Purification and characterization of alkaline protease produced by *Streptomyces flavogriseus* and its application as a biocontrol agent for plant pathogens

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Background
A protease is an enzyme that helps proteolysis: protein catabolism by hydrolysis of peptide bonds. Proteases were the first enzymes to be commercialized, partly on the account of the history of availability and on account of need.
Aim
The aim of the present work is studying the properties of the partially purified enzyme and some application of the enzyme.
Materials and methods
In this study, alkaline protease was produced by *Streptomyces flavogriseus* using the shaking method. The enzyme was partially purified by ammonium sulfate precipitation. The most active fraction was evaluated on Sephadex G-100 column chromatography. The activity of the enzyme was determined at different temperatures and different pH values using various buffers.
Results and conclusion
In this study, alkaline protease produced by *S. flavogriseus* was partially purified by ammonium sulfate precipitation at 75% w/v saturation and pooled on Sephadex G-100 with a yield of 60.85% and specific activity of 85.14 U/mg protein. The enzyme was optimally active at 45°C in 0.5 mol/l potassium phosphate buffer (pH 8.0) after 30 min of incubation and was broadly stable at 45°C for 60 min and pH 6–8. The alkaline protease relative activity was increased in the presence Mn^{2+}, Co^{2+}, K^{+}, Na^{+}, Fe^{2+}, and Mg^{2+} at concentration 1 mmol/l, respectively.

Keywords:
alkaline protease, biological control, characterization, purification, *Streptomyces flavogriseus*

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Introduction

Streptomyces is known as a producer of antibiotics. In recent years, some other products such as alkaline protease were examined regarding their pharmaceutical application. *Streptomyces* is quantitatively one of the most abundant and qualitatively the key group among soil bacterial communities [1]. A chance to efficiently colonize the rhizosphere and form desiccation-resistant spores means they are excellent candidates for biocontrol studies. Furthermore, several members of the group can produce mycolytic enzymes, siderophores, and antimicrobials as principal antifungal agents [2]. Although chitinases and glucanases are viewed as major mycolytic enzymes, few research studies have also identified the role of proteases in fungal biocontrol. The role of proteolytic enzymes manufactured by the *Trichoderma harzianum* and *Pseudomonas aeruginosa* M-1001 in biological control of fungal pathogens may be demonstrated [3,4]. Studies show that overproduction of extracellular protease by *Stenotrophomonas maltophilia* strain W81 enhanced its biocontrol efficacy up against the fungal pathogen [5]. Antifungal action of protease suggests their potential in agriculture for control of fungal phytopathogens. Such novel agricultural application will lead to increased demand of proteases, leadin to seeking out for newer sources. Although proteases have already been reported from several *Streptomyces* spp. [6,7], there are no reports on their antifungal potential. We have recently investigated the antagonistic properties of *Streptomyces* spp. against many fungal plant pathogens. Indirect evidence has suggested that *Streptomyces* spp. A6 antagonizes primarily by antibiosis, bringing about cell death, and secondly by degradation of cell wall by mycolytic enzymes, causing mycolytic cell death. The aim of this study is to recognize the ability of some *Streptomyces* spp. to produce protease enzyme and to study the properties of the partially purified enzyme.

Materials and methods

Microorganism

The *Streptomyces* spp. used in this study was isolated and identified in a previous study by the same authors as *Streptomyces flavogriseus* (Gen Bank accession number: AB723782).

Culture conditions

Inoculum preparation and fermentation conditions were as follows: the inoculum was prepared by growing *S. flavogriseus* on soy bean slants for 9 days, and then a spore suspension of optical density 0.6 at 660 nm was made by using sterile saline solution and used to inoculate experimental 250-ml Erlenmeyer flasks. Two milliliter of spore suspension was inoculated in 48-ml liquid medium, which contained (g/l) 20, sucrose; 0.5, NaCl; 2, KNO_3; 1, K_2HPO_4; 0.5, $MgSO_4$; 3, $CaCO_3$; and 0.01 for each of $FeSO_4$, $ZnSO_4$ and $MnCl_2$ in 250-ml Erlenmeyer flasks. The experimental flasks were sterilized by autoclaving at 121°C for 15 min. The cooled sterilized flasks were inoculated by 4% v/v spore sustentations. Thereafter, the flasks were incubated for 6 days at 30°C in a shaking incubator (200 rpm). At the end of fermentation period, the culture medium was centrifuged at 6000 rpm for 10 min, and the supernatant was assayed used for determination of protease activity and protein content.

