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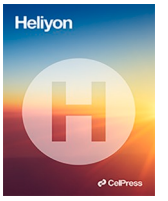

Research article

Screening of *Bacillus* spp. bacterial endophytes for protease production, and application in feather degradation and bio-detergent additive

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ABSTRACT

Research on proteases and secondary metabolites from endophytes is an area that requires attention from researchers. In this study, proteases from *Bacillus* sp. strain MHSD16 and *Bacillus* sp. strain MHSD17 endophytes were characterised, and their potential biotechnological applications were investigated. Optimum protease production was achieved when isolates were grown in media containing (g/L): glucose 10g, casein 5g, yeast extract 5g, KH_2PO_4 2g, Na_2CO_3 10g at pH 9. The crude protease extracts were active in alkaline environments, thus referred to as alkaline proteases with optimal pH of 10. Additionally, *Bacillus* sp. strain MHSD 16 and *Bacillus* sp. strain MHSD17 proteases were active at high temperatures, with optimum enzyme activity at 50 °C. Thermostability profiles of these proteases showed that the enzymes were highly stable between (40–60 °C), maintaining over 85 % stability after 120 min incubation at 60 °C. Furthermore, the enzymes were stable and compatible with various household and laundry detergents. In the presence of commercial laundry detergent, OMO® 68 % and 72 % activity was retained for *Bacillus* sp. strain MHSD16 and *Bacillus* sp. strain MHSD17, respectively, while 67 % and 68 % activity were retained in the presence of Sunlight®. The potential application for use in detergents was investigated through the removal of blood stains with the crude alkaline extracts displaying efficient stain removal abilities. Feather degradation was also investigated and *Bacillus* sp. MHSD17 exhibited feather keratin degrading properties more effectively than *Bacillus* sp. MHSD16.

1. Introduction

With the modern world placing emphasis on the importance of environmentally friendly technologies, proteases can be used as ecofriendly alternatives to replace chemicals used in the breakdown of protein rich material. Proteases are a group of enzymes which catalyse the hydrolysis of proteins, resulting in smaller peptide chains and/or free amino acids [1]. This class of enzymes is ubiquitous in nature, present in humans, plants, and microorganisms [2,3]. The enzymes play pertinent physiological roles [4,5] and in addition, are important industrial enzymes [6]. Proteases are applied in pharmaceuticals, food, feed, detergent industries and in the recovery of

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silver from X-ray films [7]. Furthermore, this enzyme class contributes to approximately 65 % of the overall global enzyme sales, with majority alkaline proteases [8].

Majority of the commercialised microbial alkaline proteases are isolated from *Bacillus* spp. genus with a remarkable amount applied in detergents [9]. Some of the commercial microbial proteases include keratinases, which are a group of proteases which catalyse the hydrolysis of keratin rich material [10]. The increase in poultry waste production as well as the need to recycle has largely driven research on keratinases. Studies have indicated that the annual production of feather waste production from meat processing industries is approximately 2–5 million tonnes [11,12]. In South Africa alone, about 230 million kg of feather waste is produced annually from poultry farms with majority still getting discarded into landfills or incinerated [13]. This does not only present various environmental pollution issues but also, waste of protein rich source. Chicken feathers are composed of >90 % keratin, an essential structural protein which contains various essential amino acids like threonine, cysteine, and arginine [14,15]. When appropriately recycled, keratin hydrolysates can be applied in bioactive molecules with antioxidant activities, angiotensin-converting enzyme (I) inhibitors [16] antimicrobial activities [12]and supplementation of animal feed [17]. However, due to the abundance in disulphide bonds forming cross linkages which in turn fold into β-sheets and packaged into helical structures, this renders them highly insoluble and challenging to recycle [10]. Attempts to recycle feathers into nutritional animal feed product using chemicals and steam pressure have been made [18,19]. However, the disadvantage of these methods is in compromising product quality and destruction of certain amino acids [20]. Bioconversion of chicken feathers by microbial keratinases is thus increasingly gaining popularity [21]as it is a greener approach.

On the other hand, there is an increased demand for proteases with special properties suitable for use in detergents. In comparison to harsh chemicals used in conventional laundry detergents to improve fabric cleanliness, which ultimately lead to environmental pollution, enzymes are green and biodegradable. The addition of enzymes into laundry detergents enhances the ability of detergents to remove tough stains [22]. Although enzymes have been used in detergents for decades, research for novel enzymes with increased stability in detergent formulations continues. Detergent manufacturers have outlined the criteria for ideal detergent proteases as follows: the ability to be active and maintain stability at broad alkaline pH ranges, as well as the ability to function over broad temperature ranges for cold and warm water washes [23,24].

While alkaline proteases can be isolated from various sources, microbial sources continue to be an ideal and inexpensive source. This is because isolation from animals and plants pose limitations due to climate related and ethical issues. In addition, the feasibility of meeting ever surging industrial demands by isolation from plants or animals is unlikely [6,7]. On the contrary, isolation from microorganisms confers certain advantages such as the ease of optimisation of parameters for increased production, the ability to culture in large quantities in a short period of time and genetic engineering to increase yield [25,26].

Of all microbial sources explored for production of alkaline proteases for industrial purposes, bacteria from *Bacillus* genus have been studied the most. In fact, majority of commercialised alkaline proteases have been isolated from microorganisms of the genus *Bacillus*. Although much research has been reported on the optimisation, purification, and characterisation of alkaline proteases from *Bacillus* spp. research on the quest for microbial alkaline proteases which can remain stable and maintain activity under harsh industrial environments continues. The aim of this study was to thus investigate the potential of endophytes from *Dicomaanomala* as novel sources of proteases with industrial applications. The aim was achieved by screening of *Bacillus* sp. strain MHSD16 and *Bacillus* sp. strain MHSD17 endophytes for protease production, optimising media pH, characterisation of protease and investigation of biotechnological applications in detergents and in chicken feather degradation.

2. Materials and methods

2.1. Isolation of endophytes

Two bacterial endophytes, namely *Bacillus* sp. strain MHSD16 and *Bacillus* sp. strain MHSD17 had been previously isolated by Makuwa and Serepa-Dlamini, (2021) [27]. Glycerol stocks were prepared and stored at −80 °C.

2.2. Screening for protease production

Following overnight incubation in nutrient agar, a single colony from each bacterial strain was plated onto sterile skim milk agar plates and incubated at 30 °C for 48 hours (h). The skim milk agar contained the following: (casein 0.5 %, yeast extract 0.25 %, dextrose 0.1 %, skim milk powder 2.8 % and agar 1.5 %) [28]. Production of extracellular protease was confirmed by visualisation of a clear hydrolysis zone around the colonies.

