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CELLULAR AND MOLECULAR BIOLOGY

Isolation of keratinase-producing *Bacillus* strains and enhanced enzyme production using *in vitro* mutagenesis

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Abstract: Millions of tons of feathers produced annually by the poultry industry cause environmental pollution and waste a significant source of protein. In the present study, three keratinolytic *Bacillus* strains, *Bacillus* sp. MK1, MK2, and MK3 were isolated. Some of the enzymatic properties of these keratinases were determined. The effects of some chemicals on enzyme activities were investigated. The specific activities of MK1, MK2, and MK3 were 2.76, 0.77, and 5.48 U/mg protein at 40°C, respectively, and mutant varieties were overexpressed after EtBr treatment. A comparison of keratinase activity between native and improved isolates showed that mutant variants exhibited higher activity ranging from 116 to 214%. According to BLAST analysis, the *Bacillus* sp. MK1 rDNA sequence was 96.83% similar to that of *B. subtilis* subsp. *stercoris* strain 153, *B. subtilis* strain FR10, *B. tequilensis* strain P12, and *B. subtilis* strain SRR21, and *Bacillus* sp. MK2 and MK3 16S rDNA sequences were 99.54% similar to those of *B. subtilis* strain 21M and *B. subtilis* strain NX17 sequences. The results of the enzymatic analysis of the enzymes and overexpressed mutant varieties are promising for application in the industrial production and application of the enzymes decomposition of feathers in poultry sector.

Key words: *Bacillus* sp., BLAST, characterization, keratinase, *in vitro* mutagenesis, isolation.

INTRODUCTION

Keratin is an insoluble fibrous protein and the main component in feathers (Jeong et al. 2010), hair, wool, nails, horns (Onifade et al. 1998), vertebrate skin (epidermis), hooves, and scales (Feughelman 1985). Keratins are made up of mainly two groups—α- and β-keratins (Ramani & Gupta 2004). Feathers are made of pure β-keratins (Wakil et al. 2011) that are highly insoluble and difficult to decompose (Agrahari & Neeraj 2010). Worldwide, the poultry industry produces 8–8.5 Gt of feathers annually (Manju 2012, Sah et al. 2015), which are used as landfill by either burning or burying. These processes cause problems with storage transportation, disposal of ashes, and greenhouse-gas emissions (Khodayari & Kafilzadeh 2018). Approximately 90% of the weight in feathers is made up of keratin, which does not easily decompose (Mousavi et al. 2013); therefore, it is an abundant and inexpensive source of protein.

Keratinases (EC 3.4.4.25) are proteolytic enzymes that are responsible for the hydrolysis of keratin polymers (Wakil et al. 2011). Microbial keratinases play an essential role in the hydrolysis of highly rigid, strongly cross-linked keratins (Wakil et al. 2011). Different microorganisms, including *Microsporium* sp. (Giudice et al. 2012), *Thermoanaerobacter* sp. (Kublanov et al. 2009, De Toni et al. 2002), *Bacillus* spp. (Tork et al. 2013, Mazotto et al. 2011), *B. licheniformis* (Lin et al. 1997), *B. subtilis*, *B. cereus*, *B. pumilus* (Kim et al.

2001), *Fervidobacterium* sp. (Kanoksilapatham et al. 2016), *Chryseobacterium indologenes* TKU014 (Wang et al. 2008), *Stenotrophomonas* sp. (Fang et al. 2014), *Streptomyces* sp. (Li et al. 2013), *Vibrio* sp. (Sangali & Brandelli 2000), and *Antinomadura keratinilytica* (Habbeche et al. 2014), have been reported to be keratinase producers. Keratinases are widely used in the medical, food, animal feed, and chemical industries; in basic biological sciences; and in the decomposition of poultry wastes.

Many mutation studies to improve enzyme properties such as selectivity, activity, alternate catalytic activity and thermal stability have been carried out by many research groups during past years (Otten et al. 2004). Mutations that closer to active site are more effective for many enzyme properties. Nevertheless, for a few enzyme properties, mutations far from active site are as effective as close mutations. For enantioselectivity, substrate selectivity and new catalytic activity, closer mutations improve enzymes more effectively than distant ones. However, both close and distant mutations can improve activity, thermal stability and stability against organic solvents. Enzymes contain more amino acids distant from the active site. Therefore, random mutagenesis methods produce further numbers of distant mutations than close mutations (Morley & Kazlauskas 2005).

This study was chosen to contribute to the proper utilization of the poultry feathers in the rapidly developing poultry sector. This study aimed to isolate and characterize keratinase-producing bacteria from poultry wastes, mainly feathers, and to obtain mutant varieties, using *in vitro* mutagenesis, that have increased enzyme production.

MATERIALS AND METHODS

Sample collection

Samples of soil mixed with feathers were collected from poultry farm feather dumps at the Research and Application Farm of Çukurova University, Adana, Turkey, brought the laboratory and used to isolate microorganisms.

