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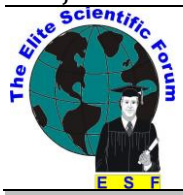

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RESEARCH ARTICLE

Kinetic Studies of Proteases from *Bacillus sp*.

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ABSTRACT

Proteases have essential qualities which have attained the attention of researches from different domains including biotechnology and have thus become important group of enzymes both for industrial use and academic research purpose. *Bacillus sp.* is commonly used for isolation and extraction of protease enzyme. This study reports the kinetics of proteases from *Bacillus sp*. Results showed that maximum/optimum activity was recorded at 60˚C temperature, 7.5 pH and 48 hours of continuous shaking at 220 rpm. The effects of different metal-ions on activity of proteases was also assessed. Lower value of K_M=0.2 obtained from Lineweaver-Burk plot for the substrate demonstrated good association/relationship between substrate and protease enzyme. Dependence of the enzyme activity on the concentration of substrate revealed a good agreement between the experimental and theoretical values. PMSF inhibition proved that the proteases produced were serine proteases. Overall, the unique characters of proteases isolated from the *Bacillus sp.* suggested this enzyme for having remarkable potential for use in different industrial applications.

INTRODUCTION

Proteases are amongst the biggest families of enzymes, holding sixty five percent of the total trade of enzymes throughout the world (Chu, 2007). The world's catalyst market reached US $123 billion in 2010, seventy five percent of these were hydrolytic enzymes and out of these hydrolytic enzymes, two-thirds were proteolytic enzymes (Dorcas and Pindi, 2016). Proteases are peculiar and found in a broad diversification of sources (Ravikumar et al., 2012). They are used in almost every field of life i.e. in food, laundry, dish washing, tannery, recovery of silk from X-rays films, protein chemistry, paper and pulp, meat processing, cheese making, waste water treatment, dehairing and bating hides, bioremediation, biosynthesis, biotransformation, textile, biocontrol agent against numerous agriculture pests, contact lens enzyme cleaners, skin ulceration brewing, diagnostic and pharmaceutical companies (Ahmetoglu et al., 2015; Jellouli et al., 2011). *Bacillus* (*B.*) *subtilis* is usually present in soils and is also regarded as hay- and/or grass-bacillus. It is rod shaped microorganism with the ability to develop and produce tough, protective endospores. These endospores can tolerate/resist extremely adverse environmental conditions. The members of *Bacillus* genus are obligatory aerobic or facultative anaerobic organisms and include both pathogenic and freely occurring species (Wanyonyi et al., 2019; Pant et al., 2015). By food and drug administration, it is generally recognized as safe (GRAS). Its capability allows easy subsequent processing to produce various products (Kabisch et al., 2013). It is highly favorable entity to produce proteases as it exhibits non-pathogenic status and is well studied example of gram-positive bacteria having excellent potential to make different types of enzymes (Ahmed et al., 2011). It is safe for humans and does not cause infection in human beings and animals, having easy excess to secrete products into extracellular environment, therefore are used in industry for bulk production of enzymes (Butt et al., 2018).

This study was aimed to produce proteases from *Bacillus* sp. followed by their characterization and to standardize the conditions for optimal production of

proteases. The differences in the production potential of proteases by the addition of various metal ions was also assessed in addition to the assessment of nature of proteases produced after the addition of various compounds including, ethylene glycol tetra acetic acid (EGTA), phenylmethylsulfonylfluoride (PMSF) and ethylene-diamine-tetra-acetic acid (EDTA).

MATERIALS AND METHODS

Proteases produced from *Bacillus sp.* were obtained from Department of Chemistry & Biochemistry, Agriculture University, Faisalabad, Punjab-Pakistan. The enzymatic activity of protease was tested at 37 ˚C using skim-milk as a substrate (Kumar et al., 2012). Tri-chloroacetic acid (TCA) was used as stop reaction. Unhydrolysed skim milk was removed by filtration through Whatman® filter-paper and solubilized skim milk concentration was determined spectrophotometrically at 275 nm. Control was also run as above but TCA was added before adding the enzyme solution. One unit of enzymatic activity is regarded as µmol of tyrosine released from 1 mL crude enzyme in 1 h incubation determined in comparison to the standard curve (Figure 1).

