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Araştırma Makalesi / Research Article

Software Development with the R Programming Language for Studying Tyrosinase Enzyme Kinetics in Giresun Chanterelle Mushrooms

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Abstract

In this study, a software application for enzyme kinetics analysis was developed using the R programming language. The software was designed utilizing the kinetic measurement values of tyrosinase enzyme from Giresun chanterelle (*Cantharellus cibarius*) mushrooms. Detailed step-by-step documentation of the software development process is provided. The program includes statistical evaluations and applies the Michaelis-Menten enzyme kinetics model to describe the enzyme kinetics of Giresun chanterelle. Initial reaction rate values were determined with a high correlation and regression coefficient ($R^2 > 0.95$). The software plotted rate values against substrate concentration, yielding Km and Vmax values of 0.25 mM and 0.0016 mM/s, respectively. These values were used to plot the theoretical curve, and its correlation with experimental data was statistically analyzed. The results demonstrate that the developed software can rapidly analyze data with minimal errors. As an open-access and extensible tool, the program can be effectively utilized and further developed for studies on enzyme activation or inhibition.

Keywords: R programming, Enzyme kinetics, Michaelis-Menten equation, Giresun chanterelle mushroom, tyrosinase.

Giresun Kantarel Mantarında Tirozinaz Enzim Kinetiğinin İncelenmesi için R Programlama Dili ile Yazılım Geliştirme

Öz

Bu çalışmada R programlama dili kullanılarak enzim kinetiği analizi için bir yazılım geliştirilmiştir. Yazılım, Giresun kantarel (*Cantharellus cibarius*) mantarındaki tirozinaz enzim aktivitesine ait kinetik ölçüm değerleri kullanılarak tasarlanmıştır. Yazılım geliştirme sürecinin ayrıntılı adım adım dokümantasyonu sağlanmıştır. Program, istatistiksel değerlendirmeler içermekte olup Giresun kantarel enzim kinetiğini Michaelis-Menten enzim kinetiği modeli ile açıklamaktadır. Başlangıç reaksiyon hızı değerleri yüksek korelasyon ve regresyon katsayısı ($R^2 > 0.95$) ile belirlenmiştir. Yazılım, hız değerlerini substrat konsantrasyonuna karşı çizerek Km ve Vmax değerlerini sırasıyla 0.25 mM ve 0.0016 mM/s olarak hesaplamıştır. Bu değerler, teorik eğrinin çizilmesinde kullanılmış ve deneysel verilerle olan korelasyonu istatistiksel olarak analiz edilmiştir. Sonuçlar, geliştirilen yazılımın verileri hızlı ve minimum hatayla analiz edebileceğini göstermektedir. Açık erişimli ve geliştirilebilir bir araç olarak, yazılım enzim aktivasyonu veya inhibisyonu üzerine yapılan çalışmalarda etkili bir şekilde kullanılabilir ve daha da geliştirilebilir.

Anahtar Kelimeler: R programlama, Enzim Kinetiği, Michaelis-Menten denklemi, Giresun kantarel mantarı, Tirozinaz.

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1. Introduction

To understand, predict, and analyze processes in biology and engineering, and to perform the necessary calculations, it is essential to first define the existing problem and identify its components. Research conducted for this purpose yields data that is used to develop conceptual models, typically framed as testable hypotheses. As scientific knowledge advances, biological processes are observed to become increasingly complex, complicating the examination and analysis of process data. To address these challenges, various algorithms and software packages have been developed. Among these, free and easily accessible programs offering open-source capabilities provide significant advantages.

R is a freely accessible software known for its simplicity of use, comprehensive documentation, tutorials, and open-access courses available for learning. It benefits from a highly active user community, making it easy to obtain support. R stands out among paid programs due to its free and open-source nature, and its ability to download numerous packages from CRAN (**C**omprehensive **R** **A**rchive **N**etwork) for various analyses. It allows users to develop their own packages, handle large datasets, create models, generate graphics, and present results in LaTeX format (Tingley, 2022). Additionally, R can process a variety of data types such as data frames, matrices, vectors, and lists, demonstrating its versatility for both academic and industrial applications. R promotes code-based analysis, ensuring that analyses are reproducible and shareable.

