Research Article

Partial characterization of extracellular protease produced by *Aspergillus* sp. isolated from soil sample

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Received: 9 June 2017 Revised: 20 July 2017 Accepted: 3 August 2017

Abstract

Background: Fungi are utilized for commercial production of extracellular enzymes, vitamins, alcohol, pigments, glycolipid and polysaccharides. Among all commercial products, protease is one of the important industrial products due to its application in food, pharmaceutical, detergent and medical sectors. **Objectives:** The purpose of present investigation was to partially characterize crude extracellular protease of wild and mutant strain of *Aspergillus* sp. to find out the stability of crude protease in chemical compounds and determination of temperature and pH optima. **Materials and methods:** Crude protease recovered from culture broth of wild and nitrous acid mutagenic strain *Aspergillus* sp. was pre-incubated at different temperature, pH, organic solvents and metal ions for 2 h thereafter protease activity and protein content were determined. **Results:** Activity of protease was optimum at pH 10.0 and 27 ºC to 37 ºC. Activity was higher in higher pH range than in acidic pH range indicates alkaline nature of enzyme. Butanol and ethanol were found excellent inducer and increased protease activity up to 57% and 37%, respectively. Protease activity was enhanced by Ca^{2+} and Mn^{2+} up to 14% and 5%, respectively for wild strain and by Mg^{2+} and Mn^{2+} up to significant level for mutant strain. **Conclusions:** crude protease of wild and mutant strain of *Aspergillus* sp. was partially characterized to find out effect of various parameters on its activity. Further characterization study can be carried out for more information of protease which can determine its utility in industries.

Keywords: characterization, *Aspergillus* sp., pH, temperature, protease activity, butanol

Introduction

Protease is one of the most imperative industrial enzymes of consideration accounting for 60% of the total enzyme market in the globe and contributes for about 40% in total global enzyme sell (Chouyyok et al., 2005). The importance of this group of enzymes, plentiful in structural diversity and mechanisms of action is reflected in the significance of their applications in industrial procedures. They have numerous applications, such as in detergent, food, pharmaceutical and textile industry (Gupta et al., 2002). In addition, they are also utilized for some medical treatments of wound and inflammation, recovery of silver from photographic film and production of digestives (Paranthaman et al., 2009). Thus, the industrial demand of proteases, with proper specificity and stability to temperature, pH, organic solvents and metal ions, keep on motivating the investigation for novel sources (Van Den Hombergh et al., 1997). Proteases with higher activity and stability within the alkaline pH range are attractive for bioengineering and biotechnological applications, particularly those from fungi and bacteria (Jellouli et al., 2009; Wang et al., 2009). Alkaline proteases are frequently utilized in the detergent industry since the pH of cleaning products is generally in the range of 9.0 to 12.0. Due to addition of proteases in the cleaning products reduces the use of other toxic solvents and corrosive compounds and decreases their influence on the climate (Castro et al., 2004).

Proteases are secreted by wide diversity of sources such as animal, plants and microbes. But they are secreted chiefly by microbes. Several microbes excrete proteases to the outside environment so as to degrade proteins; their hydrolysis products are utilized as nitrogen and carbon sources for cell growth and multiplication. Thus, microbial

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proteases are degradative in nature which catalyzes complete hydrolysis of their protein substrate (Haq et al., 2006). Microbial proteases are generally extracellular and are secreted directly into culture broth which makes purification easier (Ghildyal et al., 1985). Microbial proteinases display several distinctive features in terms of activation mechanism, mechanism of catalysis, substrate specificity, thermostability, metal ion stability and optimal pH (Rao et al., 1998).

