ISOLATION, PURIFICATION AND MASS PRODUCTION OF PROTEASE PRODUCING BACTERIA FROM SOIL.

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Abstract: Bacterial are well known for their ability to excrete enzymes into the environment. Microbial proteases play an imperative task in various biotechnological processes. Screening and isolation of protease producing strains of bacteria were carried out from soil collected near dairy in Virar. The isolates that were positive on skim milk agar (1%) were selected as protease producing strain. The organisms were tested for various biochemical tests, which lead to their tentative identification as Bacillus species producing protease enzyme. Enzyme production was carried in 100ml of production media in the fermenter at 37ºC for 48 hours at pH 8.0. Harvested protease product was partially purified by salt precipitation method and dialysed. Then protein was further characterized using SDS-PAGE.

Keywords - Bacteria, Protease, Soil, Isolates.

I. INTRODUCTION

Enzymes are delicate protein molecules necessary for lives (Gupta et al, 2002). They are biocatalysts produced by living cells to accelerate and coordinate a multitude of chemical reactions necessary to develop and sustain life processes. More than 3000 enzymes, which catalyse a wide variety of chemical reactions, are known. Among six classes of enzymes, hydrolases, proteases, amylases, cellulases and xylanases) have a wide range of biotechnological applications. Proteases constitute one of the most important groups of hydrolytic enzymes which act upon native proteins to disintegrate them into small peptides and amino acids. Microorganisms can produce protease that shares nearly 40% of the total worldwide enzyme market. Sources of proteases include all forms of life, that is, plants, animals and microorganisms. Based on their acid-base behavior, proteases are classified in to three groups, that is, acid, neutral and alkaline proteases. Acid proteases performed best at pH range of 2.0-5.0 and are mostly produced by fungi. Proteases having pH optima in the range of 7.0 or around are called neutral proteases. Neutral proteases are mainly of plant origin. While proteases having optimum activity at pH range of 8 and above are classified as alkaline proteases produced from microorganisms. Microbial enzymes find a broad spectrum of application due to their broad biochemical diversity, ease of mass culture that is, rapid growth in limited space and genetic manipulation (Adsul et al., 2007; Das and Prasad, 2010). Among the various proteases, bacterial proteases are most significant compared to animal and fungal proteases. Amongst bacteria, Bacillus species are specialized producers of extracellular proteases. Most of the commercial alkaline proteases were isolated from Bacillus species.

II. MATERIALS AND METHODS

2.1 Sample collection

Soil sample from an area near dairy, Virar (west) was collected in a sterile container and were labeled and processed on the very same day of collection.

2.2 Isolation of bacteria producing protease enzyme

The soil samples were screened for protease enzyme producing organisms by serial dilution method. After the serial dilution (10^{-2} to 10^{-6}), the last three dilutions were spread plated on the sterile Skimmed agar plate and incubated at 37 °C for 24 hours.

2.3 Screening for the best strain producing protease enzyme

All the isolated colonies showing proteolytic activity (zone of clearance) on sterile skimmed milk agar plates were further screened on sterile gelatin agar plates by gelatin clear zone method. The different isolates were spot inoculated on sterile gelatin agar plates and were incubated at 37 °C for 24 hours. After incubation the plates were flooded with 1% mercuric chloride solution. Moreover, the isolated colonies were further spot inoculated on sterile skimmed agar plates and incubated at 37 °C for 24 hours to further ensure the proteolytic activity of the organisms.

2.4 Identification of the bacteria producing protease enzyme

The screened bacteria for protease enzyme was identified based on it's cellular morphology, Gram staining, endospore staining and biochemical tests.

2.5 Media optimization for extraction of protease

The media used for optimized production of protease enzyme consisted of glucose 1% (w/v), casein 0.5%, yeast extract 0.55, KH2PO4 0.2%, Na2CO3 1%, MgSO4 . 7H2O 0.2%, and pH 8.0.ml of the media was prepared in 250 ml Erlyne Meyer flasks and sterilized using autoclave.

2.6 Mass production of alkaline protease through fermentation

The culture media used for mass production of protease contains, Dextrose 1 %(w/v), peptone 0.5%, KH_2PO_4 0.2%, $MgSO_4$. $7H_2O$ 0.2%, Casein 1% and pH 8.0. It was maintained at 37^0c for 48hrs.

2.7 Extraction of the enzyme protease

At the end of fermentation period, the whole culture broth was centrifuged at 10,000 rpm for 15 minute, to remove the cellular debrises and the clear supernatant was used as enzyme preparation.

