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Bacillus amyloliquefaciens Alkaline Protease: Potential Applications and Stabilization

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Abstract

Bacillus amyloliquefaciens alkaline protease (AL-PR) was partially purified and a 2.55 purification fold was achieved at 60% ammonium sulfate. The partially purified AL-PR was then characterized with respect to its presented activities and stabilities at variable pHs and temperatures. The partially purified AL-PR was slightly inhibited by PMSF(serine proteases inhibitor) and was completely inhibited by both EDTA (a general metalloprotease inhibitor) and 1,10 phenanthroline (a zinc metalloinhibitor), These results indicated that the enzyme might be a Zn metalloprotease. The partially purified AL-PR offered antimicrobial activity versus *Bacillus cereus, Pseudomonas aeruginosa*, and *Candida albicans*. It also offered anti-oxidant activity against DPPH with an IC_{50} of 0.59 mg/ml. Moreover, the partially purified AL-PR was proven to be safe to normal skin cells (BJ1) and it also promoted wound healing. Thus, the partially purified AL-PR has potential for wound healing applications. In order to further promote its application, it was stabilized via conjugation with variable oxidized polysaccharides. Conjugation with oxidized DEAE dextran and carboxymethyl cellulose elevated the thermal stability of the partially purified AL-PR. Nonetheless, CMC imparted a bigger stabilizing effect as it caused the AL-PR $t_{1/2}$ and D-values (at 60°C) to be 5.97 fold raised.

Keywords:Alkaline protease; partial purification; antimicrobial; wound healing; stabilization

1. Introduction

Microbial proteases show various applications in different industries [1]. They are used as therapeutic agents and have an important place in the pharmaceutical industry [2]. The microbial protease enzymes are used as inflammation inhibitors, anticancer medicines, thrombolytic agents, and anticoagulants [3]. In addition, they have antibacterial activity [4,5,6]. They also serve as reagents in wound debridement to remove dead cells and treat damaged tissues [7]. Moreover, proteases have been shown to damage cell surface proteins and degrade newly formed extracellular matrix [8]. Therefore, the microbial production of highly active alkaline proteases with novel properties has been stated by many researchers [9-11].

Nonetheless, enzymes exhibit limited stability [12]. Thus, variable approaches were previously adopted to elevate enzymes stability. These included; immobilization [13,14], and incorporation of additives [15]. The additives added to stabilize enzymes could be intended to physically interact with

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them [15] or to be chemically conjugated to such enzymes. Chemical conjugation of enzymes with polysaccharides would create new inter- and intra-molecular cross-links. These multipoint cross-links would rigidify the enzymes configuration [12]. Moreover, the conjugated polysaccharides would promote enzymes hydration. These effects would eventually elevate enzymes stability [16].

Accordingly, in this study, the alkaline protease (AL-PR), produced via *Bacillus amyloliquefaciens* NRC-IB-1, was partially purified via variable methods. Afterwards, its cytotoxicity on human normal skin fibroblasts, antioxidant activity, anti-microbial activity, and wound healing activity were investigated. Finally, the protease was stabilized via conjugation with variable polysaccharides.

2. Materials and methods

2.1. Materials

Bacillus amyloliquefaciens strain NRC-1B-11 16S genetic identification was based on (16S rRNA) studies by sigma services company as following: DNA was extracted by using the protocol of Gene JET genomic DNA purification Kit (Thermo K0721), submitted in a gene bank and registered under accession number: PP034178.1.All chemicals were Analar or of analogous quality.

2.2. Methods

2.2.1. Maintenance and inoculum preparation

B. amyloliquefaciens NRC-1B-11 16S was grown and maintained by sub-culturing on nutrient agar slants at 37°C. To prepare the inoculum a loop full of bacterial culture was transferred to sterile nutrient broth (50 ml) then incubated at 35°C, 160 rpm for 24 h. Bacterial strain were routinely grown on nutrient broth medium at 35°C for 24 h and preserved at -80°C in 50%(v/v) glycerol and cultured every 3 months for maintenance [17].

2.2.2. Alkaline protease production process and extraction of the crude alkaline protease

The previously prepared inoculum (10%) [17] was inoculated into production medium containing soy bean 5g/50 ml, distilled water , pH10 in conical flask and incubated at 35°C on a shaker adjusted at 160 rpm, for 3 days [17]. The contents were then centrifuged at 10,000 rpm at 4˚C for 15 minutes. The supernatant containing crude alkaline protease (AL-PR) was used for enzyme assays.

2.2.3. Assay of AL-PR activity

The AL-PR activity of the culture supernatant was determined according to the method presented by Amin et al. [17] by using casein as a substrate. A mixture of 500 µl of 1% (w/v) of casein in 50 mM phosphate buffer, pH 8, and 200 µl enzyme were incubated in a water bath at 40°C for 20 minutes. Then, the enzyme reaction was terminated by the addition of 1 ml of 10% (w/v) trichloroacetic acid (TCA) and was kept at room temperature for 15 minutes. The reaction mixture was centrifuged to separate the un-reacted casein at 10,000 rpm for 5 minutes at 4°C. The supernatant was mixed with 2.5 ml of 0.4 M Na_2CO_3. One ml of 3-fold diluted Follin Ciocalteu phenol reagent was then added. The resulting solution was incubated at room temperature for 30 minutes and absorbance of the blue color developed was measured at 660 nm against a reagent blank using a tyrosine standard [18]. The Optical densities were converted to the equivalent grams of tyrosine using a standard curve of authentic tyrosine concentration (10-100 micrograms/ml) that was treated similarly to experimental enzyme-catalyzed reaction mixtures.

