector (TDC). The operational temperature of injection port, oven and detector were 140, 120 and 140^0C, respectively. TCD-2014 was used to detect hydrogen The GC column for H_2 detection was Grace Porapak Q 80/100, 1/8". Nitrogen was used as a carrier gas. The retention time of H_2 was 0.83 minute.

A standard curve was needed for the quantification of H_2 present in the gas samples. To prepare H_2 with known concentrations, empty bottles (62 ml) sealed with grey butyl stoppers were used. Bottles were degassed for 15 min and were pressurized (3 psi) with N_2 for 5 min. Pure H_2 (100%, v/v) was used to fill into a 62 ml empty serum bottle that was degassed for 30 min before pressurizing with H_2 (5 psi) for 5 min. From the bottle containing 100% H_2, different volumes of H_2 gas were taken using a gastight syringe (HAMILTON, Nevada, USA) and then added to different 62- mL bottles filled with 100% N_2 (with an overpressure of 0 after releasing extra N_2 gas using a syringe needle) to prepare different concentrations of H_2 at 0, 0.01, 0.1, 1, 2 and 4% (v/v). For each concentration, two bottles were prepared. Since the total volume of each bottle was 62 ml, to obtain H_2 concentration of 0, 0.01, 0.1, 1, 2 and 4% (v/v) required the addition of 0, 6.2, 62, 620, and 1240 and 2480 µL of 100% H_2, respectively. Then, 5 mL deionized water was injected into the bottles to keep a positive pressure in the headspace. A gas sample of 100

µL was injected into the GC by using a gastight syringe for measuring the corresponding peak area of H_2.

After obtaining the peak area for each known sample, a standard curve was drawn (see figure 3.3) in order to calculate percentage of H_2 present in further unknown gas samples. To analyze the data, fit a line over the standard curve. For each unknown sample the peak area which was displayed in Y-axis was measured. The concentration of H_2 in unknown sample was the value on the X-axis. The following formula was used to calculate the percentage of produced hydrogen.

$$\text{Percentage of Hydrogen } (\%, \text{v/v}) = \frac{\text{Peak area}}{1\text{E} + 06}$$

To calculate the pressure inside each bottle, pressure was measured by using a low-pressure diaphragm gauge (The Weiss Series- NY, USA).

2.3 Scanning Electron Microscope

The purpose of doing this experiment was to observe whether cell size would change when *D. fermentans* was cultivated on different concentrations of yeast extract. The procedure for preparing samples for SEM was as follows (D. Weber, personal communication): After 20 h, 50 mL of growth culture was centrifuged at 3,000 rpm, 19° C for 25 min. Afterwards, the cell were re-suspended in 0.2 M phosphate buffer (pH= 6). Phosphate buffer was prepared by mixing 87.7 mL of 0.2 M monobasic sodium phosphate with 12.3 mL of 0.2 M dibasic sodium phosphate. The cell suspension was filtered through a 0.8 µm pore size 13 mm in diameter (Catalog number: 312-0009. Bio-Rad Labs, CA, USA). The cells collected on the filter were fixed in 2.5% glutaraldehyde in phosphate buffer overnight at 4° C. Then the sample was dehydrated by

passing through a series of different concentrations of acetone in water (10% for 10 min, 20% for 10 min, 50% for 10 min, 70% for 20 min and 100% for 20 min). Cells were critical point dried, gold coated and observed with a Hitachi model S-4500 SEM. This procedure was repeated three times: Twice the above procedure was followed and the other time the culture was filtered directly, omitting centrifuging part from the procedure.

Four cultures bottles were used which contained of (A) 0.2 g/L yeast extract and 5 g/L starch, (B) 2 g/L yeast extract and 5 g/L starch, (C) 4 g/L yeast extract and 5 g/L starch, and (D) 10 g/L yeast extract and 5 g/L starch, respectively.

Chapter 3

Results

3.1 Growth of *D. fermentans*

D. fermentans was able to grow on medium containing both yeast extract (0.2 g/L) and starch (5 g/L) at 82^{0}C and pH 6 (Fig. 3.1). It had a generation time of 7.2 h and its maximum cell density was about 1.26×10^8 per mL. It took approximately 20 hour for *D. fermentans* to enter the log phase, which had a period of about 20 hours. The overpressure of the headspace remained fairly unchanged at about 7.5 psi (Fig. 3.2).

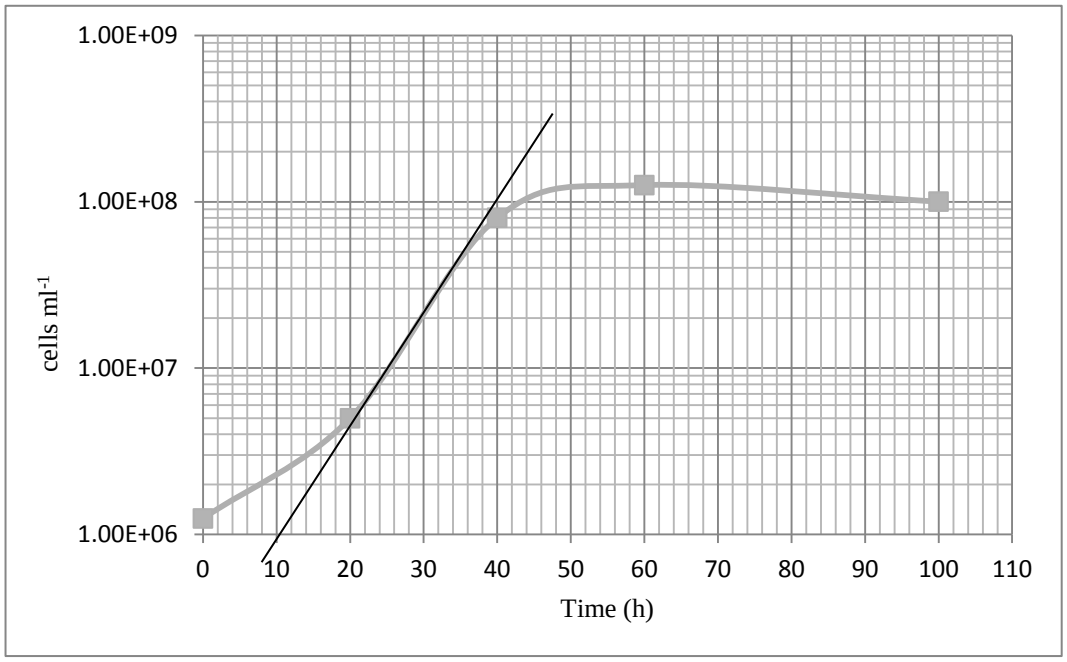

Figure 3.1 Growth of *D. fermentans* on yeast extract (0.2 g/L) and starch (5 g/L) at 82^{0}C and pH 6.0. Culture medium of 50 ml in a 160 ml serum bottle contained 1% (v/v) inoculum and was incubated without shaking. The linear line indicates how the data were chosen for the calculation of generation time.

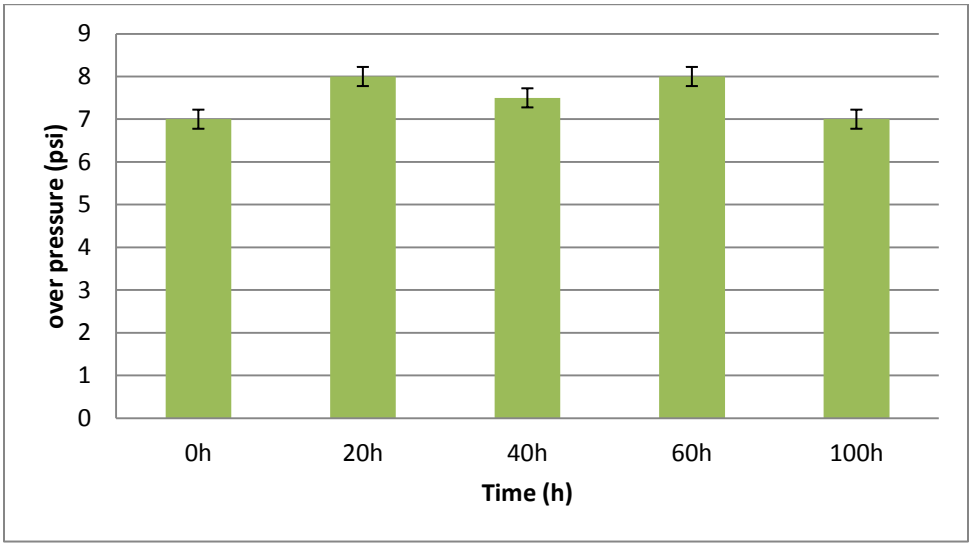

Figure 3.2 Change of the gas pressure in the headspace during the growth of *D. fermentans* on yeast extract (0.2 g/L) and starch (5 g/L) at 82^{0}C and pH 6.0. The average pressure of two culture bottles during the period of 100 hours is shown.

3.2 Effect of pH on growth

It was very difficult to adjust medium pH between 4.2 and 5.7 without using the help of a buffer. Phosphate was not an appropriate buffer for the medium due to precipitation. It seemed that $CaCl_2$ would react with phosphate ions, especially when the amount of buffer was increased. Then, HEPES was selected to buffer culture media. 10 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid]) was added culture media.