2.3. Optimisation of pH and incubation period for protease production

The strains were fermented for production of the protease. Briefly, freshly plated colonies of each strain were inoculated into 100 mL of sterilised Luria Bertani (LB) broth at 30 °C for 24 h agitating at 150 rpm. Ten percent of the culture was inoculated into sterile protease production media containing the following (g/L): glucose 10g, casein 5g, yeast extract 5g, KH_2PO_4 2g, Na_2CO_3 10g [29]and pH adjusted to 7.5, 8 and 9 separately. The cultures were grown for a period of 7 days in different flasks for each strain, at 30 °C agitating at 150 rpm. Flasks were removed daily and the media with cell growth decanted into sterile falcon tubes then centrifuged at 8000 rpm for 10 minutes (min) at 4 °C in order to harvest the crude alkaline protease. The cell free supernatant which contained the crude enzyme extract was kept on ice and used for further experiments.

2.4. Protease activity assay

For quantification of the alkaline protease activity, the Folin's Ciocalteau method was used with slight modifications [30]. Briefly, 2 % casein (Sigma-Aldrich) was prepared in 50 mM NaOH-Glycine buffer, at pH 10.5. In an Eppendorf tube, 3 mL of the casein was added to 1 mL of crude enzyme. For the control, no enzyme was added in the Eppendorf tube. All samples were then incubated at 37 °C for 30 min to allow for the enzyme to digest the casein. Thereafter, the reaction was stopped by the addition of 4 mL of cold 0.4 M trichloroacetic acid (TCA). The samples were centrifuged at 8000 rpm for 10 min at 4 °C. From the supernatant, 1250 μL was drawn and added to 250 μL of 0.4 M Na_2CO_3 and 250 μL of 10-fold diluted Folin's Ciocalteau reagent. The resulting mixture was then incubated for 30 min in the dark at 37 °C and the absorbance was measured at 680 nm. To determine the unknown concentration of tyrosine liberated during the enzymatic reaction, a tyrosine standard was conducted according to Sigma-Aldrich protocol. One unit (U) of enzyme activity was defined as the amount crude enzyme utilised to release 1 μg of tyrosine.

Enzyme activity was calculated using the following formula [31]:

$$X = \frac{(\mu moles\ of\ tyrosine\ released) \times V1 \times V2 \times n}{\mathrm{V3} \times t.}$$

μmoles of tyrosine released, obtained from the standard curve.
V1 is the total volume used in assay (substrate + enzyme + TCA) in mL.
V2 is the total volume of enzyme used in mL.
n is the dilution factor of the test sample
t is the reaction time in min.
V3 equals to the amount used in colorimetric detection.

2.5. Characterisation of crude enzyme

2.5.1. The effect of pH and pH stability

The optimal pH for the crude enzyme activity was determined according to Si et al., 2018 [32]at pH range of 7.5 and 12. The following buffers were prepared at 0.1 M: phosphate-citrate buffer, pH 7.5, Tris-HCl pH 8–9, and glycine-NaOH pH (11–12) and 2 % (w/v) of casein was added to each buffer. The pH stability was determined by pre-incubating the enzyme without substrate, in the above-mentioned buffers for 24 h at room temperature [33]. The residual activity was then measured under standard assay conditions. A control reaction was conducted in each case, without the addition of the crude enzyme.

2.5.2. The effect of temperature and thermostability

Determination of the optimal temperature for enzyme activity was conducted according to (Farhadian et al., 2019) [34]. Briefly, the effect of temperature was studied between a range of 30–80 °C. The enzyme was incubated with 2 % casein at varying temperatures (30–80 °C) for 30 min. Thereafter, the enzyme activity was determined under standard assay conditions. Thermostability of the enzyme was determined by pre-incubation of the enzyme between 40 and 60 °C for 120 min. Aliquots were drawn at every 30-min interval and the residual activity was measured.

2.5.3. The effect of surfactants

The ability of the crude enzyme to maintain activity in the presence of surfactants was assayed by pre-incubating the enzyme with different surfactants prepared at varying concentrations; 1 and 5 % of ionic and non-ionic surfactants (Tween 80, Triton X-100, and sodium dodecyl sulphate (SDS)) were prepared. The crude enzyme extract was pre-incubated in the presence of these surfactants for 30 min at 40 °C and the residual activity was determined by performing the standard protease assay. A control sample was prepared in the absence of surfactants [35].

2.5.4. The effect of inhibitors

The effect of different protease inhibitors was investigated by pre-incubating the crude enzyme with varying concentrations of inhibitors. Briefly, 1 mM and 5 mM of the following inhibitors: β-mercaptoethanol, ethylenediamine tetra-acetic acid (EDTA), and dithiothreitol (DTT) were used [36]. Equal ratio of inhibitor to enzyme were pre-incubated at 37 °C for 30 min. Samples without the inhibitors were taken as controls. Following this, the residual activity was determined according to the standard enzyme assay.

2.5.5. Compatibility with detergents

To determine the crude enzyme's compatibility with detergents, the enzyme was pre-incubated in the presence of liquid and solid commercial detergents. Powder laundry detergents: OMO® and Sunlight® (Unilever, South Africa) were prepared at a concentration of 7 mg/mL while liquid detergents Sunlight® dishwashing liquid and Handy Andy® multipurpose cleaner (Unilever, South Africa) were prepared at a concentration of 1 % (v/v). The endogenous enzymes within these detergents were first deactivated by incubation at 65 °C for 1 h, thereafter, the detergents were pre-incubated with the crude enzymes at 37 °C for 1 h. The residual activity was determined by conducting the standard protease assay, samples without the detergents were taken as negative control [37].

2.5.6. Stain removal efficacy

To determine the crude enzyme's potential as an additive in laundry detergent and its efficacy in removal of protein rich stains,

pieces of white cotton cloths (4 × 4cm) were cut and stained with 500 μL of laked horse blood and subjected to different treatments (with or without the enzyme) according to previous studies [21,38] with some modifications. After the cloths had been stained, they were then dried in the oven dryer at 40 °C for approximately 5 h. Following this, the cloths were transferred into different falcon tubes with each falcon tube containing the different treatments. The following treatments were used: 20 mL of tap water, 20 mL of enzyme only from the two different endophytes at 452 U/mL and 232 U/mL for *Bacillus* sp. strain MHSD16 and *Bacillus* sp. strain MHSD17, respectively. In addition, commercial *Bacillus licheniformis* protease, 15 mL of heat deactivated OMO® (Unilever, South Africa) detergent and 5 mL of enzyme and 20 mL of non-heat deactivated OMO® at 7 mg/mL. The falcon tubes containing the different solutions and stained cloths were then incubated at 25 °C for 30 min agitating at 80 rpm to simulate washing. After 30 min, cloths were rinsed under running tap water and dried at room temperature (±25 °C) overnight. Pictures were then taken to visualise the cleanliness of the cloths.

2.5.7. Efficacy in chicken feather degradation

White broiler chicken feathers were collected from a poultry farmer in Cosmo City, South Africa. Whole feathers were washed three times using running tap water and rinsed in distilled water three times. The washed feathers were then dried in an oven dryer for 6 h at 37 °C. Feather degradation was done using the crude enzyme extract of the two endophyte strains: *Bacillus* sp. strain MHSD16 and *Bacillus* sp. strain MHSD17 at 232 U/mL and 452 U/mL, respectively. For enzymatic degradation, 200 mL of each crude alkaline protease was added into Erlenmeyer flasks containing 2g of chicken feathers. The negative control contained 2g of feathers in 200 mL of distilled water. All flasks were incubated at 30 °C agitating at 150 rpm for 5 days. After 5 days, the solutions containing the feathers were filtered using 90 mm, grade 292 filter papers (Sartorius). The non-degraded feathers were then dried at room temperature overnight and weighed the following day to determine feather weight loss. The following formula derived from previous studies [28, 39] was used to determine the percentage of feather degradation:

$$\%\ Degradation = \frac{TF - RF}{\mathrm{TF}}\ X\ 100$$

TF is the total feather weight added initially into the flasks.

