

Increment in protease activity of *Lysobacter enzymogenes* strain by ultra violet radiation

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ABSTRACT

Background and Objectives: Increasing the amount of protease from microbial sources is in the focus of attention. Random mutagenesis by physical methods like ultraviolet (UV) radiation is a cost effective and convenient procedure for strain improvement. Therefore, in the present study attempts were made to investigate the effect of UV radiation on *Lysobacter enzymogenes* in order to increase its protease activity.

Materials and Methods: UV mutagenesis was induced in *L. enzymogenes* fresh culture at the distance of 20 cm from light source for different exposure times of 70, 90, 150 and 200 seconds. The mutated isolates were randomly cultured from the nutrient agar medium to casein agar plate, as a selective medium. The primary screening was performed by observing hydrolysis of casein in the plate and the secondary screening was carried out on skim milk agar on the basis of zone of hydrolysis using bacterial supernatants. Quantification of protease activity was done by Anson's method using tyrosine as standard.

Results: UV radiation resulted in obtaining 12 mutants out of 100 examined *L. enzymogenes* strains with increased protease activity. The mutant M2, at 90s exposure time was selected as the best mutant bacterium which produced 1.96 fold more protease over the parent strain.

Conclusion: Random mutation by UV radiation is a simple and convenient method to increase the protease activity of *Lysobacter enzymogenes*. Furthermore, it seems that the middle time of exposure to UV, 90 s, was the best time because it can induce mutagenesis but did not hamper the bacteria growth and viability.

Keywords: *Lysobacter enzymogenes*; Protease activity; Random mutagenesis; Ultraviolet radiation

INTRODUCTION

Microbial proteases are the most notable commonly used enzymes in a wide number of industrial processes including food, laundry detergents, pharmaceutical and biotechnology (1, 2). In this regard, *Lysobacter enzymogenes* which is a ubiquitous environmental bacterium was registered as a producer of extracellular proteases. The bacterium is emerging as a potentially novel biological control agent and a new source of bioactive metabolites including lytic enzymes (3). Amongst the naturally occurring proteases in *L. enzymogenes*, lysyl endopeptidase attracts

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attention especially in biotechnology as it precisely hydrolyzes lysyl peptide bonds of nonterminal amino acids. It is highly specific and preferentially cleaves Lys-I-Xaa bonds, including Lys-I-Pro (4, 5). This enzyme is widely used in research as a standard laboratory reagent for enzyme digestion to study the primary structure of proteins (6).

Microorganisms represent an excellent source of proteases, however, these metabolites are not overproduced in nature, but they must be overproduced in the pharmaceutical industry and biotechnological developments. Genetic manipulations are used in industry to obtain strains that produce hundreds or thousands of times more than that produced by the originally isolated strain. These strain improvement programs traditionally employ mutagenesis followed by screening or selection (7). Mutagenesis is carried out in two randomized and targeted methods. Random mutagenesis is a process by which changes in genes occur by applying physical (types of beams and heat) and chemical factors (types of chemicals), which can lead to achieve desirable characteristic (increase the protease activity several times) or causing the appearance of negative features that may lead to the death of the bacterium (8, 9).

Ultraviolet irradiation (UV) is one of the most commonly known mutagenic agents and it is also very easy to take precautionary measures against it (10). In the past, a randomized mutagenic technique with UV radiation has been used to increase the production of various enzymes in bacteria and fungi (11). It gives a high proposition of pyrimidine dimers and includes all types of base pair substitutions (12). In the present study, a randomized mutagenic induction technique by UV was used to increase protease activity of *L. enzymogenes* which may lead to obtain industrial strain for production of lysyl endopeptidase. After optimization of the nutritional requirements for enzyme production, the hyper-producer strains of protease were selected.

MATERIALS AND METHODS

Bacterial strain and cultivation media. The Gram negative bacterium *L. enzymogenes* subsp. *enzymogenes* with the ATCC number of 29486TM was purchased from ATCC (USA). Bacterial cells were revived by suspending the powder in nutrient broth (Merck, Germany) and cultivating aerobically

at 33°C ± 1°C. Based on previous study performed by Kuhlman et al. 2009, 1% glucose, 0.01% of each mono- and dibasic potassium phosphate, and 0.02% magnesium sulphate were added to culture media to enhance growth rate and protease production (13).

