# Enhanced Milk-Clotting Enzyme Production of *Mucor Miehei* Mutated by UV and LiCl

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**Abstract.** Milk clotting enzyme is an important enzyme in cheese industry, to obtain an industrial strain with high milk-clotting enzyme activity, Mucor miehei was used as the origin strain and mutagenized by Ultraviolet (UV) and UV combining lithium chloride(LiCl), respectively. The result shows that lower concentrations of LiCl can increase the survival rate of the spores treated with UV, while higher concentration of LiCl can increase the mortality of the spores treated with UV. The highest positive rate (reached 32.17%) was achieved in the compound mutation, which was higher than that of the UV mutagenesis (21.45%). On the basis of multiple mutation, a mutant UV-LiCl-6 was screened. The milk-clotting activity of UV-LiCl-6 reached 2703.58 SU/mL and was 57.70% higher than that of the parent strain. Results of the pass-generation test indicated that the strain had good genetic stability and may be ideal strains for the production of milk-clotting enzyme. Additionally, it was demonstrated that compound mutagenesis with UV and LiCl is an effective mutation method for the breeding of enzyme-producing strains.

## Introduction

Milk clotting enzyme is a key enzyme in cheese industry[1], which can cause milk proteins to curdle by selectively breaking up peptide bonds between certain amino acids within the Kappa casein chain[2]. Milk clotting enzyme not only clots the milk but also play an important role during cheese maturation, which is a vital and complex process for the balanced development of flavour and texture[3]. The cheese industry has traditionally used calf rennet for clotting milk—the first step in cheese making[4]. The worldwide increase of cheese production and the reduced supply of calf rennet have led to a search for novel sources of rennet. [5]. Attention has been focused on the production of milk-clotting enzyme from microbial sources for use as rennet substitutes[6], because its production is cheaper, biochemical diversity is greater, shorter fermentation period and genetic modification is easier[7]. Most commercial milk clotting enzyme are produced from Mucor pusillus, Mucor miehei and Endothia parasitica[8].

Many research studies on the enhancement of milk-clotting enzyme production have been reported in recent years, most of them dealt with the screening of wild strains and the influence of the fermentation conditions[9]. However, there are few reports about mutagenesis programs for screening milk-clotting enzyme overproducing mutants. Compound mutagenesis of UV and LiCl is easy and simple to handle and has been successfully applied in breeding of

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mutants with improved properties [10]. There are no reports about mutagenesis for screening of mutants with high activity milk-clotting enzyme using this method. In the present study, compound mutagenesis of UV and LiCl was performed to improve the yield of milk-clotting enzyme from Mucor miehei. In addition, the parameters of compound mutagenesis of UV and LiCl and characteristics of the mutant were studied.

## Materials and Methods

### Microorganism and Medium

The microorganism employed in this study was Mucor miehei HL-1 gifted by GanSu HuaLing Biological Technology Co. Ltd.

Potato dextrose agar (PDA) slants were used for sporulation and storage.

Solid state fermentation medium: 10g wheat bran was taken in 250 mL conical flask and moistened with 10 mL distilled water containing 2.5% glucose(w/w), 1.0% NH4NO3 (w/w), 1.5% whey powder (w/w). The pH of the medium was ajusted to 6.0.

### Mutation with UV Treatment

The time of UV irradiation was designed at 30, 60, 90, 120, 150, 180, 210 and 240 seconds, respectively. The lethality rates and the positive mutation rate calculated by using a method of Zhang et al [11].

### Compound Mutation of UV and LiCl

The method of compound mutation of UV and LiCl was employed by using a modified method of Zhang et al [11]. The concentration of LiCl was designed as 0.5%, 1%, 1.5%, 2% and 2.5%.

### Hereditary Stability of Mutant Strains

The genetic stability of milk-clotting enzyme producing strains was determined by continuous passage experiment. Following culture on PDA slants at 30 ℃ for 4-5 days of mutant isolate was transferred to solid state fermentation in static cultivation at 30 ℃ for 4 days; then the milk-clotting enzyme activity of the supernatant was tested. This process was repeated 8 times.

### Milk-clotting Activity Determination

The milk-clotting activity was determined as described by Arima and Iwasake[12].

### Proteolytic Activity Determination

The proteolytic activity of the enzyme was assayed as modified Anson's method[12].

## Results and Discussion

### Optimum Parameter Determination of UV

The lethality rates and positive mutation rates appeared to correlate with the irradiation time of UV. The effects of UV irradiation to strain HL-1 on the lethality rates and the positive mutation rates are shown in Fig 1.

It indicated that the lethality rate increased along with the UV irradiation time increased from 30 to 240 seconds. With 30 seconds of exposure to UV, the lethality rate of Mucor miehei was 32.14%, and after 240 seconds it was 100%. The positive mutation rate had a close relationship with the lethality rate. The distribution of the positive mutation rate was shown in Fig.1. The highest positive mutation rate (reached 21.45%) was achieved, when the UV irradiation time was 90 seconds and the lethality rate was 78.57%. This fact confirmed the theory that the highest positive mutation rate was obtained when the lethality rate of the microorganism ranges from 70% to 80%[11]. Hence, the UV irradiation time of 90 seconds was chosen as the optimum parameter of UV mutagenesis in the follow experiments.