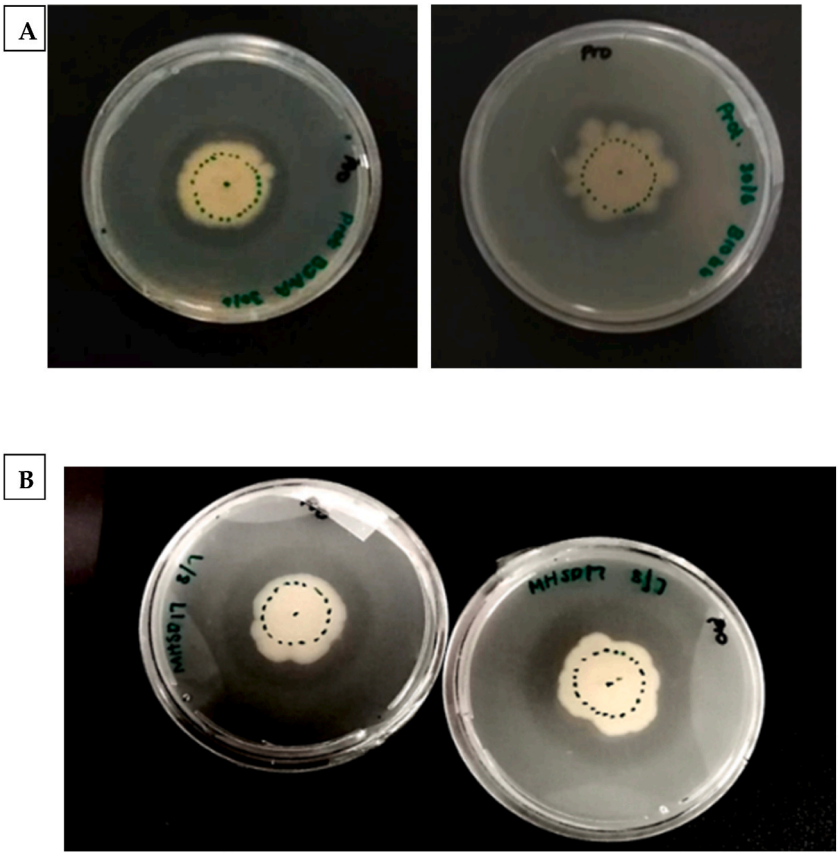

Fig. 1. The presence of hydrolysis zones, seen as transparent areas around the spread plated colonies on skim milk agar plates after 48 h of incubation at 30 °C for (A) *Bacillus* sp. strain MHSD16 and (B) *Bacillus* sp. strain MHSD17. The dotted lines indicate colony growth after 24 h of incubation.

RF is the residual feather weight after enzymatic degradation.

2.6. Statistical analysis

Each quantitative experiment was conducted in triplicates, the mean and standard deviation were calculated using Microsoft Excel, 2021 (Microsoft 365). The mean and standard deviation of the mean are reported herein, with the standard deviation indicated in error bars. Analysis of variance (ANOVA) test was also conducted using Microsoft Excel, 2021 to determine the significance of difference between the two strains, significant differences were accepted at $p < 0.05$ (Supplementary Table 1).

3. Results and discussion

3.1. Screening for protease production

Strains MHSD16 and MHSD17 showed positive production of protease. This was visualised by the presence of a clear zone around the colonies (Fig. 1). The colonies were plated on skim milk agar plates and after 24 h of incubation at 30 °C, a clear zone of hydrolysis had formed. This showed that the endophytes were able to express a protease-like enzyme and hydrolyse the protein in milk, casein. Casein is responsible for the white color in milk and when hydrolysed, it turns transparent. This thus confirms the production of extracellular proteases by the bacteria. From (Fig. 1A and B) below, it is evident that the *Bacillus* sp. strain MHSD17 strain exhibited higher proteolytic activity than the *Bacillus* sp. strain MHSD16, as seen by a wider zone of hydrolysis, which was measured to be ±1.5 cm after 48 h.

3.2. Optimisation of pH media and incubation period for protease production

The environment in which micro-organisms are cultured has been shown to play a vital role in the production of enzymes, transport of substances across the cell membrane and other metabolic processes [1]. It is therefore pertinent to optimise the pH for increased yield of protease production. Additionally, due to the close relationship between protease production and the use of nitrogenous components in the media, the fluctuation of media pH during the fermentation process might be a key indicator of the beginning and end of protease production [40]. Herein, bacterial endophytes were cultured in media at three different pH values of 7.5, 8 and 9. An increase in protease production was noted with an increase in media pH. Optimal protease production was achieved at pH of 9. High production was also noted at pH of 7.5, however, the production yield reached optimal levels at pH 9. This suggested that both strains MHSD16 and MHSD17 endophytes were viable in alkaline conditions and capable of alkaline protease production. The results obtained

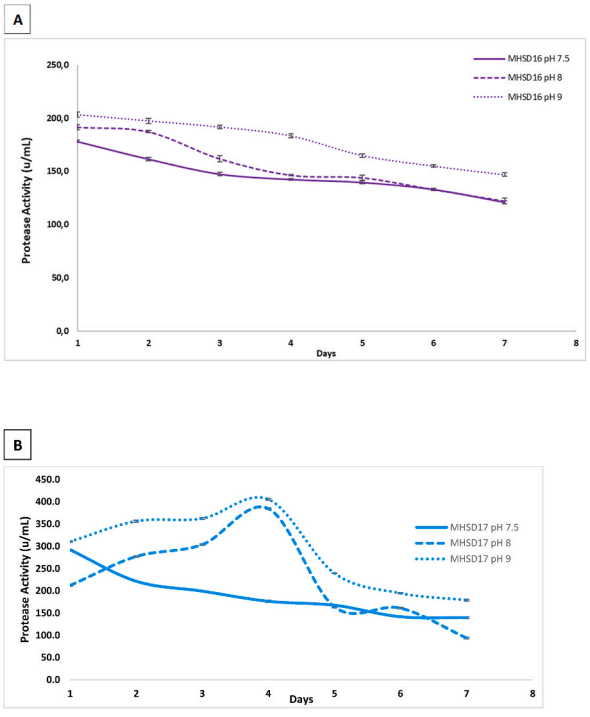

Fig. 2. The effect of initial media pH and incubation period on protease production in *Bacillus* sp. strain MHSD16 **(A)** and *Bacillus* sp. strain MHSD17 **(B).** Cultures were grown in media with a pH range starting from 7.5 to 9.0.

here are similar to those reported in a previous study [29], where maximum protease production obtained was at pH 9. Similarly, studies conducted previously [41,42] in *Bacillus* sp. APP1 reported optimal protease production at pH 9.

