Effects of temperature and carbon-to-nitrogen ratio on the growth rate of *Candida tropicalis* and *Candida utilis* in a furfural-based wastewater

by

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ABSTRACT

Batch tests were used to determine the effects of temperature and carbon-to-nitrogen ratio (C/N) on the maximum specific growth rate of two species of yeast: *Candida tropicalis* and *Candida utilis*. Raw wastewater was obtained from the effluent stream of the furfural production facility at the Quaker Oats Company in Cedar Rapids, Iowa. The first set of batch tests varied temperatures from 27°C to 45°C. However, no growth was observed at 45°C, so the effective temperature range was from 27°C to 40°C. During the first set of batch tests, the C/N ratio was held constant at 10:1. The second set of batch tests varied the carbon-to-nitrogen ratio between 10:1 and 1000:1 compared to the raw wastewater ratio of approximately 2575:1. During the second set of batch tests, the temperature was held constant at 35°C. Samples periodically taken from the batch tests were analyzed for total suspended solids (TSS), volatile suspended solids (VSS), and chemical oxygen demand (COD).

A 4^{th} order Runge-Kutta method was employed to solve two differential equations relating substrate removal to biomass growth over time. Experimental results indicate that the maximum specific growth rate (μ_{max}) for both yeast increased as the batch temperature neared 35°C and 33°C for *C. tropicalis* and *C. utilis*, respectively. The values of μ_{max} for the aforementioned temperatures were 0.42 h^{-1} for *C. tropicalis* and 0.38 h^{-1} for *C. utilis*, and μ_{max} decreased as the batch temperatures deviated from the aforementioned optimum temperatures. The results from varying the C/N ratio indicate that μ_{max} is significantly increased for C/N ratios less than 25:1. Between the ranges of 25:1 and 10:1, μ_{max} increases from 0.18 to 0.41 h^{-1} and from 0.20 to 0.42 h^{-1} for *C. tropicalis* and *C. utilis* respectively.

The μ_{max} for *C. tropicalis* and *C. utilis* is sensitive to both temperature and C/N ratio and optimizing these two operating parameters would entail further laboratory research or a pilot study.

CHAPTER 1
HISTORY

Before civilization began and wastewater was ever collected or channeled, biological water treatment only occurred naturally. Countless organisms break down scores of naturally occurring materials both aerobically and anaerobically. It was not until modern civilization and an increase in population density that human-created wastes were collected. In fact, in 500 B.C. all refuse had to be disposed of at least one mile from the city limits of Athens, Greece (Barbalace, 2000). Biodegradation as we know it today was far from discovery.

In the mid 1800's, wastewater was discharged into open ditches or directly into streams as a means for removing the odorous wastes. It was not until 1842 during the industrial revolution when environmental conditions were first linked to the health of the local populace. Specifically, Edwin Chadwick published "The Sanitary Condition of the Labouring Population of Great Britain" (Chadwick, 1842). He recognized that "atmospheric impurities produced by decomposing animal and vegetable substances, by damp and filth, and close and overcrowded dwellings," were linked to illness. He also found that "...where those circumstances are removed by drainage, proper cleansing, better ventilation, and other means of diminishing atmospheric impurity, the frequency and intensity of such disease is abated; and where the removal of the noxious agencies appears to be complete, such disease almost entirely disappears" (Chadwick, 1842).

As the condition of rivers, lakes and swamps deteriorated due to the introduction of human wastes, communities began looking for ways to treat the wastewater prior to discharge. Initially, treatment facilities were no more advanced than open irrigation fields, but better methods of treatment were soon developed and implemented. For instance, in 1887 the first municipal wastewater treatment plant in Frankfurt, Germany employed primary treatment such as grit removal, debris screens, and settling tanks. Furthermore, secondary treatment such as trickling filters, oxidation ponds, and Imhoff sludge digestion tanks were in place by 1906 (Seeger, 1999).

It was not until 1914 that flocculant biomass sludge was recycled back into the aerobic treatment vessel. Arden and Lockett described what is now known as the principle of "activated sludge" (FAO 2003).

Before 1940, a majority of the wastewater came from domestic origin. As the world grew and continues to grow more industrious, a larger percentage of the wastewater is derived from industrial practices. In contrast to domestic wastes, many chemicals released into the municipal collection systems by industry are difficult to treat with conventional systems. Sometimes, these industrial pollutants must be pretreated before entering the municipal waste stream or be treated onsite with advanced treatment processes. These advanced processes may include solids flotation, chemical precipitation, filtration, chemical oxidation, biological nutrient removal, disinfection, carbon adsorption, air scrubbing and more (Tchobanoglous et al., 2003).

In an activated sludge system, a certain sludge age must be maintained for proper system operation and waste treatment. Sludge age is regulated by wasting some of the settled biomass collected in the secondary clarifier. Traditional activated sludge systems are hosts to mainly bacteria, which have little or no monetary value. In fact, it costs considerable amounts of money to dispose of the excess bacterial sludge. So, as organic materials are broken down into less complex chemicals, biomass is produced and simultaneously creates disposal costs. However, the creation of a valuable byproduct in place of bacterial biomass eliminates disposal costs and produces additional income. This income is worth recovering and can be achieved by producing a valuable biomass such as yeast. Yeast are high in protein content (30-60%) and can be used for animal feed or for human consumption if the quality and purity is adequate. Yeast, unlike bacteria, are not pathogenic and have the ability to live in low-nutrient conditions. As a result, decreasing the nutrient addition to the wastewater stream saves additional costs.

CHAPTER 2
INTRODUCTION

Overview of Aerobic Treatment

The goals of biological wastewater treatment are to transform biodegradable elements into satisfactory and dischargeable end products, remove nutrients such as nitrogen and phosphorus, reduce dissolved and suspended solids, and decrease trace organic compounds. The oxidization of carbonaceous species into biomass, water, and carbon dioxide is the main pathway. All of these goals can be accomplished by aerobic and facultative microorganisms in a series of system components that make up the aerobic treatment process.

The processes involved in industrial treatment wholly depend upon the characteristics of the wastewater. First, preliminary treatment such as screening and grit removal removes large constituents and quickly settling, solid particles that may interfere with subsequent treatment operations. Once separated, these coarse components are disposed of in an appropriate landfill. Second, primary sedimentation removes a portion of the organic matter that is contained in smaller suspended solids. Third, advanced primary treatment may be employed to increase the removal of organic matter. This level of treatment involves chemical addition or filtration. Fourth, secondary treatment is defined as the removal of biodegradable organic matter, in both solid and solution form, through the use of microbiological organisms (Tchobanoglous et al., 2003). Last, tertiary treatment is employed to remove residual suspended solids by the use of granular filtration or microscreens. Nutrient removal may follow the secondary treatment component of the system and is considered a form of tertiary treatment. Disinfection prior to discharge is also considered part of the tertiary level of treatment.

Aerobic secondary treatment has great flexibility in its operational unit. The biomass can be fixed or suspended growth or a combination of the two in series. Fixed biomass is grown on the surface of the packing material contained in a packed-bed or fluidized-bed reactor. Suspended biomass can be grown in a batch, complete-mix, or plug-flow reactor. The

following paragraphs summarize the characteristics of the five aforementioned aerobic treatment reactors:

Attached Growth Reactors

- Attached Growth Reactors
    - Packed-bed reactors such as trickling filters are filled with material having a high surface area to volume ratio. The wastewater is "trickled" over the surface of the packing material and flows vertically downward to a collection basin where a portion is recycled back to the influent stream and the rest proceeds through the remainder of the system. As the carbonaceous portion in the wastewater flows past the biomass growing on the surface of the packing material, it is taken up by the microorganisms and oxidized (assuming required nutrients are present).

    - Fluidized-bed reactors are run in an upflow configuration where air and wastewater are injected at the bottom. The flowrate of the air and water control the extent of expansion and, hence, the porosity of the packing material. The biomass attached to the packing material utilizes the organic material and nutrients for cell synthesis and maintenance as they flow through the bed.

Suspended Growth Reactors

- Suspended Growth Reactors
    - The batch reactor uses a static volume of wastewater and biomass mixed together for a specified period of time to degrade organic matter. In a sequencing batch reactor, when the mixing phase stops, the biomass and other suspended solids are allowed to settle out. The treated supernatant is then drawn off the top of the reactor leaving the biomass behind to degrade the next batch of incoming wastewater. This method is referred to as the "fill and draw" method.

- The complete-mix reactor is a continuous flow reactor in which solution is continuously flowing into and out of. While the wastewater is in the complete-mix reactor, it is uniformly mixed with the recycled biomass and solids from the subsequent sedimentation tank.

- Plug-flow reactors are those in which particles pass through in the same order they entered. Typical plug-flow reactors have a high length to width ratio in which mixing is minimal. This assures that particles remain in the reactor for the same length of time as the theoretical detention time. Since particles are in the reactor for the same amount of time, there is no "short-circuiting" possible. Short-circuiting happens when an organic particle escapes the reactor before spending the entire theoretical detention time being oxidized.

Advantages to Aerobic Treatment

- Wide range of wastewater constituents that can be treated
- Less sensitive than anaerobic treatment to toxic inhibitors
- Less heat input required
- Better effluent quality than anaerobic treatment
- Biological nutrient removal
- Degradation of chlorinated organics

Disadvantages to Aerobic Treatment

- Lower loading rates than anaerobic treatment
- No methane production
- Large energy input to aerate reactor
- Larger sludge production due to higher specific growth rate
- Additional nutrient requirements
- Oxygen requirement

Factors Affecting Aerobic Microbial Growth

Oxygen Level

The microorganisms involved in advanced aerobic treatment processes are either strict aerobes or facultative anaerobes. Strict aerobes can grow only in the presence of adequate amounts of oxygen. Aerobic organisms need oxygen because it is the terminal electron acceptor in respiration. Suitable levels of dissolved oxygen for aerobic treatment typically fall at or above 2.0 mg/L. Some microorganisms can grow in lower oxygen concentrations and these are known as facultative anaerobes. Facultative anaerobes can grow either in the presence or absence of oxygen. This flexibility is due to the fact that facultative anaerobes can utilize compounds besides oxygen, such as sulfate, nitrate, or carbon dioxide, as electron acceptors (Bitton, 1999). The half-saturation coefficient for dissolved oxygen (K_o) is the level of dissolved oxygen at which microbial growth is inhibited. Sinclair and Ryder (1975) found the K_o value for *C. utilis* to be 0.08 mg O_2/L compared to 0.15 mg O_2/L for *Citrobacter sp.* (Lau et al. 1984). These values suggest that dissolved oxygen concentrations must be very low before having impacts on microbial growth (Grady et al., 1999).

pH

The pH of a wastewater stream is important because it affects the viability of certain types of microorganisms. For example, bacteria prefer neutral conditions while fungi and yeast prefer acidic environments and cyanobacteria grow best in alkaline surroundings. pH also effects physical aspects of microorganisms. Specifically, pH affects the ionization of chemicals and ultimately affects the ability of some cells to uptake certain nutrients into the cell (Bitton, 1999).

Temperature

Temperature has a large influence on the growth rate of microorganisms. Furthermore, certain microbes only grow in specific temperature ranges. Psychrophiles thrive in temperatures below 20°C, mesophiles grow between 25-40°C, thermophiles grow between 45-60°C and stenothermophiles enjoy temperatures from 60°C to more than 100°C. In aerobic biological wastewater treatment, however, mesophiles are the dominant

microorganism due to the large costs of maintaining a system at an elevated temperature (Davis and Cornwell, 1998).

Temperature influences the metabolic activities of the microorganisms as well as gas-transfer rates. It can also influence the rates of enzymatically catalyzed reactions (Grady et al. 1999). The Arrhenius equation relates microbial growth to temperature in a general equation as follows:

$$k=Ae^{-u/RT} \quad (1)$$

where k=temperature dependent rate coefficient (h^{-1}), A=constant, u=temperature coefficient (kJ/mole), R=gas constant (8.3143 J/°K·mole), and T=absolute temperature (°K) (Bitton 1997). This equation was first applied in 1889 when quantifying temperature effects during the enzymatic hydrolysis of sugar (Grady et al. 1999). Rearranging the Arrhenius equations for two rate coefficients at differing temperatures yields Equation 2.

$$\ln(k_1/k_2)=u(T_1-T_2)/(RT_1T_2) \quad (2)$$

Since the product of T_1 and T_2 does not vary much, a constant "C" has been used to describe the term such that $C=u/(RT_1T_2)\approx 0.0015u$. The value of C is found by plotting ln k vs. T and the resulting slope is C. A simplified version Equation 2 is shown in Equation 3.

$$k_1=k_2\, e^{C(T_1-T_2)} \quad (3)$$

Since $C=\ln(\Theta)$, Equation 4 is commonly used since considerable research has been accomplished to determine approximate values of Θ for aerobic heterotrophic growth.

$$k_1=k_2\, \Theta^{(T_1-T_2)} \quad (4)$$

In a more detailed and focused equation, the reaction rate at 20°C is used as a reference from which reaction rates at other temperatures can be found. The temperature can be in °C or °K since it is only the difference in temperature that we are concerned with. The following equation performs this conversion with a known temperature-activity coefficient (Θ):

$$k_T = k_{20}\,\Theta^{(T-20)} \qquad (5)$$

where k_T=reaction rate coefficient at T, (°C), k_{20}=reaction rate coefficient at T=20°C, Θ=temperature-activity coefficient, and T=temperature (°C). Values of Θ can vary between 1.02 and 1.25 for biological systems. Most studies on the influence of temperature reported a value of Θ =1.094 for aerobic heterotrophs (Grady et al., 1999). Tchobanoglous et al. (2003) suggest that Θ be between 1.02 and 1.10. For this project, it is assumed that Θ =1.056 as suggested by Davis and Cornwell (1998).

Since the reaction rate coefficients for C*andida tropicalis* and C*andida utilis* are not specifically known at 20°C, the rate coefficients at differing temperatures can be related with Equations 4 and 5.

For this project, the decay coefficient at 20°C (k_d) is assumed to be 0.05 d^{-1} or 0.002083 h^{-1} (Tchobanoglous et al., 2003). Therefore, the values of k_d used in the temperature variation portion of this project were calculated using Equation 5 and are displayed in Table 1.

Temperature (°C)	Decay Coefficient (k_d)
27	0.00313
30	0.00373
35	0.00499
37	0.00561
40	0.00668

TABLE 1- Decay Coefficients and Corresponding Temperatures

Nutrient Requirements

Certain key nutrients and micronutrients are needed for microbial synthesis of enzymes and cell maintenance. Nutrients such as nitrogen and phosphorus are the two major nutrients necessary for cell growth and maintenance. These nutrients are characterized as macronutrients. If the concentration of nitrogen or phosphorus is not sufficient, it can limit the rate of substrate removal. When quantifying the growth rate of a particular microorganism, it is necessary that the batch test not be limited by nutrients. Assuming a μ_{max} of 0.50 h^{-1}, it has been shown that nitrogen will not be limiting if the nitrogen concentration is higher than 1.0 mg/L as N above the stoichiometric nitrogen requirement for a specific wastewater (Goel and Gaudy, 1969). The same holds true for phosphorus concentration. The phosphorus concentration should exceed the stoichiometric concentration by a few tenths of a mg/L as P to avoid rate limiting conditions (Grady et al., 1999).

Since enzymes are composed of amino acids and amino acids contain amine groups and amine groups are those that contain nitrogen, nitrogen is an essential component to enzyme production. Enzyme production is especially important for *C. utilis* because it has a relatively high concentration of amino acids in the protein (Lawford et al., 1979). As a result, *C. utilis* can metabolize a wide range of saccharides (Shay and Wegner, 1985). Yeast are heterotrophic and, therefore, require an external source of organic molecules as an energy source. For example, brewers yeast (*Saccharomyces cerevisiae*) utilizes sugar (sucrose) as an energy source. Grady and Daigger (1999) report that 0.122mg of NH_4^+ is needed per mg of biomass chemical oxygen demand (COD) formed for heterotrophic biomass growth. This equates to 0.087 mg as N per mg of biomass COD formed. These values were calculated stoichiometrically based on the assumption that biomass is represented by the molecular formula $C_5H_7O_2N$. Given the molecular formula of $C_{12}H_{87}O_{23}N_{12}P$, which also includes phosphorus, about 12.2g of nitrogen and 2.3g of phosphorus are required per 100g of cell biomass (Tchobanoglous et al., 2003).

Phosphorus requirements can be estimated as approximately one-fifth of the nitrogen requirement on a mass basis (Grady et al. 1999). Therefore, one-fifth of 0.087 mg N is equal

to 0.0174 mg P per mg of biomass COD created. Assuming that there were no Phosphorus Accumulating Organisms (PAOs) present, the amount of phosphorus released during cell lysis was equal to the amount required for cell growth and maintenance.

Many nutrients other than nitrogen and phosphorus are needed by microorganisms for cell functions. However, these nutrients are required in such small quantities that they are not taken into account during the stoichiometric analysis. As a result, the nutrients are referred to as micronutrients. The necessary quantities of these micronutrients are also disagreeable since cations are adsorbed at different rates by different species of biomass and, hence, appear to require more than is really necessary. Micronutrients include calcium, potassium, magnesium, sulfur, sodium, chloride, iron, zinc, manganese, copper, molybdenum, and cobalt (from largest to smallest in approximate quantity required) (Grady et al., 1999).

Goals of the Current Research Project

The purpose of this research was to ultimately define the optimum growth conditions for *C. tropicalis* and *C. utilis* with respect to temperature and carbon-to-nitrogen ratio. Knowing how the yeast react to variation in temperature and carbon-to-nitrogen ratio aids in the maximization of the growth rate. Maximizing the growth rate is important because it translates into a greater quantity of valuable material and better effluent quality through substrate removal. Alternatively, the reaction of the yeast to temperature and carbon-to-nitrogen ratio variation could assist in aiming for a specific growth rate to balance biomass growth and effluent quality. Growth rate may be limited, for instance, because the yeast biomass may be contaminated by the influent and would need to be disposed of. Therefore, it would be critical to decrease the biomass growth rate. The current research assists an operator in adjusting a system's conditions to achieve the desired growth rate.

CHAPTER 3
BACKGROUND

Project Background

The objective of the current research project was to evaluate the effects of temperature and carbon to nitrogen ratio on the growth rate of *C. tropicalis* and *C. utilis* in a furfural-based wastewater. The source of the wastewater used in this project was the Quaker Oats furfural production facility in Cedar Rapids, Iowa. This high-strength, high-temperature waste stream had low pH and low suspended solids. The soluble chemical oxygen demand (SCOD) ranged from 14,000-17,000 mg/L and biochemical oxygen demand (BOD) ranged from 8,000-12,000 mg/L. The temperature of the waste stream after it had passed through a cooling tower was approximately 110°F (43°C) and the pH was 2.5. The nitrogen and phosphorus content of the raw wastewater was extremely low, so these nutrients needed to be added to supplement the biological degradation by the yeast. The concentration of furfural in the waste stream was 180 mg/L. Therefore, the chemical properties of the wastewater showed favorable growth conditions for yeast and fungi that thrive in low pH and low nutrient environments.

Characteristics of Furfural

Furfural or furfuraldehyde (C_4H_3OCHO) is a viscous, colorless liquid that, when exposed to air, turns a reddish-brown color. It boils at 162°C and commercial preparation of furfural involves the dehydration of cornstalks and corncobs and husks of oats and peanuts. It is commonly used as a solvent in the petroleum industry and as a precursor in the manufacture of pesticides, phenolfurfural resins, and tetrahydrofuran. Furthermore, tetrahydrofuran is an important material used in the preparation of nylon.

With a high-strength wastewater such as the one used in this project, anaerobic biological treatment is typically the most feasible course of action. Anaerobic treatment provides several benefits over aerobic activated sludge systems. First, lower cell synthesis rate translates into less sludge production and lower sludge disposal costs. Second, methane gas

generation provides a significant power supply. Last, anaerobic treatment provides the ability to achieve a higher organic loading rate because the oxygen transfer rate does not limit the reactions.

Despite all the benefits of anaerobic treatment, furfural is mutagenic, and therefore toxic to bacteria and animal cell systems (BIBRA Toxicology International, 1991). In addition, aliphatic aldehyde and amines are hardly degraded under anaerobic conditions (Kameya et al., 1995). Therefore, standard anaerobic treatment with bacteria cannot be successfully used to dissipate the organic discharge into the local wastewater collection and treatment system.

Some yeast are capable of growing in the absence of oxygen. Specifically, *Candida spp.* are capable of the process of degrading sugars into alcohol and carbon dioxide along with glycerol and succinate without oxygen (Feldmann, 2001). This process is known as fermentation and is the metabolic pathway used in brewing beer and wine. However, since alcohol production was not a goal of the current research project, anaerobic fermentation was not the metabolic pathway of choice.

Inhibitory Properties of Furfural

In the case of harvesting yeast, however, aerobic treatment has one main advantage over anaerobic treatment. The higher specific growth rate of an aerobic system, that was once a disadvantage when dealing with a mixed bacterial culture, transforms into an advantage. A higher specific growth rate generates more biomass and results in a greater yield of yeast that can be sold as a protein supplement to animal feed companies.

Nigam (1998) found the protein content of *C. utilis* single celled protein (SCP) to be 55.3% crude protein and 52.2% true protein when grown on pineapple cannery effluent. Forage (1978) and Prior et al (1981) also reported similar values. The higher protein concentration suggests that this yeast would be suitable as a supplement in cattle feed (Nigam 1998). The biomass produced in sugar cane refinery effluent had a lower total protein content of 31.3% (Pessoa et al., 1996).

In the case of selling a protein derived from a furfural-based wastewater, an important quality control issue must be addressed. Since furfural is a toxic and mutagenic chemical to animal cell systems, it must be assured that there are not significant amounts of biosorption in the yeast cells (BIBRA Toxicology International, 1991).

It has been found that the growth rates of yeast such as *C. tropicalis* and *C. utilis* have shown to be inhibited by increasing concentrations of furfural. Palmqvist et al. (1998) analyzed the inhibitory effects of furfural and acetic acid. The following entry discusses the growth rate (μ) and yield (Y_x) effects of acetic acid, furfural, and furfural combined with acetic acid.

> "μ was not significantly influenced by acetic acid alone (up to 10 g/L), whereas furfural (0–3 g/L) decreased this response variable in the whole concentration range tested. Acetic acid and furfural interacted negatively on μ. Like μ, the growth yield (Y_x) was not significantly influenced by acetic acid alone in the concentration range tested. In contrast to the effect on μ, however, the effect of furfural up to 2 g/L on Y_x, in the absence of acetic acid, was a slight stimulation with a maximum yield obtained at around 1 g/L, according to the fitted statistical model. Higher concentrations however decreased the yield. No significant influence was detected on the fermentation of either bakers' yeast or ATCC 96581 by acetic acid (up to 10 g/L) or furfural (up to 2 g/L) in the study of the three yeast." (Palmqvist et al., 1998)

In addition, it has been found that the specific growth rate of other yeast species like *S. cerevisiae* decreases with increasing furfural concentration within the range of 0-2 g/L. However, due to its rapid bioconversion the inhibitory effects of furfural decrease with increasing cell mass concentration (Boyer et al., 1992; Navarro, 1994). Furfural inhibition was transient, causing a pronounced lag phase before the exponential growth phase while the furfural was being metabolized. Furfural is metabolized to furfuryl alcohol, a less inhibitory compound. At the end of the lag phase, the maximum growth rate was 85–95% of the uninhibited maximum growth rate (Palmqvist et al., 1998).

It was determined by High Performance Liquid Chromatograph (HPLC) that the raw wastewater from the Quaker Oats facility contained approximately 180 mg/L of furfural. This value was significantly less than the 2 g/L tested in the aforementioned research. Therefore, based on inhibitory effects, yeast appear to be prime candidates for degrading furfural-based wastewater produced by manufacturers.

Temperature is another main concern regarding the biodegradation of the furfural-based wastewater. It costs money to run a cooling tower to bring the wastewater from temperatures as high as 110°F (43°C) down to the mesophilic range of 25-40°C. Hottiger et al. (1987) ran batch tests on *S. cerevisiae* between 27-40°C; and Lim et al. (2003) and Ortiz et al. (1996) both performed batch tests at 30°C. It appears from these research groups that the temperature is less than 40°C, therefore, Quaker Oats must cool the wastewater temperature so that it is in the optimum growth range for *C. tropicalis* and *C. utilis*. The cooling may be achieved by the airflow within an aeration basin. A decrease in the temperature of the effluent wastewater costs additional money, so finding the optimum temperature at which each species of yeast grows is a practical exercise. Optimizing the growth temperature of the yeast will eliminate unnecessary cooling of the process' waste effluent and consequently reduce cooling costs.

Furfural production involves the dehydration of corn stalks and other cellulose containing materials. Since these products have extremely low amounts of nitrogen included in them, the resulting wastewater stream is also nutrient deficient. Specifically, nitrogen is very dilute and the high strength of the wastewater results in an extremely high C/N ratio. As a result, for microorganisms to survive and thrive on the carbonaceous portion of the waste stream, a minimum amount of nitrogen must be present for cell synthesis and maintenance.

