Effect of Environmental Factors on Growth Kinetics of Biocontrol Agent *Bacillus thuringiensis* Bacterium using 2L and 5L A+ Sartorius Stedim Biostat® Fermentation Systems

C. S. Richardson, D. Upadhyay, S. Mandjiny, and L. Holmes

Abstract — *Bacillus thuringiensis* (Bt) is a soil-dwelling, Gram-positive bacterium that is used as a biological pesticide and used to genetically engineer plants due to the toxic proteins it produces. *B. thuringiensis* was studied in batch cultures to determine the specific growth rates and doubling times. The purpose of this experiment was to research the growth kinetics of *Bacillus thuringiensis* in a 2L bioreactor and a 5L bioreactor containing growth media at different environmental conditions. Fermentation parameters were controlled by utilizing a Sartorius Stedim Biostat® A+ bioreactor system for bacterial growth. The environmental conditions included temperature, agitation, and aeration. The specific growth rates of *B. thuringiensis* were determined. The optimal conditions for the 2L bioreactor were 200 RPM, 30°C, 1.5 VVM, and with the highest specific growth rate 0.30 hr and the shortest doubling time 2.3 hr. For the 5L bioreactor, the optimal conditions were 150 RPM, 30°C, 1.5 VVM, and with the highest specific growth rate 1.2 hr and the fastest doubling time 0.6 hr.

Index Terms — *Bacillus thuringiensis*, bioreactor, environmental conditions, and growth kinetics.

I. INTRODUCTION

Bacillus thuringiensis (Bt) is a Gram-positive, soil-dwelling bacteria that creates proteins that are toxic to insects when ingested; specifically, toxic to larvae. The same proteins are not toxic to mammals due to the inability of activation. There are several variations of Bt which target a variety of insects such as beetles, mosquitos, black flies, caterpillars, and moths [1]. These toxic proteins are known as Cry and Cyt toxins (crystal proteins). Crystal proteins are formed as parasporal crystalline inclusions during stationary phase of growth [2]. The life cycle of Bt is defined by two

phases: vegetative cell division and spore development or sporulation cycle. Vegetative cells are rod-shaped and divide into identical daughter cells while sporulation involves asymmetric cell divisions [2]. The life cycle of Bt in the insect begins with the crystal/spore being ingested by the larvae. The crystal dissolves and toxins are activated by an endotoxin. The toxin binds to the gut receptors (epithelium). The spores germinate and the bacteria grows, killing the larvae. Bt is commonly used as an insecticide due to its toxicity to insects. It is also the primary gene in genetically modifying several food crops, so they produce the toxin independently [2]. Bacterial growth phases must be understood to understand the results and findings of this testing. There are four distinct bacterial growth phases: the lag phase, exponential (log) phase, stationary phase, and the death phase [3]. The lag phase is the stage that allows for the adaptation necessary for bacteria cells to utilize the environmental parameters [4]. The exponential or logarithmic phase reflects a constant rate of cell division. Stationary phase is the stage when bacteria population remains constant meaning cell division continues while some bacteria cells die (cessation). The final phase is the death phase which is when the bacteria cells experience an exponential decrease in the number of living cells and where cell division ceases [3]. Certain Bt strains have been used for several decades as biological insecticides to control agricultural pests. Recently, Bt has been used in genetically modifying crops to control agricultural pests [5]. This is achieved through the cloning of the crystal protein genes and their expression. This focus is due to the increase in demand for the bacterium and its crystal proteins. For bacterial growth, seven environmental conditions are taken into consideration: nutrition, moisture, temperature, time, aeration, pH, and salt levels. In this report, the variables tested were temperature, aeration, and agitation. In this study, two bioreactors, a 2 L and a 5 L were used. The bioreactors allowed for continuous operation, temperature, agitation, and aeration control. Another advantage of utilizing bioreactors in fermentation testing is upscaling for possible mass production [6]. This study was performed with the goal of understanding these conditions to properly upscale for mass production. Bacterial isolation was done weekly using the streak plate technique.

