A STUDY ON THE GROWTH AND METABOLIC ACTIVITY OF *STREPTOMYCES VENEZUELAE*

by

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LIST OF ABBREVIATIONS AND SYMBOLS USED

ATP	Adenosine triphosphate
A_{bot}	Surface area of the bottom plate (m^2)
A_{Lid}	Surface area of the lid (m^2)
A_{wa}	Surface area of wall above the media (m^2)
A_{wu}	Surface area of wall in contact with the media (m^2)
AU	Arbitrary Units
CFU	Colony Forming Units
C_p	Specific heat capacity of air ($J \cdot g^{-1} \cdot K^{-1}$)
dH_2O	De-ionized water
Dia_o	Outer diameter of the bottom plate (m)
Dia_i	Inner diameter of the bottom plate (m)
h_{bi}	Convection coefficient for media to bottom plate ($J \cdot s^{-1} \cdot m^{-2} \cdot {}^{\circ}C^{-1}$)
h_{bo}	Convection coefficient for bottom plate to ambient air ($J \cdot s^{-1} \cdot m^{-2} \cdot {}^{\circ}C^{-1}$)
h_{li}	Convection coefficient for exhaust gas to lid ($J \cdot s^{-1} \cdot m^{-2} \cdot {}^{\circ}C^{-1}$)
h_{lo}	Convection coefficient for lid to ambient air ($J \cdot s^{-1} \cdot m^{-2} \cdot {}^{\circ}C^{-1}$)
h_{wui}	Convection coefficient for media to lower wall ($J \cdot s^{-1} \cdot m^{-2} \cdot {}^{\circ}C^{-1}$)
h_{wuo}	Convection coefficient for lower wall to ambient air ($J \cdot s^{-1} \cdot m^{-2} \cdot {}^{\circ}C^{-1}$)
h_{wai}	Convection coefficient for exhaust gas to wall above media ($J \cdot s^{-1} \cdot m^{-2} \cdot {}^{\circ}C^{-1}$)
h_{wao}	Convection coefficient for wall above media to ambient air ($J \cdot s^{-1} \cdot m^{-2} \cdot {}^{\circ}C^{-1}$)
ISP	International *Streptomyces* Project
K_{pyx}	Thermal conductivity of Pyrex glass ($J \cdot s^{-1} \cdot m^{-1} \cdot {}^{\circ}C^{-1}$)
K_{Steel}	Thermal conductivity of steel ($J \cdot s^{-1} \cdot m^{-1} \cdot {}^{\circ}C^{-1}$)
Lt	Lag time from inoculation to initial growth of bacteria (hours)
L_{wa}	Length of wall in contact with exhaust gases (m)
L_{wu}	Length of wall in contact with media (m)
M_{air}	Mass flow of air ($g \cdot s^{-1}$)
MOPS	3-(N-morpholino) propanesulfonic acid
MYM	Malt-extract Yeast-extract Maltose
OD_{600}	Optical density at 600 nm
pH	Potential Hydronium
pK_a	Acid dissociation constant
Q_{bottom}	Heat energy lost through the bottom of the reactor ($J \cdot s^{-1}$)
$Q_{exhaust}$	Heat energy lost with exhaust gases ($J \cdot s^{-1}$)
Q_{known}	A known source of thermal energy ($J \cdot s^{-1}$)
Q_{lid}	Heat energy lost though the lid of the reactor ($J \cdot s^{-1}$)

Q_{media}	Heat energy stored in media (J·s^{-1})
$Q_{metabolism}$	Heat energy generated by bacteria metabolism and activity (J·s^{-1})
Q_{mix}	Heat energy generated through agitation (J·s^{-1})
$Q_{reactorloss}$	Heat energy lost through the lid, bottom and walls of the reactor (J·s^{-1})
Q_{total}	Total heat loss calculated from the bioreactor (J·s^{-1})
Q_{wall}	Heat energy lost through the reactor wall in contact with media (J·s^{-1})
$Q_{wallabove}$	Heat energy lost through the reactor wall in contact with exhaust gas (J·s^{-1})
R squared	Residual sum of squares
SEM	Scanning Electron Microscopy
SLt	Lag time from inoculation to initial consumption of free glucose (hours)
Th_w	Thickness of the reactor wall including the bottom plate (m)
Th_L	Thickness of the reactor lid (m)
TEM	Transmission electron microscopy
t	Time from inoculation (hours)
T_{amb}	Temperature of the ambient room air (°C)
T_{bo}	Temperature at the bottom of the Pyrex glass (°C)
T_{ex}	Temperature of exhaust gases leaving the reactor (°C)
T_{li}	Temperature at the inside of the steel lid (°C)
T_{lo}	Temperature at the topside of the steel lid (°C)
T_m	Temperature of the media (°C)
T_{wao}	Temperature of the outer wall in contact with exhaust gases (°C)
T_{wai}	Temperature of the inner wall in contact with exhaust gases (°C)
T_{wuo}	Temperature of the outer wall in contact with media (°C)
U_{bot}	Overall heat transfer coefficient for the bottom of the bioreactor (s·m^2·°C ·J^{-1})
U_{lid}	Overall heat transfer coefficient of bioreactor lid (s·m^2·°C ·J^{-1})
U_{wa}	Overall heat transfer coefficient for the wall above media (s·m^2·°C ·J^{-1})
U_{wu}	Overall heat transfer coefficient for wall contacting media (s·m^2·°C ·J^{-1})
μ_{max}	The specific growth rate (hours^{-1})
μ_g	The substrate consumption rate (hours^{-1})

ACKNOWLEDGMENTS

ABSTRACT

The bacteria *Streptomyces venezuelae* produce the novel antibiotic jadomycin. The study of growth characteristics and metabolic behavior of the bacteria are necessary to scale up antibiotic production and facilitate further research. In this study, a method for producing consistent inoculum was developed that showed good repeatability when used in growth trials. The rod shaped spores of *Streptomyces venezuelae* were determined to be approximately 0.8 x 0.2 μm with a smooth surface type. The effects of temperature and pH on bacterial growth and substrate consumption were examined in a 7 L bioreactor. Of the range of parameters tested (28, 32, 36 °C, and media pH of 5, 7 and 9), 32 °C with a media pH of 7 yielded the highest rate of growth (μ_{max} of 1.43 hours^{-1} with a lag time of 7.7 hours). The results of all trials showed that free glucose was consumed before the maltose, which was the major sugar substrate in the media. The initiation of exponential bacterial growth occurred after rapid consumption of free glucose. A heat balance analysis was also performed over the bioreactor to identify the heat generated through agitation, losses over the vessel and the heat of metabolism from *Streptomyces venezuelae*. Under normal operating parameters 33 - 24 % of the heat generated through mixing was lost with the exhaust gas, while 56 - 64 % was lost through the bioreactor wall. The heat of mixing was calculated to be 1.62 J·s^{-1} while the maximum amount of heat generated by *Streptomyces venezuelae* metabolism and activity during a growth trial was 2.28 J·s^{-1} for 60 x 10^9 CFU·mL^{-1}.

CHAPTER 1   INTRODUCTION

Increasing antibiotic resistance by pathogenic bacteria is cause for concern, as the rate of discovery for new antibiotics has been in decline (Hopwood, 2007). *Streptomyces* is a genus of soil bacteria responsible for the production of many clinically useful antibiotics (Kieser *et al.*, 2000) and has been the subject of many studies in the quest for novel antibiotics (Hopwood, 2007). The species *Streptomyces venezuelae* is capable of producing the broad-spectrum antibiotics chloramphenicol and jadomycin, as secondary metabolites. A method to chemically synthesize chloramphenicol was discovered in 1947 allowing it to become the first antibiotic to be produced synthetically on a large scale (White, 1950). *Streptomyces venezuelae* has since been found to produce the jadomycin family of antibiotics when environmentally stressed and in the presence of specific amino acids (Rix *et al.*, 2003). The jadomycins are proven to be effective against bacteria and yeast cells while also showing promise in cancer research (Jakeman *et al.*, 2004).

The techniques currently used to produce jadomycin are difficult to scale-up. The typical method of jadomycin production in the laboratory is a batch process typically utilizing volumes of media less than 250 mL in shake flasks (Jakeman *et al.*, 2006). In this process, the population of *Streptomyces venezuelae* bacteria is increased in growth media before being used to inoculate jadomycin production media where there is little or minimal bacterial growth. With this process there is uncertainty concerning operating conditions needed for the most efficient growth of bacteria prior to the jadomycin production stage. Therefore further study is required in order to scale up bioreactor growth and facilitate commercial antibiotic production.

Due to the rapid exponential reproduction rate of bacteria, it is necessary to work with a controlled inoculation between trials when studying the effect of environmental parameters on bacterial growth. To compare trials accurately, the cells must be in the same physiological condition and with a comparable number of cells (Kieser *et al.*, 2000). The preparation of unfrozen spore inocula can be used to ensure that cells are in a uniform state prior to germination and growth (Kutzner and Nitsch, 1970 and Kieser *et al.*, 2000). Spores can be

separated from vegetative cells through selective filtering techniques (Parada, 1975). Scanning electron microscopy (SEM) and laser particle sizing techniques can also be used to characterize the resultant spore inoculum.

Bacterial growth rates are dependent upon many variables including the inoculum, dissolved oxygen concentration, type of media, agitation rate, temperature, pH *etc*. The consumption of substrates such as sugars is necessary for growth and is therefore also affected by these parameters. Currently, there are conflicting reports on the ideal temperature at which to grow *Streptomyces venezuelae* (Wang and Vining, 2003 and Jakeman *et al*., 2006). Also, no studies have been found that have examined the effects of pH on the growth of *Streptomyces venezuelae* in pilot scale bioreactors.

A heat balance analysis can be performed on a bioreactor system to determine the amount of heat generated and lost to the environment during normal operation. This information is useful for identifying possible changes to the current system that would result in a more efficient operation. The methodology can also be used to assess the energy efficiency of larger similarly configured bioreactors during scale-up. In addition, heat balance models can be used to determine the heat of metabolism of organisms within the bioreactor.

In this study, a method for producing consistent inoculum was developed that would be suitable for growth trials and long term storage. The effects of temperature and pH on bacterial growth were examined through a series of growth trials where the growth of bacteria and substrate consumption were monitored. A heat balance analysis was also performed over the bioreactor to identify the heat generated through agitation, losses over the vessel, and the heat of metabolism from *Streptomyces venezuelae*.

CHAPTER 2   OBJECTIVES

The overall aim of this study was to examine the effect of environmental parameters on *Streptomyces venezuelae* growth in a bioreactor system and determine the heat of metabolism in order to facilitate increased biomass production and improved reactor design.

The specific objectives of this study were to:

1. Develop a procedure to create a consistent inoculum of *Streptomyces venezuelae* for growth trials and long term storage.
2. Examine the effect of temperature and pH on the growth and substrate utilization of *Streptomyces venezuelae* in a batch operated bioreactor.
3. Perform heat balance analysis on the bioreactor system in order to determine:
   i. The heat of mixing from the bioreactor.
   ii. The heat losses from the bioreactor.
   iii. The metabolic heat produced by *Streptomyces venezuelae*.

CHAPTER 3 LITERATURE REVIEW

3.1 *Streptomyces* Genus

Streptomyces are gram positive bacteria of the Actinobacteria phylum which have a complex growth and secondary metabolite production similar to filamentous fungi (Flärdh, 2003). Bacterial growth occurs through extension and branching to form vegetative mycelia (Figure 3.1). After the formation of the mycelium, the bacteria may differentiate through the production of aerial hyphae that septate into spores (Claessen *et al*., 2006). Despite their characteristic similarities to fungi, the cell walls of vegetative cells are structurally and chemically similar to those of other gram-positive bacteria (Bradley and Ritzi, 1968).

3.1.1 Sporulation

Streptomyces sporulation is believed to be linked to stresses that induce secondary metabolite production such as the exhaustion of available nutrients. At this stage in the *Streptomyces* lifecycle, the colony of mycelial bacteria forms aerial hyphae. Continued aerial hyphae growth is maintained though the utilization of the vegetative mycelium until the multigenomic tips of the aerial hyphae growth undergo mitosis-like segregation of bacterial chromosomes and plasmids to yield unigenomic prespore compartments (Schwedock *et al*., 1997). Images of Streptomyces mycelia, aerial and sporulating hyphae are shown in Figure 3.2. When grown on solid media, *Streptomyces* will typically form characteristic round colonies with a depression in the middle (Figure 3.3). When conditions become unfavorable for growth, aerial hyphae extend upwards and sporulate as seen in Figure 3.4 (Ryding *et al*., 1999). *Streptomyces venezuelae* is one of the few *Streptomyces* species that will sporulate in liquid media (Kieser *et al*., 2000), allowing the secondary metabolites produced during the sporulation process to be more easily recovered. The exospores produced during sporulation are resistant to desiccation; however they are sensitive to extremes in pH and temperature (Davis and Chater, 1990).
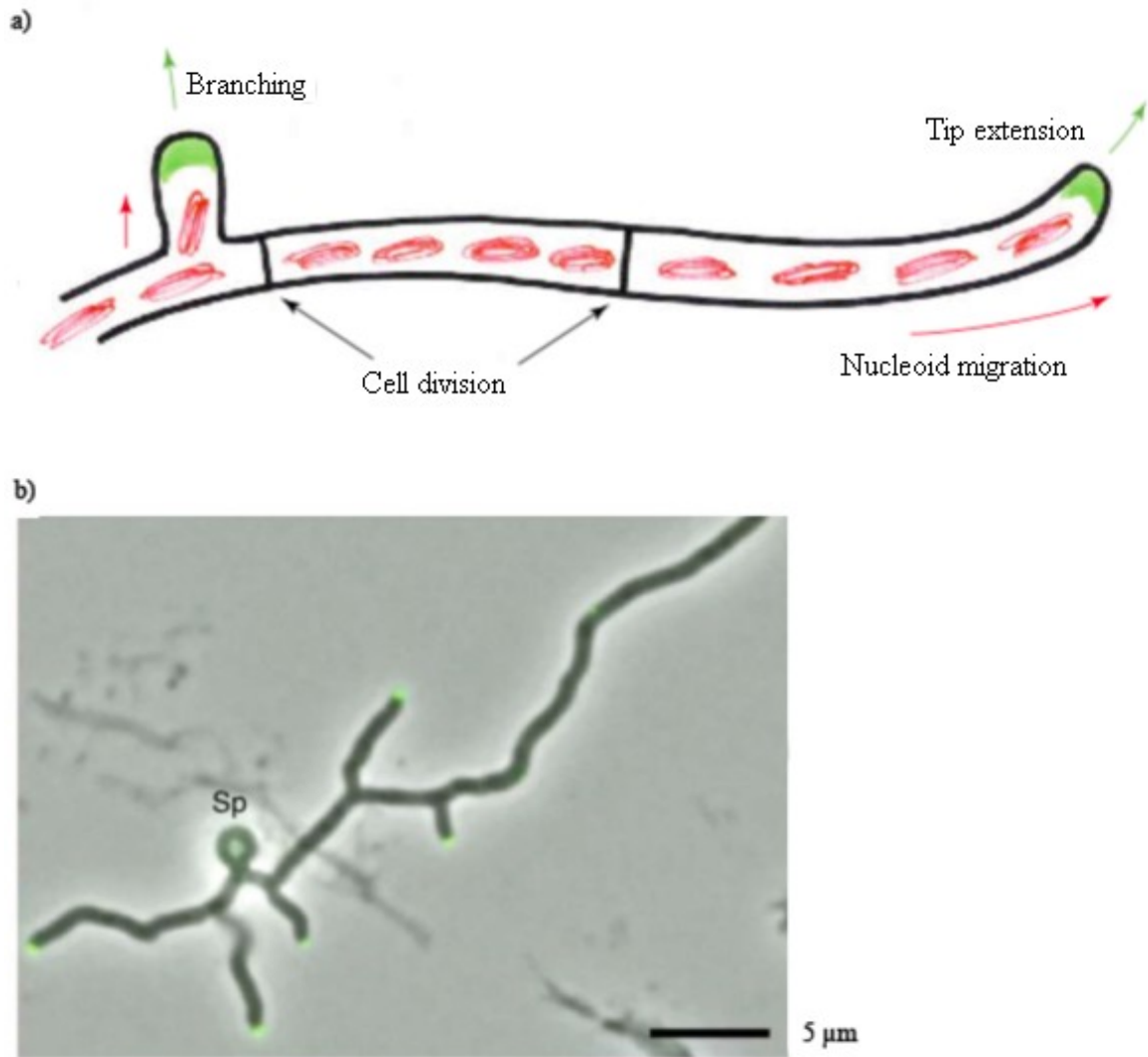
Figure 3.1   *Streptomyces* mycelia formation from an individual spore (Flärdh, 2003). (a) An illustration depicting the methods of growth by *Streptomyces* hyphae including tip extension and cellular branching; (b) A phase-contrast image of *Streptomyces* mycelium growing from of a spore (Sp).
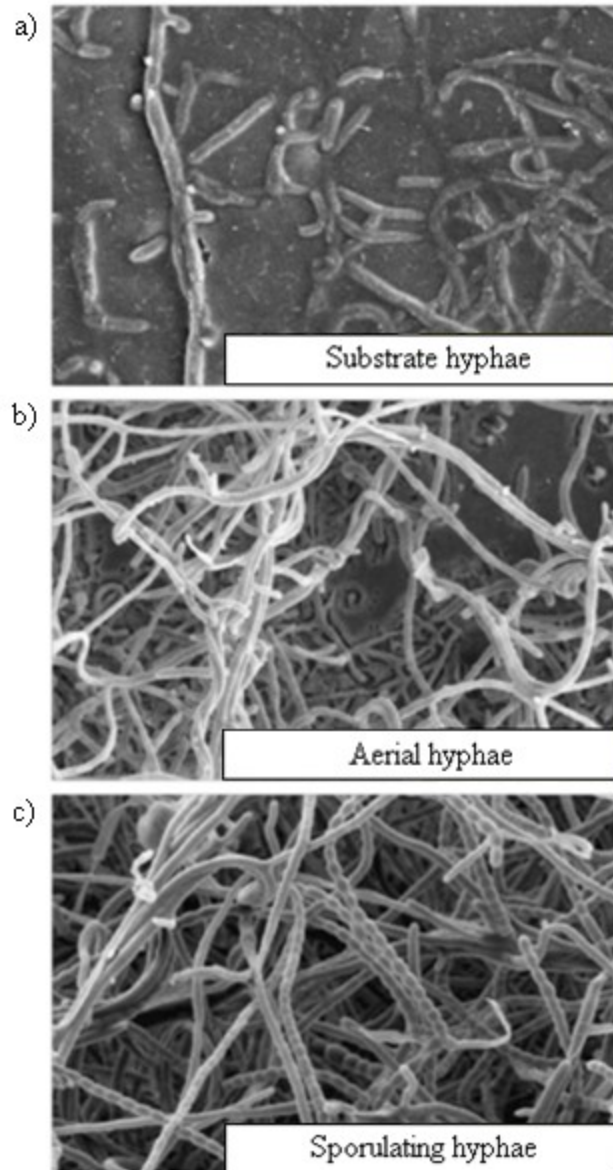
Figure 3.2   SEM image of *Streptomyces* sporulating hyphae (Claessen *et al.,* 2006). (a) Substrate hyphae forming a filamentous mycelium; (b) aerial hyphae rising form from the mycelia; (c) Aerial hyphae forming chains of exospores.
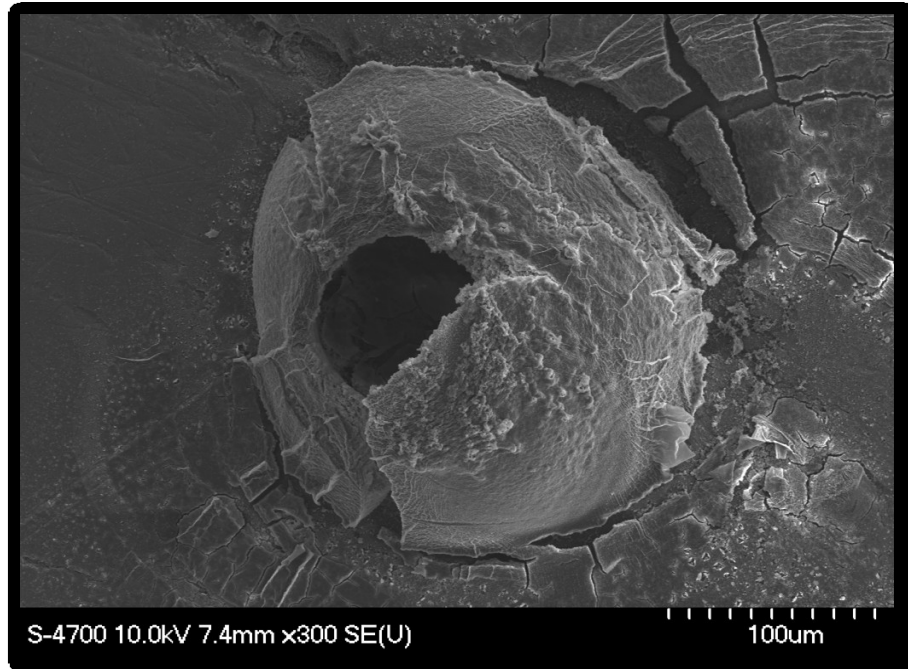
Figure 3.3   Image of a *Streptomyces venezuelae* colony grown on a solid surface captured with the cold field scanning electron microscope at the Institute for Research in Materials Dalhousie University, N.S., Canada.
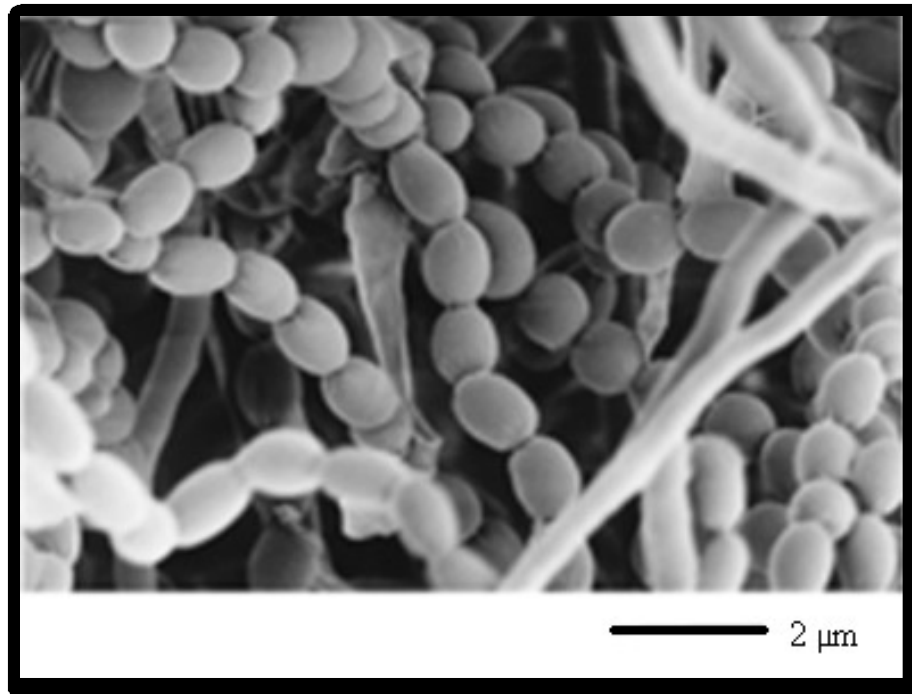
Figure 3.4   *Streptomyces coelicolor* hyphae after sporulation (Ryding *et al*., 1999)

The work of Dietz and Mathews (1971) classified *Streptomyces* spores into five groups based upon spore surface characteristics as observed through scanning electron microscopy (SEM) and transmission electron microscopy (TEM). The five classifications presented are smooth, warty, spiny, hairy, and rugose as shown in Figure 3.5.

The size of *Streptomyces* spores vary between species (Tresner *et al.,* 1960), and can be dependent upon several factors. For example, the approximate size of *Streptomyces albus* spores range from 0.85 - 1.07 μm and is dependent on the relative humidity of their environment (Górny *et al.,* 2003). When isolating spores of a particular species through filtration, the size of the spores is an important factor (Parada, 1975).

3.1.2   Secondary Metabolites

Metabolites are compounds produced by a cell through the process of cellular metabolism. Primary metabolites are compounds such as proteins and nucleic acids that are used directly in cellular growth and reproduction. Secondary metabolites are not used directly for growth, however they often give an organism a competitive advantage in a particular environment. Secondary metabolite production is often regulated and only initiated through environmental stress (Young, 2005). Like most soil bacteria, *Streptomyces* produce numerous biologically active secondary metabolites such as the molecule geosmen. Geosmen, produced by *Streptomyces* species such as *Streptomyces coelicolor* has an earthy aroma and flavor; it is responsible for the earthy taste of vegetables such as beets and a contributor to the strong scent of disturbed soil. The first antibiotic discovered by Sir Alexander Fleming in 1928 (Fleming, 1929) facilitated the discovery of many other secondary metabolites with similar properties. These compounds have been isolated from both fungi and bacteria (particularly the *Streptomyces* genus) and are extremely useful in medicine for their microbicidal properties (Hopwood, 2007).
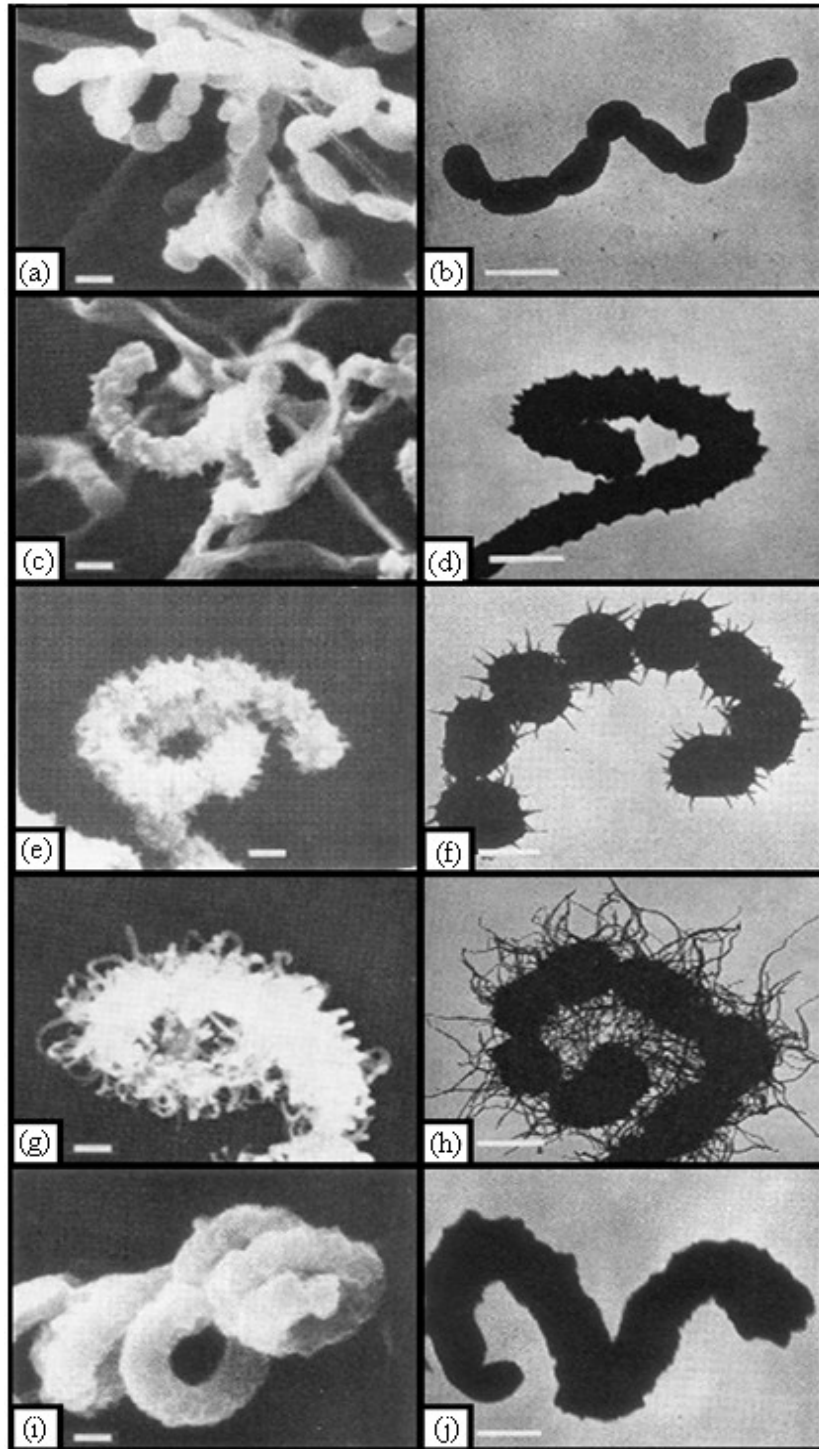
Figure 3.5   Phase-contrast and low-magnification SEM images of spore surface types. (Dietz and Mathews, 1971). Smooth (a - b), warty, (c - d), spiny, (e - f), hairy (g - h), and rugose (i - j).

3.2 *Streptomyces Venezuelae* and Antibiotic Production

Streptomyces are the largest antibiotic producing bacterial genus in microbiology (Watve *et al.*, 2001) and are often distinguished by their secondary metabolite production. *Streptomyces venezuelae* are known to produce chloramphenicol and jadomycins as secondary metabolites (Figure 3.6). The production of chloramphenicol by *Streptomyces venezuelae* was discovered in 1947 and was used clinically for several decades until toxic effects such as aplastic anemia resulted in the drug being rarely prescribed (Sheridan *et al.,* 2008). A second antibiotic family produced by *Streptomyces venezuelae*, the jadomycins, were discovered and initially linked to growth at high temperatures (Ayer *et al.,* 1991). Jadomycins hold promise both as broad spectrum antibiotics (Jakeman, 2004) and also in cancer research as aurora kinase inhibitors.

3.2.1 Chloramphenicol

The molecular structure of chloramphenicol (D-(-)*threo*-2-dichloracetamido-1-*p*-nitrophenyl-1,3-propanediol) as shown in Figure 3.6 (a) is a relatively simple structure and was one of the very first antibiotics to be produced synthetically (White, 1950) as described by Fodor (1955). The antimicrobial effects of chloramphenicol result from the specific shape of the molecule. Chloramphenicol inhibits the production of bacterial protein by directly interfering with substrate binding during synthesis. This process does not interfere with other metabolic processes and is dependent on the steric conformation of the molecule (Jardetzky, 1963). Work conducted on the biosynthetic production of chloramphenicol by Gottlieb *et al.* (1954) found production usually commenced 24 hours after inoculation and that the peak production of chloramphenicol by the bacteria occurred at the time the primary carbon source was consumed (usually 60 hours). Gottlieb *et al.* (1954) also reported that compounds structurally similar to chloramphenicol such as phenylserinol and phenylserine increased the production of the antibiotic when added to the media provided the steric conformation was similar to chloramphenicol or precursors. The antibiotic was produced during every trial attempted, although the yield was greatly affected by compounds added. It was noted that the

physiology of *Streptomyces venezuelae* did not vary between periods of growth and antibiotic production during the study.

3.2.1 Jadomycin

Individual jadomycins within the jadomycin family of antibiotics are named according to the type of molecular side-chain that is attached to the structure (Figure 3.7). Jadomycins are currently researched as a possible cancer treatment due to their function as aurora kinase inhibitators that block the phosphorylation of histone in a dose-dependent manner (Fu *et al.,* 2008). Jadomycin inhibits aurora-B activity by competing for adenosine triphosphate (ATP) in the kinase domain; this behavior induces apoptosis in tumor cells without negative side effects (Fu *et al.,* 2008).

The production of jadomycin has been linked to the heat shock response of *Streptomyces venezuelae* (Doull *et al.,* 1994) where the cellular response following an increase in temperature prompts the expression of specific genes. Other means of shocking the bacteria such as the presence of ethanol was found to have similar effects to increased temperature (Singh, 1992). Ethanol shock was found to be more effective than heat shock in inducing jadomycin production in *Streptomyces venezuelae* (Sing, 1992) making it the preferred method for laboratory scale jadomycin production for repeatability and ease of administration.

