BIOTRANSFORMATION OF PHENOL USING IMMOBILIZED POLYPHENOL OXIDASE FROM BANANA STEM

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A project report submitted in partial fulfillment of the requirements for the award of the bachelor degree of Chemical Engineering (Biotechnology)

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To my beloved father and mother

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ABSTRACT

Polyphenol oxidase(PPO) is an enzyme that can easily find in most of the plants. It is a browning agent that cause from catalyzing the oxidation of various phenolic compounds to the corresponding quinones which lead to the formation of melanin pigment. PPO commonly used as biocatalyst in a biotransformation reaction and in this study is converting phenol to less harmful compound. The objective of this study was to study the basic kinetics of batch and continuous reactors. By conducting biotransformation using polyphenol oxidase extracted from banana stem. The enzyme immobilized using Ca-Alginate in the forms of beads. Five different phenol concentrations were transformed using different enzyme loadings for batch mode while different flow rates were used in continuous mode. Samples were analyzed using High Performance Liquid Chromatography (HPLC) at 254nm, using methanol and water (20:80) as mobile phase at 1mL/min. The space time of a batch reactor was determined and weight of catalyst loading was estimated for continuous reactor.

ABSTRAK

‘Polyphenol Oxidase’ (PPO) adalah enzim yang boleh dijumpai dengan mudah dalam kebanyakan tumbuhan. Ia merupakan agen penguningan yang disebabkan dari memangkin pengoksidaan pelbagai sebatian fenolik ke kuinon yang menyebabkan pembentukan pigmen melanin.PPO umum digunakan sebagai biokatalis pada reaksi biotransformasi dan dalam kajian ini adalah untuk menukar sebatian fenol kepada sebatian kurang berbahaya. Tujuan kajian ini adalah untuk mempelajari asas kinetik reaktor sesekumpul dan kontinu dengan melakukan biotransformasi menggunakan ‘Polyphenol Oxidase’ dari batang pisang. Enzim diperangkap menggunakan Ca-Alginat dalam bentuk manik-manik. Lima kepekatan fenol yang berbeza kepekatan ditranformasi menggunakan enzim yang berbeza untuk fasa sesekumpul sementara halaju berbeza digunakan di fasa kontinu. Sampel dianalisis dengan ‘High Performance Liquid Chromatography’ (HPLC) pada 254nm, dengan menggunakan metanol dan air (20:80) sebagai fasa gerak pada 1mL/min.Waktu ruangan sebuah reaktor sesekumpul ditentukan dan berat mangkin dianggarkan untuk reaktor kontinu.

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PBR	-	Packed bed reactor
PFR		Plug flow reactor
PPO		Polyphenol oxidase
F_{A0}	-	Molar flow rate
$-r_A$	-	Reaction rate
C_A		Phenol concentration
k		Rate constant
α		Reaction order
K_m		Michealis constant
V_{max}		Maximum rate of reaction for a given total enzyme
X_A	-	Conversion
mM		milimolar
w/v		Weight over volume percentage
nm		nanometer
rpm		Round per minute
mL		Mililiter
mL/min		Mililiter per minute
^{0}**C**		Degree celcius
%		percentage

CHAPTER I

INTRODUCTION

1.1 Background of Study

The insertion of a hydroxyl group into aromatic compounds is a useful yet unusual reaction. It can be achieved biocatalytically using polyphenol oxidase (PPO). Polyphenol oxidase (PPO) (EC 1.14.18.1), which is widely distributed in the plant, is a copper-containing enzyme and is responsible for the enzymatic browning reaction occurring in many plants and vegetables damaged by inappropriate handling, resulting in bruising or indentations [11]. The enzyme is a tetramer containing four gram atoms of copper per molecule [6], and two binding sites for aromatic compounds including phenolic substrates. There is also a distinctly different binding site for oxygen, the copper site [10]. The copper is probably in the cuprous state; inactivation of the enzyme is associated with increase in Cu^{2+}. In the presence of molecular oxygen, PPO catalyzes the o-hydroxylation of monophenols to odiphenols and oxidation of the o-diphenols to o-quinones (Figure 1). Polyphenol oxidase (PPO) is an enzyme that does not need extensive purification and easily extracted from various inexpensive sources [16]. PPO has been investigated in numerous sources, e.g. in apple [14], artichoke head [8], grape [15] aubergine [6], mulberry [4], lychee [9], banana [3], and Anamur banana [12]. Biotransformations is a process where a biological agent either the whole cell or an isolated enzyme involves in catalyzing the reaction. Such biotransformations systems may be used for environmentally benign biocatalysis of synthetic reactions, bioremediation of pollutants, or waste beneficiation, a combination of these in which the biological agents convert

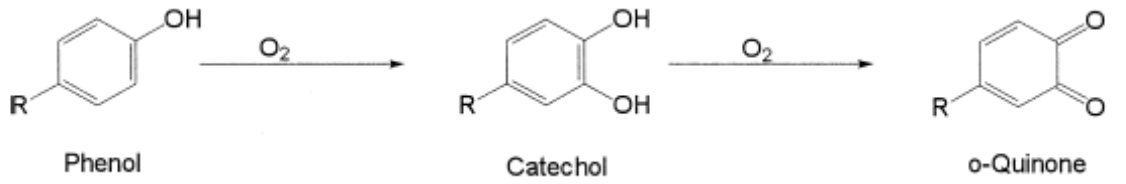


Figure 1:Hydroxylation of phenols and oxidation of catechols catalyzed by polyphenol oxidase

industrial residues to useful chemical products. In each case, suitable biocatalysts, and suitable bioreactor systems, each with particular characteristics, are required. In biotransformation processes, immobilization or stabilization of the biological agent provides clear advantages to the processes. One of it is,it can be reuse hence reduce the cost. Biocatalyst development requires identification of the enzyme and source of the enzyme [17]. Immobilization of PPO on certain membrane supports and under optimal conditions can prolong the enzyme activity and facilitate the production of catechol products, and minimizing quinone formation and obviating the need for reduction of the quinones [17].The aim of this research is to study the basic kinetics of biotransformation of phenol in batch and continuous reactor studies.

1.2 Problem Statement

1.2.1 Transforming phenol to less harmful compound due to its characteristics as hazardous pollutant.

1.2.2 Finding an effective and feasible technology in water treatment process.

1.3 Scope of Study

1.3.1 Extraction of polyphenol oxidase (PPO) from banana stem

1.3.2 Transforming phenol to less harmful compound.

1.3.3 Determination of the most efficient biocatalyst system

1.4 Objectives

1.4.1 To extract polyphenol oxidase from banana stem.

1.4.2 To do polyphenol oxidase immobilization.

1.4.3 To conduct the batch and continuous reactor operation for biotransformation of phenol.

1.4.4 To study bioreactor kinetic modelling.

1.5 Rationale and Significant

1.5.4 Society

1.5.4.1 Create an efficient cost and reliable system for effluent treatment.

1.5.1.2 Prevent from any fatal health complication.

1.5.5 Environment

1.5.5.1 Preserving aquaculture from hazardous pollutant.

CHAPTER II

LITERATURE REVIEW

2.1 Biodegradation of Phenol

Generally aromatic compounds are broken down by natural bacteria. However, polycyclic aromatic compounds are more recalcitrant. Derivatisation of aromatic nuclei with various substituents particularly with halogens makes them more recalcitrant.The critical step in the metabolism of aromatic compounds is the destruction of the resonance structure by hydroxylation and fission of the benzoid ring which is achieved by dioxygenase-catalysed reactions in the aerobic systems. Based on the substrate that is attacked by the ring cleaving enzyme dioxygenase, the aromatic metabolism can be grouped as catechol pathway, gentisate pathway, and proto catechaute pathway. In all these pathways, the ring activation by the introduction of hydroxyl groups is followed by the enzymatic ring cleavage. The ring fission products, then undergoes transformations leading to the general metabolic pathways of the organisms. Most of the aromatic catabolic pathways converge at catechol. Catechols are formed as intermediates from a vast range of substituted and nonsubstituted mono and poly aromatic compounds. Aerobically, phenol also is first converted to catechol, and subsequently, the catechol is degraded via ortho or meta fission to intermediates of central metabolism. The initial ring fission is catalysed by an ortho cleaving enzyme, catechol 1, 2 dioxygenase or by a meta cleaving enzyme catechol 2,3 dioxygenase, where the product of ring fission is a cis-muconic acid for the former and 2-hydro cis muconic semi aldehyde for the latter [13]. In this study,we used polyphenol oxidase as

an agent on transforming phenol. Polyphenol oxidase is a (EC 1.14.18.1) monoxygenase which catalyses the O-hydroxylation of phenols and the oxidation of O-dihydric phenols to O-quinones using molecular oxygen. Laccase are phenol oxidases which utilize molecular oxygen. They are known to have the ability to oxidize polyphenols, meta substituted phenols, diamines and a variety of other components [15]. The mechanism by which polyphenol oxidase catalyses the conversion of monophenols to O-quinones involves the hydroxylation of monophenols followed by dehydrogenation to form O-quinones. These quinines undergo spontaneous nonenzymatic polymerization in water, eventually forming water insoluble polymers whichcan be separated from water by filtration [8]. There were various reports on the exploitation of polyphenol oxidase in the detoxification of the phenols. The interest in polyphenol oxidase had been fueled by their potential uses in detoxification of environmental pollutants [2]. Production of useful chemicals from lignin [3] by polyphenol oxidase was also reported. [11] reported a polyphenol oxidase from the white rot fungus *Trametes trogii*. It was an enzyme with molecular weight 70 KD. The purified enzyme oxidised a number of phenolic compounds. This multicopper oxidases had a wide range of substrate specificity. Of the various enzymes acting on phenol, polyphenol oxidase was the most important one probably because of its increasing demand in lignin degradation [11]. The non specific nature of the polyphenol oxidase was also discussed by Schneider et al. (1999)[21]. Immobilised polyphenol oxidase on chitosan coated polysulphone capillary membranes were used for improved phenolic effluent bioremediation [9]. They also highlighted the removal of quinones and other polymerized products using chitosan. Polyphenol oxidases were widely distributed in many plants and fungal species [18]

2.2 Polyphenol Oxidase

Polyphenol oxidase (PPO) (EC 1.14.18.1), which is widely distributed in the plant and animal kingdoms, is a copper-containing enzyme and is responsible for the enzymatic browning reaction occurring in many plants and vegetables damaged by improper handling, resulting in bruising, compression or indentations [23]. In the presence of molecular oxygen, PPO catalyzes the o-hydroxylation of monophenols to odiphenols (monophenolase activity) and oxidation of the o-diphenols to o-quinones (diphenolase activity) [6]. PPOs are very important enzymes in the food industry, due to their involvement in the enzymatic browning of edible plants, which is highly undesirable. Enzymatic browning impairs the sensory properties and marketability of the product and also lowers the nutritional value [12].

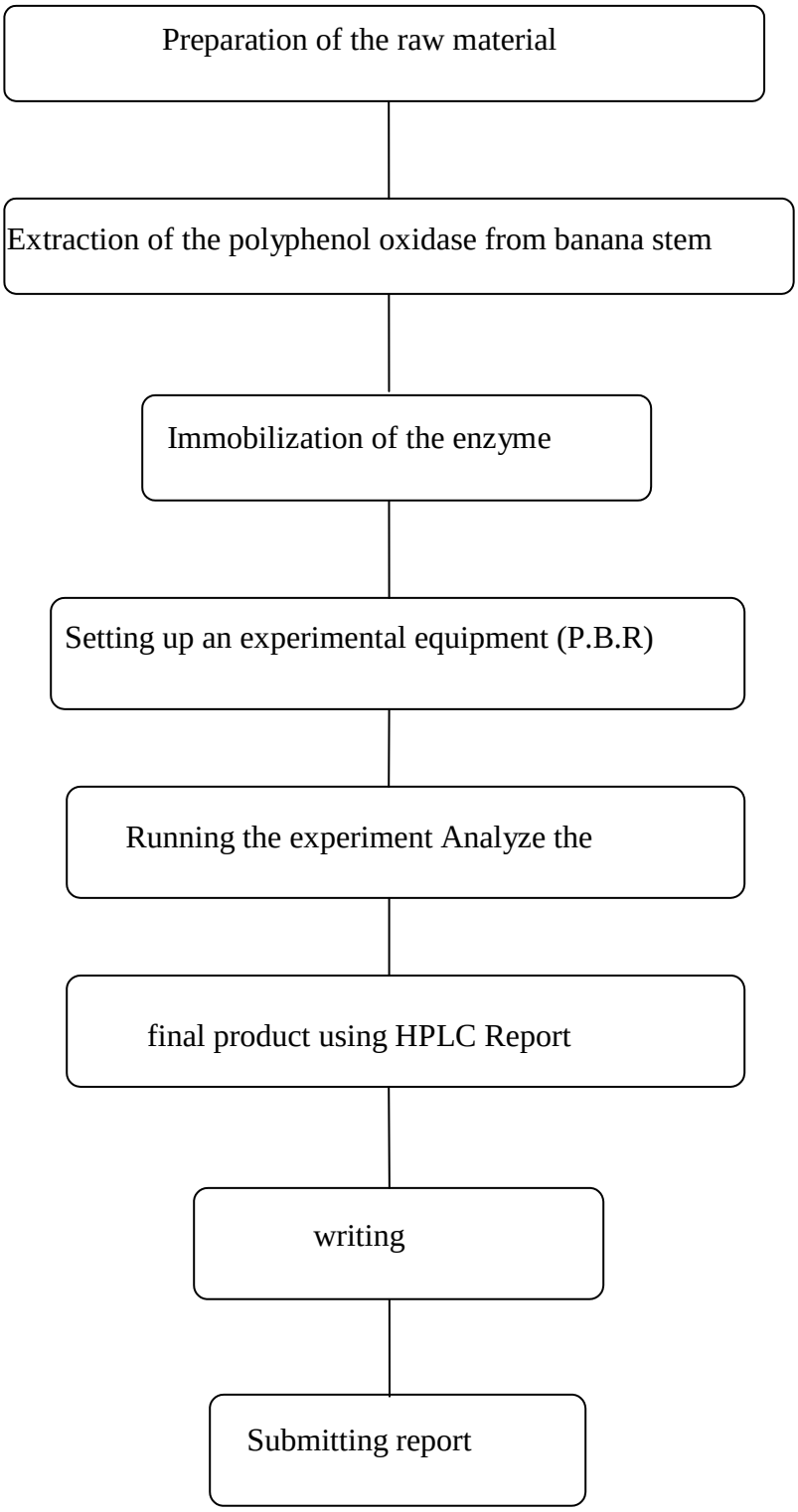
CHAPTER III

MATERIALS AND METHODOLOGY

3.1 Chemicals and Apparatus

i. Sodium Alginate
ii. Calcium Chloride
iii. Barium Chloride
iv. Ascorbic Acid
v. Triton X-100
vi. Monobasic phosphate
vii. Dibasic phospate
viii. Sodium Chloride
ix. Beaker
x. Burette
xi. Peristaltic pump
xii. HPLC
xiii. Thermocol

3.2 Overall Methodology



3.3 Methodology (details)

3.3.1 Collection

Collection of banana stem from Kg Gambang, Kuantan. Banana tree was chopped down and the middle part of the stem was taken as raw material for enzyme extraction.