R provides numerous functions and advantages for bioengineering studies and the analysis of resulting data. It significantly contributes to specific areas such as adsorption, purification, enzyme activity determination, extraction, fermentation, kinetic data analysis, genetic analyses and bioinformatics, drug delivery, and release kinetics. Its extensive package and library support enable the efficient processing and analysis of large datasets in these fields. R's powerful statistical analysis and modeling capabilities allow for the accurate and efficient evaluation of complex biological data in bioengineering. Additionally, R's advanced graphics and data visualization features facilitate the understanding and effective presentation of analysis results. R provides several advantages in biological data analysis over Excel, such as superior big data processing capabilities, more efficient, rapid, and accurate performance of complex tasks like statistical analysis, modeling, and hypothesis testing, and practical application of various data through code. Packages like ggplot2 enhance data visualization and the effective presentation of analysis results (Hartvigsen, 2021).

Effectively and accurately evaluating enzyme kinetics is crucial not only for understanding biological systems but also for industrial applications. A classical approach in enzyme kinetics is the application of the Michaelis-Menten equation, which describes the dependency of the reaction rate catalyzed by an enzyme on substrate concentration. Under default conditions, data analysis can often

be explained by the Michaelis-Menten equation. However, in cases where these conditions are not met, more complex enzymatic models can be employed. Several software programs such as DynaFit (Kuzmič, 1996), KinTek (Johnson, 2009), ENZO (Bevc et al., 2011), PENNZYME (Schremmer et al., 1984) have been developed to analyze enzyme kinetics data. However, assumptions in these models can lead to misleading results in classical Michaelis-Menten equation analyses (Aledo, 2022). The use of these software programs can be costly, challenging to understand for beginners, and may complicate analysis without fundamental knowledge. Therefore, researchers often opt for general-purpose software like GraphPad Prism and Microsoft Excel for enzyme kinetics analysis. However, these software packages can be time-consuming and prone to human error. Additionally, methods such as unweighted regression analysis may lead to misleading results in kinetic parameters.

Chanterelle mushrooms (locally known as “tavuk mantarı”) grown in Giresun are naturally found in the region's humid and forested areas. This mushroom stands out with its bright yellow-orange colors and umbrella-shaped structure. Chanterelles have a firm and resilient texture, with a strong aroma and a slight peppery taste. The climate and soil structure of Giresun facilitate the efficient growth of chanterelle mushrooms (Peksen et al., 2016). Typically harvested in the autumn months, they hold significant culinary importance in the region due to their delicious and nutritious properties. Chanterelle mushrooms are known for their high levels of antioxidants and are rich in vitamins A, C, D, and B groups. They also contain enzymes of the oxidoreductase class, such as tyrosinase, which play a crucial role in the mushroom's biochemical processes (Muszynska et al., 2016). These enzyme activities contribute to the oxidation of phenolic compounds, preservation of the mushroom's color, and enhancement of its nutritional value.

The aim of this study is to develop a step-by-step software using the R programming language to model enzyme reactions based on the classic Michaelis-Menten model, with a statistical approach to accurately determining model parameters such as Vmax (maximum velocity) and Km (Michaelis-Menten constant). The software was developed based on kinetic data obtained from a study on the tyrosinase enzyme activity of chanterelle mushrooms grown in Giresun. The goal is for the software to be practical for use in other enzyme activity and inhibition studies, and to be adaptable for modeling complex enzyme mechanisms.

2. Materials and Methods

2.1. Setting Up R and RStudio Programs and Generating Commands

The open-access R programming language and its simplifying interface, RStudio, can be downloaded and installed from their respective links (R Core Team, 2020; https://www.r-project.org) and (RStudio Team, 2016; https://www.rstudio.com/), allowing users to upgrade to the latest versions.

In RStudio, there are four panels. In the top-left panel, there is a "script file" section where lines of code can be entered as a script. RStudio allows multiple script files to be open in this section and creates tabs to help navigate them. The bottom-left panel is the "console," where commands can be typed and their outputs can be viewed (or "command prompt"). The top-right panel displays a section (environment) that shows variables and their assigned values, along with tabs for history, connections, and applications. The bottom-right panel contains sections for files, plots, packages, and help files (Figure 1).

