Remove Ethidium bromide by EtBr destroyer and Mutagenicity of End-products

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## Abstract

Two pieces of 2％ agarose gel are taken and soaked in solution of 10μg/mL EtBr （ethidium bromide） and 10μg/mL EtBr that contained EtBr destroyer respectively. Five minutes later, through UV box observation, fluorescence is found on one of film that soaked in 10μg/mL EtBr solution; however, another film that soaked in 10μg/mL EtBr with EtBr Destroyer is not fluoresced. Furthermore, the above film that soaked in 10μg/mL EtBr is re-soaked in EtBr Destroyer solution; Five minutes later, the result is similar to the above experiment, the fluorescence is disappeared. Therefore, it is concluded if EtBr is added EtBr Destroyer and then decomposed into end-products that is non-double bond which cannot produce fluorescence. The above coloring film is left in normal temperature for 24 hours and 48 hours and re-observed the fluorescence. It is resulted that after EtBr Destroyer treatment, the EtBr coloring agarose film cannot produce the retro-EtBr. Ames test analysis is used to study mutagenicity of EtBr and its decomposed product and it is found there is no growth restraint for *Salmonella typhimurium* TA97、TA98 、TA100、TA102 or TA1535 bacteria when the concentration of EtBr is taken between 10μg/mL ~0.25μg/mL. In the meantime, EtBr Destroyer does not restrain the growth of the above five strains either. For mutagenic test, the five standard strains in 5μg/mL EtBr solution will be increased over 2 times colonies due to mutation. However, nothing is happened and there is no difference between colony number and negative control group in same concentration of EtBr + EtBr Destroyer solution. In conclusion, the study shows EtBr can be neutralized by EtBr Destroyer and the end-product after neutralization does not have mutagenicity.

# I. Introduction

Ethidium bromide, EtBr, posses flat structure and can be inserted into DNA or RNA helix structure to be integrated closely. It also appears reddish orange fluorescence chemical substance under ultraviolet light. Therefore, it is used as an agent for DNA and RNA in molecular biological experiment. As it can be conjugated with DNA that affects the cloning process of cell DNA and lead to mutation; therefore, it is regarded as one of toxic carcinogens or mutagen. Normally, activated carbon or sodium hypochlorite is used to remove EtBr; however, the cost of the activated carbon is too expensive that general labs cannot afford it. Although sodium hypochlorite treatment can reduce hazard of EtBr, the waste chemical liquid will produce second hazard to our environment. In addition, sodium hypochlorite destroys 98% EtBr only that may create reversion occurrence. Thus, an effective EtBr Destroyer to removal Ethidium bromide, EtBr has been researched and developed. It is expected to reduce the hazard of EtBr to lab personnel as well as environment. This experiment will evaluate the effectiveness of EtBr Destroyer for Ethidium bromide, EtBr and the mutation test for activated ingredients.

# II. Material and Method

## 1. Material :Medium and Reagent

(1.) Nutrient broth culture solution, hereinafter NB solution.

Per liter solution contains 8g Difco bacto nutrient broth and 5g NaCl that is sterilized by 121℃ 15PSI pressure for 20 minutes and then stored in 4℃ for future use.

(2.) Nutrient agar plate, hereinafter NA medium.

15g agar is dissolved in NB solution and is sterilized by high temperature and pressure. And then it is respectively packed into 100x15mm sterile culture plate (25ml) while it is hot. After condensing under normal temperature, stored in 4℃ freezer for future use.

(3.) 50 times of Volgel-Bonner salts solution, hereinafter 50xVB salt.

Per liter solution contains 10g $MgSO_4$, 100g citric acid monohydrate, 500g $K_2HPO_4$, $NaHNH_4PO_4$ and 175g $4H_2O$. To preheat 670ml distilled water up to 45℃ when disposing. And then to mix and add each element continually one by one when the former element has been dissolved completely. Finally, to add distilled water into the volume until it is 1 liter. To sterilize the solution by high temperature and pressure and then to store in freezer for future use.

(4.) Minimal glucose agar plates, hereinafter MA plate, for mutation test.

15g agar is added into 930mL distilled water and sterilized by high temperature and

pressure and then add sterile 50xVB salt 20mL and sterile 40％ glucose 50mL. After mixing evenly, to pour it into 100x15mm sterile culture plate (25mL) to let it condense under normal temperature. And then pour it in culture plate under 37℃ environment for one day of culturing to be used.