Protease activity assay

Quantitative assay of protease was as follows: protease activity in the culture filtrate of *S. flavogriseus* was assayed by the method of Tsuchida *et al.* [8] with some modification by using casein as the substrate. Overall, 100 µl of enzyme solution was added to 900 µl of substrate solution [2 mg/ml w/v of casein in 10 mmol/l Tris-HCl buffer (pH 8)]. The mixture was incubated at 37°C for 30 min. Reaction was terminated by the addition of 1 ml of 10% (w/v) trichloroacetic acid, and then the reaction mixture was allowed to stand in ice for 15 min to precipitate the insoluble proteins. The supernatant was separated by centrifugation at 12 000 rpm for 10 min at 4°C; the acid-soluble product in the supernatant was neutralized with 5 ml of 0.5 mol/l Na_2CO_3 solution. Then, 0.5 ml of three-fold diluted Folin–Ciocalteau reagent was added and allowed to stand for 30 min in room temperature. The developed color was measured at 660 nm after adding 0.5 ml of three-fold diluted Folin–Ciocalteau reagent. All assays were done in triplicate. One protease unit is defined as the amount of enzyme that releases 1 µg of a tyrosine ml/min under the aforementioned assay conditions. The specific activity is expressed in the units of enzyme activity mg/protein.

Protein determination

The protein concentration was determined by the method of Lowery *et al.* [9] with bovine serum albumin as standard.

Purification and characterization of alkaline protease

Partial purification of the protease

Ammonium sulfate precipitation: The extracellular protease enzyme was subjected to different ammonium sulfate precipitation. The concentrations of ammonium sulfate were 30, 50, 75, and 100% (w/v). The precipitated fractions were collected by centrifugation at 10 000 rpm, and the pellet obtained of each fraction was resuspended in a minimal volume of 100 mmol/l Tris-HCl, pH 8.0.

Sephadex G-100 gel filtration

The 75% (w/v) ammonium sulfate fraction was subjected to gel filtration on a Sephadex G-100 (Sigma-Aldrich Chemie Gmb, NJ, USA) column (3 cm×100 cm) equilibrated with 25 mmol/l Tris-HCl, pH 8.0. Enzyme fractions of 5 ml were collected at a flow rate of 25 ml/h with the same buffer. Protein content and protease activity were measured. Fractions showing alkaline protease activities were pooled. The purity was checked by Coomassie blue-stained SDS PAGE. Protein concentration was determined by the method of Lowery *et al.* [9].

Gel electrophoresis and molecular weight of alkaline protease

Five milliliter from fraction IV of purification was withdrawn and heated at 95°C for 2 min; each 20 µl was loaded in a lane. The solution was pipetted into the assembled vertical slab gel unit in the casting mode leaving 2 ml from the front. A layer of distilled water was pipetted on the top of solution. The gel was allowed to polymerize at room temperature, and the water was poured from the upper surface. The stacking gel solution was prepared and added onto the top of separating gel.

Influence of pH and temperature on protease activity and stability

The optimum pH of the purified enzyme was studied in 6.0–10.0 pH range using 0.1 mol/l buffers (PBS, pH 6.0–7.5; Tris-HCl buffer, pH 8.0–8.5; carbonate-bicarbonate buffer, pH 9.0–11.0) at 100 mmol/l. For the measurement of pH stability, the enzyme was kept at room temperature for 1 h in different buffers (PBS,

pH 6.0–7.5; Tris-HCl buffer, pH 8.0–8.5; carbonate-bicarbonate buffer, pH 9.0–11.0) at 100 mmol/l, and the residual alkaline protease activity was determined under standard assay conditions.

To analyze the effect of temperature, the activity was tested at different temperatures (40–70°C) for 15 min at the optimum selected pH of 8.5 from the previous experiment.

Thermal inactivation was examined by incubating the purified enzyme at 30, 35, 40, 45, 50, 55, 60, 65, 70, and 75°C for 60 min. Aliquots were withdrawn at desired time intervals to test the remaining activity at 45°C and pH 8.5. The nonheated enzyme was considered as a control (100%).

