OPTIMIZATION OF NUTRITIONAL AND CULTURE CONDITIONS FOR IMPROVED PROTEASE PRODUCTION BY *ASPERGILLUS NIDULANS* AND *ASPERGILLUS FLAVUS*

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ABSTRACT

Aspergillus sp. is considered to be a good source of protease. Medium optimization is essential for each isolate in order to maximise the protease production. In the present study, different media were evaluated for maximising protease biosynthesis under submerged fermentation (SmF) using *A. nidulans* LCJ249 and *A. flavus* LCJ253. Among the six media tested, Medium 6 was found to be the best medium for maximum protease production in both *A. nidulans* LCJ249 (1178.0 U/mL) and *A. flavus* LCJ253 (950.6 U/mL). Effect of essential nutrient parameters like carbon, nitrogen and casein and other culture conditions such as incubation time, inoculum size, pH and shaking and static conditions were evaluated by the conventional one factor at a time approach. In the case of *A. nidulans* LCJ249, a medium containing 20 g/L glucose, 15 g/L malt extract, 5 g/L casein, initial medium pH of 7 and inoculum size of 30 mg/L favoured maximum protease production. Similarly, in *A. flavus* LCJ253, a medium containing 20 g/L starch, 20 g/L peptone, 15 g/L casein, initial medium pH of 7 and inoculum size of 10 mg/L favoured maximum protease production. In both the cultures, protease production was higher under shaking condition than in static conditions. The study showed that the optimised medium with optimal conditions enhanced protease production when compared to the original medium.

Keywords: Proteases, submerged fermentation, *Aspergillus*, medium optimization

INTRODUCTION

Proteases hydrolyse a wide variety of proteins (**Genckal and Tari, 2006**). Proteases find application in detergent, meat, protein, brewing, photographic, leather and dairy industries (**Anwar and Saleemuddin, 1998**). Proteases are classified into six prominent groups such as serine proteases, cysteine (or) sulphydral proteases, aspartic proteases, metalloproteases, threonine proteases and glutamic acid proteases (**Son and Kim, 2002**) and based on their optimum pH activity they are classified as acid, neutral, and alkaline proteases (**Asoodeh and Abadi, 2012**). Among the microorganisms used for the protease production, filamentous fungi are extensively exploited because of their capacity to grow on many substrates and produce various enzymes (**Lekha and Lonsane, 1994**).

Submerged fermentation (SmF) involves the production of enzymes in liquid medium. In SmF, scale-up from laboratory level to industry is simple, with parameters being more easily monitored and controlled (**Rao et al., 1998**). Submerged fermentation is preferred for the production of proteases due to steady protease production, definite medium and easier downstream processing (**Sandhyaa et al., 2005**). Several physical and nutritional factors influence the production of protease and hence optimization of these factors is essential to improve the yield and make the process cost-effective (**Rani et al., 2012**). Optimization by conventional methods (one factor at a time) is done by varying one particular parameter at a time while keeping the other parameters constant (**Wu and Hang, 2000**).This method helps to assess the importance of a specific parameter on the enzyme production.

The objective of our work was to optimize nutritional and physical conditions for enhancing protease production by *A. nidulans* LCJ249 and *A. flavus* LCJ253 using 'one factor at a time' method under submerged fermentation and scaling up of the production using the modified conditions.

MATERIAL AND METHODS

Culture and maintenance

A. nidulans LCJ249 and *A. flavus* LCJ253 used for this study were isolated from soil and leaf litters collected from Loyola College Campus, Chennai. The above cultures were maintained on potato dextrose agar plates at 4 °C and sub-cultured periodically.

