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Growth of B. methanolicus MGA3 and B. licheniformis in artificial seawater media

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GROWTH OF *B. METHANOLICUS* MGA3 AND *B. LICHENIFORMIS* IN ARTIFICIAL SEAWATER MEDIA

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ABSTRACT

GROWTH OF *B. METHANOLICUS* MGA3 AND
B. LICHENIFORMIS IN ARTIFICIAL SEAWATER MEDIA

by Chi On Chiu

While prior research on *Bacillus methanolicus* strain PB1 demonstrated growth in artificial seawater-based medium, no work has been carried out to date with *Bacillus methanolicus* strain MGA3. In this work, *B. methanolicus* strain MGA3 achieved a maximum optical density (OD) of less than 0.1 when growing in artificial seawater. The maximum OD reached 1.04 in artificial seawater medium with the addition of 2mM of each of the following reported osmoprotectants: glycine betaine, choline, proline, carnitine, glutamate, trehalose, sucrose, and methionine. In the absence of osmoprotectants, a stepwise increment in sodium chloride content in the growth medium enabled strain MGA3 to reach a maximum OD of 3.87 in minimal yeast medium at a final concentration of 25 g/l NaCl. A culture contaminant, *Bacillus licheniformis,* was identified during the research. With the use of a factorial design of experiment (DOE) and a mixture DOE, it was determined that glycerol and yeast extract served as carbon sources for the growth of *B. licheniformis* in minimal yeast medium. After adaptation, the high salt-adapted organism grew in artificial seawater with the addition of 2mM trehalose and methionine.

DEDICATION

I dedicate this thesis to my father, Yee-bor Chiu, my mother, Lai-ching Chi Chiu, my sisters, Jean, Lowell, Polly Chiu, my love, Cherie Chu, my classmate, Tim C.K. Pun, my friend, Trevor Pardee, and brothers and sisters from my church, Bay Area Peace Evangelical Church. The patient, encouragement and prayer from all of you made this thesis possible.

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CHAPTER ONE
INTRODUCTION

1.1 Biocatalysis with Microorganisms

Limited fossil organic carbon stocks, environmental considerations, and cost factors have contributed to the promotion of biocatalysis for enabling the production of building blocks and end-products in the pharmaceutical and chemical industries [1]. Biocatalysis is the use of enzymes or whole cells as catalysts in chemical reactors. Fermentation is a typical example, which transforms renewable carbon, through the use of living microorganisms, into biomass and metabolites such as alcohols, ketones, vitamins, antibiotics, amino acids, and other commodity and fine chemicals. In a typical fermentation, the organisms convert sugar into the desired product. However, many other carbon sources can also be used, including synthesis gas, methane, methanol, and other hydrocarbons. By means of biochemical and genetic engineering, some non-native compounds, such as indigo (originally from plant) and catechol, can now be produced by fermentation from renewable carbon [2].

1.2 Bioreactors for the Production of Chemicals

Bioreactors provide a convivial environment for organisms to grow and produce a desired product through their metabolic activity. To achieve this purpose, the bioreactor must prevent the interaction of the live culture with the reactor operators and the open atmosphere. Typical bioreactor systems are outfitted for the control of temperature, dissolved oxygen, pH and tank level, and usually have a scheme for preventing excessive foam development. The size of bioreactors ranges from milliliter volumes to 500 m^3. In addition, in order to continuously improve the productivity of bioreactors, many of the large chemical companies have developed in-house biocatalysis engineering groups, or spun off companies, or established sub-companies devoted to the development and use of biocatalysis in chemical production.

Batch and continuous-flow stirred tank reactors are two categories of cultivation methods for bioprocesses. Most commercial bioprocesses are fed-batch systems. The shorter process time in batch systems prevents back mutation from the specialized productive stain to the less productive native stain. In a continuous system, on the other hand, the less productive variant may dominate the reaction culture, and the productivity is adversely affected. In addition, since the demand of many fermentation products is difficult to project, the flexibility of batch systems provide a better choice than continuous

systems that may be dedicated to a single product only [3]. The choice of reactor depends on experience, availability and other subjective reasons. An important consideration in process design is the requirement of oxygen. For organisms with a high oxygen demand, the transfer of oxygen from the gas phase to the liquid phase is critical to the success of the culture. In that case, the design of the reactor system must be able to provide a high oxygen transfer to the culture media.

1.3 Methanol as a Fermentation Feed Source

Methanol has many great advantages for being a fermentation feedstock. Compared to glucose and soybean oil, methanol has a lower market price per ton and higher biomass yield at the same time as shown in Figure 1 [2, 34-36]. In addition, the development of Gas to Liquid technology (GTL) may further reduce the production cost of methanol by converting natural gas or methane into methanol [4]. Sometimes, the natural gas that is released from offshore oil refineries is flared, resulting in the expulsion of greenhouse gases to the atmosphere. GTL technology proposes to use natural gas for the production of useful chemicals, by first converting it to methanol through a two-step process [5]. The use of this technology to generate feedstocks for fermentation has been proposed recently [6].

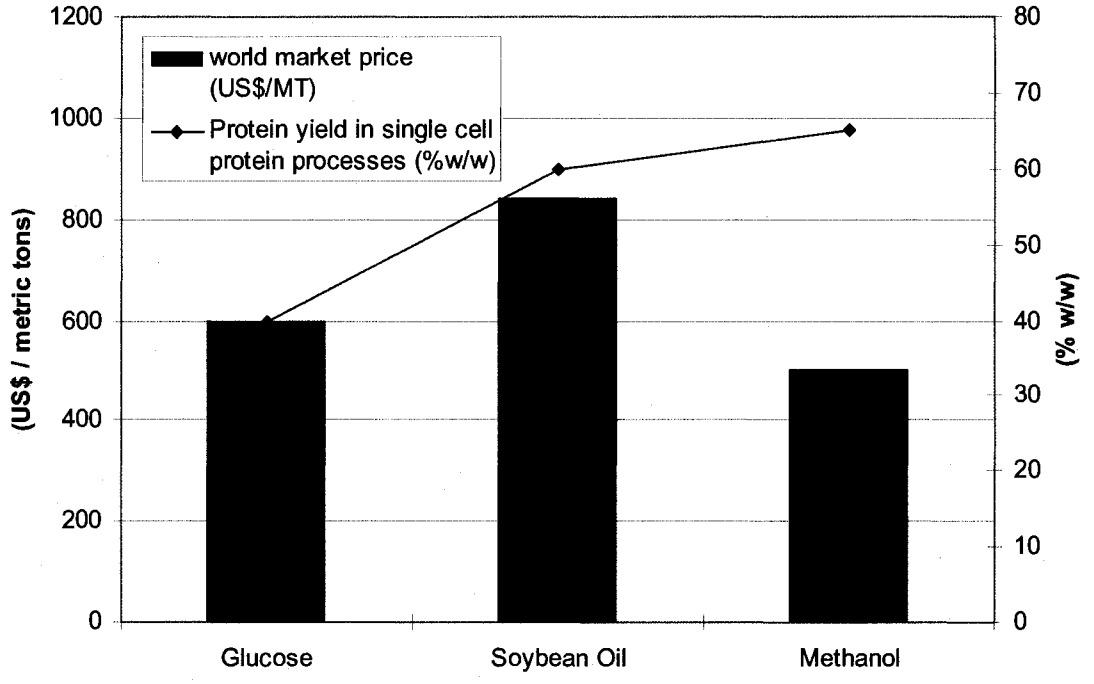

Figure 1. Price and protein yield comparisons for common growth substrates for biocatalysts [2, 34-36].

1.4 Fresh Water and Desalination

Bacterial fermentation takes place in an aqueous media. The typical fermentation media contains salts, such as phosphate salts, potassium salts, sodium salts, and other metal-based compounds. The concentration of salts is about 0.9% for most bacteria. Usually the media is made from fresh desalinated water and the appropriate salts and other components are added to it. However, of all the Earth's water, only 1 percent is fresh water available for humans to drink; 97 percent of the Earth's water is salt water, and 2 percent is

frozen [7]. Seawater contains about 2.6% salt, making the salt concentration too high for most bacterial fermentations.

The cost of desalinating seawater varies widely from country to country. The type of desalination processes used, and the scale of the plant mainly dominate the unit cost of desalted water. The unit cost of desalted water for a large-scale plant varies from US$0.4 – 1.0 per 1000 liters, and that for small-scale plant can be more than US$1.5 per 1000 liters [8]. Reverse Osmosis (RO) produces 10,000 cubic meters per day is considered as large scale, while the same capacity in Multi-stage Flash distillation (MSF) is classified as small scale [8], which operates at 23-27 kWh/m^3 of distilled water [9].

The use of bacteria that can grow in water with the salt concentration found in seawater is proposed to reduce the operating cost of fermentation for the production of commodity chemicals from off-shore oil rigs. The operating cost of fermentation is the sum of the cost for carbon source and media components, cooling water to maintain the operating temperature, air compression for the oxygen source, and labor costs of the operator. With bacteria that can grow in salt water, there is a significant reduction in the cost of the fermentation broth that would not need to be first desalinated prior to media preparation.

1.5 Characteristics of *Bacillus methanolicus*

Bacillus methanolicus is a new species of bacteria found in the early 1990s. Analysis of 16S rRNA sequences indicated that it is distinct from *Bacillus brevis* but closely related to *B. firmus* and *B. azotoformans* [10]. The thermotolerance and the methanol utilizing ability of this species generated great interest in exploring the use of *B. methanolicus* for research in biotechnology.

1.5.1 Thermotolerance

Bacillus methanolicus can grow optimally between 37 °C and 60 °C. Specifically, it has a preferred growth temperature from 50 °C to 53 °C. The relatively high growth temperature is a cost advantage for the production of commodity chemicals because of the lower demand for cooling water in the high temperature process [11].

1.5.2 Methanol as a Carbon and Energy Source

Bacillus methanolicus is a bacterium that can utilize methanol as a carbon and energy source. Compared to glucose, the price of methanol is relatively cheap and stable. In addition, methanol is toxic for most microorganisms, and less contamination occurs in the reaction broth [2].

CHAPTER TWO
LITERATURE REVIEW

Biocatalysis is a new trend for both novel and traditional chemicals production. For drugs and fine chemicals production, certain bioprocesses are carried out in low reaction volumes because of the high selling value of the product. On the other hand, there are needs for large-scale bioprocesses of either bulk or fine chemicals production [1]. Challenges for large-scale bio-based industrial processes include raising productivity and stability of the process, but also reducing the cost of production. While genetic engineering can serve to improve yield and selectivity by modifying the genetic make-up of microorganisms, little has been published directly about other methods to improve cost effectiveness of large-scale microbial fermentation. Generally speaking, common considerations on production cost of large-scale fermentation include the price of feedstock, the utility cost of maintaining appropriate operation conditions, and the purification cost of reaction broth or downstream products, which are the considerations shared by all chemical processes.

Due to the continuing increases in raw material prices, use of methanol as fermentation feedstock is a new trend in biotechnology research. One possible technique to further reduce the price of this feedstock is the development of Gas-To-Liquid

technology [4]. To further reduce costs with GTL technology, seawater can be used as the water source for reaction media.

In this project, the growth of *Bacillus methanolicus* in seawater-based medium was the main subject of study. Literature discussing GTL technology, marine biotechnology, and bacteria growth under hyperosmolality conditions will be reviewed. In addition, literature on the development of an optimized growth medium for *Bacillus methanolicus* will be discussed.

2.1 Gas to Liquid Technology as a Source of Methanol

In the fermentation industry, methanol is known as a pure, high energy but cheap feedstock. It can be produced from natural gas by using GTL technology. Basically, methane is first mixed with steam and converted to syngas ($CO + H_2$) with a catalyst. The syngas is then converted to methanol by an oxygenation method [5, 12]. However, the usage of GTL technology coupled with large-scale fermentation for production of chemical commodities has received very little attention, and thus no literature was found for this application.

2.2 Marine Biotechnology -- Use of Seawater

Marine biotechnology offers tremendous potential for new product development in pharmaceuticals, food, and bioremediation [13]. Marine bacteria have been demonstrated to produce drugs or valuable chemicals [13, 14]. For example, the antitumor agent alteramide A could be extracted from marine bacterium called *Halichondria okadai,* which is grown on sponge [13]. While a great number of valuable chemicals were reported to have been extracted from marine organisms, none of these chemicals had developed as a commercialized drug approved by the FDA. Major obstacles are the inability to harvest enough of the source organism and the instability of the organisms in large-scale culture [13].

2.3 Growth Medium of *Bacillus methanolicus*

In 1989, Schendel *et al.* [15] demonstrated the production of L-lysine by the growth of *Bacillus methanolicus* in a shake flask and a fed-batch reactor. The medium used for the shake flask cultures was named minimal salts (MS) medium, and the composition is shown in Table 1. In addition, other components were added to study the effect of the bacterial growth. The minimal vitamin (MV) medium was the MS medium supplemented with vitamins as shown in Table 2. Minimal yeast (MY) medium was the MV medium

supplemented with yeast exact in the concentration of 0.5 g/L. Trace metal (TM) solution, as shown in Table 3, was required in all MS, MV and MY solutions. Methanol was fed as the energy and carbon source in both the fed-batch reactor and the shake flask experiments. The medium composition used in the shake flask was slightly different from the one in the fed-batch reactor and the reason for this deviation was unknown. TM solution was also added with the methanol feed of the fed-batch reactor. In the shake flask experiment, the maximum growth rate in MY medium was 0.8 h^{-1}, and that in MV was 0.48 h^{-1}, with the yield of 0.48 g of dry cell weight per g methanol, and the same result occurred in the fed-batch experiment under the methanol concentration of 100mM. In addition, out of all vitamins used in these experiments, Schendel *et al.* found that only biotin and vitamin B_{12} were vital for the growth of *Bacillus methanolicus*.

Table 1. Minimal salts medium [15].

Compound	Concentration (g/L)
K_2HPO_4	3.8
NaH_2PO_4-H_2O	2.8
$(NH_4)_2SO_4$	3.6
$MgSO_4$-$7H_2O$	0.5

Table 2. List of supplemental vitamins [15].

Compound	Concentration (μg/L)
thiamine hydrochloride	50
D-calcium pantothenate	50
riboflavin	50
nicrotinamide	50
biotin	20
folic acid	20
vitamin B_{12}	1

Table 3. Trace metal solution [15].

Compound	Concentration (mg/L)
$MnSO_4 \cdot H_2O$	0.2
$CaCl_2 \cdot 2H_2O$	5.3
$FeSO_4 \cdot 7H_2O$	2.00
$ZnSO_4 \cdot 7H_2O$	0.2
$CoCl_2 \cdot 6H_2O$	0.04
$CuSO_4 \cdot 5H_2O$	0.04
$Na_2MoO_4 \cdot 2H_2O$	0.047
H_3BO_3	0.03

Cue *et al.* [16] used a similar medium for the cultivation of *Bacillus methanolicus* for genetic manipulation. In addition, tryptone soytone broth (TSB: 1.5% tryptone, 0.5% soytone, and 86mM NaCl) was also included in the medium. However, the detailed

composition of other supplements in the cultivation medium was not specified in that paper. Similar to the study in Cue *et al.* [16], Shinichi *et al.* [17] also used TSB as cultivation medium for the bacteria, but the composition of TSB was slightly different than the one used by Cue *et al.*

In 2000, Schendel *et al.* [18] claimed a patent for the production of glutamate by using *Bacillus methanolicus*. Schendel *et al.* used shake flask and fed-batch cultures to study the growth of the bacteria with MS and MV medium. In the shake flask experiments, 3 different types of carbon sources (2% methanol, 1.6% methanol + 40mM sodium acetate, and 1.52% mannitol) were used without the enhancement of trace metals or vitamins in the feed, and it was found that using mannitol resulted in the highest yield of glutamate. In the fed-batch experiments, various compositions of trace metals were added with methanol feed to evaluate effects on biomass growth and glutamate production.

Pluschkell's research also demonstrated the production of glutamic acid with *Bacillus methanolicus* [11]. A shake flask and a continuous stirred tank reactor (CSTR) were used, and the reaction medium used was based on the study of Schendel *et al.* in 1989. In the continuous culture, methanol was added with MS, TM, biotin, and vitamin B_{12} for maintaining the steady state condition with the concentration ranging from 100mM to 300mM methanol.

Wang [19] used an adaptive control method on the methanol feed for the growth of *Bacillus methanolicus* in MY medium. In this study, a shake flask was first used to determine the optimal methanol concentration in the initial feed based on the biomass and initial specific growth rate. In addition, chemostat experiments were done to determine the yield coefficients on methanol and oxygen. Using these values for the initial settings, Wang then used an adaptive control for the methanol feed for fed-batch fermentation of *B. methanolicus*.

Cheung [20] investigated the growth of *Bacillus methanolicus* PB1 in a shake flask with 4 different media: 1) MY medium, 2) trypticase soy broth, 3) seawater, yeast extract, TM, and methanol, 4) artificial seawater (shown in Table 4), yeast extract, TM and methanol. Cheung adapted the bacteria (Strain PB1aST) to grow in medium made with seawater without the addition of any deionized water [22]. There was little effect of NaCl on the growth rate of *Bacillus methanolicus* in different media; however, the final dry biomass concentration in the shake flask cultures was reduced from that of the non-adapted strain PB1 grown in low-salt media. Cheung also increased the concentration of yeast extract above the 0.5 g/L used in the Schendel *et al.* media to improve the growth characteristics of strain PB1aST in seawater-based media. A comparison of the growth in natural seawater-based medium with different concentrations of yeast extract is shown in

Figure 2. The ratio of methanol consumed per biomass unit (g/g) in the flasks during this experiment (Figure 3) shows that with 2.0 g/L of yeast extract in the medium, the bacteria utilized the most methanol of any concentration of yeast extract. While the use of yeast extract for a large scale process was not preferred due to cost considerations, this result suggested that further optimization of the medium could result in improved growth characteristics of *B. methanolicus* at high salt concentrations.

Table 4. Compositions of artificial seawater in Cheung's study [20].

