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PROTEASE PRODUCTION BY *PENICILLIUM* SP. LCJ228 UNDER SOLID STATE FERMENTATION USING GROUNDNUT OILCAKE AS SUBSTRATE

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ABSTRACT

The production of alkaline protease by *Penicillium* sp. LCJ228 was studied under solid state fermentation. Oilcakes such as groundnut oilcake, mahua oilcake, sesame oilcake, cotton seed oilcake and coconut oilcake were used as substrates. The best substrate for maximum protease production was found to be groundnut oilcake and a high protease activity was observed on the 6th day of growth. The effect of medium components and culture conditions for maximizing protease production was investigated by one factor at a time method. Glucose (20 g/L) was found to be the best carbon source for maximum protease production. Yeast extract (15 g/L) was found to be the best nitrogen source and casein (5 g/L) was a suitable inducer. A pH value of 9.0, an inoculum size of 2 g/kg solid substrate and a moisture content of 80% were optimal for maximum protease production. After optimization of conditions, a 2.2 fold increase in the protease production was observed. The study proved that *Penicillium* sp. LCJ228 was able to produce a very high level of protease under SSF using inexpensive oilcakes.

Key words: *Penicillium* sp., alkaline protease, solid state fermentation, oilcakes.

INTRODUCTION

Proteases are important industrial enzymes, which contribute about 60% of the total world enzyme market (Kumar et al. 2012; Rao et al. 1998). They find application in several industrial and biotechnological processes. The major application of proteases are in detergents, meat processing, cheese making, recovery of silver from photographic film and certain medical treatments for inflammation and virulent wounds (Mohan et al. 2005; Paranthaman et al. 2009). Proteases are classified as alkaline, acid and neutral protease according to the range of pH at which they show maximum activity (Leng and Xu, 2011). Alkaline proteases especially those from fungi such as *Aspergillus* and *Penicillium* species are considered the most commercially important among the microbial proteases (Dixit and Verma, 1993; Malathi and Chakraborthy, 1991; Rani et al. 2012). Fungal proteases have greater advantages over bacterial protease as they are normally GRAS (Genetically regard as safe) strains. Moreover, fungal strains can utilize low cost material, high protease can be produced and fungal enzymes can be easily recovered. Fungal mycelium can also be easily removed by filtration (Prakasham et al. 2006; Viswanatha et al. 2010). Protease production by fermentation can be carried out by both submerged fermentation (SmF) and solid state fermentation (SSF). SSF is simple because of the use of inexpensive medium components and higher yield of enzyme (Pandey et al. 2001). SSF is suitable for fungi as they have low moisture requirements for their growth. Various agro-wastes such as oilcakes and husks which are cheaper can be used as substrates and thus reduce the cost of enzyme production (Gnanadoss et al. 2011; Mudgett et al. 2001). The product can also be obtained in a highly concentrated form as compared to the protease recovered by submerged fermentations.

The present study was aimed to optimize the protease production by *Penicillium* sp. LCJ228 under SSF by using a suitable substrate and by amending suitable carbon source, nitrogen source and inducers. The influence of physical parameters such as incubation time, pH, inoculum size and moisture content was also standardized.

MATERIALS AND METHODS

2.1.MICROORGANISM

Penicillium sp. LCJ228 used in this study was isolated from rice mill waste using standard procedures and the pure culture was maintained on Potato Dextrose Agar (PDA) slants at 4 °C.

2.2.SUBSTRATES

Different oilcakes such as groundnut oilcake, mahua oilcake, sesame oilcake, cotton seed oilcake and coconut oilcake were screened for protease production under solid state fermentation.

2.3.FERMENTATION CONDITIONS AND ENZYME EXTRACTION

The oilcakes were soaked overnight and water was drained off and shade dried. SSF was carried out in 200 mL stoppered bottle containing 10 g of the dried substrates which were moistened with water and autoclaved at 121˚C for 30 min. After sterilization, the bottles were cooled and inoculated with one fungal mycelium disc (6 mm). The inoculated flasks were incubated for 9 days at room temperature. After sufficient growth of the fungi, extraction of the enzyme was done by adding 50 mL sterile distilled water into the bottles. The bottles were kept in the shaker for 24 h. The contents were filtered and then centrifuged at 10,000 rpm for 10 min. The supernatant was used for protease assay.

