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Optimization and Detergent Compatibility of Protease Produced from *Aspergillus oryzae* by Utilizing Agro Wastes

Komal Farooq, Zahid Anwar,* Waseem Khalid,* Shoaib Hasan, Fareed Afzal, Muddassar Zafar, Usman Ali, Othman Alghamdi, Ammar AL-Farga, and Saleh M. Al-maaqar*

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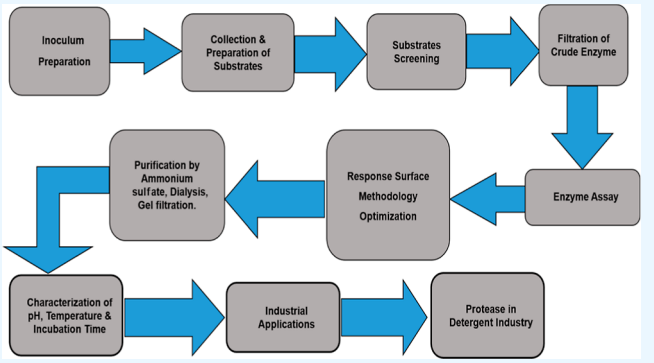
ABSTRACT: The biotechnological process called solid-state fermentation (SSF) was applied for hyper production of protease by using a fungal strain called *Aspergillus oryzae*. From screening of 9 different local substrates (peanut shell, wheat bran, guava leaves, sugar cane bagasse, rice polish, wheat straw, corn straw, reed grass, and rice straw), peanut shells serve as the best substrates for protease production under optimized cultured conditions. The varying physiochemical parameters such as pH (2−9.5), temperature (30−52 °C), incubation time (1−10 days), inoculum size (1−8 mL), moisture level (20−125%), and substrate concentration (1−7 g) were optimized by response surface methodology (RSM). The highest activity of protease was recorded to be 1101.778 U/mL at 660 nm using peanut shell was optimum at pH 8, temperature 52 °C, incubation time 8 days, inoculum size 2 mL, moisture level 20%, and substrate concentration 2 g. The crude form of enzymes produced were further purified through ammonium sulfate precipitation, dialysis, and gel filtration chromatography. Then, purified enzymes were characterized at different pH, temperature, and incubation time. For characterization of purified protease, pH, temperature, and incubation time were 8, 52 °C, and 8 days for peanut shell and was done by one factor at a time method. Hence, isolated enzymes were alkaline in nature, i.e., alkaline proteases. Then, protease produced from peanut shells was applied to locally available detergents to increase their catalytic activity for strain removal. At last, the final results were interpreted in the form of 3D surface and contour plots using Microsoft Excel 2013 and Minitab 17 software. In conclusion, the utilization of *A. oryzae* and peanut shell as the substrate in the biotechnological process of SSF demonstrated successful hyper production of alkaline protease. The optimized conditions resulted in high enzyme activity and showcased the potential application of the isolated enzymes in improving the catalytic activity of locally available detergents.



1. INTRODUCTION

The proteases are the enzymes of choice due to their various applications in large industries.[1] The microbial proteases are the most studied enzymes from all classes of enzymes because they belong to the largest group of hydrolytic enzymes that are applicable on a commercial scale.[2] As microbial systems are used for the production of proteases, they are classified into three main types including neutral, alkaline, and acidic proteases. Alkaline proteases are more important than neutral and acidic proteases due to commercial use and their specific production. The alkaline proteases work efficiently when alkaline pH conditions are applied.[3] The alkaline proteases consist of serine residues, which are located at their active site. The bioindustry is mainly dependent on alkaline proteases because they are particularly suitable in a wide range of applications. The other factor that is very important for use of alkaline proteases in the bioindustry includes their high activity and stability even during extreme physiological conditions. The alkaline proteases are capable of performing their functions properly under inhibitory compounds and in the presence of high temperatures and high pH.[4]

More than 25% of the protease is produced from a major class of mesophilic fungi that includes *Aspergillus candidus*, *Aspergillus flavus*, *Aspergillus fumigatus*, *Aspergillus melleus*, *Aspergillus niger*, *Aspergillus oryzae*, *Aspergillus sojae*, *Aspergillus sulphurous*, and *Aspergillus sydowi*.[5,6] Such diverse types of fungi result in the production of a variety of proteases that are applicable in various catalytic reactions.[7] The fungal strain for the industrial scale production of proteases is filamentous

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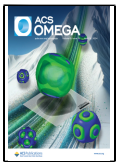

Table 1. RSM Results for Optimization of Protease Produced from the Peanut Shell

pH	temperature (°C)	incubation time (days)	inoculum size (mL)	moisture level (%)	substrate concentration (g)	absorbance at 660 nm	protease production (U/mL)
2	45	5	5	60	4	0.129	333.458
5.5	37	5	5	60	4	0.133	352.666
5.5	45	5	2	100	2	0.108	232.616
5.5	45	5	7	20	2	0.188	616.776
5.5	45	10	7	100	6	0.131	343.062
5.5	45	2	2	20	6	0.132	347.864
5.5	52	5	5	60	4	0.079	93.358
9.5	45	5	2	100	6	0.209	717.618
8	52	8	2	20	2	0.289	1101.778
4	52	1	7	20	6	0.162	491.924
5.5	45	5	5	60	4	0.102	203.804
5.5	45	5	7	100	2	0.221	775.242
4	30	8	8	60	4	0.098	184.596
8	30	1	4	125	4	0.134	357.468
5.5	45	5	1	60	4	0.214	741.628
8	52	1	4	60	7	0.205	698.41
8	30	8	5	60	4	0.071	54.942
4	30	1	5	60	4	0.171	535.142
4	52	8	5	60	1	0.197	659.994
5.5	45	5	5	60	4	0.123	304.646

fungi, such as *A. oryzae,* because they can be grown easily relative to other fungi on inexpensive substrates. Due to the unique nature, nutritional requirements, and cosmopolitan of *A. oryzae*, it is mainly utilized for the maximum production of proteases.[8] It is also suitable for a large variety of solid substrate bioconversion because of the requirement of less energy, lesser amount of liquid, and low cost for enzyme production on large scale. So, it is logical to use this genus by optimization of different factors like inoculum size, moisture level, incubation time, incubation temperature, substrate concentration, and pH for the study of production of protease.[9]

