PRODUCTION, OPTIMIZATION AND MOLECULAR CHARACTERIZATION OF ALKALINE PROTEASE FROM BACILLUS *AUSTRALIMARIS*

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ABSTRACT

The industrial prospective of alkaline protease hold extensive application in various industries like tannery, detergent, food and leather industries. In the present study 25 different Bacterial species were isolated from garden soil, Pollachi, Coimbatore. From this potential isolates were screened for their potential against gram positive and gram negative organisms. The isolate showing the property of alkaline protease was then identified by 16S RNA sequencing and species were identified as *Bacillus australimaris*. The isolate was cultivated in submerged fermentation in Horikoshi -I (alkaline medium) and further enhanced by optimization of cultural parameters (pH, temperature, Carbon, Nitrogen, Aeration and Agitation). The maximum alkaline protease enzyme production from this isolate were observed at pH 9 at 50°C. Ammonium sulphate and Glucose are designated a good source for alkaline protease production by *Bacillus australimaris.* A Bacillus auralimatis has been used for optimal production through media standardization and appropriate purification methods for produce an alkaline protease. Molecular weight was found as 25 kDa. Following 48 h of incubation, a protease production profiling study indicated a maximum enzyme activity (147.9 U/ml).The enzyme extract was immobilized using calcium alginate for industrial application.

Keywords: Alkaline protease, Enzyme, *Bacillus*, Immobilization, Optimization.

1. INTRODUCTION:

Industrial enzymes played a significant role in biotechnological processes. The constant stream of novel products depend heavily on industrial enzymes, and there is a significant need for industrial production of enzymes. New applications have boosted demand for enzymes, and the Industry is focusing more on environmental protection. The usage of microbial enzymes has grown

significantly in various industries over the last few years, including chemical, textile, pharmaceutical, paper, food, and agriculture manufacture. Rao et al., 1998 reported that the nature of the enzyme's allows it to change the structure of proteins by breaking peptide links [Rao et al., 1998]. Bacillus sp. are the maximum energetic extracellular alkaline protease fabricators used in the commercial sector. Proteases play an important role in both physiological and industrial processes.The *Bacillus* genus is essential for the production of economically relevant industrial product.The organism forms a tough, rod-like structure with a protective endospore to withstand extreme environmental conditions (Dubal et al. 2008, Sekhon 2010). These organisms are facultative anaerobes that can exist in the presence/absence of oxygen

Alkaline protease (EC.3.4.21-24.99) is an important economic resource which is active at alkaline pH levels of 9 to 11 [Varela et al, 1996]. Three percent of the proteins are alkaline serine proteases, which are inactivated by phenyl methane sulfonyl fluoride (PMSF). This enzyme can be found in water, soil and extreme alkaline pH 9.0–12.0 environment. In addition to detergent contamination [Hsiao et al, 1994] dried fish, and sand soil, alkaline proteases can be separated from other proteases for various applications. Among the serine proteases, alkaline proteases are most commonly used in the detergent industry [Adinarayana et al, 2003]. Using a Phenyl Sepharose 6 Fast Flow column, the protease produced by bacteria has been concentrated tenfold with an 82% yield in one step [Ansha et al, 2005]. Their primary use is explored in the detergent industry and accounts for 30% of the World's total enzyme production [Venugopal et al, 2016] due to the pH range of 9.0–12.0 of laundry detergents. Alkaline proteases are cleaning additives used in detergent formulations along with other hydrolytic enzymes to help break down of proteins [Rao et al, 1998, Khan et al, 2011]. Alkaline proteases are also utilised in a wide range of other industrial processes, such as the extraction of silver from old X-ray film, leather processing, medicines, protein processing, food processing, diagnostic reagents, soy processing, and peptide synthesis [Horikoshi 1971, Abd-Rahman et al, 2006]. Thus, the need in industry for highly selective, and active alkaline proteases that are stable in wide range pH, temperature, and organic solvents continues to fuel the development of novel enzymes.This paper presents the results of a screening procedure we used to isolate and identify alkaline-producing bacteria from environmental sources.

2. MATERIALS AND METHODS:

2.1.Sample collection and Processing:

In pollachi, agriculture land close to the topslip hills were used to gather soil samples. Serial dilution and spread plate methods were the techniques used to isolate the target bacteria..The samples were serially diluted up to a 10^5 dilution, distributed on nutrient agar plates, and incubated for 24 hours at 37°C. On the basis of visual and biochemical traits, including endospore staining, the colonies were recognised as B. subtilis. Colonies were subcultured and kept to produce a pure culture on nutrient agar plates. This cultures was then transferred to a medium (pH 8.0) containing 1% glucose, 0.5% casein, 0.55% yeast extract, 0.2% KH2PO4, 1% Na2CO3, 0.2% MgSO47H2O, and pH 8 and incubated at 37 °C with constant shaking for propagation.

2.2. Screening of Bacterial isolate:

Solid substrate gelatin was added to nutritional agar medium at 1% (w/v), sterilized, and then poured into Petri plates. A sterile cork borer was used to create wells, and 100-200µl of cell-free culture filtrate was then aseptically poured into each well before incubation at 37°C. After 12 hours, the proteolytic zone's diameter was established by observing clear zones surrounding the wells caused by the hydrolysis of substrates in the presence of inhibitor solution.

2.3. Taxonomy of *Bacillus* sps isolate:

Microbiologists and biotechnologists have widely accepted the process of identifying and assigning a scientific name to an isolated bacterial species or to a new strain of a previously identified species using their 16S r RNA gene sequences (Chaudhary, 2012).

