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Optimization of Keratinase Enzyme synthesized by *Micrococcus luteus* using Taguchi DOE Method

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ABSTRACT
Keratinase is an important enzyme used for degradation of the keratinous wastes, especially slaughterhouse and poultry-derived wastes, that cause environmental pollution. In the current study, optimum conditions for keratinase production by *Micrococcus luteus* Y23-18 strain were investigated using Taguchi DOE L9 orthogonal array. For this purpose, the selected environmental factors were initial pH, incubation temperature and time. The optimal conditions were obtained as pH 9.5, temperature 30˚C and 3 days. The obtained results showed that keratinase activity was enhanced approximately 2.3-folds (34.95 U mL^{-1}) when compared with the unoptimized conditions (15.33 U mL^{-1}). As a result, *M. luteus* Y23-18 is an effective keratinase producer microorganism and Taguchi design of experiment is a useful tool for optimization.

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Key words
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Micrococcus luteus Tarafından Sentezlenen Keratinaz Enziminin Taguchi DOE Yöntemi Kullanılarak Optimizasyonu

ÖZET
Keratinaz, keratinöz atıkların, özellikle çevresel kirliliğe yol açan mezbaha ve kümes hayvancılığı kökenli atıkların parçalanmasında kullanılan önemli bir enzimdir. Mevcut çalışmada, *Micrococcus luteus* Y23-18 suşu tarafından keratinaz enziminin üretiminin Taguchi DOE L9 ortogonal dizisi kullanılarak optimizasyonu araştırılmıştır. Bu amaçla seçilen çevresel faktörler, başlangıç pH değeri, inkübasyon sıcaklığı ve zamandır. Optimal şartlar 9.5 pH değeri, 30˚C sıcaklık ve 3 gün olarak belirlenmiştir. Elde edilen sonuçlar keratinaz aktivitesinin, optimize edilmeyen durumla (15.33 U mL^{-1}) karşılaştırıldığında yaklaşık olarak 2.3 kat (34.95 U mL^{-1}) arttığını göstermiştir. Sonuç olarak, *M. luteus* Y23-18 etkili bir keratinaz üretici mikroorganizmadır ve Taguchi deney dizaynı optimizasyon için kullanışlı bir araçtır.

Mikrobiyoloji

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INTRODUCTION
Keratin is an insoluble fibrillar protein that has high resistance against proteolytic digestion in the skin of birds, reptiles, and mammals. It has two types, the first one is acidic Type I and the other one is neutral Type II (Scott & Untereiner, 2004). Keratinase (EC 3.4.4.25) is an important enzyme that catalyses the hydrolysation of the keratin (Sharma & Kango, 2021; Vidmar & Vodovnik, 2018). An increase in the population of the world causes environmental pollution, hence the elimination of the waste becomes more important for human health. Keratin-originated industrial materials, especially slaughterhouse and poultry wastes, require particular elimination methods depending on their natural structure. In this context, side industrial wastes are critical, like cattle hair and chicken feather, etc. There are approximately 1,5 billion cattle in the world (Murray-Tortarolo & Jaramillo, 2020) and waste cattle hair has not been used for commercial protein source for animal feedstock (Cai et al., 2022). However, cattle hair has valuable bioactive peptides to be recycled (Etemadian

et al., 2021). Chicken meat and chicken-derived products have been consumed continually all over the world TUIK (TUIK Turkey Statistical Institute, 2022) reported the chicken meat consumption to be 582444 tons which was derived from 330211 chicken, during January-March 2022 period in Turkey. The poultry wastes are keratin-rich materials and the chicken feathers contain ß-keratin (38%), α-keratin (41%), and amorphous keratin (21%) (Daroit & Brandelli, 2014). The poultry wastes consist of infertile eggs, feathers, empty shells and dead embryos that have dangerous structure for the environment (Chen & Jiang, 2014; Nowak et al., 2017; Prabakaran & Valavan, 2021; Simpson, 1991). Keratinous wastes are processed various techniques including alkaline extraction, ionic fluids, oxidative reduction, steam flash explosion, microwave radiation, thermal hydrolysis, enzymatic hydrolysis, and microbial fermentation (Martinez et al., 2020); however, keratinase-based degradation of these wastes is more sustainable and environmentally friendly (Zhang et al., 2022).

