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ORIGINAL ARTICLE

Effect of bioprocess parameters on alkaline protease production by locally isolated *Bacillus cereus* AUST-7 using tannery waste in submerged fermentation

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Abstract

Present study aims to enhance protease production by employing locally isolated bacterial strains using animal skin (beef) wastes as a substrate. A proximate analysis of the substrate (skin waste) was carried out to know the percentage of protein, fats, and ash. To understand the particle size, shape, and other structural changes, scanning electron microscope (SEM) and Fourier transform infrared spectroscopy (FTIR) of both untreated and treated samples were performed. The substrate showed 83.81% crude protein, 11.23% crude fat, and 4.96% ash contents. SEM and FTIR analysis revealed changes in treated samples as compared to that untreated skin waste (control). In 140 isolated strains, 60 strains showed clear zone on 1% skim milk agar medium. The selected strains were cultured for protease assay on a production medium. The maximum protease (249.65 IU/ml) producing strain was identified as *Bacillus cereus* AUST-7 morphologically, biochemically, and based on 16S rDNA gene sequencing. The bioprocess parameters like aeration rate, shaking speed, and incubation period were optimized in lab scale bioreactor of 1.5 L capacity. During scale-up in a bioreactor (1.5 L), 2 volumes of aeration rate per volume of liquid medium per minute (VVM) aeration, shaking speed of 500 rpm, and 24 h of incubation period yielded maximum protease (1917.5 IU/mL) under submerged fermentation. The results depicted that defatted skin waste could be utilized as a potential substrate for the cost-effective production of alkaline protease.

Keywords *Bacillus cereus* · Tannery waste · SEM · FTIR · Protease production · Bioreactor

1 Introduction

Enzymes are known to be functional proteins responsible for prevailing biochemical reactions in living cells by lowering the activation energy [1]. Enzymes convert different substrate molecules into selective and effective products within multifarious matrices [2]. Proteases have a 65% share among all of the commercial enzymes in the market [3]. The global demand during the years 2014–2019 is increasing at the growing rate of 5.3%, and this increase is further expected. It degrades peptide bonds of protein molecules [4, 5]. Proteases are classified as alkaline, metallo, thiol, and acidic proteases. Among these groups, alkaline proteases have a wide range of applications in different industries. It plays an important role in biotransformation, surfactants, detergents, organic fertilizers, leather processing, waste treatment, and silver recovery from X-ray film [6–9]. Proteases are known to be one of the most significant proteolytic enzymes that are produced by a broad array of microorganisms including

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bacteria, fungi, and various animal, and plant tissues [10]. Microbial sources of alkaline proteases are remarkably steady and are produced at a large scale. In multiple fields, the enzymes attracted important commercial interest due to their high activity and stability at alkaline pH. Although a huge number of microorganisms produce alkaline proteases, however, to explore high protease-producing strains and their optimization regarding various parameters is always desired [11].

Bacillus genus is a large phenotypically, heterogeneous assembly of Gram variable spore-forming, facultative anaerobes or aerobic bacteria [12]. *Bacillus* species are employed in many pharmaceuticals, medical, agricultural, and industrial progression due to their broad range of physiologic properties and capability to produce antibiotics, metabolites, and a multitude of enzymes. Nutraceuticals like vitamins (cobalamin, inositol, and riboflavin) and carotenoids are produced by different species of *Bacillus* and have been applied for the production of health-related supplements for human usage [13, 14]. Protease is mainly produced by *Bacillus horikoshii*, *Bacillus licheniformis*, *Bacillus firmus*, *Bacillus sphaericus*, *B. cereus*, *Bacillus subtilis*, and *Bacillus alcalophilus* [15]. *Bacillus* sp. is isolated commonly from the soil, air, and decomposing residues of plants and water. However, the soil is the main habitat for *Bacillus*, and survival in soil for a long time is expected [16]. Conventionally, the identification of bacteria is carried out based on the physiological, biochemical, and morphological features of the species. The conventional method of identification is time-consuming and has low accuracy [17–19]. A molecular approach based on 16S rRNA genome sequence is used as an alternative technique for the identification of bacteria [20].

A massive amount of untreated industrial waste dumped on the surface of the earth on daily bases causes environmental pollution in developing countries. Generally, there is not a single industry that is pollution free in developing countries. The leather industries also caused environmental pollution by disposing of organic wastes. Tanning processing produces a significant number of by-products and wastes in the form of gaseous, liquid, and solid which causes major environmental pollution by total dissolved solids (TDS), biological oxygen demand (BOD), chemical oxygen demand (COD), sulfates, heavy metals, and chlorides [21, 22]. The main constituents of this tannery waste are protein. If these proteins and other chemicals which are present in the chemically treated protein are not consumed properly, it will pose a dangerous pollution problem to the ecosystem [23]. These wastes may be used to produce biomolecules using microbial strains. The microorganisms represent an exceptional source of protease owing to their extensive biochemical diversity and susceptibility to genetic manipulation. Among the various proteases, bacterial proteases are generally most noteworthy than others such as animal and fungal proteases [24, 25]. The main objectives of the present research work were the isolation of the proteolytic *Bacillus* strain and the utilization of tannery wastes as a substrate. Solid wastes such as beef skin were collected from the leather industrial area of District Kasur near Lahore, Punjab, Pakistan to produce alkaline protease that is mainly used in the leather industry as a dehairing and de-fleshing agent. Before the fermentation process, the skin and hides were washed and removed fat contents. FTIR and SEM studies were employed to understand the structural changes in both untreated (control) and treated (defatted) skin waste. Control and treated skin (tannery) wastes were also screened for optimum production of alkaline proteases. Furthermore, the optimization of parameters in bioreactors was also carried out.