Several physical and chemical factors influence the growth of microorganisms in different artificial media.[10] On the laboratory level, the culture media is used for the purpose of microbial growth. The culture media is based on the nutrient composition, as the composition of nutrients plays a vital role in the culture medium for the microbial growth.[11] The standardized media are used in the research laboratories for the purpose of specific growth of microorganisms. A well-defined composition of potato dextrose agar (PDA) is commonly used in research laboratories for a wide range of fungal growth. In all the types of PDA media, agar as a solidifying agent must be used.[12,13]

The present research work is mainly focused on overcoming environmental pollution by utilizing local biomass. The pollutants that are created from the agro wastes cause serious problems for humans as well as aquatic life. To overcome the danger of pollution, the scientists used the concept of recycling of waste material to produce useful products which are cost-effective and economically beneficial. Some of the substrates that have been used for enzyme production included starch, steam pretreated willow, steamed rice, corn flour, wheat flour, cassava flour, mustard oil cake, coconut oil cake, rapeseed cake, peanut meal, apple pomace, sweet sorghum pulp, sugar beet pulp, aspen pulp, palm oil mill waste, cassava waste, tea waste, banana waste, coconut coir pith, corncobs, saw dust, grapevine trimmings dust, rice husk, rice straw, wheat straw, gram bran, maize bran, rice bran, wheat bran, and sugar cane bagasse. So, these agro wastes served as substrates for many companies such as the enzyme industry for the production of novel enzymes such as proteases, also called pectinases. The biodegradable nature of enzymes made them the best contributors for environmental cleanliness and a green environment as they replaced the harsh chemicals to such a great extent that the enzyme industry started getting much attention toward world research technologies. Human genome contained about 2% proteases. Proteases were also referred as proteolytic enzymes.[14]

After selection of fermenting organism, fermentation method, and substrate, the next important step was to maximize the yield of enzymes to meet the future demand of the enzyme industries as 60% of these industries depend on proteases. In the literature, different methods were available for the optimization of the products in which there were dependent and independent variables that were kept for studying the effect of different parameters on enzyme production. This method involved a technique in which one variable kept changing while the other variable was kept constant. But it did not give a precise result due to consumption of more reagents and increased cost of the procedure. The other statistical method called response surface methodology (RSM) was quite suitable for this research. This method was already practiced and interpreted by many research scientists and regarded as the best method for the study of mechanism and optimization of factors and end products. Ultimately, this method has overcome the problem from the previous method then results deduced to check which environmental factor involved in the hyper production of enzymes from which biomass with reference to the industrial applications.[15] The aim of this study was to investigate the application of solid-state fermentation (SSF) using *A. oryzae* and various substrates for the hyper production of protease, optimize the process parameters through RSM, purify and characterize the enzymes, and explore their potential

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application in the enhancement of catalytic activity in locally available detergents.

2. RESULTS AND DISCUSSION

2.1. Screening of the Best Agro Industrial Substrate. During the process of SSF, the fungal strain of *A. oryzae* could produce a maximum amount of protease from the screening of nine different biomasses. Spectrophotometric analysis showed that peanut shell was capable of producing a maximum yield of protease at 660 nm.

2.2. Optimization of Nutritional and Physio-Chemical Parameters. The influence of nutritional and physio-chemical variables was studied by RSM by applying optimum conditions for the substrate, moisture level, inoculum size, incubation time, temperature, and pH for hyper production of protease from the peanut shell, as shown in Table 1.

2.2.1. Effect of Substrate. In this study, agro-industrial biomass, such as the peanut shell, was used as a substrate to study its effect on enzyme production by following SSF. The substrate concentration plays an important role in protease production from *A. oryzae* and it greatly affected the production of protease. The substrate concentration was measured in grams for agro-industrial biomasses, such as 1, 2, 4, 6 and 7 g, as shown in RSM Table 1. This is according to (Ogino)[16] optimum conditions.

Substrate optimization for the peanut shell. A total of 40 trials of biomasses were measured and autoclaved, and then optimum conditions were given to all trials according to RSM. After the enzyme assay, a maximum activity of 1101.778 U/mL was observed at 2 g of fermented peanut shell.

2.2.2. Effect of Moisture Content. In this study, the moisture level played an important role in protease production for optimized fungal growth. The moisture level was maintained according to the substrate concentration in all autoclaved flasks. The levels of moisture for all 40 trials were designed by RSM as 20, 60, 100, and 125% for agro-industrial biomasses.

2.2.2.1. Moisture Content Optimization for the Peanut Shell. The protease enzymes produced from the fermented substrates were assayed and the highest enzyme production was recorded, as shown in RSM in Table 1 for the peanut shell. The substrates showed different quantities of enzymes with respect to the moisture level. The highest enzyme production of 1101.778 U/mL was recorded for fermented peanut shells at the 20% moisture level.

2.2.3. Effect of Inoculum Size. In this research, the organism of choice for optimum protease production was *A. oryzae*. Inoculum media was prepared in 500 mL flasks for substrates separately. Then, inoculated media were incubated to obtain optimal production of spores. Different quantities of inoculum, such as 1, 2, 4, 5, 7, and 8 mL, were added in all 40 trials according to RSM in Table 1 for biomasses and the effect of inoculum concentration was checked on enzyme production for agro industrial substrates.

2.2.3.1. Inoculum Size Optimization for the Peanut Shell. The biomass of peanut shell was inoculated with fungal spores and was given optimal growth conditions for a maximum of 10 days. After that, the enzymes from the fermented substrates were extracted, and the protease assay was done. It was recorded that the inoculum size played a pivotal role during SSF for protease production. The maximum protease activity from the peanut shell was recorded as 1101.778 U/mL when the inoculum volume was 2 mL. Optimum substrate quantity and inoculum volume were required for fungal culture growth.

2.2.4. Effect of Fermentation Time. According to the RSM in Table 1, a certain time period was provided to all 40 trials for the fermented substrate peanut shell. Each day, a flask was taken out and maximum enzyme activity was analyzed after enzyme assay. Different time periods to be 1, 2, 5, 8, and 10 days were provided to fungal spores to grow in an optimum culture medium to show its maximum activity for protease production. Then, the highest enzyme activity in U/mL was recorded for the agro industrial substrates.

2.2.4.1. Time Period Optimization for the Peanut Shell. Fermentation time for the optimum production of protease was determined after the enzyme assay. It was found that the optimum incubation time was 8 days for peanut shells, as it yielded 1101.778 U/mL of protease.

2.2.5. Effect of Temperature. One of the most important parameters in SSF for protease production is incubating temperatures. In order to get the highest protease yield, different levels of temperatures, such as 30, 37, 45, and 52 °C, were designed in RSM in Table 1, and their effects were shown on fermented substrates such as on the peanut shell. Then, the enzyme assay was done for all 40 RSM trials to have the highest protease activity.

