ABSTRACT

LU, HAO. Glucose Synthesis in a Protein Based Artificial Photosynthesis System. (Under the direction of Dr. Wenqiao Yuan).

Photosynthesis, by converting light, CO₂, and water into carbohydrates, provides the foundation for biofuels production. Artificial photosynthesis applies the same fundamental principles of the natural process to convert solar energy into ATP and sugars. The research about glucose synthesis in artificial photosynthesis system has been studied. However, artificial photosynthesis is known to be affected by the reaction conditions, which have not been examined in details in the literatures. **The goal of this study was to investigate artificial photosynthesis as a potential alternative to the glucose production for biofuels. The first objective** was to understand the effect of LED light intensity, light color and illumination pattern on the growth and bacteriorhodopsin (BR) content of *Halobacterium halobium*. The photobioreactor system was used to study the light effect on bacterial growth. Results showed that bacterial growth and BR content increased with increasing LED white light intensity. LED blue light was found to be the best for bacterial growth. It was also observed that 12 h LW - 12 h DK cycle illumination pattern lagged the bacteria growth but help for obtaining higher value of bacterial growth and BR content. Fade pattern was better than jump pattern for bacterial growth and BR formation. **The second objective** was to develop and compare several predictive models, including three-parameter models (Quadratic, Logistic and Gompertz) and four-parameter models (Schnute and Richards) to describe the growth of *Halobacterium holobium* as a function of temperature or light, on the basis of statistical analysis. The Gompertz model was considered statistically sufficient to describe the growth of *Halobacterium halobium* as a function of temperature or light. In addition, the biological growth parameters, including specific growth rate (μ_m), lag time (λ), and asymptote (A)

changes under Gompertz modeling were evaluated. **The third objective** was to understand the accumulation and ATPase activity of F_0F_1 ATP synthase in thermophilic bacteria *Bacillus* PS3 affected by culture conditions, including the type of nitrogen source and the concentrations of polypeptone and sodium chloride in the culture medium, as well as the medium pH and aeration rate. The highest yield and ATPase activity of purified enzymes were obtained when adding polypeptone as the nitrogen source. Contrarily, sodium chloride concentration had little effect on the growth of PS3 or F_0F_1 ATP synthase accumulation. Medium pH and aeration rate showed significant effects on the accumulation and activity of F_0F_1 ATP synthase. **The fourth objective** was to develop and compare several predictive models to describe the lag time, specific growth rate and asymptote of *Bacillus* PS3 as a function of temperature, on the basis of statistical analysis. The modified Ratkowsky model was the most suitable to describe both the growth rate and asymptote as a function of temperature. The hyperbolic model was selected to describe the lag time as a function of temperature. The above chosen models combined with Gompertz equation were used to predict bacteria growth, and the peak value prediction error was 7.78%. Finally, **the fifth objective** was to understand glucose synthesis of a protein-based artificial photosynthesis system affected by operating conditions, including the concentrations of reactants, reaction temperature, and illumination. Results from non-vesicle-based glyceraldehyde-3-phosphate (GAP) and glucose synthesis showed that the initial concentrations of ribulose-1, 5-bisphosphate (RuBP) and adenosine triphosphate (ATP), lighting source, and temperature significantly affected glucose synthesis. Glucose synthesis in the vesicle-based artificial photosynthesis system reconstituted with bacteriorhodopsin, F_0F_1 ATP synthase and polydimethylsiloxane – methyloxazoline - polydimethylsiloxane triblock copolymer was successfully demonstrated.

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Glucose Synthesis in a Protein Based Artificial Photosynthesis System

by
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DEDICATION

This work is dedicated to my parents, Mr. Qiyu Lu and Mrs. Jianling Li, for their genuine love, support, and to my wife, Mrs. Jingjing Wang, who came with me from China, and gave me the precious love and encourage. This work is also dedicated to my grandparents, Mr. Wuzun Li, and Mrs. Hemin Wang, who gave me the most precious love in my young ages.

BIOGRAPHY

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CHAPTER 1 - Introduction

Many countries have been motivated to find alternative energy sources because of the increasing fossil fuel prices, energy supply security and environmental concern. Especially in developed countries, these aspects are to be considered as the main drives to an increasing interest in biofuels (Stefan, 2009).

Photosynthesis, by converting light, CO₂, and water into carbohydrates, provides the foundation for food and fuel production (Peter, 2002; Dennis et al., 1998; Thorsten et al., 1999). For decades, scientists are devoting to the research on mimicking the natural process of photosynthesis, called artificial photosynthesis. It applies the fundamental principles of natural photosynthesis processes to convert solar energy into ATP and sugars (Bard et al., 1995; Thomas, 1989). ATP serves as the most important biological high-energy compound and has been formed artificially by coupling photon-induced proton motive force (Stoeckenius et al., 1982) and the key enzyme, F₀F₁ ATP synthase (Choi et al., 2006). Glucose is obtained at the end through the ATP motivated carbon fixation reactions (Choi et al., 2006).

Glucose synthesis through artificial photosynthesis has several advantages. (1) Reduce excess CO₂ (Barton et al., 2008). Research about artificial photosynthesis is designed to convert CO₂ into carbohydrate compound (glucose) or fuels (such as CO₂ to CO, CH₃OH and CH₄, etc). In this way, large amount of CO₂ could be reduced and used. (2) Environmental friendly. The raw materials used in artificial photosynthesis are mainly CO₂, H₂O and light. They can be reused or regenerated by processing (Sato et al., 2011). (3) Simple production system. Compared with complex glucose and O₂ producing system from natural photosynthesis, artificial photosynthesis is designed to achieve similar results by applying

simpler systems or components (Devens et al., 2009). So overall reactions and producing systems are simplified.

While some progress has been made in mimicking photosynthesis in artificial systems, some key components applied in the artificial photosynthesis are very expensive, e.g., enzymes F_0F_1 ATP synthase (Choi et al., 2006) and bacteriorhodopsin (Gali et al., 1998). However, these enzymes can be purified from bacteria and many researches have been conducted related to bacteria culturing and enzymes purification (Lee et al., 1998; Kagawa et al., 1979). Therefore, if the enzyme purification with higher enzymatic activity is achieved, glucose could be realized through artificial photosynthesis in an economical way.

1.1. Problem statement

This project was focused on the glucose synthesis in a protein based artificial photosynthesis system. In this system, glucose can be synthesized through enzymatic and ATP motivated CO_2 fixation reactions. However, this process is known to be affected by the reaction conditions, have not been examined in details by researchers. In order to understand this process, the effect of reactants conditions, including temperature, light type and concentration of reactants on glucose synthesis within the artificial photosynthesis system need to be studied. Also, the two key enzymes, BR and F_0F_1 ATP synthase can be purified from thermophilic bacteria *Bacillus* PS3 and halophilic bacteria *Halobacterium halobium*. So far researchers usually focused on bacteria culturing and enzyme purification processes. But little work has been done to study culture conditions on bacterial growth and enzyme concentration. In addition, the growth of the bacteria as a function of temperature or light need to be modeled, in order to accurately predict the bacteria yield.

1.2. Research objective

First of all, glucose synthesis in a protein-based artificial photosynthesis system affected by operating conditions was detailed studied. Then the effects of light and culture condition on the growth BR content of *Halobacteria halobium*, and F_0F_1 ATP synthase activity in *Bacillus* PS3 were also evaluated. As part of this project two kinetic models for the prediction of above two bacteria as a function of temperature or light were developed.

This work was divided into five main objectives:

- 1) To understand the effect of LED light intensity, light color and illumination pattern on the growth and BR content of *Halobacterium halobium*.
- 2) To develop and compare several predictive models that better describe the growth of *Halobacterium holobium* as a function of temperature or light;
- 3) To examine the accumulation and ATPase activity of F_0F_1 ATP synthase in thermophilic bacteria *Bacillus* PS3 affected by culture conditions;
- 4) To develop and compare several predictive models that better describe the growth of *Bacillus* PS3 as a function of temperature;
- 5) To investigate the effect of reaction conditions, including temperature, light type and concentration of reactants on glucose synthesis within a protein based artificial photosynthesis system.

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CHAPTER 2 – Review of literature

2.1 Glucose synthesis in artificial photosynthesis system

Artificial photosynthesis is one of the methods that convert solar energy into ATP and glucose (Gordon et al., 2007; Lynd et al., 1991). There are mainly two research directions related to artificial photosynthesis. Hydrogen and electricity is one of the method that can be potentially used in glucose synthesis in artificial photosynthesis. Huang (2012) proposed nine kinds of dark reaction pathways, that NADH is regenerated from hydrogen and electricity, rather than ATP. These methods combined with solar photovoltaic or solar hydrogen technology, highly improve the efficiency of the dark reaction. Zhang and Huang (2011) proposed electricity-carbohydrate-hydrogen (ECHO) cycle, which is noval, high-energy efficiency, biological CO₂ fixation pathways to fill the gaps from electricity and hydrogen to sugar. The two major roles of the ECHO cycles are storage of electricity or hydrogen and production of high-purity hydrogen for hydrogen fuel cell vehicles. They also had a hypothesis about biological CO₂ fixation process related to artificial photosynthesis. The process is mediated by non-natural cascade enzymes from high concentration CO₂ from power stations. Still, ATP is more used in artificial photosynthesis since glucose is synthesized driven by ATP in natural process of photosynthesis. The process can be divided into two parts: the light-dependent reactions and the light-independent reactions (also referred to as the dark reactions) (Lu et al., 2015). In light-dependent reactions, ATP synthesis is catalyzed by F₀F₁ ATP synthase (Elston et al., 1998). Direct evidence has been found that the proton gradient generated by electron transfer complexes and light-harvesting proteins provides the energy for

the synthesis of ATP (Weber et al., 2003), which is used to fix carbon from carbon dioxide into the energy storing carbon compounds in the dark reactions (Lu et al., 2015).

These years, researchers have developed different reconstituted system for the artificial photosynthesis studies. The beginning approach was the reconstitution of the system by using the closed phospholipid (lipid) bilayer membranes (Racker, 1972; Gromet et al., 1975; Hauska et al., 1980). Huang et al. (1979) studied the direction of proton pumping by reconstituting bacteriorhodopsin (BR) within exogenous phospholipids and the result indicated the direction of proton pumping was from the outside to the interior of the vesicles. Richard et al. (1995) studied the ATP production at a range of molar ratios of F₀F₁ ATP synthase: BR between 1:0 and 1:1.7 by reconstituted with the lipid, and they found that the initial rate of ATP synthesis increased with the level of ATP bound to the enzymes. Pal et al. (1983) purified membrane-bound (pyrophosphatase) PPase from *R. rubrum* chromophore and incorporated PPase into phospholipid vesicles in the presence of PPi and Mg²⁺. PPase can functions as the H⁺ pump to drive the synthesis of ATP from ADP and Pi. However, lipid membranes have an innate instability undesirable for the production of useful bioelectronics devices (Choi et al., 2006). Moreover, working devices using proteins as active elements requires an easily producible support which does not denature the protein but instead maintains its functionality. Hence, so much attention has been attracted to the self-assembling, amphiphilic ABA triblock copolymer membrane/vesicle as a potential building material in the fabrication of biosensors and artificial photosynthesis studies (Discher et al., 2002; Nardin et al., 2000; Sauer et al., 2001; Choi et al., 2006). These ABA copolymers, composed of a hydrophobic layer [B] set between two hydrophilic layers [A], are analogous to the typical lipid bilayer (Sauer et al., 2001).

Copolymer was found desirable for long-time using while maintaining functionality of the molecule inside such as proteins. Choi et al. (2006) used a self-assembled, amphiphilic ABA triblock copolymer (PEtOz-PDMS-PEtOz) embedded with BR or purple membrane (PM) and F₀F₁ ATP synthase. Copolymers were created and functioned as mesophyll layer of real leaves for photosynthesis light reactions. The vesicle reconstructed had the function of pumping H⁺, which drives the F₀F₁ ATP synthase to convert ADP to ATP. Also, Wendell et al. (2012) successfully achieved ATP synthesis, carbon fixation and glucose synthesis in an artificial photosynthesis system reconstituted by F₀F₁ ATP synthase, BR and copolymer.

2.2 Proton gradient pump in artificial photosynthesis

Proton pump plays a central role in biological energy transductions (Philip et al., 1987). The physiological function is to generate a transmembrane H⁺-electrochemical potential difference, which is employed to energize the energy-coupling membranes of mitochondria, lipid, to synthesize ATP (Harold, 1986). So far several approaches have been studied related to the proton gradient pump. The first one is the membrane-bound pyrophosphate (PPi) from *R. rubrum* chromatophore (Margareta et al., 1999). In isolated chromophores, PPi was used to drive several energy requiring reactions (Margareta, 1969). Proton movement, which was induced by light in chromophores, was shown by addition of PPi in the dark to be linked also to the inorganic pyrophosphatase (PPase), thus showing it to be a H⁺-PPase (Nyren et al., 1983). Secondly, several approaches have been followed to measure the functional dependency of the rate of ATP synthesis on the transmembrane pH difference, produced by acid-base transition (Georg et al., 1998). Vesicles were incubated in an acidic medium until all the ions were completely equilibrated across the membrane. Rapid mixing with a second medium rapidly

changed the external concentrations while the internal concentrations remain constant for a short time. Therefore, the pH_{out} and the pH_{in} can be measured by glass electrode. The problem of this method is that ATP synthesis must be measured in a short time interval after mixing so that the initial conditions remain constant during the reaction time (Turina et al., 1991).

Third, there are a group of enzymes, like delta-rhodopsin expressed by *Haloterrigena turkmenica* (Kiyotaka et al., 2011) and *Bacteriorhodopsin* (Hampp et al., 2000), they all could act as proton gradient pumps under certain light conditions. Bacteriorhodopsin (BR), the natural photosynthetic protein locates in purple membrane of bacteria *Halobacterium Halobium*, has emerged as a prime candidate for bio-molecular device applications. BR has a seven-transmembrane helical structure, function as a light driven proton pump. The seven transmembrane helices make an internal cavity for the proton transport pathway and a binding pocket for a retinal chromophore. A photochemical reaction cycle of BR begins at light-induced isomerization of the retinal from the all-trans to the 13-cis configuration (Katsumi et al., 2000), which processes the L state going through the J and K intermediates. The proton of the Schiff base is transferred to amino acid Asp 85 in the extracellular region (Leonid et al., 1993), producing an unprotonated state of the Schiff base (M state). Another proton is transferred to the Schiff base from amino acid Asp 96 in the cytoplasmic region (Peyroula et al., 2015), and this makes the Schiff base protonated again. This state is referring to N state. After that, the retinal becomes an all-trans state in O and then returns to the initial state.

Due to this unique photoelectric property, BR has become vital to the future applicability of nanobiological devices in the area of artificial photosynthesis (Hampp et al., 2000). Choi et al (2005) successfully reconstituted a multiprotein system by bacteriorhodopsin, F_0F_1 ATP

synthase and polymersome. Results showed maximum proton pumping activity (ΔpH) of -0.09 was measured in the 60 min green light illumination and this result suggests that bacteriorhodopsin is working as a light-driven transmembrane proton pump.

2.3 ATP synthesis in artificial photosynthesis

In artificial photosynthesis, ATP synthesis is usually catalyzed by F_0F_1 ATP synthase (Manuel et al., 2004). It consists of two portions, membrane-embedded F_0 and soluble F_1 (Soga et al., 2011; James et al., 2015). In the simplest version of this enzyme, the subunit compositions are ab_2c_{10-15} (F_0) and $\alpha_3\beta_3\gamma\delta\epsilon$ (F_1). This enzyme could catalyze the formation of ATP from ADP and phosphate, and this reaction is driven by conversion of Gibbs free energy derived from a transmembrane electrochemical proton gradient (Manuel et al., 2004).

Due to this unique property, F_0F_1 ATP synthase has been largely used in artificial photosynthesis research. For example, Choi et al. (2006) presented a solvent-free membrane protein reconstitution method in polymersomes and compared the functionality of BR and F_0F_1 ATP synthase both in solvent-free and solvent systems. In the solvent system, the drop-wise polymer–protein–organic solvent (ethanol) mixture was added to an aqueous solution, leading to the formation of proteopolymersomes. They also applied solvent-free system (dissolution of polymer into water) by controlling the aqueous solution. Their results showed that the use of solvent free systems eliminated the problem of protein denaturation during protein reconstitution into polymersomes. Hazard et al. (2002) reported a fast and simple purification method for thermophilic F_0F_1 ATP synthase with higher ATPase activity. The enzymes were purified by stepwise process from bacteria *Bacillus* PS3. Stable electrochemical gradient and

ATP were generated by incorporating F₀F₁ ATP synthase and BR into liposomes (Hazard et al., 2002).

In recent years, some researchers found that ϵ -subunit of the F₀F₁ ATP synthase, in particular its C-terminal domain, exerts an inhibitory effect both for ATP synthesis and ATP hydrolysis, and deletion of this domain has been shown to increase the synthesis activity (Masaike et al., 2006), probably by preventing the formation of the inhibited form (Soga et al., 2011). Thus, Soga et al. (2011) developed simple procedure for the preparation of active proteoliposomes by using a mutant F₀F₁ ATP synthase lacking an inhibitory segment of the ϵ -subunit. They also studied the kinetic analysis of ATP synthesis, and results showed that Michaelis-Menten constants for the substrates ADP and inorganic phosphate (Pi) were 13 μ M and 0.55 mM, which are order of magnitude lower than previous values of 0.3-0.4 mM for ADP and 6-10 mM for Pi (Bald et al., 1998).

2.4 Purification of F₀F₁ ATP synthase from Bacteria *Bacillus* PS3

The proton-translocating protein F₀F₁ ATP synthase, is a transmembrane protein that catalyzes ATP synthesis (Kagawa, 1978; Kagawa et al., 1979; Richard et al., 1995). The catalytic mechanism of F₀F₁ ATP synthase has been studied extensively (Daniela et al., 1999; Yoshida et al., 2001; Elston et al., 1998; Wang et al., 1998). This enzyme is composed of two sectors, F₀ and F₁ (Elston et al., 1998; Pederson et al., 1993). The former is composed of three different subunits designated as a, b and c, with the molecular weight of 23.48, 12.30 and 7.34 kDa (Schneider et al., 1984). Its function is to conduct the proton go across the membrane. The latter contains five subunits which are α , β , γ , δ and ϵ , with the molecular weight of 54.59,

51.94, 31.78, 19.66 and 14.33 kDa (Vignais et al., 1984). Purified F_1 has been the object of intensive research on the mechanism of ATP hydrolysis (Wang et al., 1998; Boyer, 1997).

Preparation of F_0F_1 ATP synthase has been reported from several bacteria, including thermophilic PS3 (Yoshida et al., 1975; Kagawa et al., 1979; Kagawa et al., 1979; Sone et al., 1975), *E. coli* (Foster et al., 1979), *Streptococcus faecalis* (Leimgruber, et al., 1981), *Mycobacterium phlei* (Lee et al., 1976), and *Clostridium Pasteurianum* (Clarke et al., 1979). From above sources, enzymes purified from bacteria PS3 has been characterized the most (Richard et al., 1995). Richard et al. (1995) purified F_0F_1 ATPase from *Bacillus* PS3 by using the chromatography method, and they studied the ATP production at a range of molar ratios of F_0F_1 ATP synthase: BR between 1:0 and 1:1.7 by reconstituted with the lipid, and they found that the initial rate of ATP synthesis increased with the level of ATP bound to the enzymes. Kagawa et al. (1979) obtained F_0F_1 ATP synthase with specific ATPase activity of $17.6 \text{ } \mu\text{mol min}^{-1} \text{ mg}^{-1}$ by using five purification steps, including membrane washing, cholate dissolving, triton X-100 extracting, DEAE eluting and agarose eluting. Yasuo et al (1991) conducted the purification and crystallization studies on F_1 subunit, its α and β subunits, for the crystallographic study. By applying dye-ligand chromatography, each protein was purified from PS3 and the purity was about 99%. Due to the increase purity, F_1 could be crystallized. In contrast, the subunits α and β failed to crystallize.

2.5 Bacteria *Halobacterium halobium* and BR growth under light illumination

Halobacterium halobium is a bacterium that occupies a special ecological niche and it can live only in solutions containing more than 12% salt (Walther et al., 1979; Aharon et al., 2001). The cell membrane of the bacteria is surrounded by a wall made up mainly of protein, no lipid,

and small amounts of carbohydrate (Richard, 1977). The purple membrane and red membrane constitute the plasma membrane of this bacteria (Dieter et al., 1974). Purple membrane contains the membrane-bound proton pumping protein bacteriorhodopsin (BR), which could use light energy to translocate protons across the membrane; the electrochemical gradient is then used to make ATP, and subsequently to energize many of the normal functions of the cell (Janos and Andrew, 2001; Hartmut and Dieter, 1980; Dickopf and Heyn, 1997). The red membrane forms by far the largest part of the membrane in well-oxygenated cells ((Richard, 1977)). It has lower density than the purple membrane, and the color of the red membrane fraction is mainly due to the C₅₀-carotenoid *bacterioruberin* (Michael, et al., 1970; Dieter et al., 1974).