Fungi elaborate a broad diversity of protein degrading enzymes than bacteria. Potent protease producing fungi are: *Aspergillus niger* (Paranthaman et al., 2009), *Aspergillus candidus* (Nasuno and Onara, 1972), *Aspergillus awamori* (Melikoglu et al., 2013), *Aspergillus flavus* (Kranthi et al., 2012), *Aspergillus clavatus* (Silva et al., 2011), *Aspergillus oryzae* (Vishwanatha et al., 2009), *Rhizopus oryzae* (Kumar et al., 2005), *Candida albicans* (Ergin and Semra, 1994), *Fusarium solani* (Olivieri et al., 2004), *Penicillium godlewskii* (Sindhu et al., 2009), *Penicillium chrysogenum* (Haq et al., 2006), *Hirsutella rhossiliensis* (Wang et al., 2009) and commercial protease producing bacteria are: *Bacillus amyloliquefaciens* (Vasantha et al., 1984), *Bacillus pumilus* (Sangeetha et al., 2011), *Bacillus altitudinis* (Madhuri et al., 2012), *Bacillus cereus* (Rathakrishnan and Nagarajan, 2011), *Bacillus laterosporus* (Usharani and Muthuraj, 2010), *Bacillus thuringiensis* (Sugumaran et al., 2012), *Bacillus licheniformis* (Al-Shehri and Mostafa, 2004), *Bacillus subtilis* (Chouyyok et al., 2005), *Serratia marcescens* (Romero et al., 1998), *Nocardiopsis dassonvillei* (Kim et al., 1993), *Vibrio fluvialis* (Venugopal and Saramma, 2006) and *Pseudomonas fluorescens* (Kalaiarasi and Sunitha, 2009).

The culture conditions of the production medium play a very important role in the growth and production of desired metabolites. The most significant amongst these are the ingredients of medium, pH, temperature and shaking speed. The pH has been reported as having strong influence on microbial protease production because it can reduces the availability of nutrients by ionization of nutrient molecules (Al-Shehri and Mostafa, 2004). Another significant factor for protease production is temperature. It has been reported by Tunga (1995) that higher temperature reduces the growth of protease producing microbes.

Recently we have isolated an alkalophilic fungus *Aspergillus* sp. which produces extracellular protease. In view of the above background, extracellular protease enzyme produced by submerged fermentation of *Aspergillus* sp. was partially characterize to determine the effect of various parameters (temperature, pH, organic solvents and metal ions) on enzyme activity and stability.

Materials and methods

Production of protease

We have already isolated wild and nitrous acid mutagenic strain of proteolytic fungus *Aspergillus* sp. from local soil sample of Newai town. Extracellular protease production was carried out by inoculation of 100 ml of production medium with 1 ml of spore suspension of 6 days old Petri plate culture of wild and mutant strain of *Aspergillus* sp. followed by incubation at proper conditions. The production medium of following composition (g/L) was utilized: K_2HPO_4, 1; yeast extract, 10; $MgSO_4.7H_2O$, 0.2; glucose, 20; KH_2PO_4, 1; pH 7.0. Crude protein extract was recovered from culture broth after separation of fungal mycelium pellets by filtration and centrifugation at 3 days of incubation. This mycelium free crude protein lysate of both the strains was utilized for partial characterization study.

Influence of temperature on protease activity and stability

It was determined by pre-incubation of crude protein extract at different temperatures (27 ºC, 37 ºC, 50 ºC and 60 ºC) for 2 h thereafter protease activity (Tsuchida et al., 1986) was determined by performing protease assay at corresponding temperatures (27 ºC, 37 ºC, 50 ºC and 60 ºC). Protein contents (Lowry et al., 1951) were determined.

Influence of pH on protease activity and stability

Potassium phosphate buffer (50mM) of various pH (4, 5, 6, 7, 8, 9 and 10) was prepared and used in this study. Equal amount (1:1 v/v) of crude enzyme and buffer (pH is different) were mixed and pre-incubated at optimum temperature for 2 h thereafter protease activity was estimated by conducting protease assay at corresponding pH (4, 5, 6, 7, 8, 9 and 10).

Influence of organic solvents on protease activity and stability

Crude enzyme was mixed (1:1 v/v) with 10% solution of organic solvents (methanol, butanol, ethanol and acetone) followed by pre-incubation at optimum temperature for 2 h thereafter protease activity was determined in which protease assay was carried out at optimum temperature 37 ºC and pH. Residual activity was estimated by dividing the protease activity of tests with protease activity of control (crude enzyme without any pre-incubation with organic solvents).

Influence of metal ions on protease activity and stability

Metal ions (5mM) used for the investigation were Ca^{2+}, Mg^{2+}, Ba^{2+}, Zn^{2+}, Cu^{2+} and Mn^{2+}. Crude protease was mixed (1:1 v/v) with metal ion and pre-incubated for 2 h thereafter

protease activity (assay was performed at optimum temperature and pH) and protein contents were determined. Control was prepared without any exposure to metal ions and used for calculation of residual activity.