The enzyme activity was expressed in terms of enzyme units. One unit of protease was defined as the amount of enzyme that releases one microgram of tyrosine per ml enzyme per minute under the standard conditions of the assay.

2.2.4. Quantitative estimation of protein

The quantitative estimation of protein was determined by the method of Lowry et al. [18] using bovine serum albumin as the standard.

2.2.5. Partial purification of AL-PR

The clear culture filtrate obtained by growing *B. amyloliquefaciens* in the fermentation medium was precipitated by ethanol, acetone, and ammonium sulfate to select the most suitable ones.

2.2.5.1. Fractional precipitation by ethanol or acetone

The crude enzyme was precipitated by cold ethanol or acetone at concentrations of 20%, 40%, 60%, and 80% at 4°C using magnetic stirring. Precipitates of the fractions were dissolved in 10 ml. of distilled water and finally, the

protease activity and protein content were determined in each fraction as mentioned before.

2.2.5.2. Fractional precipitation by ammonium sulfate

The crude protease was precipitated from the supernatant using solid ammonium sulfate (20–80%) under cooling and continuous stirring. The precipitated protein was dissolved in a small amount of 50 mM phosphate buffer (pH 8) to obtain a concentrated enzyme solution. The dissolved protein was dialyzed with the same buffer for 24 h at 4 °C to remove ammonium sulfate. The fractions were saved at -20°C for protein and enzyme activity determination.

2.2.6. Characterization of the partially purified enzyme

2.2.6.1. Effect of pH on enzyme activity and stability

The optimum pH for AL-PR activity was tested in the reaction mixture at a pH range of (3-10) using 50 mM sodium citrate buffer (pH 3-5), sodium phosphate buffer (pH 6-8), and Tris-HCl buffer(pH 9-10).

For pH stability, the enzyme preparations were pre-incubated at different pH (3-10) with different buffer concentrations (0.05, 0.1M) for 24 h at 4 ˚C. Then, the residual activity was determined.

2.2.6.2. Effect of temperature on activity and stability

The optimal temperature for the enzyme activity was measured by incubating the reaction mixtures at different temperatures (30-65˚C). Thermal stability assays were conducted by pre-incubating the enzyme at different temperatures of 30, 40, 50, and 60 ˚C for different incubation periods of 15, 30, and 60, and the relative activity was tested. The unheated enzyme was to define 100% activity.

2.2.6.3. Effect of protease inhibitors on enzyme activity

To characterize the mechanism of the partially purified AL-PR [19], it was incubated for 10 min at 50°C in the presence of phenylmethanesulfonyl fluoride (PMSF), ethylendiaminetetraacetic acid (EDTA) and 1,10-phenanthroline (2, 4, 6, 8, and 10 mM). The activity assayed in the absence of protease inhibitors was defined as the reference control and the residual activities were estimated.

2.2.7. Potential applications of partially purified AL-PR enzyme

2.2.7.1. .Antimicrobial activity

Qualitative evaluations were carried out in nutrient agar plates. The inoculations of pathogenic microorganisms used in this study were Gram-positive bacteria [*Bacillus cereus (*ATCC 6629) & *Staphylococcus aureus (*ATCC 6538)], Gram-negative bacteria [(*Escherichia coli* (ATCC25922), *Pseudomonas aeruginosa*(ATCC27853) & *Proteus mirabillus*(ATCC9240)], and pathogenic yeast [(*Candida albicans*(ATCC 10231)]. The inoculations were prepared from fresh overnight broth cultures using a nutrient broth medium that was incubated at 37°C [20]**.** The inoculums sizes of these pathogenic strains were adjusted to approximately 0.5 McFarland standard (1.5×10^8 CFU /ml) [21], 25.0 µl inoculum size of each microorganism strain was then separately inoculated into each plate containing 20.0 ml of the sterile nutrient agar medium (NA). AL-PR samples were prepared at 3mg/ml protein content. These samples were either applied as such (100%) or diluted as 25%, 50%, and 75% before being applied into 0.9 cm well of the inoculated agar plates (agar well diffusion method). The seeded plates were placed in the refrigerator for one hour for more diffusion of these samples, followed by incubation at 37 ºC for 24 h and zones of inhibition (ZI) were measured in mm**.** The samples were passed to another test for the (%) reduction in colony forming unit CFU. This was performed by applying different protein concentrations of the AL-PR (0.75, 1.5, 2.25, and 3 mg/ml) on the tested pathogenic strains using the shake flask method and then the (%) reduction in bacterial count was estimated via the turbid metric measurement technique. The CFU of the tested strain after being treated with the AL-PR sample compared to the number of the microorganism cells surviving in the control flask after 24 hours incubation period and at 37 ºC [22]. All results were expressed according to the following equation

Relative Reduction (%) = (A- B /A) × 100

A: the number of microorganisms present in the control flask contains pathogenic strains only without any treatment .

B: the number of microorganisms present in tested flasks after applying tested sample concentrations of the sample.

2.2.7.2. Anti-oxidant activity using DPPH radical scavenging ability

The AL-PR was assayed for its anti-oxidant activity using DPPH radical-scavenging activity at different concentrations (0.5: 3 mg/ml) of the enzyme.

