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Multichannel Simultaneous Determination of Activities of Lactate
Dehydrogenase

by

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CHAPTER ONE

INTRODUCTION

Capillary electrophoresis

Electrophoresis as a very powerful separation technique¹ has been well known and applied in the separation of charged species based on differential migration in an applied potential field. One of the earliest demonstrations of the advantages of using small-diameter silk fibers (internal diameter about 15 μ m) for determination of 100pg of RNA contained within a single cell was provided by Virtanen.² He pointed out that the use of small-diameter fibers allowed concentration of the analyte into a small volume for improved detection. In early 1980's, Jorgenson and his colleagues first used a fused capillary to separate charged species.^{3,4} After about 20 years of development, capillary electrophoresis (CE) has become one of the most powerful separation methods for small samples. Capillary electrophoresis offers several exciting features: 1) attaining highly efficient and extremely fast separations of both ionic and neutral compounds; 2) requiring very small sample for one analysis ($\sim$ nl); 3) using the capillary tube acting as a microreactor to carry out microreactions, after which microscale separation and quantification are executed.

A wide spectrum of samples has been analyzed by CE. Not only can the small ions be separated and analyzed by CE coupled with different detection schemes, but also neutral molecules and the large biologically interesting molecules can be analyzed with different modes of capillary electrophoresis.

Even though researchers have developed many Capillary electrophoresis techniques, the instrument generally consists of three parts. A capillary is required for the separation, a high-voltage power supply is required to drive the separation, and a detector is required to determine the presence and amount of analyte (Figure 1).

Figure 1. Typical Capillary Electrophoresis setup

The separation mechanism is dependent on the electroosmotic flow and the migration of ions in the electric field.⁵ The total velocity of each molecule is the vector sum of the electrophoretic velocity (v_{ION}) and the velocity imparted by electroosmotic flow (v_{EOF}):

$$v_{total} = v_{EOF} + v_{ION}$$

It is important to realize that these are vector sums, in that they are directional (aligned with or opposed to the electric field). This can be used to great advantage in CE, because all molecules that have a net velocity toward the detector will eventually be observed. Thus, the presence of electroosmotic flow allows the separation of both negatively and positively charged species in the same run.

Depending on the analytes, several modes of CE techniques can be selected to perform quick, efficient analyses. As categorized by Patrick Camilleri⁶, the most common modes are 1) open tubular or capillary zone electrophoresis (CZE)⁴; 2) micellar

electrokinetic capillary chromatography (MECC);^{7,8} 3) capillary gel electrophoresis; 4) capillary isoelectric focusing, and 5) capillary isotachopheresis.^{9,10} To date, CZE has been the most popular CE mode. Since each zone of analytes migrate at a different speed, both cations and anions can be determined in the same run with CE. Molecules and ions of identical charge to mass ratio, such as DNA and proteins, have very similar electrophoretic mobilities in free buffer solution. Capillary gel electrophoresis (CGE) can provide the capability of resolving these analytes due to a different separation mechanism.^{11,12} The gel filled capillary can obstruct different sizes of analytes to different extents, or the entangled gel molecules provide a sieving effect, in which different sizes of molecules penetrate the gel medium at different speeds and are separated. The high separation efficiency and the high throughput in DNA separation by CGE, coupled with various sensitive detecting methods, have increased the pace of potentially sequencing the human genome.

Terabe and his coworkers^{7,8} developed micellar electrokinetic chromatography (MEKC) by adding modifiers (e.g. SDS) into the CE running buffer for separating neutral compounds, as well as some ions and ion pairs, which are difficult to separate with CE. The buffer modifiers, usually surfactants, form micelles when their concentrations are higher than the critical micellar, the analytes are partitioned between background electrolyte and micelles. Thereby, the separation is achieved based upon partitioning differences between analytes. The process is in fact chromatographic and not electrophoretic, even though the electroosmotic flow still plays a very important role in transporting the solution through the capillary. People also call it micellar electrokinetic chromatography (MEKC).

Capillary electrochromatography, or capillary liquid chromatography¹³⁻¹⁵ does not belong to capillary electrophoresis with the concern of the separation mechanism, but there are still some similarities between CEC and CE, since both of them use an electric field to drive the liquid carrying the analytes through the capillary tube. In CEC, capillaries are packed with a conventional HPLC stationary phase (even though the particles may be much smaller due the small capillary inside diameter). The analytes are

separated based upon their different partition ratios between the electrolyte mobile phase, and the packed stationary phase. With this technique, neutral, ion and ion pair analytes can be separated. In addition, the electric field can be used to achieve additional selectivity and efficiency.