Results and discussion

Temperature optima and thermostability

Crude protease from wild strain exhibited higher protease activity (336.11±12.83 U/ml) and specific activity (28.44±3.81 U/mg) when it was pre-incubated and protease assay was carried out at 27 ºC whereas protease of mutant strain demonstrated higher protease activity (336.15±2.14 U/ml) at 37 ºC and specific activity (49.64±0.57 U/ml) at 27 ºC. The activity of protease was higher when protease assay was performed at 27 ºC thereafter it declined with the rise of assay incubation temperature (37 ºC and 50 ºC) and reached to lowest at 60 ºC. The descending order of protease activity and thermostability for wild lipase was as follows: 27 ºC > 37 ºC > 50 ºC > 60 ºC whereas for mutant strain it was 37 ºC > 27 ºC > 50 ºC > 60 ºC (Figure 1).

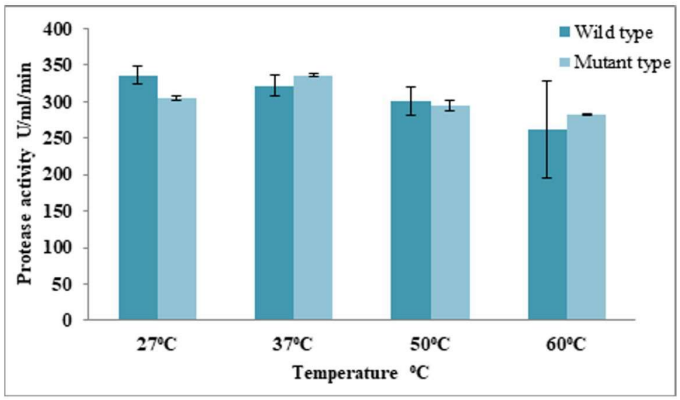

Figure 1. Determination of temperature optima for protease of wild and mutant strain of *Aspergillus* sp.

Muthulakshmi et al. (2011) reported optimum temperature of 30 ºC for *Aspergillus flavus* protease. Higher protease activity at elevated temperature (45-50 ºC) has been reported by Chandrasekaran and Sathiyabama (2014) for *Alternaria solani*, Sharma et al. (2016) for local soil fungal isolate, Kalpana Devi et al. (2008) for *Aspergillus niger*, Lario et al. (2015) for *Rhodotorula mucilaginosa* L7, Yin et al. (2013) for *Aspergillus niger* and Zanphorlin et al. (2011) for *Myceliophthora* sp. Optimum temperature 55-60 ºC has been reported by Souza et al. (2017) for *Aspergillus foetidus*, Mothe et al. (2016) for *Bacillus caseinilyticus* and Cui et al. (2015) for marine bacteria whereas Nai-Wan et al. (2014) reported extremely higher temperature 75 ºC for *Rhizopus oryzae*.

pH optima

Activity of crude protease from wild and mutant strain of *Aspergillus* sp. was lowest at pH 4.0 (14.15±0.23 U/ml for wild strain and 16.74±1.98 U/ml for mutant strain). It was gradually increased with the rise of pH of assay and reached to maximum (213.47±8.29 U/ml for wild strain and 224.08±0.96 for mutant strain) when protease assay was performed at pH 10.0 (Figure 2). Crude protease demonstrated higher activity in the alkaline pH range than in acidic and neutral pH range. These results suggest that protease of *Aspergillus* sp. is capable of working optimum at higher pH. Fungus can be utilized for bulk production of alkaline protease which can find applications in detergent industry where reactions are carried out at higher pH.

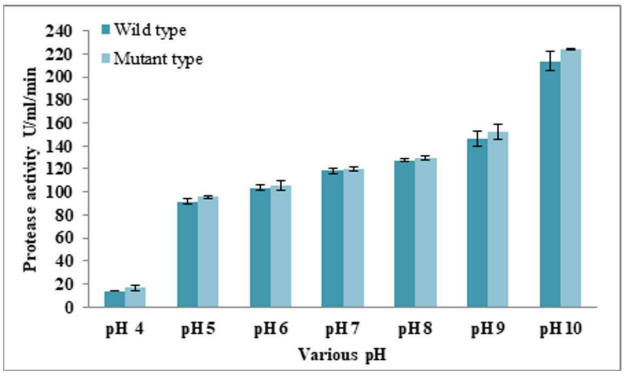

Figure 2. Determination of pH optima for protease of wild and mutant strain of *Aspergillus* sp.

