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Isolation of Protease Producing Bacteria (*Bacillus spp.*) From Soil and Water Samples of Gondar Town, Ethiopia.

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ABSTRACT

Microbial proteases are hydrolytic enzymes widely used in many industrial processes and management of wastes. This study was conducted with the aim of screening for potent protease-producing bacteria from soils and water samples. The screening of bacterial strains and protease expression were evaluated under different optimal conditions like pH, temperature, carbon source, nitrogen source, inoculum size, moisture content under solid state fermentation. The optimum protease production time for three isolates was found to be 48 h corresponding to a protease activity. The optimum temperature of protease production for both Ew-9 and Sw-11 was 37°C, Whereas 40°C was the optimum for Ds-7. In all cases, pH 7 was the optimum for production of protease with activities corresponding to 13.8 U/ml, 12.5 U/ml and 10.2 U/ml for Ds-7, Ew-9 and Sw-11, respectively. The Carbon sources wheat bran and Nitrogen sources, casein gave maximum activity. Furthermore, 0.2 M NaCl concentration was found to give better protease for isolates Ds-7 and Sw-11 corresponding to 6.8 U/ml and 4.9U/ml respectively. The best percentage of inoculum level for maximum production of protease was 10% in all isolates. The moisture content of the medium 1:3 w/v bran to moistening agent ratio was found to be the best for maximum protease production in all isolates. It can be concluded that the isolated three *Bacillus spp*. appears from soil and water samples have substantial potential for application in various proteolytic processes.

Keywords: *Bacillus sp.;* enzyme activity; protease; carbon source; nitrogen source;

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INTRODUCTION

The global environment is gradually deteriorating because of the socio-economic activities of humankind such as processing industries. Many industrial processes cause adverse changes in the immediate environmental change and therefore being challenged by society. Of these, leather industries and the increased number of feathers generated by commercial poultry processing may represent a pollution problem and needs adequate management [1]. Leather processing involves a series of unit operations. At each stage, various chemicals are used and varieties of materials are expelled (2). Depilation or dehairing of hides and skins in leather industry is traditionally done with chemical methods using lime, sodium sulfide, etc, which contributes to 80-90% of the total pollution load in the leather industry and generates noxious gases as well as solid wastes, e.g. hydrogen sulfide and lime [2]. Therefore, leather industry is one of the industries looking up to enzymes to reduce the impact of tanning processes on the environment [3].

Enzymes play crucial roles in different applications: in producing the food we eat, the clothes we wear, the drugs we need, the detergents we use, even in producing fuel for our automobiles, etc. Apart from use in various production processes towards greater efficiency, enzymes are also important in reducing both energy consumption and combating environmental pollution. The enzymatic treatment destroys undesirable pigments, increases the skin area and thereby clean hide is produced. Bating is traditionally an enzymatic process involving pancreatic proteases. However, recently, the use of microbial alkaline proteases has become popular. Alkaline proteases speed up the process of dehairing, because the alkaline conditions enable the swelling of hair roots; and the subsequent attack of protease on the hair follicle protein allows easy removal of the hair [4,5].

Proteases are hydrolytic enzymes found in every organism to undertake important physiological functions. These include: cell division, regulating protein turnover, activation of zymogenic performance, blood clotting, lysis of blood clot, processing and transport of secretory proteins across membrane, nutrition, regulation of gene expression and virulence factors. Proteases differ in their specific activities, substrate specificities, pH and temperature optima and stability, active site, and catalytic mechanisms. All these features contributed in diversifying their classification and practical applications in industries involving protein hydrolysis [6].

Ethiopians are believed to have been practicing traditional leather processing since their ancient civilizations. This local knowledge has been transferred through generations and is being widely practiced these days to process leathers of cattle origin for making their shoes, clothes, beds, cushions and many other items primarily among the rural communities. They use small ponds usually on the sides of rivers and embed the leather for a period of time to remove hairs. However, very little has been done to promote this work through the application of biotechnology. At present, there are few scientific reports available in Ethiopia on the potential microbial isolates that can be used in the process of dehairing. Therefore, there is a need to investigate the role and contribution of microorganisms during traditional leather processing. Based on the effect, the main objective of the study was to isolate the potential protease producing microorganisms from ponds used in leather processing around Gondar town and to optimize production conditions (Incubation time, pH, Temperature, C and N sources, Moisture level, NaCl concentration and Inoculum size) of the isolates for maximum protease production through solid state fermentation.

MATERIALS AND METHODS

Sample Collection

Samples were collected from traditional leather processing ponds/wastes (water from Seveha and Enfraz and soil from Dashin stagnant pond used for dehairing) and was kept in sterile tubes in refrigerator, at 4°C until used for further investigation.

