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RESEARCH ARTICLE

EFFECT OF CARBON, NITROGEN SOURCES AND INDUCERS ON PROTEASE PRODUCTION BY *PENICILLIUM CITRINUM* LCJ222

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ABSTRACT

Protease production by *Penicillium citrinum* LCJ222 was studied using the six different basal media. Among them, Medium 6 proved to be the best for protease production. Nutritional factors such as carbon, nitrogen, chemical and natural inducer sources were optimized using the selected medium. The protease production was high when lactose was used as a carbon source and yeast extract as the nitrogen source. Among the inducers, natural inducers enhanced maximum protease production when compared to the chemical inducers. Maximum protease activity was observed with black gram husk followed by red gram husk as natural inducers. In the case of chemical inducers, casein served as the best inducer while other chemical inducers inhibited the growth and protease activity. The culture medium with a pH of 9 maximized the production of protease.

INTRODUCTION

Penicillium species have the ability to secrete industrially important proteases which are stable and withstand various environmental conditions. *Penicillium* sp. is a preferred source of enzymes due to their rapid growth and the limited space requirement for their cultivation. In addition, downstream processing and enzyme recovery are simple and easy (Oztruk *et al*., 2009) with *Penicillium* sp. Several species of *Penicillium* such as *P. chrysogenum, P. restrictum, P. dupontii* (Sharma *et al.,* 1980) and *P. griseoroseum* (Haq *et al.,* 2004) have been reported to produce proteases. The cost of protease production is a major drawback in the utilization of enzymes in different industrial applications. Selection of a suitable fermentation technique and optimization of culture conditions are therefore considered important in enzyme productivity. Submerged fermentation is the best choice for microbial enzyme production (Barredo 2005). Process control and easy recovery of extracellular enzymes are the significant features in submerged fermentation. At present, 90% of the commercial microbial enzymes, including proteases are produced by submerged fermentation (Aguilar *et al.,* 2008). Nearly 30 to 40% of the cost of industrial enzymes depends on the cost of the culture medium (Joo *et al*., 2002). Hence it is important to screen the components of the medium to make the processes cost-effective and economically feasible at a commercial scale. Several agricultural substrates or byproducts that are rich in proteolytic inducers have been successfully used for proteolytic enzyme production in submerged and solid state fermentation (Sandhya *et al*., 2005). Cultural conditions (physical, chemical and nutritional factors) play a major role in the production of extracellular proteases by microbes (Kim *et al*., 1998; Seong *et al*., 2004). Nutritional factors such as the carbon sources, nitrogen sources and inducers significantly influence the proteolytic enzyme production (Akhtar *et al*., 2013). Medium pH is one of the main factors for the protease production. The pH of the fermentation medium has significant effect on the protease production (Al-Shehri, 2004). Fungal proteases are active over a wide pH range from 4 to 10. The present study focuses on screening different culture media for maximizing the yield of protease and also focuses on the optimization of different carbon, nitrogen sources, inducers and the medium pH for enhancing proteolytic enzyme production by *Penicillium citrinum* LCJ222.

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MATERIALS AND METHODS

Microorganism

Penicillium citrinum LCJ222 (Accession no. KF414682) (Jenitta and Gnanadoss, 2014) was used for this study. The

fungus was maintained on a Potato Dextrose Agar (PDA) slants and stored at 4 °C and sub-cultured every month.

Culture condition and medium preparation

P. citrinum was cultivated in six different culture media for the production of protease. Medium 1: (g/L) Glucose - 1, KH_2PO_4 - 1, $MgSO_4$ - 0.2, $CaCl_2$ - 0.2, NaCl - 0.2 (Gradisar *et al*., 2005), Medium 2: (g/L) Malt extract - 1, glucose - 6, yeast extract - 1, peptone - 2, K_2HPO_4 - 0.5, $MgSO_4$ - 0.5, $FeSO_4$ - 0.01 (Srinubabu *et al*., 2007), Medium 3: (g/L) Maltose - 10, yeast extract - 0.4, KH_2PO_4 - 5, NaCl - 2 (Tsuchiya *et al*., 1992), Medium 4: (g/L) Malt extract - 20, fructose - 5, thio urea - 5 mg, KH_2PO_4 - 1, KCl - 1, $FeSO_4.7H_2O$ - 0.02, $MgSO_4.7H_2O$ - 0.05 (Saravanakumar *et al*., 2010), Medium 5: (g/L) Sucrose - 30, KCl - 0.5, $FeSO_4$ - 0.01, $MgSO_4$ - 0.5, K_2HPO_4 - 1, $NaNO_3$ - 2 (Charles *et al*., 2008), Medium 6: (g/L) Yeast extract - 5, peptone -5, glucose - 10, casein - 20 (Namasivayam *et al.,* 2010). Experiments were carried out in 250 mL conical flasks containing 100 mL of sterilized respective media separately. One fungal mycelial disc was used for the inoculation of the flask. The culture containing flasks were then incubated for 10 days on an orbital shaker at 120 rpm at room temperature.

