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Extracellular Keratinolytic Proteases from An Alkalophilic *Streptomyces albidoflavus* TBG-S13A5: Enhanced Production and Characterization

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An alkalophilic strain of *Streptomyces albidoflavus* TBG-S13A5 secreted keratin hydrolysing proteases in submerged batch cultures in basal broth of pH 10.5 at 30±2^{0}C with white chicken feathers as inducer substrate. Enhanced protease production of around six fold was achieved with modified nutritional parameters *viz*., sodium nitrate (0.24%), magnesium sulphate (0.03%) and feather (0.8%). Under solid state fermentation maximum protease production was on 7thday (~52 U/gds). The protease activity of the crude enzyme was studied in relation to various factors. The crude enzyme was active and stable in neutral and alkaline conditions. Maximal protease activity was at pH 9.0 and in the temperature range of 60-70^{0}C. Protease activity was partially inhibited individually by the protease inhibitors PMSF (Phenylmethylsulfonyl fluoride) and EDTA (Ethylene diamine tetra aceticacid). The crude enzyme was generally tolerant to the detergents and solvents tested. Protease activity was partially inhibited by the reducing agent, β-Mercaptoethanol(β-ME). Metal ions like $BaCl_2$, $MgCl_2$ and $MgSO_4$ increased protease activity whereas it was strongly inhibited by $HgCl_2$, $ZnSO_4$ and $CuSO_4$.

Key words: *Streptomyces albidoflavus,* Keratin hydrolysing proteases, Solid state fermentation.

The importance of keratin degradation or the organisms and enzymes associated with this process can be well appreciated under the light of the fact that worldwide, several billion tones of feather keratin is generated as waste by-product at poultry processing plants annually[1]. Unlike other agroindustrial wastes like cellulose, utilization of protein rich keratin wastes is very limited. Left unattended, this poses environmental issues like pollution and disease epidemics[2]. Specific class of keratin degrading proteases (keratinases), secreted by several soil microorganisms, offer great advantage over conventional feather treatment processes in transforming the recalcitrant keratin wastes to nutritionally rich, palatable protein feed and pharmaceutically valuable rare amino acids[3]. Challenging issues like solid waste management can be well handled by anaerobic digestion of poultry waste to generate natural gas for fuel[4]. These applications demand large-scale production

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of keratinases that in turn relies upon potent strains and their cultivation conditions, to favour enzyme production in commercial yields.

In vitro, microbial keratinases were largely produced in mineral salts medium with keratinous substrates. Enzyme production is regulated by microbial cell metabolism, hence the culture conditions for optimum enzyme production differ significantly with the media components and the biochemical nature of the microbial strain. A very few studies emphasised optimizing cultivation conditions for keratinase production with respect to nutritional parameters[5,6]. An alkalophilic soil actinomycete, TBG-S13A5, isolated from partially degraded keratin waste and identified as a strain of *Streptomyces albidoflavus* was found to be a potent keratin (feather) degrader. Apart from keratinolytic properties, the crude enzyme of TBG-S13A5 showed dehairing and destaining properties. Aiming to enhance the production of keratinolytic protease, in the present investigation, we have studied the influence of nutritional parameters such as carbon, nitrogen, mineral sources and their concentration on keratinolysis by this strain under submerged fermentation. Also, we have evaluated its keratinolytic ability under solid state fermentation. Preliminary characterization of the crude enzyme was also done.

MATERIALS AND METHODS

Media components, agar, salts, bases, acids and solvents used throughout the study were of analytical grade purchased from Sigma-Aldrich Inc (USA), Hi Media (Mumbai), Qualigens (Mumbai), and SRL (Mumbai). White Chicken feathers (from a local poultry farm), fine wool (from Himalayan breed) and keratin powder (Hi-media, India) were used as inducer keratin substrates. Azocasein, protease assay substrate and protease inhibitors *viz*., E-64 (Trans-epoxysuccinyl-L-leucylamido-(4-guanidino) butane), PMSF and EDTA were purchased from Sigma-Aldrich Inc (USA).

