Production, Purification, and Characterization of Alkaline Protease from *Aspergillus flavus* and its Compatibility with Commercial Detergents

Amtul W. Wajeeha,[a] M. Javaid Asad,[a] Raja Tahir Mahmood,[b] Tayyaba Zainab,[a] Sidrah Nazir,[a] Jehangir Khan,[c] Muhammad B. Shah,[d] Maqsood Ahmed,[b] Syed L. Shah,[e] Muhammad Ismail,[e] Nasib Zaman,[f] Dawood Ahmed,[g] M. Imran Khan,[h] and Muhammad Rizwan [f]

Aspergillus flavus was used to produce alkaline protease. Solid state fermentation (SSF) strategy was adopted to explore the most favorable physical and nutritional conditions for enzyme production. Maximum production was achieved at pH 6.0 and a temperature of 30 °C after 84 h of growth period. For the optimization of the chemical parameters, different carbon and nitrogen sources were used including glucose, fructose, sucrose, ammonium sulphate, and urea. Maximum production was observed with 0.3% concentration of all the compounds. Ammonium sulphate salting out and gel chromatography was used to purify the enzyme. The enzyme was completely precipitated out at 80% saturation. The value of V_{max} was 3.9 U/mL, while the value of K_m was 1.9 mg/mL. The enzyme was tested for its compatibility with a few famous detergents available on the market. With the alkaline protease under study, the enzyme displayed a maximum retention of its activity i.e. 80.8% in the presence of commercial detergent Surf excel. The activity dropped down to 61.5% when the enzyme was allowed to work in the presence of another locally used detergent, *i.e.*, Bonus. Protease production from *A. flavus* was carried out on rice bran and wheat bran and the wheat bran gave better results.

Contact information: a: Department of Biochemistry, Pir Mehr Ali Shah, Arid Agriculture University, Rawalpindi, Pakistan; b: Department of Biotechnology, Mirpur University of Science and Technology, Mirpur-10250 AJK, Pakistan; c: Department of Biosciences, University of WAH, WAH Pakistan, d: Department of Plant Breeding & Genetics, Balochistan Agriculture College Quetta, Pakistan; e: Pakistan Museum of Natural History, Garden Avenue Shakarparian Islamabad (Pakistan), f: Center for Biotechnology and Microbiology, University of Swat, KPK, Pakistan, g: Department of Medical Laboratory Technology, The University of, Haripur, KPK, Pakistan, h: Department of Mathematics & Statistics, University of Agriculture Faisalabad;
* Corresponding author: javaidasad@uaar.edu.pk

INTRODUCTION

Enzymes are the catalysts that regulate many chemical reactions occurring in the living body and are called biocatalysts (Raimi *et al*. 2011). Proteases are enzymes that promote the cleavage of peptide bonds through hydrolysis (Franco *et al*. 2017). They can be acidic, neutral, or alkaline. Alkaline proteases show the maximum rate of reaction at high pH values. They can withstand high temperature as well. Therefore, they have become of crucial importance to many industries. Alkaline proteases have found their largest use in domestic detergents. Proteases in the detergent industry account for approximately 25% of the total global enzyme sales (Demain and Adrio 2008). Other fields utilizing these

enzymes include leather processing, the food industry, waste management, and silk degumming. Proteases can be harvested from plants, animals, or microorganisms, but the most efficient source is the microbes. Fungal strains have been successfully employed for this purpose. Fungi have many advantages over other microorganisms, such as their mycelia can be easily separated, and enzyme downstream processing is easier (de Souza *et al*. 2015). Many species of Aspergillus have been employed for this task (Devi *et al*. 2008). Researchers are competing for novel and more productive strains for protease production (Kumara and Takagi 1999). The current study aims to obtain a high yield of alkaline protease from *Aspergillus flavus* by estimating the most suitable conditions and checking the compatibility of the enzyme with commercial detergents.

EXPERIMENTAL

Fermentative Organism

Aspergillus flavus was used as fermentative organism for the production of alkaline protease. It was obtained from the Industrial and Environmental Biotechnology Laboratory, University Institute of Biochemistry and Biotechnology (UIBB), PMAS-Arid Agriculture University, Rawalpindi, Pakistan and was cultured on Potato Dextrose Agar media (PDA) having pH 5.5. All of the chemicals used were of analytical grade and purchased from Sigma Aldrich (St. Louis, Missouri, USA).

Preparation of Fungal Inoculum

The fungal inoculum was prepared for solid state fermentation (SSF) by transferring a loop full of *A. flavus* spores from the pure culture into 100 mL of liquid potato dextrose broth (pH 5.5). It was incubated in a shaking incubator to obtain 10^7 to 10^9 spores/mL for 4 to 5 days.