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

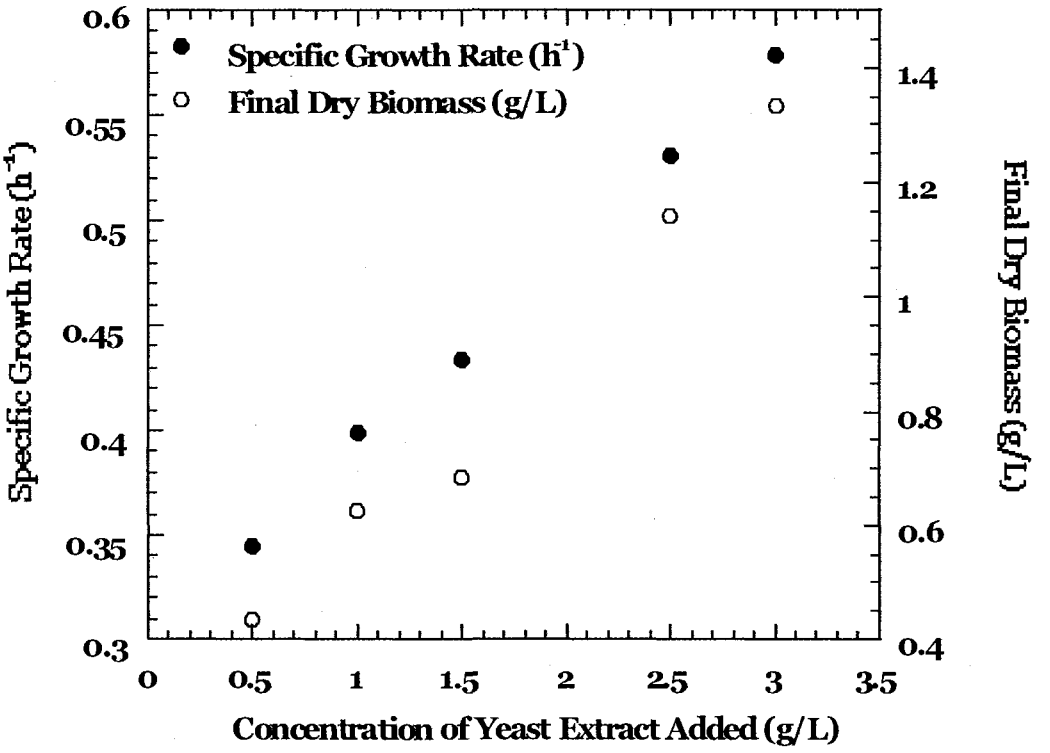

Figure 2. The effect of added yeast extract on the specific growth rate and final dry biomass of strain PB1 grown in Pacific Ocean Seawater-based MY media with methanol as growth substrate. (• Specific growth rate; o Final dry biomass) [22]

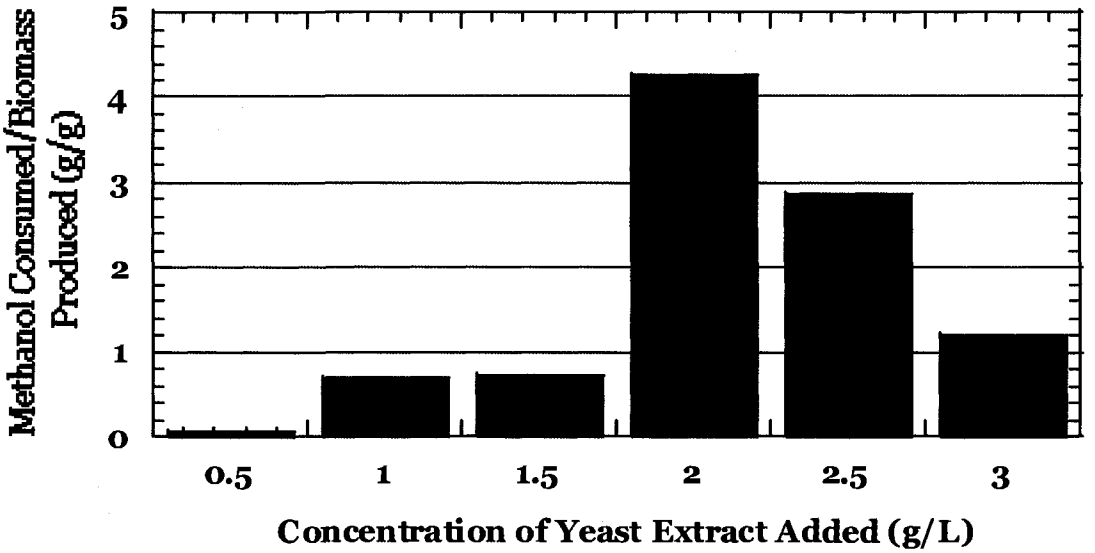

Figure 3. The effect of yeast extract concentration on methanol uptake in Pacific Ocean seawater-based medium. Initial methanol concentration was 1% (vol/vol). [22]

2.4 The Growth of Bacteria under High Salinity and the Effect of Osmoprotectants

In natural habitats, changing osmolality of the living environment is one major challenge for bacteria survival. Under high salinity, the growth of bacteria is usually inhibited due to cellular dehydration by low water activity from the environment. Failure to develop a defensive system to the changing environment can eventually cause the death of the bacteria [21]. In order to prevent the rapid efflux of water from the bacteria cell, one common response of high salt tolerant bacteria is the accumulation of some compatible solutes to restore positive turgor. Such solutes include K+, amino acids (e.g. glutamate,

proline), amino acid derivatives (e.g. methionine), quaternary amines (e.g. betaine, carnitine), sugars (e.g. sucrose, trehalose) and tetrahydropyrimidines [23-31]. Some examples of osmoprotectants (OP) in different studies are shown in Table 5.

Glycine betaine, also called betaine, is one of the most commonly studied osmoprotectants. Glycine betaine is a quaternary amine and it is synthesized by a two-step oxidation of choline via betaine aldehyde. Many studies showed that glycine betaine had a strong positive effect on the growth of bacteria under hyperosmolality [23-25, 27-31]. For example, Baliarda *et al.* [23] showed that with 2mM of glycine betaine, the growth rate of *T. halophila* increased 5.5 times more than the growth rate in the absence of any osmoprotectant.

In addition, many studies also showed that choline had a positive effect on the growth of bacteria under high salinity [23-25, 31]. Choline is not an osmoprotectant itself, but it is the precursor of glycine betaine in biosynthesis. In high osmotic environments, many bacteria bring choline into the cell as the substrate for the synthesis of glycine betaine which will protect the cell from cellular dehydration [21].

Table 5. Examples of the effect of osmoprotectants.

Author(s)	OP	Concentration	Bacteria	Growth Effect	Conditions
Baliarda *et al.* [23]	glycine betaine	2mM	*P. pentosaceus*	growth rate increase (0.100 h^{-1})	0.8 M NaCl
			T. halophila	growth rate increase (0.130 h^{-1})	2M NaCl
	choline		*P. pentosaceus*	growth rate increase (0.086 h^{-1})	0.8 M NaCl
			T. halophila	growth rate increase (0.065 h^{-1})	2M NaCl
	proline		*P. pentosaceus*	growth rate slightly increase (0.082 h^{-1})	0.8 M NaCl
			T. halophila	no effect (0.019 h^{-1})	2M NaCl
	no OP		*P. pentosaceus*	growth rate = 0.074 h^{-1}	0.8 M NaCl
			T. halophila	growth rate = 0.020 h^{-1}	2M NaCl
Wood and Sorensen [26]	glycine betaine, proline	1mM	*N. europaea*	no effect	0.1M – 0.3M NaCl
Fletcher and Csonka [27]	glycine betaine, methionine	1mM	*S. typhimurium*	growth rate increase (37°C, 45 °C)	0.3M – 0.6M NaCl
				decrease viable count (50 °C / 15mM H_2O_2)	0.3M NaCl
Pichereau *et al.* [28]	glycine betaine	1mM	*E. coli*	growth rate increase	0.8M NaCl
			R. meliloti	growth rate increase, restore growth to unstressed level	0.5M NaCl
Caldas *et al.* [30]	glycine betaine, choline	1mM	*E. coli*	increase colony-forming ability	0-12% NaCl

Glutamic acid and proline are also effective osmoprotectants among many different amino acids. Glutamic acid is usually transported from the medium, and proline can be either transported from the medium or be synthesized by the bacteria cell itself. Under high salinity, many bacteria rapidly accumulate glutamic acid, coupling with potassium ions to form the electrolyte pair K-glutamate, inside the cell to counteract the hyper osmotic stress [25, 31]. On the other hand, some gram positive bacteria accumulate proline as a more effective osmoprotectant than the electrolyte pair K-glutamate [25]. Pichereau *et al.* also point out that the stable accumulation of K-glutamate could be deleterious for cellular functions, and this can be relayed by the accumulation of other osmoprotectants, such as proline and glycine betaine, to decrease the level of the charged solute K-glutamate [28].

However, the addition of osmoprotectants in the medium does not always have a positive effect on relieving the hyperosmotic stress condition [26]; in some cases, the addition of osmoprotectants could have a toxic effect on the growth of bacteria [28].

CHAPTER THREE
RESEARCH HYPOTHESIS AND OBJECTIVES

3.1 Research Hypothesis

Bacillus methanolicus strain PB1aST was able to grow in seawater-based medium in a shake flask with similar growth characteristics to strain PB1 in low salt media. The hypothesis for this research was that the growth of *B. methanolicus* strain MGA3 by using artificial seawater-based medium should also be feasible. Likewise, the growth of the strain MGA3 in high salt conditions might be improved by modifying the culture media through addition of osmoprotectant compounds.

3.2 Research Objectives

The first objective of this thesis was to study the effect of osmoprotectants on the growth of *Bacillus methanolicus* strain MGA3 in artificial seawater medium. The second objective of this thesis was to cultivate the high salt-adapted strain of MGA3 by using stepwise increments of sodium chloride concentration in minimal yeast medium.

CHAPTER FOUR
MATERIALS AND METHODS

4.1 Revivification of Frozen Culture

Bacillus methanolicus strain MGA3 was stored as a frozen culture with 30 % glycerol at –80 ^{o}C. The frozen culture was revived in a shake flask by using trypticase soy broth, minimal yeast or artificial seawater as the growth medium. Sections 4.2.1 to 4.2.3 describe the compositions of each medium. A volume of 50 ml of the growth medium was used to thaw 1ml of the frozen culture (2% vol/vol). The shake flask culture was stored in an incubator with a rotational speed of 200 rpm for 8 to16 hours. The temperature of the incubator was set at 50 ^{o}C at atmospheric pressure. Unless otherwise specified, all frozen cultures in this research were revived with the same medium as the culture was grown in before freezing. The culture was incubated until reaching the middle or the end of the exponential phase, and then was transferred to other medium for further study.

4.1.1 Revivification of Spore Suspension Solution

Bacillus methanolicus strain MGA3 could be stored as a spore suspension solution between 4 ^{0}C and room temperature. The standard protocol of revivifying the spore suspension solution is described as follows:

1) Extract 50 µL of *Bacillus methanolicus* strain MGA3 spore suspension, and transfer it into a sterile tube.
2) Incubate the tube at 80 °C for 15 minutes.
3) Transfer the 50 µl spore suspension into a 50ml of fresh pre-warmed MY media.
4) Incubate the pre-culture at 50 °C and 275 rpm overnight.
5) Make frozen cultures from the exponential phase pre-culture for later use.

4.2 Preparation of Culture Media

4.2.1 Minimal Yeast Medium

The MY medium was one of the growth media used in this research. It was used to revive the frozen culture of *Bacillus methanolicus* strain MGA3, and used as growth medium for the study of the baseline growth profile mentioned in section 4.3.2.1. The compositions of the MY medium are shown in Tables 6 to 8.

Biotin and vitamin B_{12} were sterilized by filter-sterilization (Corning 0.22 µm bottle filter) to prevent denaturation by high temperature. All other solutions were sterilized by autoclaving at 121 °C for 20 minutes. With the addition of 1.5 % wt/vol agar powder (Difco Laboratories), the medium could be solidified as MY agar plates which were used for the isolation of bacteria colonies. Furthermore, the addition of 25 g/L of

sodium chloride in MY medium was used to study the growth profile of the bacteria under high salinity, as described in section 4.3.2.1.

Table 6. Compositions of minimal yeast medium.

Compound	Concentration (g/L)
$NaH_2PO_4 \cdot H_2O$	1.89
$K_2HPO_4 \cdot 3H_2O$	6.47
$(NH_4)_2SO_4$	3.6
yeast extract	0.5
$MgSO_4 \cdot 7H_2O$	0.25
trace metal (1000X, see Table 7)	1 ml/L
vitamin solution (10000X, see Table 8)	0.1 ml/L
methanol (HPLC grade)	3.168 (4 ml/L)

Table 7. Compositions of trace metal solution (1000X).

Compound	Concentration (g/L)
$MnSO_4 \cdot H_2O$	0.2
$CaCl_2 \cdot 2H_2O$	5.3
$FeSO_4 \cdot 7H_2O$	2.00
$ZnSO_4 \cdot 7H_2O$	0.2
$CoCl_2 \cdot 6H_2O$	0.04
$CuSO_4 \cdot 5H_2O$	0.04
$Na_2MoO_4 \cdot 2H_2O$	0.047
H_3BO_3	0.03
citric acid	1.5

Table 8. Compositions of vitamin solution (10000X).

Compound	Concentration (mg/L)
biotin	20
vitamin B_{12}	4

4.2.2 Tryticase Soy Broth

Trypticase soy broth was used for the growth of *Bacillus methanolicus* in many previous studies [16, 17, 20, 22]. In this research, this medium was used in the revivification of the frozen culture of the organism. It was purchased as powder form (BBL^{TM} Brand). When in use, it was mixed with de-ionized (DI) water to the concentration of 30 g/L. The composition of TSB medium is shown in Table 9.

The TSB medium was sterilized by autoclaving at 121 ^{o}C for 20 minutes before use. Once again, the addition of 1.5 % wt/vol agar powder (Difco Laboratories) was carried out to prepare TSB agar plates which were used for the isolation of bacteria colonies.

Table 9. Composition 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

4.2.3 Artificial Seawater Medium and Osmoprotectants

Artificial seawater was used as a medium to study the growth of bacteria under high salinity. The formulation of the artificial seawater medium (ASM) from Cheung [20] was

used in this study, and the composition of this medium was mentioned in Table 4 in section 2.3. The ASM was supplemented with yeast extract, vitamins, and trace metals as described in Table 10. Osmoprotectants were added according to the experimental plan described in Section 4.3.2.3. The list of osmoprotectants used in this study is shown in Table 11.

Due to the formation of precipitate during heating at high temperature, the artificial seawater could not be autoclaved. Therefore, it was sterilized by filter-sterilization (Corning 0.22 μm bottle filter). Other media components, except the vitamin solution, were sterilized by autoclaving at 121 °C for 20 minutes. Osmoprotectants, biotin, and vitamin B_{12} were sterilized by filter-sterilization (Corning 0.22 μm bottle filter).

Table 10. Compositions of artificial seawater medium.

Compound	Concentration (g/L)
NaCl	24.6
KCl	0.67
$CaCl_2$-$2H_2O$	1.36
$MgSO_4$-$7H_2O$	6.29
$MgCl_2$-$6H_2O$	4.66
$NaHCO_3$	0.18
yeast extract	0.5 or 2.0
methanol (HPLC grade)	3.168 (4 ml/L)
trace metal (1000X, see Table 8)	1 ml/L
vitamin solution (10000X, see Table 9)	0.1 ml/L

Table 11. List of osmoprotectants and the concentration being tested.

Osmoprotectants	Concentrations (mM)
glycine betaine	2
choline	2
proline	2
carnitine	2
glutamate	2
trehalose	2
sucrose	1
methionine	1

4.3 Batch Growth Experiments by Shake Flask

4.3.1 Experimental Conditions and Parameters of the Bacterial Growth

The growth of *Bacillus methanolicus* strain MGA3 in artificial seawater was performed in batch growth by using shake flasks, as described in section 4.3.2. In each shake flask experiment, the revivification of the frozen culture was performed as described in section 4.1, and a 2% vol/vol exponential-phase inoculum was transferred to the fresh media. The reaction culture volume was set to 50 ml using 250 ml triple-baffled shake flasks. All shake flask experiments were performed in an incubator (Innova 2100 Platform Shaker) with the speed of 200 rpm at 50°C and 1 atm.

4.3.1.1 Optical Density and Biomass Concentration

During the shake flask experiments, the optical density of the culture broth was measured by a UV/Visible spectrophotometer (Milton Roy Spectronics 1001 Plus) with the wavelength set at 500 nm. The optical density data were used as measures of biomass concentration by Equation 1.

$$\text{Dry biomass (g/L)} = m * OD_{500} \qquad \text{Equation 1}$$

where *m* is the slope of the plot for dry biomass versus optical density.

Since the same organism was being studied, the *m* value from Pluschkell's study [11] was adopted for the estimation of biomass concentration (i.e. $m = 0.176 \pm 0.004$). In addition, many studies [19, 20] have shown that optical density, in the range of 0.1 to 0.5, has a linear relationship with the cell mass concentration. Thus, all samples for the optical density measurements were diluted with de-ionized water to ensure the reading kept within this range. De-ionized water was used as the blank for the measurement.

4.3.1.2 Specific Growth Rate

With the use of the correlation in Equation 1, the specific growth rate in the exponential phase was determined by Equation 2.

$$\mu_{net} \equiv \frac{1}{X}\frac{dX}{dt} \qquad \text{Equation 2}$$

where X is biomass concentration (g/L), t is time (h), and μ_{net} is the specific growth rate (h^{-1}).

Plotting the optical density data to an integral of equation (2) gives the specific growth rate from the slope of the plot of the natural log of biomass concentration [ln (X)] against growth time (t).

4.3.1.3 Residual Methanol Concentration and the Growth Yield on Substrate

The residual concentration of the substrate, methanol, in the fermentation culture was an important parameter. The data were used to estimate the growth yield on substrate by Equation 3.

$$Y_{X/S} \equiv -\frac{\Delta X}{\Delta S} \qquad \text{Equation 3}$$

where S is the substrate concentration (g/L), and X is the biomass concentration (g/L).

The change of biomass concentration (ΔX) was determined by comparing the change of optical density during the fermentation. The relationship of optical density and the biomass concentration was described in section 4.3.1.1. The change of substrate concentration (ΔS) was determined by Equation 4.

$$\Delta S = \text{(Final methanol concentration – Initial methanol concentration)} + \text{(Methanol loss due to evaporation)} \quad \text{Equation 4}$$

In order to determine the methanol consumption only by the growth of the organism, the methanol loss due to evaporation should be considered. The loss was determined by placing a control flask next to the corresponding flask.

4.3.2 Stages of Shake Flask Experiments

4.3.2.1 Stage 1: Baseline Growth Profile in High Salinity Conditions

The goal of this stage was to determine the effect of high salinity to the growth of the organism. Four types of growth media were tested:

1) Minimal yeast medium,
2) MY medium with 25 g/L of sodium chloride,
3) Artificial seawater medium with 0.5 g/L yeast extract, and
4) Artificial seawater medium with 2.0 g/L yeast extract.

Each flask was supplemented with the required vitamins, trace metal solution, and methanol as described in sections 4.2.1 and 4.2.3. Each condition was repeated three times and there were 12 experimental runs in total. The effect of high salinity on the growth of

the organism was observed. The maximum OD and specific growth rate data were determined, and these data were compared to experiments with modification of the fermentation culture compositions.

4.3.2.2 Stage 2: Screening of Osmoprotectants

At this stage, the effect of osmoprotectants on the growth of the organism in artificial seawater medium was studied. Using a Plackett-Burman design of experiment (DOE), the effect of each chemical could be determined by relatively few experiments. However, the disadvantage of this type of DOE was that it was not able to distinguish the effect from the interaction of two factors or more.

The list of OP for the study was described in section 4.2.3.1. With the use of the statistical software Minitab 15, an 8-factor-12-run of Plackett-Burman DOE was created. With the addition of 4 center points and 3 replicates to the DOE, there were 48 runs of experiments in total. For those chemicals that showed a significant positive effect on the growth of the bacteria, a mixture DOE was performed on them to see if the growth of the bacteria could be further improved

4.3.2.3 Stage 3: Optimization of OP Compositions by Mixture DOE

Mixture DOE is a class of response surface experiments that investigate products containing several components that may have blending properties among them. In general, there are 3 different types of mixture DOE [32], as described in Table 12.

Table 12. Types of mixture DOE.

Type 1: Mixture only	It is the DOE that analyzes the relative proportions of the components only
Type 2: Mixture-process variable	It is the DOE that analyzes the relative proportions of the components and process variables. Process variables are factors that are not part of the mixture but may affect its blending properties.
Type 3: Mixture-amount	It is the DOE that analyzes the relative proportions of the components and the total amount of the mixture

Each type of these DOE's can also be further classified as simple lattice, simple centroid, and extreme vertices designs [32]. In this research, a mixture-amount DOE was performed, and the effect of osmoprotectants due to the amount of the mixture in addition to the blending properties of the components would be studied.

4.4 Determination of the Residual Methanol in Fermentation Cultures

4.4.1 Settings of the High Performance Liquid Chromatography System

An Agilent 1100 High Performance Liquid Chromatography (HPLC) system was used to analyze the residual methanol concentration in fermentation cultures. The column used in this research was the BioRad-Aminex HPX-87H Ion Exclusion Column 300mm x 7.8mm (Catalog # 125-0140), and the HPLC system was equipped with a Refractive Index (RI) detector (Waters 410 system). The RI detector identified the change of refractive index of the column eluent, which varied with its composition throughout the run time. The Agilent system quantified these signals and constructed a RI profile for each sample. In this research, the HPLC system was set at isocratic flow. The system settings are listed in Table 13.

Table 13. Settings of the Agilent 1100 HPLC system.

