Microbial Growth Kinetics of a Defined Mixed Culture: Genomic Assay Application

by

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Abstract

Microbial growth kinetics is often used to optimize environmental processes owing to its linkage to the breakdown of substrate (contaminants). However the quantification of bacterial populations in the environment is difficult due to the challenges of monitoring a specific bacterial population within a diverse microbial community. Batch experiments were performed for both single and dual cultures of *Pseudomonas putida* and *Escherichia coli* K12 to obtain Monod kinetic parameters (μ_{max} and K_s). The growth curves obtained by the conventional methods (i.e., dry weight measurement and absorbance reading) were compared to that obtained by quantitative PCR (qPCR) assay. We used qPCR assay to detect and quantify each strain's growth separately in the mixed culture reactor because conventional method was not capable of differentiating species. This work describes a novel genomic approach to quantify each species in mixed culture and interpret its growth kinetics in mixed system. We anticipate that the adoption of genomic assay can contribute significantly to traditional microbial kinetics, modeling practice, and the operation of bioreactors, where handling of complex mixed cultures is required.

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List of Abbreviations

ADP	adenosine-5'-diphosphate
ATP	adenosine-5'-triphosphate
DNA	deoxyribonucleic acid
E. coli K12	*Escherichia coli* K12
gDNA	genomic DNA
G-6-P	D-glucose-6-phosphate
G6P-DH	glucose-6-phosphate dehydrogenase
HK	enzyme hexokinase
K_s	half saturation constant
L-B medium	Luria-Bertani medium
μ	specific growth rate
μ_{max}	maximum specific growth rate
NADP	nicotinamide-adenine dinucleotide phosphate
NADPH	reduced nicotinamide-adenine dinucleotide phosphate
O.D.	optical density
PAH	polycyclic aromatic hydrocarbon
PCR	polymerase chain reaction
P. putida	*Pseudomonas putida*
qPCR	quantitative polymerase chain reaction

QC-PCR	quantitative competitive PCR
rrn	ribosomal RNA operon
TCA	tricarboxylic acid cycle
TSB medium	triptic soy medium
TSS	total suspended solids
16S rDNA	16S ribosomal DNA
16S rRNA	16S ribosomal RNA
DO	dissolved oxygen

Chapter 1: Introduction

Microbial growth kinetics is the relationship between the specific growth rate (μ) of a microbial population and the substrate concentration (S). It is an indispensable tool in the field of microbiology to address gaps of knowledge in the physiology; genetics and ecology of microorganisms, in addition to further develop the growing field of biotechnology (Kovarova-Kovar 1998). Despite more than half a century of research, many fundamental questions about the validity and application of growth kinetics are still unanswered(Koutinas 2011). Quantification of bacterial populations in the environment is difficult due to the challenges of isolating and identifying a specific bacterial population within a diverse microbial community (Schwartz 2000). Bioprocesses like the biodegradation of pollutants or wastewater treatment processes are optimized by the study of their microbial growth kinetics (Mateles 1969; Baltiz 1996; Arthur 2011). Development and optimization of these bioprocesses requires continuous information about the kinetic parameters of the microorganisms used in those processes (Ordaz 2009). Recent ecologically oriented studies in the area of microbial growth and biodegradation kinetics demonstrated that many fundamental questions in this field are still in need to be discovered, established and exploited(Kovarova-Kovar 1998). Korabora expresses that this state of affairs is probably the consequence of stagnation in the area of microbial growth kinetics during the past three decades, in which the interest of many microbiologists was attracted by rapidly developing areas such as molecular genetics or the biochemistry of the degradation of xenobiotics. Considerable attention has been paid to the modeling aspects of both growth and substrate removal (biodegradation) kinetics. Most studies almost totally neglected the facts that in nature microorganisms grow mostly with mixture of substrates, that growth may not be

controlled by only a single nutrient and that kinetic properties of a cell might change due to adaptation (Kovarova-Kovar 1998).

Several mathematical expressions are available for describing the rate of biotransformation. One of the most prominent is the Monod expression (Bakins 1998). This relatively simple empirical model proposed by Monod half a century ago continues to dominate the field of microbial growth kinetics (Egli 2009). The extraction of the Monod parameters involves the measurement of the limiting substrate as well as the biomass growth (Kovarova-Kovar 1998). Biomass can be quantified in three basic ways: total suspended solids (TSS) as grams of dry or wet weight per liter of sample, counting the number of viable/dead cells per ml, or monitoring the optical density of the sample (Monod 1949; Wang 2011). In the last method, the absorbance of the sample measured in a spectrometer is correlated to either the dry weight or the number of cells per volume (Wang 2011). The application of these methods for mixed culture samples is inefficient as none is capable to discriminate the growth of individual cultures in mixed samples and therefore neglect the microbial interaction that can significantly affect the bioprocess.

Increasing advances of technology in molecular techniques have lately been used to address problems that were encountered in past research. Techniques such as polymerase chain reaction (PCR) and quantitative PCR (qPCR) provide a way to detect specific genes at very low concentrations. (Lee 2008) A common way of using these tools to detect the presence of specific strains of microorganism is to target for the rRNA gene (Schwartz 2000). The approach of measuring the abundance of a specific bacterial population is to determine the concentration of 16S ribosomal RNA genes unique to that population can be an alternative to biomass quantification. Two bacterial strains were selected with similar kinetic parameters: *P. putida*,

widely studied soil bacterium, K_s = 4 - 9 mg/L (Ordaz 2009) and *E. coli* K12, used in numerous genomic research, K_s = 7 mg/L(Egli 2009).

In the present work, a genomic assay was developed for the analysis of biomass quantification for a pure culture and mixed culture batch reactor. Microbial kinetic parameters were calculated and compared between absorbance and genomic analysis. This work presents a novel approach to quantify cell density of a specific strain in mixed culture kinetics samples by genomic assay analysis.

Chapter 2: Literature Review

This chapter provides an overview of the literature of the Monod microbial growth kinetics and the challenges encountered in mixed cultures systems, to include the conventional approaches for the quantification of microbial growth. It is also introduced the gene quantification assay as an alternative for growth monitoring. In addition, the model bacteria, *P. putida* and *E. coli K12*, are introduced.

2.1. Microbial Growth kinetics: Biodegradation

Biodegradation is a feasible alternative for remediation of contaminated areas. Biological degradation of a chemical usually implies a breakdown by living microorganisms to more simple compounds or simpler by-products. These microorganisms are mostly heterotrophic and require energy and organic carbon sources and nutrients for their growth. In some cases the chemicals (pollutant or contaminants) may be the source of organic carbon and energy. Microbial degradation of pollutants and other organic chemicals is widely used in many restoration processes and is commonly represented by the Monod's equation (Cerniglia 1993; Novotny 2003; Chauhan 2008; Haritash 2009; Ordaz 2009).

Development and optimization of new bioprocesses requires continuous information about the kinetic and stoichiometric parameters of the microorganisms used in the processes (Spikema 1998; Ordaz 2009). Substrate consumption can be linked to microbial growth and its kinetics is predicted by Monod's kinetics. Microbial growth kinetics is the relationship between the specific growth rate (μ) of a microbial population and the substrate concentration (S). Although bioremediation has a high rate of success, its kinetics is not fully understood (Robinson 1983). The addition of pollutants-degrading microorganisms has proven successful for remediation processes; nevertheless there are numerous cases where this strategy fails (Schwartz

2000). Analysis of such failures is often hindered because densities of pollutant-degrading organisms were not measured. Obtaining this measurement through traditional culturing techniques requires a culturing strategy which isolates only the inoculums (Schwartz 2000).

2.2. Monod Kinetics

The area of microbial growth kinetics consists not just of the simple dynamic recording of biomass increase as a function of time in a culture but it is more the extraction of parameters that allow to quantitatively formulate general principles, to construct mathematical models that allow to describe and predict microbial growth processes and that provide a basis for further experimentation (Egli 2009). Classic models assume that growth kinetics is governed by the extracellular concentration of a single nutrient, though under environmental and biotechnological conditions, microbial cells utilize mostly mixtures of substrates (Alexander 1985).

J. Monod in 1942 refined and calibrated the method for quantifying microbial growth using turbidimetry and demonstrated how growth can be mathematically described in terms of growth yield, specific growth rate, and substrate concentration (Monod 1949; Mateles 1969; Egli 2009). During the last half century, the concepts in microbial growth kinetics have been dominated by the relatively simple empirical model proposed by Monod. The Monod model (equation 1) differs from the previous growth models in the way that it introduces the concept of growth-controlling or limiting substrate. For most applications, it has turned out that growth or degradation phenomena can be described satisfactory with the Monod's model (equation 1) and its kinetic parameters (Egli 2009). In this model the specific growth rate (μ) is linked to the concentration of growth controlling substrate (S) via two parameters: the maximum specific growth rate (μ_{max}) and the substrate affinity constant (K_s).

$$\mu = \mu \max\left(\frac{S}{Ks+S}\right) \quad (1)$$

2.3. Single and Mixed Culture

Since 1950 much effort was spend on either obtaining additional supportive experimental data to the Monod's model or to formulate alternative kinetics models. In the 1950's the principles of microbial competition for a common nutrient were formulated based on Monod's growth kinetic principles. In this decade also, the continuous culture technique was introduced and the kinetics of microbial growth in open systems was formulated on Monod's model (Alexander 1985).

