Improvement of *Pichia kudriavzevii* Egyptian isolate for keratinase production

Bigad E. Khalil[a], Hayam F. Ibrahim[b], Nagwa M. Abd El-Aziz[a]

Departments of [a]Microbial Genetic, [b]Genetics and Cytology, Biotechnology Research Institute, National Research Centre, Cairo, Egypt

Correspondence to Bigad E. Khalil, PhD, Department of Microbial Genetics, Biotechnology Research Institute, National Research Centre, Dokki, Giza 12622, Egypt. Tel: +20 102 562 9343; fax: +20 233 370 931; e-mail: bigad1982@gmail.com

Received: 29 December 2021
Revised: 23 February 2022
Accepted: 27 February 2022
Published: 21 July 2022

Egyptian Pharmaceutical Journal 2022, 21:192–206

Background and objective
Keratinases are gaining considerable momentum in green technology because of their endowed robustness and multifaceted application potentials, such as valorization of keratinous agro-waste. Therefore, the production of novel keratinases from relative yeasts grown in agro-waste formulated medium is cost-effective and imperative for the sustainability of thriving bioeconomy.

Materials and methods
A total of 51 yeast isolates were isolated from 10 different poultry farms and assayed for keratinase-specific activity. Molecular identification of the high-efficiency keratinase-producing yeast isolate was done by PCR amplification, employing sequencing of internal transcribed spacer regions of yeast. Mutagenesis induction with ethidium bromide, ultraviolet, and ethyl methane sulfonate (EMS) was done in a multistep mutation-induction process for creating super keratinase-productive mutants. Response surface methodology optimization of culture conditions for high-productive mutant was carried out using different parameters such as incubation time, pH, carbon sources, and nitrogen sources to test keratinase activity. Inter-simple sequence repeat (ISSR-PCR) was applied to study the genetic diversity of isolated *Pichia kudriavzevii* YK46 compared with their five mutants.

Results and conclusion
The results indicated that the isolate with the highest keratinase activity was isolate no. 46, which recorded 164.04 U/ml. It was identified as *P. kudriavzevii* and was submitted to NCBI under accession number 'OK092586'. It was named as *P. kudriavzevii* YK46. Results of mutagenesis showed that the best keratinolytic efficiency mutant was designated as EMS-37, which showed an activity of 211.90 U/ml. After response surface methodology optimization of culture conditions for mutant EMS-37, the maximum keratinase activity was noted after an optimized condition at pH 5, 72 h of incubation time, 2.5% glucose, and 2.5% beef extract (as carbon and nitrogen sources), with an activity of 240.172 U/ml (Run3). Inter-simple sequence repeat showed that the highest total and polymorphic with unique bands were revealed in the mutant EMS-37, with 82 and 54 bands, respectively, whereas the mutant EMS-56 showed 72 and 44 bands, respectively, compared with the wild-type strain *P. kudriavzevii* YK46, with 86 and 58 bands, respectively. The data obtained showed that mutant EMS-37 was the highest producer of keratinase enzyme. It had seven unique bands. These bands might be related to the increase in the productivity of keratinase enzyme.

Keywords:
inter-simple sequence repeat-PCR, internal transcribed spacer, keratinase, mutagenesis, mutation, response surface methodology

Egypt Pharmaceut J 21:192–206

1687-4315

Introduction

As a byproduct of poultry waste, a million tons of feathers are produced annually from the poultry industries [1]. Owing to improper handling, more than five million tons of feather wastes are dumped into baren lands, which leads to a negative effect on environment, thereby leading to various environmental pollutants [2]. Feather is made up of a rich source of protein called keratin, and as keratin cannot be used directly, so physical and chemical treatments are required to destroy certain amino acids for better digestibility and get converted into a digestible dietary protein for animal feed [3,4]. Feather pollution can be controlled by alternate use of feather wastes [5], but the drawback behind these methods is that they are high-energy consuming and destruct certain other amino acids such as methionine, lysine, and tryptophan [5,6], which affects protein quality [7]. Many researchers have isolated

microorganisms like fungi, yeasts, actinomycetes, and several bacterial species such as *Bacillus sp.* and *Pseudomonas sp.* that can produce keratinase, which indicates that they can be used to produce keratinase, which can degrade keratin found in the feather with a metabolic reaction [8–11]. Microbial degradation of feather improves the nutritional quality of the feather meal and is a good alternative for physio-chemical treatment [12,13]. Among all of the proteolytic enzymes, keratinase has gained attention in the past few years owing to its potential application mainly in the hydrolysis of keratin. Keratin and keratinases have various applications in biotechnology like feed for cattle, removal of hairs and feathers in leather and poultry industries, to generate natural gas, in textile industries for shrink proofing of wool, and for removing blockages in sewage systems [14–16]. So, the present study was conducted to isolation and assay keratinase-producing yeast isolates. Molecular identification was employed for high-efficiency keratinase-producing yeast isolates. The production of keratinase enzyme was increased by genetic improvement of yeast strain using physical mutagenesis like ultraviolet (UV) and chemical mutagenesis like ethidium bromide (Eth.Br.) and ethyl methane sulfonate (EMS). For optimization, response surface methodology (RSM) was carried out to optimize cultural conditions for the overproduction of keratinase-producing yeast strain. Moreover, inter-simple sequence repeat (ISSR-PCR) markers were applied to study the genetic diversity of wild-type yeast strain compared with their mutants.

Materials and methods

Collecting samples and preparation of keratin powder

A total of 10 poultry waste samples were collected from 10 different farmers in Cairo, Egypt, from a depth of 5–10 cm, in sterile containers. All samples were collected on the same day. Chicken feathers obtained from local poultry waste samples were used to prepare keratin powder according to literature studies [17–20].

Culture media and isolation of keratinase-producing yeasts

The basic medium used for isolation and fermentation of the feather-degrading microorganisms was according to Duarte *et al.* [17]. Yeast peptone glucose (YPG) agar medium (Himedia, West Chester, Pennsylvania, USA) was used for yeast preparations. The keratinolytic producing yeast strain YK46 used in this study was collected from local poultry waste farmers in Cairo, Egypt. It was isolated on isolation agar medium and defined as keratinase-producing yeast based on their morphological difference as reported by Duarte *et al.* [17]. It was further subcultured on a YPG agar slant for further studies.

Preparation of crude enzyme extract and quantitative keratinase activity

A pure single colony of freshly selected yeast isolate, which was grown on YPG agar medium, was aseptically transferred for keratinase production using fermentation medium according to Duarte *et al.* [17]. After incubation, the supernatant containing the enzyme extract was used in quantitative keratinase assay. Keratinase enzyme activity was measured using keratin powder as a substrate. In brief, 1.0 ml of cell-free supernatant (crude enzyme) was incubated with 10-mg keratin powder in 1.5-ml phosphate buffer (pH 7.4) and incubated at 37°C for 1 h as reported by Duarte *et al.* [17].

Determination of protein content and residual hydrolysates

Protein content was determined as described by Bradford [21] using bovine serum albumin as a standard. The residual hydrolysates were composed of undigested feathers and cells. The residual hydrolysate's weight was determined according to Cai *et al.* [19].

Molecular identification of keratinolytic yeast

For DNA extraction, the extraction method was applied according to the instructions of Applied Biotechnology Co., Ismalia, Egypt. The yeast isolate was identified based on partial sequencing of the internal transcribed spacer (ITS) region, using the universal primers as follows: forward primer ITS1 (5′-TCC GTA GGT GAA CCT GCG G-3′) and reverse primer ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′). PCR product was purified, dyed, and sequenced as described previously [22]. PCR condition was 95°C for 3 min, 30 cycles of denaturation at 95°C for 30s, annealing at 50°C for 30 s, and extension at 72°C for 1 min/1 kbp, with final extension 72°C for 5 min. The PCR product was purified using a MEGA Quick-Spin total fragment DNA purification kit as instructed by the manufacturer. Sequencing was performed by the Sanger method. The fragment produced was around 356 bp. The obtained sequence was subjected to BLAST queries using the 'blastn' algorithm implemented at NCBI to determine the putative identity of the strain (http://www.ncbi.nlm.nih.gov/BLAST). Based on sequence homology, the first hit on BLAST was *Pichia kudriavzevii* with

accession number OK092586 [23]. The phylogenetic tree was constructed using MAFFT alignment [24]. https://mafft.cbrc.jp/alignment/server/phylogeny.html.

