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High keratinase production and keratin degradation by a mutant strain KR II, derived from *Streptomyces radiopugnans* KR 12

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Abstract
This study aimed to purify and characterize a new feather-degrading enzyme. Twenty bacterial isolates were recovered from different sources on feather-meal medium. The best keratinase producer KR12, isolated from a poultry farm, was selected for the further experiments. Physiological and biochemical studies indicated that the bacterium KR12 belongs to genus *Streptomyces*. 16S rRNA analysis confirmed this result since the KR12 was similar by 98.8% to *Streptomyces radiopugnans*. The enzyme was purified usingSephadex G-75 and DEAE (diethylaminoethyl)-Sepharose chromatographic columns. The purified enzyme had a molecular weight of 32 kDa, defined by sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis (SDS-PAGE). The enzyme was active between 30 and 60°C. The enzymatic activity of the purified enzyme was enhanced by Zn^{2+}, Na^{+}, Ca^{2+}, K^{+}, and inhibited by Fe^{2+}, Cu^{2+}, and EDTA, demonstrating that keratinase from *Streptomycesradiopugnans* KR 12 belongs to metallokeratinases. Keratinase production was enhanced using UV radiation. Three recombinant mutants were obtained, mutant KR II, produced a high keratinase enzyme (3 fold) than the wild strain. *Streptomycesradiopugnans* KR12 and its mutant are promising for keratinase production and many biotechnological applications.

Keywords: Keratinase, 16S rRNA, Mutation, Streptomyces, SDS PAGE

INTRODUCTION

Keratins are the most abundant polymer found in nature. It is a filamentous structure protein stabilized by several cross-linking disulfide bonds, hydrophobic interaction and hydrogen bonds that make keratin more stable and resistant degradation by the usual proteolytic enzymes [1]. For the previous reason, feathers, as well as keratin-rich products, are slightly used as a nutritional protein supplement for feedstuffs because the process of feather meal production requires significant amounts of energy and costs a lot of money. In addition, this process can destroy some types of amino acids yielding a product with an inadequate nutritional value. Nonetheless, feathers do not accumulate in nature due to the presence of some microorganisms able to degrade this complex structural keratin in environment [2].

Keratinases (EC 3.4.99.11) have a special interest because of their effects on insoluble keratin and a variety of protein substances [3]. Keratin degrading enzymes have many significant applications in biotechnological procedures that involve keratin-rich wastes from leather and poultry manufacturing, via the development of non-polluting methods.[1]After hydrolytic degradation, feathers can be transformed to feedstuffs, glues, fertilizers and rare amino acids sources such as serine, cysteine, and proline [2].

Several bacteria, actinomycetes, and fungi are keratinase producers [4]. Keratinases have been detected in several species of fungi such as *Microsporum*[5], *Trichophyton*[6] and in bacteria including genera*Bacillus*[7, 8, 9, 10]and *Streptomyces* [11, 12].

Induced mutagenesis is a conventional and effective technique used for enhancing strains to boost the productivity of some commercially important microbial metabolites. Cosmic rays, high vacuum, intense magnetic field, and microgravity stimulated chromosomal abnormalities leading to genetic alterations in microorganisms [13]. Various genetically engineering events such as mutation, conjugation, protoplast fusion, protoplast transformation, and recombinant DNA techniques were used to improve the productivity of *Streptomyces*to antibiotics and enzymes [14].

The objective of the current work was to isolate and identify an efficient keratinolytic bacterial isolate, showing elevated feather degradation with a potential application in biotechnological processes. The purified keratinase enzyme was characterized andthe effect of some factors modulating its activity was studied. Keratinase production was over-expressed using UV radiation and promised recombinant mutant KRII was selected for more keratinase production.