Optical detection in CE

The often nano-liter range of analytes available in the capillary electrophoresis requires highly sensitive and fast response detectors, which do not disturb the electric field for running CE. Usually absorbance detection and fluorescence detection are used.

UV-Vis Detection

Among the advantages associated with UV-Vis absorbance detection for CE is its nearly universal nature and easy coupling for on-capillary detection. With multiwavelength UV-Vis absorbance detection available, additional chemical selectivity can be obtained. Many other techniques have been developed and applied to overcome the low sensitivity of UV-Vis absorbance measurements for CE analysis. According to Beer's law, the optical absorbance of a sample, the signal associated to a certain concentration of sample, is directly proportional to the optical pathlength through which the absorbance is measured. So, increasing the pathlength becomes the most popular way to improve the sensitivity of UV-Vis absorbance detection. Chervet et al.¹⁶ designed and manufactured Z-cells by bending the capillary to extend the optical pathlength from 75 μ m to 3mm. An alternative method to increase the optical pathlength was to use a bubble-shaped cell for measurement. This kind of detection cell is commercially available with 3-time expansion¹⁷ and was extensively studied by Xue, Y. and Yeung, E.S.¹⁸. Another alternative to gain a longer optical pathlength^{19, 20} was to perform electrophoresis in "flattened" channels (square and rectangular capillaries).

The double beam optical approach has been the most popular detection scheme in commercial instruments to compensate for source intensity fluctuations. A second detection channel of no analyte serves to monitor the instantaneous variations in the light source, and then can be used to normalize the measurements in the sample channel. Based on this idea, a double-beam laser absorption was used to achieve lower LOD by reducing the noise level.²¹⁻²³ With the double-laser beam absorbance system, a LOD of 2×10^{-8} M malachite green was achieved.

Based on the above facts, we prefer to use UV absorbance as our detection method. Especially, deep-UV (200-220nm) detection of organic and biologically important compounds, like the reactants (NADH) and products (NAD⁺) of this experiment, makes this method the best choice.

Enzyme Assay

The study of enzymes has always been very important for modern clinical chemists and biochemists. Usually, determining the activity of an enzyme is more important than determining its amount. The determination of enzyme activities can help people diagnose diseases and direct the invention of new pharmaceutical products. Hence, enzymes are commonly quantified by their biological activity under selected conditions. Many successful methods like *immunoassay* have been developed for this purpose. If the sample amount of enzyme is small, capillary electrophoresis will exhibit its competitive advantages. But all of those methods, including the single-capillary electrophoresis method discussed by Xue and Yeung²⁹, can only process one sample at a time. If scientists want to do control experiments, they will have to do them separately. Due to the variation of experimental conditions and the relatively high sensitivity of this type of experiment to the environment, the differences of these two experiments will cause errors when the control experiment is used to correct the original experiment results. Also the whole procedure will be time-consuming. Exactly the same conditions for these two separate experiments are impossible to make, but if the control experiments

and original experiments can be processed at the same time and in environments that are as close as possible, the results will be more meaningful and persuasive. That is the original purpose of our experiment.

Lactate dehydrogenase (LDH) has been found to be a very valuable enzyme in diagnosing different diseases, such as liver disease, myocardial infarction, etc²⁴. Both LDH activity and the LDH isoenzyme patterns in serum are potential biomarkers for different cancers. Furthermore, an investigation by Bottomley et al. indicated the possibility of using LDH activity to classify leukemia.²⁵ With such importance of LDH, quick measurement of LDH activity becomes very necessary. Usually, a reaction by substrate, NADH and pyruvate, is used to determine the activity of LDH. This method was also used in our experiments to reach our goal.

Regnier and coworkers who utilized capillary electrophoresis to perform an enzyme assay in 1993 proved that capillary electrophoresis could be a powerful tool to analyze enzyme activity²⁶⁻²⁸. Determination of activity of LDH has been studied in quite detail. Xue, Q.F. and Yeung, E. S. have demonstrated that UV absorption capillary electrophoresis can be used for LDH enzyme assay²⁹, even though a slightly different mechanism was used in this experiment.