(5.) Soft agar or called top agar for mutation test.
To mix 0.75% agar and 0.5% NaCl, and then packed 2mL in tube (13㎜（ψ））×100㎜. After high temperature and pressure sterilized, stored in freezer for future use. Before use, dissolve it by heat and store in 45℃ water. (Note: agar should be avoided heating and condensing repeatedly to prevent mutation that will condense difficultly.)

(6.) 0.5mM Histidine/biotin solution for mutation test.
Ingredients per 100mL solution is as below,
D-Biotin: 12.2mg
L-Histidine・HCl 10.5mg
Biotin is uneasily dissolved in normal temperature but can be dissolved by heat. After biotin dissolved, histidine is added. The final solution is sterilized by 0.22μm vacuum filter membrane and store in 4℃ freezer for future use. 0.2mL soft agar is added 0.2mL final solution for each experiment.

(7.) 0.1M Histidine-HCl to test his- gene property.
2.1g Histidine-HCl is dissolved in 100mL sterile water and sterilized by 0.22μm vacuum filter membrane. After that, to store in 4℃ freezer for future use.

(8) Ampicillin to test R.
0.8g Ampicillin is dissolved in 100mL 0.22N NaOH solution and then sterilized by 0.22μm vacuum filter membrane to store in 4℃ freezer for future use.

(9) Crystal violet to test rfa property.
0.1g Crystal violet solution is added in 100mL sterile water and the container is packed with foil to keep away from sunlight and then to store in 4℃ freezer for future use.

(10) Master plates, called Ampicililn plates, to test R, his and microorganism.

The ingredient per liter in medium is as below,
Agar: 15 g
50×VB Salts: 20 mL
40% Glucose: 50 mL

0.1M Histidine-HCl: 2.6 mL
0.5mM Biotin: 6 mL
0.8% (w/v)Ampicillin: 3.15 mL

Agar is dissolved in 930mL distilled water and sterilized by high temperature and pressure. And then sterile glucose, 50xVB Salts and histidine solutions are added and mixed evenly. When the solution temperature lowers to 50℃ approximately, sterile biotine and ampicillin solutions are added and mixed evenly and then pour into 100x15mm culture plate 25mL/plate). After condensed under normal temperature, to reverse the plate in 37℃ culture box for a day to be used. The unfinished master plates can be stored in plastic bag in 4℃ freezer. It can be stored for 2 months approximately.

(11) 0.2M pH7.4 phosphate buffer:
0.2M $NaH_2PO_4 \cdot H_2O$ (27.6g/l) and 0.2M $NaHPO_4 \cdot 7H_2O$ (53.6 g/l) are mixed in ration 19：81 and adjusted the pH value up to 7.4 to be sterilized by autoclave for future use.

(12) The Disposal of Standard Mutagen 4-nitroquinoline-N-oxide（4-NQO.
0.3mg is dissolved in 100ml DMSO solution. After mixing evenly, it is sterilized and filtered by 0.22 μm microporous membrane.

(13) EtBr Destroyer

(14) Mouse Liver Activated Enzyme Preparation
a. Mouse Enzyme Inducement
Chemical compound as polychlorinated biphenyls (PCBs), Phenobarbital and methylcholanthrene (MCA) can stimulate mouse liver to produce a large number of microsome activated enzyme. As Ames (1975) stated that PCBs-Aroclor 1254 have the best inducement. The method is to dissolve Aroclor 1254 in corn oil to form 200mg/mL concentration. And then take dose of 500mg/ kg body wt to inject into an abdomen of adult male mouse that weighed 200g (*Rattus norvegicus*, Spraque-Dawley). On the 5$^{th}$ day, the mouse is dissected. Before 12 hours of dissection, no more feeding except water.

b. Dissection and Take the Liver Out
To ensure the cleanness and activation of enzyme extraction, all experimental equipment and reagent are sterilized by high autoclave except knife, scissors and forceps are sterilized by alcohol lamp. Following sterilization, dry them and store in 4℃ freezer for future use.

