Enhanced production, partial purification, and characterization of alkaline thermophilic protease from the endophytic fungus *Aspergillus ochraceus* BT21

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Background and objective
Endophytic fungi are thought to be a potential source for biologically active compounds such as enzymes, especially proteases which find their application in modern biochemical industries. The aim of this study was to optimize the production conditions of protease from *Aspergillus ochraceus* BT21, which was previously isolated from the Egyptian medicinal plant *Ruprechita salicifolia*. The produced protease was optimized, partially purified and characterized.

Materials and methods
A. ochraceus BT21 was identified by 18 S rRNA under the accession number of MN564896 in gene bank. The physicochemical parameters of the fermentation medium were optimized. Furthermore, the harvested protease was concentrated and partially purified by ethanol fractionation and then characterized to detect the enzyme identity.

Results and conclusion
The protease production increased by about 7.5-fold (3644.9 U/mg) after applying the final optimized fermentation medium, which contains dextrin 30, peptone 2, K_2HPO_4 1, $MgSO_4$ 0.5, KCl 0.5, and $FeSO_4$ 0.01(g/l) at 35°C, pH 8.0 and 150 rpm using 6% inoculum size after 6 days of incubation. The purification results showed that the highly recovered fraction was at 60% ethanol concentration with a purification fold of 4.3 and enzyme recovery of 36.5%. The enzyme was thermotolerant with an optimum temperature of 50°C and optimum pH of 8.0. Furthermore, it was observed that there was a reverse relationship between the metal ion concentration and the enzyme relative activity. Finally, the data indicated that casein and human blood were the most suitable substrates for this enzyme, indicating that the enzyme can work in alkaline conditions and has thermotolerance properties with high affinity toward the blood substrate, which makes it a potential candidate for detergent formulation to facilitate blood stain removal.

Keywords:
alkaline protease, characterization, endophytic fungi, partial purification, production

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Introduction

Enzymes are biocatalysts generated by living cells to bring about specific biochemical reactions forming parts of the metabolic pathways of the cells and they are essential for life [1]. More than 3000 different enzymes have been identified and a lot of them being utilized in biotechnological and industrial applications [2]. The protease enzyme constitutes two-thirds of the total enzymes used in various industries including meat tenderization, detergents, cheesemaking, dehairing, baking, contact lens cleaners, waste management, and silver recovery [3]. Proteases can be categorized according to the amino acids found in the catalytic site into five classes, aspartic, cysteine, glutamic, serine, and threonine proteases, or as metalloproteases if a metal ion is required for catalytic activity [4]. They are classified as acid, neutral, and alkaline enzymes based on their pH. Alkaline proteases constitute 60–65% of the global industrial market because of their activity and stability at an alkaline pH [5].

Proteases are created by a wide scope of microorganisms including bacteria, fungi, molds, and yeasts, which play an essential role in biotechnology for the generation of intracellular and extracellular enzymes on an industrial scale [3]. Microbial proteases are preferred over plant and animal sources because they are commonly less expensive to produce, their enzyme yields are more predictable and controllable, in contrast to plant and animal tissues,

and contain less potentially dangerous byproducts [6]. Fungal proteases is preferred than those from bacteria because they have a wide popularity in fermentation industry [3]. The endophytic fungi are thought to be a potential source of biologically active secondary metabolites [7] such as enzymes, antibiotics, and anticancer compounds [8]. Filamentous fungi can effectively secrete proteases which find their application in modern and biochemical industries such as foodstuff processing, improvement of detergents, pharmacologically active products, medical therapy, textiles, and molecular biology [9]. Many species of strains are documented to produce proteases including *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus melleu*, *Scedosporium apiospermum*, *Chrysosporium keratinophilum*, *Fusarium graminarum*, and *Penicillium griseofulvin* [10]. Furthermore, to achieve high levels of enzymatic yield, the perfect fermentation technology is needed. The cultivation medium and its components must be tested as well as the process parameters such as incubation temperature, pH, inoculum size, incubation time, and agitation speed [11].

The objective of this study was to evaluate the optimized conditions for maximum production, partial purification, and characterization of extracellular alkaline protease from an endophytic fungus (*Aspergillus ochraceus* BT21), which was isolated from the Egyptian medicinal plant collected from El-Orman Garden and previously identified as *Ruprechita salicifolia* at the National Research Center, Cairo, Egypt. The nutritional medium components such as carbon and nitrogen were optimized in addition to the physical parameters of culture conditions. The enzyme was partially purified by ethanol precipitation and characterized.

Materials and methods

The microorganisms

The 10 endophytic fungi used in this study were previously isolated from medicinal plants collected from El-Orman Garden, Cairo City, Egypt, and screened for protease production after 6 days of incubation using Dox medium [12].

Maintenance of fungi

The fungi were cultured in Czapek-Dox agar slants, which contained the following ingredients (g/l): sucrose 30, $NaNO_3$ 3, K_2HPO_4 1, $MgSO_4$ 0.5, KCl 0.5, $FeSO_4$ 0.01, and agar 20 [13]. Then the cultures were incubated at 28°C for 7 days and kept at 4°C.

Genetic identification of the most enzyme-producing fungus using 18S rRNA method PCR amplification and sequencing

The active strain was genetically identified according to Sigma Scientific Services Company method as follows: DNA extraction was made by Quick-DNA Fungal/Bacterial Microprep Kit (Zymo research #D6007). Then PCR cleanup to the PCR product was made by using the GeneJET PCR Purification Kit (Thermo K0701) (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Finally, the sequencing was made to the PCR product on GATC Company by an ABI 3730xl DNA sequencer using ITS1 and ITS4 forward and reverse primers [14].