Keratinases hydrolyse the recalcitrant structural keratins (Gupta et al., 2013). Keratinases are generally used in leather finishing processes, industrial waste water treatments, cosmetic industry, and organic fertilizer. Microbial keratinases are produced by bacteria, commonly, Gram positive bacteria like *Bacillus halotolerans* (Devi et al., 2022), *B. subtilis* (Zhang et al., 2022), *Microbacterium*, and *Micrococcus luteus* (Laba et al., 2015) etc.

Statistical tools are preferred due to their possibility for random experiments when compared with classical one-factor-at- a time (OFAT, namely one- variable- at- a time, OVAT) method. OFAT is a confidential optimization technique for specific requirements of the growth conditions and valuable product formation using addition or depletion of the factors, or causes variations in environmental conditions. However, all the experiments were done respectively. On the contrary, statistical optimization methods usually present randomly-organized experimental runs to gain interaction between the factors. Use of statistical designs offers the opportunity for rapid screening and shows the individual roles of each factor (Canlı Taşar, 2020; Canli Tasar, 2022; Farid et al., 2013) and the Taguchi DOE method increases the robustness of products with high quality and less labour (Kivak, 2014; Rao et al., 2008).

MATERIALS and METHOD

All of the medium components were purchased from Sigma (USA) and Merck (Germany).

Microorganism and medium

The keratinase producer bacteria were isolated from contaminated soil of a local slaughterhouse in Erzurum, Turkey. Among twenty-five keratinase producing-microorganisms, skim milk agar plates were employed for their protease activities (data not shown). The greatest clear zone was produced by a bacterium named HP2, hence it was further used and identified as *Micrococcus luteus* Y23-18 strain using 16S rRNA sequence analysis (Figure 1). ITS1 and ITS4 primers were used for amplification of the ITS gene region for sequencing under in vitro conditions at Macrogen (Netherlands) and pGEM-T Easy Vector Systems (Promega UK) were used for cloning of the PCR products. BioEdit was employed to determine and analysis the results from the database. The nitrogen source was used as powdered ram horn that was obtained from a local slaughterhouse. The ram horns were dried, milled and powdered. The keratinase production medium was designated as following (g L^{-1}): 10 glucose, 3 powdered ram horn, 1 KH_2PO_4, 0,2 $MgSO_4$, 0,1 $CaCl_2$. For inoculum preparation, the same medium was used in 250 mL Erlenmeyer flask containing 100 mL of medium, and one loopful of 24-h-old *M. luteus* Y23-18 strain from a nutrient agar plate was added. The flask was incubated at 30˚C and 200 rpm for 24 h. 0,5 mL of suspension (1.5_{600nm}) was used as the inoculum material for each experiment.

Enzyme Assay

The keratinase enzyme activity was calculated using colorimetric method. The keratin azure (azokeratin) was preferred for assays with a bit of modification (Gonzalo et al., 2020; Letourneau et al., 1998; Suntornsuk & Suntornsuk, 2003). Keratin azure solution was prepared with 0,4 g of keratin azure to 100 mL of 0.01 M (pH 7,5) Tris-HCl buffer. The reaction mixture consisted of 1 mL enzyme source (culture filtrate) and 1 mL of keratin azure solution. Then, the tubes were taken to incubation at 50˚C in a shaking incubator at 200 rpm for an hour. At the end of the incubation time, the reaction mixture was allowed to boil for 5 min, then the mixture was centrifugated at 5000 x g for 20 min. The supernatant was used for spectrophotometrically measurement for the release of the azo dye at 595 nm. On the other hand, the same process was run for the control without incubation. One-unit (U) keratinase enzyme activity was determined as the enzyme amount that caused 0.1 absorbance increase between the sample and the control at 595 nm in an hour under the experimental conditions. All the experiments were done at 200 rpm agitation speed in 50 mL medium-containing flasks. Minitab® 19.1.1 Statistical Software (United States) was used. All the experiments were run three times and the means were taken.

Taguchi methodology

For Taguchi DOE methodology, L9 orthogonal array was preferred for the optimization of keratinase production using initial pH of the medium, temperature and time factors at three levels (Table 1).

