Molecular characterization of alkaline protease-coding gene from *Bacillus licheniformis* MK90 mutants with biofilm inhibitory activity

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Background
Enzymes are organic materials that accelerate biochemical processes without themselves undergoing change. They can be produced by plant, animal, fungi, and bacteria. Bacterial proteases are much favorable than any other sources, because bacteria can grow quickly and can be easily cultivated in laboratory.
Objective
To isolate and screen bacteria from soil samples for their ability to produce alkaline protease, and to improve the alkaline protease production followed by evaluation of its antimicrobials and antibiofilm activity.
Materials and methods
Sample collection was carried out from different locations in Egypt. Isolation of bacteria from soil samples was done using serial dilution method on skim milk agar. All isolated bacteria were screened for their ability to produce protease enzyme. The bacterial isolate showing maximum alkaline protease activity was identified using 16 S rRNA genetic identification. To induce mutations, ultraviolet (UV) irradiation was used. The most active mutant strains were selected for production, purification, and characterization of alkaline protease followed by evaluation of alkaline protease antimicrobial and antibiofilm activity.
Results and conclusion
Three UV mutants (MT2, MT4, and MT26) out of 48 displayed proteolytic activity more than other mutants and wild type (WT). *Bacillus* alkaline extracellular protease gene was genetically characterized through isolation of the genomic DNA of *Bacillus licheniformis* MK90 WT, and the best protease-producing UV mutants were followed by amplification, sequencing, and analyses. WT strain and best protease-producing mutants were compared at proteomic level through sodium dodecyl sulfate polyacrylamide gel electrophoresis for total cellular proteins. Then protease enzyme of WT and mutants was purified and characterized. This study reports that the *B. licheniformis* protease was active at an alkaline pH and wide range of temperatures (40–60°C), reflecting its potential application in detergent and laundry industries. On the contrary, the antibiofilm activity of the protease enzymes was evaluated toward four pathogenic bacterial strains, i.e., *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Bacillus subtilis,* and the results showed that proteases from *B. licheniformis* MK90 may be useful for controlling biofilm formation by some pathogenic bacteria.

Keywords:
antibiofilm, *Bacillus licheniformis* MK90, mutagenesis, Bacillus alkaline extracellular protease

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Introduction

Enzymes are organic materials in the cells of living organisms in small concentrations that are able to accelerate biochemical processes without themselves undergoing change. They can catalyze the reaction without necessarily starting it [1]. Recently, extracellular hydrolytic enzymes like proteases, amylases, DNases, lipases, pullulanases, and xylanases were found to be very useful in many applications processes such as food industry, biomedical sciences, feed additive, and chemical industries [2].

Based on their optimal pH, proteases are classified into three groups: 'alkaline, acidic, and neutral proteases.' Acid proteases work at pH ranged from 2.0 to 5.0, and they are almost only synthesized by fungi. The second group is neutral proteases that act at pH ranged from 7.0 to 8.0, and they are mainly produced by plants, and finally, alkaline proteases, with pH from 8 and above

[3]. Proteases are widely distributed in nature, and they are found in all living organisms such as plants, animals, and microbes [4]. The microbial proteases are much favorable than any other sources because microbes can grow quickly and easily cultivated in laboratory [5].

Recently, high degree of concern has been directed for using alkaline protease for many reasons; they have high hydrolytic activity, have high degree of substrate specificity [6], and are active in a neutral to alkaline pH [7]. Alkaline proteases are one of the most important enzymes used in industrial applications, like pharmaceuticals, detergents [8,9], meat tenderizers, leather, protein hydrolysis, food products, and waste recycling. Microbial origin is the most common sources of alkaline proteases. They can be produced by bacteria [10], actinomycetes [11–13], yeast [14], and fungi [15]. Bacterial proteases are of utmost importance owing to their properties and also bacteria can easily be genetically modified in the laboratory. Alkaline proteases produced by *Bacillus* spp. have shown high activity and stability at different temperature and pH ranges [16–19]. Recently, genetic modification of bacteria has been proved to be an efficient tool to optimize the production and the quality of enzymes [20]. This can be achieved by DNA modification through mutation induction by using chemical or physical mutagens [21].

Bacterial biofilms are aggregate of bacteria in a self-producing matrix [22]. This process makes bacteria able to strongly attach on many living and nonliving surfaces. Bacterial biofilms are considered as one of the major hindrances against medication and control of infectious disease. It makes microbes resistant to antimicrobial agents [23]. Bacterial biofilm was found to be highly resistant to host immune defenses and antibiotics [24]. Biofilm matrix consists of different macromolecules known as extracellular polymeric substances. Extracellular polymeric substance serves as a tertiary biofilm structure, which facilitates cell-to-cell communication and surface adhesion [25]. For a long time, polysaccharides were thought to be the major component of the biofilms [26]. Recently, new studies have been reported that proteins also play a very important role in the biofilm formation [27]. Despite many trials being conducted to eradicate biofilm synthesis, there is still undeniable need to discover an efficient way to control biofilm formation by bacteria.

The introduced study was carried out to improve enzyme production and quality via genetic modifications of Bacillus strain isolated from hot springs of Sinai through ultraviolet (UV) mutagenesis. Furthermore, the application of the produced enzymes as antibiofilm against four pathogenic bacteria was also studied.

Materials and methods

Sample collection and isolation

Sample collection was carried out from different locations in Egypt. Samples were kept and stored at 4°C. Isolation of bacteria from soil samples was done by suspending of 1 g in 9-ml sterilized distilled water, followed by serial dilutions in sterile distilled water [28]. Overall, 50 µl of certain dilution was added on skim milk agar with following components (g/l): peptone, 5.0; NaCl, 5.0; agar, 20; and skimmed milk, 100, with pH 8, and incubated at 50°C for 3 days.

Qualitative screening of alkaline protease activity

To screen all isolated bacteria for their ability to produce protease enzyme, casein agar medium was used with following components (g/l): casein, 5.0; glucose, 5.0; and agar 20 [29,30]. All isolated bacterial isolates were inoculated aseptically on the plates, and then the plates were incubated in inverted position at 37°C for 24–72 h. The appearance of clear zone (zone of proteolysis) indicates positive result.

Enzyme production

To produce alkaline protease enzyme, the most potent bacterial strains based on the qualitative screening were grown on yeast extract casein broth medium [31], with the following composition (g/l): glucose, 10.0; casein, 5.0, yeast extract, 5.0; KH_2PO_4, 2.0; K_2HPO_4, 2.0; and $MgSO_4.7H_2O$, 1.0. After inoculation, growth cultures were incubated for 72 h in a shaking incubator (170 rpm) at 37°C. The pH of the medium was adjusted to 9.0–9.5. After incubation, the fermented cultures were centrifuged at 8,000 rpm, at 4°C, for 20 min, and the clear supernatant was used as the crude enzyme.

Enzyme assay

The enzyme assay was measured by modified method of Yang *et al.* [32]. The activity was measured using casein as a substrate at a concentration of 1% w/v in 50 mM glycine-NaOH buffer (pH 10). The reaction mixture was monitored by adding of 0.5 ml of enzyme solution to 2.5-ml casein solution and incubation for 1 h at 30°C. To terminate the reaction after the incubation time, 2.5 ml of 0.44 M trichloroacetic acid solution was added, and after 10 min, the mixture was centrifuged at 8000 rpm for 10 min. An aliquot of

0.5 ml of supernatant was mixed with 2.5 ml of 0.5 M Na_2CO_3 and 0.5 ml of Folin–Ciocalteu's phenol solution and kept for 30 min at room temperature, and the optical densities were measured at 660 nm.

Protein determination

Protein concentration was measured according to Lowry *et al.* [33], and protein concentration was followed by its absorbance at 280 nm.

Molecular identification of the most potent isolate

The bacterial isolate showing maximum alkaline protease activity and maximum proteolytic zone size was selected and identified using 16S rRNA genetic identification [34]. The sequence produced was analyzed by using BLAST, and the phylogenetic tree was constructed using MEGA 7 (available at https://www.megasoftware.net) software.