The scavenging activity for DPPH free radical was measured according to Zhao et al. [23] with some modifications. Samples (100µl) were mixed with 900µl of 0.1 mM DPPH solution in methanol. The mixture was shaken vigorously and allowed to reach a steady state for 30 min in dark at a temperature of 37ºC. Decolourization of DPPH was determined by measuring the absorbance at 517 nm, and the DPPH radical scavenging was calculated according to the following equation:

% Scavenging activity = $(A_1-A_2/A_1) \times 100$

Where $\mathbf{A_1}$ was the absorbance of the DPPH solution without extract and $\mathbf{A_2}$ was the absorbance of DPPH with the extract. Ascorbic acid was taken as the standard. All the tests were performed in triplicate.

2.2.7.3. Cytotoxicity on human normal skin fibroblast cell line (BJ1)

The cytotoxicity was assessed by MTT assay (the mitochondrial-dependent reduction of yellow MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide)) to purple formazan [24].Procedure: All the following procedures were done in a sterile area using a Laminar flow cabinet biosafety class II level (Baker, SG403INT, Sanford, ME, USA). Cells were suspended in Dulbecco's Modified Eagle Medium /Nutrient mixture F-12 (DMEM-F12) with1% antibiotic-antimycotic mixture (10,000U/ml Potassium Penicillin, 10,000µg/ml Streptomycin Sulfate and 25µg/ml Amphotericin B) and 1% L-glutamine at 37 ºC under 5% CO_2. Cells were batch cultured for 5 days, then seeded at a concentration of 10 $x10^3$ cells/well in fresh complete growth medium in 96-well microtiter plastic plates at 37 ºC for 24 h under 5% CO_2 using a water-jacketed Carbon dioxide incubator (Sheldon, TC2323, Cornelius, OR, USA). Media was aspirated, fresh medium (without serum) was added, and cells were incubated either alone (negative control) or with different concentrations of the sample (0.78 to 100 µg/ml) (the partially purified alkaline protease enzyme) to give a final concentration of (100 µg/ml). After 48 h of incubation, the medium was aspirated, 40µl MTT salt (2.5µg/ml) was added to each well and incubated for a further four hours at 37ºC under 5% CO2. To stop the reaction and dissolve the formed crystals, 200µL of 10% Sodium dodecyl sulfate(SDS) in deionized water was added to each well and incubated overnight at 37ºC. A positive control composed of 100µg/ml was used as a known cytotoxic natural agent that gives 100% lethality under the same conditions [25].

The absorbance was then measured using a microplate multi-well reader (Bio-Rad Laboratories Inc., model 3350, Hercules, California, USA) at 595nm and a reference wavelength of 620nm. A statistical significance was tested between samples and negative control (cells with the vehicle). DMSO is the vehicle used for the dissolution of samples and its final concentration in the cells was less than 0.2%. The percentage of change in viability was calculated according to the formula: (Reading of sample / Reading of negative control) -1) x 100.

2.2.7.4. Wound healing activity

The migration rates of BJ-1 cells were assessed by the scratch assay method. The cell density of "2×10^5 cells" was seeded into each well of a 24-well plate and incubated with complete medium at 37°C and 5% CO_2. After 24h of incubation, the monolayer confluent cells were scrapped horizontally with a sterile P200 pipette tip. The debris was removed by washing with phosphate buffer saline (PBS). The cells were treated with samples (the partially purified AL-PR enzyme) with concentrations of 62.5, 125, 250, and 500μg/ml. The cells without treatment were used as a negative control. The scratch induced that represented the wound was photographed at 0 h using phase contrast microscopy at ×40 magnification at 0 h, before incubation with the samples(the partially purified alkaline protease enzyme). After 24 h of incubation, the second set of images was photographed. To determine the migration rate, the images were analyzed using "image J" software, and the percentage of the closed area was measured and compared with the value obtained at 0 h. An increase in the percentage of the closed area indicated the migration of cells [26], alternatively, the wound closure rate over time can be as the percentage of area reduction or wound closure [27]. .

Wound closure (%) =

$$\frac{(\text{Measurement at 0h} - \text{Measurement at 24h})}{\text{Measurement at 0 h}} \times 100$$

2.2.8. Stabilization of partially purified AL-PR

Diethyl-amino-ethyl (DEAE) dextran, polyethylene glycol (PEG) of 2000 Da molecular weight, carboxymethyl cellulose (CMC), gum arabic, dextran sulfate, and sodium alginate were oxidized via sodium periodate in order to establish covalent conjugates with the partially purified AL-PR. This was performed as a modification Wehaidy et al. [12]. In brief, one gram of the respective polysaccharide was dissolved in 0.25 M sodium periodate solution (40ml). This mixture was rotated for 6 hours in a 37°C shaker-incubator. Afterwards, 1.2 ml ethylene-glycol was added and was allowed to interact for 1h. The mixture was then dialyzed overnight in order to eliminate any residual periodate moieties, and it was air dried. 0.06 g of the attained powdered oxidized polysaccharides was mixed with 1 ml partially purified AL-PR and 4 ml of the regular assay buffer. This mixture was left overnight in the fridge, and then its activity was estimated (initial activity) and was compared to the activity of a control specimen that was analogously processed but without the incorporation of the oxidized polysaccharide. The control and the conjugated samples were then incubated at 60°C, and after specified durations their residual activities were estimated and presented relative to the initial activity of each respective sample. The Log values of these residual activity percents were then plotted against the incubation duration (in min). The first order thermal-denaturation rate constants (k_d) were reckoned from the slopes in these plots. Afterwards, the half lives ($t_{1/2}$) and D-values were reckoned as follows [17]:

$t_{1/2}=\ln 2/k_d$

D-value= $\ln 10/k_d$

3. Results and discussion

3.1. Partial purification of AL-PR

The crude extract of the enzyme was subjected to precipitation using ammonium sulfate, acetone, and ethanol. It was observed that ammonium sulfate (fraction 60%) was the best one as it reached an activity recovery of giving 21.2 % and a purification fold of 2.5 (Table 1). Ammonium sulfate precipitation has been utilized in the purification of nearly all commercial enzymes [28]. The surface tension of water increases with the addition of ammonium sulfate, and this leads to an increase in hydrophobic interaction between water and protein. To reduce the contact with water, protein decreases its surface area, and this causes its precipitation [29]. Noteworthy, Senphan et al. [30] reported that ammonium sulfate at 40-60% showed the highest purity of protease from the hepato-pancreas of white shrimp (*Liptopenaeus vannamei*) compared to other concentrations of ammonium sulfate.

3.2. Effect of pH on enzyme activity and stability

The activity of partially purified AL-PR was determined at a wide range of pH (3-10). The enzyme activity was high between pH 6 and pH 10 where more than 80 % of activity was retained in this range (Figure 1). The optimum pH was 8.0. However, a significant decrease was observed in enzyme activity above pH 10 and below pH 6. The optimum pH for AL-PR enzyme was similar to that reported by Qamar et al. [31]. AL-PR was highly stable in phosphate buffer (0.1M, pH8), and retained more than 80% of original activity at pH 6 and pH 10 (Figure 2). Generally, the stability of proteins including proteases is related to their net charge at a particular pH [32]. The stability of proteases over a wide range of pH makes them suitable for industrial applications.

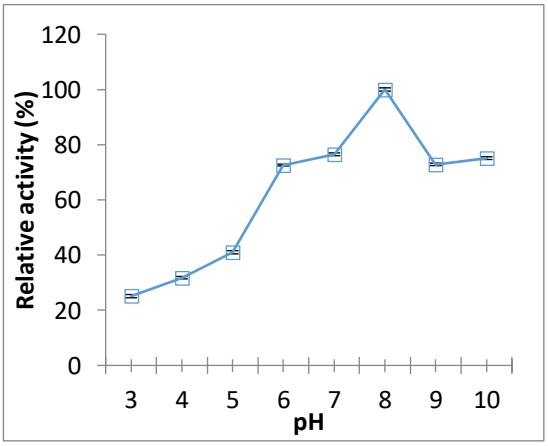


Figure 1. Effect of different pH values of the reaction mixture on activity of partially purified AL-PR (Mean±S.E.)

Table 1: Partial purification of alkaline protease

Methods	The protein of fraction (mg/f)	Total activity (U/f)	Recovered activity (%)	Specific activity (U/mg protein)	Purification fold
Culture filtrate	27	26487.3	100	981	1
Ammonium sulfate saturation (%)					
20	2.5	1124.6	4.24	379.8	0.38
40	1.1	1769.3	6.67	1357.2	1.3
60	1.9	5641.1	21.2	2505.2	2.55
80	0.9	1260.9	4.7	1185.2	1.2
Acetone					
20	1.8	145	0.54	80.55	0.082
40	1.19	434	1.63	364.7	0.37
60	1.13	1573.7	5.94	1392.6	1.41
80	0.9	74.3	0.28	82.55	0.084
Ethanol					
20	2.09	26.5	0.1	12.679	0.012
40	1.18	52	0. 19	44.06	0.044
60	1.9	4050	15.2	2131.5	2.17
80	0.8	150	0.566	187.5	0.19

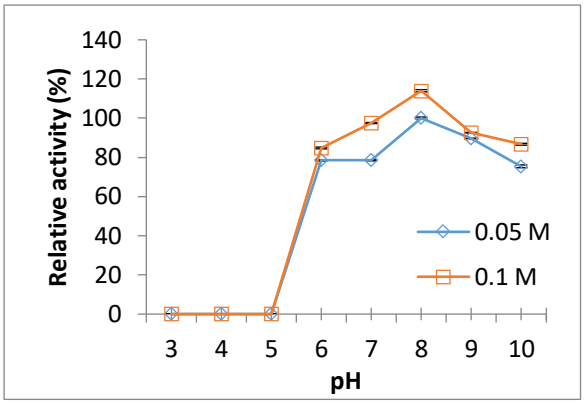


Figure 2: pH stability of partially purified AL-PR at different buffer molarities (Mean±S.E.)

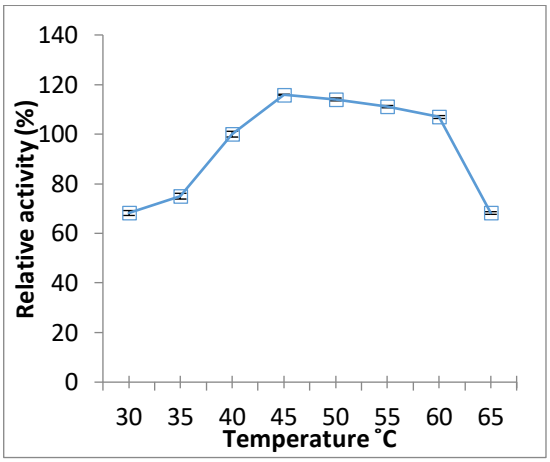


Figure 3: Effect of different temperatures of the reaction mixture on the partially purified AL-PR (Mean±S.E.)