In order to produce jadomycin, bacteria are often populated within typical growth media such as Malt-extract, Yeast-extract, Maltose (MYM) media. The cells are subsequently isolated and washed with deionized water prior to being used as inoculum for antibiotic production media (Jakeman *et al.,* 2006). While the growth media often contains excess nutrients with some variability in the sources, the production media components are often carefully controlled. As in the production of chloramphenicol, specific amino acids are added to jadomycin production media to stimulate the formation of the jadomycin family of antibiotics. For example, Jakeman *et al.* (2006) added the amino acid isoleucine to buffered production media containing glucose, trace minerals and salt solutions to facilitate the production of jadomycin B. The components of the amino acid is incoperated into the structure of the jadomycin molecule (Rix *et al.,* 2003).

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(a)

Chloramphenicol

(b)
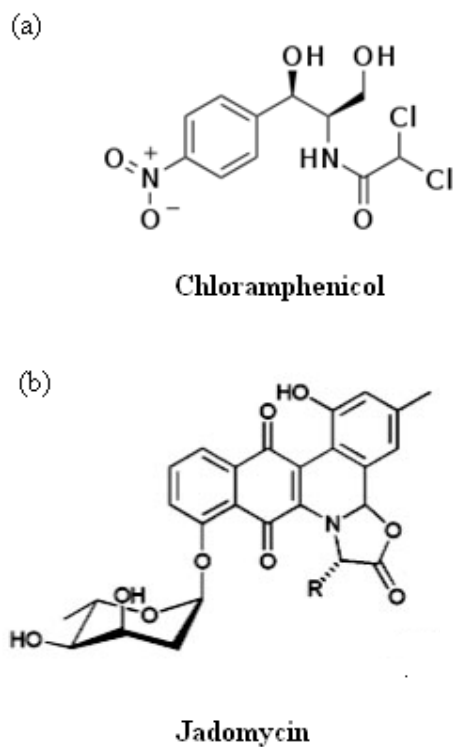
Jadomycin

Figure 3.6   Molecular structure of (a) chloramphenicol (b) jadomycin
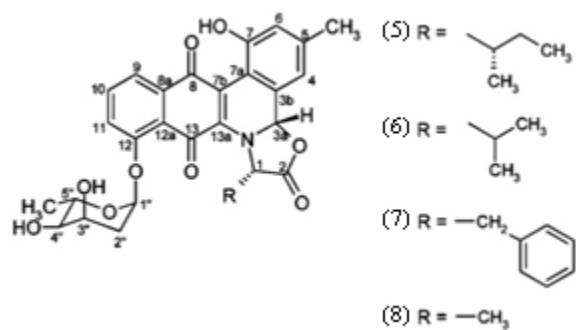
Figure 3.7   The side chains of jadomycins B (5), V (6), F (7), & Ala (8) (Rix *et al.*, 2003).

3.2   Inoculum

When conducting reproducible experiments involving microbial growth, the preparation and quality of the inocula are extremely important. When *Streptomyces* mycelia are used in the inoculum, numerous uncontrolled variables arise. These are age, stress level and growth stage of the cells, all of which must be controlled or accounted for (Kieser *et al.,* 2000). This problem becomes exacerbated when attempting to initiate trials over a period of several months as even small fluctuations in the population size of the inoculum can result in large changes to the growth rate. *Streptomyces* vegetative inocula are often prepared through the addition of several colonies sometimes from numerous starter plates to small amounts of media. This is subsequently incubated until sufficient biomass is produced, as determined through optical density measurements of the media. This method of vegetative inoculum used by Jakeman *et al.* (2004) is effective at acquiring biomass; however it does not provide the consistent inoculum required for comparative trials. The number of cells within the inoculum is difficult to directly assess and the inconsistent manner of initially adding cells can result in cells at different stages of growth or stress level.

Another method of preparing an inoculum is to harvest spores from the desired culture and store under conditions not favorable for germination. The cells within a spore suspension remain in a consistent state while still providing an "instant inoculum" (Kutzner and Nitsch, 1970). This technique also allows long term storage of a viable inoculum that should yield consistent results. By suspending the spores in a soft agar spore suspension, Kutzner and Nitsch (1970) have demonstrated that a suspension of *Streptomyces* spores will remain viable as an inoculum in excess of a year.

3.3   Factors that Affect *Streptomyces* Growth

The growth of a microorganism is highly dependent upon the surrounding environmental conditions. Every organism has a range of ideal conditions at which members of its species will grow optimally. Temperature, pH and dissolved oxygen concentration (among other factors) within the media is often carefully controlled in industrial

applications as deviations from the optimal conditions often have profound effects on the growth rate and activity of the organism (Shuler and Kargi, 1992).

3.3.1 Temperature

The behavior of bacterial growth is influenced by numerous factors such as environmental conditions and the physiological state of the bacteria. The temperature of the environment directly affects the activity and growth of cells; every species has an ideal temperature for growth that is influenced by its physiology (Shuler and Kargi, 1992). This optimum temperature for growth may not be the same as the temperature at which metabolites are produced most efficiently. *Streptomyces venezuelae* ISP 5230 are mesophilic bacteria, meaning their optimal growth temperature is between 15 and 40 °C. For every increase of 10 ºC closer to the optimum growth temperature, the growth rate of bacteria approximately doubles (Truelstrup-Hansen, 2008). If the mean media temperature is above optimal, the rate of growth is reduced. The temperatures used for *Streptomyces venezuelae* growth have been cited as 30 ºC ± 2 ºC (Wang and Vining, 2003 and Jakeman *et al*., 2004). At 42 ºC *Streptomyces venezuelae* express heat shock genes that stimulate sporulation and antibiotic production (Singh, 1992).

3.3.2 pH

Similarly to temperature, the hydronium ion concentration (pH) of the media influences the growth rate of bacteria by affecting the activity of cellular enzymes (Shuler and Kargi, 1992). To achieve the highest growth rate, current literature recommends growing *Streptomyces venezuelae* in media with pH maintained between 6.0 - 7.8 (Jakeman *et al*., 2004). The optimal pH levels are often dissimilar when maximizing growth rate and highest product yield (Shuler and Kargi, 1992). However, bacteria are often capable of growth within a large range of pH. Studies on the *Streptomyces* species by Kontro *et al*. (2005) have found the optimal pH ranges of growth and sporulation for *Streptomyces* species to be between 5.5 and 11.5. The study performed by Kontro *et al*. (2005) also found that the media strongly influences the acid tolerance of *Streptomyces* species. When grown at pH 4 in media that included yeast extract most species included in the studies were unable to grow, while at pH 7 the majority of species showed maximum

growth rate on most types of media. To buffer the pH of growth medium to near neutral values, the buffer MOPS is commonly used for this species of *Streptomyces* (Kendrick *et al.*, 1982). The buffer has a logarithmic acid dissociation constant (pK_a) of 7.2, making it ideal for buffering near neutral solutions. While studying the effects of carbon sources on antibiotic production by *Streptomyces venezuelae,* MOPS was added to jadomycin B production media by Jakeman *et al.* (2006) however, no buffer was included in the MYM agar used for growth throughout the study. Glazebrook *et al.* (1990) examined the effect of different carbon sources on *Streptomyces venezuelae* sporulation in minimal LS media. In this media, MOPS was sufficient to maintain the pH of the media at near neutral values for every carbon source except glucose. During growth in glucose-based LS media, a high production of oxo-acids was observed which resulted in the inhibition of growth.

3.3.3   Dissolved Oxygen

The dissolved oxygen available to bacterial cells within the media can be a limiting factor if the rate of consumption exceeds supply (Shuler, 1992). There is a critical oxygen concentration unique to each bacteria species, above which the growth rate is independent of the media dissolved oxygen concentration. This value is typically 5 - 10 % of the maximum saturation level, however the maximum saturation is influenced through the presence of dissolved salts, organics, and the temperature of the media (Shuler and Kargi, 1992).

The production of secondary metabolites can also be influenced through dissolved oxygen concentration. During antibiotic production by *Streptomyces clavuligerus,* increased oxygen saturation above the critical dissolved oxygen concentration resulted in additional product and biomass yield (Yegneswarant *et al.*, 1991) up to the maximum saturation limit of oxygen in the media.

3.3.4   Substrate

The substrate used for growth can affect the growth rate of bacteria as well as its characteristics. For example, Kontro *et al.* (2005) found that *Streptomyces* species behaved as acidophiles, neutrophiles or alkalophiles, depending on the media used for

growth. Most species would grow optimally at near neutral or slightly alkaline conditions on media that included yeast extract (Kontro *et al.,* 2005).

Streptomyces venezuelae is grown using many carbon sources including glycerol, glucose, lactose, mannose, maltotriose, maltose starch *etc*. The effect of these carbon source on growth and secondary metabolite production is dependent upon several factors such as pH (Kontro *et al.,* 2005). For example, studies on the sporulation of *Streptomyces venezuelae* in liquid media by Glazebrook *et al.,* (1990) found that while maltose supported the growth and sporulation of *Streptomyces venezuelae,* glucose significantly inhibited sporulation. Specifically, when grown in minimal LS media using glucose as the primary carbon source, the pH of the media dropped rapidly with the production of oxo-acids by the bacteria. The increased acidity likely contributed to growth inhibition, however the glucose was reported to also have an inhibitory effect on sporulation that was un-related to the increased acidity of the media. As the jadomycin family of antibiotics is linked to the sporulation pathway (Wang and Vining, 2003), it is evident that the media has a large effect on the growth and antibiotic production by *Streptomyces venezuelae*. Work by Jakeman *et al.* (2006) found that the carbon source added to the media also has an impact on the antibiotic production by *Streptomyces venezuelae*. The effect of several sugars on jadomycin B production was examined and significant differences in antibiotic production between sugars was found, (Figure 3.8) where glucose was found to be optimal.
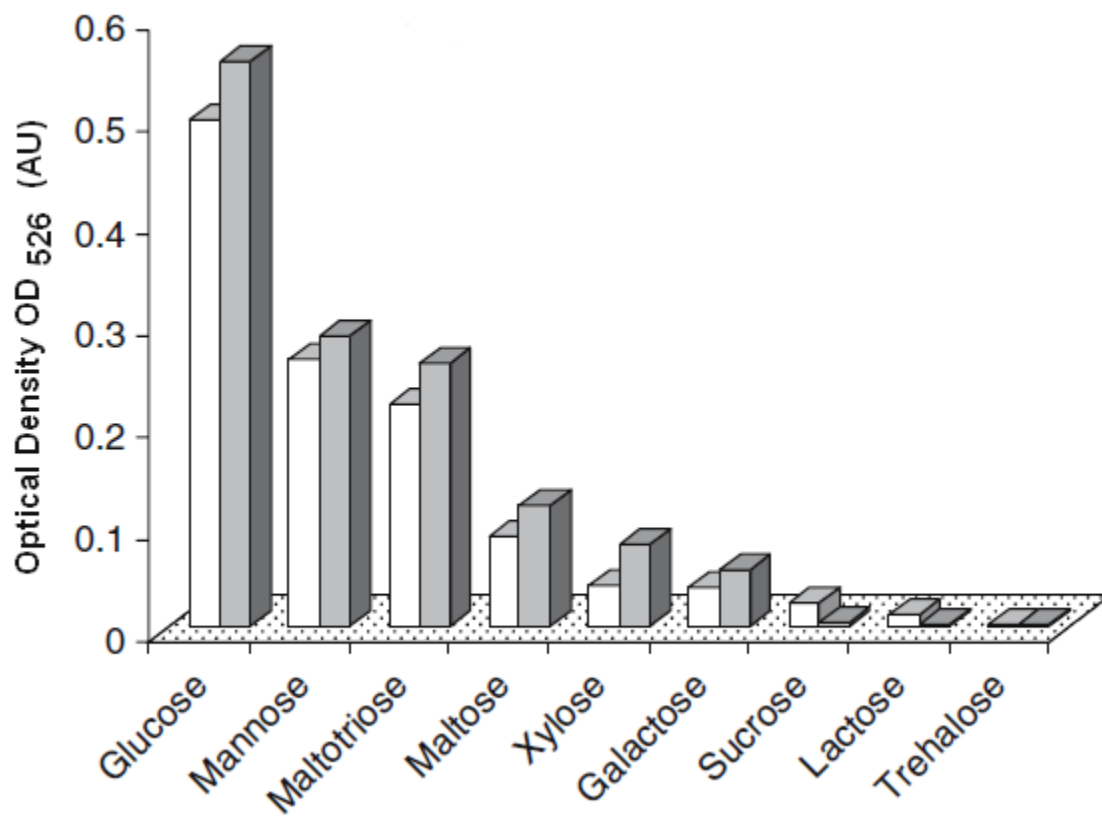
Figure 3.8   Effects of carbon source on jadomycin B production after 24 h and 72 h as depicted on clear and shaded bars, respectively (Jakeman *et al.,* 2006).

3.4   Heat Balance

As discussed in Section 3.3.1, the environmental temperature influences bacterial behavior, however the metabolism and activity of bacteria also affects media temperature. Typically 50 - 60 % of energy generated during the metabolism of nutrients within a system is converted to heat energy (Shuler and Kargi, 1992). The metabolism of sugar is a large source of thermal energy that must be accounted for in bioreactor design (Ben-Hassan *et al.*, 1992). *Streptomyces venezuelae* aerobically metabolize sugars for energy and the production of metabolites. In industry, the amount of substrate used by the microorganism for growth and product synthesis determines the economic viability of the process. The heat energy produced can be determined through the utilization of a heat balance model (Ghaly *et al.*, 1992). Once the heat of mixing is determined, the energy generated by *Streptomyces venezuelae* can be determined. The work by Ghaly *et al.* (1992) on the heat generated through mixing and metabolism during lactose fermentation showed that the temperature of media increased from 22 to 32 °C with bacterial growth (Figure 3.9). This increase in temperature was due to the heat generated by the yeast (7.4 $J \cdot s^{-1}$) as well as the heat of mixing (1.01 $J \cdot s^{-1}$). The amount of heat generated by the organisms in a bioreactor can be estimated by modeling the heat flux over the boundary of the enclosed system. This mathematical model or "heat balance" balances the thermal energy generated within a system with the energy stored and lost from the system (Ben-Hassan *et al.*, 1992). To utilize a heat balance in this manner, a trial must first be performed on the system during normal steady state operation to assess the heat generation during operation of the bioreactor (Burdock *et al.*, 2008).

A heat balance can be performed on a bioreactor to determine the amount of thermal energy generated within the system during normal operation due to mixing. To model the system under various operating conditions the reactor can be run at several agitation speeds (e.g. 200, 400 and 600 rpm) until a state of thermal equilibrium is reached. At thermal equilibrium the amount of energy generated within the bioreactor is equivalent to the amount of energy lost; this state of equilibrium can be used to eliminate unknown parameters within the heat balance model. Figure 3.10 represents sources of energy within a typical bioreactor.
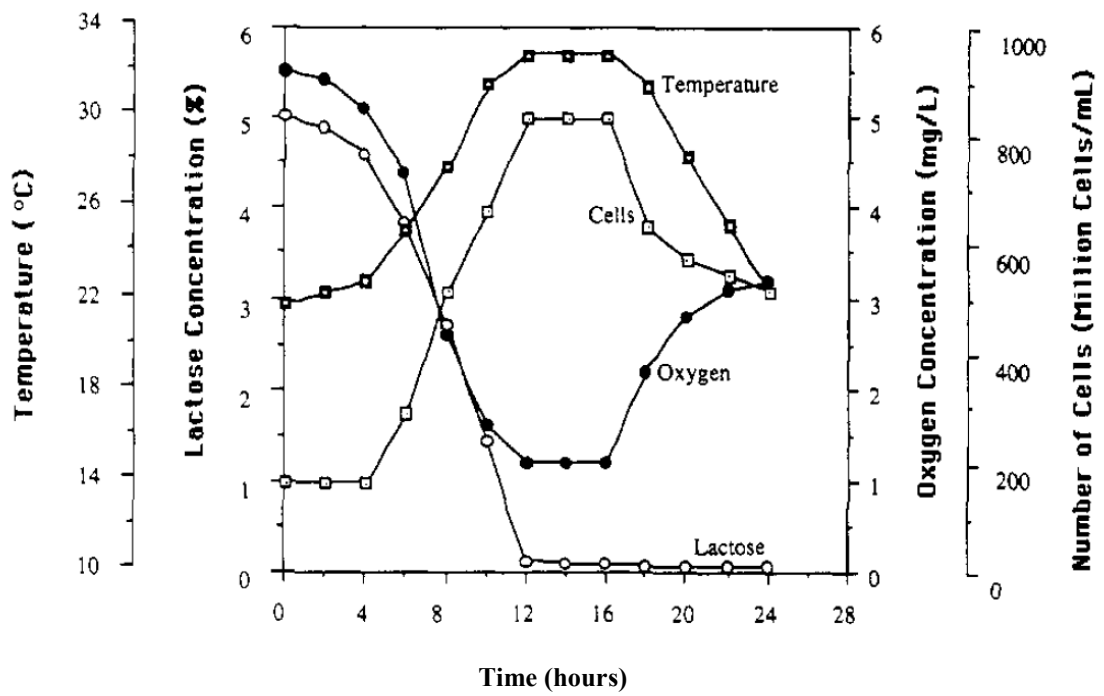
Figure 3.9   Yeast, lactose, dissolved oxygen and temperature measurements
during lactose fermentation (Ghaly *et al.*, 1992).

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Energy is generated by mixer agitation and microbiological activity while energy is lost to the ambient laboratory atmosphere mainly through convection over bioreactor components and with the exhaust gases. When a bioreactor is located in an enclosed laboratory without windows or forced airflow, thermal losses from the bioreactor can be modeled as natural convection. All significant thermal losses and gains for the reactor vessel must be considered as described by the master heat balance (Equation 1). Within this equation the terms Q_{mix} and $Q_{metabolism}$ describe possible sources of thermal energy within the media while $Q_{exhaust}$, $Q_{wallabove}$, Q_{wall}, Q_{bottom}, and Q_{lid} describe the energy lost from the exhaust gases and components of the bioreactor respectively. The term Q_{media} accounts for the energy utilized/released during changes in the temperature of media. When the bioreactor is warming from ambient temperature, a portion of the energy generated will be lost to the environment while the rest will be stored and used to increase media temperature. When the temperature of the media is above ambient and there is no internal thermal generation, energy will continue to be lost from the system as the temperature of the media cools. During periods of thermal equilibrium when the media temperature is at a steady state, Q_{media} becomes zero as all energy generated within the system is lost to the environment. To attain the temperature measurements required to model the energy losses over the bioreactor, sensors can be installed within and around the bioreactor system.

$$Q_{mix} + Q_{metabolism} = Q_{media} + Q_{exhaust} + Q_{wallabove} + Q_{wall} + Q_{bottom} + Q_{lid} = Q_{total} \qquad (1)$$

Where:

Q_{bottom}	= Heat energy lost through the bottom of the reactor (J·s^{-1})
$Q_{exhaust}$	= Heat energy lost with exhaust gases (J·s^{-1})
Q_{lid}	= Heat energy lost through the lid of the reactor (J·s^{-1})
Q_{media}	= Heat energy stored in media (J·s^{-1})
$Q_{metabolism}$	= Heat energy generated by bacteria metabolism and activity (J·s^{-1})
Q_{mix}	= Heat energy generated by agitation (J·s^{-1})
Q_{wall}	= Heat energy lost through reactor wall in contact with media (J·s^{-1})
$Q_{wallabove}$	= Heat energy lost through reactor wall in contact with exhaust (J·s^{-1})
Q_{total}	= Total heat generation within the bioreactor (J·s^{-1})
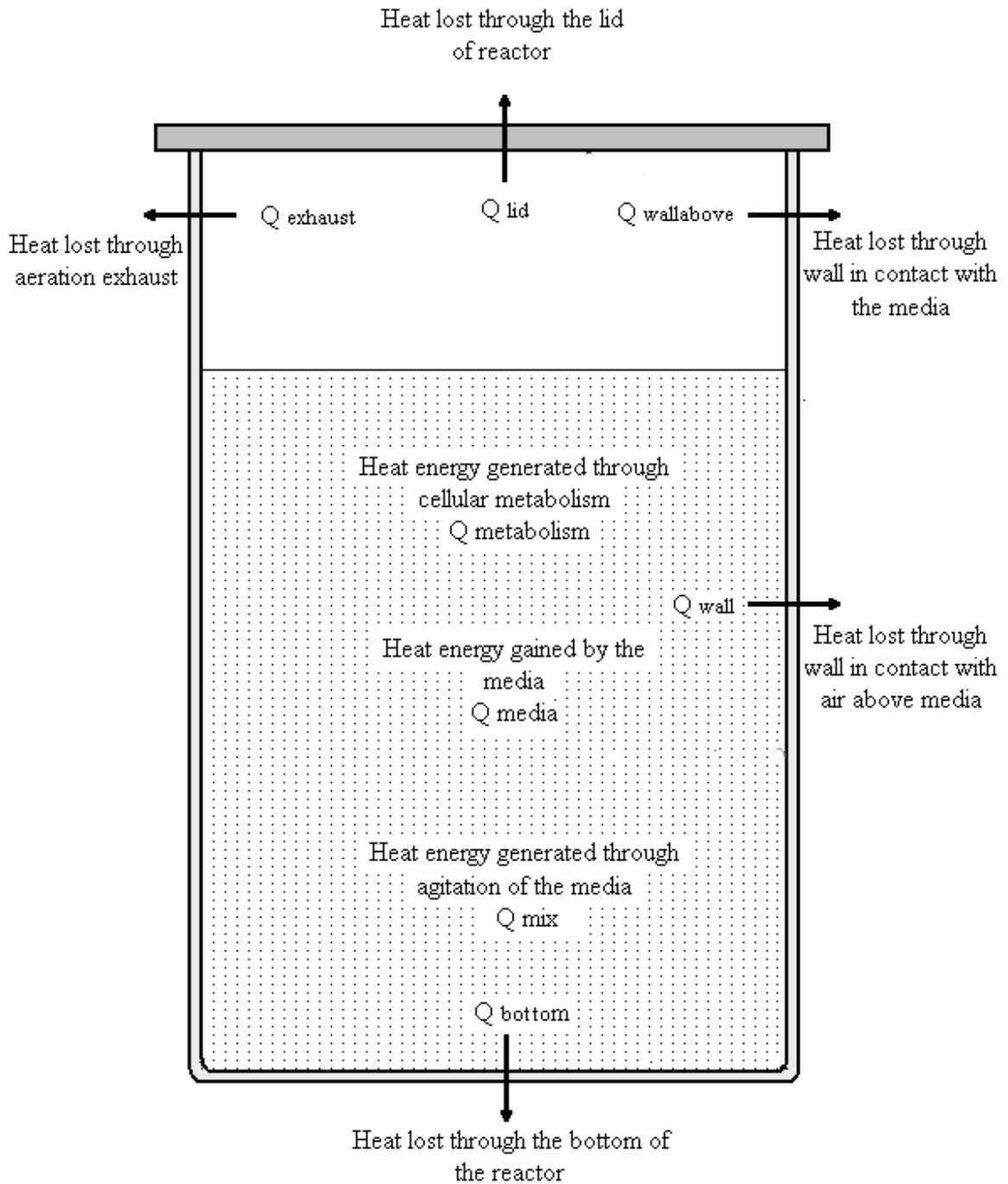
Figure 3.10 Known sources and sinks of thermal energy from a bioreactor.

The amount of heat loss from each component of a bioreactor can be calculated utilizing measured differences in temperature, the thermal resistance of the material and the surface area over which the energy transfer occurs. The heat lost from each bioreactor component are presented in Equations 2 – 6 (Ben-Hassan *et al.*, 1992). The overall heat transfer coefficients must be calculated for each bioreactor component and are dependent upon the orientation of the surface and manner of contact with the media and ambient air. The heat transfer coefficients describing energy transfer between components can be determined theoretically using methods described by Burdock *et al.* (2008) and the known physical properties of the bioreactor materials (Holman *et al.*, 2002). These heat balance parameters as well as the specifications of a typical bioreactor are detailed in Figure 3.11 and the equations are developed in the following sections.

$$Q_{exhaust} = M_{air} C_P (T_{ex} - T_{ae}) \tag{2}$$

$$Q_{wallabove} = U_{wa} A_{wa} (T_m - T_{amb}) \tag{3}$$

$$Q_{wall} = U_{wu} A_{wu} (T_m - T_{amb}) \tag{4}$$

$$Q_{bottom} = U_{bot} A_{bot} (T_m - T_{amb}) \tag{5}$$

$$Q_{lid} = U_{lid} A_{lid} (T_{ex} - T_{amb}) \tag{6}$$

Where:

A_{bot}	=	Surface area of the bottom plate (m^2)
A_{lid}	=	Surface area of the lid (m^2)
A_{wa}	=	Surface area of wall above the media (m^2)
A_{wu}	=	Surface area of wall in contact with the media (m^2)
C_p	=	Specific heat of water (J·g^{-1}·$^{\circ}$K^{-1})
M_{air}	=	Mass flow of air (g·s^{-1})
T_{amb}	=	Temperature of the ambient room air ($^{\circ}$C)
T_{bo}	=	Temperature of the pyrex glass bottom ($^{\circ}$C)
T_{ex}	=	Temperature of exhaust gases leaving the reactor ($^{\circ}$C)

T_{lo}	=	Temperature at the topside of the steel lid (°C)
T_m	=	Temperature of the media (°C)
T_{wao}	=	Temperature of the outer wall in contact with exhaust gases (°C)
T_{wuo}	=	Temperature of the outer wall in contact with media (°C)
U_{bot}	=	Overall heat transfer coefficient for the bottom of the bioreactor ($s \cdot m^2 \cdot °C \cdot J^{-1}$)
U_{lid}	=	Overall heat transfer coefficient of bioreactor lid ($s \cdot m^2 \cdot °C \cdot J^{-1}$)
U_{wa}	=	Overall heat transfer coefficient for the wall above media ($s \cdot m^2 \cdot °C \cdot J^{-1}$)
U_{wu}	=	Overall heat transfer coefficient for wall contacting media ($s \cdot m^2 \cdot °C \cdot J^{-1}$)

Figure 3.11 Physical locations of heat balance parameters on a typical bioreactor.

Where:

Dia_i	=	Inner diameter of the bottom plate (m)
Dia_o	=	Outer diameter of the bottom plate (m)
h_{bi}	=	Convection heat transfer coefficient for media to bottom plate ($J{\cdot}s^{-1}{\cdot}m^{-2}{\cdot}{^\circ}C^{-1}$)
h_{bo}	=	Convection heat transfer coefficient for bottom plate to ambient air ($J{\cdot}s^{-1}{\cdot}m^{-2}{\cdot}{^\circ}C^{-1}$)
h_{wai}	=	Convection coefficient for exhaust gases to portion of wall above media ($J{\cdot}s^{-1}{\cdot}m^{-2}{\cdot}{^\circ}C^{-1}$)
h_{wao}	=	Convection coefficient for the portion of wall above media to ambient air ($J{\cdot}s^{-1}{\cdot}m^{-2}{\cdot}{^\circ}C^{-1}$)
h_{wui}	=	Convection coefficient for media to lower wall ($J{\cdot}s^{-1}{\cdot}m^{-2}{\cdot}{^\circ}C^{-1}$)
h_{wuo}	=	Convection coefficient for lower wall to ambient air ($J{\cdot}s^{-1}{\cdot}m^{-2}{\cdot}{^\circ}C^{-1}$)
h_{li}	=	Convection coefficient for exhaust gas to lid ($J{\cdot}s^{-1}{\cdot}m^{-2}{\cdot}{^\circ}C^{-1}$)
h_{lo}	=	Convection coefficient for lid to ambient air ($J{\cdot}s^{-1}{\cdot}m^{-2}{\cdot}{^\circ}C^{-1}$)
K_{pyx}	=	Thermal conductivity of Pyrex glass ($J{\cdot}s^{-1}{\cdot}m^{-1}{\cdot}{^\circ}C^{-1}$)
K_{Steel}	=	Thermal conductivity of Steel ($J{\cdot}s^{-1}{\cdot}m^{-1}{\cdot}{^\circ}C^{-1}$)
L_{wa}	=	Length of wall in contact with exhaust gases (m)
L_{wu}	=	Length of wall in contact with media (m)
M_{air}	=	Mass airflow ($g{\cdot}s^{-1}$)
Th_w	=	Thickness of the reactor wall (m)
T_{li}	=	Temperature of the inner lid (${^\circ}C$)
T_{wai}	=	Temperature of the inner wall above the media (${^\circ}C$)

3.4.1 Heat Transfer through the Bottom of the Bioreactor

The overall heat transfer coefficient of the bottom plate, (U_{bot}) is calculated (Equation 7) by determining the heat transfer coefficients between the media, bottom plate and ambient air. As the media within the reactor is continuously stirred, the convection heat transfer coefficient (h_{bi}) from the media to the bottom plate will be through forced convection for which the thermal resistance is known to be significantly smaller than natural convection. Thus, h_{bi} can be neglected and U_{bot} can be calculated using Equation 8.

When energy transfer from the reactor bottom to the air takes place through natural convection, the heat transfer coefficient (h_{bo}) can be determined by modeling the bottom of the reactor as a heated plate facing downward (Equation 9). The formula is valid when the Rayleigh number for the film temperatures experienced during the experiment is less than 10^9, indicating laminar flow (Holman, 2002).

$$U_{bot} = \left(\frac{1}{\left(\dfrac{1}{h_{bi}}\right) + \left(\dfrac{Th_w}{K_{Pyx}}\right) + \left(\dfrac{1}{h_{bo}}\right)} \right) \tag{7}$$

$$U_{bot} = \left(\frac{1}{\left(\dfrac{Th_w}{K_{pyx}}\right) + \left(\dfrac{1}{h_{bo}}\right)} \right) \tag{8}$$

$$h_{bo} = 0.59\left(\frac{T_{bo} - T_{amb}}{Dia_o}\right)^{\wedge 0.25} \tag{9}$$

3.4.2 Heat Transfer through the Wall of the Bioreactor

In a typical bioreactor, part of the wall will be in contact with the media while the rest of the wall will be in contact with exhaust gas after it passes through the media. The energy transfer through the portion of wall contacting the media behaves in a similar manner to the bottom plate. The forced convection coefficient from the media to the wall (h_{wui}) is known to be far greater than the term for natural convection on the outside of the wall h_{wuo}, therefore the overall heat transfer coefficient (U_{wu}) is calculated using Equation 10. The natural convection coefficient from the wall (h_{wuo}) can be modeled as a heated vertical cylinder (Holman, 2002) as described by Equation 11.

The section of reactor wall above the media is in direct contact with heated exhaust gas after it passes through the media. The equation for the overall heat transfer coefficient (U_{wa}) is calculated as Equation 12 where the convection coefficient h_{wao} (Equation 13) is modeled in the same manner as h_{wuo} (Equation 11).

This section of bioreactor exhibits different thermal characteristics dependent upon the activation of the aeration system. When aeration is activated the exhaust gas is stirred inducing forced convection, therefore $h_{wao} \ll h_{wai}$ and the overall heat transfer coefficient is calculated as Equation 14.