To cut the mouse neck with bloodletting and leave it on dissecting tray with limbs fasten. To spray 70~95% alcohol or wipe iodine on its chest and then to dissect and stick its skin to both sides tightly. To re-spray the alcohol on mouse muscle and replace clean scissors and forceps to dissect the liver.

c. Liver Activated Enzyme (S9 fraction) Preparation
Dissected liver is weighed and washed by ice-cold 0.15M KCI several times. Do not leave any blood to prevent haemoglobin affecting P450 enzyme activation of cytochrome. 0.15M KCI (3mL/g per liver weighed 15g) is added. First of all, liver is cut up and grinded by tissue grinder. And to be centrifuged by 9000xg (Becjman L5-65 centrifuge, Roter Type30, 10,000rpm) for 10 minutes. Take upper layer liquid as S9 Fraction and pack in freeze tube (1mL) to store in -80℃ low temperature freezer. S9 Fraction is unfrozen in normal temperature before use; however, it should put in ice to maintain the enzyme activation.

d. S9 mixed liquid Preparation:
The ingredient per 50mL S9 mixed liquid for experiment is as below:
Ingredient per 50mL:
1、 Rat liver S9 (Aroclor-1254-induced): 2.00 mL
2、 0.4M $MgCl_2$-1.65M KCl: 1.00 mL
3、 1M glucose-6-phosphate: 0.25 mL
4、 0.1M NADP: 1.00 mL
5、 0.2 M phosphate buffer, pH 7.4: 25.00 mL
6、 sterile distilled $H_2O$: 19.75 mL

**2. Method**
(1) Observe Fluorescence by UV Light
Two piece of agarose gel are soaked in10μg/mL EtBr solution and 10μg/mL EtBr that contained EtBr Destroyer solution respectively for 5 minutes and then observe the fluorescence in UV box. Furthermore, the above film that soaked in 10μg/mL EtBr solution will be re-soaked in 10μg/mL EtBr that contained EtBr destroyer for 5 minutes and then observe the fluorescence. Following 24 hours and 48 hours leave the film in UV box again to observe whether reversion appears.

(2) Preparation of Test Organism
The mutation strain for this experiment is *Salmonella typhimurium* TA97, TA98, TA100, TA102 and TA1535. To take 0.1mL -70℃ preserved bacterium liquid to inoculate nutrient broth. Medium tube is wrapped by foil and leave in 35℃ incubator for 24 hours for cultivation. After that, experiment is carried out immediately.

(3) Toxicity Test

EtBr and EtBr Destroyer against *Salmonella typhimurium* TA97, TA98, TA100, TA102 and TA1535 are tested in accordance with Ames method (1983).

To test whether there is inhibition for strain growth. 1.5mL sterile centrifuge tube is put in ice bath for future use. The sampling treatment is as below:

(1) 0.5mL S9 mix (+S9 mix) + 0.1mL bacterium liquid; （2）S9 mix（-S9 mix）+ 0.1mL bacterium liquid replaced by 0.5 mL DMSO solution; （3）0.5 mL S9 mix is added respectively and then added 0.1 mL 10, 5, 1, 0.5, 0.25μg/mL concentration of EtBr+0.1 mL bacterium liquid respectively; (4）0.1 ml EtBr destroyer +0.1mL bacterium liquid. Following that, culture it in 35℃ incubator for 20 minutes. The mixed solution after culture will be diluted to about 2-3×$10^2$ cfu/mL colony numbers. To take 1mL diluent with Nutrient agar to proceed mix-diluting test. Each experiment shall be repeated for 3 times.

(4) Mutation Test

EtBr Destroyer against *Salmonella typhimurium* TA97, TA98, TA100, TA102 and TA1535 are tested in accordance with Ames method (1983).

The outcome of strain mutation: the experimental sample treatment is as below, 1.5mL sterile centrifuge tube is put in ice bath. （1）S9 mix (0.5 mL)（+S9 mix）; （2）S9 mix（-S9 mix）replaced by DMSO solution; （3） 0.1mL of 5μg /mL EtBr; （4）0.1 mL EtBr destroyer that contained 5μg /mL EtBr; （5）4-nitroquinoline-N-oxide（4-NQO）of 3μg/plate for positive control group; （6）0.1mL oolong tea on the market for negative control group and then add 0.5mM histidine/biotin (0.2 mL) with overnight cultivation *Salmonella typhimurium* TA97, TA98, TA100, TA102 and TA1535 bacterium（0.1 mL）. And then leave it in 35℃ incubator for 20 minutes, and then add dissolved 0.75% of Top Agar 4 mL (45℃）. After mixing evenly, pour in Minimal glucose agar plates for condensation. After condensation, leave in 35℃ incubator for 48 hours to count the colony.