Culture conditions and inoculum preparation

Spore suspensions of 10^6 CFU/ml from each isolate were allowed to grow at 28°C in 250 ml Erlenmeyer conical flasks contained 50 ml of Dox medium at 200 rpm for 3 days. Then, the production medium was inoculated with 3 ml of the vegetative inoculum, which was then shaken at 200 rpm for 6 days at 28°C. After that protease activity and total protein as well as final pH were determined in cell-free supernatants.

Optimization of protease production

Screening of different fermentation media

Five different media were examined for protease production by using the maximum producing organism. The media contained the following ingredients (g/l):

M1: glucose 10, peptone 5, yeast extract 1, $MgSO_4$ 0.5, and KH_2PO_4 1 [15].
M2: glucose 20, peptone 10 [16].
M3: yeast extract 3, malt extract 3, glucose 10, and peptone 5 [17].
M4: sucrose 30, $NaNO_3$ 3, K_2HPO_4 1, $MgSO_4$ 0.5, KCl 0.5, and $FeSO_4$ 0.01 [18].
M5: glucose 30, yeast extract 2, peptone 10, $NaNO_3$ 3, $MgSO_4$ 0.5, KCl 0.5, and KH_2PO_4 0.5 [19].

All the previous media were incubated with shaking at 200 rpm at 28°C for 6 days. Enzymatic activity was determined after 2, 4, and 6 days of incubation.

Investigation of carbon sources

Different carbon sources (lactose, molasses, arabinose, dextrin, sorbose, glucose, starch, and rice bran) were investigated by replacing the carbon source (sucrose) of the best selected fermentation medium obtained from the previous step (Dox medium M4) with 3% (w/v) of each carbon source. The flasks were incubated at 28°C in a shaker at 200 rpm for 6 days. Furthermore, the best

selected carbon source (Dextrin) was optimized at different concentrations (from 1 to 7%).

Investigation of nitrogen sources

A number of supplementary nitrogen sources were tested for optimum protease production by supplying some inorganic sources (ammonium hydrogen phosphate, ammonium nitrate, ammonium sulfate, urea) and some organic sources (casein, yeast extract, meat extract, soybean, peptone, and tryptone) instead of sodium nitrate in the best medium (Dox medium M4) at a level equivalent to 0.3% (w/v). Different concentrations of peptone (1–5 g/l) as the best nitrogen source were investigated for maximum production of protease.

Effect of initial medium pH

The effect of initial pH on protease production was examined by adjusting the culture pH at different pH ranges from 4.0 to 10.0 at 1.0 unit interval with 1 N HCl/NaOH before sterilization. The optimized media with the above pH values were inoculated with the organism and incubated at 28°C at 200 rpm for 6 days. The contents were centrifuged, and protease specific activity was checked in the cell-free extract.

Effect of different degrees of temperature

The ability of the organism to produce protease at different degrees of temperature was investigated. The fungus was subjected to various temperature degrees ranging from 25 to 40°C and the specific activity of the enzyme was evaluated.

Effect of inoculum size

The impact of inoculum density was tested by inoculating the production media with spore and vegetative suspensions of the fungus at a range of 2–10% V/V of each one. Then protease production was determined in cell-free supernatants as described previously.

Effect of agitation speed

The fungal cultures were incubated at 100–250 rpm with 50.0 unit variation.

Enzyme assay

To determine protease activity, azocasein substrate was used at a final concentration of 2.0% [20] modified according to Cabral *et al.* [21]. The reaction mixture that contains the enzyme, substrate, and 100 mM Tris-HCl buffer at pH 8.5 was incubated for 30 min at 37°C and then a 0.5 ml of 15% (w/v) trichloroacetic acid was added. After the incubation of that mixture for 15 min in ice bath, it was centrifuged and the reading of the supernatant was measured at 366 nm. One unit of proteolytic activity was defined as μg azocasein hydrolyzed per min under standard assay conditions according to Brock *et al.* [22].

Determination of protein

Protein contents were determined using standard bovine serum albumin [23].

Partial purification of protease

Ethanol fractionation and precipitation

The concentrated crude enzyme which is obtained from the final optimized medium was precipitated by cold ethanol at concentrations of 0–40%, 40–60%, 60–80%, and 80–90% at 4°C using magnetic stirring. Precipitates of the fractions were dissolved in phosphate buffer (pH 7.0) and assayed for protease activity and protein.

Characterization of the partially purified enzyme

Effect of pH on enzyme activity

Different pH ranges from 3.0 to 11.0 were examined for the detection of enzyme activity. The assay was applied at a buffer concentration of 100 mmol/l using standard buffers: sodium citrate buffer (pH 3.0–4.5), sodium acetate buffer (pH 5.0–6.0), sodium phosphate buffer (pH 6.5–7.5), Tris-HCl buffer (pH 8.0–9.0), and glycine-NaOH buffer (pH 10.0–11.0).

Effect of temperature on enzyme activity

To detect the maximum activity of the enzyme, different temperatures ranges from 20 to 80°C were applied.

Effect of some metals on enzyme activity

To test the effect of silver and copper metals on the activity of the partially purified enzyme, different concentrations at 1, 5, and 10 mmol/l were preincubated with the enzyme for 30 min, followed by addition of the substrate and detection of activity.