2.4. TIME COURSE EXPERIMENT

The production of protease by *Penicillium* sp. LCJ228 was studied by incubating the inoculated SSF bottles for a total period of 12 days. The enzyme was extracted at intervals of 3 days and protease activity was measured.

2.5.OPTIMIZATION OF PROTEASE PRODUCTION

Production of protease by *Penicillium* sp. LCJ228 was studied by optimizing suitable nutritional factors such as carbon source, nitrogen source, inducer and other physical parameters such as incubation time, pH, inoculum size and moisture content.

2.5.1.EFFECT OF NUTRITIONAL FACTORS ON PROTEASE PRODUCTION

The addition of different concentrations of glucose (suitable carbon source) ranging from 5 to 30 g/L on the protease production by *Penicillium* sp. LCJ228 was studied. Similarly, the influence of different concentrations of yeast extract (suitable nitrogen source) in the range of 5 to 30 g/L and casein (inducer) concentrations ranging between 5 and 30 g/L was evaluated for optimum production of protease by *Penicillium* sp. LCJ228. All the experiments were performed at room temperature. For each experiment, the protease activity was determined at the end of the 6th day of incubation.

2.5.2.EFFECT OF PHYSICAL FACTORS ON PROTEASE PRODUCTION

The production of protease by *Penicillium* sp. LCJ228 was studied under different physical conditions namely, pH (4 to 10), inoculum size (1 to 5 g/kg) and initial moisture content (40 to 90%). The experiments were carried under room temperature. The protease activity was measured at the end of the 6th day of incubation.

2.6. PROTEASE ASSAY

Protease activity was determined spectrophotometrically using casein as the substrate for the enzyme. A mixture of 500 µL of 0.5% (w/v) of casein (pH 7) in 0.2 M phosphate buffer of 300 µL (pH 7) and 200 µL crude enzyme extract was incubated at room temperature for 10 min. After 10 min, the enzyme reaction was terminated by the addition of 1 mL of 5% (w/v) trichloroacetic acid (TCA). The reaction mixture was then centrifuged to separate the unreacted casein at 10,000 rpm for 15 min. Then 1 mL supernatant was mixed with 5 mL of 0.4 M Na_2CO_3. To this solution, 1 mL of 3-fold diluted Folin Ciocalteu's reagent was added. The resulting solution was incubated in the dark for 30 min at room temperature and absorbance was measured at 660 nm. Protease activity was calculated according to the method described by Sigma Aldrich method. One unit of protease activity was defined as the amount of enzyme liberating one µ/mole of tyrosine/mL/min. The protein content was estimated by following the method described by Lowry et al. (1951) using bovine serum albumin as the standard. Protease activity was expressed as units per gram dry substrate (U/g ds).

RESULTS AND DISCUSSION

Protease producing *Penicillium* sp. LCJ228 has been isolated from rice mill waste. Enhancement of protease production was studied by selection of oilcake, fermentation time, amount of carbon, nitrogen and inducer supplementation, pH of substrate, inoculum size and initial moisture content of substrate. The results of these parameters are discussed below.

3.1.SELECTION OF SUITABLE OILCAKE FOR SSF

The choice of an appropriate substrate is of great importance for the successful production of protease under SSF. In the present study, groundnut oilcake, sesame oilcake, mahua oilcake, cotton seed oilcake and coconut oilcake were used as substrate for protease production by *Penicillium* sp. LCJ228 under SSF. The results showed that groundnut oilcake was the best substrate for protease production with 2728.3 U/g ds of protease activity and this was statistically significant at 5% level. When *Penicillium* sp. LCJ228 grown on sesame oilcake and mahua oilcake, the fungus produced 2103.2 U/g ds and 1982.4 U/g ds of protease activity, respectively. Oilcakes have been widely reported to be the best substrates for protease production under SSF (Nagamani et al. 2012). Gnanadoss et al. (2011) reported that *Aspergillus niger* produced the maximum amount of protease utilizing cotton seed oilcake as substrate. This indicates that each fungus has its preferred substrate for maximal enzyme production under SSF. The production of protease in the present study was probably enhanced due to the higher protein content of the substrate.