Ultraviolet mutagenesis

To induce mutations in the most potent alkaline protease producer *Bacillus licheniformis* MK90, UV irradiation was used according to modified method of Khedr *et al*. [34] where bacterial cell suspensions were exposed to UV irradiation at a distance of 20 cm for 3, 5, 7, and 10 min. After UV irradiations, the treated suspensions were kept for 1 h in dark place, and then 1 ml from treated cells with suitable dilution was incubated on LB supplemented with casein and minimal casein [35].

Electrophoresis

'Sodium dodecyl sulfate polyacrylamide gel electrophoresis' (SDS-PAGE) was used to separate proteins with relative molecular mass more than 10 kD [36]. According to Laemmli [36], Harlow and Lane [37], and Sambrook and Russell [38], SDS-PAGE was prepared for analysis of the expressed proteins [39].

DNA isolation and designing of primers

The genomic DNA of the *B. licheniformis* wild type (WT) and the highest three alkaline protease producing UV mutants was isolated using 'EasyPure bacteria genomic DNA kit.' To measure the complete nucleotide sequence of the *Bacillus* alkaline extracellular protease (BAEP) gene, two primers were selected via 'NCBI primer design tool' and named Khedr-F (5-GTGCATCCTCTTCCGGTCAA-3) and Khedr-R (5-TTGCCATCGAGGTTCCTGAC-3). For this purpose, information was derived on conserved sequences around coding nucleotide sequences (upstream and downstream) of the complete alkaline protease genes of three *Bacillus* sp. on GenBank. The individual nucleotide sequences were aligned by CLUSTALW online software to identify the conserved sequences (Kyoto University Bioinformatics Center).

PCR amplification of Bacillus alkaline extracellular protease

PCRs were carried out in a programmable DNA thermal cycler PCR system. PCR reaction mixture was optimized to 25 µl that contains 40 ng (6 µl) of template (bacterial) DNA solution and 8.5 µl of master mix, which includes dNTPs mix, $MgCl_2$, Taq polymerase, and PCR buffer. Primers were added separately after preparation from lyophilized stock (1 µmol/l of each primer). The amplified DNA product along with a DNA marker [gene ruler 100 bp DNA ladder (SM0241)] was separated by electrophoresis using 1% agarose gel in TAE. The gel was stained with ethidium bromide, and the banding profile was recorded using UV-gel documentation system. The amplified DNA product was purified and sequenced.

Partial purification of alkaline protease and characterization

After the fermentation, the cells of WT and mutant strains were removed from the crude supernatant by centrifugation at 12 000 g for 10 min. The proteins in the extracellular extract of the WT and mutant strains were precipitated by salting out with ammonium sulfate fractionation. The calculated volume of ammonium sulfate was combined with culture supernatant with constant stirring in cooling condition to obtain 40, 60, and 80% saturation. The obtained precipitate recovered by centrifugation (10 000 g for 10 min at 4°C) was dissolved in a low-quantity citrate-phosphate buffer (pH 7.5) and then dialyzed against the same buffer in cellophane bag in refrigerator for further times to ensure complete elimination of the salt, as checked by reaction with barium chloride. The fractions with the maximum proteolytic activity were pooled and used for subsequent characterization.

Effect of pH on activity and stability of partially purified alkaline protease

The optimum pH for proteolysis activity was determined in the reaction mixture along with pH range of 5–11 using 50 mmol/l of citrate-phosphate buffer (pH 4.0–8.0) and carbonate-bicarbonate buffer (pH 9.0–11.0). For pH stability, the enzyme preparations were pre-incubated at different pH levels (5.0–11.0) for 6 h at 30°C. Then, the residual activity was determined at the optimum pH value.

Effect of temperature on activity and stability of partially purified alkaline protease

The optimal temperature for the proteolysis activity was measured by incubating the reaction mixtures in 50 mmol/l glycine-NaOH (pH 10.0) at different temperatures (30–80°C) for 30 min. The relative enzyme activity was calculated as the percentage of its maximal activity. Thermal stability assays were conducted by preincubating the enzyme at 40, 50, 60, 70, and 80°C. Aliquots were drawn at desired time intervals, and the remaining activity was tested at 50°C, pH 10.5. The unheated enzyme was used to define 100% activity.

Antimicrobial activity of partially purified protease

The antimicrobial activities of the partially purified enzymes were measured using the agar well diffusion method with some modification [28].

Antibiofilm activity

To measure the biofilm inhibitory activity of the partially purified enzymes, MTP assay was carried out using 96-well flat-bottom polystyrene titer plates and four clinical microbes, i.e., '*Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Escherichia coli*, and *Bacillus subtilis,*' according to Christensen *et al.* [40]. In brief, each well was filled with 180 µl LB broth, 10 µl of overnight pathogenic bacteria, and 20 µl of the sample, along with control (without test sample), and incubated at 37°C for 24 h. After incubation, the content of the wells was removed, and the wells were washed with 200 µl of phosphate buffer saline pH 7.2 to remove the floating bacteria. For staining, 200 µl/well of crystal violet (0.1%, w/v) was added. To remove the excessive stain, the wells were washed with 200 µl/well deionized water and kept for drying. Furthermore, dried plates were washed with 95% ethanol, and optical density was determined at optical density of 570 nm by using a Spectrostar Nano Microplate Reader (BMG Labtech GmbH, Allmendgrun, Germany).

Results and discussion

Isolation and screening of protease producer

Protease-producing bacteria were isolated and purified on casein medium. After 24 h of incubation, the bacterial growth with a clear zone around was marked as a positive producer. Table 1 summarizes the proteolytic zone size of each bacterial isolate. Asha and Muthusamy [41] screened 30 bacterial isolates on skim milk agar plates and found that all of them had proteolytic activity. Nayab *et al.* [42] isolated 70 protease-producing bacterial strains out of 100 isolates on skim milk agar plates, and these isolates were diverse between higher proteolytic and lower proteolytic isolates. The most potent isolate among the rest of the isolates was isolate ASH70 with clear zone of 17 mm, followed by two isolates ASH85 and ASH100, with 16 and 15 mm of clear zones, respectively. The most potent strains were cultured in the production media at 37°C, pH 8.0, for 24 h, and assayed quantitatively, and the cell-free supernatant of isolate ASH70 displayed the highest protease activity (33.3 U/ml). Several investigations reported that the optimum pH of alkaline proteases ranged from 8 to 11 [43–47].

Table 1 Screening protease productivity by proteolytic zone (mm) on casein agar for 24 h of incubation at 37°C