Similar to present findings, pH 9-10 has been reported for optimum protease activity by Chandrasekaran and Sathiyabama (2014) for *Alternaria solani,* Kalpana Devi et al. (2008) for *Aspergillus niger*, Sharma et al. (2016) for local soil fungal isolate, Zanphorlin et al. (2011) for *Myceliophthora* sp. and Cui et al. (2015) for marine bacteria. Mothe et al. (2016) reported pH 8.0 for *Bacillus caseinilyticus*.

An optimum pH 5.0 has been reported by Muthulakshmi et al. (2011) for *Aspergillus flavus*, Souza et al. (2017) for *Aspergillus foetidus* and Lario et al. (2015) for Antarctic yeast *Rhodotorula mucilaginosa* L7 whereas optimum pH 3.4 by Nai-Wan et al. (2014) for *Rhizopus oryzae* and pH 2.5 by Yin et al. (2013) for *Aspergillus niger*.

Impact of organic solvents

Similar trend of protease activity and residual activity was observed for both wild and mutant strain. Ethanol and butanol exhibited stimulatory effect on the activity of protease. Among all solvents, butanol was found excellent inducer of protease activity and increased activity up to 51% (71.69±1.76 U/ml) for wild strain and up to 57% (76.31±1.93 U/ml) for mutant strain as compared to protease activity of control (47.49±1.94 U/ml for wild strain and 48.62±0.59 U/ml for mutant strain). Protease retained

Table 1.Effect of organic solvents on protease activity and stability of wild and mutant strain of *Aspergillus* sp.

Organic solvents	Protease activity (U/ml/min)	Residual activity (%)	Protease activity (U/ml/min)	Residual activity (%)	Specific activity (U/mg)	
	Wild strain		Mutant strain		Wild strain	Mutant strain
Control	47.49±1.94	100	48.62±0.59	100	11.77±0.53	22.93±0.28
Methanol	43.86±1.15	92.35	45.28±1.15	93.13	13.36±0.93	14.90±0.30
Ethanol	60.89±5.24	128	67.02±0.63	137.84	13.99±0.75	21.43±0.30
Butanol	71.69±1.76	151	76.31±1.93	157	13.92±0.73	18.27±0.58
Acetone	38.44±3.28	81	39.99±1.27	82.25	17.33±0.83	18.69±0.72

Table 2.Effect of metal ions on protease activity and stability of wild and mutant strain of *Aspergillus* sp.

Metal ions	Protease activity (U/ml/min)	Residual activity (%)	Protease activity (U/ml/min)	Residual activity (%)	Specific activity (U/mg)	
	Wild strain		Mutant strain		Wild strain	Mutant strain
Control	121.07±3.81	100	117.82±2.81	100	34.74±0.46	37.76±0.80
$CaCl_2$	138.34±2.21	114.26	100.60±1.15	85.38	39.07±5.02	27.68±1.58
$MgSo_4$	99.14±0.06	81.88	137.72±1.69	116.89	45.52±1.38	33.35±0.83
$BaCl_2$	93.67±1.22	77.36	96.78±1.89	51.20	42.15±2.15	43.66±2.07
$ZnSo_4$	79.29±2.64	65.49	86.31±0.98	73.25	37.92±1.23	39.64±1.94
$CuCl_2$	95.41±3.45	78.80	95.88±3.31	81.37	31.14±2.32	37.35±0.53
$MnCl_2$	127.53±2.48	105.33	131.59±2.66	111.68	32.42±0.36	38.21±1.28

93% and 81% of its activity when it was pre-incubated for 2 h with methanol and acetone, respectively (Table 1). The present results indicate that crude protease was found stable in all organic solvents with excellent stability in ethanol and butanol. Stimulatory effect of butanol might be due to that butanol interacted with enzyme and increased catalytic activity of the enzyme.

