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ARTICLE

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# The use of isothermal titration calorimetry for the assay of enzyme activity: Application in higher education practical classes

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**Abstract**

Determination of enzyme activity is crucial for discovery, research, and development in life sciences. The activity of enzymes is routinely determined using spectrophotometric assays that measure rates of substrate consumption or product formation. Though colorimetric-based detection systems are simple, rapid, and economical to perform, the majority of enzymes are unsuitable for this technique as their substrates/products do not absorb in the UV or visible range. This limitation can be addressed by the use of coupled-enzyme assays or artificial chromogenic substrates; however these approaches have their own drawbacks. Here, we describe a method based on the use of an isothermal titration calorimeter (ITC) to measure the heat produced or absorbed during any enzyme-catalyzed reaction. The concept of calorimetric enzyme assays was demonstrated for the determination of enzyme hexokinase activity, which cannot be monitored colorimetrically without first coupling it to another enzymatic reaction. The assay is suitable for incorporation into undergraduate laboratory classes, providing students with an appreciation for; the versatility and ease of use of ITC assays; ITC as a flexible generic method for exploring the functional characteristics of uncharacterized enzymes; an activity detection parameter suitable for enzymes that either have no straightforward colorimetric methods available or require the use of nonartificial chromogenic substrates.

**KEYWORDS**

calorimetry, biotechnology laboratory class experiment, enzyme activity assay, Michaelis–Menten kinetics

## 1 | INTRODUCTION

Enzymes are generally assayed for activity by using spectroscopic techniques, such as monitoring changes in light absorbance following exposure to substrates and/or products, as these are consumed or formed respectively during an enzyme-catalyzed reaction. However, most enzyme-catalyzed reactions cannot be followed

colorimetrically as neither the substrates nor products absorb in the UV–Vis range. Furthermore, spectroscopy is a highly nonspecific approach, since many compounds absorb in the 220–300 nm UV range, and there is a risk that a nonspecific sample component may artefactually contribute to the absorbance. To overcome these limitations, coupled-enzyme assays have routinely been employed in which a noncolorimetric reaction is combined with another detectable enzyme-catalyzed colorimetric reaction.[1,2] Sometimes more than two coupling enzymes are needed to get an absorption signal. Some disadvantages of coupled assays include; (1) the nonavailability and high cost of purified commercial enzymes and reagents, and (2) difficulty in optimizing reaction conditions (coupling enzyme concentrations, pH, co-factors, and temperature) with minimal lag phase so that only the enzyme reaction to be monitored is rate-limiting.[1,2] Other problems for coupled assays can arise from enzyme inhibition caused by high substrate concentrations and product formation as well as variable enzyme stabilities.

Some substrates have been altered to become chromogenic or fluorogenic by linking them to small molecules or dyes.[3] The disadvantage of chromogenic substrates is that these artificial substrates do not reveal the real values of Michaelis–Menten parameters ($k_{cat}$, $K_m$, $K_i$) as compared to natural substrates. In addition, enzyme assays based on viscosity, electrochemical signal (pH, oxygen etc.), photometry (fluorescence, turbidimetry), chromatography, radioactivity, and gel-electrophoresis have also been described.[4] However, these noncolorimetric methods have their own drawbacks that include working with radiation, lower sensitivity, expensive reagents, requirement for large amounts of enzyme and being tedious and lengthy in their operation.

Calorimetry is a relatively simple to use alternative detection method for exploring interactions and binding characteristics of both small molecules and biopolymers. Heat is a universal signal generated during both protein-ligand binding and enzyme-catalyzed reactions. This heat can be detected directly by isothermal titration calorimetry (ITC) in turbid solutions, suspensions, as well as multiple phase systems, without requiring chemical labeling.[5,6] Currently available semi- and fully automated ITC equipment (Supplementary Figure S1) is highly sensitive, with the ability to detect change in heat as low as 0.1 μcal $sec^{-1}$ and requires relatively low reaction volumes (280 μl), which limits the cost of reagents and enzymes.[7] Using any substrate (natural or artificial), calorimetric determination of Michaelis–Menten parameters ($k_{cat}$ and $K_m$) of an enzyme can be completed within 10 min, whereas typical spectrophotometric/fluorometric procedures may take hours and involve the precise preparation of several substrate solutions.[8] Furthermore, various mechanisms in enzyme kinetics (such as Michaelis–Menten vs. Hill, monophasic vs. biphasic reactions), which involve product inhibition[9] and allosteric substrate activation,[10] can also be readily explored using calorimetry.