# Result

## 1. EtBr Coloring Result

The illustration outcome shows that the film soaked in 10μg /mL EtBr solution for 5 minutes, fluoresce is observed in UV box (Left Illustration 1) and no fluoresce is observed for the film soaked in 10μg /mL EtBr that contained EtBr Destroyer (Right Illustion2). The film that soaked in 10μg /mL EtBr for 5 minutes is re-soaked in EtBr Destroyer solution for 5 minutes again but the existing fluoresces disappears. It is presumed that EtBr is dissolved by adding EtBr Destroyer; therefore, the colored film cannot bright fluoresce in UV box. Furthermore, leave the above colored film under normal temperature for 24 hours and no fluoresce is re-observed that proves

no EtBr reversion happened when the EtBr agarose colored film having EtBr Destroyer treatment.

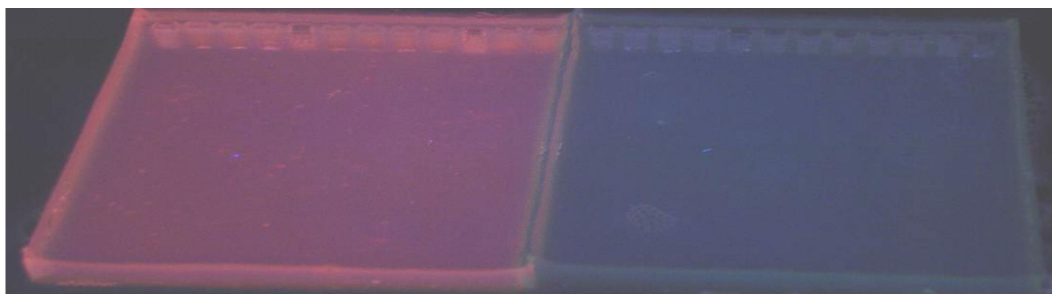


Illustration 1: the left one shows fluorescence in UV box when 10μg /mL EtBr is colored after 5 minutes. The right photo shows no fluorescence in UV box when 10mg/mL EtBr is in EtBr Destroyer solution.

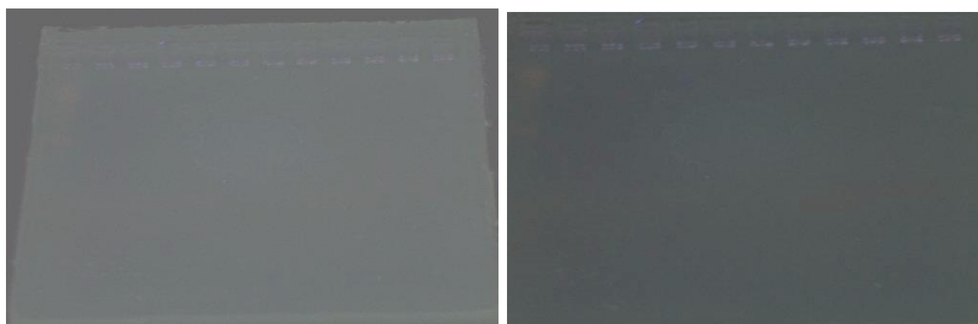


Illustration 2: the film coloring in 10μg /mL EtBr for 5 minutes and then put into EtBr Destroyer solution, the previous fluorescence disappears (Left). Leave the colored EtBr film in normal temperature for 24 hr and 48 hr. No fluorescence is observed (Right).

## 2. Toxic Test Result

Table 1 shows that when EtBr concentration is between 10μg ~0.25μg with EtBr Destroyer do not have any growth inhibition effect on *Salmonella typhimurium* TA97, TA98, TA100, TA102 or TA1535 strain. (Table 1)

Table 1: The strain toxic test on sample to *Salmonella typhimurium* TA97, TA98, TA100, TA102 and TA1535.

| | TA97 | TA98 | TA100 | TA102 | TA1535 |
|---|---|---|---|---|---|
| +S9 | 58.60.61 | 42.40.48 | 55.58.48 | 114.121.117 | 77.76.70 |
| -S9 | 55.57.62 | 42.48.44 | 53.46.42 | 112.108.114 | 75.70.73 |
| 10μ g EtBr | 58.55.61 | 34.31.37 | 42.36.39 | 117.104.105 | 63.65.53 |
| 5 μ g EtBr | 53.56.50 | 40.37.42 | 50.47.43 | 102.106.107 | 72.69.67 |
| 1 μ g EtBr | 57.56.60 | 46.42.38 | 53.47.50 | 114.116.101 | 74.77.73 |
| 0.5 μ g EtBr | 62.55.57 | 44.47.49 | 52.54.49 | 122.124.102 | 80.83.74 |
| 0.25 μ g EtBr | 57.66.58 | 48.42.43 | 55.58.50 | 112.132.121 | 70.72.69 |
| EtBr destroyer 100 μ 1 | 54.52.51 | 40.42.47 | 49.51.42 | 106.108.104 | 77.72.75 |

