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Experiment 14C, Enzyme Kinetics

1 Introduction

Enzymes are of fundamental importance in cell metabolism. Such enzymes are proteins that increase the rate of a specific chemical reaction. For a cell to operate accurate chemical reactions have to occur very specifically for a time restricted period and in a specific amount. This demanding task is ensured with enzymes, also known as biocatalysts.

Generally proteins are linear chains of amino acids connected together with peptide bonds in a very specific and predefined sequence. However enzymes often interact with non-peptidic factors (cofactors and coenzymes). Their task is to regulate the "conversion" of a specific chemical compound (substrate). Each enzyme recognizes its substrate with the binding of this substrate to the active center of the enzyme. The interaction between the substrate and the enzyme is the done by hydrogen bonds, charge/charge contacts, charge/dipole contacts and hydrophobic interactions.

The efficiency of an enzyme under specific conditions is discussed in the topic of enzyme kinetics. The terms "maximum rate" ($v_{\max}$) of a reaction and the "Michaelis constant" (K_{M}) are fundamental for the theoretical Michaelis–Menten enzyme kinetics model. In the easiest case of this model, an enzyme E catalyzes the conversion of a substrate S into the product P with the formation of an enzyme-substrate complex ES as intermediate. This intermediate complex ES can either decompose back to E and S or, with a certain probability, react to the product P (1).

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P \qquad (1)$$

The mathematical relation between the concentration of a substrate [S] and the reaction rate v for such a general reaction scheme (1) can be formulated as an equation (2) depending on $v_{\max}$ (3) and K_{M} (4).

$$v = v_{\max} \cdot \frac{[\mathrm{S}]}{K_{\mathrm{M}} + [S]} \qquad (2)$$

$$v_{\max} = k_2 \cdot ([\mathrm{E}] + [\mathrm{ES}]) = k_2 \cdot [\mathrm{E}]_{\mathrm{sat}} \qquad (3) \qquad K_{\mathrm{M}} = \frac{k_{-1} + k_2}{k_1} \qquad (4)$$

The function (2) giving the corresponding rate v for each substrate concentration [S] is hyperbolic with the value $v_{\max}$ as horizontal asymptote when the graph approaches as $[\mathrm{S}] \to \infty$ $\left(\lim_{[\mathrm{S}]\to\infty} v([\mathrm{S}]) = v_{\max}\right)$. Thus for high substrate concentration the reaction rate is limited to $v_{\max}$. The Michaelis constant K_{M} is defined as the substrate concentration at half $v_{\max}$ (independent of the enzyme concentration) and therefore has the unit of a molar concentration (M mole per liter, mM or μM). K_{M} is also a value for the affinity of an enzyme to a specific substrate, the smaller K_{M} the higher the affinity.

In fact the curve (fig. 1) of our function (2) shows that when the substrate concentration is very small, each substrate molecule finds an enzyme to undergo a reaction. Or mathematically if $K_{\mathrm{M}} = 1$ μM and [S] = 0.001μM, [S] in the bottom of the fraction can be neglected (the difference between 1.00 μM and 1.01 μM can be neglected). The reaction rate is approximately proportional to the substrate concentration. However this is only the case until K_{M} is reached. Inserting K_{M} = [S] in to our

equation (2) gives us $v_{\max}\frac{K_M}{K_M + K_M}$ thus $\frac{1}{2}v_{\max}$. For substrate concentration [S] being very high K_M can be neglected giving $v_{\max}\frac{[S]}{[S]}$, thus $v = v_{\max}$. However it has to be noted that the curve can also look very different. An example is the O_2 binding curve of haemoglobin which has a sigmoid behaviour. In this case oxygen is not only the substrate but also an effector and when an oxygen binds to one of the four active sites of haemoglobin the oxygen affinity of the remaining three is enhanced due to a conformational change of a subunit.