Effect of different substrates on enzyme activity

The partially purified enzyme was incubated with three different substrates (casein, gelatin, and blood as a source for fibrin and hemoglobin) for 60 min in a shaking water bath. Under the assay conditions, the substrates were used at the same volume and concentration. Then enzyme activity was detected and the relative activity was calculated.

Statistical analysis

Results are expressed as the mean±SE of three independent astrocytic culture preparations performed in triplicate.

Results

Generally, microbial production of enzymes depends on the environmental conditions of the fermentation medium including chemical and physical parameters of the culture such as pH value, temperature, agitation speed, medium nutrients and substrates, inoculum size, etc. This study investigated different parameters of the medium to maximize protease production by *A. ochraceus* BT21 followed by enzyme partial purification and characterization.

A number of fungi isolated from the medicinal plants were screened for their efficiency to produce protease enzyme. The most active isolate was identified as *A. ochraceus* BT21 and chosen for further investigations.

Genetic identification of the most potent fungus

The 18S rRNA (ribonucleic acid) sequencing technique was used to identify the fungal isolate on a molecular level. The phylogenetic tree (Fig. 1) demonstrated that the fungus was in a close relation to *A. ochraceus* strain BT21. Similarity of the sequencing and multiple alignments confirmed that the fungus identity was 99% with *A. ochraceus* and it was submitted in a gene bank and registered under the accession number: MN564896.

Optimization production of *A. ochraceus* BT21 protease

Effect of different fermentation media

The results in Table 1 show that medium number (4) was superior for maximum yield of the enzyme at all incubation times, where the specific activities recorded 319.9, 376.8, and 488.6 U/mg after 2, 4, and 6 days, respectively, while media (2, 3, and 5) were less favorable to be utilized by the fungus for the production of active enzymes.

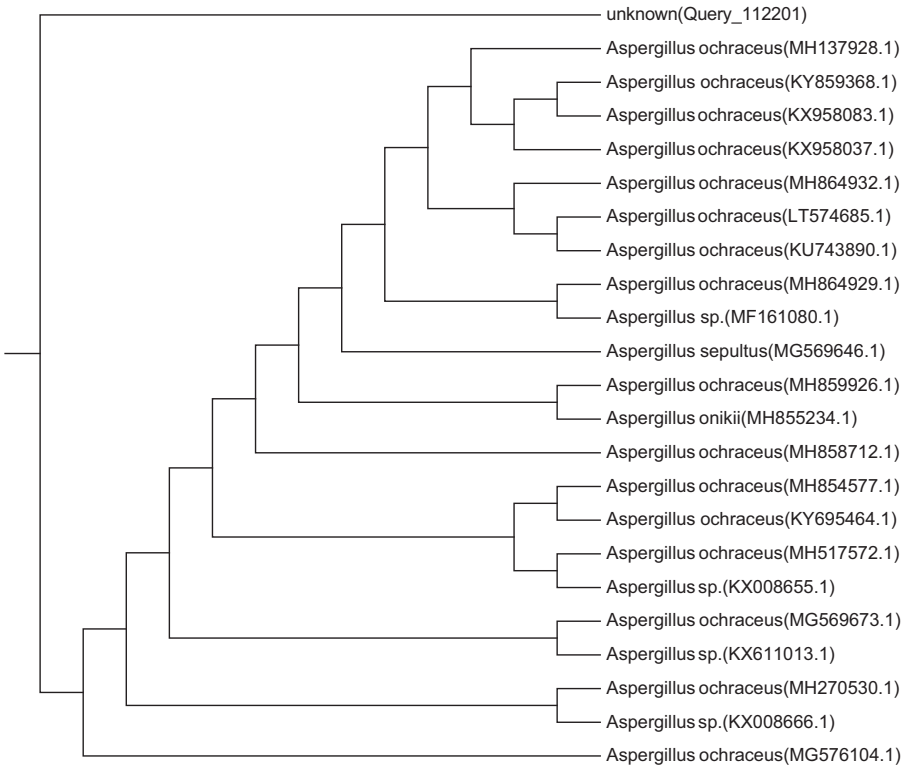
Role of carbon substrates

A number of carbon nutrients were chosen and screened in the fermentation medium for protease production. The results presented in Table 2 confirmed the vital importance of dextrin as a potent inducer of the enzyme, which exhibited 671.4 U/mg followed by arabinose (617.6 U/mg). On the other hand, moderate activity (424.4, 489.1, and 495.7 U/mg) was obtained by the addition of rice bran, lactose, and sucrose, respectively, in the fermentation medium. Moreover, it was noticed that the use of sorbose decreased the activity of the enzyme to its lowest value (135.9 U/mg).

Impact of dextrin levels

To improve and evaluate the production of protease from *A. ochraceus* BT21, six concentrations of dextrin were studied. It was evident from

Figure 1



Phylogenetic tree of the fungal isolate (*Aspergillus.ochraceus* MN564896).

