4

Results and Discussion

Microorganisms are the most significant sources for intracellular and extracellular enzyme production on an industrial scale and serve as a preferred source because of their fast growth and limited space required for their cultivation. For the elevated output of desirable enzymes, choice of the appropriate organism is very important. Many of the microorganisms produce more than one kind of protease enzyme. It is mostly produced from different bacteria and are also acknowledged as industrially important and occupies about 60% of the total enzyme sale (Asha and Palaniswamy, 2018). Culture conditions play an important role on the growth and production of protease by bacteria. As compared to plants and animals, microorganisms represent an attractive source of protease as they can be cultured in large quantities in a relatively short time by established fermentation methods and also produce an abundant, regular supply of the desired product. Microbial enzymes have a longer shelf life and can be stored under less than ideal conditions for weeks without significant loss of activity. Microbial proteases are generally extracellular in nature and they are directly secreted into fermentation broth by the producer, thus compared to proteases obtained from plants and animals, microbial proteases simplify the downstream process (Sharma *et al.,* 2014).

Enzymes are considered as 'Green Chemicals' and their role in industries is well established (Rai and Mukherjee, 2010). Proteases are one of the commercially important groups of extracellular microbial enzymes widely used in several industries such as detergent, textile, food, leather, chemical and silk. Inspite of its demand, microbial protease production is limited due to the need of cost-effective substrates (Maruthiah *et al.,* 2015).

Bacillus species are specific producers of extracellular protease. A number of proteases have already been isolated, purified and characterized from several *Bacillus* strains due to their wide temperature, pH tolerance and stability (Benkiar *et al.,* 2013).

Bacillus subtilis is one of the most widely used bacteria for the production of industrially important protease enzyme. These are more specific producers of extracellular proteases that can be cultivated under extreme temperature and pH conditions to give rise to products which are stable in a wide range of harsh environments (Padmapriya and Williams, 2012).

The demand for highly active preparations of proteases with appropriate specificity and stability over a wide range of pH, temperature and retention of activity in the presence of ions, surfactants and organic solvents continues to stimulate the search for new enzyme sources (Doddapaneni *et al.,* 2009). Newer enzymes with novel properties which can further enhance industrial processes that employ the current enzymes is always in demand.

Keeping these aspects in view, the present study entitled **'Characterization, Immobilization and Applications of Extracellular Protease from *Bacillus* sp. ASASBT isolated from Termite Soil'** has been carried out with the following objectives:

- Isolation and screening of protease-producing bacteria from different environmental soil samples
- Optimization of media components for the production of extracellular protease
- Purification and characterization of the isolated protease
- Immobilization studies of purified protease
- Application studies of protease

The experiment was designed in six phases:

Phase I consisted of isolation of bacteria from different environmental soil samples and screening them for the presence of enzymes. **Phase II** involved the identification of protease producing bacteria and optimization of media components for maximum protease production. **Phase III** comprised of the purification and molecular characterization of protease. **Phase IV** outlines the biochemical characterization of protease. **Phase V** consisted of the immobilization of protease and **Phase VI** involved the various applications of the purified, immobilized and commercial protease.

The findings of the study are discussed under the following headings:

4.1 Phase I – Isolation of bacteria from different environmental soil samples and screening for enzymes

4.1.1 Isolation of bacteria

4.1.2 Screening for enzymes

4.1.3 Assay of enzymes

4.2 Phase II – Identification of selected protease-producing bacteria and optimization of media components for protease production

4.2.1 Identification of protease-producing bacteria

4.2.1.1 Morphological and biochemical characterization of the selected bacterial isolate TMS1

4.2.1.2 Scanning Electron Microscopy (SEM) analysis of the selected bacterial isolate TMS1

4.2.1.3 16S rRNA gene sequencing analysis of the selected bacterial isolate TMS1

4.2.1.4 Identification of protease gene

4.2.2 Optimization of media components for protease production

4.2.2.1 Qualitative method

4.2.2.2 Quantitative method

4.2.2.3 Effect of incubation time on protease production

4.2.2.4 Effect of pH on protease production

4.2.2.5 Effect of temperature on protease production

4.2.2.6 Effect of inoculum size on protease production

4.2.2.7 Effect of carbon sources on protease production

4.2.2.8 Effect of nitrogen sources on protease production

4.2.2.9 Effect of agitation speed on protease production

4.2.2.10 Effect of natural substrates on protease production

4.3 Phase III – Purification and molecular characterization of protease

4.3.1 Purification of protease

4.3.1.1 Precipitation with ammonium sulphate

4.3.1.2 Dialysis

4.3.1.3 Ion exchange chromatography using AKTA FPLC system

4.3.1.4 Gel filtration chromatography using AKTA FPLC system

4.3.1.5 Summary of purification profile of protease at various stages of purification

4.3.2 Molecular weight of purified protease

4.3.2.1 SDS polyacrylamide gel electrophoretogram

4.3.2.2 Confirmation of protease activity by zymography

4.3.2.3 Identification of protein by MALDI-TOF/TOF analysis

4.4 Phase IV – Biochemical characterization of purified protease

4.4.1 Effect of pH on activity and stability of purified protease

4.4.2 Effect of temperature on the activity and stability of purified protease

4.4.3 Effect of substrate concentration on the activity of purified protease

4.4.4 Effect of metal ions on purified protease activity

4.4.5 Effect of inhibitors on purified protease activity

4.4.6 Effect of surfactants and oxidizing agents on purified protease activity

4.4.7 Effect of organic solvents on purified protease activity

4.4.8 UV-visible spectrum of purified and commercial protease

4.4.9 Fourier Transform Infrared (FTIR) spectroscopy of purified and commercial protease

4.5 Phase V – Immobilization of purified protease

4.5.1 Determination of immobilized protease activity

4.5.2 Characterization of immobilized protease

4.5.2.1 Effect of pH on activity of immobilized protease

4.5.2.2 Effect of temperature on activity of immobilized protease

4.5.2.3 Storage stability of immobilized protease

4.5.2.4 Reusability of immobilized protease

4.5.2.5 Surface morphology of immobilized protease

4.6 Phase VI – Applications of protease

4.6.1. Applications in detergent industry

4.6.1.1. Action of protease on stains

4.6.1.2. Preparation of enzyme-based detergent cake

4.6.2. Applications in leather industry

4.6.2.1. Action of protease on animal skin

4.6.3. Applications in pharmaceutical industry

4.6.3.1. Antibacterial activity of protease

4.6.3.2. Clot lysis activity of protease

4.6.4. Applications in photographic industry

4.6.4.1. Silver recovery from waste x-ray film

4.6.5. Applications in textile industry

4.6.5.1. Activity of protease on degumming of silk

PHASE - I

4.1 Phase I - Isolation of bacteria from different environmental soil samples and screening for enzymes

Bacteria from different sources, namely, termite mound soil (TMS), organic waste degraded soil (OWS), textile effluent degraded soil (TES) and marine soil (MRS) samples were isolated, screened and assayed for the presence of the enzymes amylase, cellulase, protease and lipase.

4.1.1 Isolation of bacteria

Bacteria isolated by Serial Dilution method from the four soil samples (TMS, OWS, TES and MRS) produced morphologically distinct colonies as shown in Plate 1.

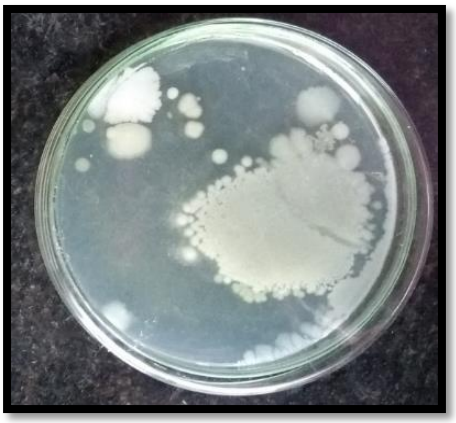
Plate 1

Colonies produced by selected soil samples

A – Termite Mound Soil (TMS)

B – Organic Waste degraded Soil (OWS)

C – Textile Effluent degraded Soil (TES)



D – Marine Soil (MRS)

From this, each colony was taken and further isolated and named accordingly. A total of 45 bacterial colonies were obtained. They were designated from TMS1 to TMS16 for Termite Mound Soil samples, OWS1 to OWS9 for Organic Waste degraded Soil, TES1 to TES13 for Textile Effluent degraded Soil and MRS1 to MRS7 for Marine Soil.

Table 3 shows the naming of the above.

Therefore, a total of 45 bacterial colonies were isolated from four different soil samples.

4.1.2 Screening for enzymes

The developed 24-hour old single colonies were picked up and streaked on sterile nutrient agar plates. They were further purified by quaternary streaking and then screened for the presence of enzymes - amylase, cellulase, protease and lipase by observing the zone of clearance. The results of this are shown in Table 3.

The table clearly reveals that, from the **45** bacterial isolates screened, **5** isolates showed the presence of **amylase** (TMS2, OWS4, TES9, TES10, TES11), **12 cellulase** (TMS1, TMS2, TMS13, TMS14, OWS2, OWS3, OWS7, TES6, TES7, TES9, TES11, MRS7), **14 protease** (TMS1, TMS4, TMS9, TMS11, TMS15, OWS2, OWS5, OWS9, TES2, TES5, TES10, TES12, MRS2, MRS6) and **10 lipase** (TMS3, TMS9, TMS10, OWS6, OWS7, OWS8, TES3, TES8, MRS5, MRS6). It is also clear that TMS showed maximum number of enzyme secreting colonies (16 numbers) followed by TES (13 numbers).

It can be inferred that, of the 45 bacterial isolates screened, 5 isolates showed the presence of amylase, 12 cellulase, 14 protease and 10 lipase. The presence of amylase was more in TES samples as compared to the other samples. Similarly, presence of cellulase was more in TMS and TES samples. Protease and lipase were also present more in TMS samples as compared to the other samples. It can thus be inferred that TMS was the best source of amylase, cellulase, protease and lipase.

Isolation and screening of enzymes from various soil samples were also reported by several other research workers. A total of seventy bacterial isolates were isolated from termite mound soil, out of these twenty-four isolates showed proteolytic activity (Devi and Thakur, 2018). Selvam *et al.,* (2011) reported that fifty-six marine actinomycetes were isolated from marine sediments and of these, three strains quantified as effective producers of cellulase, amylase and lipase. According to Duza and Mastan, (2013) also, a total of seventeen bacterial strains were isolated from different soil samples of Andhra Pradesh and out of these, two isolates were found to have lipase producing activity, two cellulase producing activity, eight protease producing activity and nine amylase producing activity.

Another report by Rupali, (2015) mentions the screening and isolation of protease-producing bacteria from soil samples collected from different areas such as farm soil, garden soil and oil spilled area of Burhanpur. Similarly, Narendra *et al.,* (2012) reported that twenty-five microorganisms were isolated from various fields near to Ravulapalem village, East Godavari District, Andhra Pradesh in which five isolates were protease positive. Thus, they suggested that Indian soil is best for the protease-producing bacteria. Another report states that a total of fifty-three bacterial isolates from different areas of Karachi were isolated, out of which twenty-five isolates were alkaline protease producers (Siddalingeshwara *et al.,* 2010).

4.1.3 Assay for enzymes

The levels of various enzymes secreted by the isolates are recorded in Table 3.

Table 3

Screening and assay of enzymes present in bacteria isolated from termite mound, organic waste degraded, textile effluent degraded and marine soil samples from various places of Tamil Nadu

S.No.	Sample Code	Amylase		Cellulase		Protease		Lipase	
		Screening	Assay (U/ml)	Screening	Assay (U/ml)	Screening	Assay (U/ml)	Screening	Assay (U/ml)
Termite Mound Soil (TMS)									
1	TMS1	-	64.97	+++	207.64	+++	258.36	+	95.31
2	TMS2	+++	106.31	+++	143.28	-	45.62	+	69.93
3	TMS3	-	58.16	+	74.25	-	03.97	+++	143.20
4	TMS4	-	23.75	-	08.69	+++	114.82	++	136.38
5	TMS5	-	37.59	-	29.34	-	31.76	++	121.57
6	TMS6	++	93.44	++	98.42	-	07.44	-	28.18
7	TMS7	-	12.38	-	69.16	-	63.96	-	16.25
8	TMS8	+	83.94	+	82.45	-	14.73	-	45.43

S.No.	Sample Code	Amylase		Cellulase		Protease		Lipase	
		Screening	Assay (U/ml)	Screening	Assay (U/ml)	Screening	Assay (U/ml)	Screening	Assay (U/ml)
9	TMS9	-	35.48	-	01.63	+++	198.19	+++	182.64
10	TMS10	-	19.23	-	75.05	-	36.46	+++	177.79
11	TMS11	+	81.72	-	33.53	+++	174.58	-	20.86
12	TMS12	-	2.09	-	18.80	-	27.63	-	41.91
13	TMS13	-	64.72	+++	128.00	-	10.29	++	118.40
14	TMS14	++	99.27	+++	118.28	-	53.61	++	105.55
15	TMS15	-	49.56	-	01.60	+++	190.75	-	36.04
16	TMS16	+	76.84	-	08.50	-	01.56	-	22.32
Organic Waste degraded Soil (OWS)									
17	OWS1	-	28.96	-	23.05	-	1.96	-	16.74
18	OWS2	-	5.38	+++	164.87	+++	176.53	-	27.31
19	OWS3	-	57.13	+++	119.50	-	34.93	-	41.17
20	OWS4	+++	149.47	++	81.92	-	12.86	++	105.95
21	OWS5	+	86.21	+	66.83	+++	143.72	+	83.82
22	OWS6	-	12.97	-	18.64	+	88.26	+++	116.09
23	OWS7	-	32.64	+++	185.27	-	26.37	+++	162.27
24	OWS8	++	127.48	++	95.04	-	9.42	+++	175.38
25	OWS9	-	35.74	-	37.16	+++	159.63	-	33.46
Textile Effluent degraded Soil (TES)									
26	TES1	++	87.07	++	88.34	-	73.83	-	22.67
27	TES2	-	50.67	-	17.90	+++	204.98	-	03.51
28	TES3	++	96.32	-	52.09	+	62.45	+++	146.23
29	TES4	-	23.08	-	0.12	-	02.78	-	09.75

S.No.	Sample Code	Amylase		Cellulase		Protease		Lipase	
		Screening	Assay (U/ml)	Screening	Assay (U/ml)	Screening	Assay (U/ml)	Screening	Assay (U/ml)
30	TES5	-	48.61	-	10.68	+++	166.39	++	95.08
31	TES6	-	65.24	+++	107.67	-	15.61	-	10.83
32	TES7	+	78.13	+++	128.25	-	11.29	+	85.15
33	TES8	-	14.67	+	74.32	-	68.37	+++	174.18
34	TES9	+++	108.86	+++	186.29	-	39.24	-	13.42
35	TES10	+++	210.89	-	1.37	+++	150.27	-	01.69
36	TES11	+++	157.42	+++	206.04	-	21.88	-	0.92
37	TES12	+	75.35	++	81.57	+++	163.71	-	26.78
38	TES13	-	19.26	-	43.39	-	09.67	-	02.14
Marine Soil (MRS)									
39	MRS1	+	71.85	-	0.68	+	81.32	++	116.73
40	MRS2	++	100.39	-	10.54	+++	183.92	-	67.12
41	MRS3	-	14.67	+	86.79	-	54.30	-	18.24
42	MRS4	+	66.08	++	99.12	-	45.52	+	76.17
43	MRS5	-	42.51	-	24.45	-	35.76	+++	152.39
44	MRS6	+	61.28	+	78.09	+++	208.78	+++	136.04
45	MRS7	-	35.12	+++	109.28	-	25.06	-	13.89

+ (Zone of hydrolysis), ++ (Minimum hydrolysis), +++ (Maximum hydrolysis), - (No hydrolysis zone)

From the table, it is obvious that amylase was produced maximally by TES10 bacteria (210.89 U/ml) followed by TES11 (157.42 U/ml). TMS1 bacteria recorded the highest amount of cellulase (207.64 U/ml) followed by TES11 (206.04 U/ml). Protease was found to be secreted maximally by TMS1 bacteria (258.36 U/ml) followed by MRS6 bacteria (208.78 U/ml). Lipase was maximally produced by TMS9 bacteria (182.64 U/ml) followed by TMS10 bacteria (177.79 U/ml).

Proteases are the most studied microbial enzymes at the industrial, commercial, pharmaceutical analytical and diagnostic levels.

Hence, among the 14 isolates which secreted protease in high amounts, TMS1 was selected for further studies since it showed the highest protease activity.

The presence of enzymes in soil samples as observed in the present study is supported by similar studies. Selvam *et al.,* (2011) screened and quantified the industrial enzymes amylase, cellulase and lipase. A serine type of protease-producing *Bacillus* sp. was isolated from marine soil samples and its extracellular enzyme production properties studied by Devi *et al.,* (2013) and Mohapatra *et al.,* (2003). It revealed that this strain produces more than one extracellular enzyme such as protease, lipase, amylase and gelatinase depending upon the nutritional conditions the production of industrial enzymes like amylase, carboxymethylcellulase and protease. High activities of the three enzymes were recorded in bacterial strains belonging to the genera *Bacillus* and *Alcaligenes.* Another study by Tomova *et al.,* (2014), isolated 24 bacterial strains under aerobic conditions from terrestrial samples at two locations in maritime Antarctica. Among the isolates, producers of proteases, ureases, polygalacturonases, β-glucosidases, phytases and ribonucleases were detected. Yet another work by Vakilwala and Patel, (2017) reported that, a total of 14 bacterial colonies were isolated from soil of various regions of Udwada and all the 14 showed zone of clearance on casein agar plate which indicates their ability to produce protease enzyme.

Phase I - Highlights of the findings

- **All the soil samples (TMS, OWS, TES and MRS) showed the presence of amylase, cellulase, protease and lipase.**
- **Of the 45 isolates obtained, 14 showed good protease activity.**
- **TMS1 gave the highest protease activity and was thus selected for further studies in the following phase.**

Phase – II

4.2 Phase II - Identification of selected protease-producing bacteria and optimization of media components for protease production

Protease plays a significant role in industrial biotechnology. Microbes are the important source for enzyme production due to their rapid growth. Microbial enzymes have extensive applications in industries and medicine. Now-a-days industries are looking for new microbial strains in order to produce different enzymes to meet the current enzyme requirements (Hamza, 2017).

4.2.1 Identification of protease-producing bacteria

From Phase I, Termite Mound Soil (TMS1) which produced the highest amount of protease was selected and further sub-cultured.

Plate 2 showed the isolated bacterial colonies from TMS1. They were then identified based on morphological and biochemical characterization, confirmed by Scanning Electron Microscopy (SEM), 16S rRNA gene sequencing analysis and identification of protease gene.

Plate 2

Isolated colonies of TMS1 on nutrient agar plate by quadrant streak method

4.2.1.1 Morphological and biochemical characterization of the selected bacterial isolate TMS1

The results of the morphological and biochemical characteristics of the bacteria TMS1 isolated from termite soil are presented in Table 4.

Table 4

Morphological and biochemical characterization of the selected bacterial isolate TMS1

Description	Inference
Morphological characteristics	
Size	Moderate
Configuration	Circular
Appearance	Shiny
Elevation	Convex
Pigmentation	White
Margin	Entire
Texture	Rough
Optical property	Opaque
Gram staining	Positive rods
Endospore staining	Positive
Motility	Motile
Biochemical characteristics	
Indole Production Test	Negative
Methyl Red Test	Negative
Voges Proskauer Test	Positive
Citrate Utilization Test	Positive
Triple Sugar Iron Agar Test	Negative
Urease Test	Positive
Catalase Test	Positive
Oxidase Test	Negative
Starch Hydrolysis Test	Positive
Nitrate Reduction Test	Positive
Gelatin Liquefaction Test	Positive

The data reveals that the isolate showed moderate sized white, circular shiny, rough opaque and convex colonies at the end of 48 hours incubation. They were also found to be gram-positive, rod-shaped, motile and spore producing. The bacterial isolate was positive for Voges Proskauer, citrate utilization, urease, catalase, starch hydrolysis, nitrate reduction and gelatin liquefaction tests. However, Indole production, methyl red, triple sugar iron agar and oxidase tests were negative.

Thus, considering the morphological and biochemical characteristics observed, it can be said that the phenotypic characteristic of the selected strain TMS1 belongs to the genus *Bacillus* sp. according to the Bergey's manual of systematic classification.

The ability of *Bacillus* sp. isolated from soil samples to secrete protease in the present study is in accordance with several other studies. A number of protease-producing bacterial strains were isolated from soil samples collected from in and around Hyderabad, India and among the different strains, *Bacillus* sp. was isolated and selected for further studies because it showed to be the best producer of extracellular protease (Doddapaneni *et al.,* 2009). Similar findings were reported by Mazer *et al.,* (2012) also, who identified *Bacillus subtilis* BP-36 based on morphological and biochemical characteristics. Another study based on the reports given by Rathod and Pathak (2014), mentioned that protease-producing isolates (LW8) had morphological, microscopic, biochemical and physiological attributes and antibiotic sensitivity was similar to that of *Bacillus*, hence the microorganism isolated was found to be *Bacillus alcalophilus.*

4.2.1.2 Scanning Electron Microscopy (SEM) analysis of the selected bacterial isolate TMS1

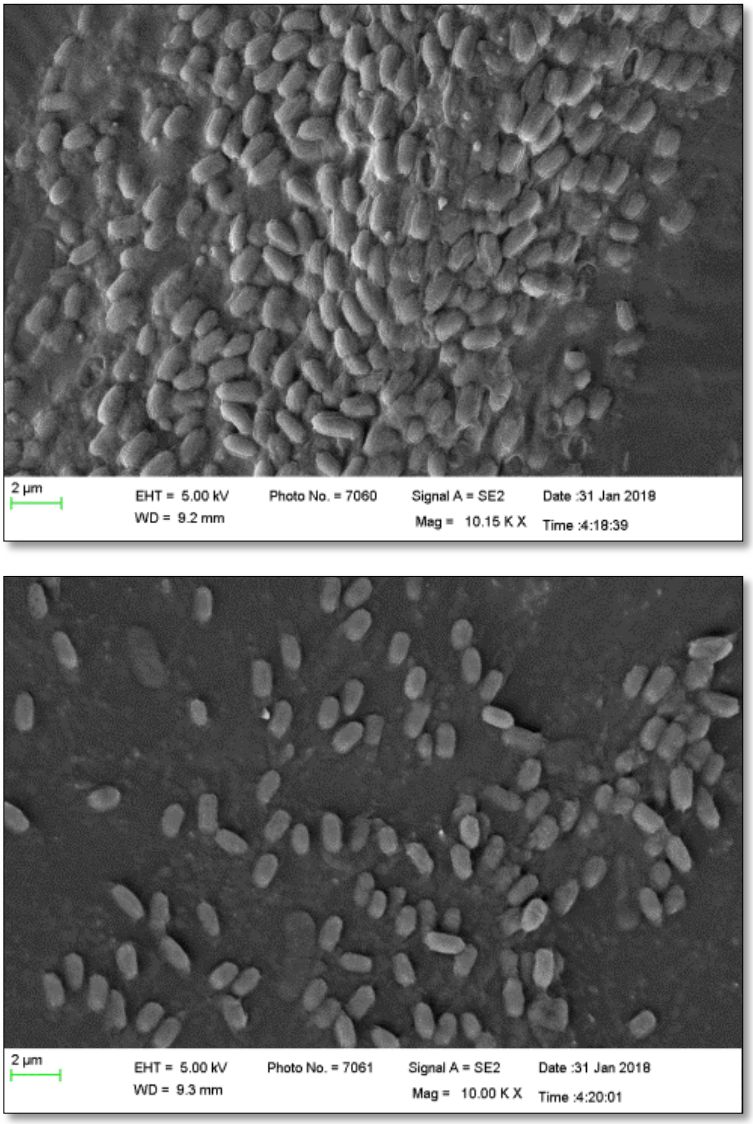
Scanning electron microscopy was done in order to observe the particular shape of the single cell of bacterium.

Plate 3 shows the scanning electron micrograph (10000X magnification) of the selected bacterial isolate TMS1 isolated from termite mound soil.

It is clear from the picture that the cells were single, rod shaped bacteria.

Plate 3

Scanning electron micrograph of the selected bacterial isolate TMS1



The results of the above SEM studies are supported by several similar studies. Devi *et al.*, (2013) reported that the SEM results of the protease-producing microbial strain isolated from marine soil samples, revealed long rod-shaped morphology which belonged to the *Bacillus* sp. According to Manivasagan *et al.,* (2013) also, *Streptomyces* sp. MAB18 isolated from marine sediments of Cuddalore coast, showed a scanning electron microscopic view where the aerial mycelium was grey, spiral sporophores with loops and hooks and oblong conidia with smooth surface. Another study indicated that, scanning electron microscopic view of *Bacillus* sp. NPST-AK15 showed motile, short rods, with

lengths of about 1.5 µm and 0.5 µm in diameter and the cells existing as single, paired or short chains (Ibrahim *et al.*, 2015).

4.2.1.3 16S rRNA gene sequencing analysis of the selected bacterial isolate TMS1

The DNA from the selected bacterial isolate TMS1 was isolated and the 16S rRNA region was amplified using the primers 8F and 1541R. The amplified product was eluted using Bioserve Gel Elution Kit and sequenced using ABI3130 capillary electrophoresis. The obtained 16S rRNA sequences of bacterial isolate were aligned using Chromas software.

Figure 6 represents the final aligned sequence of the bacterial isolate TMS1. The obtained nucleotide sequence of the bacterial isolate was submitted to the NCBI GenBank database and the assigned accession number is **MF618325.**

Figure 6

16S rRNA sequence of the selected bacterial isolate TMS1


>contig
TCATCTGTCCACACCATTCGGCGGCTAGAGACTACCTAAAAGGTTACCTCACCGACTT
CGGGTGTTACAAACTCTCGTGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTAT
TCACCGCGGCATGCTGATCCGCGATTACTAGCGATTCCAGCTTCACGCAGTCGAGTTG
CAGACTGCGATCCGAACTGAGAACAGATTTGTGGGATTGGCTTAACCTCGCGGTTTCG
CTGCCCTTTGTTCTGTCCATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGAT
GATTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCACCTTAGAGTGCCC
AACTGAATGCTGGCAACTAAGATCAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACA
TCTCACGACACGAGCTGACGACAACCATGCACCACCTGTCACTCTGCCCCCGAAGGGG
ACGTCCTATCTCTAGGATTGTCAGAGGATGTCAAGACCTGGAAAGGTTCTTCGCGTTG
CTTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCCGTCAATTCCTTTGAGT
TTCAGTCTTGCGACCGTACTCCCCAGGCGGAGTGCTTAATGCGTTAGCTGCAGCACTA
AGGGGCGGAAAACCCCCTAACACTTAGCACTCATCGTTTACGGCGTGGACCTACCAGG
GTATCTAATCCTGTTCGCTCCCCACGCTTTCGCTCCTCAGCGCAGTTAAAAAACAAGAA
AGTCGCCTTCGCCACTGGTGTCCTCCAAATCTTCTAACGCATTTTCACCGCTAACACGT
GGAATTCCACTCTCCTCTTTCTGCACTCAGTTCCCCAGTTTCCAATGACCCCTCCCGGT
TTGGAGCGGGGGTTTTCCACATCAGACTTAAAGAAACCCGCCTGGCGAGCCCTTTACG
CCCAATAAATTTCCAGAACACGCTTTGCCACCTACGATTTACGCGGCTGCTGGCACGT
GTTTAGCCGTGGGCTTTTCGTGGTAAGTACGGTCAGTACCGCCCTTAATTCGAACGGT
ACTTTGTTTCTTTCCTAAACAACAAGAGCTTATTACGTATCGAAAACCTTCATTCACTC

The final aligned 16S rRNA sequence was compared with the 16S rRNA sequences present in the NCBI database using BLAST to further identify the species of the organism. The homologous search indicated that the strain shared 94% identity with

Bacillus subtilis, which is shown in Figure 7. The neighbor joining phylogenetic tree was constructed using the type of *Bacillus* sp. strains and given in Figure 8.