The result shows that EtBr concentration is between is between 10μg ~0.25μg with EtBr Destroyer do not have any growth inhibition effect on *Salmonella typhimurium* TA97, TA98, TA100, TA102 or TA1535 strain.

### 3. Mutation Test Result

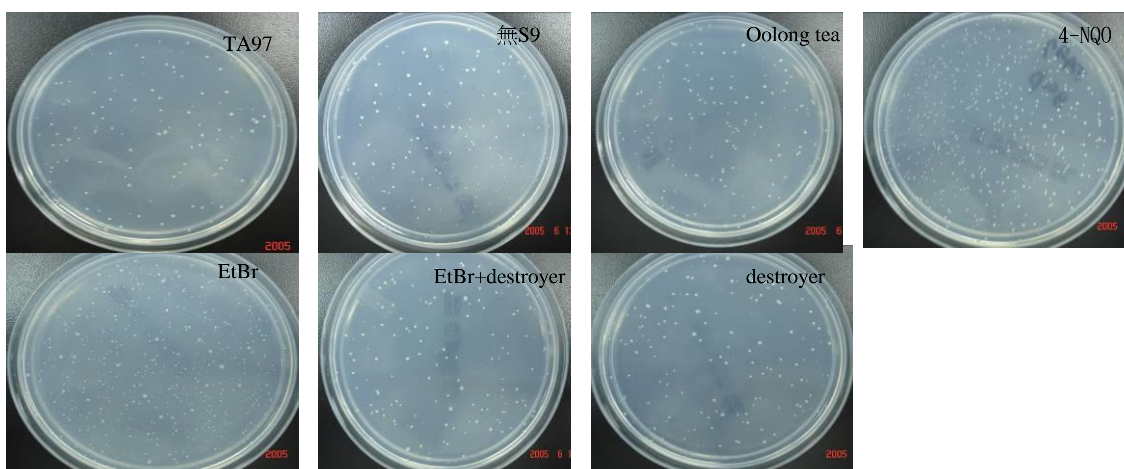
Table 2 ~ 6 shows that there is no difference on colony number among TA97, TA98, TA100, TA102 and TA1535 with +S9, S9, EtBr+Destroyer and oolong tea. This proves that the strain does not have any mutation. However, the colony number is significantly doubled up after adding EtBr and standard mutagen 4-NQO. This demonstrates that strain mutation will be induced by EtBr.

Table 2: The mutation test of EtBr Destroyer to TA 97 microorganism.

| | TA97(+S9) | TA97(-S9) | Oolong tea | 4-NQO | EtBr | EtBr+destroyer | destroyer |
|---|---|---|---|---|---|---|---|
| 1 | 107 | 87 | 111 | TNTC | TNTC | 112 | 103 |
| 2 | 104 | 80 | 108 | TNTC | TNTC | 114 | 107 |
| 3 | 102 | 84 | 106 | TNTC | TNTC | 101 | 101 |

As the result shows that the colony number of mutation are not significantly increased on the test of TA 97 strain to +S9,-S9, EtBr+Destroyer and oolong tea. This shows that the strain is not mutated.

Illustration 3: The mutation test of EtBr Destroyer to TA 97 microorganism.



