ORIGINAL RESEARCH PAPER

Botany

KEY WORDS: Solid state fermentation, *Penicillium griseofulvum* LCJ231, mahua oil cake, protease production

MAHUA OIL CAKE AS THE NOVEL AND INEXPENSIVE SUBSTRATE FOR THE PRODUCTION OF PROTEASE BY PENICILLIUM GRISEOFULVUM LCJ231 UNDER SOLID STATE FERMENTATION

X. Josephine Jenitta — PG and Research Department of Botany, Government Arts College for Men Autonomous, Nandanam, Chennai-600 035, Tamil Nadu, India

K. Perinbam* — PG and Research Department of Botany, Government Arts College for Men Autonomous, Nandanam, Chennai-600 035, Tamil Nadu, India
*Corresponding Author

ABSTRACT

Solid state fermentation is an alternative method that has gained more attention of researchers over the past 20 years. In solid state fermentation excellent nutritional value of the oil cakes employed as the substrates for protease production. In this study evaluates the feasibility of using mahua oil cake as a substrate for the production of protease by *Penicillium griseofulvum* LCJ231 under solid state fermentation. The mahua oil cake is very cheap agricultural by-product. The influences of components of the medium and culture conditions such as substrate moisture content, pH and inoculum size for maximizing the protease production was examined by one-factor-at-a-time method. An interesting results were examined through this study is addition of all the nutritional factors (starch, yeast extract and casein) did not enhance the protease production. Only the substrate with water produced the maximum amount of protease. A pH of 8.0, 6g/kg of an inoculum and 80% of the moisture content were secreted maximum protease production. This study proved that *Penicillium griseofulvum* LCJ231 was able to produce a very high level of protease using inexpensive mahua oil cake as the substrate under SSF.

INTRODUCTION

Proteases are served as the multifunctional enzymes accounting for nearly 60% of the whole enzyme market (Woods *et al.,* 2001, Kumar *et al.,* 2018). These proteases are frequently used in detergent, leather, pharmaceutical, food, brewing, meat, dairy and photographical industries (Patil *et al.,* 2004, Chang *et al.,* 2004, Basu *et al.,* 2008, Paranthaman *et al.,* 2009, Ramakrishna *et al.,* 2010). Since based on these applications, there has been renewed attention in the discovery of proteases with novel properties.

Microorganisms are evidence for great potential for protease production due to their broad biochemical diversity and their susceptibility to genetic manipulation. Fungal proteases have an advantage over bacterial proteases as mycelium can be removed easily by the method of filtration (Kranthi *et al.,* 2012). Protease producing fungi are normally recognized as generally regarded as safe (GRAS) (Schuster *et al.,* 2002, Soccol *et al.,* 2003, Singh *et al.,* 2023). Fungi such as *Rhizopus*, *Aspergillus*, *Mucor*, *Peniciliium*, *Trichoderma* species have the ability to produce proteases under suitable growth conditions (Banerjee *et al.,* 2001, Suraishkumar *et al.,* 2003, Haq and Mukhtar, 2004, Barbosa-Tessman *et al.,* 2015).

Solid State Fermentation (SSF) is that there is nearly no free water in the solid substrate (Chen and Xu, 2004, Takashi *et al.,* 2009, Manan and Webb, 2017). Substrate of the solid material not only performs as a physical structure for the microbial growth, but it also provides a carbon, nitrogen source and growth factors for the microorganisms (Pandey *et al.,* 2010). SSF method is the simple method of the fermentation process and can use agricultural wastes are the substrates for the enzyme production (Srinophakun *et al.,* 2008, Sakinah *et al.,* 2022). Solid state fermentation is the solution for solving the energy crisis and environmental pollution (Chen and Qui, 2010, Chen and He, 2012).

Utilization of agro-waste residues as substrates provides an alternative path and value addition to these (Pandey *et al.,* 2009, Vijayaraghavan and Vincent, 2012). Microorganisms take all the nutrients present in the solid substrate for their growth as well as for the protease production. More number of agricultural and industrial wastes are used as the substrates such as tannery solid waste (Kumar *et al.,* 2009), coffee pulp and coffee husk (Pandey *et al.,* 2000), shrimp shell waste (Wang and Yeh, 2006), cow dung (Vijayaraghavan and Vincent, 2012), defatted soybean cake (Germano *et al.,* 2003), wheat bran (Kranthi *et al.,* 2012, Ahmed *et al.,* 2011), combination of soybean and wheat flours (Wang *et al.,* 2005) for enzyme production.