2.4. Identification isolation by 16s sequencing;

On nutrient broth, a pure colony of bacteria was cultivated overnight. Cell lysis was used to extract DNA from bacteria and the 16S rDNA was amplified in a thermocycler using specific primers Eppendorf. Forward: 5-AGAGTTTGATCCTGGCTCAG-3c Reverse: 5¢-TACCTTGTTACGACTT 3 An automated sequencer was used to sequence the amplified 16S rDNA PCR product. The 16S rDNA sequence was compared to other sequences using the internet search engine BLAST (http://www.ncbi.nlm.nih.gov/blast/). According to report of author, the isolate was identified through a BLAST search using the highly aligned sequence [Ansha et al, 2005].

2.5. Phylogenetic analysis:

The sequences were compared to 16S rRNA sequences available in public databases from BLAST (http://www.ncbi.nlm.nih.gov/ BLAST/, NCBI, Bethesda, MD, USA), and gene homology was found. CLUSTAL-X Multiple Sequence Alignment Program (Strasburg, France) aligned 16S rRNA sequences of isolates with those of similar organisms obtained from GenBank. PHYLIP was used to carry out the phylogenetic analysis, and the TreeView tool was used to create phylogenetic trees using the neighbor-joining method. A bootstrap study was carried out to ensure that the branching pattern was repeatable.

2.6.Submerged fermentation for *Bacillus* sps cultivation

Horikoshi-I alkaline medium (Glucose – 10 g, Peptone – 5 g, Yeast extract – 5 g, KH2PO4 – 1 g, MgSO4.7H2O – 0,2 g, Na2CO3 – 5 g, pure water – 1000 ml) was used for production of enzyme under controlled environment . The *Bacillus* sps inoculated into flasks with a loop of cultures in a autoclaved medium (pH 10) incubation at 37°C for 48 hours with 120 rpm and continuous agitation. In the liquid media, matt bacteria growth was observed after 48 hours. In order to eliminate the microbiological matt, the material was filtered using what man filter paper. The content was centrifuged at 10000 rpm for 10 minutes [Rao et al, 1998]. The extracted clear supernatant was used as crude enzyme for further study.

2.7. Enzyme assay:

Protease production was triggered by a number of conditions, including the ionic chemical used in the industrial process, temperature, and handling. When assessing enzyme with 0.5 percent substrate and reaction mixture, the apparent advantage of employing modified Anson's approach was used. The reaction mixer was then incubated for one hour at 50°C. The assay was stopped after 10 minutes with 3ml of cold TCA solution. By spinning the enzyme solution at 8,000 rpm, a clear solution was obtained. The yield was measured spectrophotometrically at 280nm to obtain the economically significant enzymes. The amount of enzyme liberated was estimated and compared to a standard using Anson's approach. Under typical assay conditions, one unit (U) of enzyme activity indicates the quantity of enzyme necessary to liberate one gram of tyrosine [Anson, 1938]. A bovine serum albumin standard curve was used for protein quantitation in Lowry's method [Lowry et al, 1951] to obtain the specific activity of enzymes.

2.8 Molecular weight Identification:

On a Modular electroblotting system with a 10% polyacrylamide concentration, the Laemmli (1970) method for denaturing SDS polyacrylamide gel electrophoresis (SDSPAGE) was applied. This is done to determine the homogeneity of the protease enzyme and estimate its molecular mass using a marker for fermented protein molecular weight [Laemmli, 1970].

2.9 Optimization of Cultural parameters:

In order to accomplish this, isolates that can produce alkaline proteases and also remain relatively stable in the operating conditions were pursued. The production of protease was studied at different pH ranges (7-9) and temperatures (30-50°C) using Horikoshi-I alkaline medium. A study of the effects of different carbon sources was also conducted. The Box-Behnken experimental design was selected for the investigation as it aids in analysing the linear, quadratic, and cross product impacts of these components, each of which varied at these levels, and comprises three replication centres [Govarthanan et al, 2015].

2.10 Partial Purification of Protease:

Pharmaceutical and other medical applications require pure protease preparations. Crude protease preparations are typically used for commercial purposes. It is necessary to purify enzymes to explore its applications and understand its structure [Razzaq et al, 2019].

2.10.1. Ammonium sulphate fractionation and Dialysis:

Ammonium sulphate crystal was added to the crude extract to achieve an infiltration rate of 80% (salting in/ salting out). The saturated solution was centrifuged at high speed to separate debris, which was then suspended in 0.1 percent Tris-Hcl buffer (pH 9) and the buffer was dialyzed as well at 4 °C.

2.10.2. Fractionation by Column chromatography technique:

After pre-equilibration with 0.2 M phosphate buffer pH 7.2, the enzyme was eluted from a Sephadex G-200 column (Pharmacia 2.6 x 70 cm). The gel column preparation and fractionation method were carried out. A total of thirty fractions (5 ml) were gathered (each of 5ml). A new dialysis bag was used to collect and concentrate the sharp peaks created by the sephadex G200 column. Ten fractions (5mL each) were collected, and Protein content and enzyme activity were determined separately for each fraction to determine the specific activity of the enzyme.

2.11. Characterization of alkaline protease enzyme:

2.11.1. Effect of pH on protease activity and stability:

Under conventional test conditions, the impact of pH on protease activity was determined over a pH range from 6.0 to 10.0. By pre-incubating the purified protease with different pH levels for 30 minutes and measuring the residual activity, the pH stability of the purified protease was confirmed [Jameel et al, 2011]

2.11.2. Effect of temperature on protease activity and stability:

The standard assay technique was carried out at temperatures ranging from 20°C to 70°C to investigate the impact of temperature on protease activity. Thermal stability was estimated by pre incubating the protease at 20°C, 30°C, 37°C, 40°C, 50°C, 60°C, and 70°C for 30 minutes and measuring residual activity [Liu et al. 2013].

