WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 5.045
Volume 3, Issue 10, 1008-1018. Research Article ISSN 2277– 7105

ISOLATION AND IDENTIFICATION OF PROTEASE FROM *BACILLUS* SPECIES USING SDS-PAGE

Huda Jameel Makki[1], Ganesh Dama[2], Prasad M.P[3], Balasubramanian Sathyamurthy[4]*

[1]Department of Biochemistry, Bangalore City College, Bangalore – 560043.

[2]Department of Applied Genetics, Indian Academy Degree College, Centre for Research and Post Graduate Studies, Bangalore – 560043, India.

[3]Director, Sangenomics Research Lab Pvt Ltd, Bangalore.

[4]Associate Professor, Department of Biochemistry, Reva University, India.

Article Received on
26 September 2014,

Revised on 21 Oct 2014,
Accepted on 14 Nov 2014

***Correspondence for Author**
Balasubramanian Sathyamurthy
Associate Professor, Department of Biochemistry, Reva University, India.

ABSTRACT

This investigation was carried out to isolate and identification of protease producing bacteria from different sources like soil, water and fruit samples. The colonies producing protease were identified as *Bacillus* strains by standard microbiological techniques. The *Bacillus* strains were grown on nutrient broth for the isolation of protein. The isolated proteins were precipitated using ammonium sulphate. The proteins got precipitated at 50% and 60% saturation of ammonium sulphate. Eluted proteins were then identified using SDS-PAGE with standard protein marker. SDS-PAGE results shown, with 50% ammonium sulphate precipitation showed high amount of proteins with multiple bandings with different molecular weights and with 60% ammonium sulphate precipitation showed low amount of proteins as They had faint bands with multiple bandings pattern of different molecular weights.

KEYWORDS: *Bacillus*, Protease, SDS-PAGE, Proteins, Microbiological techniques.

INTRODUCTION

Bacillus is a rod-shaped, gram-positive bacterium that belongs to the family Bacillaceae. Bacillus consists of both non-pathogenic and pathogenic species. In certain conditions, Bacillus can produce oval endospores that are not actually true spores, but which the bacteria can reduce themselves to and remain in an inert state for very long periods. Alpha amylase used in the starch hydrolysis and protease subtilisin is used in detergents and the restriction

enzyme BamH1 is used in DNA research. Proteases from microbial sources are particularly important to the global nitrogen and carbon cycles in the recycling of proteins and such activity tends to be regulated by nutritional signals in these organisms [1]. Many species of Bacillus can produce large amounts of enzymes which are made use of in different industries. Proteases derived from the Bacillus species are widely used in the pharmaceutical and leather industry and also find application in the bioremediation processes [2-4]. Several species of Bacillus that are involved in the protease production are Bacillus Mojavensis, Bacillus Subtitles, Bacillus Cereus, Bacillus Sterothermophilus and Bacillus Megaterium [5-10]. Proteases are produced from high yielding bacterial strains including species of Pseudomonas fluorescens, Bacillus and Aeromonas hydrophilia grown under submerged culture conditions. Among this Bacillus is the most important group of bacteria that involved in the enzyme industry and this bacterium is also known for producing proteolytic enzymes quite effectively. Microbial enzymes are more preferred to those from both plant and animal sources because they are very cheaper to produce, and their enzyme contents are more stable. [11]

MATERIALS AND METHODS

Sample Collection

Strains of Bacillus spp, were isolated from different sources like soil, fruit and water samples.

Isolation of Bacillus

The samples were streaked on Nutrient agar plates and incubated at 37 ºC for 24 – 48 hours. After incubation the colonies isolated were characterized by morphology of the colonies and Gram's staining. The colonies which show similar characteristics to Bacillus species were isolated individually by quadrant streaking on nutrient agar plate. These cultures were then maintained in nutrient agar slants for further studies.