This study was designed to optimize the production of protease by *Penicillium griseofulvum* LCJ231 under SSF by using a suitable substrate. The influence of nutritional factors (carbon source, nitrogen source and inducer) and physical parameters such as incubation time, pH, inoculum size and moisture content were also evaluated.

MATERIALS AND METHODS

Fungal Strain

In this study used *Penicillium griseofulvum* LCJ231 (Accession no. KF414683) was isolated from cotton seed oil cake (Jenitta and Gnanadoss, 2014). This culture was maintained on Potato Dextrose Agar (PDA) at 4°C.

Substrates

The type of twelve different substrates such as rice bran, wheat bran, coconut oil cake, sesame oil cake, groundnut oil cake, mahua oil cake, cotton seed oil cake, neem oil cake, black gram husk, red gram husk, green gram husk and chickpea husk were screened for the production of protease under solid state fermentation.

Solid state fermentation

The 10 g of weighed substrates of husks were soaked with water at overnight and the oil cakes were soaked with 5 h. The excess amount of water was drained off and dries it. The solid state fermentation was carried out in 200 mL stopper bottle. The substrates containing bottles were autoclaved at 121°C for 30 min. then the bottles were cooled and inoculated with one mycelia disc (4 mm). The inoculated bottles were incubated for 9 days at room temperature.

Enzyme Extraction Method

The enzyme was extracted from the substrate using 0.2 M Glycine-NaOH buffer (pH 9.0). 50 mL of Glycine-NaOH buffer was added into the substrates containing bottles. Then the bottles were kept in an orbital shaker for 24 h. After that, the whole contents were filtered using muslin cloth and then the filtrate was centrifuged at 10,000 rpm for 15 min. The clear supernatant was served as the enzyme source for protease assay.

Procedure for protease assay

The protease activity was assayed using casein as a substrate

by the modified method of Keay and Wildi (1970). 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 (Lowry *et al.*, 1951). 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.

Enzyme activity calculation

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

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

Time course study

The production of protease by *Penicillium griseofulvum* LCJ231 was tested by incubating the inoculated solid state fermentation bottles with the substrate for a total period of 15 days. The enzyme was extracted every 3 days intervals and protease activity was also estimated.

The influences of various factors on the production of protease

The protease production by *Penicillium griseofulvum* LCJ231 was studied. The selection process of suitable nutritional factors such as carbon source, nitrogen source and inducers and physical factors such as incubation time, pH, inoculum size and moisture content for maximum protease production was optimized through this study.

Effect of nutritional factors on the production of protease

Effect of carbon concentrations on the production of protease

The impact of various concentrations of the starch (suitable carbon source) ranging from 5 to 30 g/L on the protease production by *Penicillium griseofulvum* LCJ231 was evaluated.

Effect of nitrogen source concentration on the production of protease

In the same way, different concentrations of yeast extract (suitable nitrogen source) in the range from 5 to 30 g/L were tested by *Penicillium griseofulvum* LCJ231 for the enhancement of protease production.

Effect of inducer concentration on the production of protease

The selective inducer of casein concentrations in the range of 5 to 30 g/L was studied for maximum production of protease by *Penicillium griseofulvum* LCJ231. All these experiments were carried out at room temperature. The end of the 9th day of incubation protease activity was measured.

Effect of physical parameters on the production of protease

Effect of pH on the production of protease

The effect of initial pH on protease production by *Penicillium griseofulvum* LCJ231 was evaluated by adjusting the pH of distilled water ranging from 4 to 10. The pH was adjusted using 0.1 N NaOH and 0.1 N HCL.

Effect of inoculum size on the production of protease

The inoculum concentration on the protease production was studied by *Penicillium griseofulvum* LCJ231. An inoculum size range of 1 to 6 g/kg was used in this study.

Effect of initial moisture content on the production of protease

Optimum initial moisture content for protease production was determined by adjusting the initial moisture content of the fermentation substrate to varying levels of 40 to 100%. The protease assay was determined at the end of the 9th day of incubation.

