ISOLATION & EVALUATION OF PROTEASE PRODUCING BACTERIA FROM SOIL SAMPLES OF DISTRICT MARDAN, PAKISTAN

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ABSTRACT

Microbial proteases, widely used in various industrial processes and commercial applications, are hydrolytic enzymes of significant importance. This study aimed to isolate and characterize efficient protease-producing bacteria from soil samples collected in Mardan. Screening for proteolytic activity on skim milk agar and casein agar yielded six potent bacterial isolates. These were identified as five Bacillus species and one cocci species based on morphological and biochemical characteristics. The optimum protease production time for the isolates was determined to be 24 hours, with proteolytic activities ranging from 4 mm to 12 mm. The isolate P6 exhibited the highest activity (12 mm), while P11 showed the least (4 mm). Optimal conditions for protease activity were found to be 37°C and pH 9 for all isolates, with activity declining at higher temperatures. Substrate specificity tests using casein, soybean flour, and gelatin revealed that the isolates demonstrated superior proteolytic activity on soybean flour and casein. The effect of various additives on protease production was also investigated. Media supplemented with MgCl2 resulted in maximum proteolytic activity compared to other metallic ions tested (CaCl2 and NaCl). However, NaCl was found to be effective for some isolates, particularly P6 Bacillus sp., which consistently showed the highest protease activity across all parameters tested. This study identifies several promising protease-producing bacterial strains, with P6 Bacillus sp. emerging as the most potent. Further characterization and purification of these enzymes at the molecular level are recommended for their potential industrial applications.

Key Words: Proteases, Soil bacteria, proteolysis, optimization, Evaluation, Mardan

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INTRODUCTION

Enzymes are known as biocatalyst because they are highly efficient to speed up any chemical reactions and most of the protein catalysts are synthesized by living organisms, some enzymes can work even in extreme temperatures (Nadeem *et al*. 2009). Without the presence of enzymes, the chemical reaction in biological cells would occur too slowly. During chemical reaction it remain unchanged and can be used again and again in specific reaction but any malfunctioning or under production of enzymes can lead to severe diseases (Abdullah *et al*. 2006).

Proteases belongs to the class of enzymes known as hydrolases that play important role with respect to their physiological functions as well as their commercial applications.75% of hydrolases enzymes are used in industries. Out of which 40% are protein degrading enzymes (Shaheen *et al*. 2008). Proteolytic enzymes are ubiquitous in nature, consider as most essential for cell growth and differentiation. Industrially point of view Proteases represent one of the three largest groups important enzymes (Lakshmi *et al*. 2014). It naturally occurs in all living organisms. They are involved in a different type of physiological reactions from simple digestion of food to complex break down of dead organic matter. According to their function they are present in different parts of human body. Protease also determine the lifetime of different proteins which works in hormones, antibodies and thus regulate the switching off and switching on mechanism of genes in living organisms (Kumari *et al*,2012).

Extracellular proteases are most valuable commercially valuable, the most significant are those which are obtained from microorganisms, compared with animal and fungal proteases. And among microbes, soil bacteria are specific producers of extracellular proteases. These extracellular proteases are widely applied in pharmaceutical, leather, laundry, food and waste processing industries (Das *et al*, 2010). Among different types of bacteria *Bacillus* strains are the main source of industrial micro-organisms, which possess almost all the characteristics required for applications (Rao *et al*., 1998).

Enzymes which are commonly occur in microbes are protease, pectinase, amylase and cellulase which are considered as industrially important due to their hydrolytic action to degrade the natural substances. As animal and plant proteases are insufficient to meet the current need of world demand so it diverts the interest in microbial proteases. Today most of the proteases available in the market are extracted from microbes. Microbial proteases dominate the world market because they can be easily cultured in a short period of time and produce the desired products. So, two-third share of industrial proteases are derived from microorganisms (Gupta *et al*. 2002, Vishwantha, 2009).

Among hydrolytic enzymes microbial proteases have been studied extensively which are intracellular and extra cellular both. Intracellular are important for various metabolic functions such as maintenance of protein pool, maturation of enzymes and proteins and sporulation etc. Extra cellular are applied commercially to assist protein degradation in different industrial processes (Gupta *et al*, 2002).

Today several commercial products available in the market are based on bacterial proteases. These proteases are produced by different species of bacteria such as *Pseudomonas, Bacillus, Flavobacterium, Clostridium, Staphylococcus aureus and species belonging to Streptomyces* (Nirmal*et al*. 2011). But most important source which secrete different types of proteases is the bacteria which belons to genus *Bacillus* beacuase it bears great capability to withstand at high temperature as well as extreme pH. (Kumar & Vats.2010, Hema and Shiny *et al*. 2002, Kiran *et al*. 2002).

Microorganism living in soil play key role in different biological functions such as cycling of nitrogen, sulphur, and the decomposition of organic matter. Besides all these biological activities the bacteria living in soil also play important role in the production of different enzymes, of which the most important one is protease enzyme which are used commercially in different industrial products (Pankhurst *et al*. 1997).

Some extra cellular proteases are known to produce by mesophilic bacteria which are obtained from the soil in which most *Bacillus* species were obtained from soil and used in the production of extra cellular proteases, so the mesophilic bacterium belongs to the genus *Bacillus* are most suitable to produce extracellular proteases (Abdullah *et al*. 2006).

Protein plays an important role in the human diet for providing essential amino acids. They also constitute the most important raw materials out of which the complex structures of the body are built. Their nutritional quality depends on their amino acid content and on the physiological utilization of specific amino acids after digestion, absorption, and minimal obligatory rates of oxidation. In nutrition, proteins are broken down in the stomach during digestion by enzymes known as proteases into smaller polypeptides to provide amino acids for the body, including the essential amino acids

that cannot be biosynthesized by the body itself. All living cells maintain a rate of protein turnover by continuous, albeit balanced, degradation and synthesis of proteins. Catabolism of proteins provides a ready pool of amino acids as precursors of the synthesis of proteins. Intracellular proteases are known to participate in executing the proper protein turnover for the cell (Walsh, 2002; Whitford, 2005).

Proteases find huge prospective in various food and feed industrial applications such as in dairy industry (milk protein; casein and whey protein hydrolysis for use in cheese flavor development), baking industry (treatment of flour in the manufacture of baked goods and enhancement of dough texture, flavor, and colour in cookies, *etc.*), brewing industry, soy protein hydrolysis, soy sauce manufacture gelatine hydrolysis, meat protein recovery, fish protein hydrolysis and meat tenderization and improves digestibility of animal feeds (Gupta 2002,Sumantha*et al*,, 2006, Ikram, 2008; Nadeem, 2009). The proteases produced by GRAS (generally regarded as safe) microbes such as *Bacillus subtilis*, *Mucormichei*, and *Endothia parasitica* have been used in cheese production. They are also involved in lactose reduction and flavor modification in dairy applications (Gupta *et al*., 2002a; Sumantha *et al*., 2006; Ikram, 2008; Nadeem 2009; Vishwanatha, 2009; Ray, 2012).

Proteases are used in brewing industry for extracting sufficient proteins from malt, barley and in the production of brewing wort protease are used to solubilize protein from barley adjuncts, thereby releasing peptides and amino acids which can fulfill the requirement of the nitrogen supply. The proteolytic enzymes are used in chill proofing, a treatment designed to prevent the formation of precipitates during cold storage. In beer, hazes are formed due to the presence of proteinaceous substances which also precipitate the polyphenols and oligosaccharides. Hydrolysis of the protein components prevents aggregation of the insoluble complex and hence used as seasoning materials from the foods containing various proteins, the degradation of the turbidity complex resulting from protein in fruit juices and alcoholic liquors, and the improvement of quality of protein-rich Sumantha *et al*, 2006).

