“Enzyme Test Bench”: a biochemical application of the multi-rate modeling

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Abstract. In the expanding field of “white biotechnology” enzymes are frequently applied to catalyze the biochemical reaction from a resource material to a valuable product. Designed by evolution to catalyze the metabolism in any life form, they selectively accelerate complex reactions under physiological conditions. Modern techniques, such as directed evolution, have been developed to satisfy the increasing demand on enzymes. Applying these techniques and also rational protein design, the improvement of enzymes’ activity, selectivity and stability is the aim. To tap the full potential of these techniques, it is essential to combine them with adequate screening methods. Nowadays a great number of high throughput colorimetric and fluorescent enzyme assays are applied to measure the initial enzyme activity with high throughput. However, the prediction of enzyme long term stability within short experiments is still a challenge. A new high throughput technique for enzyme characterization with specific attention to the long term stability, called “Enzyme Test Bench”, is presented. The concept of the Enzyme Test Bench consists of short term enzyme tests conducted under partially extreme conditions to predict the enzyme long term stability under moderate conditions. The technique is based on the mathematical modeling of temperature dependent enzyme activation and deactivation. Adapting the temperature profiles in sequential experiments by optimum non-linear experimental design, the long term deactivation effects can be purposefully accelerated and detected within hours. During the experiment the enzyme activity is measured online to estimate the model parameters from the obtained data. Thus, the enzyme activity and long term stability can be calculated as a function of temperature. The results of the characterization, carried out in micro liter format applying short term experiments of hours, are in good agreement with the long term experiments of a week carried out in 1L stirred tank reactors. Thus, the new technique allows for both: the enzyme screening with regard to the long term stability and the choice of the optimal process temperature. This article gives a successful example for the application of multi-rate modeling, experimental design and parameter estimation within biochemical engineering. At the same time, it shows the limitations of the methods at the state of the art and brings the current problems to the attention of the applied mathematics community.
1. **Introduction and state of the art**

The Enzymes are mostly proteins evolutionary functionalized by evolution to catalyze metabolism reactions. Characterized by structure and folding they accelerate complex reactions with high purity (selectivity) under physiological temperature and pressure. In the expanding field of “white biotechnology” enzymes are frequently applied to catalyze the biochemical reaction from a resource material (substrate) to a valuable product (product). Chemical reactions requiring several steps under extreme conditions in expensive reactors or laborious by-product separation are increasingly replaced by enzymatic reactions. Modern techniques like directed evolution have been developed to satisfy the increasing demand on enzymes [14], [15]. Applying these techniques together with rational protein design, the aim is to improve the enzymes’ characteristics.

![Directed evolution. A simplified scheme.](image)

