Chemical mutation of *Bacillus subtilis* RRM1 increases L-asparaginase activity

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Strain improvement through chemical mutagenesis can be used for improving enzyme activity. An investigation has been carried out on the enzyme activity improvement of *Bacillus subtilis* RRM1 induced by the chemical mutagens, ethyl methanesulfonate, 5-bromouracil, and ethidium bromide. Different concentrations of mutagens were applied to produce improved strain for increasing L-asparaginase activity. Qualitative and quantitative assays of L-asparaginase on the mutant bacterial isolates showed that the enzyme activity could increase from 3.18 to 12.87 U/mL. Hence, as compared to the wild type strains, it indicated that induced mutation enhanced the ability of *Bacillus subtilis* RRM1 strains to produce more L-asparaginase enzyme up to 4.09-fold.

Keywords: mutation, *Bacillus subtilis* RRM1, L-asparaginase.

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Introduction

L-asparagine amidohydrolase or L-asparaginase (E.C.3.5.1.1) is an enzyme that catalyzes the hydrolysis of L-asparagine to aspartic acid and ammonia by breaking amide bonds [1]. L-asparaginase enzymes are widely found in animal tissues, bacteria, plants, and rat serum. *Escherichia coli*, *Erwinia carotovara*, *Enterobacter aerogenes* are the primary bacterial producers for commercial L-asparaginase [2].

L-asparaginase enzymes in the food field can prevent the formation of acrylamide by converting L-asparagine to aspartic acid [3]. Acrylamide is a carcinogenic compound that can be found in processed foods due to high temperatures processes (100-120°C). L-asparaginase can reduce the formation of acrylamide by hydrolyzing L-asparagine in raw material foods. Furthermore, L-asparaginase enzyme is also an agent for treating lymphoblastic leukemia [4].

The production of the L-asparaginase enzyme as an anti-cancer of leukemia by microbes has the disadvantage of having little enzyme yield and poor enzyme stability, making it challenging to produce for industrial-scale. Optimization of the production of L-asparaginase enzymes needs to be increased so that the enzymes produce more so that they can be produced on an industrial scale. One way to increase enzyme production is through mutagenesis techniques [5].

Mutation techniques, according to Venkatanagaraju *et al*. [6], can be used to increase bacterial culture and the efficiency of enzyme production. Mutations are sudden and random changes in the genetic materials (genomes, chromosomes, and genes). At mutations, there are two types of mutagens namely physical and chemical mutagens. The physical method mostly used UV as a mutagen. The chemical method mainly used ethane methane sulfonate (EMS), bromouracil (BU) and ethidium bromide (EtBr). A study by Ho and Chor [7] indicated that chemical mutagens were more preferable than that of UV to increase enzyme activity. It is also supported by Meraj *et al*. [8], which found that chemical mutagens failed to produce high enzyme activity on *B. substilis*.

This study used *B. subtilis* RRM1 bacteria, which is mangrove endophytic bacteria *Rhizophora mucronata*. *B. subtilis* RRM1 is reported to produce the L-asparaginase enzyme but has little results. Therefore, this research was carried out by using mutation techniques using chemical mutagens ethyl methane sulfonate, 5-bromouracil, and ethidium bromide to increase the enzyme activity of L-asparaginase.

Materials and Methods

Materials

B. subtilis RRM1 is a collection of Laboratory of Microbiology, Faculty of Fisheries and Marine Science, Brawijaya University. Ethyl methanesulfonate (EMS), 5-Bromouracil (BU), and Ethidium bromide (EtBr) were purchased from Sigma (Sigma-Aldrich, Saint Louis, MO, USA). All materials for these experiments were analytical grade.