3.2. EFFECT OF INCUBATION PERIOD ON PROTEASE PRODUCTION

The incubation period is directly proportional to the production of enzymes and other metabolites. The effect of incubation time on protease production by *Penicillium* sp. LCJ228 using groundnut oilcake as a substrate is shown in Fig. 1. The maximum protease production (2899.1 U/g ds) was observed on the 6th day of incubation and decreased thereafter. In agreement with the present results, Santhi (2014) also observed the maximum protease production by *A. niger* MTCC 281 on the 7th day of incubation and decreased further. Similarly, in *A. flavus*, maximum protease production was achieved between the 5th and 7th day of incubation and declined thereafter (Malathi and Chakraborthy, 1991). Further increase in incubation time resulted in decreased protease production and this can be attributed to the decrease in supply of nutrients to the microorganism, competition and accumulation of toxic metabolites (Romero et al. 1998).

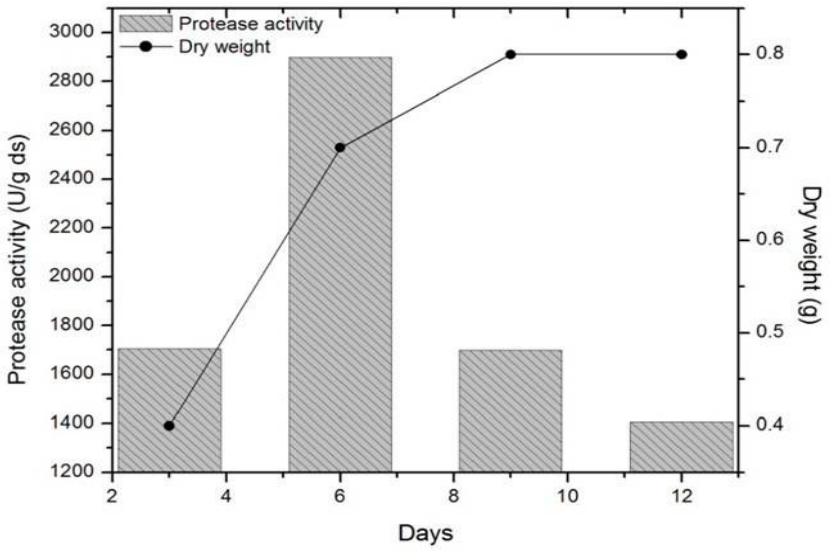

Figure 1
Growth and protease production by Penicillium sp. LCJ228 on different days

3.3.EFFECT OF NUTRITIONAL FACTORS ON PROTEASE PRODUCTION

Studies have shown that the production of extracellular enzymes is highly influenced by the availability of additional nutrient sources (Wang et al. 2005; Nagamani et al. 2012; Rani et al. 2012). Addition of carbon, nitrogen and inducers to the solid substrate also significantly enhances protease

production in SSF. Depending on the nature of the substrate and their concentration and additional supplementation of carbon, nitrogen and inducers may repress or induce enzyme production.

3.3.1.EFFECT OF GLUCOSE AS CARBON SUPPLEMENTATION ON PROTEASE PRODUCTION

The choice of the carbon source has a major influence on the production of protease. Based on the preliminary studies, glucose was found to be the best carbon source for maximum protease production by *Penicillium* sp. LCJ228 (data not shown). Wang and Lee (1996) and El-Shore et al. (1997) also reported that glucose proved to be the best carbon source for improving the productivity of the protease by *Conidiobolus coronatus* and *Aspergillus niger*. In the present study, different concentration of glucose ranging from 5 to 30 g/L were studied and control without supplementation of glucose was also maintained. The results showed that 20 g/L of glucose significantly ($p<0.05$) enhanced maximum protease production with 4155.2 U/g ds of protease activity and declined beyond this concentration. The decline in higher concentration of glucose may be due to the repression exerted by excessive amount of metabolizable sugar in protease production. Repression of synthesis and induction of protease by glucose are extensively reported by Taragano et al. (1997). The inducing effect of protease production by *Penicillium* sp. LCJ228 may due to the reason that glucose is a monosaccharide and it is readily available for the metabolism of the fungus for enzyme production.