ITC is a popular research tool for binding studies, but its value for enzyme assays has not caught the attention of teachers, and few undergraduate courses (particularly biotechnology and biomedical) include calorimetry techniques in either their theoretical or laboratory practical components. Within this paper a simple calorimetric determination of enzyme kinetics parameters is described, using a bisubstrate enzyme, hexokinase (HK) as an example.[11] The calorimetric kinetic parameters for both glucose and ATP were determined and compared with previously published kinetic parameters.[8,12] Furthermore, the utility of the experiment for pedagogy was demonstrated, by using it as a practical example in the Biochemistry and Molecular Biology Lab course for undergraduate students, in the School of Biotechnology and Biomolecular Sciences (BABS) at The University of New South Wales (UNSW), Sydney, Australia.

## 1.1 | Theory of colorimetric versus calorimetric enzyme assay

The activity of HK is routinely determined by coupling it to glucose-6-phosphate dehydrogenase (G6PDH) and measuring the formation of NADH at 340 nm in a continuous spectrophotometric (colorimetric) assay.

$$\begin{aligned}&\mathrm{Hexose(e.g.,glucose)+ATP}\\&\rightarrow\mathrm{hexose\,6-phosphate+ADP(HK)}\end{aligned}$$

$$\begin{aligned}&\mathrm{Glucose\,6-phosphate+NAD^{+}\rightarrow 6-phospho}\\&\mathrm{-glucono-1,5-lactone+NADH(G6PDH)}\end{aligned}\qquad(1)$$

Hexokinase assays can also be performed in the reverse direction, in which ATP can be measured by coupling to luciferase and glucose can be detected by coupling to oxidase/peroxidase.[13]

By contrast to colorimetric assays, ITC measures the heat change (calorimetric) resulting from an enzyme-catalyzed chemical reaction at a constant temperature (Supplementary Figure S1).

$$\begin{aligned}&\mathrm{Rate(v)\ of\ reaction\ determined\ by\ a\ calorimetric\ assay}\\&=(\Delta Q/\mathrm{t})\times(1/\Delta H^{*}\,\mathrm{V})\end{aligned}\qquad(2)$$

where $\Delta Q/t$ represents the change in heat per unit time; $\Delta H$ represents the total heat absorbed/released when the substrate is mixed with the enzyme in the ITC and $V$ is the total volume of reaction mixture.

$$\text{The rate}(\text{v},\text{U}/\text{ml}) \text{ of reaction determined by a colorimetric assay} = (\Delta A/t) \times (1/\varepsilon) \times V \quad (3)$$

where $\Delta A/t$ represents the change in absorbance per unit time; and $\varepsilon$ is the extinction coefficient of NADH (light absorbed by 1 mM solution of NADH). Note that the equations for determining the rate of reaction using calorimetric (Equation 2) or colorimetric (Equation 3) assays are similar except that $\Delta A/t$ and $\varepsilon$ in the photometric assay replaces $\Delta Q$/t and $\Delta H$ in the calorimetric assay.

