Dose-response curves in Industrial Biotechnology

NCS-2012
Thomas A Poulsen
Novozymes A/S

Novozymes

- Develops, produces and sells industrial enzymes
- Spun out of NovoNordisk in 2000
- 5800 Employees world wide
- 700 products in 130 countries within more than 30 different industries
- Sales ~ 1E10DKK (1.3E9 EUR)

AGENDA

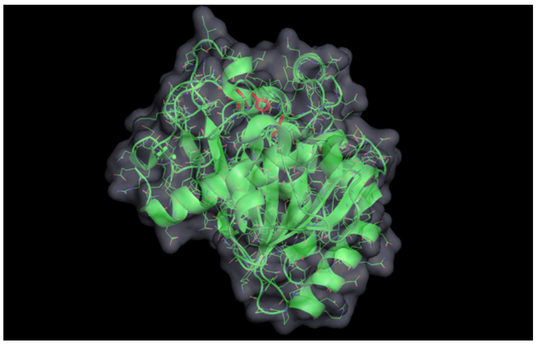
- Novozymes
- Laundry
- pKa-values / Enzyme kinetics

Novozymes Products

Environmental impact comparisons

Tergotometer Wash

An enzyme



Washing is complicated

$$\frac{d\text{Fat}}{dt} = f(\text{fat, fat-type, variant, dose, detergent, detergent conc, pH, temperature, swatch, ...}))$$

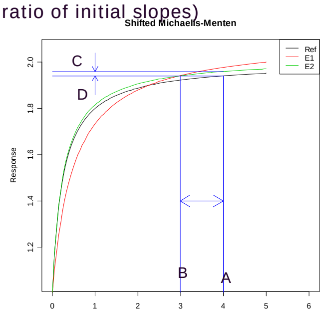
Could we study dependence on some of the parameters separately?

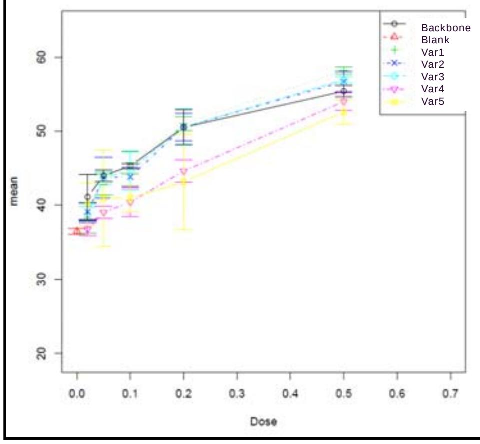
Parameters of interest

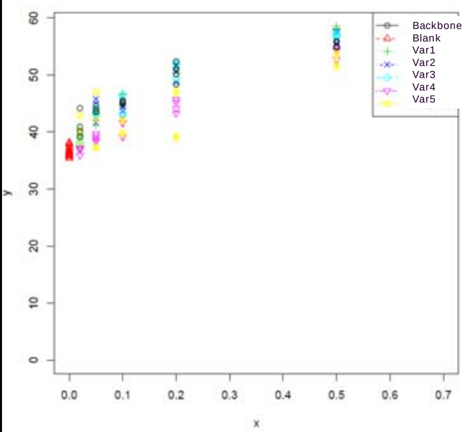
- RP: relative performance
- IF: Improvement factor (ratio of initial slopes)
- SF: Substitution Factor

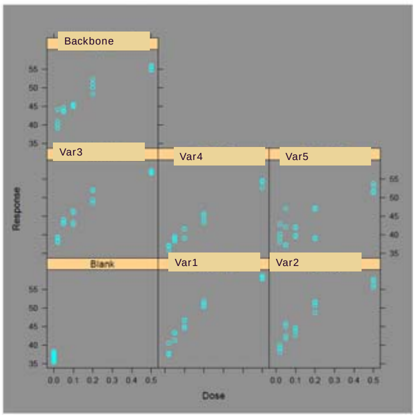
SF = A/B

RP= C/D







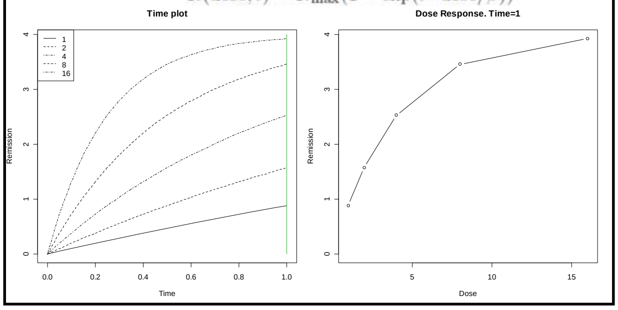


Backbone

Decay model

$$\frac{dR}{dt} = k_{\text{variant}} \cdot \text{dose} \cdot (1 - R/R_{\max})$$

$$R(\text{dose}, t) = R_{\max}(1 - \exp(t \cdot \text{dose}/\beta))$$



Estimates from drc

```
> summary(dd.drc)

A 'shifted Michaelis-Menten' model was fitted.

Parameter estimates:
```

