Purification Characterization and Optimization Fermentation Parameters of Alkaline Protease Enzyme produced from *BACILLUS SUBTILIS* Strain BS.HK

Muhammad Aftab Akhtar, Amber Afroz, Nadia Zeeshan*
Department of Biochemistry and Biotechnology University of Gujrat, Gujrat, Punjab, Pakistan

***Corresponding Author**
Dr Nadia Zeeshan*
Associate Professor
Department of Biochemistry and Biotechnology
University of Gujrat. Pakistan
ORCID: 0000-0003-0701-7078

Muhammad Aftab Akhtar
Ph D scholar
Department of Biochemistry and Biotechnology
University of Gujrat

Amber Afroz
Assistant Professor
Department of Biochemistry and Biotechnology
University of Gujrat.

Acknowledgement:

The research conducted under the Higher Education Commission funded project # NRPU 20-4571.

Abstract: In this study a strain of *Bacillus sp.* Capable of producing protease enzyme was isolated from leather processing tannery soil, upon characterization four strain which shows maximum protease activity on skimmed milk- agar plates were further analyzed by protease assay and only one strain which shows the the maximum protease activity was selected for further analysis. It was identified as *Bacillus subtilis* BS.HK through 16SrRNA gene sequencing and submitted to GenBank under the accession #OP604265.1. Alkaline protease enzyme was produced from this strain bysubmerged fermentation technique. The highest yield of alkaline protease enzyme production was attained within a 24-hour fermentation period in a basic medium with glucose and Casein as the best Carbon and Nitrogen sources. The optimal conditions for the production of alkaline protease by BS.HK were determined as temperature 37°C, pH 9.5, and an agitation speed of 140 rpm. The enzyme was purified upto 7.40 folds by $(NH4)_2SO_4$ ammonium sulphate precipitation and then purified by Gel filtration chromatography upto 40.62 folds. The Kinetic parameter study revealed that the purified enzyme has molecular weight of 35KDa exhibits optimum activity at 50℃, pH 9.5, and shows high stability at 60℃ and pH 8-12 for 1 hour. The enzyme display remarkable activity with lower concentration of casein as substrate having Km 0.992 µM and Vmax 19.72 U/ml. The inhibitors PMSF and EDTA significantly reduce enzyme activity through different mechanisms. Different surfactant like Triton X-100 and Tween-80 have milder inhibitory effects. Metal ions can either enhance or inhibit enzyme activity, additionally,the anticipation of physicochemical characteristics indicated that the serine protease from BS.HK strain exhibits exception stability, and resilience to alkaline conditions. The purified enzyme from *B. subtilis* BS.HK completely dehaird cow skin without damiging the structural components of skin as compared to chemically dehaired skin. In conclusion, alkaline protease seems to be an important enzyme for leather processing industry because of its thermostability at high temperature and alkali stability at alkaline pH.

Keywords-Protease, Bacillus subtilis, Submerged Fermentation, Enzyme Purification, Kinetic Parameters, Alkaline Protease

1 INTRODUCTION

Proteases a diverse class of enzymes reffered to as peptidyl-peptide hydrolases, play crucial role in breaking down protein molecules by cleaving their peptide bond through hydrolysis [1].Proteases, are categorized into four distinct

groups depending on their mode of : acidic proteases, alkaline proteases, thiol proteases and metallo proteases[2]. Among these various protease types, alkaline proteases hold significant importance due to their extensive applications across a wide range of industries. A wide array of microorganisms possess evidential potency for the production of alkaline protease enzymes when cultivated under favorable growth conditions [3]. Among these microorganisms, bacteria constitute the predominant group of alkaline protease producers, Bacterial proteases have gained significant attention due to their stability under harsh conditions, which makes them suitable for industrial processes. Within the bacteria the *Bacillus* genus being particularly prominent, including *Bacillus subtilis*, have been known to produce proteases of industrial significance [4]. This is due to the rapid growth characteristics of Bacillus species and their ability to thrive in confined cultivation spaces, making them an ideal source for these enzymes [3]. In this study, we purpose to isolate a protease producing bacterial strain from soil, identify it and subsequently produce, purify, and characterize an alkaline protease using a submerged fermentation technique and used this purified enzyme in leather processing industry.

2. MATERIAL AND METHOD

2.1. Isolation and Identification of Protease Producing Bacterial Strain

Soil samples were collected from leather processing area geographical location 32°29'08''N, 74°29' 37"E Sambrial, District Sialkot. The serial dilution and spread plate technique using a M-9 minimal medium containing casein as nitrogen source, was used for the isolation of protease producing bacterial species. Plates were incubated at 37°C for 12 hours, and colonies showing maximal proteolytic activity were selected [5].Morphological identification (gram-staining) was done by Smith & Hussey, 2005[6]. Genomic DNA was extracted by the protocol represented by Neumann et al., 1992 [7]. Molecular identification was confirmed through 16S rRNA gene amplification and sequencing by using 27F and U1492R primers [8].

2.2. Submerged Fermentation for Alkaline Protease Production

Bacterial strain was cultured in M-9 minimal media with Casein as Nitrogen source and Glucose as Carbon source, under submerged fermentation conditions. The composition of M-9 media is as follows; M9 salt was prepared separately consists of ;di-sodium hydrogen phosphate ($Na_2HPO_4.7H_2O$) 1.3%potassium di hydrogen phosphate (KH_2PO_4) 0.3%,sodium chloride (NaCl) 0.5%,ammonium chloride NH4Cl 1% all these compounds were dissolved in 800 ml water and then made volume of total 1 liter. 1M MgSO4,was prepared in stock and autoclave for 15 min at temperature 121°C 15 psi pressure for complete sterilization, glucose 0.5 % was prepared and autoclave separately, calcium chloride ($CaCl_2$) 1 M was prepared and autoclave separately, Casein protein (Sigma Aldrich) 0.5% was as nitrogen source for bacterial growth. For preparation of 50 ml M9 minimal media 20 ml of M9 salt was taken from stock solution and added in 250ml Erlenmeyer flask, then 2ml of 0.5% glucose was taken from stock and then 0.2 ml of 1M ($MgSO_4$) was added, then 10 µl of 1 M $CaCl_2$ was added and then filled volume up-to 50 ml pH 8.5 was set and then 0.25 g of casein protein was added and sealed with cotton plugs and autoclave for 15min at 121°C and 15 psi pressure. The culture parameters including incubation period, pH, , temperature, agitation, carbon and nitrogen source were optimized to increase growth and Enzyme production.

2.3. Protease Assay

The assessment of enzymatic activity was conducted using casein from Sigma Aldrich as the substrate. In this process, 1 ml of 0.5% casein, dissolved in a 50 mMTris-HCl solution at pH 8, was mixed with 1 ml of the appropriately dilute protease enzyme solution. This reaction mixture was then incubated at a temperature of 50°C for a duration of 20 minutes. To halt the enzymatic reaction,1 ml of 10% TCA (Tri-Chloro Acetic Acid) solution was added to each sample, which was subsequently paced on ice for approximately 10 to 15 minutes. The supernatant was separated by centrifugation at 4,500 X g for 10 minutes at 4°C, and its absorbance was measured at 280nm as per the methodology outlined by Manavalan et al. in 2020 [9]. The quantification of enzyme activity was defined as one unit, signifying the enyme quantity needed to release one microgram (µg) tyrosine amino acid per minute under specified essay conditions, following the criteria established by Bouacem et al. in 2015 [10]. Additionally, the total protein contents of samples was determined by using the Bradford assay [11].

2.4. Optimization of Fermentation Parameters

2.4.1. Effect of incubation Time period on enzyme production

The impact of varying incubation time period on growth was investigated by subjection of the culture medium to various time intervals of 12 hours from 12 -72 hours. Following each time interval, the activity of enzyme was calculated to determine the best optimal incubation period that provides yields the highest production of alkaline protease enzyme.

2.4.2. Effect of pH on Growth and Enzyme Production

The best pH for the production of alkaline protease enzyme was ascertained by varying the initial pH levels of the growth medium at different points (ranging from 7.0 to 11.0, with increments of 0.5). Prior to sterilization, each growth medium's pH was carefully adjusted.

2.4.3.Effect of Temperature on Growth and Enzyme Production

The influence of different incubation temperatures, spanning from 25 to 50°C, on enzyme production was investigated by by subjecting the culture medium to a range of cultivation temperatures. Cell biomass and protease activity was assessed at each temperature to observe the effects.

2.4.4 Effect of Agitation speed on growth and Enzyme Production

The influence of different agitation speeds on cell growth and enzyme production were studied by incubating the culture medium at different speeds ranging from (60 -160 rpm with the interval of 20)under optimized standard conditions, and enzyme activity was measured to identify the best agitation speed. The optimal agitation speed that would yield maximum alkaline protease production and promote the growth of bacteria.

2.4.5. Effect of Different Carbon Sources on Cell Growth and Enzyme Production

The influence of various carbon sources on cell growth and enzyme production from *B.subtilis BS.HK* was determined by employing 1.0% (w/v) of different soluble carbon sources (Fructose, Glucose, Lactose, maltose, starch and Galactose) in the culture media to identify the best carbon source for growth of bacteria and enhancing production of alkaline protease enzyme[12].

2.4.6. Effect of Different Nitrogen Sources on Growth and Enzyme Production

A variety of different Nitrogen sources, including organic compounds and inorganic salts were explored to determine the most suitable nitrogen source for *Bacillus subtilis* for producing proteolytic enzymes. Initially, the culture medium was enriched with various organic nitrogen sources such as peptone, tryptone, yeast extraxt, casein and gelatin, each at a concentration of 1.0% (w/v). Among the different Inorganic nitrogen sources , compounds like sodium nitrate, potassium nitrate, $(NH_4)2HPO_4$, $(NH4)2SO_4$ and ammonium nitrate $(NH4NO_{3)}$ also at 1.0% (w/v)concentration , were tested to determine the most effective nitrogen source for producing proteolytic enzymes by *Bacillus subtilis* BS.HK.

