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Enhancing keratinase production of a native *Bacillus paralicheniformis* FUM-2 through random mutagenesis using a chemical agent and ultraviolet

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Abstract

Daily accumulation of feather waste due to the development of the poultry industry is a critical issue, considering the environmental and health hazards. Among feather waste disposal methods, biodegradation is a practical, cheap, and environment-friendly method. This study aimed to enhance the keratinolytic activity of FUM-2 isolate, a native keratinolytic bacterium, using mutations and culture improvement. The isolate was identified as *Bacillus paralicheniformis* based on biochemical, morphological, and molecular analysis. Using the one factor at a time (OFAT) method, the isolate produced more keratinase at 45 °C, pH 11, 2% carbon substrate concentration, 75% aeration rate, and 48 hours incubation. Also, it was able to degrade both α and β keratin. Culture improvement increased enzyme production from 507.6 U/mL to 1706.4 U/mL. In random mutation using ultraviolet radiation, three mutants were isolated, and a 10-minute UV-treated mutant of UVF2-D showed a 36.3% increase in keratinolytic activity compared to its parent. In the next round of mutagenesis by ethidium bromide, seven double mutants were isolated from the UVF2-D mutant, among which the EUF2-D2 double mutant showed a 79% increase in keratinolytic activity. No viable mutants were isolated from the wild strain using ethidium bromide. Our results show that many organisms can potentially produce beneficial products that could be improved using random mutagenesis and optimization methods, which are more useful for strain improvement than complex and costly molecular techniques.

1. Introduction

One of the main wastes produced by the poultry processing industry is feathers, an organic solid waste that pollutes the environment with several million tons of waste annually worldwide (Govarthanan et al., 2015; Pandey et al., 2019). Feathers contain about 90% keratin, an insoluble fibrous protein composed of α- keratin, β- keratin, and amorphous keratin (Singh & Kushwaha, 2015; Purchase, 2016). The structure of α-keratin's protein is helical, one of the common secondary structures in proteins. The β-keratin's protein structure is in the form of β sheets, which are mostly disulfide bonds with less hydrogen and

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hydrophobic bonds. Extensive hydrophobic interactions and hydrogen and disulfide bonds make the protein resistant to common proteolytic enzymes such as trypsin, pepsin, and papain (McKittrick et al., 2012).

Physical, chemical, enzymatic, and biological treatments are generally used for feather hydrolysis. Except for biological treatment, these are expensive, require a lot of energy, and degrade essential amino acids (Bokveld et al., 2021; Bhari et al., 2020; Govarthanan et al., 2011). Biological treatments, such as microbial hydrolysis, offer higher nutritional value and quality and have been proposed as an alternative for degrading and producing powder feather meals.

Keratinases have recently attracted particular attention in biotechnology research due to their numerous industrial applications in food, textile, pharmaceutical, and cosmetic industries (Singh & Kushwaha, 2015; Bhari, 2020; Vidmar, 2018; Nnolim et al., 2020). Microbial keratinases are serine or metalloproteinase enzymes that are able to fully degrade insoluble keratins and produce hydrolysis proteins (Gupta et al., 2013; Pahua-Ramos et al., 2017).

Keratinolytic microorganisms have been isolated from various sources such as soil, decomposing organic matter, poultry farms, and slaughterhouses (Rodrigues da-Silva, 2018; Daroit & Brandelli, 2014). Various groups of microorganisms, including bacteria such as actinomycetes, some Bacillus species, yeasts, and Dermatophytes, have been reported to produce keratinase (Govarthanan et al., 2015; Jaouadi et al., 2015; Lateef et al., 2015; Tamreihao et al., 2019). Mutations in enzyme-producing microorganisms, applied to improve enzyme productivity of microbial strains, are usually performed using classical mutagenesis techniques such as exposing microbes to physical mutagens like UV rays, X-rays, and Ɣ-rays, and chemical mutagens like EtBr, NTG, and EMS (Parekh et al., 2000). Mutations are used to overproduce various industrial enzymes such as protease, glucoamylase, lipase, keratinase, and cellulase (Haki & Rakshit, 2003; Bapiraju et al., 2004). Traditional mutagenesis has been proven to create strains that significantly increase enzyme production and activity, which can be used for microbial keratinase production and activity (Abd El-Aziz et al., 2023).