Staining Techniques & Biochemical Tests

Staining procedure makes differences between bacterial cells by imparting different colors to different bacteria are termed as staining techniques. Different staining techniques and biochemical tests like (Gram Stain, Bacterial spore staining, Acid-fast stain, Indole production test, Citrate utilization test, Methyl red test ,Voges – Proskauer test, Catalase test, Urease test, Casein hydrolysis, Glucose fermentation, Starch hydrolysis, Nitrate reduction test) have been performed for identification of *Bacillus spp.*, according to Bergey's Manual.

Purification of Intracellular Enzyme

Ammonium Sulphate Precipitation

The different steps of protein purification were carried out at 4ºC. The precipitation of the proteins was executed according to the chart of Gomori 1955. 30ml of the broth centrifuged at 10000rpm for 20minutes and the pellet was lysed using HEPES buffer. The samples were brought to 50% (w/v) saturation with solid ammonium sulphate and leave it for overnight at 4ºC. After ultracentrifugation at 10,000 rpm for 20 minutes the obtained precipitate was discarded and only the pellets was collected and dissolved it in 1ml of 0.1M TrisHcl buffer for further purification through dialysis.

Dialysis

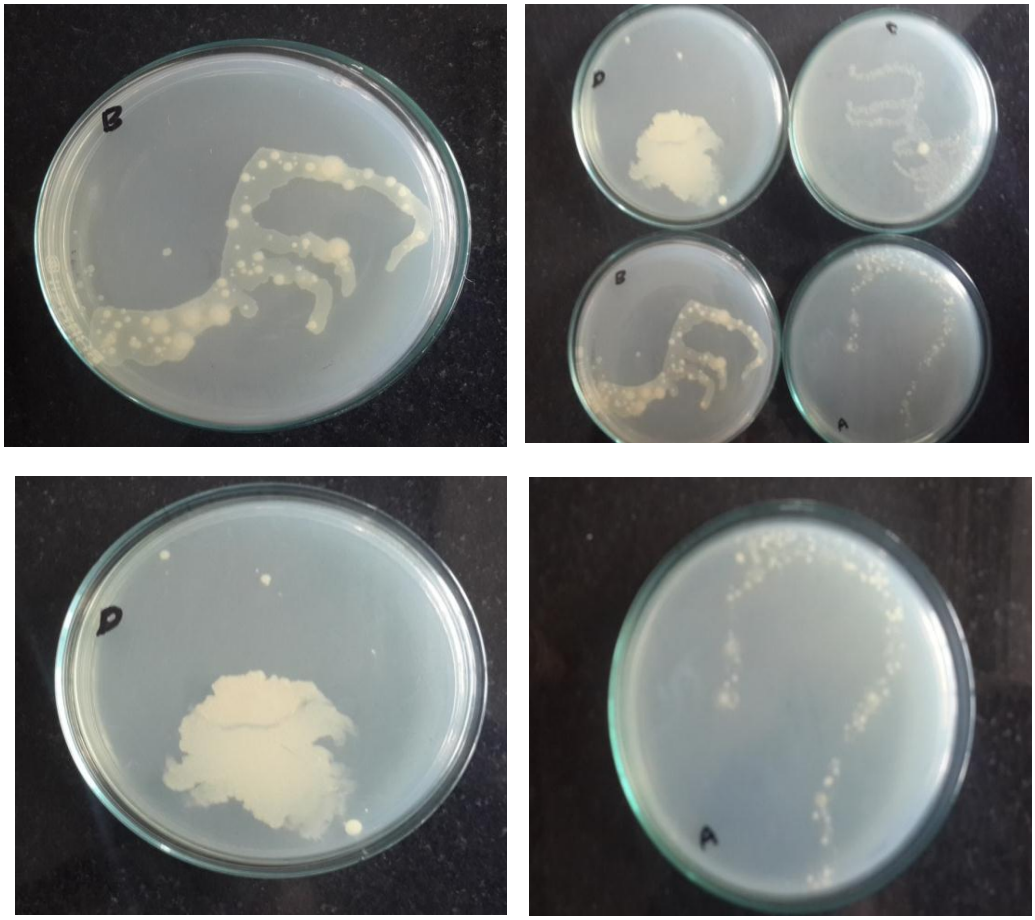
The resultant ammonium sulphate precipitate (in solution) was introduced to a special plastic bag called the dialysis tube. Dialysis was carried out to clear of all the traces of the ammonium sulphate. Then 1ml of the sample was loaded in the dialysis tube and was kept in an upside- down position in a 500 ml beaker containing. And it was kept on a magnetic stirrer for 24h at room temperature and after every 6h the Tris HCl buffer in the beaker was changed.