Recently, 96-capillary electrophoresis has become a very powerful tool for analyzing many samples in a very short time. One high-throughput 96-capillary array electrophoresis system using UV absorption detection which is able to do everything commercial single-capillary electrophoresis machines can do only with higher throughput was invented by Edward S. Yeung and Xiaoyi Gong at Iowa State University³⁰. With this instrument, 96 different conditions can be easily individually applied to 96 capillaries to do the enzyme assay. The detection on all capillaries is done with the same light source at the same time. So the drift of the light source will not affect the comparison. The detection windows of these capillaries are packed side by side, so the detection conditions are as similar as possible. Such control experiments were done to find out what the best condition for LDH activity is, the result would be more reliable.

In this experiment, the different conditions focused on the pH values of reaction buffers and the concentration of LDH.

With this detection method, similar experiments can also be done to determine the best conditions for the activities of other enzymes. The wide application of this method can be expected in the near future.

CHAPER TWO

EXPERIMENTAL

Instrumentation

A total of 96 fused-silica capillaries (50- μm i.d., 150- μm o.d.; Polymicro Technologies, Phoenix, AZ) packed side by side with 50-cm effective length and 70-cm total length were used for separating reactants, products and enzymes. A uniform window region was created after packing by using an excimer laser beam to burn off the polyamide coating. At the ground (exit) end of the capillary array, the capillaries were bundled together to allow simultaneously buffer filling and rinsing. At the injection end, the capillary array was spread out and mounted on a copper plate to form an 8 $\times$ 12 format with dimensions that fitted into a 96-well preloaded plate for sample introduction. In addition, 96 gold-coated pins (Mill-Max Mfg. Corp.) were located next to the capillary tips to serve as individual electrodes. Even though CCD has a lot of advantages, but it has small electron well capacity (0.3 million electrons), its Limit of Detection is limited by the high shot noise in absorption detection. Also, the overwhelming amount of data per exposure and the presence of mechanical slits to restrict the light paths make it an unsuitable detector for this experiment. Instead, photodiode arrays (PDA) are much better choices than CCD. PDA have been used in HPLC and CE systems for providing absorption spectra of the analytes in real time. Different elements in the array were used to image different axial locations in one capillary tube to follow the progress of the separation. PDA's large electron well capacity (tens of million electrons) makes it superior to CCD as an absorption detector. 0.2 ml enzyme-clean 96-well preloaded plates were used as reactors for carrying out enzyme reactions. Other optic items are used to optimize the detection conditions. A 254nm mercury lamp was used for UV

absorption detection. A voltage of +11 kV (~157V/cm) was applied across the capillary for separation and for driving the reactant and product zones through capillaries.

Reagents

Sodium pyruvate (99+%) was obtained from Sigma (St. Louis, MO, USA). β -Nicotinamide Adenine dinucleotide, reduced form (β -NADH) was also obtained from Sigma. Both solutions were freshly prepared and kept in refrigerator before experiment. NADH solution was covered by black tape to prevent exposure to light. L-lactate dehydrogenase (LDH-5(M_4) 98+% isoenzyme, suspension in 2.1 M $(\text{NH}_4)_2\text{SO}_4$) was also from Sigma. Bacteria-free 0.2ml 96-well preloaded plates were from Marsh Biomedical Products, Inc, (Rochester, NY, USA). Sodium Phosphate monobasic ($\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$) was from Fisher (Fair Lawn, NJ, USA). All water used in this experiment was purified by Millipore water purification system and heated to 200°C for 30 minutes to make sure that there is no enzyme contamination. During the period of incubation, the plates were covered by plastic film to prevent the vaporization of water in reaction solution to make sure that the concentration of enzyme and substrate were as stable as possible, but it could be found that the vaporization still cause some problems. This is also good to avoid the contamination from the air.

CHAPTER THREE

SEPARATION AND DETECTION

As mentioned in Chapter one, UV-Vis absorbance is the best detection method. A PDA was used as the detector for the UV-Vis absorbance detection.