Solid State Fermentation

For SSF, two substrates, *i.e.* wheat bran and rice bran, were screened for the production of alkaline protease for 8 days. Wheat bran showed higher activity with better fungal growth and was selected as the substrate for further study. It was collected from the local market and dried to zero moisture level and ground into a fine powder (mesh size 40 mm). The required amount (2 to 10 g) of the dried, ground substrate was placed in a 100-mL flask. Distilled water was then added, which maintained the moisture level at 50% (w/v) of the substrate. The flasks were cotton plugged and autoclaved at 121 °C for 15 min. The sterile solid substrate was inoculated with an inoculum of *A. flavus*, using a sterile pipette. The flasks were placed in the incubator at the required temperature for desired incubation time (according to the experimental design).

Enzyme Extraction

After the incubation, the flask containing the SSF culture was removed and 50 mL of distilled water was added. The culture was mixed and placed in the shaking incubator at 30 °C and 120 rpm for 60 min for homogenization. The mixture was filtered through Whatman No. 1 filter paper. After filtration, the mixture was centrifuged at 10,000 rpm for 15 min at 4 °C. The supernatant was stored as a crude enzyme at 4 °C and the pallet was discarded.

Alkaline Protease Assay

Protease activity in the crude enzyme extract was determined using the protocol given by Chandrasekaran *et al.* (2015), using casein as the substrate. Tris-HCl buffer (0.1 M and pH 8.5; Sigma Aldrich, St. Louis, USA, was used to make 0.65% casein solution. A total of 5 mL of this solution was placed into all the vials, and they were kept at 37 °C for 5 min to 10 min. Then, 1 mL of crude enzyme was collected from each of the enzyme samples and added into its respective reaction vial. No enzyme was placed in the blank vial. It was homogenized by gentle shaking and placed at 37 °C for 30 min. To stop the reaction, 5 mL of chilled trichloroacetic acid (TCA), 10%; solution was poured into all of the vials including the blank vial. The tubes were again kept for 30 min at room temperature. The solution from the vials was sieved through 0.45-µm syringe filters. Then, 5 mL of 0.5 M sodium carbonate (Na_2CO_3) was added to 2 mL of the filtrate. The filtrate was taken in separate vials. Sodium carbonate solution was prepared by dissolving 26.5 g of the salt in 500 mL of the solution. For superior results, 1 mL of Folin-Ciocalteu (FC) phenol (2-fold diluted) reagent was added instantly after the sodium carbonate. The resulting solutions were placed in very dim light for 30 min at room temperature and a brilliant blue color was achieved. The absorbance of the blue color compound was measured at 660 nm against a reagent blank using tyrosine standard. One protease unit was defined as the amount of enzyme that releases 1 µg of tyrosine per minute at pH 8.5 at 37 °C (Lowry *et al.* 1951).

Optimization of Physical Parameters for Maximum Enzyme Production

Growth parameters, including incubation period, pH, temperature, inoculum size, and substrate size, were optimized for the maximum production of alkaline protease through SSF using rice bran and wheat bran as substrates. Response surface methodology (RSM) was employed to design optimization experiments under central composite design (CCD). For this purpose, JMP 13.0 software (by SAS institute, Cary, NC, USA) was used, which designed 28 experiments with two central points. RSM is a statistical tool that was used to design experiments for studying the effect of various independent parameters on single dependent parameter. It reduced the number of treatments as well as time period for the research. The program analyzed the interaction between various parameters (independent) during their effect on the other parameters.

Optimization of Carbon and Nitrogen Source for Maximum Enzyme Production

Glucose, fructose, sucrose, ammonium sulfate, and urea were used as the carbon and nitrogen sources. The RSM was used under CCD, there were 28 experiments with two central points to optimize the 5 parameters simultaneously.

Ammonium Sulphate Precipitation Method

Proteins in the crude enzyme extract were purified by adding ammonium sulphate according to different saturation levels of the salt including 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, and 85%. The samples were left overnight at 4 °C. Later the samples were centrifuged for 15 min, at 4 °C, and at 10,000 rpm. The pallets were dissolved in 1.5 mL of Tris-HCl buffer (pH 8.5). Both the supernatant and the pallet were subjected to alkaline protease assay.

Gel Filtration Chromatography

Gel filtration chromatography was performed for the enzyme purification. Sephadex G-100 resin (bead size 20 to 50 µm) was used for gel filtration chromatography, and it was packed in 3 cm×100 cm column. The gel was equilibrated with 0.05 M tris-HCl buffer (pH 8) and eluted with the same buffer at a flow rate of 30 mL/h. Total 30 elusions (1 mL) were collected and first five were discarded and other 25 elusions were assayed for the alkaline protease activity.