SDS PAGE of Protein Extract

One dimensional Sodium dodecyl sulfate polyacrylamide gel electrophoresis was carried out according to Laemmli (1970). Concentration of 10% separating gel and 5% stacking gel were prepared, in the eppendorf tube 35µl of each crude sample were mixed with 30µl of sample loading buffer. The samples which are containing with the loading buffer were heated in the dry bath at 95˚C for 5 minutes for denaturation. After denaturation, the denatured protein samples were centrifuged at 5000rpm for 5 minutes to precipitate out the debris. 35µl from each sample and 20µl of standard marker were loaded into the gel wells. According to the standard protocol samples were run at 120V with 25mA in 1X running buffer. When dye reaches the bottom of the gel, remove it out from the electrophoresis apparatus and the gel was fixed with 10% TCA. Protein bands were visualized by overnight staining with 0.25% CBB-250. Protein bands were observed after destaining the gel in the next day using destaining solution.

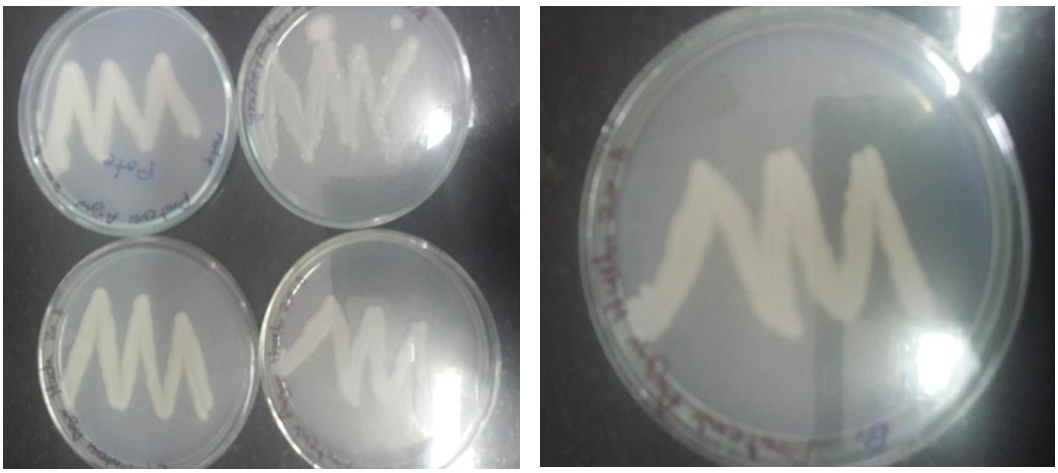
RESULTS AND DISCUSSION

Isolation of *Bacillus* from different samples: The microorganisms were isolated on Nutrient agar medium from different sources like soil and fruits.