RESULTS AND DISCUSSION

The fungal strain of *Penicillium griseofulvum* LCJ231 was isolated from cotton seed oil cake. The enhancement of protease was evaluated by suitable substrate selection, incubation time, carbon, nitrogen and inducer supplementation, pH, inoculum size and initial moisture content of the substrate.

Suitable substrate selection for solid state fermentation

The selection of an ideal agricultural by-product for enzyme production is the major role in SSF process. This process depends on the cost and substrate availability (Pandey *et al.,* 2000). In this study, twelve different agro-wastes were used as the substrate for protease production by *Penicillium griseofulvum* LCJ231 under solid state fermentation. Maximum protease production (420.0 U//g ds) was observed with mahua oil cake (Table 1). Followed by groundnut oil cake produced more amount of protease (355.18 U/g ds). These two substrates were selected for this culture.

In this paper deals with the protease production using mahua oil cake as the substrate. The result presented here is highly significant because more reports not presented on the use of mahua oil cake as the substrate for the production of protease. Based on this fact, mahua oil cake can be effectively utilized as an ideal substrate for the production of protease due to its low cost and availability.

Table 1: Screening Of Suitable Substrate For Protease Production

Substrates	Protease activity (U/g ds)
Rice bran	69.86± 0.87
Wheat bran	77.28± 1.35
Coconut oil cake	188.30± 0.99
Sesame oil cake	237.30± 4.32
Groundnut oil cake	355.18± 0.65
Mahua oil cake	420.00± 1.78
Cottonseed oil cake	196.14± 0.67
Neem oil cake	220.78± 3.13
Black gram husk	59.36± 0.99
Red gram husk	76.58± 3.08
Green gram husk	80.85± 4.10
Chickpea husk	12.78± 0.86

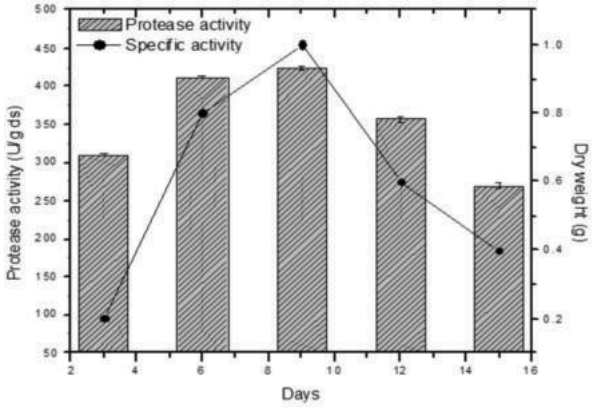

Fig. 1 Growth and protease production by *P. griseofulvum* LCJ231 using mahua oil cake as substrate

Time Course Study

The influence of incubation time on protease production by *Penicillium griseofulvum* LCJ231 was evaluated. In the present

study, maximum protease production was observed on the 9th day of incubation with the protease activity of 424.38 U/g ds (Figure 1). The protease activity was gradually decreased with increasing the time of incubation. This result clearly indicated protease was produced in the log phase of the fungal growth for utilization of nutrients present in the oil cake. Sumantha *et al.* (2006) also agree the growth of the fungus utilized the nutrients present in the solid substrate. The fungal log phase may vary from one organism to another. Maharshi and Thaker (2012) reported 96 h onwards upto 288 h showed the log phase in *Fusarium* sp.

Effect Of Nutritional Factors On The Production Of Protease

The additional nutrient sources influenced the production of protease (Wang *et al.,* 2005, Nagamani *et al.,* 2012, Rani *et al.,* 2012). Additional nutritional sources of carbon, nitrogen and inducers may or may not be enhances the protease production due to the nutrient present in the used substrate.

Effect Of Starch As A Suitable Carbon Source On The Production Of Protease

The suitable selection of the carbon source has a major point on the protease production. Based on the preliminary studies, starch found to be the good carbon source for the production of protease by *Penicillium griseofulvum* LCJ231 (data not shown). Puri *et al.* (2002) also found starch as the best carbon source for maximum protease production by *Bacillus* sp.

