Mutation Technique for Increasing the Production of Antibacteria *Lactobacillus plantarum* TGR-2

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ABSTRACT

Production of antibacteria of ***Lactobacillus plantarum*** *TGR-2 was carried out by mutation technique using UV radiation and chemical mutagenic agent (acrydine orange (AO), ethyl methane sulfonate (EMS), and N-methyl-N-nitro sulfonate nitrosoguanidine (NTG). As a marker was applied chloramphenicol for 10 ppm. Total 214 mutants were obtained from all of the treatments and then screened based on their activities against* ***Staphylococcus aureus*** *FNCC 0047 as bacterial indicator, using agar diffusion and turbidimetric assay techniques. Results showed that 24 mutants could produce higher amount of antibacteria and two of them, NTG and acrydine orange treatments, had higher stability than others. Characterization of two mutants, determination of biochemistry traits and optimization of antibacterial (bacteriocin) production were done. The product showed that UV-15, EMS-10, AO-6 and NTG-21 mutants could produce antibacterial substance 3,90, 4.40, 3.40 and 4.17 AU/ml for 16, 12, 12, and 16 hours incubation, respectively, compared to Lactobacillus plantarum TGR-2 which produced 1.07 UA/ml for 24 hours incubation.*

INTRODUCTION

Adding preservation agent to foodstuffs is one of the methods to lengthen their shelflife. Many strains of lactic acid bacteria (LAB) has been used in fermentation of various fermented foodstuffs such as milk, meat, fruits, vegetables, and others. These foodstuffs are preserved by LAB by acidifying effect, as a result of sugar conversion into organic acids, especially lactate and acetic acid. Furthermore, LAB are found to produce antibacteria substances such as hydrogen peroxide, diacetyl, hypothiosianite and bacteriocin. Bacteriocin of LAB are of a great industrial importance, mainly in food fermentation process and have become a major area of study in the search for new and safe preservatives (Chasy, 1987 and Gillialand, 1985).

Bacteriocin are defined as protein with intraspecific antagonistic effects or possessing activity as bactericide with a narrow spectrum activity and synthesis of these proteins is encoded on plasmids (Daeschel, 1989 and Eckner, 1992).

Lactobacillus plantarum TGR-2 was one of LAB isolated from traditionally fermented raw cassava (*growol*) from Indonesia (Rahayu, *et al.* 1996), and this isolate could produce antibacterial substance which classified as bacteriocin (Djaafar, *et al.* 1996). Unfortunately, the production of bacteriocin was still very low, furthermore this research trying to apply mutation technique for increasing production of antibacteria substance.

MATERIALS AND METHODS

Microorganism

Lactobacillus plantarum TGR-2 was isolated from growol (fermented raw cassava) in Yogyakarta, Indonesia. The previous study this isolate was identified as *Lactobacillus plantarum* and *Lactobacillus pentosus complex*, but after DNA-DNA homology test was done ultimately determined as *Lactobacillus plantarum* TGR-2. Culture was stored at - 80°C in the cryovial containing glycerol 10% and grown in TGE broth before use it. Bacterial indicators were *Staphylococcus aureus* FNCC 0047 and *Enterococcus faecalis*, both of

them were grown in MRS agar and TSA medium and also prepared by IUC Food and Nutrition, Gadjah Mada University, Yogyakarta, Indonesia.

Marker

Marker selection was done using *Gradient plating method*, using MRS agar and three kinds of antibiotic, that are chloramphenicol, tetracycline and ampicilin. Minimum Inhibitory Concentration (MIC) Test was done in MRS agar by adding selected antibiotic on several concentration treatments. Poured of *L. plantarum* TGR-2 on plate and incubated at 30°C for two days. Observed the inhibition the growth of *L. plantarum* TGR-2 and determined the MIC value.