Since BR has the unique characteristics that translocate protons across the membrane, it has recently been used for bioelectronics device design and artificial photosynthesis (Oesterhelt et al., 1991; Boucher et al., 1996; Lanyi and Luecke, 2001; Chen, 1993; Lu et al., 2015). As a large quantity of BR is in demand for research and development, there are more researchers focusing on *Halobacterium halobium* culturing and BR purification from the bacteria. Among these bacteria culturing protocols, they all share similar characteristics that bacteria culturing requires not only the high salt concentration, but also continuous light illumination (Hartmann et al., 1980). For example, Henry (1982) cultured the bacteria under 160 W of fluorescent lighting in the first day, and an additional 500 W of incandescent light was added from the second through the fourth day during pigmentation (BR) development. Lee et al (1998) recycle-cultured bacteria *Halobacterium halobium* by using the halogen lamp

as the light source. In addition, Oesterhelt et al. (1974) cultured the bacteria in a MicroFerm fermentor with illumination by the daylight fluorescent tubes.

Some researchers also conducted some research to compare the light and dark effect on bacteria growth. Valera et al. (1983) studied the slightly aerated cultures of *Halobacterium halobium* by using three different levels of illumination, in darkness, illuminated at 5000 Lux and 10000 Lux. Results showed that light was acting as an important energy source for the cell growth, and the population density of the culture receiving 10000 Lux and 5000 Lux were about fourfold and twofold higher than of non-illuminated culture. Hartmann et al, (1980) found that photophosphorylation of BR controlled *Halobacterium halobium* growth and this light-dependent growth could continue until BR was diluted to an extent that no longer allowed a sufficient rate of photophosphorylation. They found that cells containing large amounts of BR grew in light, whereas cells containing no BR (inhibitor nicotine inside) did not.

2.6 References

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CHAPTER 3 – The effect of light intensity, light color, and light illumination pattern on the growth and bacteriorhodopsin content of *Halobacterium halobium*

Abstract

The objective of this study was to understand the effect of LED light intensity, light color and illumination pattern on the growth and bacteriorhodopsin (BR) content of Halobacterium halobium. The photobioreactor system was applied to study the light effect on bacteria growth. Results showed that bacterial growth and BR content increased with increasing white LED light intensity. LED blue light was found to be the most efficient for bacterial growth. It was also observed that 12 h LW - 12 h DK cycle illumination pattern lagged the bacteria growth but help for obtaining higher value of bacterial growth and BR content. Fade pattern was better than jump pattern for bacterial growth and BR formation. Halobacterium halobium growth and BR content are highly associated under the illumination conditions. The highest dry cell weight of bacteria halobacterium halobium and BR content were $1.84 \pm 0.005 \text{ g l}^{-1}$, and $11.76 \pm 0.292 \text{ mg l}^{-1}$, under the 2000 Lux LED white light illumination. LED green light (170 Lux) was found saved 84% energy consumption for bacterial growth and increased 784% BR productivity compared with LED white light (2000 Lux).

3.1 Introduction

Halobacterium halobium is a halophilic bacterium that occurs naturally in salt lakes and salterns (Walther et al., 1979; Aharon et al., 2001). The purple membrane and red membrane constitute the plasma membrane of this bacteria (Dieter et al., 1974). Purple membrane contains the membrane-bound proton pumping protein bacteriorhodopsin (BR), which could

use light energy to translocate protons across the membrane, and thereby generates an electrochemical gradient. The energy stored in the gradient could be used for ATP synthesis and other energy-requiring functions (Janos and Andrew, 2001; Hartmut and Dieter, 1980; Dickopf and Heyn, 1997). The red membrane has lower density than the purple membrane, and the color of the red membrane fraction is mainly due to the C₅₀-carotenoid bacterioruberin (Michael, et al., 1970; Dieter et al., 1974).

Since BR has the unique characteristics that translocate protons across the membrane, it has recently been used in electronic applications, culminating in the development of numerous prototypes of devices that include Fourier-transform holographic associative processors (Oesterhelt et al., 1991), biosensors (Boucher et al., 1996; Lanyi and Luecke, 2001), and protein-based retinal prostheses (Chen, 1993). In addition, BR could also be used in light reaction part in artificial photosynthesis, acting as proton gradient translocator for ATP synthesis. Then ATP can be used to fix carbon from carbon dioxide into the energy storing carbon compounds in the dark reaction part (Lu et al., 2015). For instance, Choi and Montemagno (2006) studied the BR pumping activity and ATP synthesis in a bubble architecture and demonstrated *in vitro* biochemical synthesis inside the water channels of foams using engineered artificial organelles. Lu et al. (2015) studied glucose synthesis in the vesicle-based artificial photosynthesis system reconstituted with BR, F₀F₁ ATP synthase and polydimethylsiloxane-methyloxazoline-polydimethylsiloxane triblock copolymer. They found that the initial concentrations of ribulose-1, 5-bisphosphate (RuBP) and ATP, lighting source, and temperature significantly affected glucose synthesis.

As a large quantity of BR is in demand for research and development, there are more researchers focusing on *Halobacterium halobium* culturing and BR purification from the bacteria. Among these bacteria culturing protocols, they all share similar characteristics that bacteria culturing requires not only the high salt concentration, but also continuous light illumination (Hartmann et al., 1980). For example, Henry (1982) cultured the bacteria under 160 W of fluorescent lighting in the first day, and an additional 500 W of incandescent light was added from the second through the fourth day during pigmentation (BR) development. Lee et al (1998) recycle-cultured bacteria *Halobacterium halobium* by using the halogen lamp as the light source. In addition, Oesterhelt et al. (1974) cultured the bacteria in a MicroFerm fermenter with illumination by the daylight fluorescent tubes.

However, both the bacteria yield and BR content can be affected by light conditions, e.g., light intensity, light color, illumination pattern, which have not been examined in details. Thus the main objective of this research was to understand the effect of varying light conditions, including light intensity (dark, 120, 780, 1430 and 2000 Lux), light color (blue, red and green), and illumination pattern (white light-dark flash, 12 h white light-12 h dark cycle, fade and jump) on bacteria *Halobacterium halobium* growth and BR accumulation. The energy efficiency, shown by bacteria dry cell weight and BR content, at the above conditions were also evaluated and compared.

3.2 Materials and methods

3.2.1 Microorganism

Bacteria *Halobacterium halobium* was purchased from American Type Culture Collection (#29341, Manassas, VA, USA) and kept under -80 °C. To begin with, bacteria (1 ml) were reactivated in a 10-ml glass tube containing 5-ml sterilized culture medium (with 250.0 g NaCl, 5.0 g HY Case SF, 5.0 g yeast extract, 20.0 g MgCl₂ 6H₂O, 2.0 g KCl and 0.2 g CaCl₂ 2H₂O in 1-l distilled water, pH = 7.6). Then they were inoculated at 37 °C for 3-4 days, and one drop of the culture was then cultured in a polystyrene petric dish (Fisher, PA, USA) containing the above culture media plus 15%-20% agar (w/w, pH = 7.6). They were then kept in an incubator at 30 °C for 4-5 days before being transferred into a 4 °C refrigerator and kept until experiments. All chemicals were purchased from Fisher Scientific (Pittsburgh, PA, USA) except for HY Case SF, which was purchased from Sigma (St. Louis, MO, USA).

3.2.2 Bacteria culture

To study the light effect, *H. halobium* was cultured in a photobioreactor system (PBR, University of Texas at Austin Culture Collection, Austin, TX, USA). The system consisted of three parts: (1) A 2-l cylindrical glass container with the dimensions of 26 cm (H) × 10 cm (D). (2) A magnetic stirrer underneath the glass container. (3) A UTEX RGB-LED spectral illumination platform customized with various LED light colors (white, green, blue and red), adjustable light intensities (ranging from 0 to 2300 Lux) and various illumination patterns (white-dark flash, jump, and fade). The platform was placed surrounding the tank. In the experiments, the platform was controlled by an infrared receiver (IR) remote. The peak

wavelengths of the three LED light colors were as follows: LED blue (LB, 470 nm), LED green (LG, 525 nm), LED red (LR, 625 nm). The illumination patterns of the platform included white-dark flash (quickly switching between white LED light illumination and dark, 0.2 s lasting for both white LED light illumination and dark), jump (illumination quickly cycling among red, green and blue colors, 0.2 s lasting for each color light) and fade (illumination slowly cycling between blue, green, red and other transitional colors with wavelength changes from 450 to 700 nm, 0.5 s lasting for each color light). Dark condition (DK) served as the control.

3.2.3 Analytical methods

The starter culture from polystyrene petric dish was transferred to a 250-ml culturing flask containing 150-ml medium. After culturing for 5 days at 37 °C, the starter culture was transferred to the 2-l PBR containing 1.3-l of the above culture medium. At every 8 or 12 hours, the bacteria sample (200 µl) was taken for optical density (OD) measurement by using a Synergy MX microplate reader (BioTek, Vermont, USA) via absorbance change at 660 nm. Here, the typical linear relationship between OD and *Halobacterium halobium* dry cell weight is shown in Fig. 3.1. BR content was measured as described by Lee et al. (1998). Briefly, bacteria cells from 2-ml culture broth were harvested by centrifugation, and were lysed in 1-ml deionized H₂O containing DNase (0.01 mg). After vortexing, the lysate was mixed with 4 M NaOH and 4 M NH₄OH in the ratio of 9:0.5:0.5 (lysate:NaOH:NH₄OH) in the dark. The absorbance at 568 nm (A₅₆₈) was first measured in the dark (A₅₆₈⁰). Then the mixture was exposed to light illumination for 24 h to remove retinal from purple membrane, and again A₅₆₈

was measured (A_{568}^{24}). As the molecular weight of BR is 26 kDa and the molar extinction coefficient is $63,000 \text{ M}^{-1}\text{cm}^{-1}$, the content of BR was determined by the following equation:

$$\text{BR (mg l}^{-1}\text{)} = 26000 \times (A_{568}^0 - A_{568}^{24}) / 63000.$$
 Light intensity for each light source was measured by a luxmeter. Energy consumption of the PBRs was measured by a P3 P4400 Kill-A-Watt electricity usage meter.

Fig. 3.1. Typical linear relationship between optical density and *halobacterium halobium* dry cell weight at all experiments (Original one and 100% diluted after bacteria culturing are included). The linear equation is $y = 3.522x - 0.0746$. ($R^2 = 0.9965$).

3.2.4 Statistical analysis

The results of the present study were expressed as mean of two replicates $\pm$ standard deviation. Statistical analysis was performed by one-way ANOVA and Turkey test with SAS (Cary, NC, USA). A p-value of 0.05 was considered significant.

3.3 Results and discussion

3.3.1 The effect of LW light intensity

The effect of LW light intensity on *halobacterium halobium* growth is shown in Fig. 3.2 (A). and Table 3.1. Significant differences were observed within bacterial growth and the bacteria grew better in LW light (highest OD = 0.52 ± 0.01) than in the dark (highest OD = 0.28 ± 0.01). These differences were probably related to the energy absorption of pigmentation inside of the bacteria. Photoheterotrophic bacteria *Halobacterium halobium* contains pigment carotenoid and rhodopsin-like pigment BR, which exhibit the strong light absorption at 470 nm and 570 nm wavelength in the visible spectrum (Daniel and John, 2002; Walther, 1985; Hiroaki et al, 1986; Roberto et al, 1982). The light energy absorbed by these two pigments can be used to produce ATP through photophosphorylation in rhodopsin system inside of the bacteria (Helgersson et al., 1983). Then the synthesized ATP is used to build bacteria structures and other bio-molecules by carbon fixation with added organic culturing materials (Danon et al., 1974). Thus more energy in higher intensities LW light can be absorbed by these two pigments, and used for the bacterial growth. These results were consistent with the findings by Hartmann, et al. (1980) who studied the effect of light conditions on the growth and BR content of bacteria *halobacterium halobium* and demonstrated that the growth of bacteria was higher under illumination than in dark conditions. In the dark, their OD value and cell count number were 0.18 and 1.5×10^8 after 100 hours culturing. However, under two 150-W lamps illumination, OD value and cell count number were increased to 0.80 and 8×10^8 , respectively. Similar results were also found by Valera, et al (1983). They studied the slightly aerated cultures of *Halobacterium halobium* by using three different levels of illumination, in darkness,

illuminated at 5000 Lux and 10000 Lux light. Results showed that light was acting as an important energy source for the cell growth, and the population density of the culture receiving 10000 Lux and 5000 Lux were about fourfold and twofold higher than of non-illuminated culture.

BR content in the dark condition and various LW light intensities are shown in Fig. 3. 2 (B) and Table 3.1. Again, significant differences were observed and higher LW light intensities improved BR formation. The highest BR content was measured as 11.76 ± 0.29 mg/l under 2000 Lux LW illumination. BR is one of the rhodopsin-like pigments and its formation and accumulation is light-dependent (Henry, 1982). Henry (1982) cultured the bacteria under 160 W of fluorescent lighting on the first day. However, an additional 500 W of incandescent light was added from the second through the fourth day during pigmentation (BR) development. In addition, some researchers found that the growth of *halobacterium halobium* was mediated by BR. Hartmann et al (1980) found that photophosphorylation of BR controlled *halobacterium halobium* growth and this light-dependent growth could continue until BR was diluted to an extent that no longer allowed a sufficient rate of photophosphorylation. They found that cells containing large amounts of BR grew in light, whereas cells containing no BR (inhibitor nicotine inside) did not. In our study, the growth of bacteria and BR content confirmed that the growth of *halobacterium halobium* was mediated or related with BR. A strong linear correlation between OD and BR content is shown in Fig. 3.3. This suggests that *halobacterium halobium* growth and BR content are highly associated under the illumination conditions in PBRs.

Fig. 3.2 (A) *Halobacterium halobium* growth in the dark and varying intensities of LED white light; (B). BR content in the dark and varying intensities of LED white light

Table 3.1. Maximum bacterial OD value and BR content in different light conditions

	Dark	120 Lux	780 Lux	1430 Lux	2000 Lux
Peak OD	0.28 ± 0.01 ^d	0.34 ± 0.01 ^c	0.41 ± 0.02 ^b	0.50 ± 0.01 ^a	0.52 ± 0.01 ^{a*}
Peak BR content (mg/l)	8.36 ± 0.14 ^d	9.15 ± 0.21 ^c	9.90 ± 0.33 ^b	11.14 ± 0.29 ^a	11.76 ± 0.29 ^a

*Different letters indicate significant differences ($p < 0.05$).

Fig. 3.3 Linear correlation between peak dry cell weight of *halobacterium halobium* and BR content

3.3.2 The effect of LED light color

Halobacterium halobium growth under different LED light colors is shown in Fig. 3.4 (A). Among these colors, LB obtained better results towards *halobacterium halobium* growth than LW, LG and LR, which were all better than in the dark. The reason is because the pigmentation formation in the bacteria. *Halobacterium halobium* contains a number of carotenoids in the red membrane. The pigment carotenoid exhibits strong light absorption in the blue portion (450 nm - 500 nm) of the visible spectrum (Daniel and John, 2002; Walther, 1985; Hiroaki et al, 1986; Roberto et al, 1982). Thus under the illumination of LB light, *halobacterium halobium* could efficiently absorb the light energy and the energy could be used for bacterial growth. Statistical analysis results confirmed that bacteria grew differently in the following order: LB > LW $\approx$ LR $\approx$ LG > DK (Table 3. 2).

Results from Fig. 3. 4 (B) and Table 3. 2 showed that the effect of LED light color on BR content was not significant, although with illumination resulted in higher BR content than in the dark. This result confirms the conclusion that BR's formation is light-dependent and higher intensity of light improved BR formation, compared with dark condition.

Fig. 3.4. (A) *Halobacterium halobium* growth in the dark and different color of the LED light;

(B). BR content in the dark and different color of the LED light

Table 3.2. Maximum bacterial OD value and BR content in different color of light conditions

	Dark	LW (120 Lux)	LG (170 Lux)	LB (160 Lux)	LR (178 Lux)
Peak OD	0.28 $\pm$ 0.01 ^c	0.34 $\pm$ 0.01 ^b	0.32 $\pm$ 0.01 ^b	0.38 $\pm$ 0.01 ^a	0.33 $\pm$ 0.02 ^b
Peak BR content (mg/l)	8.36 $\pm$ 0.14 ^b	9.15 $\pm$ 0.21 ^a	8.87 $\pm$ 0.14 ^a	8.86 $\pm$ 0.29 ^a	9.30 $\pm$ 0.29 ^a

*Different letters indicate significant differences (p<0.05).

3.3.3 The effect of illumination pattern

Halobacterium halobium growth and BR content, under three illumination patterns, including continuous LW light illumination, LW-DK flash and 12h LW-12 DK cycle conditions are shown in Fig. 3. 5 (A), (B), and Table 3.3. Results clearly showed that in continuous LW light illumination condition, the highest bacterial growth (0.52 ± 0.01) and BR content (11.76 ± 0.29 mg/l) were obtained, because continuous LW illumination had the most energy input which was critical for bacterial growth (Valera, et al., 1983).

Fig. 3. 5 (A) and (B) also illustrate the comparison of the bacterial growth and BR content between LW-DK flash and 12 h LW-12 h DK cycle. It is noted that when exposed to 12 h LW-12 h DK illumination, bacteria growth and BR content were higher in the light time, and decreased in the dark. The step-wise bacteria growth and BR content increased in the 12 h LW-12 h DK pattern (e.g., Fig. 3.5 A and B, 72-84 h and 84-96 h) reflects nutrient uptake in the light (72-84 h) and a slowdown of this activity during the dark interval (84-96 h) (Miyake et al., 1999). This activity delays the bacterial growth but helps for obtaining higher value of bacterial growth and BR content. This result was consistent with the finding by Miyake et al

(1999), who reported that the photosynthetic bacterium *Rhodobacter sphaeroides* RV cells grew slowly during dark periods but survival and was restored when illumination started again.

Fig. 3.5. (A) *Halobacterium halobium* growth under different illumination pattern of illumination; (B). BR content under different illumination pattern of illumination (The light intensities of LW were controlled at 2000 Lux in all three conditions)

Table 3.3. Maximum bacterial OD value and BR content in different illumination patterns.

	LW light	LW-DK flash	12h LW-12h DK cycle
Peak OD	0.52 ± 0.01^a	0.35 ± 0.03^c	0.41 ± 0.01^b
Peak BR content (mg/l)	11.76 ± 0.29^a	9.26 ± 0.21^c	9.90 ± 0.14^b

*Different letters indicate significant differences ($p < 0.05$).

The effect of fade and jump pattern on bacterial growth and BR content can be seen from Fig. 3.6 (A), (B), and Table 3.4. The highest bacterial growth (0.38 ± 0.01) and BR content (9.75 ± 0.14 mg/l) of and were obtained in the fade pattern. However, when illuminated under the jump pattern, bacterial growth and BR content were relatively lower (0.35 ± 0.01 and 9.29 ± 0.014 mg/l). This indicates that fade pattern is better for bacterial growth and BR formation.

The reason is that the jump pattern only provides basic colors illumination (LG, LR and LB). During the illumination, bacteria growth and BR formation could be maintained due to nutrient uptake from the culturing media. However, bacterial growth and BR formation were decreased in the dark period (Miyake et al., 1999). Differently, fade pattern is the illumination regime that provides continuous all-colors effect. Adsorption wavelength of pigment BR (500-650 nm, with the adsorption peak at around 568 nm) and carotenoid (400-500 nm, with the adsorption peak at 470 nm) are completely covered by this large scale illumination pattern (wavelength from 450 to 700 nm). Hence, BR and carotenoid could efficiently absorb the light energy from all-colors LED light illumination and the energy could be used to bacterial growth.

Fig. 3.6. (A) *Halobacterium halobium* growth under jump and fade pattern of illumination; (B). BR content under jump and fade pattern of illumination

Table 3.4. Maximum bacterial OD value and BR content in jump and fade patterns.

	Jump	Fade
Peak OD	0.34 ± 0.01^b	0.39 ± 0.01^a
Peak BR content (mg/l)	9.29 ± 0.14^b	9.75 ± 0.14^a

*Different letters indicate significant differences ($p < 0.05$).

3.3.4 Energy Efficiency in *Halobacterium halobium* culturing under various illumination type

In the current study, although LED light intensity, LED light color and illumination pattern are different issues to discuss, the energy consumption can be accounted for when comparing the *Halobacterium halobium* growth and BR content across different lighting conditions. Here, specific growth efficiency and BR productivity in *Halobacterium halobium* culturing under various illumination type are shown in Table 3.5. Similar to Akkerman et al. (2002), energy consumption per unit of bacteria dry cell weight, was used as an index of specific growth efficiency in this study. Results showed that the specific growth efficiency (kJ/g) ranged from 733 to 4636. In other words, this was the amount of energy (kJ) required to produce 1 g of bacteria under the various lighting conditions. The ranking, from most to least productive, was LG (170 Lux) > LR (178 Lux) > LW (120 Lux) > LB (160 Lux) > Fade > 12h LW-12h DK > Jump > LW (780 Lux) > LW (1430 Lux) > LW (2000 Lux) > LW-DK flash (Table 5).