Component	Settings
column and RI detector temperature	50 °C
guard column	BioRad-Microguard Cation H refill (30mm x 4.6mm, Catalog # 125-0129)
mobile phase	0.01N H_2SO_4
maximum pressure	103 bars
mobile phase flow rate	0.6 ml / min
injection volume	20 µl
run time	30.0 min

4.4.2 Sample Preparation Procedures

Fermentation culture samples were taken at the beginning and periodically until the OD of the culture reached its maximum value. Since the samples were run in an acidic environment, proteins tended to precipitate out of the solution, and the guard column of the HPLC system would clog. To prevent this from happening, the following protein precipitation method was run before the samples were loaded onto the HPLC system:

1) Extract 1.5 ml of fermentation culture from the shake flasks.
2) Remove cell debris by centrifuging the sample with 13000 rpm for 5 minutes. The temperature of the centrifuge was set at 5 ^{o}C.
3) Extract 1mL of the fermentation supernatant, and add 30 µl of 70% perchloric acid.
4) Vortex the sample and hold it on ice for 10 minutes.
5) Centrifuge the sample again as described in step (2) with the same settings.
6) Transfer the sample supernatant to an HPLC vial and load the sample to the HPLC system.

CHAPTER FIVE
DATA AND ERROR ANALYSIS

5.1 Optical Density Measurements

The optical densities for all culture samples were measured according to section 4.3.1. All optical density measurements were performed three times at each sample point, and the average value of these data points and their standard errors were reported.

5.2 Linear Curve Fit Analysis

The specific growth rate (μ_{net}) of the bacteria was determined by plotting the natural log of biomass concentration [ln (X)] against growth time (t), according to Equation 2. During the exponential growth phase, the natural log of biomass concentration [ln(X)] had a linear relationship with the growth time, as described by Equation 5.

$$Y = mX + C \qquad \text{Equation 5}$$

Therefore, in order to determine the specific growth rate, the linear fitting of data from these two parameters was required. The specific growth rate and its standard error were determined by the regression analysis of Microsoft EXCEL. To avoid error, only

exponential phase data were chosen for the fitting. Bhattacharya *et al.* [33] found that if the interval for the maximum growth rate was misidentified, the entire experiment could be futile.

5.3 Error Analysis in Design of Experiments

5.3.1 Alpha-Level and Confidence Interval

In this research, the statistical software Minitab release 15 was used to design the DOE experiments, and analyze all data. During the DOE analysis, the alpha-level (α), also called significance level, was set at 0.10; which means the confidence interval of the DOE analysis was 90%. The alpha-level of 0.10 is a common choice in DOE practice. The alpha-level was also set at 0.05 for more developed processes, or at 0.01, resulting in confidence levels of 95% and 99%, respectively. The alpha-level was indicated with a red line in each DOE analysis. All responses were analyzed in standardized effect, which is also called the t-statistics of the analysis of variance (ANOVA).

5.3.2 Residual Plots

After obtaining a final model in DOE, three different types of residual plots were constructed:

1) Normal probability plot of residuals.
2) Plot of residuals versus the fitted values.
3) Plot of residuals versus the order of the data.

For each DOE analysis, these plots were reviewed to certify the following assumptions of the model error term:

1) The errors are normally distributed.
2) The error is the same for every design point. In other words, the error variance remains constant for different factor level combinations and the error variance does not change with the values of the predicted response.
3) The errors are independent. That means no correlation exists between the errors; each error is independent of all other errors.

CHAPTER SIX
RESULTS AND DISCUSSION

6.1 Initial Work on the Growth of the Organism

6.1.1 Technical Challenges during the Transfer of the Pre-Culture

At the early stage of this research project, there was a major technical problem regarding the growth of the bacteria. After the revivification of the frozen culture with TSB according to the procedure in section 4.1, the bacteria could not grow in fresh MY solution well. The experiments were repeated several times, and the maximum optical densities of the cultures in these experiments were all below 1.0, which indicated biomass produced was far below the values reported from the literature [10, 11, 15]. When the revivification procedure was performed with MY solution, the same phenomenon was observed again. While the maximum optical density of the bacteria could reach as high as 3.0 for the pre-culture, the growth could not be sustained after transferring to fresh MY solution with the same composition and the maximum optical density was also below 1.0. It was speculated that the residual glycerol, which was added to make frozen culture, might affect the growth of the bacteria. Therefore, instead of following procedures mentioned in section 4.3.2, several investigational experiments were performed. These investigations were described in section 6.1.2.

6.1.2 Investigations with Design of Experiments

6.1.2.1 Full Factorial DOE

A full factorial DOE was first performed to investigate and study if there could be any effect of different amounts of yeast extract, glycerol, and methanol on the growth of the bacteria in the MY medium. The temperature and the shaker speed were set to 50°C and 200 rpm respectively. The detailed experimental matrix is shown below in Table 14.

Table 14. A 3-factor-2-level factorial DOE with 2 center points.

Run Order	Methanol (ml / 50ml of culture)	Yeast Extract (0.25 g/L)	Glycerol (ml / 50ml of culture)
1	0.30	no	0.00
2	0.00	yes	0.30
3	0.15	no	0.15
4	0.30	no	0.30
5	0.30	yes	0.30
6	0.00	no	0.00
7	0.00	yes	0.00
8	0.30	yes	0.00
9	0.00	no	0.30
10	0.15	yes	0.15

The growth profiles are showed in Figure 4. The maximum optical density and the specific growth rate were the responses of this DOE. The data are shown in Table 15 and the DOE analyses for these two responses are shown in Figures 5 and 6. Residual plots of

these two responses are showed in Figures 7 and 8, showing that the assumptions mentioned in section 5.3.2 were valid.

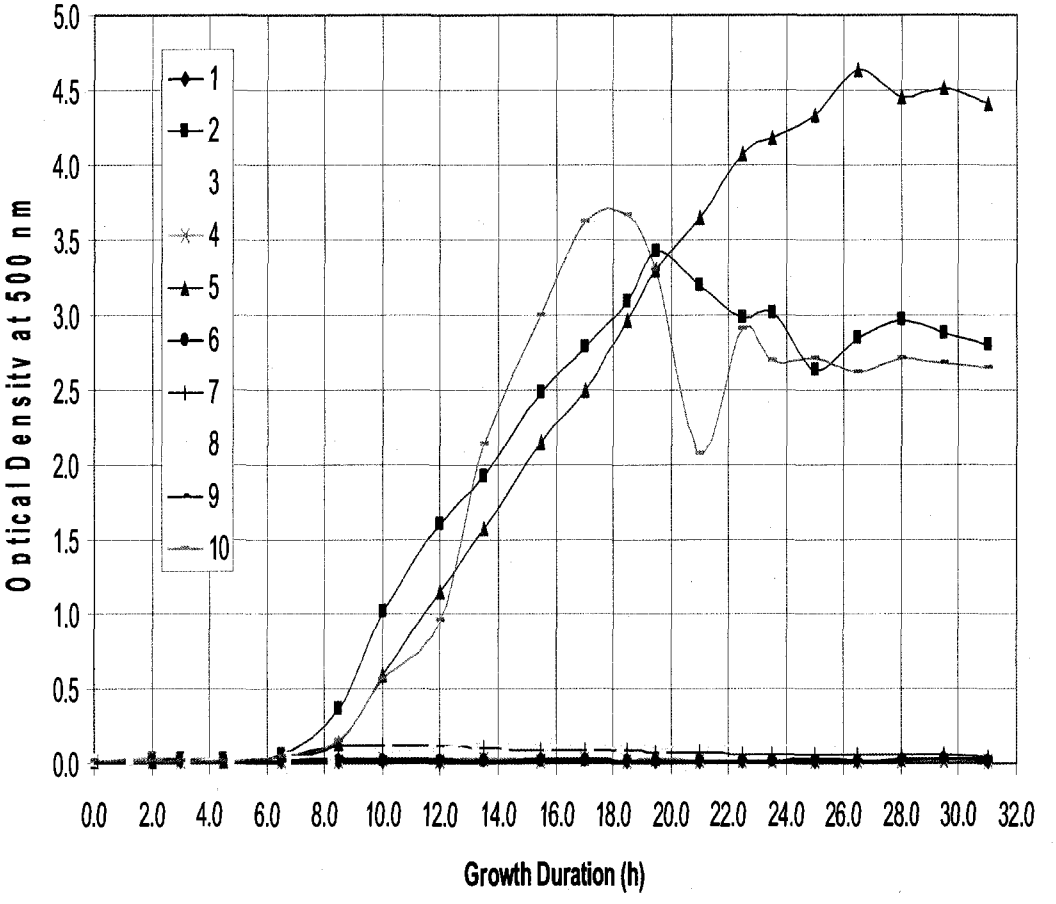

Figure 4. The growth profile of the full factorial DOE as per Table 14 criteria.

Table 15. Specific growth rate and maximum OD in the factorial DOE.

Run Order	Specific Growth Rate (h^{-1})	Standard Error of Specific Growth Rate (h^{-1})	Maximum OD	Standard Error of Maximum OD
1	0.3466	0.0379	0.027	0.0010
2	0.1206	0.0060	3.430	0.0251
3	0.3270	0.0464	0.029	0.0015
4	0.4377	0.0355	0.026	0.0015
5	0.1125	0.0048	4.630	0.0379
6	0.5148	0.0622	0.019	0.0010
7	0.4024	0.0105	0.124	0.0046
8	0.2644	0.0203	0.131	0.0035
9	0.2648	0.0287	0.027	0.0012
10	0.3590	0.0270	3.670	0.0115

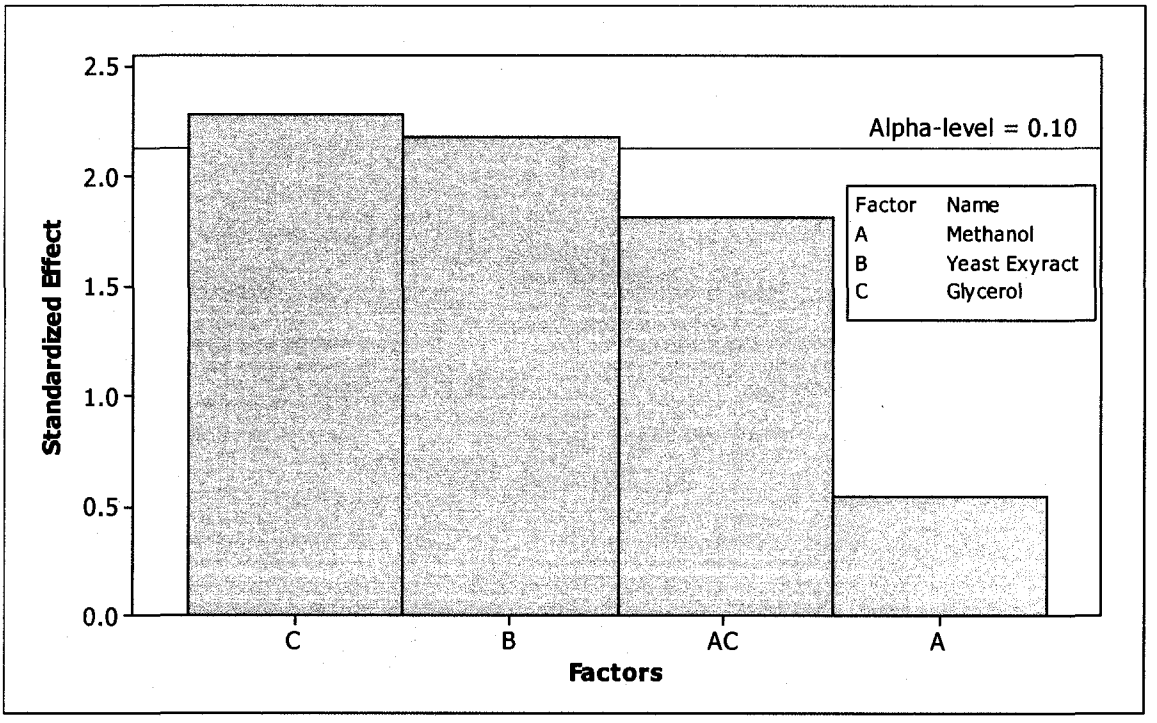

Figure 5. Analysis of the specific growth rate in the full factorial DOE.

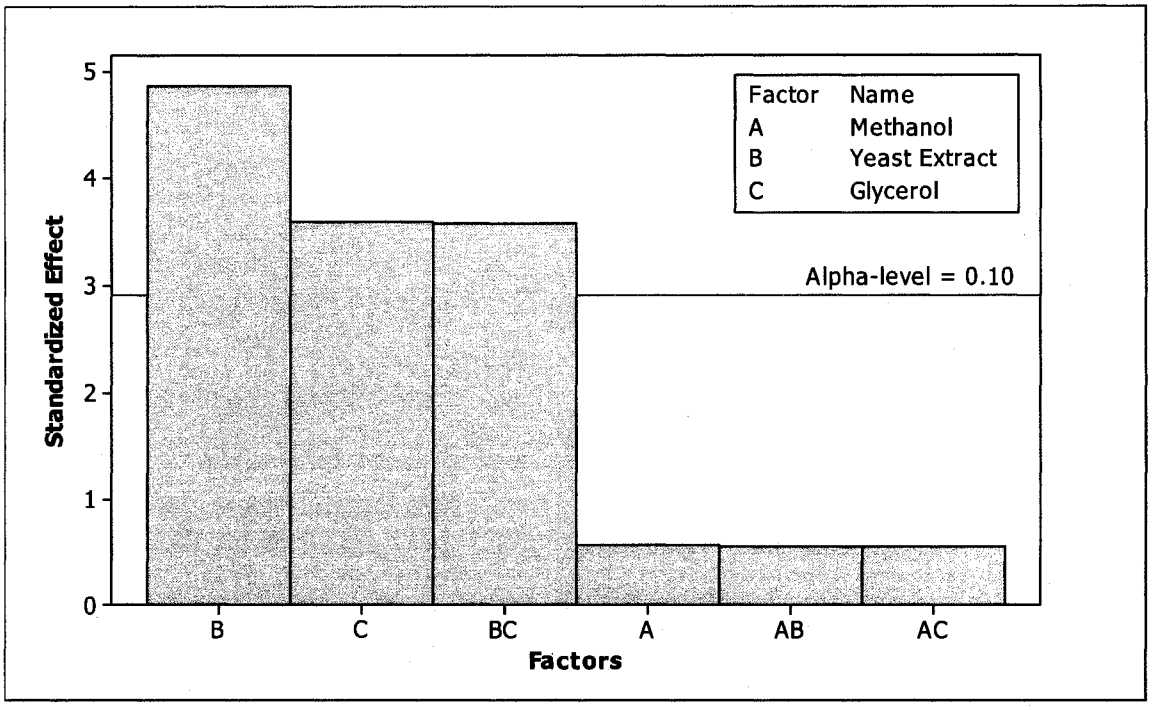

Figure 6. Analysis of the maximum OD in the full factorial DOE.

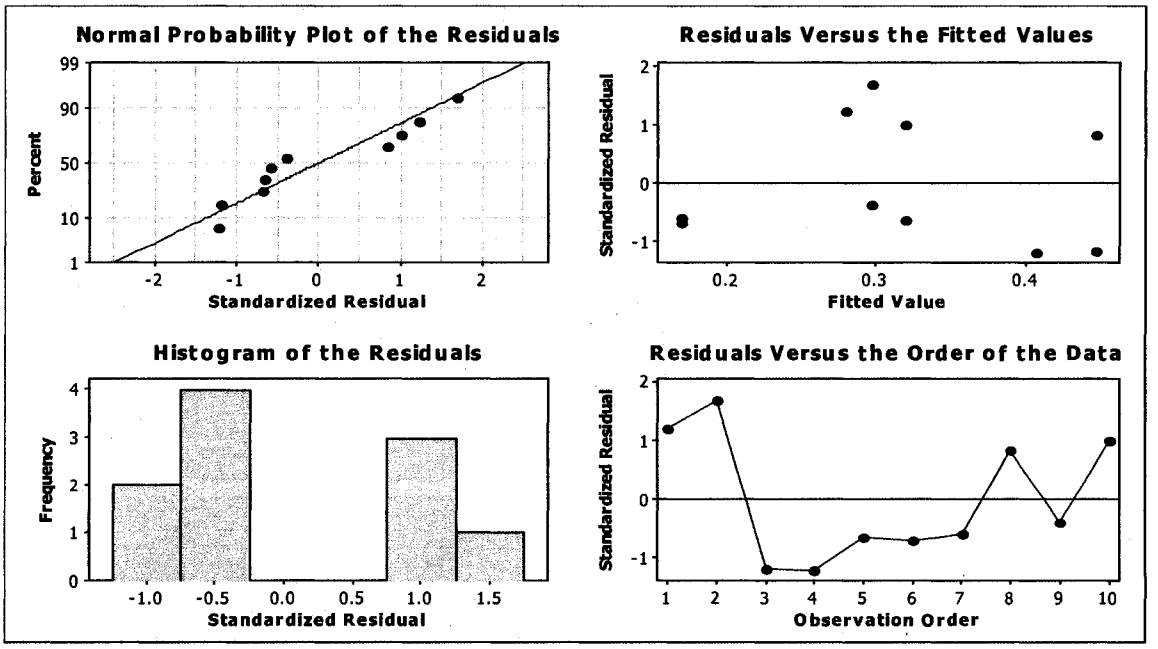

Figure 7. Residual plots for the specific growth rate in the full factorial DOE.

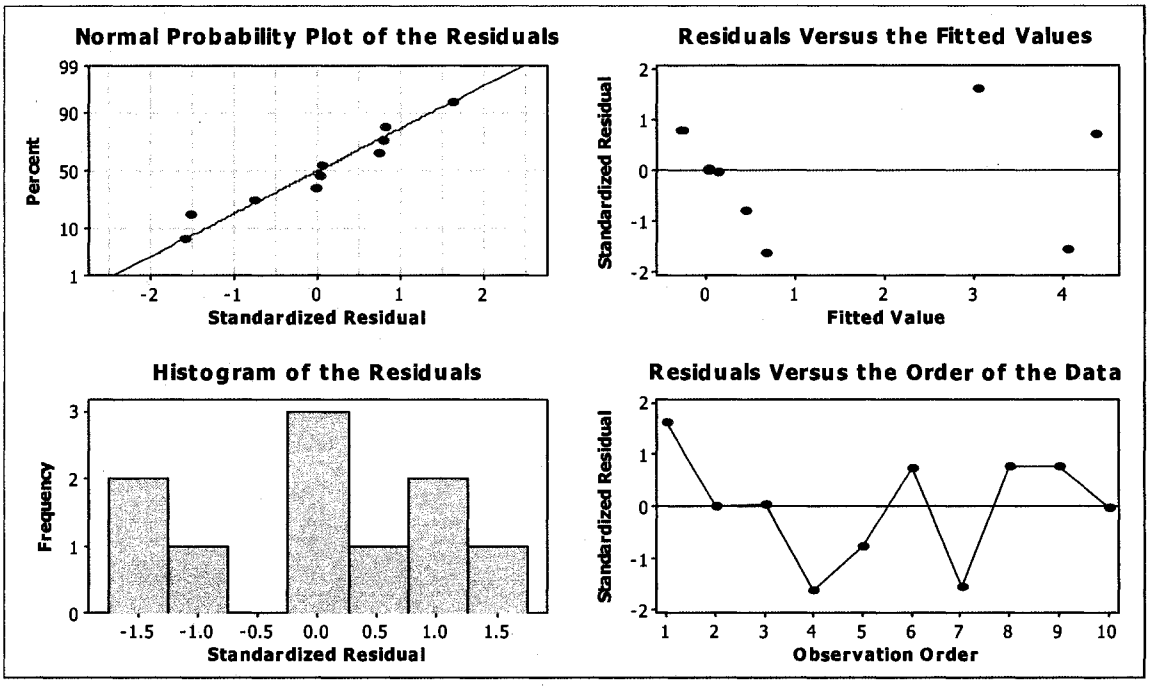

Figure 8. Residual plots for the maximum OD in the full factorial DOE.

The analysis shows that methanol did not have statistical significance with regard to either the specific growth rate or the maximum OD. According to Figures 5 and 6, yeast extract and glycerol were the only components that affected both the specific growth rate and the maximum OD in a statistically significant way. In addition, in the analysis of the maximum OD in Figure 6, there was a significant interaction effect between the glycerol and the yeast extract on the growth of the bacteria investigated. It also indicated that glycerol, with the presence of yeast extract, could act as a carbon source for the growth of the bacteria.