Effect of metal ions and inhibitors on alkaline protease activity

The effect of various metal ions (1 and 10 mmol/l) on enzyme activity was investigated using $CaCl_2$, $MnCl_2$, $ZnSO_4$, $CoSO_4$, $CuSO_4$, NaCl, KCl, $FeSO_4$, and $MgSO_4$. The effects of enzyme inhibitors on protease activity were studied using EDTA, iodoacetate, and PMSF. The purified enzyme was preincubated with some inhibitors for 30 min at 45°C and then the remaining enzyme activity was measured.

Results and discussion

Purification of alkaline protease

The crude enzyme was first extracted by ammonium sulfate precipitation with saturation ranging from 30 to 100 (w/v). The precipitated protein increased along with increasing saturation. The enzyme activity attained a maximum value at saturation of 75% w/v. The specific activity and the recovery rate was 45.6 U/mg and 63.5% respectively. The active fraction was purified by subjected to Sephadex G-100. Fractions were eluted by 25 mmol/l Tris-HCl at pH 8. The elution profile of alkaline protease is illustrated in Fig. 1. The purification fold was 36.4 and had a recovery rate of 60.9% (Table 1).

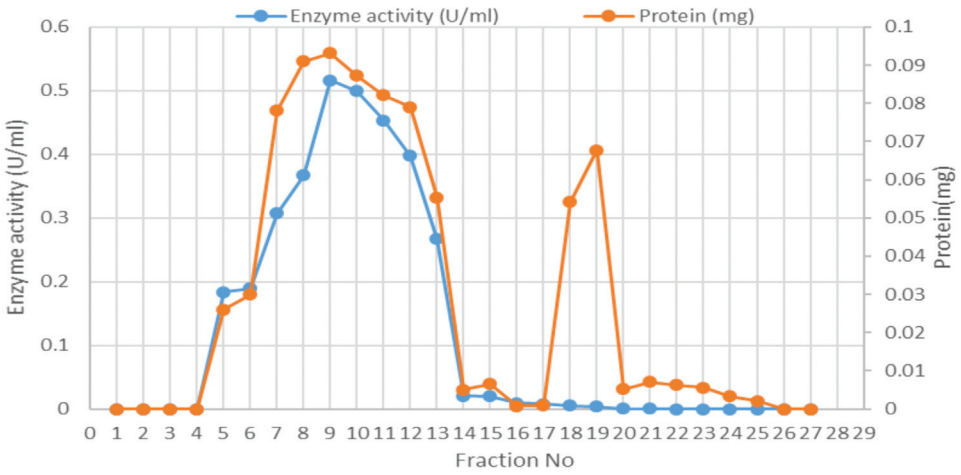
Some properties of the purified enzyme

The alkaline protease is active at pH ranging from 6.5 to 9.5, with optimum pH at 8 (Fig. 2). The enzyme showed high enzyme activity at pH 8 (100% relative activity) when glycine-NaOH buffer 0.05 mol/l was used, but good activity appeared within the range 7–9.5 (80–82% of relative activity of its high activity). The increase in molarity than 0.05 mol/l decreased the enzyme activity up to 20% (Fig. 3). The enzyme was stable at pH 6–8 when kept at room temperature for 1 h. It remained almost 77% of its activity at pH 4.5 and 76% at pH 9.5 (Fig. 3). Similar findings were reported by Si *et al.* [10], but are not in agreement with that obtained by Xin *et al.* [11] and Touioui-Boulkour *et al.* [12]; they used 0.5 mol/l glycine NaCl at pH 9.0 and 10, respectively.

Time course and profiles of alkaline protease reaction

Time course and profiles of alkaline protease reaction (Fig. 4) indicate that the rate of tyrosine release increased as the reaction time increased up to 30 min; thereafter, no additional hydrolysis products were liberated. Gul *et al.* [13] and Thebti *et al.* [14] reported the optimum time of incubation of maximum protease activity was 20 min. Touioui-Boulkour *et al.* [12] reported that the optimally time of alkaline protease activity produced by Streptomyces spp.