The nutrient properties of the raw wastewater shown in Table 2 indicate that, for an acceptable C/N ratio, nitrogen is lacking. Therefore, nitrogen must be added in the form of ammonia so that yeast can readily utilize it in cell processes. Yeast can directly assimilate ammonia into amino acids such as glutamine. Glutamine is absolutely required as a

precursor in the synthesis of asparagine, tryptophane, histidine, arginine, carbamoyl phosphate, CTP, AMP, GMP, glucosamine, and NAD (Feldmann, 2001).

However, adding chemicals that cost significant amounts of money to a wastewater treatment system could be careless without first determining an approximate dosage. If too much nitrogen is added to the process, then excess nitrogen will simply wash out of the system with the treated effluent. As a result, more money is spent on supplements than necessary. Conversely, if too little ammonia-nitrogen is added, the full capacity for yeast growth may not be met and the wastewater may not receive optimum levels of treatment.

Examples of Yeast in Wastewater Treatment Processes

To demonstrate that the use of *C. tropicalis* and *C. utilis* for biological wastewater treatment and simultaneous protein production is feasible, this section reviews research that has been performed on the topic.

The yeast *Yarrowia lipolytica* ATCC 20255 was used to biodegrade a wastewater produced by an olive oil production operation. The yeast was found to reduce the COD level by 80% in 24 hours and produce a useful biomass of 22.45 g/L. Considering the fact that the initial COD of the raw wastewater is between 100,000 and 200,000 mg/L, 80% removal efficiency is a significant savings on both cost of discharge and the capacity of nearby treatment plant that are receiving the waste stream (Scioli and Vollaro, 1997). For a wastewater with such high strength, a typical aerobic activated sludge system will produce inordinate quantities of bacterial biomass. Even the low growth coefficients of an anaerobic contact system treating such waste would produce significant amounts of waste sludge. Therefore, aerobically treating such high strength wastewater while creating a valuable, protein-rich byproduct is ideal.

Recent issues regarding fuel supply, price, and the emissions from vehicular exhaust has sparked interest in alternative fuels. Ethanol, for example, has been fermented from corn and the idea of producing ethanol from molasses by flocculating yeast for use as an alternative energy source was evaluated by Kida et al. (1997). Their studies found that ethanol could be

produced at a rate of 25,000 mg $h^{-1}L^{-1}$ of reactor volume with a final ethanol concentration of 63,000 mg/L. Although less efficient than fossil fuels with respect to power, ethanol burns significantly cleaner and thus, reduces greenhouse gas emissions. Alternative energy sources to fossil fuels are becoming more abundant and cost-effective as technology in the market advances. However, in this particular study, the end use of the biomass itself was not discussed. Perhaps to make this entity less costly, the yeast biomass could be sold to help support the ethanol production operation.

Lim et al. (2003) conducted a study to investigate the feasibility of using fish-processing wastewater as a means for supporting *Candida rugopelliculosa* as feed for the rotifer, *Brachionus plicatilis*. *B. plicatilis* was, in turn, used as diet for the fish fry being raised at the hatchery. *C. rugopelliculosa* was cultivated in aerated continuous stirred tank reactors, which were maintained at a temperature of 30°C and controlled at a Ph of 4.0. Using Monod kinetic equations and a 4^{th} order Runge-Kutta method to evaluate the growth rate, μ_m was found to be $0.82\pm0.22h^{-1}$ and K_s was 690±220 mg SCOD/L. Since this is a closed-loop system where the waste stream is used to feed the head of the process, heavy metals must be monitored to assure that they are not accumulating in the fish.

Another market where yeast use has proven beneficial is in the milk and dairy sector. The acceptance of dry yeast by ruminants is high. Thus, using them to feed milk cows significantly enhances the protein content of the milk and the digestibility of the dry matter (D'Arce et al., 1985).

These four instances of yeast production from waste streams show how versatile and beneficial the microorganisms can be. They can produce valuable byproducts such as ethanol or can be fed to larger organisms. Furthermore, they are tolerant of high strength wastes as was demonstrated by the research performed by Scioli and Vollaro (1997) on olive oil wastewater. As more is learned about the process controls and growth conditions of yeast, more opportunities will arise where they may be employed. Furthermore, with regards to the current research project, yeast proved to be prime candidates for the degradation of

furfural-based wastewater. *C. tropicalis* and *C. utilis* are not inhibited by furfural as dramatically as bacteria are and they are able to degrade high-strength wastewater both aerobically or anaerobically. Since aldehydes do not degrade well anaerobically and in this case were contained in a high-strength wastewater, yeast appeared to be a strong choice for the situation.

CHAPTER 4
MATERIALS AND METHODS

The materials and methods used in the present research project are outlined in this chapter. The materials section outlines the equipment and chemicals utilized over the course of the work. The methods section covers the procedures used to grow the inoculant and to prepare batch tests for temperature and C/N ratio variation. Furthermore, the methods section covers the lab procedures used in analyzing samples for ammonia and Total Kjeldahl Nitrogen (TKN).

Materials

The table on the following page (Table 2) lists the materials utilized over the course of the research project. The items with stock or model numbers will have them listed in the second column.

Raw Wastewater	Magnetic Stir Plate
Distilled Water	Vacuum Filtration Pump
Candida tropicalis	Refrigerator
Candida utilis	Desiccator
0.5N NaOH Solution	Incubator
NH_4Cl	Balance
K_3PO_4	HPLC
$CaCl_2$	105°C Oven
$MgCl_2$	550°C Furnace
EDTA Ammonium Probe Solution	250 ml Erlenmeyer Flasks
Nutrient Broth	Magnetic Stir Bars
Ph Meter	Foam Flask Stoppers
Ammonium Meter	Alcohol Thermometer
Filter Paper (GFC)	

TABLE 2- List of Materials Used

Methodology

Raw Wastewater Sampling and Storage

The raw wastewater was sampled directly from the wastewater effluent stream at the Quaker Oats furfural production facility in Cedar Rapids, Iowa. The faucet from which the samples were taken receives wastewater after it has gone through the cooling tower. 15-gallon containers with screw-on caps were filled with the raw wastewater and transported to the laboratory at Iowa State University via pickup truck. Upon arrival, the containers were moved into the laboratory and the Ph was adjusted to approximately 1.5 with stock sulfuric acid (H_2SO_4). This was done to preserve the wastewater and prevent biological growth prior to use. One pickup truck load of wastewater would typically last 6-8 months, and this is the reason behind why two different samples of wastewater had to be used for the temperature and nutrient variation phases of this project.

Batch Test Overview

Batch tests were performed to investigate the effects of temperature and carbon to nitrogen ratio on the growth rate of *C. tropicalis* and *C. utilis*. By employing batch tests, it is possible to observe the four distinct growth phases of a microbial population. The four growth phases of microorganisms are the lag, exponential growth, stationary, and death phases.

The lag phase is the period of time when the microbial cells adjust to the new environment. The duration of the lag phase depends upon the cell's prior history, exposure to chemical agents, and culture medium. In the case of *C. tropicalis* and *C. utilis*, the lag phase lasted anywhere from 6 to 39 hours. The exponential growth phase, also known as the log phase, occurs once the cells have acclimated to the new environment and begin new cell synthesis. Cells in the exponential growth phase are more sensitive to chemical and physical factors than in the stationary phase. Due to a lack of nutrients and electron acceptors and donors, cell reproduction equalizes with the death rate and the cell population reaches the stationary phase. Following the stationary phase is a period where the decay rate is higher than the growth rate. This phase is known as the death phase and is distinguished by increased cell lysis (Bitton, 1999).

Inoculum Preparation Procedure

To acclimate each of the two species of yeast to the furfural in the wastewater, the inoculants were grown in a diluted sample of the raw wastewater. This acclimation was used to reduce the aforementioned lag time associated with microbial growth and effectively reduces the duration of each batch test. First, both *C. tropicalis* and *C. utilis* were grown on a sterile agar plate at room temperature (27°C) until sufficient colonies formed. Colonies of sufficient size for sampling usually formed within a week. Second, nutrient broth was prepared with distilled water and YM nutrient broth, then autoclaved at 250°F for 30 minutes. The precaution was taken to autoclave the nutrient broth to minimize the possibility of competitive microorganisms. However, the raw wastewater was not sterilized in any of the batches because of the possibility of altering its chemical composition. Inoculating the raw wastewater with a relatively high concentration of yeast gives the yeast a competitive advantage over any other viable organism that may be present in the raw wastewater.

Next, the autoclaved nutrient broth was inoculated with its respective species of yeast from a colony on the agar plate. The inoculated nutrient broth was, then, grown on a stir plate in an incubator at 35°C for four days. After four days, the nutrient broth culture was removed from the incubator and stored in a refrigerator at 4°C. The culture grown in the nutrient broth was then added to a diluted volume of raw wastewater that had been dosed with nutrients (N, P, Ca, and Mg). Calcium and magnesium were added at the concentration of 1.0 g/L and 0.5 g/L respectively. The carbon to nitrogen to phosphorus (C/N/P) ratio of the raw wastewater was attuned to 100:10:1 prior to inoculation, Ph adjustment, and dilution of the solution. Once the supplemented wastewater had been inoculated and diluted, the solution was placed in an incubator at 35°C for four days. After four days, the acclimated yeast culture was removed from the incubator and placed in a 4°C refrigerator for storage. The same flasks of acclimated yeast inoculant were used throughout the temperature variation portion of the project.

Batch Test Procedure for Temperature Variation

The acclimated yeast inoculants prepared according to the procedure explained in the previous section of this report were used to inoculate temperature-varied batch tests. The batch tests were conducted at temperatures of 27, 30, 35, 37, 40, and 45°C. Since yeast are mesophilic microorganisms, the temperatures were selected to cover the mesophilic temperature range. The C/N/P ratio of each batch test was controlled at 100:10:1 so that nitrogen was not a limiting growth factor. To assure each batch received the same nutrient dosage, a four-gallon container of raw wastewater was mixed with the proper amounts of nutrients. This four-gallon container was used to supply wastewater for all of the temperature varied batch tests.

To begin a batch test, 45 mL of nutrient-dosed wastewater and 36.3 mL of yeast inoculant were combined in a 1.0 L beaker. A value of 50 mg/L for an initial biomass concentration was arbitrarily selected as the initial Total Suspended Solids (TSS) concentration. Therefore, the 36.3 mL volume of inoculant was the result of the 620 mg/L inoculant TSS concentration and the initial batch test TSS concentration of 50 mg/L. In addition, a volume of 36.3 mL at 620 mg/L translates into 22.5 mg of biomass added to each 450 mL batch test.

After the inoculant was added to the nutrient-dosed wastewater, the pH was then adjusted from 2.5 to 4.5 with 0.5N sodium hydroxide (NaOH) solution. An initial pH of 4.5 was selected because, as was mentioned earlier, yeast thrive in acidic conditions. Although the initial pH was controlled, the pH during the batch test was neither controlled nor monitored. Once the pH was adjusted, the sample was diluted ten times (10^{-1}) to 450 mL and divided into three sterile, 250 mL Erlenmeyer flasks. The flasks contained a magnetic stir bar and were capped with a cotton stopper to allow for oxygen transfer within the vessel. Once the solution was divided and the flasks capped, they were placed in an incubator previously set at the appropriate temperature for that particular batch test. To minimize the possibility of allowing foreign microorganisms into the flasks, flasks were sampled until empty. Reducing the number of times the cotton stopper is removed reduced the chance for bacteria and fungi to enter and contaminate the solution. This procedure was utilized for both species of yeast.

Thirty (30) mL samples were taken every 4-10 hours depending upon the phase of the microbial growth. For instance, during the lag phase yeast do not grow rapidly, if at all. Therefore, samples were initially taken every 10 hours. However, once the exponential growth phase began, samples were taken every 4-5 hours to obtain a more accurate representation of the growth rate and COD removal rate. After the apparent density of the yeast solution had ceased to increase in opacity, sampling was stopped. Immediately after each sample was taken, the sampling container was labeled with the date, time, and yeast species and then stored in a freezer. When all the samples had been collected and frozen from a batch test, they were thawed at room temperature and immediately analyzed for total suspended solids (TSS), volatile suspended solids (VSS), and COD.

Batch Test Procedure for C/N/P Ratio Variation

The procedure for initiating a batch test evaluating the effects of C/N/P ratio on the growth rate of yeast is similar to the one described in the previous section. However, over the course of this segment of the project, the original acclimated inoculants ran out and were prepared again. The new sample of wastewater used to acclimate the yeast was of a higher concentration than the original wastewater sample (17,000 mg/L), so more nutrients had to be added to reproduce a C/N/P ratio of 100:10:1 than were added to the original inoculants. The inoculants were prepared in the same manner as before and resulted in a similar suspended solids concentration. Therefore, the previous inoculant dosage of 36.3 mL per batch test was held constant. Although the same biomass was added to each batch test, the initial solids concentration was considerably higher (~100 mg/L). The increase in suspended solids was due to the use of a second batch of wastewater with an elevated COD concentration in comparison to the original four-gallon container used in the temperature-variation batch tests.

The temperature of the batch tests varying C/N/P was selected to be 35°C based upon preliminary results obtained in the temperature variation portion of the project. C/N/P ratios ranging from 100:0:1, 100:1:1, 100:3:1, and 100:5:1 resulted from gradually increasing the

C/N ratio from zero until the growth rates of the yeast were similar to those exhibited in the 35°C temperature-varied batch tests with a C/N/P of 100:10:1.

The sampling procedure used for the C/N/P-variation batch tests was the same sampling procedure used for the temperature-variation batch tests. Samples were labeled and subsequently stored in a freezer until being analyzed for COD and suspended solids concentrations. All samples were stored in a freezer for no more than one week before being analyzed.

Lab Analysis Procedures

All of the samples taken from the batch tests were analyzed using the procedures outlined in Standard Methods (American Public Health Association, 1998) for TSS, VSS, and soluble COD. Standard Methods was also referenced when analyzing samples for ammonia and TKN. Ammonia concentration was measured using the ammonia probe listed in the Materials and Methods section of this paper. Four standards (0.1, 1, 10, and 100 mg/L) were analyzed along with the samples. Table 3 summarizes the results of the baseline nitrogen and phosphorus sample analysis. The raw wastewater has a SCOD concentration of approximately 14,500-17,000 mg/L and results in an extremely high C/N ratio between 2200 and 2575.

Sample	TKN mg/L as N	NH_3-N mg/L as N	Organic-N* mg/L as N	Total Phosphorus mg/L as P
Raw Wastewater	6.6	1.5	5.1	6.7
C. tropicalis Inoculant	1590	1406	184	29.0
C. utilis Inoculant	366	336	30	106.9

TABLE 3- Baseline TKN, Ammonia, and Total Phosphorus Concentrations

- Organic-N was found by subtracting the NH_3-N from TKN.

Data Analysis Procedures

The procedure for analyzing the data collected is similar to that used by Lim et al. (2003) for their research on yeast growth in fish-processing wastewater. A 4^{th}-Order Runge-Kutta

approach was taken to simultaneously solve two differential equations relating substrate uptake and biomass growth through Monod growth kinetics. Specifically, a Microsoft Excel spreadsheet created by Barbeau (1996), was referenced and modified to fit the current situation. The original spreadsheet input was the oxygen uptake rate (OUR) from a respirometer whereas the current research input was SCOD and VSS concentration (Ellis et al., 1996).

The Monod equation has been extensively used to accurately model microbial growth in batch and continuous culture (Tobajas et al., 2003). Therefore, it has been selected for use in the current project as well. Equations (6) and (7) describe the relationship between biomass concentration, X (mg/L), yield, Y (%), maximum specific growth rate, μ_{max} (h^{-1}), substrate concentration, S (mg/L), and the half-saturation coefficient, K_s (mg/L). The equations are simultaneously solved by varying μ_{max} (h^{-1}) and K_s (mg/L) to minimize the squared error between the Runge-Kutta model and the observed data.

$$\frac{dx}{dt} = \frac{(\mu_{max} \cdot S) \cdot X}{(K_s + S)} - k_d \qquad (6)$$

$$\frac{ds}{dt} = - \frac{(\mu_{max} \cdot S)}{(K_s + S)} \cdot \frac{X}{Y} \qquad (7)$$

The maximum specific growth rate (μ_{max}) is the maximum rate at which new cells are produced per biomass already present. μ_{max} is a function of the initial substrate concentration and environmental conditions. Since μ_{max} is a rate coefficient, it is directly affected by temperature. The half-saturation constant, K_s, is the substrate concentration where μ is equal to one-half of μ_{max}. K_s describes how substrate concentration affects the specific growth rate (Grady et al., 1999).

The yield is the ratio of the amount of biomass produced to the amount of substrate consumed in the oxidation process. It is a parameter that may be approximated stoichiometrically by assuming a chemical composition for biomass, possibly $C_5H_7O_2N$, and

the primary electron donor. The number of moles of biomass produced is multiplied by the molecular weight of biomass and then divided by the number of moles of electron donor used in the balanced chemical equation multiplied by its molecular weight.

When selecting the time increment (Δt) for the model it was observed through trial and error that Δt's greater than 0.5 hours produced highly variable and inaccurate results. For simplicity and accuracy, Δt for the model was set at 0.5 hours for all of the data analysis. 0.5 hours proved to be small enough to provide satisfactory results.

The biomass yield (Y) of each batch test was calculated based on the increase in volatile suspended solids and the removal of bsCOD. By definition, the yield is the mass of biomass COD formed per mass of substrate COD removed. Therefore, this value could simply be calculated for each batch test. Since the TSS concentration in the raw wastewater was so low, it was assumed that the increase in VSS concentration was directly due to biomass growth. It was further assumed that 1-gram of biomass produced 1.42 grams of COD (Tchobanoglous et al., 2003). Therefore, biomass yield could be calculated in terms of COD.

Before the model could be compared to the observed data, the observed data had to be adjusted for the non-biodegradable soluble microbial products (NBSMP) that arose from biomass growth. The products probably remained in tact because neither species of yeast had the capability to produce the enzyme necessary to degrade them. These soluble microbial products likely consisted of enzymes and high molecular weight organic chains from the membranes of lysed cells and were assumed to be non-biodegradable by either species of yeast in the present conditions. The NBSMP was accounted for by dividing the remaining soluble COD by the change in biomass concentration. Therefore, a linear relationship was assumed between NBSMP and biomass growth. From this procedure, an average rate of NBSMP production per mass of biomass formed was established. Next, the point at which biomass increased (i.e. the lag phase ended), the NBSMP rate was multiplied by the biomass concentration (mg/L COD) to get a NBSMP concentration in terms of COD. The NBSMP COD was then subtracted from the observed soluble COD value to obtain a true

biodegradable soluble COD (bsCOD) value. The bsCOD value was then used to create the Runge-Kutta model. Biodegradable soluble COD is used to quantify concentration because "it easily relates to the stoichiometry of substrate oxidized or used in cell growth" (Tchobanoglous et al., 2003).

Finally, the "Solver" function of Microsoft Excel was employed to iterate through the function until the model converged on values of μ_{max} and K_s that resulted in the least squared error. Therefore, these μ_{max} and K_s values were assumed to be the most accurate approximation of the kinetic parameters for the given conditions. The Runge-Kutta procedure was performed on the lab sample data of each batch test run, and the results of each were compared. The "Results and Discussion" chapter of this paper displays the data in both tabular and graphical form.

Growth Rate Calculation

The equations utilized in the calculation of the Monod kinetic parameters for *C. tropicalis* and *C. utilis* are summarized in this chapter. After the initial observed data (S_0, X_0, Y and lag time) and assumed data (k_d, μ_{max}, and K_s) are input into the following equations, the sum of squared error (SSE) between the adjusted observed data and theoretical output is minimized by iterating through the calculation until values of μ_{max} and K_s are converged upon. It was assumed that 85% of the VSS in the samples was biomass and that there are 1.42 mg/L of COD per gram of biomass. The COD value of the biomass was denoted as $X_{observed}$ in the following procedure.

After several runs using trial and error, it was found that the Runge-Kutta model output better results with smaller time interval (Δt, h^{-1}). Δt was held constant at 0.5 hours for each batch test.

$$k_{01} = \Delta t\left[\left(\frac{\mu_{max} \cdot S_{t-1}}{K_s + S_{t-1}}\right) - k_d\right] \cdot X_{t-1} \qquad (8)$$

$$k_{02} = -k_{01}/Y \tag{9}$$

$$k_{11} = \Delta t\left[\frac{(\mu_{\max}\cdot(S_{t-1}+1/2\cdot k_{02})(X_{t-1}+1/2\cdot k_{01})}{K_s + S_{t-1}+1/2\cdot k_{02}}\right] \tag{10}$$

$$k_{12} = -k_{11}/Y \tag{11}$$

$$k_{21} = \Delta t\left[\frac{(\mu_{\max}\cdot(S_{t-1}+1/2\cdot k_{12})(X_{t-1}+1/2\cdot k_{11})}{K_s + S_{t-1}+1/2\cdot k_{12}}\right] \tag{12}$$

$$k_{22} = -k_{21}/Y \tag{13}$$

$$k_{31} = \Delta t\left[\frac{(\mu_{\max}\cdot(S_{t-1}+1/2\cdot k_{22})(X_{t-1}+1/2\cdot k_{21})}{K_s + S_{t-1}+1/2\cdot k_{22}}\right] \tag{14}$$

$$k_{32} = -k_{31}/Y \tag{15}$$

Once the above eight kinetic rate factors are calculated, they were input into Equations 16 and 17. The output from these equations became the next line of substrate and biomass concentrations for a given time "t".

$$S_t = S_{t-1} + 1/6\cdot(k_{02} + 2\cdot k_{12} + 2\cdot k_{22} + k_{32}) \tag{16}$$

$$X_t = X_{t-1} + 1/6\cdot(k_{01} + 2\cdot k_{11} + 2\cdot k_{21} + k_{31}) \tag{17}$$

The length of the lag phase was entered manually by examining the observed sample data. Once the cumulative batch test time passed the lag phase, the calculation from Equation 8 to 17 was initiated using an IF/THEN statement in Microsoft Excel. Prior to the SSE step, the observed data was adjusted for the production of non-biodegradable soluble microbial

products (NBSMP) by the yeast. These non-biodegradable products left a COD remainder after each batch test. The remainder was divided by the increase in biomass to obtain a ratio of NBSMP (mg/L) produced per mg/L of biomass. The ratio was multiplied by the biomass produced for the observed data point and then subtracted from the observed substrate concentration. Equation 18 shows the equation used and all values are in terms of COD.

$$S_{t_{adjusted}} = S_{t_{observed}} - \left(\frac{S_{remainder}}{TotalX_{produced}} \right) \cdot X_{t_{observed}} \tag{18}$$

Once the data was modeled, it was compared to the adjusted observed data by squaring the difference between the substrate concentrations and biomass concentrations. These differences were squared and then added together to obtain the SSE for a particular data point. Equation 19 displays the equation utilized.

$$SSE = \sum \left[(S_{t_{adjusted}} - S_{t_{theoretical}})^2 + (X_{t_{observed}} - X_{t_{theoretical}})^2 \right] \tag{19}$$

After the SSE was calculated for all of the observed data versus the theoretical data, the Solver function of Microsoft Excel was employed to minimize the SSE by adjusting the values of μ_{max} and K_s. When the Solver function converged on μ_{max} and K_s, the values were recorded for that particular batch test and used in the graphical and tabular additions to this paper.

CHAPTER 5
RESULTS AND DISCUSSION

The results of the preceding methodology are reported and discussed in this chapter. Upon the completion of the lab procedures and data analysis, the results were compiled and presented in the following section of this paper. The results and discussion were divided into two distinct headings for temperature and C/N ratio variation. The results obtained in this experiment were not specifically analyzed for a statistical confidence interval, but the standard deviation of both μ_{max} and K_s for the two yeast species combined was computed for each condition. The results of these calculations are displayed in Table 4.

Effects of Temperature Variation on the Growth Rate

As previously mentioned, temperature plays a significant role in the existence of all microorganisms and *C. tropicalis* and *C. utilis* are no exception. The sensitivity of each species to was evident as the growth rate of each decreased at both ends of the specified temperature range (27-40°C). Furthermore, neither species of yeast showed growth in the batch test performed at 45°C. Several retrials were started at 45°C, but none showed growth after extended time periods. Table 4 summarizes the combined average μ_{max} (h^{-1}) and K_s (mg/L) values for both species of yeast at each temperature tested. It should be noted that the μ_{max} and K_s values ranged from 0.23-0.41 (h^{-1}) and 1191-1834 (mg/L) respectively. The standard deviation for μ_{max} and K_s ranged from 0.01-0.08 (h^{-1}) and 29-311 (mg/L) respectively.

Temperature (C)	Average		Standard Deviation		First Order Rate
	μ_{max}	K_s	μ_{max}	K_s	μ_{max}/K_s
27	0.23	1191.18	0.08	311.22	1.97E-04
30	0.34	1408.88	0.02	49.69	2.43E-04
35	0.41	1789.75	0.01	153.79	2.30E-04
37	0.37	1488.22	0.01	17.29	2.46E-04
40	0.33	1834.48	0.04	29.86	1.81E-04

TABLE 4- Average μmax and Ks Values as Temperature Varied

Although neither *C. tropicalis* nor *C. utilis* showed signs of biomass growth at 45°C, there are yeast that can survive such conditions. These yeast are referred to as thermotolerant yeast. Barron et al. (1995) researched the yield coefficient of *Kluyveromyces marxianus* on media containing milled paper and commercial cellulase at 45°C. The yield achieved by *K. marxianus* was 21% of the theoretical yield.

