Biodegradation of Formaldehyde-Releasing Preservatives

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SUMMARY

A strain of Pseudomonas aeruginosa was isolated from the spoiled product and its characteristics on various formaldehyde-releasing preservatives were investigated.

This strain, P. aeruginosa FR, could utilize 1.0% of imidazolidinyl urea and 0.2% of DMDM hydantoin as a sole carbon and nitrogen source in the minimal salts medium. With the growth of the strain in minimal salts medium containing imidazolidinyl urea, formic acid was initially accumulated according to the decrease of formaldehyde concentration. It was suggested that formaldehyde dehydrogenase was involved in this oxidation process and could catalyze formaldehyde, imidazolidinyl urea, DMDM hydantoin and quaternium-15, but not bronopol.

MICs of this strain to each preservative were 0.03% in formaldehyde, 1.0% in imidazolidinyl urea, 0.2% in DMDM hydantoin, 0.2% in quaternium-15 and 0.1% in EDTA-2Na. But the MICs were diminished about ten times when 0.01% of EDTA-2Na was added to the preservative systems.

In actual challenge test, the eyeliner and the pack which contained paraben and imidazolidinyl urea were not able to be protected from this strain, but when 0.05% EDTA-2Na was added the products were sufficiently preserved.

INTRODUCTION

The spoilage of cosmetic products by microorganisms has been the serious problem in economical and health-care aspects. Especially in the O/W emulsion systems, water and various soluble organic compounds have provided favorable environment for the growth of microorganisms. Also due to the current trends using natural ingredients and the increased resistance of microorganisms to antimicrobial agents, the selection of effective preservative system has been more difficult than ever before.

Over the last 20 years, several new preservatives have been introduced and some of these have already gained wider acceptance. Among them, formaldehyde-releasing preservatives have been occupied as a main portion and widely used in the cosmetic industry [1] Most common properties of formaldehyde-releasing preservatives were highly soluble in water, but less soluble in oil and more effective against bacteria than against fungi and yeast. These preservatives were not used alone for the preservation, but recommended in combination with other preservatives such as paraben. It has been well known that the preservative systems of paraben with formaldehyde-releasing preservative provided effective antimicrobial activity against current contamination problems, including Pseudomonas aeruginosa [2]

One of the most important microorganism causing spoilage problems was the strains of Pseudomonads, especially Pseudomonas aeruginosa, which were widely distributed in nature and highly adaptable. It has been shown to be able to grow in distilled water [3] and even in disinfectant solution [4,5]

This paper described that one strain of Pseudomonas aeruginosa was isolated from the emulsion product with paraben and imidazolidinyl urea as preservatives, and its behaviors on the various formaldehyde-releasing preservatives in the minimal salts medium were investigated.

In conclusion, to protect emulsion system from this highly resistant strain, the new preservative system was proposed.

EXPERIMENTAL PROCEDURE

1. Materials
   Bronopol (Boots Co. Ltd.)
   DMDM hydantoin (Glyco Chem. Inc.)
   Quaternium-15 (Dow Chem. Co.)
   Imidazolidinyl urea (Sutton Lab. Inc.)
   Ethylenediaminetetraacetic acid disodium salt (Hayashi Pure Chem.)
   Formaldehyde (Junsei Chem. Co.)

2. Idnetification of the isolate
   The isolate was identified by following the standard techniques with reference to Bergey's manual of determinative bacteriology (6) and other (7).

3. Total viable count
   The viable counts were performed on Tryptic Soy Agar (TSA: Difco Lab.) using pour plate method (8).

4. Cultivation of microorganism

To examine the isolate's ability to utilize the formaldehyde-releasing preservatives as a sole carbon and nitrogen source, each preservative was added to minimal salts medium. The composition of minimal salts medium was 7.6g of $Na_2HPO_4 \cdot 12H_2O$, 2.0g of KH_2PO_4, 0.02g of $MgSO_4 \cdot 7H_2O$ and 0.017g of $FeCl_3 \cdot 6H_2O$ in 1 liter of distilled water. The inoculum was grown in Nutrient broth (Difco) for 24 hours at 37°C. After centrifugation, the pellets were washed twice with sterile saline. 0.1 Ml of culture suspension was then inoculated into 200ml of minimal salts medium containing 1.0% of imidazolidinyl urea or 0.2% of DMDM hydantoin, and incubated at 30°C on a reciprocal shaker.