Organism and culture conditions

Streptomyces albidoflavus TBG-S13A5 produced keratin hydrolysing proteases in basal broth (NH_4Cl- 0.5 g/L; $CaCl_2$- 0.22 g/L; $MgSO_4.7H_2O$- 0.2 g/L; K_2HPO_4- 0.3 g/L; KH_2PO_4- 0.4 g/L and yeast extract -0.1 g/L, pH-10.5) containing 0.25% of raw white chicken feathers (autoclaved separately and added aseptically to basal broth before inoculation). Broth was inoculated with 1% (v/v) ($4x10^7$ CFU/mL) of five day old seed culture (grown in yeast extract malt extract (YEME)) broth and grown for five days on an orbital shaker at 120 rpm at 30±2°C. The supernatant after centrifugation at 8000 rpm for ten minutes was used as crude enzyme preparation.

Enzyme assay and protein determination

Protease assay and keratinase assay were done using azocasein (2% in 50 mM Tris buffer pH 9.0) and keratin powder (0.5% in 50 mM Tris-HCl buffer, pH 9.0) respectively as substrates as described by Sarath and coworkers[7]. One unit of protease activity was defined to be the amount of enzyme required to produce an absorbance change of 1.0 in a 1cm cuvette under the conditions of assay. Protease and keratinase activities were expressed in Units/mL of culture filtrate. The concentration of soluble protein in the culture supernatant was estimated following the methodology of Lowry *et al*.,[8] and expressed in mg/mL of culture broth. Changes in the pH of the culture medium were measured using a digital pH meter (Cyber Scan, Germany).

Influence of nutritional parameters on protease production

Carbon sources (diethyl ether-sterilized) *viz*., glucose, sucrose, fructose, galactose, lactose, glycerol and maltose, each were added (1% w/v) to basal broth to study their effect on protease production. The basal broth originally contained 0.05% ammonium chloride. Protease production was evaluated after replacing ammonium chloride with inorganic nitrogen sources (at concentrations equivalent to 0.01% of nitrogen) like sodium nitrate, potassium nitrate, calcium nitrate and ammonium sulphate. Concentration of the best nitrogen source (sodium nitrate) was varied from 0-0.4% in the basal broth and its effect on protease production was studied. Effect of different chloride ions was studied by replacing calcium chloride with barium chloride, sodium chloride and potassium chloride (0.02% w/v) in the original broth. Effect of variations in concentration of calcium chloride (0-0.1%) and magnesium sulphate (0-0.05%) on enzyme production and keratin degradation were

studied. Trace salt solution ($CuSO_4.5H_2O$- 0.64%, $FeSO_4.2H_2O$- 0.11%, $MnCl_2.2H_2O$- 0.79% and $ZnSO_4.7H_20$- 0.15%) was added to basal broth (50-400 µL) to study its effect on enzyme production. Apart from feather, keratin substrates like hair and wool were tried at 0.25% level to induce keratinolytic activity. Protease production on increased substrate concentration was studied with feather by adding it from 0.1 to 1 % level to the basal broth. Finally, a comparison of protease and keratinase activity was done with substrates like wool and keratin powder at their optimal concentration, in modified basal broth ($NaNO_3$- 2.4 g/L; $CaCl_2$- 0.2g/L; $MgSO_4.7H_2O$- 0.3g/L; K_2HPO_4- 0.3g/L; KH_2PO_4- 0.4g/L and yeast extract -0.1g/L, pH-10.5). Enzyme assay, soluble protein concentration and pH of the culture filtrate were estimated on 5th day after inoculation.

Solid state fermentation

Sterilized feather (0.5 g) taken in 250 mL flasks were seeded with 1 mL inoculum ($4x10^7$ CFU/mL) and moistened with 2 mL modified basal broth. Fermentation was carried out for ten days at 30°C in an incubator. Proteolytic enzymes were extracted at two days interval from the fermented solids with dist. H_2O (30 mL/flask), after stirring. The supernatant collected after removing the solids after centrifugation at 8,000 rpm for ten minutes was used for assay, soluble protein estimation and pH studies. Protease activity was expressed in units per gram dry weight of substrate (U/gds).