Effect of nutritional conditions

Selection of liquid medium for protease production

Production of protease by submerged fermentation was studied in six different basal medium. Medium 1 (**Gradisar et al., 2005**) g/L: Glucose - 1, KH_2PO_4 - 1, $MgSO_4$ - 0.2, $CaCl_2$ - 0.2, NaCl - 0.2; Medium 2 (**Srinubabu et al., 2007**) 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, pH - 8.3; Medium 3 (Tsuchiya *et al.*, 1992) g/L: Maltose - 1, Yeast Extract - 0.04, KH_2PO_4 - 0.5, NaCl - 0.2, pH - 8.5; Medium 4 (**Saravanakumar et al., 2010**) g/L: Malt Extract - 20, Fructose - 5, Thio Urea - 0.005, KH_2PO_4 - 1, KCl - 1, $FeSO_4.7H_2O$ - 0.02, $MgSO_4.7H_2O$ - 0.5, pH - 8; Medium 5 (**Charles et al., 2008**) g/L: Sucrose - 30, KCl - 0.5, $FeSO_4$ - 0.01, $MgSO_4$ - 0.5, K_2HPO_4 - 1, $NaNO_3$ - 2, pH - 8; Medium 6 (**Namasivayam et al., 2010**) g/L: Yeast Extract - 5, Peptone - 5, Glucose - 10, Casein - 20, pH - 7.

About 100 mL of each medium was dispensed into 250 mL conical flask and autoclaved at 121˚C for 15 min. A pinch of nalidixic acid was added to avoid bacterial contamination. Under sterile condition, two mycelial discs from a fully grown fungal plate were inoculated into the conical flask. The flasks were incubated on an orbital rotary shaker set to 30 °C and 120 rpm. Aliquots of the culture filtrate were collected for determination of extracellular protease activity.

Optimization of essential nutrients

Various nutritional parameters for protease production were optimized by the "one factor at a time" method which involves changing only one independent variable, while fixing the others at certain levels in the original medium. Each subsequent factor was examined after taking into account the previously optimized factors.

The effect of carbon sources on protease production was studied by supplementing the media with separately with glucose, sucrose, galactose, maltose, starch and lactose at a concentration of 10 g/L. Original medium served as a control. The most suitable concentration of carbon source for *A. nidulans*

LCJ249 and *A. flavus* LCJ253 was further determined using the most suitable carbon source in the range of 5 to 30 g/L.

The effect of nitrogen source on protease production was studied by supplementing the medium with three organic nitrogen (malt extract, peptone, and yeast extract) and three inorganic nitrogen (ammonium sulphate, ammonium chloride and ammonium nitrate) sources. These nitrogen sources replaced the original nitrogen source (yeast extract, peptone and casein) available in the medium. The original medium served as control. The optimum concentration of the suitable nitrogen source for protease production by *A. nidulans* LCJ249 and *A. flavus* LCJ253 was carried out by varying the concentrations of the suitable nitrogen source in the range of 5 to 30 g/L.

The effect of the inducer casein on protease production by *A. nidulans* LCJ249 and *A. flavus* LCJ253 was evaluated. The effect of different concentrations of casein ranging from 5 to 30 g/L was also studied.

Optimization of culture conditions

The optimal time of incubation for maximum protease production by *A. nidulans* LCJ249 and *A. flavus* LCJ253 was determined by incubating the flasks containing the medium for a period of 16 days. Aliquots of the culture filtrate were drawn every 2 days and used for the determination of extracellular protease activity. The biomass was also simultaneously harvested and used for the determination of fresh weight and dry weight.

The effect of different inoculum size on protease production by *A. nidulans* LCJ249 and *A. flavus* LCJ253 was evaluated using various inoculum sizes ranging from 10 to 60 mg/100 mL. Mycelial discs (6 mm) were inoculated into the conical flask containing culture medium. The amount of mycelium on agar plug was determined, based on which inoculum size was calculated. The protease activity was determined at the end of the study period.

The effect of initial pH of the medium on protease production by *A. nidulans* LCJ249 and *A. flavus* LCJ253 was studied under different pH range ranging from 4 to 9. The pH of the production medium was adjusted by adding 1N NaOH and HCl.

The effect of static and shaking condition on protease production was studied by incubating under static and shaking condition (120 rpm) at 30 °C.