The rate at which substrate concentrations $[S]_t$ change is determined using Equation 4, from which Michaelis–Menten parameters ($V_{max}$ and $K_m$) are determined.[8]

$$\text{Rate}\,(v) = \left(V_{max}.[S]_t\right)/\left(K_m + [S]_t\right) \quad (4)$$

The turnover number per unit time ($k_{cat}$) is determined from Equation 4 using the known enzyme concentration [E].

$$k_{cat} = V_{max}/[E] \quad (5)$$

## 2 | MATERIALS AND METHODS

Purified yeast HK was purchased from Merck (Australia). All other reagents and buffers were purchased from Sigma-Aldrich/Merck and were of ACS/reagent grade. The water used in all experiments described here was highly purified with carbon and ion exchange cartridges, to a minimum resistivity of 18mΩ, using a MilliQ water purification system (Merck-Millipore, Sydney, Australia). A MicroCal microcalorimeter ($ITC_{200}$) was used for the calorimetry experiments described here, (GE Healthcare, Australia). Raw ITC data was analyzed using ITC specific Origin Software, for the determination of enzyme kinetics parameters. TeamViewer was downloaded from https://www.teamviewer.com/en/ to allow remote access of the live experiment from the ITC site to the teaching Lab.

The experiment was performed in single injection mode (SIM) at 25°C with stirring at 800 rpm; initial delay 60 s; injection rate 18 µl in 90 s for all assays; spacing between two assays, 500 s; filter period 5 s; cell volume, 204 µl and syringe volume, 40 µl. HK assays were performed by either varying glucose or ATP concentrations as substrates. For determining Michaelis–Menten parameters for glucose, the ITC cell contained HK and a saturating concentration of ATP solution (Table 1a), whereas the titration syringe contained glucose solution (Table 1b). To determine the Michaelis–Menten parameters for ATP, the ITC sample cell contained HK and a saturating concentration of glucose solution (Table 2a) whereas the syringe contained the ATP solution (Table 2b). Other than the enzyme in the ITC cell and the substrate in the syringe, both cell and syringe solutions contained identical buffer, additives, and co-factors, so as to avoid superfluous heat due to dilution of non-identical diluents (known as heat of dilution, HOD). The first injection via syringe into the cell was followed by a second identical injection to determine any product inhibition due to the accumulation of glucose-6-phosphate and ADP. The HOD was determined by replacing the HK solution with water. The HOD was subtracted from the experimental heat data. Protocol examples and ITC operations are shown in the Supplementary material and additional experimental details are given in Supplementary Figures S2 and S3. Some tips for troubleshooting common problems are given in Supplementary Table S1.

### 2.1 | Data analysis

Once the titration was completed, the data were analyzed using Origin (included with the Microcal ITC). The analysis

**TABLE 1a** Cell compartment solution containing hexokinase and saturating concentration of ATP for determining Michaelis–Menten parameters for glucose.

| Solutions | Stock concentration | Final concentration | Volume needed/400 µl |
|---|---|---|---|
| HEPES/NaOH, pH 8 | 1 M | 50 mM | 20 µl |
| $MgCl_2$ | 600 mM | 24 mM | 16 µl |
| KCl | 1 M | 100 mM | 40 µl |
| ATP | 0.014 g/0.5 ml = 50 mM | 10 mM | 80 µl |
| [a]TCEP, pH 8 | 500 mM | 1.25 mM | 1 µl |
| Hexokinase (yeast, 54 kDa) | 1 µg/µl diluted 4× to 0.25 µg/ml | 1.25 ng/µl (23.3 nM) (0.0000233 mM) | 2 µl |
| Milli-Q water | - | - | 241 µl |
| Total volume | - | - | 400 µl |

[a]Prepared fresh just before use as it is very unstable in aqueous solution.

**TABLE 1b** Syringe compartment solution containing glucose for injection.

| Solutions | Stock concentration | Final concentration | Volume needed/100 µl |
|---|---|---|---|
| HEPES/NaOH, pH 8 | 1 M | 50 mM | 5 µl |
| KCl | 1 M | 100 mM | 10 µl |
| Glucose | 200 mM | 10 mM | 5 µl |
| Milli-Q water | - | - | 80 µl |
| Total volume | - | - | 100 µl |

**TABLE 2a** Cell compartment solution containing hexokinase and saturating concentration of glucose for determining Michaelis–Menten parameters for ATP.