2.5. Purification of Alkaline Protease

The crude protease enzyme was extracted from the fermentation broth and subjected to a series of purification steps, which involves the precipitation with (70%) ammonium sulfate and subsequent ion exchange chromatography,and then by gel filtration chromatography. The purification of crude extracellular proteases from *Bacillus subtilis* BS.HK species involved a well-defined protocol, ensuring the attainment of homogeneity. The process comprised two key steps: salting out with ammonium sulfate and size exclusion chromatography using a Sephadex G-75 column, all conducted at a temperature of 4°C. Firstly, the enzyme was precipitated by gradually adding ammonium sulfate (reaching 84% saturation) and subsequently centrifuged at 11000 g for 15 min at 4°C. The resulting pellet contains protein which was re-suspended in 50mM Tris-HCl (pH 8.5) buffer, and then dialysis was performed in the same buffer using nitrocellulose membrane. The soluble protein was added into gel filtration coloumn containg Sephadex G-100 coloum from Sigma(USA) which was pre-equilibrated with 50mM Tris-Cl buffer(pH 8.5). The elution of the protein was carried out at a steady flow rate of 1.0 ml per minute. To concentrate the active fractions, centricon (with a cutoff of 10 kDa) was employed. The final purified enzyme underwent analysis through 12% (w/v) (SDS-PAGE), [13].

2.6. Characterization of Alkaline Protease Enzyme

The purified alkaline protease enzyme underwent various characterizations for following parameters;

2.6.1. Determination of Kinetic Parameters and Substrate Specificity

The enzyme's activity was tested using different dilution of casein protein (0.1-2.0%) as substrate to determine its specificity. Michaelis - Menten equation was employed for the determination of Km and Vmax.

2.6.2. Effect of pH on Enzyme Activity and Stability

The effect of pH of purified alkaline protease was calculated by measuring the activity of enzyme at various pH values ranges from (6-12) by using dissolving 0.65% casein protein as substrate in various buffer solutions; 0.05 M phosphate

buffer having (pH 6-7), Tris-Cl buffer having(pH8-9) and glycine-NaOH buffer having (pH 10-12). The pH stability was assessed by incubating the enzyme across a range of pH values from (6-12) in various buffer solutions at 50°C for 1 hour. Subsequently the residual activity was measured following standard assay protocols [14].

2.6.3. Effect of Temperature on Enzyme activity and Thermostability

Effect of temperature on the activity and of purified alkaline protease was examined by incubating the reaction mixture containing enzyme and 1% casein solution at various temperatures ranging from 30°C to 80°C. The thermostability of purified enzyme was also assessed by incubating it at temperature ranging from 30°C-80°C. After that enzyme activity was determined each incubation by comparing activity with the non-heated enzyme as 100%.

2.6.4. Effect of metal ions and Inhibitors

The effect of different inhibitors and metal ions concentration on enzyme activity was investigated by incubating the enzyme solution 0.1mM to 1mM concentration of inhibitors and activators for 30min at 40°C and % activity was measured by standard method [15].

2.7. Removal of Hairs from Animal Hide

Dehairing of animal skin was done by incubating the (2 X 2 inch) pieces of animal hide with alkaline protease enzyme, sodium sulfide and water at 37°C for 12 hours. After 12 hours of incubation check the skin either hair are removed or not.

3. RESULTS

3.1. Isolation and Identification of Protease Producing Bacterial Strain

The protease-producing bacterial strain isolated from soil samples was confirmed to be gram positive *Bacillus sp.* based on morphological identification including gram staining, as shown in Fig. 1(a),this particular strain displayed the widest zone diameter of 18mm on agar plate containing skimmed milk as shown in Fig. 1(b).

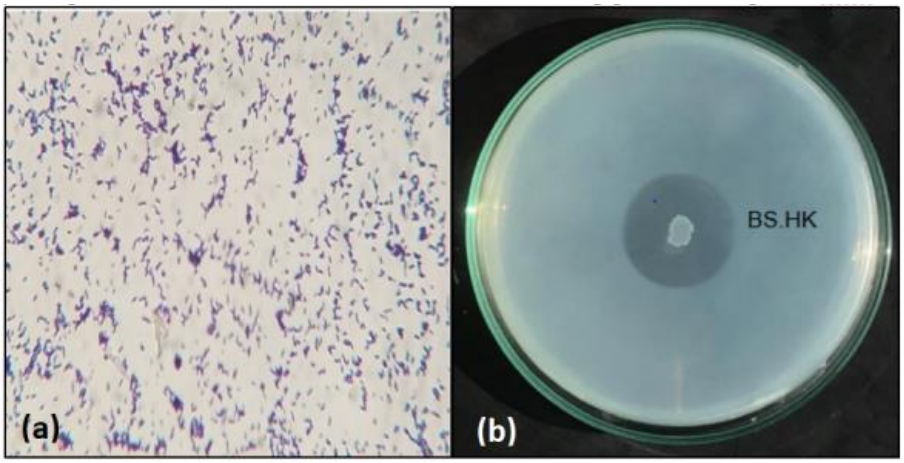

Fig. 1.(a) Result of gram staining under light microscope with magnification (1000X) shows gram positive Bacillus bacteria, **(b)** Proteolytic activity of *Bacillus subtilis* BS.HK on skim milk agar plate shows clear zone of hydrolysis of approximately 18mm

3.2. Isolation of Genomic DNA

About 5ml of overnight pure culture of gram positive *Bacillus sp.* in LB media. The extraction of bacterial DNA was performed using a liquid media method as described earlier. Band of purified genomic DNA was shown on 1 % agarosegel under UV light as shown in Fig. 2(a).

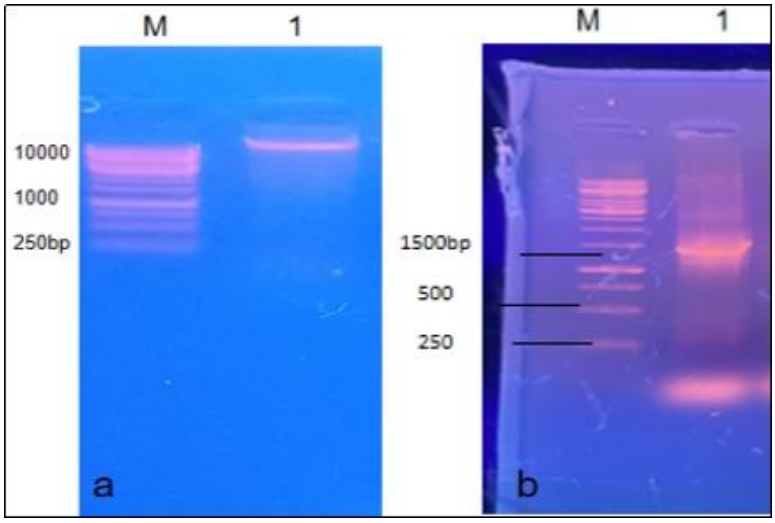

Fig.2. DNA and PCR amplified product on 1% agarose gel under UV visualizer, (a) Lane (M) represents the 1kB gene ladder, lane (1) shows genomic DNA isolated from *B.subtilis* BS.HK (b) 16S rRNA gene amplification byPCR using 27F and U1492R primers set, lane (M) represents the 1kB gene ladder lane (1) showing the size of 1500bp on 1% agarose gel

3.3. 16S rRNA Gene Amplification

by using a set of universal primers 27F forward primer and U1492R reverse primer from highly conserved region was used for amplification of full length 16S RNA gene from the smaller subunit. A 1.5 kb fragment of 16S rRNA gene from the *Bacillus sp.* BS.HK was successfully amplified. The results of the 1% agarose gel electrophoresis demonstrated gene amplification as shown in fig. 2(b). The amplified bands were almost 1500bp. The phylogenetic relationship of identified *Bacillus subtilis strain* BS.HK was dtermined by using NCBI BLAST and the sequences having similarity of 99% was used for multiple sequence alignment and construction of phylogenetic tree using MEGA-XI software that employed neighbour joining method for construction of tree Fig.3 Shows the phylogenetic tree.

