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Full Length Research Paper

Production and characterisation of protease enzyme produced by a novel moderate thermophilic bacterium (EP1001) isolated from an alkaline hot spring, Zimbabwe

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A moderately thermophilic bacterium designated EP1001 isolated from an alkaline hot spring (conditions in the hot spring: temperature 53-54°C and pH 9.3) in Zimbabwe was investigated for protease production. Studies to determine the growth and protease enzyme production by the novel bacterium were carried out in ordinary batch, regulated batch and fed-batch modes of fermentation using a BioFlow III 3 L fermentor. Biomass production and protease production were dependent on fermentation mode. Higher biomass formation and protease production were obtained in fed-batch fermentations compared with that in ordinary and regulated batch cultivations. The protease enzyme was found to be a thermostable alkaline serine protease with optima activity at 75°C and pH 10. The enzyme had a half life of 45 min at 80°C and 12 h at 70°C. It was stable over the pH range of 5.0 to 11.0. The enzyme was inhibited by phenylmethane-sulfonyl fluoride and EDTA but not by N-Tosyl-L phenanylalanine chloromethyl, iodoacetamide and O-phenathroline. The ions Ca^{2+} and Fe^{2+} at 0.5 and 2.5 mM concentration were stimulatory, while Mg^{2+} and Mn^{2+} had little effect on the enzyme activity. The enzyme produced by bacterium (EP1001) was concluded to be an alkaline protease that requires calcium and iron ions for its activity.

Key words: Protease production, thermophilic bacterium, alkaline hot springs, alkaline protease.

INTRODUCTION

Extracellular proteases are naturally produced by microorganisms mainly to degrade large polypeptides in the medium into peptides and amino acids before cellular uptake. Man has commercially exploited such enzymes to assist in protein breakdown in various industrial processes. Protease enzymes constitute one of the most important groups of industrial enzymes being extensively used in the food, pharmaceutical, protein hydrolysis, detergent, cheese-making, brewing, photographic, baking, meat and leather industries and inclusions in animal and human food as digestive aids (Synowiecki, 2010; Seiffzadeh et al., 2008; Dias et al., 2008). About 75% of world sales of industrial enzymes applications are hydrolytic enzymes of which proteolytic enzymes constitutes about 60% (Ningthoujam et al., 2009; Ningthoujam and Kshetri, 2010; Chu, 2007; Rai et al., 2010).

Protease enzymes are produced commercially from plants, animals and microbial sources. Microorganisms offer an attractive source of protease enzymes because they can be cultured in large quantities in a short period of time using established fermentation techniques, they produce an abundant, regular supply of the desired product and they can be genetically manipulated easily

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than plants and animals (Gupta et al., 2002; Dabananda and Kshetri, 2010; Tambekar et al., 2009). In addition, the protein products they produce are more stable than those from plants and animals.

Microorganisms found in many different environments are routinely screened in the quest of identifying more attractive sources of useful proteases. Novel groups of microorganisms found living under extreme conditions such as thermophilic hot springs and volcanic and geothermal regions have been found to have unique features of considerable industrial and scientific interests (Chen et al., 2006). Thermophilic bacteria have become an attractive source for thermostable industrial enzymes for many reasons (van den Burg, 2003). Thermostable enzymes show a higher degree of resistance to protein denaturants e.g., detergents, extreme pH and organic solvents when compared to analogous mesophilic enzymes.

Production of many metabolites by microorganisms is influenced significantly by the mode of cultivation used. There are two major methods for cultivation of micro-organisms, viz., batch and continuous cultivation. Fermentation of proteases is regulated by varying the C/N ratio and can be scaled-up using fed-batch, continuous or chemostat approaches by prolonging the stationary phase of the culture. In fed-batch cultivation, there are essentially two phases, a growth phase in which the cells are grown to maximum biomass and then a production phase in which the limiting carbon source and other requirements for product formation are fed to the fermentor in a relatively concentrated liquid form at a pre-calculated rate. There are many advantages offered by the fed-batch culture technique, which include a means of overcoming undesirable effects like inhibition or repression of synthesis of the desired product by certain substrates, production of more biomass and products, catabolite repression and glucose effect.

Before assessing the biotechnological potential of any enzyme, prior knowledge of various fermentation parameters and characterization of those biochemical properties having some relevance to industrial application is of utmost importance (Gupta et al., 2002). The choice of a protease for an application depends in part on its specificity for peptides bonds, and pH and temperature activity and stability, response to inhibitors and metal ions, cost and availability of the enzyme (Rai et al., 2010). Alkaline proteases are particularly important because they are both stable and active under harsh conditions, such as temperature ranging from 50 to 90 ºC, high pH, and the presence of surfactants (Alqueres et al., 2007; Chen et al., 2006). Serine alkaline proteases are found widespread in fungi and bacteria and owe their name to the fact that serine is an essential amino acid of their active centre. They are endoproteases that have broad substrate specificities and their molecular weights are in the range 20 to 50D (Akel et al., 2009).