In the absence of exhaust gases, the air above the media is not agitated and therefore there are additional thermal resistances from the air within the chamber to the inner wall (Equation 12). The natural convection coefficient for this section is approximated as Equation 15:

$$U_{wu} = \left(\frac{1}{\left(\frac{\ln\left(\frac{(Dia_o / 2)}{(Dia_i / 2)} \right)}{2\pi K_{pyx} L_{wu}} \right) + \left(\frac{1}{h_{wuo}} \right)} \right) \tag{10}$$

$$h_{wuo} = 1.42 \left(\frac{T_{wuo} - T_{amb}}{Dia_o} \right)^{0.25} \tag{11}$$

$$U_{wa} = \left(\cfrac{1}{\left(\cfrac{1}{h_{wai}} \right) + \left(\cfrac{\ln\left(\cfrac{(Dia_o / 2)}{(Dia_i / 2)} \right)}{2\pi K_{pyx} L_{wa}} \right) + \left(\cfrac{1}{h_{wao}} \right)} \right) \qquad (12)$$

$$h_{wao} = 1.42 \left(\frac{T_{wuo} - T_{amb}}{Dia_o} \right)^{0.25} \qquad (13)$$

$$U_{wa} = \left(\cfrac{1}{\left(\cfrac{\ln\left(\cfrac{(Dia_o / 2)}{(Dia_i / 2)} \right)}{2\pi K_{pyx} L_{wa}} \right) + \left(\cfrac{1}{h_{wao}} \right)} \right) \qquad (14)$$

$$h_{wai} = 1.42 \left(\frac{T_{ex} - T_{wai}}{Dia_i} \right)^{0.25} \qquad (15)$$

3.4.3   Heat Transfer through the Lid of the Bioreactor

The overall heat transfer coefficient for the lid of the bioreactor (U_{lid}) is described by Equation 16, where the heat transfer coefficient for the inner chamber (h_{li}) is calculated as a plate facing downwards (Equation 17) and the outer heat transfer coefficient (h_{lo}) calculated as a plate facing upwards (Equation 18).

As with the portion of the wall above the media, the activation of the aeration system induces forced convection between the exhaust air and the lid reducing the overall heat transfer calculation to Equation 19.

$$U_{lid} = \left(\cfrac{1}{\left(\cfrac{1}{h_{li}} \right) + \left(\cfrac{Th_L}{K_{Steel}} \right) + \left(\cfrac{1}{h_{lo}} \right)} \right) \qquad (16)$$

$$h_{li} = 0.59 \left(\frac{T_{ex} - T_{Li}}{Dia_i} \right)^{0.25} \tag{17}$$

$$h_{lo} = 1.32 \left(\frac{T_{Lo} - T_{amb}}{Dia_o} \right)^{0.25} \tag{18}$$

$$U_{lid} = \left(\frac{1}{\left(\frac{Th_L}{K_{Steel}} \right) + \left(\frac{1}{h_{lo}} \right)} \right) \tag{19}$$

CHAPTER 4 METHODS AND MATERIALS

4.1   Reagents

During all experiments with *Streptomyces venezuelae,* variations of MYM (D-maltose, yeast-extract, malt-extract) media were used to culture and support bacterial growth. This media used by Jakeman *et al.* (2006) and others is a complex media that is known to support growth of *Streptomyces venezuelae* on solid (agar) surfaces and in liquid media. All components of this media were supplied by BioShop (Burlington, ON, Canada). To culture *Streptomyces venezuelae* on agar Petri plates, MYM agar was prepared (Table 4.1). When *Streptomyces venezuelae* was cultured in 250 mL shake flasks, liquid MYM media was used (Table 4.2). The buffer 3-(N-morpholino) propanesulfonic acid (MOPS) supplied through BioShop was added to the media by Burdock (2009) to maintain neutral media pH in shake flask experiments where external pH control was impractical. When the liquid MYM media was used in the bioreactor, the media was prepared without a buffering compound (Table 4.3), as the pH within the reactor vessel was controlled through an external proportional-derivative pH controller.

To control the acidity of the media in the bioreactor, 6 M sodium hydroxide (NaOH) and 6 M hydrochloric acid (HCl) solutions were used. The acid solution was also used as a hydrolyzing agent prior to the measurement of D-glucose sugars. Sodium hydrogen carbonate powder (NaHCO$_3$) was also necessary to neutralize the 6 M HCl to facilitate measurements with a sugar analyzer. To reduce foam formation within the bioreactor during operation, approximately 5 mL of silicone emulsifier antifoam C supplied by Dow Corning (Midland, MI, U.S.) was added to the bioreactor over the course of each growth trial as required. During the preparation of the soft agar spore suspension, dilute (1:10000) aqueous Triton X solution ($C_{14}H_{22}O(C_2H_4O)_n$ n = 9 - 10, supplied by BioShop) was used to isolate spores from colony growth in Petri plates. Once harvested, spores were stored in an aqueous solution of 1.25 g·L^{-1} agar to keep the spores in suspension.

Table 4.1   MYM agar composition.

Component	Chemical Formula	Amount
De-ionized water	H_2O	0.5 L
Maltose	$C_{12}H_{22}O_{11}$	2 g
Agar	N/A	7.5 g
Yeast Extract	N/A	2 g
Malt Extract	N/A	5 g

(Jakeman *et al.*, 2006)

Table 4.2   MYM media components for 250 ml shake flasks.

Component	Chemical Formula	Amount
De-ionized water	H_2O	250 mL
Maltose	$C_{12}H_{22}O_{11}$	1 g
MOPS	$C_7H_{15}NO_4S$	0.475 g
Yeast Extract	N/A	1 g
Malt Extract	N/A	2.5 g

(Burdock, 2009)

Table 4.3   MYM media components for bioreactor.

Component of MYM growth media	Chemical Formula	Amount
De-ionized water	H_2O	5 L
Maltose	$C_{12}H_{22}O_{11}$	20 g
Yeast Extract	N/A	20 g
Malt Extract	N/A	50 g

4.2 Media Preparation

MYM agar was prepared by combining the maltose, yeast-extract, malt extract and agar (listed in Table 4.1) with sterilized deionized water while heat and agitation were provided through a heat plate/agitator (Model 310T, Fisher Scientific, Ottawa, Ontario, Canada). The media was added to autoclave-safe glass bottles, the necks plugged with cotton wool and the tops covered with aluminum foil prior to sterilization in a Sterile-Max steam sterilizer (Harvey Sterile-Max™ steam sterilizer, Thermo Fisher Scientific, Waltham, MA, U.S.) at 121 °C for 15 minutes. The MYM agar was then aseptically divided into individual Petri plates before the media cooled and solidified. The plates were either inoculated immediately after cooling or sealed and stored at 1 - 3 °C in the laboratory refrigerator. MYM media for use in 250 mL shake flasks and the bioreactor was prepared from media components listed in Tables 4.2 and 4.3 respectively using the same heat plate/agitation and sterilization procedure as described above. MYM liquid media was then added aseptically to pre-sterilized shake flasks or the pre-sterilized bioreactor prior to inoculation. A soft agar solution was required for the standardized spore inoculum. This was prepared by dissolving 1.25 g·L^{-1} agar in sterilized de-ionized water at 60 °C.

4.3 Bacteria

A stock plate of *Streptomyces venezuelae* was originally obtained from Dr. Jakeman, College of Pharmacy, Dalhousie University (Halifax, N.S., Canada). Stock plates of *Streptomyces venezuelae* were prepared from the original plate and used as the bacteria source for experiments. To prepare the stock plates, several colonies were isolated aseptically from the original plate and transferred into 250 mL shake flasks containing buffered MYM broth (Table 4.2). The neck of the flask was plugged with cotton wool and aluminum foil was used to seal the top before incubation in a rotary shaker (Series 25, New Brunswick Scientific Co. Inc., New Brunswick, NJ, U.S.) at 30 °C, 250 rpm for 24 hours. After the incubation, MYM agar plates were streaked with a loopful of culture from the flasks using a disposable inoculation loop and incubated until visible colonies formed (approximately 24 h). At this point the colonies appeared smooth and slightly

translucent as characteristic for vegetative cells. Plates that were created to harvest spores were instead incubated for an additional 13 days. The colonies would then become opaque and crusty indicating the presence of aerial hyphae and spores rather than vegetative cells (Kieser *et al.*, 2007).

4.4   Bioreactor System

The bioreactor used in this study ("Microferm® Fermentor" New Brunswick Scientific Co., Inc. New Brunswick, NJ, U.S.) consisted of a 7 L pyrex glass cylinder reactor vessel capable of aseptic operation while providing agitation, filtered airflow, temperature regulation and antifoam addition. Several ports were available for sampling, reagent addition and placement of sensors through a stainless steel top plate; these ports were sealed with steel plugs when not in use to maintain an aseptic environment. Agitation was supplied to the media through three impellers distributed evenly along a central rotating shaft (Figure 4.1). For all growth and heat balance trials, the three impellers were distributed equally in 5 L of media. Heating/cooling water was circulated through a stainless steel u-tube, while filtered air was supplied to the bioreactor through the bottom of an additional steel shaft. Further details regarding temperature, pH and dissolved oxygen components and monitoring are given in Sections 4.4.1-4.4.4.

Sterilization of the bioreactor occurred before each use. This involved the disassembly of the reactor vessel (Figure 4.1). The parts of the vessel that could be sterilized in an autoclave were hand washed with anti-microbial soap and sterilized with the Sterile-Max steam sterilizer at 135 °C for 3 minutes. The components that could not sustain the high temperatures required for sterilization (i.e. tubing, wires, and the pH and dissolved oxygen sensors) were cleaned using 99.99 % ethanol solution in a positive pressure aseptic environment supplied by an in-house laminar fume hood. Once sterilized, all components were assembled and the media added while within the laminar fume hood.
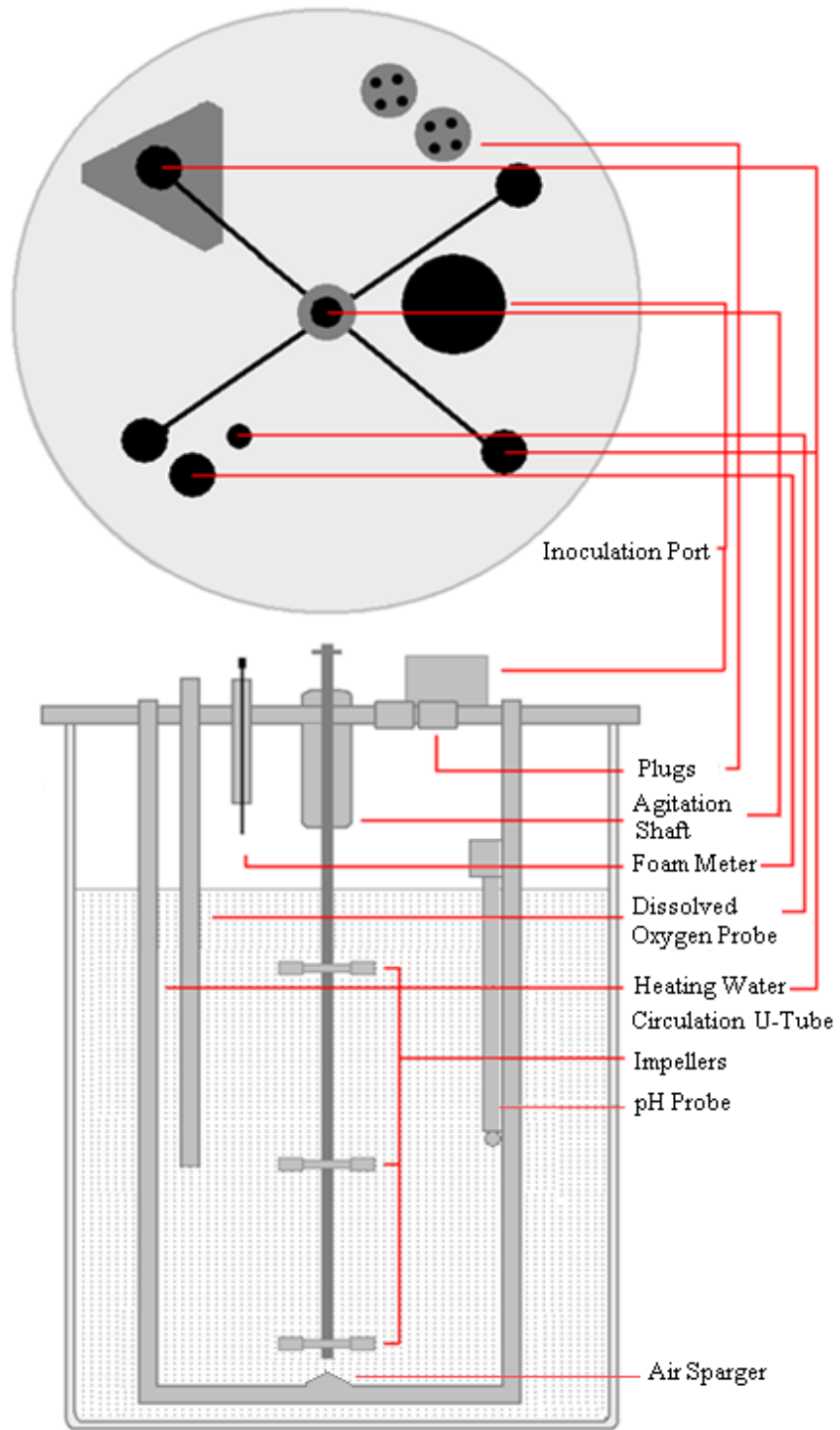
Figure 4.1   Diagram of Microferm Fermentor reactor vessel.

4.4.1 Temperature

The bioreactor used circulated water with an built-in electric heater in series to control the temperature of the system. Energy transfer occurred within the reactor by circulating the fluid through a stainless steel u-tube submerged within the bioreactor media (Figure 4.1). The temperature in the bioreactor was controlled with a temperature controller (53602-0 temperature controller, Fenwal® Inc., Lake Zurich, IL, U.S.). Seven thermistors (NTC thermistor, General Electronics, Edison, NJ, U.S.) were used to record temperatures at points on and around the bioreactor (Figure 4.2). These temperatures have been previously defined in Section 3.4 and locations depicted in Figure 4.2.

Thermistors T_{ex} and T_m were housed in clear plastic tubes with their ends heat sealed. T_{ex} was suspended in the airspace above the media and T_m was placed within the media. All other thermistors were attached to surfaces on the bioreactor using tape and string to ensure contact. The thermistor sensors were incorporated into an amplified Wheatstone bridge circuit to provide a signal proportional to the sensor temperature and so that very small changes in temperature could be detected. The temperature signal was calibrated using a mercury thermometer that was marked to a precision of 0.2 °C (Thermo Fisher Scientific, Waltham, MA, U.S.).

The signals from the thermistors were collected using a 6008 USB National Instruments data acquisition device supplied by National Instruments Co. (Austin, TX, U.S.). However only two analog input ports on the device were available, therefore two multiplexor integrated circuits (4052 Multiplexor, Unisonic Technologies Co., Ltd, Liaoning, China) were used to select two new sensors every 20 s. The program "THERM.C" (Appendix A) controlled the two multiplexor integrated circuits through three digital input/output ports in a parallel circuit. The signals from the multiplexors were amplified through the use of an operation amplifier (LM224J Low Power Quad Operational Amplifier, National Semiconductor, Santa Clara, CA, U.S.).
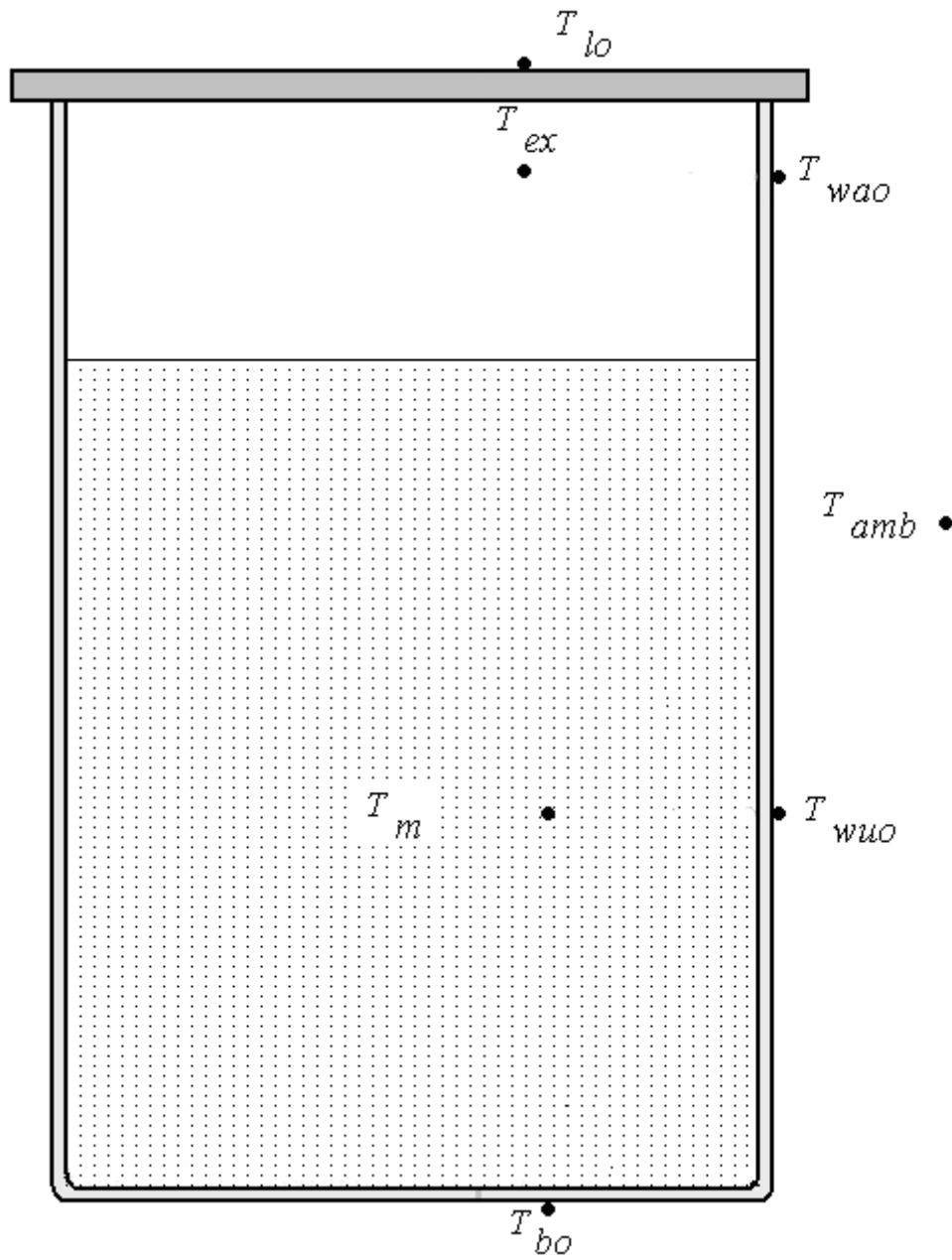
Figure 4.2   Thermistor locations on and around the bioreactor.

4.4.2   pH

A pH probe (Accumet 13-620-299, Thermo Fisher Scientific, Waltham, MA, U.S.) was used to measure the pH within the bioreactor and a Proportional-Derivative pH controller (PD pH controller, Bioengineering AG, Wald, Switzerland) was used to maintain the pH of the media at the desired set-point. The probe was attached to the outside of the heating water u-tube using sterilized steel wire. The pH probe was calibrated prior to every bioreactor experiment. Two 3-roller peristaltic pumps added 6 M hydrochloric acid (HCl) and 6 M sodium hydroxide (NaOH) into the bioreactor as required. At a set point of pH 7, the controller was able to maintain the pH between 6.5 and 7.5 during the experiments.

4.4.3   Dissolved Oxygen

A dissolved oxygen probe (M1016-5002, New Brunswick Scientific Co. Inc., New Brunswick, NJ, U.S.) was used to measure dissolved oxygen levels in the bioreactor. Laboratory air was supplied to the bioreactor at 103 kPa (15 psi) and passed through a long flexible tube that was submerged in a circulatory water-bath to maintain the air at room temperature. The air was then passed through a 7μm filter (SS-4TF, Nupro Industries Co, Boca Raton, FL, U.S.) to remove any microbial contamination prior to entering the bioreactor. The flow rate of air entering the bioreactor for all experiments was set at a level that was 1.5 times the flow rate required to maintain saturated dissolved oxygen levels in the media for the duration of each experiment. Empirical testing showed that dissolved oxygen levels did not deviate far from saturated levels even under full biological load. For example, during an experiment conducted at 30 °C, the dissolved oxygen levels remained near the saturation level of 7.5 mg·L^{-1}. The dissolved oxygen probe was connected to the N.I. data acquisition device for data logging purposes and calibrated through the correlation of voltage with published saturation tables (Radtke *et al.*, 1998).

4.4.4 Data Acquisition

The signals from the thermistors and the dissolved oxygen probe were acquired using the N.I. data acquisition device. Two of the four analog input ports on the device were utilized for temperature monitoring, while the remaining two ports were for dissolved oxygen monitoring. A custom program "THERM.C" (Appendix A) was written in National Instruments Lab Windows™/CVI Programming Environment to aid in data acquisition."THERM.C" was configured to sample all of the analog ports on the data acquisition device every second (temperature readings were averaged over a 20 s period). These values were written to the data file "thermrecord.txt" in comma-delimited style. The corresponding engineering units were calculated and displayed on the graphical user interface (Figure 4.3). "THERM.C" was also programmed to calculate the heat lost from the bioreactor using heat balance formulae in real-time.

4.5 Procedure for *Streptomyces Venezuelae* Enumeration

Optical density is often used to assess the growth of bacteria in media, the transmission of light through media is affected by bacteria that scatter and absorb light. Previous work on *Streptomyces venezuelae* by researchers such as Jakeman *et al.* (2006) used the optical density of media at 600 nm to assess bacteria growth. A calibration curve was prepared to determine the relationship between optical density at this wavelength (OD_{600}) and the number of active cells represented by colony forming units ($CFU \cdot mL^{-1}$). Before the curve was established, a UV visible light scanning spectrometer (Cary 50Bio , Varian Inc., Palo Alto, CA, U.S.) was used to verify the suitability of using a wavelength of 600 nm for measuring of *Streptomyces venezuelae* growth. For all other growth trials the OD_{600} was sampled using a Genesys 20 visible light spectrometer, (Thermo Fisher Scientific, Waltham, MA, U.S.) due to availability.
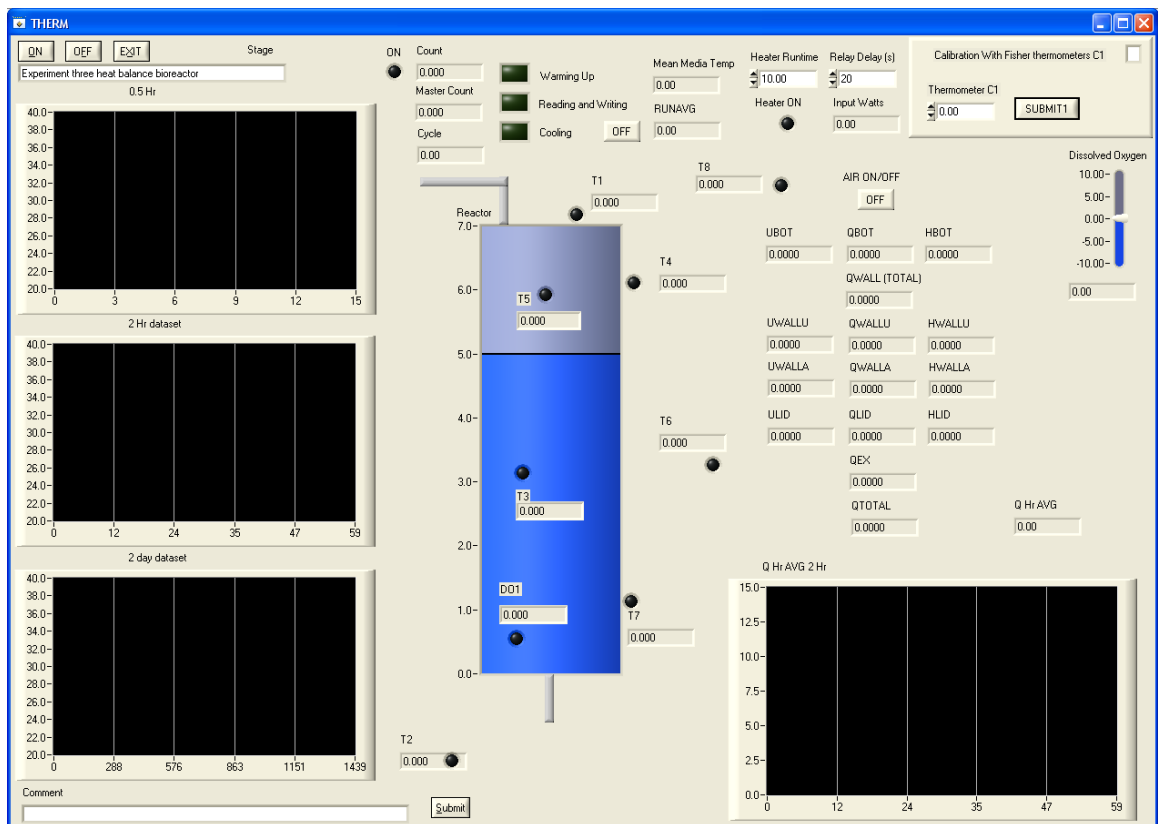
Figure 4.3   Graphical user interface for "THERM.C".

During a typical bioreactor growth trial, samples were taken hourly after inoculation. The OD_{600} was then measured and samples diluted (where appropriate) so that the bacteria population could be determined using spread plate culturing techniques to count the number of colony forming units present. The level of dilution for each sample was determined by using a hemocytometer (Neubauer hemocytometer, Millscience & Millseth Co., Hong Kong, China) to count previously dyed and immobilized cells with the aim of achieving between 300-3000 cell mL^{-1} (30-300 cells per Petri plate) for accurate counting. An aliquot of 0.1 mL from each diluted sample was added to three MYM agar plates and spread across the surface using a flame sterilized glass spreader. The plates were incubated inverted in a VWR incubator (Sheldon Manufacturing Inc. Cornelius, OR, U.S.) at 30 °C for 28 hours. The number of $CFU \cdot mL^{-1}$ was counted and the resulting relationship correlated with the optical density at 600 nm. To ensure accuracy the plates were grown in triplicate with control plates.

4.6   Procedure for Sugar Monitoring

Utilization of the sugar substrate by bacteria was measured during the experiments. In MYM broth, D-glucose is predominantly found as maltose where maltose is comprised of two D-glucose monomers. The D-glucose content of the media was measured through enzymatic oxidization using a YSI 2700 select sugar analyzer (YSI Inc., Yellow Springs, OH, U.S.) with a D-glucose membrane. Samples were analyzed for free glucose and hydrolyzed glucose content. The free glucose was obtained from the samples taken directly from the bioreactor whereas the hydrolyzed glucose content was measured after samples underwent acid hydrolysis. During acid hydrolysis, 4 mL of each sample was combined with an equal portion of 6 M HCl and heated at 90 °C for 2 hours. The acid hydrolysis converted the ether linkages between the two D-glucose subunits into hydroxy groups, consuming 1 equivalent of H_2O per maltose unit. Repeated testing showed that after two hours no further glucose became available indicating no further hydrolysis occurred.

4.7 Preparation of a Standardized Spore Inoculum

During the development of a soft agar spore inoculum, methods from literature were tested and adapted empirically. Spores were initially collected using techniques described by Kieser *et al.* (2000) and isolated by adapting the filtration method described by Parada (1975) to accommodate spores of *Streptomyces venezuelae*. The final procedure for the creation of a *Streptomyces venezuelae* soft agar spore suspension used in this study is described below and summarized in Figure 4.4.

To form a spore inoculum, vegetative cells from a stock plate (Section 4.3) were used to inoculate a 250 mL shake flask containing maltose yeast-extract malt-extract (MYM) growth broth (Table 4.2). The flask was incubated in an incubator shaker (Series 25, New Brunswick Scientific Co. Inc., New Brunswick, NJ, U.S.) at 30 °C and 250 rpm for 24 hours. Three plates of MYM agar (Table 4.2) were subsequently inoculated with 0.1 mL of this solution using the spread plate technique (Truelstrup-Hansen, 2008) and were incubated at 30 °C for 14 days. Translucent gel-like colonies formed on the plates after 12 - 48 hours of incubation, turning crusty and opaque after 12 - 14 days indicating sporulation had occurred.

The spores were first harvested from the plates by adding 1-3 mL of dilute (1:10,000) Triton X aqueous solution to each plate using an auto-pipette and removing the spores with gentle agitation. The spore/surfactant mixture was then gravity-filtered through Whatman 40, ashless 8 μm filter paper (Whatman plc, Springfield ME, U.K.) that had been wetted with approximately 5 mL of sterilized de-ionized water that was subsequently discarded. Next, the solution was vaccum filtered through Whatman 42 ashless 2.5 μm filter paper (wetted as described above) to remove large debris and vegetative cells. During this filtration approximately 2 mL of sterilized de-ionized water was added to facilitate the filtration. Finally, to remove particles smaller than the spores, the solution was passed through a Whatman 0.45 μm filter that was wetted with only 1 mL sterilized de-ionized water and the spores were collected onto the surface of the filter. This step took considerably longer than previous filtrations and the apparatus was sealed to prevent contamination. Once collected onto the filter surface the spores were re-suspended from the 0.45 μm filter into a minimal amount of sterilized de-ionized water to ensure that a solution of a high spore

concentration was achieved. Subsequent tests indicated that inocula with lower concentrations of spores would result in growth trials with greater variability. For storage, the spore solution was combined with 1.25 g/L agar solution at 60 °C to create a soft spore suspension which was immediately cooled and maintained at 1- 4 °C. The steps for this procedure are summarized in Figure 4.4. For all bioreactor trials including bacteria, 1.0 mL of the spore solution was used to inoculate 5 L of MYM media. The samples were viewed using Scanning Electron Microscopy (SEM) and the presence, size, and visible properties of the spores analyzed. A laser particle size analyzer (Nanotrac particle size analyzer, Microtrac, Montgomeryville, PA, U.S.) was used as a quick measurement of particle size and distribution within the media to help develop the filtering techniques.

Small scale growth trials completed in 250 mL shake flasks were also used to verify the quality and consistency of the inoculum. For these experiments, 1 mL of the spore suspension was used to inoculate the 250 mL of MYM media (Table 4.2). Flasks were then incubated at 32 °C and 250 rpm in a rotary shaker for approximately 60 hours. Although long term viability of stored inoculum was not investigated in this study, the work completed by Kutzner and Nitsch (1970) indicates that the inocula are likely to be viable in excess of a year after preparation.