Hydrolysis of food proteins is widely employed for value addition through improvement of nutritional characteristics, retarding deterioration, improvement of functional properties and removal of toxic or inhibitory ingredients. Proteases are used to hydrolyse proteins from plants and animals to produce hydrolysates of well-defined peptide profiles of high nutritional value. These protein hydrolysates play an important role in blood pressure regulation and are used in infant food formulations specific therapeutic dietary products and the fortification of fruit juices and soft drinks. (Genckal, 2004; Ikram, 2008).

The enzymatic treatment destroys undesirable pigments, increases the area of the skin and thus clean hide is produced. Bating is traditionally an enzymatic process involving pancreatic proteases. However, recently, the use of microbial alkaline proteases has become popular. Proteases such as alkaline speed up the process of dehairing, because the alkaline setting facilitate the puffiness of hair roots; and the ensuing attack of protease on the hair follicle protein allows easy elimination of the hair (Gupta, *et al.*, 2002, Genckal, 2004; Ikram, 2008; Nadeem, 2009; Ray, 2012).

Enzymes plays vital role in the detergent industry for their ability to remove stains and dirt from different surfaces and to bring unique remuneration that cannot otherwise be acheived through conventional detergent technologies. Applications of detergent proteases have grown significantly, and the largest relevance is in domestic laundry detergent formulations. The detergent which are made from these enzymes are inexpensive and not harmful to the fabric quality. Morever protease enzymes improve the status of laundary detergents which lead to advancement in their commercial and domestic applications (Gupta, 2002, Genckal, 2004, Ikram, 2008, Nadeem, 2009, Ray, 2012).

Medical products are developed by using the alkaline proteases in them which increase their significancy. These mainly include activity of elastocity which are imply to prepare the for the treatment of many accidental injuries such as burns, purulent wounds, carbuncles, furuncles and deep abscesses, as thrombolytic agent having fibrinolytic activity (Gupta, *et al,*2002, Genckal, 2004; Ikram, 2008, Nadeem, 2009, Ray, 2012).

In the bioprocessing of used X-ray or photographic films for silver recovery proteases such as alkaline play important role to prevent unpleasant pollutions because these silver recovery can be done by burning of films which affect health of other living organism by causing undesirable change and pollution. So it is more suitable to extract such type of silver by using proteolytic enzymes which is eco-friendly method. Proteolytic hydrolysis of gelatin not only helps in extracting silver, but also the polyester film base can be recycled (Gupta *et al;* 2002a; Genckal, 2004; Nadeem, 2009; Ray, 2012).

Protease enzymes have been widely used for the creation of dipeptides and tripeptide, regioselective sugar esterification and dia-stereoselective hydrolysis of peptide esters. Several advantages of

Enzymatic peptide can be seen over chemical methods e.g., to increase the solubility of nonpolar substrates reactions can be performed stereospecifically and reactants do not require side-chain protection, or shifting thermodynamic equilibria to favor synthesis over hydrolysis. Enzymatically synthesized small peptides (usually di or tripetides) are being used successfully for human and animal nutrition and as pharmaceuticals and agrochemicals (Gupta, *et al*, 2002a, Genckal, 2004).

Some of the important non-caloric sweetener aspartame, the lysine sweet peptide, kyotorphin, angiotensin, enkephalin and dynorphin and some nutritional dipeptides and tripeptides are examples of pharmaceutical products in which proteases are used extensively. Protease are also used in the production of biodegradable films, coatings and glues from keratinous waste products like hair, feathers, skin, fur, animal hooves, horns etc. for compostable packaging, agricultural films or edible film applications. For stable dispersion and useful application, the Keratin structure is chemically modified and hydrolyzed to enhance the quality of such enzymes (Gupta, *et al*, 2002a, Genckal, 2004).

Silk industry is one of the least explored areas for the application of protease enzyme and only a few application of these enzymes have been filed describing the use of proteases for the degumming of silk. Production of Sericin, which is about 25% of the total weight of raw silk, is consider as expensive item because of its costly extraction from the fibroin material and then twist setting process by using of starch fibers. This process is generally facilitated in now a day by using proteolytic enzymatic activity which can easily proceed the whole process without any hurdle specially the process of degumming the silk prior to dyeing (Gupta, *et al*,2002a, Genckal, 2004, Nadeem, 2009, Ray, 2012).

The global environment is gradually worsening day by day due to the socio-economic activities of humankind such as dispensation of industries. Most of the industrial activities and human practices cause unpleasant changes in the instantaneous ecological change and therefore being challenged by society. Mainly leather producing industries and the amplified number of feathers generated by saleable poultry processing may represent a wide variety of different pollution problem and needs tolerable administration. In this view, alkaline protease applications in the administration of wastes from different industries and household actions unbolted a innovative era in the employ of proteases in squander managing prcesses. Proteinaceous waste are solubilized by using proteolytic enzymes which help to lower the biological oxygen demand of aquatic systems. For the management of waste feathers from poultry slaughterhouses the alkaline protease from *B. subtilus* was used to overcome the industrial hazards (Gupta, *et al*, 2002, Genckal, 2004, Ikram, 2008, Nadeem, 2009, Ray, 2012).

Keeping in view the importance of proteases, current study is designed to isolate and evaluate protease producing bacteria from soil samples of District Mardan.

2. MATERIALS AND METHODS

Sample collection:

For the isolation of protease producing bacteria the soil samples were collected from different areas such as garden, field, pond side, road side and garbage areas of Mardan District. The texture of the soil was noted, and all the samples were collected from 7cm below the surface and then stored in sterile plastic bags at 4^{o}C.

Isolation of protease producing bacteria:

The techniques used for isolation of bacterial strain were serial dilution and spread plate method on skimmed milk agar (1%). The zone of hydrolysis will be noted for each sample (indicate protease production). 1 gm of soil from each sample was weighed and serial dilutions were prepared in 10 ml of distilled water. Optimum dilutions from 10^{-3} to 10^{-6} were spread on the screening medium and incubated at 37°C for 24 and 48 hours. The bacterial isolates having clear zones were taken as protease producers.

Composition and preparation of the medium:

Skimmed-milk = 1 gm/ 100 ml distilled water.
Agar = 1.5gm/ 100 ml distilled water.

Streak plate Method:

The colonies showing highest zones of hydrolysis were selected for further screening; these 24 and 48 hrs old cultures were streaked on the same skimmed milk agar media and incubated for 24 hours at 37°C in order to obtain the pure cultures.

Characterizations of Isolates of bacteria:

The identification of bacteria will be carried out by morphological studies i.e. staining including Gram staining, motility test, Acid Fast test, Endospore staining, Cultural characterization on skimmed milk agar plates like colony morphology that is shape, size, margin elevation etc.

Macroscopic Characterization of Bacterial Isolates:
The characterization of colonies such as margin, design, distance from the ground, tincture and shape were investigated directly through direct observation on 24 hrs old skimmed milk agar plate.

Microscopic Characterization of Bacterial Isolates:
The gram staining and endospore staining, and acid-fast staining techniques were applied to study the bacterial isolates under the microscope (Harley and Prescott, 2002).

Biochemical Characterization of Isolates:
The sample of bacterial culture were again re-streaked on to a skimmed milk agar plates and incubated for 24 hrs at 37°C. From the resulting culture the colonies showing clear zones were selected for Catalase (Harley and Prescott, 2002) and motility tests (Murray *et al*., 2007) of bacterial isolates.

Qualitative Screening of Selected Isolates for the confirmation of Proteolytic activity:
For the screening and evaluation of bacterial isolates having proteolytic activity. These bacterial isolates were streaked on Luria casein agar(1%) plates and incubated at 37^{o}C for 24 hours. After 24 hours the zone of clearance appeared around the bacterial colonies were measured in millimeters and the protease producers were shifted to nutrient agar slants and preserved at 4^{o}C.

Optimization of selected isolates For their Proteolytic activity

Effect of Time on the Production of Protease
To determine the time for maximum production of protease, the 24 h growth isolate were inoculated in to the medium as before and incubated at 37°C for 24-72. The proteolytic activity of selected isolates was checked after the incubation of 24, 48 and 72 hours based on appearance of clear zones around the cultures and the zones diameter were measured in millimeter.

