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Application of Design-of-Experiments Procedures to Optimize Efficiently Pretreatment of Lipase for Use in a Nonaqueous Reaction

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Abstract

A variety of different pretreatments can improve the performance of enzymes in nonpolar reaction media. These pretreatments have primarily been studied in isolation; however, interactions between some pairs of pretreatments are known to exist. The presence of these interactions complicates the design of an optimum multifactor pretreatment. Modern design-of-experiments techniques allow the simultaneous optimization of two or more variables. To improve the performance of lipase in a model reaction, we used a technique called the method of steepest ascent to optimize three variables simultaneously: pretreatment pH and sodium phosphate concentration, and the concentration of acetic acid (one of the reactants) in the reaction mixture. In only 26 experimental runs, this optimization process determined a combination of variable settings that yielded a reaction product approx 180 times faster than achieved with untreated enzyme. Evidence is presented to demonstrate that locating this optimum with single-factor experiments would be inefficient. This article demonstrates the efficiency of the method of steepest ascent particularly for evaluation of enzymatic reaction conditions exhibiting significant interactions.

Index Entries: Nonaqueous enzymology; lipase; pretreatment; optimization; lyophilization; esterification; fragrance.

Introduction

Nonpolar media can be superior, in many respects, to traditional aqueous media for the operation of enzyme-catalyzed reactions *(1–3)*. Enzymes,

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however, typically retain only a small portion of their activity in nonpolar media. This low activity is a primary obstacle to industrial adoption of nonaqueous enzymatic processes.

Several factors prevent enzymes in nonpolar environments from achieving their full catalytic potential *(4,5)*. A factor that is often overlooked is that a nonpolar reaction medium does not buffer the reaction mixture and enzyme against a change in pH, as a traditional buffered aqueous reaction medium would. Consequently, in reactions involving short-chain organic acids as substrates, enzymes in nonpolar environments may be further inhibited by the substrate itself *(6–8)*. It is believed that water-soluble substrates partition themselves into the microaqueous phase surrounding the enzyme, resulting in a very acidic local environment for the enzyme *(9)*. Although many different types of reactions catalyzed by enzymes in nonpolar environments have been reported in the literature, reactions with short-chain organic acids as substrates have consistently reacted slowly and given low product yields, unless the concentration of organic acid was kept very low *(7,8,10)*.

The catalytic performance of enzymes in nonpolar environments can be greatly improved by applying a pretreatment to the enzyme before its introduction to the reaction mixture *(4,11–16)*. Pretreatment involves dissolving commercial enzyme in aqueous solutions or emulsions containing various additives, including buffers, salts, acid, base, substrate analogs, or nonpolar materials. The enzyme solution is then frozen and lyophilized to remove the water and volatile additives *(3)*.

Typically, each type of pretreatment variable has been studied and optimized individually *(17)*. For example, Persson et al. *(4)* found that the catalytic rate of a lipase could be improved up to 46-fold by pretreatment with the optimum concentration of potassium chloride. Additionally, Yang et al. *(18)* found that the catalytic rate of subtilisin could be improved 100-fold by optimizing the pH of the pretreatment solution.

Studying the effects of different pretreatments individually neglects possible interactions between pretreatments. For example, in our previous work *(19)*, lipase pretreated without any buffer salts performed optimally when the pH of the pretreatment solution was adjusted to 11.0; lipase pretreated with 80% (w/w) sodium phosphate had a broad pH optimum, from 8.5–11.0; and lipase pretreated with 90% (w/w) sodium phosphate had an optimum at pH 8.5 and was almost completely inactive when pretreated at pH 11.0. In this case, the utility of optimizing either pH or [NaPhos] in isolation would be very limited.

These types of pretreatment interactions complicate the problem of pretreatment optimization; optimization of each pretreatment variable in isolation can fail to locate the global optimum. Modern design-of-experiments techniques can achieve simultaneous optimization of several variables, using a minimum number of experimental runs.