To determine the best pH amongst those were tested, *D. fermentans* was inoculated in the culture medium without adding any buffers. The pHs which were verified were as follow: 2, 3.8, 4.2, 5.7, 6.1, 6.3, 6.5, 6.8, 6.9, 7.2, 7.5 (Table 3.1). The culture medium of 50 mL in a 160 mL serum bottle contained 1% (v/v) inoculum at different pHs from 2 to 7.5. All experiments were done at 82°C. It showed that the optimal growth pH was 7.5 (Table 3.1). The best growth was observed in the bottle which had the pH of 7.5 at the beginning of the experiment. pH of this bottle reached 6.1 in log phase, so pH 6.0 was chosen as the best pH for further growth experiments when a buffer was used for stabolizing the pH of the growth media.

Table 3.1 pH variation without adding buffer to culture media.

pH before autoclave	pH after autoclave + adding Na_2S	Final pH (after 120 hours)	Generation time (h)	Maximum cell density (cells/ml)
1.5	2	2	No growth	1×10^6
2.5	3.8	3.8	No growth	2×10^6
2.5	4.2	4.2	No growth	1.25×10^6
3	5.7	4.9	No growth	1.5×10^6
4.5	6.1	5.3	No growth	1.25×10^6
5	6.5	5.5	17.04	2.5×10^6
5.5	6.8	5.5	20.1	4.58×10^7
6.5	7.5	6.1	5.4	6.45×10^7

3.3 H_2 standard curve

H_2 produced during the growth of *D. fermentans* was measured using the GC. The retention time of H_2 was determined to be approximately 0.83 min. A calibration curve for known amount of H_2 was plotted (Fig. 3.3). This experiment was repeated twice to get the most linear graph. Table 3.2 showed that different standard H_2 samples were injected to the GC and the corresponding peak area recorded. The equation derived from the standard curve was used in further H_2 measurement calculation.

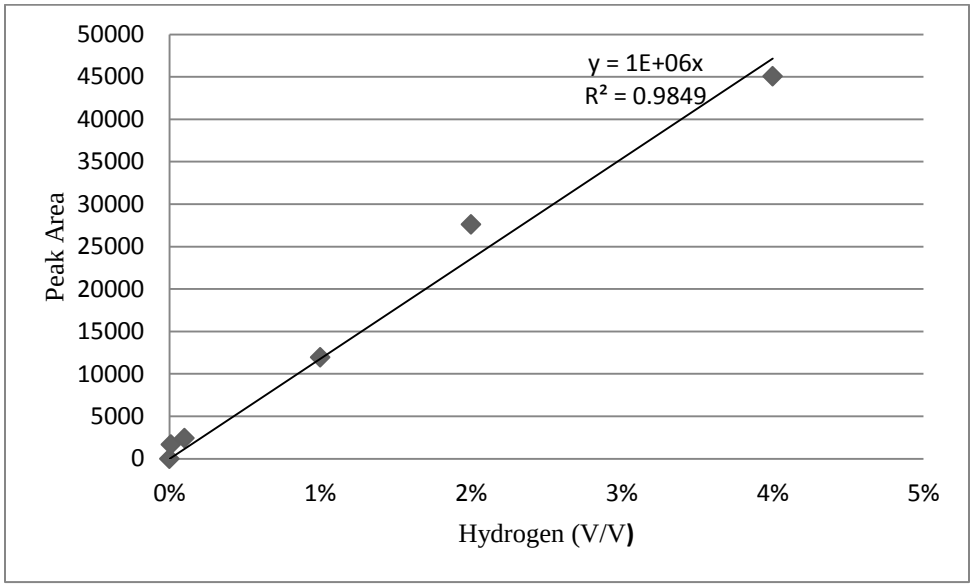

Figure 3.3 The standard curve for H_2 determination. By applying the equation of the graph, further calculations for hydrogen production were done.

Table 3.2 Different standard H_2 samples were injected to GC machine to get a standard curve. The experiment was repeated twice (sample 1 and 2).

Gas	Retention Time	Peak Area
PURE H_2. NO1	0.835	1,308,213
0.01% H_2. NO1	ND	ND
0.1% H_2. NO1	0.832	1,549
1% H_2. NO1	0.833	1,2826
2% H_2. NO1	0.833	2,852
4% H_2. NO1	0.833	29,170
PURE H_2.NO2	0.835	1,304,534
0.01% H_2. NO2	0.829	1,680
0.1% H_2. NO2	0.836	2,434
1% H_2. NO2	0.818	11,967
2% H_2. NO2	0.837	27,615
4% H_2. NO2	0.833	45,067

3.4 Effect of different substrates on the growth of *D. fermentans* and the production of H_2

The substrates used for the experiments were yeast extract, vitamin solution, cellobiose, arabinose, starch, xylan, cellulose, avicel. The following section detail the results obtained.

3.4.1 Effect of yeast extract on the growth of *D. fermentans*

The amounts of yeast extract which were added to culture media were as follow: 0, 0.05, 0.1, 0.15, 0.2, 0.25, 0.4, 1, 2, 3, 4 g/L, respectively. The experiments were done at 82°C and pH 6. In the absence of yeast extract, no growth of *D. fermentans* was observed.

By adding 0.05, 0.15 and 0.1 g/L yeast extract, with or without starch, no good growth of *D. fermentas* could be observed. The optimal growth of *D. fermentans* was observed when the yeast extract was present at a concentration of 2 g/L (Table 3.3). The shortest generation time was 4.6 hours and the highest cell density was 3.15×10^8 cells/mL. More yeast extract did not result in better growth (Table 3.3).

The production of H_2 from the cultures listed in Table 3.3 was also measured (Fig. 3.4 and Fig. 3.5). The highest amount of H_2 was produced when *D. fermentans* grew in the presence of yeast extract at both concentrations 2 and 3 g/L (Fig. 3.4). However, maximum H_2 production was achieved when *D. fermentans* was incubated in the presence of yeast extract at a concentration of 4 g/L after incubation at 130 hours (Fig 3.5).

The requirement yeast extract was determined in the previous experiment by eliminating the yeast extract, indicating that yeast extract was essential for growth of *D. fermentas*. In the following experiments the effect of different amounts of yeast extract on growth in absence of starch were evaluated (experiment was started by adding 0.05 g/L yeast extract). Table 3.3 displays the different amounts of yeast extract which were added to each bottle. Furthurmore, generation time and maximum growth rate of each bottle was calculated.

The effect of elimination of starch from culture media: before 70 hours under best condition (4 g/L yeast extract) *D. fermentans* was able to produce up to 3% (v/v) hydrogen but it could produce up to 9% (v/v) hydrogen after 130 hours (Table 3.5, Figure 3.4 and 3.5).

Table 3.3 Effect of different amounts of yeast extract on the growth of *D. fermentans* at 82^0C and pH 6.0. Starch as one of the carbon sources was eliminated in the experiments.

Substrates	Generation Time (hour)	Initial cell density (Cells/mL)	Maximum cell density (Cells/mL)
0.05 g/L yeast extract	22.3	1.35×10^6	2.5×10^6
0.1 g/L yeast extract	14.7	1.5×10^6	6.5×10^6
0.15 g/L yeast extract	21.6	1×10^6	5.75×10^6
0.2 g/L yeast extract	10.0	1.75×10^6	1.73×10^7
0.25 g/L yeast extract	20.1	1.75×10^6	1.95×10^7
0.4 g/L yeast extract	15.3	2.5×10^6	4.45×10^7
1 g/L yeast extract	6.2	1.75×10^6	9.43×10^7
2 g/L yeast extract	4.6	1.25×10^6	3.15×10^8
3 g/L yeast extract	5.3	2×10^6	1.97×10^8
4 g/L yeast extract	5.9	1.5×10^6	1.14×10^8

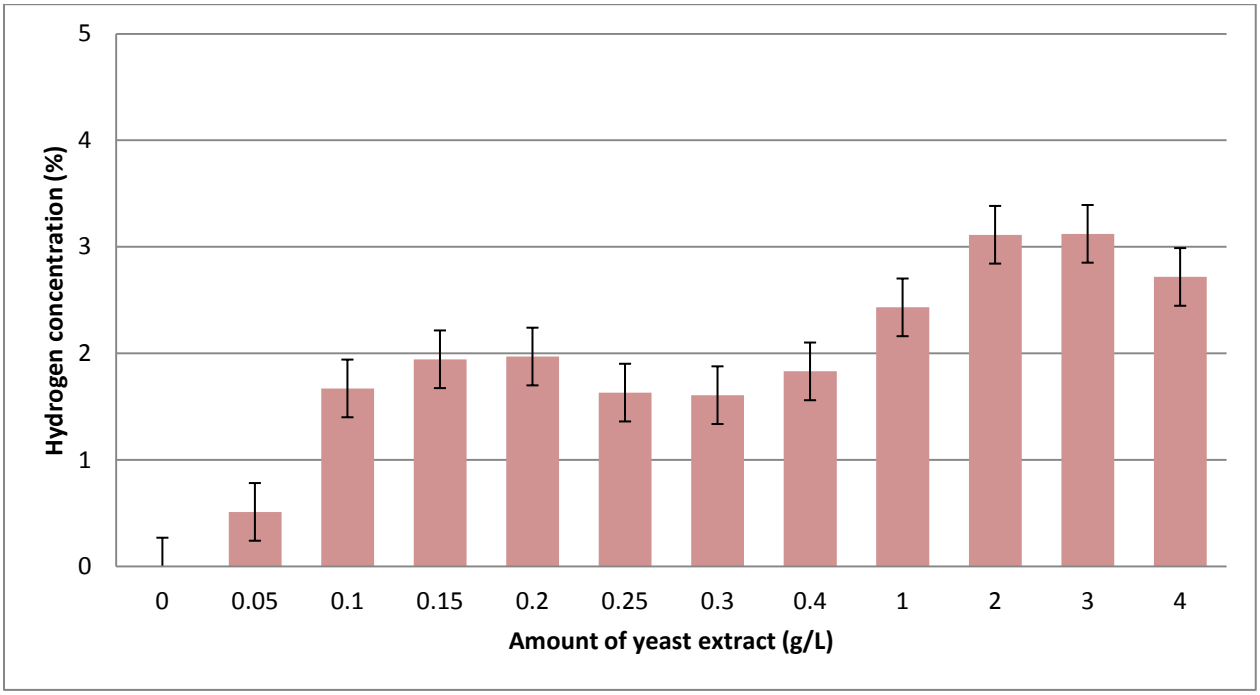

Figure 3.4 Production of H_2 by *D. fermentans* at the incubation time of 70 h at 82^{o}C and pH 6.0.

