

**Hydrogen Production and Utilization of
Agricultural Residues by *Thermotoga* Species**

by

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Abstract:

Hydrogen can be a renewable energy source to replace conventional fossil fuels. Compared to current hydrogen production processes by consuming fossil fuels, biological hydrogen production has the advantage of being environmentally friendly because of the use of renewable and low value biological materials. Some hyperthermophiles, such as *Thermotoga* species, are capable of producing hydrogen during growth. In this study, *Thermotoga maritima*, *Thermotoga neapolitana* DSM 4359 and DSM 5068, were used to investigate their potential in converting selected sugars (glucose and xylose) and complex carbon sources (cellulose, starch, xylan and agricultural residues, such as barley straw, corn stover, soybean straw, wheat straw and corn husk) to hydrogen. In addition, factors which influenced growth and hydrogen production were studied, and optimal conditions for hydrogen production were obtained. All three *Thermotoga* species could grow in the presence of mono sugars (glucose, xylose) and complex carbohydrates (starch, xylan, milled corn husk). They all could produce hydrogen in the presence of micro-molar level of oxygen without addition of any reducing agents in the growth medium. Compared to the slight inhibition caused by L-lactate accumulation during the growth, gradual pH decreases were the main reasons to inhibit both growth and hydrogen production of *T. neapolitana* species. Increasing the initial pH of the growth medium to 8.5 and stabilizing the pH by 50 mM Tris buffer resulted in higher growth and hydrogen production of *T. neapolitana* strains. Adjusting the medium pH at early stationary phase also increased the hydrogen production, and fewer enhancements to the growth. The pH control methods also resulted in higher conversion efficiency (converting glucose to H₂) of *T. neapolitana* strains from 2.2 to 3.6 (H₂/glucose), which was approximately 90% of the theoretical efficiency (4 moles H₂ produced from 1 mole glucose). The expression of hydrogenases of *T. neapolitana* strains could also be increased by the pH control methods. *Thermotoga* species could grow and produce hydrogen using agricultural residues, such as corn husk, achieving 60% growth and hydrogen production as compared to that

from glucose. With pH control methods, hydrogen production by *T. neapolitana* strains from corn husk was higher than that from glucose without pH control. These results indicated that the pH was the main factor to affect both hydrogen production and growth of *T. neapolitana* species, and optimal conditions for hydrogen production could be achieved by using pH control methods. Selected agricultural residues could be utilized for biological hydrogen production by *Thermotoga* species with minimum pre-treatment, and the pH control methods could result in a higher hydrogen production compared to that from glucose. Further studies on the continuous growth and hydrogenases of *Thermotoga* species are needed for better understanding of the hydrogen production mechanisms.

Key words: hydrogen production, *Thermotoga neapolitana*, pH control, corn husk.

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Chapter 1

Introduction

1.1: An overview

The global energy consumption are mostly from fossil fuels since more than 90% of the world's energy needs are derived from fossil oil, natural gas and coal (Lay et al. 1999), which eventually leads to foreseeable depletion of current energy resources and contributes to global climate warming mainly due to the emission of pollutants, such as CO, CO₂, NO_x, SO_x, C_xH_x, soot and ash. These compounds released to the atmosphere result in environmental pollution. In order to solve these problems, efforts have been focused on development of clean and sustainable energy resources, which are renewable, non-polluting and capable of meeting the global energy needs. The integrated development of renewable energy resources may fit these requirements. The fossil fuels can be substituted, at least in part, by a number of alternative energy sources, such as hydrogen gas or alcohols. Hydrogen is one of the most abundant elements on earth, but, unfortunately, not readily available in nature. Compared to alcohol, hydrogen gas has advantages, such as clean end product (water only) and high energy production (hydrogen 120 MJ/kg, natural gas 33-35 MJ/kg, ethanol 21 MJ/kg) (Thomas 2000). Hydrogen gas is not only utilized as a source of energy, but also widely used as feedstock for the production of chemicals, hydrogenation in food industry, and production of electronic devices, processing steel, desulfurization and reformulation of gasoline in refineries. The major problems in utilization of hydrogen gas as a fuel are poor availability in nature and lack of inexpensive, energy efficient hydrogen production methods.

Approximately 50 million tones of hydrogen are produced world wide annually with a growth rate of nearly 10% per year (Winter 2005). Near 99% of the hydrogen gas is produced from fossil

fuels, while chemical production and some other renewable energy sources account for the rest (Hart et al. 1999). Conventional hydrogen gas production methods are steam methane reforming (SMR), partial oxidation (POX) and gasification. These processes use methane and hydrocarbons as substrates to produce hydrogen and release CO₂. Pyrolysis is another process to convert hydrocarbons to hydrogen gas without producing CO₂. These energy intensive processes require very high temperature (higher than 850°C) and consume fossil fuels. Water electrolysis is the cleanest reaction to produce hydrogen by directly splitting water into hydrogen and oxygen. This process is widely used in small scale hydrogen production, but still limited by electrical power supply, de-ionized water availability and costly electrodes. Compared to these technically matured hydrogen production processes, biological hydrogen production is still at its early stage of research and far from being a commercial process, because of its low conversion efficiency and high price. In recent years, biological hydrogen production has drawn considerable attention since it is not only environmentally friendly, but also provides a new way to produce renewable energy resources using solar energy, water, biomass and other organic substrates at ambient temperature and pressure. Combined with the waste recycling, biological hydrogen production as a “green technology” is a promising approach to meet the increasing energy needs as a substitute for fossil fuels.

1.2 Biological hydrogen production

Biological hydrogen production processes can be classified into three types: bio-photolysis, photodecomposition and fermentation. In these processes, microorganisms and key enzymes, such as nitrogenases and hydrogenases, are involved in the hydrogen evolution.

Bio-photolysis is a process by which H₂ and O₂ are simultaneously produced from water by photosynthetic microbes, such as microalgae and cyanobacteria, which use the same process found in plants and algal photosynthesis. The difference between these processes is that bio-

photolysis generates hydrogen gas instead of CO₂. Bio-photolysis or photosynthesis involves the absorption of light by two distinct photosynthetic systems operating in series: water splitting and O₂ evolving system (photosystem II or PSII) and a second photosystem (PSI) that generates the reductant used for CO₂ or H₂ formation (Ramachandran and Menon 1998). In this coupled process, two photons (one per photosystem) are used for each electron removed from water and used in CO₂ reduction or H₂ formation. In plants, there is no hydrogenase available, so there is no hydrogen gas production. Some microbes, such as microalgae and cyanobacteria, have hydrogenase or nitrogenase enzymes, and produce hydrogen gas only in certain conditions (Benemann 1997). Although bio-photolysis attracted considerable attention as a promising biological hydrogen production method, the evolution of oxygen is the major disadvantage because the accumulation of oxygen will inhibit, or even inactivate the anaerobic hydrogen evolution processes. The removal of oxygen during the process is essential for hydrogen production from bio-photolysis.

Photodecomposition (photo-fermentation) is an attractive process by which photosynthetic bacteria use light as an exogenous energy source to drive the hydrogen evolution. These bacteria grow in the light under anaerobic conditions on a variety of organic substrates. Photosynthetic bacteria are considered as the most promising microbial system for the biological hydrogen production (Jun et al. 1999). Compared to bio-photolysis of microalgae and cyanobacteria, the advantages of hydrogen production through photo-decomposition by photosynthetic bacteria are as below:

- (1) No O₂ produced, that means no O₂ inactivation of different biological systems.
- (2) Comparatively high theoretical hydrogen conversion rate and production,
- (3) Ability to consume organic substrates, which can be a potential process used in organic waste treatment.

On the other hand, one of the disadvantages of photodecomposition is that the enzyme involved in the hydrogen production is nitrogenase, whose turnover rate (from proton to H₂) is much lower than that of hydrogenase. The limitation of hydrogen evolution still needs to be solved.

Fermentation is a pathway in which NADH (or another reduced electron acceptor that is generated by oxidation reactions in the pathway) is re-oxidized by the metabolites produced by the pathway (White 1996). Fermentative microorganisms can use either biomass synthesized from photosynthesis or other fermentable substrates. Hydrogen can be produced under dark, anaerobic growth conditions on carbohydrate-rich substrates, giving organic fermentation end products, H₂ and CO₂. Hydrogenase is the key enzyme for the hydrogen production. In fermentative hydrogen production, carbohydrate-rich substrates can be directly fermented to H₂, CO₂ and organic acids. This hydrogen production can be influenced by the environmental conditions, such as temperature, pH, substrates, hydrogen partial pressure and metal ions.

Compared to the light-driven processes, less attention has been drawn by fermentative hydrogen production because it requires organic substrates and produces other by-products. However, fermentative hydrogen production has several advantages:

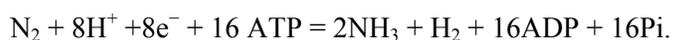
- (1) Fermentation process can produce hydrogen without the requirement of solar energy.
- (2) Fermentative microorganisms have comparatively high rate of hydrogen production.
- (3) Fermentative microorganisms have good growth rate during hydrogen production.
- (4) Fermentative microorganisms do not produce oxygen and have no inhibition to the hydrogenase.
- (5) Combined with hydrogen production, fermentative microorganisms can be used in organic waste treatment.

Another process called “combined process” that comprises photosynthetic and non-photosynthetic microorganisms is also very interesting. Both photosynthesis and fermentation can produce hydrogen during the processes, while non-photosynthetic microorganisms may be able to utilize the biomass produced by photosynthetic microorganisms. This process appears to be most promising once the growth condition and interaction of different microorganisms has been optimized. Many microbial species from microalgae, cyanobacteria, photosynthetic bacteria and fermentative bacteria are representatively used for biological hydrogen production (Table 1).

1.3 Hydrogenase

All processes of biological hydrogen production are fundamentally dependent upon the presence of hydrogen-producing enzymes. It is possible that the quantity or inherent activity of these enzymes could limit the overall process. Hydrogen-producing enzymes catalyze the simplest chemical reaction: $2\text{H}^+ + 2\text{e}^- = \text{H}_2$. However, a survey of all presently known enzymes capable of hydrogen evolution shows that they contain complex metal-clusters as active sites and that the active enzyme units are synthesized in a complex process involving auxiliary enzymes and protein maturation steps. At present, four enzymes that carrying out this reaction are known: nitrogenase, Fe-hydrogenase, NiFe hydrogenase and metal free hydrogenase.

Nitrogenase is a two component protein system that uses Mg ATP (2 ATP/e⁻) and low-potential electrons derived from reduced ferredoxin or flavodoxin to reduce a variety of substrates. In the absence of other substrates, nitrogenase continues to turnover, reducing protons to hydrogen. H₂ is formed as a by product during ATP-dependent reduction of nitrogen by the cells:



H₂ evolution by nitrogenase requires considerable ATP in addition to reductants. ATP, reductants and electrons are provided by either photo-synthesis or by degradation of sugars (Prince and Kheshgi 2005). Since 1 molecular H₂ production costs 8e⁻ and 16ATP, the turnover rate is much

Table 1: Microorganisms used for biological hydrogen production:

Type of microbes	Representative species	substrate	Major enzyme
Microalgae	<i>Chlamydomonas</i> sp. (Greenbaum 1990) <i>Scenedesmus obliquus</i> (Schnackenberg et al. 1993)	Water, CO ₂	Hydrogenase
Cyanobacteria	<i>Anabaena</i> sp. (Banerjee et al. 1989; Kumar et al. 1991) <i>Aphanothece halophytico</i> (Belkin et al. 1978) <i>Mastidocladus laminosus</i> (Bothe et al. 1990) <i>Nostoc muscorum</i> (Spiller et al. 1978) <i>N. spongiaeforme</i> (Vyas and Kumar 1995) <i>Oscillatoria Miami BG7</i> (Kumazawa and Mitsui 1981) <i>Phormidium valderianum</i> (Bagai and Madamwar 1998) <i>Plectonema boryanum</i> (Sarker et al. 1992) <i>Westiellopsis prolifica</i> (Vyas and Kumar 1995)	Water, CO ₂	Nitrogenase and hydrogenase.
Photosynthetic bacteria	<i>Chromatium</i> sp. (Ohta et al. 1981) <i>Chlorobium limicola</i> (Gogotov et al. 1991) <i>Chloroexu aurantiacus</i> (Gogotov et al. 1991) <i>Halobacterium halobium</i> (Khan and Bhatt 1991) <i>Rhodobater</i> sp.(Fascetti et al 1998; Krahn et al 1996) <i>Rhodopseudomonas</i> sp. (Singh and Srivastava 1991; Vincezini et al 1982) <i>Rhodospirillum rubnum</i> (Zurrer and Bachofen 1982) <i>Thiocapsa roseopersicina</i> (Gogotov et al. 1991)	Organic substrate	nitrogenase
Fermentative bacteria	<i>Clostridium butyricum</i> (Tanisho et al. 1987) <i>C.pasteurianum</i> (Brosseau and Zajic 1982) <i>Citrobacter intermedius</i> (Brosseau and Zajic 1982) <i>Desulfovibrio vulgaris</i> (Bothe et al. 1990) <i>Enterobacter aerogenes</i> (Tanisho et al. 1983) <i>E. cloacae</i> (Kumar and Das 1999) <i>Escherichia coli</i> (Tanisho et al. 1987)	Organic substrate	hydrogenase

lower than that of hydrogenase. The reaction using nitrogenase is not very promising in hydrogen production (Kumar and Kumar 1991). Hydrogenase is an enzyme that is involved in the hydrogen production of microalgae, cyanobacteria, photosynthetic bacteria and fermentative microorganisms. The term “hydrogenase” does not refer to a single enzyme, but a class of enzymes. Hydrogenases are enzymes that catalyse the oxidation of hydrogen to protons and the

reduction of protons to hydrogen. NiFe hydrogenase, which is usually thought to be functioning as an “uptake” hydrogenase, is an enzyme whose normal metabolic function is to derive reductants from H₂ to proton. Although this membrane-bound enzyme plays an opposite role in hydrogen production, it is still an essential enzyme in bio-hydrogen processes and great attention has been paid (Pierik et al. 1998).

Fe-hydrogenase is also called reversible or classical hydrogenase. This cytoplasmic enzyme functions to remove excess reducing equivalents during fermentations carried out by strict anaerobic bacteria and the periplasmic enzymes function normally in hydrogen oxidation. These enzymes contain a unique complex Fe–S center in which one of the Fe atoms is complexed with CO and CN (Pierik et al. 1998). The highly reactive nature of this cluster, together with the proposed formation of an iron-hydride intermediate during proton reduction, may make the search for an oxygen stable hydrogenase a rather elusive goal (Peters et al. 1998). Since this enzyme is directly related to the hydrogen gas evolution, it is important to characterize this enzyme and the factors which can influence the catalytic direction and activities of Fe-hydrogenase (hydrogen uptake or evolution), including the partial pressure of hydrogen.

Metal free hydrogenase, also referred to an iron–sulphur cluster-free hydrogenase, was first reported in 1990 and it has an important structural difference with Fe-hydrogenase and NiFe hydrogenase (Zirngibl et al. 1990; Hartmann et al.1996). It was described as an H₂-forming methylenetetrahydromethanopterin dehydrogenase (Hmd) used to catalyze the reversible reduction of N⁵, N¹⁰-methenyltetrahydromethanopterin (methenyl-H4MPT) with H₂ to N⁵, N¹⁰-methylenetetrahydromethanopterin (methylene-H4MPT) (Berkessel et al. 1995; Hagemeyer et al. 2000). The purified Hmd contains two iron molecules per homodimer, but no acid-labile sulphur, indicating that it is devoid of iron–sulfur clusters. Based on the current reports, it was known that the presence of the iron was essential for the activation of H₂, and Hmd was not a “metal free”

hydrogenase, as previously proposed (Thauer et al. 1996; Schwoerer et al. 1993; Lyon et al. 2004).

In bio-hydrogen metabolic pathway, hydrogen gas is produced by consuming solar energy, water, organic substrates, which can be considered as renewable resources. Although these biological hydrogen systems provide a wide range of approaches to generate hydrogen gas and many progresses have been made, none of them are considered to be practical or commercial applications in the anticipated future. Low hydrogen production, low conversion efficiency, high production cost and considerable difficulty in separation and purification of the hydrogen from the gas phase, limit biological hydrogen production to be a competitive renewable energy generation process. Increasing the hydrogen production rate and the conversion efficiency, and using less expensive materials will be the only way to make biological hydrogen production a commercial process. The factors that influence the hydrogen production and conversion efficiency during the biological processes need to be studied; and the use of low value materials need to be tested.

The biological hydrogen production processes can be influenced by many factors, such as the oxygen inhibition, temperature, pH shifting, substrates consumption and end products accumulation. The first problem for the anaerobic bio-hydrogen production process is oxygen concentration, especially for photolysis, because photolysis produces hydrogen and oxygen at the same time. Approaches were applied to overcome this problem, include the use of oxygen scavengers or absorbers, and significant research efforts to increase oxygen tolerance of enzymes (hydrogenases or nitrogenases) are underway (Peters et al. 1998). Another important factor to influence the hydrogen production is pH value of the medium. The pH value of the growth condition not only influences the growth of the hydrogen-evolution microorganisms, but also directly influences the metabolic pathway to the produce hydrogen (Samir et al. 2004). With the accumulation of the end products, especially the organic acids produced during the fermentation,

the pH value of the growth condition will decrease to a level at which the growth and hydrogen production will be inhibited (Ueno et al. 1996). The optimization of the hydrogen producing condition is one of the most attractive aspects within the biological hydrogen production research (Samir et al. 2004).

1.4 Agricultural residues

The substrates used in biological hydrogen production should be rich in carbohydrate content, biodegradable, easy to collect and cost efficiency. Pure carbohydrates, such as glucose, sucrose or starch, are not suitable because of their high cost. The waste biomass residues from the forest-related industry and agricultural industry seem to be the most promising resources because of their large annual production and low cost (Abedi et al. 2001). Among all biomass residues produced, forest-related industry accounts for 65%, whereas 33% comes from the residues of agricultural crops (Strehler and Stuetzle 1987). Compared to forest-related residues which can be used in low value wood-industry and paper industry, agricultural residues are easier to collect and have lower value (Hoogwijk et al. 2003). Conversion technologies for the production of energy from agricultural residues can be classified as biological (fermentation) or thermal processes (burning, pyrolysis and gasification). Since burning the residues produces less energy and more pollution, pyrolysis and gasification consume energy to maintain high temperature, so, these processes are not ideal conversion technologies for renewable energy production. Comparatively, biological conversion technology using agricultural residues is a better choice to produce liquid and gaseous energy carriers, for example, ethanol, methane, and hydrogen gas (McKendry 2002).

As one of the most abundant biomass resource, agricultural industry produces more than 3 billion tones commodities world wide annually (FAO yearbook 2005-2006), and about 60% of which come from wheat, rice, barley, maize and soybean (Table 2). Among all the agricultural harvests, about one half or more productions are agricultural residues (Unger 1994). The main components

of agricultural residues are three types of polymeric compounds: cellulose (35-50%), hemicelluloses (20-35%) and lignin (10-25%) (Wyman 1994). Cellulose is the most abundant component of plant biomass and is found in nature almost exclusively in plant cell walls, although it is produced by some animals (e.g., tunicates), as well as a few bacteria (Lynd et al. 2002). Cellulose is chemically composed of glucose monomers only with the linkage of β -1, 4 (Starch is also composed of glucose only with the linkage of α -1, 4) (Brown et al. 2000). Despite great differences in composition and anatomical structure of cell walls across the plant cells, high cellulose content—typically in the range of approximately 35 to 50% of plant dry weight—is a unifying feature (Lynd et al. 1999). In most cases, however, the cellulose fibers are embedded in a matrix of other structural biopolymers, primarily hemicelluloses, comprising 20% to 35% of plant dry weight, and lignin, comprising 5% to 30% of plant dry weight (Brown et al. 2000). An important feature of cellulose, which is unusual in the polysaccharide world, is the crystalline structure. That means cellulose is synthesized in nature as individual molecules (linear chains of glucosyl residues) which undergo self-assembly at the site of biosynthesis. Approximately 30 individual cellulose molecules are assembled into larger units known as elementary fibrils, which are packed into larger units called microfibrils, and these are assembled into the familiar cellulose fibres (Lynd et al. 1999).

Hemicelluloses are the second most common polysaccharides in nature and represent about 20–35% of plant biomass. Unlike cellulose, hemicelluloses are not chemically homogeneous. Hemicelluloses are heterogeneous polymers of pentoses (xylose, arabinose), hexoses (mannose, glucose, galactose) and sugar acids. Hardwood hemicelluloses contain mostly xylans, whereas softwood hemicelluloses contain mostly glucomannans (McMillan 1993). Xylans, as a major component of hemicelluloses, are heterogeneous polysaccharides with a backbone consisting of β -(1, 4)-linked D-xylosyl residues with several side-groups attached to the main chain. The glucomannans, on the other hand, are heteropolymers of glucopyranose and mannopyranose

Table 2: Production of main agricultural commodities in 2004 (FAO yearbook 2005-2006; in 1,000 tones):

product	Wheat	Rice	Barley	Maize	Rye	Oat	Millet	Sorghum	Potatoes	Soybean
Canada	25860	---	13186	8836	418	3683	---	---	5171	3048
U.S.A.	58738	10470	6091	299917	210	1679	342	11555	20686	85013
world	629873	608368	153830	724515	17650	25828	27767	57924	330125	206408

joined by $\beta - (1, 4)$ linkages with $\beta - (1, 6)$ branches to other substituents. The glucomannans and xylans are often grouped together and called hemicelluloses (Saha 2003).

Lignin is a large, complex polymer derived mainly from the dehydrogenative polymerization of cinnamyl alcohols. The three principal alcohols are *p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol, although other monomers can also be used in lignin biosynthesis (Whetten et al. 1998). Within a lignin polymer, the monolignol residues are interconnected by a variety of bonds that can bond to polysaccharides, such as cellulose, xylan and protein, resulting in multifarious and sturdy three dimensional structures (Lynd et al. 1999). The macromolecular properties and structural characteristics of lignin make it very difficult to be biodegraded.

Cellulose, hemicellulose and lignin are three main polymers to consist the cell wall of agricultural residues, and the total complex of these polymers is often referred as lignocellulose (McMillan 1993). Cellulose is built up as fibres that are partially arranged in a crystalline structure, integrated with hemicellulose and embedded in a matrix of lignin. Compared to glucose and starch, the main problem encountered with the biological utilization of lignocellulose is the inaccessible structure because the sugar availability of polymers is very low. To solve this problem, the pre-treatment of agricultural residues is required in biological conversion processes. The pre-treatment and hydrolysis of lignocellulose can be carried out physically (e.g. milling or steam treatment),

chemically (e.g. by acid or alkaline hydrolysis), enzymatically, or through a combination of these methods (Badal and Saha 2003). Considering the cost and pollution caused by these methods, simpler and more cost-efficient methods need to be developed.

1.5 Hyperthermophiles and *Thermotoga* species

Many microorganisms were reported to have the ability to produce hydrogen during fermentation (Table 1). Fermentative hydrogen production usually proceeds from the anaerobic glycolytic breakdown of sugars. The majority of microbial hydrogen production is driven by the anaerobic metabolism of pyruvate, formed during the catabolism of various substrates. The overall yield in these metabolic pathways is relatively low: only one to two hydrogen molecules are produced per molecule of pyruvate. For example, *Clostridium butyricum* (Tanisho et al. 1987) and *C. pasteurianum* (Brosseau and Zajic 1982) are reported to produce about 2 mole H₂ from 1 mole glucose during fermentation. In fact, this is a natural consequence of that fermentation have been optimized by evolution to produce cell biomass, not hydrogen. Also, in many organisms, the actual yield of hydrogen is reduced by hydrogen recycling due to the presence of one or more uptake hydrogenases, which consumed a proportion of the hydrogen production. On the other hand, the ratio as 2 moles H₂ produced from 1 mole glucose is far from theoretical maximum hydrogen conversion efficiency. Some microorganisms, such as *Enterobacter aerogenes* (Tanisho et al. 1983) and *E. cloacae* (Kumar and Das 1999; Kumar et al. 2001), have the ability to produce 2.2 to 3 mole H₂ by consuming 1 mole glucose. Based on the current reports, the breakdown to pyruvate produces 2 moles H₂ per mole glucose, and additional H₂ (in theory 2 moles per mole) can be produced by the action of NADH:ferredoxin oxidoreductase, which recycles the NAD and produces Fd_(red) that can drive hydrogen evolution. In theory, these fermentations are capable of generating 4 H₂ per glucose (Thauer et al. 1977)

Hyperthermophiles, including bacteria and archaea, normally cannot grow at temperatures lower than 60°C, representing the organisms at the upper temperature border of life (Stetter 1999). This characteristic enable them to be well adapted to their biotopes, such as water in volcanic areas, like solfataric fields and hot springs; in marine environment, such as shallow submarine hydrothermal systems, abyssal hot vents ('Black Smokers') and active seamounts. Hyperthermophiles belong to phylogenetically distant groups and may represent rather ancient adaptations to heat (Huber et al. 1990). Normally, they have maximum growth between 80°C and 110°C (Huber and Stetter 2001). Due to their heat adaptation, there is an increasing interest in hyperthermophiles for applications in biotechnological processes. Compare to mesophiles, the high growth temperature by hyperthermophiles not only decreases the risk of contamination, but also be helpful to the degradation of organic substrates, which are essential for the biological hydrogen production. In addition, the enzymes purified from hyperthermophiles maintaining activities at high temperature have broad industrial application (Huber et al. 2000).

Some hyperthermophilic species from *Pyrodictium*, *Hyperthermus*, *Thermoproteus*, *Desulfurococcus*, *Staphylothermus*, *Thermococcus*, *Thermotoga*, *Thermosipho*, *Fervidobacterium*, *Pyrococcus*, produce hydrogen by using peptides or carbohydrate as substrates (Adams and Kelly 2001). Among all these microorganisms, *Thermotoga* genus shows great potential in hydrogen production and has been well studied. *Thermotoga* genus is one of the deepest branches of the bacteria domain in the phylogenetic tree (Bocchetta et al. 2000), and belongs to *Thermotogales* order (Fardeau et al. 1997). *Thermotoga* species are rod-shaped cells surrounded by a characteristic sheath-like structure ('toga') over ballooning at the ends (Rachel et al. 1997). Based on the study, *Thermotoga* species have the ability to ferment various carbohydrates, like glucose, starch and xylan, forming acetate, L-lactate, H₂ and CO₂ as by products and end products (Huber et al. 1986). Currently, all the *Thermotoga* species are found to have the ability to produce hydrogen during the growth (Belkin et al. 1986; Van Niel and., Budde 2002; Suellen and Van

Ooteghem 2001). *Thermotoga elfii* (Van Niel and Budde 2002), *T. maritima* (Schroder et al. 1994), *T. neapolitana* (Suellen and Van Ooteghem 2001) have been studied in different aspects, especially the enzymes involved in carbohydrates utilization and hydrogen production. Compared to other species, *T. maritima* and *T. neapolitana* strains have attracted more attention than others because of their special properties (Bronnenmeier et al. 1995; Bok et al. 1998).

T. maritima was originally isolated from geothermal heated marine sediment at Vulcano, Italy (Huber et al. 1986). *T. neapolitana* has two different strains: *T. neapolitana* DSM 4359 was isolated from shallow marine sediment in a volcanic region near Lucrino, Bay of Naples, Italy (Belkin et al. 1986). *T. neapolitana* DSM 5068 was isolated from continental solfataric springs at Lac Abbe in Djibouti, Africa (Windberger et al. 1989) *T. maritima* and *T. neapolitana* are closely related and both of them ferment different carbohydrates: glucose, xylose, galactose, lactose, maltose, ribose, starch, xylan and so on. Cellulolytic and hemicellulolytic enzymes have been isolated from *T. maritima* and *T. neapolitana* (Bronnenmeier et al. 1995; Bok et al. 1998). These enzymes make it possible for *T. maritima* and *T. neapolitana* to utilize the lignocellulose-content substrates, including agricultural residues, to produce hydrogen. *T. maritima* and *T. neapolitana* DSM 4359 produce acetate, lactate, L-alanine which are accumulated in the liquid phase, while H₂ and CO₂ are produced and released in the gas phase as end products when glucose is used as substrate (Ravot et al. 1996). The growth and hydrogen production of *Thermotoga* species using different substrates and the optimization of hydrogen production are described in this thesis.

1.6 Objectives of the study

The goal of this study was to examine the growth and hydrogen production of *Thermotoga* species, and their capability of utilizing agricultural residues.

- (1) To examine the growth and hydrogen production of *Thermotoga* species influenced by different factors, including the substrate, temperature, salt concentration, initial pH and oxygen concentration in the gas phase. The utilization of different substrates and the accumulation of various end products were also investigated.
- (2) To optimize the conditions for the hydrogen production of *Thermotoga* species, especially *T. neapolitana*, the cell density, hydrogen production, conversion efficiency and hydrogenases activities were determined.
- (3) To test the capability of utilizing agricultural residues by *Thermotoga* species, in this part, the minimum pre-treatment was applied and the hydrogen production was optimized. The results obtained should show if it is possible to utilize low value biomass, instead of expensive substrates, to produce hydrogen without intensive pre-treatment.

Chapter 2

Material and methods

2.1 Organisms

Thermotoga maritima (DSMZ 3109), *T. neapolitana* (DSMZ 4359) *T. neapolitana* (DSMZ 5068) were obtained from the DSMZ - Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Mascheroder Weg 1b 38124 Braunschweig Germany.

2.2 Growth of *Thermotoga maritima*

The growth medium including trace mineral solution was prepared as previously reported (Verhagen and Adams 2001). *T. maritima* was routinely grown in a 160 ml serum bottle with 50 ml medium under anaerobic condition at 80°C unless otherwise specified. The composition of the basal medium (gram per liter): NaCl (Fisher Scientific, Canada), 20; (NH₄)₂CO₃ (Fisher Scientific, Canada), 1.14; KCl (BDH Inc), 2; MgCl₂·6H₂O (Fisher Scientific, Canada) 1.42; MgSO₄·7H₂O (Fisher Scientific, Canada), 1.72; CaCl₂·2H₂O (EM Science), 0.05; Yeast extract (EM Science), 2.50; Trace mineral solution, 10 ml; Glucose (Fisher Scientific, Canada), 4; 0.05% Resazurin (Sigma) , 1 ml. The medium pH was adjusted to 6.8.

The composition of the trace mineral solution (gram per liter): Nitrilotriacetic acid (Sigma), 1; EDTA (J.T. Baker Chemical Co), 0.29; MnSO₄·H₂O (Sigma), 0.5; FeCl₃·H₂O (J.T. Baker Chemical Co), 1.1; Na₂WO₄·H₂O (Sigma), 0.3; NiCl₂·H₂O (Sigma), 0.2; CoSO₄·H₂O (Sigma), 0.1; ZnSO₄·H₂O (BDH Inc), 0.1; CuSO₄·H₂O (Sigma), 0.01; Na₂MoO₄·H₂O (Sigma), 0.01.