They are generally active at neutral and alkaline pH with an optimum pH between 7 and 12. They are commonly inhibited by diisopropyl fluorophosphates (DIFP) or phenyl methyl sulphonyl fluoride (PMSF).

Due to growing market and potential uses of proteases, there is ongoing interest in the isolation of new bacteria species that produce proteolytic enzymes with novel properties suitable for industrial applications. Underexploited regions and niche habitats are likely to yield such enzymes. In our search for protease producing thermophilic bacteria that could be put into use for industrial purposes, a novel bacterium was isolated from Chimanimani alkaline hot springs, Zimbabwe. The hot springs in Zimbabwe have not been investigated in detail as sources of microorganisms that produce extracellular enzymes (Zvauya and Zvidzai, 1995; Mawadza et al., 2000; Zvidzai and Zvauya, 2001). The overall aim of this study was to investigate the production of biomass and protease enzyme by EP1001 isolate in batch and fed-batch cultivation and characterization of the enzyme.

MATERIALS AND METHODS

Microorganism used

The bacterium isolate EP1001 used in this study was isolated from hot springs (conditions in the hot spring: temperature 53-54°C and pH 9.3) in Chimanimani, Zimbabwe. The bacterium is a new genus of the γ-proteobacteria that is distantly related to *Xanthomonas* group according identification done by DSMZ (Accession number DSM ID 99-846) (Parawira and Zvauya, unpublished). The bacterium was maintained on nutrient agar plates at 4°C for short term storage and subcultured every four weeks. For long term storage it was kept at -80°C in nutrient broth containing sterile glycerol (50% v/v).

Bioreactor cultivations

All ordinary, regulated and fed-batch fermentations were carried in a 3-litre BioFlow III fermentor (New Brunswick Scientific, USA) with a working volume of 2 litres.

Ordinary and regulated batch fermentations

The fermentor vessel, headplate, accessories and medium were autoclaved at 121°C for 45 min. The medium used was modified M162 medium (Zvidzai and Zvauya, 2001) containing (gL-1) 4.0 tryptone and 2.0 yeast extract, the other components were the same as in the original M162 medium.

The fermentor conditions for the uncontrolled (ordinary) batch fermentation were; agitation speed of 900 rpm, airflow rate of 30 L/h, temperature 40°C and initial pH 8.0. For the regulated or controlled batch cultivation, the pH was maintained at 8.0 automatically by addition of acid (2.5 M HCl) or alkali (2.5 M NaOH) as required. The pH was also monitored by measuring the pH of offline samples by a pH meter (Jenway, 2010). Foaming was prevented by addition of sterile antifoam 289 solution (Sigma Chemicals) as required.

Preparation of precultures

Precultures at 10% of the working volume were prepared by

inoculating 200 ml of modified M162 medium with a loopful of the pure isolate from an agar plate in 1000 ml Erlenmeyer flasks. The flasks were incubated for 24 h at 45°C, 200 rpm in a New Brunswick Scientific Innova 4000 incubator shaker. The precultures were microscopically checked for contamination using a Zeiss Olympus Phase Contrast Microscope. The preculture was used to inoculate 2000 ml of modified M162 medium in a 3-litre BioFlow III fermentor (New Brunswick Scientific, USA). The experiments were done in duplicate.

Fed-batch fermentation

1. *Growth phase*

The fed-batch culture was initiated as a regulated batch culture in the 3-litre BioFlow III fermentor, with a working volume of 2 litres. An overnight inoculum at 10% for the fed batch culture was prepared in the same way as for regulated batch mentioned above. Fed Batch growth phase cultivation was done at 45°C (optimum temperature determined for growth), pH was maintained at 8.0 by adding of either 2.5 M HCl or 2.5 M NaOH as and when required.

2. *Feeding phase*

Feeding of concentrated M162 complete medium and also of glucose alone in different fed batch reactors at a flow rate of 20 ml per hour was begun after 8 h of cultivation when the cells had just entered the stationary phase. The feed flow rate was controlled by a Masterflex pump, (Cole-Parmer Instrument, Co). The temperature was changed from 45°C (optimum temperature determined for growth) to 40°C (optimum temperature determined for protease enzyme production) on the start of feeding the medium. All the other conditions were the same as in batch cultivation.

Sampling and sample analyses

The biomass concentrations and the protease activities were measured by off-line assays of culture samples collected at hourly intervals during cultivations.

Samples were spun immediately after collection at 1500 rpm for 5 min in an ultracentrifuge (Hetich Mikroliter, Depex) and then frozen in 1.5 ml Eppendorf tubes at -20°C until required for analysis. Contamination was checked during cultivation by observing the samples on a Zeiss Olympus Phase Contrast Microscope.

Biomass determination

Biomass was determined by measuring the absorbance (OD) at 600 nm using a UV-120-02 spectrophotometer (Shimadzu). All samples giving an OD higher than 0.7 were diluted with distilled water for accurate measurement.