3.3.2 Extraction of the polyphenol oxidase from banana stem

Sample (banana stem) was diced into small parts. The diced stem then grounded in liquid N_2. After that, the frozen sample suspended in phosphate buffer (0.2M, pH7) added with 0.01% of ascorbic acid and 1% Triton X-100 for 20minutes. Solution then centrifuged at 12000rpm for 30minutes at 4^0C and the supernatant (crude enzyme) was collected and stored at 4^0C

3.3.3 Immobilization of the enzyme

Enzyme was immobilized in Ca-Alginate in the form of beads. Initially 3% w/v sodium alginate ($NaC_6H_70_6$) solution was prepared. For batch mode, three different concentration of enzyme were prepared. For continuous mode, one set of enzyme prepared. The crude enzyme was mixed with sodium alginate solution and slowly stirred till homogenous. After that 1000mL solution containing barium chloride ($BaCl_2$) and calcium chloride ($CaCl_2$) at 5:3 ratio was prepared. The solution of sodium alginate and enzyme was dripped into the stabilizing solution of barium chloride and calcium chloride using syringe. Formed beads then let to harden for 20minutes. Finally, the harden beads was washed using distilled water and kept at 4^0C [7].

3.3.4 Setting up and running the experiment

3.3.4.1 Batch reactor study.

For the batch mode experiment, we use three enzyme concentrations; 5mL, 7mL and 10mL with five phenol concentrations; 0.2mM-1.0mM. We placed phenol solution in a beaker along with magnetic stirrer and thermocol. Beads containing enzyme were placed on the top of the thermocol but immersed in the solution. The stirrer was set to optimum speed and sample was taken at 10minutes interval for an hour.

3.3.4.2 Continuous (Packed Bed Reactor) study.

In continuous mode, we ran the experiment using three flow rates which were 0.5rpm (7.92mL/min), 0.7rpm (11.1mL.min) and 0.9rpm (14.3mL/min). Five phenol concentrations still transformed using one beads enzyme concentration. The beads were put in the burette and the phenol solution was pumped through the column using peristaltic pump. The dimensions of the column, porosity of the beads were determined. Samples were taken at 5minutes interval for half an hour.

3.3.5 Analyze the final product using HPLC

All the samples were quantified using High Performance Liquid Chromatography (HPLC) Agilent series 1100. C-18 RP column were used and detected at 254nm wavelength and mobile phase used was methanol/water (20:80) at 1mL/min.

CHAPTER IV

RESULT AND DISCUSSIONS

4.1 Introduction

In this chapter, a review and thorough discussion will be performed from the results of the experiment that has been done.

4.2 Results

The result for this experiment is divided into two parts; the first one is for batch data while for the second one is for the continuous data.

4.2.1 Batch Experimental Data

From the batch modes of experiment, the data were plotted and the linear trend line was applied to compare the linear equation; y= mx + c with ln $-\frac{dC\,a}{dt} = \ln k_A + \alpha \ln C_A$ where y=ln $-\frac{dC_A}{dt}$, m= α, x= ln $C_{A,}$ and c= ln k_A. After we compare and analyze the data from the experiment, we then find the space time for each reaction rate form the data.