Figure 1 displays the data in Table - 4 in graphical form and Figure 2 displays the average yield versus temperature. The trend lines of μ_{max} and the first-order rate coefficient interpret the raw data by the least squared error method. Microsoft Excel computed an equation to describe the trend line and the R-squared (R^2) value associated with that trend line. The R^2 is a statistic employed in regression analysis that measures how much variance has been explained by the regression model and is typically employed as a measure of "goodness of fit." Specifically, it is the proportion of the total variance in the dependent variable that can be explained by the independent variable (McClelland, 1999). When calculating the R^2 value of a trend line, the distance of the actual "y" value from the predicted trend line y-value is used. R-squared ranges from 0 to 1, and the higher the value of R^2, the more variance in y is described by the independent (x) variable. For instance, if all of the y-values lied on the trend line, the R^2 value would be equal to 1. In the case of this experiment, the two independent variables are temperature and carbon to nitrogen ratio (described later).

Through the Runge-Kutta method of solving differential equations, the values of the two kinetic variables, μ_{max} and K_s, both displayed parabolic relationships with respect to temperature. With R^2 values of 0.96 and 0.83 respectively, there appears to be a strong correlation between both μ_{max} and temperature and μ_{max}/K_s and temperature within the selected temperature range. The equation describing the trend line for μ_{max} was differentiated to find the temperature at which the relative maximum specific growth rate occurs. As a result, the derivative indicated that the peak of the average maximum specific growth rate occurred at a temperature of 35.1°C. Later in this section, both species of yeast underwent individual evaluations to determine the effect temperature had on each. In general, it has

been observed that the growth rate doubles with each 10°C increase in temperature up to the optimum temperature (Tchobanoglous et al., 2003).

The average biomass yield found at each temperature also showed a strong correlation with temperature as both μ_{max} and μ_{max}/K_s did. With the exception of one outlying point at 37°C, the yield follows a smooth parabolic curve increasing between 27-34°C and decreasing from 34-40°C as shown in Figure 2. The trend line for the yield was generated in the same manner as the trend lines for μ_{max}. In Figure 2, if the trend line excluded the outlying point at 37°C, the yield trend line had a high R^2 value (0.996). The derivative of the equation of the trend line indicated a peak yield at a temperature of 34.5°C, which was nearly the same temperature at which average μ_{max} peaks (35.0°C). Therefore, from the data collected in this experiment, it appeared that biomass yield increased with temperature from 27-34.5°C and decreased with higher temperatures.

Since yield is a stoichiometric factor between substrate utilization and biomass production, the rates of the two activities are proportional. Therefore with a higher yield, more biomass is produced per quantity of substrate utilized. So the energy harnessed by the yeast is being more efficiently transferred into biomass. Different microorganisms use different pathways of synthesis and degradation, so they have differing efficiency of energy generation and, therefore, yield. Furthermore, biomass grown in complex wastewater streams has a higher yield coefficient than biomass grown in simpler media. Other factors influencing the yield coefficient are the type of terminal electron acceptor and pH (Grady et al. 1999).

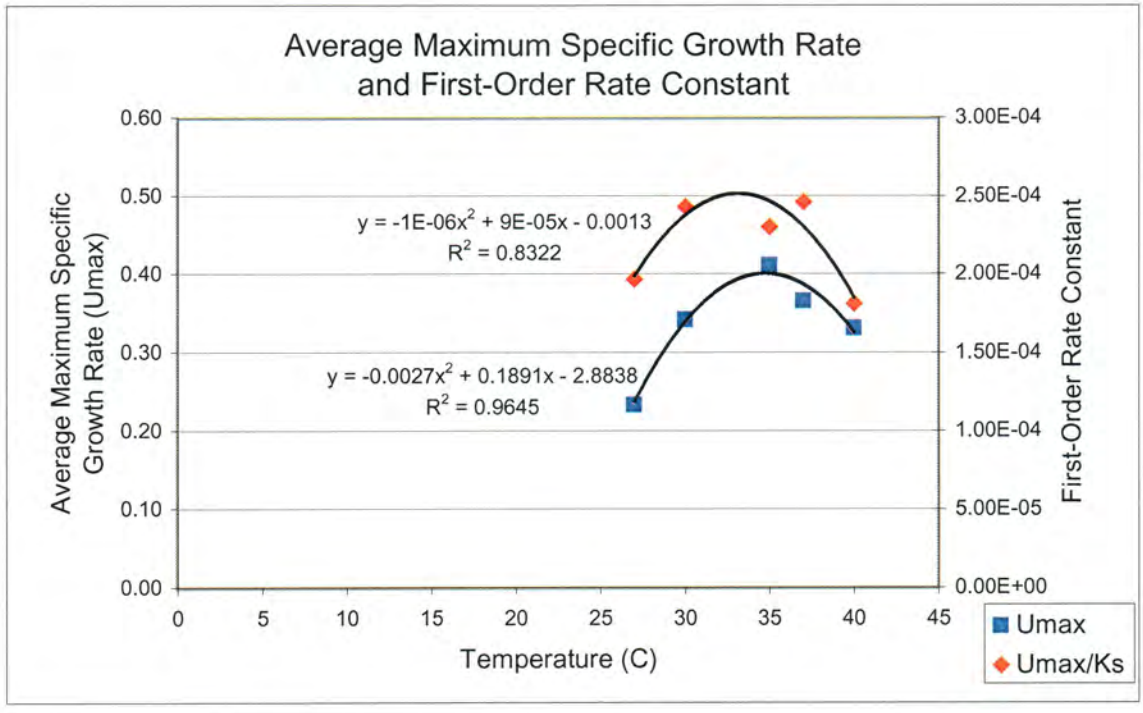

FIGURE 1- Average Max. Specific Growth Rate and First-Order Rate Constant

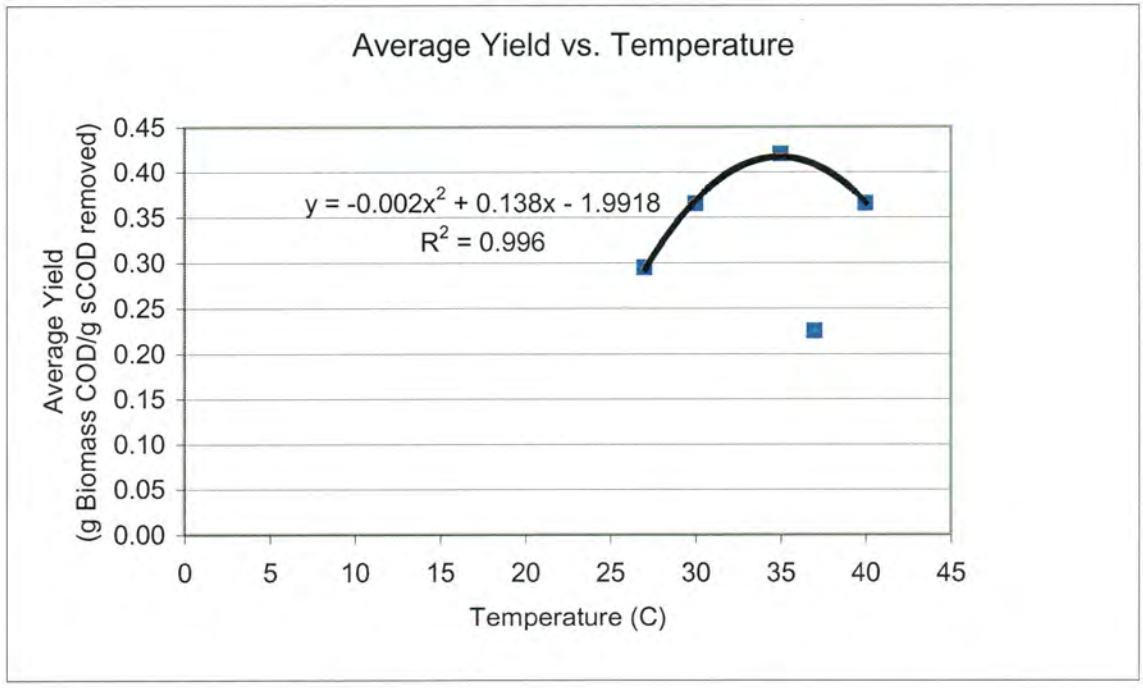

FIGURE 2- Average Yield vs. Temperature

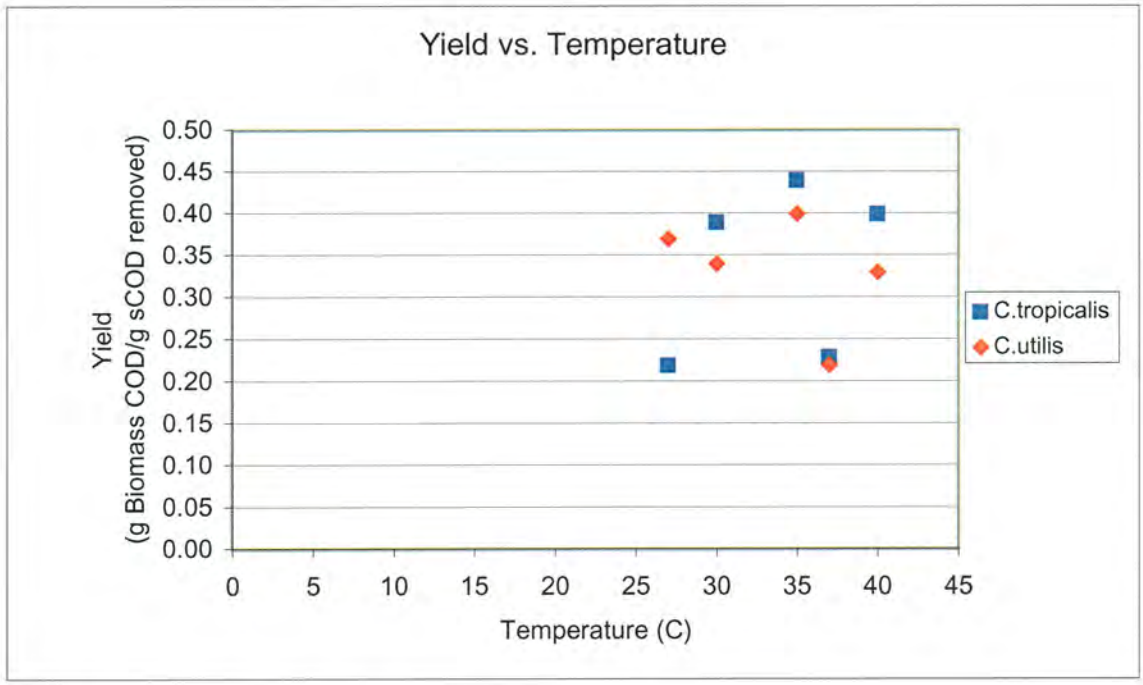

FIGURE 3- Yield vs. Temperature

Ahmad and Holland (1995) investigated the growth rate of *C. utilis* using glucose as substrate over a temperature range of 26-34°C. They reported that temperatures above 34°C had adverse affects on cell metabolism. Specifically, μ_{max} increased from 0.40 to 0.96 (h^{-1}) over the temperature range of 26 to 34°C respectively. Once the temperature exceeded 34°C, the opposite effects on maximum specific growth rate were observed, and μ_{max} decreased.

The observations of μ_{max} made over the course of this research project showed similar patterns to the results reported by Ahmad and Holland (1995). To demonstrate, Figure 1 displays the combined average maximum specific growth rate and the first-order reaction rate constant of *C. tropicalis* and *C. utilis* versus temperature. In Figure 1, the average μ_{max} increased between 27-35°C and decreased between 35-40°C. The first-order rate constant showed a similar tendency with the rate peaking at a temperature slightly less than 34°C.

In the present investigation of the reactions of both *C. tropicalis* and *C. utilis* to temperature variation, *C. utilis* appears to be slightly less sensitive to temperature change than *C.*

tropicalis as shown in Figure 4. This is denoted by the trend line of *C. utilis* being wider and flatter than the trend line of *C. tropicalis*. And the differentiation of the equations of the trend lines reveals that the peak μ_{max} occurs at 35.1°C and 33.3°C for *C. tropicalis* and *C. utilis* respectively. However, error may be associated with these values because only one batch test per temperature was sampled. Completing more batch tests per set of conditions would increase the statistical accuracy of the results. The graphical figures depicting the substrate removal and biomass production for each species and each batch test can be found in Figures 6 through 27 in Appendix A.

The optimum temperature for mesophilic bacterial growth is around 35°C and the microorganisms begin to die off between 40-45°C (MacKinney, 1962). These characteristics were also observed in *C. tropicalis* and *C. utilis*, which showed that they could be regarded as mesophilic microorganisms as well.

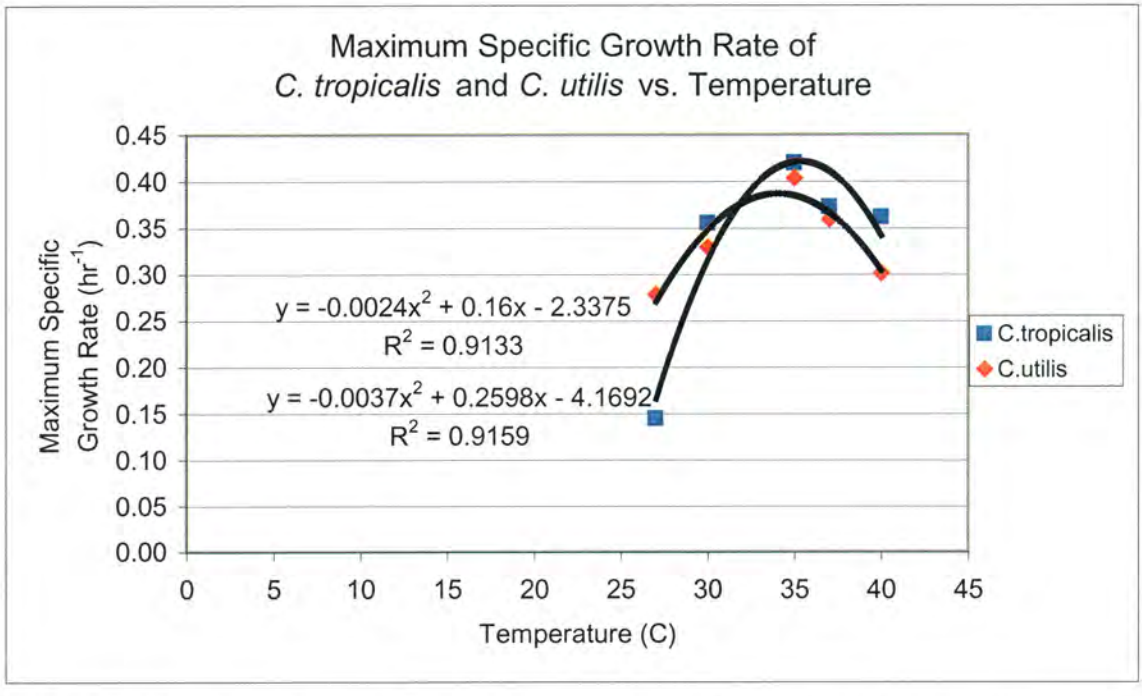

FIGURE 4- Max. Spec. Growth Rate of *C. tropicalis* and *C. utilis* vs Temperature

Trehalose Concentration Variation with Temperature

A question that must be addressed is the question of whether or not the increase in VSS concentration is actually an increase of the same magnitude in cell quantity. In yeast cells, trehalose is believed to function as a protectant against physical and chemical stressors. Trehalose is a carbohydrate stored in increasing amounts during periods of increasing temperature. The trehalose content also reduces as the temperature returns to a less stressful level (Hottiger et al., 1987). Ling et al. (1995) also investigated this topic using batch tests at varying temperatures. They counted the number of cells and measured the trehalose concentration at each temperature (30, 33, and 35°C) and found that the concentration did, in fact, increase with increasing temperature. However, the increased trehalose concentration was only found to occur during the exponential growth phase of *S. cerevisiae* and other yeast. Once the stationary growth phase was reached, the concentration of trehalose leveled off at a maximum. Then, during the death phase the trehalose content decreased with a decrease in number of viable cells. From these findings, the conclusion was drawn that trehalose may act as a "thermoprotectant" in yeast.

Therefore, if the trehalose concentration increased as temperature increased, it may be reasonable to assume that the average mass of an individual yeast cell increased as the batch temperature increased. If this is the case, then the masses weighed in the solids analysis of this project may not be as consistent between temperatures as first assumed. The yeast samples taken at higher temperature would indicate higher TSS and VSS concentrations and would be interpreted as having higher yield.

As was noted in the methods and materials section (Chapter 5) of this paper, the samples were taken from the incubator and immediately stored in a freezer. Over the course of freezing, and prior to solids analysis, the temperature decreased and the yeast may have released trehalose. If the trehalose was, in fact, released during freezing, it would negate any concerns over consistent average cell mass between temperatures. Kandror et al. (2003) demonstrated an adaptive response in yeast that was activated below 10°C and increased tolerance to low temperatures and freezing. Since trehalose is recognized as a

thermoprotectant, the response involved a "dramatic accumulation" of trehalose. The trehalose concentration increased as the temperature decreased below 10°C in order to protect the yeast cell from below-optimum temperatures.

Although the possibility of increased trehalose content in the yeast biomass exists, the determination of maximum specific growth rate is also based upon substrate removal. One of the assumptions relating to Monod growth kinetics is that the rate of biomass growth is proportional to the rate of substrate removal. Therefore, it is not known if the elevated trehalose concentrations significantly affect the maximum specific growth rate. Manginot et al. (1998) researched the nitrogen requirement of different strains of yeast and found that that the trehalose content of the different yeast ranged from 8.5–12.5% (by weight) at a temperature of 24°C. From the aforementioned findings of Ling et al. (1995), it is likely that the trehalose content was higher than this range at temperatures between 27-40°C. In fact, Wiemken (1990) found the concentration of trehalose to reach up to 35% of the dry weight of the cells. As a result, the values of μ_{max} in the present project may be slightly inaccurate, as they do not account for the elevated trehalose concentrations with increasing temperatures.

Effects of Carbon-to-Nitrogen Ratio on the Growth Rate

Mentioned earlier in this paper (Chapter 2), nitrogen is one of two major nutrients necessary for cell growth and maintenance with phosphorus being the other. Without sufficient nitrogen, the maximum specific growth rates of microorganisms are negatively affected. Liebig's law, "the law of the minimum," states that one nutrient restricts the maximum quantity of biomass that can be produced by a system, thus leaving all other nutrients in excess. This rule has also been applied to biomass growth. For instance, individual nutrient concentrations can be adjusted relative to each other such that the limiting nutrient in the growth media determines the maximum cell density that can be achieved in a culture (Egli and Zinn, 2003).

Carbon and nitrogen play a large part in fermentation because these nutrients are directly linked to the formation of the biomass. Casa Lopez et al. (2003) reported the effects of

different carbon and nitrogen sources and the C/N ratio on the fermentation *Aspergillus terreus* ATCC 20542 for producing lovastatin. Lovastatin is an ingredient used in cholesterol lowering medication and is more readily produced when nitrogen is the limiting nutrient. The influence of the C/N ratio was evaluated over the range of 14:1 to 50:1 compared with the ratio of 14:1 selected to correspond with the C/N ratio of the *A. terreus* biomass. It was concluded that the metabolic pathways for the synthesis of lovastatin from carbon were slower than the pathways for the synthesis of biomass from carbon. As a result, nitrogen limiting, and therefore growth suppressing conditions aid in the production of lovastatin by diverting more carbon to its synthesis.

However, to maximize the growth rate and biomass synthesis, nitrogen requirements must be met accordingly. Manginot et al. (1998) studied the nitrogen requirements of different strains of *S. cerevisiae* and made the assumption that yeast with higher nitrogen requirements would also have higher nitrogen content. Much to their surprise, the strains with the highest nitrogen requirements did not have the highest nitrogen content. In fact, when more nitrogen was taken up, biomass dry weight increased, and this synthesis was primarily related to an increase in glycogen content within the cells (Manginot et al. 1998).

Nitrogen requirements during the stationary growth phase vary depending on the strain. These differences appeared to be strain specific since they were independent of the nitrogen source and almost unaffected by the composition of the initial growth medium. A complete explanation for these differences was not attempted, but the nitrogen requirements of the different strains were more related to biomass synthesis than to final nitrogen content of the yeast cells. Strains with the lowest nitrogen demand were also the ones with the highest specific substrate utilization rates. Therefore, these strains appeared to be able to use nitrogen for protein synthesis more efficiently than the others (Manginot et al., 1998).

An important variable with respect to nitrogen addition is the form that it is added to the growth media. The three typical laboratory nitrogen supplements used are urea, ammonia, and nitrate. In the case of this research project, nitrogen was added in the form of

ammonium chloride, which dissociates in water to the ammonium ion. This is important because ammonia is the form in which microorganisms incorporate nitrogen during growth (Grady et al., 1999). Studies have been performed to analyze which nitrogen source produces higher growth rates and urea was found to be superior (Zhang et al. 1999 and others).

The effect of the C/N ratio on μ_{max} of *C. tropicalis* and *C. utilis* can be observed in Figure 5. In the present research project the C/N ratio was observed over a range of approximately 10:1 to 1000:1. Figure 5 demonstrates that the maximum specific growth rate appears highly sensitive to C/N ratio when it is less than 30:1. Specifically, the growth rate approximately doubles when the C/N ratio decreases from 30:1 to 10:1. From 30:1, the yeast do not respond as dramatically to increasing C/N ratios. This conclusion was drawn as the maximum specific growth rate decreased rapidly as the C/N ratio increased from 10:1 to 30:1. The change in growth rate significantly leveled off as the C/N ratio increased above 30:1. This is important to the topic at hand because it provides an approximate relationship between C/N ratio and μ_{max} for lower C/N ratios. As a result, nitrogen could be added to a unit process to obtain a desired growth rate. It would also be insightful to study a C/N ratio lower than 10:1 to see how the maximum specific growth rate would be affected.

Figure 6 was a plot of the half-saturation coefficient versus C/N ratio. The points followed a similar pattern as the plots seen in Figure 5. As C/N ratio increased K_s decreased. The rate of decline in K_s was dramatic at low C/N ratios, and it leveled off at C/N ratios greater than 75. Figure 7 showed the first-order rate coefficient while varying the C/N ratio for both species of yeast. There was considerable scatter in the data, but it appeared that the first-order rate coefficient increased from a C/N ratio of 0 to 100 and decreased for higher C/N ratios. The values seen in Figures 6 and 7 are the values shown in Table 5 on the following page.