Characterization of Alkaline Protease

The enzyme reaction was conducted at different values of pH (6.5 to 11.5) and temperature (25 to 50 °C) to calculate the optimum pH and temperature. Tris-HCl buffer was used to maintain pH from 6.5 to 8.5 and from 9.5 to 11.5 sodium bicarbonate buffer was used.

Estimation of Kinetic Parameters

The reaction of the enzyme was checked for multiple casein concentrations made in tris-HCl (0.1 M) buffer at optimum values of pH and temperature. The results obtained were used for the calculation of K_m (mg/mL), and V_{max} (Units/mL/min) calculated based on Michaelis-Menten model.

Alkaline Protease Compatibility with Commercial Detergents

Commercial detergent solutions (7 mg/mL) Surf Excel, Arial, Brite, and Bonus were prepared in distilled water. These solutions were boiled for 10 min to inactivate any already present proteases.

After cooling, the reaction mixture was made by adding 5 mL of 0.65% casein made in tris-HCl buffer (0.1 M, pH 7.5) and 1 mL of detergent solution. After 10 min of incubation, 1 mL of enzyme solution was added to all the reaction mixtures. These were kept at 45 °C for 30 min. After 30 min, the reaction was stopped *via* the addition of 5 mL of 10% of TCA solution.

The mixture was left again at room temperature (25°C) for 30 min and filtered through Whatman No. 1 filter paper. Then, 2 mL of the filtrate was transferred to a separate tube. Next, 5 mL of sodium carbonate (0.5 M) was added, followed by 1 mL of 0.5 M FC reagent. The mixture was incubated at room temperature for 30 min. and OD was noted at 660 nm.

Alkaline Protease Production on Wheat Bran and Rice Bran

The production of alkaline protease was checked at the optimum level of all variables, *i.e.* pH 6, temperature 30 °C, time period 84 h, inoculum size 3.5 mL, substrate 6 g, and 0.3% of each of the carbon and nitrogen sources. The experiment was conducted in triplicate with wheat bran and rice bran.

Statistical Analysis

A statistical analysis of the experimental data was conducted using JMP Response Surface Design and Surface Plot graphs.

RESULTS AND DISCUSSION

A. flavus Culture

  A. flavus was cultured on a PDA petri plate. After 2 days of growth, the immature colonies appeared velvety and white. The mature colonies after 5 days of incubation appeared dark green. For the preservation of fungus for the current study, its slants were prepared. There are less chances of contamination in slant culture due to less surface area.

