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Production and characterization of partially purified thermostable alkaline protease by *Bacillus subtilis* SFL for blood destaining and dehairing applications

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ABSTRACT

Microbial proteases have been preferred over animal and plant proteases because of their basic properties and ease of production. *Bacillus subtilis* SFL, an alkaline-thermal protease-producing bacterium was isolated from different sources of wastewater and identified using morphological, biochemical, and molecular methods. The 16S rDNA sequence has been deposited in GenBank with accession number OP714187. Partial purification of alkaline protease was performed by two method (ammonium sulfate preciptation and organic solvent preceptation) precipitation by 60% ammonium sulfate and ethyl acetate by the ratio (1:1) and column chromatography (gel filtration) by using a sephadex G-100. The partially purified alkaline-thermal protease of *Bacillus subtilis* SFL was at stable 40°C and pH 8.0. Our results show that 40 g/l of meat extract and 12 g/l of xylose serve as the best nitrogen and carbon sources respectively for the production of this enzyme. The effect of tested metal ions indicated that $Mg^{+2,}$ $Ca^{+}2$, Na^{+}, Fe^{+2}, Cu^{+2}, Co^{+2} and Cd^{+2} inhibited the activity of the protease from *Bacillus subtilis* SFL. The crude and partially purified protease from *B. subtilis* SFL substantially degraded red blood cells, distained blood color, dehaired cow skin and decomposed cow hair.

Introduction

Enzymes are biocatalysts proteins that are produced by all living cells (Namasivayam *et al*., 2011). They are necessary for all kinds of life and perform critical functions in daily living (Aaisha and Barate, 2016). Proteases are biological macromolecules that are simple destructive enzymes with numerous scientific, catalytic, analytical, and commercial applications (Neurath and Walsh, 2011). Proteases are a type of industrially important enzyme. Proteases catalyse the hydrolytic breakdown of big protein molecules' peptide bonds, converting them to amino acids or tiny peptides. Proteases are the most prominent enzyme class, accounting for 60% of the overall enzyme industry (Sharma *et al*., 2017). Based on pH differences, proteases are generically classed as acidic protease, neutral protease, and alkaline protease (Flores-Gallegos *et al*., 2019).

Proteases are also categorized according to their source, which can be plants, animals, bacteria, or fungi (Jisha *et al*., 2013). *Bacillus*, *Micrococcus*, *Pseudomonas*, and *Streptomyces*, among others, have been reported to express alkaline proteases under a variety of physiochemical and nutritional settings throughout the last two decades (Hashem *et al*., 2015; Khajuria *et al*., 2015). Because of their durability at high temperatures and pH levels, commercial proteases are typically isolated from *Bacillus* strains (Ibrahim *et al*., 2015; Al-Hakim *et al*., 2018). In comparison to plant, animal, and fungal proteases, bacterial proteases are the most important. This is due to their extracellular nature, high production yield, restricted space and short culture period, and genetic manipulation potential (Breithaupt 2001; Selvamohan and Sherin, 2010).

Due to distinctive qualities such as high stability and relatively low substrate specificity, alkaline protease accounts for two-thirds of the protease market (Matkawala *et al*., 2021). Proteases play a significant role in biopharmaceuticals like contact lens cleansers and enzymatic debriders (Anwar and Saleemuddin, 2000). Proteolytic enzymes promote spontaneous healing by rapidly eliminating necrotic material in the topical treatment of skin ulcers (Sjodahl *et al*., 2002). We isolated, tested, and discovered thermo-tolerant alkaline bacteria capable of generating alkaline protease in this study. Several physicochemical parameters were optimized to maximize alkaline protease production. The crude and partially purified enzymes were used to degrade red blood cells, dehair cow skin, and decompose cow hair.

Materials and methods

Isolation of thermal alkaliphiles bacteria from different samples

This study was carried out on different samples collected from different sources including bloody soil (soil saturated with blood from abattoirs), vegetable wastewater, poultry waste, and animal waste from Sharkia and Ismailia governorate. Serial dilutions were performed as described by Aftab *et al*. (2006). Plates were incubated at 40°C for 24–48 h. Plates giving single colonies with clear inhibition zone were selected for the next step. The isolation media used from Microgen company, composed of the following ingredients (g/l): 15.0 peptic digest of animal tissue, 5.0 sodium chloride, 1.5 beef extract, 1.5 yeast extract, and 15.0 agar. The medium was supplemented with skimmed milk (zero fats). NaOH (0.1N) was added to the medium prior to sterilization to adjust the final pH to approximately 8.0.

Qualitative screening of alkaliphilic isolates for the production of alkaline protease

Qualitative screening was performed by detecting the diameter of

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the formed clear inhibition zone (mm). Single colony isolates were cultured on alkaline skimmed milk nutrient agar media at 40°C (Ibrahim *et al*., 2007). Clear zones diameters were measured with a ruler after 24 and 48 h incubation. Pure cultures were then obtained on alkaline skimmed milk nutrient agar slants.