To prepare the trace mineral solution, nitrilotriacetic acid was first dissolved in de-ionized water and pH was adjusted to 7.0 using NaOH (EMD Chemical Inc), then each component was added separately. Final pH was adjusted to 7.0.

For the growth in 50 ml medium, 0.135 ml 15% cysteine-HCl (Fisher Scientific, Canada) solution, 0.185 ml 1M KH_2PO_4 (BDH Inc) solution was added before inoculation. One ml 1 M $\text{Na}_2\text{S}_2\text{O}_3$ (J.T. Baker Chemical Co) solution was added if required. All the stock solutions were transferred with sterilized 1 ml sterile syringes (BD).

2.3 Stock solution preparation

2.3.1 1 M KH_2PO_4 (pH 7) solution

3.4 g KH_2PO_4 was dissolved in 20 ml de-ionized water, and the pH was adjusted to 7 using 10 M NaOH solution. After autoclaving the solution in a 50 ml serum bottle, the bottle was degassed and filled with N_2 in the gas phase, then stored at room temperature.

2.3.2 15% cysteine-HCl solution

Autoclaving 25 ml de-ionized water in a 50 ml serum bottle, the bottle was degassed for 30 minutes; then pressurized with nitrogen at 1.5 psi. After opening the bottle and quickly adding 3.75 g cysteine-HCl, the bottle was sealed again and further degassed and pressurized with nitrogen gas, then stored at room temperature.

2.3.3 1 M $\text{Na}_2\text{S}_2\text{O}_3$ solution

A solution of 1 M $\text{Na}_2\text{S}_2\text{O}_3$ solution was prepared by adding 6.205 g $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$ in 25 ml de-ionized water, which was filtered using a sterile syringe filter (25 mm 0.45 μm sterile Nylon syringe filter) (Fisher Scientific, Canada). The bottle containing sterilized solution was further degassed and pressurized, then stored at room temperature for up to 1 week.

2.3.4 15% glucose solution

After adding required amount of glucose in de-ionized water, the solution was filter-sterilized using a sterile syringe filter. The solution was degassed and pressurized, then stored at room temperature.

2.4 Growth of *T. neapolitana*

The growth medium for both *T. neapolitana* strains including trace mineral solution was prepared as previously reported (Van Ooteghem et al. 2002 and DSMZ website: <http://www.dsmz.de/microorganisms/html/media/medium000343.html>). *T. neapolitana* was routinely grown in a 160 ml serum bottle with 50 ml medium at 77°C under anaerobic condition unless otherwise specified.

2.4.1 Medium I for growing *T. neapolitana* (gram per liter):

KH₂PO₄(BDH Inc), 0.5; trace mineral solution (DSMZ141),15 ml; NiCl₂·6 H₂O(Sigma), 2 mg; NaCl, 20; artificial sea water, 250 ml; yeast extract, 0.5; Resazurin, 1 mg; Na₂S·9H₂O (Fisher Scientific, Canada),0.5; substrates were added as required and pH was adjusted to 7.0.

2.4.2 Trace mineral solution (DSMZ141)

Dissolve 1.5 g nitrilotriacetic acid in 1000 ml de-ionized water and adjust pH to 6.5 with a solution of 1 M KOH. Add the following minerals into the solution as below (gram per liter): MgSO₄·7H₂O (J.T. Baker Chemical Co), 3; MnSO₄·2H₂O (Sigma), 0.5; NaCl,1; FeSO₄·7H₂O (J.T. Baker Chemical Co), 0.1; CoSO₄·7H₂O (Sigma), 0.18; CaCl₂·2H₂O (EM Science), 0.1; ZnSO₄·7H₂O (BDH Inc), 0.18; CuSO₄·5H₂O (J.T. Baker Chemical Co), 0.01; KAl(SO₄)₂·12 H₂O (J.T. Baker Chemical Co.), 0.02; H₃BO₃ (Sigma), 0.01; Na₂MoO₄·2H₂O (Sigma), 0.01; NiCl₂·6H₂O (Sigma), 0.025; Na₂SeO₃·5 H₂O (Sigma), 0.3 mg. Adjust pH to 7 with KOH (BDH Inc). Stored at 4°C.

2.4.3 Artificial sea water

Dissolve NaCl, 27.7 g; MgSO₄·7H₂O, 7 g; MgCl₂·6H₂O, 5.5 g; KCl, 0.65 g; NaBr (Sigma), 0.1 g; H₃BO₃, 30 mg; SrCl₂·6H₂O (Sigma), 15 mg; citric acid (Sigma), 10 mg; KI, 0.05 mg; CaCl₂·2H₂O, 2.25 g in 1000 ml de-ionized water. Stored at room temperature.

2.4.4 Medium II for growing *T. neapolitana* (gram per liter) (Van Ooteghem's et al. 2002)

NH₄Cl (J.T. Baker Chemical Co), 1; K₂HPO₄, 0.3; KH₂PO₄, 0.3; MgCl₂·6 H₂O, 0.2; CaCl₂·2H₂O, 0.1; NaCl, 10; KCl, 0.1; yeast extract, 2; trypticase Soy Broth (TSB) (BD), 2; vitamine solution (DSMZ141), 10 ml; trace mineral solution (DSMZ 141), 10 ml; substrates were added as required. pH was adjusted to 7.

2.4.5 Vitamin solution (All chemicals are from Sigma)

Biotin, 2 mg; folic acid, 2 mg; pyridoxine-HCl, 10 mg; thiamine-HCl·2H₂O, 5 mg; riboflavin, 5 mg; nicotinic acid, 5 mg; D-Ca-pantothenate, 5 mg; vitamin B₁₂, 0.1 mg; p-Aminobenzoic acid, 5 mg; lipoic acid, 5 mg were dissolved in 1000 ml de-ionized water. Filter sterilized then stored at 4°C.

2.4.6 pH control methods

The medium pH was adjusted by adding 1 M NaOH or KOH solution, 1 M HCl-Triz buffer before incubation or by adding 1 M NaOH solution into the medium during the growth.

2.5 Procedures for preparing anaerobic medium

Fifty ml medium was transferred into a 160 ml serum bottle then the bottle was stopped using a grey butyl stopper (Snap-on, 20 mm Fisher Scientific, Canada) and sealed using an aluminum seal (20 mm CS/M, Fisher Scientific, Canada) with a hand crimper (Wheaton). After autoclaving (20 minutes), 25 G 5/8 disposable needles (BD) and 25 mm syringe filters (0.22µm MCE sterile,

Fisher Scientific, Canada) were used to connect the bottle to a manifold that was connected with a Welch vacuum pump (Model 1402B-80 Thomas Industries Inc. USA) and a nitrogen gas tank (Praxair Canada). Oxygen in the gas phase and liquid phase was removed by degassing (30 minutes) and pressurizing with nitrogen. After that, 3 minutes of pressurizing and 3 minutes of degassing were followed and repeated three times; then 10 minutes flushing and 5 minutes pressurizing with 1.5 psi nitrogen were applied.

2.6 Growth of *T. maritima* and *T. neapolitana* in 500 ml medium

The media and procedures described in the sections above (2.2 and 2.4) were also used for the large scale growth (500 ml). A one liter flask (Pyrex, Mexico) containing 500 ml medium was autoclaved for 30 minutes. The flask was sealed with a black rubber stopper (# 7) that had an access port with a sterile grey butyl stopper and an aluminium cap. The degassing and pressurizing procedure were the same as described in the preparation of 50 ml medium bottles.

2.7 Inoculum preparation for *T. maritima* and *T. neapolitana*

The inoculum cultures were freshly prepared at their late log phase of growth when the cell density was higher than 10^8 per ml.

2.8 Aseptic procedures for preparing growth medium and transferring inoculated cultures

The sterile stock solution or prepared culture was transferred using 1 or 5 ml sterile syringes (BD). During the processes of degassing the autoclaved medium, a 25 mm syringe filter (0.22 μ m MCE sterile, Fisher Scientific, Canada) was used to filter the nitrogen. The surface of the bottles' stopper was rinsed using 75% ethanol solution before transferring the stock solution or culture.

2.9 Monitoring of the growth

The growth of the microorganisms was monitored by direct cell counting using a Petroff-Hausser bacteria counting chamber (0.02 mm-deep, Hausser Scientific) under a Nikon Eclipse E600 phase-contrast light microscope (Nikon Co) equipped with 15× eyepieces and a 40× objective lens. In batch culture, the cell number was obtained from two parallel experiments (culture bottles) and the average value was used.

2.10 Determination of reducing sugar using DNS method

A solution of 0.4 M NaOH (Fisher Scientific, Canada) was prepared and a required amount dinitrosalicylic acid (DNS, Sigma) was dissolved in the solution to achieve a final concentration of 1% (w/v). The solution was stored at 4°C. Reducing sugar in the culture supernatant during growth was determined using the modified DNS method previously described (Bernfield 1995). A reaction mixture containing 0.1 ml sample and 0.5 ml 1% DNS solution was incubated at 95°C for 15 minutes, and then, 0.4 ml de-ionized water was added to make a total volume of 1 ml. The absorbance of the mixture was measured at 540 nm using a Genesys 10uv spectrophotometer. Reducing sugar concentration was calculated using a standard curve that was prepared using glucose as standard substrate. For each sample, the absorbance was obtained from two parallel experiments and the average value was used.

2.11 Determination of H₂ concentration

Gas Chromatograph (GC) Model 910 (Buck Scientific, East Norwalk, CT, USA) was used to quantitatively measure H₂ production in the gas phase. A thermal conductive detector (TCD), 6 feet 1/8 inch TMTM, 60/80 molecular sieve 5A and S.S column (Supelco Co, Bellfonte, USA) were installed in the GC for determination of H₂ evolution. Nitrogen (Praxair ON) was used as carrier gas, the pressure was 11 psi and the flow rate was 16 ml per minute. The temperature of the injector was 110°C, the temperature of the TCD was 100°C and the temperature of the oven was 60°C. A gas sample (50 µl) taken from the gas phase of the culture bottle was injected into

the GC injector immediately using a 50 μ l air tight micro-syringes (Hamilton Co. Reno, Nevada, USA). Under these conditions, the retention time of H₂ was about 0.45 minute and the peak-related data was recorded and calculated by the program Peak Sample 2.66. Duplicate analyses were carried out for each sample. Hydrogen concentration in the gas sample was calculated from the peak area using a standard curve, which was obtained using the samples that were prepared with the following procedures: A 50 ml sealed serum bottle was degassed and filled with nitrogen. The over pressure of nitrogen was released by inserting a disposable needle 25G 5/8 (BD) into the grey stopper. Another sealed serum bottle was prepared with same procedures, but filled with hydrogen. Different amounts of hydrogen gas were removed from the hydrogen bottle to the nitrogen bottles using a gas tight syringe and a series of hydrogen concentration samples from 0 to 10% were prepared. During the procedure, the same volume of de-ionized water as the hydrogen removed was added into the hydrogen bottle to keep a positive pressure in the hydrogen bottle. By using the different concentration of hydrogen samples, the standard curve was created. The molar hydrogen production from the culture can be calculated from the hydrogen concentration (% v/v) using following calculation: percentage (% v/v) of hydrogen in gas phase \times volume of gas phase of 110 ml \times pressure measured by the gauge, divided by the molar volume of hydrogen (22,400 ml/mol at 25°C). The amount of hydrogen was then converted to mmol by multiplying 1000, and the hydrogen concentration (mM) was calculated by the hydrogen production (mmol) divided by the culture volume (0.05 liter). For each sample, the measurement was duplicated and the average value was used.

2.12 Cell mass collection

Cells in the culture medium were harvested using a centrifuge. Fifty ml, 250 ml or 500 ml Sorvall centrifugation tubes (Kendro Laboratory Products, USA) were used depending on the culture volume. The Sorvall GSA (Sorvall Instruments) rotor and Sorvall SS-34 rotor (Du Pont Instruments) were used in Sorvall RC-5B refrigerated super-speed centrifuge (Du Pont

Instruments). The centrifugation temperature was set between 4 to 10°C and the speed was 8,000 r.p.m (GSA rotor: 10444 ×g; SS-34 rotor: 7719 ×g). The cells were stored at -80°C before use.

2.13 Collection and physical pretreatment of agricultural residues

The agricultural residues, barley straw, corn stover, soybean straw, wheat straw were provided by Dr. Duane Falk, Department of Plant Science, University of Guelph. Corn husk was purchased from a local store in Waterloo. All these agricultural residues were milled into powder using a Cyclone sample mill (UDY Corporation, Fort Collins Co. USA) equipped with a copper screen (0.25mm diameter opening). The agricultural residues powders were stored in 1200 ml plastic collection bottles (UDY Corporation, Fort Collins Co. USA) at room temperature.

2.14 Preparation of cell free extracts (CFE)

The frozen cells were put into a serum bottle with a stir bar, and then the bottle was stoppered by a grey butyl stopper and capped with an aluminium seal. The bottle was degassed and pressurized by nitrogen gas. Lysis buffer (pH 7.8) was prepared, containing 50 mM Tris (Fisher Scientific, Canada), 5% glycerol (Fisher Scientific, Canada), 2 mM SDT (sodium dithionite, Fisher Scientific, Canada), 2 mM DTT (dithiothreitol, Fisher Scientific, Canada), 1 mg /ml lysozyme (Sigma) and a trace amount DNase I (Sigma), then degassed to remove the oxygen in the gas phase. Five to ten times anaerobic lysis buffer (volume to the weight of the cells) was transferred to the bottle, then the cell suspension was stirred using a magnetic stirrer at 37°C for approximately 2 hours. The lysis mixture (crude extract) was transferred into 1.5 ml plastic microtubes (DiaMed Lab Supplies Inc.) and centrifuged at 10,000 r.p.m for 20 minutes using an Eppendorf 5415 centrifuge. The clear supernatant (CFE) was transferred to a 10 ml serum bottle. After the bottle was sealed, the manifold was used to remove the oxygen in gas phase. The CFE was used for further experiments. The CFE was stored either at -20°C (for less than 1 week) or -80°C (for longer than 1 week) if storage was required,.

2.15 Protein determination

Bio-Rad Protein assay dye reagent concentrate was used to determine the protein concentration in the crude and cell free extract (Bradford 1976). The assay mixture including 20 μ l sample, 200 μ l dye reagent concentrate (Bio-Rad Laboratories, Inc) and 780 μ l de-ionized water was immediately mixed by vortex mixture, and the absorbance at 595 nm was measured using a Genesys 10 vis spectrophotometer (Thermo Spectronic) after 30 minutes incubation at room temperature. A series of protein samples (BSA, Pharmacia Co) with the concentrations from 0.5 mg /ml to 10 mg /ml were used to obtain a standard curve. The linear relationship between protein concentration and the absorbance at 595 nm was obtained. For each sample, the absorbance was obtained from two parallel experiments and the average value was used.

2.16 Hydrogenase determination

Hydrogenase catalyzes the reversible oxidation of hydrogen gas according to the equation: $2\text{H}^+ + 2\text{e}^- = \text{H}_2$. The hydrogen evolution activity and the hydrogen oxidation activity of hydrogenase were measured using the methods described previously (Ma and Adams 2001). All the measurements were duplicated and the average value was used.

2.16.1 Hydrogen evolution assay

Hydrogen evolution activity of hydrogenase is measured by monitoring dithionite-reduced methyl viologen (MV) dependent hydrogen production. A 2 ml assay mixture was added into an 8 ml sealed glass vial, containing 100 mM buffer (EPPS: pH 7, 8, 9; CAPS: pH 10, 11), 1 mM methyl viologen (MV) and 10 mM sodium dithionite (SDT) (Fisher Scientific, Canada). The vial was placed in a shaking water bath (140 rpm) (Rose scientific Ltd.) at 80°C, and the reaction was initiated by adding the enzyme after 4 minutes pre-incubation. The hydrogen production was

measured by gas chromatography (see section 2.11) every 2 minutes. One unit of activity is equal to 1 μmol hydrogen produced per minute.

2.16.2 Hydrogen oxidation assay

Hydrogen oxidation activity of hydrogenase is measured by monitoring H_2 -dependent reduction of benzyl viologen (BV) at 580 nm at 80°C using a Genesys 10 UV-spectrophotometer (Thermo Spectronic). A 2 ml assay mixture was added into a stoppered glass cuvette (3 ml), containing 100 mM buffer (EPPS: pH 7, 8, 9; CAPS: pH 10, 11), 1 mM benzyl viologen with 100% hydrogen in the gas phase. After 4 minutes pre-incubation, the reaction was initiated by adding the enzyme. The absorbance of the reduced benzyl viologen was monitored using the spectrophotometer and a molar absorptivity ($7,800 \text{ M}^{-1}\text{cm}^{-1}$) was used to calculate the concentration of reduced benzyl viologen produced. One unit of enzyme activity is equal to 1 μmol H_2 oxidized or 2 μmol benzyl viologen reduced per minute.

Chapter 3

Results

3.1. Growth and hydrogen production of *Thermotoga* species

Three *Thermotoga* species, *Thermotoga. maritima*, *Thermotoga neapolitana* DSM 4359 and DSM 5068, could grow and produce hydrogen on glucose, xylose, starch and xylan, but not on cellulose. The growth and hydrogen production varied when different *Thermotoga* species and substrates were used. During the growth, hydrogen was produced in log phase and early stationary phase, but no hydrogen was produced in the late stationary phase. Detailed results are described in the following sections.

3.1.1 Growth of *T. maritima*

T. maritima could grow on glucose, xylose, starch and xylan (Fig.1) (final cell density 5×10^8 /ml). Growth was also observed in the absence of any added carbon source (control, final cell density 2.6×10^8 /ml), which was due to the presence of yeast extract (0.25% w/v) in the medium (Fig.1). The similar growth to the control was observed when cellulose was added into the medium. The doubling time of *T. maritima* was 0.33 hour when using glucose as carbon source. Hydrogen was produced along with the growth of *T. maritima* on various substrates (Fig.2). The hydrogen level was 15% to 17% (v/v) when using glucose, xylose, starch and xylan as substrates (Fig.2). Hydrogen level was only about 7% when the medium did not contain any added carbon source or in the presence of cellulose (Fig.2), indicating that there was no hydrogen production from cellulose. This result is consistent with the observation that *T. maritima* cannot grow on cellulose (Huber et al. 1986).

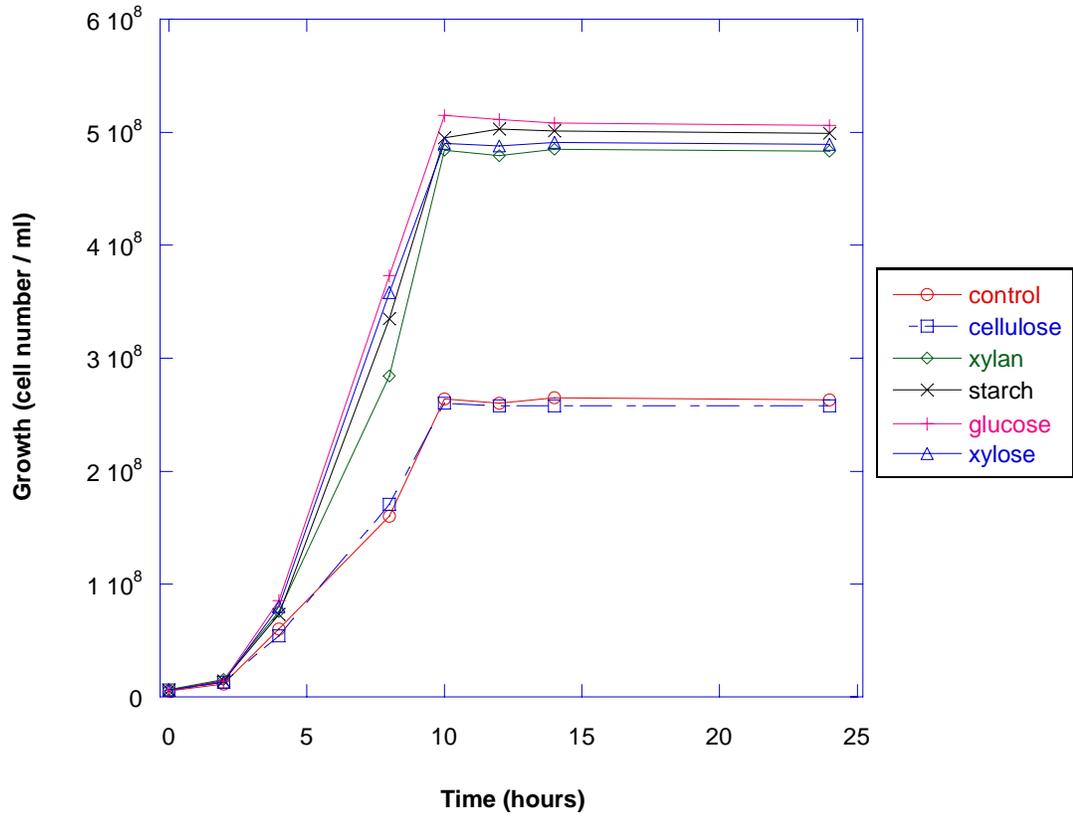


Figure 1: Growth of *T. maritima* in the presence of simple sugars and complex carbohydrates at 80°C. (“control” means no extra sugar or other carbon sources added except yeast extract)

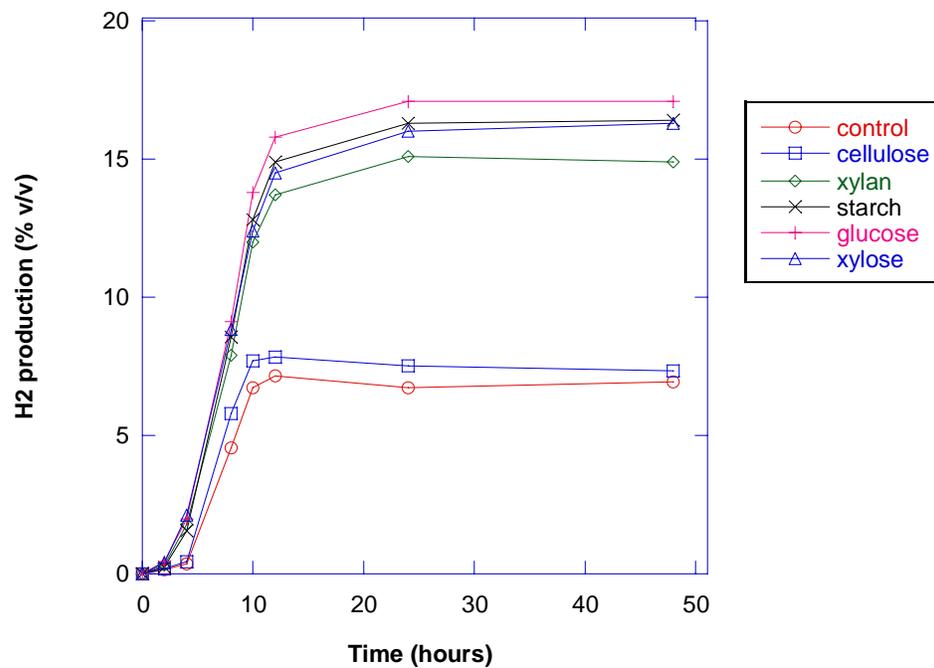


Figure 2: Hydrogen production of *T. maritima* in the presence of simple sugars and complex carbohydrates at 80°C.

3.1.2 Growth of *T. neapolitana* DSM 4359 on medium I

T. neapolitana DSM 4359 could grow on glucose, xylose, starch and xylan (Fig.3) (final cell density 2.9×10^8 /ml). Growth was also observed in the absence of any added carbon source (control, final cell density 1.6×10^8 /ml), which was due to the presence of yeast extract (0.05% w/v) in the medium (Fig.3). A similar growth to the control was observed when cellulose was added in the medium. The doubling time of *T. neapolitana* DSM 4359 was 0.71 hour when using xylose or glucose as carbon source in this medium. Hydrogen was produced along with the growth of *T. neapolitana* DSM4359 on various substrates (Fig.4). The hydrogen production was 12% when using glucose and xylose as substrates (Fig.4), however, hydrogen produced was only about 1.7% when the medium did not contain any added carbon source (Fig.4). In the presence of cellulose in the medium, the hydrogen produced was only 3.8%. The results from all growth experiments indicated that *T. neapolitana* DSM 4359 could not grow on cellulose, although, a slight increase of hydrogen production by *T. neapolitana* DSM 4359 was observed when cellulose was present in the medium.

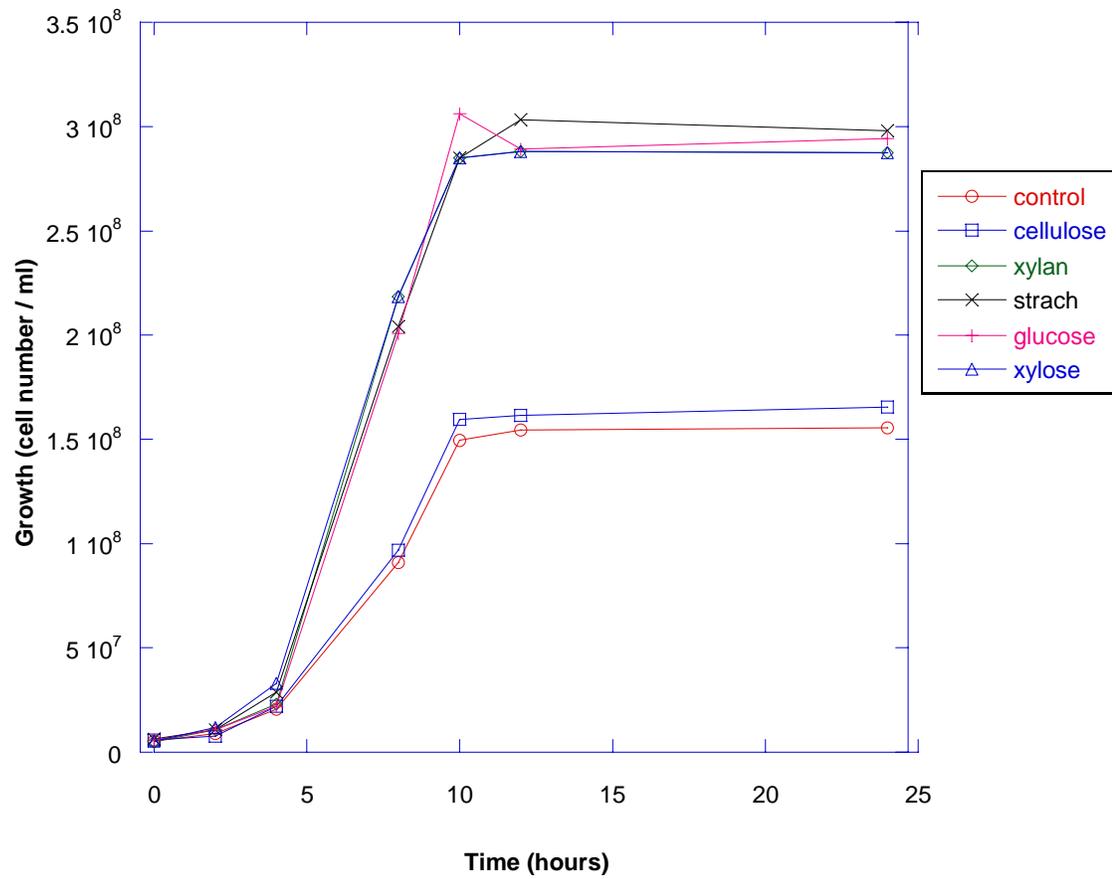


Figure 3: Growth of *T. neapolitana* DSM 4359 in the presence of simple sugars and complex carbohydrates at 77°C. (“control” means no extra sugar or other carbon sources added except yeast extract)

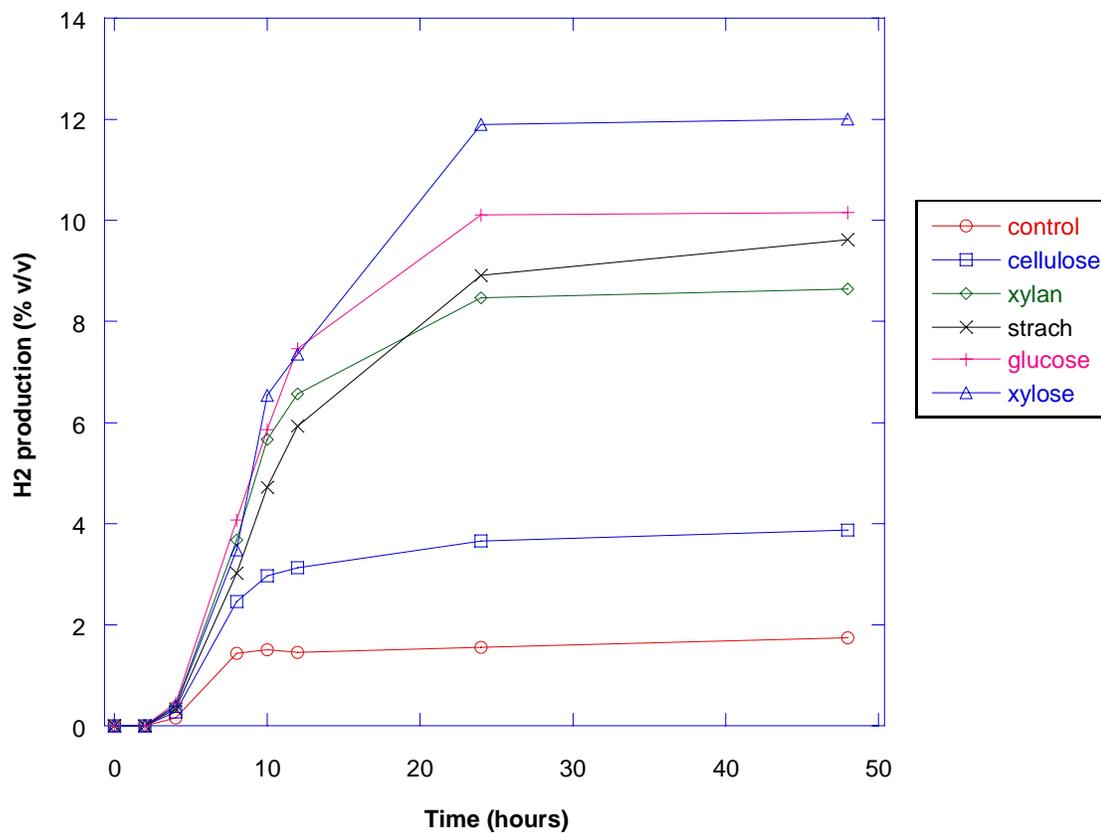


Figure 4: Hydrogen production of *T. neapolitana* DSM 4359 in the presence of simple sugars and complex carbohydrates at 77°C.

