JMBFS

Journal of Microbiology, Biotechnology and Food Sciences

International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

OPTIMIZATION OF EXTRACELLULAR ACID PROTEASE PRODUCTION FROM *ASPERGILLUS NIGER* BY FACTORIAL DESIGN

*Vishalkirti Vijay Kalaskar, Narayanan Kasinathan, Volety Mallikarjuna Subrahmanyam * and Josyula Venkata Rao*

Address(es):
Department of Pharmaceutical Biotechnology, Manipal College of Pharmaceutical Sciences, Manipal University, Manipal -576 104, India.

*Corresponding author: vm.subra@manipal.edu

doi: 10.15414/jmbfs.2014.4.2.132-136

ARTICLE INFO

Received 10. 8. 2014
Revised 19. 8. 2014
Accepted 22. 8. 2014
Published 1. 10. 2014

Regular article

OPEN ACCESS

ABSTRACT

The cultural conditions for acid protease production by *Aspergillus niger* was optimised using factorial design experiments and one factor-at-a time approach. In the production medium casein served as substrate and protease activity was measured in terms of tyrosine yield. The yield was further improved through UV mutation. Tyrosine yield amounted to 29.22 mg / g on casein substrate. Protease from this microbial strain was mesophilic. The enzyme was stable over a wide temperature range (30 to 45 °C) with maximum activity at 40 °C and showed better activity under acidic conditions. Also the extracted protease exhibited good tolerance towards organic solvents, surfactants and showed an increase in activity in presence of Ca^{2+}. UV mutant showed 37.2 % increase in yield over the natural selectant and had better pH and thermostability. SDS PAGE showed 27.5 kDa as molecular size of the enzyme. This was further confirmed by zymography and gel filtration (Sephadex G-50). An improved enzyme yield and a better pH and thermal stability were achieved by using a combination of cultural condition optimization and mutation technique.

Keywords: Fractional factorial design, optimization, protease, UV radiation

INTRODUCTION

Among the industrial enzymes, proteases occupy a prominent role in having wide applications. They are commercially exploited for both therapeutic and non therapeutic applications (**Johansson *et al.*, 2008**). All the organisms are capable of producing protease irrespective of their kingdom. Among various organisms, microbes serve as the best source for large scale production of proteases as they can be easily scaled up to meet the requirements in terms of volume and purity (**Rauf *et al.*, 2010**). Among the three types of proteases *viz.*, acid, neutral and alkaline, acid proteases are mainly found in fungi and only to a small extent in bacteria (**Alnahdi, 2012**). Acid proteases are mainly explored in food industry as seasoning material and for their ability to clot proteins (**Sumantha *et al.*, 2006**). Acid proteases are sometimes referred as "mould proteases" as moulds are the most important producer of acid protease (**Ramamurthy *et al.*, 1991**). Filamentous fungi are the main class of microorganism that are commercially used for production of acid protease. These organisms can be cultured on a simple medium to produce industrially useful enzymes that are mainly extracellular in nature (**Vishwanatha *et al.*, 2010**). Various species of *Aspergillus*, *Rhizopus* and other moulds have been reported to produce acid protease (**Rauf *et al.*, 2010**). Fermentation process economics can be improved through optimization of cultural conditions and by genetic manipulation of the microbial strain (**Parekh *et al.*, 2000**).
Many acid proteases do not have commercial viability owing to lower yields and instability. Also their therapeutic applications are not explored considerably. The objective of our study was to increase the yield of an acid protease produced by *Aspergillus niger* that had exhibited declotting, dehairing and anti-inflammatory activities (**Kalaskar *et al.*, 2010**) by fractional factorial design experiments and one factor at a time approach methods. pH and thermostability, tolerance towards various metal ions, organic solvents and surfactants of the partially purified enzyme were also investigated.

MATERIAL AND METHODS

Chemicals and Reagents

Sephadex G-50, DEAE-Sepharose was purchased from Sigma Aldrich, India, and Protein marker- from Genei Pvt. Ltd. Bangalore. Media components *viz.*, peptone, yeast extract, soluble starch, potato dextrose agar were from Himedia (India). Ammonium sulphate, magnesium sulphate, Folin- Ciocalteu's phenol reagent and other fine chemicals from Merck Pvt. Ltd. Mumbai