Solid State Fermentation

  As reviewed by Muthulakshmi *et al*. (2011), SSF was found to be an inexpensive and highly productive way of producing important enzymes including alkaline proteases. Agricultural byproducts can be used as cheap raw material for the synthesis of industrially important enzymes and chemicals. Rice bran was chosen as the substrate for *A. flavus* growth in SSF (Silva *et al.* 2005).
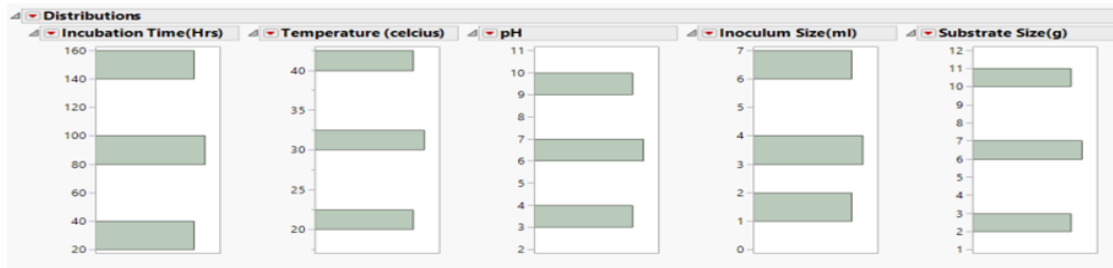
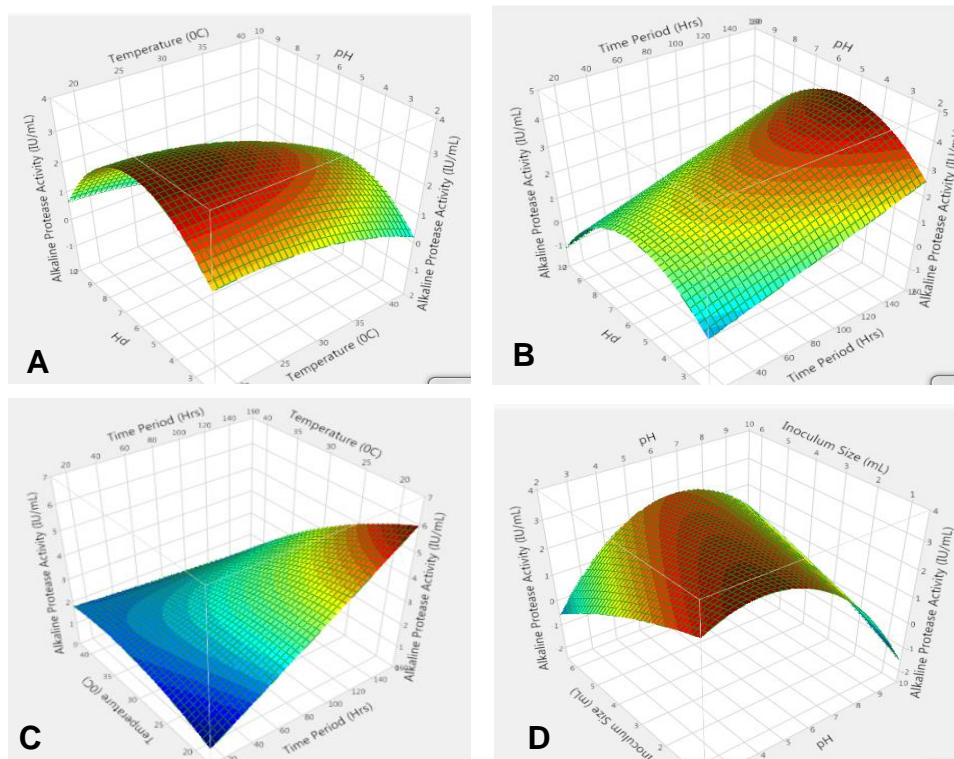**Fig. 1.** Response surface design showing the distribution for physical factors

Fig. 2. 3D response surface graphs showing interaction between (a) Temperature & pH (b) Incubation time & pH (c) incubation time & temperature (d) pH & inoculum size

Optimization of Parameters for _A. flavus_ Growth and Enzyme Production

Physical parameters – Optimization and interaction

　　　A statistical analysis of the data by CCD and distribution analysis showed that the optimum pH and temperature for enzyme production were 6.0 and 30 °C, respectively. The highest activity was observed in the presence of 6g substrate, 3.5 mL of fungal inoculum and after 84 h (Fig. 1).

　　　Interaction among these parameters and the impact on enzyme production was studied. The results showed a positive interaction between various parameters (Fig. 2a-d). This positive interaction can be observed from the umbrella like graph and the peak of the graph indicates the highest activity at the values on the x- and y-axis. The protease activity decreased on both sides of the peak value.

