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Isolation and Characterization of Bacteria Associated with Tannery Effluent and Their Protease Producing Ability

Jehan Zeb[a], Nadia Zeeshan[a], Ghazalah Yasmin[b]* and Umbreen Javed Khan[c]
[a]Department of Biochemistry and Molecular Biology, University of Gujrat, Gujrat-Pakistan
[b]Government Graduate College For Women, Jehlum-Pakistan
[c]Government Graduate College for Women, Model Town, Lahore-Pakistan

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Abstract. The bacteria producing proteases are important for the industries. The proteases are utilized commercially in leather, detergent, pharmaceutical and food industry. The proteases are hydrolytic enzymes which can degrade protein into peptides and amino acids. In leather industry proteases are employed to remove the redundant parts from the animal hide especially hairs to make fine quality of the products. The proteases also prove as green environmental approach. In present study a total of 40 bacterial isolates were recovered from the soil samples of the tannery and screened for proteolytic activity on casein ager plates, among them three isolates were selected with good activity. The morphological and biochemical characteristic features were used to identify the strains; the different conditions of the culture and medium were optimized to check the protease activity. The best proteolytic activity was observed at temperature of 37 °C, pH 8.5, 6% casein concentration as substrate and casein as nitrogen sources as well at 12 h of incubation. The maximum activities by SZ2, SZ1 and SZ3 observed 16.53U/mL, 8U/mL and 7.16U/mL, respectively. The goat skin was treated with proteases from these isolates, complete dehairing observed after 12 h of incubations. Present study was conducted to identify microbes from local tannery, to find out most efficient strain for protease production and to use these enzymes in leather industry.

Keywords: proteases, green environmental approach, tannery, dehairing

Introduction

The environment of earth in good excellence is necessary for life. The protection of environment demanded the use of alternative techniques. The two major issues of pollution, the wastes discarding and industrial effluents are observed (Glick and Pasternak, 1994). Biocatalytic practices deal with more favorable environmentally sound procedures (Lens *et al*., 2004). The majority of enzymes are produced from micro-organisms as 40% of proteases are of microbial origin (Rani *et al*., 2012).

The proteases are enzymes that catalyze peptide bond in proteins and mostly isolated from *Bacillus* sp., *Aspergillus* sp. and *Amycolatopsis* sp. The low cost, high production and efficient activity of proteases make them very significant. So, they are used in leather industry, food industry and wastewater treatment (Kumar and Sharma, 2019). The proteases from microbe make two-thirds of their commercial production and account for more than 60% sale of total enzyme sales (Haider *et al*., 2019). The *Bacillus* sp. is well known for producing most commercial proteases which are neutral, alkaline in nature and showed substrate specificity (Khan, 2013). *Bacillus* produces extracellular proteases so found applicable in different industries (Pastor *et al*., 2001). The micro-organisms especially, bacteria are easy way to get proteases because they can be culture by fermentation method in large quantities in a short time (Pradeep *et al*., 2012).

Tanning is mainly identified as the high-water intake industry which releases huge amount of wastewater containing many compounds that converts skin of animals (Zhao *et al*., 2022). The leather industry has economic importance as it provides employment and foreign exchange by the means of its products. 20% of raw skin is converted to finished leather goods during tanning, while 80% result in effluents. The lime and sulphides are conventionally used for dehairing in tanning but it causes pollution and purification of dehairing solution is very difficult and expensive. So, enzymes are environmentally friendly alternative in un-hairing. Khambhaty (2020) observed about 70 un-hairing proteases and keratinases from bacteria and fungi.

*Author for correspondence;
E-mail: ghazalahrizwan@gmail.com

The environmentally safer laws are becoming strict and leather industry has to use alternate to minimize its negative impact on environment. The use of enzymes in tanning is efficient tool in this regard (Biðkauskaitë *et al*., 2021).

Present work was conducted to identify microbes in local tannery for the isolation of enzymes from most efficient strain so that to produce protease for the use in leather industry.