2 Materials and methods

2.1 Collection and pretreatment of industrial skin waste

Tannery waste (industrial beef skin) was collected from a leather processing area, in District Kasur, Pakistan. The skin waste was processed in the industry for leather preparation. The collected skin waste was pretreated before it was used as a substrate for protease production because many chemicals and salts were applied on its surface in the tannery industry. The skin waste was cut into small pieces and washed with tap water. For the removal of absorbed salts and chemicals, skin waste was treated with 1.25% of ammonium chloride for 3 h [26]. Then, it was washed again with tap water and suspended in tap water for 2 h. After that, it was dried in an oven at 60 °C.

2.2 Proximate analysis and structure of treated and untreated industrial skin waste

For the estimation and removal of fat, it was treated with n-hexane for 7 h in the Soxhlet apparatus (AOAC, 2016) [27]. After the complete removal of fat, it was dried at 60 °C and ground to make powder. The protein content of industrial skin waste dried powder was determined following the Kjeldahl method. The ash contents were estimated from the weight loss which occurs through the complete rusting of defatted skin waste sample at a high temperature (usually 500 to 600 °C) through volatilization of organic materials according to the standard method (AOAC, 2016) [28]. Fourier transform infrared spectroscopy (FTIR) of fatted and defatted industrial skin wastes was performed by Agilent Technologies Cary 630 model at Food and Biotechnology Research Center PCSIR, Laboratories Complex

Lahore, Pakistan. Scanning electron microscopy (SEM) of fatted and defatted industrial skin wastes was conducted by S-3700N (Hitachi) at 10 kV with 500 magnification mode by field emission scanning electron microscope (FESEM) at the Pakistan Institute of Technology for Minerals and Advanced Engineering Materials (PITMAEAM) PCSIR, Lahore, Pakistan.

2.3 Isolation and screening of microbial strain

The soil samples were collected in sterilized bottles from three different sampling sites (slaughterhouse, fish processing areas, and poultry processing area) in Lahore City for isolation of proteolytic bacteria, in Food and Biotechnology Research Center at PCSIR Lahore, Pakistan. Each collected soil sample was diluted separately by adding 10 g of soil in sterilized normal saline (90 mL). The samples were serially diluted up to 10^{-5} after shaking for 1 h at 37 °C and allowed to heat shock at 80 °C for 15 min to kill all other vegetative cells except *Bacillus* species. Each diluted soil sample was inoculated on the skim milk agar medium (g/L skim milk 10, agar 15, peptone 1, NaCl 5, pH 8.5) and incubated at 37 °C for 24 h. Separated colonies that indicate a prominent clear zone on skim milk agar were again inoculated on the same medium and incubated at 37 °C for 48 h to re-visualize the proteolytic activity. Then, the selected strains were cultured on the production medium having the following composition: (g/L) industrial skin waste (defatted) 10, glucose 5, NaCl 0.5, $MgSO_4.7H_2O$ 0.5, and K_2HPO_4 5.0. Inoculate 2% (v/v) of 24 h of old culture in nutrient broth in the production medium and incubate for 48 h at 37 °C at 100 rpm in a shaker. Initially, the pH of the production medium was adjusted to 8.5. The production medium was centrifuged at 5000 rpm for 15 min at 4 °C after 48 h of fermentation and supernatant was used for enzyme assay.

2.4 Protease assay

The enzyme assay was conducted according to the slightly modified method of Sumita et al. [21]. The crude enzyme (1 mL) was mixed with 2 mL of 1% casein solution in a glycine–NaOH buffer (pH 10) and incubated for 30 min at 40 °C. Then, 3 mL of 10% trichloroacetic acid (TCA) solution was added to stop the reaction. The mixture was then centrifuged at 9000 rpm for 10 min. The absorbance was measured at 280 nm against the blank. Tyrosine standard curves of different concentrations were used to calculate the standard factor. The absorbance was measured at 280 nm against the blank. Tyrosine standard curves of different concentrations were used to calculate the standard factor.

$$\text{Enzyme Activity} = \frac{\text{Standard Factor} \times \text{Absorbance}}{\text{Time}}$$

2.5 Identification of the selected bacterial strain

2.5.1 Biochemical and morphological characteristics of the bacterial strain

The selected isolated strains were identified with the help of recommended method in Bergey's manual of determinative bacteriology [27] and Scott's diagnostic microbiology [28]. The maximum protease-producing strain was also preceded for molecular 16S rDNA identification.