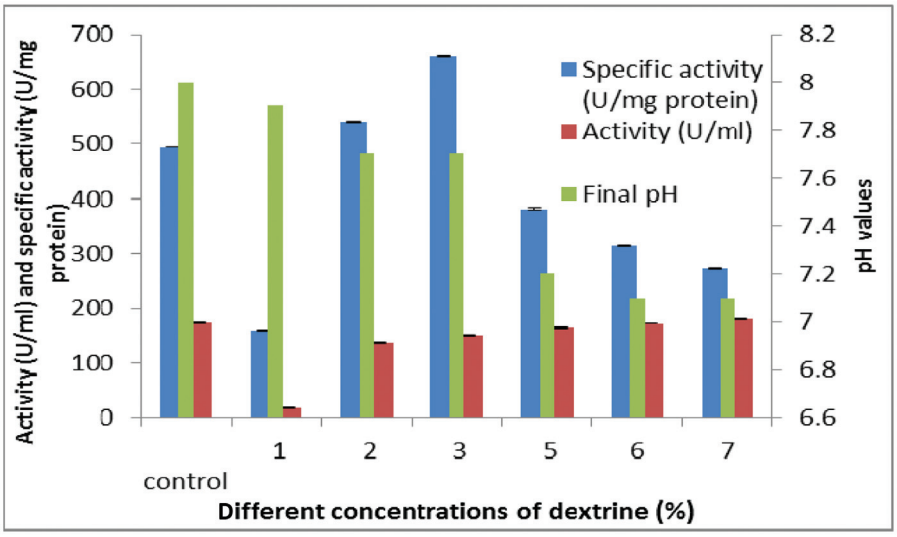
Table 1 Effect of different media on protease production

	Enzyme-specific activity (U/mg) and activity (U/ml)/(days)					
	2 days		4 days		6 days	
Medium no.	Activity	Specific activity	Activity	Specific activity	Activity	Specific activity
1	197.0±1.0	289.1±0.2	226.1±0.4	309.7±0.12	214.1±183	273.1±1.40
2	234.8±0.5	145.5±0.5	252.4±0.8	176.5±0.56	263.2±1.5	152.1±1.75
3	226.4±0.4	229.6±0.76	214.4±1.4	177.2±0.80	170.4±1.0	135.9±0.6
4	209.2±1.03	319.9±0.12	169.2±1.9	376.8±0.9	167.6±0.65	488.6±0.8
5	268.4±1.4	118.8±0.63	244.0±0.7	110.4±1.4	299.6±0.2	141.9±1.6

Table 2 Effect of different carbon sources on protease production

Carbon sources	Specific activity (U/mg protein)	Activity (U/ml)	Final pH
Control (sucrose)	495.7±0.5	170.9±1.5	7.5±0.1
Lactose	489.1±0.65	107.0±0.5	7.1±0.02
Molasses	340.2±1.2	263.3±0.9	7.9±0.05
Arabinose	617.6±1.8	174.8±2.1	7.6±0.05
Dextrin	671.4±0.45	153.1±0.2	7.0±0.05
Sorbose	135.9±0.75	128.9±1.8	7.3±0.03
Glucose	187.4±1.7	119.1±1.15	7.1±0.04
Starch	218.6±1.9	145.2±0.2	7.0±0.02
Rice bran	424.4±2.0	156.1±0.7	7.5±0.04

Figure 2



Effect of different concentrations of dextrin on protease production.

Fig. 2 that the sharp increase in enzyme potency (660 U/mg) was achieved by supplying the fermentation medium with 3% dextrin, followed by 2% dextrin, which recorded 540.9 U/mg. On the contrary, the low and high levels of dextrin (1 and 7%) caused a clear decrease in enzyme activity (160.0 and 272.4 U/mg), respectively.

Effect of different nitrogen sources

Different sources of organic and inorganic nitrogen ingredients were examined in the fermentation media of the fungus to assess the one most suitable for optimum production. Data presented in Table 3 confirm a high activity of the enzyme by using organic nitrogen sources particularly peptone, which achieved a maximum activity (1151.3 U/mg) followed by soybean (1082.2 U/mg). On the other hand, addition of inorganic sources gave either a moderate production (891.7 U/mg) by urea or low enzyme efficiency (190.1 U/mg) by the addition of ammonium nitrate.

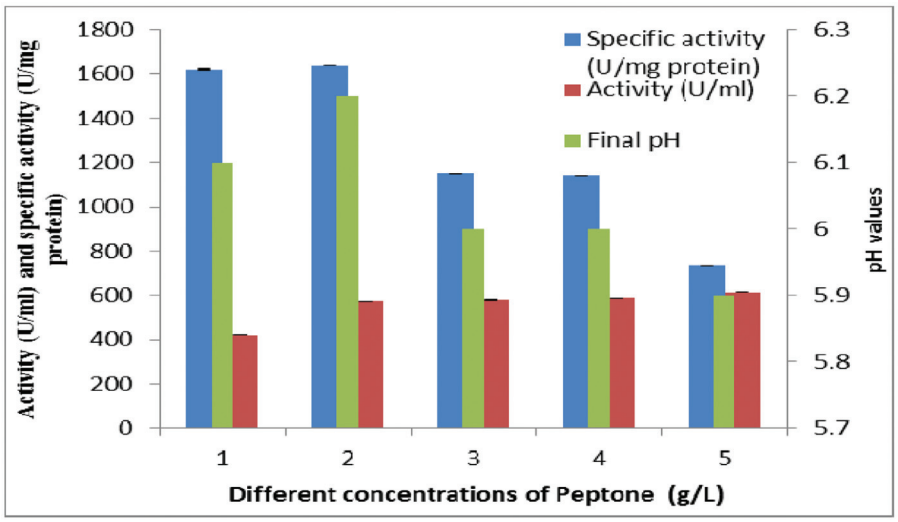
Effect of different concentrations of peptone

Five concentrations of peptone (1–5) g/l were examined in the production medium (Fig. 3). The presented data