Effect of Temperature on the Production of Protease
The optimum temperature for protease production was determined by incubating the culture at different temperatures (i.e. 25, 37 and 45°C), at pH 7 for 24 hours. At the end of incubation period the degradation of skimmed milk agar media was observed by observing zone formation around the bacterial colonies which was the indication of protease production at different optimized temperature conditions.

Effect of pH on the Production of Protease
The effect of pH on the growth of bacterial isolates was investigated by adjusting the pH of the growth medium to pH 5.0, 7.0 and 9.0. For this the medium was prepared in 3 separate 250ml Erlenmeyer flasks each contain 100ml volume of medium (skimmed milk agar) and the pH was adjusted in range 6.0, 7.0 and 9.0 with 1N NaOH and 0.1 HCl. Then all the flasks were autoclaved at 121°C for 30 minutes. After autoclaving the media was cooled and pour into 18 petri plates each 6 labelled for the respective pH (6.0, 7.0 and 9.0) after solidifying the medium was streaked with selected protease producers and incubated at 37°C for 24 hours. The zone of hydrolysis was noted from each plate in order to determine the highest proteolytic activity shown by protease producers after 24 hours of incubation.

Effect of NaCl concentration on the production of protease
NaCl was added at concentration 0.5% into the protease production medium. For this the medium was prepared in 250ml Erlenmeyer flask which contain 100ml media (2.5 gm skimmed milk agar and 0.5 gm NaCl) and autoclaved at 121°C for 30 minutes. After cooling the media was poured into Petri plates and 18 hrs old culture were streaked on these plates then it was incubated at 37°C for 24 hours after incubation period the growth of isolates were observed, and the zone of inhibition was measured with the help of measuring scale in millimeters which was the indication of proteolytic activity by their producers.Effect of concentration of NaCl was studded by considering results of the protease activity, higher activity of protease was due to higher production of protease.

Effect of metallic ions on the production of Protease
The effect of metallic ions was studied by growing the bacterial culture in the presence of chemicals containing divalent cations such as MgCl2 and CaCl2 at concentration of 0.2%. The culture was incubated for 24 hours and the bacterial strain from each plate were subsequently analyzed for protease activity and compared with one another on the basis of appearance of clear zones around the producer's colonies. Effect of each metallic ion on the growth of bacterial isolates was studied depending on the production of protease after analyzing the protease activity by measuring the zones of inhibition produced by each isolate.

Effect of Different Substrates on the production Of Protease:

To analyze the effect different substrates on protease production (i.e casein, gelatin and soybean flour 1%) were used in skimmed milk agar medium as a suitable substrate for the growth of protease producing isolates. For this purpose, 3 separate 250ml flasks were taken each contained 100ml media (2.5% skimmed milk agar and 1% substrate) each flask was labeled according to substrate name (casein, gelatin and soybean flour). After oven sterilization the medium was poured into sterilized petri plates and the selected isolates were streaked in a straight line on these plates incubated at 37°C for 24 hours. The enzyme activity produced by each isolate was visualized as formation of clear zones around the bacterial colonies due to the hydrolysis of substrates which was the indication of protease production by the producers on different substrates. The diameter of each proteolytic zone was measured in millimeters.

Statistical Analysis:

Statistical analysis of the presented study were performed through spss software and microsoft excel and all the numerical values were generated through one way ANOVA Test

RESULTS

Screening for Protease Producing Isolates

In present study a total 93 bacterial isolates showed hydrolytic activity from all the five soil samples. Out of these 27 isolates were considered as different protease producer strains on the basis of appearance of colonies. Among these 29, 6 bacterial isolates were isolated from field soil, 6 from garbage soil, 6 from pond side area soil, 5 isolates were from garden soil and 5 from road side area, all these isolates were able to produce protease in varying degree of concentration (Table 1.2). Out of these 29 bacterial isolates 6 bacterial isolates displayed highest zone of inhibition were selected for further study in order to evaluate their efficient proteolytic activity.

Table 1.2: Protease producing bacterial isolates obtained from five soil samples

Sample Source	Isolate	Diameter of Zone of Clearance (mm)
Field Soil	P1	26mm
	P2	23mm
	P3	22mm
	P4	9mm
	P5	8mm
	P6	28mm
Garbage area soil	P7	26mm
	P8	12mm
	P9	15mm
	P10	7mm
	P11	21mm
	P12	6mm
Pond side soil	P13	15mm
	P14	6mm
	P15	2mm
	P16	1.5mm
	P17	6mm
	P18	8mm
Garden soil	P19	6mm
	P20	7 mm
	P21	16 mm
	P22	12 mm
	P23	9 mm

Road side area soil	P24	2 mm
	P25	1 mm
	P26	3 mm
	P27	4mm

Macroscopic Characterization of the Bacterial Isolates:

The macroscopic analysis of selected bacterial isolates indicated that all the selected isolates possessed circular shape, smooth texture and entire margins of their colonies. Most of the isolates e.g P1, P2, P6, P11 indicated raised elevetaion of their colonies whereas in the case of P3 and P7 the colonies were flat. Size morphology of the selected isolates revealed that 3 isolates P1, P2 and P6 had average colony size of 0.75 mm while isolate P3 had 0.85 mm coclony size, P7 had colony size of 0.95 mm and P11 had average colony size of 1mm. Most of the bacterial isolates (P2, P3, P6, P11) appeared transparent on their growth medium while isolate P1 appearance was creamy white and strain P7 had creamy yellow appearance on skim milk agar (Table 1.3).

Table: 1.3; The Macroscopic characterization of selected isolates

Colony Morphology	**Selected Isolates**					
	P1	**P2**	**P3**	**P6**	**P7**	**P11**
Relative Size	0.75mm	0.75mm	0.85mm	0.75mm	0.95mm	1mm
Shape	Circular	Circular	Circular	Circular	Circular	Circular
Texture	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth
Margin	Entire	Entire	Entire	Entire	Entire	Entire
Elevation	Raised	Raised	Flat	Raised	Flat	Raised
Color	Creamy White	Transparent	Transparent	Transparent	Creamy Yellow	Transparent

Microscopic Characterization of the Bacterial Isolates:

The selected isolates were further subjected to microscopic characterization. Gram staining of bacterial isolates revealed that isolate P1, P2, P3 and P7 were identified as gram negative *Bacillus spp.* while P6 was recognized as gram positive *Bacillus* strain, whereas P11 isolate was identified as gram negative *cocci* (Figure 1). Most of the bacterial isolates were identified as positive endospore forming species however strain P11 was identified as non-sporulated bacteria. Similarly the acid fast staining of selected isolates was positive for 5 bacterial strains (P1, P2, P3, P4, P7) while it was negative for P11 isolate. Motility test indicated that 5 isolates (P1, P2, P3, P4, P7) were motile except P11 which was non motile (Table 1.4).

