View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by AJOL - African Journals Online

African Journal of Biotechnology Vol. 10(43), pp. 8630-8640, 10 August, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB10.1611
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

Oxidant and solvent stable alkaline protease from *Aspergillus flavus* and its characterization

Santosh Kumar Yadav[1], Deepali Bisht[1], Shikha[2] and Nandan Singh Darmwal[1]*

[1]Department of Microbiology (Centre of Excellence), Dr. Ram Manohar Lohia Avadh University, Faizabad, Uttar Pradesh, India.
[2]Department of Environmental Science, Babasaheb Bhimrao Ambedkar University, Lucknow, Uttar Pradesh, India.

Accepted 24 March, 2011

The increase in agricultural practices has necessitated the judicious use of agricultural wastes into value added products. In this study, an extracellular, organic solvent and oxidant stable, serine protease was produced by *Aspergillus flavus* MTCC 9952 under solid state fermentation. Maximum protease yield was obtained when the strain was grown under wheat bran and corn cob mixture (1:1) incubated for 48 h at pH 9.0 and temperature 37°C with 50% of initial moisture content. The partially purified enzyme showed wide range of pH optima (8.0-12.0) and pH stability (7.0-12.0), whereas, optimum temperature was 40°C and was stable over a wide range of temperature 30-45°C. The protease was extremely stable towards several organic solvents. The enzyme retained 80% of its original activity in the presence of non ionic and ionic surfactants and 100% with 10% H_2O_2 after 1 h of incubation at 30°C. In addition, the enzyme showed excellent compatibility with some commercial powder detergents. The compatibility of our protease with several detergents, oxidants and organic solvents suggests its possible use in detergent industry and peptide synthesis.

Key words: Alkaline protease, solid state fermentation, PMSF, organic solvent.

INTRODUCTION

Proteases are commercially important enzymes (Ferrero et al., 1996; Kumar et al., 1999) which account for approximately 60% of global industrial enzyme sales (Rao et al., 1998). Proteases occur widely in plants and animals, but commercial proteases are produced exclusively from microorganism (Chutmanop et al., 2008). *Aspergillus, Penicillium* and *Rhizopus* are widely used for protease production since several species of these genera are regarded as safe (GRAS) strains (Pandey, 1992). *Aspergillus* has ideally been an organism of choice for bulk production of industrial enzymes, as the fungi can be grown on relatively inexpensive agricultural wastes (Bergquist e t al., 2002).

Enzyme production can be carried out by both submerged fermentation (SmF) and solid-state fermentation (SSF), the latter being a technique of choice (Pandey et al., 2001; Sandhya et al., 2005). Alkaline serine proteases (EC 3.4.21) are active and stable at neutral to alkaline pH (7-12), and find extensive applications in protein chemistry and protein engineering as well as in industries such as detergents, leather, protein recovery, meat tenderization etc. (Lauer et al., 2000; Johnvesly and Naik, 2001).

Alkaline proteases with novel properties, such as stability with organic solvents, are in great demand for their increasing application in organic synthesis (Gupta and Khare, 2006). In view of the above, we report here the production of alkaline protease by *Aspergillus flavus* MTCC 9952, with an aim to find a set of culture conditions using agro industrial wastes and characterize the properties of partially purified enzyme leading to the development of detergent formulations and for peptide synthesis in non aqueous media.

*Corresponding author. E- mail: dr_nsdarmwal@yahoo.co.in. Tel: 05278 247350.

Abbreviations: SmF, Submerged fermentation; **SSF,** solid-state fermentation; **PMSF,** phenylmethylsulfonyl fluoride; **TCA,** trichloroacetic acid; **EDTA,** ethylene-diaminetetraacetic acid; **SDS,** Sodium dodecyl sulphate.

Materials and methods

Isolation and screening of microorganism

Forty six fungal strains were isolated from soil samples of different habitats using modified Warcup method (Warcup, 1951). Soil suspension (0.2 ml) was spread on medium containing (gl^{-1}); peptone, 10.0; dextrose, 40.0; skimmed milk, 50.0; rose Bengal, 33.0 mg and plates were incubated at 37°C for 48 h. Fungal colonies showing zone of hydrolysis were isolated, purified and screened quantitatively under SSF for alkaline protease production. Wheat bran (5 g) was dispensed in 250 ml Erlenmeyer flasks, moistened with 20 ml of Czapek dox solution (containing g/l: $NaNO_3$, 25; KCl, 0.5; $MgSO_4$, 0.5; KH_2PO_4, 1.0; pH 9.0), sterilized, inoculated with 1 ml of spore suspension (OD-1.5-1.6 at 540 nm) and incubated at 37°C for 4 days.

Maintenance, medium and preparation of inoculums

The culture was routinely maintained on malt extract-glucose-agar slants (containing g/l: malt extract, 20; glucose, 20; KCl, 0.5; $MgSO_4$, 0.5; KH_2PO_4, 1.0; pH 9.0) and stored at 4 °C. Prior to each experiment, the fungus was transferred to fresh slants and incubated at 37 °C for 4 days. The spores from fully sporulated slant were dislodged and dispersed in 10 ml of 0.1 % (v/v) Tween 80 with inoculum loop under aseptic conditions. The spore suspension was used as inoculum (1 ml), viable spores in the suspension being determined by serial dilution followed by plate count ($1x10^7$ spores/ml).

A Study of process variables on protease production

Effect of following variables on protease production was studied sequentially: agro-industrial wastes (wheat bran, WB; rice bran, RB; corn cob CC; pigeon pea bran, PB and black gram bran, BgB) were studied alone and in combinations (ratio of 1:1). The effect of incubation period (24 to 168 h) was studied followed by different ratios of WB and CC. The media were moistened to 44.4 to 87.5 % initial moisture and incubated at 32 to 45 °C. The effect of initial pH (8.5-10.5), different inoculum levels ($1x10^4$ to $1x10^9$ spores/gram of solid substrate, gss) were also evaluated for the optimum enzyme yield.

Supplementation of different carbon sources were studied at the level of 100 mg/gss. Similarly several organic, inorganic nitrogen sources and metal ions were also tested at the level of 50 mg/gss, 20 mg/gss and 0.5 mg/gss, respectively.

All experiments were carried out in triplicates and the results are presented as the mean of three independent observations.