The BLAST search based on the 16S rRNA gene sequence analysis and Neighbor-Joining method, phylogenetic tree using similar type strains revealed that the isolated bacterial strain TMS1 shared high similarity with *Bacillus subtilis.*

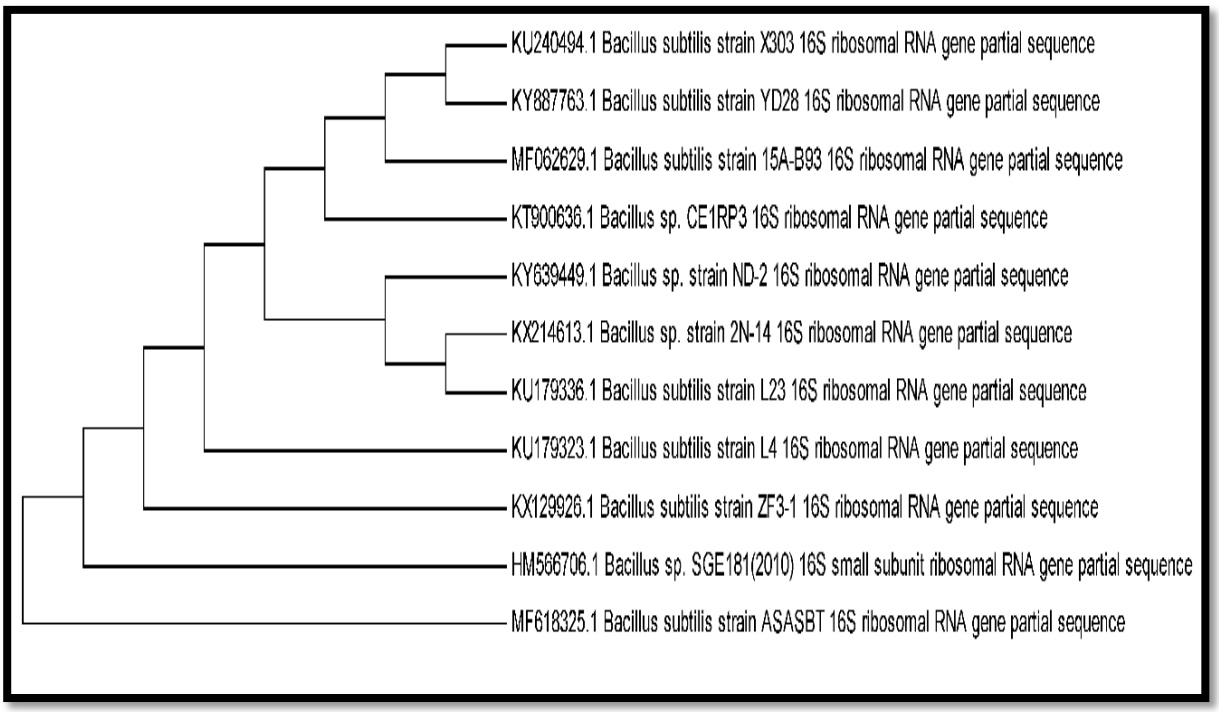
Figure 7

BLAST output from rRNA database of NCBI for the query 16S rRNA sequence of the isolate TMS1

Description	Max score	Total score	Query cover	E value	Ident	Accession
Bacillus sp. 13900 16S ribosomal RNA gene, partial sequence	1672	1672	100%	0.0	94%	JN874767.1
Bacillus subtilis strain CAF3 16S ribosomal RNA gene, partial sequence	1668	1668	100%	0.0	94%	KY806228.1
Bacillus subtilis strain 55C1-1 16S ribosomal RNA gene, partial sequence	1666	1666	100%	0.0	94%	JN366797.1
Bacillus sp. N14(2011) 16S ribosomal RNA gene, partial sequence	1666	1666	100%	0.0	94%	HQ601003.1
Bacillus sp. M1(2010) strain M1 16S ribosomal RNA gene, partial sequence	1666	1666	100%	0.0	94%	GQ340518.1
Bacillus subtilis 16S ribosomal RNA gene, partial sequence	1666	1666	100%	0.0	94%	DQ187378.1
Bacillus sonorensis strain T1Ni 16S ribosomal RNA gene, partial sequence	1664	1664	99%	0.0	94%	KY419149.1
Bacillus subtilis subsp. inaquosorum 16S ribosomal RNA gene, partial sequence	1664	1664	100%	0.0	94%	KP126518.1
Bacillus subtilis subsp. natto strain CGMCC 2108, complete genome	1663	16518	100%	0.0	94%	CP014471.1
Bacillus sp. XY5 16S ribosomal RNA gene, partial sequence	1663	1663	99%	0.0	94%	KF986307.1
Bacillus subtilis subsp. natto BEST195 DNA, complete genome	1663	16518	100%	0.0	94%	AP011541.2
Bacillus subtilis strain 30L1-3 16S ribosomal RNA gene, partial sequence	1663	1663	100%	0.0	94%	JN366796.1
Bacillus subtilis strain 30L2-1 16S ribosomal RNA gene, partial sequence	1663	1663	100%	0.0	94%	JN366756.1
Bacillus tequilensis strain YC5-2 16S ribosomal RNA gene, partial sequence	1663	1663	100%	0.0	94%	HM770882.1
Bacillus sp. M95(2010) strain M95 16S ribosomal RNA gene, partial sequence	1663	1663	100%	0.0	94%	GQ340487.1
Bacillus sp. M67(2010) strain M67 16S ribosomal RNA gene, partial sequence	1663	1663	100%	0.0	94%	GQ340486.1
Bacillus sp. TT46(2010) strain TT46 16S ribosomal RNA gene, partial sequence	1663	1663	100%	0.0	94%	GQ340471.1
Bacillus subtilis strain CW14, complete genome	1661	16612	100%	0.0	94%	CP016767.1

Figure 8

Neighbor-Joining tree showing the phylogenetic position of the isolate TMS1



According to Bozoglu *et al.,* (2013) and Rajeswari and Bhuvaneswari, (2016) the bacterial strains belonging to the same genus which exhibit less than 97% 16S rRNA gene sequence similarities should be considered as members of different species and that 16S rRNA gene sequence analysis was inadequate in discriminating the closely related species.

In the present study, the isolate TMS1 exhibited 94% similarity with the species *Bacillus subtilis.* Therefore, 16S rRNA gene sequence analysis and phylogenetic method were not sufficient to identify the isolated strain at species level. The present data should be supported by more advanced genomic analysis methods for the identification at the strain level.

Hence the isolate TMS1 was identified as *Bacillus* sp. and named as *Bacillus* sp. ASASBT.

The results obtained in the present study are in agreement with those of other research workers. Tambekar *et al.,* (2015), stated that the phylogenetic position of the

bacterial strain isolated from Lonar lake belongs to the genera *Bacillus* and may be *Bacillus flexus, Bacillus pseudofirmus* and *Bacillus cereus*. The results of Annamalai *et al.,* (2014); Uttatree and Charoenpanich, (2018), states that the 16S rRNA gene sequence and phylogenetic analysis of the strain CAS 5 and BUU1 isolated from marine wastes was closest to *Bacillus alveayuensis* and *Staphylococcus saprophyticus*. According to Ravi *et al.,* (2015) also, the 16S rRNA sequence of the bacterial strain JRK-3 isolated from soil sample exhibited 99% similarity with *Bacillus subtilis.*

4.2.1.4 Identification of protease gene

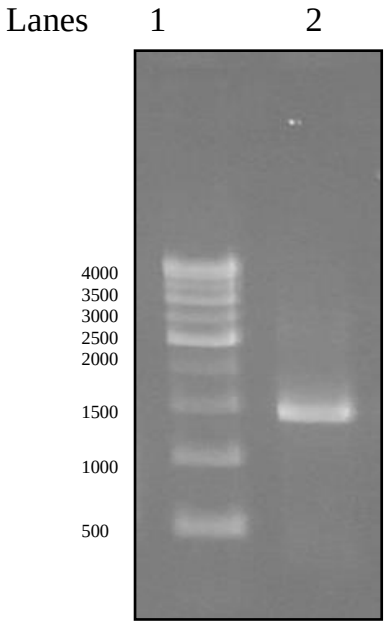
The genomic DNA extracted from the *Bacillus* sp. ASASBT is shown in Plate 4 (a). The extracted DNA was used as a template for the protease gene amplification. A pair of primers (Pro1 F: ATGTGCGTGAAAAAGAAAAATGTG; Pro1 R: TTAGTTAGAAGCTGCTTGAACGTT) for amplifying protease genes was designed based on the conserved nucleotide sequences and used to amplify a protease gene from *Bacillus* sp. ASASBT. Plate 4 (b) shows the amplified protease gene in 1% agarose gel

Plate 4 (a)

Genomic DNA of *Bacillus* sp. ASASBT loaded in 1% agarose gel

Plate 4 (b)

Amplified protease gene in 1% agarose gel



The size of PCR amplified product is ~1.2kb
Lane 1 : Mid Range DNA Ladder
Lane 2 : Amplified gene

From the Plate, it is clearly understood that the obtained PCR product showed approximately 1.2 kb, which was run on 1% agarose with Mid Range DNA ladder. This confirmed the presence of protease gene in the isolated *Bacillus* sp. ASASBT.

Thus, it is evident that, protease is present in the isolated *Bacillus* sp. ASASBT.

The above observations are in accordance with several other studies. Baweja *et al.,* (2016), reported that the amplified protease gene from *Bacillus pumilus* MP 27 was 1152 bp. According to Zilda *et al.*, (2018) also, for the protease gene amplified from BII-1 (*Bacillus subtilis)* using a designed degenerated primer, the sequence result showed that the amplified gene shared 99% and 100% similarity with those from *Bacillus subtilis* and *Geobacillus thermophiles* in the GenBank database. Similar work was also explained by Lv *et al.,* (2012).

4.2.2 Optimization of media components for protease production

Optimization is one of the most important criteria in developing a new microbial process. The various parameters influencing enzyme production during submerged fermentation were optimized. The aim was to optimize each parameter individually and subsequently combine the optimal conditions so as to obtain an increase in the yield of the enzyme. Enzymes are highly beneficial because they reduce activation energy of a reaction thereby making the reaction process more efficient with reduced energy consumption. By optimizing the parameters, it is possible to ensure more efficient functioning of enzymes (Parameswaran *et al.*, 2013).

The identified *Bacillus* sp., ASASBT was again screened qualitatively for the presence of protease and quantitatively for the production of protease using basal media and the media components were then optimized for maximum protease production.

4.2.2.1 Qualitative method

Plate 5 shows the zone of clearance of *Bacillus* sp. ASASBT on Skim milk agar and Caesin agar.

Plate 5

Zone of clearance of *Bacillus* sp. ASASBT on Skim milk agar and Caesin agar

a) Skim milk agar

b) Caesin agar

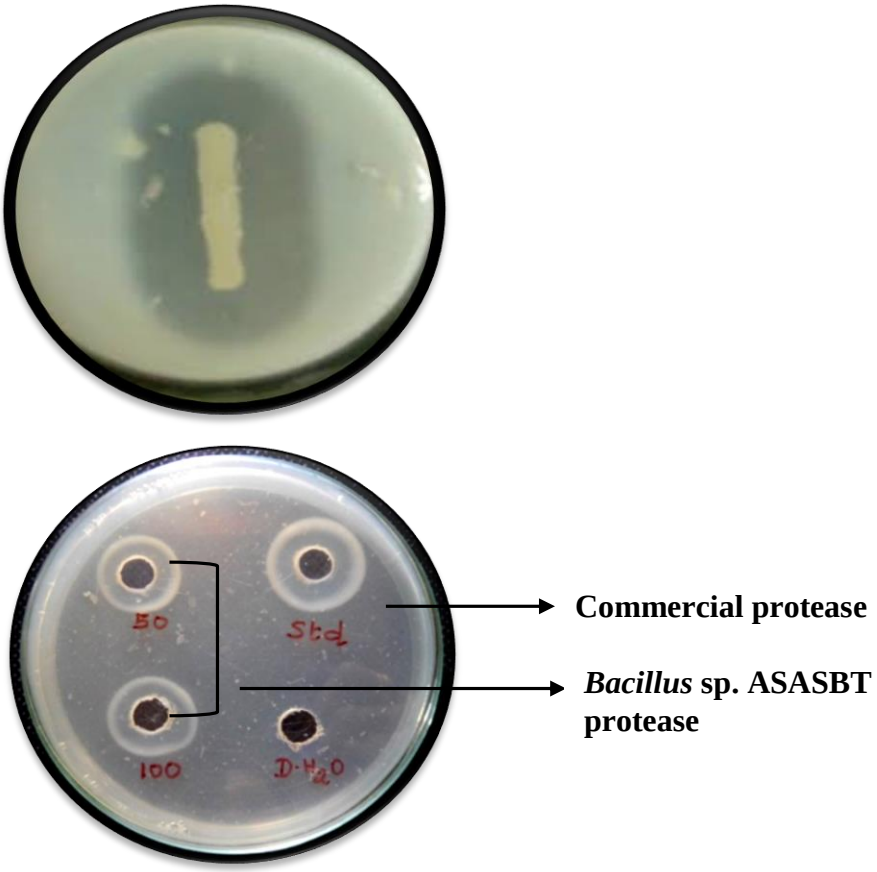


Plate 5 (a) shows that there was maximum zone of clearance around the single streak of *Bacillus* sp. ASASBT which was inoculated into skim milk agar medium at 7.0 pH. A similar trend was observed in casein agar medium at pH 7.0 also as shown in Plate 5 (b). Here, the selected isolate *Bacillus* sp. ASASBT when compared with commercial protease. At 100 µl concentration of the commercial protease the zone of clearance of 7 mm was obtained, whereas, in the selected isolate at 50 µl concentration 4 mm zone of clearance and at 100 µl concentration 6 mm was obtained. In the well with sterile distilled water which acted as a control, no zone of clearance occurred.

This confirms the presence of protease in the isolated bacterial strain *Bacillus* sp. ASASBT.

Several studies have been done for screening of new isolates for proteases production. The proteolytic activity of different *Bacillus* strains on skim milk agar and gelatin agar were also reported by Alnahdi, (2012). Ayaz, (2012) also demonstrated that a total of seven bacterial isolates were isolated from sea water and sediment in Jeddah, Saudi Arabia and all of them when screened and quantitatively assayed for the production of protease using gelatin agar media, seven of them proved to be the best producers of extracellular protease forming larger clear zones than all the others. Another researcher, Gupta *et al.,* (2005) isolated bacterial colonies from environmental samples and screened their ability to produce protease using skim milk agar and reported that the *Streptomycin* sp. CD3 produced protease to the maximum when compared to the other isolated strains. Yet another report given by Devi and Thakur, (2018) also described that the out of 24 bacterial isolates NTS 49, NTS 78 and BTS 17 from termite mound soil exhibited maximum proteolytic activity.

4.2.2.2 Quantitative methods

a) Biomass

The growth of *Bacillus* sp. ASASBT in basal medium is shown in Table 5.

It is obvious from the table that the exponential growth of cells at constant rate continued upto 48 hours, starting from 0.753 mg/ml at 0 hours to 1.464 mg/ml after 48 hours. The specific growth rate during exponential phase was observed to be 0.015 h^{-1} under optimal shaking. The maximum biomass concentration of 1.464 mg/ml was attained at 48 hours and then there was a gradual decrease in the biomass concentration as well as the specific growth rate from 72 hours onwards starting from 1.391 mg/ml to 1.186 mg/ml after 120 hours.

Thus at 48 hours of fermentation time, maximum biomass concentration was obtained.

Table 5

Biomass production of *Bacillus* sp. ASASBT

Fermentation Time (h)	Biomass Concentration/yield (mg/ml)
0	0.753
12	0.908
24	1.152
36	1.346
48	1.464
72	1.391
96	1.270
120	1.186

Values are the mean of triplicates

Similar findings as above were reported by Soundra *et al.*, (2012), who mentioned that a high level of extracellular thermostable activity of protease in *Bacillus* sp. was observed after 48 hours incubation. In the report given by Umayaparvathi *et al.*, (2013) also, the maximum biomass yield was recorded at 36 hours of incubation period of *Bacillus cereus* SU12.

b) Protease activity

The protease activity in the cell free culture supernatant was also determined the results of which are depicted in Figure 9.

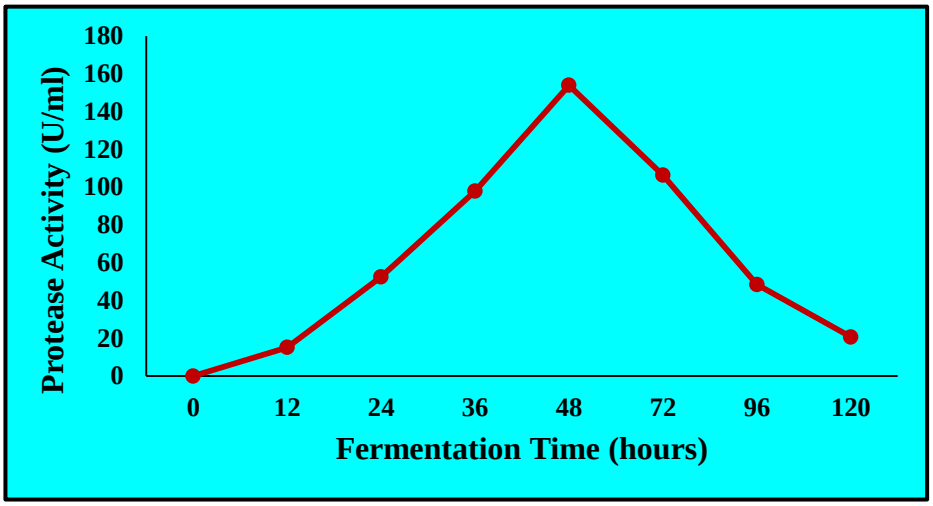
It can be observed from the figure that the enzyme secretion started during the early exponential phase of growth, but reached substantial levels only by the end of the exponential growth phase.

The activity increased from 0 hours to 48 hours, where it reached the maximum (153.89 U/ml) at 48 hours of incubation at 37°C. Further the activity started to decrease with increase in time period. A similar trend was seen for biomass production also.

Therefore, it can be stated that maximum protease activity is show at 48 hours of fermentation time. The results further suggest that improved levels of protease production could be possible with high active production of biomass.

Figure 9

Protease activity of *Bacillus* sp. ASASBT



Values are the mean of triplicates

Several investigations have been done for the screening of new isolates for protease production by other researchers. The use of skim milk agar for the isolation of protease producing bacteria has also been reported earlier by Boominadhan *et al.,* (2009) and Haile & Gessesse, (2012). These results were in concordance with the studies of Jayashree *et al.,* (2014) who showed that among the strains isolated from thorn forest soil samples from Tamil Nadu, the strain *Methylobacterium* sp MSF 46 exhibited a large zone of clearance around the well on gelatin agar indicating the extracellular protease activity in the hydrolysis of gelatin. The cell free supernatant also depicted significantly higher protease activity than other isolates. The report given by Mohsin *et al.,* (2011), stated that among a total of 118 bacterial isolates screened from soil, *Bacillus* sp. being predominant was used for protease production.

The different parameters optimized for maximal enzyme production is as follow;

4.2.2.3 Effect of incubation time on protease production

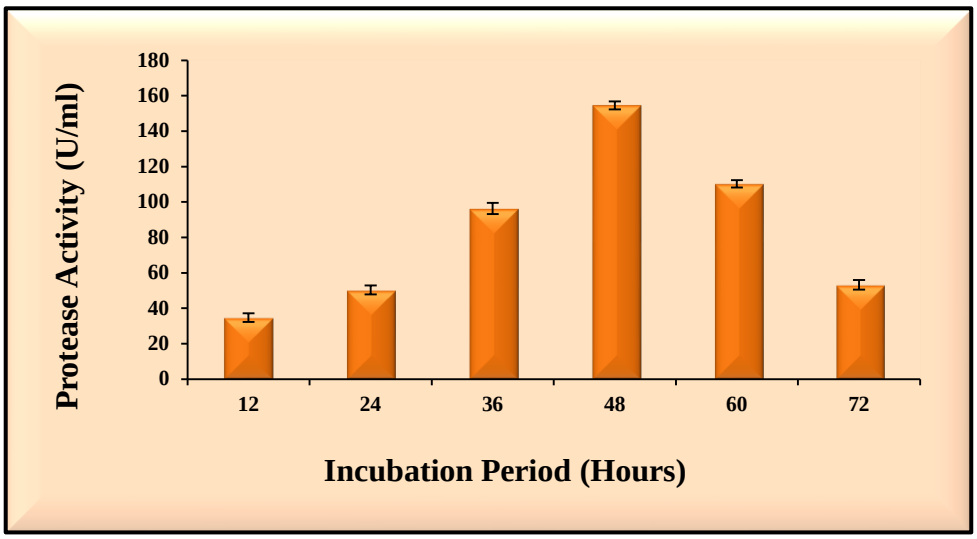
Study of different incubation time reveals when the selected organism is capable of producing maximum level of enzyme. The production of enzyme is dependent on incubation period and active growth of the bacterial strain. The bacterial culture was

incubated at 37°C for 72 hours in an orbital shaker with 150 rpm and analyzed for protease activity every 12 hours.

Figure 10 shows the effect of incubation period on protease production of *Bacillus* sp. ASASBT.

Figure 10

Effect of incubation period on protease production of *Bacillus* sp. ASASBT



Values are the mean ± SD of triplicates

It can be seen from the figure that there was a gradual increase in protease production from 0 to 48 hours, reaching a maximal activity of 154.54±2.33 U/ml. After this there was a decrease in activity with time upto 72 hours, when the activity reached 53.28±2.73 U/ml.

Thus, the maximum protease activity was observed at 48 hours of incubation. Hence, from these results, it can be suggested that prolonged incubation and static conditions reduce protease production.

The present results are in agreement with the studies of several others. Kaur *et al.,* (1998) stated that enzyme production was related to growth kinetics and therefore there is a correlation between incubation period and production of enzyme. According to Sai Smita *et al.,* (2012), during the early growth phase of 6 to 18 hours, protease production showed slower rate after which it increased sharply reaching a maximum value at 48 hours and further incubation resulted in a sharp decline of enzyme production. Similar findings

were also reported by some researchers, who mentioned that maximum enzyme production was observed at 48 hours of growth (Olajuyigbe and Ajele, 2005; Rathod and Pathak, 2014).

4.2.2.4 Effect of pH on protease production

Microorganisms are sensitive to modifications in hydrogen ion concentration. The medium pH is one of the most significant environmental factors affecting the growth of bacteria and many enzymatic processes and transportation of various components across the cell membrane. Gerald, (1975) described that the change in enzyme activity with varying pH levels is due to changes in ionization of the enzyme and the substrate or the enzyme-substrate complex. Therefore, to optimize the pH for better protease production, in the present study, growth media were taken at varying pH ranging from 5.0 to 10.0 and the enzyme activity studied.

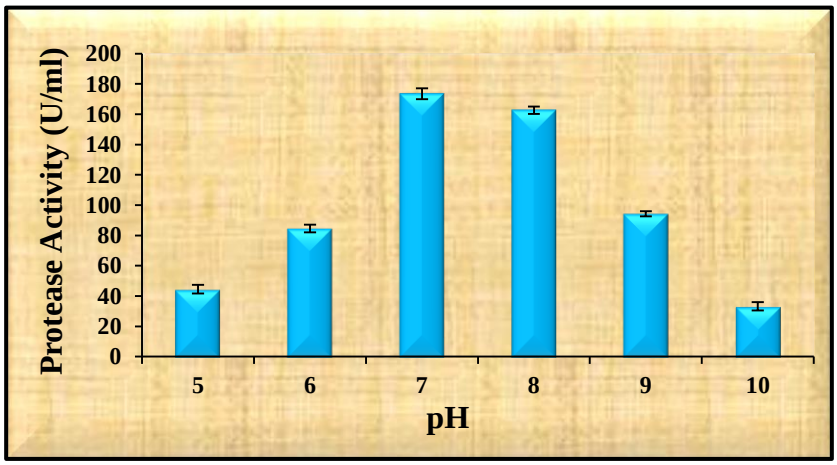
Figure 11 shows the effect of pH on protease production of *Bacillus* sp. ASASBT.

It is revealed from the figure that the maximum protease production (173.53±1.97 U/ml) was obtained at pH range 7.0 followed by pH 8.0 (162.62±2.39 U/ml) as shown in Figure 12. At pH 5.0, 6.0, 9.0 and 10.0, the production of protease by *Bacillus* sp. ASASBT declined upto 33.25±2.77 U/ml. This is based on the physiological character of the *Bacillus* sp. ASASBT in the culture medium.

Hence, at pH 7.0 the *Bacillus* sp. ASASBT showed highest protease production.

Figure 11

Effect of pH on protease production of *Bacillus* sp. ASASBT



Values are the mean ± SD of triplicates

These observations are in accordance with other similar studies. Kanekar *et al.,* (2002), Dorcas and Pindi, (2016), Vasantha and Subramanian (2012), Muthulakshmi *et al.,* (2011), El-Safey and Abdul-Raouf, (2004) reported the optimum pH for production of protease from *Bacillus cereus, Pseudomonas* sp., *Bacillus* sp. and *Bacillus subtilis* respectively to be 7.0. Jadhav *et al.,* (2014) and Prabhavathy *et al.,* (2012) also indicated that the optimum pH was found to be 7.5 for the maximum production of protease from *Bacillus subtilis.* The bacteria isolated from soil samples of Kohat District, Pakistan showed maximum protease activity at pH 9.0 (Shah *et al.,* 2014).

4.2.2.5 Effect of temperature on protease production

Temperature is also an important environmental parameter. In the fermentation process, it is a crucial parameter which has to be controlled. There is a linkage between enzyme synthesis and energy metabolism which is controlled by oxygen uptake and temperature. The enzyme activity of a microorganism is strongly influenced by the optimum temperature. It also regulates the extracellular and intracellular synthesis.

To optimize the temperature for higher protease production of *Bacillus* sp. ASASBT, the growth medium was incubated at various temperatures ranging from 20°C to 70°C and the protease activity observed.

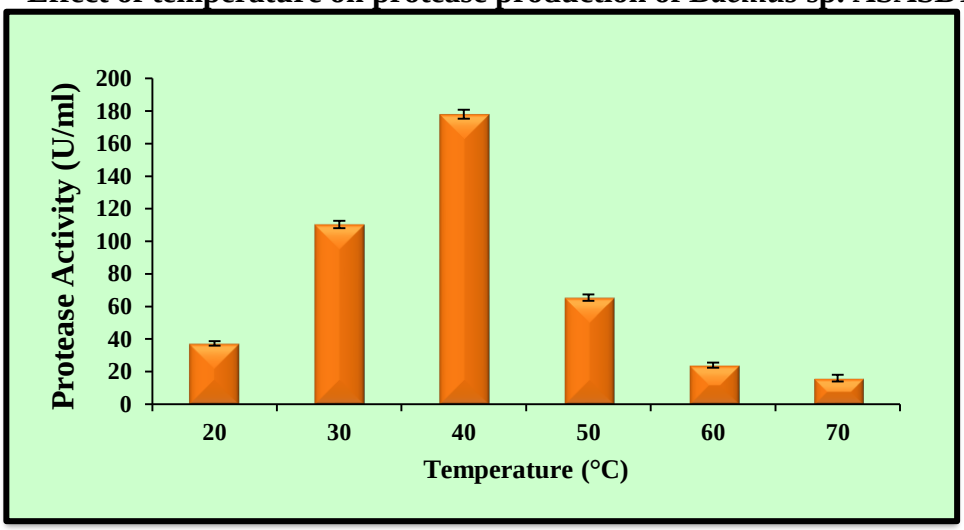
Figure 12 depicts the effect of temperature on protease production of *Bacillus* sp. ASASBT.

The results revealed that with increase in temperature, the protease activity also increased and reached a maximum value (178.13±2.75 U/ml) at 40°C. After this, there was a gradual decline in the enzyme activity which reached a minimum level (15.94±2.07 U/ml) at 70°C.

Thus, the optimum temperature for maximum protease production by the *Bacillus* sp. ASASBT was 40ºC.

Figure 12

Effect of temperature on protease production of *Bacillus* sp. ASASBT



Values are the mean ± SD of triplicates

The present results are similar to those of other workers. Agrawal *et al.,* (2012) and Ibrahim *et al.,* (2015) reported that the optimum temperature for protease production and cell growth of *Bacillus* sp. was found to be 40°C. According to Sai Smita *et al.,* (2012) also, the protease enzyme production was found to be maximum at 37°C, which showed that the bacterial strain APB1 isolated from different areas of Orissa state is temperature dependent for the production of enzyme. The results of Kumar *et al.,* (2012) revealed protease activity to be 80.56 U/ml at 45°C for the protease production by newly isolated *Bacillus subtilis.* The optimum growth and proteolytic activity of the three isolates from soil and water in Gondar, Ethiopia were shown to be between 37°C - 45°C (Bizuye *et al.,* 2014). In a similar study by Abdelnasser *et al.,* (2007), the optimum temperature for proteolytic activity of protease producing bacteria was found to be 37°C - 50°C.

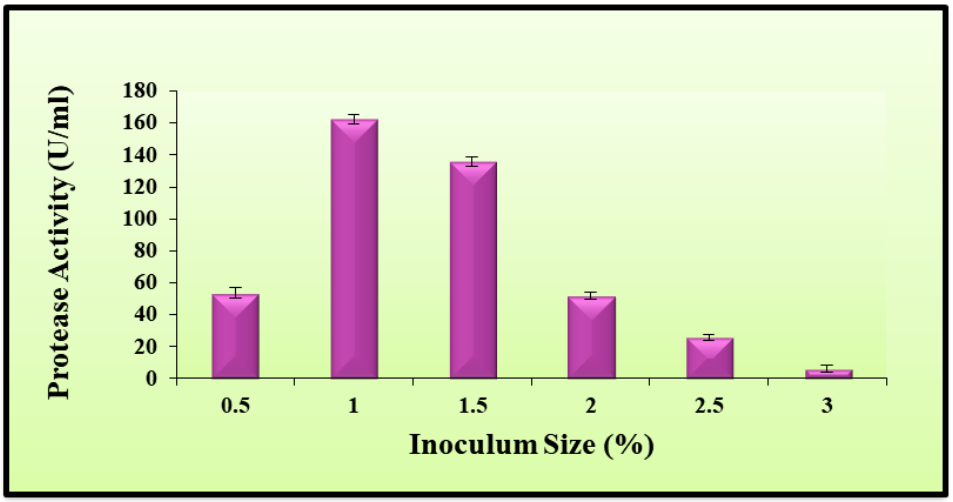
4.2.2.6 Effect of inoculum size on protease production

Among the various factors which influence enzyme production by microorganisms, the size of inoculum plays a significant role. Therefore, in the present study, the production medium was seeded with different inoculum concentrations of (0.5% to 3%) and the effect of these concentrations on the activity of protease when incubated at 37ºC for 48 hours was observed.

Figure 13 exhibits the suitable inoculum size for maximum protease production by *Bacillus* sp. ASASBT.

Figure 13

Effect of inoculum size on protease production of *Bacillus* sp. ASASBT



Values are the mean ± SD of triplicates

It is revealed from the figure that, at 1.0 %, the protease production was 162.21±3.07 U/ml. This was followed by 1.5 % with 135.63±2.82 U/ml of protease activity. However, the protease production started decreasing when the inoculum size was less than 1.0 % and above 1.5 % as depicted in Figure 13. The decrease in protease production below 1.0 % inoculum size may be because the growth of *Bacillus* sp. ASASBT was not sufficient enough to produce a good amount of protease. The decrease in enzyme activity for 1.5 % inoculum size may be due to the shortage of nutrients and dissolved oxygen available in the medium for the growth of more bacteria.