During the development of the soft agar spore inoculum many samples were prepared for SEM. During the preparation of the inoculum used during the growth trials, three 0.01 mL samples were taken to assess the quality of the inoculum during the various stages of the preparation process. The samples were viewed using a cold field emission scanning electron microscope (S-4700, Hitachi, Mississauga, Ontario, Canada) located at the Institute for Research in Materials (Dalhousie University, Halifax, N.S., Canada). To prepare the samples for SEM imaging the three 0.01 mL samples were placed onto SEM studs, allowed to dry and then coated in 180-200 nm gold. Sample 1 was obtained after initial collection from Petri plates. Sample 2 was obtained after filtration through the 8 μm filter paper and sample 3 was obtained after all filtration steps and final re-suspension in de-ionized water. The location of the samples taken throughout the preparation process is shown in Figure 4.4.
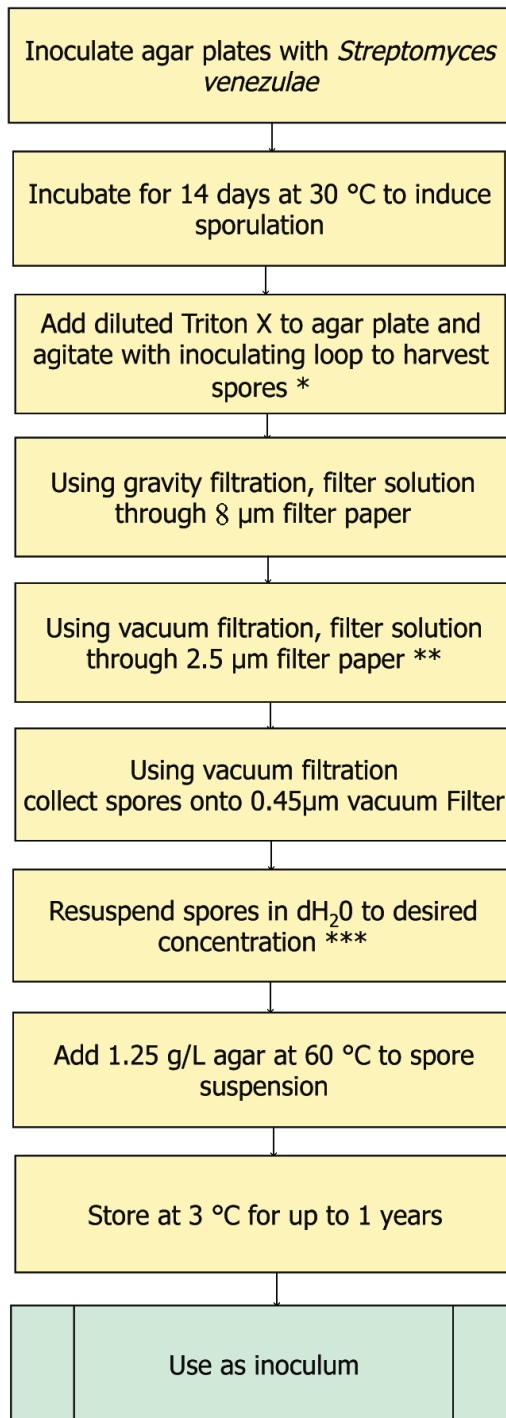
Figure 4.4   Flow chart summarizing the soft agar spore suspension method for producing standardized spore inoculum

*   SEM Sample 1 taken
**   SEM Sample 2 taken
***   SEM Sample 3 taken

4.8 Procedure for Temperature and pH Bioreactor Growth Trials

The bioreactor was first sterilized as described in Section 4.4. Once re-assembled, 5 L of MYM media (Table 4.3) was added aseptically and the unit sealed and connected to the bioreactor support structure. Before inoculation the media, temperature and pH were brought to trial specifications. At this point the bioreactor was inoculated with 1 mL of prepared soft spore inoculum added aseptically though the inoculation port on the lid of the bioreactor. Aeration was provided for all experiments at a steady rate of 2000 cm^3·min^{-1} and the agitation supplied at 400 rpm. For the next 12-16 h the temperature and pH of the media was measured continuously through the data acquisition system and maintained using the pH controller and bioreactor heating system. The free and hydrolyzed glucose concentrations and OD_{600} were monitored manually from samples taken on an hourly basis.

In order to examine the effect of temperature, the above procedure was used with the pH set to 7 and temperatures of 28, 32 and 36 °C for each trial. The effect of pH was investigated by completing additional bioreactor experiments at 32 °C with pH 5 and 9 specified for each trial. To assess variation between trials, one set of conditions (pH of 7 and temperature of 32 °C) was performed three times.

4.9 Procedure for Bioreactor Heat Balance

A heat balance analysis was performed on the bioreactor system in order to determine the heat of mixing (Q_{mix}) and the metabolic heat produced by *Streptomyces venezuelae* ($Q_{metabolism}$) during growth. In order to facilitate this, several experiments were conducted so that the terms in the master heat balance equation (Equation 1) could be determined. The theoretical equations used for the analysis relating to Equation 1 have been discussed in Section 3.4.

4.9.1 Heat Balance Trials

The master heat balance equation (Equation 1) contained too many unknown variables to solve for with a single experiment. Therefore three sets of trials were completed to isolate

and solve for the parameters. The simplified heat balance equation that applied to the experiments are given in equations 20-23.

$$Q_{mix} = Q_{wallabove} + Q_{wall} + Q_{bottom} + Q_{lid} \qquad (20)$$

$$Q_{exhaust} = Q_{mix} - Q_{wallabove} - Q_{wall} - Q_{bottom} - Q_{lid} \qquad (21)$$

$$Q_{reactorloss} = Q_{wallabove} + Q_{wall} + Q_{bottom} + Q_{lid} - Q_{exhaust} \qquad (22)$$

$$Q_{metabolism} = Q_{exhaust} + Q_{wallabove} + Q_{wall} + Q_{bottom} + Q_{lid} - Q_{mix} \qquad (23)$$

For all heat balance trials the bioreactor water heating system was disabled. Trial 1 experiments utilized sterilized media under aseptic conditions and operated without aeration thereby creating a closed system. Through the elimination of $Q_{metabolism}$ and $Q_{exhaust}$ from Equation 1, the energy generated though mixing (Q_{mix}) becomes the sum of thermal losses at steady state (Equation 20), which can be measured using the measured temperatures and heat balance equations (Section 3.4 and 4.4). During the experiments for Trial 2, the aeration system was activated which introduced the term describing energy lost though exhaust ($Q_{exhaust}$) as described by Equation 21. As a comparison between trials the amount of energy lost though the components of the bioreactor ($Q_{reactorloss}$) was determined through the use of Equation 22. To determine the energy generated though the aerobic metabolism of sugars by *Streptomyces venezuelae* metabolism within the media ($Q_{metabolism}$) bacteria were introduced for Trial 3. The $Q_{metabolism}$ was inferred through the heat balance model (Equation 23), as any additional energy generation within the bioreactor over normal operating conditions would be attributed to biological metabolism and activity. The experimental conditions for the three trials are summarized in Table 4.4. Experiments relating to Trials 1 and 2 were run at agitation speeds of 200, 400 and 600 rpm. At each agitation speed the system was allowed to come to thermal equilibrium. This was maintained for a period of one hour and the temperatures averaged for the heat balance calculations. It was found that ambient temperature deviations between trials did not affect the amount of heat generated within the system, however minor deviations in the ambient air temperature during each experiment

caused slight variations in the amount of thermal loss from the system at any given time. The Trial 3 experiment was run at 400 rpm only and followed the procedure described in Section 4.8 except the media was initially at ambient temperature and the water heating system was disabled. During Trials 1 and 2 the media was kept at a pH of 2 to discourage growth while media at a pH of 7 was used for Trial 3.

During Trials 1 and 2, the reactor was allowed to reach thermal equilibrium (steady state) with the environment at three agitation speeds (200, 400 & 600 rpm). This period of thermal equilibrium was maintained for a period of one hour over which the amount of energy loss for each component of the bioreactor was calculated. A retrial was performed at each agitation speed to ensure the method was consistent. To ensure accuracy, the energy loss from the system was averaged over the hour period. A sample calculation for Trial two at 600 rpm is shown in Appendix B.

Table 4.4   Summary of conditions used for heat balance experiments.

Parameter	Trial 1	Trial 2	Trial 3
Aeration (2000 $cm^3 \cdot min^{-1}$)	No	Yes	Yes
Bacteria (1 mL inoculum)	No	No	Yes
Agitation Speed (rpm)	200,400,600	200,400,600	400

4.9.2   Validation of Heat Balance

To validate and correct the heat balance model, additional validation experiments were completed using a submersible electric resistance heater (Elite 60W submersible, Hagen, Baie d'Urfe, QC, Canada) with a known energy output (Q_{known}) in place of $Q_{metabolism}$. To describe the balance of energy inputs and outputs within the system, Equation 24 was used where $K_{balance}$ is a correlation constant between the model and validation trial. Several trials were completed with set thermal outputs (0, 1.5 and 3 $J \cdot s^{-1}$) and each trial was run until thermal equilibrium was reached. The terms in Equation 24 were calculated and compared to the results of the heat balance model for previous trials. If the model perfectly predicted the amount of energy entered into the system through Q_{known}, then $K_{balance} = 1$. Equation 25 was rearranged to solve for $K_{balance}$.

$$Q_{known} + K_{balance} * Q_{mix} = K_{balance} * Q_{reactorloss} \tag{24}$$

$$K_{balance} = \frac{Q_{known}}{(Q_{reactorloss} - Q_{mix})} \tag{25}$$

To find the correlation constant, the program THERM.C was modified to control the submersible heater through the use of a 6008 USB National Instruments data acquisition device. A 5 V signal activated a solid state relay connecting the heater to a wall-mounted 120 V power source. An amp-meter was connected in series and a voltmeter connected in parallel to determine the amount of power utilized by the heater. When fully operational, the heater provided 60 W of power, however the program modulated the signal to pulse on/off, allowing a specific amount of power to be delivered over time. A trial was completed with the operating parameters identical to Trial 3 with the set values of Q_{known} to replace the heat generated by the bacteria. From this trial the amount of energy generated within the bioreactor above the normal operating parameters (Q_{known}) was calculated via the heat balance (Equation 24). To properly emulate the thermal generation and dispersal of bacteria both agitation (400 rpm) and aeration (2000 cm^3·min^{-1}) were required during the trial. During the first validation trial an average of 1.5 J·s^{-1} thermal energy was added to the media in addition to the operating conditions (400 rpm and 2000 cm^3·min^{-1} of air). This condition was maintained until a state of thermal equilibrium was reached for a period of one hour. An additional trial of 3.0 J·s^{-1} was completed to properly establish the correlation.

CHAPTER 5 RESULTS

5.1   Optical Density vs. CFU·mL⁻¹ Calibration Curve

To verify the appropriateness of using a wavelength of 600 nm for *Streptomyces venezuelae* detection, the optical density of samples taken throughout a bioreactor growth trial was measured at wavelengths 325 - 800 nm. This is shown in Figure 5.1 where samples were taken at 0, 4, 6, 11 and 12 hours after inoculation. The optical density of the media was approximately 0.04 - 0.08 AU and there was no detectable change to the optical density immediately after inoculation (zero hours). At wavelengths of 550 - 800 nm the optical density of the tested samples was in a detectable range indicating that OD_{600} is appropriate for *Streptomyces venezuelae.* A calibration curve was then created to correlate OD_{600} measurements with CFU·mL⁻¹ from plate counts to quantify *Streptomyces venezuelae* growth (Figure 5.2). An exponential equation was fitted to the experimental data (Equation 26) with an R squared value of 0.998 indicating a good fit.

$$N(t) = 356.11 * e^{(OD_{600}*2.96)} \hspace{4cm} (26)$$

Where:

|		
N(t)	=	Number of bacteria at time *t* (CFU·mL⁻¹)
OD_{600}	=	Optical density at 600 nm (AU) at time *t*
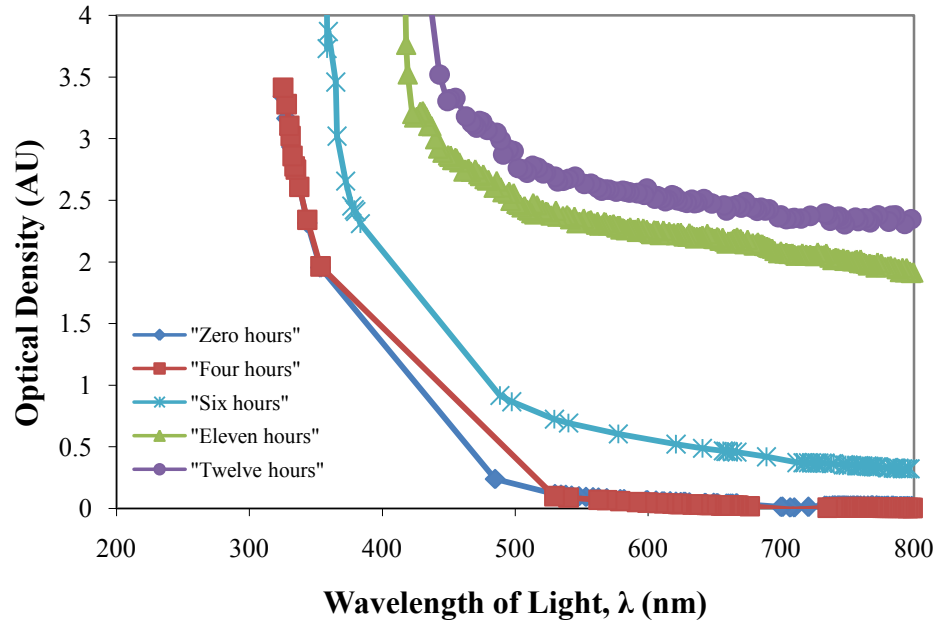
Figure 5.1   Optical densities of samples taken throughout a bioreactor growth trial
over a range of wavelengths.
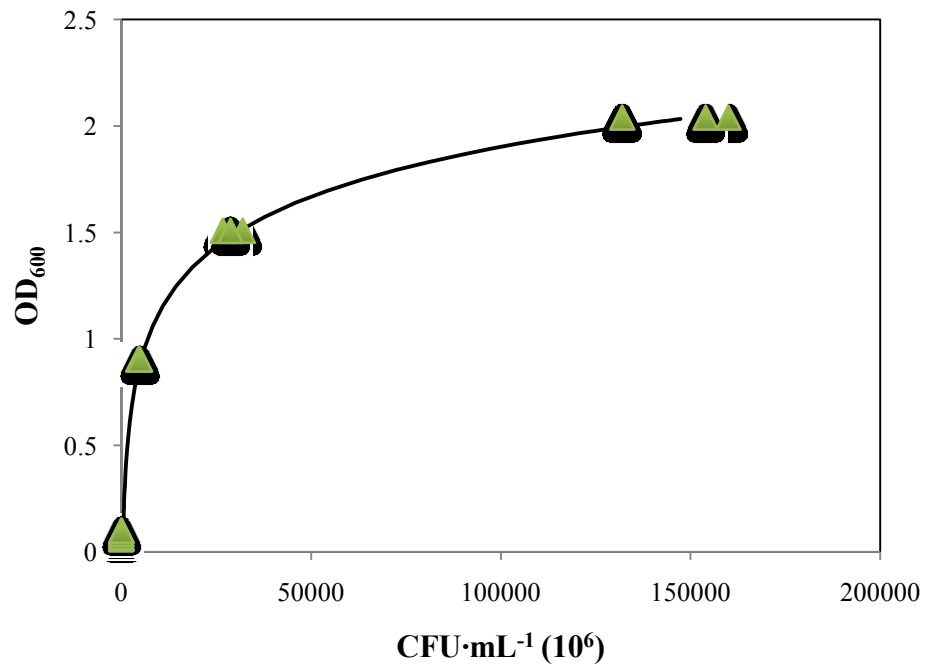
Figure 5.2   Optical density vs. CFU·mL^{-1}calibration curve.

5.2   Spore Inoculum

During the development of the soft agar spore suspension, Scanning Electron Microscopy (SEM) was used to gauge the effectiveness of filtering techniques. The images helped access the quality, concentration and size of the spores. To assess the consistency of the suspension as an inoculum, a series of growth trials were conducted.

5.2.1   SEM Analysis

SEM was used to observe the *Streptomyces venezuelae* spore suspension at several stages during the inoculation preparation process. As described in Section 4.7, a high concentration of spores was isolated from *Streptomyces venezuelae* stock plates to use in a standardized inoculum. A sample from the final inoculum is shown in Figure 5.3. The material from the initial stock plates of *Streptomyces venezuelae* (Sample 1) is shown in Figure 5.4. This image appears to include mycelia fragments and debris as well as individual spores. Figure 5.5 was taken from the spore suspension after filtering though both 8 and 2.5 μm pore size filters (Sample 2). Within this sample there were fewer contaminants than in Figure 5.4. However, a large number of smaller unknown particles were observed (debris). Figure 5.6 is an image of the spore suspension formed after collection onto a 0.45 μm filter and subsequently re-suspended in de-ionized water (Sample 3). The SEM image shows a much higher spore concentration than the previous samples without the previously observed debris.

As the final sample was mostly free of observable debris and large particles (Figure 5.3), the spore suspension was deemed acceptable for use in growth trials and the average size of spores was determined using the SEM images (Figure 5.7). After the 0.2 μm thickness of the gold coating was taken into consideration, the size of the spores was calculated to be 0.8 x 0.2 μm on average. The size distribution of particles within the final spore suspension was also determined through the use of a Nanotrac particle size analyzer, as shown in Figure 5.8, where two samples were measured in duplicate trials.
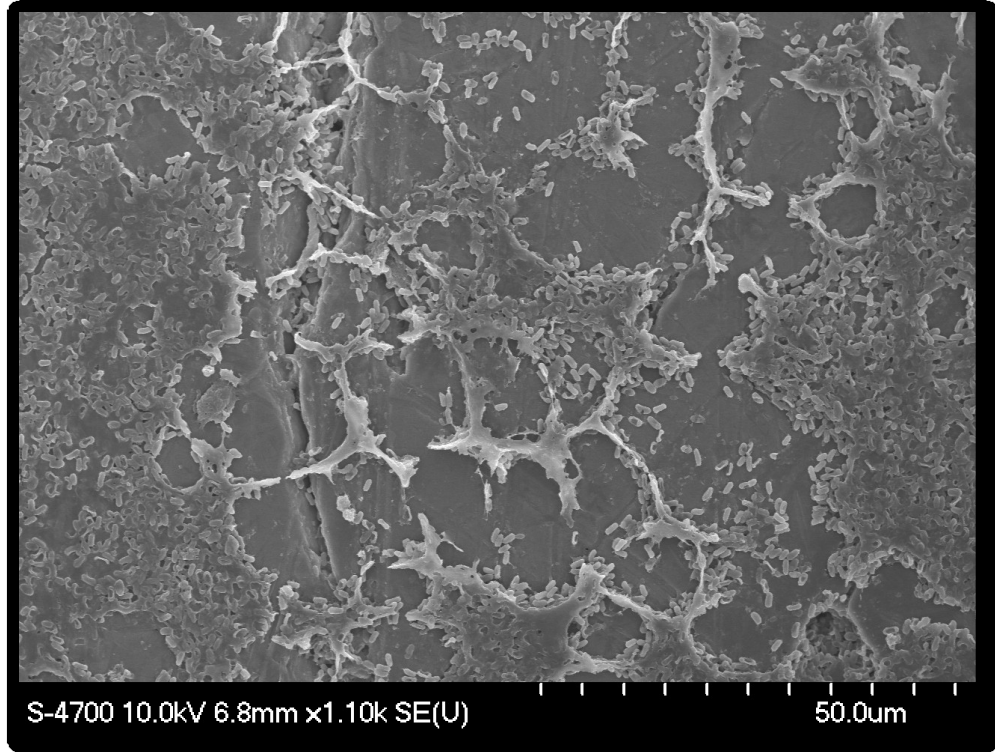
Figure 5.3   SEM image of final *Streptomyces venezuelae* spore inoculum.
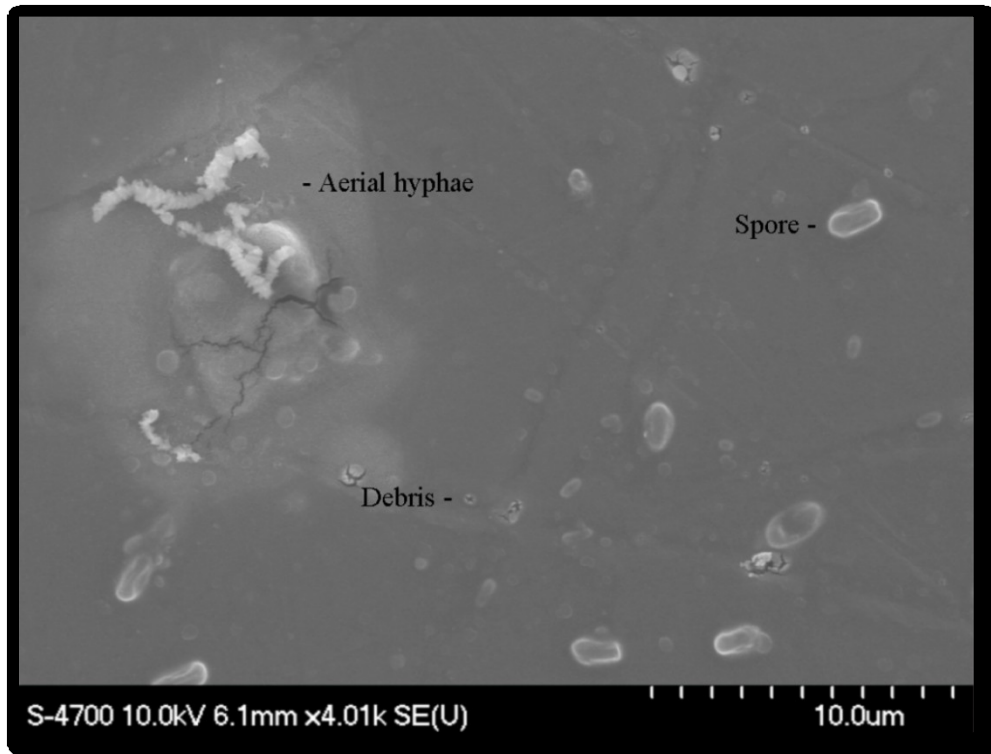
Figure 5.4   SEM image of spores and colony fragments obtained from initial stock plate.
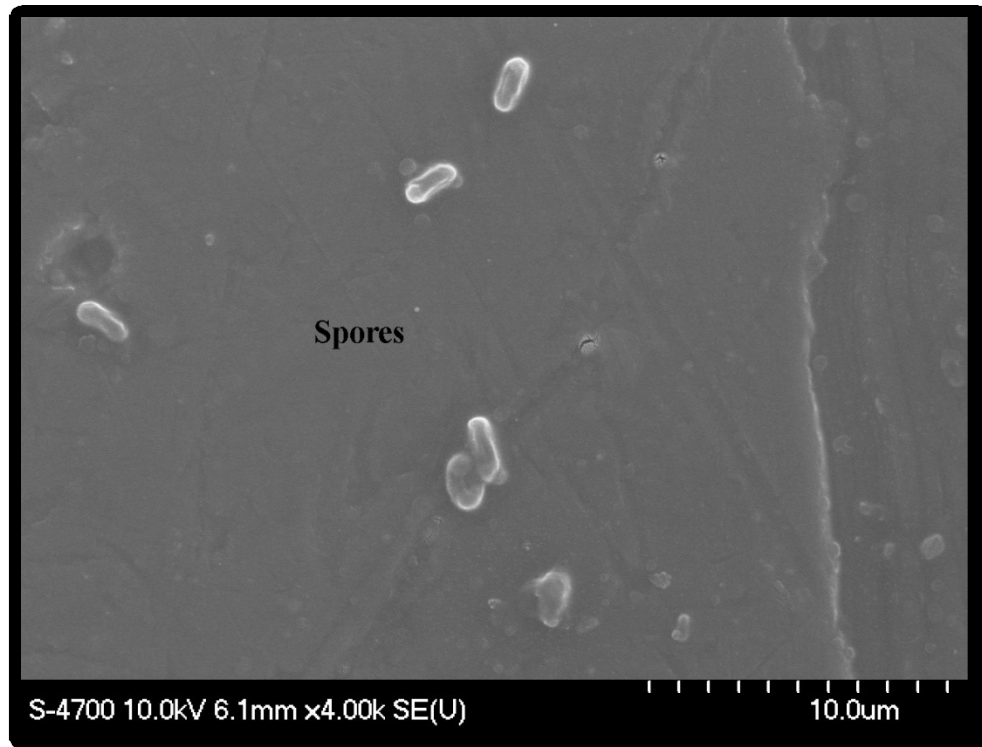
Figure 5.5   SEM image of solution after filtering through 8 and 2.5 µm filter paper.
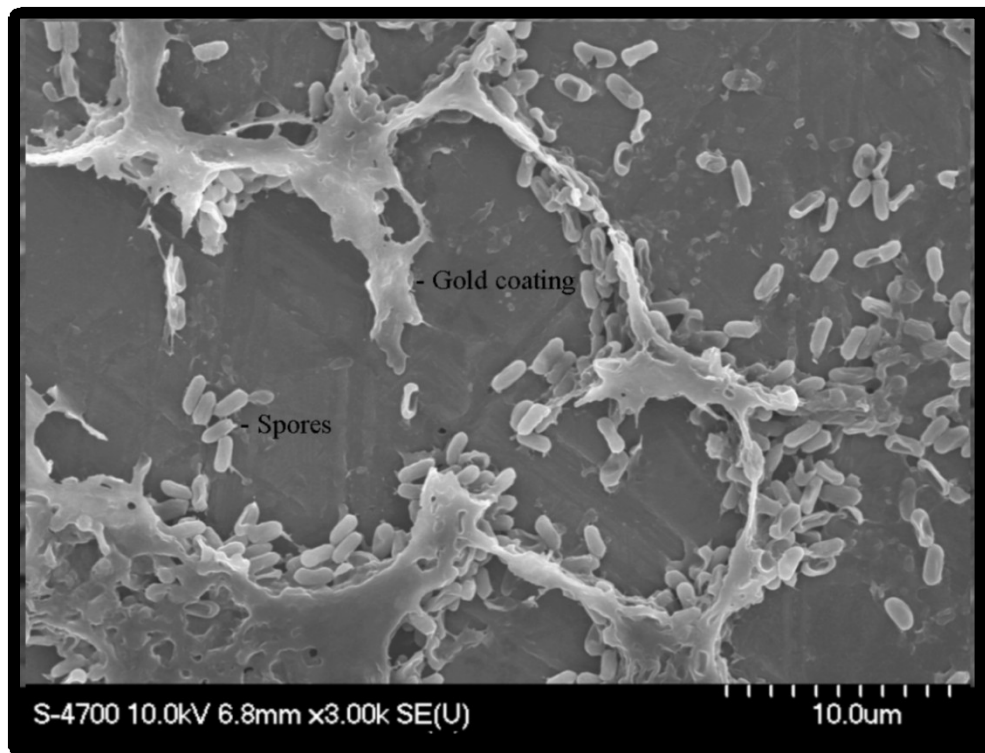
Figure 5.6   Concentrated spores in de-ionized water with debris removed.
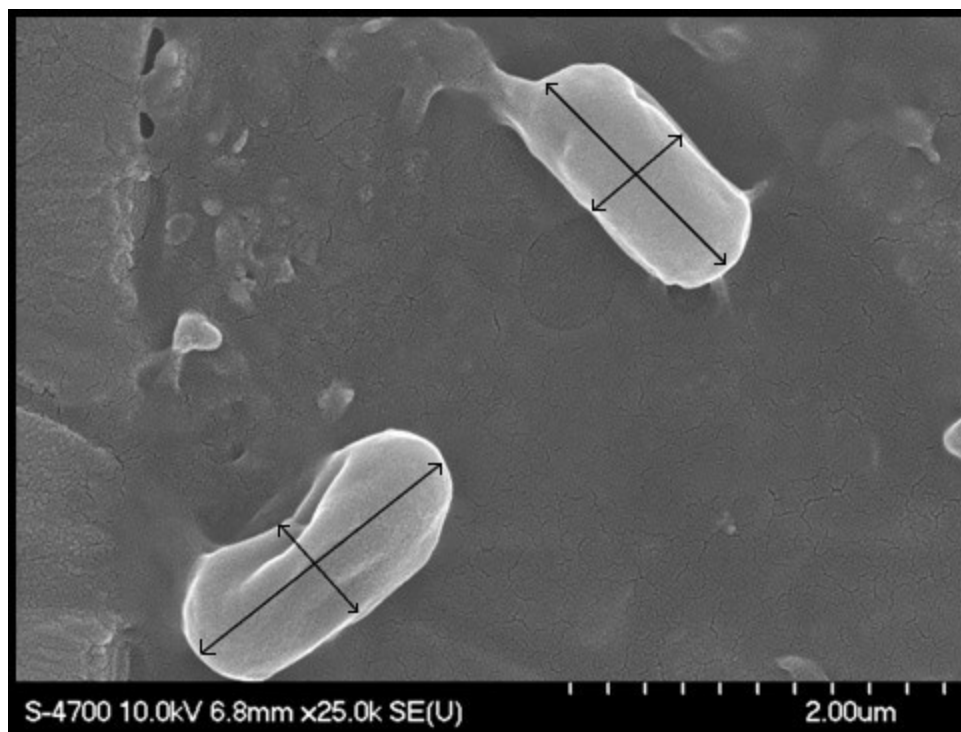
Figure 5.7   SEM image of *Streptomyces venezuelae* spores with size dimensions.
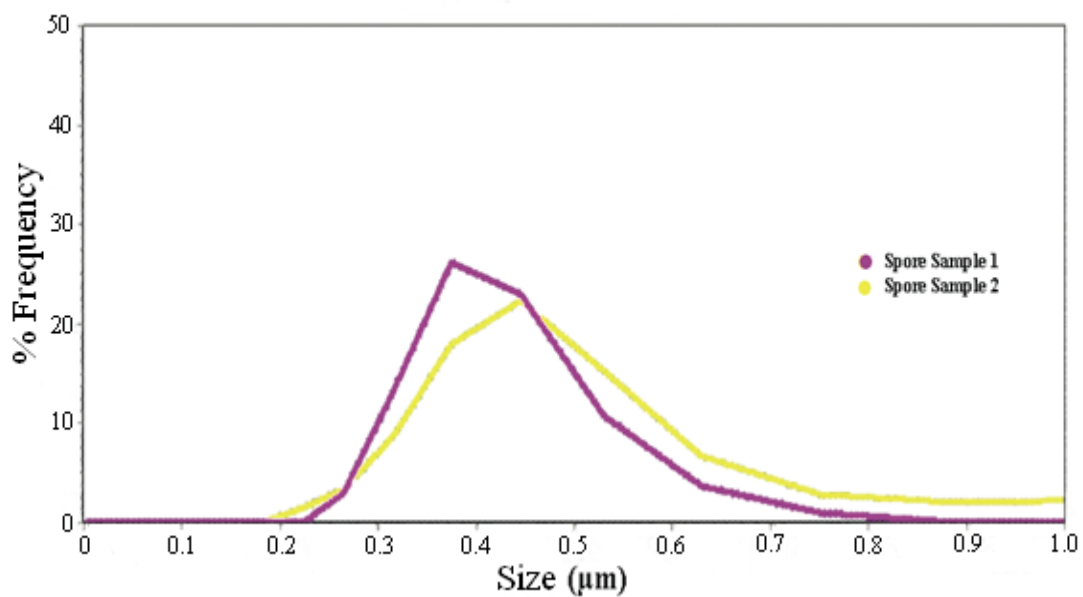
Figure 5.8   Particle size distributions of two spore samples using the Nanotrac analyzer.

5.2.2 Inoculum Validation

A 100 mL quantity of soft agar spore suspension was prepared for use as a standardized inoculum for all the bioreactor experiments in this study. To access the quality and consistency of the inoculum, three sets of growth trials in shake flasks were conducted with identical conditions as described in Section 4.7. These trials were performed at least a week apart to ensure the inoculum would not degrade over time. Figure 5.9 compares the growth of bacteria with time from the three trials using the standardized spore inoculum.

Using the statistical software analysis package Systat® Version 5.2,1 (Systat Software Inc., Richmond, CA), a modified Gompertz model (Speers *et al.*, 2003) was used to fit the experimental growth data. The software performed a Gauss-Newton non-linear regression using least squares estimation to fit the model. The modified Gompertz model is given in Equation 27. The fitted curves are shown on Figure 5.9 and calculated parameters are summarized in Table 5.1.

.

$$N_{(t)} = \frac{N_{(0)} - N_{(inf)}}{(1 + e^{(-B(t-M))})} + N_{(inf)} \tag{27}$$

Table 5.1   Calculated parameters for the "Number of Bacteria" model.