Characterization of crude protease enzyme

Enzyme source

The isolate TBG-S13A5 was grown for five days (120 rpm; 30±2°C) in the modified basal broth ($NaNO_3$- 2.4 g/L; $CaCl_2$- 0.2g/L; $MgSO_4.7H_2O$- 0.3g/L; K_2HPO_4- 0.3g/L; KH_2PO_4- 0.4g/L and yeast extract-0.1g/L, pH-10.5) with 0.8% feather. The cells and debris were harvested by centrifugation at 8,000 rpm for 10 min at 4°C and the supernatant was used as crude enzyme solution.

Influence of pH on protease activity was assayed by incubating the enzyme solution with 0.2% azocasein in buffers of different pH (Tris-HCl: 7.0, 8.0, 9.0 and glycine-NaOH: 10.0 and 11.0). The effect of pH on stability was studied after incubating the enzyme solution in these buffers overnight at 4°C. Influence of incubation temperature on protease activity was studied by incubating the reaction mixture in water bath adjusted to temperatures from 20-80°C at an interval of 10°C for 1 h. For thermal stability studies, the enzyme solution was kept incubated at different temperatures (20-80°C at an interval of 10°C) in a water bath for 30 min and then assayed for protease activity. Stability of enzyme over a period of eight days was studied incubating the enzyme solution at room temperature (30±2°C).

Effect of protease inhibitors (10 mM) *viz*., PMSF, EDTA and E64, reducing agent like β-ME (0.1-0.5% v/v), detergents like Triton X-100 (0.1-0.5% v/v) and sodium dodecyl sulphate (0.1-0.5% w/v), solvents (1%,10%,50%v/v) *viz.,* dimethysulphoxide, isopropanol and acetonitrile and metal ions (10 mM) *viz*., $CaCl_2$, $BaCl_2$, $MgCl_2$, $HgCl_2$, $MnCl_2$, $FeCl_3$, $CuSO_4$, $MgSO_4$ and $ZnSO_4$, on protease activity was studied by pre-incubating crude enzyme with these chemicals for 30 minutes at 30±2°C and then assaying for protease activity.

Statistical analysis

The data were calculated from atleast three observations and presented as mean ± standard deviation. Analysis of variance was done using Genstat DE 3 followed by Least Significant Difference (LSD) test for multiple comparisons with the level of significance chosen at $P < 0.05$.

RESULTS AND DISCUSSION

Influence of nutritional parameters on protease production

The influence of various nutrient sources on protease production by *Streptomyces albidoflavus* TBG-S13A5 is given in Table 1. All the carbon sources under study *viz*., glucose, glycerol, mannitol, fructose, lactose, sucrose, maltose and galactose, strongly inhibited protease production and feather degradation. This might be due to catabolite repression as had been reported for most keratinolytic strains[9]. Keratinases, in most cases were produced using keratin as the sole source of carbon and nitrogen[2,10]. A few workers had reported that supplementation of basal media with carbon sources like galactose, glucose, glycerol and starch (1% w/v) proved favourable for keratinase production[5]. However, with *Streptomyces* sp. TBG-S13A5, carbon source supported biomass production but protease production and keratin

Table 1. Effect of selected nutritional parameters on protease production by *Streptomyces albidoflavus* TBG-S13A5