Protease activity assay

The protease activity was assayed in duplicate with cell-free culture supernatants, using azocasein as the substrate (Kole et al., 1988). Enzymatic hydrolysis of azocasein produces stable dye-labelled peptides and amino acids into the reaction mixture which can be measured easily.

Azocasein protease activity was measured by incubating 1 ml of culture supernatant and 1 ml of 0.5% (w/v) azocasein (Sigma) in 0.2 M Tris-hydroxymethyl amino methane hydrochloride (Tris-HCl) buffer (pH 7.4) in an incubator (Innova, New Brunswick Scientific) at 75°C for one hour. The reaction was stopped by adding 2 ml of 10% (w/v) trichloroacetic acid (Kole et al., 1988). The test tubes were allowed to stand for 30 min at room temperature. The mixture was thoroughly mixed using a vortex mixer (VF2, Jankel and Keunkel Kika Larbotechnik) before being centrifuged at 3 000 rpm for 10 min to remove a yellow precipitate. The absorbance of the supernatant was measured at 440 nm using a Shimadzu UV-120-2 spectrophotometer. The activity of the protease was expressed in arbitrary units, where 1 unit of activity is equivalent to change in optical density of 0.01 nm per min at 440 nm. The enzyme assays were done in duplicate for each sample.

Protease enzyme characterization

Optimum temperature and thermal stability of protease enzyme

Cell-free enzyme solutions from ordinary batch cultivation were used in studying temperature relationships of the enzyme produced by this isolate. Optimum temperature for the enzyme activity was determined as follows; 1 ml of azocasein substrate (0.5% w/v in Tris-HCl buffer (0.2 M at pH 7.4) and crude enzyme supernatant (1 ml) in test tubes were incubated at specified temperatures (40-95°C) for 1 h. Azocasein protease activity was measured after 1 h as described above. The temperature stability was determined by incubating the crude enzyme solutions at different temperatures (50-80°C) for 24 h. Samples were collected at intervals and kept frozen at -20°C and later assayed for residual protease activity at 75°C and pH 7.4. This experiment was done in duplicate.

Optimum pH and pH stability of protease enzyme

Cell-free enzyme solutions from ordinary batch cultivation were used in studying pH relationships of the enzyme produced by this isolate. The optimum pH for protease activity was determined by carrying out protease enzyme assay with 0.5% (w/v) azocasein prepared in different buffers of pH range 4.0 to 11.0 (0.1 M citrate-0.2M phosphate, pH 4.0-6.0; 0.2M phosphate pH 7-8; and 0.1M carbonate-bicarbonate, pH 9-11). The pH stability was determined by diluting the crude enzyme to 50% v/v with the respective buffer solutions of pH 4.0 to 11.0 and then incubating at 25°C for 24 h. Samples were collected from these enzyme/buffer mixtures at 2 h intervals and kept frozen at -20°C until required for residual protease determination. This experiment was done in duplicate.

Effect of Inhibitors on protease activity

In order to find out the type of protease produced by the bacteria, several compounds known to inhibit various proteases were investigated for their effect on the hydrolysis of azocasein by the protease. Two different final concentrations of 1 and 2.5 mM of phenylmethane-sulfonyl fluoride (PMSF), o-phenathroline, N-Tosyl-L phenanylalanine chloromethyl (TPCK) and iodoacetamide, all dissolved in dimethyl sulfoxide (DMSO), were mixed with the protease enzyme. Residual protease activity was determined at 75°C and pH 10.0. The established temperature and pH optimum conditions were used to assay protease activity when testing the effects of inhibitors on the enzyme activity. The residual protease activity was calculated with reference to activity of controls incubated in the absence of the inhibitors and DMSO used as solvent in this study. The experiment was carried out in duplicate.

Table 1. Maximum biomass and protease production in different batch fermentation types. Time (h) refers to the time when highest biomass and protease activity was observed.

Fermentation Type	Time (h)	Biomass (OD600 nm)	Protease activity (units/ml)	Specific protease activity (units/ml/OD)
Ordinary batch	5	1.6	1.2	0.75
Regulated batch	6	2.7	1.4	0.52
M162 medium	20	3.3	5.23	1.58
Fed-batch				
Glucose fed-batch	22	2.9	4.44	1.53

The ordinary and regulated batch all used the M162 medium as in the M162 fed-batch and the other fed batch was with glucose.

Effect of metal ions on protease activity

The effect salt chloride solutions of Ca^{2+}, Mn^{2+}, Mg^{2+}, Co^{2+}, Zn^{2+}, Hg^{2+}, Fe^{2+}, Ag^{2+}, and Cu^{2+} on protease activity were investigated. Final concentrations of 0.5 and 2.5 mM of the salts were used. The individual assay mixtures contained either no divalent metal ions (control) or 0.5 or 2.5 mM of the salt solutions. The established temperature and pH optimum conditions were used to assay protease activity when testing the effects of divalent metal ions on the enzyme activity. The degree of inhibition or stimulation of protease activity in the assay was determined after one hour and expressed as a percentage of the control.