Cultivation conditions

The fungal organism used in the study belonged to *Aspergillus niger* (**Kamath *et al.*, 2010**). The microorganism was grown on Potato Dextrose agar (PDA) medium for 10 days at 28°C and then stored at 4°C.
A spore suspension (spore count in the range of 7-10 x 10^5 cfu) prepared using sterile water was transferred to glucose – yeast extract medium (**Kamath *et al.*, 2010**) and maintained at 150 rpm with pH 7.0 and 28°C for 2 days. Production medium consisting of soluble starch (1%), casein (1.5%), peptone (1%), potassium nitrate (1%), potassium dihydrogen phosphate (0.1%), dipotassium hydrogen phosphate (0.1%), magnesium sulphate (0.1%) and calcium carbonate (0.1%) and distilled water q.s. was seeded at 10% level and incubated as in the case of inoculum. The harvest was centrifuged at 4000 g for 10 min and the supernatant was employed for further studies.

Assay of protease

The proteolytic activity was determined using Folin- Ciocalteu's method. Casein was used as substrate. Tyrosine was used as standard. The tyrosine standard was prepared by dissolving 10 mg of tyrosine in 50 ml of 50 mM phosphate buffer (pH 7). A mixture of 5 ml casein substrate (0.6 g casein dissolved in 100 ml distilled water having 6% of 1.2 M lactic acid solution) and 1 ml of the enzyme solution was used. The reaction was carried out at 37°C for 10 min and stopped by addition of 5 ml of 0.44M trichloroacetic acid. The filtrate (2 ml) was treated with 5ml of 0.2M sodium carbonate and 1 ml Folin and ciocalteaus phenol reagent. The absorbance was measured at 660nm (Shimadzu, Japan) (**Folin and Ciocalteu, 1927**). One unit of protease activity (U) was defined as the amount of enzyme which releases one µg of tyrosine per min at 37°C.

Optimization of production conditions

The production medium components were optimized using factorial design (using Mintab 16, trial version) and one factor at a time method. Effect of the seven medium components (soluble starch, combined nitrogen source (casein, peptone and potassium nitrate), phosphate (di-potassium hydrogen phosphate and potassium di-hydrogen phosphate, calcium carbonate and cobalt sulphate) on

protease yield was studied by fractional factorial design. The design included eight runs (Table 1).

Table 1 Fractional Factorial Design for seven factors with eight trials

Std Order	Soluble starch	Casein, Peptone, KNO_3	K_2HPO_4, KH_2PO_4	$MgSO_4$	$CaCO_3$	$FeSO_4$	$CoSO_4$	EY*
1	1 (2)	1 (2)	1 (0.2)	-1 (0.02)	1 (0.2)	-1 (0.02)	-1 (0.02)	24.8
2	-1 (0.2)	1 (2)	1 (0.2)	1 (0.2)	-1 (0.02)	1 (0.2)	-1 (0.02)	23.56
3	-1 (0.2)	-1 (0.2)	1 (0.2)	1 (0.2)	1 (0.2)	-1 (0.02	1 (0.2)	7.02
4	1 (2)	-1 (0.2)	-1 (0.02)	1 (0.2)	1 (0.2)	1 (0.2)	-1 (0.02)	2.64
5	-1 (0.2)	1 (2)	-1 (0.02)	-1 (0.02)	1 (0.2)	1 (0.2)	1 (0.2)	28.38
6	1 (2)	-1 (0.2)	1 (0.2)	-1 (0.02)	-1 (0.02)	1 (0.2)	1 (0.2)	2.57
7	1 (2)	1 (2)	-1 (0.02)	1 (0.2)	-1 (0.02)	-1 (0.02)	1 (0.2)	24.99
8	-1 (0.2)	-1 (0.2)	-1 (0.02)	-1 (0.02)	-1 (0.02)	-1 (0.02)	-1 (0.02)	2.21

*EY- Enzyme yield (Amount of tyrosine released (mg/ gram of substrate)

Among the variables studied, carbon and nitrogen sources which were found to be significant were further optimised. This is to identify their level at which the maximum yield could be obtained. Carbon source was tested at 0.5% - 2.25% level and for nitrogen, it was 0.5% - 2%. After identifying the level of carbon and nitrogen sources, the effect of other carbon sources (fructose and lactose) and nitrogen sources (peptone, tryptone and soyabean casein digest) in place of the previously used carbon and nitrogen source was studied by one-factor at a time approach. Enzyme from optimized medium was precipitated using 50% ammonium sulphate and dialysed for 10 h against 0.1M Tris- HCl buffer with pH- 5 before using for further studies (**Kamath *et al*., 2010**).