3.1.3 Growth of *T. neapolitana* DSM 4359 on medium II

T. neapolitana DSM 4359 could grow on glucose, xylose, starch and xylan (Fig.5) (final cell density 7.4×10^8 /ml). Growth was also observed in the absence of any added carbon source (control, final cell density 2.6×10^8 /ml), which was due to the presence of yeast extract (0.2% w/v) and TSB (0.2%) in the medium (Fig.5). A similar growth to the control was observed when cellulose was added in the medium. The doubling time of *T. neapolitana* DSM 4359 was 0.64 hour when using xylose or glucose as carbon source in this medium. Hydrogen was produced along with the growth of *T. neapolitana* DSM4359 on various substrates (Fig.6). The hydrogen produced was up to 20.6% when using glucose, xylose, starch and xylan as substrates (Fig.6), the hydrogen produced was only about 7.5% when the medium did not contain any added carbon source (Fig.6). In the presence of cellulose, the hydrogen production was the same as the control. These results indicated that *T. neapolitana* DSM 4359 could not grow on cellulose and no hydrogen was produced from cellulose.

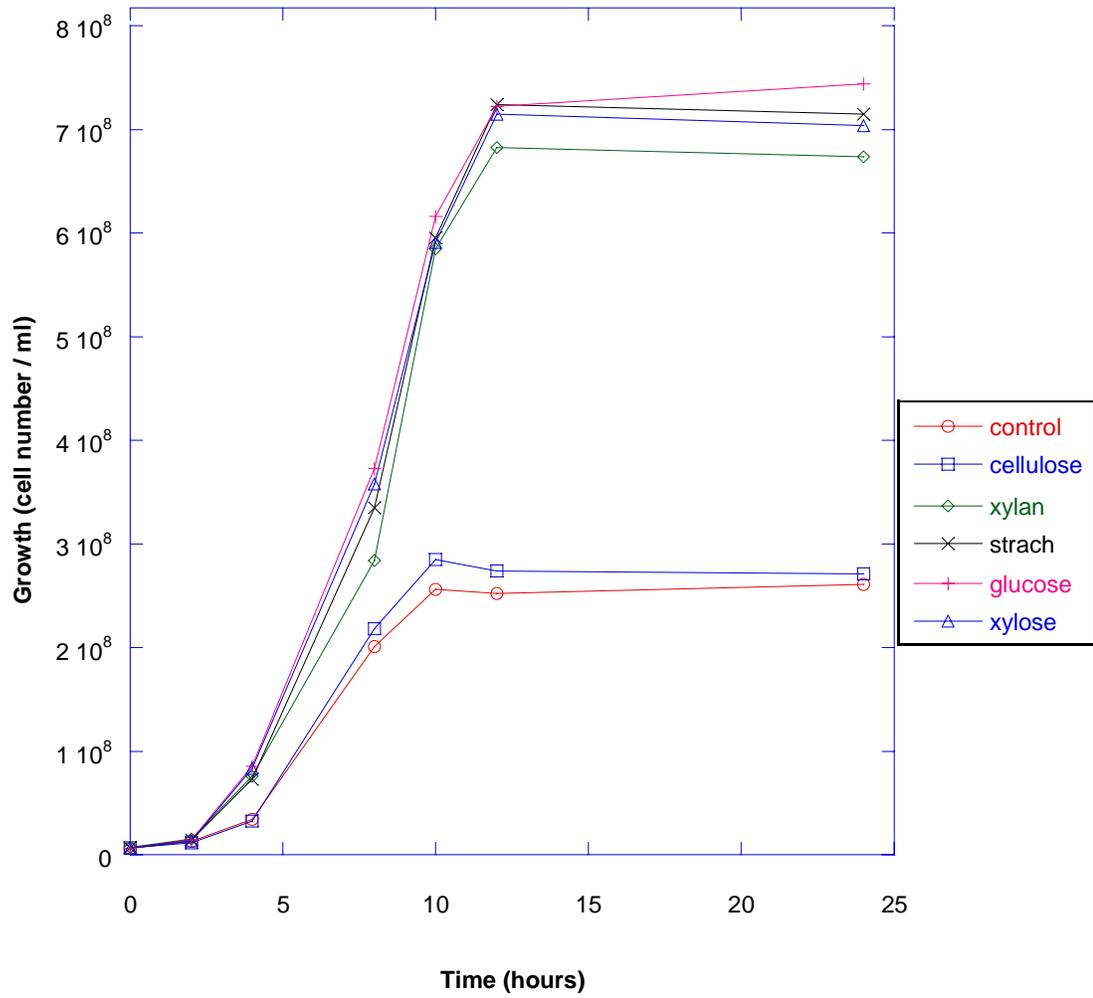


Figure 5: Growth of *T. neapolitana* DSM 4359 in the presence of simple sugars and complex carbohydrates at 77°C. (“control” means no extra sugar or other carbon source added except yeast extract and TSB)

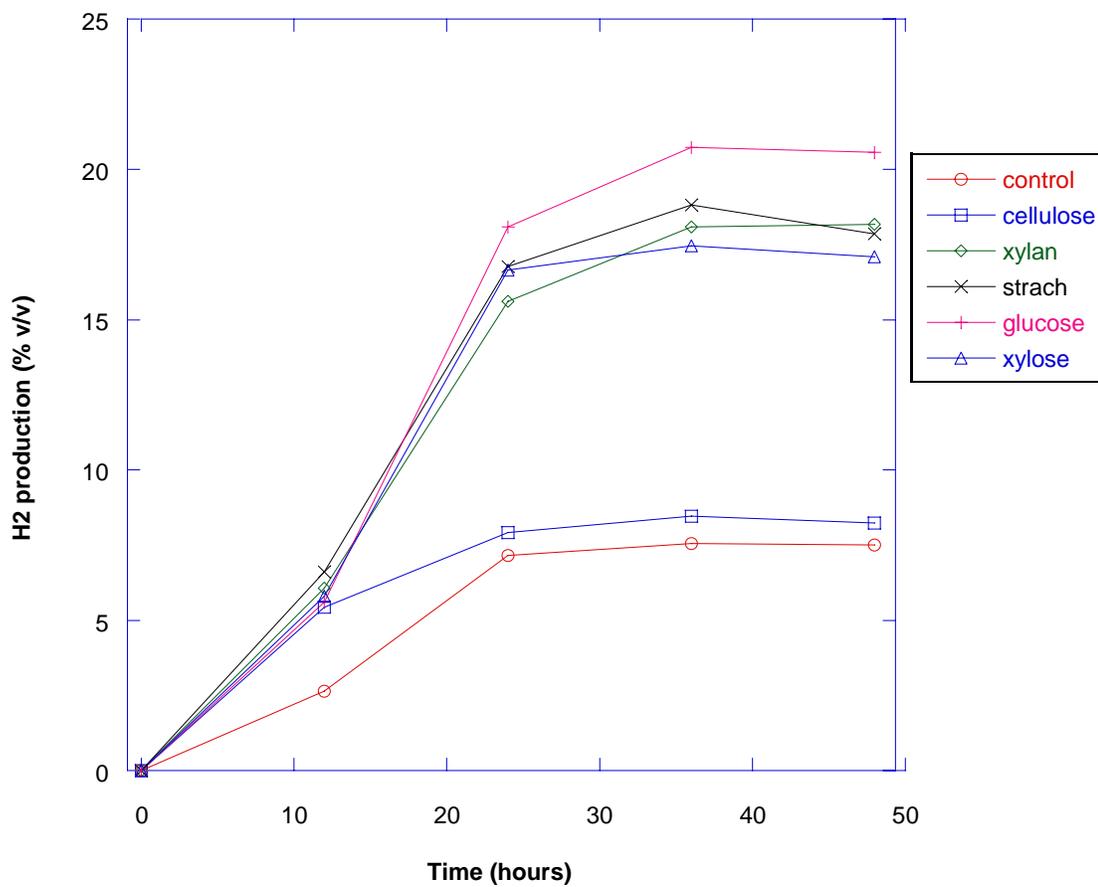


Figure 6: Hydrogen production of *T. neapolitana* DSM 4359 in the presence of simple sugars and complex carbohydrates at 77°C.

3.1.4 Growth of *T. neapolitana* DSM 5068 on medium II

T. neapolitana DSM 5068 could grow on glucose, xylose, starch and xylan (Fig.7) (final cell density 7.5×10^8 /ml). Growth was also observed in the absence of any added carbon source (control, final cell density 2.5×10^8 /ml), which was due to the presence of yeast extract (0.2% w/v) and TSB (0.2%) in the medium (Fig.7). A similar growth to the control was observed when cellulose was added in the medium. The doubling time of *T. neapolitana* DSM 5068 was 0.60 hour when using xylose as carbon source. Hydrogen was produced along with the growth of *T. neapolitana* DSM 5068 on various substrates (Fig.8). The hydrogen produced was 21% when using xylose and glucose as substrates (Fig.8); hydrogen production was only about 5.8% when the medium did not contain any added carbon source (Fig.8). In the presence of cellulose, the hydrogen production was only 7.9% (Fig.8). The results from all growth experiments indicated that *T. neapolitana* DSM 5068 could not grow on cellulose, although a slight increase in hydrogen production by *T. neapolitana* DSM 5068 was observed when cellulose was added in the growth medium.

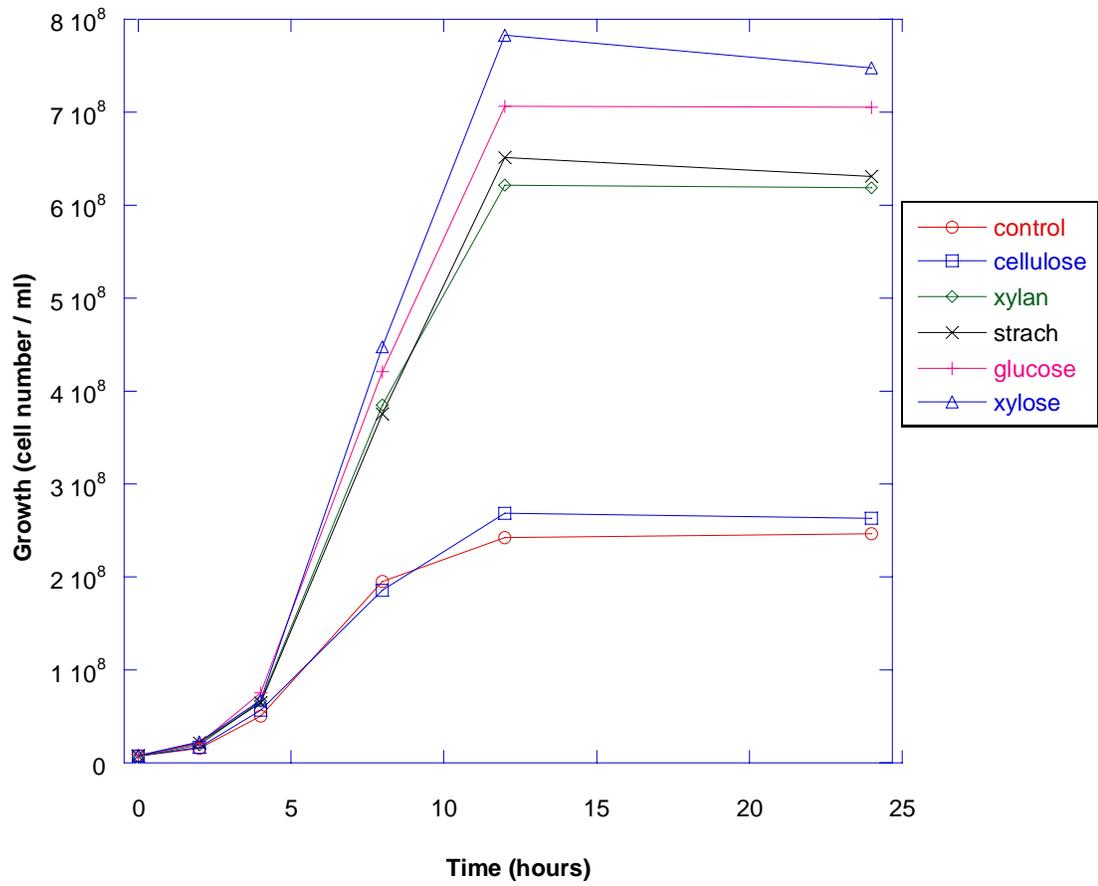


Figure 7: Growth of *T. neapolitana* DSM 5068 in the presence of simple sugars and complex carbohydrates at 77°C.

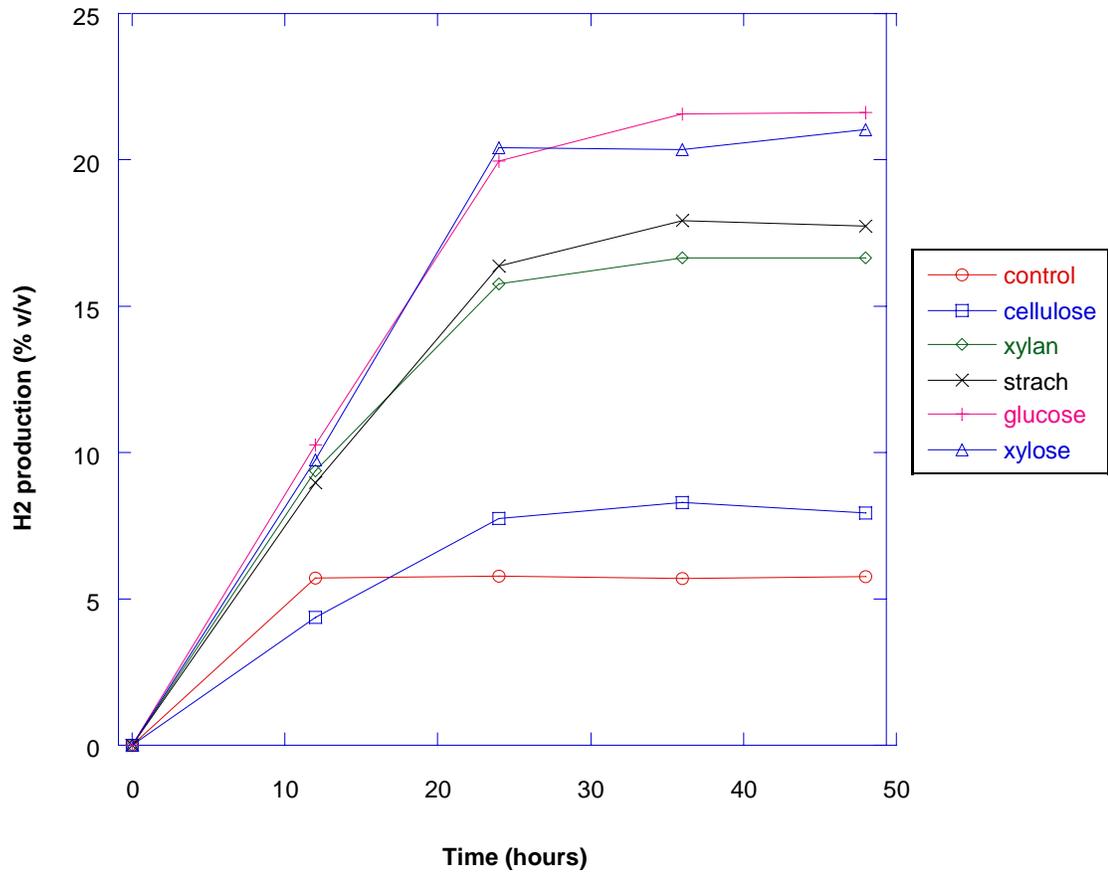


Figure 8: Hydrogen production of *T. neapolitana* DSM 5068 in the presence of simple sugars and complex carbohydrates at 77°C.

Table 3: Generation time, highest cell density and hydrogen production of *T. maritima*, *T. neapolitana* DSM 4359 and DSM 5068 grown on different carbon sources.

	<i>T. maritima</i>	<i>T. neapolitana</i> DSM 4359 on medium I	<i>T. neapolitana</i> DSM 4359 on medium II	<i>T. neapolitana</i> DSM 5068 on medium II
Generation time (hrs) and sugar used	0.33 glucose & xylose	0.71 xylose	0.64 glucose & xylose	0.60 xylose
The highest cell density (cells /ml) and sugar used	5.1×10^8 glucose	2.9×10^8 glucose	7.4×10^8 glucose	7.5×10^8 glucose & xylose
The highest hydrogen production (% v/v) and sugar used	17.1% glucose	12% xylose	20.6% glucose	21% glucose & xylose
Generation time (hours, control)	0.88	0.87	0.68	0.60
Cell density (cells / ml, control)	2.6×10^8	1.6×10^8	2.6×10^8	2.5×10^8
Hydrogen production (% v/v, control)	6.9%	1.7%	7.5%	5.8%

In conclusion, *T. neapolitana* strains could grow better on medium II than that of DSMZ medium I. *T. neapolitana* strains had longer generation time, better growth and higher hydrogen production than *T. maritima* when medium II was used (Table 3). Compared to complex carbon sources, mono-sugars could support better growth and higher hydrogen production in all *Thermotoga* species tested (Table 3).

3.2: Effects of temperature, pH, salt concentration and oxygen concentration on the growth and hydrogen production of *Thermotoga* species

The growth and the hydrogen production of *Thermotoga* species could be influenced by temperature, initial pH, salt concentration, oxygen concentration, as reported previously (Huber et al. 1986; Childers et al.1992; Windberger et al. 1989; Ravot et al. 1996; Van Ooteghem et al. 2002). In this project, however, detailed study was focused on *T. neapolitana* DSM 4359, and comparative study was done with *T. neapolitana* DSM 5068 because both strains had very similar growth requirements. *T. maritima* was also grown and used for comparison. Results are described in the following sections.

3.2.1 Effects of the temperatures on the growth and hydrogen production of *T. neapolitana* DSM 4359

The optimal temperature for the growth of *T. neapolitana* DSM 4359 was reported to be 77°C (Childers et al.1992), and this result was verified in this study (Fig.9). The growth (about 6×10^8 /ml) and hydrogen production (20% v/v) were very similar when the incubation temperature was ranging from 70°C to 80°C (Fig. 9 and Fig.10). No growth was observed at 90 °C and there was a very poor growth at 60°C (Fig.9).

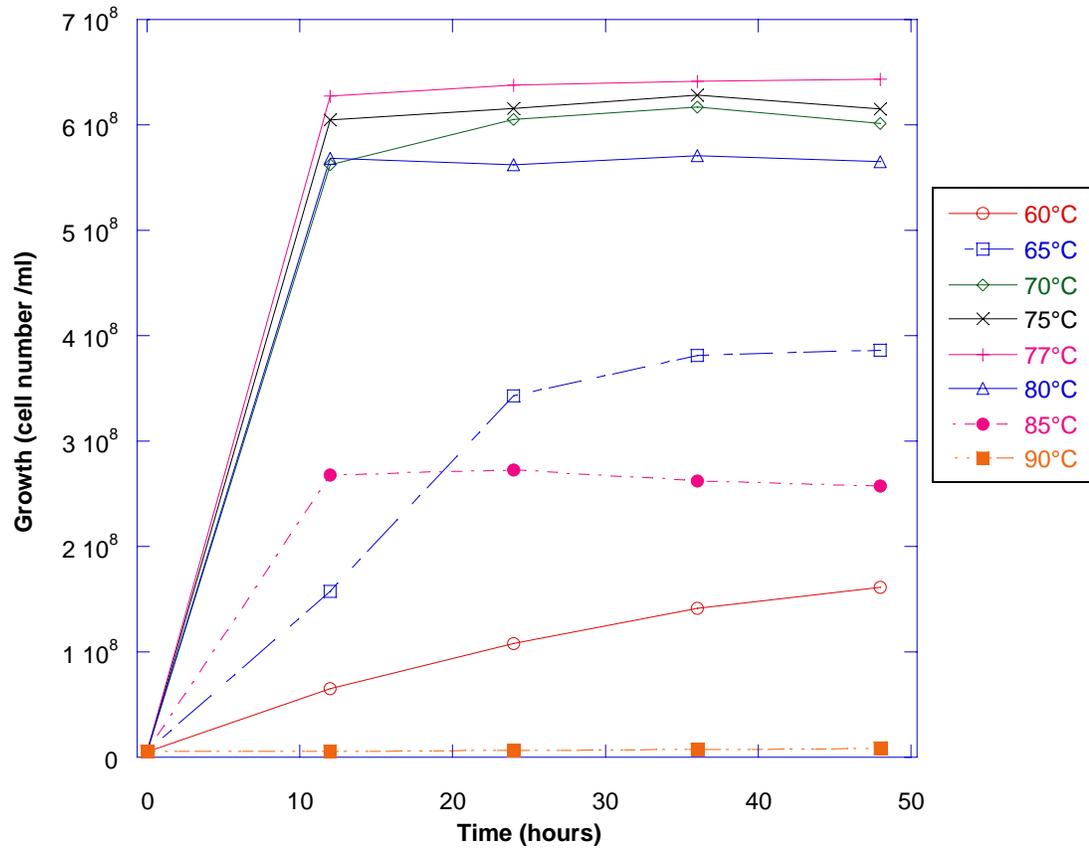


Figure 9: The growth of *T. neapolitana* DSM 4359 at different incubation temperatures.

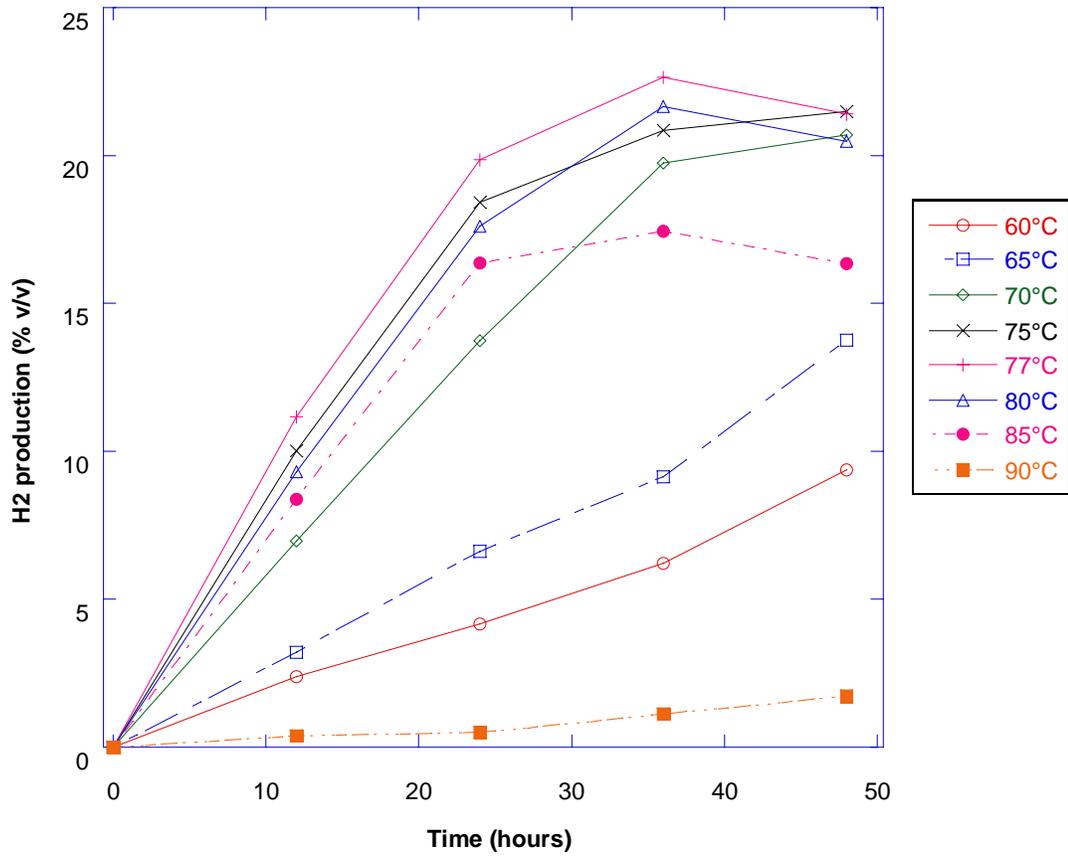


Figure 10: The hydrogen production of *T. neapolitana* DSM 4359 at different incubation temperatures.

3.2.2 Effects of initial pH of the media on the growth and hydrogen production of *T. neapolitana* DSM 4359

The growth of *T. neapolitana* DSM 4359 could be influenced by initial pH and the reported optimal pH for growth was 7 (Childers et al.1992). In this study, when the initial pH was decreased below 7, the growth and hydrogen production also decreased. The growth and hydrogen production were completely inhibited when the initial pH decreased to 5.5 (Fig.11 and Fig.12). On the other hand, when the initial pH increased, the growth rate was decreased slowly within the initial pH range from 7 to 8.5 (Fig.11), and the doubling time was about 0.7 hour and the final cell density was higher than 6×10^8 / ml after 12 hours incubation; both final cell density and hydrogen production increased along with the increasing initial pH value. The initial pH higher than 8.5 was not suitable for *T. neapolitana* to grow because the growth was poor (Fig.11). When the initial pH was 9, the doubling time was 2 hours and the cell density was 3×10^8 / ml after 48 hours incubation. When the initial pH was 9.5, the doubling time increased to 12 hours and the cell density was 7×10^7 / ml after 48 hours incubation (Fig.11). For hydrogen production, when initial pH was in the range from 7 to 8.5, the hydrogen production was higher than 20% (v/v) after 36 hours incubation. The initial pHs higher than 8.5 or lower than 7 could not achieve a comparable hydrogen production, even after 48 hours incubation (Fig.12).

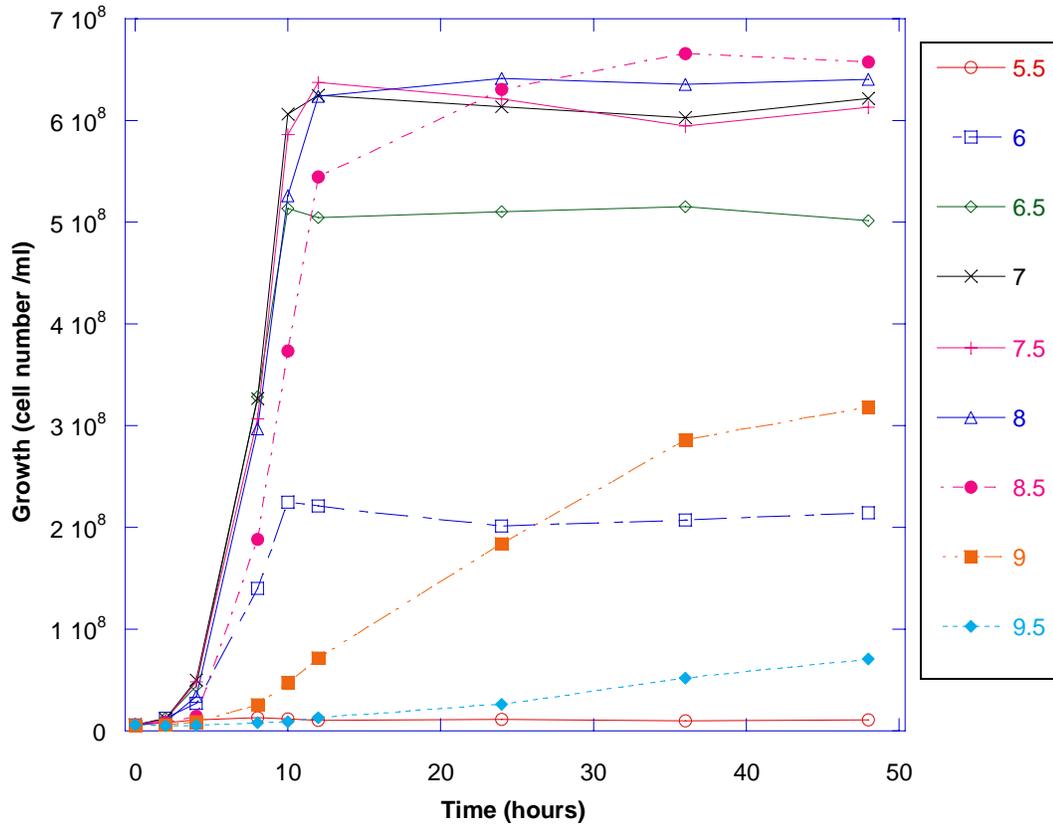


Figure 11: The growth of *T. neapolitana* DSM 4359 at different initial pH value incubated at 77°C. (The pH value was adjusted using 1 M HCl or 1 M NaOH solution)

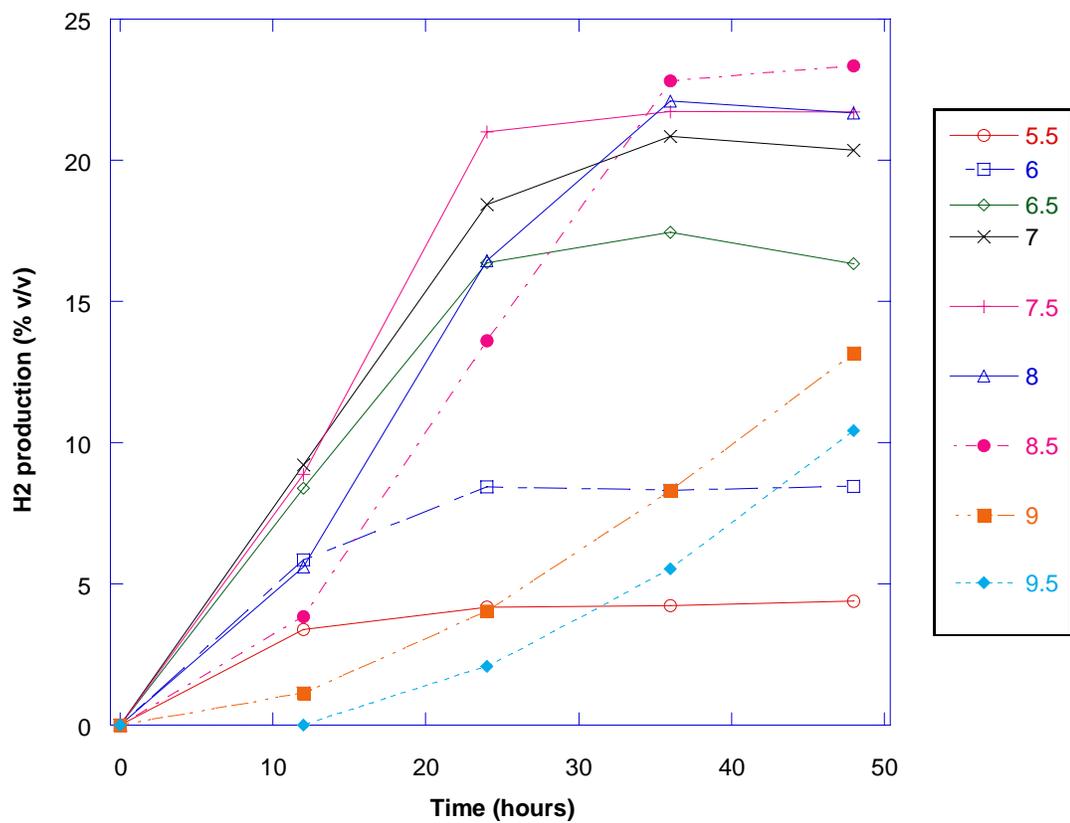


Figure 12: The hydrogen production of *T. neapolitana* DSM 4359 at different initial pH value incubated at 77°C.