In Fig. 1 the simplified scheme of directed evolution is presented. Applying microbiological tools, the genes from enzymes found in the nature are isolated, mutated and expressed. By these means diverse recombinant enzymes are created. The selection (screening) is carried out in the next step to find enzymes of desired characteristics. These selected enzymes can be further improved in an iterative way. Therefore, the screening procedure plays an important role defining the direction of the evolutive enzyme changes [30], [15]. Being carried out in microreactor arrays in micro liter format (microtiter plates) the modern optical screening techniques provide for the testing of the initial reaction rate with high throughput. However, the initial reaction rate is neither sufficient to design an industrial process nor to select the optimal enzyme. Under process conditions (in situ) enzymes show a complex temperature dependent activity and deactivation behavior (stability). This behavior is illustrated in Fig. 2. The reaction rate of two enzyme candidates is presented over time. For both enzymes the reaction rate decreases due to deactivation effects with process duration. Comparing the product formation for the time period given, enzyme one (dashed line) features a higher turnover, represented by the area under the curve, and should be preferred. However, selecting enzymes accordingly to the state of the art by the initial reaction rate, enzyme two would be chosen. Hitherto, the long term stability was not considered within industrial screening process [27] despite its crucial impact on important process and economical parameters, such as total turnover number or space-time yield. The strongest argument against the long term experiments is their duration of weeks or months, especially for stable and, thus, the most promising candidates. Such long time experiments are not compatible with the high throughput screening approach.
[6], [7] and [12] propose an elegant approach to determine the long term enzyme stability within a short time. Similar to the breaking tests employed in material science, the enzyme behavior is observed under partly extreme conditions to predict the long term behavior under more moderate operating conditions. This approach assumes that the kinetics of the enzyme deactivation can be described mathematically. Typically, the temperature dependence of those deactivation reactions is assumed to be governed by the Arrhenius law. Thus, the long term deactivation effects can be accelerated by enhanced temperature and monitored within a short time. Typically, the experiments include temperature ramps. Measuring the enzyme activity continuously while the temperature increases, different effects (e.g.: Arrhenius activation at low temperatures, reversible and irreversible unfolding at elevated temperatures) are observed and their temperature dependence is determined. At the end of the procedure the parameterization of the activation and deactivation model is performed, resulting in the prediction of enzyme turn over or space-time yield as a function of temperature and process duration. Thus, whereas a long time experiment would deliver information on the enzyme stability only at a chosen temperature, the new approach predicts the temperature dependent behavior, allowing for the optimization of process temperature with respect to the enzyme long term stability. The principle is demonstrated in Fig. 3. Here, an objective function (e.g. turnover number) is mathematically modeled as a function of process conditions, mainly temperature and time. Carrying out short experiments labeled by an asterisk in the figure, the model parameters are determined and the temperature dependent behavior predicted.

Based on this approach, it was attempted to include the long term stability determination into the enzyme screening process. On the one hand, the aim was to scale down the original experimental equipment into the screening compatible microtiter plate format. On the other hand, the existing approach was extended from intuitive experimental design (mostly temperature ramps) to computer aided optimum experimental design with respect to the enzyme deactivation model.
Objective, e.g. space time yield

Fig. 3: The applied principle of model based enzyme characterization. The objective function (turnover number) is modelled as a function of time and the control (temperature). Parameterizing the model by short term experiments (asterisk), the objective function is optimized.

2. Model based enzyme characterization

Within the enzyme characterization procedure, the following quantities are considered:

- differential states: \( y(t, z(t), u(t), q, p) \)
- algebraic states: \( z(t, y(t), u(t), q, p) \)
- experimental controls: \( u(t), q \)
- model parameters: \( p \)
- observable: \( \eta \)
- measurement error: \( \sigma(t, y(t), z(t), u(t), q, p) \)
- model response: \( h(t, y(t), z(t), u(t), q, p) \)
- coupled constraints w.r.t.: \( u(t), q, h(t, y(t), z(t), u(t), q, p) \)

The system is experimentally observed by the observable \( \eta \), which is measured with a time and system states dependent standard deviation \( \sigma \). Within the model \( \eta \) is mapped by the model response \( h \). A part of the states, controls \( u(t), q \) (e.g. temperature or initial concentrations) can be adjusted within an experiment. In addition to states, the model equations include physical constants and system specific coefficients \( p \). The aim of the model based enzyme characterization procedure is to identify the model equations and parameters to map \( \eta \) by \( h \) with sufficient accuracy with respect in the measurement error.