2.2.5.1. Temperature Optimization for the Peanut Shell. After the enzyme assay, the highest activity of 1101.778 U/mL was shown by protease produced from the peanut shell when the fermented temperature was kept at 52 °C. The further increase in the temperature inhibited the fungal growth and ultimately the protease production was reduced from substrates. So, the temperature should be maintained to get the highest yield of protease.

2.2.6. Effect of pH. Different pH values were adjusted in different buffer solutions and were given to all fermented media inside 40 flasks for the substrate peanut shell. Different pH values, such as 2, 4, 5.5, 8, and 9.5, were designed in RSM in Table 1 and were provided in SSF to all fermented media. The pH optimization for the peanut shell. After the protease assay, the enzyme activity was measured for all 40 trials, and then the highest enzyme activity was recorded in U/mL. From the recorded results in RSM in Table 1, it was cleared that the activity of the enzyme produced from agro industrial substrates gradually increased with increase in the pH of the medium. The highest enzyme activity of 1101.778 U/mL was measured by the peanut shell fermented media when the pH was kept at 8. So, pH affected enzyme activity a lot, and results showed that the enzymes produced from sources were alkaline in nature.

2.3. Statistical Analysis by RSM. The Minitab 17 Software was used to optimize the investigational variables, such as pH, temperature, incubation time, inoculum size, moisture level, and substrate concentration for the selected biomasses, i.e., peanut shell, as shown in RSM table.

2.3.1. Response Surface Regression for Peanut Shell. To analyze the significance of the experimental data, response surface design was used that represented enzyme activity (U/mL) versus pH, temperature, incubation time, inoculum size, moisture level, and substrate concentration.

2.3.2. Regression Equation in Uncoded Units for Peanut Shell. The following formula is used for the calculation of the production of protease from the peanut shell substrate.

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$$\text{Enzyme activity}\left(\frac{\text{U}}{\text{mL}}\right) = 745 + 371\ \text{pH} - 18\ \text{temperature}\ (^{\circ}\text{C}) - 308\ \text{incubation time(days)} - 788\ \text{inoculum size(mL)} + 51.0\ \text{moisture level(\%)} - 375\ \text{substrate concentration(g)} + 62.8\ \text{pH} \times \text{pH} - 0.71\ \text{temperature}(^{\circ}\text{C}) \times \text{temperature}(^{\circ}\text{C}) - 33.7\ \text{incubation time(days)} \times \text{incubation time(days)} + 76.79\ \text{inoculum size(mL)} \times \text{inoculum size(mL)} - 0.534\ \text{moisture level(\%)} \times \text{moisture level(\%)} + 218.9\ \text{substrate concentration(g)} \times \text{substrate concentration(g)} - 3.9\ \text{pH} \times \text{temperature}(^{\circ}\text{C}) - 27.01\ \text{pH} \times \text{incubation time(days)} + 37.4\ \text{pH} \times \text{inoculum size(mL)} + 2.75\ \text{pH} \times \text{moisture level(\%)} - 227\ \text{pH} \times \text{substrate concentration(g)} + 17.16\ \text{temperature}(^{\circ}\text{C}) \times \text{incubation time(days)}$$

2.3.3. Interpretation of Protease Activity for Peanut Shell. The contour and three-dimensional (3D) surface plots represent the models for linear relation, square relation, and two-way interaction of factors. The *p*-values for optimized parameters and for these models are presented in minitab17 RSM in Table 2 and depicts that the smaller the *p*-values, the more significant will be results. The *R*-square value for protease production from the peanut shell came out as 99.63%, which indicates that the results are highly significant and experimental values are accurate, more acceptable, and have the least error in the whole experimental work by utilizing the substrate peanut shell for the production and optimization of alkaline protease.

2.4. Statistical Graphs (3D Surface Plots and Contour Surface Plots). After the response surface design was analyzed in Minitab 17 Software, the contour surface graphs and 3D surface graphs were created for the alkaline protease obtained from the peanut shell. The predicted graphical data show the effect of two optimized factors on the enzyme activity. The graphical results for substrates showed the effect of one variable on the enzyme activity. For example, the maximum enzyme activity is obtained at a certain optimum level with the increase of one variable. After that enzyme activity started to decline in case of selected substrates, as shown in Figure 1 statistical graphs.

Table 2. Analysis of Variance for Peanut Shell[a]

source	DF	adj. SS	adj. MS	*F*-value	*P*-value
model linear	18	1,359,910	75,551	14.86	0.005
	6	458,092	76,349	15.02	0.005
pH temperature (°C)	1	58,612	58,612	11.53	0.182
incubation time (days)	1	1706	1706	0.34	0.666
inoculum size (mL)	1	97,633	97,633	19.20	0.143
moisture level (%)	1	134,607	134,607	26.47	0.122
substrate concentration (g)	1	88,082	88,082	17.32	0.150
square	1	6014	6014	1.18	0.473
pH*pH	6	506,426	506,426	16.60	0.005
temperature (°C)*temperature (°C)	1	36,450	36,450	7.17	0.228
incubation time (days)*incubation time (days)	1	1585	1585	0.31	0.676
inoculum size (mL)*inoculum size (mL)	1	12,904	12,904	2.54	0.357
moisture level (%)*moisture level (%)	1	305,457	305,457	60.08	0.082
substrate concentration (g)*substrate concentration (g)	1	76,940	76,940	15.13	0.160
2-way interaction	1	30,671	30,671	6.03	0.246
pH*temperature (°C)	6	216,022	216,022	7.08	0.005
pH*incubation time (days)	1	635	635	0.12	0.784
pH*inoculum size (mL)	1	48,682	48,682	9.57	0.199
pH*moisture level (%)	1	1291	1291	0.25	0.703
pH*substrate concentration (g)	1	1256	1256	0.25	0.706
temperature (°C)*incubation time (days)	1	213,49	21,349	4.20	0.289
error total	1	154,811	154,811	30.45	0.114
	1	5085	5085		
	19	1,364,994			

[a] R^2 = 99.63%.

2.5. Purification. *2.5.1. Ammonium Sulfate Precipitation.* The activity of protease was determined at 660 nm through a spectrophotometer after ammonium sulfate precipitation. The results showed that the maximum protease from the peanut shell was precipitated at 60%. That percentage having 0.176 nm absorbance from the substrate was selected for further analysis. Table 3 indicates the precipitation of protease from the peanut along with the optical density (OD).

2.5.2. Dialysis. After precipitation of enzyme, the samples of more purified enzyme were taken out from the dialysis bag and were analyzed at 660 nm. The enzyme activity of protease produced from peanut shells was recorded to be 299.844 U/mL.