| Solutions | (stock solution) | (working solution) | Volume needed/400 µl |
|---|---|---|---|
| HEPES/NaOH, pH 8 buffer | 1 M | 50 mM | 20 µl |
| $MgCl_2$ | 600 mM | 24 mM | 16 µl |
| KCl | 1 M | 100 mM | 40 µl |
| Glucose | 200 mM | 10 mM | 20 µl |
| [a]TCEP, pH 8 | 500 mM | 1.25 mM | 1 µl |
| Hexokinase (yeast, 54 kDa) | 1 µg/µl diluted 4x to 0.25 µg/ml | 1.25 ng/µl (23.3 nM) (0.0000233 mM) | 2 µl |
| Milli-Q water | - | - | 301 µl |
| Total volume | - | - | 400 µl |

[a]Prepared fresh just before use as it is very unstable in aqueous solution.

**TABLE 2b** Syringe compartment solution containing ATP for injection.

| Solutions | Stock concentration | Final concentration | Volume needed/100 µl |
|---|---|---|---|
| HEPES/NaOH, pH 8 buffer | 1 M | 50 mM | 5 µl |
| KCl | 1 M | 100 mM | 10 µl |
| $MgCl_2$ | 600 mM | 24 mM | 4 µl |
| ATP | 50 mM | 10 mM | 20 µl |
| Milli-Q water | - | - | 61 µl |
| Total volume | - | - | 100 µl |

is fully automated and essentially consisted of following steps.

1. Subtraction of HOD reference from the experimental data (Supplementary Figure S4a). The area under the curve is the apparent heat of reaction ($\Delta H$).
2. Conversion of data to [S] versus rate and truncation of data within [S] = 0 and $V_{max}$ (Supplementary Figure S4b).
3. Fitting of experimental data to a model. In the present case, the data were fitted to Michaelis–Menten Equation for both glucose and ATP as substrates (Figure 1b,c,e,f). In cases where the data does not conform to Michaelis–Menten kinetics, and a different model, such as the Hills Equation, is deemed more applicable, the [S] versus rate data can be exported to the Enzyme Kinetics Module 1.1 of Sigma-Plot 8.02 Software[9] or any other enzyme kinetics software. Examples of different stages of data analysis are shown in supplementary materials (Supplementary Figure S4).

## 3 | RESULTS AND DISCUSSION

A broader appreciation of the utility of calorimetry in the biotechnology and biochemistry fields, will provide a more complete and contemporary education in analytical techniques to tertiary students. Furthermore, the relative simplicity of calorimetry makes it highly adaptable for use in bioscience practical class pedagogy. The approach used in this study demonstrated the application of