Mutation Techniques

Mutation was performed with Ultra Violet radiation (Eisentadt, *et al.* 1994). Cultured *L. plantarum* TGR-2 for 24 hours was centrifuged at 3500 rpm for 20 minutes. Pellet was suspended in sterilized water and put on plate and then lighted on 254 nm for 30 seconds until 20 minutes. Samplings were done periodically and then poured in the MRS agar added with antibiotic marker as selection technique, the raising colonies indicated mutants. Chemicals mutation was performed using EMS, ethyl methane sulfonate; Acrydine orange, and NTG, N-methyl-N-nitro-N-nitro-soguanidin). Cultured *L. plantarum* TGR-2 for 24 hours was centrifugation 3500 rpm for 20 minutes. Pellet was suspended in buffer solution (phosphate for EMS and AO, whereas NTG using maleate buffer) and added with 100-300 ppm of mutagenic agent and incubated for 5-15 minutes. Samplings were taken 1 ml periodically, for dilution purposes with peptone-water solution. One ml of diluted sample was poured in MRS agar containing marker and incubated at 30°C for 2-3 days, the raising colonies indicated mutants.

Mutants selection

Selection was done based on the inhibitory activities of mutans against bacterial indicators, using the diffusion agar and turbidimetric assay. The turbidimetric assay was used to determine antibacteria activity of neutralized supernatant of mutants *L. plantarum* TGR-2. One ml of five time concentrated supernatant was added to one ml of tryptic soy broth (TSB, Difco), double strength, pH 6,5 and inoculated with 1% of *Staphylococcus aureus* (OD_{600} = 1.2) as bacterial indicator, then incubated at 37°C for 24 h. The growths were periodically determined by measurement of turbidity using Spectrophotometer. Agar diffusion method (Spelhaug and Harlander, 1989), medium Trypticase soy agar (TSA) 15 ml, containing 1% bacterial indicator *S. aureus* FNCC 0047 24 h incubation, poured on plate. Cylinder (6 mm diameter) were put on solidified medium and fill with 100 ul supernatant culture of mutant and then incubated at cool room for 1 h, finally incubated the plate at 37°C for 24 h. Inhibitory activity of antibacteria will be shown by raising of clear zone around of cylinder.

Mutant Stability Test

The higher activity of mutant were tested in their stability (Breidt, *et al.* 1993), by transferring the cultured mutant age for 48 h without marker and then incubated at 30°C for 24 h. The next step was taken samples 2% (v/v) and transferred into new MRS medium. These work were done for 8 times. Samplings were done periodically and antibacterial activities were determined as s parameter of its stability.

Optimization of antibacteria production.

Optimization product was focused on incubation time, i,g. 0,4,8,12,16,20 and 24 h. Antibacterial activities were tested using bacterial indicator *S, aureus*. Scaling up was done by fermentor 2 liter.

RESULTS AND DISCUSSION

Chemicals, substrates, produced pigment, antibiotics, amino acids and others could be used as markers to detect resulted mutants on the medium. In this research, antibiotics were applied to detect the mutants and three kind of antibiotics, chloramphenicol, ampicilin, and tetracycline were tested by *gradient plating technique* on their effect to *Lactobacillus plantarum* TGR-2, results depicted in Table 1.

Table 1. **The growth of Lactobacillus plantarum TGR-2 by Gradient plating technique, at 40 ppm concentration of each antibiotics.**

No.	Antibiotics	Growth
1	Chloramphenicol	+++
2	Ampicilin	–
3	Tetracycline	+++
4	Untreated	+++++

+) growth
–) no growth

L. plantarum TGR-2 could not grow in 40 ppm of ampicilin but could grow on part of plate medium agar containing chloramphenicol or tetracycline, it means that both of them could be used as a markering mutant of *L. plantarum* TGR-2 by looking for the minimum inhibitory concentration of those antibiotics. Based on the other characters of both antibiotics chloramphenicol has resistancy to heat effect, can be autoclaving until 121°C for 15 minutes, but tetracycline can not. The application of chloramphenicol much be more advantage than tetracycline for this purpose.

Minimum inhibitory concentration (MIC) of chloramphenicol was tested and results were depicted in Table 2. MIC means that minimal concentration of antibiotic which can inhibits microbial growth (Angus, et al. 1987). Bordering concentration of this antibiotic were 5 ppm (still grow) and 10 ppm (not grow). For application of this concentration was chosen that 10 ppm of chloramphenicol would be fitted concentration for markering the mutant. Mechanism inhibition of this antibiotic is on the peptide bond formation because of its binding to ribosome (Franklin and Snow, 1989).