3.3. The effect of temperature on activity and stability

The temperature activity profile and the stability of partially purified AL-PR were summarized in Figure 3 and Figure 4. The enzyme was highly active at temperatures from 40 to 60 °C with maximum activity at 45°C. Noteworthy, the AL-PR of *B. subtilis* also exhibited its maximum activity at 40°C [33]. As regards to the thermal stability of the partially purified AL-PR (Figure 4), it kept 26.14% activity following 1 h incubation at 60°C . This thermal stability was finer than that presented by the *B. licheniformis* AL-PR which kept 7.7% activity after 1 h incubation at 59°C [34]. Such elevated stability would favor the application of the AL-PR produced herein.

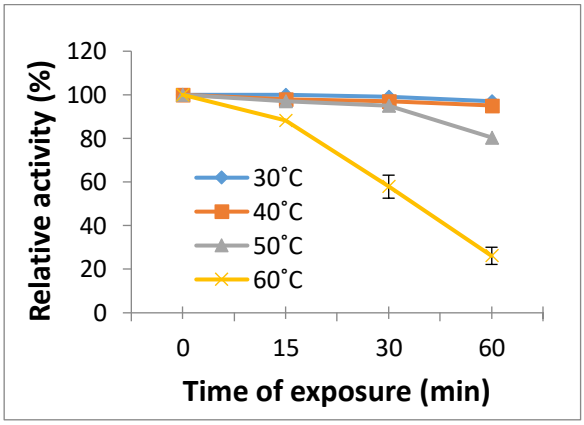


Figure 4.: Thermal stability of the partially purified AL-PR

Table 2: Effect of protease inhibitors on enzyme

Inhibitors	Residual activity (%)				
	2mM	4mM	6mM	8mM	10mM
Control	100	100	100	100	100
EDTA	9.73±0.051	9.55±0.032	9.43±0.010	8.38±0.047	1.96±0.032
1,10-phenanthroline	5.77±0.052	5.86±0.084	5.23±0.05	4.64±0.026	4.19±0.049
PMSF	97±0.1	95±0.113	91.1±0.09	90.8±0.08	88.4±0.08

3.4. Effect of protease inhibitors on enzyme activity

The results presented in table (2) showed that little inhibition was observed with PMSF (inhibitor of serine proteases [19]). Thus, AL-PR doesn't belong to the serine protease. However, significant levels of inhibition were observed following incubation with either the chelating agents; EDTA or 1,10-phenanthroline even at low concentration (2 m M). Noteworthy, 1,10-phenanthroline exhibits escalated affinity for zinc [19]. Thus, the *B. amyloliquefaciens* alkaline protease (AL-PR) can be classified as a Zn metalloprotease type. Similarly, the *Flavobacterium frigidimaris* ANT34-7 metalloprotease was inhibited by both EDTA and 1,10-phenanthroline [19].

3.5. Potential applications of partially purified AL-PR enzyme

3.5.1. Antimicrobial activity

An open wound is the best medium for microbial colonization. Antibiotics help in preventing bacterial infection. However, most antibiotics are broadly acting; thus, they prevent the growth or kill both good and bad bacteria. Moreover, the recurrent use of antibiotics can trigger bacterial resistance. More than 70% of the bacteria that are responsible for wound infections display resistance to at least one of the antibiotics used in the clinic [35]. *Staphylococcus aureus*, *E. coli*, *Pseudomonas species*, and *Candida albicans* are multi-resistant bacteria that are involved in skin infections [36]. The number of multidrug-resistant bacteria is increasing at an alarming rate, i.e. bacteria are gaining resistance to all known classes of natural and synthetic antibiotics. Thus, there is an urgent need for new therapeutic alternatives. The majorities of infected wounds are, generally, polymicrobial and are usually contaminated by pathogens found in the surrounding environment, i.e. membranes. In the initial stages of wound formation, gram-positive organisms, specifically *S. aureus* are predominant. In the later stages, gram-negative organisms, *E. coli, Proteus mirabillus*, *Pseudomonas species*, and *Candida albicans* are observed and tend to break deeper layers of skin causing tissue damage. Therefore, these types of bacteria were used as target microorganisms to determine the antimicrobial activity of partially purified AL-PR enzyme. The anti-microbial activity of the enzyme was first determined by the agar well diffusion method. The results indicated that the enzyme (100%, 3mg/ml) had a bacteriostatic effect against *B. Cereus* (18mm), *P. aeruginosa* (16mm), and *C. albicans* (17mm). *Proteus mirabillus* was also partially inhibited. However, *E.coli*, and *S. aureus* offered negative results (Table 3 & Figure 5). These results are similar to those of Philip et al. [37] who reported the presence of antimicrobial activity against *Candida albicans, Saccharomyces cerevisiae*, and others through the production of hydrolytic enzymes including proteases.

Furthermore, the microbial susceptibility of the assayed microbes was tested under different concentrations of the enzyme ranging from 0.75 to 3 mg/ml (3mg/ml was the 100%) (Table 3). It was observed that the microbial sensitivity increased with the increase of enzyme concentration with minimum inhibitory concentration at enzyme concentration 0.75 mg/ml (25%) for *B. Cereus*, *P. aeruginosa*, and *C. albicans.* Nonetheless, bacterial growth inhibition does not mean bacterial death, this method with a bacteriostatic effect.