Protease production under different nutritional conditions
Effect of carbon sources and their concentrations on protease production

Protease production was studied separately in liquid medium containing different carbon sources such as glucose, galactose, lactose, maltose, starch and sucrose. The effect of different concentration of the best carbon source in the range of 5 - 30 g/L in the fermentation medium was also evaluated.

Effect of nitrogen sources and their concentrations on protease production

The effect of different nitrogen sources such as yeast extract, beef extract, peptone, ammonium chloride, ammonium sulphate and sodium nitrate on the protease production by *P. citrinum* LCJ222 was also evaluated. The influence of different concentration of the best nitrogen source ranging from 5 - 30 g/L was also examined for maximizing the production of protease.

Effect of inducers and their concentrations on protease production

The effect of various inducers on proteolytic enzyme production was determined using different chemical inducers such as Bovine Serum Albumin (BSA), casein, egg albumin and gelatin and natural inducers namely groundnut oil cake, mahua oil cake, sesame oil cake, black gram husk, green gram husk and red gram husk. The effect of chemical inducer concentrations (0 - 30 g/L) were also tested for maximizing the protease production. Similar studies were carried out by replacing the chemical inducers with natural inducers in the fermentation liquid medium for enhancing protease production.

Effect of medium pH on protease production

The optimum pH for the growth of the isolate was determined by culturing the organism in medium set to pH values ranging from 4 - 10. The effect of medium pH in the range of 4 - 10 on proteolytic enzyme production was also tested.

Comparison study of original and optimized medium for protease production

Protease production by *Penicillium citrinum* LCJ222 in optimized and original medium (Namasivayam *et al.,* 2010) was studied. The optimized production medium contained the following components (g/L): Lactose – 20, Yeast extract – 25, Casein – 10, Black gram husk – 10 in addition to the original medium components. The flasks containing sterilized medium were inoculated with one mycelial disc each and incubated at room temperature on a rotary shaker. The culture supernatant was used to determine the activity of proteolytic enzyme.

Protease enzyme assay

Protease activity was measured by the modified method of Keay and Wildi (1970) with casein as the substrate. 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). The reaction mixture was incubated at room temperature for 10 min and the reaction was arrested by adding 1 mL of 2.5% trichloroacetic acid (Keay and Wildi, 1970). The reaction mixture was then centrifuged at 8000 rpm for 10 min. To the supernatant, 5 mL of 0.4 M Na_2CO_3 and 1 mL of 3-fold diluted Folin Ciocalteau's phenol reagent were added. The solution was incubated at room temperature for 30 min and the absorbance of the blue color developed was read at 660 nm (Lowry *et al*., 1951). Tyrosine was used as a standard. One unit of enzyme activity was defined as the amount of enzyme that liberated 1µg of tyrosine from substrate (casein) per minute under assay conditions.

Protease activity was calculated using following calculation:

$$\text{Protease activity (U/mL)} = \frac{(\mu\text{mole tyrosine equivalent released} \times 7)}{0.2 \times 10 \times 4}$$

7 = Total volume of assay (mL)
10 = Time of assay (mins)
0.2 = Volume of enzyme used (mL)
4 = volume used in colorimetric determination (mL)

Protein determination

Protein was estimated following the method described by Lowry *et al.* (1951).

RESULTS

Effect of different media on protease production

Production of protease was evaluated using six different basal media. The production of protease reached a maximum between the 4^{th} and 6^{th} day of incubation in all six culture media used in this study and declined thereafter. Among the different media studied, Medium 6 enhanced maximum protease production in *P. citrinum* LCJ222 up to 810.6 U/mL (Fig. 1).