Table 2. **Effect of chloramphenicol concentration to the growth of Lactobaccilus plantarum TGR-2.**

No.	Chloramphenicol Concentration (ppm)	Growth Observation
1	0	+++++
2	1.25	+++++
3	2.50	+++
4	5.00	+
5	10.00	–
6	20.00	–
7	40.00	–

+) growth
–) no growth

The number of mutant was 214 consist of 21 mutants from UV radiation treatment, 23 mutants from ethyl methane sulfonate (EMS) mutagenic treatment, 22 mutants from acrydine orange (AO) mutagenic treatment, and 148 mutants from N-methyl-N-nitro-N-Nitrosoguanidine (NTG) treatment. All of the mutants were selected based on the antibacterial activity and stability, and results showed that mutant UV-15, AO-6 and NTG-21 could produce maximum antibacterial bacteriocin 16, 12, 12 and 16 hours, respectively. This time production was shorter than wild type strain itself *L. plantarum* TGR-2, that was 24 hours, and these mutants had antibacterial activities 3.96 AU/ml, 4.40 AU/ml, 3.40 AU/ml, and 4.17 AU/ml compared with *L. plantarum* TGR-2 was 1.07 AU/ml. These data showed that mutation techniques could improve the production of antibacterial bacteriocin of *L. plantarum* TGR-2. The number of resulted mutants from mutagenic agents was NTG treatment, in case due to the two active group of methyl and N-nitroso. Methyl group is alcylating group cause form of methyl group bounding to oxygen on C-6 atom from guanine-base and form Omethyl-guanine (Adams, 1986). Nitroso (N-NO) group can react to amine compound of cell and reform intermediate compund alcanedizotic which can react into center nucleofilic in DNA, finally it will disturb the replication process (Gichner and Velemminsky, 1988). Frequency of mutation is around $7.4/10^3$, especially NTG mutagenic agent had higher frequency compared with other mutagenic agents.

Mutant with the high amount of bacteriocin production were tested on their stability based on the Breidt method (Breidt, *et al.*, 1993) and results showed that all mutants had low stability after transfering the culture two or three time. Breidt examine mutant resistant to hogh concentration of nisin of *Leuconostoc mesenteroides* LA108, it did not show their character of log phase alternation of the even transferring culture until 14 times (around 100 generations). The decreasing bacteriocin production of mutants of *Lactobacillus plantarum* TGR-2 was assumed that back mutation process occurred. Data is depicted in Figure 1.

Those results above also indicated that there were differences between mutants and wild type strain. Confirmation mutant also could be done concerning some biochemistry and physiologic characters. By using 20 kind of sugars and amylum some differences could be observed that NTG-21 could not be able to ferment arabinose and melibiose; AO-6 mutant could not be able to ferment manose; and EMS-19 mutant could not be able to ferment galactose, but wild type it could. Be assumed that aldolase enzyme which plays an importance role on the glucose metabolism process is unwork (Holzafel and Wood, 1995). Differences on the physiological character was represented by growing of mutant and wild type at different growth temperature, data is shown in Table 3.