Isolation of Proteases producing microorganisms

Isolation of protease producing microorganisms were carried out after enrichment using solid state fermentation medium containing (g/g) wheat bran, 10; K_2HPO_4, 0.1; $MgSO_4$, $7H_2O$, 0.02; $CaCl_2$, 0.01; and

casein, 1.0 in a 250ml Using Erlenmeyer flask, moistening agent (distilled water) were added in such a way to give final bran to moisture ratio of 1:3, thoroughly mixed, and autoclaved at 121 ºC for 15 minutes. Then, each flask was inoculated with 10 % (v/w) aliquots of mud suspensions as inoculums and incubated at 37 °C for 5 days [7].From thoroughly mixed enriched fermented solid substrate of each sample, 1g was taken and suspended into 30 ml glass tube containing 5 ml of sterilized distilled water. Then the glass tube was placed on a 121 rpm shaker for 30 minutes at room temperature. This suspension was serially diluted (10^{-1} to 10^{-12}) and spread on agar plates. Individual colonies were isolated and screened for protease production.

Screening for Protease Production

Screening of isolates for protease production was carried out using casein-yeast extract peptone (CYP) agar medium containing (g/l): casein, 10; bacteriological peptone, 5; yeast extract, 1; K_2HPO_4, 1; $MgSO_4.7H_2O$, 0.2; $CaCl_2$, 0.1; and agar, 15 [8]. After inoculation, the plates were incubated at 37 °C for 48 hours. Formation of halo zone around the colonies, resulting from casein hydrolysis was taken as positive for proteolytic activity. These colonies were isolated and streaked repeatedly in fresh plates until single uniform colonies were obtained.

To select an isolate which gives protease with high activity, a loop full of culture from agar plate was taken and inoculated into 30 ml glass tube containing 5 ml of alkaline protease production medium and incubated overnight at 121 rpm at room temperature. Then, 5 % (v/v) of the 16 hr inoculum was inoculated into 50 ml of same medium kept in 250 ml Erlenmeyer flask and incubated with rotary shaking (121 rpm) at room temperature for 5 days. Five ml of the fermented broth was taken and centrifuged at 6000 rpm for 5 minutes and the cell free supernatant was used as enzyme source.

Preparation of Substrates for Solid State Fermentation

Agricultural residues namely wheat bran and rice bran were collected from local market in Gondar town. Before using these substrates for fermentation, the unnecessary parts of the substrates such as adhered surface dust particles were first separated and removed by washing with distilled water. After that, the remaining useful materials were sun-dried and ground to small size in a mixer grinder and finally used as a substrate for the production of protease under SSF [9].

Solid State Fermentation (SSF)

SSF medium containing (g/g): wheat bran, 10; K 2HPO4, 0.1; MgSO4. 7H2O, 0.02; CaCl2 0.01; and casein, 1.0 was prepared in a 250 ml Erlenmeyer flask and the solid substrate to moistening agent ratio was adjusted to 1:3, unless stated otherwise. After autoclaving, sterile sodium carbonate was added to give a final concentration of 10% (w/w), inoculated and incubated at 37°C for 5 days. From the fermented substrate, alkaline protease was harvested by soaking the fermented solid with ten volumes of distilled water per gram solid substrate (wheat bran), in shaking (121rpm) condition for 30 minutes at room temperature [9]. At the end of the extraction, the suspension was hand squeezed through a double layered muslin cloth and the particulate materials clarified by centrifugation at 10,000 rpm for 5 minutes.

Determination of the Protease Activity of Selected Isolates

Protease assay

Protease activity was determined using casein as a substrate as described by Hema and Shiny (2012). The reaction mixture contained a total volume of 2 ml which in turn was composed of 1 ml of 1% casein in 50 mM sodium phosphate buffer (pH 7) and 1 ml enzyme solution. After 20 min of incubation at 37°C, the reaction was terminated by adding 2 ml of 10% trichloroacetic acid (TCA) and again incubated at 37°C for 20 min. After separation of the un-reacted casein precipitate by centrifugation at 10000 rpm for 15 min, 0.5 ml of clear supernatant was mixed with 2.5 ml of 0.5M Na_2CO_3 and 0.5 ml of 1N Folin-Ciocalteau's phenol reagent. After incubation for 20 min at 37°C, absorbance was measured at 660 nm against a reagent blank. One unit of protease activity is defined as the amount of enzyme that releases 1 µg amino acid equivalent to tyrosine per min under the standard assay conditions [10].

$$\text{Units /ml} = \frac{\mu \text{ mole of tyrosine x reaction vol.}}{\text{Sample vol x reaction time x vol. assay}}$$

Tyrosine calibration curve

As a reference to protease enzyme activity, tyrosine standard curve was generated using an appropriate amount of tyrosine diluted in water. The suitably diluted samples (0.1 – 1.5 mg/ml) were treated similar to the experimental enzyme catalyzed reaction mixture and then were measured using a spectrophotometer at a wavelength of 660 nm [11].

Optimization of the Growth Conditions for Production of Protease

Effect of time on the production of protease

To determine the optimum period for maximum production of protease, the culture in the medium containing wheat bran, peptone, yeast extracts, casein, $CaCl_2$, K_2HPO_4 and $MgSO_4$ was incubated at 37°C for 24-72 hrs and the protease activity was determined at 12 h intervals. Thus 2 ml of culture broth was collected after each interval and protease activity was determined as described above in section 2.5.1.