2.11.3. Effect of substrate concentrations on protease activity and stability:

To determine the optimal casein concentration, various ranging from 0.5% to 2% were used (Casein in 0.2M phosphate buffer of pH -10). The protease was pre-incubated with various concentrations of casein at 37°C for 30 min to determine the specific activity and effective substrate concentration [Liu et al. 2013].

2.11.4. Effect of Time on protease activity and stability:

The standard test protocol was used to calculate the influence of time on protease activity. Casein in 0.2M phosphate buffer with a pH of 10.0 was used as the 1% substrate, and different time intervals between 5 and 50 minutes were used to incubate the mixture before recording the highest amount of activity [Liu et al. 2013].

2.11.5. Effect of Metal ions on protease activity and stability:

The metal ions like Hg2+, Ca2+, Mg2+, Ba2+, Mn2+, Cu2+, Fe3+, and Zn2+ solutions, all with a concentration of 2 mM, were combined with 50 µL of protease solution. It was also evaluated by mixing 50 µL of pure protease solutions with 5 mM Tween 80 and 2.5 mM ethylene diamine tetra-acetic acid (EDTA) (Sigma). After one hour of storage at 37 °C, the relative activity values were calculated. To calculate the results, distilled water was used as the control [Liu et al. 2013]

2.11.6. Characterization of Protease by FTIR:

One of the known techniques for characterising proteins and peptides is Fourier-transform infrared (FTIR) spectroscopy. A number of different infrared absorption bands, or amide bands, are produced by the repeating amino acid building blocks that make up the backbone of proteins and peptides. These bands include both chemical and structural information. As a result, FTIR is commonly employed to analyse and measure the secondary structures of proteins [Byler et al, 1986]. A High Throughput Screening eXTension (HTS-XT) equipment connected to a Tensor 27 spectrometer was used to make FTIR measurements (Thermo Fisher). With a spectral resolution of 4 cm1 and an aperture of 5.0 mm, the spectra were captured in the range between 3495.26-649.90 [Kristoffersen, 2020].

2.12. **Immobilization of Protease Enzyme:**

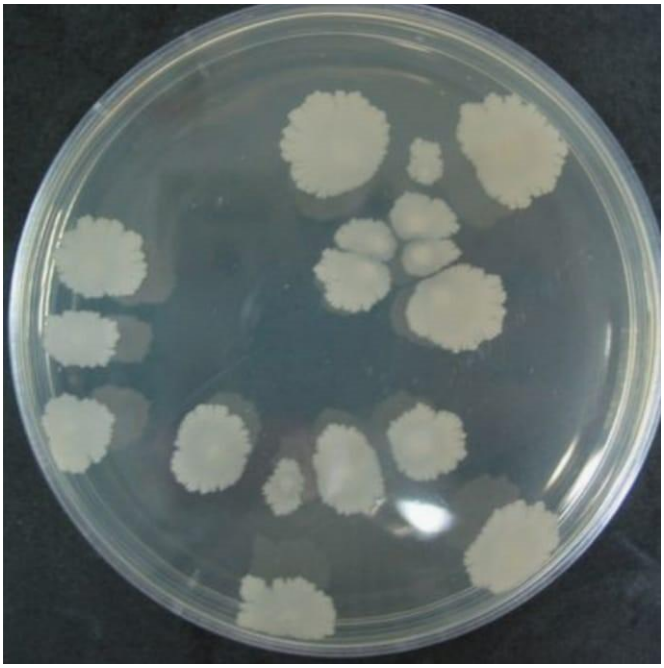
In a 1:2 ratio, the bacterial cell suspension was added to a 2 percent sodium alginate solution and gently vortexed to ensure full mixing. Drop by drop, from a safe distance, the resultant solution was added to 30ml of 0.2 M CaCl2. Colorless transparent gel beads with a diameter of 2-5 mm were obtained (Fig 10). They were left in the CaCl2 solution for 30-60 minutes before being thoroughly cleansed in double distilled water and stored at -110°C for a maximum of one week [Anupama et al, 2012].

3. RESULT AND DISCUSSION:

3.1. Isolation and Identification of isolate:

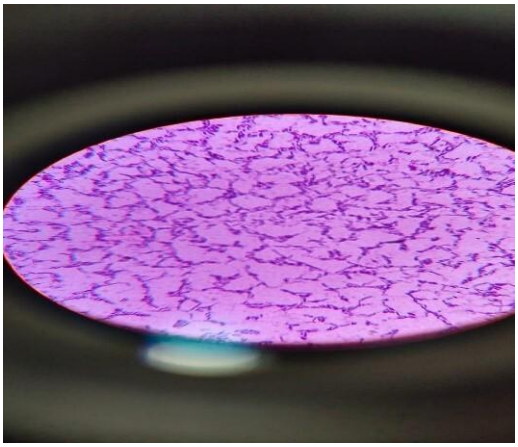
The Potential 25 strains were isolated from soil and named as *BL1 , BL2, BL3….BL25* respectively. Among the 25 isolates three isolates were selected for further study based on zone production (Fig 1). The isolated species BL5, BL8 and BL10 are identified as gram-positive (Fig.2), strictly aerobic, rod-shaped and motile (Table 1). After 48 h of incubation at 37°C on LB medium, colonies are visualized as light yellow round translucent and 2-3 mm in diameter. Three isolates were positive for the enzyme oxidase, Bacillus is the most prolific and prevalent source of alkaline protease among alkalophilic bacteria [Anupama et al, 2012, Lowry et al, 1951].The characteristic features tested for the isolates BL5, BL8 and BL10 were listed in the table 1 for comparative analysis. All the three isolates showed hydrolysis of casein and starch.