Material and Methods

Screening, isolation and maintenance of proteolytic bacteria. The soil samples of shavings, trimmings and leather splits were collected from local commercial tannery wastes from city Sambaryal of district Sialkot Punjab, Pakistan. The casein hydrolyzing bacteria were screen from the soil by culture enrichment method and pore plate method, the two to three grams of the samples were taken. The modified Han's (MH) medium was used with casein as nitrogen source the composition of media was ammonium sulphate 0.1%, magnesium sulphate 02%, calcium chlorite 01%, dipotassium hydrogen phosphate 05%, potassium dihydrogen phosphate 05%, yeast extract 2%, casein 5% (Kasana *et al*., 2008). The pure colonies were spotted on the duplicate plates eight colonies were picked from each sample and labeled 1 to 8. After incubation at 37 °C for 12 h, formation of clear hydrolysis zone was observed that showed the proteases activity. The diameter indicated the potential of protease production of each colony. This is called primary screening (Kannan, 2002) selected for secondary screening.

Identification of selected strain. The strains were identified by the gram staining, colony morphology, shape of bacteria and motility. The biochemical tests were performed by the rapID™ ONE system kit like urea, arginine, ornithine, lysine, aliphatic thiol, fatty acid ester, sugar aldehyde, sorbitol, ρ-nitrophenyl-β, d glucoronide, σ-nitrophenyl-β, d galactoside, ρ-nitrophenyl-β, d glocoside, ρ-nitrophenyl-β, d-xyloside, ρ-nitrophenyl-n-acetyl-β, d-glucosaminide, malonate, proline β-naphthylamide, γ-glutamyl- β- naphthylamide, pyrrolidonyl- β- naphthylamide, adonitol, trytophane, oxidase, catalase and glucose test that based on the microbial degradation of the specific substrate.

Isolation of crude enzyme. For the production of proteases the liquid fermentation process was performed. The incubation was carried at 37 °C for 12 h at 100 rpm. The fermented culture was centrifuged at 4500 rpm for 20 min at 4 °C. The filtrate was crude enzymes.

Protease assay. The mixture was prepared by dissolving the 1 mL of substrate (casein) and same volume of the crude enzyme, incubated at 60 °C for 20 min then 2 mL of the trichloro acetic acid (TCA) was added and centrifuge at the 4500 rpm for 10 min. The supernatant was observed at 280 nm in the spectrophotometer. The reference of the sample was also prepared to check the proper efficiency of the enzyme with same composition and volume except the incubation time that is necessary for the activity of the enzyme.

Process of optimization for maximum protease production. The parameters like temperature, pH, substrate concentration and nitrogen source were optimized for selected bacterial strains for the production of proteases. The fermentation performed for 12 h. In present study the temperature range was from 28, 33, 37, 42 °C and pH range from 6.5, 7.5, 8.5 and 9.5. The substrate (casein) concentration in media ranged from 2, 3, 4, 5 and 6%. The ammonium chloride, sodium nitrate, yeast extract, peptone and casein are nitrogen sources which examined for their effect on protease production

Enzymatic dehairing. The skin of goat was cut to approximately 2 inches and incubated with the 15 mL of partially purified crude protease at 37 °C, with different incubation period of 2 h, 4 h, 6 h, 8 h and 12 h, respectively. The skin was checked for dehairing activity at different incubation periods. After incubation the blunt knife was used to hair removed from the skin.

Results and Discussion

Initially fourty isolates of five samples were cultured but only three were selected for their diameter of hydrolysis zone and proteases activity. The size of hydrolysis zone was 30 mm, 6 mm and 5 mm and protease activity 16 U/mL, 8 U/mL and 7.16 U/mL, observed by SZ2, SZ1 and SZ3, respectively. The isolate SZ2 showed maximum protease activity (Table 1).

