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A 96-WELL-PLATE–BASED METHOD FOR THE ESTIMATION OF ALPHA-AMYLASE ACTIVITY USING MINIATURISES 3,5-DINITROSALICYLIC ACID (DNSA) COLORIMETRIC METHOD

NOOR SYAFFINAZ NOOR MOHAMAD ZIN[1,2], NUR AIN SABRINA AZMI[2], NURSARAH SYAMIMI ANUAR[1,2], SYAHIRAH AIN SHAFIE[1,2], MUHAMAMMAD AIMAN FARIS MAZNAN[1,2], YONG MENG GOH[2,3] and NURDIANA SAMSULRIZAL[1*]

[1]*Faculty of Applied Sciences, Universiti Teknologi MARA, 40450 Shah Alam, Selangor, Malaysia*
[2]*TuAH Industrial Research Lab with Bio Fluid Sdn Bhd, Faculty of Applied Sciences, Universiti Teknologi MARA, 40450 Shah Alam, Selangor*
[3]*Department of Preclinical Sciences, Faculty of Veterinary Medicine, Universiti Putra Malaysia, Serdang, Selangor, Malaysia*
[*]*E-mail: nurdiana7251@uitm.edu.my*

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ABSTRACT

The DNSA assay has been widely employed for the *in vitro* detection and quantification of alpha-amylase inhibitory activity. However, the conventional method is associated with inconsistencies between protocols and requires a large volume of samples and other assay reagents that can compromise accurate quantitation. Therefore, the study aimed to develop a reliable, simple, and rapid analytical method for determining α-amylase activity. The developed method was carried out in 96-well microplates with a total volume of 250 µL and a total assay time of 1 hr, including pre-incubation. The method was validated for linearity, the limit of detection (LOD), the limit of quantitation (LOQ), and precision. A higher coefficient of determination (R^2) value was observed for the developed method as compared to the conventional method (0.9983 ± 0.0003 vs 0.9667 ± 0.0383). The coefficient of variation (CV%) of each data point was less than 15%, indicating excellent data precision. The optimum assay conditions were identified at 2 U/mL of enzyme solution and 5% (w/v) starch solution at 50 °C incubation temperature with an IC_{50} value of 0.026 ± 0.005 mg/mL. It is concluded that the developed method is practical, precise, and accurate for estimating α-amylase inhibitory activity and would provide reproducible results.

Key words: 3,5-Dinitrosalicylic Acid (DNSA), 96-well plate, alpha-amylase enzyme assay, alpha-amylase inhibition

INTRODUCTION

The enzyme alpha-amylase is essential for the breakdown of carbohydrates. Alpha-amylase inhibition has been shown to slow down the rate of glucose absorption, hence preventing postprandial hyperglycemia. (Kifle & Enyew, 2020). The 3,5-dinitrosalicylic acid (DNSA) colorimetric assay is one of the widely used techniques for assessing alpha-amylase enzyme inhibition. The 3,5-dinitrosalicylic acid (DNSA) colorimetric assay is one of the widely used techniques for determining the inhibition of the alpha-amylase enzyme. The Miller (1959) DNSA assay requires a significant amount of chemical consumption, a large number of samples, and several labor-intensive and time-consuming processes (Negrulescu *et al*., 2012). In consequence of those limitations, several studies have been conducted in developing an adapted protocol using a microtiter plate. These include the studies conducted by Goncalves *et al*. (2010), Negrulescu *et al*. (2012), and Zbîrcea *et al*. (2016). The use of a microtiter plate for the quantification of reducing sugar carries one major benefit which is the rapid screening of large numbers of samples that could reduce labor, time, and reagents consumption (Bener *et al*., 2018).

The application of the microtiter plate system in the DNSA assay involves a heating step in the reaction. The heating step is important to accelerate the reaction, as well as to aid in color development (Negrulescu *et al*., 2012). Goncalves *et al*. (2010), Negrulescu *et al*. (2012) and Zbîrcea *et al*. (2016) replaced the traditional incubation in boiling water bath step with heating in the oven, microwave, and thermomixer, respectively. However, the modified method by Goncalves *et al*. (2010) and Zbîrcea *et al*. (2016) could be costly as a heat-resistant crystal polyester microtiter plate and thermomixer are

* To whom correspondence should be addressed

required. Meanwhile, the thermal energy distribution could be varied among different microwave models as suggested by Negrulescu *et al*. (2012), which could eventually affect the reaction and color formation of the DNSA assay. This study was therefore conducted to improve the DNSA assay method adaptation using a microtiter plate by maintaining the conventional heating in the water bath step.

