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Growth of Bacillus methanolicus PB1 in seawater and isolation of its plasmid

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GROWTH OF *BACILLUS METHANOLICUS* PB1 IN SEAWATER AND ISOLATION OF ITS PLASMID

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ABSTRACT

GROWTH OF *BACILLUS METHANOLICUS* PB1 IN SEAWATER AND ISOLATION OF ITS PLASMID

by Louis Yip-Yan Cheung

Bacillus methanolicus, a gram-positive thermotolerant methylotroph, has interesting properties for large-scale production of bio-products such as L-lysine and glutamic acid. Preliminary experiments were done to test the ability of *B. methanolicus* PB1 (ATCC 51375) to grow in high salt concentration. The results from studies with strain PB1 showed little salt-inhibition of growth at the concentration of NaCl in seawater that is approximately 25 g/L. The ability of *B. methanolicus* PB1 to grow in seawater-based medium using methanol as a carbon and energy source was also tested. Through adaptation, PB1 was able to grow in minimal media with 100% seawater using methanol as carbon and energy source with good yields and similar growth rates as compared with media prepared in de-ionized water. The 20.5-kilo base pair plasmid DNA was found to be present in *B. methanolicus* PB1. This may further allow us to genetically modify the bacteria for production of some other amino acids or bio-molecules via seawater and methanol.

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CHAPTER 1.0 INTRODUCTION

1.1 Growth Potential of the Biotechnology Industry

Due to the increased understanding of the human genomic sequence and advances in recombinant technologies, gene products can be expressed in genetically engineered microbial or animal cell hosts [1]. The developments of processes for recombinant therapeutic proteins and other commercially important natural products have demonstrated the turning of genomic discoveries into reality [1-3]. L-lysine, for example, is a commercially important natural product because it is nutritionally important for livestock and human beings of which are unable to synthesize L-lysine. The development of biologically derived pharmaceuticals represents the strongest growth area in biotechnology. Recombinant pharmaceuticals, such as recombinant antibodies for cancer treatments, play an important role in improving health care and raising the quality of life. An increasing demand has been predicted with annual growth rates varying between 5 to 30% [5]. The research and development expenses were found to have an 18% increase between 2000 and 2001 [6]. The worldwide biotechnology market is expected to be worth US$38 billion by 2006 [7].

1.2 Over-expression Technology

Obtaining sufficient quantities of a bio-product of interest is always one of the major problems in the fermentation research industry. For example, a 10-L culture of *E. coli* producing DNA polymerase I may have a final titer of only 7 mg of protein [8].

Many additional proteins are also present in the culture. Thus, it is rare that the product of interest can be recovered in large amount and in pure form from the culture. In recent years, the development of over-expression technology has largely eliminated these difficulties [8].

Over-expression technology is combined with genetic engineering and recombinant DNA technology. The initial goal is to insert a DNA segment containing a gene of interest into an autonomously replicating DNA that is called an expression vector. Transforming an expression vector into a host organism such as *E. coli* can result in replication of large amounts of the vector and the inserted DNA segment, when the host organism replicates. If the cloned DNA segment is positioned properly with the protein synthesis regulatory sequences, including a promoter, terminator, ribosome binding site, large amounts of protein specified by that gene can be expressed [8].

1.3 What is an Over-expression Vector?

A plasmid-based over-expression vector, as shown in Figure 1, is a circular DNA that contains a replication origin where DNA replication is initiated. The replication origin allows the DNA to replicate autonomously in a host organism. The typical size of the plasmid-based over-expression vector ranges from 1 to 200 kilo base (kb) pairs. It may give benefits to its host by providing function such as antibiotics resistance. The antibiotics resistance gene, such as amp^R gene, within the plasmid always contributes to the antibiotics resistance. The amp^R gene confers resistance to ampicillin, an antibiotic derived from penicillin. Plasmids that are presented in one or a few copies per host are

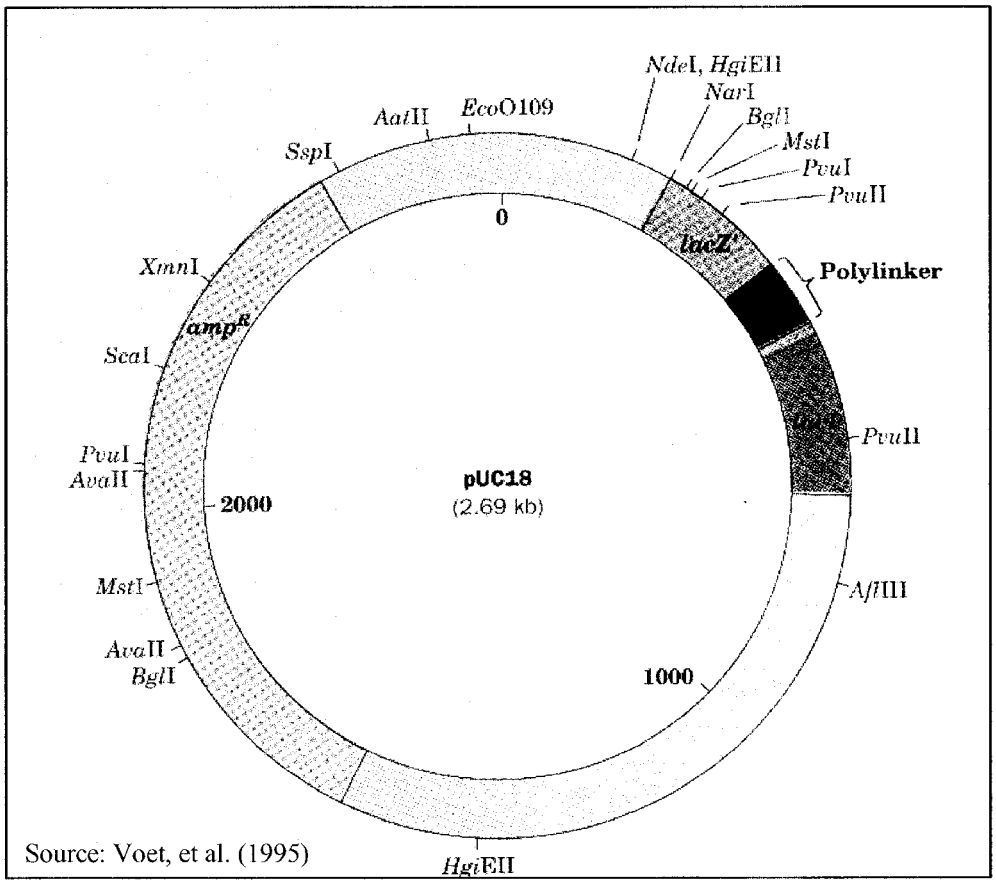

Figure 1. An example of plasmid-based cloning vector. PUC18 is a plasmid derived from *E. coli* [8].

said to be under stringent control. On the other hand, plasmids that are presented in 10 to as many as 700 copies per cell are said to be under relaxed control. The control of replication has therefore divided the bacteria two categories: bacteria with low copy number of plasmid and bacteria with high copy number of plasmids. Plasmids under relaxed control are commonly used in molecular cloning. Plasmid vector also contains a number of restriction endonuclease sites. Segments of DNA can be inserted into these restriction sites by a technique called gene splicing. Many other plasmid vectors also contain a short segment of DNA known as polylinker. The polylinker is strategically synthesized to contain a variety of restriction endonuclease sites that are unique within the plasmid [8].

1.4 Construction of a Recombinant DNA Plasmid by Gene Splicing

A plasmid vector contains a number of restriction endonuclease sites where it can be cut by specific restriction enzymes. Restriction enzymes cut duplex DNA at specific sites. This reaction yields single-stranded ends that are complementary to each other. Therefore, a foreign DNA fragment can be inserted into a plasmid vector by using the same restriction enzyme for cutting. Figure 2 shows the details of the construction of recombinant DNA plasmid by gene splicing.

The complimentary ends of the two DNAs can be specifically joined through the action of an enzyme called DNA ligase [8]. If the cloned DNA segment is positioned with the controlling sequence of protein synthesis properly within the expression vector,

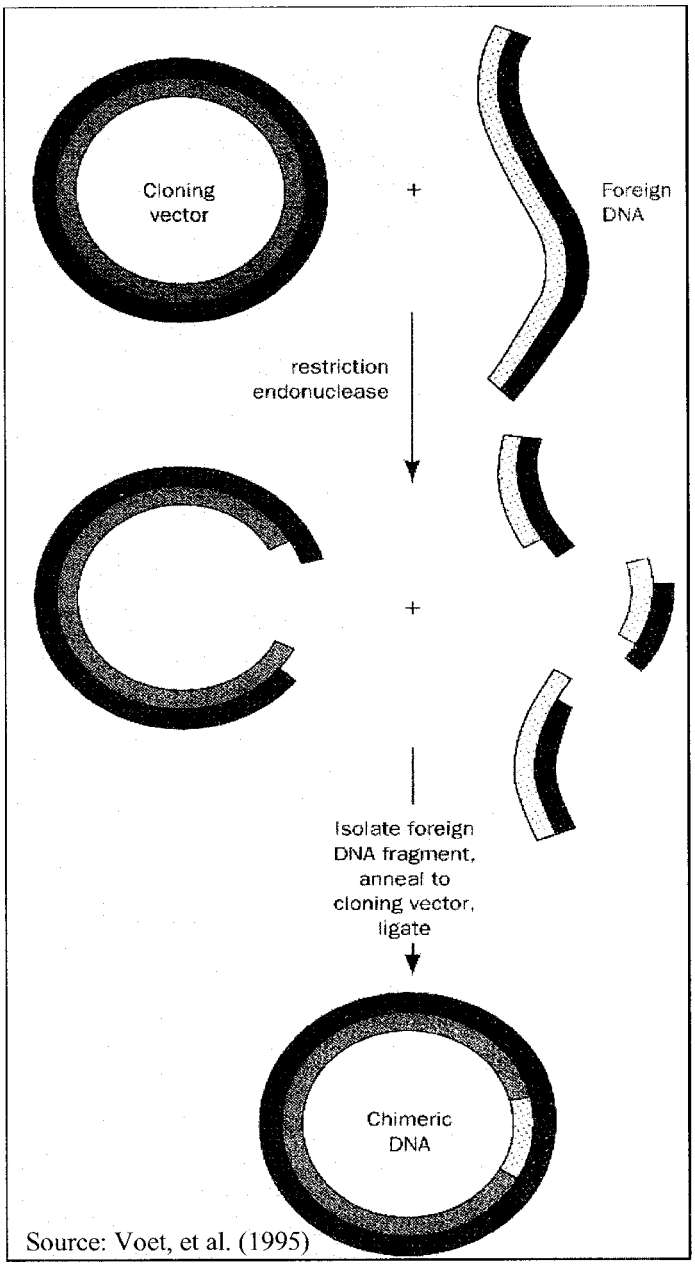

Figure 2. Construction of a Recombinant DNA Plasmid by Gene Splicing [8].

large amounts of protein specified by that gene can be expressed when the expression vector is transformed into a host organism.

1.5 *Bacillus methanolicus*

1.5.1 Gram-Positive Bacteria

Bacteria can be divided into two major groups: gram-positive and gram-negative. *B. methanolicus* belongs to the gram-positive group. The distinction between gram-positive and gram-negative bacteria is their cell wall structure. The gram-positive cell wall consists mostly of peptidoglycan and teichoic acid. Gram-positive cell wall is much thicker when comparing to gram-negative cell wall. A gram-negative cell wall is a multi-layered structure and is quite complex [9]. Figure 3 shows the schematic diagram of gram-positive and gram-negative bacteria.

A gram-positive cell wall mainly consists of peptidoglycan. It makes up as much as 90% of the gram-positive cell wall. However, gram-positive cell walls lack the outer membrane as in gram-negative cell walls. A gram-negative cell wall consists of an outer membrane that is composed of lipopolysaccharide, lipoprotein, and other macromolecules. Peptidoglycan only makes up of 10% of the gram-negative cell wall. Although gram-negative cell walls have a multi-layer structure, they are generally thinner than gram-positive cell walls [9].

1.5.2 Thermophilic Bacteria

Thermophilic bacteria typically grow in moderately high temperatures ranging from 50°C to 65°C. *B. methanolicus* is an example of a thermophile. Its optimal growth

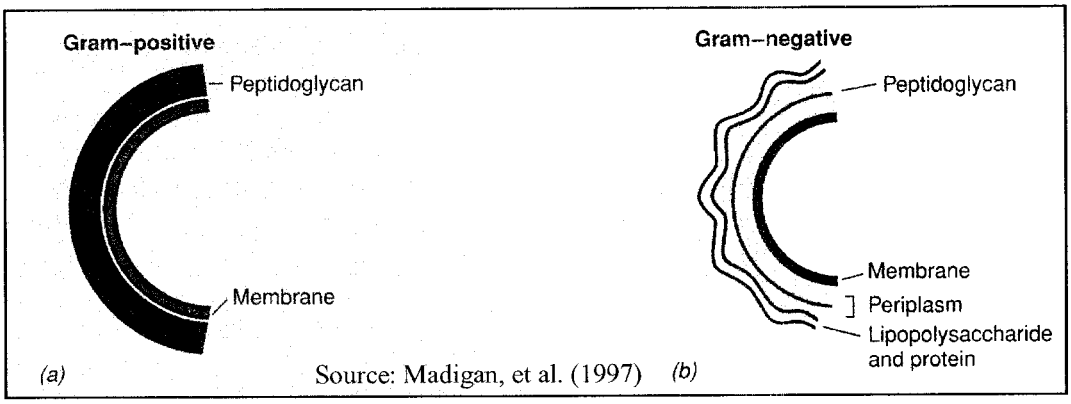

Figure 3. Basic structure of (a) gram-positive, (b) gram-negative cell wall [9].

temperature is 50 to 53°C. Besides thermophiles, microorganisms that grow at low, midrange, and very high temperature, are defined as psychrophiles, mesophiles, and hyperthermophiles, respectively. The relations between temperature and growth rate of a typical psychrophile, thermophile, mesophile, and hyperthermophile are shown in Figure 4.

Thermophilic microorganisms are of interest for many industrial and biotechnological applications. In terms of the advantages of bio-product production from thermophilic bacteria, the cooling cost for the large-scale fermentor can be significantly reduce. During the growth of bacteria in large-scale fermentor (i.e. 450 m^3), the flow rate of cooling water required for maintaining the fermentation temperature at 50°C is much less than the 37°C fermentation temperature [10]. The overall operation cost of the fermentor can therefore be reduced because of the reduced cooling water cost. Furthermore, enzymes manufactured from thermophiles are generally more thermo-stable than enzymes produced from mesophiles. Enzymes from thermophiles are capable of catalyzing biochemical reactions at elevated temperature. Taq polymerase is an example of heat-stable enzyme used in the polymerase chain reaction that is one of the most common and powerful biotechnology techniques. Taq polymerase is isolated from the hyperthermophile *Thermus aquaticus* [9].

1.5.3 Methylotrophic Bacteria

Methylotrophic bacteria are capable of using methanol, which is a reduced one-carbon compound, as carbon and energy sources. *B. methanolicus* is an example of a

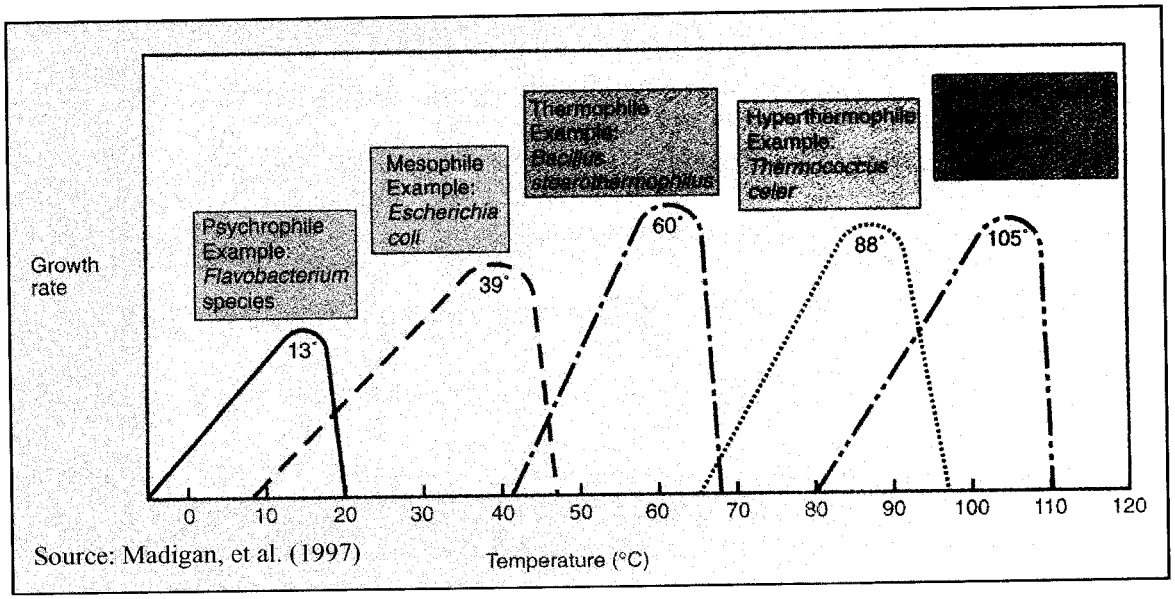

Figure 4. Relation of growth rates to different temperature classes of microorganisms. [9]

methylotrophic bacteria. It oxidizes methanol for energy by use of its NAD-dependent methanol dehydrogenase gene [11-12].

In addition to thermophilic microorganisms, methylotrophic bacteria are also of interest for many industrial and biotechnological applications. Methanol is a relatively cheap and readily available feedstock. Its price is relatively stable. Thus, the nutrient supplements cost for the methylotrophic bacterial fermentation can be significantly reduced as compared with organisms that grow on glucose. Furthermore, the residual methanol can be easily isolated from products after fermentation is completed. The downstream purification cost of the products can also be reduced [2]. The cross-contamination problems in the reactor may be reduced because methanol is toxic for most organisms.

CHAPTER 2.0 LITERATURE REVIEW

2.1 History of *Bacillus methanolicus*

In 1992, a research team at University of Groningen, Haren, The Netherlands, studied 14 strains of gram-positive bacteria that are able to use methanol as a carbon and energy source. All of them were aerobic and thermotolerant organisms. They have the capability to grow at a temperature between 35 and 60°C. The DNA-DNA hybridization studies, 5S and 16S rRNA sequence analysis, and physiological characteristics revealed that those 14 strains of bacteria gather as a well-defined group. Thus, a distinct new species was formed. The name *Bacillus methanolicus* (*B. methanolicus*) was proposed for this new species. The PB1 strain was deposited as type strain in the National Collection of Industrial and Marine Bacteria as NCIMB 13113 and American Type Culture Collection as ATCC 51375 [11].

2.2 Commercial Usefulness of *B. methanolicus*

B. methanolicus has a broad potential usefulness in the fermentation industry for the production of amino acids, single-cell proteins, polysaccharides, and vitamins from methanol [3,10-11]. L-lysine, for example, is an amino acid that is produced through the biosynthetic system of *B. methanolicus* [2-3]. L-lysine is a member of the aspartate family of amino acids. L-lysine is primarily used as a supplement for livestock feed. It is nutritionally important for livestock because their bodies are unable to synthesize L-lysine. They must have this amino acid supplied as part of their diet [2]. Although L-

lysine can also be produced through fermentation processes that use strains of *Corynebacterium glutamicum* or *Brevibacterium lactofermentum*, production of L-lysine from *B. methanolicus* has an advantage. Enzymes and proteins produced by thermophilic bacteria, *B. methanolicus*, for example, are generally stable at elevated temperatures. They are also stable against pH variation or organic solvents [3-4, 9]. Based on the review in the commercial usefulness of *B. methanolicus*, the further studying of these bacteria is warranted.