C/N Ratio (adjusted)	*C. tropicalis*		First Order	C/N Ratio (adjusted)	*C. utilis*		First Order
	u_{max}	K_s	u_{max}/K_s		u_{max}	K_s	u_{max}/K_s
200	0.02	200	9.25E-05	1000	0.1	254	3.94E-04
67	0.14	314	4.50E-04	91	0.15	172	8.71E-04
29	0.20	675	2.89E-04	32	0.19	599	3.13E-04
18	0.24	247	9.54E-04	20	0.28	642	4.43E-04
10	0.42	1681	2.50E-04	10	0.40	1898	2.13E-04

TABLE 5- μ_{max}, K_s, and the First-order Rate Constant for *C. tropicalis* and *C. utilis*

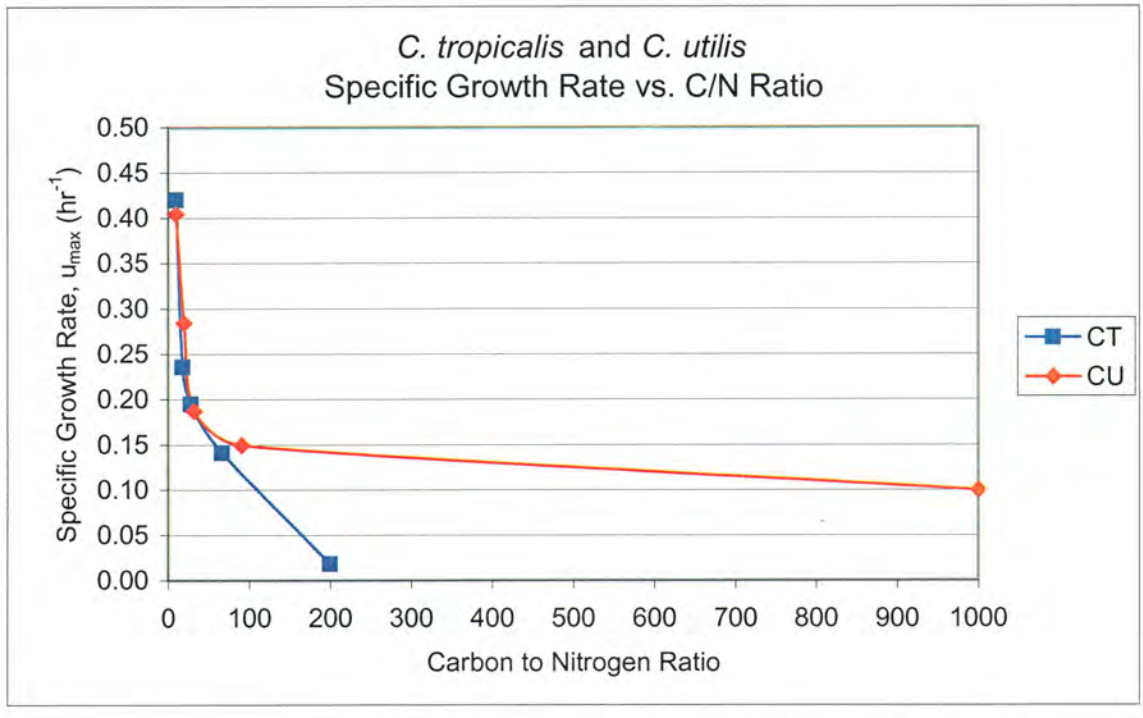

FIGURE 5- Max. Spec. Growth Rate of *C. tropicalis* and *C. utilis* vs C/N Ratio

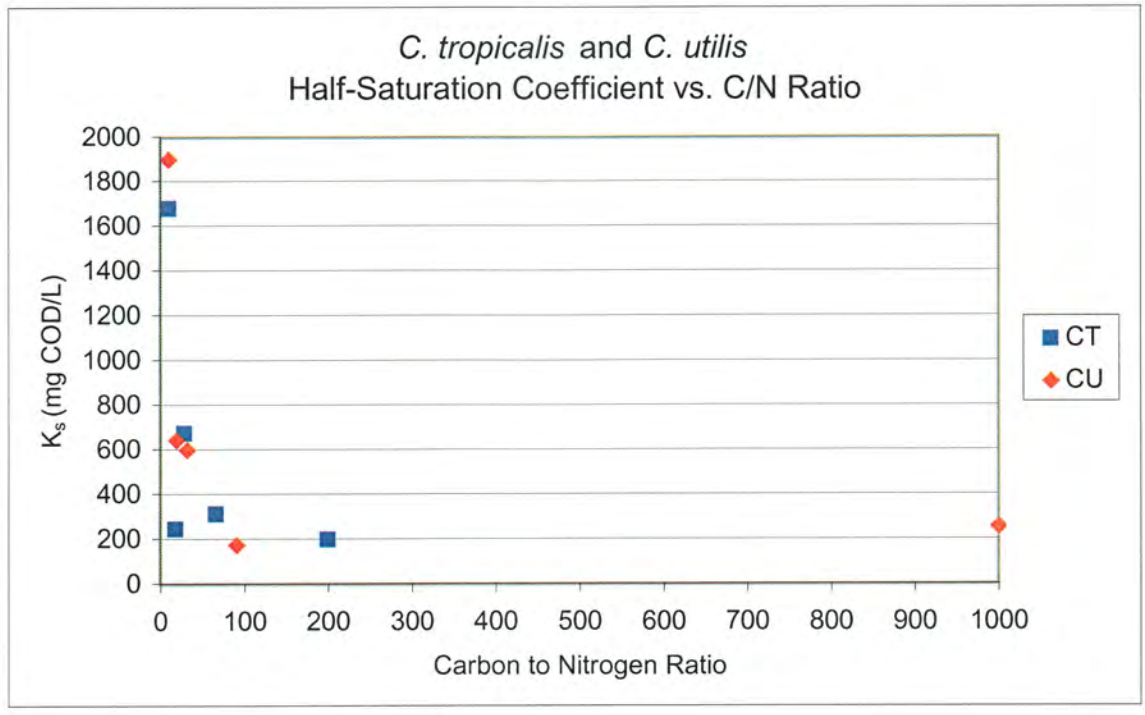

FIGURE 6- *C. tropicalis* and *C. utilis* Half-saturation Coefficient vs. C/N Ratio

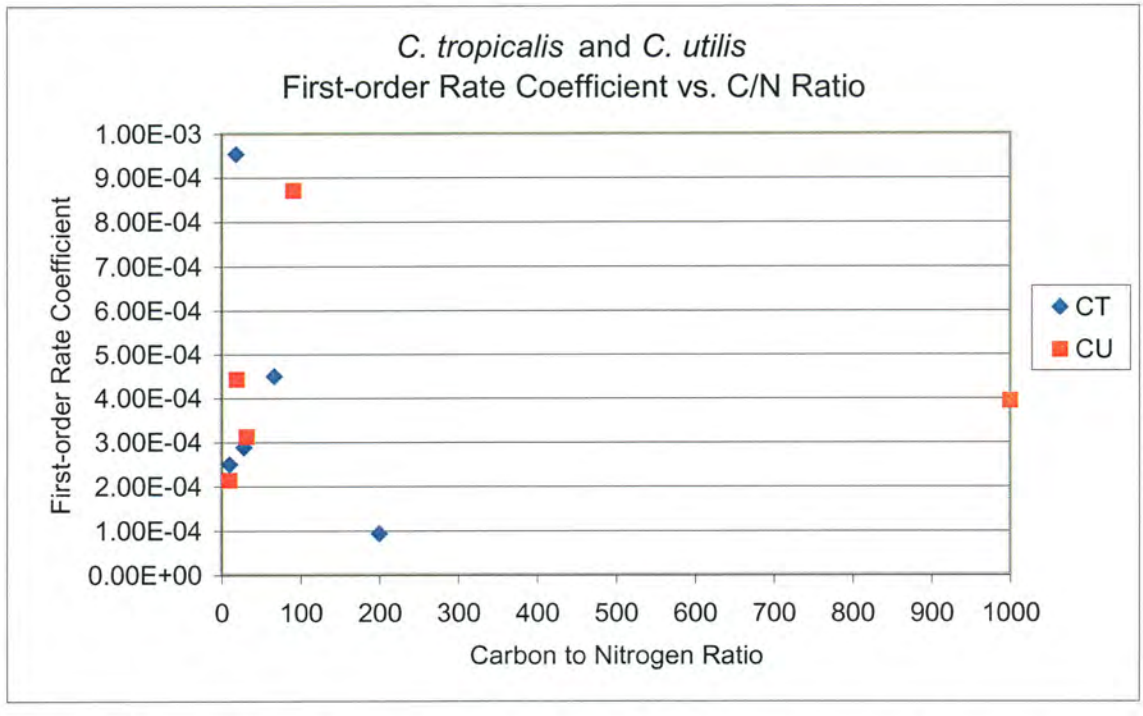

FIGURE 7 - *C. tropicalis* and *C. utilis* First-order Rate Coefficient vs. C/N Ratio

CHAPTER 6
ENGINEERING SIGNIFICANCE

In order to have a purpose, research must be applicable to outside areas. Simply knowing the maximum specific growth rate of yeast under specific growth conditions does little more than reaffirm or contradict the results of other research. Therefore, the significance of the present research to the environmental engineering field is covered in the following section.

The present research project holds biological, economic, and engineering significance. In addition, each facet of this research has ties to each of the three aforementioned areas of importance. No single property or characteristic can be important to one area without somehow affecting another. They are all interrelated so this section will default to referring to the engineering significance of this research.

This research project is important to the field of biological research in the respect that it provides a description of how *C. tropicalis* and *C. utilis* respond to variation in temperature and C/N ratio in terms of kinetic coefficients (μ_{max}, K_s, and Y). It also describes ranges of temperature and C/N ratio in which the yeast will grow and thrive. The viability of the yeast in these conditions was evaluated based on μ_{max}. So, a higher μ_{max} translated into an environment better fit for yeast reproduction.

Yeast such as *C. tropicalis* and *C. utilis* are important to the engineering community. These yeast can be utilized to degrade problematic industrial wastewater streams such as those from furfural production facilities. The yeast can also degrade wastewaters that are toxic to bacteria or for high-strength wastewaters that do not break down well in anaerobic conditions. In addition to COD reduction, yeast yield a significant amount of biomass under aerobic conditions. The biomass contains protein ranging between 30 and 50% by weight. Depending on the properties of the chemical(s) being treated in the wastewater stream, the yeast may be settled out of solution and used as feedstock supplement. Intuitively, if the chemical being treated is toxic to humans or livestock and significant amounts are adsorbed by the yeast, it will not be possible to sell the biomass for feedstock supplement. The

following point directly relates the engineering and economic importance of this research project: if the wastewater stream is benign with respect to toxicity in both humans and yeast, then considerable cost savings can be made on biomass disposal by either giving or selling the biomass to farmers. Also related to the biological significance of the present research, it has been found that mixing yeast biomass with livestock feed increases the animal's digestion capabilities as well as the protein content of the cattle and goat milk.

This research derived relationships between μ_{max} and temperature for two species of yeast. From these relationships, an optimum operating temperature for an aerobic reactor could be determined. Due to the fact that the influent wastewater was 110°F, a cooling tower was in place. The airflow through the cooling tower could be regulated to optimize the temperature of the influent wastewater. Thus, the temperature within the reactor could be directly controlled by airflow through the cooling tower.

This project also explained how the C/N ratio affected the growth rate of *C. tropicalis* and *C. utilis*. From this relationship, a growth rate could be selected and a correlating C/N ratio found. For a given wastewater stream having a known influent nitrogen concentration, it would be possible to supplement the influent with a nitrogen source in an accurate and efficient manner to achieve the desired μ_{max} for the yeast.

In summary, this research provided relationships between the temperature and μ_{max} and C/N ratio and μ_{max}.

CHAPTER 7
CONCLUSIONS

The information and insight brought forth by this paper is summarized in the following chapter. The chapter discusses the effects of temperature and C/N ratio on μ_{max} of *C. tropicalis* and *C. utilis.*

Evaluation of the temperature effects on μ_{max}

The objectives of this research project were to evaluate the effects of temperature and C/N ratio on the maximum specific growth rate of *C. tropicalis* and *C. utilis.* The wastewater treated in this project exited the facility at a relatively high temperature, so observing the effects that temperature has on both species of yeast was important. From the evidence collected, the specific growth rates of both species of yeast are sensitive to temperatures within the mesophilic range and do not grow in temperatures outside of that range. Therefore, it can be concluded that *C. tropicalis* and *C. utilis* are mesophilic microorganisms. *C. tropicalis* achieves its maximum μ_{max} at a temperature of 35.1°C while *C. utilis* reaches its peak at 33.3°C. The μ_{max} for these respective temperatures and species are 0.42 and 0.38 h^{-1}. The optimum temperatures achieved in the present research agree with many studies that have been performed at 30 and 35°C.

Evaluation of the C/N ratio effects on μ_{max}

The raw wastewater stream used in this project had a high C/N ratio of 2575:1, which makes it difficult for yeast to support cell maintenance and reproduction. Therefore, it is critical that the C/N ratio be decreased to more habitable levels to promote growth. The data obtained in this project indicated that C/N ratios below 30:1 significantly increased the maximum specific growth rates of both species of yeast. Decreasing the C/N ratio from 30:1 to 10:1 increased the μ_{max} of *C. tropicalis* from 0.20 to 0.48 h^{-1} and that of *C. utilis* from 0.18 to 0.41 h^{-1}. Therefore, from a plant operator standpoint, it would be advised that the C/N ratio be lower than 30:1 to promote yeast growth. On the other hand, the high C/N ratio could be used to select for yeast and prevent bacterial contamination in the reactor. Yeast

yield would decrease in this case, but bacterial growth would be diminutive. Perhaps periodic C/N ratio increases in a system could sufficiently select for yeast without having to retard the growth rate at all times. These questions are beyond the scope of this project and could be answered in further research efforts.

