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Increasing the Production of L-asparaginase from *Bacillus substilis* RRM-1 by UV-Mutation

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Increasing the Production of L-asparaginase from *Bacillus substilis* RRM-1 by UV-Mutation

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Abstract. L-asparaginase is an enzyme that can hydrolyze L-asparagine to L-glutamic acid and ammonia. This enzyme has essential usage for food and health. *Bacillus subtilis* RRM-1 produces enzyme L-asparaginase, yet the production of L-asparaginase from B. *substilis* RRM-1 still relatively low. Hence, the effort to increase the production of the enzyme is needed. For increasing the enzyme production, the bacteria were mutated using UV rays to increase the production of L-asparaginase. UV rays (15 watts) were exposed to bacteria with a distance of 15 cm and a contact duration of 30, 60, 90, 120 min. Survival rates of bacteria were calculated, and mutant bacteria were analyzed for their ability to produce L-asparaginase. The mutants examined were taken from a 120-minute mutation treatment, which produced six isolates (UV1, UV2, UV3, UV4, UV5, UV6). Mutant UV1 and UV6 showed better results in producing the enzyme L-asparaginase. Moreover, UV6 produces an activity of 8.02 ± 1.22 U / ml. UV6 production is higher than that of *B. subtilis* RM-1 wild type (3.18 ± 0.65 U / ml). Mutations with UV can produce mutants that can increase enzyme production by three times.

1. Introduction

L-asparagine amidohydrolase or L-asparaginase (E.C.3.5.1.1) is an enzyme that catalyzes the hydrolysis reaction of L-Asparagine to aspartic acid and ammonia by breaking the amide bonds [1]. L-asparaginase enzyme is found in animal, bacterial, plant, and rat serum. L-asparaginase in large quantities was produced by bacteria such as *Escherichia coli*, *Erwinia carotovora*, *Enterobacter aerogenes* [2]. Commercially available L-asparaginase was purified from *E. coli* and *E. carotovora.*

L-asparaginase enzyme in food can prevent the formation of acrylamide by converting L-Asparagine to aspartic acid [3]. Acrylamide is a carcinogenic compound causing cancer. Acrylamide found in foods that are processed with high temperatures (>100-120°C), such as shredded fish products [4]. L-asparaginase can be used as an antineoplastic agent for treating lymphoblastic leukemia [5].

The production of L-asparaginase enzyme needs to be increased to provide the potency for industrial-scale production. One alternative to increase enzyme production is through mutagenesis techniques [6]. Strain improvement can be carried out through chemical and physical methods. For a

physical method, a less chemical and dangerous agent is applied. The physical method uses ultraviolet (UV) irradiation, is accounted as effective, efficient, and environmentally friendly.

This study used bacterium Bacillus subtilis RRM-1, which is an endophytic bacterium of *Rhizophora mucronat*a [7] to produce L-Asparaginase . In previous studies, the *Bacillus subtilis* RRM-1 bacterium was reported to be able to produce the enzyme L-asparaginase but had a small production yield. Therefore in this study, we would like to increase the production of the L-asparaginase enzyme from *B. subtilis* RRM-1 using mutations during the physical method.

2. Materials and methods

Bacillus subtilis RRM-1. was obtained from our previous work [7].

2.1. Pre-culture of bacteria

Bacillus subtilis RRM-1 was cultured on slant Luria Bertani agar (LBA). LBA consists of 0.05 gram yeast extract; bacteriological agar 0.15 gr; peptone 0.1 gr, NaCl 0.1 gr . The LBA media was then weighed and dissolved in 10 mL dH_2O, then homogenized and boiled. Boiled LBA media was then sterilized using an autoclave for 15 mins, at a temperature of 121°C, and 1 atm. Bacteria were picked from the stock and inoculated onto LBA media. Plates were then incubated in an incubator at 34°C for 24 hours.

2.2. Bacterial mutation with UV

Bacillus subtilis RRM-1 was grown using a shaker incubator at 150 rpm and 28°C for 48 hours. 4 mL of *B. subtilis* RRM-1 that had been refreshed in LB Broth media was then put in vial bottles for each treatment. Bacteria were mutated using ultraviolet (UV) (15 W) at a distance of 15 cm for 0, 30, 60, 90, and 120 minutes. Afterward, the mutated bacteria were incubated overnight in a dark place to avoid photo-reactivation. 1 mL of each incubated sample was then properly diluted before spreading it on LB Agar media.

2.3. Survival rate calculation

The rate of bacteria that survives against UV mutations was calculated using the Total Plate Count (TPC) method using the following equation:

$$SR = \frac{N0 - Nt}{N0} x\ 100\% \quad (1)$$

where: N0 = CFU of non-mutated bacterial and Nt = CFU of mutated bacteria

2.4. L-asparaginase assay

Enzyme activity was investigated with two different methods, i.e., semi-quantitative and quantitative analyses. The semi-qualitative analysis was done by following the method of Mahajan et al. [8]. Bacteria were cultured in liquid media with 0.007% Bromothymol Blue. Furthermore, bacteria were centrifuged, and the color of the cell-free liquid media was compared to a color standard (Figure 1) to identify enzyme production based on the developed color.