Analytical methods

Enzyme extraction

Fermented bran (5 g) was soaked in 50 ml of sterile distilled water and the resulted slurry was agitated on a rotary shaker (120 rpm, 2h, and 30 °C). Slurry was finally removed by filtering and centrifuging at 10000 xg for 10 min at 4 °C. The resulting supernatant was recovered which served as crude enzyme extract.

Enzyme assay

Protease activity was determined as described by Hagihara et al. (1958), 1ml of suitably diluted enzyme was mixed with 1ml of casein (1% w/v prepared in 50 mM carbonate-bicarbonate buffer of pH 10.0). The mixture was incubated at 37 °C for 10 min. The reaction was quenched by adding 3 ml of 10 % pre-chilled trichloroacetic acid (TCA).

The reaction was allowed for 30 min to completely precipitate the proteins. The contents of the reaction tubes were filtered through Whatman No. 1 filter paper and absorbance of the filtrate was read at 275 nm which was extrapolated against tyrosine standard curve. A unit of protease activity was defined as the amount of enzyme liberating 1µg tyrosine/ml/min under the assay conditions. The protease activity was reported as per gram of solid substrate (gss) used for protease production.

Estimation of total soluble protein

The total soluble protein content was estimated following Lowry's method (Lowry et al. 1951) using BSA as standard.

Partial purification of enzyme

The supernatant was separated into three fractions by adding chilled acetone in ratio of 1: 0.8, 1:2 and 1:3 (enzyme extract: acetone). The precipitated proteins were separated by centrifugation at 10000 xg at 4 °C for 15 min, left for some time to evaporate the traces of acetone and dissolved in minimum amount of Tris-HCl buffer (50 mM, pH 9.0) and stored at 4 °C for further study.

Characterization of the enzyme

Effect of temperature and pH on enzyme activity and stability

The optimum temperature for protease activity was determined by incubating the reaction mixture at different temperatures (20-65 °C). Thermal stability was assayed by pre-incubating the enzyme at different temperatures (30-50 °C) for 30 and 60 min and residual protease activity was determined at temperature 37 °C and pH 10.0.

The optimum pH was assayed by determining the proteolytic activity at different pH by using the following 50 mM buffer solutions (Sodium phosphate, pH 6.0 - 8.0; Tris-HCl, pH 9.0; glycine/NaOH, 10.0-12.0). For pH stability the enzyme was pre-incubated (1:1) with above mentioned buffers at 30 °C for 1 h and thereafter the residual protease activity was determined under standard assay conditions.

Effect of cations, inhibitors, surfactants and oxidizing agents on enzyme activity/stability

The effect of various metal ions (5 mM) on enzyme activity was investigated using NaCl, KCl, $CaCl_2$, $MnCl_2$, $ZnCl_2$, $CoCl_2$, $CuSO_4$, $FeSO_4$, $NiCl_2$, $HgCl_2$, $PbCl_2$ and $MgSO_4$ in the reaction mixture. The effects of some surfactants (Triton X-100, Tween 40, Tween 80, and SDS) and oxidizing agents (H_2O_2 and sodium perborate) on enzyme stability were also studied by pre-incubating the enzyme for 30 min and 1 h, at 30 °C. The residual protease activity was measured under standard assay conditions.

The effects of enzyme inhibitors (2.5 mM) on protease activity were studied using phenylmethylsulfonyl fluoride (PMSF) and ethylene-diaminetetraacetic acid (EDTA). The enzyme was pre-incubated with inhibitors for 30 min at 30 °C and the residual enzyme activity was estimated under standard assay conditions.

Effect of organic solvents on enzyme stability

Enzyme was incubated with different hydrophilic and hydrophobic

Table 1. Effect of type of solid substrate and incubation period on protease production.

Type of substrate	Mean[a] enzyme activity (U gss^{-1})					
	48 h	**72 h**	**96 h**	**120 h**	**144 h**	**168 h**
WB	244±18	420±22	735±66	563±35	410±28	397±34
RB	249±18	355±28	460±48	381±12	210±58	249±16
PB	473±25	789±50	526±45	382±26	338±62	289±43
BgB	552±28	814±55	703±25	546±44	486±42	422±22
WB+RB	131±20	473±44	552±32	276±38	149±18	236±30
PB+WB	486±32	697±52	670±18	407±60	368±21	328±30
WB+CC	815±44	447±30	565±40	315±52	289±15	263±17
BgB+WB	434±40	605±62	780±60	552±22	499±54	381±21
BgB+CC	302±38	604±50	473±80	289±30	210±32	209±35

$p^b < 0.02^c < 0.02^c < 0.02^c < 0.02^c < 0.02^c < 0.02^c$. [a] Average of three independent experiments; [b] The P value refers to the comparison of protease activity with wheat bran to other substrates. The comparison is among the values within the column; [c] All the values in the coloumn are statistically significant.
WB, wheat bran; RB, rice bran; PB, pea bran; BgB, black gram bran; CC, corn cob.

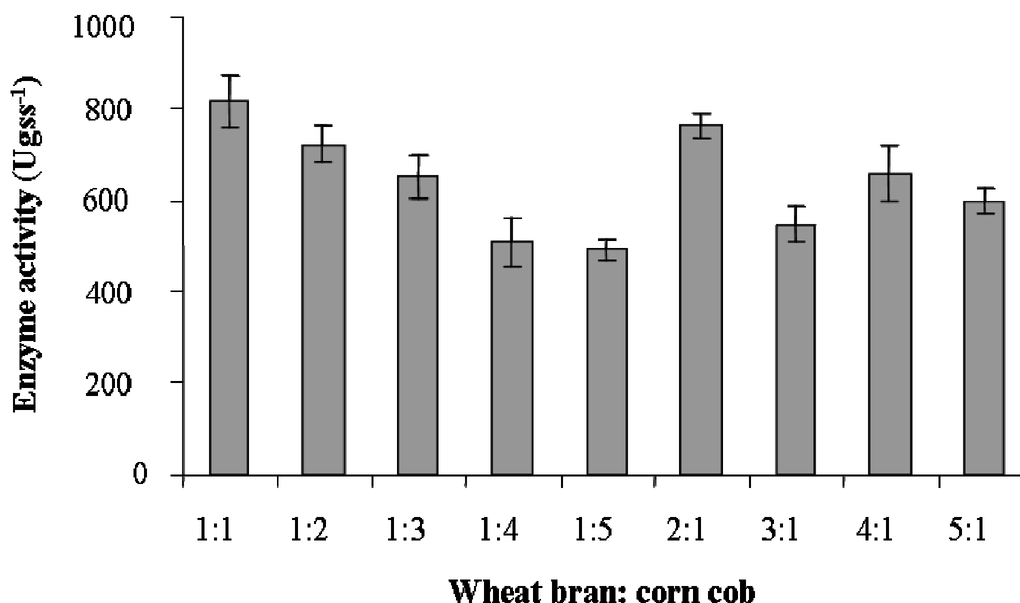

Figure 1. Effect of ratio of wheat bran and corn cob on protease production. An average of three observations, bars indicate the standard error, $p < 0.02$. The p value refers to the comparison of protease activity at 1:1 ratio of WB to CC with other combinations. All the comparisons are statistically significant.

organic solvents (benzene, chloroform, hexane, xylene, toluene, ethanol, acetone, diethyl ether, butanol and methanol) at a concentration of 25 % at 30 °C for 1 h and residual protease activity was estimated by standard assay method. Enzyme without organic solvent was taken as a control.