Preparing bacterial cell suspension. Initially, a single clone of the bacterium transferred into 5 ml of sterilized nutrient broth medium and incubated at 33°C ± 1°C with agitation at 180 rpm until observing the opacity of the seed culture. Then the bacterial culture was inoculated into 50 ml nutrient broth and incubated for another day. After reaching the OD_{600nm} to 1, 106 times dilution was performed using sterilized phosphate buffer saline (PBS) or nutrient broth as diluent.

Exposure to UV radiation. UV irradiation method was performed using a modified protocol described by Zarif et al. (14), and Ghazi et al. (15) based on *L. enzymogenes* growth characteristics. Briefly, to induce mutation, 2 ml of the bacterial suspension which prepared in the previous step was poured into the cell culture plates and exposed to the UV lamps (two 15-w germicidal lamps at 254 nm, G15TB, Philips, Japan). Bacterial suspension was exposed to UV light at the distance of 20 cm from the source for different time intervals (70, 90, 150 and 200 seconds) in the dark in order to avoid photo reactivation process. After exposure, 0.1 ml of mutated bacterial suspension was inoculated on the nutrient agar medium and cultured for 24 h at 33°C, to obtain single colonies.

Study of survival rate after UV exposure. In order to investigate the effect of UV radiation on the bacterial viability, bacterial colony-forming units (CFU) counting was performed to determine the bacterial death rate caused by UV radiation. For this, after exposing the bacterial suspension to UV (70 and 200 s), serial dilutions (8 to 10 times dilutions) are prepared and 0.1 ml of diluted mutated bacterial were inoculated onto agar medium to count the number of alive colonies, obtaining a single colony, to compare with the wild type strain. Dilutions which yielded colonies ranging from 30-300 were selected and counted and compared with the control .

Mutants screening based on protease activity. Initial screening stage is performed to select colonies with clear hydrolysis zone of casein as sub-

strate based on the method described by Yokota, et al. (16) with some modifications. For this, single mutated clones were selected randomly from nutrient agar medium and cultured on a 2.5% skim milk agar plates (Merck, Germany). By observing the transparent zone resulting from protein degradation, these colonies went through a secondary screening to compare casein hydrolysis ability with the wild type. In the next step, screening was done quantitatively by measuring and comparing the diameter of the transparent zones of the mutated and wild strain. The overnight cultures were prepared for each single mutated colony and 20 µL of each inoculum was transferred at the center of wells which was made in the skim milk agar plates. In each plate, one well devoted to wild type bacterium and one for nutrient broth without bacteria as negative control. The plates were incubated at 33°C ± 1°C at least for 48 hours then the diameter of hydrolysis zone was measured and compared with wild type bacteria. The procedure was done again in triplicates for those mutants that showed larger zones' diameter than the wild strain.

Enzyme activity. Finally, the pre-cultures was prepared for each selected mutant strain with higher protease activity and then inoculated at 1% W/V into enzyme production media (50 ml in 250 ml Erlenmeyer flasks) the flasks were incubated at 37°C for 2, 8, 10, 16, 24 and 48 h in a shaking incubator at 180 rpm. At the end of each period, the cultures were centrifuged (6000 rpm, 10 min) and the supernatants were used for the determination of proteolytic activity. Total protease activity was measured using casein as substrate by the Sigma's protease activity assay which is a modified version of Anson Method (17, 18). One mL of the culture supernatant was mixed with 2 mL 0.05 M phosphate buffer (pH 7.0 adjusted with phosphoric acid) containing 0.65% casein, and incubated for 30 min at 37°C. The reaction was stopped by adding 2 mL 110mM Trichloroacetic acid. After 30 min stand at 37°C, the precipitate was removed by centrifugation at 6000 rpm for 10 min. 1 mL of the supernatant was treated with 5 mL 0.5 M NaCO₃ and 1 mL of 0.5 M Folin–Ciocalteu reagent (1:1). After 20 min of waiting in the dark at room temperature the optical density of the sample was measured at 660 nm. A standard curve was generated using solutions of 10-500 µg mL⁻¹ tyrosine. One unit of protease activity was defined as the amount of enzyme required to liberate 1 µg mL⁻¹ tyrosine under the experimental conditions used.