S. No.	Nutritional parameters	Protease activity (U/mL)	Soluble protein (mg/mL)	Final pH
1.	*Carbon source* (1% w/v)			
a	Control	0.149±0.007	0.139±0.006	8.70±0.05
b	Glucose	0.009±0.002	0.290±0.005	7.24±0.05
c	glycerol	0.036±0.004	0.244±0.006	6.76±0.05
d	Mannitol	0.026±0.002	0.325.±0.009	7.26±0.06
e	Galactose	0.006±0.003	0.244.±0.007	6.55±0.06
2.	*Nitrogen source* (0.01% w/v of N)			
a	Control	0.111±0.006	0.114±0.010	8.86±0.06
b	NH_4Cl	0.129±0.004	0.146±0.005	8.82±0.06
c	$(NH_4)_2SO_4$	0.150±0.007	0.155±0.007	9.01±0.05
d	$NaNO_3$	0.224±0.007	0.168±0.005	8.72±0.07
e	$Ca(NO_3)_2$	0.021±0.009	0.111±0.005	6.75±0.06
f	KNO_3	0.217±0.008	0.152±0.009	8.71±0.04
3.	*Concentration of* $NaNO_3$ (% w/v)			
a	Control	0.117±0.009	0.105±0.007	8.84±0.07
b	0.08	0.208±0.006	0.167±0.008	8.78±0.06
c	0.24	0.338±0.006	0.237±0.008	8.26±0.05
d	0.42	0.268±0.006	0.171±0.006	8.18±0.07
4.	*Chloride ions* (0.02% w/v)			
a	Control	0.146±0.006	0.181±0.008	8.29±0.05
b	$BaCl_2$	0.073±0.007	0.153±0.006	8.72±0.04
c	$CaCl_2$	0.306±0.004	0.228±0.007	8.18±0.07
d	NaCl	0.107±0.010	0.267±0.007	8.56±0.06
e	KCl	0.072±0.009	0.233±0.009	8.56±0.03
5.	*Concentration of* $CaCl_2$ (% w/v)			
a	Control	0.155±0.006	0.180±0.006	8.30±0.05
b	0.02	0.312±0.005	0.222±0.009	8.05±0.07
c	0.04	0.165±0.006	0.120±0.007	8.31±0.02
d	0.06	0.105±0.006	0.095±0.008	6.71±0.03
e	0.08	0.027±0.006	0.079±0.006	5.43±0.05
6.	*Concentration of* $MgSO_4$ (% w/v)			
a	Control	0.163±0.007	0.181±0.006	8.48±0.05
b	0.01	0.312±0.010	0.189±0.005	8.42±0.06
c	0.03	0.367±0.009	0.246±0.008	8.32±0.03
d	0.05	0.318±0.008	0.209±0.006	7.96±0.07
7.	*Trace salt solution* (µL/ 100mL)			
a	Control	0.367±0.008	0.246±0.006	8.34±0.08
b	50	0.311±0.006	0.306±0.004	8.52±0.06
c	100	0.228±0.006	0.286±0.007	8.68±0.06
d	300	0.010±0.005	0.142±0.009	6.81±0.08
8.	*Keratin substrate* (0.25% w/v)			
a	Control	0.037±0.006	0.081±0.007	8.55±0.06
b	wool	0.484±0.011	0.272±0.005	8.21±0.05
c	Hair	0.106±0.006	0.078±0.008	8.50±0.06
d	Feather	0.360±0.006	0.233±0.009	8.19±0.04
9.	*Concentration of feather* (% w/v)			
a	Control	0.016±0.004	0.091±0.005	8.84±0.06
b	0.2	0.363±0.009	0.230±0.006	8.18±0.06
c	0.4	0.527±0.007	0.299±0.007	8.35±0.06
d	0.8	0.899±0.010	0.433±0.008	8.42±0.06
e	1	0.865±0.008	0.420±0.007	8.52±0.05

degradation were negligible.

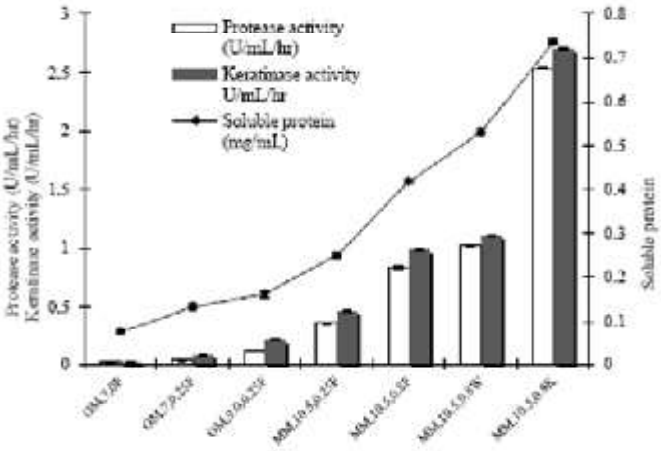
In this study, originally we used ammonium chloride 0.05% as inorganic nitrogen source as followed by Letourneau *et al.,*[11] for keratinase production from *Streptomyces* sp.. Among the different inorganic nitrogen sources supplied, maximum production of 0.224 U/mL was recorded with sodium nitrate followed by potassium nitrate (0.217 U/mL). Calcium nitrate proved inhibitory for keratin degradation. Sodium nitrate was tried at different concentrations and 0.24% of this inorganic nitrogen favoured highest protease secretion (0.338 U/mL). Even without any inorganic nitrogen source reasonably good proteolytic activity was recorded. Among the different chloride ions tested, calcium chloride (0.02%) recorded maximum production (0.3 U/ml). Increasing the concentration of $MgSO_4$ from 0.02% to 0.03% slightly improved the protease production. Concentration of magnesium sulphate beyond 0.03% was not effective. Feather degradation was poor in the absence of $MgSO_4$ and $CaCl_2$ though sufficient growth could be observed, suggesting the importance of these salts for enzyme production or activity. Noval and Nickerson[12] reported that increasing the initial concentration