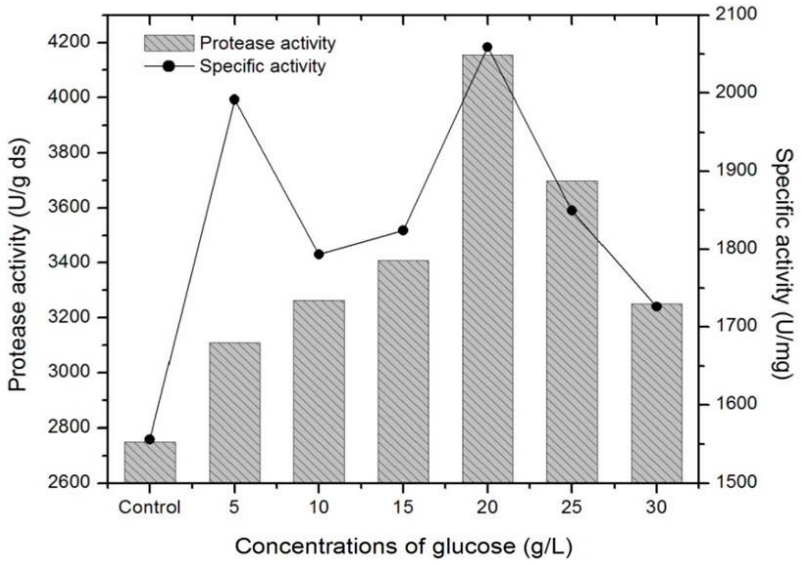

Figure 2

Effect of different glucose concentration added to groundnut oilcake on the protease production by Penicillium sp. LCJ228 on the 6th day of incubation

3.3.2.EFFECT OF YEAST EXTRACT AS NITROGEN SUPPLEMENTATIONS ON PROTEASE PRODUCTION

Addition of nitrogen sources to the solid substrates has been reported to augment protease production in SSF and this was proved in the present study. In the preliminary studies, different nitrogen (both organic and inorganic) sources were analyzed and yeast extract was found to be the best nitrogen source for protease production by *Penicillium* sp. LCJ228 (data not shown). The effect of different concentrations of yeast extract (5 to 30 g/L) added to the solid substrate was studied and results showed that 15 g/L of yeast extract enhanced maximum protease production (4279.8 U/g ds) by *Penicillium* sp. LCJ228 with a 5% level of significance. Phadatare et al. (1993) also reported the enhancement of protease production by the use of yeast extract. Several other studies also suggest that yeast extract is a suitable nitrogen sources which stimulate protease production in *Aspergillus terreus* (Ashour et al. 1996). The production of protease probably was enhanced due to the high protein and amino acid components in the yeast extract.

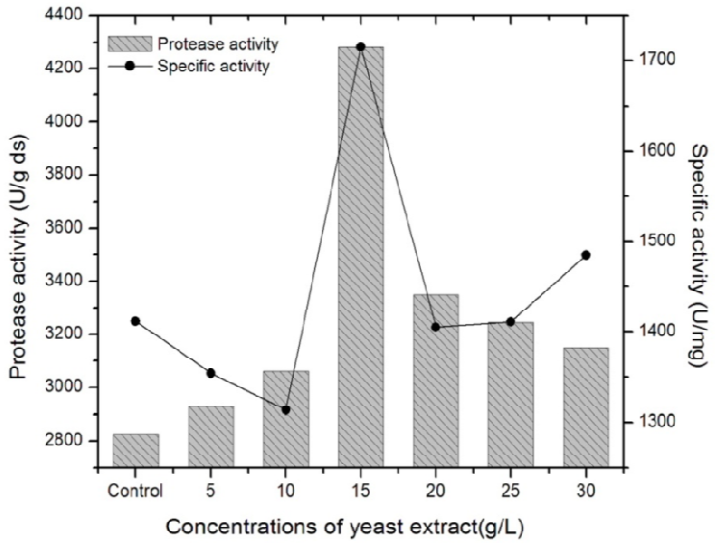

Figure 3

Effect of different yeast extract concentration added to groundnut oilcake on the protease production by Penicillium sp. LCJ228 on the 6th day of incubation

3.3.3.EFFECT OF CASEIN AS INDUCER ON PROTEASE PRODUCTION

The present study proved that casein in a best inducer for protease synthesis by *Penicillium* sp. LCJ228. The effect of different concentrations of casein ranging from 5 to 30 g/L on protease production by *Penicillium* sp. LCJ228 was studied. Results showed that the lower concentration (5 g/L) of casein significantly ($p<0.05$) enhanced maximum protease activity (4012.7 U/g ds) whereas the higher concentration (30 g/L) of casein inhibited protease production with 2800 U/g ds of protease activity. Similar findings were also reported by Battaglino et al. (1991) and Kamath et al. (2010). As casein is a protein, its role in induction of protease synthesis is evident from these results.