2.3 Characteristics of *B. methanolicus* Strain PB1

B. methanolicus strain PB1 is a gram-positive thermotolerant methylotroph that is capable of using methanol, which is a reduced one-carbon compound, as carbon and energy source. PB1 and MGA3 are the most common *B. methanolicus* strains studied by researchers. Studies have shown that the 16S prokaryotic ribosomal RNA (rRNA) sequences of PB1 and MGA3 have a 99% match [11]. The degree of similarity in rRNA sequences between the two organisms indicates their relative phylogenetic relationship [9]. Thus, the 99% match in 16S rRNA sequences in PB1 and MGA3 indicates that they belong to the same species. Therefore, the published information from these two *B. methnolicus* strains may be comparable and can be used to guide research on both strains.

2.3.1 Physical Properties of *B. methnanolicus*

B. methanolicus MGA3 was found to be a rod shaped bacteria that ranges from 0.8 to 1.0 by 2.5 to 4.5 μm in sizes. The colonies produced on agar plates are colorless, translucent, circular, and convex in shape. Endospores are also found within the cells

that are oval and 0.8 to 1.0 μm by 1.1 to 1.2 μm in size [2]. Endospores are differentiated forms that are very resistant to heat and cannot be destroyed easily, even by harsh chemicals [9]. Although the physical properties of the PB1 strain are not reported in the literature, PB1 may possess similar characteristics as MGA3. At least, endospores are also found within the *B. methanolicus* PB1 [3].

2.3.2 Optimal Growth Temperature of *B. methanolicus*

B. methanolicus is a thermophilic bacteria. Its optimal growth temperature was found to be 50 to 53°C, while the growth can occur from 37 to 60°C. According to Schendel's research group, the growth rate of *B. methanolicus* strain MGA3 at 37°C is equal to half of the μ_{max} at 50°C [2]. μ_{max} is defined as the maximum specific growth rate of the bacteria [13]. The PB1 strain is believed to share the same optimal growth temperature with MGA3 strain because of the similarity in their16S rRNA sequence homology

2.3.3 The Growth Media for *B. methanolicus*

2.3.3.1 Strain PB1

Shinichi and his research group in Japan reported the recipes of the growth media for *B. methanolicus* strain PB1. According to Shinichi et al., PB1 was able to grow in a medium composed of 1.5% Bacto tryptone, 0.5% Bacto soytone, and 0.5% NaCl [3]. On the other hand, Schendel et al. reported that strain MGA3 did not grow well in trytone/soytone-based nutrient broth or on tryptone/soytone-based nutrient agar [3].

2.3.3.2 Strain MGA3

Schendel and his research group also carried out growth studies of *B. methanolicus* strain MGA3 in different growth media [2]. The minimal salts (MS) medium was prepared according to Table 1. The minimal vitamin (MV) medium was the MS medium supplemented with thiamine hydrochloride, D-calcium pantothenate, riboflavin, and nicotinamide, each at 50 μg/L; biotin and folic acid, each at 20 μg/L; and vitamin B_{12} at 1 μg/L. The minimal yeast extract (MY) medium was the MV medium supplemented with yeast extract at 0.5 g/L. Both MV and MY medium contained 0.5% (vol/vol) methanol.

Studies showed that growth of MGA3 in MY medium in the 14-liter reactor at 50°C resulted in a maximum growth rate of 0.8 h^{-1}, while growth on MV medium resulted in a maximum growth rate of 0.48 h^{-1}. This showed that the MY medium may be a better growth media for MGA3. Results indicated that strain MGA3 grew poorly in nutrient broth or on nutrient agar. Furthermore, within the eight vitamin components in MV medium, biotin and vitamin B_{12} were required for growth. The growth was at optimal near pH 7.0; however, growth did not occur at pH 5.5 [2]. The strain MGA also possesses antibiotic resistance to neomycin, chloramphenicol, and erythromycin [10].

2.3.3.3 Suggestions of Further Studies of the Growth Media for Strain PB1

Extensive studies of the growth media for MGA3 have been done. However, the recipes of the growth media for strain PB1 still need to be clarified. The optimal pH and the methanol concentration of the growth media for strain PB1 were not reported in the literature. Since PB1 and MGA3 may grow similarly because of their 16S rRNA

Table 1. The Composition of Minimal Salts (MS) Medium, in 1L of Distilled Water.

Chemicals	**Amount Added**
K_2HPO_4	3.8 g
$NaH_2PO_4 \bullet H_2O$	2.8 g
$(NH_4)_2SO_4$	3.6 g
$MgSO_4 \bullet 7H2O$	0.5 g
$FeSO_4 \bullet 7H_2O$	2 mg
$CuSO_4 \bullet 5H_20$	40 μg
H_3BO_3	30 μg
$MnSO_4 \bullet H_2O$	200 μg
$ZnSO_4 \bullet 7H_2O$	200 μg
$Na_2MoO_4 \bullet 2H_2O$	47 μg
$CaCl_2 \bullet 2H_2O$	5.3 mg
$CoCl_2 \bullet 6H_2O$	40 μg

sequence homology, the PB1 strain is believed to share the same growth conditions and media as described for strain MGA.

2.4 Plasmid Studies of *B. methanolicus*

2.4.1 Current Information on *B. methanolicus* pBM1 plasmid

In 2000, a pBM1 plasmid isolated from *B. methanolicus* strain PB1 was patented by Shinichi and his research group, a Japanese research group of Ajinomoto Co., Inc., a global food and amino acid products company. The pBM1 plasmid has the size of about 20.5 kilo base pairs and undergoes an autonomic replication in *B. methanolicus*. The research group also generated a restriction enzyme cutting map, as shown in Figure 5, of the pBM1 plasmid [14]. The plasmid map has given useful information on the possible gene splicing locations at the pPB1 plasmid. Table 2 summarizes the size of the resulting DNA fragments when pPB1 plasmid is cut by varies restriction enzymes.

2.4.2 Suggestions of Further Studies on *B. methanolicus* pBM1 plasmid

Plasmid studies with *B. methanolicus* have so far been very limited. Although the restriction enzyme cutting map of pPB1 plasmid was found in the literature, very little information is reported about the mode of replication, stability and the functions of the pPB1 plasmid. Increasing our knowledge of the pPB1 plasmid will provide useful tools for the genetic studies of *B. methanolicus*. The genetic manipulation of *B. methanolicus* can therefore be facilitated if the complete gene library of the pPB1 plasmid were characterized.

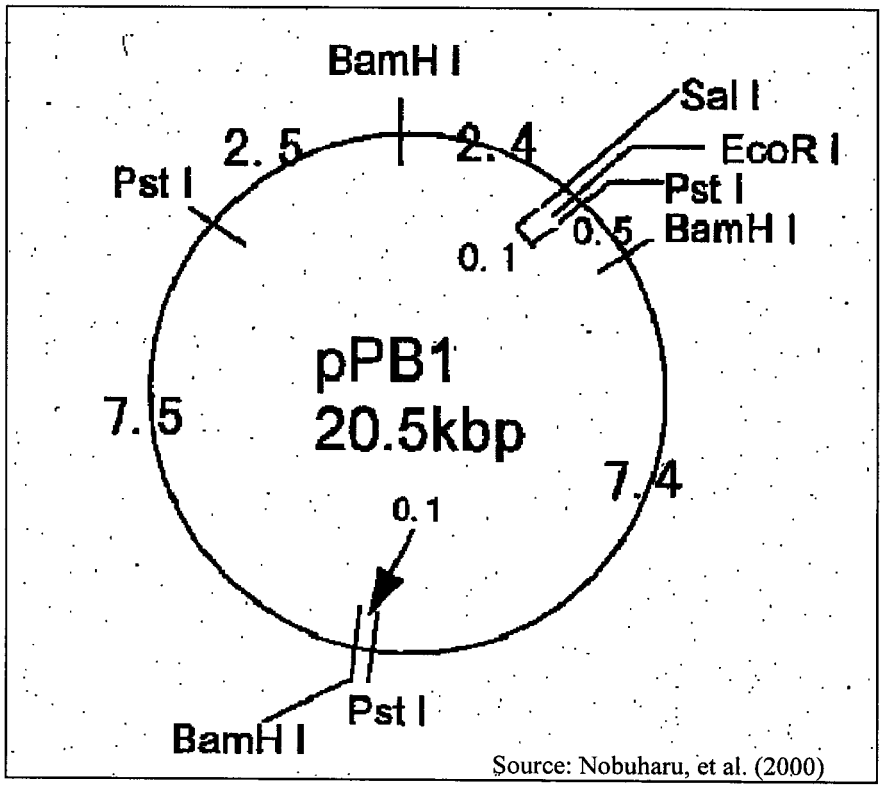

Figure 5. The pBM1 Plasmid from *B. methanolicus* Strain PB1 [14].

Table 2. The Size of the Digested DNA Fragments of pBM1 Plasmid [14].

Restriction Enzyme	**Size of DNA fragments (kbp)**
BamHI	3.0, 7.5, 10.0
SalI	20.5
EcoRI	20.5
PstI	5.0, 7.9, 7.6
BamHI, PstI	0.1, 0.5, 2.5, 2.5, 7.4, 7.5
BamHI, SalI	0.6, 2.4, 7.5, 10.0
BamHI, EcoRI	0.6, 2.4, 7.5, 10.0
SalI, EcoRI	20.5
SalI, PstI	0.1, 4.9, 7.5, 8.0
EcoRI, PstI	20.5

2.5 Plasmid DNA extraction from *B. methanolicus* strain PB1

The pPB1 plasmid extracted from *B. methanolicus* strain PB1 has been shown to possess the size of about 20.5 kilo base pairs [14]. Plasmids of more than 50 kilo base pairs are considered as large plasmids whereas plasmids of less than 10 kilo base pairs are characterized as small plasmids [15]. Therefore, the pPB1 plasmid is considered a mid-size plasmid. DNA, especially from large size plasmids, is very sensitive to mechanical disruption such as vigorous pipetting and harsh lysis procedures. Therefore, gentle plasmid DNA extraction should be followed [15-16].

Plasmid extraction with the addition of lysozyme has been suggested as a gentle cell lysis procedure for gram-positive bacteria [15-16]. The addition of isosmotic solution of sucrose and lysozyme aid in breaking down the gram-positive cell wall. The resulting spheroplasts are then lysed by the addition of SDS that is a detergent. This method minimizes the mechanical stresses that are necessary to extract the plasmid from the interior of the bacteria [16].

2.6 Transformation of *B. methanolicus* strain PB1

The procedure for transformation of *B. methanolicus* strain PB1 was not reported. Cue and his research group, on the other hand, reported a standard transformation protocol for *B. methanolicus*. They reported that typical transformation techniques, such as heat shock and electroporation, could not be used on *B. methanolicus* because of its gram-positive cell wall properties. While the efficiency of transformation for *B. methanolicus* was low when comparing to other gram-negative bacteria, the

transformation procedures reported by Cue and his research group is still comparable to those reported for gram-positive bacteria, such as *Bacillus subtilius* [10].

2.7 Summary of Literature Review

Although *B. methanolicus* has been studied by several groups, the information on genetic and biochemical characterization of *B. methanolicus* are still very limited. The literature reviews have provided information about the optimal growth temperature and media for the bacteria [2, 10]. Information on plasmid DNA extraction has also been suggested [15-16].

The establishment of an expression system in *B. methanolicus* is expected to possess a great advantage in the fermentation industry for large–scale production of recombinant products [10, 12, 17-19]. It also has a broad potential usefulness in the fermentation industry for the production of amino acids, single-cell proteins, polysaccharides, and vitamins from methanol [10-11]. As a result, a better understanding of the plasmid harbored by this economically important microorganism, *B. methanolicus*, will hopefully lead to the development of the more reliable bacterial strains for the production of biological products in large scale fermentation. Therefore, the isolation and characterization of the pPB1 plasmid of *B. methanolicus* is significant.

CHAPTER 3.0 RESEARCH HYPOTHESES AND OBJECTIVES

3.1 Research Hypotheses

- *B. methanolicus* strain PB1 possesses a 20.5 kilo base pairs plasmid that is called pPB1. The plasmid can be purified from the bacteria and characterized.
- Natural seawater may be used to prepare culture media for *B. methanolicus* strain PB1 as this bacteria has been shown to be salt-tolerant.

3.2 Research Objectives

The first objective of this thesis was to study the feasibility of using natural seawater as culturing medium for *B. methanolicus* strain PB1. The effect of sodium chloride concentration on the growth of *B. methanolicus* strain PB1 was first observed in trypticase soy broth and minimal media. Since the inhibitory effect from sodium chloride concentration on the order of that found in natural seawater was not strong, the growth of *B. methanolicus* strain PB1 in seawater with methanol as carbon source was also further studied.

The second objective of this thesis was to develop a standard protocol for extracting the pPB1 plasmid from *B. methanolicus* strain PB1. *B. methanolicus* strain PB1 is a gram-positive bacteria. A vigorous bacterial lysis method was developed in order to isolate the plasmid out from the gram-positive cell. The plasmid was characterized by the restriction enzyme digestion method.

CHAPTER 4.0 MATERIALS AND METHODS

4.1 Bacterial Strain

B. methanolicus strain PB1 was purchased from American Type Culture Collection (ATCC). The PB1 strain was originally deposited in the National Collection of Industrial and Marine Bacteria and American Type Culture Collection as NCIMB 133113 and ATCC 51375, respectively. The bacteria were shipped freeze-dried and were reconstituted according to the instructions provided from ATCC [20].

Trypticase soy broth was used to re-suspend the freeze-dried culture. Initially, 0.3 mL of the liquid medium was aseptically added to the freeze-dried material. The mixture was mixed gently and transferred to 5 mL of trypticase soy broth in a 10 mL culture tube (Falcon, Cat# 352032) and incubated at 50°C with shaking at 200 rpm in an incubator shaker (New Brunswick, Model G25). Most freeze-dried cultures normally can be grown in a few days; however, a prolonged lag period may sometimes be observed [21]. The bacterial growth was indicated by the turbidity of the liquid medium. Single colonies were further isolated from trypticase soy agar plates and expanded into liquid trypticase soy broth. Frozen culture was also prepared by adding 30% of glycerol into the bacteria culture. The mixture was further transferred into 1.7 mL cryo-tubes for long-term storage at –80°C freezer.

4.2 Preparation of Culture Media

4.2.1 Trypticase Soy Broth

Trypticase soy broth (BBL^{TM} Brand, Cat# 211768), also known as soybean-casein digest broth, was purchased in powder form. The recommended concentration of trypticase soy broth is 30 grams per liter of de-ionized water. The approximate formula of trypticase soy broth is stated in Table 3. Sodium hydroxide was used to adjust the pH to 7.5. The medium was sterilized by autoclaving (Hirayama, Model HA-MII) at 121°C for 20 minutes. Trypticase soy agar plates were prepared by solidifying the trypticase soy broth with the addition of 1.5% (wt/vol) agar powder (Difco Laboratories, Cat# 0140-01). The autoclaved mixture was cooled down to approximately 50°C after autoclaving and was poured into petri dishes.

4.2.2 Minimal Yeast Extract (MY) Medium

The minimal yeast extract (MY) medium for *B. methanolicus* was prepared with methanol as the carbon source. Minimal salts (MS) solution was prepared according to Table 4. Yeast extract, trace metals, magnesium sulfate, and vitamins were added to the MS solution as growth supplements according to the amounts listed in Table 5 and 6. The yeast extract and magnesium sulfate were prepared in stock solutions as 50 g/L and 1 M, respectively. The preparation of minimal medium is summarized in Table 7 [21].

All solutions, except the biotin and vitamin B_{12} solution, were sterilized by autoclaving at 121°C for 20 minutes. Biotin and vitamin B_{12} can be denatured by high temperature during autoclaving; therefore, the vitamin solution was sterilized by filter-

Table 3. Approximate Formula of Trypticase Soy Broth.

Compound	Concentration (g/L)
Pancreatic Digest of Casein	17.0
Papaic Digest of Soybean Meal	3.0
Sodium Chloride	5.0
Dipotassium Phosphate	2.5
Dextrose	2.5

Table 4. Minimal Salt (MS) Solution Used in The Preparation of Minimal Yeast Extract Medium.

Compound	Concentration (g/L)
$K_2HPO_4 \bullet 3H_2O$ [MW=174.19]	6.47
$NaH_2PO_4 \bullet H_2O$ [MW=137.99]	1.89
$(NH_4)_2SO_4$ [MW=132.14]	2.0

Table 5. Trace Metal (TM) Solution Used in The Preparation of Minimal Yeast Extract Medium.

Compound	Concentration (g/L)
$FeCl_2 \bullet 4H_2O$ [MW=198.83]	3.977
$ZnCl_2$ [MW=136.29]	0.136
$MnCl_2 \bullet 4H_2O$ [MW=197.92]	9.896
$CaCl_2 \bullet 2H_2O$ [MW=147.02]	7.351
$CuCl_2 \bullet 2H_2O$ [MW=170.49]	0.027
$CoCl_2 \bullet 6H_2O$ [MW=237.95]	0.040
$Na_2MoO_4 \bullet 2H_2O$ [MW=241.96]	0.048
H_3BO_3 [MW=61.84]	0.030

Table 6. Vitamin Solution Used in The Preparation of Minimal Yeast Extract Medium.

Compound	Concentration (mg/L)
Biotin	20
Vitamin B_{12}	1.0

Table 7. Preparation of Minimal Yeast Extract Medium for the Growth of *B. methanolicus* Strain PB1.

Medium Component	Amount Added (mL)
Minimal Salt Solution	1000
Yeast Extract (50g/L)	10
Methanol (99.5%)	10
$MgSO_4 \bullet 7H_2O$ (1M)	1.0
Trace Metal Solution	1.0
Vitamin Solution	1.0

sterilization (Corning 0.22 μm Bottle Filter, Cat# 430769). The storage temperature of the vitamins solution is 4°C.

Methanol was added into the minimal yeast extract medium at 1% (vol/vol) of which the methanol concentration is equivalent to 7.94 g/L or 248 mmol.

4.2.3 Seawater-Based Medium

Pacific Ocean seawater was collected from the shoreline of Santa Cruz (California) during a high-tide condition. In order to separate the sediment from Pacific Ocean seawater, the seawater was initially filtered through filter paper (Whatman Brand, Cat# 1001917). The seawater was then filter-sterilized (Corning 0.22 μm Bottle Filter, Cat# 430769).

Methanol was added into the Pacific Ocean seawater at 1% (vol/vol) as the carbon source for *B. methanolicus*. Yeast extract, trace metals, and vitamins solution, that were prepared according to Chapter 4.2.2, were also added to the seawater as supplements. Magnesium sulfate was not added to the seawater. According to chemical compositions of the artificial seawater, seawater was known to have the magnesium sulfate concentration at 6.29 g/L. Therefore, further addition of magnesium sulfate was not required for bacteria growing in seawater. The preparation of seawater-based culturing medium is summarized in Table 8.