Table 1.4: Microscopic analysis of selected isolates

Staining Method	**Selected Isolates**					
	P1	**P2**	**P3**	**P6**	**P7**	**P11**
Gram Staining	G-ve, Rod	G-ve, Rod	G-ve, Rod	G+ve, Rod	G-ve, Rod	G-ve, *Cocci*
Endospore Staining	+ve	+ve	+ve	+ve	+ve	-ve
Acid Fast Staining	+ve	+ve	+ve	+ve	+ve	-ve
Motility Test	Motile	Motile	Motile	Motile	Motile	Non-motile

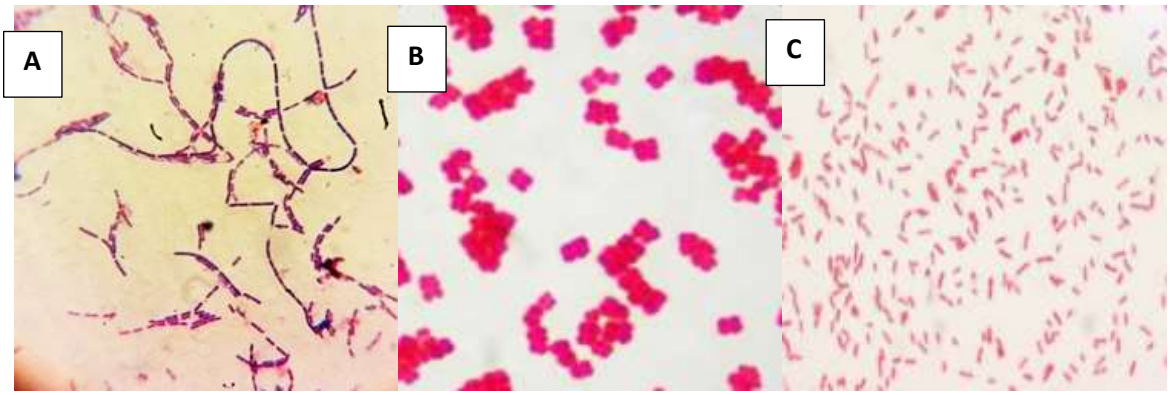

Figure 1: The microscopic analysis shows that selected isolates belongs to (**A**) Gram+ve rods of *Bacillus*, (**B**) gram -ve Cocci and (**C**) Gram –ve *Bacillus*

Biochemical Analysis of Bacterial Isolates:
Biochemical characterization of selected bacterial isolates have been done through catalase test and starch hydrolysis test and the results have been shown in table 1.5. Catalase test indicated that 4 isolates P1, P2, P3, P6 were catalase positive whereas 2 isolates P7 and P11 were catalase negative. All the selected bacterial strains were positive for starch hydrolysis test.

Table 1.5: The Biochemical analysis of selected isolates

Biochemical Test	**P1**	**P2**	**P3**	**P6**	**P7**	**P11**
Catalase test	+ve	+ve	+ve	+ve	-ve	-ve
Starch hydrolysis test	+ve	+ve	+ve	+ve	+ve	+ve

Protease producing isolates Evaluation For their Proteolytic Activity:
The qualitative analysis of the six selected isolates revealed that they can produce protease at varying levels. These selected isolates were tested on a specific medium (Luria Casein agar) for their proteolytic activity and on that specific media each of them exhibit different size of zone of inhibition which clearly indicated that all of them are best protease producing strains. The proteolytic bacterial isolates were detected through platting the samples on media containing. The sample source and the diameter of zone of inhibition was measured for each selected proteolytic isolate (Figure 2).

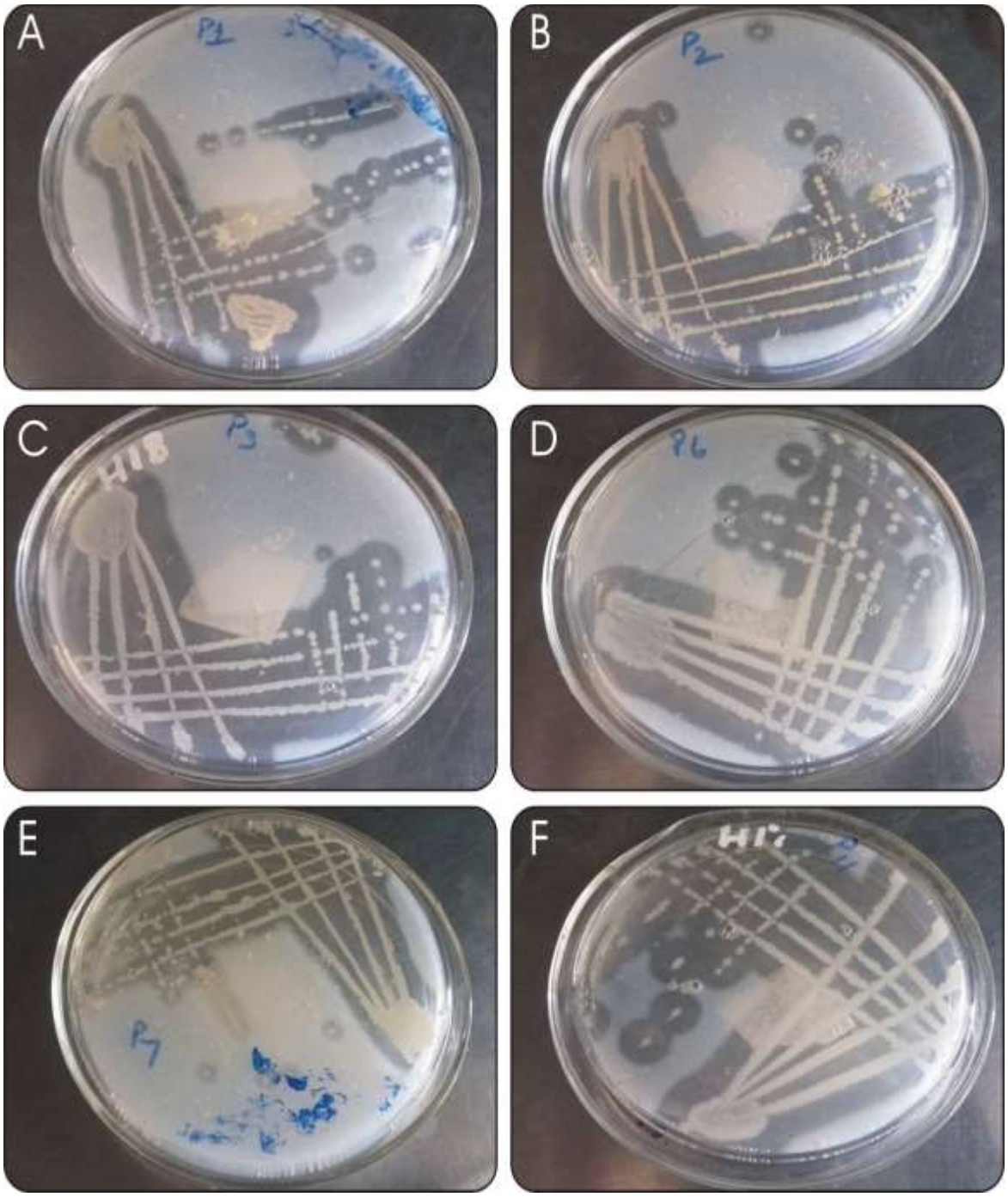

Figure 2: Petri Plates showing Zone of inhibition for selected isolates (A) P1 (B) P2 (C) P3 (D) P6 (E) P7 and (F) P11.

Optimization of selected isolates on different parameters for proteolytic activity:

Effect of incubation time on protease production

In the present study the optimum protease production time for all the selected isolates was found to be 24 hourscorresponding to protease activity of 12mm, 11mm and 10mm for isolates P6, P3 and P7 (See **Table 1.6**). While, a gradual decrease in enzyme units was observed with increase in incubation period (from 48-72 hours) which clearly suggests that the enzyme production may be growth related innature. The maximum enzyme production wasobserved during continuous growth of the culture at the late exponential phase and earlystationary phase of the growth. One-way ANOVA test indicates that it is statistically <0.002 significant.