The present work uses the lipase-catalyzed esterification between citronellol and acetic acid as the model reaction. We chose to vary simultaneously pretreatment pH, pretreatment sodium phosphate concentration ([NaPhos]), and acetic acid concentration ([acetic]) in the reaction mixture. From our previous work *(19)*, as well as unpublished results, these variables are known to display interacting effects.

The results of the present study demonstrate how simultaneous optimization using the method of steepest ascent can quickly determine a combination of pretreatment and reaction condition settings that yield high reaction performance. Challenges encountered and approaches for utilizing this method for nonaqueous enzymology are discussed.

Materials and Methods

Our methods are described in detail elsewhere *(19)*. We provide herein a brief review of these methods, as well as modifications intended to scale down the experiments and conserve lipase.

Chemicals

DL-Citronellol (95%), citronellyl acetate (CA) (83.6%), dodecane (>99%), glacial acetic acid (ACS reagent), *n*-hexane (>99%), sodium phosphate, and lipase from *Mucor miehei* (minimum of 4000 U/mg) were purchased from Sigma (St. Louis, MO).

Pretreatment of Lipase

Each aqueous pretreatment solution was prepared by first adding sodium phosphate salts to 18 mL of water. Water-insoluble citronellol (5 µL) was added to the solution and homogenized using vigorous stirring and an ultrasonic bath. The pH of the solution was adjusted to the specified pH (±0.03) by adding small aliquots of 0.1–1.0 *M* NaOH or HCl. Lipase (17.8 mg) was gently dissolved in the solution and the pH was checked and readjusted if necessary. The solutions were frozen in a bath of liquid nitrogen and lyophilized for 20 h on a 4.5-L freeze dryer (Labconco, Kansas City, MO). Ninety percent of the dry material (containing 16 mg of enzyme) was recovered from the flask, compacted with a metal spatula, weighed, and transferred to a reaction vial. To control the absorption of atmospheric water, the compaction and transfer were performed rapidly inside a plastic tent that was purged continuously with dry air, so that the environment inside the tent had a relative humidity of 16–18%. To prevent degradation, the enzyme preparation was tightly sealed and stored in a freezer until use. Differences in the procedure that we used here, compared with the procedure used in our previous work, may have resulted in pretreated lipase with different levels of hydration.

Reaction

The reaction was a lipase-catalyzed esterification between citronellol and acetic acid in hexane to produce CA. The stock reaction mixture (0.250 *M* DL-citronellol, 0.200 *M* dodecane in *n*-hexane) was stored over a mixture of anhydrous sodium acetate and sodium acetate trihydrate (5 g of each salt). Immediately before use, acetic acid was added to the reaction mixture.

Reactions were carried out in 5.5-mL glass vials fit with gas-tight sampling valves. Reaction mixture (2.5 mL) was added to the vial containing the pretreated enzyme. The vial was placed in a 37°C incubator and shaken at 325 rpm using a rotary shaker, to suspend the enzyme. Samples of 1–1.5 µL were periodically removed for analysis.

Gas Chromatography Analysis

The progress of the reaction was monitored by gas chromatography analysis using dodecane as the internal standard. The measure of enzyme performance was based on the rate of generation of the desired product, CA. The concentration of CA was measured just as the reaction was started, and at three additional time points during the course of the reaction.

The data were used to calculate relative performance (RP), a measure of catalytic activity. RP was defined as follows:

$$\mathrm{RP} = \left(\frac{\text{Rate of CA production with pretreated lipase} \div \text{Pretreated lipase concentration}}{\text{Rate of CA production with untreated lipase} \div \text{Untreated lipase concentration}} \right) \quad (1)$$

The interpretation of RP is straightforward: lipase with an RP of 8 catalyzes a reaction eight times faster than untreated lipase (i.e., lipase used as received from a commercial supplier without any pretreatment).

