Effect of Irradiation on Heat Resistance of Thermophilic Bacterial Spores in Cultivated Mushrooms

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양송이內 好熱菌 胞子의 熱抵抗性에 對한 放射線照射의 効果

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Summary

Pure culture strain of Desulfotomaculum nigrificans spore was artificially added to fresh cultivated mushroom, irradiated, and spore counts on sulphite agar and reinforced clostridium agar after different heat treatment were compared between the unirradiated and irradiated mushrooms.

Irradiated mushroom showed 0.7 and 0.9 log cycle of decrease in spore count for reinforced clostridium agar and sulphite agar respectively with the heat treatment of 100℃ for 30 min. while 0.9 and 1.5 log cycle of decrese were observed with 121℃ for 10 min. This indicates the synergistic effect of 0.2 and 0.6 log cycle on reinforced clostridium agar and sulphite agar respectively by the combined treatment of higher temperature and irradiation. D value and heat sensitivity of D. nigrificans and C. thermosaccharolyticum were also determined.

Heat treatment of 115℃ for 10 min. and 110℃ for 10 min. in addition to 80℃ for 10 min. for D. nigrificans and C. thermosaccharolyticum respectively were selected as proper temperature.

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With D. nigrificans, D value of irradiation with heat treatment of 80℃ for 10 min. and 115℃ for 10 min. in addition to 80℃ for 10 min. were 2.20 and 1.78 respectively while with C. thermosaccharolyticum these were 2.54 and 1.72 respectively with additional 110℃ for 10 min. Both strains showed noticeable synergistic effect with combined treatment of heat and irradiation. Sensitizing factors of D. nigrificans and C. thermosaccharolyticum were 1.67 and 1.56 respectively.

INTRODUCTION

Mushrooms are known to be one of the most nutritive vegetable food items. They contain 4.9% of protein (on dry matter basis), 3.6% of carbohydrate, and 0.2% of fat(Kovacs and Vas, 1972). However, these are highly perishable and could be stored only one day at 10℃ in prime guality as a fresh mushroom (Wahid and Kovacs, 1980). Numerous attemps, therefore, were made on the way of preservation.

Since Staden(1964) introduced ionizing radiation as a new method of shelf-life extension of mushroom, several workers(Gill et al. 1969, Yamaguchi and Campbell 1973, Stanek 1978, Skou et al, 1974, Salkova et al. 1978) tried this irradiation method to improve the quality of fresh cultivated mushrooms.

Canned mushroom is one of important exporting processed vegetable items in some Asian countries such as Korea and Taiwan. Although export is gradually increasing every year in these countries, the quality of this products sometimes gives canning industry problems of spoilage due to growth of thermophilic spoilage bacterial spores which survived the heat treatment. The most important spoilages are "Flat sour", "T. A (thermophilic anaerobes)", and "Sulfide stinker" which are caused by Bacillus stearothermophilus, Clostridium thermosaccharolyticum and Desulfotomaculum nigrificans respectively. Bacillus stearothermophilus is a facultative anaerobe and the other two are obligate anaerobes. C. thermosaccharolyticum produces large quantity of hydrogen and carbon dioxide and gives products a "cheesy" odour while D. nigrificans produces hydrogen sulfide. Hydrogen sulfide is very soluble in water and react with any iron present to form iron sulfide which gives characteristic black color (Speck, 1976). Among above three thermophilic anaerobes, D. nigrificans were studied more extensively than the other two (Doores, 1983).

Lin and Lin(1970) also found that D. nigrifcans was a troublesome spoilage organism of canned mushrooms in Taiwan. These workers made a modification of beef extract tryptone iron medium for the detection and recovery of this microorganism and simple plate counting procedure was introduced. Donnelly and Busta(1980) who studied on heat resistance of D. nigrificans spores in soy protein infant formula preparations used spent mushroom compost infusion as a sporulation medium. In this experiment, above two media, beef extract tryptone iron and spent mushroom compost infusion, were tried as well. This study was carried out to see the effect of irradiation on the microbiological guality of fresh mushroom in comparison with unirradiated mushroom together with artificially contaminated fresh mushroom. The effect of irradiation on two thermophilic bacterial spores, Desulfotomaculum nigrificans and Clostridium

thermosaccharolyticum was investigated as well in this experiment.