In this study, various concentrations of starch ranging from 5 to 30 g/L were tested for maximum protease production. Without supplementation of starch was used as the control. The results proved without the addition of starch produced maximum protease and this data was significantly ($p<0.05$) proved in Table 2. The substrate gave the sufficient amount of nutrients to the culture. The mahua oil cake contains 68.3% of the fixed carbon (Kureel *et al.,* 2009). Kumar *et al.* (2018) reported that Mahua oil cake is rich in sugar.

Effect Of Yeast Extract On The Production Of Protease

The addition of nitrogen sources to the substrates has been reported to increase the protease production. In the preliminary studies, yeast extract was the best nitrogen source for protease production (data not shown). The same finding was reported by Nadeem *et al.* (2008).

The effects of various concentrations of yeast extract (5 to 30 g/L) supplemented to the solid substrate was tested and results showed that control without the addition of yeast extract enhanced the maximum protease production (160.18 U/g ds) by *Penicillium griseofulvum* LCJ231 represented in Table 2. This result was significant at a 5% level. In mahua oil cake more number of organic substances is present. These are act as the nitrogen source and gave the nutrients to the fungus. Kureel *et al.* (2009) reported that 90.8% of the organic matter is present in the mahua oil cake. Mahua seed cakes were found to be rich in nutrients, especially nitrogen source (Kumar *et al.,* 2018).

Table 2: Effect Of Nutritional Factors Concentrations On Protease Production By *Penicillium Griseofulvum* LCJ321

Variables	Protease activity (U/g ds)
Control	160.18± 9.05
Starch concentration (g/L)	
(p 0.932)	
5	150.10± 5.54a
10	153.90± 5.93a
15	154.92± 6.22a
20	157.50± 0.67a
25	156.13± 5.54a
30	150.63± 9.43a
Yeast extract concentration (g/L)	
(p 0.001**)	
5	99.34± 0.19a
10	118.77± 0.30c
15	118.59± 2.54c
20	115.53± 4.27bc
25	100.86± 4.08ab
30	91.92± 0.00a
Casein concentration (g/L)	
(p 0.006**)	
5	119.28± 5.43c
10	109.44± 9.81c
15	102.64± 1.25ab
20	99.23± 7.77ab
25	98.95± 1.16ab
30	93.08± 1.94a

Effect Of Casein As The Inducer On The Production Of Protease

The effect of casein on the protease production by *Penicillium griseofulvum* LCJ231 was studied at different ranging from 5 to 30 g/L. The results represented that the addition of casein to the substrate produced very low amount of protease as compared to the control (160.18 U/g ds), that is without the addition of inducer (Table 2). This result was significantly ($p<0.05$) proved with the statistical analysis. The reason is the oil cake of mahua contain very rich amount of proteins. These proteins are served as the nutrients to *Penicillium griseofulvum* LCJ231 and produced a maximum protease production. This result proved the cost effectiveness of the solid state fermentation. Mahua oil cakes are the good and cheap source of proteins and also contain essential amino acids (Kureel *et al.,* 2009). Kumar *et al.,* (2018) proved 19.68% of proteins present in Mahua oil cake.

Effect of physical parameters on the production of protease

Under solid state fermentation the protease production was significantly enhanced by physical factors such as pH, temperature, moisture content and concentrations of inoculum (Srinubabu *et al.,* 2007, Gnanadoss *et al.,* 2015). These factors are differing from one species to another species. Therefore optimizations of these conditions were important for the enhancement of protease production.

Effect of pH on the production of protease

The pH strongly influences the process of enzymes (Paranthaman *et al.,* 2009). Generally fungi can grow over a wide range of pH. In this study, the results examined the pH 8 produced maximum protease production (166.71 U/g ds) showed in Table 3. The data was proved at 5% level of significant. In the same way Munawar *et al.* (2014) reported that the protease activity was high at alkaline level by *A. terreus*. Gnanadoss *et al.* (2015) also proved the pH of 9 produced the maximum amount of protease by *Penicillium* sp. using groundnut oil cake as the substrate.

Effect of inoculum size on the production of protease

The effect of inoculums size on protease production by *Penicillium griseofulvum* LCJ231 was tested. In the fermentation process, inoculums size is an important biological factor (Norliza and Ibrahim, 2005, Pandey *et al.,* 2005). An exponential increase in the protease production was recorded with increasing the size of the inoculums (Table 3). Similar finding was also reported by Patil *et al.* (2004). The present study clearly indicates that the oil cake have the large number of nutrients in it. So that reason only large amount of inoculums also not affects the protease production.