Results

Isolation and screening

The alkaline protease production profile of forty six fungal strains was studied and the fungal strain NSD08, giving identified as *Aspergillus flavus* at Indian Institute of Microbial Technology, Chandigarh and given an accession No. MTCC 9952 was selected for further studies.

An effect of type of substrate and incubation time on protease production was studied. Results (Table 1) revealed a significant variation in incubation time with respect to the type of substrate used. However, a comparable level of protease activity was obtained with the substrates WB + CC (815 $Ugss^{-1}$), BgB (814 $Ugss^{-1}$)

Table 2. Effect of initial moisture content on protease production

Initial moisture content (%)	Mean[a] Enzyme activity (U gss^{-1})
44.4	1091 ± 62
50.0	1377 ± 82
66.66	1195 ± 108
75.0	982 ± 58
80.0	805 ± 62
83.33	783 ± 72
85.7	477 ± 55
87.5	349 ± 59

$p^b < 0.02^c$; [a] Average of the three independent experiments; [b] the P value refers to the comparison of protease activity at 44.4% initial moisture content with other values (50-87.5 % moisture content) to other substrates. The comparison is within the values among the column; [c] all the values in the coloumn are statistically significant.

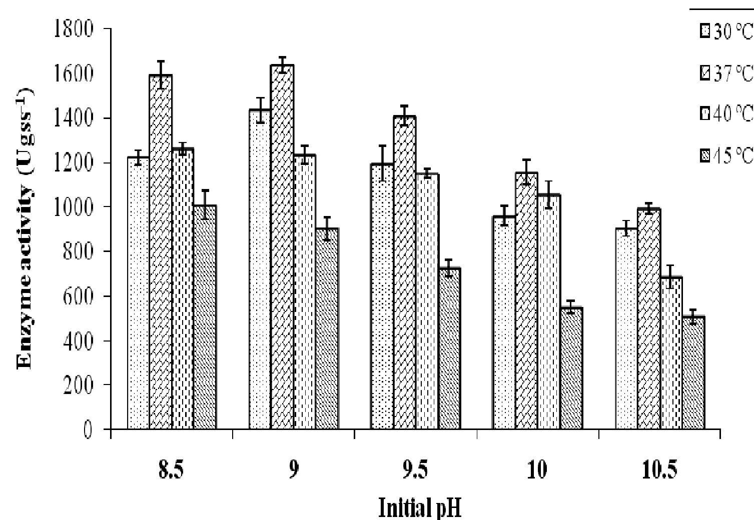

Figure 2. Effect of pH and temperature on alkaline protease production. An average of three observations, bars indicate the standard error, p < 0.02. The p value refers to comparison of protease activity at different pH and temperatures. All comparisons are statistically significant.

and WB (735 $Ugss^{-1}$), after 48 h, 72h, and 96 h, respectively, probably due to its ability to use diverse substrates. With a view to select the best combination, mixtures of WB and CC were investigated. The ratio of 1:1 (WB: CC) gave maximum production (807 U/gss) followed by 2:1 with enzyme units of 763 U/gss (Fig 1).

The effect of initial moisture content on protease production by *A. flavus* suggested an approximate 50 % of initial moisture content as optimum for protease production (Table 2).

Effect of inoculum size on protease production

A gradual increase in the enzyme activity was observed with increasing concentration of spores in the inoculum (data not shown). $1x10^8$ spores/g substrate was found to be optimum (1621 U/gss), however, further increase in the inoculum size reduced the enzyme activity.

Combined effect of pH and temperature on enzyme production

The effect of pH and temperature on alkaline protease production is shown in Fig 2. The fungus is capable to produce significant amount of enzyme in the temperature range 32-40 °C and pH range 8.5 to 10.0. Maximum protease production was obtained at 37 °C and pH 9.0 (1640 U/gss).

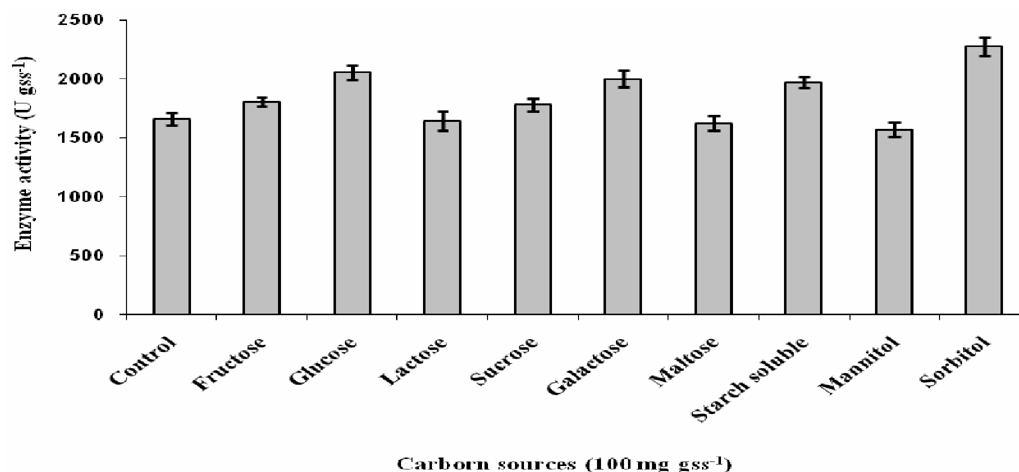

Figure 3. Effect of additional carbon sources on protease production. An average of three observations, bars indicate the standard error, $p< 0.02$. The p value refers to the comparison of protease activity at control (without any carbon source) with other carbon sources amended in the medium. All the comparisons are statistically significant.

