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Research Article

Optimization of Culture Conditions and Inducers for Improved Protease Production by *Penicillium griseofulvum* LCJ231 under Submerged Fermentation

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ABSTRACT

The production of protease by *Penicillium griseofulvum* LCJ231 under submerged fermentation was studied with an objective to improve the production through medium optimization. Important nutritional and physical parameters were optimized for maximizing the protease production. The most suitable carbon source, nitrogen source and inducer for maximizing the protease production were studied. It was found that starch, yeast extract and casein were the suitable carbon source, nitrogen source and inducer respectively. The present study also explored the utilization of several agro-wastes as low-cost natural inducers for protease production. The addition of black gram husk as an inducer successfully enhanced the protease production (145.12 U/mL). Maximum production of the protease enzyme was found in the culture medium with initial medium pH of 8 and 2 g/L of inoculum. The results obtained in the present study demonstrate the potential use of the cheap and abundantly available black gram husk for the induction of proteases and thus offer a new approach for industrial enzyme production.

Keywords: Protease; Submerged fermentation; Medium optimization; *Penicillium griseofulvum*

[I] INTRODUCTION

Enzymes are biocatalysts produced by living organisms to bring about specific biochemical reactions which form a part of the cellular metabolism. Enzymes are exploited in the detergent, food, pharmaceutical, diagnostics, and fine chemical industries. Today, more than 3000 different enzymes have been identified of which several of them find application in biotechnology and industries [37]. Even small improvements in biotechnological enzyme production processes are considered significant for commercial production and process [36].

Microbial proteases are extracellular enzymes that catalyse proteolysis by hydrolysis of the peptide bonds that link amino acids together in the polypeptide chain. Proteases are the most valuable commercial enzymes and account for 60% of the total enzyme market [34]. Microbial

proteases are widely used in several industrial sectors such as food, detergent, pharmaceutical, chemical, leather, textile, silk, waste treatment and agriculture industries [5,12,20,29,44].

Large scale production of proteases in industries is mainly carried out under submerged fermentation, where enzyme recovery is a very simple process [38]. Under submerged fermentation, a number of conditions have been described to stimulate proteolytic enzyme production. These conditions include composition of the medium, type and concentration of carbon and nitrogen sources and process parameters such as pH, temperature and agitation speed [11,16,23,32].

The nitrogen source in the production medium affects the growth and extracellular protease production [6,7]. Protease production is also influenced by inducer substrates. Certain proteases are produced and secreted only in the presence of proteins in the medium [2].

The optimization of a suitable fermentation medium is a critical factor as it significantly influences the yield of proteases. Generally the optimization process starts with the conventional (one-factor-at-a-time) method. In this method the medium components are optimized by modifying one-factor-at-a-time while keeping the other variables as constant [13,28]. Conventional optimization method is simple and helps in the selection of significant factors enhancing protease production.

Formulation of a cost-effective fermentation medium is considered important. Thirty to forty percent of the cost involved in the production of industrial enzymes depends on the cost of the growth medium [17,19,39]. Utilization of cheap material like agro-waste as an inducer in the liquid media is an alternative approach for protease production which reduces the expense [1,35].

The present study deals with the optimization of nutritional and physical conditions for the production of extracellular proteases by *Penicillium griseofulvum* LCJ231 under submerged fermentation using one-factor-at-a-time method.

[II] MATERIALS AND METHODS

2.1 Fungal strain

Protease producing fungi *Penicillium griseofulvum* LCJ231 (Accession no. KF414683) which was isolated from cotton seed oil cake [15] was used in this study. The culture was grown and maintained in Potato Dextrose Agar (PDA) at 4°C and sub- cultured at regular intervals.

2.2 Assay for protease activity

The protease activity was assayed using casein as the substrate by the modified method of Keay and Wildi [18]. The reaction mixture contained 200 µL of crude enzyme extract, 500 µL of casein (0.5%) and 300 µL of 0.2 M Glycine-NaOH buffer (pH 9.0). The reaction mixture was incubated at room temperature for 10 min and the reaction was arrested by the addition of 1 mL of 2.5% trichloroacetic acid. The reaction mixture was then centrifuged at 8000 rpm for 15 min. To 1 mL of supernatant, 5 mL of 0.4 M Na_2CO_3, 1 mL of 3-fold diluted Folin and Ciocalteau's phenol reagent were added. The solution was incubated at room temperature for 30 min and the absorbance of the blue colour developed was read at 660 nm [21]. Tyrosine was used as the standard. One unit of enzyme activity was defined as the amount of enzyme that liberated 1 µg of tyrosine from substrate (casein) per minute under assay conditions.