The *Bacillus* sp. ASASBT thus showed maximum protease production with 1.0 % inoculum size.

The above results obtained are in agreement with several previous investigations, which revealed that the production of protease decreased when the inoculum size was below or above the optimum size. Suganthi *et al.*, (2013) reported that the maximum protease production by *Bacillus licheniformis* was observed at 1 % inoculum size. Shine *et al.*, (2016) reported that the optimized inoculum size for alkaline protease

production by *Bacillus cereus* RS3 isolated from desert soil was shown to be 4 %. Production of protease by the selected mutant of *Bacillus subtilis* (BS-90) was optimized with the inoculum size of 1 % (Mohsin *et al.*, 2017). According to Irfan *et al.*, (2009) an optimum inoculum size of 2 % showed maximum enzyme activity. Mabrouk *et al.*, (1999) and Kanekar *et al.*, (2002) also reported that an inoculation ratio of 2 % to 5 % is optimum for *Bacillus* type of strain.

4.2.2.7 Effect of carbon sources on protease production

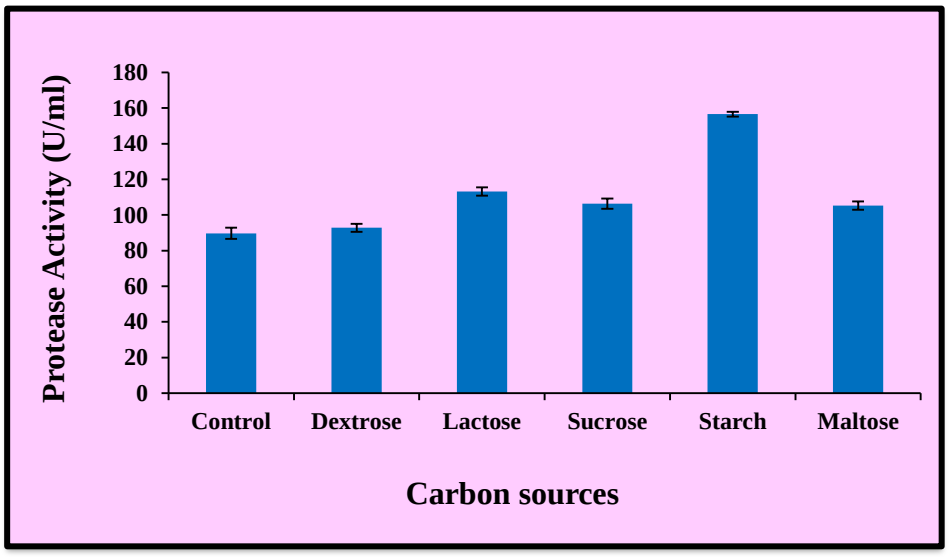
Nutrient sources are important factors for enzyme production. The addition of carbon source in the form of either monosaccharides or polysaccharides could have a major impact on the production of enzymes to a great extent (Sudharshan *et al.*, 2007).

The enzyme yield of *Bacillus* sp. ASASBT is also affected by the type of carbon source used in the production medium. Since carbon is one of the primary nutrients for bacteria, different carbon sources like dextrose, lactose, sucrose, starch and maltose were analyzed for protease production.

Figure 14 represents the protease activity when various carbon sources were used in the medium.

Figure 14

Effect of carbon source on protease production of *Bacillus* sp. ASASBT



Values are the mean ± SD of triplicates

It is understood from the figure that optimum production of protease (156.56±1.33 U/ml) was observed when starch served as the carbon source. This was followed by lactose (113.21±2.31 U/ml), sucrose (106.29±2.88 U/ml), maltose (105.21±2.35 U/ml) and dextrose (92.82±2.26 U/ml). Therefore, the results of the present study indicate that different carbon sources have a different effect on protease production by *Bacillus* sp. ASASBT.

Hence, starch was found to be the best carbon source for protease production.

The finding of the present study that maximum protease production was observed using starch as the carbon source is on par with the work of Chauhan and Gupta, (2004) and Vonothini *et al.*, (2008) who reported that *Bacillus* sp. and *Streptomyces roseiscleroticus* produce maximum protease in the presence of starch. Similar results were reported by Darani *et al.,* (2008), who said that use of 5 % (w/v) starch as carbon source led to maximum protease production by *Bacillus* sp. Kumar *et al.,* (2004) also said that the *Bacillus clausii* strain No. 58 grew well in various starch-based carbon sources at a concentration of 0.5 % (w/v) and gave maximum production of protease, followed by wheat bran and wheat flour. Another study by Ozdenefe *et al.,* (2017), confirmed that the best carbon source for maximum enzyme production from *Bacillus subtilis* was 1 % waste bread followed by sucrose, starch, glucose, lactose, maltose, dextrose, fructose and carboxymethylcellulose.

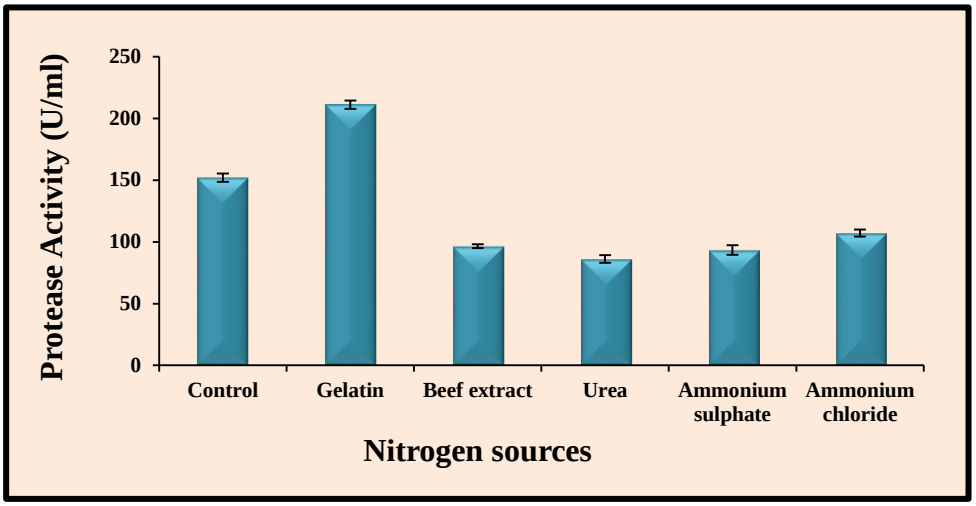
4.2.2.8 Effect of nitrogen source on protease production

Nitrogen also serves as an important nutrient source next to carbon for enzyme production. In microorganisms, both organic and inorganic nitrogen are metabolized to produce amino acids, nucleic acids, proteins and cell wall components. Hence, different nitrogen sources like gelatin, beef extract, urea, ammonium sulphate and ammonium chloride were used as nitrogen sources for protease production in the medium and the one which gives maximum activity of the enzyme identified.

Figure 15 shows the results of the effect of various nitrogen source on protease production.

Figure 15

Effect of nitrogen source on protease production of *Bacillus* sp. ASASBT



Values are the mean ± SD of triplicates

It can be inferred from the figure that gelatin is the best nitrogen source for the medium as it increases the production of protease upto 211.08±3.29 U/ml. This might be due to efficient metabolization of gelatin by the protease mainly due to its proteinaceous nature. Accordingly, moderate enzyme activity was detected in the control (152.02±3.45 U/ml), then followed by ammonium chloride (107.15±2.91 U/ml), beef extract (96.49±1.5 U/ml), ammonium sulphate (93.51±3.92 U/ml), urea (86.18±3.14 U/ml). However, inorganic nitrogen sources proved to be unfavorable, where the production of enzyme has been drastically reduced to 50% of the maximum output level, in case of ammonium sulphate and ammonium chloride.

Therefore, the maximum protease production of *Bacillus* sp. ASASBT was observed when gelatin was used as the nitrogen source.

The present study was in accordance with several other research work. Dissanayaka and Rathnayake, (2019), reported that *Geobacillus* sp. showed a maximum production of protease when gelatin was used as the nitrogen source. According to Chuprom *et al.*, (2016), among the various nitrogen sources studied, gelatin showed the maximum protease production. Manikandan *et al.*, (2009) and Patel *et al.*, (2005) stated that increased protease production could be achieved after using gelatin as nitrogen source in a fermentation process with haloalkaliphilic *Bacillus* sp. and *Bacillus alcalophilus.*

4.2.2.9 Effect of agitation speed on protease production

Aeration of the culture is one of the most important parameters affecting microbial growth and enzyme production.

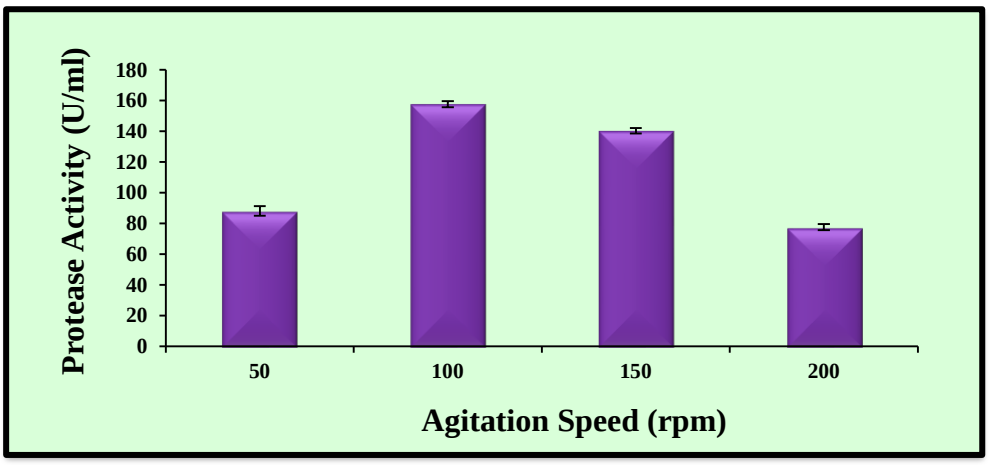
The effect of aeration on protease production by *Bacillus* sp. ASASBT was investigated by incubating the culture at various agitation speeds ranging from 50 to 250 rpm. Figure 16 represents the effect of agitation speed on protease production of *Bacillus* sp. ASASBT.

The selected isolate *Bacillus* sp. ASASBT was grown in a culture media containing starch and gelatin as the carbon and nitrogen sources respectively and the protease activity studied. The maximum protease activity was found to be 157.69±1.99 U/ml at 100 rpm agitation speed after 24 hours of incubation. This was followed by 150 rpm agitation speed (140.37± 1.75). This may be because, at this speed, the aeration of the culture medium was increased which would have led to sufficient supply of dissolved oxygen in the media. Nutrient uptake by bacteria also has been increased, thereby resulting in increased protease production. It was also revealed that, decrease in agitation rate drastically lowered the total protease production. Nearly 50 percent reduction in production of protease was detected beyond 100 rpm agitation speed.

Thus, at 100 rpm agitation speed, the production of protease from *Bacillus* sp. ASASBT revealed maximum.

Figure 16

Effect of agitation speed on protease production of *Bacillus* sp. ASASBT



Values are the mean ± SD of triplicates

High agitation speed creates a reduction in protease production due to cell lysis, excessive cell permeability related to abrasion by shear forces and limited oxygen in dense pellets leading to formation of extracellular polysaccharides (Patil and Chaudhari, 2011).

These findings are supported by those of other research workers. The optimum agitation speed of *Bacillus* sp. was found to be 100 rpm (Patil and Chaudhari, 2013; Genckal and Tari, 2006). According to Shine *et al.,* (2016), the protease production by *Bacillus cereus* RS3 isolated from desert soil showed an increase in protease production with increase in the agitation rate upto 170 rpm. Padmapriya and Williams, (2012) reported that, *Bacillus subtilis* grown in culture medium containing whey protein and starch showed maximum protease activity at 180 rpm agitation speed after 24 hours incubation. The findings of Joshi *et al.,* (2008) showed that, at 200 rpm speed was optimum for the protease production from *Halophilic* bacterium MBIC3303.

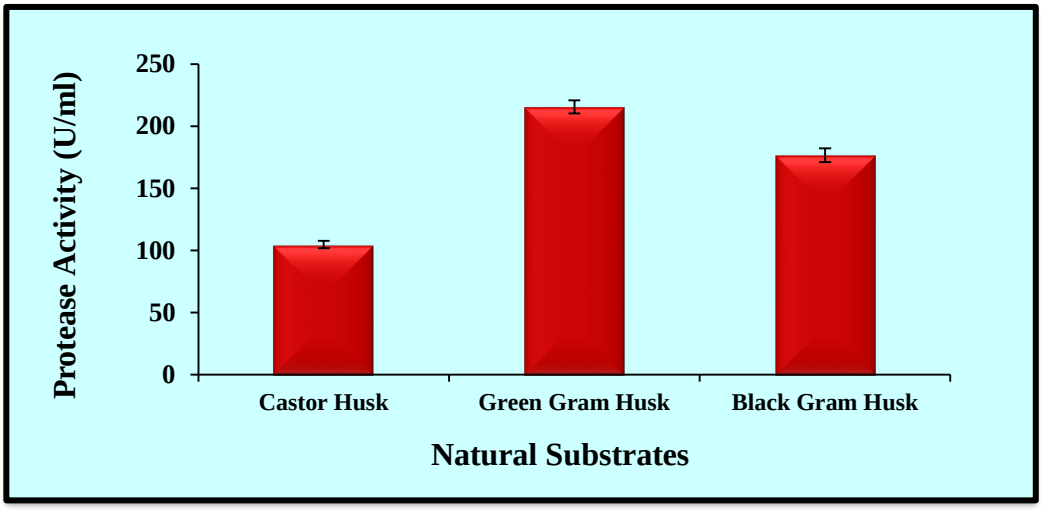
4.2.2.10 Effect of natural substrates on protease production

In solid state fermentation, the substrates play an important role in the growth of the microorganisms. It becomes essential to select the suitable solid substrate for better growth and maximum product formation (Mukherjee *et al.,* 2008).

Figure 17 presents the protease activity when castor husk, green gram husk and black gram husk were used as natural substrates.

Figure 17

Effect of natural substrates on protease production of *Bacillus* sp. ASASBT



Values are the mean ± SD of triplicates

Among the different substrates tested, green gram husk showed the highest protease production (215.56±2.96 U/ml) followed by black gram husk (176.69±1.95 U/ml) and castor husk (104.72±2.98 U/ml).

Hence, green gram husk proved to be the best natural substrate for maximum production of protease.

Similar findings were reported by Ramakrishna *et al.,* (2012), who stated that maximum production of protease from *Bacillus subtilis* KHS-1 (MTCC NO-10110) was found when green gram husk was used as the substrate. The use of agricultural wastes as in the present study is supported by the findings of several other studies also. According to Prakasham *et al.,* (2006) and Vanitha *et al.,* (2014), efforts have been directed to reduce the enzyme production cost through improving the yield and use of cost free or low-cost feed stocks or agricultural by products and maximum protease production was got when green gram husk was used as substrate. Dutta *et al.,* (2014) also reported that maximum protease production by *Acinetobacter calcoaceticus* occurred with cattle feed as substrate.

Phase II - Highlights of the findings

- **The morphological characteristics of the isolate TMS1 showed motile, endospore forming, Gram-positive rod-shaped bacteria.**
- **The phenotypic characteristics of the isolate belongs to the genus *Bacillus*.**
- **The phylogenetic analysis based on the 16S rRNA gene sequencing using the neighbor-joining method indicated that the isolate TMS1 was *Bacillus* sp. and designated as *Bacillus* sp. ASASBT.**
- **Optimization of media components for protease production showed higher activity of the enzyme for an incubation period of 48 hours at pH 7.0, 40ºC temperature and with starch as the carbon source and gelatin as the nitrogen source and an agitation speed of 100 rpm with green gram husk as the natural substrate.**

4.3 Phase III - Purification and molecular characterization of protease

4.3.1 Purification of protease

The most important requirement for the study of any protein or enzyme its purification. Protein/enzyme purification is a multistep process intended to isolate a single type of protein from a complex mixture. Autolysis is one of the inherent problems faced in the purification of enzymes (Wingfield, 2016).

The various steps followed for the purification of protease isolated from *Bacillus* sp. ASASBT are ammonium sulphate precipitation, dialysis, ion exchange chromatography and gel filtration chromatography. The results are presented as follows:

4.3.1.1 Precipitation with ammonium sulphate

For purification of protease, ammonium sulphate precipitation is often used as the first purification step and concentration procedure. The supernatant obtained from the culture filtrate was maintained at optimum conditions and was fractionated by precipitation with varying concentrations of ammonium sulphate (0-100%). After this, the total protease activity, total protein content, specific activity, purification fold and recovery percentage of the protease was studied.

Table 6 depicts the purification profile of protease from varying concentrations of ammonium sulphate precipitation.

From the table it is observed that, the total protease activity and total protein content of all the precipitated samples decreased on comparison with the crude enzyme sample (4294.19 U and 381.29 mg). The maximum protease activity was exhibited by the 40 - 60 % ammonium sulphate precipitated sample (3497.00 U) as compared to other samples. In the case of protein content also, the 40 - 60 % ammonium sulphate precipitated sample showed the highest activity (104.00 mg).

Specific activity is a measure of enzyme purity. The 40 - 60 % ammonium sulphate precipitated sample recorded the maximum value for specific activity (33.62 U/mg) also on comparison with the specific activity of the crude enzyme sample (11.26 U/mg).

The recovery percentage of all the ammonium precipitated samples exhibited a detectable decrease when compared to the crude enzyme sample (100 %). Among the ammonium sulphate precipitated samples, the highest recovery percentage was observed by the 40 - 60 % sample (81.44 %).

Similarly, like specific activity, the 40 - 60 % ammonium sulphate precipitated sample also exhibited increase in purification fold (2.99) on comparison with the crude enzyme sample (1.00).

Since the 40 - 60 % of ammonium sulphate precipitated protease showed maximum specific activity and purification fold as compared to the other samples, it was selected for further purification.

Table 6

Purification profile of ammonium sulphate precipitated fractions of protease from *Bacillus* sp. ASASBT

Samples	Total protease activity (U)	Total protein content (mg)	Specific activity (U/mg)	Recovery percentage (%)	Purification fold
Crude	4294.19	381.29	11.26	100	1.00
Ammonium sulphate precipitation 0-20%	415.50	54.50	7.62	9.67	0.68
20-40%	1835.00	98.00	18.72	42.73	1.66
40-60%	3497.00	104.00	33.62	81.44	2.99
60-80%	1764.00	91.00	19.38	41.08	1.72
80-100%	932.50	63.00	14.80	21.71	1.31

Values are the mean of triplicates

The findings of the present study are in line with the reports of other similar studies indicating maximum activity of protease at 60 % saturation of ammonium sulphate (Venkat *et al.,* 2014). According to Gayatri Devi and Hemalatha (2014) also, 60 %

ammonium sulphate precipitation revealed highest protease activity, protein content, specific activity and purification fold. Josephine *et al.*, (2012) another researcher stated that the specific activity and purification fold of partially purified protease isolated from the *Bacillus* sp. obtained from 50 % and 70 % ammonium sulphate fractionation was found to be maximum with a high purification fold. The alkaline protease from *Bacillus subtilis* 168 isolated from food industry waste was partially purified with 100 % saturated ammonium sulphate (Vanitha *et al.*, 2014). The report of Abirami *et al.*, (2011) also mentioned that the crude protease enzyme from *Penicillium janthinellum* and *Neurospora crassa* was saturated at 70 % ammonium sulphate with maximum total activity, total protein and purification fold.

4.3.1.2 Dialysis

The 40 - 60 % ammonium sulphate precipitated fraction which exhibited the highest specific activity, recovery percentage and purification fold was further purified by dialysis to remove the salts present in the sample.

Table 7 shows the specific activity of dialyzed protease from *Bacillus* sp. ASASBT.

Table 7

Purification profile of dialyzed protease from *Bacillus* sp. ASASBT

Parameters	40 – 60 % Ammonium sulphate precipitated	Dialyzed
Total protease ctivity (U)	3497.00	1314.80
Total protein content (mg)	104.00	36.00
Specific activity (U/mg)	33.62	36.52
Recovery percentage (%)	81.44	30.61
Purification fold	2.99	3.24

Values are the mean of triplicates

From the table, it is understood that the protease activity of 40 - 60 % ammonium sulphate precipitated fraction had decreased from 3497.00 U to 1314.80 U. Similarly, the

protein content of the dialyzed samples also showed a decrease (36.00 mg) when compared with the ammonium sulphate precipitated sample (104.00 mg) respectively on dialysis.

As regards specific activity, the dialyzed sample registered a higher value (36.52 U/mg) on comparison with the 40 - 60 % ammonium sulphate precipitated sample (33.62 U/mg). The increase in specific activity of the dialyzed sample ensures the progress in purification of protease.

From the recovery percentage, it can be noticed that, the dialysate showed a decrease (30.61 %) on comparison with 40 - 60 % ammonium sulphate precipitated samples (81.44 %). Since the recovery percentage is related to the protease activity, it decreases with decrease in the protease activity.

It is evident from the table, that the dialysate recorded a higher value of 3.25 for the purification fold as compared to the 40 - 60 % ammonium sulphate precipitated sample with a value of 2.99.

This result confirms the increase in the purification fold after dialysis. Besides, the purification fold is known to be directly proportional to the specific activity of an enzyme, since it increases with increase in the specific activity.

The increase in the specific activity and purification fold after dialysis in the present study agrees with the results of another study by El-Safey and Abdul-Raouf, (2004) who stated that dialysis of ammonium sulphate precipitated protease of *Bacillus subtilis* isolated from water increased its specific activity and purification fold. Similar findings were also reported by El-Safey and Ammar, (2003). In the study of Li *et al*., (2018) he stated that, ammonium sulphate precipitation and dialysis of protease isolated from tamarillo fruit showed increase in the specific activity. According to Mothe and Sultanpuram, (2016) after 60 % ammonium sulphate saturation and purification by dialysis of protease enzyme, the specific activity and purification fold were increased. Similar findings were reported by Qureshi *et al.,* (2018), who reported the dialyzed sample to have an increased specific activity and decreased protease activity and protein content which confirmed the level of purification.

4.3.1.3 Ion exchange chromatography using AKTA FPLC system

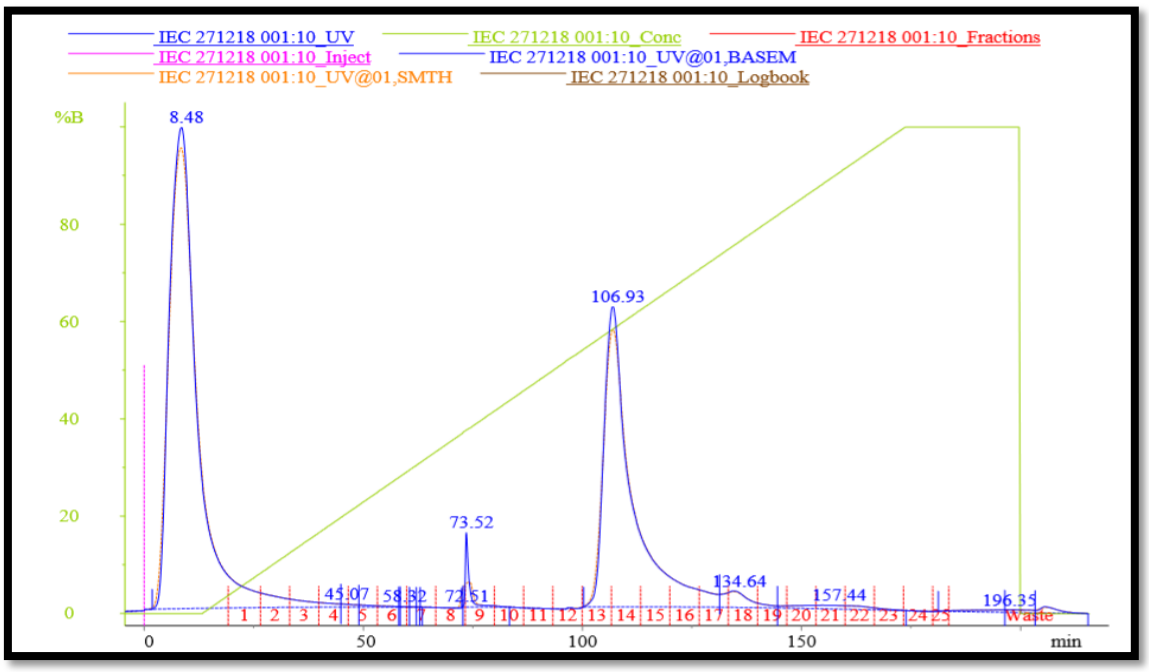
The concentrated sample obtained from dialysis was loaded onto a Hi Trap Q FF column of AKTA FPLC system, equilibrated with phosphate buffer at pH 7.4 and the fractions collected.

Figure 18 indicates the elution profile of dialyzed protease sample on ion exchange Hi Trap Q FF column.

It can be noticed from the figure that a total of 25 fractions (1.5 ml each) were eluted. There was a gradual increase in the protease activity in fraction 8 and fraction 9 then a gradual decrease. This increase may be due to washing of the column and subsequent elution of unbound proteins. However, on treatment of the column with a linear gradient of sodium chloride at a concentration of 60 %, the activity of protease increased from fraction 13 upto fraction 16 with the fraction 14 giving the highest. After this there was a decrease in the protease activity upto fraction 19 after which there was no activity detected. The fractions showing active peaks were pooled together and used for further studies.

Figure 18

Ion exchange chromatogram of purified protease from *Bacillus* sp. ASASBT



The purification profile of ion exchange eluted active protease fraction is represented in Table 8, which depicts the total protease activity, total protein content, specific activity, recovery percentage and purification fold.

From the table, it can be expressed that, there was a decrease in the total protease activity (948.57 U) and total protein content (8.91 mg) of the ion exchange purified sample when compared to the total protease activity and total protein content of the dialyzed sample (1314.80 U and 36.00 mg) respectively. This decrease was around 35 folds.

However, the total protease activity and total protein content were lowered for the active protease fraction and its specific activity was increased (106.46 U/mg) when compared to that of the dialyzed enzyme extract (36.52 U/mg). This increase in specific activity is thus indicative of the progress in purification of the enzyme.

Similar to the total protease activity and total protein content, the recovery percentage (22.08 %) of the active protease fractions of ion exchange chromatography represents a decrease on comparison with the recovery percentage of the dialyzed sample (30.62 %).

In view of purification fold, the active fraction of the ion exchange purified sample showed an increase which was around 9.45 folds when compared to that of the dialyzed sample (3.25).

From the above results, it can be deduced that ion exchange purified protease showed the highest specific activity and purification fold.

Table 8

Purification profile of ion exchange purified protease from *Bacillus* sp. ASASBT

Parameters	Dialyzed	Ion exchange purified
Total protease ctivity (U)	1314.80	948.57
Total protein content (mg)	36.00	8.91
Specific activity (U/mg)	36.52	106.46
Recovery percentage (%)	30.62	22.08
Purification fold	3.25	9.45

Values are the mean of triplicates

The present findings are supported by several other researchers. Benmrad *et al.,* (2016), stated that the detergent-stable serine alkaline protease from *Trametes cingulate* fungal strain CTM10101 was isolated and purified through various stages of purification and showed the purification fold of 6.27. Similar findings were also reported by Mechri *et al.,* (2017), the maximum purification fold was appeared at UNO Q-6 FPLC system. Aliei and Arabaci, (2018) also stated that the affinity chromatogrphic purification of serine protease from strawberry (*Fragaria ananassa*) showed maximum enzyme activity and increase in purification fold.

Compared to the earlier studies, the purification protocol followed in the present study yielded more purity with increased specific activity.

4.3.1.4 Gel filtration chromatography using AKTA FPLC system

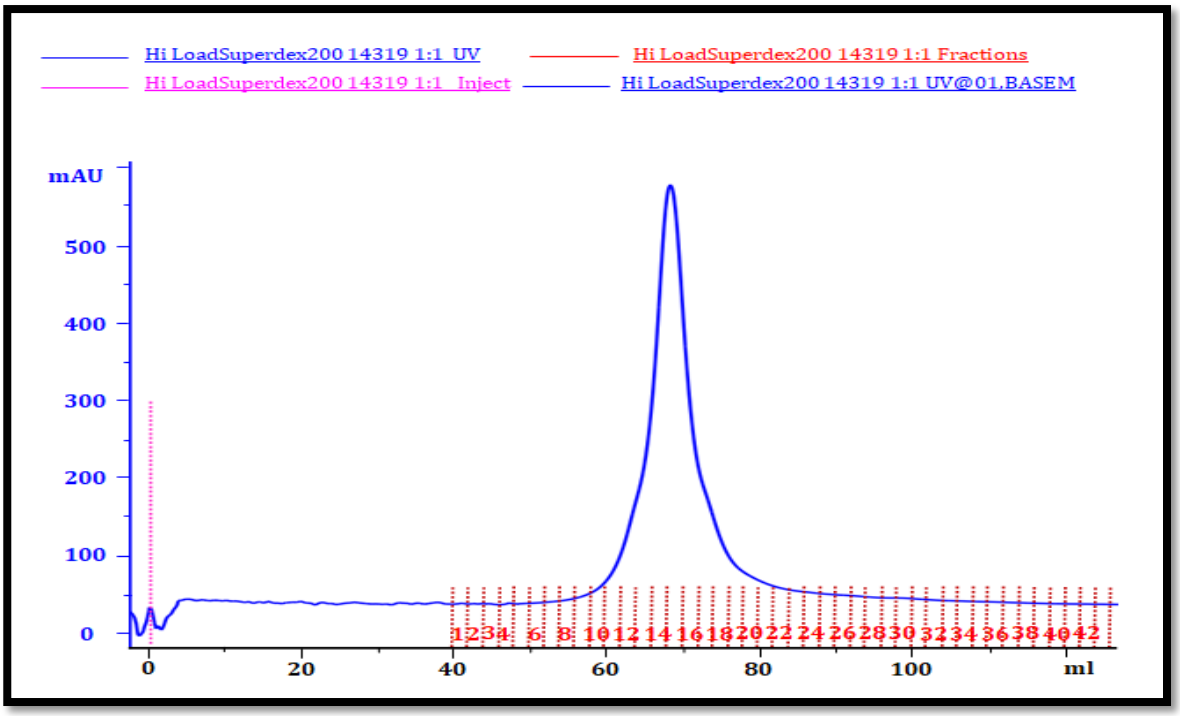
Size exclusion / Gel filtration chromatography was used to separate the molecules according to their hydrodynamic diameter or hydrodynamic volume. It is generally a low-resolution chromatographic technique used for the final step of purification. The concentrated fraction obtained from ion exchange chromatography was then loaded onto Superdex-200 10/300 GL column fitted to FPLC system and the fractions collected.

