Medium Optimization for Acid protease production from *Aspergillus* sps under Solid state fermentation and mathematical modelling of protease activity

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ABSTRACT

Present study reports the production and optimization of process and nutritional parameters for extracellular acid protease from new isolate *Aspergillus* sps under solid state fermentation and modelling of protease activity for a set of process parameters. On the economic feasibility of fermentation, wheat rawa was selected as the main solid substrate for protease enzyme production. The optimal values of factors influencing the production were found to be moisture content 60% (v/w), incubation temperature 32±2°C, inoculum level 10% (v/w), incubation period 5 days and pH 5.0. The nutritional variables such as fructose and chickpea meal enhanced the protease enzyme production. The cost of fermentation was reduced by the replacement of optimized carbon source (fructose) with vegetable waste, potato peel. Linear polynomial models were developed expressing the relationship between acid protease activity and various fermentation factors. This was the first report on the design of batch fermentation media for production and optimization of acid protease in *Aspergillus* sps on wheat rawa as basal medium.

Key words: *Aspergillus* sps, acid protease, media optimization, economy, polynomial model.

INTRODUCTION

Proteases are complex enzymes which are responsible for the hydrolysis of protein molecules [1]. These are the important group of industrial enzymes accounting to 60% of the total worldwide enzyme sales due to their potential industrial applications (Fig. 1) [2-9].
Protease enzymes are classified as acid, neutral and alkaline enzymes based on pH. Proteases of fungal origin have an advantage over bacterial protease as mycelium can be easily removed by filtration. Acid proteases are stable and active in the pH ranges of 2 – 6 and are mainly secreted by several fungal species of *Mucor*, *Aspergillus*, *Penicillium* and *Rhizopus* [4, 10]. Currently enzymes are produced by SmF (submerged Fermentation) and SSF (solid state fermentation). The SSF is currently receiving significant attention as it uses inexpensive substrates and yields higher volumetric productivity [11-14]. A novelty in fermentation medium design is necessary to increase protease enzyme production with low cost solid substrates. However, several reports are available on alkaline protease production and a few reports on acid protease production. The isolated fungal culture *Aspergillus* sps from soil polluted with abattoir waste is able to secrete extracellular acid protease in submerged fermentation [15]. In view of potential applications of acid protease enzyme and economy of production, an attempt was made to produce and optimize the acid protease production from *Aspergillus* sps through solid state fermentation with following objectives

- Screening of suitable wheat substate on economic basis
- Study of effect of process parameters viz., moisture content, pH, tempearture, inoculum level and incubation period
- Optimization of various carbon and nitrogen sources
- Development of mathematical model explaining effects of pH, temperature, moisture content, inoculum size, and incubation period
MATERIALS AND METHODS

Inoculum preparation

The mesophilic fungal culture was isolated from soil contaminated with abattoir waste collected from rural areas of Tirupati, A.P. India. The isolated fungal culture was identified as *Aspergillus* sps based on its morphological and microscopic characteristics and these values matched with values in standard reference book compendium of soil fungi [16]. Further the culture was screened on casein agar medium for protease production. The fungal inoculum was prepared by addition of 10ml of 0.1% triton X-100 solution to the 7th day old slant and was shaken well to obtain homogeneous spore suspension.

Solid state fermentation

The wheat based substrates (10g) viz., wheat bran, wheat rawa, wheat grass powder, and wheat flour were used as main carbon substrates in the present study. It was moistened with 20ml of salt mineral solution (pH 6.0) comprised of: KH$_2$PO$_4$ 1, K$_2$HPO$_4$ 2, MgSO$_4$ 1, CaCl$_2$ 0.1 and ZnSO$_4$ 0.01 and then sterilized. Then each flask was inoculated with 20% (v/w) spore suspension of *Aspergillus* sps and incubated at room temperature for 5 days. Crude protease was extracted by the addition of 50ml of various solvents viz., water, and 50% aqueous mixtures of various solvents such as ethanol, methanol and amyl alcohol to fermented solids and then, contents were mixed at speed 150 rpm at room temperature for 1 hour. Later, the contents of the flasks were filtered using Whatman No.1 filter paper and the filtrate was used for the assay of crude protease enzyme by modified Anson’s method [17]. Protein content was analyzed by Lowry’s method [18]. The mycelia dry weight was determined according to method reported by [19]. A unit of protease activity (U) was defined as the amount of enzyme liberating µg tyrosine per ml of enzyme per min of incubation time. The protease activity was reported enzyme unit per gram of solid substrate, (U g$^{-1}$). All the solid state fermentations were conducted in duplicate in 250ml Erlenmeyer flasks and the results were the mean and standard deviation of two trials. Wheat rawa and distilled water were fixed as main substrate and extraction solvent in further experiments.