MATERIAL and METHODS

Isolation and screening of feather-degrading bacterial isolates

Samples of poultry farm wastes, soil, water, fodder, and feather were collected from Jeddah, Saudi Arabia. Serial dilutions of each sample were prepared and approximately 0.1 ml of the appropriate dilution was spread on feather-meal medium composed of (g/l): delipidated feather meal as the only carbon and nitrogen source, 10.0, NaCl, 0.5 K_2HPO_4, 0.3; and KH_2PO_4, 0.4, agar, 20, (pH 7.5). Incubation was carried out at 37°C for 5-7 days and colonies were selected and conserved on nutrient agar medium at 4°C. Proteolytic

activities of the bacterial isolates were measuredafter culturing on skimmed milk agar plates. To observe the hydrolysis zone, 10% trichloroacetic acid (TCA) was put on the skimmed milk agar plate [15].The growth of bacterial isolate in broth medium was estimated by evaluating the number of colonies forming units (CFU/ml) on nutrient agar plates. Pure cultures were stored in 50% glycerol (v/v) at −80ºC [16].

Taxonomical studies

Morphological, physiological and biochemical characterization

International *Streptomyces* Project (ISP) media were performed as reported inShirling and Gottlieb [17]. Morphological and physiological characters were defined following the method reported by Williams et al. [18]. Morphology of spores and mycelia was shown by both light microscope and transmission electron microscope (XL30-ETEM). Physiological examinations were performed at 30°C [19]. Different carbon and nitrogen sources were also used to test the ability of the microbial isolate to grow on different sources of carbon and nitrogen. The isomer of diaminopimelic acid (DAP) and the entire composition of cell sugar were analyzed according to the techniquedefined by Hasegawa et al.[20]. Fatty acid methyl esters were detected [21]by gas chromatography.The phospholipid kinds were estimated by two-dimensional thin-layer chromatography [22].

Molecular characterization

KR12 genomic DNA was obtained byQIAamp DNA Mini Kit. About 3 µl of genomic DNA was amplified in a 100 µl reaction by using the hot star master mix from QIAGEN (2X). The design of the primers was relayed on the extremely -conserved region of 16S rRNA from a great variety of bacterialstrains [23, 24]. PCR technique was used to amplify 16S rRNA gene using forward primer 5′- AGTTTGATCATGGTCAG-3′ and reverse primer 5′-GGTTACCTTGTTACGACT 3’. The PCR was achieved by using DNA thermal cycler (Perkin Elmer, USA). Reaction conditions included one cycle with five minutes of DNA denaturation at 95°C, followed by 35 thermal cycles of DNA denaturation at 95°C for 2 min., 48°C (1 min), and 72°C (4 min), and one other cycle with a final 20-min chain elongation at 72°C. The amplified PCR product of 1.500bp was purified and sequenced using big dye terminator cycle sequence kit. The obtained sequence was analyzed using DNA sequencer ABI PRISM 310 genetic analyzer (Perkin Elmer, USA). Sequence data were submitted to GenBank database. The DNA sequence was compared to the GenBank database in the NationalCenter for Biotechnology Information (NCBI) using the BLAST program.

Keratinase assay

Activity of keratinase was estimated using the method established by Letourneau *et al*.[25].A mixture of 0.2 ml of enzyme sample and 0.8 ml of 0.4 % (w/v) of keratin azure (Sigma Aldrich, Germany) in 50 mM phosphate buffer was incubated under the following conditions: 60 °C with 150 revs/ min. at pH 8.5 for 1 h. One unit of enzyme activity was expressed as the quantity of enzyme leading to an increase in absorbance at 595 nm (A595) of 0.1 after interaction with the keratin azure for 1 h. [26].