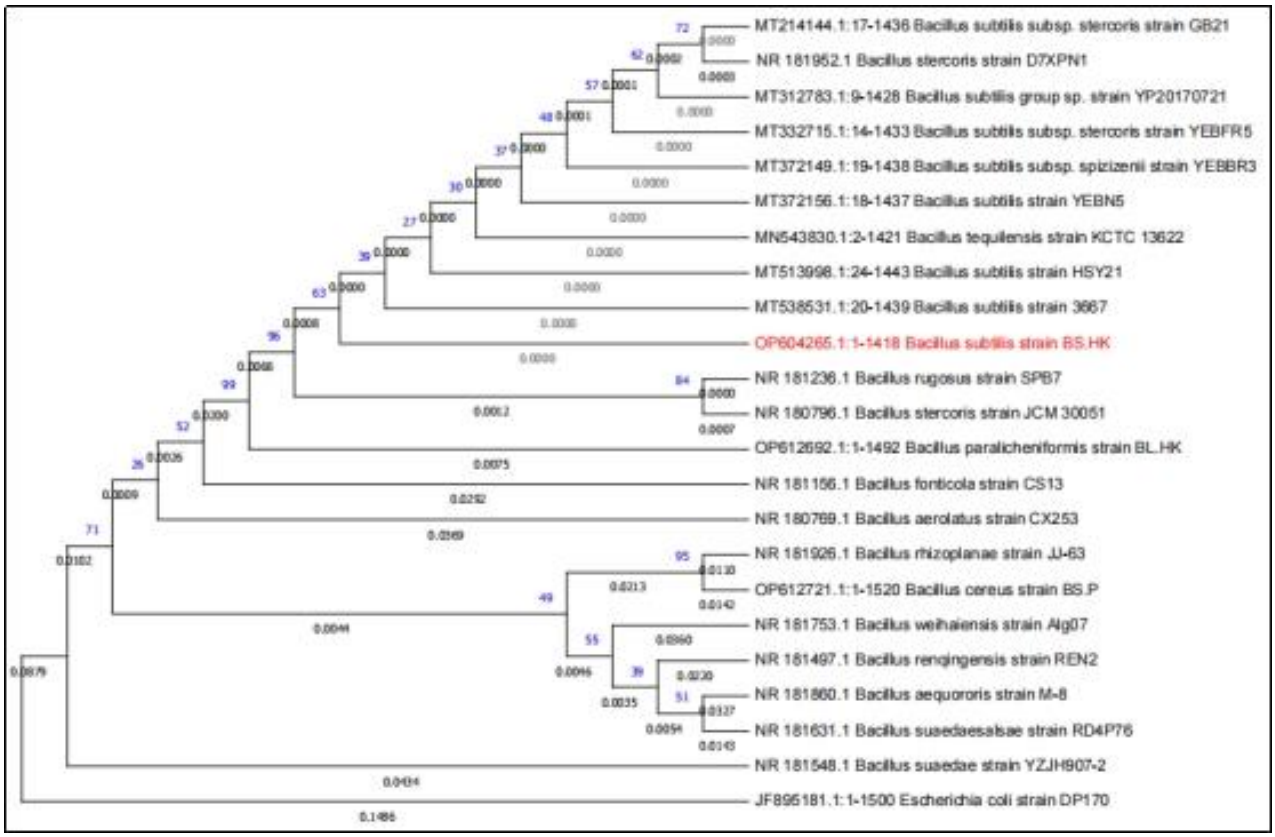

Fig. 3. The phylogenetic relationship of Bacillus sp. within the Bacillus genus was determined by using Neighbour joining method and bootstrap values were calculated from 1000 replicates. The analysis was based on 16S rRNA nucleotide sequences of the following Bacillus strains: *Bacillus subtilis subsp.stercoris* strain GB21, *Bacillus stercoris*strain D7XPN1, *Bacillus subtilis group sp.* strain YP20170721, *Bacillus subtilis subsp.stercoris* strain YEBFR5, *Bacillus subtilissubsp.spizizenii* strain YEBBR3, *Bacillus subtilis* strain YEBN5, *Bacillus tequliensis*strain KCTC 13622, *Bacillus subtilis*strain HSY21,*Bacillus subtilis*strain 3667, *Bacillus subtilis* strain BS.HK, *Bacillus rugosus* strain SPB7,*Bacillusstercoris* strain JCM30051, *paralicheniformis strain* BL.HK, *Bacillus subtilisfonticola*strain CS13, *Bacillus aerolatus* strain CX253, *Bacillus rhizoplanae*strain JJ-63, *Bacillus cereus* strain BS.P, *Bacillus welhalensis* strain Alg07, *Bacillus renqingensis* strain REN2, *Bacillus aequorosis* strain M-8, *Bacillus suaedaesalsae* strain RD4P76, *Bacillus suaedae* strain YZJH907-2, *Escherichia coli* strain DP170.

3.4. Alkaline Protease Production

Submerged fermentation of *B.subtilis* BS.HK resulted in significant alkaline protease production, with maximum activity observed under optimized conditions.

3.4.1. Effect of Incubation Period

The initial cell biomass at 12 hours is 1.72 g/L and enzyme activity is 156U/ml. Over the next 12 hours (from 12 to 24 hours), the biomass increases significantly to 3.85 g/L and enzyme activity to 874.31U/ml, indicating rapid microbial growth. Between 24 and 36 hours, there is a slight decrease in cell biomass from 3.85 g/L to 3.82 g/L and activity decreases to 802.09U/ml, which could indicate stabilization or slowing of growth. After 36 hours, the cell biomass continues to gradually decrease reaching 3.06 g/L at 72 hours and enzyme activity continues to decline progressively over time, reaching 532.77 U/ml as shown in Fig.4 (a). This suggests that the microbial population is declining or not growing as rapidly and decreases, the enzyme production also decreases.

3.4.2. Effect of Incubation Temperature on Protease Production

Incubation temperature importantly affects the cell growth and enzyme production in *Bacillus subtilis BS.HK.* At lower temperatures (25°C and 28°C), the cell biomass is relatively low, with values of 1.02 g/L and 1.09 g/L, respectively. As the temperature increases from 31°C - 37°C, there is a significant increase in cell biomass, reaching a peak of 3.83 g/L at 37°C. This indicates that the microorganisms are most productive in terms of growth at this temperature. Beyond 37°C, the cell biomass starts to decline, reaching 2.02 g/L at 50°C. This decline is likely due to the adverse effects of higher temperatures on microbial growth. Enzyme activity shows a similar trend to cell biomass in response to temperature. At lower temperatures (25°C and 28°C), enzyme activity is relatively low (273.22 U and 313.6 U, respectively). As the temperature increase from 31°C to 37°C, there is a substantial increase in enzyme activity, with the highest activity of 878.13 U observed at 37°C. Beyond 37°C, enzyme activity starts to decline, dropping to 185.32 U at 50°C as shown in Fig.4 (b). This decrease in enzyme activity is likely due to the denaturation or inactivation of the enzyme at higher temperatures.

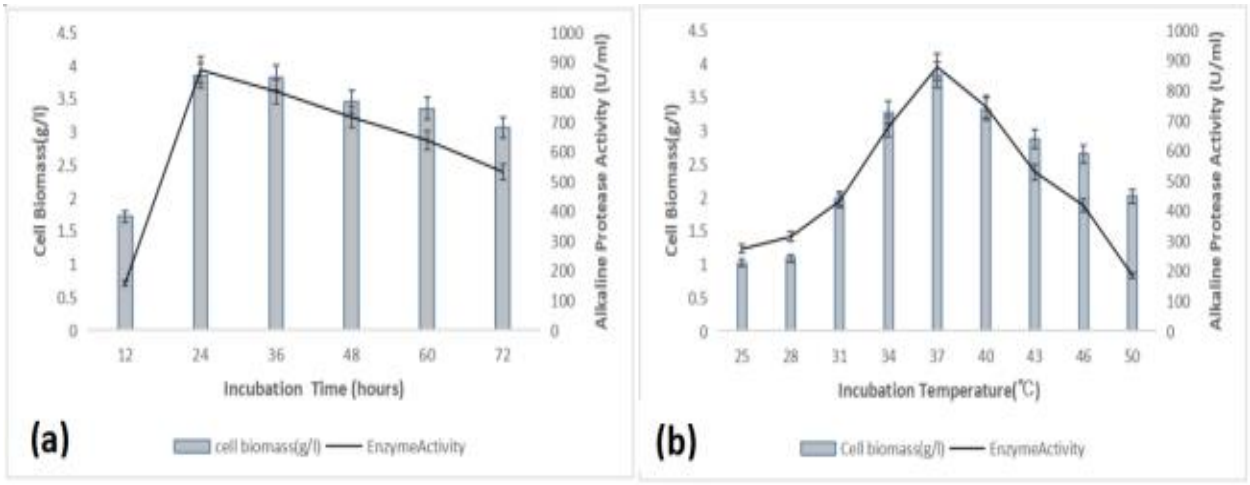

Fig.4 .(a) Effect of incubation time period, **(b)** Effect of incubation temperature on growth and Enzyme activity of alkaline protease enzyme from *B.subtilis* BS.HK at pH 9.5, 140 rpm in shake flask cultures, Bars represents SD

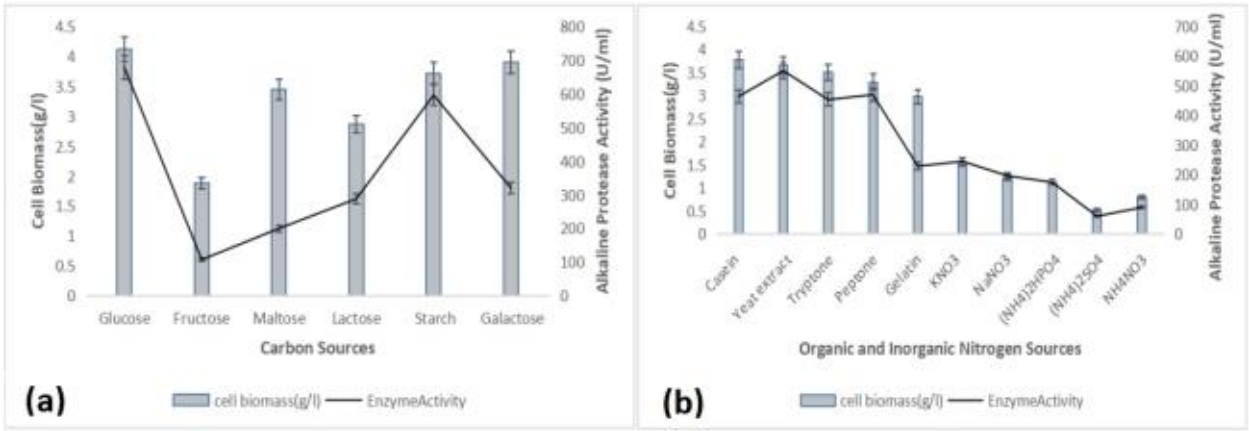

Fig. 5. (a) Effect of Different Carbon sources **(b)**Effect of different Nitrogen sources on growth and specific activity of alkaline protease enzyme produced from *B.subtilis* BS.HK pH of culture media was set at 9.5, temperature 37°C,agitation speed 140 rpm incubation time 24 hours in shake flask cultures, Bars represents ±SD

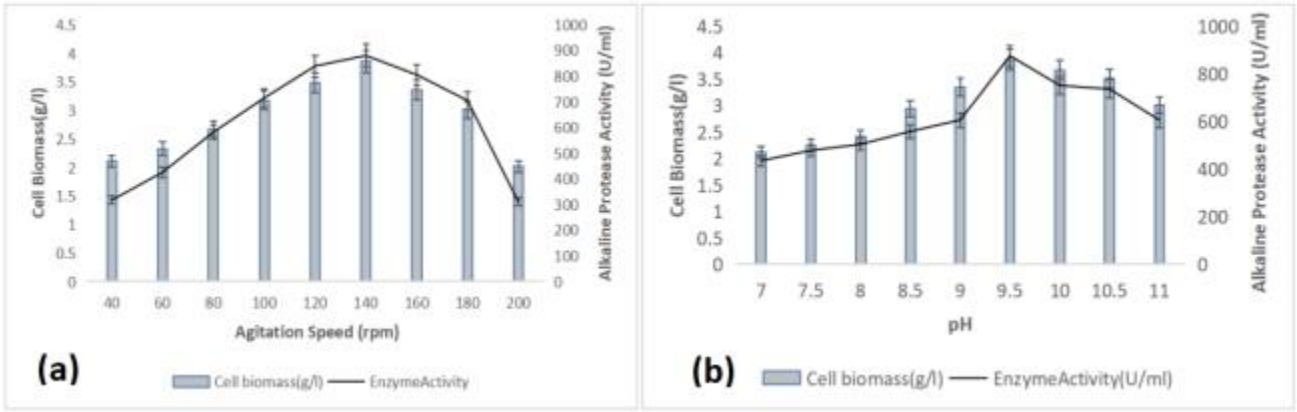

Fig.6 .(a) Effect of agitation speed, **(b)** Effect of pH on growth and enzyme activity of alkaline protease enzyme from *B.subtilis* BS.HK after 24 h of incubation , temperature 37°C in shake flask cultures, Bars represents SD

3.4.3. Effect of Different Carbon Sources

Production of Alkaline protease enzyme from bacteria is highly influenced by the carbon source used. Among the carbon sources tested, glucose resulted in the highest cell biomass production at 4.12 g/L, followed by Galactose at 3.9 g/l and starch at 3.72 g/L and Maltose at 3.45 g/L. Fructose and Lactose produced lower cell biomass, with Fructose being the lowest at 1.88 g/L. Glucose led to the highest enzyme activity at 677.64 U/ml. Starch and Maltose also exhibited substantial enzyme activity at 595.82 and 200.09U/ml, respectively. Galactose and Lactose showed moderate enzyme activity. Fructose resulted in the lowest enzyme activity at 107.22 U/ml. When considering both cell biomass and enzyme activity, Glucose and Starch appear to be the most favorable carbon source for promoting both microbial growth and enzyme production. Glucose stands out as the best carbon source for maximizing enzyme activity, with the highest enzyme activity observed among all the carbon sources. Glucose is effective in promoting high cell biomass production and substantial enzyme activity, making it a strong candidate for overall fermentation performance as shown in Fig. 5(a). On the other hand, Fructose resulted in the lowest cell biomass and enzyme activity compared to other carbon sources.

