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# A Simple Assay for Measuring Catalase Activity: A Visual Approach

## SUBJECT AREAS:

TECHNIQUES AND  
APPLICATIONS

BIOCHEMICAL ASSAYS

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**In this study, an assay that combines the ease and simplicity of the qualitative approach for measuring catalase activity was developed. The assay reagents comprised only hydrogen peroxide and Triton X-100. The enzyme-generated oxygen bubbles trapped by Triton X-100 were visualized as foam, whose height was estimated. A calibration plot using the defined unit of catalase activity yielded the best linear fit over a range of 20–300 units (U) ( $y = 0.3794x - 2.0909$ ,  $r^2 = 0.993$ ). The assay precision and reproducibility at 100 U were 4.6% and 4.8%, respectively. The applicability of the assay for measuring the catalase activity of various samples was assessed using laboratory strains of *Escherichia coli*, catalase-deficient isogenic mutants, clinically isolated Shiga toxin-producing *E. coli*, and human cells. The assay generated reproducible results. In conclusion, this new assay can be used to measure the catalase activity of bacterial isolates and human cells.**

Catalase is a ubiquitous antioxidant enzyme that degrades hydrogen peroxide into water and oxygen<sup>1</sup>. Several pathogens produce catalase in order to defend themselves against attacks by hydrogen peroxide, a weapon commonly used by the host's immune system, in addition to oxidative stress. A previous report has in fact demonstrated that a catalase-deficient mutant pathogen was more susceptible than its wild-type strain to the oxidative stress induced by hydrogen peroxide and immune cell attacks (which involve hydrogen peroxide)<sup>2</sup>. It is thus useful to measure the catalase activity of pathogens in order to gain a better understanding of the underlying mechanisms of their pathogenicity, including their resistance towards oxidative stress.

*Escherichia coli* has 2 catalase enzymes, hydroperoxidase I (HPI) and HPII, which catalyze the dismutation of hydrogen peroxide to water and oxygen<sup>1</sup>. The *katG* gene product, HPI, is transcriptionally induced during logarithmic growth in response to low concentrations of hydrogen peroxide<sup>3</sup>. However, HPII, encoded by *katE* and positively regulated by *rpoS*, is not peroxide inducible and is a key player in survival during the stationary phase and other stresses<sup>4,5</sup>. Several studies have focused on these 2 catalases, as they play pivotal roles in protecting cells against the effects of oxidative stress<sup>6</sup>. Because HPII activity varies among clinical isolates, it is necessary to distinguish between the activities of HPI and HPII in order to investigate the activity of the RpoS-encoded stress response gene *rpoS*.

To date, several methods have been described for measuring catalase activity in bacteria. One of the simplest qualitative procedures involves determination of the enzyme's presence in the test bacterial isolate by using hydrogen peroxide, which is broken down to bubble-producing O<sub>2</sub> by catalase-positive bacteria<sup>7,8</sup>. On the other hand, quantitative approaches focusing on careful measurements include colorimetric and spectrophotometric assays<sup>9–14</sup>. There are several limitations inherent in these methods, including cumbersome procedures and high cost, despite the availability of convenient kits. Furthermore, during quantitative analyses, although the reaction occurs spontaneously, the enzyme increases the reaction rate considerably. Therefore, while processing a large number of samples, the initial and final reaction rates differ among different samples, thus producing ambiguous results. This necessitates the development of a simple and cost-effective method for measuring catalase activity.