RESULTS

Primary assessment of *L. enzymogenes* colonies and proteolytic activity. Initially, shape of single colonies appeared on the skim milk agar plates for 48 hours was investigated. The pale yellow colored colonies with a clear zone of casein hydrolysis were observed on the nutrient agar medium containing 2% casein (Fig. 1A). Then, the proteolytic activity of *L. enzymogenes* was assessed by culturing the wild type microorganism in the wells made in 2.5% skim milk agar as triplicate (Fig. 1B). The average clearance zone of the wild type calculated as 5.1 millimeter (mm).

CFU counting after exposure to UV radiation. The viability assessment of bacterial suspension exposed to UV lamps showed that the longer time of exposure (200 seconds) results in more survival decrease in contrast to shorter exposure time (70 seconds) (Table 1). For both exposure times, the third dilution was selected as the best dilution (30-300 clones) and the bacterial count was performed based on this dilution (Table 1). The lethality rate of 54.4% and 69.4% were observed after exposure of 70 and

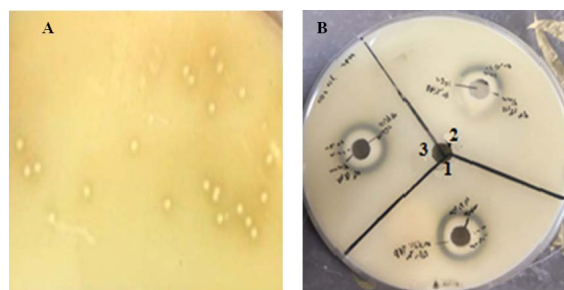


Fig. 1. The colony appearance and casein hydrolysis activity of *L. enzymogene*. A. Single colonies of *L. enzymogene* with a hollow zone of casein hydrolysis around the colonies. B. Casein hydrolysis activity of *L. enzymogenes* by cultivating bacterial suspension in the well have made in skim milk agar.

Table 1. Effect of UV exposure on the survival rate of *L. enzymogenes*.

Bacterial Suspension	CFU/ml	Survival percentage
Wild type bacteria	16 × 10 ⁵	100
Mutant bacteria (70s UV exposure)	73 × 10 ⁴	45.6
Mutant bacteria (200s UV exposure)	49 × 10 ⁴	30.6

200 seconds at the distance of 20 cm from the UV source.

Selection of mutants with increased proteolytic activity. In the present study, bacterial suspensions were separately exposed to UV radiation for 70, 90, 150 and 200 seconds. Then 25 single colonies which obtained from different times of exposure were selected randomly and cultures into skim milk agar plates. Initially mutants with casein hydrolysis zone around themselves were selected at the first screening step. Some colonies did not show proteolytic activity after UV treatment (Fig. 2A). Then proteolytic activity of each mutant strain was compared with wild type bacteria by applying bacterial suspensions into wells made in the skim milk agar plates (Fig. 2B). Mutated bacteria which showed larger hydrolysis zone in contrast to wild type conducted to secondary screening step. In this step, supernatants were obtained from logarithmic phase of mutant and wild type bacteria. Then the diameter of hydrolysis zone in mutant bacteria were compared with wild type supernatant (Fig. 3). Totally, among 100 examined mutated bacteria, three mutants from each time of exposure (70, 90, 150, 200) were selected which showed larger diameter zone in contrast the wild type (Table 2). Among all mutant bacteria, M2 strain which obtain from bacteria exposed to UV for 90 seconds showed the highest proteolytic activity (10 mm, Table 2).