Protease production in original and optimised medium

Protease production by *A. nidulans* LCJ249 and *A. flavus* LCJ253 in the optimised and original medium (**Namasivayam *et al*., 2010**) was compared. The modified medium for *A. nidulans* LCJ249 contained the following components (g/L): Malt Extract - 15, Glucose - 20, Casein - 5 and the modified media for *A. flavus* LCJ253 contained (g/L): Peptone - 20, Starch - 20, Casein - 15. Fermentation was carried out in Erlenmeyer flasks (1L) containing 500 mL of the modified production medium. The medium containing flasks were inoculated with the mycelia discs. The flasks were incubated at room temperature for 1 week in a rotary shaker at 120 rpm. 10 mL of the culture filtrate was drawn on the peak day and centrifuged at 3600 rpm for 10 min. The supernatant was used as a crude enzyme for protease activity.

Protease assay

Protease activity was determined spectrophotometrically using casein as the substrate. A mixture of 500 μL of casein (0.5% w/v) , 300 μL of 0.2 M phosphate buffer (pH 7) and 200 μL crude enzyme extract was incubated at room temperature 37 °C for 10 minutes. After 10 minutes, the enzyme reaction was terminated by the addition of 1 mL of 5% (w/v) trichloroacetic acid (TCA). The reaction mixture was then centrifuged at 10,000 rpm for 15 minutes to separate the unreacted casein. To 1 mL of the supernatant, 5 mL of 0.4 M Na_2CO_3 and 1 mL of 3-fold diluted Folin Ciocalteu's reagent were added. The resulting solution was incubated in the dark for 30 minutes at room temperature and absorbance was measured at 660 nm (**Lowry *et al*., 1951**).

Enzyme activity (1 Unit) was defined as the amount of enzyme liberating one μ/mole of tyrosine/mL/min under the defined conditions.

$$\text{Units/ml Enzyme activity} = \frac{(\mu/\text{mole tyrosine equivalents released}) \times 7}{0.2 \times 4 \times 10}$$

7 = Total volume (in millilitres) of assay
10 = Time of assay (in minutes) as per the Unit definition
0.2 = Volume of Enzyme (in millilitres) of enzyme used
4 = Volume (in millilitres) used in Colorimetric Determination (Sigma Aldrich method).

Protein assay

Protein content of the extract was estimated following the method described by **Lowry *et al*. (1951)** using bovine serum albumin as the standard.

### Statistical analysis

Each parameter was analyzed with three replicates and a standard deviation (SD) was calculated and data are expressed ± SD of three replication.

RESULTS

Selection of liquid medium for protease production

The protease production was significantly different in all the 6 media tested. Among the six media studied, Medium 6 favoured maximum protease production in both *A. nidulans* LCJ249 and *A. flavus* LCJ253 as shown in Figure 1. In Medium 6, a maximum protease activity of 988.4 (U/mL) and 950.6 (U/mL) was recorded in *A. nidulans* LCJ249 and *A. flavus* LCJ253 on the 6th day of incubation. Results also proved that Medium 1 did not favour protease production in both the cultures.
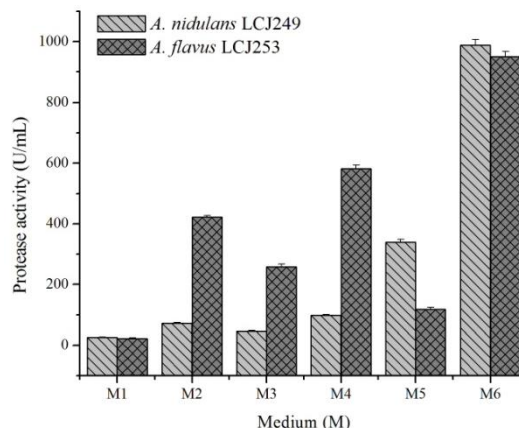
Figure 1 Influence of different medium compositions on the protease production by *A. nidulans* LCJ249 and *A. flavus* LCJ253