Nowadays, it is a priority to find new strains that can produce more enzymes under simpler cultivation conditions. Improving the strains with simple and low-cost methods such as mutation can also help us to obtain suitable isolates for industrial production. The present study aimed to find new native keratinolytic bacteria that can produce higher keratinase and enhance the production and activity of the enzyme through culture medium improvement and mutation.

2. Materials and Methods

2.1. Feather meal preparation

The feathers purchased from a local poultry farm were initially washed multiple times with water and detergent, degreased with chloroform: ethanol (1:1 v/v) for 1 hour, rinsed again with water to remove residual solvent, dried at 60 °C for 24 hours, powdered by a mixer (Amin Asia Company, Iran) and used as feather meal (Pahua-Ramos et al., 2017).

2.2. Culture medium

Feather meal broth (FMB) was used as follows (g/L): 0.5 NaCl, 0.1 $MgCl_2.6H_2O$, 0.4 K_2HPO_4, 0.3 KH_2PO_4, 1% feather meal at an initial pH of 7.5, and for feather meal agar (FMA), agar was added at a final concentration of 1.5% to the FMB.

2.3. Isolation and screening

A suspension was prepared from the contaminated soil of a poultry farm near Mashhad, Iran. Then, ten serial 10-fold dilutions of 10-1 to 10-10 were prepared in PBS, and 100 µL of each serial dilution was plated onto FMA medium and incubated at 37°C. Colonies were screened for the halo zone, isolated, and cultured in FMB medium for a second screening. Isolates with the best result were selected for further experiments.

2.4. Microorganism and medium culture

Bacteria from the previous steps were identified based on morphological, biochemical, and molecular methods and evaluated for their keratinolytic activity and production. DNA extraction was performed using a CinnaGen kit (Cinnagen, Iran), and PCR was done using an Amplicon kit (Amplicon Co, Denmark) with universal primers. PCR products were electrophoresed on 1% agarose gel. PCR products were sequenced, and data were analyzed using NCBI and Ez-Taxon databases. Then, phylogenetic analysis was performed using MEGA software (version 11), MUSCLE software, and the Neighbor-joining model, and finally, the phylogenetic tree was drawn.

To produce the enzyme, FMB medium was inoculated using overnight culture of FUM-2 isolate (2% v/v) and incubated for five days at 37°C in a shaker incubator with 150 rpm. Next, at 24 h time intervals, 2 mL of the culture sample was collected and centrifuged, and then 100 µL of supernatant was used to estimate the enzyme activity.

2.5. Assay of keratinolytic activity

The keratinase activity assay was performed according to Rammni and Gupta method with some modifications (Sanghvi et al., 2016; Kumar et al., 2021; and Parinayawanich et al., 2021). Feather powder was used as a substrate to measure the keratinase activity of the FUM-2 isolate. The procedure was as follows: Briefly, 20 mg of feather powder was suspended in 4 mL Tris-HCl buffer (50 mM, pH 8) and stirred thoroughly. Next, 1 mL of the crude enzyme was added and incubated at 37°C for 60 min in a shaker incubator at 150 rpm. Then, the reaction was stopped by adding trichloroacetic acid (2 mL, 10% v/v), centrifuged at 13,000 rpm for 10 min, and the supernatant was filtrated to remove unused substrates. An uninoculated culture medium was used as a control. Next, to evaluate the keratinase activity, the increase in absorbance at 280 nm was measured using a UV-160 spectrophotometer (Eppendorf Co., German).

The experiments were performed three times, and the average results were analyzed. The amount of enzyme that resulted in an increase in absorbance at 280 nm of 0.01 per 60 min was defined as one unit of keratinase activity.

2.6. Improvement of keratinase production

The culture medium was improved using the one factor at a time method and seven variable factors. Factors and different values used for FUM-2 improvement are shown in (Table 1).