Results from Table 3.5 showed that higher LW light intensities resulted in lower specific growth efficiency, and 120 Lux LW light illumination obtained highest ranking for specific growth efficiency (2245 kJ/g). This is because even though higher intensities LW light could improve bacterial growth, the efficiency of photon absorption from LW light by bacteria is limited. Light intensities higher than those required for the saturation of photon absorption, could result in dissipation and loss of the excess absorbed energy as the heat (Anastasios et al., 2009). Besides the light absorptions part, the carbon fixation by using organic materials from the culture media also limits the overall process. When light intensity is increased above the

energy conversion efficiency, the rate of the carbon fixation reaches a plateau, and reflecting the fact that brighter light and a faster rate of absorption incoming photons does not translate into a greater productivity by the cells. So limitation in the rate of whole process is imposed upon by the carbon fixation (Melis et al., 1999). Additional limitations may be imposed by the relatively slow rate of photophosphorylation process after the light energy is absorbed (Hartmann et al., 1977). So some absorbed energy from higher intensity of LW light would be wasted in the whole process, and result in lower specific growth efficiency.

From Table 3.5, it can also be found that LG light (170 Lux) obtained better results towards specific growth efficiency than similar intensity of LW (120 Lux), LB (160 Lux) and LR (178 Lux). These are probably two reasons for this result. First of all, bacteria *Halobacterium halobium* contains a large number of rhodopsin-like pigment BR, which exhibit the strong light absorption in the green portion (500-650 nm, with the adsorption peak at 570 nm) of the visible spectrum. So the light energy can be efficiently absorbed by BR under LG light illumination, and the more energy could be used for bacterial growth. This bacteria also contains relatively lower content of pigment carotenoid. So the energy absorption efficiency of carotenoid in LB light is lower than BR in LG light. In addition, the sequence of typical luminous efficacy (a measure of how well a light source produces visible light, lumen/energy) of colored LED light is green > red > blue (Yukio et al., 2010). The findings from this part confirm the conclusion that for the same lumen or intensity of LED light provided, blue and green LED light would consume higher and lower energy (Table 3.5).

In addition, Table 3.5 shows that 12h LW-12h DK illumination pattern obtained the lower ranking towards specific growth efficiency, compared with LW-DK flash pattern, even though

both of these patterns shared the same illumination and dark period. Here, flashing LED resemble standard LED but it contains an integrated multivibrator circuit that causes the LED to flash between white and dark (Hagan et al., 2002). In multivibrator, while one of the capacitors is charging, current flows to the base of the transistor, making LW light working. When the capacitor is charged, it stops conducting and switches off the transistor, and then the other capacitor begins to charge, leading to dark period. So even during the dark period of LW-DK flash pattern, the capacitor in multivibrator is still charging, and this activity would consume more energy, thus leading to lower specific growth efficiency.

The effect of fade and jump pattern on specific growth efficiency can be seen from Table 3.5. Results showed that fade pattern obtained the better specific growth efficiency. This is because adsorption wavelength of BR (500-650 nm, with the adsorption peak at 570 nm) and carotenoid (400-500 nm, with the adsorption peak at 470 nm) are completely covered by this large scale illumination pattern (wavelength from 450 to 700 nm). Energy from the light could be largely adsorbed and efficiently used for bacterial growth.

In addition, energy consumed divided by BR content was used as an index of energy efficiency (kJ l/mg) ranged from 93 to 729. Since abovementioned results suggest that a strong linear correlation was obtained between bacterial growth and BR content (Fig 3.3), the ranking for specific BR productivity is similar to specific growth efficiency at above illumination conditions.

The efficiency ratio, when compared with LW light (2000 Lux), suggests that using LG light (170 Lux) as the light source can save 85% ($1 - 733/4608 = 84\%$) energy expenditure when yielding the same level of bacteria production. In the meantime, by using LG light (170

Lux), specific BR productivity was also improved as 7.84 times ($729/93 = 784\%$) as the one in LW light (2000 Lux). Thus, to save both energy and costs, it is advisable to use LG as the light source when culturing *Halobacterium halobium*.

Table 3.5 Energy efficiency, shown by dry cell weight and BR content, in *halobacterium halobium* cultured under various illumination type

Illumination type ¹	Energy ² (kJ)	Dry cell weight (g)	Specific growth efficiency ³ (kJ/g)	BR content (mg/l)	Specific BR productivity ⁴ (kJ l/mg)
LW (120 Lux)	2650	1.18±0.002 ^g	2245	9.15±0.210 ^c	290
LW (780 Lux)	5841	1.44±0.002 ^d	4056	9.90±0.332 ^b	590
LW (1430 Lux)	7344	1.78±0.002 ^b	4126	11.14±0.292 ^a	659
LW (2000 Lux)	8571	1.84±0.005 ^a	4608	11.76±0.292 ^a	729
LG (170 Lux)	821	1.12±0.020 ⁱ	733	8.87±0.144 ^d	93
LR (178 Lux)	2298	1.16±0.007 ^h	1981	9.30±0.292 ^c	247
LB (160 Lux)	3940	1.29±0.005 ^e	3054	8.86±0.292 ^d	445
LW-DK flash	5702	1.23±0.002 ^f	4636	9.26±0.210 ^c	616
12h LW-12h DK	4821	1.48±0.030 ^c	3258	9.90±0.144 ^b	487
Jump ⁵	4032	1.23±0.017 ^f	3278	9.29±0.144 ^c	434
Fade ⁶	3917	1.25±0.022 ^f	3133	9.75±0.144 ^b	402

¹ Illumination type: LW: LED white light; LG: LED green light; LR: LED red light; LB: LED blue light; LW-D flash: LED white light-dark flash; 12h LW-12h D: 12h LED white light-12h dark.

² Product of voltage, ampere and illumination time.

³ Specific energy efficiency = energy consumption per unit of bacterial dry cell weight. Rank by specific growth efficiency in ascending order.

⁴ Specific BR productivity = energy consumption per BR content. Rank by specific BR productivity in ascending order.

⁵ Energy of jump pattern ranges from 6.9 to 7.1 W.

⁶ Energy of fade pattern ranges from 6.4 to 7.2 W.

Different letters indicate significant differences ($p < 0.05$).

3.4. Conclusions

This study for the first time indicated that the growth and BR content of *Halobacterium halobium* were affected by LED light intensity, LED light color and illumination pattern in UTEX PBRs. Bacterial growth and BR content were increased with LW light intensity. LB

light (160 Lux) was found to be the best for bacterial growth. It was observed that 12 h LW - 12 h DK cycle and fade pattern of illumination obtained higher value of bacterial growth and BR content. LG light was found to have the best result when comparing specific growth efficiency and BR productivity in *Halobacterium halobium* culturing under various illumination type.

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CHAPTER 4 – Modeling growth of bacteria *Halobacterium halobium* as a function of temperature and light

Abstract

The objective of this study was to develop and compare several sigmoidal models, including three-parameter models (Quadratic, Logistic and Gompertz) and four-parameter models (Schnute and Richards) to describe the growth of bacteria Halobacterium halobium as a function of temperature or light. The models were compared statistically by using t test and F test. In the t test, confidence bounds for parameters were used to distinguish among models. For the F test, the lack of fit of the models was compared with the prediction error. The Gompertz model was 100% accepted by the t test and 97% accepted by the F test when the temperature effects were considered. Results also indicated that the Gompertz model was 94% accepted by the F test when the growth of Halobacterium halobium was studied under varying light intensities. Thus the Gompertz model was considered statistically sufficient to describe the growth of Halobacterium halobium as a function of temperature or light. In addition, the biological growth parameters, including specific growth rate (μ_m), lag time (λ), and asymptote (A) changes under Gompertz modeling were evaluated.

4.1 Introduction

Predictive microbiology is an area of research in microbiology that has been gaining an increasing significance as a tool for microbial growth prediction (Huang et al., 2013). Mathematical models have been developed to describe microbial growth under varying physical or chemical conditions, such as temperature (Amir and Sinclair, 1991), light (Eroglu,

et al., 2010), pH (Rosso et al., 1995), and water activity (Davey, 1989). In order to build such predictive models, growth of bacteria has to be measured. Bacterial growth often shows a phase in which the specific growth rate begins at a value of zero and then accelerates to a maximal value (μ_m) in a certain period of time, resulting in a lag time (λ). In addition, growth curves contain a final phase in which the rate decreases gradually and finally change into zero, so that an asymptote (A) is obtained (Zwietering, et al., 1990). To describe such a curve by reducing measuring data or the number of parameters, growth models were developed and studied by many researchers (Table 4.1), such as the three-parameter models of Quadratic (Buchanan et al., 1997), Gompertz (Buchanan et al., 1997), and Logistic (Fujikawa et al., 2004), and the four-parameter models of Richards (Baranyi et al., 1994), Stannard (Skinner et al., 2007), Schnute (Francis, 1995), and others (Whiting and Cygnarowics, 1992). The abovementioned models were usually described by using the dry cell weight or the number of cells as a function of time (Zwietering, et al., 1990).

Table 4.1. Models commonly used to describe bacteria growth

Model	Equations ^a
Logistic	$y = \frac{A}{\{1 + \exp [\frac{4\mu_m}{A} (\lambda - x) + 2]\}}$
Gompertz	$y = A \exp\{- \exp [\frac{\mu_m \cdot e}{A} (\lambda - x) + 1]\}$
Quadratic	$y = ax^2 + bx + c$
Richards	$y = A\{1 + v \cdot \exp(1 + v) \cdot \exp[\frac{\mu_m}{A} (1 + v)^{(1 + \frac{1}{v})}(\lambda - x)]\}^{(-1/v)}$
Schnute	$y = (\mu_m \frac{(1 - b)}{a}) \left[\frac{1 - b \cdot \exp(a \cdot \lambda + 1 - b - ax)}{1 - b} \right]^{1/b}$

^a μ_m , λ , A, a, b, c and v are parameters of the growth models., where μ_m , λ , and A are maximal growth rate (slope of the tangent line at the inflection point), lag time (the x-axis intercept of this tangent line), asymptote (the ultimate value of $\ln (OD/OD_0)$) respectively and the others are model coefficients.

Halobacterium halobium is a halophilic bacterium living naturally in salt lakes (Walther et al., 1979; Aharon et al., 2001). This bacterium contains proton pumping protein bacteriorhodopsin (BR), which could use light energy to translocate protons across the membrane. Purified BR could be used in bioelectronic device design (Oesterhelt et al., 1991) and artificial photosynthesis (Lu et al., 2015). Previous research usually focused on *Halobacterium halobium* culturing and BR purification from the bacteria (Henry, 1982; Lee et al., 1998; Oesterhelt et al., 1974). However, so far little research has been conducted to study this bacterium under varying temperature and light conditions. Besides, modeling the growth of *Halobacterium halobium* as a function of temperature or light was not available in the literature.

Thus, the objective of this work was to develop and compare several predictive models to describe the growth of *Halobacterium holobium* as a function of temperature or light, on the basis of statistical analysis. In addition, the biological growth parameters, including specific growth rate, lag time, and asymptote of the bacteria under the chosen model were also evaluated.

4.2 Materials and methods

4.2.1 Microorganism

Bacteria *Halobacterium halobium* was purchased from American Type Culture Collection (#29341, Manassas, VA, USA) and kept under -80 °C. To begin with, bacteria (1 ml) were reactivated in a 10-ml glass tube containing 5-ml sterilized culture medium (with 250.0 g NaCl, 5.0 g HY Case SF, 5.0 g yeast extract, 20.0 g MgCl₂ 6H₂O, 2.0 g KCl and 0.2 g

CaCl₂ 2H₂O in 1-l distilled water, pH = 7.6). Then they were inoculated at 37 °C for 3-4 days, and one drop of the culture was then cultured in a polystyrene petric dish (Fisher, PA, USA) containing the above culture media plus 15%-20% agar (w/w, pH = 7.6). They were then kept in an incubator at 30 °C for 4-5 days before being transferred into a 4 °C refrigerator and kept until experiments. All chemicals were purchased from Fisher Scientific (Pittsburgh, PA, USA) except for HY Case SF, which was purchased from Sigma (St. Louis, MO, USA).

4.2.2 Bacteria culture

The starter culture from the polystyrene petric dishes was transferred to a 250-ml culturing flask containing 150-ml culture medium in a water bath. During the bacteria growth, fluorescent light (intensity = 2500 Lux) was applied above the water bath. Ten different temperatures including 21.2, 25.1, 29.2, 33.0, 37.1, 41.2, 45.3, 49.2, 53.2, and 57.0 °C with three replications each were studied.

To study the light effects, *Halobacterium halobium* was cultured in a photobioreactor system (University of Texas at Austin Culture Collection, Austin, TX, USA) by varying the intensity of the LED light. The system was consisting of three parts: (1) A 2-l cylindrical glass container with the dimensions of 26 cm (H) × 10 cm (D). (2) A magnetic stirrer underneath the glass container. (3) An LED spectral illumination platform customized with adjustable light intensity (ranging from 0 to 2030 Lux). The platform was placed surrounding the container. The starter culture from the polystyrene petric dish was first transferred to a 250 ml flask containing 150-ml culture medium. After culturing for 5 days at 37 °C, the starter culture was transferred to the 2-l photobioreactor system containing 1.3-l of the above culture medium.

Eight different light intensities, including 0, 120, 370, 570, 780, 1520, 1780, and 2030 Lux were studied with duplications.

At appropriate time intervals (depending on the time), the bacteria sample (200 ul) was taken for optical density (OD) measurement using a Synergy MX microplate reader (BioTek, Vermont, USA) via absorbance change at 660 nm. The growth curve was defined as the logarithm of the bacterial dry cell weight as a function of time. Here, the typical linear relationship between OD value and *Halobacterium halobium* dry cell weight was obtained and shown in Fig. 4.1. Thus, cell growth was expressed as the logarithm of the OD value as a function of time.

Fig. 4.1 The typical linear relationship between OD value and *halobacterium halobium* dry cell weight. The correlation equation is $y = 3.522x - 0.0746$. ($R^2=0.9965$)

4.2.3 Model development and comparison

The models (nonlinear equations) were fitted to the data of all growth curves in Matlab by nonlinear regression (with a Marquardt algorithm). Biological growth parameters, including specific growth rate, lag time and asymptote of all growth curves were estimated. Also, the set

of parameters (a, b, c and v) with the lowest residual sum of squares (RSS) and their 95% confidence bounds were calculated.

For each model, the RSS alone does not give enough information because different models have different number of parameters. Models with a greater number of parameters usually have a lower RSS. So a better method here is to determine whether it is worthwhile to add more parameters to lower RSS. Therefore, data fitted models were compared statistically by the use of the t test and F test.

First of all, all groups of data were fitted by the Schnute model and therefore parameters a and b with minimum and maximum confidence bounds were estimated. The Schnute model is a comprehensive and initial model that encompasses all of the other simpler models. As shown in Table 4.2, values or ranges of the Schnute parameters a and b are given, resulting the transformation of other models. The 95% confidence bounds of the different parameters were calculated with the values given by the t test.

Table 4.2. Selection of the model based on Schnute model

Values of a and b	Model	No. of parameters
$a > 0, b = 0$	Gompertz	3
$a > 0, b < 0$	Richards	4
$a > 0, b = -1$	Logistic	3
$a = 0, b = 0.5$	Quadratic	3
$a = 0, b = 1$	Linear	2
$a = 0, b = 0$	t th power	2
$a < 0, b = 1$	Exponential	3

The Logistic, Gompertz, Quadratic, Richards, and Schnute models were first used to fit all groups of data. Then parameters in each model were estimated by Matlab. Each predicted growth value was calculated based on the estimated parameters and the growth model. By comparing the difference between the measured value and the predicted one, RSS for each model was calculated. Under the assumption that the four-parameter Schnute model exactly predicts the number of bacteria, RSS of the Schnute model is used to estimate the prediction error, the deviation of the predicted value from the measured one. Whether a three-parameter model can be sufficient to describe the data could then be statistically validated with an F test. Here, the difference between the RSS values for the three and four parameter models was compared to the RSS of the four-parameter model. This difference is the profit obtained from adding the extra parameter. If this profit is much smaller than the prediction error calculated from Schnute model, adding more parameter is not worthwhile, as it would not be observable. However, if this profit is much greater than the prediction error, it is worthwhile to add the extra parameter. The following is then calculated:

$$f = \frac{(RSS_2 - RSS_1)/(DF_2 - DF_1)}{RSS_1/DF_1} \text{ tested against } F_{\frac{DF_2 - DF_1}{DF_1}}$$

where DF_1 is the number of degrees of freedom from the Schnute model and equals n points minus 4 (n means total number of datum points in the experiment), and DF_2 is the number of degrees of freedom from the three-parameter model and equals n points minus 3. So F test can be transformed into:

$$f = \frac{RSS_2 - RSS_1}{RSS_1/DF_1} \text{ tested against } F_{\frac{1}{DF_1}}$$

where RSS_1 is the RSS from four-parameter Schnute model. RSS_2 is the RSS from the three-parameter model, and it can be calculated by the following equation:

$$RSS_1 \text{ or } RSS_2 = \sum_{i=1}^n [y_i - f(x_i)]^2$$

where y_i is the i th value of the variable to be predicted, x_i is the i th value of the explanatory variable, and $f(x_i)$ is the predicted value of y_i .

4.3. Results and discussion

4.3.1 *Halobacterium halobium* growth under varying culture temperatures and light intensities

Figure 4.2 shows that the culture temperature had considerable influences on *Halobacterium halobium* growth. With the increase of culture temperature from 21 to 37 °C, bacterial growth (highest OD value) increased from 0.088 to 0.493 at 168 h. This result was consistent with other researchers that 37 °C was the optimum culture temperature for *Halobacterium halobium* (Henry, 1982; Lee et al., 1998; Oesterhelt et al., 1974). When the temperature was higher than 37 °C, bacterial growth was inhibited and the highest OD value was reduced to only 0.174 at 57 °C. This may be due to a variety of reasons such as inactivation or denaturation of proteins and instability or no synthesis of RNA inside of the bacteria (Ratkowsky et al., 1982). Also, the cytoplasmic membrane of the bacteria, which is composed of lipids and proteins that are often glycosylated may collapse at higher culturing temperatures. There were some evidences to suggest that higher temperature could decompose or break the glycosylated lipid component, making the cytoplasmic membrane of the bacteria loose and even collapsed (Quinn, 1988).

Fig. 4.2. *Halobacterium halobium* growth at varying culture temperatures (each point is the average of three replicates)

Figure 4.3 shows *Halobacterium halobium* growth under varying light intensities. Results suggested that the bacteria grew better in LED white light (highest OD = 0.524) than in the dark (highest OD = 0.282), and higher light intensities improved bacterial growth. This result was consistent with previous findings that higher light intensities were favored by bacteria *Halobacterium halobium* (Hartmann et al., 1980; Henry, 1982).

Fig. 4.3 *Halobacterium halobium* growth at varying light intensities. (Each point is the average of two replicates)

4.3.2 t test of *Halobacterium halobium* growth modeling as a function of temperature

Modeling results of the parameters estimation for 30 groups of growth data can be seen from Table 4.3. With the use of Schnute model t test value, the 95% confidence bounds for the parameters a and b were calculated. The lower 95% confidence bounds of the parameter a ($a_{\min}$) ranged from 0.014 to 0.145, which suggested that parameter a was greater than 0. Then the lower and higher 95% confidence bounds of parameter b were also estimated and shown in Table 4.3 and Fig. 4.4. In all cases, value of zero was within the 95% confidence bounds of b ($b_{\min} < 0$ and $b_{\max} > 0$). So it can be concluded that parameters $a > 0$ and $b = 0$ were obtained in all 30 groups of data, and these were suitable for the Gompertz model. Thus the Gompertz model was 100% accepted in all cases by the t test (Table 4.4). In a similar way, the Richards model ($a > 0$ and $b < 0$) was also 100% suitable for all the cases. However, the Logistic model was only 21% accepted by t test analysis, since the value of -1 (Fig. 4.4 shows) was much less frequent within the confidence bounds (only 7 times). Even no case was found to be accepted in the Linear, Quadratic, t th power and Exponential models and thus these models were rejected.