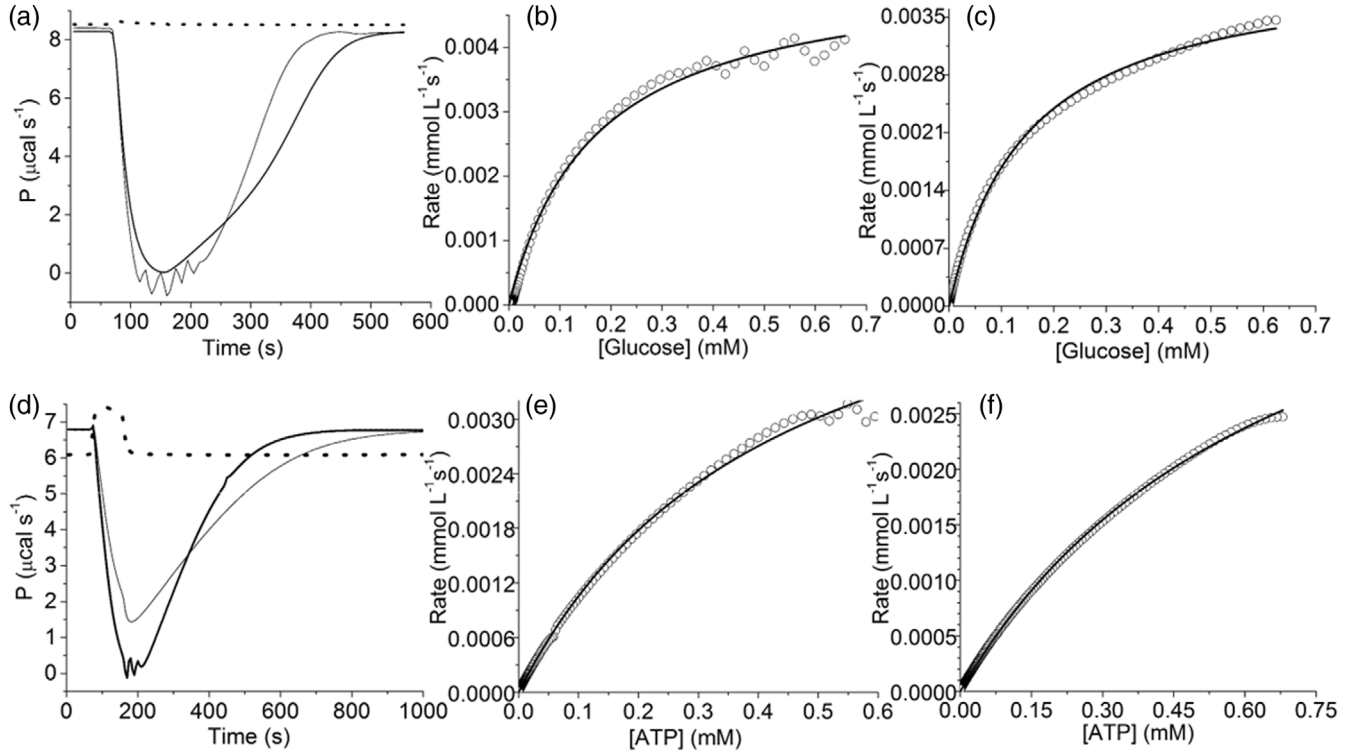


**FIGURE 1** Determination of kinetic parameters in pH 8 buffer at 25°C using isothermal titration calorimeter (ITC). Plots of hexokinase activity using glucose (a–c) and ATP (d–f) as substrates. (a) and (d), Raw ITC data showing exothermic heat flow (thermal power) as a function of time of heat-of-dilution (HOD) (dotted line), and two successive substrate injections (thin solid curve, 1st injection; thick solid curve, 2nd injection). A second identical injection was repeated to check for product inhibition. (b,c,e,f), kinetic plots of experimental data (circle) and line of best-fit to Michaelis–Menten equation (solid line). (b) and (e), Michaelis–Menten plots for 1st injections; (c) and (f), Michaelis–Menten plots for the 2nd injections. The upper dotted lines (a) and (d) are the HOD (in the absence of hexokinase) which was subtracted from the experimental curve and subsequently converted to rate (Supplementary Figures S3 and S4)

calorimetry for assaying enzymes and determination of Michaelis–Menten parameters ($V_{max}/k_{cat}$, $K_m$) and its suitability for inclusion in the laboratory components of undergraduate "biochemistry-based" courses. The ITC method was demonstrated using HK but can be readily adapted for determining the activity of other enzymes, such as carbamate kinase. The Michaelis–Menten kinetic values determined by calorimetry for both glucose (Figure 1a–c; Table 3a) and ATP (Figure 1d–f; Table 3b) as substrates were compared with previously published values (Table 3). Examples of experimental setup, data acquisition, and handout for students are shown in supplementary materials (Supplementary Tables S2 and S3).

## 3.1 | Single-injection mode is simpler than multiple-injection mode (MIM)

SIM was employed to determine the kinetic values of HK, although MIM can also be used.[8] SIM has the advantage that the apparent heat of reaction, $\Delta H$ (area under the curve in Supplementary Figure S4a) and kinetic parameters are determined in the same experiment whereas for MIM, a separate experiment is needed to determine $\Delta H$, requiring more time and additional reagents and enzyme.

