



Enhanced fusidic acid transdermal delivery achieved by newly isolated and optimized *Bacillus cereus* Keratinase



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ABSTRACT

The expanding interest in bioremediation of poorly degradable wastes has led to the discovery of many microbial enzymes capable of degrading recalcitrant substances such as keratinaceous wastes that are produced in vast quantities on daily basis. Such enzymes don't only work as a bioremediation tool but also have multiple beneficial applications. Hence, environmental samples were collected from sewage water, soils, animal bodies and feces in order to isolate keratinase producing organisms. Keratinolytic isolates were isolated from sewage water; soils; animal bodies; animal feces, and identified both traditionally and molecularly through 16S-rRNA sequencing to be *Bacillus cereus* strain. Produced keratinase was purified by centrifugation, ammonium sulfate precipitation, and HPLC, then assayed using Azokeratine based analysis. keratinase quantification yielded a 420 ± 1.63 U/mL. Optimum production was obtained at 40 °C, pH 7, 3 days incubation, 0.5 % substrate, 0.4 g/l magnesium ion, 2% v/v inoculum, 0.5 g/l NaCl, 0.4 g/l K₂HPO₄, and 0.3 g/l KH₂PO₄. Production was increased by 1.9 fold after acclimatization to reach 809 ± 2.49 U/mL in only 2 days. Thermal and pH stability testing revealed the effectiveness of the isolated keratinase over a wide range of temperatures at neutral pH. Finally, isolated keratinase enhanced fusidic acid topical penetration to treat induced deep skin bacterial infection in mice. A 1.4 fold decrease in treatment period and a 2 log cycle reduction in the viable count of *Staphylococcus aureus* were noticed in keratinase/fusidic acid treated mice compared to mice treated with fusidic acid alone. This study shed some light on a simple keratinase production optimization technique and suggested a promising medical application of this enzyme as a drug delivery agent.

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1. Introduction

The human population is increasing round the clock, that is accompanied by increased consumption of various nutritional elements (especially animal proteins) and hence, accumulation of tremendous amount of wastes. One of the highly overlooked wastes is keratinaceous ones that are produced in vast quantities from slaughterhouses such as feathers, horns, hooves, wool, etc. Keratin rich wastes are tolerant to all known proteases (pepsin, trypsin, and papain) owing to tight packing of its protein chains, and immense stability resulting from hydrogen bonds, hydrophobic interactions, and disulfide bonds [1]. Conventional disposal methods lead to environmental damage caused by omission of

toxic sulfur fumes and toxic residues accumulation in soil due to incineration or land burial, respectively.

A novel approach for safe disposal suggests the implication of keratinase enzyme that is capable of degrading complex keratin molecules into simpler peptides and amino acids [2]. Several organisms have been reported to produce keratinase particularly *Bacillus* species [3] with variable levels of keratinase production. Several trials were conducted to increase the produced amount of the enzyme through manipulation of production conditions. Beside its bioremediation role other applications have been reported regarding agriculture, textile, or industrial field.

The concern of this study is to isolate a novel keratinolytic organism, assay the produced keratinase and augment the production process through culture conditions optimization and acclimatization approach [4], along with a novel application of keratinase enzyme in transdermal drug delivery system with the potential to enhance topical antibiotics delivery into deep skin infection.

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2. Material and methods

2.1. Samples collection and preparation

Samples were collected from multiple ecological sites in Egypt including farm soils, sewage water, and animal feces [5,6] in sterile 50 mL falcon tubes. Samples were cultured in tryptone soya broth then serially diluted up to 10^{12} CFU/mL. One loopful from each dilution was then streaked on nutrient agar plates. Separate colonies were streaked on slants for further purification and identification.

2.2. Screening of keratinase producing organisms

A basal salt media (BSM) containing keratin as a sole source of carbon and nitrogen was prepared [7] with some modification, to prepare one liter BSM the following was mixed into double distilled water: KH_2PO_4 0.3; NaCl 0.5; MgSO_4 0.2; K_2HPO_4 0.4 (g/l); pH 7.0 ± 0.2 . A 100 mL aliquote was transferred to 250 mL erlenmeyer flasks followed by adding 1% w/v finely chopped white chicken feathers (WCF). The media was sterilized by autoclaving at 121°C for 15 min. BSM was inoculated with one loopful from each isolate separately and incubated at 37°C for 5 days, isolates that showed visible keratin (feathers) degradation was preserved for identification.