Effect of temperature on the production of protease

The optimum temperature for protease production was determined by incubating inoculated production media at different temperatures (i.e. 25, 30, 37, 40, 45 and 50°C), for as long as the protease activity began to drop from the maximum. Protease activity was then determined for each culture every 12 hours till a decline in activity was observed by taking a cell free supernatant and testing using the method of [10].

Effect of pH on the production of protease

The effect of pH on the production of protease was investigated by adjusting the pH of the production media to 5.0, 6.0, 7.0, 8.0, and 9.0 and incubating at 37°C for as long as the protease activity began to drop from the maximum Adjustment of pH was done using 1N NaOH and 0.1N HCl solutions. Following this, protease activity was determined for each culture every 12 hours till a decline in activity was observed using the method of [10].

Effect of carbon source on the production of protease

Glucose, rice bran, wheat bran, and sucrose were used as carbon sources. The cultures were incubated at 37°C for as long as the protease activity began to drop from the maximum [12]. Protease activity for each culture was then determined every 12 hours using the method of [10].

Effect of nitrogen source on the production of protease

Two different sources of nitrogen, viz. organic nitrogen and inorganic nitrogen were tested for their potentials to enhance protease production. The production medium was initially supplemented with different organic nitrogen sources such as yeast extract, peptone, casein, each at 1% (w/v) and inorganic nitrogen sources such as, $(NH_4)_2SO_4$, and NH_4Cl at 1% (w/v) were tested after incubating culture at 37°C for as long as the protease activity began to drop from the maximum [12]. The effect was studied by determining the protease activity for each culture every 12 hours till a decline in activity was noted as in sections 2.5.1.

Effect of NaCl concentration on the production of protease

NaCl was added at various concentrations, i.e. 0.0, 0.2, 0.4, 0.6 and 0.8M, into the protease production medium and assay for crude enzyme (protease) activity was carried out by incubating each culture for as long as the protease activity began to drop from the maximum [13]. Effect of concentration of NaCl was

studied by determining the protease activity for each culture every 12 hours till a decline in activity was noted as in sections 2.5.1.

Effect of moisture level on protease production

The effect of moisture level on protease production of the selected bacterial isolates (1% inoculum) was determined by adding moistening medium to wheat bran at level of 1:2, 1:3, 1:4 and 1:5 (w/v). SSF medium was incubated at 37°C and the crude enzymes were harvested to determine protease activity every 12 hours until the activity began to show a decline. The procedures used to determine the activity of the crude enzyme were similar to those shown in sections 2.5.1.

Effect of inoculum size on protease production

The effect of inoculum size on protease production of the selected bacterial isolates was assessed by inoculating the SSF medium (a medium composed of a1:3 wheat bran to moistening agent ratio) with inoculum size of 5%, 10%, 15% and 20%. Protease activity was determined for each culture using the same procedures indicated in sections 2.5.1.

RESULTS AND DISCUSSION

Isolation, Screening and Selection of Protease Producing Bacteria

Isolation and screening of protease producing bacteria

Based on colony morphology, a total of 147 colonies were isolated from the three different sample sources. Out of the total isolated colonies, 85 colonies (57.8%) were positive for protease production (Table1). As it can be seen from Table 1, the isolates showed a great variation in the size of the clear zone of hydrolysis they produced on milk agar plates ranging from the least 1 mm to the largest 20 mm. This indicates the possible capability of the isolates to produce potential protease, which could be used for different industrial applications. In addition, the capability of the isolates to hydrolyze milk agar is in good agreement with the earlier reported 1-20mm diameter of clear zone formed on casein agar [14,15,16].

Table 1. Screening of the 15 protease producing isolates

Sample sources	Positive isolates	Zone of clearance(mm)	Protease activity after 48hrs (U/ml)
Soil samples collected From Dashen Brewery	Ds-6	11	14.6
	Ds-7	**14**	**24.1**
	Ds-8	08	9.2
Water sample from Enfraz	Ds-9	12	17..7
	Ds-10	10	12.5
	Ew-5	11	9.7
	Ew-7	13	12.3
	Ew-8	09	7.0
	Ew-9	**14**	**14.7**
	Ew-11	12	11.5
	Sw-7	09	9.5
	Sw-8	10	10.6
Water sample from Seveha	Sw-9	13	12.1
	Sw-10	11	11.0
	Sw-11	**15**	**14.0**

Selection of the best protease producing bacteria

From the total of 85 positive isolates, 15 isolates with relatively large clear zone of hydrolysis were selected for further investigation. The selection of potent bacteria was done by comparing the isolates with each other in terms of both their diameter of clear zone of hydrolysis and their protease activities (Table1).

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The results showed that the isolates with higher clear zone of hydrolysis also gave higher protease activities (Table1). This step resulted in selection of three potentially potent isolates, i.e Ds-7 from Dashen soil, Ew-9 from Enfraz water and Sw-11 from Seveha water (Figure 1).