Table 4.3. Statistical-analytical data for 30 growth curves of *Halobacterium halobium*

Temp (°C)	$a_{\min}^{a,b}$	$b_{\min}^{a,b}$	$b_{\max}$	f value		F table ^c	RSS ₂		
				Gompertz	Logistic		Gompertz	Logistic	Richards
21.2	0.015	-0.358	1.886	0.400	0.800	7.709	0.011	0.012	0.010
21.2	0.016	-0.354	1.804	2.621	3.310	7.709	0.048	0.053	0.037
21.2	0.014	-0.357	1.892	0.848	2.780	7.709	0.040	0.071	0.037
25.1	0.016	-0.416	1.902	1.034	7.977	4.667	0.095	0.142	0.093
25.1	0.019	-0.427	1.925	2.116	6.651	4.667	0.150	0.195	0.148
25.1	0.022	-0.388	1.965	0.118	0.236	4.667	0.111	0.112	0.111
29.2	0.040	-0.188	1.388	2.869	6.694	4.451	0.187	0.223	0.181
29.2	0.038	-0.194	1.313	1.878	6.669	4.451	0.201	0.252	0.192

Table 4.3 Continued

29.2	0.027	-0.187	1.314	3.490	5.742	4.451	0.182	0.202	0.172
33.0	0.068	-0.427	0.726	1.906	10.059	4.414	0.188	0.265	0.176
33.0	0.071	-0.417	0.742	2.185	7.179	4.414	0.194	0.242	0.190
33.0	0.068	-0.423	0.734	0.918	15.061	4.414	0.103	0.180	0.102
37.1	0.089	-0.620	0.843	5.417	22.083	4.351	0.122	0.202	0.098
37.1	0.087	-0.623	0.812	3.256	8.837	4.351	0.150	0.186	0.132
37.1	0.080	-0.601	0.839	0.870	6.377	4.351	0.144	0.182	0.142
41.2	0.090	-1.091	0.602	2.222	25.926	4.351	0.030	0.062	0.029
41.2	0.112	-0.670	0.609	3.333	19.333	4.351	0.035	0.059	0.032
41.2	0.129	-0.718	0.707	2.885	6.923	4.351	0.119	0.140	0.109
45.3	0.094	-0.315	1.134	1.463	2.927	4.351	0.044	0.047	0.042
45.3	0.117	-0.382	0.905	1.243	1.921	4.351	0.188	0.194	0.180
45.3	0.109	-0.415	0.902	0.648	3.148	4.351	0.223	0.250	0.218
49.2	0.084	-0.705	0.645	2.485	7.846	4.451	0.149	0.190	0.141
49.2	0.082	-0.674	0.716	1.631	2.061	4.451	0.217	0.222	0.213
49.2	0.080	-0.710	0.644	1.473	6.007	4.451	0.163	0.203	0.154
53.2	0.102	-1.105	0.833	1.528	7.067	4.451	0.194	0.252	0.189
53.2	0.066	-1.118	0.821	1.591	3.099	4.451	0.222	0.240	0.210
53.2	0.124	-1.073	0.633	3.008	4.054	4.451	0.153	0.161	0.139
57.0	0.129	-1.033	1.032	1.717	2.821	4.667	0.120	0.129	0.117
57.0	0.112	-1.002	0.938	0.990	2.352	4.667	0.113	0.124	0.111
57.0	0.145	-1.012	1.238	1.324	2.046	4.667	0.119	0.125	0.110

^a a and b are Schnute parameters.

^b min and max are 95% confidence bounds.

^c Right-tail probability (α) = 0.05.

Fig. 4.4 Confidence bounds of b for *Halobacterium halobium* data fitted with the Schnute model (Experiment number, Exp. No).

Table. 4.4. t test results for *Halobacterium halobium* growth based on the Schnute model

Values of a and b	Model	No. (% of total) of results accepted with:	
		t test	F test
a>0, b=0	Gompertz	30 (100%)	29 (97%)
a>0, b<0	Richards	30 (100%)	
a>0, b=-1	Logistic	7 (21%)	13 (43%)
a=0, b=1	Linear	0 (0)	
a=0, b=0.5	Quadratic	0 (0)	
a=0, b=0	<i>t</i> th power	0 (0)	
a<0, b=1	Exponential	0 (0)	

4.3.3 F test of *Halobacterium halobium* growth modeling as a function of temperature

F test results for the Gompertz and Logistic models, as well as F table values are shown in Table. 4.3. With the use of F test, the values calculated were compared with F table values, which are the critical ones of the F-distribution for false-rejection probability ($\alpha = 0.05$). For the Gompertz model, F test values were all lower than F table values except for 1 case, and accepted results were 97% of all of the cases (Fig. 4.5). This 3% rejection level may be expected with a 95% confidence level. However, for Logistic model, it was accepted by F test with only 43% (13 out of 30). This lower value suggested that the Logistic model may not be suitable to describe the bacterial growth. In addition, Richards model was not considered here since it has the same number of degree of freedom with Schnute model.

RSS values of Gompertz, Logistic and Richards models are also shown in Table 4.3. Results showed that RSS values for the four-parameter models (Richards) were always lower than the RSS values for the three-parameter models (Gompertz and Logistic). Also, RSS₂ values of Richards model were always smaller than the Gompertz model (Table 4.3). But the

differences between the two models were small and the Gompertz model was still fitting the data acceptably (Fig. 4.6). In all cases, RSS_2 of the Gompertz model were all smaller than the Logistic model, which suggested that the former was better fitted with the data.

Fig. 4.5. Results of an F test for *Halobacterium halobium* growth modeling

Fig. 4.6. Growth curves of *Halobacterium halobium* fitted with Gompertz, Logistic and Richards models (at 37.1°C)

So, results from the t test and F test of *Halobacterium halobium* growth modeling (under temperature effect) showed that the Gompertz model was 100% accepted by the t test and 97% accepted by the F test (Table. 4.4); therefore, the Gompertz model can be regarded as sufficient to describe the growth curves of *Halobacterium halobium*. The Logistic model, however, seemed not to be sufficient to describe the data, since it was only accepted in 21% of the cases by the t test and in 43% of the cases by the F test with *Halobacterium halobium* growth.

4.3.4 t test of *Halobacterium halobium* growth modeling as a function of light

Modeling results of the parameters (a and b) estimation for 16 groups of growth data can be seen from Table 4.5. It can be found that value of zero was within the 95% confidence bounds of a ($a_{\min} < 0$ and $a_{\max} > 0$) except for four cases. Also, value of 0.5 was within the 95% confidence bounds of b ($b_{\min} < 0.5$ and $b_{\max} > 0.5$). So it can be concluded that parameters $a = 0$ and $b = 0.5$ were obtained in 12 groups of data, and these were suitable for the Quadratic model. So the Quadratic model was accepted in most of the cases (75%) by t test (Table 4.6). However, for Gompertz and Richards models, they were only 25% accepted by the t test for all the cases. Even no case was found to be accepted by other models (Logistic, Linear, Exponential and t th power).

Table 4.5. t test analysis for 16 growth curves of bacteria *Halobacterium halobium*

Light intensity (Lux)	$a_{\min}^a$	$a_{\max}$	$b_{\min}$	$b_{\max}$
0	0.022	0.034	-0.022	0.623
0	0.010	0.044	-0.020	0.623
120	-0.021	0.027	0.220	0.560
120	0.019	0.040	-0.130	0.568

Table 4.5 Continued

370	0.013	0.046	-0.119	0.585
370	-0.021	0.032	0.220	0.595
570	-0.021	0.028	0.226	0.602
570	-0.022	0.027	0.222	0.637
780	-0.019	0.022	0.457	0.670
780	-0.013	0.018	0.432	0.668
1520	-0.016	0.019	0.366	0.637
1520	-0.014	0.016	0.360	0.630
1780	-0.013	0.019	0.354	0.733
1780	-0.014	0.020	0.349	0.730
2030	-0.015	0.019	0.467	0.723
2030	-0.014	0.020	0.463	0.700

^a min and max are 95% confidence bounds.

Table. 4.6. t test results for *Halobacterium halobium* based on Schnute model

Values of a and b	Model	No. (% of total) of results accepted with:	
		t test	F test
a>0, b=0	Gompertz	4 (25%)	15 (94%)
a>0, b<0	Richards	4 (25%)	
a=0, b=0.5	Quadratic	12 (75%)	15 (94%)
a>0, b=-1	Logistic	0 (0)	
a=0, b=1	Linear	0 (0)	
a=0, b=0	<i>t</i> th power	0 (0)	
a<0, b=1	Exponential	0 (0)	

4.3.5 F test of *Halobacterium halobium* growth modeling as a function of light

In addition, F test results for Gompertz and Quadratic model, as well as F table values are shown in Table 4.7. For the Quadratic model, F test values were all lower than F table values except for 1 case, and accepted results were 94% of all of the cases. This 6% rejection level

may be expected with a 95% confidence level. At the same time, the Gompertz model was also 94% accepted by F test for all the cases. This result suggested that Gompertz model was also suitable to describe the bacterial growth.

RSS values of Gompertz, Quadratic and Richards models are also shown in Table 4.7. Results showed that RSS values for the four-parameter models (Richards) were always lower than the RSS values for the three parameter models (Gompertz and Quadratic). RSS₂ differences between the Gompertz and Quadratic models were very small and these two models fitted the data acceptably.

Table 4.7. F test analysis for 16 growth curves of *Halobacterium halobium*

Light intensity (Lux)	f value		F table *	RSS ₂		
	Gompertz	Quadratic		Gompertz	Quadratic	Richards
Dark	2.429	2.429	4.451	0.016	0.016	0.014
Dark	1.789	1.789	4.451	0.021	0.020	0.019
120	7.792	4.250	4.451	0.035	0.030	0.031
120	3.988	1.259	4.451	0.100	0.087	0.085
370	3.676	2.757	4.451	0.045	0.043	0.041
370	1.434	4.301	4.451	0.090	0.104	0.085
570	1.102	1.731	4.451	0.115	0.119	0.111
570	3.178	4.766	4.451	0.127	0.137	0.114
780	4.220	3.450	4.279	0.071	0.069	0.063
780	3.932	2.949	4.279	0.137	0.132	0.120
1520	1.250	3.500	4.301	0.093	0.102	0.090
1520	3.907	3.084	4.301	0.126	0.122	0.114
1780	2.953	3.938	4.325	0.073	0.076	0.070
1780	3.102	4.295	4.325	0.101	0.106	0.090
2030	2.007	3.860	4.325	0.149	0.161	0.140
2030	2.222	2.453	4.325	0.153	0.146	0.145

* Right-tail probability (α) = 0.05.

Thus, results from t test and F test of *Halobacterium halobium* growth modeling (under light effect) showed that the Quadratic model was 75% accepted by the t test and was 94% accepted by the F test; therefore, the Quadratic model can be regarded as sufficient to describe the growth curves of *Halobacterium halobium*. However, this model has its drawback that the equation only contains simple mathematical parameters (a, b and c), and parameters with the biological meaning (μ_m , λ and A) are not included. Thus, Quadratic model has no biological meaning since it could not exhibit growth parameters change with the culturing time. However, Gompertz model not only exhibits growth parameters (μ_m , λ and A) with a biological meaning, but also has the better result for F test analysis (94% accepted of all cases). Even though this model was not well accepted by t test, it was chosen to better describe the growth of bacteria *Halobacterium halobium* as a function of light.

Here, three-parameter models gave no difficulties in finding the least-square parameters. In most of the situations, the Gompertz model can be regarded as the best model to describe the growth data of *Halobacterium halobium* as a function of temperature or light. Though four-parameter model (Richards model) obtained smaller RSS, the Gompertz model is still recommended to use because a less correlated model is more stable. Also, fewer-parameter models could have higher degree of freedom, which is important to predict bacterial growth condition by using a smaller number of experiments.

4.3.6 Biological growth parameters of *Halobacterium halobium* under Gompertz modeling

Fig. 4.7 (A) shows the specific growth rate change under Gompertz modeling affected by temperature. With the increase in culture temperature from 21.2 to 37.1 °C, the specific growth rate increased from 0.006 to 0.022 h⁻¹. When the temperature was beyond this point (37.1 °C), bacterial growth was inhibited and the specific growth rate gradually reduced. This may be due to inactivation or denaturation of proteins, instability or no synthesis of RNA at higher culturing temperature (Ratkowsky et al., 1982). Fig. 4.7 (B) shows the specific growth rate change under Gompertz modeling affected by light. With the increase in culture light intensity from 0 to 2030 Lux, the specific growth rate increased from 0.012 to 0.019 h⁻¹.

Fig. 4.7. Specific growth rate of *Halobacterium halobium* under Gompertz modeling. (A) Specific growth rate change as a function of temperature. (B) Specific growth rate change as a function of light

Fig. 4. 8 (A) shows the asymptote change under the Gompertz model affected by temperature. Below 37.1 °C, asymptote value increased with increasing temperatures, and the highest asymptote value was 1.757. Then the asymptote decreased due to denaturation of

proteins at higher temperatures. These results indicated that temperature had considerable influences on asymptote of bacterial growth. Fig. 4.8 (B) shows the asymptote change of bacteria under the Gompertz model affected by light. With the increase in culture light intensity from 0 to 2030 Lux, asymptote increased from 1.55 to 2.09, meaning that higher light intensities were preferred.

Fig. 4. 8. Asymptote of *Halobacterium halobium* under Gompertz model. (A) Asymptote change as a function of temperature. (B) Asymptote change as a function of light

In this part, the lag time data showed a large prediction error between the replicated samples. To limit the influences, a logarithmic transformation was used on the data obtained from Gompertz modeling, since this method could reduce the difference between the replicates. Fig. 4.9 (A) shows the lag time change under the Gompertz model affected by temperature. Results showed that lag time ($\ln \lambda$) decreased from 3.27 to 1.36 h with increased temperatures from 21.2 to 49.2°C. After that, lag time ($\ln \lambda$) increased due to denaturation at higher temperatures. This result was consistent with previous findings by Kandhai et al., (2006) and Wijtzes et al., (1995). They studied and modeled bacterial growth of *Enterobacter sakazakii* and *Lactobacillus curvatus* as a function of temperature, and results showed that lag

time of these two bacteria decreased with the increasing temperature conditions. In addition, the hyperbolic equation was found to better describe the relationship between the lag time and temperature. Fig. 9 (B) shows the lag time change of bacteria under the Gompertz model affected by light. With the increase in culture light intensity from 0 to 2030 Lux, lag time ($\ln \lambda$) decreased from 1.90 to 1.07 h.

Fig. 4.9. Lag time change of *Halobacterium halobium* under Gompertz modeling. (A) Lag time change as a function of temperature. (B) Lag time change as a function of light

4.4 Conclusions

This study for the first time applied three-parameter models (Quadratic, Logistic and Gompertz) and four-parameter models (Schnute and Richards), to describe the growth of bacteria *Halobacterium halobium* as a function of temperature or light. They were compared statistically by using t test and F test. Results showed Gompertz model was considered statistically sufficient to describe the growth of *Halobacterium halobium* affected by temperature or light. In addition, the biological growth parameters change under Gompertz modeling were evaluated. Results showed that when the temperature effect was considered, the biological growth parameters, including specific growth rate (μ_m) and asymptote (A)

increased with the increase of culturing temperatures, and then decreased due to inactivation or denaturation of proteins in higher temperatures. Lag time (λ) decreased with increased of culturing temperatures. When the light effect was considered, both specific growth rate (μ_m) and asymptote (A) increased with the increase of light intensities, while lag time (λ) was decreased.

4.5 Acknowledgements

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CHAPTER 5 - The effect of culture conditions on the accumulation and activity of F_0F_1

ATP synthase in thermophilic bacteria *Bacillus* PS3

Abstract

*The objective of this study was to understand the accumulation and ATPase activity of F_0F_1 ATP synthase in thermophilic bacteria *Bacillus* PS3 affected by culture conditions, including the type of nitrogen source and the concentrations of polypeptone and sodium chloride in the culture medium, as well as the medium pH and aeration rate. The highest yield and ATPase activity of purified enzymes were obtained when adding polypeptone as the nitrogen source. Contrarily, sodium chloride concentration had little effect on the growth of PS3 or F_0F_1 ATP synthase accumulation. Medium pH and aeration rate showed significant effects on the accumulation and activity of F_0F_1 ATP synthase. The highest yield and activity of purified F_0F_1 ATP synthase were 1.631 g l^{-1} and $7.99 \mu\text{mol min}^{-1} \text{ mg}^{-1}$, under the culture conditions of pH 7, aeration of 3 l min^{-1} and 0.3% NaCl and 1.2% (w/w) polypeptone concentrations. The F_0F_1 ATP synthase purified from bacteria PS3 was characterized by SDS-PAGE.*

5.1. Introduction

The proton-translocating protein F_0F_1 ATP synthase, purified from the thermophilic bacteria *Bacillus* PS3, is a transmembrane protein that catalyzes ATP synthesis (Kagawa, 1978; Kagawa et al., 1979; Richard et al., 1995). The catalytic mechanism of F_0F_1 ATP synthase has been studied extensively (Daniela et al., 1999; Yoshida et al., 2001; Elston et al., 1998; Wang et al., 1998). This enzyme is composed of two sectors, F_0 and F_1 (Elston et al., 1998; Pederson et al., 1993). The former is composed of three different subunits designated as a, b and c, with

the molecular weight of 23.48, 12.30 and 7.34 kDa (Schneider et al., 1984). Its function is to conduct the proton go across the membrane. The latter contains five subunits which are α , β , γ , δ and ϵ , with the molecular weight of 54.59, 51.94, 31.78, 19.66 and 14.33 kDa (Vignais et al., 1984). Purified F_1 has been the object of intensive research on the mechanism of ATP hydrolysis (Wang et al., 1998; Boyer, 1997).

F_0F_1 ATP synthase has been largely studied in ATP motivated glucose synthesis in artificial photosynthesis (Choi et al., 2006; Choi et al., 2006; Hazard et al., 2002; Lu et al., 2015). Artificial photosynthesis can generally be divided into two parts: the light-dependent and the light-independent reactions. In light-dependent reactions, ATP synthesis is catalyzed by F_0F_1 ATP synthase. Proton gradient generated by electron transfer complexes and light-harvesting proteins provides the energy for the synthesis of ATP. In light-independent reactions, ATP is used to fix carbon in carbon dioxide into the energy storing carbon compounds and then glucose, catalyzed by a suit of enzymes.

Preparation of F_0F_1 ATP synthases has been reported from several bacteria, including *Bacillus* PS3 (Yoshida et al., 1975; Kagawa et al., 1979; Kagawa et al., 1979; Sone et al., 1975), *E. coli* (Foster et al., 1979), *Streptococcus faecalis* (Leimgruber, et al., 1981), *Mycobacterium phlei* (Lee et al., 1976), and *Clostridium Pasteurianum* (Clarke et al., 1979). From above sources, enzymes purified from *Bacillus* PS3 has been characterized the most (Richard et al., 1995). Kagawa et al. (1979) obtained F_0F_1 ATP synthase with specific ATPase activity of 17.6 $\mu\text{mol min}^{-1} \text{mg}^{-1}$ by using five purification steps, including membrane washing, cholate dissolving, triton X-100 extracting, DEAE eluting and agarose eluting. Hazard et al. (2002) reported a fast and simple purification method for thermophilic F_0F_1 ATP

synthase with a higher ATPase activity of $41 \text{ } \mu\text{mol min}^{-1} \text{ mg}^{-1}$. Only one column purification (DEAE) step instead of two was used to obtain high-activity enzyme. They also successfully incorporated F_0F_1 ATP synthase and BR into liposomes and measured stable electrochemical gradient and ATP generation. Sone et al. (1975) purified F_0F_1 ATP synthase from the membrane of the bacterium PS3 in the presence of triton X-100 and found that ATPase activity of enzyme was maximal at 70°C at pH 8.6 and this activity was dependent on Mg^{2+} , Co^{2+} , or Mn^{2+} .

However, both the yield and ATPase activity of the purified F_0F_1 ATP synthase can be affected by culture conditions, e.g., the nitrogen source, the concentrations of polypeptone and sodium chloride in the culture medium, medium pH, and the aeration rate during bacterial cultivation, which have not been examined in details. Thus the main objective of this research was to understand the effect of different culture conditions, including different nitrogen source (polypeptone, peptone, potassium nitrate and ammonium chlorides), polypeptone concentration (0, 0.4, 0.8 and 1.2%, w/w, once we knew it was the optimum nitrogen source), sodium chloride concentration (0, 0.1, 0.3, and 0.5%, w/w), aeration rate (2, 3, 4, 5 l min^{-1}) and medium pH (6, 7 and 8) on thermophilic bacteria *Bacillus* PS3 accumulation and ATPase activity of purified F_0F_1 ATP synthase. The purified F_0F_1 ATP synthase from the optimum culture condition was characterized with sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE).

5.2. Materials and methods

5.2.1 Microorganism

Thermophilic bacteria *Bacillus* PS3 was obtained from Drs. Toshiharu Suzuki and Masasuke Yoshida (Waseda University, Japan) and kept under -80 °C for reservation. To begin with, PS3 (1 ml) was reactivated in a 10-ml glass tube containing 5-ml sterilized culture medium (0.8% polypeptone, 0.4% yeast extract and 0.3% sodium chloride, w/w). Then they were inoculated in an incubator (Eppendorf, NY, USA) at 68 °C. After shaking for 12-15 h, this culture was transferred to a 250-ml flask containing 50-ml abovementioned medium. After shaking for 12 h, one drop of the culture was cultured in 60 mm × 15 mm polystyrene petric (Fisher, PA, USA) containing 0.8% polypeptone, 0.4% yeast extract, 0.3% NaCl and 15%-20% agar (w/w). They were then kept in another incubator under 30 °C. After slowly growing for 2-3 days, they were transferred into a 4 °C refrigerator.