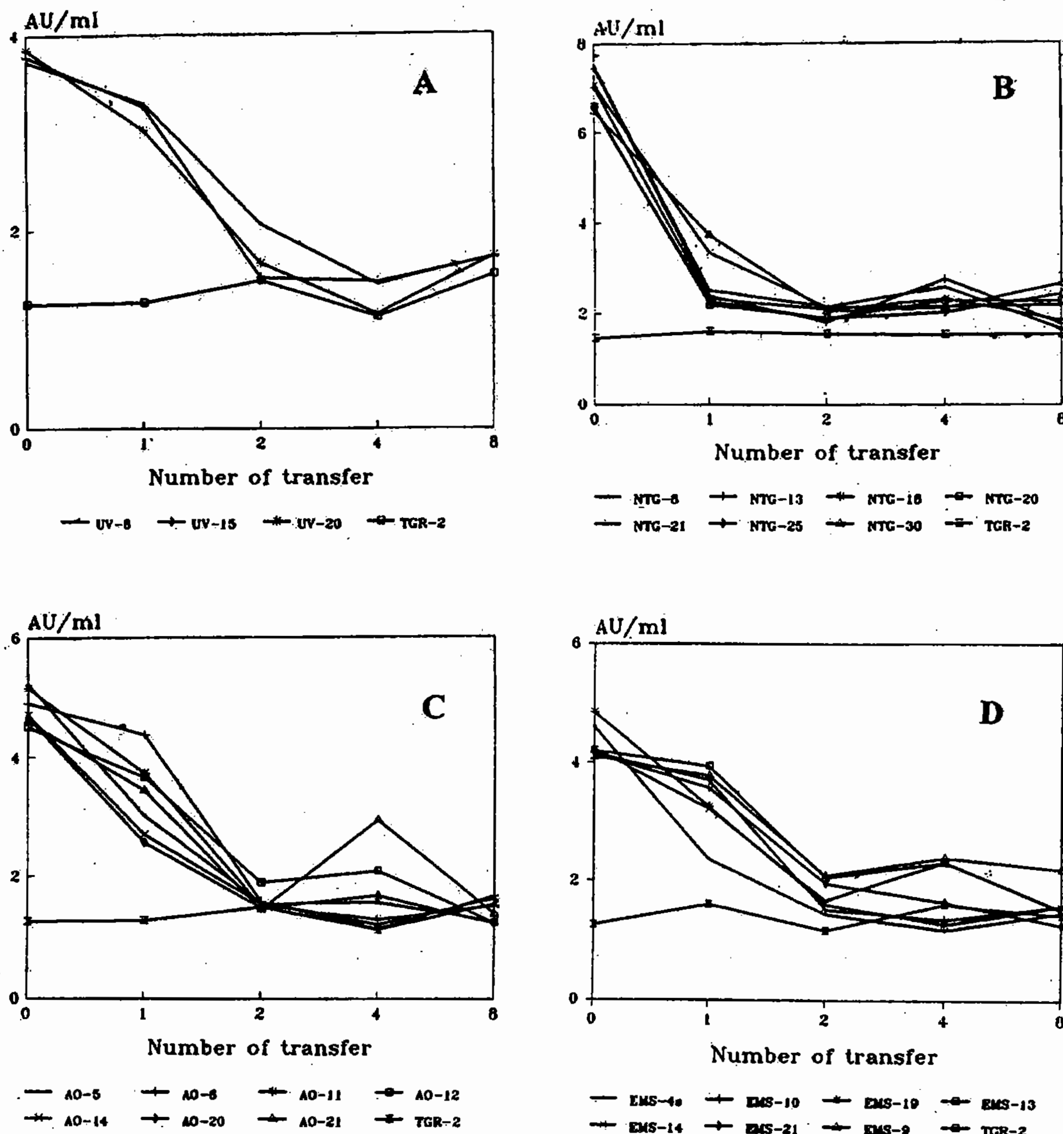

Figure 1. **Stability of suspected mutants of L. plantarum TGR-2**
A - AV mutants
B - NTG (N-methyl-N-nitro sulfonate nitrosoguanidine) mutants
C - AO (acrydine orange) mutants
D - EMS (ethyl methane sulfonate) mutants

Optimization production of mutants UV-15, AO-6, EMS-10, and NTG-21 were done using 1000 ml, fermentor results is shown in Figure 2. Time course optimum condition for bacteriocin production each mutant were slightly different, mutant UV-15 obtained the maximum production at 16 hours and number of product was 3.96 AU/ml. Increasing production compared to wild type was 3.7 times. Mutant EMS-10 obtained the maximum production at 12 hours and number of product was 4.40 AU/ml, increasing production compared to wild type was 4.11 times. Mutant AO-6 obtained the maximum production at 12 hours and number of product was 3.40 AU/ml, increasing production compared to wild type was 3.18 times. The last mutant, NTG-21 obtained the maximum production at 16 hours and number of product was 4.17