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APPENDIX

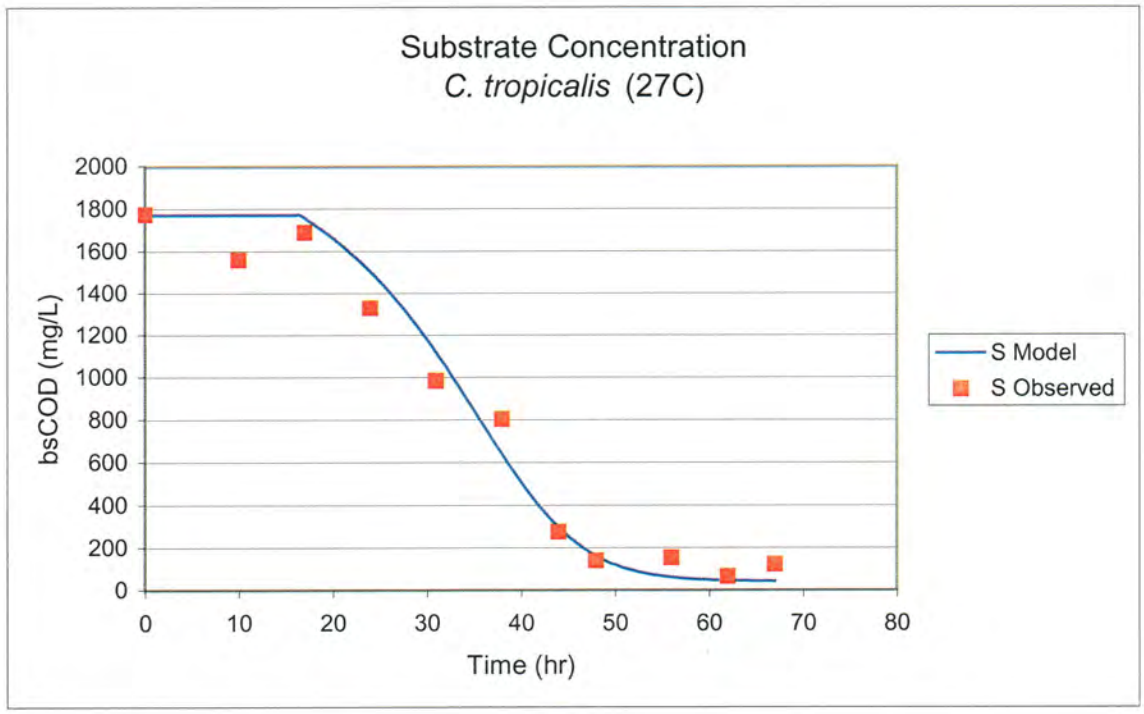

FIGURE 8- Substrate Concentration of *C. tropicalis* at 27°C

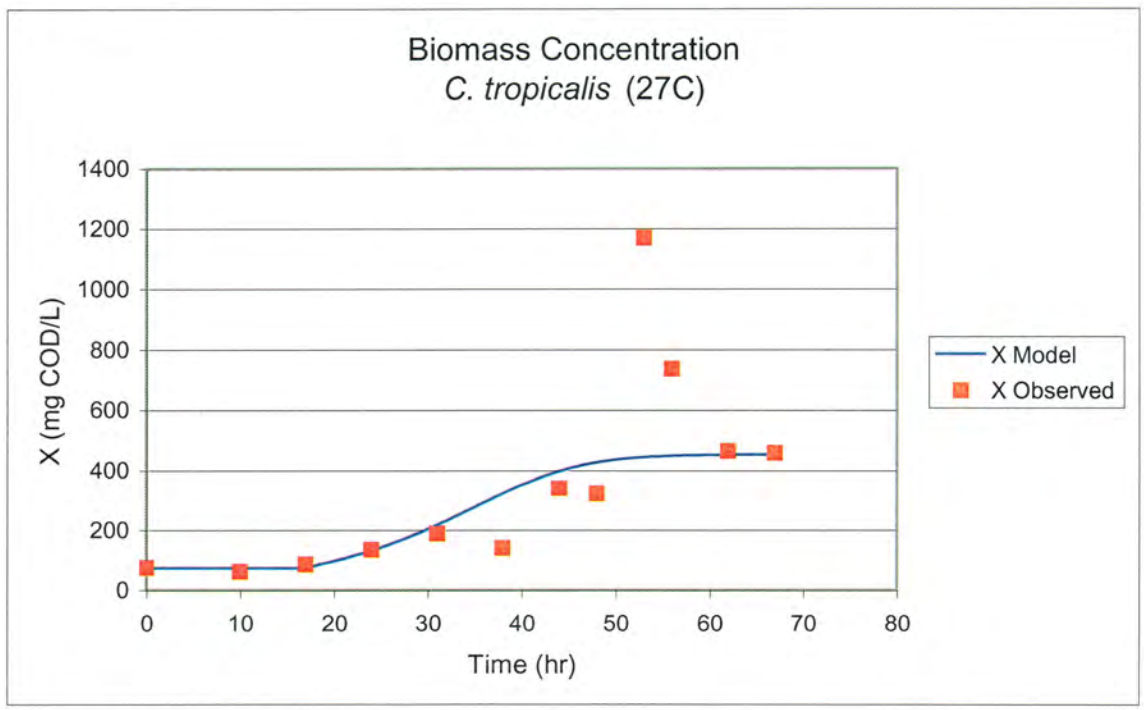

FIGURE 9- Biomass Concentration of *C. tropicalis* at 27°C

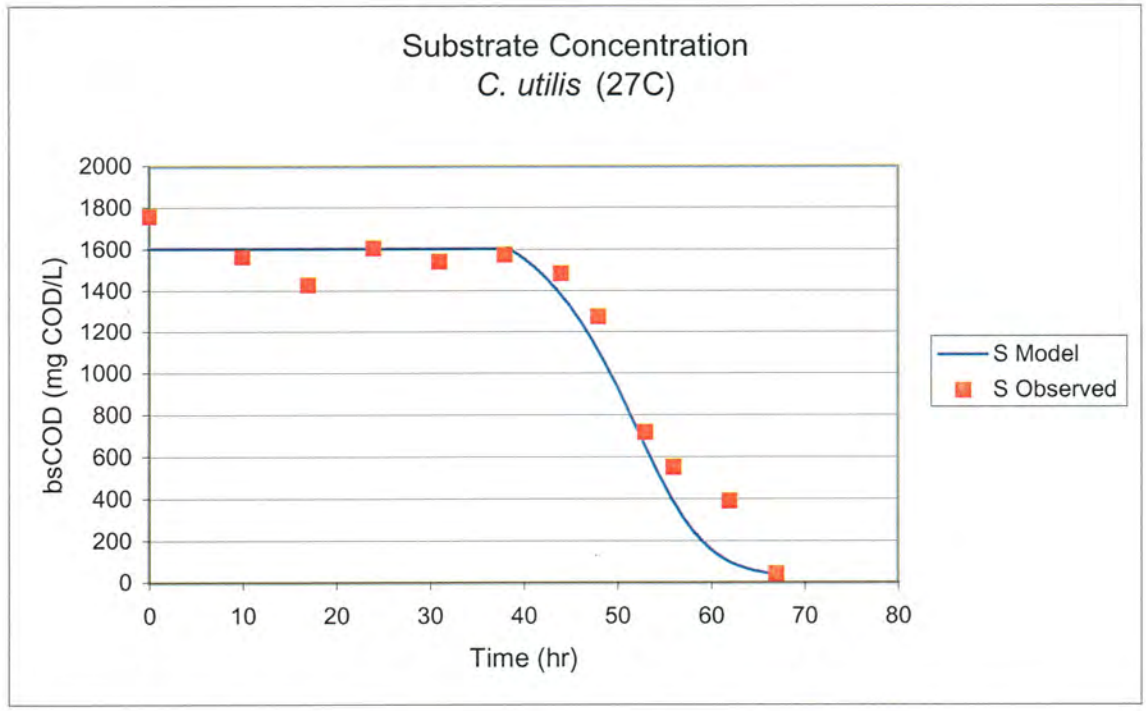

FIGURE 10- Substrate Concentration of *C. utilis* at 27°C

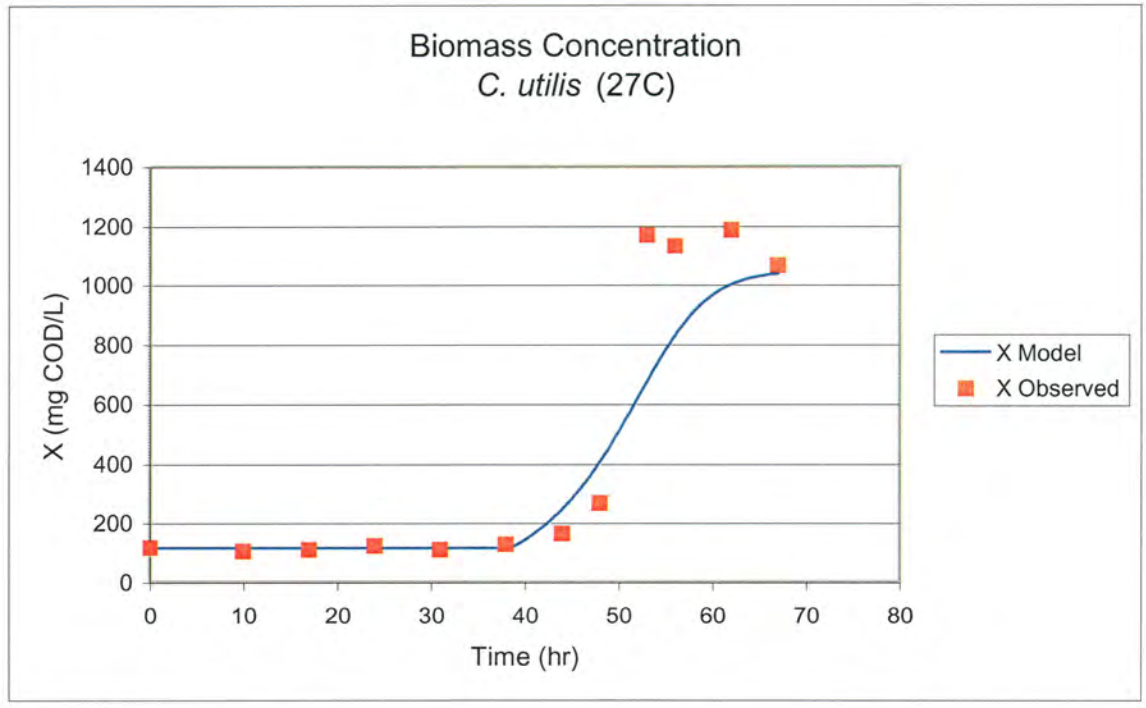

FIGURE 11- Biomass Concentration of *C. utilis* at 27°C

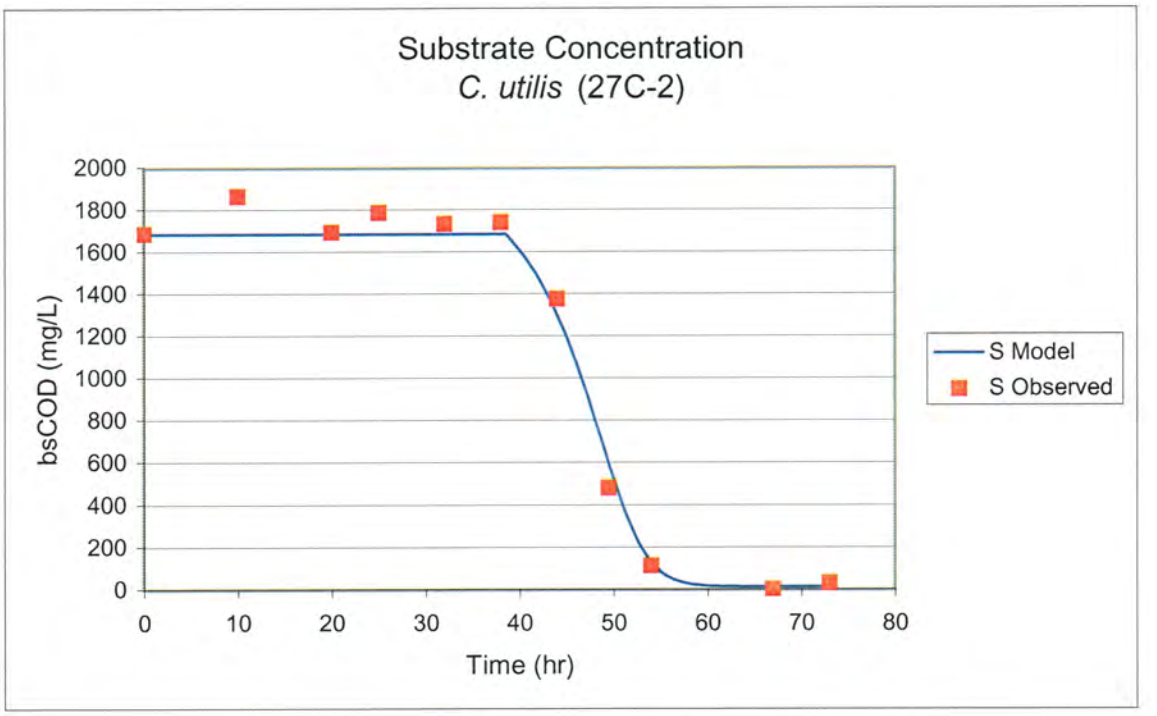

FIGURE 12- Substrate Concentration of *C. utilis* at 27˚C-2

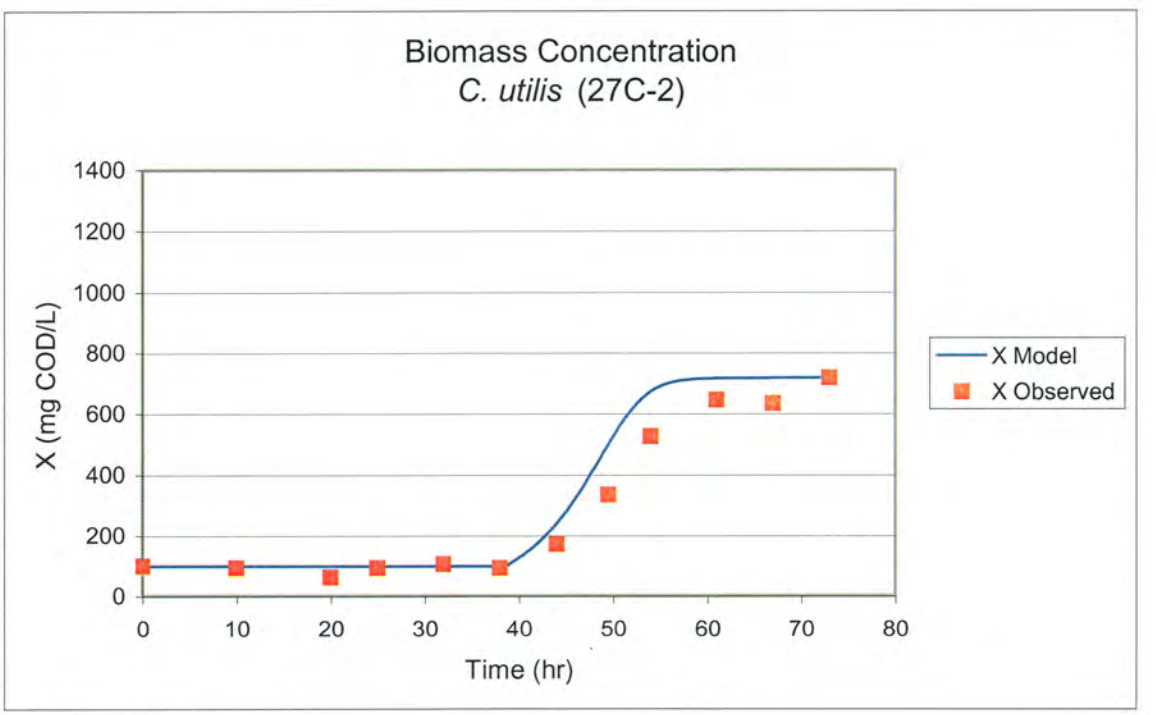

FIGURE 13- Biomass Concentration of *C. utilis* at 27˚C-2

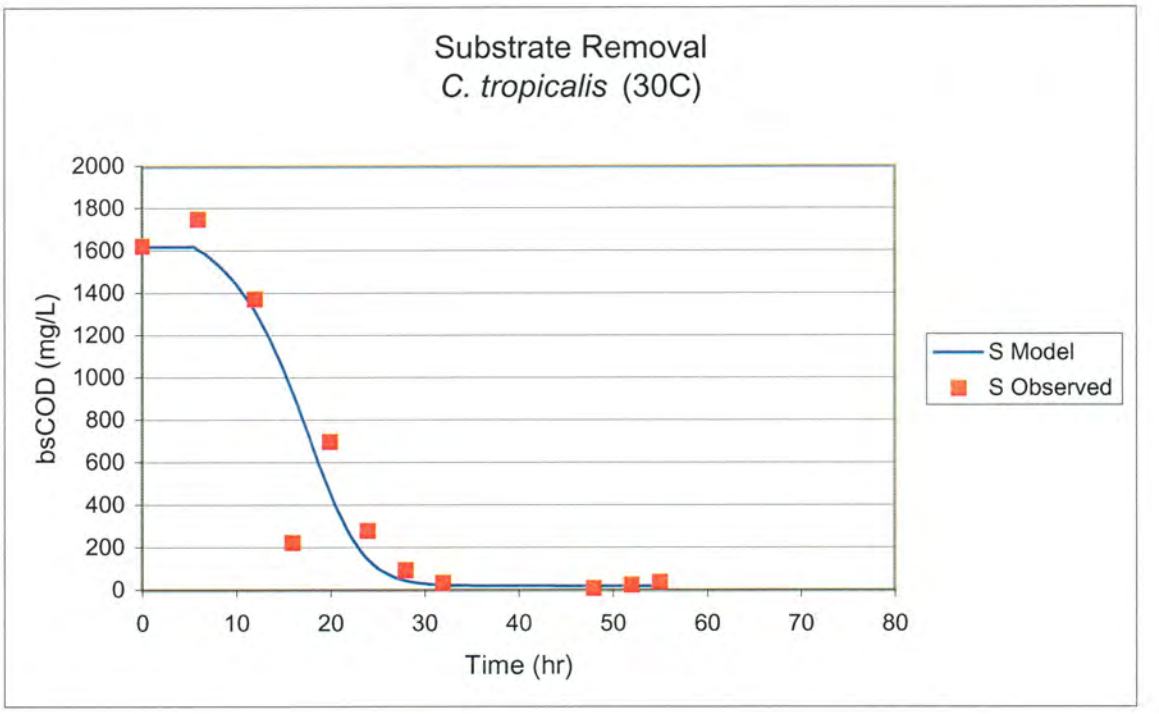

FIGURE 14- Substrate Concentration of *C. tropicalis* at 30°C

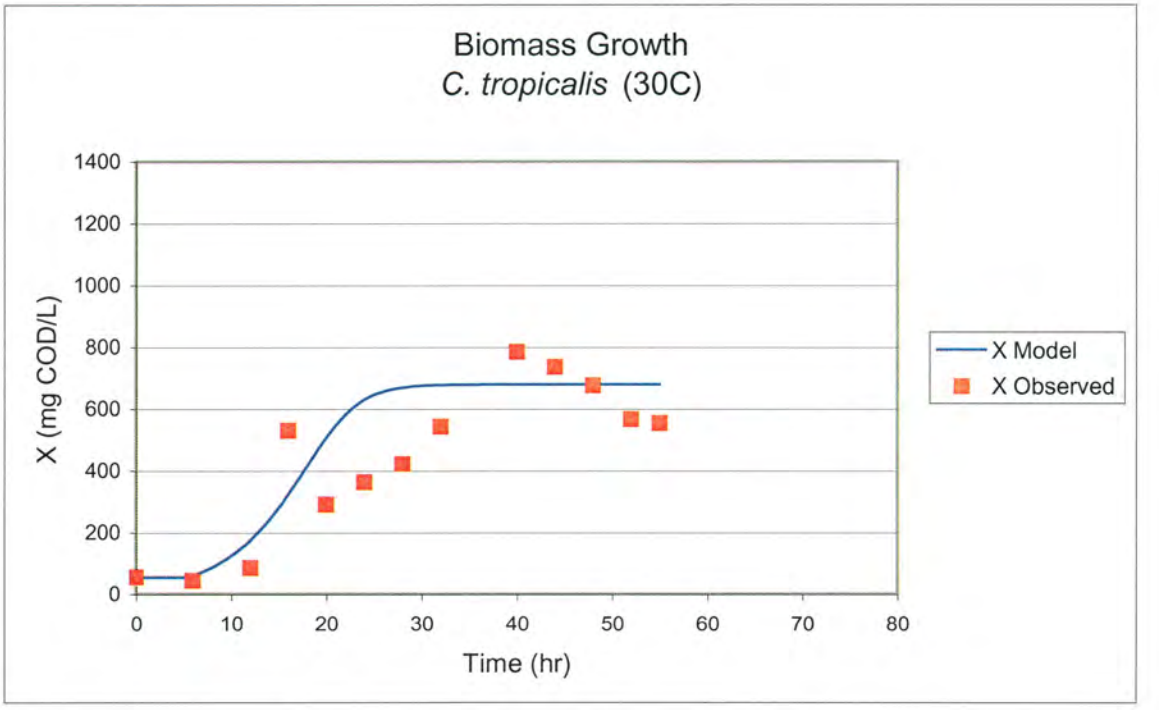

FIGURE 15- Biomass Concentration of *C. tropicalis* at 30°C

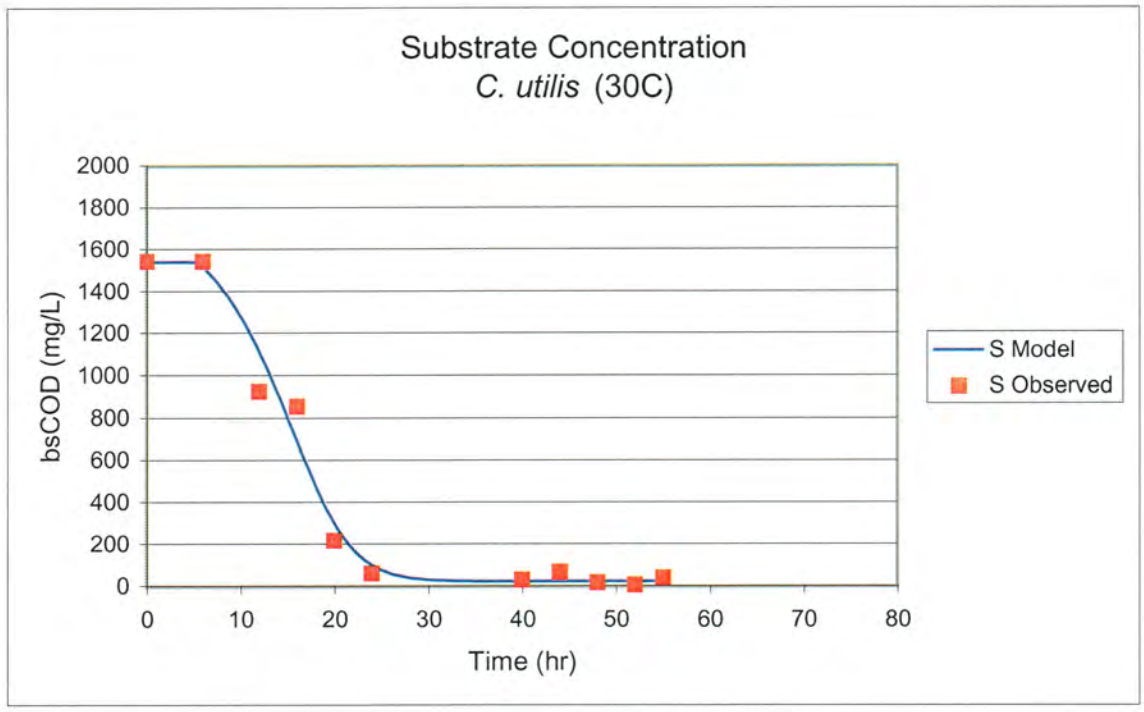

FIGURE 16- Substrate Concentration of *C. utilis* at 30˚C

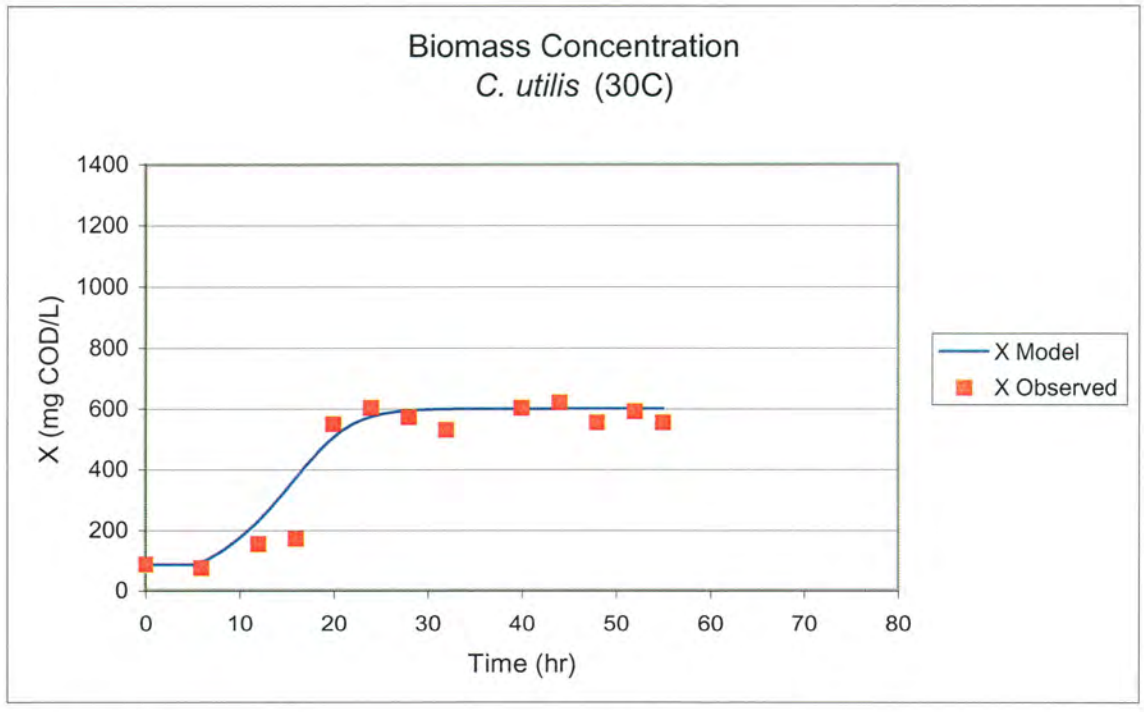

FIGURE 17- Biomass Concentration of *C. utilis* at 30˚C

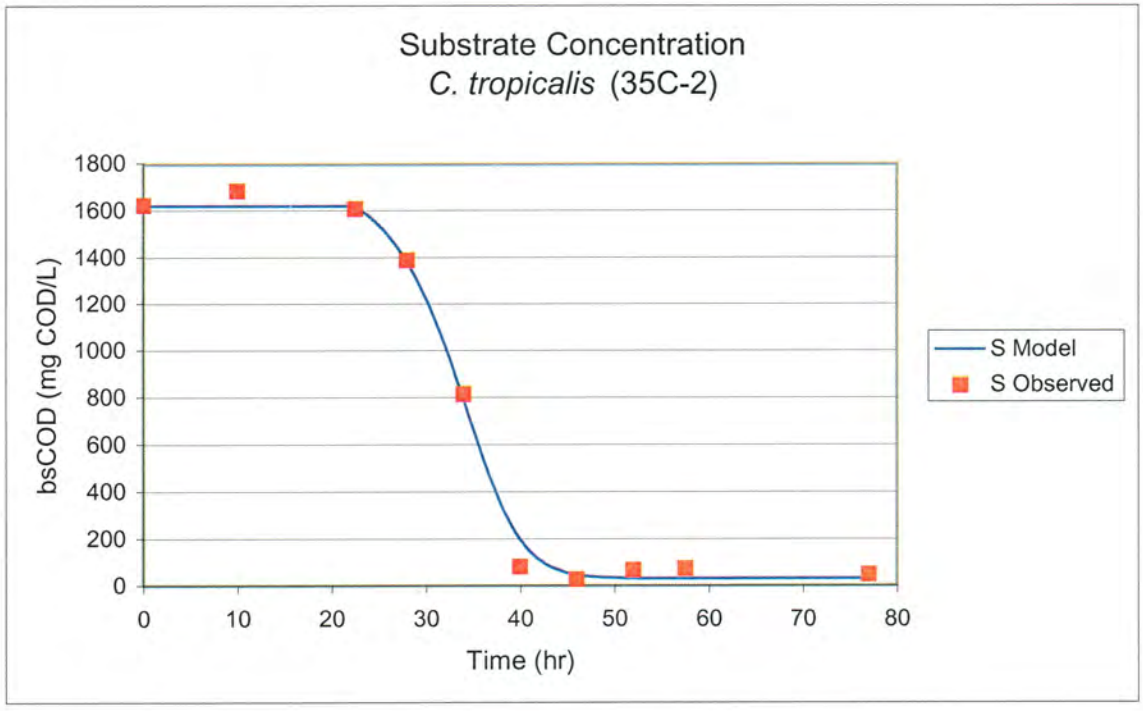

FIGURE 18- Substrate Concentration of *C. tropicalis* at 35°C

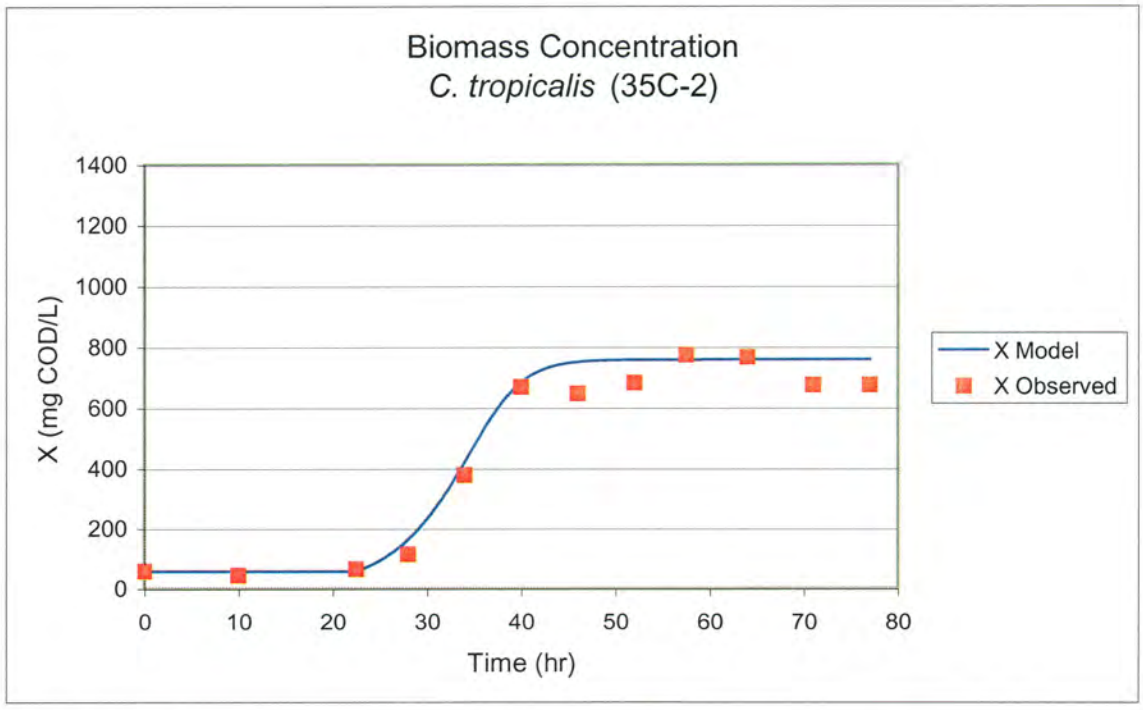

FIGURE 19- Biomass Concentration of *C. tropicalis* at 35°C