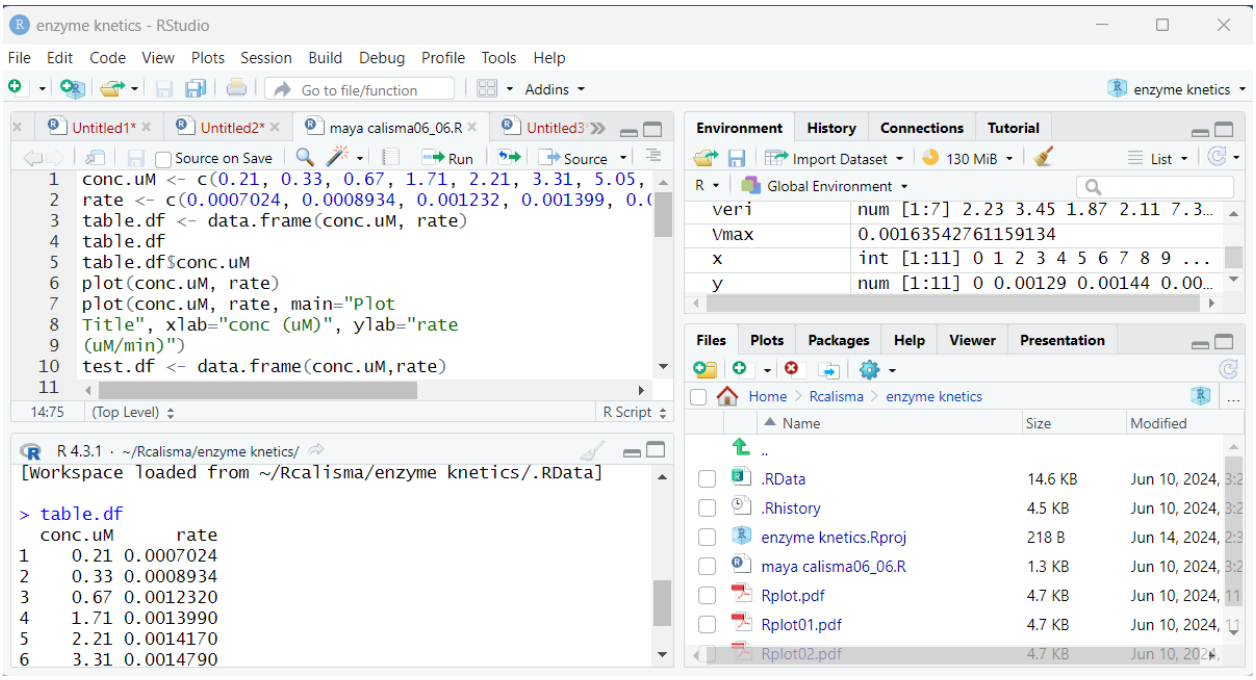

Figure 1. Four panels of RStudio

The R program operates on a package-based system, requiring users to install and activate desired packages according to their application needs. For this study, to create graphics, the ggplot2 package needs to be installed and activated, while for data manipulation, packages such as data.table, tidyr, tidyverse, and broom are necessary.

Commands for enzyme kinetics analysis have been constructed step-by-step with detailed explanations, enabling users to understand fundamental concepts and create customized software tailored to their specific needs.

2.2. Preparation of Crude Enzyme and Collecting Enzyme Kinetics Data

Tyrosinase enzyme was extracted from Giresun chanterelle (*Cantharellus cibarius*) mushrooms. For this purpose, 10 g of mushrooms were blended in 100 mL of 10 mM phosphate buffer (pH 7.0) at 25°C for 3 minutes. The solution was then filtered using a 0.22 µm hydrophilic syringe filter (PVDF) to remove large mushroom particles. Thus, a crude tyrosinase extract was obtained and kept on ice (+2°C) for analysis. To determine enzyme activity, reactions with catechol were conducted at room temperature following the procedure described by Flurkey et al., 2017. A 10 mM stock catechol solution was prepared using 0.5 mM orthophosphoric acid, and substrate initial concentrations (ranging from 0.33 to 7.10 mM) were adjusted with appropriate dilutions. The reaction volume was 1.5 mL, with the enzyme amount fixed at 125 µL. Absorbance values (A410) were recorded every 10 seconds for 130 seconds using a UV-visible spectrophotometer (Mapada 1100) at 410 nm.