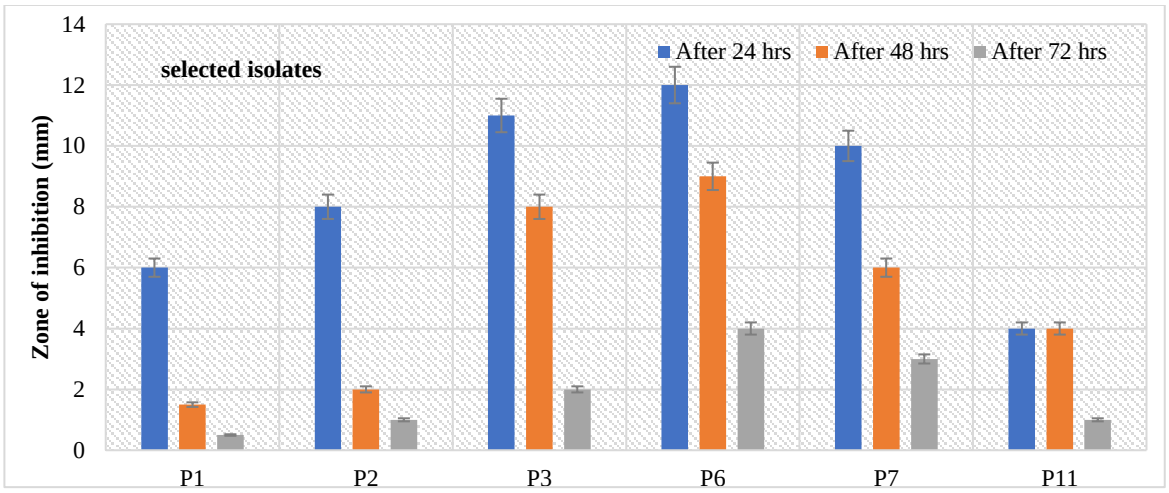

Figure 3: Effects of incubation time on protease production

Effect of temperature on selected isolates

As part of the initial selection criteria, the protease activity for each of the isolates was determined and measured by growing the cultures at different temperatures and the zone of inhibition was also measured in mm unit. The statistical results obtained from one-way ANOVA test showed that the following model is <0.24 non-significant.

At room temperature (37ºC) the protease production activity was observed to be maximum for isolate P6, P3 and P11 (20mm, 14mm and 12mm) as compared to 25ºC and 45ºC temperatures. But it was also noted that the P6 isolate shows a higher protease activity at 25ºC and 45ºC temperatures. A similar increase in protease activity was also observed in P11 isolate at 45ºC temperature (**Figure 4**).

The optimum temperature for P1, P2, P6 and P7 was found to be 37-45°Ccorresponding to the zone of inhibition of14-30mm, 12-16mm, 12-20mm and 10-20mm respectively. In all the selected isolates, there was progressive decline of enzyme production observed after their respective optimum temperatures and less enzyme production was observed up to 45°C.

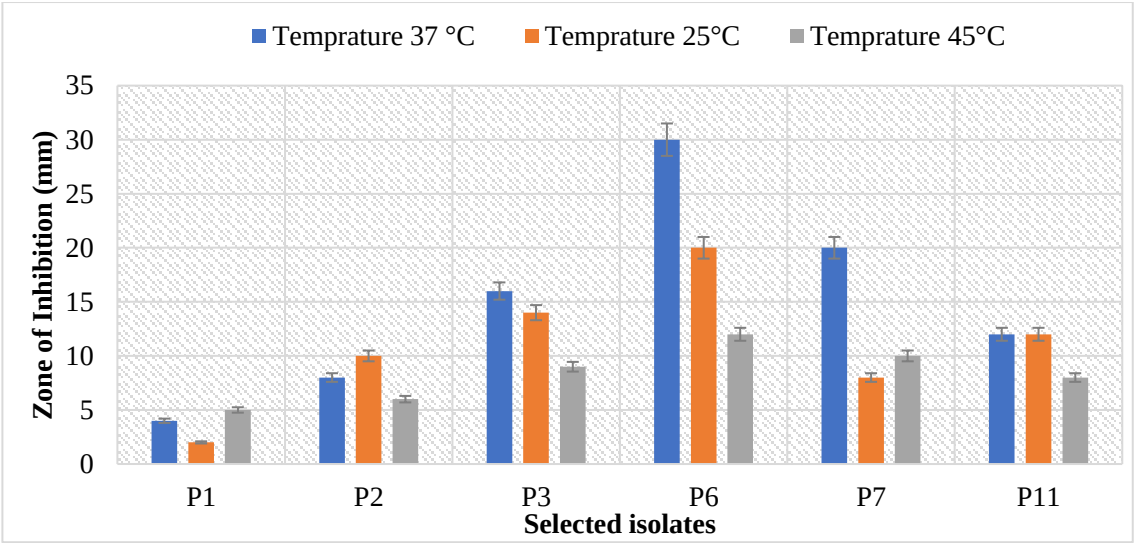

Figure 4: Effect of different tempratures on selected isolates

Effect of pH on selected isolates

The optimal observed pH value for protease production of the selected isolates was 9. At pH 9, theprotease activities for P1, P3, P6 and P7 were 6mm, 12mm, 14mm, 10mm respectively. However, on pH 5-7 the there was an increase in protease activity also observed for isolate P3 (8mm, 6mm), P6 (10mm, 12mm). Hence from these findings it can be concluded that the optimal pH for protease activity ranges from 7-9 (See **Table1.8**and **Figure5**). For statistical analysis one-way ANOVA was applied with statistically significant value of <0.20.

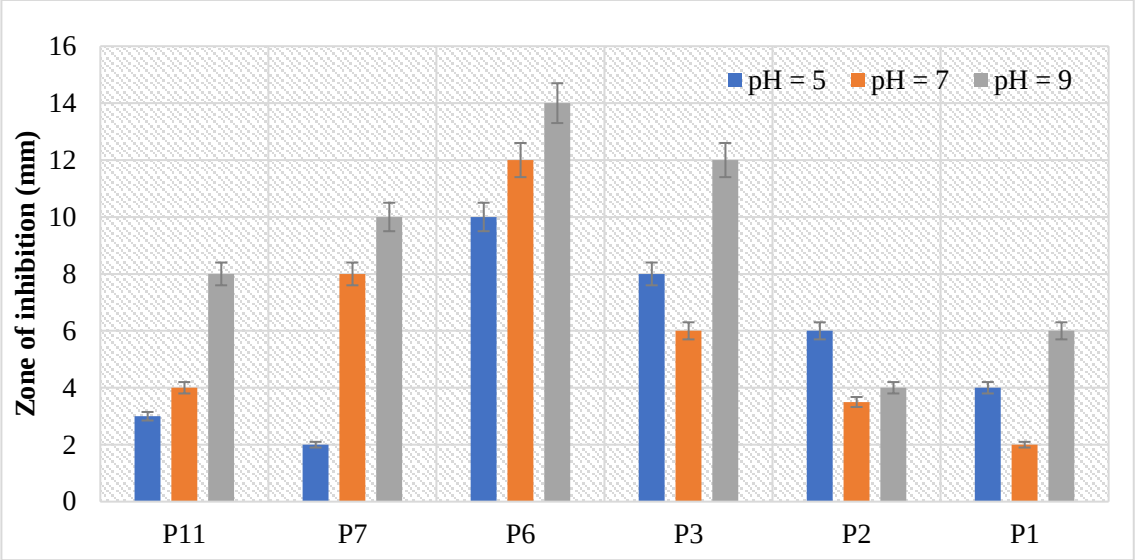

Figure 5: Showing effects of different pH on the selected isolates

Evaluation of different substrates for protease production

Gelatin, casein and soya-bean flour hydrolysis as substrate is helpful for the differentiation zone of hydrolysis produced by different bacterial species. These isolates were screened for protease activity by using the skim milk agar plates which contains gelatin, soya-bean and casein as substrate (1%) and incubated at 37°C for24 h. The proteolytic activity was detected and measured by the presence of clear zone of hydrolysis on gelatin agar (**Figure 6**).

While, Casein is frequently used as substrate for proteolytic activities due to its easy availability and stability under storage situations. Because of its complex arrangement and casual structure, this substrate endures proteolysis with all existing proteolytic enzymes without any requirement for prior denaturation. The amount of hydrolysis is usually determined, after acid precipitation of the unchanged casein by measurement of the clearance zone.

The soya bean extracts and soya beans flour are considered as good inducers for production of protease. The best clearance activity was observed for P6, P3 and P7 isolates that was 19mm, 16mm and 15mm inhibitory zones respectively on soya-bean flour substrate. For statistical analysis one-way ANOVA test was done which showed that the following model is <0.05 significant.