Quantitative screening of thermo-alkaline protease positive isolates

The pure isolates colonies that gave a positive result on a qualitative screening medium through giving larger clear inhibition zone were selected and tested quantitatively by culturing them in alkaline skimmed milk broth medium (Ibrahim *et al*., 2007). The pH of the medium was adjusted to 8.0 after that autoclaved at 121°C for 30 min. A 25 ml volume of alkaline protease screening medium in 100 ml flask was inoculated by 1 ml 10^6 CFU/ml selected bacterial isolate, then the cultures were maintained in a static incubator at 40°C for 72h at aseptic conditions. At the end of each fermentation period, the broth was filtrated and centrifuged at 5,000 rpm for 20 min at 4°C. The clear supernatant was used as a crude enzyme.

Alkaline protease assay

Takami *et al.* (1989) technique was used to assess alkaline protease activity in cell-free supernatant. As a substrate, 1% casein was dissolved in a 50 mM glycine-NaOH buffer (pH 9). The assay was usually carried out in 0.5 mL of properly diluted enzyme and 2.5 mL of casein solution. At 660 nm, the optical density of the solutions was evaluated in comparison to a sample blank.

Determination of total soluble protein (Lowry et al., 1951)

This protein determination method, commonly known as the Lowry method, employs this concept to measure proteins in biological materials. Protein reacts with folin phenol reagent to generate a blue colour in this method. A UV spectrophotometer (Shimazu model 1024UV) was used to test the absorbance of this blue colour solution at 750 nm. A standard curve with a concentration range of 0.1-0.5 mg/ml bovine serum albumin (crystalline BSA) was created using bovine serum albumin.

Identification of most potent isolated bacteria

Bacterial isolates from vegetable wastewater were prepared fresh. The separated colonies' total DNA was extracted. Sambrook and Russell (2001) described the process for extracting DNA from a new colony. PCR was used to amplify the gene encoding 16S rRNA using universal primers (front primer [F27] 5'-AGAGTTTGATCCTGGCTCAG-3' (Chénbey *et al*., 2000) and reverse primer [R1492] 5'-GGTTACCTTGTTACGACTT-3' (Turner *et al*., 1999). These primers bind to a generally conserved area and allow for the amplification of a 1500-bp fragment. A Gene-Amp PCR system 9600 thermal cycler (Perkin Elmer) was used for PCR amplification. The following were the amplification conditions: 94°C for 10 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing-extension at 56°C for 1 minute, 72°C for 1 min and an extension at 72°C for 10 min. The PCR product was purified using the Fermentas GeneJETTM PCR Purification Kit. GATC Biotech AG (Konstanz, Germany) used an ABI 3730xl DNA sequencer to partially sequence the amplified DNA fragment using forward and reverse primers. The 16S rDNA sequences discovered in this investigation have been uploaded to the NCBI web server (www.ncbi.nlm.nih.gov). The Basic Local Alignment Search Tool (BLAST) programme (http://www.ncbi.nlm.nih.gov/blast) was used for sequence analysis and comparison to known sequences (Altschul *et al*., 1997). Using software (http://www.clustal.com), a phylogenetic tree of the isolated bacteria was generated based on 16S rDNA data.

Fermentation process for alkaline protease production

After the quantitative screening of protease producing bacteria, *Bacillus subtilis* SFL was selected among the bacterial isolates for studying the factors affecting alkaline protease production. A bacterial cell suspension was prepared by adding 5 ml sterile distilled water to 18-30 h old slant culture and emulsified with a sterile needle.

Nutritional factors

100 ml Erlenmeyer conical flasks containing 25 ml of prepared media (yeast extract casein production media) were used. Each flask was sealed with a cotton plug and inoculated with 0.5 ml inoculum 10^6 CFU/ml (0.5 McFarland) after cooling under sterile conditions. Flasks were then incubated at 40°C in a static incubator for 48 h. At the end of incubation period, the culture broth was filtrated and centrifuged at 5.000 rpm for 20 min using cooling centrifuge, and the filtrate was used directly for enzymatic activity, protein, specific activity, and final pH determinations. Duplicate flasks were used for each condition. The components of the yeast extract casein production medium described by Rao and Narasu, (2007) were as following (g/l): 10.0 glucose, 5.0 casein, 5.0 yeast extract, 1.0 KH_2PO4, 1.0 K_2HPO4 and 1000ml H_2O. The medium was supplemented with 46 ml/l of skimmed milk (zero fats). The media pH was adjusted to 8.0 using (0.1 N) NaOH before sterilization.

Effect of different carbon sources

Different carbon sources were tested to demonstrate the effect of carbon source on the production of alkaline protease. Seven carbon sources were added to the medium (yeast extract and casein medium) at a concentration of 1% instead of glucose (control). Carbon sources were glucose (control), cellulose, lactose, maltose, xylose, sucrose and fructose. This experiment was carried out on two media, a prepared medium (yeast extract casein production media) and skimmed milk prepared medium. The effect of different concentrations of optimal carbon source was determined.