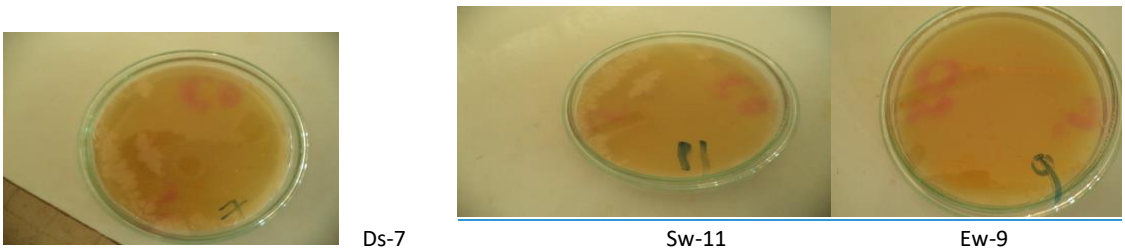

Figure 1 . Zone of hydrolysis of casein by the three selected isolates

Phenotypic Characterization of the Bacterial Isolates

The isolates Ds-7, Ew-9 and Sw-11 were identified as spore-forming bacterial species that belong to the genus *Bacillus* based on the information obtained from Bergey's Manual of Classification of Determinative Bacteriology (Table 2.). Although members of the genus *Clostridium* are also spore-forming bacteria, the 3 isolates of this study do not belong to this genus as they are catalase positive and capable of growing under aerobic condition in the ordinary incubator.

Table 2. Morphological and biochemical characteristics of the selected bacterial isolates

Parameters	Bacterial Isolates		
	Ds-7	**Ew-9**	**Sw-11**
Cell shape	Long, Rod	Long, Rod	Short, Rod
Cell arrangement	Chain	Pair	Pair
Colonial pigmentation	White	Red	White
Gram staining	+ve	+ve	+ve
Endospore staining	+ve	+ve	+ve
Motility test	+ve	+ve	+ve
Catalase test	+ve	+ve	+ve
Indole production	-ve	-ve	-ve
H_2S production	-ve	-ve	-ve
Starch hydrolysis	+ve	+ve	+ve
Gelatin hydrolysis	+ve	+ve	+ve
Casein hydrolysis	+ve	+ve	+ve
Gas production	-ve	-ve	-ve
Urea hydrolysis	-ve	-ve	-ve
Glucose fermentation	+ve	+ve	+ve
Sucrose Fermentation	+ve	+ve	+ve

Key: +ve = Positive, -ve = Negative

Effect of Culture Conditions on Protease Production under SSF

Effect of time course on protease production

In the present study, the optimum time for protease production from the three isolates was found to be 48 hrs with protease activities of 3.5 U/ml, 2.7 U/ml, and 4.2 U/ml, for Ds-7, E-9 and S-11, respectively (Fig. 2). After 48 hours of incubation time, there was neither further increase nor a pronounced drop in protease production. This might be due to the decrease in microbial growth associated with the depletion of available nutrient, loss of moisture content, production of toxic metabolites and autolysis caused by the protease produced [17].

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These results are in accordance with observations made by [18,19,20] where maximum enzyme production was observed during growth of the culture at the late exponential phase and early stationary phase of the growth and thereafter the number of viable cells decreased due to depletion of readily available carbon sources and other nutrients.

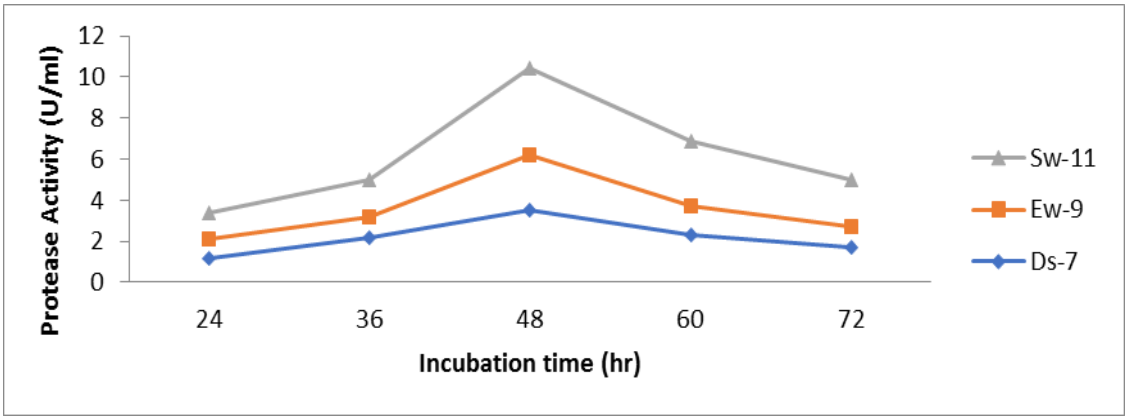

Figure 2 . Effect of incubation time on protease production

Different kind of incubation time (24-72 Hrs) taken in X axis and Protease activity was taken in taken in Y axis. (U/ml).