Ethidium bromide, ethyl methane sulfonate, and ultraviolet mutagenesis

The wild-type yeast *P. kudriavzevii* YK46 was cultured on YPG broth medium at 28°C for 48 h. Then, 10 ml of culture was centrifuged at 9000*g* at 4°C for 10 min to separate the cell biomass. Then, the cell biomass pellet was resuspended in 10 ml (0.9%) of sterilized saline. For UV-induced mutagenesis, sterile petri dishes containing 4 ml was exposed to UV light (UV-dispensing cabinet fitted with 15-W lamps with about 90% of its radiation at 265 nm) [25]. The plates were placed 30 cm away from the center of UV light source and exposed to UV light. For Eth. Br. and EMS-induced mutagenesis, 10 µg/ml Eth.Br. [26] and 3% EMS [17] were added and incubated at 37°C for 60 min. Then, the UV plates were incubated in dark overnight to avoid photoreactivation. After treatments, cells were washed with sterile saline after being obtained from the centrifugation at 2800*g* for 15 min. Successive serial dilutions were prepared, and 0.1-ml aliquot suspension was spread on YPG agar plates [17,27] and incubated at 28°C for 48 h. Then, *P. kudriavzevii* YK46 mutants appeared after the incubation time, which were subjected to quantitative keratinase-specific activity to select high-efficiency keratinase-producing mutants as reported by Duarte *et al.* [17].

Medium optimization conditions and experimental design by response surface methodology statistical analysis for keratinase production improvement

The basic medium was optimized with various factors that influence keratinase production. The various physicochemical parameters of fermentation were optimized, which included pH (5, 6, 7, 8, and 9), incubation time (1, 2, and 3 days), 0.5% concentration of carbon sources (glucose, sucrose lactose, xylose, and fructose), and 0.5% concentration of nitrogen sources (tryptone, peptone, beef extract, malt extract, and yeast extract) according to Barman *et al.* [28]. To identify suitable carbon and nitrogen sources for keratinase production, the enzyme assay was carried out at 28°C, shaken 200 rpm, 48-h incubation, pH 5, and 1% feather.

In the RSM study, four autonomous factors (pH, incubation time, glucose %, and beef extract %) were represented at two levels, high concentration (+1) and low concentration (−1), in 30 runs, as shown in Table 3. Each row represents a trial run, and each column represents an independent variable concentration. With the insignificant ones eliminated, a smaller and more fitting collection of factors was obtained and analyzed. The design of 30 experiments and the coded and uncoded levels of the four investigated independent variables are listed in Table 4. The second-order observed model can be achieved from the experimental data and the relationship between the response yield (keratinase activity) and the variables through the polynomial regression analysis. The form of the second-order polynomial model is as follows:

$$Y_{Activity} = ß_0 + ß_1X_1 + ß_2X_2 + ß_3X_3 + ß_4X_4 + ß_{12}X_1X_2 + ß_{13}X_1X_3 + ß_{14}X_1X_4 + ß_{23}X_2X_3 + ß_{24}X_2X_4 + ß_{34}X_3X_4 + ß_{11}X_1^2 + ß_{22}X_2^2 + ß_{33}X_3^2 + ß_{44}X_4^2$$

where Y activity is the predicted production of keratinase (U/ml), and X_1, X_2, X_3, and X_4 are the independent variables corresponding to the chosen affecting factors. $ß_0$ is the intercept; $ß_1$, $ß_2$, $ß_3$, and $ß_4$ are linear coefficients; $ß_{11}$, $ß_{22}$, $ß_{33}$, and $ß_{44}$ are quadratic coefficients; and $ß_{12}$, $ß_{13}$, $ß_{14}$, $ß_{23}$, $ß_{24}$, and $ß_{34}$ are cross-product coefficients [29].

Statistical analysis

The statistical analysis of the model was performed to assess the analysis of variance. Statistical significance of the model equation was determined by Fisher's test value, and the proportion of variance explained by the model was given by the assessment of multiple coefficients for each variable. The quadratic models were showed as contour plots (3D), and the response surface curves were generated by using Design expert 11 program.

Molecular markers inter-simple sequence repeat-PCR for wild-type and mutant yeast strains

ISSR-PCR analysis was applied according to Zietkiewicz *et al.* [30] and procured from University of British Columbia (UBC, Biotechnology Laboratory, Vancouver, Canada) based on core repeats anchored at the 5′ or 3′ end as in. The genomic DNA of six yeasts was amplified using Taq-DNA PCR as in the manufacturer's instructions master mix (amaR one PCR) for ISSR primers. The PCR reaction consisted of a 5-min incubation period at 94°C followed by 40 cycles of 94°C/50 s (45°C)/1 min, and 72°C/1 min, with a final extension step of 72°C/10 min. The PCR product was separated by 1.5% agarose gel electrophoresis using a TAE buffer and 0.004% red safe dye Table 1.

Table 1 The inter-simple sequence repeat-PCR primer name and sequences

Primers	Sequence	Annealing
ISSR-152	5′-(AG)8YC-3′	45°C
ISSR-153	5′-(AG)8YG-3′	
ISSR-154	5′-(AC)8YT-3′	
UBC-857	5′-(AC)8YG-3′	
UBC-851	5′-(GT)8YG-3′	
ISSR-157	5′-CGCGATAGATAGATAGATA-3′	
ISSR-158	5′-GACGATAGATAGATAGATA-3′	
ISSR-836	5′-(GT)6YG-3′	

ISSR, inter-simple sequence repeat; UBC, University Of British Columbia.

Results

Isolation and quantitative keratinase assay of yeast isolates

A total of 51 single yeast colonies were isolated from 10 different poultry waste samples collected from 10 different farmers in Cairo, Egypt. Using serial dilution method, each sample was spread on isolation agar medium and incubated at 28°C for 2 days. Selection of yeasts was performed based on their morphological difference and then stored on YPG agar slants for further study. The obtained 51 yeast isolates were assayed for keratinase production, and specific activities, and the results showed a broad range of keratinase-specific activity. Of them, only one isolate showed the highest keratinase-specific activity, which was isolate no. 46, recording 164.04 U/ml. Another keratinase assay of activity for all isolates was protein contents, which were measured at A_{28o} as indicated by residual feather measurements. Among all of them, the isolate with the highest keratinase activity found in this study was no. 46 recorded in basic medium, with residual feather weight of 150±0.017 mg and protein content of 1.450 mg/ml. It was capable of carrying out 1% feather degradation completely within 48 h, and then, it was named as YK46, as shown in Fig. 1.

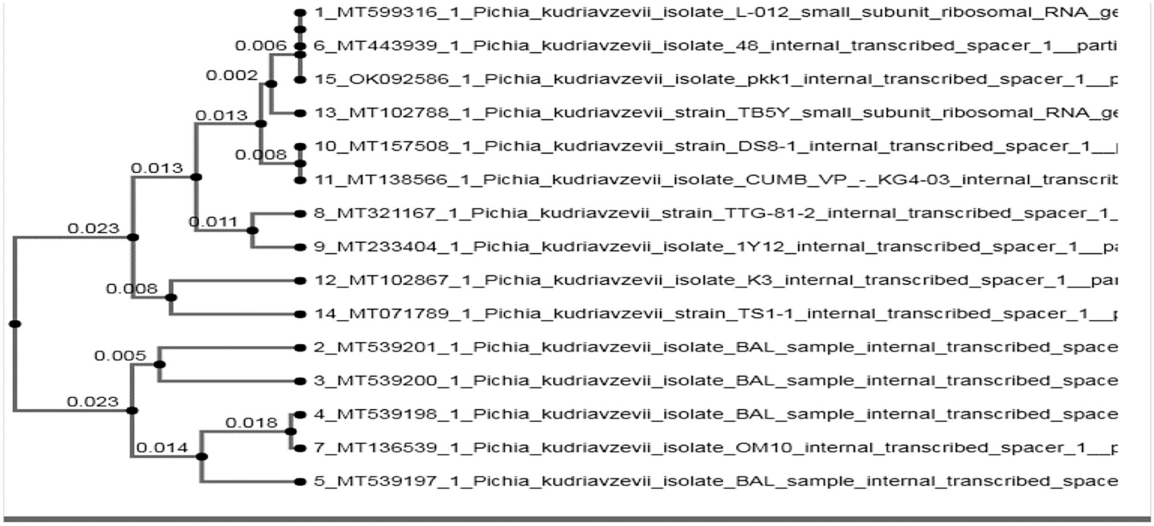
Figure 1

Colony morphology of yeast isolateYK46 on yeast peptone glucose (YPG) agar medium.

Molecular identification of yeast YK46 using internal transcribed spacer region gene alignment in Genbank (Blast)

Nucleotide's sequence of isolate YK46 ITS regions was performed using Blast program. Nucleotide alignment of isolate YK46 ITS regions gene in GenBank (Blast) under accession number (OK092586) showed 100% similarity with *P. kudriavzevii*. Phylogenetic tree showed that the isolate YK46 was found in the same group and closely related to *P. kudriavzevii* depending on ITS region sequence, and then, it was named *P. kudriavzevii* Y'K46, as shown in Fig. 2.