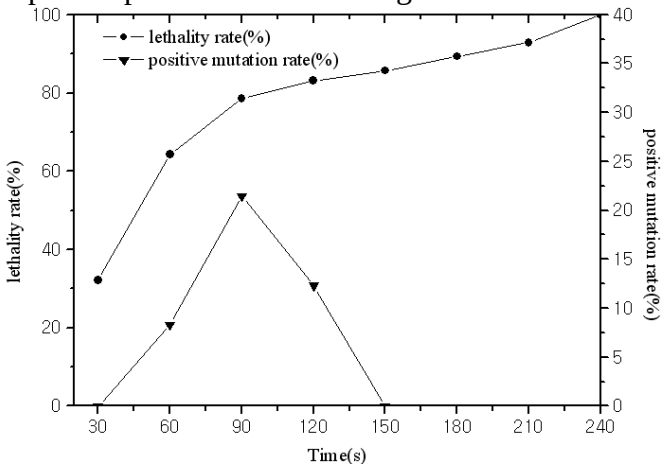


Fig.1 The lethality rate (●) and positive mutation rate (▼)of Mucor miehei spores of UV treatment.

## Screening Result of UV Treatment

Five mutants with high milk-clotting activity were isolated and inoculated into solid state fermentation medium after Mucor miehei (HL-1) spores were treated with UV repeatedly. Table 1 showed milk-clotting activity of five mutants obtained in primary screening. The high milk-clotting activity mutant, UV-13, the milk-clotting activity was about 42.85% higher than that of the origin strain, while the proteolytic activity reduced by 13.66%. The ratio of milk-clotting activity to proteolytic activity reached 271.11.

Table 1 The Screening Results of UV Treatment

| Strains | Milk-clotting Activity(SU/mL) | Proteolytic activity(U/mL) | MCA/PA |
|---|---|---|---|
| HL-1 | 1714.28 | 10.54 | 162.60 |
| UV-2 | 2099.73 | 9.88 | 214.61 |
| UV-13 | 2448.98 | 9.10 | 271.11 |
| UV-19 | 2123.89 | 9.64 | 220.36 |
| UV-20 | 2195.79 | 8.67 | 254.06 |
| UV-31 | 2228.41 | 8.32 | 268.09 |

## Optimum Parameter Determination of Multiple Mutated By UV and LiCl

Fig.2 showed that the lethality rate of the spores appeared to correlate with the different LiCl concentrations between 0.5 and 3% (w/v). When the concentration of LiCl increased from 0.5 to 1%, the lethality rate increased from 34.48 to 65.52%, implying that lower concentrations of LiCl can increase the survival rate of the spores treated with UV. When the concentration of

LiCl increased from 1.5 to 2.5%, the lethality rate increased from 82.76 to 93.10%, indicating that higher concentration of LiCl can increase the mortality of the spores treated with UV.

The relationship between the positive mutation rate and the concentration of LiCl was shown in Fig.2. It revealed that the positive mutation rate increased sharply along with the concentration of LiCl increased from 0.5% to 1.5%. When the concentration of LiCl was 1.5%, the highest positive rate (reached 32.17%) was achieved, which was higher than the highest positive mutation rate of the spores treated with UV (21.45%). When the concentration of LiCl increased from 1.5 to 2.5%, the positive mutation rate decreased from 32.17% to 0. In the next step, the LiCl concentration of 1.5 % was chosen as the optimum mutagenic agent dose for UV and LiCl treatment.

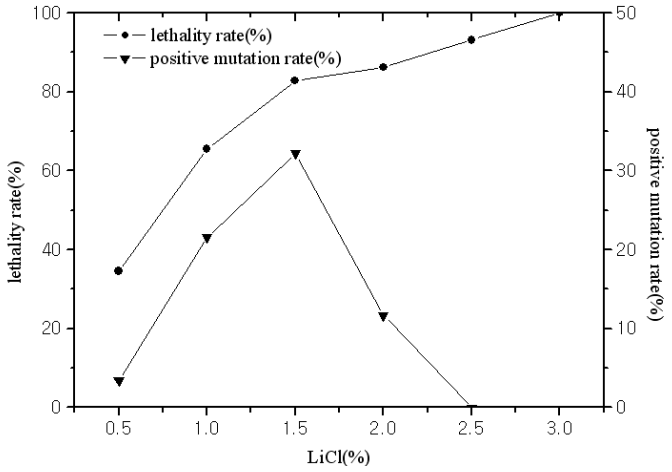


Fig.2 The lethality rate (●) and positive mutation rate (▼) of Mucor miehei spores after multiple mutation by UV and LiCl.