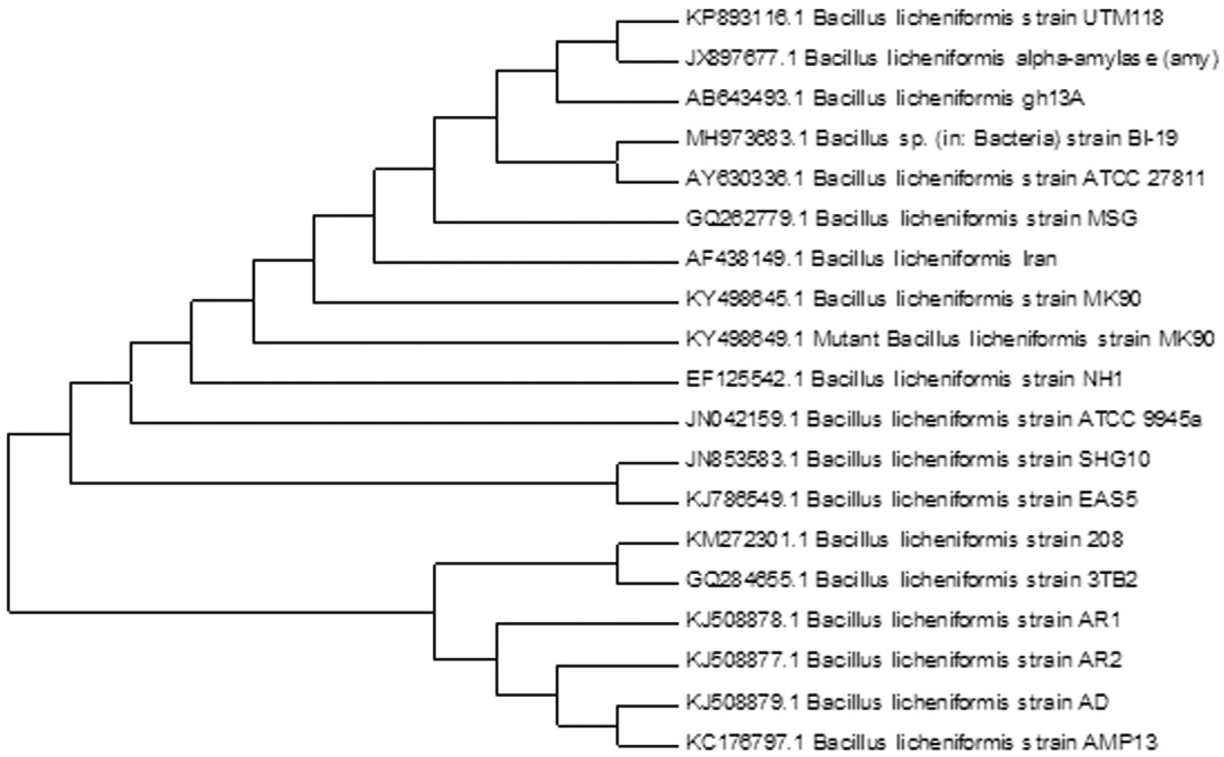
Isolate code	Source	Proteolytic zone (mm)
ASH5	Clay soil cultured with rice	16
ASH6	Clay soil cultured with rice	15
ASH7	Clay soil cultured with rice	14
ASH75	Clay soil cultured with rice	10
ASH2	Clay soil cultured with rice	8
ASH85	Clay soil cultured with rice	16
ASH70	Hot spring of Sinai	17
ASH1	Clay soil cultured with wheat	10
ASH87	Clay soil cultured with wheat	12
ASH88	Clay soil cultured with wheat	13
ASH89	Clay soil cultured with wheat	14
ASH90	Clay soil cultured with wheat	14
ASH91	Clay soil cultured with wheat	10
ASH92	Clay soil cultured with wheat	12
ASH93	Clay soil cultured with wheat	11
ASH96	Clay soil cultured with wheat	11
ASH86	South Sinai soil	12
ASH94	South Sinai soil	13
ASH95	South Sinai soil	12
ASH97	South Sinai soil	12
ASH100	South Sinai soil	15

Screening protease productivity by clear zone (mm) on casein agar for 24 h of incubation at 37°C.

Molecular identification of the most potent alkaline protease producer

Bacterial isolate ASH70 was identified by 16S rDNA [34] as *B. licheniformis* MK90, and the sequence was deposited in the gene bank (accession number KY366269.1). Sequence was analyzed versus others on Gene bank database through online BLAST tool

Figure 1



Phylogenetic relationship between *Bacillus licheniformis* MK90 and other members of *Bacillus* sp.

to determine the similarity score (http://www.blast.ncbi.nlm.nih.gov/Blast). The obtained result confirmed a very close similarity of the 16S rDNA gene sequence with 97.73% homology of the isolate ASH70 with *B. licheniformis*. The phylogenetic tree was constructed using MEGA 7 program [48] and neighbor-joining method [49] (Fig. 1).

Mutagenesis by ultraviolet treatment

Cyclobutane pyrimidine dimers and pyrimidine (6–4) pyrimidone photoproducts at dipyrimidine sites (two pyrimidine (Py) bases are adjacent in nucleotide sequence of DNA) are DNA mutations considered as damaged and induced by UV irradiation. These UV lesions resulted from photochemical reaction; their efficiency depends on the wavelength, following direct UV energy absorption by DNA bases [50]. Volff *et al.* [50] noted that nitrosoguanidine and UV rays induced mutation in the structural genes coding enzymes responsible for the biosynthesis. However, most prokaryotes and all eukaryotes have highly conserved regions called protein systems that recognize DNA mismatches, and many DNA damages play significant roles in maintenance of genetic stability [51]. These long patch mismatch-repair systems minimize DNA replication mistake rates 100-fold to 1000-fold, by identifying and repairing base/base and insertion/deletion-loop out mismatches that flee from proofreading by DNA polymerase. Martinez and Baquero [52] explained that the mutation process in bacterial cells does not occur regularly. Many factors affect the rate and type of mutants that can be chosen under pressure. Furthermore, the appearance of mutations that produce mutator phenotypes in bacteria and the ability of some antibiotics to increase mutability are more complicated to understand the effects of population dynamics on the emergence of antibiotic-resistant mutants in bacteria.

Moreover, Liu *et al.* [53] used too low UV doses to make SOS functions; most incorrect bases versus occasional photoproducts may be eliminated by mismatch repair. However, in heavily irradiated (SOS-induced) cells, mismatch repair is limited and corrects only some photoproduct/base mismatches, so UV mutagenesis remains fundamental. Strain *B. licheniformis* as a parental strain or WT was treated with UV radiation at wavelength 254.5 for different periods of time 3, 5, 7, and 10 min, and all the available colonies appeared after UV treatment were isolated. All colonies were grown on casein agar medium and tested for their ability to produce protease enzyme. Based on the enzymatic activity results of the produced mutant strains, three mutants (M2, M4, and M26) were considered as the highest alkaline protease producers

Table 2 Proteolytic zone on skim milk agar medium owing to protease activity of random ultraviolet mutants and their wild type for 72 h of incubation at 37°C

Mutants	Skimmed milk minimal agar medium (mm)			
	24 h	48 h	72 h	Growth
M1	–	–	–	Low
M2	14	18	22	High
M3	–	6	8	Low
M4	9	11	14	High
M5	5	6	8	Low
M6	8	8	8	Low
M8	–	–	–	Low
M9	7	7	7	Low
M10	10	12	14	Medium
M11	9	10	10	Medium
M12	–	–	–	Low
M13	–	–	–	Low
M14	7	7	8	Low
M15	8	12	18	High
M16	–	–	–	Low
M17	–	–	–	Low
M18	–	–	-	Zero
M19	9	9	9	Medium
M20	7	7	8	Medium
M21	7	8	10	Medium
M26	17	19	21	High
M25	9	9	9	Medium
M27	–	–	–	Low
M28	–	–	–	Low
M29	–	–	–	Low
M37	–	–	–	Low
M38	9	10	11	High
M41	–	–	–	Low
M42	–	–	–	Low
M43	8	10	12	Medium
M44	7	7	8	Medium
M45	–	–	–	Low
M46	–	–	–	Low
M47	–	–	–	Low
M48	–	–	7	Low
WT	7	9	9	Medium

WT, wild type.

with specific activity (22.4, 40.9, and 51.8 U/ml, respectively). These results were nearly identical when compared with qualitative screening of the resultant mutants (Tables 2 and 3).