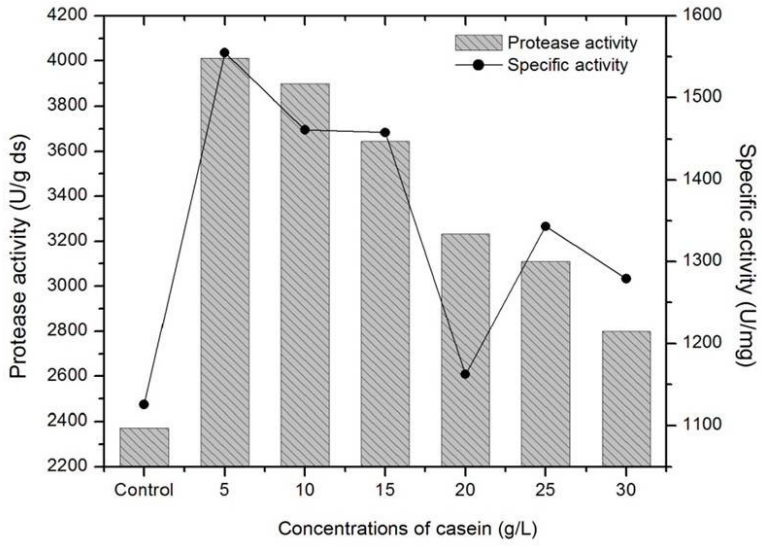

Figure 4

Effect of different casein concentration added to groundnut oilcake on the protease production by Penicillium sp. LCJ228 on the 6th day of incubation

3.4.EFFECT OF PHYSICAL FACTORS ON PROTEASE PRODUCTION

The production of protease under SSF is significantly influenced by physical factors such as pH, temperature, moisture content and inoculum concentration etc. (Srinubabu et al. 2007) and these factors vary widely from species to species. It is

therefore important to optimize these conditions for maximum protease production.

3.4.1.EFFECT OF pH ON PROTEASE PRODUCTION

The pH of the culture medium strongly influences many enzymatic processes and transport of various components across the cell membranes, which in turn support the cell growth and enzyme production (Paranthaman et al. 2009). Usually, fungi grow over a wide range of pH. In the present study, the results showed that initial pH when alkaline, can increase both the proliferation of mycelium as well as protease production in *Penicillium* sp. LCJ228. A maximum protease production (4177.6 U/g ds) was observed at pH 9 with a 5% level of significance (Table 1). Similarly, Munawar et al. (2014) reported alkaline protease activity by *A. terreus* grown on wheat bran. The alkaline proteases have enormous potential in industrial applications. Moreover, production of protease at high pH reduces contamination risk during fermentation processes (Gessesse and Mamo, 1999).

3.4.2.EFFECT OF INOCULUM SIZE ON PROTEASE PRODUCTION

The amount of inoculum used to culture the microorganism in SFF influence protease production. In the present study, the production of protease by *Penicillium* sp. LCJ228 increased with an increase in inoculum size up to a level (2 g/kg) after which enzyme yield was reduced (Table 1). Statistical analysis also showed that 2 g/kg inoculum size was significant at 5% level. When the amount of mycelium increased, it rapidly consumed majority of the substrate for growth, hence decreasing enzyme synthesis (Carlile et al. 2001; Sathya et al. 2009).