Quantifying protease activity of wild type and mutated *L. enzymogenes*. Protease activity of wild type and the mutated *L. enzymogenes* which showed the highest protease activity was checked in liquid media based on equivalent deliberated micromoles of tyrosine. The protease activity of wild type (147 IU ml⁻¹) was attained at OD₆₀₀ = 0.4, while for the mutant M2, UV 90s was 289.66 IU mL⁻¹ (Table 3).

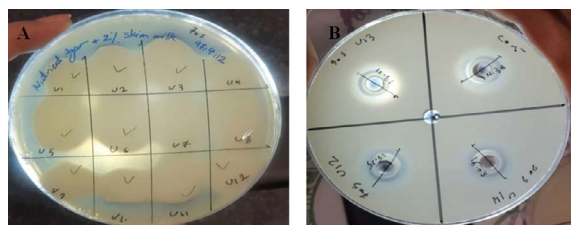


Fig. 2. Primary screening step for selecting mutants with enhanced caseinolytic activity. As it is shown in the picture some mutants lose their proteolytic activity after exposing to UV radiation.

Maximum enzyme production was achieved in the middle of the logarithmic phase of parental type and mutant strain.

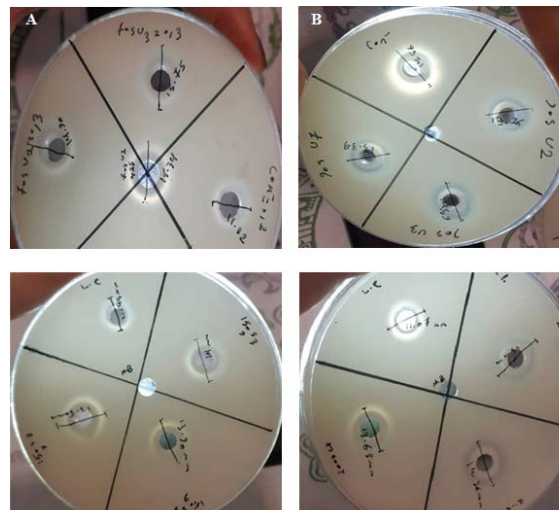


Fig. 3. Secondary screening step for selecting *L. enzymogenes* mutants with enhanced proteolytic activity. Representative photo of the casein hydrolysis by supernatant obtained from logarithmic phase of strains which primarily selected after exposure to UV for A. 70 seconds, B. 90 seconds, C. 150 seconds, and D. 200 seconds.

In each plate, 3 well were used for supernatant of mutant bacteria (as triplicate), one for supernatant of wild type *L. enzymogenes* and a well in the center used as negative control (nutrient broth).

Table 2. Selected mutated *L. enzymogenes* strains with enhanced protease activity in contrast to the wild type bacteria.

Bacterial supernatants	Hydrolysis zone diameter (mm)	Increment relative to the wild type
Wild type	5.1 ± 0.5	-
<i>L. enzymogenes</i>		
70 s UV exposure		
M1	9.75 ± 0.3	1.91
M2	9.34 ± 0.2	1.83
M3	9 ± 0.31	1.76
90 s UV exposure		
M1	7.26 ± 0.35	1.42
M2	10 ± 0.4	1.96
M3	9.51 ± 0.32	1.86
150 s UV exposure		
M1	8 ± 0.3	1.56
M2	7.9 ± 0.21	1.54
M3	7.5 ± 0.25	1.47
200 s UV exposure		
M1	7.63 ± 0.28	1.49
M2	9 ± 0.31	1.76
M3	8.36 ± 0.33	1.63

Table 3. Protease activity and biomass of parental strain and mutant M2 (90s exposure) strain.