Effect of temperature on the production of protease

The optimum temperature for production of proteases by isolates Ew-9 and Sw-11 was found to be 37°C, which resulted in protease activities of 10.1 U/ml and 9.0 U/ml, respectively. Whereas for isolate Ds-7, maximum protease production (9.3 U/ml) was obtained at 40°C. However, a considerable decrease in protease activity was observed with further increase in temperature beyond the maximum in all three isolates (Fig.3). It might be due the fact that at high temperature, the growth of the bacteria was hindered.

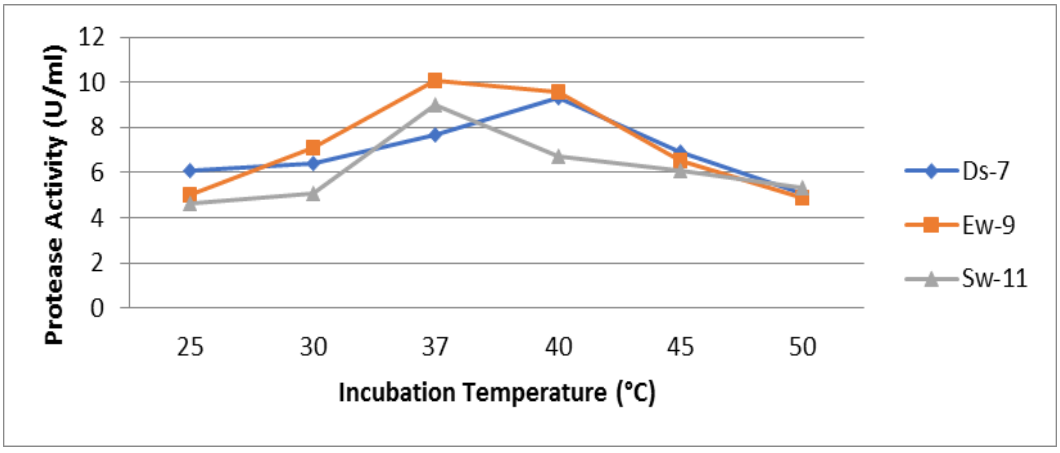

Figure 3. The effect of incubation temperature on protease production

Different kind of Tempreature (25-50) taken in X axis and Protease activity was taken in taken in Y axis. (U/ml).

According to the report of Abdel Nasser *et al*. [21] high temperature may inactivate the expression of the gene responsible for the synthesis of protease enzyme. At relatively low temperature (< 25°C), protease production was very low probably due to slow growth of the bacterial isolates at low temperature. Several reports indicate that maximum protease production was achieved at 35-40°C for certain *Bacillus* spp. [20,22,23]. On the basis of the temperature requirement for maximum protease production, it can be gathered that isolates Ds-7, Ew-9 and Sw-11 belong to the mesophilic protease producers.

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Effect of initial pH on the production of protease

The optimum pH for protease production for the three isolates was 7.0 although the enzyme was active in the pH range of 7- 11 (Fig. 4).

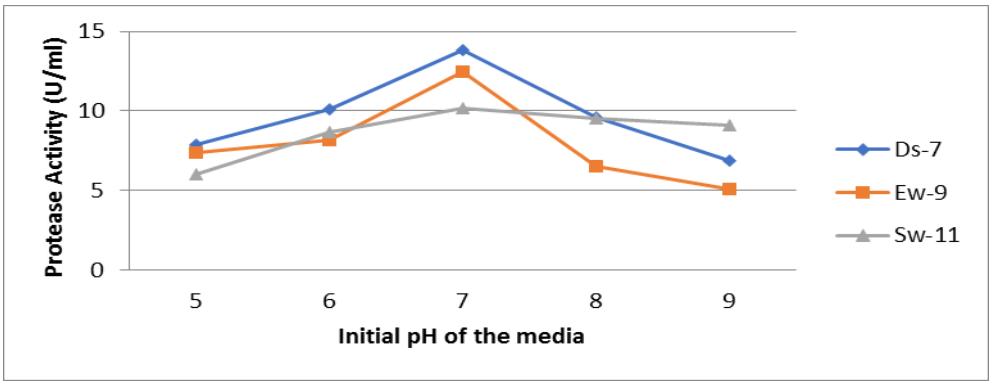

Figure 4. The effect of initial pH of the media on protease production

Different kind of pH (5-9) taken in X axis and Protease activity was taken in taken in Y axis. (U/ml).

At pH 7, the protease activities for Ds-7, Ew-9 and Sw-11 were 13.5 U/ml, 12.5 U/ml and 10.2 U/ml, respectively. However, from the survey of literature it can be seen that the optimum pH range for protease production is generally between 7 and 9 [24, 25] .

Further increase in initial pH values resulted in the decrement of protease production. This might be because the isolates had preference for neutral pH to optimally grow in the medium [26]. Normally, *Bacillus spp*. prefer neutral or slightly alkaline or a range between 6.8 and 7.2 pH for protease production at the initial stage of fermentation [27] . For bacteria isolated from mesophilic environments, reports from earlier studies revealed that an optimum pH for protease production was pH 7 [28,29].