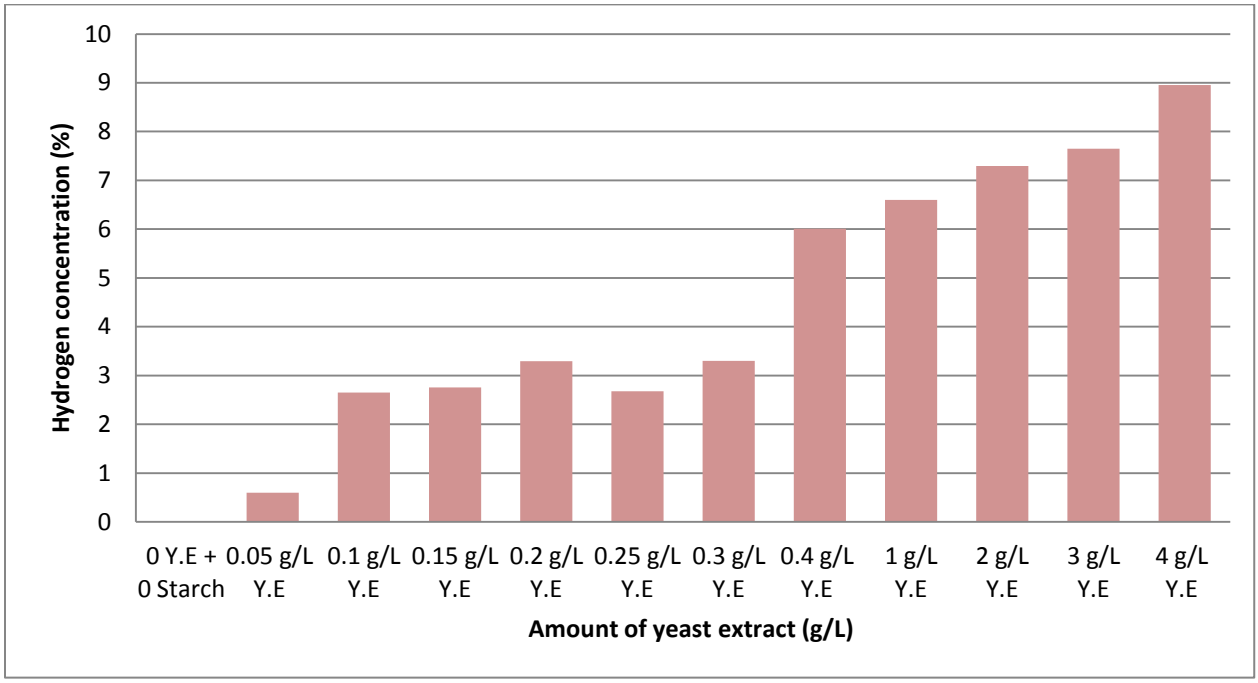

Figure 3.5 Production of H_2 by *D. fermentans* at the incubation time of 130 h at 82^{0}C and pH 6.0.

3.4.2 Effect of different amount of starch on the growth of *D. fermentans*

Yeast extract (0.2 g/L) and 1, 2, 3, 4 g/L starch were added to each bottle. Two control bottles were also prepared (one without starch and one with 5 g/L starch). The experiments were carried out at 82°C and pH 6. *D. fermentans* could grow on culture medium containing starch or without starch (Fig. 3.6 and Table 3.4). It also produced H_2, and almost all the samples showed the maximum hydrogen production at 120 hours (Fig. 3.7).

Apparently, all charts did not show too many differences in growth by decreasing the amount of starch. Even the amount of H_2 production stayed almost the same in all five experiments except the bottle which contained no starch in it. Two control bottles were prepared for this experiment, one without starch and the other one with 5g/L starch (all bottles contained 0.2 g/L yeast extract).

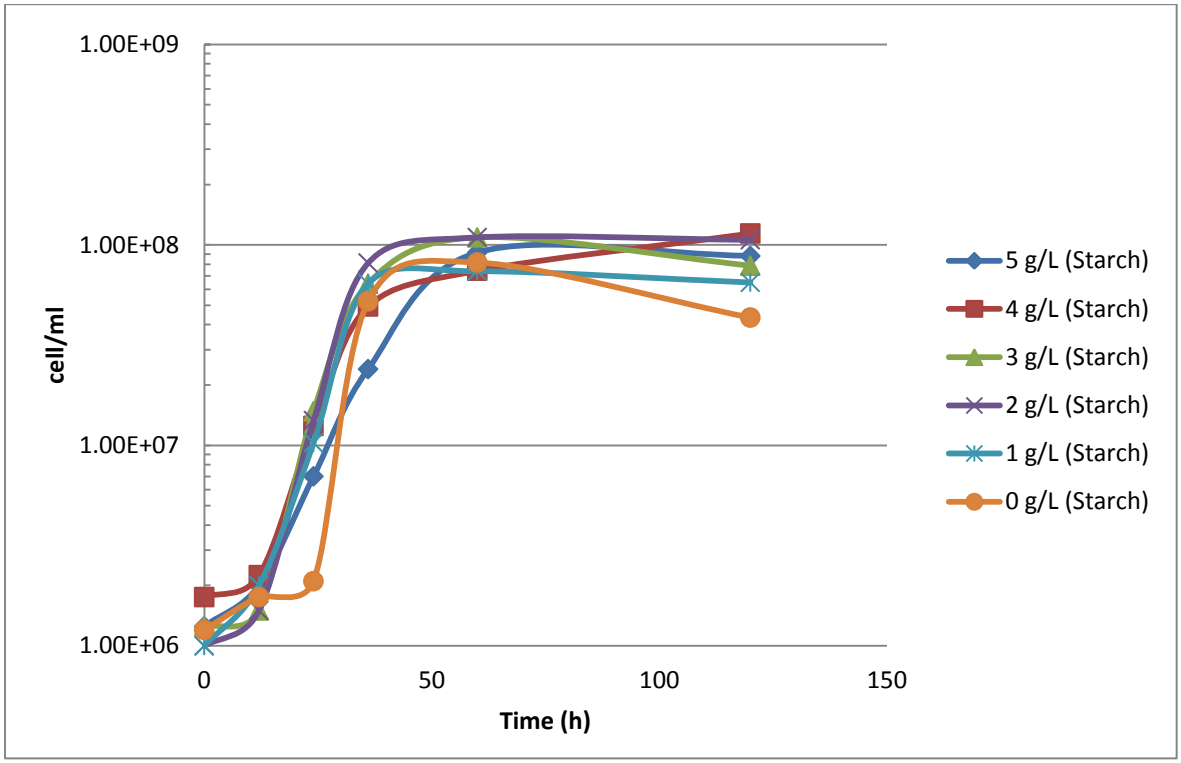

Figure 3.6 Growth of *D. fermentans* on yeast extract (0.2 g/L) and different amount of starch (from 1 to 5 g/L) at 82° C and pH 6.0.