Stability studies and characterization

Effect of temperature, pH, organic solvents and surfactant

A pH range of 3-9 and temperature in the range 20 -70°C were used. The pH stability was determined by pre incubation of extracted protease with buffers in pH range 3-9 for 12 and 24 h. The effect of surfactants was studied using a reaction medium having the extracted enzyme (1 ml) incubated with 0.1 ml of sodium do-decyl sulphate (SDS) and Tween-80 (1% w/v) for 1 h at 4°C and assaying for activity. Ethanol, acetone, dimethyl formamide (DMF) and dimethyl sulfoxide (DMSO) were used at 5% and 10% levels to check the stability. These solvents were added in reaction mixtures containing 1ml enzyme and 5 ml casein solution.

Effects of metal ions and enzyme inhibitors on protease activity

The effect of various metal ions (Ca^{2+}, Mg^{2+}, Fe^{2+}, Cu^{2+}, and Zn^{2+}) at 2 mM level on enzyme activity was next investigated.

Substrate specificity of protease

Bovine Serum Albumin (BSA), gelatin and casein solutions (at 2% w/v level) were used as substrates. The enzyme was incubated with each of these substrates and assayed.

Determination of molecular weight

The molecular weight of the extracted protease was confirmed using zymography (10% resolving gel). Substrate i.e., casein (1% w/v) was added to PAGE and the extracted protein was electrophorosed. After separation was completed, the gel was soaked in triton-X 100 (2.5% v/v) for 20 min. The gel was then stained with 0.1% Coomassie brilliant blue R-250 for 4 h after which it was detained. Enzyme activity was analyzed by incubating the gel in 50 mM glycine HCl buffer pH-5 for 12 h.

Purification of extracted enzyme

Protein present in the supernatant was precipitated using ammonium sulphate at 50% saturation level. The precipitated protein was dialyzed overnight in Tris-buffer (0.1M buffer pH- 5). The dialyzed protein was loaded onto a sephadex G-50 column (1.3x 74 cm) which was previously washed and equilibrated with 0.1 M Tris-HCl (pH 5) and eluted with the same buffer. The flow rate was 0.8 ml/min and 4.0 ml fractions were collected. Each of the collected fractions was checked for presence of protein by measuring the absorbance at 280 nm. The fractions showing the presence of protein was analyzed for protease activity using the standard assay procedure described earlier. The fraction showing protease activity was lyophilized and the molecular weight was identified using SDS-PAGE by comparing with the standard protein ladder.

Mutation studies

Initially the organism was subjected to natural selection. A spore suspension in the range 1-10 x 10^6 cfu was prepared in phosphate buffer (pH 7.0) and plated on PDA medium. The plates were incubated for 72 h at 27 °C. Colonies having different morphological features were selected, transferred to PDA slants and incubated. These cultures were tested for their enzyme producing capacity by measuring the tyrosine released. The natural selectant yielding maximum product was used for UV irradiation. Two ml of spore suspension was transferred to an 80 mm Petri dish and exposed to UV rays for 8 min at distance of 26 cm from a UV lamp (Philips, 254 nm). The suspension was then plated using PDA medium and incubated for 72 h at 27 °C. The survivors from plates having 99 % kill were transferred to PDA slants and incubated. The strains were then tested for enzyme activity (**Zambare, 2010, Ellaiah *et al*., 1986**).

RESULTS AND DISCUSSION

Optimization of production conditions

The fungal growth in culture medium was a mixture of mycelium and pellets. The fractional factorial study showed that casein, Peptone, KNO_3, Co^{2+}, Ca^{2+} and Mg^{2+} had positive effect on enzyme yield i.e., addition of these parameters improved the enzymes yield. The remaining factors i.e., soluble starch, Fe^{2+} and K^+ had negative effect i.e., presence of these factors reduced the enzyme yield. Among all the 7 factors tested the combination of nitrogen sources had maximum effect on enzyme yield. More than 97% of the protease yield was dependent on it (Table 2).

Table 2 Effect and percentage contribution of each factor on protease yield

Term	Effect	Coefficient	Sum of squares	% contribution
Soluble starch	-1.5430	-0.7715	4.7617	0.485548
Casein, Peptone, KNO_3	21.8210	10.9105	21.821	97.1068
K_2HPO_4, KH_2PO_4	-0.0670	-0.0335	-0.067	9.15 x 10^{-4}
$MgSO_4$	0.0610	0.0305	0.007442	7.59 x 10^{-4}
$CaCO_3$	2.3750	1.1875	11.2813	1.15034
$FeSO_4$	-0.4670	-0.2335	0.436178	0.0444768
$CoSO_4$	2.4370	1.2185	11.8779	1.21119

Soluble starch, combination of nitrogen sources, Co^{2+} and Ca^{2+} had were found to be significant factors affecting enzyme yield (Table 3).