Figure 2



The phylogenetic tree of *Pichia kudriavzevii* YK46 accession no. OK092586 and the other isolates in GenBank.

Pichia kudriavzevii YK46 multistep mutation induction for keratinase production improvement

In the first step of mutation induction, parent *P. kudriavzevii* YK46 strain was treated with Eth.Br. to improve its keratinase enzyme productivity. After exposure time, a total of 61 *P. kudriavzevii* YK46 mutants survived. Then, they were assayed for keratinase activity, and only three mutants exhibited high efficiency in keratinase-specific activity. The results showed that mutant E-49 was the most hyperactive mutant, showing a higher keratinase-specific activity of 185.0 U/ml, with residual feather of 120±0.011 mg and protein content of 1.574 mg/ml, over the wild-type strain *P. kudriavzevii* YK46 (164.04 U/ml), as shown in Table 2. Mutant E-49 was then treated with UV in the second step of mutation induction. After exposure, a total of 45 mutants survived and were assayed for keratinase production. Only three mutants showed low efficiency in kerarinase assay. The results showed that mutant UV-16 was the most hyperactive mutant, giving 181.4 U/ml, with residual feather of 130±0.083 mg and protein content of 1.280 mg/ml, which was less than the wild-type mutant E-49 (185.0 U/ml). Mutant UV-16 was treated with EMS in the third step of mutation induction. After exposure time, a total of 60 mutants survived and were assayed for kerarinase production. Only five mutants showed high efficiency of keratinase activity. The results concluded that mutant EMS-37 was the most hyperactive mutant, giving 211.9 U/ml, with residual feather of 100±0.024 mg and protein content of 1.405 mg/ml, over the wild-type mutant UV-16 (181.4 U/ml), as shown in Table 2.

Medium optimization conditions by response surface methodology of selected mutant ethyl methane sulfonate-37 for keratinase production improvement

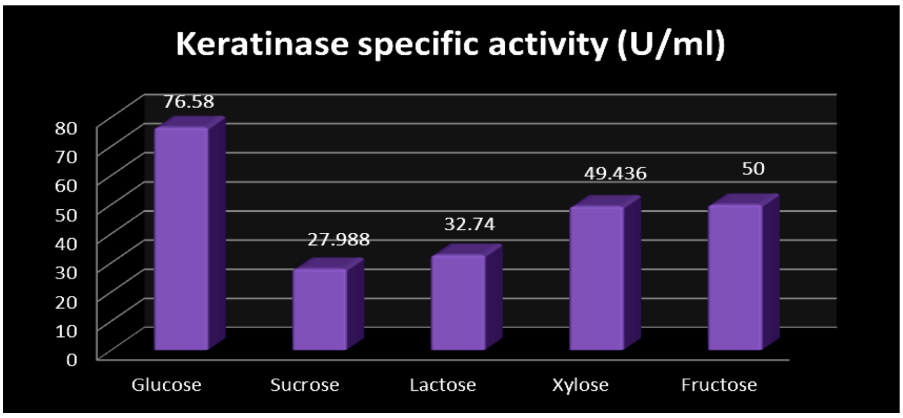
To identify the most suitable carbon and nitrogen sources for keratinase production by the mutant EMS-37, different carbon and nitrogen sources were tested. The results showed that the maximum keratinase assay was recorded in the glucose-supplemented medium (76.58 U/ml) and beef extract-supplemented medium (113.74 U/ml), as shown in Figs 3 and 4. Statistical and mathematical analyses of multivariable data obtained from RSM are one of the most important tools to improve and optimize keratinase production by yeast mutant EMS-37. In the RSM, a central composite model was selected for keratinase production using a 30-run experimental design on mutant EMS-37. A 30-trial matrix with four factors and three levels (−1, 0, and +1) including three replicates at the central point was performed. Table 3 illustrates the independent variables with coded matrix, responses, as well as experimental and predicted values for keratinase activity. The modification in the enzyme activity was observed during the 30 runs of the experiments. The alteration within the enzyme activity was owing to the different conditions in the experiment in each run, reflecting the importance of statistical optimization of

Table 2 Estimation of keratinase-specific activity produced by yeast strain *Pichia kudriavzevii* YK46 and their mutants after 48 h of incubation at 28°C

Pichia kudriavzevii YK46 strain	Keratinase-specific activity (U/ml)	Residual feather (mg)	Protein content (mg/ml)
First step ethidium bromide* mutagenesis			
Parent *Pichia kudriavzevii* YK46 strain	164.04	150±0.017	1.450
E-mutants			
E-34	171.0	130±0.032	1.320
E-49	185.0	120±0.011	1.574
E-56	179.0	140±0.076	1.509
Second step UV mutagenesis			
Parent E-49	185.0	120±0.011	1.574
UV mutants			
UV-16	181.4	130±0.083	1.280
UV-30	157.0	160±0.034	1.448
UV-43	169.8	150±0.065	1.357
Third step EMS** mutagenesis			
Parent UV-16	181.4	130±0.083	1.280
EMS mutants			
EMS-17	192.3	110±0.043	1.361
EMS-29	188.5	120±0.021	1.023
EMS-37	211.9	100±0.024	1.405
EMS-44	183.2	130±0.070	1.300
EMS-56	186.0	120±0.063	1.421

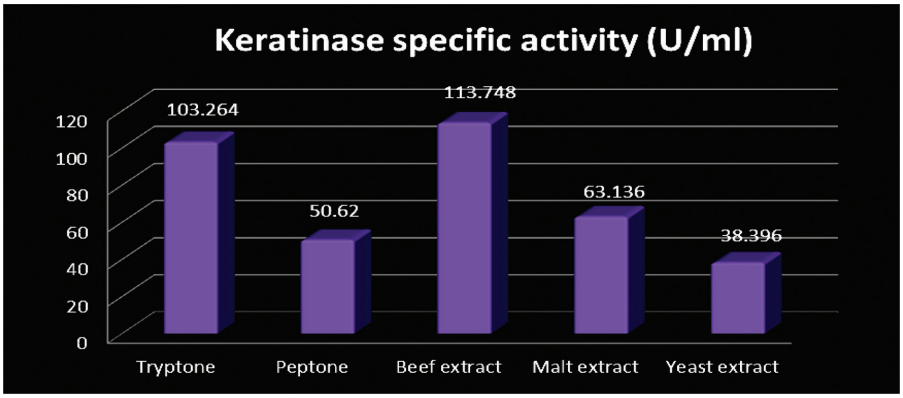
EMS, ethyl methane sulfonate; UV, ultraviolet. *Ethidium bromide: 10 μg/ml concentration. **EMS: 3% concentration.

Figure 3



Medium optimization conditions by supplementing different carbon sources of selected yeast mutant EMS-37. EMS, ethyl methane sulfonate.

Figure 4



Medium optimization conditions by supplementing different nitrogen sources of selected yeast mutant EMS-37. EMS, ethyl methane sulfonate.

fermentation condition over the traditional fermentation methodology. The maximum keratinase activity was obtained after an optimized culture condition at pH 5, 72 h incubation time, 2.5% glucose, and 2.5% beef extract with activity of 240.172 U/ml (Run3). The determination coefficient (R^2) showed the accuracy of the model. R^2 was 0.9981, explaining 99.5% of the variability in the response could be accounted for the model and was considered as a significant correlation. Therefore, the present value of R^2 confirms the reliability of the current model for keratinase production and exhibited a good correlation between the experiment and theoretical values. A second-order final equation in terms of actual factors is as follows:

$$Y, \text{Activity} = 140.98 - 43.38X_1 + 25.29X_2 + 8.55X_3 + 9.55X_4 - 9.02X_1X_2 - 6.40X_1X_3 - 6.02X_1X_4 + 4.76X_2X_3 + 4.30X_2X_4 + 5.34X_3X_4 - 23X_1^2 + 7.66X_2^2 + 0.1844X_3^2 - 7.35X_4^2$$

Where Y represents response or keratinase yield, and X1, X2, X3, and X4 are pH, incubation time, glucose percentage, and beef extract percentage, respectively.