Figure 1

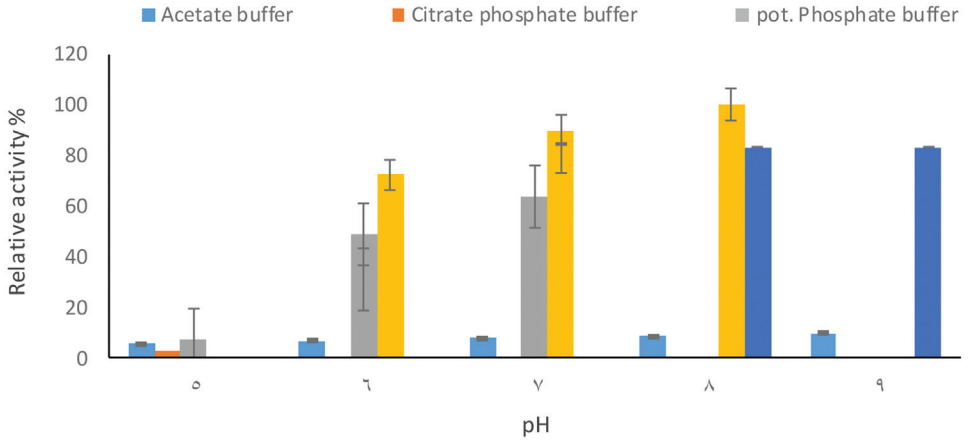


Elution profile of alkaline protease from *Streptomyces flavogriseus* on Sephadex G-100 gel filtration chromatography.

Table 1 Purification of alkaline protease from *Streptomyces flavogrisus*

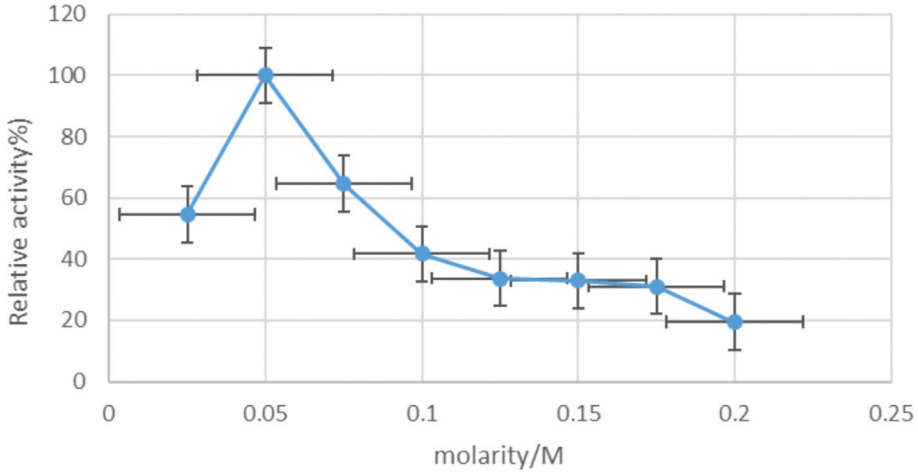
Purification steps	Total protein (±SD) (mg)	Total activity (±SD) (U)	Specific activity (±SD) (U/mg protein)	Enzyme recovery (%)	Purification fold
Crude enzyme	2329	5442	2.34	100	1.00
Ammonium sulphate 75% w/v	758	34566	45.60	63.50	19.50
Sephadex G-100	38.9	3312	85.14	60.85	36.43

Figure 2



Effect of different pH at different buffers on enzyme activity.

Figure 3

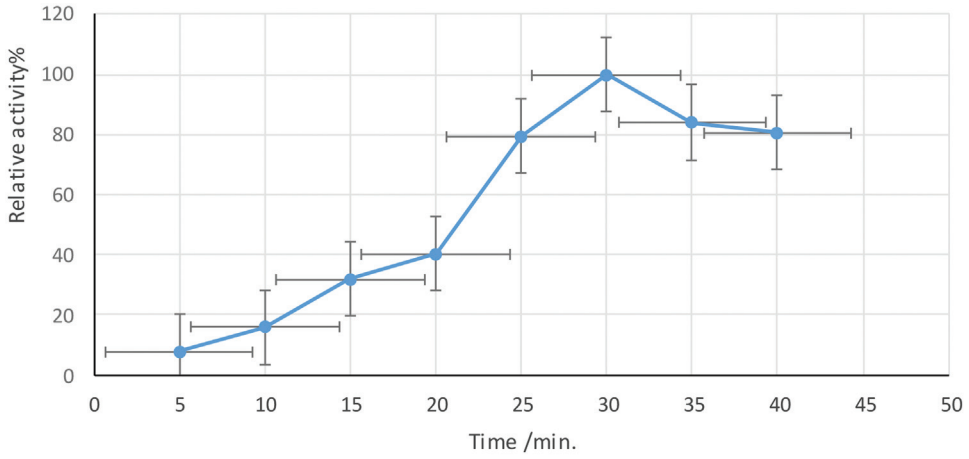


Effect of phosphate buffer molarity (pH 8) on alkaline protease activity.

strain AH4 was 15 min. Similar results were recorded for alkaline protease from *Streptomyces clavuligerus* strain Mit-1 and *Streptomyces griseous* [15,16]. These authors mentioned that the enzyme activity increased as the incubation time increased and the nonlinear increase of enzyme activity is caused by the multifunctional.