Effect of different carbon sources on the production of protease

Among the various carbon sources used in this study, the easily available complex carbon sources like wheat bran and rice bran were found to be the best for protease production by the selected isolates. Wheat bran showed maximum enzyme production which was even better than that produced on glucose for isolates Ds-7 (20.0 U/ml) and Ew-9 (12.9 U/ml), whereas glucose was better for isolate Sw-11 (12.8 U/ml) (Fig 5).

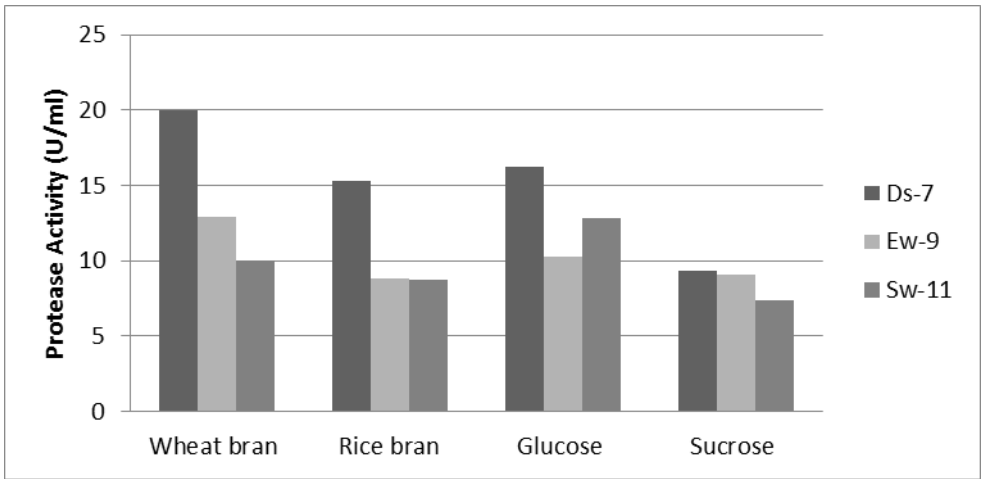

Figure 5. The effect of different carbon sources on protease production.

Different kind of carbon source taken in X axis and Protease activity was taken in taken in Y axis. (U/ml).

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Microbial growth medium for enzyme production at industrial scale takes about 30-40% of the production cost [30]. By using wheat bran alone, appreciable amount of protease can be produced with reduced cost. The production of large amount of protease from complex carbon sources suggests the presence of enough nutrients in wheat bran that promote enzyme production and support very little growth of the isolates.

Effect of nitrogen source on the production of protease

Effect of various nitrogen sources (organic and inorganic nitrogen sources) on protease production of the three selected isolates (i.e. Ds-7, Ew-9 and Sw-11) was also examined. It was observed that the growth medium containing casein yielded highest activity in all isolates (i.e.33.5 U/ml, 37.6 U/ml and 33.7 U/ml for Ds-7, Ew-9 and Sw-11, respectively). This was followed by peptone, yeast extract, ammonium sulphate and ammonium chloride (Fig.6).

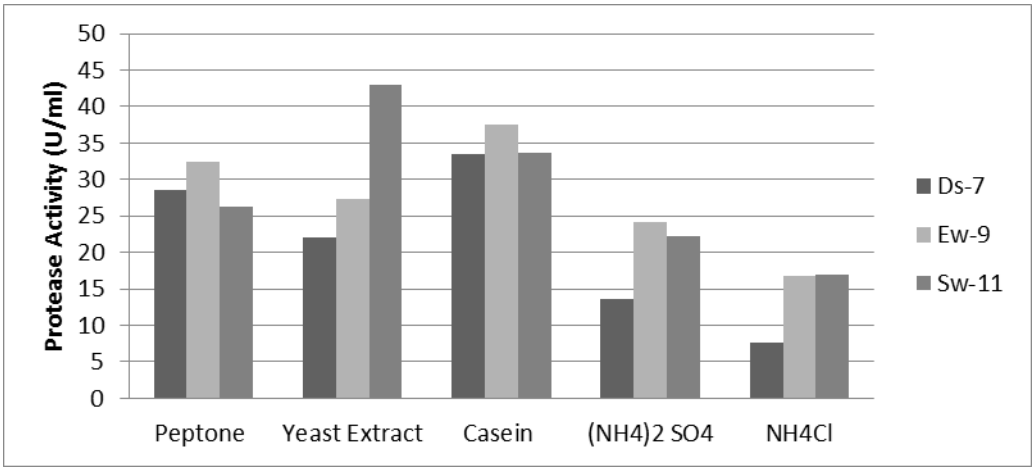

Figure 6. Effect of different nitrogen sources on protease production

Different kind of Nitrogen source taken in X axis and Protease activity was taken in taken in Y axis. (U/ml).

As shown in the above figure, organic nitrogen sources (casein, yeast extract and peptone) enhance protease production better than inorganic nitrogen sources (ammonium sulphate and ammonium chloride). This maximum protease production by casein, peptone and yeast extract might be due to the presence of high nutritional amino acids in these organic nitrogen sources. By contrast, least production of protease was observed in SSF medium supplemented with ammonium sulphate and ammonium chloride, respectively. These findings were generally in agreement with the results reported by [31]. The low level protease production might be due to the inability of the bacterial isolates to utilize these nitrogen sources or due to the inhibitory effect of the inorganic N sources. In connection with this, Niadu and Devi,[32] also reported the repressing ability of inorganic nitrogen sources in Bacillus isolate.