Figure:1 Photomicrograph shows Isolated *Bacillus australimaris colonies on* nutrient agar plate.



Colonies are viewed as light yellow round translucent and 2-3 mm in diameter

Figure:2 Microscopic view of *Bacillus australimaris* Positive and rod shaped



The isolates identified as gram-positive, strictly aerobic, rod-shaped bacterium

Table 1: Parameters used to identify the isolates:

Test	BL5	BL8	BL10
Colony Morphology	Pale white translus colony	Pale white translus colony	Pale white translus colony

Gram Staining	Positive rod	Positive rod	Positive rod
Motility	Positive	Positive	Positive
Indole	Negative	Negativve	Negative
VP	Negative	Positive	Negative
Catalase	Positive	Negative	Negative
Oxidase	Positive	Positive	Positive
Casein hydrolysis	Positive	Positive	Positive
Starch hydrolysis	Positive	Positive	Positive

BL8 and BL10 showing closely related results , BL8 showing catalase , casein and starch hydrolysis positive.

3.2 Screening of protease producing microbes by caseolytic activity

The protease production activity were assessed through hydrolytic activity of casein. The proteolytic activity of the organism was measured using casein agar and expressed as the diameter of the clear zone in mm. Three isolates out of twenty four isolates were used in the current investigation, the presence of a clear zone around the colony after incubation showed hydrolytic activity with the zone of inhibition measuring 32mm, 28mm and 16 mm respectively (Fig.3). These observations are consisting with Afshan Jameel et al, 2011 reported that with a clear zone diameter of 42 mm, isolate No. 5 had the highest level of proteolytic activity, followed by isolate No. 4, isolate No. 2, isolate No. 3, and isolate No. 1, with clear zones of 25 mm and 18 mm, respectively [Deng et al, 2010].

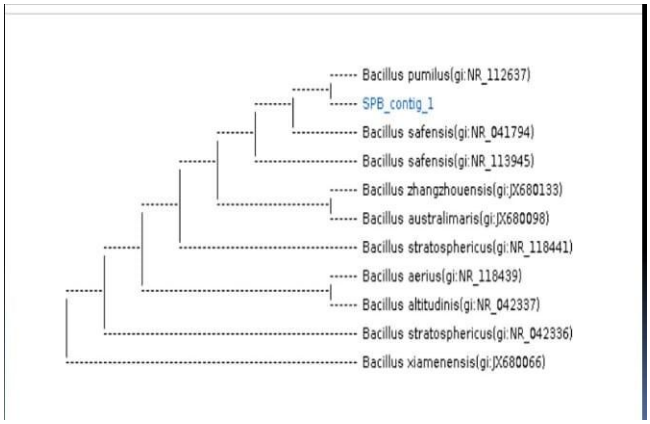
Fig 3: Zone of hydrolysis of Bacillus Sps BL5 (32mm) on casein agar

Clear zone around the colony showing hydrolytic activity with the zone of inhibition measuring 32mm

3.3.1. Identification of isolates by 16s rna sequencing:

Molecular identification of the chosen bacterial isolate BL5 was done using isolation of genomic DNA amplified by PCR, and visualised using agarose gel electrophoresis. The results of a blast analysis revealed that BL5 was 97.86% identical to Bacillus ***australimaris*** at the nucleotide level and 97.87% homologous to those strains at the rRNA level. Based on the 16S rRNA gene sequences of isolate BL5 and its related nucleotide sequences, a phylogenic tree was built using the mega x programme and neighbor-joining method, as shown in Fig. 4. Collectively those results suggested the isolate to be identified as *Bacillus australimaris* based on phylogenetic analysis of nucleotide sequences on the basis of 16s rRNA using BLAST.

Fig 4: Phylogenetic analysis of *Bacillus auralimaris*

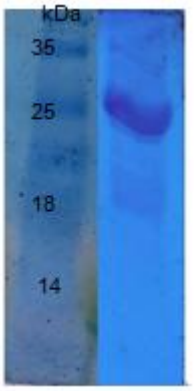


The Phylogenetic tree showing at the nucleotide level and 97.87% homologous to Bacillus *australimaris* at the rRNA level.

3.3.2. Molecular weight identification

SDS-PAGE revealed a single band indicating a homogeneous preparation, confirming the enzyme purity. Based on marker similarity, the enzyme's weight of 25 kDa (Fig. 5) was determined.

Fig 5: Molecular weight of alkaline protease on SDS PAGE.