Variable	Variable Description	Effect on Curve
$N_{(t)}$	Number of CFU·mL^{-1} at time (t)	Variable with respect to time (t)
$N_{(inf)}$	Number of CFU·mL^{-1} at time infinity	Lower asymptote
$N_{(0)}$	Number of CFU·mL^{-1} at time zero	Upper asymptote
M	Time to point of inflection	Inflection point of sigmoid
B	Slope at inflection point of sigmoid	Slope at inflection point
t	Time t	Progression of the curve
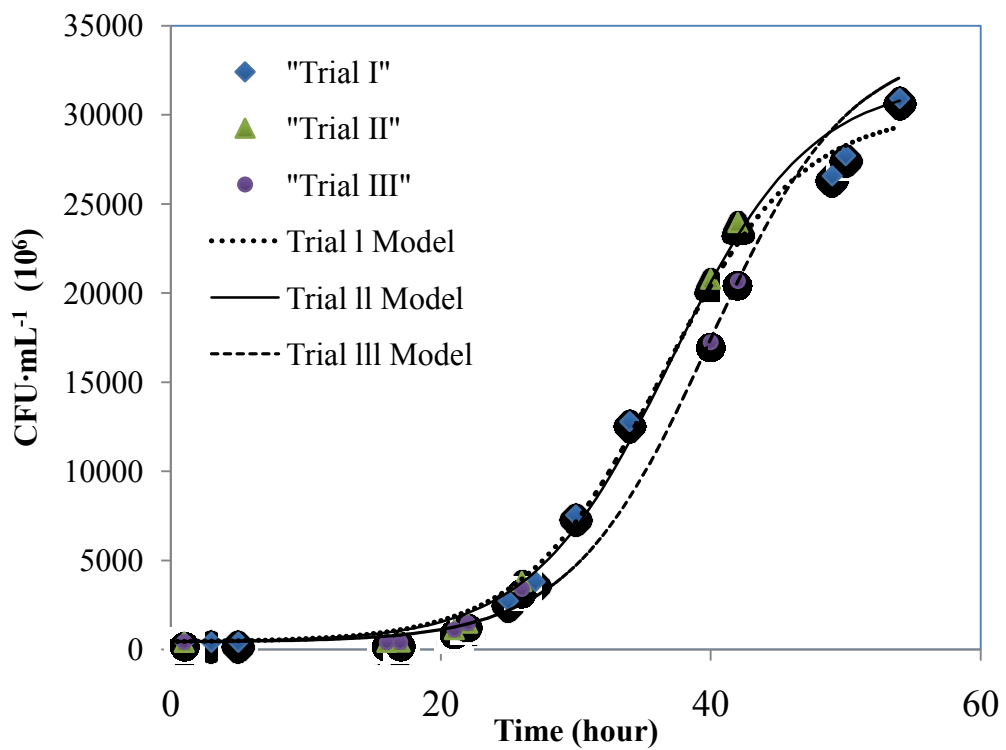
Figure 5.9   Comparison of *Streptomyces venezuelae* growth from spore inocula in shake flasks.

The specific growth rate μ_{max} was also calculated for each of the trials with the method used by Cann (2010). This method used Equation 28 to determine μ_{max}. Calculation of μ_{max} was achieved from the fitted model curve. The equation was applied to the exponential growth portion of the model which appeared linear when plotted on a semi-log plot. Table 5.2 shows $\mu_{max,}$ for each of the inoculum validation trials with the calculated asymptotic standard deviation for the non-linear fit. The lag time (Lt), also shown in Table 5.2 was visually attained from the semi-log plot as the period prior to exponential growth.

$$\mu_{max} = \frac{(Log_{10}(N_{(t)}) - Log_{10}(N_{(0)})) \cdot 2.303}{(t - t_0)} \qquad (28)$$

Where:

$N_{(t)}$	=	Number of CFU·mL^{-1} at time (t)
$N_{(0)}$	=	Number of CFU·mL^{-1} at time (Lt)
t	=	Time from inoculation (hour)
t_0	=	Lt (hour)

Table 5.2   Growth regression parameters from inoculum validation experiments

Parameter	Trial 1	Trial 2	Trial 3
$N_{(inf)}$ (CFU·mL^{-1})	30265	31913	34147
B(hour^{-1})	-0.194	-0.212	-0.187
M (hour)	36.36	37.02	39.92
μ_{max*} (hour^{-1})	0.135	0.137	0.138
Lt (hour)	14	14	14.5

* Asymptotic standard deviation = 0.0013 hour^{-1} with 25 data points.

5.3   Bioreactor Operating Parameter Experiments

In order to assess the variation between trials, one set of conditions supported by the literature was completed three times. The results from these trials were used to indicate the typical repeatability of experiments and help to assess the effect of manipulated parameters in later trials.

5.3.1   Repeatability

The three identical trials (R1, R2, and R3) were completed using media at 32 ºC, neutral pH, maximum dissolved oxygen saturation and agitated at 400 rpm in the bioreactor. The CFU·mL^{-1} vs. time data for the three trials are plotted on Figure 5.10 showing sigmoidal behavior characteristic of bacterial growth. During trial R3 there is a delay in the exponential growth of bacteria at approximately 14 hours where the pH control failed for approximately 0.5 hours. This temporary failure did not seem to significantly affect the overall growth rate or total number of bacteria produced (Figure 5.10). To model the growth over the trial curves were fitted to the data using the modified Gompertz model (Equation 27). The fitted curves are also shown on Figure 5.10 and the parameters calculated for the non-regression are given in Table 5.3. The specific growth rate and lag time (μ_{max} and Lt respectively) as calculated in Section 5.2.2 were used to compare trials and are also shown in Table 5.3. The lag time (Lt) between each trial was determined to be within 0.5 hours. The standard asymptotic standard deviation of μ_{max} between trials was 0.003 hour^{-1} (Table 5.3) indicating the growth rates were similar.
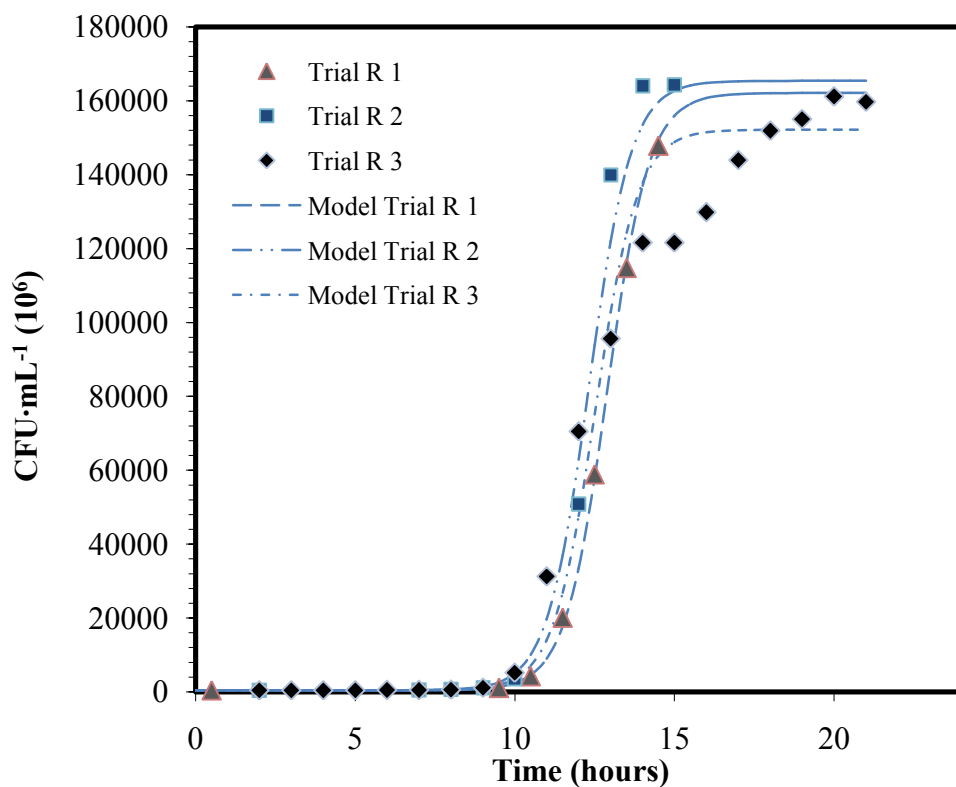
Figure 5.10   Bacterial growth during three identical bioreactor trials

At 32 °C and a media pH of 7.

Table 5.3   Growth regression parameters from repeatability trials

Parameter	R 1	R 2	R 3
$N_{(inf)}$ (CFU·mL^{-1})	162087	165375	152184
B (hour^{-1})	-1.450	-2.528	-0.822
M (hour)	12.89	12.33	12.52
μ_{max*} (hour^{-1})	1.436	1.436	1.431
Lt (hour)	8.0	7.5	7.5

* Asymptotic standard deviation = 0.003 hour^{-1} with 36 data points

Experimental data for hydrolyzed and free glucose measurements over the course of the experiment are shown in Figures 5.11 and 5.12 respectively. The maltose specifically added to the media as the primary energy source was measured in the form of hydrolyzed glucose. The number of measurements for hydrolyzed glucose is two less than the absorbance readings for bacterial growth as the samples required two hours for complete hydrolysis (Section 4.6). The consumption of hydrolyzed glucose appeared to follow a sigmoidal consumption curve, however full data was not available for every trial due to time constraints.

Table 5.4 indicates that free glucose was likely available in the media through the addition of the malt extract. Figure 5.12 shows a sigmoid consumption curve for free glucose, therefore the experimental data was modeled using the modified Gompertz model (Equation 29). The parameters for this regression are described in Table 5.5. Additionally the rate of the glucose consumption (μ_g) as described by Equation 30 and the time until substrate consumption was detected (*SLt*) for each trial was determined. The model parameters, free glucose consumption rates and substrate consumption lag times for the three repeatability trials are shown in Table 5.6. Figure 5.13 combines the free and hydrolyzed glucose consumption with the growth models for the three repeatability trials. The free glucose present in small quantities was completely consumed at the onset of each experiment while the maltose in the form of hydrolyzed glucose showed signs of initial consumption after the free glucose had been consumed. As the test for free glucose was quick and consistent, this parameter was used as a comparison between trials.
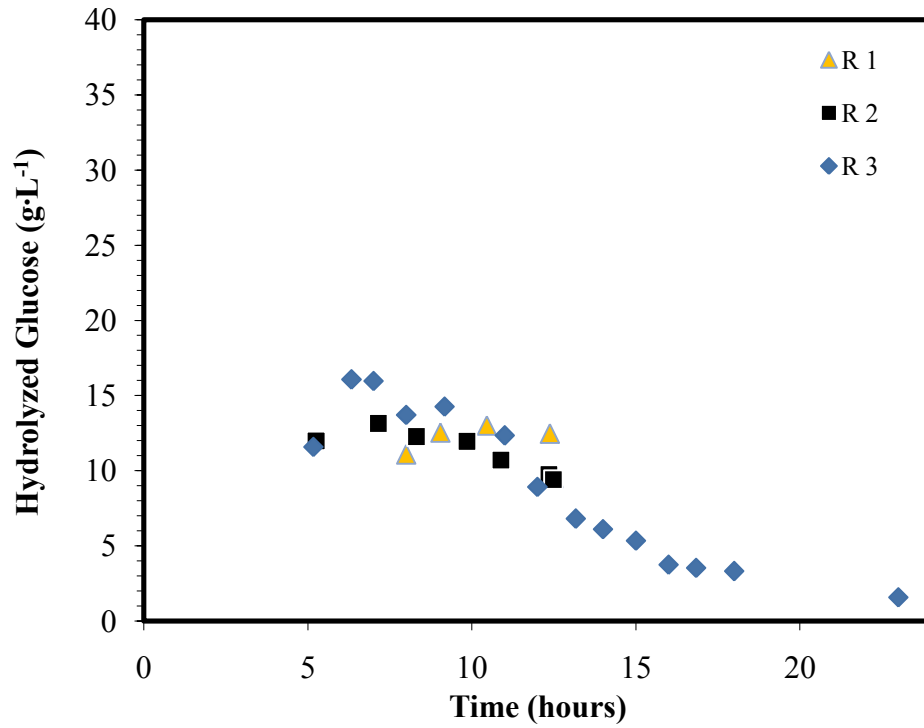
Figure 5.11   Consumption of hydrolyzed glucose during repeatability trials
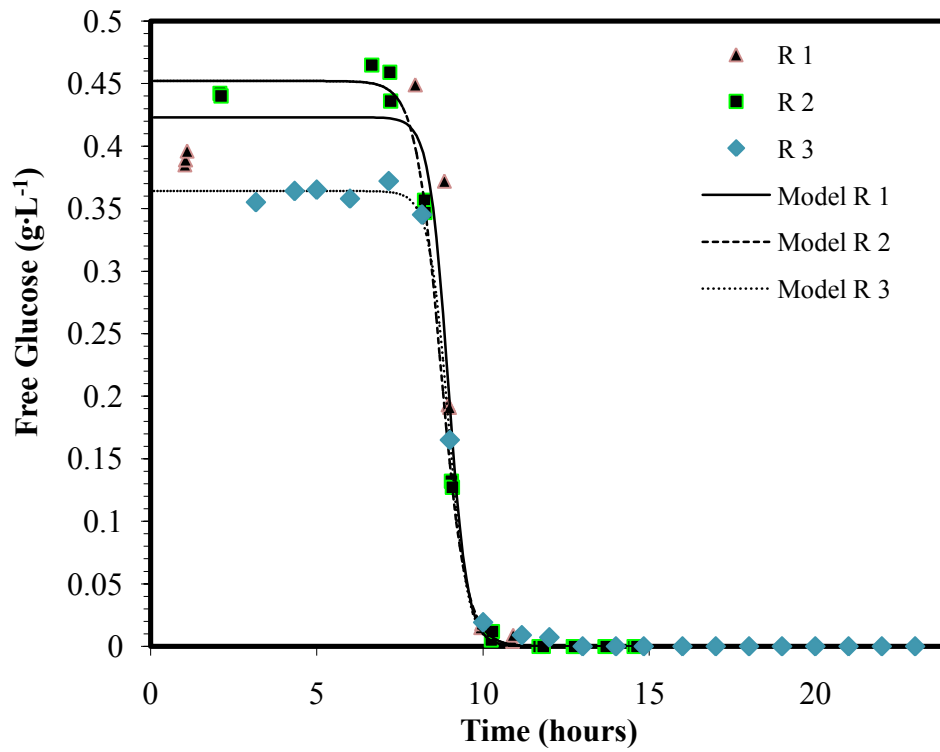
Figure 5.12   Consumption of free glucose during repeatability trials

60

Table 5.4   Carbon sources within MYM media

Component	Chemical Formula	Amount
D-Maltose*		
Glucose	$C_6H_{12}O_6$	0.6 %
Maltose	$C_{12}H_{22}O_{11}$	95 % min
Maltotriose	$C_{18}H_{32}O_{16}$	1 %
Malt extract**		
Fructose	$C_6H_{12}O_6$	1-2%
Glucose	$C_6H_{12}O_6$	7-10%
Sucrose	$C_{12}H_{22}O_{11}$	1-3%
Maltose	$C_{12}H_{22}O_{11}$	39-42 %
Maltotriose	$C_{18}H_{32}O_{16}$	10-15 %
Higher Saccharides	N/A	25-30 %
Yeast extract***		
Nitrogen content	N/A	8 – 12 %
Protein content	N/A	3.0 – 5.2 %
Amino nitrogen content	N/A	50 – 75 %
Carbohydrate content	N/A	4 – 13 %

Sources: *   BioShop label located on bottle
           **   Hickenbottom , 2007
           ***   EURASYP, 2010

$$G_{(t)} = \frac{G_{(0)} - G_{(inf)}}{(1 + e^{(-B(t-M))})} + G_{(inf)} \qquad (29)$$

Table 5.5   Parameters for free glucose model.

Variable		Variable Description	Effect on Curve
$G_{(t)}$	=	Glucose g/L at time (t)	Variable with respect to time (t)
$G_{(inf)}$	=	Glucose at time infinity	Lower asymptote
$G_{(0)}$	=	Glucose at time zero	Upper asymptote
M	=	Time to point of inflection	Inflection point of sigmoid
B	=	Slope at inflection point	Slope at inflection point

$$\mu_g = \frac{(Log_{10}(G_{(t)}) - Log_{10}(G_{(0)})) \cdot 2.303}{(t - t_0)} \qquad (30)$$

Where:

$G_{(t)}$	=	Glucose g·L^{-1} at time (t)
$G_{(0)}$	=	Glucose g·L^{-1} at time (SLt)
t	=	Time from inoculation (hour)
t_0	=	SLt (hour)

Table 5.6   Free glucose model parameters from repeatability trials.

Parameter	R 1	R 2	R 3
$G_{(0)}$ (g·L^{-1})	0.423	0.452	0.364
B (hour^{-1})	-3.5	-2.657	-3.429
M (hour)	8.95	8.723	8.953
μ_{g*} (hour^{-1})	-1.354	-1.206	-1.078
SLt (hour)	8.0	7.8	7.5

* Asymptotic standard deviation = 0.138 hour^{-1} with 52 data points
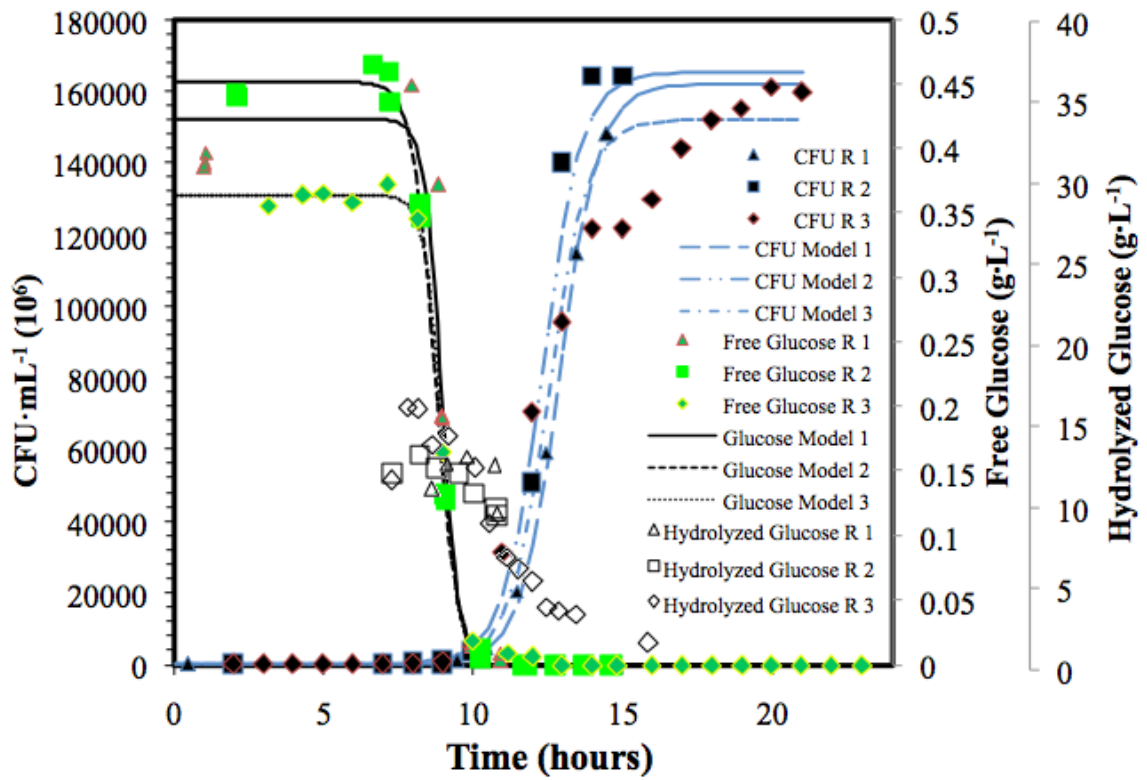
Figure 5.13 Sugar and bacterial growth measurements during repeatability trials.

5.3.2   Temperature

The effect of temperature on *Streptomyces venezuelae* growth and activity was examined by modifying the temperature of the bioreactor as the manipulated variable during growth trials. Trials were initially completed at temperatures of 28 & 32 ºC. An additional trial at 36 ºC examined the effect of higher temperatures on *Streptomyces venezuelae* growth.

Experimental data are shown (Figure 5.14), where 32 °C data from Section 5.3.1 are compared to growth trials at 28 and 36 °C. The modified Gompertz model was used to fit the data and Lt and μ_{max} determined as in the previous section. The model parameters, Lt and μ_{max} are shown in Table 5.7. Single ANOVA statistical analysis (Microsoft® Office Excel® 2007, Microsoft, Redmond, WA, U.S.) was performed to determine if μ_{max} for trials at 28 and 36 ºC were statistically different at the 95 % confidence level from the trials at 32 ºC. The P-values for this analysis are also shown in Table 5.7. As the P- Value for the μ_{max} of the 28 and 36 ºC is less than 0.05, the growth rates were considered significantly different at the two pH levels evaluated.

Experimental data for hydrolyzed and free glucose were determined as previously discussed and are shown in Figures 5.15 and 5.16, respectively. The modified Gompertz model was used to model the free glucose consumption Equation 29 and the μ_g was determined using Equation 30 (Table 5.8). The shortest Lt occurred during the temperature trial at 36 ºC, however during this trial the media colour became lighter and began to emit a sweet odor. Possible explanations for this behavior are discussed in Section 6. Finally, the consumption of free and hydrolyzed glucose is shown with bacterial growth in Figure 5.17.
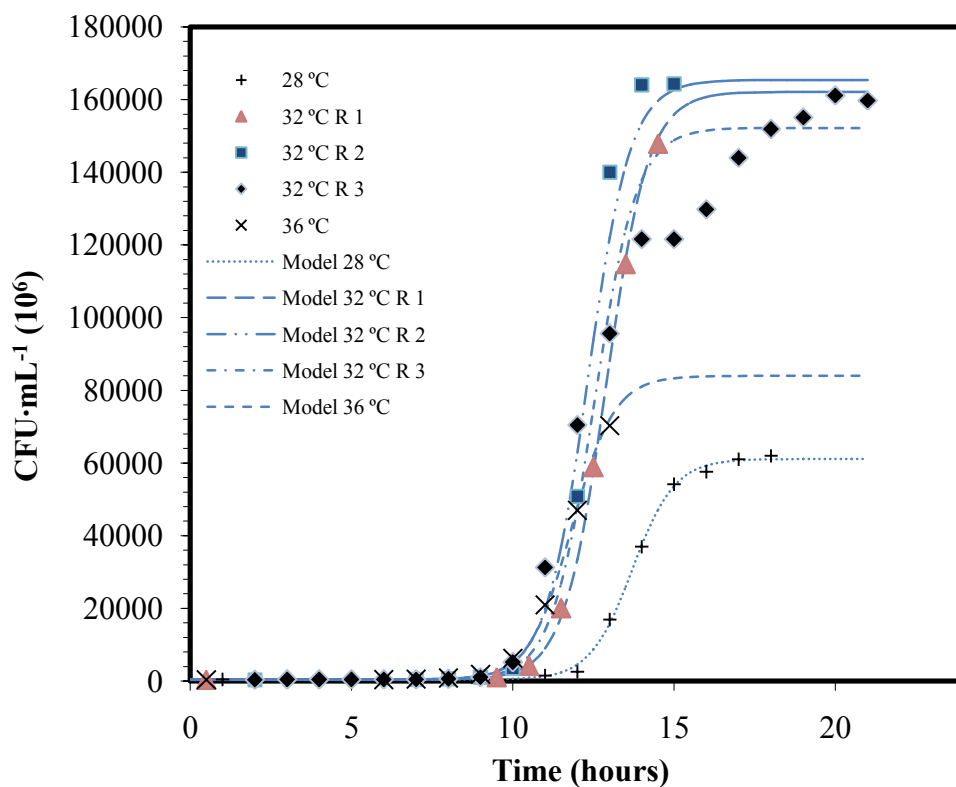
Figure 5.14 Bacterial growth measured during temperature and repeatability trials.

Table 5.7   Growth regression parameters from temperature trials.

Parameter	28 ºC	32 ºC[†]	36 ºC
$N_{(inf)}$ (CFU·mL^{-1})	61107	159882	83984
B (hour^{-1})	-1.53	-1.60	-1.38
M (hour)	13.7	12.6	11.8
μ_{max} (hour^{-1})	1.24	1.43	1.26
P-Value for μ_{max}	0.0003	N/A	0.0004
Lt (hour)	10.5	7.7	7.5

†Averaged values
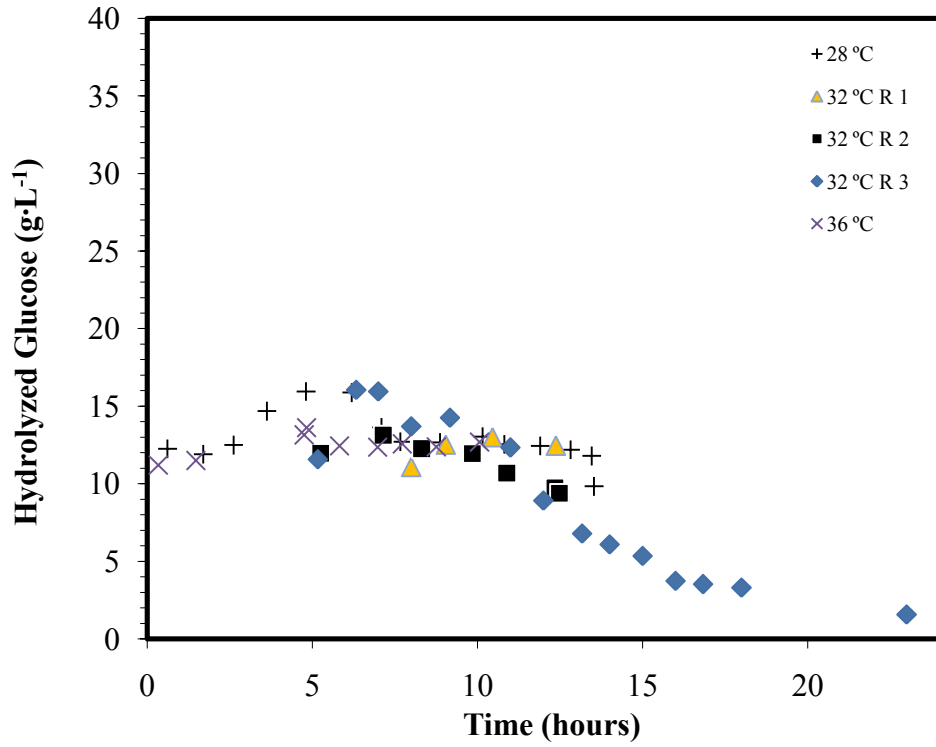
Figure 5.15   Hydrolyzed glucose measured during temperature and validation trials.
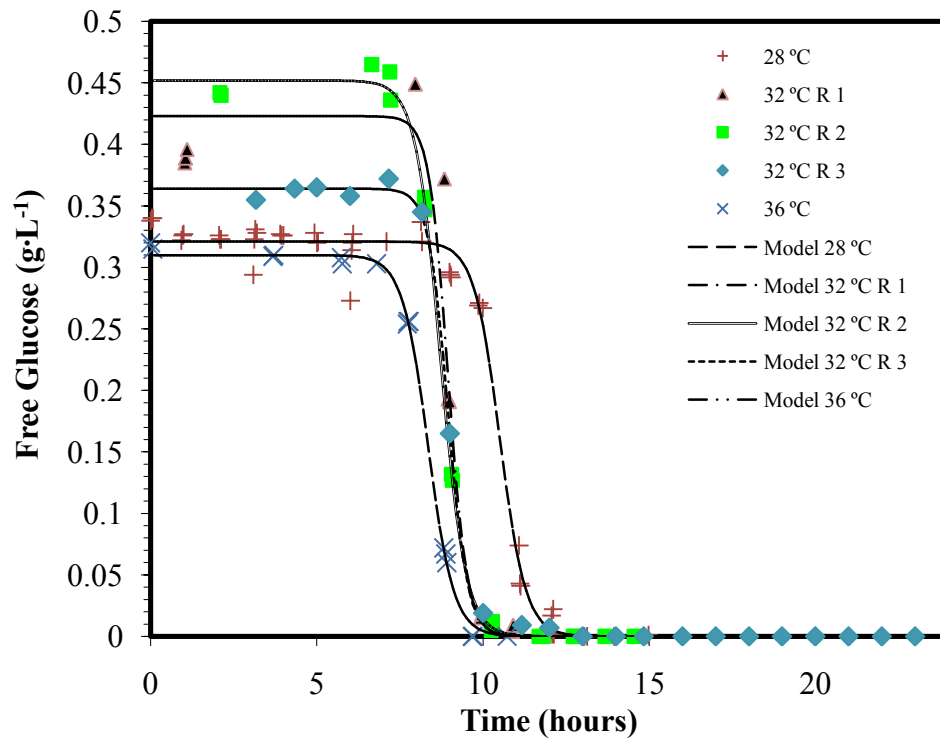
Figure 5.16 Free glucose measured and modeled during temperature and validation trials.

Table 5.8   Free glucose regression parameters from temperature trials.

Parameter	28 ℃	32 ℃†	36 ℃
$G_{(0)}$ (g·L^{-1})	0.321	0.4227	0.31
B (hour^{-1})	-2.567	-6.201	-2.578
M (hour)	10.51	8.875	8.349
μ_g (g·L^{-1}·hour^{-1})	-1.22	-1.212	-1.012
SLt (hour)	10.0	7.8	6.9

†Averaged values
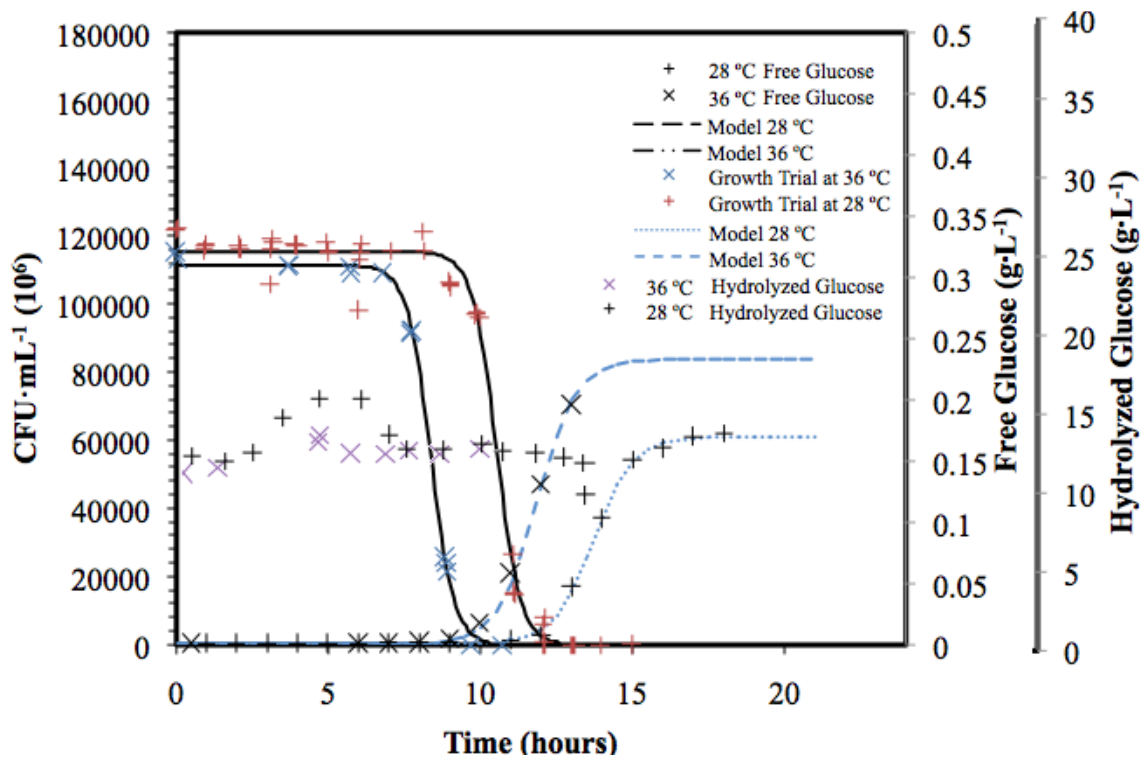
Figure 5.17 Sugar and bacterial growth measurements during temperature trials at 28 and 36 °C.

5.3.3 pH

Three trials were completed to assess the effect of alkaline, neutral and acidic conditions on the growth and substrate consumption by *Streptomyces venezuelae*. All trials were conducted as in the previous section at 32 °C. The test conducted using acidic media conditions at pH of 5 inhibited growth to such extent that growth (as a function of optical density) was not detected for the duration of the experiment.