RESULTS AND DISCUSSION

Biomass and protease production in ordinary, regulated and fed-batch fermentations

The maximum biomass and protease production in ordinary, regulated and fed-batch fermentations are summarized in Table 1. The fermentation type (batch, fed-batch, continuous cultivation) have been found to have an influence on growth and exoproteases production (Gupta et al., 2002).

Highest biomass was produced in fed-batch fermentation compared to ordinary and regulated batch fermentation. There was a biomass increase of 69% in regulated batch fermentation compared with ordinary batch fermentation. This increase in biomass in regulated batch demonstrated the importance of controlling pH and aeration in the cultivation of the bacteria which led to improved growth of the bacteria. There was an increase in biomass of 81% and 106% during glucose fed-batch and concentrated M162 medium fed-batch fermentation respectively when compared to ordinary batch fermentation. The concentrated M162 medium and glucose feed were initiated after 8 h when glucose concentration was at lowest steady levels of 8.5 µmol/ml and the bacterium was in the stationery phase.

The protease production of ordinary, regulated and glucose fed-batch and concentrated M162 medium fed-batch are also presented in Table 1. More protease enzyme activity was obtained during regulated batch fermentation (1.4 units/ml) than in ordinary batch fermentation (1.2 units/ml) representing a 15% increase in enzyme production. The higher protease activity observed in regulated batch cultivation was due to higher biomass production since the specific protease activity in the regulated batch fermentation was lower than in ordinary batch cultivation. The bacteria appeared to continue to produce the enzyme for long periods in the stationery phase in the regulated batch culture than in ordinary batch culture fermentation. The explanation for the stable amounts of protease activity in the stationery phase in the regulated batch is that there was probably a steady release of cell-associated protease into the culture fluid or the conditions in the stationery phase were favorable to protease production by this bacterium. Fed-batch experiments with concentrated M162 medium and glucose were made to stimulate and extend the stationery phase.

The protease activity levels in both fed-batch fermentations surpassed the ordinary and regulated batch fermentations. The protease activity increased by 266 and 356% during glucose fed-batch and concentrated M162 medium fed-batch fermentations compared to ordinary batch fermentation. The specific protease activity (units/ml/OD) also increased by 104 and 110% during glucose fed-batch and concentrated M162 medium fed-batch fermentations compared to ordinary batch fermentation. The higher protease activity levels observed in concentrated M162 medium fed-batch compared to glucose fed-batch cultivation was due to the higher biomass formation in the former. To maximize protease production in batch and fed-batch fermentations therefore requires an optimal combination of cell mass concentration and specific protease production.

Similar increases in protease activity in fed-batch fermentations have been reported for protease production by *Bacillus* species (Mao et al., 1992; Singh et al., 2004; Beshay and Moreira, 2005). Mao et al. (1992) reported higher alkaline protease production (49%) in fed-batch fermentation compared to ordinary batch fermentation of *Bacillus licheniformis*. Kole et al. (1988b) also reported more protease production by *Bacillus sub-*

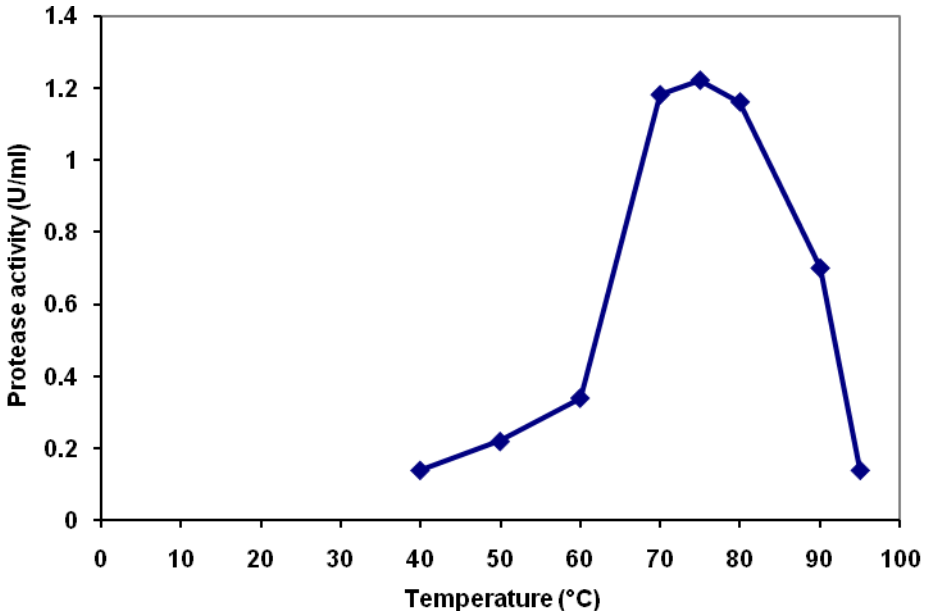

Figure 1. Azocasein protease activity from EP1001 isolate as a function of assay temperature.

tilis in oxygen-controlled, glucose fed-batch fermentations. Although protease production is an inherent property of all organisms, only those microbes that produce a substantial amount of extracellular protease have been exploited commercially.