Morphological and biochemical identification. The bacterial colony of SZ1, SZ3 was *Streptococcus* and SZ2 was *Bacillus* the SZ1, SZ3 were gram positive, while SZ2 gram negative (Table 01). The rapID™ ONE system kit used for biochemical tests of isolates, diverse colours in the kit and + - signs indicated their abilities

to consume them as carbon and nitrogen source or not (Table 2). Arginine, lysine, ornithine, aliphatic thiol, fatty acid ester, sager aldehyde , sorbitol, ρ-nitrophenyl-β,d-xyloside, ρ-nitrophenyl-n-acetyl-β, d-glucosaminide, γ -glutamyl-, β- naphthylamide, pyrrolidonyl, β- naphthylamide positive isolates are SZ 1, SZ 2, SZ3 and these isolates showed negative results for the urea, tryptophan and ρ-nitrophenyl-β, d glucoronide. The SZ2 is further tested positive for σ- nitrophenyl- β, D-galactoside, adonitol and negative for the proline β-naphthylamide, malonate. Bases on these tests, few more tests (oxidase, catalase, glucose and mannitol fermentations) were carried out. The SZ2 was oxidase positive by the appearance of diffusible green colour (Fig. 4) and its glucose fermentation was negative. Therefore, SZ2 was recognized as *Pseudomonas aeruginosa*, while SZ1, SZ3 which showed positive results for catalase, mannitol by the appearance of distinguished white cluster (Fig. 4) identified as *Staphylococcus* spp.

Optimization of different production parameters for proteases activity. ***Effect of temperature.*** The protease activity in culture supernatant gradually increased by the isolates as fermentation temperature was increased from 28 °C to 37 °C (Fig. 1) but further increase in temperature up to 42 °C decreases the activity which represented that 37 °C optimum temperature for enzymatic activity that was 16.53 U/mL, 8 U/mL and 7.16 U/mL by SZ2, SZ1 and SZ3, respectively. The different studies confirmed that the proteases production by various microbes showed maximum activity at the 37 °C (Ifrij *et al*., 2002). Economically important proteases are secreted by the genus *Bacillus* (Hashmi *et al*., 2022). *Bacillus licheniformis* N-2 showed proteolytic activity at 37 °C (Nadeem *et al*., 2008).

Table 1. Characteristics of the protease producing isolates (bacteria)

Isolate	Colony colour	Gram +/-	Shape (colony)	Shape (cell)	Protiolytic index	Activity(U/mL)
SZ 1	Violet	+	Round	*Staphylococcus*	6 mm	08
SZ 2	Pink	-	Rod	*Bacillus*	30 mm	16
SZ 3	Violet	+	Round	*Staphylococcus*	5 mm	7.16

Table 2. Biochemical properties of the protease producing isolates (bacteria)

Biochemical test	Isolate (SZ 1)	Isolate (SZ 2)	Isolate (SZ 3)
Urea	-	-	-
Arginine	+	+	+
Ornithine	+	+	+
Lysine	+	+	+
Aliphatic thiol	+	+	+
Fatty acid ester	+	+	+
Suger aldehyde	+	+	+
Sorbitol	+	+	+
ρ-Nitrophenyl-β, dglucoronide	-	-	-
σ-Nitrophenyl-β, dgalactoside	+	+	-
ρ-Nitrophenyl-β, dglocoside	-	+	-
ρ-Nitrophenyl-β, d-xyloside	+	+	+
ρ-Nitrophenyl-n-acetyl-β, d-glucosaminide	+	+	+
Malonate	+	-	+
Proline β- naphthylamide	+	-	+
γ -Glutamyl- β- naphthylamide	+	+	+
Pyrrolidonyl- β- naphthylamide	+	+	+
Adonitol	-	+	+
Trytophane	-	-	-

Akcan and Uyar (2011) viewed many bacterial species showed proteases activity at 37 °C.

Effect of pH. The protease activity in culture supernatant increased with an increase of the pH of culture medium from pH 6.5 to 8.5 (Fig. 2). There was a decrease in protease activity by further increasing the pH of the culture medium from 8.5 to 9.5. So, higher protease activity was at pH 8.5, the maximum enzymatic activity was 16.8 U/mL, 8.6 U/mL and 7.6U/mL by SZ2, SZ1 and SZ3, respectively. The observed that optimum pH for the. For the maximum activity of proteases generating bacteria, pH range varies from 8 to 9 (Jayasree *et al*., 2010; Mukherjee *et al*., 2008; Giongo *et al*., 2007) and reported that alkaline proteases showed keratinolytic activity for dehairing during tanning.