Table 1. Factors and Values Used for Improvement of FUM-2 Isolate

Factors	Values
Incubation time	24, 48, 72, 96 and 120 h
Temperature	20, 30, 37, 40, 45, 50, 55 and 60 °C
pH	6, 7, 8, 9, 9.5, 10, 10.5, 11, 11.5 and 12
Different concentration of feather substrate	0.25, 0.5, 1, 1.5, 2 and 2.5 % w/v
Additional carbon sources	glucose, mannitol, starch, lactose, sucrose and dextran (1%, w/v)
Nitrogen sources	ammonium nitrate, ammonium chloride, tryptone, yeast extract and peptone (1%, w/v)
Inoculum size	1-6 %, v/v
Aeration rates	25, 50 and 75% at 150 rpm

2.7. Substrate specificity

The ability of the selected isolate to use different keratinous substrates was investigated. To do this, 1% (w/v) of the three different substrates, poultry feathers (β-keratin), ostrich feather (β-keratin), and human hair (α-keratin), was added to the optimal culture conditions (45 °C, pH 11, 150 rpm) and enzyme activity was measured as mentioned above.

2.8. UV-mutagenesis (Physical mutagenesis)

Mutation was performed in both liquid and solid media using a 15 W UV lamp with a wavelength of 260 nm. In the solid method, the cells were

harvested from an overnight culture, washed three times using normal saline by centrifuging at 6500 rpm, and resuspended to the equivalent of a 1 McFarland standard in normal saline. Six serial 10-fold dilutions of 10^{-1} to 10^{-6} were then made, and 100 µL of each dilution were plated onto Skim Milk agar, exposed to UV rays for 10, 15, and 20 minutes at a distance of 30 cm, and incubated at 37 ℃ for six days. In the liquid method, 3-4 ml of bacterial suspension were diluted to the equivalent of a 1 McFarland standard, poured onto sterile glass plates, and mutagenized using the abovementioned method. Next, under red light, six serial 10-fold dilutions of 10^{-1} to 10^{-6} were made, and 100 µL of each dilution was plated onto Skim Milk agar and incubated at 37 ℃ for six days. Untreated plates were used as a control. Data from the inhibition zone and the colony diameter were recorded for 6 days at 24-hour time intervals. Colonies with a halo zone larger than the parent isolates were selected as the mutant isolates and cultured in a nutrient agar medium for further work (Lateef et al., 2015; Javed et al., 2013).

2.9. EtBr- mutagenesis (Chemical mutagenesis)

To do this, overnight cultures of the bacterial strains, FUM-2, and physical mutagenic strains were centrifuged at 6500 rpm for 10 minutes, and supernatants were removed. The pellets were washed three times with sterile normal saline, resuspended in sterile normal saline to reach turbidity of 1 McFarland, and distributed into microtubes. Then, they were treated with ethidium bromide at concentrations of 50, 100, and 150 µg/mL and kept at room temperature for 45, 90, and 120 minutes. Next, the microtubes were centrifuged at 13,000 rpm for 10 minutes, and the supernatants were removed. Then, ethidium bromide was eliminated by washing pellets using sterile normal saline to stop mutagenesis. Next, serial dilutions of bacteria were prepared, and 100 µL of each dilution was spread on Skim Milk agar and incubated at 37 ℃ for six days (Soliman et al., 2016; Suribabu et al., 2014). Untreated plates were used s a control. The diameter of the halo zone around the colonies and the diameter of the colonies were measured and recorded using the same method mentioned above. The colonies with a halo zone larger than the parent isolates were selected as mutant isolates and cultured in a nutrient agar medium for further work. At the second screening, selected mutants and wild-type (FUM-2) were cultured in basal medium (FMB inoculated), incubated under optimal conditions of 45 ℃, pH 11, and 150 rpm for 48 h, and the enzyme activity was measured.

2.10. Improvement of culture conditions for the selected mutants

Culture conditions were optimized and compared to the wild-type results obtained under optimal conditions to enhance the keratinolytic activity of mutants (Table 2).

Table 2. Factors and Values Used for Improvement of Mutants

Factors	Values
Incubation time	24, 48, 72, 96 and 120 h
Different concentrations of feather substrate	0.25, 0.5, 1, 1.5, 2 and 2.5 % w/v
Aeration rates	25, 50 and 75% at 150 rpm
Inoculum size	1-6 %, v/v 1x, 2x, 3x, 4x, 5x and 6x