Effect of different nitrogen sources

Different organic and inorganic nitrogen sources were tested. Nitrogen sources in the prepared medium (casein and yeast extract medium) were casein + yeast extract (control), casein, yeast extract, meat extract, beef extract, sodium nitrate, ammonium sulphate and ammonium phosphate that were added separately with equimolecular weight. The effect of these nitrogen sources on enzymatic activity was determined. The effect of different concentrations (10-60 g/l) of the meat extract as optimal nitrogen source has also been demonstrated.

Partial purification of alkaline protease from Bacillus subtilis SFL

Partial purification of alkaline protease had been carried out by two methods where:

Precipitation by ammonium sulphate: by adding different concentrations of ammonium sulfate (20, 40, 60, and 80%) to cell free culture medium after filtration and centrifugation at 5.000 rpm using cooling centrifuge through keeping in an ice bath and slowly add solid ammonium sulfate to the ice-cold enzyme solution with gentle stirring until ammonium sulfate is completely dissolved. The solution was then left in the refrigerator overnight and then centrifuged at 5000 rpm for 20 min in cooling centrifuge. The supernatant was separated from the precipitate to detect enzymatic activity.

Organic solvent precipitation: different organic solvents were used as acetone, methanol, ethanol and ethyl acetate for partial purification of

alkaline protease by ratio 1:1 (v/v).

Column chromatography (Gel filtration)

The ethyl acetate (organic layer) gave the highest enzymatic activity where it completely concentrated and dissolved in 750 µl buffer (0.05M glycine-NaOH buffer, pH 9.0) then subjected to a gel filtration column where elution was performed using a Sephadex G-100 column with 0.05M glycine-NaOH buffer (pH 9.0) at flow rate of 30 ml/h. Collected fractions were analyzed for protein and alkaline protease activity. The most active fractions were selected and pooled for subsequent analysis and characterization.

Characterization of the partially purified alkaline protease from Bacillus subtilis SFL

Effect of incubation period on partially purified enzyme activity

Same volumes of partial purified alkaline protease were incubated with buffered substrate (casein) for different incubation periods (20, 40, 60, 80 and 100 min) and the enzyme activity was determined.

Effect of incubation temperature on partially purified enzyme activity

This experiment was performed to find the optimal temperature for the activity of *Bacillus subtilis* SFL enzyme. The optimum incubation temperature was verified by incubating partially purified enzyme with the substrate (casein solution) at 20, 30, 40 and 50°C.

Effect of enzyme concentration on partially purified enzyme activity

In this experiment, different concentrations of partially purified enzyme were added to 2.5 ml of casein solution. Concentrations used to detect optimal enzyme concentration were 0.3, 0.4, 0.5, 0.6 and 0.7 ml to 2.5 ml of 1% reaction substrate mixture.

Effect of substrate concentration on partially purified enzyme activity

Partially purified enzyme activity was detected at different concentrations of casein solution. Concentrations used were 1.5, 2, 2.5, 3 and 3.5 ml and were added to 0.5 ml of filtrate (enzyme solution).

Effect of different pH values on the stability of partially purified enzyme

In this experiment, similar aliquots of the partially purified enzyme were adjusted separately to different pH values (6- 8 phosphate buffer and 9-11 glycine NaOH buffer) using different buffers. Then the activity of the enzyme was detected by incubating an aliquot of the enzyme with other components of the reaction mixture.

Thermal stability

In this experiment, the similar aliquots of the partially purified enzyme were firstly incubated separately at different temperatures (40, 50, 60, 70 and 80°C) for 20 min. Then the activity of the enzyme was detected after incubating prewarmed enzyme aliquots with the other components of the reaction mixture after cooling.

Effect of different metals ions on partially purified enzyme activity

The effect of different metal ions on the activity of partially purified enzyme was tested. Various inorganic salts with different cations Mg^{2+}, Ca^{2+}, Fe^{2+}, Cu^{2+}, Co^{2+}, Na^{+} and Cd^{2+} were separately added to the reaction mixture (added to casein solution and enzyme solution) at concentration of 10.0 mM. Control was prepared by the same procedure without the addition of metal ions, and enzyme activity was measured in the presence and absence of metal ions as control.

Applications

Blood stain removal (Najafi *et al*., 2005)

Three clean pieces of white cotton clothes were soaked in different concentrations (0.5, 2, 1 v) of blood then each piece containing blood was separately soaked in same concentration (2 ml) reaching the total volume to 4 ml using dist. water, at room temperature for 8, 16 and 24 h. The cloth was cut to the same size (1 x 2 cm^2) and no detergent was used. The experiment was taken place for both crude enzyme and partially purified enzyme. Control (4^{th} cotton piece) was treated with distillated water and red blood cell by ratio (1:1) (v/v).