Optimization Procedure

A response surface method known as the method of steepest ascent *(20)* was used to find efficiently a high-performance combination of variable settings. The starting point for this process was a set of conditions that had produced some of the highest RP values in our previous work, specifically, pH 8.5, 80% sodium phosphate, 5 µL of citronellol, 20 h of lyophilization, and 0.125 *M* acetic acid in reaction mixture *(19)*. For each of the three variable conditions, settings both higher and lower than the starting point were selected. Using these settings, a 2^3 full factorial design with four additional center points was designed and the experiments were performed. The results were analyzed, and if it was found that a first-order regression model could appropriately fit the data, the direction in which the response surface increased most rapidly (the gradient vector) was determined. Single experimental runs were performed at a number of dif-

ferent distances along this direction, outside the factor space of the initial factorial experiment. Typically, with this method, the response will increase for a short distance along the gradient, reach a peak, and then drop. The process was then repeated by constructing a new factorial experiment around the performance peak, determining whether a first-order model fit, and so on. Cycles of this procedure continued until one of the factorial experiments produced results that were not well described by a first-order model. At this point, the final factorial design was augmented with additional axial and center points, to allow the estimation of second-order effects, and a more complex model was developed. The approximate location of the optimum could be derived from this model.

Results and Discussion

The initial factorial experiment was designed to investigate the region of "factor-space" surrounding the starting point (Fig. 1). Analysis of this experiment showed all three main effects, [NaPhos], [acetic], and pH, as well as the interactions pH × [acetic] and [NaPhos] × [acetic], to be significant at $\alpha = 0.05$. The greatest RP resulted from a pretreatment with the highest [NaPhos] (90% [w/w]), the highest pH (pH 9.0), and the lowest [acetic] (0.075 *M*). The effect of increasing [acetic] was a surprisingly large decrease in RP; all conditions that included the highest [acetic] (0.175 *M*) resulted in an RP ≤0.52. This created a problem for the method of steepest ascent. This method requires one to fit a first-order model to the data from the factorial experiment and find the direction of most rapid increase (the gradient vector) according to this model. One then follows this vector through factor-space, conducting a number of experiments with conditions specified by points along the vector. First, the effect of decreasing [acetic] was so great that following the gradient any significant distance would require experiments with zero or negative [acetic]. Second, very low [acetic] is undesirable, from a practical perspective, because acetic acid is one of the reactants and, thus, is readily exhausted. Dilute systems have either very high volumes or very low yields. Our approach to solving this problem was to set [acetic] at the lower value of 0.075 *M*, from this point on, continuing the optimization with only [NaPhos] and pH as variable factors.

Using the regression coefficients from the factorial analysis, we constructed the following first-order model ([NaPhos] units are % [w/w]):

$$RP = -117.9 + 0.41[NaPhos] + 23.1(pH) \quad (2)$$

The gradient of this equation is

$$\nabla RP = 0.41\mathbf{i} + 23.1\mathbf{j} \quad (3)$$

in which **i** is a unit vector in the positive [NaPhos] direction, and **j** is a unit vector in the positive pH direction.

An early version of the given regression model, produced immediately after the first round of optimization, contained an error and was a

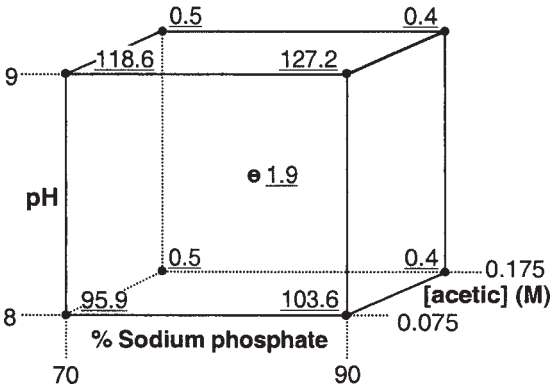

Fig. 1. Experimental design and measured relative performance (underlined numbers) from first step of optimization. ɵ represents the center point of the factorial design.

poor fit of the data. Consequently, the gradient vector calculated from this initial regression equation was also in error. Although this procedural error was initially thought to have derailed the optimization process, the use of the off-sequence operating conditions served the same purpose, and the resulting process and the ultimate outcome of the process were unaffected.