3.2.3 Effects of salt (NaCl) concentration on the growth and hydrogen production of *T. neapolitana* DSM 4359

Concentration of NaCl used for the growth of *T. neapolitana* DSM 4359 was 1% w/v (Van Ooteghem et al. 2002) or 2% w/v (Childers et al.1992). Based on the results described below, when the salt concentration was 1% to 2%, the cell density of *T. neapolitana* DSM 4359 achieved higher than 6×10^8 / ml after 12 hours incubation. When the salt concentration was 3% w/v, *T. neapolitana* DSM 4359 could only achieved about 5×10^8 / ml cell density. *T. neapolitana* DSM 4359 could not grow when the salt concentration was higher than 5% or absent (Fig.13). For hydrogen production, the hydrogen production was about 20% (v/v) in the gas phase when the salt concentration was 1% and 2% w/v (Fig.14).

3.2.4 Effects of oxygen on the growth and hydrogen production of *Thermotoga* species

The anaerobic bacterium, *T. neapolitana* DSM 4359 was reported to be able to grow under micro aerobic condition (Van Ooteghem et al. 2002). In this study, *T. neapolitana* DSM 4359, *T. neapolitana* DSM 5068 and *T. maritima* were grown in the presence of different oxygen concentrations in the gas phase. *T. neapolitana* strains could grow on the medium without the addition of any reducing agents, such as cysteine-HCl, and the growth and hydrogen production were almost the same as that on the medium with reducing agents (Fig.15; Fig.16; Fig.17; Fig.18). The inhibition of growth and hydrogen production caused by the oxygen in the gas phase became greater along with the increase of the oxygen concentration in the gas phase, and the growth and hydrogen production were very low (cell density lower than 3×10^8 / ml, hydrogen level lower than 8% v/v) when the oxygen concentration was 1% (v/v).The growth and hydrogen production were entirely inhibited when the oxygen concentration increased to 2% (v/v) in the gas phase (Fig.15; Fig.16; Fig.17; Fig.18). Compared to *T. neapolitana* strains, the growth and hydrogen production of *T. maritima* were completely inhibited when the oxygen concentration was 0.5% (v/v) (Fig.19 and Fig.20). *T. neapolitana* strains showed better oxygen tolerance than *T. maritima* did.

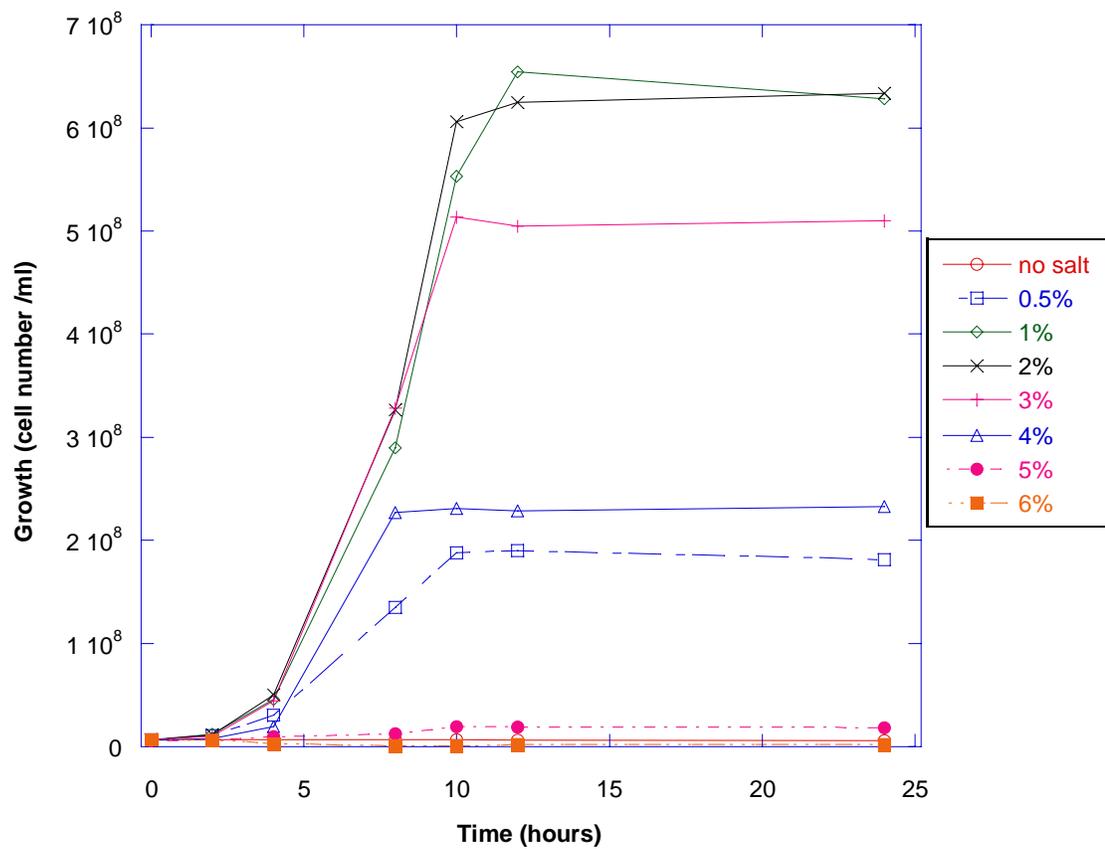


Figure 13: The growth of *T. neapolitana* DSM 4359 on different NaCl concentrations.

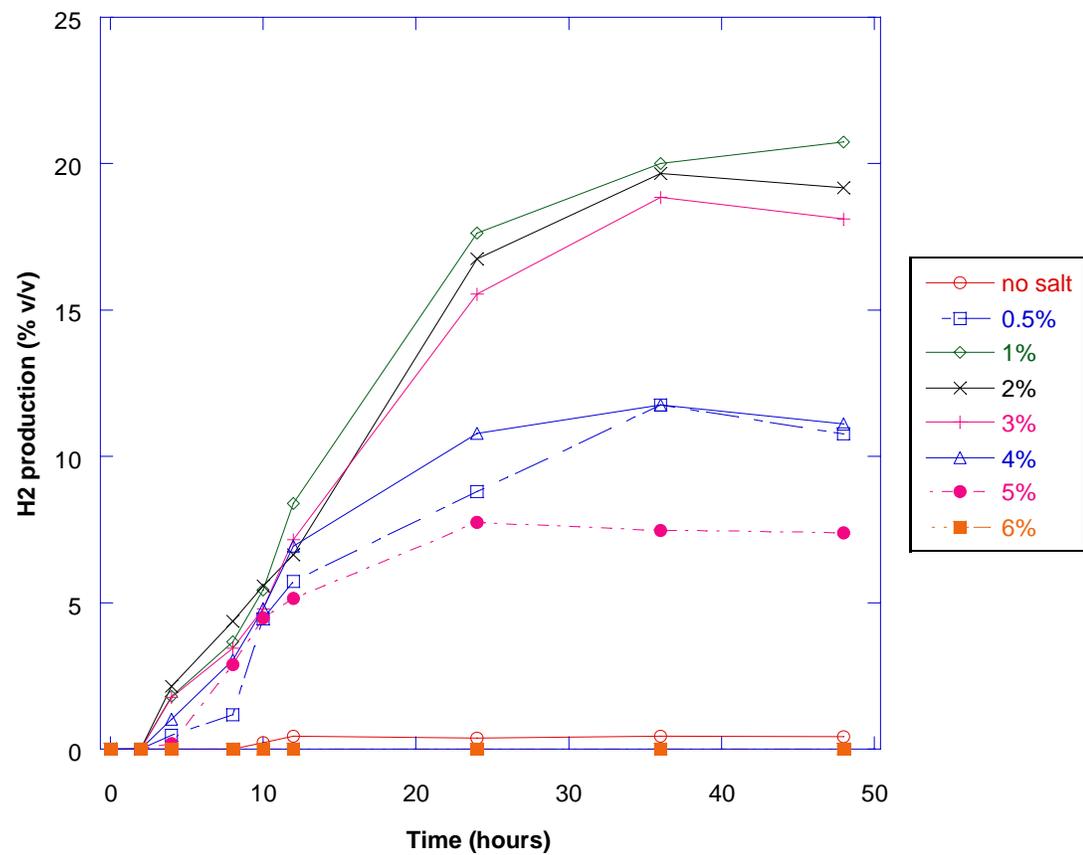


Figure 14: Hydrogen production of *T. neapolitana* DSM 4359 on different NaCl concentrations.

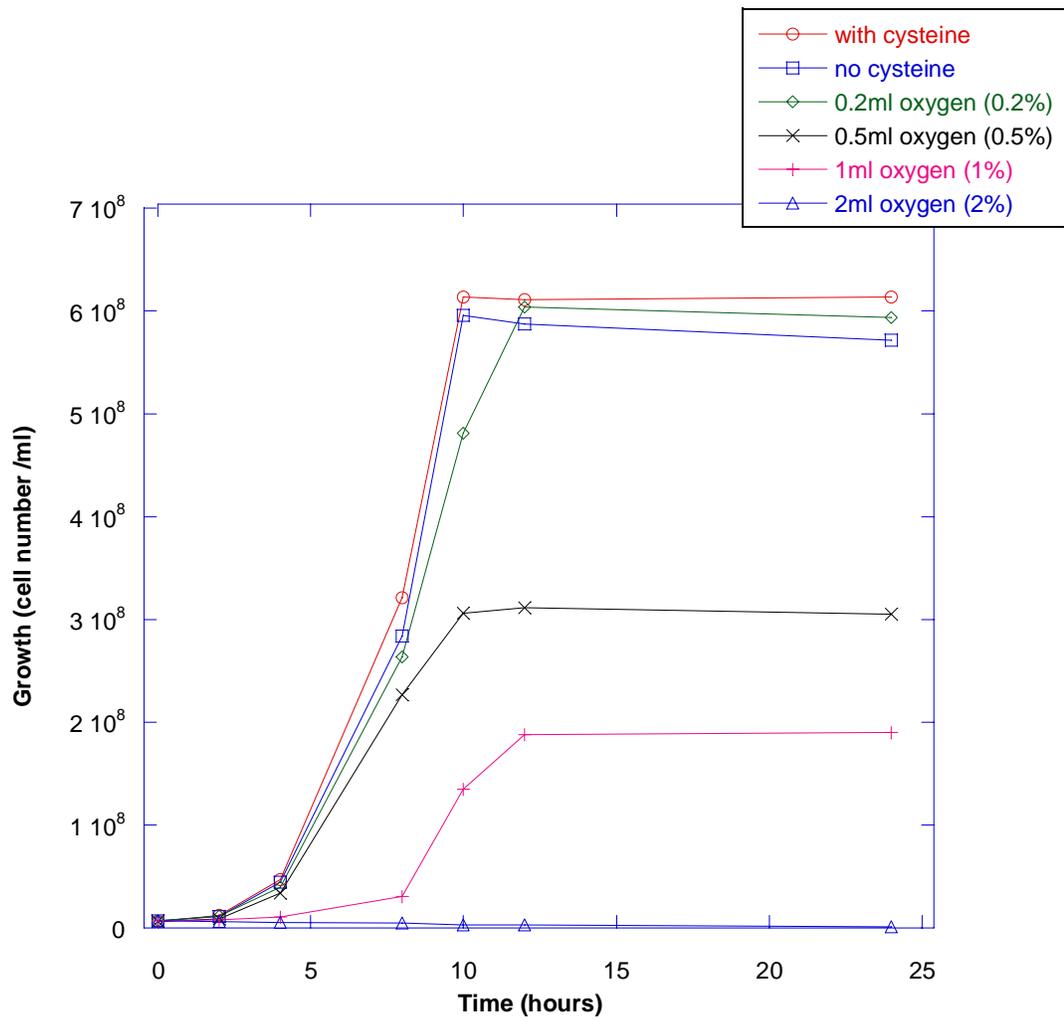


Figure 15: The growth of *T. neapolitana* DSM 4359 in the presence of different oxygen concentrations in the gas phase.

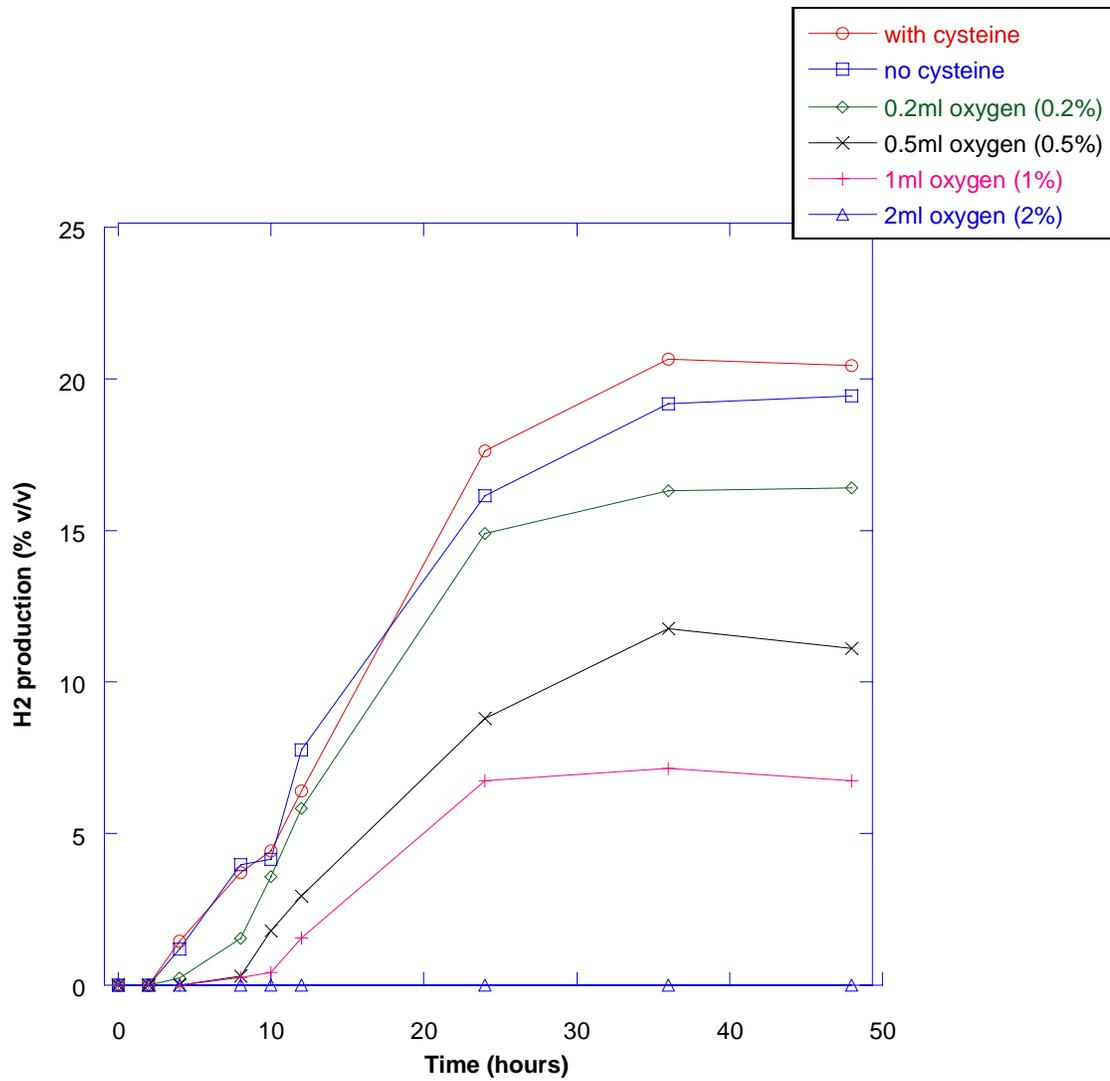


Figure 16: The hydrogen production of *T. neapolitana* DSM 4359 in the presence of different oxygen concentrations in the gas phase.

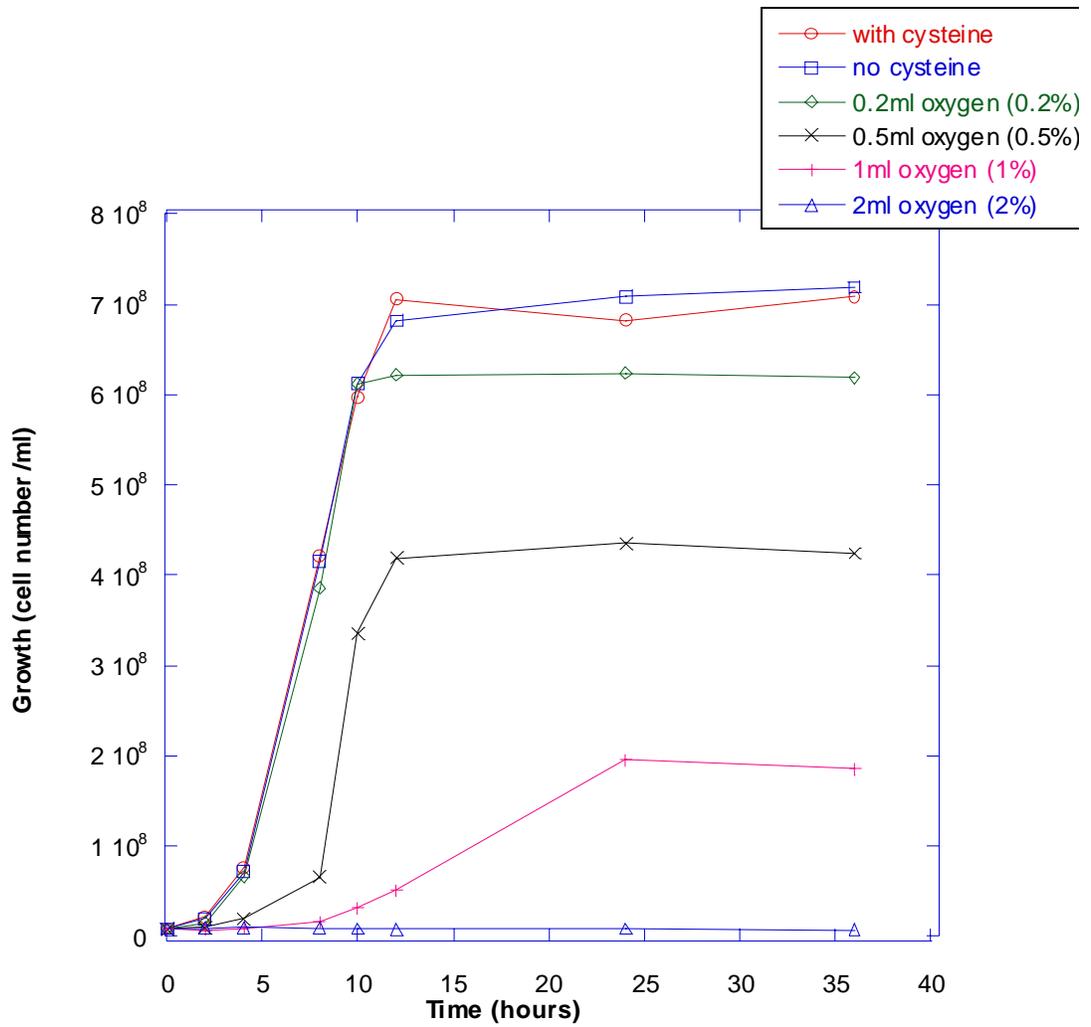


Figure 17: The growth curves of *T. neapolitana* DSM 5068 in the presence of different oxygen concentrations in the gas phase.

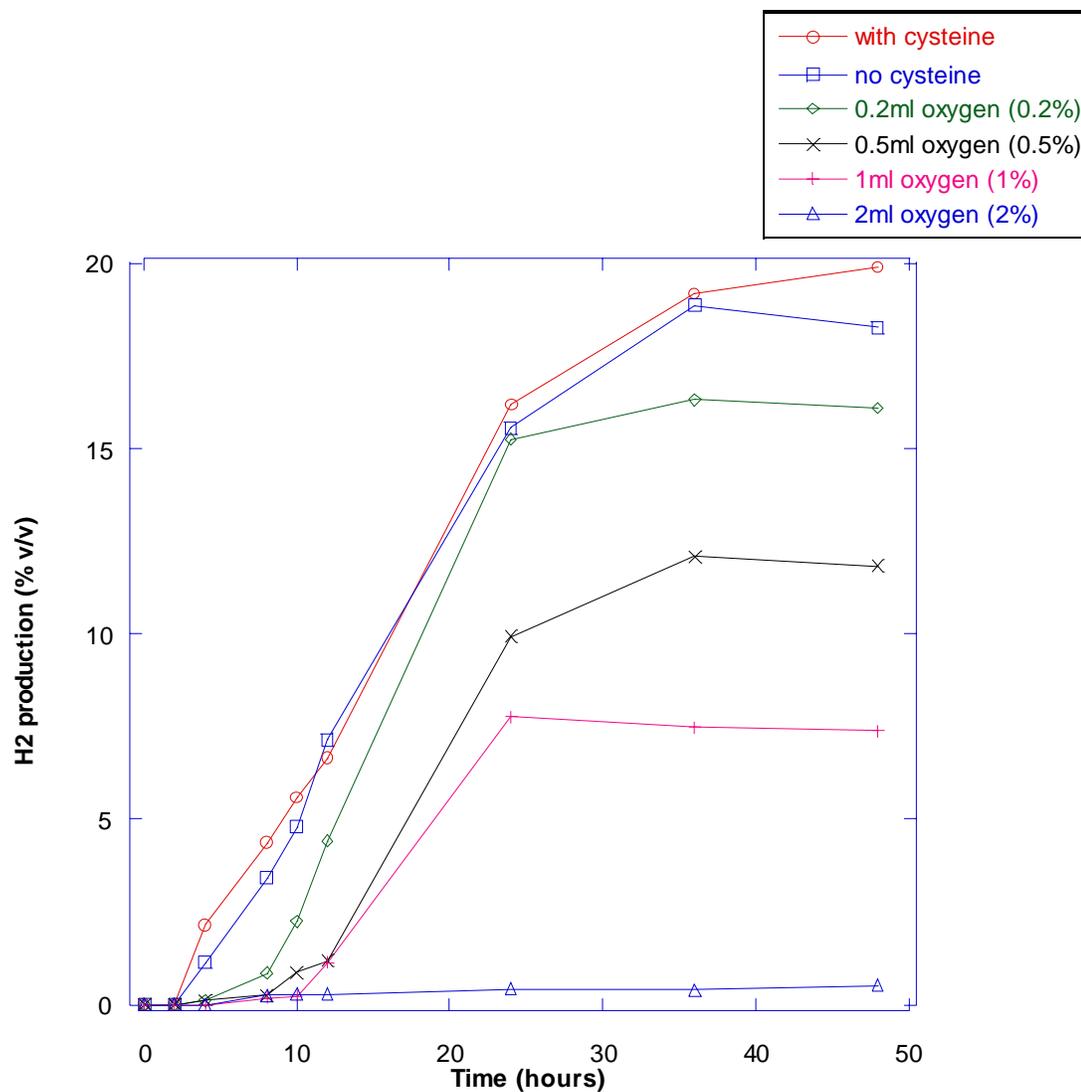


Figure 18: The hydrogen production of *T. neapolitana* DSM 5068 in the presence different oxygen concentrations in the gas phase.

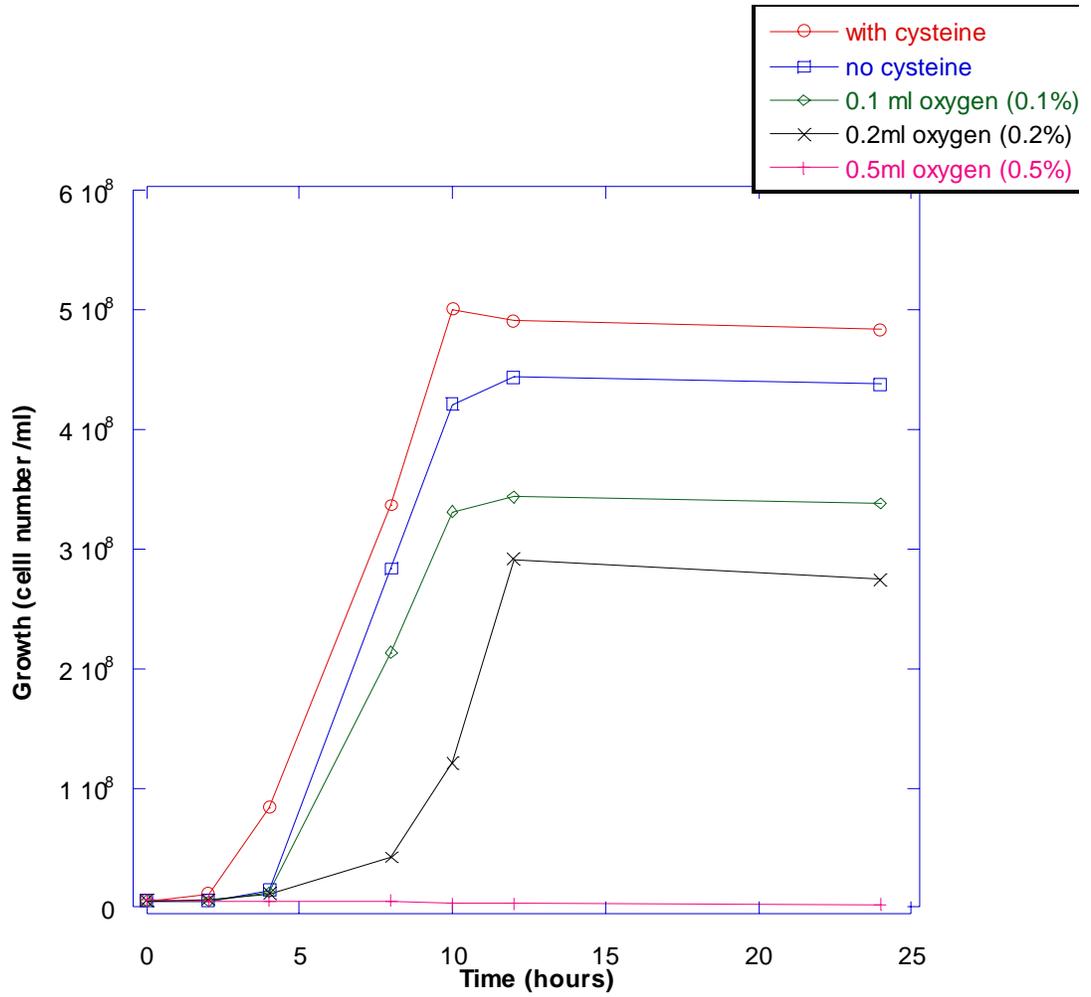


Figure 19: The growth of *T. maritima* in the presence of different oxygen concentrations in the gas phase.

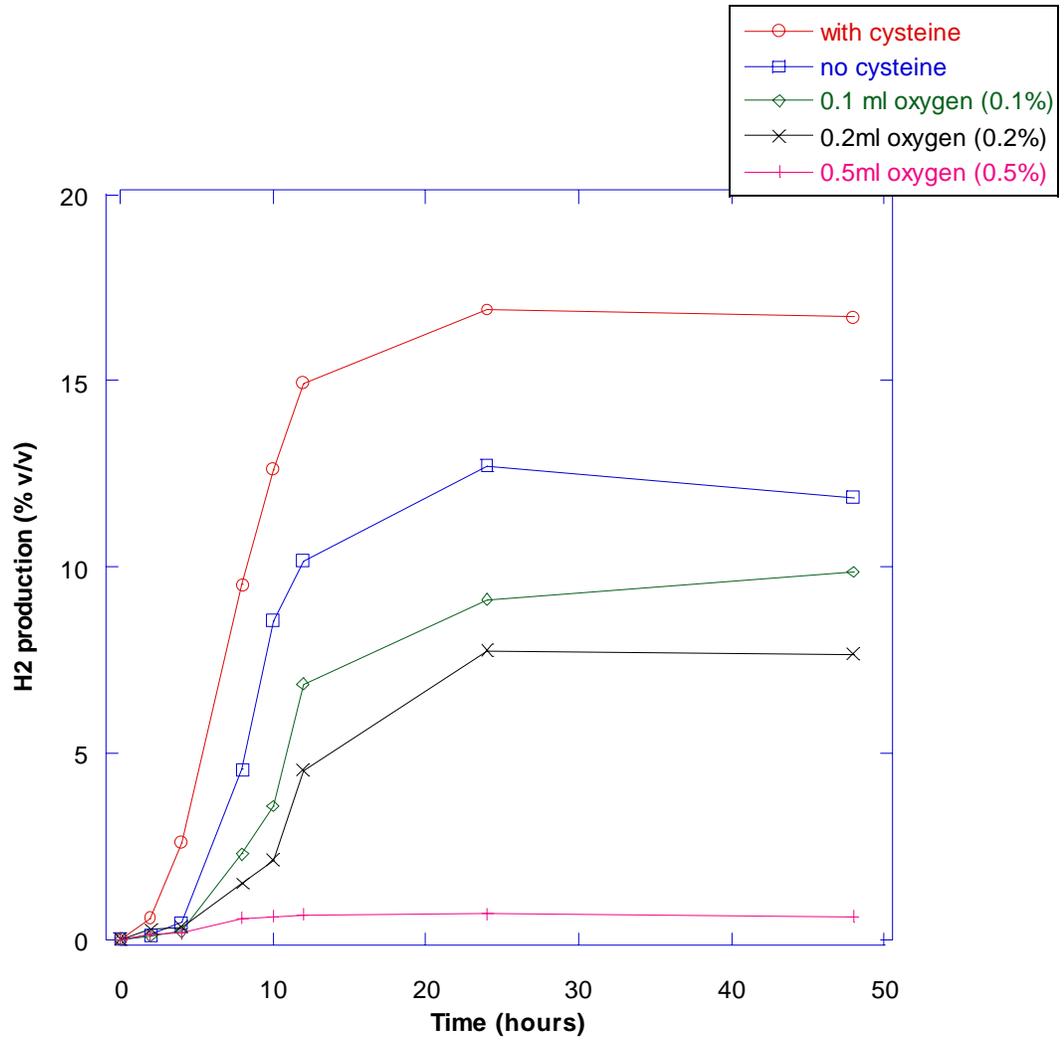


Figure 20: The hydrogen production of *T. maritima* in the presence of different oxygen concentrations in the gas phase.

3.3 Effects of the substrates and the end products on growth and hydrogen production of *T. neapolitana* strains

In batch culture, the yeast extract and glucose were both utilized by *T. neapolitana* strains, the end products such as lactate, acetate and alanine were produced during the growth and hydrogen production.