Protease enzyme characterization

Hot springs can serve as sources of thermophilic bacteria that produce stable extracellular protease enzymes. The choice of a protease for an application depends in part on its specificity for peptides bonds, activity and pH and temperature activity and stability, response to inhibitors and metal ions, cost and availability of the enzyme.

Optimum temperature and thermal stability of protease enzyme produced by bacterial EP1001 isolate

The protease enzyme produced by bacterium EP1001 had an optimum activity at 75°C using azocasein as substrate at pH 7.4 as shown in Figure 1. Protease activity was low at temperature below 60°C and decreased sharply above 80°C due to enzyme denaturation. The thermostability of the crude protease enzyme from EP1001 isolate is shown in Figure 2. The enzyme was relatively stable at 60 and 70°C with half-life around 12 h of incubation. It was more stable at 50°C with a half-life of 16 h and retaining about 80% of its initial activity after 5 h of incubation. Thermostability is one of the most important characteristics of enzymes that can be employed in industrial processes involving high temperatures. The commercially available serine proteases are also active at pHs and temperature ranging between 8.0 to 12.0 and 50 to 60°C, respectively (Seifzadeh et al., 2008).

Optimum pH and pH stability of protease enzyme produced by bacterial EP1001 isolate

The protease activity towards azocasein in various buffers ranging from pH 4.0 to 11.0 is shown in Figure 3. The protease enzyme had high activity between pH 9 and 11, with highest at pH 10 indicating that enzyme is an alkaline protease. This property makes the protease enzyme from EP1001 a suitable candidate for use in the detergent industry. The optimum pH of this enzyme is comparable to several proteases reported that have an optimal pH close to 10 (Seifzadeh et al., 2008; Patel et al., 2006). Generally commercial proteases from microorganisms have maximum activities in the alkaline pH range of 8.0 to 12.0 (Genckal and Tari, 2006). All currently used detergent enzymes are alkaline and thermostable in nature and possess high pH optima (Saeki et al., 2007).

The stability of the protease enzyme at different pH values is shown in Figure 4. The enzyme was most stable over the pH range 5 to 9, where the residual protease activities as a percentage of initial activity were above 70% after 24 h of incubation. Alkaline enzymes are stable within this pH range (Zvidzai and Zvauya, 1995).

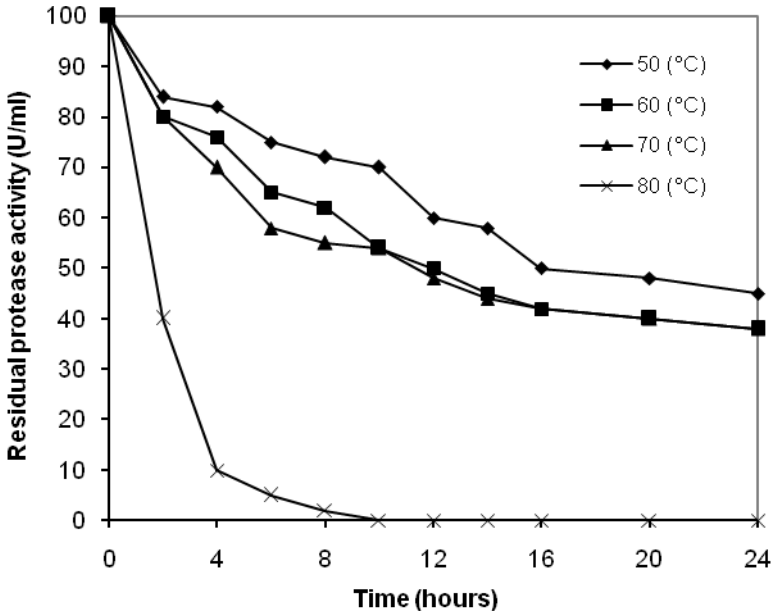

Figure 2. Stability of protease enzyme from EP1001 isolate as a function of temperature. Cell-free enzyme samples were incubated for 24 h at a temperature range of 50-80°C. Residual protease activity is the protease activity assayed after incubation as a percentage of initial enzyme activity at different temperatures.