A number of influential models had been published to predict single culture microbial growth kinetics in laboratories. The majority of them are modified Monod saturation type models containing additional constants, such as for maintenance, for diffusion of the growth-controlling substrate to the cell surface, for the presence of multiple substrate uptake systems, or for the presence of a minimum substrate concentration required for growth (Alexander 1985).

The estimation of kinetic parameters for pure cultures is traditionally performed through batch or chemostat (continuous) cultures (Kovarova-Kovar 1998; Ordaz 2009). Mateles conducted continuous culture experiments to determine whether the results obtained with pure culture could be reproduced in natural populations. The result was qualitatively the same as that found with the pure culture but possibly representing a shift in the microbial population. Heterogeneous populations in batch culture showed the sequential uptake pattern found in diauxic growth (Mateles 1969). Marazioti developed a model to predict the behavior of defined mixed cultures using a kinetic model based on previously developed models for each bacterium

separately and compared with the results from mixed culture kinetics. He presented that the majority of kinetic studies are performed with mixed (and not well-defined) populations systems, such as activated sludge. Because of the interactions between various microbial species and substrate, modeling of such systems has presented serious difficulties. The microbial populations in a mixed culture system used for kinetic experiments, besides their specificity due to their origin, often vary in a laboratory reactor over time of the experiment, making reproducibility of kinetics results virtually impossible and therefore limiting the applicability of the estimated parameters to the studied conditions (Marazioti 2003).

2.4. Microbial Growth Quantification: Estimation of Growth

2.4.1. Bacteria Densities and Cell Concentrations

There are several techniques employed for the estimation of bacterial density and cell concentrations. For the estimation of bacterial density, the basic method is the determination of the dry weights. Monod emphasized that this method is accurate only if relatively large amounts of cell can be used and it is employed mainly as a check of other indirect methods (Monod 1949).

Cell concentration determinations are performed either by direct counts (total counts) or by indirect (viable) counts. The value of the first method depends on technical details and its interpretation depends on the properties of the strain and media and is unequivocal only to organisms which do not tend to remain associated in chains or clumps (Monod 1949; Marazioti 2003).

Indirect counts, so called viable, are made by planting out suitable dilutions of the culture on solid media. The method has an additional difficulty, as it gives only the number of cells

capable of giving rise to a colony on agar under conditions widely different from those prevailing in the culture (Monod 1949). Viable counts retain the undisputed privilege of being by far the most sensitive method and of alone permitting differential counting in the analysis of complex populations (Monod 1949).

2.4.2. Indirect Chemical methods

Various indirect chemical methods have been used for the estimation of microbial growth kinetics. Nitrogen determinations are generally found to check satisfactorily with dry weights. When cultures are grown on media containing an ammonium salt as sole source of nitrogen, estimations of the decrease of free ammonia in the medium appear to give adequate results (Monod 1949). Estimations of metabolic activity (oxygen consumption, acid production) may be convenient, but their use id obviously very limited (Monod 1949; Mateles 1969). Respirometry allows the indirect measurement of substrate consumption rates by monitoring the biological oxygen consumption rate under well defined conditions (Spanjers 1999; Ordaz 2009). Pulse respirometry consists of measuring the dissolved oxygen (DO) concentration during the transient state observed after the injection of a defined concentration of substrate into the system and is one of the most promising respirometry techniques (Kong 1994; Vanrolleghem 1995; Riefler 1998; Ordaz 2009). Pulse respirometry has been often used to characterize mixed cultures applied to wastewater treatment and activated sludge (Ordaz 2009).

2.4.3. Optical Density

Nevertheless, the most widely used methods for bacterial density are based on determinations of transmitted or scattered light. It should be noted that in spite of the widespread use of the optical techniques, not enough efforts have been made to check them against direct

estimations of cell concentrations or bacterial densities. Furthermore a variety of instruments, based on different principles, are in use. The readings of these instruments are often quoted without reference to direct estimations as arbitrary units of turbidity, the word being used in an undefined sense (Monod 1949).

The instruments best fitted for the purpose appear to be those which give the readings in terms of optical density (log I_o/I). With cultures well dispersed, it is generally found that optical density remains proportional to bacterial density throughout the positive phases of growth of the cultures (Monod 1942). When this requirement is fulfilled, optical density determinations provide an adequate and extremely convenient method of estimating bacterial density (Monod 1949; Lee 2008).

2.5. Genomics

Molecular typing methods targeting the 16S rRNA gene (rrn) are widely used to investigate microbial communities in various environments (Crosby 2003; Lee 2006). The culture-independent techniques, such as terminal restriction fragment length polymorphism, denaturing gradient gel electrophoresis, single strand conformation polymorphism, fluorescence in situ hybridization, and the most recently developed real-time polymerase chain reaction (PCR), have provided powerful tools to particularly look into a mixed culture system (LaParra 2000; Ueno 2001; Klatt 2003; Lee 2006; Yu 2006).

2.5.1. PCR and qPCR

The polymerase chain reaction (PCR) can detect very low concentrations of 16S ribosomal genes in soil (or medium) and therefore measure low populations densities. PCR

procedure for quantification of bacterial populations is inappropriate because the amplification efficiencies vary between environmental samples or template concentrations (Reysenbach 1992; Suzuki 1996; Polz 1998; Schwartz 2000). Biases such as variations in amplification efficiencies or product-generated plateaus due to consumption of necessary reagents can be avoided by using a quantitative competitive PCR (QC-PCR) protocol (Diviacco 1992; Siebert 1992; Schwartz 2000). The QC-PCR technology offers fast and reliable quantification of any target sequence in a sample (Burgos 2002; Lee 2006). QC-PCR is particularly well suited for monitoring the population density of an inoculated organism (Schwartz 2000). While many methods are available for quantification of nucleic acids, real-time PCR is at present the most sensitive and accurate method (Ferre 1992; Klein 2002; Lee 2006).

2.6. Model Bacteria

2.6.1. *Pseudomonas putida*

The genus *Pseudomonas* represents a physiologically and genetically diverse group with a great ecological significance (Widmer 1998; Zago 2009; Mulet 2010). *Pseudomonads* are ubiquitous microorganisms found in all major natural environments and in intimate association with plants and animals (Zago 2009). Migula in 1894 described the genus as of gram-negative, rod shaped microorganisms (Palleroni 1984; Wu 2011). A prominent property of some species or strains is their metabolic versatility, making them attractive candidates for use in bioremediation (O'Sullivan 1992; Keel 1996; Widmer 1998; Wu 2011). *Pseudomonas* has the potential to degrade aromatic hydrocarbons that range in size from single ring to polycyclic aromatic (e.g., naphthalene) (Zago 2009; Wu 2011). Polycyclic aromatic hydrocarbons (PAHs) are toxic and carcinogenic compounds so widely distributed in the environment to motivate the study of the

microbial metabolism of these compounds to develop bioremediation technologies. *Pseudomonas putida* metabolizes the naphthalene to salicate, which then converted to cathechol, followed by ortho- or meta-cleavage to TCA cycle intermediates (Zago 2009). Furthermore, *P. putida* is used as a biocontrol agent for the *Fusarium* wilt pathogen to control black root disease of tobacco in large scale biotechnological application (Zago 2009).

2.6.2. *Escherichia coli* K12

Escherichia coli are normal inhabitants of the colons of virtually all warm-blooded mammals. *E. coli* belong to the taxonomic family known as *Enterobacteriaceae*, which is one of the best-defined groups of bacteria. The *E. coli* K12 is a debilitated strain which does not normally colonize in the human intestine. It has also been shown to survive poorly in the environment, has a history of safe commercial use, and is not known to have adverse effects on microorganisms or plants. Because of its wide use as a model organism in the research areas of microbial genetics and physiology, and its use in industrial applications, *E. coli* K12 is one of the most extensively studied microorganisms (EPA 1997).

2.6.3. 16S rRNA

One approach to measure the abundance of specific bacterial population is to determine the concentration of 16S ribosomal genes unique to that population (Schwartz 2000). Although the design of genus-specific 16S rRNA gene PCR primers depends on both a well-defined molecular taxonomy and a representative collection of target sequences (Widmer 1998), it is a powerful tool for genus assignments; still it does not discriminate sufficiently at the inter-species

level (Yamamoto 2000; Mulet 2010). For the purpose of this study, 16S rRNA is a feasible target to discriminate between two model bacteria.

Chapter 3: Material and Methods

3.1. Microbial cultures

Escherichia coli, E. coli K12 (DSM 5911) was grown aerobically in Difco^{TM} Luria-Bertani (L-B) medium (Sparks, MD) at 37 ^oC. *Pseudomonas putida, P. putida* (DSM 8368) was grown aerobically in Bacto^{TM} Triptic Soy (TSB) medium (Sparks, MD) at 37 ^oC. Both strains were revived in 1.0 mL of their respective growth medium and incubated at 37 ^oC until life cycle reached stationary phase. Each bacteria growth was monitored by absorbance at 550 nm using a Nanodrop 1000 spectrophotometer (Thermo Scientific). After initial growth, the strains were continued growing in 125 mL of medium aerated by an orbital shaker (GallenKamp) at 240 rpm in ambient temperature, until the stationary phase was reached for continuing to transfer in preparation for kinetic experiments. Prior kinetic experiments, each culture was transferred to Amresco^{TM} M9 medium (Solon, OH) with 20% glucose concentration as the seeding reactors.