4.2.4 Artificial Seawater-Based Medium

Artificial seawater (ASW) was prepared according to Table 9 [22]. It was filter-sterilized before use. Procedures from Table 8 were followed for the preparation of artificial seawater-based culturing medium.

Table 8. Preparation of Pacific Ocean Seawater-Based Medium for the Growth of *B. methanolicus* Strain PB1.

Medium Component	Amount Added (mL)
Pacific Ocean Seawater	1000
Yeast Extract (50g/L)	10
Methanol (99.5%)	10
Trace Metal Solution	1.0
Vitamin Solution	1.0

Table 9. Approximate Formula of Artificial Seawater.

Compound	Concentration (g/L)
NaCl	24.6
KCl	0.67
$CaCl_2 \bullet 2H_2O$	1.36
$MgSO_4 \bullet 7H_2O$	6.29
$MgCl_2 \bullet 6H_2O$	4.66
$NaHCO_3$	0.18

4.2.5 Tap Water-Based Medium

Tap water was collected from a water tap in the bio-processing lab at San Jose State University. It was filter-sterilized before use. Tap water from the bio-processing lab at SJSU is known is be hard water that contains a variety of mineral salts. Therefore, the tap water may compose of the necessary minerals to support the growth of *B. methanolicus* strain PB1. Procedures from Table 8 were followed for the preparation of tap water-based medium as culturing medium.

4.3 Bacterial Growth in Batch Condition

The bacterial growth curves were determined for *B. methanolicus* strain PB1 growing in trypticase soy broth without methanol added, and in minimal yeast extract medium, seawater, artificial seawater, and tap water with methanol added. The growth curves were determined with optical density measurements coupled to information from the dry biomass measurement.

4.3.1 Acclimation of the Bacteria in Different Growth Medium

In order to assure the bacterial cells were acclimated with the growth media and their corresponding carbon sources, the bacteria was transferred 2 successive times in each medium before actual inoculum was used for experiments. The small-scale acclimation steps were done in the 250 mL Erlenmeyer flask (Pyrex Brand, Cat# 4980) with 20% liquid volume of growth media. The growth media were inoculated with 2% (vol/vol) of inoculum.

4.3.2 Batch Growth Condition

The 1 liter baffled shaker flasks (Kimax Brand, Cat# 25630-1000) with metal vent caps were used to perform all the batch growth experiments. The liquid volume was set to be at 200 mL in the flask. In order to increase the reproducibility of the experiments, the culture medium was always inoculated with 2% (vol/vol) of 10 hours inoculum. The culture medium was always pre-warmed to 50°C before the addition of inoculum. The culturing condition was set to be at 50°C with shaking at 200 rpm in the incubator shaker (New Brunswick, Model G25). Extra care to maintain sterility was followed since the culturing media for *B. methanolicus* strain PB1 did not contain antibiotics for selection.

4.3.3 Cell Sampling and Optical Density Measurements

A 1 mL sample was taken every 60 to 120 minutes from each individual flask. The optical densities of the samples were measured with the UV/Vis Spectrophotometer (Hewlett Packard, Model 8452A) at 500 nm wavelength. The linear range of the UV/Vis Spectrophotometer was previously found to be around 0.2 to 0.9 at 500 nm. Therefore, de-ionized water was used to dilute samples having optical densities that were larger than 0.9. De-ionized water served as the blank for all experiments.

4.4 Determination of the Bacterial Dry Cell Weight With Respect to Optical Density

Eight 250 mL Erlenmeyer flasks with 50 mL of trypticase soy broth were inoculated with 2% (vol/vol) overnight culture. All of them were incubated at 50°C with shaking at 200 rpm. The flasks were taken out from incubator at different time intervals ranging from 0 to 600 minutes. In other words, bacteria samples from different growth

phases were obtained in different flasks. Optical densities of the samples were measured according to Section 4.3.3. Afterwards, the samples from each flask were transferred into individual 50 mL centrifuge tubes (Falcon, Cat# 352070) and centrifuged down for 15 minutes at 6000 rpm in a centrifuge (Forma Scientific Inc., Rotor Model 86-11). The supernatants were discarded and the bacterial pellets were re-suspended in 10 mL of de-ionized water. The samples were centrifuged and the supernatants were discarded. The bacterial pellets were again re-suspended in 3 mL of de-ionized water and transferred onto aluminum weight boats (Fisherbrand, Cat# 08-732). Additional 2 mL of de-ionized water was added into each individual tubes to collect the remaining bacteria. They were dried in the 50°C incubator overnight. The net bacterial dry cell weight was measured by an electronic weight balance (Mettler AE 200) after the pellet was completely dried.

4.5 Determination of the Bacterial Growth in Media with Different Concentrations of Sodium Chloride

Preliminary experiments were carried out to test the ability of *B. methanolicus* strain PB1 to grow in a salt concentration higher than normal saline up to the concentration of artificial seawater. The tests were carried out by culturing *B. methanolicus* in media with the addition of different amounts of sodium chloride.

4.5.1 Trypticase Soy Broth

Trypticase soy broth was prepared according to Chapter 4.2.1. Trypticase soy broths with the addition of varying amounts of sodium chloride were prepared according to Table 10. The inoculation procedures for batch growth experiments as described in

Table 10. Determination of the Growth of *B. methanolicus* Strain PB1 in Trypticase Soy Broth/Minimal Yeast Extract Medium with Different Amount of Sodium Chloride.

	Concentration of Sodium Chloride in Trypticase Soy Broth/ Minimal Medium (g/L)	Volume (mL)	Sodium Chloride Added (g)
#1	0	200	0
#2	5	200	1
#3	10	200	2
#4	15	200	3
#5	20	200	4
#6	25	200	5

Chapter 4.3.2 were followed. The cells were sampled and their optical densities were measured according to Chapter 4.3.3.

4.5.2 Minimal Yeast Extract Medium

Minimal yeast extract medium was prepared according to Chapter 4.2.2. MY media with the addition of different amounts of sodium chloride were prepared according to Table 10. The inoculation procedures for batch growth experiments as described in Chapter 4.3.2 were followed. Samples were taken and their optical densities were measured according to Chapter 4.3.3.

4.6 Determination of the Bacterial Growth in Pacific Ocean Seawater, Artificial Seawater, and Tap Water Based Medium

Pacific Ocean seawater, artificial seawater, and tap water based medium were prepared according to Chapter 4.2.3, 4.2.4, 4.2.5, respectively. Methanol was used as the carbon source for culturing *B. methanolicus* in Pacific Ocean seawater, artificial seawater, and tap water based medium. The supplements as described in Chapter 4.2.3 were also used.

4.6.1 Pacific Ocean Seawater Diluted with Different Amounts of De-ionized Water

Pacific Ocean seawater was diluted into de-ionized water according to Table 11. The de-ionized water was sterilized by autoclaving at 121°C for 20 minutes before the dilution with seawater. The growth supplements were added according to Chapter 4.2.3. Methanol was added at 1% (vol/vol) concentration into Pacific Ocean seawater as carbon source. Bacteria previously grown in 100% Pacific Ocean seawater were used as

Table 11. Determination of the Growth of *B. methanolicus* Strain PB1 in Pacific Ocean Seawater Diluted with Different Ratio of De-ionized Water.

	Ratio of Pacific Ocean Seawater/De-ionized Water	Pacific Ocean Seawater Added (mL)	De-ionized Water Added (mL)
#1	100% / 0%	200	0
#2	80% / 20%	160	40
#3	60% / 40%	120	80
#4	40% / 60%	80	120
#5	20% / 80%	40	160
#6	0% / 100%	0	200

inoculum. Similar procedures as described for batch growth experiments in Chapter 4.3.2 and 4.3.3 were followed.

4.6.2 Pacific Ocean Seawater with Different Amount of Yeast Extract as Supplement

Yeast extract was added into Pacific Ocean seawater-based medium according to Table 12. The 100% Pacific Ocean seawater was used to prepare the culturing medium for this experiment. The growth supplements were added according to Chapter 4.2.3 except the amount of yeast extract added was varied from 2 mL to 12 mL, which represented a final yeast extract concentration of 0.5 g/L to 3 g/L in the culturing medium. Methanol was added at 1% (vol/vol) concentration into Pacific Ocean seawater as carbon source. Bacteria previously grown in 100% Pacific Ocean seawater-based medium were used as inoculum. Procedures as described for batch growth experiments in Chapter 4.3.2 and 4.3.3 were then followed.

4.7 Determination of Osmolality

The osmolality, which is defined as the number of osmoles of solute particles per kilogram of H_2O, was determined for all growth media using The Advanced™ Micro-Osmometer (Advanced Instruments, Model 3MO Plus). The Advanced™ Micro-Osmometer determines the osmotic concentration of solution by means of the freezing-point measurement. The osmometer was calibrated using the 50 mOsm/kg H_2O (Advanced Instruments, Cat# 3MA005) and 850 mOsm/kg H_2O (Advanced Instruments, Cat# 3MA085) standard solutions according to the operation manual from manufacturer.

Table 12. Determination of the Growth of *B. methanolicus* Strain PB1 in Pacific Ocean Seawater-Based Medium with Different Amount of Yeast Extract as Supplement.

	Concentration of Yeast Extract in Pacific Ocean Seawater (g/L)	Pacific Ocean Seawater Added (mL)	Yeast Extract Added (mL) *Stock Conc. is at 50g/L
#1	0.5	200	2
#2	1.0	200	4
#3	1.5	200	6
#4	2.0	200	8
#5	2.5	200	10
#6	3.0	200	12

The osmolality of samples were measured using the Advanced™ 20-Microliter Sampling System Kit (Advanced Instruments, Cat# 3MO825) in accord with the standard operating procedures [23].

4.8 Determination of Methanol Concentration

In order to monitor the substrate (methanol) concentration during the bacteria growth, the residual methanol concentration in the samples was determined by the Biochemistry Analyzer (YSI, Model 2700 Select™). The YSI Biochemistry Analyzer determines the methanol concentration based on its immobilized enzyme biosensor technology. Special enzyme membranes (YSI, Cat# 2725) and carbonate buffer (YSI, Cat# 1579) were installed to the Biochemistry Analyzer for this application. The standard operation procedures were followed for the determination of methanol concentration in the samples [24].

4.9 Plasmid pPB1 from *B. methanolicus* strain PB1

4.9.1 Extraction of Plasmid

The protocol from Qiagen QIAprep Spin Miniprep Kit (Qiagen, Cat# 27104) was used as the basis for the extraction of plasmid from *B. methanolicus*. The Qiagen QIAprep Spin Miniprep Kit is designed for the isolation of plasmid from gram-negative bacteria; therefore, modification of the standard protocol is required for the isolation of plasmid from *B. methanolicus* that is a gram-positive bacteria [25]. The lysis of *B.*

methanolicus strain PB1, a gram-positive bacteria, required the addition of lysozyme (ICN, Cat# 10083101) into the lysis buffer [26-29].

B. methanolicus strain PB1 was grown overnight for 10 hours in 50 mL trypticase soy broth. The standard inoculation procedures from Chapter 4.3.2 were followed. The cells were harvested by centrifuging in a 50 mL centrifuge tube (Falcon, Cat# 352070) at 5000 rpm and 4°C for 10 minutes in a centrifuge (Forma Scientific Inc., Rotor Model 86-11). The supernatant was discarded and the pellet was re-suspended in 4 mL of Qiagen Buffer P1 (50 mM Tris Cl, 10 mM EDTA, 100 μg/mL RNase A) with the addition of lysozyme. In order to determine the optimal working concentration of lysozyme, the lysozyme concentration in Buffer P1 was varied from 0 to 10 mg/mL. The mixture was incubated at 50°C for 20 minutes. Afterwards, 4 mL of Qiagen Buffer P2 (200mM NaOH, 1% SDS) was added into the mixture. The solution was mixed by inverting the tube for 4 – 6 times. The solution was mixed gently to prevent shearing of plasmid DNA. The mixture was then incubated at room temperature for 5 minutes. Afterwards, 5.6 mL of Qiagen Buffer N3 was added. Buffer N3 is a buffer that has a proprietary formula from Qiagen. This buffer aids in the binding of plasmid DNA onto the silica membrane of the Qiagen QIAprep Spin Column. The mixture was mixed gently by inverting the tube 4 – 6 times and incubated at room temperature for 2 minutes. After centrifugation at 5000 rpm and 4°C for 20 minutes, the supernatant was decanted to the QIAprep Spin Column with the 2 mL collection tube. The column was spun at 10000 rpm for 45 seconds in a micro-centrifuge (Thermo IEC, Micromax Centrifuge). Afterwards, the flow-through from the 2 mL collection tube was discarded. The steps mentioned

previously were repeated until all the supernatant was added into and centrifuged through the column. The column was then washed with the addition of 0.75 mL Buffer PB. The flow-through was discarded after centrifugation at 10000 rpm for 45 seconds. The washing step was repeated 4 times. The column was further washed 5 times by 0.8mL of Buffer PE. The flow-through was again discarded after centrifugation at 10000 rpm for 45 seconds. The column was centrifuged for an additional 1 minute to remove the residual elution buffer. The column was then transferred to a new micro-centrifuge tube. Finally, the DNA was eluted by the addition of 50 μL Buffer EB (10 mM Tris Cl, pH 8.5). The column with the addition of Buffer EB was incubated at 50°C for 10 minutes before eluting the DNA by centrifugation. The incubation time was designed to facilitate the solubility of the high molecular weight pPB1 plasmid in the elution buffer. Finally, the DNA was eluted through the column through centrifugation at 10000 rpm for 3 minutes. The plasmid DNA was stored in 4°C for further analysis as described in Chapter 4.9.2 [25].

4.9.2 Restriction Digestion of Plasmid

Enzymes and buffers used for the plasmid digestion were ordered from New England Biolabs (NEB). The reactions were set up according to a patent from a Japanese research group [14]. The experimental set-up is summarized in Table 13. The reaction volume for restriction digestion was set as 20 μL. According to the experimental protocols in the NEB catalog, the volume of restriction enzyme should not exceed 10% of the final reaction volume. The mixtures were incubated at 37°C overnight in order to allow for complete digestion [30].

Table 13. Restriction Digestion Analysis of pPB1 Plasmid.

		Single Restriction Enzyme Digestion			
	Component	#1	#2	#3	#4
	Plasmid DNA	5 μL	5 μL	5 μL	5 μL
Restriction Enzymes	BamH I	2 μL	---	---	---
	Sal I	---	2 μL	---	---
	EcoR I	---	---	2 μL	---
	Pst I	---	---	---	2 μL
Buffers	NEB Buffer 2 (10X)	2 μL	---	2 μL	---
	NEB Buffer 3 (10X)	---	2 μL	---	2 μL
	BSA (100X)	0.2 μL	0.2 μL	0.2 μL	0.2 μL
	DI H_2O	10.8 μL	10.8 μL	10.8 μL	10.8 μL
	TOTAL VOL.	20 μL	20 μL	20 μL	20 μL

		Double Restriction Enzyme Digestion					
	Component	#5	#6	#7	#8	#9	#10
	Plasmid DNA	5 μL	5 μL	5 μL	5 μL	5 μL	5 μL
Restriction Enzymes	BamH I	1.5 μL	1.5 μL	1.5 μL	---	---	---
	Sal I	---	1.5 μL	---	1.5 μL	1.5 μL	---
	EcoR I	---	---	1.5 μL	1.5 μL	---	1.5 μL
	Pst I	1.5 μL	---	---	---	1.5 μL	1.5 μL
Buffers	NEB Buffer 2 (10X)	2 μL	---	2 μL	---	---	---
	NEB Buffer 3 (10X)	---	2 μL	---	2 μL	2 μL	2 μL
	BSA (100X)	0.2 μL	0.2 μL	0.2 μL	0.2 μL	0.2 μL	0.2 μL
	DI H_2O	9.8 μL	10.8 μL	10.8 μL	10.8 μL	10.8 μL	10.8 μL
	TOTAL VOL.	20 μL	20 μL	20 μL	20 μL	20 μL	20 μL

4.9.3 Gel Electrophoresis Analysis

The DNA samples digested with restriction enzymes from Chapter 4.9.2 were analyzed by gel electrophoresis. The agarose gel (FisherBrand, Cat# BP 1356-100) was prepared at 0.8% (wt/vol) concentration with the addition of ethidium bromide (Fisherbrand, Cat# BP103-5). The λ DNA/Hind III Fragments DNA ladder (GibcoBRL, Cat# 15612-013) and the DNA Ladder I (GeneChoice, Cat# 62-6108-01) that served to determine the size of DNA fragments between 125 to 23,130 base pairs and 200 to 12,000 base pairs, respectively, were used. 5 μL of digested DNA mixed with 1 μL of 6X gel-loading buffer (Fisherbrand, Cat#BP633-5) was loaded into each lane of the agarose gel. In order to separate the high molecular weight DNA on the agarose gel, the gel was ran overnight (10 hours) at 40 volts in a gel apparatus with the power supply.

The digital image of the agarose gel was observed and taken by a digital gel documentation system (UVP Ultraviolet Transilluminators). Procedures for taking the digital image are contained in the user manual [31].

CHAPTER 5.0 DATA AND ERROR ANALYSIS

5.1 Optical Density Measurements

The optical densities for all the samples were measured using the UV/Vis Spectrophotometer as described in Chapter 4.3.3. The optical density measurement was taken twice for each sample and the average value was used for data analysis.

5.2 Conversion of Optical Density to Dry Biomass Concentration

In order to present the cell concentration in a more direct way, the optical density data were reported in terms of dry biomass (g/L), which is described in the Results and Discussion section. The optical density measurements were converted into dry biomass concentration based on the linear relationship found from the experiment that was done according to Chapter 4.4.

5.3 Linear Curve Fit Analysis

The linear curve fit was done by Kaleida Graph (Synergy Software, Version 3.0), a mathematical analysis computer software. Equation 1 was used as the basis for the linear curve fit.

$$y = mx + c \qquad \text{Equation 1}$$

The errors and R^2 values generated from Kaleida Graph were reported and used to determine the goodness of fit and error of the experiment.

5.4 Analysis for Specific Growth Rate

In order to find the specific growth rate (μ) of the bacteria, the optical density data collected during the early exponential phase of the bacteria growth were chosen. The specific growth rate in a batch reactor is defined as in Equation 2.

$$\mu = \frac{1}{X}\frac{dX}{dt} \qquad \text{Equation 2}$$

X and t are defined as cell mass concentration and time, respectively. Equation 3 is obtained by rearranging Equation 2.

$$\mu = \frac{d(\ln X)}{dt} \qquad \text{Equation 3}$$

Equation 3 was used as the basis for obtaining the specific growth rate (μ) during the exponential growth phase of the bacteria. By plotting ln(X) versus time, the specific growth rate (μ) was obtained from the slope of the linear fit line. The errors and relative variance (R^2) values generated from Kaleida Graph have been reported.

5.5 Analysis for Size of DNA Fragments on Agarose Gel

The advanced analysis function of the LabWorks Software from Ultra-Violet Product (UVP), Inc. was used to analyze the size of DNA fragments on agarose gel. The digital image of the gel was first taken. The software automatically detected the DNA bands and identified the size of the DNA using DNA ladder I (GeneChoice, Cat# 62-6108-01) as reference. Detailed procedures for image analysis were referred to the user manual [31].