Enzyme characterization

Bacterial isolate KR12 was grown in feather meal broth for 4 days at 37°C and undegraded solid materials were removed from culture medium by filtration through glass wool. Bacterial cells were taken away by centrifugation at 5000 rpm for 10 minutes. Keratinase enzyme was precipitated from the culture filtrate by 80% NH_4SO_4 at 4°C for 24 h. The precipitated protein was gathered by centrifugation at 5000 rpm for 40 min and dissolved in a minimum quantity of 25 mM potassium phosphate buffer, pH 5.8. Desalting was carried out by dialysis with a cellulose dialysis bag (3500 Da pore-size) in 25mM potassium phosphate buffer at pH 5.8. This step followed by dissolving the enzyme in 2 ml distilled water and applying on carboxymethyl cellulose. The active fractions were gathered and lyophilized. Sephadex G75column chromatography was used for purification of the enzyme and gave final product with an improved purity. purification step removed proteins that may reduce keratinase activity. The active fractions were used for the detection of the molecular mass and enzyme characters. Protein content (mg/ml) was assayed using Bradford test [27].

Molecular weight determination

Keratinase molecular weight was determined by the method described by Laemmli [28]. The gel composed of 12.5% separating and 4% stacking gel was stained in 0.15% w/v Coomassie brilliant blue R-250 in methanol-acetic acid-water (40: 10: 50, by volume) for 4 h. Destaining was carried out in the same solvent without the dye. The molecular weight was estimated with reference to low molecular weight protein standard (Amersham Pharmacia) ranged from 14-96 kDa.

Effect of pH and temperature on enzyme activity and stability

Enzyme activity was evaluated at different pH by using 50 mM Tris-HCl (pH 4 –8), 50 mM potassium phosphate (pH 8 –10). Likewise, the influence of temperature was determined at a temperature ranged from 25 to 80°C. All tests were performed by incubating the solution of the purified enzyme at different temperature levels and in different pH for 30 min., and the enzyme activity was evaluated [29].The effect of metal ions and EDTA (serine protease inhibitor) on enzyme activity was evaluated by pre-incubating the enzyme for 30 min at 45ºC with some metal ions and EDTA with 1 mM (inhibitor) individually at the final concentration indicated prior to substrate addition, the relative enzyme activity was calculated.

Substrate specificity

Powder milk, wool, keratin, collagen and bovine serum albumin (BSA) were used as a substrate. The tested substrate at concentration 0.5% in 20 mM phosphate buffer (pH 7.5) was mixed with 10 µg of the purified keratinase, the mixture was incubated at 40ºC for 60 min. The reaction was stopped by the addition of an equal volume of 15% trichloroacetic acid (TCA). Then,centrifugation was performed at 10000g for 10 min. at 4ºC. Five- hundred microliter from the reaction mixture was added to 1.0 ml of Cd-ninhydrin reagent and incubated for 5 min. at 84ºC. After cooling,absorbance was estimated at 507 nm [30]. One unit of enzyme activity was measured as the quantity of enzyme that could causethe release equivalent to 1 µmol of tyrosine after 60 min of reaction [31].

UV-induced mutagenesis

For UV-induced mutagenesis, the wild-type bacterium was cultured on starch nitrate broth at 37°C for 48 h. Spore suspension (4 ml) was put into sterile Petri dishes and exposed to UV light (UV-dispensing cabinet fitted with 15 W lamps with about 90% of its radiation at 265 nm). The plates were put at 30 cm away from the center of UV light source and exposed to UV light for 20 min. Then, the UV-exposed plates were incubated overnight in the dark to avoid photoreactivation. 4 ml of spore suspension was spread on feather meal plates and incubated at 37ºC for 7 days. The obtainedcolonies were selected and grown on skimmed milk plates for 48 hr**.**

Statistical analysis

Each test has three replicates. Variable and standard deviation means were documented and *t*- student test was performed to evaluate any significant differences between the results of control and the treated samples.