2.5.3. Chromatography. After the fractions of enzyme were collected from the peanut shell, the enzyme activity was calculated. The highest enzyme activity of protease taken from the peanut shell was 251.824 U/mL.

2.6. Characterization of Physio-Chemical Parameters. Purified protease was characterized and the effect of different physical parameters like pH, temperature, and incubation time were studied on the enzyme activity.

2.6.1. pH Characterization. The purified protease was kept at pH 2−10 for 60 min and then the enzyme assay was done.

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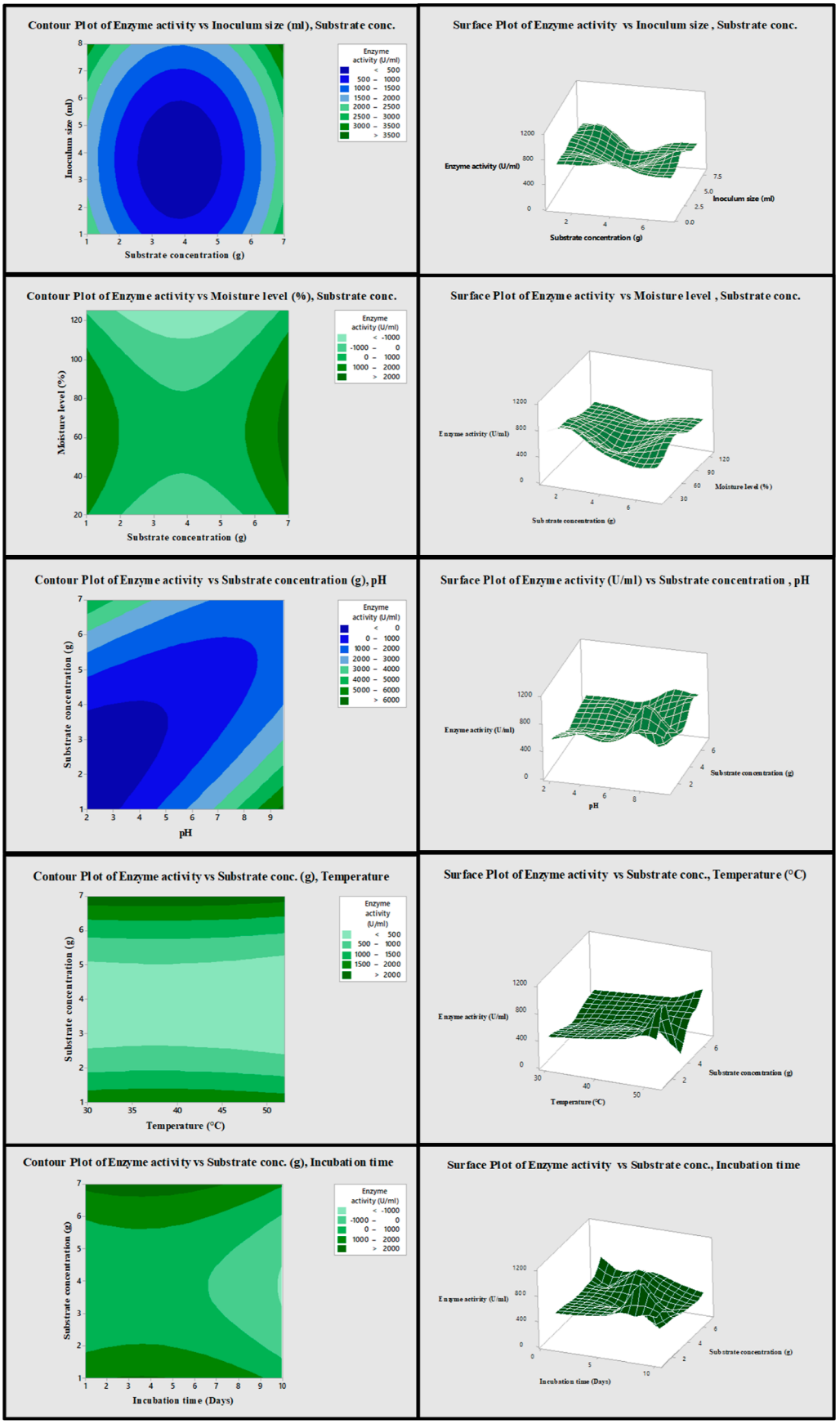

Figure 1. continued

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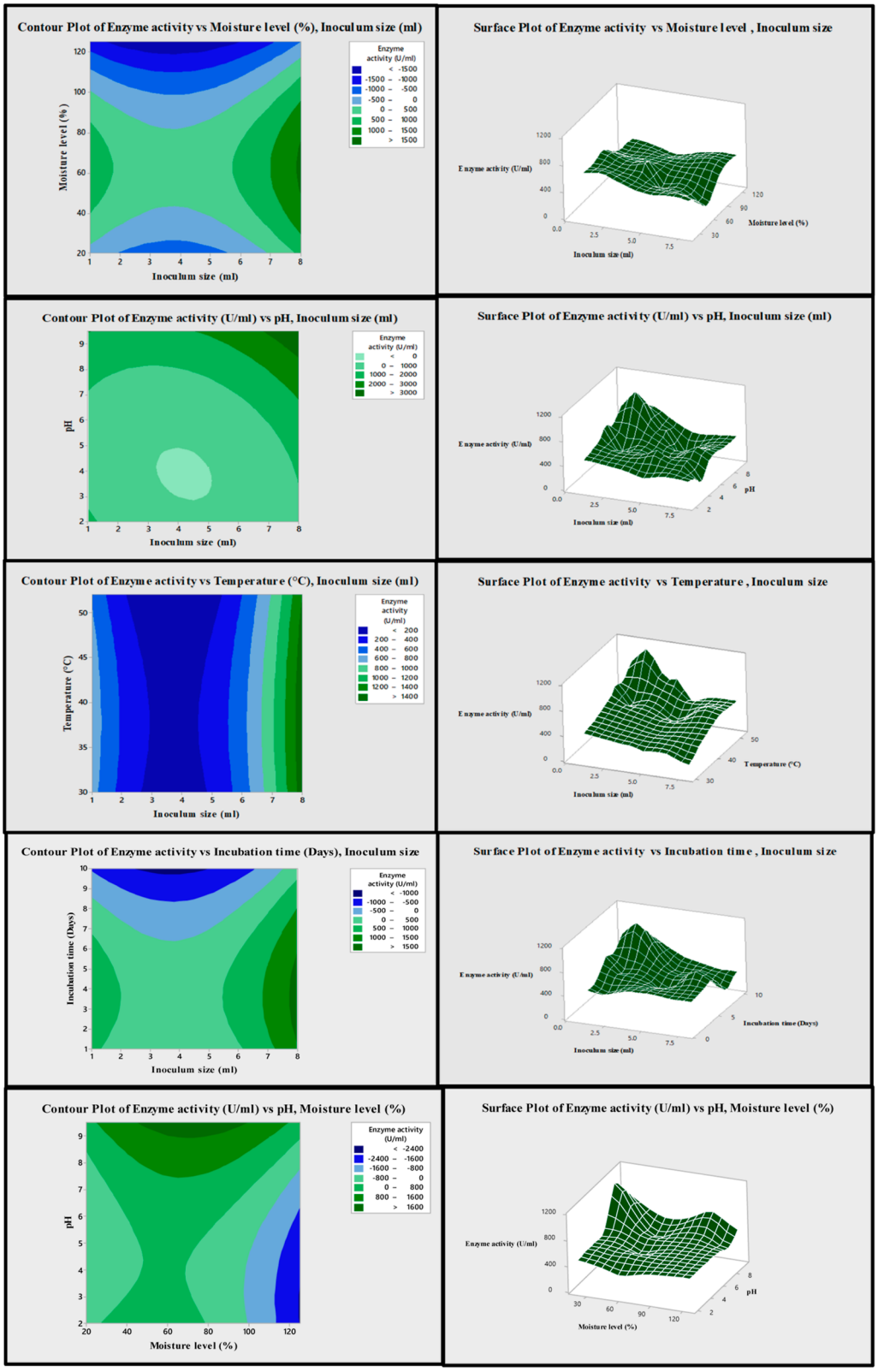