5. Analytical method

At time intervals, the culture medium was collected and formaldehyde concentration was determined with acetyl acetone reagent (9) using fluoroescence spectrophotometer model 204-S from Hitachi.
The calibration curve was measured by freshly prepared standard solution of formaldehyde.

The residual imidazolidinyl urea was estimated with phenylhydrazine method [10, 11] The detailed procedure was as follows; 1 ml of 0.5N NaOH was added to each sample and heated in boiling water bath for 10 minutes. After cooling to room temperature, 2.5ml of 0.5N HCl and 1ml of phenylhydrazin hydrochloride (33%) solution were added to the each sample and heated in boiling water bath for 2 minutes. After it was chilled with Ice-NaCl solution for 20 minutes, 3ml of cold HCl and 1 ml of 2% potassium ferrocyanide were added and allowed them for 30 minutes to development of the color and then measured the absorbance at 525nm with spectrophotometer (Hitachi model 200-10).

The formic acid which was accumulated in the culture media, was measured as follows; A 100ml portion of medium culture was centrifuged to remove the cells and the supernatant was acidified to pH 2.0 with diluted sulfuric acid, and distilled with steam. About 200ml of distillate was adjusted to pH 10.0 again with diluted NaOH solution and concentrated to less than 5ml by heating in water bath under reduced pressure. Immediately prior to injection into the gas chromatography, the sample was acidified with 0.25ml of concentrated phosphoric acid and brought to volume.
The gas chromatography analysis [12] was carried out by a Varian model 3700 gas chromatography with flame ionization detector. The glass column was packed with Porapak Q (100/120 mesh, Waters Associates Inc.) and the conditions were; column temperature, 170°C; inlet temperature, 210°C; detector temperature, 200°C. The flow rate for the carrier gas,

helium, was 30 CC/min and for the detector gas, air and hydrogen, were 300 and 30 CC/min respectively.

6. Preparation of crude enzyme

Bacterial cells grown in 1.0% of imidazolidinyl urea were harvested by centrifugation at 1,600 x g for 20 minutes and the collected cells were washed with M/15 phosphate buffer (pH 7.0) and resuspended with the same buffer.

This suspension was subjected to sonic oscillation with the Fisher ultrasonic disintegrator for 5 minutes.

Cell debris was removed after centrifugation at 30,000 x g for 1 hour at 4°C. The clear supernatant solution was used as a crude enzyme.

7. Assay of formaldehyde dehydrogenase

Formaldehyde dehydrogenase activity was assayed spectrophotometrically[13] at 340nm by measuring the formation of $NADH_2$ consequent upon the oxidation of formaldehyde to formic acid.

The reaction mixture contained, in 1-ml volume; 0.1ml of triethanolamine -HCl buffer (1M, pH 8.5), 0.05ml of glutathion (0.067M), 0.03ml of NAD (0.012M), 0.04ml of $NH_2OH \cdot HCl$ (0.02M) and enzyme.

After 2 minutes of preincubation, 0.05ml of HCHO (0.02M) or corresponding formaldehyde-releasing preservatives was added to start the reaction. All substrates used in this experiment were freshly prepared.

8. Determination of MIC

The broth dilution method[14] was employed to determine minimum inhibitory concentrations (MIC) of this strain against various formaldehyde-releasing preservatives alone or combination with ethylenediaminetetraacetic acid disodium salt (EDTA-2Na). To compare the resistance to preservatives, P. aeruginosa NCTC 10490 was used.

A series of tubes containing various concentrations of the tested preservatives in nutrient broth were inoculated with 24 hour-cultured organism. After culturing for 48 hours at 37°C, the presence of viable growth was checked.

9. Actual challenge test for new formulation

Two emulsified preparations, the eyeliner and the pack, were selected to check the new preservative system. Also two kinds of the inocula, the one was the contaminated products itself and the other was cultured on nutrient broth, were prepared to compare the adaptability. Initial inoculum level was approximately 1×10^6 cells per gram of test products and the other detailed procedure for the challenge test was conducted by the CIFA recommendation[15]

RESULTS AND DISCUSSION

One strain of the microorganism was isolated from the claimed eyeliner containing 0.25% of methyl paraben, 0.1% of propyl paraben and 0.7% of imidazolidinyl urea as a preservative system.