3.2. Experimental design of bioreactor

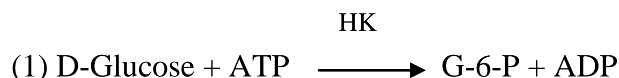
Microbial growth experiments at different initial substrate concentration were conducted for *P. putida* and *E. coli* K12 in batch reactors. The batch experiment for *P. putida* growth was performed in a 500 mL flask with 300 mL Amresco^{TM} M9 medium (Solon, OH) (Koutinas 2011). M9 broth contained sodium phosphate dibasic, potassium phosphate monobasic, sodium chloride, and ammonium chloride (pH 7.2) with an additional supplement of 1M $MgSO_4$, 1M $CaCl_2$, and D-glucose with an initial glucose concentration 175 mg/L. For another single culture growth batch, the growth kinetics of *E. coli K12* was performed in a 500 mL flask with 300 mL M9 broth with the same medium specifications as the former experiment. We measured the biomass growth using absorbance and genomic assay as well as glucose consumption for the kinetics of single culture and substrate.

Seeding of single culture reactors with pure cultures only followed after growth reached the stationary phase in M9 medium. The life cycle for *P. putida* was recorded as 7 days and *E. coli* K12 had a life cycle of 24 hrs. Each pure culture was then centrifuged for 10 min at 5000 rpm. The supernatant was discarded and the pellet was resuspended in deionized water to a volume of 10 mL. For washing purposes, we centrifuged the later at 5000 rpm for an additional 5 min to eliminate all nutrients from seeding reactor. The pellet was then transferred to the kinetic reactors and homogenized manually.

Following the single culture experiments, mixed culture was examined for the kinetics of microbial growth. The batch experiment for dual culture (1:1 ratio) of *P. putida* and *E. coli* K12 was performed in a 500 mL flask with 300 mL of M9 medium and an initial glucose concentration 175 mg/L. After the reactors were inoculated with pure cultures they were continuously aerated at 240 rpm by an orbital shaker. The bacteria were transferred from the seeding reactor (20% glucose) and washed as described previously.

3.2.1 Substrate

Glucose concentration in the samples was measured using R-Biofarm substrate- D-glucose enzymatic kit. The D-glucose concentration was obtained as a result of the following principle. D-Glucose is phosphorylated to D-glucose-6-phosphate (G-6-P) in the presence of the enzyme hexokinase (HK) and adenosine-5'-diphosphate (ADP) (Reaction1)

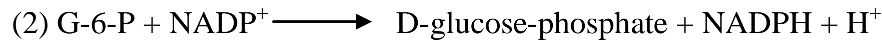


$$(1)\ \text{D-Glucose} + \text{ATP} \xrightarrow{\text{HK}} \text{G-6-P} + \text{ADP}$$

In the presence of the enzyme glucose-6-phosphate dehydrogenase (G6P-DH), G-6-P is oxidized by nicotinamide-adenine dinucleotide phosphate (NADP) to D-Glucose-6-phosphate

with the formation of reduced nicotinamide-adenine dinucleotide phosphate (NADPH) (Reaction 2)

G6P-DH



(2) G-6-P + $NADP^+$ ⟶ D-glucose-phosphate + NADPH + H^+

The amount of NADPH formed in this reaction is stoichiometric to the amount of D-glucose. The increase in NADPH is measured by means of its light absorbance at 334 nm wavelength. After determining the absorbance difference of the blank and sample, equation 2 was used to calculate the concentration:

$$c = \frac{V * MW}{\in * d * v * 1000} \; X \, \Delta A \qquad (2)$$

It follows for D-Glucose

$$c = \frac{3.020 * 180.16}{6.3 \; * 1.0 * 0.1 * 1000} \; X \, \Delta A$$

For the determination of the D-Glucose using the UV-method enzymatic kit, we measured the absorbance at 340 nm wavelength using a MDS SpectraMax M2 spectrophotometer (Sunnyvale, CA). The initial absorbance was measured 3 min after adding 0.100 mL of sample to 1 mL of solution #1 in a 1.00 cm light path cuvette (Plastibrand ®). Solution #1 consisted of triethanolamine buffer, pH approximated 7.6; NADP; ATP and magnesium sulfate. The second measurement was performed 15 min after adding 0.020 mL of solution #2 to initiate the reaction. Solution #2 was a suspension consisting of hexokinase and glucose-6-phosphate dehydrogenase.

3.3. Microbial growth kinetics

Each batch culture experiment was monitored as a function of time. Two main variables measured were: (1) the consumption of the growth-controlling substrate (D-Glucose) and (2) the increase in biomass concentration by three quantification methods: total suspended solids (TSS), optical density and genomic assay. TSS measurement was obtained using the dry weight method. Cells were separated from the medium by filtration using Watman qualitative filter membranes, 0.45 μm in pore size, in duplicates with a sample volume of 10 mL. Vacuum was applied to pull the liquid through the membrane and the wet weight of the culture was measured immediately after all medium had been pulled through. The cell paste was then dried in an oven at 100 ^{o}C for 24 hrs. Dry weight was then recorded in order to calculate the difference in weight for biomass calculations. ptical density measurement was obtained by the absorbance produced at 550 nm wavelength using a MDS SpectraMax M2 spectrophotometer in a Nunc™ 96 well cell culture plate (Roskill, Denmark). Triplicates were measured with a sample volume of 300 μL. Immediately after sampling, 2 mL of each sample were stored for DNA extraction at -80 ^{o}C.

The kinetics parameters μ_{max} and K_s were calculated from the data using nonlinear curve fit regression in Excel software. This method involves manual data entry and graphing of data, followed by curve fitting and displaying the resulted curve fit on top of the data. This process minimizes the value of the square sum of the differences between the data and the fit (Brown 2001).

3.4. Genomic DNA extraction

Genomic DNA (gDNA) was extracted from each sample following the manufacturer's protocol using Fast DNA kit for soil (MP Biomedicals). 1.5 mL samples were used for this method and involved mixing by centrifugation and filtering steps in conjunction to the addition of solutions provided by the kit. A lysing matrix was used for cell rupture with sodium phosphate buffer and MT buffer. A protein precipitator solution (PPS), Binding matrix and SEWS-M solutions were also provided. The gDNA was eluted in 100 µL of DNase/Pyrogen-Free water for further analysis. Genomic DNA concentration was measured using Nanodrop 1000 spectrophotometer. To confirm the presence of genomic DNA extracted from samples gel electrophoresis was conducted in a 1% agarose gel solution with a 1 µL sample volume and 0.5 µL Biorad nucleic acid sample buffer 5X. A Biorad molecular ruler 50 - 2000 bp ladder was to determine the size of the DNA band.

3.5. PCR assay

All PCR amplifications were conducted in an Applied Biosystem 2720 Thermal Cycler. Each PCR reaction mixture for *E. coli* K12 was prepared using 5 µL Buffer (10X), 3 µL of $MgCl_2$ (25 mM), 4 µL dNTPs (2.5 mM), 2.5 µL of forward primer (10 µM), 2.5 µL of reverse primer (10 µM), 0.4 µL of *Taq* polymerase (5 U/µL), 2 µL of gDNA (1 µL). The thermal cycling protocol was as follows: initial denaturation 94 ^{o}C for 5 min, followed by 30 cycles of 30 sec at 94 ^{o}C, 30 sec at 55 ^{o}C; 30 sec at 72 ^{o}C and 7 min 72 ^{o}C. Primers targeting 16S rRNA of *E. coli* K12 (101 bp) were f: 5'-GCTACAATGGCGCATACAAA-3' and r: 5'-TTCATGGAG TCGAGTTGT TGCAG-3'(Lee 2006).

Each PCR reaction mixture for *P. putida* was prepared using 5 µL of Buffer (10X), 3 µL of $MgCl_2$ (25 mM), 2.6 µL dNTPs (2.5 mM), 4 µL of forward primer (10 µM), 4 µL of reverse primer (10 µM), 3.7 µL of *Taq* polymerase (5 U/µL), 10 µL of gDNA (1 µL). The thermal cycling protocol was as follows: initial denaturation 95 ^{o}C for 5 min, followed by 30 cycles of 45 sec at 94 ^{o}C, 1 min at 66 ^{o}C; 1 m at 74 ^{o}C and 10 min 74 ^{o}C. Primers targeting 16S rRNA of *P. putida* (990 bp) f: 5'-GGTCTGAGAGGATGATCAGT-3' and r: 5'-TTAGCTCCACCTCG CGGC-3' (Widmer 1998).

For both PCR products from pure culture we conducted gel electrophoresis in a 2% agarose gel solution to confirm that the product size was in accordance with the referenced publication. *E. coli* K12 band was imaged as reported by Lee et al. at the 101 bp region and the *P. putida* band was imaged as reported by Widmer et al. in the 990 bp region (Widmer 1998; Lee 2008).