Optimization of essential nutrients

To optimize the most suitable carbon source for protease production by *A. nidulans* LCJ249 and *A. flavus* LCJ253, experiments were carried out using glucose, sucrose, galactose, maltose, starch and lactose in Medium 6 and the results are presented in Table 1. The result showed that glucose enhanced significantly higher protease production (1183.0 U/mL) in *A. nidulans* LCJ249, when compared to other carbon sources. Maltose also moderately favoured protease production in *A. nidulans* LCJ249. In the case of *A. flavus* LCJ253, the liquid medium containing starch as the carbon sources favoured maximum amount of protease (1474 U/mL) and the next suitable carbon source was lactose.

The suitable nitrogen source for the protease production by *A. nidulans* LCJ249 and *A. flavus* LCJ253 was studied using organic (malt extract, peptone and yeast extract) and inorganic nitrogen sources (ammonium sulphate, ammonium chloride and ammonium nitrate) and the results are presented in Table 1. The results showed that in general organic nitrogen favoured better growth high protease production when compared to inorganic nitrogen sources. In the case of *A. nidulans* LCJ249, maximum protease production of 1134.0 U/mL was recorded in the medium amended with malt extract on the 6th day of culture. Whereas in *A. flavus* LCJ253, the medium amended with peptone favoured a maximum protease production 1129.8 U/mL on the 6th day. In all the experiments, protease activity was observed from the first day of inoculation and peak activity was observed on the 6th day and declined thereafter.

The effect of different concentrations of glucose (most suitable carbon source) was studied to ascertain the most suitable concentration for protease production by *A. nidulans* LCJ249 and the results are shown in Table 2. Results showed that 20 g/L of glucose significantly increased the yield of protease (833 U/mL) when compared to other concentrations. It was initially observed that the production of protease increased with increase in the concentration of glucose but declined when the concentration was increased beyond 20 g/L. The effect of different concentration of malt extract (suitable nitrogen source) on the protease production by *A. nidulans* LCJ249 was studied and it was found that malt extract concentration of 15 g/L was ideal for maximum protease activity of 1212.4 U/mL (Table 2).

Table 1 Influence of essential nutrients on the protease production by *A. nidulans* LCJ249 and *A. flavus* LCJ253 on the sixth day.

Nutrients	*A. nidulans* LCJ249	*A. flavus* LCJ253
Carbon Sources	**Protease activity (U/mL)**	
Glucose	**1183.0 ± 35.2**	504.0 ± 14.0
Galactose	742.0 ± 22.1	1076.6 ± 33.4
Lactose	651.0 ± 15.2	1376.2 ± 32.8
Maltose	786.8 ± 20.8	883.4 ± 27.6
Starch	683.2 ± 18.7	**1474.2 ± 31.3**
Sucrose	620.2 ± 16.9	784.0 ± 15.5
Nitrogen Sources		
Yeast extract	819.0 ± 25.4	868.0 ± 30.2
Peptone	841.4 ± 28.2	**1129.8 ± 31.1**
Malt extract	**1134.0 ± 33.5**	805.0 ± 14.9
Ammonium nitrate	834.4 ± 22.0	302.4 ± 10.5
Ammonium chloride	684.6 ± 16.8	488.6 ± 12.2
Ammonium sulphate	459.2 ± 16.7	141.4 ± 8.1

Table 2 Influence of various concentrations of glucose and malt extract on the protease production by *A. nidulans* LCJ249 on the sixth day.