Isolation of keratinolytic bacteria

One gram of soil sample containing feathers pieces and collected from chicken farm was added to 10 mL sterile distilled water in a 100-mL flask and shaken for 1 min. The mixture was allowed to settle at room temperature for 10 min, after which 0.5 mL soil supernatant was transferred into a sterile microcentrifuge tube and incubated at 80°C for 10 min to destroy any vegetative bacteria. The sample was then plated on skim-milk agar (0.8% skim milk, 1% peptone, 1% meat extract, 0.5% NaCl, 1.5% agar) (Mohamedin 1999) and incubated for 24 h at 37°C to detect proteolytic bacteria with a clear zone around colonies, which indicates protease production. Protease-positive bacteria were collected using sterile toothpicks and transferred onto feather–meal agar plates comprising 0.5 g/L NH_4Cl, 0.5 g/L NaCl, 0.3 g/L K_2HPO_4, 0.4 g/L KH_2PO_4, 0.1 g/L $MgCl_2.6H_2O$, 0.1 g/L yeast extract, 10 g/L feather meal, and 15 g/L agar at pH 7.5 (Kim et al. 2001) for secondary screening of any keratinolytic activity. Again, the keratinolytic bacteria were confirmed by the formation of a clear zone around the colonies. Glycerol stocks (20% v/v) of selected isolates were prepared and stored at -20°C for long-term preservation.

Identification of keratin-degrading bacteria

Carbohydrate fermentation of the isolates was identified using the Analytical Profile Index to identify enteric Gram-negative rods (API 20E). In addition, 16S rDNA isolate sequencing

was conducted by the RefGen Biotechnology Company (Ankara University Technology Development Zone, Ankara, Turkey) through a service procurement. The sequences were compared by aligning them with existing 16S rDNA sequences from the National Center for Biotechnology Information (NCBI) GenBank database, and BLASTN software (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to identify the bacteria.

Keratinase measurements

Keratinase was measured using the modified method of Akan (2010). One unit (U, µmol/min) of enzyme activity was defined as the amount of the enzyme that catalyzes the conversion of 1 µmol of substrate per minute under defined the specified conditions of the assay method in absorbance at 595 nm (A 595). Results are shown as a percentage of relative activity comparing to that of control (native isolates, 100%) for Figure 5, comparing to that of maximum activity (maximum activity accepted as 100%) for Figure 6, and comparing to that of control (no additive) for Table I.

Precipitation of extracellular enzymes

Bacterial isolates were cultivated in feather–meal broth at 37°C for 72 h, after which they were removed by centrifugation at 2380x g for 10 min. The cell-free supernatants were filtered through the Whatman-5 filter paper. A 70% volume of the cold ethanol (96% v/v) was added to the samples and incubated at -20°C for 24 h, after which they were centrifuged at 8000x g for 20 min and stored at 4°C to determine the keratinolytic activity (Akan 2010).

Effect of different temperatures on keratinase activity

The effect of different temperatures on keratinase activity was determined by observing the enzyme reactions at different incubation temperatures. One milliliter of enzyme precipitation from each isolate + 1 mL glycine–NaOH buffer + 1 mL keratin azure (Sigma) suspension (4 mg/mL in 10 mM Tris HCl, pH 7.5)

Table I. Effect of some chemicals on the activity of *Bacillus* sp. MK1, MK2, and MK3 keratinases.

Chemicals	Relative activity* (%)					
	MK1		MK2		MK3	
	1 mM	*5 mM*	*1 mM*	*5 mM*	*1 mM*	*5 mM*
Control	100	100	100	100	100	100
PMSF	114	119	127	130	143	157
EDTA	84	59	72	54	56	38
Urea	121	124	109	116	118	128
SDS	87	71	91	86	107	117
$MgCl_2$	102	91	118	146	74	64
$CaCl_2$	130	137	116	134	127	153
$ZnCl_2$	71	48	112	119	36	8
KCl	100	104	107	102	103	94
Tween 80	113	104	101	106	104	109
Triton X-100	103	105	92	83	114	119

***Each value is the mean of three independent determinations.**

was added to sterile test tubes and incubated at temperatures ranging from 30 to 100°C for 1 h. Keratin azure and glycine–NaOH buffer (1:2 v/v) was prepared as the blank. After incubation, all samples were centrifuged at 4000x g for 15 min at 4°C to remove any substrates. Absorbance was measured at 595 nm against the blank. The temperature experiments were repeated three times and the average values were calculated ((RV1+RV2+RV3)/3; RV: Repeated Value).

Effect of pH on keratinase activity

The following buffers were used in the reactions: citrate (pH 4.0–6.0), sodium phosphate (pH 6.0–8.0), glycine–NaOH (pH 8.0–10.0), and borax–NaOH (pH 10.0–13.0). One mL enzyme precipitation of each isolate + 1 mL buffer + 1 mL keratin azure suspension (4 mg/mL) was added to sterile test tubes and incubated at 37°C for 1 h. Keratin azure and buffer (1:2 v/v) used as the blank. After incubation, all samples were centrifuged at 4000x g for 15 min at 4°C to remove any substrates. Absorbance was measured at 595 nm against the blank. The temperature experiments were conducted in triplicate, and the average values calculated as mentioned above.

Temperature and pH stability

The temperature stability of the enzymes was determined by pre-incubating the enzyme precipitates at temperatures ranging from 30 to 100°C for 30 min. For pH stability, the enzyme precipitates were pre-incubated in buffers at pH values from 7.0 to 13.0 at room temperature for 30 min. The temperature and pH stability experiments were conducted as described above.

Effect of chemicals on keratinase activity

The effect of various chemicals on enzyme activity was determined by pre-incubating the enzyme precipitates with different chemicals in both 1 mM and 5 mM concentrations for 30 min at room temperature. The following chemicals were used: phenylmethylsulfonyl fluoride (PMSF), ethylenediaminetetraacetic acid (EDTA), urea, sodium dodecyl sulfate (SDS), $MgCl_2$, $CaCl_2$, $ZnCl_2$, KCl, Tween 80, and Triton X-100. The residual activities were determined at 40°C and pH 9.0 for MK1 and MK3 keratinases and 8.0 for MK2 keratinase, as described above. The control without chemicals was considered 100% and the relative activities were calculated for the control.