Effect of inoculum size on protease production

The size of inoculum plays an important role in the production of high protease [33].In the present study, 10% was found to be an optimum inoculum size for amylase production for all isolates (i.e 12.5 U/ml, 11.3 U/ml and 13.6 U/ml for isolates Ds-7, Ew-9 and Sw-11, respectively) shown in Figure 7.

In this study, inoculum size higher or lower than 10% has been shown to decrease protease production. The decrease in protease yield at lower inoculum size might be due to the longer time required by the bacterial isolates to grow to an optimum number to utilize the substrate and form the desired product. On the other hand, the low protease production at higher inoculum size (>20%) might be due to the stressful conditions created by the microbial cells such as depletion of nutrients, pH fluctuation, change in availability of oxygen and competition for limited resources [34,31].

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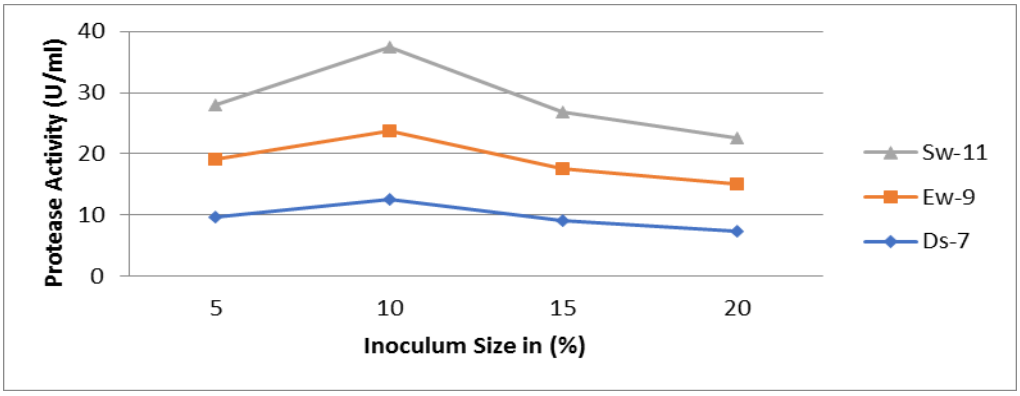

Figure 7. The effect of inoculum size on protease production.

Inoculum size (%) taken in X axis and Protease activity was taken in taken in Y axis. (U/ml).

Effect of moisture content on protease production

The effect of moisture level on enzyme production was determined by growing the bacterial isolates on wheat bran supplemented with moistening agent (distilled water) at different ratios (w/v). In all isolates, maximum protease activity was shown in Figure 8 at moisture content 1:3 (i.e 9.5 U/ml, 11.9 U/ml and 10.5 U/ml for isolates Ds-7, Ew-9 and Sw-11 respectively).

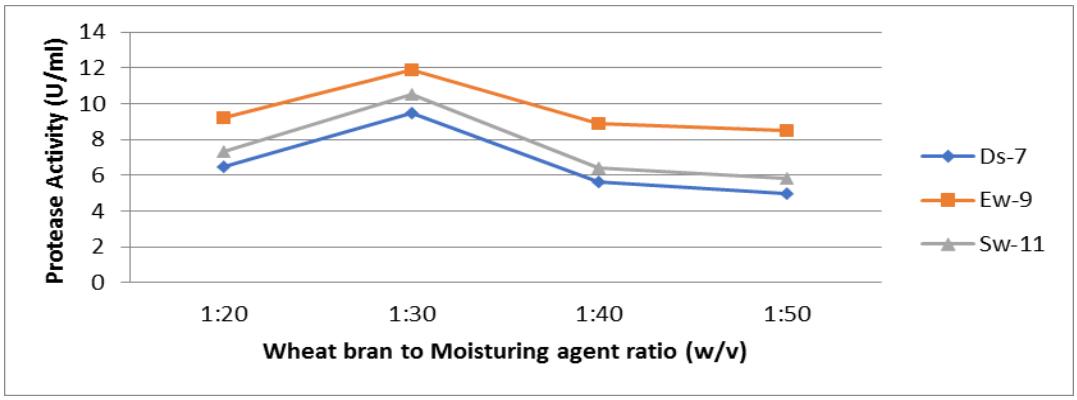

Figure 8. Effect of moisture level on protease production

Wheat bran moisture ratio taken in X axis and Protease activity was taken in taken in Y axis. (U/ml).

Among several factors that are essential for microbial growth and enzyme production under solid-state fermentation, moisture level is one of the most critical factors [35, 36]. In the present study, in all isolates, high enzyme activity was obtained when the substrate to moisture ratio maintained at 1:3. In all isolates, any further increase or decrease of moisture ratio from the optimum (1:3) resulted in a slight decline of enzyme production. This slight reduction of enzyme yields at low moisture level might be due to clumping of solid particles, reduction in solubility of the nutrients of the substrate, low degree of swelling and higher water tension [36]. The low enzyme activity at high moisture level (at 1:5) might be due decreased oxygen availability and steric hindrance of the growth of the isolates by reduction in porosity of the wheat bran [36].