Table 3 Significance of each factor on protease yield

Source	Sum of Squares	Degree of freedom	Mean Square	F value	p-value Prob > F
Soluble starch	980.24	5	196.0	883.86	0.0011
Casein, Peptone, KNO3	4.76	1	4.76	21.47	0.0436
K2HPO4, KH2PO4	8.978 x 10^{-3}	1	8.978 x 10^{-3}	0.040	0.8592
CaCO3	11.28	1	11.28	50.86	0.0191
CoSO4	11.88	1	11.88	53.55	0.0182
MgSO4	7.442 x 10^{-3}	1	7.442 x 10^{-3}	1.066 x 10^{-3}	0.9755
FeSO4	0.44	1	0.44	0.062	0.8149

Among these four parameters carbon and nitrogen sources were studied further (Table 4).

Table 4- Effect of various combinations of soluble starch and Peptone, Casein and potassium nitrate on enzyme yield

Soluble starch %	Peptone, Casein KNO_3	Yield mg/ gram of substrate
0.5	0.5	10.69
1.25	0.75	11.69
1.0	1.0	18.57
0.75	1.25	21.53
1.5	1.5	23.34
2.25	1.75	34.75
2.0	2.0	34.01

Fig. 1 and Fig. 2 show the effect of various concentrations of these four factors *viz*., soluble starch, combination of nitrogen sources, Co^{2+} and Ca^{2+} ions on enzyme yield.

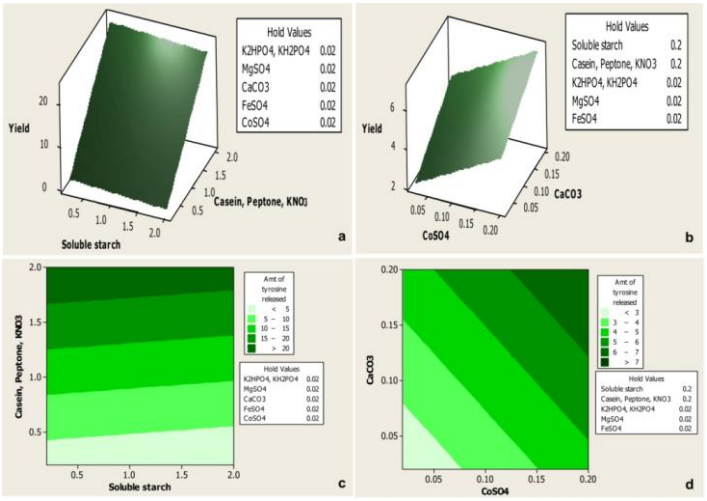

Figure 1 Surface and contour plot showing effect of selected nutrients on enzyme yield. **a**. Surface Plot of yield (Amt of tyrosine) vs Casein, Peptone, Sol starch. **b.** Surface Plot of Amt of tyrosine released vs CaCO3, CoSO4. **c.** Contour Plot of Amt of tyrosine vs Casein, Peptone, Sol starch, **d.** Contour Plot of Amt of tyrosine released vs CaCO3, CoSO4

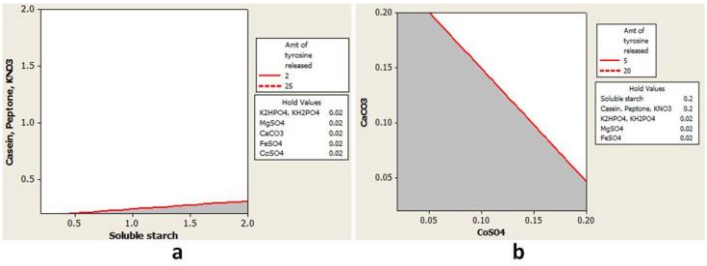

Figure 2 Overlaid contour plot showing effect of selected nutrients on enzyme yield. **a**. Overlaid contour plot showing the effect of various combination of soluble starch and combined casein peptone and potassium nitrogen to obtain an enzyme yield between 2 to 25 U/ml when the remaining nutrient levels are kept at their lowest level. **b.** Overlaid contour plot showing the effect of various combination of $CoSO_4$ and $CaCO_3$ to obtain an enzyme yield between 5 to 20 U/ml when the remaining nutrient levels are kept at their lowest level