Incubation period was optimised concurrently with media pH. This parameter has a crucial impact on enzyme production and was reported to be dependent on other variables such as inoculum size, the type of microorganism under study and temperature. Herein, the effect of incubation period was studied over 7 days. It can be seen from (Fig. 2A), after one day of incubation, *Bacillus* sp. strain MHSD16 showed optimal protease production. As incubation period progressed, there was a linear decline in the production of protease, with day 7 having the lowest protease production. In contrast, *Bacillus* sp. strain MHSD 17 exhibited exponential enzymatic production from day 1 to day 3, with the 4th day showing optimum production. After day 4, there was a gradual decrease in enzymatic production, the lowest production was recorded after 7 days of incubation (Fig. 2B). The decline in protease production in the latter stages of the fermentation process could be linked to the potential decrease or depletion of nutrient sources or accumulation of toxic by-products [43]. Results of strain MHSD16 are similar to those previously reported [44] for *Bacillus licheniformis* and for *Bacillus pumilus* where maximum alkaline protease activity was observed after 24 h of incubation. Results obtained for strain MHSD17 coincide with those reported for *Bacillus licheniformis* [45], and *Bacillus tropicus* LS27 [43], where optimal protease production was recorded after 96 h of incubation. Information on optimal incubation period varies in the literature with some studies reporting optimal protease production after 24 h [46,47], 40 h [48], and for some keratinases, 72 h of optimal incubation period has been reported [39] while another study reported optimal keratinase production after 5 days of incubation [49].

3.3. Characterisation of crude alkaline protease

3.3.1. The effect of temperature on enzyme activity and temperature stability

Enzymes which show activity in high temperature conditions are ideal for industrial purposes. Moreover, the ability to maintain stability in high temperatures is an ideal feature for industrial enzymes [50,51]. In this study, the crude alkaline protease extracts from both endophytes showed appreciable activity in the range between 40 and 60 °C with maximum activity seen at 50 °C. At the optimum activity temperature of 50 °C, the crude enzymatic extract of the *Bacillus* sp. strain MHSD17 showed almost double the protease activity (452.9 U/mL) (Fig. 3A), compared to the extract of the *Bacillus* sp. strain MHSD16 (232.4 U/mL. Optimal protease activity at 50 °C was also reported by studies previously conducted [30] in *Bacillus* sp. DEM07, and [52] in *Bacillus circulans.*

Protease activity gradually decreased beyond 50 °C (Fig. 3A), reaching the lowest activity at 80 °C. A decline in activity at higher temperatures is due to the conformational changes in catalytic sites which disrupts intramolecular forces that are essential for proper folding of proteins [53,54]. Thermal stability was studied between 40 and 60 °C for 2 h (Fig. 3B), and residual activity was determined

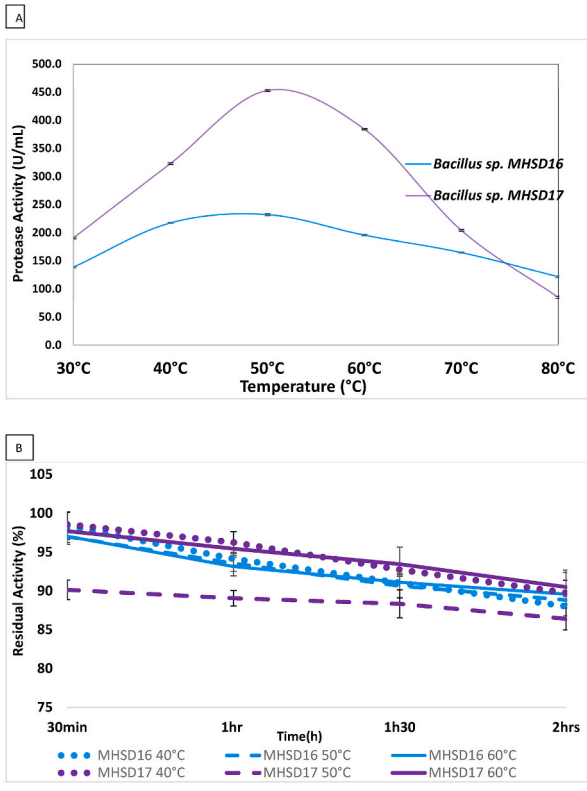

Fig. 3. (A) shows the temperature activity of the crude proteases between 40 and 80 °C. Both proteases have maximum activity at 50 °C. The temperature stability is shown in (B), and *Bacillus* sp. strain MHSD17 had the highest thermostability at 40 °C.

at 30-min intervals over the 2 h duration. Notable thermal stability was observed in both strains MHSD16 and MHSD17 crude protease extracts. For strain MHSD16, over 90 % of activity was observed after 90 min of incubation at temperatures between 40 and 60 °C. However, after 120 min of incubation, residual activity was above 80 %. Strain MHSD17 crude protease also exhibited exceptional thermal stability. Over 90 % of activity was observed after incubation for 90 min at 40 °C, while 89 % activity was observed after 2 h. At 50 °C, over 86 % activity was observed after 2 h incubation while at 60 °C, 90 % of activity was observed after the 2 h incubation period. This strongly suggested that the enzyme was thermostable and could be used in mild to harsh temperature conditions. When compared to partially purified protease BAKer from *Bacillus* sp. AD-AA3 [55], where the residual activity after 2 h of incubation was 65 % and 18 % at 40 °C and 50 °C, respectively, the proteases herein indicate thermostability.

3.3.2. The effect of pH on enzyme activity and pH stability

The effect of pH on enzyme activity was studied at a range between 7.5 and 12. The crude alkaline protease extract was assayed in substrate prepared in different pH buffers. The results obtained after the protease was assayed at different pH levels are shown in (Fig. 4A). There was an increase in enzymatic activity from 7.5 to 9, with maximum activity observed at pH 10 for both strains MHSD16 and MHSD17 proteases. After pH 10, there was a decrease in enzyme activity. Optimal enzymatic activity in *Bacillus mojavensis* A21 [56], *Bacillus* sp. DEM07 [54], protease BPKER from *Bacillus* sp [55]. and protease from *Bacillus subtilis* RD7 [35]. For an enzyme to be a suitable candidate for laundry detergent additives, activity in alkaline environments is crucial [57]. This suggested that the above proteases could be key candidates for additives in laundry detergents. In an extreme alkaline condition of pH 12, a decline in enzymatic activity and stability was observed in proteases from *Bacillus* sp. strain MHSD16 and strain MHSD17. The enzymes maintained only 47 % and 45 % (Fig. 4B) of their initial activity. However, the pH stability herein is much greater than that reported previously [58]. In these studies, the half-lives of the proteases at optimum pH were at 18 h. In comparison, protease from *Bacillus* sp. strain MHSD16 retained over 50 % of its relative activity after 24 h while that of strain MHSD17 retained 47 % of its relative activity after incubation at optimal pH 10.5 for 24 h. This suggested that the proteases studied herein were more stable than those studied previously [58,59].