2.7 Ammonium Sulfate Precipitation

Ammonium Sulphate Precipitation is a simple and effective means of fractionating proteins. It is based on the fact that at high salt concentrations the natural tendency of proteins not to aggregate is overcome, since the surface charges are neutralized. Charge neutralization means that proteins will tend to bind together, form large complexes and hence are easy to precipitate out by mild centrifugation. Since each protein will start to aggregate at a characteristic salt concentration, this approach provides a simple way of enriching for particular proteins in a mixture.

The amount of ammonium sulfate to be added to the solution in order to increase % saturation level was calculated from the table mentioned Table 1 below.

Initial concentration of ammonium sulphate (% saturation)	Final concentration of ammonium sulphate (% saturation)																
	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90	95	100
	Gram solid ammonium sulphate to add to 100ml of solution																
0	10.7	13.6	16.6	19.7	22.9	26.2	29.5	33.1	36.6	40.4	44.2	48.3	52.3	56.7	61.1	65.9	70.7
5	8	10.9	13.9	16.8	20	23.2	26.6	30	33.6	37.3	41.1	45	49.1	53.3	57.8	62.4	67.1
10	5.4	8.2	11.1	14.1	17.1	20.3	23.6	27	30.5	34.2	37.9	41.8	45.8	50	54.5	58.9	63.6
15	2.6	5.5	8.3	11.3	14.3	17.4	20.7	24	27.5	31	34.8	38.6	42.6	46.6	51	55.5	60
20	0	2.7	5.6	8.4	11.5	14.5	17.7	21	24.4	28	31.6	35.4	39.2	43.3	47.6	51.9	56.5
25		0	2.7	5.7	8.5	11.7	14.8	18.2	21.4	24.8	28.4	32.1	36	40.1	44.2	48.5	52.9
30			0	2.8	5.7	8.7	11.9	15	18.4	21.7	25.3	28.9	32.8	36.7	40.8	45.1	49.5
35				0	2.8	5.8	8.8	12	15.3	18.7	22.1	25.8	29.5	33.4	37.4	41.6	45.9
40					0	2.9	5.9	9	12.2	15.5	19	22.5	26.2	30	34	38.1	42.4
45						0	2.9	6	9.1	12.5	15.8	19.3	22.9	26.7	30.6	34.7	38.8
50							0	3	6.1	9.3	12.7	16.1	19.7	23.3	27.2	31.2	35.3
55								0	3	6.2	9.4	12.9	16.3	20	23.8	27.7	31.7
60									0	3.1	6.3	9.6	13.1	16.6	20.4	24.2	28.3
65										0	3.1	6.4	9.8	13.4	17	20.8	24.7
70											0	3.2	6.6	10	13.6	17.3	21.2
75												0	3.2	6.7	10.2	13.9	17.6
80													0	3.3	6.8	10.4	14.1
85														0	3.4	6.9	10.6
90															0	3.4	7.1
95																0	3.5
100																	0

Table 1: Precipitation of the enzyme using ammonium sulphate method.

2.8 Dialysis

Dialysis is the process of separating molecules in solution by the difference in their rates of diffusion through a semipermeable membrane. It will elute out salts. After dialysis, the sample was removed from the membrane and stored in a sterilized container.

2.9 Molecular weight determination by SDS-PAGE

The molecular weight of the purified protein was determined under denaturing conditions by subjecting the samples to 12% SDS-PAGE. SDS-PAGE is an electrophoresis method that allows protein separation by mass. The protein samples were run along with standard molecular weight marker and bands are visualized with silver staining.

2.10 Protein estimation procedure

The estimation of protein was carried out using Folin's Lowry method. Most protein estimation techniques use Bovin Serum Albumin (BSA) universally as a standard protein, because of its low cost, high purity and ready availability. The method is sensitive down to about 10 µg/ml.

2.11Protease activity by casein digestion assay

The protease activity was determined according to the casein digestion unit (CDU) method and tyrosine was used as a standard. Proteases break peptide bonds. It is often necessary to measure and/or compare the activity of proteases. This non-specific protease activity assay is used as a standardized procedure to determine the activity of proteases for quality control purposes. In this assay, casein acts as a substrate. When the protease we are testing digests casein, the amino acid tyrosine is liberated along with other amino acids and peptide fragments. Folin & Ciocalteus Phenol, or Folin's reagent primarily reacts with free tyrosine to produce a blue colored solution, which is measured at an absorbance value at 660 nm colorimetrically.