Figure 19 represents the elution profile of the size exclusion chromatography of ion exchange purified protease.

A total of 42 fractions (1.5 ml each) were eluted. From the figure it clearly indicates that the active peaks from Superdex 200 column fitted to FPLC showed maximum total enzyme activity and protein content in fractions 12 - 20. Peaks 14 to 16 fractions depict the highest total enzyme activity. The fractions before 10 and after 20 recorded lower protease activities. Hence these fractions were pooled together and one part of this was taken for studying the purification profile.

Figure 19

Gel filtration chromatogram of purified protease from *Bacillus* sp. ASASBT



The purification profile of Superdex-200 column eluted protease fractions is shown in Table 9.

Table 9

Purification profile of gel filtration purified protease from *Bacillus* sp. ASASBT

Parameters	Ion exchange purified	Gel filtration purified
Total protease ctivity (U)	948.57	696.43
Total protein content (mg)	8.91	5.46
Specific activity (U/mg)	106.46	127.55
Recovery percentage (%)	22.08	16.21
Purification fold	9.45	11.32

Values are the mean of triplicates

From the table, it can be clearly stated that, there was a decrease in the total protease activity (696.43 U) of the Superdex 200 purified sample when compared to the ion exchange purified enzyme sample (948.57 U).

A similar trend as that of total protease activity was followed in total protein content also (8.91 mg to 5.46 mg). The active protease fractions showed a lower value (5.46 mg) on comparison with that of the ion exchange purified (8.91 mg) sample.

Though the total protease activity and total protein content were lowered for the active protease fraction, its specific activity was increased (127.55 U/mg) when compared to that of the ion exchange purified enzyme sample (106.46 U/mg). Therefore, the increase in specific activity confirmed the progress in purification of the enzyme.

Like total protease activity and total protein content, the recovery percentage (16.21 %) of the active protease fractions of Superdex 200 recorded a decrease on comparison with the recovery percentage of the ion exchange purified sample (22.08 %).

Similar to specific activity, the purification fold of Superdex 200 purified sample also exhibited an increase in value of 11.32 when compared to that of the ion exchange purified sample (9.45).

Therefore, it can be concluded that gel filtration purified protease revealed a higher specific activity and purification fold than the ion exchange purified sample.

Similar studies as above were reported by Deng *et al.,* (2010), who showed that surfactant-stable high-alkaline protease from *Bacillus* sp. B001 which has been serially purified using AKTA FPLC, recorded increase in specific activity and purification fold. Based on the findings of Anbu, (2013) and Secades *et al.,* (2001), reported that the solvent stable extracellular protease from *Bacillus koreensis* (BK-P21A) was purified by gel filtration chromatography using AKTA FPLC system with Superdex 200 and Superdex 75 column, exhibited maximum specific activity with the purification fold. Another study by Dorra *et al.,* (2018) also stated that the alkaline protease produced by *Bacillus halotolerans* strain CT2 revealed increase in purification fold and specific activity.

4.3.1.5 Summary of purification profile of protease at various stages of purification

The summary of purification profiles of the sequentially purified ammonium sulphate precipitated protease from *Bacillus* sp. ASASBT reveals the total protease activity, total protein content, specific activity, recovery percentage and purification fold subjected to various stages of purification.

Table 10 shows the summary of purification profile of gel filtration purified ammonium sulphate precipitated protease.

Table 10

Summary of purification profile of protease at various stages of purification

Purification steps	Total activity (U)	Total protein (mg)	Specific activity U/mg	Recovery percentage (%)	Purification fold
Crude	4294.19	381.29	11.26	100	1.00
Ammonium sulphate precipitated (40-60%)	3497.00	104.00	33.62	81.44	2.99
Dialyzed	1314.80	36.00	36.52	30.62	3.25
Ion exchange purified	948.57	8.91	106.46	22.08	9.45
Gel filtration purified	696.43	5.46	127.55	16.21	11.32

From the table, it is understood that there was a decrease in the total protease activity with every step of purification (from 4294.19 U to 696.43 U). The total protein content of the gel filtration purified sample also showed a decrease from 381.29 mg to 5.46 mg with each step of purification. On comparing the total protein content with the total protease activity, it can be deduced that as the protease activity of all the samples decreased, their protein content decreased. As regards specific activity, with each step of

purification, the specific activity increased starting from 11.26 U/mg to 127.55 U/mg. The increase in specific activity ensures the progress in purification of the protease. Similarly, purification fold of the enzyme increased with increase in purification (from 1.00 to 11.32). The recovery percentage decreased with each stage of purification from 100 % to 16.21 %.

Thus, it can be deduced that with increase in purification of the enzyme, the specific activity and purification fold also increased.

The results of the present study are in agreement with the reports of other similar studies. The findings of Qureshi *et al.,* (2018) described that the purification of organo-solvent tolerant protease from *Bacillus* sp. BBXS-2 isolated from soil sample, showed on increase in specific activity and purification fold after ion exchange chromatography. Bacha *et al.,* (2017) reported that the purification profile of a newly isolated serine protease from *Rhamnus frangula* (shrub) purification with the Sephadex G-50 gave a high recovery yield and a purification fold. Zivkovic *et al.,* (2010) and Gaur *et al.,* (2010) also stated that, protease from *Pseudomonas aeruginosa* after gel filtration and DEAE-cellulose column chromatography, the specific activity of protease increased with increase in purification fold.

4.3.2 Molecular weight of purified protease

4.3.2.1 SDS polyacrylamide gel electrophoretogram

SDS-PAGE is the most commonly used gel electrophoretic technique for proteins, because it provides an easy way to estimate the number of polypeptides in a sample and thus evaluate the complexity or purity of the sample (Davey and Lord, 2003).

The ammonium sulphate precipitated, dialyzed, ion exchange purified and gel filtration purified protease alongwith protein markers were run on SDS-PAGE. The purified protease appeared as a single band on SDS - PAGE stained with Commassie Brilliant Blue and Silver staining.

a) Coomassie Brilliant Blue staining

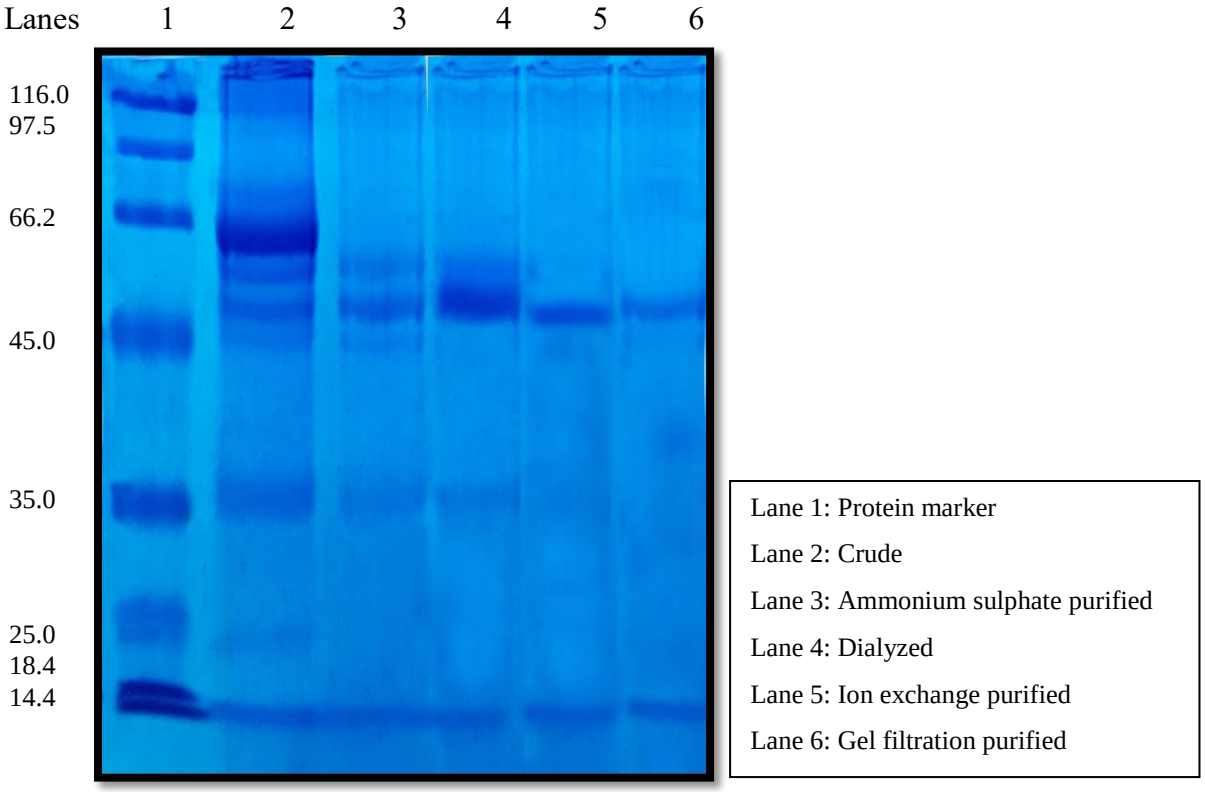
Plate 6 presents the SDS-PAGE pattern of Coomassie Brilliant Blue stained protease.

The plate shows the electrophoretic pattern of the protein marker in Lane 1, crude in Lane 2, ammonium sulphate precipitated in Lane 3, dialysate in Lane 4, Ion exchange purified in Lane 5 and Gel filtration purified in Lane 6. The protein marker used had a molecular weight ranging from 14.4 to 116.0 kDa. A series of bands appeared in Lane 2, indicating the presence of other proteins apart from protease. However, Lanes 5 and 6 showed only a single band. On comparison of Lanes 3 and 4, it can be inferred that the electrophoretogram of the gel filtration purified sample showed a single band when compared to the ammonium sulphate precipitated and dialyzed samples indicating the progress in purification of the isolated protease.

From the above observations it can be inferred that the molecular weight of the protease from *Bacillus* sp. ASASBT could be 46 kDa by SDS-PAGE - Coomassie Brilliant Blue staining.

Plate 6

SDS PAGE of protease – Coomassie Brilliant Blue staining



b) Silver staining

The presence of a single band on Coomassie Brilliant Blue stained gel filtration purified protease was confirmed with Silver staining. Plate 7 shows the SDS-PAGE pattern of Silver stained protease.

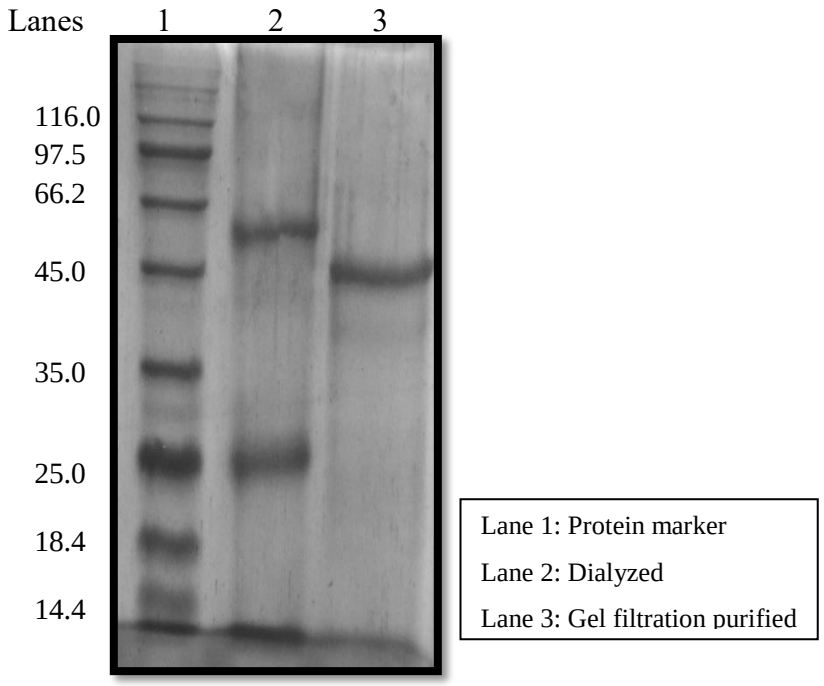
From the Plate, it clearly indicated the electrophoretogram of the Silver stained protein marker in Lane 1. Lane 2 indicates the dialyzed protease which is partially purified and in Lane 3 the finally gel filtration purified protease in Lane 3.

The protein marker used in Lane 1 had a molecular weight ranging from 14.4 to 116.0 kDa. It is quite obvious from the picture that two bands were present in Lane 2 indicating the presence of only a partially purified enzyme. On the other hand, a single clear band was observed in Lane 3 which clearly indicates the homogeneity of the purified protease.

Thus, it can be concluded that the apparent molecular weight of protease from *Bacillus* sp. ASASBT could be 46 kDa by Silver staining – SDS-PAGE.

Plate 7

SDS-PAGE of protease – Silver staining



The molecular weight of the purified protease as derived at in the present study was more or less similar to the molecular weight of the protease from *Bacillus cereus* MCM B-326 (45 kDa) (Zambare *et al.,* 2007). According to Maruthiah *et al.,* (2015), the molecular weight of the protease isolated from a marine *Bacillus* sp. APCMST-RS3 using marine shell wastes was 40 kDa. Dipasquale *et al.,* (2008) also reported that the molecular weight of an alkaline protease isolated from *Bacillus thermantarcticus* M1 was found to be 42 kDa. The purified protease from *Bacillus* sp. SB12 was homogenous on non-denaturing PAGE and showed a single band with molecular weight of 41 kDa (Briki *et al.,* 2016).

4.3.2.2 Confirmation of protease activity by zymography

Zymography is a rapid assay method that detects nanograms of proteins, in contrast to SDS-PAGE which detects microgram quantities and it is a very sensitive technique. Here the isolated enzyme acts on casein and hydrolyzes it to produce white bands. Zymography uses an SDS-substrate gel, for detecting the proteolytic activity of enzymes and the presence of protease inhibitors in polyacrylamide gels (Ktari *et al.,* (2012) and Nasri *et al.,* (2011)).

The purified *Bacillus* sp. ASASBT protease was studied by zymography using casein as the substrate to confirm the presence of protease and its ability to hydrolyze the casein. Plate 8 depicts the zymogram of purified protease.

Plate 8

Zymogram of gel filtration purified *Bacillus* sp. ASASBT protease

The picture reveals the zymographic studies of the active fractions obtained from gel filtration Superdex 200 column chromatography purified protease which showed a single clear hydrolyzed portion on the stained agarose gel that is, a clear band of lysis against a deep blue background. The proteolysis confirms the presence of protease.

Based on SDS-PAGE and zymogram analysis, it can be suggested that purified protease from *Bacillus* sp. ASASBT is a monomeric protein comparable to those previously reported for other proteases from various *Bacillus* strains.

The findings of the present study are in line with the report given by Doddapaneni *et al.*, (2009) who stated that a *Bacillus cereus* protease developed a single band in zymogram. The zymogram staining of *Halobacillus karajensis* protease revealed one clear zone of proteolytic activity against the blue background (Heidari *et al.,* 2009). Briki *et al.*, (2016), Annamalai *et al.,* (2014) and Anbu (2013) also stated that purified protease from the *Bacillus* sp. showed a clear zone of proteolytic activity on zymogram.

4.3.2.3 Identification of protein by MALDI-TOF/TOF analysis

The Coomassie Brilliant Blue-stained protein band corresponding to 46 kDa protein was excised from the polyacrylamide gel and it was digested by trypsin. The resulting peptide masses were analyzed by MALDI-TOF/TOF mass spectrometry, where acquired with a range of (500 to 3500 *m/z*).

Figure 20 presents the molecular mass of peptides of the purified enzyme/protein were analyzed by the method of MALDI-TOF/TOF Mass spectrometry.

From the figure it can be said that the peptide mass fingerprinting (PMF) of the protein after digestion with trypsin yielded 14 prominent *m/z* peaks. It shows that each spectrum was produced by accumulating data from 500 consecutive laser shots.

Figure 20

Peptide mass fingerprint spectra of purified protease from *Bacillus* sp. ASASBT

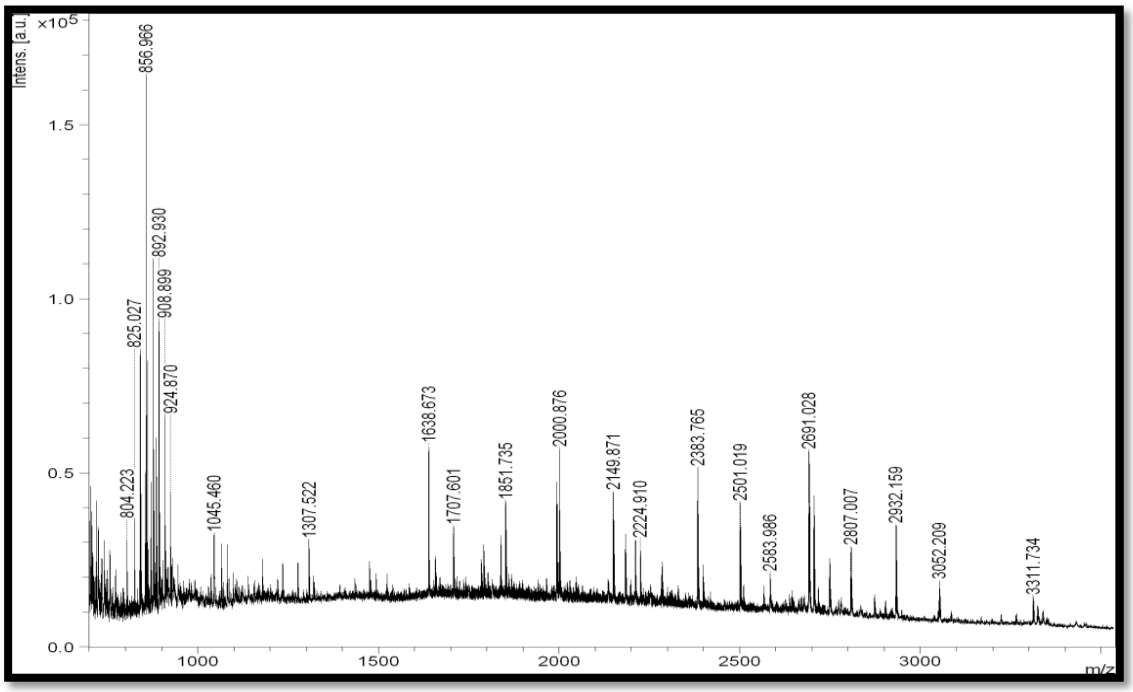


Figure 21 (a) and (b) depicts the Mascot search results and concise protein summary report.

Figure 21 (a)

Mascot score histogram of purified protease from *Bacillus* sp. ASASBT

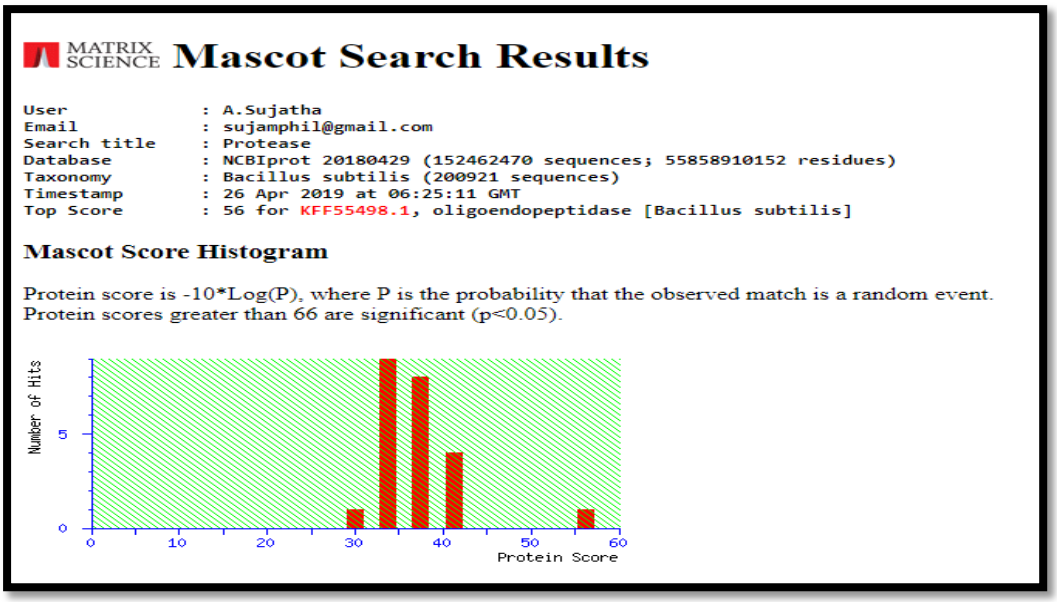
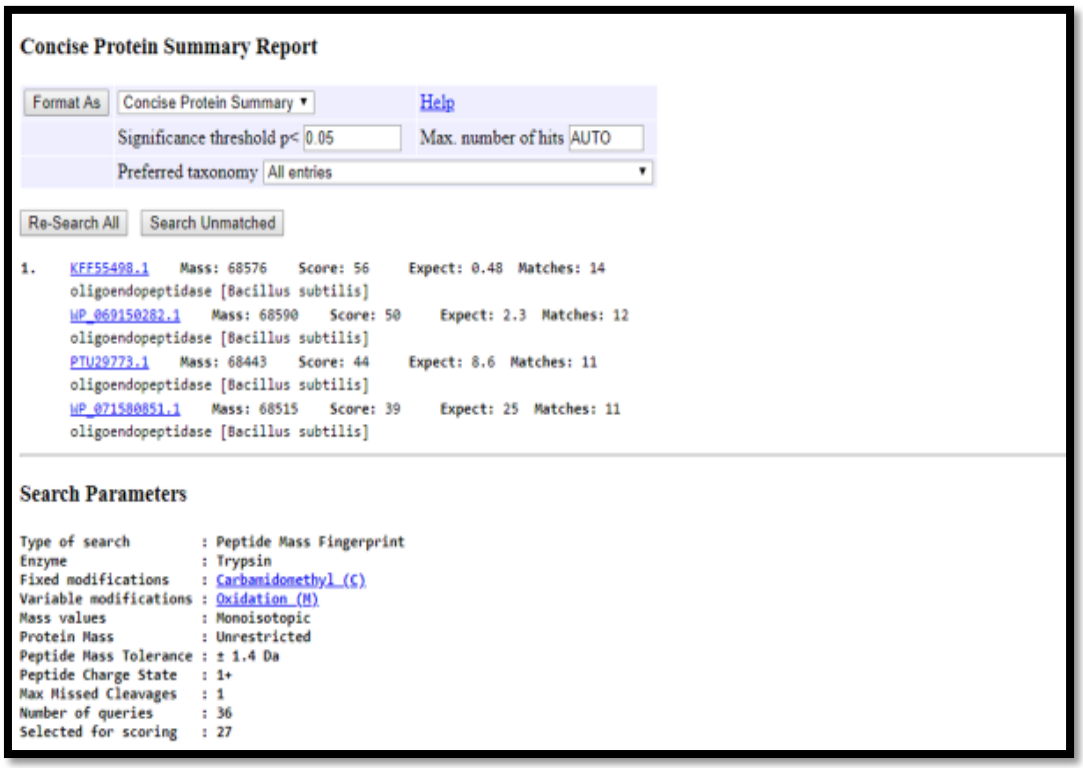


Figure 21 (b)

Protein sequence coverage of purified protease from *Bacillus* sp. ASASBT



Concise Protein Summary Report

Format As: Concise Protein Summary ▾ Help

Significance threshold p< 0.05 Max. number of hits AUTO

Preferred taxonomy: All entries ▾

Re-Search All | Search Unmatched

1. KFF55498.1 Mass: 68576 Score: 56 Expect: 0.48 Matches: 14
oligoendopeptidase [Bacillus subtilis]
WP_069150282.1 Mass: 68590 Score: 50 Expect: 2.3 Matches: 12
oligoendopeptidase [Bacillus subtilis]
PTU29773.1 Mass: 68443 Score: 44 Expect: 8.6 Matches: 11
oligoendopeptidase [Bacillus subtilis]
WP_071580851.1 Mass: 68515 Score: 39 Expect: 25 Matches: 11
oligoendopeptidase [Bacillus subtilis]

Search Parameters

Type of search : Peptide Mass Fingerprint
Enzyme : Trypsin
Fixed modifications : Carbamidomethyl (C)
Variable modifications : Oxidation (M)
Mass values : Monoisotopic
Protein Mass : Unrestricted
Peptide Mass Tolerance : ± 1.4 Da
Peptide Charge State : 1+
Max Missed Cleavages : 1
Number of queries : 36
Selected for scoring : 27

It is understood from the figures that the singly charged monoisotopic peptide masses were searched against Swiss-Prot and NCBIProt databases by utilizing the MASCOT server 2.2 search engine (www.matrixscience.com, Matrix Science Ltd., USA, UK, Japan) upto one missed tryptic cleavage was considered and the mass tolerance for monoisotopic peptide masses was set to 1.2 Da. The obtained spectra were compared with the mascot software. The identical protein showed 56% of match with oligoendopeptidase (KFF55498.1) as annotated protein. Three experimental peptide masses matched to the oligoendopeptidase protein.

The results of peptide fragments obtained from MALDI-TOF/TOF followed by Mascot search analysis confirmed that the purified protein fraction was an oligoendopeptidase.

Thus, the results implied that the purified protease from *Bacillus* sp. ASASBT belonged to the class of metalloprotease.

Similar findings as the above are in accordance with other studies also, as reported by several researchers. Mothe and Sultanpuram (2016), stated that the MALDI-TOF/TOF peptide mass fingerprinting of *Bacillus caseinilyticus* protein after digestion with trypsin yielded eight prominent *m/z* peaks. The *m/z* values were identified and found to be similar to peptidase S8 and S53 subtilisin from *Bacillus cellulosilyticus.* Similar findings were reported by Enling *et al.*, (2017) also, who performed MALDI-TOF-TOF/MS of an alkaline protease from *Micrococcus* sp. isolated from the South China Sea, which showed it might belong to the peptidase S8 family. MALDI-TOF-MS analysis of alkaline, thermostable and organic solvent stable protease from a wild and mutant of *Bacillus* sp. also confirmed that the purified protein was protease in wild and peptidase in mutant strain respectively (Thakur *et al.*, 2018).

Phase III - Highlights of the findings

- **After four step purification, the protease showed higher specific activity and purification fold.**
- **The molecular weight of the purified protease was found to be 46 kDa.**
- **MALDI-TOF/TOF analysis identified the isolated enzyme to be an oligoendopeptidase which belongs to the metalloprotease family.**

4.4 Phase IV - Biochemical characterization of purified protease

The purified protease from *Bacillus* sp. ASASBT was characterized. The following factors were studied to get the conditions for optimal activity of the purified protease - effect of pH, temperature, concentration of substrate, metal ions, inhibitors, surfactants, oxidizing agents and organic solvents. Spectral analysis which gives additional details for characterization of purified protease was also studied.

4.4.1 Effect of pH on activity and stability of purified protease

Enzymes are proteins and they are sensitive to changes in pH. They may denature or alter in extreme levels of hydrogen ions either acidic or alkaline. The pH at which an

enzyme has the highest activity is known as optimum pH. The protease that can be used in detergent industries should have two characteristics - stability at alkaline pH and compatibility with detergent (Niyonzima and More, 2015). To study the effect of pH on activity and stability of purified protease isolated from *Bacillus* sp. ASASBT was incubated at different pH levels 3.0 to 10.0.

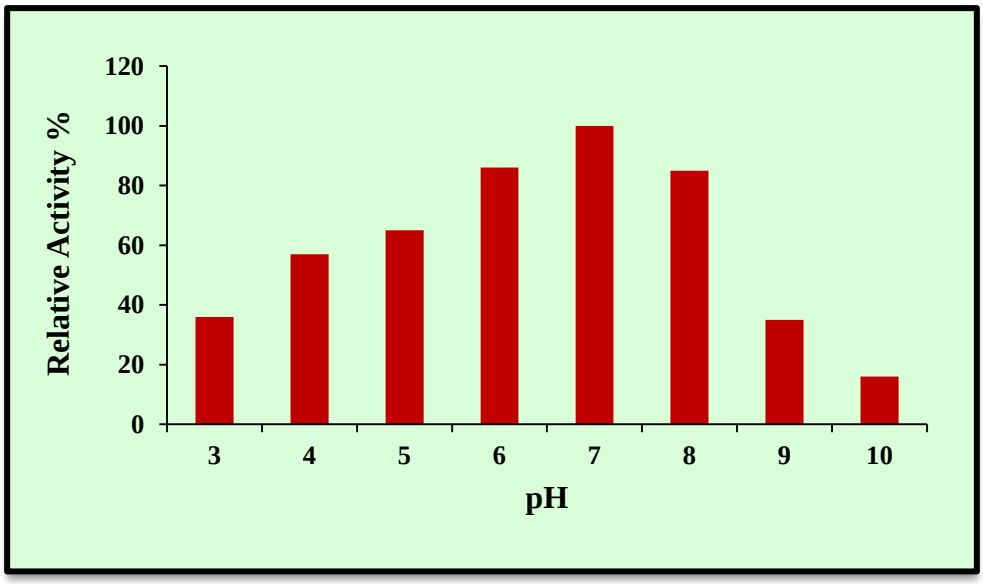
The effect of varying pH on the relative activity of purified protease isolated from *Bacillus* sp. ASASBT was studied and the results are recorded in Figure 22.