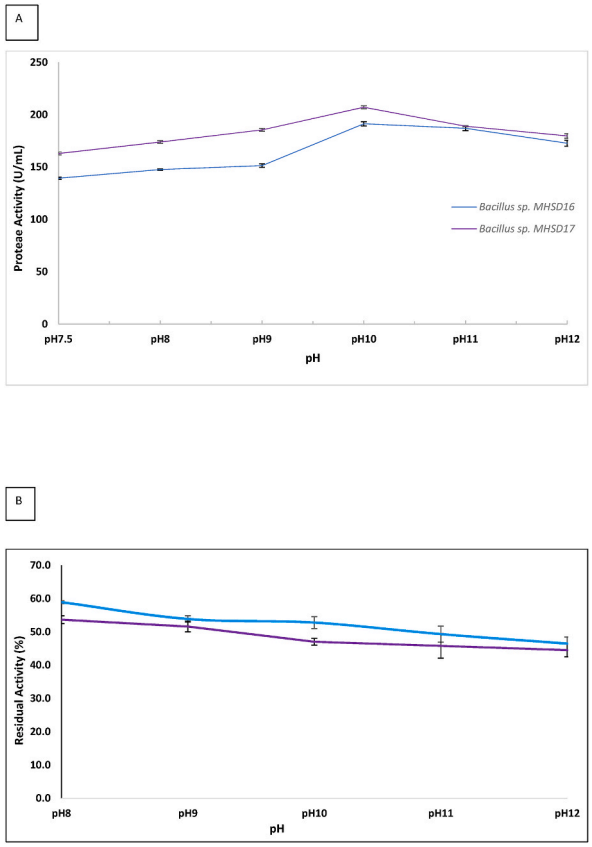

Fig. 4. The effect of pH on enzymatic activity. (A) shows that proteases from both *Bacillus* sp. strains MHSD 16 and MHSD 17 have optimal activity at pH 10. (B) demonstrates the proteases' residual activity after being pre-incubated in different buffers overnight at ±25 °C. *Bacillus* sp. strain MHSD16 showed the highest stability in all pH ranges in comparison to protease from *Bacillus* sp. strain MHSD17.

3.3.3. Effect of surfactants on enzyme activity

Enzymes need to be active in the presence of surfactants to be considered potential detergent additives [9], laundry detergent formulations can constitute up to 50 % of surfactants. Surfactants are amphiphilic molecules, and are used for their foaming, solubilising, wetting, dispersing and emulsifying properties [59]. Herein, the effect of surfactants on enzyme activity was studied by pre-incubating the enzyme in the presence of non-ionic surfactants (Tween 20 and Triton X-100) and anionic surfactant; sodium dodecyl sulphate (SDS) for 30 min at 37 °C, thereafter, the residual activity was measured by the standard enzyme assay. For strain MHSD16 protease (Fig. 5), above 70 % of activity was observed after incubation in the presence of 1 % and 5 % Tween 20 while 79 % and 66 % of enzyme activity was observed in the presence of 1 mM and 5 mM Triton X-100, respectively. The protease showed appreciable activity in the presence of these surfactants. On the other hand, SDS, an anionic surfactant, decreased the enzyme activity the most, with only 64 and 58 % of enzyme activity observed after pre-incubation with 1 and 5 %, respectively. For strain MHSD17 (Fig. 5) protease, 93 % of activity was observed in the presence of 1 % Tween and 54 % in the presence of 5 % Tween. This endophyte's protease showed exceptional activity in the presence of 1 % Tween. It also showed exceptional activity in the presence of 1 % Triton X-100, with 85 % activity observed from its initial activity after pre-incubation while 66 % of activity was observed in the presence of 5 % Triton X-100. The protease from *Bacillus* sp. strain MHSD17 retained greater activity in the presence of 1 % anionic surfactants when compared to the protease from *Bacillus* sp. strain MHSD16. However, at a concentration of 5 % Tween 20, strain MHSD16 protease retained the highest activity.

It has been reported that surfactants interact with proteases and can bring about an activation or inhibition of enzyme activity [25]. Numerous studies have shown that anionic surfactants such as SDS led to a decline of enzyme activity [60–62]. This could be because anionic surfactants induce an increase in autoproteolysis of proteases which in turn reduces their activity [63]. Another study on the effect of anionic surfactants on protease activity suggested that the decline in protease activity in the presence of anionic surfactants is because of the ability of the anionic surfactants' hydrophobic moieties to interact with the hydrophobic inner active site of proteases via attraction of electrostatic charges and van der Waals's forces [64]. This interaction may result in micelle formation thus bringing about changes in protease conformation and surface properties which then inactivates the protease [64]. In addition, SDS has also been reported to decrease enzyme activity through interaction of its charged head group with positively charged side chains of proteins [84]. Herein, strain MHSD17 protease showed exceptional activity in the presence of 1 % of Tween and Triton X-100, and appreciable activity in the presence of SDS thus making it a potential candidate for application in laundry detergents.

3.4. The effect of inhibitors and chelating agent on enzyme activity

Enzyme inhibitors are compounds which possess the ability to modify catalytic properties of enzymes, thereby slowing down rates of reactions or in some instance, inhibiting overall enzyme activity [65]. Generally, inhibitors are categorised according to the class of enzymes they target, for example, serine protease inhibitors, also known as serpins [66], target the serine class of proteases. Majority of alkaline proteases are reported to be inhibited by phenyl-methyl-sulfonyl-fluoride (PMSF) and diisopropylfluorophosphate (DFP) which target the serine class of proteases [1]. In this study, crude alkaline proteases from strains MHSD16 and MHSD17 were studied and because they were not purified, may contain various classes of protease inhibitors. Briefly, the effect of protease inhibitors on protease activity was studied by pre-incubating the enzymes for 30 min at 37 °C with β-mercaptoethanol, Dithiothreitol (DTT) and Ethylenediaminetetraacetic acid (EDTA), a chelating agent, at 1 and 5 mM concentrations and the residual activity was measured. For strain MHSD16, the addition of β-mercaptoethanol at 1 mM had almost no impact on enzyme activity as 98.4 % of activity was retained (Fig. 6). This suggested that the crude alkaline protease from strain MHSD16 was not highly dependent on the cysteine residues and

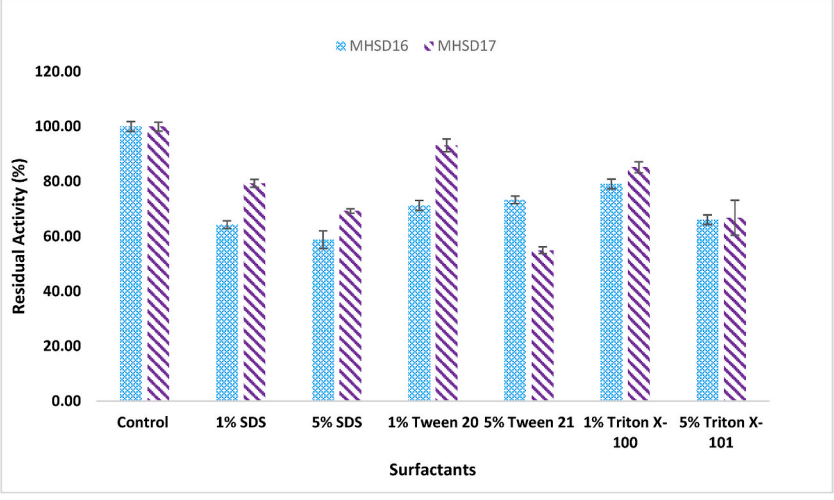

Fig. 5. The effect of ionic and anionic surfactants on enzyme activity. At 1 % concentration, the anionic surfactant (SDS) decreased enzymatic activity more than the other surfactants at the same concentration. The proteases showed the highest stability in the presence of 1 % Tween 20.