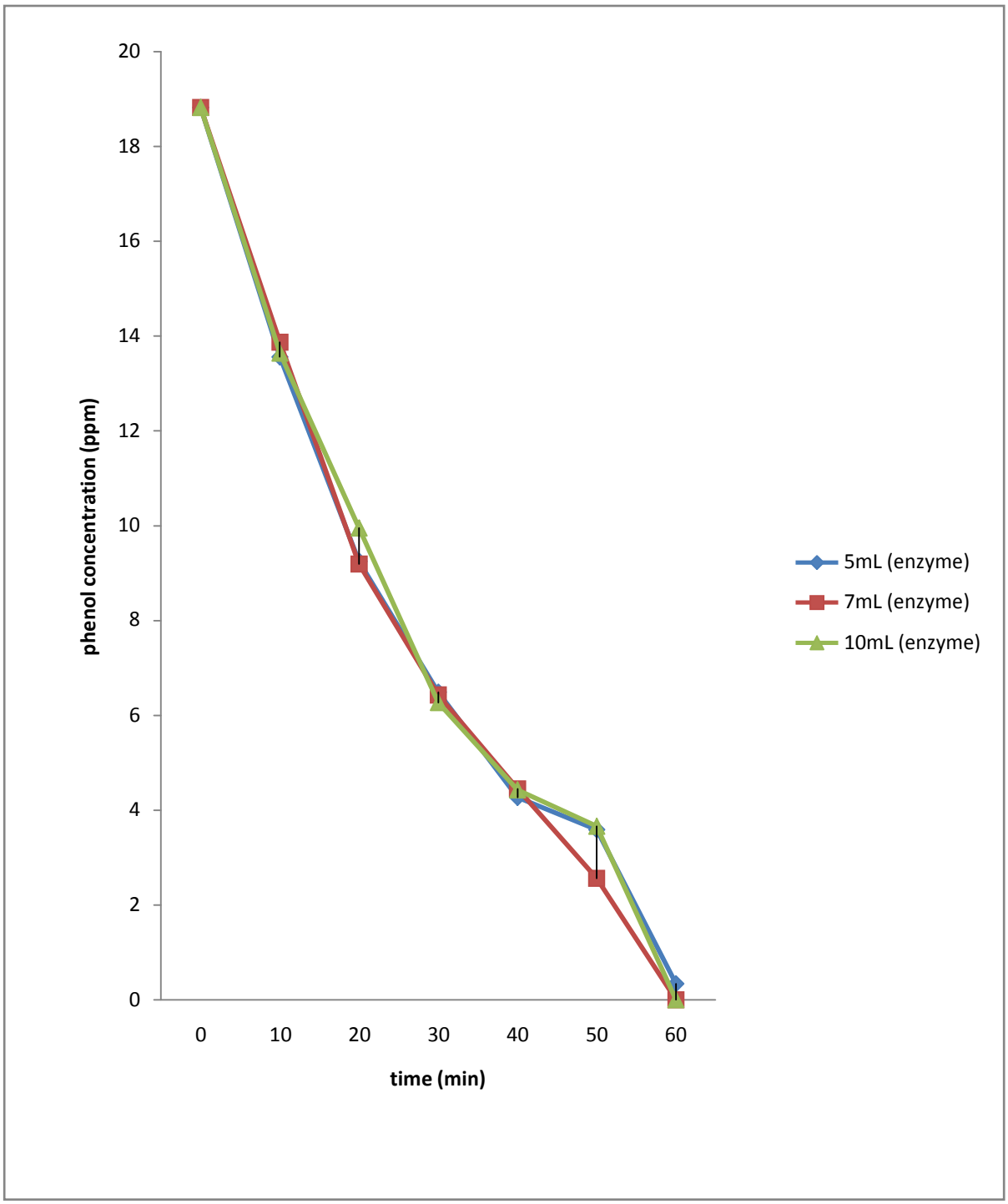


Figure 4.1 Phenol (0.2mM) concentration profile versus time

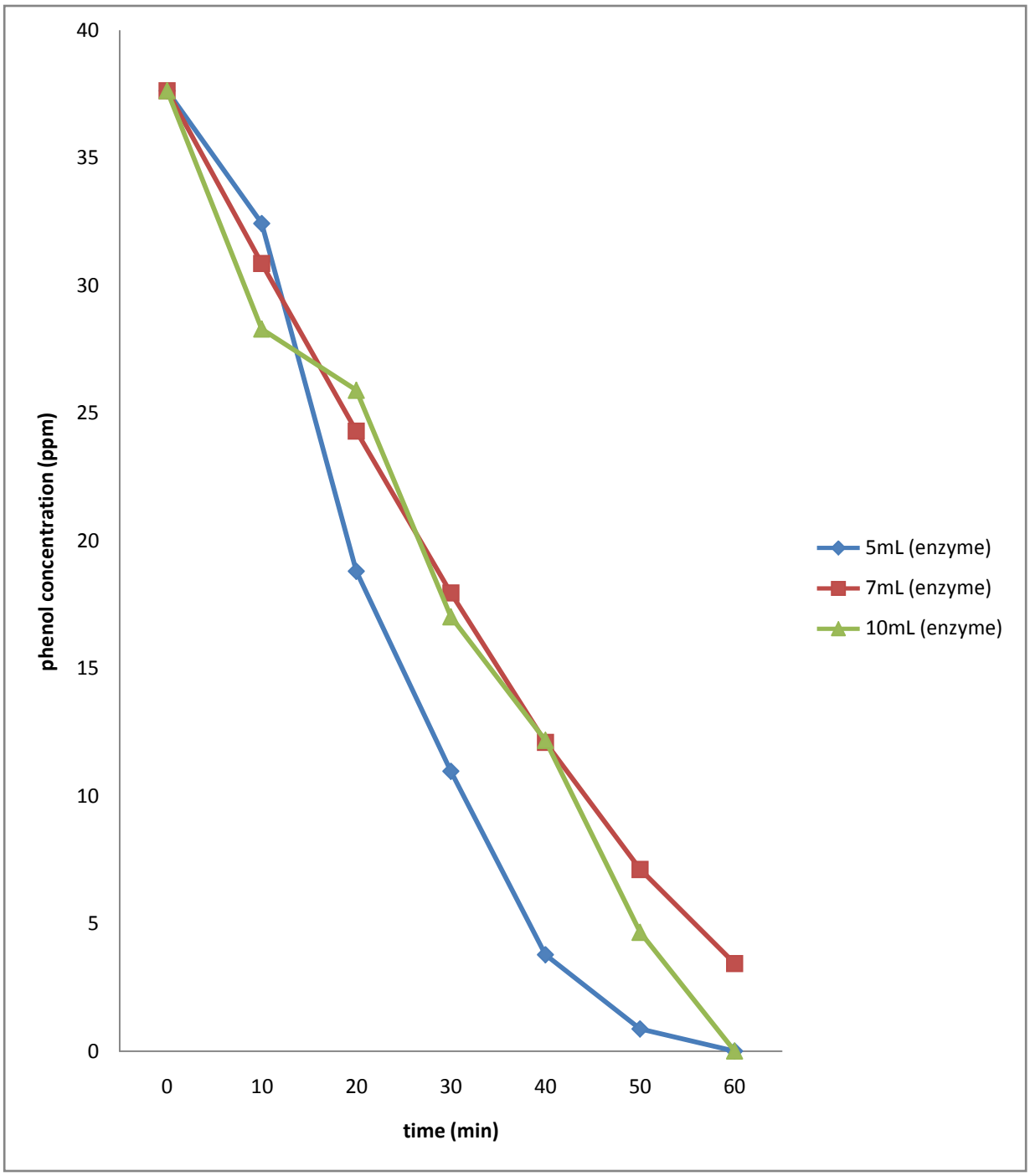


Figure 4.2: Phenol (0.4mM) concentration profile versus time

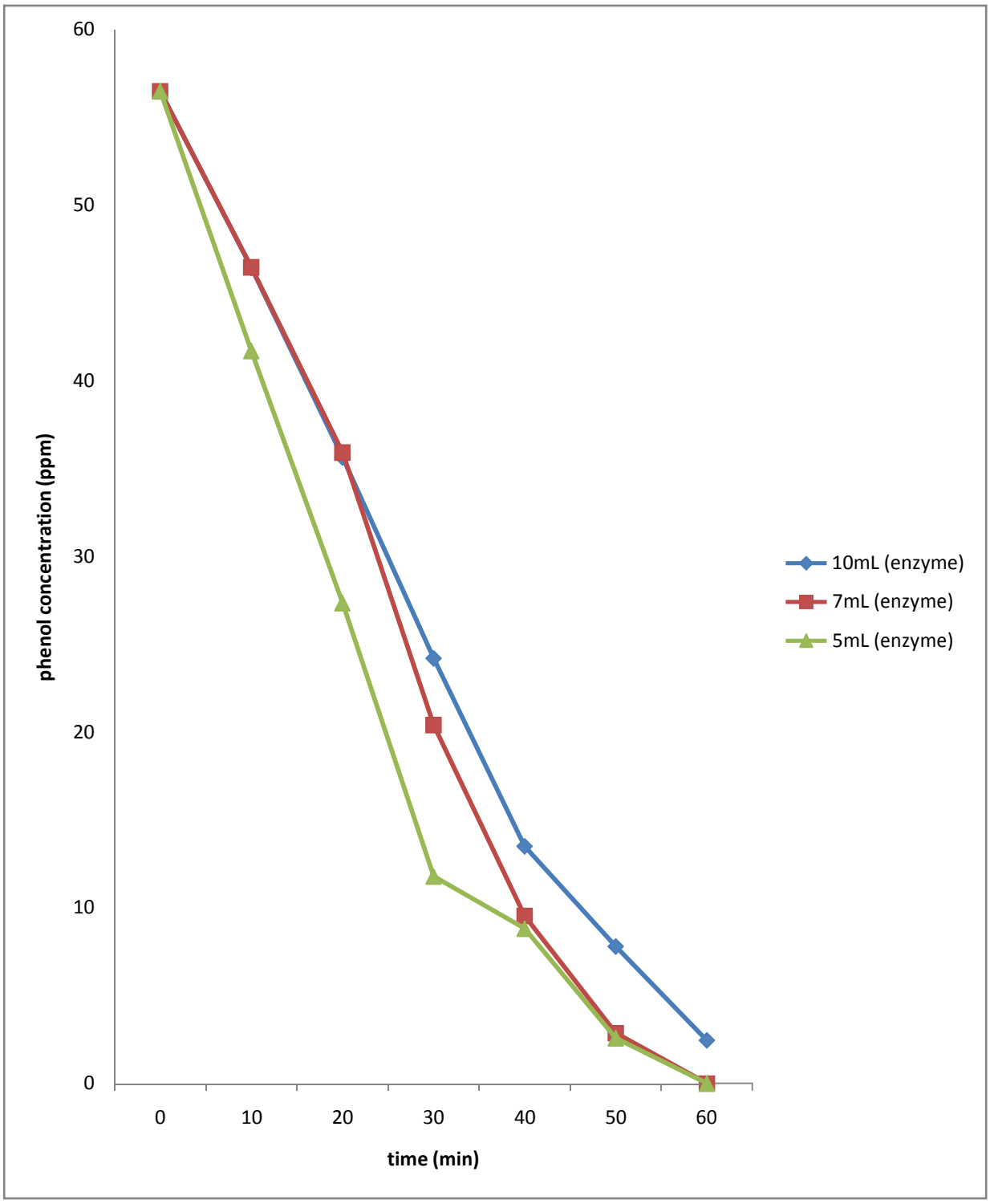


Figure 4.3: Phenol (0.6mM) concentration profile versus time

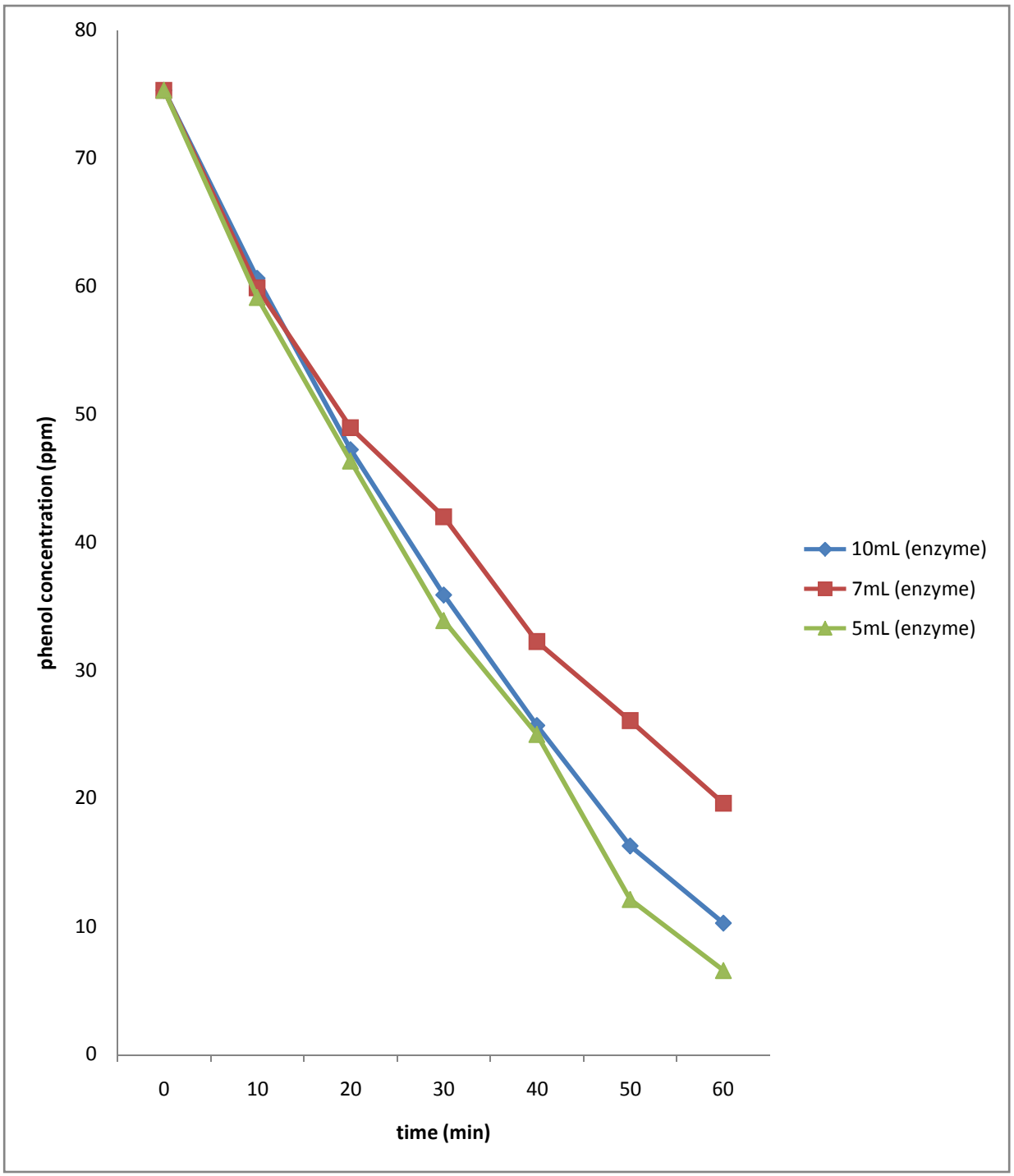


Figure 4.4: Phenol (0.8mM) concentration profile versus time

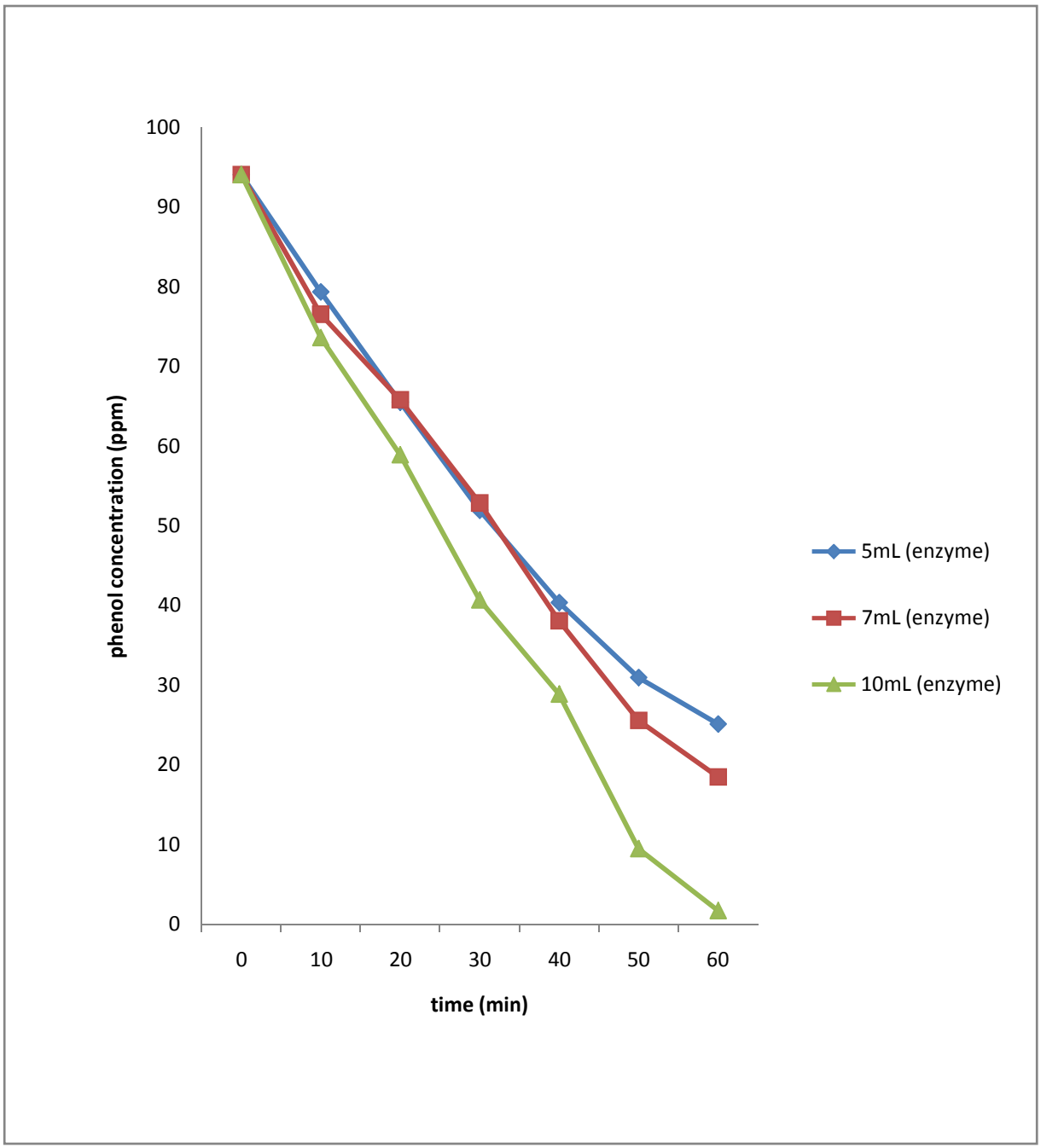


Figure 4.5 : Phenol (1.0mM) concentration profile versus time

Table 4.1: Rate constant for each reaction

enzyme	Concentration(ppm)	Rate constant
5ml	0.2	2.9153795
	0.4	1.75892003
	0.6	1.24620134
	0.8	0.78513469
	1	0.32719466
7ml	0.2	2.01234357
	0.4	2.16495592
	0.6	1.73811293
	0.8	0.63311703
	1	0.76705252
10ml	0.2	2.66658866
	0.4	3.42431003
	0.6	3.95151876
	0.8	1.33189135
	1	2.17341573