It was identified as Pseudomonas aeruginosa on the basis of following bacteriological features; Gram negative, motile, rod, diffusing characteristic greenish-blue pigment on T S. Agar, oxidase and catalase positive, gelatin liquefaction, hydrolysis of arginine, growth at 41'C and produced heat-stable alkaline phosphatase.

From now on, the isolate was called as P. aeruginosa FR.

When P. aeruginosa FR was grown in minimal salts media containing 1.0% of imidazolidinyl urea as a sole carbon and nitrogen source, the change of number of viable cells, the concentration of formaldehyde and imidazolidinyl urea are shown in Fig. 1.

As shown in Fig. 1, the total viable counts decreased during the adaptation period of about 3 days, and then rapidly increased and the number of living cells reached to 10^7 cell/ml after 7 days.

The concentration of formaldehyde in the media decreased after 3 days that viable cell number began to increase, and eventually dropped to zero within 7 days.

But merely exhaustion of the formaldehyde concentration did not represent the degradation of imidazolidinyl urea itself, so we used the phenylhydrazine method to determine the residual concentration of imidazolidinyl urea in the culture media. Until now this method has not been applied to measure the imidazolidinyl urea, but in our experiment the calibration curve of it was found to be linear with concentration over the 0.1 to 1.0 mg/ml.

As shown in Fig. 1, the change of the concentration of imidazolidinyl urea did not occur within 6 days. But since then the formaldehyde concentration dropped to zero, the imidazolidinyl urea was degraded up to the 60% over several days and no further degradation.

As the cells grew, the pH of culture media decreased according to the exhaustion of the formaldehyde concentrations.

There were several possibilities to change the pH, but a certain acidic metabolites might be formed during the initial stage of degradation. The initial metabolite was identified as formic acid by G. C. analysis.

The correlation between the pH of culture medium, the concentration of formaldehyde and formic acid is shown in Fig. 2.

As shown in Fig. 2, formation of formic acid coincided with decrease of formaldehyde content, and maximum amount of formic acid in the culture medium reached about 90mg/100ml on 7 days and rapidly disappeared next 2 days. After 7 days, the pH of culture medium rapidly increased and reached

to 9.0. This was probably ue to the degradation of formic acid formed initially and decomposition of imidazolidinyl urea which led to formation of ammonia. At that time, because of the high pH of medium this strain could not degrade the remained materials and nearly 40% of imidazolidinyl urea remained in the culture medium.

Growth of P. aeruginosa FR on other formaldehyde-releasing preservative, DMDM hydantion, was examined and the result is shown in Fig. 3.

This strain could utilize 0.2% of DMDM hydantoin for growth, but not above concentration. Compared with the imidazolidinyl urea, the time for exhaustion of formaldehyde was similar to that of imidazolidinyl urea, but the number of viable cell remained at low level and reached only 10^6 cells/ml.

The crude enzyme was prepared from P. aeruginosa FR at logarithmic phase in imidazolidinyl urea medium and its substrate specificities against various formaldehyde-releasing preservatives were examined. The results are shown in Fig. 4.

As shown in Fig. 4, formaldehyde, imidazolidinyl urea, DMDM hydantoin, and quaternium-15 were served as substrates, but not bronopol.

The difference of the rate of $NADH_2$ formation between each substrate was presumably related to the rate of releasing formaldehyde.

But the bronopol, which has been generally accepted as a member of formaldehyde-releasing preservatives, could not be served as a substrate. It is thought that formaldehyde dehydrogenase activity might be inhibited by bacteriocidal mechanism of bronopol for the thiol-containing enzyme [16] But in our experiment bronopol could not inhibit the activity of this enzyme.
The difference of our results with other's[17, 18] were probably due to the difference of method which used to detect the free formaldehyde. But the detection condition of enzymatic method which we employed was milder than that of any other chemical method and similar to the actual situation.

We thought that this enzyme was very useful not only in defining the formaldehyde-releasing preservatives but also in studying the kinetics of formaldehyde released from its doner.