PCR and DNA sequence analysis of BAEB

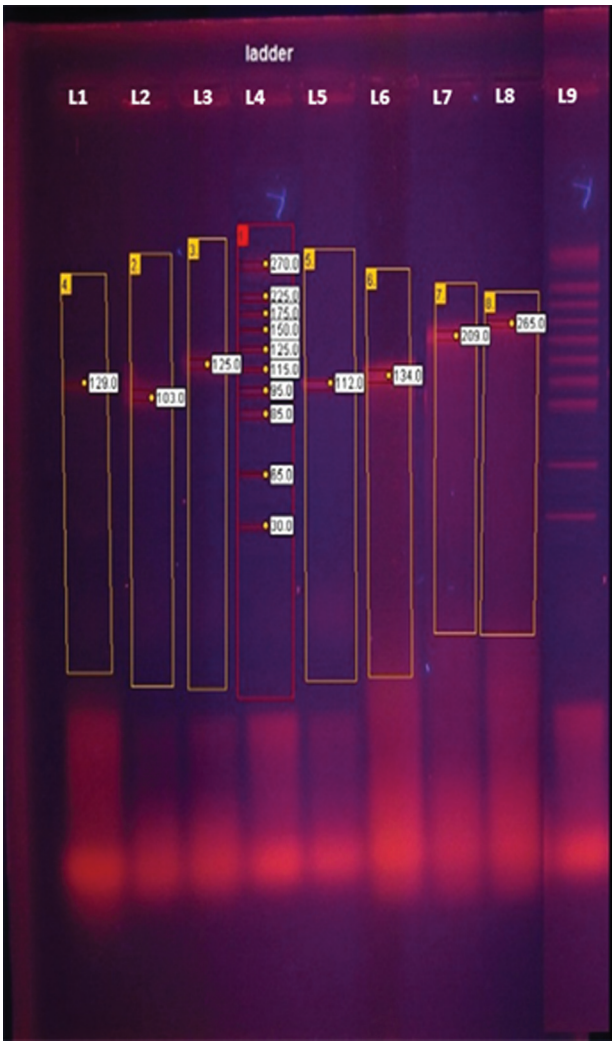
The resulting four PCR products of parental strain and its three mutants were sequenced. Sequencing of amplified protease gene showed 557 bps in WT and its three UV mutants, as showed on agarose gel electrophoresis (Fig. 2), with 100% similarity, without any base pair change or single nucleotide polymorphism. The apr gene was obtained by PCR using the genomic DNA of *B. licheniformis* CICIM B5102 as a template.

Table 3 Quantitative screening of alkaline protease of random ultraviolet mutants and wild type

Isolate	Total protein	Specific activity (U/ml)
M2	2.11	22.4
M4	1.9	40.9
M10	1.55	18
M11	1.66	15
M14	1.69	10.5
M15	1.89	18
M19	1.68	13.5
M20	1.71	15
M26	1.85	51.8
M25	1.68	13.5
M38	1.61	18
M43	1.69	18
M44	1.59	15
WT	0.7	33.3

WT, wild type.

Figure 2



Electrophoretic patterns of partially amplified alkaline protease gene from *Bacillus licheniformis* MK90 and its UV mutants with DNA 100 bp ladder; amplified product was estimated to be nearly 557 bp. Lanes 1 and 2 from left (*Bacillus licheniformis* MK90 wild type), lane 3 (Mutant 2), lane 4 (DNA ladder), lane 5 and lane 6 (Mutant 4), lane 7 and lane 8 (mutant 26), and lane 9 (DNA ladder). UV, ultraviolet.

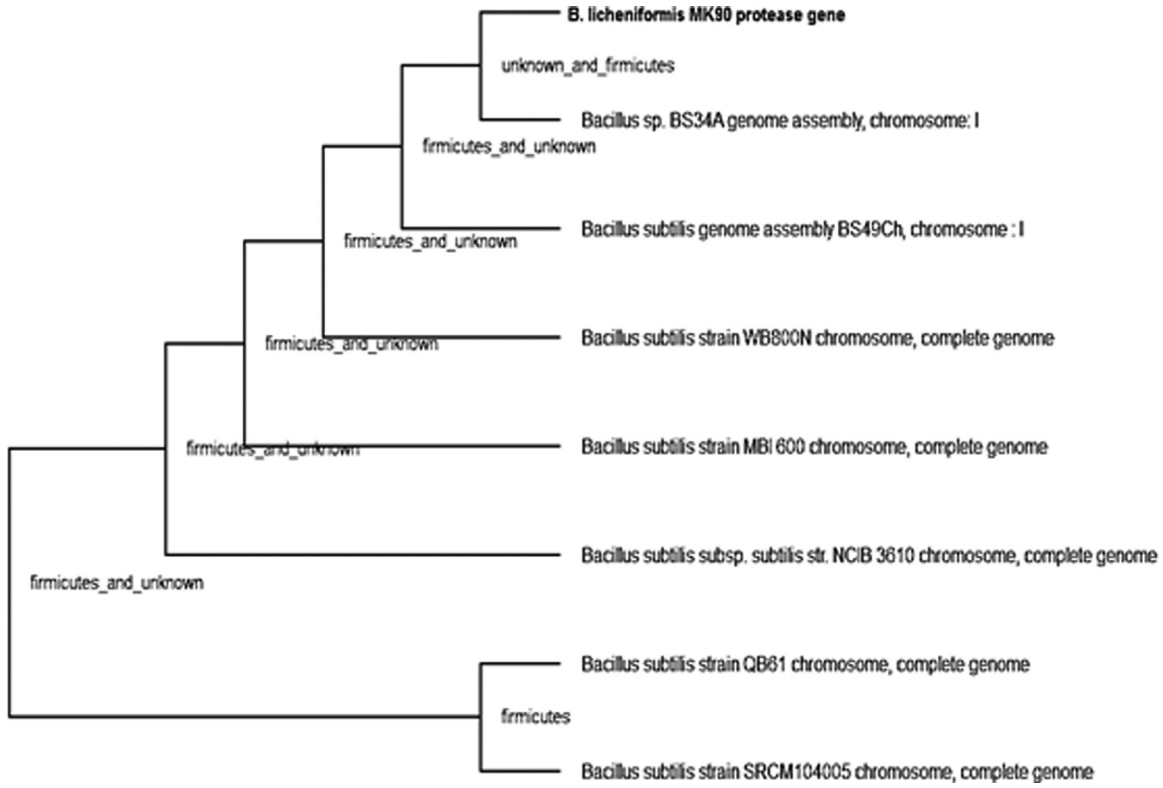
PCR product was 1.8 kb bp of the apr gene [54]. The sequencing reactions were carried out by Sanger Sequencing Technology on Applied Biosystems automated DNA sequencer, model ABI 3730XL DNA Analyzer (Applied Biosystems, Beverly, MA, United States; service provided by Macrogen Inc., South Korea). The sequence analyses and alignments were performed by NCBI-BLAST programs of the National Center for Biotechnology Information [55]. The resultant gel electrophoresis bands were analyzed through Gel-Analyzer software. Khedr *et al*. have isolated, amplified, and sequenced AmyE gene that coded a thermo-stable α-amylase enzyme from *B. licheniformis* MK90; the gene was estimated to be approximately 1539 bp and encoded a 515-residue protein, composed of a 25-amino acid putative signal peptide and a 490-amino acid mature protein [34]. Phylogenetic tree was constructed on the basis of *B. licheniformis* MK90 incomplete sequence of BAEP and other related *Bacillus* sp. using NCBI-BLAST online and modified through tree viewer software (Fig. 3).

Nucleotide partial sequences of BAEP from the parental strain (WT) and its three mutants were compared with each other using online Cluster W beside jalview, version 2 (a multiple sequence alignment editor and analysis workbench) (Fig. 4). The three nucleotide sequences measured nearly 557 bp. The four nucleotide sequences were identical without any mutations in any one of them. Kaur *et al*. [56] isolated and amplified the alkaline protease gene of *Bacillus circulans,* and the amplified sequence was estimated to be approximately 1,360 bps.