There is a variety of protocols conducted by different studies for the determination of the inhibitory activity of alpha-amylase. These included the concentration of alpha-amylase enzyme solution, percentage of starch solution as well as the incubation temperature of the reaction (Nyambe-Silavwe *et al*., 2015). The protocol inconsistencies could lead to undesirable results when it is applied in a different environment. Hence, this study also aims to determine the optimum *in vitro* alpha-amylase inhibition protocol with the employment of the proposed DNSA assay method.

MATERIALS AND METHODS

Materials

All chemicals including sodium potassium tartrate, sodium hydroxide, 3,5-dinitrosalicylic acid, D-(+)-maltose monohydrate, soluble starch, sodium phosphate dibasic, sodium phosphate monobasic, sodium chloride, and alpha-amylase from human saliva were purchased from Sigma-Aldrich, USA.

Preparation of DNSA reagent

The DNSA reagent was prepared according to the protocol demonstrated previously by Nyambe-Silavwe *et al*. (2015). Warm water was used to prepare a DNSA concentration of 96 mM, while sodium potassium tartrate was dissolved in 2 M sodium hydroxide to get final molarity of 5.3 M. The sodium-potassium tartrate solution may be more easily dissolved when heated to 80 °C. The final DNSA reagent was prepared by mixing 12.0 mL of warm water, 8.0 mL of 96 mM DNSA solution, and 20.0 mL of 5.3 M sodium potassium tartrate solution.

Maltose calibration curve – The proposed and conventional methods of DNSA assay

The DNSA assay for maltose calibration was conducted according to Malpani and Manjunath (2013) with slight modification. A serial dilution of D-(+)-maltose monohydrate standards were prepared ranging from 10-0 mg/mL with distilled water. The detailed protocol of both proposed and conventional methods of DNSA assay conducted in this study is illustrated in Figure 1.

Partial method validation

A partial method validation approach was used to determine the analytical quality of the proposed methodology. The linearity, limit of detection (LOD), and limit of quantification (LOQ) were computed by the selected range of the maltose calibration curve plotted (0 - 10 mg/mL, n = 6). The linearity test was conducted by determining a linear regression equation ($y = mx + c$), slope (m), y-intercept (c), and coefficient of determination (R^2) (Curbani *et* al., 2019). Based on the data obtained, the mean and standard deviation were calculated to be used for the LOD and LOQ calculations.

The LOD is defined as the lowest concentration of an analyte in a sample that can be detected, but not necessarily measured (Shrivastava & Gupta, 2011). In contrast, the LOQ is the lowest analyte concentration that can be measured with acceptable precision and accuracy under the specified test conditions. The LOD & LOQ were obtained based on the calibration curve and calculated according to the equation demonstrated previously by Şengül (2016) as follows.

Equation 1:

$$LOD = \frac{3.3 \times SD\ of\ y-intercept}{slope\ (m)}$$

Equation 2:

$$LOQ = \frac{10 \times SD\ of\ y-intercept}{slope\ (m)}$$

Utilization of the developed method for the optimization of the alpha-amylase enzyme reaction and inhibition by acarbose tablet

The validated proposed method was employed to determine the optimum condition for α-amylase enzyme reaction. The conditions tested were the concentrations of the enzyme (2, 4, 6, 8, 10 U/mL) and starch (1, 2, 3, 4, 5% w/v) incubated at three different temperatures, including room temperature (Oyedemi *et* al., 2017), 37 °C (Oluwagunwa *et* al., 2021) and 50 °C (Xiao *et al*., 2006). An amount of 20 µL of 0.02 M sodium phosphate buffer containing 0.006 M of sodium chloride (pH 6.9) was mixed with 20 µL of soluble starch and 20 µL of α-amylase enzyme solution. The reaction mixture was incubated for 30 min at the respective incubation temperature. An amount of 20 µL of DNSA reagent was added to the mixture and incubated in an 85 °C water bath for 15 min. The mixture was then diluted with 170 µL of distilled water. Absorbance was recorded at 540 nm wavelength with a microplate reader.