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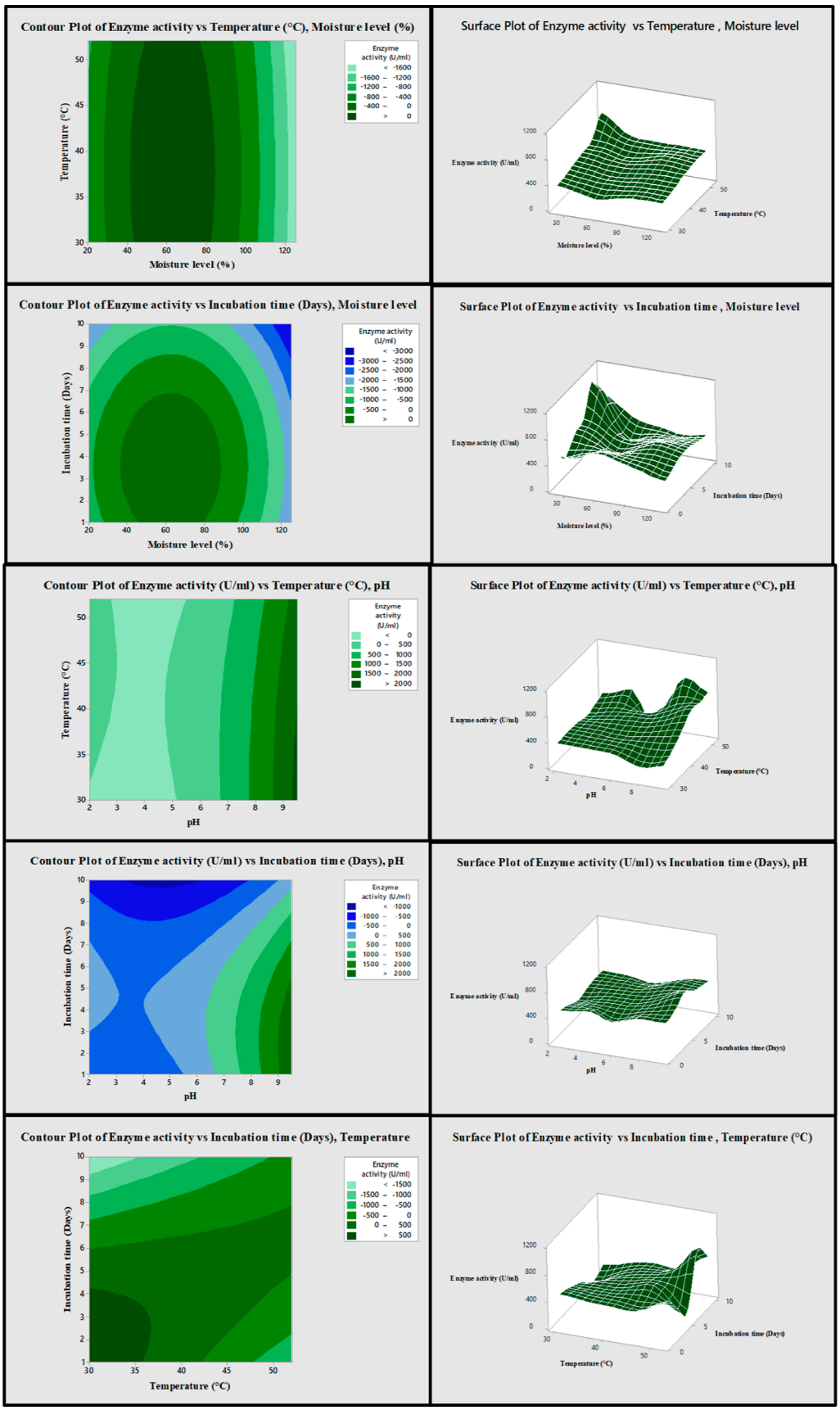

Figure 1. Contour and 3D surface plots for protease from the peanut shell.

The highest protease activity was obtained at 8 pH from substrate samples, which demonstrated that pH affects enzyme activity a lot and enzymes produced were alkaline in nature. Optimum pH of the protease from the peanut shell. In Figure

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ACS Omega 2024, 9, 17446−17457

Table 3. OD of Protease Produced from the Peanut Shell after $(NH_4)2SO_4$ Precipitation

percentage of $(NH_4)_2SO_4$ (%)	absorbance at 660 nm
10	0.203
20	0.179
30	0.218
40	0.327
50	0.267
60	0.176
70	0.185
80	0.221
90	0.210
100	0.209

2, the activity profile of protease and highest protease activity were obtained at 8pH from the peanut shell and alkaline protease produced from it.