	Estimate	Std. Error	t-value	p-value
Vm: Var1	32.615745	3.282793	9.935365	7.318e-18
Vm: Var2	29.550114	3.122574	9.463382	2.153e-16
Vm: Var3	29.302216	2.949913	9.933246	7.406e-18
Vm: Var4	114.722392	88.791193	1.292047	0.1987
Vm: Var5	72.908070	132.299997	0.551081	0.5825
Vm: Backbone	23.184242	1.974928	11.739285	2.643e-22
K: Var1	0.272534	0.059043	4.615852	9.447e-06
K: Var2	0.254111	0.059345	4.281943	3.622e-05
K: Var3	0.240404	0.054443	4.415731	2.131e-05
K: Var4	2.915903	2.608818	1.117710	0.2658
K: Var5	1.868360	4.201424	0.444697	0.6573
K: Backbone	0.143834	0.032735	4.393952	2.324e-05
y0:(Intercept)	37.095820	0.352603	105.205718	1.068e-125

Estimated residual variance: 2.987747

Surface adsorption

- Langmuir equation
  - Models adsorption to surface

$$S^* + P \rightleftharpoons SP$$

$$K = \frac{[SP]}{[S^*][P]}$$

$$\theta = \frac{\alpha \cdot P}{1 + \alpha \cdot P}$$

  - Same form as Michaelis-Menten Equation

First order model

```
> summary( nlsList(Response ~ y0 + a*(1-2**(-Dose*T))| Enzyme, data=d,
subset=d$Enzyme!='Blank',start=list(y0=50, a=5, T=2)))
Call:
  Model: Response ~ y0 + a * (1 - 2^(-Dose * T)) | Enzyme
   Data: d
```

y0

	Estimate	Std. Error
Var1	50.39845	0.2671837
Var2	51.93990	0.1986147
Var3	51.35220	0.4210537
Var4	51.96671	0.2707380
Var5	50.24382	0.2662588
Backbone	49.70936	0.3759703
Backbone	50.05580	0.2384233
	50.33227	0.2762553

a

	Estimate	Std. Error
Var1	3.827892	0.2522510
Var2	3.676799	0.1982280
Var3	4.025224	0.3926118
Var4	4.119330	0.2547631
Var5	5.243593	0.2516272
Backbone	4.249818	0.3554718
Backbone	4.385852	0.2436900
	4.029864	0.2589698

T

	Estimate	Std. Error
Var1	5.740147	1.1720258
Var2	4.818542	0.8899828
Var3	6.842588	1.8143273
Var4	5.871807	1.1074860
Var5	5.705709	0.8518683
Backbone	5.690506	1.4835772
Backbone	4.623022	0.8937416
	6.074128	1.1616943

Michaelis Menten Model

```
> summary( nlsList(Response ~ y0 + a*Dose/(c+Dose)| Enzyme, data=d,
subset=d$Enzyme!='Blank',start=list(y0=50, a=5, c=.5)))
Call:
  Model: Response ~ y0 + a * Dose/(c + Dose) | Enzyme
   Data: d
```

y0

	Estimate	Std. Error
Var1	50.04645	0.4437219
Var2	51.71227	0.2983783
Var3	50.76364	0.8180342
Var4	51.60433	0.4475471
Var5	49.77212	0.4396434
Backbone	49.21679	0.6584128
Backbone	49.67558	0.3785044
	49.99411	0.4554571

a

	Estimate	Std. Error
Var1	4.992783	0.3267290
Var2	4.835225	0.2730711
Var3	5.301707	0.6103626
Var4	5.357709	0.3284898
Var5	6.838845	0.3249970
Backbone	5.583683	0.4784864
Backbone	5.773523	0.3251463
	5.221463	0.3325169