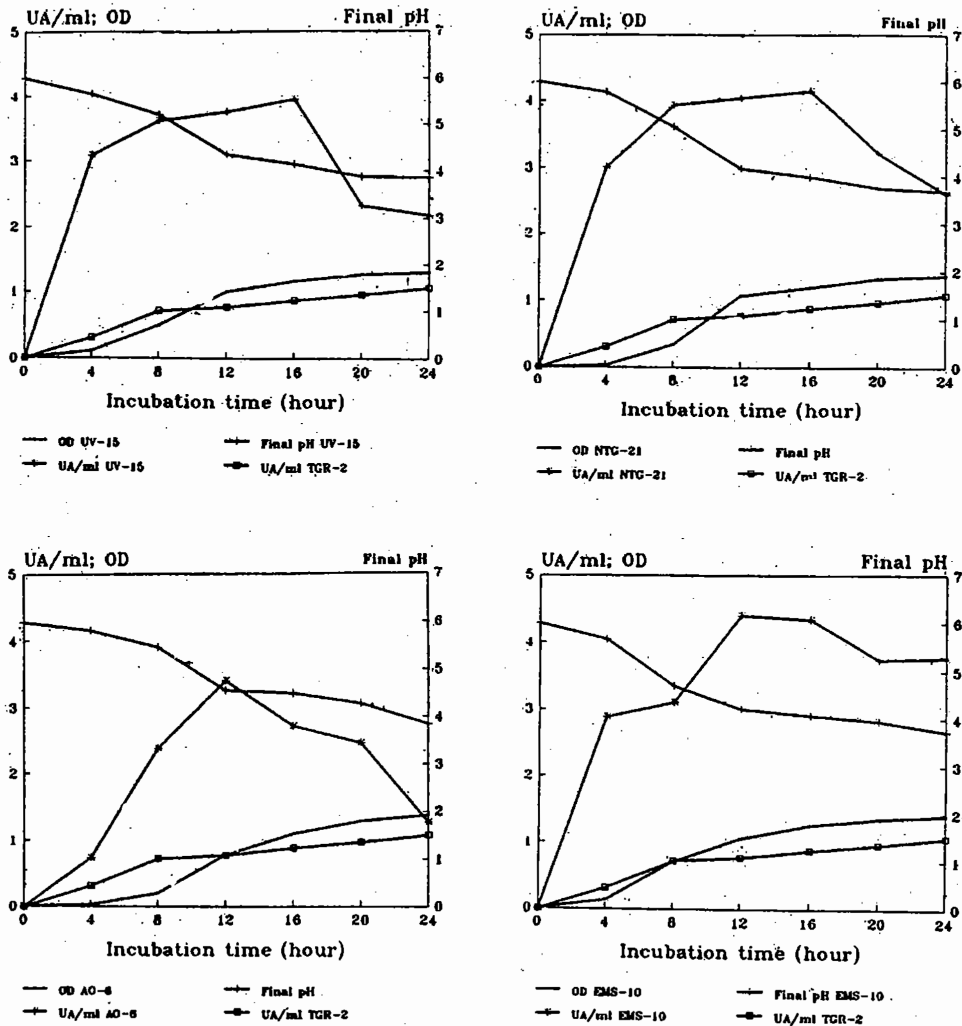

Figure 2. **Production of antimicrobial substance by mutants of *L. plantarum* TGR-2**

AU/ml, was 3.9 times, white *Lactobacillus plantarum* TGR-2 could produce bacteriocin 1.07 AU/ml of for 24 hours incubation.

CONCLUSION

Mutation techniques using UV-radiation, AO, EMS and NTG could increase the production of anti microbial substance. The mutants UV-15, AO-6 and NTG-21 produced antibacterial substances three to four times higher than the *Lactobacillus plantarum* TGR-2.

Maximum production of antibacterial substance by UV-15 and NTG-21 were at 12 hours, whereas AO-6 and EMS-10 produced antibacterial substance at 16 hours. Scaling up AO-6 mutant using 2 liter, fermentor the maximum production of the substance was 3.34 AU/ml after 12 hours, whereas *Lactobacillus plantarum* TGR-2 was 1.97 AU/ml after 20 hours incubation time.

Table 3. **Effect of growth temperature between mutants and *L. plantarum* TGR-2**

No.	Microbes	Growth Temperature (°C)		
		15	30	45
1.	**TGR-2**	+	+++	+++
2.	UV-8	–	+++	+
3.	UV-15	–	+++	+
4.	EMS-10	–	+++	+
5.	**EMS-19**	–	+++	–
6.	AO-5	–	+++	+
7.	**AO-6**	–	+++	+
8.	**NTG-21**	–	+++	–
9.	NTG-30	–	+++	–

+) growth
–) no growth

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