The reaction was chosen as follows:

Because of the inhibition of pyruvate for the catalysis of LDH, an optimal value of concentration of pyruvate is important. At pH 7.2 and 25°C, 2 mM pyruvate is normally suitable for the M₄ isoenzyme. So in reaction buffers (20 mM phosphate, pH ranges from 5.8 ~ 8.0)³¹, 2.0 mM NADH and ~ 2.0 mM pyruvate were added as the substrates for the enzymatic reaction.

At first, the reaction was allowed to progress for a prescribed amount of time. Then hydrodynamic injection was used to introduce reactants, products, and enzymes into the 96 capillaries. A solution of 10 mM phosphate with a pH of 8.0 was used as the separation buffer. The reactants were easily separated from enzymes due to their significantly different mobilities. Without the catalysis of enzymes, the reaction stopped. At the same time, products were also separated from reactants due to their different mobilities.

Since we used a low concentration of enzyme ($5 \times 10^{-10} \sim 1 \times 10^{-8}$ mM), the amount of LDH is much less than the amount of substrates available for the catalyzed

reaction thus we can consider this reaction to be pseudo-first-order. For these conditions, the amount of NAD^+ formed during a given period of time at a fixed temperature is linearly proportional to the LDH concentration, more precisely to LDH activity. Therefore, the LDH activity can be quantified by measuring the amount of NAD^+ formed during the fixed incubation period.

In this work, we integrated the peak areas of NAD^+ peaks and NADH peaks. Because the injection method was hydrodynamic injection, we had to consider the effects caused by different migration times on the peak areas. By dividing the peak areas by the corresponding migration times, more accurate peak areas were calculated and used to determine the percentage of NADH reacted. Because NAD^+ and NADH both strongly absorb 254 nm UV light while LDH isoenzymes do not contribute much to the background at this wavelength, 254 nm mercury lamp was used. By measuring the absorption coefficients of NAD^+ and NADH at 254 nm, we converted the areas of NAD^+ peaks to the corresponding areas of NADH peaks. From the ratio between the converted NADH peak areas and the integrated NADH peak areas, percentage of NADH reacted could be calculated. Since background reaction exists, we did control reactions and deducted the effect of background reaction.

CHAPTER FOUR

SAMPLE PREPARATION AND INJECTION

Sample Preparation

20mM phosphate buffers with pH values of 5.8, 6.3, 6.5, 6.7, 7.0, 7.3, 7.6, and 8.0 were prepared separately. 175 μ l buffer solution, 10 μ l enzyme solution, 10 μ l 40mM NADH and 5 μ l 90mM pyruvate were added to the 96 reaction wells to make the reaction solutions. The final concentrations of enzyme in those solutions were 5×10^{-10} M, 2×10^{-9} M, 3×10^{-9} M, 4×10^{-9} M, 5×10^{-9} M, 6×10^{-9} M, 7×10^{-9} M, 8×10^{-9} M, and 1×10^{-8} M, respectively. For every concentration of enzyme, eight different buffer solutions with pH of 5.8, 6.3, 6.5, 6.7, 7.0, 7.3, 7.6, and 8.0 were used as the reaction buffer. At the same time, for every reaction buffer solution with different pH values, 9 different concentrations of enzyme were studied. Reaction was completed at room temperature. We incubated the reaction solution for 30 minutes, 128 minutes, 180 minutes, 480 minutes, and 1477minutes.

Sample Injection

After each incubation, we hydrodynamically injected a portion of the reaction solution into 96 capillaries at the same time. The reason for using hydrodynamic injection instead of using electrokinetic injection is that electrokinetic injection would have caused more errors. These significant errors are due to different conditions from capillary to capillary and different migration velocities of NADH and NAD^+ . Two sets of injection experiments were done to prove that electrokinetic injection would cause more errors than hydrodynamic injection. Solutions were prepared with same concentrations of NAD^+ and NADH. Then solutions were injected separately with hydrodynamic injection and electrokinetic injection. By integrating the peak areas of NAD^+ and NADH, 9 sets of injected sample amounts were compared. The results were shown in the following figures (Figure 2-3):