Table 2. Effect of different chemicals on the protease activity of the crude extracellular enzyme of *Streptomyces albidoflavus* TBG-S13A5

S. No.	Substance group	Substance	Conc.	Residual protease activity (%)
1	Control			100.00
2	Inhibitors	PMSF	1(mM)	43.28±1.28
			10	36.93±1.26
		EDTA	1	35.94±0.97
			10	31.47±1.12
		E64	1	99.56±1.39
			10	97.23±2.54
3	Reducing agent	β-Mercaptoethanol	0.1(%v/v)	54.32±1.04
			0.3	41.96±1.04
			0.5	32.14±1.00
4	Detergents	Triton X-100	0.1(%v/v)	99.55±1.18
			0.3	96.88±1.18
			0.5	108.93±1.68
		SDS	0.1	92.26±1.04
			0.3	110.27±1.22
			0.5	87.35±1.16
5	Solvents	DMSO	1(% v/v)	103.20±1.71
			10	105.15±1.71
			50	111.19±1.41
			1	108.35±1.39
			10	75.49±1.16
			50	57.37±1.16
		Acetonitrile	1	104.26±1.62
			10	121.67±1.34
			50	86.15±1.74
6	Metal ions	$CaCl_2$	10(mM)	74.84±1.57
		$BaCl_2$	10	111.01±1.26
		$MgCl_2$	10	122.17±1.42
		$HgCl_2$	10	13.99±0.94
		$MnCl_2$	10	57.08±0.94
		$FeCl_3$	10	90.72±1.26
		$CuSO_4$	10	26.42±0.79
		$MgSO_4$	10	104.87±1.10
		$ZnSO_4$	10	29.72±0.94

of calcium chloride or magnesium sulphate in the basal salt medium shortened the latent period for wool digestion by *Streptomyces fradiae* 3739. A few workers had used trace salts such as $FeSO_4$ and $ZnSO_4$ in broth for keratin degradation using fungal strains[13,14]. We observed inhibition of protease production and feather hydrolysis by TBG-S13A5 in broth containing trace salt supplements.

Among the natural keratin substrates, wool (0.25%) induced maximal protease production (0.484U/mL). Human hair was not degraded by this isolate. A few workers have recorded the differential ability of keratinolytic isolates to attack α and β keratin[12,15]. In hard keratins the rate of hydrolysis corresponds roughly to the "hardness" i.e. cystine content. Nails, feather or wool are therefore cleaved more easily than human hair. Among mammalian hairs, those with a large medulla are least resistant[16]. Around 1-1.5% of feather for optimal keratinase production has been suggested for most bacteria and fungi[17, 18]. It is demonstrated that high feather concentration cause substrate inhibition or repression of keratinase production



OM - Original minerals salts broth; MM - Modified minerals salts broth,
7,9 & 10.5 - pH of the browth ; 0.25 & 0.8 % of substrate, F-Feathers, W-Wool, K - Keratin

Fig. 1. A comparative study of enzyme production by TBG-S13A5 with modifications in culture conditions