Obviously $v_{\max}$, as the enzyme amount becomes a limiting factor, can only be observed with very high ($\rightarrow \infty$) substrate concentration in a Michaelis-Menten diagram. To easily get the values of $v_{\max}$ and K_M a transformation (5) of the Michaelis-Menten equation can be done. This representation is a double inversion of the the data. The value of $\frac{1}{v_{\max}}$ is where the line intercepts the ordinate and $\frac{1}{-K_M}$ where the line intercepts the abscissa. This plotting method is called Lineweaver-Burk diagram (fig. 2) and such approximation of the data was widely used in the past, because the model to calculate reliable approximation of a maximum reaction rate where not yet known or difficult.

$$\frac{1}{v} = \frac{K_M}{v_{\max}} \cdot \frac{1}{[S]} + \frac{1}{v_{\max}} \qquad (5)$$

The activity of enzymes can be reduced by other substances called inhibitors. There are reversible and irreversible inhibitions, the latter cannot be undone. The enzyme is blocked permanently as the inhibitor is bound to the enzyme and locks the enzyme's active site. Reversible inhibitors can disengage and the enzyme can catalyse reactions again. There are two different types of reversible inhibitors: competitive (isosteric) and non-competitive (allosteric). A competitive inhibitor binds to the active site of the enzyme (where the substrate should bind) and is in a competition with the substrate. In the Michaelis-Menten theory K_M has a different value under these conditions but the $v_{\max}$ stays the same (fig. 1).

A non-competitive inhibitor binds to an enzyme not at its active site and therefore does not compete with the substrate. Instead it causes a structural change of the protein. In the Michaelis-Menten theory then $v_{\max}$ is decreased but K_M remains unchanged (fig. 1). This can also be observed in a Lineweaver-Burk plot (fig. 2).

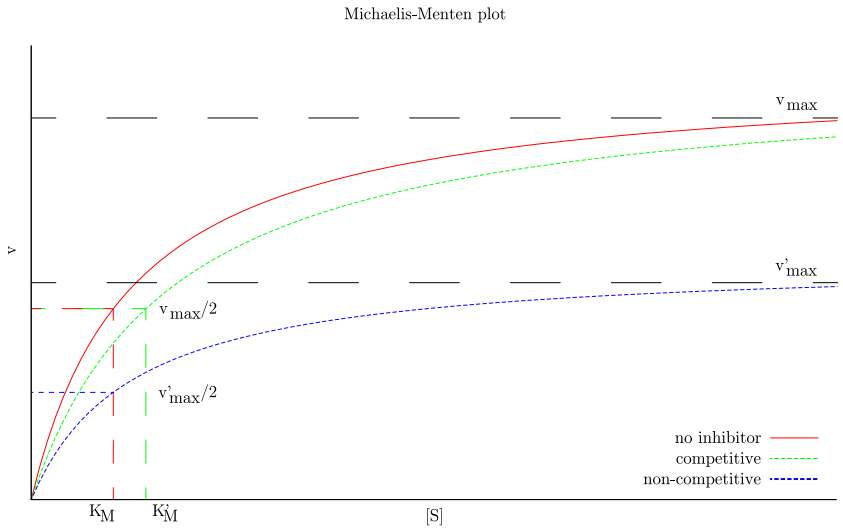

Figure 1: Michaelis-Menten diagram

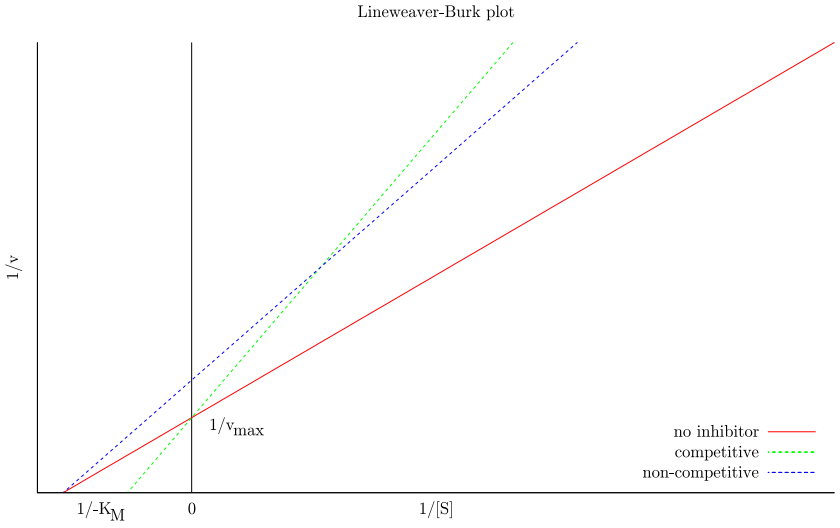

Figure 2: Lineweaver-Burk diagram

There are many different enzymes and inhibitors for specific reactions such the already mentioned haemoglobin or MAO (monoamino oxidase) and its inhibitor (MAOI). We will work with the enzyme catalse, freshly obtained from Swiss potatoes. Catalase accelerates the decomposition of hydrogen peroxide (our substrate) to water and oxygen (6). We are going to explore the activity of ice-cooled catalse, in particular in environments with different pH and in interaction with the reversible catalase inhibitor hydroxylamine.