Zanphorlin et al. (2011) reported stimulatory effect of isopropanol and inhibitory effect of acetone, butanol and methanol (20% v/v) on protease activity of *Myceliophthora* sp. Cui et al. (2015) reported that acetone, methanol and isopropanol (25% v/v) increased protease activity up to 41%, 38% and 1%, respectively for marine bacteria. Sharma et al. (2016) reported that n-butanol increased protease activity up to 5.70% and 35.71%, respectively for two local soil fungal isolates, while acetone and methanol decreased protease activity.

Impact of metal ions

Calcium chloride, manganese chloride and magnesium sulfate demonstrated stimulatory effect on the activity of protease from wild and mutant strain of *Aspergillus* sp. For wild strain, Ca^{2+} and Mn^{2+} increased protease activity up to 14% (138.34±2.21 U/ml) and 5% (127.53±2.48 U/ml), respectively as compared to activity of control (121.07±3.81 U/ml) whereas for mutant strain, Mg^{2+} and Mn^{2+} increased protease activity up to 17% (137.72±1.69 U/ml) and 11% (131.59±2.66 U/ml), respectively when compared to 117.82±2.81 U/ml of control. Crude protease of wild strain retained 82%, 79%, 77% and 65% of its activity after 2 h pre-incubation with Mg^{2+}, Cu^{2+}, Ba^{2+} and Zn^{2+}, respectively whereas protease of mutant strain retained 85%, 81%, 73% and 51% of its activity with Ca^{2+}, Cu^{2+}, Zn^{2+} and Ba^{2+}, respectively (Table 2). The stimulatory effect of Ca^{2+} might be due to that Ca^{2+} is required as co factor for optimum functioning of protease. The present results specify that crude protease was found stable in all metal ions except Ba^{2+} in which protease activity was reduced to half after 2 h pretreatment.

Chandrasekaran and Sathiyabama (2014) reported that protease of *Alternaria solani* retained significant activity in presence of Ca^{2+}, Mg^{2+}, Mn^{2+} while Na^{+} and K^{+} showed inhibitory effect on protease activity. Similarly stimulatory effect of Ca^{2+}, Mg^{2+} and Mn^{2+} (5mM) was reported by Zanphorlin et al. (2011) for *Myceliophthora* sp. Nai-Wan et al. (2014) reported that 1mM Ca^{2+} did not show any effect on proteolytic activity of *Rhizopus oryzae* while Mg^{2+}, Mn^{2+}, Zn^{2+} and Cu^{2+} decreased protease activity. Mothe et al. (2016) reported stimulatory effect of Ca^{2+}, Mg^{2+}, Fe^{2+} and K^{+} for *Bacillus caseinilyticus*. Cui et al. (2015) reported

increased protease activity of marine bacteria with Ca^{2+}, Mn^{2+}, Zn^{2+}, Cu^{2+} and Na^{+}. Inhibition of activity of *Aspergillus niger* protease by Ag^{+}, Sn^{2+}, Fe^{3+} and Sb^{3+} has been reported by Yin et al. (2013). Kalpana Devi et al. (2008) reported increment in protease activity of *Aspergillus niger* up to 5% after pre-incubation with Ca^{2+} whereas Sharma et al. (2016) reported 23% increased activity for local soil fungal isolate with Ca^{2+}.

Conclusions

Aspergillus is a genera of filamentous fungi and recognized as generally regarded as safe (GRAS). Its different species are well known to produce variety of extracellular enzymes including proteases which find some promising applications in various industries ranging from food, dairy, textile, pharmaceutical, detergent, textile, silver recovery etc. The present study was aimed to partially characterize crude protease of wild and mutagenic strain of *Aspergillus* sp. pH optima of protease from both strains was found to be 10.0 whereas temperature optima for protease of wild and mutant strain was 27 ºC and 37 ºC, respectively. Butanol and ethanol showed stimulatory effect on protease activity and among metals, Ca^{2+}, Mg^{2+} and Mn^{2+} enhanced protease activity up to significant level.

Acknowledgements

We are thankful to Professor Aditya Shastri, Vice-Chancellor, Banasthali University for providing research facilities in the Department.

Declaration of interest

The authors report no declarations of interest.

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