The model validation

The validity of the proposed model was estimated by prediction of yeast mutant EMS-37 keratinase production for each trail of the matrix. The experimental results in Table 3 show that the maximum observed keratinase production (240.172) was very close to the predicted value (241.07) run3. Results obtained by analysis of variance for production of keratinase by yeast mutant EMS-37 are given in Table 4. It showed that the model was highly significant with an F value of 572.16 for keratinase production and showed that it was a significant model, which is evident from Fisher's F test along with a very low probability value (P model $>F$=0.0001). Values of 'Prob>F' that are less than 0.05 signify significant model, and at the same time, a relatively lower coefficient of variation (2.07%) was indicated, implicating a better precision and reliability of the experiments carried out. The results obtained from this study revealed that agreement between the actual values and the predicted values and all factors have a significant

Table 3 Design of different trials of the response surface methodology for independent variables and responses by yeast mutant ethyl methane sulfonate-37

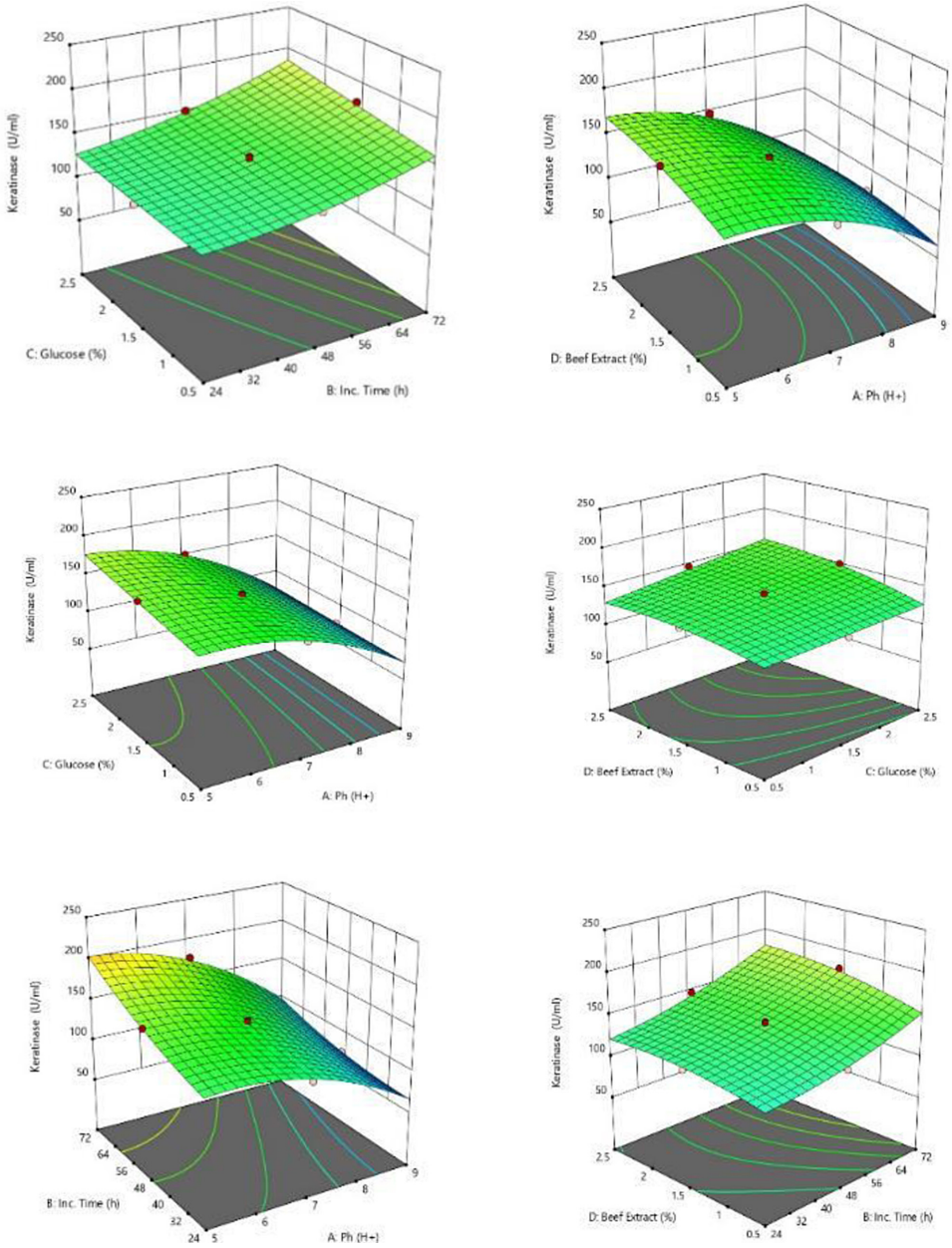
Run	Factor 1 A: ph H+	Factor 2 B: Inc. time h	Factor 3C: glucose %	Factor 4 D: beef extract %	Actual value keratinase U/ml	Predicted value	Residual
C	5 (−1)	24 (−1)	2.5 (+1)	2.5 (+1)	151.73	154.33	−2.60
2	5 (−1)	24 (−1)	2.5 (+1)	0.5 (−1)	124.024	121.12	2.90
3	5 (−1)	72 (+1)	2.5 (+1)	2.5 (+1)	240.172	241.07	−0.8978
4	7 (0)	48 (0)	1.5 (0)	1.5 (0)	142.536	140.98	1.56
5	5 (−1)	72 (+1)	0.5 (−1)	0.5 (−1)	162.028	161.92	0.1063
6	9 (+1)	24 (−1)	0.5 (−1)	0.5 (−1)	70.632	67.54	3.09
7	7 (0)	48 (0)	1.5 (0)	1.5 (0)	139.504	140.98	−1.47
8	7 (0)	48 (0)	2.5 (+1)	1.5 (0)	151.023	149.72	1.31
9	7 (0)	48 (0)	1.5 (0)	1.5 (0)	141.004	140.98	0.0270
10	9 (+1)	48 (0)	1.5 (0)	1.5 (0)	74.024	74.60	−0.5791
11	9 (+1)	24 (−1)	2.5 (+1)	0.5 (−1)	50.213	51.66	−1.45
12	9 (+1)	24 (−1)	0.5 (−1)	2.5 (+1)	53.482	55.33	−1.85
13	7 (0)	48 (0)	1.5 (0)	1.5 (0)	139.589	140.98	−1.39
14	9 (+1)	72 (+1)	2.5 (+1)	0.5 (−1)	85.362	85.11	0.2535
15	9 (+1)	72 (+1)	0.5 (−1)	2.5 (+1)	86.218	86.93	−0.7103
16	7 (0)	48 (0)	1.5 (0)	1.5 (0)	141.359	140.98	0.3820
17	5 (−1)	24 (−1)	0.5 (−1)	2.5 (+1)	125.213	123.27	1.94
18	9 (+1)	72 (+1)	2.5 (+1)	2.5 (+1)	110.254	111.44	−1.19
19	5 (−1)	72 (+1)	2.5 (+1)	0.5 (−1)	190.253	190.67	−0.4147
20	7 (0)	48 (0)	1.5 (0)	0.5 (−1)	120.321	124.08	−3.76
21	5 (−1)	24 (−1)	0.5 (−1)	0.5 (−1)	110.34	111.41	−1.07
22	7 (0)	48 (0)	0.5 (−1)	1.5 (0)	131.581	132.61	−1.03
23	5 (−1)	48 (0)	1.5 (0)	1.5 (0)	162.214	161.35	0.8604
24	5 (−1)	72 (+1)	0.5 (−1)	2.5 (+1)	190.149	190.97	−0.8185
25	7 (0)	48 (0)	1.5 (0)	1.5 (0)	141.026	140.98	0.0490
26	7 (0)	48 (0)	1.5 (0)	2.5 (+1)	147.22	143.18	4.04
27	7 (0)	72 (+1)	1.5 (0)	1.5 (0)	177.248	173.92	3.33
28	7 (0)	24 (−1)	1.5 (0)	1.5 (0)	120.298	123.35	−3.05
29	9 (+1)	24 (−1)	2.5 (+1)	2.5 (+1)	62.892	60.80	2.09
30	9 (+1)	72 (+1)	0.5 (−1)	0.5 (−1)	82.291	81.95	0.3408

Table 4 Analysis of variance for keratinase production by yeast mutant ethyl methane sulfonate-37

Source	Sum of squares	DF	Mean square	*F* value	*P* value
Model	55900.95	14	3992.93	572.16	<0.0001*
A-Ph	33865.47	1	33865.47	4852.69	<0.0001
B-Inc. Time	11509.02	1	11509.02	1649.17	<0.0001
C-Glucose	1317.37	1	1317.37	188.77	<0.0001
D-Beef extract	1641.00	1	1641.00	235.14	<0.0001
AB	1303.01	1	1303.01	186.71	<0.0001
AC	654.73	1	654.73	93.82	<0.0001
AD	579.26	1	579.26	83.00	<0.0001
BC	362.55	1	362.55	51.95	<0.0001
BD	295.42	1	295.42	42.33	<0.0001
CD	456.09	1	456.09	65.35	<0.0001
A^2	1370.43	1	1370.43	196.37	<0.0001
B^2	151.84	1	151.84	21.76	0.0003
C^2	0.0881	1	0.0881	0.0126	0.9121
D^2	139.86	1	139.86	20.04	0.0004
Residual	104.68	15	6.98		
Lack of fit	98.12	10	9.81	7.48	0.0192*
Pure error	6.56	5	1.31		
Cor total	56005.63	29			

R^2=0.9981; coefficient of variation=2.07%; >*F*<0.05. *Significant at probability.