CHAPTER 6.0 RESULTS AND DISCUSSIONS

6.1 Growth Behavior of *B. methanolicus* Strain PB1

6.1.1 Revivification of Freeze-Dried Culture

B. methanolicus strain PB1 freeze-dried culture was received from ATCC and was stored at 4°C for about a month before re-hydration. The freeze-dried culture was re-hydrated according to Chapter 4.1; however, the revivification was unsuccessful after several attempts. According to the technical support services from ATCC, the *B. methanolicus* strain PB1 freeze-dried culture has to be re-hydrated immediately upon receiving the shipment. Although ATCC suggests the freeze-dried culture that is not re-hydrated immediately can be stored at 4 °C or lower, the revivification of freeze-dried culture of *B. methanolicus* strain PB1 after prolong storage was impossible.

A second shipment of *B. methanolicus* strain PB1 (Lot#1376151) was received from ATCC and was immediately re-hydrated according to Chapter 4.1. Bacteria growth was observed after 44 hours of incubation in trypticase soy broth at 50^0C.

6.1.2 Preparation of Frozen Glycerol Stock

The frozen glycerol stocks were prepared according to Chapter 4.1. The 18-hour overnight culture was initially used to prepare the glycerol frozen stocks. One vial of frozen stock was thawed and inoculated into trypticase soy broth in 1% (v/v) for testing the viability of bacteria in frozen stock. However, no growth was found after 48 hours incubation. This indicates that the 18-hour culture may have been too old for the preparation of frozen stocks.

The second batch of bacteria frozen stock was prepared using a 6-hour culture with an optical density of approximately 2. The viability of the frozen stock was also tested through inoculation into trypticase soy broth in 1% (v/v). The bacteria were able to grow; however, it exhibited a 12-hour lag phase.

6.1.3 Preparation of the Inoculum

Problems of culturing *B. methanolicus* strain PB1 in batch mode were experienced during the initial stage of the experiment. Prolonged lag phase or the death of the bacteria was sometimes observed after transferring the bacteria into fresh medium. *B. methanolicus* strain PB1 growing in trypticase soy broth experienced a more stable growth behavior as trypticase soy broth is a nutrient rich medium. The bacteria were able to stay alive in trypticase soy broth for as long as 28 hours; however, bacteria of older than 28 hours could never be retrieved by re-seeding into fresh medium.

A more fragile growth behavior was observed for *B. methanolicus* strain PB1 growing in minimal medium. The bacteria grew poorly during the initial transfer from trypticase soy broth to minimal yeast extract media; the second generation of *B. methanolicus* strain PB1 in minimal yeast extract medium usually grew well. This suggests that time is needed for the bacteria to adapt to an alternate carbon source, i.e. methanol in minimal yeast extract medium. Bacteria older than 18 hours could never be retrieved by re-seeding into fresh minimal yeast extract medium. Studies have shown that the residual methanol concentration of the inoculum has a significant effect on the viability of the bacteria in fresh medium [21]. If the residual methanol concentration were low in the inoculum, the bacteria would be exposed to a sudden step change in

methanol concentration when it is seeded into fresh medium. Due to the potential toxicity of methanol, bacteria from an inoculum with a low residual methanol concentration may die during the sudden exposure to high methanol concentration in fresh medium [21].

Due to the instability of *B. methanolicus* strain PB1, a standard inoculation protocol as described in Chapter 4.3.2 was strictly followed in order to increase the repeatability of the experiments. In order to prevent the sudden step change in temperature, media were also pre-warmed before inoculation with the bacteria.

6.2 Correlation of Optical Density and Bacterial Dry Cell Weight

The growth of *B. methanolicus* strain PB1 in culture suspensions was quantitatively determined by the optical density measurement at 500 nm wavelength throughout the experiments carried out in this paper. In order to present the cell concentration in a more direct way, the optical density data is reported in terms of dry biomass (g/L). Therefore, an initial experiment was carried out to correlate the bacterial dry cell weight with respect to the optical density reading at 500 nm wavelength according to method described in Chapter 4.4.

A linear relationship was found between dry biomass and optical density based on the regression analysis as shown in Figure 6. The R^2 value of the linear fit curve was found to be 0.9099, which is an acceptable value for a typical regression line. The conversion factor used for correlating optical density at 500 nm and dry biomass was generated as shown in Equation 4.

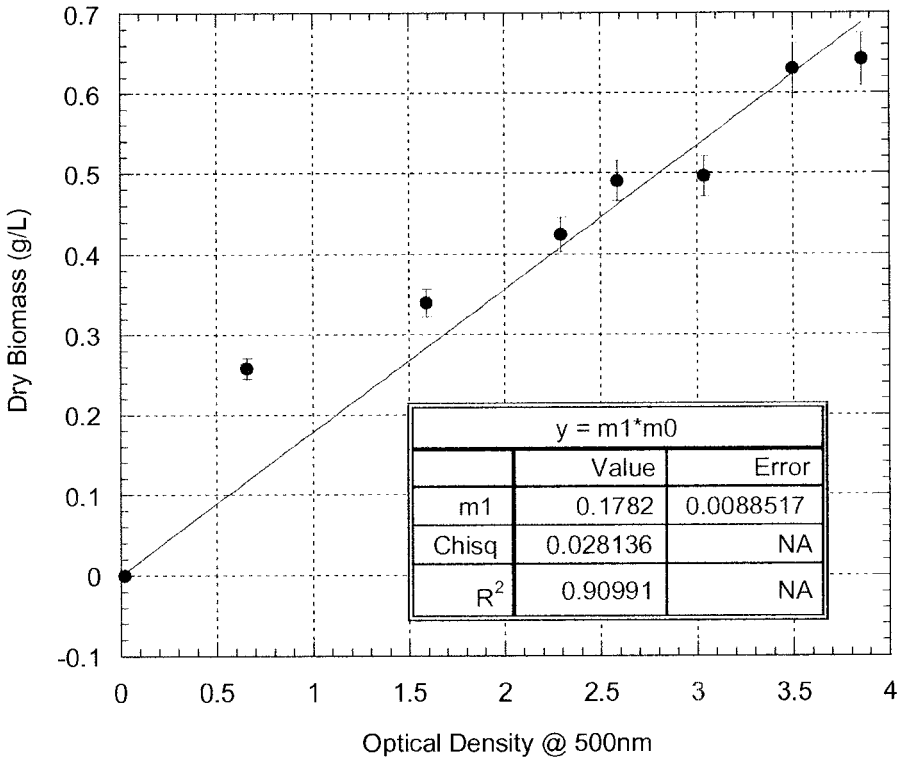

Figure 6. The Linear Correlation of Dry Biomass and Optical Density for *B. methanolicus* Strain PB1 growing in Trypticase Soy Broth.

$$\text{Dry Biomass}\,(g/L) = [0.1782 \times (\text{OD}\,@\,500nm)] \pm 0.008852 \qquad \text{Equation 4}$$

Although this conversion factor was determined from *B. methanolicus* growing in trypticase soy broth, similar conversion factor was found from *B. methanolicus* growing in minimal salt medium by both Pluschkell [21] and Wang [32]. Their conversion factors were reported as 0.176•OD500 ± 0.004 g/L and 0.1432•OD500 ± 0.0046 g/L, respectively. The optical density data obtained from all the experiments were converted to and reported as dry biomass (g/L) based on the relationship shown in Equation 4.

6.3 The Effect of Sodium Chloride on the Growth of *B. methanolicus* Strain PB1

Preliminary experiments were carried out to test the ability of *B. methanolicus* strain PB1 to grow in high salt medium. Trypticase soy broth and minimal medium with sodium chloride concentrations ranging from 0 to 25 g/L were prepared according to Chapter 4.5.1 and Chapter 4.5.2, respectively.

6.3.1 Osmolality of Trypticase Soy Broth and Minimal Yeast Extract Medium

The osmolalities of trypticase soy broth and minimal medium were determined by the Advanced™ Micro-Osmometer according to Chapter 4.7. The osmolality data are summarized in Figure 7 and Table 14.

In trypticase soy broth, the osmolality was found to be 279 mOsm/kg H_2O in the absence of sodium chloride. The osmolality increases to 1140 mOsm/kg H_2O when 25 g/L of sodium chloride is added to the media. A linear relationship is found between osmolality and sodium chloride concentration based on the linear regression analysis.

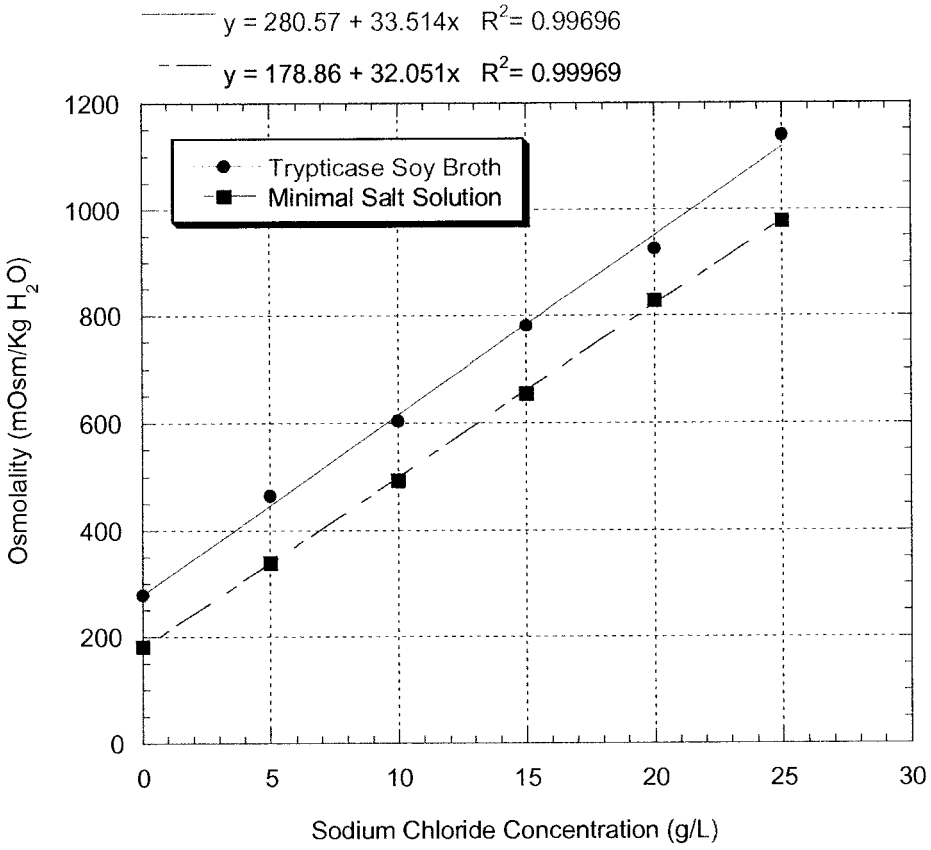

Figure 7. The Correlation of Osmolality and Sodium Chloride Concentration in Trypticase Soy Broth and Minimal Salt Solution.

Table 14. The Correlation of Osmolality and Sodium Chloride Concentration in Trypticase Soy Broth and Minimal Salt Solution.

	Osmolality (mOsm/kg H_2O)	
Salt Added (g/L)	Trypticase Soy Broth	Minimal Salt Solution
0	279	182
5	466	340
10	604	494
15	781	654
20	927	828
25	1140	979

The osmolality is increased by 33.5 mOsm/kg H_2O when 1 gram of sodium chloride is added into 1 liter of trypticase soy broth.

In minimal salt solution with sodium chloride concentration ranging from 0 to 25 g/L, the osmolality is found to range from 182 to 979 mOsm/kg H_2O. Based on the linear regression analysis, the osmolality of minimal salt solution increases by 32.1 mOsm/kg H_2O per 1 gram addition of sodium chloride into 1 liter of solution.

According to the ingredient information from BBL™, the manufacturer of trypticase soy broth powder, trypticase soy broth is known to have approximately 5 g/L sodium chloride initially. Therefore, the osmolality of trypticase soy broth is found to be relatively higher than minimal salt solution. In order to represent the salt concentration in a standardize way, the salt concentrations of trypticase soy broth and minimal media were all reported as osmolality during data analysis.

6.3.2 The Growth of *B. methanolicus* strain PB1 in High Salt Media

The effect of sodium chloride on the growth of *B. methanolicus* PB1 was observed under batch culture conditions as described in Chapter 4.3. Each individual shaker flask with 200 mL medium was inoculated with 4mL (2% v/v) of 10 hours overnight culture. The inoculum was initially grown in medium without the addition of sodium chloride.

The time course of the growth of *B. methanolicus* strain PB1 in trypticase soy broth and minimal yeast extract media with the addition of sodium chloride are shown in Figure 8 and Figure 9, respectively. The specific growth rates for the *B. methanolicus* are determined according to Chapter 5.4 and summarized in Table 15. The final dry biomass

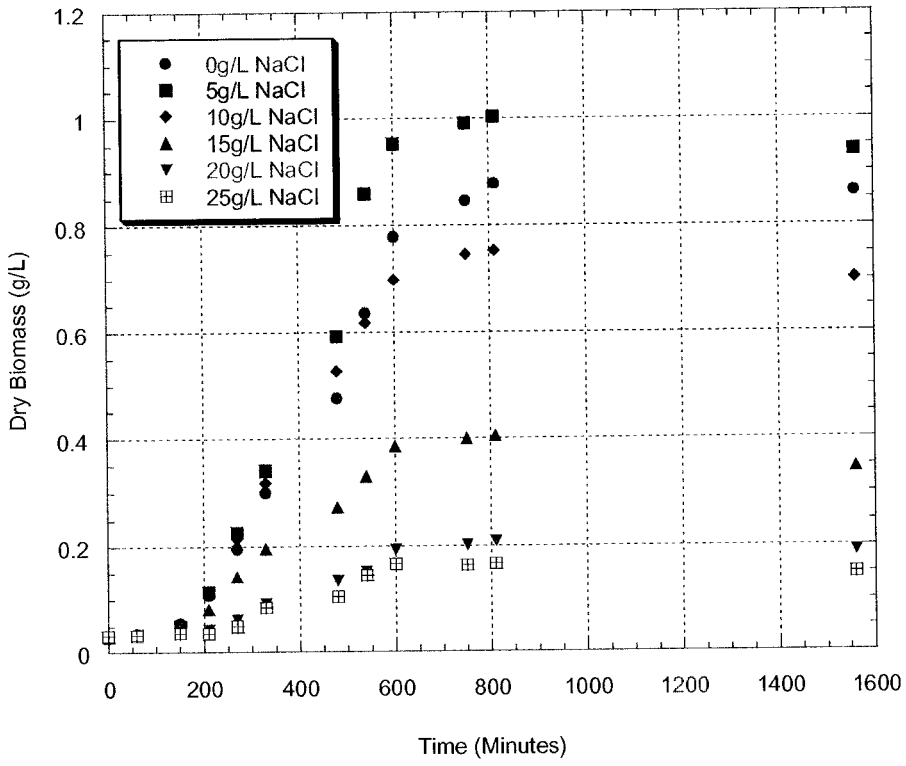

Figure 8. The Effect of Sodium Chloride on the Growth of *B. methanolicus* Strain PB1 in Trypticase Soy Broth (Without Using a Pre-Adaptation Method).

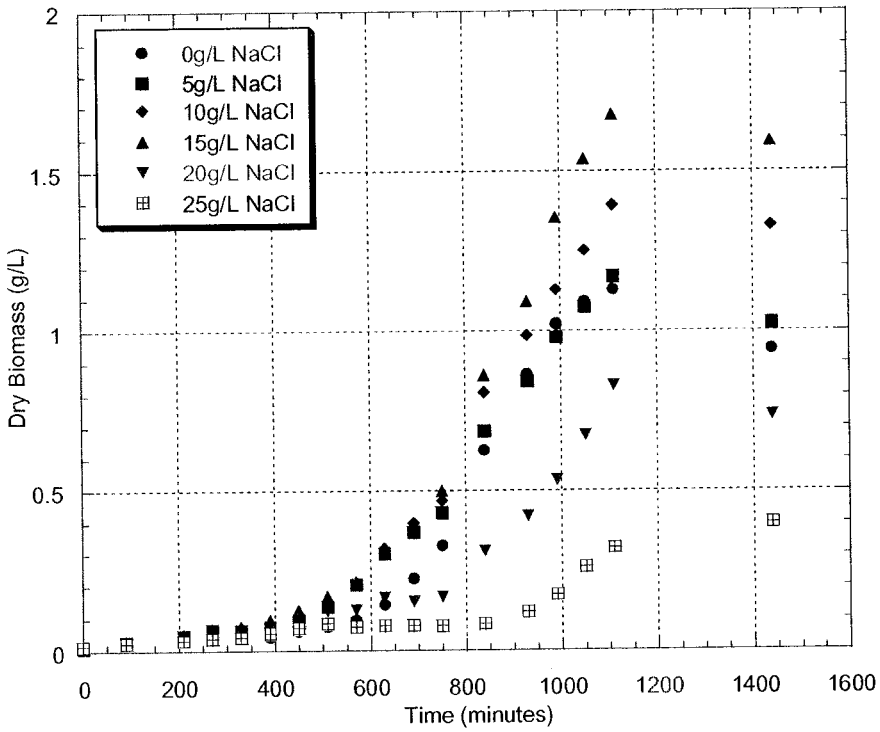

Figure 9. The Effect of Sodium Chloride on the Growth of *B. methanolicus* Strain PB1 in Minimal Yeast Extract Media

Table 15. The Effect of Sodium Chloride on the Specific Growth Rate of *B. methanolicus* Strain PB1 in Trypticase Soy Broth and Minimal Yeast Extract Medium.

	Specific Growth Rate (min^{-1})		Specific Growth Rate (hr^{-1})	
NaCl Concentration (g/L)	Trypticase Soy Broth	Minimal Yeast Extract Medium	Trypticase Soy Broth	Minimal Yeast Extract Medium
0	0.005413 ± 0.0006185	0.004950 ± 0.0002645	0.3248 ± 0.03711	0.2970 ± 0.01587
5	0.006076 ± 0.0007644	0.003917 ± 0.0002472	0.3646 ± 0.04586	0.2858 ± 0.01483
10	0.005410 ± 0.0008619	0.004081 ± 0.0002361	0.3246 ± 0.05171	0.3008 ± 0.01417
15	0.004197 ± 0.0005652	0.004170 ± 0.0001504	0.2518 ± 0.03391	0.3214 ± 0.009024
20	0.003551 ± 0.0002650	0.003207 ± 0.0001857	0.2131 ± 0.01590	0.2418 ± 0.01114
25	0.003546 ± 0.0003544	0.001991 ± 0.0003378	0.2128 ± 0.02126	0.1787 ± 0.02027

concentrations are also summarized in Table 16. Results show that the inhibitory effect of sodium chloride on the growth of *B. methanolicus* strain PB1 is not strong. The complete inhibition of growth is not found. The specific growth rate and final dry biomass of *B. methanolicus* strain PB1 growing in trypticase soy broth and minimal yeast extract medium are compared and shown in Figures 10 and 11, respectively.