The optimum condition obtained for the alpha-amylase enzyme reaction was further validated with the determination of IC_{50} of acarbose tablet against the alpha-amylase enzyme. An amount of 20 µL of 0.02 M sodium phosphate buffer with 0.006 M of sodium chloride (pH 6.9) (control) or 20 µL of acarbose tablet

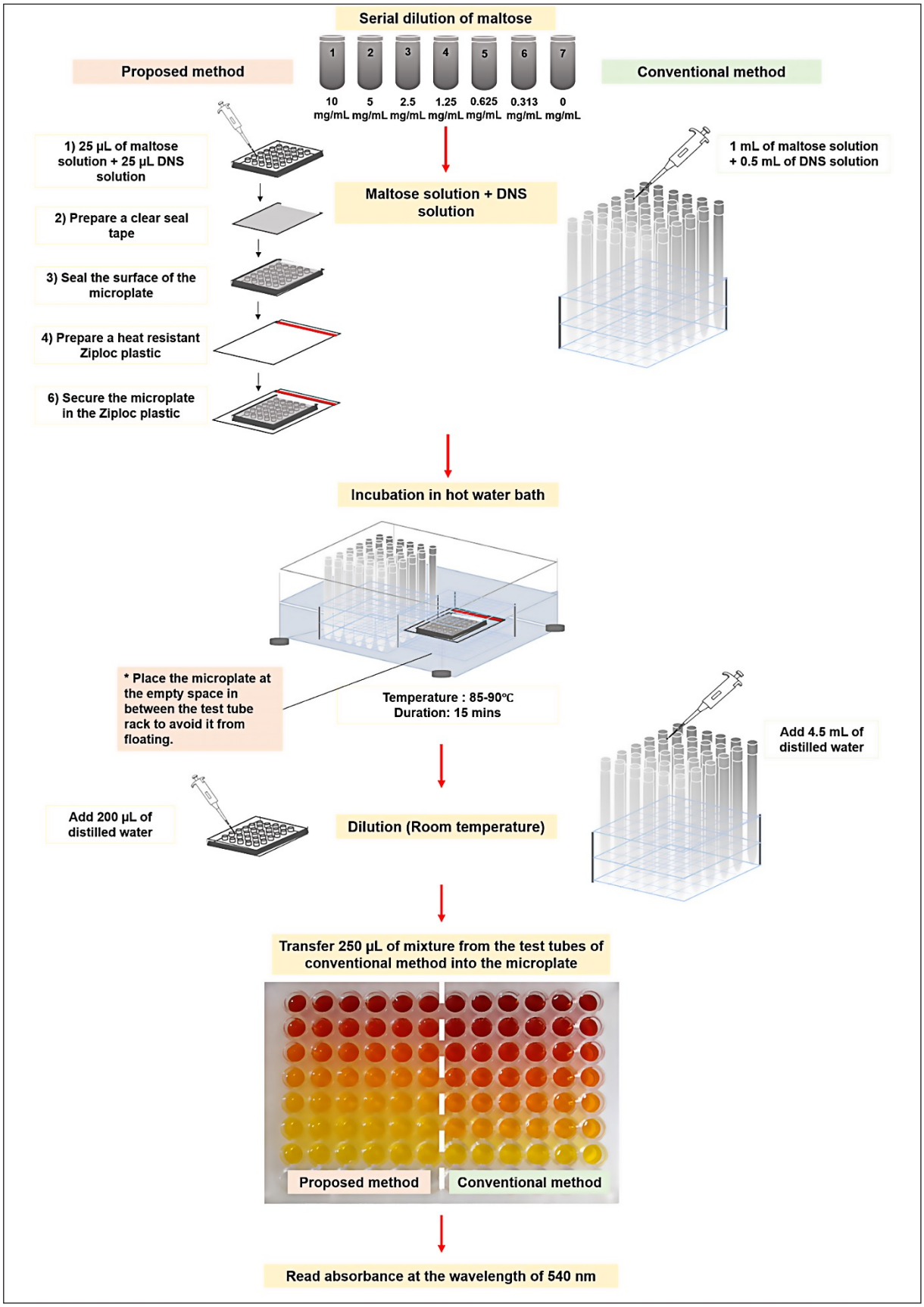

Fig. 1. The proposed and traditional method of DNS assay using maltose

(0.02 – 1.25 mg/mL) (test sample) were mixed with 20 µL of alpha-amylase enzyme solution. The mixture was incubated for 15 min at the optimum temperature obtained. An amount of 20 µL of the starch solution was then added and further incubated for 30 min. The reaction mixture was then treated with DNSA and diluted with distilled water as mentioned above. The percentage of enzyme inhibition was calculated by using the absorbance reading recorded according to the following formula (Poovitha & Parani, 2016):

Equation 3:

$$Percentage\ of\ inhibition\ (\%) = \frac{Abs\ of\ control - Abs\ of\ test\ sample}{Abs\ of\ control} \times 100\%$$

The percentage of inhibition calculated was subjected to the determination of the half-maximal inhibitory concentration (IC_{50}) value using GraphPad Prism 8 (GraphPad Software, San Diego). The IC_{50} value was obtained from the least square fit of the inhibitor concentration vs response (three parameters) model where, the X-axis is the concentration of acarbose tablet, and the Y-axis is the percentage of inhibition.