The enzyme activity was calculated as follows.

$$\text{Protease activity (U/mL)} = \frac{(\mu\text{mole tyrosine equivalent released x total volume of assay})}{0.2 \times 10 \times 4}$$

10 = Reaction timing (in min)
0.2 = Volume of enzyme used (in milliliters)
4 = Volume used in colorimetric determination (in milliliters)

2.3 Optimization of culture conditions for protease production

Penicillium griseofulvum LCJ231 was grown in the culture medium described by Namasivayam *et al.* [25]. The influence of carbon source, nitrogen source, inducers, pH and inoculum size on the production of protease was studied by modifying the medium. In all the experiments the un-optimised medium served as the control.

The protease production was carried out in Erlenmeyer flask (250 mL) containing 100 mL of the production media inoculated with one mycelial disc (4 mm). The flasks were maintained at room temperature for 10 days on an orbital shaker (120 rpm). The culture medium was harvested after an interval of 24 hrs and centrifuged at 7000 rpm for 15 min to obtain the supernatant. This served as the enzyme source and the protease activity was assayed.

2.3.1 Effect of carbon sources and their concentrations on the production of protease

The influence of different carbon sources on protease production by *Penicillium griseofulvum* LCJ231 was studied. The carbon source in the medium was replaced with different carbon sources such as galactose, lactose, maltose, starch and sucrose at a concentration of 10 g/L and the protease was assayed every alternate days. The best carbon source was selected and the effect of varying concentrations (5 to 30 g/L) was also investigated. The carbon source which supports high yield of protease production by *Penicillium griseofulvum* LCJ231 was chosen for further study.

2.3.2 Effect of nitrogen sources and their concentrations on the production of protease

The influence of different nitrogen sources was determined by supplementing both organic nitrogen sources (beef extract, peptone, yeast extract) and inorganic nitrogen sources (ammonium chloride, ammonium sulphate and sodium nitrate) at a concentration of 5 g/L to the production medium and the protease enzyme was assayed every alternate days. The best nitrogen source was selected and the influence of various concentrations (5 to 30 g/L) was studied to maximize the protease production.

2.3.3 Effect of inducers and their concentrations on the production of protease

The effect of various inducers on protease production by *Penicillium griseofulvum* LCJ231 was studied. The production medium was supplemented with different inducers such as bovine serum albumin (BSA), egg albumin, casein and gelatin at a concentration of 20 g/L and its effect was tested. Also, the effect of varying concentrations (5 to 30 g/L) of the best inducer on protease production was studied. The un-optimized medium served as a control. The best inducer which supports high yield of protease production by *Penicillium griseofulvum* LCJ231 was chosen for further study.

The influence of various natural inducers such as groundnut oil cake, mahua oil cake, sesame oil cake, black gram husk, green gram husk and red gram husk on the production of protease was studied at a concentration of 20 g/L. This study was carried out by replacing the chemical inducers with natural inducers in the liquid medium for enhancing protease production.

2.3.4 Effect of initial pH on the production of protease

The effect of initial medium pH on protease production by *Penicillium griseofulvum* LCJ231 was evaluated. The medium pH was adjusted from 4 to 10 using 0.1N HCL and 0.1N NaOH. The flasks were inoculated and incubated on an orbital shaker (120 rpm) at room temperature. The protease activity was determined on alternate days.

2.3.5 Effect of inoculum size on the production of protease

The effect of inoculum size on the production of protease by *Penicillium griseofulvum* LCJ231 was studied. The production medium was inoculated with different mycelial discs of various sizes ranging from 1 to 6 g/L. The inoculated flasks were incubated on an orbital shaker (120 rpm) at room temperature.

Statistical analysis

The obtained data were statistically analyzed and expressed with one way analysis of variance (ANOVA). The statistical analysis was carried out using SPSS 11.5 software.

[III] RESULTS AND DISCUSSION

Optimization of medium components plays an important role in improving the enzyme yield which makes the enzyme production cost-effective and economically feasible. Similarly, Choudhary and Jain [9] also optimized culture conditions for increased protease production. Moon and Parulekar [22] reported that the production of protease was significantly

promoted by the culture conditions and they also reported that each microorganism needed different medium components for protease production. Similarly, Kumar and Takagi [20] also reported that each microorganism has its own conditions for maximizing the enzyme production.