2.1. Characterization scheme

Fig. 4 illustrates the enzyme characterization procedure. Based on the previous knowledge, such as in the literature or from earlier experiments with structurally comparable enzymes, a model of temperature dependent enzyme activation and deactivation is built up. Initially, intuitive experiments are carried out to get a first overview of the enzyme behavior. Based on these experimental results, the
parameter estimation is performed to get a first parameterized model candidate. The results of the parameter estimation are evaluated with regard to the quality of the fit (reproducibility of the experimental results by the simulation of the preset experimental conditions using estimated parameters) and their significance (effect of the parameter variation on the fit quality [3]). Typically, further experiments are necessary. Based on the model candidate, optimum experimental design is carried out, to obtain experiments of high sensitivity to the estimated parameters. The obtained experiments are carried out and parameters are estimated again. This iteration loop is repeated until the condition of sufficient fit and parameter significance is fulfilled. If both are sufficient, a cross validation test is carried out. This test consists in the design and the conduct of one more experimental set. In contrast to parameter estimation no fitting of model coefficients is carried out in this case. Instead, the experimental results are compared to the model prediction for the correspondent experimental conditions. By these means, the power of prediction of the parameterized model is proven. As the requirements of good data fit, parameter significance and predictivity are fulfilled, the enzyme is assumed to be characterized and its long term stability can be predicted.

Fig. 4: The flowchart of the enzyme characterization procedure.
2.2. Multi-rate aspects of the modeling

In modeling the relevant enzyme catalyzed systems, the multi-rate aspects must be taken into account (see Fig. 5). Different processes occur with different velocities relative to the experimental duration and measuring interval. For instance, the reversible enzyme folding (change between different populations) takes place on the time scale of $10^{-9}$-$10^{-3}$ s. Thus, the dynamics of such processes is too fast to be observed. Therefore, the enzyme folding is assumed as infinitely fast equilibrium and modeled as algebraic equations. The observable within an enzymatic reaction is strongly connected to the reaction rate of product formation. Thus, the enzyme concentration is adjusted in a way to assure a measurable product formation within the whole experiment. The product formation is consequently modeled as a differential equation. The irreversible enzyme long term deactivation effects are slow processes, which cannot be observed on the relevant experimental time scale of minutes or hours. Within an industrial process of weeks or months, however, these effects cannot be neglected. To detect such effects in experiments, they consequently must be accelerated. For these purposes a control is needed to influence the deactivation. Moreover, the observed system exhibits many reactions, such as product formation, changes between enzyme populations and long term deactivation effects. All these mechanisms are measured by only one observable in the experiment. Consequently, the system is potentially underestimated. Therefore, controls are required to increase the number of observable system states. Influencing the most reactions, e.g. product formation, enzyme folding and long term deactivation in accordance with the Arrhenius law (eq. 1), temperature represents such a universal control [12]. The formulation of the Arrhenius law used here is mathematically equivalent to the conventional formulation but numerically more convenient [4].

Fig 5: Enzyme behaviour as a multi-rate process.
2.3. Modeling

Fig. 6 shows the applied model scheme of the considered reaction. The substrate molecule S reacts to the product molecule P. The uncatalyzed reaction at the reaction rate $k_c$ is very slow at low temperatures ($T<50^\circ C$). A much faster reaction takes place under catalytic agency of the active enzyme population $e_N$ with the reaction rate $k_e$ ($k_e e_N >> k_c$):

$$\frac{d[P]}{dt} = k_c + k_x \cdot [e_N]$$

$$[P](t = 0) = 0$$

Fig 6: Scheme of the considered reaction (a) and the applied enzyme deactivation (b), modelled as temperature dependent chemical reactions.

$$k = k_\infty \cdot e^{\frac{E}{RT}}$$

where

- $k$: reaction rate  [mol/L/s]
- $k_\infty$: frequency factor  [mol/L/s]
- $E$: activation energy  [J/mol]
- $R$: molar gas constant  [J/mol/K]

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The scheme of the enzyme deactivation is shown in fig. 6b. Considering different deactivation mechanisms from the literature [2], [6], [9], [11], [13], [16], [22], [25], [23], [26], four enzyme populations are defined: active enzyme, reversibly deactivated enzyme, irreversibly deactivated enzyme, irreversibly deactivated agglomerates. The arrows in the figure depict the change from population to population as chemical reactions with reaction constants $k$, which are assumed to behave in accordance with the Arrhenius law. We assume that all reactions, except of the agglomeration process, are the first order reactions. Here, the reaction order $n$ is one of the model parameters, if agglomeration is observed in experiments. Although further deactivation mechanisms are found in the literature [1], [21], [25], [26], [34], the number of fractions was limited to assure the applicability of the model (eq. 3). However, if previous knowledge or experimental results suggests further deactivation mechanisms, these mechanisms can be additionally implemented into the model.