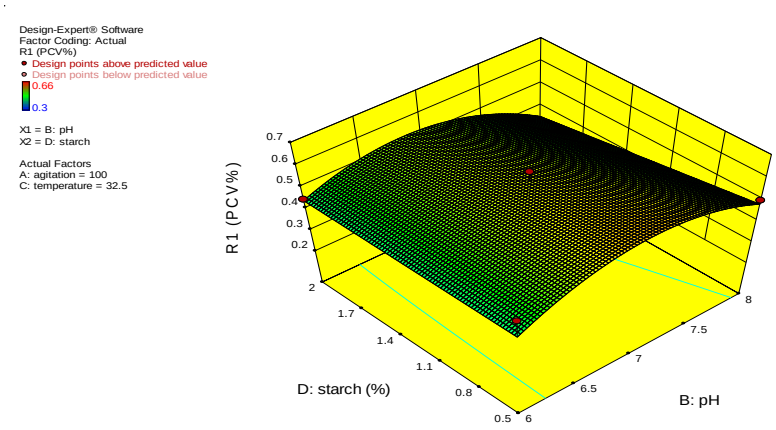


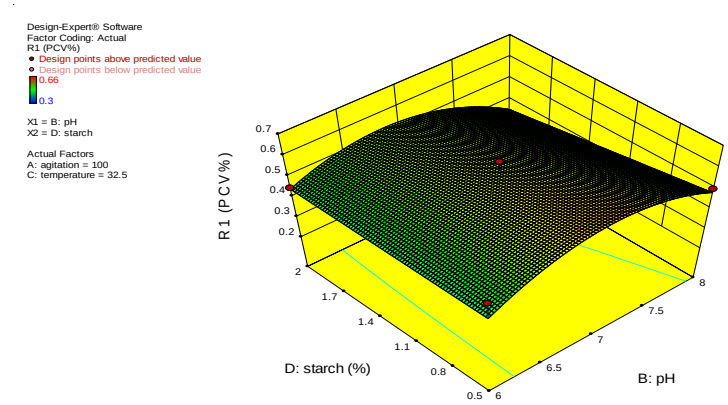
SDS-gel polyacrylamide electrophoresis (SDS-PAGE) showing the purified enzyme.

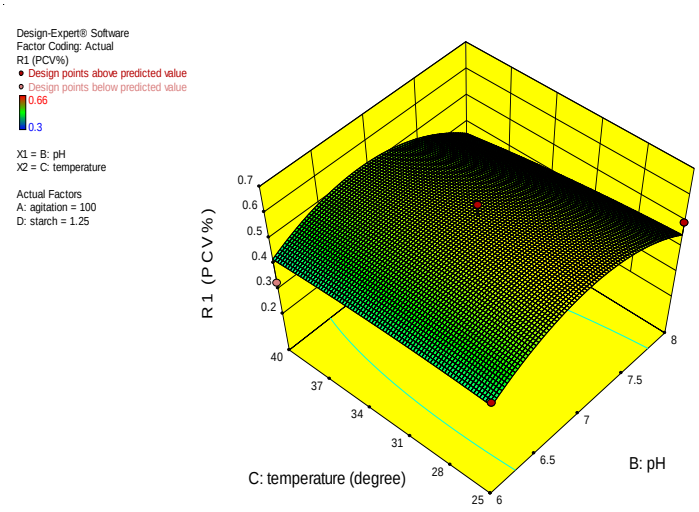
3.4 Optimization of Cultural parameters:

The agitation, pH, starch, and incubation period impacts on alkaline production by *Bacillus auralimaris* were shown individually and in combination using three-dimensional (3D) response surface plots. Fig 6. Two factors' impacts were shown in each 3D figure, with the other variable held at a middle level. The interactions between pH, temperature, starch, and incubation period were minimal. (Fig 6).

Fig: 6 Response surface 3D plots showing individual and interactive effects of variables on enzyme production by *Bacillus auralimaris*







Two factors' impacts were shown in each 3D figure, with the other variable held at a middle level. The interactions between pH, temperature, starch, and incubation period were minimal.

3.5 Partial Purification of Protease:

Table 2 depicts the purification steps for alkaline protease from *Bacillus australimaris*. The alkaline protease purification was recovered 71%, resulting in a fourfold purification by ammonium sulphate precipitation (salting out). The main advantage of ammonium sulphate over other chemicals is that it has higher molarities, which allows it to coagulate and sediment the majority of protein impurity without generating heat [Venugopal et al, 2016]. Increased ammonium sulphate concentrations are known to inhibit or limit microbial growth and protect proteins from denaturation [Chaudhary, 2012]. We are able to increase the percentage of purity gradually up to 38% with 28.66 fold of purification after DEAE cellulose column chromatography. Ammonium sulphate fractionation was described by as a suitable approach for purifying alkaline protease from *Vibrio fluvialis*.

Table 2 Purification of alkaline protease from culture filtrate of *Bacillus australimaris*

Fraction	Total vol (ml)	Activity (U/ml)	Protein (mg/ml)	Total Protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Recovery (%)
Culture filtrate	100	36.0	7.2	720	3600	5.0	1.0	100
Ammonium sulfate precipitation	30	76.1	3.4	102	2283	22.38	4.366	71.34
Dialysis	15	82.3	1.23	18.55	1235	66.81	12.94	38.39
DEAE cellulose	10	93.2	0.63	6.3	932	147.9	28.66	29.12