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II. MATERIAL AND METHODS

A. Bacterial Isolation and Inoculation

Bacillus thuringiensis (Bt) used in this study was obtained from Presque Isle Cultures (Presque Isle, PA USA). All isolations and inoculations were performed under a hood. Bacterial isolation was achieved using the streak plate technique on nutrient agar. The plates were incubated for 24 hours at 34 °C. The bacteria culture was Gram stained to ensure that isolation was achieved.

B. Preparation of Bioreactor

The media used in the bioreactor was Nutrient Broth (NB) containing 16 g of nutrient broth in the 2 L bioreactor and 36 g in the 5 L bioreactor, 1 mL of antifoam in both, and 2 L of distilled water in the 2 L bioreactor and 4.5 L of distilled water in the 5 L. The pH of the NB was adjusted before being adding to the bioreactors. The loaded bioreactors were autoclaved, and the sterile condensers were appropriately attached and set the conditions for the fermentation.

Fig. 1. Image of the bioreactor assembled. The bioreactor is ready for bacteria inoculation.

C. Experimental Parameters

Three growth parameters were tested: temperature (°C), agitation (RPM), and air flow (VVM). All experiments were conducted at a pH of 6.5. The parameters were tested in three conditions as shown in Table 1 and Table 2. Whichever condition produced the greatest growth was kept for the testing of the other conditions. The parameter conditions were separated into three separate designs. Design 1 tested the conditions of temperature: 24, 27, 30, and 33 °C with agitation set at 100 rpm and the air flow being set at 1.0 vvm. Design 2 tested agitation speed at 50, 150, 200, and 250 rpm with temperature being set at 30 °C and air flow set at 1.0 vvm. Design 3 tested the air flow rate at 0.25, 0.5, 1.5, and 2.0 vvm with temperature set at 30 °C and agitation at 150 rpm.

D. Experimental Designs

For the 5 L bioreactor, the first design kept the agitation speed at 100 rpm and the aeration at 1.0 vvm with the temperature varying from 24 °C to 33 °C increasing by three units. The second design kept the temperature constant at 30 °C (highest specific growth) and the aeration at 1.0 vvm with the agitation varying from 50 rpm to 250 rpm increasing by 50 units. The final design kept the temperature constant at 30 °C and the agitation at 150 (highest specific

growth) with the aeration varying from 0.25 vvm to 2.0 vvm increasing by 0.5 vvm.

For the 2 L bioreactor, the designs were kept the same as the 5 L except for the aeration variation design. The temperature was kept at 30 °C and the agitation was kept at 200 rpm since that showed the highest specific growth in the 2 L.

TABLE I: EXPERIMENTAL DESIGNS FOR 5L BIOREACTOR

Parameters	Temperature Variation	Agitation Variation	Aeration Variation
Temperature (°C)	24, 27,30,33	30 (Highest SGR)	30
Agitation (RPM)	100	50,150,300, 250	150 (Highest SGR)
Aeration (VVM)	1.0	1.0	0.25,0.5,1.5, 2.0

TABLE II: EXPERIMENTAL DESIGNS FOR 2L BIOREACTOR

Parameters	Temperature Variation	Agitation Variation	Aeration Variation
Temperature (°C)	24,27,30,33	30 (Highest SGR)	30
Agitation (RPM)	100	50,150,200, 250	200 (Highest SGR)
Aeration (VVM)	1.0	1.0	0.25,0.5,1.5, 2.0

E. Determination of Specific Growth Rates (SGR) and Doubling Times (DT)

Specific growth rate (SGR) is defined as the increase in bacterial cell mass per unit time during the exponential phase. The most common unit used, and used in this experiment, is reciprocal hours (h^{-1}). Doubling time (DT) is the time it takes for the growth of the bacteria in a culture to double. The specific growth rates and doubling times were calculated by using Microsoft ®Excel software. All data were entered into Excel and a scatterplot graph was made to determine specific growth rates and doubling times. The specific growth rate was determined by using data points on the graph that had linear growth.