Figure 5



Response surface plot of the interaction effect of (a) incubation time, pH (b) beef extract%, pH (c) glucose %, pH (d) beef extract %, incubation time (e), glucose %, incubation time, and (f) beef extract % and glucose % on keratinase production by yeast mutant EMS-37. EMS, ethyl methane sulfonate.

response on the keratinase production data, as presented in Table 4. The statistical optimization increased the biosynthesis of keratinase about 1-fold of that of the basal medium (U/ml). The influence on the yield of keratinase imposed by the factors and reciprocity between them is represented in Fig. 5. Response surface curves were designed to reveal the interaction between different variable factors and determine the optimum level of each variable for maximum response. Each figure indicated the effect of two factors, whereas the other factors were fixed at zero levels; the highest response value was detected at the result points glucose% 2.5, beef extract% 2.5, and pH 5 for 72 h.

Molecular markers (inter-simple sequence repeat-PCR) for strain *Pichia kudriavzevii* YK46 and their mutants

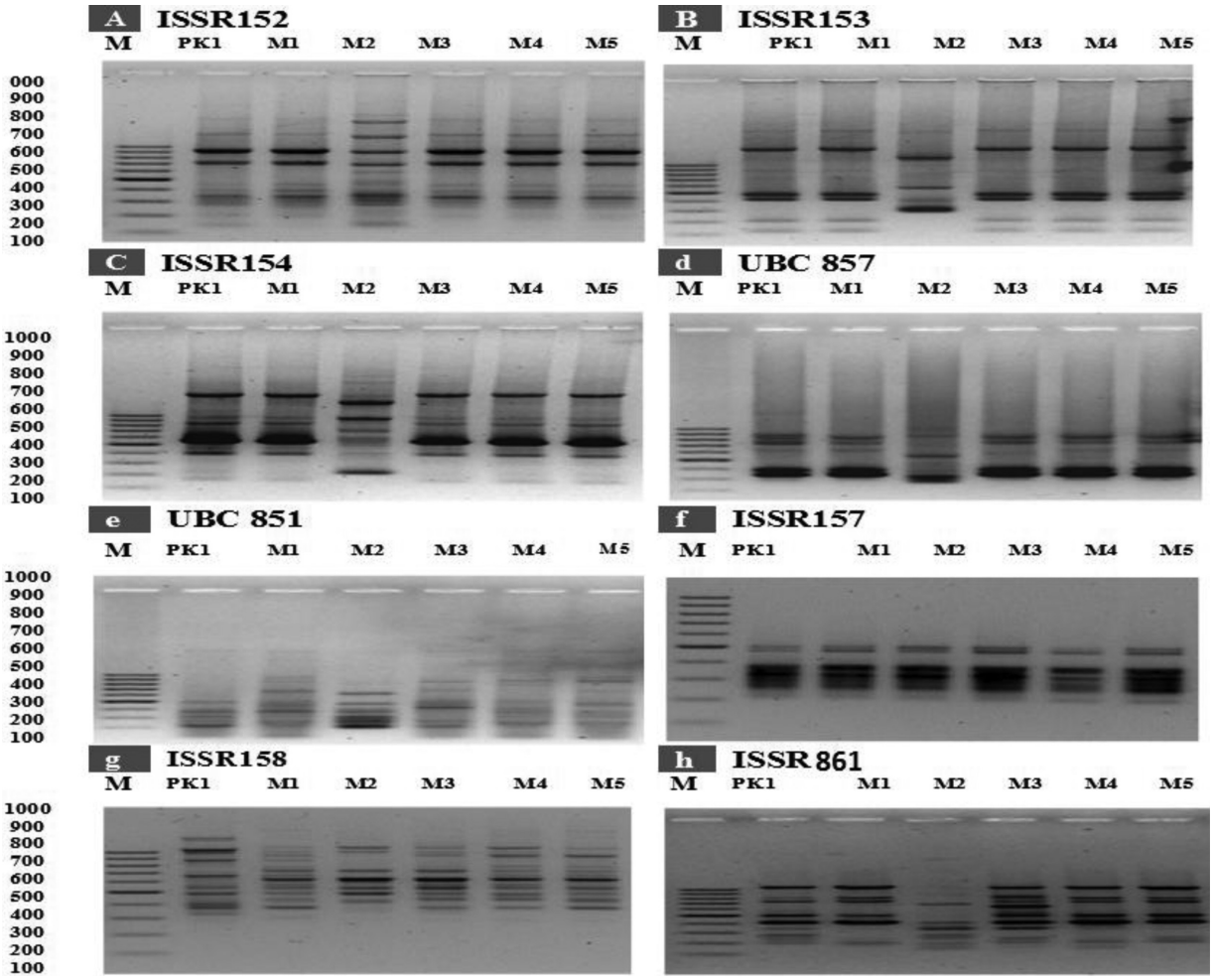
Molecular description using ISSR primer analysis profile of strain *P. kudriavzevii* YK46 and mutants. A total of 8 ISSR primers ISSR-152, ISSR-153, ISSR-154, UBC-857, UBC-851, ISSR-157, ISSR-158, and ISSR-861 produced a total of 150 markers. Moreover, the result indicated that 122 bands of them were polymorphic with unique bands displaying 81% polymorphism, as shown in Table 5 and Fig. 6. The highest number of total bands were displayed in the strain *P. kudriavzevii* YK46 (wild type) with 86 bands followed by the mutant EMS-37 with 82 bands, as

Table 5 Band variation and polymorphism percentage in six yeast samples using eight inter-simple sequence repeat primers

Primers 1	Total bands	Molecular size (bp)	Number of monomorphic bands	Number of polymorphic with unique bands	Uniq bands	Polymorphism %
ISSR-152	22	2463-151	4	18	7	81.81
ISSR-153	18	3197-138	2	16	7	88.88
ISSR-154	17	3573-167	5	12	5	70.58
UBC-857	18	1532-298	1	17	10	94.40
UBC-851	24	2109-170	0	24	9	100
ISSR-157	8	1026-209	8	0	0	0
ISSR-158	25	1725-123	6	19	8	76
ISSR-861	18	1184-125	2	16	8	88
	150		28	122		81

ISSR, inter-simple sequence repeat.

Figure 6



The inter-simple sequence repeats (ISSR) amplification pattern obtained for six samples of yeast strain *Pichia kudriavzevii* YK46 and their five mutants: (a) primer ISSR-152, (b) primer ISSR-153, (c) primer ISSR-154, (d) primer UBC-857, (e) primer UBC-851, (f) primer ISSR-157, (g) primer ISSR-158, and (h) primer ISSR-861.

shown in Table 6 and Fig. 7. The number of polymorphic with unique bands appearing in *P. kudriavzevii* KY46 (wild type) and EMS-37 were 58 and 54 bands, respectively. However, the EMS-56 displayed the lowest number of total bands and polymorphic unique bands with 72 and 44 bands, respectively, as shown in Tables 6 and 7 and Figs 7 and 8.

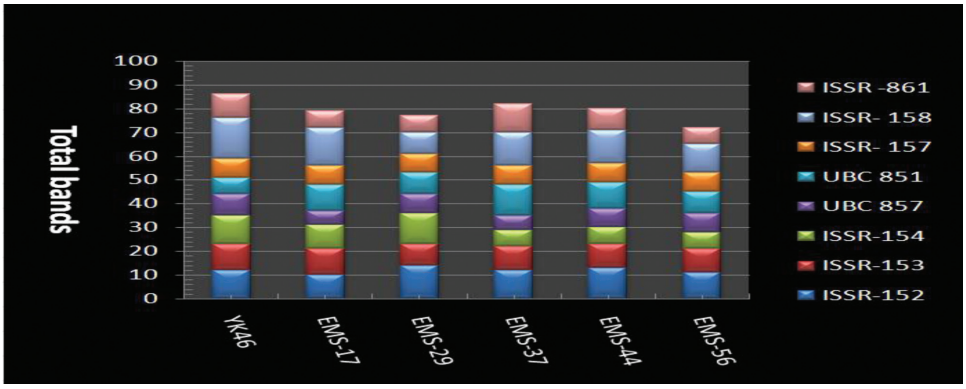
In the ISSR analysis to detect the change amplified bands of the selected mutant DNA against the wild

Table 6 The total bands generated from each primer for all yeast strain *Pichia kudriavzevii* YK46 (wild type) and their five mutants

Total bands	Primers								
Genotypes	ISSR-152	ISSR-153	ISSR-154	UBC-857	UBC-851	ISSR-157	ISSR-158	ISSR-861	Total
Pichia kudriavzevii YK46	12	11	12	9	7	8	17	10	86
EMS-17	10	11	10	6	11	8	16	7	79
EMS-29	14	9	13	8	9	8	9	7	77
EMS-37	12	10	7	6	13	8	14	12	82
EMS-44	13	10	7	8	11	8	14	9	80
EMS-56	11	10	7	8	9	8	12	7	72

EMS, ethyl methane sulfonate; ISSR, inter-simple sequence repeat.