Table 3. Effect of nitrogen sources on enzyme production by *A. flavus*

Organic nitrogen	Mean[a] enzyme activity (Ugss[1])	Inorganic nitrogen	Mean[a] enzyme activity (U gss^{-1})
Control	2540 ± 74	Control	3110 ± 95
Peptone	2570 ± 102	NH_4Cl	2942 ± 125
Tryptone	2485 ± 151	$(NH_4)_2HPO_4$	3640 ± 110
Gelatin	2279 ± 96	$(NH_4)_2SO_4$	3304 ± 97
Yeast extract	2680 ± 120	KNO_3	3580 ± 121
Casein	2129 ± 125	HH_2CONH_2	2791 ± 160
Malt extract	2923 ± 155	NH_4NO_3	3491 ± 221
Corn steep liqour	2583 ± 81	$NH_4H_2PO_4$	3505 ± 173

$p^b < 0.02^c$ $p^b < 0.02^{c;}$ [a] Average of the three independent experiments; [b] the P value refers to the comparison of protease activity of control to the organic and inorganic nitrogen sources added to the medium. The comparison is among the values within the column; [c] All the values in the column are statistically significant.

Effect of additional carbon source on protease production

Fig 3 represents the effect of addition of carbon sources on protease production by *A. flavus.* Maximum yield was obtained with sorbitol (2277 U/gss), however, sufficient amount of enzyme (2054 U/gss) was produced by this strain with glucose, followed by galactose (1949 U/gss). Further increase in sorbitol concentration increased the enzyme yield (2531 U/gss) at concentration of 200 mg/gss, while higher concentration of sorbitol showed inhibitory effect on enzyme yield which might be due to catabolite repression.

Effect of additional nitrogen sources on protease production

Although seven organic nitrogen sources (50 mg/gss) were investigated to observe their effect on protease production (Table 3), only malt extract had an inductive effect on enzyme production (2923 U/gss). Therefore, different concentrations of malt extract were investigated and a 23 % increase in protease production (3136 U/gss) was obtained at the level of 100 mg/gss, over the control. Few inorganic nitrogen sources (20 mg/gss) were also incorporated in the fermentation medium and results revealed that di-ammonium hydrogen orthophosphate was the best (Table 3) with enzyme units of 3640 U/gss.

Effect of metal salts on enzyme production

The effect of different metal ions on protease production revealed that $MnCl_2$ supported the maximum enzyme production (4091 U/gss), followed by $FeSO_4$ (3855 U/gss), whereas, $CuSO_4$, $ZnSO_4$ and $CoCl_2$ inhibited the

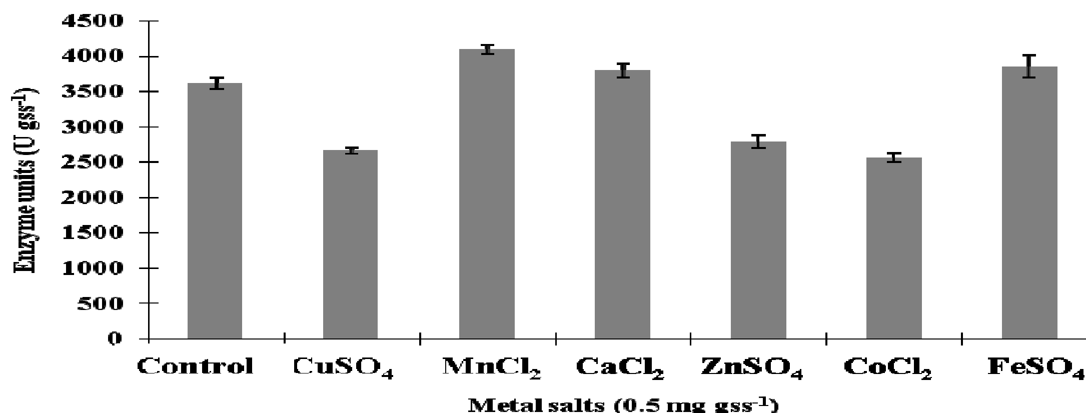

Figure 4. Effect of metal salts on protease production. An average of three observations, bars indicate the standard error, $p < 0.02$. The p value refers to the comparison of protease activity at control with other metals added in the medium. All the comparisons are statistically significant.

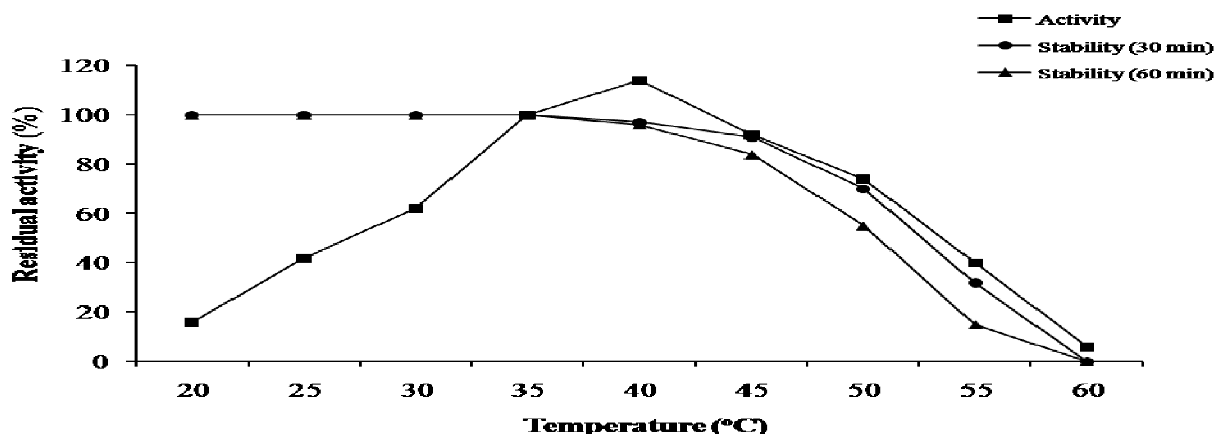

Figure 5. Effect of temperature on enzyme activity and stability. An average of three observations, standard deviation for all values is < ±5 %.

production. $CaCl_2$ insignificantly (4 %) reduced the enzyme production (Figure 4).

Characterization of enzyme:

Effect of temperature on enzyme activity and stability

The temperature profile of *A. flavus* protease activity elaborated retention of 100 % activity at 40 °C while 92, 74 and 41 % residual activities were recorded at 45, 50 and 55 °C, respectively. However, increase in temperature beyond 55 °C resulted in complete inactivation of enzyme.