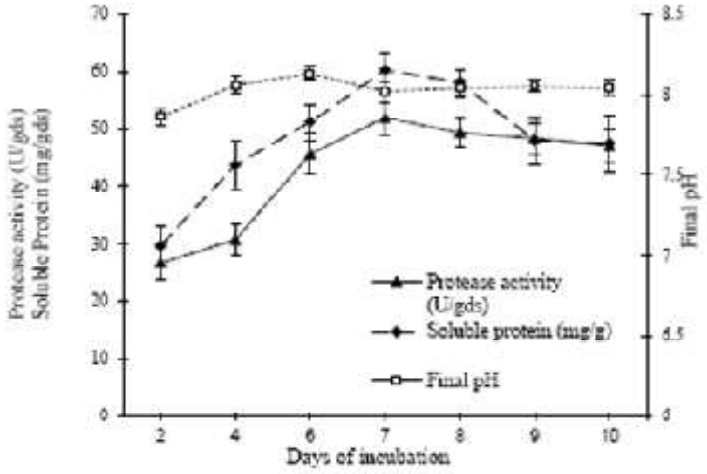


Fig. 2. Kinetic profile of protease production by TBG-S13A5 under solid state fermentation

resulting in low percentage of degradation. Protease production of TBG-S13A5 increased in positive correlation with substrate (feather) concentration upto 0.8%. Optimal substrate (white chicken feather) concentration was 0.8% and with modified nutritional parameters protease and keratinase activities were ~0.9 and 1.0 U/mL respectively. Keratin powder (Hi-Media), when used as substrate at this concentration, induced more keratinase/protease production, approximately 2.5 times higher than that obtained with feather as substrate (Fig. 1).

Solid state fermentation

SSF using feather meal[19] and hammer-milled feathers[20] has been reported previously. An attempt was made to check the suitability of raw feather as substrate in SSF for this study. *Streptomyces* TBG-S13A5 degraded feather under these conditions with maximal activity on 7th day. Production was around 52 U/gds which is around three times higher than that obtained with *Streptomyces* sp. 594 under SSF with feather meal[19]. Protease activity and soluble protein measured over a period of 10 days after incubation (Fig. 2.).