Ethidium bromide mutagenesis

Keratinolytic isolates were grown overnight on Luria-Bertani-broth (LB) medium (1% tryptone, 0.5% yeast extract, 1% NaCl, pH 7.5). Bacterial cells were pelleted by centrifugation at 1900x g for 10 min. Pellets were washed three times with 5 mL LB medium and dissolved in 5 mL LB medium after a final centrifugation, after which 100 µL bacterial suspension was spread onto an LB agar (1.5% agar) plate, and 10 µL ethidium bromide (EtBr, 10 mg/mL) was dropped onto the plates and allowed to dry for 15 min. After overnight incubation at 37°C, single colonies from the edge of the EtBr zone were picked up using sterile toothpicks and transferred onto the feather–meal agar plate. After incubating for 24 h, nine overexpressing mutant colonies were selected for further study according to the diameter of their activity zone.

RESULTS

Three keratinolytic bacteria were isolated and identified as *Bacillus* because they germinated from spores under aerobic conditions (Remize 2017) and from the results of the API 20E test. The isolates were surrounded by clear zones, which indicated keratinase activity on a feather–meal

agar plates after incubating for 24 h at 37°C (Figure 1). Based on BLAST analysis of 16S rDNA sequences (Figure 2), *Bacillus* sp. MK1 showed the 16S rDNA gene sequence closest to those of *B. subtilis* subsp. *stercoris* strain 153, *B. subtilis* strain FR10, *B. tequilensis* strain P12, and *B. subtilis* strain SRR21 (96.83%) and both *Bacillus* sp. MK2 and MK3 showed sequences similar to those of *B. subtilis* strain 21M and *B. subtilis* strain NX17 (99.54%) (Figure 3). The results of comparing the nucleic acid sequences of the three 16S rDNA with each other suggested that the MK2 16S rDNA sequence showed a sequence highly similar (99.77%) to that of MK3 16S rDNA. On the other hand, the MK1 16S rDNA showed a sequence that was 96.72% similar to that of MK2 16S rDNA and 96.39% similar to that of MK3 16S rDNA.

Mutagenesis

After mutagenesis, approximately 100 mutant colonies of each isolate were collected from the EtBr dropped agar plates (Figure 4) and compared to the wild-type strains. Among these mutant bacteria, nine overproducer mutant variants were selected according to activity-zone diameters on the skim-milk agar plates. A comparison of relative activity between native and improved isolates showed that mutant variants exhibited higher activity ranging from 116 to 214% (Figure 5).

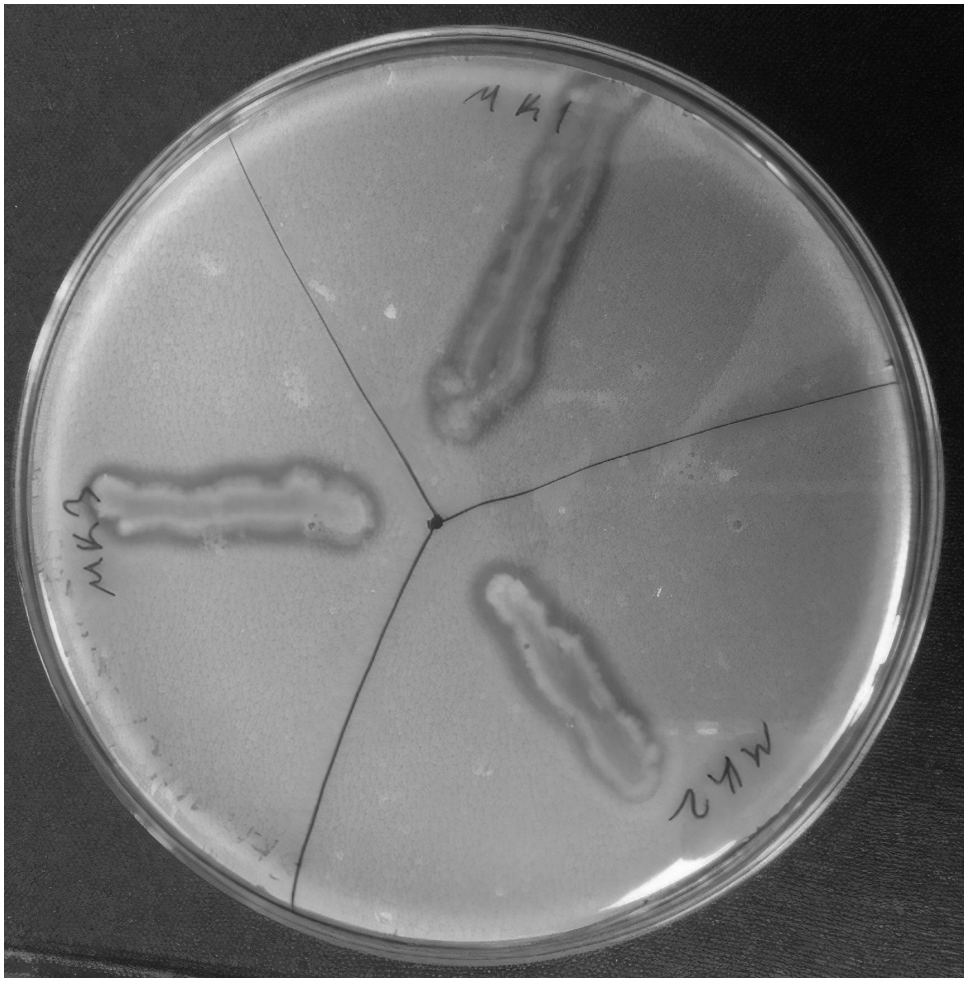

Figure 1. Production of clear zones in feather–meal agar plates using keratinolytic isolates.