$$2H_2O_2 \xrightarrow{\text{catalase}} 2H_2O + O_2 \qquad (6)$$

2 Experiments

In teams of two (my partner was Céline Delay) we run 4 experiments to see how the catalase enzyme performs under various conditions. We always measured the time needed (in seconds) for 30 μl enzyme mixture put on a filter paper to raise this filter for 1 cm in a beaker filled with a specific H_2O_2 (substrate) concentration. The emerging of the filter paper is possible due to produced O_2. As catalase is the engine behind this O_2 production the time needed to raise the filter paper is an indicator for the enzyme's activity. We measure the emerging rate v as $\frac{1}{t}$, t being our time measured. The shorter the time, the better the reaction rate and and vice versa.

2.1 Dependence from the Enzyme Concentration

In the first experiment we had to find the ideal enzyme / ice water concentration. We made four mixtures according to table 1. We then had to evaluate the our ideal enzyme / ice water mixture, the one being nearest to an emerging time of 30 seconds. Those individual results depend on the preparation of the potato and the amount of it used.

	E1	E2	E3	E4
cell extract ml	40	20	10	0
ice water ml	0	20	30	40
units ml^{-1}	100	50	25	0

Table 1: Enzyme mixtures

Our results (tab. 2) are displayed in in a diagram (fig. 3) and E2 has been chosen as our ideal cell extract / ice water mixture.