In trypticase soy broth, the specific growth rate and final dry biomass of *B. methanolicus* PB1 are found to be approximately 0.32 ± 0.037 h^{-1} and 0.88 g/L, respectively, in the absence of sodium chloride. The specific growth rate and final dry biomass increase to 0.36 ± 0.046 h^{-1} and 1.00 g/L, respectively, at 5 g/L sodium chloride concentration. The addition of 5 g/L sodium chloride in trypticase soy broth (466 mOsm/kg H_2O) seems to have provided an optimal growth condition for *B. methanolicus* PB1. The specific growth rate then gradually reduced from 0.32 ± 0.052 h^{-1} to 0.21 ± 0.021 h^{-1} as the sodium chloride concentration is increased from 10 g/L to 25 g/L. On the other hand, the final dry biomass is reduced significantly from 1.00 g/L to 0.16 g/L as the sodium chloride concentration increases. Under the high salt concentration (25g/L NaCl), the specific growth rate is only reduced by 42% to 0.21 ± 0.021 h^{-1}; the final dry biomass is significantly reduced by 84% from the optimal to 0.16 g/L. Therefore, the results show that the high salt concentration (25 g/L NaCl) has given a larger effect on final dry biomass as compared with the specific growth rate.

In minimal yeast extract medium, the specific growth rate of *B. methanolicus* PB1 is found to be approximately 0.29 ± 0.016 h^{-1} in the absence of sodium chloride and at 248 mM methanol. It gradually increases to 0.32 ± 0.009 h^{-1} as the sodium chloride

Table 16. The Effect of Sodium Chloride on the Final Dry Biomass of *B. methanolicus* Strain PB1 in Trypticase Soy Broth and Minimal Yeast Extract Medium.

	Final Dry Biomass (g/L)	
NaCl Concentration (g/L)	Trypticase Soy Broth	Minimal Yeast Extract Medium
0	0.8780	1.1275
5	1.0031	1.1697
10	0.7532	1.3904
15	0.4051	1.6744
20	0.2096	0.8298
25	0.1675	0.3208

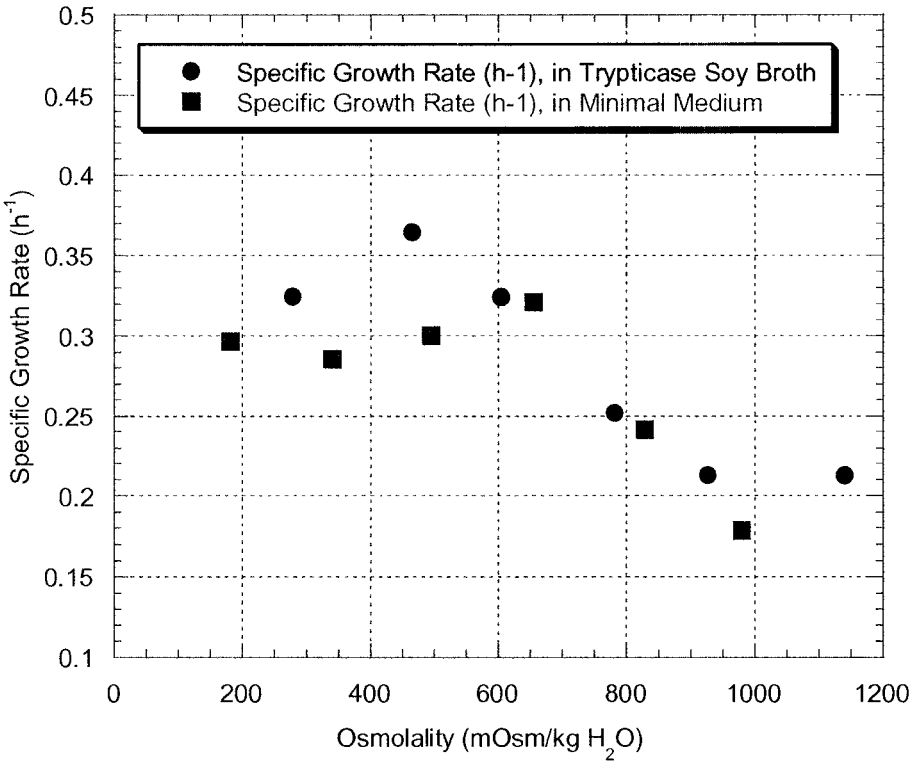

Figure 10. The Effect of Sodium Chloride on the Specific Growth Rate of *B. methanolicus* Strain PB1.

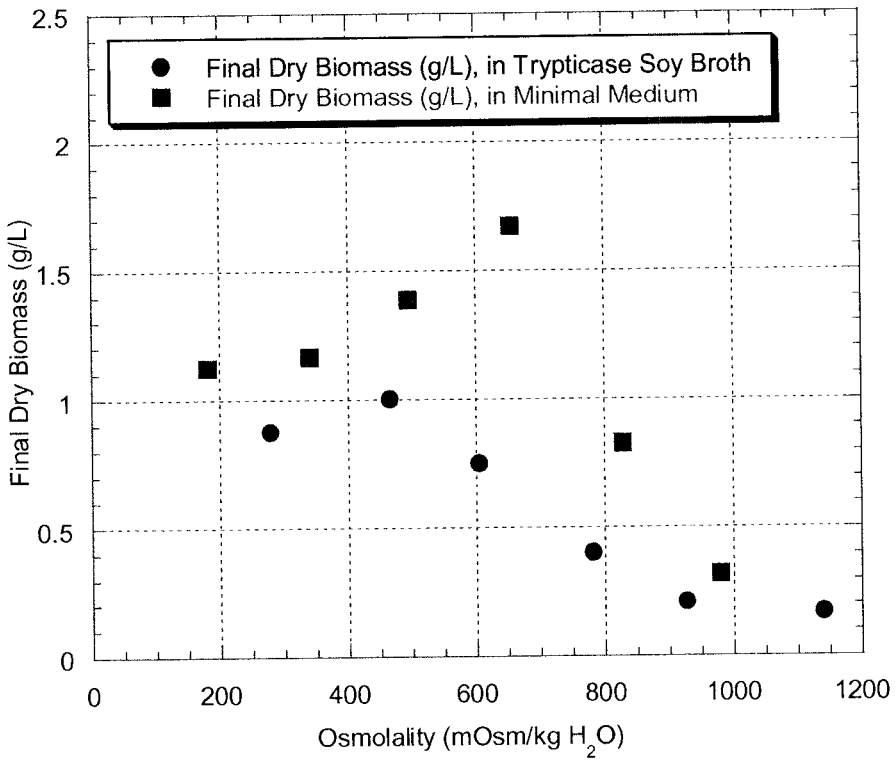

Figure 11. The Effect of Sodium Chloride on the Final Dry Biomass of *B. methanolicus* Strain PB1.

concentration increases to 15 g/L. As the sodium chloride concentration increases from 15 g/L to 25 g/L, the specific growth rate is decreased by 44% from the optimal to 0.18 ± 0.020 h^{-1}. Minimal yeast extract medium with the addition of 15 g/L sodium chloride (654 mOsm/kg H_2O) seems to provide the optimal condition for the growth of *B. methanolicus* PB1. As expected, the final dry biomass has the same trend as the specific growth rate. The final dry biomass gradually increases from 1.1 g/L to 1.67 g/L as the sodium chloride concentration increases from 0 g/L to 15 g/L. The final dry biomass then drops 81% from the optimal to 0.32 g/L as the sodium chloride concentration increases from 15 g/L to 25 g/L.

The results show that *B. methanolicus* PB1 has a similar specific growth rate in trypticase soy broth and minimal yeast extract medium. In terms of final dry biomass, the minimal yeast extract medium provides a more optimal growth condition for *B. methanolicus* PB1. The bacteria achieved a higher cell density in minimal yeast extract media with 248 mM methanol as compared with trypticase soy broth. As Schendel et al. reported that *B. methanolicus* strain MGA3 grew poorly in tryptone/soytone-based nutrient broth, *B. methanolicus* strain PB1 also seems to exhibit the same growth behavior as strain MGA3 [2].

Based on the specific growth rate data, *B. methanolicus* PB1 can initially grow slowly under high salt concentration (20 to 25 g/L NaCl); however, it cannot reach a higher final dry biomass. Since the inoculum was initially grown in medium without the addition of sodium chloride, lysing of the bacteria may occur due to sudden exposure to the high osmotic stress [33-39]. On the other hand, the growth of *B. methanolicus* PB1

seems to improve by a medium osmotic stress. The addition of up to 5 g/L and 15 g/L of sodium chloride into trypticase soy broth and minimal yeast extract medium, respectively, facilitate the growth of *B. methanolicus* PB1. In terms of osmolality, *B. methanolicus* PB1 grows best in trypticase soy broth at approximately 466 mOsm/kg H_2O and minimal medium at approximately 654 mOsm/kg H_2O. This growth improvement may be explained by the accumulation of the intracellular organic components in the original inoculum. The bacteria that were initially grown in medium without the addition of sodium chloride (279 mOsm/kg H_2O in trypticase soy broth and 182 mOsm/kg H_2O in minimal medium) might have developed certain intracellular osmotic pressure due to the secretion and accumulation of amino acid inside the cells. Therefore, the slight increase in osmolality in the fresh medium may provide them a better condition for growth. The prevention of the sudden osmotic shock may help the bacteria to grow in medium with high salt concentration [33-39].

6.3.3 The Growth of *B. methanolicus* strain PB1 in High Salt Media Using a Pre-adaptation Method

Based on the conclusion drawn from Chapter 6.3.2, a pre-adaptation method was used to facilitate the growth of *B. methanolicus* PB1 in high salt concentration. The pre-adaptation method avoids the sudden osmotic shock to the bacteria [33-39]. The procedures for carrying out the pre-adaptation of the inoculum are summarized in Figure 12. The bacteria were initially grown in trypticase soy broth with no sodium chloride. It was then transferred to medium with 5 g/L sodium chloride after the 12-hour incubation. The bacteria were subsequently transferred into fresh medium with a higher level of sodium chloride after each 12-hour incubation period.

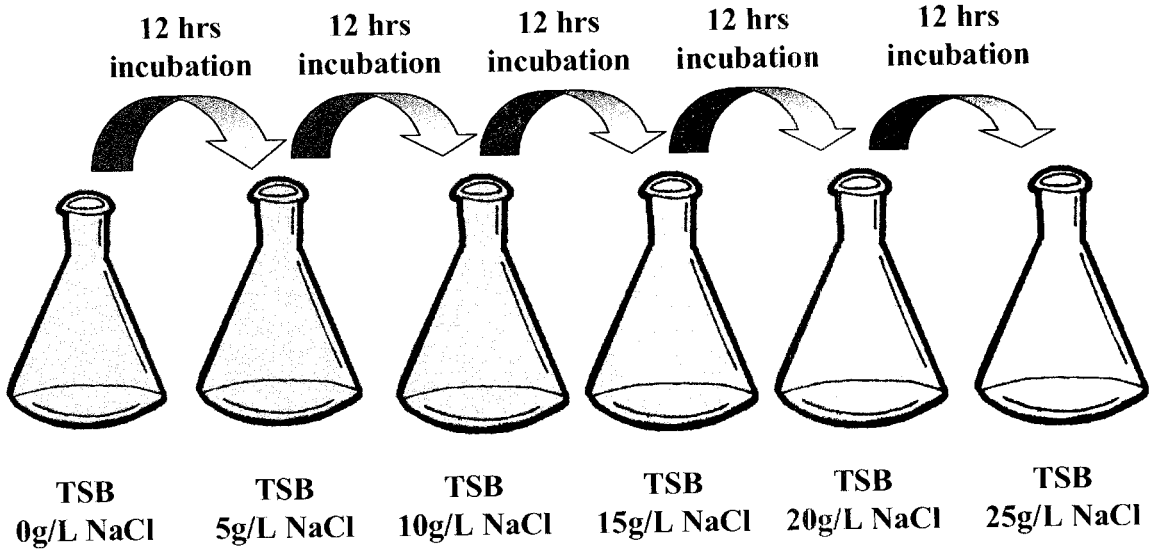

Figure 12. Preparation of Inoculum Using a Pre-adaptation Method.

This step-by-step pre-adaptation method provides a gradual way for the bacteria to adapt to high salt concentration. The sudden and steep step change in osmotic pressure for the bacteria is prevented. It has been suggested by Munro et al. that the pre-adaptation method allows the bacteria to develop an osmo-regulation mechanism and modify the structure of their outer membrane, although we have not confirmed this with *B. methanolicus* [33-36]. Therefore, the bacteria may adapt to high salt environment slowly through the gentle pre-adaptation method.

Trypticase soy broth with sodium chloride concentrations ranging from 0 to 25 g/L prepared according to Chapter 4.5.1 were used. The bacteria prepared according to Figure 13 were inoculated into their corresponding fresh medium. The time course of the growth of *B. methanolicus* strain PB1 in trypticase soy broth using the pre-adaptation method is shown in Figure 13. The specific growth rates for the *B. methanolicus* are determined according to Chapter 5.4 and summarized in Table 17. The final dry biomass concentrations are also summarized in Table 18.

Results show that *B. methanolicus* strain PB1 is able to grow well in medium with high salt concentration by applying the pre-adaptation method for the preparation of inoculum. The specific growth rate and final dry biomass of *B. methanolicus* PB1 growing in trypticase soy broth with 25 g/L NaCl are improved from 0.21 ± 0.021 h^{-1} to 0.40 ± 0.045 h^{-1} and 0.17 g/L to 0.73 g/L, respectively. In Figure 14, the specific growth rates of *B. methanolicus* strain PB1 growing in trypticase soy broth with and without the use of the pre-adaptation method are compared. When the inoculum was prepared without the use of the pre-adaptation method, the specific growth rates gradually reduced

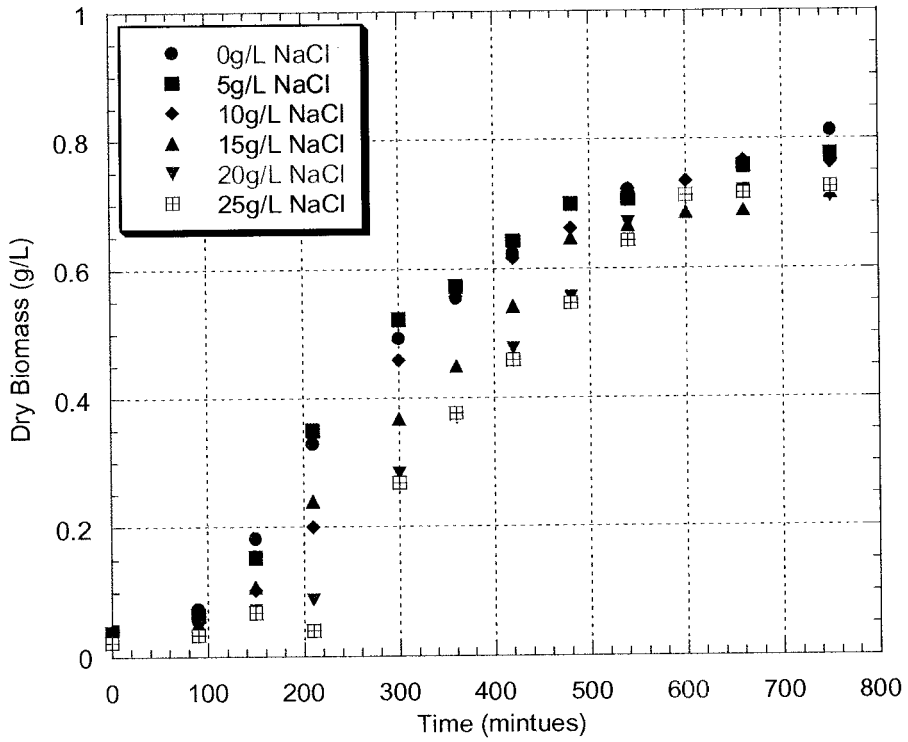

Figure 13. The Effect of Sodium Chloride on the Growth of *B. methanolicus* Strain PB1 in Trypticase Soy Broth (Using a Pre-Adaptation Method).

Table 17. The Effect of Sodium Chloride on the Specific Growth Rate of *B. methanolicus* Strain PB1 in Trypticase Soy Broth (Using the Pre-adaptation Method).

NaCl Concentration (g/L)	Specific Growth Rate (min^{-1})	Specific Growth Rate (hr^{-1})
0	0.006043 ± 0.001176	0.3626 ± 0.07056
5	0.006650 ± 0.001346	0.3990 ± 0.08076
10	0.007526 ± 0.0008840	0.4516 ± 0.05304
15	0.006933 ± 0.001054	0.4160 ± 0.06324
20	0.006345 ± 0.0005975	0.3807 ± 0.03585
25	0.006602 ± 0.0007562	0.3961 ± 0.04537

Table 18. The Effect of Sodium Chloride on the Final Dry Biomass of *B. methanolicus* Strain PB1 in Trypticase Soy Broth and Minimal Yeast Extract Medium (Using the Pre-adaptation Method).

NaCl Concentration (g/L)	Final Dry Biomass (g/L)
0	0.8126
5	0.7773
10	0.7627
15	0.7172
20	0.7102
25	0.7257

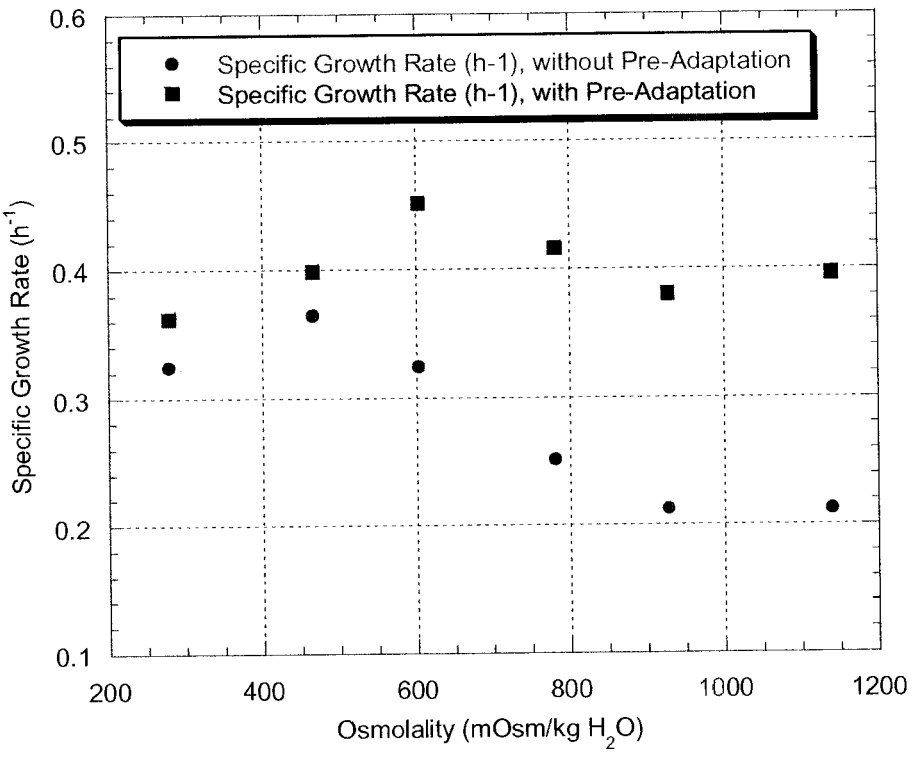

Figure 14. The Effect of Sodium Chloride on the Specific Growth Rate of *B. methanolicus* Strain PB1 in Trypticase Soy Broth (with and without the use of pre-adaptation method).

from 0.36 ± 0.04666h^{-1} to 0.21 ± 0.021 h^{-1} as the sodium chloride concentration is increased from 5g/L to 25g/L. On the other hand, when the inoculum was prepared with the use of pre-adaptation method, the specific growth rates were found to be very steady at approximately 0.40 h^{-1}. Figure 15 also shows the final dry biomass of *B. methanolicus* strain PB1 growing in trypticase soy broth with and without the use of the pre-adaptation method. Under the high salt concentration (25 g/L NaCl), when the pre-adaptation method was not used to prepare the inoculum, the final dry biomass is significantly reduced by 84% to 0.16 g/L. On the other hand, when the pre-adaptation method was used to prepare the inoculum, the significant decrease in final dry biomass is not found. *B. methanolicus* strain PB1 achieved a final dry biomass of approximately 0.71 to 0.81 g/L in trypticase soy broth with sodium chloride concentration ranging from 0 to 25 g/L.