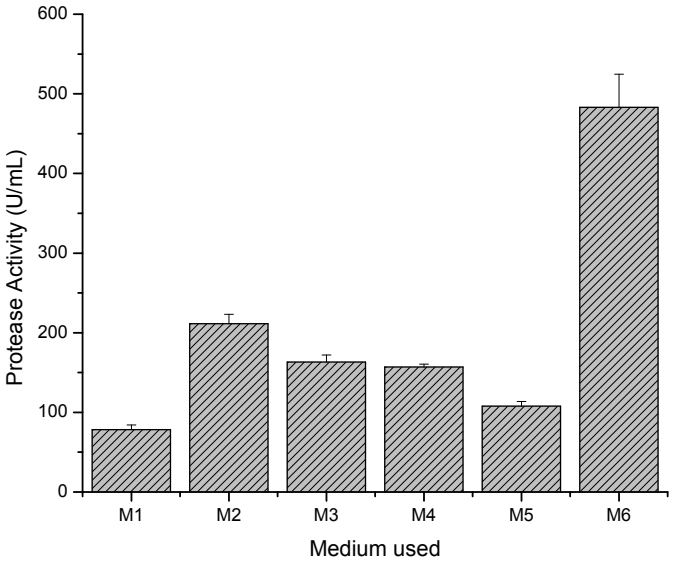

Fig. 1. Effect of different media on protease production by *Penicillium citrinum* LCJ222

(M1- Medium 1, M2- Medium 2, M3- Medium 3, M4- Medium 4, M5- Medium 5, M6- Medium 6)

Effect of nutritional factors on protease production

The protease production in *P. citrinum* LCJ222 varied with different carbon and nitrogen sources used in the experiment (Table 1). Protease activity was highly influenced by the carbon sources. Maximum protease activity was observed with addition of lactose (1277.5 U/mL), followed by starch, sucrose and maltose with a activity of 1271.2 U/mL, 1181.6 U/mL, 1177.4 U/mL, respectively. Among the different concentration of lactose, 20 g/L concentration of lactose enhanced maximum protease activity (2074.1 U/mL) (Table 2). Protease production by *P. citrinum* LCJ222 was evaluated using media containing different nitrogen sources. The fungus was able to use all the nitrogen sources tested, however maximum protease production was observed in the presence of yeast extract (1443.4 U/mL). Protease production was further maximized by the addition of 25 g/L of yeast extract (1903.0 U/mL) to the fermentation medium (Table 1 and Table 2).

Table 1. Effect of carbon, nitrogen sources on protease production by *P. citrinum* LCJ222

Carbon sources 10 (g/L)	Protease Activity (U/mL)
Glucose	0999.6± 07.91
Galactose	1073.8± 55.43
Lactose	1277.5± 84.14
Maltose	1177.4± 02.95
Sucrose	1181.6± 09.89
Starch	1271.2± 75.20
Nitrogen sources 5 (g/L)	Protease Activity (U/mL)
Control	0826.0± 24.04
Beef extract	0923.3± 16.82
Peptone	1292.7± 56.14
Yeast extract	1443.4± 13.85
NH_4CL_2	1208.7± 56.14
NH_4SO_4	0913.1± 56.99
$NaNO_3$	0925.4± 27.71

Table 2. Effect of different concentration of lactose and yeast extract on protease production by *P. citrinum* LCJ222

Lactose (g/L)	Protease Activity (U/mL)
Control	0920.8± 25.00
5	0810.6± 51.46
10	1177.3± 36.62
15	1826.3± 12.86
20	2074.1± 08.87
25	1677.2± 58.39
30	1316.7± 76.22
Yeast extract (g/L)	Protease Activity (U/mL)
Control	1486.0± 10.88
5	0548.8± 13.85
10	0695.7± 03.95
15	0783.2± 08.90
20	1190.6± 49.70
25	1903.0± 07.35
30	1302.6± 39.40

The effect of different inducers on protease production indicated that some of the inducers were capable of enhancing the proteolytic enzyme activities as compared to the controls without inducers (Table 3). Among the inducers, only casein was the efficient inducer for protease production (1157.1 U/mL). Other inducers such as BSA, egg albumin and gelatin failed to induce the production of protease. The influence of varying concentration of casein on protease production under submerged fermentation is presented in Fig 2. The maximum protease production was recorded at 10 g/L casein concentration, beyond which the activity gradually decreased.
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 was studied. Results showed that black gram husk was the best natural inducer for protease production with a maximum activity of protease (1813.7 U/mL) as shown in Table 4. Natural inducers enhanced the maximum amount of protease production when compared to the chemical inducers.