Thus, the antimicrobial activity of the partially purified AL-PR was also determined by the shake flask method for all tested organisms to obtain the bactericidal effect of the enzyme. The results in Table 4 indicated that the enzyme had a bactericidal effect against *B. cereus, P. aerginosa., C. albicans*, *P. mirabillus, E. coli*, and *S. aureus.* The higher concentrations of the enzymes manifested a bactericidal effect because the reduction value reached 100 % after 24 h and this effect was acting in a dose-dependent manner [38]. One advantage of this antibacterial action of the partially purified enzyme is that it allows wound healing to continue without bacterial interference

3.5.2. Antioxidant activity

The antioxidant activity of the partially purified AL-PR was assessed using DPPH radical scavenging. The result presented in Table 5 clearly showed that the maximum scavenging rate reached 86.2±0.43% with an enzyme concentration of 3mg/ml. The antioxidant activity also was found to be dependent on the enzyme concentration and the IC_{50} was 0.59 mg/ml suggesting that the applied enzyme exhibited good radical scavenging activity [39].

These results suggested that the enzyme exhibited an interesting radical scavenging activity. Furthermore, the antioxidant activity coupled with antibacterial, antifungal, antiallergic, and

Table 3: Diameter of inhibition zones presented by different concentrations of the partially purified AL-PR

Samples Test bacteria		No.	1	2	3	4
		Code (%)	25.0	50.0	75.0	100.0
1-	*Escherichia coli*		NiL	NiL	NiL	NiL
2-	*Pseudomonas aeruginosa*		13.0	15.0	15.0	16.0
3-	*Bacillus cereus*		12.0	13.0	15.0	18.0
4-	*Proteus mirabillus*		NiL	NiL	NiL	12.0
5-	*Staphyllococus aureus*		NiL	NiL	NiL	NiL
6-	*Candida albicans*		11.0	13.0	16.0	17.0

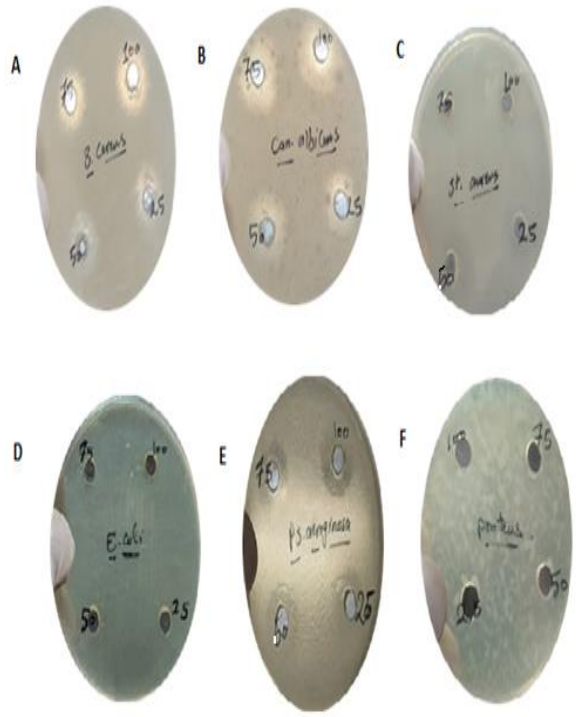


Figure 5: Antimicrobial activity of A) *B. cereus*, (ATCC 6629), *B) Candida albicans*ATCC 10231) C) *S. aureus (ATCC 6538*)) D) *E.coli* (*ATCC25922*), E) *P. aeruginosa* (*ATCC27853*) and *Proteus mirabillus* (ATCC9240)

anticarcinogenic agents could be a helpful therapeutic agent in promoting wound healing [40]. DPPH radical scavenging is an assay that is based on the reduction of the absorbance of a methanolic DPPH solution at 517 nm in the presence of electrons or a proton-donating substance [23]. The results suggest that probably the alkaline protease consists of amino acid composition, structures and hydrophobicity has electron or proton-donating abilities, and could react with free radicals to convert them to more stable products.

3.5.3. Cytotoxicity on human normal skin fibroblast cell line (BJ1)

All the tested concentrations of the enzyme (0.78 to 100 µg/ml) using MTT assay had no cytotoxicity towards BJ1 (normal skin cells). Thus, the partially purified AL-PR is safe for skin cells, and this enzyme could be suitable for wound healing applications.

3.5.4. Wound healing activity

The impact of different concentrations of the enzyme on the migration of fibroblast cells was shown in Table 6 and Figure 6. After 24 h of incubation, increased cell migration was observed, especially when the enzyme concentration was 500 μg/ml. This concentration achieved a migration rate of 66.3%, and this resulted in the observed decreased wound gap. Therefore, the partially purified AL-PR

Table (4): (%) CFU reduction of bacterial strain cells by partially purified alkaline protease enzyme after 24 h incubation period and at 37 ºC using the shake flask method applying Turbidimetric measurement technique.

Tests bacteria	CFU reduction%			
	A concentration of samples %	25.0	50.0	75.0	100.0*
Escherichia coli		69.36	70.59	74.01	88.73
Pseudomonas aeruginosa		100.0	100.0	100.0	100.0
Bacillus cereus		100.0	100.0	100.0	100.0
Proteus mirabillus		67.0	78.90	89.05	100.0
Staphyllococusaureus		87.00	89.31	89.73	95.18
Candida albicans		100.0	100.0	100.0	100.0

*The initial concentration of the sample (protein content 3mg/ml) (100%)

Table 5: Scavenging activity of partially purified AL-PR (Data are presented as mean±SD)

Enzyme concentration(mg/ml)	***DPPH % Inhibition***
0.5	**48.02±1.03**
1	**58.30±0.52**
1.5	**62.07±0.40**
2	**70.17±1.26**
2.5	**78.35±0.31**
3	**86.24±0.42**
Ascorbic acid (1mg/ml)	**97.5±0.74**

offers potential for wound healing applications. Several recent reviews detail the main roles of proteases in wound healing [8,41, 42, 43]. Noteworthy, proteases especially metalloproteinases (MMPs) were proved to be involved in wound healing [44]. Proteases are enzymes that act on proteins by breaking them down into peptides and amino acids. Thus, proteases can breakdown damaged extracellular matrix (ECM) proteins and foreign materials so that new tissue can form and wound closure can occur in an orderly fashion [8]

Table 6. Effect of the partially purified alkaline protease enzyme on in vitro scratch assay using BJ-1 cells human fibroblast.