## 3.2 | HK kinetic values are consistent with published values

For glucose as a substrate, the $K_m$ values were 0.14 ± 0.02 and 0.17 ± 0.007 mM, which are comparable with previously published values (0.072–0.12 mM) that were determined using a variety of methods such as colorimetry, mass-spectrometry and calorimetry (Table 3a). Similarly, the $k_{cat}$ values were 294 ± 13 and 246 ± 4 $sec^{-1}$, which again compare well with the published values determined using calorimetric (270 $sec^{-1}$) and colorimetric (450 $sec^{-1}$) methods (Table 3a).

For ATP as substrate, the $K_m$ values were also comparable with published values (Table 3b). Although, $k_{cat}$ values were not available for ATP, comparable $V_{max}$ values have been reported for both glucose and ATP

**TABLE 3a** Kinetic parameters of hexokinase at 25°C, pH 8 with glucose as substrate.

| Parameter | Experiment 1 | | Experiment 2 | | Literature | |
|---|---|---|---|---|---|---|
| | 1st injection | 2nd injection | 1st injection | 2nd injection | 12,14 | 8 |
| $K_m$ (mM) | 0.14 ± 0.02 | 0.14 ± 0.01 | 0.17 ± 0.007 | 0.15 ± 0.003 | 0.11 ± 0.04 (colorimetry)<br>0.12–0.13 (pH 7.2, 22°C) MS | 0.072 (calorimetry)<br>0.10 (colorimetry) |
| $k_{cat}$ ($sec^{-1}$) | 294 ± 13 | 257 ± 8 | 246 ± 4 | 197 ± 1.4 | $V_{max}$ with Glucose and ATP as substrates is similar | 270 (calorimetry)<br>450 (colorimetry) |
| $\Delta H$ (kcal) | −9.9 kcal | −10 | −10.1 | −10.2 | na | ng |

Abbreviations: NA, not applicable; NG, not given; MS, mass-spectrometry based assay.

**TABLE 3b** Kinetic parameters of yeast hexokinase at 25°C, pH 8 with ATP as substrate.

| Parameter | Experiment 1 | | Literature | |
|---|---|---|---|---|
| | 1st injection | 2nd injection | 12 | 14 |
| $K_m$ (mM) | 0.43 ± 0.01 | 0.70 ± 0.007 | 0.36 ± 0.1 | 0.1 mM (pH 7.2, 22°C) MS |
| $k_{cat}$ ($sec^{-1}$) | 264 ± 3.5 | 240 ± 1.6 | ATP = Glucose | |
| $\Delta H$ (kcal) | −10.6 | −10.8 | na | |

Abbreviations: NA, not applicable; ND, not determined; NG, not given; MS, mass-spectrometry based assay.

substrates[12] which is consistent with the present findings as the $k_{cat}$ values for both glucose (Table 3a) and ATP (Table 3b) are similar.

## 3.3 | Calorimetry can also determine product inhibition in the same experiment

The kinetic values (Table 3a) were also determined by delivering similar amounts of glucose into the cell (2nd injection) that already contained the reaction products (Equation 1) from the 1st injection, with view to determine any product inhibition. Using glucose as a substrate, no difference was observed in $K_m$ between the 1st and 2nd injections, however, $k_{cat}$ values decreased following the 2nd injection implying noncompetitive product inhibition.[14] In the case of ATP, the $K_m$ increased whereas the $k_{cat}$ decreased slightly after the 2nd injection implying competitive product inhibition, which has been previously observed in studies using noncalorimetric assays.[14]