6.1.2.2 Mixture DOE

After the analysis of the factorial DOE, a mixture DOE was performed to further investigate the blending effect on these three components, and the impact of changing the total amount of carbon sources. An extreme vertices design of mixture DOE was performed with two different amounts of carbon sources: 0.089 g and 0.340 g. The experimental conditions were the same as the previous factorial DOE in section 6.1.2.1 (i.e. 50 °C and 200 rpm). The detailed experimental plan is shown in Table 16, and the graphical representation of this DOE design is shown in Figure 9.

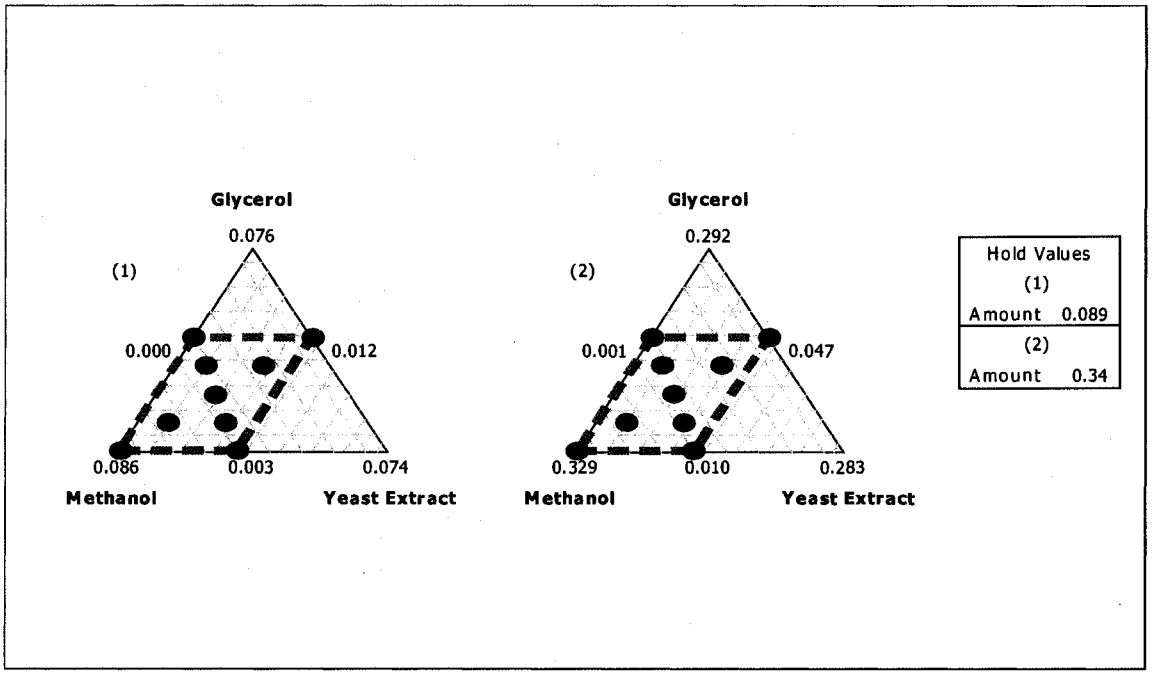

Figure 9. Graphical representation of the extreme vertices mixture DOE. The amounts of carbon sources are in grams.

Table 16. An extreme vertices mixture DOE with two different amounts of carbon sources.

Run Order	Glycerol (g)	Methanol (g)	Yeast Extract (g)	Total Amount of Carbon Sources (g)
1	0.129	0.117	0.094	0.340
2	0.013	0.068	0.008	0.089
3	0.013	0.051	0.025	0.089
4	0.044	0.045	0.000	0.089
5	0.003	0.086	0.000	0.089
6	0.010	0.205	0.125	0.340
7	0.034	0.047	0.008	0.089
8	0.050	0.258	0.032	0.340
9	0.129	0.179	0.032	0.340
10	0.003	0.054	0.033	0.089
11	0.168	0.171	0.001	0.340
12	0.089	0.188	0.063	0.340
13	0.034	0.031	0.025	0.089
14	0.168	0.047	0.125	0.340
15	0.010	0.329	0.001	0.340
16	0.050	0.196	0.094	0.340
17	0.044	0.012	0.033	0.089
18	0.023	0.049	0.017	0.089

The growth profile is shown in Figure 10. The maximum OD and the specific growth rate were responses of this DOE. The data are shown in Table 17, and the DOE analyses for these two responses are shown in Figures 11 and 12.

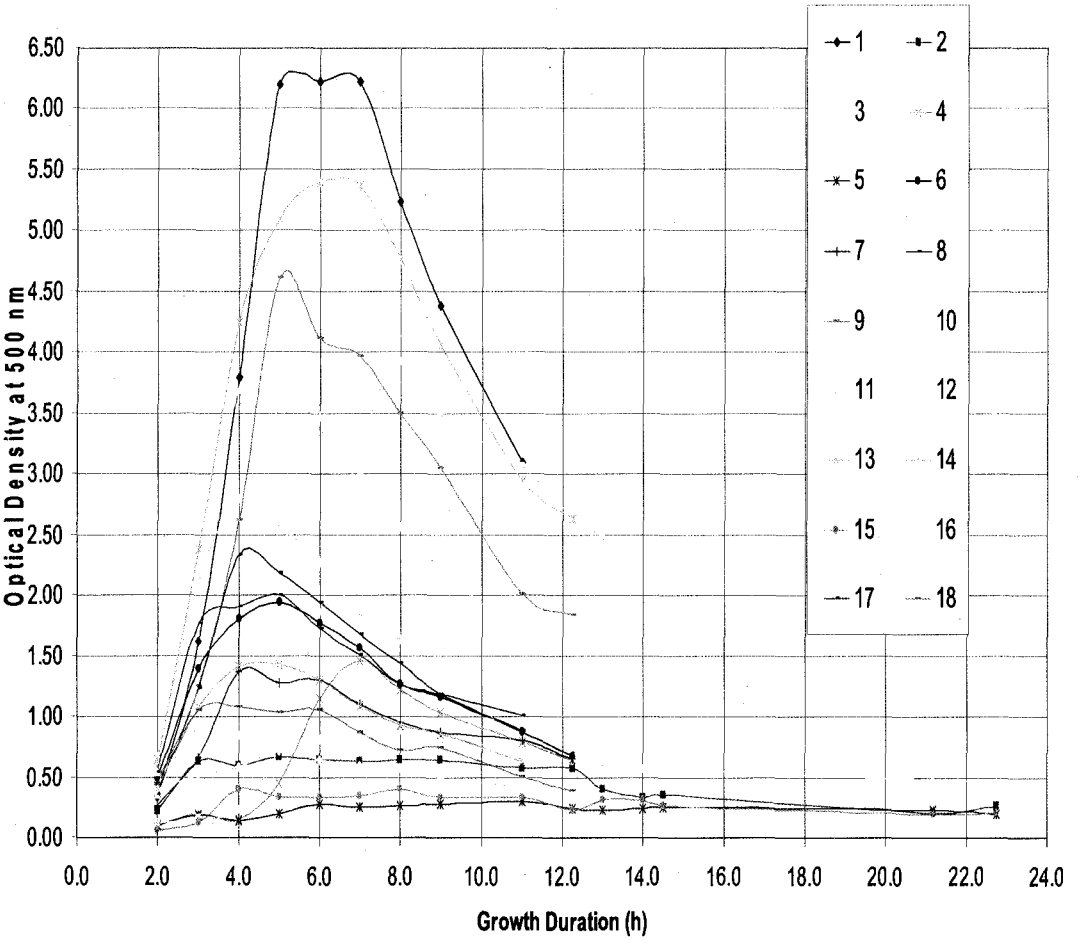

Figure 10. The growth profile of the mixture DOE as per Table 16 criteria.

Table 17. Specific growth rate and maximum OD in the mixture DOE.

Run Order	Specific Growth Rate (h^{-1})	Standard Error of Specific Growth Rate (h^{-1})	Maximum OD	Standard Error of Maximum OD
1	0.9652	0.0791	6.22	0.0400
2	1.0521	0.0263	0.67	0.0058
3	0.9491	0.0193	0.93	0.0100
4	0.9383	0.0226	1.46	0.0153
5	0.5878	0.0321	0.28	0.0000
6	1.0225	0.0159	1.94	0.0100
7	0.8309	0.0260	1.37	0.0100
8	0.9359	0.0651	2.34	0.0458
9	0.6525	0.0197	4.61	0.0306
10	0.1848	0.0111	0.71	0.0115
11	0.8835	0.0450	3.92	0.0611
12	0.9623	0.0693	3.7	0.0872
13	0.9933	0.0203	1.43	0.0153
14	0.9466	0.0803	5.36	0.0917
15	1.1239	0.0786	0.40	0.0100
16	1.1519	0.0131	2.79	0.0757
17	1.1872	0.0225	2.00	0.0625
18	0.8792	0.0398	1.08	0.0321

Figure 11 indicates the specific growth rate profile at different levels of carbon source tested. At the low carbon source level (0.089 g), the specific growth rate was favorable with a high portion of glycerol, a medium amount of yeast extract, and less methanol, specifically, 49.4% glycerol, 37.1% yeast extract, and 13.5% methanol. On the other hand, at the high carbon source level (0.34g), the specific growth rate was more favorable with a high portion of methanol, and less glycerol and yeast extract, specifically,

14.7 % glycerol, 27.6 % yeast extract, and 57.7 % methanol. Overall, the total amount of carbon sources did not have significant impact on the specific growth rate.

Figure 12 indicates that the organism could achieve a higher OD when the proportions of both methanol and yeast extract were low. This was observed at both low and high amounts of carbon sources. However, much higher OD values were observed at the high amount of carbon sources than at the low level.

Residual plots of these two responses are attached in Figures 13 and 14, showing that the assumptions mentioned in section 5.3.2 held in this experiment.

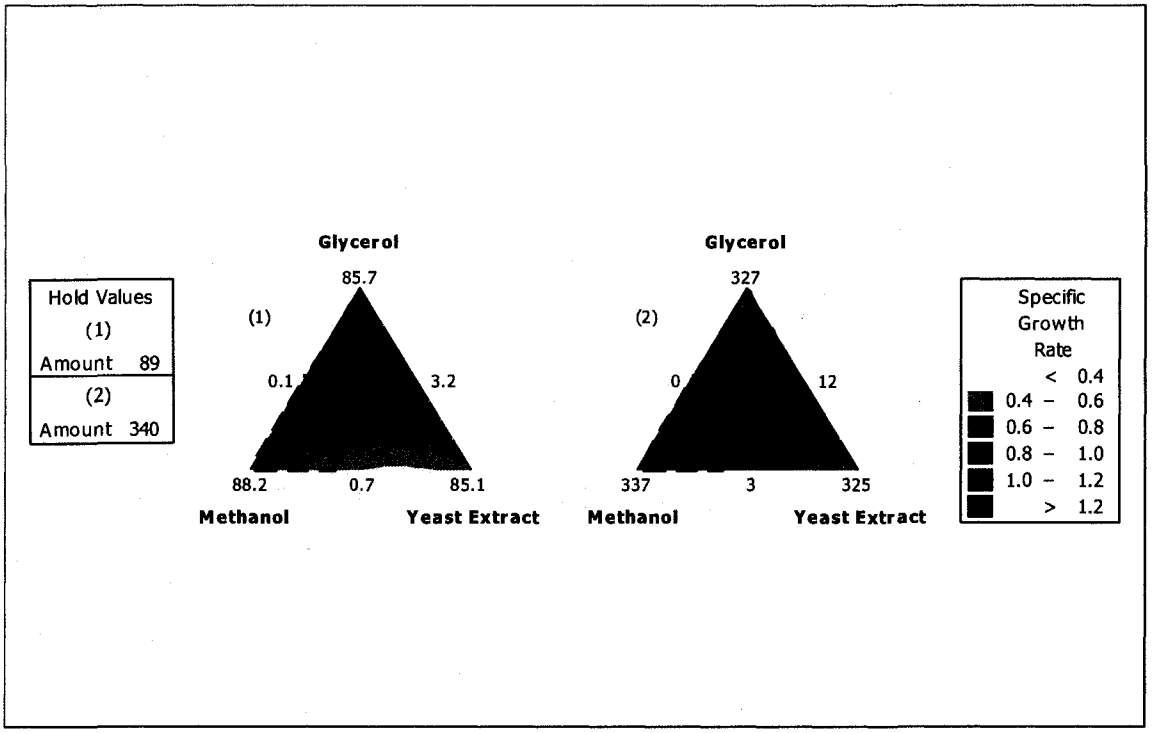

Figure 11. Analysis of the specific growth rate in the mixture DOE. The amounts of carbon sources are in mini-grams.

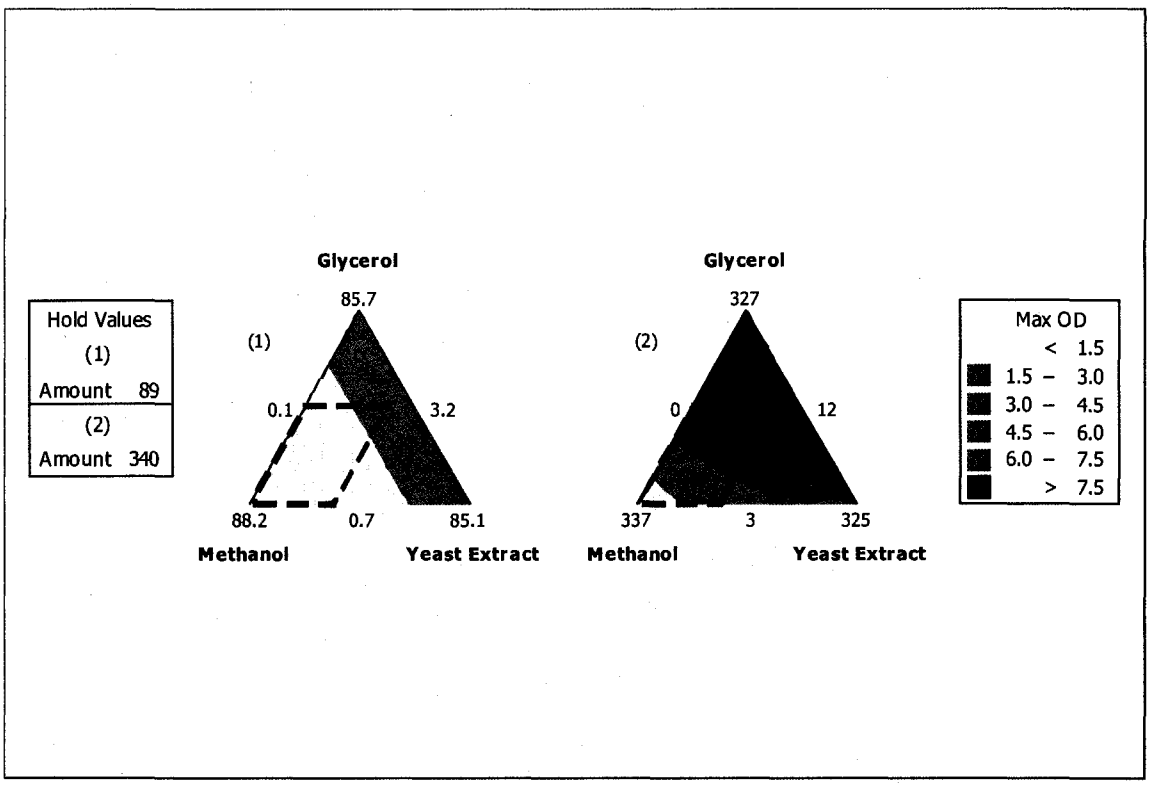

Figure 12. Analysis of the maximum OD in the mixture DOE. The amounts of carbon sources are in mini-grams.

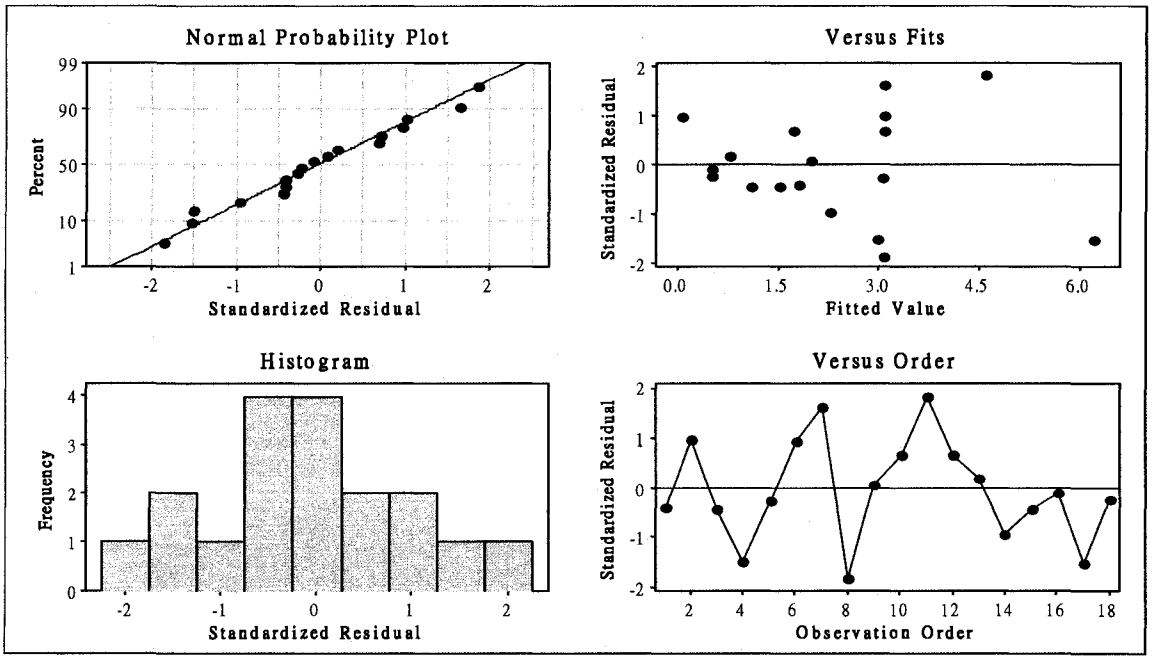

Figure 13. Residual plots for the maximum OD in the mixture DOE.

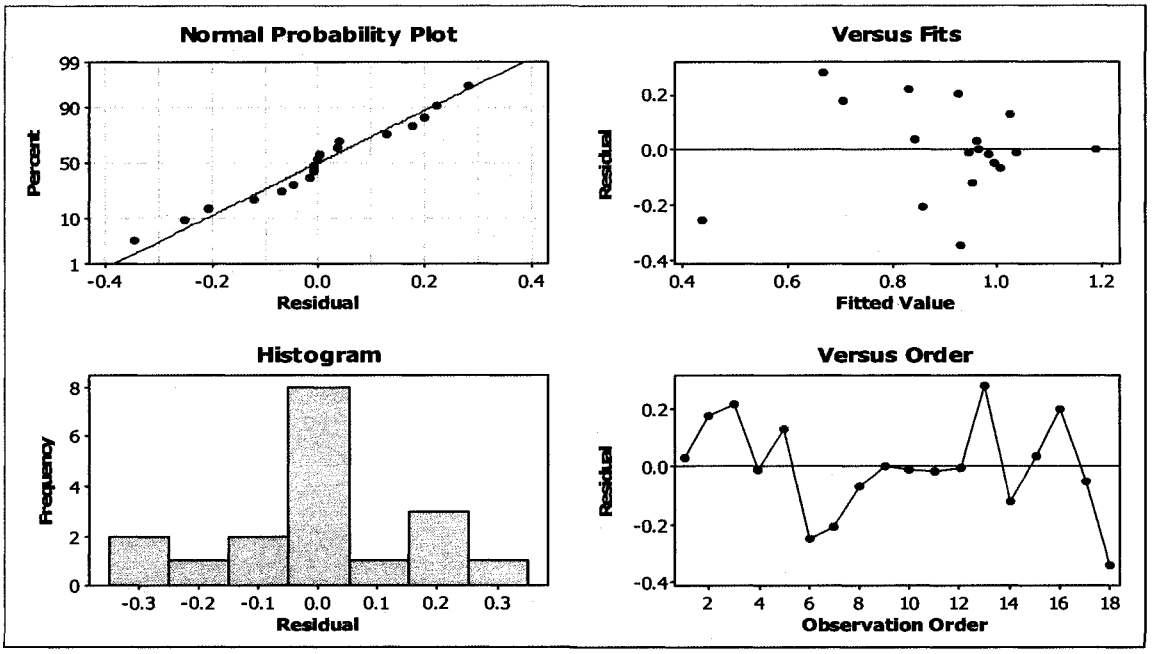

Figure 14. Residual plots for the specific growth rate in the mixture DOE.

