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Purification and characterization of thermostable alpha-amylase from *Geobacillus* sp. DS3 from Sikidang Crater, Central Java, Indonesia

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ABSTRACT Amylases are considered the most essential enzymes in biotechnology since they are widely utilized in the textile, food processing, and detergent industries. It is necessary to explore extracellular enzymatic activity in several microorganisms to discover a new potential application from amylases. In a previous study, thermophilic bacteria *Geobacillus* sp. DS3 isolated from Sikidang Crater, Dieng Plateau, Central Java, Indonesia showed amylase activity in starch medium at 70 °C. This study aimed to purify and characterize the thermostable alpha-amylase from *Geobacillus* sp. DS3. The alpha-amylase was produced and purified using ammonium sulfate and DEAE Sephadex A-25 column. The enzyme activity was determined using the 3,5-dinitrosalicylic acid (DNS) method. *Geobacillus* sp. DS3 optimally produced the alpha-amylase at 60 °C for 15 h. The alpha-amylase exhibited high enzymatic activity in 40–60% saturated ammonium sulfate extract. The molecular weight of the enzyme was estimated to be 58 kDa. The thermostable alpha-amylase showed activity at the optimum temperature of 50 °C in 200 mM sodium phosphate buffer pH 7.0. The enzyme was inhibited by EDTA, PMSF, 2-ME, and mostly by $HgCl_2$. The Km and Vmax of the pure enzyme were 235.43 mM and 1428.57 U/mL, respectively. The result suggested that the purified thermostable alpha-amylase from *Geobacillus* sp. DS3 offers potential application in areas of the food industry, such as the bakery industry.

KEYWORDS alpha-amylase; thermostable enzyme; *Geobacillus* sp. DS3; purification; characterization

1. Introduction

Amylases are applied for about 25% in several industries such as textile, detergent, and food processing. Alpha-amylases are enzymes that hydrolyze polysaccharides such as starch and cleave the α-1,4-glycosidic linkages randomly to produce glucose, maltose, and oligosaccharides. In sugar production such as HFCS (High Fructose Corn Syrup), alpha-amylases are used for liquefaction at high temperature. Therefore, the process requires enzyme that is stable at high temperature. The use of alpha-amylase during liquefaction shows a significant improvement in the production of sugar industry to economize the process (Burhan et al. 2003). Every 10 °C increasing temperature, the reaction rates may approximately double (Zamost et al. 1991). The enzyme stability at the high temperature could make the amount of the enzyme needed can be reduced or the conversion time can be shorter. The use of thermostable enzyme also reduces risk of contamination by microorganisms, and the higher temperatures can increase the solubility of polymeric substrates such as carbohydrates (Turner et al. 2007; Kambourova 2018).

Thermophilic microorganism usually produces enzymes that have stability at a high temperature of more than 40 °C known as thermozyme (Vieille and Zeikus 2001). Thermophilic bacteria like *Bacillus* and *Thermus* usually produce amylase thermostable. According to Bhatt et al. (2020), alpha-amylase, the thermozyme from *Bacillus velezensis* KB 2216 showed the optimum temperature at 55 °C and pH 5.5. The two isolates, BR 002 and BR 015, from the Bora hot spring, Center Sulawesi showed the alpha-amylase activities at 50 °C and 70 °C, respectively (Gazali and Suwastika 2018). *Geobacillus* sp. DS3 isolated from Sikidang Crater, Dieng Plateau, Central Java, Indonesia grew well at around 50 to 70 °C (Witasari et al. 2010). *Geobacillus* sp. is a Gram-positive bacteria, produces spores and lives in environments such as rock, land, deep-sea at the temperature of 45-70 °C, and hot spring. In the previous study, the thermostable alpha-amylase gene from *Geobacillus* sp. DS3 was heterologously expressed in *E. coli* BL21(DE3) (Cahyono 2020), unfortunately, the purification and characterization of its enzymes have not been carried out yet. Nevertheless, thermostable serine alkaline protease from *Geobacillus* sp. DS3 has been previously characterized and it showed the

purified protease enzyme was optimum at 70 °C and pH 9.6 (Phon et al. 2022).

In this study, the native alpha-amylase produced from *Geobacillus* sp. DS3 was purified and biochemically characterized for optimum temperature, pH, cofactors, inhibitors, and kinetic enzyme.