3.4.4. Effect of Different Nitrogen Sources

Synthesis of alkaline protease is highly influenced by the nitrogen source used in the media. Among the nitrogen sources tested, Casein resulted in the highest cell biomass production at 3.78 g/L, followed closely by Yeast Extract at 3.65 g/L and Tryptone at 3.51 g/L. The cell biomass gradually decreases with other nitrogen sources, with $(NH_4)_2SO_4$ resulting in the lowest biomass at 0.53 g/L. For enzyme activity, Yeast extract led to the highest enzyme activity at 551.1 U/ml. Casein and Peptone also showed relatively high enzyme activity at 464.11 and 470.3 U/ml, respectively. Gelatin, KNO_3, and $NaNO_3$ exhibited moderate enzyme activity. The lowest enzyme activity was observed with the ammonium-based nitrogen sources, with $(NH_4)_2SO_4$ having the lowest activity at 60.77 U/ml. When considering both cell biomass and enzyme activity, it appears that Yeast Extract, Casein and Tryptone are the most favorable nitrogen sources for promoting both microbial growth and enzyme production as shown in Fig. 5(b).

3.4.5. Effect of Agitation Speed

The study focusing on production of protease and cell growth of *B. subtilis* BS.HK, various agitation speeds were tested while incubating the M9 medium on standard conditions. The consequences revealed that highest levels of cell proliferation reaches to 3.86 g/l, and protease activity reaches 880.31 U/ml were achieved at an agitation speed of 140 rpm. However, beyond this optimal agitation speed of of 140 rpm, both protease activity and cell growth declined. When the agitation rate was increased to 160 rpm, there was a noticeable decline in protease activity and cell growth. However, the culture demonstrated an elevation in specific activity as agitation rates were raised.

, with the highest specific activity recorded at 140 rpm as shown in Fig. 6(a). Conversely, the lowest specific activity was observed at 40 rpm which is (127.89 U/mg). As a result, based on these findings, 140 rpm was determined to be the best condition for achieving the maximum production of alkaline protease by *B. subtilis* BS.HK in shake flask experiments. This selection is supported by the simultaneous enhancement of both cell growth and protease activity, making it the most favorable operational parameter for this particular bioprocess.

3.4.6. Effect of pH on Cell Growth and Enzyme production

There is a general trend of increasing cell biomass enzyme activity with increasing pH levels from pH 7 to pH 9.5.The highest cell biomass and enzyme activity is achieved at pH 9.5, with a value of 3.86 g/ and 875.4 U/ml. Beyond pH 9.5, the cell biomass starts to decrease, reaching 3.01 g/L at pH 11. Like cell biomass, enzyme activity decreases at pH 10.5 and pH 11, although it remains relatively high compared to lower pH levels. The optimal pH range for *B. subtilis* strain BS.HK, is pH 9.5 being the most favorable for both cell biomass production and enzyme activity as shown in Fig. 6(b).

3.5. Purification of Alkaline Protease

In the initial stage crude enzyme extract, has total activity of 3,219,421 units and a total protein content of 7501 mg. The specific activity, which is the amount of enzyme activity per unit of protein (U/mg), was 417.54 U/mg. This is the starting point for purification process, and it's given a purification fold of 1 because it represents the initial state. The yield at this stage is 100% because there is no loss of any enzyme activity or protein yet. After the ammonium sulfate [$(NH_4)_2SO_4$] precipitation step at 70% saturation, total protein content were reduced to 675 mg but retained 1,545,651 units of enzyme activity. The specific activity increased significantly to 1324.12 U/mg. The purification fold at this stage is 7.40, indicating that enzyme in the sample is enriched by 7.4 times compared to the crude extract. However, the yield has dropped to 71%, indicating that some protein was lost during the precipitation step. In the final purification step of gel filtration chromatography (Sephadex G-100) was used and, the total protein contents was further reduced to 26 mg while retaining 123,862 units of enzyme activity. The specific activity increased dramatically to 22,310.74 U/mg, indicating that the enzyme is highly purified and concentrated in this step. The purification fold at this stage is 40.62, indicating an important improvement in enzyme activity as compared to the crude extract as shown in (Table. 1). However, the yield has dropped to 14%, suggesting that a substantial amount of protein was removed during the gel filtration step as shown in table. 1. The alkaline protease was successfully purified, as evidenced bySDS-PAGE which represents the molecular weight of alkaline protease is approximately 35KD also shown confirmed by zymogram assay on casein agar plate as shown in fig. 7(b).The elution profile of (gel purification on Sephadex G-100)alkaline protease enzyme is shown in Fig. 7(a).

Table.1 The alkaline protease enzyme purification by through meticulous process involving ammonium sulfate precipitation and subsequent gel filtration chromatography us Sephadex G-100

Steps of Purification	Total Activity(U)	Total Protein(mg)	Specific Activity(U/mg)	Purification folds	Yield (%)
Crude Enzyme	3219421	7501	417.54	1	100
$(NH_4)_2SO_4$ Precipitation (70%)	1545651	675	1324.12	7.40	71
Sephadex G-100	123862	26	22310.74	40.62	14

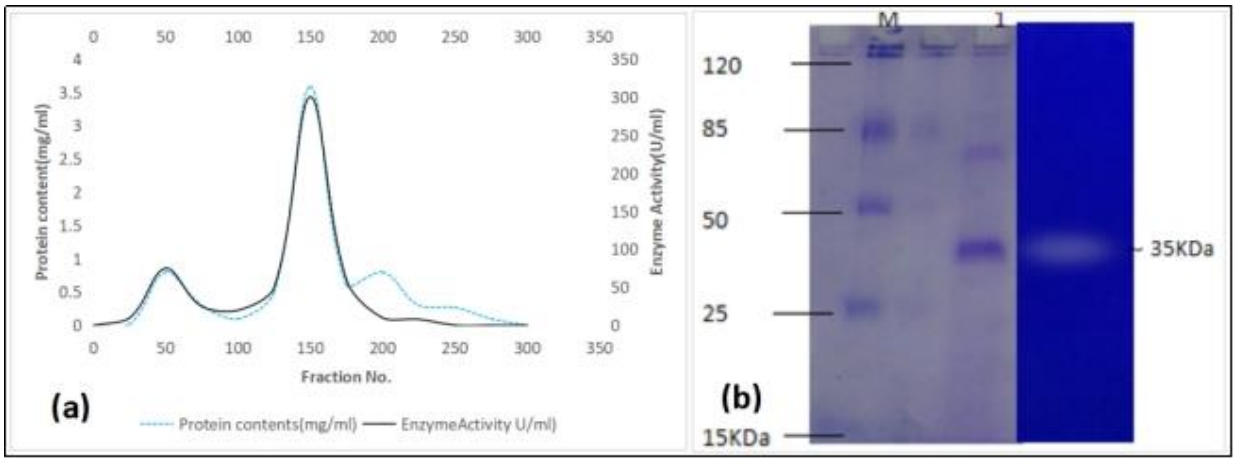

Fig.7 .(a) Elution profile and of purified alkaline protease enzyme produced from *B. subtilis* BS.HK gel filtration chromatography by using Sephadex G-100, **(b)** SDS-PAGE analysis Lane M contains a protein marker and Lane 1was loaded with purified enzyme, and zymogram analysis on casein agar plate shows the zone of hydrolysis

3.6. Characterization of Alkaline Protease Enzyme

3.6.1. Substrate Specificity

Kinetic study of purified alkaline protease revealed thata reduced Km value incates a robust binding affinity between protease with its substrate. The purified alkaline protease from *Bacillus subtilis* BS .HK exhibited a broad substrate specificity, with high activity Vmax 19.72 and Km 0.992 µM against different concentrations of protein as a substrate as shown in Fig. 8 (a and b).