2.3. Purification steps of produced keratinase

Keratinase positive cultures were subjected to a three steps purification process in order to obtain the enzyme in a relatively purer form. The process started with centrifugation at the speed of $9503 \times g$ at 4°C for 15 min to precipitate cells and other macro residues, followed by protein precipitation from the clear supernatant through 60 % ammonium sulfate saturation accompanied by continuous stirring and overnight incubation at 4°C . The produced suspension was subsequently centrifuged for 30 min at 4°C at $9503 \times g$ and pellet was retrieved and dissolved into 40 mL of sodium phosphate buffer (0.1 M, pH 7.5). Eventually, the obtained solution was further purified using HPLC by loading it onto a Sephacryl S-200 column (30 cm). Column was first equilibrated with 0.1 M sodium phosphate buffer (pH 7.5) and then elution began using 0.1 M sodium phosphate buffer (pH 7.5) at a flow rate of 0.5 mL/min. 2.0 mL fractions were gathered and subjected to keratinase assay [8].

2.4. Keratinase assay using azokeratin technique

Keratinase activity was measured as a function of its ability to liberate fluorescence upon breaking down of fluorescent Azokeratine (Sigma, Germany) [9]. Reaction mixture contained 0.8 mL Tris–HCl buffer (0.1 M) pH 9.0, and 100 mg Azokeratine, a suspension was formed by mixing the components for 15 min. A 0.2 mL purified enzyme was then added and the mixture was incubated at 37°C for 25 min. The reaction was then terminated by adding 0.2 mL of 10% w/v tri-chloro acetic acid (TCA) and centrifuged at 4°C – 10,000 RPM for 15 min [10]. Blank was prepared in the same manner but with adding TCA prior to the enzyme addition. Absorbance of the supernatant was recorded at 440 nm. Keratinase activity unit was defined as the enzyme amount that results in a 0.01 unit increase in absorbance at 440 nm/minute.

2.5. Identification of the recovered Keratinolytic isolates

Keratinolytic isolates were tentatively identified by microscopical examination and biochemical testing following Bergy's manual, then molecularly identified using 16S rRNA universal

primer pair F- (5'-CAGGCCTAACACATGCAAGTC-3') R- (5'-CGGCGWGTGTACAAGGC-3'). Obtained sequences were identified using BLASTN tool from NCBI.

2.6. Optimization of keratinase production conditions

In order to determine the required culture condition to obtain optimum keratinase production, a number of conditions were tested including: various incubation temperature ranging from 10°C – 60°C , multiple pH from 3 to 10, and different incubation period from 2 to 7 days. Various concentrations of keratin ranging from 0.25 g% – 8 g% w/v were also tested, along with different inoculum sizes ranging from 0.25 to 5% v/v obtained from a 0.5 McFarland solution (equivalent to 5×10^6 CFU/mL). All inoculums were obtained from a 0.5 McFarland bacterial suspension. Salt constituents of BSM (NaCl, KH_2PO_4 , and K_2HPO_4) were also evaluated for their optimal concentration by testing various concentrations ranging from 0.1 % – 0.7 % w/v.

2.7. Effect of metal ions supplementation on keratinase production

Several trials were conducted to evaluate metal ions influence on keratinase production by supplementing culture media with different concentrations of Ca^{2+} , Fe^{3+} , Zn^{2+} , and Mg^{2+} ions (as sulfate salts). The used concentrations were 0.1, 0.2, 0.4, 0.8, 1.6 g/l.

2.8. Effect of secondary carbon and nitrogen sources on keratinase production

Glucose, mannose, sucrose, dextrin, and soluble starch were included in the media separately as a second carbon sources in order to test their influence on keratinase production at 5% w/v concentration. Similarly yeast extract 2.23 g/l, urea 0.4 g/l, ammonium sulfate 1.1 g/l, and potassium nitrate 1.69 g/l were used as extra nitrogen sources. Samples of 2 mL were withdrawn every 24 h for 7 days to monitor keratinase production.

2.9. Acclimatization of keratinolytic isolate for augmented keratinase production

Acclimatization process was employed with some variation from [11] to increase the produced enzyme via continuous passing the keratinolytic isolates through 20 cycles of culturing and sequential sub-culturing on BSM. Each cycle consists of 3 days of incubation followed by withdrawal of bacterial inoculum for the next cycle. Standard culture conditions were applied. Keratinase assay was conducted every 24 h.