Dehairing of cow skin

Cow skin (1×2 cm^2) was incubated separately with different concentrations of enzyme with dist. water by ratio (1:1, 1:2, 1:3 v) of crude and partially purified alkaline protease from *Bacillus subtilis* SFL at room temperature for 8, 16 and 24 h. At the same time, a control was constructed containing all components except the enzyme.

Degradation of cow skin hair

Untreated thin freshly cow skin hair was used in this experiment. Cow hair suspended in 2 ml of different concentrations of enzyme with dist. water by ratio (1:1, 1:2, 1:3 v) of crude alkaline protease from *Bacillus subtilis* SFL. The reaction mixture was run at room temperature for 24h. Control hair was treated with distilled water only. The treated hair was examined under the microscope at 10 and 40x.

Results

Isolation and qualitative screening of alkaline protease-producing bacteria

All bacterial isolates were morphologically examined for colony surface, color, edge, shape, and clear zone diameter which was measured after 24-48h. Gram staining and the shape of the bacterial colony were microscopically examined for all tested bacteria isolates. The bacterial isolates that gave the highest proteolytic zone diameter in the qualitative screening were selected for quantitative screening using an alkaline skimmed milk nutrient broth medium. Isolate number 28 was selected for further study because it showed the highest alkaline protease productivity.

Molecular identification of the selected bacterial isolate

The bacterial isolate was identified, and the classification was confirmed by 16S rDNA sequencing. The expected size of the PCR product was 1500 bp, the PCR product was sequenced on an ABI 3730xl DNA Sequencing System using forward and reverse primers at GATC Biotech AG. The resulting DNA sequences of the PCR were compared to published sequences using the Basic Local Alignment Search Tool (BLAST) program (http://www.ncbi.nlm.nih.gov/blast), and eluted PCR product of SFL investigate whether homologs to the sequence of the GenBank data. The 16S rDNA sequence of the eluted PCR product of bacteria strains of SFL using forward and reverse primers; investigated in this study are over 99 % identical on a nucleotide level and closely related at the nucleotide level. Isolates are closely related to or belong to the species *Bacillus subtilis*. The sequence was deposited under the accession number SUB12203991 seq OP714187 in the NCBI GenBank database. The cluster analysis also

showed a similarity to *Bacillus subtilis* in Fig. 1.

Fig. 1. Phylogenetic tree of sequence of 16S rDNA sequence similar to *Bacillus subtilis*.

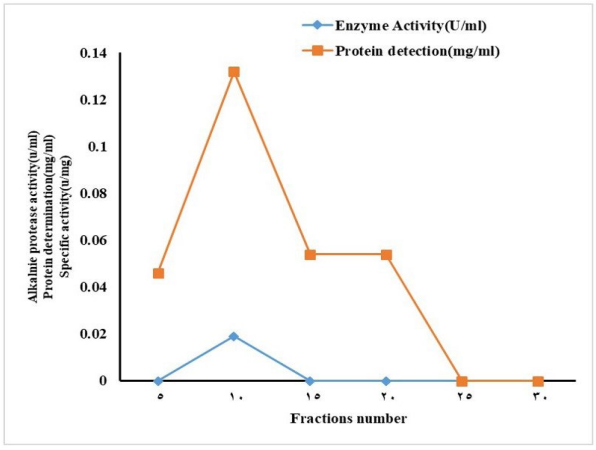

Fig 2. Purification of alkaline protease by sephadex G-100 on gel column chromatography.

Effect of different carbon sources on production of alkaline protease by Bacillus subtilis SFL

Different carbon sources were tested separately at a concentration of 1% in the prepared media and the other components were kept without change. The shown results in Table 1 indicated that xylose was the best carbon source to stimulate enzyme production at both media. It exhibited an activity of 0.420 U/ml with specific activity of 0.306 U/mg protein in presence of milk. The arrangement of other carbon sources by enzymatic activity at skimmed milk medium is as following: sucrose, lactose, glucose, maltose, cellulose and fructose with the lowest enzymatic activity of 0.229U/ml and a specific activity of 0.12 U/mg protein. In Table 2, xylose was the best carbon source at free milk media with nearly half activity with milk as substrate.

Effect of different concentrations of xylose on production of alkaline protease by Bacillus subtilis SFL

In Table 2 a concentration of 12.0 g/l showed a maximum alkaline protease activity of 0.508 U/ml with a specific activity of 0.373 U/mg protein.

Effect of different nitrogen sources on production of alkaline protease by Bacillus subtilis SFL

From organic and inorganic sources, Table 3 shows that the greatest enzymatic activity was in meat extracts. It exhibited an enzymatic activity of 1.179 U/ml and specific activity of 0.65 U/mg protein. Also, the lowest enzymatic activity (0.239 U/ml) with a specific activity 0.14 U/mg protein was in sodium nitrate.