The optimum level for carbon source was 2.25%, while for nitrogen sources it was found to be 1.75%. Among organic nitrogen supplements, peptone was most significant producing 28.88 mg of tyrosine/ g of substrate and there was a decrease in yield with tryptone and soyabean casein digest broth sequentially. Carbon source played crucial role in the enzyme production. The yield did not vary much between soluble starch and fructose and based on economic considerations soluble starch was used in enzyme production. SDS-PAGE of the the protein separated using sephadex G that had protease activity showed a single band at 27.5 kDa. Zymography further confirmed that the molecular weight of the acid protease from the present isolated could be 27.5 kDa (Fig. 3).

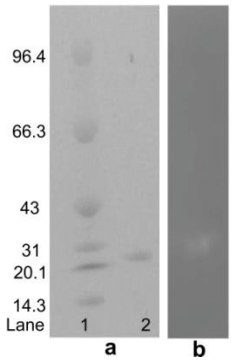

Figure 3 SDS PAGE and Zymogram. **a.** SDS-PAGE of protease purified by gel filtration. Lane 1 represents the standard protein ladder. Lane 2 represents the test sample. **b.** Casein zymogram. The clear band indicates that the substrate i.e., casein was broken into its constituent units by the protease. Therefore, except the portion of the PAGE where the casein was degraded, the remaining portions of the PAGE were stained. This indicates that the acid protease obtained in the present study have a molecular weight of 27.5 kDa in which is similar to the molecular weight observed in lane 2.

Effect of temperature, pH, organic solvents and surfactant

Enzyme stability analysis at varied temperatures showed that the enzyme is a mesophilic protease which remains active at temperatures ranging from 30 to 45 °C (Fig 4a). The enzyme activity was optimum at 40 °C and 96.85% activity was retained even after 1 h incubation (Fig 4b).

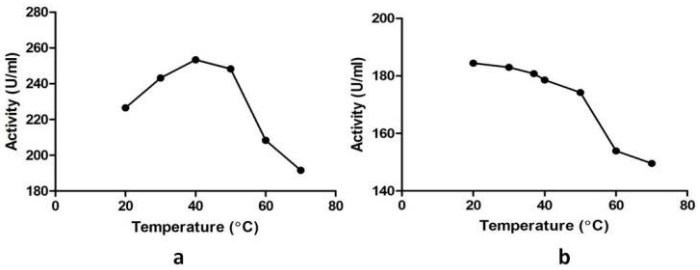

Figure 4 Relationship between temperature and enzyme activity. a. Effect of temperature on enzyme activity. The study showed that protease is mesophilic with maximum activity observed at 37 °C. b. Effect of temperature on enzyme stability. The enzyme was stable at wide temperature range retaining more than 80% of the activity at 70 °C

The effect of pH on enzyme activity was tested in the pH range from 3.0 to 9.0 and optimal pH was found to be 5.0 retaining 46.36 % even after 24 h at pH 5 (Fig 5).

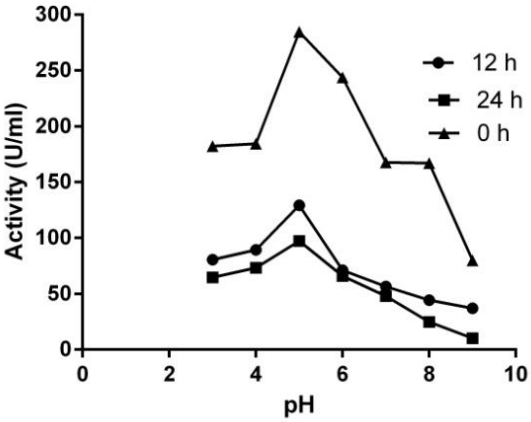

Figure 5 Relationship between pH and enzyme activity. Effect of pH on enzyme stability after 0, 12 and 24 h incubation.

The enzyme retained its activity when solvent was added at 5% level. However when the solvent was increased to 10% level, the activity decreased. The enzyme was quite stable with DMF at 5 % level while there was decrease in activity with DMSO and acetone. In presence of SDS, the enzyme retained 60.71% activity while 49.67% activity was observed with Tween- 80.

Effect of metal ions

Enzyme activity increased proportionately as the concentration of Ca^{2+} ions were increased from 2 mM to 10 mM. While enzyme activity was almost stable in presence of Mg^{2+} ions, it decreased in presence of $Fe^{2+,}$ Cu^{2+} and Zn^{2+} ions.