The thermal stability profile of the enzyme showed an initial retention of 96 % activity after 1 h of incubation at 40 °C, whereas, 84 and 53 % of residual activity was obtained at 45 and 55 °C, respectively (Fig 5).

Effect of pH on enzyme activity and stability

The activity profile of the *A. flavus* protease at different pH showed that the enzyme was active over a wide range of pH range 7-12, with maximum activity recorded between pH 8.0 – 12.0 (Fig 6).

The pH stability profile also showed the considerable stability of the enzyme between pH range 7.0 to 10.0 and showed 73 and 62 %
residual activity at pH 11 and 12, respectively.

Effect of metals ions and inhibitors on enzyme activity/stability

The effect of metal ions and inhibitors on enzyme activity showed that among all the metals ions tested, only Fe^{++}, stimulated the activity by 1.74 fold, whereas, Hg^{++} and Cu++ showed a complete inhibition of the enzyme activity

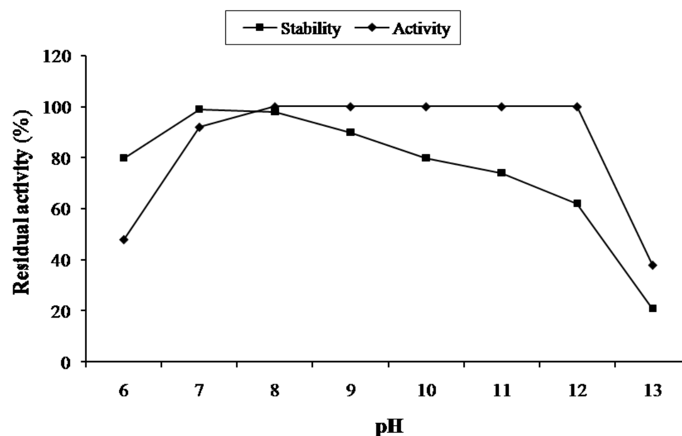

Figure 6. Effect of pH on enzyme activity and stability. An average of three observations, standard deviation for all values is < ±5 %.

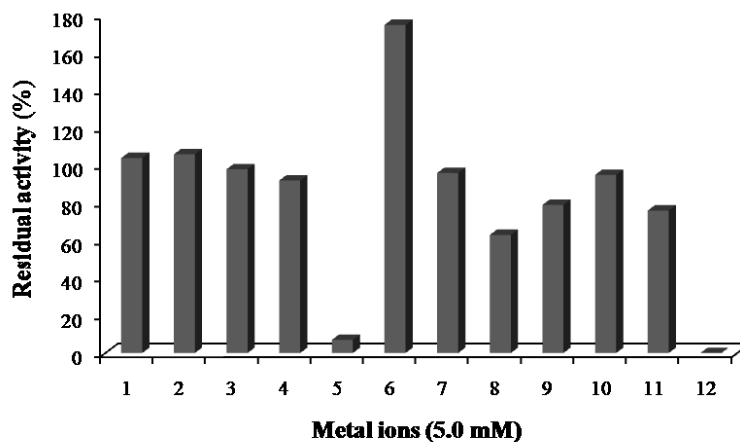

Figure 7. Effect of metals on enzyme activity. An average of three observations, standard deviation for all values is < ±5 %. 1: NaCl; 2:KCl; 3: $CaCl_2$; 4:$CoCl_2$; 5: $CuSO_4$; 6: $FeSO_4$; 7:$MgSO_4$; 8: $MnCl_2$; 9: $NiCl_2$; 10:$Pb(CH_3COO)_2$; 11: $ZnSO_4$; 12: $HgCl_2$.

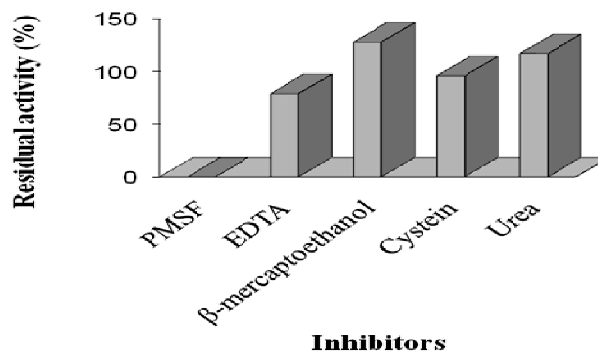

Figure 8. Effect of inhibitors on enzyme activity. An average of three observations, standard deviation for all values is < ±5 %.

Table 4. Effect of detergents and oxidizing agents on enzyme activity.

Detergent/Oxidizing agent	mean[a] residual activity (%)	
	30 min	60 min
SDS (0.1 %)	82	82
Tween 80 (1.0 %)	94	78
Tween 40 (1.0 %	81	75
Triton X 100 (1.0 %)	84	81
Ghari (1.0 %)	58	55
Ariel (1.0 %)	42	38
Surf excel (1.0 %)	46	32
H_2O_2 2.5 %	100	100
5.0 %	99	99
10.0 %	99	99
15.0 %	45	37
Sodium perborate (3.0 %)	42	34

$p^b < 0.02^c < 0.02^c$; [a] Average of the three independent experiments; [b] The P value refers to the comparison of protease activity of control residual activity after incubation with the reagents. The comparison is among the values within the column; [C] all the values in the column are statistically significant.

Table 5. Effect of organic solvents on enzyme stability

Organic solvent	Log P value	Mean[a] residual activity (%)
Control	-	100
Benzene	2.0	89.2
Chloroform	2.0	105.3
Hexane	3.5	94.3
Xylene	3.1	83.6
Toluene	2.5	91.7
Ethanol	-0.24	83.2
Acetone	-0.21	97.1
Diethyl ether	0.83	89.2
Butanol	0.80	96.2
Methanol	-0.82	81.7

$p^b < 0.02^c$; [a] Average of the three independent experiments; [b] the P value refers to the comparison of protease activity of control residual activity after incubation with the reagents. The comparison is among the values among the column; [c] all the values in the column are statistically significant.

(Fig 7). Despite being a serine protease addition of Ca++ ion showed only 2 % loss of enzyme activity.