Statistical analysis

All data were collected in six replicates and presented as mean ± SD. Two sample t-test was conducted to determine the mean differences in the slope, y-intercept, R^2, LOD, and LOQ between the proposed and conventional methods. Two-way ANOVA was conducted to compare the mean of maltose production with varying enzyme concentrations incubated at three different incubation temperatures. Meanwhile, one-way ANOVA was conducted to determine the mean of varying starch concentrations. The *p*-value less than 0.05 ($p<0.05$) was regarded as the criterion for significance for all analyses. All statistical analyses mentioned were conducted using Minitab 19 (Minitab Inc., US).

RESULTS AND DISCUSSION

Figure 2 showed the maltose calibration curve comparing the proposed and conventional methods of DNSA assay across the three days of assay trials, with six replicates for each concentration. According to the maltose calibration curves plotted, the linear equation for each day of trials is tabulated in Table 1. Partial validation steps including the linearity, precision, LOD, and LOQ were determined according to the maltose standard calibration curve.

The linearity parameters indicate the ability of an analytical method to produce results that are directly proportional to the concentration of the analyte present in the samples, in a known range of concentrations (Negrulescu *et al*., 2012). In this study, the calibration curve for each method was obtained from the linear relation between maltose concentration range between 0-10 mg/mL with a 95% confidence level. The linearity of the calibration curves was measured by six replicates of standards at seven different concentration levels across three days of trials.

The slope, y-intercept, R^2, LOD, and LOQ obtained from each method were compared by a two-sample t-test and illustrated in Table 2. No significant differences were observed between the slope, y-intercept, and R^2 parameters of both the developed and conventional methods ($p>0.05$). A stronger linear relationship was demonstrated by the developed method where the R^2 value is closest to 1.0 as compared to the conventional method. No significant differences can also be observed in the LOD and LOQ concentrations ($p>0.05$). These findings revealed that the employment of the developed method for estimating reducing the sugar by DNSA assay would not cause much difference in the data obtained but improve the working experience with fewer resources, time, and energy consumption.

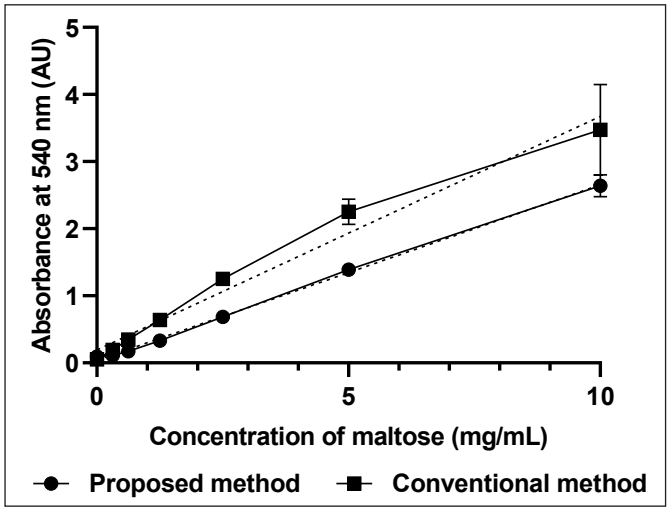

Fig. 2. The maltose standard calibration curve plotted by mean ± SD of the 6 replicates across the 3 days of trials of the proposed and conventional methods

Table 1. The linear equations of the maltose standard calibration curves comparing the proposed and conventional methods

Day	Proposed method	Conventional method
1	y = [0.2411 ± 0.0056]x + [0.0378 ± 0.0066]	y = [0.2561 ± 0.0050]x + [0.2714 ± 0.0069]
2	y = [0.2723 ± 0.0037]x + [0.0399 ± 0.0077]	y = [0.3912 ± 0.0082]x + [0.1659 ± 0.0120]
3	y = [0.2711 ± 0.0021]x + [0.0386 ± 0.0024]	y = [0.3965 ± 0.0032]x + [0.1452 ± 0.0120]