Following PCR amplification the products were purified using the Zymo DNA Clean and Concentrator 5 kit following manufacturer's protocol and the purified product was imaged for validation. The concentration of the product was measured using the Nanodrop 1000 spectrophotometer and the corresponding copy number was calculated using equation 1. (Lee 2006)

3.6. qPCR assay

All qPCR reactions were conducted in an Applied Biosystems Step One real-time PCR system. For *E. coli* K12*,* all runs were performed in duplicates, and each reaction mixture was prepared using an Applied Biosystems Fast SYBR Green Master Mix in a total volume of 20 µL: 12 µL DNAse and RNAse free water (GIBCO Ultra Pure^{TM} Distilled Water), 1.0 µL of each

primer (final concentration 0.5 μM), 4.0 μL SYBR Green and 2.0 μL template DNA. The thermal cycling protocol was as follows: initial denaturation for 10 min at 95 ^{o}C followed by 35 cycles of 5 sec at 95 ^{o}C, 5 sec at 60 ^{o}C, and 10 sec at 72 ^{o}C. The fluorescence signal was measured at the end of each extension step at 72 ^{o}C. (Lee 2008) For *P. putida*, all runs were performed in triplicates, and each reaction mixture was prepared using SYBR Green in a total volume of 20 μL: 2.2 μL DNAse and RNAse free water, 1.4 μL of each primer (final concentration 0.5 μM), 10.0 μL Absolute SYBR Green and 5.0 μL template DNA. The thermal cycling protocol was as follows: initial denaturation for 15 min at 95 ^{o}C followed by 40 cycles of 10 sec at 95 ^{o}C, 15 sec at 65 ^{o}C, and 20 sec at 72 ^{o}C. The fluorescence signal was measured at the end of each extension step at 72 ^{o}C (Widmer 1998).

Chapter 4: Results and discussion

4.1. qPCR calibration curve

Calibration curves for both genes were constructed for growth quantification. dsDNA target fragments were produced via PCR reaction and were additionally purified with the Zymo kit to be used as template for the calibration curves. Figure 1 shows the electrophoresis images for the optimization and validation of the template used in the *E. coli* K12 calibration curve. Figure 1 (a) illustrate the serial dilutions of the PCR product, which was used to confirm the product size as in the 100 bp (Lee 2008). The 10^{-1} dilution was selected as the preferred product. Depicted in Figure 1 (b) is the 10^{-1} dilution after purification to corroborate the presence of desired DNA. Similar results were obtained for the *P. putida* PCR product and purification in the electrophoresis image showing the band at 1000 bp region (Widmer 1998).

Serial dilutions from each strain template were used to construct the qPCR calibration curve (i.e. 5.8×10 to 5.8×10^8). The gene copy number from each PCR products was calculated based on the equation (3) below (Whelan 2003).

$$gene\ copy = \frac{6.02E23\left(\frac{copy}{mol}\right) \cdot DNA\ amount\ (g)}{DNA\ lenght\ (bp) \cdot 660\ \left(\frac{\frac{g}{mol}}{bp}\right)} \qquad (3)$$

This study successfully constructed individual calibration curves for each model bacteria. R^2 value (larger than 0.9) and its range of quantification for each model bacteria are presented in Table 1. Sensitivity for *E.coli* K12 was recorded as $8.4 \times 10^4 - 8.4 \times 10^{12}$. On the other hand, for *P. putida* the range of quantification was 5.8 to 5.8×10^8. The small sensitivity for the *E. coli* K12 can be addressed and optimized using plasmids as template instead of PCR product.

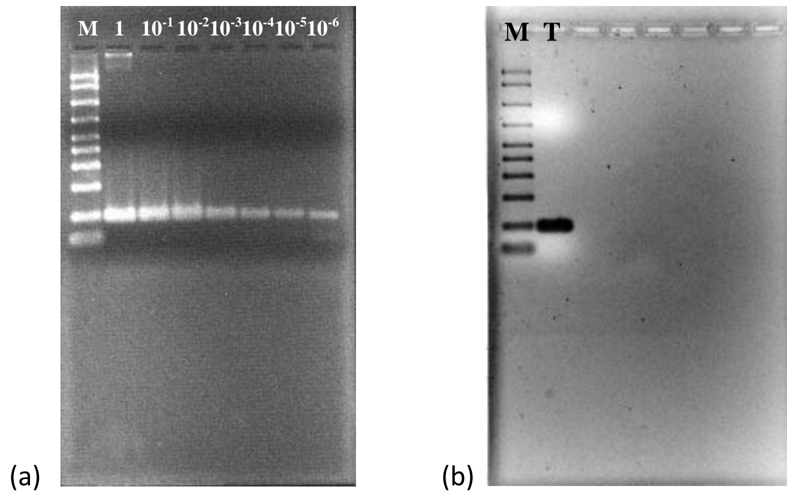


Figure 1. Optimization of template for qPCR calibration curve. The results are visualized in 1% agarose agar. A photograph of a 1% (wt/vol) agrose gel showing the PCR amplicon fragment size was in the 100 bp position. (a) *E. coli* K12 PCR product and subsequent serial dilutions (b) T denotes the PCR product after a purification step.

Table 1. Q-PCR calibration curve for genomic assay components and efficiencies

	Name of strain	
	P. putida	***E. coli* K12**
Primers		
forward	5’-GGTCTGAGAGGATGATCAGT-3’	5’-GCTACAATGGCGCATACAAA-3’
reverse	5’-TTAGCTCCACCTCGCGGC-3’	5’-TTCATGGAGTCGAGTTGTTGCAG-3’
Product size	990 bp	101 bp
Target	16s rRNA	16s rRNA
Reporter molecule	SYBR-Green	SYBR-Green
Range of quantification	$5.8 - 5.8 \times 10^9$	$8.4 \times 10^4 - 8.4 \times 10^{12}$
R^2	0.976	0.995

Note. Primers, product size, target and reporter information obtained from Widmer 1998 for *P. putida* and Lee 2008 for *E. coli* K12.

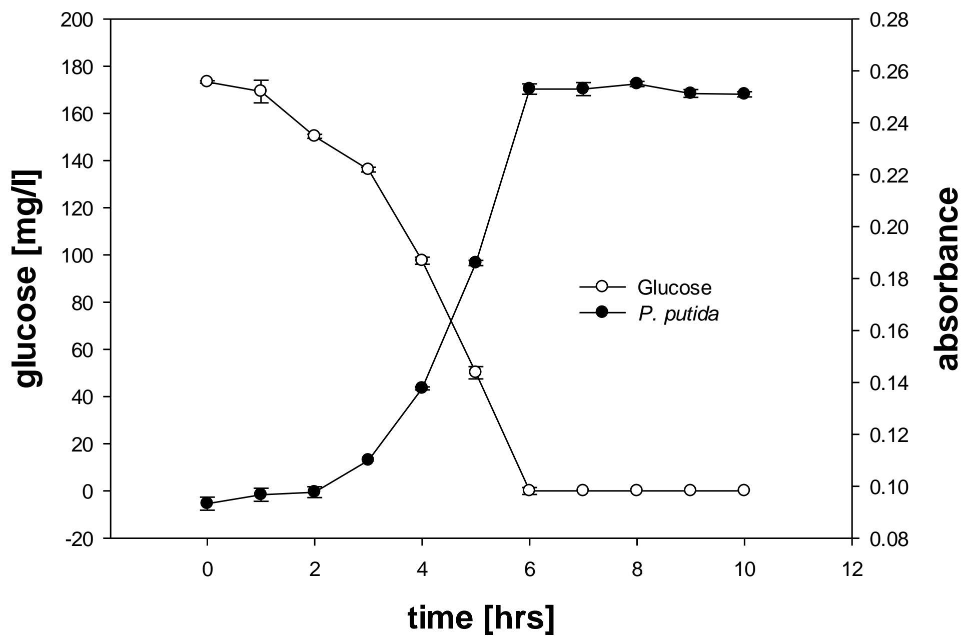
4.2. Kinetic experiments for single culture and substrate

The biomass and substrate were measured as a function of time in order to obtain growth kinetic parameters in single culture and substrate system. The biomass of *P. putida* and *E. coli* K12 was quantified using two methods described previously: optical density and genomic assay. Single culture data compiled at different initial substrate concentrations showed that the optimal substrate initial concentration of 250 mg/L of glucose for *P. putida* and 175 mg/L of glucose for *E. coli K12*. Figures 2 and 3 illustrate the data obtained for single culture reactors with initial glucose concentration of 175 mg/L. Figure 2a and 2b show the growth and glucose depletion curves obtained from *P. putida* and *E. coli K12* reactors, respectively. It is presented that ideal growth curves were obtained for both bacteria in single culture and substrate systems. Figure 3a and 3b show the biomass growth measured by absorbance and genomic assay for *P. putida* and *E. coli K12* respectively. The trend of the curve shows all three phases of growth: lag, exponential growth and steady state for *P. putida* and the exponential growth phase for the *E. coli K12* reactor. All growth curves reproduced the ideal curve with stationary phase reached only at the depletion of glucose.

4.3. Monod kinetics in single culture system.

Single culture reactors with different initial glucose concentrations were monitored to obtain the Monod kinetic parameters. Biomass and substrate were measured as a function of time using absorbance and enzymatic kit respectively. Figure 4a through 4d show the microbial growth and depletion curves for the *P. putida* reactors at four different concentrations. The data obtained was then analyzed using nonlinear curve fit to obtain kinetic parameters.