	E1	E2	E3	E4
$\bar{t}$	19	25.3	47.6	–

Table 2: Enzyme mixtures

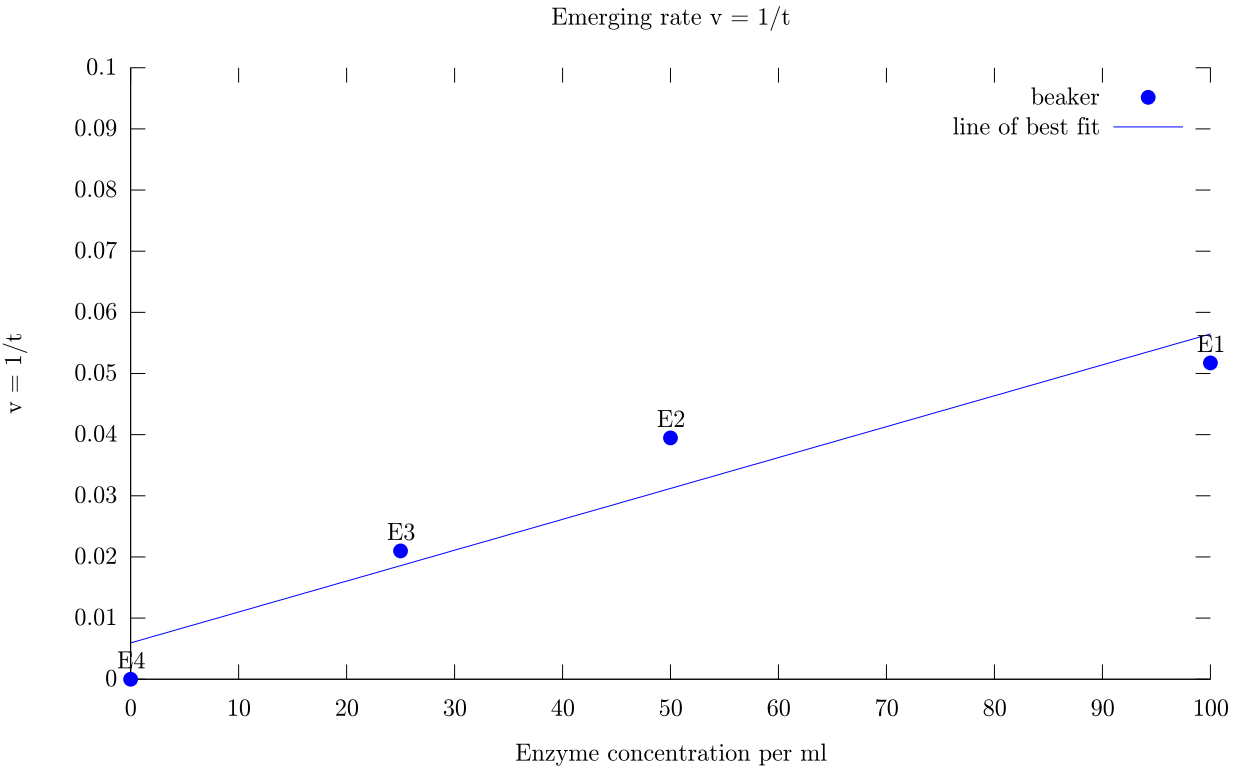

Figure 3: Ideal cell extract / ice water mixture

2.2 Determination of the Michaelis Constant K_M

In a second experiment we want to see the enzyme's activity in different substrate concentrations. With 35% H_2O_2 we created substrate solutions of different concentration and again measured the emerging time (tab. 3). The obtained data is displayed as Michaelis-Menten (fig. 4) and Lineweaver-Burk diagram (fig. 5) together with an approximated nonlinear / linear function.

m% H_2O_2	2.5	1.5	1.0	0.8	0.5	0.2
[S]	0.000735	0.000441	0.000294	0.000235	0.000147	0.000059
t_1	20	23	21	30	48	128
t_2	24	19	24	29	52	108
$\bar{t}$	22	21.0	22.5	29.5	50.0	118.0

Table 3: substrate concentration vs. emerging time

The Michaelis-Menten diagram shows a value of 0.069 cm·s^{-1} as v_{max} and a value of 0.25 μM as K_M. The Lineweaver-Burk plot gives a value of 0.178 cm·s^{-1} as v_{max} and a value of 1.17 μM as K_M.