RESULTS

Isolation of feather-degrading bacterium

Twenty bacterial isolates were recovered from different samples of poultry farm wastes, soil, water, fodder and feather on a feather-meal medium. Keratin activity was evaluated by growing the isolates on both skimmed milk and feather meal plates. Five isolates recorded the highest growth on both feather meal and skimmed milk media (Table 1). The growth and keratinase production of the five isolates were determined in the liquid medium using the feather as the sole carbon and nitrogen sources (Table 1). Growth in liquid medium was ranged from $0.28x10^4$–$0.81x10^4$ CFU/ml. Maximum keratinase production (3.26IU/ml) was obtained by the isolate KR12 which was chosen for further studies.

Table 1. Growth and keratinase production of the five selected bacterial isolates

Isolate	Bacterialgrowth			
	Solid media (Diameter of the clear zone, mm)		Feather-meal broth medium (CFU/ml $x10^4$)	Keratinase enzyme (U/ml)
	Feather-meal agar medium	Skimmed milk		
KR1	09	23	0.28	15.6
KR2	11	30	0.81	22.0
KR3	08	29	0.67	17.0
KR4	1 2	31	0.72	17.4
KR12	1 8	44	0.91	33.6

Identification and characterization of keratinase – producing isolate (KR12)

The selected bacterium was identified and characterized by recording different morphological and physiological characters [18].The growth of the isolate KR12 was observed using different agar media. The growth was heavy, moderate or scanty (poor) as shown in Table (2).

Table 3 summarized the morphological characteristics of the selected isolate KR12. Microscopic examination of the selected actinomycete KR12 displayed substrate and aerial mycelium bearing a spiral chain of conidia which had a spiny surface (Fig 1A). The spore diameter was varied from 5-6 and 7-9 µm. No zoospore, sporangium, sclerotic or fragment hyphae were observed (Table 3). Some physiological characteristics were documented in Table 4. The growth of the selected bacterial isolate was determined under aerobic condition. No melanin pigment was noticed. Temperature growth range was limited from 15 to 45°C. The isolate KR12 was resistant to several antibiotics such as penicillin, cephalosporin, and kanamycin whereas it was susceptible to rifampin and tetracyclines. Glucose, D-xylose, mannitol, arabinose, D-galactose, raffinose, and fructose are good carbon sources, whereas, poor growth was obtained using L-Rhamnose. Appropriate sources of nitrogen are ammonium chloride, potassium nitrate, sodium nitrate, amino acids, casein, and peptones, while, sodium nitrite is not a suitable nitrogen source for bacterial growth. Investigation of entire bacterial cell hydrolysates, revealed the existence of only L-isomer of diaminopimelic acid (L-DAP), in addition to glucose, demonstrating a wall chemotype I. Entire cell sugar pattern was glucose, signifying whole cell sugar pattern as type A. Since the phospholipids analysis of the isolate revealed the presence of phosphatidylinositol (PI) and no phosphatidylcholine (PC) or phosphatidylethanolamine (PE), the phospholipids (P) pattern was classified as type PII. Saturated fatty acids were estimated in the strain KR12 with no mycolic acids were present. The previously obtained results revealed that the actinomycete isolate (KR12) belongs to the genus *Streptomyces* and identified as *Streptomyces radiopugnans* KR12. The sequence of 16S rRNA gene was compared to the GenBank database in the National Center for Biotechnology Information (NCBI) using the BLAST program. It was similar to *Streptomyces radiopugnans by 98.8%,* by 97% to *Streptomyces*sp, by 94% to both*Streptomyces cellulose, Streptomyces ochraceiscleroticus,* and *Streptomyces albulus*as shown in Fig.(1).The partial sequence of 16S rRNA gene was put in GenBank database (accession number JN037694).