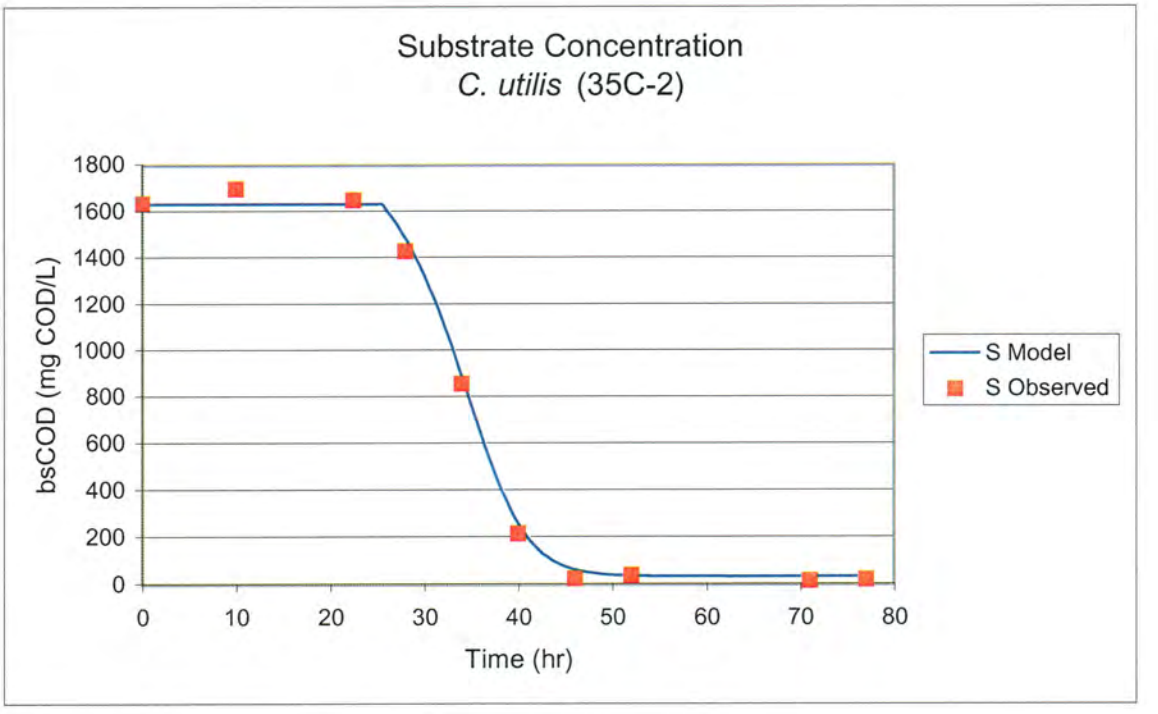

FIGURE 20- Substrate Concentration of *C. utilis* at 35˚C

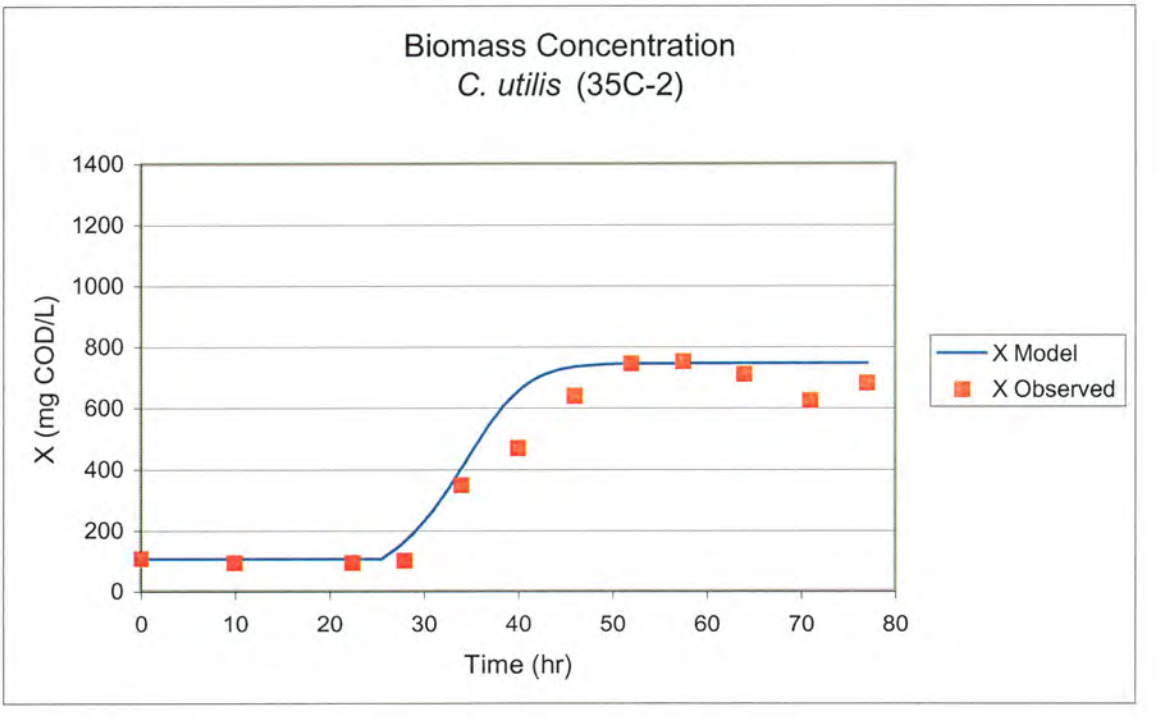

FIGURE 21- Biomass Concentration of *C. utilis* at 35˚C

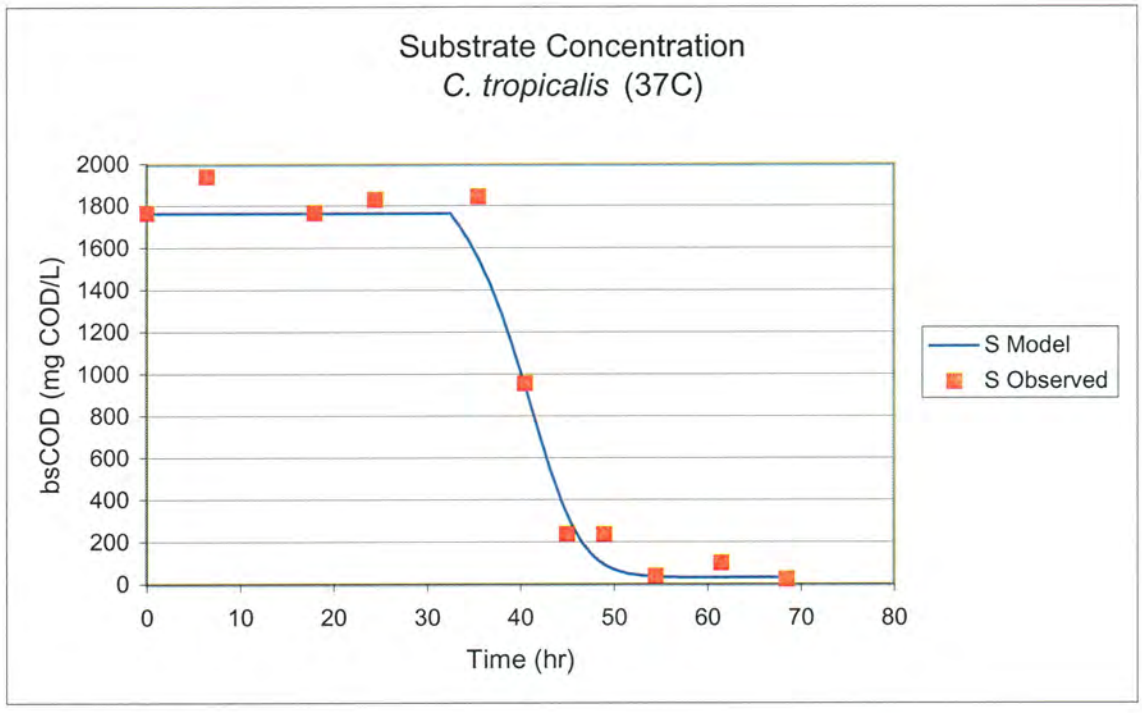

FIGURE 22- Substrate Concentration of *C. tropicalis* at 37°C

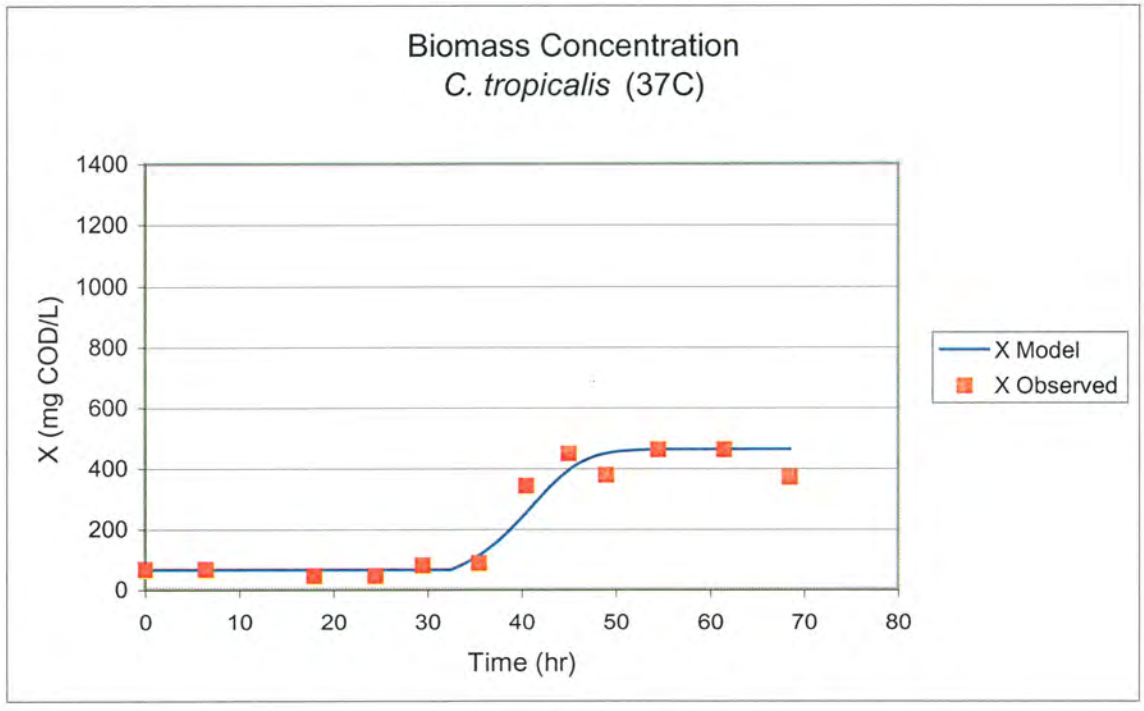

FIGURE 23- Biomass Concentration of *C. tropicalis* at 37°C

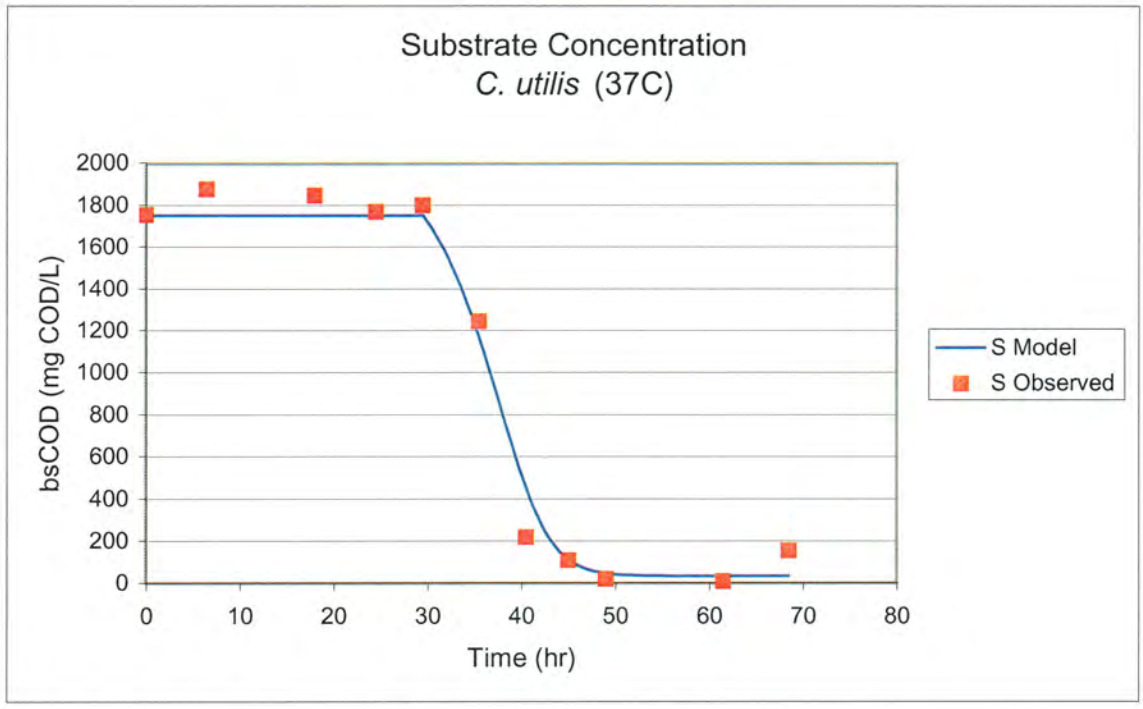

FIGURE 24- Substrate Concentration of *C. utilis* at 37°C

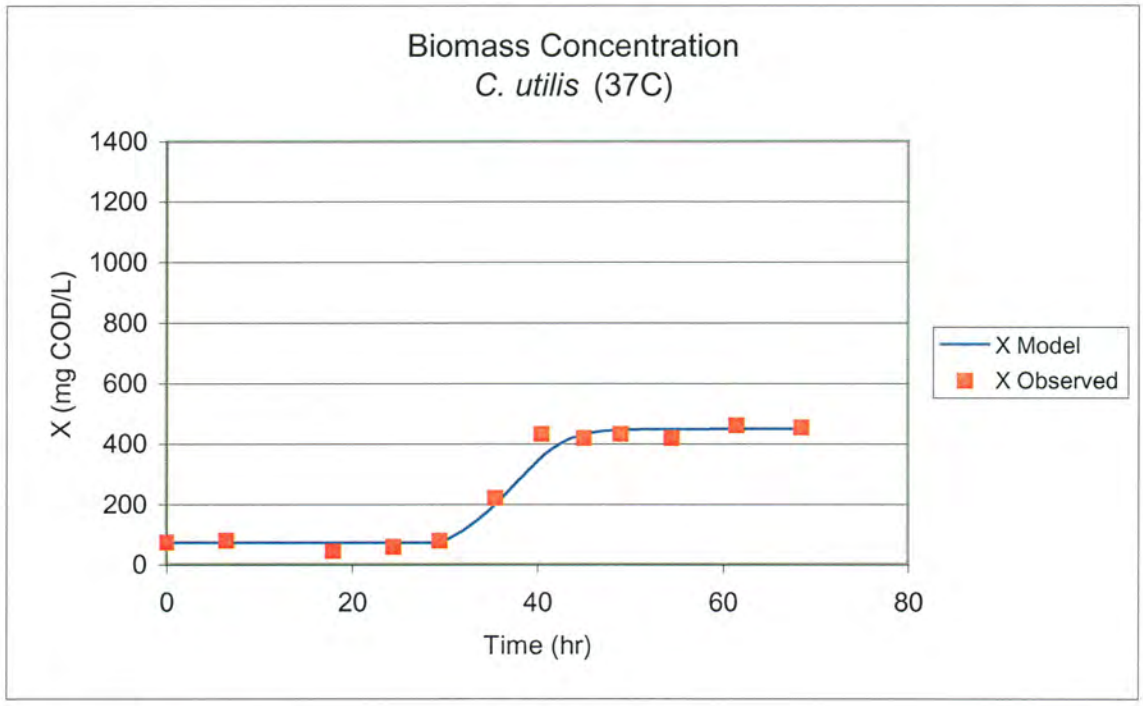

FIGURE 25- Biomass Concentration of *C. utilis* at 37°C

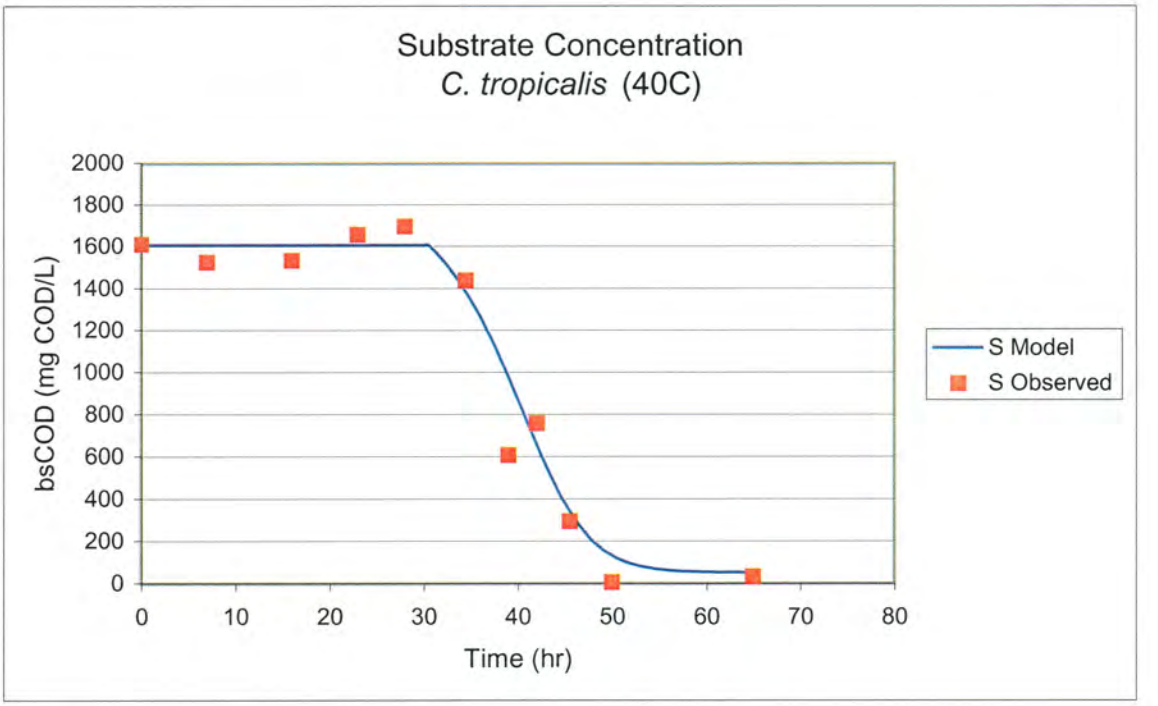

FIGURE 26- Substrate Concentration of *C. tropicalis* at 40°C

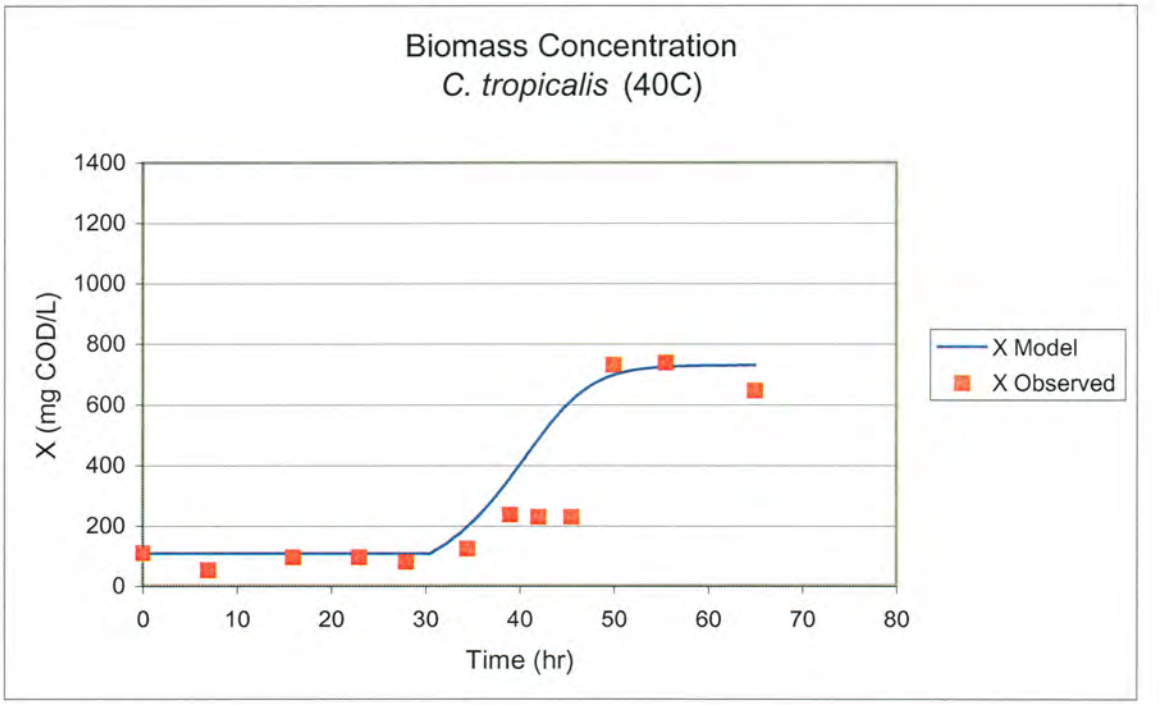

FIGURE 27- Biomass Concentration of *C. tropicalis* at 40°C

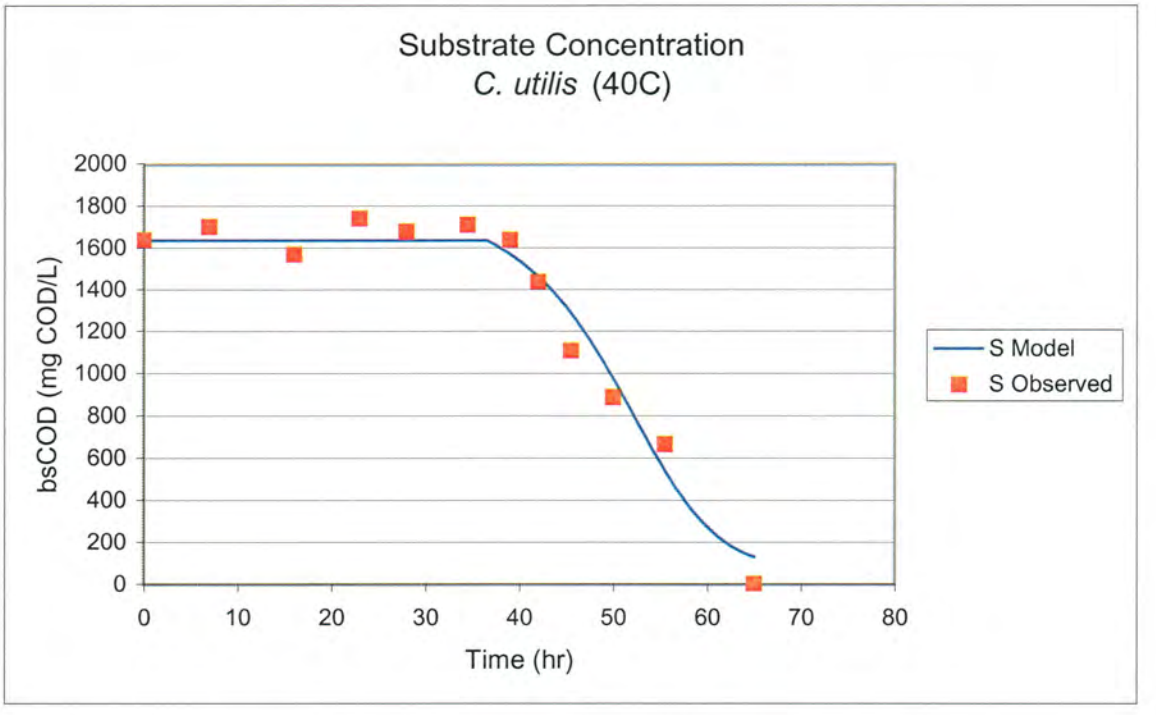

FIGURE 28- Substrate Concentration of *C. utilis* at 40°C

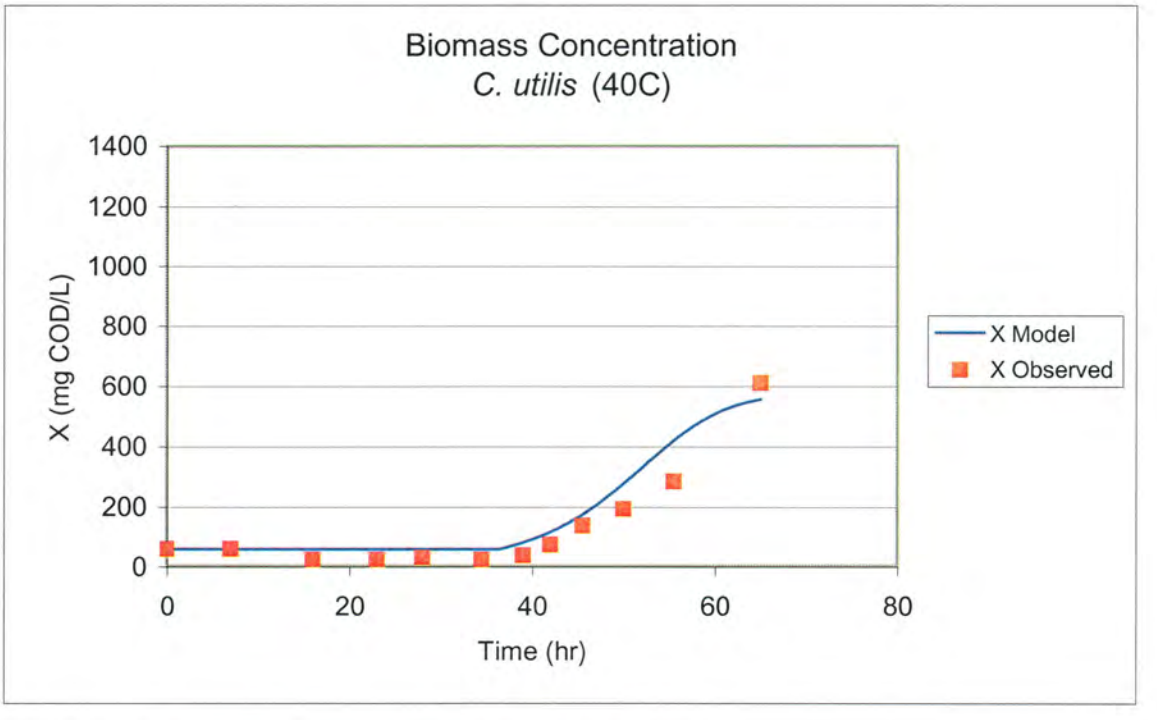

FIGURE 29- Biomass Concentration of *C. utilis* at 40°C

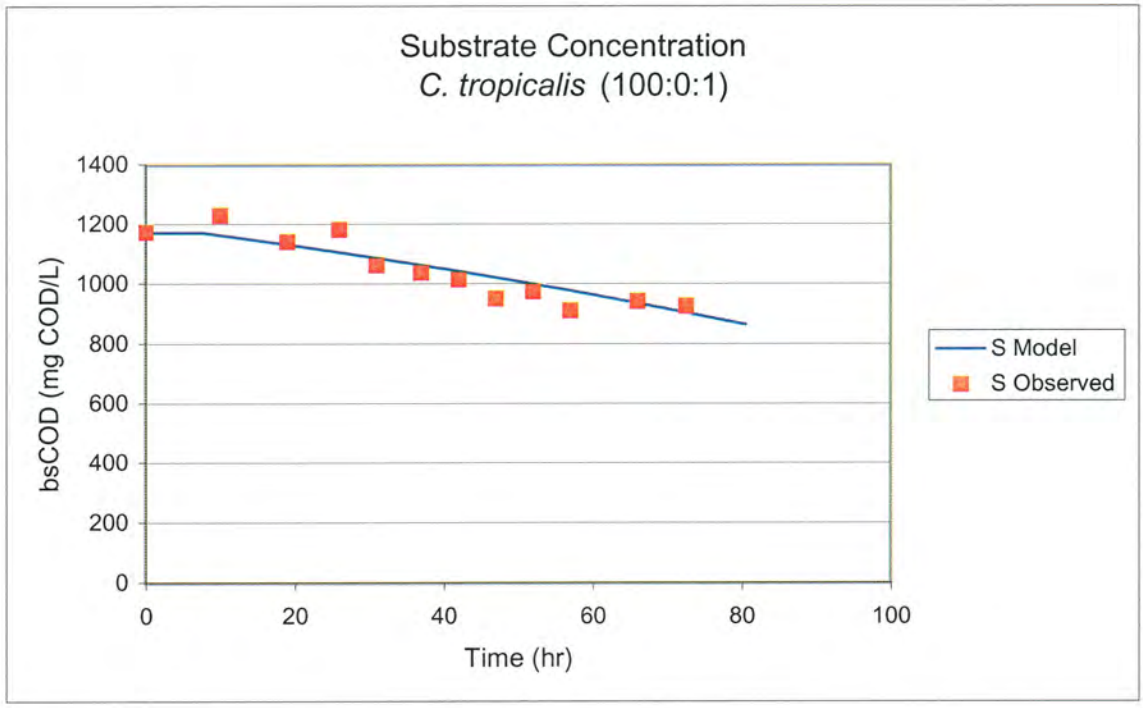

FIGURE 30- Substrate Concentration of *C. tropicalis* at (100:0:1)

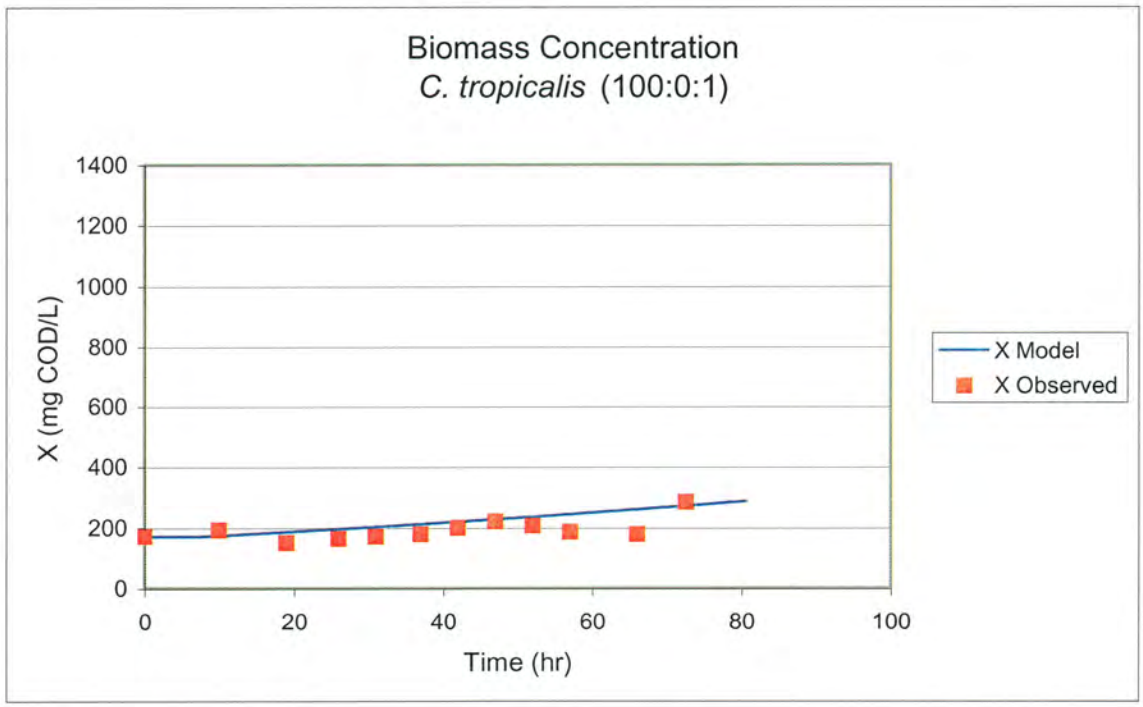

FIGURE 31- Biomass Concentration of *C. tropicalis* at (100:0:1)