(a)



(b)

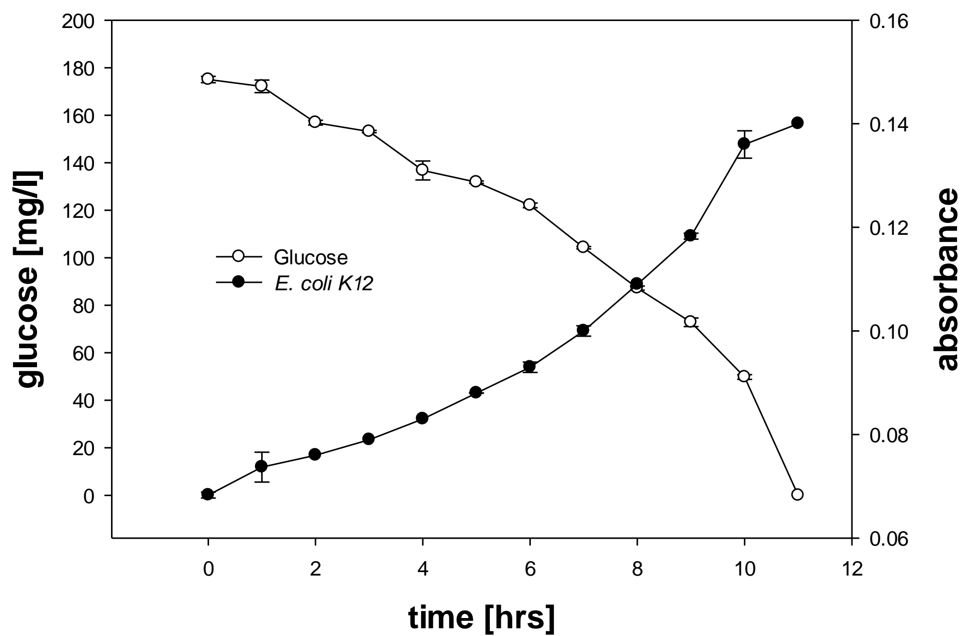
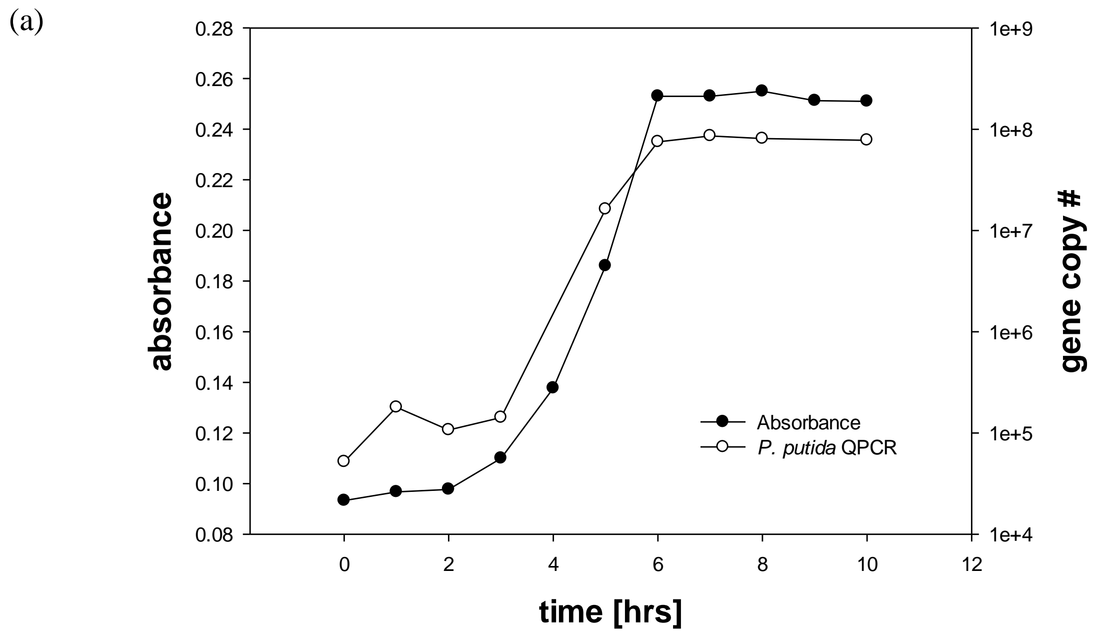


Figure 2. The results for single culture and substrate systems. It is presented the growth curves and substrate (glucose) removal for single culture reactors: (a) *P. putida* and (b) *E. coli K12*.

(a)



(b)

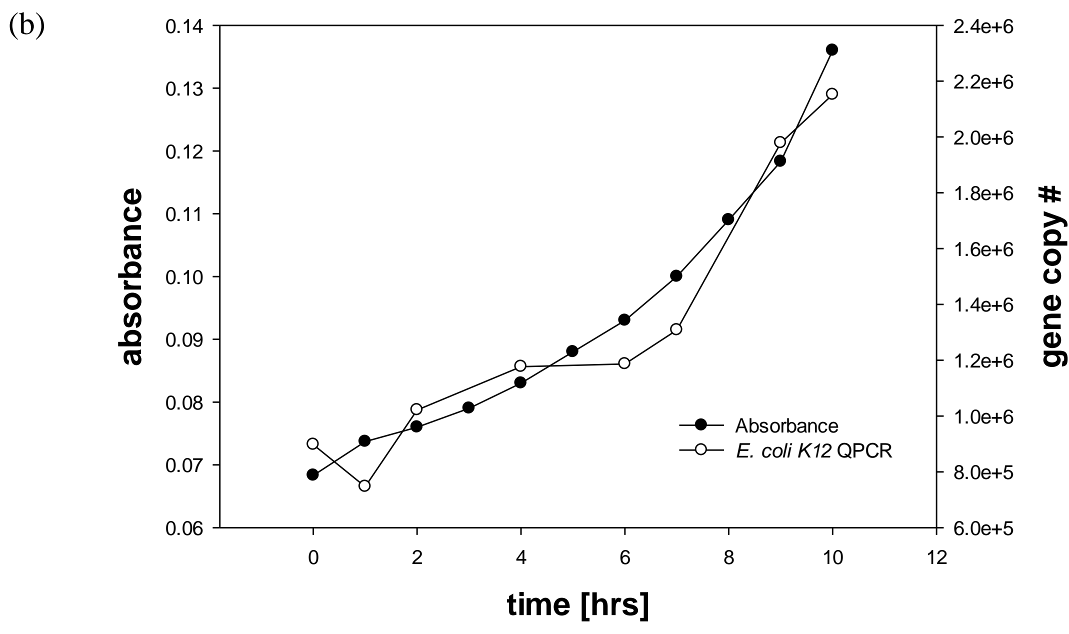


Figure 3. The results for single culture systems for growth curve analysis measured by absorbance at 550 nm wavelength and genomic assay. (a) *P. putida* and (b) *E. coli K12*.

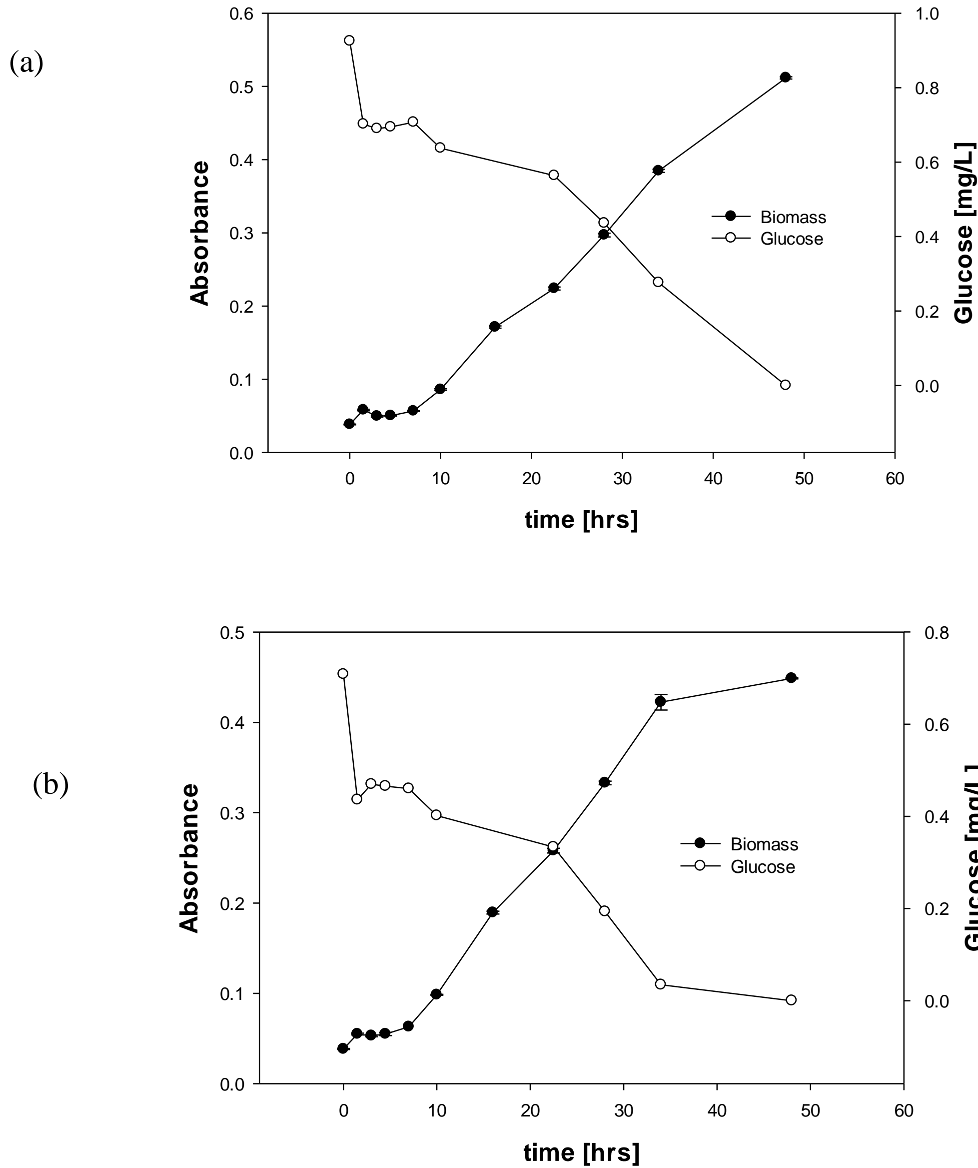
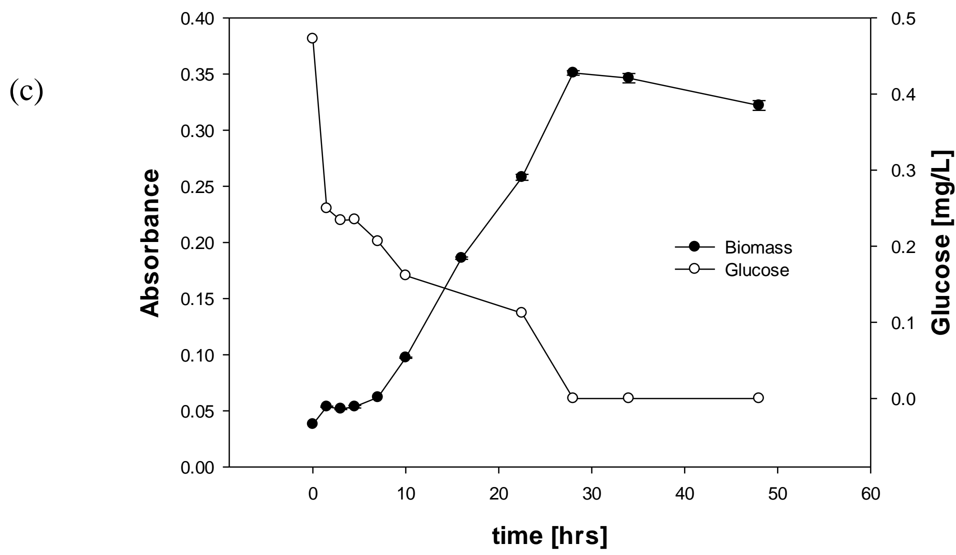