Table 1

Effect of pH, inoculum size and moisture content on the production of protease by Penicillium sp. LCJ228 on the 6th day of incubation

Parameters	Protease Activity (U/g ds)
pH	
4	2491.1±12.45^{a}
5	2785.4±20.85^{ab}
6	2802.0±15.01^{bc}
7	3047.2±18.88^{cd}
8	3476.6±28.21^{de}
9	**4177.6**±10.74^{f}
10	3875.2±19.36^{e}
Inoculum size (g/kg)	
1	3986.4±23.96^{bc}
2	**4255.6**±12.76^{c}
3	3801.0±15.24^{b}
4	3764.5±31.35^{b}
5	3210.8±12.84^{a}
Moisture content (%)	
40	1978.1±5.89^{a}
50	2076.4±18.32^{b}
60	2507.5±15.01^{b}
70	3689.2±28.46^{d}
80	**4371.6**±23.04^{e}
90	3789.3±11.39^{d}
100	2544.2±7.63^{c}

*** denotes significance at 1% level; different alphabet between parameters denotes significance at 5% level using Tukey's multiple comparison test; ± indicates the standard deviation of three replicates*

3.4.3.EFFECT OF MOISTURE CONTENT ON PROTEASE PRODUCTION

Moisture content is another important factor influencing the production of protease under SSF. The influence of initial moisture content ranging from 40 to 100% was determined in the present study. The results showed that the protease yield was significantly ($p<0.05$) high (4371.6 U/g ds) with 80% initial moisture content of substrate and declined thereafter (Table 1). This decline in protease production by *Penicillium* sp. LCJ228 beyond 80% may due to the insufficient assessment of the microbes to utilize the nutrients availability for growth and enzyme production (Kumar and Takagi, 1999). The present study reported slightly higher moisture content for maximum protease production when compared with certain other reports. This may be due to the difference in the nature of the solid substrate used for fermentation. After optimization of nutritional and physical parameters for protease production, a

2.2-fold increase in the protease production was observed.

CONCLUSION

Penicillium sp. LCJ228 proved to be an effective producer of alkaline protease by solid state fermentation. The study also concluded that the groundnut oilcake was found to be a potential and a cost-effective substrate for alkaline protease production by *Penicillium* sp. LCJ228. The optimization study also provides the basic information for further development for large scale production of industrially important alkaline protease by *Penicillium* sp. LCJ228.

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REFERENCES

1. Ashour SA, EL Shore HM, Metwally M and Habib SA. Fungal fermentation of Whey incorporated with certain supplements for the production of protease. Microbios. 1996; 86: 59-69.
2. Battaglino RA, Huergo M, Pilosof AMR and Bartholomai GB. Culture requirements for the production of protease by *Aspergillus oryzae* in solid state fermentation. Appl. Microbiol. Biotechnol. 1991; 35: 292-296.
3. Carlile MJ, Watkinson SC and Goody GW. The fungi. Second Ed. Academic Press, London. 2001: pp.475-476.
4. Dixit G. and Verma SC. Production of alkaline protease by *Penicillium griseofulvin*. Indian J. Microbiol. 1993; 33: 257-260.
5. EI-Shore HM, Taragano V, Sanchez VE and Pilos AMR. Microscopic growth of filamentous fungi on solid substrate explained by a microscopic approach. Biotech. Lett. 1997; 19: 233-236.
6. Gessesse A and Mamo G. High-level xylanase production by an alkaliphilic *Bacillus* sp. using solid-state fermentation. Enzyme Microb. Technol. 1999; 25: 68-72.
7. Gnanadoss JJ, Robert R and Jebapriya RG. Production of protease from *Aspergillus niger* and *Mucor mucedo* under submerged and solid state fermentation. Int. J. Cur. Res. 2011; 3: 75-78.
8. Kamath P, Subramanyam VM, Rao JV and Raj PV. Optimization of cultural conditions for protease production by a fungal species. Indian J. Pharm. Sci. 2010; 72: 161-166.
9. Kumar CG and Takagi H. Microbial alkaline proteases: from bioindustrial viewpoint. Biotech. Adv. 1999; 17: 561-594.
10. Kumar MDJ, Rameetha R, Lincy L, Priyadarshini S, Sandhiyachittybabu and Kalaichelvan PT. Destaining and dehairing capability of partially purified *Bacillus subtilis* protease from optimized fermentation medium. Asian J. Exp. Biol. Sci. 2012; 3: 613-620.
11. Leng XW and Xu Y. Improvement of acid protease production by a mixed culture of *Aspergillus niger* and *Aspergillus oryzae* using solid-state fermentation technique. Afr. J. Biotechnol. 2011; 10: 6824-6829.
12. Lowry OH, Rosebrough NJ, Farr AL and Randall R. Protein measurement with Folin Phenol Reagent. J. Biol. Chem. 1951; 193: 265-275.
13. Malathi S and Chakraborthy R. Purification of alkaline protease by a new *Aspergillus flavus* isolate under solid substrate fermentation conditions for the use as a depilation agent. Appl. Environ. Microbiol. 1991; 57: 712-716.
14. Mohan FN, Dileep D and Deepthi D. Potential application of protease isolated from *Pseudomonas auriginosa* PD100. Biotechnol. Ind. 2005; 8: 197-203.
15. Mudgett RE. Solid-state fermentations. In Manual of Industrial Microbiology and Biotechnology, Asiatech Publishers Inc, New Delhi. 2001.
16. Munawar TM, Aruna K and Swamy AVN. Production, purification and characterization of alkaline protease from agro industrial wastes by using *Aspergillus terreus* (AB661667) under solid state fermentation. International Journal of Advanced Research in Engineering and Applied Sciences. 2014; 3: 12-23.
17. Nagamani B, Lakshmi MVV, Sridevi V and Rajani P. Production of Protease from Sesame Oilcake by *Penicillium chrysogenum* under Solid State Fermentation. ijCEPr. 2012; 3: 137-141.
18. Pandey A, Soccol CR, Rodriguez-Leon JA and Nigam P. Solid State Fermentation in