2. Materials and Methods

2.1. Materials

Geobacillus sp. DS3 was isolated from Sikidang, Dieng crater, Center of Java, Indonesia. The bacteria were cultured in Luria Bertani (LB) broth containing 1% tryptone, 0.5% yeast extract (Oxoid, Cheshire, UK), and 1% NaCl (Merck KGaA, Darmstadt, Germany). Medium for alpha-amylase production was contained tryptone and agar bacteriological (Oxoid, Cheshire, UK), soluble starch, $CaCl_2.2H_2O$, $MgSO_4.7H_2O$, NaCl, and K_2HPO_4 (Merck KGaA, Darmstadt, Germany).

2.2. Preliminary assay of alpha-amylase activity of Geobacillus sp. DS3 on starch agar medium

To investigate the alpha-amylase expression, *Geobacillus* sp. DS3 were inoculated and incubated on solid medium containing soluble starch. The medium contained 0.5% tryptone, 1% soluble starch, and 2% agar bacteriological. After 24 h of incubation at 50 °C, the medium was dropped by 2% Iodin Lugol. The clear zones were formed around *Geobacillus* sp. DS3 colony after incubation at 50 °C for 24 h indicated amylase activity.

2.3. Effect of time and temperature for alpha-amylase expression using starch liquid medium

Alpha-amylase expression from *Geobacillus* sp. DS3 was induced using 1% soluble starch in liquid medium containing 0.5% tryptone, 0.1% $CaCl.2H_2O$, 0.02% $MgSO_4.7H_2O$, 0.1% NaCl, and 0.1% K_2HPO_4. A single colony from the solid medium was inoculated into a liquid medium and incubated for 24 h at 50 °C known as fresh culture. Each 5 mL liquid medium contained 500 µL of fresh culture then incubated at 50 °C at various times (0, 3, 6, 9, 12, 15, 18, 21, and 24 h). Afterward, various temperatures (30, 40, 50, 60, 70, 80, and 90 °C) were tested at optimum incubation time.

2.4. Alpha-amylase activity assay

The activity of alpha-amylase was analyzed with the DNS method (Xie et al. 2014). A hundred microliter of the crude enzyme was mixed with 400 µL 0.1 M acetic buffer and 500 µL of 1% soluble starch. Then, it was incubated for 10 min at 50 °C. Five hundred microliter of the sample was then added with 500 µL with DNS and then boiled for 5 min. After that, the mixture was cooled, and the absorbance was measured using GENESYS 140/150 Vis/UV-Vis Spectrophotometers at 540 nm wavelength. One unit of alpha-amylase activity was defined as the amount of enzyme which releases 1 µmol of reducing sugar per min. The activity was measured by an equation as shown below.

Enzyme activity

$$= \frac{Glucose\ conc.\ (mg/mL) \times Reaction\ vol.\ (mL) \times Dilution\ factor}{Mr\ Glucose \times Incubation\ time\ (min) \times Enzyme\ vol.} \quad (1)$$

2.5. Protein concentration

The protein concentration was measured based on the Bradford methods (Bradford 1976). Ten microliter of the enzyme was mixed with 1 mL of Bradford reagent and incubated for 5 min at room temperature. The absorbance was measured using GENESYS 140/150 Vis/UV-Vis Spectrophotometers at 595 nm. The standard solution used a BSA solution with various concentrations (0.25-1.4 mg/mL) to obtain the equation linear regression.

2.6. Alpha-amylase purification using DEAE Sephadex A-25 column

The purification of the enzyme was performed based on (Febriani et al. 2019). The starch liquid medium was centrifuged for 10 min at 4 °C at 3046 × g to obtain the supernatant. The supernatant was then added with ammonium sulfate at various saturation ranges as 0-20%, 20-40%, 40-60%, 60-80%, and 80-100%. The sample was then stirred overnight and then centrifuged for 20 min at 4 °C, 3046 × g to obtain a pellet at each fraction. The pellet was dissolved with 20 mM sodium phosphate buffer pH 7.5 and further dialyzed with the same buffer overnight.