6.1.3 Microbial Analysis of the Bacteria

The two DOE analyses in section 6.1.2 indicated that glycerol had an important role in the growth of the organism, which was in contradiction to one previous literature reference. According to the study performed by Schendel *et al.* in 1990 [15], glycerol was not utilized when *Bacillus methanolicus* strain MGA3 was grown in the MY medium, which was essentially the same type of medium used in this study. In order to confirm the identity of the bacteria used, a MY agar plate that contained the bacteria colonies was sent to an outside laboratory, Microbial Insights Inc., to analyze the 16sRNA sequences of the bacteria.

Based on the DNA sequences in the Ribosomal Database Project, the sample had a similarity index = 0.956 to the bacteria called *Bacillus licheniformis*. The similarity index was above 0.900, and it suggests that the organism grown and stored in the flasks was not *B. methanolicus*. The actual 16sRNA sequence obtained from Microbial Insights is shown in Figure 15. This result indicates that the bacteria frozen culture stored at San Jose State University Engineering Lab 109 was contaminated with *Bacillus licheniformis*. As a result, all the experiments performed from section 6.1.1 to 6.1.2 were the fermentation of *B. licheniformis* in MY medium, not *B. methanolicus.*

AGAGTTTGATCCTGGCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAA
GTCGAGCGGACCGACGGGAGCTTGCTCCCTTAGGTCAGCGGCGGACGGGTGA
GTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGG
GCTAATACCGGATGCTTGTTTGAACCGCATGGTTCAAACATAAAAGGTGGCTTT
TCGCTACCACTTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGGGGTAAC
GGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACAC
TGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTT
CCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTT
TCGGATCGTAAAACTCTGTTGTTAGGGAAGAACAAGTACCGTTCGAACAGGG
CGGTACCTGACGGTACCTACCAGAA

Figure 15. The 16sRNA sequence of the bacteria sample.

6.2 New Stock of *Bacillus methanolicus* Strain MGA3

6.2.1 The Growth of the New Stock in MY Medium

A new stock of *Bacillus methanolicus* strain MGA3 was sent from North Carolina State University (Professor Michael Flickinger). It had been stored as a spore suspension solution that could be maintained between 4 ^{0}C and room temperature. Upon the arrival of the spore suspension solution, it was revived by following the standard protocol described in the section 4.1.1, and frozen cultures were made at the exponential phase of the pre-culture. In addition, the growth of the bacteria in fresh MY solution as described in section 4.2.1 was also verified with two subsequent transfers of the inocula after each culture reached exponential phase. The experimental matrix is shown in Table 18.

Table 18. Experimental matrix of the growth of the new stock in MY medium.

Flask Number	Description
S	pre-culture
1	first transfer of inoculums
2	second transfer of inoculums

As shown in Figure 16 and Table 19, the specific growth rate of the fresh bacterial culture was about 0.4 h^{-1}, and the maximum OD was more than 2.7 for all flasks. This was consistent with the growth pattern reported by literature references [11, 19, 20]. Therefore,

the frozen cultures that were made from the exponential phase flask S culture would be used to execute experimental plans outlined in section 4.3.2.

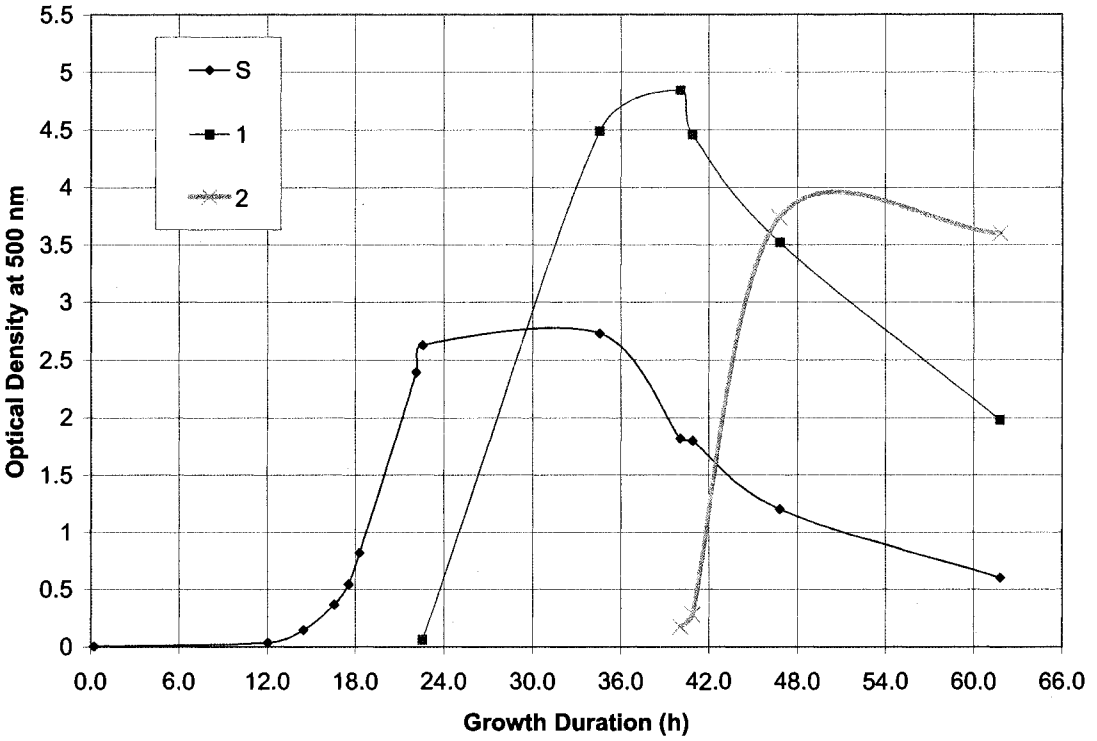

Figure 16. The growth profile of the new stock of *Bacillus methanolicus* strain MGA3 in MY medium.

Table 19. Specific growth rate and maximum OD in the growth of the new stock in MY medium.

Run Order	Specific Growth Rate (h^{-1})	Standard Error of Specific Growth Rate (h^{-1})	Maximum OD	Standard Error of Maximum OD
S	0.4898	0.0204	2.73	0.0404
1	0.3604	0.0081	4.84	0.0416
2	0.4456	0.0051	3.74	0.0252

6.2.2 The Growth Profile of the *B. methanolicus* Strain MGA3 in High Salt Conditions

The effect of high salinity for the growth of *B. methanolicus* strain MGA3 was studied according to the plan described in section 4.3.2.1, and the detailed experimental matrix is listed in Table 20. The experimental conditions were set according to section 4.3.1. The change of optical density over time is shown in Figure 17. The maximum OD and the specific growth rate are listed in Table 21.

Table 20. Experimental matrix of the growth in high salt conditions.

Run Order	Medium
1	minimal yeast medium (MY-1)
2	high salt MY (HM-1)
3	artificial seawater medium (AS-1)
4	artificial seawater medium with 2.0 g/L yeast extract (ASY-1)
5	high salt MY(HM-2)
6	minimal yeast medium (MY-2)
7	artificial seawater medium (AS-2)
8	artificial seawater medium with 2.0 g/L yeast extract (ASY-2)
9	artificial seawater medium (AS-3)
10	high salt MY (HM-3)
11	minimal yeast medium (MY-3)
12	artificial seawater medium with 2.0 g/L yeast extract (ASY-3)

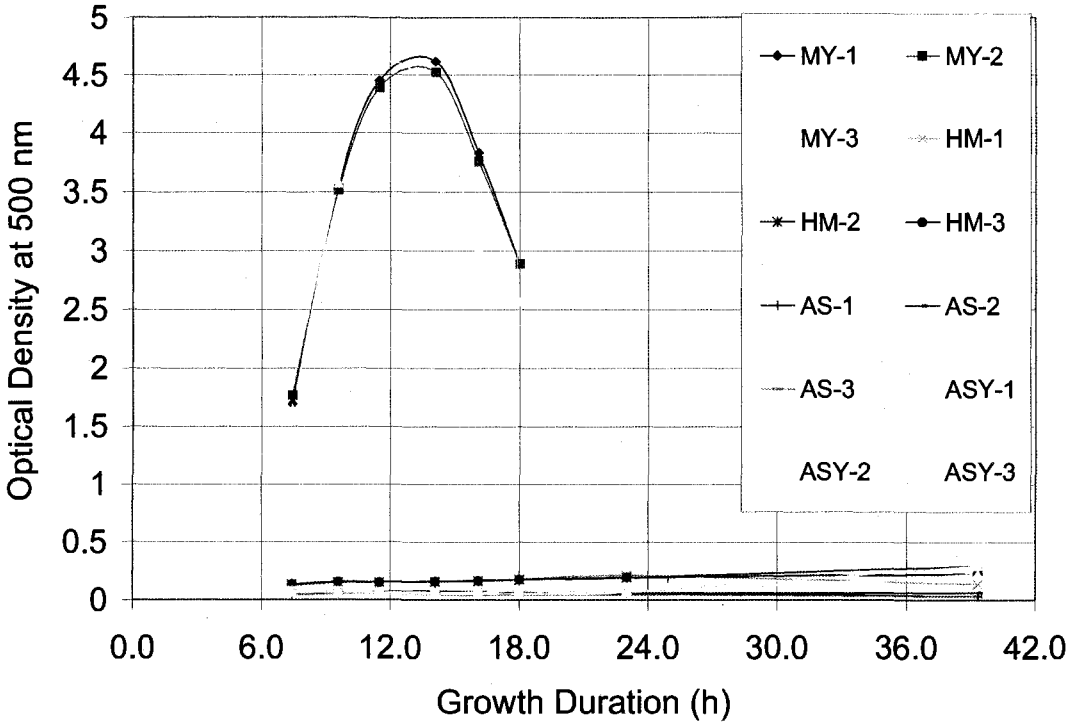

Figure 17. The growth profile of *Bacillus methanolicus* strain MGA3 as per criteria in Table 20.

Table 21. Maximum OD and specific growth rate in the growth profile experiments.

Run Order	Maximum OD	Standard Error of Maximum OD	Specific Growth Rate (h^{-1})	Standard Error of Specific Growth Rate (h^{-1})
1	4.62	0.0231	0.3427	0.0129
2	0.214	0.0042	0.0306	0.0026
3	0.071	0.0021	0.0441	0.0150
4	0.208	0.0010	0.0575	0.0058
5	0.295	0.0000	0.0234	0.0010
6	4.52	0.0153	0.3173	0.0052
7	0.08	0.0012	0.0297	0.0039
8	0.295	0.0030	0.0626	0.0081
9	0.062	0.0006	0.0632	0.0151
10	0.226	0.0021	0.0216	0.0025
11	4.58	0.0153	0.3561	0.0063
12	0.226	0.0017	0.0660	0.0072

All flasks with fresh MY medium (MY1 to MY3) had maximum ODs of about 4.5, and specific growth rates were around 0.31 to 0.35 h^{-1}. These data were consistent with the result of flask 1 in section 6.2.1. However, cultures in high salt MY medium (HM1 to HM3) and in high yeast artificial seawater medium (ASY1 to ASY3) showed little growth. The maximum ODs of all these flasks were less than 0.3 after an excessive inoculation time of more than 24 hours. Flasks containing artificial seawater medium with 0.5 g/L yeast extract (AS1 to AS3) had very poor growth. The maximum OD was less than 0.1, and specific growth rate was around 0.02 to 0.06 h^{-1}. Results from all these high salt conditions showed biomass generation significantly less than reported in the literature [11, 19, 20].

6.3 The Growth of the High Salt-Adapted Organism

6.3.1 The Adaptation Experiment with Stepwise Increment of Artificial Seawater

Results in Section 6.2.2 showed that the growth of *Bacillus methanolicus* strain MGA3 in artificial seawater medium was not favored, and it was doubtful if the addition of osmoprotectants could improve the growth of the organism in artificial seawater medium to a level comparable to that in the MY medium. Therefore, before testing the growth of the organism in the presence of any osmprotectant, the organism was put through a step-by-step adaptation process. It was the same strategy adopted by Cheung [20];

however, the growth medium used for the adaptation was modified. The frozen culture of the organism was first grown in MY medium. After 12 hours of incubation, it was transferred to a medium with 80% MY and 20% artificial seawater medium. The bacteria were subsequently transferred into fresh medium with a higher portion (i.e. 20% more) of artificial seawater medium after each 12-hour incubation period. Each flask had 2.0 g/L yeast extract and 98.9 mM initial methanol concentration. The amount of trace metals and vitamins added were same as described in section 4.2.1. At the end of the 12-hour incubation of the flask with 100% artificial seawater medium, frozen cultures were collected and used for experiments described in section 4.3.2.2. All six flasks had ODs more than 2.0 at the end of the 12-hour incubation time, and it was highly possible that the addition of OPs could improve the growth of the organism to a level that was comparable to the value in minimal yeast medium.

6.3.2 Screening DOE of Osmoprotectants

After the adaptation experiment, frozen cultures of the high salt-adapted bacteria were grown with the addition of OPs. In order to determine which OP had a significant effect on the growth of the organism, a Plackett-Burman DOE was performed for the screening. As mentioned in section 4.3.2.2, there were 48 experimental runs. The detailed

experimental plan is attached in Appendix A, and the optical density profiles are attached in Appendix B. The maximum OD and specific growth rate of the organism in each flask are documented in Tables C1 to C3 in Appendix C. These data were used to analyze the impact of the OPs. Figures 18 and 19 indicate the effect of OPs on the maximum OD as the response, and Figures 20 and 21 indicate the effect on the specific growth rate.

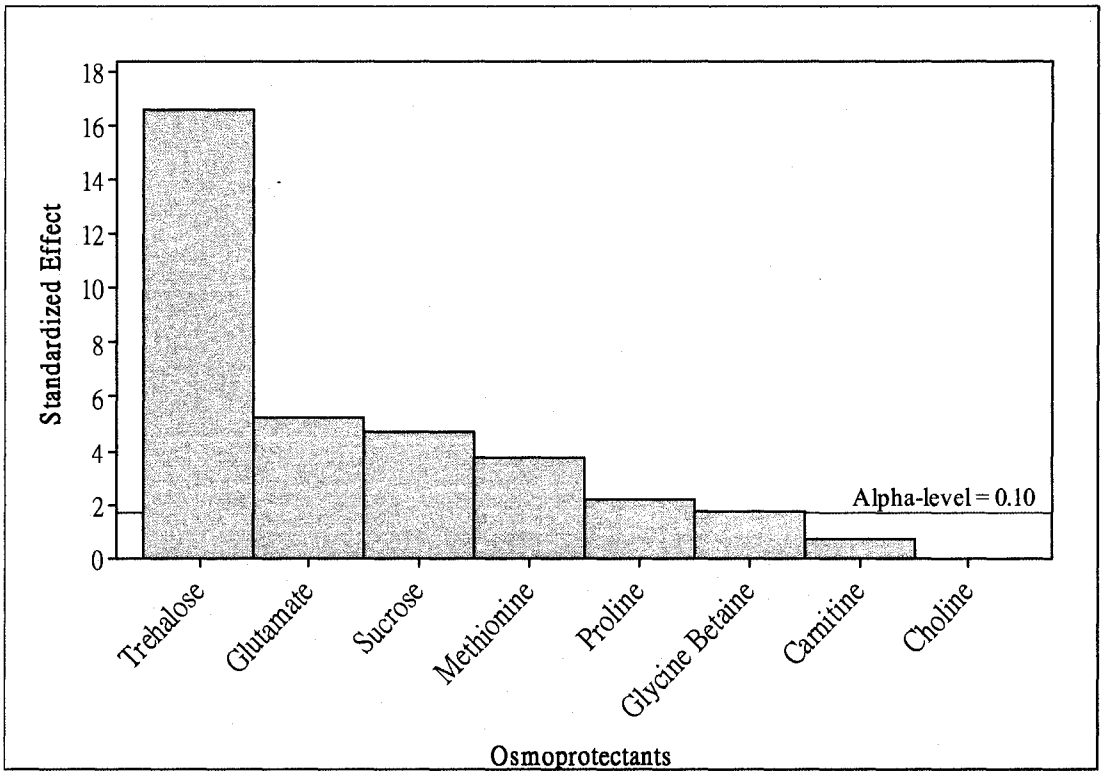

Figure 18. Analysis of the maximum OD in the screening DOE.

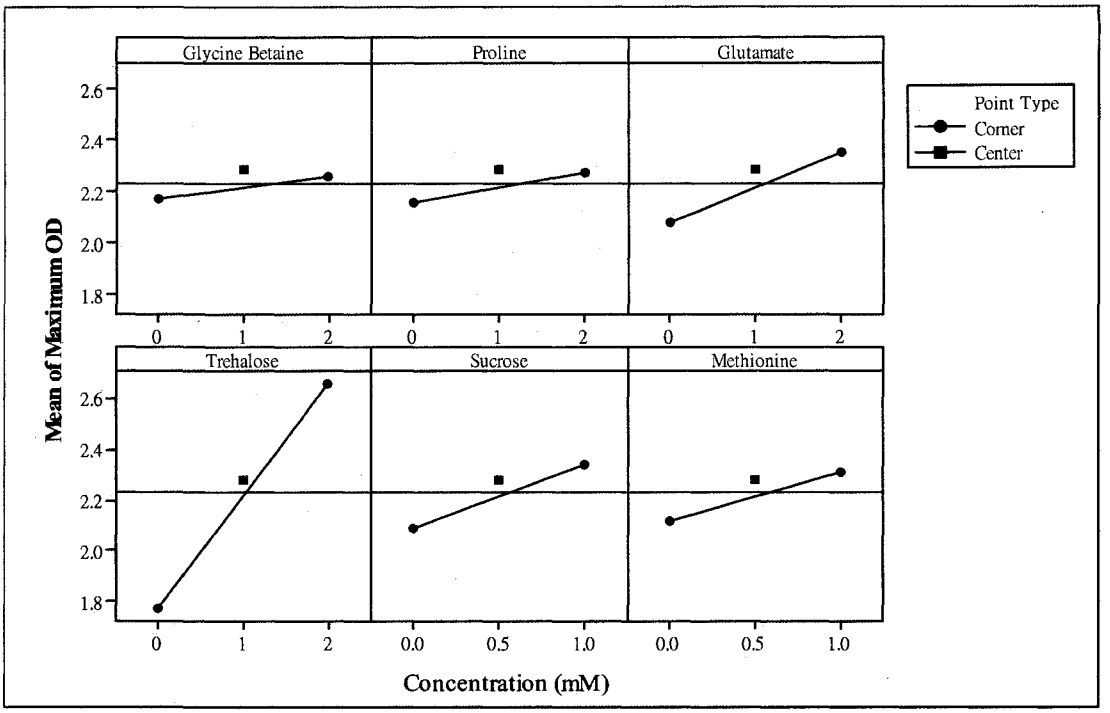

Figure 19. Main effects plot for maximum OD in the screening DOE.