Similar results were observed for *B. methanolicus* strain PB1 grown in minimal yeast extract media. In previous experiments without the use of pre-adaptation method, the final dry biomass dropped 81% from 1.67 g/L to 0.32 g/L as the sodium chloride concentration was increased from 15 g/L to 25 g/L in the minimal yeast extract media. When the pre-adaptation method was used to prepare the inoculum, the bacteria achieved a final dry biomass of approximately 1.0 g/L to 1.4 g/L as shown in Figure 16.

As a conclusion from the pre-adaptation experiment, *B. methanolicus* strain PB1 is able to develop a strategy to live in high salt environment through a gentle step by step pre-adaptation method. The specific growth rate and final dry biomass of *B. methanolicus* strain PB1 growing in high salt concentration (25 g/L) are improved by using the pre-adaptation method. The results show that the ability of *B. methanolicus*

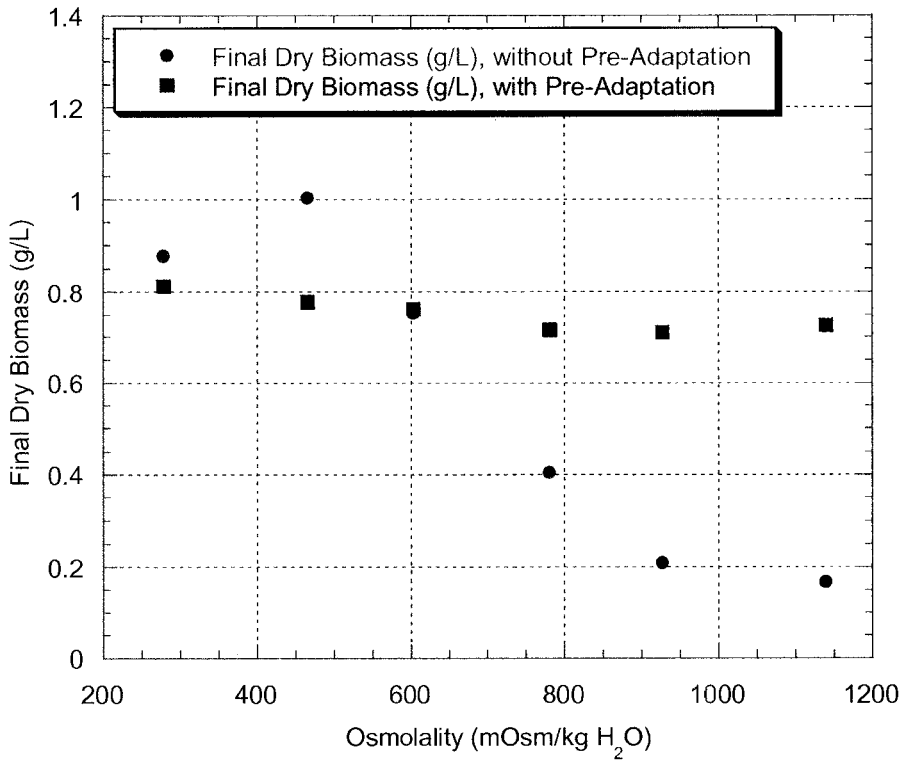

Figure 15. The Effect of Sodium Chloride on the Final Dry Biomass of *B. methanolicus* Strain PB1 in Trypticase Soy Broth (with and without the use of pre-adaptation method).

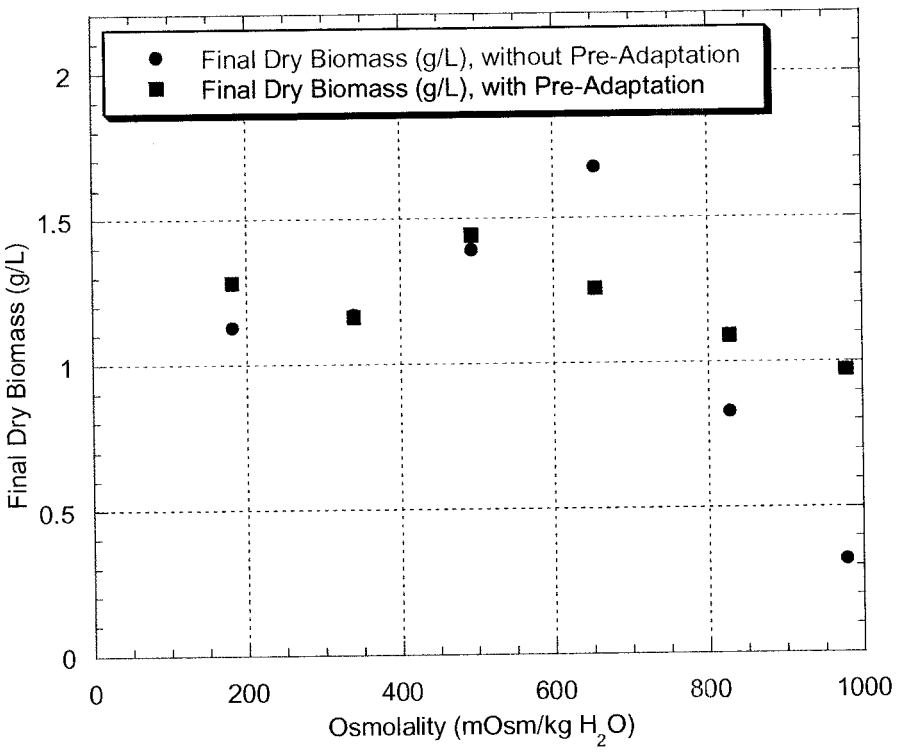

Figure 16. The Effect of Sodium Chloride on the Final Dry Biomass of *B. methanolicus* Strain PB1 in Minimal Yeast Extract Medium (with and without the use of pre-adaptation method).

PB1 to grow at a high salt concentration is closely related to its previous growth condition. As a result, it may be possible to adapt *B. methanolicus* strain PB1 to stress in seawater. The pre-adaptation method may help *B. methanolicus* strain PB1 to grow well in alternate and harsh environments, i.e. seawater [33-39].

6.4 The Growth of *B. methanolicus* strain PB1 in Seawater-Based Medium

6.4.1 Osmolality of the Pacific Ocean Seawater

Seawater was diluted in DI water at different ratios. The different dilutions of seawater were designed to test the ability of *B. methanolicus* growing in seawater-based medium. Their osmolalities were determined by using The Advanced™ Micro-Osmometer as described in Chapter 4.7. Pacific Ocean Seawater (100%, without the dilution with DI water) was found to have an osmolality of 981 mOsm/kg H_2O. This osmolality value was found very similar to minimal salt solution with the addition of 25 g/L of sodium chloride that is 979 mOsm/kg H_2O. The data were summarized in Figure 17 and Table 19.

6.4.2 Osmolality of Artificial Seawater

Artificial seawater was prepared according to Chapter 4.2.4. Its osmolality was found to be 971 mOsm/kg H_2O. It was similar to the osmolality of Pacific Ocean seawater that is 981 mOsm/kg H_2O. Since the quality and composition of Pacific Ocean seawater may vary over time, the bacteria growth conditions may not be consistent when using different batches of Pacific Ocean seawater to prepare the culturing medium.

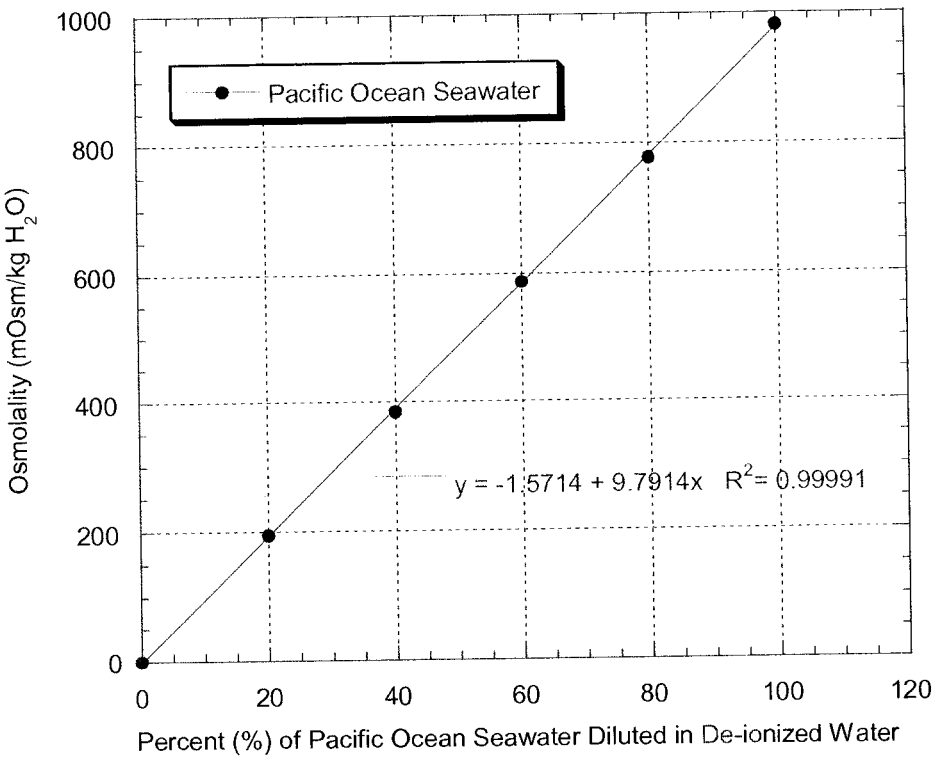

Figure 17. Osmolality of Pacific Ocean Seawater Diluted in De-ionized Water

Table 19. Osmolality of Pacific Ocean Seawater Diluted in De-ionized Water.

Ratio of Pacific Ocean Seawater/De-ionized Water	Osmolality (mOsm/kg H_2O)
100% / 0%	981
80% / 20%	778
60% / 40%	588
40% / 60%	385
20% / 80%	196
0% / 100%	0

6.4.3 Pre-adaptation of *B. methanolicus* strain PB1 in Seawater-Based Medium

The inoculum was prepared based on the concepts from the pre-adaptation experiment as shown in Figure 12. *B. methanolicus* was first prepared in minimal yeast extract medium without the addition of sodium chloride. It was subsequently transferred into minimal yeast extract medium with 15 g/L NaCl after 12 hours of incubation. Finally, *B. methanolicus* from minimal yeast extract medium with 15 g/L NaCl was used to inoculate media prepared in Pacific Ocean seawater with methanol as carbon source. The bacteria was incubated at 50^0C overnight.

The growth of *B. methanolicus* in seawater-based medium was shown by the turbidity of the solution. A control flask with the same medium was also inoculated with the un-adapted bacteria. The turbidity of the un-adapted culture was not as high as the adapted culture after the overnight incubation. Based on the optical density value measured by the UV/Vis spectrophotometer, the value from the un-adapted cells was obviously lower than the adapted cells. Therefore, results showed that *B. methanolicus* was able to grow well in the seawater-based medium by using this simple one step-pre-adaptation method.

6.4.4 The Growth of *B. methanolicus* strain PB1 in Pacific Ocean Seawater and Artificial Seawater Based Medium

The growth of *B. methanolicus* strain PB1 in Pacific Ocean Seawater and artificial seawater based medium were compared under batch growth conditions as described in Chapter 4.6. Two separate shaker flasks with 200 mL of Pacific Ocean seawater and artificial seawater based medium were inoculated with 4 mL (2% v/v) of 10 hours overnight culture. The inoculum was prepared according to Chapter 6.4.3.

The time course of the growth of *B. methanolicus* strain PB1 in Pacific Ocean seawater and artificial seawater is shown in Figure 18. The specific growth rates are determined according to Chapter 5.4. *B. methanolicus* strain PB1 growing in Pacific Ocean seawater and artificial seawater were found to have the same specific growth rate that is calculated to be 0.33 h^{-1} with standard error of 0.042 and 0.033 h^{-1}, respectively. The final dry biomass for bacteria growing in Pacific Ocean seawater-based medium and artificial seawater-based medium were found to be 0.44 g/L and 0.40 g/L, respectively.

These results show that *B. methanolicus* strain PB1 is able to utilize growth medium prepared in seawater-based medium. *B. methanolicus* strain PB1 can grow well initially in seawater-based medium; however, it cannot achieve a high cell density in the medium. The specific growth rate and final dry biomass of *B. methanolicus* are found to be similar when it was cultured in medium prepared in both Pacific Ocean seawater and artificial seawater.

6.4.5 The Growth of *B. methanolicus* strain PB1 in Tap Water

Tap water from the bio-processing laboratory of San Jose State University is known to be hard water that contains a variety of mineral salts, i.e., calcium chloride and magnesium sulfate [40]. According to Table 8, calcium chloride and magnesium sulfate are some of the major compounds in the minimal salt solution for the growth of *B. methanolicus*. Therefore, the tap water may compose of the necessary mineral salts to support the growth of *B. methanolicus* strain PB1.

The growth of *B. methanolicus* strain PB1 in Pacific Ocean seawater and tap water based medium from San Jose State University was observed in batch condition as

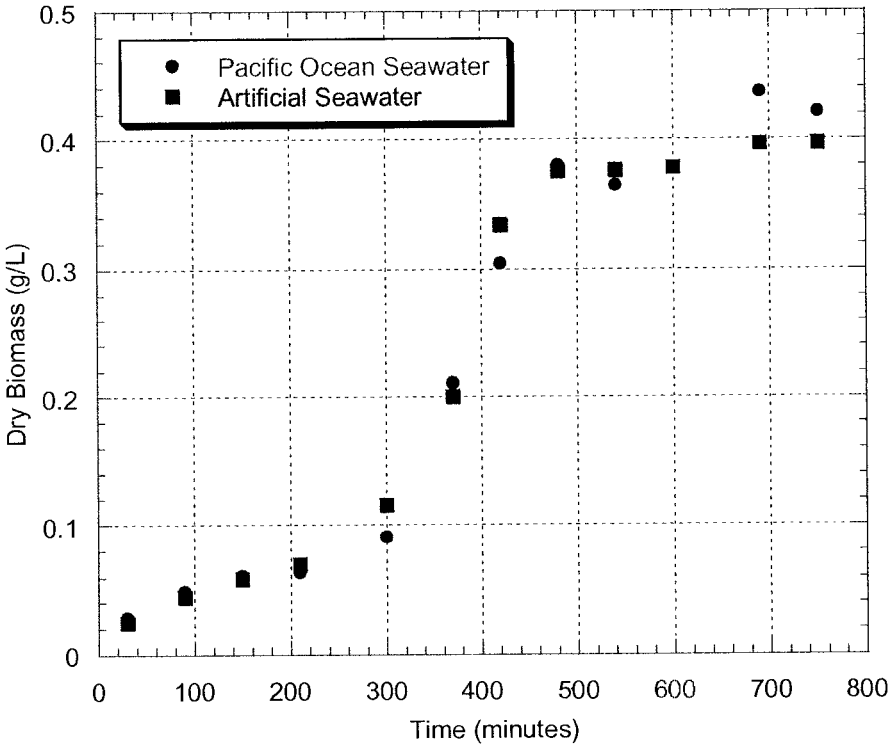

Figure 18. The Growth of *B. methanolicus* Strain PB1 in Pacific Ocean Seawater and Artificial Seawater Based Medium.

described in Chapter 4.6. The inoculum was prepared according to Chapter 6.4 and was inoculated into the medium at 2% v/v ratio. The inoculum was previously grown in minimal yeast extract medium without the addition of sodium chloride.

The time course of the growth of *B. methanolicus* strain PB1 in Pacific Ocean Seawater and tap water based medium is shown in Figure 19. The specific growth rates are determined according to Chapter 5.4 and found to be $0.37 \pm 0.052\ h^{-1}$ and $0.23 \pm 0.0084\ h^{-1}$, respectively, for bacteria growing in Pacific Ocean seawater and tap water. The final dry biomasses of *B. methanolicus* strain PB1 growing in Pacific Ocean seawater and tap water based medium are found to be 0.40 g/L and 0.30 g/L, respectively.

Results show that *B. methanolicus* strain PB1 is able to utilize tap water as growth medium. The bacteria can initially grow slowly in tap water-based medium; however, it cannot achieve a high cell density in the medium. Precipitation was also found after the 19 hours incubation. Therefore, the precipitation may affect the absorbance reading at the final time point at Figure 19. Although results show that tap water can be used as culturing medium for *B. methanolicus* strain PB1, tap water is not an ideal medium because of the precipitation problems. Furthermore, the minerals in tap water vary from location to location, using tap water-based medium for the growth of *B. methanolicus* strain PB1 is not repeatable.

6.4.6 The Growth of *B. methanolicus* strain PB1 in Pacific Ocean Seawater Diluted with Different Ratios of De-Ionized Water

The growth of *B. methanolicus* strain PB1 in Pacific Ocean seawater diluted with different ratios of de-ionized water was observed under batch growth conditions as described in Chapter 4.6.1. The dilution of seawater with de-ionized water decreases the

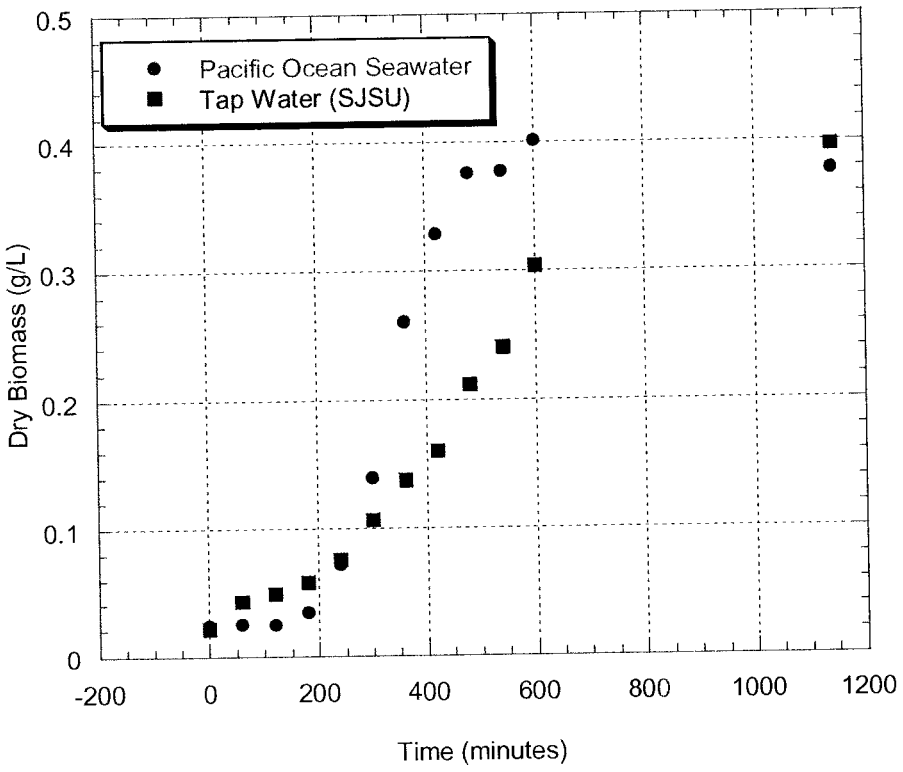

Figure 19. The Growth of *B. methanolicus* Strain PB1 in Pacific Ocean Seawater and Tap Water (SJSU) Based Medium.

osmolality of the medium and would be expected to affect the growth of *B. methanolicus* strain PB1 because of the decrease in osmotic stress. Six shaker flasks were prepared according to Table 11 and were inoculated with 4 mL (2% v/v) of 10 hours overnight culture. The inoculum was initially grown in 100% seawater according to Chapter 6.4.3.