Experimental data are shown (Figure 5.18), where data from Section 5.3.1 with media pH of 7 are compared to growth trials with pH of 9. The modified Gompertz model was used to fit the data, while Lt and μ_{max} values were determined as in Section 5.3.1. The model parameters, Lt and μ_{max} are shown in Table 5.9. Single ANOVA statistical analysis (Microsoft® Office Excel® 2007, Microsoft, Redmond, WA, U.S.) was performed to determine if μ_{max} for the trial at pH of 9 was statistically different at the 95 % confidence level from the trials at pH of 7. The P-values for this analysis are shown in Table 5.9, as the P- Value comparing the μ_{max} of pH 7 trials is less than 0.05, the growth rates are considered significantly different.

Experimental data for hydrolyzed and free glucose was determined as previously discussed and are shown in Figures 5.19 and 5.20 respectively. The modified Gompertz model was used to model the free glucose consumption using Equation 29 and the μ_g was determined using Equation 30 (Table 5.10). The combined free glucose, hydrolyzed glucose and bacterial growth data are shown in Figure 21.
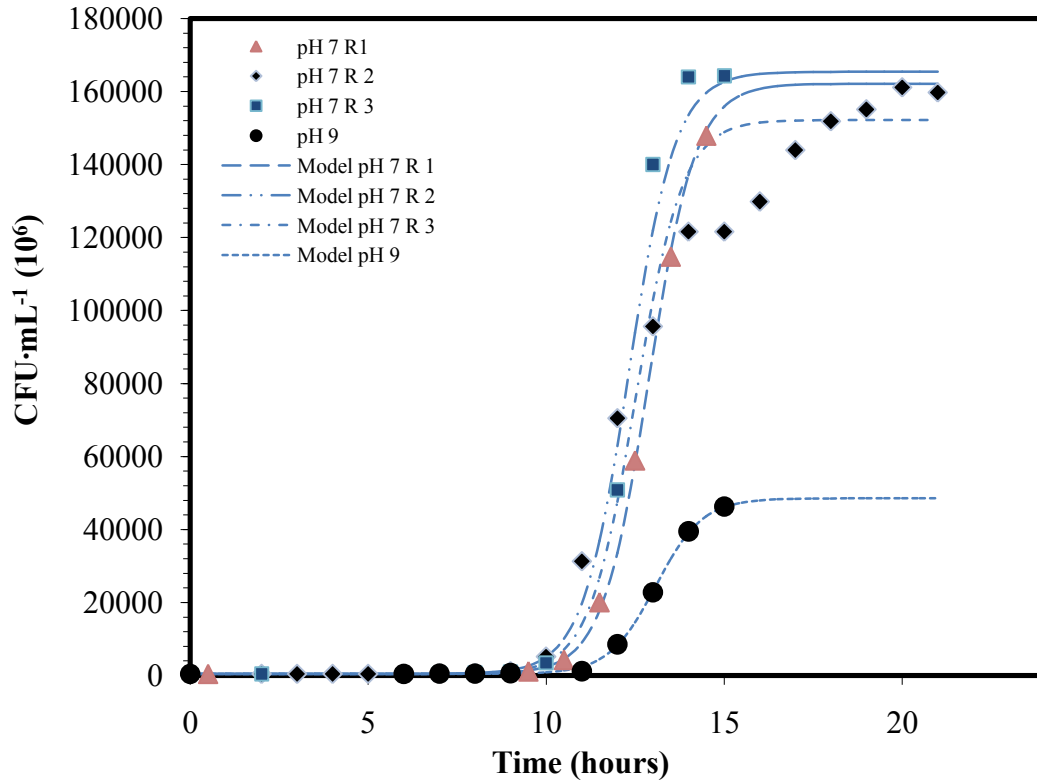
Figure 5.18   Bacterial growth measured during pH trials.

Table 5.9   Growth regression parameters from pH trials.

| Parameter	pH 7[†]	pH 9
$N_{(inf)}$ (CFU·mL^{-1})	159882	48523
B(hour^{-1})	-1.600	-1.571
M (hour)	12.58	13.08
μ_{max} (hour^{-1})	1.434	1.181
P-Value for μ_{max}	N/A	0.0002
Lt (hour)	7.67	8

†Averaged values
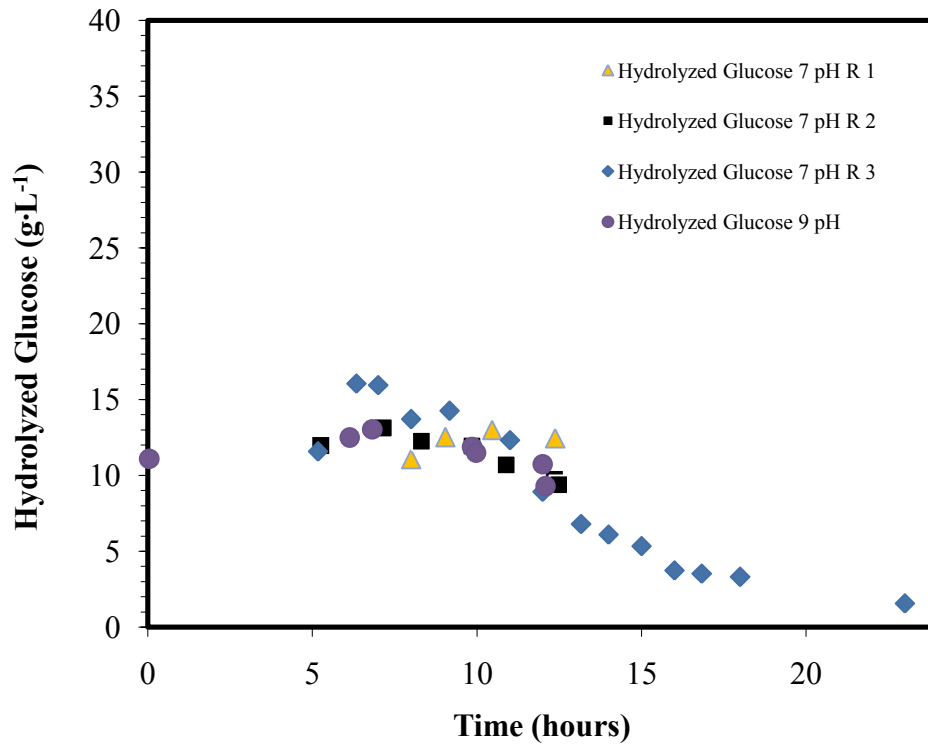
Figure 5.19   Hydrolyzed glucose measurements during pH trials.
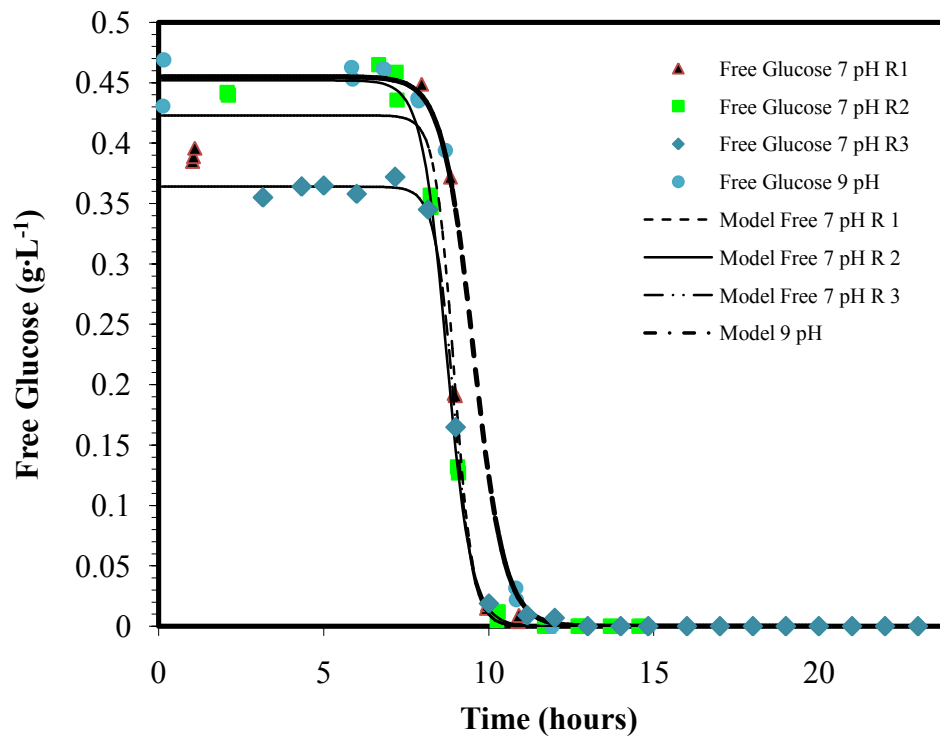
Figure 5.20   Free glucose measurements and model during pH trials.

Table 5.10   Free glucose regression parameters from pH trials

Parameter	pH7[†]	pH9
$G_{(0)}$ (g·L^{-1})	0.4227	0.455
B (hour^{-1})	-6.201	-2.121
M (hour)	8.875	9.538
μ_{g*} (g·L^{-1}·hour^{-1})	-1.212	-1.192
SLt (hour)	7.8	9

†Averaged values
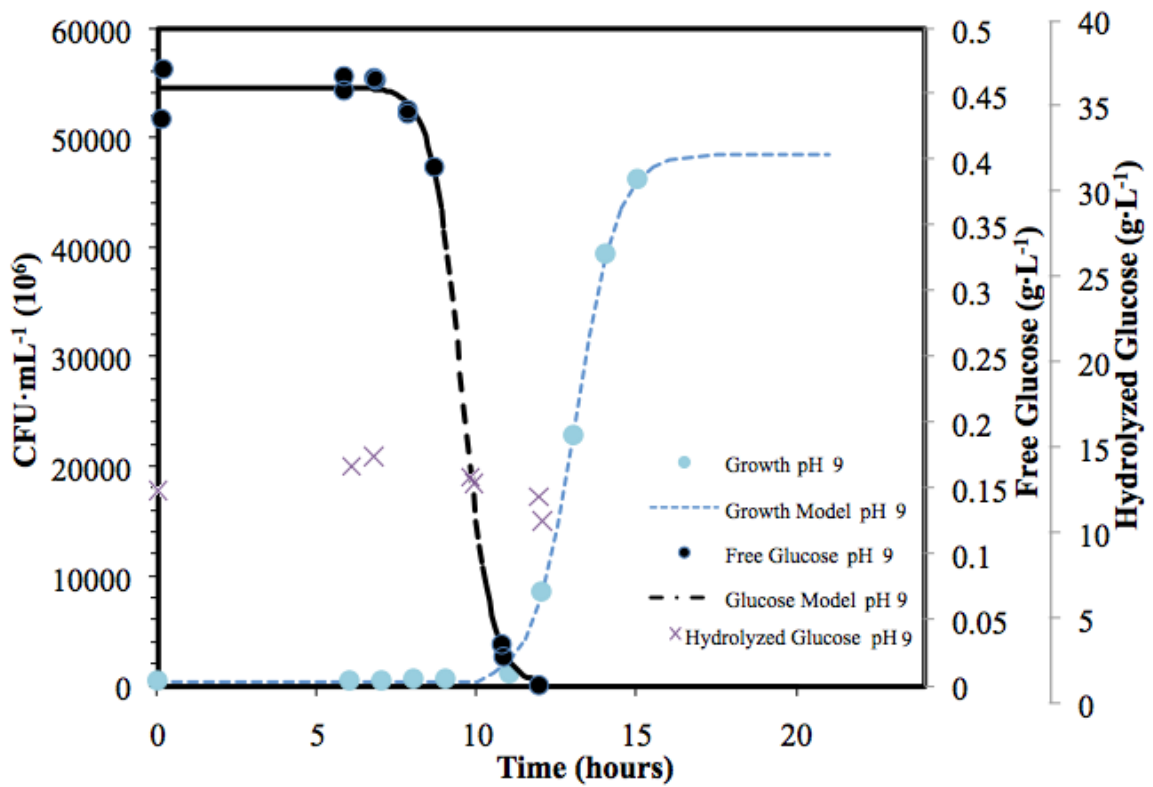
Figure 5.21   Sugar and bacterial growth measurements at a pH of 9.

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5.4   Bioreactor Heat Balance

Heat balance trials were run to isolate the sources of thermal generation within the bioreactor according to Equation 1. The first trial was conducted without aeration or bacteria to determine the amount of energy generated through mixing within the bioreactor. The second was completed with aeration while still excluding bacteria to determine the amount of energy lost with exhaust gases. The third trial, which included both aeration and live bacteria was conducted to determine the amount of energy generated with *Streptomyces venezuelae*. Finally, a validation trial was conducted in an identical manner to the third trial, only with a known energy source supplied by a heater in place of live bacteria. The known heat source allowed the heat balance to be calibrated to accurately reflect the thermal characteristics of the bioreactor and all prior results to be adjusted accordingly. The heat balance results for $Q_{reactorloss}$ in the following sections have been adjusted to reflect $K_{balance} = 1.2$ as determined through the validation trial and Equation 23. Further details regarding the validation results are given in Section 5.4.5.

The results of the heat balance validation trial are summarized in Table 5.11. Using set energy inputs of 0, 1.5, and 3 $J{\cdot}s^{-1}$, it was expected that the heat balance validation (Equation 24) would give this amount of energy in addition to the energy generated through operating parameters (Q_{mix}) at steady state. The results of the validation trial indicate that slightly more heat was generated within the bioreactor than was accounted for. The validation trial shows that the heat balance accurately predicted approximately 83 % of the energy generated within the bioreactor giving a $K_{balance}$ of 1.2 using Equation 25; a possible explanation for this is discussed in Section 6. In all future results the "corrected" energy is defined as the energy reported by the heat balance corrected to this validation. Due to the discrepancy between the predicted energy generation via the heat balance and the actual energy input, the results of each trial has been corrected.

5.4.1   Trials Without Aeration, Without Bacteria

During the first trial, the reactor was allowed to reach thermal equilibrium (steady state) at each agitation speed (200, 400, and 600 rpm). There was no aeration, no bacteria and the water heater was deactivated, providing no thermal generation except through

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mechanical mixing. Results from the heat balance are summarized in Table 5.11 where corrected energy values for Q_{mix} reflect the results of the validation trial (Section 5.4.5) From these trials (without aeration), the majority (93.1 - 95.1 %) of thermal loss occurred though the walls of the bioreactor with a negligible amount lost though the top plate. This is shown graphically in Figure 5.22. During this trial, the speed of agitation did not have a significant effect on the distribution of energy loss. At the highest agitation speed (600 rpm) it was calculated through Equation 20, that the agitation of the media generated approximately 0.03 - 1.35 J·s^{-1} of energy at agitation speeds of 200 - 600 rpm. Throughout these experiments it took in excess of 24 hours for the 5 L of media to reach thermal equilibrium.

5.4.2 Trials With Aeration, Without Bacteria

These trials were conducted similarly to those in Section 5.4.1, with the addition of aeration at 2000 cm^3·min^{-1} activated at the onset of the experiment. Results for the heat balance are also summarized in Table 5.11 During the second trial (with aeration) the distribution of energy loss changed noticeably with agitation speed (Figure 5.23), with more energy lost through the upper wall and the lid. Through the use of Equation 21, the amount of thermal loss through aeration ($Q_{exhaust}$) was calculated to be 0.01-0.32 J·s^{-1} at agitation speeds of 200 - 600 rpm.

5.4.3 Comparison of Trials With/Without Aeration, Without Bacteria

The energy loss from the components of the bioreactor ($Q_{reactorloss}$) during trial two (Section 5.4.2) was less than trial one (Section 5.4.1) as 30 - 41 % of the total energy generated through agitation was removed with the exhaust gases (dependent upon agitation speed). In Figure 5.24, the energy loss over the entire reactor is plotted against the speed of agitation for the trials without and with aeration. It is evident that the amount of heat lost through the bioreactor components during each trial increased as an exponential function in relation to agitation speed (Figure 5.24). Exponential equations were fitted to the experimental data using EXCEL. $Q_{reactorloss}$ for the heat balance trial without aeration is described through Equation 31 while Equation 32 describes the trial with aeration.

$$Q_{reactorloss} = e^{0.0095 \bullet t - 5.3479} \tag{31}$$

$$Q_{reactorloss} = e^{0.0099 \bullet t - 5.9739} \tag{32}$$

Table 5.11   Energy lost through components of Microferm bioreactor

for experiments with and without aeration.

Agitation Speed (rpm)	Q_{Wall}		Q_{Lid}		Q_{Bottom}		$Q_{exhaust}$	Q_{mix}	$Q_{reactorloss}$	Corrected $Q_{reactorloss}$
	$J{\cdot}s^{-1}$	$\%^{\dagger}$	$J{\cdot}s^{-1}$	$\%^{\dagger}$	$J{\cdot}s^{-1}$	$\%^{\dagger}$	$J{\cdot}s^{-1}$	$J{\cdot}s^{-1}$	$J{\cdot}s^{-1}$	$J{\cdot}s^{-1}$
Without Aeration										
200	0.03	94.0	0.00	0.0	0.00	6.0	0	0.03	0.03	0.04
400	0.23	95.1	0.00	0.1	0.01	4.9	0	0.24	0.24	0.28
600	1.26	93.1	0.03	2.0	0.07	4.8	0	1.35	1.35	1.62
With Aeration										
200	0.02	96.1	0.00	0.0	0.00	3.9	0.01	0.03	0.02	0.02
400	0.12	93.8	0.00	1.0	0.01	5.2	0.11	0.24	0.13	0.16
600	0.93	90.9	0.05	5.0	0.04	4.2	0.32	1.35	1.02	1.24

†Percentage of $Q_{reactorloss}$
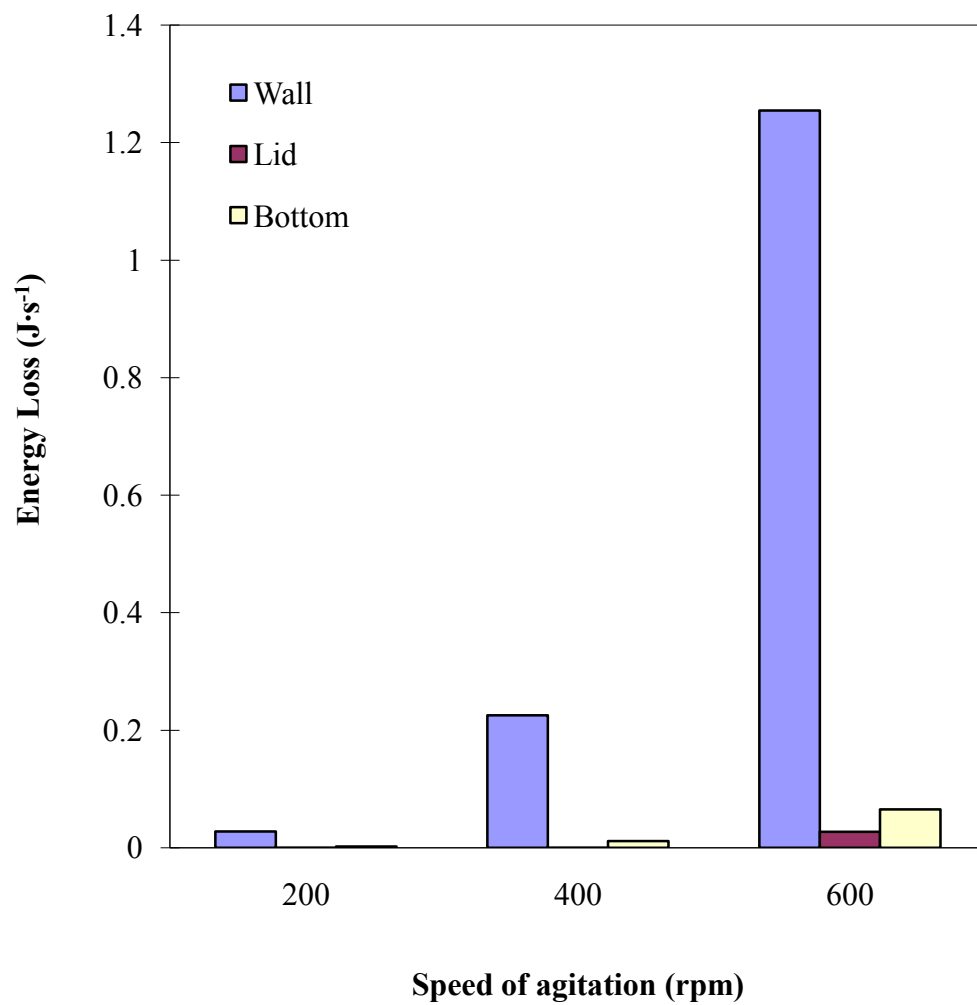
Figure 5.22   Energy loss through bioreactor components for trials without aeration.
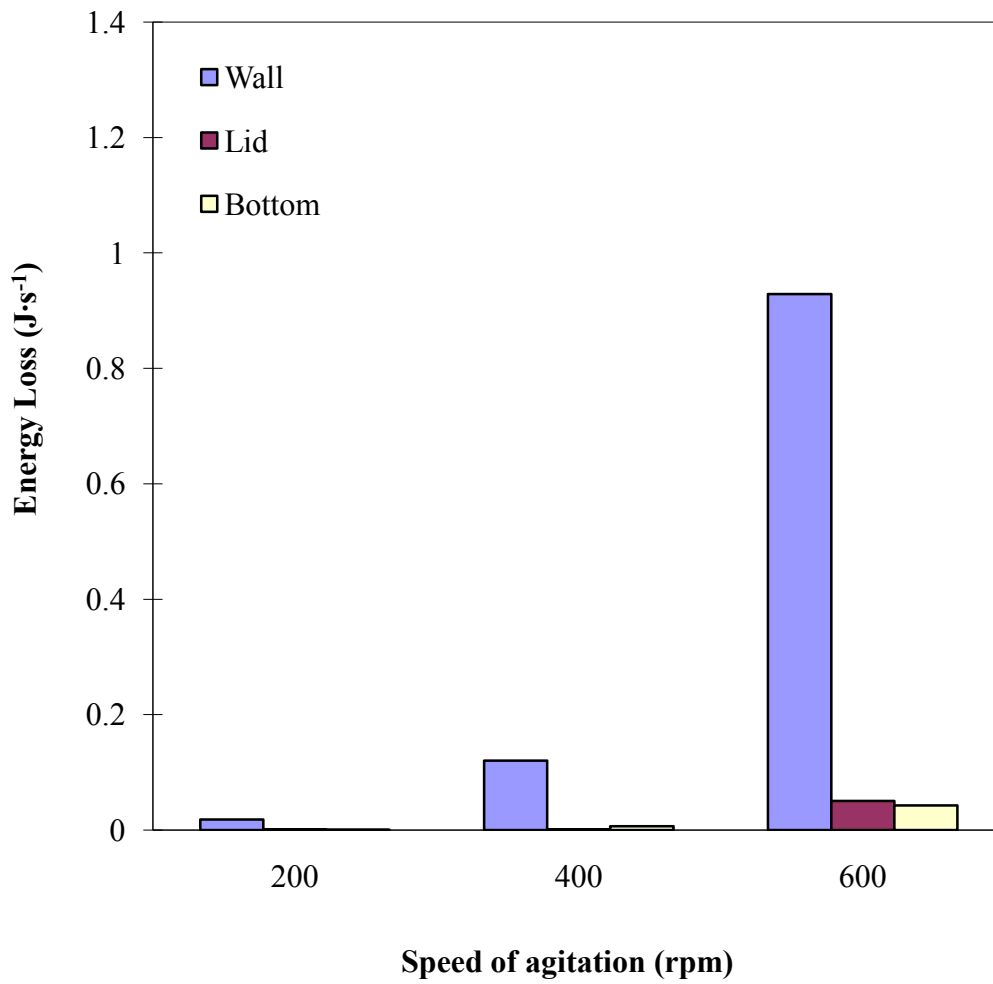
Figure 5.23   Energy loss through bioreactor components for trials with aeration.
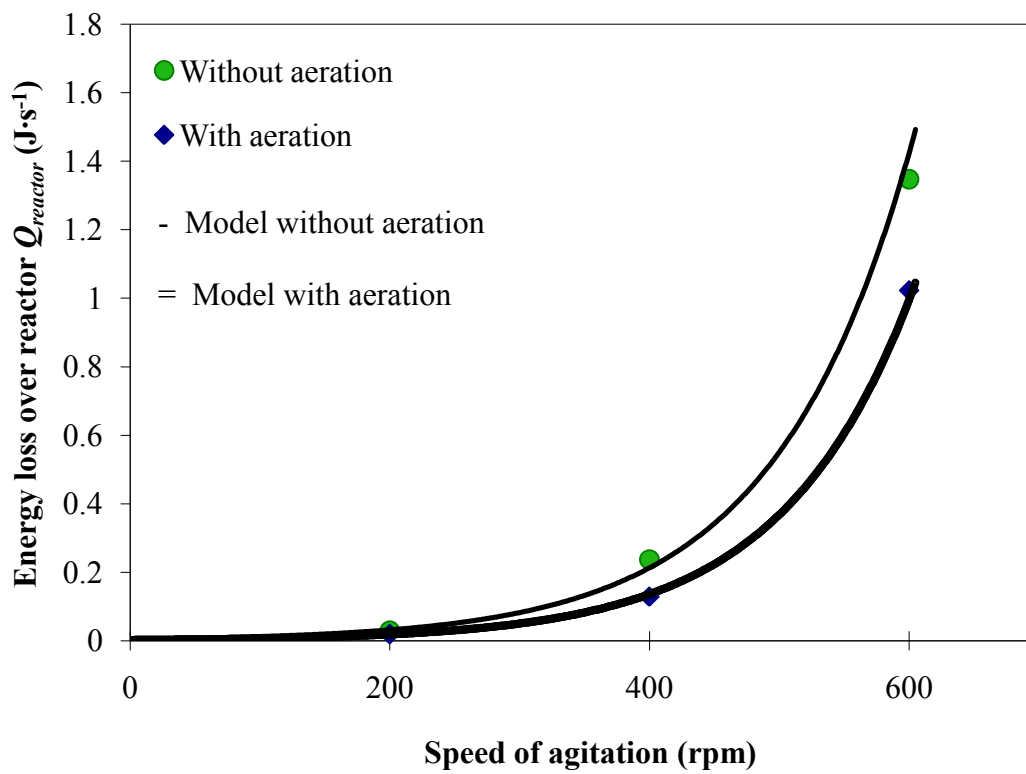
Figure 5.24 Modeled energy loss ($Q_{reactorloss}$) at several agitation speeds
for heat balance trials with and without aeration.

5.4.4 Bacterial Growth Trial

A trial with pH of 7, aeration at 2000 $cm^3 \cdot min^{-1}$ and 400 rpm agitation was conducted to determine the amount of energy produced by the bacteria in the bioreactor. The media was initially at ambient temperature and the water heating system was disabled. Growth was measured and modeled similarly to the operating parameter trials (Section 5.3) The model parameters and μ_{max} for this trial are shown in Table 5.12. Additionally, using Equation 23, the thermal generation of bacteria $Q_{metabolism}$ within the bioreactor was determined to be 2.281 $J \cdot s^{-1}$ from $Q_{reactorloss}$ after Q_{mix} and $Q_{exhaust}$ were taken into consideration and the correction factor ($K_{balance}$) was applied (Table 5.13). The temperature and energy generation ($Q_{reactorloss}$) during the growth trial are shown in Figure 5.25 while the growth and substrate consumption for the trial is depicted in Figure 5.26. These are discussed in Section 6.3.2.

Table 5.12   Growth model parameters for bacterial growth heat balance trial.

Parameter	Metabolism Trial
$N_{(inf)}$ (CFU·mL^{-1})	65595
B (hour^{-1})	-0.234
M (hour)	31.06
μ_{max} (hour^{-1})	0.136

Table 5.13   Energy lost through components of Microferm bioreactor
for trials with aeration and bacteria.

Agitation Speed (rpm)	Q_{total} (J·s^{-1})	$Q_{reactorloss}$ (J·s^{-1})	Calculated $Q_{metabolism}$* (J·s^{-1})	Corrected $Q_{metabolism}$ (J·s^{-1})
With Bacteria				
400	2.030	0.129	1.901	2.281

*$Q_{metabolism} = Q_{total} - Q_{reactorloss}$
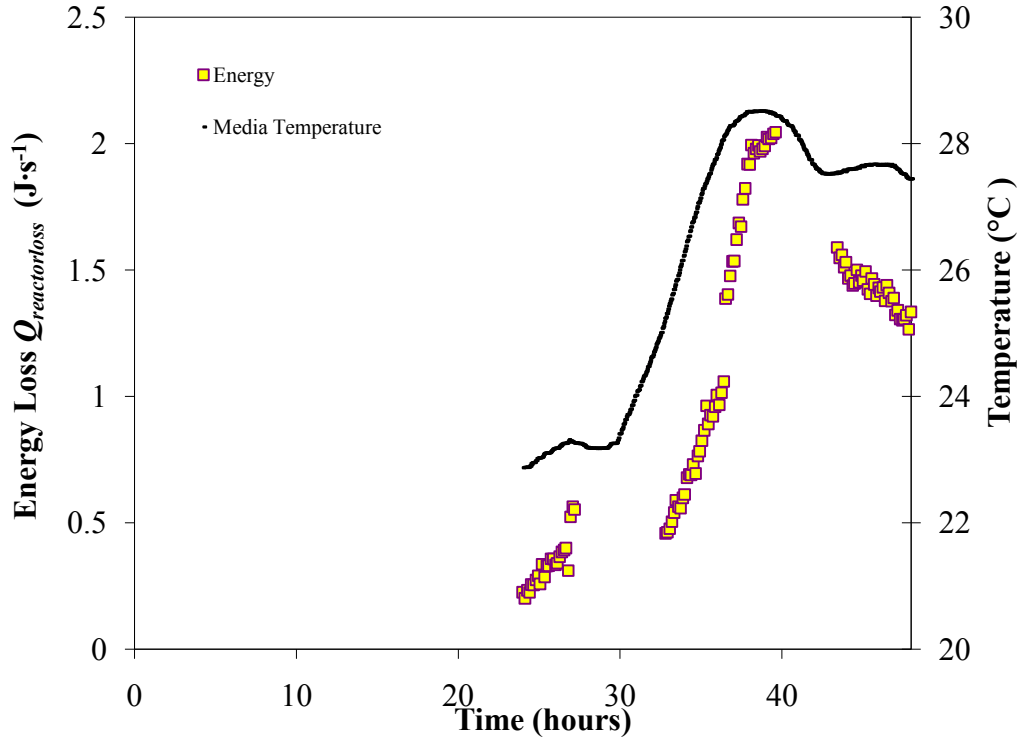
Figure 5.25 Temperature and energy generation within bioreactor.
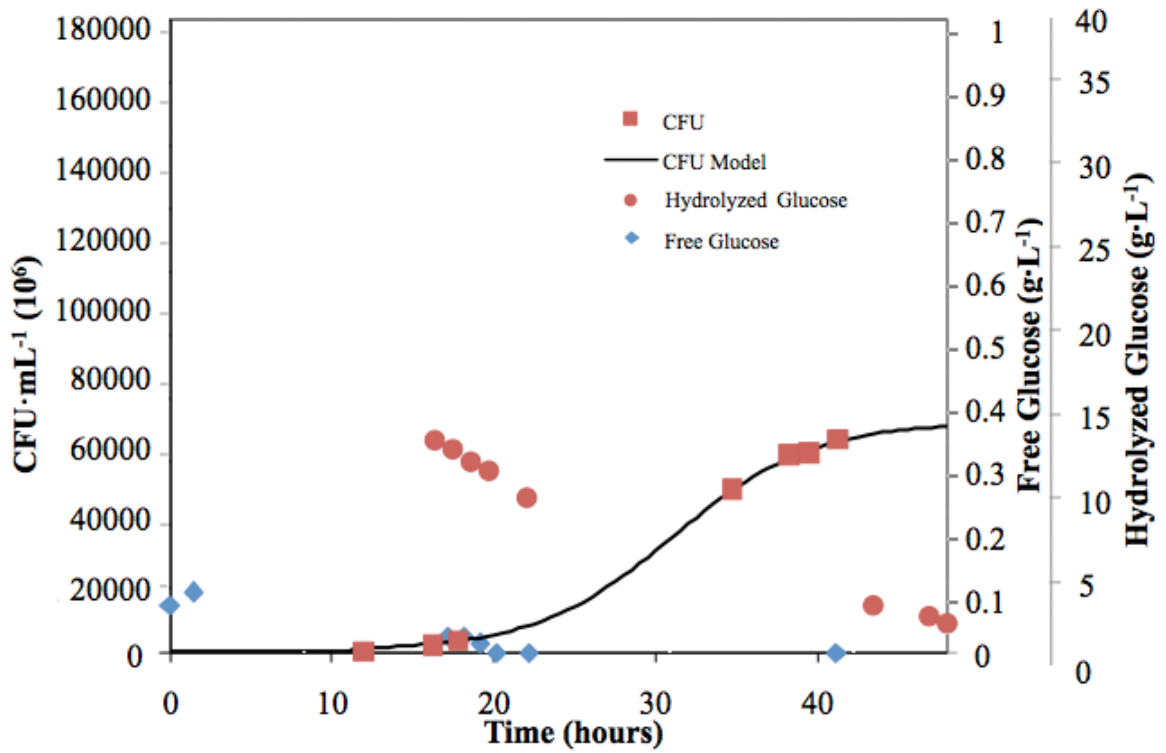
Figure 5.26 Bacterial growth and substrate consumption within bioreactor

5.4.5 Validation

In Table 5.14, the actual energy supplied to the bioreactor by the heater is shown with the calculated Q_{known} from Equation 23. To account for the operating parameters the Q_{known} was adjusted for $Q_{reactorloss}$ values previously determined in Section 5.4.1. By comparing the actual energy input with calculated Q_{known} the percentage of energy input that was predicted ranged from 81 to 87 % for the 1.5 and 3 $J \cdot s^{-1}$ trials, respectively. Therefore a $K_{balance}$ of 1.2 was used to correct the results of previous heat balance trials.