Chemical parameters – Optimization and interaction

　　　The data obtained were processed by CCD and surface plots to obtain the optimum concentration of chemicals used and to study the interaction among the carbon nitrogen sources. The analysis of 3D response surface graphs showed that activity reached up to 6.5 IU/mL/min, while the most suitable concentrations were 0.4% glucose, 0.2% fructose, 0.35% urea, and 0.2% ammonium sulfate (Fig. 3).
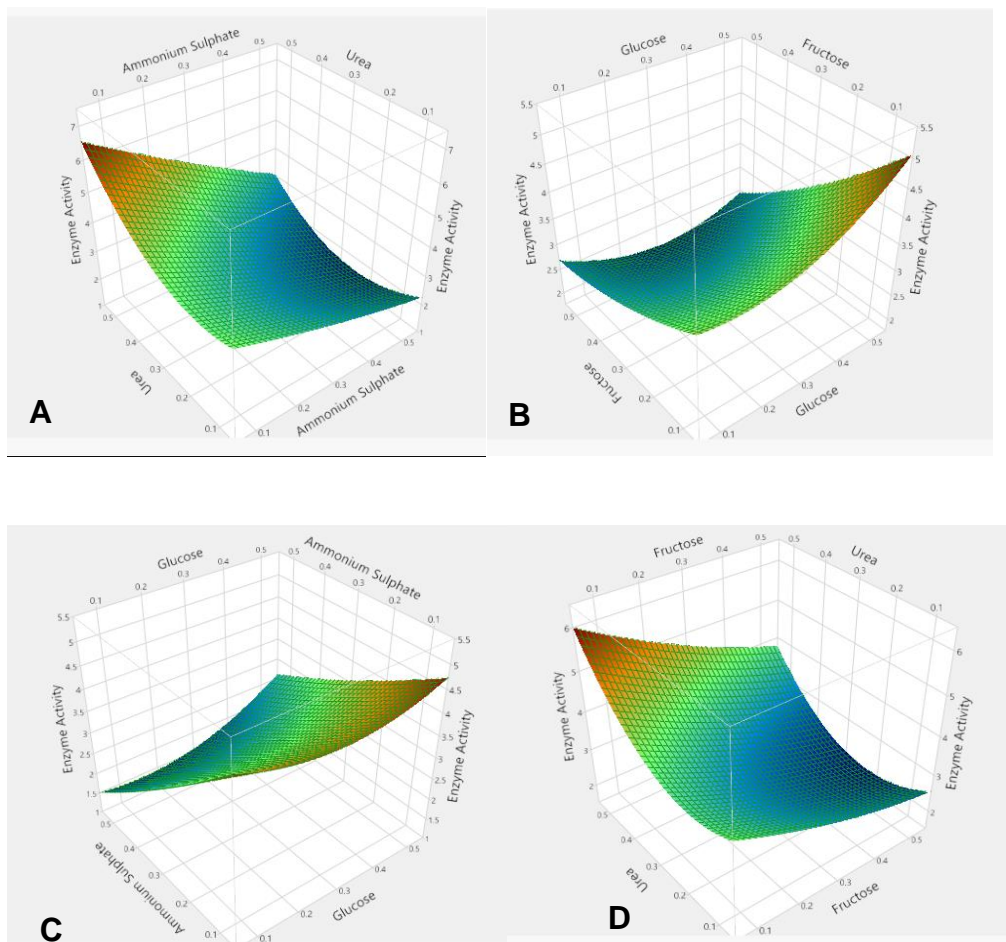
Fig. 3. 3D response surface graphs showing effect various carbon and nitrogen sources on alkaline protease through interaction between (a) glucose & fructose, (b) ammonium sulfate & urea, (c) glucose & ammonium sulfate, and (d) fructose & urea

Ammonium sulphate purification

      Partial purification of the enzyme was carried out at different percentages of ammonium sulphate (10% to 85%) with 2 mL volume of crude enzyme for each % age. Maximum enzyme precipitation was observed at 80% saturation level. The pallet showed the higher alkaline protease activity compared to supernatant, indicating the precipitation of enzyme by salting out (Fig. 4).

Gel filtration chromatography

      Sephadex G-100 (Sigma Aldrich, St. Louis, USA) was used to prepare the chromatographic column and calibrated with tris – HCl buffer (pH 8.5) and eluted with the same buffer. Thirty eluted samples, each of 1 mL volume, were taken. First, 5 elution samples were discarded, and the next were numbered as elusion samples 1 to 25 and were tested for their enzyme activity (IU/mL). Among the elusions, sample No. 6 gave the highest alkaline protease activity (Fig. 5).
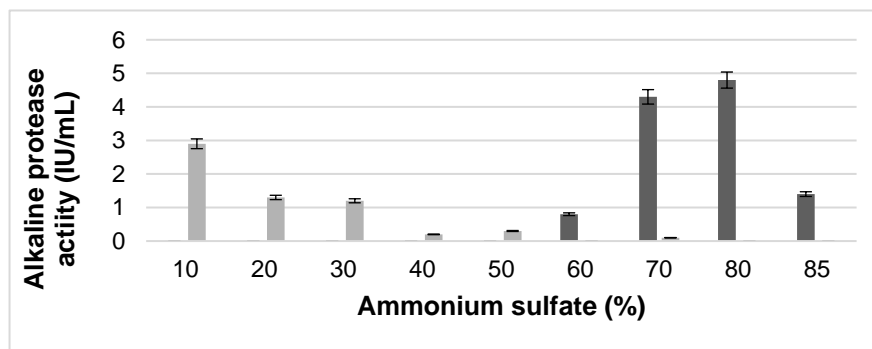