The time course of the growth of *B. methanolicus* strain PB1 in Pacific Ocean seawater diluted in different ratio of de-ionized water is shown in Figure 20. The specific growth rates for *B. methanolicus* strain PB1 are determined according to Chapter 5.4 and summarized in Table 20. The final dry biomasses are also summarized in Table 21. The specific growth rates and final dry biomass values are also plotted and compared in Figure 21.

The results of this experiment show that the specific growth rate and final dry biomass for *B. methanolicus* strain PB1 grown in 100% Pacific Ocean seawater are 0.41 ± 0.041 h^{-1} and 0.40 g/L, respectively. These results are comparable to the results from Chapter 6.4.4 of which the specific growth rate and final dry biomass for *B. methanolicus* strain PB1 growing in 100% Pacific Ocean seawater are found to be 0.33 ± 0.042 h^{-1} and 0.44 g/L, respectively. In Chapter 6.4.5, the specific growth rate and final dry biomass of *B. methanolicus* strain PB1 growing in the same condition are found to be 0.37 ± 0.052 h^{-1} and 0.40 g/L, respectively. Based on the results determined from these chapters, the specific growth rate and final dry biomass data are found to be comparable. As a result, the growth of *B. methanolicus* strain PB1 in Pacific Ocean seawater is stable and repeatable over the time period of our experimentation.

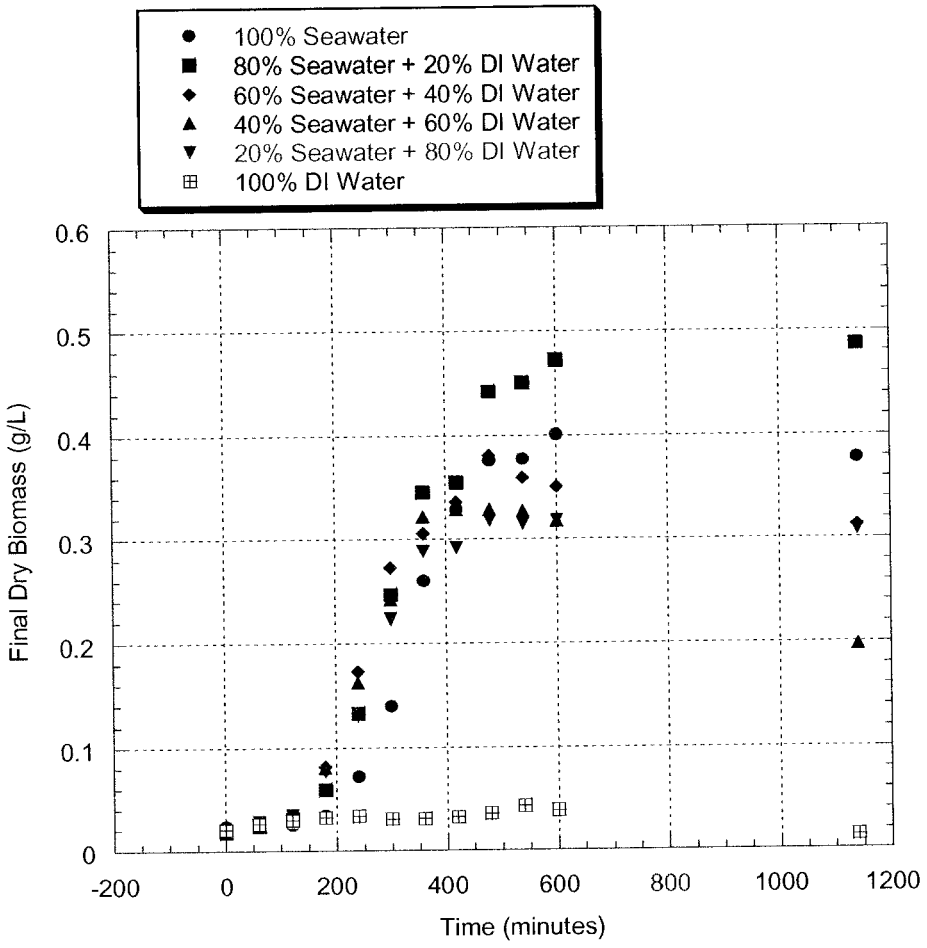

Figure 20. The Growth of *B. methanolicus* Strain PB1 in Pacific Ocean Seawater Diluted with Different Ratio of De-ionized Water.

Table 20. The Specific Growth Rate of *B. methanolicus* Strain PB1 in Pacific Ocean Seawater Based Medium.

	Ratio of Pacific Ocean Seawater/De-ionized Water	Osmolality (mOsm/kg H_2O)	Specific Growth Rate (min^{-1})	Specific Growth Rate (hr^{-1})
#1	100% / 0%	981	0.006882 ± 0.0006901	0.4129 ± 0.04141
#2	80% / 20%	778	0.006714 ± 0.0008075	0.4028 ± 0.04845
#3	60% / 40%	588	0.005890 ± 0.0009519	0.3534 ± 0.05711
#4	40% / 60%	385	0.005605 ± 0.0009276	0.3363 ± 0.05566
#5	20% / 80%	196	0.005445 ± 0.0007940	0.3267 ± 0.04764
#6	0% / 100%	0	0.0006841 ± 0.0001814	0.04105 ± 0.01088

Table 21. The Final Dry Biomass of *B. methanolicus* Strain PB1 in Pacific Ocean Seawater Based Medium.

	Ratio of Pacific Ocean Seawater/De-ionized Water	Final Dry Biomass (g/L)
#1	100% / 0%	0.4014
#2	80% / 20%	0.4720
#3	60% / 40%	0.3508
#4	40% / 60%	0.3178
#5	20% / 80%	0.3181
#6	0% / 100%	0.03891

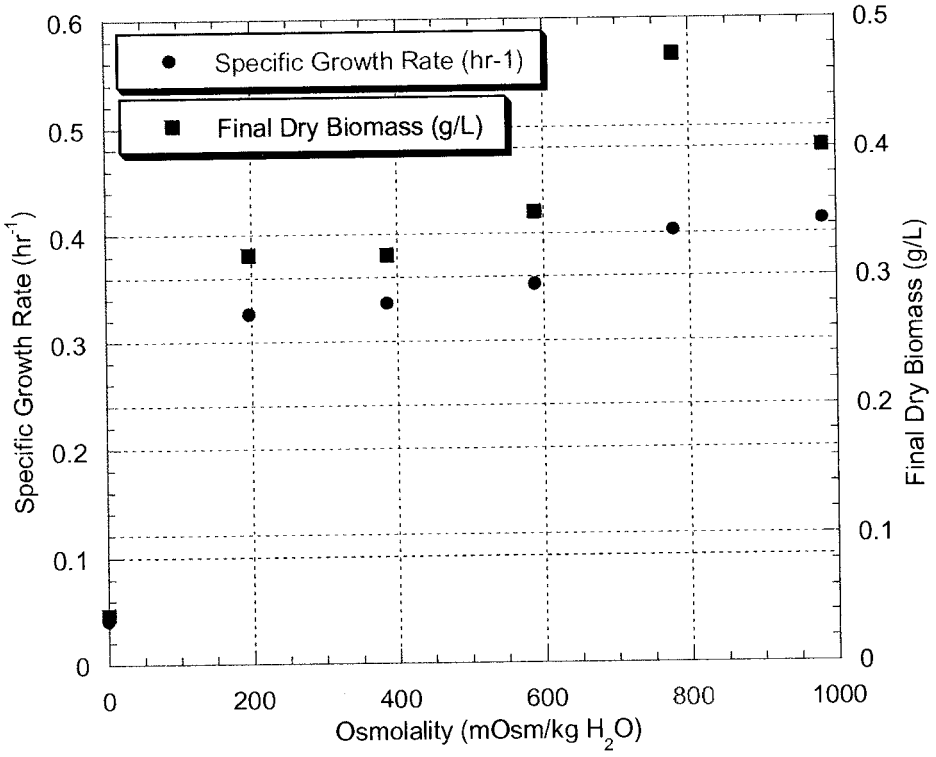

Figure 21. The Specific Growth Rates and Final Dry Biomasses of *B. methanolicus* Strain PB1 growing in Pacific Ocean Seawater Diluted with Different Ratio of De-ionized Water

Based on Figure 21, the specific growth rate of *B. methanolicus* strain PB1 decreases as the Pacific Ocean seawater is diluted with high ratios of de-ionized water. The specific growth rate decreases from 0.41 to 0.33 h^{-1} as the osmolality decreases from 981 to 196 mOsm/kg H_2O. The range of the decrease is not significant. In terms of the final dry biomass, the medium with 80% seawater/20% DI water is found to provide the optimal condition for *B. methanolicus* strain PB1. The final dry biomass is found to be 0.47 g/L when *B. methanolicus* strain PB1 grows in this medium. The final dry biomass decreases from 0.47 to 0.32 g/L as the osmolality decreases from 778 to 196 mOsm/kg H_2O. The specific growth rate and final dry biomass is found to be 0.041 h^{-1} and 0.039 g/L, respectively, when *B. methanolicus* strain PB1 grows in 100% de-ionized water of which the osmolality is equal to zero. The bacteria did not grow well in pure de-ionized water although methanol, yeast extract, trace metals, vitamin B_{12} and biotin were served as growth supplements. The result clearly shows that *B. methanolicus* strain PB1 needs salts to support its growth, as some metal salts are important cofactors for enzymes in metabolic pathways.

The results show that the medium with 80% seawater/20% DI water, which has an osmolality of 778 mOsm/kg H_2O provides the optimal condition for the growth of *B. methanolicus* strain PB1. It provides *B. methanolicus* with specific growth rate and final dry biomass at 0.41 h^{-1} and 0.47 g/L, respectively. This medium provides the necessary mineral salts for the growth of bacteria; on the other hand, it provides an optimal osmolality for the bacteria growth.

6.4.7 The Growth of *B. methanolicus* strain PB1 in Pacific Ocean Seawater with Different Amounts of Yeast Extract as Supplement

Although the medium with 80% seawater/20% DI water based medium with 248 mM methanol provided an optimal growth environment for *B. methanolicus* strain PB1, the final cell density is still very low. Furthermore, the use of 20% de-ionized water may still increase the cost for preparation of cultural medium. Therefore, the growth of *B. methanolicus* strain PB1 in 100% Pacific Ocean seawater based medium at high cell density is our main interest. In this chapter, the growth of *B. methanolicus* strain PB1 was observed in 100% Pacific Ocean seawater based medium with different amounts of yeast extract as supplements. Yeast extract composes of a variety of amino acids and peptides. It is a nutrient-rich compound and also contains carbon, energy, and electron sources. Therefore, the optimal concentration of added yeast extract for high cell density growth may be determined for the growth of *B. methanolicus* strain PB1 in 100% Pacific Ocean seawater.

The growth of *B. methanolicus* strain PB1 in Pacific Ocean Seawater based medium with the addition of different amounts of yeast extract was observed in batch condition as described in Chapter 4.6.2. Six shaker flasks were prepared according to Table 12 and were inoculated with 4 mL (2% v/v) of 10 hours overnight culture. The inoculum was initially grown in 100% seawater based medium according to Chapter 6.4.3.

The time course of the growth of *B. methanolicus* strain PB1 in Pacific Ocean seawater based medium with the addition of different amounts of yeast extract as supplements is shown in Figure 22. The specific growth rates for *B. methanolicus* strain

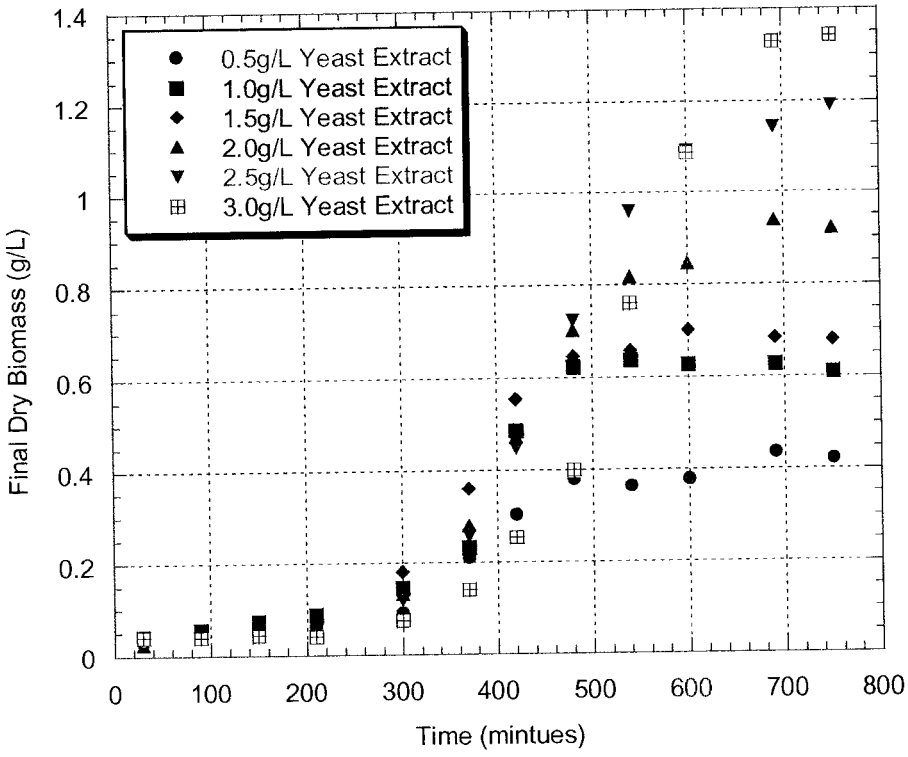

Figure 22. The Growth of *B. methanolicus* Strain PB1 in Pacific Ocean Seawater Based Medium With Different Amount of Yeast Extract as Supplements.

PB1 are determined according to Chapter 5.4 and summarized in Table 22. The final dry biomass values are also summarized in Table 23. The specific growth rates and final dry biomass values are also plotted and compared in Figure 23. The residual methanol concentrations of the media were also measured by the YSI Biochemistry Analyzer during the bacteria growth. The results are presented in Figure 24. The relations between methanol consumed and dry biomass yield in media with different amount of yeast extract are also presented in Figure 25.

Results show that *B. methanolicus* strain PB1 is able to achieve higher specific growth rate and final dry biomass as the concentration of yeast extract in Pacific Ocean seawater based medium is increased up to 3 g/l. The specific growth rate and final dry biomass are found to be 0.34 ± 0.092 h^{-1} and 0.44 g/L, respectively, when the yeast extract concentration is at 0.5 g/L in Pacific Ocean seawater based medium. The specific growth rate and final dry biomass are improved to as high as 0.58 ± 0.019 h^{-1} and 1.33 g/L, respectively, when the yeast extract concentration is increased to 3.0g/L in Pacific Ocean seawater. This result is very much comparable to *B. methanolicus* strain PB1 growing in minimal yeast extract medium of which the optimal specific growth rate and final dry biomass are found to be 0.32 ± 0.009 h^{-1} and 1.67 g/L, respectively. Although Pacific Ocean seawater with yeast extract concentration at 3.0 g/L provides an optimal condition for the growth of *B. methanolicus* strain PB1, the bacteria is found to utilize yeast extract as part of its carbon and energy sources. The bacteria consumed only 1.6 g of methanol during the growth period according to Figure 25.

Table 22. The Specific Growth Rate of *B. methanolicus* Strain PB1 in Pacific Ocean Seawater Based Medium With Different Amount of Yeast Extract as Supplements.

	Concentration of Yeast Extract in Pacific Ocean Seawater (g/L)	Specific Growth Rate (min^{-1})	Specific Growth Rate (hr^{-1})
#1	0.5	0.005743 ± 0.001538	0.3446 ± 0.09228
#2	1.0	0.006651 ± 0.001340	0.3991 ± 0.08040
#3	1.5	0.007238 ± 0.001273	0.4343 ± 0.07638
#4	2.0	0.007772 ± 0.001065	0.4663 ± 0.07638
#5	2.5	0.008845 ± 0.0008161	0.5307 ± 0.04897
#6	3.0	0.009638 ± 0.0003175	0.5783 ± 0.01905

Table 23. The Final Dry Biomass of *B. methanolicus* Strain PB1 in Pacific Ocean Seawater Based Medium With Different Amount of Yeast Extract as Supplements.

	Concentration of Yeast Extract in Pacific Ocean Seawater (g/L)	Final Dry Biomass (g/L)
#1	0.5	0.4363
#2	1.0	0.6259
#3	1.5	0.6839
#4	2.0	0.9376
#5	2.5	1.1432
#6	3.0	1.3305

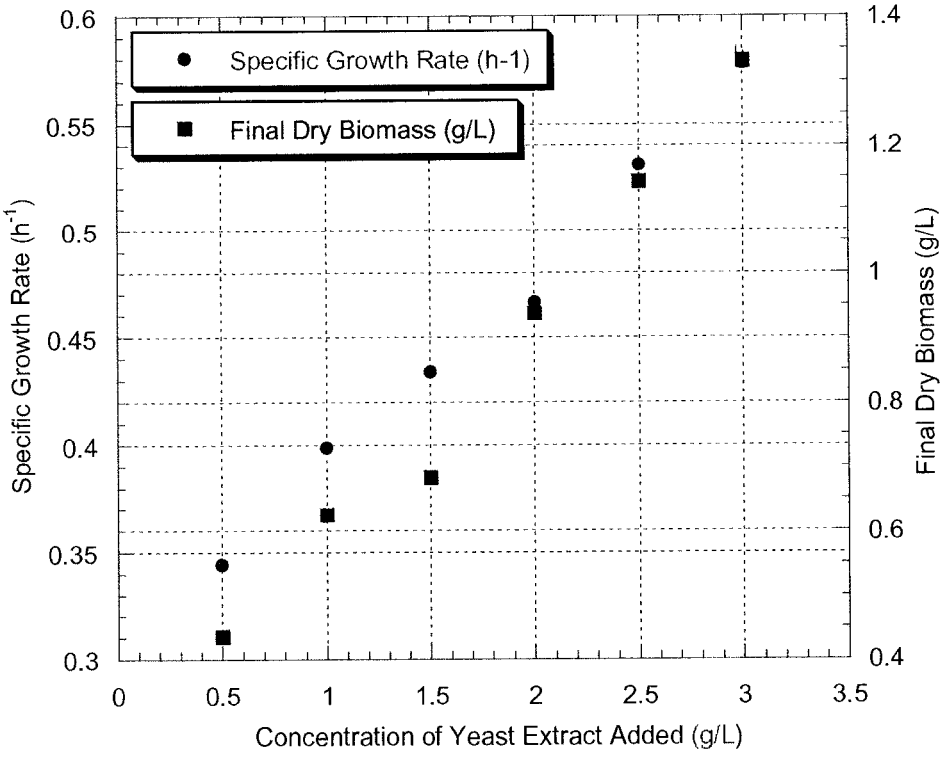

Figure 23. The Specific Growth Rates and Final Dry Biomasses of *B. methanolicus* Strain PB1 growing in Pacific Ocean Seawater Based Medium With Different Amount of Yeast Extract as Supplements.