Chemical mutation with EMS, BU, and EtBr

Chemical mutagenesis followed the methods of Suribabu *et al*. [9] and Javed *et al*. [10]. A loopful of *B. subtilis* RRM1 was inoculated into 250 mL of Erlenmeyer with 50 mL of LB Broth medium. Bacterial was incubated at shaker incubator at a speed of 150 rpm at 30°C for 48 hours. Aliquot of *B. subtilis* RRM1 (1 mL) was transferred into 1.5 mL vial for each treatment. The vials were added with EMS mutagen to reach a final concentration of 1.2 mg/mL, 3.1 mg/mL, and 6.2 mg/mL, respectively, and then were treated for 60 min. The vials were washed twice with 0.9% NaCl and were centrifuged at 10,000 rpm for 10 min at 4°C. Then the samples were diluted (10^{-8} - 10^{-10}) and spread onto LB agar. The samples were then incubated at 30°C for 48 hours. Chemical mutations with BU and EtBr were conducted with the same steps. The only difference in the mutagens was final concentration. Concentrations for both BU and EtBr mutagens were 0.2 mg/mL, 2.0 mg/mL, and 4.0 mg/mL. The mutant bacteria showed the different patterns of L-asparaginase activity. Mutant bacteria which showed higher enzyme production than that in control group accounted as potential mutant.

Survival rate of bacteria

After incubation, the survival rate of *B. subtilis* RRM-1 was then calculated using the Total Plate Count method. The desired survival rate was 1%. The survival rate calculation was following the modification of Ifadah [11] methods using equation 1 and 2 below.

$$Survival\ rate\ (Sr) = \frac{CC - CM}{CC}\ x\ 100\% \quad (1)$$

$$Survival\ rate\ (\%) = 100 - Sr \quad (2)$$

Where *CC* is total colony in control; *CM* is total colony in mutant; *Sr* is survival rate (%)

Qualitative assay of L-Asparaginase

Qualitative analysis of L-asparaginase enzyme activity using a method by Mahajan *et al*. [12] was modified by a standard color scale to determine the optimization of L-asparaginase enzyme production. Bacterial culture was grown on modified Czapek Dox medium (6 g/L Na_2HPO_4, 2 g/L KH_2PO_4, 0.5 g/L NaCl, 20 g/L L-asparagine, 2 g/L glycerol, 0.2 g/L $MgSO_4 \cdot 7H_2O$, 0.005 g/L $CaCl_2 \cdot 2H_2O$, 0.007% BTB, pH 5.5) at 30°C for 24

hour. The plates were incubated in the shaker incubator at 120 rpm. To obtain a clear supernatant, the culture media were centrifuged at 10,000 rpm for 10 min at 4°C.

Quantitative assay of L-Asparaginase
Bacteria were cultured at 50 mL of LB broth and incubated at shaker incubator at 30°C for 48 hours. The supernatant was then separated by centrifugation at 10,000 rpm for 10 min. The supernatant was dialyzed in phosphate buffer for 24 hours at 4°C. Then the enzyme activity was calculated using the Nesller method by Prihanto and Wakayama [13]. One unit of enzyme activity is defined as 1 mmol of ammonia released per minute.

Results and Discussion

Bacillus subtilis RRM1 is an L-asparaginase-producing bacterium isolated from mangrove, *Rhizophora mucronata* from the region of Tuban, East Java, Indonesia. Our previous study (data not published) revealed that *Bacillus subtilis* RRM1 produced L-asparaginase which specific only on L-asparagine. The activity of L-asparaginase toward L-glutaminase accounted for 10-12% of L-asparaginase [14]. It is an interesting character because most L-asparaginase is not only active toward L-asparagine but also on L-glutamine substrate. Hence, the study on enzyme production is needed to be done.

We investigated the efficiency of mutations by using different concentrations of mutagens. The survival rate results of mutation can be seen in Table 1. The application of mutagens showed that the higher concentration the mutagen used, the lower the survival rate. The lowest survival rate of the mutant bacteria was selected [15]. However, low growth bacteria on the plates reduces the opportunity to achieve an appropriate number of mutants. The lower survival rate about 1% was chosen for the next study. The fewer bacteria that survived, the more likely it has become mutants. Inadequate mutagen will reduce the possibility of acquiring bacterial mutants.