Effect of substrate. Microorganisms growth and enzyme action are affected by different concentrations of the substrate. The protease activity in culture supernatant increased with an increase concentration of the casein in culture medium from 0.2% to 0.6% (Fig. 3) which represented the proteolytic activity range from 16 U/mL, 8 U/mL and 7.5 U/mL by bacterial isolates SZ2, SZ1 and SZ3, respectively. The microbes used casein as nitrogen source to produce extracellular proteases (Fikret *et al*., 2011; Mukherjee *et al*., 2008).

Effect of nitrogen sources. The protease activity in culture supernatant increased with casein in culture medium as nitrogen source that showed high enzymatic activity was 16 U/mL, 07 U/mL and 6.9 U/mL by SZ2, SZ1 and SZ3 respectively (Fig. 4.). NH_4Cl, $NaNO_3$,

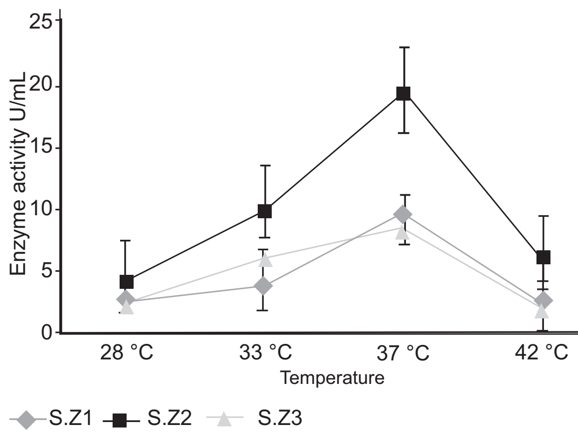

Fig. 1. Enzyme activity of the isolates at different temperatures.

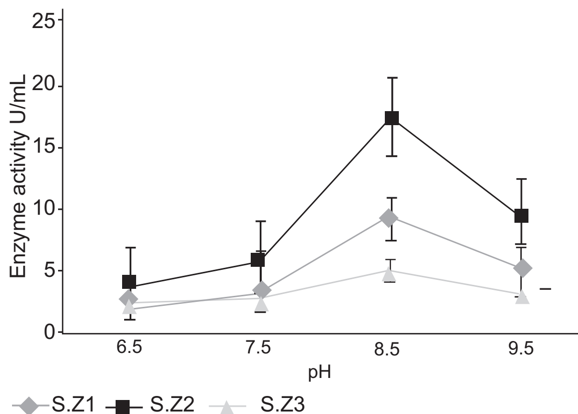

Fig. 2. Enzyme activity of the isolates at different pH.

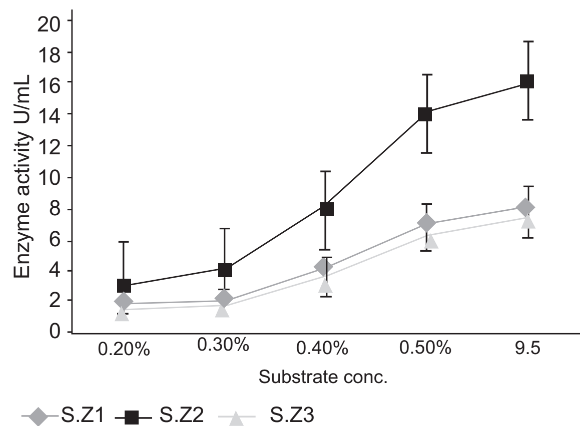

Fig. 3. Enzyme activity of the isolates at different substrate concentrations.