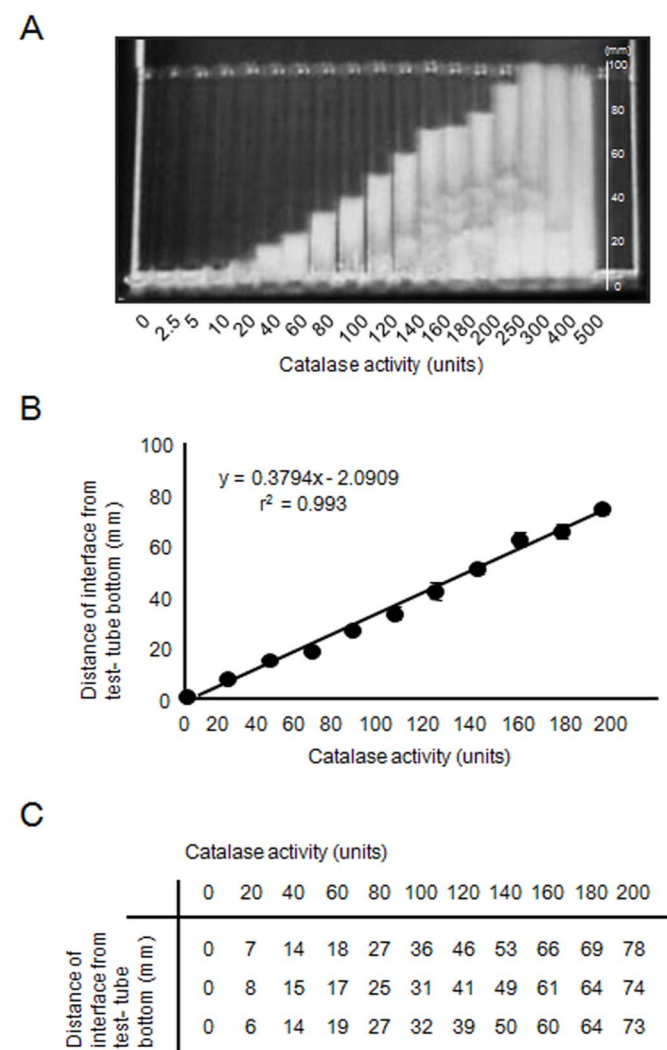
In order to address these challenges, in this study, we developed an assay that combines the ease and simplicity of the qualitative approach for measuring catalase activity. The assay uses simple and readily available reagents, namely hydrogen peroxide, Triton X-100, and catalase. We applied this assay to clinical isolates and laboratory strains of *E. coli* and its derivatives carrying mutations in the catalase genes or in their regulatory factors, and human cells.



The underlying principle of this approach is that the oxygen bubbles generated from the decomposition of hydrogen peroxide by catalase are trapped by the surfactant Triton X-100. The trapped oxygen bubbles are then visualized as foam, the test-tube height of which is measured to quantify the catalase activity.

## Results

In the present study, by employing our novel approach, we measured the catalase activity by quantifying the trapped oxygen gas, which is visualized as foam. The oxygen gas generated by the catalase–hydrogen peroxide reaction in test tubes is shown in Fig. 1A, where the height of foam developed in the tubes was measured. The calibration



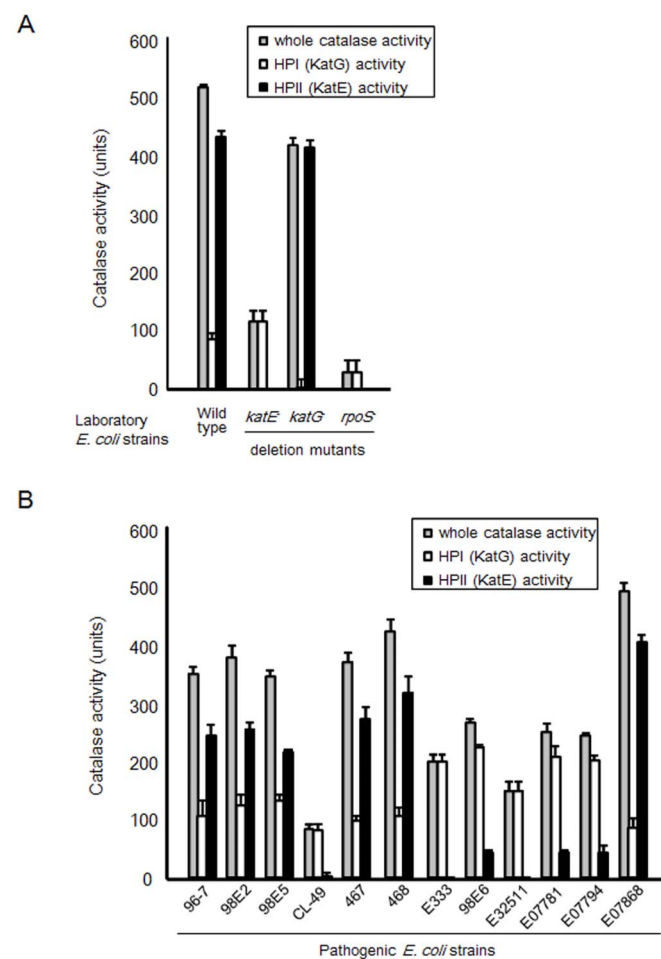
**Figure 1** | Image showing the height of foam developed in each test tube following the reaction of catalase with hydrogen peroxide, and the calibration curve plotted using the defined unit of catalase activity. (A) Image of test tubes showing foam developed as a result of catalase activity. Each solution of concentration of catalase (100- $\mu$ L) was added in a Pyrex tube (13 mm diameter  $\times$  100 mm height, borosilicate glass; Corning, USA). Subsequently, 100  $\mu$ L of 1% Triton X-100 and 100  $\mu$ L of undiluted hydrogen peroxide (30%) were added to the solutions and mixed thoroughly and were then incubated at room temperature. Following completion of the reaction, the height of O<sub>2</sub>-forming foam in the test tube was measured using a ruler. (B) The best linear fit between the foam heights and catalase activity was observed over a range of 20–200 U ( $y = 0.3794x - 2.0909$ ,  $r^2 = 0.993$ ). Mean values are shown ( $n = 3$ ). Error bars represent the standard deviation. (C) The height of foam generated after mixing catalase, Triton X-100, and H<sub>2</sub>O<sub>2</sub>.