Concentration (g/L)	*A .nidulans* LCJ249	
	Protease activity (U/mL)	
	Glucose	**Malt extract**
Control	546.0 ± 20.2	884.8 ± 22.5
5	397.6 ± 10.1	701.4 ± 20.8
10	445.2 ± 15.6	964.6 ± 33.6
15	422.8 ± 12.8	**1212.4 ± 40.1**
20	**833.0 ± 23.1**	1071.0 ± 38.4
25	509.6 ± 15.6	870.8 ± 20.9
30	656.6 ± 13.8	981.4 ± 22.7

The effect of different concentrations of starch on the protease production by *A. flavus* LCJ253 showed that 20 g/L starch resulted in a higher yield of protease of 1393 U/mL (Table 3). It was observed that initially the production of protease increased with increase in the concentration of starch but declined when the concentration was increased after 20 g/L. In *A. flavus* LCJ253, peptone concentration of 30 g/L enhanced a maximum protease activity of 2594 U/mL closely followed by 20 g/L peptone (2528 U/mL). Taking into consideration the cost factor, 20 g/L peptone was chosen as optimum for the production of protease in the case of *A. flavus* LCJ253 (Table 3).

Table 3 Influence of various concentrations of starch and peptone on the protease production by *A. flavus* LCJ253 on the sixth day.

Concentration (g/L)	*A.flavus* LCJ253	
	Protease activity (U/mL)	
	Starch	**Peptone**
Control	842.6 ± 20.1	820.4 ± 22.5
5	641.2 ± 15.2	1334.2 ± 34.6
10	721.0 ± 18.4	1274.0 ± 33.8
15	826.0 ± 22.5	1719.2 ± 39.4
20	**1393.0 ± 30.8**	2528.4 ± 44.1
25	725.2 ± 14.3	2279.6 ± 31.5
30	1145.2 ± 28.6	**2594.2 ± 42.7**

The effect of different concentrations of casein (inducer) on protease activity in both the cultures was studied. In *A. nidulans* LCJ249, 15 g/L casein enhanced maximum protease activity (1346 U/mL) as shown in Figure 2. Likewise, in *A. flavus* LCJ253, 15 g/L casein induced maximum protease production (1489 U/mL). The production of protease increased with increase in the concentration of casein but declined when its concentration exceeded 20 g/L.

Optimization of culture conditions

The protease activity was recorded every 2 days in order to determine the optimum incubation period for maximum production of protease. In both the isolates (*A. nidulans* LCJ249 and *A. flavus* LCJ253), maximum protease production was observed on the 6th day of incubation at room temperature. In *A. nidulans* LCJ249, a protease activity of 1261.4 U/mL and a protease activity of 1580.4 U/mL in *A. flavus* LCJ253 were observed on the 6th day. Prolonged incubation period decreased the enzyme activity (Table 4).
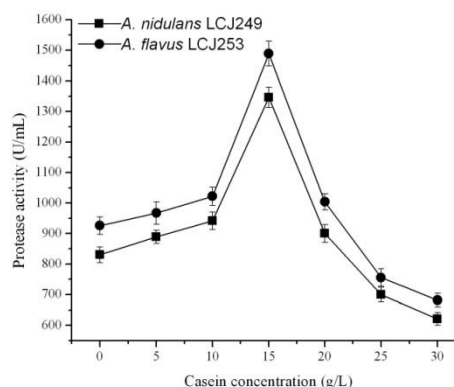
Figure 2 Influence of casein concentration on the protease production by *A. nidulans* LCJ249 and *A. flavus* LCJ253

Table 4 Influence of culture conditions on the protease production by *A. nidulans* LCJ249 and *A. flavus* LCJ253

| Culture conditions	*A.nidulans* LCJ249	*A.flavus* LCJ253
	Protease activity (U/mL)	
Incubation time (Days)		
2	1009.4 ± 38.1	735.2 ± 25.2
4	1117.2 ± 42.5	1404.0 ± 40.6
6	**1261.4 ± 40.6**	**1580.4 ± 45.1**
8	1012.2 ± 38.1	597.4 ± 11.7
10	964.6 ± 20.9	526.2 ± 10.8
pH		
4	810.6 ± 15.2	1180.2 ± 38.1
5	978.6 ± 13.1	1495.2 ± 32.5
6	1153.6 ± 33.4	2080.4 ± 41.2
7	**1289.4 ± 38.1**	**2097.2 ± 40.3**
8	1096.2 ± 29.5	1908.2 ± 30.7
9	960.4 ± 26.1	1930.6 ± 30.2
Inoculum size (mg)		
10	841.4. ± 33.2	**2223.2 ± 40.8**
20	950.6 ± 36.4	1402.8 ± 35.1
30	**1258.0 ± 41.2**	1397.2 ± 33.0
40	1068.2 ± 40.9	1358.0 ± 28.9
50	1118.6 ± 43.6	1170.4 ± 22.1
60	1157.2 ± 35.4	1099.0 ± 30.5
Static and shaking conditions		
Static	1351.0 ± 41.3	1965.6 ± 46.5
Shaking	**2262.4 ± 54.9**	**2059.4 ± 60.2**