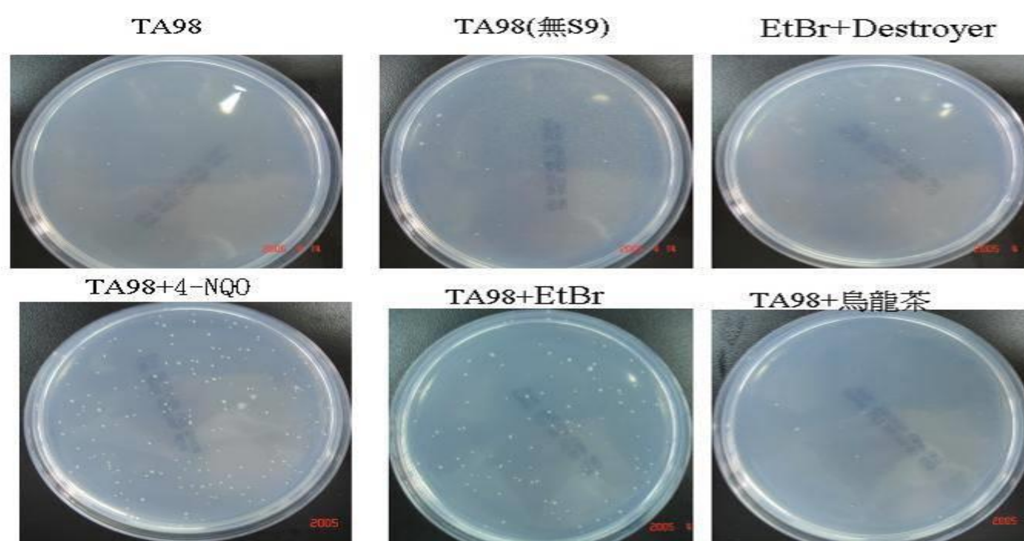

As Table 2 shows that the colony number of mutation are not significantly increased on the test of TA 98 strain to +S9,-S9, EtBr+Destroyer and oolong tea. This illustration shows that the strain is not mutated.

Table 3: The mutation test of EtBr destroyer to TA 98 microorganism.

| | TA98(+S9) | TA98(-S9) | EtBr+Destroyer | 烏龍茶 | 4-NQO | EtBr |
|---|---|---|---|---|---|---|
| 1 | 10 | 8 | 12 | 10 | 183 | 168 |
| 2 | 8 | 10 | 9 | 9 | 172 | 167 |
| 3 | 10 | 14 | 13 | 12 | 184 | 165 |

As the result shows that the colony number of mutation are not significantly increased on the test of TA 98 strain to +S9,-S9, EtBr+Destroyer and oolong tea. This shows that the strain is not mutated.

Illustration 4: The mutation test of EtBr Destroyer to TA 98 microorganism.



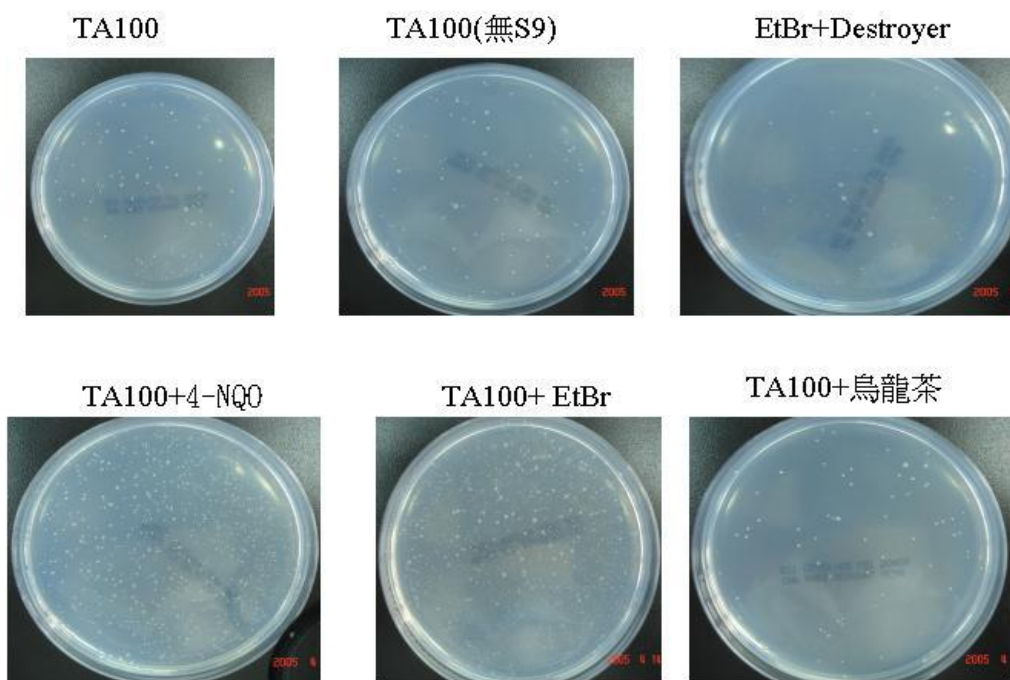

As Table 2 shows that the colony number of mutation are not significantly increased on the test of TA 98 strain to +S9,-S9, EtBr+Destroyer and oolong tea. This shows that the strain is not mutated.