2.5.2 Molecular characterization

Genomic DNA was isolated from a bacterial culture of maximum protease-producing strain, and then, ribosomal DNA was amplified [29] using universal primers. A thermocycler was used for the execution of PCR amplification. Amplified outputs were observed on 1.2% agarose gel electrophoresis, and then, DNA was cleared and purified using a GenJET™ kit. The purified amplicons were then commercially sequenced. The sequences of nucleotides were then aligned by using NCBI BLAST and were deposited in GeneBank. The sequence retrieved was ordered using CLUSTAL W 1.81 [30]. A neighbor-joining method in MEGA 5.0 (Molecular Evolutionary Genetics Analysis, version 5.0) software was employed for the phylogenetic tree construction [31].

2.5.3 Potential of Bacillus cereus AUST-7 for protease production employing fatted and defatted industrial skin waste

To estimate the potential of *Bacillus cereus* AUST-7 for protease production, the *B. cereus* AUST-7 strains were cultured on the production medium having the following composition: (g/L) industrial skin waste (defatted) 10 or industrial skin waste (fatted) 10, glucose 5, NaCl 0.5, $MgSO_4.7H_2O$ 0.5, and K_2HPO_4 5. Inoculate 2% (v/v) of 24 h of old culture in nutrient broth in the production medium and incubate for 48 h at 37 °C at 100 rpm in a shaker. Initially, the pH of the fermentation medium was adjusted to 8.5. The production medium was centrifuged at 5000 rpm for 15 min at 4 °C after 48 h of fermentation and supernatant was used for enzyme assay as described earlier.

2.6 Bioreactor scale optimization

The bioreactor experiments were conducted in a 2 L laboratory-scale bioreactor (B. Braun Germany) with a working volume of 1.5 L. The fermenter is equipped digitally with a temperature probe, dissolved oxygen electrode, pH electrode, and with dual six blades turbine impeller. The already optimized composition of medium (g/L) 20 industrial skin waste (defatted), 10 molasses, 0.5 NaCl, 0.5 $MgSO_4.7H_2O$, 5

K_2HPO_4, and 5 $NaNO_3$ was used to optimize process parameters. The effect of various parameters such as volume of air flow rate required per volume of liquid medium per minute (0.5, 1, 1.5, 2, 2.5 VVM) incubation period (24, 48, 72, 96 h) and shaking speed of fermenter (200, 300, 400, 500, 600 rpm) was studied.

2.7 Statistical analysis

The SPSS software was used for the comparison of means by post hoc test. The particle size of the samples was measured with the help of Java-based ImageJ software. A neighbor-joining method in MEGA 5.0 (Molecular Evolutionary Genetics Analysis, version 5.0) software was employed for the phylogenetic tree construction.

3 Results

In the present study, pretreatment of the industrial animal (beef) skin waste including cutting, washing with water, and treatment with ammonium chloride was carried out. The pretreated industrial skin wastes after removing salts/chemicals were ground in fine powder. The proximate composition (crude fat, crude protein, and ash contents) of industrial skin waste was presented in Table 1. The substrate has the highest percentage (83.81%) of crude protein whereas the lowest percentage (4.96%) of ash. The pretreated and defatted animal skin wastes after 11.23% fat extraction were used as substrate in further study.

Table 1 Proximate analysis of the skin waste

Serial no	Components	Percentage
1	Crude protein	83.81%
2	Crude fats	11.23%
3	Ash	4.96%

3.1 Morphological study and particle size distribution

The study of particle size, shape, distribution of particles, and surface morphology of control (untreated skin waste) and treated (defatted skin waste) samples was carried out using a field emission scanning electron microscope (FESEM). The particle size of the samples was measured with the help of Java-based ImageJ software. Various particle sizes were calculated as 32 µm, 49 µm, 52 µm, and 63 µm on the surface of the control sample (Fig. 1a) that contains fats and proteins of different shapes and sizes. These fats and proteins are distributed in a non-uniform fashion and highly agglomerated. Similarly, the treated (defatted) sample has a smooth and clear surface with a few crakes because of the lack of fat molecules (Fig. 1b). Fermentation of substrate having fat content (untreated) indicated lesser protease enzyme activity (214.86 IU/mL as compared with the treated (defatted) substrate (663.26 IU/mL).