\[
\frac{d[e_D]}{dt} = k_{RD} \cdot [e_R] + k_{AD} \cdot [e_N] + k_{AAagg} \cdot [e_N]^n + k_{RAagg} \cdot [e_R]^{n_2}
\]

\[
\frac{d[e_R]}{dt} = k_{AR} \cdot [e_N] - k_{RD} \cdot [e_R] - k_{RA} \cdot [e_R] - k_{RAagg} \cdot [e_R]^{n_2}
\]

\[
\frac{d[e_N]}{dt} = k_{RA} \cdot [e_R] - k_{AD} \cdot [e_N] - k_{AR} \cdot [e_N] - k_{AAagg} \cdot [e_N]^n
\]

(3)

With respect to irreversible deactivation, reactions from both, the active and the reversibly deactivated state [22] or the reaction exclusively from the reversibly deactivated state [13] are modeled in the literature. Here the modeling according to [13] is presented. The irreversibly deactivated enzyme ($e_D$) is generated from the reversibly deactivated state ($e_R$) with the reaction rate $k_{RD} \cdot k_{AD}$, $k_{AAagg}$, $k_{RAagg}$=0) and is described by the differential equation:

\[
\frac{d[e_D]}{dt} = k_{RD} \cdot [e_R]
\]

(4)

with the initial condition, based on the assumption of the initially completely active enzyme (enzyme storage stability is assumed):

\[ [e_D] (t = 0) = 0 \]

(4a)

Furthermore, in accordance with [7], the adjustment of the thermodynamic equilibrium between the reversibly deactivated enzyme $e_R$ and the active enzyme $e_N$ is assumed to be of infinite speed. Thus, it is modeled as an algebraic equation defining the temperature dependent equilibrium constant of reversible enzyme deactivation $K_R$:

\[
K_R = \frac{[e_R]}{[e_N]}
\]

(5)

The deactivation model is completed by the conservation equation:
For the special case of an isotherm ($T=\text{const}$) fed batch with substrate control ($[S]=\text{const}$) process, the eq. 2 and 4 can be solved analytically, to calculate the product concentration for a certain process period $t$:

$$[P] = k_e \cdot t + [e_N](0) \cdot \frac{k_i}{k_D K_R} \left(1 - e^{-\frac{h_0 K_R}{1 + K_R}}\right)$$

Moreover, the important process characteristics, such as the turn over number ($\text{TN}$) or the total turn over number ($\text{TTN}$, as turn over number as $t \to \infty$) can be calculated:

$$\text{TN} = \frac{[P]}{[e_N](0)} = \frac{k_i}{k_D K_R} \left(1 - e^{-\frac{h_0 K_R}{1 + K_R}}\right)$$

$$\text{TTN} = \frac{k_i}{k_D K_R}$$

2.4. Proof of concept

An experimental setup called Enzyme Test Bench was engineered to carry out the experiments in accordance with the enzyme characterization scheme presented here [28]. The high throughput equipment provides for the fast and homogeneous temperature control in the microtiter plate, non-invasive online monitoring of the reaction and automated operation mode. Moreover, the defined experimental conditions (e.g. ideal mixing) allow for the scale up from the microtiter plate format on the micro liter scale to the lab format on the liter scale.