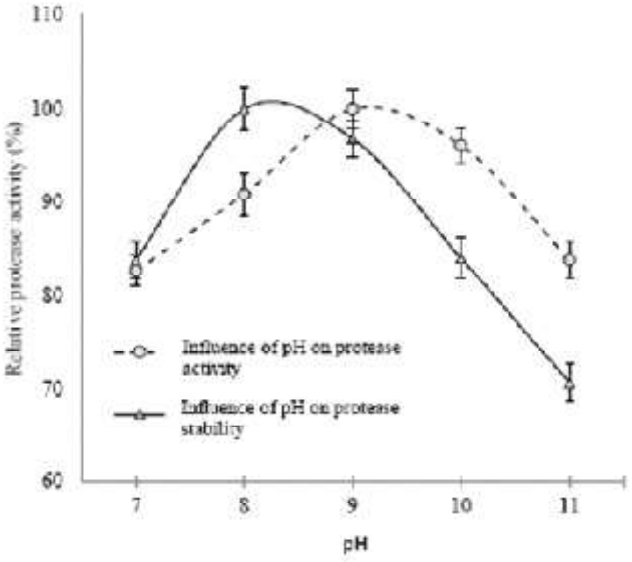


Fig. 3. Influence of pH on protease activity and stability

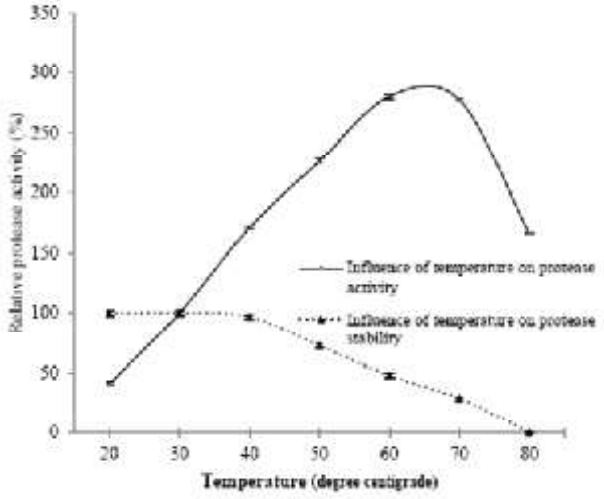


Fig. 4. Influence of temperature on protease activity and stability

Characterization of crude enzyme

The crude enzyme was active over a wide range of pH. Maximum protease activity was recorded with pH 9.0. At pH 7.0 to 11.0, it retained more than 80% of the activity. The enzyme solution was stable in neutral to alkaline pH. It was most stable from pH 8.0 to 9.0 (Tris-HCl) (Fig. 3). Enzyme solution was active at wide temperature range from 20-80°C and the maximal activity was recorded from 60-70°C. The enzyme was stable when treated at temperatures up to 40°C. Activity started declining at higher temperatures from 50-80°C (Fig. 4). The crude enzyme showed exceptional stability at room temperature for more than a week time (Fig. 5). Extracellular alkaline proteases that are active at high alkaline pH and temperature showed better compatibility with most of the commercial laundry detergents with respect to temperature stability and enzyme activity[21].

Inhibitor studies with the crude enzyme of TBG-S13A5 suggested the presence of serine and metalloproteases. A serine protease inhibitor, PMSF inhibited more than 50% and 60% respectively at 1 mM and 10 mM concentration. More than 60% activity was reduced by metal chelating agent, EDTA at 1 and 10 mM concentrations (Table 2). No significant reduction in activity was recorded with either 1 or 10 mM of E-64 (cysteine protease inhibitor). Presence of different proteinases especially keratinases (that have the ability to bind and hydrolyze solid substrates) will be more advantageous as additives for hard surface cleaners. β-Mercaptoethanol partially inhibited protease activity. Enzyme solution was generally tolerant to the detergents and solvents tested (Table 2), thus proving its suitability for detergent applications. The protease activity is stimulated in the presence of metal ions like Mg^{2+} and Ba^{2+} and is strongly inhibited by Hg^{2+}, Zn^{2+} and Fe^{2+} (Table 2). The metalloproteases present in the mixture might have got stimulated in the presence of Mg^{2+} and Ba^{2+} thereby increasing the overall activity.

In conclusion, this study reports the positive influence of nutritional parameters in improving the production of keratin hydrolyzing proteolytic enzymes by the alkalophilic strain *Streptomyces albidoflavus* TBG-S13A5. Possessing good inducible keratinolytic potential, this strain has been proven ideal for solid state fermentation of chicken feathers. Studies on biochemical and physiochemical properties of crude extracellular enzyme, shows its workability in alkaline and high temperature environment that goes with most industrial processes. Thus this strain can effectively be exploited to transform cheaper keratin substrates like feather wastes to valuable amino acids or peptides with a simultaneous production of different proteolytic enzymes suited for industrial applications.

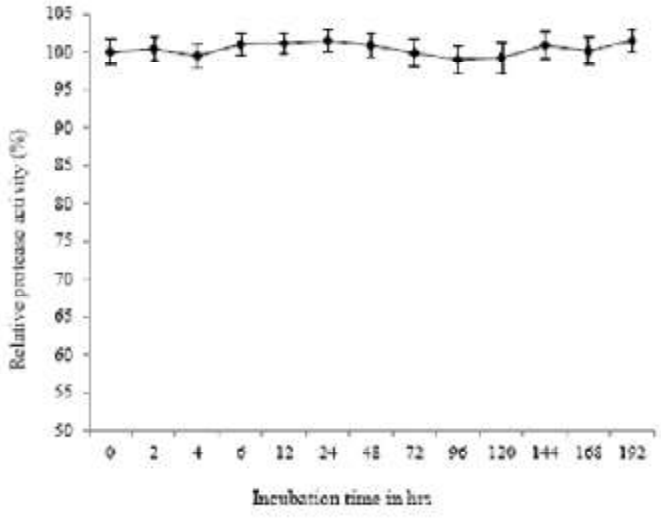


Fig. 5. Enzyme stability at room temperature (30±2°C)

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