## 3.4 | Demonstration of ITC experiment in teaching lab

Due to the high cost of the ITC equipment, it is likely that research or teaching laboratories will have access to only a single ITC unit. Consequently, some consideration is needed as to the best ways of incorporating use of calorimetry into practical classes. For smaller classes, one option may be to have several staggered experiments, with 3–4 students participating per experiment. However, for larger classes of more than 30 students, or even for smaller classes where time constraints may not allow multiple serial experiments, another approach is an on-line demonstration of the technique, with the equipment controlled remotely. The current study utilized just such an on-line approach; the experiment was set up by loading appropriate solutions into the cell and titration syringe (Tables 1 and 2) of the ITC (located in another building) and remotely controlling the ITC via TeamViewer from the teaching lab computer. The input of ITC running parameters, data acquisition, and data analysis were all performed in real time in the teaching lab, while students were able to continue with other experiments. Importantly, at the end of the experiment, students also had access to (substrate) versus rate (vol) data which could be analyzed manually by conversion to linear forms (Eadie-Hofstee plot, vol versus vol/[S]; Lineweaver-Burk plot, 1/[S] versus 1/vol; Hanes-Wolf, [S] versus [S]/vol) or by using other computer programs. Furthermore, the kinetic values determined by calorimetric analysis can be compared to the kinetic values determined by traditional colorimetric methods by students and presented in their report. Additional material to guide students discussion is provided in supplementary materials, including calorimetric data generated from our experiment that can be used by students for comparison purposes (Appendix).

### 3.5 | Benefits and limitations of calorimetry

The ongoing characterization of novel enzymes has been neglected due to the recent surge in research focus on the accumulation of genomic data. It is estimated that ~50% of the sequences in the databases have uncertain, unknown, or incorrectly annotated functions that require biochemical characterization.[15] The next step following the discovery of unique enzymes, requires that characterization of their activity using natural substrates. The activity assay methodology should be generally applicable, rapid, simple in complexity, low cost and not require synthesis of new chromogenic substrates. Currently, photometric assays fulfill most of these criteria but for characterizing most novel enzyme reactions are limited by the need to include artificial chromogenic substrates that are either not available or are difficult/costly to prepare. Heat being a universal signal can be used to detect almost any enzyme activity using natural substrates via calorimetric analysis. ITC allows for rapid assays where $V_{max}/k_{cat}$, and $K_m$ can be determined in only 600–1000 s (Figure 1a,d).

There are, however, a few limitations of ITC, including high cost of the equipment (US $ 100–150 K per unit) depending on the supplier and periodic maintenance expenditure, due mostly to titration syringe breakage. In rare cases, where the enzyme reaction is symmetric (i.e., the type and number of bonds broken in reactants are identical to bonds formed in products), no net heat is generated. An example of this is the following reaction catalyzed by L-arginine: glycine amidinotransferase which was reported to have failed to generate any heat signal.[16]

$$\text{Arginine} + \text{hydroxylamine} \rightarrow \text{Ornithine} + \text{hydroxyguanidine}(\text{amidinotransferase}).$$

## 4 | CONCLUSIONS AND FUTURE DIRECTIONS

Calorimetry is a versatile technique amenable to the direct analysis of the great majority of enzymes/substrates. Furthermore, as a relatively simple and rapid technique to implement, it is particularly useful for use in practical teaching programs, either in-person or online via remote control of the equipment. A good proportion of the current generation of students graduating in the various bioscience disciplines will be facing the challenge of understanding the activity of the vast and ever-growing huge number of unannotated enzymes. Characterization of the activity of these enzymes will be essential to determining their role in cellular metabolism. Having prior awareness and understanding of calorimetric analysis of enzymes will also be very useful in the future experimental characterization of novel and industrially important enzymes. The use of this technology can potentially lead to unexpected discoveries that promise unlimited benefits in the field of medicine, environment, and industry. It is noteworthy that ITC can also be used to demonstrate macromolecule-ligand binding. This aspect of ITC is used extensively in pharmaceutical industries for drug discovery. Both modes of calorimetry (enzyme kinetics and binding) have recently become very relevant in R&D due to Covid-19 pandemic. The versatility of ITC gives educators an option to enrich their curricula though the provision of understanding and experience in the use of a cutting edge analytical technique that will be relevant to many of their students' prospective employers.

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## CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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