Table 5.14 Results of validation trial

Input Energy	Q_{total}	$Q_{reactorloss}$	Calculated Q_{known}*	Percentage Predicted†	$K_{balance}$
$(J \cdot s^{-1})$	$(J \cdot s^{-1})$	$(J \cdot s^{-1})$	$(J \cdot s^{-1})$	(%)	
0	0	0	0	100	1
1.5	1.34	0.13	1.21	81	1.2
3	2.73	0.13	2.60	87	1.2

†The percentage of known energy input calculated by the heat balance

*$Q_{known} = Q_{total} - Q_{reactorloss}$

CHAPTER 6 DISCUSSION

For this study, a pilot scale Microferm bioreactor was used to investigate the growth of *Streptomyces venezuelae.* Trials were completed in order to develop a standardized method of inoculation, to investigate the effect of temperature and pH on growth and to determine the metabolic heat produced by the bacteria. The results from these trials are discussed in the following sections.

6.1   Inoculum

A method to create a consistent inoculum for use in growth trials was developed. Using the exospores of *Streptomyces venezuelae* as an inoculum, the physiological state, age and stress of the initial cells was controlled. The spore preparation method (Figure 4.4) preserved the unigenomic exospores of *Streptomyces venezuelae* in a soft agar medium. According to Kutzner and Nitsch (1970), the soft agar suspension provides consistent spore dispersion and should remain viable up to one year. The suspension was easily manipulated through the use of auto-pipettes allowing the inoculums to be easily and aseptically introduced to the bioreactor.

6.1.1   Spore Sizing

Spores of *Streptomyces venezuelae* appeared to be rod-shaped with a smooth surface characteristic as defined by Dietz and Mathews (1970). Bradley and Ritzi (1968), reported a mosaic pattern on the surface of *Streptomyces venezuelae* that was not seen in any of the SEM images of the spore concentration (Section 5.2.1), however this was likely obscured by the gold coating applied to the sample prior to imaging. The average spore size was determined to be approximately 0.8 x 0.2 μm by SEM. This size is slightly smaller than the approximately 1 μm diameter round spores of *Streptomyces echinatus* and larger than the 0.6 μm diameter spiral shaped spores of *Streptomyces hygroscopicus* as reported by Dietz and Mathews (1970). Based on SEM, the size of *Streptomyces venezuelae* spores appears to be very regular (Figure 5.6), this finding is consistent with the results of Górny *et al.* (2003).

The Nanotrac particle size analyzer was used to confirm SEM size analysis and provide quick spore size estimation. In this system, a laser detects the effect of particle Brownian motion on light scattering while software determines approximate particle size and distribution. A key limitation of the Nanotrac instrument was that objects were assumed to be spherical for size calculations. As *Streptomyces venezuelae* spores are rod shaped this yielded a range of values that were averaged over the 5 minute sampling period. This resulted in a bell curve shaped size distribution between the length and diameter of the bacteria. Once this was taken into consideration, the spore sizes measured with the Nanotrac particle size analysis were consistent with the 0.8 x 0.2 μm dimensions determined through SEM analysis. While the Nanotrac instrument provided a quick assessment particle size within a medium it did not yield the shape or characteristics of the particles. For this analysis, SEM was necessary to yield information to verify the presence of rod-shaped spores.

6.1.2   Inoculum Validation

The validation trial with the spore inoculum indicates that the method provides a consistent inoculum by showing similar growth curves during identical shake flask experiments completed weeks apart (Section 5.2.2). The asymptotic standard deviation of the growth curve μ_{max} during these trials was 0.0013 hours^{-1} indicating the growth was consistent. A consistent lag time (*Lt*) of approximately 14 hours was also experienced throughout these trials. In addition, a consistent lag time was experienced during the bioreactor physiological repeatability trials (Section 5.3.1). These identical growth trials resulted in a 0.003 hours^{-1} asymptotic standard deviation of μ_{max}. While this is higher than the observed asymptotic standard deviation for μ_{max} between shake flask trials (0.0013 hours^{-1}), the deviation remains very low. Unfortunately other parameters cannot be compared between bioreactor and 250 mL shake flask growth trials due to differences in aeration, agitation and pH control. Specifically, aeration was not provided to the shake flasks and agitation was provided by swirling the flasks. These effects resulted in μ_{max} for the shake flask trials which was considerably lower (0.14 hour^{-1} vs. 1.43 hour^{-1}) than for the bioreactor trials with identical temperature and pH parameters.

6.2   Effects of Environmental Conditions

Before trials examining the effects of temperature and pH on *Streptomyces venezuelae* growth and substrate utilization were completed, the amount of variation between trials was determined. This was accomplished through the completion of three identical growth trials using the pilot scale bioreactor. Using the variation between these trials, the effects of manipulated parameters could be assessed (Section 5.3.1). The three identical repeatability trials were completed using media at 32 ℃, neutral pH, maximum dissolved oxygen saturation and agitated at 400 rpm. The results of the three trials indicate that the standard deviation for μ_{max} between growth trials was very low (0.003 hours^{-1}) as shown in Table 5.2 indicating that these trials showed good repeatability. The standard deviation of the free glucose consumption rate (μ_g) between trials was 0.138 hours^{-1}, which was much higher than the standard deviation of μ_{max} (0.003 hours^{-1}). However, the rate of consumption was much higher (Table 5.6) as observed in Figure 5.13 and therefore more susceptible to error based on sampling frequency as fewer points could be obtained during the rapid decrease in free glucose.

6.2.1   Temperature

The effect of temperature on *Streptomyces venezuelae* growth and activity was examined by modifying the temperature of the bioreactor as the manipulated variable during three otherwise identical growth trials completed at 28, 32 and 36 ℃. Of the tested temperatures, the most favorable for growth was 32 ℃, at this temperature the μ_{max} was highest (1.434 hours^{-1}) with a short average lag time of 7.7 hours. This resulted in the most bacteria produced in the least amount of time. These results are somewhat surprising as several *Streptomyces venezuelae* researchers often enumerate the bacteria at lower temperatures of 28 - 30 °C (Wang and Vining, 2003 and Jakeman *et al.*, 2006). While growth at 36 ℃ initially had a lower *Lt*, the reduced μ_{max} and lower number of bacteria produced offset this advantage (Table 5.7). The sweet smelling odor produced during this trial may be due to secondary metabolite production initiated through heat shock (Hopwood, 2007).

During growth trials, the sugars in the MYM media supplied carbon and energy for cell growth and respiration. Table 5.4 indicated that although the majority of sugars were in the form of maltose, detectable amounts of glucose were present in the media from the malt extract. Therefore, both free and hydrolyzed glucose levels were measured where "free glucose" represents the glucose present as impurities from the malt extract and hydrolyzed glucose represents free glucose with the addition of glucose from hydrolyzed maltose and any other complex sugars incorporating glucose in their structure.

Figure 5.15, which shows hydrolyzed glucose consumption over time indicated that for all temperatures, there is a fairly constant level of hydrolyzed glucose (11-16 $g \cdot L^{-1}$) between hours 0 - 10 of the growth trials. After the 10th hour, the amount of hydrolyzed glucose begins to decline in a manner that seems related to growth (Figure 5.14). Figure 5.16 shows that the free glucose follows a sigmoidal consumption curve. In this graph there appears to be high variation in the amount of free glucose present, however the variation is only ± 0.075 $g \cdot L^{-1}$. This variation may be due to measurement errors in media preparation or the measurement precision of the sugar analyzer. The rate of substrate consumption during temperature trials (Table 5.8) as represented by μ_g appears sigmoidal and ranges from -1.2 to -1.0 $hours^{-1}$. However the consumption of free glucose was very rapid and the sampling frequency of 1 hour was likely insufficient to model this parameter, resulting in a standard deviation of 0.138 $hours^{-1}$. As all substrate consumption rates fall within this deviation, μ_g was not used as a point of comparison between trials. However the substrate lag time (*SLt*) was found to be shorter at higher temperatures with values of 10, 7.8 and 6.9 hours obtained at 28, 32, and 36 ℃ respectively. This parameter indicated when the bacteria had reached numbers sufficient to detect their effect on the substrate and always coincided with their transition into exponential growth phase (Figure 5.14). Comparison of the bacterial growth and free glucose consumption graphs (Figure 5.14 and 5.16 respectively) shows the same characteristic behavior for all temperatures. As the bacteria transitioned through the exponential growth phase, there is an increase in the rate of free and hydrolyzed glucose consumption. This trend appears to be affected by temperature, as at 28 ℃ the initial

transition occurs at approximately 11 hours where at 32 and 36 ºC this occurs at approximately 8 hours. From the hydrolyzed glucose results taken over the duration of a growth experiment (Figure 5.15), the hydrolyzed glucose decreases once the free glucose is fully consumed. This indicates that on *Streptomyces venezuelae* preferably utilizes glucose over maltose. This may be because it is not necessary for *Streptomyces venezuelae* to produce the enzyme maltase when metabolizing glucose. A study by Glazebrook *et al*. (1990) compared the growth *Streptomyces venezuelae* on MYM media to growth and sporulation on Glucose, Yeast-Extract, Malt-Extract (GYM) media and found that MYM media was far superior for bacterial growth. It was hypothesized that this was due to the high formation of acids when grown in glucose-based medium. These results are interesting as glucose was shown by Jakeman *et al.* (2006) to be superior to maltose during jadomycin B production (Section 3.3.4).

6.2.2 pH

To assess the effect of acidic, neutral and alkaline conditions on *Streptomyces venezuelae*, growth trials were completed at pH levels of 5, 7 and 9. As normal *Streptomyces* growth and activity results in lowered media pH, 6 M NaOH was added to the bioreactor to counteract this and maintain the selected pH. Of the tested pH, growth was most favored at a media pH of 7. This result is supported through the work of Kontro *et al*. (2005) who found that most species of *Streptomyces* grew optimally at neutral pH when yeast extract was used as a substrate. The results of trials at pH of 9 indicate alkaline conditions reduced the growth rate (μ_{max}) of *Streptomyces venezuelae* from 1.43 to 1.18 hours^{-1} as well as total number of bacteria produced during the trial (Figure 5.18). As shown in Table 5.9 the P-value for the trial at a pH of 9 was 0.0002 indicating significance between this trial and those conducted at a pH of 7. With acidic media conditions at pH of 5, growth was not detected for the duration of the experiment. Without detected growth, this trial was deemed a failure. It is possible that the growth of bacteria was completely inhibited by the acidic conditions.

While the growth rate appeared retarded by alkaline conditions, the consumption of free and hydrolyzed glucose was less affected (Figure 5.19 and 5.20 respectively). The

higher pH of 9 also seems to have had a less pronounced effect on the Lt and SLt as the values were within an hour of those at neutral pH (Tables 5.10 and 5.11 respectively). Figure 5.21 shows the growth and consumption of sugars during the trial at pH 9, although the rates are reduced each variable behaves in a similar manner to the validation trials at pH 7. As in the temperature trials the free glucose was consumed at the onset of each growth trial as seen in Figure 5.21. It was observed that the consumption of free glucose preceded both detectable consumption of hydrolyzed glucose and exponential growth.

6.3   Heat Balance

It was determined that the amount of heat generated by the pilot scale bioreactor is significant and can be predicted through the heat balance model. The energy produced by bacteria within bioreactor was also determined and should be taken into consideration during future growth trials involving *Streptomyces venezuelae*.

6.3.1   Heat Generated Through Bioreactor Operating Conditions

The energy generation within the bioreactor due to agitation (Q_{mix}) was found to be 0.03 – 1.62 J·s^{-1} at 200 – 600 rpm with the majority (91 - 94 % of $Q_{reactorloss}$) being lost through the walls of the reactor (Figures 5.22 and 5.23). The amount of energy lost through the reactor components ($Q_{reactorloss}$) during heat balance trials 1 and 2 is summarized in Figure 5.24 and Table 5.11. From Figure 5.24 it is evident that the energy generated through mixing increases exponentially with agitation speed. The exponential fits to this data (Equations 31-32) results in an R squared value of 0.99-1.0 indicating a good fit. A similar study performed by Burdock *et al.* (2008) on a 17 L pilot scale bioreactor determined the energy generation for 10 L of media agitated at 250 rpm to be 4.9 J·s^{-1}. Differences in bioreactor design prohibit the direct comparison of energy generation, however the similar magnitude and proportion of aeration loss at high energy levels agree with the findings of this study. Specifically Burdock *et al.* (2008) reported that at 4.9 J·s^{-1} (26.5 % of Q_{total}) of the energy was lost with aeration while this study found that the proportion of energy lost through aeration increased with agitation speed from 33 % at 0.03 J·s^{-1} (200 rpm) to 24 % at 1.62 J·s^{-1} (600 rpm). Repeated tests indicated that changes

in ambient room temperature did not affect the energy generation within the bioreactor as predicted by the heat balance.

6.3.2 Heat Generated by *Streptomyces Venezuelae*

The metabolism of sugars by *Streptomyces venezuelae* is an exothermic reaction from which heat was released. The heat energy produced was determined through the utilization of the heat balance model performed on the Microferm bioreactor. Previous heat balance trials examined the amount of heat generated through agitation of the media (Q_{mix}). As the amount of thermal generation through agitation within the bioreactor was known, the excess thermal generation was attributed to the metabolism and activity of *Streptomyces venezuelae* ($Q_{metabolism}$).

Figure 5.26 shows that the heat generated within the bioreactor continued to rise until the bacteria entered stationary phase. The heat produced by *Streptomyces venezuelae* in 5 L of media was measured to be 2.281 $J \cdot s^{-1}$ at approximately 60×10^9 $CFU \cdot mL^{-1}$. When combined with the energy generated through mixing (Q_{mix}) and the energy lost through exhaust ($Q_{exhaust}$), this energy was sufficient to bring the temperature of the media from 24.6 °C to 28.5 °C. The heat generated by the bacteria continued to increase with bacterial numbers until the exponential growth phase ended. The stationary growth phase likely coincides with low hydrolyzed glucose levels within the media. As steady state is required for the heat balance to yield accurate results, only the peak energy production was reported once the bacteria had entered into steady state for a period of one hour (Figure 5.26). The μ_{max} of bacteria within the reactor for this trial was calculated to be 0.136 $hour^{-1}$ (Table 5.12) and was considerably lower than the other growth trials. This was likely due to the lack of temperature control throughout the trial as lower temperatures yielded a lower μ_{max} as detailed in Section 6.2.1. The total number of bacteria produced during the trial was similar to the population achieved during the 28 °C growth trial (Figure 5.14).

The thermal generation by *Streptomyces venezuelae* was dependent on the number of cells and increased with population until the bacteria entered stationary phase (Figure 5.26). Work by Ghaly *et al.* (1992) on the heat generated through mixing and metabolism

during lactose fermentation by *Kluyveromyces fragilis* shows a very similar curve (Figure 3.9). The thermal generation through mixing and microbial growth increased at a near constant rate in both studies while the microbes were in the exponential growth phase (Figures 5.26 and 3.9). When the microbes entered the stationary phase, the thermal energy generation stabilized and a constant temperature was held until the microbes entered the death phase at which time the temperature began to fall. In both studies, the microbes entered the stationary phase when the primary substrate was nearly consumed (Figures 5.26 and 3.9). Both *Kluyveromyces fragilis* and *Streptomyces venezuelae* growth resulted in increased temperature of the media. The reported $Q_{metabolism}$ of *Streptomyces venezuelae* (2.281 J·s^{-1} with 60x10^9 CFU·mL^{-1}) was calculated during the steady state conditions experienced during temperature stabilization. This is the maximum thermal generation over the course of the fermentation. This heat generation is reported as the heat balance is only accurate at steady state conditions as explained in Section 3.4 due to the energy storing capacity of bioreactor components and the media.

6.3.3   Heat Balance Validation Trial

During the heat balance validation trial a discrepancy was found between the energy supplied to the bioreactor and the energy calculated using the theoretical model. A discrepancy was expected as the model relied on ideal conditions, materials and in some cases simplified parameters. For example, the lid was modeled as a flat plate however, the actual plate contained numerous plugs and areas of variable thickness. Additionally, the heat balance relied upon temperature measurements from fixed positions on and around the bioreactor. It was assumed that these temperatures were representative of the bioreactor area however, there was likely variation across the surface resulting in variable heat flux. Finally, the baffles and agitation shaft were likely sinks of thermal energy, however these were not included in the model. These additional sinks of thermal generation likely contributed to the discrepancy observed in the validation trial. A discrepancy of approximately 20 percent between the actual energy generation and the modeled heat transfer was found which was significant, however a large discrepancy was expected as the balance relied on ideal conditions and several assumptions. When the

metabolism of bacteria and heat losses over the bioreactor was reported in previous sections the energy was corrected to reflect the discrepancy found during the validation trial.

CHAPTER 7 CONCLUSION

The soft agar spore preparation developed during this study was used as a consistent inoculum for *Streptomyces venezuelae* growth trials. Using SEM images captured during the development process, the size of *Streptomyces venezuelae* spores was determined to be 0.8 x 0.2 μm with a smooth surface characteristic. Repeatability trials show that the standardized spore suspension was successful in providing bacterial growth with similar lag time of approximately 14 hours when grown in shake flasks.

The effects of temperature on the growth and substrate consumption by *Streptomyces venezuela*e in a bioreactor show statistically significant effects. Media temperature maintained at 28 and 36 °C resulted in slower growth rates compared to 32 °C (μ_{max} of 1.24 and 1.26 hours^{-1} compared to 1.43 hours^{-1}, respectively). The growth trial at 36 °C resulted in a shorter lag time (*Lt*) than at 28 and 32 °C, however the higher temperature also resulted in a lower overall bacterial population than at 32 °C. Additionally, at the higher temperature the media began to emit a sweet odor, possibly indicating heat stress and the production of secondary metabolites. Growth at 32 °C is considered the most favorable due to the high μ_{max} observed and total number of bacteria generated. The effect of temperature on the consumption of free glucose during these trials seemed to be related, as the initiation of exponential growth was always preceded by the rapid consumption of free glucose. At 28 °C the consumption of free glucose was not detected until 10.5 hours after inoculation while at 32 °C the *SLt* was 7.8 hours. During each trial the growth and consumption of sugar followed typical behavior that was consistently repeated. In every trial the free glucose was consumed before the maltose, which was the major sugar substrate in the media.

The pH of the media in the bioreactor also affected the growth rate of *Streptomyces venezuela*e. At pH 7, the μ_{max} of *Streptomyces venezuelae* was the most favorable of the tested pH levels. In alkaline conditions (pH of 9) the μ_{max} was lower than during trials at a pH of 7 as was the total number of bacteria attained. The substrate lag time (*SLt)* for alkaline conditions was nearly identical to the *SLt* at neutral pH. In acidic

media conditions (pH of 5), the growth trial was determined to have failed, as there were no detectable changes to the media over the duration of the experiments.

The heat balance model was used to calculate the amount of thermal energy generated by the bioreactor during normal operating parameters at steady state. The amount of energy generated within the reactor through agitation (Q_{mix}) was found to be dependent upon agitation speed and was found to be $0.03 - 1.616$ J·s^{-1} at $200 - 600$ rpm. The heat balance can be used to predict how a change in operating parameters or physical characteristics of the bioreactor will affect the amount of heat lost from the system. Heat losses from the bioreactor occurred over the walls (91-96 % of $Q_{reactorloss}$), top (0 - 5 %) and bottom (4 - 6 %). Losses from the exhaust gases ($Q_{exhaust}$) were typically 33 to 24 % of Q_{total} and were dependent upon agitation speed.

The maximum heat of metabolism produced by *Streptomyces venezuelae* in 5 L of media was measured to be 2.281 J·s^{-1}. This energy generation combined with heat generated through agitation was sufficient to bring the temperature of the media to 28.5 °C from 24.6 °C for a period of one hour. The net energy production by bacteria is greater than the energy generated through agitation and must be taken into consideration in the design of bioreactors intended to work with *Streptomyces venezuelae.*

Although this study has examined several key areas of *Streptomyces venezuelae* growth, there are still many difficulties to overcome in the scaling up of biomass and jadomycin production. The bioreactor modifications can be used to support additional experiments with *Streptomyces venezuelae* as suggested below.

As discussed in Section 4.4.1, the media temperature directly affects the growth of bacteria, and every species has an ideal temperature for growth that is influenced by its physiology (Shuler, 1992). The work completed in this study found that when grown at 28, 32 and 36 ºC, the growth of *Streptomyces venezuelae* was most favorable at 32 ºC. As temperature was shown to have a large effect on the growth and behavior of *Streptomyces venezuelae,* the author recommends additional temperature trials at 30 and 34 ºC to further study the effect of temperature on *Streptomyces venezuelae* growth.

When scaling up bioreactor volume, it becomes necessary to introduce agitation to maintain homogeneity within the system. Mechanical agitation is often used, as it is easily adjusted and facilitates high rates of diffusion (Shuler, 1992). Additionally increased agitation creates shear forces within the media. Shear disperses bubbles, increases submersion time and reduces bubble size thereby facilitating gas availability to cells (Shuler, 1992). While often necessary in larger systems, agitation can also be detrimental to cell growth when the shear rate causes physical damage to cells. Prior to increasing antibiotic production the effect of agitation on *Streptomyces venezuelae* growth and antibiotic production should be examined in detail.

This study was based upon a series of batch processes where all substrate was introduced at the beginning of the trial. In continuous processes nutrients are continuously added to a system while cells and products are removed. There are several types of continuous processing techniques including fed batch with intermittent feeding regimes, chemostat reactor designs with constant chemical environment and liquid levels, and turbidostat reactor designs with constant liquid and biomass volume (Doran, 1995). In a continuous process the cell cultures are maintained for long periods of time. The process will often enter a state of equilibrium that can be exploited for the maximum yield of product with the minimal substrate requirement. It may be possible engineer a continuous process for jadomycin production involving continuous growth of *Streptomyces venezuelae.* Such a process would enable biomass or products to be extracted continuously.

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```
//Program written by Andrew MacIntosh to monitor 7 or 8 thermistors, media dissolved
//oxygen concentration, control a heater, and perform HB calculations.
//Contact at ajmacint@dal.ca for questions
#include <NIDAQmx.h>
#include <ansi_c.h>
#include <cvirte.h>
#include <userint.h>
#include "THERM.h"
#include "DAQanaloginProject.h"
#include "DAQanalogOUTfivevolts.h"
#include "DAQTaskInProject.h"
#include "DAQTaskInProjectdigitalrelaycontrol.h"
//global variables
static int thermpanel;TaskHandle analogtaskhandle;TaskHandle analogtaskhandleout;TaskHandle
digitalportone;
TaskHandle digitalportzero;int
STARTFLAG=0;int num_sample;
double count=1double mastercount=1;double relaycount=0;double relaydelay;double heatdelay;double
watts;
double UBOT, UWALL, UWALLA, UWALLU, ULID, UEX, UTOTAL, QBOT, QWALL, QWALLA,
QWALLU, QLID, QEX, QTOTAL, HBOT, HWALL, HWALLA, HWALLU, HLID, HEX, HTOTAL,
AIRd;
FILE * athermrecord;
char USERCOMMENTS[100] = {"Experiment three heat balance bioreactor"};
char comment[101];char print1[101]= {"Warming all sensors"};;char print2[101]= {"Preparing and reading
bank T1 & T5"};char print3[101]= {"Preparing and reading bank T2 & T6"};char print4[101]= {"Preparing
and reading bank T3 & T7"};char print5[101]= {"Preparing and reading bank T4 & T8"};char print6[101]=
{"cooling all sensors"};
 //double variables
double inputarray[4];double analogout[2];double tracers[10];uInt8 port1[3];uInt8 port0[2];
double CALT1,CALT2; //calibration vars
double DOV1, PHV1, DO1, DO2;//dissolved oxygen vars
double V0, V1, V2, V3, V4, V5, V6, V7, V8;//temp voltage/inputed values
double T0, T1, T2, T3, T4, T5, T6, T7, T8;;//calculated temperatures from voltages
```

```
double T1SUM=0;double T2SUM=0;double T3SUM=0;double T4SUM=0;double T5SUM=0;double
T6SUM=0;
double T7SUM=0;double T8SUM=0;
double V1SUM=0;double V2SUM=0;double V3SUM=0;double V4SUM=0;double V5SUM=0;double
V6SUM=0;
double V7SUM=0;double V8SUM=0;
int SUMON;
double meanmon=0, summon1=0, summon2=1,sumrun=0, MMT,cyclek,
average1=0,average2=0,average3=0,average4=0,average5=0,average6=0,average7=0,average8=0,average9
=0,average10=0,average11=0,average12=0,average13=0,average14=0,average15=0,average16=0,average1
7=0,average18=0,average19=0,average20=0;
//prototypes
void usbinit(void);
void hbcalculations(void);
//main program
int main (int argc, char *argv[])
{
        if (InitCVIRTE (0, argv, 0) == 0)
                return -1;          /* out of memory */
        if ((thermpanel = LoadPanel (0, "THERM.uir", THERMPANEL)) < 0)
                return -1;
        usbinit();
        //hbcalculations();
        SetCtrlVal (THERMPANEL, THERMPANEL_COMMENT_2, USERCOMMENTS);
        DisplayPanel (thermpanel);
        RunUserInterface ();
        DiscardPanel (thermpanel);

        return 0;
}
/*---------------------------------OFF BUTTON---------------------------------------------*/
int CVICALLBACK OFF_CB (int panel, int control, int event,
                void *callbackData, int eventData1, int eventData2)
{switch (event)
        {case EVENT_COMMIT:
        STARTFLAG=0;
```

```
                analogout[0]=0;
                analogout[1]=0;
        DAQmxWriteAnalogF64 (analogtaskhandleout, 1, 0, 2, DAQmx_Val_GroupByChannel,
analogout, &num_sample, 0);
                        port1[0] = 1; //inhibit
                        port1[1] = 0; //B
                        port1[2] = 0; //A
                        DAQmxWriteDigitalLines (digitalportone, 1, 1, 2,
DAQmx_Val_GroupByChannel, port1, &num_sample, 0);
                        port0[0] = 1; //Relay
                        port0[1] = 1; //Relay
                        DAQmxWriteDigitalLines (digitalportzero, 1, 1, 2,
DAQmx_Val_GroupByChannel, port0, &num_sample, 0);
        SetCtrlVal (THERMPANEL, THERMPANEL_LED, 0);
                        break;
        }return 0;
}
/*---------------------------------ON BUTTON----------------------------------------*/
int CVICALLBACK ON_CB (int panel, int control, int event,
                void *callbackData, int eventData1, int eventData2)
{switch (event)
        {case EVENT_COMMIT:
        STARTFLAG=1;
        port0[0] = 1; //Relay
        port0[1] = 1; //Relay
        DAQmxWriteDigitalLines (digitalportzero, 1, 1, 2, DAQmx_Val_GroupByChannel, port0,
&num_sample, 0);


        SetCtrlVal (THERMPANEL, THERMPANEL_LED, 1);
                        break;
        }return 0;
}
/*---------------------------------EXIT BUTTON----------------------------------------*/
int CVICALLBACK EXIT_CB (int panel, int control, int event,
                void *callbackData, int eventData1, int eventData2)
{switch (event)
```

```
{case EVENT_COMMIT:
                STARTFLAG=0;
                port1[0] = 1; //inhibit
                port1[1] = 0; //B
                port1[2] = 0; //A


                DAQmxWriteDigitalLines (digitalportone, 1, 1, 2,
DAQmx_Val_GroupByChannel, port1, &num_sample, 0);
                port0[0] = 1; //Relay
                port0[1] = 1; //Relay
                DAQmxWriteDigitalLines (digitalportzero, 1, 1, 2,
DAQmx_Val_GroupByChannel, port0, &num_sample, 0);
                analogout[0]=0;
                analogout[1]=0;
                DAQmxWriteAnalogF64 (analogtaskhandleout, 1, 0, 2,
DAQmx_Val_GroupByChannel, analogout, &num_sample, 0);
                fclose (athermrecord);
                DAQmxClearTask (analogtaskhandle);
                DAQmxClearTask (analogtaskhandleout);
                DAQmxClearTask (digitalportone);


                QuitUserInterface (0);
                break;
        }return 0;
}
int CVICALLBACK Tick_CB (int panel, int control, int event,
                void *callbackData, int eventData1, int eventData2)
{switch (event)
        {case EVENT_TIMER_TICK:


                if (STARTFLAG==1)
                        {
                        SetCtrlVal (THERMPANEL, THERMPANEL_COUNTY, count);
                        SetCtrlVal (THERMPANEL, THERMPANEL_COUNTY_2,
mastercount);
                        /*------Initilize--------------------------*/
                        if (count>0 && count<2)
```