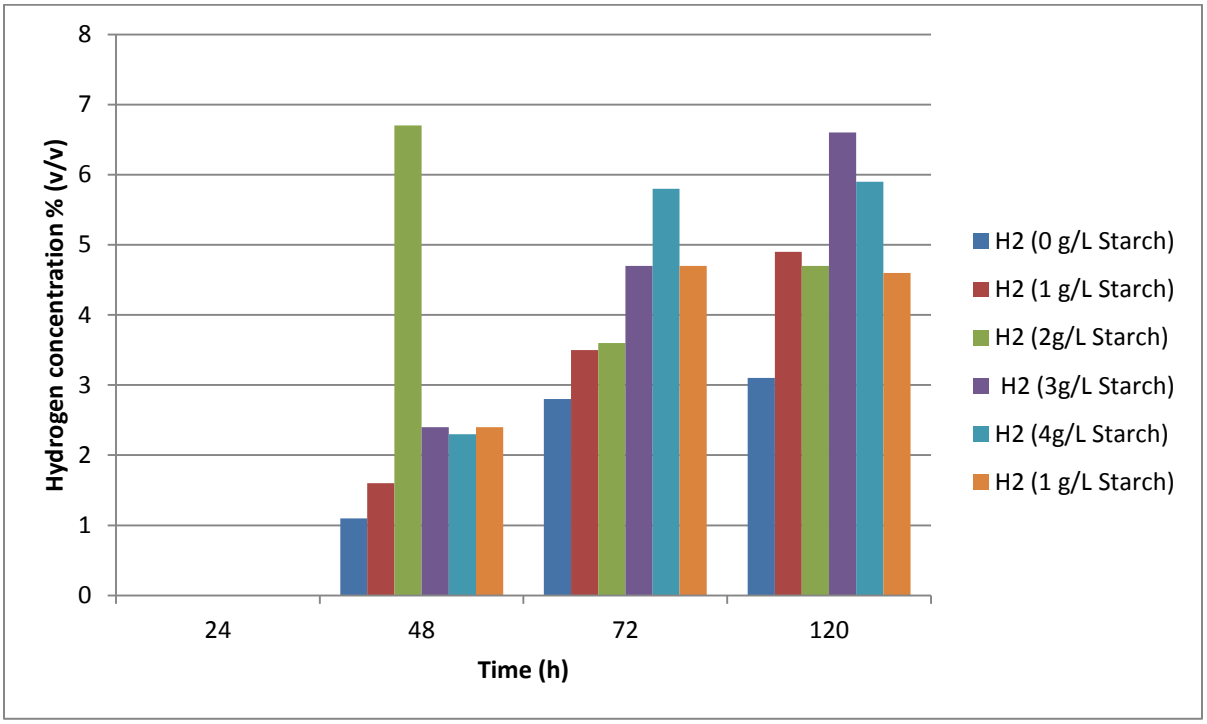

Figure 3.7 Comparison of the production of H_2 when *D. fermentans* was grown on starch at different concentration at 82^{0}C and pH 6.0. H_2 was expressed in % (v/v) in the headspace of the culture bottles.

Table 3.4 Generation time of *D. fermentans* grown on different amount of starch at 82° C and pH 6.0.

Substrates	Generation Time (hour)	Initial cell density (Cell/mL)	Maximum cell density (Cells/mL)
0.2 g/L yeast extract and 5 g/L starch	6.02	1.25×10^{6}	9.15×10^{7}
0.2 g/L yeast extract and 4 g/L starch	5.6	1.75×10^{6}	1.14×10^{8}
0.2 g/L yeast extract and 3 g/L starch	7.2	1.25×10^{6}	1.09×10^{8}
0.2 g/L yeast extract and 2 g/L starch	4.18	1.00×10^{6}	1.09×10^{8}
0.2 g/L yeast extract and 1 g/L starch	4.7	1.00×10^{6}	7.4×10^{7}
0.2 g/L yeast extract and 0 g/L starch	5.2	1.20×10^{6}	8.17×10^{7}

3.4.3 Effect of other substrates and yeast extract on the growth of *D. fermentans* and the production of H_2

Four or 10 g/L of each of the carbon sources other than starch were added to culture medium. The carbon sources which were used in this part were: CMC, cellobiose, avecil, cellulose, L-arabinose, filter paper and xylan. The experiments were done at 82°C and pH 6. Two seed-cultures were used in these experiments. Seed culture A was cultivated in culture medium with 0.2 g/L yeast extract and 5 g/L starch and the other one (which is called B) was just cultivated with 0.2 g/L yeast extract. To balance the culture bottles for centrifugation and eliminate the effect of nutrient carry over, the seed culture was washed. Reduced substrate free medium was added (it did not have any effect on microorganism growth and had the same osmotic pressure). A needle was inserted into the bottle septum to release the overpressure (~30 sec); these procedures were done under the fume hood. The bottles were put in a special adaptor, and samples were centrifuged at 19^0 C, 25 min and 3,000 rpm (Thermo Scientific Sorvall RC 6 PLUS, rotor SLA-3000 - from USA). Supernatant was sucked out with a 20 ml needle syringe; simultaneously N_2 was filled to maintain pressure in the bottle and to keep an anaerobic condition inside the bottle.

Not all subsrates tested supported the growth of *D. fermentans* (Table 3.5). The best growth was observed for the conditon with 0.2 g/L yeast extract and 4 g/L xylan in the growth medium, which gave the lowest generation time of 3.6 h and a relatively high cell density of 2.83×10^8 per mL. The production of H_2 was also measured from the cultures. The amount of H_2 produced increased when incubation time was longer (Fig. 3.8 and 3.9).

Table 3.5 Effect of different substrates and yeast extract (0.2 g/L) on the growth of *D. fermentans* at 82^{0}C and pH 6.0. The culture A bottles were inoculated with the inoculum culture that contained both yeast extract and starch and the culture B bottles were inoculated with inoculum culture that were contained only yeast extract.

Substrates	Generation Time (hour)	Initial cell density (cells/mL)	Maximum cell density (cells/mL)
0 yest extract and 5 g/L starch only	No growth	1. 5× 10^6	No growth
0.2 g/L yeast extract (A)	6.2	3.5× 10^6	2. 1× 10^7
0.2 g/L yeast extract(B)	20.0	3.25× 10^6	2.3× 10^7
0.2 g/L yeast extract and 4 g/L CMC(A)	18.0	2. 75× 10^6	1.89× 10^8
0.2 g/L yeast extract and 4 g/L CMC(B)	4.7	2. 25× 10^6	1.76× 10^8
0.2 g/L yeast extract and 4 g/L Cellobiose(A)	5.6	1.00× 10^6	2.05× 10^7
0.2 g/L yeast extract and 4 g/L Cellobiose(B)	6.5	1.25× 10^6	1.93× 10^7
0.2 g/L yeast extract and 4 g/L Cellulose(A)	4.5	1.00× 10^6	2.63× 10^7
0.2 g/L yeast extract and 4 g/L	11	1.75× 10^6	5.53× 10^7

Cellulose(B)			
0.2 g/L yeast extract and 4 g/L Avicel(A)	3.7	2.50×10^{6}	6.5×10^{7}
0.2 g/L yeast extract and 4 g/L Avicel(B)	16.4	1.50×10^{6}	6.08×10^{7}
0.2 g/L yeast extract and 4 g/L Arabinose(A)	4.8	1.25×10^{6}	2.23×10^{7}
0.2 g/L yeast extract and 4 g/L Arabinose(B)	7.0	1.75×10^{6}	4.23×10^{7}
0.2 g/L yeast extract and 4 g/L filter paper(A)	7.7	1.50×10^{6}	1.5×10^{7}
0.2 g/L yeast extract and 4 g/L filter paper(B)	5.6	3.25×10^{6}	1.27×10^{7}
0.2 g/L yeast extract and 4 g/L xylan(A)	10.3	2.00×10^{6}	2.83×10^{8}
0.2 g/L yeast extract and 4 g/L xylan(B)	3.6	1.25×10^{6}	2.23×10^{8}
0.2 g/L yeast extract and 5 g/L starch(A)	4.4	2.75×10^{6}	1.76×10^{8}
0.2 g/L yeast extract and 5 g/L starch(B)	ND	2.5×10^{6}	6.5×10^{7}