Effect of different concentrations of meat extract on alkaline protease production by Bacillus subtilis SFL

Table 4 shows the effect of different meat extract concentrations on alkaline protease production by *B. subtilis* SFL. It was found that the meat extract at a concentration of 40 g/l showed the highest alkaline protease production (1.46 U/ml and 1.01 U/mg protein).

Table 1. Effect of different carbon sources on production of alkaline protease by *Bacillus subtilis* SFL with skimmed and free skimmed milk as substrate after 48 h at 40°C.

Carbon source (-1%)	Final pH		Enzyme activity (U/ml)		Protein determination (mg/ml)		Specific activity (U/mg protein)	
	Skim	Free skim	Skim	Free skim	Skim	Free skim	Skim	Free skim
Glucose(control)	6.41	5.86	0.28	0.24	1.61	1.57	0.17	0.15
Cellulose	8.25	8.19	0.24	0.24	1.83	1.59	0.13	0.15
Lactose	6.95	7.45	0.30	0.24	1.43	1.5	0.21	0.16
Maltose	5.63	6.05	0.26	0.21	1.79	1.61	0.14	0.13
Xylose	6.96	6.79	0.42	0.28	1.37	1.54	0.31	0.18
Sucrose	6.59	5.81	0.31	0.17	1.36	1.57	0.23	0.11
Fructose	5.51	5.65	0.23	0.27	1.87	1.56	0.12	0.17

Table 2. Effect of different concentrations of xylose as the optimal carbon source on alkaline protease production by *Bacillus subtilis* SFL.

Xylose concentration (g/l)	Final pH	Enzyme activity (U/ml)	Protein determination (mg/ml)	Specific activity (U/mg protein)
4	7.15	0.40	1.47	0.27
6	7.36	0.40	1.44	0.28
8	7.27	0.42	1.41	0.29
10 (control)	6.85	0.42	1.40	0.30
12	7.33	0.51	1.36	0.37
14	6.98	0.41	1.41	0.29

Partial purification of alkaline protease production by Bacillus subtilis SFL

Ammonium sulfate precipitation

The results in Table 5 showed that an ammonium sulfate concentration of 60% gave the best alkaline protease activity of 0.281U/ml with a specific activity of 0.93 U/mg protein.

Precipitation with different organic solvents

The results reported in Table 6 showed that ethyl acetate was the best solvent, giving an enzymatic activity of 0.29 U/ml for the upper layer (solvent) and 0.192 U/ml for the filtrate. According to Tables 6 and 7, ethyl acetate was better than ammonium sulfate 60% in partial purification of enzyme.

Sephadex G100 gel filtration column

In Fig. 2, the highest protein activity and enzyme activity were attended to fraction number 10.

Characterization of partially purified alkaline protease from Bacillus subtilis SFL

The results in Fig. 3 indicated that alkaline protease from *Bacillus subtilis* SFL showed optimal activity after an incubation period of 40 min at 30°C with concentration of 0.5 ml and the optimal substrate concentration was 2 ml. The enzyme was stable at temperature of 40°C, at pH 8.0. The activity of partially purified alkaline protease was tested in the presence of 10 mM of different metal ions and compared to control (without metal ions). The results in Fig 4 showed that all the metal ions inhibited the activity of partially purified enzyme.

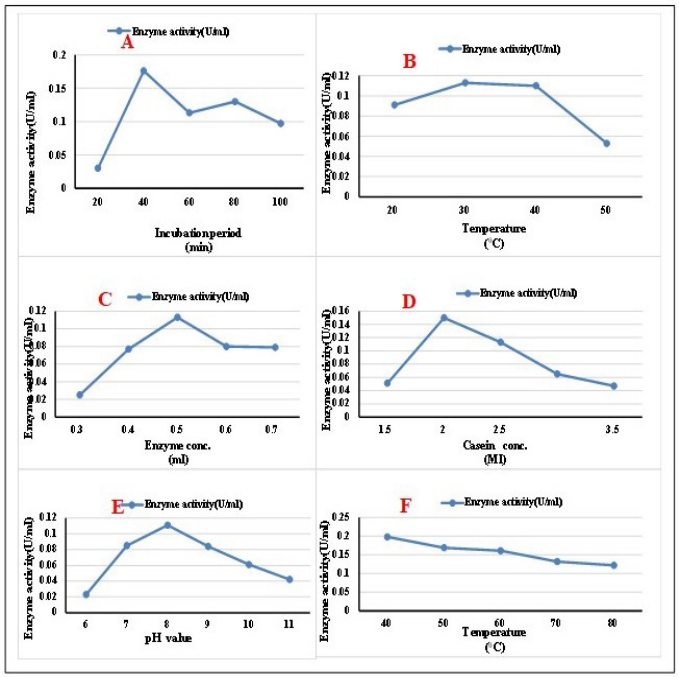

Fig. 3. Characterization of partially purified alkaline protease from *Bacillus subtilis* SFL where A: effect of incubation period on the enzyme, B: effect of temperature on the enzyme, C: effect of enzyme concentration on the enzyme, D: effect of casein concentration on the enzyme, E: effect of pH stability on the enzyme and F: effect of temperature stability on the partially purified alkaline protease.