3.3.1 Growth and end products of *T. neapolitana* DSM 4359

At the late stationary phase (72 hours incubation), the concentrations of L-lactate and acetate were found to be approximately 4 mM and 12 mM, respectively. However, the concentration of L-lactate and acetate were about 8 mM and 24 mM respectively after 72 hours incubation when 50 mM Triz buffer was used to stabilize the pH (Fig.21). Under this condition, the cell density and hydrogen production also increased about 2 fold (Fig.21). It seemed that the end products were increased proportionally related to the growth.

3.3.2 Effects of the end products accumulation and substrates consumption during the growth of *T. neapolitana* DSM 4359 and *T. neapolitana* DSM 5068

To study the influences of the consumption of the substrates and the accumulation of end products during the growth and hydrogen production of *T. neapolitana* strains, different concentrations of acetate, alanine and L-lactate were added in the growth medium before inoculation. At the late stationary phase, the gas phase was flushed with N₂ to remove the H₂ and CO₂ in order to study the influence of the end products accumulation in the gas phase. In another parallel experiment, 1 ml 5% yeast extract solution and 0.5 ml 15% glucose solution were added individually into each batch bottle after late log phase to study the influences of the substrates consumption. Cell density and hydrogen production were determined. It was observed that acetate and alanine did not affect the growth and hydrogen production of *T. neapolitana* strains when the concentration was lower than 50 mM (Fig.22; Fig.23; Fig.24; Fig.25).

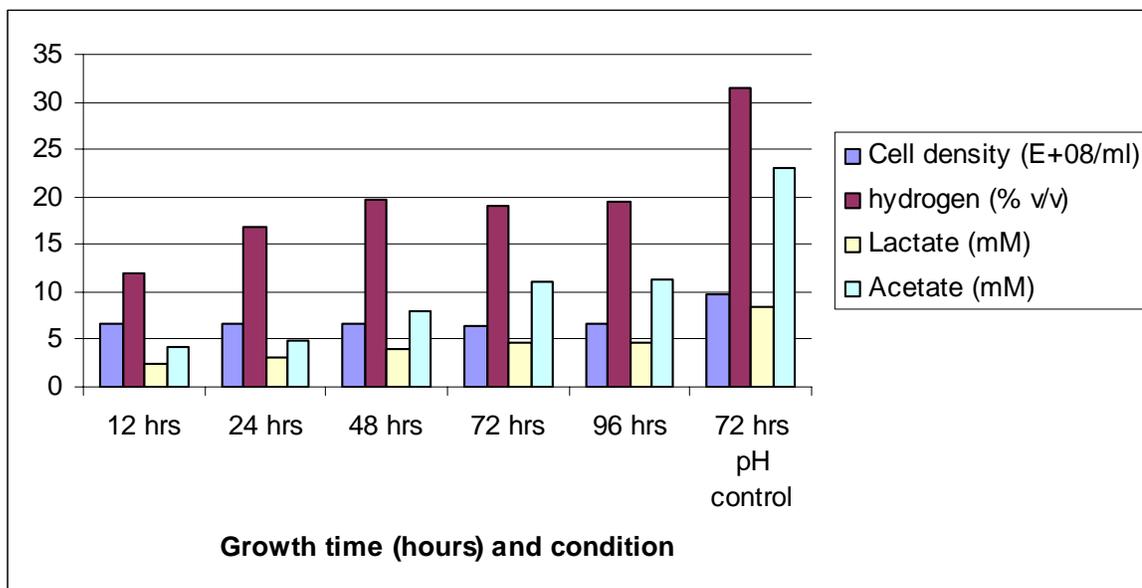


Figure 21: The growth and end products of *T. neapolitana* 4359 on different conditions.

Note: “12 hrs” means after 12 hours incubation; “72 hrs pH controls” means 72 hours incubation with 50 mM Triz buffer in the medium to stabilize the pH.

Slight inhibition of hydrogen production of *T. neapolitana* strains appeared only when the acetate concentration increased to 100 mM (Fig.22; Fig.23; Fig.24; Fig.25). The presence of L-lactate showed very little inhibition of the growth and hydrogen production of *T. neapolitana* strains when the concentration was 5 mM. But when the L-lactate concentration increased to 10 mM, greater inhibition was observed, and the inhibition was very significant when the L-lactate concentration was increased to 20 mM (Fig.22; Fig.23; Fig.24; Fig.25). Compared to the effect of initial pH, the inhibition caused by L-lactate was not dramatic (Fig.26 and Fig.27). L-lactate caused greater inhibition of the hydrogen production by *T. neapolitana* DSM 4359 than that of the growth (Tab.4 and Tab.5).

The initial pH range from 7 to 8.5 was suitable for *T. neapolitana* strains to grow and produce hydrogen. The inhibition caused by L-lactate was greater when the initial pH was low (Tab.4 and Tab.5). This indicated that, in this initial pH range, the high pH value could decrease the inhibition caused by L-lactate.

The hydrogen partial pressure was reported to be able to inhibit the growth of *T. neapolitana*, and elemental sulfur was always required for alleviating this inhibitory effect (Childers et al.1992). However, the cell density and hydrogen production did not increase after flushing the gas phase (Fig.22; Fig.23; Fig.24; Fig.25). This might be due to the hydrogen concentration in the gas phase of the culture bottles was not high enough, and may have no inhibitory effect to the growth and hydrogen production, or some other factors inhibited the growth of *T. neapolitana* strains, such as the metabolic end products in the growth media. The growth and hydrogen production did not increase after adding extra glucose, indicating that the consumption of glucose was not a limited factor (Fig.22; Fig.23; Fig.24; Fig.25). Further experiments showed that actually only part of the glucose initially added was consumed during the growth (Table 7 and Table 8), so, adding extra glucose adding resulted in no increase in growth or hydrogen production. Addition of extra yeast extract increased the cell density and hydrogen production slightly, indicating that the depletion of yeast extract was partially responsible for the inhibition of the growth and hydrogen production of *T. neapolitana* strains (Fig.22; Fig.23; Fig.24; Fig.25).

In conclusion, both L-lactate and depletion of yeast extract might be the factors resulting in inhibitory effects on the growth and hydrogen production of *T. neapolitana* strains.

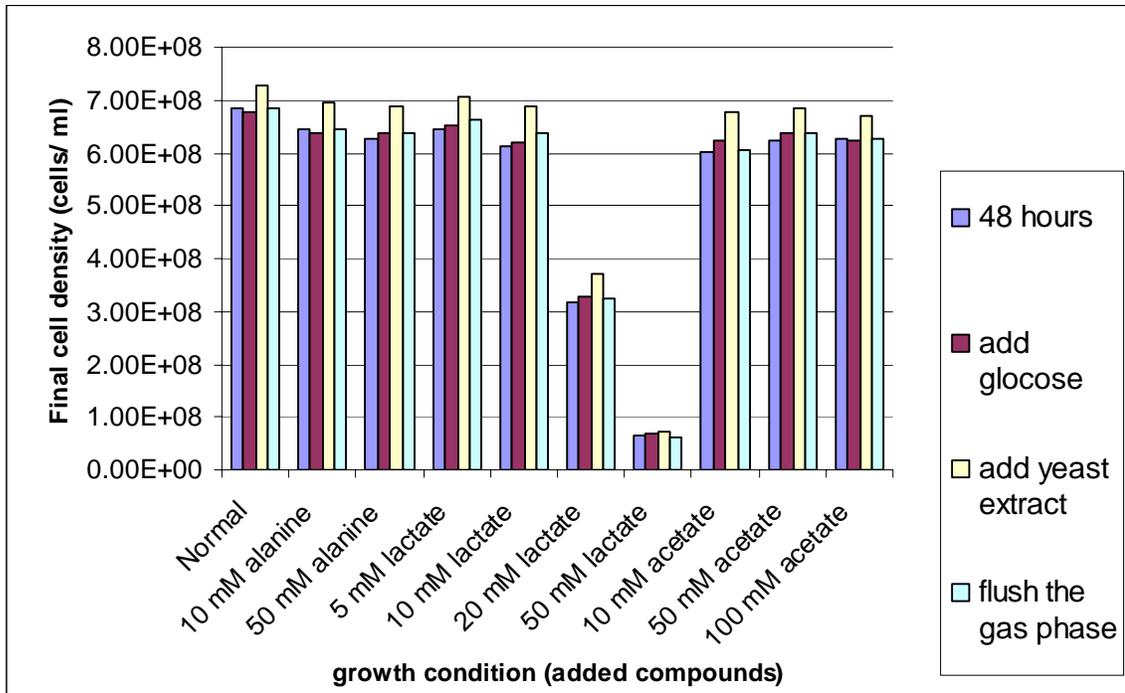


Figure 22: The growth of *T. neapolitana* DSM 4359 grown in the presence of L-lactate, acetate and alanine. Notes: “48 hours” means after 48 hours incubation; “Add glucose” and “add yeast extract” mean that the extra glucose and yeast extract were added separately to different batch bottles; “Flush the gas phase” means after 48 hours incubation, the gas phase was exchanged with N₂ by flushing about 15 minutes until no hydrogen could be detected. The cell density and hydrogen concentration were determined 24 hours after the flushing.

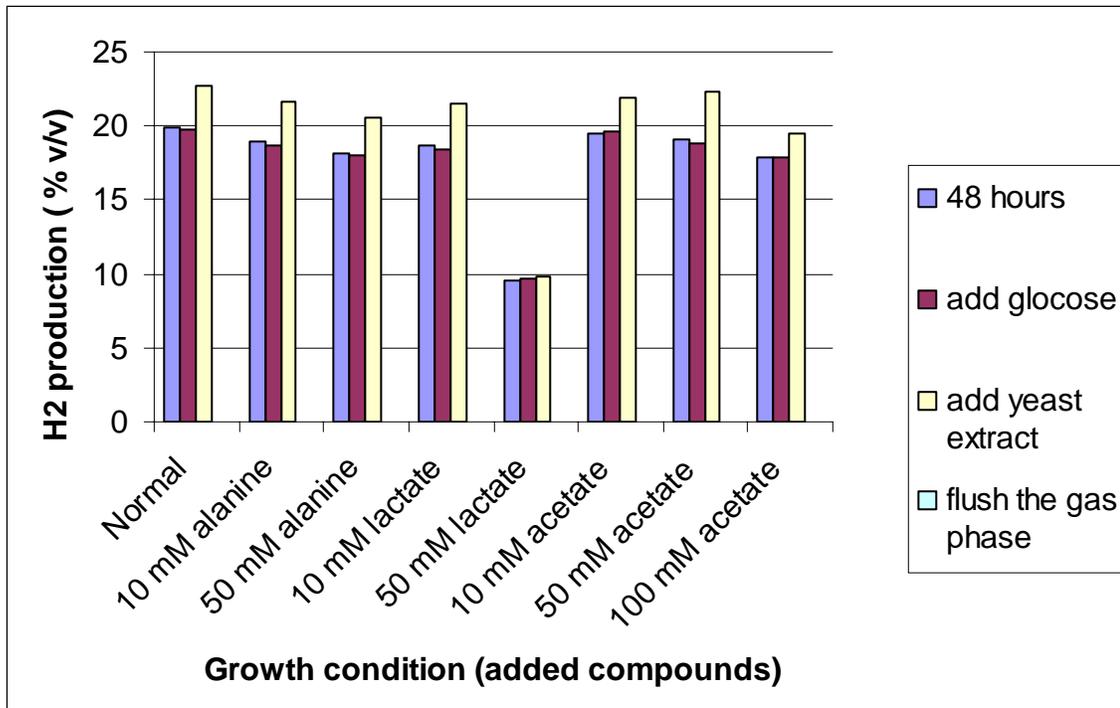


Figure 23: The hydrogen production of *T. neapolitana* DSM 4359 grown in the presence of L-lactate, acetate and alanine. Note: The hydrogen concentration was 0 after the flushing the gas phase by nitrogen.

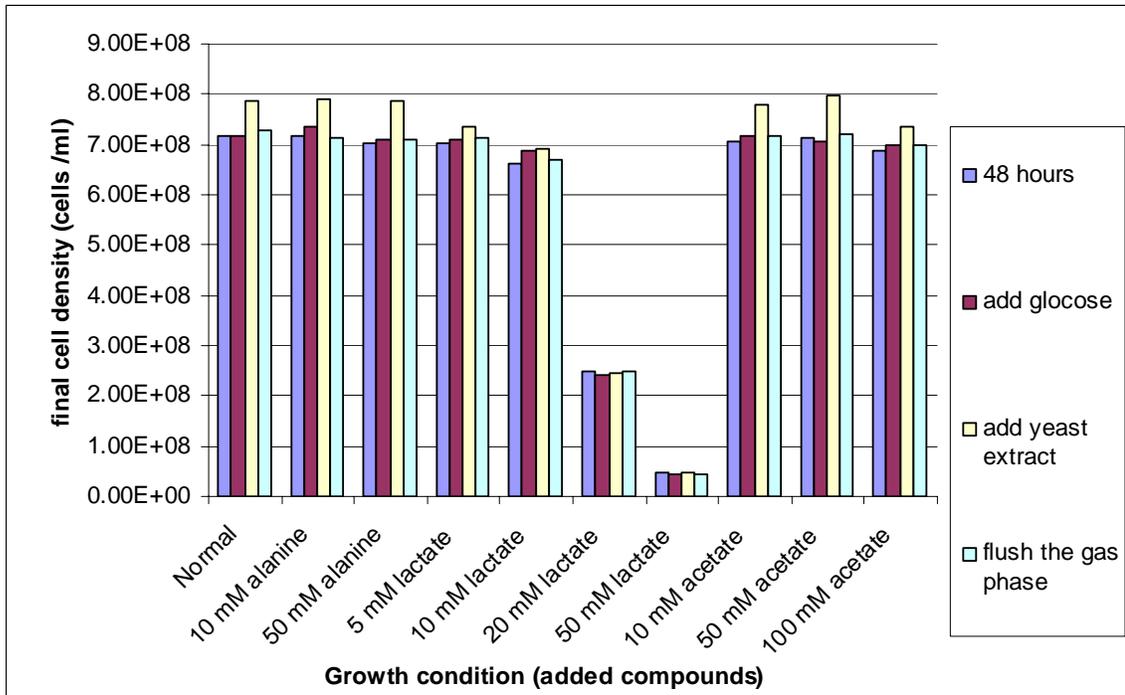


Figure 24: The growth of *T. neapolitana* DSM 5068 in the presence of L-lactate, acetate and alanine.

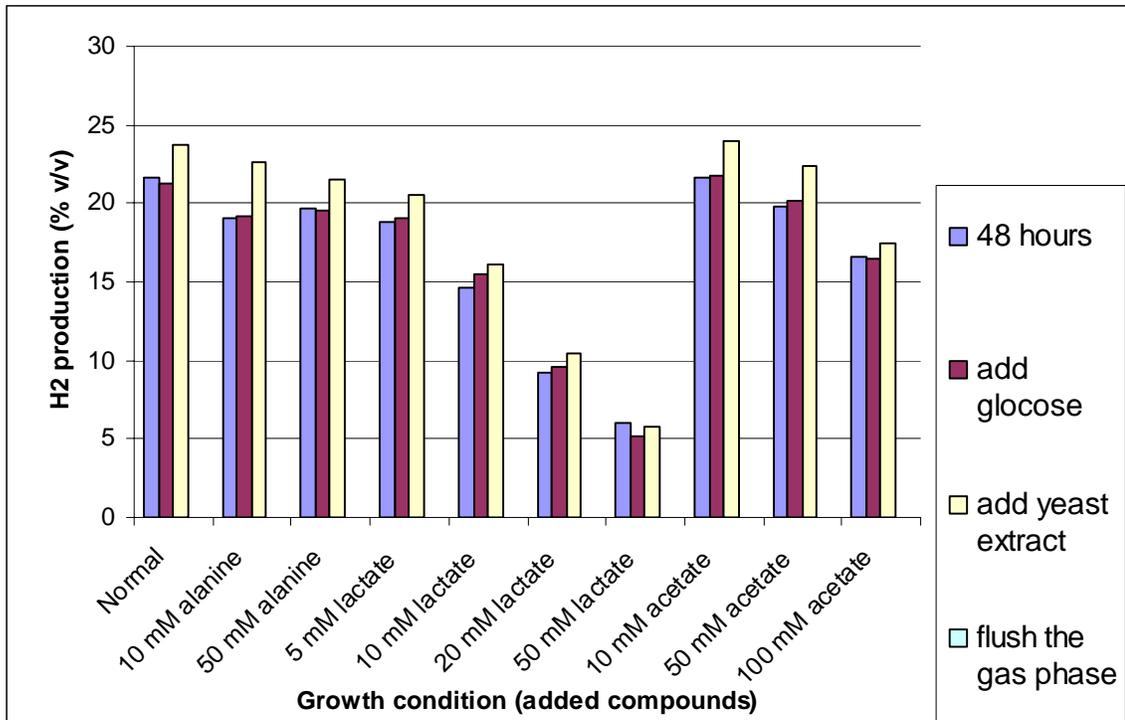


Figure 25: The hydrogen production of *T. neapolitana* DSM 5068 grown in the presence of L-lactate, acetate and alanine.

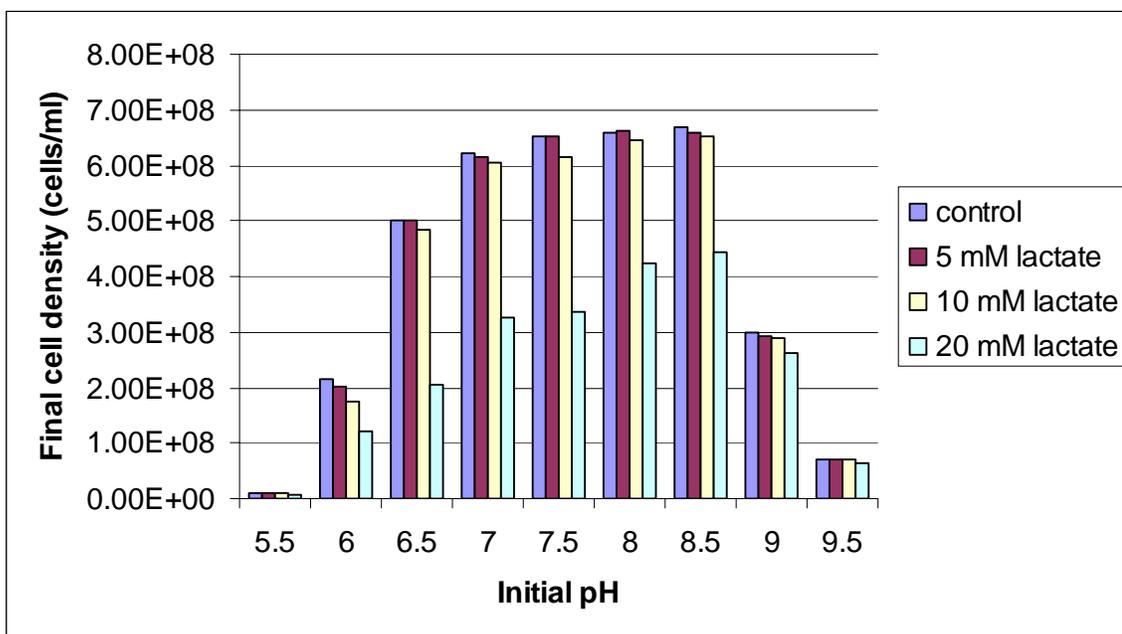


Figure 26: The growth of *T. neapolitana* DSM 4359 in the presence of different L-lactate concentrations and at different initial pHs.

Table 4: Comparison of growth of *T. neapolitana* DSM 4359 in the presence of different L-lactate concentrations at different initial pHs.

Initial pH	5.5	6	6.5	7	7.5	8	8.5	9	9.5
control	100%	100%	100%	100%	100%	100%	100%	100%	100%
5 mM lactate	92.9%	94.0%	99.8%	98.7%	99.7%	100%	98.9%	98.5%	98.5%
10 mM lactate	78.1%	81.7%	96.3%	97.5%	94.3%	97.9%	97.9%	96.4%	97.8%
20 mM lactate	56.6%	56.4%	40.8%	52.3%	51.7%	64.2%	66.6%	87.6%	88.7%

Note: “control” means no extra L-lactate added; 100% equals to the value of growth or hydrogen production in the culture without extra L-lactate.

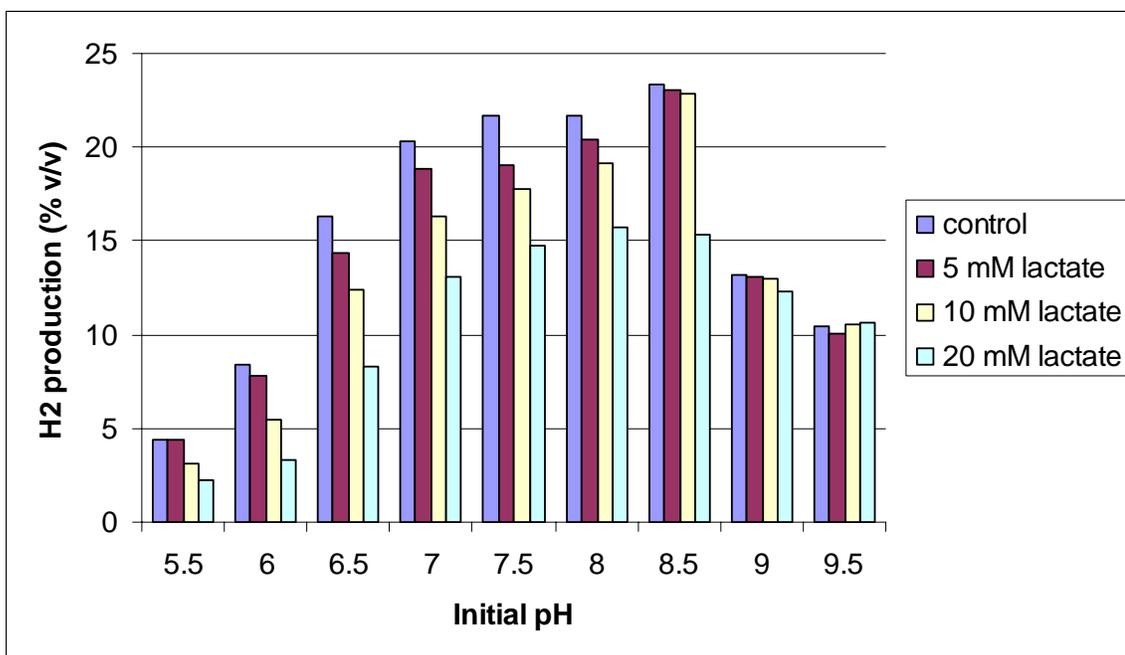


Figure 27: The hydrogen production of *T. neapolitana* DSM 4359 in the presence of different L-lactate concentrations and at different initial pHs.

Table 5: Comparison of the H₂ production of *T. neapolitana* DSM 4359 in the presence of different L-lactate concentrations and different initial pHs.

Initial pH	5.5	6	6.5	7	7.5	8	8.5	9	9.5
control	100%	100%	100%	100%	100%	100%	100%	100%	100%
5 mM lactate	99.9%	92.8%	87.9%	92.8%	87.6%	94.3%	98.6%	99.6%	96.6%
10 mM lactate	72.3%	64.4%	76.1%	80.0%	81.7%	88.1%	97.8%	98.9%	100%
20 mM lactate	50.1%	39.3%	51.1%	64.4%	67.8%	72.3%	65.7%	93.3%	102%

Note: “control” means no extra L-lactate added; 100% equals to the value of growth or hydrogen production in the culture without extra L-lactate.

3.4 Increased hydrogen production of *T. neapolitana* strains by pH control

To achieve optimal growth and hydrogen production, Triz buffer and addition of NaOH solution were used for stabilizing the pH of the medium during the growth. Various initial pHs were tested to obtain an optimal condition. The Triz buffer was added in the medium before inoculation. Different amounts of 1 M NaOH solution were added to the growth medium for maintaining a steady pH during the growth.

3.4.1 Effect of Triz buffer

During the growth of *T. neapolitana* DSM 4359, the pH of the medium decreased from 7 to 5 after 48 hours incubation. When the initial pH of the medium was 7, 1 or 10 mM Triz buffer present in the growth medium did not show any obvious influence on the pH changing. When 50 mM Triz buffer was used, the pH was stabilized and decreased from 7 to 6 after 48 hours incubation (Fig.30). The final cell density increased from 6×10^8 /ml to 7.4×10^8 /ml (Fig.28), and the hydrogen production increased from 20% (v/v) to 24 % (v/v) (Fig.29). The Triz buffer concentration should be 50 mM or higher, in order to achieve obvious effect on stabilizing the decrease of pH during the growth.

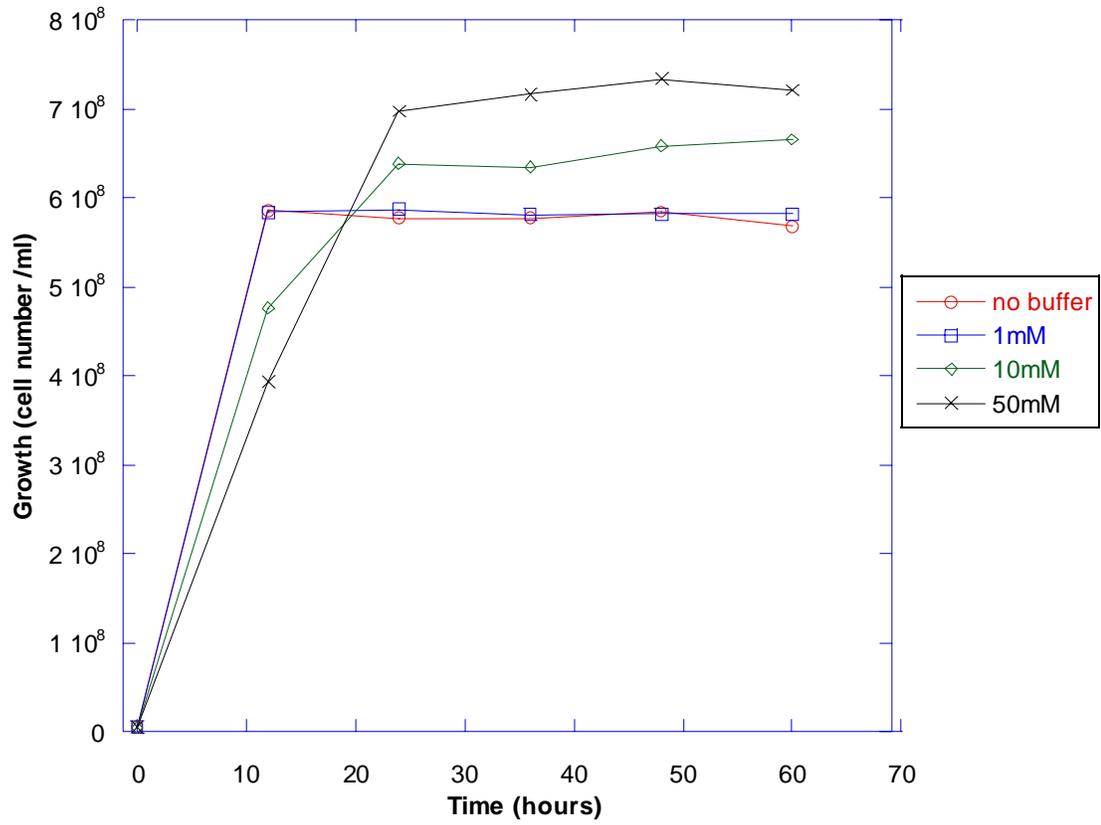


Figure 28: The growth of *T. neapolitana* DSM 4359 in the presence of Triz buffer when the initial pH was 7.

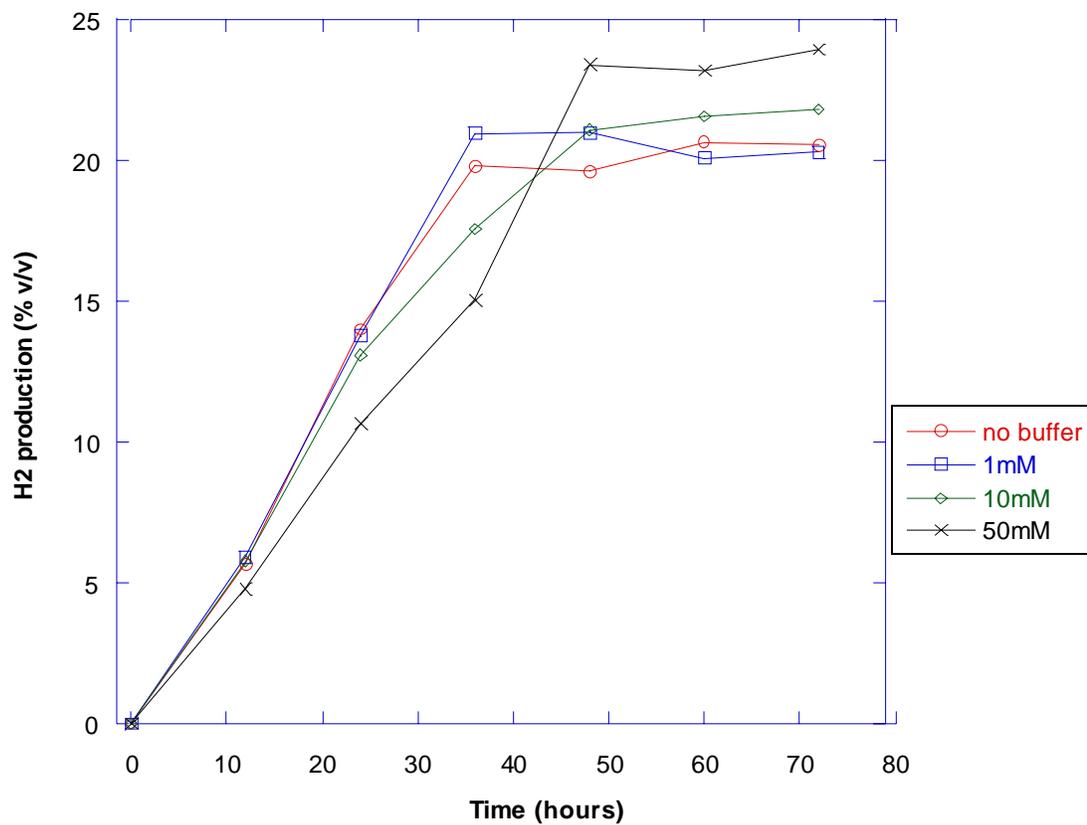


Figure 29: Hydrogen production of *T. neapolitana* DSM 4359 in the presence of Triz buffer when the initial pH was 7.