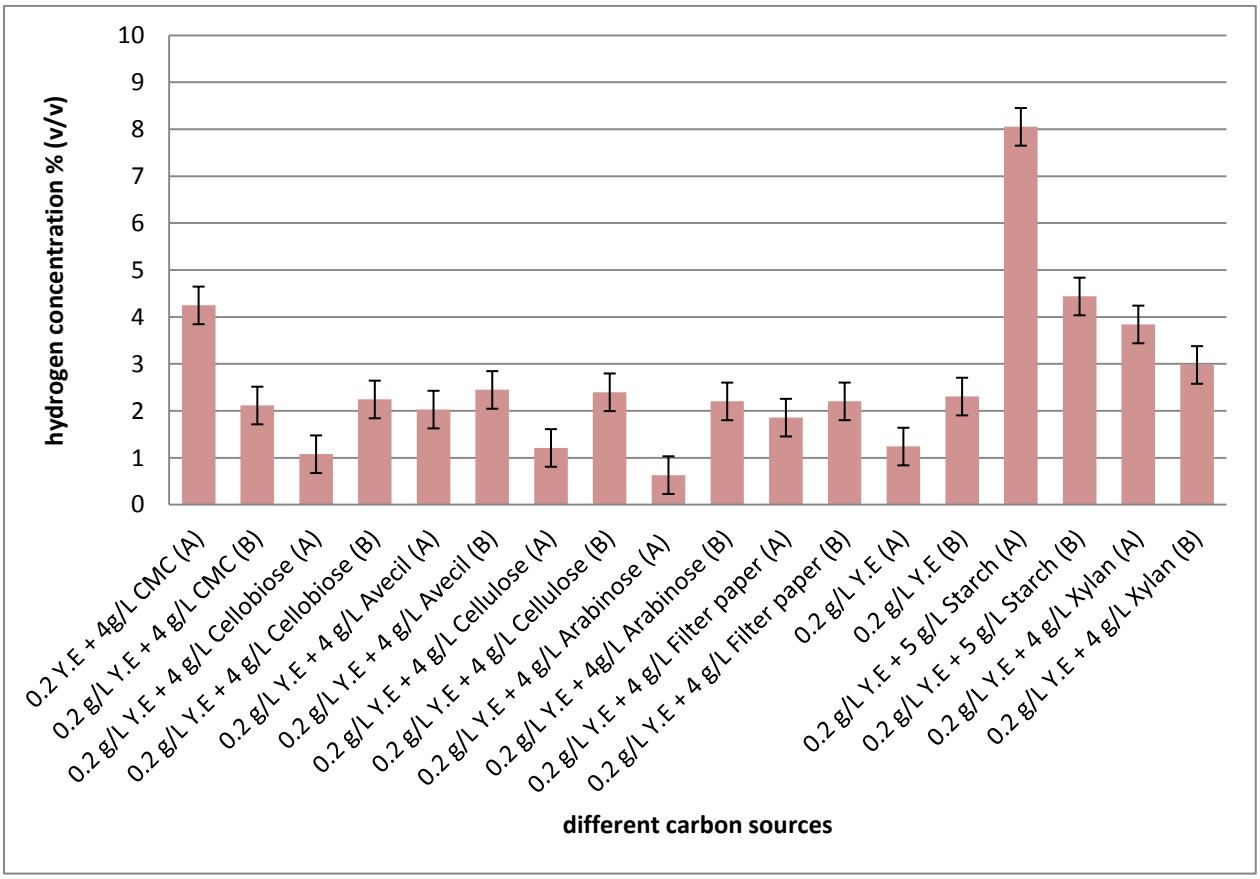

Figure 3.8 Production of H_2 when *D. fermentans* was grown with an incubation time of 100 hours at 82^{0}C and pH 6.0. H_2 was expressed in % (v/v) in the headspace of the culture bottles.

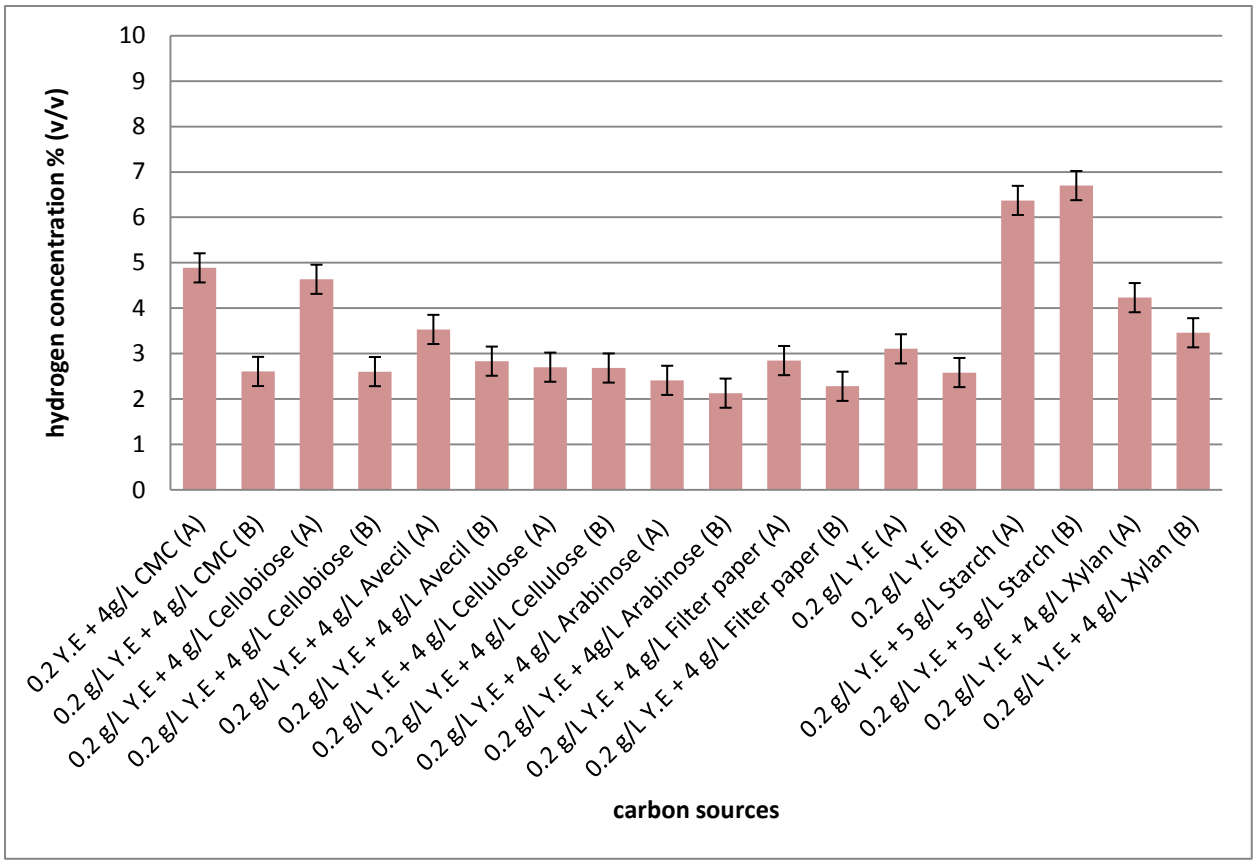

Figure 3.9 Production of H_2 when *D. fermentans* was grown with an incubation time of 150 hours at 82^{o}C and pH 6.0. H_2 was expressed in % (v/v) in the headspace of the culture bottles.

Although the maximum growth was observed in bottles which contained xylan or CMC, maximum hydrogen was observed in the bottle which contained starch. This meant that the presence of starch in culture media increased the amount of H_2 production. On the other hand, on incubation time of 100 hours, more hydrogen production was observed in the bottles which were inoculated by seed culture which had starch in it (A), it meant that some starch carry over occurred in the A bottles and more hydrogen was produced. At incubation time of 150 hours, the hydrogen production for both bottles was almost the same, which it meant that with the time passing the microorganism tried to utilize other carbon sources to produce hydogen.

The average pressure that was calculated from each bottle was 7.5 psi, so the total amount of H_2 produced could be converted to mM in the growth media from the measurement of percentage H_2 produced. The results showed that xylan or CMC could be good substitutes for starch. They had the highest amount of growth and had slightly higher hydrogen production compared to other bottles (Table 3.5). By eliminating starch as one of the carbon sources generation time of *D. fermentans* has been changed although, no significant changes were seen in maximum cell density (Table 3.5).

In the following experiments, effect of 0.2 g/L yeast extract, 5 g/L starch and 4 or 10 g/L of other carbon sources have been investigated. The amount of hydrogen in each sample after 24, 48, 72 and 120 hours was detected repectively using the GC, all the experiments were done in pH 6 medium and at 82° C.

By adding one carbon source in addition to 5 g/L starch and 0.2 g/L yeast extract, slightly higher amounts of hydrogen production were observed. But the bottles which included CMC had 12%

hydrogen production, a considerable amount. By adding 4 or 10 g/L cellobiose, the generation time was decreased to approximately 2.5 hours (Table 3.6 and 3.7). No other differences were observed in the growth of other bottles.

Table 3.6 Growth of *D. fermentans* on various carbon sources in the presence of starch (5g/L) and yeast extract (0.2 g/L) at 82^{0}C and pH 6.0

Substrates	Generation Time (h)	Initial cell density (cells/mL)	Maximum cell density (cells/mL)
5 g/L starch	ND	1.5×10^{6}	1.42×10^{8}
4 g/L CMC	7.2	1.25×10^{6}	1.5×10^{8}
10 g/L CMC	4.5	1.2×10^{6}	9.93×10^{7}
4 g/L cellobiose	2.4	1.75×10^{6}	9.35×10^{7}
10 g/L cellobiose	2.5	1.75×10^{6}	9.93×10^{7}
4 g/L avicel	4.4	1.5×10^{6}	1.9×10^{8}
10 g/L avicel	4	1.75×10^{6}	1.59×10^{8}
4g/L cellulose	4	2×10^{6}	1.03×10^{8}
10g/L cellulose	7.5	1.25×10^{6}	1.59×10^{8}
4g/L xylan	5.5	1.25×10^{6}	2.11×10^{8}
10 g/L xylan	7.7	1.00×10^{6}	9.15×10^{7}

Table 3.7 Hydrogen production by *D. fermentans* grown on various carbon sources in the presence of starch (5 g/L) and yeast extract (0.2 g/L) at 82^{0}C and pH 6.0

Substrates	H_2 production at 24 h	H_2 production at 48 h	H_2 production at 72 h	H_2 production at 120 h
5 g/L starch	0	2.4%	3.8%	6.4%
4 g/L CMC	0	1.1%	3.9%	12%
10 g/L CMC	0	0.9%	4.03%	8.7%
4g/L cellobiose	0	2.4%	4.7%	6.4%
10 g/L cellobiose	0	2.3%	5.8%	9.6%
4 g/L avicel	0	2.6%	5%	7.3%
10 g/L avicel	0	2.2%	4.9%	7.2%
4g/L cellulose	0	2.3%	4.1%	5.5%
10g/L cellulose	0	3%	5.5%	6.3%
4g/L xylan	0	ND	5.3%	9.4%
10 g/L xylan	0	0.53%	3.5%	8.7%

By decreasing the amount of starch from 5 to 1 g/L, the microorganism was still growing very well, although, H_2 production was decreased slightly (Figure 3.7).