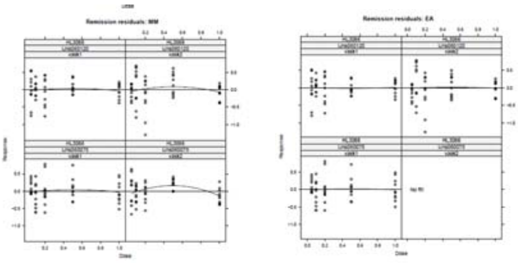
c

	Estimate	Std. Error
Var1	0.2007349	0.07587586
Var2	0.2698033	0.08752647
Var3	0.1426877	0.07499205
Var4	0.1983283	0.06988408
Var5	0.2033743	0.05610701
Backbone	0.1878077	0.09003560
Backbone	0.2548282	0.08417483
	0.1935510	0.07004698

Conclusions

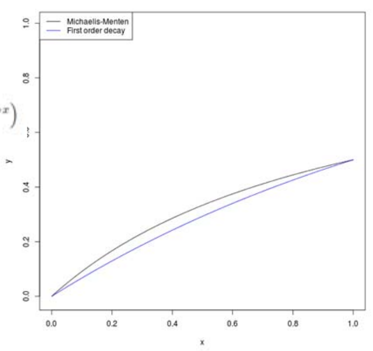
- Model choice not always obvious
- Both models are plausible
- No significant difference beween models
- Needs more experiments
- But the project is closed

Residuals



$$M(x, v, k) = \frac{v\,x}{k + x}$$

$$E(x, a, b) = a\left(1 - 2^{-bx}\right)$$



2.1 Shifted Michaelis Menten model

The (shifted) Michaelis-Menten model (4) has a number of nice features.

$$y = B + \frac{M \cdot x}{K + x} \tag{4}$$

where y is the cleaning, B is the cleaning by detergent alone, M is the asymptotic maximum, and K is the dose giving half of $M - B$. The enzyme dose is measured in x.

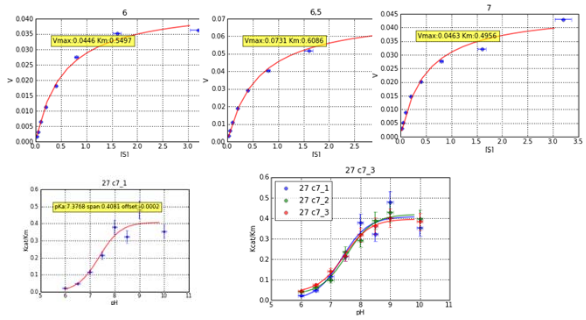
If we consider two enzymes (1 and 2) at the same dose, the relative performance is given in eq (5)

$$RP = \frac{y_1 - B}{y_2 - B} = \frac{M_1 \cdot x}{K_1 + x} \frac{k_2 + x}{M_2 \cdot x} = \frac{M_1}{M_2} \cdot \frac{K_2 + x}{K_1 + x} \tag{5}$$

Asymptotically this is

$$RP \longrightarrow_{x \to \infty} \frac{M_1}{M_2}, \quad RP \longrightarrow_{x \to 0} \frac{M_1}{M_2} \cdot \frac{K_2}{K_1} = IF \tag{6}$$

Measuring pKa-values



Enzyme pKa values

Titration curves are of the general form (1), as illustrated in figure 1.

$$f(x) = \frac{1}{1 + a^{(b-x)}} \tag{1}$$

$$f'(x) = \frac{a^{(b-x)} \log(a)}{(1 + a^{b-x})^2} \tag{2}$$

In chemistry, b is the pKa value, x the pH, and $a = 10$. From the general form, we see that the slope at the pKa is $\log(10)/4 = 0.58$.

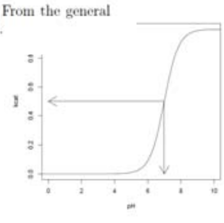
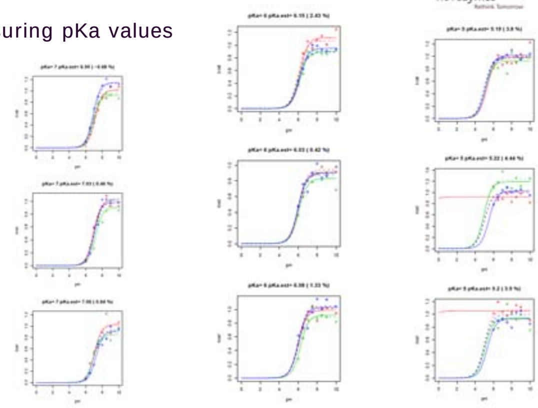


Figure 1: Titration curve of the case pKa=7

Measuring pKa values



Labour effort (50 variants)

- Traditional MM:
  - 8 pH values
  - 8 substrate concentrations
  - 3 replicates
  - = 192 kinetic curves per variant