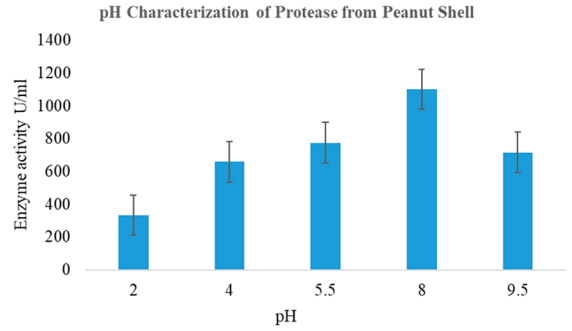

Figure 2. pH Characterization of protease from the peanut shell.

2.6.2. Temperature Characterization. The purified protease was incubated at different temperatures of 30−52 °C for 60 min, and the highest enzyme activity was recorded after the enzyme assay.

In Figure 3, the highest activity profile for protease and demonstrated that protease produced from the peanut shell

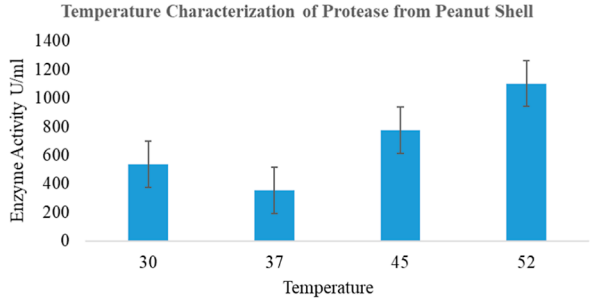

Figure 3. Temperature characterization of protease from a peanut shell.

was fully active when incubated at 52 °C. So, the optimum temperature for the highest activity of alkaline protease was 52 °C.

2.6.3. Fermentation Time Characterization. Time of incubation affects enzyme activity a lot, and its characterization was done for alkaline protease obtained from the peanut shell. Then, enzymes were assayed and results were recorded, also reported by Coral.[17] Optimum fermentation time of protease from a peanut shell is shown in Figure 4. The enzyme activity

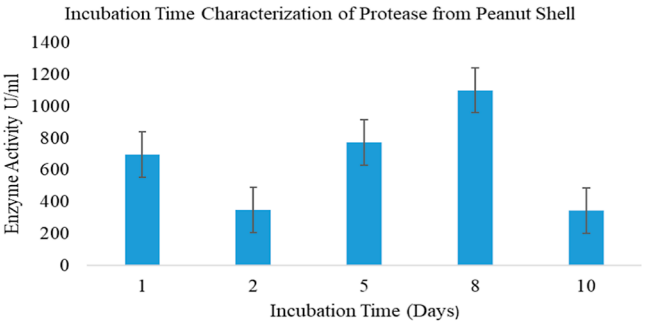

Figure 4. Incubation time characterization of protease from the peanut shell.

profile of alkaline protease showed that a maximum of 8 days were required for it to be fully active. So, optimum incubation time for alkaline protease produced from the peanut shell was 8 days when it was kept at 52 °C.

2.7. Industrial Applications. *2.7.1. Detergent Industry (Protease from Peanut Shell).* Protease is an important additive in detergents that can remove blood and protein stains from the surfaces of clothes. Purified protease produced from the peanut shell was applied to different brands of locally available detergents to check its compatibility with detergents. The highest relative increase in protease activity was observed with Ariel, which was 150 U/mL, as shown in Figure 5. This revealed the potential of protease from *A. oryzae* to be used as a suitable additive in laundry, which is also reported by Maurer.[18]

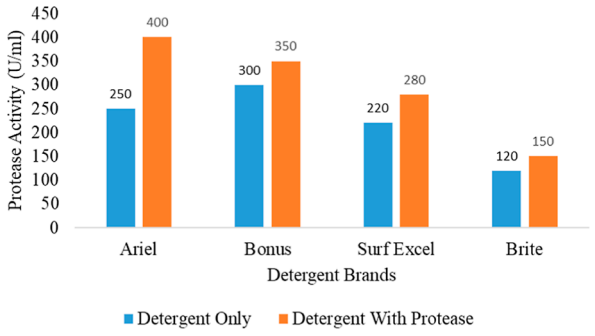

Figure 5. Protease as an additive in the detergent industry.

3. MATERIALS AND METHODS

3.1. Microorganism. The fungal strain of *A. oryzae* was obtained from the culture collection of Biochemistry Laboratory M-12, Nawaz Sharif Medical College, University of Gujrat Hafiz Hayat Campus, Pakistan. The pure colonies of fungal strain were grown on agar slants using a PDA medium at 35 °C in a shaking incubator for 3 days. It is a filamentous and eukaryotic fungi capable of producing spores through asexual production.

3.2. Preparation of Inoculum of *A. oryzae*. Potato dextrose media was used to obtain the spores of fungi. The PD media is suitable for the growth of fungus, which was prepared in 250 mL flasks. The pure colony from the agar slants was inoculated on PD media in the 250 mL flasks and placed in a shaking incubator for 72 h at 35 °C. After 3 days, the homogeneous suspension of fungal spores were obtained. The

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inoculum preparation was performed under a laminar air flow hood to avoid contamination.

3.3. Collection and Preparation of Substrates. The agro industrial waste was collected from the surroundings of Rahwali and Ghakhar, Pakistan. A total of nine substrates were collected from different local substrates (peanut shell, wheat bran, guava leaves, sugar cane bagasse, rice polish, wheat straw, corn straw, reed grass, and rice straw). They were all washed with tap water and dried under sunlight. After drying, all of the local biomass was crushed into a fine powder using a grinding machine available in Industrial Biotechnology lab P-112 of BAB department, University of Gujarat, Hafiz Hayat Campus, Pakistan. All of the substrates in the powder form was stored in an airtight container. The collected biomass used as the substrate along with their scientific names are given the peanut shell (*Arachis hypogaea*), wheat bran (*Triticum aestivum*), guava leaves (*Psidium guajava*), sugar cane bagasse (*Saccharum officinarum*), rice straw (*Oryza sativa*), reed grass (*Phragmites australis*), rice polish (*O. sativa*), wheat straw (*T. aestivum*), and corn straw (*Zea mays*).

3.4. Screening of Best Suitable Substrate for Protease Production. About 5 g of each of the nine substrates were added in seven Erlenmeyer flasks (250 mL) and were autoclaved at 121 °C for 15 min. Then, all of the flasks were taken out and biomass was inoculated with 3 mL of spores of fungi inside a laminar air flow hood. Then, all of the flasks containing biomass and inoculum were kept in a shaking incubator at 30 °C for 3 days.