3.5 Cell size change of *D. fermentans* growing on yeast extract

Under light microscope coccal cells of *D. fermentans* were observed. In culture medium that contained 0.2 g/L yeast extract and 5 g/L starch, the cell diameter was approximately 1 µm. This result was confirmed by using electron microscopy as well. By adding more yeast extract to the culture medium cell size changed dramatically, especially at high concentrations. Increasing the amount of yeast extract up to 10 g/L resulted in the increase in the cell size up to 3 µm. Although the cell size changing was directly related to the amount of yeast extract, no significant change in number of cells was observed. This phenomenon might happen because of saturation of growth substrates. The same fact happened when the amount of carbon sources was increased from 4 to 10 g/L.

The cell size of *D. fermentans* increased when the concentration of yeast extract increased from 0.2 g/L to 10 g/L. The cell had a diameter of about 1 µm when grown on yeast extract of 0.2 g/L and 5 g/L starch (Fig. 3.10). Although the sample was washed several times with acetone, precipitation of the culture media could still be observed. Based on Prevalova et al (2005) results, this microorganism should have one flagellum. The flagellum could not be observed due to the centrifuging process or the different steps of sample preparation.

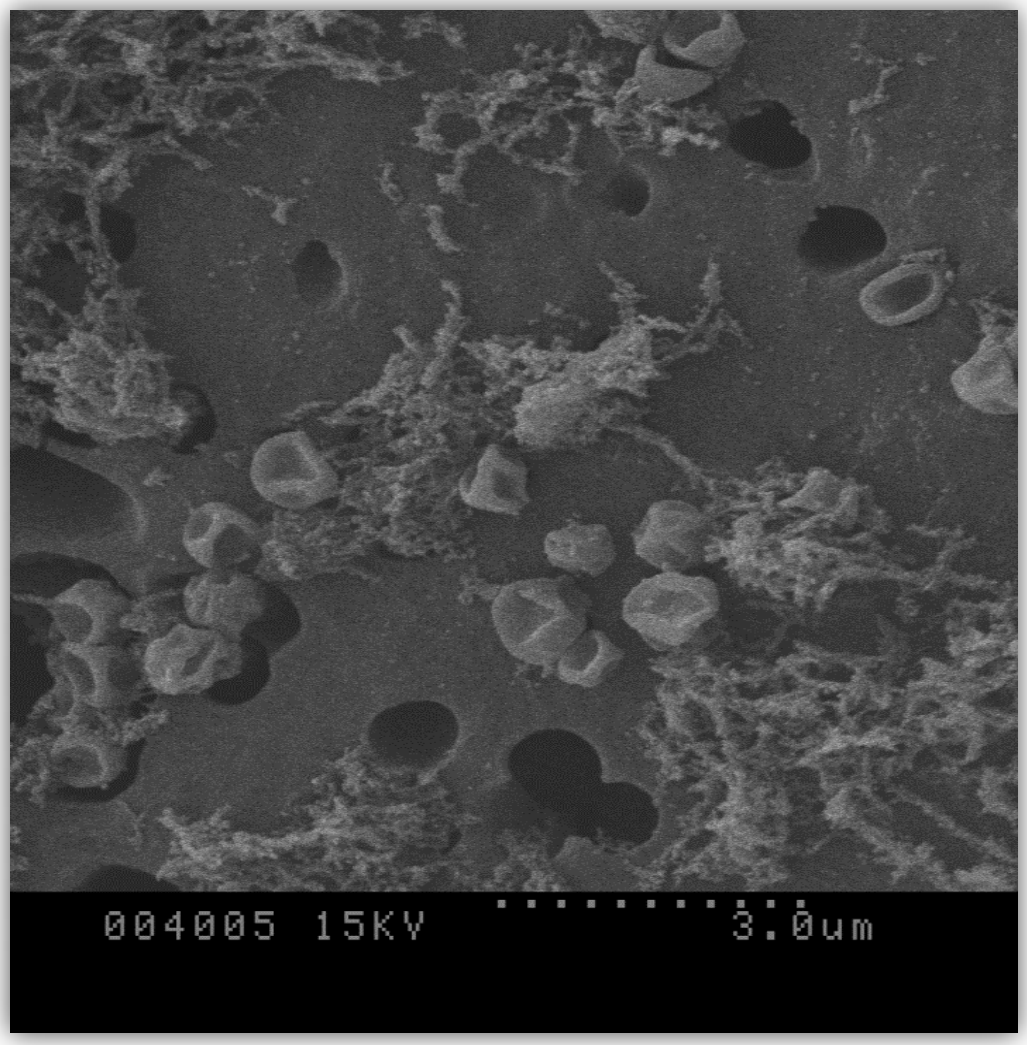

Figure 3.10 Cell size of *D. fermentans* was about 1 µm in diameter when it was grown on yeast extract (0.2 g/L) and starch (5 g/L) at 82^{o}C and pH 6.0.

The cell size increased to about 1.25 µm in diameter when the concentration of yeast extract increased to 2 g/L (Fig. 3.11).

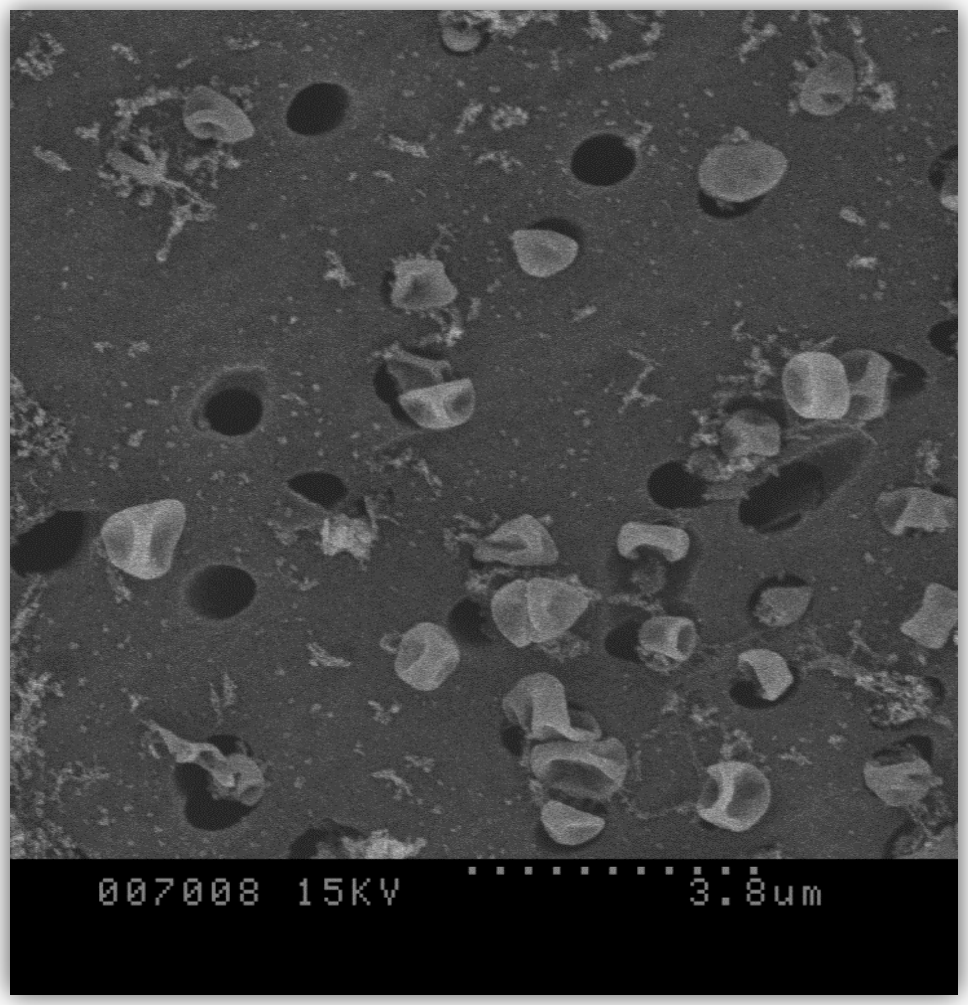

Figure 3.11 Cell size of *D. fermentans* was about 1.25 µm in diameter when it was grown on yeast extract (2 g/L) and starch (5 g/L) at 82^{0}C and pH 6.0.

The cell size increased to about 3 µm in diameter when the concentration of yeast extract increased to 10 g/L (Fig. 3.12). More cells could be observed because the cell size was much bigger than the pores of filter so they were captured in the upper side of filter. The filter pore size was 0.8 µm, which could cause loss of some cells. *D. fermentans* cells noticeably collapsed (Figures 3.10- 3.12), even after repeating the experiment. It was supposed that removal from the acetone for some minutes caused this phenomenon but afterwards, the samples were kept in acetone until the last minute of CPD. The same results were obtained even with this method. The experiment was done once without centrifuging the sample. In this case no cells were detected because of the coverage of the cells by a layer of precipitated culture medium.