2.10. Determination of keratinase thermal and pH stability

Thermal stability profile was established by initially incubating 2 mL of enzyme solution in pH 7 (50 mM Tris–HCl buffer) at various temperatures ranging from 4°C to 70°C . Then withdrawing samples from each temperature tested at the following intervals 15, 30, 60, 120, 240, 480, 960 min. Finally 0.1 mL from each temperature at every time interval was withdrawn and the residual keratinase activity was measured [7].

Similarly pH stability profile was established by incubation at various pH values ranging from 3 – 11. To obtain pH 3–5 citric acid buffers were used, for pH 6–8, Sodium phosphate buffers were used, while for pH 9–10 sodium carbonate buffers were used.

2.11. Detection of keratinase gene in Bacillus cereus isolate

NCBI Primer designing tool (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>) was used to design a primers pair specific for

Bacillus cereus strain Wu6 ker-6 keratinase gene F- (5'-ATCTGTCCGAGCTAATGCT-3') R- (5'-AAGTTAGGGGATGTCGTTGT-3') 55 °C, and another pair for *Bacillus thuringiensis* ker-S3KUBOT gene F- (5'-AATCTGTCCGAGCTAATGTC -3') R- (5'- TTGGCGAATTGTGTATTGC -3') 55 °C.

2.12. Keratinase role in enhanced transdermal drug delivery

The designed animal model complied with the ARRIVE guidelines and was carried out in accordance with the Egyptian National Institute of Health guide for the care and use of Laboratory animals. Twenty one BALB/c female mice weighing around 15–20 g had their back side shaved using Braun 5427 Cruzer 5 (china) one day prior to conducting the experiment. On day 0, a 2×10^9 CFU/mL *Staphylococcus aureus* ATCC 25,729 suspension was prepared in phosphate buffer saline (PBS), then 50 μ L (equivalent to 1×10^8 CFU) were injected subcutaneously into each mouse. Mice were then kept at their cages and given food and water ad libitum. On day1, upon manifestation of the signs (abscess and boils) treatment commenced twice daily according to the following protocol: mice were equally distributed into 3 groups (A, B, and C), group A was treated with keratinase enzyme solution for 5 min followed by fucidic acid cream, group B was treated with fucidic acid cream only (control 1), and group C was not treated with any drug so as to act as a blank (control 2). Complete healing periods were recorded for each group. On day 4, representative samples from each group were euthanized then a 2×2 cm piece from each mouse back was cut, homogenized and subjected to viable count experiment on Mannitol salt agar plates.

2.13. Statistical analysis

All experiments were carried out in triplicates and obtained values were expressed as the mean \pm standard deviation. In order to determine significant differences, *t*-test or one-way analysis of variance (ANOVA) was performed. Results were considered significant at P value < 0.05.

3. Results

3.1. Keratinolytic isolates screening and keratinase quantification

A total of fifty isolates showed keratinolytic activity indicated by visible degradation of WCF after a minimum of three days with others requiring an entire 5 days to show complete lysis. Isolate

number 4 (from soil) showed the highest persistent production of keratinase (420 ± 1.63 U/mL) with a relatively rapid complete lysis of WCF in 3 days only.

3.2. Keratinolytic isolate identification

Preliminary identification suggested that Isolate 4 belongs to *Bacillus* genus (Supplementary Fig. 1). Molecular identification using 16S-rRNA confirmed this finding and showed that isolate 4 is similar to *Bacillus cereus* strain 151,007-R3_I05_21_26 F with 96.88 % identity. Sequence data was uploaded to NCBI database under the accession number MT543036. A phylogenetic tree of keratinolytic isolate 4 and other similar species, based on 16S-rRNA analysis, was established using NCBI tool (Supplementary Fig. 2)

3.3. Effect of culture incubation temperature and pH on *B.cereus* keratinase production

A relatively higher keratinase production was recorded at temperatures ranging from 30 °C–50 °C when compared to lower and higher temperatures with maximum production recorded at 40 °C (Table 1).

B. cereus isolate was capable of producing the highest keratinase when the initial pH of the culture media was adjusted to 7. However, production started at low pH as low as 3 and continued till pH 11 (Table 1).

3.4. Effect of substrate concentration and incubation period on *B. cereus* keratinase production

B. cereus isolate was able to produce keratinase when supplemented with different substrate concentrations starting from 0.25 % w/v till 10 % w/v however the highest production was recorded at 0.5 % w/v after which a significant decline in activity was recorded as shown in (Table 1). As for the optimum incubation period to recover the produced keratinase, a three days period were sufficient to obtain the maximum production as shown in (Table 1).