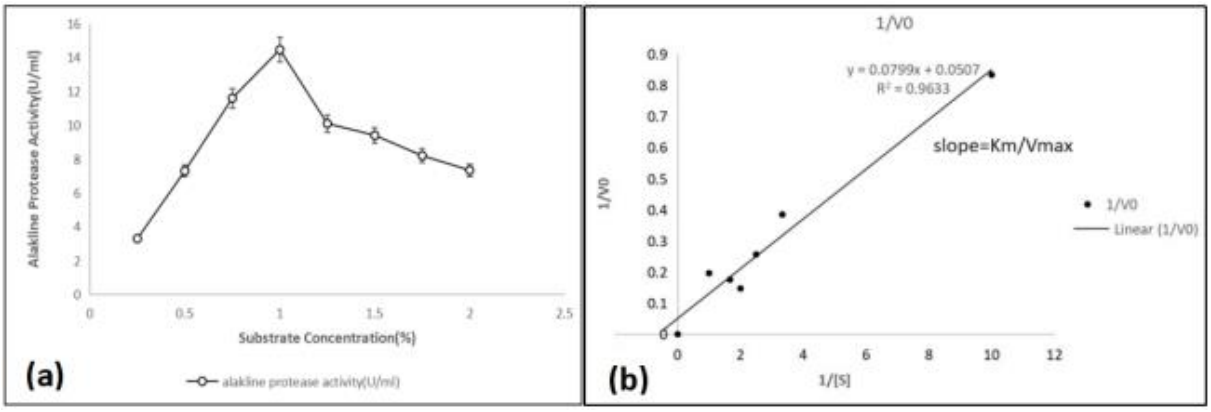

Fig.8 .(a) effect of substrate concentration on enzyme activity, **(b)** Line weaverburk plot of enzyme activity and substrate concentration of enzyme produced from *B. subtilis* BS.HK for varied degree of substrate concentrations (0.1-2.0µM) indicating Km and Vmax

3.6.2. PH Optimum and Stability

The enzyme displayed highestactivity of 16.3 (U/ml) at pH 9.5 , and it exhibited remarkable stability from pH 8-12 and shows 100% activity at pH 10.5. The enzyme shows its maximum activity at pH 9.5, where it reaches 16.3 U/ml. This pH level is the enzyme's "optimum pH," at which it is most efficient in catalyzing proteolytic reactions. Alkaline protease activity generally increases as the pH becomes more alkaline (above pH 7). The enzyme's activity steadily rises from pH 7 (3.9 U/ml) to pH 9.5 (16.3 U/ml), indicating that it is well-suited for alkaline environments. Beyond its optimal pH of 9.5, the activity of enzyme starts to decline. This decrease in activity is evident as the pH continues to rise, reaching its lowest point at pH 12 (6.1 U/ml) as shown in Fig. 9(a). Extremely high pH can denature enzymes or affect their active site, leading to reduced catalytic efficiency. The enzyme is stable at pH 10.5, where the residual activity is 100%. The enzyme's

activity generally increases as the pH becomes more alkaline (above pH 7). The trend starts from pH 7 (30.1% residual activity) and continues to increase steadily up to pH 10.5 (100% residual activity), indicating that the enzyme is well-suited for alkaline environments. Beyond the optimum pH of 10.5, the enzyme's residual activity starts to decline. This decrease in activity is evident as the pH continues to rise, reaching its lowest point at pH 13 (39.8% residual activity)as shown in Fig. 9(b). This characteristic is valuable for industrial applications where the enzyme needs to function under varying pH conditions.

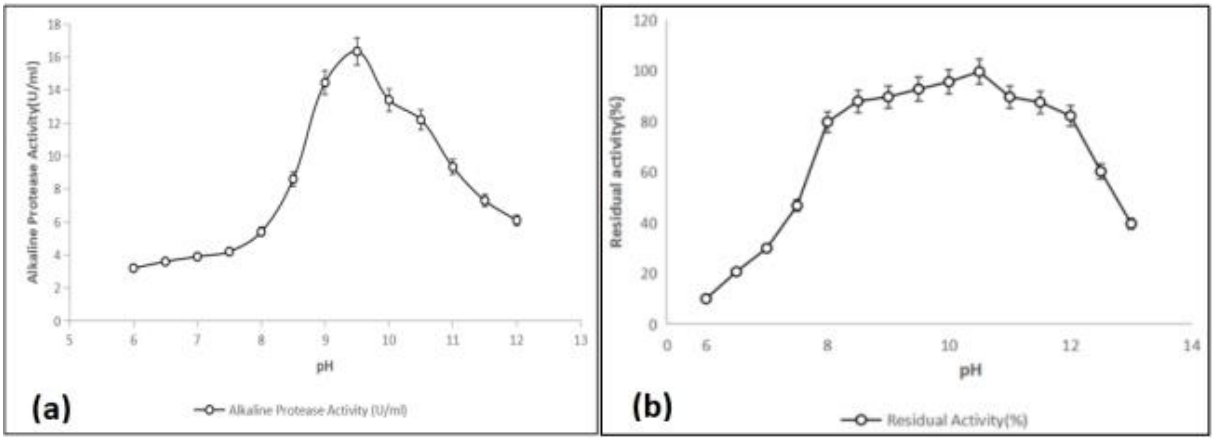

Fig.9. (a) The Effect of pH on activity of alkaline protease enzyme, **(b)** Effect of pH on Stability of alkaline protease enzyme, bars represents SD

3.6.3. Temperature Optima and Stability

The enzyme from *B.subtilis* BS.HK exhibits its maximum activity at 50°C, where the enzyme's activity is 16.3 U/ml. This temperature is the enzyme's "optimum temperature," indicating that it is most efficient at catalyzing reactions at this specific temperature. The enzyme's activity increases as the temperature rises from 30°C to 50°C. The trend shows a steady increase in enzyme activity as the temperature increases, indicating that the enzyme is more active and efficient at higher temperatures within this range. Beyond the enzyme's optimum temperature of 50°C, the enzyme's activity begins to decline. This decrease in activity is evident as the temperature continues to rise, reaching its lowest point at 80°C (3.5 U/ml) as shown in Fig. 10(a). Extremely high temperatures can denature enzymes, causing them to lose their structure and activity. As the temperature increases from 40°C to 60°C, there is only a slight reduction in enzyme activity. At 50°C, the enzyme still retains 100% residual activity, and at 60°C it retains 97% of its residual activity. This suggests that the enzyme is relatively stable and active at moderately elevated temperatures. Beyond the enzyme's optimum temperature range, a substantial drop in activity occurs. At 70°C, the enzyme's residual activity decreases to 40%, indicates that the enzyme is less stable and less active at this higher temperature. At 80°C, the enzyme's residual activity drops significantly to only 4% as shown in Fig. 10(b). This steep decline in activity at this temperature suggests that the enzyme is highly sensitive to extreme heat and likely undergoes denaturation, losing its structure and, consequently, its catalytic activity.

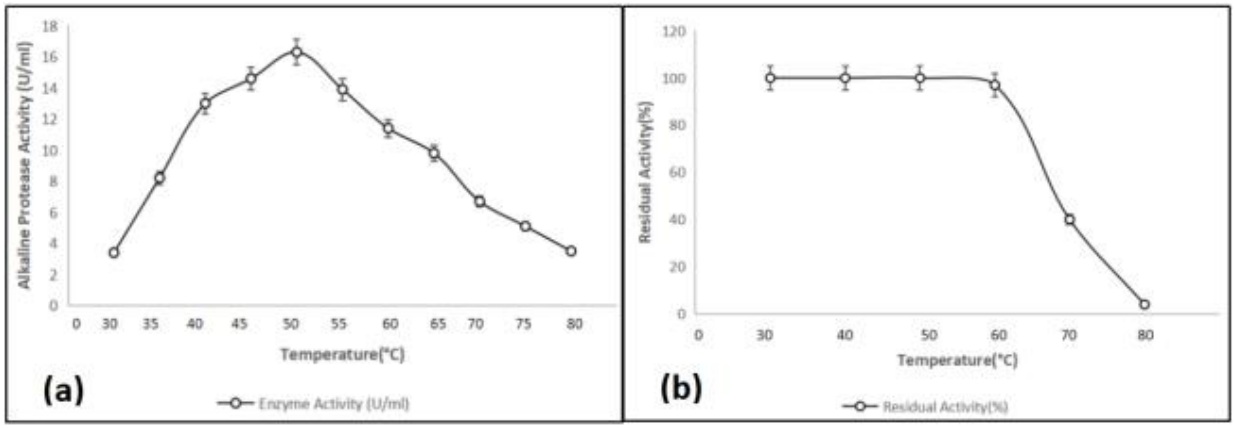

Fig.10. (a) Effect of temperature on enzyme activity,bars represents SD **(b)**Effect of temperature on stability of enzyme bars represents SD

3.6.4. Effect of Inhibitors and metal ions

PMSF (Phenylmethylsulfonyl fluoride) is a strong irreversible serine protease inhibitor. It modifies the active site of the enzyme by covalently binding to a serine residue, thereby greatly reducing enzyme activity to 20%. EDTA is a chelating agent that bind to the metal ions, which are often cofactors for enzymes. By chelating essential metal ions, it can inhibit enzyme activity, but in this case, it only reduces it to 70%. Surfactants like Triton X-100 and Tween-80; they reduce the activity to 46% and 71% respectively but not as significantly as the inhibitors above. Some enzymes require metal ions as cofactors for catalytic activity. Copper (Cu2+) serve as a cofactor for this enzyme so these ions enhance enzyme activity upto 96%. Calcium (Ca2+) can stabilize enzyme structure or participate directly in the enzyme's catalytic activity. Calcium ions significantly enhance enzyme activity upto 125%. Cobalt and iron and zinc ions have a moderate enhancing effect. Co2+ 85%, Fe2+ 80%, Zn2+ 94%. Magnesium ions significantly enhance enzyme activity to 114%. Mercury and aluminum ions have a moderate effect on enzyme activity having 90% Activity and Al3+ has 92% Activity. Manganese (Mn2+) ions significantly reduce enzyme activity to 50% as shown in Fig. 11. This suggests that manganese interfere with the enzyme's catalytic mechanism or disrupt its structure.

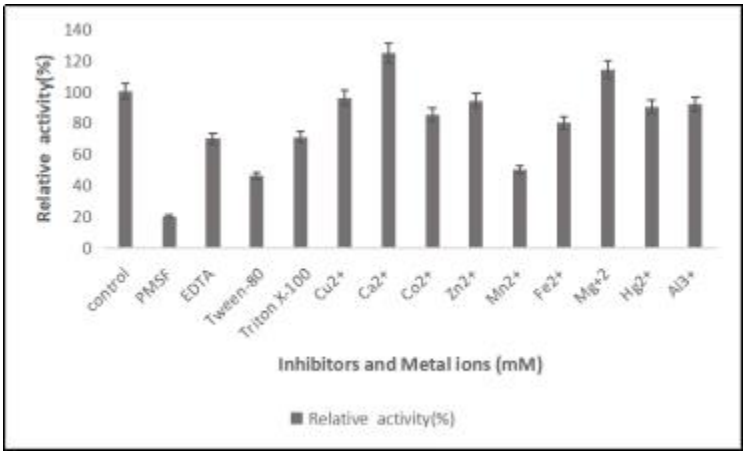

Fig.11. Effect of inhibitors and metal ions on alkaline protease activity, Bars represents SD

3.7. Dehairing of Animal Skin

The purified enzyme from *Bacillus subtilis* BS.HK demonstrated its efficacy in completely dehairing cow skin without compromising the structural integrity of the skin, in contrast to chemically dehaired skin as shown in Fig. 12.