3.2 FTIR analysis of the substrate

FTIR spectrum of skin sample showed major peaks at 3280, 2918, 2849, 1742, 1541, 1459, and 1235 Cm^{-1} that were attributed to -OH, $-CH_2$, -CH, -C=O, -NH (bending), $-CH_2$ (bending), and -C-O functional groups, respectively. The only major difference is between the spectra of skin samples and treated samples in the intensities of the vibrations at 2918 cm^{-1} (asymmetric $-CH_2$ stretch), at 2849 cm^{-1} (symmetric $-CH_2$ stretch), and at 1742 cm^{-1} (C=O stretch). These vibrations are associated with the presence of lipids, fatty acids, triglycerides, etc. The vibration at 1648 cm^{-1} was assigned to the -C=O stretch of the amide bond of protein, and the vibration at 1531 cm^{-1} was attributed to amide, which could originate from -N–H bending and -C-N stretching of the protein amide group (Fig. 2A). After extraction of fats with hexane, the intensities of peaks at 2919, 2849, and 1742 cm^{-1} were decreased significantly which demonstrates that lipid

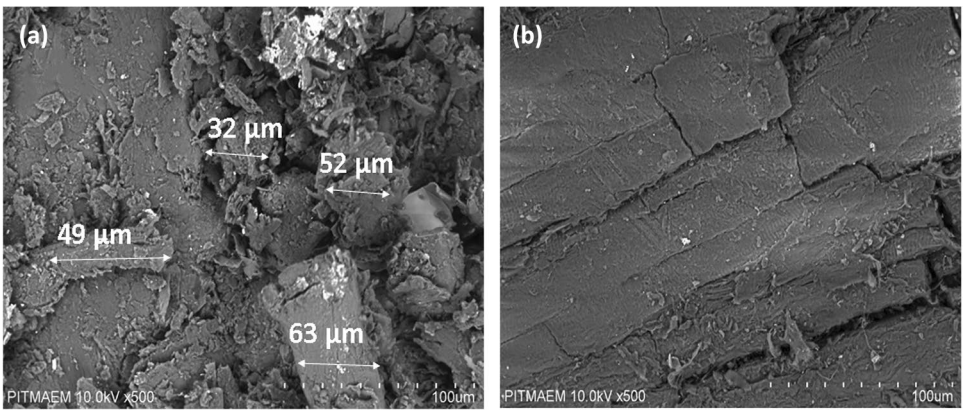

Fig. 1 **A** SEM study of the control sample and **b** treated (defatted) sample

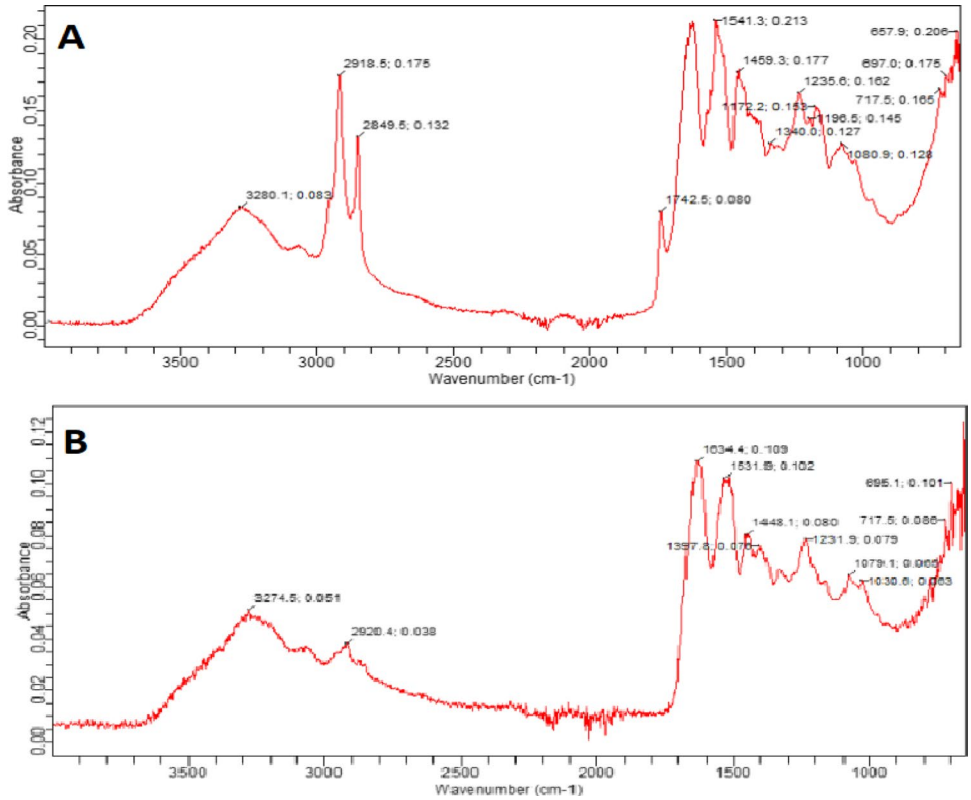

Fig. 2 **A** FTIR of control (skin waste) sample and **B** treated (defatted) skin waste

and fatty acid molecules were removed from the skin sample upon extraction with hexane (Fig. 2B).