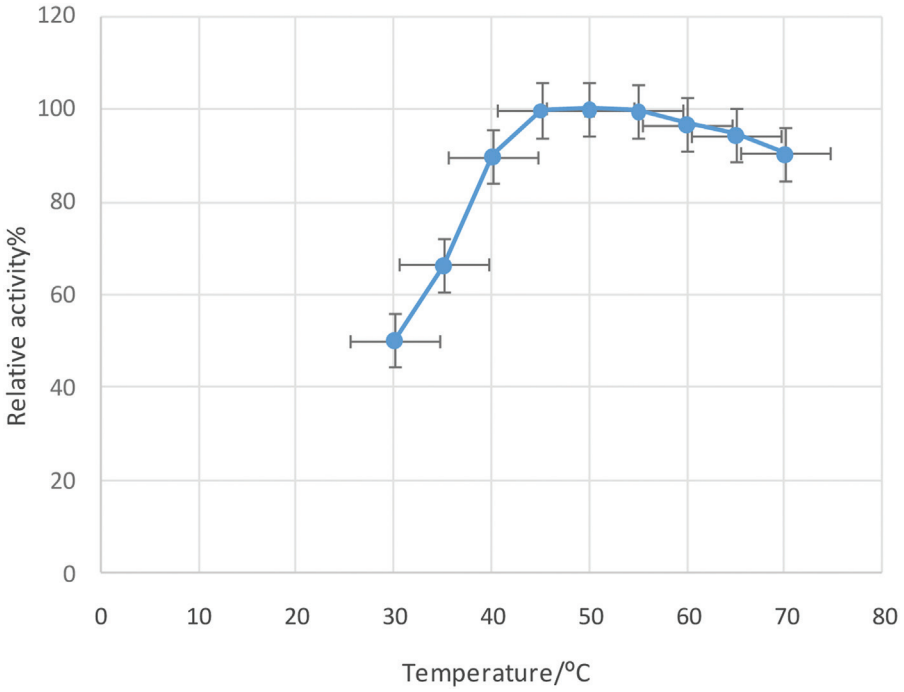
The optimum temperature of the purified enzyme was 45–55°C, and it showed 89 and 94% activity at 40 and 65°C, respectively (Fig. 5). The alkaline protease was also found to be completely stable at 45°C up to 60 min, at 50 and 55°C, the activity was decreased to 98 and 95% respectively. The prolonged temperature and time decreased the activity of protease (Fig. 6). Many authors studied the effect of pH and temperature on purified alkaline protease activity and stability. The optimum temperature and pH for protease activity from *Streptomyces* spp. were 60°C and pH 11.5, and the enzyme was stable up to 50°C and between pH values of 4.0 and 12 [17], whereas protease from *Streptomyces clavuligerus* Mit-1

Figure 4



Effect of reaction time on alkaline protease activity.