Amino acid sequence alignment

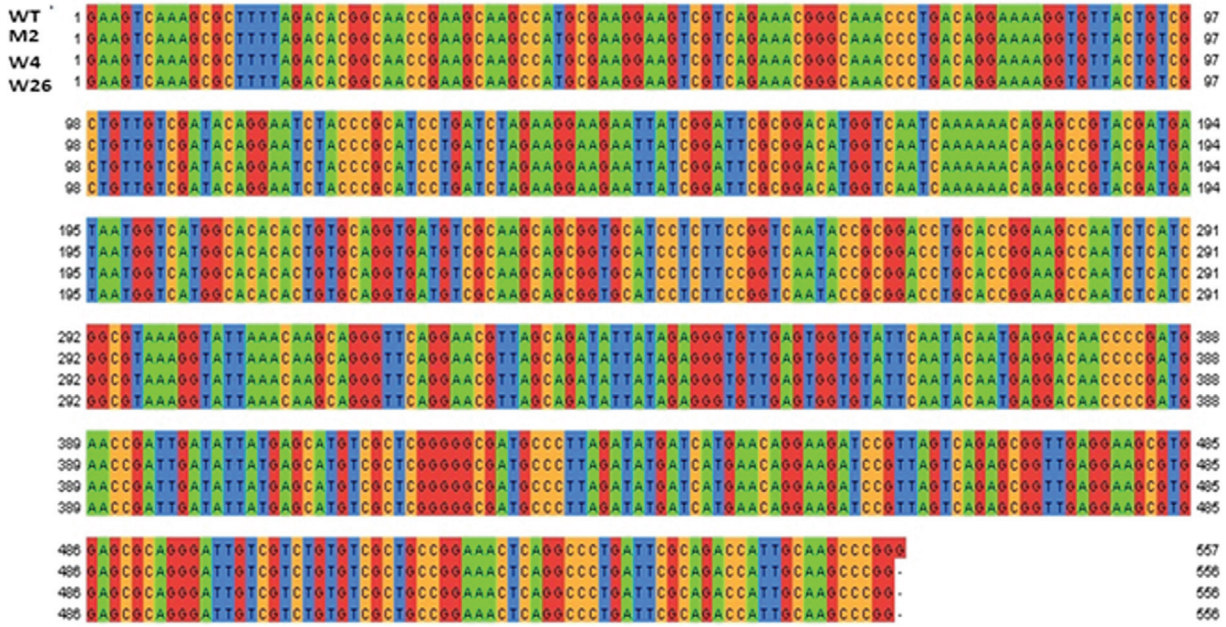
The corresponding predicted protein (amino acid) sequence of BAEP gene was also blasted into NCBI-blast (protein) function, available at http://www.ncbi.nlm.nih.gov/BLAST, for identifying any nucleotide change (induced UV mutagenesis). The four sequences of parental strain and its three mutants were analyzed via SnapGene viewer, version 3.1.2.156 software, to translate the nucleotide sequence of genes into amino acid sequences to observe the effect of UV in the gene expression and amino acid sequence of enzyme. The 557 bp coded for 185 amino acids. Amino acid sequences resulting after software analysis are presents in Fig. 5 and showed no change in amino acid sequences within three mutants against WT sequence. Amino acid symbols were coded according to SnapGene software (from GSL Biotech; available at https://www.snapgene.com/) amino acid table.

Figure 3



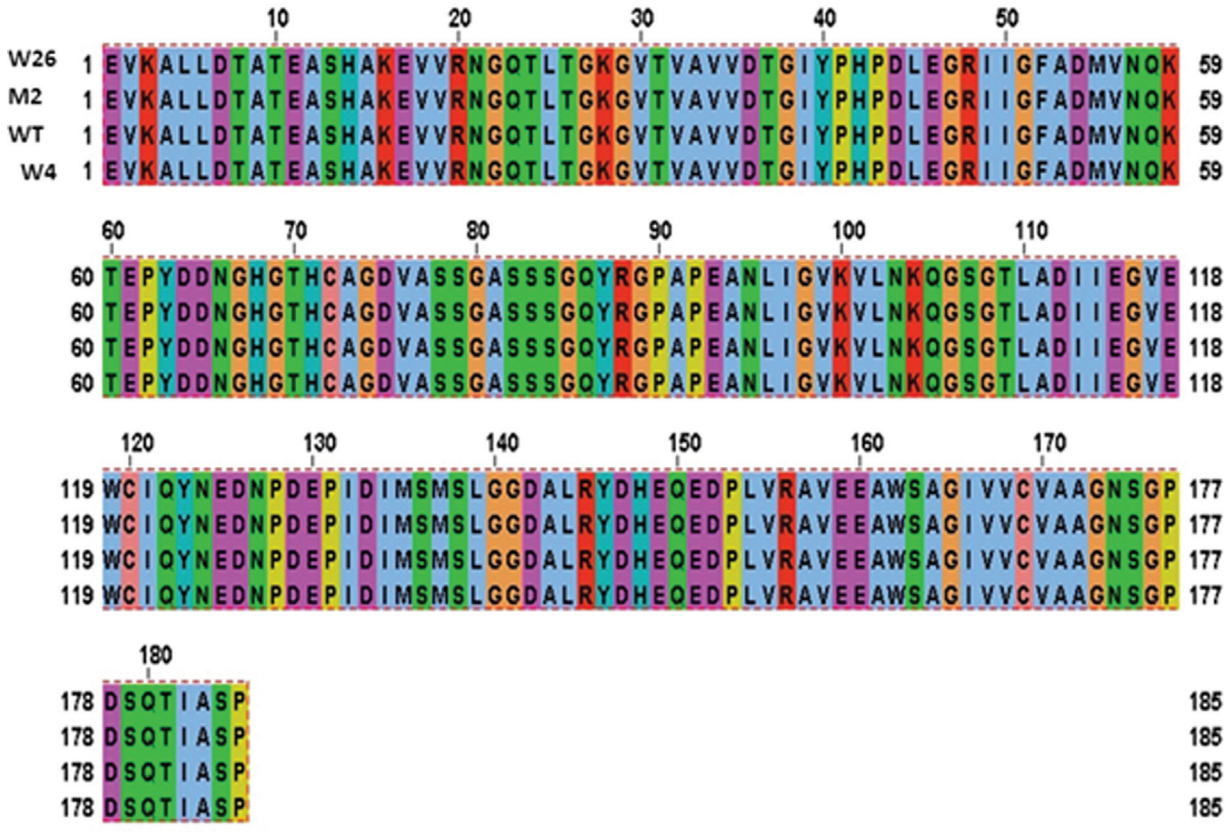
Phylogenetic tree constructed according to *Bacillus licheniformis* MK90 protease gene and other members of *Bacillus* sp. carried out by NCBI-BLAST online software.

Figure 4



DNA alignment through CLUSTAL OMEGA online between protease gene sequence from parental strain (row 1) and the two UV mutants. Mutant 1 (row 2), mutant 2 (row 3), and mutant 3 (row 4) using Jalview software. UV, ultraviolet.

Figure 5

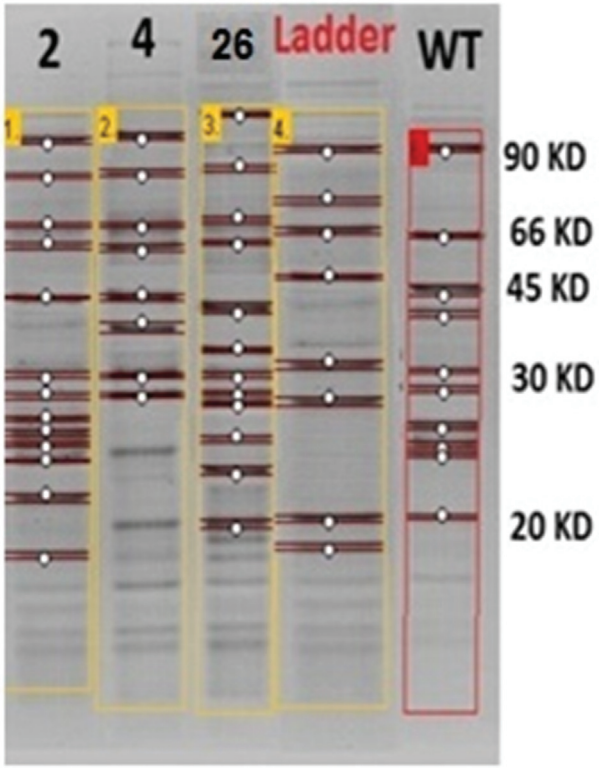


Multiple alignments of amino acid sequence for parental strain WT (row 3) and the best three mutants (rows 1, 2, and 4). WT, wild type.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis of alkaline proteases of wild-type *Bacillus licheniformis* MK90 and selected mutants

This step was carried out to monitor changes in mutants against WT, as no changes were obtained on the level of amplified sequence of BAEP gene. The total cellular protein profile of the higher enzyme-producing mutants M2, M4, M26 and parental strain was performed using SDS-PAGE with protein marker, and the results are presented in Fig. 6. Gel was analyzed with gel-analyzer 2010 software, and results showed remarkable differences between WT and the selected mutants. WT showed 10 bands, whereas M4 showed eight bands, M26 showed 12 bands, and M2 showed 13 bands. Proteomic analysis via SDS-PAGE showed remarkable band changes among mutants themselves and WT. Different protein patterns refer to mutation of DNA of tested mutants, and these mutations are constant, which are transferred through generations along subculturing. Protease molecular weight of WT and its mutants was nearly 30 kD in comparison with bovine serum albumin) ladder. Similar results had been noted by Kumar and Takagi [57], as their microbes produce alkaline protease enzyme with molecular weight ranged from 15 to 30 kD. Nearly similar results were also achieved by Barth and Gaillardin [58], when they worked on *Yarrowia lipolytica,* which produced alkaline extracellular protease with 32 kD. Marine yeast *Aureobasidium pullulans* also produces alkaline protease with the same molecular weight of *Yarrowia lipolytica* [59].