a

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  1 ATGGGCGGCC CCGTCAATCC TTGAGTTCAG TCTTGCGGAC
 41 CGTACTCCCC AGGCGGAGTG CTAATGCGTA GCTGCAGCAT
 81 AAGGGCGGAA ACCCCTACAC TAGCCATCAT CGTTTACGGC
121 GGTGGACTAC AGGGTATCTA ATCCTGTCGC CTCCCCACGC
161 TTCGCTCCTC AGCGTCAGTT ACAGACCAGA GGAGTCGCAT
201 CGCACTGGTG TCCTCCACAT CTCTACGCAT TTCACCGCTA
241 CACGTGGAAT CACTCTCCTC CTCTGCACTC AAGTTCCCCA
281 GTTCCAATGA CCCTCCCCGG GTGAGCCGGG GGCTTTCACA
321 TCAGACTAAG AAACCGCCTG CGAGCCCTTT ACGCCCAATA
361 ATTCCGGACA ACGCTTGCCA CCTACGTATT ACCGCGGCTG
401 CTGGCACGTA GTTAGCCGTG GCTTTCTGGT TAGGTACCGT
441 CAAGGTACCG CCCTATTCGA ACGGTACTTG TTCTTCCCTA
481 ACAACAGAGC TTTACGATCC GAAAACCTTC ATCACTCACG
521 CGGCGTTGCT CCGTCAGACT TTCGTCCATT GCGGAAGATT
561 CCCTACTGCT GCCTCCCGTA GGAGTCTGGG CCGTGTCTCA
601 GTCCCAGTGT GGCCGATCAC CCTCTCAGGT CGGCTACGCA
641 TCGTTGCCTT GGTGAGCCGT TACCTCACCA ACTAGCTAAT
681 GCGCCGCGGG TCCATCTGTA AGTGGTAGCC GAAGCCACCT
721 TTTATGTTTG AACCATGCGG TTCAAACAAC CATCCGGTAT
761 TAGCCCCGGT TTCCCGGAGT TATCCCAGTC TTACAGGCAG
801 GTTACCCACG TGTTACTCAC CCGTCCGCCG CTAACATCAG
841 GGAGCAAGCT CCCATCTGTC CGCTCGACAT GCAACACTAG
881 GCGGTACCTG TTACTGTAC
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b

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  1 GGGACGTAAG GTAGCGGCTA GTGTTTGCAT GTCGAGCGGA
 41 CAGATGGGAG CTTGCTCCCA TGATGTTAGC GGCGGACGGG
 81 TGAGTAACAC GTGGGTAACC TGCCTGTAAG ACTGGGATAA
121 CTCCGGGAAA CCGGGGCTAA TACCGGATGG TTGTTTGAAC
161 CGCATGGTTC AAACATAAAA GGTGGCTTCG GCTACCACTT
201 ACAGATGGAC CCGCGGCGCA TTAGCTAGTT GGTGAGGTAA
241 CGGCTCACCA AGGCAACGAT GCGTAGCCGA CCTGAGAGGG
281 TGATCGGCCA CACTGGGACT GAGACACGGC CCAGACTCCT
321 ACGGGAGGCA GCAGTAGGGA ATCTTCCGCA ATGGACGAAA
361 GTCTGACGGA GCAACGCCGC GTGAGTGATG AAGGTTTTCG
401 GATCGTAAAG CTCTGTTGTT AGGGAAGAAC AAGTACCGTT
441 CGAATAGGGC GGTACCTTGA CGGTACCTAA CCAGAAAGCC
481 ACGGCTAACT ACGTGCCAGC AGCCGCGGTA ATACGTAGGT
521 GGCAAGCGTT GTCCGGAATT ATTGGGCGTA AAGGGCTCGC
561 AGGCGGTTTC TTAAGTCTGA TGTGAAAGCC CCCGGCTCAA
601 CCGGGGAGGG TCATTGGAAA CTGGGGAACT TGAGTGCAGA
641 AGAGGAGAGT GGAATTCCAC GTGTAGCGGT GAAATGCGTA
681 GAGATGTGGA GGAACACCAG TGGCGAAGGC GACTCTCTGG
721 TCTGTAACTG ACGCTGAGGA GCGAAAGCGT GGGGAGCGAA
761 CAGGATTAGA TACCCTGGTA GTCCACGCCG TAAACGATGA
801 GTGCTAAGTG TTAGGGGTTT CCGCCCCTTA GTGCTGCAGC
841 TAACGCATTA AGCACTCCGC CTGGGGGAGT ACGGTCCGCA
881 AGACTGAAAC CCAA
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c