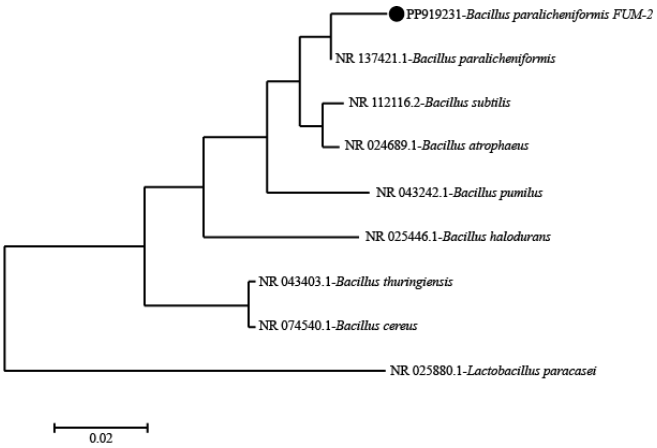
2.11. Statistical analysis

Statistical analysis was performed using one-way and Tukey HSD analysis of variance with Dunnett's post-hoc test at 95% confidence level and $p < 0.05$.

3. Results and Discussion

Out of five keratinase-producing bacteria isolated from contaminated soil of a poultry farm near Mashhad, Iran, the best keratinase producer, FUM-2, was selected for further experiments. Biochemical, morphological, and molecular analysis identification of the isolate indicated that the isolate belonged to *Bacillus* spp. with a 99.4% similarity to *Bacillus paralicheniformis* KJ-16 (Fig. 1) and from now on named *Bacillus paralicheniformis* FUM-2 (accession number PP919231) in the text.

Figure 1. The phylogenetic tree of *Bacillus paralicheniformis* FUM-2; *Lactobacillus paracasei* NR 025880 designated as Outgroup.



3.1. Improvement of enzyme production

At 45 °C and 48 h of incubation time, the strain showed a 59.9% increase in keratinase production (from 507.6 to 847 U/mL), which drastically decreased after 120 h of incubation (Fig. 2A and 2B) as the medium probably ran out of the substrate. In line with our results, Abdel-Fattah et al. (2018) also reported the maximum keratinolytic activity at 42 °C for *Bacillus licheniformis*. The available data show that although some keratinase enzymes are more active at acidic or high alkaline pH, most microbial keratinases are active in the range of neutral to alkaline pH with an optimum pH of 7.5 to 9 (Nnolim et al., 2020; Borhani et al., 2019; Daroit & Brandelli, 2014). To be useful and usable in industry, proteases must be stable and active in a wide range of pH. Many proteases, especially keratinases, show high stability at a wide pH range, making them suitable for biotechnological application in various industrial sectors, especially in detergent and leather industries (Moridshahi et al., 2021; Salwan & Sharma, 2019). The results showed that although there was no statistically significant difference ($p<0.05$) in enzyme activity over a wide range of pH (6-11.5), the strain was able to produce keratinase at the mentioned pH range with the maximum activity at pH 11 (Fig. 2C).

The bacterium was also able to degrade and use both α and β keratin without the need for any additional carbon and nitrogen sources (940 U/mL) (Figs. 2D and 2E), which agrees with results obtained by Mahta et al. (2014), Moridshahi et al. (2020), and Mini et al. (2015). Simple and available carbon and nitrogen sources reduce the consumption of complicated proteins such as keratin (Mehta et al., 2014). Also, excess carbon and nitrogen sources reduce enzyme production in some microorganisms, possibly due to the catabolite repression mechanisms in different bacteria (Brandelli et al., 2010).

A substrate concentration of up to 2% resulted in optimal keratinase production (Fig. 2 F). A 75% aeration rate and 2-3% inoculum size provided the best condition for keratinolytic activity (Figs. 2G and 2H). Culture improvement using the one factor at a time method, the enzyme production increased up to 3.36 fold from 507.6 U/mL to 1706.4 U/mL. Based on the results, temperature and aeration rate significantly affected keratinolytic activity with 66.8% and 37.6% increase in enzyme production, respectively.

3.2. Substrate specificity

Different keratinous materials (1% w/v) were tested, and the chicken and ostrich feathers were the best for keratinase production, with enzyme production of 972 U/mL and 958.5 U/mL, respectively. The hair substrate with a 299 U/mL enzyme production shows the ability of the isolate to use and degrade both α and β-keratin substrates. As shown in Fig. 3, there was no significant difference ($p <0.05$) between enzyme yield from chicken and ostrich feathers.

3.3. Physical and chemical mutagenesis

The results showed that the physical mutagenesis (UV radiation) worked better in liquid than solid medium. Mutated strains could grow and form a halo zone only at 10- and 15-minute UV exposure but not at 20 minutes.