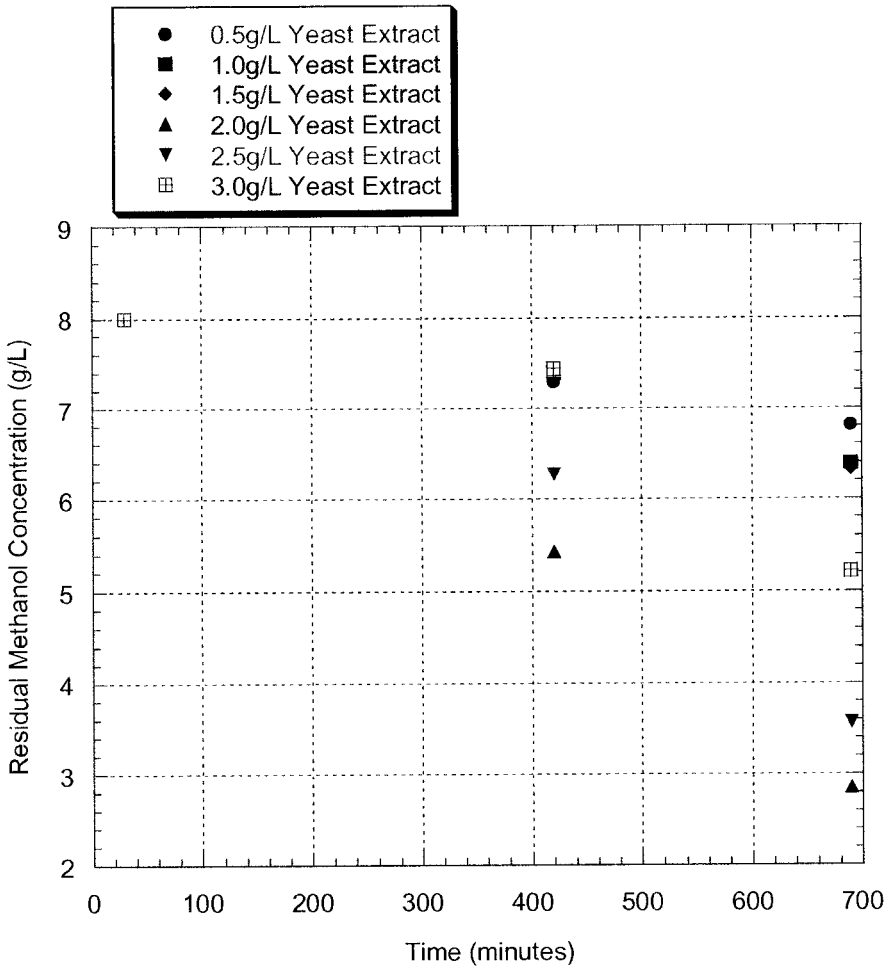

Figure 24. The Residual Methanol Concentration of Media When *B. methanolicus* Strain PB1 growing in Pacific Ocean Seawater Based Medium With Different Amount of Yeast Extract as Supplements.

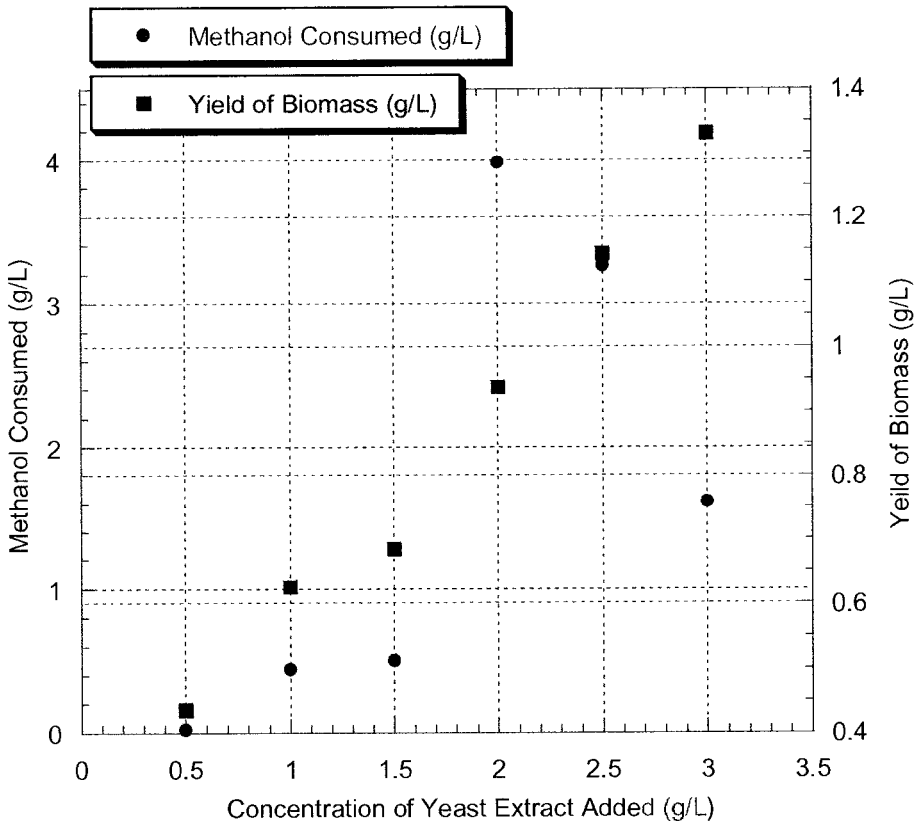

Figure 25. The Relations Between Methanol Consumed and Dry Biomass Yield in 100% Pacific Ocean Seawater Based Medium With Different Amount of Yeast Extract as Supplements.

On the other hand, *B. methanolicus* strain PB1 growing in Pacific Ocean seawater based medium with 2.0 g/L yeast extract is found to utilize the most methanol. The bacteria consumed 3.98 g/L methanol during the growth period. In this condition, the 2.0 g/L yeast extract concentration is just enough to help *B. methanolicus* strain PB1 initiating the growth and utilizing methanol as carbon and energy sources. The specific growth rate and final dry biomass are found to be 0.47 ± 0.076 h^{-1} and 0.94 g/L, respectively.

In this experiment, the results clearly show that the growth of *B. methanolicus* strain PB1 in Pacific Ocean seawater based medium is facilitated by the addition of yeast extract. The amino acids, growth factors and cofactors from yeast extract seem to facilitate the growth of *B. methanolicus* growing at high salt concentration. However, it appears that yeast extract instead of methanol is the preferred carbon and energy source for *B. methanolicus* strain PB1 at a yeast extract concentration above 2.0 g/l. Thus, Pacific Ocean seawater based medium with addition of 2.0 g/L yeast extract is found to be optimal in a medium with 248 mM methanol for the objective of high cell density and maximum methanol usage. The yield of biomass on methanol, $Y_{x/s}$, at this condition is found to be 0.24 g cells/g methanol.

6.5 The pPB1 Plasmid from *B. methanolicus* Strain PB1

6.5.1 The Extraction of the pPB1 Plasmid

The pPB1 plasmid was successfully extracted from *B. methanolicus* strain PB1 by following the procedures described in Chapter 4.9.1. Four sets of 50 mL overnight

cultures were set up. The same plasmid isolation procedures were followed for the four different sets except different amount of lysozyme was used to lyse the bacteria. The lysozyme concentration in Buffer P1 was varied from 0 to 10 mg/mL. The purified plasmid DNAs were analyzed on a 0.8% agarose gel. The picture of the gel is shown in Figure 26.

The size of the plasmid DNA is found to be approximately 20-kilo base pairs when comparing to the λ Hind III DNA marker. Results show that the brightness of the DNA band, of which the intensity increases as the DNA is more concentrated, increases as the bacteria was lysed with an increased amount of lysozyme. This shows that the gram-positive cell wall of *B. methanolicus* strain PB1 could be lysed by adding as little as 2.5 mg/L lysozyme into Buffer P1; however, the addition of 10 mg/L lysozyme into Buffer P1 seemed to provide a good condition for the lysis of the bacteria. Lane#4 shows the absence of the DNA products. This clearly shows that the plasmid cannot be released from the gram-positive cell wall without the addition of lysozyme. Lysozyme is proved to be an important lysis reagent for the gram-positive bacteria.

Since the Qiagen QIAprep Spin Miniprep Kit is designed for the isolation of plasmid from gram-negative bacteria, i.e. *E. coli*, modification of the standard protocol is necessary for the isolation of plasmid from *B. methanolicus*. According to the Qiagen QIAprep Miniprep Handbook, 1 to 5 mL of overnight culture is enough for isolation of DNA from bacteria that has high plasmid copy number. However, a large culture volume was harvested for the preparation of the bacterial pellet because *B. methanolicus* strain PB1 was assumed to have low plasmid copy number. Plasmid DNA isolated from only 5

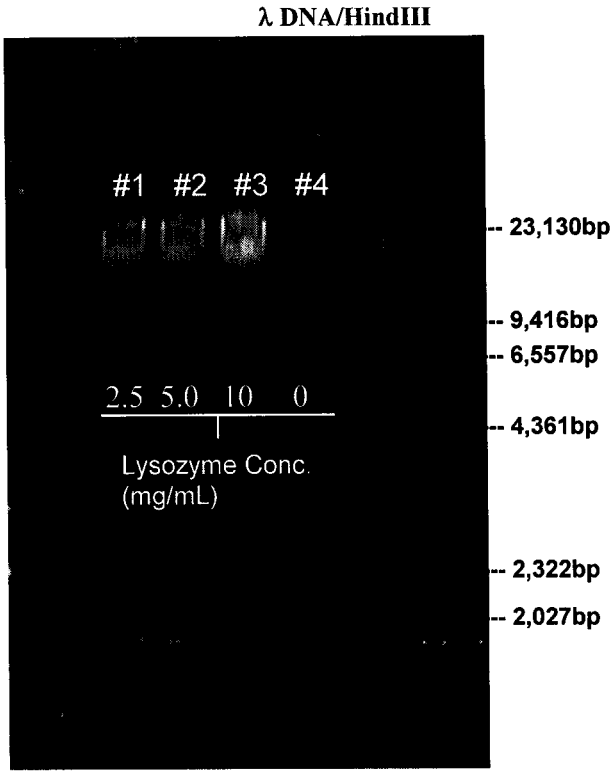

Figure 26. The Purified pPB1 Plasmid DNA from *B. methanolicus* Lysed with Different Amount of Lysozyme. (Lane#1: 2.5 mg/mL; Lane#2: 5 mg/mL; Lane#3: 10 mg/mL; Lane#4 - Control: 0 mg/mL)

mL of overnight *B. methanolicus* culture may not be enough for visualization on an agarose gel. According to Chapter 4.9.1, plasmid DNA was purified from 50 mL of overnight culture that is 10 times more than the suggested volume. As a result, the buffers used for re-suspending (Buffer P1), lysing (Buffer P2), neutralizing (Buffer N3), and washing (Buffer PB & Buffer PE) the bacteria DNA were also used approximately 10 times more than the suggested volume.

6.5.2 The Restriction Enzyme Digestion of the pPB1 Plasmid

The plasmid DNA isolated from *B. methanolicus* strain PB1 from previous chapter was characterized by restriction enzyme digestion. The restriction digestion of the plasmid was set up according to a patent from a Japanese research group [14] and was discussed in Chapter 6.5.3. Single and double digestions of the plasmid were set up according to Table 13.

The digested fragments were successfully separated by gel electrophoresis. The results of the single and double digestions of the plasmid are shown in Figure 27 and 28, respectively. The λ Hind III DNA marker and DNA ladder I, which are manufactured from GibcoBRL and GeneChoice, respectively, were used as the references of the DNA sizes. The sizes of the separated DNA fragments were analyzed by the LabWorks Software according to Chapter 5.5. The values are summarized in Table 24.

The values analyzed by the LabWorks Software are found to have minor difference from the published values in the Japanese Patent [14]. Due to the resolution limitations of the commercial DNA markers and the separation limitations of the large size DNA on agarose gel, the prediction of the DNA sizes by gel electrophoresis may not

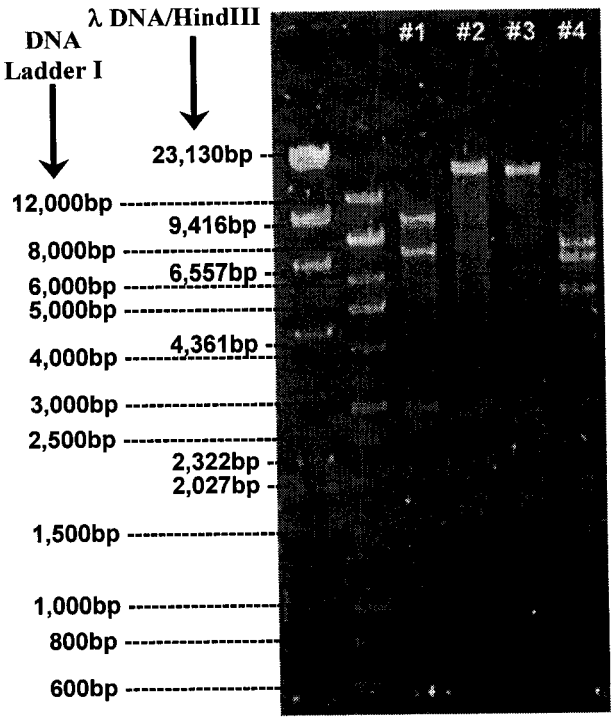

Figure 27. The Single Digestion of pPB1 Plasmid. (Lane#1: BamHI; Lane#2: SalI; Lane#3: EcoRI; Lane#4: PstI)

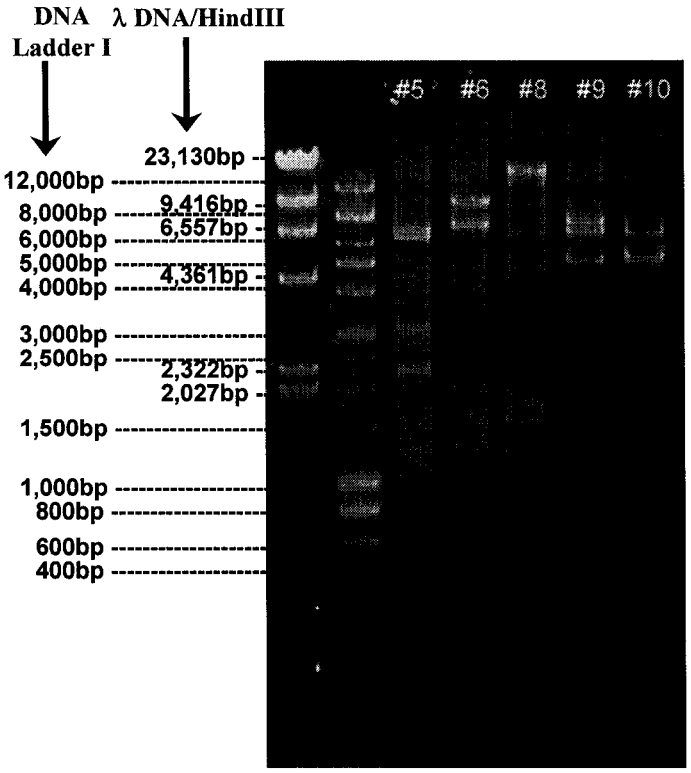

Figure 28. The Double Digestion of pPB1 Plasmid. (Lane#5: BamHI + PstI; Lane#6: BamHI + SalI; Lane#8: SalI + EcoRI; Lane#9: SalI + PstI; Lane#10: EcoRI + PstI)

Table 24. The Size of the Digested DNA Fragments of pBM1 Plasmid.

	Size of DNA fragments (kilo base pairs)	
Restriction Enzyme	**Values analyzed from this paper**	**Values published in literature [14]**
BamHI	3.0, 7.2, 8.9	3.0, 7.5, 10.0
SalI	19.8	20.5
EcoRI	19.0	20.5
PstI	5.5, 7.0, 7.8	5.0, 7.9, 7.6
BamHI, PstI	2.3, 3.2, 6.9, 7.1	0.1, 0.5, 2.5, 2.5, 7.4, 7.5
BamHI, SalI	0.8, 2.0, 7.4, 10.0	0.6, 2.4, 7.5, 10.0
SalI, EcoRI	1.9, 18.0	20.5
SalI, PstI	5.2, 6.9, 7.6	0.1, 4.9, 7.5, 8.0
EcoRI, PstI	1.9, 5.4, 6.8	20.5

be very accurate. The accuracy on predicting the sizes of large DNA fragment is especially a problem because the range of separation decreases as the size of the DNA fragment increases. Although the predication of the size of the digested DNA fragments may not be accurate, the restriction enzyme digestion results have given valuable information to prove the existence of the plasmid.

CHAPTER 7.0 CONCLUSIONS AND FUTURE RESEARCH

7.1 Conclusions

Preliminary experiments were done to test the ability of *B. methanolicus* PB1 (ATCC 51375) to grow in media with high salt concentration. *B. methanolicus* was grown in trypticase soy broth with NaCl concentration ranging from 0 to 25g/L. The results showed that the growth rate decreased by 34% in media with a salt concentration equal to that in seawater, as compared with the 40% decrease in minimal yeast extract media prepared in de-ionized water. The ability of *B. methanolicus* PB1 to grow in Pacific Ocean seawater using methanol as a carbon and energy source was also tested at different ratios of seawater/de-ionized water. Initially, the medium with 80% seawater and 20% DI water provided the highest dry biomass yield. Through adaptation, PB1 was able to grow in 100% seawater-based medium using methanol as carbon and energy source with good yields and similar growth rates as compared with media prepared in de-ionized water. Results also show that the growth of *B. methanolicus* PB1 in seawater-based medium can be facilitated by the addition of yeast extract.

A standard protocol was successfully developed to isolate the pPB1 plasmid from *B. methanolicus* strain PB1. Lysozyme was found to be necessary for isolation of plasmid from gram-positive bacteria. The plasmid was characterized with restriction enzyme digestion. The size of the plasmid is found to be approximately 20-kilo base pairs. Results are comparable to the information published in a Japanese patent, although minor differences are found.

7.2 Future Research

L-lysine and glutamic acid are known to be the metabolic by-products from *B. methanolicus*. If *B. methanolicus* is able to utilize seawater and methanol as the growth medium and substrate efficiently, this should be an economic way to produce L-lysine and glutamic acid as commercial products. The presence of the DNA plasmid may also enable the genetic modification of the bacteria for production of some other amino acids or bio-molecules via seawater and methanol.

As a result, future research should be focused on optimizing the growth components in seawater-based medium for *B. methanolicus* strain PB1, optimizing its growth conditions in large-scale bioreactor, quantifying the production of L-lysine and glutamic acid while the growth of *B. methanolicus* strain PB1 is in seawater-based medium. Furthermore, the pPB1 plasmid should also be studied in depth. The complete sequence of the plasmid should be clarified before we can study *B. methanolicus* strain PB1 at the molecular level.

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