Effect of process parameters

The effect of various parameters on protease enzyme production was studied. The impact of inoculum density was employed by inoculating production media with a range of 0.5 – 5ml of spore suspension. The effects of temperature and incubation time on protease production were studied by conducting the bioprocess at temperature range 25 - 40°C and incubation period range of 3 – 9 days. To investigate the influence of initial moisture content, the step was performed with the range of initial moisture content (50 to 90 % (v/w)). The influence of initial pH of medium was conducted on enzyme production with pH range 3 – 11.

Effect of various carbon and nitrogen sources:

To enhance the acid protease production, wheat rawa was supplemented with one gram of various carbon sources viz., glucose, galactose, fructose, dextrose, sucrose, lactose, cellulose, starch. Further fermentation medium was designed with one gram (10% (w/w)) of various nitrogen sources such as soya bean meal, corn meal, skim milk powder, lobia white meal, chick pea meal, yeast extract, tryptone, peptone, sodium nitrate, potassium nitrate and ammonium sulphate. To utilize the waste and to lower the cost of
fermentation, the study was performed using one gram of fruit and vegetable waste such as pomegranate fruit peel, mango fruit peel, carrot peel and potato peel in place of fructose.

**RESULTS AND DISCUSSION**

**Effect of various wheat substrates on acid protease production**

Present experimental study reported the design of low cost fermentation medium required for acid protease production by conventional method (OFAT: one factor at a time). The crucial step in SSF is the selection of suitable solid substrate since it acts as support to cells and also supplies nutrients to biomass [20]. Protease production was initiated with agro industrial substrates viz. wheat grass, wheat rawa, and wheat flour and wheat bran. All the tested substrates supported both fungal growth and protease enzyme production. Maximum protease activity (230.15 U g\(^{-1}\)) was obtained with an expensive substrate, wheat grass, while minimum enzyme production (4.04 U g\(^{-1}\)) was observed with wheat bran and wheat flour (Fig. 2) and moderate enzyme production (133.45 U g\(^{-1}\)) was noted with wheat rawa. With a view to design low cost medium, wheat rawa was selected as main substrate for acid protease production from *Aspergillus* sps. Wheat bran, wheat flour and wheat rawa were used as solid substrates for alkaline protease production by *T. thalpophilus* PEE 14 [21]. In the present study, it was observed that higher wheat bran (10 g) may be inhibitory to protease enzyme yield.

![Fig. 2. Effect of various wheat materials on protease production](image)

Similarly, [22] reported that the presence of larger amounts of wheat bran did not improve protease production.

**Influence of extracting solvent**

The effect of different solvents on protease extraction was listed in Table 1.
Table 1: Effect of extraction solvent on protease activity

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Protease activity, U g(^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water (50ml)</td>
<td>133.14±7.21</td>
</tr>
<tr>
<td>50% Ethanol</td>
<td>124.71±3.02</td>
</tr>
<tr>
<td>50% Methanol</td>
<td>140.71±7.81</td>
</tr>
<tr>
<td>50% Amylalcohol</td>
<td>149.15±8.89</td>
</tr>
</tbody>
</table>

Values are mean ± SD (Standard Deviation)

Highest protease activity (149.41 U g\(^{-1}\)) was noticed with 50% aqueous mixture of amylalcohol while 90% of this value (133.41 U g\(^{-1}\)) was obtained with distilled water. On economic feasibility of fermentation, distilled water was chosen as suitable solvent for the extraction of crude protease from fermented solids.

**Effect of inoculum level on protease production**

The dependency of protease activity on inoculum level was depicted in Fig. 3.