In order to determine the nature of the protease, enzyme activity was measured in the presence of different enzyme inhibitors (Fig 8). The enzyme was found to be strongly inhibited by PMSF indicating that the enzyme is a serine protease. The chelating agent (EDTA) moderately inhibited (22%) the enzyme activity. However, β-mercaptoethanol and urea enhanced the protease activity by 27 and 17 %, respectively. Cystein, On the other hand, showed slight inhibition (4 %) in protease stability.

Effect of oxidizing agents and surfactants on protease stability

As shown in Table 4 the enzyme was appreciably stable in the presence of non-ionic surfactants like Tween 80 and Triton X-100. The strong anionic surfactant, sodium

dodecyl sulphate (SDS, 0.1 % w/v) caused a moderate inhibition (18 %) in enzyme activity. In addition, protease retained 48 and 100 % of its activity after incubation for 1 h at 30 °C in the presence of 3 % (w/v) sodium perborate and 10 % (v/v) hydrogen peroxide, respectively.

Effect of organic solvents on enzyme stability

It is clear from Table 5 that enzyme was remarkably stable with all the solvents used. The maximum enzyme activity was found in the presence of chloroform (105.3 %) followed by acetone (97.1 %) and butanol (96.2 %). However, other solvents showed moderate inhibition (about 20 %) in enzyme activity.

Discussion

Forty six fungal strains were screened for their alkaline protease production profile. Fungal strain *Aspergillus flavus* (NSD 08), was identified and selected for further studies.

The composition of substrate in the SSF and incubation time has a marked effect on the protease production. Our results revealed that a ratio of 1:1 (WB: CC) gave maximum production (815 U/gss) followed by the ratio 2:1 with enzyme units of 763 U/gss recorded after 48 h of incubation. Malathi and Chakraborty (1991) and Agrawal et al (2004) obtained maximum protease yield using wheat bran as substrate after 48 and 72h of incubation, with *A. flavus* and *Penicilium* sp, respectively. However, Sumantha et al (2005) reported production of a metalloprotease on mixed substrate after 48 h of incubation.

The moisture content of the fermentation medium is well known to have a profound effect on both, the fungal growth as well as the enzyme production under SSF. Since presence of water in the medium makes the nutrients more readily accessible. The effect of initial moisture content on protease production by *A. flavus* suggested an approximate 50 % of initial moisture content as optimum for protease production. Maximum protease production with 55 and 63 % initial moisture content have also been reported earlier by *Penicillium* LPB-9 (Germano et al, 2003) and *A. flavus* IMI 327634 (Malathi and Chakraborty, 1991), respectively. Protease yield was found to reduce sharply below or above optimum level, as at lower moisture content the substrate would dry out and inhibit the growth of the fungus, while higher level of moisture content might waterlog the substrate which in turn affects the O_2 diffusion resulting in lower level of protease production (Bogar et al, 2003).

We observed a gradual increase in the enzyme activity with increasing concentration of spores in the inoculum. An inoculum size of $1x10^8$ spores/g substrate was found to be optimum (1621 U/gss), however, further increase in the inoculum size reduced the enzyme activity.

Conflicting reports are available on protease activity of fungi in relation to inoculum size. For *R. oryzae* NRRL 21498, $2x10^5$ spores/g wheat bran has been reported as optimum spore concentration (Tunga et al, 1998), whereas, Agrawal et al (2005) reported $1.0x10^{10}$ spores/g substrate as optimum.

It is well established that extracellular pH and temperature is important for cell growth and enzyme production (Kumar and Tagaki 1999). Our fungal isolate was capable of producing significant amount of enzyme in the temperature range 32-40 °C and pH range 8.5 to 10.0. Maximum protease production ($p < 0.02$) was obtained at 37 °C and pH 9.0 (1640 U/gss). Anandan et al (2007) also reported maximum protease production by *A. tamarri* at pH 9.0 but at temperature 30 °C, however, Negi and Banerjee (2006) found optimum protease production at temperature 37 °C and pH 7.0 for *A. awamori*.

Glucose has been universally suggested as the best carbon source (Mehrotra et al., 1999, Srinubabu et al, 2007) as it can be easily metabolized by the microorganisms (Ashour et al, 1996). Adequate amount of enzyme (2054 U/gss) was produced by the isolated strain with glucose, however, sorbitol was found to be best source and maximum yield of enzyme (2531 U/gss) was obtained at a concentration of 200 mg/gss, while the inhibitory effect at higher concentrations might be due to catabolite repression.

Out of the seven organic nitrogen sources investigated, only malt extract had an inductive effect and production increased by 23 % (2923 U/gss). Our results are in accordance with earlier reports where complex nitrogen sources induced protease production by different microbial species (Pandey et al, 2000, Prakasham, et al, 2006).

Few inorganic nitrogen sources were also incorporated in the fermentation medium and results revealed that di-ammonium hydrogen orthophosphate was the best with enzyme units 3640 U/gss, while urea was the poorest one and other sources did not have any significant effect. Our report is in accordance with Srinubabu et al. (2007) who also reported di-ammonium hydrogen ortho-phosphate for protease production by *A. oryzae*, whereas, Johnvesly and Naik (2001) observed nitrates as best nitrogen sources.

It is well known fact that the metal ions in the fermentation medium greatly influence the protease production by microbes (Varela et al. 1996). Results revealed that $MnCl_2$ stimulated the enzyme production (4091 U/gss), followed by $FeSO_4$ (3855 U/gss), whereas, $CuSO_4$, $ZnSO_4$ and $CoCl_2$ inhibited the production. $CaCl_2$, however, showed insignificant effect. In earlier studies different metals have been reported for protease production with different microbes (Abidi et al. 2008; Vijayanand et al. 2010).

The partially purified enzyme elaborated a 3 fold increase increase in specific activity. A 100 % retention of protease activity was recorded at 40 °C while 92, 74 and 41 % residual activities were recorded at 45, 50 and

55 °C, respectively. However, an increase in temperature beyond 55 °C resulted in the inactivation of enzyme. Wang et al (2005) and Tremacoldi et al (2007) also reported alkaline protease from *Aspergillus* sp. having optimum activity at 40 °C.

The thermal stability profile of the enzyme showed an initial retention of 96% activity after 1 h of incubation at 40 °C, whereas, 84 and 53 % of residual activities were scored at 45 and 55 °C, respectively. It also showed that protease from *A. flavus* was relatively thermostable when compared to other reports for the same organism (Tremacoldi et al 2007, Tunga et al, 2003).