Figure 6



SDS-PAGE analysis for total protein of the most potent producer mutants (M2, M4, and M26) in comparison with wild type (WT). SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Alkaline protease purification and characterization

Among several mutant strains of *B. licheniformis* isolated after UV-mutagenesis, M26, M4, and M2 strains were selected for further characterization. Proteolytic activity in culture supernatants of *B. licheniformis* and its mutants was evaluated during cultivation period (96 h). Enzyme production during incubation period by WT and mutant strains grown in casein medium showed a linear increase on protease production and biomass yield, reaching optimum values at 3 days for the WT, whereas the maximum enzyme activity was noted after 2 days for the mutant strains (data not shown). However, on additional increase of incubation time, a detectable decrease in both enzyme productivity and biomass could be observed, and this might relate to nutrient limitation or accumulation of inhibiting substances [60]. The highest enzyme activities were obtained with M26 and M4 mutants, which produced 1.5-fold and 1.2-fold more protease productivity, respectively, as compared with WT strain. Nevertheless, the mutant strain M2 showed a notable increase in the growth rate with 1.4-fold decreases in enzyme yield, and this is owing to the high protein level produced by this mutant.

B. licheniformis protease was then subjected to precipitation by ammonium sulfate (partial purification) from crude culture supernatant as described in 'Materials and methods.' As can be seen in Table 4, ammonium sulfate salting out followed by dialysis led to a 1.87-fold and 2.24-fold increase of specific activity for M26 and M2 mutant strains, respectively, with 40% ammonium sulfate saturation. Meanwhile, the maximum fractions for the WT and M4 mutant strain were obtained at 60% ammonium sulfate saturation with 1.98-fold and 2.66-fold increase of specific activity.

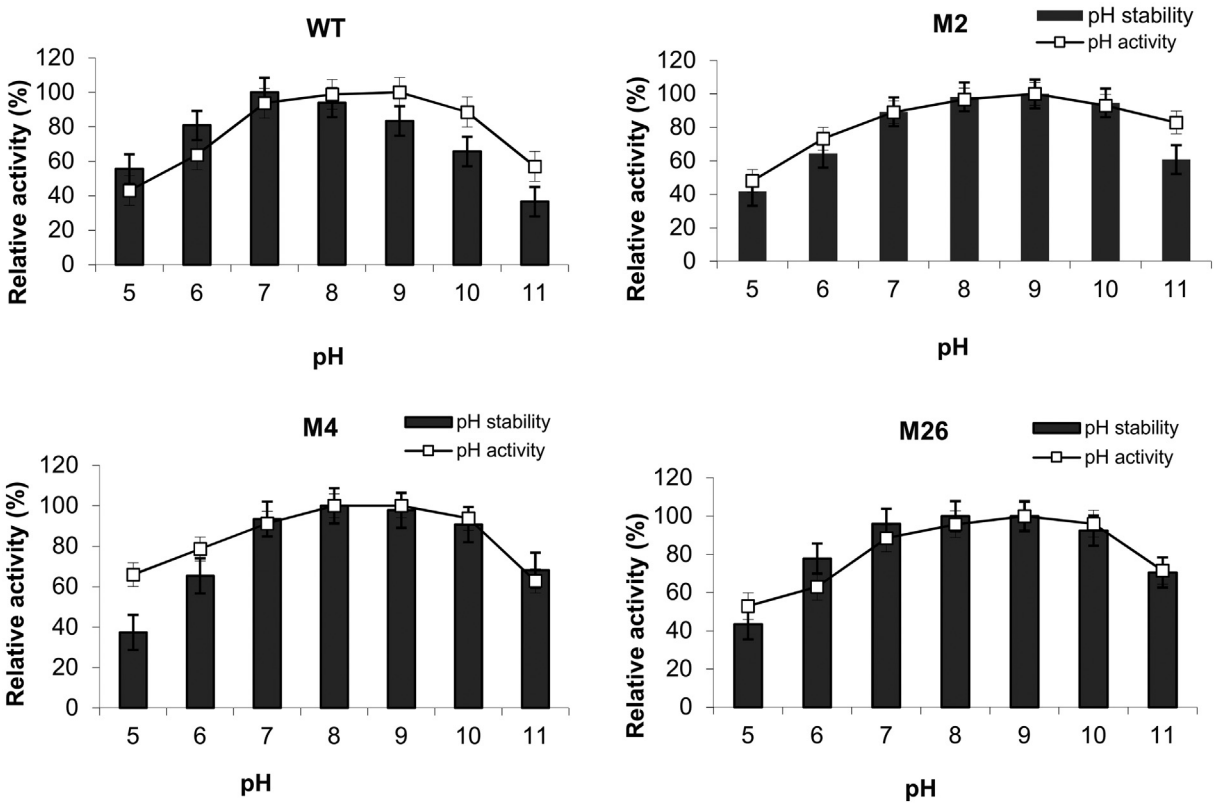
Further studies were implemented to characterize the WT and mutants of *B. licheniformis* extracellular protease. The effect of pH on the catalytic enzyme activity was tested in pH range 5–11 using different buffer systems. As shown in Fig. 7, protease has been found to be active at broad pH intervals (7.0–10.0) with respect to the maximal pH activity at eight and nine for WT and mutant strains, respectively. On the contrary, the pH stability of the enzyme was showed maximally

Table 4 Partial purification of alkaline proteases via salting out with ammonium sulfate

Methods	WT		M2		M4		M26	
	SA (U/mg)	PF	SA (U/mg)	PF	SA (U/mg)	PF	SA (U/mg)	PF
Culture filtrate	33.3	–	22.4	–	40.9	–	51.8	–
0–20	56.7	1.70	33.1	1.47	83.7	2.04	68.3	1.31
20–40	64.7	1.94	50.3	2.24	96.1	2.34	97.3	1.87
40–60	66	1.98	44	1.96	108.8	2.66	64.7	1.24
60–80	20.7	0.62	11.8	0.52	32.1	0.78	42.3	0.81

Partial purification of alkaline protease via salting out with ammonium sulfate. PF, purification fold; SA, specific activity; WT, wild type.