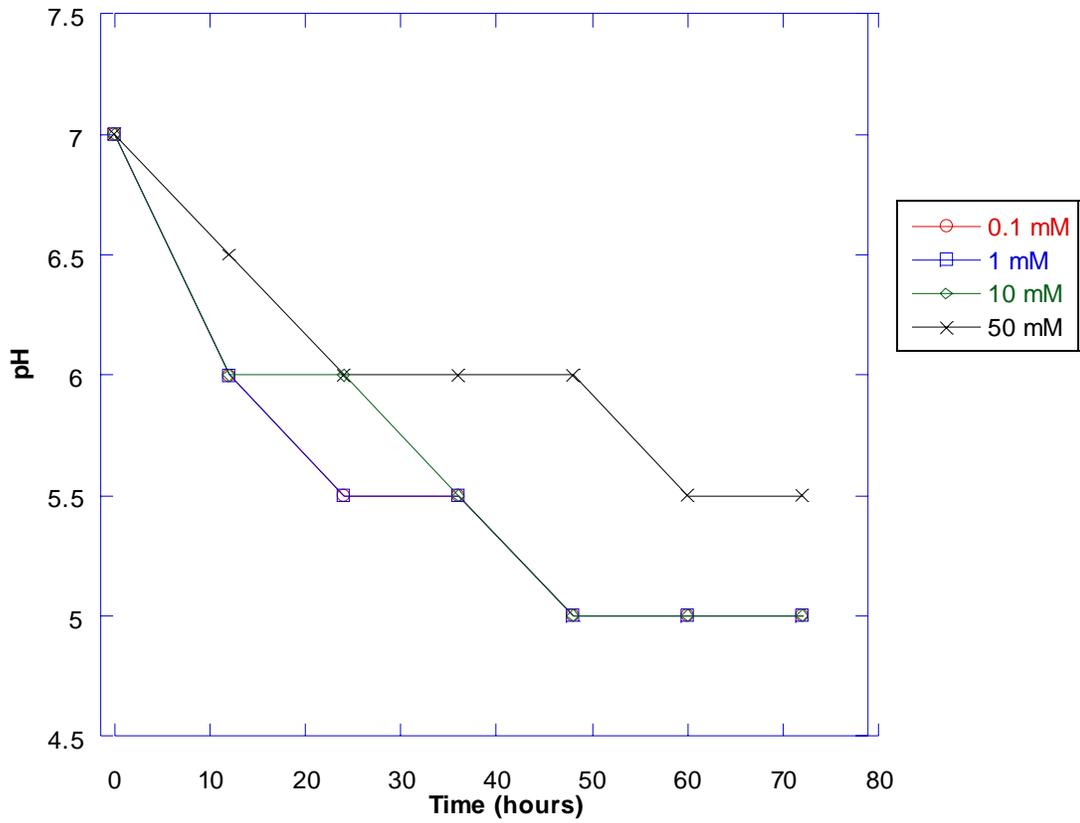


Figure 30: The change of pH in the medium during the growth of *T. neapolitana* DSM 4359 in the presence of Triz buffer when the initial pH was 7.

3.4.2 Effects of 50 mM Triz buffer at different initial pHs on the growth and hydrogen production of *T. neapolitana* DSM 4359

When the pH of the growth medium was stabilized in the presence of 50 mM Triz buffer, *T. neapolitana* DSM 4359 could tolerate initial pH from 6 to 9, but the growth and hydrogen production were very poor when the initial pH was 6 or 9. An optimal pH range between 8 and 8.5 was observed (Fig.31). A series of experiments had been done to test the growth and hydrogen production of *T. neapolitana* DSM 4359 grown in the presence of Triz buffer (50-200 mM) at different initial pH (6.5-8.5). The medium with initial pH of 8.5 and stabilized by 50mM Triz buffer resulted in the highest cell density (1.1×10^9 / ml) and the highest hydrogen production (35% v/v). The hydrogen production rate was quite stable during the incubation (Fig.31 and Fig.32). Although higher buffer concentration could achieve higher hydrogen production, the lower rate of growth and hydrogen production made it less attractive for hydrogen production (Table 6). Based on the results obtained, the suitable pH range for *T. neapolitana* DSM 4359 was from 7 to 8.5. When the medium with initial pH of 8.5 in the presence of 50 mM Triz buffer, the pH of the medium decreased from 8.5 to 7 after 60 hours incubation, which was the period of time for hydrogen production (Fig.32 and Fig.33).

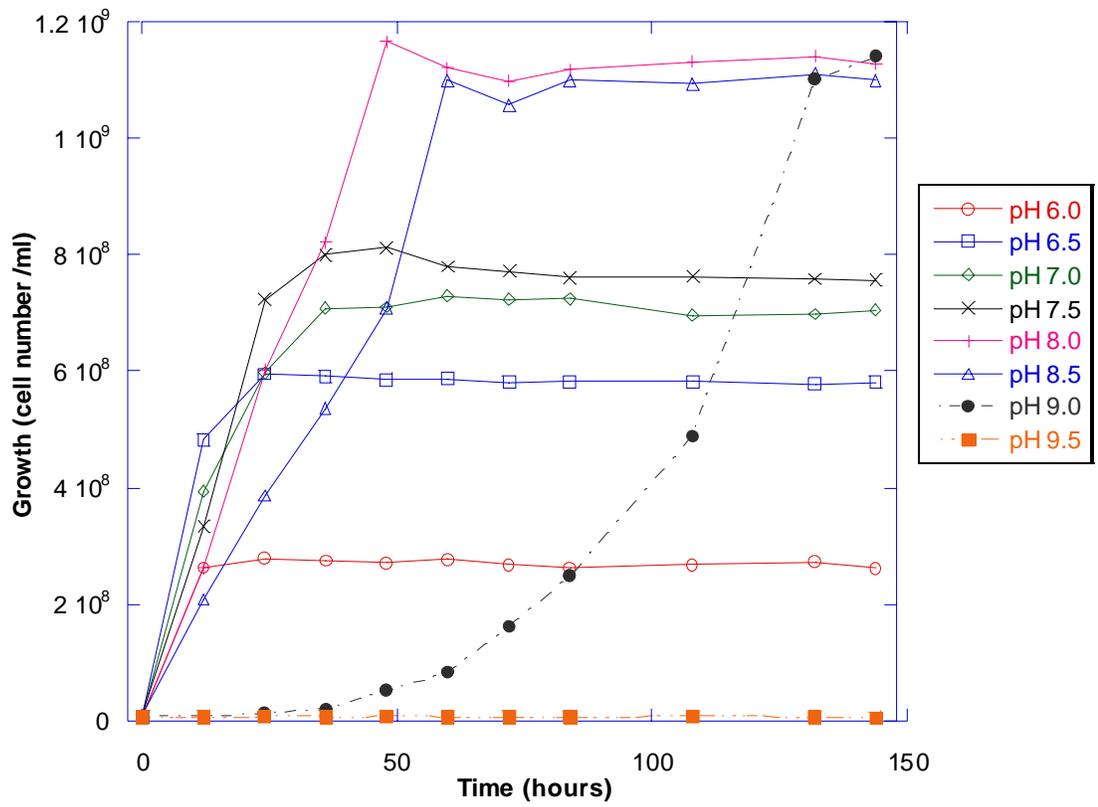


Figure 31: The growth of *T. neapolitana* DSM 4359 in the presence of 50 mM Triz buffer at different initial pHs.

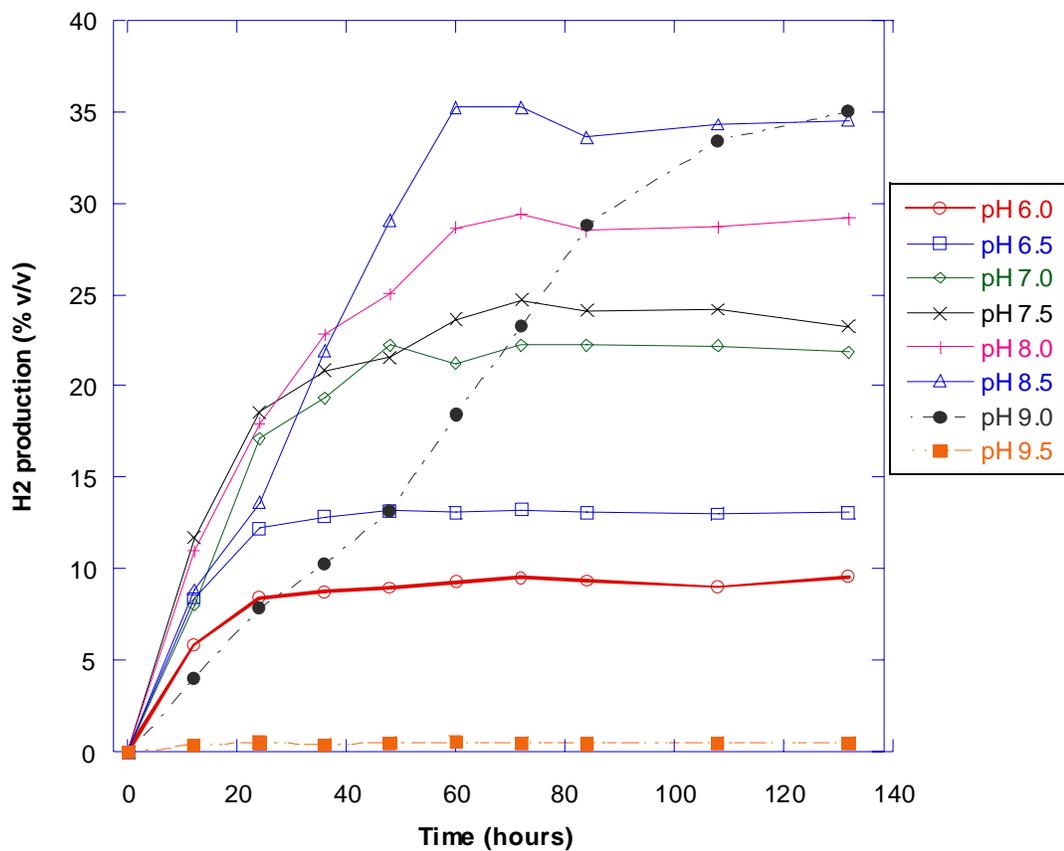


Figure 32: The hydrogen production of *T. neapolitana* DSM 4359 in the presence of 50 mM Triz buffer at different initial pHs.

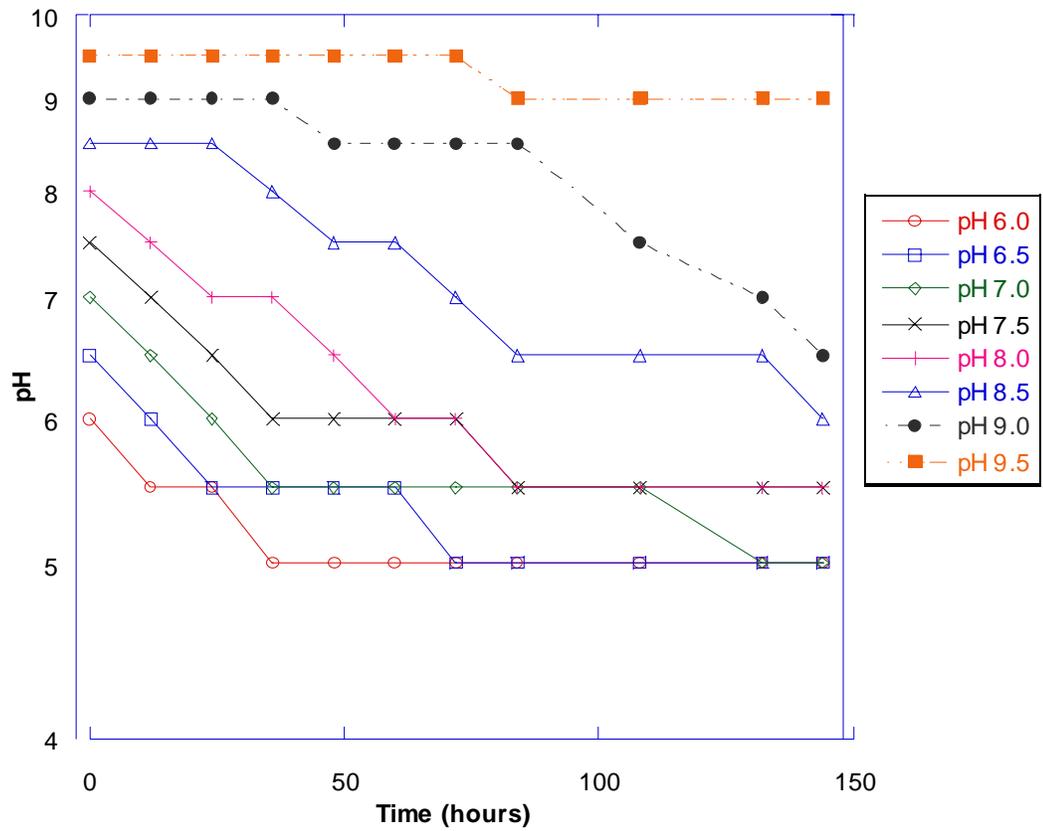


Figure 33: The pH change of the media during the growth of *T. neapolitana* DSM 4359 in the presence of 50 mM Tris buffer at different initial pHs.

Table 6: The growth, hydrogen production and pH change during the growth of *T. neapolitana* DSM 4359 in the presence of Triz buffer at different initial pHs.

	pH (growth stop)	Hours (growth stop)	Final cell density/ml	pH (H ₂ production stop)	Hours (H ₂ production stop)	H ₂ production mmol
6.5 (50mM)	6.0	24	5.82×10 ⁸	5.5	36	0.77
6.5 (100mM)	6.0	24	5.28×10 ⁸	5.5	48	0.83
6.5 (200mM)	6.0	36	6.50×10 ⁸	5.5	60	0.85
6.5 (300mM)	6.0	36	6.93×10 ⁸	5.5-6.0	60	0.87
7.0 (50mM)	6.0	36	7.05×10 ⁸	5.5-6.0	48	1.42
7.0 (100mM)	6.0	36	7.89×10 ⁸	5.5-6.0	60	1.23
7.0 (200mM)	6.0-6.5	48	8.56×10 ⁸	6.0	72	1.33
7.0 (300mM)	6.5	48	8.98×10 ⁸	6.5	72	1.60
7.5 (50mM)	6.0	36	7.56×10 ⁸	6.0	48	1.54
7.5 (100mM)	6.5-7.0	36	8.55×10 ⁸	6.0-6.5	60	1.97
7.5 (200mM)	7.0-7.5	48	9.02×10 ⁸	6.5-7.0	84	2.18
7.5 (300mM)	7.0-7.5	60	9.50×10 ⁸	7.0	108	2.36
8.0 (50mM)	7.5	36	1.13×10 ⁹	6.0	60	2.06
8.0 (100mM)	7.5	48	1.11×10 ⁹	6.5	84	2.21
8.0 (200mM)	8.0	60	1.09×10 ⁹	7.0	132	2.68
8.5 (50mM)	8.0	36	1.13×10 ⁹	6.5	60	2.48
8.5 (100mM)	8.0	84	1.12×10 ⁹	7.0	108	2.75

Note: The hydrogen concentration (% v/v) has been converted to amount (mmol) in each bottle (50 ml medium in 160 ml serum bottle).

$$[\text{Amount (mmol)} = (1.1 \times C) \times (14.6 + C \times 0.2157) / 14.6 / 22.4]; C = \text{H}_2 \text{ concentration \% (v/v)}$$

3.4.3 Effect of the use of NaOH solution for adjusting the pH

When *T. neapolitana* DSM 4359 was grown on the medium at initial pH of 7, the pH decreased to 6 after 18 hours incubation (early stationary phase), then adjusted back to 7 by adding 1.2 ml 1 M NaOH solution. In another trial, under the same condition, the medium pH decreased to 5.5 after 36 hours incubation (late stationary phase), then adjusted back to 7 by adding 1.5 ml 1 M NaOH (Fig.36). The cell density, hydrogen production and medium pH were determined 12 hours after the adjustment. When the pH was adjusted at early stationary phase, final cell density was slightly increased and the hydrogen production was increased from 20% (v/v) to 30% (v/v) (Fig.34 and Fig.35). No obvious effect to the growth and hydrogen production was found when using NaOH solution to adjust the pH at the late stationary phase. These results indicated that the pH adjustment by NaOH solution at early stationary phase could obviously increase the hydrogen production of *T. neapolitana* DSM 4359 but not the growth. The pH adjustment at late stationary phase resulted in no increase to the growth or hydrogen production.

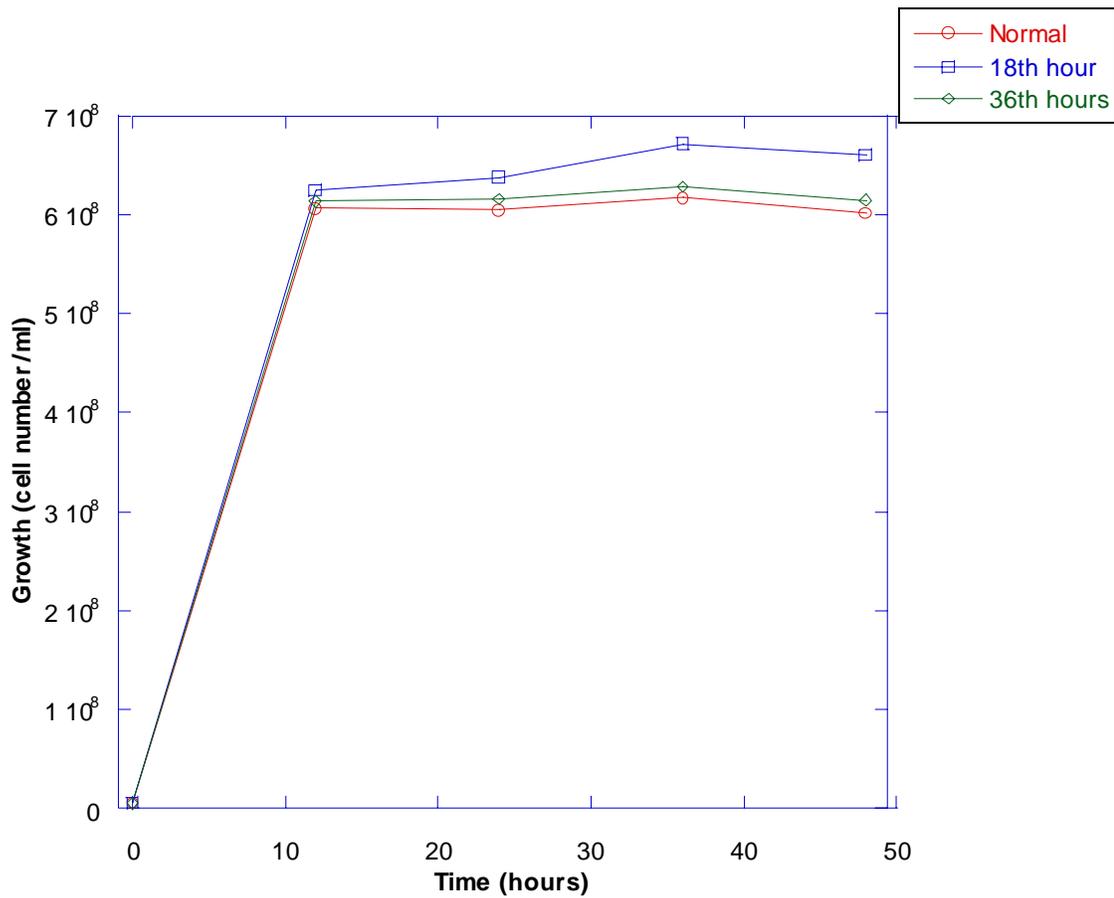


Figure 34: The growth of *T. neapolitana* DSM 4359 when pH was adjusted using NaOH.

Note: “18th hours” means that the medium pH was adjusted back to 7 by adding 1.2 ml 1 N NaOH solution at the incubation time of 18 hours (early stationary phase); “36th hours” means that the medium pH was adjusted back to 7 by adding 1.5 ml 1 N NaOH solution at the incubation time of 36 hours (late stationary phase).

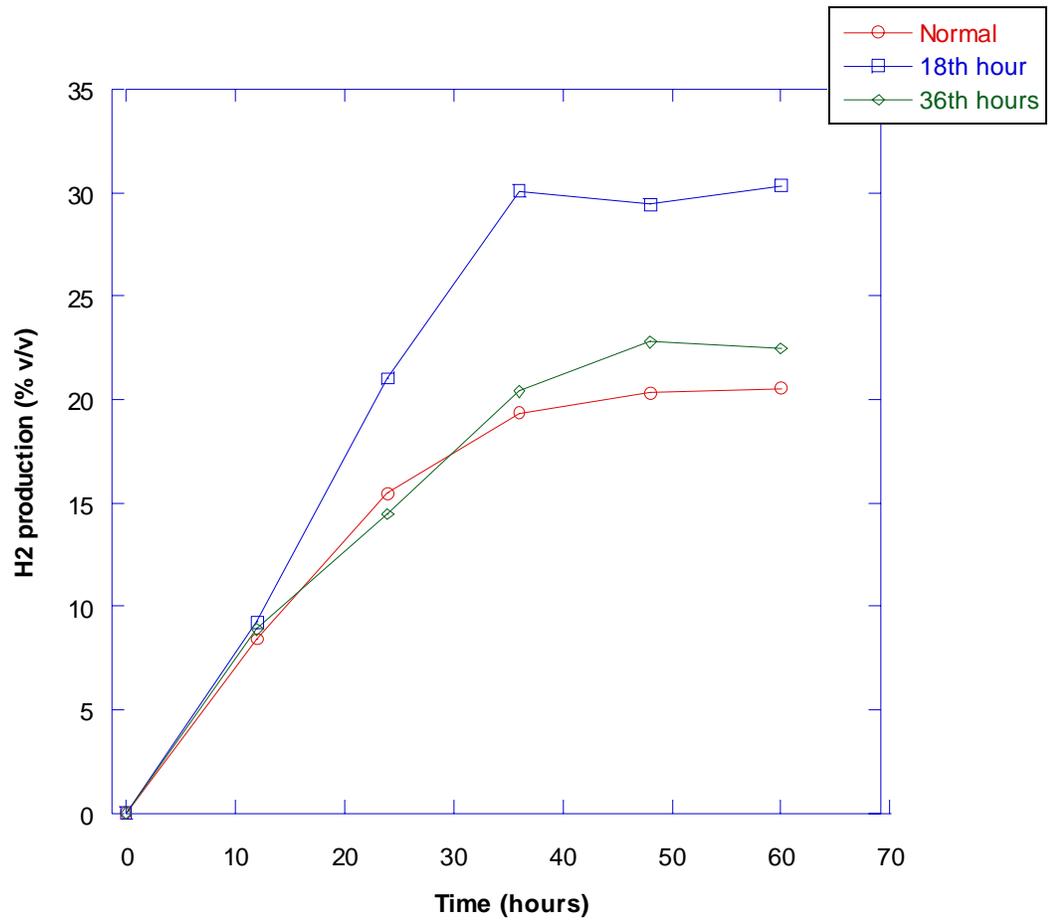


Figure 35: Hydrogen production of *T. neapolitana* DSM 4359 when pH was adjusted using NaOH.

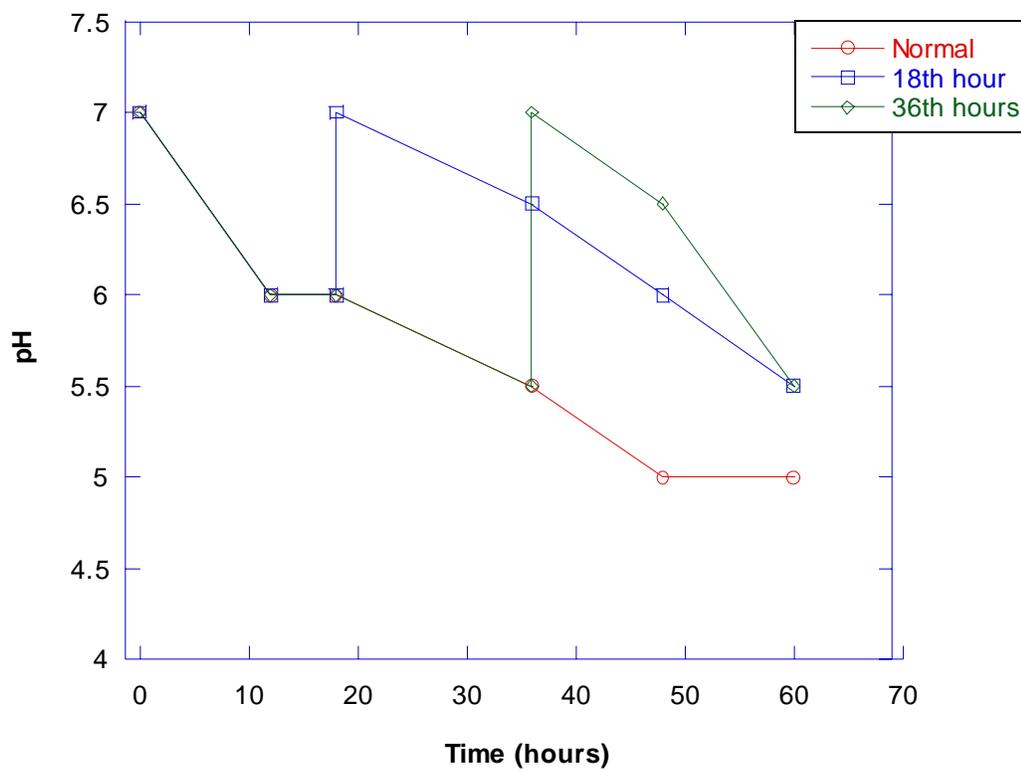


Figure 36: pH change of the medium during the growth of *T. neapolitana* DSM 4359 when NaOH was used to adjust the pH.

3.4.4 Efficiency of hydrogen production of *T. neapolitana* species

The hydrogen production (mmol per 50 ml medium) and glucose consumption were determined to obtain the conversion efficiency of *T. neapolitana* species. The hydrogen produced from yeast extract and TSB was subtracted. For *T. neapolitana* DSM 4359, the hydrogen production was increased from 1.24 mmol to 2.5 mmol when 50 mM Triz buffer was present (initial pH 8.5), and the conversion efficiency increased from 2.24 to 3.6, which was 90% of the theoretical conversion efficiency from glucose to hydrogen (Table 7). When NaOH was used for adjusting the pH at the early stationary phase, the hydrogen production was increased to 2.12 mmol and the conversion efficiency was increased to 3.38, or 85% of the theoretical conversion efficiency (Table 7). For *T. neapolitana* DSM 5068, the results were quite similar. The hydrogen production was increased from 1.4 mmol to 3.34 mmol when 50 mM Triz buffer was present (initial pH 8.5), and the conversion efficiency increased from 2.76 to 3.70, or 92% of the theoretical conversion efficiency from glucose to hydrogen (Table 8). When NaOH was used for adjusting the pH at early stationary phase, the hydrogen production was increased to 2.91 mmol and the conversion efficiency was increased to 3.56, or 89% of the theoretical conversion efficiency (Table 8). These results indicated that the use of Triz buffer or NaOH to control the pH could not only increase the growth and hydrogen production of *T. neapolitana* species, but also increase the conversion efficiency from glucose to hydrogen.

Table 7: Hydrogen production and conversion efficiency of *T. neapolitana* DSM 4359 (per 50 ml medium)

	Hydrogen production (mmol)	Glucose consumption (mmol)	Hydrogen production from yeast extract and TSB(mmol)	Conversion efficiency (Hydrogen/glucose)
Without pH control	1.24 ±0.05	0.34 ±0.01	0.48 ±0.02	2.24 ±0.15
pH stabilized by 50 mM Tris buffer	2.50 ±0.16	0.54 ±0.02	0.56 ±0.03	3.60 ±0.30
pH adjusted using NaOH solution (about 18 hr)	2.12 ±0.09	0.48 ±0.02	0.50 ±0.02	3.38 ±0.19

Note: The data showed in the table were the hydrogen and glucose amount (mmol) in each bottle (50 ml medium in 160 ml serum bottle).

Table 8: Hydrogen production and conversion efficiency of *T. neapolitana* DSM 5068 (per 50 ml medium)

	Hydrogen production (mmol)	Glucose consumption (mmol)	Hydrogen production from yeast extract and TSB(mmol)	Conversion efficiency (Hydrogen /glucose)
Without pH control	1.40 ±0.06	0.37 ±0.01	0.38 ±0.03	2.76±0.16
pH stabilized by 50 mM Tris buffer	3.34 ±0.13	0.72 ±0.02	0.69 ±0.04	3.70 ±0.18
pH adjusted using NaOH solution (about 18 hr)	2.91 ±0.17	0.63 ±0.02	0.67 ±0.04	3.56 ±0.26

3.5 Hydrogenase activity of *T. neapolitana* species

Both hydrogen evolution activity and hydrogen oxidation activity of hydrogenases from *T. neapolitana* DSM 4359 and DSM 5068 were determined. The cells were grown on different conditions and collected at different growth phases (Table 9).

Table 9: Collected cells of *T. neapolitana* species.

	Late log phase	Early stationary phase	Late stationary phase
Without pH control	After 10 hours incubation	After 18 hours incubation	After 36 hours incubation
pH controlled using 50 mM Triz buffer	After 12 hours incubation	After 24 hours incubation	After 48 hours incubation
pH adjusted using 1 M NaOH	After 10 hours incubation	Adjust pH to 7 at 18 hours and collected at 36 hours.	Adjust pH to 7 at 36 hours and collected at 48 hours.

3.5.1 The hydrogenase activity of *T. neapolitana* DSM 4359

When *T. neapolitana* DSM 4359 was grown without pH control, the specific hydrogen evolution activity was 0.95 U/mg, and specific hydrogen oxidation activity was 1.25 U/mg at late log phase; both hydrogenases activities were similar at early stationary phase (Table 10). At the late stationary phase, the specific hydrogen evolution activity decreased to 0.66 U/mg, and specific hydrogen oxidation activity decreased to 0.86 U/mg (Table 10). When NaOH was used for adjusting pH back to 7 at 18 hours and the cells were collected at 36 hours, the specific hydrogen evolution activity was 0.87 U/mg and specific hydrogen oxidation activity was 1.17 U/mg, which were 30% higher than the hydrogenase activities at the late stationary phase without pH control. When using NaOH to adjust pH back to 7 at 36 hours and the cells were collected at 48 hours, the specific hydrogen evolution activity was 0.72 U/mg and specific hydrogen oxidation activity was 0.89 U/mg, which were almost the same as the hydrogenase activities at late stationary phase without pH control (Table 10). These results indicated that using NaOH to adjust pH at early

stationary phase could increase the expression of the hydrogenases, but the adjustment at late stationary phase had no such effect.