Figure 2. The changes of keratinase activity of *Bacillus paralicheniformis* FUM-2 under the effect of different factors, A) temperature, B) incubation times, C) pH, D) carbon source, E) nitrogen source, F) substrate%, G) aeration and H) various inoculum; a, b, c, and d, indicated the statistical difference.

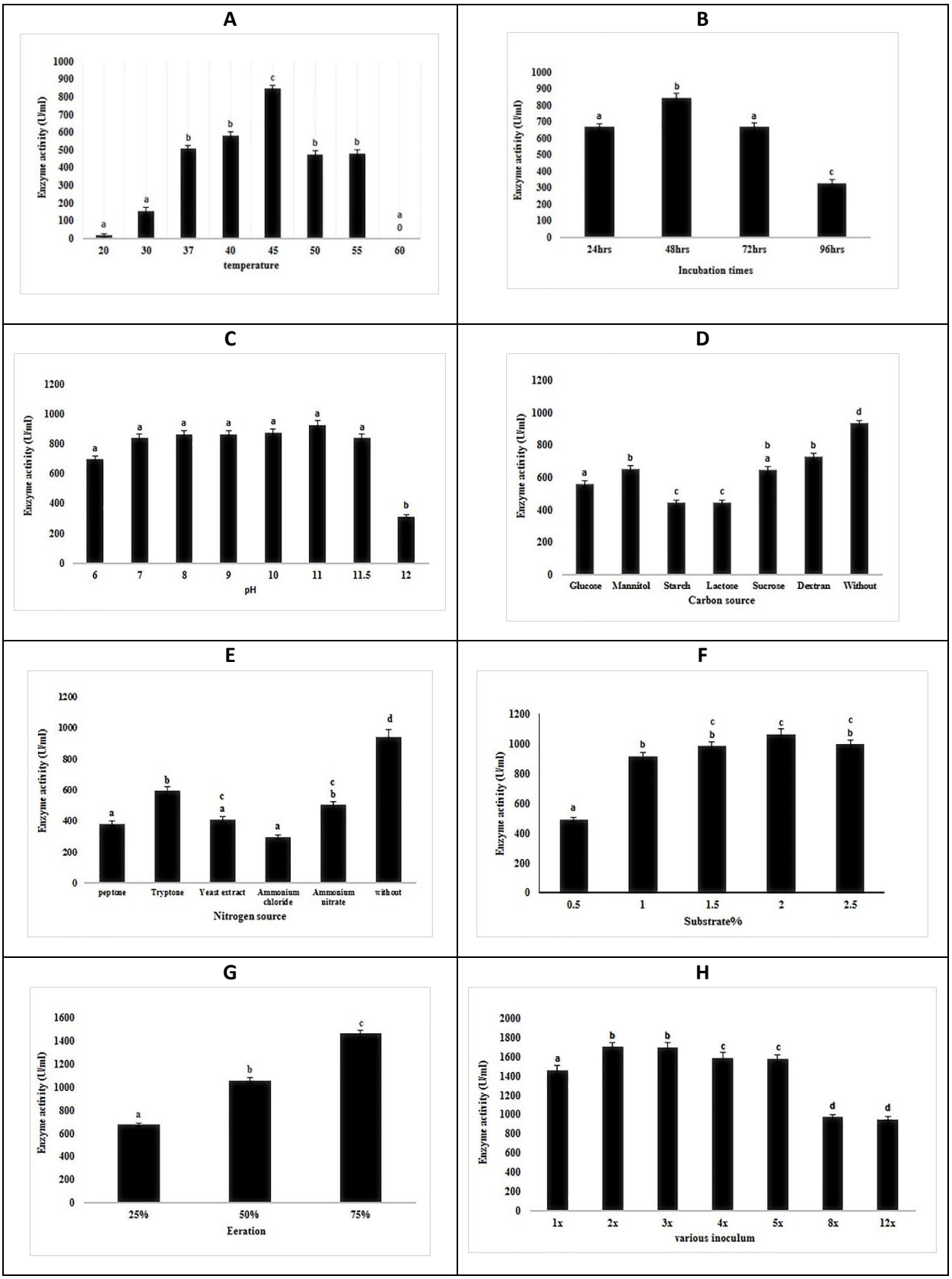
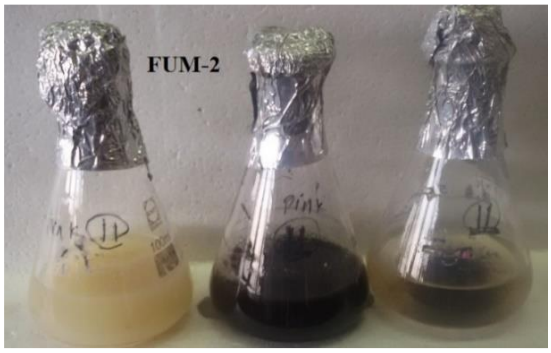
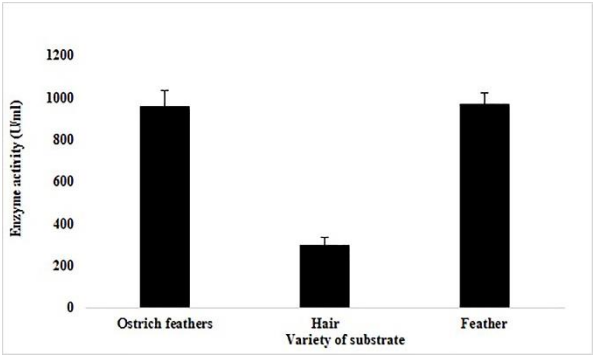