Table 3 Effect of different nitrogen sources on protease production

Nitrogen sources	Specific activity (U/mg protein)	Activity (U/ml)	Final pH
Control (sodium nitrate)	662.4±0.4	160.4±1.75	8.0±0.01
Ammonium hydrogen phosphate	204.4±0.8	84.4±2.0	3.0±0.05
Ammonium nitrate	190.1±1.5	54.80±0.7	4.5±0.03
Ammonium sulfate	291.1±0.5	72.4±0.5	3.0±0.03
Urea	891.7±1.3	285.3±1.6	7.0±0.02
Casein	927.5±0.3	442.7±1.4	6.5±0.05
Yeast extract	749.2±1.4	591.6±0.8	6.6±0.05
Meat extract	985.1±0.4	550.5±0.3	6.5±0.04
Soybean	1082.2±0.6	597.2±1.6	6.0±0.03
Peptone	1151.3±1.4	549.2±1.2	6.0±0.02
Tryptone	664.9±0.5	428.5±0.4	6.9±0.01

Figure 3



Effect of different concentrations of peptone on protease production.

exhibited that high activities of the enzyme (1619.9 and 1637.8 U/mg) were produced at low levels of peptone (1 and 2 g/l, respectively). The proteolytic activity was significantly reduced (734.6 U/mg) at the highest concentration of the nitrogen source (5 g/l).

Effect of pH

To determine the pH optima for protease production, initial pH of the fermentation medium was adjusted at different pH values that ranged from 4.0 to 10.0 (Fig. 4). It was observed that the enzyme-specific activity increased as the pH of the medium increased and the maximum production was obtained at pH 8.0 (1890.5 U/mg). Moreover, it was shown that further increase above pH 8.0 resulted in a decrease in protease specific activity.

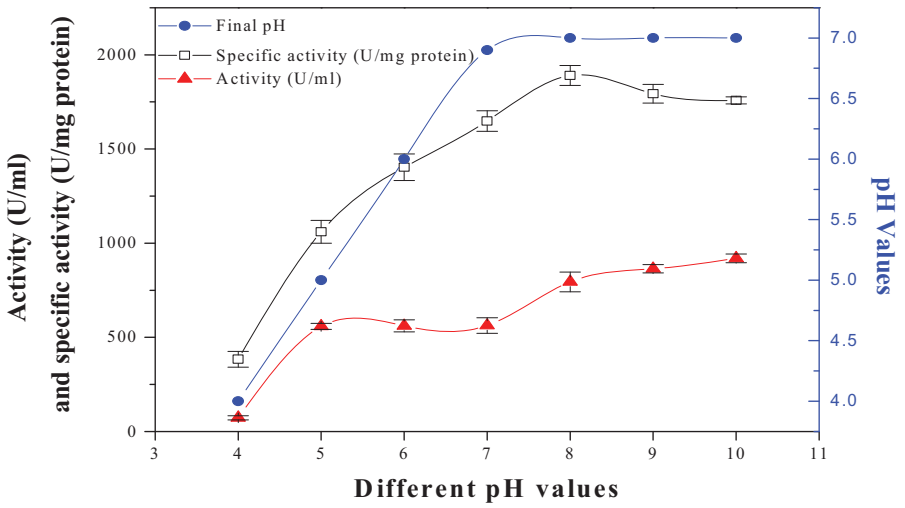
Effect of temperature

To study the production of protease under the effect of different temperature values, the inoculated medium was incubated at different temperatures that ranged from 20 to 40°C. It was found that the enzyme production increased to reach its maximum value (2354.9 U/mg) at 35°C (Fig. 5). A further increase in temperature resulted in a decrease of enzyme-specific activity, where the producing strain was sensitive to temperatures above and below the optimum value.

Effect of inoculum level

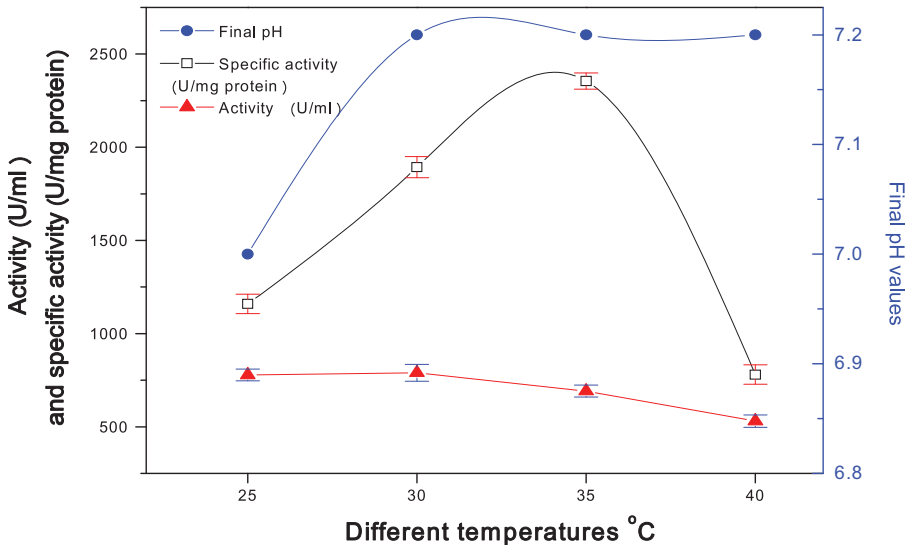
The dependence of protease production on inoculum level was investigated by testing different volumes (2–10% v/v) of both spore and vegetative inoculums (Table 4). The maximum yield of the enzyme (2616.3 and 3133.0 U/mg) was obtained by 6% v/v inoculum size of vegetative and spore cells, respectively; then a slight decrease in productivity was obtained by increasing the inoculum volume. Further, it was observed that the spore suspension was more effective than the vegetative one.

Figure 4



Effect of different pH values on protease production.