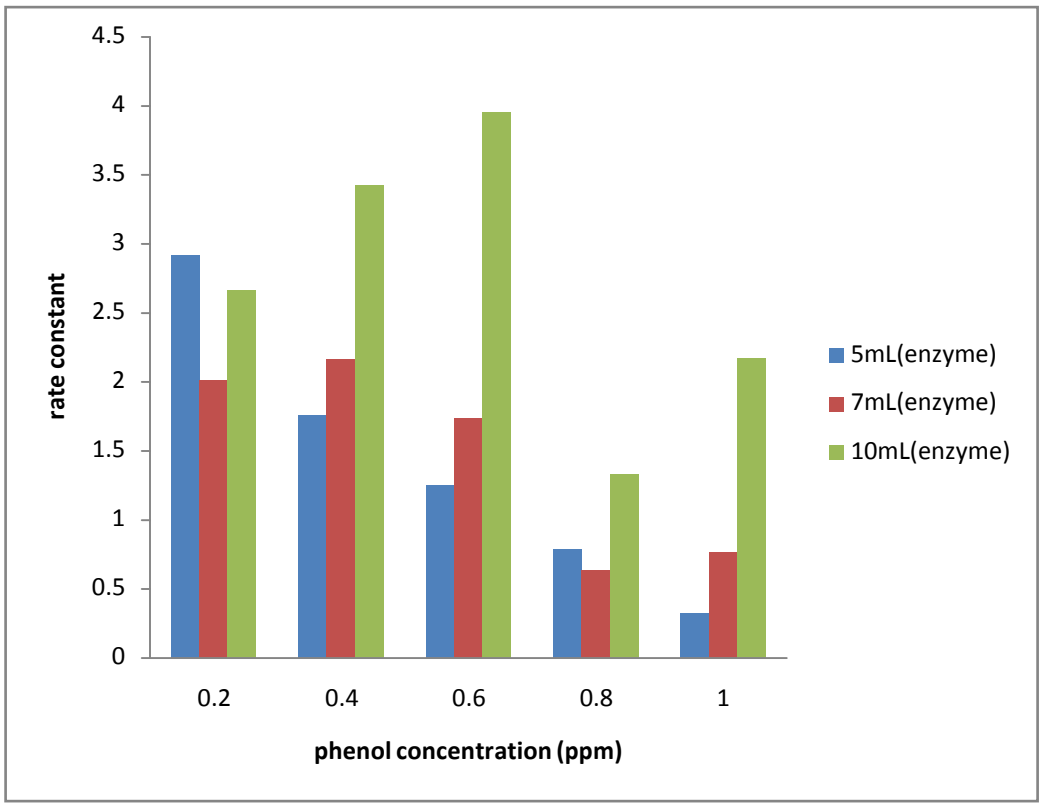


Figure 4.6: Rate constant plotted for each reaction

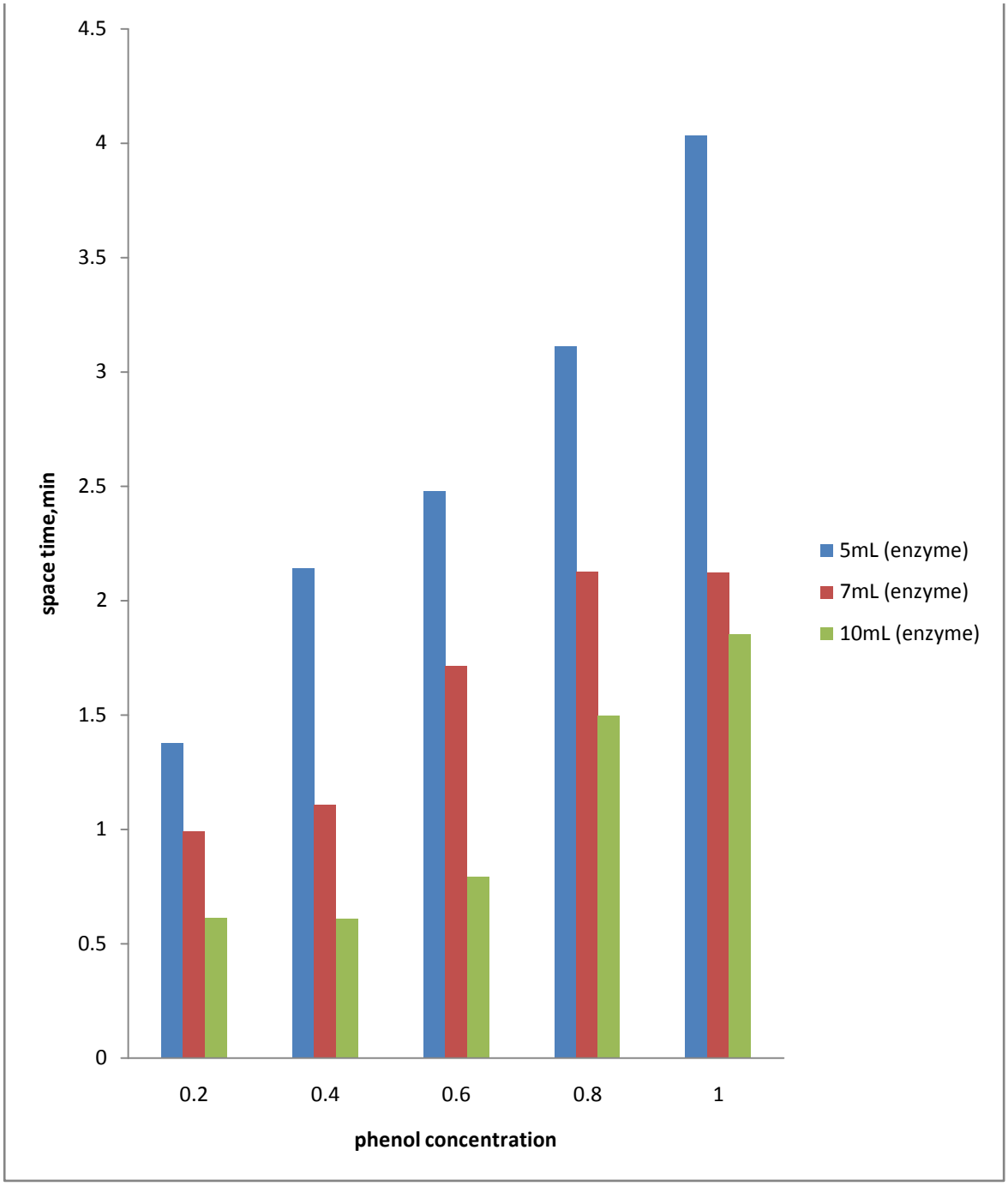


Figure 4.7: Space time of each reaction against different enzyme volumes.

4.2.2 Continuous Experimental Data

For continuous mode, the data were plotted to find the approximate value of weight catalyst needed for the experiment. The area under the graph is equal to approximate value of the catalyst volume. The area was analyzed using Simpson`s Rule. In real situation, the enzyme was coated outside of the beads surface rather than immobilized it. This method needs less amount of enzyme but since biocatalyst was used, entrapment in Ca-Alginate beads seems to be a more practical method in immobilizing the enzyme due to protein denature.