Treatment	Dose (μg/ml)	Length between the scratch (μm) 0h	24 h	Cells migration %rate
Negative control	-	641.37	503.56	21.4
Samples	62.5	763	494.07	35.2
	125	733.52	318.3	56.5
	250	790.38	305.89	61.29
	500	601.86	202.7	66.3

3.6. Stabilization of the partially purified AL-PR

Variable polysaccharides were oxidized and were utilized to covalently conjugate AL-PR .The activities of the attained oxidized polysaccharides AL-PR conjugates were recorded after overnight incubation with the respective polysaccharide and were considered as their initial activities (Table 7).

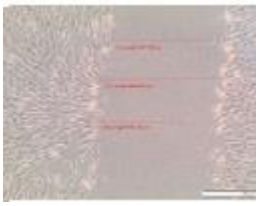
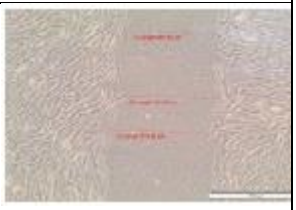
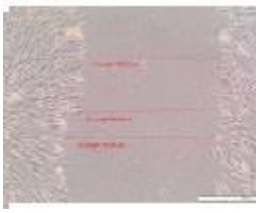
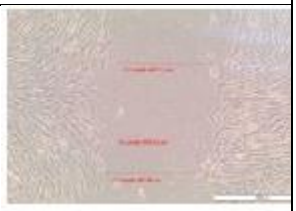
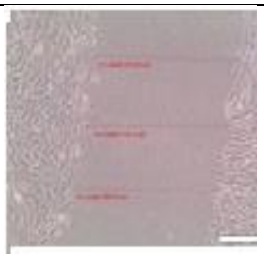
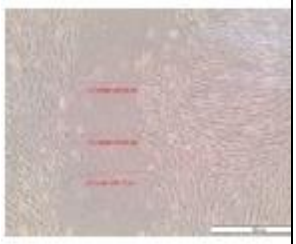
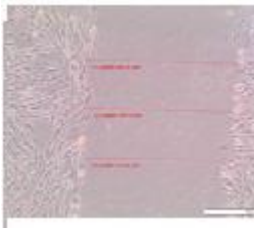
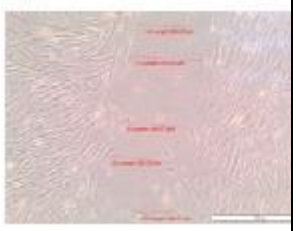
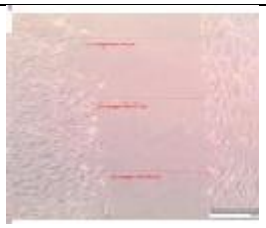
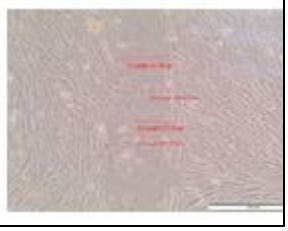
	0 hr	24 hr
Control μg/ml		
62.5		
125		
250		
500		

Figure 6: Images from the in vitro scratch assay experiment at different time points (0 and 24 hr) of BJ1 fibroblast cells treated with partially purified alkaline protease at different concentrations (62.5, 125, 250, and 500 μg/ml)

The thermal stabilities of these conjugates were also evaluated at 60°C (Figure 7) as thermal stability is critical for enzymes actual application [45]. The K_d values of the conjugates were then reckoned from slopes of Figure 8 and then the $t_{1/2}$ and D-values were reckoned (Table 7). Amidst the investigated polysaccharides, the oxidized CMC and DEAE dextran elevated the partially purified AL-PR thermal stability. Moreover, they elevated its initial activity (Table 7). The oxidized DEAE dextran conjugated AL-PR initially offered 138.13% activity as compared to the control specimen. Moreover, it offered 74.86 min $t_{1/2}$ and 248.68 min D-value after its 60°C thermal incubation whereas 65.52 min $t_{1/2}$ and 217.66 min D-value were recorded for the control. As regards to the oxidized CMC conjugated AL-PR, it initially offered 106.95% activity, and its $t_{1/2}$ and D-value amounted to 391.49 min and 1300.52 min, respectively. These values were 5.97 folds larger than those of the control. The elevated thermal stability of the attained conjugates could be regarded to the inter and intra-molecular cross-links which were newly created after the conjugation with the oxidized [12] CMC and DEAE-dextran. In a somewhat similar situation, pectin, amylopectin, and two altered dextrans were oxidized and utilized to conjugate the *B. stearothermophilus* AL-PR. Nonetheless, only pectin elevated the thermal stability of that AL-PR. The oxidized pectin conjugated *B. stearothermophilus* AL-PR offered a 2.98 fold larger $t_{1/2}$ than that offered by its control [16]. This stabilizing effect was lesser than that recorded herein after conjugation with the oxidized CMC (Table 7)

In an attempt to further elevate the AL-PR thermal stability, different CMC concentrations were investigated, and the activity kept after 1h 60°C thermal incubation was assessed. Figure 9 revealed that the residual activity increased upon raising the CMC weight till 0.1 g where 97.75% activity was kept. Raising the CMC weight to 0.15g reduced the retained activity to 91.54%. Thus, 0.1g CMC was the optimal weight to be mixed with AL-PR specimen. Noteworthy, the un-conjugated AL-PR kept only 26.14% activity after its 1 h 60°C incubation (Figure 7). On another occasion, raising the concentration of galactose, which was used to stabilize β-galactosidase, from 0.05 to 0.15 M elevated the β-galactosidase thermal stability at 60 °C [15].