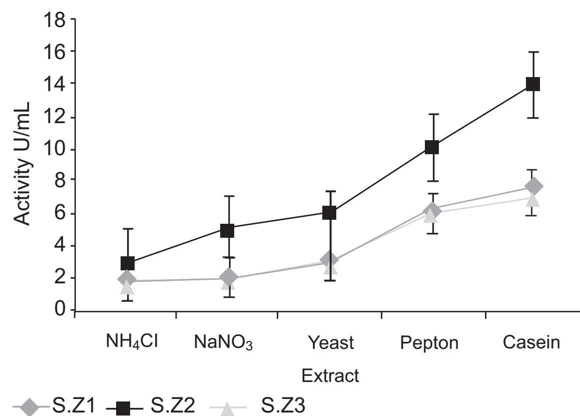

Fig. 4. Enzyme activity of the isolates at different nitrogen sources.

yeast extract and peptons were also used as nitrogen sources but proteolytic activity was enhanced with casein. Casein and pepton are more important than ammonium chloride and sodium nitrate to produce of extracellular proteases (Uyar *et al*., 2011). Present study also confirmed that casein is more important because it act as substrate and nitrogen source.

Dehairing. The maximum dehairing activity by the proteases was observed by SZ2, SZ1 and SZ3 respectively at 4, 6, 8 and 12 h incubation, respectively (Fig. 5a-5d). When crude enzyme extraction of bacterial colony SZ2 was applied for dehairing of goat skin, maximum activity was detected at 12 h incubation, the same isolate showed maximum activity in enzyme assay at different pH, substrate concentrations and nitrogen sources. Jaouadi *et al*. (2010) viewed the importance of gram positive *Bacilli* species in production of proteases involve in the dehairing. Around 70 unhairing proteases and keratinases have been isolated from bacteria and fungi (Khambhaty, 2020).

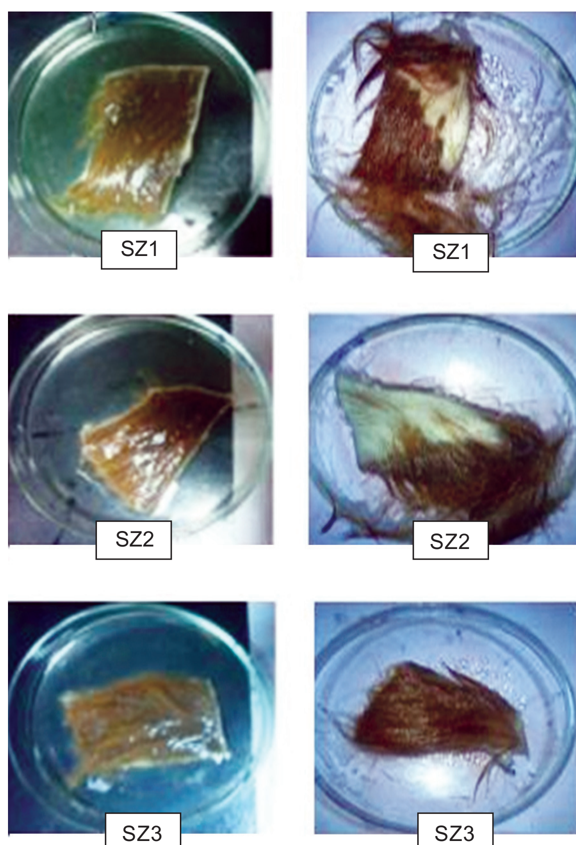

Fig. 5a. De-hairing activity by the protease at 4 h of incubations-1.

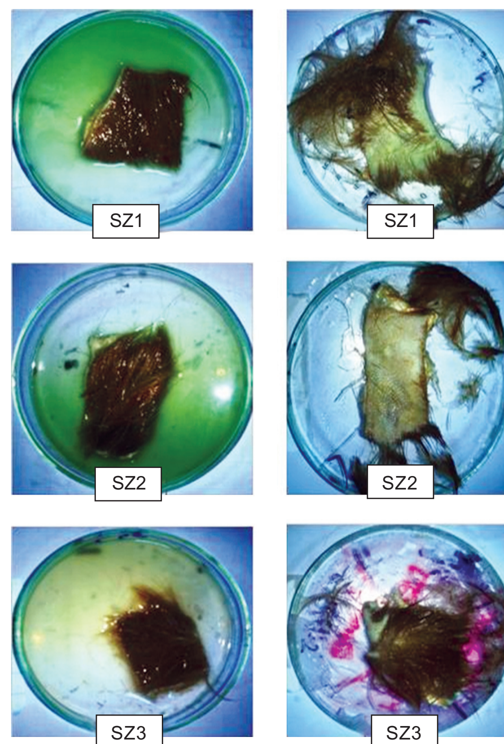

Fig. 5b. De-hairing activity by the protease at 4 h of incubations.