It is clear from the figure, that there was a gradual increase in the relative activity of purified protease from pH 3.0 (36 %) and this reached a maximum at 7.0 (100 %). The activity then started to decrease slowly thereafter from pH 8.0 to 10.0 (85 % to 16 %).

This confirms the optimum pH of the isolated protease to be 7.0 since the relative activity at this pH was 100 %.

Figure 22

Effect of pH on activity of purified protease



Values are the mean of triplicates

The findings of the present study were similar to the reports of several other researchers. Maruthiah *et al.*, (2015) stated that the pH optima for haloalkalophilic organic solvent tolerant protease from a type of marine *Bacillus* sp. APCMST-RS3 isolated from

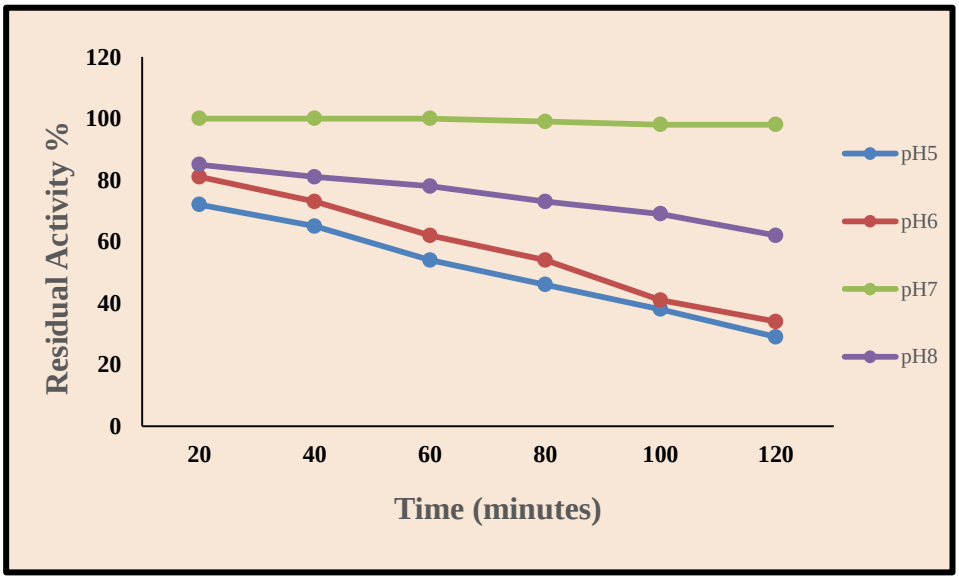
marine shell was around pH 7.0. Another report by Zivkovic *et al.*, (2010) also reported that the optimum pH of protease isolated from *Pseudomonas aeruginosa* ATCC 27853 was found to be 7.0. *Bacillus majovensis* A21 also registered maximum activity at pH 9.0 (Haddar *et al.*, 2010).

Proteases considered for industrial applications should have activity and stability over a vast range of temperatures and pH extremes for prolonged time periods. The thermal stability of the purified protease enzyme was assessed by pre incubating the enzyme at various levels of pH ranging from 5.0 - 8.0 for 2 hours.

The residual activity for pH stability profile of purified protease was calculated and depicted in Figure 23.

Figure 23

Effect of pH on stability of purified protease



Values are the mean of triplicates

It is clear from the figure that the results revealed the significant differences in the stability of purified protease at different pH with increase in time period. The purified protease was more stable at pH 7.0 and retained 100 % residual activity up to one hour and at the end of two hours incubation it maintained at 98 % activity. At pH 5.0 and 6.0 enzyme maintained 60 % activity up to one hour, thereafter it lost the activity drastically

and maintained 30 % activity at the end of two hours incubation. At pH 8.0 the residual activity of 78 % at one hour and 62 % was observed at two hours incubation. However, the stability of the protease was low at a lower pH (pH 5.0).

From the above findings, it can be stated that the purified protease isolated from *Bacillus* sp. ASASBT was more stable at pH 7.0. The high activity and stability exhibited by the enzyme at this pH represents an important attribute that provides support for its potential applications in industries.

The above finding confirming the optimum pH of the isolated purified protease to be pH 7.0 is in concurrence with various studies. Usharani and Muthuraj, (2009) stated that the optimum activity and stability of protease from *Bacillus laterosporus* was at pH 7.0. Similar results were reported on stability of protease from *Bacillus* sp. B001 by Deng *et al.,* (2010) which was found to be highly stable between pH range of 5.0 - 12.0. Benmrad *et al.,* (2016) also stated that the protease from fungi *Trametes cingulate* CTM10101 showed high stability in the pH range of 7.0 - 12.0.

4.4.2 Effect of temperature on the activity and stability of purified protease

The rate of an enzymatic reaction increases with increase in temperature. The temperature at which the enzyme activity is maximum is called as optimum temperature. Beyond the optimum temperature, the enzyme gets denatured due to its proteinaceous nature, thereby decreasing the rate of the reaction constantly (Daniel *et al.,* 2009).

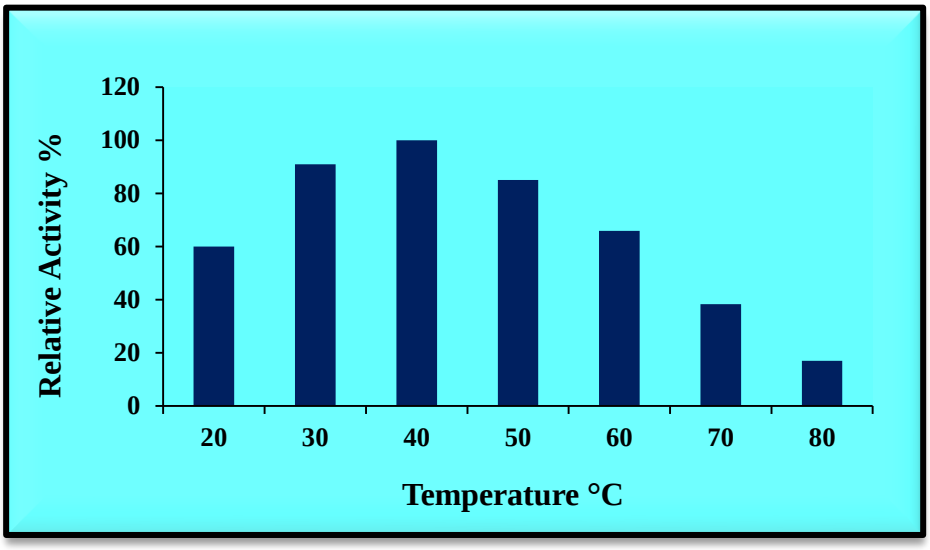
The effect of temperature on the activity of the purified protease isolated from *Bacillus* sp. ASASBT is depicted in Figure 24.

From the figure it clearly indicates that there were differences in the relative activity of the purified protease at various temperatures from 20°C to 80°C. There was a gradual increase in their relative activity from 20°C to 40°C, with a maximum activity of 100 % at 40°C, followed by 85 % relative activity at 50°C. A sudden decrease in relative activity was observed with further increase in temperature from 40°C to 80°C.

Hence, it can be inferred that the purified protease has an optimum temperature of 40°C with a relative activity of 100%.

Figure 24

Effect of temperature on the activity of purified protease



Values are the mean of triplicates

The findings of the present study are in agreement with the results of Josephine *et al.*, (2012) who reported, 40°C as the optimum temperature for maximum protease activity produced from a *Bacillus* SNR01 and also Bhaskar *et al.*, (2007) stated that the protease from a type of *Bacillus proteolytics* showed maximum activity around 40-50°C.

The thermal stability profile of purified protease was studied by pre incubating the protease enzyme at different temperatures ranging from 30-60°C for 2 hours.

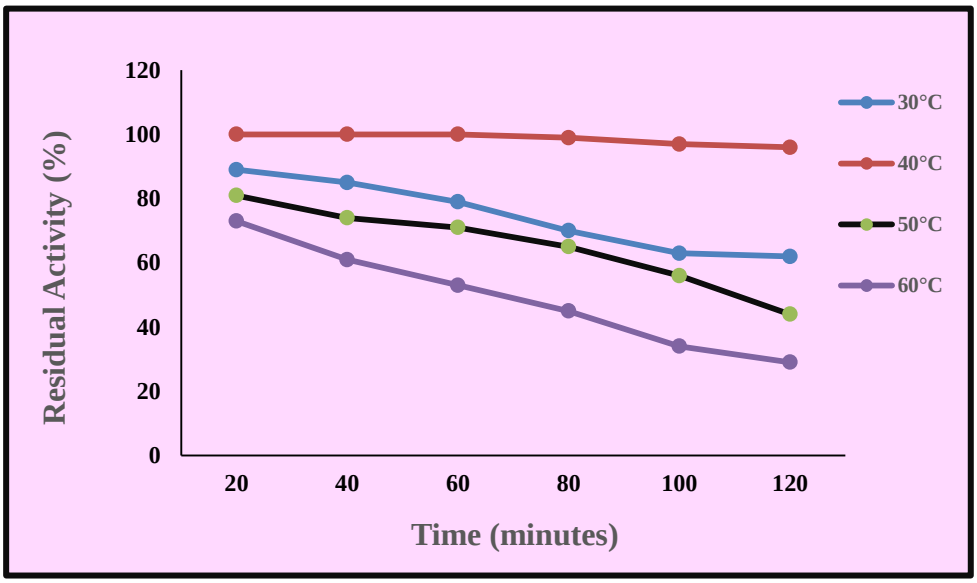
Figure 25 represents the effect of temperature on stability of purified protease.

From the figure it is clear that the stability of the isolated enzyme varies with temperature and hours of incubation. 100 % activity was exhibited at 40°C even after two hours of incubation. This was followed by 79 % activity at 30°C for one hour and 62 % activity for two hours of incubation. At 50°C, the activity showed 71 % for one hour and 44 % for two hours incubation and 53 % activity at 60°C for one hour after which the residual activity decreases.

This confirms that 40°C is the optimum temperature for protease activity since the enzyme retains 100 % activity which is stable for two hours.

Figure 25

Effect of temperature on stability of purified protease



Values are the mean of triplicates

The above results are in accordance with those of Rajkumar *et al.,* (2011) who reported that the stability of protease from *Bacillus megaterium* RRM2 was found to be between temperature 50°C and 60°C. Adinarayana *et al.,* (2003) also reported that protease from a newly isolated *Bacillus subtilis* PE-11 showed the optimum temperature to be 60°C for maximum activity and stability (100 %).

4.4.3 Effect of substrate concentration on the activity of purified protease

Kinetic studies of enzymes constitute information pertaining to rates of activation and inactivation of enzymes and actually gives the rate at which a process occurs. The important characteristics of proteases is their ability to discriminate among competing substrates and utility of these enzymes often depends on their substrate specificity (Shankar *et al.*, 2011; Dutta *et al.,* 2005). Measurements of enzymatic reactions are used to characterize enzymes with regard to their substrate affinities and maximal reaction rates. However, the speed of any fastidious reaction being catalyzed by a particular enzyme can only reach a certain maximum value. This rate is known as V_{max} while K_m is the

concentration of substrate at which half of the maximal velocity is obtained (Ahmed *et al.*, 2011). V_{max} and K_m indicate the level of affinity the enzyme has for its substrate.

Figures 26 and 27 present the Michaelis-Menten and Line weaver Burk plots of the purified enzyme obtained from *Bacillus* sp. ASASBT.

From Figure 26 it is clear that, the purified protease activity increased with increase in the substrate concentration to a certain level and then remained stable giving a hyperbolic curve. The K_m and V_{max} of the purified protease were calculated to be 0.84 µM and 92.36 µM respectively. In the Lineweaver-Burk plot (Figure 27) -1/ K_m and $1/V_{max}$ were found to be -1.219 and 0.0108 respectively. The K_m value was found to be very low which showed high affinity of the enzyme towards substrate.

Thus, the isolated purified protease was apparently suited to being a good scavenger due to its low K_m. Lower the K_m, stronger is the binding affinity of the substrate to the enzyme. From the above findings, it can be concluded that the K_m values obtained by the purified protease lower and it has a strong affinity towards the substrate.

Figure 26

Michaelis-Menten plot for purified protease

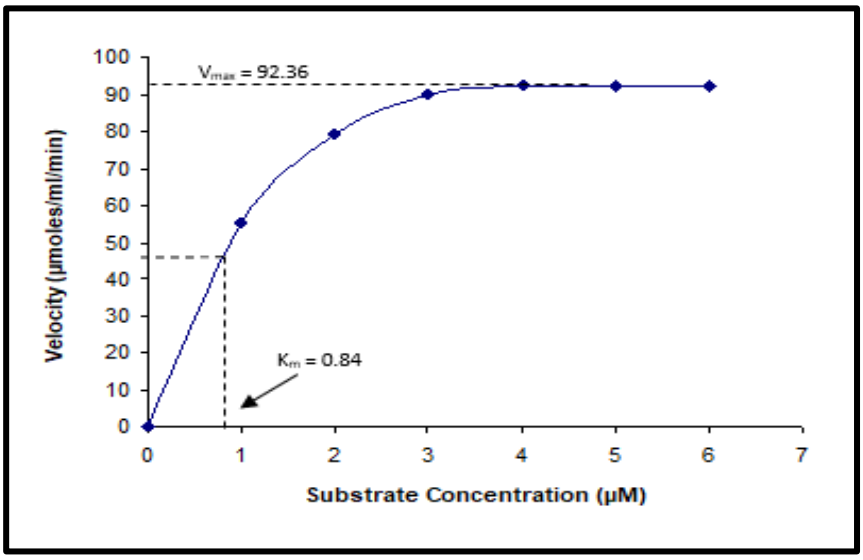
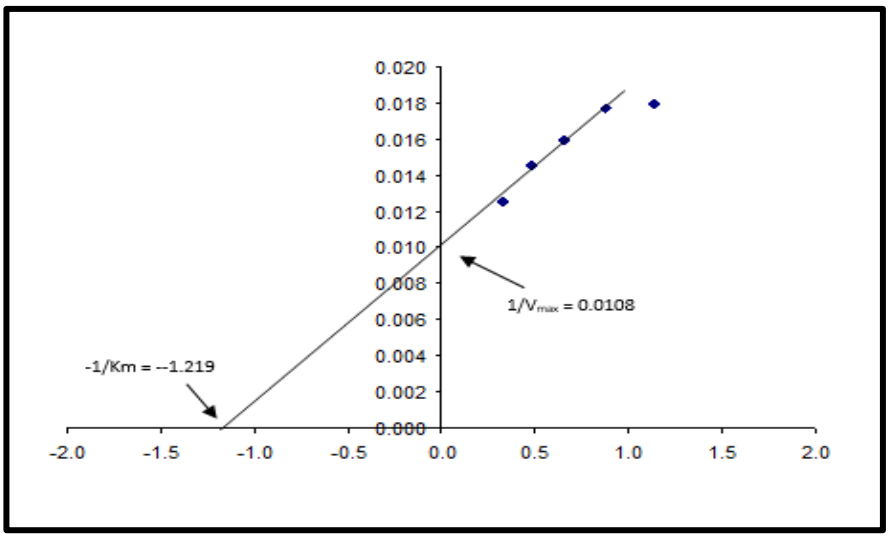


Figure 27

Lineweaver-Burk plot for purified protease



The above observations are in accordance with the findings of several other researchers. Maruthiah *et al.,* (2015) reported that the *Bacillus* sp. APCMST-RS3 from marine shell wastes showed K_m of 0.6666 g/l, *Bacillus circulans* from agronomical waste showed K_m of 0.597 mg/ml (Rao *et al.,* 2009a), *Bacillus clausii* GMBAE42 from compost had a K_m of 1.8 mg/ml and V_{max} 11.50 µM/min (Kazan *et al.*, 2005).

4.4.4 Effect of metal ions on purified protease activity

Many bacterial proteases normally require a divalent cation or a combination of such cations for activity and stability. Identification of appropriate metal ions for activity and thermo stability of the enzymes are very important for their applications at commercial levels (Nadeem *et al.*, 2007). Metal ions may be part of the active sites of enzymes and participate directly in catalytic reactions. They play an important role in enzyme activity particularly in stimulating protease activity. Magnesium and sodium normally act as a salt or ion bridge conserving histidine in active sites and maintaining the enzyme molecule's rigid conformation (Dzubiella, 2008). These phenomena indicates that

protease enzyme requires metallic ions as cofactors. Especially divalent metal ions which positively influence the activity of protease.

The impact of different metal ions on purified protease enzyme activity which included calcium chloride, magnesium chloride, manganese chloride, copper chloride, zinc chloride and sodium chloride at concentrations of 1mM and 5mM were studied and the results shown in Table 11.

Table 11

Effect of different metal ions on purified protease activity

Metal ions	Concentration	Relative activity (%)
Control	----	100
Calcium chloride (Ca^{2+})	1mM	86
	5mM	92
Magnesium chloride (Mg^{2+})	1mM	98
	5mM	100
Manganese chloride (Mn^{2+})	1mM	51
	5mM	46
Copper chloride (Cu^{2+})	1mM	35
	5mM	28
Zinc chloride (Zn^{2+})	1mM	91
	5mM	98
Sodium chloride (Na^{+})	1mM	68
	5mM	70

Values are the mean of triplicates relative to the control samples

From the table, relative activity of the purified protease on different metal ions can be observed. Among the metal ions studied, the highest activity was noted in the presence of magnesium chloride (Mg^{2+}) at a concentration of 1 mM (98 %) and 5 mM (100 %)

followed by zinc chloride (Zn^{2+}) 5 mM (98 %) and calcium chloride (Ca^{2+}) 5 mM (92 %). Lowest activity was in the presence of copper chloride (Cu^{2+}) 1 mM (35 %) and 5 mM (28 %), followed by manganese chloride (Mn^{2+}) 1 mM (51 %) and 2 mM (46 %) and sodium chloride (Na^{+}) 1 mM (68 %) and 5 mM (70 %).

Thus, from the above results it can be deduced that, magnesium chloride and zinc chloride increased the activity of purified protease from *Bacillus* sp. ASASBT to the maximum and copper chloride decreased the activity of the enzyme to the maximum extent.

The effect of metal ions on the purified protease as reported in the present study is supported by the work of Hamza, (2017) who stated that the activity of protease isolated from *Bacillus sp. Cab*44 increased in the presence of magnesium chloride. Similarly, Anbu (2013) also reported that the extracellular protease activity from *Bacillus koreensis* (BK-P21A) was greatly stimulated by calcium chloride. According to Adinarayana *et al.*, (2003) majority of the purified proteases from various species of *Bacillus* are stimulated by calcium chloride, magnesium chloride and manganese chloride ions. The findings of Mechri *et al.,* (2017) also showed that addition of zinc sulfate, ferrous sulfate and calcium chloride at 2 mM concentration enhanced the enzyme activity. However, in another report, metal ions like manganese chloride and calcium chloride were found to be potent enhancers (Siddalingeshwara *et al.,* 2010).

4.4.5 Effect of inhibitors on purified protease activity

Protease inhibitors are molecules that block the activity of proteases and typically function on classes of proteases with similar mechanisms of action. Protease inhibitors can either be in the form of proteins, peptides or small molecules (Ritchie, 2013). The effect of different class specific protease inhibitors on the purified protease from *Bacillus* sp. ASASBT were investigated.

Table 12 depicts the inhibitory effect of various concentrations of inhibitors on proteolytic activity of purified protease.

From the table it can be observed that, of all the inhibitors, the maximum inhibition was exhibited by EDTA at 5 mM concentration (15 %) followed by the same inhibitor at

1 mM concentration (23 %). However, pepstatin at 1 mM and PMSF also at 1 mM concentration inhibited the enzyme activity only to a minimum (94 % and 86 % respectively) as compared to the other inhibitors. The enzyme inhibition studies can provide high insight into the nature of the enzyme, its cofactor requirements and the nature of the active center.

Thus, it clearly indicates that the purified protease belongs to the family metalloprotease.

Table 12

Effect of inhibitors on purified protease activity

Protease Inhibitors	Concentrations (mM)	Relative activity (%)
Control	---	100
PMSF	1	86
	5	79
EDTA	1	23
	5	15
Pepstatin	1	94
	5	82
Indoacetic acid	1	80
	5	72

Values are the mean of triplicates relative to the control samples

Similar kind of results as discussed above were observed by Jain *et al.*, (2012) who reported that EDTA strongly inhibited protease activity in *Bacillus* sp. According to Annamalai *et al.*, (2014), protease isolated from *Bacillus alveayuensis* CAS 5 using marine wastes belongs to the family metalloprotease and the metalloprotease inhibitor EDTA significantly inhibited the protease activity at both the concentrations 1mM and 5mM. Similar inhibitory effects with EDTA at concentrations 0.1 mM, 1 mM and 10 mM were observed by Jellouli *et al.*, (2008) and Wang *et al.*, (2009). A decrease in the enzyme activity by EDTA confirms that metal ions may be necessary for activity or stability of the enzyme (Nilegoankar *et al.*, 2002).

4.4.6 Effect of surfactants and oxidizing agents on purified protease activity

In addition to activity and stability at high pH range and various temperatures, protease incorporated into detergent formulations must be compatible and stable with all commonly used detergent compounds like surfactants, bleaching agents and other additives, which might be present in the formulation. Most of the commercial detergent proteases are stable in the presence of various detergent components. However, most of them are unstable in the presence of oxidizing agents (Jain *et al.,* 2012). Thus, it is necessary to search for new proteases with novel properties from the many different sources as available.

The effect of the influence of anionic surfactant SDS and cationic surfactant CTAB at 0.25% and 0.05% (w/v), non-ionic surfactant Triton X-100 and oxidizing agent H_2O_2 at 0.5% and 2.5% (v/v) on purified protease activity was measured.

Table 13 reveals the effect of surfactants and oxidizing agents on purified protease activity.

The table shows that the enzyme was highly stable in the presence of the non-ionic surfactant 0.5 % and 2.5 % (v/v) Triton X-100 with 92 % and 91 % activity respectively. In addition, the protease enzyme was relatively stable in the presence of the strong anionic surfactant, approximately 89 % and 48 % of its initial activity in the presence of 0.05 % and 0.25 % (w/v) concentration of SDS respectively. The cationic surfactant CTAB showed 52 % and 18 % of the enzyme activity at 0.05 % and 0.25 % (w/v) concentration.

In addition, the investigations on the effect of the oxidizing agent H_2O_2 on purified protease enzyme showed that the enzyme activity was influenced by H_2O_2 and its relative activity was 51 % and 55 % in the presence of 0.5 and 2.5 % (v/v) concentrations of the surfactant respectively. The highest stability of the protease enzyme in the presence of oxidizing agents is a very important characteristic for its main use in detergent formulations.

Therefore, the purified protease was highly stable in the presence of non-ionic surfactant Triton X-100, which is commonly used in biochemical applications to solubilize proteins.

Table 13

Effect of surfactants and oxidizing agents on purified protease activity

Additives	Concentration (%)	Relative activity (%)
Control	----	100
SDS	0.05 (w/v)	89
	0.25 (w/v)	48
CTAB	0.05 (w/v)	52
	0.25 (w/v)	18
Triton X-100	0.5 (v/v)	92
	2.5 (v/v)	91
H_2O_2	0.5 (v/v)	51
	2.5 (v/v)	55

Values are the mean of triplicates relative to the control samples

The results obtained in the present study are in agreement with those of other research workers. Patil *et al.,* (2016) and Bajaj *et al.,* (2013) stated that the *Bacillus circulans* MTCC 7942 and *Bacillus cereus* NS-2 protease was stable in the presence of Triton X-100, hydrogen peroxide and SDS. Anbu *et al.,* (2013) also reported that protease from *Bacillus koreensis* BK-P21A was moderately stable in the presence of hydrogen peroxide. The metalloprotease isolated from *Aeromonas caviae* showed an inhibitory effect in the existence of the cationic surfactant – CTAB and the enzyme showed maximum activity in the presence of Triton X-100 (Laishram and Pennathur, 2016).

4.4.7 Effect of organic solvents on purified protease activity

The use of proteases in organic media has been one of the most important novelties of catalysis in the last few years and expected to play a great role in the upcoming years. One of the major concerns in this regard has been their instability and low-activity in organic media, since proteases are suitable for use as bioactive peptides in pharmaceutical and ester synthesis under non-aqueous conditions (Vulfson *et al.,* 2001).

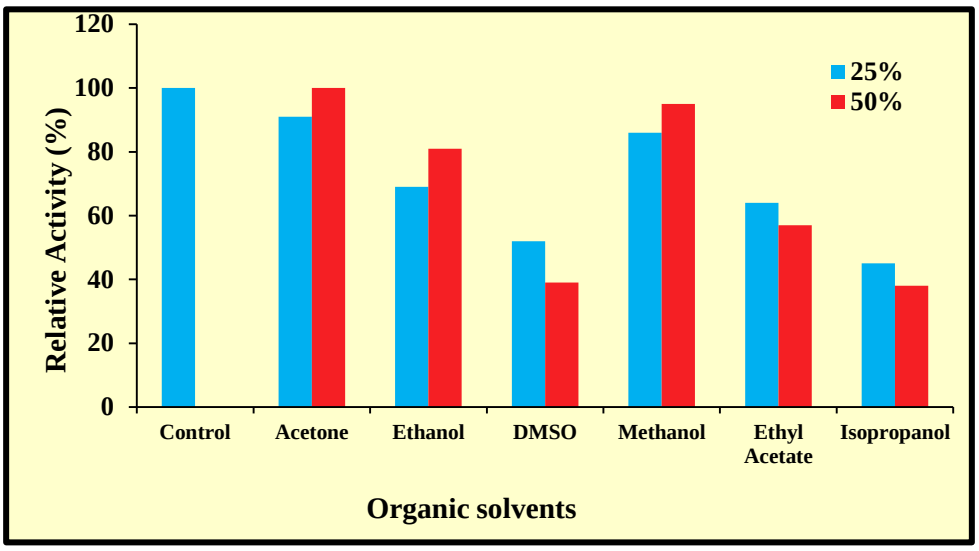
The effect of various organic solvents such as acetone, ethanol, DMSO, methanol and ethyl acetate on purified protease and its stability were investigated and the results shown in Figure 28.

The figure revealed that the enzyme showed increased activity (91 % and 100 %) in the presence of both concentrations 25 % and 50 % of acetone. This was followed by methanol with 86 % and 95 % activity and ethanol with 69 % and 81 % activity respectively. However, the activity of the enzyme decreased in the presence of organic solvent like isopropanol which gave only 45 % and 38 % of enzyme activity for 25 % and 50 % respectively. DMSO also exhibited low enzyme activity of 52 % and 39 % respectively for 25 % and 50 % concentrations.

Thus, this study evidenced that, purified protease from *Bacillus* sp. ASASBT had maximum tolerance in the presence of acetone, methanol and ethanol.

Figure 28

Effect of organic solvents on purified protease activity



Values are the mean of triplicates relative to the control samples

The present finding that methanol, acetone and ethanol increased the activity of protease is similar to several reports given by other researchers. Jellouli *et al.*, (2011) stated that protease from *Bacillus licheniformis* was stable with organic solvents like ethanol, diethyl-ether, methanol and hexane. According to the reports of Mesbah and Weigel (2014) also alkaline protease from *Alkalibacillus* sp. recorded maximum protease

activity in the presence of ethanol and methanol. Another report by Dorra *et al.*, (2018) stated that protease from *Bacillus halotolerans* strain CT2 showed a good stability in the presence of hexane, methanol, ethanol, acetone and dichloromethane. Protease from *Bacillus* sp. showed maximum activity in the presence of ethanol, ethylene glycol, butanol, xylene and perchloroethylene according to Sana *et al.*, (2006).