Absorbance (OD _{600nm})	Protease Activity (IU /ml)*	
	Wild type <i>L. enzymogenes</i>	Mutated (M2, 90 s UV exposure)
0.2	65.5	131.2
0.4	147.4	289.66
0.5	74.1	199.66
0.6	80.7	236.33
0.8	74.11	185.8

*Values are shown as means of triplicates

DISCUSSION

Proteases are one of the most important industrial enzymes which account for about 60% of total enzymes worldwide sales (10). Microorganisms are a major source of proteases due to their rapid growth, simple growth conditions, specific metabolic processes and wide distribution (19). In this regard, *L. enzymogenes* is a putative protease producing strain which is the commercial source for lysyl endopeptidase. This enzyme is used to perform a variety of techniques required in proteomics (20). However, microorganisms usually produce the desired product only to the extent of their own requirements, and for commercial use, the strain needs to be modified to increase production. Random mutagenesis is a common method used to obtain optimized strains with enhanced industrial capability.

In the present study, random mutagenesis was induced using UV radiation as a physical agent. UV radiation is not an ionizing radiation but it can react with DNA. The excitation of electrons in the DNA molecule often results in the formation of pyrimidines dimers (especially thymine). Some of these random mutations can lead to obtain a mutant strains with improved characteristics (21). Using UV radiation in the present study resulted in isolating mutant strains with enhanced protease activity. In details, in all exposure times to UV radiation (70, 90, 150, 200 seconds), a number of mutant bacteria were selected based on increased protease activity in contrast to wild type bacteria. However, it should be noted that as the exposure time increased, the bacterial survival rate decreased.

In the last decades, enhancing protease activity of microorganisms has gained a big interest among researchers and many studies have been conducted

to achieve this goal. In a study performed in 2015, Wang and his colleagues have found a mutant *Bacillus subtilis* S1-4 strain with enhanced extracellular protease activity about 2.5-fold higher than the wild-type strain by combining ultraviolet irradiation and N-methyl-N'-nitro-N-nitrosoguanidine treatment for mutagenesis (19). In another study carried out in 2017, the protease activity of *Aspergillus terreus* CJS-127 was increased using physical and chemical mutagenesis. Mutation using UV radiation leads to obtain a strain with maximum protease production of 163.57 U / ml, which was 2.30-fold of wild type protease activity (22). In the same way, another study was conducted with the aim of increasing protease production in two bacterial species of *Bacillus* sp. and *Micrococcus* variants using UV mutagenesis. Similar to the present study, the ability of protease production was evaluated by transferring a single clone isolated from both bacterial species (wild and mutated) into skim milk plates. Protease activity was quantitatively evaluated by measuring the release of tyrosine in supernatant culture due to the reaction of the protease with casein as a substrate by the Anson's method. This indicates that protease production is increased about 2.5-fold of wild-type (10). In parallel to mentioned studies, UV mutagenesis in the present study also resulted in obtaining 12 strains with enhanced protease activity. Among these selected mutant strains, M2 strain from 90s UV exposure showed the highest increment of protease activity (1.96 fold) in contrast to wild type *L. enzymogenes*.

Strain improvement gains a considerable interest in biotechnology especially in the field of developing improved enzyme producing strains. In this regard, the process of mutagenesis may cause alteration by changing binding affinity, specificity, catalytic rate, thermo stability and other physicochemical characteristics of the target enzyme. However, in random mutagenesis choosing the appropriate screening method is very critical to identify such alternations. For instance, in a study performed by Taguchi et al. a cold-adapted protease was isolated by evolutionary engineering based on sequential *in vitro* random mutagenesis on the target gene and an improved method of screening (23). Alternatively, the target protein can be purified from the mutant strains then the desired features can be compared to the wild type protein. Considering this approach, Wang et al. purified a serine protease from the mutated isolate (UMU4) *Bacillus subtilis* S1-4 and its catalytic properties were

characterized. The protease purified from UMU4 showed higher hydrolytic activity towards casein over a wide range of temperatures (50°C-75°C) and pHs (6-12) ranges in contrast to the wild type (19). Here, we reported that strain improvement by UV exposure is very useful and convenient method for increasing protease production in *L. enzymogenes*. These strains, together with optimization of environmental conditions, can have a significantly higher protease titer than wild type strains. In the next steps, Endopeptidase Lys-C one of the most important proteases of *L. enzymogenes* will be purified and examined further to evaluate its characteristics in contrast to the wild type protease.

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