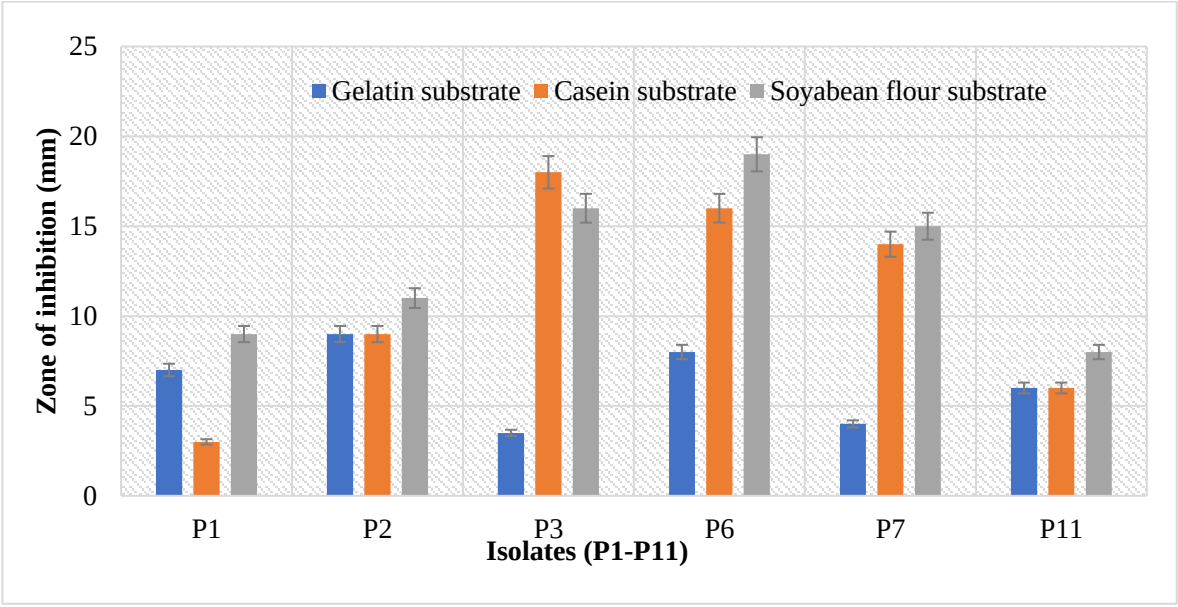

Figure 6: Protease production at different substrates

The proteolytic activity for Casein was measured to be higher than that of Gelatin for isolate P3, P6 and P7, 18mm, 16mm, 14 mm correspondingly. While, the isolate P1 and P11 shows less protease

activity on Casein substrate of about 3mm and 6mm. The maximum protease production activity was observed for P2 isolate (9mm) followed by P6 (mm) on Gelatin substrate. The lower zone of clearance activity or proteolytic activity of hydrolysis for Gelatin substrate was observed for P3 and P7 isolates of 3.5mm and 4mm respectively (**Figure 6**).

Effect of NaCl(0.5%) on protease production

The NaClwas used at 0.5% concentration for determination of optimumlevelrequired to produce protease by the selected isolates. It was found that the maximum activity was observed in isolates P3, P6 and P7 (12mm, 14mm and 10mm respectively). While, the isolates P11, P2 and P1 were resulted in 6mm, 8mm and 9mm of zone of inhibition respectively. The highest activity was observed in P6 isolate, though it was also observed that in all isolates 0.5% of NaCl concentration resulted in the least production of protease (**Figure 7**).

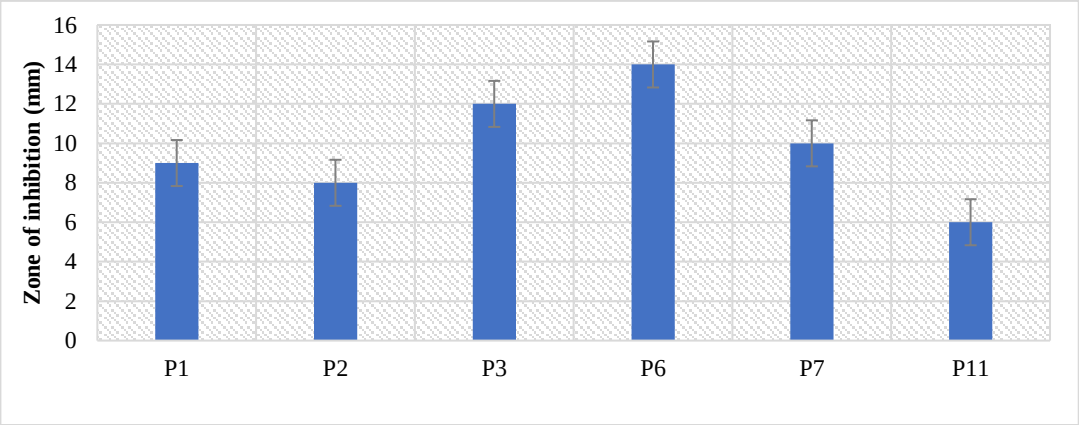

Figure 7: Effect on 0.5% NaCl on protease production

Effects of Metallic ions on protease activity

The effect of metal ions ($MgCl_2$ and $CaCl_2$) on the protease activities of selected six isolates is shown in**Table1.10** below. The outcomes suggested that the proteases of P7, P6,P1 and P2 isolatesare activated by $MgCl_2$at 0.2% concentration and inhibited by thesemetal ions at 16mm, 14mm, 12mm and 10mm respectively. The less clearance zone was observed for P11 and P3 isolates that were recorded to be 6mm and 8mm. The $CaCl_2$shows less activity in comparison to $MgCl_2$, it inhibited the protease activity on P6 isolate (10mm) at 0.2% concentration. At 0.2% concentration the $CaCl_2$showed the highest inhibitory effect followed by a gradual decrease in its activity for other isolates (**Figure 8**). For statistical analysis one-way ANOVA was applied with statistically significant value of <0.02.

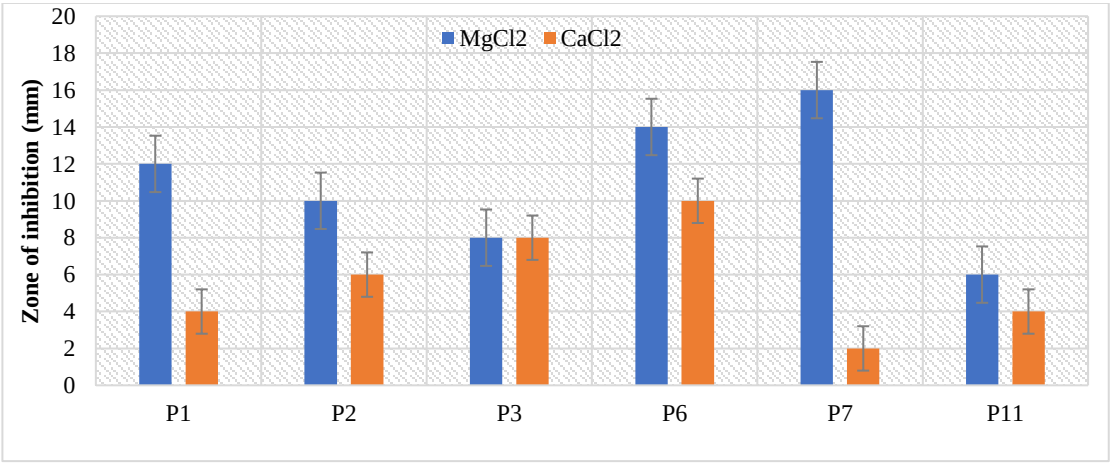

Figure 8: Effects of different Metallic ions on protease production

DISCUSSION

Protease enzyme play significant role in commercial and industrial applications and therefore consider as champion of all enzymes due to its widely distributed quality in nature. Though it is obtained from different sources but mostly microorganisms are reported as a preferred source of proteases, because of their rapid growth and best results of protease production. The production of protease enzyme is an inherent capacity of all

microbes, but mostly bacterial species are known for the assemblage of protease (Gupta *et al* .2002b).