Figure 7



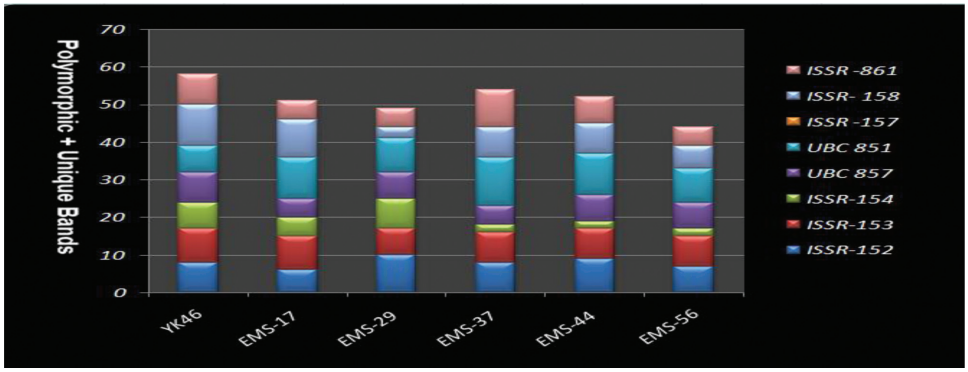
The relationship between total bands of the different primers used for the detection of six samples of yeast strain *Pichia kudriavzevii* YK46 (wild type) accession number OK092586 and their five mutants.

Table 7 Polymorphic with unique bands produced from each primer for all amplified fragments from yeast strain *Pichia kudriavzevii* YK46 (wild type) and their five mutants

	ISSR-152	ISSR-153	ISSR-154	UBC-857	UBC-851	ISSR-157	ISSR-158	ISSR-861	Total
Pichia kudriavzevii Yk46	8	9	7	8	7	0	11	8	58
EMS-17	6	9	5	5	11	0	10	5	51
EMS-29	10	7	8	7	9	0	3	5	49
EMS-37	8	8	2	5	13	0	8	10	54
EMS-44	9	8	2	7	11	0	8	7	52
EMS-56	7	8	2	7	9	0	6	5	44

EMS, ethyl methane sulfonate; ISSR, inter-simple sequence repeat.

Figure 8



The relationship between polymorphic with unique bands and polymorphism percentage of eight ISSR primers used for the detection of six yeast samples of yeast strain *Pichia kudriavzevii* YK46 (wild type) accession number OK092586 and their five mutants. ISSR, inter-simple sequence repeat.

type strain *P. kudriavzevii* YK46, many different variations were detected and recorded. Moreover, five mutants compared with the wild-type strain, and many different variations were detected and recorded. On the contrary, the highest unique bands appeared in EMS-29 followed by *P. kudriavzevii* YK46 and EMS-37 with 31, 13, and 7, respectively, whereas the lowest total bands were revealed in EMS-17, EMS-44, and EMS-56 with 2, 2, and 1 band, respectively.

The results showed that the mutant EMS-37 had the highest production of keratinase enzyme and also featured with seven unique bands. These bands appeared in primer UBC-851 with two bands with molecular sizes 706 and 356 bp, primer ISSR-158 appeared with two bands with molecular sizes 1192 and 304 bp, and primer ISSR-861 showed three bands with molecular sizes of 882, 637, and 497 bp, as shown in Table 8. These bands show a possibility related to the production of keratinase enzyme. This possibility deserves to be studied in the future.

Genetic similarity

Data shown in Table 9 recorded 15 pairwise comparisons to debate the genetic relationships among six *P. kudriavzevii* genotypes detected in terms of similarity. The genetic similarity ranged from 0.921 to 0.443, and the highest value of genetic similarity was 0.921 among EMS-44 and EMS-56, and the lowest value of similarity was 0.443 between EMS-29 and EMS-56, respectively, whereas the other values were in between.

Cluster analysis (phylogenetic tree)

Results of cluster analysis are shown in Fig. 9. All *P. kudriavzevii* genotypes were divided into two main clusters. Cluster I included EMS-29 only. Cluster II included two subclusters. The subcluster one was divided into two sub-subclusters. The sub-subcluster one included one group EMS-56 and EMS-44, and the sub-subcluster two included EMS-37 only. However, the subcluster II was divided into two sub-subclusters EMS-17 and *P. kudriavzevii* YK46, respectively.

Table 8 The unique bands and its molecular size produced from each primer for all amplified fragments from yeast strain *Pichia kudriavzevii* YK46 (wild type) and their five mutants

Primer name	Ms	*Pichia kudriavzevii* YK46	EMS-17	EMS-29	EMS-37	EMS-44	EMS-56	Unique bands
ISSR-152	2463	–	–	+	–	–	–	1
	2043	–	–	+	–	–	–	1
	1407	–	–	+	–	–	–	1
	1107	–	–	+	–	–	–	1
	773	–	–	+	–	–	–	1
	617	–	–	–	–	+	–	1
	272	–	–	+	–	–	–	1
ISSR-153	693	+	–	–	–	–	–	1
	544	–	–	+	–	–	–	1
	639	–	–	+	–	–	–	1
	792	–	–	+	–	–	–	1
	874	–	–	+	–	–	–	1
	369	–	–	+	–	–	–	1
	274	–	–	+	–	–	–	1
ISSR-154	2992	–	–	+	–	–	–	1
	3573	–	–	+	–	–	–	1
	695	–	–	+	–	–	–	1
	563	–	–	+	–	–	–	1
	463	–	–	+	–	–	–	1
UBC-857	1532	+	–	–	–	–	–	1
	1454	+	–	–	–	–	–	1
	999	–	–	+	–	–	–	1
	896	+	–	–	–	–	–	1
	821	+	–	–	–	–	–	1
	533	–	–	+	–	–	–	1
	434	–	–	+	–	–	–	1
	363	–	–	+	–	–	–	1
	333	–	–	+	–	–	–	1
	298	–	–	+	–	–	–	1

(*Continued*)

Table 8 (*Continued*)

Primer name	Ms	*Pichia kudriavzevii* YK46	EMS-17	EMS-29	EMS-37	EMS-44	EMS-56	Unique bands
UBC-851	784	–	–	–	–	+	–	1
	706	–	–	–	+	–	–	1
	601	–	–	+	–	–	–	1
	471	+	–	–	–	–	–	1
	418	–	+	–	–	–	–	1
	381	–	–	+	–	–	–	1
	356	–	–	–	+	–	–	1
	306	–	–	–	–	–	+	1
	464	–	–	+	–	–	–	1
ISSR-157		–	–	–	–	–	–	1
ISSR-158	1412	+	–	–	–	–	–	1
	1192	–	–	–	+	–	–	1
	1071	+	–	–	–	–	–	1
	871	+	–	–	–	–	–	1
	367	+	–	–	–	–	–	1
	294	+	–	–	–	–	–	1
	263	–	+	–	–	–	–	1
	304	–	–	–	+	–	–	1
ISSR-861	882	–	–	–	+	–	–	1
	679	–	–	+	–	–	–	1
	637	–	–	–	+	–	–	1
	533	+	–	–	–	–	–	1
	497	–	–	–	+	–	–	1
	249	+	–	–	–	–	–	1
	242	–	–	+	–	–	–	1
	158	–	–	+	–	–	–	1
		13	2	31	7	2	1	56 bands

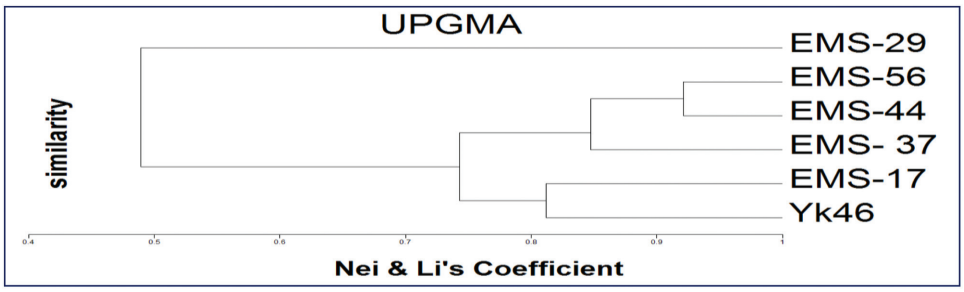
EMS, ethyl methane sulfonate; ISSR, inter-simple sequence repeat.