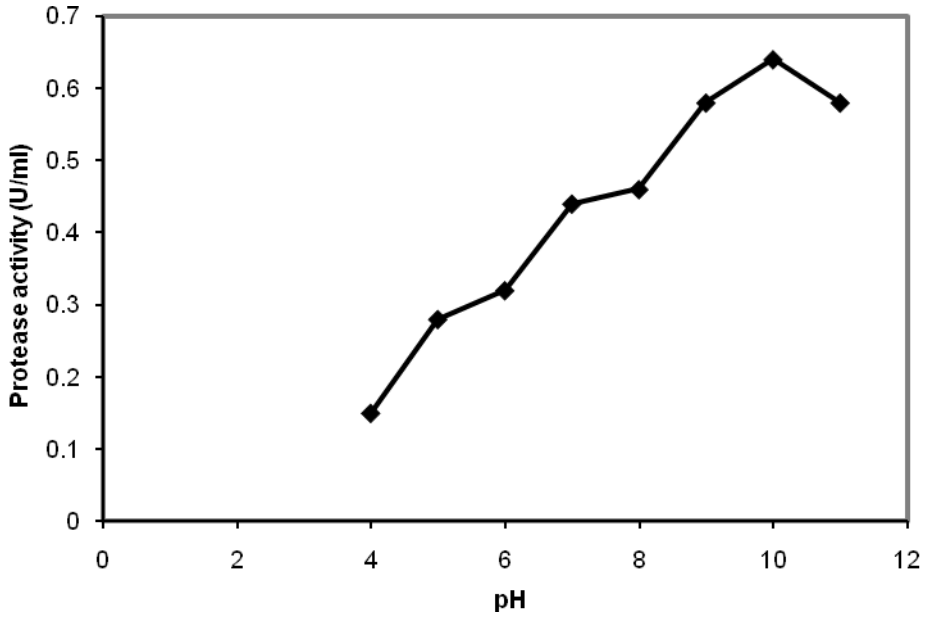

Figure 3. Azocasein protease activity from EP1001 isolate as a function of assay pH. The protease activity towards azocasein in various buffers ranging from pH 4.0 to 11.0.

The protease enzyme was most stable at pH 8.0 where residual activity was nearly 90% after 24 h of incubation. At high alkaline pH values of 10.0 and 11.0 the residual protease activity was around 50% after 24 h of incubation. The stability of this protease enzyme over a broad pH range is comparable to that of other alkaline proteases (Saeki, et al., 2007; Seifzadeh et al., 2008).

The stability of this protease enzyme over a broad

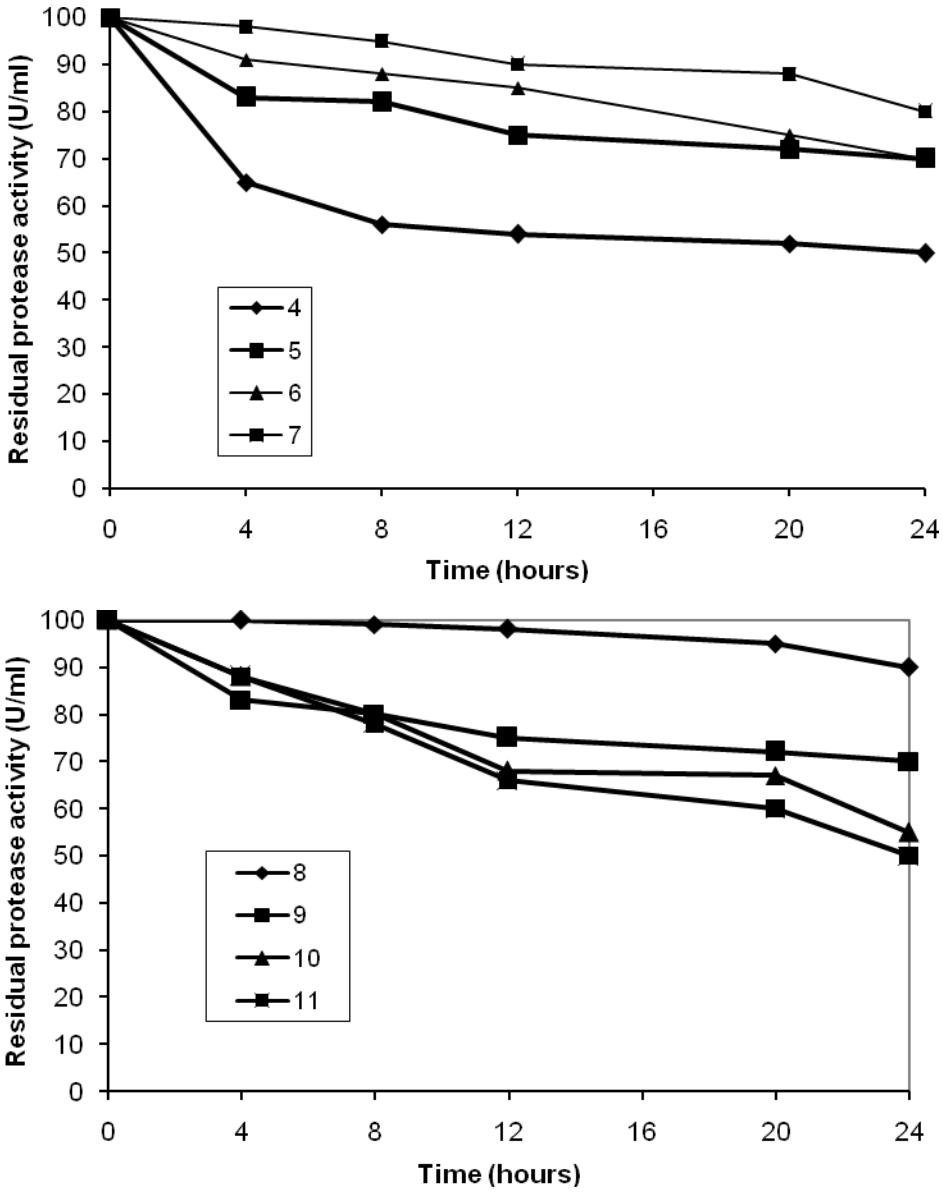

Figure 4. Stability of protease enzyme from EP1001 isolate as a function of pH after incubation for 24 h in buffers of pH values 4.0-11.0. Residual protease activity as a percentage of initial enzyme activity at each pH.