5.2.2 Bacteria culture conditions

Thermophilic bacteria PS3 was cultured in the medium containing 0.8% polypeptone, 0.4% yeast extract, and 0.3% NaCl (w/w, pH = 7). The starter culture from polystyrene petric (all the bacteria colonies) in a 300 ml flask (100-ml medium inside) was inoculated in an incubator shaker at 68 °C. After shaking for 12-15 h, a portion of the starter culture (about 40 ml) was transferred to a 1-liter flask containing 360 ml of the above medium. After shaking for 12-14 h, this culture was transferred to a 5 liters Bioflo/CelliGen 115 fermenter (Eppendorf, NY, USA) containing 3.6 liters of the culture medium. The fermenter was controlled at 68 °C and aerated by Whisper 30-60 air pump (Tetra, USA) with filter-sterilized air. Different nitrogen

sources (polypeptone, peptone, potassium nitrate and ammonium chlorides), polypeptone concentrations (0, 0.4, 0.8 and 1.2%, w/w, once we knew it was the optimum nitrogen source), sodium chloride concentrations (0, 0.1, 0.3, and 0.5%, w/w), aeration rates (2, 3, 4, 5 l min⁻¹) and medium pH (6, 7 and 8) were studied.

5.2.3 F₀F₁ ATP synthase purification from bacteria PS3

After culturing, bacteria PS3 was separated from the medium by RC-3B refrigerated centrifuge (Dupont, GA, USA). The cells were then re-suspended in 70-ml purification buffer (50 mM Tris-sulfate, pH 8.0, 5 mM paminobenzamidine, 40 mM ϵ amino n-carproic acid, 0.5 mM DTT, 0.5 mM EDTA). Lysozyme (10 mg) was added into the beaker and they were stirred for 30 min at 37 °C. DNA was hydrolyzed by incubation of lysed cells with 0.5 ml of 1 M MgCl₂ and 0.2 mg of DNase I. After 2 hours, the resulting lysate was centrifuged by WX Ultra 80 centrifuge (Fisher, PA, USA) at 18000 g for 30 min. The precipitate membrane was homogenized and washed three times with 20 ml purification buffer and sedimented at 26000g for 30 min because thorough washing was essential to remove soluble proteins. The washed membranes of the thermophilic bacterium were re-suspended in the purification buffer containing 1.5 % sodium cholate and 1.5% sodium deoxycholate (w/w) and 0.25 M Na₂SO₄, at a protein concentration of 8-10 mg ml⁻¹. After incubation at room temperature for 60 min with stirring, the mixture was centrifuged at 100000 g for 40 min. The yellow supernatant fraction which contained approximately 20%-30% of the ATPase was discarded. Then the precipitate was re-suspended in 2-3 ml solubilization buffer (purification buffer containing 0.1 M sucrose, 15% glycerol) and frozen overnight at -80°C. Extracted membranes were thawed and the protein concentration was determined. The volume of solubilization buffer needed to

bring the protein concentration down to 10 mg ml⁻¹ was determined. After that solid n-dodecyl β -D-maltoside was added to this solubilization buffer such that the final concentration of detergent was 30 mM. The sample and buffer were combined and sonicated by a sonicator (Fisher, PA, USA) on ice at an output of 100 W for 1 min, then stirred at room temperature for 30 min, and centrifuged at 108000 g for 60 min. Finally, purified F₀F₁ ATP synthase was collected from the supernatant part of the solution. Tris-sulfate, ϵ amino n-carproic acid, DTT, MgCl₂, Na₂SO₄, sucrose and glycerol were purchased from Fisher Scientific (Pittsburgh, PA, USA). Paminobenzamidine, EDTA, n-dodecyl β -D-maltoside, DNase I and sodium cholate were purchased from Sigma (St. Louis, MO, USA).

5.2.4 SDS-PAGE

The running buffer was prepared by adding 1.51 g Tris-Base, 7.2 g glycine and 0.5 g SDS into 500 ml DI water. The pH of the running buffer was adjusted to 8.3 by adding 0.5 M NaOH solution. Then sample buffer was prepared by adding 2 g SDS, 1 ml 2-mercaptoethanol, 0.6 g Tris-base, 30 ml glycerol and 0.002 g bromophenol blue into 100 ml DI water. After that, 20- μ l sample buffer and 20- μ l low molecular mass standard were mixed with 20 μ l sample solution in the microcentrifuge tubes and they were incubated on the heat block at 90°C for 10 min. The incubated samples were centrifuged for 1 min. Molecular mass standard with (8 μ l) and samples (20 μ l) were loaded into the 10% precise Tris-glycine gel. After that, 150 ml running buffer was added into Owl electrophoresis system connected with power supplies. The gel was run at 200 V, 30 mA until the bromophenol blue tracking dye approached the bottom of the gel. This electrophoresis process was kept in 60-90 min. Then the gel was disassembled from the plate and was rinsed 3 $\times$ 5 minutes with 100-200 ml DI water. Gelcode blue reagent was

added for staining and this process was kept for several hours. After that, the gel was destained with DI water 3-4 times. The Owl electrophoresis system (Catalog Number: P81), 10% precise Tris-glycine gels (Catalog Number: PI1879433A), gelcode blue reagent (Catalog Number: 24592), power supplies (Catalog Number: FB 300) and other analytical chemicals were purchased from Fisher Scientific (Pittsburgh, PA, USA). Low molecular mass standard was purchased GE Healthcare (Catalog Number: 17-0446-01, Alpharetta, GA, USA).

5.2.5 Analytical methods

The thermophilic bacteria *Bacillus* PS3 growth was monitored hourly by using a Synergy MX microplate reader (BioTek, Vermont, USA) via absorbance change at 600 nm (Sone et al., 1991). The typical linear relationship between optical density and PS3 dry cell weight was shown in Fig. 5.1. Protein concentration was determined by the method of Lowry et al. (1951). Total ATPase activity was defined as the enzyme catalyzed the turnover of 1 μ mol of ATP per minute under reaction conditions and was determined following Kagawa et al. (1979). Specific ATPase activity was calculated by total ATPase activity over protein amount.

5.2.6 Statistical analysis

The results of the present study were expressed as mean of two replicates $\pm$ standard deviation. Statistical analysis was performed by one-way ANOVA and Turkey test with SAS (Cary, NC, USA). A p-value of 0.05 was considered significant.

Fig. 5.1. Typical linear relationship between OD and PS3 dry cell weight at all experiments (Original one and 100% diluted after bacteria culturing are included). The linear equation is $y = 3.1185x - 0.0004$. ($R^2=0.9945$)

5.3. Results and discussion

5.3.1 The effect of nitrogen source

Peptone and polypeptone were better nitrogen sources for PS3 growth, as shown in Fig. 5.2 and Table 5.2. Compared with potassium nitrate and ammonium chloride, peptone is the main nitrogen sources in culture media by providing plenty of amino acids, oligopeptides, vitamins and trace elements, which are the most nutrient-filled habitats for growing microorganisms and cells (Zhang, et al., 1994; Keay, et al., 1977; Bassi, et al., 2007). Similar result was also found by Zhang et al. (1994). They found that addition of peptone into the culture media resulted in over 20% increase of WuT3 hybridoma cell densities. Fig. 5.2 also shows that PS3 grew fastest when polypeptone was supplied in the medium. Polypeptone is a mixture of peptones made up of equal parts of pancreatic digest of casein and extra peptic digest of animal tissue. Polypeptone can be used in the culture medium as a superior source of nutrients for the bacterial growth, and it is better than just individual peptones used (Bassi, et

al., 2007). Similar results were also found by Pokhrel et al. (2007). They studied the effect of various nitrogen sources, including peptone, polypeptone and other inorganic chemicals on the production of mycelia in terms of dry weight, exo-polysaccharide (EPS) by *Lyophyllum decastes*, and results showed that less mycelial and EPS yields were achieved from peptone compared with polypeptone.

The total protein content, total ATPase activity and specific ATPase activity of the proteins obtained in every step of purification of the four nitrogen sources are shown in Table 5.1. As expected, the total protein content and total ATPase activity decreased when the proteins were more purified, but the specific ATPase activity increased in polypeptone or peptone enriched media. Polypeptone medium resulted in the highest protein content (52.03 mg), total ATPase activity ($413.12 \mu\text{mol min}^{-1}$) and specific ATPase activity ($7.94 \mu\text{mol min}^{-1} \text{mg}^{-1}$) among all the nitrogen sources tested.

Fig. 5.2. The growth curves of PS3 in 3.6-liter culture medium (pH=7, aeration of 3 l min^{-1}) containing 0.4% yeast extract, 0.3% NaCl and varying nitrogen sources. Nitrogen concentration for polypeptone, peptone, potassium nitrate and ammonium chloride were maintained at the same level.

Table 5.1. F₀F₁ ATP synthase purification in different nitrogen source

Nitrogen source	Purification step	Total protein content (mg)	Total ATPase activity (umol/min)	Specific ATPase activity (umol/min/mg)
Polypeptone	Cell lysate	1014.70	1308.96	1.29
	Washed membranes	293.42	980.02	3.34
	Detergent extract	178.47	542.41	3.04
	Solubilized enzyme	52.03	413.12	7.94
Peptone	Cell lysate	766.70	843.37	1.10
	Washed membranes	157.18	462.11	2.94
	Detergent extract	120.13	330.35	2.75
	Solubilized enzyme	47.12	179.98	3.82
KNO ₃	Cell lysate	710.83	710.83	1.00
	Washed membranes	132.23	372.88	2.82
	Detergent extract	91.47	288.14	3.15
	Solubilized enzyme	30.61	82.35	2.69
NH ₄ Cl	Cell lysate	699.88	699.88	1.00
	Washed membranes	141.01	401.87	2.85
	Detergent extract	72.43	201.35	2.78
	Solubilized enzyme	26.00	61.62	2.37

Table 5.2. Maximum bacterial OD value and enzyme activity in different nitrogen source

Nitrogen source	Polypeptone	Peptone	KNO ₃	NH ₄ Cl
Peak OD	0.49 ± 0.01 ^a	0.35 ± 0.01 ^b	0.28 ± 0.01 ^c	0.24 ± 0.01 ^d
Specific ATPase activity (umol/min/mg)	7.94 ± 0.42 ^a	3.82 ± 0.33 ^b	2.69 ± 0.32 ^c	2.37 ± 0.26 ^c

*Different letters indicate significant differences ($p < 0.05$).

5.3.2. The effect of polypeptone concentration

Fig. 5.3 shows PS3 growth in varying concentrations of polypeptone. PS3 grew better when adding more polypeptone into the culture medium (Table 5.4). It can be noted that PS3 grew only a little (optical density from 0.07 to 0.20) without polypeptone in the medium.

Stepwise purification of F₀F₁ ATP synthase from the above cultured PS3 was summarized in Table 5.3. For all three series of experiments (0.4%, 0.8%, and 1.2%, w/w), the purification steps appeared to increase specific ATPase activity of the enzyme. Highest protein content (56.98 mg), total ATPase activity (455.31 $\mu\text{mol min}^{-1}$) and specific ATPase activity (7.99 μmol

min⁻¹ mg⁻¹) were obtained from 1.2% polypeptone (w/w) among all the nitrogen sources used. Nitrogen is a key factor among all nutrients to support bacterial growth since it is used for the synthesis of proteins, amino acids, DNA, and RNA of the bacterium (Henriksen *et al.*, 1999). In addition, increasing N availability also stimulated the enzyme activities, that the exocellulase ($R^2 = 0.85$) and endocellulase ($R^2 = 0.60$) activities were correlated with the estimates of the active microbial biomass (Henriksen *et al.*, 1999).

Fig. 5.3. The growth curves of PS3 in 3.6-liter culture medium (pH=7, aeration of 3 l min⁻¹) containing 0.4% yeast extract, 0.3% NaCl and varying polypeptone concentrations.

Table 5.3. F₀F₁ ATP synthase purification in different polypeptone concentration

Polypeptone (w/w)	Purification step	Total protein content (mg)	Total ATPase activity (umol/min)	Specific ATPase activity (umol/min/mg)
0%	Cell lysate	236.61	217.68	0.92
	Washed membranes	65.39	113.12	1.73
	Detergent extract	44.88	52.96	1.18
	Solubilized enzyme	31.02	46.22	1.49
0.4%	Cell lysate	741.39	874.85	1.18
	Washed membranes	166.88	532.35	3.19
	Detergent extract	127.99	359.65	2.81
	Solubilized enzyme	51.03	234.24	4.59
0.8%	Cell lysate	1014.70	1308.96	1.29
	Washed membranes	293.42	980.02	3.34
	Detergent extract	178.47	542.41	3.04
	Solubilized enzyme	52.03	413.12	7.94

Table 5.3 Continued

1.2%	Cell lysate	1120.66	1501.69	1.34
	Washed membranes	309.62	990.77	3.20
	Detergent extract	180.27	535.39	2.97
	Solubilized enzyme	56.98	455.31	7.99

Table 5.4. Maximum bacterial OD value and enzyme activity in different polypeptone concentration

Polypeptone concentration (w/w)	0%	0.4%	0.8%	1.2%
Peak OD	0.52 ± 0.01 ^a	0.49 ± 0.01 ^b	0.39 ± 0.01 ^c	0.20 ± 0.01 ^d
Specific ATPase activity (umol/min/mg)	7.99 ± 0.16 ^a	7.94 ± 0.42 ^a	4.59 ± 0.23 ^b	1.49 ± 0.26 ^c

*Different letters indicate significant differences ($p < 0.05$).

5.3.3. The effect of sodium chloride concentration

The effect of sodium chloride concentrations on PS3 growth can be seen from Fig. 5.4. Results showed that no significant differences were observed within bacterial growth. In Table 5.5, the specific ATPase activities of purified enzymes and the total protein content were similar at different NaCl concentrations in culture medium. Statistical analysis results revealed enzyme activities had no significant difference by adding different concentration of sodium chloride into the culturing medium (Table 5.6). These results indicate that NaCl had little effect on the yield of ATPase activity of F_0F_1 ATP synthase.

Fig. 5.4. The growth curves of PS3 in 3.6-liter culture medium (pH=7, aeration of 3 l min⁻¹) containing 0.8% polypeptone, 0.4% yeast extract and varying sodium chloride concentrations.

Table 5.5. F₀F₁ ATP synthase purification in different sodium chloride concentration

Sodium chloride (w/w)	Purification step	Total protein content (mg)	Total ATPase activity (umol/min)	Specific ATPase activity (umol/min/mg)
0%	Cell lysate	1102.29	1388.88	1.26
	Washed membranes	299.58	991.61	3.31
	Detergent extract	160.96	526.34	3.27
	Solubilized enzyme	55.04	394.09	7.16
0.1%	Cell lysate	1000.24	1350.33	1.35
	Washed membranes	280.26	950.07	3.39
	Detergent extract	160.06	475.39	2.97
	Solubilized enzyme	55.03	379.15	6.89
0.3%	Cell lysate	1014.70	1308.96	1.29
	Washed membranes	293.42	980.02	3.34
	Detergent extract	178.47	542.41	3.04
	Solubilized enzyme	52.03	413.12	7.94
0.5%	Cell lysate	889.70	1201.09	1.35
	Washed membranes	299.36	999.87	3.34
	Detergent extract	180.48	597.40	3.31
	Solubilized enzyme	47.03	358.34	7.62

Table 5.6 Maximum bacterial OD value and enzyme activity in different sodium chloride concentration

Sodium chloride (w/w)	0%	0.1%	0.3%	0.5%
Peak OD	0.45 ± 0.02 ^a	0.47 ± 0.02 ^a	0.49 ± 0.01 ^a	0.47 ± 0.02 ^a
Specific ATPase activity (umol/min/mg)	7.16 ± 0.38 ^a	6.89 ± 0.51 ^a	7.94 ± 0.42 ^a	7.62 ± 0.26 ^a

*Different letters indicate significant differences (p < 0.05).

5.3.4 The effect of medium pH

Fig. 5.5 describes the effect of medium pH on PS3 growth. Neutral pH was found to be optimum whereas lower or higher pH reduced bacterial growth. This result suggests that medium pH affected bacterial membrane function and the uptake of various nutrients (Shu et al., 2004). In Table 5.7 and Table 5.8, media with pH= 7 resulted in the highest protein content (52.03 mg) and specific ATPase activity (7.94 umol min⁻¹ mg⁻¹) among all the pH tested. These results suggest that pH could not only affect bacterial growth, but also change the nature of the proteins inside. Medium pH could affect the secondary and tertiary structure of the proteins. Higher or lower pH can denature the protein sometimes (Yang et al., 1993). In addition, pH could affect the state of ionization of acidic or basic amino acids. If the state of ionization in a protein is altered, then the 3-D shape of the protein can be altered. This can lead to altered protein recognition or an enzyme might become inactive (Yang et al., 1993).

Fig. 5.5. The growth curves of PS3 in 3.6-liter culture medium (aeration of 3 l min⁻¹) containing 0.8% polypeptone, 0.4% yeast extract and 3% NaCl at varying pH.

Table 5.7. F₀F₁ ATP synthase purification in different medium pH

pH	Purification step	Total protein content (mg)	Total ATPase activity (umol/min)	Specific ATPase activity (umol/min/mg)
6	Cell lysate	734.91	720.21	0.98
	Washed membranes	140.86	400.05	2.84
	Detergent extract	100.94	296.76	2.94
	Solubilized enzyme	31.85	72.61	2.28
7	Cell lysate	1014.70	1308.96	1.29
	Washed membranes	293.42	980.02	3.34
	Detergent extract	178.47	542.41	3.04
	Solubilized enzyme	52.03	413.12	7.94
8	Cell lysate	756.85	749.28	0.99
	Washed membranes	169.95	407.88	2.40
	Detergent extract	90.89	302.66	3.33
	Solubilized enzyme	30.12	87.34	2.90

Table 5.8 Maximum bacterial OD value and enzyme activity in different medium pH

Medium pH	6	7	8
Peak OD	0.31 ± 0.01 ^c	0.49 ± 0.01 ^a	0.33 ± 0.01 ^b
Specific ATPase activity (umol/min/mg)	2.28 ± 0.28 ^c	7.94 ± 0.42 ^a	2.90 ± 0.22 ^b

*Different letters indicate significant differences (p < 0.05).

5.3.5 The effect of aeration rate

The growth curves of the thermophilic bacteria PS3 under four aeration conditions are shown in Fig. 5.6. Results clearly show that higher aeration rate improved bacterial growth because higher aeration rate was preferred by aerobic bacteria PS3 (Feng, et al., 2003). In Table 5.9, with the increase in aeration rate from 2 to 3 l min⁻¹, specific ATPase activity of the purified enzyme also increased from 4.95 to 7.94 $\mu\text{mol min}^{-1} \text{mg}^{-1}$. The specific ATPase activities had no significant differences at aeration rates of 4 and 5 l min⁻¹ (Table 5.10). Since more power would be required for a higher level of aeration, 3 l min⁻¹ was considered to be acceptable in this study.

Fig. 5.6. The growth curves of PS3 in 3.6 liter culture medium (pH=7) containing 0.8% polypeptone, 0.4% yeast extract and 3% NaCl at varying aeration rates.

Table 5.9. F₀F₁ ATP synthase purification in different aeration rate

Aeration (l min ⁻¹)	Purification step	Total protein content (mg)	Total ATPase activity (umol/min)	Specific ATPase activity (umol/min/mg)
2	Cell lysate	839.20	1032.22	1.23
	Washed membranes	274.68	791.08	2.88
	Detergent extract	136.94	391.66	2.86
	Solubilized enzyme	39.95	197.76	4.95
3	Cell lysate	1014.70	1308.96	1.29
	Washed membranes	293.42	980.02	3.34
	Detergent extract	178.47	542.41	3.04
	Solubilized enzyme	52.03	413.12	7.94
4	Cell lysate	993.96	1500.88	1.51
	Washed membranes	330.83	1131.45	3.42
	Detergent extract	177.33	500.07	2.82
	Solubilized enzyme	63.08	472.45	7.49
5	Cell lysate	1343.82	1760.41	1.31
	Washed membranes	335.45	1174.09	3.50
	Detergent extract	186.85	551.22	2.95
	Solubilized enzyme	61.05	485.38	7.95

Table 5.10 Maximum bacterial OD value and enzyme activity in different aeration rate

Aeration rate (l min ⁻¹)	2	3	4	5
Peak OD	0.41 ± 0.01 ^d	0.49 ± 0.01 ^c	0.52 ± 0.01 ^b	0.57 ± 0.02 ^a
Specific ATPase activity (umol/min/mg)	7.16 ± 0.38 ^b	6.89 ± 0.51 ^b	7.94 ± 0.42 ^a	7.62 ± 0.26 ^a

*Different letters indicate significant differences (p < 0.05).