3.1 Effect of carbon sources and their concentrations on the production of protease

The influence of different carbon sources on the production of protease by *Penicillium griseofulvum* LCJ231 was studied. The production medium supplemented with different carbon sources was employed to study their effect on the protease production. Among the different carbon sources studied, maximum protease production (91.22 U/mL) was observed in the culture medium supplemented with starch which significantly ($p<0.05$) stimulated the protease production (Table 1). Palanivel *et al.* [30] reported that using starch as the carbon source resulted in maximum protease production by *Aspergillus* strain KH17. However, there was also a report that monosaccharides are known to influence the enzyme production [42]. Wang and Lee [45] reported that maximum protease production was observed when the medium was supplemented with glucose in *Aspergillus niger*. Similiarly Chellapandi [8] reported that fructose and glucose proved to be the best carbon sources for improving the productivity of protease from *Aspergillus flavus* and *Aspergillus terrus*. The study thus proves the uniqueness of each fungi towards their carbon requirement.

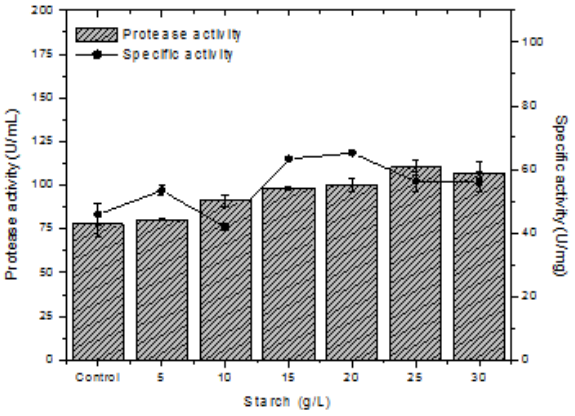

Fig. 1 Effect of various concentrations of starch on protease production by *Penicillium griseofulvum* LCJ231 on the peak day

The effect of various concentrations of starch ranging from 5 to 30 g/L on the protease production by *Penicillium griseofulvum* LCJ231 was also studied. Maximum protease production (110.27 U/mL) was obtained in fermentation medium with 25 g/L of starch (Figure 1) at 5% level of significance. Likewise, in a previous report, Palanivel *et al*. [30] obtained maximum protease production by *Aspergillus* strain KH17 with 1% of starch.

Table 1: Effect of Carbon and Nitrogen sources on the production of protease by *Penicillium griseofulvum* LCJ231

Factors	Protease activity (U/mL)
Control	77.18±0.57
Carbon sources (10g/L)	**(p 0.001**)**
Galactose	79.40± 3.21^{cd}
Lactose	23.51± 8.75^{a}
Maltose	73.00± 1.16^{cd}
Sucrose	57.26± 7.29^{bc}
Starch	91.22± 0.28^{d}
Nitrogen sources (5g/L)	**(p 0.000**)**
Beef extract	113.36± 0.98^{c}
Peptone	86.21± 0.97^{b}
Yeast extract	136.12± 0.50^{c}
Ammonium chloride	25.71± 6.02^{a}
Ammonium sulphate	22.48± 3.20^{a}
Sodium nitrate	5.84± 0.09^{a}

** denotes significance at 1% level; * denotes significance at 5% level; significance between the factors at 5% level using Tukey's multiple comparison test is depicted in the form of alphabets: ± indicates the standard deviation of the three replicates

3.2 Effect of nitrogen sources and their concentrations on the production of protease

The effect of different nitrogen sources on protease production by *Penicillium griseofulvum* LCJ231 was evaluated by incorporating three different organic and three different inorganic nitrogen compounds in the production medium. Maximum protease production of 136.12 U/mL was observed when yeast extract was used as the nitrogen source which was followed by beef extract (113.36 U/mL) and peptone (86.21 U/mL) respectively (Table 1). Nadeem *et al*. [24] reported enhanced protease activity in culture medium containing yeast extract as a nitrogen source. In this study, organic nitrogen sources enhanced the protease production when

compared with the inorganic nitrogen sources. Similar results were reported by Narayana and Vijayalakshmi [26] and Radha *et al.* [33].

Among the nitrogen sources tested, yeast extract induced maximum protease production. The effect of various yeast extract concentrations ranging from 5 to 30 g/L was tested on the production of protease by *Penicillium griseofulvum* LCJ231. Addition of yeast extract at a concentration of 15 g/L significantly ($p<0.05$) enhanced maximum protease activity (148.63 U/mL) as shown in Figure 2. Nehra et al., [27] reported that casein along with yeast extract was found to be the best nitrogen source for the production of alkaline protease under submerged condition for *Aspergillus* sp. In the present study, the production of protease was gradually decreased when yeast extract concentration was increased above 15 g/L.