3.3 Isolation, screening, and identification of microbial strain

In the present study, 140 *Bacillus* isolates were isolated from different soil samples of various areas of Lahore. Out of 140 isolates, 60 (42.85%) isolates showed a prominent clear zone on 1% skim milk agar medium. For qualitative estimation of protease, the selected isolates were cultured on the production medium. The *B. cereus* AUST-7 shows maximum proteolytic activity (249.65 IU/mL and 45 mm of clear zone) followed by *B. cereus* AUST-2 and *B. cereus* AUST-3 (208.76 and 188.60 IU/mL, respectively). Based on biochemical profiling (Table 2), it was observed that sixty protease-producing isolates belong to 8 different species of genus *Bacillus*, namely, *B. subtilis* (7), *B. licheniformis* (9),

Table 2 Biochemical and morphological characterization of proteolytic *Bacillus* strains

	Tests	Bacterial strains							
		B. subtilis	*B. licheniformis*	*B. cereus*	*B. polymyxa*	*B. pumilus*	*B. brevis*	*B. macerans*	*B. coagulans*
	G. staining	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve
	Mortality	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve
	Morphology	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods
Hydrolysis	1. Casein	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve
	2. Gelatin	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve
Biochemical	Citrate utilization	+ ve	+ ve	+ ve	+ ve	+ ve	− ve	− ve	− ve
Tests	VP test	+ ve	+ ve	+ ve	+ ve	+ ve	− ve	− ve	+ ve
	Oxidase	+ ve	− ve	+ ve	+ ve	+ ve	+ ve	− ve	+ ve
	Indole	− ve	− ve	− ve	− ve	− ve	− ve	− ve	− ve
	Catalase	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve
	Growth on MacConkey agar	+ ve	+ ve	− ve	+ ve	+ ve	− − ve	− ve	+ ve

B. cereus (11), *Bacillus polymyxa* (5), *Bacillus pumilus* (7), *Bacillus brevis* (7), *Bacillus macereans* (6), and *Bacillus coagulans* (8) as shown in Table 3. The identification of the maximum protease-producing strain was re-confirmed by 16S rDNA and identified as *B. cereus* AUST-7. The phylogenetic analysis of this strain revealed that it shared 99% homology with *Bacillus cereus* strain (NR_112630) (Fig. 3).

3.4 Screening of sample (fatted) and treated (defatted) substrate

To study the protease-producing potential of *B. cereus* AUST-7, fatted and defatted industrial skin waste was used in the production medium. *B. cereus* AUST-7 yielded maximum protease 663.26 ± 9.39 IU/mL in the presence of defatted skin waste in comparison with fatted skin waste of 214.86 ± 17.46 IU/mL as described in Table 4. The reason to choice defatted skin waste over the untreated counterpart for protease production is that fats (or lipids) are not substrates for protease; therefore, it is understandable that there was little protease activity in high-fat content substrates than defatted substrates. Based on this, further experimental work was carried out using defatted skin wastes.

3.5 Bioreactor studies

Different bioprocess parameters were studied one by one in fermenter to achieve maximum production of protease enzyme. Firstly, incubation period was optimized by giving each batch different incubation period of 24, 48, and 72 h, respectively, while keeping aeration and shaking speed constant. Similarly, at static condition and the absence of

Table 3 Production of protease by various strains of *Bacillus* sp. in submerged fermentation

Serial no	Bacterial strain	Enzyme activity IU/mL	Serial no	Bacterial strain	Enzyme activity IU/mL
1	*Bacillus subtilis* AUST-1	130.23	31	*Bacillus polymyxa* AUST-4	106.19
2	*Bacillus subtilis* AUST-2	109.09	32	*Bacillus polymyxa* AUST-5	104.49
3	*Bacillus subtilis* AUST-3	100.32	33	*Bacillus pumilus* AUST-1	98.64
4	*Bacillus subtilis* AUST-4	106.25	34	*Bacillus pumilus* AUST-2	102.97
5	*Bacillus subtilis* AUST-5	110.93	35	*Bacillus pumilus* AUST-3	108.43
6	*Bacillus subtilis* AUST-6	101.03	36	*Bacillus pumilus* AUST-4	105.78
7	*Bacillus subtilis* AUST-7	108.09	37	*Bacillus pumilus* AUST-5	80.35
8	*Bacillus licheniformis* AUST-1	113.43	38	*Bacillus pumilus* AUST-6	98.20
9	*Bacillus licheniformis* AUST-2	106.98	39	*Bacillus pumilus* AUST-7	111.23
10	*Bacillus licheniformis* AUST-3	65.95	40	*Bacillus brevis* AUST-1	110.93
11	*Bacillus licheniformis* AUST-4	120.43	41	*Bacillus brevis* AUST-2	101.32
12	*Bacillus licheniformis* AUST-5	113.63	42	*Bacillus brevis* AUST-3	91.32
13	*Bacillus licheniformis* AUST-6	105.05	43	*Bacillus brevis* AUST-4	96.65
14	*Bacillus licheniformis* AUST-7	101.45	44	*Bacillus brevis* AUST-5	121.04
15	*Bacillus licheniformis* AUST-8	100.61	45	*Bacillus brevis* AUST-6	132.91
16	*Bacillus licheniformis* AUST-9	85.48	46	*Bacillus brevis* AUST-7	112.03
17	*Bacillus cereus* AUST-1	180.23	47	*Bacillus macerans* AUST-1	99.42
18	*Bacillus cereus* AUST-2	208.76	48	*Bacillus macerans* AUST-2	123.54
19	*Bacillus cereus* AUST-3	188.60	49	*Bacillus macerans* AUST-3	107.08
20	*Bacillus cereus* AUST-4	120.65	50	*Bacillus macerans* AUST-4	128.30
21	*Bacillus cereus* AUST-5	135.48	51	*Bacillus macerans* AUST-5	133.69
22	*Bacillus cereus* AUST-6	110.79	52	*Bacillus macerans* AUST-6	119.51
23	*Bacillus cereus* AUST-7	249.65	53	*Bacillus coagulans* AUST-1	117.82
24	*Bacillus cereus* AUST-8	131.31	54	*Bacillus coagulans* AUST-2	51.31
25	*Bacillus cereus* AUST-9	126.45	55	*Bacillus coagulans* AUST-3	63.69
26	*Bacillus cereus* AUST-10	147.62	56	*Bacillus coagulans* AUST-4	89.43
27	*Bacillus cereus* AUST-11	170.26	57	*Bacillus coagulans* AUST-5	102.17
28	*Bacillus polymyxa* AUST-1	108.54	58	*Bacillus coagulans* AUST-6	69.77
29	*Bacillus polymyxa* AUST-2	134.50	59	*Bacillus coagulans* AUST-7	96.49
30	*Bacillus polymyxa* AUST-3	120.21	60	*Bacillus coagulans* AUST-8	108.52