Figure 4.1. The results of single culture and substrate system for *P. putida* in batch reactor with four initial glucose concentrations: (a) 1000 mg/L (b) 750 mg/L



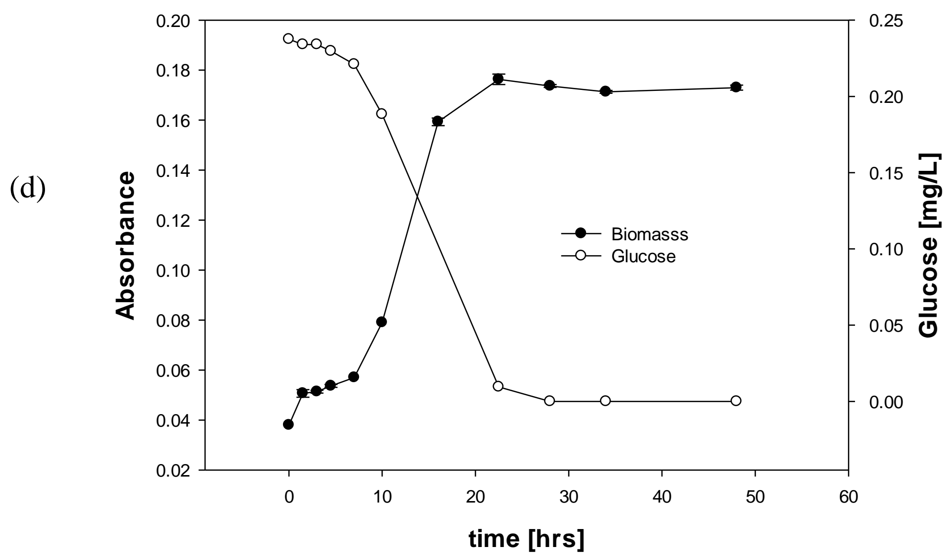


Figure 4.2. The results of single culture and substrate system for *P. putida* in batch reactor with four initial glucose concentrations: (c) 500 mg/L (d) 250 mg/L

4.4. Determination of biomass in culture system.

Single culture and substrate systems at different glucose concentrations (750 to 250 mg/L) were performed to monitor the biomass growth using TSS, optical density and genomic assay and to determine the validity of the genomic assay analysis. Plots from the data measured by absorbance and genomic assay replicated the ideal microbial growth curve; however the plots produced by TSS data failed to display growth trends. Figure 5 summarizes the three growth patterns measured in this experiment for *P. putida*. The results obtained from TSS data can be attributed to the small sample volume, which may had compromise the effectiveness of the TSS method (Monod 1949). As reported by Lee, the monitoring of variations in rrn copy number with growth can be applied in a time-course study, in mixed as well as pure culture systems (White 2000; Klappenbach 2001; Lee 2008). The data confirms that genomic assay is a vial alternative for microbial growth measurements as it shows a similar growth as measured by the most traditional method of absorbance.

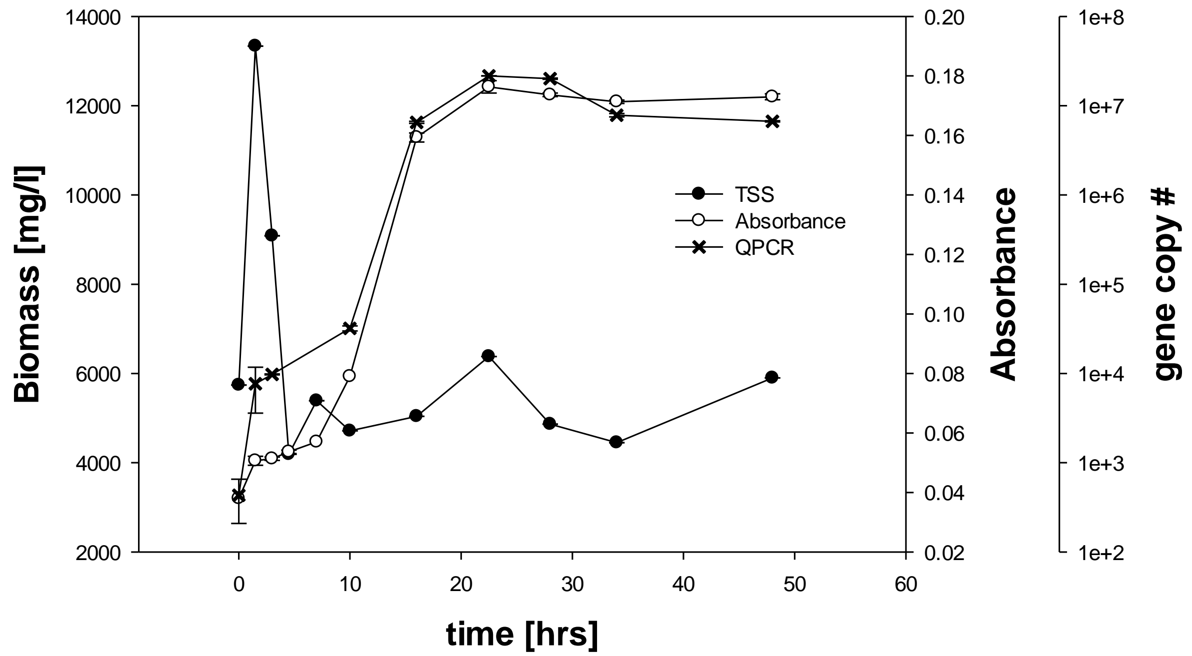


Figure 5. Microbial growth (*P. putida*) measured using TSS, absorbance and qPCR. The data presented is from the batch reactor with initial substrate concentration of 250 mg/L.

4.5. Kinetics in mixed culture system

4.5.1 Mixed culture system

Kinetics for the mixed culture was conducted following the procedure presented previously. *P. putida* and *E. coli K12* biomass was measured by absorbance and qPCR assay. D-Glucose concentration was also measured as described for the other experiments. The growth and substrate depletion curves for the mixed culture are presented in Figure 6. As expected in this study, the overall growth measured by absorbance and substrate consumption followed the similar trend presented in the single culture systems.

4.5.2 Monod kinetics

Kinetic parameters, μ_{max} and K_s, were calculated for single and mixed culture system. Table 2 summarizes the kinetic parameters calculated from the presented experiments and the reference values published in equivalent studies. The comparison indicates that the kinetic parameters obtained from the batch experiments are reasonably fit to the reference values. Figure 6 depicts the Monod curves fitted from the obtained values using a nonlinear fit regression. Linear regression or Lineweaver-Burk method has been found to give a deceptively good fit, even with unreliable data points, and thus some authors recommended against its use. Nonlinear regression analysis is reported to yield better parameter estimations in laboratories experiments (Grady 1999). Robinson presented that when substrate consumption is linked to growth, the number of catalytic units, or activity, increases over time. Assuming that the initial concentration is greater than that which gives one-half of the maximum growth rate, an increase in activity concomitant with substrate consumption yields an S-shape substrate depletion curve, or sigmoidal kinetics (Robinson 1983). Our data followed sigmoidal kinetics as shown in Figure 7.