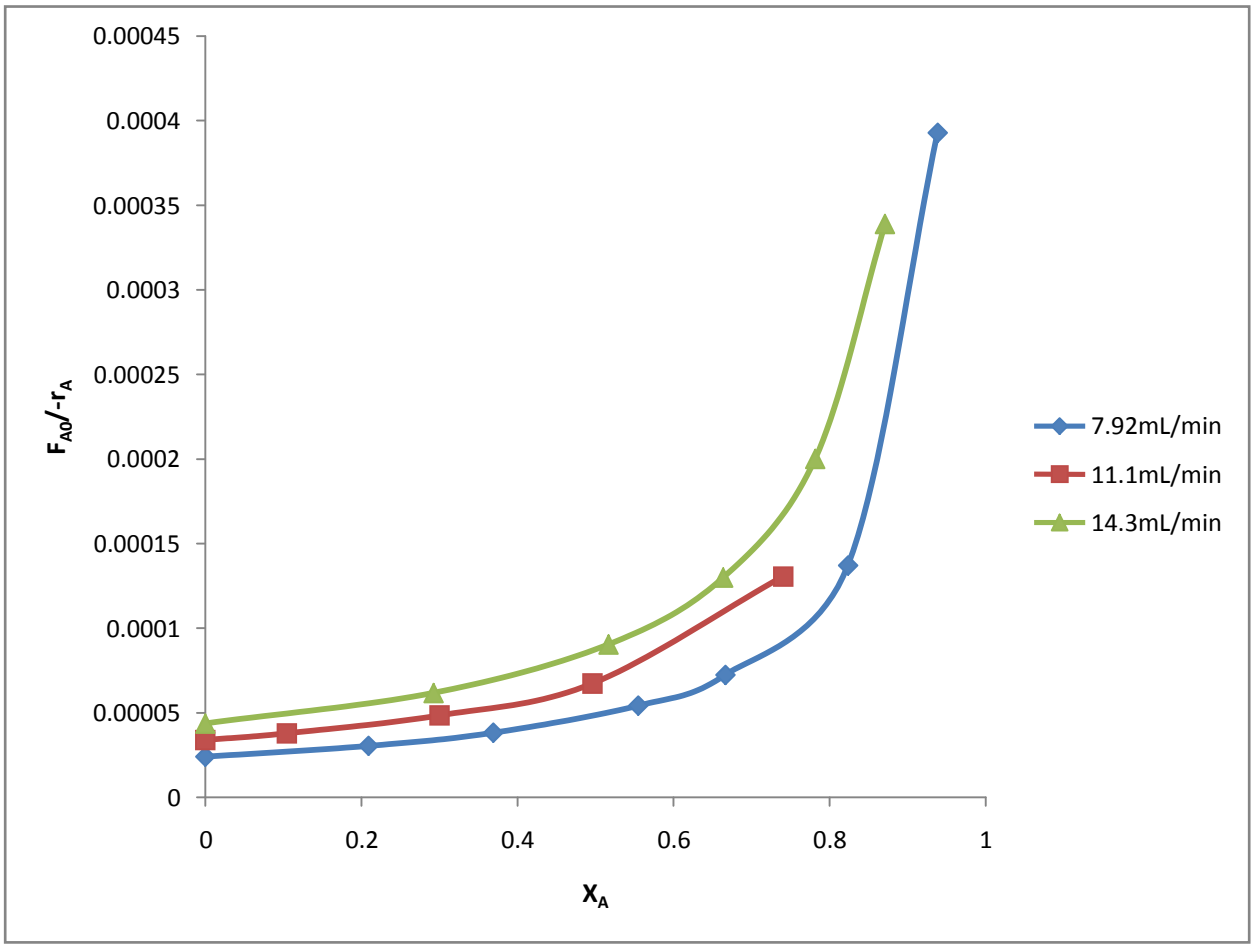


Figure 4.8 : Levenspiel plot for phenol (0.2mM) in continuous mode

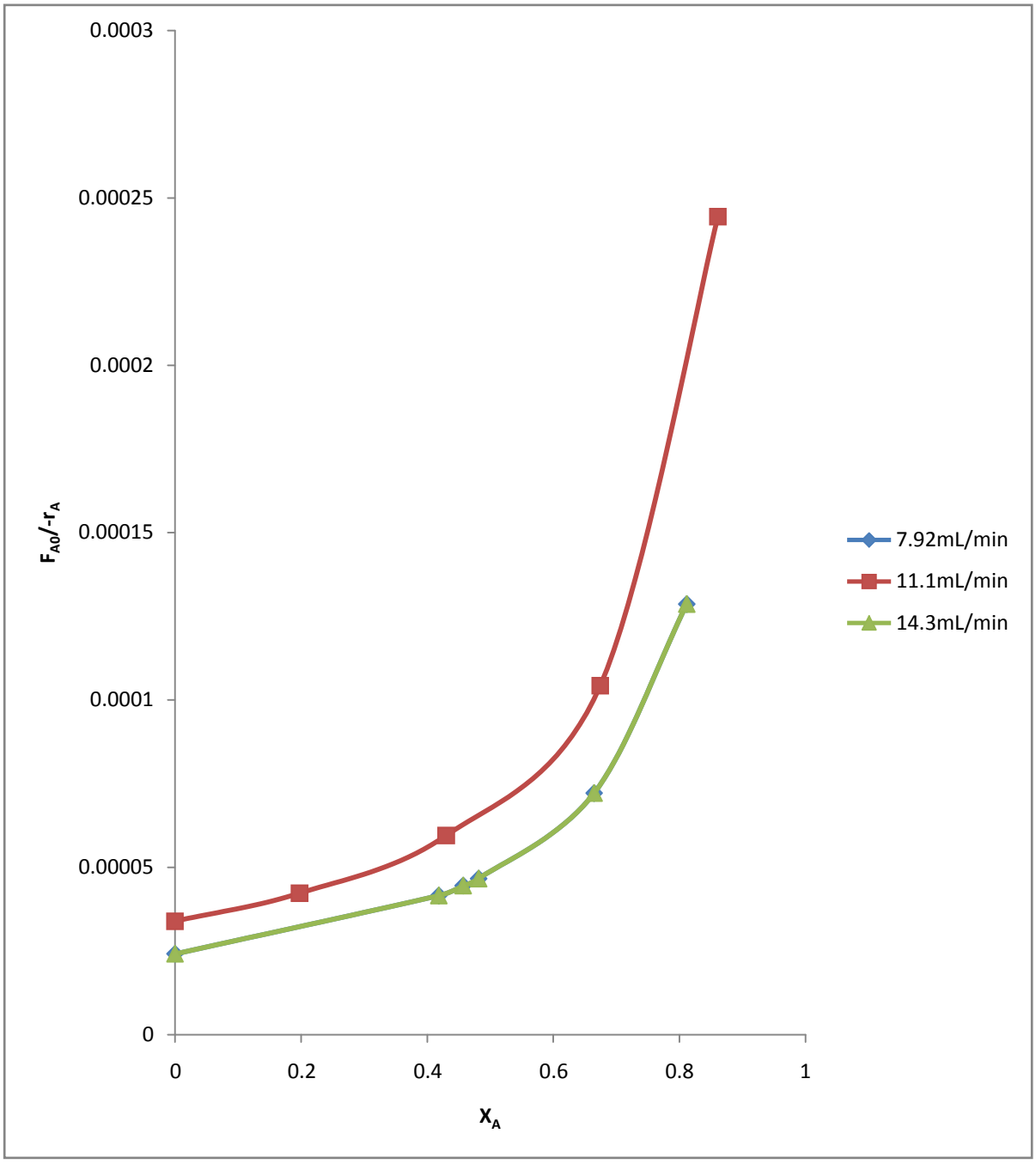


Figure 4.9: Levenspiel plot for phenol (0.4mM) in continuous mode

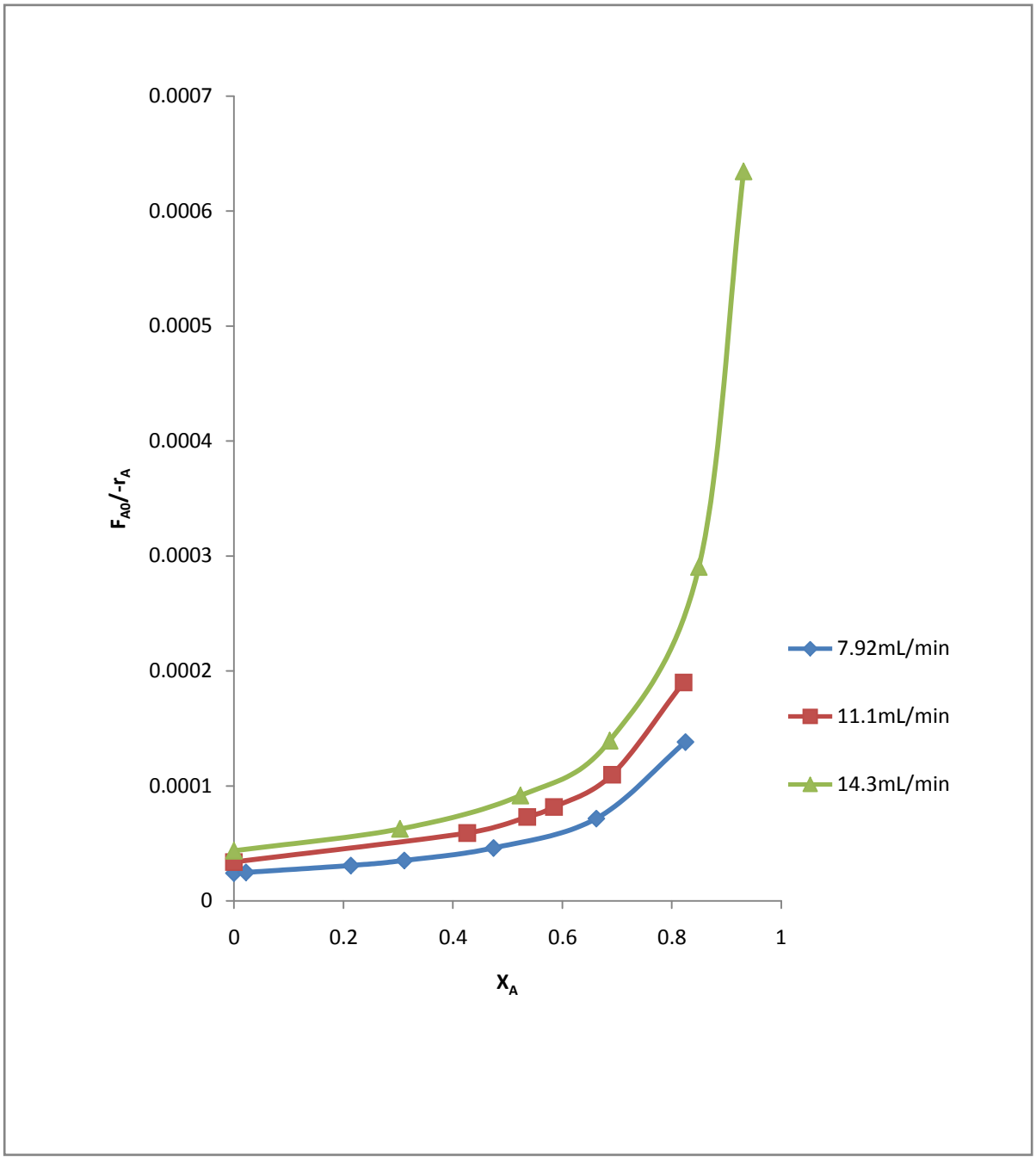


Figure 4.10: Levenspiel plot for phenol(0.6mM) in continuous mode

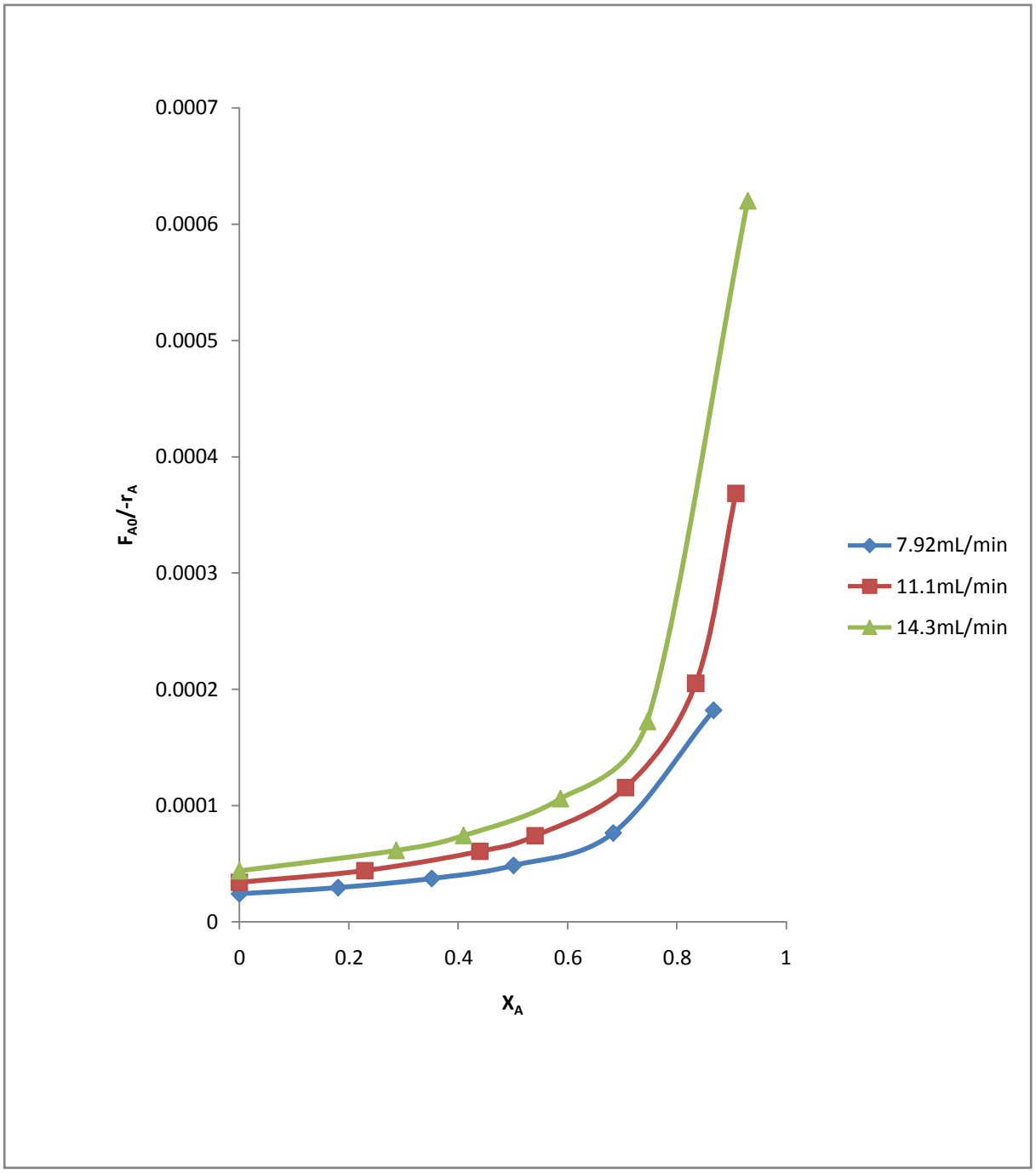


Figure 4.11: Levenspiel plot for phenol (0.8mM) concentration in continuous mode

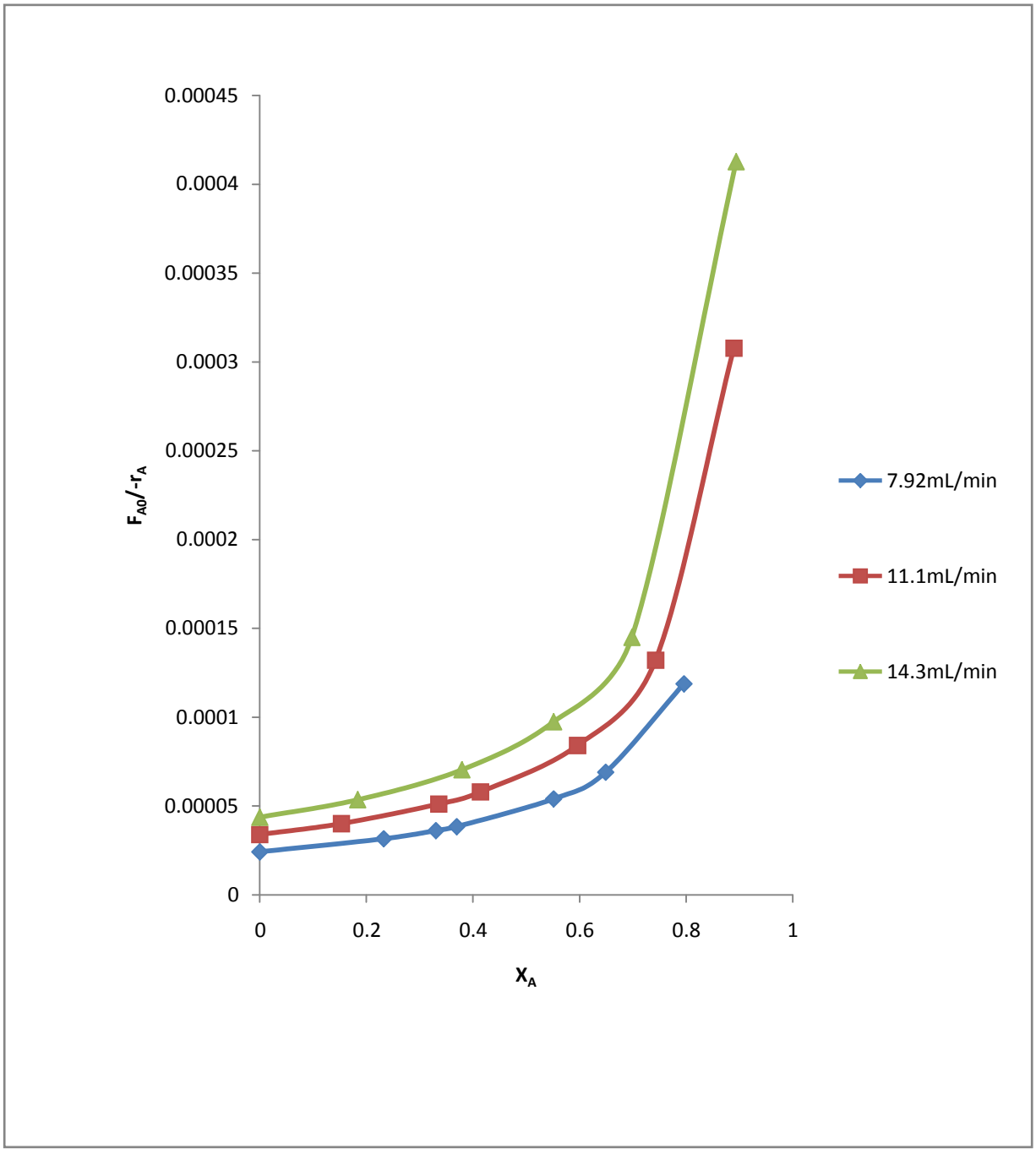


Figure 4.12: Levenspiel plot for phenol (1.0mM) concentration in continuous mode

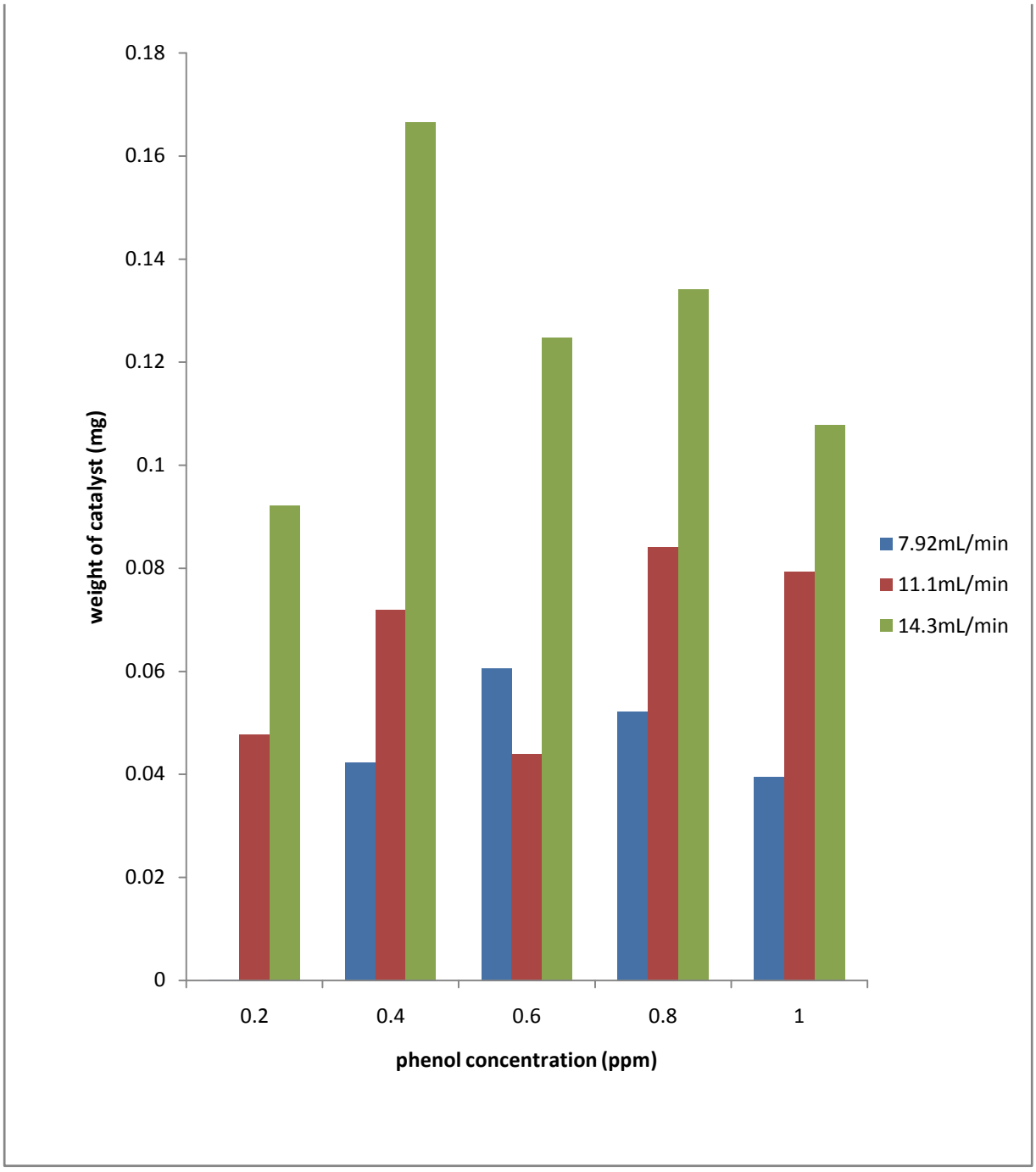


Figure 4.13 : Weight of catalyst in packing for each phenol concentration

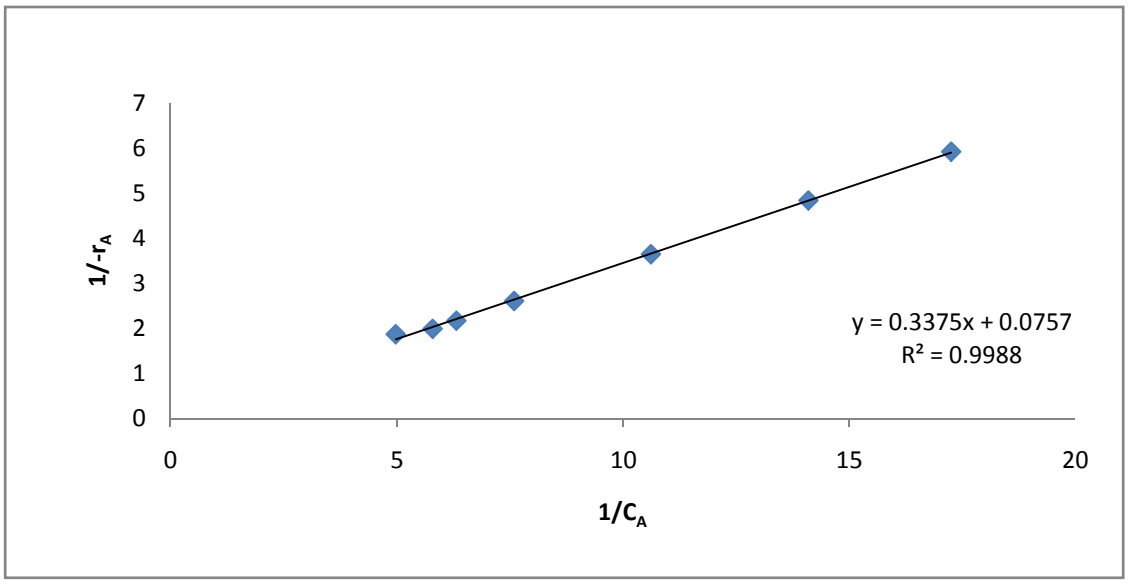


Figure 4.14: Lineweaver-Burk plot for 0.2mM phenol at 7.92mL/min

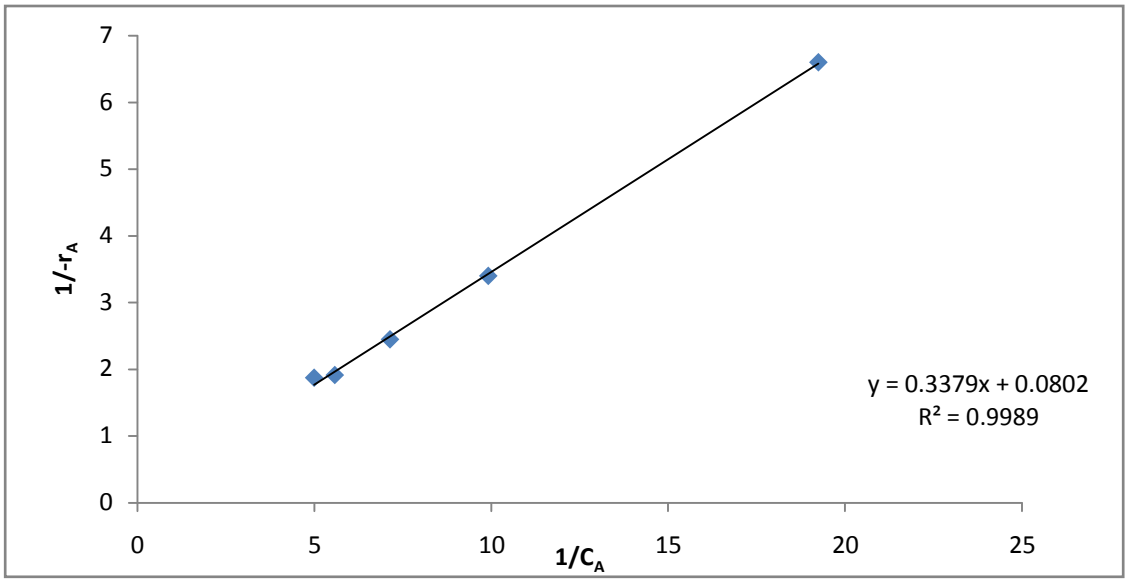


Figure 4.15: Lineweaver-Burk plot for 0.2mM phenol at 11.1mL/min

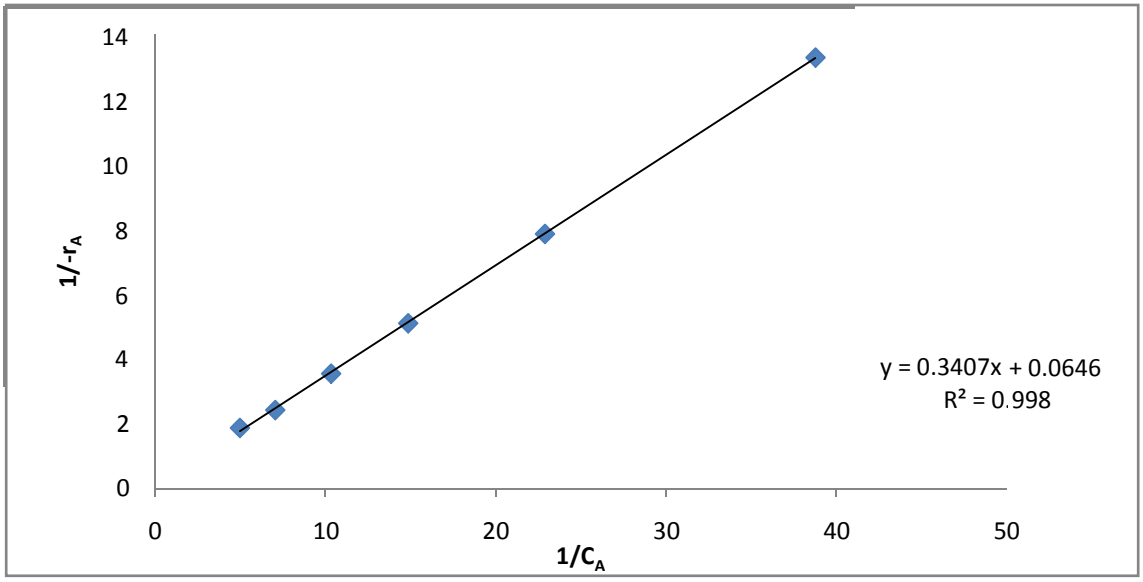


Figure 4.16: Lineweaver-Burk plot for 0.2mM at 14.3mL/min

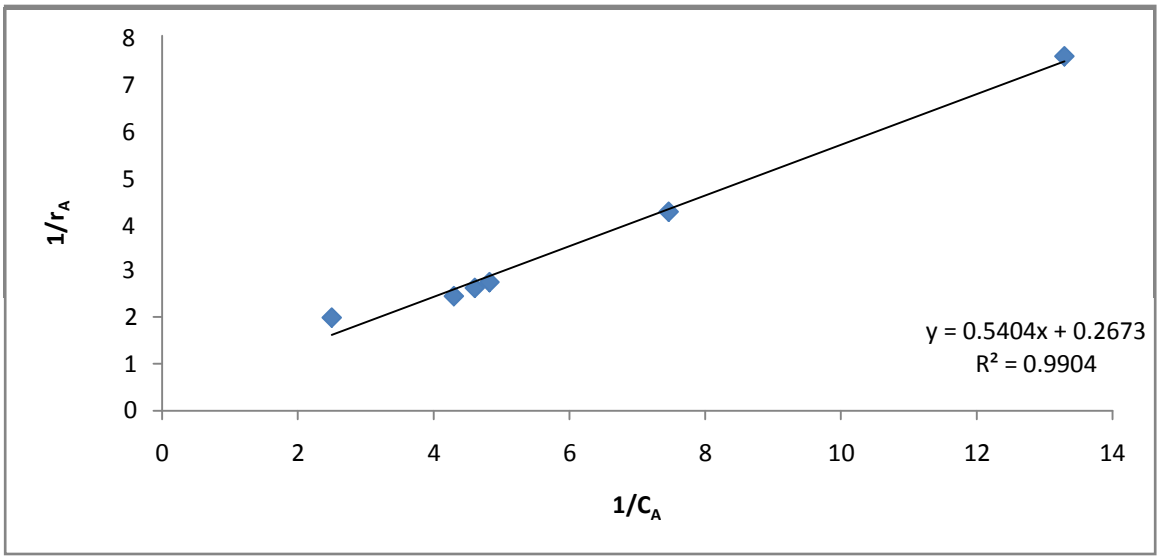


Figure 4.17: Lineweaver-Burk plot for 0.4mM phenol at 7.92mL/min

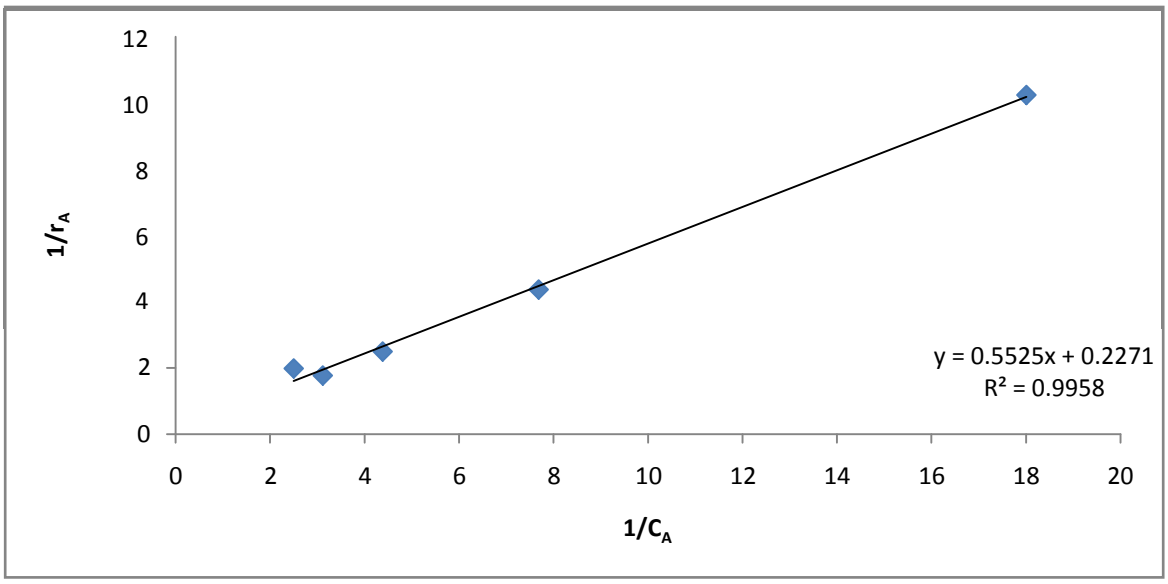


Figure 4.18: Lineweaver-Burk plot for 0.4mM at 11.1mL/min

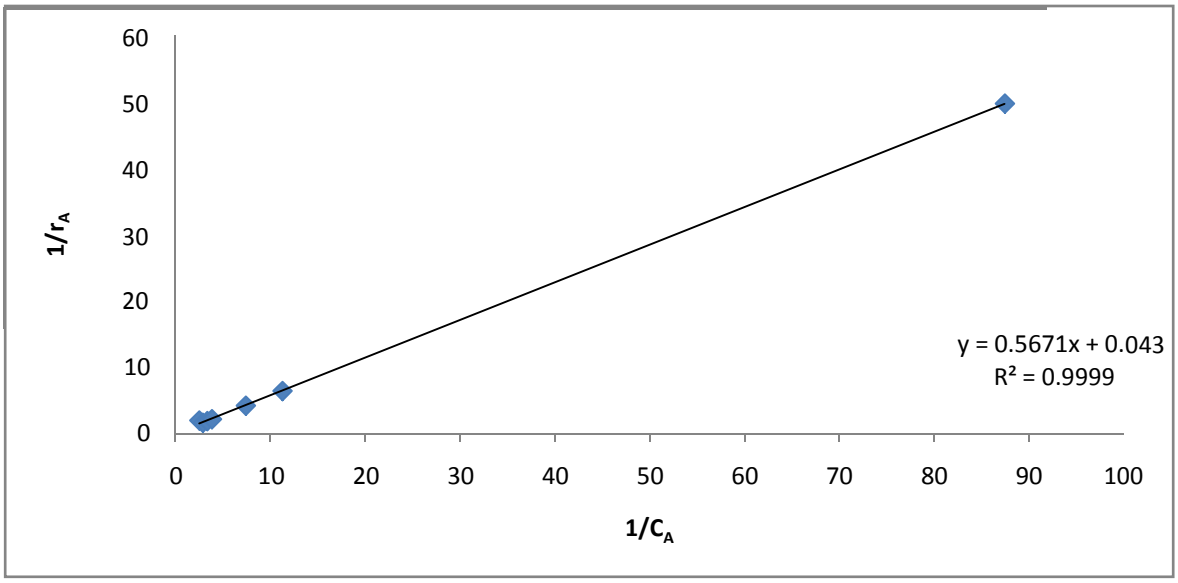


Figure 4.19: Lineweaver-Burk plot for 0.4mM phenol at 14.3mL/min

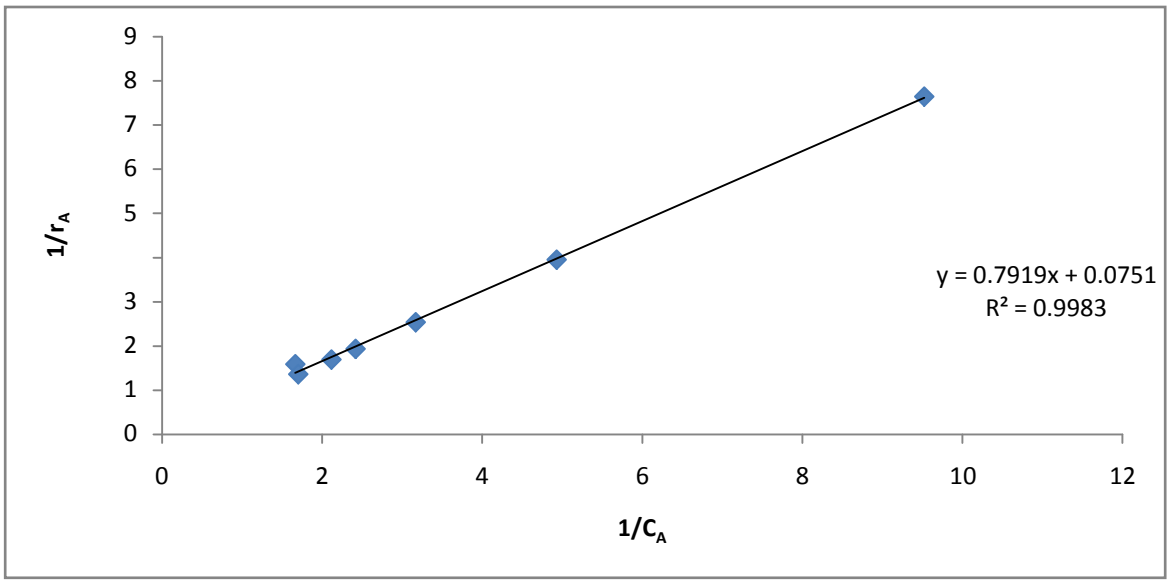


Figure 20: Lineweaver-Burk plot for 0.6mM at 7.92mL/min

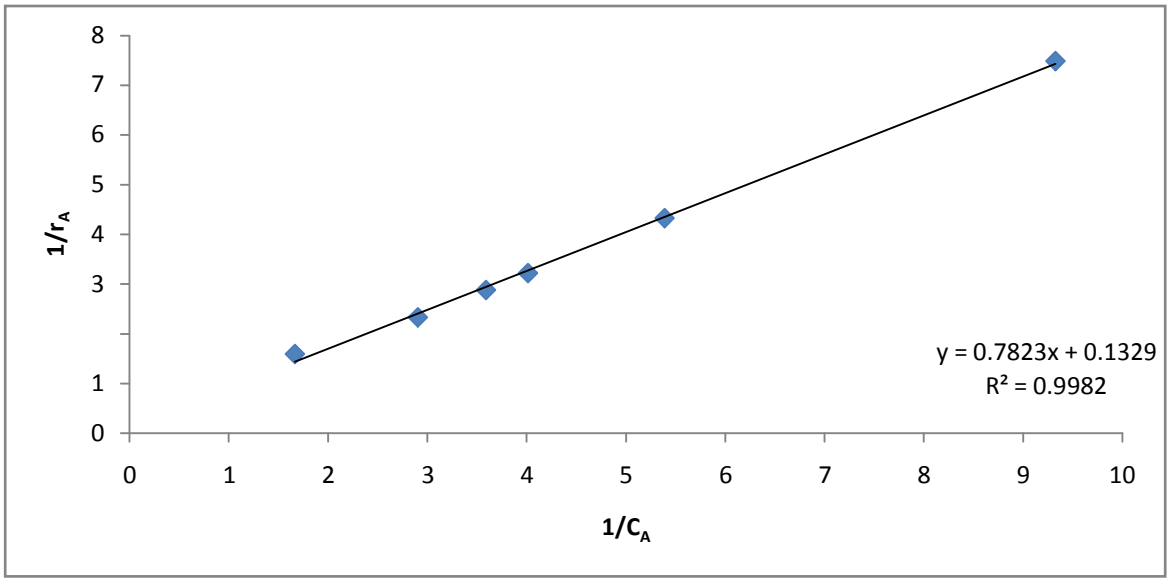


Figure 21: Lineweaver-Burk plot for 0.6mM phenol at 11.1mL/min

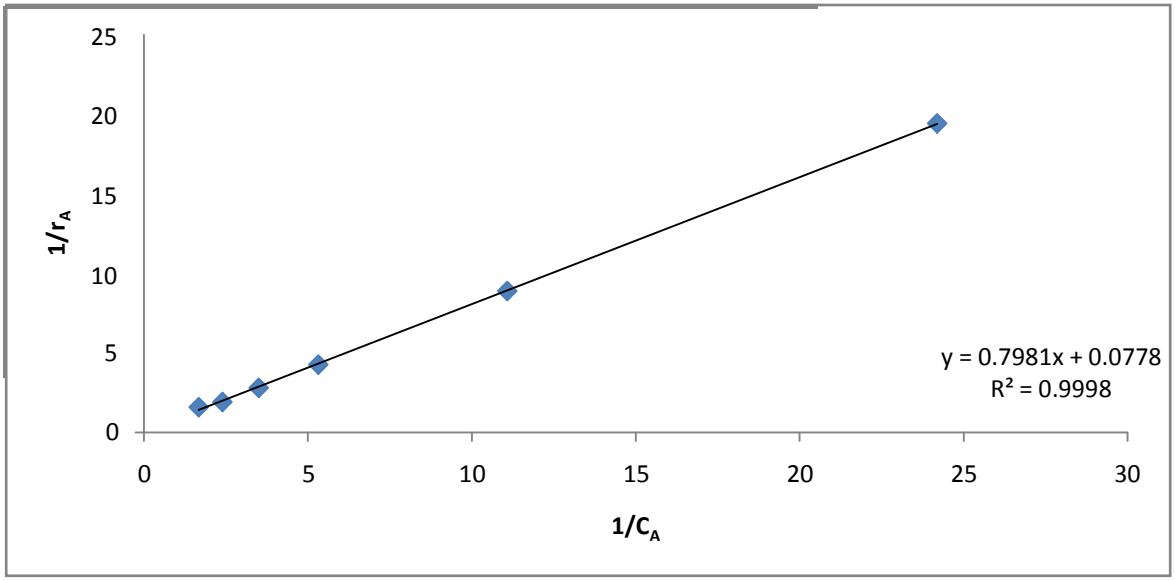


Figure 4.22: Lineweaver-Burk plot for 0.6mM phenol at 14.3mL/min

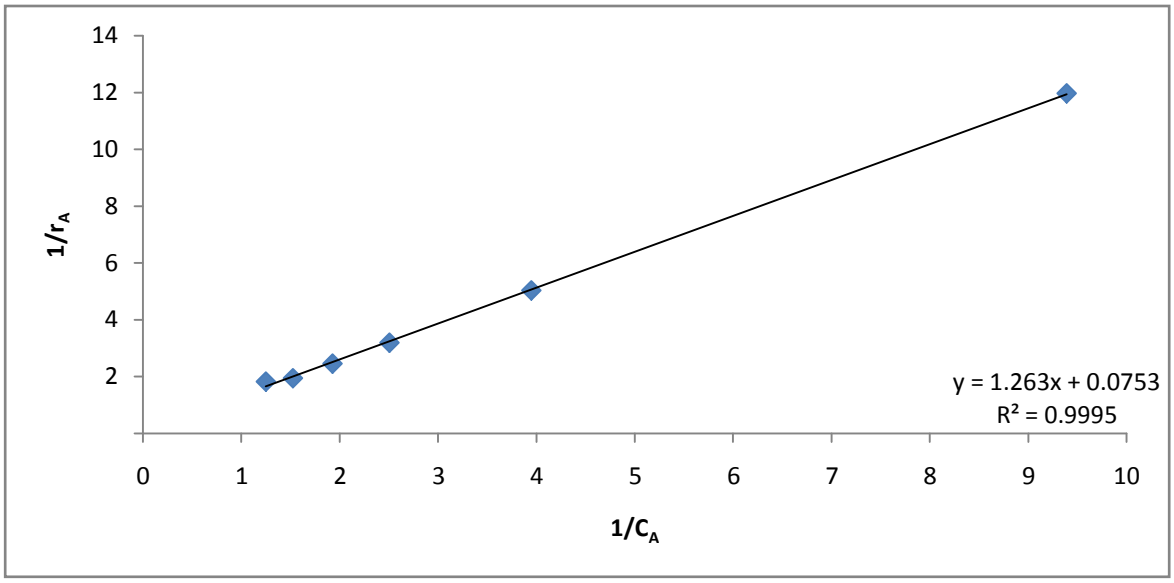