Table 2. Linearity, LOD, and LOQ of the DNS proposed and conventional methods for the determination of reducing sugar (maltose)

Characteristics	Proposed method	Conventional method	*p*-value
Slope (m)	0.2615 ± 0.0177	0.3479 ± 0.0795	0.208
y-intercept (c)	0.0388 ± 0.0011	0.1942 ± 0.0677	0.058
Regression coefficient (R^2)	0.9983 ± 0.0003	0.9667 ± 0.0383	0.289
Range of maltose (mg/mL)	0 – 10	0 – 10	n/a
LOD (mg/mL)	0.1373 ± 0.1367	0.0966 ± 0.0067	0.658
LOQ (mg/mL)	0.1516 ± 0.1096	0.2930 ± 0.0205	0.159

The reproducibility of the proposed method is also supported by the excellent data precision illustrated by the percentage of CV from each data point. Tiwari and Tiwari (2010) illustrated that good data precision must demonstrate a CV of less than 15%. According to Table 3, both proposed and conventional methods demonstrated the percentage of CVs of less than 15% in each data point.

The reliability of the proposed DNS method was validated by the optimization of alpha-amylase reaction assay. The optimum assay conditions including incubation temperature, the concentration of α-amylase, and the concentration of starch were determined and presented in Figures 3a and 3b. Figure 3a revealed a significantly higher (p<0.05) product produced from the α-amylase enzyme reaction incubated at 50 °C temperature as compared with the room and 37 °C temperatures. This is in contrast with the most reported papers including Keharom *et al*. (2016), Simair *et al*. (2017), and Oluwagunwa *et al*. (2021) which showed the ability of alpha-amylase enzyme to react at the range of 37-39 °C temperatures. However, Xiao *et al*. (2006) demonstrated that the alpha-amylase enzyme was mostly active after 30 min of incubation at 50 °C. These could be contributed by the influence of different types of incubators. A water bath could transfer heat more efficiently as compared to air in the incubator oven (Shehu *et al*., 2019). These could also be explained by the formula of the rate of conductive heat transfer through a slab of material as shown below (Ling *et al*., 2021):

Equation 4:

$$P = kA\frac{dT}{dx}$$

where P, is the power or rate of heat transfer in watts or kilocalories per sec, A and d, surface area and thickness of the slab of material, T, temperature difference across the slab, and x, coordinate in the direction of heat flow and k, the thermal conductivity of the material. Given that the thermal conductivity (k) of water and air are 0.6 W/m.°C and 0.023 W/m.°C, respectively with the other parameters kept constant, the rate of heat transfer in water would be higher than in air. Hence, these explain the differences in the optimum alpha-amylase enzyme reaction upon the use of different types of incubators.

The optimum alpha-amylase concentration determined was 2 U/mL. This is due to the insignificant changes in the product obtained even with the further increment of alpha-amylase concentration. Figure 3b showed the significantly highest product produced with the 5% (w/v) starch concentration. Taken together, the data suggest that the optimum conditions for alpha-amylase assay by the DNS proposed method are 2 U/mL of the enzyme, 5% (w/v) of starch with 50°C incubation temperature. The optimum assay condition utilized for the determination of the inhibitory activity of alpha-amylase by acarbose tablet resulted in the IC_{50} of 0.008 ± 0.001 mg/mL (Figure 3c).

CONCLUSION

The developed 96-well-plate–based method tested in this study is reliable and would provide reproducible results. The utilization of the proposed method for DNSA assay showed that the optimum condition for *in vitro* α-amylase enzyme reaction were 2 U/mL of enzyme and 5% (w/v) of starch with 50 °C incubation temperature.

Table 3. Mean CV (%) of absorbance obtained from each maltose concentration across the three days of assay trials

Concentration of maltose (mg/mL)	Mean CV (%)	
	Proposed method	Conventional method
0.00	3.97 ± 1.30	6.32 ± 4.21
0.31	3.73 ± 0.73	3.72 ± 1.78
0.63	6.86 ± 5.89	4.44 ± 3.87
1.25	1.93 ± 0.32	4.50 ± 2.44
2.50	2.01 ± 1.05	2.05 ± 0.41
5.00	1.61 ± 0.40	2.95 ± 1.46
10.00	1.55 ± 0.77	1.42 ± 0.87