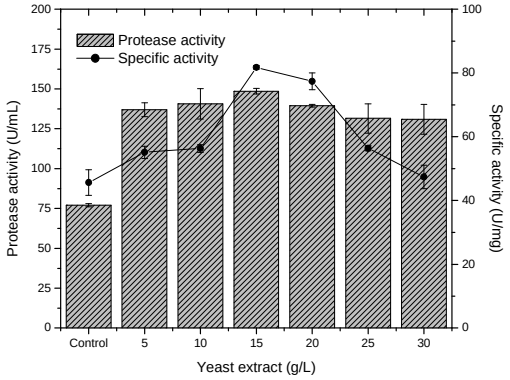

Fig. 2 Effect of various concentrations of yeast extract on protease production by *Penicillium griseofulvum* LCJ231 on the peak day

3.3 Effect of inducers and their concentrations on the production of protease

Influence of different inducers on the production of protease by *Penicillium griseofulvum* LCJ231 was evaluated. In this study, protease production was significantly ($p<0.05$) enhanced when casein was used as an inducer with 126.01 U/mL of protease activity and the results are presented in Table 2. Generally, casein is a protein rich substrate, which supports high protease production. Nehra *et al*. [27] also reported that casein was the most suitable inducer for protease production by *Aspergillus oryzae*.

The concentration of casein at various levels (5 to 30 g/L) was also evaluated. Casein at a concentration of 15 g/L significantly ($p<0.05$) yielded the maximum amount of protease (125.40 U/mL) as shown in Figure 3. Niyozima and More [28] reported maximum protease production at a casein concentration of 1.5%. In another study, 2% of the casein was reported as the best inducer for protease production by *Conidiobolus* species [41].

The influence of different natural inducers on the production of protease was studied. Addition of black gram husk as a natural inducer into the medium supported high amount of protease (145.12 U/mL) and it was significant ($p<0.05$). This was followed by groundnut oilcake, red gram husk, mahua oilcake supported high protease production (144.57 U/mL, 135.77 U/mL and 129.59 U/mL) respectively (Table 2). Natural inducers showed best response by producing increased amount of protease by *Penicillium griseofulvum* LCJ231 when compared to chemical inducers when they replaced chemical inducers. Black gram husk contains rich amount of proteins. These proteins are very helpful for the protease production. Bhattacharya *et al.* [3] used mahua flowers as a low cost natural inducer for protease production. Likewise, soy cake was found to be the best agro-based inducer for protease production [4,31].

Table 2: Effect of Chemical and Natural inducers on the production of protease by *Penicillium griseofulvum* LCJ231

Factors	**Protease activity (U/mL)**
Chemical inducers (20 g/L)	**(p 0.000**)**
BSA	84.69± 0.19[a]
Casein	126.01± 4.57[b]
Egg albumin	76.79± 1.25[a]
Gelatin	85.16± 0.07[a]
Natural inducers (20 g/L)	**(p 0.020*)**
Groundnut oil cake	144.57± 9.04[b]
Mahua oil cake	129.59± 8.45[ab]
Sesame oil cake	117.97± 0.57[a]
Black gram husk	145.12± 9.04[b]
Green gram husk	121.68± 0.96[ab]
Red gram husk	135.77± 0.48[ab]

** denotes significance at 1% level; * denotes significance at 5% level; significance between the factors at 5% level using Tukey's multiple comparison test is depicted in the

form of alphabets: ± indicates the standard deviation of the three replicates

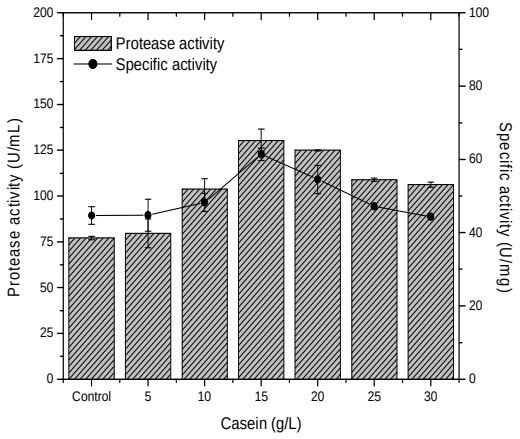

Fig. 3 Effect of various concentrations of casein on protease production by *Penicillium griseofulvum* LCJ231 on the peak day