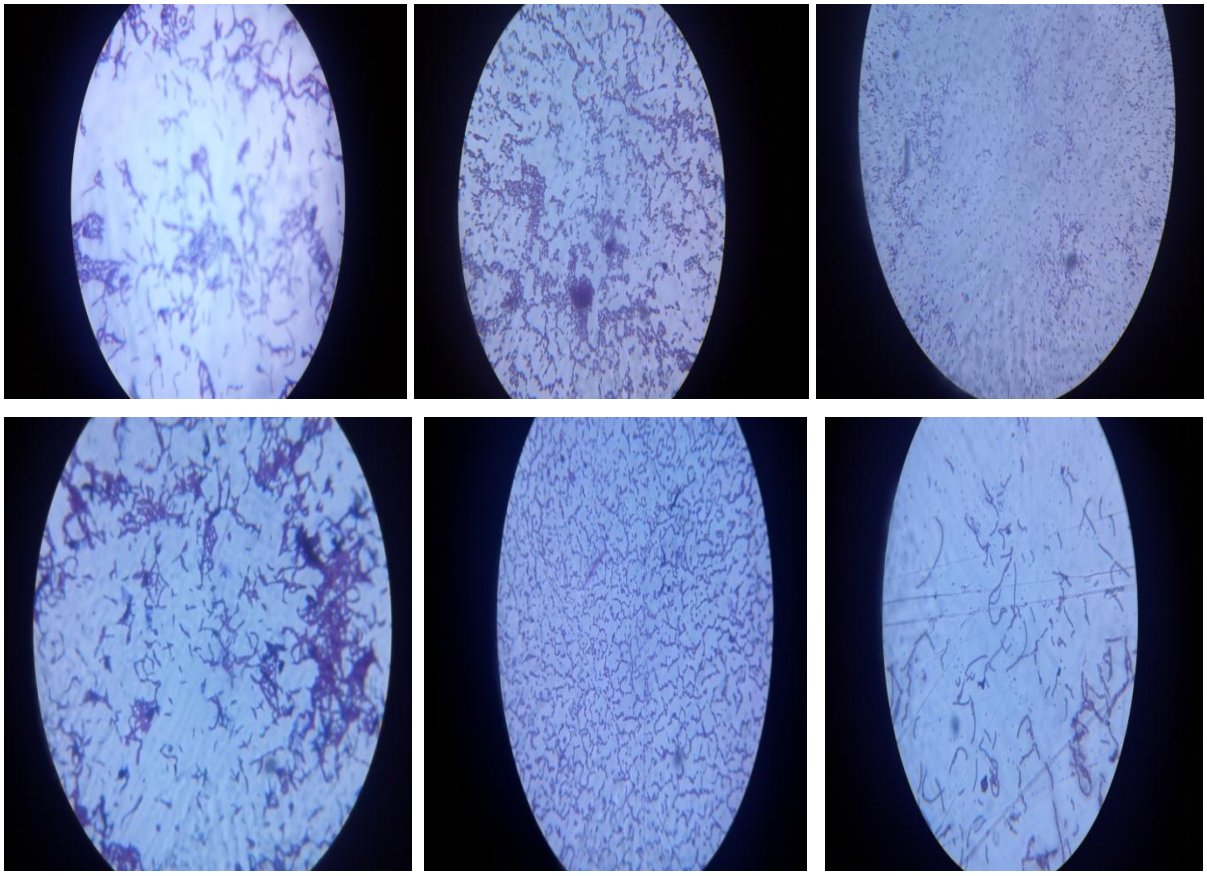
Screening for Protease production

The microorganisms isolated were individually screened for the production of protease by plating them on protease containing medium where they formed zone of clearance around the colonies. The colonies producing protease were identified by standard microbiological techniques. The *Bacillus spp*., which produced protease were found from 4 different soil sources and grape waste.



Identification of microorganisms

The microorganisms producing protease were usually found to be *Bacillus spp.*, the identification was done by the standard microbiological techniques (Gram's staining and Biochemical tests).



Gram Staining

The Gram staining results showed that all the selected colonies were gram positive rods.