MATERIALS AND METHODS

Microbiological examinations (Oxoid Manual, 1982)

For microbiological examinations follo ving media and incubation conditions were used.

Total mesophilic aerobic bacterial cell count : pour plates with tryptone soya broth yeast extract agar, incubated aerobically at 30℃.

Thermophilic aerobic spore count : pour plates with dextrose tryptone agar, heat treatment of 100℃ for 30 min., incubated aerobically at 55℃.

Thermophilic anaerobic, gas-forming spore count : liguid culture tubes with liver broth, heat treatment of 100℃ for 30 min., incubated anaerobically at 55℃.

Thermophilic anaerobic, H_2S producing spore count : pour plates with sulfite agar, heat treatment of 100℃ for 30 min., added with membrane filtration sterilized 2㎖ ferric citrate solution and 1㎖ sodium sulphite solution into 100 ㎖ melted basis medium, incubated anaerobically at 55℃.

Total mesophilic anaerobic bacterial cell count : pour plates with Schaedler agar, incubated anaerobically at 37℃.

Mesophilic aerobic spore count : pour plates with TSBYA, heat treatment of 80℃ for 10 min., incubated aerobically at 30℃.

Mesophilic anaerobic spore count : pour plates with Schaedler agar, heat treatment of 80 ℃ for 10 min., incubated anaerobically at 37℃.

Organisms and sporulation

Culture strain : Desulfotomaculum nigrificans (ATCC 7946), Clostridium themosaccharolyticum (ATCC 7956), both obtained from Unilever Ltd., the Netherlands.

Sporulation : A piece of lyophilized culture was first transfered from culture vial to 10㎖ B. E. T. I broth using 3㎜ loop and incubated anaerobically at 55℃ (stock culture). After approximately 48 hours incubation, all stock culture was poured into 500㎖ spent mushroom compost sporulation medium. Before pouring, oxygen was removed either by aeration of nitrogen for approximately 5 minutes using sterile pipet or heat for approximately 10 minutes in boiling water on hot plate.

Sporulation medium (Donnelly and Busta, 1980) : 100g of spent mushroom composts obtained from a local commercial grower and a hobby grower were separately suspended in 500 ㎖ water and autoclaved for 20 minutes at 121℃. The suspensions were incubated at 55℃ for 24 hours for the germination of any spores survived, reautoclaved and reincubated. After filtration through a cheesecloth, the volume was readjusted to 500㎖ and reautoclaved. The infusions were refiltered through fluted porous filter paper, volume was readjusted to 500㎖, reautoclaved and kept in room temperature until use.

Artificial mushroom contamination : 50㎖ mushroom compost infusion culture was diluted with 450㎖ tap water to make 500㎖ diluted culture. To this culture suspension, 250g of fresh cultivated mushroom was added and exhausted by repeated evacuation in a vacuum tin sealer for approximately 5 minutes to make liquid thoroughly penetrated to mushroom. Whole of soaked mushroom (430g) were divided into two equal parts : half was

irradiated at 2.5 kGy and the rest was used as control.

Comparison of C. thermosaccharolyticum spore counting method between reinforced clostridium ager pour plate method and liver broth M. P. N method

From two different pure culture flasks, spent mushroom compost infusion and clostridium broth, 1㎖ each of spore suspension was taken and decimal dilutions were made by putting into 9㎖ peptone water. Each suspension tubes were heat treated at 100℃ for 30 minutes to kill vegetative cells, 1㎖ was put into liver broth, covered with plain agar, up to 10^{-4} dilutions were made in three tubes of liver broth for M. P. N determination. This liver broth M. P. N method was compared with pour plating reinforced clostridium agar on pure culture strain (ATCC. 7956) of C. thermosaccharolyticum grown each in spent mushroom compost infusion and clostridium broth which was recommended by American Type Culture Collection.

However, the counts of liver broth M. P. N method and R. C. A pour plate method were so disagreeable on pure culture strain of both sporulation media, spent mushroom compost infusion and clostridium broth, that further application of liver broth M. P. N method was not considered.

Preparation of clostridium broth for sporulation (Pheit and Ordal, 1967)

5g peptone, 5g yeast extract together with 5g L-arabinose were dissolved in 900㎖ distilled water, autoclaved at 121℃ for 20 minutes and 100㎖ of filter sterilized sporulating salt mixture solution were added. Formula of salt mixture for sporulation is as follows.