The specific activity, recovery (%) and purification fold were estimated by the following equations:

$$\text{Specific activity (U/ml)} = \frac{\text{Protease activity (U/ml)}}{\text{Protease content (mg/ml)}}$$

III. Result and Discussion

3.1 The soil samples were screened for protease enzyme producing organisms by serial dilution method (Fig 1). After the serial dilution (10-2 to 10-6), the last three dilutions were spread plated on the sterile Skimmed agar plate and incubated at 37 °C for 24 hours.

Fig.1: Serial dilution of soil sample.

3.2 Different colonies showing zone of clearance around them were obtained on sterile skimmed milk agar plates on incubation at 37^0c for 24hrs after performing a standard plate count of the soil sample. This indicates that they may be organisms producing proteases. (Fig.2)

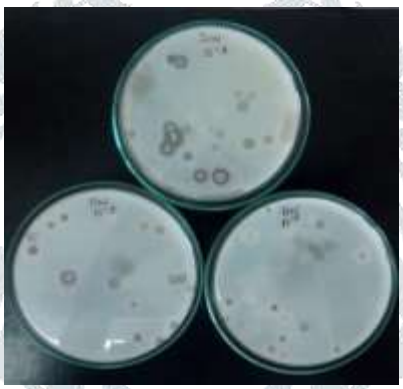

Fig.2: Sterile skimmed milk agar plates of different dilutions (10-4, 10-5 and 10-6) showing zone on clearances around them.

3.3 Colonies that showed good zone of clearance from the three plates were selected and streaked on sterile nutrient agar slants (Fig. 3).

Fig.3: Selected colonies streaked on sterile Nutrient agar slants

3.4 In order to confirm this proteolytic activity and to screen for the best strain producing extra cellular protease, the isolates obtained on sterile skimmed milk agar plates were further spot inoculated on sterile gelatin agar plate and also sterile skimmed milk agar plate and they were incubated at 37^0C for 24hrs.

On flooding the gelatin agar plates after incubation with 1% $HgCl_2$ solution after the incubation period, zone of clearance was observed around the spotted colonies.

Also, the skimmed milk agar plate showed a better zone of clearance around the spotted colonies. Thus, the isolates showing a larger and better zone of clearance was chosen for studies. (Fig.4 and 5)

Fig 4: Zone of clearance around spotted isolates on sterile Gelatin agar plate.

Fig.5: Zone of clearance around spotted isolates on Sterile Skimmed milk agar plate.

3.5 The gram's morphology, endospore forming characteristic of the chosen organism was done and also its biochemical characteristic studied. The organism was found to be gram positive rods in chains and positive for endospore production. (Fig. 6 and 7)

Fig.6: Gram staining of the isolate

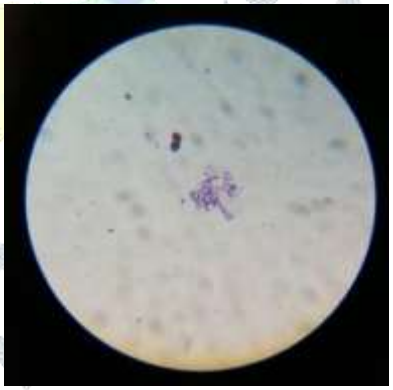

Fig.7: Spore staining of the isolate.

3.6 Colony characteristics of the chosen isolate on sterile Nutrient agar plate and incubated at 37^0c for 24hrs.

Characteristics	**Results**
Size	Medium
Shape	Circular
Colour	Off white
Opacity	Opaque
Elevation	Slightly elevated
Consistency	Butyrous
Grams morphology	Grams positive rods in chains

3.7 The biochemical characteristics of the organism is tabulated as follows:

3.7.1 Sugar Fermentation tests

Sugar Fermentation Interpretation (A C= Arobic conditions & An C= Anerobic conditions) fig: 8

Sugar Fermentation											
Dextrose		Lactose		Sucrose		Manitol		Maltose		Xylulose	
A C	An C	A C	An C	A C	An C	A C	An C	A C	An C	A C	An C
+	+	+	+	+	+	-	-	-	-	+	+

Fig.8: Sugar Fermentation
(Left: Arerobic conditions and Right image Anerobic conditions

3.7.2 IMViC test

The following table shows the result for IMViC test

Indole Test	Methyl Red Test	Voges Proskauer Test	Citrate Test
+	+	+	-

Fig. 9: Indole Test

Fig. 10: Methyl Red Test

Fig 11: Voges Proskauer Test

Fig. 12: Citrate Test

3.7.3 Catalase

The test was found to be positive. (Fig. 13)

Fig. 13: Catalase Test

3.7.4 Starch hydrolysis

The test was found to be positive. (Fig. 14)

Fig. 14: Starch Hydrolysis

From the obtained biochemical results, it can be tentatively concluded that the organism belongs to *Bacillus species* and further it has to be confirmed by 16s rRNA method.