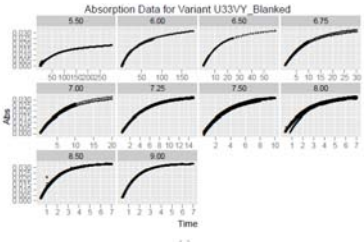
Enzyme kinetics

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E + P$$

$$\begin{aligned} \dot{c} &= k_1\, e\, s - (k_2 + k_3)\, c \\ \dot{p} &= k_3\, c \end{aligned}$$

Reagent	Timedep. conc.	Initial conc.
Enzyme	e	e_0
Substrate	s	s_0
Enzyme-substrate complex	c	0
Product	p	0

Reader curves



Enzyme Kinetics Michaelis-Menten

$$\begin{aligned} \dot{c} &= k_1\, e\, s - (k_2 + k_3)\, c \\ \dot{p} &= k_3\, c \end{aligned}$$

$$e_0 = e + c$$

$$\dot{c} = 0$$

$$c = \frac{e_0\, s}{\frac{k_2 + k_3}{k_1} + s} = \frac{e_0\, s}{K_M + s}$$

$$V = \dot{p} = k_3\, c = k_3\, e_0 \frac{s}{K_M + s} = V_{\max} \frac{s}{K_M + s}$$

Enzyme Kinetics: Substrate depletion

$$V = \dot{p} = k_3\, c = k_3\, e_0 \frac{s}{K_M + s} = V_{\max} \frac{s}{K_M + s}$$

$$\dot{p} = -\dot{s}$$

$$\dot{s} = -k_3\, e_0 \frac{s}{k_M + s}$$

$$\dot{s} = -k_3\, e_0 \frac{s}{k_M + s} = -\frac{k_3 e_0\, s}{k_M} + \frac{k_3 e_0\, s^2}{{k_M}^2} - \frac{k_3 e_0\, s^3}{{k_M}^3} + \cdots$$

$$\dot{s} = -\frac{k_3 e_0}{k_M} s \qquad s(t) = s_0 \exp(-\frac{k_3}{k_M} e_0\, t)$$

$$\boxed{p = s_0 \left(1 - \left(1 - \frac{e_0}{k_M}\right) e^{(-D\,t)}\right)}$$

pKa values. Substrate depletion results

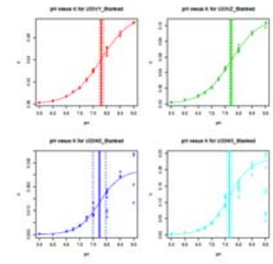
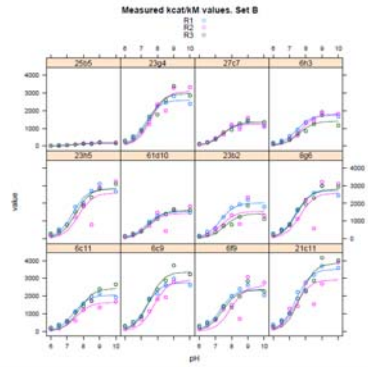


Figure 1: Models from cleaned data, *excluding* points marked by circles. Solid lines indicate estimated pK_a and the dashed lines are 19.6 standard errors.

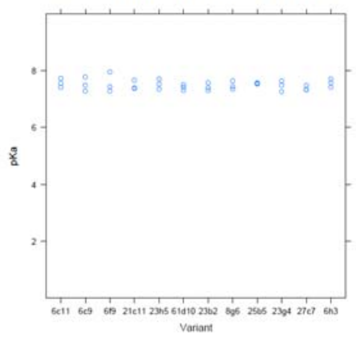
Labour effort (50 variants)

- Traditional MM:
  - 8 pH values
  - 8 substrate concentrations
  - 3 replicates
  - = 192 kinetic curves per variant
- Substrate depletion:
  - 8 pH values
  - 3 substrate concentrations
  - 3 replicates
  - = 48 kinetic curves per variant

Michaelis-Menten titration curves



pKa values based on measured kcat/kM values. Set B



Thanks to

- Jens E Nielsen, UCD / Novozymes
- Helen Webb, UCD
- Damien Farrell, UCD
- Esben P Friis, Novozymes
- Benjamin Høyer, DTU, Novozymes

- Ref (pKa): *Biochemistry, 2012, 51 (26), pp 5339–5347*
- Animation package: Yihui Xie

Conclusions

- pKa-values are difficult to measure
  - And even harder to change
- Is is important to consider analysis as part of experiment design
- Non-linear analysis can save ¾ of the work