Figure 7

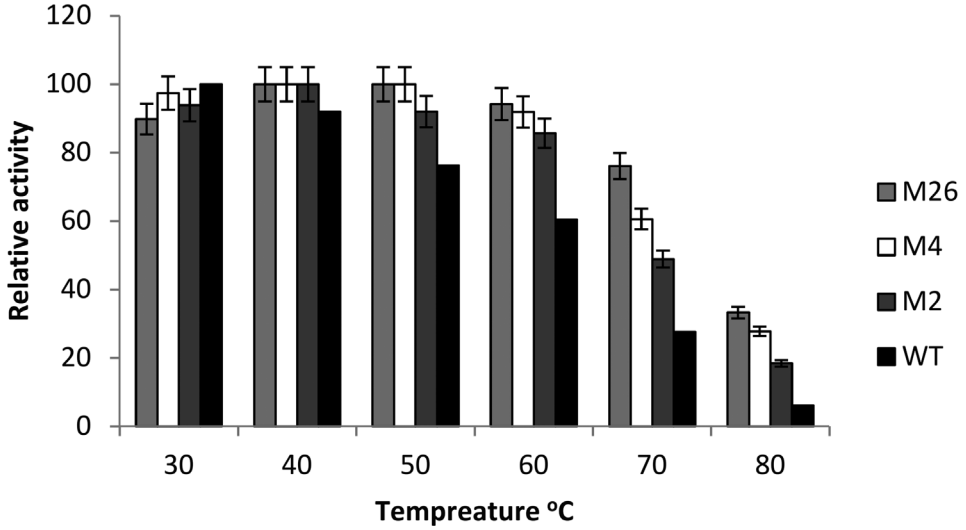


Effect of pH on alkaline protease activity and stability.

at pHs ranging from 7.0 to 8.5 of the WT, whereas enzyme derived from mutant strains exhibited its significant catalytic stability in the broad range of pH 7.0–10.0. A slight decrease in the enzyme activity was observed at pH 11, whereas at pH 5, the enzyme retained approximately 64.5, 65.4, and 77.8% of their initial activity of each of M2, M4, and M26, respectively, reflected the significant inhibitory influence on protease activity at acidic pH conditions (5 : 6).

These results revealed that the enzyme was alkaline protease, particularly for the mutant strains. Our results are consistence with several earlier reports showing pH optima being close to pH 9.0 [61–63]. However, this variety of optimum catalysis is relatively wide as compared with most previous reports [61,63]. Generally, commercial proteases from *Bacillus* sp. have maximum activities within the alkaline pH range of 8.0–12.0 [64,65]. In other words, the enzyme activity of mutant strains was very stable when the pH was between 7.0 and 11.0. However, the enzyme activity of the WT decreased rapidly when incubated in pH 11.0 (57%). The proteases from mutant strains have become stable between pH 8.0 and 11.0., with maximal stability at the pH range of 8.0–10. The induction of protease from the mutant strains of *B. licheniformis* was found to be varying from that relating to other *Bacillus* sp., which exhibits a strong loss of alkaline protease stability at pH 10.0 and 11.0, respectively [61,62]. In accordance with our results, the alkaline proteases from *Bacillus* sp. have also been considered to be stable with the same pH range [62]. These results indicate the enzyme derived from

Figure 8



Effect of temperature on alkaline protease activity.

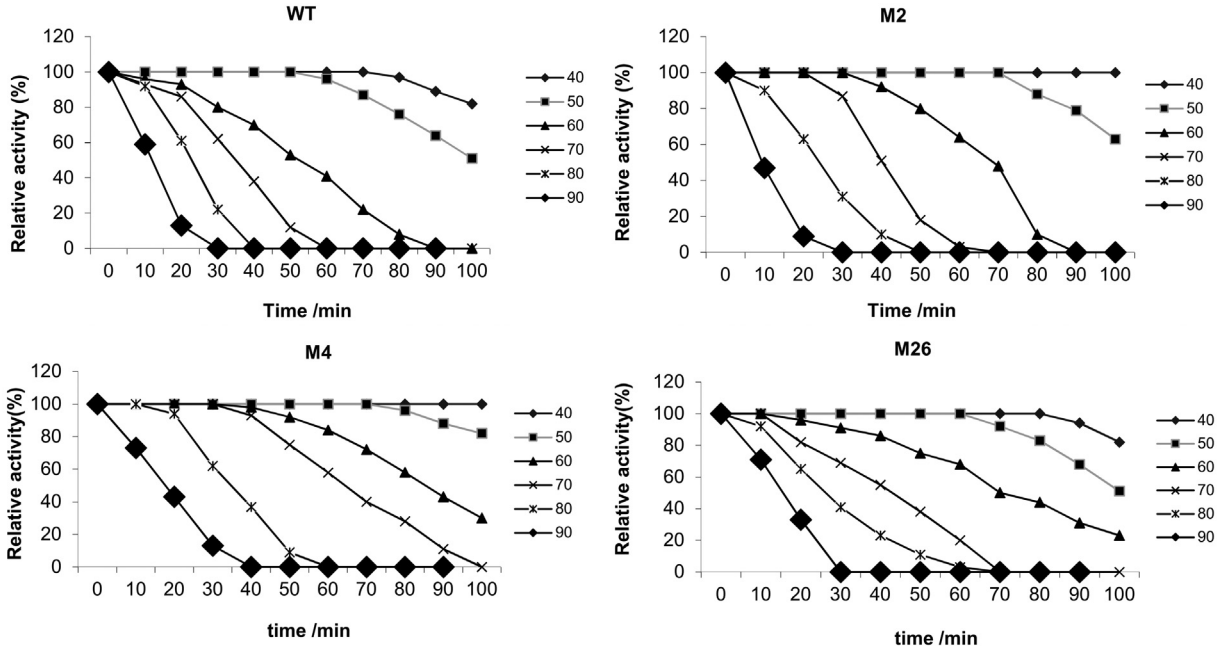
the mutant strains is more desirable for potential industrial applications, such as detergent industries, which needs alkaline pH stability [63]. The high enzyme activity in alkaline conditions can be a significant factor required in almost all detergent enzymes, where the pH range of detergents is between 9.0 and 10.5. It evidenced that the enzyme produced by mutant strains could presumably be beneficial in the detergent industries. Meanwhile, the enzyme is more effective at alkaline conditions in comparison with the detergent enzymes mainly used in the industry, like those produced by *B. licheniformis* (Alcalase) and by *B*acillus *clausii* (Savinase), which has a maximal catalytic activity at pH 8.5 [63].

The thermo-dependence curve of protease activity indicated that the enzyme exhibited activities between 30 and 40°C, with its highest activity at 30°C for the WT (Fig. 8). In contrast, the optimal assay temperature for the mutant strains was noticed in the range between 40 and 50°C, with its maximum activity at 40°C for M2 and M4 strains and 50°C for M26 mutants. The enzyme activity lost about 62, 21, 18, and 36% of its initial activity at a reaction temperature of 60°C for WT, M26, M4, and M2 strains, respectively.

The thermostability investigations in the partially purified protease revealed that the protease from mutant strains was definitely stable within temperatures of 50° C after 1-h incubation. The enzyme remained fully active after 100-min incubation at 40°C. Even at 60°C, the remainder protease activity was 40, 68, 84, and 64%, for WT, M26, M4, and M2, respectively, where the enzyme lost more than 60% of its initial activity for the WT (Fig. 9). These results confirm that the enzyme could be utilized under moderate thermal conditions. Furthermore, after heating the partially purified enzyme at 70°C for 1 h, the enzyme still retained approximately 57.9% of its original activity. Close to these results, the optimal temperature for the protease activity was achieved at 60°C, which had been observed in *Bacillus* cereus by Ghorbel *et al.* [66], *Bacillus pumilus* by Kumar [47], and *Bacillus mojavensis* by Beg and Gupta [67]. Our results were better than that reported from *B. licheniformis* MP1 [68], whereas the enzyme activity was retained fully even after incubation for 2 h at 50°C, in contrast remained only 41 and 5% after being incubated for 1 h at 60°C and 20 min at 70°C, respectively. This thermal stability would have been a preference of this enzyme application in the industry sectors including laundry detergent formulations.

Close to our results, proteases from *Geobacillus* sp. PA-9, *B. mojavensis*, and *Bacillus* sp. SSR1 have been shown to display an optimum temperature of 65, 60, and 50°C, respectively [65,69]. As reported previously, the higher optimum temperature of 60°C has been reported for alkaline proteases belonging to *Bacillus stearothermophilus* F1 and *Bacillus* sp. JB-99 [63]. The alkaline proteases produced by the GUS1 strain are highly stable at 70°C. After 60 min of incubation at 70°C, the enzymes retained 100% of their original activities [65]. Khajeh *et al.* [70] reported that, in case of thermophilic proteases, activation is obtained,

Figure 9



Thermal stability of alkaline protease.

and this might be related to the stimulation of flexibility in their relatively rigid structure.