Table 2. Cultural characteristics of the bacterial isolate KR 12

Media	Growth	Color of aerial mycelium	Color of substrate mycelium	Color of soluble pigment
Starch-nitrate agar	Heavy	Gray	Dark gray	Pale gray
Glucose Asparagine agar	Heavy	Gray	Dark gray	Gray
In-organic salts-starch iron agar (ISP-4)	Moderate	Pale gray	Yellowish-gray	Gray
Tyrosine agar medium (ISP-7)	Moderate	Pale gray	Gray	Gray
Yeast extract-malt extract agar (ISP-2)	Moderate	Dark gray	Yellowish gray	Gray
Oatmeal agar (ISP-3)	Moderate	Gray	Dark gray	Gray
Glycerol-asparagine agar (ISP-5)	Moderate	White	Yellowish white	Gray
Bennet agar	Scanty	Pale gray	Gray	Gray
Omura agar	Scanty	pale yellow	Yellow-brown	Gray

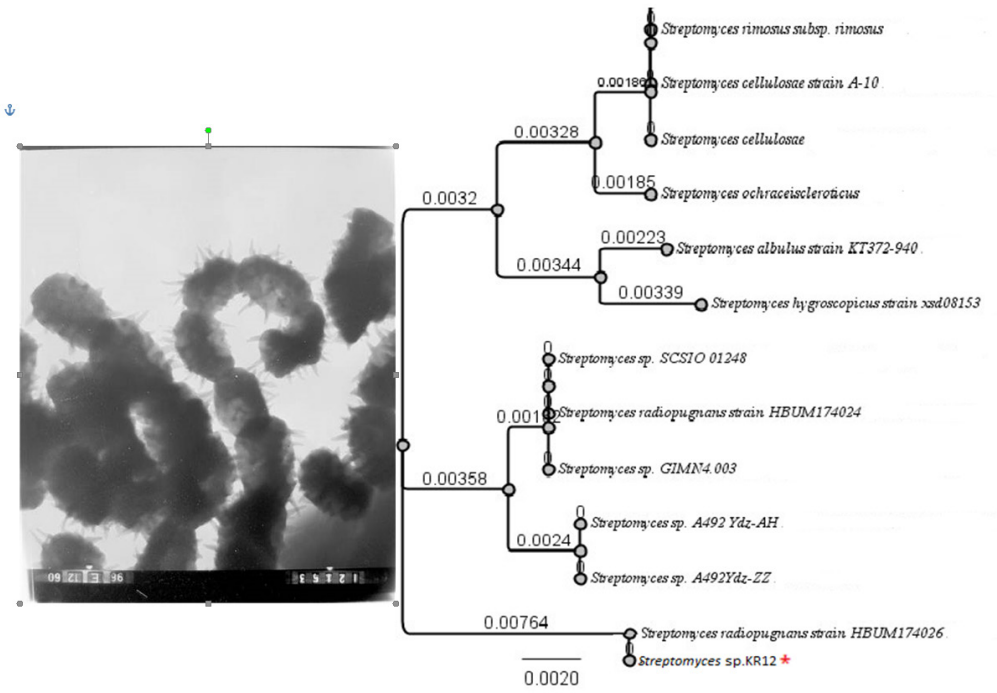

Figure 1. A: Transmis sion electron microscope X 60 000 of KR12. **B**: Phylogenetic tree of 16S rRNA of *Streptomyces radiopugnans* KR12and allied bacteria. The branching pattern was generated by the neighbor-joining method. Bar Jukes-Cantor distance of 0.002.

Table 3. Morphological characters of the bacterial isolate KR12

Tested character	**Results**
Gram stain	Gram +ve
Source of isolation	Soil
Motility of spore	Absent
Shape of spore	Cylindrical (5-6 and 7-9 µm)
Spore chain morphology	Spirals
Spore Surface ornamentation	Spiny
Number of spore/ chain	22-35
Aerial hyphae and Substrate mycelium	Well developed
Zoospore, Sporangium, Sclerotia, Fragmented mycelia	Absent

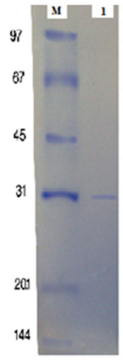

Figure 2. Molecular weight of keratinase isolated from *Streptomyces radiopugnans*KR12. by SDS-PAGE, M: protein standards employed were phosphorylase (97kDa), albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa), lane 2: Purified keratinase.