Different studies showed difference in optimum moisture content needed for production of protease. Saxena and Singh [33] reported that 1:3 moisture content as an optimum moisture ratio for enzyme production from *Bacillus* species, which was in agreement with the present study. On the other hand,Salwa *et al*.[37] reported that the optimum moisture levels required for enzyme production by *Bacillus cereus* and *Bacillus* species were 1:2 and 1:2.5, respectively. These reports demonstrated slightly lower moisture ratio for maximum enzyme production compared to the result obtained in the present study. This might be due to the difference in the nature of the solid substrates used for fermentation.

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Effect of NaCl concentration on the production of protease

Figure 9 shows the various NaCl concentrations (i.e. 0, 0.2, 0.4, 0.6, 0.8M) were used to determine the optimum level required for the production of protease by the three selected isolates (i.e. Ds-7, Ew-9 and Sw-11).It was observed that the growth medium containing 0.2M NaCl yielded the maximum protease production in isolates Ds-7 and Sw-11 corresponding with 6.8 U/ml and 4.9 U/ml respectively. Whereas, for isolate Ew-7 0.4M of NaCl was resulted in maximum protease activity (i.e 4.9 U/ml).

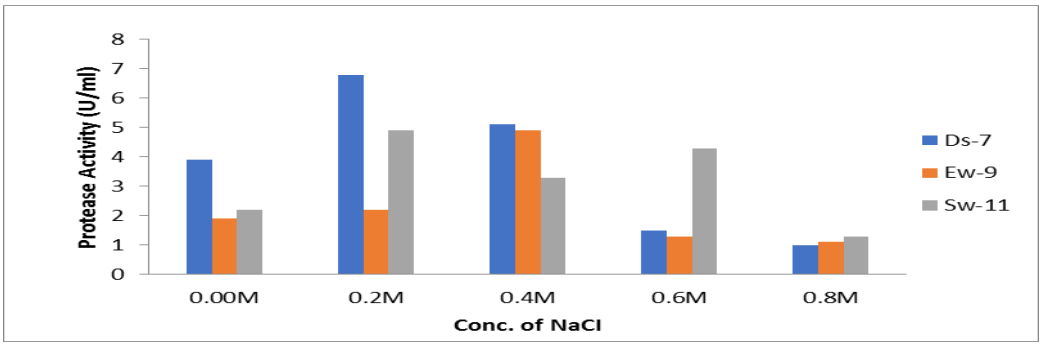

Figure 10. Effect of NaCl concentration on the production of protease

Concentration of NaCl (M) taken in X axis and Protease activity was taken in taken in Y axis. (U/ml).

The present study considerably well agreement with the study conducted by (Huang *et al*.[38)]who reported on halophilic and alkaliphilic bacterial isolates at 4M NaCl.

CONCLUSION

In this study the screening of potent protease producing bacteria (*Bacillus spp.)* from three different sample sources (water from Seveha and Enfraz and soil from Dashin) and to optimize their cultivation condition for maximum protease production. From a total of 147 pure bacterial colonies, 85 (57.8%) were found as protease positive, out of the 85 protease positives, Based on the results of different morphological, physiological and biochemical tests done, these isolates were found to be members of the genus *Bacillus spp*. Time courses of protease production in all isolates indicate that the production increases as time increases was harvested after 48 hrs in all isolates. Isolates Ew-9 and Sw-11 produce maximum protease at 37°C, whereas 40°C was optimum for isolate Ds-7. All isolates produced maximum protease at pH 7 when compared to some slight acidic and alkaline pH. Isolate Ds-7 and Ew-9 gave maximum protease in medium supplemented with wheat bran whereas isolate Sw-11 gave high protease in the present of glucose. The effect of nitrogen sources indicated that in all isolates organic nitrogen sources resulted in maximum protease production as compared to inorganic nitrogen sources. In isolate Ds-7 and Ew-9 maximum protease was obtained in a medium containing casein, whereas yeast extract gives maximum protease in isolate Sw-11. The production curve of effect of different size of inoculums on protease production revealed that protease production increased when the percent of inoculums increased up to the optimum and decreased beyond the optimum size. In all isolates maximum protease were harvested in 10% v/v inoculums. The effect of moisture level on protease production indicated that protease production increased with increased bran to moistening agent till optimum decreased beyond the optimum and all isolates give maximum protease at 1:3 v/w bran to moisture ratio. On the other hand, production of protease is also influenced by the concentration of NaCl on the growth media. The optimum NaCl concentration was found to be 0.2 M for isolates Ds-7 and Sw-11 but 0.4M was optimum for isolate Ew-9. The isolated new source of protease producing bacteria, from the soil and water samples that are collected from traditional leather processing ponds might be an alternative source for the potential industrial applications.

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