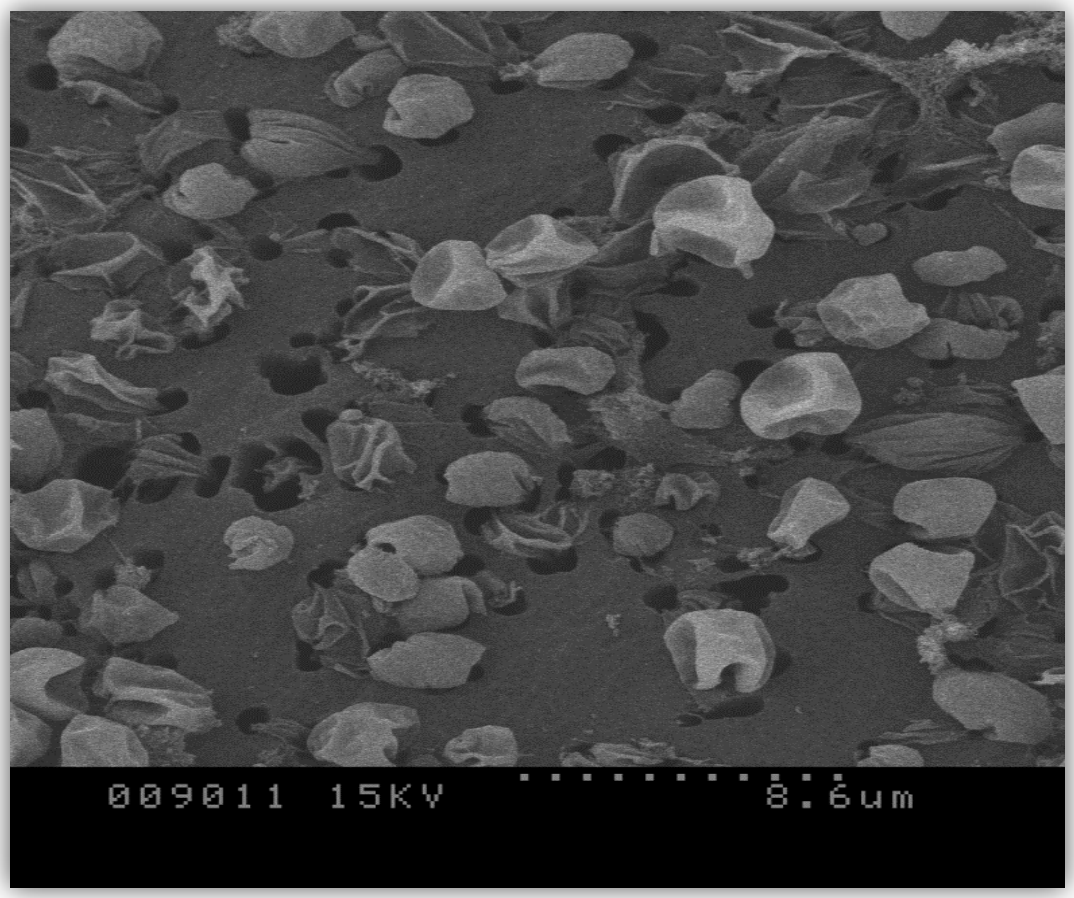

Figure 3.12 Cell size of *D. fermentans* was about 3 µm in diameter when it was grown on yeast extract (10 g/L) and starch (5 g/L) at 82^{0}C and pH 6.0.

Chapter 4

Discussion

4.1 Growth of *D. fermentans* on different substrates and the production of H_2

H_2 can be produced from a wide range of waste materials. Microorganisms that are able to do this process do not suffer from inhibitory impact of oxygen due to the anoxygenic process of fermentation (Nguyen et al., 2008). Microbial fermentation can potentially convert a variety of biomass-based substrates into H_2 at high rates (Adams et al., 1990). H_2 is a clean energy source with high energy content and is considered as the 'energy for the future'. Hydrogen is not as readily available as other fuels; consequently, other procedures are required to be done for its production. There are different methods of hydrogen production, such as steam reforming of hydrocarbons and partial oxidation of fossil fuels in high temperature, but these procedures are costly. Biological procedures are much better compared to chemical ones. Utilizing rich carbohydrate such as cellulose or starch is a good way to serve the purpose. Cellulose and micro-crystalline cellulose in mesophilic conditions can be used for hydrogen production by microorganisms (Kapdan et al., 2006). Mesophilic microorganisms are capable of producing H_2 at cultivation temperatures between 30 and 45^0C. However, H_2 yields are generally higher in hyperthermophiles than mesophiles (Vieille et al., 2001). The high growth temperatures for hyperthermophile may improve processing of carbohydrates to H_2. Hyperthermophiles are equipped with a wide array of glycan-degrading enzymes for producing biomass in their hydrothermal habitats. High temperature may help eliminate certain enzymatic and intermediate cooling steps (Verhagen et al., 1999).

Production of hydrogen at high temperature has several advantageous such as no need for reactor cooling, risk of contamination, a higher reaction. Starch and sucrose are accessible in large amounts in plants. Glucose and xylose are the main monomeric sugars and hemicellulose and

cellulose are the most abundant biomaterials. These substrates are mainly used for hydrogen production. H_2 and carbon dioxide will be produced from the microorganisms which are able to utilize glucose. *D. fermentans* is not able to utilize glucose, which is likely due to a lack of transporter as it could use cellobiose that could be hydrolyzed into glucose in the cells. It was not clear what all end products of *D. fermentans* were, which will be a future project to make that determination. However, it would be expected that it might share some similarities to other microorganisms (Verhaart et al., 2010) (Table 4.1 and Table 4.2).

In 2005, Perevalova et al reported the H_2 production by *D. fermentans* grown on amygdalin, dextran, fructose, lactose, laminarin, lichenan, maltose, ribose, starch and microcrystalline cellulose (Avicel). This was confirmed by the results from this study as *D. fermentans* could use cellulose, cellobiose, carboxymethyl cellulose, arabinose, filter paper and xylan to produce H_2 up to 12% (v/v).

Peptides are common substrates of anaerobic organotrophic hyperthermophilic archaea, while carbohydrates are metabolized by a limited number of hyperthermophilic archaeal species (Kengen et al., 1994). *Pyrococcus furiosus* can ferment maltose and cellobiose. *Pyrococcus woesei*, *D. amylolyticus*, *Thermococcus stetteri* and *Acidilobus aceticus* are able to grow fermentatively on starch (Perevalova et al., 2005). *Pyrococcus glycovorans* was able to grow fermentatively on glucose, cellobiose and starch. Hydrogen production (12%) by *D. fermentans* when grown on 4 g/L CMC, 5 g/L starch and 0.2 g/L yeast extract is comparable by *Kelebsielleoxytaca HP1* (12.3%) and *Thermoanaerobacterium* (17%). *Thermotoga maritima* is a heterotrophic, hyperthermophilic bacterium and ferments various carbohydrates (Jenney et al., 2008).

Table 4.1 Utilization of carbohydrates by microorganisms (Verhaart et al., 2010)

Organism	Temp(^{0}C)	Culturing Type	Substrate	End products	H_2/hexose (molecule /molecule)
Thermoanaerobacterium saccharolyticum YS485	55	Batch	Cellobiose	Acetate, lactate, Ethanol	0.87
Thermoanaerobacterium thermosaccharolyticum PSU-2	60	Batch	Starch	Acetate, ethanol, Butyrate	2.8
Clostridium thermocellum ATCC 27405	60	Chemostat	α-Cellulose	Acetate, lactate, ethanol, formate	1.65
Thermotoga elfii DSM 9442	65	Batch	Sucrose	Acetate	3.3
Thermotoga neapolitana DSM 4359	80	Batch	Glucose	Acetate, lactate	3.3
Caldicellulosiruptor saccharolyticus DSM 8903	70	Batch	Sucrose	Acetate, lactate	3.3

Thermoanaerobacter tengcongensis JCM11007	75	Batch	Starch	Acetate, ethanol	2.8
Thermococcus kodakaraensis TSF100	85	Batch	Glucose	Acetate	3.3
Pyrococcus furiosus DSM 3638	90	Batch	Maltose	Acetate, butyrate	2.9
Thermotoga maritima DSM 3109	80	Batch	Glucose	Acetate	3.2

Table 4.2 Yields and rates of bio-hydrogen production from pure carbohydrates by continuous dark fermentations (Kapdan et al., 2006)

Organism	Carbon source	H_2	$\%H_2$
Klebsiella oxytoca HP1	Glucose	1 mol/mol glucose	16.7
E. cloacae II T-BT 08	Glucose (1%)	2.2 mol/mol glucose	
E.coli	Glucose (20 g/L)		
H.alvei	Glucose (10 g/L)		
Klebsiella oxytoca HP1	Sucrose (50 mM)	1.5 mol/mol sucrose	12.3
C. pasteurium (dominant)	Sucrose (20 g COD/L)	4.8 mol/mol sucrose	
E. cloacae II T-BT 08	Sucrose (10 g/L)	6 mol/mol sucrose	28
Thermoanaerobacterium	Cellulose (5 g/L)	102 mL/g cellulose	23
Clostridium sp.	Microcrystalline cellulose (25 g/L)	2.18 mmol/g cellulose	18
E. aerogenes	Starch (20 g glucose/L)	1.09 mol/mol glucose	
Thermoanaerobacterium	Starch (4.6 g/L)	92 mL/g starch	17
C. pasteurianum	Starch (24 g/L)	106 mL/g starch	19
C. acetobutyricum	Glucose	2 mol/mol glucose	50
Clostridium sp.	Glucose (20 g COD/L)	1.7 mol/mol	42.6

		glucose	
Clostridium sp.	Glucose (20 g COD/L)	1.7 mol/mol glucose	42.6
Clostridium sp.	Glucose (10 g/L)		60
E. aerogenes HO39	Glucose (10 g/L)		
Klebsiella oxytoca HP1	Sucrose (50 mM)	3.6mol/mol sucrose	
C. butyricum + E. aerogenes	Starch (2%)	2.5 mol/mol glucose	
Thermococcus kodakaraensis KOD1	Starch (5 g/L)	3.33 mol/mol starch	‹10
C. termolacticum	Lactose (29 mmol/L)	3 mol/mol lactose	86