Table 4. Physiological characteristics of the isolates KR 12

Character	Reaction
Melanin production	
- Tyrosine agar	-ve
- iron agar	-ve
Lecithinase	-ve
Lipase	+ve
Chitinase	-ve
Gelatinase	+ve
Pectinase	+ve
H_2S production	-ve
Growth temperature:	10 – 45ºC
Tolerance to NaCl:	5-15%
pH range	5-9
Resistance to antibiotic Penicillin	+
Cephalosporin	+
Kanamycin	+
Rifampin	-
Tetracycline	-

ve: Negative result, +ve: Positive result,
+:Resistance, : Sensitive

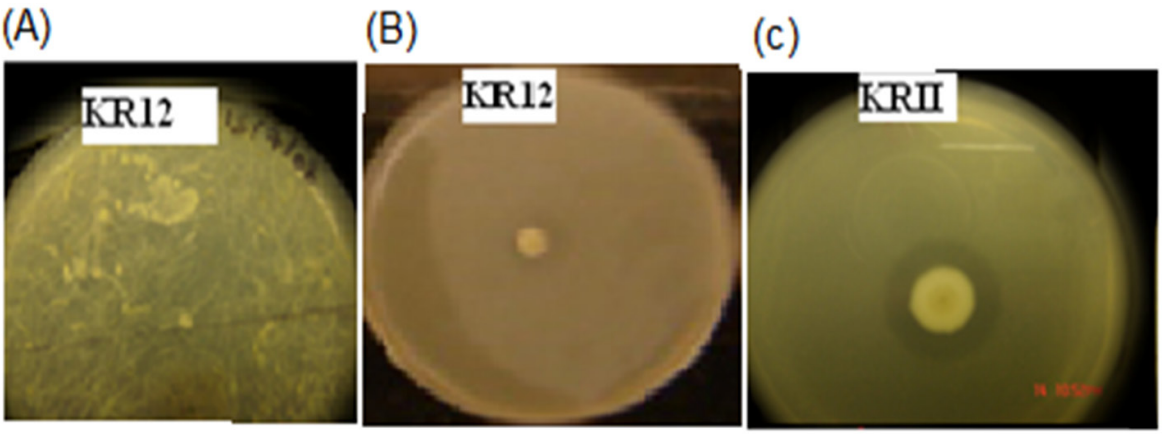

Figure 3. The selected actinomycete KR12 grown on feather meal agar (A), on skimmed milk agar plat (B). The recombinant mutant KRII on skimmed milk plat(C)