Fig. 4. Alkaline protease activity in supernatant (light) and pellet (dark), (SD represented by bars)
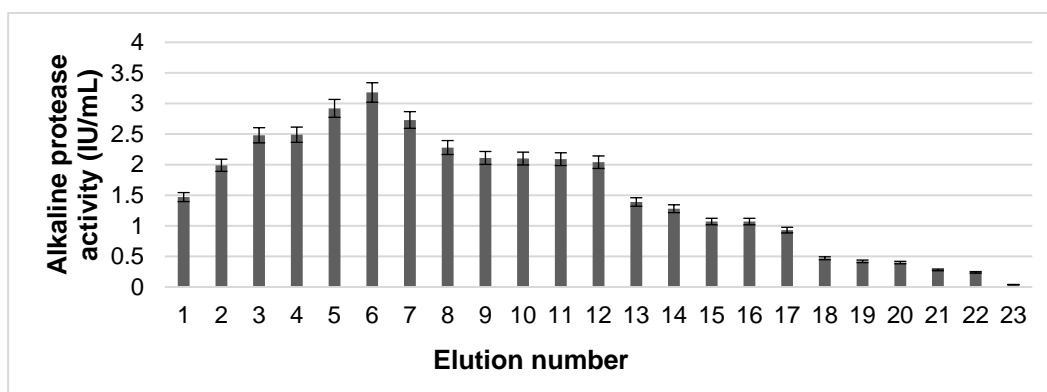

Fig. 5. Alkaline protease activity in eluted samples after gel filtration chromatography (SD represented by bars)

Characterization of Alkaline Protease from *A. flavus*

      The alkaline protease was characterized for its optimum temperature by performing an assay at various temperature. The activity increased from 25 °C and reaches to maximum at 45 °C, indicating its optimum temperature (Fig. 6). Similar results were obtained by Franco *et al*. (2017), where the highest value of enzyme activity was seen within the temperature range of 45 to 55 °C. Yadav *et al*. (2011) reported the maximum enzyme activity of protease from *A. flavus* at 40 °C, whereas, a maximum activity was

reported at 50 °C by experimenting with protease from *A. flavus* (Muthulakshmi *et al.* 2011).

Experiments for optimum pH showed the highest protease activity at pH 7.5 (Fig. 7). The study by Yadav *et al.* (2011) showed that the protease extracted from *A. flavus* remains active in the pH range of 7 to 12, while according to Muthulakshmi *et al.* (2011) the protease from another *A. flavus* isolate showed maximum activity at pH 7. The enzyme was active within the pH range of 7 to 8 as similarly reported by Chandrasekaran *et al.* (2015).

The effect of substrate, *i.e.* casein (2 mg/mL to 20 mg/mL) was used to calculate K_m and V_{max}. The value of V_{max} was come out 3.9 U/mL, while the value of K_m was 1.9 mg/mL (Fig. 8).
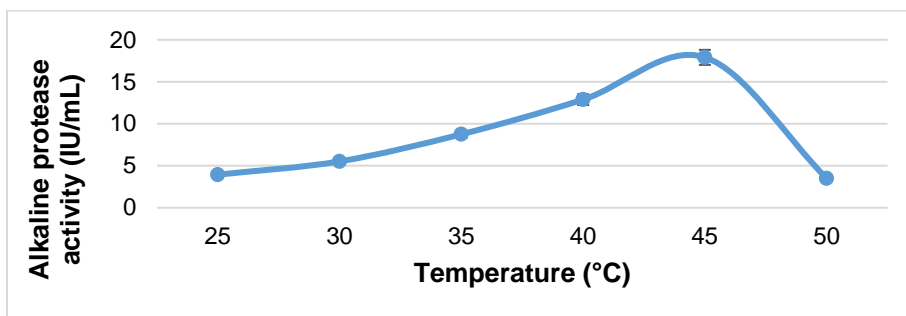
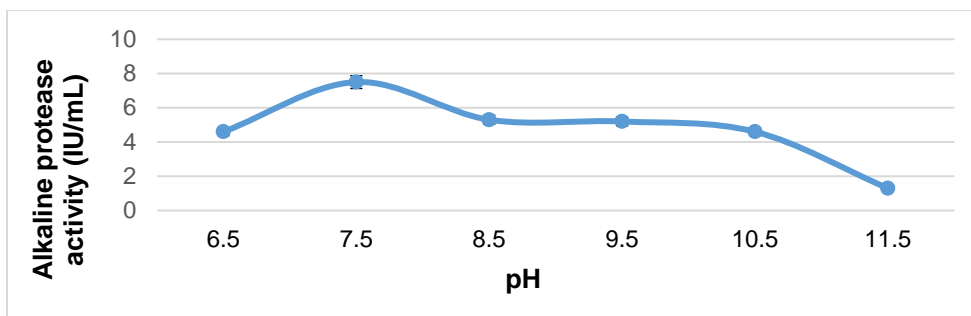**Fig. 6.** Effect of temperature on alkaline protease activity (SD represented by bars)