Colony Characteristics

A

Dilution	Colony	Form	Size (cm)	Elevation	Margin	Pigment	Gram staining		Endospore staining
							+Ve/-Ve	Rodes/Cocci	
10^{-3}	1	Irregular	1	Flat	Ridges	Creamish white	+Ve	Both	+Ve
	2	Irregular	0.6	Slightly elevated	Ridges	Creamish white	-Ve	Rodes	+Ve
	3	Irregular	0.4	Slightly elevated	Ridges	Creamish white	+Ve	Rodes	-Ve
	4	Irregular	0.2	Flat	Ridges	Yellow	-Ve	Both	-Ve
	5	Irregular	0.2	Flat	Ridges	Creamish white	+Ve	Rodes	+Ve
	6	Irregular	0.2	Slightly	Ridges	Pale	-Ve	Both	-Ve

				elevated		yellow			
	7	Irregular	0.4	Flat	Ridges	Creamish white	+Ve	Rodes	+Ve
	8	Irregular	0.1	Flat	Ridges	Creamish white	+Ve	Rodes	-Ve
	9	Irregular	0.8	Flat	Ridges	Yellow	+Ve	Both	-Ve
10^{-5}	1	Irregular	0.7	Flat	Ridges	Creamish white	+Ve	Rodes	+Ve
	2	Irregular	0.5	Flat	Ridges	Pale yellow	+Ve	Rodes	+Ve
	3	Irregular	0.5	Flat	Ridges	Creamish white	+Ve	Rodes	+Ve
	4	Irregular	0.2	Slightly elevated	Ridges	Yellow	+Ve	Both	-Ve
	5	Irregular	0.1	Flat	Ridges	Creamish white	+Ve	Rodes	+Ve

B

Dilution	Colony	Form	Size (cm)	Elevation	Margin	Pigment	Gram staining		Endospore staining
							+Ve/-Ve	Rodes/cocci	
10^{-2}	1	Circle	0.5	Flat	Entire	Pale yellow	-Ve	Cocci	+Ve
	2	Irregular	0.3	Flat	Ridges	Creamish white	+Ve	Rodes and cocci	+Ve
	3	Irregular	1.5	Slightly elevated	Ridges	Creamish white	+Ve	Rodes	+Ve
	4	Irregular	0.5	Flat	Ridges	Creamish white	+Ve	Rodes and cocci	-Ve
	5	Irregular	0.2	Slightly elevated	Ridges	Pale yellow	+Ve	Cocci	+Ve
	6	Irregular	0.3	Flat	Ridges	Creamish white	-Ve	Rodes	+Ve
10^{-4}	1	Irregular	0.5	Flat	Ridges	Creamish white	-Ve	Rodes	+Ve
	2	Circle	0.1	Flat	Entire	Creamish white	+Ve	Rodes	-Ve

C

Dilution	Colony	Form	Size (cm)	Elevation	Margin	Pigment	Gram staining		Endospore staining
							+Ve/-Ve	Rodes/cocci	
10^{-2}	1	Circle	0.4	Flat	Entire	Creamish white	+Ve	Rodes	+Ve
	2	Irregular	0.8	Flat	Ridges	Creamish white	+Ve	Rodes	+Ve
	3	Irregular	0.6	Flat	Ridges	Creamish white	+Ve	Rodes	+Ve
	4	Irregular	0.4	Slightly elevated	Ridges	Yellow	+Ve	Rodes	-Ve

	5	Irregular	0.30	Flat	Ridges	Creamish white	-VE	Rodes and cocci	-Ve
	6	Irregular	0.6	Flat	Ridges	Creamish white	+Ve	Rodes	+Ve
	7	Irregular	0.3	Flat	Ridges	Translucent	-Ve	Rodes	-Ve
	8	Irregular	0.3	Flat	Ridges	Creamish white	+Ve	Rodes and cocci	+Ve
10^{-4}	1	Circle	0.5	Flat	Entire	Creamish white	-Ve	Rodes and cocci	-Ve
	2	Irregular	0.2	Flat	Ridges	Translucent	+Ve	Rodes	+Ve
	3	Irregular	0.1	Elevated	Ridges	Yellow	+Ve	Rodes	+Ve

Biochemical Tests

Indole Production Test

Fig.1 shows absence of the cherry red ring formation indicated that all strains were negative for Indole test.