A gradual increase in protease activity was observed for inoculum level in the range of 0.5 – 1.0 ml and was reduced significantly. The optimum inoculum ratio was found to be 10% (v/w) with the maximum enzyme production (141.45 U g\(^{-1}\)). It was observed that the highest inoculum level (5.0 ml) suppressed protease activity (13.95 U g\(^{-1}\)). The optimal value of inoculum size was in good agreement with general industrial inoculum size range (1 to 10%) [23].

**Influence of incubation period**

The enzyme production increased linearly from 72 to 120h of fermentation time but further increased period showed reduction of enzyme activity (Fig. 4). These results were in accordance with reports of [10]. The decrease in enzyme productivity after 120h of incubation time was due to the inactivation of protease by other constituent proteases.
Optimization of temperature
Maximum enzyme production (148 U g\(^{-1}\)) was observed at temperature of 32±2\(^{0}\)C while minimum protease production (88 U g\(^{-1}\)) was noticed at 40\(^{0}\)C (Fig.5).

The producing strain was sensitive to temperature above and below the optimum. The optimum temperature indicated that isolated Aspergillus sps was mesophilic fungi. Similarly, several authors have reported that the suitable incubation temperature for protease enzyme from Aspergillus sps was in the range 31- 35\(^{0}\)C [7, 10, 24].

Effect of initial substrate moisture content
The availability of water in the substrate can influence the nutrient accessibility to fungal strain and oxygen diffusion. The natural moisture content of wheat rawa is too low to support the
fungal growth as well as enzyme production. Therefore the initial moisture content of substrate was adjusted with salt and mineral solution as 50 to 90 % (v/w) and the results were given in Table 2. The initial moisture level of 60% yielded the peak acid protease activity 154.39±12 while the low enzyme activity obtained with control (135.21 U g\(^{-1}\) ). [22] reported that the optimum initial moisture level for combination of wheat bran and rice bran is about 50% for protease production using Aspergillus oryzae. Results of present study revealed that a slightly higher moisture level (60%) was effective for wheat rawa.

Table 2: Effect of initial moisture content for protease production

<table>
<thead>
<tr>
<th>Moisture content, % (v/w)</th>
<th>Protease activity, U g(^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>135.21±5.23</td>
</tr>
<tr>
<td>50</td>
<td>143.12±11.47</td>
</tr>
<tr>
<td>60</td>
<td>154.58±3.36</td>
</tr>
<tr>
<td>70</td>
<td>131.21±15.53</td>
</tr>
<tr>
<td>80</td>
<td>104.21±14.67</td>
</tr>
<tr>
<td>90</td>
<td>99.48±8.17</td>
</tr>
</tbody>
</table>

* Values are mean ± SD (Standard Deviation)

These results were in accordance with observations made by [6] on wheat bran.

**Role of initial pH**

In shake flasks, it is difficult to control the pH of medium during fermentation, hence only initial pH of fermentation medium was studied to monitor the protease production in pH range 3 – 10 (Fig. 6). Maximum production was noticed at pH 5.0 with an activity of 166.72 U g\(^{-1}\). Evaluation of data at different pH conditions indicated that pH 4 to 5 was suitable for protease production from Aspergillus sps and revealed the producing fungal strain was able to secrete large amounts of extracellular acid protease. Similar report was made by [15] for protease production from the same fungal culture in submerged fermentation.

![Fig. 6: Effect of initial pH of fermentation medium](image-url)
Optimization of carbon and nitrogen sources
The protease activity can be enhanced with supplementing the media with one gram of various carbohydrates. Results indicated that maximum protease activity of 287.89 U g\(^{-1}\) was obtained for monosaccharide, fructose (Fig. 7).

Fungal culture was able to utilize most of the tested carbon sources except cellulose. The activity of protease with disaccharides (sucrose and lactose) and with polysaccharides (starch and cellulose) was same as that of control. Optimum concentration of fructose was found to be 10% (w/w) (Table 3).

<table>
<thead>
<tr>
<th>Concentration of fructose, %(w/w)</th>
<th>Protease Activity, U g(^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>286.92±11.65</td>
</tr>
<tr>
<td>2</td>
<td>240.64±15.57</td>
</tr>
<tr>
<td>3</td>
<td>199.23±16.01</td>
</tr>
</tbody>
</table>

*Values are mean ± SD (Standard Deviation)*

Further medium was improvised by the addition of one gram of nitrogen source. Among all the tested nitrogen sources, maximum protease activity of 657.51 U g\(^{-1}\) was obtained with tryptone. Almost the same product yield was obtained with the cheap material, chick pea meal (Fig. 8).