Sigmoidal kinetics is predicted by Monod kinetics, but it was shown that nonlinear regression analysis generally provided better estimates of the parameters than did the least-squares analysis of the linearized data (Robinson 1983).

Koutinas stated that when no substrate interactions are identified (single substrate system), simple Monod terms can be added in sum kinetics (Koutinas 2011). A simple additive relationship between the kinetic parameters from single culture systems was created to validate or reject this relationship for mixed culture systems. Based on the experimental design, we proposed an additive model to predict mixed culture Monod parameters. Equation 4 shows the hypothesized model to predict mix culture kinetics from single culture reactors.

$$\mu_{max}\ (mix) = \mu_{max}\ (\textit{E.coli}) + \mu_{max}\ (\textit{P.putida})$$
$$K_s\ (mix) = K_s\ (\textit{E.coli}) + K_s\ (\textit{P.putida}) \qquad (4)$$

Table 2 presents the result from adding the respective parameters of each single culture data. We concluded that there is a significant difference between the predicted and measured values for both kinetic parameters. From literature we understand that variations of reported values between different laboratories can be attributed to confounding factors between experimental setup. Regular transfer of cells in the exponential phase into new medium frequently results in a slight increase of μ_{max}, a well-known effect referred as 'training', probably due to selection of fast growing mutants, but sometimes also to improved experimenter's handling (Egli 2009). K_s may also vary with substrate concentration if multiple uptake systems with different affinities for the compound are present. The activity of high affinity transport systems would be expected to increase relative to uptake systems of lower affinity as substrate concentration decreases. In addition, organisms capable of producing different uptake systems may change the relative rates at which high- and low- affinity systems are synthesized as a

function of substrate concentration. Presumably, over several generations of growth at low substrate concentrations, progeny cells would be enriched for high-affinity transport systems. In either case, the value of K_s estimated by nonlinear regression might be expected to decrease with declining substrate concentration (Alexander 1985).

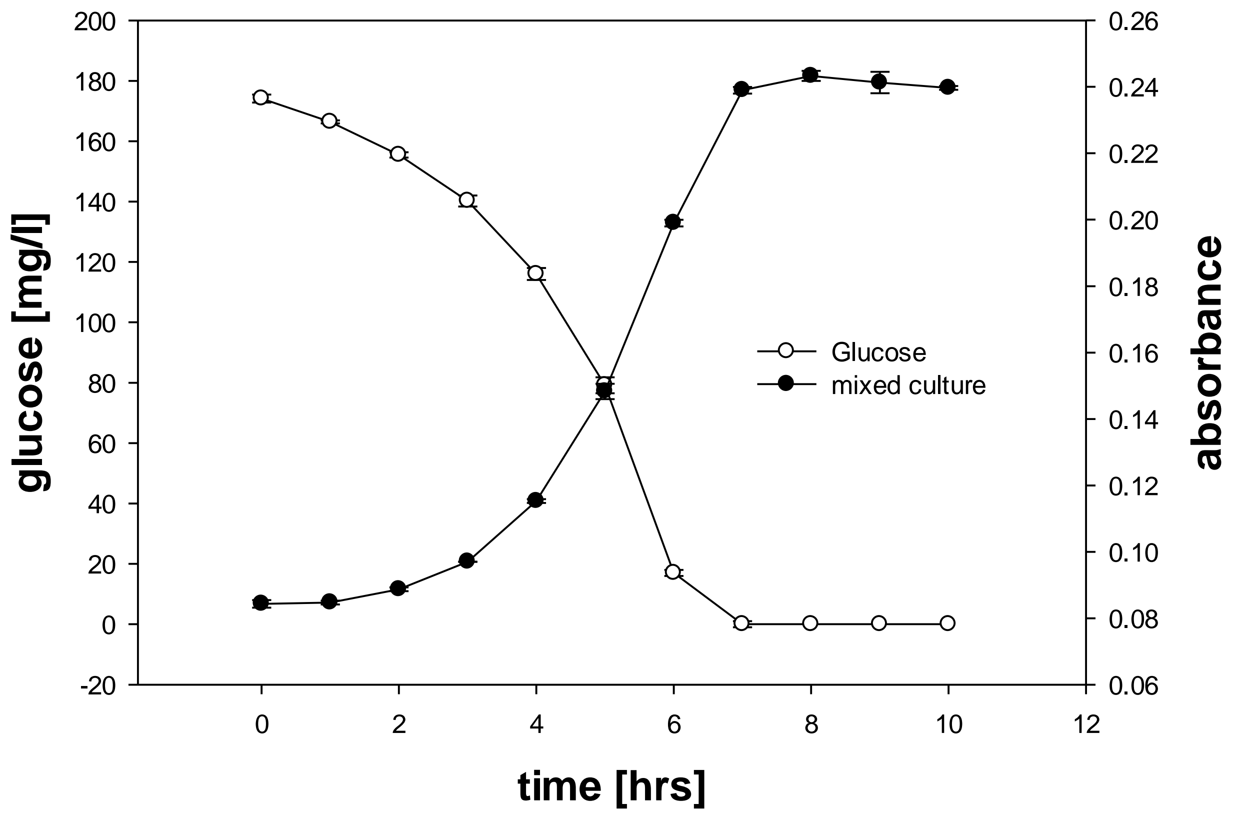


Figure 6. The results for mixed culture system. Absorbance determined by a spectrophotometer at 550 nm wavelength indicates the average biomass in the mixed culture reactor.

Table 2. Monod kinetic parameters for single culture and mixed culture kinetics.

		kinetic parameters	
Name of strain	**Description**	**K_s**	**μ_{max}**
***E. coli* K12**	this study	10.0029 mg/L	0.0990 hr^{-1}
	Egli, 2009	7.1600 mg/L	0.7600 hr^{-1}
P. putida	this study	39.0501 mg/L	0.0444 hr^{-1}
	Ordaz 2009	4.86-9.30 mg/L	0.014-0.20 hr^{-1}
mixed culture	this study	57.279 mg/L	0.1065 hr^{-1}
	model	49.053 mg/L	0.1434 hr^{-1}

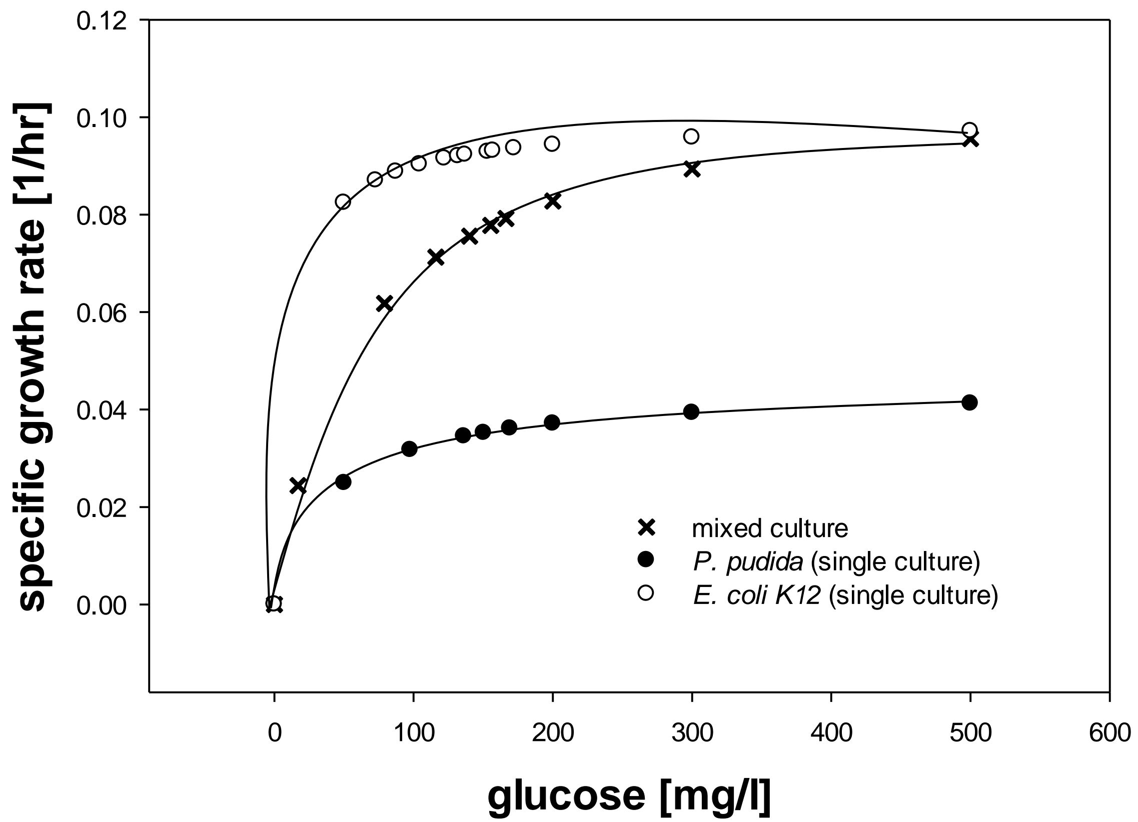


Figure 7. The Monod fit for single cultures and mixed culture batch reactors. Biomass was measured by absorbance for parameters derivation using nonlinear curve fitting.