Time course experiments showed that the protease activity increased with increase in time and the peak protease activity was observed on the 6th day. The protease activity decreased thereafter. The effect of initial pH (4 to 9) of the medium on protease shows that initial pH of 7 was the best pH for the production of protease by *A. nidulans* LCJ249 and *A. flavus* LCJ253. In *A. nidulans* LCJ249, a protease activity of 1289.4 U/mL and in *A. flavus* LCJ253, a protease activity of 2097.2 U/mL was observed at pH 7 (Table 4). However, protease activity was present at all pH evaluated in this study. The effect of different inoculum size on protease production by *A. nidulans* LCJ249 and *A. flavus* LCJ253 was studied. In *A. nidulans* LCJ249, the maximum protease activity of 1258 U/mL was observed with addition of 30 mg of inoculum size. Whereas in *A. flavus* LCJ253, the maximum protease production of 2223.2 U/mL was obtained with 10 mg of inoculum size (Table 4). The culture medium became more viscous by increasing the inoculums size.

In *A. nidulans* LCJ249 when compared to the static condition, shaking condition favoured maximum protease production of 2262.4 U/mL on the 6th day of incubation. In *A. flavus* LCJ253 also shaking condition favoured maximum protease production of 2059.4 U/mL on the 6th day of incubation (Table 4).

Protease production in original and optimised medium

Protease production by *A. nidulans* LCJ249 and *A. flavus* LCJ253 in the original and the optimised medium was studied. In *A. nidulans* LCJ249, the modified medium showed a maximum protease activity (1267.0 U/mL) on the 6th day when compared to the original medium (961.8 U/mL). Likewise, in *A. flavus* LCJ253, the protease production was 1583.4 U/mL in the modified medium when compared to the original medium where it was 679.0 U/mL (Figure 3). The results showed that the optimised medium enhanced the protease production in both the cultures.
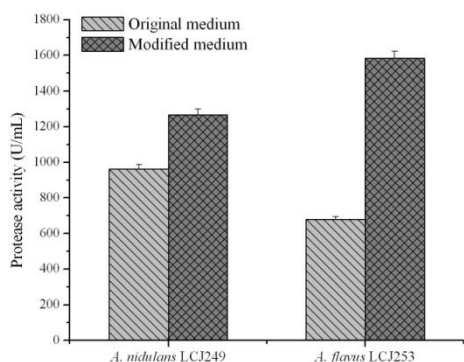
Figure 3 Protease production by *A. nidulans* LCJ249 and *A. flavus* LCJ253 in the original and optimised medium

DISCUSSION

Fungal proteases are produced extracellularly which make it easy to be recovered from the fermentation medium (**Sandhya** *et al.,* **2005; Murthy and Naidu, 2010**). Filamentous fungi also have the potential to grow under varying environmental conditions such as fermentation type, time, pH, temperature and utilizing various sources of substrate as nutrients (**Ikram-ul-haq** *et al.,* **2006**). Proteases obtained from *Aspergillus* sp. have been declared to be safe. *Aspergillus niger* has been reported to produce proteases (**Paranthaman** *et al.,* **2009**). Similarly, protease production has been reported in *Aspergillus oryzae* and *Aspergillus fischeri* (**Murthy and Naidu, 2010**; **Saravanakumar** *et al.,* **2010**). The optimization of medium components is carried out to maintain a balance between the various medium components and thus minimizes the amount of unutilized components at the end of the fermentation process (**Kumara** *et al.,* **1999**). Several researchers have reported different methods to optimize the production of protease. **Saravanakumar** *et al.* (**2010**) reported that the medium composition and some physical factors such as the pH, fermentation period and temperature greatly influence the extracellular protease production. **El Enshasy** *et al.* (**2008**) emphasized that for the improvement of yield of proteases, the optimization of the production medium and the physical conditions are to be considered to develop an economically feasible technology. Better fermentation conditions will enhance higher production of proteases.