Figure 5



Effect of different temperatures on protease production.

Table 4 Effect of different inoculum sizes on protease production

	Specific activity (U/mg protein)		Activity (U/ml)		Final pH	
Inoculum size (v/v %)	Vegetative	Spores	Vegetative	Spores	Vegetative	Spores
2	2163.3±2.8	2588.7±3.0	694.9±0.8	694.9±0.7	6.3±0.1	6.2±0.1
4	2343.2±1.9	2839.4±1.6	688.8±0.9	713.8±0.9	6.10±0.05	6.3±0.12
6	2616.3±2.2	3133.0±1.9	696.6±1.1	740.5±1.3	6.2±0.04	6.2±0.15
8	2546.9±2.5	3101.4±2.1	709.5±1.2	766.9±1.2	6.0±0.15	6.0±0.04
10	2476.0±1.3	3045.7±2.6	721.5±0.9	827.3±1.3	6.3±0.1	6.0±0.03

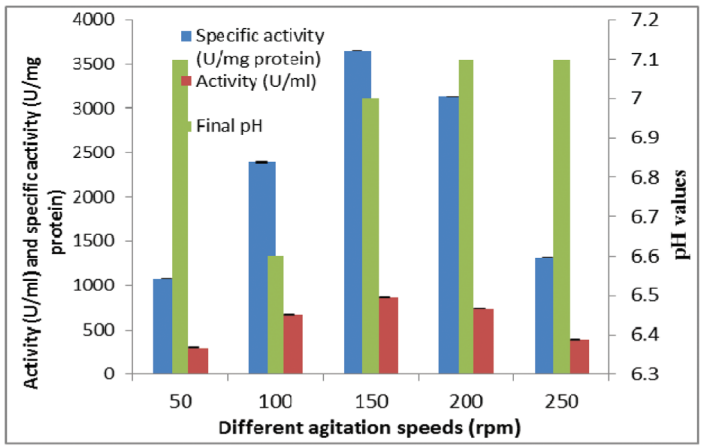
Effect of agitation rates

The effect of different agitation speeds on protease production was determined by applying different rpm (50–250) (Fig. 6). The maximal protease production (3644.9 U/mg) was obtained at 150 rpm.

Partial purification of protease

The concentrated crude enzyme was precipitated by cold ethanol using different concentrations (0–40%, 40–60%, 60–80%, and 80–90%). This step led to increase the purification fold to 4.3 with an enzymatic recovery of 36.5% at a concentration of

Figure 6



Effect of different rpm on protease production.

Table 5 Ethanol fractionation profile

Purification steps	Total activity (U)	Total protein (mg)	Specific activity (U/mg protein)	Purification fold	Recovery (%)
Crude extract	71 680 ±6.5	20.41 ±1.1	3512.0 ±5.5	1.0	100
Ethanol fractionation at 40%	100 +1.4	3.0+0.4	33.3 ±3.5	0.01	0.001
60%	26 144 ±21.2	1.73 ±0.6	15 112.1 ±35.4	4.3	36.5
80%	3560 ±2.3	4.58 ±0.6	777.3 ±3.8	0.22	0.05
90%	292 ±1.8	0.76 ±0.1	384.2 ±18.0	0.11	0.004

40–60% (F60%) (Table 5), while the other fractions gave lower purification folds, therefore the F60% will be selected for further study.

Characterization of the partially purified protease

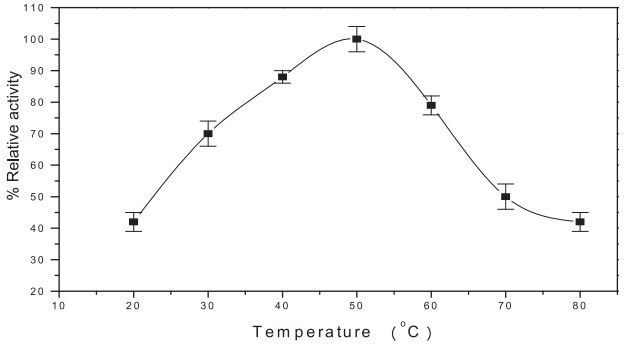
Effect of different temperatures on protease activity

The enzyme activity was tested at a temperature range of 20–80°C (Fig. 7). The maximum activity was achieved at 50°C and the enzyme was retained almost of its activity at a wide temperature range from 30 to 60°C.

Effect of pH on protease activity

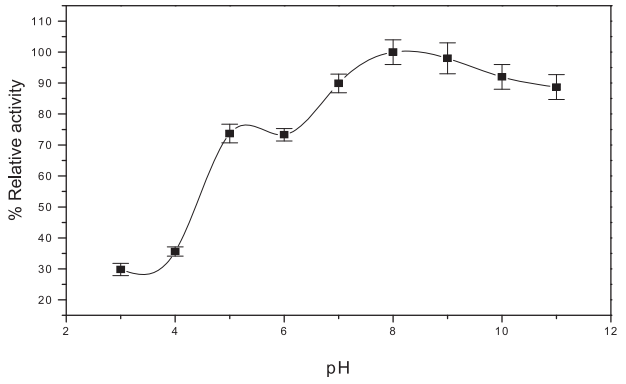
To investigate the influence of pH on protease activity (F60%), different pH values in the range from 3.0 to 11.0 were studied. Data shown in Fig. 8 indicate that the optimum pH was at 8.0 and the enzyme maintained its activity at a broad pH range from 5.0 to 11.0.

Figure 7



Effect of different temperatures on protease activity.