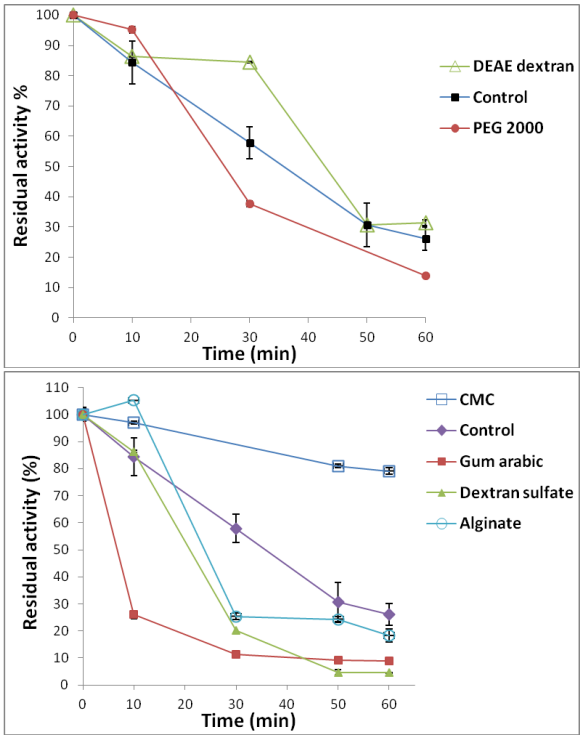


Figure 7: Activity percents kept by the control and the conjugated AL-PR specimens after the thermal incubation at 60°C (mean±S.E.)

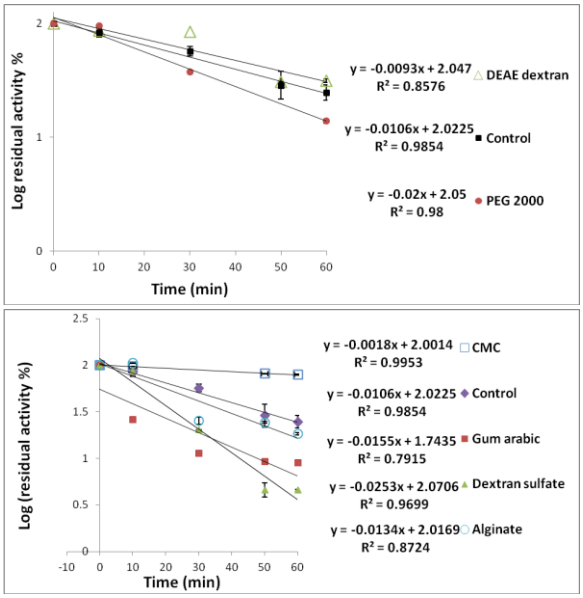


Figure 8: Log values of the activity percents, which were kept by the control and the conjugated AL-PR specimens after the thermal incubation at 60°C, plotted versus incubation period to reckon k_d values (mean±S.E.)

Table 7: Influence of polysaccharide conjugation on the initial activity and thermodynamic parameters of the partially purified AL-PR

Oxidized polysaccharide	Intial activity (%)	k_d (min^{-1})	$t_{1/2}$ (min)	D-value (min)
Control	100.00	0.0106	65.52	217.66
DEAE dextran	138.13±0.39	0.0093	74.86	248.68
PEG 2000	64.90±0.27	0.0152	45.75	151.98
CMC	106.95±2.74	0.0018	391.49	1300.52
Gum arabic	45.14±1.12	0.0155	44.64	148.28
Dextran sulfate	126.85±0.78	0.0253	27.42	91.09
Alginate	101.01±1.12	0.0134	51.71	171.77

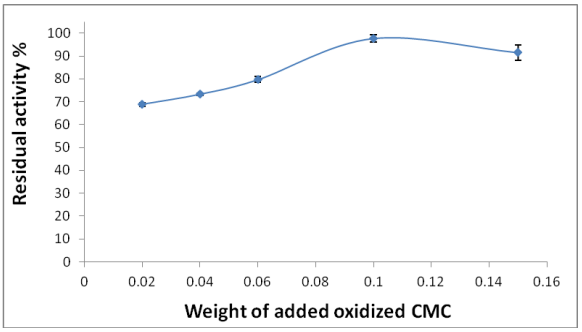


Figure 9 : Effect of varying the oxidized CMC weight on the residual activity of AL-PR after 1h thermal incubation at 60°C (mean±S.E.)

Conclusions

The partially purified AL-PR attained herein holds potential for wound healing applications owing to its wound healing promoting traits, its antimicrobial, and antioxidant traits. Moreover, its safety was verified. Its stabilization was also successful particularly via the oxidized CMC, and this would extend its life time and encourage its utilization.

Conflicts of interest

There are no conflicts to declare.

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