Table 9 Genetic similarity matrix for the six genotypes of *Pichia kudriavzevii* using eight primers of inter-simple sequence repeat

Similarity matrix	*Pichia kudriavzevii* Yk46	EMS-17	EMS-29	EMS-37	EMS-44	EMS-56
Pichia kudriavzevii Yk46	1.000					
EMS-17	0.812	1.000				
EMS-29	0.515	0.538	1.000			
EMS-37	0.702	0.770	0.491	1.000		
EMS-44	0.687	0.792	0.459	0.877	1.000	
EMS-56	0.684	0.821	0.443	0.818	0.921	1.000

EMS, ethyl methane sulfonate.

Figure 9



UPGMA-based dendrogram showing the genetic relationship among six genotypes of *Pichia kudriavzevii* using eight primers of ISSR. ISSR, inter-simple sequence repeat.

Discussion

In the present study, we have recently reported the isolation of keratinolytic wild-type yeast *P. kudriavzevii* KY46, collected from 10 local poultry waste farmers in Cairo, Egypt, which produced a higher extracellular keratinase activity, recorded as 164 U/ml of keratinolytic-specific activity after 48 h. The results also reported the usage of three efficiency mutagenesis such as EMS, UV, and ethidium bromide as a tool to modify original wild-type strain *P. kudriavzevii* KY46 in multistep mutation induction for keratinase production improvement. It resulted in a collection of *P. kudriavzevii* KY46 mutant strains, yielding one mutant EMS-37 with higher keratinolytic-specific activity than the wild-type strain *P. kudriavzevii* KY46, which recorded 211.9 U/ml. Thus, the present study indicated that using EMS, Eth.Br., and UV mutagenesis to induced mutation were in favor of keratinase production improvement. This simple method provided strains that produced more enzyme than the wild type from which it was derived. These results were agreement with the results obtained from Duarte *et al*. [17] which reported the isolation of a keratinolytic producing yeast strain *Candida parapsilosis* from poultry farm. The strain was treated with EMS to produce mutants with a higher keratinase-specific activity. Moreover, they reported the collection of 500 mutants, which were compared with the wild type of *C. parapsilosis*, producing 80 U/ml, whereas the mutant strains I7, H36, and J5 recorded 130, 110, and 140 U/ml of keratinase-specific activity, respectively. As reported by Cai *et al*. [19], an isolate of *Bacillus subtilis* keratinase-producing bacterial strain was treated by chemical mutagenesis N-methyl-N-nitro-N-nitrosoguanidine and resulted in the isolation of KD-N2 mutant strain, which showed twice the keratinase-specific activity of wild strain [31]. It was reported that the isolation of *Bacillus pantothenticus* mutants increased (1.44-fold) of alkaline protease production over the parent strain of *B. pantothenticus* through mutation induction [32]. The isolated *Pseudomonas sp.* JNGR242 was treated by UV and resulted in an isolation mutant, which increased 2.5-fold in alkaline protease production.

The effect of improvement parameters under examination was assessed to acquire the most extreme yield of enzyme production. This technique was utilized in the optimization of environmental conditions necessary for production of keratinase. Applying RSM, at the same time, the determination of the main and interaction effects of different environmental factors on keratinase production as performed. Results showed that the maximum keratinolytic activity for mutant EMS-37 was observed at pH 5, 72 h incubation time, 2.5% glucose, and 2.5% beef extract, recording an activity of 240.172 U/ml. There are many reports for evaluation of the perfect culture conditions for enzyme production using RSM method. These results agree with Dutta and Banerjee [33], who used the technique RSM to optimize production of keratinase by *Bacillus cereus* and reported that RSM can yield improvement as well as the enzyme production cost; Sivakumar *et al*. [34], who also used the RSM technique to optimize strain *Aspergillus niger* for invertase enzyme production growing on agricultural wastes defined as low-cost; and Ire *et al*. [35], who also used the method of RSM to optimize fungi *Penicillium bilaiae* for acidic protease production. RSM is gaining attention owing to its suitability in effectively aggregating optimal conditions for multivariable conditions [36]. The use of RSM in optimizing bioprocess techniques has been heralded as an effective way of determining the most suitable parameters for the production process [37]. Moreover, Salim and colleagues reported the usage of the RSM method to improve and optimize laccase enzyme activity by fungus *Penicillium chrysogenum*. The maximum laccase-specific activity after optimization in cultivation at 32°C and for 5 days was recorded at 7.9 U/ml. Thus, the RSM technique was more efficiency than the classical optimization method, which recorded 6.0 U/ml in laccase enzyme-specific activity.

ISSR-PCR technique was used to study the genetic diversity and relationships between *P. kudriavzevii* strains. ISSR-PCR also has been successfully employed in genetic diversity studies, of the selected mutant DNA against the wild strain *P. kudriavzevii* (YK46), many different variations were detected and recorded. Moreover, five mutants were compared with the wild type. This result was in agreement with Adawy *et al*. [38], who used the ISSR analysis to detect the changes in the nucleotide sequence of the selected mutant and fusant DNA against the wild isolate (*C*andida *tropicalis*, ShY-1 strain). ISSR is a simple, inexpensive technique and has high reproducible profiles. The results showed that the mutant EMS-37 had the highest production of keratinase enzyme and also featured with seven unique bands appeared in primer UBC-851, with two bands at 706 and 356 bp, primer ISSR-158 also showed two bands at 1192 and 304 bp, and in addition, primer ISSR-861 also showed three bands at 882, 637, and 497 bp. These bands show

a possibility related to the production of keratinase enzyme; this possibility deserves to be studied in the future. ISSR results agree with Hashoosh *et al.* [39], and they found that the used RAPD-PCR to differentiate between 24 isolates of *A. niger* isolated from different sources (soil, seeds, powdered milk, and factory wastewater). Similar observations were reported by Sharma and Devi [16], which used RAPD and ISSR to differentiate between seven isolates of *Alternaria solani* and their pathogenic capability [40]. Moreover, they used RAPD marker diagnostic potential in identifying highly virulent isolates but ISSR markers in identifying the isolates with different virulence against these two pests of tobacco crops. However, Gupta *et al.* [15] applied ISSR technique to monitor *Saccharomycopsis fibuligera* in the fermentation process. Their study supports the recommendation to use ISSR markers for routine discrimination of yeast isolates. The results showed that the highest values of genetic similarity observed between EMS-44 and EMS-56 were 0.921, whereas the lowest value of similarity was 0.443 between EMS-29 and EMS-56, respectively. The results of cluster analysis divided all *P. kudriavzevii* genotypes into two main clusters. The cluster I included EMS-29 only, and the cluster II included two subclusters. The subcluster one was divided into two sub-subclusters. The sub-subcluster one included one group EMS-56 and EMS-44, and the sub-subcluster two included EMS-37 only. However, the subcluster two was divided into two sub-subclusters EMS-17 and *P. kudriavzevii* YK46, respectively. These results are in agreement with Salim *et al.* [41], who used the ISSR marker to illustrate the genetic similarity between *Aspergillus* isolates. Moreover, Salim *et al.* [37] studied the invertase enzyme productivity from different *Aspergillus spp.* and divided the genotypes into three major clusters and subclusters based on RAPD-PCR primers.

Conclusion

In the current study, some yeast strains were assayed for keratinase production. *P. kudriavzevii* was the most promising isolate for keratinase enzyme production, which recorded 164 U/ml. Physical UV and chemical EMS and Eth.Br. mutagenesis were successfully used to increase the expression of keratinase in strain *P. kudriavzevii*. In this study, a mutant strain called EMS-37 showed higher keratinolytic activity (211.9 U/ml) of their wild-type strain *P. kudriavzevii* (164 U/ml). RSM seems to the prospective technique for optimization of microbial enzymes production after a combination between various factors. The data obtained from this study has demonstrated a significant increase in extracellular keratinase production by strain *P. kudriavzevii* through RSM. Keratinase production was the result of a synergistic combination of effective parameter interactions such as at pH 5, 72 h incubation time, 2.5% glucose, and 2.5% beef extract with activity of 240.172 U/ml. The optimized conditions for keratinase enzyme production as reported in this study can be of several advantages, with a wide range of industrial applications such as the animal feeding. The use of *P. kudriavzevii* for the keratinase production seems to be a promising candidate, with an Eukaryotic origin. ISSR-PCR markers have the potential technique for differentiation and study the genetic diversity and relationships between *P. kudriavzevii* strains. ISSR-PCR indicated that the highest total and polymorphic with unique bands were revealed in the mutant EMS-37 with 82 and 54 bands, respectively, whereas the mutant EMS-56 showed at 72 and 44 bands compared with the wild-type strain *P. kudriavzevii* YK46 at 86 and 58 bands, respectively. The data obtained showed that mutant EMS-37 was the highest producer of keratinase enzyme and had seven unique bands. These bands show a possibility related to the production of keratinase enzyme; this possibility deserves to be studied in the future.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

References

1 Azeredo L, Lima M, Coelho R, Freire D. Thermophilic protease production by *Streptomyces* sp. 594 in submerged and solid-state fermentations using feather meal. J Appl Microbiol 2006; 100:641–647.