The pellet was then loaded onto a DEAE Sephadex A-25 column (20 cm × 1 cm). The column was equilibrated with a 20 mM sodium phosphate buffer pH 7.5. A thousand microliter enzyme was loaded into the column and eluted using stepwise gradients elution of 0.1 M, 0.25 M, and 0.5 M NaCl in 20 mM sodium phosphate buffer pH 7.5. Each fraction consisted of 1.5 mL volume and each elution gradient contained 20 fractions. The fractions were analyzed for absorbance at 280 nm. The major peak was then subjected for SDS-PAGE analysis.

2.7. SDS-PAGE analysis of purified protein

The molecular weight of purified thermostable alpha-amylase was detected by SDS-PAGE using the Laemmli method (Laemmli 1970). The samples were prepared by mixing purified enzyme and SDS loading buffer, heated at 95 ºC for 5 min to denature the protein, and stored on ice to stop the denaturation. The SDS-PAGE gel consisted of 12% resolving gel and 7% stacking gel. Protein bands were visualized by overnight staining with bromophenol blue (Merck KgaA, Darmstadt, Germany). Protein bands were observed after destaining the gel the next day using the destaining solution consisting of methanol and acetic acid.

2.8. Temperature and pH optimum

2.8.1 Temperature optimum

The temperature optimum of the crude enzyme was performed at various temperatures of 30, 40, 50, 60, 70, and

80 °C using 200 mM sodium phosphate buffer pH 7.5. After incubation, the alpha-amylase activity of each sample was analyzed with the DNS method.

2.8.2 pH optimum

The pH optimum of the crude enzyme was conducted from 4.0-9.0 with different buffers such as 200 mM acetic acid buffer pH 4.0-5.6, 200 mM sodium phosphate buffer pH 6.0-8.0, and 200 mM Tris-HCl buffer pH 8.0-9.0. The alpha-amylase activity assay was applied as mentioned above.

2.9. Effect of cofactors and inhibitors

The cofactors used in this study were $CaCl_2.2H_2O$, $MgCl_2.6H_2O$, and KCl. The inhibitors were ethylenediaminetetraacetic acid (EDTA), phenylmethylsulfonylfluoride (PMSF), 2-mercaptoethanol (2-ME), and $HgCl_2$. The enzyme was mixed with the cofactors and inhibitors, and the residual activity of the enzyme was measured. Both cofactors and inhibitors were prepared in two concentrations, 1 mM and 5 mM for each.

2.10. Enzyme kinetic assay

The kinetic assay was determined by using 100 µL of enzyme and several concentrations of soluble starch as substrate in the range of 5, 10, 15, 20, 25, and 30 mM at the optimum conditions of 50 °C and pH 7.0 for 1 min of incubation. The Vmax and Km were then generated from double reciprocal plots.

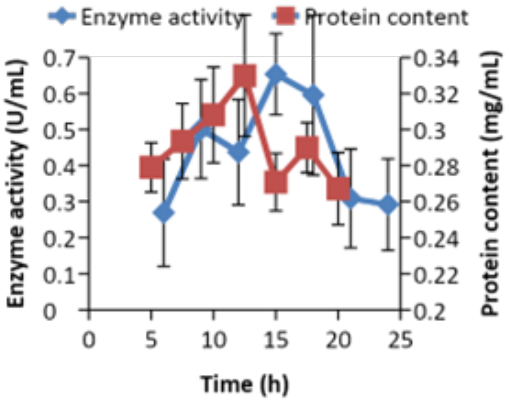
3. Results and Discussion

3.1. Preliminary assay of alpha-amylase activity of Geobacillus sp. DS3 on solid medium

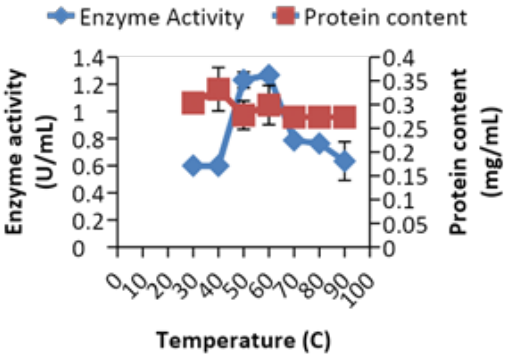
Preliminary assay using a solid starch medium was performed to investigate the expression of alpha-amylase from *Geobacillus* sp. DS3. The clear zones were formed around *Geobacillus* sp. DS3 after incubation at 50 °C for 24 h indicated that the bacteria expressed alpha-amylase (Supplementary Figure 1). According to Gazali and Suwastika (2018), it has been reported that thermobacteria obtained from hot spring in Center Sulawesi has formed a clear zone at 55 °C for 24 h of incubation. *Geobacillus* sp. NMS2 isolated from a hot water spring in Sri Lanka exhibited a clear zone with iodine test on starch-nutrient agar medium incubated at 50 °C overnight (Mathew and Rathnayake 2014).