Table 3. Effect of different nitrogen sources on production of alkaline protease by *Bacillus subtilis* SFL with skimmed milk as substrate at pH 8.0 after 48 h at 40°C.

Different nitrogen source	Final pH	Enzyme activity (U/ml)	Protein determination (mg/ml)	Specific activity (U/mg protein)
Sodium nitrate	7.27	0.24	1.66	0.14
Ammonium sulphate	6.76	0.43	1.61	0.27
Ammonium phosphate	6.09	0.57	1.93	0.29
Casein + yeast ext.	6.08	0.54	1.68	0.32
Casein	7.11	0.64	1.57	0.41
Yeast extract	6.2	0.61	1.81	0.34
Meat extract	5.82	1.18	1.81	0.65
Beef Extract	7.3	0.51	1.80	0.28

Table 4. Effect of different concentration of meat extract as the best nitrogen source on alkaline protease production by *Bacillus subtilis* SFL.

Meat extract concentration (g/l)	Final pH	Enzyme activity (U/ml)	Protein Determination (mg/ml)	Specific activity (U/mg protein)
10	7.35	0.73	1.18	0.62
20	7.55	0.99	1.55	0.64
30(control)	7.32	1.15	1.74	0.66
40	7.57	1.46	1.45	1.01
50	6.77	1.13	1.74	0.65
60	6.9	1.09	2.02	0.54

Table 5. Precipitation of alkaline protease by different concentration of ammonium sulphate.

Ammonium sulphate concentration (%)	Enzyme activity (U/ml)		Protein Determination (mg/ml)		Precipitate specific activity (U/mg protein)	Filtrate specific activity (U/mg protein)
	Precipitate	Filtrate	Precipitate	Filtrate		
20	0.14	0.00	0.39	0.04	0.35	0.02
40	0.28	0.00	0.33	0.08	0.84	0.05
60	0.28	0.02	0.3	0.21	0.93	0.07
80	No ppt	0.25	No ppt	0.34	-	0.73

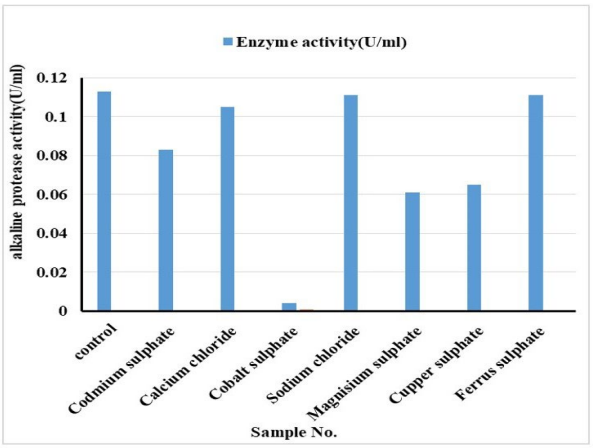

Fig. 4. Effect of different elements ions on partial purified enzyme activities produced by *Bacillus subtilis* SFL.

Potential application of crude and partially purified alkaline protease from Bacillus subtilis

Degradation of red blood cells from cotton cloth

The obtained results in Fig. 5, showed that the decreasing the blood concentration increased the degree of red blood cells decolorization when compared with control sample. Experiment was performed without added any detergent. Treatment with crude enzyme was slightly better than partial purified enzyme in blood degradation.

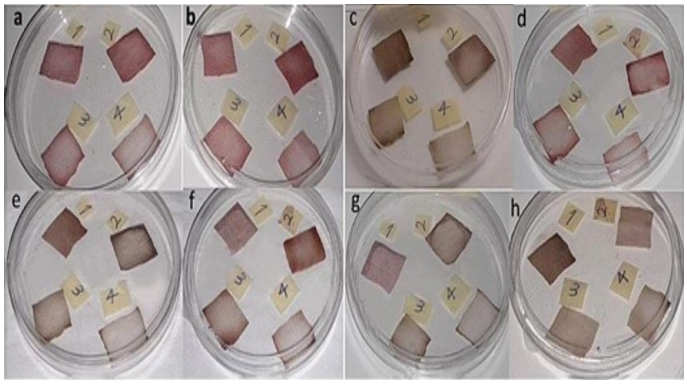

Fig. 5. Degradation of red blood cell from cotton cloth at different times using crude and partially purified alkaline protease. Where: a,c,e,g are crude enzymes, b,d,f,h are purified enzymes 1= Control (distilled water + red blood cells)(1 : 1)), 2= enzyme + red blood cells (1 : 1), 3= enzyme + red blood cell + dist. H_2O (2 : 1 : 1), 4 = enzyme + red blood cells + distilled. H_2O (2 : 0.5 : 1.5). a,b after 0 hours, c,d after 8 hours, e,f after 16 hours, g,h after 24 hours.