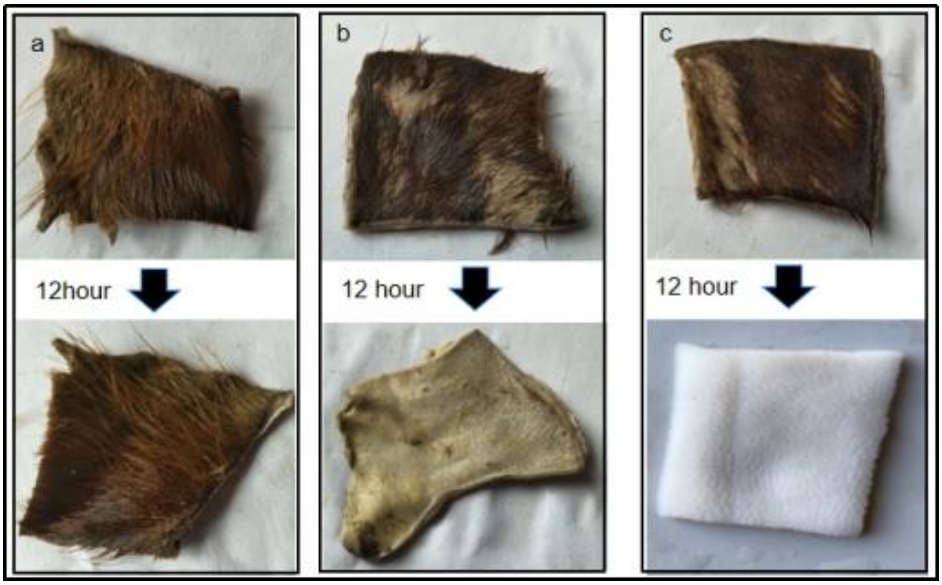

Fig.12. (a) Control skin treated with distilled water, **(b)** Chemically, (N_2S) Sodium sulphide treated skin,**(c)** Enzyme treated skin,all the samples were incubated for 12 hours

4. DISCUSSION

In this study we successfully isolated a novel bacterium from tannery effluent soil with the remarkable ability to produce a thermostable protease enzyme, the stability of which was accessed. It's noteworthy that among all the screened organisms, this particular strain displayed the widest zone diameter of 18mm, indicative of its superior protease-producing capacity, yielding an impressive protease activity level of 16.3 U/ml. Further identification of bacterium by phenotypic assessment by gram staining confirms that the isolated bacteria belong to gram positive bacillus group. The Gram stain procedure plays a pivotal role in the phenotype assessment of bacteria, serving as a crucial tool for distinguishing microbial organisms within the Bacteria domain based on their cell wall composition. Specifically, it discriminates between two major categories: Gram-positive cells, characterized by a robust peptidoglycan layer that imparts a blue to purple stain, and Gram-negative cells, featuring a comparatively thinner peptidoglycan layer resulting in a red to pink stain[15]. The amplification of 16S rRNA by PCR and sequencing was performed for the molecular identification of isolated *Bacillus sp.* BS.HK. BLAST similarity search with NCBI database shows that the isolated bacterial strain was similar to *Bacillus subtilis.* The phylogenetic analysis of *Bacillus sp. BS.HK*, along with several other *Bacillus strains*, was conducted by using neighbour joining method, reveals that *Bacillus subtilis strain BS.HK* appears to be closely related within the *Bacillus* genus. After the successful isolation and identification of *Bacillus subtilis* BS.HK as a protease-producing strain from soil, the effect several fermentation parameters were optimized for maximum growth and production of an alkaline protease from this strain through submerged fermentation. Among the optimized parameters incubation period significantly influences the production of extracellular protease [16-17]. Incubation time study revealed that maximum production of enzyme was achieved after 24 hours of incubation, after 24 hours a gradual decline in protease levels, likely attributed to factors such as denaturation, degradation, and autolysis of the protease enzyme, [18].This suggests that the microbial population is declining or not growing as rapidly and decreases, the enzyme production also decreases. In a study reported that the protease production by *B. subtilis* PE-11 started after 16h and gradually increases and reaches maximum after 28 hours [19]. Previous studies reported the incubation period may vary for the maximum production of protease from *Bacillus* species, such as *Bacillus subtilis KO* shows maximum production after the incubation of 48 hours [20]. Incubation temperature significantly affects the cell growth and production of metabolites by bacteria[21-22].The optimum temperature for growth and production of protease enzyme by *Bacillus subtilis BS.HK* was 37°C. Optimal temperatures for crude protease production vary among different bacterial strains. For instance, *B. subtilis* strain 38 displayed peak protease activity at a temperature of 47°C [23], whereas *Bacillus sp.* MIG reached its optimum temperature for protease production at a cooler 30°C [59]. On the other hand, Bacillus sp.

SIMA-2 demonstrated maximal activity of protease at a considerably higher temperature of 60°C [24], while *Bacillus licheniformis* produced its protease most efficiently at 50°C [19]. A study reported a fascinating connection between energy metabolism and production of enzymes, and this intricate relationship is intricately regulated by oxygen uptake and temperature [25]. In the case of extracellular enzymes, temperature seemed to play a significant role in their secretion, by altering the physical characteristics of cell membrane [17]. Among the other optimized parameters the pH level in the culture media significantly impacts numerous enzymatic processes and transportation of of different components through cell membranes [26]. The best pH for protease production by *B. subtilis* strain BS.HK was 9.5. The optimal pH conditions for protease activity have been documented in multiple research studies. For instance, the crude protease enzyme derived from *B. subtilis* 38 exhibits its highest activity at pH 6.5 [23]. An extracellular protease isolated from novel bacterial isolate displayed highest activity at pH 7.5[27]. Additionally, a pH study on a protease enzyme produced from thermophilic *Bacillus sp.* SIMA2 has optimum pH of 8.0 [24]. Previous study demonstrated that the pH level within a culture plays a significant role in influencing numerous biological processes involving enzymes and the transfer of diverse elements through the cellular membrane [28]. Various studies reported that the alkaline protease produced from *B.licheniformis* MP1 attain maximum production at an optimal pH of 8.0 [29] *B.circulans* and *B.infantis SKS1* was 10.0 [30, 31, 32]. The alkaline protease enzyme production is profoundly affected by the choice of Carbon source and nitrogen source employed in the culture medium [33-34]**.** Among the carbon sources evaluated, glucose emerged as the most favorable, yielding the highest cell biomass production at 4.12 g/L and the highest enzyme activity at 677.64 U/ml. In terms of nitrogen sources, casein, yeast extract, and tryptone were found as the best, with casein leading to the highest cell biomass production at 3.78 g/L and yeast extract yielding the maximum activity of enzyme about 551.1 U/ml. Overall, glucose and starch were the optimal carbon sources, while casein, yeast extract, and tryptone were the preferred nitrogen source. Consistent findings have been previously documented in studies involving glucoseas the best carbon source for the production of protease in different bacterial strains, as noted in the different works [26, 35]. Furthermore, it has been documented that the inclusion of peptone and yeast extract in the culture media significantly bolstered the production of protease by *Bacillus sp.*MA6,[36], as well as by *B. subtilis*[37,38]**.** Agitation holds a pivotal role in governing the efficient transfer of nutrients, the solubility of oxygen, the dispersion of cells, and consequently, the augmentation of aerobic metabolism in microorganisms, [39].In the context of shake flask fermentation, our investigation revealed that an agitation rate of 140 rpm emerged as the ideal condition for achieving the maximum protease production by *B.subtilis* BS.HK. After the production of alkaline protease enzyme under optimized fermentation condition, the succeeding measure is to make pure the crude enzyme. The enzyme was subsequently purified to a significant degree, with a fold increase of 7.40 through ammonium sulfate precipitation and an additional 40.62-fold purification achieved via gel filtration chromatography. The evidenced by SDS-PAGE analysis, which display the molecular weight of alkaline protease is approximately 35 KDa also shown confirmed by zymogram assay on casein agar plate. These findings align with previous research, which has indicated that serine proteases typically exhibit molecular masses ranging from 18 to 35 kDa[4]. Furthermore,[2] based on these by noting that cysteine proteases tend to have molecular masses falling within the 32 to 50 kDa range. For instance, alkaline proteases derived from Bacillus species typically possess molecular masses less than 50 kDa[40]. In another study, it was reported that the *B.subtilis* have mol. wt of 27KDa [41].The alkaline protease derived from *Bacillus* species has consistently been observed ashaving sinle band along wiyh mol. Wt falling within the range of 16 to 32 kilodaltons (kDa), [42, 43]. The partially purified enzyme was subjected to a comprehensive characterization study, which encompassed the evaluation of several crucial parameters. These included assessing the impact of temperature on enzyme activity and its stability under varying temperature conditions, as well as examining how pH levels affected enzyme activity and its stability. Kinetic study of purified alkaline protease revealed that reduced Km value signifies a potent binding affinity between the proteases and its substrate. The alkaline protease purified from BS .HK exhibited a broad substrate specificity, with Km 0.992 µM and Vmax 19.72U/ml against various concentrations of casein (0.5%) as a substrate. Previous studies reported that highest activity of alkaline protease was shown when casein was used as substrate as compared to other substrates. In a study [44], kinetic parameters from *Bacillus halodurans JB 99* conducted at an elevated temperature of 70°C and a pH level of 11.0, encompassing substrate concentrations ranging from 0.5 to 7.5 mg/ml of casein revealed that the purified protease exhibited a Km value of 3.3 mg/ml and a Vmax of 15 U/mg proteins. Similar results were reported for