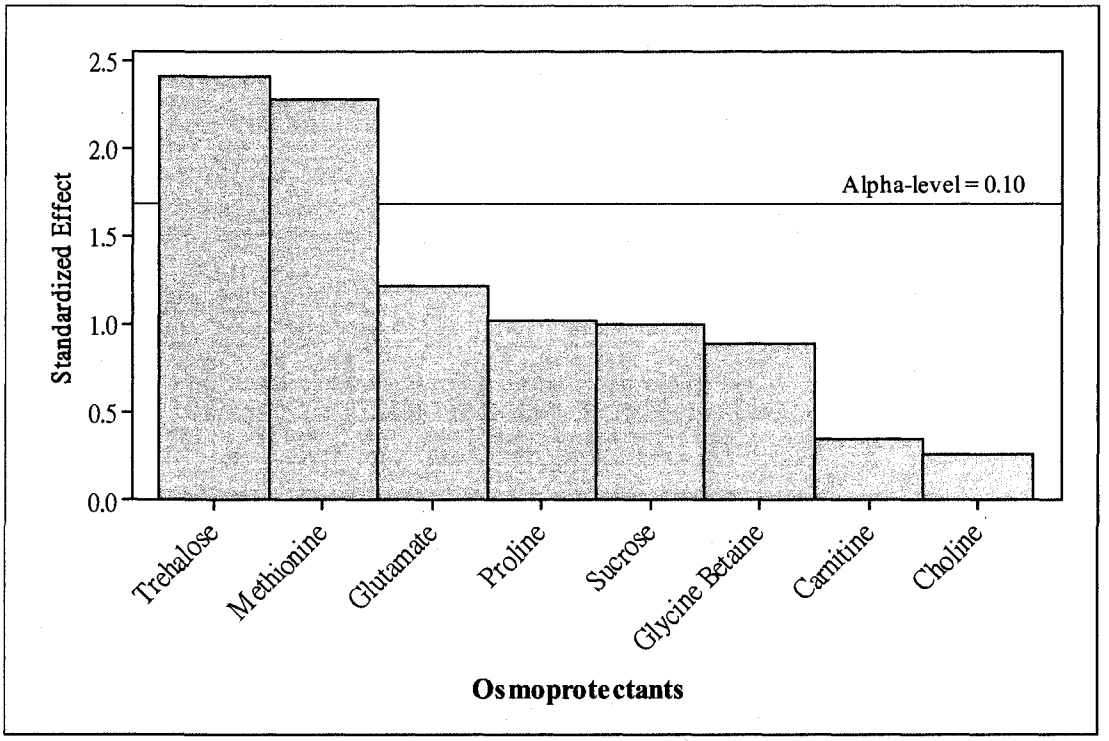

Figure 20. Analysis of the specific growth rate in the screening DOE.

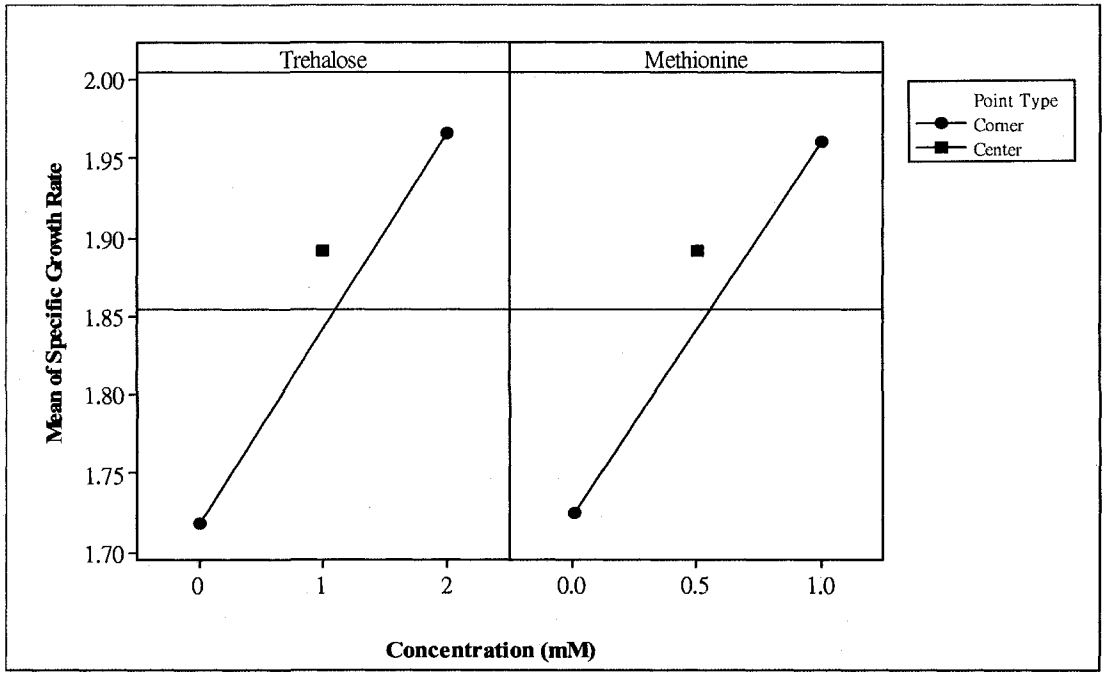

Figure 21. Main effect plot for the specific growth rate in the screening DOE.

As indicated in Figure 18, six of the osmoprotectants had a significant effect on the maximum OD. The main effects plot in Figure 19 indicates that all of these six chemicals had a positive effect on the maximum OD of the organism. Among these chemicals, trehalose had a much stronger effect than the rest. In the analysis of the specific growth rate in Figure 20, only trehalose and methionine had a statistically significant effect. The main effects plot in Figure 21 shows that both of these two chemicals had positive effects with regard to the specific growth rate of the organism. Based on these results, it was concluded that adding trehalose and/or methionine should significantly improve the growth of the high salt-adapted organism in artificial seawater medium.

6.4 Determination of Methanol Yield for the Growth of the Organism

6.4.1 HPLC Calibration Curves for Different Growth Media

In the late stage of this research, it was found that an Agilent 1100 HPLC system could be used to determine the residual methanol concentration of the fermentation culture. By using settings described in section 4.4.1, it was determined that a methanol peak consistently eluted at about 20.2 min in the refractive index profile of the HPLC analysis. The calibration curves of each growth medium are shown in Figures D1 to D5 in Appendix D.

6.4.2 The Substrate Yield of the High Salt-Adapted Organism in Artificial Seawater Medium with the Presence of 2mM Trehalose and Methionine

With the HPLC system, the methanol yield by the high salt-adapted organism obtained in section 6.3.1 could be determined. The organism was grown in artificial seawater with the addition of trehalose and methionine. The concentration of each osmoprotectant was set to 2mM. The organism was grown with 2.0 g/L yeast extract, and two different initial methanol concentrations: 4 and 20 ml/L. Each flask was accompanied by a control flask to determine the methanol loss due to evaporation.

The growth profile is shown in Figure 22, and the maximum optical density values are documented in Table 22. These results indicated that growth was not affected by the initial methanol concentration. In addition, the analysis of fermentation samples by HPLC revealed that the substrate yield of the flasks with initial methanol concentrations of 4 and 20 ml/L were 2.35 and 1.03 g cells / g methanol consumed, respectively. These yield values were different than any value report in the literature. Pluschkell's study [11] showed a yield value of 0.5 g/g, and Wang's thesis [19] showed a value of 0.46 g/g from the fermentation of *B. methanolicus* in minimal yeast medium with 0.5 g/L yeast extract and 4 ml/L initial methanol concentration. Cheung's thesis [20] generated a yield value of 0.24 g/g for growth of the high salt-adapted *B. methanolicus* strain PB1 with 2 g/L yeast extract and 10 ml/L initial methanol concentration. The yield values in this experiment suggested that methanol was not the primary carbon source in the fermentation, and that the adapted strain had consumed carbon sources other than methanol. The detailed calculation of the substrate yield is documented in Appendix E.

Table 22. Maximum optical density, substrate yield, and specific growth rate for the growth of the high salt-adapted organism in artificial seawater medium with 2mM trehalose and methionine.

Initial Methanol Concentration (ml/L)	Maximum OD	Standard Error of Maximum OD	Substrate Yield (g cell / g methanol)	Specific Growth Rate (h^{-1})	Standard Error of Specific Growth Rate (h^{-1})
4	3.28	0.0473	2.35	1.5766	0.0127
20	3.29	0.0231	1.03	1.6003	0.0065

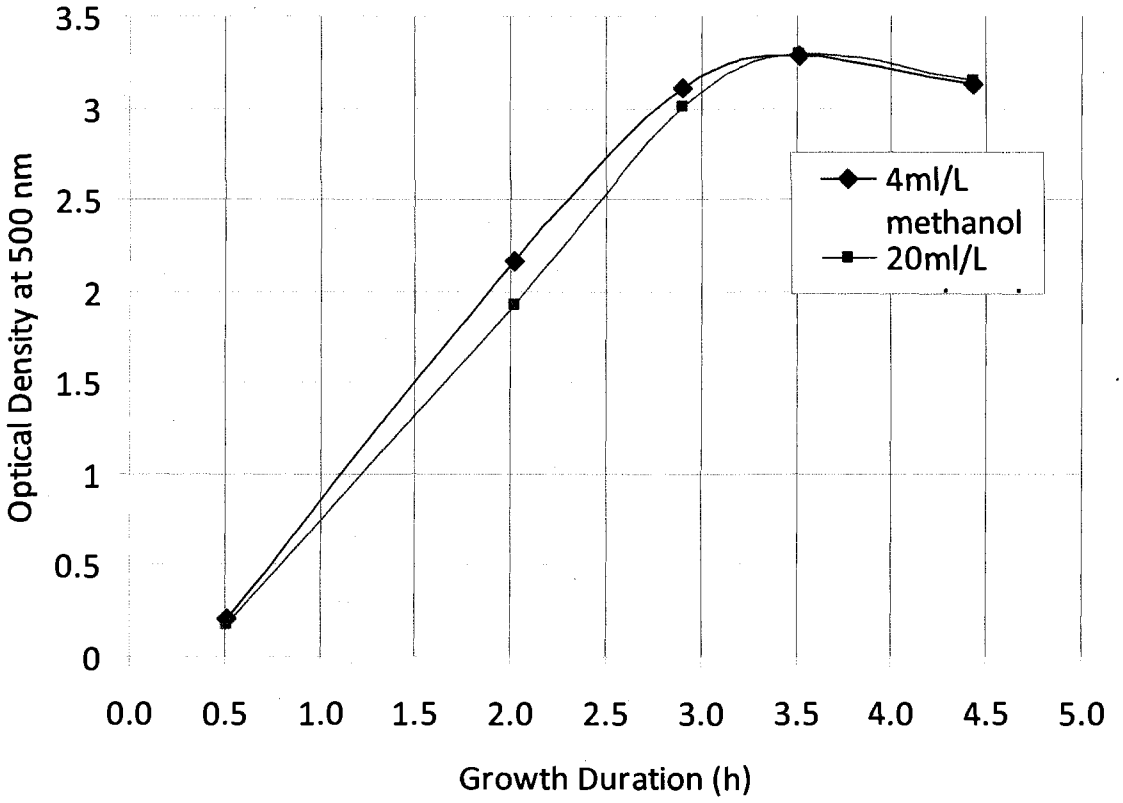

Figure 22. Growth profile of the high salt-adapted organism in artificial seawater medium with 2mM trehalose and methionine.

6.4.3 The Methanol Yield of the High Salt-Adapted Organism and the Non-Adapted *B. methanolicus* Strain MGA3 in Minimal Yeast Medium

Bacillus methanolicus is a methylotrophic bacterium. It can utilize methanol as a carbon and energy source. Results from section 6.4.2 indicated that the high salt-adapted organism behaved differently from this important characteristic when it was grown in artificial seawater medium. However, it was not known if the high salt-adapted organism would still consume methanol as its major food source in the minimal yeast medium. Therefore, the organism was grown in minimal yeast medium, and the methanol yield on the growth of the organism was calculated. In addition, the non-adapted *Bacillus methanolicus* strain MGA3 was also cultured in the same type of medium as a reference to the high salt-adapted organism. Both organisms were cultivated with 0.5 g/L of yeast extract and 4 ml/L initial methanol concentration. The experimental plan is described in Table 23.

Table 23. Experimental plan for the growth comparison.

Flask	Inoculated with
NS	non-adapted *B. methanolicus* strain MGA3
AS	high-salt adapted organism from section 6.3.1

The growth profile is shown in Figure 23, and the maximum OD values are listed in Table 24. The frozen culture of the high-salt adapted strain was thawed in 50 ml of the minimal yeast solution as a preculture. The preculture grew quite well, and the optical density was 2.5 at the time it was transferred to the fresh minimal yeast medium. However, the high salt-adapted organism did not grow well in the fresh minimal yeast medium. The maximum OD was only 0.57, which was much lower than the value for non-adapted *Bacillus methanolicus* strain MGA3. ODs of both control flasks were below 0.01.

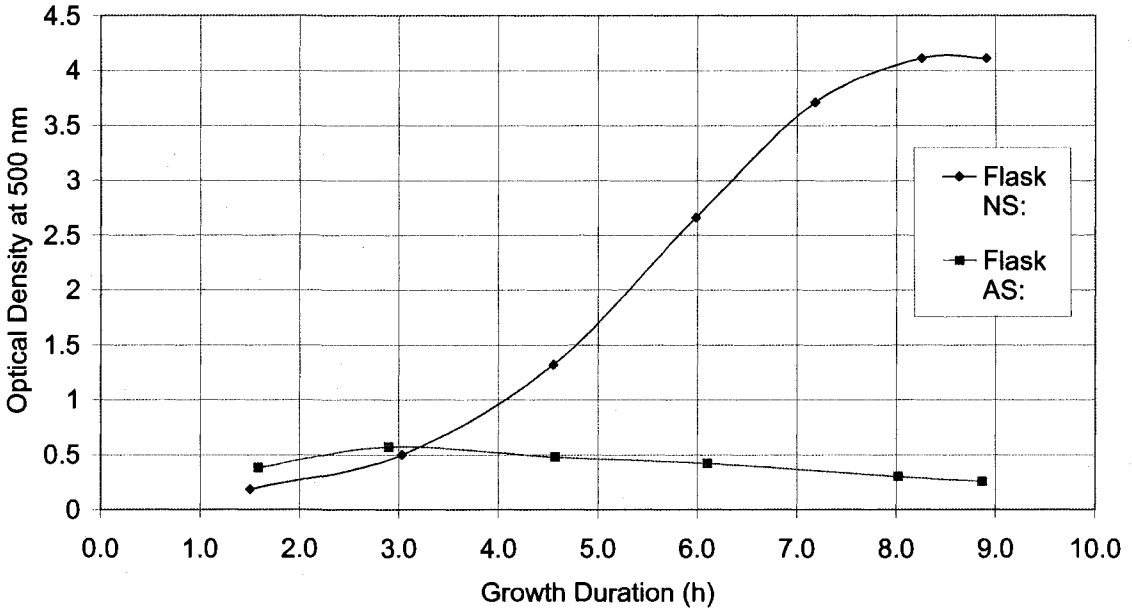

Figure 23. Growth profiles of the high salt-adapted organism and the non-adapted *B. methanolicus* strain MGA3 in minimal yeast medium.

Table 24. Maximum optical density and substrate yield in the growth comparison experiment.

Flask	Maximum OD	Standard Error of Maximum OD	Substrate Yield (g cell / g methanol)
NS	4.11	0.0321	0.42
AS	0.57	0.0115	4.36

In the analysis of fermentation samples by HPLC, it was determined that the substrate yield of the high salt-adapted organism (flask AS) was 4.36 g cells / g methanol. This value confirmed the observation in section 6.4.2 that methanol was not the primary carbon source being consumed in the fermentation. There was about 0.35 g/L of residual glycerol from the inocula. In addition to the 0.5 g/L yeast extract presented in the minimal yeast medium, these two components were more than enough to generate a biomass of OD 0.57 (equivalent to 0.1 g/L biomass). One explanation for this yield value is the presence of a contaminant in the culture, *Bacillus licheniformis*, during the adaptation process described in section 6.3.1. Nybroe *et al.* [37] and the Manachini *et al.* [38] showed that *Bacillus licheniformis* was able to grow in seawater medium. On the other hand, the substrate yield of the non-adapted *B. methanolicus* strain MGA3 was 0.42 g cells / g methanol, which was very close to the value in Wang's study [19]. This yield value was consistent with the conclusion that the non-adapted strain was not contaminated. The substrate yield calculations are documented in Appendix H.

6.5 Development of the Fermentation of the Non-Adapted *Bacillus methanolicus* Strain MGA3 in Artificial Seawater Medium

6.5.1 The Growth of the Non-Adapted *Bacillus methanolicus* Strain MGA3 with 2mM Osmoprotectants

The effect of osmoprotectants on the growth of non-adapted *Bacillus methanolicus* strain MGA3 in artificial seawater medium was determined at this stage. Previously described in section 6.2.1, the growth of the non-adapted strain MGA3 was suppressed in artificial seawater medium. However, as discussed in section 2.4, the osmotic stress in the growth environment could be alleviated by the addition of osmoprotectants, and thus the growth of the organism would be improved. After the revivification of the frozen culture in minimal yeast medium, it was transferred to the artificial seawater medium. The yeast extract concentration was 0.5 g/L, and the initial methanol concentration was 4 ml/L (98.9 mM). All eight chemicals listed in Table 11 were added to the medium at 2mM.

Compared to the growth profiles obtained in section 6.2.2 with the same condition (AS1, AS2, and AS3), Figure 24 indicates that the growth of the organism was greatly improved with the addition of the osmoprotectants. The maximum OD was 1.04 (standard error = 0.0208), and the specific growth rate was 0.2848 h^{-1} (standard error = 0.0174 h^{-1}).

These data were more than 10 times those values from the baseline profiles AS1 to AS3. In addition, the methanol yield was 0.31 g cells / g methanol consumed, which was slightly more than the yield value obtained in Cheung's study [20] but less than in Wang's study [19]. The substrate yield calculation is listed in Appendix G. The methanol yield measured in this research was evaluated in a shake flask, whereas Wang's value was obtained in a chemostat. There were more errors involved in the yield measurement in this research than in Wang's research.

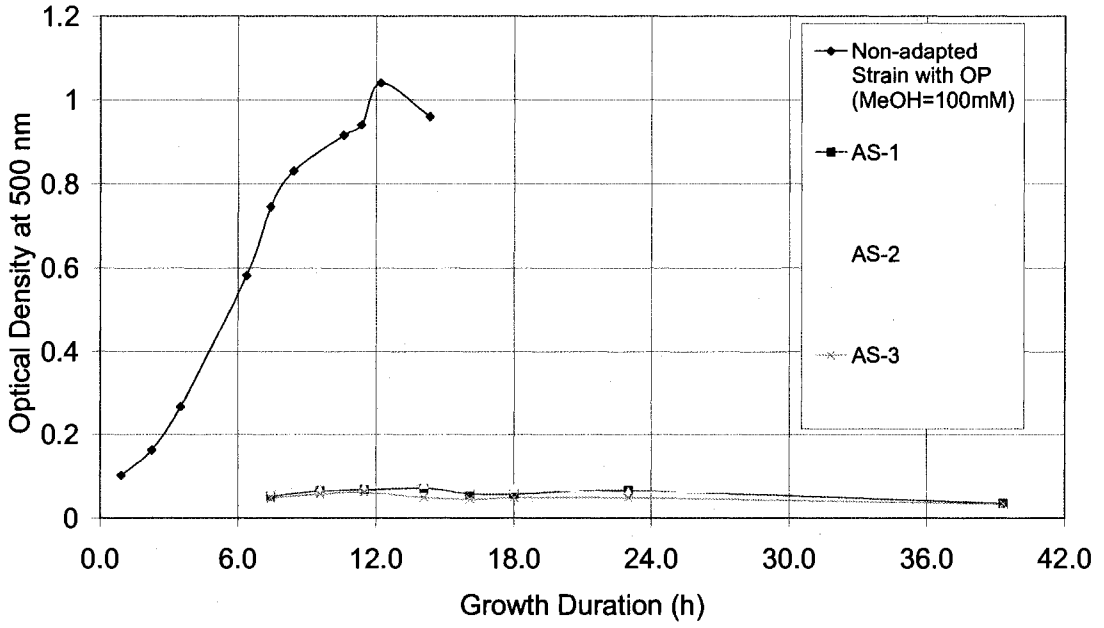

Figure 24. The growth profile of the non-adapted *B. methanolicus* strain MGA3 in artificial seawater medium with 2mM osmoprotectants.