3.5. Effect of salt concentration and inoculum size on keratinase production

When evaluating the influence of multiple concentrations of BSM salt constituents on keratinase production process, it was evidenced that K_2HPO_4 , KH_2PO_4 , and NaCl were essential for

Table 1

Determination of optimum keratinase production conditions from *B. cereus* culture media. A table showing the effect of different variables on the production of keratinase from *B. cereus* isolate culture media. Keratinase was assessed using Azokeratin technique. Optimum values are bolded. The result is the average of 3 independent experiments \pm S.D.

	Temperature (°C)							
	10	20	30	37	40	50	60	–
Keratinase unit (U/mL)	20 \pm 2.1	260 \pm 1.2	385 \pm 0.5	420 \pm 1.7	490 \pm 0.47	389 \pm 1.24	175 \pm 2.4	–
	pH							
Keratinase unit (U/mL)	3	4	5	6	7	8	9	10
	75 \pm 4.9	120 \pm 3.2	180 \pm 4.3	298 \pm 2.5	420 \pm 2.4	340 \pm 3.2	194 \pm 0.8	76 \pm 4.02
	Substrate concentration (mg/mL)							
Keratinase unit (U/mL)	0.25	0.5	1	2	4	6	8	10
	360 \pm 0.8	582 \pm 3.2	420 \pm 2.05	386 \pm 1.24	264 \pm 1.88	173 \pm 1.6	63 \pm 2.6	42 \pm 3.3
	Incubation period (days)							
Keratinase unit (U/mL)	1	2	3	4	5	6	7	8
	86 \pm 1.2	260 \pm 2.05	450 \pm 2.9	430 \pm 2.4	420 \pm 1.2	367 \pm 2.6	246 \pm 2.6	109 \pm 3.2

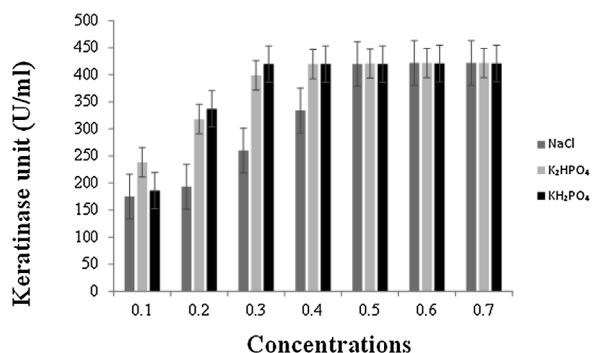


Fig. 1. The effect of BSM salts concentrations on keratinase production. A column chart showing the effect of the BSM salts variable concentrations on keratinase production. A range of 0.1 % - 0.7 % w/v of the salt constituents of BSM (NaCl, KH₂PO₄, and K₂HPO₄) were evaluated for the highest keratinase production. The produced keratinase were measured using Azokeratin technique. The experiment was repeated 3 subsequent times and the error bars represent the S.E.

obtaining the highest keratinase production at the following concentrations respectively: 0.4, 0.3, 0.5 g/l (w/v). Increasing any of the salts concentration above these values did not provide any significant increase in keratinase production (Fig. 1). The most suitable inoculum size to achieve maximum keratinase production was found to be 1–2 % v/v of 0.5 McFarland bacterial suspensions. Higher or lower volumes showed minute influence on keratinase production (Fig. 2).

3.6. Effect of metal ions inclusion on keratinase production

It was notable that both calcium and zinc ions had little to null effect on keratinase production for all concentrations. While ferric ion had a lessening effect on production of keratinase at concentrations higher than 0.4 g/l, on the other hand, magnesium ion boosted the production of keratinase when utilizing 0.4 g/l concentration above which no significant increase was observed (Fig. 3).

3.7. Effect of supplementation of the production media with secondary carbon/nitrogen source on keratinase production

All of the additional carbon sources inhibited keratinase production initially then demonstrated a detrimental effect on the production, however the inhibition period varied from three days as in the case of dextrin and mannose; four days for glucose and sucrose; or even as long as five days for soluble starch as shown in in (Fig. 4). Likewise keratinase production was completely inhibited when incorporating additional nitrogen sources for an entire 3 days as in the case of yeast extract and KNO₃; or 4 days as for urea and (NH₄)₂SO₄ after which production commenced but still affected by the additional nitrogen source as shown in (Fig. 5).