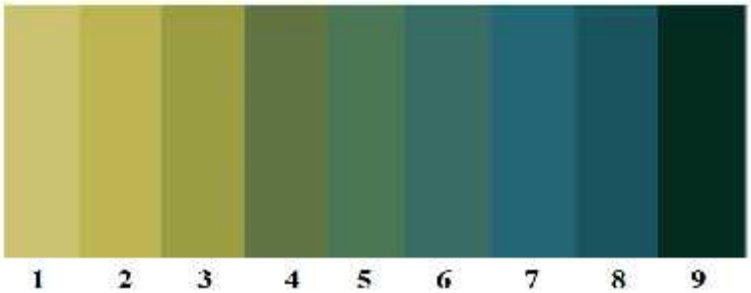

Figure 1. Standard color for semi-quantitative assay of L-asparaginase.

A quantitative L-asparaginase assay was performed using the Nessler method [9]. This method uses reagent Nestler to detect the release of ammonia in a solution. The color was read using a spectrophotometer with a wavelength of 420 nm. An enzyme unit is expressed in μmol ammonia which is released per minute under analytical conditions in one minute.

3. Results and discussion

3.1. The survival rate of bacteria

The results of the survival rate calculation for each contact time with UV are shown in Table 1. The lowest survival rate of bacteria (0.9%) was obtained from the treatment with UV contact for 120 min (D). The UV radiation is known to be able to kill bacteria. Hence, the longer the contact, the lower the survival rate of bacteria.

Table 1. The survival rate of Treated Bacteria.

	UV-Contact time (min)	Survival rate (%)
A	30	4.36
B	60	3.80
C	90	3.95
D	120	0.90

Longer contact will reduce the opportunity of getting enough mutants for further analysis. Exposure to UV for 120 minutes resulted in a lot of cell death. This can occur because the longer the bacteria are exposed to ultraviolet (UV) light; the more cells in the bacteria will mutate and disrupts bacterial metabolism and will eventually cause death. According to Miller [10], UV damages the DNA of microorganisms by forming thymine dimers. The dimer prevents microorganisms from transcribing and replicating DNA, which will ultimately cause cell death.

3.2. Production capability of L-asparaginase enzyme

Only the colonies from sample D was further investigated. Six pure mutants' isolates were investigated for their capability to produce L-asparaginase. Both semi-qualitative and qualitative assay was applied. L-asparaginase activity was characterized by changes in the color of the bacterial growth medium to become more blackish blue. Bromothymol blue (BTB) used as an indicator of color change when the medium under acidic conditions will turn yellow, and if the alkaline pH conditions will turn blue. The greater the blue zone produced, the higher the hydrolysis activity.

The analysis showed that isolates UV1 and UV 6 produced higher L-asparaginase compared to wild type, whereas four isolates (UV2-UV4) showed lower L-asparaginase production compared to wild type (Figure 2.).

Figure 2. L-asparaginase activity of *B. substilis* RRM-1 Mutant.
WT=wild type, UV1-UV6= mutant bacteria number.

A qualitative calculation of the activity of the L-asparaginase enzyme showed that the best isolate in producing L-asparaginase was UV6-isolate (Table 2). UV6 isolate produced high enzyme activity with a value of 8.02 ± 1.22. L-asparaginase activity from UV6 had almost three times of activity compared to wild type, which only had an activity of 3.18 ± 0.65.

Table 2. Result of L-asparaginase activity assay.

Semi-quantitative		
	Score	Range of Activity
WT	4	Medium
UV1	5	Medium
UV2	1	Low
UV3	1	Low
UV4	1	Low
UV5	1	Low
UV6	8	High
Quantitative		
	Activity (U/ml)	
WT	3.18±0.65	
UV1	4.17±0.38	
UV2	nd	
UV3	nd	
UV4	nd	
UV5	nd	
UV6	8.02±1.22	

An increase in enzyme production was due to spontaneous mutation on the gene, which responsible for the production of this enzyme. The results of the Erumalla et al., [11], also showed a similar result. All *Bacillus thuringiensis* mutants, which were treated with ultraviolet light, had a higher enzyme production than non-mutated *B. thuringiensis*.

4. Conclusion

The lowest survival rate of *B. subtilis* RRM-1 mutated by UV light is at a distance of 15 cm for 120 minutes. Two *B. subtilis* RRM-1 mutants, i.e. UV-1 and UV-6 showed an increase in L-asparaginase production. It was concluded that UV6 isolate was the best mutant that produces L-asparaginase with about three times higher than that of *B. subtilis* wild type.

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