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  1 GGACCGAAAA GGTAACCGCC TAAATGTTGC ATGTCGAGCG
 41 GACAGATGGG AGCTTGCTCC CATGATGTTA GCGGCGGACG
 81 GGTGAGTAAC ACGTGGGTAA CCTGCCTGTA AGACTGGGAT
121 AACTCCGGGA AACCGGGGCT AATACCGGAT GGTTGTTTGA
161 ACCGCATGGT TCAAACATAA AAGGTGGCTT CGGCTACCAC
201 TTACAGATGG ACCCGCGGCG CATTAGCTAG TTGGTGAGGT
241 AACGGCTCAC CAAGGCAACG ATGCGTAGCC GACCTGAGAG
281 GGTGATCGGC CACACTGGGA CTGAGACACG GCCCAGACTC
321 CTACGGGAGG CAGCAGTAGG GAATCTTCCG CAATGGACGA
361 AAGTCTGACG GAGCAACGCC GCGTGAGTGA TGAAGGTTTT
401 CGGATCGTAA AGCTCTGTTG TTAGGGAAGA ACAAGTACCG
441 TTCGAATAGG GCGGTACCTT GACGGTACCT AACCAGAAAG
481 CCACGGCTAA CTACGTGCCA GCAGCCGCGG TAATACGTAG
521 GTGGCAAGCG TTGTCCGGAA TTATTGGGCG TAAGGGCTCG
561 CAGGCGGTTT CTTAAGTCTG ATGTGAAAGC CCCCGGCTCA
601 ACCGGGGAGG GTCATTGGAA ACTGGGGAAC TTGAGTGCAG
641 AAGAGGAGAG TGGAATTCCA CGTGTAGCGG TGAAATGCGT
681 AGAGATGTGG AGGAACACCA GTGGCGAAGG CGACTCTCTG
721 GTCTGTAACT GACGCTGAGG AGCGAAAGCG TGGGGAGCGA
761 ACAGGATTAG ATACCCTGGT AGTCCACGCC GTAAACGATG
801 AGTGCTAAGT GTTAGGGGTT TCCGCCCCTT AGTGCTGCAG
841 CTAACGCATT AAGCACTCCG CCTGGGGGAG TACGGTCGCA
881 AGACTGAAAC CCAATA
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Figure 2. The 16S rDNA sequences generated for isolate MK1 (a), MK2 (b) and MK3 (c).

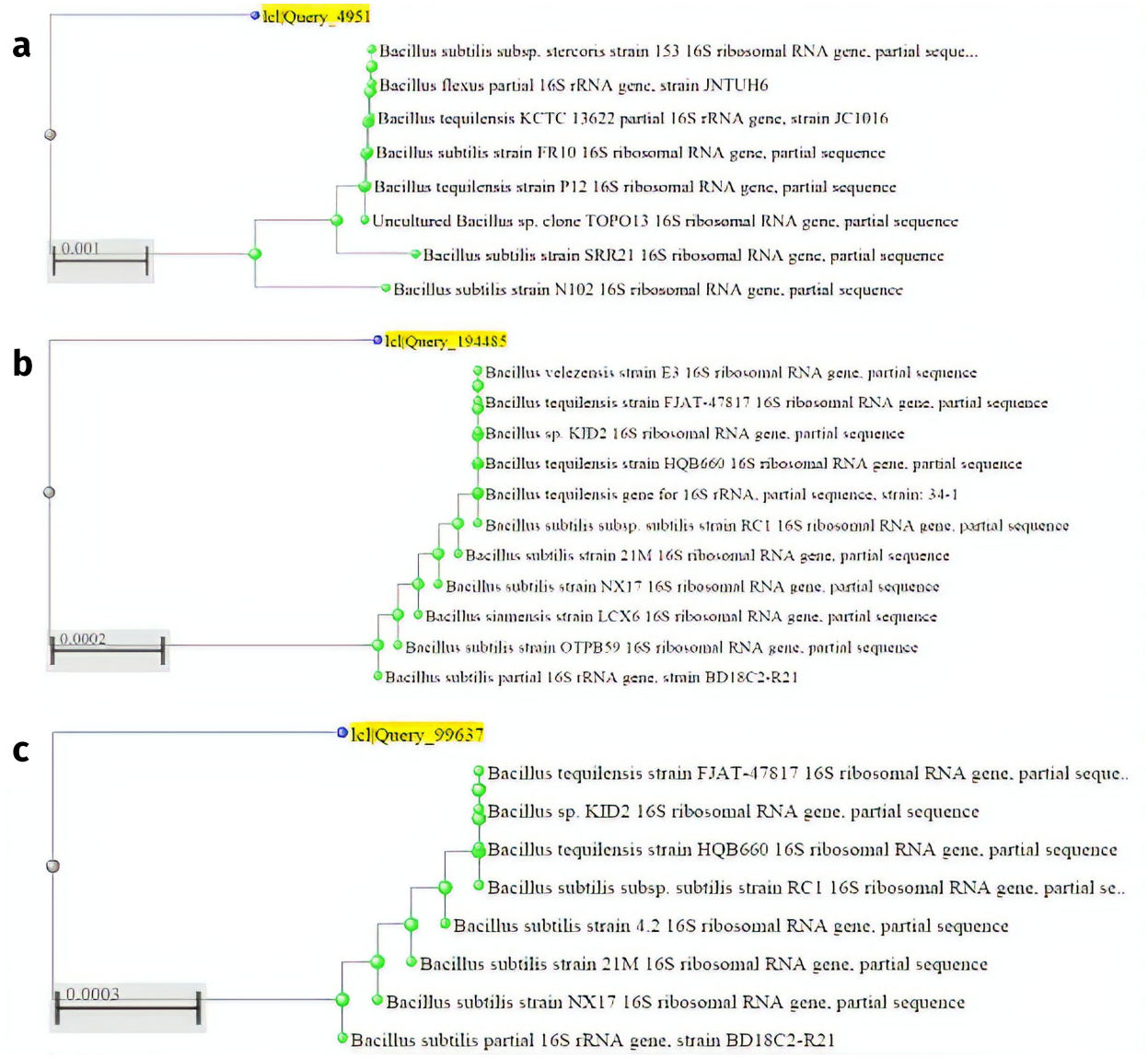

Figure 3. Phylogenetic trees for *Bacillus* sp. MK1 (a), MK2 (b), and MK3 (c) based on sequence analyses of their 16S rDNA genes.