Table 1. Survival rate of *B. subtilis* RRM1 after treating with chemical mutagens.

Mutagens	Concentration (mg/mL)	Survival rate (%)
EMS	0.0	100
	1.2	2.6
	3.1	1.41
	6.2	1.12*
BU	0.0	100
	0.2	4.5
	2.0	3.8
	4.0	1.4*
EtBr	0.0	100
	0.2	2.73
	2.0	1.82
	4.0	0.9*

* chosen treatment for colony purification.

In subsequent investigations, only the bacterial colonies that grew on EMS concentration 6.2 mg/mL, BU concentration of 4.0 mg/mL, and EtBr concentrations of 4.0 mg/mL were purified. The pure isolates obtained from EMS, BU, and EtBr treatments were 8, 6, and 6 colonies, respectively. Qualitative analysis revealed that only a few mutants were known to have higher L-asparaginase activity compared to that of control (*B. subtilis* RRM1 wild type) (Figure 1).

Figure 1. A qualitative assay of L-asparaginase activity on *B. subtilis* RRM1 mutant. **A.** mutant from EMS. **B.** Mutant from BU. **C.** mutant from EtBr.

Table 2. L-asparaginase activity of *B. subtilis* RRM1 mutant.

EMS	Isolate							
	EMS1	EMS2	EMS3	EMS 4	EMS 5	EMS 6	EMS 7	EMS 8
Activity (U/ml)	0.62	0	1.18	4.22	0.54	3.16	4.88	7.39
BU	Isolate							
	BU1	BU 2	BU 3	BU 4	BU 5	BU 6		
Activity (U/ml)	3.92	12.87	12.76	0	0	3.16		
EtBr	Isolate							
	EtBr1	EtBr 2	EtBr 3	EtBr 4	EtBr 5	EtBr 6		
Activity (U/ml)	0	7.68	0	7.64	3.08	0		

Table 2 shows the result of the qualitative assay of L-asparaginase. Not all colonies showed an increase in L-asparaginase activity. All mutagens produced colonies that lose the L-asparaginase activity (Table 2). However, some colonies showed the increase of the enzyme activities. In EMS, BU, and EtBr treatments, there were three EMS colonies (EMS4, EMS7, EMS8), three BU colonies (BU1, BU2, BU3), and two EtBr colonies (EtBr2, EtBr4), which showed higher activity than it in *B. subtilis* RRM1 WT (3.18 U/mL). The highest yield was obtained from the treatment of BU as one isolate BU2 (17.87 U/mL).

An increase of enzyme activity due to strain mutagenesis was reported in several studies. According to Amena *et al*. [16], the mutation of *Streptomyces gulbargensis* enhanced the activity of L-asparaginase by 1.49-fold. Another research by Kumar *et al*. [17] revealed that chemical mutagenesis on *Aspergillus terreus* resulted in 4.9-fold enzyme activity improvement. Similarly, our data shows that chemical mutations can increase enzyme activity. The bacteria that were mutated by using BU showed an increase in enzyme activity of 4.05-fold. The highest L-asparaginase production was shown by using BU as a mutagen. The mutation is caused by tautomerization on bacterial nucleotides. Tautomerization-induced bromouracil is the more reliable mechanism for strain and enzyme activity improvement of *B. subtilis* RRM1. DNA contacting with BU results in Guanine-BU base pair formation and is more preferable than adenine-BU. Hence, it will increase the mismatching [18, 19].

In conclusion, the strain improvement by chemical mutation successfully enhances the L-asparaginase activity. The 5-Bromouracil-mutated *B. subtilis* produced greater L-asparaginase than that of EMS and EtBr mutated bacteria. Hence, mutated *B. subtilis* RRM1 under the code BU2 (Table 2) is a potential producer for L-asparaginase. Furthermore, investigation to examine the stability of the mutated bacteria requires to be conducted.

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