Table 4: The mutation test of EtBr Destroyer to TA 100 microorganism.

| | TA100(+S9) | TA100(-S9) | EtBr+Destroyer | 烏龍茶 | 4-NQO |
|---|---|---|---|---|---|
| 1 | 118 | 95 | 91 | 86 | TNTC |
| 2 | 102 | 96 | 104 | 111 | TNTC |
| 3 | 105 | 93 | 107 | 109 | TNTC |

As the result shows that the colony number of mutation are not significantly increased on the test of TA 100 strain to +S9,-S9, EtBr+Destroyer and oolong tea. This shows that the strain is not mutated.

Illustration 4: The mutation test of EtBr destroyer to TA 100 microorganism.



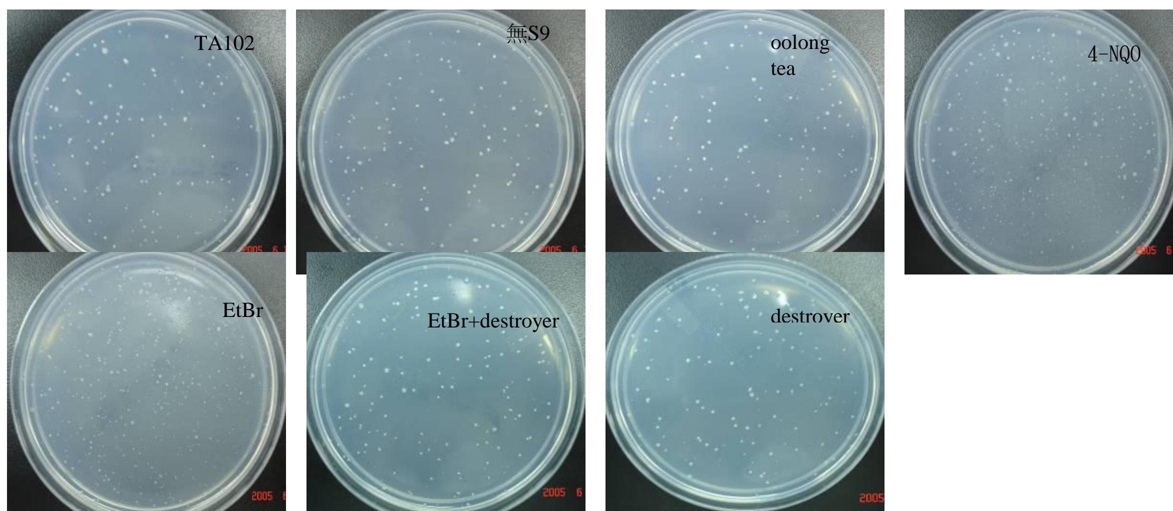

As Table 4 shows that the colony number of mutation are not significantly increased on the test of TA 100 strain to +S9,-S9, EtBr+Destroyer and oolong tea. This shows that the strain is not mutated.

Table 5: The mutation test of EtBr Destroyer to TA 102 microorganism.

| | TA102 | 無S9 | 茶 | 4-NQO | EtBr | EtBr+destroyer | destroyer |
|---|---|---|---|---|---|---|---|
| 1 | 121 | 109 | 110 | TNTC | TNTC | 107 | 104 |
| 2 | 118 | 106 | 121 | TNTC | TNTC | 115 | 111 |
| 3 | 115 | 101 | 123 | TNTC | TNTC | 111 | 107 |

As the result shows that the colony number of mutation are not significantly increased on the test of TA 102 strain to +S9,-S9, EtBr+Destroyer and oolong tea. This shows that the strain is not mutated.

Illustration 5: The mutation test of EtBr destroyer to TA 102 microorganism.