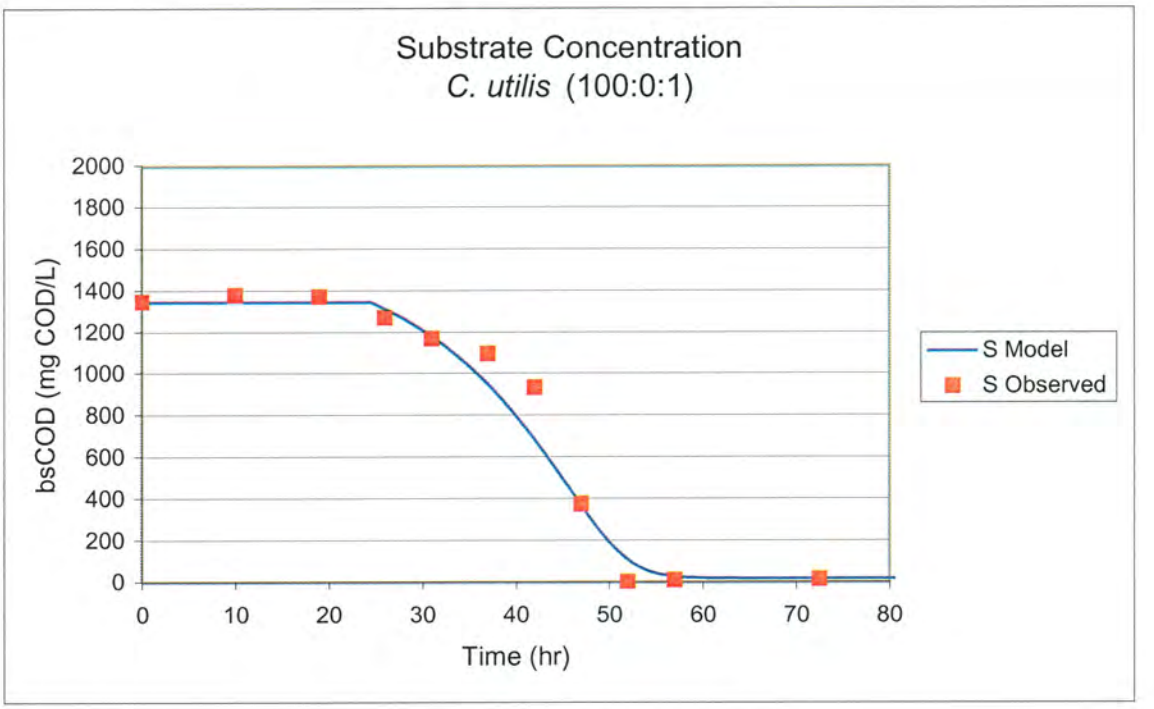

FIGURE 32- Substrate Concentration of *C. utilis* at (100:0:1)

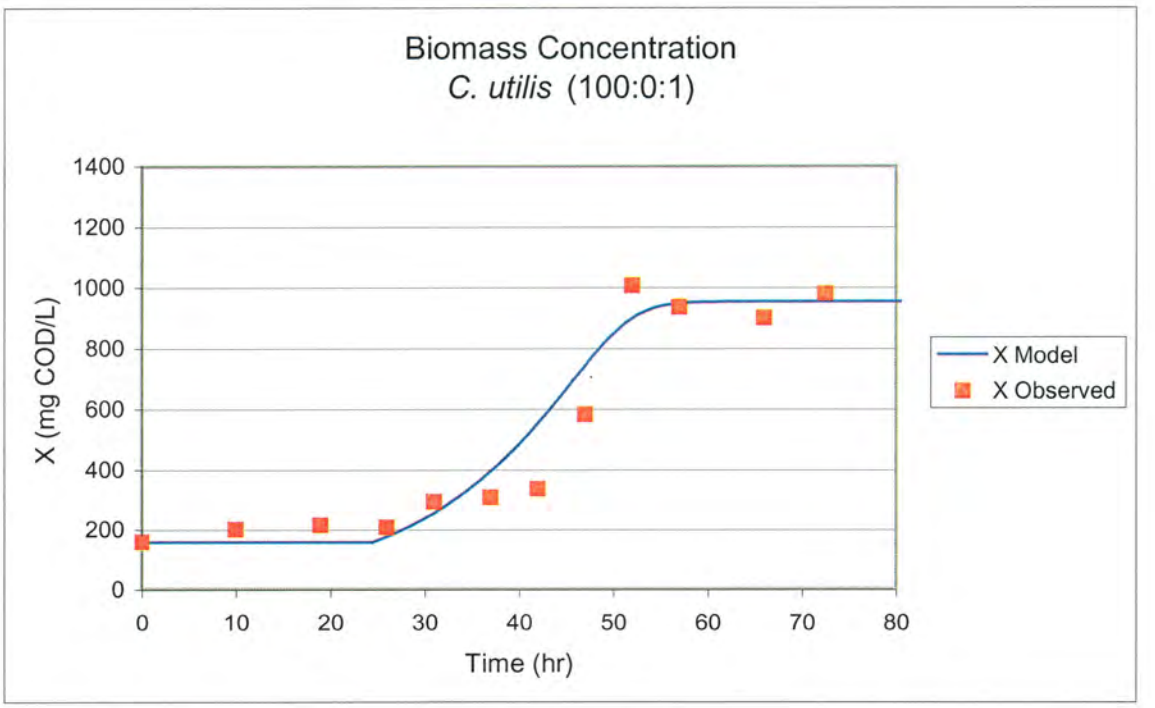

FIGURE 33- Biomass Concentration of *C. utilis* at (100:0:1)

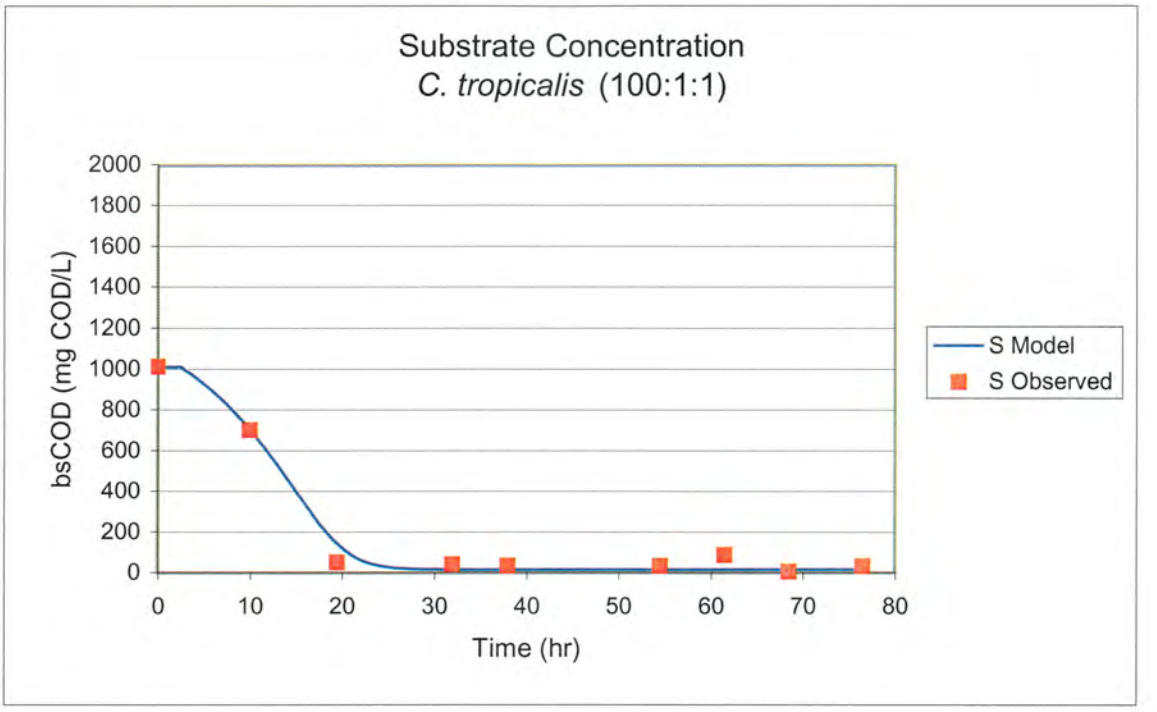

FIGURE 34- Substrate Concentration of *C. tropicalis* at (100:1:1)

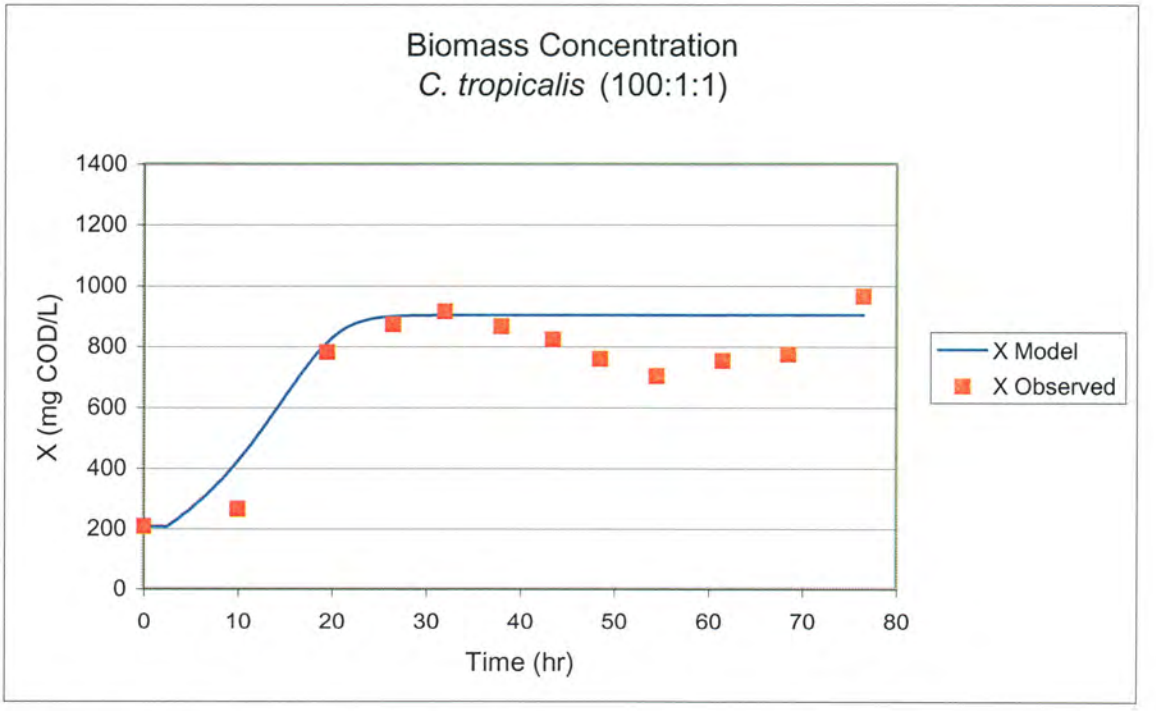

FIGURE 35- Biomass Concentration of *C. tropicalis* at (100:1:1)

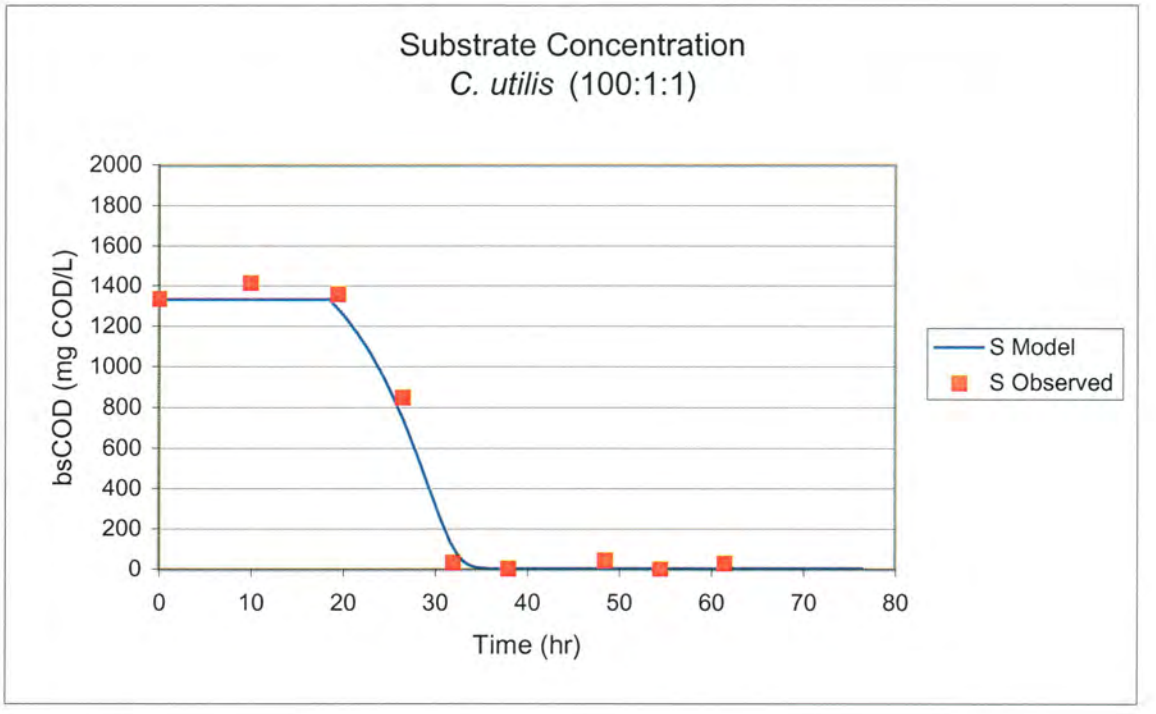

FIGURE 36- Substrate Concentration of *C. utilis* at (100:1:1)

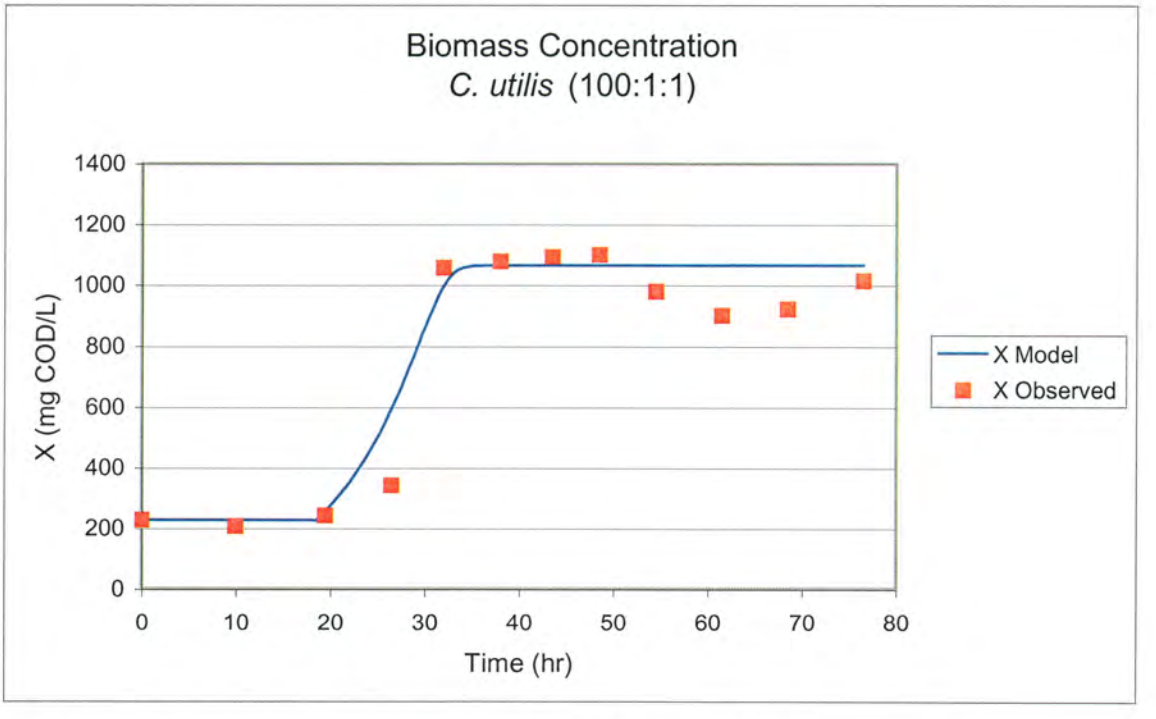

FIGURE 37- Biomass Concentration of *C. utilis* at (100:1:1)

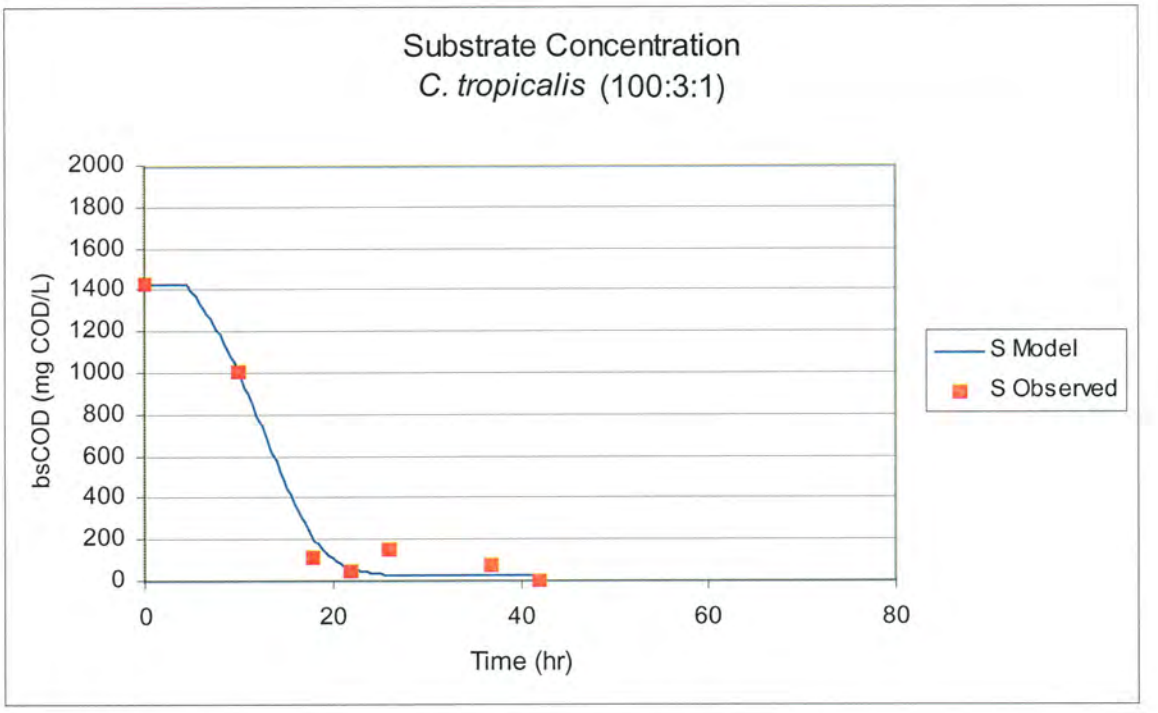

FIGURE 38- Substrate Concentration of *C. tropicalis* at (100:3:1)

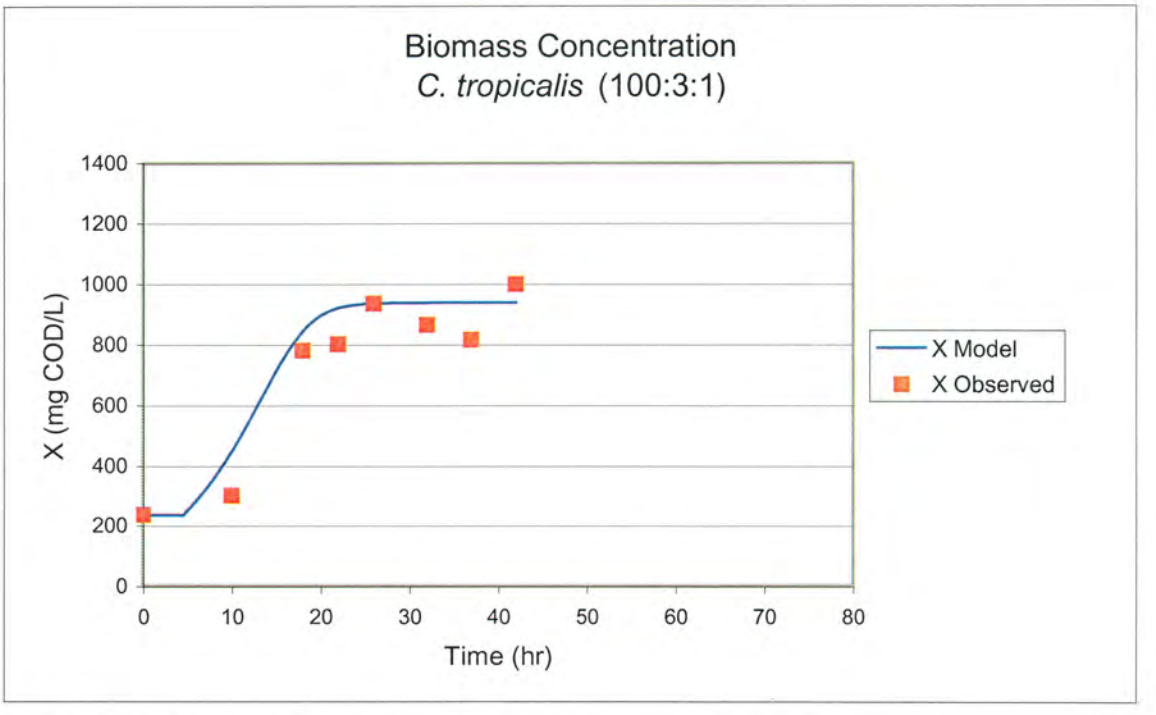

FIGURE 39- Biomass Concentration of *C. tropicalis* at (100:3:1)

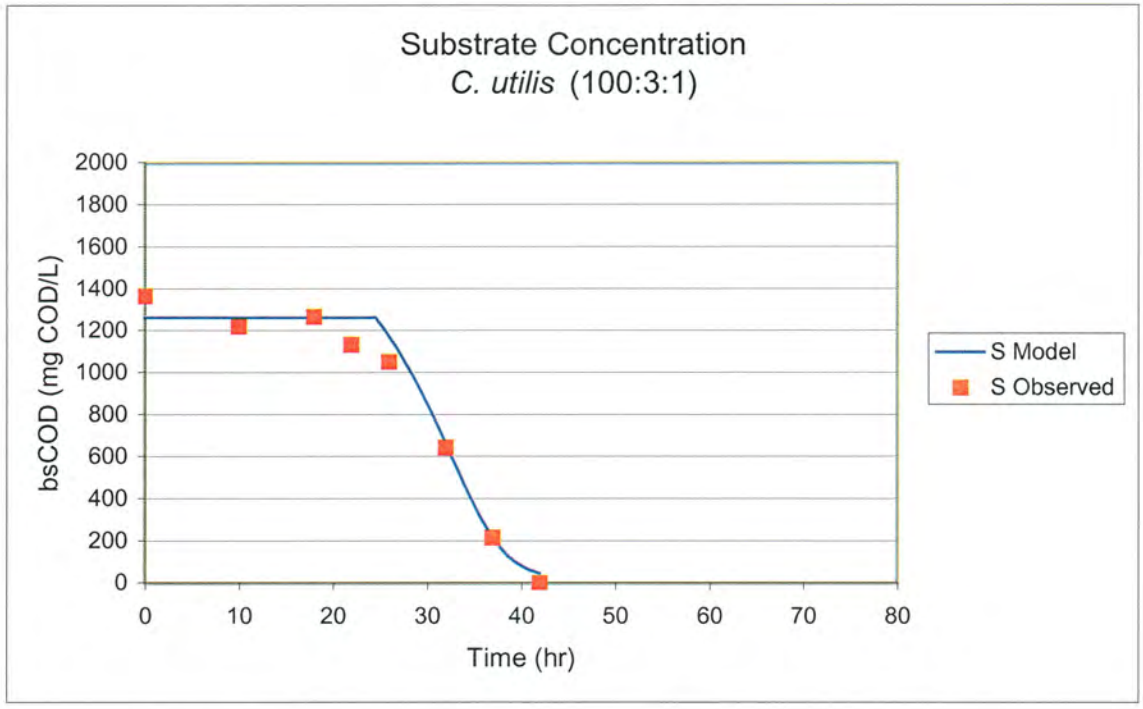

FIGURE 40- Substrate Concentration of *C. utilis* at (100:3:1)