Figure 2. Electrokinetic injection results

Figure 3. Hydrodynamic injection results

The solution with 1mM NADH and 1mM NAD⁺ was prepared and injected separately by hydrodynamic injection and electrokinetic injection into 9 different capillaries. A +11kV voltage was applied for 30 seconds to inject. For the hydrodynamic injection, a 5cm difference in height was kept for 60 seconds. Then after applying +11kV voltage to the capillaries, the NADH and NAD⁺ sample zones were driven through the detection windows. Since all the solutions had the same NADH and NAD⁺ concentrations, the similar peak areas were expected. By comparing the results shown above, the hydrodynamic injection only caused very small relative standard deviation (RSD) of 0.027 with the mean being 0.817. The electrokinetic injection caused serious errors and variations in the amounts of NADH and NAD⁺ injected. The RSD of electrokinetic injection was 0.483 with the mean of 3.66. It could be explained as below:

$$\text{Amount of NAD (injected)} \propto (\mu_{\text{eof}} + \mu_{\text{epNAD}}) \times V \times \text{Time of injection};^{32}$$

Note: μ_{eo} stands for the electroosmotic flow, μ_{ep} stands for the electrophoretic mobility of NAD⁺.

$$\text{Amount of NADH (injected)} \propto (\mu_{\text{eof}} + \mu_{\text{epNADH}}) \times V \times \text{Time of injection}$$

Note: μ_{epNADH} stands for the electrophoretic mobility of NADH.

Since the charges on NAD and NADH vary with the changes of pH values, μ_{epNADH} and μ_{epNAD} vary with the change of the pH values of buffer. For different capillaries, μ_{eo} is different. So we calculate the ratio between the injected amount of NADH and the injected amount of NAD using following equation:

$$\begin{aligned} \text{Ratio} &= (\text{Amount of NADH}) / (\text{Amount of NAD}^+) \\ &= (\mu_{\text{eof}} + \mu_{\text{epNADH}}) / (\mu_{\text{eof}} + \mu_{\text{epNAD}}) \end{aligned}$$

The results would have been affected by the conditions in different capillaries a lot. The injected amounts were not stable, even when the same voltages were applied for the same injection times.

During normal experiments, hydrodynamic injections were used. The injection ends of capillaries were kept 7 cm higher than the other end for 60 seconds.

CHAPTER FIVE

RESULTS AND DISCUSSIONS

The separation times varied from capillary to capillary, because the conditions of those capillaries were different. Some of them are so different from others that we could tell directly from the electropherograms. Even though the migration times were different, the sequence of NADH and NAD⁺ peaks was the same.

Since NAD⁺ takes more positive charges than NADH, we expected to see NAD⁺ peaks first, and then NADH peaks. After a 30-minute incubation, the portion of NADH converted to NAD⁺ was calculated using the following equations:

Amount of *NADH (reacted)* (in peak area) = [(NADH absorption coefficient at 254nm) × (NAD⁺ peak area)] / (NAD⁺ absorption coefficient at 254nm)

$$\text{Percentage of } NADH \text{ (reacted)} = \left(\frac{\text{Amount of } NADH \text{ (reacted)}}{\text{Migration time of } NADH} \right) / \left[\left(\frac{\text{Amount of } NADH \text{ (reacted)}}{\text{Migration time of } NADH} \right) + \left(\frac{\text{Amount of } NAD \text{ (remained)}}{\text{Migration time of } NAD} \right) \right]$$

As we stated before, we could use the percentage of *NADH (reacted)* to represent the activity of enzyme since this reaction is a pseudo-first-order under the conditions in this experiment.

The electrophoregrams in the previous pages showed us how the activities of LDH isoenzymes were affected by pH and the concentrations of LDH. With the concentration of LDH increasing, the peak areas of NADH (reactant) decreased compared with the peak areas of NAD⁺ (product). It means that the conversion rate of

Figure 4. Electrophoregram of 128-minute incubation of reaction solutions with different LDH concentrations in pH 6.3 buffer

Figure 5. Electrophoregram of 128-minute incubation of reaction buffers with 5×10^{-10} M LDH and different pH

NADH increased with the concentrations of LDH. Before the pH increased to 7.0, the peak areas of NADH decreased compared with the peak areas of NAD^+ . After pH reached 7.0, with pH increasing the peak areas of NADH increased compared with the peak areas of NAD^+ . The tendency was correct qualitatively.

To further prove the tendency quantitatively, we graphed the peak areas with the pH values of reaction solution and the concentrations of LDH.