Figure 8



Effect of pH on protease activity.

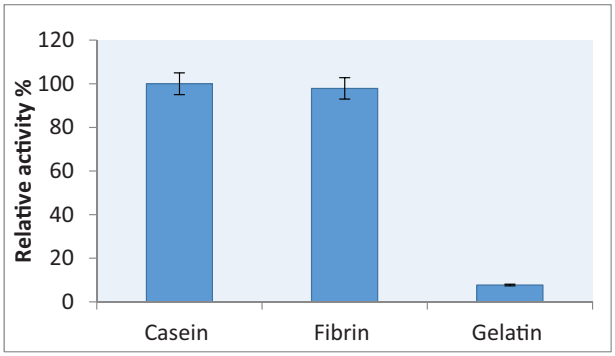
Effect of metal ions on protease activity

The F60% of the partially purified protease was preincubated with two metal ions (Ag^{+2} and Cu^{+2}) at concentrations of 1, 5, and 10 mmol/l for 30 min. Table 6 shows that the enzyme activity was inhibited with increasing metal ion concentration under the

Table 6 Effect of metal ions on protease activity

	Relative activity (%)		
Metal ions	1 mmol/l	5 mmol/l	10 mmol/l
Control	100	100	100
Ag^{+2}	97.5±2.3	69.1±1.7	62.7±0.8
Cu^{+2}	101.2±1.8	102.6±0.7	100.4±1.5

Figure 9



Effect of different substrates on protease activity.

effect of Ag^{+2} reaching 37.3% at 10 mmol/l concentration, whereas Cu^{+2} had a small stimulation of the activity (2.6%) under a concentration of 5 mmol/l.

Effect of different substrates on protease activity

The partially purified protease was targeted to hydrolyze natural proteins such as casein, gelatin, and blood as a substrate for fibrin (Fig. 9). The enzyme showed specificity toward casein (100%) followed by blood (97.9%) and a small efficiency toward gelatin (7.7%).

Discussion

Optimization of fermentation conditions

Effect of different fermentation media on protease production

Suitable cultivation conditions are very important factors for enzyme production. The medium composition including C/N ratio, and presence of easy metabolites and minerals greatly influence protease production [24]. Our results indicated that the highest yield of the enzyme (488.6 U/ml) was obtained by the use of M4 which contains sucrose, $NaNO_3$, and mineral constituents, while, a poor production of the enzyme (135.9 U/ml) was obtained by M3, which contains glucose, peptone, and lacks in mineral salts. This is in accordance with Madzak *et al.* [25] who found that sucrose is a good substance for protease productivity. Furthermore, Puri *et al.* [26] recorded that glucose inhibited the enzyme production. Also, the decrease of medium mineral salts such as $MgSO_4$ and $NaNO_3$ was found to dramatically inhibit the protease yield by *A. niger* [6].

Effect of different carbon sources

Various carbohydrates were examined as a carbon ingredient in the production medium of protease. The productivity of the enzyme was affected by the type of carbohydrate added. The stimulatory effect of dextrin was noticed at a concentration of 3%, followed by arabinose. Our observation was in agreement with the results of Ahmed [27], who used dextrin at a concentration of 3% for protease production. Moreover, Phadatare *et al.* [28] reported that the highest yield of protease was obtained by the addition of arabinose in the fermentation medium. On the other hand, addition of glucose suppressed the yield of the enzyme (187.4 U/ml) and this is in accordance with Leger *et al.* [29].

Effect of nitrogen sources

Various organic and inorganic nitrogen sources were screened for enzyme productivity and it was observed that the organic sources enhanced the production of protease [30]. Peptone was the most favorable source for a maximum yield of the enzyme followed by soybeans, while the inorganic ammonium compounds repressed the yield. This is in accordance with Kumar and Takagi [31], who found that the rapidly metabolized inorganic nitrogen sources inhibited enzyme production. Similar results with *Aspergillus Oryzae* were observed by Srinubabu *et al.* [32]. Moreover, maximum productivity was obtained with low concentrations of peptone (0.1–0.2%), while the highest concentrations decreased the activity. The same results were recorded by Rajkumar *et al.* [33], who applied 0.5% peptone using *Bacillus megaterium.*

Effect of pH

Among physical parameters, pH of the production medium can influence microbial enzymatic secretion and product stability in the culture medium [34] due to the effect on the chemical structure of the enzyme, which may cause enzyme denaturation and loss of its catalytic activity. Also, it may alter the charge and structure of the substrate consequently; the enzyme–substrate complex can no longer be formed [35]. The optimal pH varies with different microorganisms and enzymes. The produced proteases can be alkaline or acidic depending on the microorganisms and isolation source [36]. From our results, the enzyme was more active in alkaline conditions and the maximum production was at pH

8.0 (Fig. 4). A similar result was reported by Oyeleke *et al.* [37], who found that the maximum protease activity for *A. niger* and *A. flavus* was at pH 8.0. On the other hand, the maximum protease activity was detected at pH 9.0 in case of *Penicillium chrysogenum* and *A. niger* by Sethi and Gupta [38].

Effect of temperature

Incubation temperature has an effective role in the metabolic activities of an organism, and then can affect enzyme productivity [35]. Most of protease production studies that have been done with mesophiles were within the range of 25–50°C [39]. Our data shown in Fig. 5 indicated that the optimum production was noticed at a temperature of 35°C, while by raising temperature to 40°C, a minimum enzyme yield was observed that is due to the increased temperature that has adverse effects on enzymatic activities of microorganisms [40]. In a similar study, Yadav *et al.* [41] found that the suitable incubation temperature for protease production form mesophilic fungi such as *Aspergillus* sps was in the range from 31 to 35°C, while, in another study the authors obtained an optimum activity of protease from *A. niger at* 40°C [6].