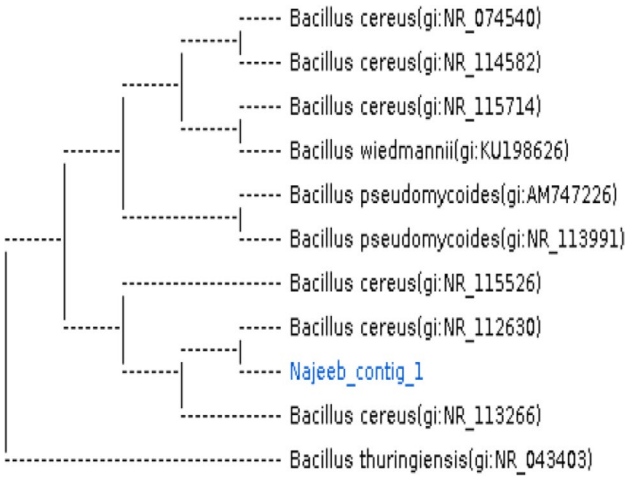

Fig. 3 Phylogenetic studies of a newly isolated *Bacillus cereus* from the soil

Table 4 Screening of sample and treated (defatted sample)

Serial no	Types of substrate	Enzyme activity ± SD
1	Sample (fatted skin waste)	214.86 ± 17.46
2	Treated sample (defatted skin waste)	663.26 ± 9.39

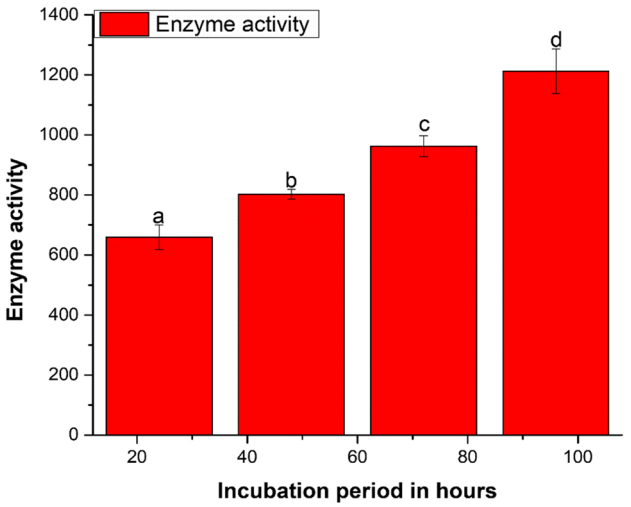

Fig. 4 Effect of incubation period at static condition without aeration on protease production

aeration, maximum activity was achieved after 96 h of incubation period as indicated in Fig. 4.

Further bioprocess parameter optimization of protease production at bioreactor level illustrated that aeration rate of 2 VVM, shaking speed of 500 rpm, and 24 h of incubation period yielded maximum production up to 1945.2 ± 44.87 IU/mL (Figs. 5 and 6). Briefly, to find best growth at different aeration rate, a set of five batches in fermenter were run having 0.5, 1, 1.5, 2, and 2.5 VVM (volume of air flow rate required per volume of liquid medium per minute) for 24-h incubation period with constant agitation rate of 300 rpm. Among that, the best results were found at 2 VVM aeration rate as shown in Fig. 5. Similarly, for optimization agitation speed, same set batches were run at constant aeration rate, i.e., 2 VVM and incubation period of 24 h with different shaking speed of 200, 300, 400, 500, and 600 rpm as shown in Fig. 6. Maximum enzyme activity was detected from the batch run on 500 rpm.