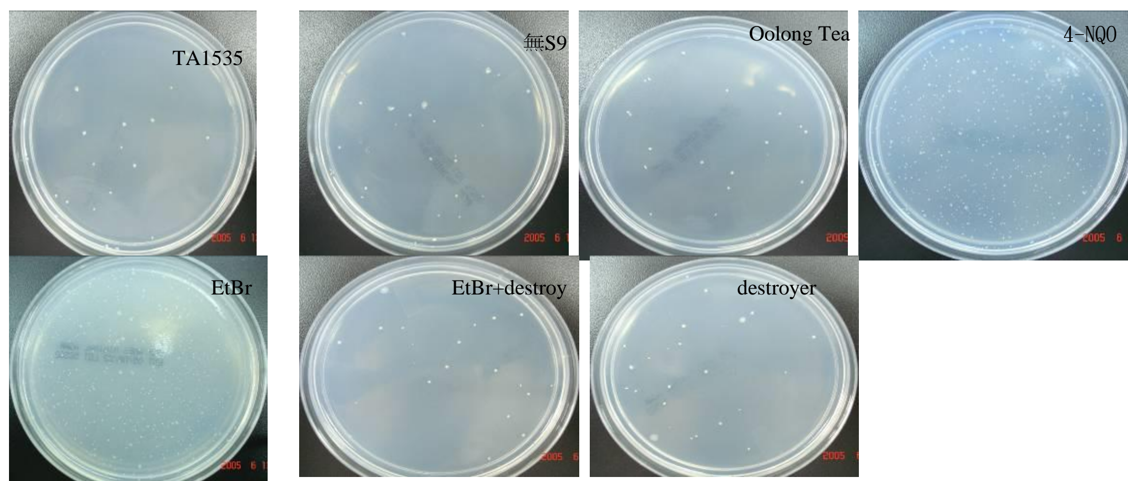

As Table 5 shows that the colony number of mutation are not significantly increased on the test of TA 102 strain to +S9,-S9, EtBr+Destroyer and oolong tea. This shows that the strain is not mutated.

Table 6: The mutation test of EtBr destroyer to TA 1535 microorganism.

| | TA1535 | 無S9 | Oolong tea | 4-NQO | EtBr | EtBr+destroy | destroyer |
|---|---|---|---|---|---|---|---|
| 1 | 25 | 20 | 20 | 406 | TNTC | 24 | 21 |
| 2 | 23 | 22 | 18 | 415 | TNTC | 28 | 25 |
| 3 | 22 | 24 | 20 | 407 | TNTC | 24 | 20 |

As the result shows that the colony number of mutation are not significantly increased on the test of TA 1535 strain to +S9,-S9, EtBr+Destroyer and oolong tea. This shows that the strain is not mutated.

Illustration 6: The mutation test of EtBr Destroyer to TA 1535 microorganism.




As Table 6 shows that the colony number of mutation are not significantly increased on the test of TA 1535 strain to +S9,-S9, EtBr+Destroyer and oolong tea. This shows that the strain is not mutated.

## Discussion

In EtBr experiment, the EtBr colored film appears reddish fluoresce under ultraviolet light; however, there is no fluoresce after EtBr having been neutralized with EtBr Destroyer. The film soaked in 1mg/ml EtBr solution for 5 minutes and re-soaked in EtBr Destroyer for 5 minutes again, the existing fluoresce disappears. This shows that EtBr is dissolved after adding EtBr Destroyer; therefore, the colored film cannot shine fluoresce. Furthermore, there is no fluorescing observed after leaving the EtBr colored film under normal temperature for 24 hours and 48 hours. This proves that the EtBr agarose film cannot produce EtBr reversion after EtBr Destroyer treatment.

From toxic experiment of Ames test, it is observed that there is no strain growth inhibition occurred when the concentration of EtBr and EtBr Destroyer are between 10μg/mL ~0.25μg/mL with *Salmonella typhimurium* TA97, TA98, TA100, TA102 and TA1535. In Ames test, it is observed that the colony number shows no difference among *Salmonella typhimurium* TA97, TA98, TA100, TA102 and TA1535 whether there is S9 mix. This shows S9 is normal. In oolong tea (negative control group), the outcome shows that there is no significantly change on colony number. This proves that the strain and experiment process are normal and free of contamination. However, the colony number is significantly increased, moreover, the increasing range is over 2 times after adding the standard mutagen 4-NQO (positive control group). According to Ames (1983) report, the mutagenicity is existence when the colony number of positive control group is 2 times over more than the one of negative control group. Therefore, the experimental outcome shows the strain sensitivity is normal. When the colony number of experimental strain is increased over 2 times that proves EtBr provides mutagenicity. And the colony number of

experimental strain is not increased if EtBr + EtBr Destroyer that proves EtBr destroyer can destroy EtBr effectively and the end-product do not provide any mutagenicity and reversion after neutralization with EtBr.

## Reference

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