Broth dilution method was used for the determination of MICs of various formaldehyde-releasing preservatives against this test strain. Table 1 showed the MICs of each preservative alone and combination with EDTA-2Na.

As shown in Table 1, P. aeruginosa FR showed considerably higher resistance to the test preservatives than the type culture strain of P. aeruginosa NCTC 10490, especially against imidazolidinyl urea.

When 0.01% of EDTA-2Na was added to each tested preservative, the MICs against both organisms were diminished about ten times than those of the

test preservative alone.

EDTA has intrinsic activity against P. aeruginosa and its sensitizing mechanism has been generally known as lytic action of the cell wall of gram negative bacteria [19]

The synergistic potentiations of EDTA with various preservatives are well known[20, 21, 22]

In our experiment, P. aeruginosa FR which has high resistance to formaldehyde-releasing preservatives also showed sensitivity to EDTA.

But such a simple technique, tube dilution method, might not provide full information about its real activity on the actual formulation. Moreover, in case of EDTA, which originally proposed as chelating agent, the antibacterial activity was greatly affected by the presence of cations [20] For this reason it was necessary to examine their synergistic effect on actual cosmetic preparation.

In actual challenge test, we used two preparations, eyeliner and pack, and their formulas are shown in Table 2.

Their relative high concentration of polymers or inorganic pigments tend to adsorb and inactivate the added preservatives.

In these experiments, we used P. aeruginosa FR cultivated as inocula in different media, nutrient broth and originally contaminated product, and each resistance to preservative system was compared. The preservative system and the results of challenge test are shown in Table 3.

When the contaminated product itself was inoculated to the test preparations, both eyeliner and pack with 0.25% of methyl paraben, 0.1% of propyl paraben and 0.7% of imidazolidinyl urea failded to kill it. As shown in Table 3, the number of P. aeruginosa FR decreased by about 10^4 cells/g during first 3 days, and then vigorously grow and survived about 10^7 cells/g throughout test period.

But the same organism grown on nutrient broth could not survive in these products.

Such a remarkable difference of resistance to the same preservative system was due to the circumstance in which they had grown. It is early reported that minor change in growth media caused great change in their resistance [23, 24]

In such a view point, the result obtined from common challenge test which inoculated with cells grown on nutrient broth or any other synthetic media would not always represent real situation.

On the other hand, when 0.05% of EDTA was added to the same products and imidazolidinyl urea concentration was reduced to 0.3%, test organisms were killed within 1 day and no P. aeruginosa were recovered over the 28-day test period.

The results of actual challenge test indicated that the paraben and imidazolidinyl urea could not provide sufficient preservative system against this

highly resistant organism, and the synergistic effect of EDTA shown in MIC test was also proved in actual cosmetic formulations.

From these results, it is clear that EDTA-2Na would provide sufficient preservative capacity to the products which are hard to be preserved and has inactivation of preservatives by interaction of preservatives and other components.

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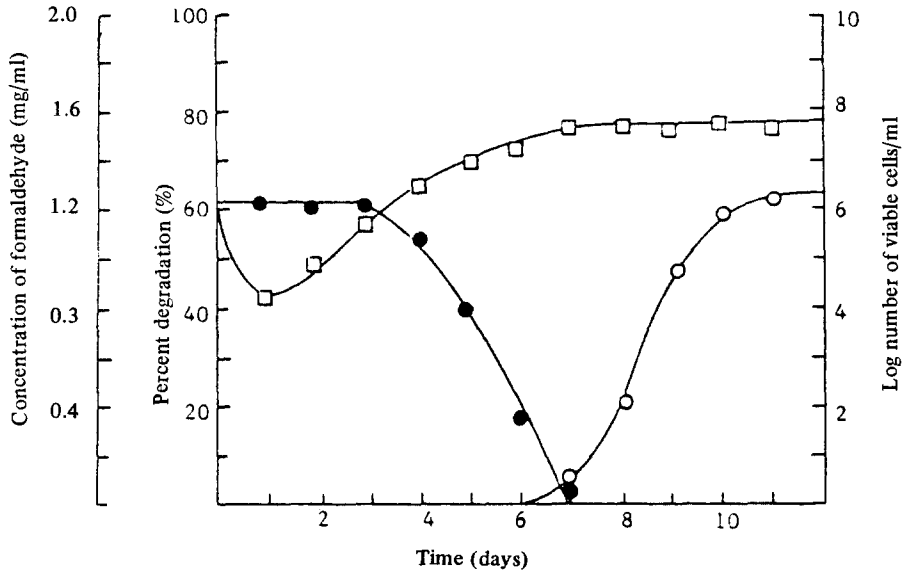