Effect of moisture content on the production of protease

Moisture content also one of the major factor for influencing the protease production under solid state fermentation. The impact of initial moisture content ranging from 40 to 100% was examined by *Penicillium griseofulvum* LCJ231. The results showed that 80% of the initial moisture content of the

substrate was produced maximum yield of protease (142.58 U/g ds) with 5% level of the significant (Table 3). Similarly Gnanadoss *et al.* (2015) reported the high protease with 80% of the moisture content level. The level of 90% onwards decreased protease activity was recorded due to the insufficient assessment of the fungi to utilize the nutrients availability for enzyme production (Kumar and Takagi, 1999).

Table 3: Effect of physical factors on protease production by *Penicillium griseofulvum* LCJ231

Variables	Protease activity (U/g ds)
pH	
(p 0.001)**	
4	143.55± 0.77a
5	154.20± 1.06ab
6	160.73± 1.16bc
7	162.31± 1.46bc
8	166.71± 3.79c
9	157.36± 0.01bc
10	153.24± 3.01ab
Inoculum size (g/kg)	
(p 0.000)**	
1	101.40± 2.71a
2	105.91± 4.15ab
3	117.49± 1.45bc
4	128.90± 0.71cd
5	129.68± 3.79cd
6	136.94± 1.47d
Moisture content (%)	
(p 0.000)**	
40	123.33± 2.05a
50	128.42± 3.50a
60	127.25± 0.28a
70	130.83± 2.43b
80	142.58± 6.61c
90	136.81± 1.35bc
100	133.64± 0.77b

CONCLUSION

In the present study, protease was produced using mahua oil cake as substrate. Due to its availability and low cost, mahua oil cake may be a best substrate for solid state fermentation. This oil cake utilization also proved the low cost and the best substrate. Because any addition of carbon, nitrogen and inducer were no need for the production protease. Mahua oil cake contain proteins in a huge level, only these proteins are sufficient for the growth of fungi and also the protease production. Physical factors also enhanced the protease production by *Penicillium griseofulvum* LCJ231.