Enzyme properties

Temperatures between 30 and 100°C were used to test keratinase activity. The maximum keratinase activity was observed at 40°C for MK1 and MK3 and 50ºC for MK2 strains (Figure 6a). The relative enzyme activities of MK1 strain were 95, 54, 36, and 27% at 50, 60, 70, and 80°C respectively, after 30 min; whereas, they were 71, 70, 23, and 9% for MK3 strain, respectively, at the same temperatures and timeframe. The relative activities were 91, 86, and 36% for 60, 70, and 80°C, respectively, for MK2 strain. All three enzymes lost most of their activity after incubation at 90 and 100°C for 30 min.

Bacillus sp. MK1 and MK3 keratinases exhibited their maximum activity at pH 9.0; whereas, MK2 exhibited it at pH 8.0 (Figure 6b). The average relative enzyme activity between pH 5.0 and 11.0 were 62, 81, and 62% for MK1, MK2, and MK3, respectively. The average relative

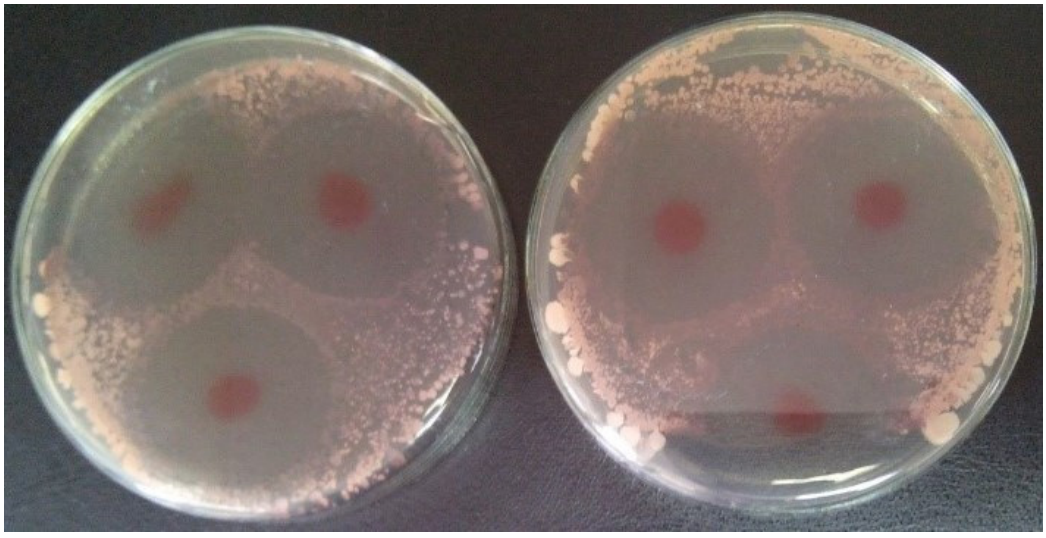

Figure 4. LB–agar plates with colony development after ethidium bromide treatment.

enzyme activity at pH 5.0–7.0 were 33, 51, and 44%, and at pH 7.0–12.0 were 60, 64, and 85% for MK1, MK2, and MK3, respectively.

The stability of keratinases at different pH levels was determined by pre-incubating the enzymes with the appropriate buffers. We found that the enzymes exhibited a significant amount of activity within the broader range of pH 8.0–11.0 (Figure 6c). The thermal stability of the enzymes was determined by measuring their residual activities after pre-incubating at various temperatures. *Bacillus* sp. MK1, MK2, and MK3 enzyme activities remained relatively stable at <60, <80, and <70°C, respectively, after 30 min but decreased rapidly beyond these temperatures (Figure 6d).

The effect of incubation time on keratinolytic activity was investigated. Maximum keratinolytic activity for all enzymes was achieved at 36 h (Figure 6e). MK1, MK2, and MK3 keratinases showed 2.76, 0.77, and 5.48 U/mg after 24 h at 40ºC, respectively. The relative specific activities are shown in Figure 6f.

The effects of different chemicals on the enzymes were studied at concentrations of 1 and 5 mM. PMSF, urea, $CaCl_2$, and Tween 80 were observed to enhance enzyme activity for all the enzymes; whereas, Triton X-100 for MK1 and MK3; $MgCl_2$, $ZnCl_2$, and KCl for MK2; and SDS for MK3. Although EDTA inhibited all three enzymes, $ZnCl_2$ inhibited only MK1 and MK3, $MgCl_2$ inhibited only MK3, and Triton X-100 inhibited only MK2 enzymes (Table I).