Substrate specificity of protease

Protease was more specific with casein while it showed only 47.49 % activity with BSA and 26.71 % with gelatin.

Mutagenesis of *Aspergillus niger*

In the present study, the fungal organism, *A.niger*, yielded 29.22 mg of tyrosine/ g substrate from initial batch experiments. The organism exhibited mixed growth of pellets and mycelia in the culture broth having enzyme activity of 11.55 U/ ml. This organism was subjected to natural selection. During natural selection strain NS-05 yielded a maximum of 34.49 mg of tyrosine /g of substrate. This strain showed a pellet form of growth with pellet size of 0.5 - 0.7 cm and enzyme activity of 13.66 U/ml. The natural selectant NS-05 was selected for UV treatment.

In the current study, among the UV exposed strains, UV-08 gave a maximum yield of 40.09 mg of tyrosine/ g substrate with activity 15.77 U/ml. Tyrosine yield was 37.2 % more than that of wild strain. Growth was in pellet form with size of 0.5-0.8 cm. The enzyme was extracted from wild, natural selectant and UV mutants and tested for pH and temperature stability. The respective extracts showed 194.8, 237.4, 255 U/ml activity. The enzyme extracted from UV-08 was more stable than wild strain protease showing 98.85% activity at 50 °C even after 1 h incubation. The stability also increased after 24 h incubation over a wide pH range (pH 3-9) (Fig. 6). Further strain improvement studies were not made as the yield and stability increase were not considerable.

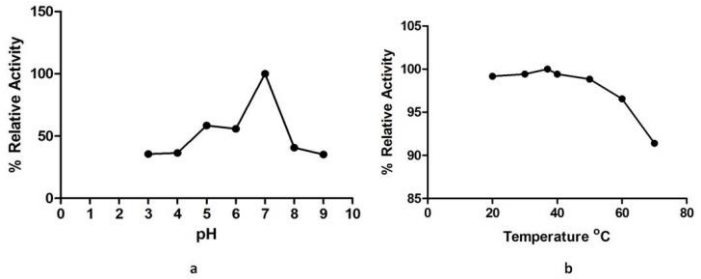

Figure 6 pH and thermo stability of the UV mutant, UV-08. **a**. pH stability of the UV mutant (UV-08). Enzyme activity is expressed in terms of percentage relative activity. pH of the control (7.0) was considered 100% and the activity at other pH was calculated in reference to the control. **b**. Temperature stability of the UV mutant (UV-08). Enzyme activity is expressed in terms of percentage relative activity. Temperature of the control (37 °C) was considered 100% and the activity at other temperatures was calculated in reference to the control.

DISCUSSION

Among the microbial kingdom acid protease are generally produced by moulds belonging to fungal kingdom (**Ramamurthy *et al*., 1991**). *Aspergillus* species are known to produce proteases (**Negi and Banerjee, 2006, Vishwanatha *et al*., 2009**). Factorial study showed that nitrogen and carbon sources played significant role in enzyme yield. Further studies showed that carbon and nitrogen at 2.25 and 1.75 % w/v respectively improved enzyme yield. Among the carbon sources tested soluble starch had maximum influence on enzyme yield. A similar effect of starch on acid protease production has been reported by Siala *et al* (**Siala *et al*., 2012**). Among the nitrogen sources peptone improved the enzyme yield. Protease production in *A. carbonarius* improved appreciably when supplemented with peptone (**Ire *et al*., 2011**). Peptones are obtained by enzymatic digestion of animal proteins and contain high amount of peptides. Proteases break down proteins and peptides. Therefore presence of peptides in the culture medium could induce production of protease (**Siala *et al*., 2012**). Also peptones are known to support rapid growth of moulds (**Yan *et al*., 2012**). Zymogram studies confirmed that extracted acid protease was low in molecular weight. The molecular weight of acid protease from *Aspergillus* species was reported to be in the range of 30 – 90 kDa (**Aalbæk *et al*., 2002, Tsujita and Endo, 1978**).