curve plotted using the defined unit of catalase activity is shown in Fig. 1B. A linear regression on the pooled data yielded the best linear fit over a range of 20–300 units (U) of catalase activity ( $y = 0.3794x - 2.0909$ ,  $r^2 = 0.993$ ); hence, all the measurements were carried out in that range. The assay precision (intraassay coefficient of variation) and reproducibility (interassay coefficient of variation) at 100 U were determined to be 4.6% and 4.8%, respectively. The assay generated reproducible results (Fig. 1B and 1C).

No reagents were used to stop the reaction, as the generation of oxygen stops naturally within 5 min. After the reaction, the generation of bubbles ceased completely, despite an additional 100  $\mu$ L of hydrogen peroxide being added to all the test tubes. To verify the specificity of the assay for measuring catalase activity, the catalase inhibitor azide was used and, as expected, foam formation was inhibited.

By applying the developed method to *E. coli* strains carrying mutations in the catalase genes or in their regulatory factors, we were able to determine the catalase activity in the *katE*, *katG*, and *rpoS* deletion mutants, as well as in the wild type (Fig. 2A), and to demonstrate the ability of the assay to accurately discriminate between the activities of HPI and HPII. Only HPI or HPII activity was observed in the *katE* and *katG* deletion mutants, respectively, and the *rpoS* deletion mutant showed only HPI activity. In contrast, both HPI and HPII activities were noted in the wild-type strain.

Furthermore, different clinical isolates were analysed for their catalase activity (Fig. 2B). HPI activity was detected in all the isolates



**Figure 2** | Catalase activity of *E. coli* strains, as determined with the newly developed method. Catalase activities of (A) laboratory strains of *E. coli* and (B) clinical isolates. Mean values are shown ( $n = 3$ ). Error bars represent the standard deviation.



tested in the study. However, the activity of HP11 varied among the isolates.

In addition, we applied the developed method to assay the catalase activity of LNCaP and PC-3 cells, which are human prostate cancer cell lines commonly used in the field of oncology. A linear regression on the pooled data yielded the best linear fit over a range of  $2.5 \times 10^6$  to  $1 \times 10^7$  LNCaP and PC-3 cells (Fig. 3A and B). The catalase activity of LNCaP cells was higher than that of PC-3 cells (Fig. 3A and B).

## Discussion

Using the method, the limit of detection for the assay was higher than that of other previous methods<sup>9–14</sup>, but the linearity, intraassay precision, and interassay precision were similar. In terms of handling, the method was simpler than other quantitative methods.