Removal of cow skin hair

Different concentrations of crude and partially purified alkaline protease were incubated with cow skin hair with diameter (1×2) for various 8, 16 and 24 h at room temperature, leading to easily removing of cow hair as compared to control. The degree of dehairing increased with the increasing of enzyme concentration and incubation time. This alkaline protease can digest collagen, the process of dehairing must be controlled to avoid reducing the quality of leather. The advantage of the enzymatic process is the reduced use of contaminants and harmful chemicals. The results represented in Fig. 6. The treatment with crude enzyme was slightly better than partial purified enzyme.

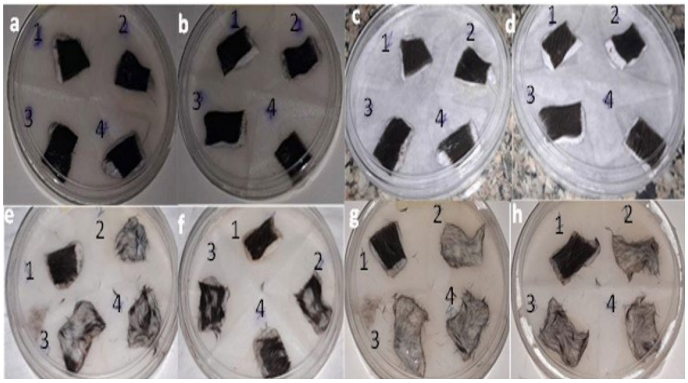

Fig. 6. Removal of cow skin hair, by using crude and partial purified alkaline protease at different times. Where: a, c,e,g are crude enzymes, b,d,f,h are purified enzymes 1= control (dist. H_2O only), 2= enzyme only, 3= enzyme + dist. H_2O (1 : 1), 4 = enzyme + dist. H_2O (1 : 2) and a,b after 0h, c,d after 8 h, e,f after 16 h, g,h after 24h.

Decomposition of cow hair

The results in Fig. 7 show that the hair degradation increases with increasing enzyme concentration.

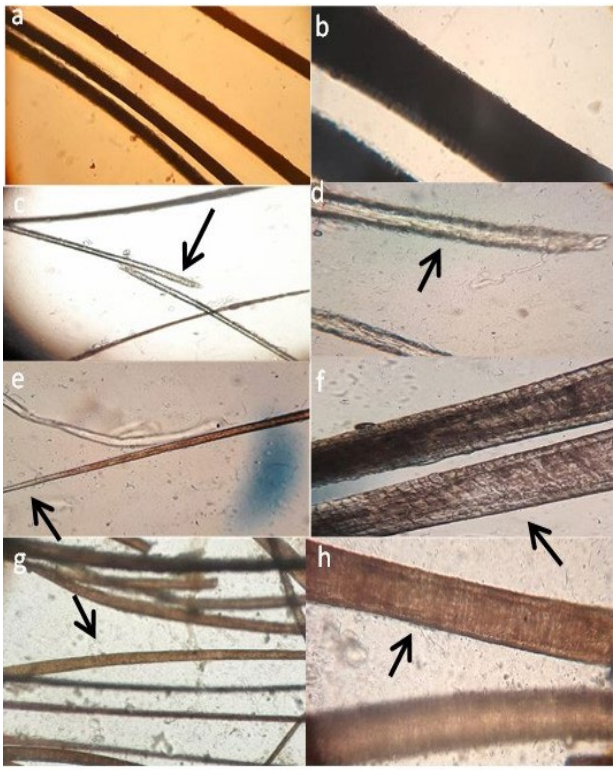

Fig. 7. Decomposition of cow hair under light microscope using crude alkaline protease at 24h. Where: a,c,e,g are magnified under by 10x magnification, b,d,f,h are magnified by 40x magnification, a,b= 1= Control (dist. H_2O only), c, d= 2= enzyme only, e,f= 3= enzyme + dist. H2O (1 : 1), g,h=4 = enzyme + distilled. H2O (1: 2).

Table 6. Effect of different organic solvent on precipitation of alkaline protease by *Bacillus subtilis* SFL.