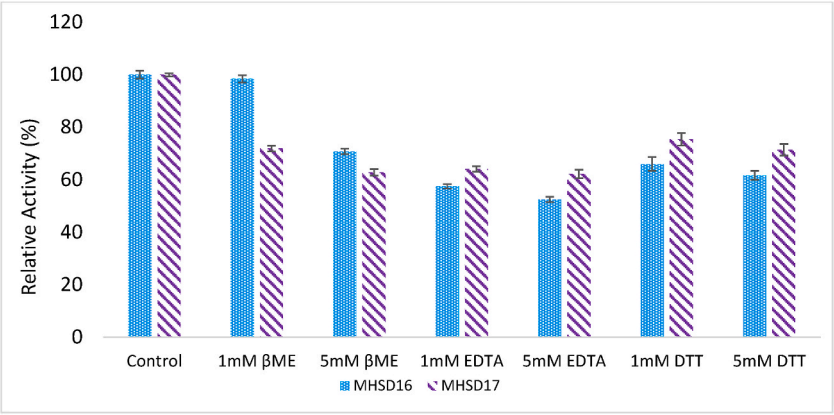

Fig. 6. The effect of protease inhibitors on protease activity. The residual enzyme activity in the control sample was taken as 100 % as it contained no inhibitors while the residual activity in other experiments were measured relative to the control sample.

sulfhydryl groups for optimal activity, or that they are further away from the active site [67]. However, at a concentration of 5 mM, 30 % of activity was inhibited. This suggested that when the concentration of β-mercaptoethanol increased, the inhibition of enzyme activity also increased. DTT is another compound which inhibits cysteine proteases like β-mercaptoethanol. It had a slightly higher inhibition ability than β-mercaptoethanol, inhibiting 34 and 39 % of enzyme activity at 1 and 5 mM concentrations, respectively (Fig. 6). This suggested that the crude alkaline protease had appreciable amounts of cysteine proteases, also known as thiol proteases. In contrast, a remarkable amount of inhibition was exerted by EDTA, reducing enzymatic activity by 43 and 48 % at 1 and 5 mM concentrations, respectively. This strongly suggested that there was a significant number of metalloproteases within this crude alkaline extract. Metalloproteases are proteases which depend on metals for their optimal activity, and are inhibited by EDTA, a metal chelator [1]. For strain MHSD17 protease, DTT and β-mercaptoethanol had almost similar effects of inhibition. The DTT inhibited enzyme activity by 29 and 35 % at 1 and 5 mM concentrations, while β-mercaptoethanol inhibited enzyme activity by 29 and 38 % at 1 and 5 mM concentrations, respectively (Fig. 6). This suggested the presence of cysteine proteases within this crude alkaline extract. In contrast, the metal chelator EDTA, reduced the enzyme activity by 38 and 34 % at 1 and 5 mM concentrations, respectively. The ability of strain MHSD17 enzyme to maintain higher activity compared to strain MHSD16 protease in the presence of EDTA makes it a better candidate for addition in laundry detergents, as EDTA is another key additive in laundry detergents, which helps with softening hard water [58].

3.4.1. Effect of powder and liquid detergents on enzyme activity

The shift towards environmentally friendlier options has seen an increase in research based on enzymes for application in detergents. An estimation of 30 % of protease enzymes are produced for incorporation in laundry detergents [22]. Proteases improve the efficacy of detergents in removal of protein rich stains. Enzyme based detergents are also beneficial due their ability to effectively remove stains at low temperatures. In addition, the enzymes have been reported to reduce water consumption during washing [62]. The compatibility of the crude alkaline proteases in this study was investigated based on the enzymes' ability to retain activity in the

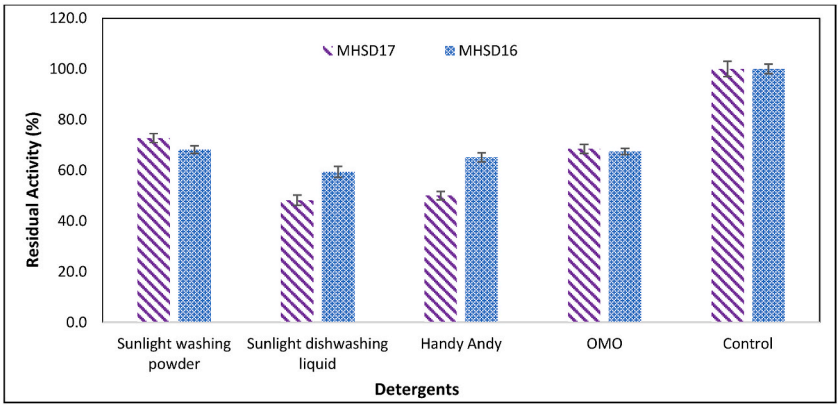

Fig. 7. Compatibility of the proteases with commercial detergents was investigated. It can be seen from the graph that the proteases maintained high stability in the presence of powder detergents (OMO® and Sunlight® washing powder) in comparison to liquid detergents (Sunlight® dishwashing liquid and Handy Andy®).

presence of various surfactants and to function optimally at alkaline pH. Briefly, 7 mg/mL of commercial detergents OMO® and Sunlight® washing powder (Unilever, South Africa) and 1 % of commercially available liquid cleaning detergents (Sunlight® dish-washing liquid and Handy Andy® multipurpose) were pre-incubated at 65 °C for 1 h to deactivate the endogenous enzymes found within these detergents [62]. Thereafter, these were pre-incubated with crude alkaline proteases for 30 min at 37 °C and the residual activity was determined by the standard protease assay. For strain MHSD16 (Fig. 7), the highest activity was obtained in the presence of powder laundry detergents at 68 and 67 % for Sunlight® and OMO®, respectively. In contrast, liquid detergents inhibited enzymatic