5.3.6 The correlation between thermophilic *Bacillus* PS3 growth and ATPase activity of purified F₀F₁ ATP synthase

The abovementioned results suggest that culture conditions affected PS3 growth and ATPase activity of F₀F₁ ATP synthase. The linear relationship between these two parameters was obtained and depicted in Fig. 5.7. The accumulated dry cell weights of PS3 were ranging from 0.630 to 1.631 g l⁻¹ and the related ATPase activity of purified F₀F₁ ATP synthase were starting from 1.49 to 7.99 μmol min⁻¹ mg⁻¹. These two pairs of data showed a strong linear

correlation. This suggests that PS3 growth and ATPase activity of F₀F₁ ATP synthase are highly associated under the above culture conditions and purification method.

Fig. 5.7. Linear relationship between dry cell weight of PS3 and specific ATPase activity of purified F₀F₁ATP synthase.

5.3.7 The characterization of the purified F₀F₁ ATP synthase

In the gelcode blue-stained SDS-polyacrylamide gel of purified protein illustrated in Fig. 5.8, bands with the expected molecular weights for each of the seven subunits can be visualized. Subunit c with the smallest molecular weight (7.34 kDa) is not shown in this figure because it is too close to the end of the gel. Other faint bands which may represent contaminating proteins or proteolysis product of the enzyme are also visible.

Fig. 5.8. SDS-PAGE of purified F₀F₁ ATP synthase. F₀F₁ ATP synthase was purified from PS3, under the culture condition at pH=7, aeration of 3 l min⁻¹ and 0.4% yeast extract, 0.3% NaCl and 1.2% (w/w) polypeptone concentration. Samples were run on 10% tris-glycine gels. (Left) Molecular weight markers. (Right) F₀F₁ ATP synthase subunits; the expected molecular weights are α, 54.60; β, 51.94; γ, 31.78; a, 23.48; δ, 19.66; b, 17.27; ε, 14.33; c, 7.34.

5.4. Conclusions

This study for the first time indicated that the accumulation and activity of F₀F₁ ATP synthase in thermophilic bacteria *Bacillus* PS3 were affected by culture conditions, including the type of nitrogen source, the concentrations of polypeptone and sodium chloride in the culture medium, as well as the medium pH and aeration rate. Polypeptone was found to be the best nitrogen source for PS3 accumulation and activity of F₀F₁ ATP synthase. Higher concentrations of polypeptone promoted the bacterial growth and ATPase activity of purified F₀F₁ ATP synthase. PS3 growth and F₀F₁ ATP synthase purification were found non-sensitive to sodium chloride incorporation. However, these two parameters were significantly affected by medium pH and aeration rate. The F₀F₁ ATP synthase purified from PS3 was verified and characterized by SDS-PAGE.

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CHAPTER 6 - Modeling the growth of thermophilic bacteria *Bacillus* PS3 affected by temperature

Abstract

*The objective of this study was to predict the growth of bacteria *Bacillus* PS3 by modeling the lag time, specific growth rate and asymptote, combined with Gompertz equation. The suitability and usefulness of different models for lag time, specific growth rate and asymptote were determined by using *F* test, by which the lack of fit of the models was compared with the prediction error. Results showed that the modified Ratkowsky model was the most suitable to describe both the growth rate and asymptote as a function of temperature. The hyperbolic model was selected to describe the lag time as a function of temperature. The above chosen models combined with Gompertz equation were used to predict bacteria growth, and the largest relative prediction error was 7.78%. These results suggested that the chosen models combined with Gompertz equation could predict *Bacillus* PS3 growth at given growth time and temperature. Peak value of bacterial growth can also be accurately predicted by using the selected models.*

6.1. Introduction

Predictive microbiology is an area of research in microbiology that has been regarded as a tool for microbial growth prediction (Huang et al., 2013). In recent years, the interest in developing mathematical models to describe the growth of microbiology has increased (Robinson, et al., 1998). The models can help to decrease the required amount of costly and time-consuming challenge tests, may help to design more efficient ways of challenge testing.

Another important feature of models is the acquisition of improved knowledge of the factors that determine microbial growth (Wijtzes, et al., 1995).

Microbial growth is dependent on intrinsic factors, such as temperature (Amir and Sinclair, 1991), pH (Rosso et al., 1995), and water activity (Davey, 1989). Among these factors, temperature is the major factor affecting microbial growth (Zwietering et al., 1994). Previous studies found that Gompertz model with three parameters (μ_m , λ , and A) better described microbial growth curves for most of the cases (Wang and Zuidhof, 2004; Gadhamshetty et al., 2010; Zeinhu, 2003). Here, μ_m , λ , and A are maximal growth rate (slope of the tangent line at the inflection point), lag time (the x-axis intercept of this tangent line), asymptote (the ultimate value of $\ln(OD/OD_0)$) respectively.

$$y = A \exp\{-\exp[\frac{\mu_m \cdot e}{A}(\lambda - x) + 1]\} \quad \text{Gompertz model}$$

There are totally three relationships towards bacterial growth and temperature: Growth rate-temperature relationship, lag time-temperature relationship, and asymptote-temperature relationship. So far, various models have been proposed to describe these relationships. Ohta and Hirahara (1977) proposed a linear relationship by plotting the square root of the rate of nucleotide breakdown in a cool-stored carp muscle and temperature, and this was described by the equation $\sqrt{r} = 0.065x + 0.518$, where x is the temperature in degrees Celsius. Also, Zwietering et al (1991, 1994) compared several explicit models for bacteria *Lactobacillus plantarum* growth, either reported in the literature or newly developed, using a lot of experimental data. Different models were compared statistically, as well as with respect to their ease of use. Zwietering et al (1991, 1994) reparameterized the models in order to obtain biologically significant parameters, such as the maximum specific growth rate (μ_m), the lag

time (λ), and the asymptotic level (A) of the growth curve. Duh and Schaffner (1993) studied and compared several mathematical models which better predict the growth rate and lag time of bacteria *Listeria innocua* and *Listeria monocytogenes* as a function of temperature. Results showed that the 4-parameter Zwietering square root model gave the best fit of all the models used to predict growth rate. The 2-parameter models predicted lag time better than the models containing more parameters. In addition, the 2-parameter square root model gave the best fit of all the models evaluated when a traditional method of determining lag time was used.

Bacteria *Bacillus* PS3 is a thermophilic bacterium, which contains the proton-translocating protein F_0F_1 ATP synthase. This enzyme has been largely studied in ATP motivated glucose synthesis in artificial photosynthesis (Hazard et al., 2002; Lu et al., 2015). So far researchers usually focused on bacteria culturing and enzyme purification processes. Kagawa et al. (1979) obtained F_0F_1 ATP synthase with specific ATPase activity of $17.6 \text{ } \mu\text{mol min}^{-1} \text{ mg}^{-1}$ by using five purification steps, including membrane washing, cholate dissolving, triton X-100 extracting, DEAE eluting and agarose eluting. Hazard et al. (2002) reported a fast and simple purification method for thermophilic F_0F_1 ATP synthase with a higher ATPase activity of $41 \text{ } \mu\text{mol min}^{-1} \text{ mg}^{-1}$. Only one column purification (DEAE) step instead of two was used to obtain high-activity enzyme.

However, so far little research has been conducted to study *Bacillus* PS3 under varying temperature conditions. Besides, modeling the growth of *Bacillus* PS3 as a function of temperature was scarce in the literature, making the prediction of this bacteria growth difficult. Thus, the objective of this study was to develop and compare several predictive models to describe the lag time, specific growth rate and asymptote of *Bacillus* PS3 as a function of

temperature, on the basis of statistical analysis. The selected models combined with Gompertz equation were used to predict bacteria growth as a function of temperature.

6.2. Materials and methods

6.2.1 Microorganism

Thermophilic bacteria *Bacillus* PS3 was obtained from Drs. Toshiharu Suzuki and Masasuke Yoshida (Waseda University, Japan) and kept under -80 °C for reservation. To begin with, PS3 (1 ml) was reactivated in a 10-ml glass tube containing 5-ml sterilized culture medium (0.8% polypeptone, 0.4% yeast extract and 0.3% sodium chloride, w/w). Then they were inoculated in an incubator (Eppendorf, NY, USA) at 68 °C. After shaking for 12-15 h, this culture was transferred to a 250-ml flask containing 50-ml abovementioned medium. After shaking for 12 h, one drop of the culture was cultured in a 60 mm × 15 mm polystyrene petric dish (Fisher, PA, USA) containing 0.8% polypeptone, 0.4% yeast extract, 0.3% NaCl and 15%-20% agar (w/w). They were then kept in another incubator under 30 °C. After slowly growing for 2-3 days, they were transferred into a 4 °C refrigerator.

6.2.2 Bacteria culturing under varying temperatures

The starter culture from the polystyrene petric dishes was transferred to a 250-ml culturing flask containing 150-ml culture medium in a water bath. In 42 experiments at 14 different temperatures, *Bacillus* PS3 was cultured from 32°C up to 84°C at steps of 4°C with three replicates each. At appropriate time intervals (depending on the time), the bacteria sample (200 ul) was taken out for optical density (OD) measurement. Bacteria PS3 cell growth was

monitored by using a Synergy MX microplate reader (BioTek, Vermont, USA) via absorbance change at 600 nm.

6.2.3 Expression of bacteria growth data

The growth curve was defined as the logarithm of the bacterial dry cell weight as a function of time. Here, the typical linear relationship between OD value and PS3 dry cell weight was obtained, and shown in Fig.6.1. Thus, Growth curve was expressed as the logarithm of the OD value as a function of time.

Fig. 6.1. Typical linear relationship between OD and PS3 dry cell weight at all experiments (Original one and 100% diluted after bacteria culturing are included). The linear equation is $y = 3.1185x - 0.0004$. ($R^2 = 0.9945$)

6.2.4 Data Fitting

The Gompertz model was used to fit the data of 42 growth curves in software Matlab by nonlinear regression. Biological growth parameters, including specific growth rate (μ_m), lag time (λ) and asymptote (A) of all growth curves were estimated. The set of parameters with their 95% confidence bounds were also calculated. Then the suitability and usefulness of different models for estimated lag time, specific growth rate and asymptote were determined by using F test. In the meantime, the parameters in each model were also calculated. After that,

the estimated values for lag time, specific growth rate and asymptote at each temperature were predicted by using the chosen models and calculated parameters. Then, a new growth curve at each temperature was predicted by substituting estimated values (lag time, specific growth rate and asymptote) and time into the Gompertz model.

6.2.5 Models for the description of bacterial growth as a function of temperature

A. Growth-temperature relations

(1) Square root model of Rathowsky (Ohta and Hirahara, 1977). This model was based on the observation that at lower temperatures the square root of the specific growth rate is linear with the temperature.

$$\mu_m = [b_1 (T - T_{\min})]^2 \quad (\text{Ratkowsky 1})$$

where μ_m is the maximal growth rate (h^{-1}), b is the Ratkowsky parameter ($^{\circ}\text{C}^{-1} \text{h}^{-0.5}$), T is reaction temperature ($^{\circ}\text{C}$), and $T_{\min}$ is the minimum temperature at which growth is observed ($^{\circ}\text{C}$). The subscript 1 relates to Ratkowsky 1.

(2) Expanded square root model of Ratkowsky (Vanimpe et al., 1992). This model was used to describe the growth rate around the optimum and the maximum temperatures.

$$\mu_m = < b_2 (T - T_{\min}) \{ 1 - \exp [c_2 (T - T_{\max})] \} >^2 \quad (\text{Ratkowsky 2})$$

where μ_m is the maximal growth rate (h^{-1}), b is the Ratkowsky parameter ($^{\circ}\text{C}^{-1} \text{h}^{-0.5}$), and c is Ratkowsky parameters ($^{\circ}\text{C}^{-1}$), T is reaction temperature ($^{\circ}\text{C}$), and $T_{\max}$ and $T_{\min}$ are the maximum and minimum temperatures at which growth are observed ($^{\circ}\text{C}$). The subscript 2 relates to Ratkowsky 2.

(3) Modified Ratkowsky model (Juneja et al., 2009). At temperature above $T_{\max}$, Ratkowsky 2 model overpredicts values of the growth rate; therefore, this model cannot be used above $T_{\max}$. The Ratkowsky model was modified so that the decline of μ_m toward $T_{\max}$ was described by an exponential function.

$$\mu_m = [b_3 (T - T_{\min})]^2 \{1 - \exp [c_3 (T - T_{\max})]\} \quad (\text{Ratkowsky 3})$$

where μ_m is the maximal growth rate (h^{-1}), b is the Ratkowsky parameter ($^{\circ}\text{C}^{-1} \text{h}^{-0.5}$), and c is Ratkowsky parameters ($^{\circ}\text{C}^{-1}$), T is reaction temperature ($^{\circ}\text{C}$), and $T_{\max}$ and $T_{\min}$ are the maximum and minimum temperatures at which growth are observed ($^{\circ}\text{C}$). The subscript 3 relates to Ratkowsky 3.

(4) Hinshelwood model. This model was based on the following assumptions. (i) The total amount of all compounds in the cell is constant, and only one enzyme reaction is rate controlling. (ii) The product of this enzyme reaction is a heat-sensitive essential biomolecule, which is irreversibly denatured at high temperatures. Both the enzyme reaction and the high temperature denaturation show an Arrhenius type of temperature dependency and are zero order.

$$\mu_m = k_1 \exp \left(-\frac{E_1}{RT} \right) - k_2 \exp \left(-\frac{E_2}{RT} \right)$$

where μ_m is the maximal growth rate (h^{-1}), T is reaction temperature ($^{\circ}\text{C}$), R is gas content, k_1 and k_2 are frequency actors (h^{-1}). E_1 and E_2 are the activation energies (J mol^{-1}) of the enzyme reaction and the high temperature denaturation, respectively.

(5) General model. The mean value of the measured data at every temperature was used in this model.

$$\mu_{m,ave} (i) = \sum_{j=1}^m \frac{\mu_m(i, j)}{m}$$

where $\mu_m (i, j)$ is the j th growth rate at T_i and $\mu_{m,ave} (i)$ is the mean growth rate at T_i .

B. Asymptote-temperature relations

(1) Expanded square root model of Ratkowsky (Vanimpe et al., 1992). This model was used to describe the asymptote around the optimum and the maximum temperatures.

$$A = < b_2 (T - T_{min}) \{ 1 - \exp [c_2 (T - T_{max})] \} >^2 \quad (\text{Ratkowsky 2})$$

where μ_m is the maximal growth rate (h^{-1}), b is the Ratkowsky parameter ($^{\circ}C^{-1} h^{-0.5}$), and c is Ratkowsky parameters ($^{\circ}C^{-1}$), T is reaction temperature ($^{\circ}C$), and T_{max} and T_{min} are the maximum and minimum temperatures at which growth are observed ($^{\circ}C$). The subscript 2 relates to Ratkowsky 2.

(2) Ratkowsky 4 model (Zwietering et al., 1991). This model was proposed at the condition that the asymptote did not show a strong dependency on temperature at the lower temperature range.

$$A = b_4 \{ 1 - \exp [c_4 (T - T_{A_{max}})] \} \quad (\text{Ratkowsky 4})$$

where A is the asymptote, b is the final level reached at low temperatures, T is reaction temperature ($^{\circ}C$), and $T_{A_{max}}$ is the maximum temperature at which growth is observed ($^{\circ}C$). The subscript 4 relates to Ratkowsky 4.

C. Lag time-temperature relations

(1) Ratkowsky 5 model (Zwietering et al., 1991). This model was based on the fact that the lag time data showed a large prediction error at lower temperatures. The logarithmic transformation was used on the experimental data and on the model equations.

$$\ln(\lambda) = \ln \left\{ [b_5 (T - T_{\min})] \{ 1 - \exp [c_5 (T - T_{\max})] \}^{-2} \right\} \quad (\text{Ratkowsky 5})$$

where λ is the lag time (h), b is the Ratkowsky parameter ($^{\circ}\text{C}^{-1} \text{ h}^{-0.5}$), and c is Ratkowsky parameters ($^{\circ}\text{C}^{-1}$), T is reaction temperature ($^{\circ}\text{C}$), and $T_{\max}$ and $T_{\min}$ are the maximum and minimum temperatures at which growth are observed ($^{\circ}\text{C}$). The subscript 5 relates to Ratkowsky 5.

(2) Hyperbolic model (Duh et al., 1993). This model was proposed since results from Duh et al. (1993) showed a hyperbolic behavior of the lag time and the temperature.

$$\ln(\lambda) = \frac{p}{(T - q)} \quad (\text{Hyperbolic})$$

The parameter q is the temperature ($^{\circ}\text{C}$) at which the lag time is infinite. The parameter p ($^{\circ}\text{C h}$) is a measure for the decrease of the lag time when the temperature is increased.

6.2.6 Comparison of the models

The models were compared statistically with the use of an F ratio test. With the general model, the prediction error was estimated by determining the deviation of the measured values from the mean value at one temperature. The sum of squares of the deviations between the data and the general model was calculated (RSS_1):

$$\text{RSS}_1 = \sum_{i=1}^k \cdot \sum_{j=1}^m [\mu_m(i, j) - \mu_{m \text{ Ave}}(i)]^2$$

(General model)

With $\mu_m(i, j)$ being the j^{th} growth rate at T_i and $\mu_{m \text{ Ave}}$ being the mean growth rate at T_i .

The sum of squares of the deviation between the data and a given growth temperature model was calculated (RSS_2).

$$RSS_2 = \sum_{i=1}^k \cdot \sum_{j=1}^m [\mu_m(i, j) - \mu_{m, pre}(i)]^2$$

(Growth-temperature model)

With $\mu_{m, pre}(i)$ being the model prediction at temperature T_i .

RSS_2 is always greater than or equal to RSS_1 . The RSS_2 of the growth-temperature model used is built up for both the prediction error and the lack of fit; therefore, the difference between RSS_2 of the model and RSS_1 (the prediction error) is calculated as an estimation of the lack of fit. If the lack of fit ($RSS_2 - RSS_1$) is much smaller than the prediction error (RSS_1), the model is adequate. If the lack of fit is larger than the prediction error, the model is not suitable. The comparison between the lack of fit and the prediction error can be quantified statistically by the f testing value.

$$f = \frac{(RSS_2 - RSS_1)/(DF_2 - DF_1)}{RSS_1/DF_1} \text{ tested against } F_{\frac{DF_2 - DF_1}{DF_1}}$$

where DF_1 is the number of degrees of freedom from the general model that equals the total number of datum points minus the number of different temperatures measured ($42 - 14 = 28$). DF_2 is the number of the degrees of freedom from the growth-temperature model that equals the number of datum points minus the number of parameters.

6.2.7 Selection of the models

First, the models were compared statistically by using the F test. With the use of F test, the values calculated were compared with F table values, which are the critical ones of the F-distribution for false-rejection probability ($\alpha = 0.05$). Also, the models with the fewest number of parameters were selected since less correlated parameter model is more stable. Fewer

parameters models could have higher degree of freedom, which is important to predict the bacterial growth condition by using a smaller number of experiments.

6.3. Results and discussion

6.3.1. *Bacillus* PS3 growth under various culturing temperatures

Fig. 6.2 (A) and Fig. 6. 2 (B) show that culture temperature had considerable influences on bacteria *Bacillus* PS3 growth. With the increase in culture temperature from 32 °C to 68 °C, bacterial growth (highest OD value) increased from 0.068 to 0.473 in 14 h. This result was consistent with other researchers that 68 °C was the optimum culture temperature for *Bacillus* PS3 (Nobuhito et al., 1983; Saiki et al., 1994). When the temperature was beyond 68 °C, bacterial growth was inhibited and the highest OD value was reduced to only 0.068 at 84 °C. This may be due to a variety of reasons such as inactivation or denaturation of proteins, instability or no synthesis of RNA at higher culturing temperature (Ratkowsky et al., 1982).

Fig. 6.2. *Bacillus* PS3 growth with trend lines at varying culturing temperatures between (A) 32 °C and 56 °C; (B) 60 °C and 84 °C. (Each point is the average value of three replicates)

6.3.2. Growth parameters estimation from the Gompertz model

The Gompertz model was used to fit the data of 42 bacterial growth curves by nonlinear regression. The growth parameters, including growth rate, lag time and asymptote, were estimated and shown in Fig. 6. 3. Results showed that the specific growth rate and asymptote increased with the increasing culturing temperature, and their highest values were 0.380 h^{-1} and 1.907, respectively, under the culturing temperature of 68°C (Fig. 6.3 (A) and (B)).

Lag time data obtained from Gormpertz modeling showed a large prediction error, especially at lower temperatures. To limit this influences, a logarithmic transformation was used on the data, and results are shown in Fig. 6.3 (C). Different with specific growth rate and asymptote change, lag time ($\text{Ln } \lambda$) showed a decreasing trend from 2.521 h to 0.111 h with increasing temperature from 36°C to 80°C . Lag time estimated for 32°C and 84°C were all equal to zero, and they were not suitable for logarithmic transformation. So the lag time data at these two temperatures were not shown in the figure.