Figure 4.23: Lineweaver-Burk plot for 0.8mM phenol at 7.92mL/min

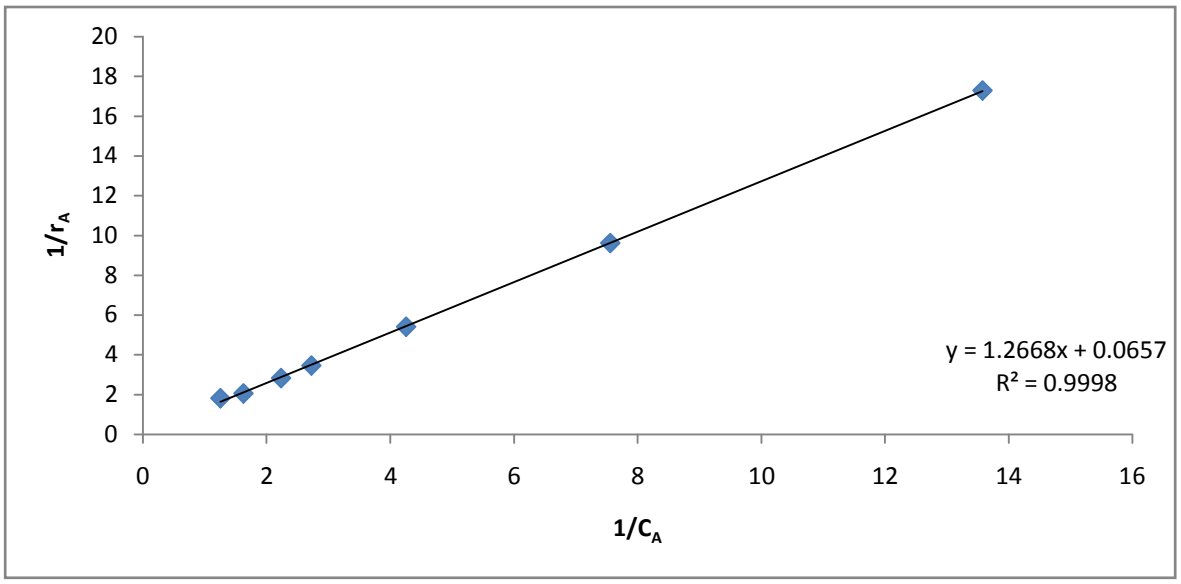


Figure 4.24: Lineweaver-Burk plot for 0.8mM phenol at 11.1mL/min

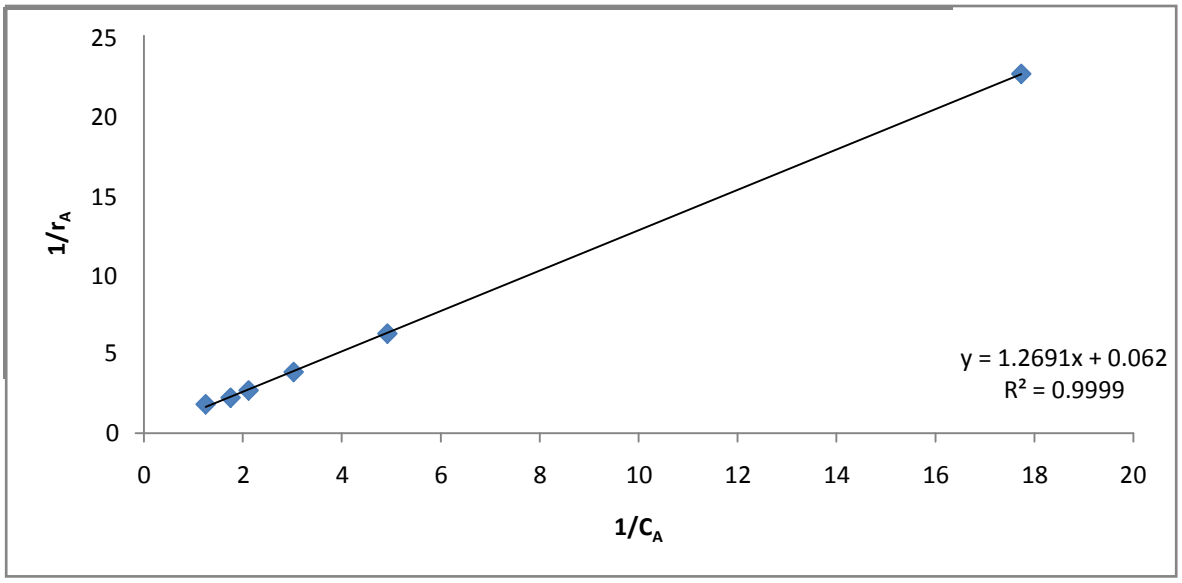


Figure 4.25: Lineweaver-Burk plot for 0.8mM phenol at 14.3mL/min

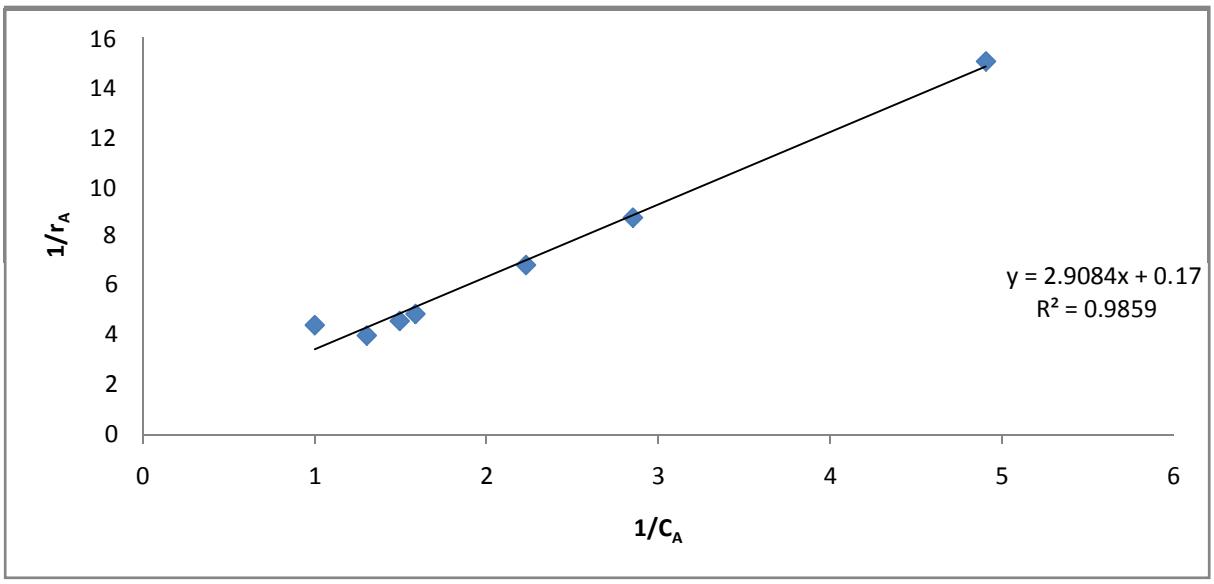


Figure 4.26: Lineweaver-Burk plot for 1.0mM phenol at 7.92mL/min

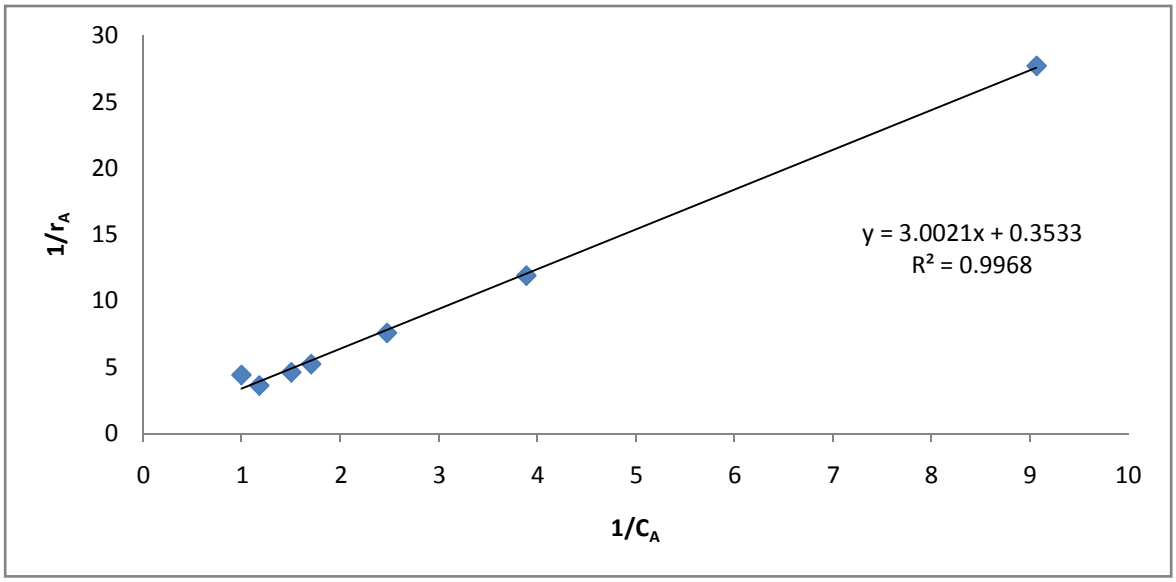


Figure 4.27: Lineweaver-Burk plot for 1.0mM phenol at 11.1mL/min

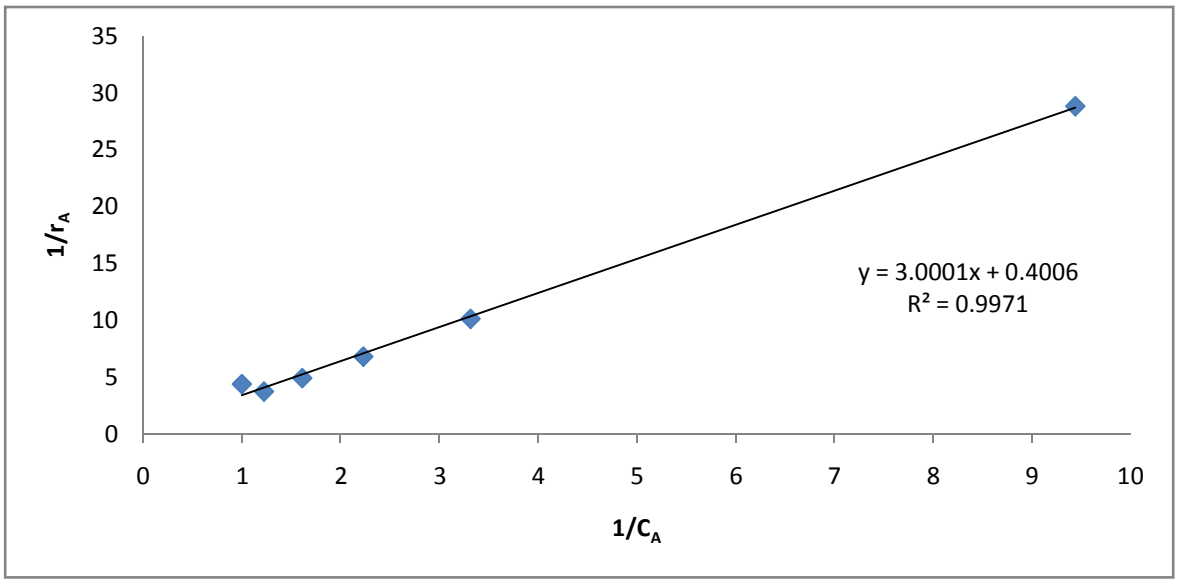


Figure 4.28: Lineweaver-Burk plot for 1.0mM phenol at 14.3mL/min

Table 4.2: Values of K_m and V_{max}

Flow rate (mL/min)	Phenol conc (mM)	Km	Vmax
7.92	0.2	4.45838838	13.2100396
	0.4	2.02169847	3.74111485
	0.6	10.5446072	13.3155792
	0.8	16.7729084	13.2802125
	1	5.62553192	1.93423598
11.1	0.2	4.21321696	12.4688279
	0.4	2.43284897	4.40334654
	0.6	5.88638074	7.52445448
	0.8	19.281583	15.2207002
	1	8.49731107	2.8304557
14.3	0.2	5.27399381	15.4798762
	0.4	5.43720038	9.58772771
	0.6	10.2583548	12.8534704
	0.8	20.4693548	16.1290323
	1	7.48901648	2.49625562

4.3 Discussion

4.3.1 Batch experimental data

All the results from batch experiment mode are shown in figure 2, table 1 and figure (A). As shown in figure 2, the concentration of each phenol solution decreased. This happened due to existence of PPO in the beads thus converting phenol. From the results, the specific reaction rate and space time of the batch reactor were determine. Theoretically, when the specific reaction rate is high, it will decrease the amount of space time of each reaction. Though the enzyme activity entrapped in the beads was not quantified, assumption can be made that the activity