Fig. 1. Growth of P. aeruginosa FR in 1% imidazolidinyl urea.
number of viable cell;
formaldehyde concentration;
percent degradation;

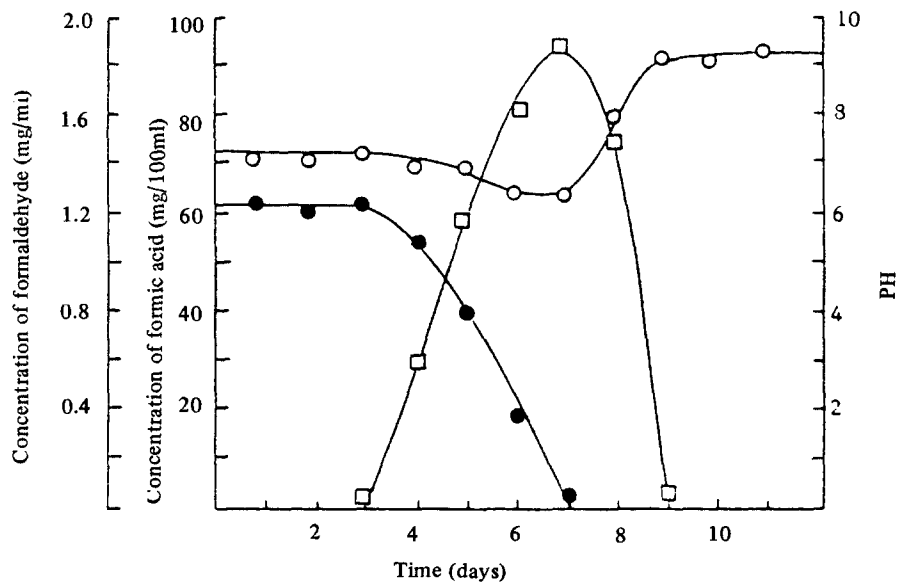

Fig. 2. Growth of P. aeruginosa in 1% imidazolidinyl urea.
formaldehyde; —●—
formic acid; —□—
pH. of media; —○—

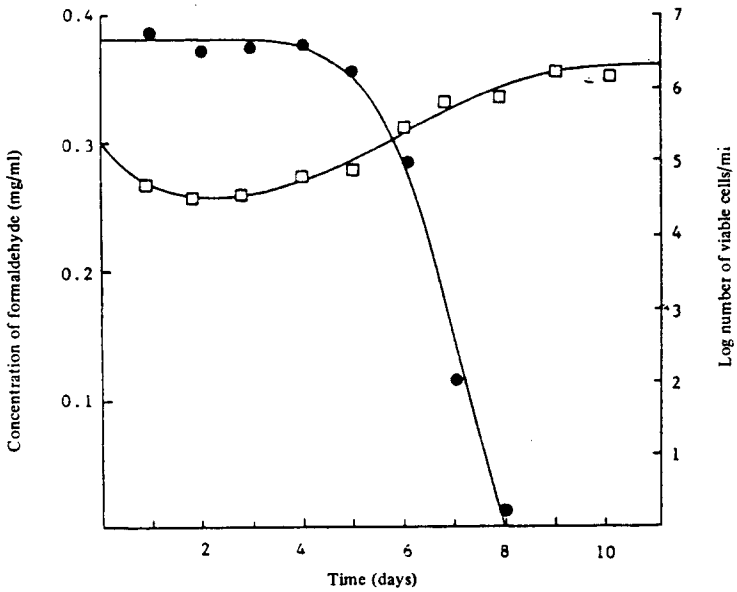

Fig. 3. Growth of P. aeruginosa FR in 0.2% of DMDM hydantoin.
number of viable cell;
formaldehyde cconcentration;