Table 3. Effect of chemical inducers on protease production by *P. citrinum* LCJ222

Chemical inducers	Protease Activity (U/mL)
Control	0928.2± 67.31
BSA	0409.5± 40.58
Casein	1157.1± 10.88
Egg albumin	0465.5± 26.72
Gelatin	0394.1± 20.78

Table 4. Effect of natural inducers on protease production by *P. citrinum* LCJ222

Natural inducers 10 (g/L)	Protease Activity (U/mL)
Control	1276.1± 24.74
Groundnut oil cake	1234.1± 06.92
Mahua oil cake	1444.1± 64.34
Sesame oil cake	1224.8± 11.15
Black gram husk	1813.7± 32.66
Green gram husk	1549.8± 68.29
Red gram husk	1566.6± 11.87

Effect of medium pH on protease production

The effect of medium pH was studied for the production of protease by *P. citrinum* LCJ222. There was a gradual increase in the amount of protease from pH 4 to 8 and maximum

production of protease was observed at pH 9 (1849.4 U/mL) (Table 5). However, pH of the fermentation medium beyond 9 resulted in a marked decrease in the production of protease. Based on the result, it was concluded that *Penicillium citrinum* LCJ222 produced an alkaline protease.

Table 5. Effect of medium pH on protease production by *P. citrinum* LCJ222

Medium pH	Protease Activity (U/mL)
4	1347.5± 34.64
5	1379.0± 09.64
6	1388.1± 20.78
7	1572.2± 39.29
8	1603.3± 10.32
9	1849.4± 47.51
10	1696.1± 28.16

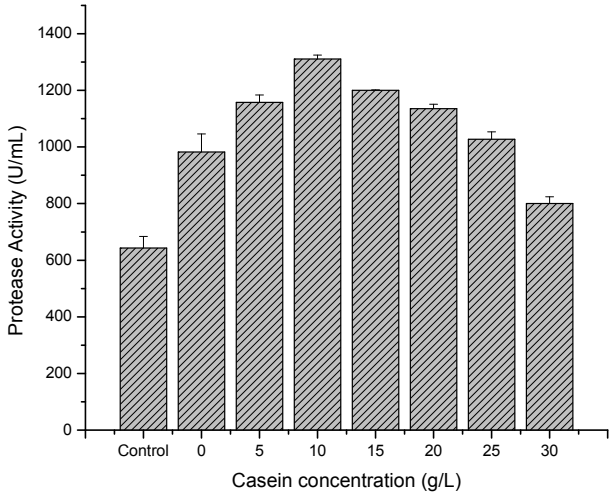

Fig. 2. Effect of different concentration of casein on protease production by *Penicillium citrinum* LCJ222

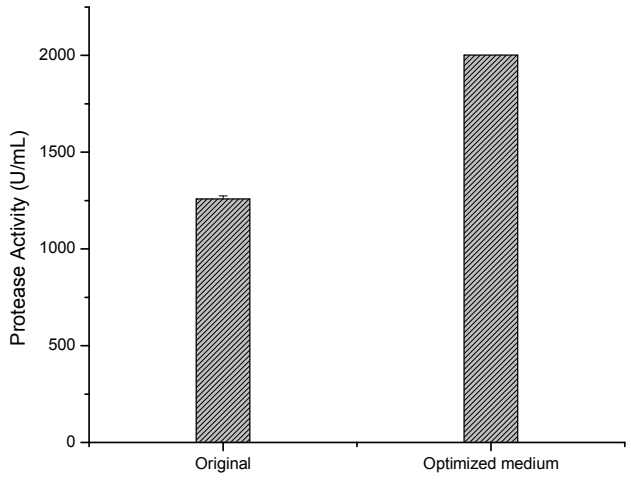

Fig. 3. Comparison of original and optimized medium for protease production by *Penicillium citrinum* LCJ222

Comparison study of original and optimized medium for protease production

Protease production by *Penicillium citrinum* LCJ222 in optimized and original medium was studied. The optimized medium showed a maximum amount of protease production of 2002.0 U/mL on the 4th day. The original medium showed 1258.6 U/mL (Fig. 3). Nearly, 1.5 fold increases in protease production was observed in optimized medium.