REFERENCES

1. Agrawal, D., Patidar, P., Banerjee, T., and Patil, S. (2004). Production of alkaline protease by Penicillium sp. under SSF conditions and its application to soy protein hydrolysis. *Process Biochemistry*, *39*(8), 977-981.
2. Ahmed, I., Zia, M. A., Iftikhar, T., and Iqbal, H. M. (2011). Characterization and detergent compatibility of purified protease produced from Aspergillus niger by utilizing agro wastes. *BioResources*, *6*(4), 4505-4522.
3. Benluvankar, V., Jebapriya, G.R. and Gnanadoss, J.J. (2015). Protease production by Penicillium sp. LCJ228 under solid state fermentation using groundnut oilcake as substrate. *Life*, *50*, p.12.
4. Chen, H. and Qiu, W. (2010). Key technologies for bioethanol production from lignocellulose. *Biotechnology Advances*, *28*(5), pp.556-562.
5. Chen, H. Z., and Xu, J. (2004). Principle and application of modern solid-state fermentation.
6. Chen, H.Z. and He, Q. (2012). Value‐added bioconversion of biomass by solid‐state fermentation. *Journal of Chemical Technology & Biotechnology,* 87(12), pp.1619-1625.
7. Chilakamarry, C.R., Sakinah, A.M., Zularisam, A.W., Sirohi, R., Khilji, I.A., Ahmad, N. and Pandey, A. (2022). Advances in solid-state fermentation for bioconversion of agricultural wastes to value-added products: Opportunities and challenges. *Bioresource technology*, *343*, p.126065.
8. Chutmanop, J., Chuichulcherm, S., Chisti, Y., and Srinophakun, P. (2008). Protease production by Aspergillus oryzae in solid‐state fermentation using agroindustrial substrates. *Journal of Chemical Technology and Biotechnology,* 83(7), 1012-1018.
9. Germano, S., Pandey, A., Osaku, C. A., Rocha, S. N., and Soccol, C. R. (2003). Characterization and stability of proteases from Penicillium sp. produced by solid-state fermentation. *Enzyme and Microbial Technology*, *32*(2), 246-251.
10. Gupta, A., Sharma, A., Pathak, R., Kumar, A. and Sharma, S. (2018). Solid state fermentation of non-edible oil seed cakes for production of proteases and cellulases and degradation of anti-nutritional factors. *J. Food Biotechnol. Res*, *2*(4), pp.1-6.
11. Haq, I., and Mukhtar, H. (2004). Biosynthesis of proteases by Rhizopus oligosporus IHS13 in low‐cost medium by solid‐state fermentation. *Journal of basic microbiology*, *44*(4), 280-287.
12. Jenitta, X. J., and Gnanadoss, J. J. (2014). Screening, molecular characterization and phylogenetic analysis of *Penicillium* sp producing high levels of protease. *International journal of current science*. 11: E 10-17.
13. Joo, H. S., Kumar, C. G., Park, G. C., Paik, S. R., and Chang, C. S. (2004). Bleach-resistant alkaline protease produced by a Bacillus sp. isolated from the Korean polychaete, Periserrula leucophryna. *Process Biochemistry*, *39*(11), 1441-1447.
14. Keay, L., & Wildi, B. S. (1970). Proteases of the genus Bacillus. I. Neutral proteases. *Biotechnology and Bioengineering*, *12*(2), 179-212.
15. Kranthi, V. S., Rao, D. M., and Jaganmohan, P. (2012). Production of Protease by Aspergillus flavus Through Solid State Fermentation Using Different Oil Seed Cakes. *Intl. J*, *3*(1), 12-15.
16. Kumar, A.G., Venkatesan, R., Prasad Rao, B., Swarnalatha, S. and Sekaran, G. (2009). Utilization of tannery solid waste for protease production by Synergistes sp. in solid‐state fermentation and partial protease characterization. *Engineering in Life Sciences,* 9(1), pp.66-73.
17. Kumar, C. G., and Takagi, H. (1999). Microbial alkaline proteases: from a bioindustrial viewpoint. *Biotechnology advances*, *17*(7), 561-594.
18. Kureel, R. S., Kishor, R., Dutt, D. and Ashutosh, P. (2009). Mahua: a potential tree borne oilseed. *National oilseeds & Vegetable oils Development board, Ministry of Agriculture, Govt. of India, Gurugaon. Pg*, 1-21.
19. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *J biol Chem*, *193*(1), 265-275.
20. Lumi Abe, C.A., Bertechini Faria, C., Fernandes de Castro, F., De Souza, S.R., dos Santos, F.C., Novais da Silva, C., Tessmann, D.J. and Barbosa-Tessmann, I.P. (2015). Fungi isolated from maize (Zea mays L.) grains and production of associated enzyme activities. *International journal of molecular sciences*, *16*(7), pp.15328-15346.
21. Manan, M.A. and Webb, C. (2017). Design aspects of solid state fermentation as applied to microbial bioprocessing. *J Appl Biotechnol Bioeng*, *4*(1), p.91.
22. Munawar, T.M, Aruna, K and Swamy, A.V.N. (2014). 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. 3: 12-23.
23. Nadeem, M., Qazi, J. I., Baig, S and Syed, Q. (2008). Effect of medium composition on commercially important alkaline protease production by Bacillus licheniformis N-2. *Food Technol Biotechnol*, *46*(4), 388-394.