Figure 5



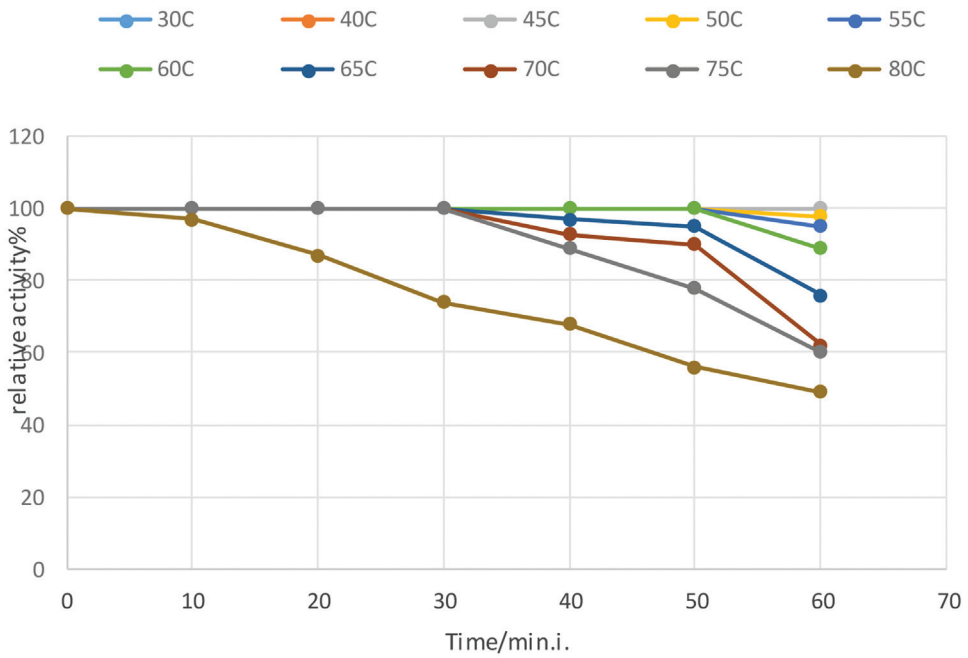
Effect of temperature on purified alkaline protease activity.

had optimum temperature at 70°C and pH at 10–11. The enzyme was stable at 50°C for 60 min. Thumar and Singh [6] and Singh and Chhatpar [18] found that the protease activity from *Streptomyces* spp. A6 exhibited highest activity at pH 7.0 and at temperature 55°C. On the contrary, Touioui-Boulkour *et al.* [12] studied the pH profile with respect to the activity of two proteases over a broad pH range (8.0–12.5), which were obtained from *Streptomyces* spp. AH4. The optimum pH for activity was at pH 10 and 12, whereas pH stability ranged between 8.5 and 12. The optimum temperature was 55 and 65°C with thermal stability below 50°C. The bacterial alkaline protease from *Micrococcus* spp. showed optimum temperature and pH at 50 and 10°C, respectively. The enzyme showed strong stability in a wide pH range (6.0–10.0), and the thermal stability showed that the enzyme retained 90% of its activity at 40°C but lost almost all activity at 50°C [19].

Effect of enzyme concentrations

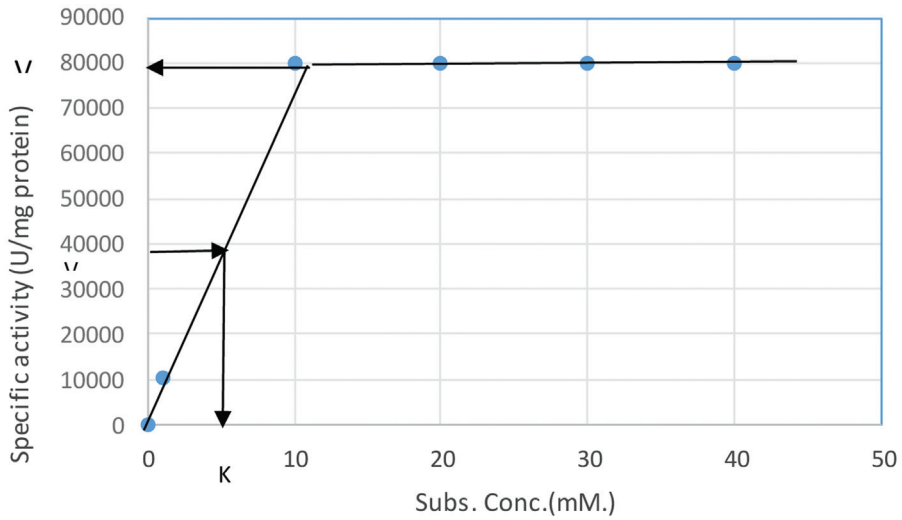
Increasing enzyme concentration will increase the rate of reaction, as more enzymes will be colliding with substrate molecules. However, this too will only have an effect up to a certain concentration, where the enzyme concentration is no longer the limiting factor.

Figure 6



Effect of temperature on purified alkaline protease stability.

Figure 7



Effect of substrate concentrations on alkaline protease activity.