6.5.2 Adaptation Experiments

6.5.2.1 Repeat Adaptation Steps in Section 6.3.1

Due to medium salt precipitation seen in the control flasks following the incubation for only two hours at 50 °C, the strategy of using mixtures of artificial seawater medium and minimal yeast medium was abandoned because the precipitates affected the optical density of the fermentation culture, and the change of optical density could not be determined.

6.5.2.2 3-Step Adaptation from Cheung's Study [19]

Cheung [19] had successfully adapted *Bacillus methanolicus* strain PB1 to grow well in both artificial seawater-based media and Pacific Ocean seawater-based media. In that study, the organism was first incubated in minimal yeast medium for 12 hours, and then subsequently transferred into MY medium with 15 g/L NaCl, and finally into the seawater medium. The yeast extract concentration was 0.5 g/L, and the initial methanol concentration was 10 ml/L. This approach was also tried on the *B. methanolicus* strain MGA3, and the results are shown in Table 25. The strain MGA3 was able to grow in the minimal yeast medium with or without NaCl. The maximum OD of the minimal yeast medium was 5.375, and that in the MY medium with 15 g/L NaCl was 5.480. However, the

growth in artificial seawater was not successful. The optical density was low throughout the 12-hour incubation, and the maximum OD was only 0.151.

Table 25. Maximum OD of the 3-step adaptation.

Growth Medium	Maximum OD	Standard Error of Maximum OD
MY	5.375	0.0382
MY with 15 g/l NaCl	5.480	0.0907
artificial seawater medium	0.151	0.0000

6.5.2.3 5-Step Adaptation

The adaptation procedures in section 6.5.2.2 were modified. Specifically, instead of transferring the organism from the MY medium with 15 g/L NaCl directly into the artificial seawater medium, it was subsequently transferred into the MY medium with 20 g/L NaCl, and then into the MY medium with 25 g/L NaCl before transferring it into artificial seawater medium. The yeast extract concentration was 0.5 g/L and the initial methanol concentration was 10 ml/L for all flasks. Table 26 summarizes the overall experimental plan.

Table 26. The experimental matrix of the 5-step adaptation.

Flask	Medium	Actual Incubation time (h)
G1	MY	12
G2	MY with 15 g/L NaCl	24
G3	MY with 20 g/L NaCl	22
G4	MY with 25 g/L NaCl	24
G5	artificial seawater medium	25

Figure 25 shows the optical density profile of the 5 flasks, and Table 27 shows the maximum OD and the substrate yield. These data show that the organism was able to grow well in high concentration NaCl MY medium, but not the artificial seawater medium. The growth profile determined in experiment G5, using the artificial seawater, was not much different than the non-adapted strain MGA3 in section 6.2.2. The maximum OD was only slightly improved to 0.147. Apparently, the adaptation process was not successful in the artificial seawater medium, and therefore the methanol yield was not determined.

The substrate yield was subsequently decreased from 0.38 g cells /g methanol to 0.21 g cells / g methanol from flasks G1 to G4. This indicated that the metabolic pathway of the organism changed with the osmotic stress of the environment, and methanol was a more favorable carbon source at the high salinity environment. Appendix H showed the detailed calculation of the methanol yield of flasks G1 to G4.

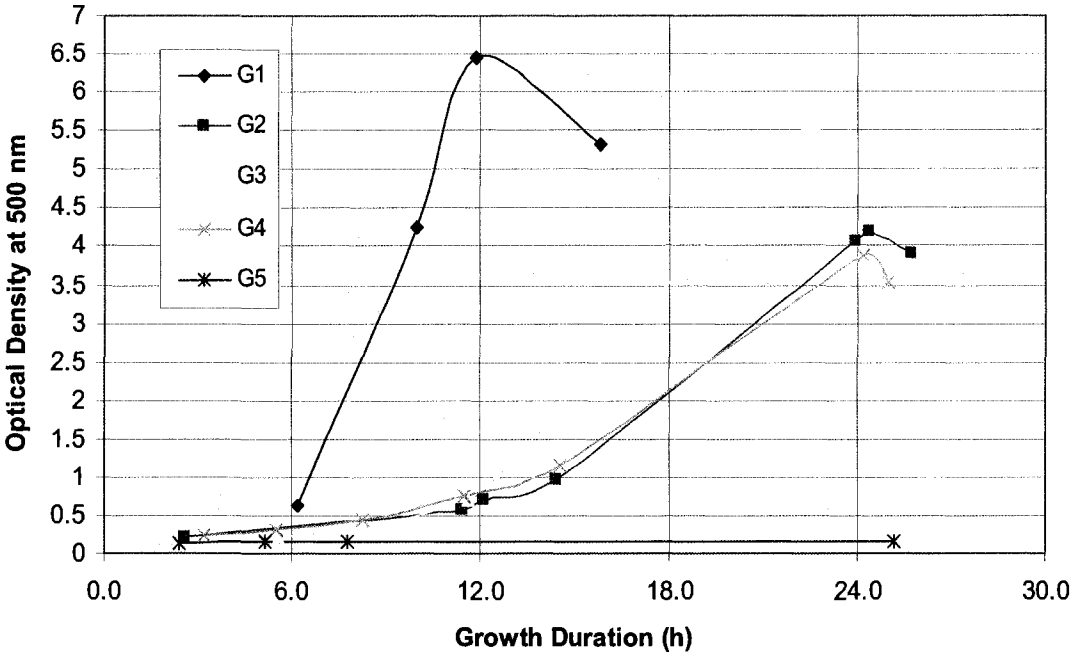

Figure 25. The OD profile of the 5-step adaptation.

Table 27. Maximum OD and substrate yield of the 5-step adaptation.

Flask	Medium	Maximum OD	Standard Error of Maximum OD	Substrate Yield (g cells /g methanol)
G1	MY medium	6.44	0.0757	0.38
G2	MY with 15 g/l NaCl	4.19	0.0153	0.26
G3	MY with 20 g/l NaCl	5.48	0.0945	0.23
G4	MY with 25 g/l NaCl	3.87	0.0666	0.21
G5	Artificial seawater	0.147	0.0006	not determined

CHAPTER SEVEN
CONCLUSIONS AND FUTURE REASEARCH

7.1 Conclusions

The growth of non-adapted *Bacillus methanolicus* strain MGA3 was very unfavorable in artificial seawater medium, and the maximum OD could not exceed 0.1. On the other hand, osmoprotectants showed a positive effect on the growth of the non-adapted strain MGA3 in artificial seawater medium, and improved the maximum OD to slightly more than 1.0. Furthermore, by using a stepwise adaptation process, the strain MGA3 was also able to achieve a maximum OD of better than 3.5 in high salt MY media (25 g/L NaCl). However, the growth could not be sustained when it was transferred into the artificial seawater medium.

Initially, the subject of this research was *Bacillus methanolicus* strain MGA3 only. However, a contaminant, *Bacillus licheniformis,* was identified in the fermentation culture; so much work in this research was actually performed on the growth of the contaminant, not from the *B. methanolicus*. Glycerol and yeast extract were consumed by the *B. licheniformis* in minimal yeast medium, and the organism apparently could not grow on methanol. With 2mM trehalose and methionine, a high salt-adapted organism, possibly *B. licheniformis*, had a better maximum OD in artificial seawater medium.

7.2 Future Research

The culture contaminant, *Bacillus licheniformis*, was able to grow to an optical density of more than 2.5 with a specific growth rate of more than 1.0 h^{-1} in artificial seawater medium in a shake flask. It could be an interesting topic to further explore its growth behavior at larger scale conditions, such as fed-batch or continuous bioreactor.

Culture contamination was the major obstacle in the research of *Bacillus methanolicus*. While it may be impossible to completely eliminate all contaminants from the environment, it is possible to develop a simple test method to validate the identity of the fermentation organism. For example, fermentation products from the organism could be identified, and quantified with HPLC and LC/MS.

While Cheung [20] was able to grow the *B. methanolicus* strain PB1 in seawater medium by a 3-step adaptation, the attempt to perform the same task on the strain MGA3 did not succeed. With further process analysis, it may be possible to grow the MGA3 in artificial seawater medium.

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APPENDICES

Appendix A DOE Plan of the OP Screening in Section 6.3.2

Table A1. Screening DOE (block 1: flasks 1 to 16).

Run Order	Glycine Betaine	Choline	Proline	Carnitine	Glutamate	Trehalose	Sucrose	Methionine
1	0	2	0	0	0	2	1	1
2	2	2	0	2	0	0	0	1
3	2	2	0	2	2	0	1	0
4	1	1	1	1	1	1	0.5	0.5
5	0	0	0	2	2	2	0	1
6	1	1	1	1	1	1	0.5	0.5
7	2	0	2	0	0	0	1	1
8	1	1	1	1	1	1	0.5	0.5
9	2	0	2	2	0	2	0	0
10	0	2	2	0	2	0	0	0
11	2	0	0	0	2	2	1	0
12	0	0	0	0	0	0	0	0
13	1	1	1	1	1	1	0.5	0.5
14	2	2	2	0	2	2	0	1
15	0	2	2	2	0	2	1	0
16	0	0	2	2	2	0	1	1

Table A2. Screening DOE (block 2: flasks 17 to 32).

Run Order	Glycine Betaine	Choline	Proline	Carnitine	Glutamate	Trehalose	Sucrose	Methionine
17	0	2	2	0	2	0	0	0
18	2	0	2	0	0	0	1	1
19	2	0	0	0	2	2	1	0
20	0	0	0	0	0	0	0	0
21	0	0	2	2	2	0	1	1
22	2	2	0	2	0	0	0	1
23	2	2	2	0	2	2	0	1
24	1	1	1	1	1	1	0.5	0.5
25	1	1	1	1	1	1	0.5	0.5
26	2	2	0	2	2	0	1	0
27	0	0	0	2	2	2	0	1
28	1	1	1	1	1	1	0.5	0.5
29	0	2	2	2	0	2	1	0
30	2	0	2	2	0	2	0	0
31	0	2	0	0	0	2	1	1
32	1	1	1	1	1	1	0.5	0.5

Table A3. Screening DOE (block 3: flasks 33 to 48).

Run Order	Glycine Betaine	Choline	Proline	Carnitine	Glutamate	Trehalose	Sucrose	Methionine
33	0	2	2	0	2	0	0	0
34	0	0	0	0	0	0	0	0
35	1	1	1	1	1	1	0.5	0.5
36	0	2	0	0	0	2	1	1
37	2	0	2	0	0	0	1	1
38	0	0	2	2	2	0	1	1
39	2	2	0	2	2	0	1	0
40	2	2	2	0	2	2	0	1
41	2	0	0	0	2	2	1	0
42	0	0	0	2	2	2	0	1
43	1	1	1	1	1	1	0.5	0.5
44	2	2	0	2	0	0	0	1
45	0	2	2	2	0	2	1	0
46	1	1	1	1	1	1	0.5	0.5
47	2	0	2	2	0	2	0	0
48	1	1	1	1	1	1	0.5	0.5

Appendix B Results of the Screening DOE in Section 6.3.2

Table B1. Maximum OD and specific growth rate of flasks 1 to 16.

Flask	Maximum OD	Specific Growth Rate (h^{-1})
1	2.87333	2.0692
2	1.64667	1.6088
3	1.89000	1.8434
4	2.26333	2.0159
5	2.57000	1.9992
6	2.17667	2.0270
7	1.74333	1.9737
8	2.28000	1.9282
9	2.48667	1.8945
10	1.66000	1.5019
11	2.38667	1.9356
12	1.26667	1.5361
13	2.24667	1.9203
14	2.90000	2.0584
15	2.05667	1.9169
16	1.85667	1.8494

Table B2. Maximum OD and specific growth rate of flasks 17 to 32.

Flask	Maximum OD	Specific Growth Rate (h^{-1})
17	1.66333	1.0884
18	2.06000	1.2255
19	2.92667	1.5814
20	1.21667	0.9408
21	2.24333	1.5335
22	1.28333	1.1751
23	2.73667	2.0099
24	2.21333	1.6459
25	2.28333	1.8812
26	2.09667	1.5306
27	2.72333	1.7226
28	2.22333	1.7364
29	2.59667	1.7500
30	2.35333	1.9086
31	2.71000	1.9304
32	2.39667	1.6987

Table B3. Maximum OD and specific growth rate of flasks 33 to 48.

Flask #	Max OD (Average)	Specific Growth rate (hr^{-1})
33	1.86333	1.4208
34	1.49667	2.4274
35	2.34667	2.5715
36	2.75333	2.2186
37	2.10667	2.1242
38	2.33667	2.3859
39	2.05333	2.4686
40	3.02667	2.5398
41	2.85000	2.2652
42	2.58333	2.5557
43	2.27667	2.2703
44	1.50000	2.3017
45	2.57000	1.4948
46	2.24333	1.4955
47	2.66000	1.5460
48	2.45667	1.5083

Appendix C Growth Profiles of the Screening DOE in Section 6.3.2

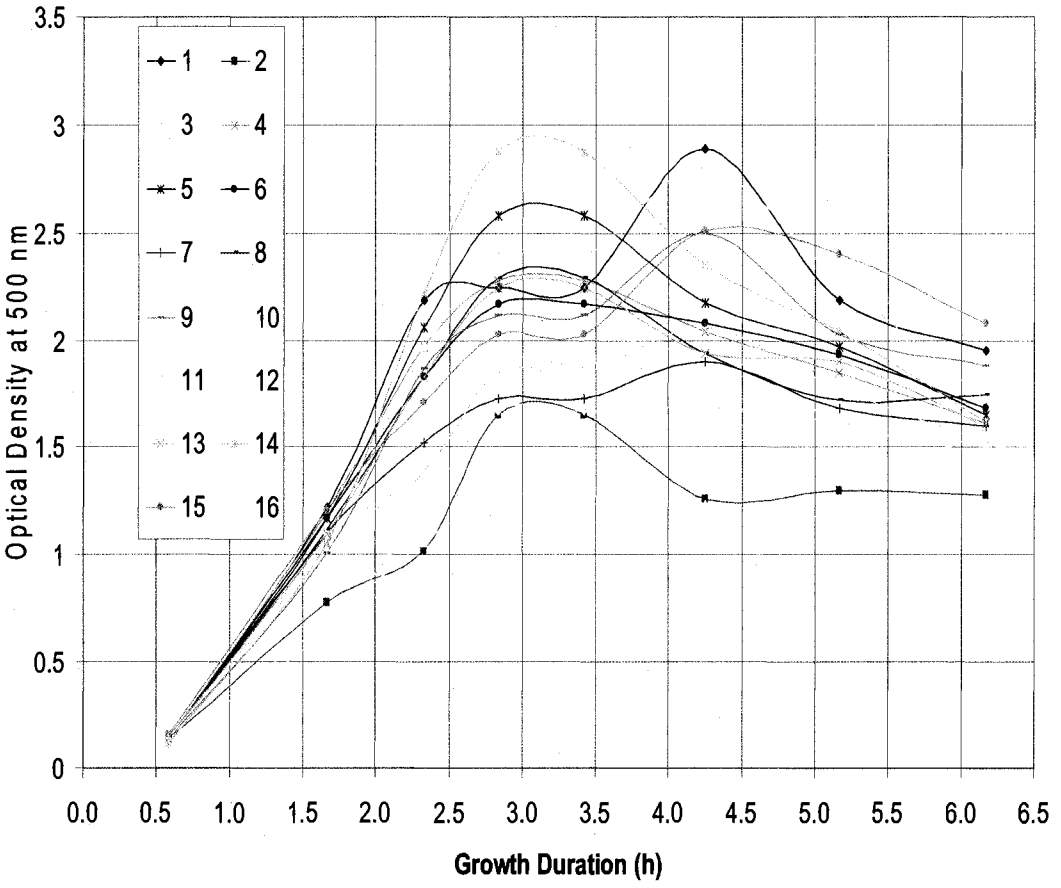

Figure C1. Growth profiles of the screening DOE block 1.

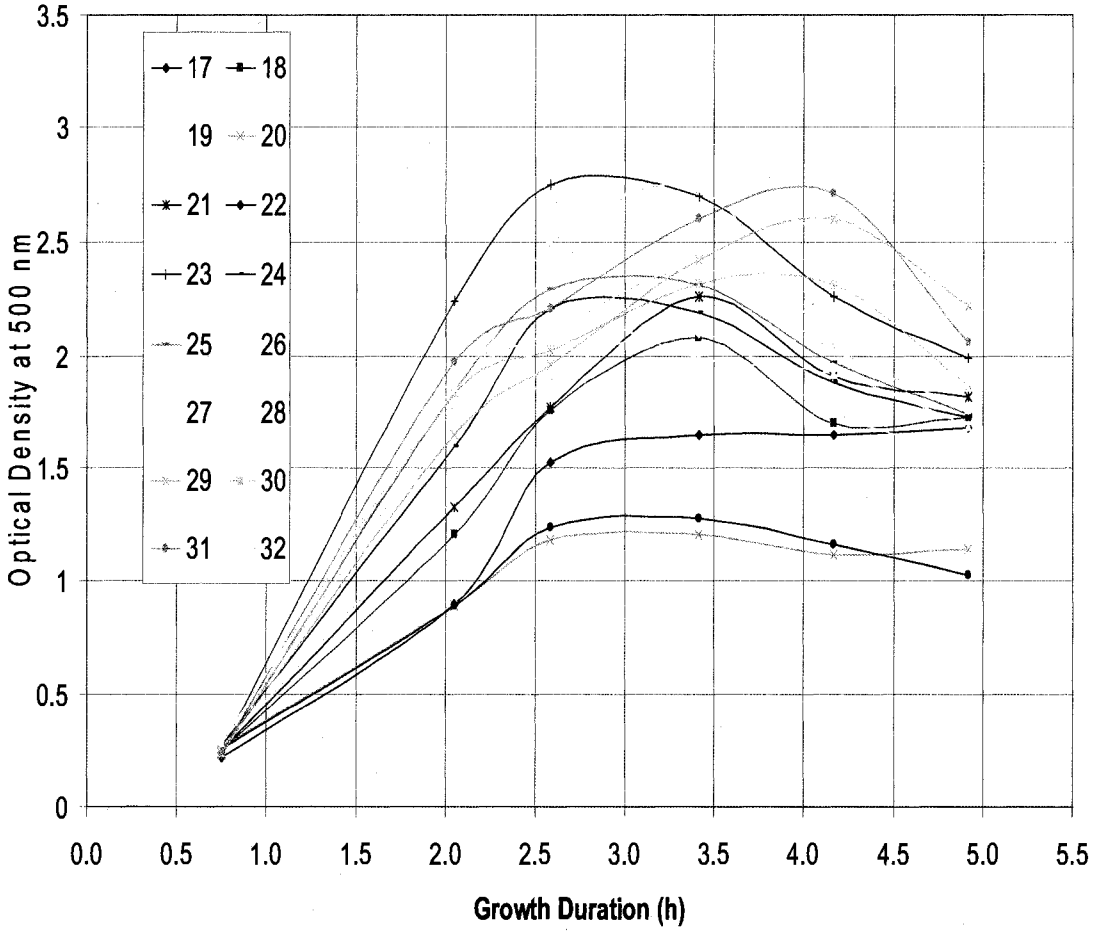

Figure C2. Growth profiles of the screening DOE block 2.