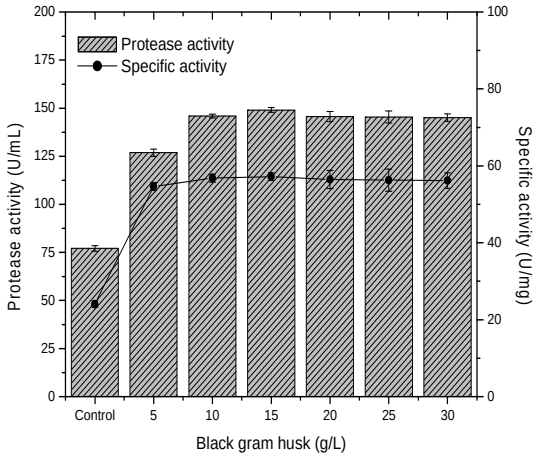

Fig. 4 Effect of various concentrations of black gram husk on protease production by *Penicillium griseofulvum* LCJ231 on the peak day

The effect of different quantity of black gram husk added to the medium was also studied. Results showed that the addition of 15 g/L of the black gram husk induced maximum protease production. Addition above 15 g/L did not result in any further increase in the protease production (Figure 4).

3.4 Effect of initial medium pH on the production of protease

In order to study the effect of pH of the medium for protease production by *Penicillium griseofulvum* LCJ231, experiments were performed with media adjusted to different initial pH ranging between 4 and 10. The maximum enzyme production of protease (148.96 U/mL) was observed at pH 8 and this was significant at 5% and results are represented in Figure 5. The enzyme yield decreased if the medium pH was higher or lower than 8.

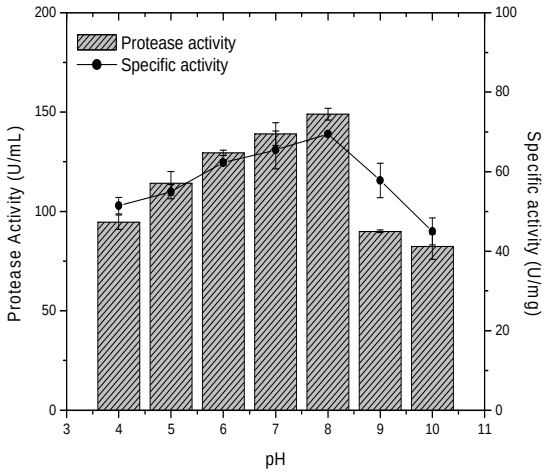

Fig. 5 Effect of medium pH on protease production by *Penicillium griseofulvum* LCJ231 on the peak day

These findings are in accordance with the reports of Sindhu *et al.* [40] and Hossain *et al.* [14] where they found that an initial pH 8 was suitable for enhanced protease production by *Penicillium godlewskii* SBSS25 and *Aspergillus flavus* respectively. Similarly, Hossain *et al.* [14] reported that a pH of 8 supported high yield of protease by *Aspergillus flavus*. Sutar *et al.* [43] also reported that the suitable pH for maximizing protease production by *Conidiobolous coronatus* was pH 8. Thus the result reveals that the pH of the medium strongly affects many enzymatic processes.

3.5 Effect of inoculum size on the production of protease

Size of inoculum is an important biological factor which determines the fungal growth and enzyme production. The influence of inoculum size on the production of protease by *Penicillium griseofulvum* LCJ231 was studied. The fermentation medium was inoculated with varying concentrations of inoculum ranging from 1 to 6 g/L. Maximum enzyme production

was observed when the medium was inoculated with 2 g/L inoculum which had significant effect ($p<0.05$), while increasing inoculum level decreased the protease production in *Penicillium griseofulvum* LCJ231 (Table 3). This was in agreement with the reports of Niyonzima and More [28] and De souza *et al.* [10], who reported that a large inoculum proved to be inhibitory to enzyme activity.

Table 3: Influence of inoculum size on the production of protease by *Penicillium griseofulvum* LCJ231

Factors	Protease activity (U/mL)
Inoculum size (g/L)	**(p 0.001**)**
1	125.19± 6.70^{bc}
2	142.44± 5.63^{d}
3	127.46± 4.46^{c}
4	122.92± 0.57^{bc}
5	114.94± 8.36^{b}
6	85.52± 0.57^{a}

** denotes significance at 1% level; * denotes significance at 5% level; significance between the factors at 5% level using Tukey's multiple comparison test is depicted in the form of alphabets: ± indicates the standard deviation of the three replicates

CONCLUSION

The production of protease by *Penicillium griseofulvum* LCJ231 was significantly increased several folds using selected nutritional and physical parameters. In the present study, cheap and easily available agro-waste, black gram husk was used as a natural inducer and yielded maximum protease production than the chemical inducer. Only few reports are available on the utilization of cheap agro-waste as an inducer for protease production. This study proves that black gram husk is a cost-effective inducer for protease production.

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