The present study was designed to determine the protease producing capability in bacterial species which were isolated from different soil samples of District Mardan KPK. A total of 29 bacterial strains were isolated due to their zone of hydrolysis around their colonies on skim milk agar medium. The use of skim milk agar as a medium for the isolation of protease producers has earlier been reported by some workers (Shehzad and Muhammad, 2014; Rupali and Tiwari, 2015).

These selected proteases producing isolates were identified on the basis of their morphological appearance and biochemical nature through different techniques and it was found that out of these 6 isolates 5 belongs to genus *Bacillus*, of which 4 members of this genus were exhibited gram negative characteristics while one member is gram positive, the remaining 1 specie belong to gram negative cocci group. Members of genus *Bacillus* were also identified as endospore forming bacteria while member of genus cocci was recognized as non-endospore forming bacteria. Many workers reported the isolation of *Bacillus* and *cocci* species from natural soil. For instance, Vakilwala and Devyani Patel, (2017) identified 14 different gram-positive and gram-negative species of genus *Bacillus* from the soil of different regions of Vapi, of which strain of gram negative *Bacillus* was selected for further analysis for maximum protease production. Similarly, in another study it was reported that mostly proteolytic bacteria are found in topsoil as compared to subsoil sample (Bach and Munch, 2000). Due to the analysis and findings of all these workers clearly indicate that protease producers are widely distributed in soil and other natural habitats because they can survive in every type of environment under different physiological conditions.

In present study the proteolytic activities of selected isolates were studied by testing them on different parameters under the optimized conditions. Before their optimization they were qualitatively screened for their proteolytic activity on a special media called Luria casein agar for the confirmation of their protease producing ability. It was found that among these 6 isolates Gram positive member of genus *Bacillus* which was assigned the name P6 yielded highest zone of inhibition followed by gram negative spices of genus *Bacillus* P3, P7, P1 and P2 while the member of genus *cocci* displayed least proteolytic activity on Luria casein agar medium. The use of special medium Luria casein for the confirmation of proteolytic activities was also reported by Shaheen *et al*. (2008) they found the proteolytic activity of *Bacillus subtilus* bacteria on Luria agar plates.

The optimum incubation time for the growth of 6 selected proteolytic bacteria was noted after 24 hours of incubation, the protease activity was gradually declined after 48 and 72 hours of incubation. This finding is comparable with the finding of Sughanti and Mageswari *et al.* (2013) who reported the highest protease producing capability in *Bacillus licheniformis* (TD4) at an incubation time of 24 hrs, which indicated the shorter period of incubation for protease production.

The optimum temperature for the growth of selected proteolytic isolates was found to be 37°C. Out of 6 selected isolates the highest proteolytic activity was shown by *Bacillus* strains P6, P7, P2, P1 and P3 at room temp 37°C while the *cocci* strain P11 has shown maximum proteolytic activity at 45°C. However, it was also noted that the gram-positive strain of *Bacillus* was found to be best protease producer even at temp 25°C and 45°C. This indicates that this strain is more stable and active producers as compare to the other strains of protease producers. The same findings in term of temperature compatibility was also given by some workers, who reported that maximum protease productivity was achieved at temperature ranging 37 to 45°C for certain *Bacillus spp* (Qadar *et al*. 2009; Kumara *et al.* 2012; Josephine *et al*. 2012). These results suggest that the selected isolates P6, P7, P3, P2 and P1 belongs to mesophilic protease producer group while the selected isolate P11 belongs to thermostable group of protease producers.

The proteolytic activities of all 6 selected isolates were measured at pH values 5, 7 and 9. Maximum activity of the selected isolates was found to be at pH 9 which indicate that the selected isolates belong to alkaline group of protease producers. This result is in agreement with other survey of literature which shows that the optimum pH for the activity of proteolytic bacteria from *Bacillus* spp. ranges 9 – 11 (Durham *et al*. 1987; Bundela and Mandal, 2013). Mostly bacterial species were investigated active protease producers at pH 9 because the enzyme protease possessed good stability over a pH range 8-10 (Gooma*et al.* 2014). The substrates used in commercial protease enzyme agitations are normally common agricultural commodities like soybean, casein, starch and ordinary sugar (Cheetham, 1995). The proteases formation is largely dependent on the condition of growth of the microbial culture and composition of nutrient medium (Fujiwara et al., 1993). In the present study, casein, gelatin and soybean flour with skim milk agar were used as

substrates for the proteolytic activity of selected isolates and maximum protease activity after 24-hour incubation of selected isolates were observed in case of soyabean and casein while in substrate gelatin the proteolytic activity was found to be least. However, *Bacillus* P6 and P3 was found to be best proteolytic producers in both casein and soyabean substrates followed by P7 isolate while P2 showed highest proteolytic activity in gelatin substrate as compared to other protease producing isolates, P1 isolate shown least proteolysis in casein substrate while P11 was observed to be not active producer in all the 3 different substrates. Overall activity was high in soyabean and casein substrate by the protease producers. These results are comparable with Shaheen *et al.,* (2008), who reported the highest proteolytic activity of *Bacillus subtilis* BS1 in soyabean and casein substrate at pH 11.

All the isolates showed proteolytic activity at 0.5% NaCl concentration. The present analysis are comparable with the result of Mostafa *et al.* (2012) who reported the proteolytic activity of *Str. pseudogrisiolus* NRC-15 in the presence of NaCl (1%) at pH 9.

The effect of metallic ions on selected bacterial strain was observed after 24 hours of incubation period. Metallic ions include CaCl2 and MgCl2 were used at concentration of 0.2% in the selected growth media The result showed that the presence of $MgCl_2$ in the growth medium enhance the proteolytic activity of selected isolates as compare to while P3 displayed equal proteolytic activity on both metallic ions. This report is in line with the work of Huang *et al.* (2003) who reported that the presence of metallic ions such as $MgCl_2$ and $CaCl_2$ enhanced the production of alkaline serine protease in *Bacillus pumilus*. Similarly, most of the extracellular protease retained their thermal stability in the presence of $CaCl_2$ which revealed that the producers can remain stable even at high temperature up-to 60 ^oC (Khan *et al.* 2011).

Over all current study revealed that protease producers are mainly found in rich fertile soil (field sample). Results indicate that best protease producer was *Bacillus* strain P6 which was more stable and active at different growth parameters while *cocci* strain P11 displayed least proteolytic activity on each optimized parameter. The other *Bacillus spp.* responds differently in different optimized conditions. All the selected strains can be used for protease production but there is a need to identify chemical nature of these protease producers and further optimize before scaling up at industrial level.

References

1. Abdullah F.M 2006, 'The production of extracellular protease using *Bacillus subtilis* effect of temperature and agitation speed', BSc Thesis, University College of Engineering and Technology, Malaysia, pp. 46
2. Bach, H. J., & Munch, J. C 2000, 'Identification of bacterial sources of soil peptidases', *Biology of Fertile Soils,* vol. 34, pp. 219-224.
3. Bundela, V. & Mandal, S. K 2013, 'Purification and characterization of an extracellular alkaline protease produced from an isolated *Bacillus subtilis*', International journal of applied Biology and Pharmaceutical technology, vol. 4, pp.112-119.
4. Cheetham, PSJ 1995, 'Principles of industrial biocatalysis and bioprocessing', *International Handbook of Enzyme biotechnology*, (Ed.), A, Wiseman, Ellis Harwood, U.K, pp. 88.
5. Das, G. and Prasad, MP 2010, 'Isolation, purification & mass production of protease enzyme from Bacillus subtilis', *International research journal of Microbiology*, vol. 1(2), pp. 26-31.
6. Durham DR 1987, 'Utility of GX as a detergent additive', *Journal of Applied Bacteriology,* vol. 63, pp. 381-386.
7. Fujiwara, N, Masui, A. & Imanaka, T 1993, 'Purification and properties of the highly thermostable alkaline protease from an alkaliphilic and thermophilic *Bacillus* sp.', *Journal of Biotechnology,* vol. 30, pp. 245–56.
8. Gupta, R, Beg, Q. K. & Lorenz, P 2002, 'Bacterial alkaline proteases molecular approaches and industrial applications', *Applied Microbiology & Biotechnol*ogy, vol. 59, pp. 15-32.
9. Gupta, R., Q.K. Beg, S. Khan & B. Chauhan 2002, 'An overview on fermentation, downstream processing and properties of microbial alkaline proteases', *Applied Microbiology & Biotechnology,* vol. 60(4), pp. 381-95.
10. Harley, J.P, & Prescott, L,M 2002, 'Laboratory Exercise in Microbiology 5th edition, The McGraw-Hill Companies', pp. 466.
11. Hema, T.A & Shiny, M 2012, 'Production of Protease Enzyme from *Bacillus Clausii*Sm3', *IOSR Journal of Pharmacy and Biological Sciences*, vol. 1, pp. 37-40.
12. Ikram, N 2008, 'Enhanced production of thermostable bacterial proteases and their