3.8 From the results obtained for media optimization for the selected isolate it was found that the bacterium showed optimum growth when dextrose was used as carbon source, yeast extract was used as nitrogen source, optimum pH was to be 8 and temperature was 37ºC.

3.8.1 Carbon source optimization

Carbon source	O.D at 660 nm
Dextrose	0.8
Sucrose	0.08
Lactose	0.19

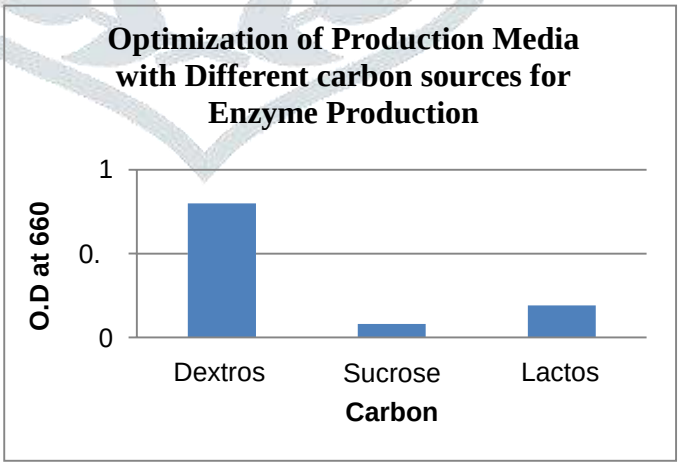

Figure 15: Optimization of production media for various Carbon sources

3.8.2 Nitrogen source optimization

Nitrogen source	O.D at 660 nm
Yeast extract	0.84
Tryptone	0.38
meet extract	0.54
Peptone	0.35