range of pH values and temperature is a desirable characteristic for commercial applications. Stability denotes the length of time over which an enzyme remains catalytically active due to the resistance of the appropriate conformation to disruption by environmental factors. Thermostability and pH stability is a highly desirable characteristic of industrial enzymes since it allows enzyme recycling, simple handling and storage.

The activity and thermostability of the protease enzyme produced by EP1001 bacteria is comparable to that of other purified and characterised thermostable protease enzymes, Table 2. However, the half-life for previous purified proteases varies from one source to another. Bacterial alkaline protease enzymes possessing these properties are primarily used in detergents, leather tanning, desizing and brewing processes.

Effect of inhibitors on protease activity

The effects of inhibitors on protease activity were carried out to characterize the protease synthesized by the bacterial isolate, Table 3. PMSF caused inhibition of 33 and 43% at 1 and 2.5 mM concentration respectively.

Table 2. Comparison of the properties of the protease from EP1001 bacteria with other alkaline serine bacterial proteases.

Source	Optimum		T1/2	Reference
	pH	Temp (°C)		
EP1001 (protease)	10	75	45 min/85°C	This study
Bacillus clausii *GMBAE 42*	11	60		Kazan et al. (2005)
Bacillus sp. GUS1	10.0	70	ND	Seifzadeh et al. (2008)
Bacillus st HUTTBS71	7.8	65	300 min/80°C	Akel et al. (2009)
Bacillus subtilis SH1	8.0	50	30 min/50°C	Dadananda and Kshetri (2010)

T1/2 = half life (thermostability), which is the heating time after which 50% of the initial activity remain when the sample was cooled, then assayed using the standard assay for protease activity as described in the work.

Table 3. Effects of inhibitors at 1 and 2.5mM concentrations on protease activity from bacterial EP1001 isolate.

Inhibitor	Assay conc. (mM)	Residual protease activity (%)	Type of protease inhibited
None (control)	none	100	
PMSF	1	67 ±1.0	Serine proteases
	2.5	57.17 ± 1.0	Serine proteases
TPCK	1	100 ± 0.0	Chymotrypsin-like serine proteases
	2.5	100 ± 0.0	Chymotrypsin-like serine proteases
O-phenathroline	1	100 ± 0.0	Metallo-proteases
	2.5	100 ± 0.0	Metallo-proteases
EDTA	1	67.17 ± 0.40	Metallo-proteases
	2.5	54/39 ± 0.20	Metallo-proteases
Iodoacetamide	1	100 ± 0.0	Cysteine-proteases
	2.5	100 ± 0.0	Cysteine-proteases

Results are given as residual percentage inhibition of enzyme activity and are an average of two assays. The protease activity of control (without inhibitor) was 0.86 units/ml. ± Standard deviations of means of duplicate figures obtained from the assays.

PMSF is a known serine protease inhibitor and reacts (sulphonation) with the serine residues of the active site in serine proteases (Beynon and Bond, 2001). Since the enzyme activity was inhibited by PMSF, it can be suggested that the bacterial EP1001 isolate produces a serine or alkaline protease. This suggestion is supported by the fact that hydrolysis of azocasein by the protease enzyme occurred at neutral to alkaline pH with optimum activity at pH 10.0. Serine alkaline proteases exhibit their optimum activity at pH range 9-11. These results are similar to what was reported for alkaline proteases by Seifzadeh et al. (2008), where 5 mM PMSF inhibited protease activity by up to 80%.

EDTA, a metal chelating agent, caused 33 and 46% inhibition at 1 and 2.5 mM concentration, respectively. The serine alkaline proteases are not generally inactivated by metal chelating agents like EDTA, but other examples alkaline proteases that are inhibited by EDTA have been reported in literature (Akel et al., 2009). These alkaline proteases probably require a metal ion for its activity and stability, and hence it was sensitive to presence of EDTA at 1 mM concentration. Ningthoujam and Kshetri recently reported alkaline proteases from a *B. subtilis* strain SH1 to be serine protease which is inhibited by EDTA (Ningthoujam and Kshetri, 2010).

TPCK, an inhibitor of chymotrypsin-like serine proteases, did not cause enzyme activity inhibition at the concentrations used in this study. Iodoacetamide and o-phenathroline caused no azocasein hydrolysis inhibition by the protease produced by the bacterial EP1001isolate. The bacteria EP1001 isolate did not produce metallo-proteases because o-phenathroline, a specific metallo-protease inhibitor did not inhibit the enzyme activity towards azocasein. The absence of inhibition of azoca-

Table 4. Effects of metal ions at 0.5 and 2.5 mm concentrations on protease activity from bacterium EP1001 isolate.