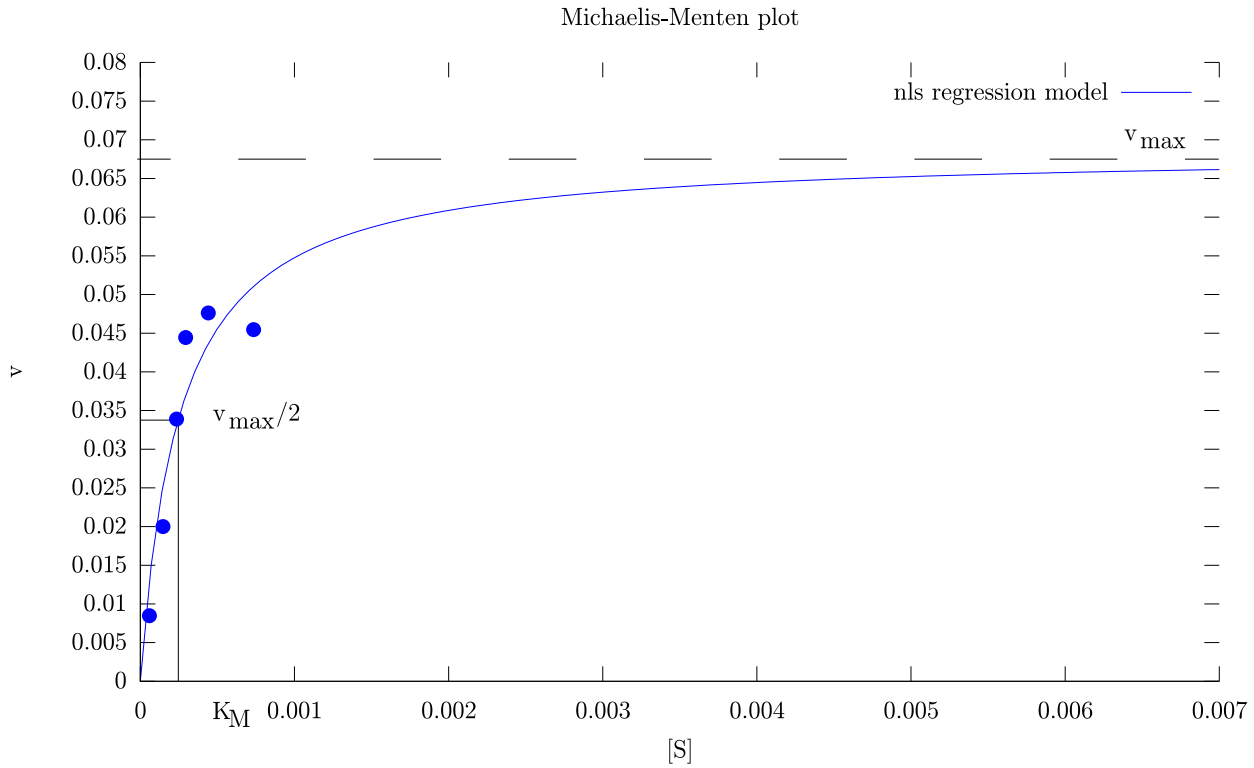

Figure 4: Michaelis-Menten diagram

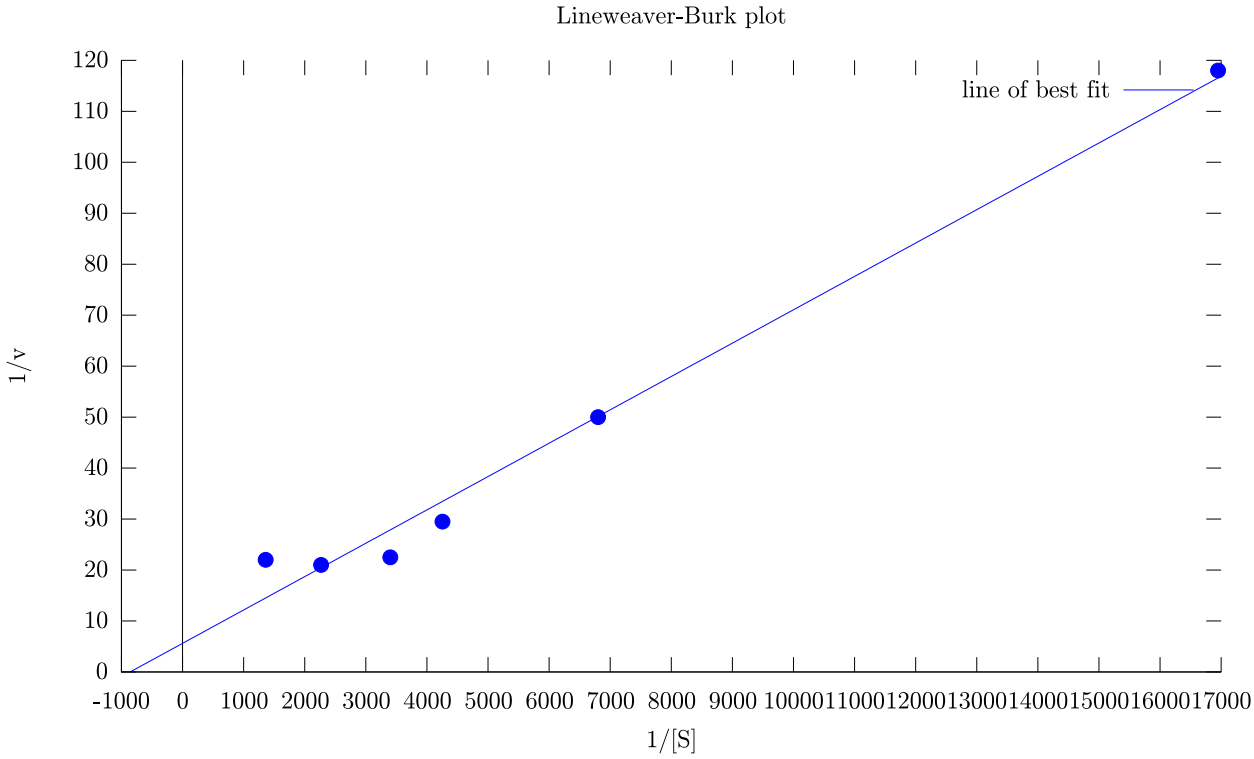

Figure 5: Lineweaver-Burk diagram

2.3 Enzyme Inhibition

In this experiment we observe the activity of catalase while adding NH_2OH (hydroxylamine, an catalase inhibitor) step by step. The concentration NH_2OH added to the enzyme and the substrate solutions and our results are summarized in table 4.

m% H_2O_2	2.5	1.5	1.0	0.8	0.5	0.2
[S]	0.000735	0.000441	0.000294	0.000235	0.000147	0.000059
$5.0 \cdot 10^{-6}\%$	24	17	26	31	52	84
$1.0 \cdot 10^{-5}\%$	23	16	28	33	45	111
$1.5 \cdot 10^{-5}\%$	21	15	27	22	41	130
$2.0 \cdot 10^{-5}\%$	24	17	29	31	45	110

Table 4: Catalase activity with hydroxylamine inhibitor

The obtained data is also displayed as Michaelis-Menten (fig. 7) and Lineweaver-Burk diagram (fig. 8) together with approximated nonlinear / linear functions (page 7 due to formatting optimization).