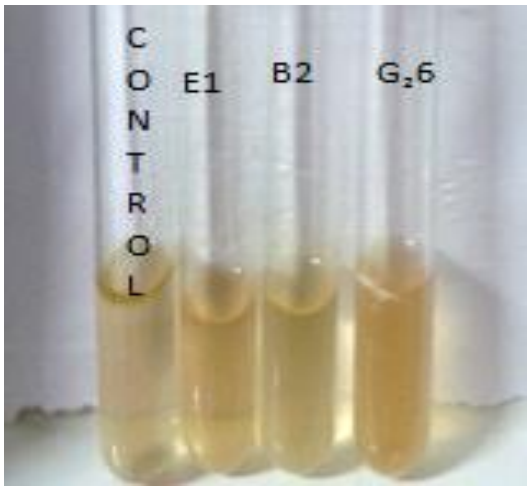


Fig.1 Indole Test.

Methyl Red Test- Fig.2 Shows development of red Colouration upon the addition of MR reagent indicated a positive test for all the strains.

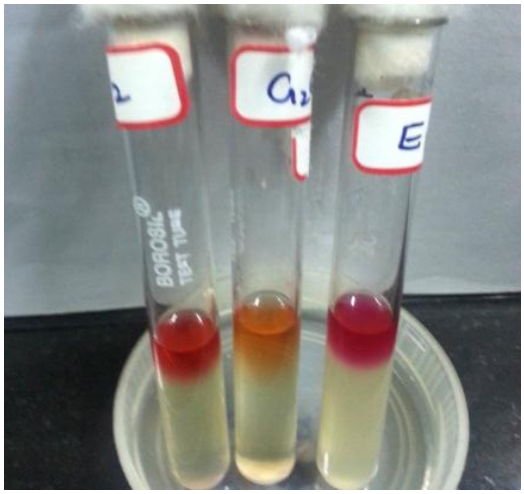


Fig.2 Methyl Red Test

Voges Proskauer Test- Fig.3 shows development of brown ring upon the addition of VP reagent indicated a positive test for all the strains.

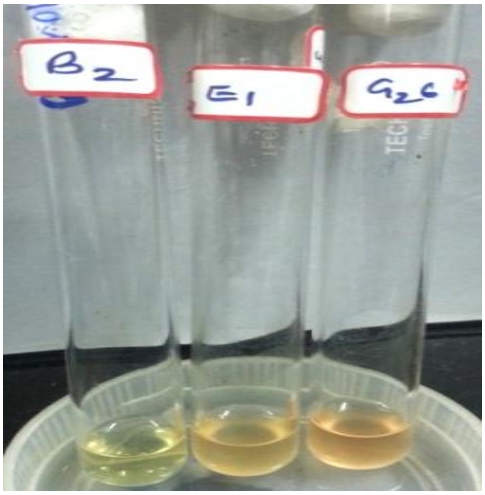


Fig.3 Voges Proskauer Test.

Citrate Utilization Test- Fig.4 all the strains showed negative result for citrate utilization test as the colour of the slants remained green after 48 hours of incubation.