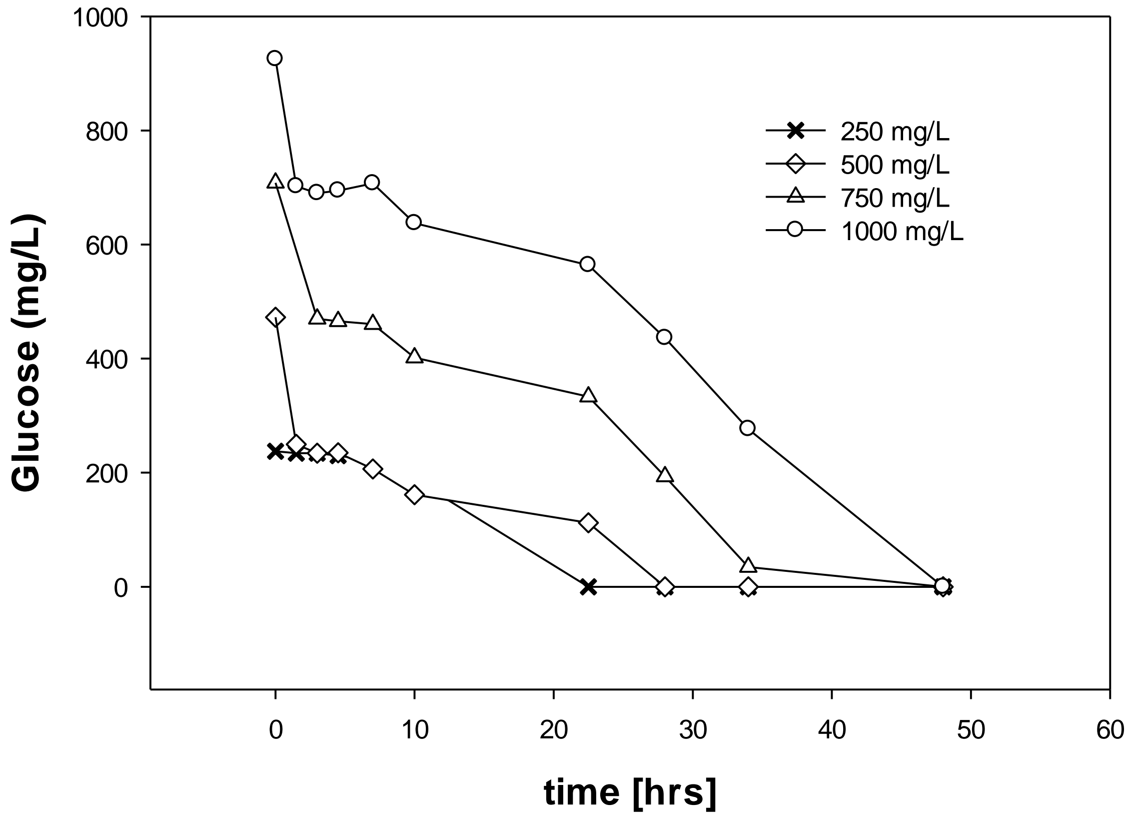


Figure 8. Mineralization of D-glucose by *P. putida* in single culture batch reactors at four different substrate concentrations. Substrate consumption yielded an S-shape substrate depletion curve.

4.5.3. Biomass changes in mixed culture system

Samples from the mixed culture system were used to examine the necessity of genomic assay that can determine individual species in the mixed culture while traditional OD measurement cannot differentiate individual species. Figure 9 illustrates that we effectively quantified each strain growth curve separately. This figure shows that traditional OD measurements cannot discriminate between strains in mixed cultures, but that genomic assay can accurately monitor growth as a function of time.

The growth curve derived from absorbance is similar to the genomic quantification of the *Pseudomonas* strain, on the other hand, the *Escherichia* strain did not present a significant growth during the time of the experiment and a decline as the substrate approached exhaustion (Figure 8). Confounding variables may be present while conducting the experiments. Simkins and Alexander noted from their results a tendency for cell quota to decrease with decreasing initial concentration of substrate and the density of *Pseudomonas* sp. cells. This may explain the decrease in DNA concentration from the mix culture reactor, because the substrate was used more slowly when provided at low level to small populations, one would expect maintenance costs to consume a greater fraction of the cell's energy, thus increasing the cell quota (Alexander 1985).

Although the genomic assay data for the *E. coli* K12 has lower fitting in comparison to the *P. putida*, one can conclude that for both reactors the growth was effectively measured using the genomic assay method proposed in this study.

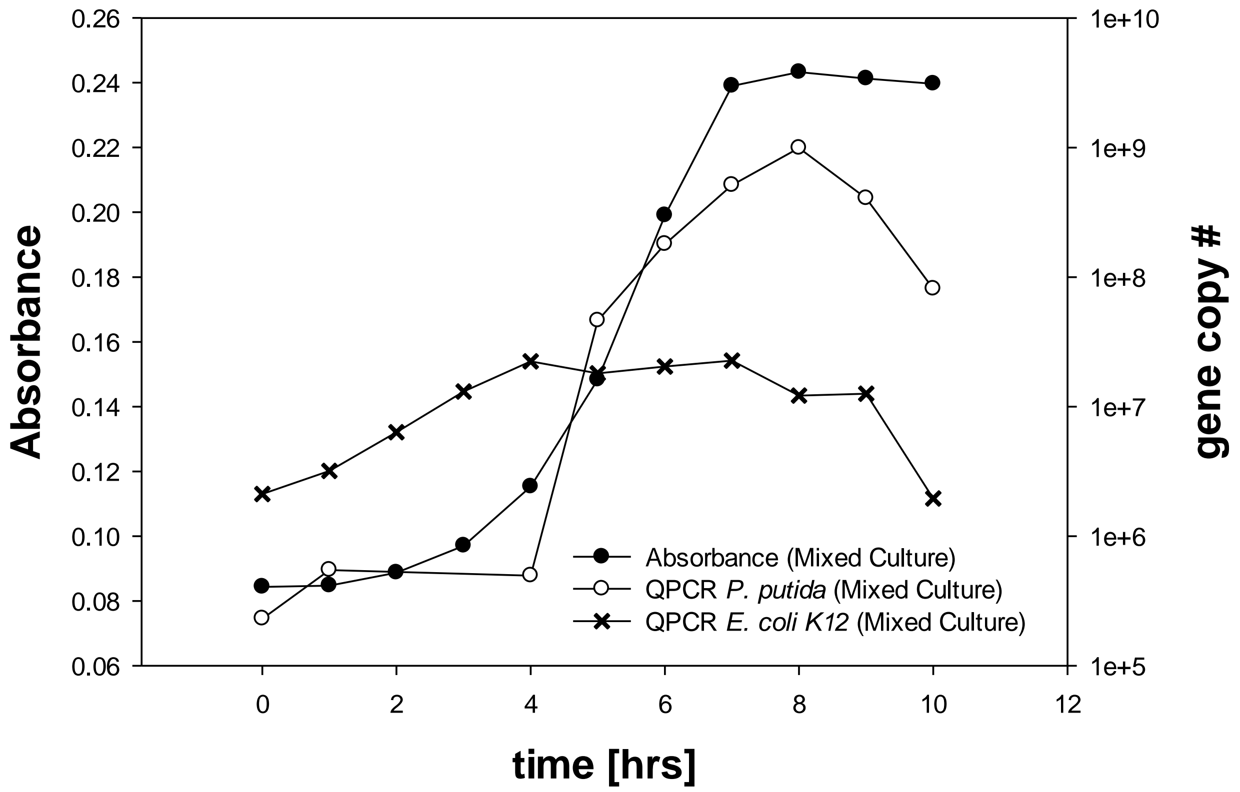


Figure 9. Growth curves for mixed culture system at a substrate concentration 175 mg/L. Two different growth curves were obtained by genomic assay and the overall microbial growth was measured by absorbance at 550 nm wavelength.

Chapter 5: Conclusions and Future Work

Much progress has been made in the field of microbial growth kinetics, but more studies are needed. The application of genomic assay in the calculation of Monod kinetic parameters will benefit those who model microbial kinetics as well as engineers looking to optimize bioprocesses. This study successfully obtained the kinetic parameters by nonlinear fit analysis for single culture and substrate systems. The kinetic parameters for mixed culture were calculated but a concise relationship to predict such parameters from the single culture reactors was not attained. In addition, genomic assays were implemented in the determination of the microbial growth in mixed culture systems. The author successfully measured two growth curves separately from the mixed culture samples by genomic assay. It is concluded that current growth quantification methods are inefficient for mixed culture reactors and genomic assay is a vial alternative to accurately quantify the individual growth of strains in mixed culture systems.

It is recommended to further elucidate the ecology of mixed culture by constructing Monod curves using genomic assay data. A study that can combine a mathematical model and gene expression with the growth kinetics of the host microorganism can be of significant application in the field of bioremediation. Subsequent experiments using the parameters obtained from this study to construct a model to simulate growth kinetics for defined mixed culture can serve as a source for the developing of models of more complex systems as those found in the environment with mixed substrates and populations.

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