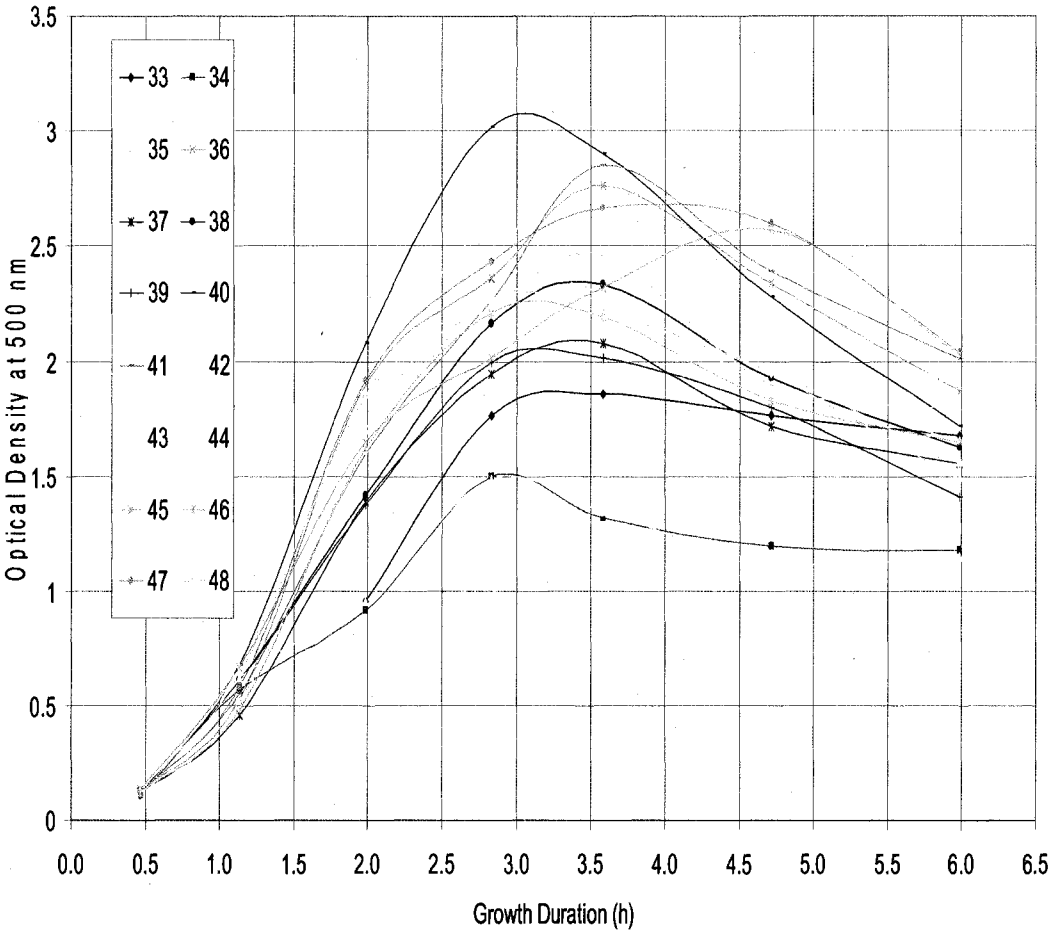

Figure C3. Growth profiles of the screening DOE block 3.

Appendix D HPLC Calibration Curves for Different Growth Medium

Several growth media were used in this research and had different impacts on the RI profile, especially on the area of the methanol peak. In order to have an accurate estimation of the residual methanol concentration in the sample, a calibration curve was constructed for each growth medium. It was done using a known amount of methanol in the growth media and integrating the area under the methanol peak of the RI profile. The concentration of methanol used to construct the calibration curve varied between 1 ml to 10ml per 1 liter of the growth medium. Based on the regression analysis, a linear relationship was found between the methanol concentration and the area under the methanol peak in the RI profile. The least square fit equation, standard error, and its corresponding R^2 value for each growth medium are listed in Table D1.

Table D1. The relationship between methanol concentration and the RI peak area at 20.2 minutes.

Growth Medium	Least Square Fit Equation [y = area of the methanol peak, x = methanol concentration (g/L)]	R^2 value	Standard Error
artificial seawater medium	y = 36 x	0.9920	0.4309
MY medium	y = 34.776 x	0.9924	0.3198
MY medium with 15 g/Ll NaCl	y = 34.811 x	0.9948	0.2691
MY medium with 20 g/L NaCl	y = 34.296 x	0.9969	0.2003
MY medium with 25 g/L NaCl	y = 34.219 x	0.9977	0.1687

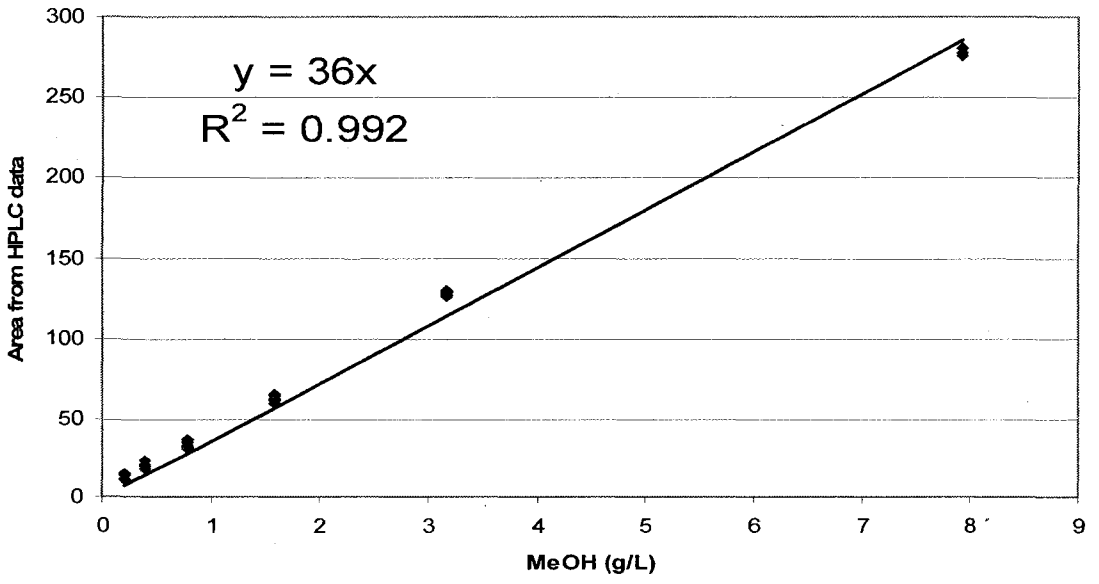

Figure D1. The HPLC calibration curve of the artificial seawater medium.

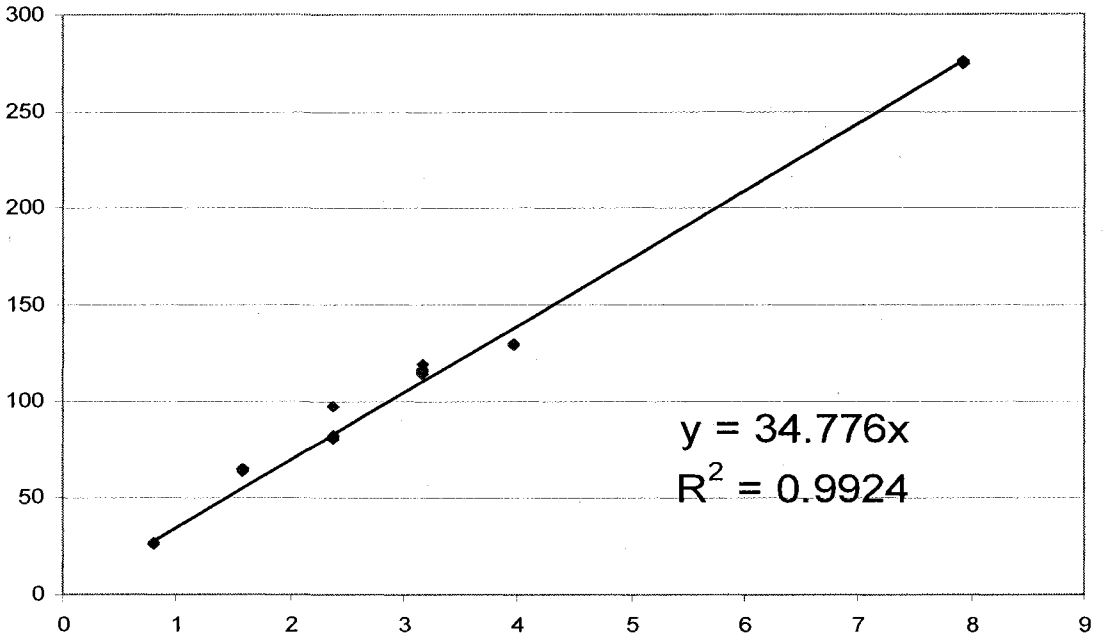

Figure D2. The HPLC calibration curve of the MY medium.

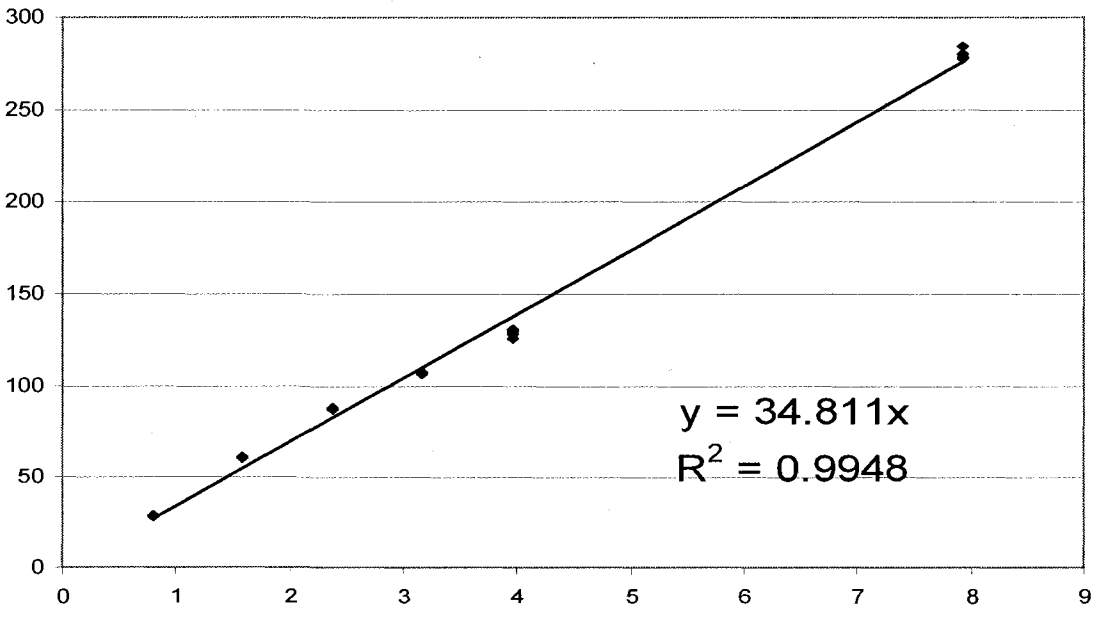

Figure D3. The HPLC calibration curve of the MY medium with 15 g/L NaCl.

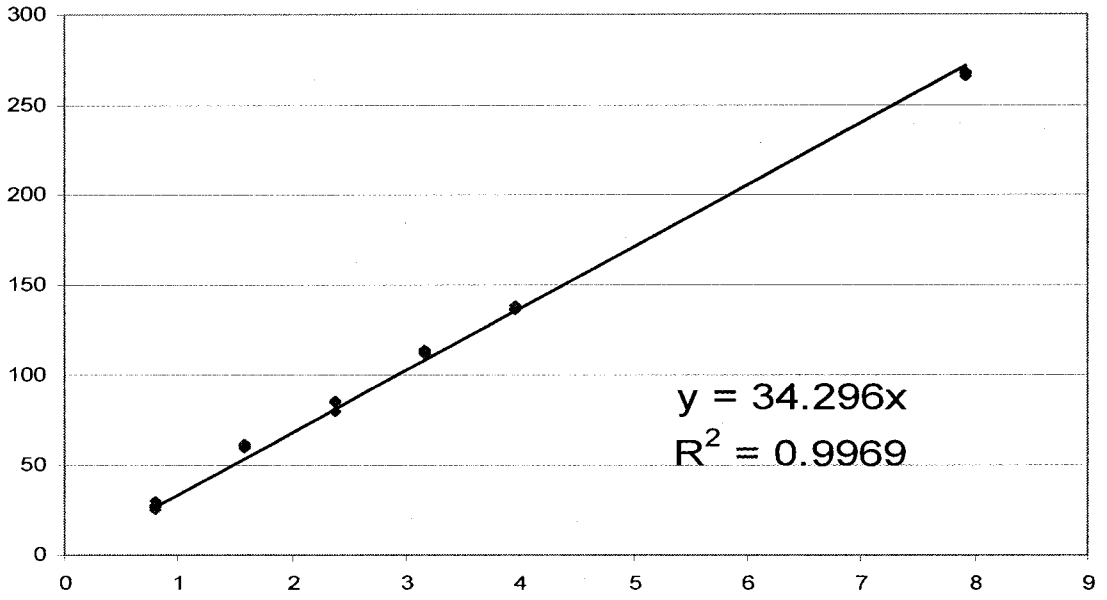

Figure D4. The HPLC calibration curve of the MY medium with 20 g/L NaCl.

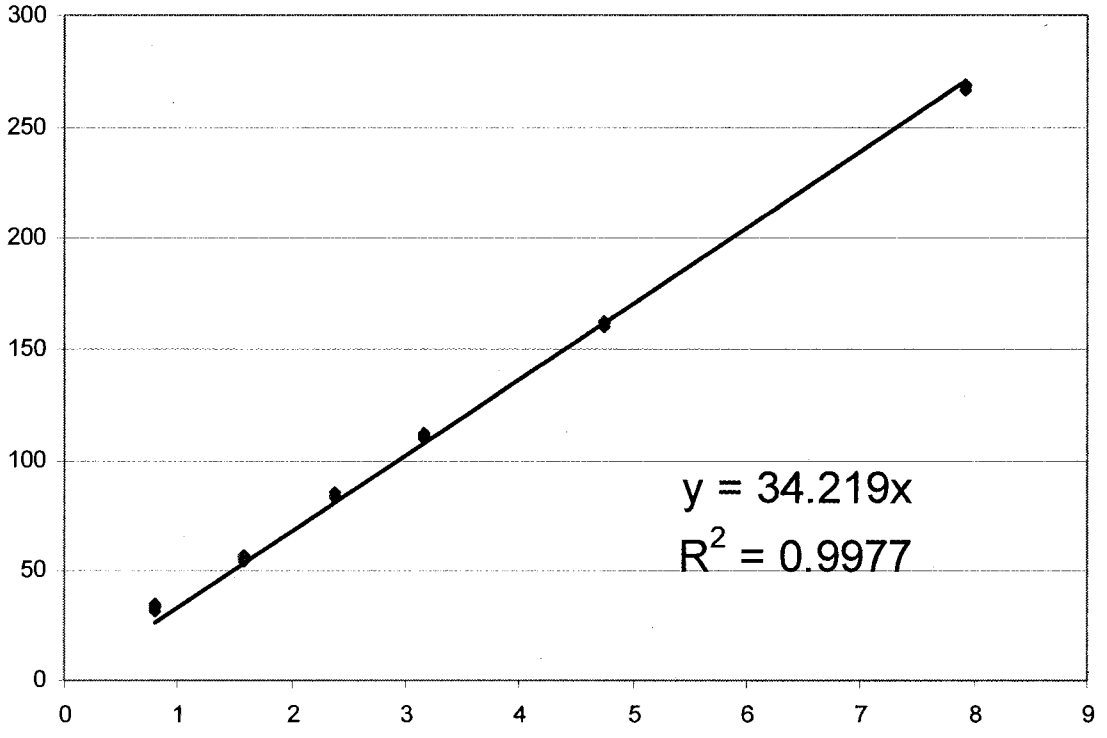

Figure D5. The HPLC calibration curve of the MY medium with 25 g/L NaCl.

Appendix E Calculation of Residual Methanol Concentration and Substrate Yield in Section 6.4.2

Since the growth medium is artificial seawater, the calibration curve in Figure D1 was used for the methanol concentration calculation.

Flask 1 (100mM methanol):

Average area of the methanol peak in Flask 1	=	93.8
Residual methanol concentration (g/L)	=	2.61
Average area of the methanol peak in control flask 1	=	102.6
Residual methanol concentration (g/L)	=	2.85
Maximum optical density	=	3.28
Biomass generated (g/L)	=	0.5773
Yield (x/S) at 100mM initial MeOH	=	0.5773 / (2.85 – 2.61)
	=	2.35

Flask 2 (500mM methanol):

Average area of the methanol peak in Flask 2	=	447.2
Residual methanol concentration (g/L)	=	12.42
Average area of the methanol peak in control flask 2	=	467.4
Residual methanol concentration (g/L)	=	12.98
Maximum optical density	=	3.29
Biomass generated (g/L)	=	0.5790
Yield (x/S) at 500mM initial MeOH	=	0.5790 / (12.98 – 12.42)
	=	1.03

Appendix F Calculation of Residual Methanol Concentration and Substrate Yield in Section 6.4.3

Since the growth medium is minimal yeast medium, the calibration curve in Figure D2 was used for the methanol concentration calculation.

Flask AS (High-salt adapted organism):

Average area of the methanol peak in Flask AS	=	79.63
Residual methanol concentration (g/L)	=	2.29
Average area of the methanol peak in control flask AS	=	78.83
Residual methanol concentration (g/L)	=	2.27
Maximum optical density	=	0.57
Biomass generated (g/L)	=	0.1003
Yield (x/S)	=	0.1003 / (2.29 – 2.27)
	=	4.36

Flask NS (Non-adapted *B. methanolicus* strain MGA3):

Average area of the methanol peak in Flask NS	=	76.5
Residual methanol concentration (g/L)	=	2.20
Average area of the methanol peak in control flask NS	=	16.7
Residual methanol concentration (g/L)	=	0.48
Maximum optical density	=	4.11
Biomass generated (g/L)	=	0.7234
Yield (x/S)	=	0.7234 / (2.20 – 0.48)
	=	0.42

Appendix G Calculation of Residual Methanol Concentration and Substrate Yield in Section 6.5.1

Since the growth medium is artificial seawater medium, the calibration curve in Figure D1 was used for the methanol concentration calculation.

Average area of the methanol peak in Flask	=	55.78
Residual methanol concentration (g/L)	=	1.549
Average area of the methanol peak in the control flask	=	77.13
Residual methanol concentration (g/L)	=	2.143
Maximum optical density	=	1.04
Biomass generated (g/L)	=	0.1830
Yield (x/S)	=	0.1830 / (2.143 – 1.549)
	=	0.31

Appendix H Calculation of Residual Methanol Concentration and Substrate Yield in Section 6.5.2.3

Flask G1 (MY medium):

Since the growth medium is minimal yeast medium, the calibration curve in Figure D2 was used for the methanol concentration calculation.

Average area of the methanol peak in Flask G1	=	64.24
Residual methanol concentration (g/L)	=	1.847
Average area of the methanol peak in control flask G1	=	167.14
Residual methanol concentration (g/L)	=	4.806
Maximum optical density	=	6.44
Biomass generated (g/L)	=	1.133
Yield (x/S)	=	1.133 / (4.806 – 1.847)
	=	0.38

Flask G2 (MY medium with 15 g/L NaCl):

Since the growth medium is minimal yeast medium with 15 g/L NaCl, the calibration curve in Figure D3 was used for the methanol concentration calculation.

Average area of the methanol peak in Flask G2	=	16.86
Residual methanol concentration (g/L)	=	0.484
Average area of the methanol peak in control flask G2	=	114.14
Residual methanol concentration (g/L)	=	3.279
Maximum optical density	=	4.19
Biomass generated (g/L)	=	0.737
Yield (x/S)	=	0.737 / (3.279 – 0.484)
	=	0.26

Flask G3 (MY medium with 20 g/L NaCl):

Since the growth medium is minimal yeast medium with 20 g/L NaCl, the calibration curve in Figure D4 was used for the methanol concentration calculation.

Average area of the methanol peak in Flask G3	=	9.38
Residual methanol concentration (g/L)	=	0.274
Average area of the methanol peak in control flask G3	=	152.86
Residual methanol concentration (g/L)	=	4.457
Maximum optical density	=	5.48
Biomass generated (g/L)	=	0.964
Yield (x/S)	=	0.964 / (4.457 – 0.274)
	=	0.23

Flask G4 (MY medium with 25 g/L NaCl):

Since the growth medium is minimal yeast medium with 25 g/L NaCl, the calibration curve in Figure D5 was used for the methanol concentration calculation.

Average area of the methanol peak in Flask G4	=	38.25
Residual methanol concentration (g/L)	=	1.118
Average area of the methanol peak in control flask G4	=	149.28
Residual methanol concentration (g/L)	=	4.362
Maximum optical density	=	3.87
Biomass generated (g/L)	=	0.681
Yield (x/S)	=	0.681 / (4.362 – 1.118)
	=	0.21