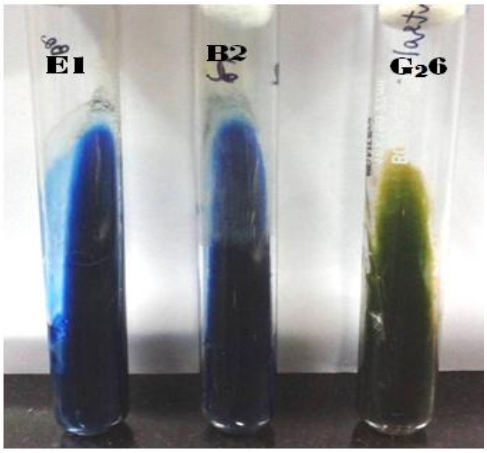
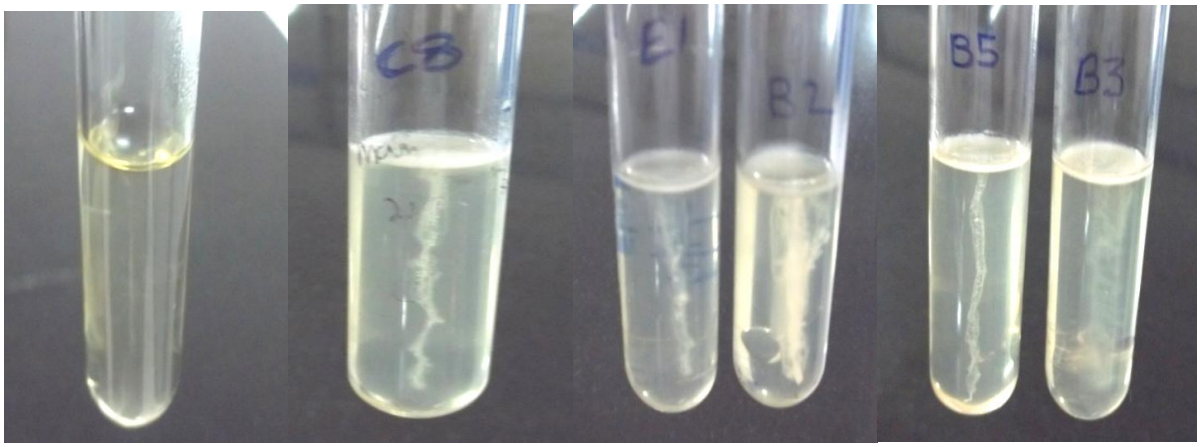


Fig.4 Citrate Utilization Test

Catalase Test- Fig.5 all samples showed positive result for catalase test.

Fig.5 Catalase Test.

Motility Test- Fig.6 all samples showed positive result for Motility Test.



Negative control **C8** **E1** **B2** **B5** **B3**

Fig.6 Motility Test.

Urease Test- All samples showed negative result for urease test.

Nitrate Reduction Test-All samples showed positive result for nitrate reduction test.

Starch Hydrolysis Test-All samples showed positive result for starch hydrolysis test.

Casein Hydrolysis Test-All samples showed negative result for casein hydrolysis test.

Tests Performed and Their Results (Table-1).

Test	Result
Catalase	-ve
TSIA	-ve
SCA	+ ve
Motility	+ ve
Indole production	-ve
Oxidase	+ ve
Starch hydrolysis	+ ve
Gas production	+ ve
MacConkey agar	Light pink
Eosin methylene blue	Colourless
Gram staining	+ ve
Shape	Rod
Spore formation	+ ve

Table.1. Shows +/- results of tests.

SDS-PAGE

The protein identification was carried out by SDS-PAGE. The precipitation for the proteins was done with 50% and 60% ammonium sulphate precipitation.

The SDS-PAGE with 50% ammonium sulphate precipitation showed high amount of proteins with multiple bandings with different molecular weights (Fig.7).

The SDS-PAGE with 60% ammonium sulphate precipitation showed low amount of proteins as they had faint bands with multiple bandings pattern of different molecular weights (Fig.8).