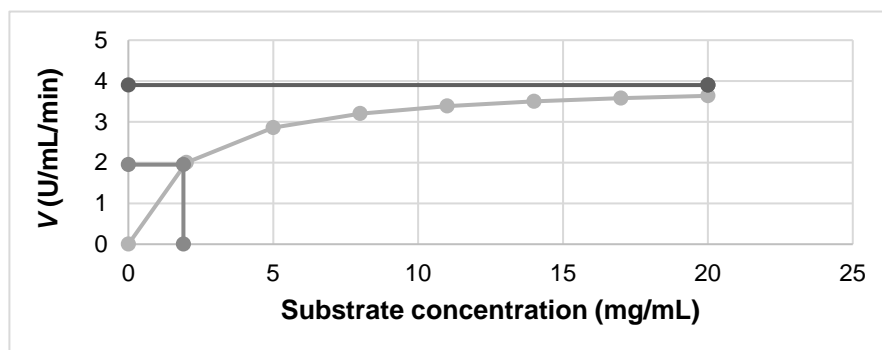**Fig. 7.** Effect of pH on alkaline protease activity (SD represented by bars)

Fig. 8. Value of K_m and V_{max} (U/mL/min)

Compatibility with Commercial Detergents

The enzyme reaction was conducted in the presence of four different commercially available laundry detergents. The enzyme retained 80.8% and 79.5% of activity with Surf

bioresources.com

Excel, and Brite, respectively (Table 1). However, the activity was decreased to 70.5% with Ariel and 61.5% in the presence of Bonus (Fig. 9).

The results showed that the enzyme worked efficiently in the presence of commercial laundry detergents. It retained much of its activity at a higher temperature and a basic pH.

Further studies are recommended for testing the stain removing capability of the alkaline protease harvested from _A. flavus_ in the presence of detergents. Moreover, future research may include modification of the enzyme through genetic manipulation to make it work in a higher basic environment.

Table 1. Protease Activity with Different Detergents

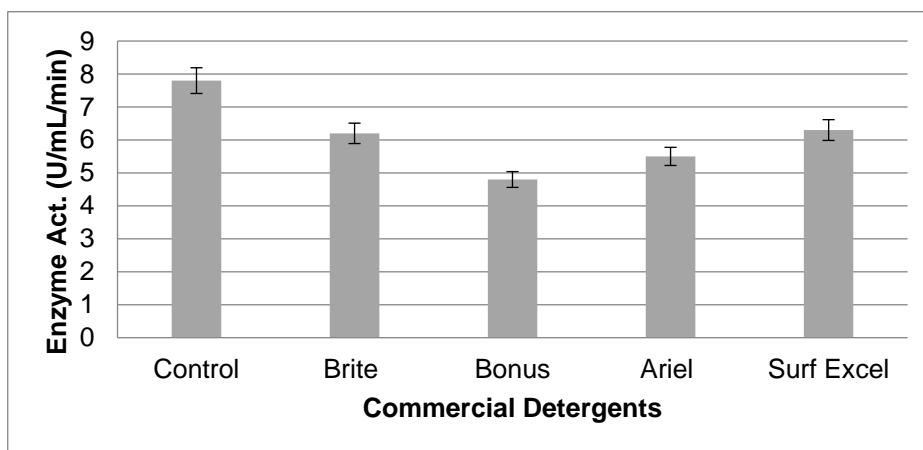| Detergent	OD at 660 nm	Control OD	Enzyme Activity (U/mL)	Activity Retained (%)
Control	0.537	0.537	7.8	100
Brite	0.431	0.537	6.2	79.5
Bonus	0.340	0.537	4.8	61.5
Ariel	0.385	0.537	5.5	70.5
Surf Excel	0.435	0.537	6.3	80.8

OD: Absorbance

Fig. 9. Compatibility of alkaline protease form _A. flavus_ with commercial detergents
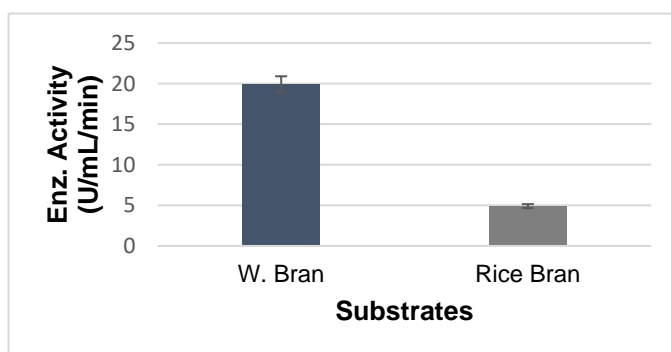

Fig. 10. Comparison of wheat bran and rice bran for alkaline protease production by _A. flavus_

Alkaline Protease Production at Optimized Conditions

The enzyme production was checked at optimized conditions on both substrates for testing the efficacy of optimization process. There was enhanced production of alkaline protease when maintaining all conditions at optimum level. Like screening results there was more activity (19.90 IU.mL/min) when wheat bran was used as substrate compared to rice bran (4.90 IU/mL/min). Similar results were observed by Muthulakshmi *et al.* (2011), where wheat bran gave the best protease production among other solid substrates (Fig. 10).