Chick pea meal was equally effective to tryptone the optimization of haloalkaliphilic protease by an Extremophile-\textit{Halobacterium} Sp. Js1 [25]. It was observed that all the inorganic nitrogen sources (sodium nitrate, potassium nitrate and ammonium sulphate) were found to be yield protease activity in the range of 550 to 615 U g\(^{-1}\). Further, acid protease enzyme activity was enhanced with optimum value of chick pea meal (Table 4).
Fig. 8. Effect of various nitrogen sources on protease activity

Table 4: Optimization of chick pea meal concentration

<table>
<thead>
<tr>
<th>Concentration of chick pea meal, % (w/w)</th>
<th>Protease Activity, U g⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>647.51±30.5</td>
</tr>
<tr>
<td>2</td>
<td>616.76±26.2</td>
</tr>
<tr>
<td>3</td>
<td>530.61±40.14</td>
</tr>
</tbody>
</table>

*Values are represented as mean ± SD (Standard Deviation)*

Table 5: Replacement of fructose with fruit and vegetable waste

<table>
<thead>
<tr>
<th>Co-carbon source (10% (w/w))</th>
<th>Protease activity, U g⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (fructose)</td>
<td>647.5±30.40</td>
</tr>
<tr>
<td>Mango peel</td>
<td>480.87±44.67</td>
</tr>
<tr>
<td>Pomegranate peel</td>
<td>620.47±64.65</td>
</tr>
<tr>
<td>Carrot peel</td>
<td>568.7±35.59</td>
</tr>
<tr>
<td>Potato peel</td>
<td>717.53±28.83</td>
</tr>
</tbody>
</table>

*Values are mean ± SD (Standard Deviation)*

Low cost fermentation medium was designed by replacing fructose with one gram of different fruit and vegetable waste (Table 5). Higher protease activity of 717.53±35 U g⁻¹ was achieved with potato peel. Among tested fruit and vegetable waste, the protease activity of carrot and mango peel was lesser than that of fructose. The best inducer was found to be potato peel. All these waste products did not show any inhibitory effect on enzyme production. [26] noted that potato peel was the best substrate for alkaline protease production from Bacillus subtilis.
Polynomial models for protease activity

The experimental data on moisture content (Table 2), inoculum size (Fig. 3), incubation period (Fig. 4), temperature (Fig. 5) and pH (Fig. 6) were used to formulate the following polynomial models using MATLAB (R2008b) 7.7.0471:

\[ PA (pH) = -0.036(pH)^5 + 1.41(pH)^4 - 18.87(pH)^3 + 101.383(pH)^2 - 183.792 pH + 138.331 \]  
\[ PA (T) = -0.019(T)^3 + 1.113(T)^2 - 11.87 T + 1 \times 10^{-4} \]  
\[ PA (t) = 3.335 (t)^3 - 65.418(t)^2 + 388.984 t - 610.456 \]  
\[ PA (I) = -0.1092I^5 - 1.984I^4 + 38.308I^3 - 191.579I^2 + 328.62I - 39.845 \]  
\[ PA (M) = -0.0257 M^2 + 2.245M + 101.9843 \]

where \( PA \) : Protease activity in \( U \  g^{-1} \), \( T \) : Temperature in \( ^0C \), \( t \) : Incubation time in days, \( I \) : Inoculum size in ml, \( M \) : Moisture content in %.

The polynomial models (Eq. 1 to 5) were found to describe the most of experimental data obtained on protease activity except for moisture content and incubation time. In a similar way, [27] have derived polynomial representations for thermostable amylase production.

CONCLUSION

The production of acid protease from \textit{Aspergillus} sps was studied on wheat rawa in solid state fermentation. The optimum combination of inexpensive main solid substrate, wheat rawa, and potato peel reduced the cost of fermentation. The protease activity was enhanced by optimization of various fermentation parameters and it was modelled by polynomial models. This study was the first report on batch fermentation of \textit{Aspergillus} sps using wheat rawa.

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REFERENCES


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