We used the conditions in the initial factorial experiment that gave the highest RP (90% sodium phosphate, pH 9.0, 0.075 *M* acetic) as the starting point for the path. The equation of a line parallel to the gradient and passing through this point is as follows:

$$\frac{[\text{NaPhos}] - 90}{19.9} = \frac{\text{pH} - 9}{2.895} \text{ or } \text{pH} = 0.1454[\text{NaPhos}] - 4.093 \qquad (4)$$

Using Eq. 4, conditions were selected for experiments along the gradient vector (*see* Table 1). Figure 2 presents the results of these experiments. In the bottom plane of Fig. 2, the box represents the area of the original factorial experiment; the dark area above this is the fitted response surface. The arrow represents the direction of steepest ascent in the pH/[NaPhos] plane. As expected, RP increases for a short distance along the gradient, after which it falls.

At this point, a central composite response surface experiment was constructed around the point that gave the highest response. This design can produce second-order models that describe a saddle- or dome-shaped surface, which is often the case when the factor area includes an optimum. Figure 3 presents a schematic of the design and the results.

Analysis of the results from the central composite did not reveal any significant effects. Figure 4 presents a visualization of the data. The surface was produced by a data-smoothing method, known as the Loess method *(21)*. It is presented here not as a predictive model, but simply as a way to interpret the data graphically. When examining the complex shape of this

Table 1
Reaction Conditions
Along Gradient Vector

[NaPhos] (% [w/w])	pH
91	9.15
93	9.44
95	9.73
97	10.02

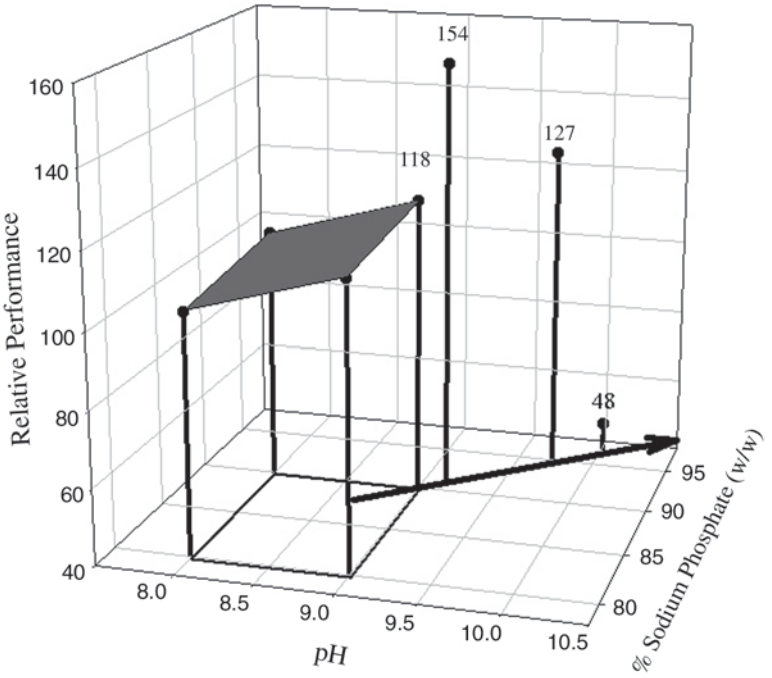

Fig. 2. Response surface from first optimization round and data points along direction of steepest ascent.

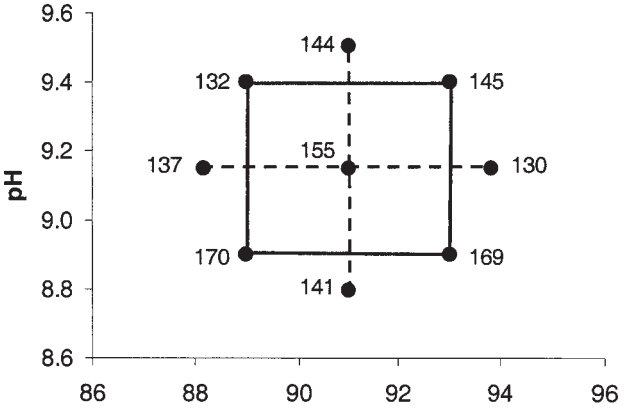

Fig. 3. Response surface design and measured relative performance.