3.8. Effect of acclimatization on keratinase production

After 20 cycles, keratinase production of *B. cereus* isolate was significantly altered and augmented by acclimatization process, the manifestation of which were: enhanced production with about 192 % increase; shorter incubation period with about 33.3 % decrease, and increased bacterial substrate capacity with 100 % as shown in (Table 2).

3.9. Thermal and pH stability profiles of *B. Cereus* isolate keratinase

B. cereus isolate keratinase demonstrated a relatively large thermal stability range by retaining up to 90 % of its activity after

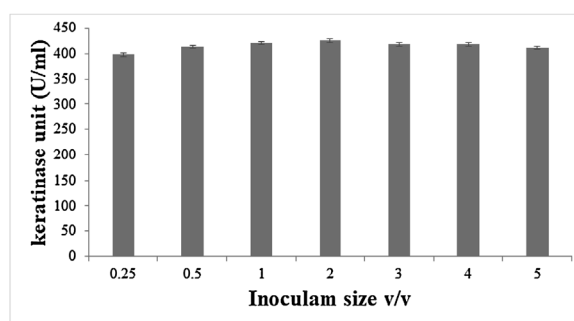


Fig. 2. The effect of inoculum size on keratinase production. A column chart showing the effect of different inoculum sizes of *B. cereus* isolate on keratinase production. Different inoculum sizes ranging from 0.25 to 5% v/v were tested. All inoculums were obtained from a 0.5 McFarland bacterial suspension. The produced keratinase were measured using Azokeratin technique. The experiment was repeated 3 subsequent times and the error bars represent the S.E.

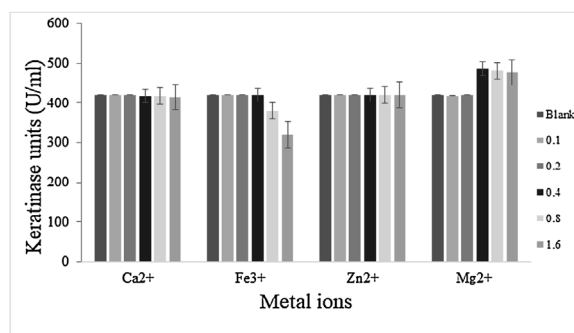


Fig. 3. Effect of metal ions on keratinase production. A column chart showing the change in keratinase production upon supplementing the media with different concentrations of metal ions. Ca²⁺, Fe³⁺, Zn²⁺, and Mg²⁺ ions were added separately with 0.1, 0.2, 0.4, 0.8, 1.6 g/l concentrations to the culture media. The produced keratinase were measured using Azokeratin technique. The experiment was repeated 3 subsequent times and the error bars represent the S.E.

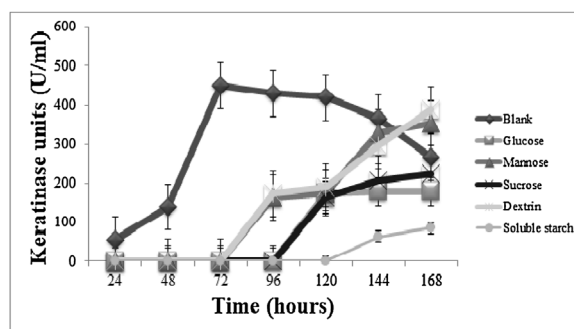


Fig. 4. Effect of additional carbon sources on keratinase production. A line graph showing the effect of addition an external carbon sources on the production of keratinase. 5% soluble starch, glucose, sucrose, dextrin and mannose were added separately to the keratin basal medium and incubated with *B. cereus* strain for 168 h (7 days). The produced keratinase were measured using Azokeratin technique. The experiment was repeated 3 subsequent times and the error bars represent the S.E.

sixteen hours of incubation at temperatures ranging from 4 °C to 50 °C. Incubation at higher temperatures had a detrimental effect on residual keratinase activity as shown in (Fig. 6). In case of pH stability determination, isolated keratinase was highly sensitive to all acidic pH that diminished its residual activity while it was maintained at pH 7 for the entire 16 h of incubation. Keratinase

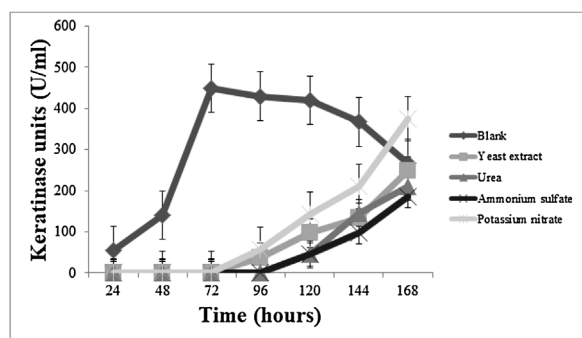


Fig. 5. Effect of additional nitrogen sources on keratinase production. A line graph showing the effect of addition of external nitrogen sources on the production of keratinase. Yeast extract, urea, ammonium sulfate and potassium nitrate were added separately to the keratin basal medium and incubated with *B. cereus* strain for 168 h (7 days). The produced keratinase were measured using Azokeratin technique. The experiment was repeated 3 subsequent times and the error bars represent the S.E.