Fig. 6.3. *Bacillus* PS3 growth parameters estimation for (A) specific growth rate; (B) asymptote; (C) lag time.

6.3.3. Model selection for growth-temperature relations

The specific growth rates as a function of temperature (42 measurements) were described and compared by using different models. RSS_2 values and the F testing values of the different growth temperature relations are shown in Table. 6.1. Some common simpler models, such as $\mu_m = 0$, $\mu_m = aT$, and $\mu_m = aT + b$, were also evaluated. Results clearly showed that the RSS_2 values decreased with an increasing number of parameters. For Hinshelwood model, the confidence intervals of such parameters (E_1 and E_2) were large (ranging from $-1.069 \times 10^6 \text{ h}^{-1}$ to $1.069 \times 10^6 \text{ h}^{-1}$), making the parameters prediction inaccurate (Table. 6.2). The prediction of the growth rate under this model was far from the measured value, especially when the temperature was above 48°C (Fig. 6.4 (D)). Results also revealed that values were not well described by a constant value or a straight line. Simple models, including $\mu_m = 0$, $\mu_m = aT$, $\mu_m = aT + b$ and Ratkowsky (with higher RSS_2), were not acceptable in this case (Fig. 6.4 (A)).

F value from Table 6.1 suggested that Ratkowsky 2 (four parameter model) and Ratkowsky 3 (four parameter model) were statistically acceptable, since the F testing values (1.69 and 1.83) were both lower than F table value (2.2). This result can also be confirmed by Fig. 6.4 (B) and (C). From the curvatures, it can be seen that Ratkowsky 2 and Ratkowsky 3 modeling trend line better described the bacterial growth rate following different temperature conditions. By comparing this two models, the prediction of Ratkowsky 3 model was too high at lower temperatures. Since the Ratkowsky 2 model had the lowest RSS_2 of all four-parameter models, and the prediction was close to the measured value, it was chosen to better describe the specific growth rate as a function of temperature.

Table 6.1. Comparison of different models describing the bacterial growth rate

Model	No. of parameters	df	RSS_2	f	F table value
$\mu_m = 0$	0	42	3.13	16.01	2.30
$\mu_m = aT$	1	41	1.37	6.33	2.10
$\mu_m = aT + b$	2	40	1.97	10.50	2.10
Ratkowsky	2	40	0.98	4.28	2.10
Ratkowsky 2	4	38	0.56	1.69*	2.20
Ratkowsky 3	4	38	0.57	1.83*	2.20
Hinshelwood	4	38	1.00	5.28	2.20
General	14	28	0.35 (RSS_1)		
$\mu_m = \mu_m(i, j)$	42	0	0.000		

Table 6.2. Results of the Hinshelwood parameter estimation

Parameter	Estimate	95% Confidence interval
k_1 (h^{-1})	8.364	-1.069 E + 06 to 1.069 E + 06
k_2 (h^{-1})	8.221	-1.069 E + 06 to 1.069 E + 06
E_1 (kJ)	151.3	-3.108 E + 05 to 3.095 E + 05
E_2 (kJ)	146.5	-3.093 E + 05 to 3.080 E + 05

Fig. 6.4. Growth rate modeled with various models. (A) Ratkowsky model; (B) Ratkowsky 2 model; (C) Ratkowsky 3 model; (D) Hinshelwood model.

6.3.4. Asymptote-temperature relations

The asymptote change of *Bacillus* PS3 as a function of temperature was evaluated with various models (Table 6.3). Results showed that RSS_2 values decreased with an increasing number of parameters, and Ratkowsky 2 model with four parameters had the lowest RSS_2 (1.48) and f value (0.51). Simpler models, $\mu_m = aT$ and $\mu_m = aT + b$ had higher values both for RSS_2 (54.82 and 22.27) and f value (92.23 and 39.21), thus they were not accepted to describe the asymptote of the bacterial growth. Similarly, Ratkowsky 4 model was also not suitable since this model was used for constant asymptote in the lower temperature range. However,

asymptote here increased with the increasing temperatures until 68 °C (Fig. 6.5). So higher values both for RSS_2 (19.17) and f value (36.46) were obtained. Here, Ratkowsky 2 model was accepted because it had the lowest RSS_2 of all models and this model better described the bacterial asymptote change.

Table 6.3. Comparison of different models describing the bacterial growth asymptote

Model	No. of parameters	df	RSS_2	f	F table value
$\mu_m = aT$	1	41	54.82	92.23	2.10
$\mu_m = aT + b$	2	40	22.27	39.21	2.10
Ratkowsky 4	3	39	19.17	36.46	2.20
Ratkowsky 2	4	38	1.48	0.51*	2.20
General	14	28	1.25 (RSS_1)		
$\mu_m = \mu_m(i, j)$	42	0	0.00		

Fig. 6.5. Asymptote of the growth modeled with (A) Ratkowsky 2 model; (B) Ratkowsky 4 model.

6.3.5. Lag time-temperature relations

The logarithm of the lag time as a function of temperature was also studied with hyperbolic, Ratkowsky and other simpler models. In this case, the simpler models ($\mu_m = aT$, $\mu_m = aT + b$) were rejected again since they had greater values for RSS_2 . Ratkowsky 5 model described the bacterial growth lag time well at lower temperatures. However, this model was not acceptable since the prediction was overestimated at higher temperatures, thus making f

value (2.40) of this model greater than F table value (2.20). The Hyperbolic model was accepted statistically to describe the lag time of the bacterial growth since f value (1.01) was smaller than F table value (2.20).

Table 6.4. Comparison of different models describing the bacterial growth lag time

Model	No. of parameters	df	RSS ₂	F	F table value
$\mu_m = aT$	1	41	42.82	76.01	2.10
$\mu_m = aT + b$	2	40	15.67	28.65	2.10
Hyperbolic	2	40	1.69	1.01*	2.20
Ratkowsky 5	4	38	1.34	2.40	2.20
General	14	28	1.18 (RSS ₁)		
$\mu_m = \mu_m(i, j)$	42	0	0.00		

Fig. 6.6. Lag time of the growth modeled with (A) Hyperbolic model; (B) Ratkowsky 5 model.

6.3.6. Model prediction and bacterial growth relations

Here, Ratkowsky 2, Ratkowsky 2 and Hyperbolic models were selected (based on F test analysis) to describe *Bacillus* PS3 growth rate, asymptote and lag time change with different temperature conditions. By using the chosen model and estimated parameters in each model, the above growth parameters at each temperature were predicted. After that, a growth curve at that temperature was described by using estimated values and Gompertz equation. The model predictions were compared with originally measured growth data, and some of the results were

shown in Fig. 6. 7 (A) and (B). Peak value obtained from the prediction and the measured one were very close in each temperature except for 36°C (the lowest) and 80°C (the highest). This is because inactivation or denaturation of proteins, instability or no synthesis of RNA, or inhibition under these conditions. Models applied here were only suitable in normal biological situations, but not valid for extreme temperature conditions. In other conditions (from 40°C to 76°C), model predictions and the measured values for maximum bacterial accumulation were close, and the largest relative error was 7.78%. These results suggested that the chosen models combined with Gompertz equation could accurately predict *Bacillus* PS3 growth at given time and temperature. Peak values of bacterial growth can also be accurately predicted by using the selected models.

Fig. 6.7. Growth data and prediction models at (A) 48°C and 56°C; (B) 52°C and 68°C. Different symbols in measured data indicate the average value of three samples.

6.4. Conclusions

This study for the first time predicted the growth of bacteria *Bacillus* PS3 as a function of temperature and time. The suitability and usefulness of different models of lag time, specific growth rate and asymptote were also determined by using F test. Results showed that the modified Ratkowsky model was most suitable model for both the growth rate and asymptote as a function of temperature. The hyperbolic model was chosen to describe the lag time change as a function of temperature. The chosen models combined with the Gompertz model were used to predict *Bacillus* PS3 growth as a function of temperature and time, and the largest relative prediction error was 7.8%.

6.5. References

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CHAPTER 7 – Glucose synthesis in a protein-based artificial photosynthesis system

Abstract

The objective of this study was to understand glucose synthesis of a protein-based artificial photosynthesis system affected by operating conditions, including the concentrations of reactants, reaction temperature, and illumination. Results from non-vesicle-based glyceraldehyde-3-phosphate (GAP) and glucose synthesis showed that the initial concentrations of ribulose-1, 5-bisphosphate (RuBP) and adenosine triphosphate (ATP), lighting source, and temperature significantly affected glucose synthesis. Higher initial concentrations of RuBP and ATP significantly enhanced GAP synthesis, which was linearly correlated to glucose synthesis, confirming the proper functions of all catalyzing enzymes in the system. White fluorescent light inhibited artificial photosynthesis and reduced glucose synthesis by 79.2% compared to the dark. The reaction temperature of 40 °C was optimum whereas lower or higher temperature reduced glucose synthesis. Glucose synthesis in the vesicle-based artificial photosynthesis system reconstituted with bacteriorhodopsin, F_0F_1 ATP synthase and polydimethylsiloxane - methyloxazoline - polydimethylsiloxane triblock copolymer was successfully demonstrated. This system efficiently utilized light-induced ATP to drive glucose synthesis and $5.2 \mu\text{g ml}^{-1}$ glucose was synthesized in 0.78 ml reaction buffer in 7 hours. Light-dependent reactions were found to be the bottleneck of the studied artificial photosynthesis system.

7.1. Introduction

Photosynthesis, by converting light, CO₂, and water into carbohydrates, provides the foundation for food and fuel production. Photosynthesis can generally be divided into two parts: the light-dependent reactions and the light-independent reactions (also referred to as the dark reactions) (Peter et al., 2002; Dennis et al., 1998; Thorsten et al., 1999). In light-dependent reactions, adenosine triphosphate (ATP) synthesis is catalyzed by F₀F₁ ATP synthase, an enzyme usually found in energy-transducing membranes, including inner mitochondrial membranes and bacterial membranes (Daniela et al., 1999; Yoshida et al., 2001). This enzyme is composed of two sectors, F₀ and F₁. The function of the former is to pump protons across the membrane. The latter catalyzes the synthesis of ATP (Elston et al., 1998; Wang et al., 1998). Direct evidence has been found that the proton gradient generated by electron transfer complexes and light-harvesting proteins provides the energy for the synthesis of ATP (Weber et al., 2003), which is used to fix carbon in carbon dioxide into the energy storing carbon compounds in the dark reactions. The dark reactions of photosynthesis involve a suite of eight enzymes (Fig. 7. 1). Ribulose-1, 5-bisphosphate (RuBP) is the initial point for this phase, and after a well-organized reaction pathway catalyzed by enzymes such as ribulose bisphosphate carboxylase and phosphoglycerate kinase, the key intermediate product glyceraldehyde-3-phosphate (GAP) is produced. Glucose can be synthesized following GAP synthesis with the catalysis of fructose-1, 6-bisphosphatase, phosphoglucose isomerase and glucose-6-phosphatase. The process of glucose synthesis from GAP is also called gluconeogenesis (Exton, 1972).

Fig. 7. 1. A schematic diagram of glucose synthesis in photosynthesis.

For decades, scientists are trying to mimick the natural process of photosynthesis, called artificial photosynthesis, applying the fundamental principles of natural photosynthesis processes to convert solar energy into ATP and sugars. ATP serves as the most important biological high-energy compound and has been formed artificially by coupling photon-induced proton motive force (Stoeckenius et al., 1982; Oesterhelt. 1998; Takematsu et al., 2008; Hampp et al., 2000; Heyn et al., 1982) and the key enzyme, F_0F_1 ATP synthase (Choi et al., 2006; Choi et al., 2006; Hazard et al., 2002). Glucose is obtained at the end through the ATP motivated

carbon fixation reactions (Fig.7.1). Artificial photosynthesis has been considered a renewable energy solution to the depleting fossil fuels (Ward et al., 1989; Pan et al., 2007; Laser et al., 2002; Krishnan et al., 2010; Goldemberg et al., 2008); therefore, it has attracted much attention in recent years. Hydrogen and electricity can be potentially used in glucose synthesis in artificial photosynthesis. Huang (2011) proposed nine kinds of dark reaction pathways, that NADH is regenerated from hydrogen and electricity, rather than ATP. These methods combined with solar photovoltaic or solar hydrogen technology, highly improve the efficiency of the dark reaction. Zhang and Huang (2012) proposed electricity-carbohydrate-hydrogen (ECHO) cycle, which is novel, high-energy efficiency, biological CO₂ fixation pathways to fill the gaps from electricity and hydrogen to sugar. The two major roles of the ECHO cycles are storage of electricity or hydrogen and production of high-purity hydrogen for hydrogen fuel cell vehicles. They also had a hypothesis about biological CO₂ fixation process related to artificial photosynthesis. The process is mediated by non-natural cascade enzymes from high concentration CO₂ from power stations. Still, ATP is more used in artificial photosynthesis since glucose is synthesized driven by ATP in natural process of photosynthesis. For example, Choi et al. (2006) presented a solvent-free membrane protein reconstitution method in polymersomes and compared the functionality of bacteriorhodopsin (BR) and ATP synthase both in solvent-free and solvent systems. In the solvent system, the drop-wise polymer–protein–organic solvent (ethanol) mixture was added to an aqueous solution, leading to the formation of proteopolymersomes. They also applied solvent-free system (dissolution of polymer into water) by controlling the aqueous solution. Their results showed that the use of solvent free systems eliminated the problem of protein denaturation during protein

reconstitution into polymersomes. Choi et al (2006) also studied the BR pumping activity and ATP synthesis in a foam architecture and demonstrated in vitro biochemical synthesis inside the water channels of the foam using engineered artificial organelles. Hazard and Montemagno (2002) reported a fast and simple purification method for thermophilic F_0F_1 ATP synthase with higher ATPase activity. Stable electrochemical gradient and ATP were generated by incorporating F_0F_1 ATP synthase and BR into liposomes (Hazard et al., 2002). Wendell et al. (2010) successfully achieved carbon fixation and glucose synthesis in an artificial photosynthesis system by using ranaspumin-2 based foam structure. However, artificial photosynthesis is known to be affected by the reaction conditions, e.g., concentration of reactants and enzymes, temperature, and light, which have not been examined in details in the abovementioned researcher. Thus, the objective of this research was to understand the effect of operating conditions, including temperature (20, 30, 40 and 50 °C), illumination type (white fluorescent light, green LED light, and in the dark), and concentration of reactants (RuBP and ATP) on glucose synthesis within a protein based artificial photosynthesis system.

7.2. Materials and methods

Experiments were carried out in two steps. In the first step, enzyme reactions of GAP and glucose synthesis were conducted separately and then combined in sequence in the reaction buffer (called non-vesicle-based glucose synthesis). The purpose of the non-vesicle-based glucose synthesis experiments was to validate the workability of all enzymes and find appropriate conditions for the second step reactions. In the second step, light and dark reactions

were combined and realized in triblock copolymer vesicles reconstituted with BR and F₀F₁ ATP synthase (called vesicle-based glucose synthesis).

7.2.1 Non-vesicle-based glucose synthesis

7.2.1.1 GAP synthesis

Non-vesicle-based GAP synthesis procedure was adopted from Wendell et al. (2010). The reaction buffer was made by adding 605.7 mg Tris-Base, 23.8 mg MgCl₂, 330 mg KHCO₃ and 39 mg dithiothreitol (DTT) into 50-ml DI water. Then 2-ml reaction buffer (pH=7.8) was mixed with 1 mg pure ATP standard (Sigma, St. Louis, MO, USA. Catalog Number: FLAAS-1VL). The above ATP solution (0.75 ml) was transferred into a 1.5-ml centrifugal tube. Then the following carbon-fixation enzymes/reagents were added in sequence to the tube: 5 units of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 0.177 mg of NADH (reduced form for nicotinamide adenine dinucleotide), 10 units of phosphoglycerate kinase (PGK), 0.625 mg of ribulose biphosphate carboxylase (RuBisCO) and 0.116 mg of RuBP. The solution was vigorously shaken for 30 seconds and then quickly transferred into a 96-well round bottom assay plate (Corning Incorporated, NY, USA. Catalog Number: 3795). They were allowed to react for 20-30 minutes with continuous shaking and the absorbance at 340 nm was measured at 20 s intervals by using a microplate reader (Synergy Mx, BioTek Instruments Inc., Winooski, VT, USA). The amount of GAP was measured by the change in absorbance at 340 nm due to the oxidation of NADH by GAPDH (Wendell et al., 2010). GAPDH, NADH, PGK, RuBisCO and RuBP were purchased from Sigma. All other chemicals were of analytical grade and purchased from Fisher Scientific (Pittsburgh, PA, USA).

7.2.1.2 GAP to glucose synthesis

Four different concentrations of GAP standard solution (0.06, 0.09, 0.11 and 0.14 mM) were measured and separately placed into a 1.5-ml centrifugal tube. Glucose was formed by the addition of 12.5 units of triose phosphate isomerase (TPI), 12.5 units of fructose -1, 6-bisphosphatase (F1, 6 BPase), 12.5 units of phosphoglucose isomerase (PGI), 0.2 mg aldolase and 0.1 units of glucose-6-phosphatase (G6Pase). They were diluted into a total volume of 1 ml by using DI water and then vibrated on a vortex mixer for 6 hours. The formation of glucose was assayed using a colorimetric assay kit (Sigma. Catalog Number: GAGO20-1KT). GAP, TPI, PGI and G6Pase were also purchased from Sigma. Aldolase was purchased from MP Biomedicals LLC (Solon, OH, USA. Catalog Number: 0215985980) and F1, 6 BPase was purchased from Cayman Chemical Co. (Ann Arbor, MI, USA. Catalog Number: 11104).

7.2.1.3 Full glucose synthesis

After the separate synthesis of GAP (starting with RuBP and ATP) and glucose (starting with GAP), non-vesicle-based full glucose synthesis (starting with RuBP and ATP) was studied under varying operating conditions, including temperature (20, 30, 40 and 50 °C), illumination type (white fluorescent light, green LED light, and in the dark), and concentrations of the reactants (RuBP and ATP). The typical reaction mixture contained 6 units of GAPDH, 0.75 mg of RuBisCO, 12 units of PGK, 0.20 mg of NADH, 0.13 mg of RuBP, 0.76 mg (1.5 μ mol) of ATP, 15 units each of TPI, F1, 6 BPase, and PGI, 0.3 mg F1, 6 BPase aldolase, and 0.15 units of glucose-6-phosphatase in a volume of 1.5 ml reaction buffer (pH=7.8).

7.2.2 Vesicle-based glucose synthesis

7.2.2.1 BR-ATP synthase vesicle preparation

The procedure for preparing BR-ATP synthase vesicles was described by Choi et al. (2006). The triblock polymer (10 mg, polydimethylsiloxane-methyloxazoline-polydimethylsiloxane, PMOXA-PDMS-PMOXA, Molecular Weight (MW) = 8400 g mol⁻¹, Polymer Source Inc., Montreal, Canada, Catalog Number: P3186) was added to 68.5 µl BR monomers (25 mg ml⁻¹, Sigma, Catalog Number: B0184-5MG) and kept vortexing for 30 min. Then 0.59 mg sodium cholate and 0.29 mg sodium deoxycholate were added into the BR/copolymer mixture and kept vortexing for 2 hours in the dark to ensure a homogeneous solution. After that, 1.98 mg sodium cholate and 0.96 mg sodium deoxycholate were added into 230 µl of F₀F₁ ATP synthase (2.225 mg ml⁻¹ × 230 µl = 511.75 µg) and kept vortexing for 1 h. Then the ATP synthase/detergent mixture was slowly transferred into the polymer/BR/detergent mixture. After vortexing for 0.5 h, this protein – polymer mixture was dropwise added to the dialysis buffer (20 mM MOPS, 50 mM Na₂SO₄, 50 mM K₂SO₄, 2.5 mM MgSO₄, 0.25 mM DTT, 0.2 mM EDTA, pH = 7.25) at the rate of 10 µl every 30s. The sample was controlled at 2 ml. Mixing by magnetic bar was provided for 2.5 h until the BR-ATP synthase vesicle was created. Non-functional multilamellar vesicles were removed by syringe filtration through a 0.2 µm membrane filter. Detergents were dialyzed by dialysis tubing (molecular weight cutoffs between 12000 and 14000, Fisher Scientific. Catalog Number: 08667A) at 4 °C in the dark for two days. All chemicals were of analytical grade and purchased from Sigma.