applications', PhD. Thesis. University of Punjab. Pakistan, pp. 158.

13. Josephine S, Ramya V, Devi N, Ganapa B, Siddalingeshwara K, G, Venugopal, N & Vishwanatha T 2012, 'Isolation, production and characterization of protease from *Bacillus Sp* isolated from soil sample', *Journal of Microbiologyl & Biotechology Research',* vol. 2(1), pp.163-168.
14. Lakshmi, B,K,M, Sri, P,R., Devi, K,A & Hemalatha, K.J 2014, 'Screening, optimization of production and partial characterization of alkaline protease from haloalkaliphilic Bacillus sp.', *International Journal of Research in Engineering & Technology,* vol.3 (2), pp.435-443.
15. Khan, M.A, Ahmad, N, Zafar, A,U, Nasir, I,A & Qadir, M.A 2011, 'Isolation and screening of alkaline protease producing bacteria and physio-chemical characterization of the enzyme', *African Journal of Biotechnology*, vol. *10*(33), pp.6203-6212.
16. Kim, E, Hong J, Yun N. & Lee, Y 2011. 'Characterization of *Bacillus Phage*-K2 Isolated from Chungkookjang, A Fermented Soybean Foodstuff', *Journal of Industrial Microbiology and Biotechnology*, pp. 39-42.
17. Kiran, K,S,P, Satyavani Y, Chandana Lakshmi M,V,V. & Sridevi V 2002, 'Production of protease Enzyme using various sources', Review: *Research Journal of Biotechnology*, vol.7(4), pp. 250-258.
18. Kumar, R. & R, Vats, 2010, 'Protease Production by *Bacillus subtilis* Immobilized on Different Matrices', *New York Science Journal*, vol. 3(7), pp. 20-24.
19. Kumara, M,S, Kashyap, N, S, Vijay. R, Rahul, T, Anuradha, M 2012, 'Production and optimization of extra cellular Protease from *bacillus* sp. Isolated from soil', *International Journal of Advanced Biotechnology and Research*, vol.3, pp.564-569.
20. Kumari, K,S,P, Satyavani Y, Lakshmi,C, M,V & Sridev,i V 2012, 'Production of protease Enzyme using various sources', *Research Journal of Biotechnology*, vol. 7 (4), pp. 251.
21. Lakshmi, B,K,M, Sri, P,R., Devi, K,A & Hemalatha, K.J 2014, 'Screening, optimization of production and partial characterization of alkaline protease from haloalkaliphilic Bacillus sp.', *International Journal of Research in Engineering & Technology*, vol.3 (2), pp.435-443.
22. Mostafa, E,S,E, Saad, M,M, Awad, H.M., Selim, M.H. & Hassan, H,M 2012, 'Optimization conditions of extracellular proteases production from a newly isolated Streptomyces pseudogrisiolus NRC-15', *Journal of Chemistry*, vol. *9*(2), pp.949-961.
23. Murray, Baron, Jorgenson, Landry & Pfaller 2007, 'Manual of clinical microbiology. 9th, edition', American society for microbiology, Washington, Dc, pp.1-10.
24. Nadeem, M, Qazi, J, I. & Baig, S, 2009, 'Effect of aeration and agitation rates on alkaline protease production by Bacillus licheniformis UV-9 mutant', Turk Journal of Biochemistry, vol. 34, pp. 89-96.
25. Nirmal, N,P, Shankar S & Laxman R,S ' Fungal Proteases: An Overview 2011', *Journal of Biotechnology & Biosciences*, vol. 1(1), pp. 1-40.
26. Pankhurst, N,W & Van Der Kraak, G 1997, ' Effects of stress on reproduction and growth of fish', Cambridge, UK: Cambridge University Press, pp. 73-93.
27. Qadar, S,A,U, E, Shireen, S, Iqbal and A, Anwar,2009, 'Optimization of Protease production from newly isolated strains of Bacillus sp. PCSIR EA-3', Indian Journal of Biotechnology, vol. 8, pp. 286-290.
28. Rao, M,B, Aparna, T,M, Mohini G,S, Vasanti D.V 1998, 'Molecular and biotechnological aspects of microbial proteases', *Microbiology & Molecular Biology Review*, vol. 62, pp. 597-635.
29. Rao, Y K, Lu S C, Liu B L & Tzeng Y, M 2006, 'Enhanced production of an extracellular protease from *Beauveriabassiana*by optimization of cultivation processes', *Journal of Biochemical Engineering*, vol. 28, pp. 57–66.
30. Ray, A 2012, 'Protease Enzyme- Potential Industrial Scope *Review', International Journal of Technology*, vol. 2(1), pp. 01-04.
31. Rupali, D 2015, 'Screening and Isolation of Protease Producing Bacteria from Soil Collected from Different Areas of Burhanpur Region (MP) India', *International Journal of Current Microbiology & Applied sciences*, vol. *4*, pp.597-606.
32. Shaheen, M, Shah, A.A, Hameed, A & Hasan, F 2008, 'Influence of culture conditions on production and activity of protease from Bacillus subtilis BS1', *Pakistan Journal of Botony*, vol. *40*(5), pp.2161-2169.
33. Shehzad, A & Muhammad, N 2014, 'Isolation and Characterization of Protease Producing Bacteria from Soil Samples of District Kohat Pakistan', *Journal of Bio-Molecular Sciences (JBMS)*, vol. *2*(1), pp.1-5.

34. Sumantha, A, C, Larroche & A, Pandey 2006, 'Microbiology and Industrial Biotechnology of Food-Grade Proteases', A Perspective. *Food Technoogyl & Biotechnology*, vol. 44 (2), pp. 211–220.
35. Tiwari, O,N, Devi, TB, Devi, K,S, Oinam, G., Indrama, T, Ojit, K, Avijeet, O & Ningshen, L 2015, 'Isolation and optimization of alkaline protease producing Bacteria from undisturbed soil of NE-region of India falling under Indo-Burma biodiversity hotspots', *Journal of Applied Biology & Biotechnology*, vol. *3*, pp. 25-31.
36. Vakilwala, M & Devyani P. 2017, 'Isolation and Screening of Protease Producing Organisms from Soil Sample', *International Journal of Research and Scientific Innovation (IJRSI)*, vol. 4, pp. 2321–2705.
37. Vishwanatha, K,S 2009, 'Acid protease from *Aspergillus oryzae*: Structure stability and enhancement of the activity by physical, chemical and molecular biological approaches',
PhD. Thesis, Central Food Technological Research Institute, Karnataka, India, pp. 266.
38. Walsh, G 2002, 'Proteins Biochemistry and Biotechnology', John Wiley & Sons, Ltd, England, pp. 547. Pergamon Press, Oxford, UK.
39. Whitford, D, 2005, 'Protein Structure and Function', John Wiley & Sons, Ltd, England, pp. 528