Figure 3. Enzyme activity of *Bacillus paralicheniformis* FUM-2 in the presence of 1% (w/v) of the three different substrate types, poultry feathers (β-keratin), ostrich feather (β-keratin) and human hair (α-keratin) in the optimal culture conditions (45 °C, pH 11, 150 rpm) (A & B).

A



B



Keratinase activity of mutants was evaluated over three days. The 15-minute UV-treated mutant of UVF2-1 showed only a 10% increase in keratinolytic activity compared to its parent, while a 10-minute UV-treated mutant of UVF2-D showed a 36.3% increase in keratinolytic activity compared to its parent (data is not shown), which is in line with results obtained by Zhang et al. (2012) of 35.5%.

Ethidium bromide was used as a chemical mutagenic agent to enhance keratinolytic activity in both the *B. paralicheniformis* FUM-2 and UVF2-D mutants. No suitable mutant was isolated from *B. paralicheniformis* FUM-2, but seven double mutants were isolated from the UVF2-D mutant, and their keratinolytic activity was measured. Accordingly, we can conclude that chemical mutagenesis is more effective in increasing the production of proteases if the isolates are first exposed to physical mutagenic agents. Zhang et al. (2012) believed that a combination of multiple mutagenic treatments (physical and chemical mutagens) increases enzyme production.