III. RESULTS

A. Temperature

Fig. 2. Effect of temperature (°C) on *Bacillus thuringiensis* specific growth rate.

For the 2 L bioreactor, the maximum specific growth rate (0.22 hr^{-1}) measured at 30 °C. fastest doubling time (3.2) was measured at 33 °C. The optimal growth from this design for the 2 L bioreactor is measured at 30 °C.

For the 5 L bioreactor, the maximum specific growth rate

(1.2 hr⁻¹) was measured at 30 °C. The shortest doubling time (0.6 hr) was measured at 33 °C. The optimal growth for temperature for the 5 L bioreactor is measured at 30 °C. Both the 2 L and the 5 L had minimum specific growth rates at 33°C.

B. Agitation

Fig. 3. Effects of agitation (RPM) on *Bacillus thuringiensis* specific growth rate.

For the 2 L, the maximum specific growth rate (0.2 hr⁻¹) was measured at 200 rpm. The shortest doubling time (3.5 hr) was measured at 250 rpm. The optimal growth for this design was measured at 200 rpm.

For 5 L bioreactor, the maximum specific growth rate (0.2 hr⁻¹) was measured at 150 rpm. The shortest doubling time (3.5 hr) was measured at 250 rpm. Both the 2 L and the 5 L had minimum specific growth rates at 250 rpm.

C. Aeration

Fig. 4. Effect of aeration (vvm) on *Bacillus thuringiensis* specific growth rate.

For the 2 L the maximum specific growth rate (0.3 hr⁻¹) was measured at 1.5 vvm. The shortest doubling time (2.3 hr) was measured at 0.5 vvm. The optimal growth was measured at 1.5 vvm. The 2 L had a minimum specific growth rate at 0.5 vvm.

For the 5 L the maximum specific growth rate (0.3 hr⁻¹) was measured at 1.5 vvm. The shortest doubling time (2.3 hr) was measured at 0.25 vvm. The optimal growth was measured at 1.5 vvm. The 5 L had a constant specific growth rate between 0.25 vvm and 0.5 vvm and was the minimum specific growth rate.

IV. DISCUSSION

A. Temperature

El-Gayar et.al [7] reported their highest growth rate at 37 °C after 3 days of fermentation while the current study showed the highest growth rate at 30 °C. El-Gayar et.al [7] showed they used whey as the medium while the medium in the current study was nutrient broth. El-Gayar et.al [7] had a longer fermentation period (3 days). This suggests that using whey medium is more effective than nutrient broth. Another study showed that the rate of bacterial mortality or the rate of Bt activity was slightly temperature dependent. Frankenhuysen [8] suggested that temperature and exposure time effect the toxicity of Bt. Specifically, they examined the influence of temperature and exposure time on the mortality of various insect nests; suggesting that if the larvae were exposed and kept in extreme environments, then the bacteria may lose its toxicity. Fankenhuyzen [8] investigated the mortality rate of larvae with an increase in temperature which might be accounted for by an increase in bacterial growth. The results showed a correlation between exposure time and temperature to the larvae death rates [8]. Similarly, the current study showed a low rate of Bt growth when the conditions were set at the lowest interval (24 °C). In addition, Promdonkey and David [9] investigated the pore forming mechanism of a cytolytic protein produced by Bt. Their study included the effect of temperature on pore formation. Promdonkey and David [9] helped outline how the toxin transfers from the bacteria into the membrane. In this case, low temperatures neither inhibited membrane binding nor closed the formed pores. However, low temperatures did inhibit oligomerization of the toxins. This study suggested that the bacterial growth has a linear correlation with temperature whereas in the current study there is a linear correlation until the highest interval (33 °C). With studying the various impacts of temperature, researchers can continue to investigate and understand the impact of temperature on *Bacillus thuringiensis* and its production.