2.4 pH Dependence of the Reaction

In our last experiment we observe the activity of catalase in environments with different pH-values. To do so, we use existing borax and phosphate buffer to get enzyme and substrate solutions with a pH of 4, 6, 8 and 9.4. In addition we already have a value for pH 7 (tab. 2). Although we did not measure the pH of the first solution, we expect it to be around 7. The obtained data is summarized in table 5 and visualized with a bar plot diagram (fig. 6).

pH	4	6	7	8	9.4
$v = 1/t$	0	0.038911	0.039526	0.028818	0.027778

Table 5: pH dependant activity of catalase

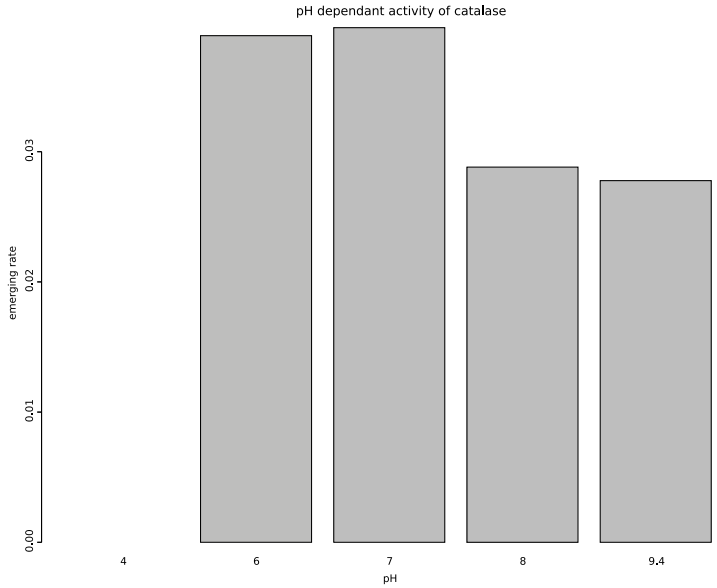

Figure 6: pH dependant activity of catalase

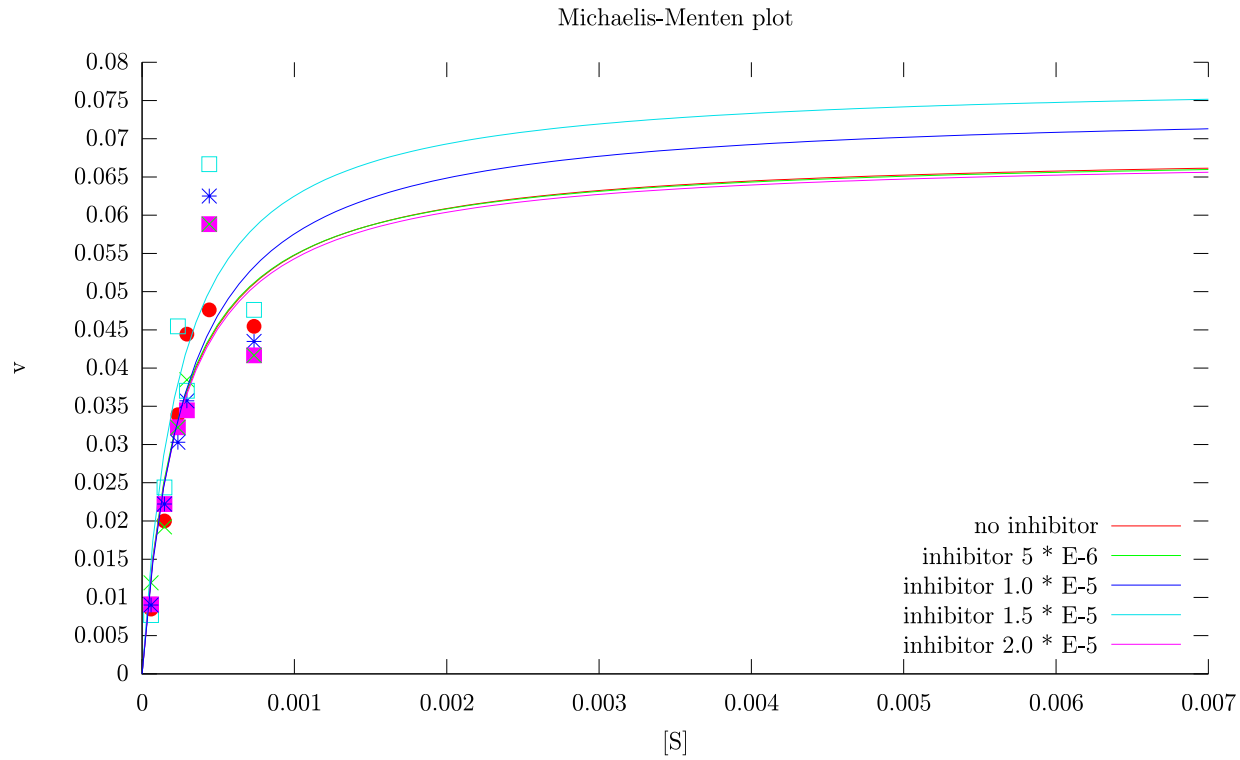

Figure 7: Michaelis-Menten diagram

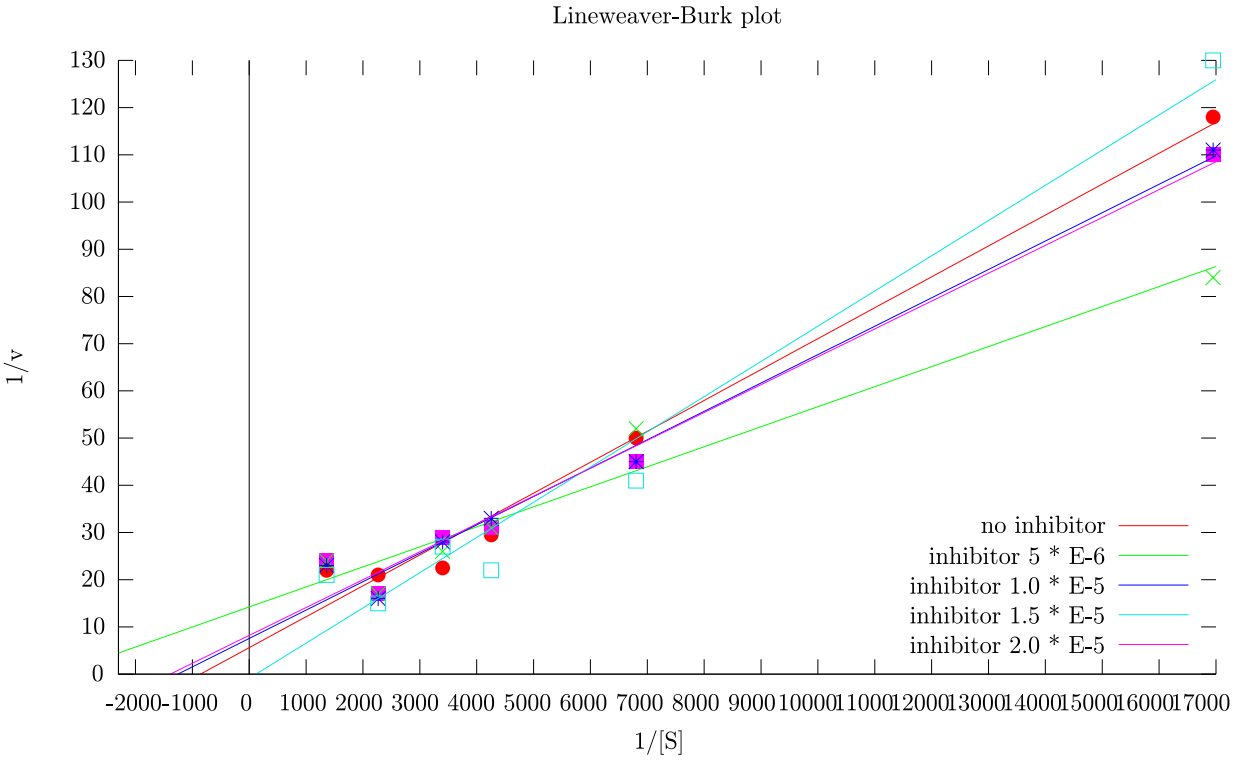

Figure 8: Lineweaver-Burk diagram

2.5 Discussion

In a first experiment we could observe how the reaction rate increases with an higher amount of enzyme. Though the approximation of our results could be linear, we do not have enough data to be certain, but in any case a linear increase of the rate up to a certain limit would make sense.