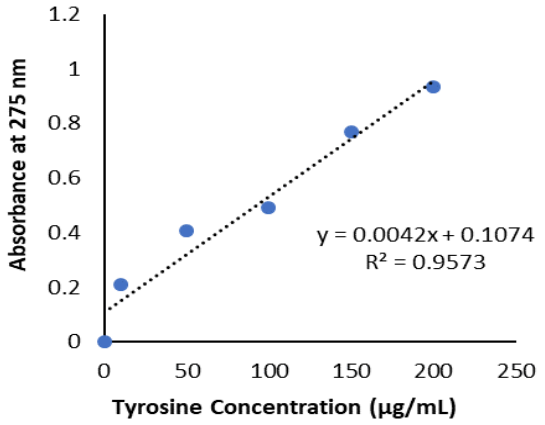

Fig. 1: Standard Curve for Tyrosine at 275 nm.

Detection of optimum temperature and pH

Optimum pH for proteases activity was evaluated by assaying the enzyme activity in phosphate buffered saline (0.2 M) with pH ranging from 5-13. The temperature for optimum activity of protease enzyme was assessed (at temperatures ranging from 20-80ºC) by following the methodology described by Kumar et al. (2012).

Effect of substrate concentration

Proteases were assayed as reported by Kumar et al. (2012) in phosphate buffer and skim milk with variable amounts (0.1 to 1.0%). The values of Vmax and Km were calculated from Line weaver - Burk plot (1/Vvs1/[S]).

Effects of different metallic ions on proteases activity

Effects of different metallic ions on activity of protease enzyme was studied after adding the respective ions (Concentration=5 mM, each) to the reaction mixture and assayed as described previously (Joshi and Satyanarayana, 2013). These were included as chloride salts of potassium, calcium, sodium, magnesium, manganese, ferric and cobalt.

Effects of EDTA, EGTA and PMSF on the activity of proteases

Effect of various compounds (EDTA, EGTA and PGMF) as activators and inhibitors on proteases activity was studied at varying concentrations i.e. 1 and 10 mM (Mothe and Sultanpuram, 2016).

RESULTS AND DISCUSSION

Proteases obtained from the bacterial strain *Bacillus sp.* were investigated in the present study for proteolytic hydrolysis under different conditions of temperature, pH and concentration of substrate in addition to assess the nature of proteases.

Optimum pH and temperature

Protease activities were evaluated at different pH levels from 5 to 13 by using phosphate buffer (0.2 M) and incubated for 30 minutes to check the role of pH on proteases production. Enzyme (protease activities) against different pH values are shown in Figure 2.

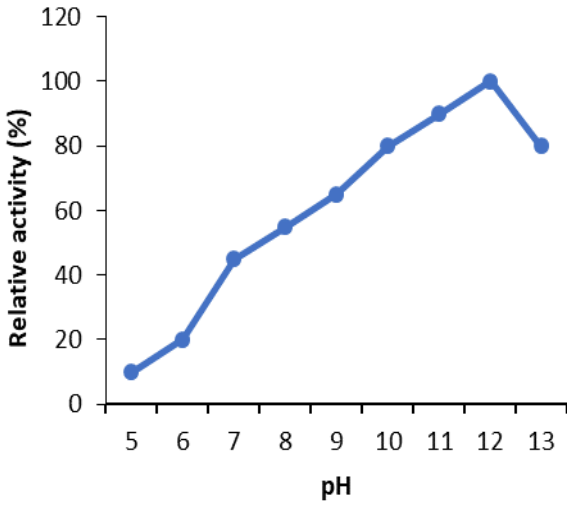

Fig. 2: Effect of pH value on the activity of *Bacillus sp.* on proteases.