Two enzymes were characterized applying the Enzyme Test Bench. The predictions of the Enzyme Test Bench were compared to the laborious long term experiment results in 1L reactors. In Fig. 7 the turn over prediction (lines) for a six days process obtained from short term experiments in the microtiter plate is compared to results of the long term experiments (symbols), for both enzymes as a function of temperature. The temperature dependent turn over number of a six days process exhibits maxima at approx. 37°C and 40°C for the mesophilic enzyme (solid line) and the thermophilic enzyme (short-dashed line), respectively. The predictions are in a good qualitative agreement with the long term experiments. With respect to turn over number, both, the choice of the optimal enzyme at a given temperature and the choice of the optimal process temperature for a given enzyme are possible. To generate the long term data (symbols) in Fig. 7 2200 h were needed applying three 1L stirred tank reactors. In contrast, the generation of the prediction data (lines) by the enzyme test bench took 120 h including modelling time. Furthermore, additional information like product, substrate and pH-influence was obtained from the enzyme test bench without additional effort. However, more time could be saved by the new technique if higher enzyme numbers would be tested simultaneously using the microtiter plate to full capacity. Interestingly, the mesophilic enzyme exhibited higher long term stability at moderate temperatures than the thermophilic enzyme. Although the thermostability is not identical with long term stability [35], the enzyme long term stability is frequently estimated by measuring the residual activity after exposing the enzyme to elevated temperatures. This method is based on the assumption, that an enzyme of higher stability at an elevated temperature also exhibits higher stability at a moderate temperature. The data in Fig. 7 definitely disprove this assumption. In fact, exposing both enzymes to a temperature of 50°C for an hour would lead to an almost complete deactivation of the mesophilic enzyme, whereas the thermophilic enzyme would still exhibit activity.
Nevertheless, the mesophilic enzyme exhibits higher turn over number at moderate temperature < 42°C than the thermophilic one.

![Graph showing turn over number prediction of a six days process. Predictions from short term experiments in microtiter plates (lines) are compared with results of long term experiments in 1L stirred tank reactor (symbols).]

Quantitatively, the prediction systematically overestimates the turn over number. This can be explained by an additional, almost temperature independent deactivation mechanism. Such deactivation mechanism might be, for example, the specific power input [10], [31], the shear stress [32], [33] or the specific gas-liquid interfacial area [8], [36]. Being temperature independent, the respective deactivation mechanisms cannot be intensified by temperature increase. Thus, their impact cannot be observed in short term experiments. The adjustment of the results predicted in short term experiments in microtiter plates to the pilot and production scale can be performed by introducing one additional temperature independent deactivation parameter.

2.5. Numeric methods of experiment design and analysis

Fig. 8 gives an overview over the problems and the methods of experimental design and analysis within enzyme characterization. Fig. 8b illustrates schematically the measured observable (reaction rate, error bars) for a preset course of control (temperature, dashed line). The qualitative course of the reaction rate conforms to the frequently observed enzyme behaviour. At low temperatures in the first trimester of the experiment, the temperature increase leads to the enzyme activation. At the elevated constant temperature in the second third of the experiment a slow dynamic enzyme deactivation is observed. The temperature increase at the end of the experiment results in a fast enzyme deactivation. This behaviour is fitted by the parameter estimation procedure (solid line). Hereby the problem of minimization of least square between the weighted observables and model responses is solved [3], [19], eq. 9). The standard deviations of the error model are the weights.
\[
\min \frac{1}{2} \sum_{i=1}^{M} \left( \frac{\eta_i - h_i(t_i, y(t_i), z(t_i), p, q)}{\sigma_i} \right)^2
\]  

(9)

Obviously, different experiment designs, represented by different courses of controls lead to different quality of parameter estimation. This is shown in a simplified example in Fig. 8a. Estimating the parameters of the Arrhenius law by an experiment of constant temperature (Fig. 8a1) would result in a good fit but in parameter values, characterized by high uncertainties (grey confidence ellipse in Fig. 8a3). The model is underestimated. In contrast, varying temperature (Fig. 8a2), it is possible to estimate the both parameters with high significance (white confidence ellipse in Fig. 8a3). The mathematical basics of experimental design are described in [19].