To determine the Michaelis Constant K_M we used two approaches, the determination with a Michaelis-Menten diagram and the determination with a Lineweaver-Burk diagram. Using the same data for both diagrams we could expect the same K_M and v_{max} values in both diagrams. However, this is not the case, but it is not a surprise either. We knew it is not possible to get a reliable v_{max} value with a Michaelis Menten diagram, as we do not have data for high substrate concentration and sometimes it is even impossible to perform such an experiment. Also the Lineweaver-Burk diagram gives us only an approximation and for a reliable value under these conditions we do not have enough data. To acquire more accurate results data from higher substrate concentration (e.g. 1.5 and 3 $mmol \cdot ml^{-1}$) would be useful.

Based on our data we can not make statement regarding the inhibitor's character (competitive or non-competitive). Our results are scattered over a large area. We can't supply an explanation for our results, but it is obvious that with the few measurements we did outlier have an immense impact. An explanation for the discrepancy from our results of the second experiment could be that the experiments were performed by different persons, each having an individual perception to measure the emerging time. An other explanation why we got such data could be that the catalase was not not cooled properly because of the lack of ice in the laboratory.
But knowing the structure of hydroxylamine we can expect it to be a competitive inhibitor as the structure is similar to hydrogen peroxide. Hydroxylamine would then act as an competitive inhibitor like carbon monoxide (CO) being a strong inhibitor of haemoglobin.

We could demonstrate the absence of enzyme reaction in an environment with a pH of 4 and slightly reduced enzyme activity in an pH 8 and pH 9.4 environment. As already mentioned in the introduction enzymes are made of amino acid chains and a change of environment (such as temperature or pH) can lead to a conformational change, disturb the correct folding of the protein or even deprotonate the amino acids and destroy the protein. Some of this has obviously been the case in the pH 4 environment, but we were surprised to see the enzyme still work under pH 9.4 conditions.

A Linear / Nonlinear Regression with R

Michaelis Menten:

```
nicolas@alice:~/stat R
> v=read.table("exp2_1.txt", header=T) ;attach(v) ; v
      conc     rate
1 0.000059 0.008475
2 0.000147 0.020000
3 0.000235 0.033898
4 0.000294 0.044445
5 0.000441 0.047619
6 0.000735 0.045455

> o=nls(rate~(alpha*conc)/(conc+beta),data=v,start=list(alpha=0.1,beta=0.01),trace=T)
0.006508449 :  0.10 0.01
...
0.0001528092 :  0.0685167738 0.0002514998
> o; summary(o)
Nonlinear regression model
  model:  rate ~ (alpha * conc)/(conc + beta)
   data:  v
    alpha      beta
0.0685168 0.0002515
 residual sum-of-squares: 0.0001528

Number of iterations to convergence: 13
Achieved convergence tolerance: 5.303e-06

Formula: rate ~ (alpha * conc)/(conc + beta)

Parameters:
       Estimate Std. Error t value Pr(>|t|)
alpha 0.0685168  0.0150126   4.564   0.0103 *
beta  0.0002515  0.0001314   1.915   0.1281
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Residual standard error: 0.006181 on 4 degrees of freedom

Number of iterations to convergence: 13
Achieved convergence tolerance: 5.303e-06
```

Lineweaver Burk:

```
nicolas@alice:~/stat R
> d=read.table("exp2_2.txt", header=T) ; attach(d) ; d
      conc  rate
1  1360.54  22.0
2  2267.57  21.0
3  3401.36  22.5
4  4255.32  29.5
5  6802.72  50.0
6 16949.15 118.0
> fit <- lm(rate ~ conc)
> fit

Call:
lm(formula = rate ~ conc)

Coefficients:
(Intercept)         conc
   5.615831     0.006545

> summary(fit)

Call:
lm(formula = rate ~ conc)

Residuals:
      1       2       3       4       5       6
 7.4798  0.5436 -5.3768 -3.9657 -0.1377  1.4568

Coefficients:
             Estimate Std. Error t value Pr(>|t|)
(Intercept) 5.6158306  3.0975812   1.813    0.144
conc        0.0065447  0.0003943  16.597 7.72e-05 ***
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Residual standard error: 5.075 on 4 degrees of freedom
Multiple R-squared: 0.9857,Adjusted R-squared: 0.9821
F-statistic: 275.5 on 1 and 4 DF,  p-value: 7.72e-05
```