The activity profile of the *A. flavus* protease at different pH showed that the enzyme was active over a wide range of pH 7-12, with maximum activity at pH 12. With regard to pH stability the enzyme was stable between pH range 7.0 to 10.0 and showed 73 and 62% residual activity at pH 11 and 12, respectively. These findings are in accordance with earlier studies (Miyaji et al, 2006), where the optimum pH for alkaline protease was 11-12, whereas, Devi et al (2008) and Charles et al (2008) reported pH optima 10.0 and 8.0, for *A. niger and A. niduans* HA01, respectively.

The effect of metal ions and inhibitors on enzyme activity showed that among all the metals ions tested, only Fe^{++}, stimulated the activity by 1.74 fold, whereas, Hg^{++} and Cu^{++} showed a complete inhibition of the activity. Despite being a serine protease addition of Ca^{++} ion showed 2% loss of enzyme activity, which reflected the independency of the enzyme on Ca^{++} ion, as most of the serine proteases have Ca^{2+} binding site(s) (Vielli and Zeikus, 2001).

The inhibition of the enzyme activity by serine protease inhibitor (PMSF) indicated that the enzyme was a serine protease. The enzyme was moderately inhibited by the chelating agent EDTA, with 78 % residual activity. However, β-mercaptoethanol and urea enhanced the protease activity by 27 and 17%, respectively, and cystein showed slight inhibition of activity. The high activity of enzyme in the presence of EDTA is very useful for application as detergent additive because chelating agents are components of most of the commonly used household detergents (Hajji et al, 2007).

A good detergent protease must be compatible and stable with all commonly used detergent compounds such as surfactants, bleaches, oxidizing agents and other additives, which could be present in the formulation (Gupta et al, 2002). The enzyme produced by the isolated strain was appreciably stable in the presence of non-ionic surfactants like Tween 80 and Triton X-100. The strong anionic surfactant (SDS) at 0.1 % (w/v) caused a moderate inhibition (18 %) in enzyme activity on the other hand Tremacoldi et al. (2007) reported that the protease from *A. clavatus* CCT2759 was strongly inhibited by SDS, Tween 80 and carbonate ion. The stability of the enzyme against SDS was lower than *A. parasiticus* protease which retained about 97% of its initial activity after 1 h incubation with 2 % SDS at room temperature (Tunga et al. 2003). In addition, protease retained 100 and 48% of its activity after incubation for 1 h at 30 °C in the presence of 10 % (v/v) hydrogen peroxide and 3 % (w/v) sodium perborate, respectively. Mei and Jiang (2005) reported valuable stability with H_2O_2 at concentration 1%, whereas, Joo et al (2003) reported 110% activity with 10% H_2O_2 after 72 h.

Most of the organic solvent stable proteases are reported from bacteria, particularly by *Pseudomonads* and *Bacillus* sp. (Gupta and Khare 2006; Fang et al. 2009). Though the alkaline protease produced by our strain is stable in presence of different organic solvents but not the strain, hence, it could be used as a biocatalyst for peptide synthesis in organic media.

Conclusion

The *A. flavus* serine protease in the present study retained its activity even in the presence of EDTA and was an oxidant stable as was evident by 100% retention of its activity in the presence of hydrogen peroxide, which makes its use ideal for detergent formulations. Agro industrial waste combinations such as wheat bran and corn cob could also be exploited as a cost effective substrate for pilot and large scale industrial applications. The application profile of this protease is extended by its stability in the presence of organic solvents, due to which it can potentially be used in organic synthesis.

Acknowledgments

Financial assistance to one of the authors (Santosh Kumar Yadav) by Council of Science and Technology, Uttar Pradesh, is thankfully acknowledged.

REFERENCES

Abidi F, Limam F, Nejib MM (2008). Production of alkaline proteases by *Botrytis cinerea* using economic raw materials: Assay as biodetergent. Process. Biochem. 43(11): 1202-1208.

Agrawal D, Patidar P, Banerjee T, Patil S (2005). Alkaline protease production by a soil isolate of *Beauveria felina* under SSF condition: parameter optimization and application to soy protein hydrolysis. Process. Biochem. 40(3-4): 1131-1136.

Agrawal D, Patidar P, Banerjee T, Patil S (2004). Production of alkaline protease by *Penicillium* sp. under SSFconditions and its application to soy protein hydrolysis. Process. Biochem. 39: 977–981.

Anandan D, Marmer WN, Dudley RL (2007). Isolation, characterization and optimization of culture parameters for production of an alkaline protease isolated from *Aspergillus tamari*. J. Ind. Microbiol. Biotechnol. 34:339–347.

Ashour SA, Shora HM, Metwally M, Habib SA (1996). Fungalfermentation of whey incorporated with certain supplements for the production of proteases. Microbio. 86:59–69.

Bergquist PL, Te'o VSJ, Gibbs MD, Cziferszky ACE, DeFaria FP, Azevedo MO, Nevalainen KMH (2002). Production of recombinant bleaching enzymes from thermophilic microorganisms in fungal hosts. Appl. Biochem. Biotechnol. 98–100:165–176.