Antimicrobial activity of *Bacillus licheniformis* MK90 extracellular protease

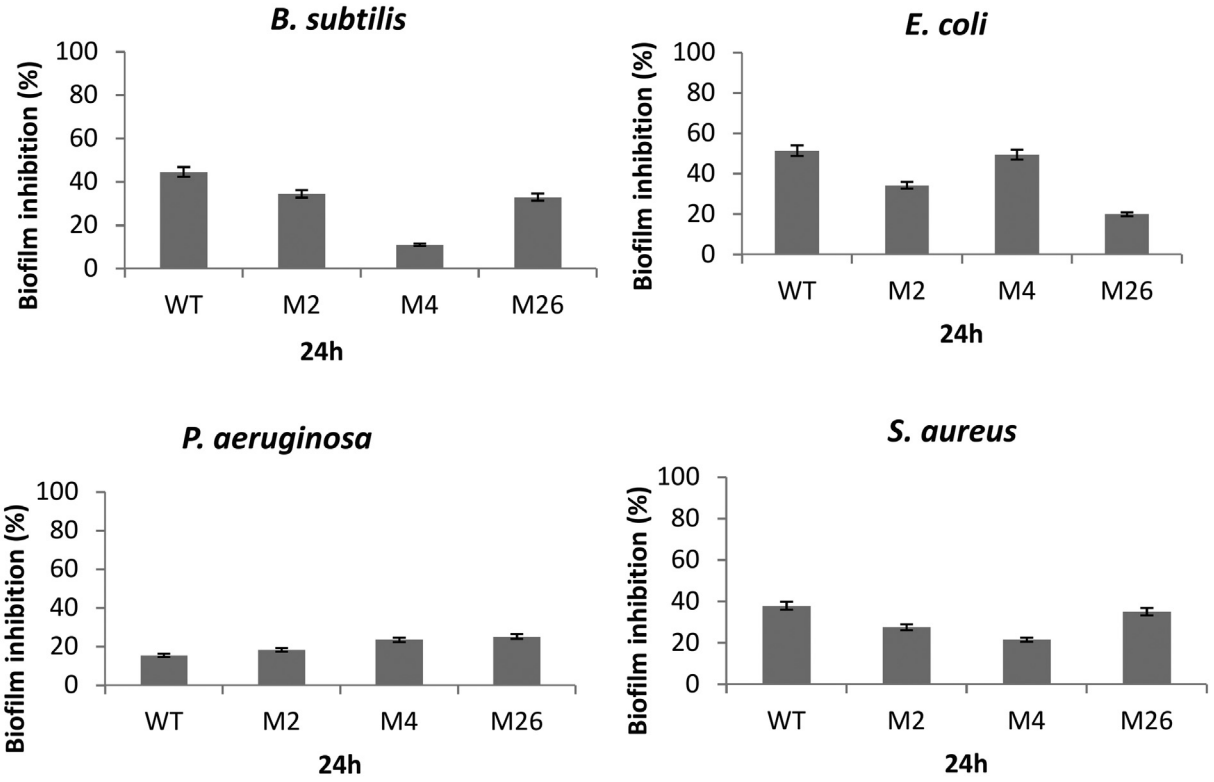
The antimicrobial activity of the extracellular proteases produced by *B. licheniformis* MK90 WT and mutants (M2, M4, and M26) was evaluated using agar well diffusion techniques against four pathogenic microbes, representing gram-negative bacteria (*S. aureus*), gram-positive bacteria (*P. aeruginosa*), yeast (*Candida albicans*), and fungi (*Aspergillus niger*). The results showed that only proteases of mutants 4 and 26 displayed a low to moderate inhibition activity against gram-positive *S. aureus* when compared with gentamycin as control. Similar results had been noted by Indarmawan *et al.* [71], when they tested the protease enzyme of *Xylaria psidii* KT30 against five pathogenic bacteria, and the protease enzyme exhibited antibacterial activity only against gram-positive bacteria.

Antibiofilm activity

Research over the past few decades has now clearly established the mechanism of bacterial biofilm formation. Studies reported that once bacterial cells attach to a surface, they secrete a complex exopolymer, consisting of proteins, polysaccharides, and nucleic acids [72–74]. This phenomenon helps bacteria to preserve their attachment to the surface and to maintain bacterial cells in close proximity to each other [75]. Recently, researchers have found that biofilm plays a very important role in the persistence of bacterial infections. They reported that bacteria become more resistant to antibiotic within a biofilm matrix compared with planktonic bacterial cells [76]. Thus far, biofilms are recognized as a remarkable source of pathogens that are involved in specific infectious diseases, such as periodontitis, osteomyelitis, dental caries, otitis media [77–79], pulmonary infections of patients with cystic fibrosis, and nosocomial infections such as urinary tract, lower respiratory tract and surgical site infections [80]. In 2012, the French association RAISIN 'Réseau d'alerte, d'investigation et de surveillance des infections nosocomiales' did a survey and reported that, most frequent bacterial pathogens associated with nosocomial infections were high biofilm producers, such as '*E. coli*, *S. aureus*, *P. aeruginosa*, and K*ebsiella* pneumonia' [81].Despite several trails to inhibit bacterial biofilm formation, no drugs are in clinical use that specifically target bacterial biofilms. Recently, new studies have reported that biofilm eradication could be achieved via enzymatic hydrolysis of matrix and adhesin proteins that play a very important role in the biofilm formation [82,83].

Recently, several reports have studied the use of proteases as antibiofilm agent by degradation of the biofilm matrix proteins that play diverse roles in biofilm backbone [84–87]. The *Staphylococcus epidermidis* serine protease has been reported to

Figure 10



Biofilm inhibition ratio of proteases produced by *Bacillus licheniformis* wild type (WT) and mutants (M2, M4, and M26) against four clinical microbes (*Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Escherichia coli*, and *Bacillus subtilis*). Biofilm was quantified using the microtiter plate test and crystal violet assay. The bars on the graph represent mean±SD as a percentage of biofilm inhibition.

inhibit the *S. aureus* biofilm formation [88]. Another example for using enzymatic eradication of biofilm formation was metalloprotease serratiopeptidase (SPEP) produced by *Serratia marcescens*, which successfully inhibited biofilm formation of both *P. aeruginosa* and *S. epidermidis* [89].

The antibiofilm activity of the extracellular proteases produced by *B. licheniformis* WT and mutants has been examined against four clinical microbes (*P. aeruginosa*, *S. aureus*, *E. coli*, and *B. subtilis*) (Fig. 10). The protease enzyme of WT reduced the biofilm formation of the strain *B. subtilis* up to 44%, followed by M2, with inhibition ratio 34%. *E. coli* biofilm inhibition was shown by proteases of WT and M4, with percentage reaching to 51 and 49%, respectively. Similar results have been shown by glycosidase pectinase and protease subtilisins A, which successfully suppressed the *E. coli* biofilm formation and enhanced its sensitivity to ampicillin [82]. On the contrary, *P. aeruginosa* biofilm displayed a very low response to proteases of *B. licheniformis* WT and mutants. However, *S. aureus* biofilm formation was inhibited over 73% by WT protease and up to 35% by M26 protease. Results showed high percentages of biofilm inhibition of protease produced *B. licheniformis* WT against most of the tested microbes. Mitrofanova *et al.* [90] reported that the proteases of *Bacillus* sp. have antibiofilm activity through the destruction of the biofilm matrix integrity.

Conclusion

The results obtained proved that UV mutagenesis is an efficient tool in improving the alkaline protease production. After the WT bacteria were subjected to UV irradiation, an increasing in the alkaline protease enzyme production from some mutants has been noted. On the contrary, sequencing and alignment of the alkaline protease enzyme genes for the WT and mutants strains showed that no change in the nucleotide sequence of the mutant strains was detected. Results also indicated that some mutants exhibited higher thermostability than the parental WT bacteria. Moreover, the biofilm inhibition activity of the alkaline protease enzyme produced by *B. licheniformis* WT and mutants has been studied, and the results showed that protease enzyme could be useful for controlling and eradication of biofilm formation process by some pathogenic bacteria.

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Conflicts of interest
There are no conflicts of interest.

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