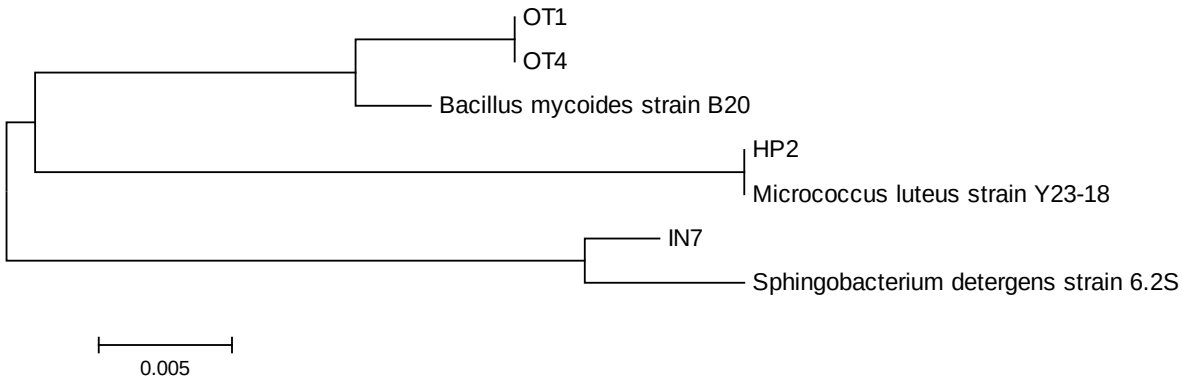

Figure 1. Neighbour joining phylogenetic tree on the basis of 16S rRNA gene sequence data of *Micrococcus luteus* 23-18 strain.

Şekil 1. Micrococcus luteus Y23-18 suşuna ait 16S rRNA gen sekans bilgisine bağlı olarak neighbour joining yöntemi ile oluşturulmuş filogenetik ağaç

Table 1. Optimization parameters and selected levels
Çizelge 1. Optimizasyon parametreleri ve seçilen seviyeler

Serial No	Factors	Level 1	Level 2	Level 3
1	pH	9	9.5	10
2	Temperature (˚C)	30	32	34
3	Time (d)	1	2	3

A full factorial design is useful to investigate all the factors at each level; however, this method is not useful depending on the large number of the factors and levels. Taguchi DOE methodology has three quality characteristics following: the larger- the better, the nominal-the better and the smaller-the better. It was aimed to enhance the keratinase activity, the larger-the better characteristic was utilized and the equation is shown below:

$S/N = -10 \log10 (1/n \sum_{i=1}^{n} \mathbf{1}/\boldsymbol{Yi}^{2})$

where S/N are performance statistics. In the equation, the n determines the number of repetitions and the *Yi* is a performance value of the i*th* experiment. S/N ratio was calculated to find out the selection of the best value by the researcher (Jean & Tzeng, 2003). Taguchi DOE method uses S/N ratio to interpret the results instead of the average values (Tan et al., 2005; Canlı Taşar, 2020).

Analysis of variance

The analysis of variance (ANOVA) was used to find out the optimal levels of the factors. The highest effects obtained from the factors had maximum impact on the keratinase activity. The effects of each parameter were indicated individually. Minitab® 19.1.1 Statistical Software (United States) was employed as the data software.

RESULTS AND DISCUSSION

The obtained results in the current study showed that keratinase production was strongly affected by the environmental conditions (Table 2). Optimal pH values for keratinase production were reported in a pH range between 5 to 10 (Abdel-Fattah et al., 2018; Bockle et al., 1995). In a previous study, maximum keratinase activity was obtained at 6.0 pH and 42˚C by *B. licheniformis* ALW1 using corn steep liquor (Abdel-Fattah et al., 2018). However, the optimal pH and incubation temperature were obtained as 9.5 and 30˚C in the current study, respectively. In another previous study, keratinase production by *M. luteus* the optimum values for pH and temperature were determined as 9.4 and 55˚C, respectively (Laba et al., 2015), which was closer to the current study except temperature. This difference may be resulted from substrates and strains.