B. Agitation

Agitation is believed to be the most important factor in maintaining the production of Bt cells and the bioavailability of Bt toxins [10]. High agitation speed can cause a stressful environment and can interrupt the bacterial growth and fermentation process. Conversely, a slower agitation speed can lead to an accumulation of bacterial cells on the bottom of the fermentation vessel which would also impact the growth. W-T et.al [11] achieved a high yield (5.5 g/L Bt cells) at the end of a five-hour cultivation period in which agitation was at 500 rpm and the aeration was 1.5 vvm. This result contrasts with the current study since the highest interval measured was 250 rpm and the specific growth rate was not the highest at this interval. W-T et.al [11] performed a second five-hour cultivation with a pulse change in agitation from 500 rpm to 300 rpm and air flow at 1.5 to 4.5 vvm. With these conditions, the production of Bt increased 1.4-fold compared to the first study. This suggested that Bt can adapt and grow in a stressful environment and that a low concentration of oxygen has little effect on Bt production. W-T et.al [11] investigated the variation in agitation and aeration during the exponential

phase and stationary phase. Once agitation speed was decreased during the stationary phase, production increased to 43% [11]. This suggested that agitation speed should not be constant through the entire fermentation process due to the different needs of each bacterial growth phases. In contrast, the current study kept the same agitation interval for the entire fermentation period. Agitation brings continuous movement within the fermenter, allowing for mass and oxygen transfer between the growth phases. Dey et.al [12] investigated the effect of agitation and aeration regarding protease production in the *Bacillus* genus. The study was performed at lab-scale 2.2 L bioreactor with a volume of 2 L medium at 37 °C for an incubation time of 84 h [12]. The maximum protease production was found with the air flow at 2 vvm and the agitation speed at 180 rpm [12]. In comparison, the results from Dey et.al [12] showed a maximum protease production at 180 rpm and the results from the current study show the maximum specific growth rate at 200 rpm. It could be suggested that if the agitation speed exceeds 200 rpm for a constant speed, then this could cause a decrease in the bacterial growth due to the impact of shear stress causing cell damage.

C. Aeration

Aeration has been described as a key factor for Bt growth, sporulation, and δ -endotoxin production since Bt is an aerobic bacterium [13]. Boniolo et.al [14] showed that higher aeration level (50% dissolved oxygen) resulted in a higher spore count and improved the toxic activity in the fermentation broth. However, the present study showed the greatest air flow rate (2 vvm) did not produce a higher Bt production than 1.5 vvm. In addition, it has been suggested that there is a correlation between aeration levels and Bt production along with various processes within the bacteria such as the failure of Bt to sporulate under low aeration levels while under the presence of high sugar concentration [15]. The study suggests that Bt has a dependability on aeration that is significant to the growth and production of toxins. Mounsef and Lteif [13] investigated the effect of aeration on the fermentation kinetics of Bt and δ -endotoxins productivity. It is apparent, that as the aeration levels increased, then the production of Bt and toxins increased. With the increase in availability of oxygen, it allows for the bacterial cells to sporulate at a faster rate ultimately increasing the production.

V. CONCLUSION

In conclusion, this study was performed to understand the optimal growth conditions for *Bacillus thuringiensis*. This study explored how *B. thuringiensis* responds to various environmental parameters with using a 2 L and 5 L bioreactor. It is imperative to understand the optimal growth conditions for *B. thuringiensis* to produce insecticides and for facilitating the process of genetically modifying crops. With this, mass production of *B. thuringiensis* could help with the production of insecticides. Further studies should be performed to understand the effects of various conditions regarding bacterial growth such as researching the correlation between temperature, aeration, and agitation to *Bacillus thuringiensis* growth phases. In addition, it has been suggested that changing parameters during the fermentation period could result in greater sporulation and

could show the bacteria's ability to adjust quickly in disrupted environments. With this, a greater understanding of *Bacillus thuringiensis* and its properties can result in a greater understanding on how to efficiently produce Bt.

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