24. Nagamani, B., Lakshmi, M. V. V. C., Sridevi, V., and Rajani, P. (2012). Production of Protease from Sesame Oil Cake by Penicillium Chrysogenum under Solid State Fermentation. *International Journal Of Chemical, Environmental And Pharmaceutical Research*, *3*(2), 137-141.
25. Norliza, A. W., and Ibrahim, C. O. (2005). The production of benzaldehyde by Rhizopus oligosporus USM R1 in a solid state fermentation (SSF) system of soy bean meal: rice husks. *Malays J Microbiol*, *1*, 17-24.
26. Pandey, A., Soccol, C. R., Nigam, P., Brand, D., Mohan, R., and Roussos, S. (2000). Biotechnological potential of coffee pulp and coffee husk for bioprocesses. *Biochemical Engineering Journal*, *6*(2), 153-162.
27. Paranthaman, R., Alagusundaram, K., and Indhumathi, J. (2009). Production of protease from rice mill wastes by Aspergillus niger in solid state fermentation. *World Journal of Agricultural Sciences*, *5*(3), 308-312.
28. Pawar, K.S., Singh, P.N. and Singh, S.K. (2023). Fungal alkaline proteases and their potential applications in different industries. *Frontiers in Microbiology*, *14*, p.1138401.
29. Puri, S., Beg, Q. K., and Gupta, R. (2002). Optimization of alkaline protease production from Bacillus sp. by response surface methodology. *Current microbiology*, *44*(4), 286-290.
30. Ramakrishna, D.P.N., Gopi, R.N. and Rajagopal, S.V., 2010. Purification and properties of an extra cellular alkaline protease produced by Bacillus Subtilis (MTCC N0-10110). *International Journal of Biotechnology & Biochemistry*, *6*(4), pp.489-501.
31. Rani, M.R., Prasad, N.N. and Sambasivarao, K.R.S. (2012). Optimization of cultural conditions for the production of alkaline protease from a mutant Aspergillus Flavus AS2. *Asian J Exp Biol Sci*, *3*, pp.565-576.
32. Sahoo, S., Rao, K. K., and Suraishkumar, G. K. (2003). Induced reactive oxygen species improve enzyme production from Aspergillus niger cultivation. *Biotechnology letters*, *25*(10), 821-825.
33. Sandhya, C., Sumantha, A., Szakacs, G., and Pandey, A. (2005). Comparative evaluation of neutral protease production by Aspergillus oryzae in submerged and solid-state fermentation. *Process Biochemistry*, *40*(8), 2689-2694.
34. Schuster, E., Dunn-Coleman, N., Frisvad, J., and Van Dijck, P. (2002). On the safety of Aspergillus niger–a review. *Applied microbiology and biotechnology*, *59*(4-5), 426-435.
35. Singhania, R. R., Patel, A. K., Soccol, C. R., and Pandey, A. (2009). Recent advances in solid-state fermentation. *Biochemical Engineering Journal*, *44*(1), 13-18.
36. Singhania, R. R., Sukumaran, R. K., Patel, A. K., Larroche, C., and Pandey, A. (2010). Advancement and comparative profiles in the production technologies using solid-state and submerged fermentation for microbial cellulases. *Enzyme and Microbial Technology*, *46*(7), 541-549.
37. Srinubabu, G., Lokeswari, N., and Jayaraju, K. (2007). Screening of nutritional parameters for the production of protease from Aspergillus oryzae. *Journal of Chemistry*, *4*(2), 208-215.
38. Sumantha, A., Larroche, C., and Pandey, A. (2006). Microbiology and industrial biotechnology of food-grade proteases: a perspective. *Food Technology and Biotechnology*, *44*(2), 211.
39. Takashi, A.N.O., Jin, G.Y., Mizumoto, S., Shahedur, R.M., Okuno, K. and Shoda, M. (2009). Solid state fermentation of lipopeptide antibiotic iturin A by using a novel solid state fermentation reactor system. *Journal of Environmental Sciences*, *21*, pp.S162-S165.
40. Tunga, R., Banerjee, R., and Bhattacharyya, B. C. (2001). Optimization of some additives to improve protease production under SSF. *Indian journal of*

experimental biology, *39*(11), 1144-1148.
41. Vijayaraghavan, P., and Vincent, S. G. P. (2012). Cow dung as a novel, inexpensive substrate for the production of a halo-tolerant alkaline protease by Halomonas sp. PV1 for eco-friendly applications. *Biochemical Engineering Journal*, *69*, 57-60.
42. Wang, R., Law, R. C. S., and Webb, C. (2005). Protease production and condition by Aspergillus oryzae in flour fermentation. *Process Biochemistry*, *40*(1), 217-227.
43. Wang, S. L., and Yeh, P. Y. (2006). Production of a surfactant-and solvent-stable alkaliphilic protease by bioconversion of shrimp shell wastes fermented by Bacillus subtilis TKU007. *Process Biochemistry*, *41*(7), 1545-1552.
44. Woods, R., Burger, M., Bevan, □., and Beachem, I. (2001). Extracellular enzyme production in *Pseudomonas flourescens*. *J. Microbiol*, *143*, 345-354.