When *T. neapolitana* DSM 4359 was grown on the medium in presence of 50 mM Triz buffer (initial pH 8.5), the specific hydrogen evolution activity was 0.87 U/mg, and specific hydrogen oxidation activity was 1.28 U/mg at the late log phase; both hydrogenase activities were similar at the early stationary phase and the late stationary phase (Table 10). These results indicated that the Triz buffer not only stabilized the pH of the medium, but also increased the activities of hydrogenases. The ratio between the specific hydrogen evolution activities to the specific hydrogen oxidation activities was not constant under different conditions at different growth phases (Fig.37). It seemed that more than one hydrogenase was involved in the metabolism of *T. neapolitana* DSM 4359.

Table 10: Hydrogenase activities of *T. neapolitana* DSM 4359

	Cell harvest (g)	CFE (ml)	Protein mg / ml	Hase evol U / mg	Hase oxid U / mg
N(10) LL	0.47	3.3	7.72	0.95	1.25
N(18) ES	0.91	7.5	12.35	0.93	1.3
N(36) LS	0.97	7.5	11.79	0.67	0.86
Triz(12) LL	0.33	2.5	8.87	0.89	1.29
Triz(24) ES	1.35	11.2	11.74	0.9	1.22
Triz(48) LS	1.41	12.7	11.21	0.83	1.12
NaOH(18/36)	0.97	7.5	12.78	0.87	1.17
NaOH(36/48)	0.95	7.5	12.36	0.72	0.89

Note: N (10) LL: without pH control, collected at 10 hours at the late log phase.

N (18) ES: without pH control, collected at 16 hours at the early stationary phase.

N (36) LS: without pH control, collected at 16 hours at the late stationary phase.

Triz (12) LL: 50 mM buffer, collected at 12 hours at the late log phase.

Triz (24) ES: 50 mM buffer, collected at 24 hours at the early stationary phase.

Triz (48) LS: 50 mM buffer, collected at 48 hours at the late stationary phase.

NaOH (18/36): adjust pH to 7 at 18 hours and collected at 36 hours.

NaOH (36/48): adjust pH to 7 at 36 hours and collected at 48 hours.

Hase evol: hydrogen evolution activity.

Hase oxid: hydrogen oxidation activity.

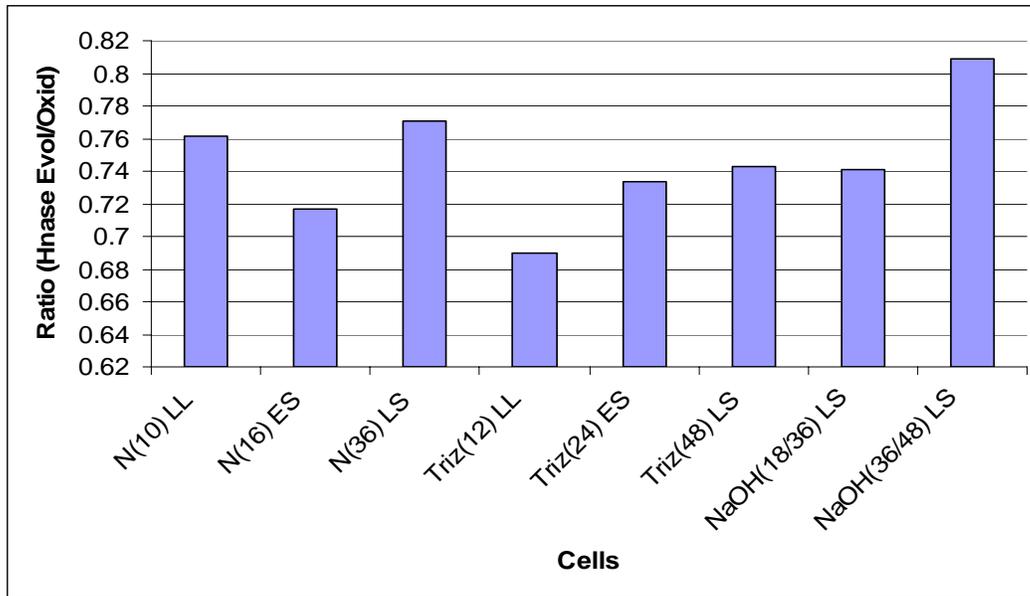


Figure 37: The ratio of the hydrogen evolution activity to hydrogen oxidation activity of *T. neapolitana* DSM 4359 hydrogenases.

3.5.2 Hydrogenase activities of *T. neapolitana* DSM 5068

When *T. neapolitana* DSM 5068 was grown without pH control, the specific hydrogen evolution activity was 0.67 U/mg and specific hydrogen oxidation activity was 1.24 U/mg at the late log phase. Both hydrogenase activities were similar at the early stationary phase (Table 11). At the late stationary phase, the specific hydrogen evolution activity decreased to 0.31 U/mg and specific hydrogen oxidation activity decreased to 0.83 U/mg (Table 11). When NaOH was used for adjusting pH back to 7 at 18 hours and the cells were collected at 36 hours, the specific hydrogen evolution activity was 0.65 U/mg and specific hydrogen oxidation activity was 1.28 U/mg, which were 100% (hydrogen evolution activity) or 50% (hydrogen oxidation activity) respectively higher than the hydrogenase activities at late stationary phase without pH control. When using NaOH to adjust pH back to 7 at 36 hours and the cells were collected at 48 hours, the specific hydrogen evolution activity was 0.66 U/mg, which was almost the same as the hydrogenase activities at the late stationary phase without pH control (Table 11). The specific hydrogen oxidation activity was 1.36 U/mg, which was the same as that of the log phase, 60% higher than

that of the late stationary phase without pH control. These results indicated that using NaOH to adjust pH at the early stationary phase could increase the activities of both hydrogenases, but the adjustment at the late stationary phase could only increase the activities of hydrogen oxidation of *T. neapolitana* DSM 5068.

When *T. neapolitana* DSM 5068 was grown on the medium in presence of 50 mM Triz buffer (initial pH 8.5), the specific hydrogen evolution activity was 0.57 U/mg and specific hydrogen oxidation activity was 1.1 U/mg at the late log phase. Both hydrogenase activities were similar at the early stationary phase and the late stationary phase (Table 11). These results indicated that the Triz buffer not only stabilized the pH of the medium, but also the activities of the hydrogenase. The ratio of the specific hydrogen evolution activity to the specific hydrogen oxidation activity was not constant under different conditions at different phases (Fig.38). It seemed that more than one hydrogenase were involved in the metabolism of *T. neapolitana* DSM 5068.

Table 11 Hydrogenase activities of *T. neapolitana* DSM 5068

	cell harvest (g)	CFE (ml)	Protein mg / ml	Hase evol U / mg	Hase oxid U / mg
N(10) LL	0.55	3.3	8.41	0.67	1.24
N(18) ES	1.17	9.7	10.51	0.66	1.34
N(36) LS	1.08	8.8	11.17	0.31	0.83
Triz(12) LL	0.48	3.1	7.94	0.57	1.1
Triz(24) ES	1.16	9	10.69	0.58	1.22
Triz(48) LS	1.27	9.5	9.73	0.53	1.14
NaOH(18/36)	0.58	3.8	8.88	0.65	1.28
NaOH(36/48)	1.2	9.7	12.11	0.66	1.36

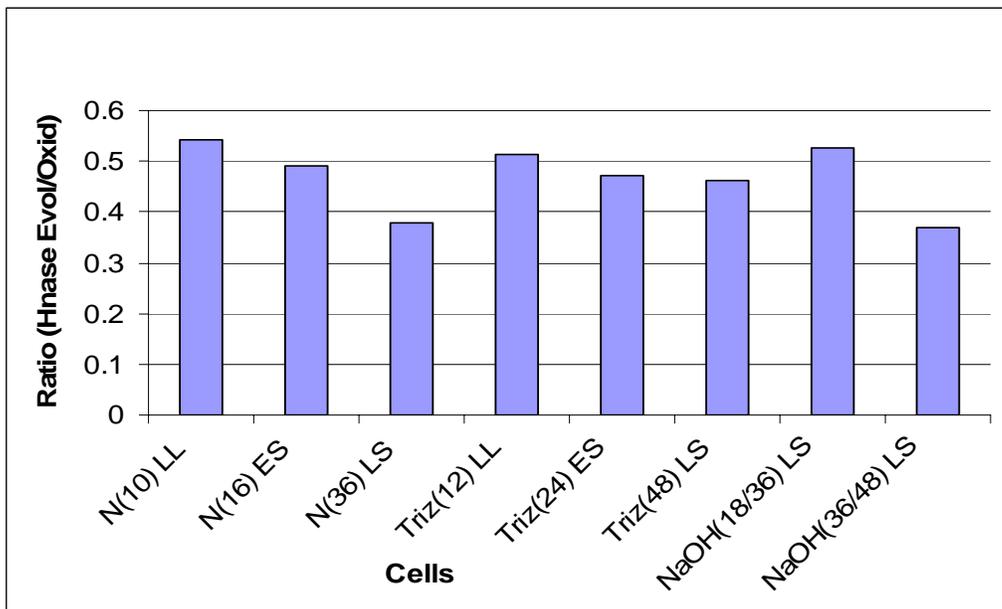


Figure 38: The ratio of the hydrogen evolution activity to the hydrogen oxidation activity of *T. neapolitana* DSM 5068 hydrogenase.

3.6 The growth and hydrogen production of *T. maritima*, *T. neapolitana* DSM 4359 and *T. neapolitana* DSM 5068 using agricultural residues as substrates

Agricultural residues, such as barley straw, corn stover, soybean straw, wheat straw and corn husk, were used as substrates to support the growth of three *Thermotoga* species, *T. maritima*, *T. neapolitana* DSM 4359 and *T. neapolitana* DSM 5068. The selected agricultural residues (0.25g /50 ml medium) were added to substitute for glucose or xylan. The growth and hydrogen concentration in the gas phase were measured.

3.6.1 *T. maritima*

When agricultural residues were used for supporting the growth of *T. maritima*, the growth on corn husk was the same as that from blank control (Fig.39). However, the hydrogen production in the gas phase was 13% (v/v), which was much higher than that from control (5% v/v) and quite close to that from glucose or xylan (15% v/v) (Fig.40). The growth on barley straw, corn stover, soybean straw and wheat straw was lower than control (Fig.39), and the hydrogen production in the gas phase was about 6% (v/v), which was also quite similar to that from the control as well (Fig.40). It seemed that barley straw, corn stover, soybean straw and wheat straw could not support the growth and hydrogen production of *T. maritima* directly; but corn husk could be used as a substrate to support the hydrogen production of *T. maritima*.

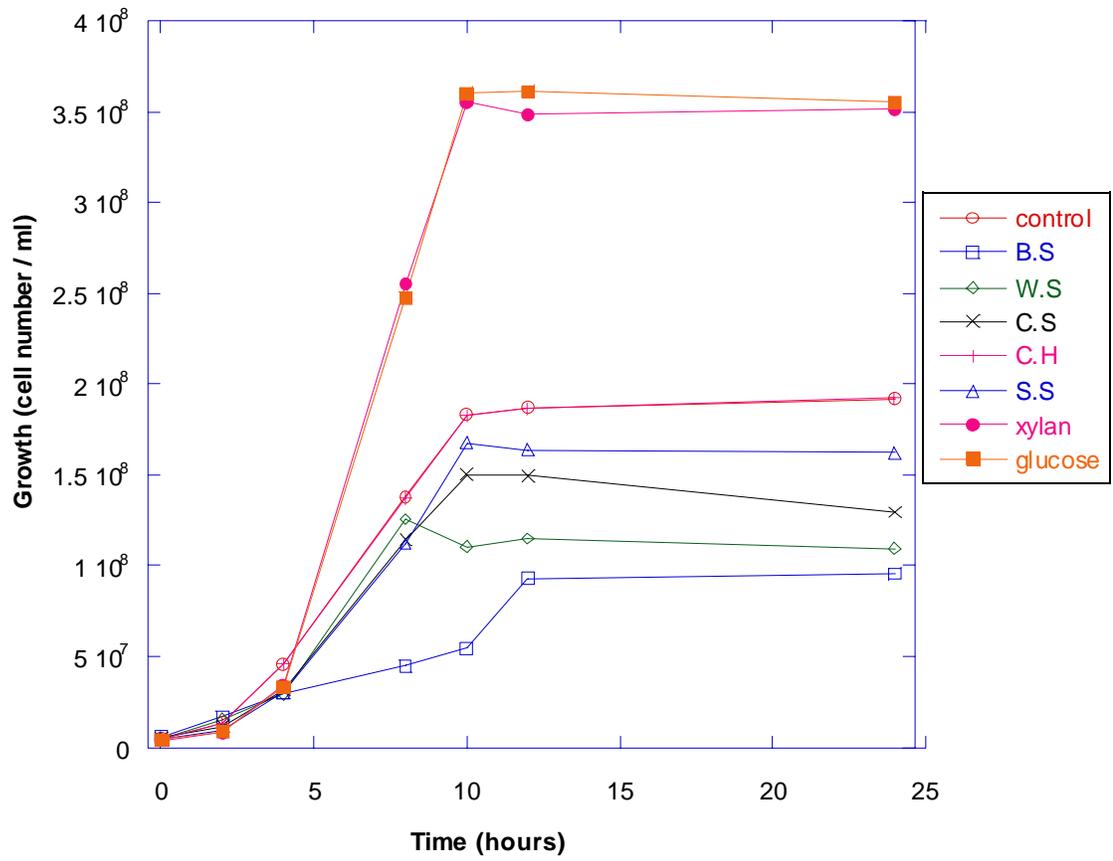


Figure 39: The growth of *T. maritima* on agricultural residues.

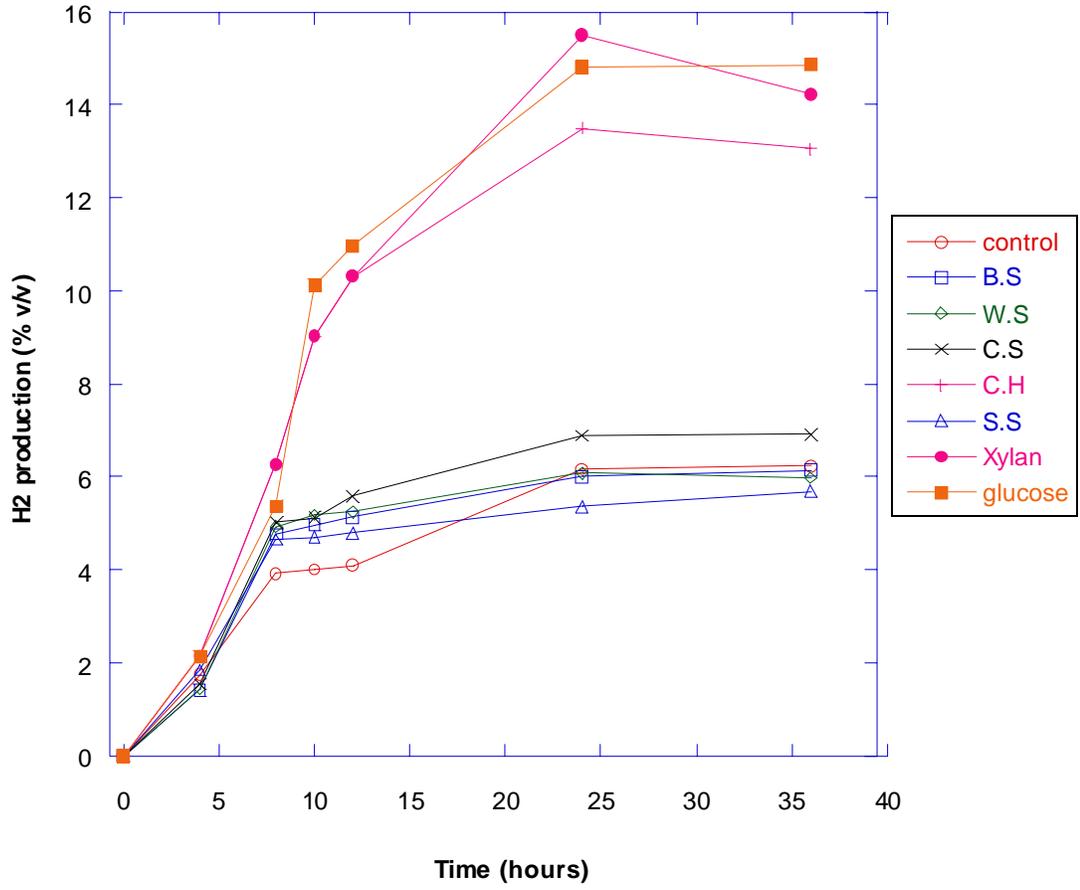


Figure 40: Hydrogen production of *T. maritima* grown on agricultural residues.

3.6.2 *T. neapolitana* DSM 4359

When agricultural residues was used for supporting the growth of *T. neapolitana* DSM 4359, the growth on corn husk achieved 4.5×10^8 /ml, which was much higher than that from blank control (2×10^8 /ml), and about 75% of that from glucose (6×10^8 /ml) (Fig.41). The hydrogen produced was 12.7% in the gas phase, which was higher than that of the control (Fig.42). The growth on barley straw, corn stover, soybean straw and wheat straw was the same as that of the control (Fig.41), and the hydrogen production produced in the gas phase was similar to that of the control (Fig.42). It seemed that barley straw, corn stover, soybean straw and wheat straw could not support the growth and hydrogen production of *T. neapolitana* DSM 4359 directly; but corn husk could be used as substrate to support the growth and hydrogen production of *T. neapolitana* DSM 4359.

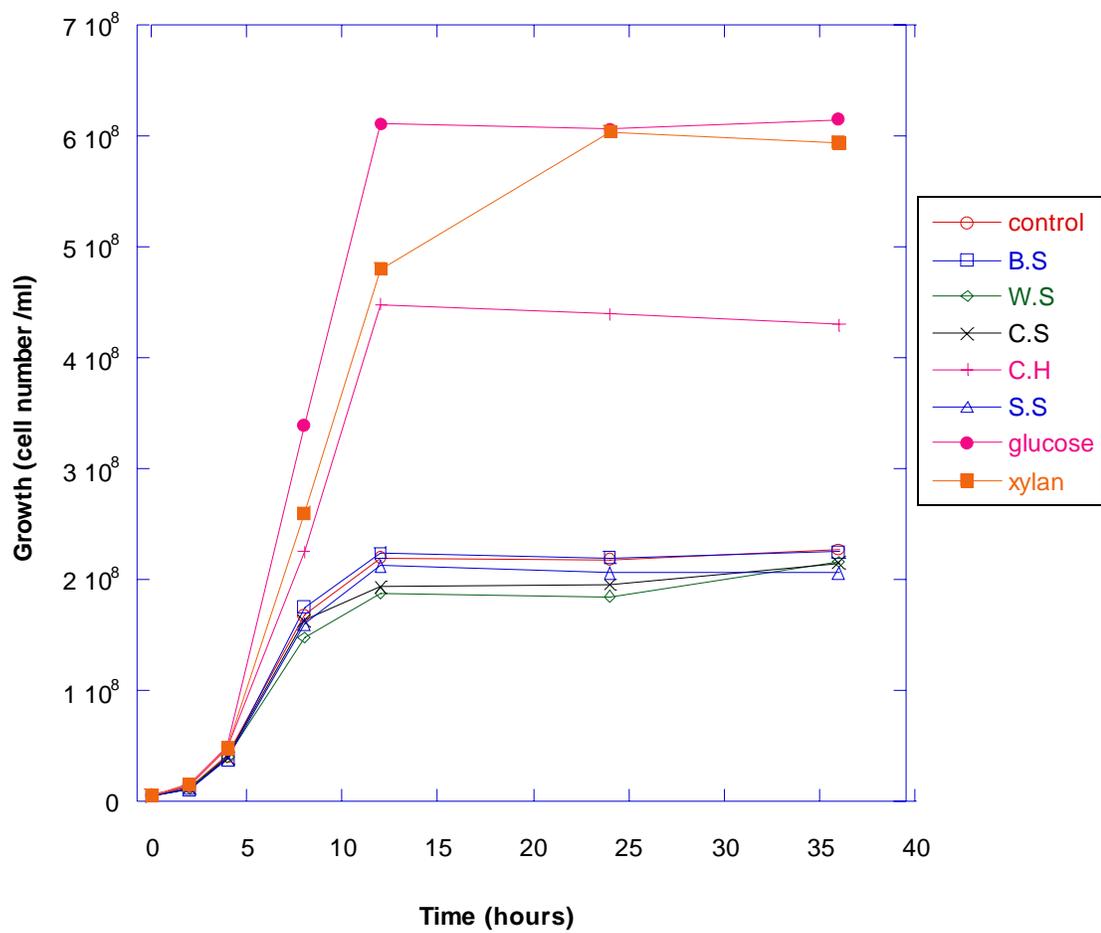


Figure 41: The growth of *T. neapolitana* DSM 4359 on agricultural residues.

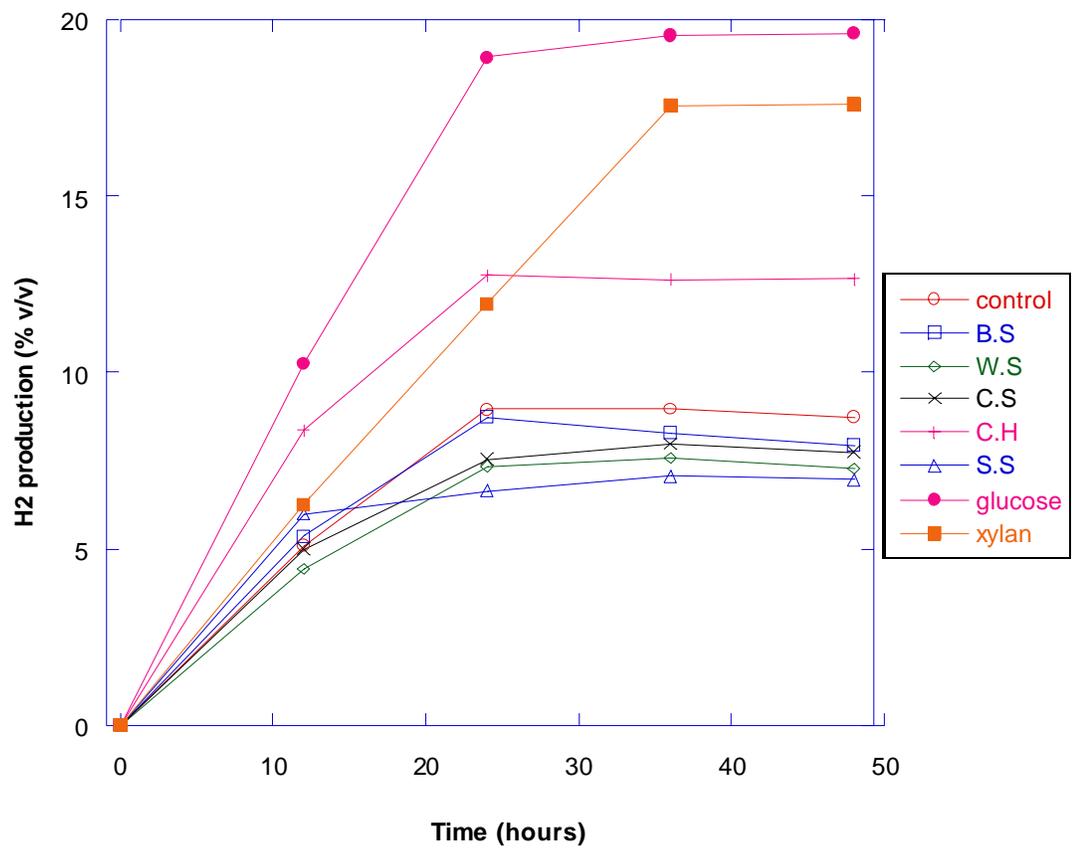


Figure 42: Hydrogen production of *T. neapolitana* DSM 4359 grown on agricultural residues.

3.6.3 *T. neapolitana* DSM 5068

When agricultural residues was used for supporting the growth of *T. neapolitana* DSM 5068, the growth on corn husk achieved 4.2×10^8 /ml, which was much higher than that of the control (2.7×10^8 /ml), and about 60% of that of glucose (7×10^8 /ml) (Fig.43). The hydrogen produced was 13% in the gas phase, which was higher than that of the control (Fig.44). The growth on barley straw, corn stover, soybean straw and wheat straw was the same as that of the control (Fig.43), and the hydrogen produced was similar of that of the control (Fig.44). It seemed that barley straw, corn stover, soybean straw and wheat straw could not support the growth and hydrogen production of *T. neapolitana* DSM 5068 directly; corn husk could be used as substrate to support the growth and hydrogen production of *T. neapolitana* DSM 5068.

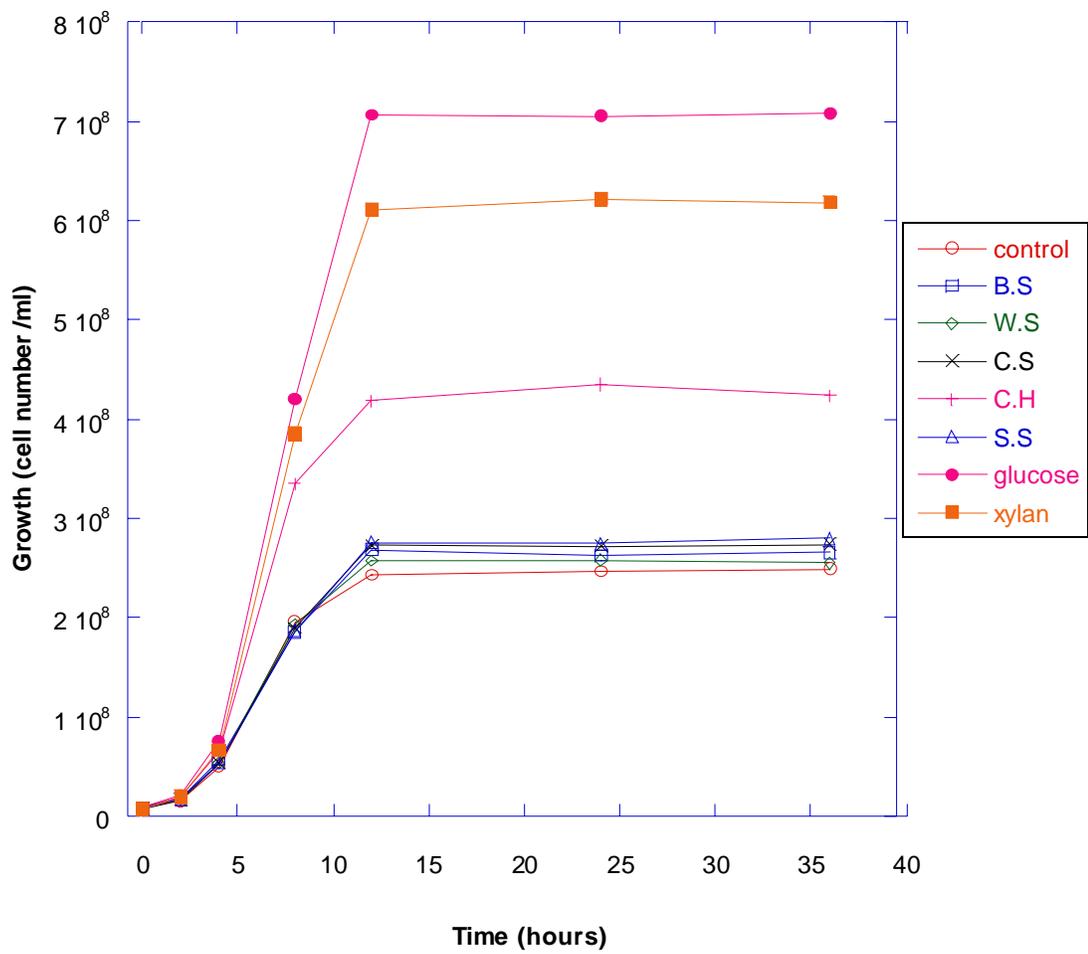


Figure 43: The growth of *T. neapolitana* DSM 5068 on agricultural residues.

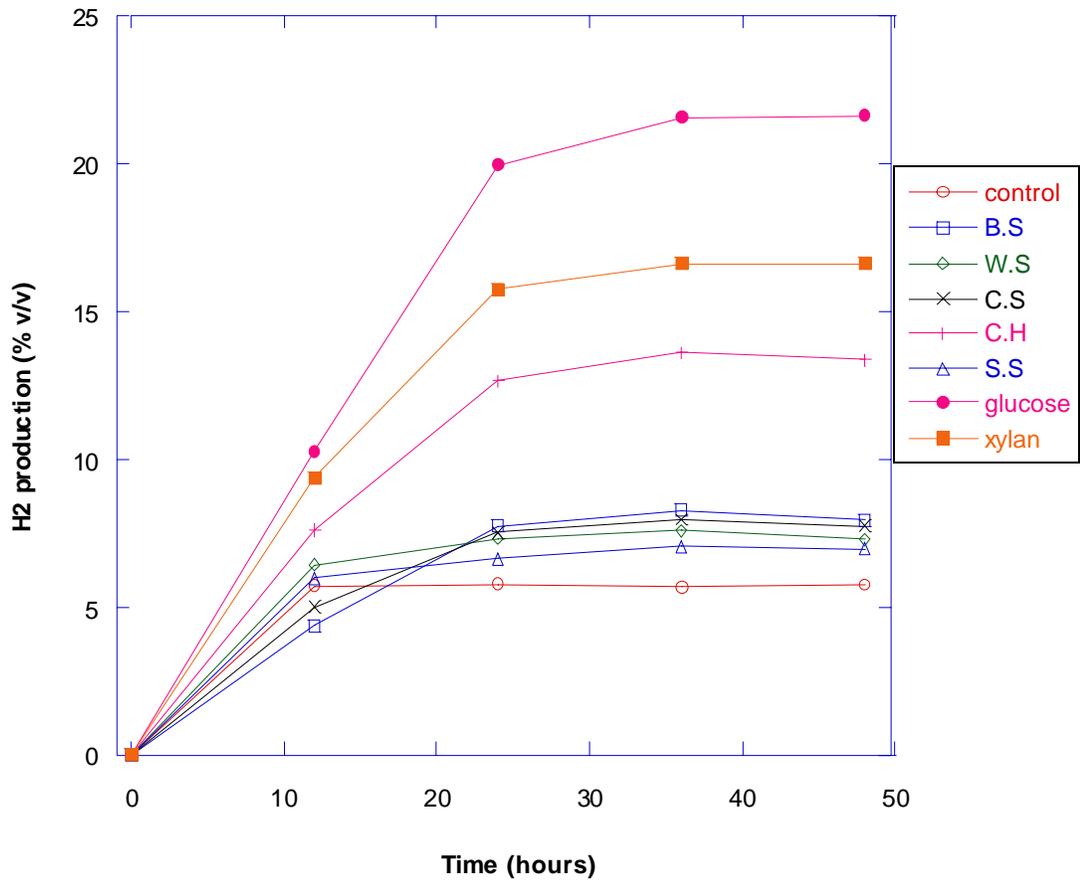


Figure 44: Hydrogen production of *T. neapolitana* DSM 5068 grown on agricultural residues.