3.5. Filtration of Crude Enzyme. After incubation for 3 days, all of the flasks were taken out from a shaking incubator and 50 mL of distilled water was added to them. Then, all of the flasks were again placed in a shaking incubator at 150 rpm for 60 min having 30 °C temperature. After 1 h, the crude enzyme was harvested using muslin cloth from 9 different substrates in small plastic bottles. Then, the enzyme assay of this harvested enzyme was done, and absorbance values of each of the 9 samples were checked at 660 nm using a ultra visible spectrophotometer. The biomass sample having the highest absorbance value was selected for further analysis.

3.6. Enzyme Assay. The harvested crude enzyme was passed through an enzyme assay called the Bradford method with slight modifications. About 1 mL of enzyme from each bottle was added into 9 different falcon tubes. After that 4 mL of 1% casein solution containing the tyrosine as the substrate was added in all falcon tubes. Then, this mixture was kept in an incubator at 35 °C for 1 h. After that 5 mL of 5% trichloroacetic acid was added in each of the falcon tubes to stop this reaction. Then, this mixture was kept at room temperature for half an hour. Later on, all falcon tubes containing the casein and enzyme was centrifuged at 4 °C for 10 min having 3000 rpm speed. As a result of centrifugation, a supernatant and pallet was formed. The pallet was discarded, and the supernatant from all falcon tubes was retained. Then, 1 mL of supernatant was taken from each falcon tube and added into 9 different test tubes. Then, 4 mL of alkaline reagent, i.e., Na_2CO_3 and 1 mL NaOH was mixed in the supernatant and this mixture was placed at room temperature for 10 min. After that, 3 mL of ninhydrin reagent was added in all test tubes and placed in a shaking incubator overnight at 35 °C and 150 rpm As a result, a reddish purple color was produced because supernatant contained free amino acids, which were released as a result of casein hydrolysis by the enzyme protease. Casein was used as a substrate because it is known to be the best substrate for protease enzymes. The 10th test tube was set to be the control in which only 1 mL of enzyme extract was added and then 3 mL of ninhydrin was added in it and placed overnight in a shaking incubator. As a result, no color was produced which indicates that no free amino acids are produced.[19]

3.7. Ultra-Visible Spectrophotometric Analysis. The enzyme activity was checked at 660 nm using a spectrophotometer.[20,21] The distilled water from the blank test tube was taken and added into the cuvette to set the spectrophotometer zero through it. Then, a few milliliters from all test tubes containing enzymes for the assay were taken into a cuvette and readings were checked from a spectrophotometer. This analysis was performed for all 9 substrates. The assay showed that the peanut shell yielded a maximum amount of protease.

3.8. Optimization of Nutritional and Physio-Chemical Parameters. The peanut shell was selected as suitable substrates to be optimized further for large-scale enzyme production from other 9 agro industrial waste biomasses. The fermentation parameters consisted of 20 trials for the peanut shell that were carried out in 40 different Erlenmeyer flasks (500 mL) by following SSF. Different factors were responsible in controlling the highest yield of the enzyme. Six different variables were selected for substrates that were pH, temperature, incubation time, inoculum size, moisture level, and substrate concentration. The different conditions by these factors were provided, and the best condition was selected under which the maximum yield of protease was obtained from the substrates as shown in the RSM Table 1. All of the 6 factors were optimized under SSF by RSM.

3.9. Statistical Methods for Optimization. All 6 factors were optimized by RSM using the selected substrates and organism *A. oryzae*. The fermentation was done in 500 mL Erlenmeyer flasks under optimal growth conditions. The selected biomasses peanut shell were measured in triplicates (1−7 g) and were added in 40 flasks and were autoclaved for 15 min at 121 °C. Then, the selected substrates were inoculated with freshly prepared media of fungi (1−8 mL) in all autoclaved flasks. Further different buffer solutions were added with pipette in all of the 40 flasks to maintain the pH (2−9.5) under a laminar air flow hood. The different ranges of moisture content (20−100%) was maintained in all of the flasks and was kept in an incubator at different temperatures (30−52 °C) for 1−10 days to check optimized pH, temperature, and incubation time for the biomasses. Later on, after the selected incubation time, each day a flask was drawn out from the incubator and 50 mL of dH_2O was added followed by stirring in a shaking incubator at 115 rpm for 1 h. Then, the extracellular enzyme was harvested in bottles. Later on, the enzyme assay was carried out and OD for all trials were recorded at 660 nm by an ultra-visible spectrophotometer. Then, the maximum protease producing parameters for substrates were selected for further analysis. The optimization parameters are given in Tables 1 and 2 by RSM.[22]

3.10. Purification. The crude enzyme from the peanut shell was purified by different techniques after production under highly optimized conditions. The purification techniques include ammonium sulfate precipitation, dialysis, and column chromatography.[23]

3.10.1. Ammonium Sulfate Precipitation. Two enzymes obtained through the peanut shell were precipitated out by salting in and salting out techniques.[24,25] For this purpose, different amounts of ammonium sulfate from 10 to 100% were

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ACS Omega 2024, 9, 17446−17457

calculated and added in 10 falcon tubes for the first enzyme and in other 10 falcon tubes for the second enzyme.[25,26] Then, 10 mL of distilled water was added in all falcon tubes separately. After that 1 mL of enzyme filtrate from the peanut shell was added separately in all falcon tubes containing ammonium sulfate and dH_2O, respectively. Then, all tubes were placed in a freezer overnight. The next day all the falcon tubes were centrifuged at 3000 rpm for 10 min. The pallet was discarded, and the supernatant was retained in small falcon tubes. Again, the supernatant was centrifuged with the same speed and time for further analysis. Later on, the ninhydrin test was performed on enzymes and enzyme activity was checked out of all falcon tubes. The process of salting in and salting out was monitored. The percentage with 0.176 nm OD for the enzymes was selected because maximum protein was precipitated in them and was used for further purification on a large scale.

3.10.2. Dialysis. The precipitated enzyme having least enzyme activity were taken out from those small falcon tubes and 5 mL of the enzyme samples were packed inside a dialysis bag carefully. The optimized pH for the enzymes was 8 pH so the bags were placed inside two beakers having sodium phosphate buffer of 8 pH separately. Then, these beakers were kept in a shaking incubator for 24 h to remove ammonium sulfate salt from the enzymes, and the more purified form of the enzyme will remain inside the dialysis bag that was used for further analysis.