DISCUSSION

New strains of *Bacillus* with keratinolytic activity, as identified by their phylogenetic relationships, were isolated from soil samples. In previous reports, the spore-forming bacterial strains with keratinolytic activity, such as *B. megaterium* (Saibabu et al. 2013), *B. subtilis* (Kazi et al. 2015), and *B. licheniformis* (Vigneshwaran et al. 2010), were isolated from various ecosystems. Keratinase enzymes exhibit different characteristics in terms of optimum pH values. *Bacillus* sp. MK1, MK2, and MK3 keratinases showed alkaline properties and stability, and keratinases with high alkalinity have been previously reported (Korkmaz et al. 2004, Akan 2010); however, these keratinases were not highly alkaline. *Bacillus* species are the most popular source of commercial alkaline proteases because they can produce large amounts of these proteases

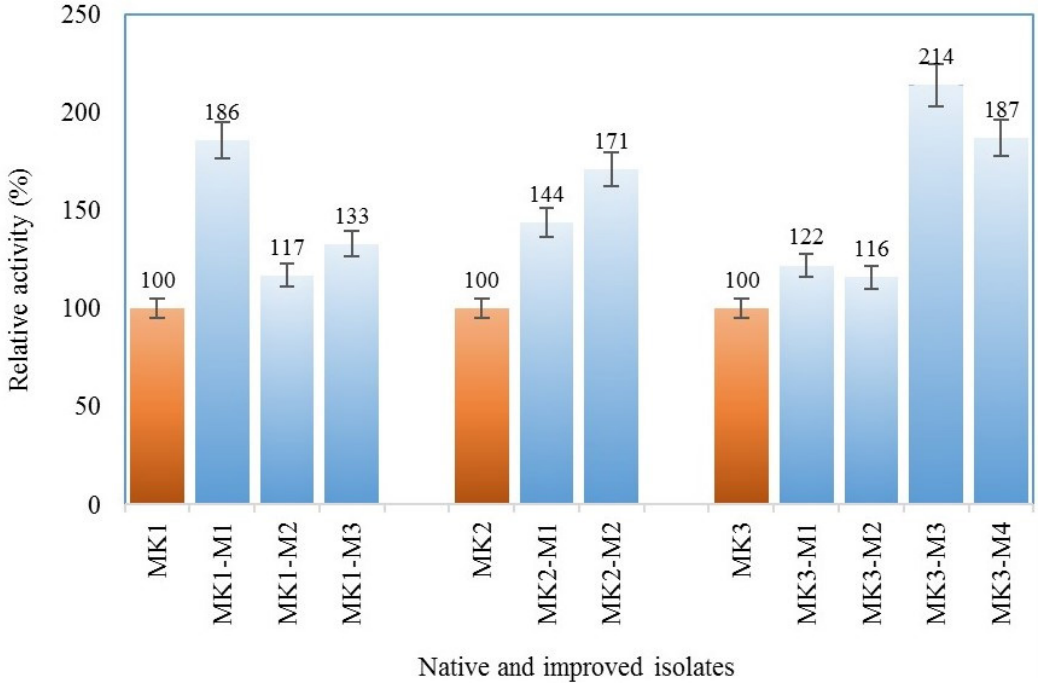

Figure 5. Comparison of relative activity between native and improved isolates.

that have significant activity and stability at high pH as well as high temperature (Fellahi et al. 2016). The majority of the identified keratinase-producing microorganisms appear to be able to hydrolyze only β-keratin in chicken feathers, and few are known to hydrolyze both α- and β-keratin (Gupta et al. 2013). To determine whether the MK1, MK2, and MK3 keratinases are enzymes included in the α- or β-keratinase group, the amino acid sequences must be detected.

The maximum keratinase activity was observed at 50°C for MK2 and 40°C for MK1 and MK3. Nevertheless, MK1 and MK2 keratinases showed 100% residual activity after pre-incubation for 30 min at 40°C; however, a slight decrease in residual activity was found after pre-incubation for 30 min at 50°C. Decreasing residual activity in MK3 keratinase was observed after pre-incubation for 30 min at 30°C. Our results are following those of Cai et al. (2008), who noted that most bacterial keratinases show optimum activity within a range of 30 to 80°C under neutral and alkali conditions between pH 7.0 and 9.5.

EDTA partially inhibited all three keratinases; however, $ZnCl_2$ inhibited MK1 and MK3 and stimulated MK2. Similarly, SDS inhibited MK1 and MK2 and stimulated MK3. In general, some heavy metal ions, such as Hg^{2+} (Thys et al. 2004), Cu^{2+} (Riffel et al. 2003), and Zn^{2+} (Thys et al. 2004) inhibit keratinase activity; however, Mg^{2+}, Ca^{2+}, and Mn^{2+} stimulate other keratinases (Nam et al. 2002). Although PMSF has been indicated to inhibit serine proteases (Shrinivas et al. 2012), it stimulated all three enzymes in this study. The active sites of metalloproteases have metal ions, such as Zn, Co, and Mg, which are responsible for the activity, and generally neutral bacterial proteases (active proteases between pH 6.0 and 9.0) are included in this group. The optimum activity of MK1, MK2, and MK3 between pH 6.0 and 9.0 and inhibition by EDTA at different rates suggest that these keratinases might be metalloproteases. $MgCl_2$ significantly stimulated MK2. The stimulation of keratinases in the presence of metal ions such as Mg^{2+} results from the formation of a salt or an ion bridge that maintains the confirmation