CONCLUSIONS

The current study aimed to produce alkaline protease from *A. flavus* and to test its compatibility with commercially available detergents.

1. The physical and nutritional parameters for enzyme production were optimized, and the enzyme production was maximum when the fungus was grown at pH 6.0 and a temperature of 30 °C, inoculum size 3.5 mL, substrate 6g for 84 h.

2. The enzyme was precipitated between 60% to 85% ammonium sulphate, while the maximum precipitation was observed at 80%.

3. The enzyme displayed maximum activity at 45 °C and retained most of its activity within the alkaline pH range of 7.5 to 10.5.

4. The enzyme was found compatible with most of the commercially available detergents and retained 60% to 80% of its activity when assayed in the presence of the detergents.

5. Wheat bran was a better substrate for the enzyme production and it is recommended that wheat bran be used for the large-scale production of alkaline protease.

6. It was concluded that the alkaline protease produced from indigenously isolated *A. flavus* can be used for further studies for the development of bio-detergents.

ACKNOWLEDGEMENTS

Special thanks to the Institute of Biochemistry and Biotechnology, PMAS-Arid Agriculture University Rawalpindi for providing the facilities to carry out this research work.

REFERENCES CITED

Chandrasekaran, S., Kumaresan, S. S. P., and Manavalan, M. (2015). "Production and optimization of protease by filamentous fungus isolated from paddy soil in Thiruvarur District Tamil Nadu," *Journal of Applied Biology & Biotechnology* 3(6), 066-069. DOI: 10.7324/JABB.2015.3610

Demain, A. L., and Adrio, J. L. (2008). "Contributions of microorganisms to industrial biology," *Mol. Biotechnol.* 38(1), 41-55. DOI: 10.1007/s12033-007-0035-z

De Souza, P. M., Bittencourt, M. L., de Freitas, C. C. M., de Almeida, R. P. C., Silveira, D., Fonseca, Y. M., Filho, E. X. F., Junior, A. P., and Magalhães, P. O. (2015). "A

biotechnology perspective of fungal proteases," *Braz. J. Microbiol.* 46(2), 337-346. DOI: 10.1590/S1517-838246220140359

Devi, K. M., Banu, A. R., Gnanaprabhal, G. R., Pradeep, B. V., and Palaniswany, M. (2008). "Purification, characterization of alkaline protease enzyme from native isolate of *Aspergillus niger* and its compatibility with commercial detergents," *Indian J. Sci. Technol.* 1(7), 7-13. DOI: 10.17485/ijst/2008/v1i7/29599

Franco, D. G., Spalanzani, R. N., Lima, E. E., Marchetti, C. R., Silva, P. O., Masui, D. C., Giannesi, G. C., and Zanoelo, F. F. (2017). "Biochemical properties of a serine protease from *Aspergillus flavus* and application in dehairing," *Biocatal. Biotransfor.* 35(4), 249-259. DOI: 10.1080/10242422.2017.1322584

Kumara, C. G., and Takagi, H. (1999). "Microbial alkaline proteases: From a bio-industrial viewpoint," *Biotechnol. Adv.* 17(7), 561-594. DOI: 10.1016/S0734-9750(99)00027-0

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, 265-275.

Muthulakshmi, C., Gomathi, D., Kumar, D. G., Ravikumar, G., Kalaiselvi, M., and Uma, C. (2011). "Production, purification and characterization of protease by *Aspergillus flavus* under solid state fermentation," *Jordan Journal of Biological Sciences* 4(3), 137-148.

Raimi, O. G., Elemo, B. O., Fatai, A. A., Bankole, H. A., Kazeem, M. I., and Banjoko, A. O. (2011). "Isolation and partial characterization of a protease enzyme from *Thaumatococcus daniellii* waste," *Afr. J. Biotechnol.* 10(16), 3186-3190. DOI: 10.5897/AJB10.2065

Silva, D., Tokuioshi, K., Martin, E. S., Silva, R. D. and Gomes, E. (2005). "Production of pectinase by solid state fermentation with *Penicillium viridicatum* RFC3," *Process Biochem.* 40, 2885–2889.