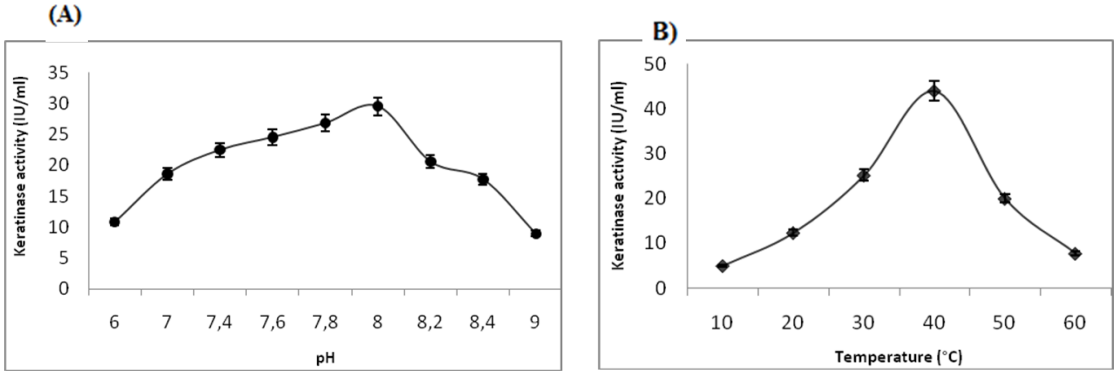

Figure 4. **A:** The pH optimum of *Streptomyces radiopugnans* KR12 keratinase using keratin azure as substrates, using 50 mM sodium acetate buffer (pH 3.5–5.5), sodium phosphate buffer (pH 5.5–7.5) and Tris–HCl buffer (pH 7.5–10.0). Each point represents the average of two experiments. **B:** Effect of different temperatureranging from 20 to 60ºC on keratinase enzyme produced from *Streptomyces radiopugnans* KR12.

The purified enzyme was obtained after precipitation by ammonium sulfate usingSephadex G-75 and DEAE (diethylaminoethyl)-Sepharose chromatographic columns. The purified enzyme had a molecular weight of 32 kDa (Fig. 2), evaluated by SDS-PAGE.

Keratinase activity was affected by several factors, especially pH values and incubation temperatures. A significant increase in keratinase activity was noticed at pH 8.0 and incubation temperatures 40ºC. In contrast, lower enzyme activity was obtained at pH 6and pH 9, and at incubation temperatures 30and 50ºC (Fig. 4A, B).The effect of metal ions and EDTA (chelating agent) on the activity of the purified enzyme was estimated. This activity was inhibited by the relatively low concentration of ($Fe^{2+}$and Cu^{2+}) whereas, Mg^{2+} exhibited no effect, (K^{+} and Ca^{2+}) have a slightly enhancing effect and (Zn^{+2} and Na^{+}) act as strongenhancers on the enzyme activity (Table 4). Chelating agents such as EDTA acts as a strong inhibitor for *Streptomycesradiopugnans*KR12 keratinase. Purified keratinase, obtained from *Streptomycesradiopugnans*KR12, was able to hydrolyze the abroad range of protein substrates. It was clear that the purified enzyme had high hydrolytic activity against keratin, wool, and powder milk, while, it had weak keratinolytic activity against collagen and bovine serum albumin (Fig. 5).

Table 5. Effect of metal ions on *Streptomyces radiopugnans* KR12 keratinase activity.

Metal ion(1mM)	Relative Activity (%)
Control	100
Na^{+}	120*
K^{+}	105
Mg^{2+}	94
Fe^{2+}	71
Cu^{2+}	80
Ca^{2+}	110*
Zn^{2+}	180*
EDTA	66

Significant results using studied- t-test at $p< 0.05$ compared with control without addition.

Keratinase production was enhanced using UV radiation. Three recombinant mutants were obtained, mutant KR II, produced a high keratinase enzyme (3 fold) than the wild strain *(Streptomycesradiopugnans*KR12).This mutant is a promised bacterium for biotechnological application especially for keratinase production (Fig. 3C).

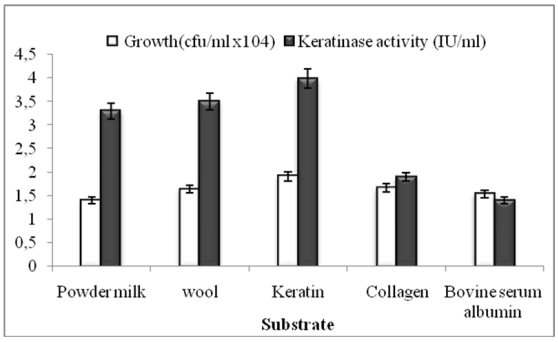

Figure 5. Substrate specificity of keratinase from Streptomyces radiopugnans KR12 on protein substrates