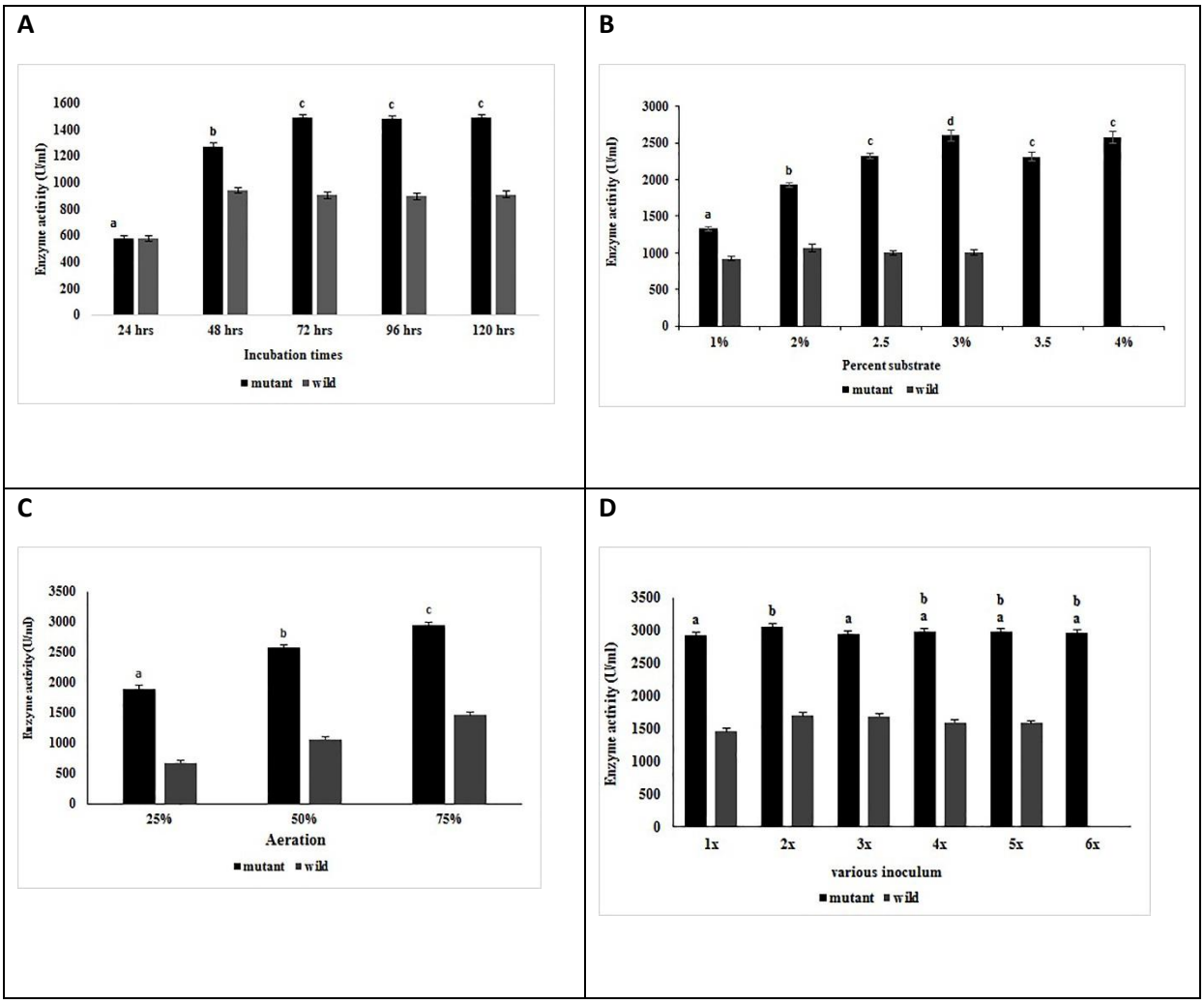
Our results showed an increased keratinolytic activity in three double mutants of EUF2-D1, EUF2-D2, and EUF2-D3 compared to their parent, amount which the EUF2-D2 double mutant showed an increase of 62% in keratinolytic activity compared to *B. paralicheniformis* FUM-2 (Table 1) in FMB medium under the conditions of pH 11.5, 45 °C, 150 rpm, 75% aeration rate, 1% feather meal, 1x inoculation size.

Mariyam et al. (2011) reported that the development of a mutation depends on the time and amount of the mutagenic agents. Our results showed that 90- and 120-minute treatments, along with 100 and 150 μg/mL of ethidium bromide, were more effective in mutagenesis.

3.4. Culture improvement of superior mutant

According to the results, the EUF2-D2 double mutant showed the highest keratinolytic activity and was therefore used for further experiments. Its keratinolytic activity was optimized using one variable at a time method, and accordingly, EUF2-D2 double mutant showed its highest keratinolytic activity on the third day of incubation at 45 °C and 3% substrate. In comparison, the double mutant isolate (3060U/mL, Fig. 4d) showed a 79.9% increase in keratinase production compared to the wild-type parent (1701U/mL, Fig. 4d) under optimal conditions (pH 11, 45 °C, 150 rpm, 75% aeration rate, 2% feather meal, 2x inoculation size) (Fig. 4). The bacterium was also able to decompose and tolerate high substrate concentrations.

Figure 4. The changes of keratinase activity of UVF2-D2 mutant and wild strain, under the effect of different factors, A) incubation times, B) present substrate, C) aeration and D) various inoculum; ■: mutant, □: wild type; a, b, c, and d, indicated the statistical difference.



4. Conclusion

In the present study, we found a native keratinolytic-producing bacterium, and using simple and cheap methods like mutagenesis and culture improvement, we were able to improve the bacterium so that it can tolerate high substrate concentration, has higher enzyme activity, and is active in a wide range of pH from a light acid to high alkaline pH, making it a valuable candidate for biotechnological purposes.

Author contribution:

All authors contributed to the study's conception and design.

Conflict of interest

The authors declare no conflict of interest

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Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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