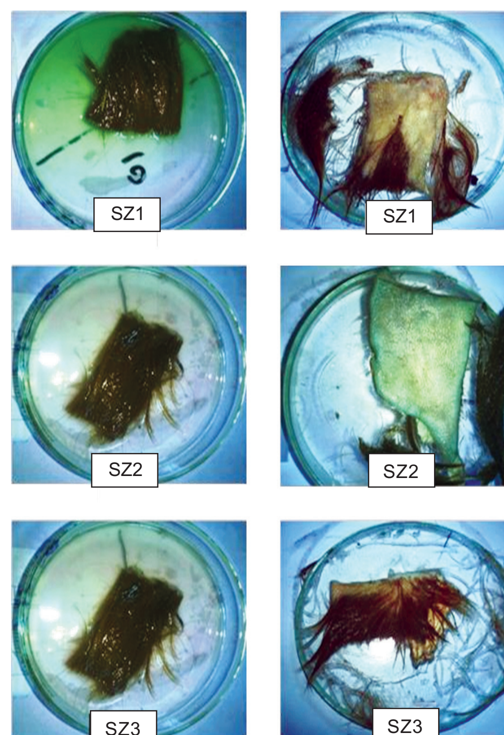

Fig. 5c. De-hairing activity by the protease at 6 h of incubations.

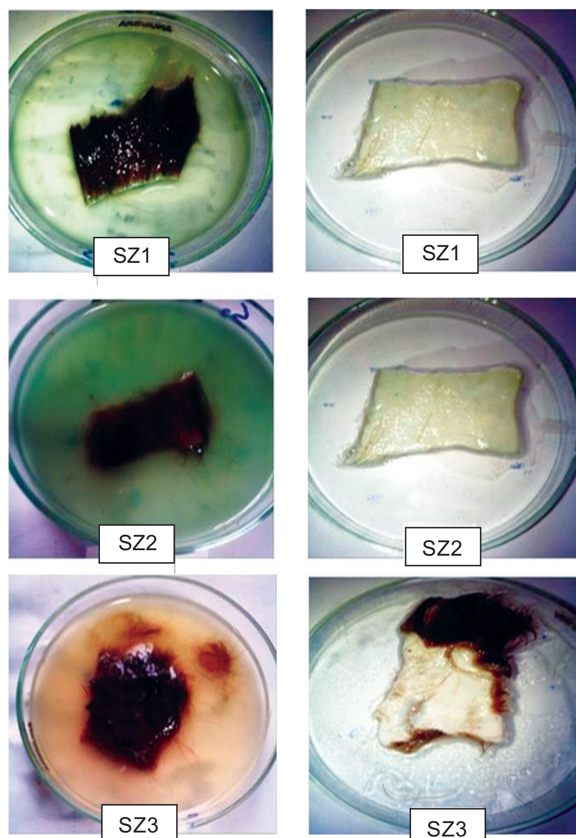

Fig. 5d. De-hairing activity by the protease at 12 h of incubations.

Conclusion

The tanneries are in confronted of environmental legislation as environmental laws become more severe and concern about negative impacts of industries. So, it is necessary to protect environment by exploring microbes for enzyme production It can be concluded from present study that bacterial isolates taken from local tannery produce proteases in alkaline culture medium while growth and production of proteases regulated by different factors of all isolates, maximum proteases activity was seen in SZ2 that was identified as *Pseudomonas aeruginosa* by morphological and chemical characterization. The proteases produced in present study isolates can be used in leather industry for de-hairing process as applied to the goat skin and maximum de-hairing was observed after 12 h of incubation.

Conflict of Interest. The authors declare that they have no conflict of interest.

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