Solvent	Enzyme activity (U/ml)		Protein determination (mg/ml)		Precipitate specific activity (U/mg protein)	Filtrate specific activity (U/mg protein
	Precipitate	Filtrate	precipitate	Filtrate		
Ethanol	0.11	0.24	0.13	0.26	0.86	0.93
Acetone	0.15	0.25	0.17	0.26	0.90	0.95
Methanol	0.14	0.27	0.18	0.28	0.79	0.98
Ethyl acetate	0.29 (upper-solvent)	0.19 (lower-layer)	0.25 (upper solvent)	0.23 (lower-) (layer)	1.16	0.83

Discussion

Microbes are important source for production of alkaline proteases. This study deals with isolation, identification and characterization of a bacterial isolate from vegetable wastewater of Sharkia province, Egypt that produces thermo-alkaline proteases. Phylogenetic tree constructed with the similar sequences showed that the *Bacillus* spp isolates were related to *Bacillus subtilis*. Media composition plays an important role in enzyme production by microorganisms. Apart from that, environmental factors such as temperature, pH value, incubation time also have a great influence on microbial metabolism (Abidi *et al*., 2008). These factors are important for promoting, stimulating, enhancing and optimizing protease production. Optimizing fermentation media for protease production is essential to obtain economically viable high yields of protease (Abd Rahman *et al*., 2005). The result showed that the media containing 12 g/l xylose as a carbon source supported highest alkaline protease production compared to other carbon sources, and among the different nitrogen sources investigated, 40 g/l meat extract supported the highest level of alkaline protease production. This result indicated that the organic nitrogen compounds favored higher amount of alkaline protease production than the inorganic ones (Azad and Hoq, 2000). In contrast, Jaswal *et al*., (2008) found that soyabean meal was better than casein, gelatin, and peptone for production of protease by *B. circulans*. The present study results were in contrast to the results obtained by Das and Parsad (2010) where they found that dextrose was the suitable carbon source for the production of protease by *Bacillus subtilis* strain. This study reports characterization of partially purified enzyme of *Bacillus subtilis* SFL obtained through using ethyl acetate as organic solvent.

The partially purified enzyme of *B. subtilis* was stable and active up to 40°C with 100% activity. This is in confirmation to Abusham *et al*. (2009) who reported that alkaline protease produced from *Bacillus subtilis* strain R have complete stability at temperature between 35 - 55°C. BY studying the effect of different pH values on stability of alkaline protease produced by *Bacillus subtilis*, it was found that alkaline protease was stable at pH 8.0. This result was in an agreement with Jaouadi *et al*. (2008) and Yildirim *et al*. (2017) who reported that protease produced from *Bacillus* pumilus CBS showed the same pH stability. Joo *et al*. (2003) have reported that alkaline protease produced by *Bacillus* clausii showing stability at pH 4.0 - 12.0. By measuring the alkaline protease activity at different incubation temperatures, the enzyme showed maximum activity at 30 0C. This result was lower than 40°C which reported by Hakim *et al*. (2018) and Ramkumar *et al*. (2018), 45°C which reported by Shaghayegh *et al*. (2015), 55 0C who reported by Gulmus and Gormez, (2020) and Zheng *et al*., (2020) and 60 0 C which reported by Iqbalsyah *et al*., (2019); Ahmad *et al*., (2020) and Hammami *et al*., (2020) for alkaline protease produced by bacteria. The optimum incubation period of partially purified enzyme from *B. subtilis* was 40 min. This result contrasted with Devi *et al*. (2008); Seifzadeh *et al*. (2008) and Al-Askar *et al*. (2015) who reported that the optimum incubation period for enzyme activity was 60 min. The present study illustrated that Ca^{2+}, Na^{1+}, Mg^{2+}, Cu^{2+}, Co^{2+}, Cd^{2+}, and Fe^{2+} act as inhibitors of the alkaline protease activity. This agrees with Venugopal and Saramma (2007) and Olajuyigbe and Falade (2014) who reported that Co^{2+}, Cd^{2+}, decrease the activity of enzyme. Additionally, Gaur *et al*. (2010) reported that Ca^{2+}, Mg^{2+}, Cu^{2+} moderately effect enzyme activity. Whereas this result in partial contrast to Gupta *et al*. (2005) and Rajkumar *et al*. (2011) who reported that activity of alkaline protease produced by *Bacillus* megaterium RRM2 and *Bacillus* sp. increased by Ca^{2+}.

In our study, some applications had been applied by using alkaline protease. The first applied experiment was degradation of red blood cells from cotton cloth where the results showed the ability of alkaline protease for removal of blood stain, this is in agreement with Vishalakshi *et al*. (2009) and Anupama and Kshipra (2012) who reported the successful removal of blood stain by alkaline protease produced by *Bacillus licheniformis* and *Streptomyces gulbargensis* respectively. Another applied experiment was removal of cow skin hair. The result showed the ability of alkaline protease to remove cow hair within 24h. Zekeya *et al*. (2019) and Moonnee *et al*. (2021) reported the ability of protease for dehairing within 48–72 h.

Conclusion

Molecular characterization was performed using 16S rDNA sequencing and identified as *Bacillus subtilis* SFL that isolated from vegetables wastewater in Sharkia province of Egypt. The partially purified enzyme was stable at 40°C and pH 8.0. Thermo-alkaline protease from *B. subtilis* SFL degraded red blood cells, removed hairs from raw cow skin and decomposed cow hairs revealing that it might be potential for biotechnological applications.

Conflict of interest

The authors confirm that there is no conflict of interests in publishing this paper.

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