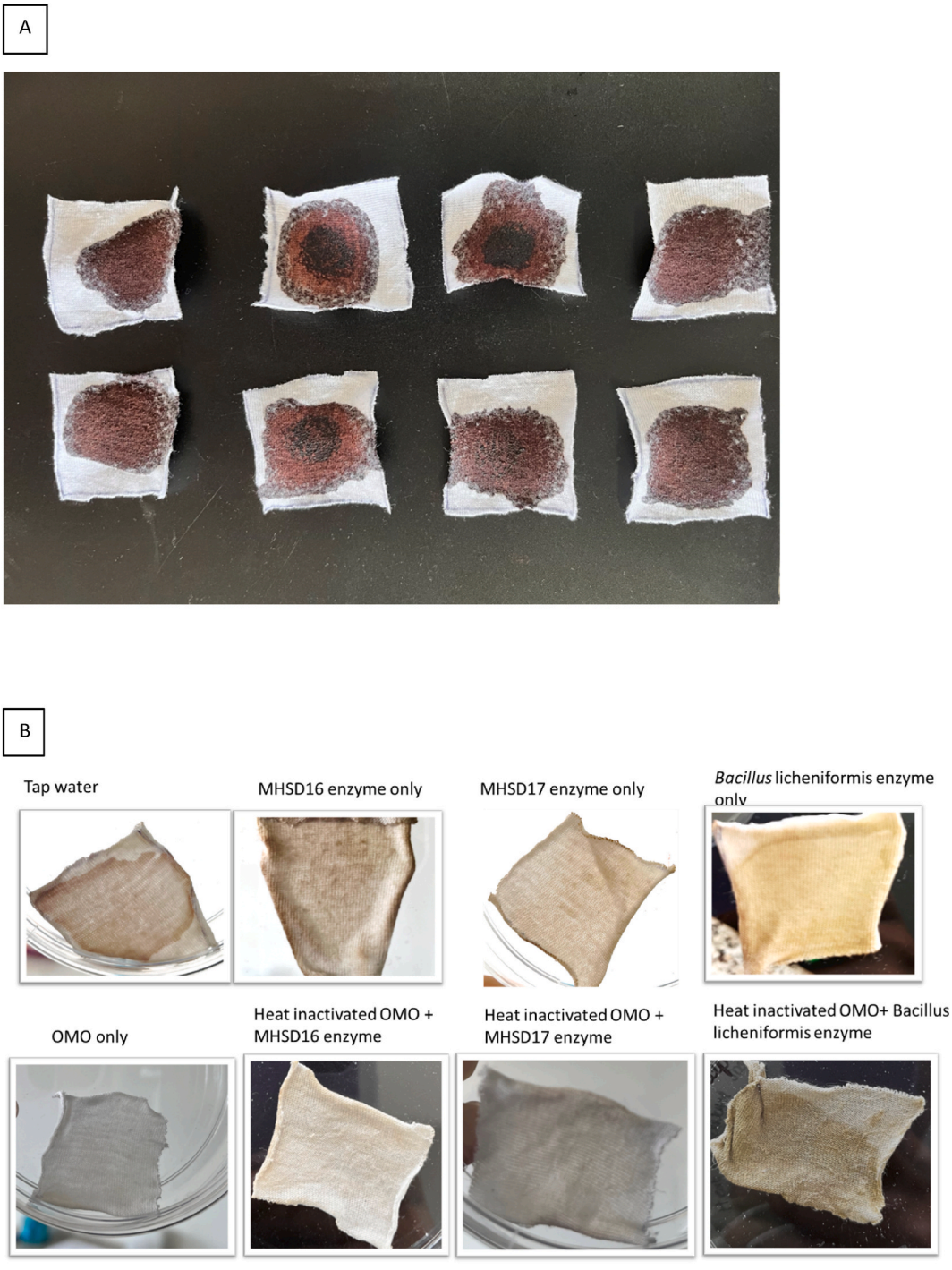

Fig. 8. (A). Depiction of cloths (4 × 4cm) with equal volumes of laked horse blood before subjection to different treatments (A) and (B) shows the cloths after subjection to various treatments. Tap water was used as a negative control and the OMO® detergent was used as a positive control.

activity more than the powder detergents, resulting in a residual activity of 65 % and the lowest at 59 % for Handy Andy® all-purpose cleaner and Sunlight® liquid dishwashing liquid, respectively. In contrast, a slightly higher activity in the presence of powder laundry detergents was obtained in *Bacillus* sp. strain MHSD17 (Fig. 7), with 72 and 68 % of activity remaining for Sunlight® and OMO®, respectively. Liquid detergents had the highest inhibitory effect on the *Bacillus* sp. strain MHSD17 protease, resulting in only 50 and 48 % of activity for Handy Andy® and Sunlight® dishwashing liquid. The differences in residual activity could be due to the difference in constituents (bleaching agents, surfactants, and chelators) of each detergent which could affect enzyme activity, such as bleaching agents, surfactants, and chelators. In addition, research has shown that autoproteolysis is highly induced in liquid detergents [62], which could explain the enhanced inhibition of enzyme activity in liquid detergents. This phenomenon has also been reported to be counteracted by addition of protease inhibitors into liquid detergents to prevent autodigestion. In the past decades, the addition of glycerine, propylene glycol, and polyethylene glycol with boric acid was a method used to reduce autodigestion [68]. Advancement in research has indicated that enzyme stability could also be enhanced by addition of ethoxylated co-surfactants in detergents [69].

3.4.2. Stain removal efficacy

The removal of protein rich stains was investigated by staining pieces of white cotton cloths (4 cm × 4 cm) with laked horse blood (Fig. 8A) and subjecting the cloths to various treatments (Fig. 8B), with or without the crude alkaline proteases. Tap water was used as a negative control and the cloth washed in OMO® washing powder as the positive control. After the wash simulation through agitation in falcon tubes, the cloths were evaluated for stain removal, cleanliness, and brightness. The cloths subjected to tap water only wash treatment had visible blood stains and were not clean, whereas the cloths treated with OMO® washing powder was the cleanest and brightest. This was expected as its main ingredients include sodium linear alkylbenzene sulfonate (LAS), sodium sulphate, sodium carbonate, and proteases as well as other enzymes such as lipases and mannase. These ingredients have surfactant, saponification abilities, which allow for thorough mixing of the detergent with water and removal of fats, oils, and protein-based stains. The proteases from *Bacillus* sp. strain MHSD16 and strain MHSD17 were much more effective in stain removal when compared to commercially available protease from *Bacillus licheniformis*, indicating their potential as additive to laundry detergents (Fig. 8B). Furthermore, the addition of proteases from *Bacillus* sp. strain MHSD16 and *Bacillus* sp. strain MHSD17 into heat inactivated OMO® produced results that are closer to those obtained from the active detergent only treatment. These observations further confirm the potential of both strains MHSD16 and MHSD17 proteases for use in laundry detergents.