of enzyme increase when the volume of crude enzyme entrapped increased. Figure (A) show different pattern of result. In batch experiment, several causes may lead to this trait. When PPO is not separated from the product, it will exhibit the enzyme activity thus increasing the space time of the reaction. The space time for an enzyme volume against phenol concentration increased as the concentration increased. This happened due to amount of phenol converted increased and the process was inhibited by the product, thus longer time needed for the reaction to complete.

4.3.2 Continuous Experimental Data

The figure 3 shows Levenspiel plot for data in continuous mode. It is to estimate the reactor sizing or to estimate the catalyst weight needed in the reactor to achieve the target conversion. The method is initially plot the $F_{A0}/-r_A$ versus conversion (X_A) and then the area under the curve were determine as it represent the reactor sizing for plug flow reactor (PFR) and represent catalyst weight in packing for packed bed reactor (PBR). Simpson Rule was applied to calculate area under the curve. As for figure 3(C), the graph represents the summary of all the weight of catalyst for each phenol concentration at three different flow rates. From the graph, the amount of catalyst

needed is increasing as flow rates increased. This happened due to less retention time caused by high velocity of fluid passed through the packed column. There is slight error can be observed in data for 0.6mM at 7.92mL/min where it shoots a bit passed data for 11.1mL/min. This may happened due to less enzyme activity entrapped in the beads. Since enzyme activity in the entrapped beads was not quantified, this