Biotechnology, Asiatech Publishers Inc., New Delhi. 2001.

19. Paranthaman R, Alagusundaram K and Indhumathi J. Production of protease from rice mill wastes by *Aspergillus niger* in Solid State Fermentation. World J. Agr. Sci. 2009; 5: 308-312.
20. Phadatare SU, Deshpande VV and Srinivasan MC. High activity alkaline protease from *Conidiobolus coronatus* (NCL 86.8.20), Enzyme production and compatibility with commercial detergents. Enzym. Microb. Technol. 1993; 15: 72-76.
21. Prakasham RS, Rao SCH and Sarma PN. Green gram husks an inexpensive substrate for alkaline protease production by *Bacillus* sp. in solid state fermentation. Bioresour. Technol. 2006; 97: 1449-1454.
22. Rani RM, Prasad NN and Sambasivarao KRS. Optimization of Cultural Conditions for the Production of Alkaline Protease from a Mutant *Aspergillus flavus* AS2. Asian J. Exp. Biol. Sci. 2012; 3: 565-576.
23. Rao MB, Tanksale AM, Ghatge MS and Deshpande VV. Molecular and biotechnological aspects of microbial proteases. Microbiol. Molecul. Biology. 1998; 62: 597-634.
24. Romero F, Garcia LA and Diaz, M. Protease production from whey at high concentration by *Serratia marcescens*. Resour. Environ. Biotechnol. 1998; 2: 93-115.
25. Santhi R. Extracellular protease production by solid state fermentation using *Punica granatum* peel waste. Indo American Journal of Pharmaceutical Research. 2014; 4: 2706-2712.
26. Sathya R, Pradeep BV, Angayarkanni J and Palaniswamy M. Production of milk clotting protease by a local isolate of *Mucor circinelloides* under SSF using agro-industrial wastes. Biotechnol. Bioprocess Eng. 2009; 14: 788-794.
27. Taragano V, Sanchez VE and Pilosof AMR. Combined effect of water activity depression and glucose addition on pectinases and protease production by *Aspergillus niger*. Biotechnol. Lett. 1997; 19: 233-236.
28. Viswanatha KS, Rao AGA and Singh SA. Acid protease production using solid state fermentation by *Aspergillus oryzae* MTCC 5341: optimization of process parameters. J. Ind. Microb. Biotechnol. 2010; 37: 129-138.
29. Wang R, Chau Sing Law R and Webb C. Protease production and conidiation by *Aspergillus oryzae* in flour fermentation. Process Biochem. 2005; 40: 217-227.
30. Wang Y and Lee M. Influence of culture and nutritional conditions on the production of protease from thermophilic strain of *Aspergillus* sp. NTU-FC671. J. Chinese Agric. Chem. Soc. 1996; 34:732-736.