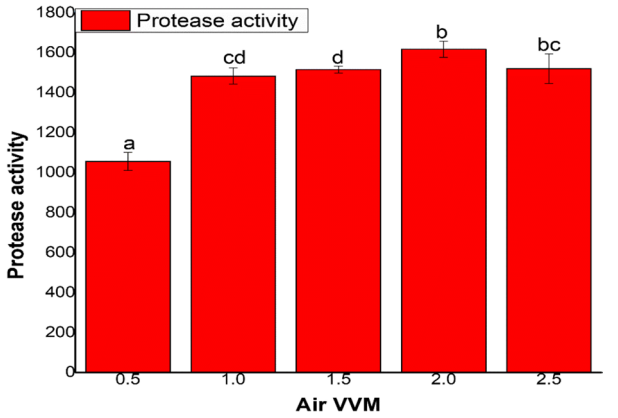

Fig. 5 Effect of aeration rate on protease production at constant shaking speed of 300 rpm and 24-h incubation period

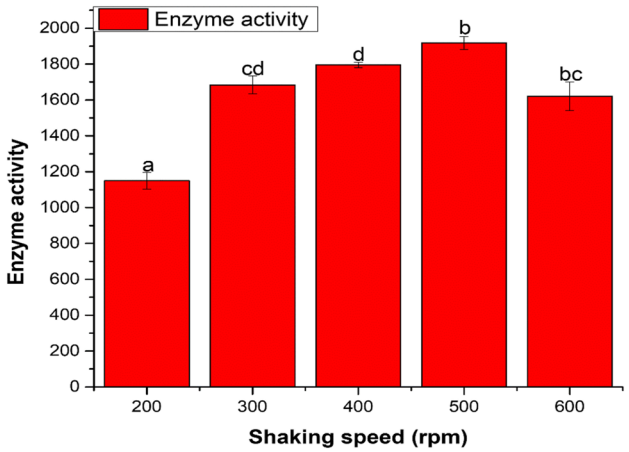

Fig. 6 Effect of agitation rate on protease production at constant aeration rate of 2 VVM and 24-h incubation period

4 Discussion

Proteases from microbial sources are stable remarkably and highly dynamic, and large-scale production in a more cost-effective way is convenient. Many microorganisms

produced proteases; however, the researchers always desired to search for higher yielding strains and optimization techniques for maximum production [11]. The present investigation includes exploring the consumption of industrial skin waste (tannery waste) as a substrate, its proximate analysis, FTIR and SEM of the skin waste sample, and defatted skin waste. The production of protease was conceded out by the newly isolated *Bacillus* strain from proteinaceous sources of soil from different areas of Lahore City of Pakistan and its identification, morphological, biochemical, and molecular characterization by 16S rDNA for the production of an alkaline protease. This study also involves the bioreactor scale optimization process such as aeration, shaking speed, and incubation period. The industrial skin (tannery) wastes were treated with 1.25% of ammonium chloride to all salts and chemicals absorbed during the manufacturing of leathers from the skin. The same procedure was conducted by Kumar et al. [33]. A similar process was carried out by Ahmad and Ansari [34] for the removal of absorbed salt and chemicals.

A group of researchers (Kumar et al. [35]) used proteinaceous solid wastes produced by leather processing industries as a substrate for the production of alkaline protease and found the maximum protease activity of 1160–1175 U/mL. Similarly, the alkaline protease was produced by a group of researchers and used *Synergistes* sp. for the production of alkaline protease, utilized tannery solid wastes as a substrate under submerged and solid-state fermentation, and found the highest activity under solid-state fermentation of 745–755 U/g. These studies were found similar to the present investigation. The concentration of protein, fats, and ash was 83.81%, 11.23%, and 4.96% as represented in Table 1. Kumar et al. [35] reported crude protein ranged from 85 to 93%, and Akwetey et al. [36] reported 91.85% of crude proteins in the cattle's hides collected from the tannery industry. Taha et al. (2016) measured ash and fates contents of 6.01 and 11.79%, respectively. SEM indicated major differences in particle size and distribution among fatted and defatted skin waste. The bending position of both samples was specified by FTIR. The vibrations associated with lipids, fatty acids, and triglycerides were at 2918 cm^{-1} (asymmetric -CH_2 stretch), at 2849 cm^{-1} (symmetric -CH_2 stretch), and at 1742 cm^{-1} (C = O stretch). These were decreased significantly upon extraction with hexane. The peaks at the regions 1500-6650 cm^{-1} represent small variations and especially at a wavelength of 1118 and 1096 cm^{-1} corresponding to the C-H bending and C-H deformations of fatty acid [37].