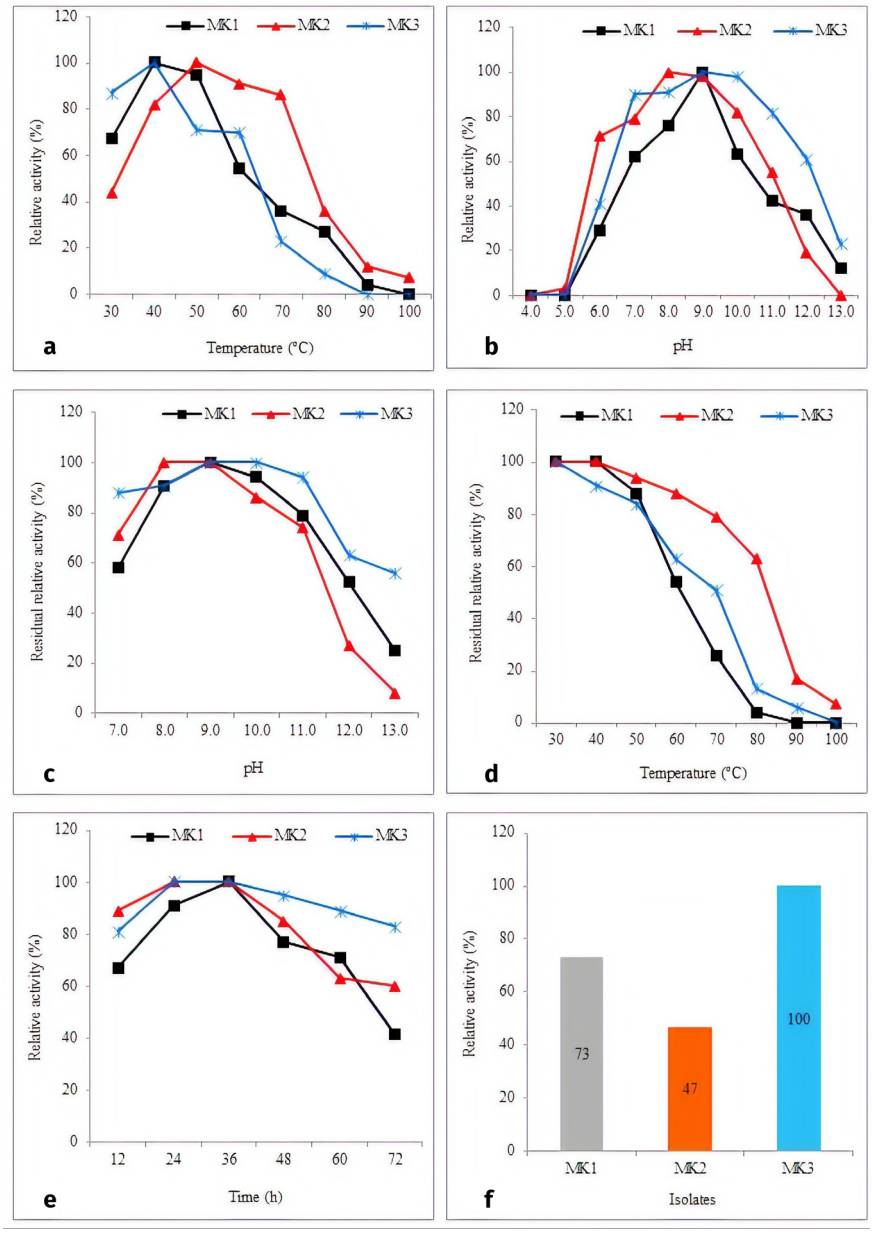

Figure 6. **Enzymatic properties of keratinases. a) Effect of temperature, b) effect of pH, c) pH stability, d) thermal stability, e) relative keratinase production at different incubation periods (12–72 h), and f relative specific activities.**

of the enzyme–substrate complex (Balaji et al. 2008). Inhibition of keratinases by metal ions has been reported to be linked to bridging between catalytic ions in the catalytic region and metal monohydroxide (Sivakumar et al. 2013). In the present study, Tween 80 slightly stimulated MK1, MK2, and MK3, and Triton X-100 slightly inhibited MK1 and MK3. It has been reported that a small number of keratinases are stimulated by the presence of detergents, such as Triton X-100, Tween 20, Tween 80, and nonionic surfactants (Purchase 2016). *Chryseobacterium gleum* (Chaudhari et al. 2013), *Actinomadura keratinilytica* Cpt29 (Habbeche et al. 2014), and *Brevibacillus* sp. AS-S10-II (Jaouadi et al. 2013) keratinases are some of these that show results similar to those with *Bacillus* sp. MK1, MK2, and MK3 keratinases.

The wild-type isolates were subjected to EtBr treatment, and overexpressing mutant

varieties were created. The keratinase activity of *Bacillus* sp. MK1, MK2, and MK3 increased to 5.62, 1.77, and 7.84 U/mg, respectively, after *in vitro* mutagenesis. Chemical and physical mutagens, such as EtBr, ethyl methanesulfonate, and ultraviolet light (UV), have been used in studies to develop overexpressing keratinase-producing mutant bacteria (Mehtani et al. 2017). The findings from our study are consistent with those of previous studies conducted by Raju & Divakar (2013), who have reported a 2- to 4-fold increase in protease production over the parent strain of *B. cereus* GD 55 strain, while Dutta & Banerjee (2006) have also observed a 2.5-fold increase in alkaline protease production by UV mutant *Pseudomonas* sp. JNGR242. Also, Azad (1994) has reported a 1000s-fold increase in enzyme activity after mutagenic treatment in *Bacillus* isolate MA6. Enhanced enzyme production after mutagenesis results from an increase in gene copy numbers and amplification of the DNA region (Cherry et al. 2009).

CONCLUSIONS

In the present study keratinolytic *Bacillus* species were isolated, and keratinases were partially characterized. Improved overexpressing mutant strains were created after *in vitro* mutagenesis by EtBr. The results of the enzymatic analysis of native and mutant enzymes are promising for application in the industrial production and application of the enzymes. In addition, the enzyme production levels and kinetic parameters of the enzymes could be improved by cloning and site-specific mutations.

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M.K. and B.D.O: performed the growth of bacterial isolates, mutagenesis studies, characterization of the enzyme; B.D.O: coordinated the study and wrote the manuscript; all authors commented on and improved the manuscript.