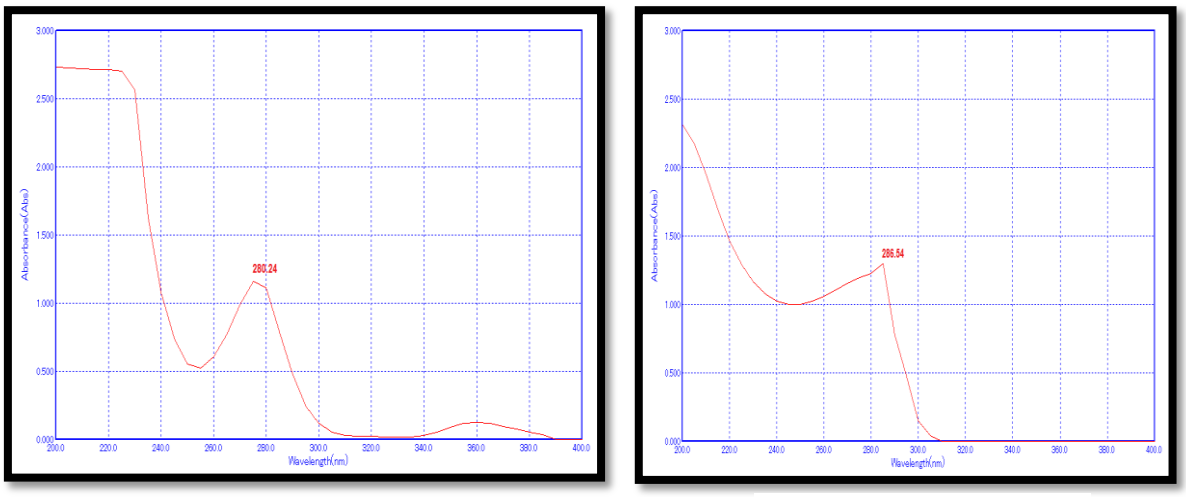
4.4.8 UV-visible spectrum of purified and commercial protease

The biological macromolecules - proteins and nucleic acids absorb light in the UV-visible region of the spectrum. Absorbance measurements are used for measuring concentrations for the detection of conformational changes, ligand binding and enzyme reactions. Generally, proteins show maximum absorption between 275 nm and 280 nm. The peptide groups of protein main chain absorb light in the far-UV range (180-230 nm). The aromatic side chains of tryptophan, tyrosine and phenylalanine also absorb light in this region and in addition, they absorb in the 240-300 nm region, this is near-UV or aromatic region. Disulfide bonds that form between two cysteine residues also show an absorbance band near 260 nm (Schmid, 2001).

Figure 29 represents the UV-visible spectrum of purified protease from *Bacillus* sp. ASASBT as compared with the commercial protease, which will show the originality of the isolated enzyme.

Figure 29

UV-visible spectrum of purified and commercial protease



Commercial protease **Purified protease**

The figure clearly indicates that purified protease showed a maximum absorption peak at 286 nm and when compared to the commercial protease enzyme, it showed a maximum absorption peak at 280 nm indicating the presence of an aromatic side chain, especially the presence of tyrosine, tryptophan, phenylalanine or their residues.

Hence, it is evident that purified enzyme showed an absorption peak at 280-286 nm corresponding to the aromatic side chains as for the commercial protease.

The above results correlate with those given by Nakashima *et al.,* (2008) who also demonstrated that the spectrophotometric assay for protease activity showed the maximum absorbance peak at 280 nm. Antosiewicz and Shugar, (2016); Yang *et al.,* (2014) also studied UV–visible spectroscopy of tyrosine side-groups in studies of protein structure and protease activity in in-vivo depicted the absorbance maxima at 245 nm.

4.4.9 Fourier Transform Infrared (FTIR) spectroscopy of purified and commercial protease

The presence of various functional groups in the purified protease was compared and confirmed by FTIR analysis with the commercially available protease.

Figures 30 and 31 present the FTIR spectra of purified and commercial protease.

From the figures, it can be observed that the characteristic infrared pattern of the peptide bonds in the purified protease isolated from *Bacillus* sp. ASASBT and commercial protease exhibits specific bond bending and stretching at specific wavelengths.

The absorption spectrum showed strong peaks at 3388.11, 3363.03, 3305.17 cm^{-1} which attributes to the O-H stretching vibration. Peaks at 1646.32, 1659.82 cm^{-1} is assigned to C=O stretching vibration, thereby indicating the presence of carbonyl compounds in the protein. The C-N stretch vibration frequencies observed at 1238.35, 1079.22, 608.57 cm^{-1} confirmed the presence of aliphatic groups (CH2- S-) and C-S stretch at 664.51 cm^{-1} confirms the existence of thiol or thio ether group in the protein. Presence of C=O group and C-N stretching in the protein sample confirms the presence of amino acids and presence of amine group in their side chains. As there is no absorbance in between the regions 2220 - 2260 cm^{-1}, it indicates that there are no cyanide groups present in the protein.

Thus, it can be inferred that both the purified and commercial enzymes showed similar bond bending and stretching. Hence, the functional groups present in the purified protease are homogenous to the commercial protease.

Figure 30

FTIR spectroscopy of commercial protease

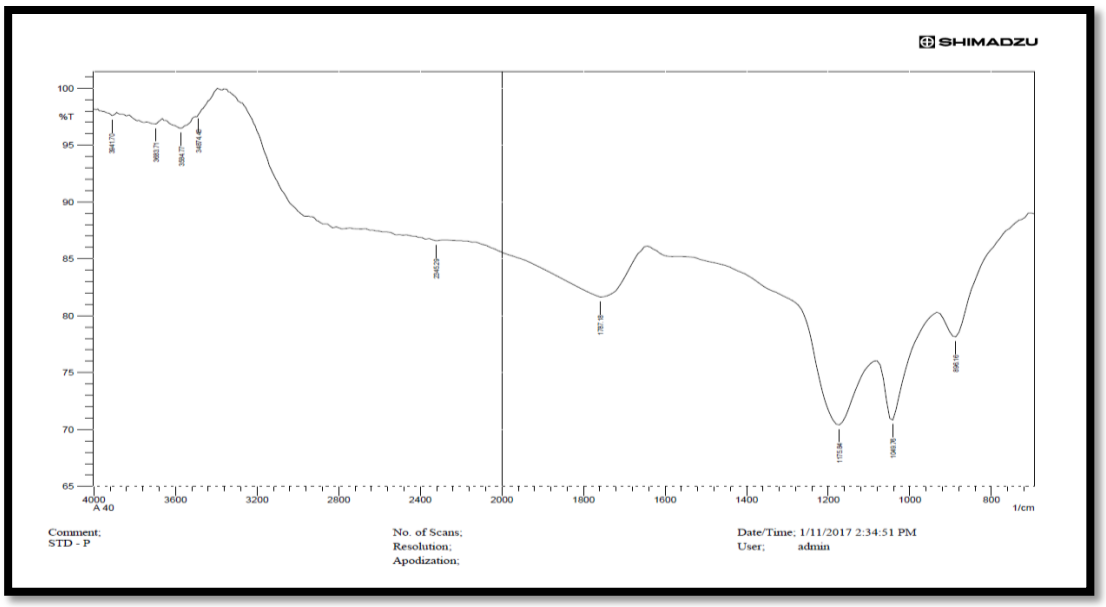
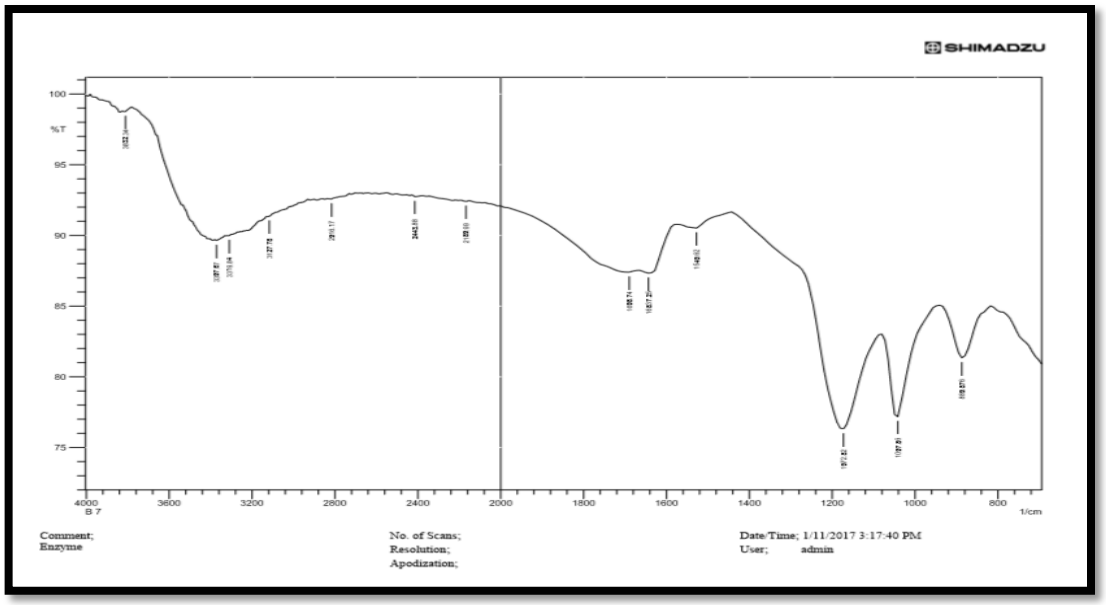


Figure 31

FTIR spectroscopy of purified protease



The findings of the present study are in accordance with those of Sinha and Khare, (2014) and Raval *et al.*, (2014) who mentioned that the secondary structural perturbations in *Bacillus* sp. EMB9 protease and seawater haloalkaliphilic bacterial protease were confirmed by FTIR spectroscopy. Similar functional groups were also observed in bromelain proteases from *Ananas comosus* with very slight variations in the IR bands both in terms of intensity and frequency (Swaroop and Viswanathan, 2013).

Phase IV - Highlights of the findings

- **Optimum pH of the gel filtration purified protease from *Bacillus* sp. ASASBT was found to be 7.0 and optimum temperature 40°C.**
- **The kinetic constant Km of the enzyme was 0.84 and Vmax 92.36 µmoles.**
- **Magnesium chloride (Mg^{2+}) and Triton X-100 increased protease activity while EDTA inhibited the protease activity maximally and hence the isolated enzyme may be a metalloprotease.**
- **The organic solvents - acetone, methanol and ethanol increased the protease activity to the maximum in both 25 % and 50 % concentrations.**
- **UV-visible spectra and FTIR analysis confirmed the presence of purified protease.**

4.5 Phase V - Immobilization of purified protease

Immobilization is employed to produce reusable enzymes and to reduce other limitations of enzymes like activity, stability, resistance to inhibitors and also to reduce contamination and thereby to improve the purity of enzymes (Barbosa *et al.*, 2015). Among all the immobilization methods, the best method to enhance the catalytic performance of an immobilized enzyme is entrapment. The entrapment method is the physical confinement of the enzyme within the polymer network and attracted scientific attention due to its eco-friendly nature, cost-effective, mild conditions required for immobilization process and nontoxic nature (Bilal *et al.*, 2017 and Rao *et al.*, 2009b). In the present study the gel filtration purified protease from *Bacillus* sp. ASASBT was immobilized using alginate-chitosan, agar-agar and calcium-alginate methods. For the

preparation of beads with proper permeability and rigidity, parameters such as concentration and molarity were optimized based on the literature (Rezakhani *et al.*, 2014, Anwar *et al.*, 2009, Sattar *et al.*, 2018). Further the immobilized beads were studied for selected parameters like pH, temperature, storage and reusability.

Plate 9 shows the alginate-chitosan, agar-agar and calcium-alginate immobilized beads of purified protease.

The alginate-chitosan method at a concentration of 2 % alginate, 2 % chitosan and 0.7 M calcium chloride was the optimum concentration for formation of spherical and stable beads which is depicted in Plate 9(a). An optimum concentration of 3 % agar-agar solution was used for agar-agar entrapment and the formation of square shaped immobilized enzyme is shown in Plate 9(b). A concentration of 2 % sodium alginate and 0.2 M calcium chloride for calcium-alginate method which depicts spherical and stable beads is represented in Plate 9(c).

Plate 9

Immobilization of purified protease

a) Alginate-chitosan method **b) Agar-agar method** **c) Calcium-alginate method**

Immobilization of enzyme using entrapment method is reported by others also. Maheswari and Priyadharshini, (2014) and Chaudhary *et al.*, (2019) studied immobilization of amylase by different entrapment methods using sodium alginate, agar, polyacrylamide, gelatin and chitosan.

4.5.1 Determination of immobilized protease activity

The activity of immobilized protease and its immobilization efficiency were studied and the results given in Table 14.

Table 14

Determination of immobilized protease activity

Immobilization methods	Immobilized protease activity (U/ml)	Efficiency of immobilization (%)
Alginate-chitosan	92.37	56
Agar-agar	86.59	49
Calcium-alginate	83.68	42

Values are the mean of triplicates

The data from the table confirms the highest activity of 92.37 U/ml and immobilization efficiency of 56 % when the enzyme was immobilized by the alginate-chitosan method as against the calcium-alginate method which gave the lowest enzyme activity of 83.68 U/ml and the lowest immobilization efficiency of 42 %. This low immobilization efficiency and activity may be due to maximum leakage of enzymes from the immobilized beads due to the larger pore size of the beads, which results in lower immobilization percentage. In the case of agar-agar, immobilization showed moderate activity and efficiency percentage (86.59 U/ml and 49 %).

Thus, it can be inferred from the table that, of all the entrapment methods, alginate-chitosan method exhibited maximum immobilized enzyme activity and efficiency.

The results of the present study were supported by several other research workers. Rezakhani *et al.,* (2014) reported that the physical immobilization (entrapment) of protease in alginate-chitosan beads exhibited reasonable stability and good activity. The findings of Anwar *et al.,* (2009) also showed that the total enzyme activity after entrapment of protease in calcium-alginate beads showed the maximum level. In agar-agar entrapped protease, the maximum activity and stability of protease was observed (Sattar *et al.,* 2018).

4.5.2 Characterization of immobilized protease

4.5.2.1 Effect of pH on activity of immobilized protease

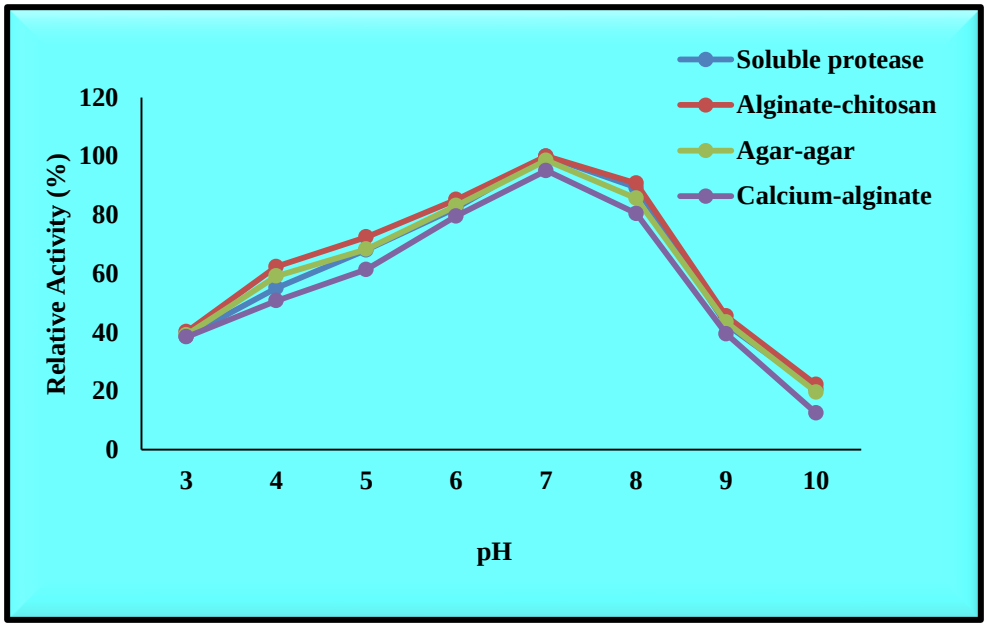
Figure 32 represents the effect of pH on the relative activity of the immobilized and soluble proteases of *Bacillus* sp. ASASBT. The activity was studied at a pH range of 3.0 - 10.0 and an optimum temperature of 40°C.

From the figure, it is evident that, there was a gradual increase in the activity of the soluble enzyme from pH 3.0 (38 %) to 7.0 (100 %), as well as all the three immobilized enzymes, alginate-chitosan showed 40 % to 100 %, followed by agar-agar 39 % to 98 % and calcium-alginate 38 % to 95 %. Then there was a gradual decrease from pH 8.0 to 10.0 in soluble and all the immobilized enzymes, which implies that the optimum pH for the activity of the immobilized enzyme is 7.0.

Even though all the three methods showed a similar trend, the alginate-chitosan method revealed a higher increase in relative activity compared to other two methods of immobilization. These results thus clearly indicate that there is no change in the optimum pH of the enzyme on entrapment.

Figure 32

Effect of pH on activity of immobilized protease



Values are the mean of triplicates

The findings of the present study that immobilization does not change the optimum pH are on par with the results of the studies of Wang *et al.,* (2011) who demonstrated that the immobilization of the enzyme glucose oxidase in alginate-chitosan microcapsules showed no change in optimal pH. Another study by Kim and Park, (2004) also stated that the alginate-chitosan immobilized α-amylase preparation has the same optimum pH as the free enzyme. Agar-agar immobilization of endo-1,4-xylanase also showed no change in the optimum pH before and after immobilization (Bibi *et al.,* 2015). It was also reported that the optimum pH of pectinase remained the same after it was covalently immobilized with agar-agar (Tuoping *et al.,* 2008). Similar findings were also expressed by Anwar *et al.,* (2009) who stated that pH of the protease enzyme from newly isolated strain of *Bacillus subtilis* KIBGE-HAS showed no changes before and after immobilization with calcium alginate and remained stable at pH 7.5. Another study by Rehman *et al.,* (2013) reported that there was no change in the pH optima of pectinase immobilized within calcium alginate beads.

4.5.2.2 Effect of temperature on activity of immobilized protease

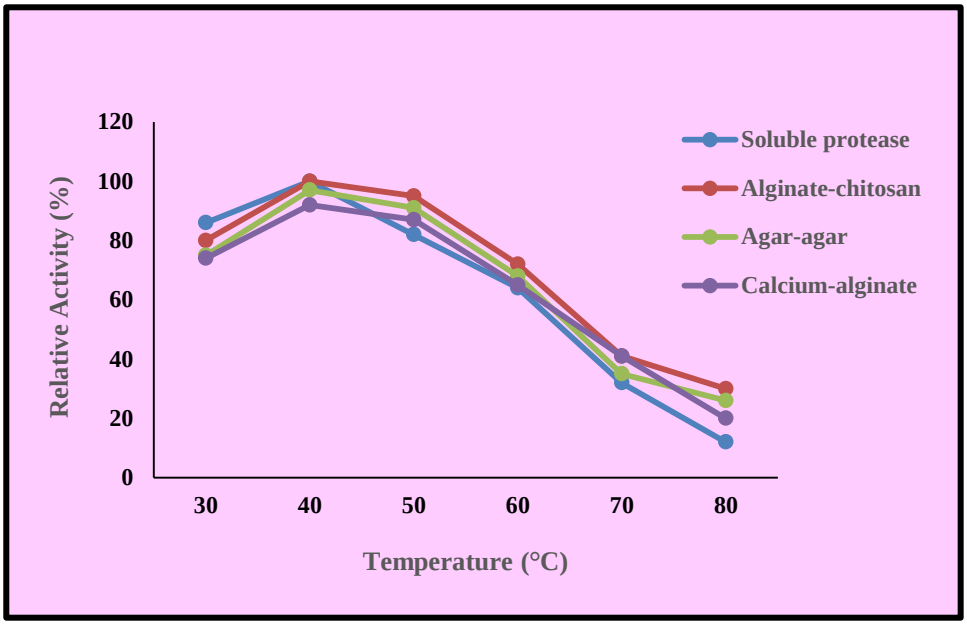
The soluble protease and entrapped protease were assayed at various temperatures ranging from 30°C to 80°C at an optimum pH of 7.

Figure 33 illustrates the optimum temperature of the immobilized enzymes and soluble enzyme.

It is understood from the figure that the activity of both soluble and all the three immobilized proteases increased from 30°C to 40°C and then gradually decreased upto 80°C. Hence, it can be inferred that the optimum temperature for the isolated enzyme is 40°C for both the soluble and immobilized forms. However, in the case of alginate-chitosan immobilized enzyme, even at 50°C there was a high activity of 95 % which was not so for the soluble protease and also the agar-agar and calcium-alginate immobilized proteases. The highest relative activity at a temperature of 40°C for alginate-chitosan method showed 100 %, followed by agar-agar 97 % and calcium-alginate 92 %. At 50°C in alginate-chitosan method exhibited 95 %, followed by agar-agar 91 % and calcium-alginate 87 % respectively. As regards the soluble enzyme, the highest relative activity was exhibited at 40°C (100%) followed by 30°C (86%).

Figure 33

Effect of temperature on activity of immobilized protease



Values are the mean of triplicates

Therefore, a shift to a higher temperature was observed in the activity of the immobilized enzymes with respect to that of the free enzyme. Hence it was difficult to determine the near optimal temperature for the immobilized and soluble enzymes.

Furthermore, it was observed that when the temperature was raised above the optimum value from 40°C to 50°C, the alginate-chitosan immobilized enzyme still retained 95% of its activity while the free enzyme showed only 82% activity. This shows the significance of immobilization.

The slight increase in temperature for maximum activity of immobilized enzyme might be due to fact that, immobilized enzyme requires greater amount of activation energy as compared to free enzyme for which it is readily available in the aqueous environment of the reaction.

The above findings are in agreement with the reports of Phadtare *et al.*, (2002) who stated that immobilization increases the optimum range of temperature to some extent and the immobilized enzyme shows greater relative activity at different temperatures as compared to free enzyme. Another report mentioned that thermal stability of an enzyme increased because of the immobilization process and could not protect the tertiary structure of the peptide from conformational changes caused by higher temperatures

(Jia *et al.,* 2013). The present results are also supported by Lu *et al.,* (2007) who stated that the optimum temperature for free laccase had maximal activity at 60°C and alginate-chitosan immobilized laccase had 65°C. A similar study by Kumar *et al.,* (2006) reported a shift in pH and temperature optima on entrapment of α-amylase in alginate beads. According to Rehman *et al.,* (2014), immobilization of pectinase from *Bacillus licheniformis* KIBGE IB-21 using agar-agar as a support found that the soluble enzyme showed maximum activity at temperature 45°C, while agar-agar entrapped pectinase showed maximum activity at 50°C. Sharma *et al.,* (2014) said that the free and agar entrapped enzymes were active at a higher temperature with no change in the optimum temperature of 50°C, whereas, optimum temperature for calcium-agar entrapped enzyme activity was shifted from 50°C to 55°C. Geethanjali and Subash, (2013) emphasized that immobilization of protease on calcium-alginate revealed no change in the optimum temperature of 40°C before and after entrapment. Calcium-alginate immobilized xylanase from *Bacillus amyloliquefaciens* SK-3 showed a shift in temperature from 60°C to 70°C with reference to free enzyme (Kumar *et al.,* 2016).

4.5.2.3 Storage stability of immobilized protease

Storage stability is an important factor for commercialization of an enzyme. The process of immobilization is a form of storage and it is advisable to immobilize enzymes because free enzymes can lose their activities soon. Generally, an enzyme in aqueous solution is not stable during storage and the activity gradually reduces (Cevik *et al.,* 2011).

Both the enzymes, soluble and immobilized were stored at 4°C and 27°C, its relative activity noted every day for 10 days to determine the storage stability of the soluble and entrapped enzyme. These temperatures were selected based on the optimal storage temperatures of the isolated protease.

Figure 34 indicates the storage stability of the soluble and immobilized proteases from *Bacillus* sp. ASASBT.

The soluble enzyme at 4°C was stable only for about 2 days with 100 % activity which gradually decreased and reached 50 % activity on the 10^{th} day. At 27°C, also the soluble enzyme exhibited 100 % activity for 1 day and this decreased to 0 % activity on the 10^{th} day.

In the case of alginate-chitosan immobilized protease, at 4°C the enzyme exhibited 100 % activity for nearly 6 days and this decreased to 80 % on the 10^{th} day. At 27°C, the enzyme recorded 100 % activity for 2 days which decreased to 40 % on the 10^{th} day.

In agar-agar immobilized protease, at 4°C, the enzyme was stable only for 3 days with a relative activity of 100% and then gradually decreased to 75 % on the 10^{th} day. At 27°C, the immobilized enzyme revealed 2 days with 100 % activity for 1 day and this decreased to 40 % on the 10^{th} day.

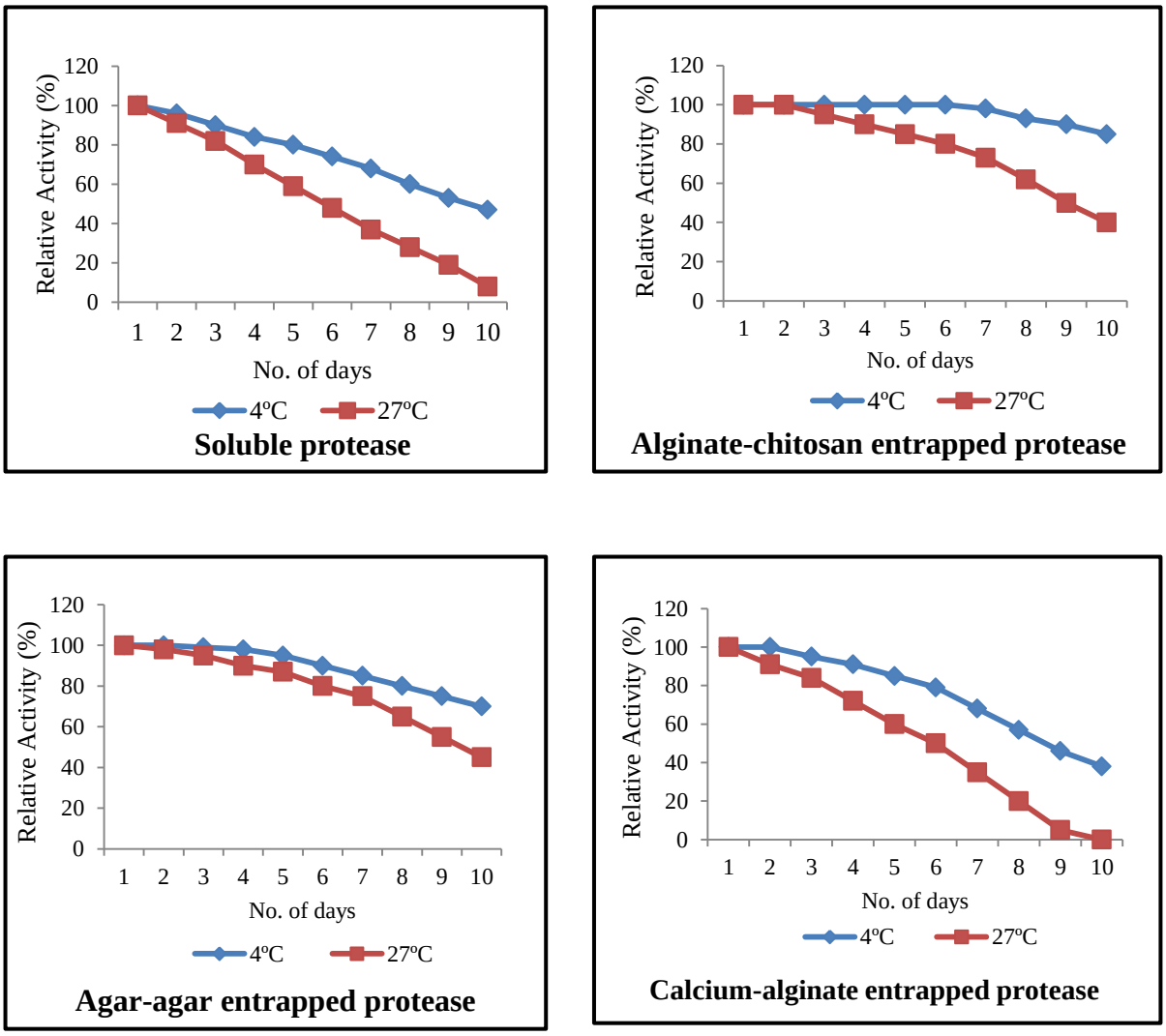
The calcium-alginate immobilized protease at 4°C was stable for about 2 days with 100 % activity, then gradually decreased and reached 40 % activity on the 10^{th} day. At 27°C, also the enzyme exhibited 100 % activity for 1 day and this decreased to 0 % activity on the 10^{th} day.

The above observations suggest that the activity was more stable at 4°C when compared to 27°C in all the enzymes and thus 4°C is the suitable temperature for storage of most of the enzymes. At 27°C the enzyme might undergo autolysis because being themselves proteins they are known to be cleaved by other protease molecules which might be the reason of inactivation of the soluble and immobilized enzymes at this temperature. Immobilization with alginate-chitosan made the enzymes stable with 100 % activity for 6 days at 4°C and this activity decreased to only 80 % on the 10^{th} day. Even at 27°C, there was 100 % activity for 2 days and this decreased to 40 % on the 10^{th} day. After this, agar-agar immobilization was the best since 100 % activity was maintained 3 days at 4°C and this become 75 % after 10 days. Alginate-chitosan was the best one for immobilization since the enzyme exhibited 100 % activity for 6 days at 4°C which decreased to 80 % on the 10^{th} day.

Enzyme protease is more stable at 4°C than 27°C in the soluble and immobilized forms. Finally, the result demonstrated that the immobilization process maintained the stability of the enzyme for a longer time than the soluble enzyme at 4°C and 27°C.

Figure 34

Storage stability of soluble and immobilized protease



The above findings which reported decrease in enzyme activity with increase in the number of days of storage is supported by the work of Thu and Krasaekoopt, (2016) who stated, when the concentration of alginate increased, the retention of entrapped protease activity increased. Chitosan also provides better stability of entrapped protease. Alginate-chitosan method showed the highest protease stability during the10 days storage at 4°C. According to Sattar *et al.*, (2018); Datta *et al.*, (2017); Bilal *et al.*, (2017) and Rodrigues *et al.*, (2013), the storage stability of the agar-agar entrapped and soluble protease at 4°C for 3 days showed maximum residual activity where, insignificant activity was retained in soluble protease. A similar study conducted by Qader *et al.*, (2007) reported, a 36% loss in

the residual activity of calcium-alginate immobilized enzyme at 30°C after 3 hours and observed 86% loss in residual activity at 40°C within 2 hours. The increase in storage stability of the immobilized enzyme was due to the alteration effects which happened after immobilizing the enzymes.