3.10.3. Column Chromatography (Gel Filtration Chromatography). A Sephadex column (G-100) was used in column chromatography obtained from the Department of Chemistry, University of Gujarat Hafiz Hayat Campus, Pakistan. The column was quarter-half filled with pure sand and then 50 g of silica gel was added on the sand inside the column. Soon, 50 mL of distilled water was added in gel. The silica gel expanded up to half of the column. Then, the column was equilibrated and washed with 5 mL of sodium phosphate buffer having pH = 8. After that, 5 mL of enzyme from a dialysis bag was loaded inside column containing sand, silica gel, and buffer. The 10 mL of purified enzyme was collected in airtight bottles. The same procedure was repeated for the other enzyme too, and OD of samples was calculated after the enzyme assayed to have the highest enzyme activity.

3.11. Characterization of Physio-Chemical Parameters. The purified enzyme samples produced from the peanut shell were characterized by incubating them at different pH, temperatures, and times to find the optimum conditions for the action of enzymes on an industrial scale.[26] The enzymes were assayed, and the highest enzyme activity was noted by varying the pH, temperature, and time at 660 nm by an ultravisible spectrophotometer.

3.11.1. pH Characterization. The 1 mL of purified enzymes of substrates were taken in test tubes and placed in different buffers of varying pH to find the optimum pH for the highest enzyme action. The enzyme assay was done after 1 h of incubation for samples to find the highest protease activity. Following this, different buffers were prepared for pH characterization.

3.11.2. Temperature Characterization. The 1 mL purified enzymes from samples were taken in test tubes and were placed in different ranges of temperatures like 30, 37, 45, and 52 °C. After 1 h of incubation, the optimum temperature for the highest protease activity was analyzed by the enzyme assay for the samples.

3.11.3. Fermentation Time Characterization. Analysis for the highest protease activity of enzymes was done by providing different days of incubation like 1, 2, 5, 8, and 10 days. Each day test tubes were taken out, and the highest enzyme activity for samples was determined after the enzyme assay.

3.12. Industrial Applications. Although the enzyme produced from the peanut shell showed more enzyme activity.[27]

3.12.1. Protease in the Detergent Industry. To investigate the compatibility of *A. oryzae* protease produced from the peanut shell, the enzyme was added to laundry detergents. For this purpose, different brands of detergents, namely, Ariel, Bonus, Surf excel, and Brite were used. Three milliliter detergent solutions of all these detergents were prepared in 4 beakers as prescribed on their respective packets and set as the control. Then, 0.4 mL sodium phosphate buffer of 8pH dissolved in 2 mL casein (used as a substrate). Then, the reaction mixture containing 2 mL casein, 3 mL of all detergent solutions, and 1 mL purified protease in 4 beakers were incubated at 52 °C for 20 min. Then, the detergent compatibility of all detergents without protease and with protease was analyzed followed by the enzyme assay as described earlier.

4. CONCLUSIONS

The main objective of this research work was based on the "From waste to health" phenomenon that was to produce protease enzyme from fungal strains by the recycling of waste biomass as substrates and converting them into useful biotechnological products by following the process of SSF for the industrial applications that will ultimately help in reducing and combating environmental pollution, including both land and water tunnel pollution. Moreover, the screening of 9 different substrates was done and concluded that peanut shells proved to be the best substrate for the hyper production of alkaline proteases. The fungal strain of *A. oryzae* generally recognized as safe was used for hyper production of enzymes. After optimizing the growth conditions such as pH, temperature, incubation time, inoculum size, moisture level, and biomass size for the selected substrates, it was reported that the highest amount of alkaline protease produced from the peanut shell as a substrate was 1101.778 U/mL after ultravisible spectrophotometric analysis at 660 nm. To optimize the fermentation parameters, an efficient statistical system called RSM was used by consuming less amount of effort, time, and resources. The crude form of enzyme produced from substrates was further purified by following the techniques of ammonium sulfate precipitation, dialysis, and gel filtration chromatography. After that, the characterization of purified enzymes from the substrate was done for pH, temperature, and incubation time. The enzyme produced from the substrate was highly active at alkaline pH 8 and showed the highest detergent compatibility with Ariel because of the over congested state of microbes and lesser amount of gaseous exchange in the case of substrate concentration for the peanut shell that was 2 g. Then, to study the effect of different parameters and to study the relationship of dependent and independent variables on enzyme production during optimization, the results were interpreted in the form of 3D surface plots and contour plots using Minitab 17 software. The characterization of purified alkaline proteases was done by the one factor at a time method using the Microsoft Excel 2013 software, and the final results were represented in the graphical form for substrates in

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ACS Omega 2024, 9, 17446−17457

minimum time with less chances of error. The research scientist produced good quality and quantity of alkaline proteases for proper application in the detergent industry. The economically beneficial production of enzymes was done not only for the enzyme industry but also for Pakistan that will ultimately result in decreasing the import rate of protease from the foreign industry. In short, by consuming cheaper resources, the industrial biotechnologist was able to produce the maximum quantity of protease on a global scale too by following the strict optimized cultured conditions.

AUTHOR INFORMATION

Corresponding Authors

Zahid Anwar − *Department of Biochemistry and Biotechnology, University of Gujrat, Gujrat 54000, Pakistan*; Email: zahid.anwar@uog.edu.pk

Waseem Khalid − *University Institute of Food Science and Technology, The University of Lahore, Lahore 54000, Pakistan; Department of Organic Chemistry, Faculty of Chemical Sciences and Technologies, University of Castilla La Mancha, 13071 Ciudad Real, Spain*; Email: waseem.khalid@uifst.uol.edu.pk

Saleh M. Al-maaqar − *Department of Biology, Faculty of Education, Albaydha University, Al-Baydha 00967, Yemen*; orcid.org/0000-0001-5184-1529; Phone: +967 777 355 628; Email: al-maaqar@baydaauniv.net

Authors

Komal Farooq − *Department of Biochemistry and Biotechnology, University of Gujrat, Gujrat 54000, Pakistan*

Shoaib Hasan − *Institute of Molecular Biology and Biotechnology (IMBB), The University of Lahore (UOL), Lahore 54000, Pakistan*

Fareed Afzal − *Department of Food Science, Government College University Faisalabad, Faisalabad 38040, Pakistan*

Muddassar Zafar − *Department of Biochemistry and Biotechnology, University of Gujrat, Gujrat 54000, Pakistan*

Usman Ali − *Department of Biochemistry and Biotechnology, University of Gujrat, Gujrat 54000, Pakistan*

Othman Alghamdi − *Department of Biochemistry, College of Science, University of Jeddah, Jeddah 21589, Saudia Arabia*

Ammar AL-Farga − *Department of Biochemistry, College of Science, University of Jeddah, Jeddah 21589, Saudia Arabia*

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.4c00274

Notes

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