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 (Alnabli, 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 (Ramanurthy 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. 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 Ca²⁺. 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

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 10⁷ 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 Ciocalteu’s 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

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

ARTICLE INFO

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 Ca²⁺. 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.
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

<table>
<thead>
<tr>
<th>Std</th>
<th>Soluble starch</th>
<th>Casein, Peptone, KNO3</th>
<th>KH2PO4, K2HPO4</th>
<th>MgSO4</th>
<th>CaCO3</th>
<th>FeSO4</th>
<th>CoSO4</th>
<th>EY*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1 (2)</td>
<td>1 (2)</td>
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<td>-1 (0.02)</td>
<td>1 (0.2)</td>
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<td>1 (0.2)</td>
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<td>-1 (0.02)</td>
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<td>-1 (0.2)</td>
<td>1 (0.2)</td>
<td>1 (0.2)</td>
<td>1 (0.2)</td>
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<td>1 (0.2)</td>
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<td>-1 (0.02)</td>
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<td>1 (0.2)</td>
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</tr>
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<td>5</td>
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<td>1 (2)</td>
<td>-1 (0.02)</td>
<td>1 (0.2)</td>
<td>1 (0.2)</td>
<td>1 (0.2)</td>
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<td>-1 (0.02)</td>
<td>1 (0.2)</td>
<td>2.57</td>
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<td>7</td>
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<td>1 (2)</td>
<td>-1 (0.02)</td>
<td>1 (0.2)</td>
<td>-1 (0.02)</td>
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<td>1 (0.2)</td>
<td>24.99</td>
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<td>-1 (0.2)</td>
<td>-1 (0.02)</td>
<td>-1 (0.02)</td>
<td>2.21</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*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 soyaean 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 10% 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 (Ca2+, Mg2+, Fe3+, Cu2+, and Zn2+) 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 electrophoresed. 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 detanned. 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 (1.3×74 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, Ellahia 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, KNO3, Co2+, Ca2+ and Mg2+ had positive effect on enzyme yield i.e., addition of these parameters improved the enzymes yield. The remaining factors i.e., soluble starch, Fe2+ 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

<table>
<thead>
<tr>
<th>Term</th>
<th>Effect</th>
<th>Coefficient</th>
<th>Sum of squares</th>
<th>% contribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soluble starch</td>
<td>-1.5430</td>
<td>-0.7715</td>
<td>4.7617</td>
<td>0.485548</td>
</tr>
<tr>
<td>KH2PO4, K2HPO4</td>
<td>-0.0670</td>
<td>-0.0335</td>
<td>-0.067</td>
<td>9.15 x 10^-4</td>
</tr>
<tr>
<td>MgSO4</td>
<td>0.0610</td>
<td>0.0305</td>
<td>0.007442</td>
<td>7.59 x 10^-4</td>
</tr>
<tr>
<td>CaCO3</td>
<td>2.3750</td>
<td>1.1875</td>
<td>11.2813</td>
<td>1.15034</td>
</tr>
<tr>
<td>FeSO4</td>
<td>-0.4670</td>
<td>-0.2335</td>
<td>0.436178</td>
<td>0.044768</td>
</tr>
<tr>
<td>CoSO4</td>
<td>2.4370</td>
<td>1.2185</td>
<td>11.8779</td>
<td>1.21119</td>
</tr>
</tbody>
</table>

Soluble starch, combination of nitrogen sources, Co2+ and Ca2+ had been found to be significant factors affecting enzyme yield (Table 3).

Table 3 Significance of each factor on protease yield

<table>
<thead>
<tr>
<th>Source</th>
<th>Sum of squares</th>
<th>Degree of freedom</th>
<th>Mean Square</th>
<th>F value</th>
<th>p-value Prob &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soluble starch</td>
<td>980.24</td>
<td>5</td>
<td>196.0</td>
<td>883.86</td>
<td>0.0011</td>
</tr>
<tr>
<td>Casein, Peptone, KNO3</td>
<td>4.76</td>
<td>1</td>
<td>4.76</td>
<td>21.47</td>
<td>0.0435</td>
</tr>
<tr>
<td>KH2PO4, K2HPO4</td>
<td>8.978 x 10^-3</td>
<td>1</td>
<td>8.978 x 10^-3</td>
<td>0.040</td>
<td>0.8592</td>
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<tr>
<td>CaCO3</td>
<td>11.28</td>
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<td>11.28</td>
<td>50.86</td>
<td>0.0019</td>
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<tr>
<td>CoSO4</td>
<td>11.88</td>
<td>1</td>
<td>11.88</td>
<td>53.55</td>
<td>0.0182</td>
</tr>
<tr>
<td>MgSO4</td>
<td>7.442 x 10^-3</td>
<td>1</td>
<td>7.442 x 10^-3</td>
<td>1.066</td>
<td>9.755</td>
</tr>
<tr>
<td>FeSO4</td>
<td>0.44</td>
<td>1</td>
<td>0.44</td>
<td>0.062</td>
<td>0.8149</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Soluble starch %</th>
<th>Peptone, Casein KNO₃</th>
<th>Yield mg/gram of substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>0.5</td>
<td>10.69</td>
</tr>
<tr>
<td>1.25</td>
<td>0.75</td>
<td>11.69</td>
</tr>
<tr>
<td>1.0</td>
<td>1.0</td>
<td>18.57</td>
</tr>
<tr>
<td>0.75</td>
<td>1.25</td>
<td>21.53</td>
</tr>
<tr>
<td>1.5</td>
<td>1.5</td>
<td>23.34</td>
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<tr>
<td>2.0</td>
<td>2.0</td>
<td>34.01</td>
</tr>
</tbody>
</table>

Fig. 1 and Fig. 2 show the effect of various concentrations of these four factors viz., soluble starch, combination of nitrogen sources, Co²⁺ and Ca²⁺ ions on enzyme yield.

![Figure 1](image1.png)

**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](image2.png)

**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 CoSO₄ and CaCO₃ 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](image3.png)

**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](image4.png)

**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](image5.png)

**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\textsuperscript{2+} ions were increased from 2 mM to 10 mM. While enzyme activity was almost stable in presence of Mg\textsuperscript{2+} ions, it decreased in presence of Fe\textsuperscript{2+}, Cu\textsuperscript{2+} and Zn\textsuperscript{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 enzyme 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 (pH 8-10). pH 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. (2011). Aspergillus niger I1 strain with enzyme activity of 11.55 U/ml. 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. (2011). 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 enzyme 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). 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.

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