Effect of substrate concentration on alkaline protease

In this experiment, the kinetic parameters such as Michaelis–Menten constant (K_m) and maximum velocity (V_{max}) of purified alkaline protease were determined by incubating the enzyme with different concentrations of soluble casein as a substrate in the range of 1 µmol/l to 40 mmol/l under optimum assay conditions. The apparent K_m of purified enzyme was calculated from a Lineweaver–Burk plot. The K_m value of alkaline protease was found to be 4.9 mmol/l (as shown in Fig. 7). Results obtained in this respect show that the enzyme showed maximal activity at a substrate level of 10 mmol/l followed by a slight decrease at higher concentrations. This indicates that the active center of the enzyme became saturated with its substrate at concentrations above 10 mmol/l. Moreover, the apparent K_m of alkaline protease was found to be 0.5 mmol/l indicating high affinity of *Streptomyces griseus* alkaline protease to its substrate (Casein) and its high therapeutic value [20,21]. In this finding, different K_m values were obtained for alkaline protease purified from various microbial sources [22,23].

Table 2 Effect of metal ions on alkaline protease activity

Concentration (mmol/l)	Metal ions relative activity (%)														
	None	K^+	Na^+	Ca^{2+}	Mg^{2+}	Ba^{2+}	Zn^{2+}	Ni^{2+}	Cu^{2+}	Mn^{2+}	Hg^{2+}	Cd^{2+}	Co^{2+}	Fe^{2+}	Cr^{3+}
1	100	126	114.6	98.6	101.1	95.9	87.9	51.4	103	170	6.7	80.7	143	112	44.2
10	100	114	162	98.8	100.2	66.4	97.3	35.1	98.7	147	0.0	11.4	101.2	99.1	23.6

Effect of metal ions and some chemicals on alkaline protease activity

The effect of some monovalent, divalent, and trivalent metal ions on protease activity is shown in Table 2. The activity was strongly activated by Mn^{2+} and Co^{2+} at 1 mmol/l concentration (170 and 143%) followed by K^+, Na^+, and Fe^{2+} at the same concentration (126, 115, and 112%, respectively). When the concentration of metal ions increased to 10 mmol/l, the activity profile was modified. The result indicates that Na^+ and Mn^{2+} activated the enzyme up to 162 and 147% followed by K^+ (114%). Hg^{2+} ions highly inhibited the enzyme activity at concentrations of 10 and 1 mmol/l (0.0 and 6.7%, respectively) followed by Cd^{2+}, Cr^{3+}, and Ni^{2+} at 10 mmol/l (11.4, 23.6, and 35.1%, respectively). The enzyme was quite stable with Mg^{2+} and Cu^{2+} ions at both concentrations. The results regarding the effect of metal ions on proteases activity had varied from one microorganism to the other. It was found that metal ions such as Hg^{2+} and Co^{2+} strongly inhibited protease activity from *Streptomyces* spp., while Ca^{2+}, Ni^{2+}, Fe^{2+}, Mg^{2+}, Na^+, and K^+ did not show any appreciable effect [10,16,17,24] and they also observed that a significant increase in protease activity from *Streptomyces clavuligerus* Mit-1 with Ca^{2+} and Cd^{2+} up to 140%. Touioui-Boulkour *et al.* [12] found that Ca^{2+}, Mg^{2+} and Mn^{2+} increased proteases activity from *Streptomyces* spp. AH4 up to 200, 150 and 130% [10,16,24].

$Ca^{2+}$and Mg^{2+} increased protease activity from *Geobacillus toebii* LBT77, whereas the enzyme was quite stable with Mn^{2+}, Na^+, K^+, and Cu^{2+} [14].

The effect of chemicals on protease activity

The results in Table 3 indicated that nonionic surfactants Tween 80, Tween 20, and Triton X-100 decreased the activity up to 78, 76, and 56%, respectively, and this feature may be used in laundry detergent industry. These results are in agreement with extracellular proteases produced by *S. flavogriseus* HS1 [19,25] with alkaline protease produced by *Micrococcus* spp. In contrast, Singh and Chhatpar [18] found that the enzyme produced by *Streptomyces* spp. A6 was activated by Triton X-100 and Tween 80 at 5% concentration. The enzyme was easily denatured by the ionic surfactant SDS which inhibited the activity completely (Table 3). The results are agreement with