Table 2

Acclimatization effect on *B. cereus* isolate keratinase production. A table showing the beneficial effect of acclimatization on the amount of produced keratinase, incubation period and keratinase lysing capacity. Twenty successive subcultures were carried out. The amount of produced keratinase was assessed by Azokeratin technique before and after acclimatization.

Factor	Before	After
Produced keratinase (U/mL)	420	809
Incubation period (Days)	3	2
Lysing capacity (mg/mL)	0.5	1

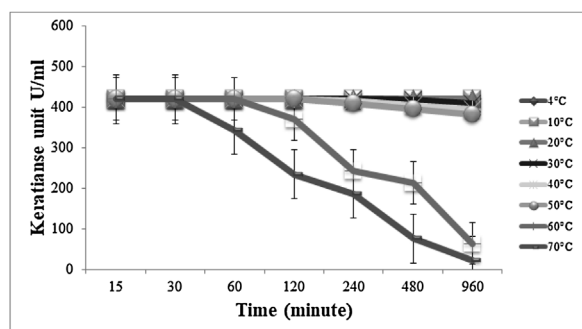


Fig. 6. Effect of temperature on the stability of keratinase produced by *B. cereus* isolate.

A line chart illustrating the keratinase activity upon incubation at various temperatures (4, 10, 20, 30, 40, 50, 60, 70 °C) for different time intervals up to 960 min (16 h). The produced keratinase were measured using Azokeratin technique. The experiment was repeated 3 subsequent times and the error bars represent the S.E.

stability was reduced in case of alkaline pH to reach about 90 % of activity after two hour incubation at pH 9 as shown in (Fig. 7).

3.10. Detection of keratinase gene in *B. Cereus* isolate

PCR analysis of our isolate confirmed the presence of keratinase gene similar to *Bacillus thuringiensis* ker-S3KUBOT as shown in (supplementary Fig. 3). A band at approximately 900+ base pair was detected from *B. cereus* isolate before and after acclimatization.

3.11. Keratinase role in enhanced transdermal drug delivery

Keratinase incorporation into treatment of group (A) resulted in a significant improvement in the treatment protocol of deep skin

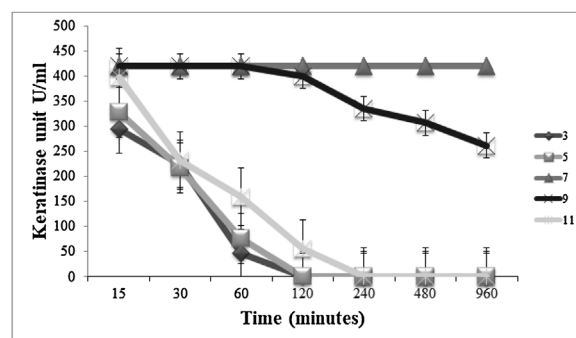


Fig. 7. Effect of pH on the stability of keratinase produced by *B. cereus* isolate. A line chart illustrating the keratinase activity upon incubation at various pH (3–11) for different time intervals up to 960 min (16 h), the produced keratinase were measured using Azokeratin technique. The experiment was repeated 3 subsequent times and the error bars represent the S.E.

infection illustrated by: a reduction in the required time for complete healing by about 30 % (from 7 days to just 5 days); and a remarkable decline in *S. aureus* viable count during the course of treatment by about 2 log cycles as shown in (Fig. 8) compared to group B and C.