```
                    {
//printf ("Warming all sensors, \n");
SetCtrlVal (THERMPANEL, THERMPANEL_COMMENT_2,
print1);

analogout[0]=5;
DAQmxWriteAnalogF64 (analogtaskhandleout, 1, 0, 2,
DAQmx_Val_GroupByChannel, analogout, &num_sample, 0);
port1[0] = 1; //inhibit
port1[1] = 0; //B
port1[2] = 0; //A
DAQmxWriteDigitalLines (digitalportone, 1, 1, 2,
DAQmx_Val_GroupByChannel, port1, &num_sample, 0);
SetCtrlVal (THERMPANEL, THERMPANEL_WARMING, 1);
SetCtrlVal (THERMPANEL, THERMPANEL_COOLING, 0);
                    }
/*-------T8&4---------------------------*/
if (count>10 && count<12)
                    {
//printf ("Preparing bank T1 & T5, \n");
SetCtrlVal (THERMPANEL, THERMPANEL_COMMENT_2,
print2);

port1[0] = 0; //inhibit o on 1 off
port1[1] = 1; //B
port1[2] = 0; //A
DAQmxWriteDigitalLines (digitalportone, 1, 1, 2,
DAQmx_Val_GroupByChannel, port1, &num_sample, 0);
                    }
if (count>15 && count<36)
                        {
//printf (" %lf \n", count);
DAQmxReadAnalogF64 (analogtaskhandle, 1, 2.0,
DAQmx_Val_GroupByChannel, inputarray, 4, &num_sample, 0);
SetCtrlVal (THERMPANEL, THERMPANEL_READING, 1);
SetCtrlVal (THERMPANEL, THERMPANEL_WARMING, 0);
V1=     inputarray[3];
V5= inputarray[2];
```

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```
                                    T8= -0.6178*V1*V1 - 16.52*V1 + 27.225; //-16.4*V1 + 26.957;//-
16.146*V1+27.395;

                                    T4=  0.5076*V5*V5 - 15.446*V5 + 26.762;//-16.642*V5 + 27.253;//-
16.146*V5+27.395;

                                    //T1=-14.678*V1 + 50.328;
                                    //T5=-15.102*V5 + 51.045;
                                    //T1= -15.813*V1 + 53.325;
                                    //T5=-15.977*V5 + 53.542;
                                    //T1= -15.813*V1 + 52.705;
                                    //T5=-15.977*V5 + 53.542;
                                    tracers[0]=T8 ;
                                    tracers[4]=T4;
                                    SetCtrlVal (THERMPANEL, THERMPANEL_T8, T8);
                                    SetCtrlVal (THERMPANEL, THERMPANEL_T4, T4);
                                    SetCtrlVal (THERMPANEL, THERMPANEL_LED_8, 1);
                                    SetCtrlVal (THERMPANEL, THERMPANEL_LED_4, 1);
                                            }
                                    /*--------------------------------*/
                                     if (count>35 && count<37)
                                            {
                                    //printf ("Preparing bank T2 & T6, \n");
                                    SetCtrlVal (THERMPANEL, THERMPANEL_COMMENT_2,
print3);
                                    SetCtrlVal (THERMPANEL, THERMPANEL_LED_8, 0);
                                    SetCtrlVal (THERMPANEL, THERMPANEL_LED_4, 0);
                                    port1[0] = 0; //inhibit o on 1 off
                                    port1[1] = 0; //B
                                    port1[2] = 1; //A
                                    DAQmxWriteDigitalLines (digitalportone, 1, 1, 2,
DAQmx_Val_GroupByChannel, port1, &num_sample, 0);
                                            }
                                    if (count>40 && count<61)
                                            {
                                    //printf (" %lf \n", count);
                                    DAQmxReadAnalogF64 (analogtaskhandle, 1, 2.0,
DAQmx_Val_GroupByChannel, inputarray, 4, &num_sample, 0);
                                    V2=  inputarray[3];
```

```
V6=      inputarray[2];
T7=  0.8726*V2*V2 - 15.311*V2 + 26.733;//-16.309*V2 + 27.523;//-
16.146*V2+27.395;

T3= 1.8405*V6*V6 - 13.478*V6 + 26.348;
//T2= -15.274*V2 + 51.459;
//T6= -15.81*V6 + 52.462;
//T2=-16.041*V2 + 53.774;
//T6= -15.661*V6 + 53.187;
//T2=-16.041*V2 + 53.774;
//T6= -15.661*V6 + 52.687;
tracers[1]= T7;
tracers[5]= T3 ;
SetCtrlVal (THERMPANEL, THERMPANEL_T7, T7);
SetCtrlVal (THERMPANEL, THERMPANEL_T3, T3);
SetCtrlVal (THERMPANEL, THERMPANEL_LED_7, 1);
SetCtrlVal (THERMPANEL, THERMPANEL_LED_3, 1);
        }
/*----------------------------------*/
if (count>60 && count<62)
        {
//printf ("Preparing bank T3 & T7, \n");
SetCtrlVal (THERMPANEL, THERMPANEL_COMMENT_2,
print4);

SetCtrlVal (THERMPANEL, THERMPANEL_LED_7, 0);
SetCtrlVal (THERMPANEL, THERMPANEL_LED_3, 0);
port1[0] = 0; //inhibit o on 1 off
port1[1] = 0; //B
port1[2] = 0; //A
DAQmxWriteDigitalLines (digitalportone, 1, 1, 2,
DAQmx_Val_GroupByChannel, port1, &num_sample, 0);
        }
if (count>65 && count<86)
        {

//printf (" %lf \n", count);
DAQmxReadAnalogF64 (analogtaskhandle, 1, 2.0,
DAQmx_Val_GroupByChannel, inputarray, 4, &num_sample, 0);
```

```
                          V3=     inputarray[3];
                          V7=     inputarray[2];
                          T6= 2.0272*V3*V3 - 13.142*V3 + 25.246;//-16.282*V3 + 27.396;//-
16.146*V3+27.395;

                          T2= -16.145*V7 + 27.178;//-16.146*V7+27.395;
                          //T3=18;//-15.335*V3 + 51.774;
                          //T7=-15.93*V7 + 52.61;
                          //T3=-16.25*V3 +53.85;
                          //T7=-16.215*V7 + 54.037;
                          //T3=-15.335*V3 + 51.774;
                          //T7=-16.215*V7 + 54.037;
                          tracers[2]= T6;
                          tracers[6]= T2 ;
                          SetCtrlVal (THERMPANEL, THERMPANEL_T6, T6);
                          SetCtrlVal (THERMPANEL, THERMPANEL_T2, T2);
                          SetCtrlVal (THERMPANEL, THERMPANEL_LED_6, 1);
                          SetCtrlVal (THERMPANEL, THERMPANEL_LED_2, 1);
                                 }
                          /*---------------------------------*/
                          if (count>85 && count<87)
                                 {
                          //printf ("Preparing bank T4 & T8, \n");
                          SetCtrlVal (THERMPANEL, THERMPANEL_COMMENT_2,
print5);
                          SetCtrlVal (THERMPANEL, THERMPANEL_LED_6, 0);
                          SetCtrlVal (THERMPANEL, THERMPANEL_LED_2, 0);
                          port1[0] = 0; //inhibit o on 1 off
                          port1[1] = 1; //B
                          port1[2] = 1; //A
                          DAQmxWriteDigitalLines (digitalportone, 1, 1, 2,
DAQmx_Val_GroupByChannel, port1, &num_sample, 0);
                                  }
                          if (count>90 && count<111)
                                  {
                          //printf (" %lf \n", count);
                          DAQmxReadAnalogF64 (analogtaskhandle, 1, 2.0,
DAQmx_Val_GroupByChannel, inputarray, 4, &num_sample, 0);
```

```
                              V4=     inputarray[3];
                              V8=     inputarray[2];
                              T5=0.889*V4*V4 - 14.928*V4 + 25.618;//-16.961*V4 + 26.547;//-
16.146*V4+27.395;

                              T1=0.457*V8*V8 - 15.08*V8 + 26.567;//-16.146*V8+27.395;//-
16.146*V8+27.395;



                              //T4=-15.295*V4 + 51.142;
                              //T8=-15.421*V8 + 51.337;
                              //T4=-15.147*V4 + 51.865;
                              //T8=-16.026*V8 + 53.541;
                              //T4=-15.147*V4 + 50.755;
                              //T8=-16.026*V8 + 52.941;
                              tracers[3]= T5 ;
                              tracers[7]= T1 ;
                              SetCtrlVal (THERMPANEL, THERMPANEL_T5, T5);
                              SetCtrlVal (THERMPANEL, THERMPANEL_T1, T1);
                              SetCtrlVal (THERMPANEL, THERMPANEL_LED_5, 1);
                              SetCtrlVal (THERMPANEL, THERMPANEL_LED_1, 1);
                                    }
                       /*------cool--------------------------*/
                       if (count>110 && count<112)
                              {
                                    /* read o2 sensors*/
                              DAQmxReadAnalogF64 (analogtaskhandle, 1, 2.0,
DAQmx_Val_GroupByChannel, inputarray, 4, &num_sample, 0);
                                    PHV1=  inputarray[0];
                              DOV1= inputarray[1];
                                DO1=DOV1 ;
                              tracers[8]=DOV1;
                              tracers[9]=PHV1;
                              SetCtrlVal (THERMPANEL, THERMPANEL_DO, DO1);
                              /*----------------------------------------*/
                                    //printf ("cooling all sensors, \n");
                              SetCtrlVal (THERMPANEL, THERMPANEL_COMMENT_2,
print6);
```

```
                    analogout[0]=0;
                    DAQmxWriteAnalogF64 (analogtaskhandleout, 1, 0, 2,
DAQmx_Val_GroupByChannel, analogout, &num_sample, 0);
                    port1[0] = 1; //inhibit
                    port1[1] = 0; //B
                    port1[2] = 0; //A
                    DAQmxWriteDigitalLines (digitalportone, 1, 1, 2,
DAQmx_Val_GroupByChannel, port1, &num_sample, 0);
                    SetCtrlVal (THERMPANEL, THERMPANEL_LED_5, 0);
                    SetCtrlVal (THERMPANEL, THERMPANEL_LED_1, 0);
                    SetCtrlVal (THERMPANEL, THERMPANEL_READING, 0);
                    SetCtrlVal (THERMPANEL, THERMPANEL_COOLING, 1);
                    PlotStripChart (THERMPANEL, THERMPANEL_THERMCHART,
tracers, 10, 0, 0, VAL_DOUBLE);
                    PlotStripChart (THERMPANEL, THERMPANEL_THERMCHART2,
tracers, 10, 0, 0, VAL_DOUBLE);
                    PlotStripChart (THERMPANEL, THERMPANEL_THERMCHART3,
tracers, 10, 0, 0, VAL_DOUBLE);
                          }
                    T1SUM=T1SUM+T1;
                    T2SUM=T2SUM+T2;
                    T3SUM=T3SUM+T3;T4SUM=T4SUM+T4;
                    T5SUM=T5SUM+T5;T6SUM=T6SUM+T6;
                    T7SUM=T7SUM+T7;T8SUM=T8SUM+T8;
                    V1SUM=V1SUM+V1;V2SUM=V2SUM+V2;
                    V3SUM=V3SUM+V3; V4SUM=V4SUM+V4;
                    V5SUM=V5SUM+V5; V6SUM=V6SUM+V6;
                    V7SUM=V7SUM+V7;V8SUM=V8SUM+V8;
                    //fprintf(athermrecord, " V1, %lf,V2, %lf,V3, %lf,V4, %lf,V5, %lf,V6,
%lf,V7, %lf,V8, %lf, T1, %lf, T2, %lf, T3, %lf, T4, %lf, T5, %lf, T6, %lf, T7, %lf, T8,
%lf,CAL1,%lf,CAL2,%lf,DOV1,%lf,DOV2,%lf,DO1,%lf,DO22,%lf,%s\n",V1,V2,V3,V4,V5,V6,V7,V8,
T1, T2, T3, T4, T5, T6, T7, T8,CALT1, CALT2,DOV1,DOV2,DO1,DO2,USERCOMMENTS);
                    //GetCtrlVal (THERMPANEL,
THERMPANEL_COMMENTBLANK, &USERCOMMENTS[0]);
                    mastercount++;
                    count++;
                          if (count==120)
```

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```
                    {
T1=T1SUM/20;T2=T2SUM/20; T3=T3SUM/20; T4=T4SUM/20;
T5=T5SUM/20;T6=T6SUM/20; T7=T7SUM/20;T8=T8SUM/20;

V1=V1SUM/20; V2=V2SUM/20; V3=V3SUM/20; V4=V4SUM/20;
V5=V5SUM/20; V6=V6SUM/20; V7=V7SUM/20; V8=V8SUM/20;

hbcalculations();
//mean media temp
average20=average19; average19=average18; average18=average17;
average17=average16; average16=average15; average15=average14;
average14=average13      average13=average12;
average12=average11; average11=average10;
average10=average9; average9=average8;
average8=average7; average7=average6;
average6=average5; average5=average4;
average4=average3; average3=average2;
average2=average1; average1=T3;
cyclek++;

MMT=(average1+average2+average3+average4+average5+average6+average7+average8+averag
e9+average10+average11+average12+average13+average14+average15+average16+average17+average18
+average19+average20)/20;
                    SetCtrlVal (THERMPANEL, THERMPANEL_MMT, MMT);
                    SetCtrlVal (THERMPANEL, THERMPANEL_CYC, cyclek);
                        GetCtrlVal (THERMPANEL, THERMPANEL_SUMON,
&SUMON);

                    if(SUMON==1)
                        {
                    summon1=summon1+T2;
                    sumrun=summon1/summon2;
                    summon2++;

                    SetCtrlVal (THERMPANEL, THERMPANEL_SUMRUN,
sumrun);
                        }
```

```
                    fprintf(athermrecord, " %lf, %lf, %lf, %lf, %lf, %lf, %lf, %lf, %lf, %lf,
%lf, %lf, %lf, %lf, %lf, %lf,CAL1,%lf,DOV1,%lf,DO1,%lf,%s,QWALLA, %lf,QWALLU, %lf,QLID,
%lf,QBOT, %lf,QEX,%lf,MMT,%lf,SUMRUN,%lf,\n",V8,V7,V6,V5,V4,V3,V2,V1, T1, T2, T3, T4, T5,
T6, T7, T8,CALT1, DOV1,DO1,USERCOMMENTS,QWALLA, QWALLU, QLID, QBOT,
QEX,MMT,sumrun);
                    GetCtrlVal (THERMPANEL,
THERMPANEL_COMMENTBLANK, &USERCOMMENTS[0]);
                    count=1;
            T1SUM=0; T2SUM=0; T3SUM=0; T4SUM=0; T5SUM=0;
            T6SUM=0; T7SUM=0; T8SUM=0;


            V1SUM=0; V2SUM=0; V3SUM=0; V4SUM=0; V5SUM=0;
            V6SUM=0; V7SUM=0; V8SUM=0;
            CALT1=0; CALT2=0;
                    }
            //reset everything
            T1=0; T2=0; T3=0; T4=0; T5=0; T6=0; T7=0; T8=0;


            V1=0; V2=0; V3=0; V4=0; V5=0; V6=0; V7=0; V8=0;
        //relay
        GetCtrlVal (THERMPANEL, THERMPANEL_RELAY, &relaydelay);
        GetCtrlVal (THERMPANEL, THERMPANEL_HEAT, &heatdelay);

        watts = 150*(heatdelay/(heatdelay+relaydelay));
        SetCtrlVal (THERMPANEL, THERMPANEL_WATTS, watts);

        if (relaycount == relaydelay+heatdelay)
        {
                relaycount=0;
        }
        if (relaycount == 0)
        {
        port0[0] = 1; //Relay off
        port0[1] = 1; //Relay off
        analogout[1]=0;
        DAQmxWriteAnalogF64 (analogtaskhandleout, 1, 0, 2,
DAQmx_Val_GroupByChannel, analogout, &num_sample, 0);
```

```c
                        //DAQmxWriteDigitalLines (digitalportzero, 1, 1, 2,
DAQmx_Val_GroupByChannel, port0, &num_sample, 0);
                        //printf ("relay off, \n");
                        SetCtrlVal (THERMPANEL, THERMPANEL_HEATLED, 0);
                        }
                        if (relaycount == relaydelay)
                        {
                        port0[0] = 0; //Relay ON
                        port0[1] = 0; //Relay ON

                        analogout[1]=3;
                        DAQmxWriteAnalogF64 (analogtaskhandleout, 1, 0, 2,
DAQmx_Val_GroupByChannel, analogout, &num_sample, 0);
                        //DAQmxWriteDigitalLines (digitalportzero, 1, 1, 2,
DAQmx_Val_GroupByChannel, port0, &num_sample, 0);
                        //printf ("relay on, \n");
                        SetCtrlVal (THERMPANEL, THERMPANEL_HEATLED, 1);
                        }
                        //printf (" %lf \n", relaycount);
                        relaycount++;
        }
                        break;
        }return 0;
}
int CVICALLBACK SUBCAL1_CB (int panel, int control, int event,
                void *callbackData, int eventData1, int eventData2)
{switch (event)
        {case EVENT_COMMIT:
                        GetCtrlVal (THERMPANEL, THERMPANEL_CAL1, &CALT1);
                        break;
        }return 0;
}
/*---------------------------------Comment submit---------------------------------------*/
int CVICALLBACK SUBCOMMENT_CB (int panel, int control, int event,
                void *callbackData, int eventData1, int eventData2)
{switch (event)
        {case EVENT_COMMIT:
```

```c
        GetCtrlVal (THERMPANEL, THERMPANEL_COMMENT, &USERCOMMENTS[0]);
                        break;
        }return 0;
}
/*--------------------------------Initilize USB and file----------------------------------*/
void usbinit(void)
{
        CreateDAQanalogInProject(&analogtaskhandle);
        DAQmxStartTask (analogtaskhandle);
        CreateDAQanalogOUTfivevolts(&analogtaskhandleout);
        DAQmxStartTask (analogtaskhandleout);
        Portonemultiplexcontrol(&digitalportone);
        DAQmxStartTask (digitalportone);
        CreateDAQTaskInProjectdigitalrelaycontrol(&digitalportzero);
        DAQmxStartTask (digitalportzero);


        athermrecord = fopen ("thermrecord.txt", "w");


         return;
}
/*--------------------------------HBcalculations-------------------------------------------*/
void hbcalculations(void)
{
        double Tlid, Tm, Tae, Tbot, Tamb, Twallu, Twalla,Tex;
        double HWALLAIN, HLIDIN, T9wallin, T9up, T9down, hup, hdown, deltah, olddeltah;
        double kwall;
        double kpyx;
        int AIR;
        double Mdot=0;
        double Cp=0;


        Tlid=T1;//23.323885;//T1normally  T1;
        Tm=T3;//25.42743559;//T2;
        Tae=T6;//T3;//spare = T3(Tae (aeration air))
        Twalla=T4;//23.47798686;//T5; used to be T4, was broken so took T5
        Tbot=T7;//24.8237499;//T7//T5 moved - use T7 instead
        Tamb =T6;//22.2932966;//T6;
```

```
Twallu=T7;//24.76582822;//T7;
Tex=T5;//23.87295966;//T8;

GetCtrlVal (THERMPANEL, THERMPANEL_AIR, &AIR);
AIRd = AIR;

//QBOT///////////////////////////////////////////////////////////////////////
QBOT = 0;
HBOT = 0;
UBOT = 0;

if (Tm>Tbot)
{
        if(Tbot>Tamb)
        {
        HBOT = 0.59*pow (((Tbot-Tamb)/0.1492), 0.25); //0.1492 = Diao
        UBOT = 1/(0.002886+(1/HBOT));//(Thw() / Kpyx() = 002886)
        QBOT = UBOT*0.0175*(Tm-Tamb);//Abot = 0.17085m
        }
}
////QWALLA/////////////////////////////////////////////////////////////////////

QWALLA = 0;
HWALLA = 0;
UWALLA = 0;

if(Tex>Twalla)
{
 if(Twalla>Tamb)
 {
HWALLA = 1.42*pow (((Twalla-Tamb)/0.09112), 0.25); //0.09112 = Wall L above media
T9down = Twalla;
olddeltah = 5000;
deltah = 1000;

        while (olddeltah > deltah)//buggered Newton's method to find REAL T9
        {
```

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```
                    T9down = T9down+.001;

                    hup = 54*T9down-1065.31;

                    hdown = -1.8202*pow(T9down,0.25)+3.86215;

                    olddeltah= deltah;

                    deltah = fabs(hdown-hup);

                              if(T9down >=Tex)

                              {

                                        T9down=Tex;

                                        deltah=1000;

                              }

                    //printf (" %lf %lf %lf %lf %lf \n ", hup, hdown, deltah, olddeltah, T9down);

                    }

          //printf (" %lf %lf \n ", Tex, T9down);

          T9down=T9down-.001;

          HWALLAIN =  1.42*pow ((((Tex-T9down)/0.09112), 0.25); //0.09112 = Wall L above media


          if(AIR == 1)

                    {

                    HWALLAIN = 10000000;

                    }

                    UWALLA = 1/((1/HWALLAIN)+0.07632+(1/HWALLA));//(ln(ro/ri)/2piKLwallabove =

0.076327)

          QWALLA = UWALLA*0.0427*(Tex-Tamb);//Awalla = 0.0427m^2

           }

          }


          //WALLU////////////////////////////////////////////////////////////////////


          QWALLU=0;

          HWALLU=0;

          UWALLU=0;


          if(Tm>Twallu)

          {

                    if (Twallu>Tamb)

                    {

          HWALLU =  1.42*pow ((((Twallu-Tamb)/0.3645), 0.25); //0.3645 = Wall L under media
```

```c
UWALLU = 1/(0.0185176+(1/HWALLU));//(ln(ro/ri)/2piKLunderwall = 0.0185176)
QWALLU = UWALLU*0.175*(Tm-Tamb);//Abot = 0.17085m^2
//printf (" %lf %lf %lf \n ", HWALLU, UWALLU, QWALLU);
        }
}

QWALL = QWALLU + QWALLA;
//QLID//////////////////////////////////////////////////////////////////////////
QLID=0;
HLID=0;
ULID=0;

if(Tex>Tlid)
{
        if(Tlid>Tamb)
        {

HLID = 1.32*pow (((Tlid-Tamb)/0.1492), 0.25); //0.1492 = Diao

T9up = Tlid;
olddeltah = 5000;
deltah = 1000;
                while (olddeltah > deltah)//buggered Newton's method to find REAL T9
        {
        T9up = T9up+.001;
        hup = 54*T9up-1065.31;
        hdown = -1.8202*pow(T9up,0.25)+3.86215;
        olddeltah= deltah;
        deltah = fabs(hdown-hup);
        if(T9up >=Tex)
                {
                        T9up=Tex;
                        deltah=1000;
                }
                }
T9up=T9up-.001;
HLIDIN =  1.32*pow (((Tex-T9up)/0.1492), 0.25); //0.1492 = Diao
```

```
if(AIR == 1)
        {
        HLIDIN = 10000000;
        }


ULID = 1/((1/HLIDIN)+0.000521+(1/HLID));//(Thlid() / Ksteel() = 0.000521)
QLID = ULID*0.0175*(Tex-Tamb);//Abot approx = Alid =  0.175m^2
        }
}


//QEX  //requires additional thermistors   (Tae)  Q=MCdeltaT(Tex-Tae)
QEX=0;
Mdot=0.43;//g/s
Cp=1;//  j/g C

if(AIR==1)
{
        if(Tex>Tae)
        {
        QEX=Mdot*Cp*(Tex-Tae);
        }
}
QTOTAL = QBOT+QLID+QWALL+QEX;
//printf (" %lf \n ", QTOTAL);
PlotStripChartPoint (THERMPANEL, THERMPANEL_QCHART, QTOTAL);
// set indicators on GUI with values
//UIND
SetCtrlVal (THERMPANEL, THERMPANEL_UBOT, UBOT);
SetCtrlVal (THERMPANEL, THERMPANEL_UWALLA, UWALLA);
SetCtrlVal (THERMPANEL, THERMPANEL_UWALLU, UWALLU);
SetCtrlVal (THERMPANEL, THERMPANEL_ULID, ULID);
//QIND
SetCtrlVal (THERMPANEL, THERMPANEL_QBOT, QBOT);
SetCtrlVal (THERMPANEL, THERMPANEL_QWALLA, QWALLA);
SetCtrlVal (THERMPANEL, THERMPANEL_QWALLU, QWALLU);
SetCtrlVal (THERMPANEL, THERMPANEL_QWALL, QWALL);
```

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```
SetCtrlVal (THERMPANEL, THERMPANEL_QLID, QLID);
SetCtrlVal (THERMPANEL, THERMPANEL_QEX, QEX);
//HIND
SetCtrlVal (THERMPANEL, THERMPANEL_HBOT, HBOT);
SetCtrlVal (THERMPANEL, THERMPANEL_HWALLA, HWALLA);
SetCtrlVal (THERMPANEL, THERMPANEL_HWALLU, HWALLU);
SetCtrlVal (THERMPANEL, THERMPANEL_HLID, HLID);
//QTOTAL
SetCtrlVal (THERMPANEL, THERMPANEL_QTOTAL, QTOTAL);
return;
}
```

APPENDIX B SAMPLE CALCULATION

A sample heat balance calculation was completed for the second heat balance trial with aeration at 600 rpm. The $Q_{reactorloss}$ at steady state is calculated to be 1.02 $J \cdot s^{-1}$. The values for temperatures and constants used are provided below:

Dia_o	=	0.1492 m
K_{pyx}	=	1.1 $J \cdot s^{-1} \cdot m^{-1} \cdot K^{-1}$
K_{steel}	=	13.8 $J \cdot s^{-1} \cdot m^{-1} \cdot K$-1
L_{wa}	=	0.0911 m
T_{amb}	=	23.358 °C
T_{bo}	=	25.084 °C
T_{ex}	=	24.751 °C
T_{li}	=	Not required °C
T_{lo}	=	24.337 °C
T_m	=	25.591 °C
T_{wai}	=	Calculated as an example, but not required °C
T_{wao}	=	24.540 °C
T_{wuo}	=	25.129 °C
Th_w	=	0.00317 m
Th_L	=	0.0076 m (3/8")

To find the calibrated signals from the bioreactor without aeration the following equations were used:

$$Q_{reactorloss} = Q_{wallabove} + Q_{wall} + Q_{bottom} + Q_{lid} \tag{22}$$

Where:

$$Q_{walllabove} = U_{wa} A_{wa} (T_m - T_{amb}) \tag{3}$$

$$Q_{wall} = U_{wu} A_{wu} (T_m - T_{amb}) \tag{4}$$

$$Q_{bottom} = U_{bot} A_{bot} (T_m - T_{amb}) \tag{5}$$

$$Q_{Lid} = U_{Lid} A_{Lid} (T_{ex} - T_{amb})$$ (6)

Calculation of Q_{bottom} :

$$h_{bo} = 0.59 \left(\frac{T_{bo} - T_{amb}}{Dia_o} \right)^{\wedge 0.25}$$ (9)

$$h_{bo} = 0.59 \left(\frac{25.084\ °C\ - 23.358\ °C}{0.1492\ m} \right)^{\wedge 0.25} = 1.088\ J \cdot s^{-1} \cdot m^{-2} \cdot °C^{-1}$$ (9)

$$U_{bot} = \left(\frac{1}{\left(\frac{Th_w}{K_{pyx}} \right) + \left(\frac{1}{h_{bo}} \right)} \right)$$ (8)

$$U_{bot} = \left(\frac{1}{\left(\frac{0.00317\ m}{1.1\ J \cdot s^{-1} \cdot m^{-1} \cdot °\ C^{-1}} \right) + \left(\frac{1}{h_{bo}} \right)} \right) = 1.085\ J \cdot s^{-1} \cdot m^{-2} \cdot °C^{-1}$$ (8)

$$Q_{bot} = U_{bot} A_{bot} (T_m - T_{amb})$$ (5)

$$Q_{bot} = U_{bot} 0.0175 m^2\ (25.591°C\ - 23.358°C) = 0.042\ J \cdot s^{-1}$$ (5)

Q_{wall}:

$$h_{wuo} = 1.42 \left(\frac{T_{wuo} - T_{amb}}{L_{wu}} \right)^{0.25}$$ (11)

$$h_{wuo} = 1.42 \left(\frac{25.129\ - 23.358}{0.3645} \right)^{0.25} = 2.108\ J \cdot s^{-1} \cdot m^{-2} \cdot °C^{-1}$$ (11)

$$U_{wu} = \left(\frac{1}{\left(\frac{\ln\left(\frac{(Dia_o / 2)}{(Dia_i / 2)} \right)}{2\pi K_{pyx} L_{wu}} \right) + \left(\frac{1}{h_{wuo}} \right)} \right)$$ (10)

$$U_{wu} = \left(\cfrac{1}{\cfrac{\ln\left(\cfrac{(0.149\ /2)}{(0.142\ /2)}\right)}{2\pi \cdot 1.1 \ \cdot 0.3645} + \left(\cfrac{1}{h_{wuo}}\right)} \right) \quad = \quad 2.026 \ \text{J·s}^{-1}\text{·m}^{-2}\ \text{·}^{\circ}\text{C}^{-1} \qquad (10)$$

$$Q_{wall} = U_{wu} A_{wu} (T_m - T_{amb}) \tag{4}$$

$$Q_{wall} = 2.026 \cdot 0.171 \cdot (25.591 \ - 23.358 \ ) \ = 0.774 \ \text{J·s}^{-1} \tag{4}$$

When the aeration was inactive and the heat transfer coefficient for the inner wall needed to be determined, the temperature of the inner wall was necessary for the calculations. This was not measured, however it could be calculated by using the known properties of the wall and measured temperatures. To determine the temperature of the inner wall, the equations for the energy transfer from the exhaust air to the wall was set equal to the energy transfer through the wall so that the temperature of the inside of the wall (T_{wai}) was the unknown variable. This step was not necessary when the aeration was activated, however for trial T_{wai} was calculated as shown below.

$$1.42\left(\frac{T_{ex} - T_{wai}}{L_{wa}}\right)^{0.25} = \frac{2\pi K_{pyx} L_{wa}}{\ln\left(\cfrac{(Dia_o\ /2)}{(Dia_i\ /2)}\right)} \cdot (T_{wai} - T_{wao})$$

$$1.42\left(\frac{24.751 - T_{wai}}{0.0911}\right)^{0.25} = \frac{2 \cdot \pi \cdot 1.1 \cdot 0.0911}{\ln\left(\cfrac{(0.149/2)}{(0.142/2)}\right)} \cdot (T_{wai} - 24.540)$$

$T_{wai} = 24.651 \ ^{\circ}\text{C}$

$$h_{wai} = 1.42\left(\frac{T_{ex} - T_{wai}}{L_{wa}}\right)^{0.25} \tag{15}$$

$$h_{wai} = 1.42\left(\frac{24.751 \ - 24.651}{0.0911}\right)^{0.25} = 1.453 \ \text{J·s}^{-1}\text{·m}^{-2}\ \text{·}^{\circ}\text{C}^{-1} \tag{15}$$

$$h_{wao} = 1.42\left(\frac{T_{wu} - T_{amb}}{L_{wa}}\right)^{0.25} \tag{13}$$

$$h_{wao} = 1.42\left(\frac{24.540 - 23.358}{0.0911}\right)^{0.25} = 2.695 \text{ J·s}^{-1}\text{·m}^{-2}\text{·°C}^{-1} \tag{13}$$

$$U_{wa} = \left(\frac{1}{\left(\frac{\ln\left(\frac{(Dia_o/2)}{(Dia_i/2)}\right)}{2\pi K_{pyx} L_{wa}}\right) + \left(\frac{1}{h_{wao}}\right)}\right) \tag{14}$$

$$U_{wa} = \left(\frac{1}{\left(\frac{\ln\left(\frac{(0.149/2)}{(0.142/2)}\right)}{2\cdot\pi\cdot 1.1\cdot 0.0911}\right) + \left(\frac{1}{2.695}\right)}\right) = \quad 2.235 \text{ J·s}^{-1}\text{·m}^{-2}\text{·°C}^{-1} \tag{14}$$

$$Q_{walllabove} = U_{wa} A_{wa}(T_m - T_{amb}) \tag{3}$$

$$Q_{wallabove} = 2.235\cdot .0427\cdot (24.751 - 23.358) = 0.152 \text{ J·s}^{-1} \tag{3}$$

$$h_{Lo} = 1.32\left(\frac{T_{Lo} - T_{amb}}{Dia_o}\right)^{0.25} \tag{18}$$

$$h_{Lo} = 1.32\left(\frac{24.337 - 23.358}{0.149}\right)^{0.25} = 2.113 \text{ J·s}^{-1}\text{·m}^{-2}\text{·°C}^{-1} \tag{18}$$

$$U_{lid} = \left(\cfrac{1}{\left(\cfrac{Th_L}{K_{Steel}} \right) + \left(\cfrac{1}{h_{Lo}} \right)} \right)$$
(16)

$$U_{lid} = \left(\cfrac{1}{\left(\cfrac{.0076}{13.8} \right) + \left(\cfrac{1}{2.113} \right)} \right) = 2.110 \text{ J·s}^{-1}\text{·m}^{-2}\text{·°C}^{-1}$$
(16)

$$Q_{Lid} = U_{Lid} A_{Lid} (T_{ex} - T_{amb})$$
(6)

$$Q_{Lid} = 2.11 \cdot .0175 \cdot (24.751 - 23.358) = 0.0514 \text{ J·s}^{-1}$$
(6)

$$Q_{reactorloss} = Q_{wallabove} + Q_{wall} + Q_{bottom} + Q_{lid}$$
(22)

$$Q_{reactorloss} = 0.152 + 0.774 + 0.042 + .0514 = 1.02 \text{ J·s-1}$$
(22)