3.6.4 The growth and hydrogen production of *T. neapolitana* strains using corn husk as substrate in the presence of Triz buffer or pH adjustment with NaOH

When corn husk was used as substrate to support the growth of *T. neapolitana* strains, pH control methods were used to test if there would be any further enhancement in the growth and hydrogen production. When *T. neapolitana* DSM 4359 was grown in the presence of 50 mM Triz buffer (initial pH 8.5), the growth was much better because the final cell density increased from 4.5×10^8 /ml to 7.1×10^8 /ml, and hydrogen production also increased from 12.7% to 21% (v/v) (Table 12). The pH adjustment using NaOH at the early stationary phase also resulted in increase in the hydrogen production to 19% (v/v), although no obvious increase in the growth was observed (Table 12). When *T. neapolitana* DSM 5068 was grown in the presence of 50 mM Triz buffer (initial pH 8.5), the growth was also much better because the final cell density increased from 4.2×10^8 /ml to 7.8×10^8 /ml, and hydrogen production also increased from 13% to 24% (v/v) (Table 12). The pH adjustment using NaOH at the early stationary phase also increased the hydrogen production to 20% (v/v), and the final cell density increased to 5.9×10^8 /ml. These results indicated that the pH control methods could enhance the utilization of corn husk to produce hydrogen by *T. neapolitana* strains.

Table 12: The growth and hydrogen production of *T. neapolitana* strains (corn husk as carbon source):

	Final cell density of DSM 4359 (cells /ml)	Hydrogen Production of DSM 4359 (% v/v)	Final cell density of DSM 5068 (cells /ml)	Hydrogen Production of DSM 5068 (% v/v)
Without pH control	4.5×10^8	12.7%	4.2×10^8	13%
pH stabilized by 50mM Tris buffer	7.1×10^8	21%	7.8×10^8	24%
pH adjusted using NaOH solution (at 18 hr)	5×10^8	19%	5.9×10^8	20%

Chapter 4

Discussion

Biological processes provide a range of approaches to generate hydrogen, including direct photolysis, indirect photolysis, photo-fermentation, and dark fermentation (Levin et al. 2004). Biological hydrogen production from anaerobic fermentation of organic substrates has the potential to be an economical and sustainable means of hydrogen production if conversion yields can be increased. Compared to other processes, fermentative hydrogen production has several advantages, such as high hydrogen production rate, high growth rate, no oxygen production and the possibility for applications in organic waste treatment. To produce hydrogen by fermentation processes, different microorganisms are used to achieve this goal. Hyperthermophiles are defined as organisms with optimal growth temperatures at about 80°C or higher (Stetter 1999; Huber and Stetter 2001). Cultivation of hyperthermophiles at high temperatures prevents contamination from ordinary microbes. Another advantage is that the solubility of various macromolecules increases at high temperatures.

The genus *Thermotoga* belongs to the order *Thermotogales*, and it is located at one of the deepest branches of the bacteria domain (Fardeau et al. 1997). *Thermotoga* species have the ability to ferment various carbohydrates like glucose, starch and xylan, forming acetate, L-lactate, H₂ and CO₂ as by products and end products (Huber et al. 1986). Among all the *Thermotoga* species, the highest hydrogen production reported is about 25%-27% hydrogen accumulated in the gas phase when *T. neapolitana* DSM 4359 was cultivated in small scale batch culture with a very high cell density of 2.78×10^9 /ml (Van Ooteghem et al. 2002). To further explore the potential of hydrogen production using various substrates, *T. neapolitana* DSM 4359 and *T. neapolitana* DSM 5068 were used in this research and *T. maritima* was used for comparative study.

4.1 The growth and hydrogen production of *Thermotoga* species

T. maritima, *T. neapolitana* DSM 4359 and DSM 5068 can grow on different carbon sources, including simply sugars and complex carbohydrates (Bronnenmeier et al. 1995; Bok et al. 1998). The results from this study showed that these micro organisms could grow on glucose, xylose, starch and xylan, but not on cellulose. Although *T. neapolitana* species have longer generation time than that of *T. maritima*, the final cell density and hydrogen production ability of *T. neapolitana* strains were greater, indicating that *T. neapolitana* strains may be more suitable for biological hydrogen production. These *Thermotoga* species could not growth on cellulose, although cellulolytic enzymes have been isolated from *T. maritima* (Bronnenmeier et al. 1995). It was reported that the cellulases from *T. neapolitana* were induced during the growth on cellobiose (Vargas and Noll 1996). However, these *Thermotoga* species could grow on xylan and produce hydrogen. The xylanases from *T. maritima* and *T. neapolitana* were purified from the cells grown on xylan and xylobiose (Bronnenmeier et al. 1995; Zverlov et al. 1996). It seems that these *Thermotoga* species have much better capacity for utilizing xylan than cellulose. This is also consistent with the results that corn husk can be used as a substrate and it contains a higher hemicellulose compared to other agricultural residues tested in this study (Hang and Woodams 1999; Wyman 1994).

The growth and hydrogen production of microorganisms can be easily affected by the growth conditions. The factors which have significant influences should be studied. In this research, the factors such as growth temperature, salt concentration, oxygen in the gas phase, the consumption of substrates, the accumulation of end products and the initial pH were studied. Based on all results obtained, the relation between the factors and the hydrogen production can be monitored and the optimization of the hydrogen production may be achieved. Although *T. neapolitana* strains were reported to have an optimal growth temperature as 77°C, *T. neapolitana* DSM 4359

can grow at a broad range of temperature from 60°C to 90°C. The best conditions for growth and hydrogen production without pH control were very similar at the temperature ranging from 70°C to 80°C with a final cell density of 6×10^8 / ml (or higher) and hydrogen production of 20% (v/v). *T. neapolitana* can grow at salt (NaCl) concentrations ranging from 0.5% to 5% (w/v), but 1% to 2% (w/v) salt present in the growth medium made no difference in the growth and hydrogen production. Such a broad adaptation of *T. neapolitana* strains makes it attractive to be considered in a practical biological hydrogen production process.

Compared to the factors discussed above, the oxygen level and pH value are more important. *T. neapolitana* strains and *T. maritima* were reported as strict anaerobic micro organisms (Huber et al. 1986; Belkin et al. 1986; Windberger et al. 1989). Although oxygen is toxic and sparse in their natural habitat, it has been reported that some *Thermotoga* species, such as *T. maritima* and *T. neapolitana* DSM 4359, could grow in the presence of micro-molar level oxygen (van Ooteghem et al. 2002; van Ooteghem et al. 2004; Yang and Ma 2007). Normally, to remove the trace amounts of oxygen in the medium, reducing agents, such as cysteine-HCl, were used. However, using these reducing agents is not economically feasible because they are relatively expensive, and the ability of microorganisms to survive under micro aerobic condition would be important for applicants in industry. Both *T. neapolitana* strains can grow and produce hydrogen without cysteine-HCl and there is no change in the cell density and hydrogen production. *T. neapolitana* strains could even survive in the presence of about 1% (v/v) oxygen in the gas phase (1 ml oxygen in 110 ml gas phase), although the inhibition caused by the oxygen is obvious, with only 30% to 40% cell density and hydrogen production remaining under this condition (1% oxygen). On the other hand, the growth and hydrogen production of *T. maritima* were completely stopped when the oxygen concentration was about 0.5% (v/v) in the gas phase. A much higher oxygen tolerance (12% in the gas phase) has been reported by van Ooteghem in 2004 (van Ooteghem et al. 2004). However, considering the differences in the growth condition, for example, cysteine-HCl was added in the

medium, the reported oxygen tolerance may not be reliable. The oxygen tolerance of *Thermotoga* species could be partially explained by the presence of an oxygen-removing system. Based on current report, the purified NADH oxidase from *T. maritima*, whose activity was depended on concentrations of both oxygen and NADH, is very efficient at removing oxygen. Although NADH oxidase from *T. maritima* could produce both H₂O₂ and H₂O during the oxygen-removing, there was no H₂O₂ produced when the oxygen concentration was low (up to 5% v/v) in a short period of time. When the oxygen concentration increased, the H₂O₂ was produced, and the production increased along with the oxygen concentration (Yang and Ma 2007). Because H₂O₂ is more toxic than molecular oxygen, this oxygen-removing system could provide an explanation why anaerobic *T. maritima* can tolerance low oxygen concentration, and only low oxygen concentration. The oxygen tolerance is very important for industrial scale of biological hydrogen production. The oxygen tolerance also shows the importance to the “combined process”, which combines photosynthetic and non-photosynthetic microorganism together. In the combined process, the oxygen sensitive fermentative microorganisms would be inhibited by oxygen produced by photosynthetic microorganisms.

The pH of the medium showed strong effects on growth and hydrogen production. Although *T. neapolitana* could grow at the initial pH ranging from 6 to 9, it could only grow best at the initial pH between 7 and 8.5 to achieve cell density higher than 6×10^8 / ml. Within this range, higher pH value resulted in longer lag phase and higher hydrogen production. The maximum hydrogen production (23% v/v) was achieved at an initial pH 8.5 with a 4 hour lag phase. When the pH increased to 9 or 9.5, the inhibition of the growth and hydrogen production were very significant. However, at high pH, *T. neapolitana* still could grow slowly, which was much better than the growth at initial pH 6 or 5.5. This indicated that the acidic condition was not favourable to hydrogen production of *T. neapolitana*. In fact, during the growth, organic acids such as lactate and acetate were accumulated in the medium and the pH of the medium was gradually decreased.

The methods to stabilize the pH during the growth have positive effects on hydrogen production, which will be further discussed in section 4.3.

4.2 The consumption of substrates and accumulation of end products

The growth of microorganisms is limited because of the consumption of the substrates and the accumulation of end products. It was reported that some *Thermotoga* species, including *T. maritima*, utilize sugars, such as glucose, to produce acetate, alanine, lactate, H₂ and CO₂ through the Embden-Meyerhof (EM), Entner-Doudoroff (ED) pathways and other electron transfer processes (Schröder, et al. 1994; Martina, 1997). The end products ratio of H₂ to CO₂ is 2 to 1 (van Ooteghem et al 2004). The yeast extract is required for the growth of *Thermotoga* species. In batch culture, yeast extract and glucose were consumed; acetate, alanine and lactate were produced and accumulated in the medium; H₂ and CO₂ were produced and accumulated in the gas phase. When *T. neapolitana* DSM 4359 cultures were incubated at 77°C at initial pH of 7, the highest cell density was 6×10⁸ /ml after 12 hours incubation and the highest H₂ production was 20% (v/v) after 36 or 48 hours incubation, and the highest acetate concentration was 12 mM and L-lactate concentration was 4 mM after 72 hours incubation. Using pH control approach (in the presence of 50 mM Triz buffer), the acetate concentration was 24 mM and L-lactate concentration was 24 mM after 72 hours incubation, which was much higher. Alanine, acetate and CO₂ did not show significant effects on the growth and H₂ production, because the addition of alanine and acetate or removing the CO₂ from the gas phase had no effect to the growth and hydrogen production. Hydrogen was reported to be an inhibitory factor to the growth and hydrogen production of *T. neapolitana* (Childer et al. 1992), however, in this study, removing the hydrogen from the gas phase did not result in any increase in the hydrogen production. This may be because both growth and hydrogen production of *T. neapolitana* were inhibited by other factors, or the hydrogen concentration was not high enough to show any inhibitory effect. Adding extra yeast extract resulted in slight increase in the growth and H₂ production, indicating that the depletion of

yeast extract in the medium might be partially responsible for the termination of the H₂ production process. Adding glucose was not necessary because there was still about 65% of initial added glucose remaining in the medium after 72 hours incubation. The inhibition caused by L-lactate was obvious when its concentration was 10 mM, which was close to the highest L-lactate concentration produced in the culture medium (8 mM). The inhibition caused by L-lactate to H₂ production was higher than the inhibition to the growth, but, in general, the inhibition was decreased when the initial pH was increased at the pH range from 6.5 to 8.5 (Table 4& table 5). When the initial pH value was lower than 6.5 or higher than 8.5, the growth of *T. neapolitana* strains was very poor. The inhibition caused by L-lactate presented in the medium may be due to the possibility that higher concentration of extra cellular L-lactate can inhibit the lactate efflux down the concentration gradient from inside the cell to outside the cell. Lactate efflux can produce a membrane potential by the symport with protons or sodium ions without ATP consumption. It was reported that the addition of lactate to the external medium prevented the formation of membrane potential, because the lactate concentration gradient was decreased (Otto R et al. 1982). The methods that can be used to decrease the L-lactate production or remove the L-lactate from the medium may need to be considered for achieving higher hydrogen production.

4.3 pH control methods

It was showed that the initial pH did have effects on both growth and hydrogen production of *T. neapolitana* strains. It was reported previously that the initial pH and pH control showed profound effect to the hydrogen production potential and hydrogen production rate (Lay JJ et al.1999; Lay JJ et al.2000; Fang HHP and Liu H. 2002; Khanal SK et al. 2004). In these studies, with continuous monitoring of pH and hydrogen production during hydrogen fermentation in the serum bottle, it is possible to determine the actual pH values that yield maximum hydrogen. Therefore, a control of pH for optimizing the hydrogen production was required. In this study, Triz buffer (pKa 8.06) was selected to stabilize the pH of the medium, because the pH range to achieve maximum

hydrogen production was 7 to 8.5. In the presence of Triz buffer, enhancement of the final cell density and hydrogen production of *T. neapolitana* DSM 4359 were observed (Table 6). The initial pH of medium was 8.5 and stabilized by 50 mM Triz buffer could achieve final cell density of 1.1×10^9 /ml, which was about 2 fold of the cell density achieved without Triz buffer, and hydrogen production was 35% (v/v) in the gas phase, which was much higher than the hydrogen production achieved without Triz buffer (20% v/v). When this pH control method was applied to *T. neapolitana* DSM 5068, the hydrogen production was 39% (v/v) in the gas phase. By converting the hydrogen production from percentage to mmol (per 50 ml medium), it was clear that pH 8.5 was the optimal initial pH for hydrogen production when the medium was stabilized by 50 mM Triz buffer. During the growth, all the hydrogen production continued when the pH remained between 8.5 to 7, which was in the range of Triz buffer's capacity. Higher pH or Triz buffer concentration resulted in much lower hydrogen production rate (Table 6). At the pH ranging from 7 to 8.5, the hydrogen was mainly produced during the late log phase and the early stationary phase, therefore, the pH stabilization during this period of time was necessary for the hydrogen production. Without buffering the pH, the log phase of *T. neapolitana* lasted only 10 hours and the pH decreased to 6 after the log phase. In the presence of 50 mM Triz buffer (initial pH 8.5), the log phase lasted about 30 hours; after the log phase and the early stationary, the medium pH was stabilized at 7 or above. Combined with the increasing of the growth, *T. neapolitana* strains with higher cell density could produce more hydrogen in a longer period of time at appropriate pH values.

Considering the cost of Triz buffer, other pH control methods were studied. To stop or slow down the pH decreasing during the growth, the pH could be not only controlled by Triz buffer, but also by alkali during the growth. Adding a certain amount of 1 M NaOH solution to adjust the pH back to 7 at early stationary phase (18 hours) without any buffer involved could also enhance the hydrogen production of *T. neapolitana* DSM 4359 from 20% to 30% (v/v), and the final cell

density was increased from 6×10^8 /ml to 6.8×10^8 /ml. The adjustment at late stationary phase (after 36 hours) only resulted in a slight increase in the hydrogen production, and the final cell density did not increase significantly after the pH adjustment. It seems that this method only resulted in increase in the hydrogen production. Although this method could not provide the same stimulating effect on hydrogen production compared to the use of Triz buffer method, adding NaOH solution is much easier to apply and it is less expensive. Adding NaOH at the late stationary phase only resulted in a slight increase in hydrogen production. Considering the complex mechanisms of such inhibition at the late stationary phase, the pH adjustment may not be sufficient for reversing the inactivation occurred at this period of time.

To achieve a practical hydrogen production process, not only high hydrogen yield needs to be considered, but also the conversion efficiency. The conversion efficiency from the substrates such as glucose to hydrogen during the fermentation of microorganisms is directly related to the efficiency of biological hydrogen production. It was reported that some *Thermotoga* species, including *T. maritima*, degrade sugars through Embden-Meyerhof (EM) and Entner-Doudoroff (ED) pathways (Schröder, et al. 1994, Martina, 1997), and EM pathway was the main metabolic pathway (85%). In a typical EM pathway, 1 mole glucose was theoretically converted to 4 moles H_2 ($C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 4H_2 + 2CO_2$). Based on the results obtained (Table 7 & 8), the pH control methods not only enhanced the hydrogen production, but also the conversion efficiency. The conversion efficiency was about 3.6 in the presence of 50 mM Triz buffer. This value is about 90% of the theoretical hydrogen conversion efficiency. The method using NaOH also resulted in the increase in the conversion efficiency from 2.2 to 3.3 (DSM 4359) and 2.7 to 3.5 (DSM 5068), respectively, which was also 80% to 88% of the theoretical hydrogen conversion efficiency. It was reported that, in batch culture, the hydrogen conversion efficiency from glucose to hydrogen was around 2.0-2.4 (Ueno et al. 2001; Fang and Liu 2002; Morimoto et al. 2004), which was quite similar to the result in this study without using pH control (about 2.2).

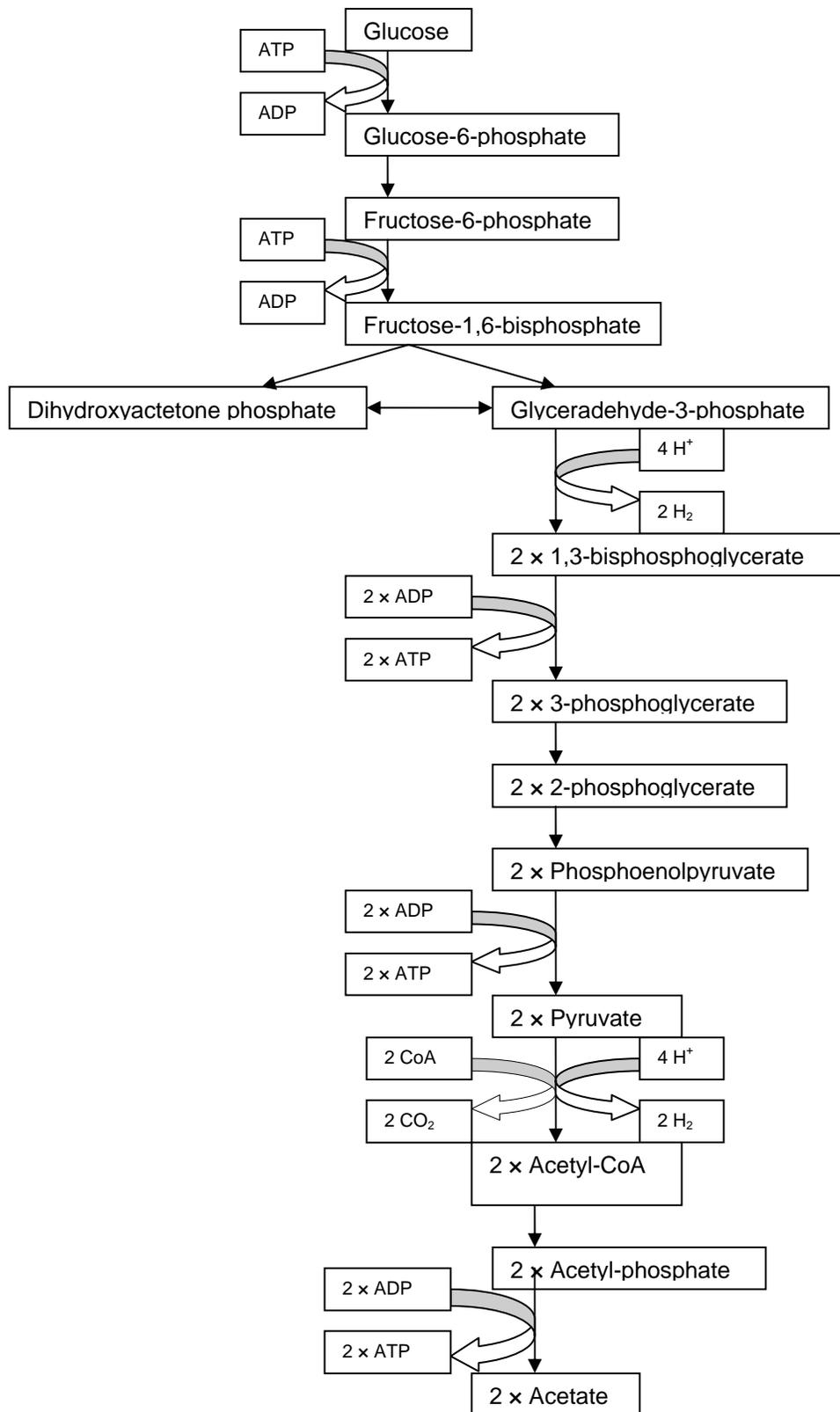


Figure 45: Embden-Meyerhof (EM) pathway for glucose fermentation to acetate, CO₂ and H₂ in *Thermotoga maritima*. (Schröder et al. 1994)

Enterobacter cloacae ITT-BY 08 achieved the highest conversion efficiency that produced 2.2 mole H₂ per mole glucose, 6 mole H₂ per mole sucrose and 5.4 mole H₂ per mole cellulose (Kumar and Das 2000). The increased conversion efficiency caused by the pH control methods should be credited to the cells that were kept active at proper pH value of the growth medium.

4.4 Hydrogenase

Hydrogenases catalyze the reversible oxidation of H₂ ($2\text{H}^+ + 2\text{e}^- = \text{H}_2$), and they have been purified from a wide variety of bacteria and archaea. Fe-hydrogenase has been purified from *T. maritima*, and the hydrogenase activities have been detected in *T. neapolitana* DSM 4359 (Juszczak et al. 1991; Kaslin et al. 1998). In this study, both hydrogenase activities (hydrogen evolution activity and hydrogen oxidation activity) were determined at different growth phases by using different pH control methods. At normal condition (without pH control), the hydrogenase activities (both directions) were quite similar between the late log phase and the early stationary phase. Compared to the log phase and the early stationary phase, hydrogenase activities were lower at the late stationary phase. Based on the results obtained, there were no growth and hydrogen production of *T. neapolitana* strains when the cells were at the late stationary phase. These results may lead to a conclusion that the expression of hydrogenase was lower in the late stationary phase due to the unfavourable growth conditions. Compared to the cells grown without pH control, the use of pH control methods, such as adding 50 mM Triz buffer, could result in increase in the hydrogenase activities (both directions) at the late stationary phase, so the hydrogenase activities (both directions) at the log phase, early stationary phase and stationary phase were very similar. This discovery, combined with the previous results that in the presence of 50 mM Triz buffer, the hydrogen production rate of *T. neapolitana* DSM 4359 was quite stable during 60 hours incubation (Fig.32), leads to another conclusion that the expression of the hydrogenase is directly related to the hydrogen production capacity. Compared to the method using Triz buffer, adding NaOH at 18 hours to adjust the pH also showed similar effect on the hydrogenase activities of *T.*

neapolitana strains in the late stationary phase. It showed that the expression of hydrogenase was related to the pH changing, and the expression of hydrogenases would not be inhibited unless the pH value of the medium was decreased to an unfavorable level (lower than pH 5.5). On the other hand, adding NaOH at 36 hours to adjust the pH only resulted in an increase in the hydrogen oxidation activity of *T. neapolitana* DSM 5068 at the late stationary phase. This indicated that when the expression of hydrogenase was inhibited by low pH value, this inhibition could not be reversed in a short period of time just by pH adjustment. The ratio of the hydrogen evolution activity and the hydrogen oxidation activity of hydrogenase from *T. neapolitana* DSM 4359 and DSM 5068 were different at different growth conditions and at different growth phases. It may indicate that more than one hydrogenase were involved in the hydrogen production process and the growth conditions had different effects to the expression of these enzymes. The enhancement of hydrogen evolution hydrogenase activities and the decrease or elimination of hydrogen oxidation hydrogenase activities may need to be considered in further studies.

4.5 The utilization of agricultural residues

In general, the raw materials used to replace the sugars in biological hydrogen production always need pre-treatment. For example, steam explosion, wet oxidation under alkaline conditions, supercritical CO₂ pre-treatment, mild and concentrated acid/alkali hydrolysis and solvent extractions are applied to the lignocellulose contained biomass to promote the accessibility of polysaccharides in the lignocellulose complex for enzymatic hydrolysis (Fan and Lee 1982; Parisi 1989; Bjerre et al. 1996; Zheng et al. 1995). In this study, only a simple pre-treatment such as milling was used, and the powder of agricultural residues was directly used as substrates in a concentration similar to that of glucose. Other pre-treatment procedures were not applied considering economic and environmental reasons. Although some selected agricultural residues, such as barley straw, corn stover, soybean straw and wheat straw, could not be utilized directly for hydrogen production under the tested conditions, *Thermotoga* species showed capacity of utilizing

corn husk as a substrate to support the growth and hydrogen production, and about 60% growth and hydrogen production were achieved compared to that from glucose. This may be due to the fact that corn husk has a higher content of hemicelluloses (44%) and less lignin (6.6%) (Hang and Woodams 1999). Compared to other agricultural residues that contain 35-50% cellulose, 20-35% hemicelluloses and 10-25% lignin (Wyman 1994), less lignin components could decrease the inaccessibility caused by the special structure of lignin embedded in the lignocellulose. Combined with the results that *Thermotoga* species could utilize xylan, but not cellulose, it seems that *Thermotoga* species had a much better capability of utilizing hemicellulose components in the agricultural residues to produce H₂. This indicated that the further selection of biomass should be focused on the candidates with more hemicellulose and less lignin. By using pH control methods, H₂ production of *T. neapolitana* species using corn husk as carbon source could achieve up to 24% (v/v) in the gas phase, which was similar (or higher) to the hydrogen production from glucose (21%) under the growth condition without pH control (Table 7). Since only trace amount of reducing sugar was detected in the medium during the growth of *Thermotoga* species on agricultural residues (including corn husk) with or without pH control, no evidence showed that the pH control methods could increase the hydrolysis of the agricultural residues in the medium. The enhancement of the hydrogen production should be contributed to the improvement of the hydrogen production capacity of *T. neapolitana* strains by using pH control methods.

Although *T. neapolitana* species could not utilize all the selected agricultural residues, it still provided a possibility to use waste biomass as a competitive alternative to the expensive sugars. The biological production of hydrogen by fermentation, using biomass as energy source, has many new developments. Many efforts were focused on the pre-treatment because a successful biological conversion of biomass to hydrogen strongly depended on the processing of raw materials to produce feedstock which could be fermented by the microorganisms (Benemann 1996; Benemann 2001). Based on the current reports, corn straw and Miscanthus, which has

similar chemical components to the agricultural residues used in this project, could be utilized by mixed culture and *Thermotoga elifii*, and possible pre-treatment methods, such as alkali extrusion, enzymatic hydrolysis and steam reforming may be required (Li and Chen 2007; Vrije rt al. 2002). All such energy consuming and environmentally polluting pre-treatments were avoided in this project and a one step hydrogen production system using agricultural residues to produce hydrogen has been investigated. Based on the current results, it is concluded that the biological hydrogen production from agricultural residues can be achieved using *Thermotoga* species.

Biological hydrogen production is still in its infancy. The current studies based on the existing technologies could only be used to study the potential for practical applications. Further research and development should be focused on the increase of the hydrogen production and conversion efficiency. A commercially competitive biological hydrogen production system must be able to provide sufficient hydrogen with minimum cost. Considerable efforts including optimization of the growth condition, removal of the end products, and genetic modification of enzymes and pathways that compete with hydrogen producing enzyme systems should be applied for the optimization of hydrogen production. The renewable, low value materials used as substrates and acceptable pre-treatment are also required to be investigated.

Chapter 5

Conclusion

5.1 *Thermotoga* species, *T. maritima*, *T. neapolitana* DSM 4359 and DSM 5068, could grow in the presence of mono sugars (glucose, xylose) and complex carbohydrates (starch, xylan). Mono sugars could support *T. neapolitana* strains to have better growth and hydrogen production than complex carbohydrates.

5.2 *T. neapolitana* strains have broad growth conditions. The range of growth temperatures of *T. neapolitana* strains to produce hydrogen was from 60°C to 90°C, and the best temperature range was from 70°C to 80°C. The range of salt (NaCl) concentrations was 0.5% to 5%, and the best concentrations was from 1%-2%. The range of the initial pH was from 6.0 to 9.0, and the best range of initial pH values was from 7 to 8.5.

5.3 Both *T. neapolitana* strains and *T. maritima* could grow and produce hydrogen in the present of micro-molar level oxygen in the absence of reducing agents. But *T. maritima* has higher oxygen sensitivity than *T. neapolitana* strains.

5.4 Compared to the slight inhibition caused by the L-lactate accumulation during the growth, the gradual pH decreasing caused by the accumulation of organic acids was the main reason for the inhibition of the growth and hydrogen production of *T. neapolitana* strains. The accumulation of acetate, alanine, H₂ and CO₂ have no considerable inhibition to the growth and hydrogen production.

5.5 The use of pH control methods could result in significant increase in the growth and hydrogen production by stabilizing the pH dropping during the growth. Increasing the initial pH to 8.5 and

using 50 mM Triz buffer could increase the growth and hydrogen production of *T. neapolitana* strains by 2 fold. The pH adjustment at the early stationary phase, by using NaOH, showed significant increase in the hydrogen production, but less increase in the growth. By using pH control methods, the conversion efficiency (converting glucose to H₂) of *T. neapolitana* strains was about 3.6, which was approximately 90% of the theoretical value (4 moles of H₂ produced from 1 mole of glucose).

5.6 The hydrogen evolution and oxidation hydrogenase activities have been detected in the *T. neapolitana* strains. The hydrogenase activities were directly related to the hydrogen production capacity and could be affected by pH control methods. The ratio of the hydrogen evolution activities and hydrogen oxidation activities indicated that more than one hydrogenase may be involved in the hydrogen production metabolism.

5.7 Some agricultural residues, such as corn husk, could be utilized by *Thermotoga* species directly. When corn husk was used as a substrate, *T. neapolitana* strains could grow and produce hydrogen, which was 60% of that from glucose under the growth conditions without pH control. With pH control, the growth and hydrogen production of *T. neapolitana* species from corn husk was similar or higher than that from glucose without pH control.

Further study

This research project was the first step to study the biological hydrogen production using low value materials such as agricultural residues by hyperthermophiles. However, to achieve higher hydrogen production rate and yield, the continuous growth of *Thermotoga* species using agricultural residues as substrates needs to be studied. Further work includes the study of pre-treatment methods of the agricultural residues, the optimization of the growth conditions related to the hydrogen production. Regarding the hydrogenase from *T. neapolitana* strains, the purification and the characterization of the enzymes are required.

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