During the reaction process, absorbance values recorded over time were plotted against time in RStudio to obtain curves. The initial linear portions of these curves were used to calculate reaction rates using RStudio. After determining the slopes, the values were divided by the extinction coefficient (ε) according to Lambert-Beer's law (which is approximately 1623 $M^{-1}cm^{-1}$ for catechol at 410 nm), and then multiplied by 1000 to obtain rate values in units of mM/s. Enzyme reaction rates against substrate concentration were graphically generated using RStudio, and the resulting curve was analyzed using the Michaelis-Menten model equation to determine model parameters.

3. Findings and Discussion

3.1. Tyrosinase Enzyme Kinetics Data

Tyrosinase enzyme kinetics data for eight different initial catechol concentrations were obtained by recording absorbance values at 410 nm every 10 seconds, as presented in Table 1. Codes S1 to S8 indicate catechol concentrations of 0.21, 0.33, 0.67, 1.71, 2.21, 3.31, 5.05, and 7.10 mM in the reaction volume, respectively.

Table 1. A_{410} values of tyrosinase enzyme kinetics at different substrate concentrations

t(s)	S1	S2	S3	S4	S5	S6	S7	S8
0.01	0.0873	0.1005	0.1175	0.1414	0.1521	0.1621	0.1671	0.1599
10	0.0987	0.1119	0.1476	0.1538	0.1751	0.1898	0.1905	0.1747
20	0.1101	0.1233	0.1577	0.1862	0.1981	0.2155	0.2159	0.2083
30	0.1215	0.1347	0.1798	0.2186	0.2211	0.2322	0.2493	0.2309
40	0.1329	0.1481	0.1979	0.2310	0.2541	0.2509	0.2687	0.2595
50	0.1443	0.1591	0.2180	0.2584	0.2687	0.2856	0.3041	0.2801
60	0.1557	0.1795	0.2481	0.2758	0.2850	0.3103	0.3125	0.3107
70	0.1671	0.2056	0.2582	0.2942	0.3131	0.3350	0.3479	0.3323
80	0.1733	0.2228	0.2793	0.3206	0.3361	0.3597	0.3703	0.3609
90	0.1925	0.2346	0.3284	0.3430	0.3591	0.3654	0.3654	0.3875
100	0.1925	0.2447	0.3309	0.3324	0.3324	0.3324	0.3324	0.3624
110	0.2133	0.2565	0.3228	0.3470	0.3470	0.3470	0.3470	0.3510
120	0.2251	0.2582	0.3231	0.3503	0.3501	0.3501	0.3501	0.3591
130	0.2295	0.2814	0.3328	0.3422	0.3423	0.3423	0.3423	0.3483

3.2. Defining Data Using R Programming

In order to evaluate enzyme kinetics, two of the most useful formats for storing data in RStudio are vectors and data frames. For instance, concentrations of a substrate at different levels can be stored in a vector named 'conc.uM' using the 'c()' function (e.g., conc.uM <- c(S1, S2, S3, ...)). Alternatively, data prepared in Excel or as a text file can be imported into RStudio.

To import enzyme kinetics data for tyrosinase, the data should be saved in a .csv format with a specified filename within the predefined working directory in R. In RStudio, navigate to the <files> tab in the bottom-right panel, select <import dataset> to locate and import the data file from the designated folder. This procedure ensures that the data is properly imported into RStudio. In this study, the data file is named 'enzimveri'. This file contains absorbance values over time for catechol substrates at various concentrations ranging from 0.21 to 7.10 mM across 8 different studies. The data should be organized to delineate these studies effectively. For this purpose, the values collected for the first substrate concentration (S1) are named "deney_1" and defined by pulling them from the "data" file.

```
deney_1 <- enzimveri %>%
select(c(1, 2)) %>%
```

The first column is labeled as "time." and the second column is labeled as "absorbance".

```
  rename(time = 1, absorbance = 2) %>%
```

R software interprets all data in a .csv file as character if there are any character's present; definitions must be made to convert this to numeric format.

```
  mutate(time = as.numeric(time), absorbance = as.numeric(absorbance))
  head(deney_1)
```