Fig 8: Applied and aspired numeric aspects of the model based enzyme characterization. A suboptimal experiment design (a1), an optimal experiment design (a2), the confidence ellipses for both experiment designs (a3). The observable (error bars), the fit curve by the parameter estimation (solid line) for a certain control course (temperature profile, dashed line) (b). Different enzyme folding kinetics, building basis for model discrimination (c). Reasons for robust experimental designs: uncertainties in the parameters (d2), testing of several candidates in parallel (d3); Concept of the adjustment of the temperature by an observable based control loop.

Further numerical methods, not applied at the state of the art, have the potential to increase the performance of the enzyme test bench. In certain cases, different models are able to map the observed experimental behaviour. For instance, in Fig. 8c the reaction kinetics in accordance with the Arrhenius law (dashed line) could be replaced by the cooperative phase transition kinetics (solid line). Both might be suitable to map the experiments. In such cases the tools of model determination would ensure the selection of the superior model. Hunter and Reiner formulated the problem of the experimental design for the model discrimination by: “Choose the experimental points which will most strain the incorrect model to jointly explain the data. For two models the problem results in the selection of controls \( u(t) \) and \( q \) to maximize the difference between the model responses relative to measurement error (eq. 10).
In future, the enzyme characterization could begin with a standard set of experiments to select the most suitable model from a model pool for the particular system. Alternatively, the tools of model reductions could be applied [18], [19]. In this case the characterization would start with an extended model, subsequently reducing it in the course of the enzyme characterization.

The robustness enhancement represents another promising approach to improve the experimental design. The robustness problem is illustrated in Fig. 8d2-3. The quality of the experimental design strongly depends on the actual parameter values. The uncertainties are not taken into account at the state of the art. Thus, at the beginning of the enzyme characterization procedure, as the parameter uncertainties are high (Fig. 8d2), suboptimal experiments are frequently designed. Comparable problems are faced, if different enzyme candidates are tested simultaneously in the same microtiter plate (Fig. 8d3). In this case the optimal experiment design for one candidate is not required, but a compromise applicable for every candidate. Later, when the most promising candidates are selected, tailor made tests for these candidates are necessary. The mathematical basics for the robust experimental design concerning the parameter uncertainties are presented in [20]. Another kind of robust experimental design would be represented by so called “controlled” experimental design (Fig. 8d1). It should be possible to adjust the course of the control state (temperature, dashed line) using the predicted course of the observable (solid line) as the set value and the measured course (error bars) as the actual value for the control loop. The mathematical basis for this strategy must yet be developed.

3. Summary and outlook
A new model based technique for high throughput enzyme characterization is presented. The technique was applied to characterize two enzymes with regard to temperature dependent long term stability. The characterization procedure, consisting of experimental designs, experiments and parameter estimations is explained. The results of the characterization, carried out in microtiter plates in a time scale of hours, are in good agreement with the results of long term experiments at different temperatures in 1L stirred tank reactors over a week. The new technique allows for both: enzyme screening with regard to long term stability and the choice of the optimal process temperature with respect to such process parameters as turn over number or space-time yield.

The performance of the technique could be enhanced by improving the applied numerical methods. In fact, the costly, manually performed parts of the modeling work should be replaced by the robust numerical experimental design, model discrimination and model reduction methods. By these means, the automation level and throughput could be increased and higher pyramiding level of experimental and modeling parts reached. For the proposed strategy of the online controls’ adjustment of controls by observables, mathematical techniques must be developed. Generally, the new concepts can be easily tested in-silico, defining a model with a set of “right” parameters. Simulating the observable and noising the results by a defined error model, in-silico experimental data are obtained. Performing parameter estimation, experimental design etc. the ability of new concepts to identify the defined model and parameters can be proven. In developing new concepts, in-silico experiments exhibit the advantage of cost saving by avoiding laborious experiments. Moreover, the quality of the results can be easily examined, since the “right” model and set of parameters are known. However, as the concepts are developed, they should be applied within enzyme screening.
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