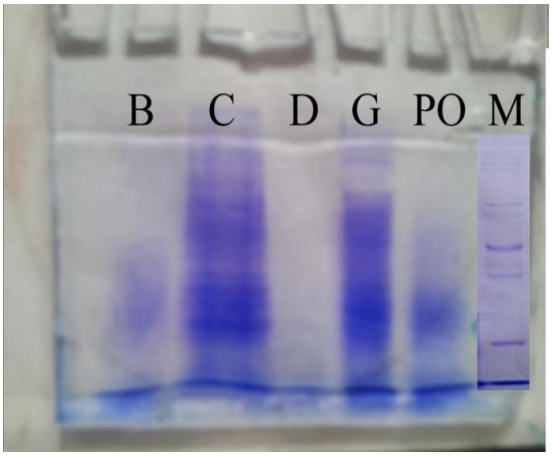


Fig.7 SDS Gel

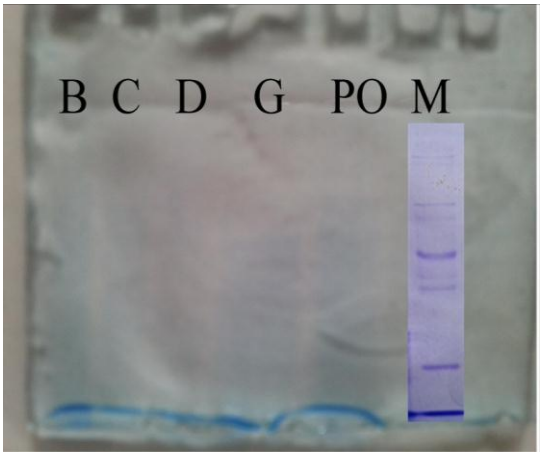


Fig.8 SDS Gel

CONCLUSION

Based on our results we conclude that, Protease producing *Bacillus* strains were isolated from the different sources like soil and fruit samples. From the strains proteins were extracted and precipitated. SDS-PAGE results shown, with 50% ammonium sulphate precipitation showed high amount of proteins with multiple bandings with different molecular weights and with 60% showed low amount of proteins as they had faint bands with multiple bandings pattern of different molecular weights. Proteases of commercial importance are produced from different sources, but proteases derived from *Bacillus* strains are widely used in the leather industry and pharmaceutical industry.

ACKNOWLEDGEMENTS

I would like to thank Department of Life Sciences, REVA University, Bangalore and Sangenomics Research lab Pvt Ltd, Bangalore for their continuous support and encouragement throughout this research work.

REFERENCES

1. Sims G K, Nitrogen Starvation Promotes Biodegradation of N-Heterocyclic Compounds in Soil. Soil Biology & Biochemistry, 2006; 38: 2478-2480.
2. Anwar A, and Saleemuddin, M. Alkaline proteases. A Review. Bioresource Technology, 1998; 6:175- 183.

3. Gupta R, Beg Q.K, Lorenz P. Bacterial alkaline proteases: molecular approaches and industrial applications. App. Microbial. Biotech, 2002; 59:15-32.
4. Wang HY, Liu DM, Liu Y, Cheng CF, Ma QY, Huang Q and Zhang YZ. Screening and mutagenesis of a novel *Bacillus pumilus* strain producing alkaline protease for dehairing. Lett. Appl. Microbial. 2006; 44: 1–6.
5. Ammar MS, El-Louboudy SS, Abdulraouf UM. Protease (s) from *Bacillus anthracis* S-44 and B. cereus var. mycoids, S-98 isolated from a temple and slaughter house in Aswan city. Az. J. Microbial, 1991; 13: 12-29.
6. Sookkheo B, Sinchaikul S, Phutrakul S, Chen ST. Purification and characterization of the highly thermostable proteases from *Bacillus stearothermophilus* TLS33. Prot.Exp. Pur, 2000; 20:142- 151.
7. Banik RM, Prakash M. Laundery detergent compatibility of the alkaline protease from Bacillus cereus. Microbial. Res, 2004; 159(2):135-140.
8. Beg QK, Gupta R. Purification and characterization of an oxidation-stable, thiol-dependent serine alkaline protease from Bacillus mojavensis. Enz. Microbial Techn, 2003; 32: 294-304.
9. Gerze A, Omay D,Guvenilir Y. Partial purification and characterization of protease enzyme from *Bacillus subtilis* and Bacillus megatherium. Appl Biochem Biotechnol, 2005; 121-124:335-45.
10. Soares VF, Castilho LR, Bon EP, Freire DM. High-yield *Bacillus subtilis* protease production by solid-state fermentation. Appl Biochem Biotechnol, 2005; 121-124: 311-9.
11. Burhan A, Nisa U, Gokhan C, Ashabil A, Osmair G. Enzymatic Properties of a novel thermostable ther-mophilic alkaline and chelator resistant amylase from an al-kaphilic *Bacillus sp* Isolate ANT-6. Process Biochemistry, 2003; 38: 1397–1403.