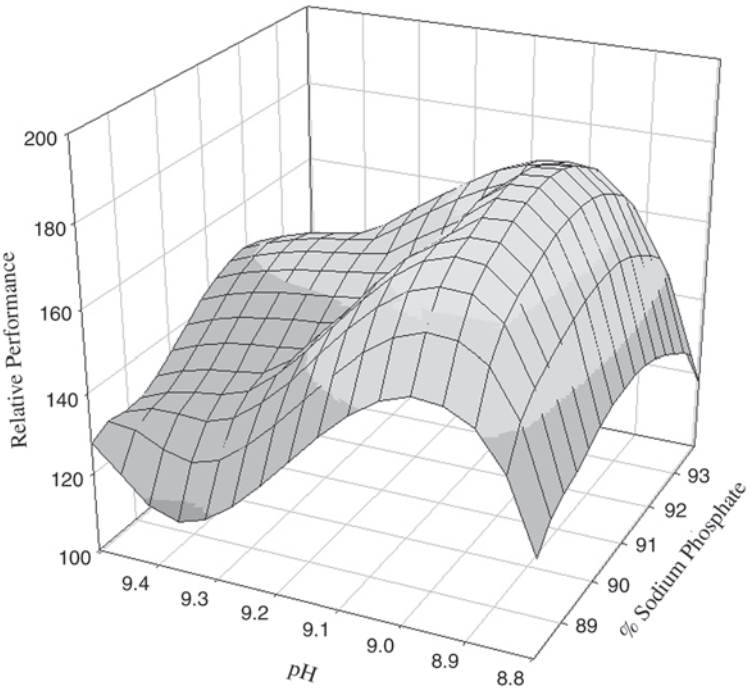

Fig. 4. Response surface generated by Loess data smoothing using a third-degree polynomial.

surface, it is not surprising that a first or second-order polynomial function could not fit the surface.

The hill shape in Fig. 4 suggests that this region is at least a local if not the global optimum for these two factors and, thus, was selected as the end point of the optimization. Based on the response surface, the optimum is located near 91% sodium phosphate, pH 8.95 and may produce an RP > 180. If further optimization were desired, one could build another experiment around the top of the hill with higher resolution (smaller factor spacing) or higher statistical power (more replications). However, by this method, further increases in RP are likely to be small. Alternately, one could use a point at the top of the hill as a starting point for optimizing other factors, such as duration of lyophilization or concentration of an active-site protectant.

Conclusion

We consider the described optimization process highly successful. The entire process required only 26 experimental runs, which were conducted over three pretreatment and three reaction days. The process led us to a combination of factors that produced performance 88 times more rapid than our initial combination. This approximate optimum probably would not be located by conducting one-factor experiments, and combining the

optimum level of each factor. If the only data available were from the single-factor experiments on [NaPhos] and pH from our previous work *(19)*, one would likely select 70% sodium phosphate at pH 8.5–11.0 as the optimum. According to the results of the present work, this combination is likely far from optimum. Additionally, the one-factor experiments cited required 29 experimental runs, making them less efficient in terms of time and resources used. The process also proved itself to be robust to errors in the determination of the direction of steepest ascent.

Our experiences, however, also highlight some potential pitfalls of such iterative, designed experiments. The experimenter is committed to his or her initial experimental protocol, from beginning to end of the designed experiment, even if it becomes apparent that protocol changes are desirable. In the present work, the pretreatment protocol from our previous work *(19)* was modified with the intention of conserving enzyme. This modification had the unintended consequence of depressing RP for any given set of pretreatment conditions. This effect became apparent only when the designed experiment was well under way. Additionally, with the method of steepest ascent, there is always the possibility that the method will lead the experimenter to a local optimum, rather than the global optimum.

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