To determine which substrate concentration each data point belongs to the data should be defined separately. For this purpose, a function named "data_set_al" has been created.

```
data_set_al <- function(data, time_col, abs_col) {
  new_data <- enzimveri %>%
    select(c(all_of(time_col), all_of(abs_col))) %>%
    rename(time = 1,
           abs = 2) %>%
    mutate(time = as.numeric(time),
           abs = as.numeric(abs)) }
```

Thus, by changing only the `abs_col` number, the data has been prepared so that it can be used separately for each experiment.

```
deney_1 <- data_set_al(data = data. rows_to_get = 1:14, time_col = 1, abs_col = 2)
deney_2 <- data_set_al(data = data. rows_to_get = 1:14, time_col = 1, abs_col = 3)
“ “ “ “
```

3.3. Generation of Enzyme Kinetics Curves

For each dataset, an absorbance graph against time was generated using the `geom_point()` function in ggplot2. A linear trendline passing through the data points was added using the `geom_smooth` function. Deviations from linearity were observed at high S concentrations (Figure 2, blue solid lines). To address this, a subset of the data was created using the `subset` command to achieve higher correlation specifically for high substrate concentration data (S3-S8). Following the addition of highly correlated linear trendlines (Figure 2., red solid lines), the slopes of these lines were recorded. The following codes were developed for these procedures:

```
deney_1 %>%
  ggplot(aes(time. abs)) +
  geom_point() +
  labs(x = "time (s)",
       y = "Absorbance at 410 nm") +
  geom_smooth(method = "lm", se = FALSE)
```

Upon completing operations for all datasets, graphs as shown in Figure 2 were obtained.