Table 3 Effect of some chemicals on protease activity

Chemical agent	Concentration [relative activity (%)]	
	1 mmol/l	10 mmol/l
Control	100	100
Surfactants		
Tween 80	78.2	38.2
Tween 20	75.8	33.1
Triton X-100	55.9	34.8
SDS	0	0
Inhibitors		
PMSF	26	9
EDTA	55	13
Iodoacetate	72	51
β-Mercaptoethanol	120	97

those obtained by Yum *et al.* [17] with alkaline serine protease from *Streptomyces* spp., with Singh and Chhatpar [18], with *Streptomyces* spp. A6 protease, and with Ghorbel *et al.* [25] with *S. flavogriseus* HS1 protease. In contrast, Touioui-Boulkour *et al.* [12] found that SDS at 2% activated alkaline proteases from *Streptomyces* spp. AH4 up to 110%. PMSF strongly inhibited the activity to 26 and 9% at 1.0 and 10 mmol/l, respectively. This confirms its belonging to the group of serine proteases. The metalloproteases inhibitor EDTA inhibited the activity by 45 and 13% at 1.0 and 10 mmol/l, respectively. The iodoacetate (alkylating agent) showed inhibition activity by 28 and 49% at the same concentrations. It reacts with cysteine residues in proteins to modify SH-groups in irreversible inhibition. These were similar to results of proteases from *Streptomyces* spp., *Streptomyces* spp. A6, *S. flavogriseus* HS1, and *Streptomyces* spp. AH4 [12,17,18,25]. However, β-marcaptoethanol activated the enzyme up to 120% at 1 mmol/l concentration, and at 10 mmol/l, the activity was lost by 3%, suggesting that the enzyme is thiol dependent, and a reversible reaction was observed when the excess of β-marcaptoethanol was used. This result is similar to the protease from *G. toebii* LBT77 [14].

Application of protease as a biocontrol agent against some plant pathogenic fungi

The protease was used as biocontrol agent against some plant pathogenic fungi in primary experiment in petri dishes at concentration of 25–75 µl. The results are

Table 4 Antifungal activity of purified protease as a biocontrol agent against some plant pathogenic fungi

Pathogenic fungi	Protease volume (μl)	Inhibition zone (mm)
Aspergillus niger	25	18
	50	36
	75	49
Fusarium oxysporium	25	15
	50	30
	75	74
Rhizoctonia solani	25	11
	50	30
	75	41
Alternaria spp.	25	11
	50	26
	75	32
Trichoderma spp.	25	7
	50	13
	75	18

illustrated in Table 4. *Fusarium oxysporium* had shown inhibition zone of 74 mm at 75 μl enzyme followed by *Aspergillus niger* (49 mm) and *Rhizoctonia solani* (41 mm). At less volumes of enzyme (i.e. 25 and 50 μl) the inhibition zone for all fungi tested was less than 75 μl concentration. *Trichoderma* and *Alternaria* were less effective in contrast to other fungi tested. These results are promising and need more experiments to use the alkaline protease in a wide range.

These results are agreement with those obtained by Singh and Chhatpar [18]; they found that purified protease (37 U/ml) from *Streptomyces* spp. A6 inhibited germination of *Fusarium udum* spores. However, El-Khonezu *et al.* [16] found that the purified protease produced by *S. griseus* inhibited the growth of *Fusarium solani* with an inhibition zone of 2.0 cm, indicating its antifungal potential [16]. The same results were confirmed by Al-Askar *et al.* [26], who found that the purified protease produced by *Streptomyces griseorubens* E44G demonstrated marked inhibitory effects against *R. solani* using disc diffusion method.

Conclusion

Alkaline protease produced by *S. flavogriseus* was partially purified by ammonium sulphate precipitation at 75% saturation and Sephadex G-100 with a yield 60.85%. Moreover, the partially purified enzyme showed a high activity at 45°C in 0.5 mol/l potassium phosphate buffer (pH 8.0) after 30 min of incubation, and stability at broad ranges of temperatures and pH. The results also showed that the presence Mn^{2+}, Co^{2+}, K^{+}, Na^{+}, Fe^{2+}, and Mg^{2+} at concentration 1 mmol/l was correlated with an increase in the enzyme activity respectively and strongly inhibited by PMSF. The K_m values was 4.9 mmol/l. The study of alkaline protease as an antifungal activity against some plant pathogenic fungi showed inhibitory effects against different fungi under study, especially *F. oxysporium* and *R. solani*.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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