4. Discussion

Keratinase enzyme is one of the valuable bacterial products that has many environmental and medical applications. In a quest to isolate and characterise a new keratinase enzyme with useful treats, samples were collected from different Egyptian environments to isolate keratinase producing bacteria. Isolate 4 (the one with highest keratinase production) excelled some bacterial isolates reported in other studies. It produced higher amount of keratinase than the 80 U/mL reported by [12] for *Chryseobacterium* species, and quite similar to keratinase units reported by [13] for *B. cereus* strain SS-8 (510.91 ± 5.15 U/mL) and strain HS-2 (400.01 ± 7.71 U/mL). Keratinase measurements showed high consistency, reproducibility, and minimum divergence which suggest the utilization of Azokeratine analysis as a reliable assay tool. Isolate 4 was confirmed to be a *Bacillus cereus* strain which is similar to the findings of [13–15], this come in accordance with the common findings that *Bacillus* species are the main keratinase producers.

The optimum incubation temperature was found to be within the range 20 °C–80 °C reported by [16] and is consistent with the 42 °C reported by [7] for *B. licheniformis*, while closely related species such as *B. thuringiensis* serovar *israelensis* showed optimal production at as low as 28 °C [8]. A reasonable amount of keratinase was produced at a large range of pH 5–9, this wide pH range facilitates the cultivation condition without the need for strict control over pH and since the optimal pH is 7, this allows for easier media preparation, and lies within the pH range 5–9 reported for different *Bacillus* species [14].

Substrate concentration was illustrated to be the most influential factors affecting keratinase production, since the slightest change in the concentration resulted in major alteration in enzyme production. This sharp decline in the activity might be owing to the increased media viscosity with more substrate addition. A similar percentage was reported by [17] that used 0.4 % w/v substrate, this percentage represents a more economic approach when compared to the 1.0 % (w/v) reported by [5,18,19]. The 3 days incubation period is considered reasonable and energy/time sparing when compared to the 4 days reported by [7,8], 5 days [17] and 7 days reported by [1].

The 1–2 % v/v inoculum size was found to be reasonable and lies within the range reported by [20,21] and similar to [22] findings

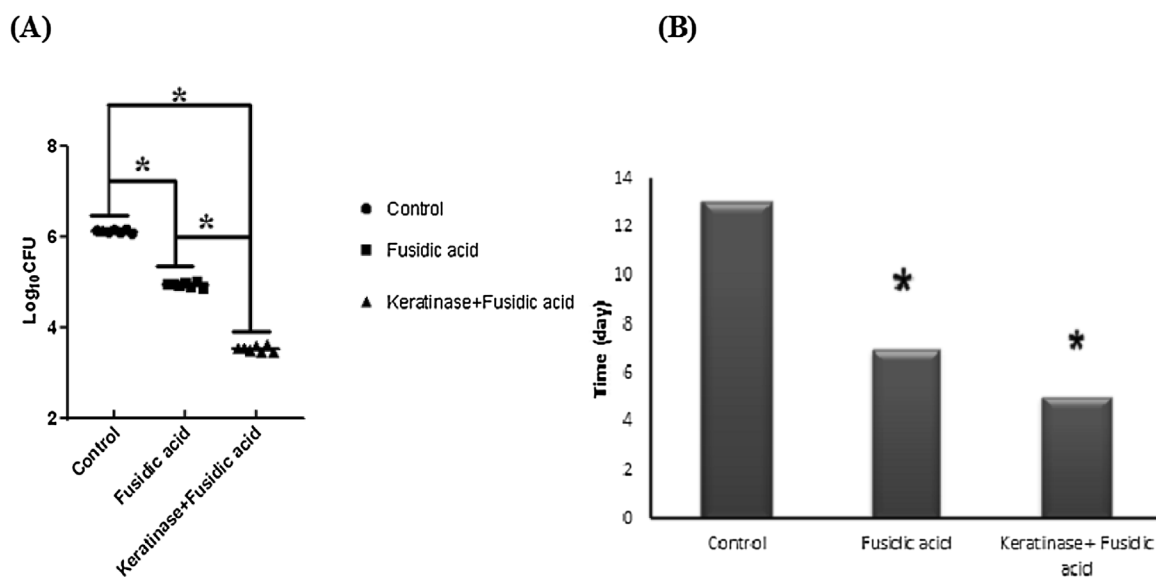


Fig. 8. In vivo assessment of keratinase as a drug delivery agent.