Among the various metals tested, protease activity increased as the concentration of Ca^{2+} was gradually increased. Increase in activity with increasing Ca^{2+} concentration could be attributed to the ability of Ca^{2+} to stabilize protease structure (**Ghorbel-Frikha *et al*., 2005**). Therefore the higher protease activity in presence of calcium observed in the present study could be due to the stabilization of the enzyme. However calcium was reported to have no effect on protease obtained from *A. niger* I1 strain, While K^{+} ion improved the activity. Also there was a slight reduction in activity when incubated with Mg^{2+} (**Siala *et al*., 2012**). In case of protease from *A. fumigatus* the enzyme activity was reported to be inhibited by divalent cations but stable in presence of monovalent cations (**Monod *et al*., 1991**). In case of *A. flavus*, Mn^{+} and Fe^{2+} increased the yield while Zn^{2+}, Co^{+} and Cu^{2+} inhibited the activity (**Yadav and Darmwal, 2011**). In the present study the extracted protease was stable against wide range of solvents. Enzyme was stable even when it was incubated with SDS (1% w/v) and Tween-80 (1% w/v). Also the enzyme was able to retain activity when ethanol, acetone, DMF and DMSO were added at 5% v/v to the reaction mixture. Protease obtained from *A. fumigatus* and *A. flavus* are reported to be tolerant to organic solvents (**Monod *et al*., 1991, Yadav and Darmwal, 2011**). Depending on the type of solvent system, activity and stability of enzymes are affected due to change in the hydration layer around the protein which results in higher compaction, thereby stabilizing the protein (**Sellek and Chaudhuri, 1999**).

UV irradiation which causes pyrimidine dimerization represents a cost effective method for inducing mutation in microbes (**Rowlands, 1984**). Protease production in *Trichoderma harzianum* was improved through UV mutation. The UV mutant proved to be a better antagonist against plant pathogens (**Szekeres *et al*., 2004**). In the current study UV mutant showed better pH and thermostability. These properties could be useful when the enzyme in employed as seasoning material.

CONCLUSION

The yield of an acid protease from *Aspergillus niger* was improved using statistical design model and mutation technique. The uv mutant, UV-08, was more stable than wild strain protease showing 98.85% activity at 50°C even after 1 h incubation. The stability also increased after 24 h incubation over a wide pH range (pH 3-9). This increase in yield along with its promising application can be made use in commercial production of protease with significant cost reduction.

Acknowledgments: We would like to acknowledge authorities of Manipal University for providing required facility to carry out this work.

REFERENCES

AALBÆK, T., REESLEV, M., ERIKSEN, S. H. 2002. Acid protease and formation of multiple forms of glucoamylase in batch and continuous cultures of *Aspergillus niger*. *Enzyme and microbial technology*, 30**,** 410-415. http://dx.doi.org/10.1016/s0141-0229(02)00006-6

ALNAHDI, H. S. 2012. Isolation and screening of extracellular proteases produced by new Isolated *Bacillus sp*. *Journal of Applied Pharmaceutical Science*, 2**,** 071-074. http://dx.doi.org/10.7324/japs.2012.2915

ELLAIAH, P., PRABHAKAR, T., RAMAKRISHNA, B., THAER TALEB, A., ADINARAYANA, K. 1986. Strain improvement studies on glucose isomerase producing *Streptomyces* sp s-011. *Indian Drugs*, 24**,** 316-318.

FOLIN, O., CIOCALTEU, V. 1927. On tyrosine and tryptophane determinations in proteins. *The Journal of Biological Chemistry*, 73**,** 627-650.

GHORBEL-FRIKHA, B., SELLAMI-KAMOUN, A., FAKHFAKH, N., HADDAR, A., MANNI, L., NASRI, M. 2005. Production and purification of a calcium-dependent protease from *Bacillus cereus* BG1. *Journal of Industrial Microbiology and Biotechnology*, 32**,** 186-194. http://dx.doi.org/10.1007/s10295-005-0228-z

IRE, F. S., OKOLO, B. N., MONEKE, A. N., ODIBO, F. J. C. 2011. Influence of cultivation conditions on the production of a protease from *Aspergillus carbonarius* using submerged fermentation. *African Journal of Food Science*, 5**,** 353-365.

JOHANSSON, B. P., SHANNON, O., BJORCK, L. 2008. IdeS: a bacterial proteolytic enzyme with therapeutic potential. *PloS one*, 3**,** e1692. http://dx.doi.org/10.1371/journal.pone.0001692

KALASKAR V. V., NARAYANAN K, SUBRAHMANYAM VM, RAO J. V, 2012. Partial characterisation and therapeutic application of protease from a fungal species, *Indian Drugs*, 49 42-46.