2 Han MW, Luo Q, Gu Q, Yu X. Isolation and characterization of a keratinolytic protease from a feather – degrading bacterium pseudomonas aeruginosa c11. Afr J Microbiol Res 2011; 6:2211–2221.

3 Riffel A, Brandelli A. Keratinolytic bacteria isolated from feather waste. Braz J Microbiol 2006; 37:395–399.

4 Abirami S, Ragavi R, Samrot AV. Utilization of keratinolytic *Lichtheimia corymbifera* AS1 for degradation of cattle hoove-a slaughterhouse waste to use in plant growth. Biointerface Res Appl Chem 2020; 10:6417–6426.

5 Dhiva S, Sreelakshmi R, Sruthi S. Optimization of biomass of keratinase producing *Bacillus* sp CBNRBT2 to utilize in whole cell immobilization for feather degradation. Lett Appl NanoBioSci 2020; 9:1339–1347.

6 Gurav R, Nalavade V, Aware C. Microbial degradation of poultry feather biomass in a constructed bioreactor and application of hydrolysate as bioenhancer to vegetable crops. Environ Sci Pollut Res Int 2020; 27:2027–2035.

7 Zerdani I, Faid M, Malki A. Feather waste digestion by new isolated strains *Bacillus* spp. in Morocco. Afr J Biotechnol 2004; 3:67–70.

8 Cai C, Zheng X. Medium optimisation for keratinase production in hair substrate by a new *Bacillus subtilis* KD-N2 using response surface methodology. J Ind Microbiol Biotechnol 2009; 36:875–883.

9 Rieger TJ, De Oliveira CT, Pereira JQ, Brandelli A, Daroit DJ. Proteolytic system of *Bacillus* sp.CL18 is capable of extensive feather degradation and hydrolysis of diverse protein substrates. Br Poult Sci 2017; 58:329–335.

10 Daroit DJ, Correa APF, Brandelli A. Keratinolytic potential of a novel *Bacillus* sp. P45 isolated from the amazon basin fish piaractus mesopotamicus. Int Biodeter Biodegr 2009; 63:358–363.

11 Chaturvedi V, Bhange K, Bhatt R, Verma P. Production of kertinases using chicken feathers as substrate by a novel multifunctional strain of Pseudomonas stutzeri and its dehairing application. Biocatal Agri Biotechnol 2014; 3:167–174.

12 Forgacs G, Alinezhad S, Mirabdollah A, Feuk-Lagerstedt E, Horvath IS. Biological treatment of chicken feather waste for improved biogas production. Int J Environ Sci 2011; 23:1747–1753.

13 Reddy MR, Reddy KS, Chouhan YR, Bee H, Reddy G. Effective feather degradation and keratinase production by Bacillus pumilus GRK for its application as bio-detergent additive. Bioresour Technol 2017; 243:254–263.

14 Brandelli A. Bacterial keratinases: useful enzymes for bioprocessing agroindustrial wastes and beyond. Food Bioprod Tech 2008; 1:105–116.

15 Gupta R, Sharma R, Beg QK. Revisiting microbial keratinases: next generation proteases for sustainable biotechnology. Crit Rev Biotechnol 2013; 33:216–228.

16 Sharma R, Devi S. Versatility and commercial status of microbial keratinases: a review. Rev Environ Sci Biotechnol 2018; 17:19–45.

17 Duarte TR, Oliveira SS, Macrae A, Cedrola SML, Mazotto AM, Souza EP, *et al.* Increased expression of keratinase and other peptidases by *Candida parapsilosis* mutants. Braz J Med Biol Res 2011; 44:212–216.

18 Wawrzkiewicz K, Lobarzewski J, Wolski T. Intracellular keratinase of *Trichophyton gallinae*. Med Mycol 1987; 25:261–268.

19 Cai C, Lou B, Zheng X. Keratinase production and keratin degradation by mutant strain of *Bacillus subtilis*. J Zhejiang Univ Sci 2008; 9:60–67.

20 Mazotto AM, Cedrola SM, Lins U, Rosado AS, Silva KT, Chaves JQ. Keratinolytic activity of *Bacillus subtilis* AMR using human hair. Lett Appl Microbiol 2010; 50:89–96.

21 Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976; 72:248–254.

22 Khattab SMR, Kodaki T. A novel production method for high-fructose glucose syrup from sucrose-containing biomass by a newly isolated strain of osmotolerant *Meyerozyma guilliermondii*. J Microbiol Biotechnol 2016; 26:675–683.

23 Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol 1990; 215:403–410.

24 Rozewicki J, Li S, Amada KM, Standley DM, Katoh K. MAFFT-DASH: integrated protein sequence and structural alignment. Nucleic Acids Res 2019; 47:5–10.

25 Tork S. High keratinase production and keratin degradation by a mutant strain KR II derived from *Streptomyces radiopugnans* KR12. J Appl Biol Sci 2018; 12:18–25.

26 Akbar S, Prasuna GR, Rasheed K. Multistep mutagenic strain improvement in *Aspergillus carbonarius* to enhance pectinase production potential. Int J Appl Biol Pharma Technol 2013; 4:92–98.

27 Kamalambigeswari RI, Alagar S, Sivvaswamy N, Kamalambigeswari RJ. Strain improvement through mutation to enhance pectinase yield from *Aspergillus niger* and molecular characterization of polygalactouronase gene. Pharm Sci Res 2018; 10:989–994.

28 Barman NC, Zohora FT, Das KC, Mowla Md G, Banu NA, Salimullah M, Hashem A. Production, partial optimization and characterization of keratinase enzyme by *Arthrobacter* sp.NFH5 isolated from soil samples. AMB Exp 2017; 7:1–8.

29 Senthivelan T, Kanagaraja J, Panda RC, Narayani T. Screening and production of a potential extracellular fungal laccase from *Penicillium chrysogenum*: media optimization by response surface methodology (RSM) and central composite rotatable design (CCRD). Biotechnol Rep 2019; 23:e00344.

30 Zietkiewicz E, Rafalski A, Labuda D. Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. Genomics 1994; 20:176–183.

31 Qattan SY. Phenol bioremediation by a genetically improved strain of *Candida tropicalis*. Pharmacophore 2020; 11:4.

32 Shikha SA, Darmwal NS. Improved production of alkaline protease from a mutant of alkalophilic *Bacillus pantotheneticus* using molasses as a substrate. Biores Technol 2007; 98:881–885.

33 Dutta JR, Banerjee R. Isolation and characterization of a newly isolated *Pseudomonas* mutant for protease production. Brazarch Biol Technol 2006; 49:37–47.

34 Sivakumar T, Shankar T, Vijayabaskar P, subramanian R. Statistical optimization of keratinase production by Bacillus cereus. Global J Biotech Biochem 2012; 6:197–202.

35 Ire FS, Aguguo VJ, Ezebuiro V. Optimization of invertase from *Aspergillus niger* grown on low cost agricultural wastes by response surface methodology (RSM). J Adv Microbiol 2018; 13:1–15.

36 Ben Mefteh F, Frikha F, Daoud A, Chenari Bouket A, Luptakova L, Alenezi FN, Belbahri L. Response surface methodology optimization of an acidic protease produced by *Penicillium bilaiae* isolate TDPEF30, a newly recovered endophytic fungus from healthy roots of date palm trees (*Phoenix dactylifera* L). Microorganisms 2019; 7:74.

37 Salim RG, Abo-Sereh NA, Khalil BE. Optimization and molecular characterization of novel Aspergillus spp. producing invertase enzyme. Egypt Pharma J 2020; 19:321–329.

38 Adawy S, Hussein Ebtissam HA, El-Khishin D. Genetic variability studies and molecular fingerprinting of some Egyptian date palm (*Phoenix dactylifera* L.) cultivars, II – RAPD and ISSR profiling. Arab J Biotech 2002; 5:225–236.

39 Hashoosh QH, Al-Araji AM, Al-Assie AH. Genetic diversity using random amplified polymorphic DNA (RAPD) analysis for *Aspergillus niger* isolates. Iraqi J Sci 2015; 56(4C):3376–3389.

40 Vianna MF, Pelizza S, Russo ML, Toledo A, Mourelos C, Scorsetti AC. ISSR markers to explore entomopathogenic fungi genetic diversity: implications for biological control of tobacco pests. J Biosci 2020; 45:1–11.

41 Salim RG, Aly SES, Abo-Sereh NA, Hathout AS, Sabry BA. Molecular identification and inter-simple sequence repeat (ISSR) differentiation of toxigenic Aspergillus strains. Jordan J Biol Sci 2019; 12:5.