(A) Scatter graph illustrating the differences in viable count values in logarithmic form of *S.aureus* obtained from each treatment group. Twenty one female mice were deep skin infected by 1×10^8 CFU of *S. aureus* then divided into three groups. The first group was kept as a negative control without treatment. The second one was treated by fusidic acid alone. The third group was given keratinase and fusidic acid. On day 4, representative samples from each group were euthanized then a 2×2 cm piece from each mouse back was cut, homogenized and subjected to viable count experiment on Mannitol salt agar plates. Student *t*-test was applied using CLC work bench software tool. *: indicates significant difference with p value < 0.05 . (B) Bar graph demonstrating the time required for complete healing and the disappearing of the symptoms for the different treatment groups. Student *t*-test was applied using CLC work bench software tool. *: indicates significant difference compared to the control group with p value < 0.05 .

that reported the use of 2% inoculum size. Inoculum size was not a key player in the keratinase production from *B.cereus* isolate culture media as increasing or decreasing inoculum size led to a maximum of about $\approx 6\%$ decline in keratinase production.

K_2HPO_4 optimal concentration is somewhat close to the 1.0 g/l as utilized by [23] for *B. licheniformis* ZJUEL31410, and much less than 10.0 and 14.0 g/l reported by [7,24] for *B. licheniformis* RG1 and ALW1, respectively. Those large differences can be attributed to the nature of the used bacterial species. A similar results were noted for NaCl optimal concentration which was significantly lesser than the 50.0 g/l suggested by [7].

The boosting effect of Mg^{2+} ion recommends its inclusion in BSM. This may be attributed to the catalytic and stabilizing effect of Mg^{2+} ion on keratinase enzyme. Similar reports were made by [10,25], however they used a mixture of different metal ions simultaneously in the assay while in this study the effect of various concentrations of each single metal ion were tested separately.

The inhibition effect produced by additional carbon sources can be justified by the relative ease of utilization of these sources when compared to keratin. This finding is similar to the reports made by [26–28]. Production started after 3–5 days suggesting depletion of the carbon source, however at lower rates depicting residual effect from the carbon source or declining in the organism population. Nitrogen sources effect is in accordance with [7,10,29] which can be also be explained by more simple catalysis, while other reports mentioned an increase in production with corn steep liquor [23] or urea [30].

Acclimatization process isolate augmentation can be justified by possible genotypic or phenotypic modifications resulting from the continuous exposure to keratin as a sole carbon and nitrogen source. Acclimatization process represents a simpler and safer technique when compared to [31,32] where the two fold increase in production was achieved after inducing mutation through physical treatment (UV radiation) and the 250 % increase, reported by [33] was reached after chemical agents mutation induction (ethidium bromide & ethyl methyl sulfonate). Moreover, such methods are unpredictable and may result in decrease of activity or

even enzyme impairment [15]. Other approaches like gene cloning technique resulted in 110–300 % increase [34], or enhancing thermal stability of the enzyme [35], but this method are usually complicated and expensive especially when compared to our acclimatization method that resulted in a comparable 192 % increase in keratinase production.

Thermal stability profile of isolate 4 keratinase depicted ease of enzyme handling and storage, and more stability compared to the one described by [7] that showed 90 % residual activity for only an hour and a half at $50^\circ C$ – $60^\circ C$ and a half-life of 2 h at $60^\circ C$. It was also more thermally stable than [8] keratinase that maintained 90 % activity at a maximum of $30^\circ C$ for only 2 h. On the other hand, pH stability profile depicted the neutral to slightly alkaline nature of the enzyme which requires maintaining keratinase pH constant at storage and using stable neutral buffers.

The improved healing capabilities of fusidic acid/ keratinase mixture on deep skin bacterial infection in mice can be justified by the keratinase ability to facilitate drug passage through stratum corneum by loosening cell attachment and increasing interstitial spaces through dissolving keratin residues in them. A related application was reported by [36] in which keratinase was used to improve antifungal drug delivery through onychomycotic nails. This application paves the way for possible utilization of keratinase in transdermal drug delivery systems for faster, easier and simpler drug penetration without the need for complex systems.

5. Conclusion

Bacillus cereus strain identified by 16S-rRNA sequencing showed a sustained high keratinase yield. Keratinase production was successfully enhanced by adjusting various culture conditions from which temperature, pH and substrate concentration which showed the highest effect. Production was further augmented by acclimatization process which represented a successful approach in inducing keratinase production with comparable results to other more complex techniques. Detection of keratinase gene opens the door for possible gene cloning into a commercial vector for

inducing higher production, and the detection of possible genetic mutation brought by acclimatization. The in vivo study illustrated a promising application of keratinase enzyme as a transdermal delivery agent that can replace other complex delivery systems.

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Ethical approval

All applicable international, national, and institutional guidelines for the use of animals were followed. This article does not contain any studies with human participants performed by any of the authors.

Declaration of Competing Interest

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.btre.2021.e00620>.

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