3.2. Effect of time and temperature for alpha-amylase expression using liquid medium

Various incubation times and temperatures were tested to obtain the optimum condition of *Geobacillus* sp. DS3 to express alpha-amylase. Alpha-amylase showed the highest activity at 15 h of incubation and then decreased gradually (Figure 1a). The decreased amylase activity might be due to protease expression by *Geobacillus* sp. DS3 (Phon et al. 2022). The optimum incubation time of 15 h was then applied to analyze the optimum temperature for alpha-amylase expression. Alpha-amylase activity increased up to 60 °C and then dropped significantly at 70°C (Figure 1b). Thus, *Geobacillus* sp. DS3 expressed alpha-amylase at optimum condition at 60 °C and 15 h incubation. The optimal temperature for *Aeribacillus pallidus* BTPS-2 growth to produce alpha-amylase was at 60 °C (Timilsina et al. 2020). Extracellular alpha-amylase produced by *Bacillus licheniformis* WF67 displayed the highest activity at 50 °C for 72 h (Saad et al. 2021). The highest thermostable alpha-amylase production from *Bacillus licheniformis* So-B3 was at 35 °C for 36 h (Fincan et al. 2021).



(a)



(b)

FIGURE 1 Effect of time (a) and temperature (b) for alpha-amylase expression from *Geobacillus* sp. DS3

3.3. Alpha-amylase partial purification

First step purification was performed using ammonium sulfate precipitation method. The activity of the enzyme after partial purification was 2.5 times higher than the crude extract. The highest alpha-amylase activity was at 40-60% saturation of ammonium sulfate (Supplementary Figure 2). It has been reported that a higher activity of alpha-amylase from *Bacillus velezensis* KB 2216 was obtained at 80% of ammonium sulfate fraction (Bhatt et al. 2020). Alpha-amylase from thermophilic *Bacillus* spp. was partially purified by ammonium sulfate precipitation

at 80% saturation (Msarah et al. 2020).

The protein extract of 40-60% saturation ammonium sulfate was then purified with anion-exchange column chromatography DEAE Sephadex A-25. The bound fractions were eluted using stepwise gradients of 0.1 M, 0.25 M, and 0.5 M NaCl in 20 mM sodium phosphate buffer pH 7.5. The fractions were collected and analyzed its absorbance at 280 nm (Supplementary Figure 4). Afterward, alpha-amylase activity was analyzed at high absorbance peak (Supplementary Figure 5). Purified alpha-amylase exhibited higher enzyme activity but lower yield (Table 1). The alpha-amylase (AA11) from *Bacillus cereus* strain SP-CH11 isolated from Chilika Lake displayed 3.2% yield from the crude enzyme (Priyadarshini et al. 2020). It has been reported that the specific activity of purified alpha-amylase from *Bacillus stearothermophillus* after three-step purifications (ammonium sulfate precipitate, ultrafiltration, and DEAE Sepharose Fast Flow) was increased over 100 fold than crude extract (Ravindran et al. 2019).

3.4. Molecular weight determination

The molecular weight of alpha-amylase from *Geobacillus* sp. was estimated to be 58 kDa (Supplementary Figure 3). In the previous study, the molecular weight of alpha-amylase from *Geobacillus* sp. DS3 expressed in *E. coli* BL21(DE3) was 60 kDa (Cahyono 2020). Alpha-amylase from *Pseudomonas balearica* VITPS19 from agricultural fields in Tamil Nadu, India showed MW of 47 kDa (Kizhakedathil and C 2021). The molecular weight of alpha-amylase from Tepidimonas fonticaldi HB23 from a thermal spring in Hammam Righa, Algeria was 48 kDa (Allala et al. 2019). Montor-Antonio et al. (2017) reported a higher molecular weight of alpha-amylase produced by recombinant than the native enzyme from *Bacillus amyloliquefaciens* JJC33M. According to Mehta and Satyanarayana (2016), the most alpha-amylases thermostable from microorganisms have molecular weight ranging from 21 to 160 kDa.