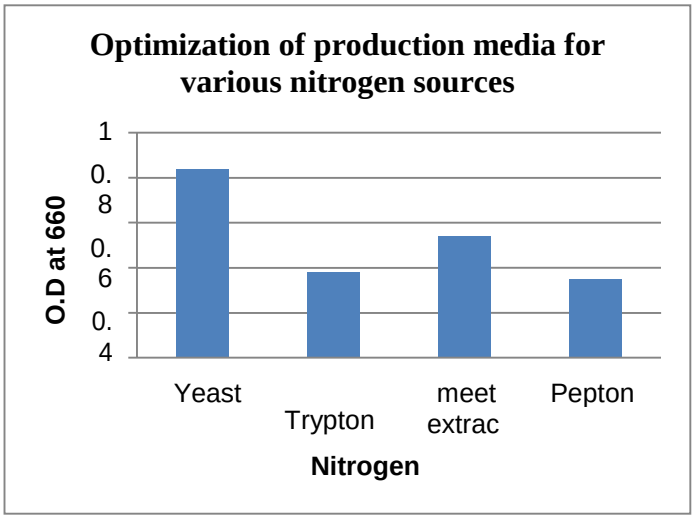

Figure 16: Optimization of production media for various nitrogen sources

3.8.3 pH optimization

pH	O.D at 660 nm.
6	0.36
7	0.43
8	0.9
9	0.79

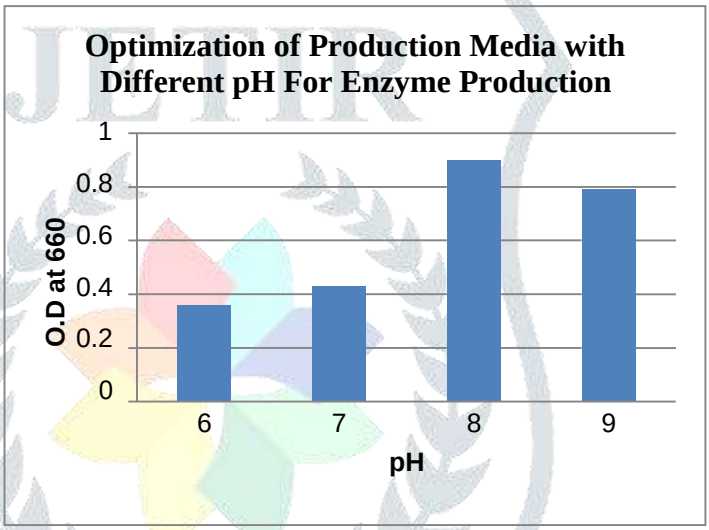

Figure 17: Optimization of production media for various pH

3.8.4 Temperature optimization

Temperature	O.D at 660 nm.
25	0.73
37	0.86
40	0.82

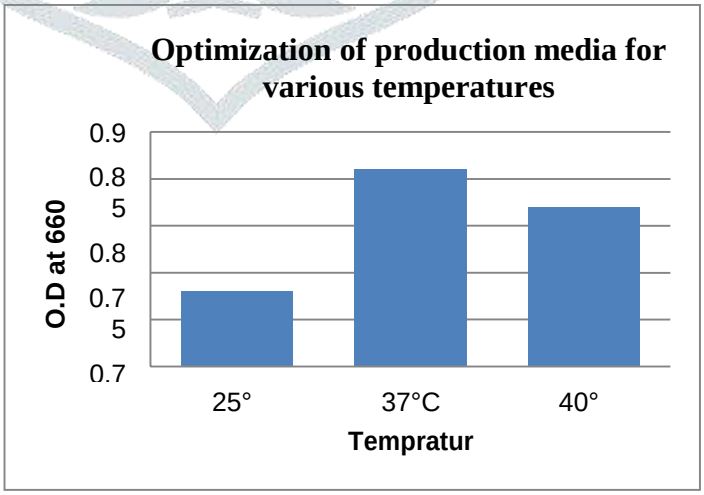

Fig 18: Optimization of production media for various Temperatures.

3.9 After optimization, the mass production was carried in optimized media, at 37°C for 48 hours at a pH of 8.0 (Fig. 19)

Fig19: Mass production of culture.

3.10 After 48 hours of incubation the culture was centrifuged and crude extract of the enzyme was obtained. The harvested protease product was treated with 50 % and 80 % ammonium sulphate for purification by salt precipitation method (Fig 20).

Fig. 20 : 50 % and 80 % ammonium sulphate for purification by salt precipitation method

3.11 Then the culture was purified by dialysis (Fig 21).

Fig. 21: Dialysis

3.12 Then the total protein content of both crude and ammonium sulfate precipitated samples were determined by Lowry's method. By the Lowry's estimation, the standardization was carried out using gradient concentrations of BSA. (Fig. 22) A graph was plotted with BSA Concentration on the x-axis and Absorbance at 660nm on the y-axis (Fig. 23) Thus, a standard graph for Lowry's estimation was obtained. With the help of the standard graph, the total protein content in each fraction was calculated.

Fig. 22: Protein estimation by Lowry's method

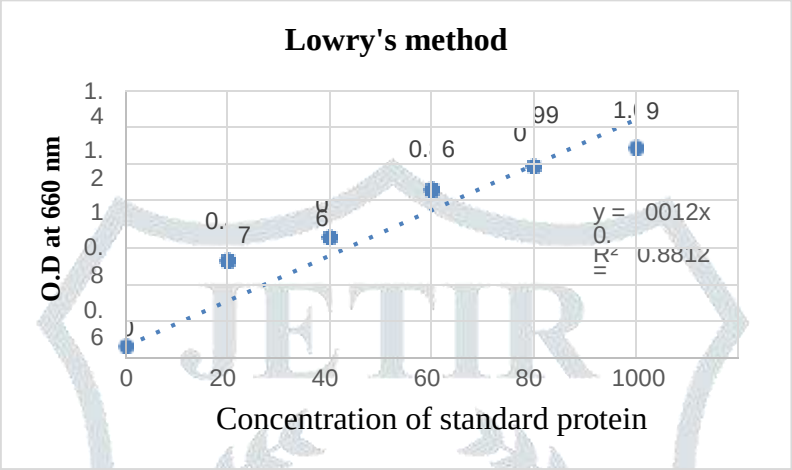

Fig.23: Graphical representation of Protein estimation by Lowry's method

The total protein content of samples is tabulated as follows:

SAMPLE	CONCENTRATION OF PROTEIN (µg/ml)
Crude extract	450
Ammonium sulphate precipitated (50%)	141.67
Ammonium sulphate precipitated (80%)	158.33
Dialysed sample (50%)	1008.33

3.13 Also, the specific activity of the crude extract and 50% dialysed samples was calculated using casein digestion assay for protease (Fig. 24). A graph was plotted with Tyrosine concentration on the x-axis and Absorbance at 660nm on the y-axis (Fig. 25) Thus, a standard graph for Protease activity by casein digestion assay was obtained. With the help of the standard graph, the protease activity was calculated.