In this experiment, relative proteases production was achieved maximum 100% at pH 12 in comparison to other pH values (Figure 2). It was observed that the produced enzymes belonged to alkaline protease group. Results of current study are in close agreement with those described by Joo et al. (2002) who reported a pH

value of 11 as best suited for maximum activity of proteases and observed that protease production decreased above pH 12. This distinct property of high pH is a common feature among all proteases of alkaline nature. Similar findings have been described earlier (Thebti et al., 2016) who observed optimal temperature and pH values for maximal proteases activity to be 95°C and 13, respectively.

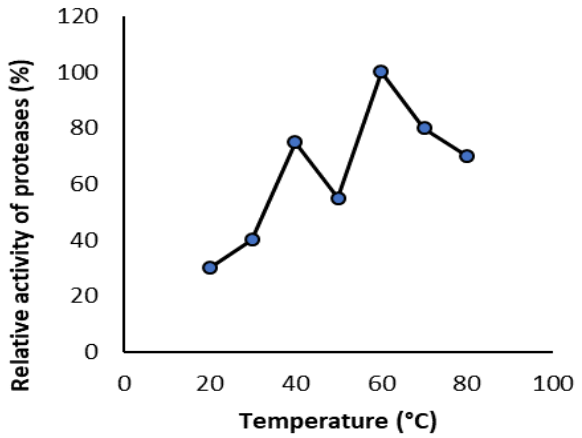

Fig. 3: Effect of temperature on the activity of *Bacillus sp.* on proteases. Relative activity was assessed at pH 12 using skim milk as substrate.

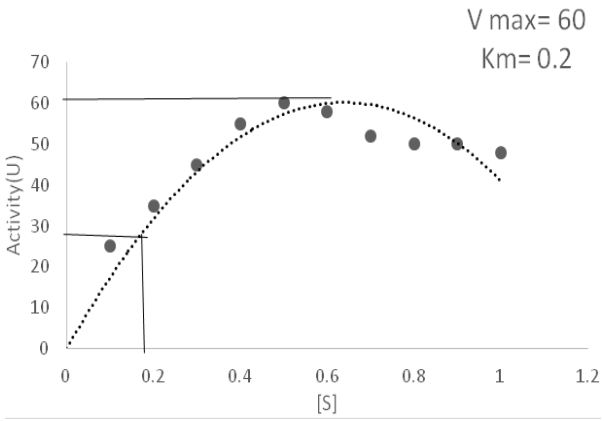

Fig. 4: Determination of Km and Vmax for proteases produced by *Bacillus sp.*

Table 1: Effect of protease inhibitors on proteases activity

Inhibitors	Concen-trations	Relative activity (%)
Control	0.0	100
EDTA	1mM	98.5
	10mM	98.1
EGTA	1mM	97.5
	10mM	96.1
PMSF	1mM	82.2
	10mM	44.9

This experimental study was conducted to check the impact of difference in temperature on proteases activity (ranging from 20 to 80°C with 10°C intervals; Asker et al., 2013; Ahmed et al., 2011). Highest protease activity was observed at 60 °C. At 80 °C the proteases were quite active. Results indicated that the optimal temperature for the protease enzymes was 60°C (Fig. 2), based upon which proteases can be speculated for a wider range of industrial applications for being thermostable at relatively higher temperature.

Current results are in accord with Savitha et al. (2011) and Sharma et al. (2011) who reported temperature optima to be 60-65°C of proteases from various sources i.e. bacterial and fungal. The enzymes showed a tendency to lose activity above 70°C to a great extent. Similar results have also been reported by Cha et al. (2005) who observed that proteases showed maximum proteolytic activity at 60°C and showed 20% activity at 80°C.

Effect of substrate concentration

Chemical changes are catalysed by different enzyme. The speed of chemical reaction is catalysed by addition of specific enzyme which optimize the rate of reaction. The rate is called Vmax and K_M is defined as concentration of the substrate at which the half of maximal velocity of reaction is achieved.

The substrate specificity of the purified proteases was examined with different concentrations (0.1-1.0 mM) of skim milk (Fig. 3). Hyperbolic curve was obtained as shown in Fig. 3 along with K_M and V_{max} values. Lower value of K_M is an indicator of excellent affinity for its substrate. Results of Muthulakshmi et al. (2011) are in complete accordance with the current results, values of K_M and V_{max} are 60 U and 0.6, respectively.