In this study, the catalase activity of various samples was measured by using the new method. When the assay was applied to *E. coli* strains carrying mutations in the catalase genes or in their regulatory factors, the expected results were obtained. When the assay was also applied to various clinical isolates of Shiga toxin-producing *E. coli*, the activity of HP11 (which is encoded by *katE* and is positively regulated by *rpoS*) was found to vary among the isolates. These findings are consistent with previous reports<sup>7,15–18</sup>. In addition, the developed method could measure the catalase activity in human cells, where the activity in LNCaP was higher than that in PC-3. This result

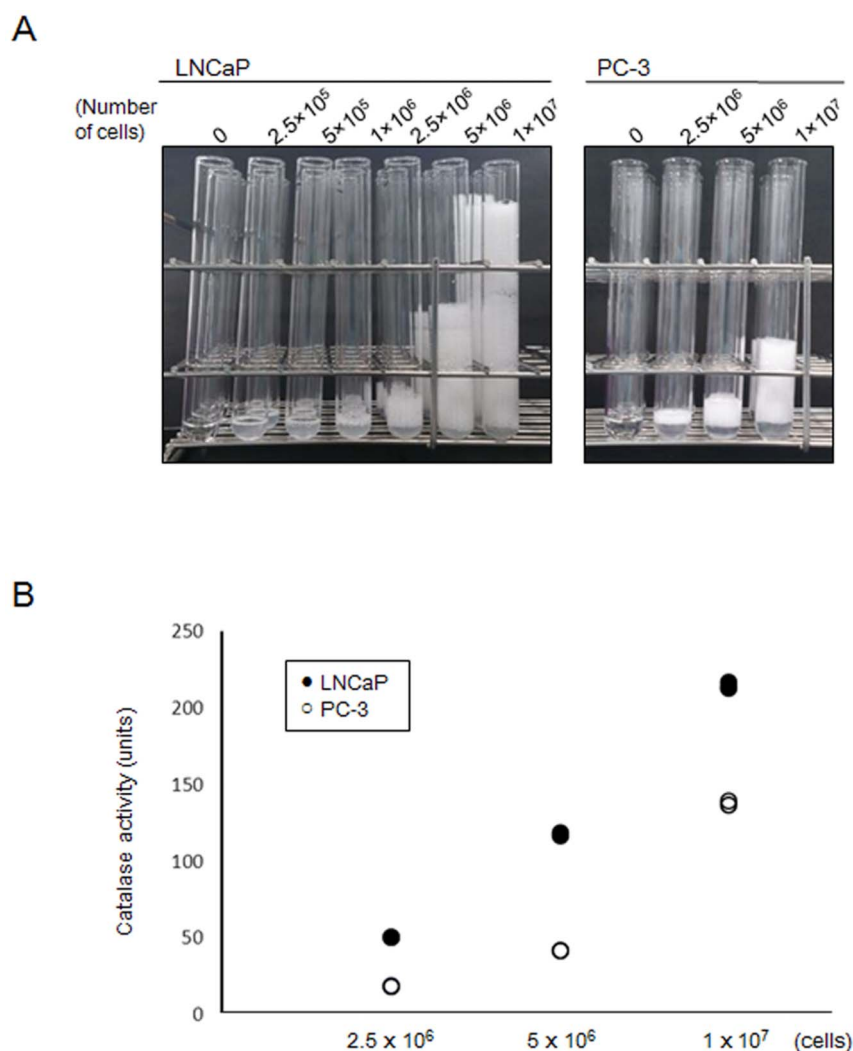
is also in accord with a past report<sup>19</sup>. Our method will be useful for easy measurement of catalase activity in various samples.

With regard to the technical aspects, the choice of surfactant was a key point for measuring the catalase activity in this assay. We tested 3 common surfactants, namely Triton X-100, Tween-20, and sodium dodecyl sulphate. Three surfactants produced similar results. Triton X-100 formed fine foam, however, other surfactants, especially SDS, formed rough foam. Therefore, we chose Triton X-100 in the assay. Next, we confirmed that the use of clean (unstained) new test tubes ensured reproducible results. Finally, this assay comprises simple procedures, in that it involves only the mixing of the bacterial suspension, Triton X-100, and hydrogen peroxide solution, without any other procedures such as the preparation of bacterial extract. In terms of costs, the cost per test of reagents used in this method is close to being gratis, and no machinery or equipment is required for the assay.