Extracellular protease production in microorganisms is strongly influenced by medium components such as carbon and nitrogen sources (**Beg** *et al.,* **2002**) and metal ions (**Varela** *et al.,* **1996**). **Banerjee** *et al.* (**1993**) reported that a complex medium has been found to be suitable for protease production and they are dependent on the nutritional requirement of the organism under consideration. *A. nidulans* LCJ249 and *A. flavus* LCJ253 had varied preference toward the carbon sources. *A nidulans* LCJ249 preferred glucose while *A. flavus* LCJ253 preferred starch for maximum protease production. There are also reports showing that different carbon sources have different influences on extracellular enzyme production by different strains (**Wang and Lee, 1996; Nehra** *et al.,* **2002**). The present results is in agreement with **Srinubabu** *et al.* (**2007**), **Tremacoldi and Carmona** (**2005**) and **Chellapandi** (**2010**) who reported glucose as the best carbon sources for improving the production of proteases by *Aspergillus flavus*, *Aspergillus clavatus* and *Aspergillus terrus.* This stimulatory effect of glucose on protease production by glucose indicates that protease production by *A. nidulans* LCJ249 may not be subject to control by catabolite repression. The stimulatory effect may also due to the fact that glucose is a monosaccharide and is readily available for the metabolism of the fungus for enzyme production. Several researchers have reported about the repression of protease synthesis and its secretion due to glucose in different microorganisms (**Tsuchiya and Kimura 1984**; **Taragano** *et al.,* **1997**). In contrast to the stimulatory effect of glucose, **Sani** *et al.* (**1992**) reported that starch/complex carbon sources supported the highest fungal growth and enzyme production in *Aspergillus* and this was evident in the present study. The influence of starch on the production of protease has also been reported by **Siala** *et al.* (**2012**). The present study is also in agreement with **Srinubabu** *et al.* (**2007**) who reported that starch and glucose will be more suitable and cost-effective carbon source for commercial protease production.

Generally in addition to an energy source and trace elements, a suitable nitrogen source in adequate quantities is essential for the rapid growth of the fungus. In the present study, organic nitrogen substances were suitable for protease production. Accordingly, malt extract enhanced maximum protease production in *A. nidulans* LCJ249 and peptone in the case of *A. flavus* LCJ253. **Banerjee** *et al.* (**1999**) reported that malt extract and yeast extract significantly induced the production of protease. In another study, addition of soybean meal in combination with peptone to growth medium has been shown to increase protease production by *A. carbonarius* (**Ire** *et al.,* **2011**). Peptone contain high

amount of peptides. Proteases break down proteins and peptides. Hence, the presence of peptides in the culture medium could have induced production of protease (**Siala** *et al.,* **2012**). Peptones are also known to sustain rapid growth of fungi (**Yan** *et al.,* **2012**). The present study also revealed that organic nitrogen source showed better protease production than inorganic nitrogen sources. This better production with organic nitrogen sources can be correlated to their capacity to induce protease production. Organic nutrients are also less expensive and supplies the necessary minerals and vitamins required for protease production. The cell growth is also further rapid and efficient when organic nitrogen sources are used, since they decrease the number of components that cells would otherwise have to be synthesized.