In a previous study using classical OFAT method, the optimal conditions were obtained as 9.0 for initial pH, similar to the current study. However, the optimal temperature and time were obtained as 40˚C and 96 hr by *Ochrobacterium intermedium* (Sharma & Kango, 2021). On the other hand, *Streptomyces pactum* DSM 40530 was reported as optimally active at pH range from 7.0 to 10.0 and for temperature from 40 to 75˚C that was not closer to the obtained results (Bockle et al., 1995).

S/N ratio determines the deviation of the quality criterions from the results (Canli et al., 2013; Sharma et al., 2005). The minimisation of the undesirable effects cause enhancement for enzyme production. The minimum keratinase activity (15.33 U mL^{-1}) was obtained from the 1st experimental design (unoptimized conditions), while the maximum activity (30.21 U mL^{-1}) was obtained from the 4th design.

Table 2. Taguchi L9 orthogonal array and keratinase activity and S/N ratios
Çizelge 2. Taguchi L9 ortogonal dizisi ve keratinaz aktivitesi ve S/N oranları

Exp. No.	pH	Temperature	Time	Keratinase (U mL^{-1})	S/N ratios
1	1	1	1	15.33	21.2632
2	1	2	2	22.11	26.8918
3	1	3	3	21.23	26.5390
4	2	1	2	30.21	29.6030
5	2	2	3	27.37	28.7455
6	2	3	1	23.15	27.2910
7	3	1	3	28.39	29.0633
8	3	2	1	20.13	26.0769
9	3	3	2	22.25	26.9466

Response data for S/N ratios and their comparison were given (Table 3). Delta value presents the changes between the maximum and minimum means for the factor. The rank value explains the rank of each Delta. Table 3 demonstrates that initial pH had more relative impact than the incubation time and incubation temperature. The analysis of variance (ANOVA) showed the rank values obtained from the basis of the amplitude of S/N variation (Table 4).

Main effects plot of S/N ratios showed the greatest impacts of the factors at optimal levels (Figure 2). The optimal levels for the factors were pH 9.5, 30˚C and 3 days for the initial pH, incubation temperature and incubation time, respectively. For prediction analyses, Taguchi DOE uses the main effects plot results. At the end of this experiment, keratinase activity was found as 34.95 U mL^{-1} which was closer to the predicted result (35.65 U mL^{-1}).

Table 3. Response table for means.
Çizelge 3. Ortalamalar için yanıt tablosu

Level	pH	Temperature	Time
1	20.56	25.64	20.54
2	26.91	23.20	24.86
3	23.59	22.21	25.66
Delta	6.35	3.43	5.13
Rank	1	3	2

Table 4. Analysis of variance for means.
Çizelge 4. Ortalamalar için varyans analizi

Source	DF	Seq SS	Adj SS	Adj MS	F	P
pH	2	60.588	60.588	30.294	10.92	0.084
Temperature	2	18.728	18.728	9.364	3.38	0.228
Time	2	45.596	45.596	22.798	8.22	0.108
Residual Error	2	5.547	5.547	2.773		
Total	8	130.459				

DF: Degree of freedom; Seq SS: Sequential sum of square; Adj SS: Adjusted sum of square; Adj MS: Adjusted mean of squares; F: F value; P: P value.

The percentage contribution of each factor on the keratinase production was shown in Figure 3. These values were calculated using sequential sum of square of a factor to the total sequential sum of square. pH had the most percentage contribution, the incubation time and temperature had less contribution among the other factors. In a prior study about glucose oxidase production by *Rhodotorula glutinis,* pH had less impact than temperature while the lowest impact was obtained from time (Canli Tasar 2022). This result might be caused by the environmental conditions depending on the target product and medium composition.

CONCLUSION

Keratinase enzyme is a commercially valuable and produced mainly by bacteria. In the current study, *M. luteus* Y23-18 strain was employed for keratinase production as the producer microorganism. Initial pH, incubation temperature and incubation time were selected as the optimization factors and Taguchi DOE was utilized. Optimized factors were determined using Taguchi L9 orthogonal array and the results showed that keratinase production is strongly affected by the environmental conditions. The optimal conditions for keratinase production by *M. luteus* Y23-18 were detected as 9.5 for pH, 30˚C for incubation temperature and 3 days for incubation time. At the end of the optimization, the maximum enzyme activity was obtained as 34.95 U mL^{-1}. Taguchi DOE presented an economical and quickly activity-enhancing way for the enzyme production process. The keratinase production was increased approximately 2.3-folds when compared with the unoptimized conditions. As a conclusion, *M. luteus* has a valuable capacity for keratinase production and Taguchi DOE is a powerful tool for optimization.