Effect of inoculum size

The inoculum size is essential for optimum microbial growth and maximum enzyme production. Table 6 shows that the maximum productivity by using vegetative and spore cell cultures was at 6% v/v inoculum size. Above and below that ratio, a reduction in protease synthesis was observed; this may be due to the insufficient quantity of mycelium in the lower inoculum but in the higher inoculum, the increase in the amount of mycelium may lead to a competition toward nutrients and reduce the dissolved oxygen [42]. Also, the highest level of inoculum rapidly consumed most of the substrate for growth purposes, and hence the enzyme yield was decreased [43]. Our result for optimum level of inoculum size was in good agreement with general industrial inoculum size range (from 1 to 10%) [44]. Radha *et al.* [45] obtained their maximum protease activity (141.45 U/g) at an inoculum level of 10% v/v by *Aspergillus* spp. Muthulakshmi *et al.* [46] noticed that the maximum protease synthesis was at 3% inoculum size by *A. flavus*.

Effect of agitation rates

It was observed that protease productivity was influenced by changing the agitation speed (Fig. 6). Enzymatic specific activity reached its maximum value when the agitation speed reached 150 rpm but it decreased in lower and higher speeds. That is because of the lack in aeration and nutrient uptake in low speed, but in high speed the cells could be damaged and its morphology may be affected due to shear stress [47]. This is in agreement with Kamath *et al.* [48], who found that the optimum protease production by *A. niger* was at 150 rpm. Furthermore, in this context, some authors reported that the optimum production of protease was in the range of 140–300 rpm [49,50].

Partial purification by ethanol fractionation

Generally, ethanol is a good precipitation agent for proteins due to its poor solubilization of these biomolecules; also, it increases the interaction forces between the protein molecules. Furthermore, the organic solvents can form a solution with water which leaves almost all water molecules away from the protein [51]. Our data indicated that an ethanol concentration of 60% increased the purification fold to 4.3 with an enzyme recovery of 36.5%. Similarly, Al Ghanimi *et al.* [51] used ethanol to precipitate protease from *Beauveria bassiana* and they got a purification fold of 2.3 with a yield of 16.3. These results are markedly different from those obtained by Dunaevsky *et al.* [52], who precipitated serine protease from *Aspergillus fumigatus* with 78% of its original activity using 50% ethanol.

Characterization of partially purified protease

Effect of temperature on protease activity

Temperature results demonstrated that the enzyme was highly active at a temperature range of 30–60°C and the optimum temperature was at 50°C supporting the thermotolerant nature of our enzyme. Previously reported studies obtained the same results with

Rhizopus *oryzae* NBRC 4749 (50°C) [53] and *Trichoderma reesei* QM9414 [54]. Higher results were obtained by Abidi *et al.* [24] using *Botrytis cinerea*, whereas Germano *et al.* [55] obtained lower optimal temperature by *Penicillium* spp.

Effect of pH on protease activity

Our results indicated that the enzyme exhibited a great activity in a wide pH range (5.0–11.0) with optimum activity at pH 8.0 indicating that it is alkaline protease. The extracellular serine protease produced by *Bacillus subtilis* was found to have maximum activity at pH 8.0 [56]. Higher pH value was exhibited at pH 9.0 of alkaline protease produced from the fungus *Pleurotus sajor-caju* which kept almost of its maximum activity at a pH range of 7.0–10.0 [57]. Lower values were recorded with R. *oryzae* MTCC 3690 at pH 5.5 [58].

Effect of metal ions on protease activity

The metal ions have varying effects on the activity of proteases produced from different microbes [53]. The influence of different concentrations of Ag^{+2} and Cu^{+2} on protease activity is shown in Table 6. The results revealed that there was a little activation by Cu^{+2} that reached 2.6%, whereas Ag^{+2} decreased the activity by 37.3%. In a similar study, Cu^{+2} had no inhibitory effect on Rhizopus pepsin produced from Rhizopus *chinensis* [59]. But there was a strong inhibition of protease from *A. niger* I1 by the addition of Cu^{+2} [60] and form *A. niger* BCRC 32720 by the addition of Ag^{+} [61].

Effect of different substrates on protease activity

Alkaline protease from *A. ochraceus* BT21 was found to have substrate specificity for casein (100%) followed by blood (97.9%) and there was a small affinity toward gelatin (7.7%). This indicates that protease can target a wide range of natural protein substrates, which is in accordance with Al Ghanimi *et al.* [51], who found that casein is the optimum substrate for protease from *B. bassiana* as compared with gelatin and bovine serum albumin. Moreover, the highest enzyme activity of *A. flavus* AP2 protease was toward gelatin [62]. Thus, it was clearly indicated that our enzyme can work efficiently in alkaline conditions at a wide range of temperatures and had high affinity toward the blood substrate; therefore, this making it a good candidate for application in detergent formulations to help in blood stain removal [57].

Conclusion

In conclusion, the high enzymatic activity suggested that *A. ochraceus* BT21 is a good producer for alkaline thermophilic protease. From our results, both physical and chemical parameters of cultivation conditions had an important role to maximize the production of our protease. Thus, these parameters could be important factors to consider to scale up production. Moreover, the characterization of alkaline protease was made to detect the enzyme properties and determine its suitable industrial and biotechnological applications.

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Conflicts of interest

There are no conflicts of interest.

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