The percentages of *NADH (reacted)* were graphed with pH values for the same concentration of LDH (Figure 6-9). The relationship between the activity of enzyme and the pH values of reaction buffer was pictured. From the graph following, it was determined that the enzyme activity increased with the increase of pH value of reaction buffer before pH value reached 7.0. After pH value reached 7, the enzyme activity decreased with the increase of pH value. So 7.0 is the best pH value for LDH catalysis, even though the concentration of LDH varied from each other. This agreed with results from the literature. It should be noticed that after being incubated for two hours, the reactions in the buffers with a pH value of 6.5 showed slightly lower reaction rates than expected. At the same time, all the other reaction solutions showed expected reaction rates. This will not affect the demonstration of this experiment within the reasonable range, the overall trend shown by this experiment was correct. Another thing which should be mentioned here is that with increasing time, the difference between the percentage of *NADH (reacted)* of the fastest three reactions became lower. That must be because the substrate, NADH, was consumed seriously. Most of NADH was converted to NAD^+ , so the reaction had been close to the equilibrium. The percentage of *NADH (reacted)* was also graphed with the concentrations of enzyme. The vertical axis represented the percentage of *NADH (reacted)*. Horizontal axis was used to stand for the concentrations of enzyme. The linear relationship between enzyme concentration and the amount of *NADH (reacted)* was obtained when the reaction time is not too long. When the reaction time is short enough, it can be assumed true that

Figure 6. 30 minute incubation results

Figure 7.128 minute incubation results

Figure 8. 180 minutes incubation results

Figure 9. 24 hours incubation results

$v_0 = \left(\frac{d[P]}{dt}\right)_{t \rightarrow 0} = \left(\frac{d[NAD]}{dt}\right)_{t \rightarrow 0} = -\left(\frac{d[NADH]}{dt}\right)_{t \rightarrow 0} \propto [\text{enzyme}]$, if the Δt is fixed, the change of the concentration of NADH should be proportional to the concentration of LDH.

Within the reasonable error, our results also agreed with this assumption. The following is the result calculated based on the 30-minute incubation (Figure 10):

Figure 10. Percentage of NADH reacted in reaction solutions with pH 6.5

The percentage of *NADH (reacted)* is proportional to the concentration of LDH when the reaction time is short enough. But after one time threshold, the linear relationship is not obeyed. Figure 11 is the result based on the 24-hour incubation. It is obvious that the relationship between percentage of *NADH (reacted)* and the

concentration of LDH is not linear any more. It is because that the assumption of pseudo first-order reaction is not right after the threshold time.

The percentage of NADH (reacted) and the reaction time were graphed. Linear relationship between the time and the change of concentration of substrate, NADH, in

Figure 11. Results of 24 hour incubation of reaction solutions with pH of 6.5

the middle part of the graph was found out. From the progress curve for the components of a simple Michaelis-Menten reaction³¹, we know that this also agreed with the results from mathematical calculation based on the M-M equation. The increase of percentage of *NADH (reacted)* was proportional to the reaction time at the first beginning since the concentration of substrate is significantly higher than the concentration of enzymes. The linear relationship was shown as the following graph (Figure 12).

The result from the graph could be explained as following. From Adrian Brown's theory, the reaction of enzyme catalysis is composed of two elementary reactions in

Figure 12. Results of 5×10^{-10} M LDH incubated for certain time.

which the substrates forms a complex with the enzyme that subsequently decomposed to products and enzymes³²

The overall rate of production of [ES] is as following:

$$\frac{d[ES]}{dt} = k_1 [E][S] - k_{-1}[ES] - k_2[ES]$$

After we apply a steady state assumption, with enough substrate, the enzymes will be saturated. The combination between enzyme and substrate reached equilibrium, the concentration of ES remains approximately constant until the substrate is nearly exhausted. That is why the increase of the percentage of *NADH (reacted)* was proportional to reaction time after reaction began. In this experiment, the time is not so long that the linear relationship is no longer observed. But eventually with time going

on, most of the NADH was converted to NAD^+ . There was not enough substrate in reaction solution, and the enzyme couldn't be saturated any more. Consequently the steady state assumption was not applicable.

Conclusion

It is very important to find the best conditions for some enzymes to do the best catalysis in current pharmaceutical industries. Based on the results above, we could say that this set-up could be widely used in finding the optimal condition for best enzyme activity of a certain enzyme. Instead of looking for the best condition for enzyme activity by doing many similar reactions repeatedly, we can complete this assignment with just one run if we could apply enough conditions.

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