3.5. pH and temperature optimum

The optimum pH of the alpha-amylase was 7.0 in 200 mM sodium phosphate buffer. This result was comparable with alpha-amylase isolated from *B. amyloliquefaciens* BH072 from honey (Du et al. 2018) and alpha-amylase from *Aeribacillus pallidus* BTPS-2 isolated from a geothermal spring in Nepal (Timilsina et al. 2020). Both exhibited the highest activity at pH 7.0. Several studies have reported alpha-amylase with higher activity in the alkaline pH range (Agüloğlu Fincan et al. 2014). Amylases that are active at an alkaline pH have potential applications in the textile and detergent industries. Unfortunately, this study showed that the alpha-amylase activity was decreased at pH 8-9. At high pH range, the ionization of amino acid groups at the active site of enzyme and the substrate is altered, affecting the binding site of the substrate on the active site (Robinson 2015).

Figure 2b showed that the activity of alpha-amylase gradually increased from 30 °C to 50 °C, then slightly decreased to 70 °C, and sharply dropped at 80 °C. The highest activity was shown at 50 °C, which means the alpha-amylase from *Geobacillus* sp. DS3 is included in the thermostable enzyme. The α-amylase from *Bacillus methylotrophicus* strain P11-2 displayed stable temperature below 50 °C, optimal at 70 °C then decreased sharply at above 70 °C (Xie et al. 2014). Each enzyme has specific optimum activity at a specific temperature and pH conditions. The optimum temperature for enzyme activity is

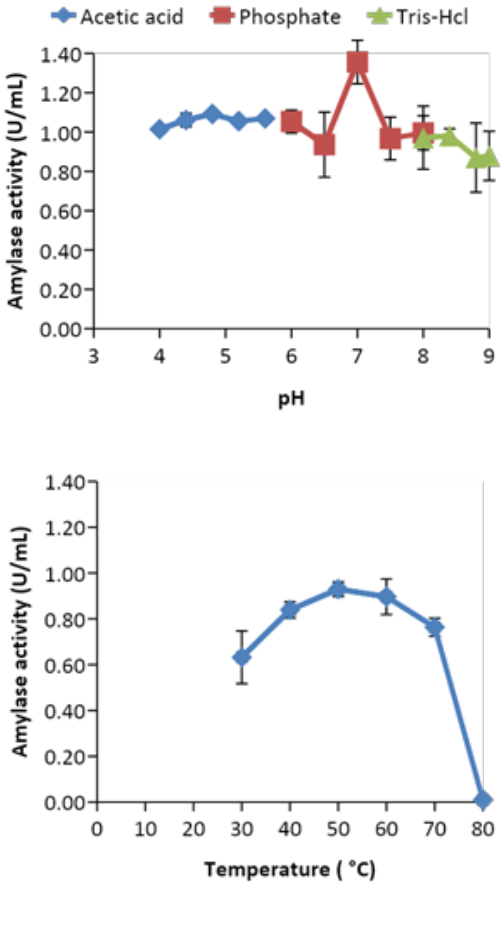


FIGURE 2 pH (a) and temperature (b) optimum of alpha-amylase

TABLE 1 The different composition of running buffer for optimization

Purification	Activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)
Crude enzyme	593.53	45.72	12.98	100.00
Ammonium sulfate 40-60%	58.32	4.92	11.85	9.83
DEAE Sephadex A-25	21.39	0.063	339.54	3.60

generally related to the temperature of the area from which the microorganisms are isolated (Febriani et al. 2019).

3.6. Effect of cofactors and inhibitors

Salts such as $CaCl_2.2H_2O$ and KCl exhibited higher activity than control, with higher concentration giving the most effective as an activator (Table 2). Mostly, alpha-amylase activity was increased in metal ions such as Ca^{2+}, Co^{2+}, and Mg^{2+} (Burhanoğlu et al. 2020). Salt like KCl has been reported to have a positive influence on alpha-amylase produced by *B. stearothermophilus* (Ravindran et al. 2019). In this study, the alpha-amylase activity was slightly decreased in the presence of $MgCl_2.6H_2O$. The presence of Mg^{2+}, Cu^{2+}, and DTT inhibited the activity of alpha-amylase from *Thermomyces dupontii* (Wang et al. 2019).