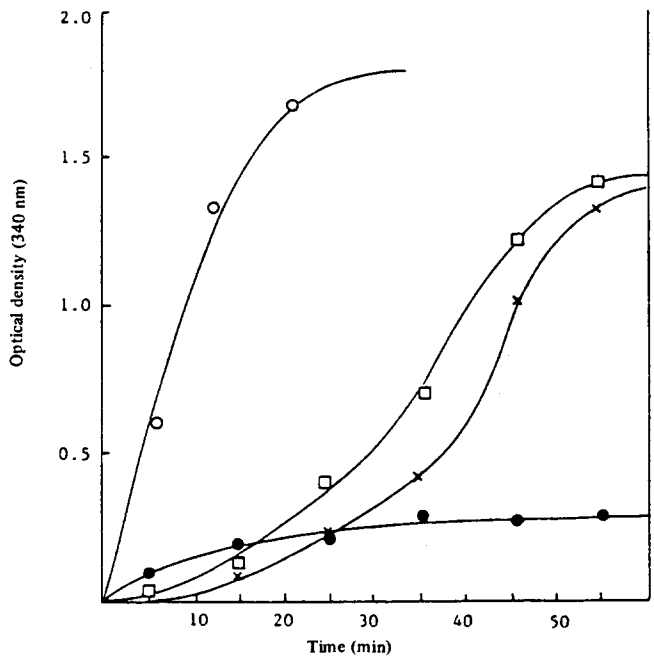

Fig. 4. Substrate specificity of formaldehyde dehydrogenase from P. aeruginosa FR.
formaldehyde;
imidazolidinyl urea;
quaternium-15;
quaternium-15;
DMDM hydantoin;

Table 1. MIC values of various preservatives against P. aeruginosa.

Preservatives	MIC (%)			
	P. aeruginosa FR		P. aeruginosa NCTC 10490	
		with 0.01% EDTA		with 0.01% EDTA
Formaldehyde	0.03	0.004	0.006	0.001
Imidazolidinyl urea	1.0	0.1	0.1	0.05
DMDM hydantoin	0.2	0.03	0.05	0.01
Quaternium-15	0.2	0.02	0.04	0.01
EDTA-2 Na	0.1	–	0.05	–

Table 2. Formulas of Eyeliner and Pack used in the challenge test

(w/w)

Eyeliner		Pack	
PVP	3.5 %	Propylene glycol and Glycerin	3.0 %
Cellulose gum	0.3 %	Emollient oil	5.0 %
Magnesium Aluminium Silicate	0.4 %	Octyl dodecanol	1.0 %
Iron Oxide (black)	12.0 %	TEA – stearate	1.5 %
Shellac wax	0.5 %	Sorbitan sesquioleate	0.5 %
Cetyl alcohol	2.5 %	Polyvinyl alcohol	13.5 %
Lanolin oil	1.0 %	Methyl paraben	0.25 %
Sorbitan Sesquioleate	0.4 %	Propyl paraben	0.1 %
TEA – Stearate	2.35%	Imidazolidinyl urea	0.7 %
Methyl paraben	0.25 %	D. I. Water	to 100 %
Propyl paraben	0.1 %		
D.I. Water	to 100 %		

Table 3. Results of challenge test

Eyeliner

cells/g

inocula / days	Imidazolidinyl urea 0.7%		Imidazolidinyl urea 0.3% + EDTA 0.05%	
	contaminated product	N.B. grown	contaminated product	N.B. grown
1	5×10^3	$< 10^2$	$< 10^2$	$< 10^2$
3	6×10^4	–	–	–
7	2.7×10^6	–	–	–
14	1.4×10^7	–	–	–
21	2.2×10^7	–		–
28	2.5×10^7	–	–	–

Pack

cells/g

inocula / days	Imidazolidinyl urea 0.7%		Imidazolidinyl urea 0.3% + EDTA 0.05%	
	contaminated product	N.B. grown	contaminated product	N.B. grown
1	1.5×10^4	$< 10^2$	$< 10^2$	$< 10^2$
3	4.2×10^6	–	–	–
7	3.5×10^7	–	–	–
14	4.2×10^7	–	–	–
21	4.5×10^7	–	–	–
28	3.7×10^7	–	–	–