DISCUSSION

Extracellular protease production in microorganisms is strongly influenced by medium components. In the present study, Medium 6 influenced maximum protease production and this result was in agreement with following previous reports (Dhevagi and Poorani, 2005). Saravanakumar *et al.* (2010) discovered that medium composition have influenced the extracellular protease production. El-Enshasy *et al.* (2008) determined that the yield of protease improvement and medium optimization need to be considered as the economically feasible development technology. Carbon and nitrogen sources are important to study the physiology and metabolism of enzyme production by fungi. The carbon and nitrogen sources have the ability to enhance the proteolytic enzymes on a commercial scale. In the present study, proteolytic activity was found to be highly influenced by carbon, nitrogen sources and inducers. Among the different carbon sources tested, lactose served as the best source for protease production. Lactose at a concentration of 20 g/L produced maximum amount of protease. Negi and Banerjee (2010) and Manivannan and Kathiresan (2007) also demonstrated that lactose increased the production of protease in *Aspergillus awamori* and *Penicillium fellutanum*.

P. citrinum LCJ222 was able to use all the nitrogen sources tested and maximum protease production was observed in the presence of yeast extract at concentration of 25 g/L. Phadatare *et al*. (1993) also reported the enhancement of protease production by the organic nitrogen source, yeast extract. Similar results were reported by Nehra *et al*. (2002) for *Aspergillus* sp. Mukhtar and Haq (2003) used peptone as the best nitrogen source for production of protease by *A. niger* and *Rhizopus oligosporous*. The selected inducers were able to enhance the protease production in this study. The chemical inducer, casein at 10 g/L concentration enhanced maximum protease enzyme. Kamath *et al*. (2010) reported casein as the best substrate for maximizing proteolytic enzyme production. Casein was found to be the effective inducer in the case of *A. oryzae* (Bataglino *et al.,* 1991). Rajamani and Hilda (1987) reported casein as the best substrate for the production of protease. Similarly Gnanadoss *et al.* (2011) also proved the significant effect of the casein on protease activity.

Different agro-wastes such as groundnut oil cake, mahua oil cake, sesame oil cake, black gram husk, green gram husk and red gram husk were evaluated for the production of protease by *P. citrinum* LCJ222 in submerged fermentation. Among all the substrates examined, black gram husk supported maximum production of protease. The reason for the highest yield with black gram husk was that it provided an adequate source of protein, carbohydrates and minerals needed by the fungi for its growth and biosynthesis of protease. Using the agricultural wastes as the natural inducers in protease production the cost of the medium can be significantly reduced. Heneri *et al*. (1988) used different protein rich substrates for the production of

protease by *A. niger* and found soybean meal as the most suitable substrate. Ahmed *et al*. (2010) reported that rice husk by-product was employed for the production of protease enzyme using submerged fermentation by *Bacillus subtilis.* Bengal gram was also found to be a suitable substrate for maximizing the protease production using *Shigella* sp. (Sankaralingam *et al*., 2011).

Medium pH plays an important role in the protease production. According to Borriss (1987), a culture medium with an initial pH of 9 to 13 maximized protease production. In the present study also, pH 9 favoured maximum production of protease in *P. citrinum* LCJ222. Similar results were also reported by Palanivel *et al.* (2013) in *Aspergillus* strain KH17.

Conclusion

In a conclusion, the present study clearly reveals that *P. citrinum* LCJ222 is an ideal fungus for protease production. The protease production ability of *P. citrinum* LCJ222 can be enhanced greatly by supplementing the basal medium with various nutritional compounds. The protease productivity seemed to be much more responsive to the inductive effect of natural inducer like black gram husk. The study also concludes that the cheaply available agro-wastes are potential substrates for the production of protease, which also reduces the production cost.

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