DISCUSSION

Microbial keratin degrading enzyme has an important role in biotechnological industries since it involved in the hydrolysis of extremely rigid, highly cross-linked insoluble structural protein (keratin) resistant to the conventional proteolytic enzymes.Many authors reported that several bacteria, actinomycetes, and fungi are keratinase producers. In the current work, approximately twenty-four bacterial isolates were recovered from diverse sources on feather meal agar medium encompassing keratin as a sole source of carbon and nitrogen. The isolate KR12 isolated from soil contaminated with poultry wastes was selected as a potent keratinase producer(3.26 IU/ml) and was identified and characterized using different morphological, physiological and biochemical events. The results were compared with that obtained from Bergey's Manual of Systematic Bacteriology [18] and revealed that this isolate was a species of the genus *Streptomyces* and classified as *Streptomyces* sp. (KR12). Phylogenetic analysis relayed on 16S rRNA sequences confirmed that this isolate belongs tothe genus *Streptomyces*. It was similar to *Streptomyces radiopugnans* by 98.8%, by 97% to *Streptomyces sp*, by 94 % to *Streptomyces cellulose, Streptomyces ochraceiscleroticus*, and *Streptomyces albulus*. The molecular mass of the purified keratinase enzyme was estimated to be 32 kDa. It was previously reported that molecular weights of keratinolytic enzymes varied from 18 to 200 kDa. The lowest value,18 kDa for *S. albidoflavus* SK 1–02[32], and the uppermost of 200 kDa for *Kocuriarosea* and *Fusariumislandicum*have been documented [33, 34]. Hence, the keratin-degrading enzyme from *Streptomyces radiopugnans* KR12 was revealed to be a novel sort of keratinase. Our result correlated with that obtained from *Streptomyces pactum* DSM 40530 (30 kDa) [35].The optimum pH andtemperature for the purified keratinolytic enzyme were pH 8.0 and40ºC, respectively, and the enzyme was stable at pH6.0-9.0 and 30-60ºC. This optimum temperature for activation was in accordance with other keratinolytic enzymes of *Streptomyces* as *S. pactum* [35].

Metal ions might perform an ion bridge that stabilizes the enzyme structure conformation or the bindingof the substrate and enzyme complex. Hence, metal ions may be involved in the maintenance of enzyme thermal stability.Our results showed that this enzyme belongs to alkaline-metalloprotease enzymes. Thermotolerant, alkali-tolerant Metallo keratinase from *Streptomyces radiopugnans* KR12 would have many applications in the bioconversion of keratinous wastes and eco-friendly dehairing in the leatherindustry.

Most of the available research on keratinolytic enzymes documented them as inducible enzymes; however, few constitutive keratin degrading enzymes have also been described [36, 37].Keratinase from *Streptomyces radiopugnans* KR12 was stimulated by keratin; therefore, it had inducible nature. Also, it possessed wider substrate degradation. Our results were compared with results previously reported [37].

A traditional mutagenesis is an effective approach using for isolating mutants that are able to yield improved products of numerous microbial enzymes as lipase and α-galactosidase [38, 39]. The same strategy could be effectively used to improve the ability of keratinase- producing strains to make this important enzyme. Mutagenesis was utilized as an effective approach to alter the original wild-type bacterial strain. Three recombinant mutants with higher keratinolytic activity were obtained using UV radiation. KRII mutant has threefold increasing in keratinase activity and displays remarkable feather-degrading abilities, therefore, it may be effective for potential biotechnological applications in keratinase production and feather waste usage.A similar result has been previously reported using the chemical mutagenic agent (N-methyl-N-nitro-N-nitrosoguanidine) to improve keratinase production [40].

Additional work can be performed to identify keratinase gene from KR12 and the increasing of its productivity by cloning it into a suitable cloning vector with a powerful promoter.

In conclusion, *Streptomyces radiopugnans* KR12 and its mutant KRII would use to improve our environment by reducing the contamination by keratin-rich wastes. Degradation of feathers by an alkali hydrolysis and steam pressure cooking would not only destroy the amino acids content but also consume large quantities of energy, so microbial keratinases are considered a promised way to overcome the accumulation of a huge quantity of keratin-rich wastes in the environment and to improve the nutritional value of animal feeds.

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