alkaline protease derived from *Bacillus licheniformis* Bl8 [45]. Their investigations confirmed that the enzyme adhered to the classical Michaelis-Menten kinetics, with an apparent KM value of 3.2 mg/ml for casein as the substrate. In a study, reported that the kinetic parameters revealed a notably low Km value of 0.03064 mM and a substantial Vmax value of 69.76 U/ml when casein was employed as the substrate. These results collectively emphasize the significance of substrate choice and the Km value in elucidating the enzymatic characteristics of alkaline proteases [46]. The pH plays an important role on the activity of an enzyme it affects the catalytic site of enzyme. We studied the effect of pH on enzyme activity and stability. The enzyme displayed maximum activity of 16.3(U/ml) at pH 9.5 , and it exhibited remarkable stability from pH 8-12 and shows 100% activity at pH 10.5. The enzyme from *B.subtilis* BS.HK exhibits its maximum activity at 50°C, where the enzyme's activity is 16.3 U/ml. The stability temperature range for purified enzyme from *B.subtilis* BS.HK is around 50-60°C, at 70°C it retains 40% of its residual activity beyond which it rapidly loses its stability and activity. Proteases known for their exceptional thermostability, sourced from different Bacillus species, have been documented to exhibit peak protease activity at varying temperature thresholds, including 45°C, 50°C, 60°C, and 65°C, as reported in multiple studies [47, 48, 49, 50]. Notably, the protease sourced from *Bacillus sp.* GUS1 has been documented to maintain its enzymatic activity at an impressive 100% level for duration of one hour at a notably high temperature of 70°C [42]. In a separate investigation, the protease derived from *Bacillus subtilis* EPE showcases remarkable stability, with its enzymatic activity remaining at 100% up to 60°C; only a modest decline of 20% is observed at 70°C [51]. Similarly, the alkaline protease originating from *B. cohnii APT5* demonstrates exceptional resilience, as it retains its full enzymatic activity at 50°C for a substantial four-hour period. Impressively, even after an extended 72-hour exposure to a milder temperature of 30°C, its enzymatic function remains unscathed [50]. It is noteworthy that the protease from *B. subtilis*, renowned for its deproteinization capabilities on crustacean wastes, exhibits an optimal temperature of 50°C. This particular protease maintains its enzymatic activity within a range from 25 to 50°C but experiences complete inactivation beyond the threshold of 60°C [52]. Furthermore, the protease known as Rand from *B. subtilis* is distinguished by its stability within the temperature spectrum of 37 to 55°C for duration of 30 minutes. Even at the challenging temperature of 60°C, this crude enzyme extract still manages to preserve a substantial 80% of its protease activity over a half-hour period [19].Lastly, another alkaline protease sourced from *B. licheniformis* is optimally active at 60°C and exhibits stability for duration of 10 minutes within temperature ranges from 30 -60°C [53].

An effect of various inhibitors and metal ions on purified alkaline from *B. subtilis* BS.HK protease revealed that PMSF and EDTA exerted significant reductions in enzyme activity through distinct mechanisms, while surfactants like Tween-80 and Triton X-100 had milder inhibitory effects. Metal ions displayed varying effects, Copper ions (Cu2+), Calcium ions (Ca2+) and Magnesium ions (Mg2+) significantly boost enzyme activity , whereas Cobalt (Co2+), iron (Fe2+), and zinc (Zn2+) ions have a moderate enhancing effect on enzyme activity. Conversely, mercury (Hg2+) and aluminum (Al3+) ions exert a moderate inhibitory effect on enzyme activity. The presence of manganese ions (Mn2+), on the other hand, significantly decreases enzyme activity, reducing it to 50%. Previous studies have shown that alkaline protease activity in *B.Subtilis* and *Bacillus megaterium RRM2* is positively influenced by the presence of Ca2+ [54, 55] but negatively affected by the presence of Hg2+, Zn2+, and Co2+[56, 57].In another study it was reported that inclusion ofMg2+, Ca2+, and Mn2+ resulted in a notable enhancement of enzymatic activity in *Bacillus sp.* MZK03. Conversely, the presence of Hg2+ exhibited an inhibitory effect on the enzyme's performance [58]. The purified enzyme from *Bacillus subtilis* BS.HK demonstrated its efficacy in completely dehairing cow skin without compromising the structural integrity of the skin, in contrast to chemically dehaired skin.This suggests that the alkaline serine protease holds great potential for the leather processing industry.

5. CONCLUSION

In conclusion, the isolation purification and characterization of protease producing *Bacillus sp.*bacteria from tannery soil revealed an excellent prospect for leather processing industry. Among the identified strains, *Bacillus subtilis BS.HK*, confirmed through 16S rRNA analysis (accession #OP604265.1), exhibited the maximum protease activity and was selected for further analysis. The production of alkaline proteae from the isolated strain was achieved through liquid state fermentation, with the highest yield observed after a 24-hour fermentation period in a basic medium. Optimal condition for production of alkaline protease enzyme were determined to be a temperature of 37°C, pH 9.5, and an agitation speed of 140 rpm. The alkaline protease production from bacteria is significantly impacted by the choice of

carbon and nitrogen sources used in the culture media. In this study, various carbon and nitrogen sources were assessed for their impact on cell biomass production and enzyme activity. Among the carbon sources evaluated, glucose emerged as the most favorable, yielding the highest cell biomass production at 4.12 g/L and the highest enzyme activity at 677.64 U/ml. In terms of nitrogen sources, casein, yeast extract, and tryptone were identified as the most favorable, with casein leading to the highest cell biomass production at 3.78 g/L and yeast extract yielding the highest enzyme activity at 551.1 U/ml. Overall, glucose and starch were the optimal carbon sources, while casein, yeast extract, and tryptone were the preferred nitrogen source. The enzyme was subsequently purified to a significant degree, with a fold increase of 7.40 through ammonium sulfate precipitation and an additional 40.62-fold purification achieved via gel filtration chromatography. The purified enzyme exhibited a mol. Wt of 35 kDa and demonstrated maximum activity at pH 9.5 and temperature 50°C, displaying remarkable stability at 60°C and within a broad pH range of 8-12 for up to 1 hour. Inhibitory studies revealed that PMSF and EDTA exerted significant reductions in enzyme activity through distinct mechanisms, while surfactants like Triton X-100 and Tween-80 had milder inhibitory effects. Metal ions displayed varying effects, either enhancing or inhibiting enzyme activity. Notably, the purified enzyme from *Bacillus subtilis* BS.HK demonstrated its efficacy in completely dehairing cow skin without compromising the structural integrity of the skin, in contrast to chemically dehaired skin. This suggests that the alkaline serine protease holds great potential for the leather processing industry due to its stability, hydrophilicity, enhanced thermostability, and resistance to alkaline environments.

ACKNOWLEDGMENTS

We would like to express our gratitude to Higher education commission (HEC) for funding the research project NRPU # 20-4571 for this research.

REFERENCES

[1]. Satyanarayana, T. (2005). Microbial diversity: current perspectives and potential applications.

[2]. Rao, M. B., Tanksale, A. M., Ghatge, M. S., & Deshpande, V. V. (1998). Molecular and biotechnological aspects of microbial proteases. *Microbiology and molecular biology reviews*, *62*(3), 597-635.

[3]. Arulmani, M., Aparanjini, K., Vasanthi, K., Arumugam, P., Arivuchelvi, M., &Kalaichelvan, P. T. (2007). Purification and partial characterization of serine protease from thermostablealkalophilic Bacillus laterosporus-AK1. *World Journal of Microbiology and biotechnology*, *23*, 475-481.

[4]. Gupta, R., Beg, Q., & Lorenz, P. (2002). Bacterial alkaline proteases: molecular approaches and industrial applications. *Applied microbiology and biotechnology*, *59*, 15-32.

[5]. Butt, M. Q., Zeeshan, N., Ashraf, N. M., Akhtar, M. A., Ashraf, H., Afroz, A., . . .Naz, S. (2021). Environmental impact and diversity of protease-producing bacteria in areas of leather tannery effluents of Sialkot, Pakistan. *Environmental Science and Pollution Research*, *28*(39), 54842-54851.

[6]. Smith, A. C., & Hussey, M. A. (2005). Gram stain protocols. *American Society for Microbiology*, *1*, 14.

[7]. Neumann, B. r., Pospiech, A., &Schairer, H. U. (1992). Rapid isolation of genomic DNA from gram-negative bacteria. *Trends in genetics: TIG*, *8*(10), 332-333.

[8]. Weisburg, W. G., Barns, S. M., Pelletier, D. A., & Lane, D. J. (1991). 16S ribosomal DNA amplification for phylogenetic study. *Journal of bacteriology*, *173*(2), 697-703.

[9]. Manavalan, T., Manavalan, A., Ramachandran, S., &Heese, K. (2020). Identification of a novel thermostable alkaline protease from Bacillus megaterium-TK1 for the detergent and leather industry. Biology, 9(12), 472.

[10]. Bouacem, K., Bouanane-Darenfed, A., Laribi-Habchi, H., Elhoul, M. B., Hmida-Sayari, A., Hacene, H., ...&Bejar, S. (2015). Biochemical characterization of a detergent-stable serine alkaline protease from Caldicoprobacterguelmensis. International journal of biological macromolecules, 81, 299-307.

[11]. Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical biochemistry*, *72*(1-2), 248-254.

[12].Hakim, A., Bhuiyan, F. R., Iqbal, A., Emon, T. H., Ahmed, J., & Azad, A. K. (2018). Production and partial characterization of dehairing alkaline protease from Bacillus subtilis AKAL7 and Exiguobacteriumindicum AKAL11 by using organic municipal solid wastes. *Heliyon*, *4*(6).

[13].Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, *227*(5259), 680-685.

[14].Phadatare, S. U., Deshpande, V. V., & Srinivasan, M. C. (1993). High activity alkaline protease from Conidioboluscoronatus (NCL 86.8. 20): enzyme production and compatibility with commercial detergents. *Enzyme and Microbial Technology*, *15*(1), 72-76.

[15].Gram, C. (1884). The differential staining of Schizomycetes in tissue sections and in dried preparations. Fortschitte der Medicin, 2(6), 185-189.

[16].Olajuyigbe, F. M., &Ajele, J. O. (2005). Production dynamics of extracellular protease from Bacillus species. African Journal of Biotechnology, 4(8), 776-779.

[17].Abd Rahman, R. N. Z., Geok, L. P., Basri, M., &Salleh, A. B. (2005). Physical factors affecting the production of organic solvent-tolerant protease by Pseudomonas aeruginosa strain K. *Bioresource technology*, *96*(4), 429-436.

[18].Chu, I. M., Lee, C., & Li, T. S. (1992). Production and degradation of alkaline protease in batch cultures of Bacillus subtilis ATCC 14416. Enzyme and Microbial Technology, 14(9), 755-761.