kind of error may arise. Figure 4 is a Lineweaver-Burk plot where it is used to find the Michaelis menten parameters; K_m and V_{max}. K_m is the Michaelis constant and is a measure of the attraction of the enzyme for its substrate while V_{max} is maximum rate of the reaction for a given total enzyme. The value of K_m and V_{max} were shown in table 2

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

Polyphenol oxidase can be used as biocatalyst in biotransformation of phenol or bioremediation of phenol-polluted water. In aqueous media the enzyme will eventually inhibited by its product quinones, but under condition where there is high enzyme concentration and the product can be separated from the reaction site, the efficiencies of the reaction can be increase. From the experimental data, basic kinetics of batch and continuous reactor were determined. The characteristics of the biocatalyst were successfully studied from the specific reaction rate from the batch data and Michaelis menten parameters for continuous reactor. Weight of catalyst loaded also estimated for continuous reactor from the Levenspiel plot

5.2 Recommendations

As for the improvement in this study, may required pure enzyme and quantifying the enzyme (PPO) activity. The amount of phenol converted over PPO activity can be determined [12]. For improving the stability of the enzyme, the addition of polyvinylpyrrolidone (PVPP) in the extraction solution since it will act as stabilizer and coat the beads with chitosan; to prevent enzyme leakage due to reuse [7].

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APPENDIX A

MATERIALS AND METHODOLOGY

Figure 1: Batch process

Figure 2: Continuous process