Metal ion	Assay conc. (mM)	Residual activity (%)	Assay conc. (mM)	Residual activity (%)
Control (no metal ion)		100		100
Ca^{2+}	0.5	126.3 ±0.13	2.5	60.46 ± 0.32
Fe^{2+}	0.5	131.46 ± 0.01	2.5	77.78 ± 0.002
Mn^{2+}	0.5	99.55 ± 0.11	2.5	54.75 ± 0.25
Mg^{2+}	0.5	96.31± 0.10	2.5	52.18 ± 0.34
Co^{2+}	0.5	64.38 ±0.04	2.5	34.84 ±0.01
Zn^{2+}	0.5	69.91 ±0.07	2.5	29.91 ±0.01
Hg^{2+}	0.5	97.44 ±0.01	2.5	38.04 ±0.04
Ni^{2+}	0.5	97.48 ±0.00	2.5	52.01 ±0.01
Ag^{3+}	0.5	76.92 ±0.02	2.5	13.68 ±0.01
Cu^{2+}	0.5	60.44 ±0.02	2.5	11.11 ±0.30

The results are an average of two assays. ± = Standard deviations of means of duplicate figures obtained from the assays.

sein hydrolysis by iodoacetamide and TPCK indicates the absence of cysteine proteases (Rai et al., 2010).

Effect of metal ions on protease activity

The influences of metal ions on the protease activity from the bacterial EP1001 isolate are shown in Table 4. The results showed that the protease enzyme was activated by Ca^{2+} and Fe^{2+} at 0.5 mM concentration and inhibited by these divalent metal ions at 2.5 mM concentration. These results suggest the need for Ca^{2+} and Fe^{2+} ions at low concentration for its activity. This is supported by the observation above that EDTA, a metal chelating agent, inhibited the enzyme activity less at lower concentration than at higher concentration. Ca^{2+} and Mg^{2+} ions were indeed found to be at low concentration (< 1.0 ppm) in the water from Chimanimani hot springs from which the bacterium was isolate (Muronde and Zvauya, unpublished data). It is not surprising that microorganisms living in alkaline environments survive in media with low concentrations of these cations but high levels are toxic. This could explain the observation that the protease enzyme from this bacterial EP1001 isolate was activated by Ca^{2+} ions at 0.5 mM concentration and inhibited at 2.5 mM Ca^{2+} ion concentration. The optimum concentration of Ca^{2+} ions for maximum activity, however, varies among the protease enzymes (Akel et al., 2009). Divalent metal ions such as Ca^{2+} are known to increase thermostability (Rai et al., 2010) but this was not investigated in this study.

Mn^{2+}, Ni^{3+} and Mg^{2+} Hg^{2+} had little effect on the protease activity at 0.5mM concentration but inhibited the enzyme at 2.5 mM concentration. The other metal ions Co^{2+}, Zn^{2+}, Cu^{2+}, and Ag^{3+} inhibited the protease activity towards azocasein at both 0.5 and 2.5 mM concentration and inhibition increased with increasing metal ion concentration. At 2.5 mM concentration, Cu^{2+} showed the highest inhibitory effect of 89%, followed by Ag^{3+} with 86%, then Zn^{2+} with 70% inhibition, Co^{2+} with 63% and Hg^{2+} with 62% inhibition. The toxic metal ions exert their toxicity by binding to a variety of organic ligands causing denaturation of proteins including enzymes.

These results are comparable with results obtained for other proteases from bacteria such as *Bacillus* that appeared to be activated and stabilized by Ca^{2+} such as *Bacillus clausii* GMBAE 42 (Kazan et al., 2005), *Bacillus* strain HUTBS71 (Akel et al., 2009). In fact Kazan et al. (2005) reported that optimum temperature for protease enzyme from *B. clausii* GMBAE 42 shifted from 60 to 70°C after addition of 5 mM Ca^{2+} ions. The characteristic results from EP1001 isolate need to be confirmed by purified protease enzyme from this bacterium. Purification, identification of its molecular size, characterization of its gene is additional information needed to classify this protease enzyme.

Conclusion

Protease production by EP1001 bacterium isolated from an alkaline hot spring in Zimbabwe was investigated in ordinary, regulated and fed-batch fermentations using a laboratory 3l fermentor. Highest biomass formation and protease production were found in fed-batch fermentations compared with that in ordinary and regulated batch fermentations. The protease enzyme was found to be a thermostable alkaline serine protease with optimum activity at 75°C and pH 10.0. The enzyme had significant activity and stability over a wide range of temperature and pH, which is an ideal characteristic for an enzyme that can be used in the detergent industry. It is worth mentioning that the protease from EP1001 bacterium isolate was characterized using crude enzyme solutions. Purification and additional characterization of this protease enzyme are necessary to confirm the

properties of the enzyme.

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