D. fermentans has a narrow growth pH range from 4.8 to 6.8, with an optimum at 6 (Perevalova et al., 2005). Without adding buffer, pH variations in the culture media were observed after adding Na_2S or during the growth. In 2004, Roychoudhury reported that there was no correlation between pH and sulfate reduction of spring waters (Roychoudhury 2004). Other metabolites like acetic acid and CO_2 produced by *D. fermentans* may decrease the pH during the growth phase (Perevalova et al., 2005). Based on the results of the first experiment, the optimum growth pH of *D. fermentans* was 7.5, but the pH dropped to 6 during growth. By dropping the pH to 6 it grew well. Furthermore, this pH was used as the best pH for the growth of *D. fermentans*. To avoid pH variations, HEPES was added to culture media. Ten mM of HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) to each 50 ml culture media was added to maintain the pH of 6. Afterwards, the pH of all bottles was adjusted at 6 before autoclaving and no fluctuation was observed. Even though the pKa of HEPES is 7.45- 7.65 at 25°C and it should work well to achieve a pH range of 6.8- 8.2, but adjustment of pH to 6 was done (Chakravarty et al., 2005). It may indicate that metabolites that would affect the pH of the media were in a relatively low concentration (< 1 mM).

The results obtained are comparable what reported by Perevalova et al in 2005. They proved that *D. fermentans* was able to grow on yeast extract (0.2 g/L) or in addition of starch (5 g/L) or avicel (5 g/L). The maximum growth is observed when both yeast extract and starch are added to culture media (Perevalova et al 2005). Their final cell yield on starch is 5×10^7 cells ml^{-1}, while the doubling time is 6.3 h, which is comparable with the results obtained from this study where the final cell yield was 1.26×10^8 cells ml^{-1} and generation time was 7.2 h.

Increase in cell size is observed for other hyperthermophiles when grown on increased concentration of yeast extract (Hao et al., 2003). The similar result was also observed for *D. fermentans* using SEM method. One of the disadvantages of using a standard method in preparing the sample of *D. fermentans* for electron microscopy was found to be the shrinkage of the cells, which was observed repeatedly by doing the experiment for a second time. The anaerobic nature of this microorganism might be one of the reasons for dehydration of cells; also utilization of different concentrations of chemical (acetone, glutaraldehyde) could have affected the dehydration procedure. Obviously the standard method for sample preparation is not the best way for electronic microscopy.

Although the images obtained from *D. fermentans* samples by using scanning electron microscopy were not as good as expected, it looks similar to what was observed for *P. fumarri* by Anderson et al in 2011. This microorganism is a crenarchaeon and it belongs to *Desulfurococcaceae* order (Anderson et al., 2011).

4.2 Future Directions

Biofuels such as biohydrogen, bioethanol, biodiesel and biobutanol might be produced from feedstocks such as sugar and starch- based crops (Henstra et al., 2007). These kinds of crops are commonly known as first-generation bioenergy crops. Trees and grasses are suggested as second-generation energy crops and the use of them will be increased in the future (Tilman et al., 2009). One of the issues in this aspect is displacement of food crop land (Antizar et al., 2008). On the other hand, by fermentation process bioethanol is produced from biodegradable carbohydrate substrate, such as corn starch and sugar cane. By acid or enzymatic pretreatment of insoluble cellulosic biomass, fermentable sugars can be obtained.

Lignocellulosic biomass by esterification and fermentation can be altered to liquid fuels through biological processes or thermochemical routes such as pyrolysis (Taylor, 2008). One of the aspects that should be considered in biofuel production from feedstocks is having lower life-cycle greenhouse-gas emission than traditional fossil fuels and with little or no competition with food production. Corn stover and straw from rice and wheat and other crop residues are rich in elements and are necessary for maintaining soil fertility and carbon stores. They can also reduce soil erosion (Tilman et al., 2009). Lignocellulosic materials are abundant and less expensive than corn grain for being used for the production of ethanol (Akin et al., 1990).

It would be attractive for future investigation on biofuel production using *D. fermentans* that can use different substrates. The biofuels that are produced from plants should not compete with food crops and also should not cause land clearing. Each year 500 million tons of feedstock is produced in United States, which can be good substitutes for fossil energy (Tilman et al., 2009). Using wastewater and other biomass as raw material for hydrogen production is interesting due to its environmentally friendly process (Ueno et al., 1995). None of the hyperthermophilic marine archaea is able to utilize crystalline cellulose efficiently but they can utilize the same glucans as well as hemicelluloses, such as xylans and mannans (Blumer et al., 2008).

Knowing the enzymes that are involved in biofuels production is critical. Activities of cellulase and hemicellulase can be determined by measuring the increase in reducing sugars produced by the enzymatic hydrolysis of the polymers (Liang et al., 2010). Metabolic pathways of *Desulfurococcus kamchatkensis* and *Desulfurococcus mucosus* are identified and these

microorganisms are in the same genus with *D. fermentans,* so probably they have similar pathways but there should be some differences in metabolic pathway of *D. fermentans* and other *Desulfurococcales* (Kublanov et al., 2009). There are different enzymes that are already extracted from hyperthermophiles. In extremophiles, cellulases which include endoglucanases, cellobiohydrolases and β-glucosidases, cleave β-1,4-glycosidic bonds of cellulose to glucose. Therefore these enzymes are interesting as potential biocatalysts for industrial applications (Park et al., 2011). One enzyme group which can be investigated in future is glycosyl hydrolases. These enzymes have the ability to hydrolyze the glycosidic bond between two or more carbohydrates (Bauer et al., 1998).

One of the enzymes that play a vital role in anaerobic metabolism is hydrogenase. This enzyme is able to catalyze the reversible oxidation of hydrogen. In 1930 first hydrogenases were discovered and they have since attracted interest for many researchers. Further understanding of the catalytic mechanism of hydrogenase might help scientists design clean biological energy sources, such as algae, that produces hydrogen (Adams et al., 1990).

Alcohol dehydrogenase (ADH) is the other enzyme involved in fermentation. In microorganisms, dissimilar from humans, normally they ferment glucose to ethanol instead of lactate. Pyruvate that is produced in glycolysis can be converted to carbon dioxide and acetaldehyde. ADH is responsible for reducing acetaldehyde to ethanol and NAD^+ is formed during this transformation (Cox et al., 2005). Some of hyperthermophiles are chemoorganotrophs such as *Thermococcus* that are able to grow on peptide-containing substrates. In *Thermococcus celer* and *Thermoccus litoralis,* glucose is converted to pyruvate by glycolysis via a modified

EM pathway. ADHs from *Thermococcus* strain ES1 contain iron and are responsible for ethanol production (Ma et al., 1994). ADHs have an effective role in alcohol, solvents and acetic acid production. ADHs from thermophiles are interesting for enzyme and alcohol production due to direct biomass fermentation to ethanol through distillation. They are also appropriate for NAD^+ and NADP synthesis. Based on cofactor specificity, there are different categories of ADHs. The most well-known one is NAD (P)-dependent (Radianingtyas et al., 2003).

The other enzymes are hemicellulases. These enzymes are able to hydrolyze hemicelluloses. They have a very important role in degradation of plant biomass. Hemicelluloses, the substrates for this enzyme, are a heterogeneous group of branched and linear polysaccharides. There are different kinds of hemicellolytic enzymes, such as endo-β-1,4-xylanase, exo-β-1,4-xylosidase, α-L-arabinofuranosidase, endo-α-1,5-arabinase, α-glucuronidase, endo-β-1,4- mannanase, exo-β-1,4-mannosidase, α-galactosidase, β-glucosidase, endo-galactanase, acetyl xylan esterase, acetyl mannan esterase, ferulic and *p*-cumaric acid esterases (Shallom et al., 1997).

Lignocellulosic biomasses are highly variable from site to site and even season to season. Various endoglucanases are required to hydrolyze crystalline cellulose to monosaccharides. They split cellulose chains internally and release shorter fragments (De Vrije et al., 2009). Cellobiohydrolases are known as cellulases, release disaccharide cellobiose units from cellulose. There are two different types of cellulolytic enzymes: one of them contained in the cellulosome and the other one is free-acting cellulases. Free acting cellulases are produced by a few anaerobic thermophilic bacteria such as *Caldicellulosiruptor* and *Thermoanaerobacter* which grow below

78^0 C. Thermophilic microorganisms that grow up to 65^0 C have cellulosomes (Blumer et al., 2008; Mandels et al., 1976).

All these enzymes can be explored in the future to understand the metabolic pathway of biofuels production. By understanding these pathways and enzymes scientists can design better solutions to develop a clean environment.

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