Fig.24: Protease activity by casein digestion assay

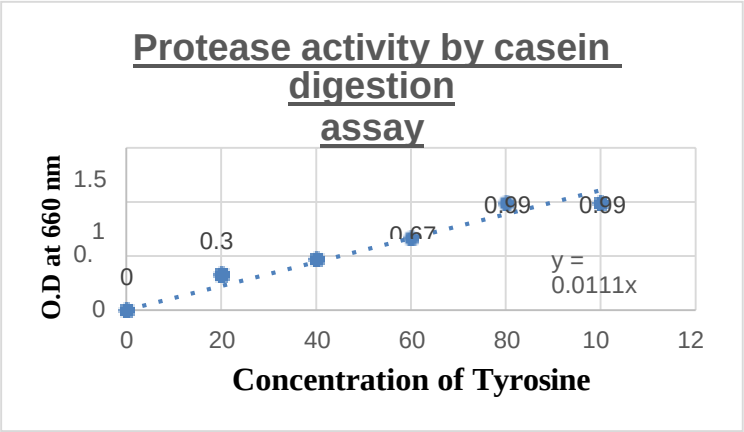

Fig.25: Graphical representation of Protease activity by casein digestion assay

The total protein content of samples is tabulated as follows:

SAMPLE	Protease activity (U/ml)
Crude extract	100.90
Dialysed sample (50%)	22.52

Specific activity (U/ml) = $\frac{\text{Protease activity (U/ml)}}{\text{Protease content (mg/ml)}}$

Steps	**Concentration of protein (**µg/ml)	**Protease activity (**U/ml**)**	**Specific activity (**U/ µg)
Crude	1.00	100.9	100.9
Dialysed sample (50%)	1008.33	22.52	0.022

3.14 Demonstration of the protein on 12% SDS-PAGE stained by Silver staining was carried out. The molecular weight of the protein was determined by running an SDS PAGE along with the standard protein marker (Lane 1)and was stained by silver nitrate staining method to visualize the protein bands develop. The crude sample (Lane 2) showed different bands of molecular weight 27KDa, 35KDa, 51KDa, 91KDa, 137KDa and 180KDa respectively, whereas two bands were obtained from the 50% Dialysed sample (Lane 3) one of molecular weight of 37KDa and the other between 137KDa and 180KDa on comparing with the standard protein marker. Generally, proteases are of the molecular weight between 30 to 80KDa. The band between 137 to 180 KDa could be of some other protein in the sample.

But further the protein can further be purified and characterized by performing dialysis, ion exchange chromatography, MALDI-TOF and protein sequencing.

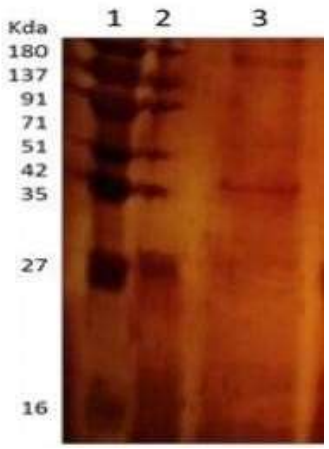

Fig.26. Siler stained 12% SDS PAGE showing the different bands of proteins
(Lane 1: standard protein marker, Lane 2: Crude sample, Lane 3: 50% Dialysed sample)

IV. CONCLUSION

From the present study it can be concluded that protease producing organisms was isolated from the soil sample and the isolated organisms was found to belong to bacillus species based on its grams morphology and biochemical characteristics but this identification can be further confirmed by molecular characterization using 16s rRNA method. The organism thus obtained was grown on culture media for the mass production of protease and the protease enzyme was extracted by centrifugation of the cultured broth.

The crude extract thus obtained was partially purified by ammonium sulphate precipitation and its molecular weight was found to be 37KDa.

This protein was further purified by dialysis and can be purified using ion exchange chromatography and further characterized using MALDI-TOF and protein sequencing.

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