[19].Abusham, R. A., Rahman, R. N. Z. R., Salleh, A. B., &Basri, M. (2009). Optimization of physical factors affecting the production of thermo-stable organic solvent-tolerant protease from a newly isolated halo tolerant Bacillus subtilis strain Rand. *Microbial Cell Factories*, *8*(1), 1-9.

[20].Younis, M. A., Hezayen, F. F., Nour-Eldein, M. A., &Shabeb, M. S. (2009). Production of protease in low-cost medium by Bacillus subtilis KO strain. *Global J BiotechnolBiochem*, *4*(2), 132-137.

[21].Banerjee, R., & Bhattacharyya, B. C. (1992). Extracellular alkaline protease of newly isolated Rhizopusoryzae. Biotechnology letters, 14, 301-304.

[22].Singh, S. K., Singh, S. K., Tripathi, V. R., Khare, S. K., &Garg, S. K. (2011). Comparative one-factor-at-a-time, response surface (statistical) and bench-scale bioreactor level optimization of thermoalkaline protease production from a psychrotrophic Pseudomonas putida SKG-1 isolate. Microbial cell factories, 10(1), 1-13.

[23].Chantawannakul, P., Oncharoen, A., Klanbut, K., Chukeatirote, E., &Lumyong, S. (2002). Characterization of proteases of Bacillus subtilis strain 38 isolated from traditionally fermented soybean in Northern Thailand. *Science Asia*, *28*(4), 241-245.

[24].Nascimento, W. C. A. d., & Martins, M. L. L. (2004). Production and properties of an extracellular protease from thermophilic Bacillus sp. *Brazilian journal of microbiology*, *35*, 91-96.

[25].Frankena, J., Koningstein, G. M., van Verseveld, H. W., &Stouthamer, A. H. (1986). Effect of different limitations in chemostat cultures on growth and production of exocellular protease by Bacillus licheniformis. *Applied microbiology and biotechnology*, *24*, 106-112.

[26].Ellaiah, P., Srinivasulu, B., &Adinarayana, K. (2002). A review on microbial alkaline proteases.

[27].Ramesh, M., Sanhita, C., Madhusmita, M., Sumana, D., Prabhat, M., Joydeep, M., . . .Chaudhuri, S. (2008). Characterization and wash performance analysis of microbial extracellular enzymes from East Calcutta Wetland in India. *American Journal of Applied Sciences*, *5*, 1650-1661.

[28].Moon, S. H., &Parulekar, S. J. (1991). A parametric study ot protease production in batch and fed-batch cultures of Bacillus firmus. *Biotechnology and bioengineering*, *37*(5), 467-483.

[29].Jellouli, K., Ghorbel-Bellaaj, O., Ayed, H. B., Manni, L., Agrebi, R., &Nasri, M. (2011). Alkaline-protease from Bacillus licheniformis MP1: purification, characterization and potential application as a detergent additive and for shrimp waste deproteinization. Process Biochemistry, 46(6), 1248-1256.

[30].Prakasham, R. S., Rao, C. S., Rao, R. S., &Sarma, P. N. (2005). Alkaline Protease Production by an Isolated Bacilluscirculans under Solid-State Fermentation Using Agroindustrial Waste: Process Parameters Optimization. Biotechnology progress, 21(5), 1380-1388.

[31].Rao, C. S., Sathish, T., Ravichandra, P., &Prakasham, R. S. (2009). Characterization of thermo-and detergent stable serine protease from isolated Bacillus circulans and evaluation of eco-friendly applications. Process Biochemistry, 44(3), 262-268.

[32].Saggu, S. K., & Mishra, P. C. (2017). Characterization of thermostable alkaline proteases from Bacillus infantis SKS1 isolated from garden soil. PloS one, 12(11), e0188724.

[33].Sen, S., Veeranki, V. D., &Mandal, B. (2009). Effect of physical parameters, carbon and nitrogen sources on the production of alkaline protease from a newly isolated Bacillus pseudofirmus SVB1. Annals of microbiology, 59, 531-538.

[34].Sharma, K. M., Kumar, R., Panwar, S., & Kumar, A. (2017). Microbial alkaline proteases: Optimization of production parameters and their properties. Journal of Genetic Engineering and Biotechnology, 15(1), 115-126.

[35].Vijayaraghavan, P., & Vincent, S. G. P. (2012). Cow dung as a novel, inexpensive substrate for the production of a halo-tolerant alkaline protease by Halomonas sp. PV1 for eco-friendly applications. Biochemical engineering journal, 69, 57-60.

[36].Azad, A. K., Shibata, H., &Hoq, M. M. (2002). Hide processing with alkaline protease from Bacillus sp. MA6. Bangladesh J. Microbiol, 19(1), 67-71.

[37].Vanitha, N., Rajan, S., &Murugesan, A. G. (2014). Optimization and production of alkaline protease enzyme from Bacillus subtilis 168 isolated from food industry waste. International journal of current microbiology and applied sciences, 3(6), 36-44.

[38].Badhe, P., Joshi, M., &Adivarekar, R. (2016). Optimized production of extracellular proteases by Bacillus subtilis from degraded abattoir waste. Journal of BioScience& Biotechnology, 5(1).

[39].Patil, U., &Chaudhari, A. (2013). Production of alkaline protease by solvent-tolerant alkaliphilic Bacillus circulans MTCC 7942 isolated from hydrocarbon contaminated habitat: process parameters optimization. International Scholarly Research Notices, 2013.

[40].Sousa, F., Jus, S., Erbel, A., Kokol, V., Cavaco-Paulo, A., &Gubitz, G. (2007). A novel metalloprotease from Bacillus cereus for protein fibre processing. *Enzyme and Microbial Technology*, *40*(7), 1772-1781.

[41].Ahmed, I., Zia, M. A., & Iqbal, H. N. (2011). Purification and kinetic parameters characterization of an alkaline protease produced from Bacillus subtilis through submerged fermentation technique. *World Applied Sciences Journal*, *12*(6), 751-757.

[42].Adinarayana, K., Raju, K. B., &Ellaiah, P. (2004). Investigations on alkaline protease production with B. subtilis PE-11 immobilized in calcium alginate gel beads. *Process Biochemistry*, *39*(11), 1331-1339.

[43].Boominadhan, U., Rajakumar, R., Sivakumaar, P. K. V., & Joe, M. M. (2009). Optimization of protease enzyme production using Bacillus sp. isolated from different wastes. *Bot. Res. Int*, *2*(2), 83-87.

[44].Shrinivas, D., &Naik, G. R. (2011). Characterization of alkaline thermostablekeratinolytic protease from thermoalkalophilic Bacillus halodurans JB 99 exhibiting dehairing activity. International Biodeterioration& Biodegradation, 65(1), 29-35.

[45].Lakshmi, G., & Prasad, N. N. (2015). Purification and characterization of alkaline protease from a mutant Bacillus licheniformis Bl8. Advances in Biological Research, 9(1), 15-23.

[46].Iqbal, M., Asgher, M., & Bashir, F. (2018). Purification and kinetic characterization of alkaline protease for UV-90 mutant of bacillus subtilis. J. Biochem. Anal. Stud, 3(1).

[47].Krishnaveni, K., Kumar, D. M., Balakumaran, M., Ramesh, S., &Kalaichelvan, P. (2012). Production and optimization of extracellular Alkaline Protease from Bacillus subtilis isolated from dairy effluent. *Der Pharmacia Lettre*, *4*(1), 98-109.

[48].Prabhavathy, G., RajasekaraPandian, M., &Senthilkumar, B. (2012). Optimization and production of extracellular alkaline protease by solid state fermentation using Bacillus subtilis. *J. Acad. Ind. Res*, *17*, 427-430.

[49].Shaheen, M., Shah, A. A., Hameed, A., &Hasan, F. (2008). Influence of culture conditions on production and activity of protease from Bacillus subtilis BS1. *Pak. J. Bot*, *40*(5), 2161-2169.

[50].Tekin, N., Cihan, A. Ç., Takac, Z. S., Tüzün, C. Y., Tunc, K., &Çökmüş, C. (2012). Alkaline protease production of Bacillus cohnii APT5. *Turkish Journal of Biology*, *36*(4), 430-440.

[51].BichThuy, D. T., & Bose, S. K. (2010). Characterization of multiple extracellular proteases produced by a Bacillus subtilis strain and identification of the strain. *International Journal of Biology*, *3*(1), 101-110.

[52].Yang, J.-K., Shih, L., Tzeng, Y.-M., & Wang, S.-L. (2000). Production and purification of protease from a Bacillus subtilis that can deproteinize crustacean wastes☆. *Enzyme and Microbial Technology*, *26*(5-6), 406-413.

[53].SEYFZADEH, S., Sajedi, R. H., &Sariri, R. (2008). Isolation and characterization of thermophilic alkaline proteases resistant to sodium dodecyl sulfate and ethylene diaminetetraacetic acid from Bacillus sp. GUS1.

[54].Gupta, A., Roy, I., Patel, R., Singh, S., Khare, S., & Gupta, M. (2005). One-step purification and characterization of an alkaline protease from haloalkaliphilic Bacillus sp. *Journal of chromatography A*, *1075*(1-2), 103-108.

[55].Rajkumar, R., Ranishree, J. K., &Ramasamy, R. (2011). Production and characterization of a novel protease from Bacillus sp. RRM1 under solid state fermentation. *Journal of microbiology and biotechnology*, *21*(6), 627-636.

[56].Olajuyigbe, F. M., &Falade, A. M. (2014). Purification and partial characterization of serine alkaline metalloprotease from Bacillus brevis MWB-01. *Bioresources and Bioprocessing*, *1*, 1-10.

[57].Venugopal, M., &Saramma, A. (2007). An alkaline protease from Bacillus circulans BM15, newly isolated from a mangrove station: characterization and application in laundry detergent formulations. *Indian journal of microbiology*, *47*, 298-303.

[58].Sayem, S. A., Alam, M., &Hoq, M. M. (2006). Effect of temperature, pH and metal ions on the activity and stability of alkaline protease from novel Bacillus licheniformis MZK03. *Proceedings-Pakistan academy of sciences*, *43*(4), 257.

[59].Gouda, M. K. (2006). Optimization and purification of alkaline proteases produced by marine Bacillus sp. MIG newly isolated from Eastern Harbour of Alexandria. *Polish Journal of Microbiology*, *55*(2), 119.