## Screening Result of Multiple Mutated by UV and LiCl

After Mucor miehei (HL-1) spores were multiple mutated by UV and LiCl repeatedly, seven mutants with high milk-clotting activity were isolated. Table 2 showed milk-clotting activity of seven mutants obtained in primary screening. Among them, mutant UV-LiCl-6 with the milk-clotting activity of 2703.58 SU/mL, exhibited about 57.70% increase in enzyme activity compared with that of the origin strain, while the proteolytic activity reduced by 27.80%. The ratio of milk-clotting activity to proteolytic activity reached 355.06. Thus, the mutant UV-LiCl-6 was selected as a preferable milk-clotting enzyme producer for further studies.

Table 2 The Screening Results of Compound Mutagenesis by UV and LiCl

| Strains | Milk-clotting Activity(SU/mL) | Proteolytic activity(U/mL) | MCA/PA |
|---|---|---|---|
| HL-1 | 1714.28 | 10.54 | 162.60 |
| UV-LiCl-6 | 2703.58 | 7.61 | 355.06 |
| UV-LiCl-7 | 2535.27 | 8.43 | 300.59 |
| UV-LiCl-11 | 2687.98 | 7.65 | 351.21 |
| UV-LiCl-14 | 2639.28 | 7.89 | 334.61 |
| UV-LiCl-17 | 2554.93 | 7.69 | 332.13 |
| UV-LiCl-20 | 2688.47 | 8.32 | 323.24 |
| UV-LiCl-21 | 2610.16 | 7.58 | 344.56 |

## Hereditary Stability of Mutant Strains

The stability of mutants is very important in industrial practice. To estimate the feasibility of this mutation for industrial application, the genetic stability of mutant UV-LiCl-6 was also investigated. After 8 generations, the milk-clotting activity of the mutant UV-LiCl-6 kept at 2711.16 SU/mL, indicating a weak influence of passage times on enzyme production of the mutant UV-LiCl-6. The average milk-clotting enzyme activity of mutant UV-LiCl-6 for 8 generations are listed in Table 3. The results indicated that the genetic stability of the mutant UV-LiCl-6 could satisfy the requirement of industrial application.

Table 3 Milk-clotting Activity of Mutant UV-LiCl-6 for Eight Generations.

| Passage times | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|---|
| milk-clotting activity(SU/mL) | 2711.10 | 2711.11 | 2711.61 | 2711.46 | 2711.32 | 2711.81 | 2712.08 | 2711.16 |

## Effect of Fermentation Time on the Production of Milk-clotting Enzyme

The time course of production of wild strain HL-1 and mutant UV-LiCl-6 for a period of 120 hours is shown in Fig.3.

In general, changes of milk-clotting activity of mutant showed the time is difference that reaches the highest enzyme activity as that of wild strain. The mutant UV-LiCl-6 began to produce milk-clotting enzyme for 48 hours. Milk-clotting activity of the mutant UV-LiCl-6 increased sharply from the 60 hours of fermentation, reached the maximum of 2706.32 SU/mL after 96 hours of fermentation, then the level of milk-clotting activity decreased in the further fermentation process. The wild strain began to produce milk-clotting enzyme for 60 hours, milk-clotting activity increased from the 72 hours, reached the maximum of 1720.21 SU/mL after 108 hours of fermentation. Results show that the mutant UV-LiCl-6 maintained a high capacity for the product milk-clotting enzyme.

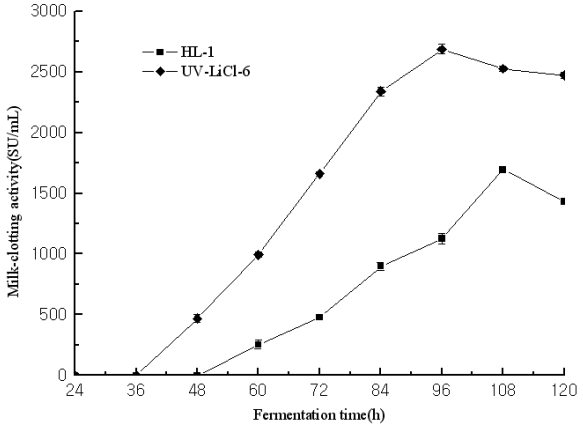


Fig.3 Time course of milk clotting enzyme production by HL-1 (◆ ) and UV-LiCl-6 (■).

## Conclusion

The research indicated that multiple mutation with optimum 90 seconds UV and the LiCl concentration of 1.5%, the mutant strain UV-LiCl-6 was screened. The activity of milk-clotting enzyme by the mutant strain UV-LiCl-6 reached a level of 2703.58 SU/mL, which was 57.70% higher than that of the wild strain, while the proteolytic activity reduced by

27.80%. The ratio of milk-clotting activity to proteolytic activity reached 355.06. The mutant was very stable and has potential application for milk-clotting enzyme production.

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