The addition of inducers such as casein increases the protease production. In the present study an increase in inducer concentration increased protease activity. Protease activity was the maximum at 15 g/L and further addition of casein decreased the activity. Similarly, **Escobar and Barnett** (**1993**) reported that the enzyme production will decrease if the substrate concentration increases. When the substrate concentration is too high, it may also prohibit the enzyme activity (**Dekleva, 1985**). **Andrade** *et al.* (**2002**) demonstrated that protease activity was at its maximum when 40 g/L of the substrate (casein) was used. The amino acids and peptides in casein may be served as specific inducers for increasing protease production by *A. nidulans* LCJ249 and *A. flavus* LCJ253.

Enzyme production is also very much dependant on the pH of the fermentation medium (**Kubackova** *et al.,* **1975**). pH influences the enzymatic processes and also plays a major role in the transport process of diverse of components across the cell membrane (**Sandhya** *et al.,* **2005; Paranthaman** *et al.,* **2009; Murthy and Naidu, 2010**). Neutral pH helps in the cell growth but acidic and alkaline pH act as an inhibitor to the cell growth and this will affect the enzyme production by the cells. **Chakrabarti** *et al.* (**2000**) proved that *Aspergillus terreus* managed to produce protease between pH 5.5 and 9.5. In the present study, protease production by *A. nidulans* LCJ249 and *A. flavus* LCJ253 was influenced by the initial pH of the medium. The best protease production was observed when the initial pH was 7 in *A. nidulans* LCJ249 and *A. flavus* LCJ253. Similarly, **Mellon and Cotty** (**1996**) reported that pH 7.5 was optimum for maximum protease production by *A. flavus*. **Ire** *et al.* (**2011**) reported that pH 6.0 as the best initial pH for the production of protease by *A. carbonarius*. The influence of pH on the protease production could be due to its effect on the stability or conformation of the plasma membrane which indirectly affects the membrane bound ribosomes involved in the synthesis of protease (**Razak** *et al.,* **1997**).

The initial inoculum size affects the growth and in turn affects the metabolite production. In the present investigation, the effect of inoculum size on the production of protease by *A. nidulans* LCJ249 and *A. flavus* LCJ253 was studied. **Raimbault and Alazard** (**1980**) reported that the decline in enzyme production with high inoculum size was related to the production of high biomass which is responsible for the reduction of the enzyme. Higher inoculum sizes have been found to negatively affect protease production in the study. This is in agreement with previous observations that reported that higher inoculum sizes need not necessarily give higher protease yield or cell growth. Higher inoculum sizes could result in the depletion of oxygen and nutrients in the culture media.

The production of protease under shaking condition was better when compared to static cultures. In the fermentation process using an aerobic culture, the oxygen affects the production of enzymes due to the changes in the metabolic pathway and metabolic fluxes (**Calik** *et al.,* **1998**). **Ducros** *et al.* (**2009**) suggested that the respiration rate of the aerobic culture is dependent on the dissolved oxygen. This is due to physiological alteration in cell metabolism (**Hwang** *et al.,* **1991**). In *A. flavus* LCJ253, shaking condition favoured protease production which is could be due to lower availability of dissolved oxygen with low mixing rates (**Potumarthi** *et al.,* **2007**). These results were consistent with the observations of **Beg** *et al.* (**2002**) for *Bacillus mojavensis* where shaking was required to stimulate protease production.

Thus protease production by *A. nidulans* LCJ249 and *A. flavus* LCJ253 under submerged fermentation were effectively enhanced by optimizing the physical and essential nutritional parameters by the conventional one factor at a time method and scaling up of the protease production was also carried out.

CONCLUSION

In this study, optimization of the culture media for maximizing protease production by submerged fermentation was carried out. The selection of a suitable production medium, carbon, nitrogen, inducer, pH, inoculum size, incubation time for protease production by submerged fermentation was carried out in a way to induce higher degree of protease production and also to minimize the operating cost. The modified medium composition and cultural conditions showed enhanced protease production compared to the original medium conditions. The modified conditions were also implemented under large scale for the production of extracellular fungal proteases.

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