Comparable results have been reported where researchers indicated the improved efficacy of blood stain removal when proteases are added into laundry detergents [70–73]. In these studies proteases were isolated from *Bacillus licheniformis* K7A, *Penicillium chrysogenium* X5, *Bacillus safensis* RH12, *Bacillus aquimaris* VITP4, respectively. We believe that proteases studied herein, isolated from *Bacillus* spp. MHSD16 and MHSD17 are equally capable of blood stain removal and are ideal candidates for addition in laundry detergents. This characteristic is valuable as enzymes are biodegradable and pose less environmental toxicity in comparison to chemicals

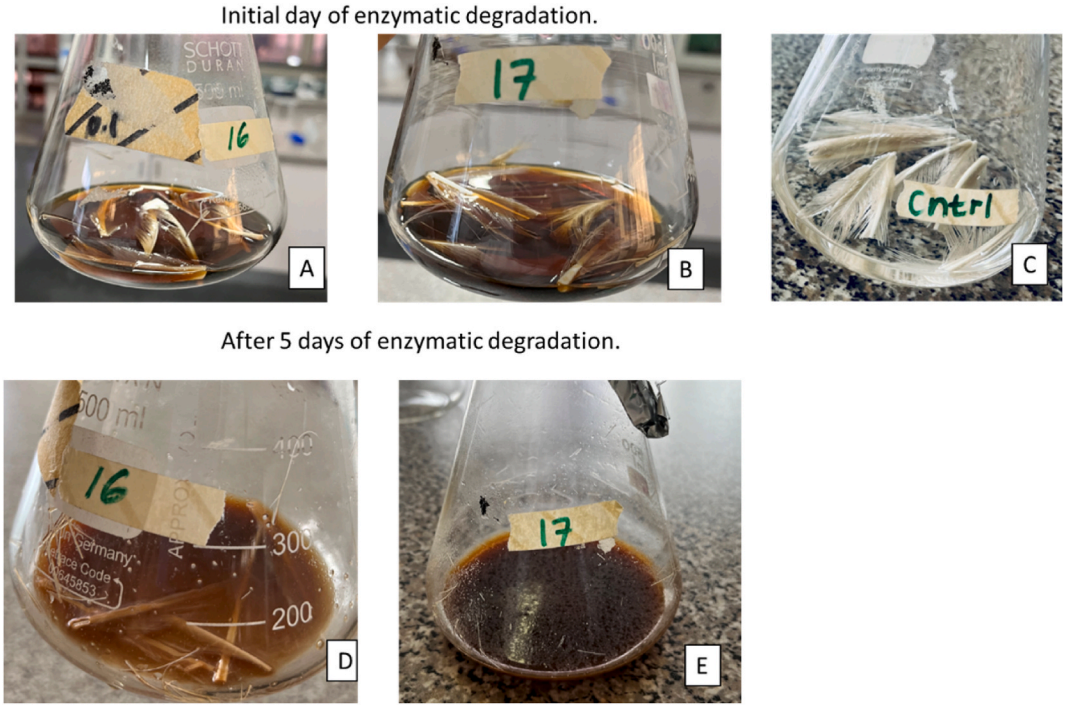

Fig. 9. (A) initial day of feather degradation in *Bacillus* sp. strain MHSD 16 and (B) MHSD17. (C) The negative control with only distilled water and (D) feather degradation after 5 days in *Bacillus* sp. strain MHSD16 and (E) *Bacillus* sp. strain MHSD17 enzymatic extracts.

used in conventional laundry detergents.

3.4.3. Feather degradation

The proteases from *Bacillus* sp. strain MHSD16 and *Bacillus* sp. strain MHSD17 were tested for their keratin degradation ability through chicken feather degradation for 5 days, wherein each crude alkaline protease was added into Erlenmeyer flasks containing feathers (Fig. 9A and B), and the negative control contained feathers in distilled water (Fig. 9C). After 5 days of incubation in crude alkaline protease from both endophytes, notable feather degradation was observed. The crude enzyme from *Bacillus* sp. strain MHSD17 led to the highest level of feather degradation amongst the two enzymes studied herein, with 80.45 % of feather degradation achieved. On other hand, feather degradation with *Bacillus* sp. strain MHSD16 enzymes resulted in 77.9 % of feather degradation (Fig. 9D). Majority of feathers treated with *Bacillus* sp. strain MHSD17 showed optimal disintegration (Fig. 9E) from the rachis and were solubilised in the crude enzyme extract and degraded by day 5. After day 5 at 30 °C agitating at 150 rpm, feathers were solubilised in the enzymatic extract, although not fully disintegrated from rachis as other feathers were still intact. Therefore, *Bacillus* sp. strain MHSD17 crude enzyme proves to be a better candidate for potential application in feather degradation.

Various methods of feather degradation have been studied with results of complete degradation varying from 24 h to days. These methods include inoculation of bacterial cultures into whole chicken feathers [74,75] while others have used heat and chemical treatment in addition to bacterial degradation [76,77]. Other studies, like herein, have used enzymatic extracts from bacteria for feather degradation. The *Bacillus mojavensis* A21 protease demonstrated the ability to completely degrade feathers after 24 h at 50 °C with 150 rpm agitation [37]. However, an addition of 2 % (w/v) sodium azide and 5 mM calcium chloride was used to enhance thermostability of the protease, therefore, could have contributed to the rapid feather degradation as the enzyme's stability in the presence of heat (50 °C) was maintained. Similarly, feather degradation by crude protease extract from *Bacillus halodurans* JB 99 [77] supplemented with 0,1 % β-mercaptoethanol enhanced the rate of feather degradation, resulting in up to 85 % degradation after 24 h, leaving the basal shaft (rachis) intact. In comparison to their experiment where β-mercaptoethanol was not used, feather degradation with β-mercaptoethanol supplementation yielded better results [78]. This is because addition of β-mercaptoethanol reduced the disulphide bonds present in keratin, thus increasing the feathers' disintegration rate. On the other hand [38], reported disintegration of feathers after 24 h and complete solubilisation using enzymatic extract of *Bacillus cereus*. However, it is worth noting that there is a distinction between 'disintegration' of chicken feathers and 'complete dissolution' of molecular keratin [79,80].

Chicken feather degradation happens optimally in the presence of live cells and reducing agents such as β-mercaptoethanol [80]. Herein, we have reported feather degradation in a cell-free environment and with the absence of reducing chemicals. Having achieved 80.45 % of feather degradation, crude enzyme extract from *Bacillus* sp. strain MHSD17 has a potential for exploration as a feather degrading enzyme (Fig. 9E).

4. Conclusion

This study has shed light on the potential industrial applications of protease extracts from *Bacillus* sp. strain MHSD16 and *Bacillus* sp. strain MHSD17 endophytes and their use as environmentally friendlier alternatives for feather degradation and bio-additives in detergents. Characterisation of the crude extracts has shown that the proteases studied herein are active in alkaline pH conditions and show appreciable thermostability while also being active in the presence of surfactants. Additionally, these crude enzymes are compatible with household and laundry detergents. Investigation of blood stain removal has indicated that the crude extracts can be applied in detergent formulations. Finally, investigation of feather degradation revealed that crude protease extract of *Bacillus* sp. strain MHSD17 was more efficient in feather degradation in comparison to that of *Bacillus* sp. strain MHSD16. The results from this study have thus helped to identify endophytes as novel sources of proteases with useful industrial applications.

Data availability statement

No data was used for the research described in the article.

CRediT authorship contribution statement

Malese Elaine Mankge: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Mehabo Penistacia Maela:** Writing – original draft, Methodology. **Adrian Mark Abrahams:** Writing – review & editing, Supervision, Investigation, Data curation. **Mahloro Hope Serepa-Dlamini:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Mahloro Hope Serepa-Dlamini reports a relationship with National Research Foundation that includes: funding grants. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e30736.

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