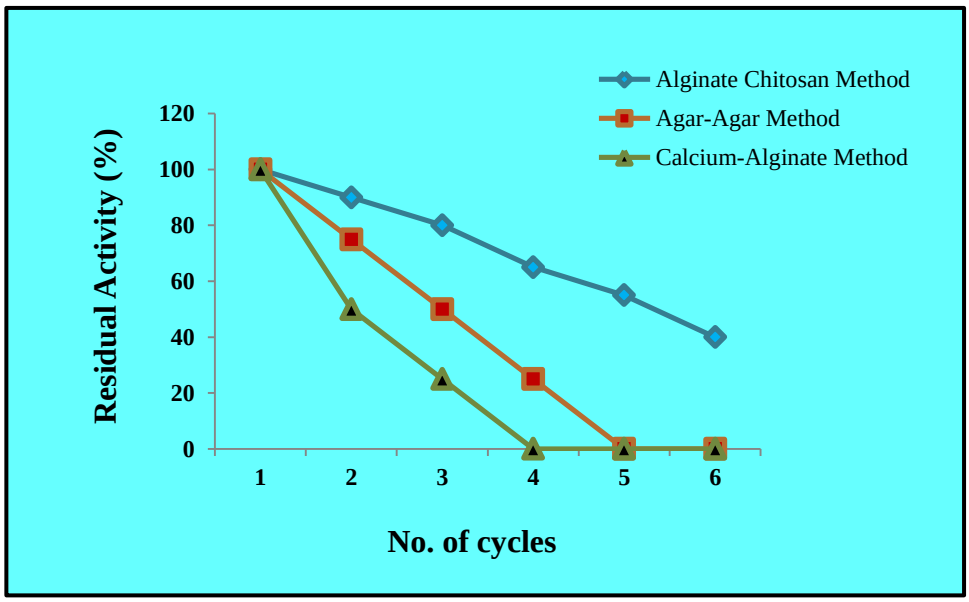
4.5.2.4 Reusability of immobilized protease

One of the most important and significant purposes of immobilizing enzymes is for its reusability and stability (Torabizadeh *et al.*, 2014).

Figure 35 shows the reusability of different types of immobilized protease.

Figure 35

Reusability of immobilized protease



Values are the mean of triplicates

The reusability of the immobilized enzymes as depicted in the figure was measured for 6 cycles. The 1st cycle residual activities were standardized as 100 %. All the enzyme exhibited 100 % activity on day 1, which gradually decreased after each reuse of the enzyme. The alginate-chitosan entrapped protease showed residual activity of 90 % in the 2nd cycle, with a gradual decrease in activity of 40 % during the 6th cycle. In the agar-agar entrapped protease, the residual activity was 75 % during the 2nd cycle and this gradually

declined in the 4th cycle to 25 % and in the 5th and 6th cycles there was no activity at all. A similar trend was followed in the calcium-alginate immobilized enzyme which showed activity only up to 3 cycles. The decrease in activity of the enzyme after each frequent use might be due to frequent washes with buffer and centrifugation after each cycle.

Therefore, among the alginate-chitosan, agar-agar and calcium-alginate immobilized methods, alginate-chitosan immobilized enzyme revealed maximum reusability.

The above findings are supported by Kumar *et al.,* (2016) who emphasized in his studies that the stability of laccase immobilized enzyme in alginate retained 36 % activity after 3 cycles. According to Bibi *et al.,* (2015), the reusability of agar-agar immobilized endo-1,4-xylanase was studied up to 6 cycles and the results showed considerable stability with more than 80 % of activity in the 2nd cycle, followed by a gradual decrease in further cycles. A similar report was given by Rehman *et al.,* (2014), who stated that agar-agar entrapped pectinase showed maximum activity till the 3rd cycle. When calcium-alginate entrapped enzyme activity was assayed for 4 cycles, the enzyme showed 80% activity during the 2nd cycle and 35 % activity in the 3rd cycle, while complete loss in activity of the entrapped enzyme was observed during the 4th cycle (Anwar *et al.,* 2009). In another study by Kumar *et al.,* (2006), the alpha-amylase entrapped in calcium-alginate beads was reused for 6 cycles with approximately 30 % loss in activity.

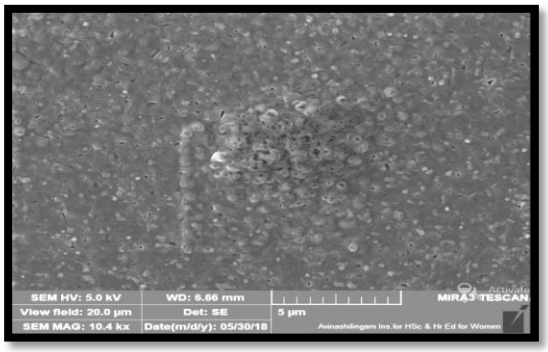
4.5.2.5 Surface morphology of immobilized protease

The surface morphology of soluble protease, alginate-chitosan immobilized, agar-agar immobilized and calcium-alginate immobilized proteases were determined by Scanning Electron Microscopy (SEM).

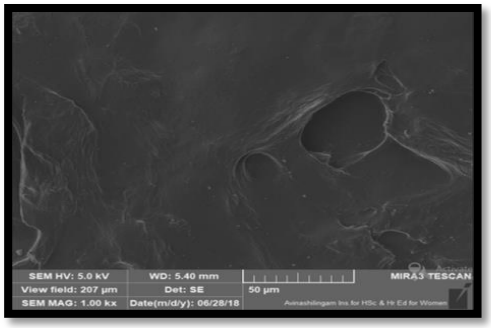
Plate 10 shows the surface morphology of the immobilized enzyme.

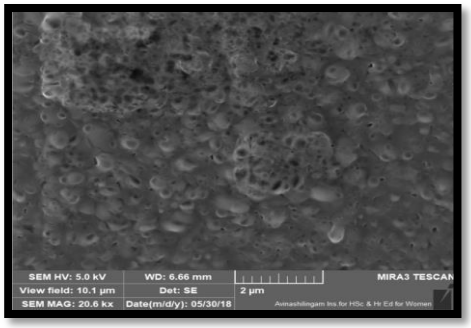
Plate 10

Scanning electron micrographs of soluble and immobilized protease

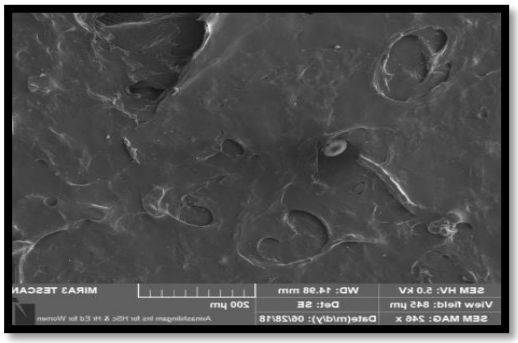


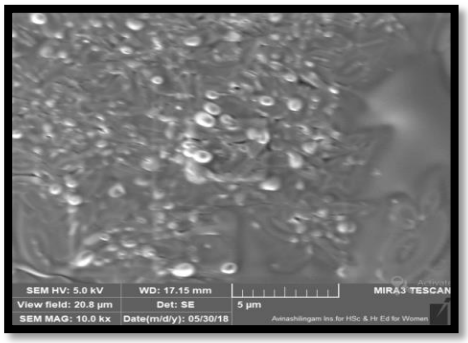
Soluble protease



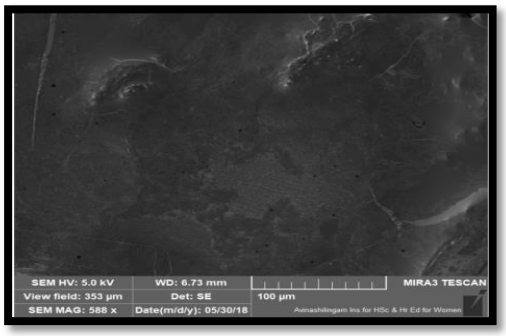


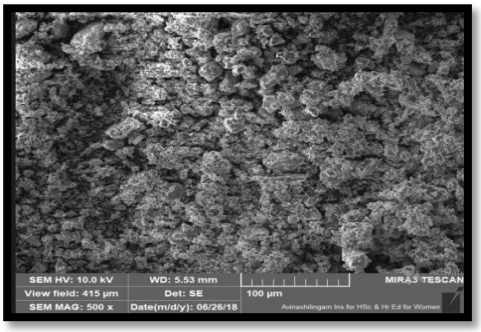
Alginate-chitosan with and without entrapped protease





Agar-agar with and without entrapped protease





Calcium-alginate with and without entrapped protease

The surface morphology of immobilized beads with and without entrapped protease has a compact structure and displayed significant difference in their structure. The SEM images revealed that alginate-chitosan, agar-agar and calcium-alginate without entrapped protease exhibited uniform even surfaces with several covered layers, whereas, alginate-chitosan, agar-agar and calcium-alginate with entrapped protease displayed heterogenous globular aggregates on the surface.

Thus, the surface morphological studies provided the evidence for significant changes in surface of the beads before and after entrapment of protease. The surface of the immobilized beads showed a mesh like compact structure.

The above results are in accordance with the reports of several other researchers. Rezakhani *et al.,* (2014) and Lu *et al.,* (2007) showed that the SEM morphology of alginate-chitosan entrapped protease had a compact structure and emulsification and gave very small microcapsules. Similarly, parallel findings according to the surface morphology of agar-agar with and without immobilized protease using SEM exhibited uniform even surface on without entrapped protease and in with entrapped protease showed several covered layers with globular aggregates (Rehman *et al.,* 2016). SEM images of calcium-alginate immobilized beads with and without xylanase were different from each other in shape and size. The beads without enzyme depicted irregular shape with cracks on the surface, whereas enzyme immobilized beads had shapes which were regular with crackles and rough surface (Kumar *et al.,* 2016). The surface morphology of the magnetic nanoparticles and protease immobilized magnetic nanoparticles and typical size distribution were clearly depicted by Sahu *et al.,* (2016) who showed the formation of agglomerates in the immobilized enzyme.

Phase V – Highlights of the findings

- **The optimum pH was 7.0 and optimum temperature was 40^{o}C for immobilized protease.**
- **Of all the immobilization methods, alginate-chitosan method showed maximum enzyme activity.**

- **The isolated enzyme was stable for six days after immobilization when stored at 4°C and two days when stored at 27°C.**
- **The immobilized enzyme could be reused upto six times.**
- **Of all the three methods, alginate chitosan method showed maximum enzyme activity in all the parameters. Hence this was selected for application studies in the following phase.**

4.6 Phase VI - Applications of protease

Proteases represent one of the three largest groups of industrial enzymes. Microbial proteases account for the largest share in the market in terms of value, followed by animal source and plant source. Microbial proteases are one of the most important group of enzymes, due to their easy availability and fast growth rate. In recent decades, microbial proteases have been recognized for their considerable applications in various industries such as detergent, leather, pharmaceutical, photographic and textile industries. They play a specific catalytic role in the hydrolysis of proteins (Singh *et al.*, (2016a); Singh *et al.*, (2016b); https://www.marketsandmarkets.com/Market-Reports/proteases-market-184780427.html).

4.6.1 Application in detergent industry

Currently, proteases constitute the largest product segment in the global industrial enzyme market. The detergent enzyme market has grown nearly 10-folds during the past 20 years. Removal of proteinaceous stains such as egg, blood, chocolate and milk are very difficult using conventional detergent methods. However, removal of such stains can be achieved by using microbial proteases. Detergents with enzymes show reduction in the stain intensity and therefore enzymes are employed as cleansing additives (Rajkumar *et al.*, 2011; Aishwarya *et al.*, 2013).

4.6.1.1 Action of protease on stains

The results of evaluation of purified and immobilized protease enzyme compared with the commercial protease enzyme for washing performance were observed.

Plate 11 shows the action of commercial, purified and immobilized protease on blood stains.

It is easily understood from the plates that the blood stains on the cloth pieces remained stable in the control even after 30 minutes of rinsing, whereas, in detergent treated samples it showed moderate reduction in the stain intensity after 30 minutes.

It can be noticed from the plate that when blood stained cloth was treated with the commercial protease alone, the stain was completely removed after 20 minutes. When treated with detergent alongwith the commercial protease, the stain was removed completely after 10 minutes.

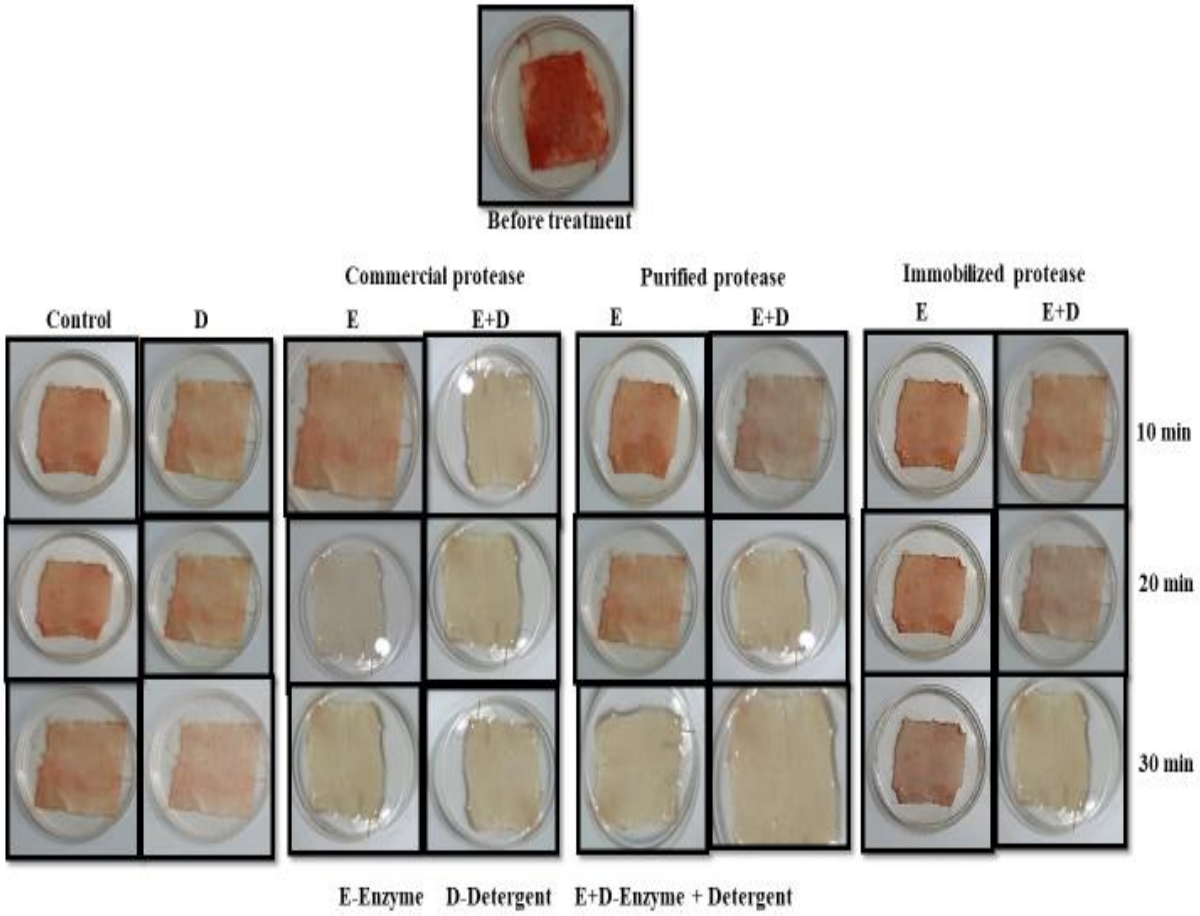
In the stained cloth treated with purified protease, the stain was completely removed after 30 minutes. In the case of both detergent and purified enzyme treated cloth, after 10 minutes, moderate removal of stain was noticed and complete removal of stain after 20 minutes.

In the cloth treated with the immobilized protease alone, there was moderate removal of stain only after 30 minutes, whereas in the cloth treated with immobilized protease and detergent, there was complete removal of stain after 30 minutes.

When compared with the commercial protease, purified protease exhibited maximum stain removal activity than immobilized protease. The blood stains were moderately removed from the cloth pieces after treatment with detergent alone, whereas, it was completely removed when treated with both enzyme and detergent for a period of 30 minutes.

Plate 11

Evaluation of commercial, purified and immobilized protease for destaining of blood stains from cloth pieces



A similar trend was followed in egg yolk stained cloth also which is represented in Plate 12.

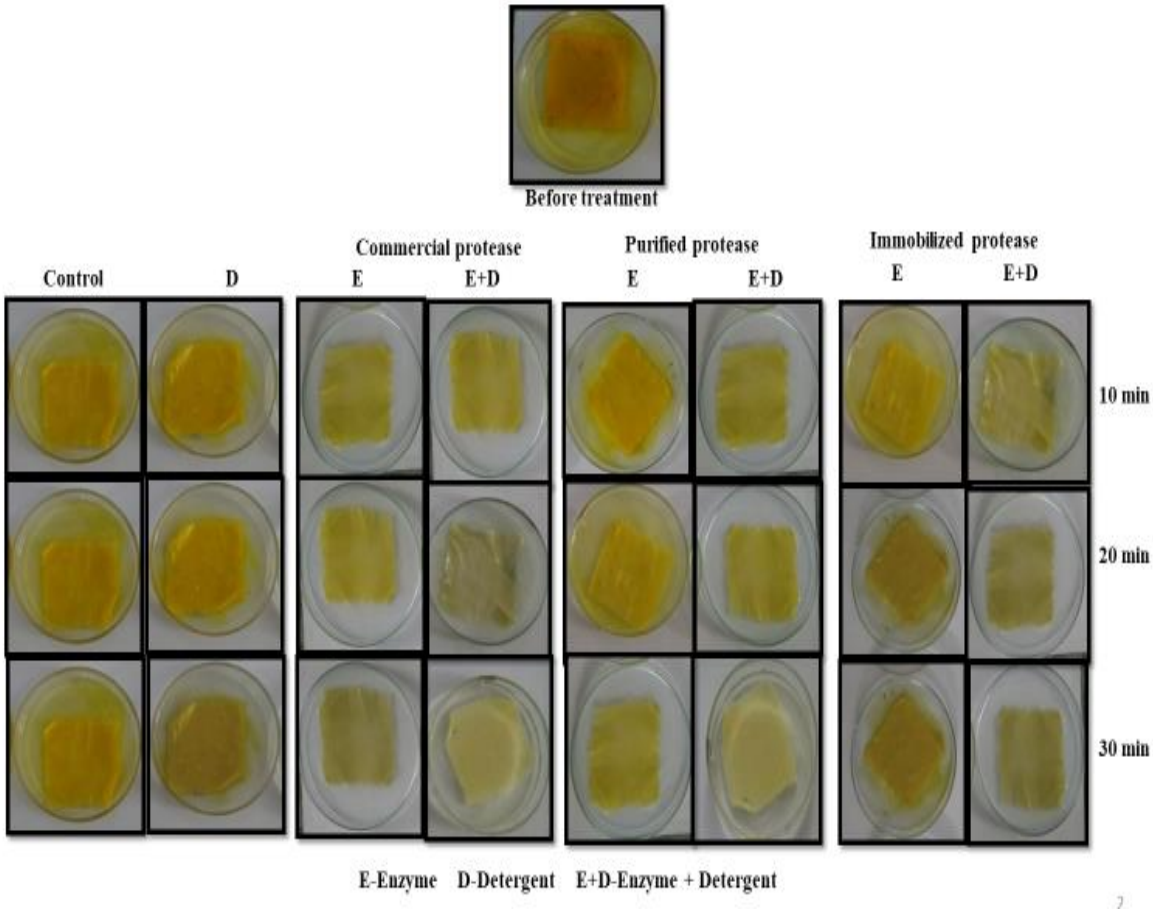
It can be noticed from the plate that when the stained cloth was treated with only distilled water, the stain was not removed. When treated with detergent alone a little stain was still retained after 30 minutes and when treated with detergent alongwith the purified enzyme and immobilized enzyme, the stain was removed completely after 30 minutes.

When the cloth was treated with detergent containing enzymes, the effect of destaining was enhanced. As compared to commercial protease, both the purified and immobilized proteases removed stains efficiently within 30 minutes duration.

Hence, the present results clearly indicate the significant role of protease produced by *Bacillus* sp. ASASBT in washing performance, in addition to maximum activity and stability indicating its application as an ingredient in commercial detergents.

Plate 12

Evaluation of commercial, purified and immobilized protease for destaining of egg yolk stains from cloth pieces



The above findings are in correlation with earlier reports. Arlumani *et al.*, (2007) reported that the addition of protease to commercial detergents significantly increased washing performance and removal of blood stains. The present study is also on par with the reports given by Puntambekar and Dake, (2017) who reported that the enzyme showed high reduction in the stain intensity compared to the detergent alone and also enzyme alongwith detergent, could enhance destaining. According to Baweja *et al.*, (2016), significant destaining was observed at 50°C and the blood stained cloth supplemented

protease with detergent and protease alone showed better destaining ability as compared to cloth washed in detergent alone. Annamalai *et al.,* (2014) stated the ability of purified protease from *Bacillus alveayuensis* combined with detergent to remove blood stains.

4.6.1.2 Preparation of enzyme-based detergent cake

Enzyme containing detergents improve the quality of fabric and keep the colour bright. They are used in small quantites as compared to synthetic chemicals. They can work at very low temperatures, are eco-friendly and completely biodegradable. Enzymes are mainly used in detergents to increase its cleaning ability (Hasan *et al.,* 2010 and Saeki *et al.,* 2007).

Since a remarkable performance in washing efficiency was noted with purified and immobilized protease from *Bacillus* sp. ASASBT, detergent cake was prepared using purified and immobilized protease and also with commercial protease.

Plate 13 shows detergent cakes with all the three forms of proteases.

The purified and immobilized protease-based detergent cakes were tested and also compared with the detergent cake prepared from commercial protease.

Plate 13

Preparation of enzyme-based detergent cake

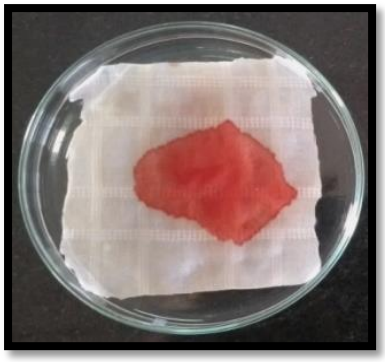
Commercial protease **Purified protease** **Immobilized protease**

The ability of blood stain removal was also checked for the purified and immobilized protease-based detergent and compared with that of the detergent cake made from commercial protease enzyme.

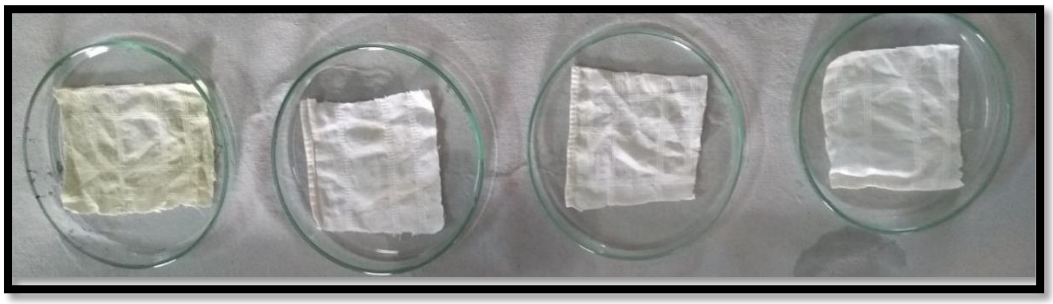
Plate 14 depicts the action of the detergent cake tested on blood stained cloth pieces.

Plate 14

Action of enzyme-based detergent cake on blood stains



Before treatment



Control **Standard protease** **Purified protease** **Immobilized protease**

It can be noticed from the plate that when the blood stained cloth was washed with commercial detergent, the stain was not completely removed. When treated with purified and immobilized protease based detergent cake, the stain was completely removed like the commercial protease enzyme within 30 minutes. Thus, the results revealed the removal of stains within 30 minutes without addition of any commercial detergents.

Thus, it can be inferred from the above findings that the isolated protease from *Bacillus* sp. ASASBT was able to remove blood stains completely and hence the enzyme can be recommended for usage in detergent industries.

The results of the present study were in accordance with the findings of Rao *et al.,* (2009b), Najafi *et al.,* (2005) and Adinarayana *et al.,* (2003) who reported that the purified

protease from microorganisms could remove blood stains very efficiently without any addition of detergents.

4.6.2 Application in leather industry

Leather industries have various advantages in terms of manufacturing capacity and export potential (Luthra, 2006). The animal skins have different processing like soaking, dehairing, deliming, etc., to prepare stabilized collagenous leather products. The conventional chemical leather processing generates large amount of environmental pollutants (Thanikaivelan *et al.,* 2004; Kanagaraj *et al.,* 2006). These processes necessitate the use of an alternative and eco-friendly process, which is accomplished by a process involving enzymes to reduce environmental pollution. Enzymatic dehairing process is gaining importance in present day scenario, significant in reduction of toxicity and improving the leather quality (Sivasubramanian *et al.,* 2008; Mamun *et al.,* 2015).

4.6.2.1 Action of protease on animal skin

Plate 15 shows the dehairing property of purified and immobilized protease from *Bacillus* sp. ASASBT and compared with the commercial protease.

It is obvious from the picture that incubation of goat skin with the commercial, purified and immobilized protease resulted in removing of the hairs, confirming the dehairing potential of the isolated protease.

It is clear that a best dehairing activity was observed in the case of commercial protease enzyme after 12 hours of incubation. Incubation of goat skin with the purified protease resulted in removing of the hairs, this confirming the dehairing potential of the isolated protease. The immobilized protease enzyme showed the considerable dehairing activity after 12 hours of incubation. In control, no hair removal was observed. From the plate, it is understandably, that a better dehairing activity was observed in the case of purified and immobilized protease enzyme on comparison with the commercial protease after 12 hours of incubation.

The above observations recommend the protease from *Bacillus* sp. ASASBT to the leather industry, based on its efficiency to remove hairs from goat skin.

Plate 15

Action of protease on animal skin

Control

Standard protease

Purified protease

Immobilized protease

The ability of protease to dehair animal skin was supported by several other studies. Briki *et al.*, (2016) reported that protease from *Bacillus* sp. SB12 had the potential to replace sodium sulfide in the dehairing process. A partially purified alkaline protease from *Bacillus megaterium* RRM2 showed efficient dehairing ability on goat skin and it was found to have accomplished this after 12 hours incubation (Rajkumar *et al.*, 2011). Another study by Dayanandan *et al.*, (2003) also demonstrated the use of *Aspergillus tamari* protease to dehair goat skins effectively.

4.6.3 Application in pharmaceutical industry

The therapeutic uses of proteases over the past several decades has provided clinical results that clearly suggest a bright future for their expanded use. Protease drugs need not solely arise from their primary proteolytic functions and can be applied in situations where they are not normally involved (Craik *et al.*, 2011). Proteases from microbial sources were detected to be effective in treatment of various diseases like cardiovascular, tumor, inflammation, cancer, blood clot lysis, etc., In addition, antimicrobial capability of protein degrading enzymes is concentrated well in most recent couple of years and proteases has been risen as important key enzymes (Ali *et al.*, 2017).

4.6.3.1 Antibacterial activity of protease

Table 15 shows the results of the antibacterial activity of commercial, purified and immobilized protease enzymes against pathogenic bacteria such as *Staphylococcus aureus* (Gram positive) and *Escherichia coli* (Gram negative).

It can be assessed from the table that the purified and immobilized protease was as effective as commercial protease. It also showed appreciable antibacterial effect against both Gram-positive and Gram-negative bacteria.

Table 15

Antibacterial activity of protease

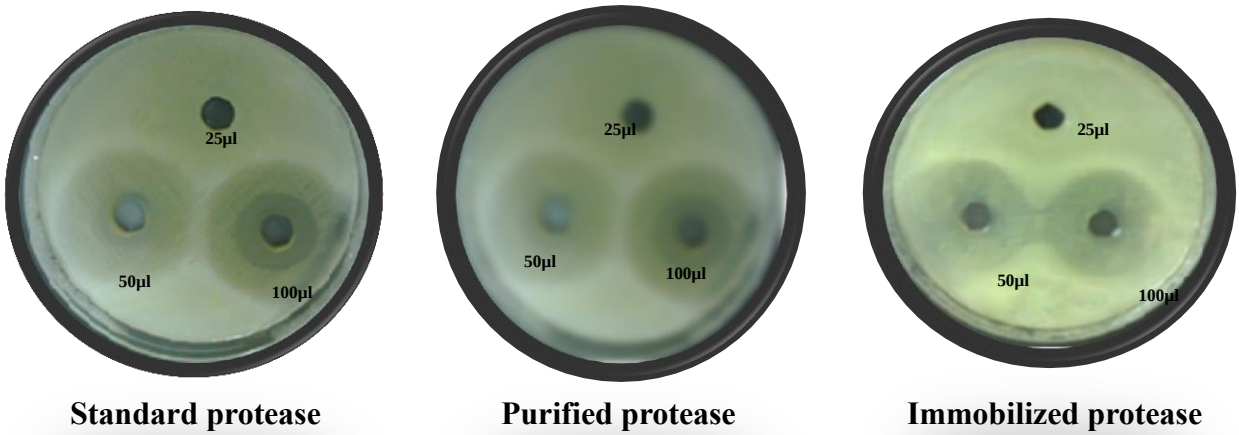
Bacteria types	Sample	Zone of inhibition (mm)		
		25µl	50µl	100µl
Staphylococcus aureus	Commercial protease	--	10	12
	Purified protease	--	7	9
	Immobilized protease	--	8	10
Escherichia coli	Commercial protease	3	4	7
	Purified protease	2	3	4
	Immobilized protease	1.5	2.7	4

The pictorial representation is given in Plate 16.