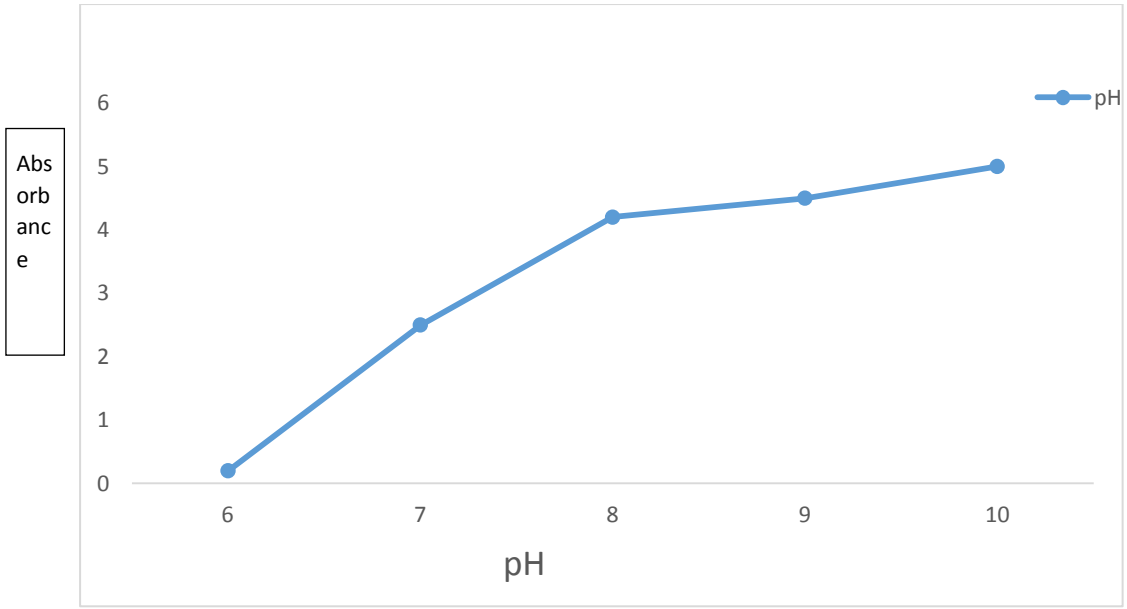
Alkaline protease yield increased 29.12% after 28.66 purification fold.

3.6 Characterization of Protease enzyme activity:

3.6.1 Effect of pH on Enzyme activity:

Maximum activity for BL5 was noted at pH – 10. Figure 7 showed the assessment of protease activity at pH 6,7,8,9&10. An increases in the activity of protease was noted at pH 8 when compared with pH 6 & pH 7. Thereafter, a slight increase is noted with maximum activity being observed at pH 10. These findings are in accordance with several earlier reports showing that pH optima of alkaline proteases of Bacilli species around 10.0 [Krishnaveni et al, 2012, Sekhon, 2010]. Most of commercial proteases mainly neutral and alkaline have been reportedly produced from the genus Bacillus [Krishnaveni et al, 2012].

Figure 7. The effect of different pH on alkaline protease

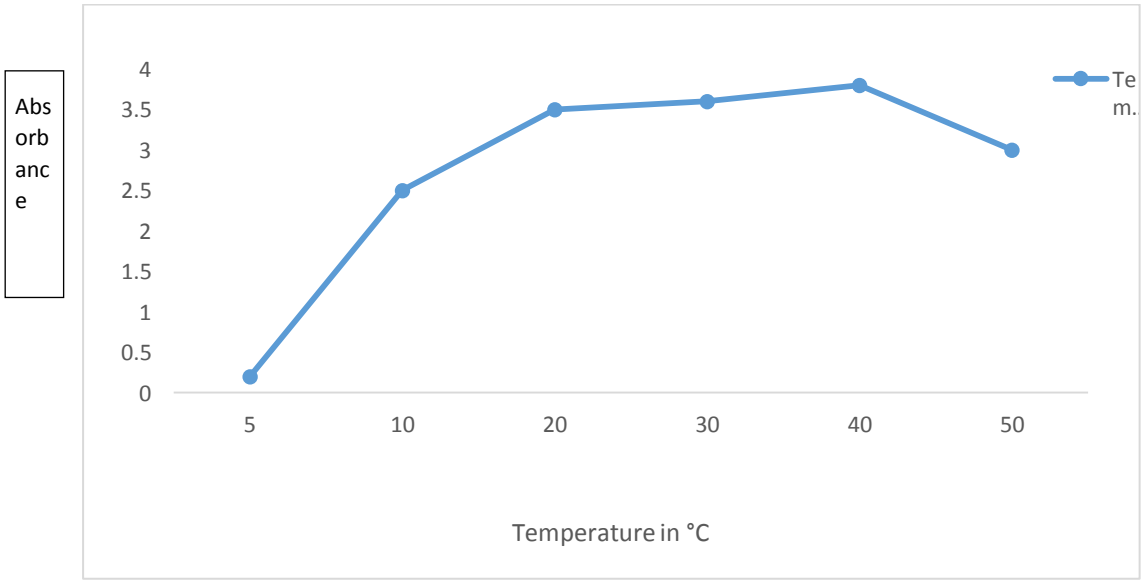


The maximum activity of alkaline protease observed at pH 10

3.6.2 Effect of Temperature on Enzyme activity:

Effect of temperature on the activity of enzymes Figure 8 showed the assessment of protease activity at temperature ranges from 5°C to 55°C (24). An increases in the activity of protease was noted at 40°C. when compared with 30°C to 40°C. Thereafter, a slight declines is noted with activity being observed at 50 °C, the maximal activity of all enzymes was observed.