KAMATH, P., SUBRAHMANYAM, V. M., RAO, J. V., RAJ, P. V. 2010. Optimization of cultural conditions for protease production by a fungal species. *Indian Journal of Pharmaceutical Sciences*, 72**,** 161-6. http://dx.doi.org/10.4103/0250-474x.65017

MONOD, M., TOGNI, G., RAHALISON, L., FRENK, E. 1991. Isoation and characterisation of an extracellular alkaline protease of *Aspergillus fumigatus*. *Journal of medical microbiology*, 35**,** 23-28. http://dx.doi.org/10.1099/00222615-35-1-23

NEGI, S., BANERJEE, R. 2006. Optimization of amylase and protease production from Aspergillus awamori in single bioreactor through EVOP factorial design technique. *Food Technology and Biotechnology*, 44**,** 257-261.

PAREKH, S., VINCI, V. A., STROBEL, R. J. 2000. Improvement of microbial strains and fermentation processes. *Applied Microbiology and Biotechnology*, 54**,** 287-301. http://dx.doi.org/10.1007/s002530000403

RAMAMURTHY, V., UPADHYAY, C. M., KOTHARI, R. M. 1991. An optimized protocol for the preparation and application of acid protease. *Journal of biotechnology*, 21**,** 187-195. http://dx.doi.org/10.1016/0168-1656(91)90270-6

RAUF, A., IRFAN, M., NADEEM, M., AHMED, I., IQBAL, H. M. N. 2010. Optimization of growth conditions for acidic protease production from *Rhizopus*

oligosporus through solid state fermentation of sunflower meal. *International Journal of Agricultural and Biological Sciences*, 1**,** 40-43.
ROWLANDS, R. T. .1984. Industrial strain improvement: mutagenesis and random screening procedures. *Enzyme and microbial technology*, 6**,** 3-10. http://dx.doi.org/10.1016/0141-0229(84)90070-x
SELLEK, G. A., CHAUDHURI, J. B. .1999. Biocatalysis in organic media using enzymes from extremophiles. *Enzyme and microbial technology*, 25**,** 471-482. http://dx.doi.org/10.1016/s0141-0229(99)00075-7
SIALA, R., FRIKHA, F., MHAMDI, S., NASRI, M., SELLAMI KAMOUN, A. 2012. Optimization of Acid Protease Production by Aspergillus niger I1 on Shrimp Peptone Using Statistical Experimental Design. *The Scientific World Journal*, 2012, 1-11. http://dx.doi.org/10.1100/2012/564932
SUMANTHA, A., LARROCHE, C., PANDEY, A. 2006. Microbiology and industrial biotechnology of food-grade proteases: a perspective. *Food Technology and Biotechnology*, 44**,** 211.
SZEKERES, A., KREDICS, L., ANTAL, Z., KEVEI, F., MANCZINGER, L. 2004. Isolation and characterization of protease overproducing mutants of *Trichoderma harzianum*. *FEMS microbiology letters*, 233**,** 215-222. http://dx.doi.org/10.1111/j.1574-6968.2004.tb09485.x
TSUJITA, Y., ENDO, A. 1978. Presence and partial characterization of internal acid protease of *Aspergillus oryzae*. *Applied and environmental microbiology*, 36**,** 237-242.
VISHWANATHA, K. S., APPU RAO, A. G., SINGH, S. A. 2009. Characterisation of acid protease expressed from *Aspergillus oryzae* MTCC 5341. *Food Chemistry*, 114**,** 402-407. http://dx.doi.org/10.1016/j.foodchem.2008.09.070
VISHWANATHA, K. S., RAO, A. G. A., SINGH, S. A. 2010. Acid protease production by solid-state fermentation using *Aspergillus oryzae* MTCC 5341: optimization of process parameters. *Journal of industrial microbiology & biotechnology*, 37**,** 129-138. http://dx.doi.org/10.1007/s10295-009-0654-4
YADAV, S. K. , DARMWAL, N. S. 2011. Oxidant and solvent stable alkaline protease from Aspergillus flavus and its characterization. *African Journal of Biotechnology*, 10**,** 8630-8640.
YAN, S., LIANG, Y., ZHANG, J., LIU, C.-M. 2012. *Aspergillus flavus* grown in peptone as the carbon source exhibits spore density-and peptone concentration-dependent aflatoxin biosynthesis. *BMC microbiology*, 12**,** 106. http://dx.doi.org/10.1186/1471-2180-12-106
ZAMBARE, V. 2010. Strain Improvement of Alkaline Protease from Improvement of Alkaline Protease from *Trichoderma Reesei* MTCC-3929 by Physical and Chemical mutagen. *The IIOAB Journal*, 1**,** 25-28.