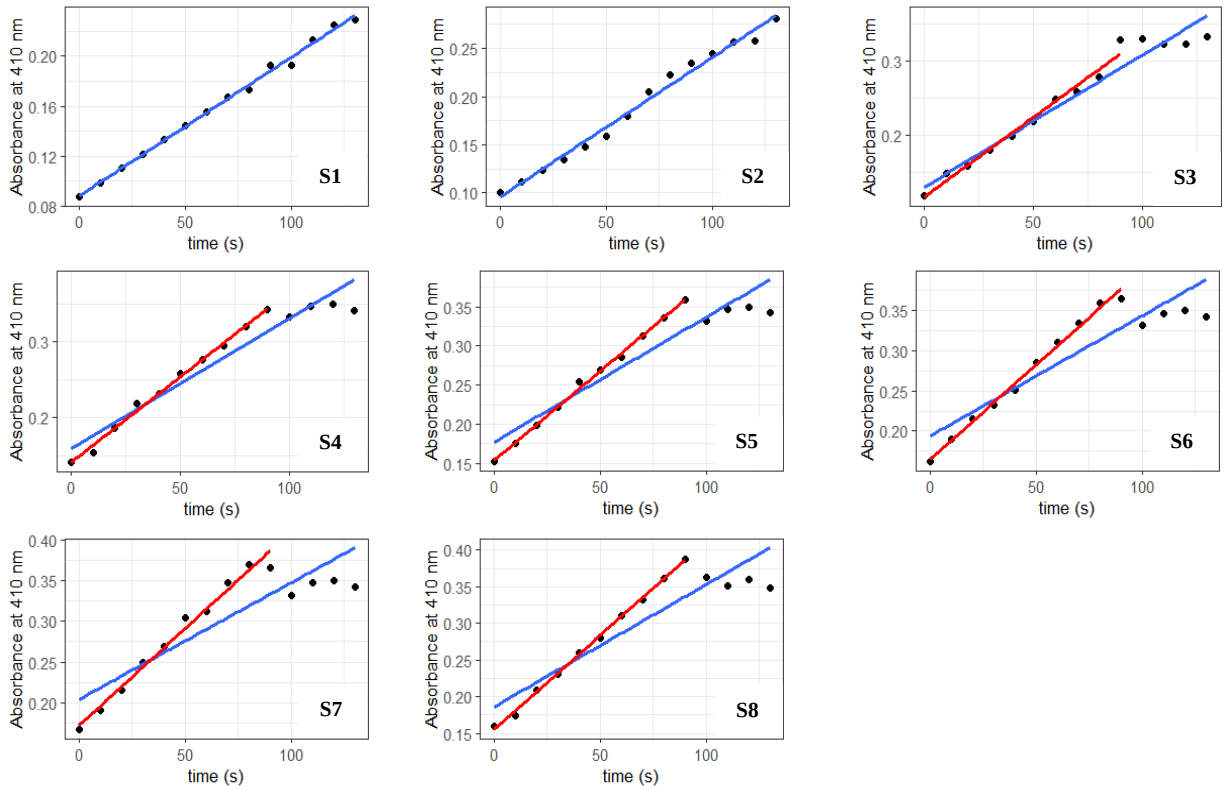

Figure 2. RStudio environment. enzyme kinetics data graphs at eight different substrate concentrations (blue lines: based on all data; red line: based on subset data).

The slopes of the linear lines generated in the graphs were determined using the "tidy" function.

```
deney_1 %>%
lm(abs ~ time, data = .) %>%
tidy()
```

Following the execution of the function, the values obtained for deney_1 data were computed including the intercept and slope (time) (Table 2).

Table 2. Parameters of the linear fit obtained for deney_1 data (RStudio data)

```
A tibble: 2 × 5
  term        estimate std.error statistic  p.value
  <chr>          <dbl>     <dbl>     <dbl>    <dbl>
1 (Intercept)  0.0880  0.00156         56.4 6.32e-16
2 time         0.00111 0.0000204       54.6 9.42e-16
```

The slope value for the deney_1 dataset was found to be 0.00111. The remarkably low standard error indicates a good fit of the data to the linear curve. Analyses were conducted for the other experimental datasets; however, as expected, the standard errors increased notably in the S3-S8 analyses due to deviations from linearity in these data (Figure 2). Therefore, data restrictions based on absorbance values or time were applied to redraw the lines (Figure 2, red lines). An example function for the S3 study was constructed as follows:

```
deney_3 <- data_set_al(data = data,  time_col = 1,  abs_col = 4)
deney_3 %>%
  ggplot(aes(time. abs)) +
  geom_point() +
  labs(x = "time (s)", y = "Absorbance at 405 nm") +
  geom_smooth(method = "lm", se = FALSE)+
  geom_smooth( data = subset(deney_3, time < 90).
  method = "lm", col="red", se = FALSE)
```

The unit of the calculated slope values should be adjusted accordingly. It has been converted to s^{-1} using the equation provided below, based on the Lambert Beer law, where ε represents the molar absorption coefficient with a value of 1623 M^{-1} cm^{-1} for catechol product at 410 nm wavelength. The path length (l) is 1 cm. C denotes the solution concentration in mol L^{-1}. Values are divided by ε and multiplied by 1000 to express them in mM.

$$A = \varepsilon * l * C \quad (1)$$

Table 3. Slopes of the adjusted trendlines and calculated rate values

Samples	Slope	Rate (mMs^{-1}) $x10^{-4}$
S1	0.00111	7.024
S2	0.00145	8.934
S3	0.00200	1.232
S4	0.00227	1.399
S5	0.00230	1.417
S6	0.00240	1.479
S7	0.00262	1.614
S8	0.00255	1.571

The rate values were defined as a data.frame based on the respective substrate concentrations and plotted against each other using the plot function. Figure 3 illustrates the dependency of enzyme reaction rate on substrate concentration. This hyperbolic curve can be described by the typical Michaelis-Menten equation and characterized by the *Vmax* parameter, representing maximum velocity in the equation. According to Figure 3, at low S concentrations, rate values show nearly linear increase; but as S concentration increases, a rectangular hyperbola formation becomes evident. *Km* is determined by the substrate concentration that gives half of the *Vmax* value and is a parameter defining the enzyme's substrate affinity. Low *Km* values indicate high substrate affinity of the enzyme.

```
concs <- c(0.21, 0.33, 0.67, 1.71, 2.21, 3.31, 5.05, 7.10)
rate <- c(0.0007024, 0.0008934, 0.001232, 0.001399, 0.001417, 0.001479. 0.001614. 0.001571)
table.df <- data.frame(concs, rate)
plot(concs, rate, main="rate vs substrate concentrations", xlab="S (mM)", ylab="rate (uM/min)")
```