Figure 8. The effect of different Temperature on alkaline protease

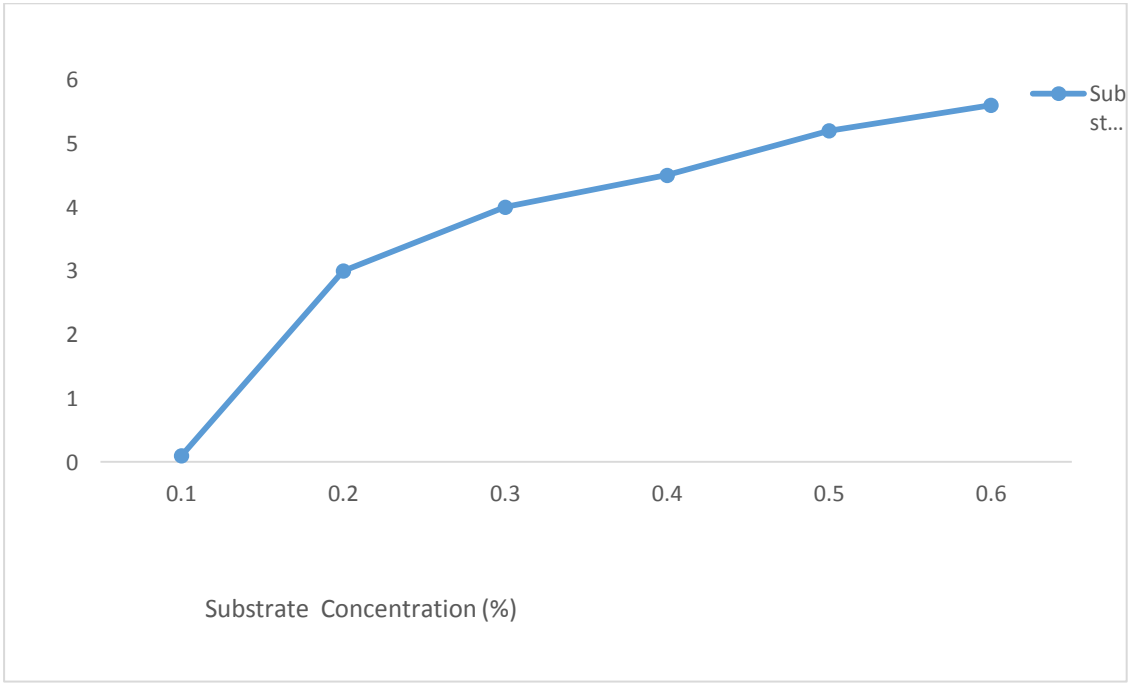


The Maximum alkaline protease activity observed at 40°C

3.6.3 Effect of substrate concentrations on protease activity and stability:

Effect of enzyme activity and substrate concentration Figure 9 portrays the impact of substrate concentration on enzyme activity. The enzyme's maximal activity was determined at a substrate concentration of 0.6%.

Figure 9. The effect of substrate concentration on alkaline protease

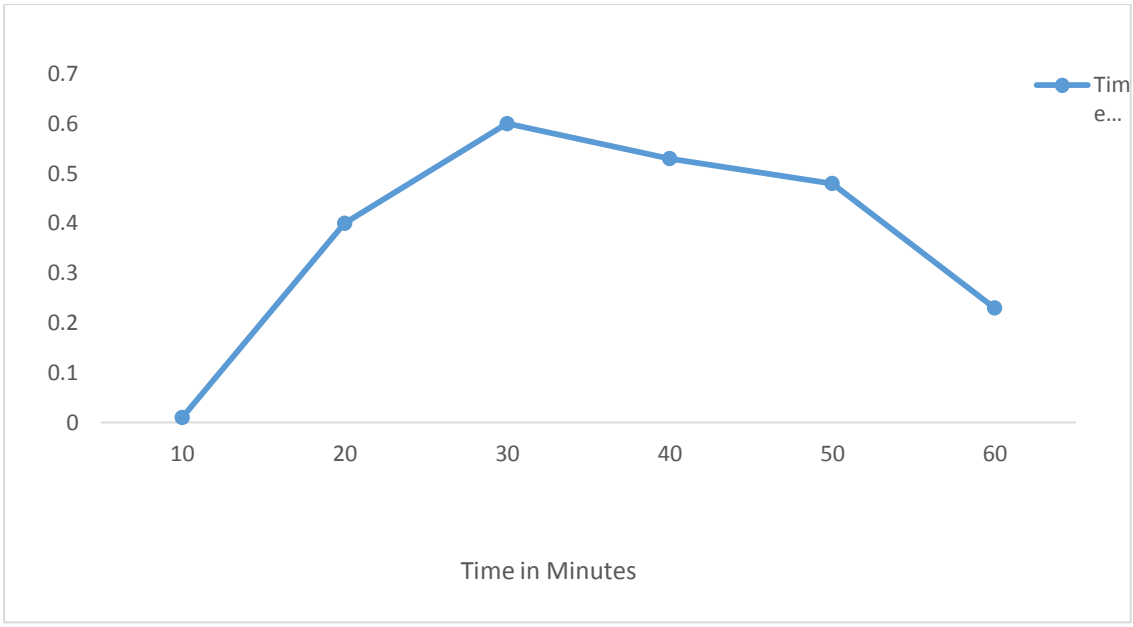


The maximum alkaline protease production observed in 0.6 gm substrate.

3.6.4 Effect of time on protease activity and stability:

Figure 10 showed the impact of incubation time on enzyme activity. Protease enzymes reached their peak activity after 30 minutes of incubation.

Figure 10. The effect of time on alkaline protease



Maximum alkaline protease activity observed in 30 minutes

3.6.5 Effect of metal ions on protease enzyme activity and stability:

To identify the cofactors which would maximize the enzyme activities, several metal ions were evaluated by Fe3+, Ba2+, and Ca2+ ions activate the enzyme and Mn2+, Hg2+, Zn2+, Pb2+, and Cu2+ ions inhibit the enzyme activity. Among the several protease inhibitors, EDTA did not completely inhibit the enzyme, as indicated in Table 3. Contrarily, the protease was severely inhibited by the Tween 80, supporting the discovery as *Bacillus auralimaris protease.*

Table 3 showed inhibitory effect of metal ions on the specific activities of protease