7.2.2.2 BR proton pumping activity measurement

To measure the internal pH change inside polymer vesicles, BR/ATP synthase–polymer mixture was formed in the presence of pyranine (Sigma, Catalog Number: H1529-1G). Untrapped pyranine and detergents were removed via overnight dialysis at 4 °C in the dark. Photo-induced activity was measured after illumination by a 5 W green LED (wavelength = 570 nm). At every 5 minutes, 40- μ l solutions were taken for fluorescence measurement on a 384-well low flange flat bottom plate (Corning Incorporated, Catalog Number: 3574). An excitation scan from 350 to 475 nm at an emission wavelength of 511 nm was performed. The pH of pyranine concentration was determined by plotting the ratio of fluorescence emission at two excitation wavelengths (emission at 456 nm excitation/emission at 402 nm excitation, or F456/402) (Hazard et al., 2002). The pyranine standard curve generated in the buffer solution was used to calculate the internal pH of vesicles in the experiments (Seigneuret et al., 1985).

7.2.2.3 ATP synthesis

To measure ATP synthesis activity, a bioluminescence assay kit (Sigma. Catalog Number: FLAAM-1VL) was used (Pitard et al., 1996). The assay procedure was adopted from Hazard and Montemagno (Choi et al., 2006). Briefly, after dialysis, the reaction solution contained 400 μ l of BR-ATP synthase-polymer vesicle solution, 40 μ l of 0.2 M ADP and 40 μ l of 0.5 M KH_2PO_4 , ADP and KH_2PO_4 , was illuminated by 5-W green LED. After incubation for 15-20 min at 30 °C under continuous illumination, ATP synthesis was initiated by the addition of 0.96 mg MgSO_4 . At the same intervals (every 10 min), 40- μ l aliquots of reaction mixture were taken and rapidly mixed with 40 μ l ATP assay mix. After that, 40- μ l sample was transferred into the

384-well low flange flat bottom plate for luminescence assays (Gain=135; read height=1.00 mm).

7.2.2.4 Glucose synthesis

The reaction buffer (780 μl , pH=7.8) containing all glucose synthesis enzymes as described in the section of full glucose synthesis was mixed with 900- μl BR-ATPase-copolymer vesicle solution and the mixture was exposed to the 5-W green LED. The formation of glucose was assayed using the same method and procedure as in the section of GAP to glucose synthesis.

7.3. Results and discussion

7.3.1. Non-vesicle based glucose synthesis

7.3.1.1 Reactant effects on GAP synthesis

The effects of varying concentrations of RuBP and ATP on GAP synthesis are shown in Fig. 7.2. It can be found that GAP synthesis was rapid in the first 10 min, and then stabilized after 20 min. In 750- μl reaction solution, when 0.5 mM RuBP and 1 mM ATP were used, the maximum GAP synthesis was 121.8 μM (20.7 $\mu\text{g ml}^{-1}$), which increased to 216.0 μM (36.7 $\mu\text{g ml}^{-1}$) and 185.9 μM (31.6 $\mu\text{g ml}^{-1}$) when the concentration of ATP (2 mM) and RuBP (1 mM) were doubled, respectively. GAP synthesis reached 272.5 μM (46.31 $\mu\text{g ml}^{-1}$) when the concentrations of ATP and RuBP were both doubled at the same time. The results were as expected because more energy (ATP) input in the reaction solution enhanced the conversion of surplus reactant (RuBP) into the product, GAP. The results shown in Fig. 7.2 confirm that all the GAP synthesis enzymes (NADH, GAPDH, PGK and RuBisCO) were working properly

and their amounts were sufficient for catalyzing the baseline reaction (0.5 mM RuBP and 1 mM ATP in 0.75-ml reaction solution).

Fig.7.2. GAP synthesis at varying concentrations of RuBP and ATP. The reaction solution (750 μ l) contained 5 units of GAPDH, 0.177 mg NADH, 10 units of PGK and 0.625 mg of RuBisCO.

7.3.1.2 From GAP to glucose

In this part of experiments, glucose was synthesized starting from GAP. Four concentrations of GAP (0.06, 0.09, 0.11 and 0.14 mM) were individually tested. Fig. 7.3(A) shows that glucose was synthesized rapidly in the first 4 h and then slowed down. Results clearly show that glucose synthesis from GAP took much longer (4 to 6 hours) than GAP synthesis (~30 min). Compared with GAP synthesis, glucose synthesis is more complicated due to the triangle-reaction-system (Fig. 7.1). Dihydroxyacetone phosphate (DHAP) is first produced by exposure to TPI and then it acts as the reactant to synthesize fructose-1, 6-bisphosphate (FBP) through the condensation reaction (Fig. 7.1). Similar result was also found

by Wong et al (1983), who mixed DHAP and TPI with aldolase and under room temperature for 24 hours to obtain FBP yield of 90%. Fig. 7.3 (A) also shows that glucose synthesis increased with increasing GAP addition. The highest glucose concentration achieved was $9.17 \mu\text{g ml}^{-1}$ when 0.14 mM GAP was added. This is as expected because GAP was the reactant in glucose synthesis. There was a linear correlation between glucose synthesis and GAP addition as shown in Fig. 7.3 (B). The results confirm that all the enzymes in glucose synthesis were working properly and their amounts were sufficient to convert up to 0.14 mM of GAP (in 1.5 ml) into glucose.

Fig. 7.3. (A) Glucose synthesis at varying concentrations of GAP. The reaction solution (1.5 ml) contained 12.5 units each of TPI, F1, 6 BPase, and PGI, 0.2 mg aldolase and 0.1 units of G6Pase. (B) The linear correlation between glucose synthesis (at 6 h) and the initial GAP concentration.

7.3.1.3 The effects of illumination on glucose synthesis

After successfully confirming the separate reactions of GAP and glucose synthesis, unified glucose synthesis experiments were conducted. A full process control group experiment was first carried out by removing one compound from the reactions. Glucose was not detected in any control sample with one compound missing, as shown in Table 7.1.

Table 7.1. The full process control group

Missing Component	Δ Absorbance (540nm)*	Calculated glucose background (nmol)
RuBisCO	-0.001	0
RuBP	0.002	0
NADH	- 0.006	0
ATP	0.005	0
PGK	-0.002	0
GAPDH	-0.003	0
TPI	0	0
F1,6 BPase	0.002	0
PGI	-0.003	0
G6Pase	0.003	0
Aldolase	0.004	0

*All absorbance values given here are within the error range of the instrument; making calculated glucose amounts in the undetectable range.

The effect of illumination on full glucose synthesis can be seen from Fig. 7.4. The highest glucose synthesis of $18.70 \mu\text{g ml}^{-1}$ was obtained in the dark. However, when illuminated by white fluorescent light, glucose synthesis was very low ($3.89 \mu\text{g ml}^{-1}$), only 20.8% of that in the dark. This indicates that white light inhibited glucose synthesis. It is well known that light regulates enzyme activities in photosynthesis systems (Powles et al., 1984). The activity change of any enzyme in the system affects the efficiency of the entire glucose synthesis process. Research conducted by Preiss et al. (1970) and Portis (1992) found that NAD^+ and RuBisCO activities were inhibited by visible light. Activity reduction of these enzymes by visible light adversely affected the synthesis of the intermediate product, GAP, which consequently reduced the synthesis of the final product, glucose. Under green LED light, glucose synthesis was 21.3% lower ($14.72 \mu\text{g ml}^{-1}$) than in the dark. Because BR could pump protons only under green lights, glucose synthesis by the BR-ATP synthase system is possible but enzymatic activities are slightly inhibited by the light.

Fig. 7.4. Glucose synthesis in the full glucose synthesis assay under varying illuminations at 30°C. The reaction solution (1.5 ml) contained 6 units of GAPDH, 0.75 mg of RuBisCO, 12 units of PGK, 375 nmol of NADH, 700 nmol of RuBP, 1500 nmol of ATP, 15 units each of TPI, F1, 6 BPase, and PGI, 0.3 mg of F1, 6 BPase aldolase, and 0.15 units of glucose-6-phosphatase.

7.3.1.4 The effects of temperature on glucose synthesis

Fig. 7.5 shows that temperature had considerable influences on glucose synthesis. With the increase of reaction temperature from 20 to 40 °C, glucose synthesis increased from 3.75 to 25.32 $\mu\text{g ml}^{-1}$ in 6 h. This significant enhancement can be attributed to the optimal catalytic activity of the enzymes at 40 °C (Dec et al., 1990; Wrba et al., 1990; Thomas et al., 1998). When the temperature was further increased to 50 °C, glucose synthesis was inhibited and the highest glucose production was reduced to 10.09 $\mu\text{g ml}^{-1}$. This may be due to the denaturation and degradation of enzymes at high temperatures, under which irreversible degradative processes occurred leading to enzyme inactivation (Daniel et al., 1996).

Fig. 7.5. Glucose synthesis in the full glucose synthesis assay at varying reaction temperatures. The reaction solution (1.5 ml) contained 6 units of GAPDH, 0.75 mg of RuBisCO, 12 units of PGK, 375 nmol of NADH, 700 nmol of RuBP, 1500 nmol of ATP, 15 units each of TPI, F1, 6 BPase, and PGI, 0.3 mg of F1, 6 BPase aldolase, and 0.15 units of glucose-6-phosphatase.

7.3.2 Vesicle-based glucose synthesis

7.3.2.1 Light-driven proton pumping activity of BR-ATP synthase-copolymer vesicles

A plot of ΔpH vs. green LED illumination time is shown in Fig. 7.6. The control of no-illumination (in the dark) is also shown. When illuminated under green LED light, BR-ATP vesicles showed quick decrease in pH in the first 25 min and the maximum pH drop was 0.078 units. The drop of pH suggests that BR pumped protons (H^+) into vesicles under green LED illumination. In contrast, there was no significant pH change in the dark, which confirms that pH changes were caused by illumination. The maximum ΔpH of -0.078 was close to the previously reported ΔpH of -0.080 by Choi et al. (2006) under similar reaction conditions. The results confirm the proton pumping functionality of the BR-ATP synthase system.

Fig. 7.6. Light-driven pH change in BR-ATP synthase-copolymer vesicles.

7.3.2.2 Photo-induced ATP synthesis in BR-ATP synthase-copolymer vesicles

Fig. 7.7. shows ATP synthesis of the BR-ATP synthase-copolymer vesicles versus reaction time. The maximum ATP synthesis was 301.2 nmol ATP per mg of ATP synthase, occurred at 40 min. Since BR was pumping protons toward the interior of the vesicle, ATP synthase had a function orientation with F_1 facing outward due to the direction of the proton motive force (Richard et al., 1995). After 40 min, ATP synthesis slightly decreased to approximately 250 nmol ATP per mg of ATP synthase. The slight drop of ATP synthesis after 40 min is in agreement with the slight decrease of proton pumping activity by BR as shown in Fig. 7.6. Apparently there was a lag of approximately 20 min in ATP synthesis compared to proton pumping, which was understandable because ATP synthesis happened only after proton gradient reached certain levels to drive ATP synthesis.

Fig. 7.7. Photo-induced ATP synthesis in green-light illuminated BR-ATP synthase-copolymer vesicles.

7.3.2.3 Glucose synthesis in BR-ATP synthase-copolymer vesicles

After demonstrating the proton pumping activity of BR and ATP synthesis of the ATP synthase constituted in the BR-ATP synthase-copolymer vesicles, all of the glucose synthesis enzymes were added to the reaction buffer containing BR-ATPase-copolymer vesicles. The solution was exposed to green LED for glucose synthesis and the results are shown in Fig. 7.8. The maximum glucose production was $5.2 \mu\text{g ml}^{-1}$ in 7 hours. As expected, glucose was not detectable without green LED illumination, as shown in Fig. 7.8 as the control. In the non-vesicle based experiments, the highest glucose synthesis was $14.72 \mu\text{g ml}^{-1}$ when 1 mM ATP was incorporated (Fig. 7.4). However, in the vesicle-based experiments, approximately only 0.1 mM ATP were generated within the vesicles, which apparently were not sufficient to drive glucose synthesis, therefore, lower amounts of glucose was synthesized. These results suggest that ATP highly affected glucose synthesis in both non-vesicle and vesicle-based reaction systems. Still, the vesicle-based enzymatic artificial photosynthesis system efficiently utilized

ATP to drive glucose synthesis because a smaller amount of ATP (about 10% of the non-vesicle system) resulted in relatively high glucose synthesis (35.3% of the non-vesicle system). Here, lower ATP generation is related to the phase region of the BR-ATP synthase-copolymer formation. Protein-polymer mixtures usually have three phases, which are vesicles, micelles and crystalline structure. Only when incorporated into the vesicles, can proteins maintain their functionality (Choi et al., 2005). Any other two structure formations could affect ATP synthesis by F_0F_1 ATP synthase. In this research, the maximum glucose production $5.2 \mu\text{g ml}^{-1}$ is lower than $10.8 \mu\text{g ml}^{-1}$ obtained by Wendell et al. (2010). However, glucose synthesis of a protein-based artificial photosynthesis system affected by operating conditions, including the concentrations of reactants, reaction temperature, and illumination were detailed studied. Moreover, higher glucose synthesis could be expected if enhancing the light reactions of artificial photosynthesis to improve proton pumping and ATP synthesis in the future.

Fig. 7.8. Glucose synthesis in BR-ATP synthase-copolymer vesicles. The 780- μl reaction buffer (pH=7.8) contained all catalytic enzymes mixed with 900 μl BR-ATPase-copolymer vesicle and was illuminated by 5-W green LED light.

7.4. Conclusions

The initial concentrations of RuBP and ATP, lighting source, and temperature significantly affected glucose synthesis. Higher concentrations of RuBP and ATP promoted glucose synthesis, but illumination (especially white fluorescent light) inhibited glucose synthesis. Glucose synthesis was found sensitive to temperature with 40 °C being optimum. Full glucose synthesis by combining the light and dark reactions in a vesicle-based artificial photosynthesis system was successfully demonstrated. This system efficiently utilized light-induced ATP to drive glucose synthesis and 5.2 $\mu\text{g ml}^{-1}$ glucose was synthesized in 0.78 ml reaction buffer in 7 hours. Light-dependent reactions were the bottleneck of the studied artificial photosynthesis system.

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CHAPTER 8 - Conclusions and Future work

8.1. Conclusions

The glucose synthesis in a protein based artificial photosynthesis system was investigated in this research. After evaluating the effect of reaction conditions on glucose synthesis, the purification and activity of two key enzymes involved in the glucose synthesis, and the effect of culture conditions on enzymes activity or content, the following conclusions were drawn:

1. The growth and bacteriorhodopsin (BR) content of *Halobacterium halobium* were affected by LED light intensity, light color and illumination pattern in the photobioreactor system. Bacterial growth and BR content increased with increasing white LED light intensity. LED blue light was found to be the most efficient for bacterial growth. It was also observed that 12 h LW - 12 h DK cycle illumination pattern lagged the bacteria growth but help for obtaining higher value of bacterial growth and BR content. Fade pattern was better than jump pattern for bacterial growth and BR formation. *Halobacterium halobium* growth and BR content are highly associated under the illumination conditions. The highest dry cell weight of bacteria *Halobacterium halobium* and BR content were $1.84 \pm 0.005 \text{ g l}^{-1}$, and $11.76 \pm 0.292 \text{ mg l}^{-1}$, under the 2000 Lux LED white light illumination.
2. Three-parameter models (Quadratic, Logistic and Gompertz) and four-parameter models (Schnute and Richards) were compared to describe the growth of bacteria *Halobacterium halobium* as a function of temperature or light. The Gompertz model was 100% accepted by the t test and 97% accepted by the F test when the temperature effects were considered. Results also indicated that the Gompertz model was 94%

accepted by the F test when the growth of *Halobacterium halobium* was studied under varying light intensities. Thus the Gompertz model was considered statistically sufficient to describe the growth of *Halobacterium halobium* as a function of temperature or light. In addition, the biological growth parameters, including specific growth rate (μ_m), lag time (λ), and asymptote (A) changes under Gompertz modeling were evaluated. When the temperature effect was considered, the biological growth parameters, including specific growth rate (μ_m) and asymptote (A) were increased with the increase of culturing temperatures, and then they were decreased due to inactivation or denaturation of proteins in higher temperatures. Lag time (λ) was decreased with increased of culturing temperatures. When the light effect was considered, both specific growth rate (μ_m) and asymptote (A) were increased with the increase of light intensities, while lag time (λ) was decreased.

3. The accumulation and ATPase activity of F_0F_1 ATP synthase in thermophilic bacteria *Bacillus* PS3 were affected by culture conditions, including the type of nitrogen source and the concentrations of polypeptone and sodium chloride in the culture medium, as well as the medium pH and aeration rate. The highest yield and ATPase activity of purified enzymes were obtained when adding polypeptone as the nitrogen source. Contrarily, sodium chloride concentration had little effect on the growth of PS3 or F_0F_1 ATP synthase accumulation. Medium pH and aeration rate showed significant effects on the accumulation and activity of F_0F_1 ATP synthase. The highest yield and activity of purified F_0F_1 ATP synthase were 1.631 g l^{-1} and $7.99 \text{ } \mu\text{mol min}^{-1} \text{ mg}^{-1}$, under the culture conditions of pH 7, aeration of 3 l min^{-1} and 0.3% NaCl and 1.2% (w/w)

polypeptide concentrations. The F_0F_1 ATP synthase purified from bacteria PS3 was characterized by SDS-PAGE.

4. Growth of bacteria *Bacillus* PS3 was predicted by modeling the lag time, specific growth rate and asymptote, combined with Gompertz equation. The suitability and usefulness of different models for lag time, specific growth rate and asymptote were determined by using F test, by which the lack of fit of the models was compared with the prediction error. Modified Ratkowsky model was the most suitable to describe both the growth rate and asymptote as a function of temperature. The hyperbolic model was selected to describe the lag time as a function of temperature. The above chosen models combined with Gompertz equation were used to predict bacteria growth, and the largest relative prediction error was 7.78%. These results suggested that the chosen models combined with Gompertz equation could predict *Bacillus* PS3 growth at any given time and temperature. Peak value of bacterial growth can also be accurately predicted by using the selected models.
5. In non-vesicle-based glyceraldehyde-3-phosphate (GAP) and glucose synthesis, the initial concentrations of ribulose-1, 5-bisphosphate (RuBP) and adenosine triphosphate (ATP), lighting source, and temperature significantly affected glucose synthesis. Higher initial concentrations of RuBP and ATP significantly enhanced GAP synthesis, which was linearly correlated to glucose synthesis, confirming the proper functions of all catalyzing enzymes in the system. White fluorescent light inhibited artificial photosynthesis and reduced glucose synthesis by 79.2% compared to in the dark. The reaction temperature of 40 °C was optimum whereas lower or higher temperature

reduced glucose synthesis. Glucose synthesis in the vesicle-based artificial photosynthesis system reconstituted with bacteriorhodopsin, F_0F_1 ATP synthase and polydimethylsiloxane - methyloxazoline - polydimethylsiloxane triblock copolymer was successfully demonstrated. This system efficiently utilized light-induced ATP to drive glucose synthesis and $5.2 \mu\text{g ml}^{-1}$ glucose was synthesized in 0.78 ml reaction buffer in 7 hours. Light-dependent reactions were found to be the bottleneck of the studied artificial photosynthesis system.

8.2. Contributions

This research covered the evaluation of proton gradient, ATP and glucose synthesis in a protein-based artificial photosynthesis system. It additionally investigated the effect of different reaction conditions on glucose synthesis in this system. This work revealed that reaction conditions, including energy (ATP), lighting source, and temperature significantly affected the glucose synthesis, suggesting that previous research related to artificial photosynthesis should be developed to improve the glucose production, thus increasing their values as potential biofuels applications.

Bacteriorhodopsin, and F_0F_1 ATP synthase are two key enzymes in artificial photosynthesis system, and the enzymatic activities and contents of these two enzymes significantly affect their performance in artificial photosynthesis system. Enzyme purification from bacteria has been investigated in a few studies, but the relationship between bacteria growth and enzyme activity or content was not investigated. From this

research it can be concluded that there was correlation between bacteria growth and enzyme activity or content. In addition, bacteria growth and enzyme activity were found to be affected by culture conditions, e.g., temperature and light.

So far little research has been conducted to study bacteria *Bacillus* PS3 and *Halobacterium halobium* under varying temperature and light conditions. Besides, modeling these two bacteria were scarce in the literatures. This research first developed models as a function of temperature or light, which could be used to accurately predict these two bacteria growth.

8.3. Recommendations for future work

Some recommendations were made for future research of artificial photosynthesis:

1. The optimization of main reactants concentrations, including CO₂, copolymer, Bacteriorhodopsin, and F₀F₁ ATP synthase, in protein-based artificial photosynthesis system needs to be considered. This research could provide correlations between the glucose, ATP production and main reactants concentrations. This could also help to understand ATP and glucose changes by modifying reactants concentration, especially to maintain relatively higher glucose production by loading fewer enzymes.
2. The improvement of enzyme activity working in artificial photosynthesis is another topic that needs to be studied. In this research, ATPase activity of F₀F₁ ATP synthase was successfully improved within stepwise enzyme purification process. However,

ATPase activity can be further improved through chromatography method, e.g., DEAE ion exchange chromatography. By using this method, F_0F_1 ATP synthase can be highly concentrated while other proteins will be washed out by running buffer. This can help to improve the enzyme performance in glucose synthesis in artificial photosynthesis system.