Plate 16

Determination of antibacterial activity of protease

Staphylococcus aureus



Standard protease **Purified protease** **Immobilized protease**

Escherichia coli

Standard protease **Purified protease** **Immobilized protease**

The commercial protease showed the highest activity against *Staphylococcus aureus* at 100 µl concentration with the zone of inhibition of 12 mm. At 50 µl it showed the zone of inhibition of 10 mm, whereas at a still lower concentration of 25 µl no activity was found. The purified protease activity against *Staphylococcus aureus* exhibited 9 mm zone of inhibition at 100 µl concentration, whereas at 50 µl concentration 7 mm zone of inhibition was occurred and at 25 µl concentration no such activity was noted. A similar trend was followed by immobilized protease also, at 100 µl with the zone of inhibition of 10 mm followed by 50 µl showed 8 mm and no zone of inhibition was found at 25 µl.

With *Escherichia coli,* the activity increased in a dose dependent manner, whereas, in commercial protease at 100 µl concentration the zone of inhibition showed 7 mm, at 50 µl shows 4 mm and 25 µl showed 3 mm. In purified protease the zone of inhibition of 4 mm at the concentration of 100 µl, followed by at 50 µl (3 mm) and 25 µl (2 mm). A similar trend was followed by immobilized protease also.

The isolated protease was efficient against the tested Gram-positive and Gram-negative bacteria thereby proving its contribution to the host defense against serious bacterial infections. Hence, this antibacterial molecule may also be effective as an alternative source of antibiotics against pathogenic microbial infections.

The present study was identical with the results of Manjusha *et al.,* (2014) who reported that, protease from *Bacillus* species showed high antibacterial activity against the pathogenic strains *Bacillus megaterium, Klebsiella pneumonia, Enterobacter aerogens, Mycobacterium tuberculosis* and *Staphylococcus epidermidis.* According to Bhaskar *et al.,* (2007) the alkaline protease of *Bacillus proteolyticus* CFR3001 showed antibacterial activity against *Escherichia coli, Listeria monocytogenes, Bacillus cereus, Yersinia enterocolitica.*

4.6.3.2 Clot lysis activity of protease

Plate 17 shows the results of various proteases (commercial, purified and immobilized protease) on clot lysis.

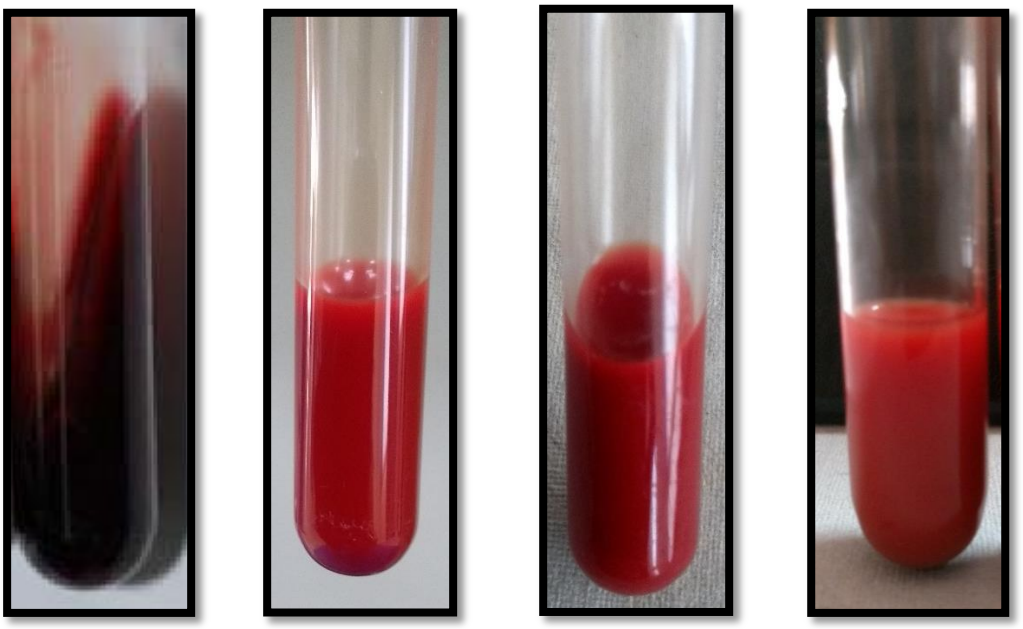
It is quite evident from the picture that the isolated protease hydrolyzed the blood clot to a liquid state completely as compared to the commercial protease.

The control sample as assumed does not show any clot lysis activity. However, the fibrinolytic activity of purified and immobilized enzyme sample showed that the highest activity of clot lysis was found with 30 minutes of incubation.

In commercial protease treated sample also, at the end of 30 minutes of incubation, a complete lysis of blood clot was obtained, suggesting that the blood clot was removed due to lysis by the fibrinolytic enzyme.

Plate 17

Clot lysis activity of protease



Control **Commercial protease** **Purified protease** **Immobilized protease**

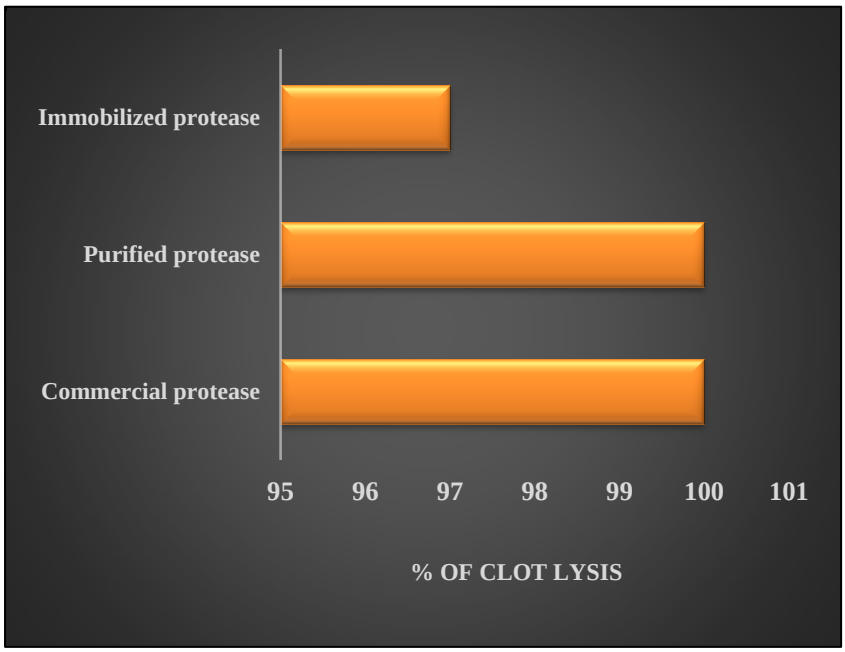
Figure 36 represents the results of the clot lysis by protease.

From the figure, the invitro clot lysis percentage showed 100% for commercial and purified proteases and 97 % for immobilized protease. This result proved that the protease enzyme secreted by *Bacillus* sp. ASASBT acts as a potent hemolytic enzyme capable of in-vitro lysis of blood clots.

This result proves that the enzyme secreted by *Bacillus* sp. ASASBT is a potent fibrinolytic enzyme capable of lysis of blood clot and thus, it can be recommended for usage in pharmaceutical industries.

Figure 36

Percent clot lysis of protease



Similar studies were also reported by several researchers. Jayakumar *et al.,* (2012) reported that the thermostable serine alkaline protease from an alkaliphilic *Bacillus pumilus* MCAS8 hydrolyzed blood clot completely within 6 hours at room temperature. Another study by Najafi *et al.,* (2005) also reported that protease from *Pseudomonas aeruginosa* PD100 showed effective lysis of blood clot. The results of fibrinolytic enzyme secreted by *Bacillus cereus* SRM-001 depicted a potent capable of invitro lysis of blood clots at the end of 3 hours (Narasimhan *et al.,* 2015). The findings of Mukherjee and Rai, (2011); Kim *et al.,* (2007) were also followed the same trend.

4.6.4 Application in photographic industry

The conventional method for silver recovery involves burning X-films directly and method is the most primitive one which generates undesirable foul smell and causes enormous environmental pollution by producing undesired emissions. The chemical method involves usage of acid and alkali and is also quite harsh and environmentally

hazardous (Marinkovic *et al.,* 2006; Ekpunobi *et al*., 2013). Moreover, the polyester film on which an emulsion of silver and gelatin is coated cannot be recovered by these methods. Therefore, to overcome these drawbacks, an enzyme-based method may be developed that may not only recover silver efficiently but also have minimal impact on the environment. Considering the eco-unfriendly nature of chemical based methods of silver extraction from X-ray films, a greener approach that is based on application of enzymes is gaining attention. The enzyme-based silver extraction from X-ray films relies more on renewable energy resources than on fossil fuel and therefore might offer an overall eco-safe process (Singh and Bajaj, 2017).

4.6.4.1 Silver recovery from waste x-ray film

Plate 18 shows the X-ray films treated with commercial, purified and immobilized proteases.

As seen in the photograph, at a pH of 7.0 and temperature of 40°C, all the three proteases hydrolyzed the gelatin layer to a small extent within 10 minutes. By 30 minutes, more of the gelatin was hydrolyzed and at the end of 60 minutes all the gelatin was hydrolyzed completely, finally resulting in increase in turbidity of the enzyme extract solution. The obtained solution which contained silver was further confirmed by a qualitative test.

Plate 19 shows the qualitative test for presence of silver.

It is clearly understood from the picture that, among all the three protease treated samples, the presence of red ring on top of the solution and black stain confirmed the presence of silver in the extracted solution. Among the three proteases tested, purified protease exhibited highest silver release followed by immobilized protease when compared to the commercial protease.

Thus, the isolated protease from *Bacillus* sp. ASASBT was capable of hydrolyzing gelatin from used X-ray films and recovering silver which could be reused.

Plate 18

Silver recovery from waste x-ray film using protease

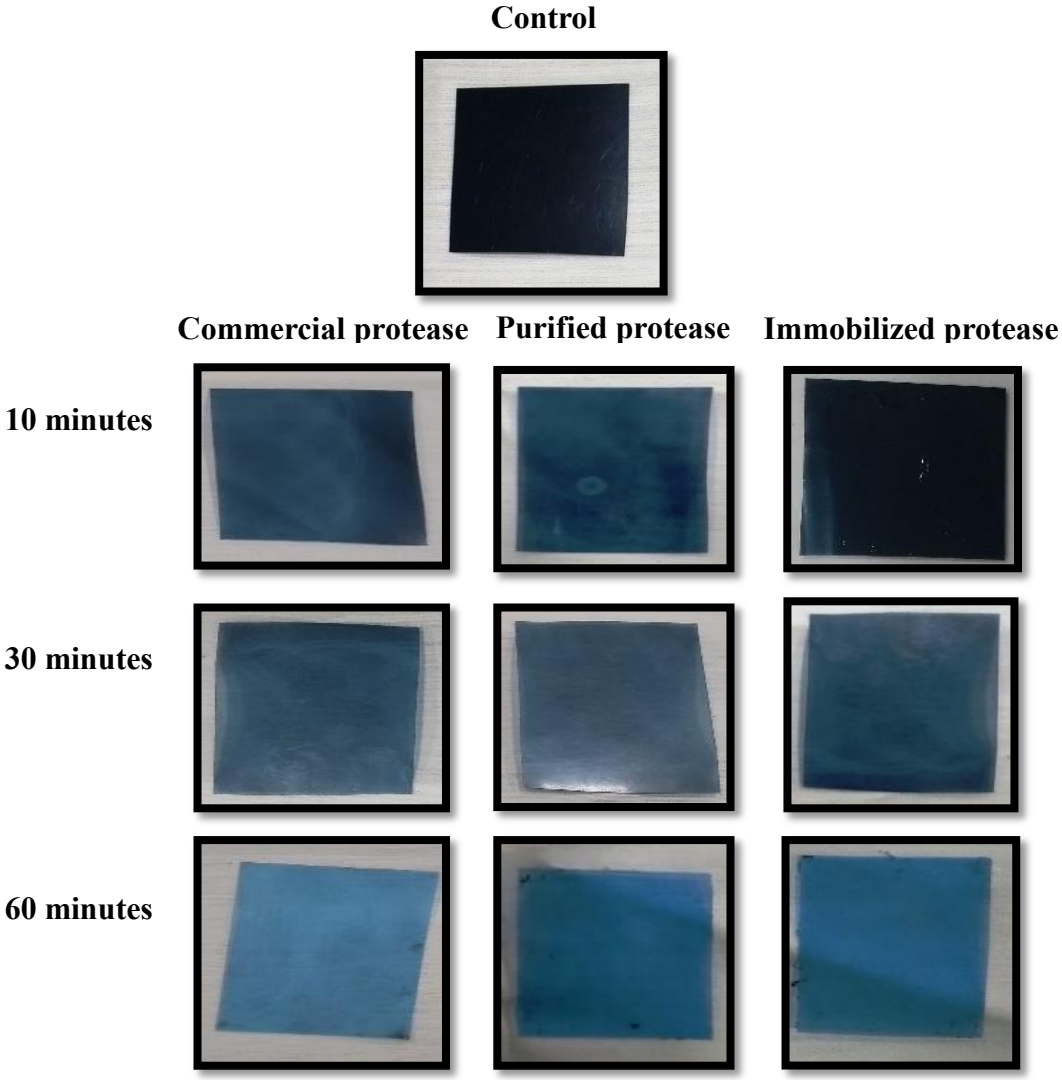
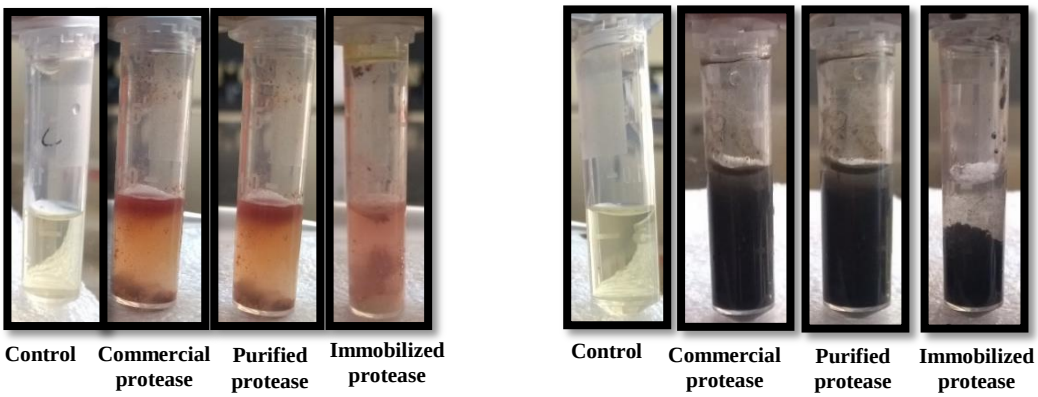


Plate 19

Qualitative test for presence of silver



Several other findings supported the above work. Proteases are reported to possess excellent gelatinolytic activity for successful recovery of silver from X-ray films. Several *Bacillus* sp. proteases from *B. lehensis* (Joshi and Satyanarayana 2013), *B. subtilis* (Kumaran *et al.*, 2013), *B. cereus* (Bajaj *et al.*, 2013; Lakshmi and Hemalatha, 2016) and *B. licheniformis* (Pathak and Deshmukh, 2012), *B. subtilis* K-5 (Singh *et al.*, 2014), *Bacillus subtilis* NCIM 2724 (Parpalliwar *et al.*, 2015) and *B. licheniformis* K-3 (Singh and Bajaj, 2017), have been demonstrated to possess good gelatinolytic activity for efficient silver recovery from X-ray films.

4.6.5 Application in textile industry

Silk is a composite material with fibroin and sericin, both are proteins accounting for about 75 % and 25 % of total silk weight (Mahmoodi *et al.*, 2011 and More *et al.* 2013). Silk processing from cocoons to the final finished clothing involves several steps, namely, reeling, weaving, degumming, dyeing and finishing. Degumming is the process where sericin, i.e. the silk gum gluing the fibroin filaments, is totally removed in order to obtain silk with desirable properties. Traditionally, degumming of silk is carried out with soap or alkali, but these methods face major limitations like, not of uniform quality, loss of strength of silk and cause environmental pollution. Therefore, there is great impetus on developing enzyme-based silk-degumming process (Nakpathom *et al.*, 2009). Proteolytic enzymes, which can cleave the peptide bonds of sericin without destroying the fibroin, may have potential for application as degumming agents.

4.6.5.1 Degumming of silk

a) Weight loss on degummed silk

Figure 37 describes the weight loss percentage of enzymatically degummed silk.

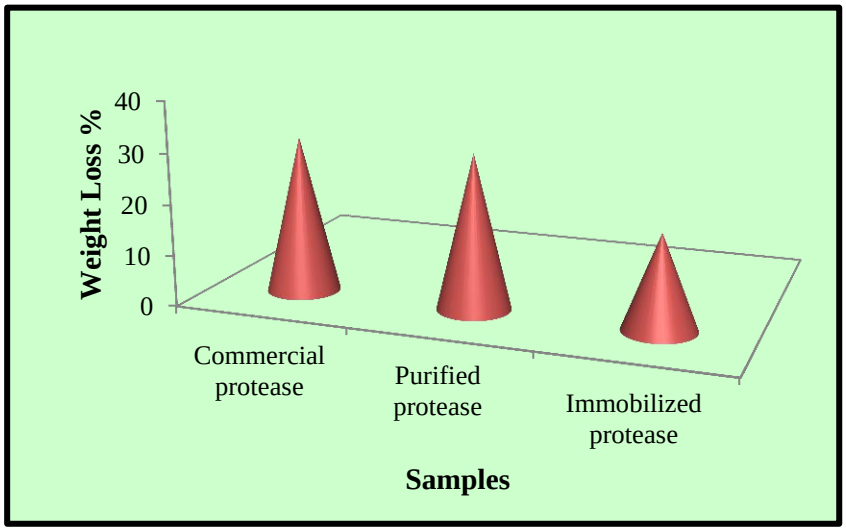
The silk fabric was weighed before and after enzyme treatment and the weight loss percentage calculated. The commercial and purified proteases perform uniform removal of sericin with 31 % and 30 % of weight loss respectively. After degumming process, the quality of silk was improved. The immobilized protease exhibited weight loss percentage of 18 %, which showed moderate activity. On comparing with commercial protease, purified protease also showed maximum removal of sericin. This result shows that the

isolated protease could be strongly recommended for degumming of silk in textile industries. Finally, the enzymatic degumming process is economic, effective and eco-friendly. It involves the proteolytic degradation of sericin, using specific proteins with minimum effect on fibroin. In enzymatic method, the silk is treated at low temperature which not only reduces energy cost but also prevents fiber weakness.

Thus, the percent weight loss on degummed silk suggest that the isolated protease showed effective degumming of silk.

Figure 37

Weight loss on degummed silk



The results of the present study are in accordance with those of Naaz *et al.,* (2017) who reported that protease isolated from *Bacillus subtilis* and *Penicillium citrinum* showed efficient degumming of silk. Similar findings were also reported by More *et al.,* (2017) and Johnny & Chinnammal, (2012).

b) Morphological characterization of degummed silk

Plate 20 shows the scanning electron micrographs of untreated and degummed silk fibres with enzymatic method using commercial, purified and immobilized proteases.

The results showed that in the case of untreated silk, the fibres were clubbed together in a bundle by means of sericin, whereas in the treated silk, the fibres have fallen

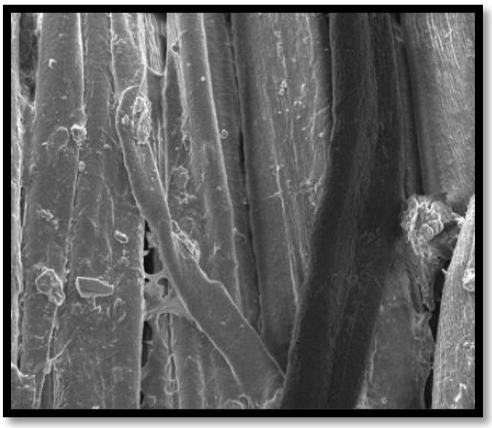
apart after degumming. Untreated silk contains sericin, which was seen as deposits on the surface of filaments. Degumming under optimal conditions resulted in the removal of the sericin deposits and separation of fibres.

Thus, it can be concluded that, all the three proteases act completely for the uniform removal of sericin from the silk fibres.

Plate 20
Morphological characterization of degummed silk

100 X **2000X**

Untreated silk



Treated with purified protease

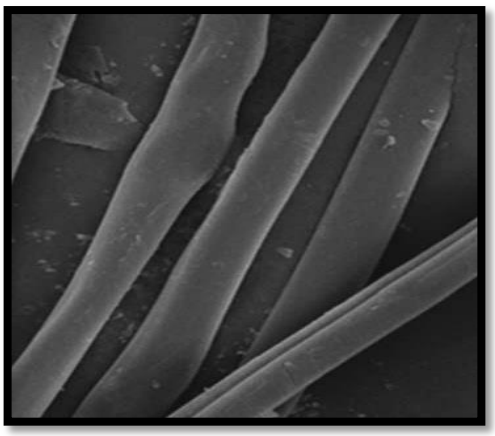
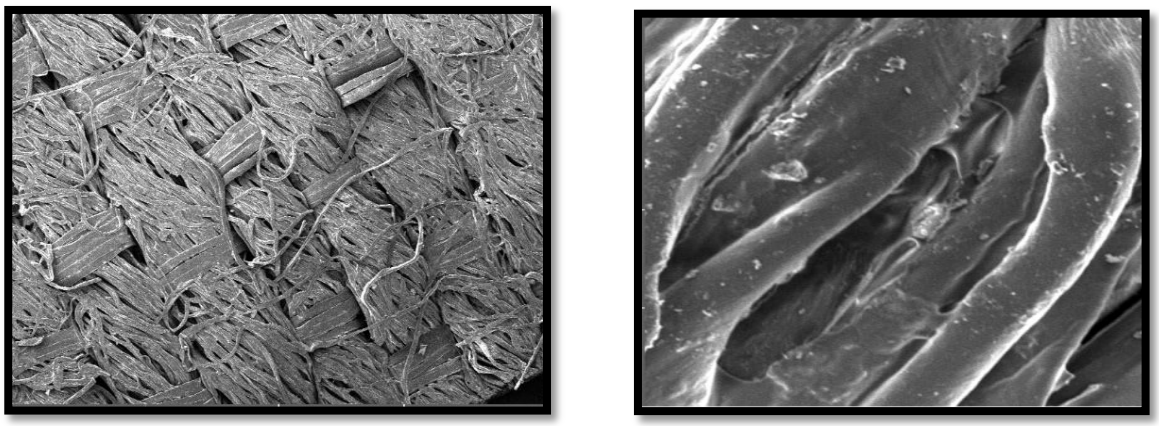
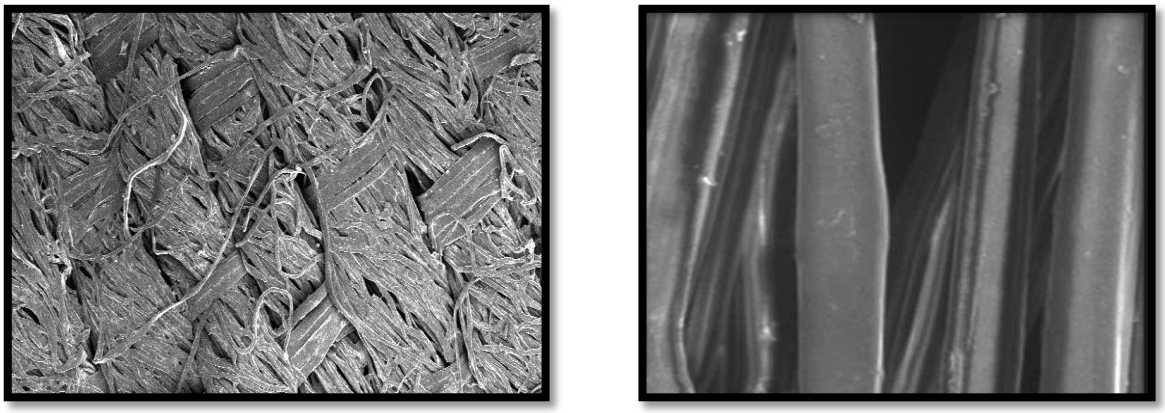


Plate 20 (Contd…)



Treated with commercial protease



The present study is in accordance with several other research works. Arami *et al.*, (2007), Nakpathom *et al.*, (2009) and More *et al.*, (2013) reported that there was no uniform sericin deposits in untreated silk, whereas, in the enzyme treated samples these were removed as a result of which the silk showed an improvement in the luster quality.

c) UV Spectra of sericin extraction from protease

Figure 38 represents the UV spectra of standard sericin and sericin extracted from standard, purified, immobilized protease and untreated degummed silk.

In the standard sericin (a), a strong characteristic peak was observed at 280 nm. The samples (b-d) also showed strong peaks at 280 nm, whereas the untreated sample (e)

showed broadening of peak as compared to other samples, indicating that no reaction has occurred when using distilled water alone. Due to the presence of peptide bonds and aromatic amino acids, proteins absorb strongly in the ultraviolet region. The peaks of commercial and purified protease extracted from silk fabric showed a spectrum close to that of the standard.

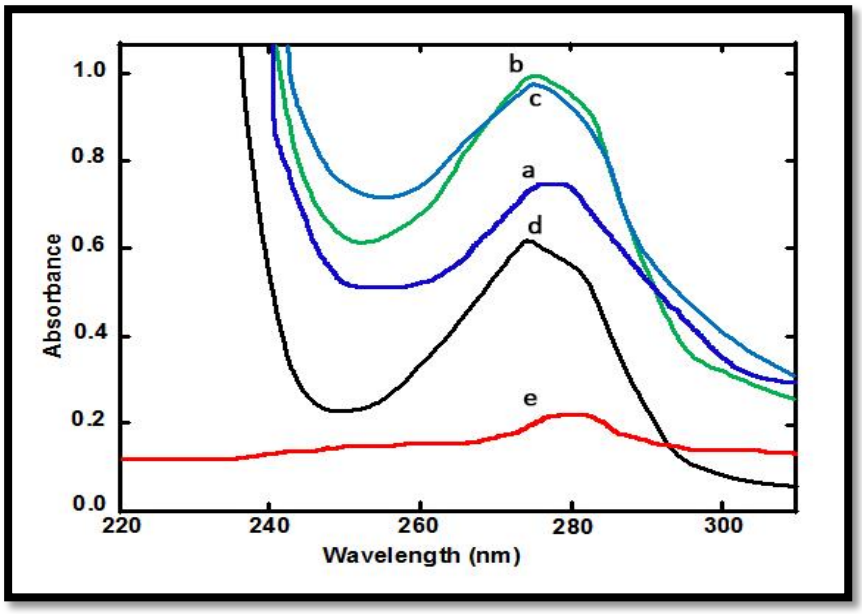
The yield, protein content and A-ratio of sericin from degummed silk using commercial, purified and immobilized protease is represented in Table 16.

It is evident that the maximum yield of 24.25 % was observed in silk treated with commercial protease, followed by silk treated with purified protease 23.72 % and a slightly less yield was obtained in silk treated with immobilized protease (20.14 %). From all the three proteases treated, extraction of A-ratio of sericin indicating that a sample source has a great impact on the quality of sericin. Standard sericin shows the maximum A-ratio of 1.75, which is close to the ideal value of 1.8. The higher the A-ratio, better the quality of sericin. In commercial protease treated sericin the A-ratio was found to be 1.70, followed by purified (1.64) and immobilized (1.46) protease treated sericin. A similar pattern was observed in the protein content also. The maximum protein content was observed in commercial protease treated silk (41.43 mg/ml), followed by purified protease treated silk (38.91 mg/ml) and immobilized protease treated silk (29.41 mg/ml) respectively.

Thus, both the quality and quantity extracted from purified and immobilized protease treated silk fabrics are found to be the best source of sericin.

Figure 38

UV-visible spectra of sericin extracted from protease



a-Standard sericin b-Standard protease c-Purified protease d-Immobilized protease e-Untreated sample

Table 16

A-ratio and protein content of different sericin samples

Source of sericin	A-ratio	Protein content mg/ml	Yield %
Standard sericin	1.75	-	-
Silk fabric treated with commercial protease	1.70	41.23	24.25
Silk fabric treated with purified protease	1.64	38.91	23.72
Silk fabric treated with immobilized protease	1.46	29.41	20.14

Therefore, the isolated protease from *Bacillus* sp. ASASBT showed maximum activity in degumming of silk and sericin was successfully recovered in good yield and also sufficient purity was occurred, thereby strongly recommends for the textile industry.

The above results are on par with the work of Gupta *et al.*, (2014) who reported that maximum extraction of sericin was obtained after the degumming of silk. Wang and Guo, (2012); More *et al.*, (2017) also reported the technical process of silk degumming and showed the highest percentage of sericin extraction.

Phase VI – Highlights of the findings

- **The purified and immobilized protease from *Bacillus* sp. ASASBT removes stains completely within 30 minutes and also has maximum efficiency of goat hair removal.**
- **The antimicrobial activity of the enzyme increased in a dose dependent manner. The purified enzyme showed maximum clot lysis percentage.**
- **The isolated protease was able to remove silver from the treated x-ray films.**
- **Purified and immobilized proteases have the ability to degum silk.**
- **Finally, both the purified and immobilized proteases could be employed in various industry-oriented applications. As compared to commercial protease, the isolated protease from *Bacillus* sp., ASASBT was very effective and hence could be used for several industrial applications.**

A summary of the above findings and the conclusions which could be drawn thereof are presented in the following chapter.