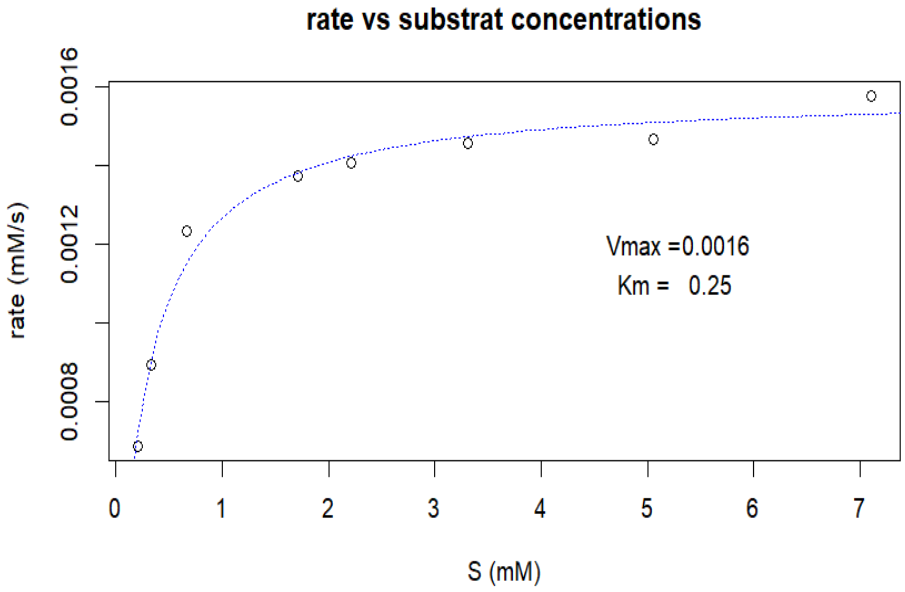

Figure 3. Change in rates dependent on substrate concentrations

The Michaelis-Menten equation is one of the fundamental equations describing the substrate-enzyme relationship:

$$V = \frac{V_{max}[S]}{(K_m+[S])} \quad (2)$$

To perform nonlinear regression using the R program, the `nls` function is employed. In this function, the equation must be defined, data selected and initial Michaelis-Menten equation's parameters (*Vmax* and *Km*) provided for fixed parameters. These initial values should be chosen considering the data of Figure 3.

```
mm.nls <- nls(rate ~ (Vmax * concs /(Km + concs)), data=table.df,
start=list(Km=0.25, Vmax=0.006))
bestfit <- nls(mm.nls, table.df, start=list(Vmax=0.006,Km=0.25))
bestfit
```

The result was presented in Rstudio is as follows:

```
Nonlinear regression model
model: rate ~ (Vmax * concs/(Km + concs))
   data: table.df
    Vmax       Km
0.001583 0.250038
residual sum-of-squares: 1.261e-08

Number of iterations to convergence: 3
Achieved convergence tolerance: 3.573e-07
```

The program has calculated Km and Vmax values by performing three different iterations, using the residual sum of squares to achieve highly accurate results. These *Km* and *Vmax* values were extracted from the model constants using the unname function for further analysis.

```
Km <- unname(coef(mm.nls)["Km"])
Vmax <- unname(coef(mm.nls)["Vmax"])
```

Using these parameters, rate values were calculated for different substrate concentration values to obtain a theoretical curve. For this purpose, x values were generated for substrate concentrations using the seq function with increments of 0.1 from 0 to 8 mM. and corresponding y values were computed. When plotted onto Figure 3, a blue dashed curve was obtained. *Km* and *Vmax* values were annotated on the graph using the text function.

```
x <- seq(0,8,0.1)
y <- (Vmax*x/(Km+x))
lines(x,y, lty="dotted", col="blue")
```

The residuals graph is used to evaluate whether the residuals in regression analysis are normally distributed and exhibit homoscedasticity. The residual analysis was performed using the `resid` function, and the graph was obtained (Figure 4a). To determine whether the residuals follow a normal distribution, a Q-Q plot was generated using the `qqnorm()` function (Figure 4b).

```
mm.resid <- resid(mm.nls)
plot(table.df$kons.mm.resid)
qqnorm(mm.resid)
qqline(mm.resid)
```