Bogar B, Szakacs G, Pandey A, Sabu A, Linden JC (2003). Production of phytase by *Mucor racemosus* in solid-state fermentation. Biotechnol. Prog. 19: 312–319.
Charles P, Devanathan V, Anbu P, Ponnuswamy MN, Kalaichelvan PT, Hur BK (2008). Purification, characterization and crystallization of an extracellular alkaline protease from *Aspergillus nidulans* HA-10. J. Basic. Microbiol. 48: 347–352.
Chutmanop J, Chuichulcherm S, Chisti Y, Srinophakun P (2008). Protease production by *Aspergillus oryzae* in solid-state fermentation using agroindustrial substrates, J. Chem. Technol. Biotechnol. 83:1012–1018.
Devi MK, Banu AR, Gnanaprabhal GR, Pradeep BV, Palaniswamy M (2008). Purification, characterization of alkaline protease enzyme from native isolate Aspergillus niger and its compatibility with commercial detergents. Ind. J. Sci. Technol. 1(7).
Fang Y, Liu S, Wang S, Lv M (2009). Isolation and screening of a novel extracellular organic solvent-stable protease producer. Biochem. Eng. J. 43(2): 212-215.
Ferrero MA, Castro GR, Abate CM, Baigori MD, Sineriz F (1996). Thermostable alkaline protease of *Bacillus licheniformis* MIR 29: isolation, production and characterization. Appl. Microbiol. Biotechnol. 45: 327–332.
Germano S, Pandey A, Osaka CA, Rocha SN, Soccol CR (2003). Characterization and stability of proteases from *Penicillium* sp. produced by solid-state fermentation. Enzyme. Microb. Technol. 32: 246–251.
Gupta A, Khare SK (2006). A protease stable in organic solvents from solvent tolerant strain of *Pseudomonas aeruginosa*. Bioresource. Technol. 97: 1788–1793.
Gupta R, Beg QK, Lorenz P (2002). Bacterial alkaline proteases: molecular approaches and industrial applications. Appl. Microbiol. Biotechnol. 59: 15–32.
Hagihara B, Matsubara H, Nakai M, Okunuki K (1958). Crystalline bacterial protease of *Bacillus subtilis*. J. Biochem. 45: 185-194.
Hajji M, Kanoun S, Nasri M, Gharsallah N (2007). Purification and characterization of an alkaline serine-protease produced by a new isolated *Aspergillus clavatus* ES1. Process Biochem. 42: 791–797.
Johnvesly B, Naik GR (2001). Studies on production of thermostable alkaline protease from thermophilic and alkaliphilic *Bacillus* sp. J99 in a chemically defined medium. Process Biochem. 37: 139–144.
Joo HS, Kumar CG, Park GC, Paik SR, Chang CS (2003). Oxidant and SDS-stable alkaline protease from *Bacillus clausii* I-52: production and some properties. J. Appl. Microbiol. 95(2): 267 – 272.
Kumar CG, Tagaki H (1999). Microbial alkaline protease: from bioindustrial viewpoint. Biotechnol. Adv. 17: 561-594.
Kumar CG, Tiwari MP, Jany KD (1999). Novel alkaline serine proteases from alkalophilic Bacillus spp: purification and some properties. Process Biochem. 34(5):441– 449.
Lauer I, Bonnewitz B, Neunier A, Beverini M (2000). New approach for separating *Bacillus subtilis* metalloprotease and amylase by affinity chromatography and for purifying neutral protease by hydrophobic chromatography. J. Chromatogr. B. 737: 277–284.
Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951). Protein measurement with Folin phenol reagent. J. Biol. Chem. 193: 265–275.
Malathi S, Chakraborty R (1991). Production of alkaline protease by a new *Aspergillus flavus* isolate under solid-substrate fermentation conditions for use as a depilation agent. Appl. Environ. Microbiol. 57: 712–716.
Mehrotra S, Pandey PK, Gaur R, Darmwal NS (1999). The production of alkaline protease by a *Bacillus* species isolate. Bioresource Technol. 67: 201-203.
Mei C, Jiang X (2005). A novel surfactant- and oxidation-stable alkaline protease from *Vibrio metschnikovii* DL 33–51. Process Biochem. 40(6): 2167-2172.
Miyaji T, Otta Y, Nakagawa T, Watanabe T, Niimura Y, Tomizuka N (2006). Purification and molecular characterization of subtilisin-like alkaline protease BPP-A from Bacillus pumilus strain MS-1. Lett. Appl. Microbiol. 42: 242–247.
Negi S, Banerjee R (2006). Optimization of amylase and protease production from *Aspergillus awamori* in single bioreactor through EVOP factorial design technique. Food Technol. Biotechnol. 44(2): 257–261.
Pandey A (1992). Recent process developments in solid-state fermentation. Process Biochem. 27: 109–117.
Pandey A, Soccol CR, Nigam P, Brand D, Mohan R, Roussos S (2000). Biotechnological potential of coffee pulp and coffee husk for bioprocess. Biochem. Eng. J. 6: 153-162.
Pandey A, Soccol CR, Rodriguez-Leon JA, Nigam P (2001). Solid-State Fermentation in Biotechnology, Asiatech Publishers Inc., New Delhi 221.
Prakasham RS, Rao CS, Sarma PN (2006). Green gram-husk an inexpensive substrate for alkaline protease production by *Bacullus* sp. in solid state culture. Bioresource Technol. 97: 1449-1454.
Rao MB, Tankasale AM, Ghatge MS, Deshpande VV (1998). Molecular and biotechnological aspects of microbial proteases. Microbiol. Mol. Biol. Rev. 62: 597–634.
Sandhya C, Sumantha A, Szakacs G, Pandey A (2005). Comparative evaluation of neutral protease production by *Aspergillus oryzae* in submerged and solid state fermentation. Process Biochem. 40: 2689–2694.
Srinubabu G, Lokeswari N, Jayaraju K (2007). Screening of Nutritional Parameters for the Production of Protease from *Aspergillus Oryzae*. E-Journal. Chem. 4(2): 208-215.
Sumantha A, Sandhya C, Szakacs G, Soccol CR, Pandey A (2005). Production and partial purification of a neutral metalloprotease by fungal mixed substrate fermentation. Food Technol. Biotechnol. 43(4): 313–319.
Tremacoldi CR, Monti R, Selistre-De-Araujo HS, Carmona EC (2007). Purification and properties of an alkaline protease of *Aspergillus clavatus*. World J. Microbiol. Biotechnol. 23: 295-299.
Tunga R, Banergee R, Bhattacharya BC (1998). Optimizing some factors affecting protease production under solid state fermentation. Bioprocess. Eng. 19: 187-197.
Tunga R, Shrivastava B, Banerjee R (2003). Purification and characterization of a protease from solid state cultures of Aspergillus parasiticus. Process Biochem. 38: 1553–1558.
Varela H, Ferrari MD, Belobradjic L (1996). Effect of medium compositing on the production by a new *Bacillus subtilis* isolate of protease with promising unhairing activity. World J. Microbiol. Biotechnol. 12: 643-645.
Vielle C, Zeikus JG (2001). Hyperthermophilic enzymes: sources, uses and molecular mechanisms for thermostability. Microbiol. Mol. Biol. Rev. 65: 1–43.
Vijayanand S, Hemapriya J, Selvin J (2010). Production and optimization of haloalkaliphilic protease by an extremophile-*Halobacterium* sp. Js1, isolated from thalassohaline environment. Global J. Biotechnol. Biochem. 5(1): 44-49.
Wang SL, Chen YH, Wang CL, Yen YH, Chern MK (2005). Purification and characterization of a serine protease extracellularly produced by *Aspergillus fumigatus* in a shrimp and crab shell powder medium. Enzyme. Microb. Technol. 36: 660–665.
Warcup JH (1951), Soil-steaming: A selective method for the isolation of ascomycetes from soil. Transactions the Brit. Mycolog. Soc. 34(4): 515-518.