In conclusion, we have developed a simple and cost-effective assay and demonstrated its feasibility in determining the catalase status of bacterial and human cells.

## Methods

**Bacterial strains and culture conditions.** Various pathogenic *Escherichia coli* strains<sup>20</sup> were employed: 96-7 (STEC), 98E2 (STEC), 98E5 (STEC), CL-49 (STEC), 467 (STEC), 468 (STEC), E333 (STEC), 98E6 (STEC), E32511 (STEC), E07781 (VT2<sup>+</sup>), E07794 (VT2<sup>+</sup>), and E07868 (VT1<sup>+</sup> and VT2<sup>+</sup>). In addition, deletion mutants of *katE* (which encodes heat-stable HP11), *katG* (which encodes heat-labile



**Figure 3** | Catalase activity of human cells, as determined with the newly developed method. In this experiment,  $10^5$ – $10^7$  cells were used. (A) Image of test tubes showing foam developed as a result of the catalase activity of LNCaP and PC-3 cells. (B) A plot of the data ( $n = 3$ ).



HPI), and *rpoS* (which positively regulates the expression of *katE* catalase), and the wild-type (BW25113) strain were also studied. All strains were obtained from the National Institute of Genetics (NIG, Mishima, Japan). The bacteria were propagated in Luria-Bertani (LB; BD Biosciences, USA) medium at 37°C, with shaking under aerobic conditions. The bacterial culture was inoculated into 10 mL of LB medium and cultured for 16 h at 37°C with shaking.

**Cell lines.** The LNCaP and PC-3 cell lines used in this study were obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured in RPMI 1640 containing 10% foetal bovine serum and Antibio-Antimycy (Life Technologies, Carlsbad, CA, USA). The cells were collected using trypsin, washed with PBS, and then used for the assay.

**Reagents and sample preparations.** The reagents used were hydrogen peroxide solution (30% (w/w) in H<sub>2</sub>O; Sigma-Aldrich, USA), 1% Triton X-100 (Sigma-Aldrich), catalase from bovine liver (2950 units/mg solid; 65% protein; 4540 units/mg protein, the product number C1345, Sigma-Aldrich), and 10 μM azide (an inhibitor of catalase activity<sup>21</sup>; Sigma-Aldrich).

Catalase powder, which was purchased from Sigma-Aldrich, was solved in 100-μL distilled pure water for preparing each concentration of catalase standards. Bacteria samples were prepared using overnight-cultured bacteria. Bacterial cells (10 mg) were suspended in 100-μL of physiological saline (diluted, if necessary). Human cells (10<sup>5</sup>–10<sup>7</sup> cells) were suspended in 100-μL of phosphate buffered saline.

**Quantification of catalase activities.** In the present method, to quantify the catalase activity, a calibration curve was plotted with the defined unit of catalase activity. Each catalase solution (100-μL) or bacterial suspension (100-μL) was added in a Pyrex tube (13 mm diameter × 100 mm height, borosilicate glass; Corning, USA). Subsequently, 100 μL of 1% Triton X-100 and 100 μL of undiluted hydrogen peroxide (30%) were added to the solutions and mixed thoroughly and were then incubated at room temperature. Following completion of the reaction, the height of O<sub>2</sub>-forming foam that remained constant for 15 min in the test tube was finally measured using a ruler.

**Discrimination of HPI and HPII activities.** In order to accurately distinguish between the activities of the heat-labile catalase HPI and heat-stable catalase HPII in *E. coli*, the bacterial suspension was divided into 2 identical aliquots. One aliquot of the bacterial suspension was subjected to heat treatment (55°C, 15 min). Finally, the residual catalase activity following heat treatment was subtracted from the total catalase activity to determine the activity of the heat-labile catalase.

**Inhibition of catalase activity.** To test the specificity of the assay for catalase, the 100 μL of physiological saline solution in the enzyme assay was replaced with 100 μL of 10 μM azide to inhibit catalase activity<sup>21</sup>.

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## Author contributions

T.I. designed the study. T.I., Y.K., A.T., S.S., K.O., I.H., K.T., Y.M. carried out experiments and discussed in detail about the obtained results. T.I. wrote the manuscript.

## Additional information

**Competing financial interests:** The authors declare no competing financial interests.

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