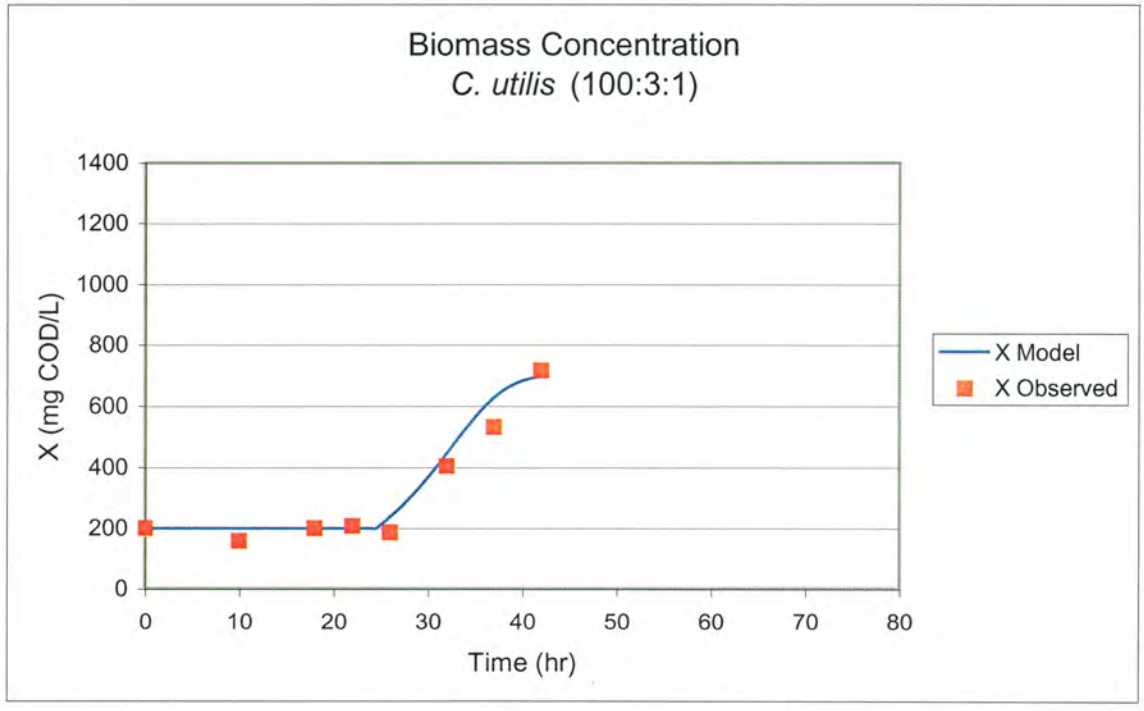

FIGURE 41- Biomass Concentration of *C. utilis* at (100:3:1)

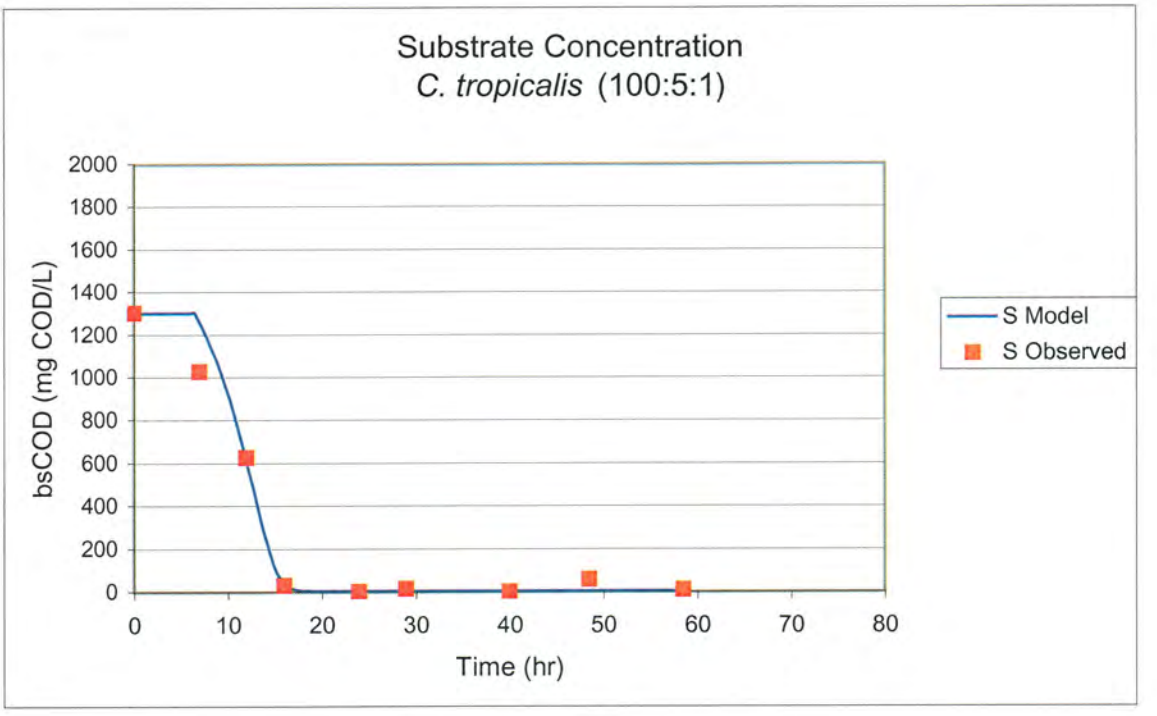

FIGURE 42- Substrate Concentration of *C. tropicalis* at (100:5:1)

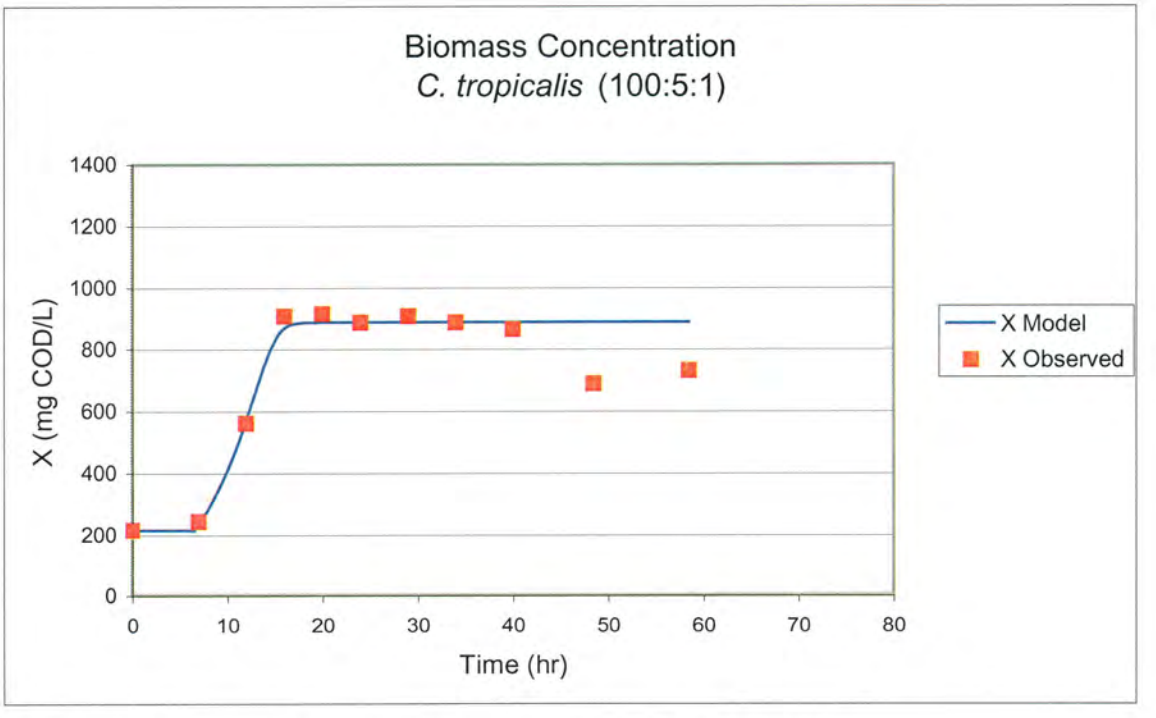

FIGURE 43- Biomass Concentration of *C. tropicalis* at (100:5:1)

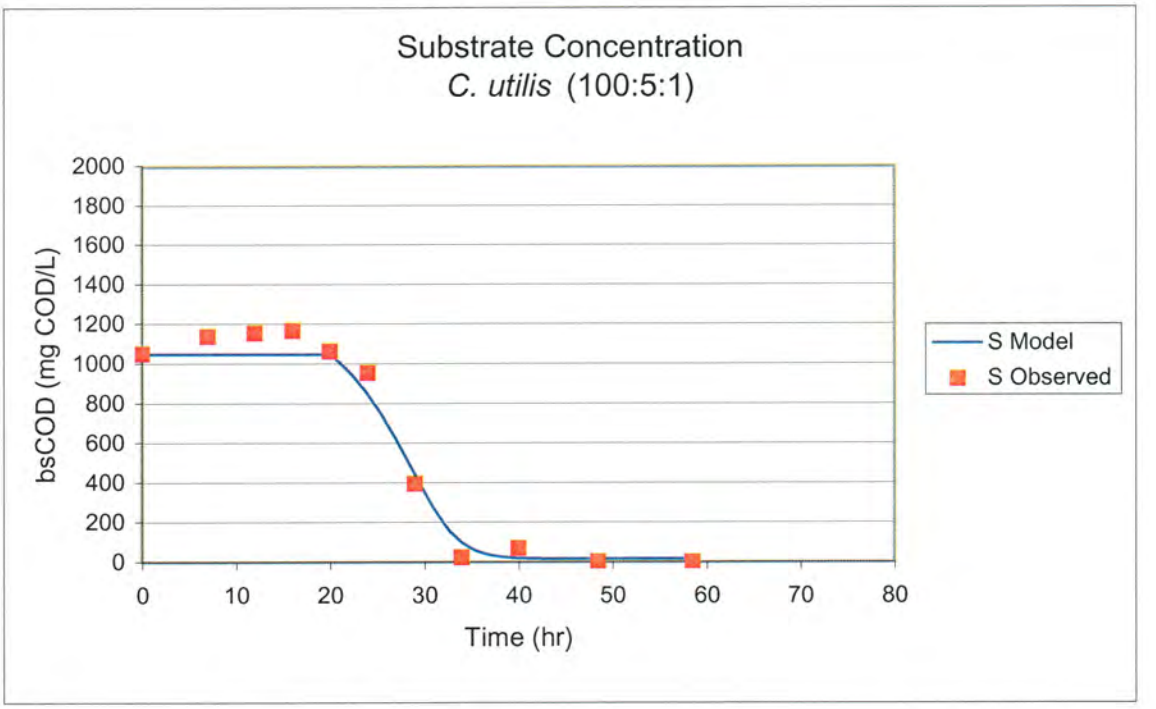

FIGURE 44- Substrate Concentration of *C. utilis* at (100:5:1)

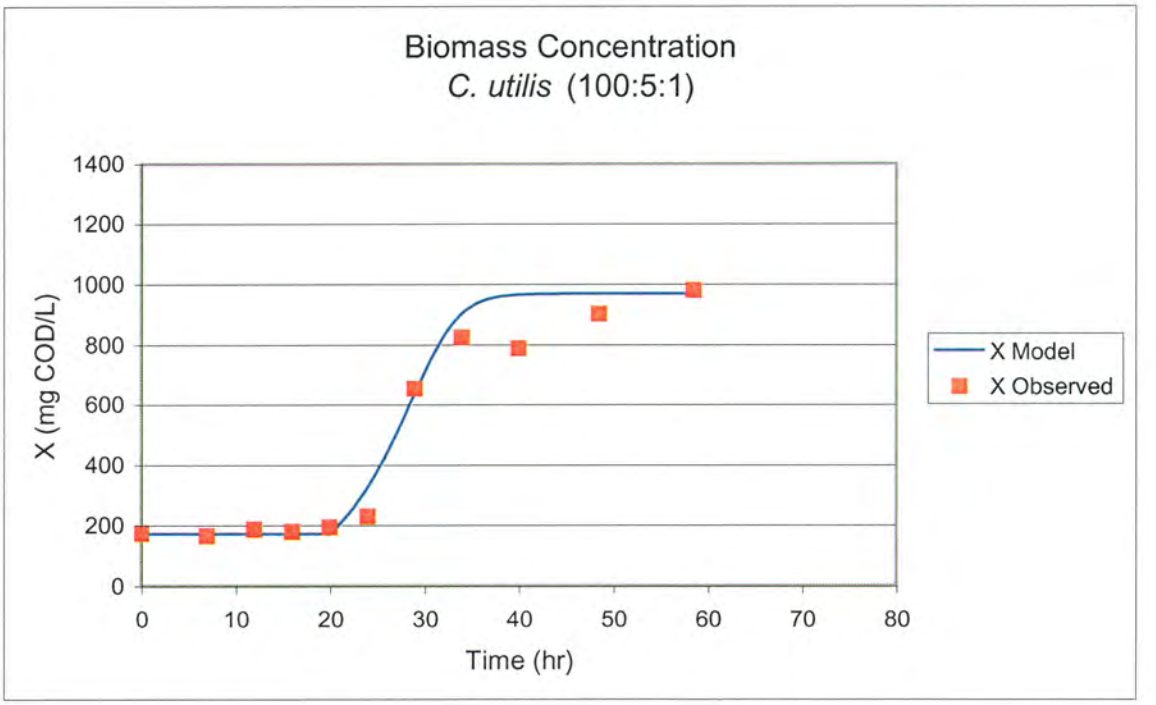

FIGURE 45- Biomass Concentration of *C. utilis* at (100:5:1)

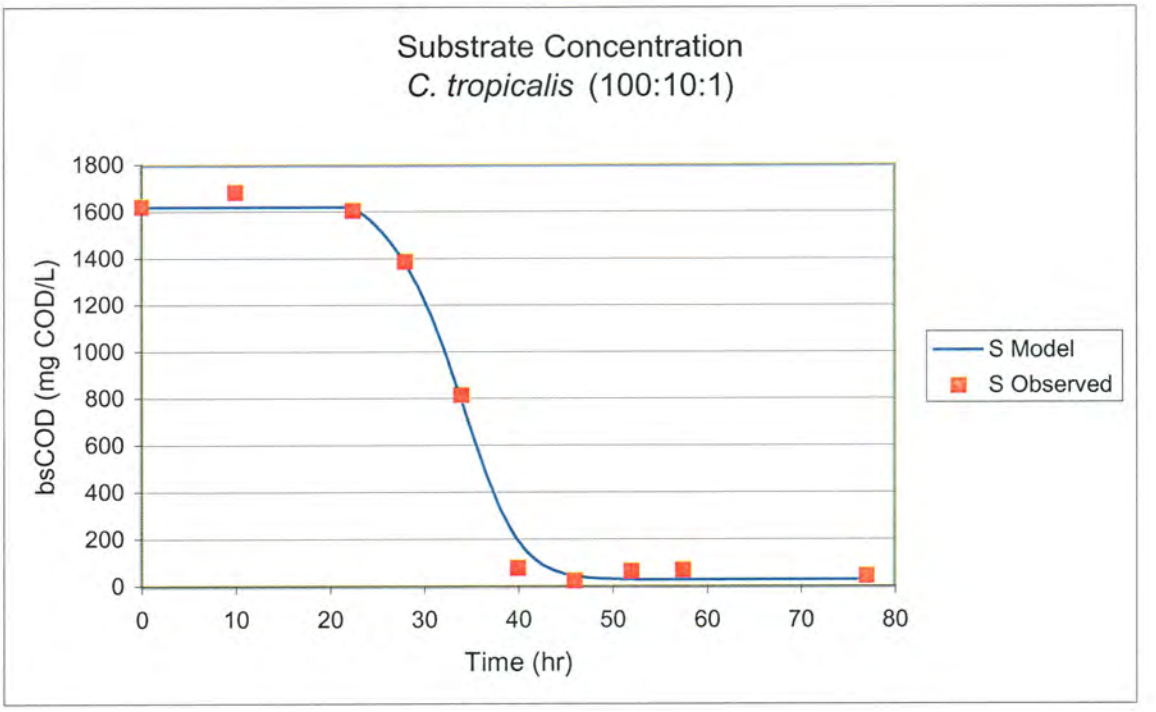

FIGURE 46- Substrate Concentration of *C. tropicalis* at (100:10:1)

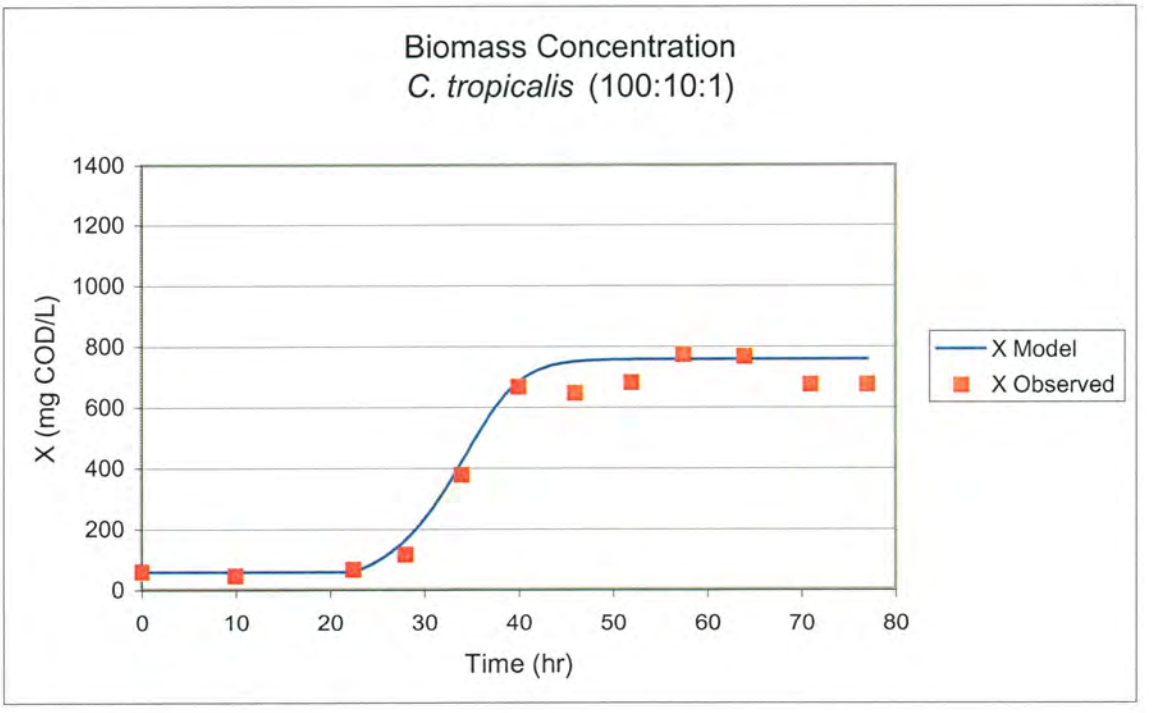

FIGURE 47- Biomass Concentration of *C. tropicalis* at (100:10:1)

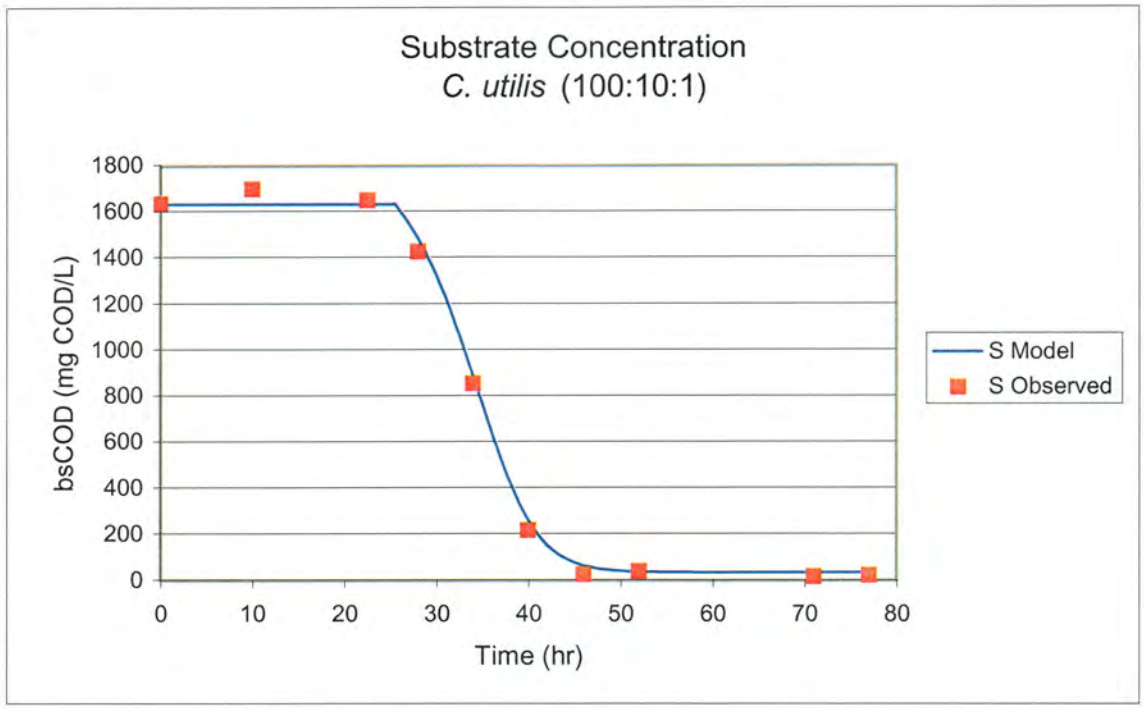

FIGURE 48- Substrate Concentration of *C. utilis* at (100:10:1)

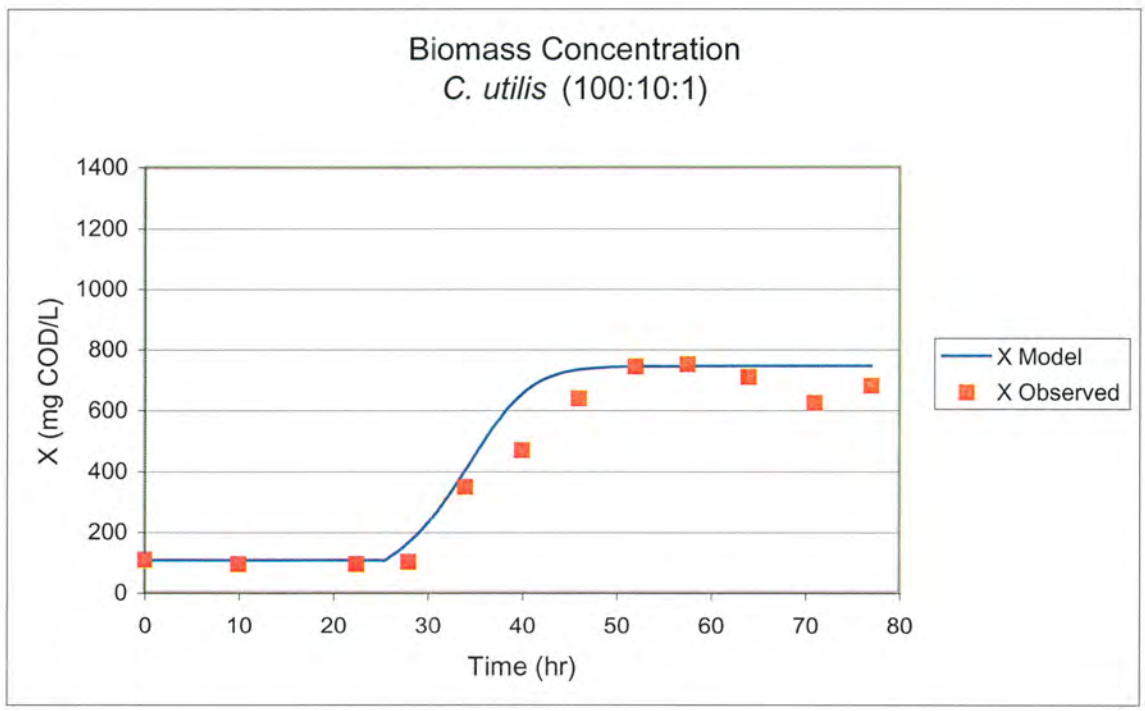

FIGURE 49- Biomass Concentration of *C. utilis* at (100:10:1)