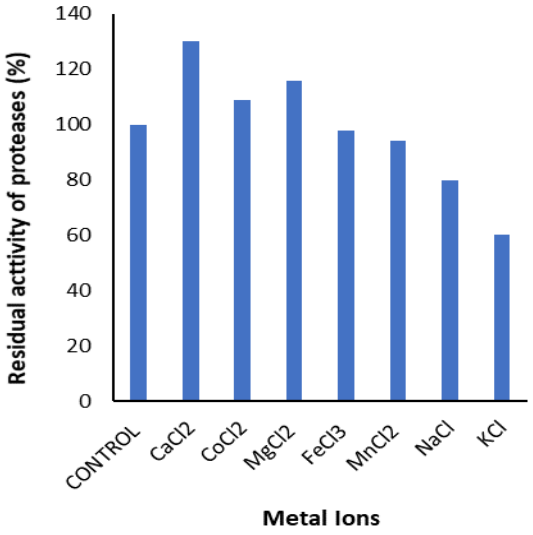

Fig. 5: Effect of metallic-ions on protease activity.

Effect of metallic ions on the activity of purified proteases

The effect of metallic ions including potassium (K^{+1}), calcium (Ca^{+2}), sodium (Na^{+1}), magnesium (Mg^{+2}), manganese (Mn^{+2}), cobalt (Co^{+2}) and ferric (Fe^{+3}) ions on the activities of proteases was also studied. Results showed that proteases activity was boosted by the introduction of 5 mM Ca^{+2}, Mg^{+2} and Co^{+2} resulting in the residual proteases activity of 130, 116 and 109 percent respectively (Fig. 4), recommended the metallic ions had a capacity to prevent the denaturation of enzymes. The metallic ions protected the enzymes from heat inactivation/ denaturation and maintained its structural conformation at high temperature. The proteases activity was slightly downturn by the addition of Fe^{+3}, K^{+}, Mn^{+2}, Na^{+} and K^{+} resulting in the relative activity 98, 94, 80 and 60%, respectively.

Previous studies (Chittoor et al., 2016; and Kavitha and Thankamani, 2012) on the effect of metal ions on the proteases have suggested they are normally stimulated in the company of divalent metal ions viz Ca^{+2}, Mg^{+2}. It could be due to activation of enzyme by metal ions. This phenomenon especially with Ca^{+2} ions is related with the stabilization of the tertiary configuration of the enzyme (Sedighi et al., 2019; Secades and Guijarro, 1999).

Effect of EDTA, EGTA and PMSF on the activity of proteases

Effect of various compounds (EDTA, EGTA and PMSF) as inhibitors on purified proteases was studied with varying concentration i.e. 1 and 10 mM. It was revealed that EDTA, EGTA and PMSF had diversified effects on protease firmness and activity. A negative correlation was detected between protease activity and increasing concentrations of PMSF (Table 1). Within contrast 1 and 10 mM EDTA and EGTA have no effect on protease activity, showing its stability in the presence of reducing agents. PMSF, the conventional inhibitor of serine protease, deactivated the enzyme at 1 mM and 45% at 10 mM, respectively. So, the protease investigated from *Bacillus subtilis* feasibly classified as serine protease. PMSF stop up the active site of protease by sulfonating the necessary serine residue. Current results are in accord to those reported by Ozcelik et al. (2014) who reported PMSF as inhibitor of the proteases. In conclusion, the smaller Km values of proteases showed high affinity of the enzyme with respective substrates which revealed that proteases obtained from *Bacillus sp*. followed the Michealis-Menton kinetics. It was also concluded that the *Bacillus sp*. possesses good quality serine type proteases which can be successfully used for industrial use.

Authors contributions

KYB performed the research work, data analysis and wrote the manuscript.KBS helped in research work. SRG conceived the idea and designed the research. MIG and MA performed the data analysis and helped in manuscript write up.

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