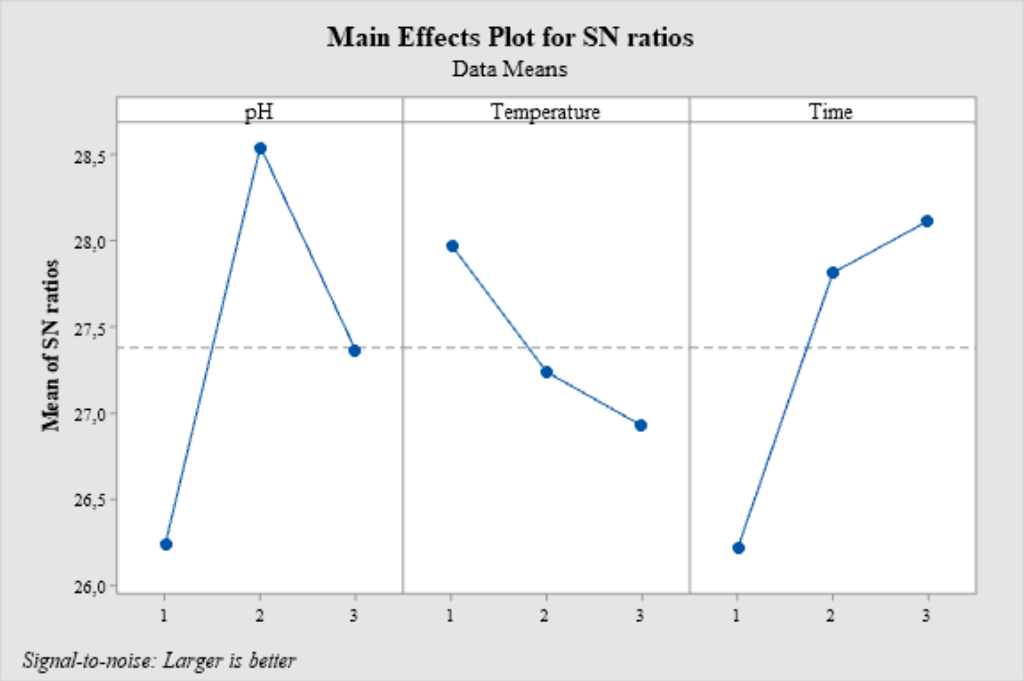

Figure 2. Main effects plots for S/N ratios
Çizelge 2. S/N oranları için asıl etki plotları

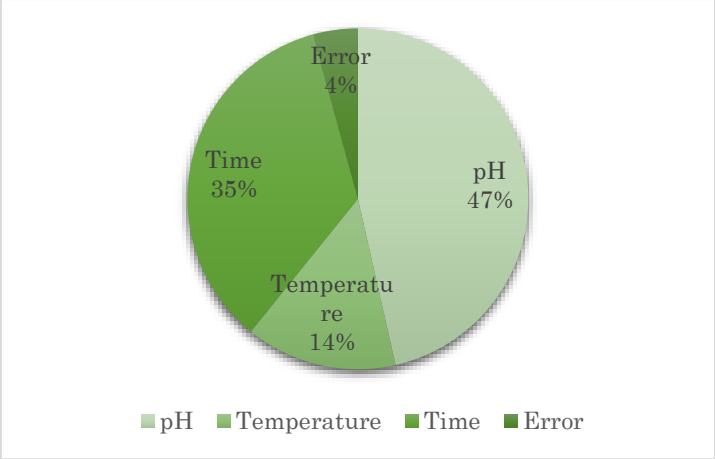

Figure 3. Percentage contribution of each factor on the keratinase production
Çizelge 3. Keratinaz üretimi üzerine etki eden her faktörün yüzde dağılımı

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Author contributions

The authors declare that they have contributed equally to the article.

Disclosure statement

The authors declared they have no conflicts of interest. The responsibility for the content and designation of the paper depends on the authors.

Ethical approval

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

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