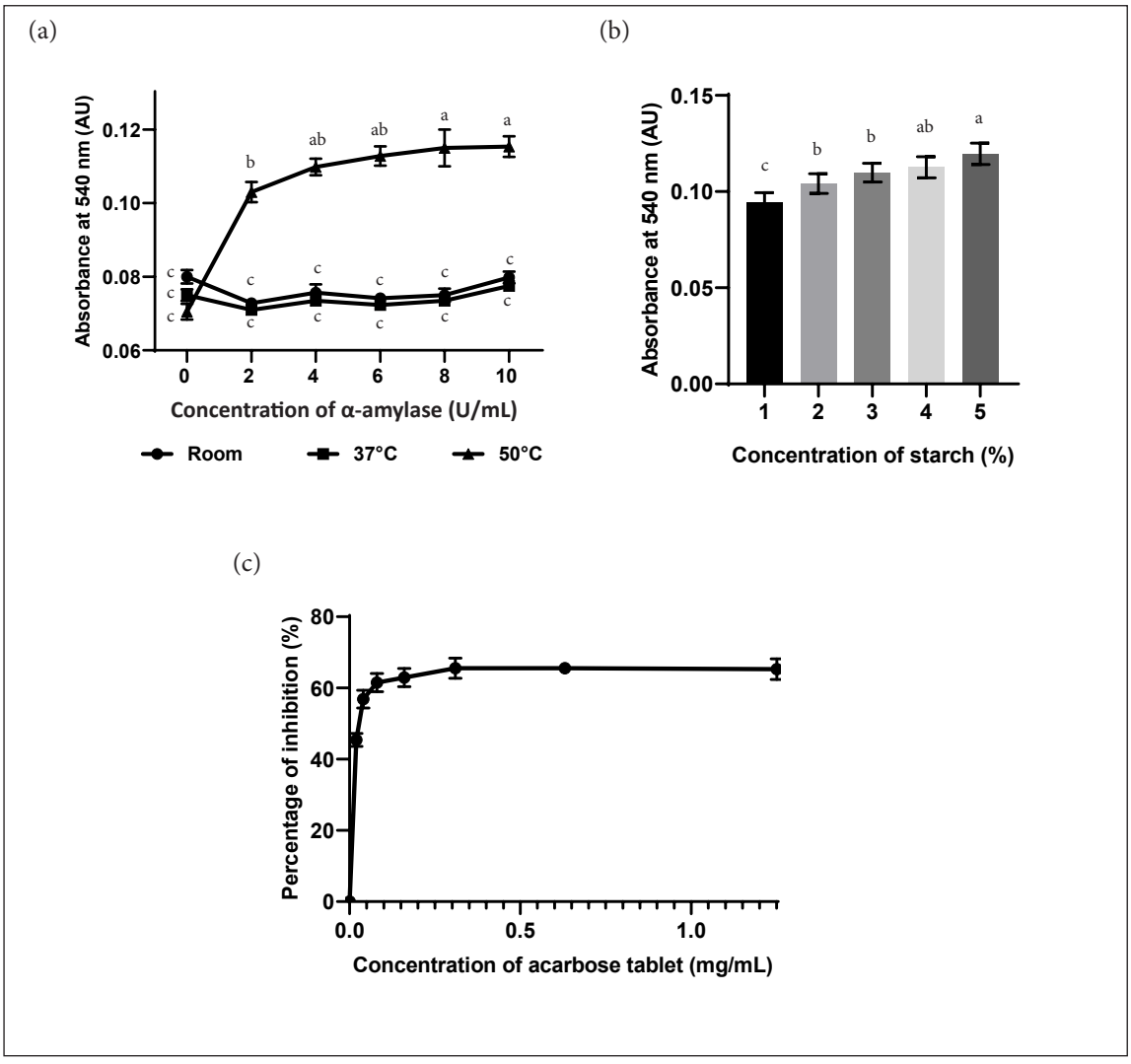

Fig. 3. (a) α-Amylase assay in different incubation temperatures with varying concentration of enzyme reacted with 1% of soluble starch solution; (b) α-Amylase assay with varying concentration of starch with 2 U/mL of enzyme in 50 °C incubation temperature; (c) Inhibition of α-amylase enzyme by acarbose tablet. Data were obtained from 6 replicates of each sample and presented as mean ± SD. Two-way factorial ANOVA and One-way ANOVA were conducted to determine the mean difference among each point in (a) and (b), respectively. Different letters (a,b,c) indicated the significant differences in population means from each data point (p<0.05), while the same letters indicated otherwise.

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CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

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