Likewise, different researchers used different substrates for the production of alkaline protease. Erwanto et al. [38] used protease-producing *Bacillus* sp. SKK11 horse gram husk was used as substrate and reported the highest protease activity of (240 U/mL) by employing maltose as a source of carbon. Govarthanan et al. [39] used four different types of substrates viz., skim milk, wheat bran, casein, and rice bran. Maximal protease activity was detected in BM2 basal medium with wheat bran after a 9.5-incubation period. The activity of extracted protease was found 341 U/mL. *Bacillus* sp. being commercially significant organisms produces an extensive variety of extracellular enzymes including proteases [38]. Various *Bacillus* spp. are involved in protease production, e.g., *Bacillus stearothermophilus*, *Bacillus megaterium*, *B. cereus*, *Bacillus mojavensis*, and *B. subtilis* [40, 41]. The proteases are produced by various microorganisms present in soil, water, and extremely alkaline environments including industrial water, desert soil, springs, and from decaying organic matter [42].

The newly isolated strain of *Bacillus cereus* AUST-7 was found to be the best producer as it possesses a prime zone of clearance on skim milk agar medium and the highest protease activity of 249.65 IU/mL followed by *Bacillus cereus* AUST-2 of activity 208.76 IU/mL. Ookkheo et al. [43] screened protease-producing bacteria from wastewater of an abattoir, and among these, *Bacillus cereuses* gave maximal protease activity of 75.79 Units/mL/min by the submerged fermentation process. Kanekar et al. [44] carried out the production of protease from *Bacillus cereus*, and cheese whey was subjected as a low-cost substrate and yielding a significantly high amount of protease (185.7 U/mL). The amount of production rate is equal to the present production of alkaline protease. Ayantunji et al. [45] isolated three strains of *Bacillus* that showed the highest potential for the production of protease and were identified as *Bacillus subtilis* NRD9, *B. subtilis* RD7, and *B. cereus* ABBA1 and via analysis and amplification of 16S rRNA genes. The results achieved from the Insilco experimental design that showed high protease activity of 138.17 U/mL, 141.28 U/mL, and 159.43 U/mL while experimental validation created a high protease activity of 163.76 U/mL, 176.00 U/mL, and 200.56 U/mL for strains NRD9, RD7, and ABBA1 respectively in an optimized medium which is closely related the result obtained in the present study by *Bacillus cereus* AUST-7 having the highest protease activity of 249.65 IU/mL followed by *Bacillus cereus* AUST-2 of activity 208.76 IU/mL. Another study reported by Rathnasamy et al. [46] isolated protease-producing bacteria from the soil containing the organic wastes, screened for production of protease on skim milk agar medium, and further confirmed the activity of protease via assay. The bacterial strain that exhibited maximal alkaline protease production was subjected to and identified by biochemical, microscopic, and 16S rRNA phylogenetic analyses as *Bacillus cereus* FT 1. The maximal yields of the enzyme were attained by the isolate at pH 9.5, 35 °C, lactose 2% as a carbon source, and 3.5% casein as a source of nitrogen after 48 h of the incubation period, and the enzyme activity was found to be 187 U/mL and is nearly equal to the present protease productivity.

Biochemically and morphologically, the newly isolated strain was identified as *Bacillus* sp., and the same result was reported by Suberu et al. [47] using biochemical and morphological tests with the help of recommended method in Bergey's manual of determinative bacteriology and Scott's diagnostic microbiology. The selected isolate was identified as *Bacillus cereus* on the base of 16S rDNA sequence analysis that includes blasting it against the available databases like RDP and NCBI. Finally, the software MEGA 5 was employed for the construction of a phylogenetic tree. By using the 16S ribosomal DNA sequencing, the bacterium with the highest protease-producing potential was classified as *Bacillus cereus*. Similarly, Asha and Palaniswamy [48] identified alkaline protease-producing isolate from industrial soil samples of poultry wastes as *Bacillus cereus*. Likewise, different researchers via 16S rDNA analysis and sequencing were able to characterize and identify novel strains [49–51].

The production of protease at the bioreactor level shows that the aeration rate of 2 VVM, stirring speed of 500 rpm, and 24-h incubation period produced a maximum production of up to 1945.2 IU/mL. A research study was conducted by Waghmare et al. [52] using *B. licheniformis* N-2 and explored the effect of aeration rate and shaking speed on the production of alkaline protease in a 2-L stirred tank bioreactor. Maximum protease yield was found at the aeration rate of 2 VVM and shaking speed of 500 rpm. All the above-stated studies are in good agreement with the obtained results in the current study.

5 Conclusions

The present research work was conducted to utilize the industrial skin (tannery) wastes and their characterization for exploitation as a substrate for alkaline protease production from *Bacillus* sp. Soil from decaying organic matter contains a huge community of microbes that can degrade organic substances and produces numerous secondary metabolites. Optimization of physical parameters for maximum growth of microorganisms for their high production is always required. It is concluded that the defatted industrial skin (tannery) wastes are a rich source of proteinaceous nutrients and good and cost-effective substrate for the production of protease from *Bacillus* sp. Another way, it can also minimize the environmental effluence of leather industries. The present effort will also be useful to exchange hazardous chemicals used in leather manufacturing industries.

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Data availability All the data generated in this research work has been included in this manuscript.

Declarations

Ethical approval Not applicable.

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