Inhibitors such as EDTA, PMSF, 2-Mercaptothenol, and $HgCl_2$ inhibited the alpha-amylase. EDTA is a general chelating agent that can remove the metal ions from the enzyme's active sites. EDTA decrease the activity of alpha-amylase by removing Ca^{2+} through chelation and destabilizing enzyme (Buonocore et al. 1976). In this study, the activity of alpha-amylase was slightly inhibited by EDTA. PMSF is a chelating agent which inhibits serine catalytic side (Nam et al. 2009). The inhibition of alpha-amylase was highest at $HgCl_2$. According to Allala et al. (2019), the inhibition by Hg^{2+} may be related to the enzyme structure-function relationship because of their thiol-depriving action, which prevents two cysteine residues from forming disulfide bonding.

TABLE 2 The different composition of running buffer for optimization

Treatment	Concentration (mM)	Alpha-amylase Activity (Unit/mL)
Control		1.35±0.11
Cofactor		
CaCl2.2H2O	1	1.41±0.12
	5	1.41±0.21
MgCl2.6H2O	1	1.32±0.16
	5	1.39±0.26
KCl	1	1.44±0.03
	5	1.61±0.19
Inhibitor		
EDTA	1	1.05±0.09
	5	0.99±0.15
PMSF	1	1.01±0.03
	5	0.84±0.14
2-ME	1	0.87±0.09
	5	0.79±0.13
HgCl2	1	0.64±0.10
	5	0.62±0.14

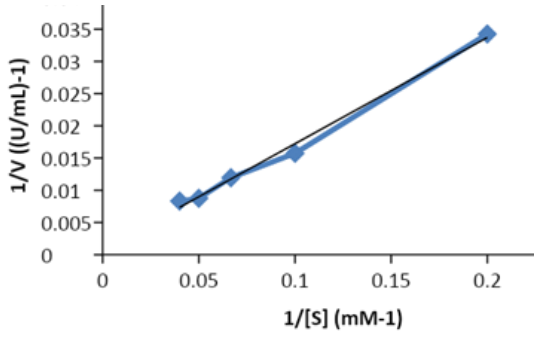


FIGURE 3 Lineweaver-Burk curve of purified alpha-amylase

3.7. Enzyme kinetic

The value of Km and Vmax of peak number 6 after DEAE Sephadex A-25 purification was 235.43 mM and 1428.57 U/mL, respectively (Figure 3). The alpha-amylase isolated from thermophilic Monascus sanguineus showed Km and Vmax of 0.055 mM and 22.07 U/mL, respectively (Tallapragada et al. 2017). Simair et al. (2017) reported the Vmax of alpha-amylase from thermophilic *Bacillus* sp. BCC 021-50 isolated from a marine environment was 5211 U/mL. The Km and Vmax of alpha-amylase from *Pseudomonas balearica* VITPS19 was reported to be 45.23 mM and 20.83 U/mL, respectively (Kizhakedathil and C 2021). Thermostable alpha-amylase from *Geobacillus* sp. DS3 showed potential candidate for food industry application, such as in the bakery industry for improving bread quality. Alpha-amylase may improve the fermentation process in the dough, promoting the Maillard reaction products formation, which in turn intensify crust color and bread flavour (El-Okki et al. 2017). The highest purified enzymes are highly required in this application process. Therefore, the future study related to purification and production of thermostable alpha-amylase for the bakery industry is necessary to be investigated.

4. Conclusions

In conclusion, thermostable alpha-amylase produced from *Geobacillus* sp. DS3 exhibited optimal enzymatic activity at 50 ºC and pH 7.0. The molecular weight was estimated to be 58 kDa. Salts like $CaCl_2.2H_2O$ and KCl enhanced its activity as cofactor. Whereas $HgCl_2$ was the most inhibitor of enzyme activity. The enzyme showed Km and Vmax of 235.43 mM and 1428.57 U/mL, respectively.

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Authors' contributions

This work was conceived and designed by LDW. Experimental work was carried out by DRW and SP. LDW, DRW, and AN contributed to data analysis and manuscript preparation. All authors have read and approved the manuscript.

Competing interests

The authors declare no conflicts of interest.

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