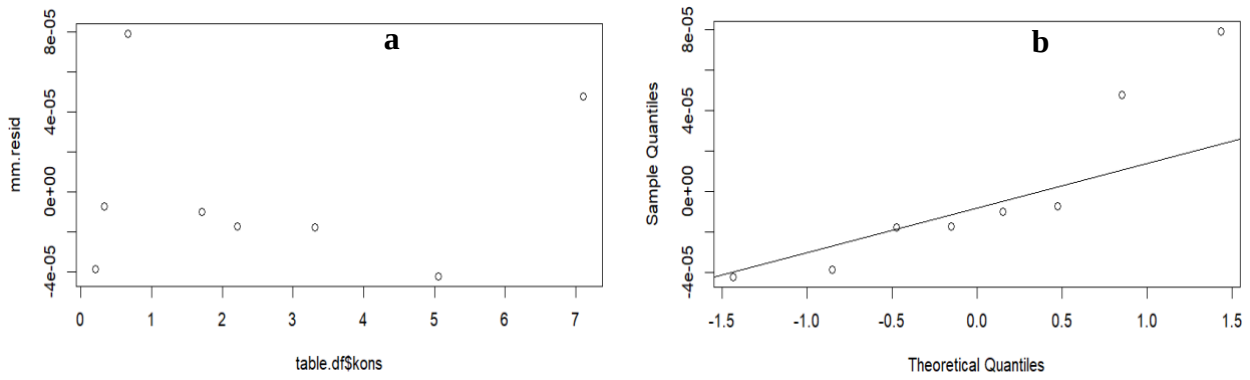

Figure 4. Residual analysis (a) and Q-Q graph (b)

The tyrosinase enzyme present in the crude extract of Giresun chanterelle mushrooms exhibits high affinity towards the catechol substrate, with a *Km* value of 0.25 mM. For mushroom tyrosinase with catechol, *Km* values reported in various studies range from 0.26 mM to 0.95 mM. The *Km* value for crude extracts obtained from *Agaricus bisporus* mushrooms was determined to be between 0.75 and 0.933 mM, indicating high affinity of the tyrosinase enzyme for the substrate (Zaidi et al., 2014). These values are reported as 0.90 and 0.85 mM for *Pycnoporus sanguineus* and *Lentinula edodes* mushrooms, respectively (Halaouli et al., 2005; Kanda et al., 1996). The *Vmax* value was calculated as 0.0016 mM s^{-1}. If this unit is converted, it is expressed as 98.89 µM min^{-1}, and the activity of 1 mL crude extract is expressed as 1186.68 µmol/min/mL. This value is reported as 2518 µmol min^{-1} mL^{-1} for *Agaricus bisporus* mushroom purified with ammonium sulfate (Kaur et al., 2022). For pure

mushroom tyrosinase (Sigma 34 U mg^{-1} mushroom tyrosinase, 145 µg in 1 mL of 0.1 mM acetate buffer), the *Vmax* value is reported as 174 mM min^{-1} (Rodríguez-Sevilla et al., 2014).

The *Km* value may decrease with enzyme purification, likely due to the removal of potential inhibitors and activators present in the crude extract. Similarly, the *Vmax* value can increase with purification. However, the purification steps may induce conformational changes, potentially damaging the enzyme structure and reducing its activity, which would affect both *Km* and *Vmax* values.

4. Conclusions and Recommendations

In this study, an R software program was developed for the analysis of enzyme activity using a mathematical model equation. The model parameters indicate the enzyme's efficiency and affinity for the substrate used. Comparing these values across different studies shows variability, likely due to differences in experimental setups, enzyme sources, and methods used to purify and characterize the enzyme. Therefore, in studies on enzyme activity, it is crucial to rapidly evaluate experimental data and make comparisons in a more consistent manner. The R program developed in this study will address this need. As the software includes a basic programming structure, it can be revised with different codes and approaches to meet various requirements.

Authors' Contributions

All authors contributed equally to the study.

Statement of Conflicts of Interest

There is no conflict of interest between the authors.

Statement of Research and Publication Ethics

The author declares that this study complies with Research and Publication Ethics.

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