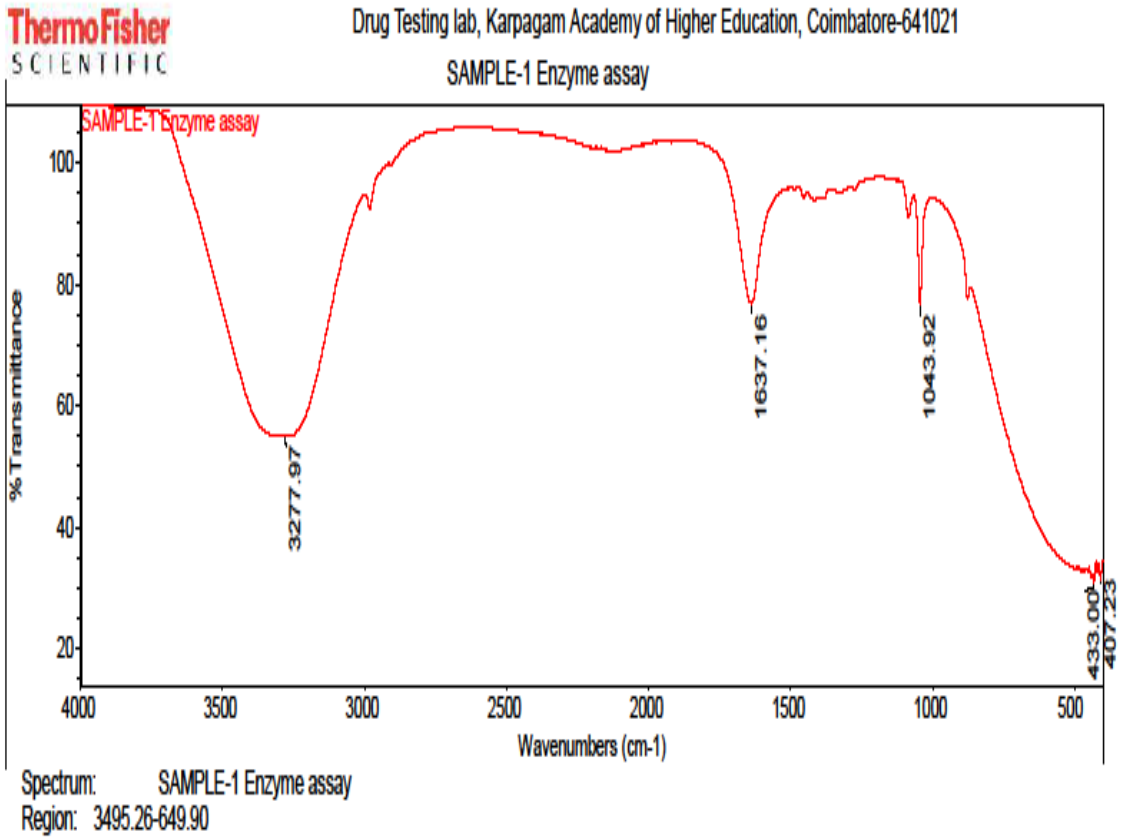
Metal Ion	Concentration (mM)	Activity (%)
Mn2+	2	69
Hg2+	2	56
Zn2+	2	72
Fe3+	2	100
Pb2+	2	78
Ba2+	2	96
Cu2+	2	76
Ca2+	2	101
EDTA	2.5	95
Tween 80	2.5	40

Alkaline protease showing 100 and 101 % of inhibitory activity at 2mM concentration of Fe3+ and Ca2+respectively.

3.6.6. Characterization of Protease by FTIR:

Two absorption peaks were visible in the purified enzymes' FTIR spectra. The spectrum shows that the mixture contained 1637 and 3277 which indicates the presence of functional groups such as carboxylic acid and hydroxyl group (OH Stretching) (Carboxylic acid). In the region of 1043.92 represents the presence of anhydride group in the enzymes.

Figure 10 showed IR Spectra of Protease enzyme

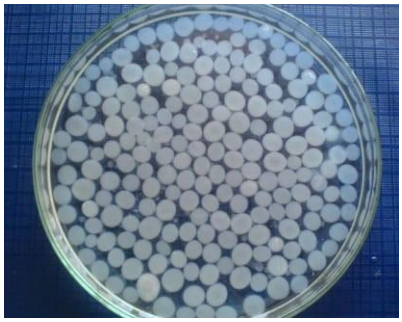


3.6.7. Immobilization of Protease enzyme:

Using multiple sequential substrate hydrolysis experiments, Ca-alginate immobilised protease showed good recyclability potential. With a 1–2.0% gelling agent (Na-alginate) and 2.0% binder (CaCl2), and 200–400 mg/L of protease concentration, the greatest immobilisation yield (> 60%) was achieved (Figure 11). In various carriers employed for immobilisation, enzyme activity was assessed. The most effective supports used for the enzyme immobilisation were calcium alginate. They reported casein hydrolysis rates of 0.0210 and 0.0186 g/mL/min, respectively, and enabled proteolytic activity was

significantly higher than that of free enzyme. Although less enzymatically active than silica the other carriers nonetheless permitted immobilisation. The stability and functional life of the enzyme are increased by the simple, repeatable adsorption approach. Our repass are in agreement with Qamar et al, (2020) reported that polysaccharide (Na-alginate) and a binder (CaCl2), and 400–600 mg/L of protease concentration, the greatest immobilisation yield (> 70%) was achieved [Qamar et L, 2020].

Figure 11: Immobilization of alkaline protease enzyme by sodium alginate



Calcium alginate beads showing 60% of yield.

4. Conclusion:

The present study identified a bacterial protease enzyme and characterized for exerting maximal specific activities at different pH, temperature and substrate concentration. Further, the cofactors which could activate or inhibition were identified which can be used for potential applications. The immunization method identified to be ideal for this enzyme can be vitilized in several industries.

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