$CaCl_2$, $2H_2O$	0.01%
$(NH_4)_2SO_4$	0.1%
$MgSo_4$	0.01%
$MnSO_4$, H_2O	0.01%
$ZnSO_4$, $7H_2O$	0.0005%
$CuSO_4$, $5H_2O$	0.0005%
$MoSO_4$	0.0001%
$FeSO_4$, $7H_2O$	0.00005%

To be dissolved in 100㎖ distilled water, and filter sterilized.

Determination of D value and heat sensitivity of pure culture strains, D. nigrificans and C. thermosaccharolyticum.

3㎖ or 5㎖ each of D. nigrificans or C. thermosaccharolytium pure culture strain was put into 6 sterile cotton plugged T. D. T tubes (1㎝ in diameter, 10㎝ in length) with rubber stopper and mounted on stainless steel dipping apparatus. Each tube was heated in oil bath at 80℃ for 10 minutes to kill all the vegetative cells and irradiated at the range of 0 kGy to 4.5 kGy in 0.75 kGy intervals. Each tube was divided into 2 and the half were further heated at 115℃ for 20 minutes (D. nigrificans) or 110℃ for 20 minutes (C. thermosaccharolyticum). After heat treatment, all the tubes were immediately cooled in ice water and plated in triplicate with decimal dilution of 10^{0} to 10^{-4}.

Radiation treatment

Irradiation was carried out with the 50 kCi cobalt-60 γ-irradiation source in pilot plant, Wageningen, The Netherlands. Samples of mushroom and spore suspensions were aerobically packaged and irradiated at various

dose levels at ambient temperature.

RESULTS AND DISCUSSIONS

Effect of irradiation on heat resistance of thermophilic bacterial spores in pure culture suspension.

Since it has been demonstrated that a combined treatment of radiation and heat reduced heat requirement for improving the quality of products due to synergistic effect, a sequence of application of irradiation and heat were carried out on pure culture strains of D. nigrificans and C. thermosaccharolyticum spores as well.

To compare the spore population of D. nigrificans in two different infusions, hobby and commercial spent mushroom infusion, and in pure cultrues collected on reinforced clostridium agar and brain heart infusion, four different culture samples were plated on sulphite agar. The collected spores were provided by harvesting colonies on reinforced clostridium agar and brain heart infusion agar by spread plate inoculation. C. thermosaccharolyticum produced in commercial spent mushroom compost infusion and clostridium broth were counted on reinforced clostridium agar. The results were shown on Table. 1.

Table 1. Spore production of two strains in various infusions.

Infusion	c. f. u ml^{-1}	
	D. nigrificans	C. thermosaccharolyticum
spent mushroom compost infusion, commercial	4.8×10^5	2.8×10^5
spent mushroom compost infusion, hobby	3.4×10^5	-
Clostridium broth	-	40.0×10^5
collected spores on reinforced clostridium agar	2.5×10^3	-
collected spores on brain heart infusion agar	1.2×10^3	-

The spore counts on spent mushroom compost were 10^5 in both types of commercial and hobby while those grown and collected on two agar media, reinforced clostridium agar and brain heart infusion agar, were both 10^3. Hence, it was confirmed that both commercial spent mushroom compost infusion and hobby infusion had enough population of spores to carry out the experiment of determining the D value and heat sensitivity.

To see the effect of combined treatment of irrdiation and heat on D. nigrificans spore, heat treatment of 100℃ for 60 minutes as first trial and irradiation doses from 0 kGy to 4.5 kGy were applied and the results were given on Table 2.

Table 2. Survival of D. nigrificans spore after heat shock of 80℃, 10min. plus combination of irradiation and additional heat treatment (commercial compost infusion, old)

Dose kGy	c. f. u ml^{-1}		*lethality of heat treatment (logN80℃, 10 min, -logN100℃, 30min)
	80℃ for 10 min.	80℃, 10min. plus 100℃, 60min.	
0	4.3×10^{4}	6.6×10^{4}	−0.19
0.75	2.3×10^{4}	3.1×10^{4}	−0.13
1.5	5.4×10^{3}	6.0×10^{3}	−0.05
2.25	2.9×10^{3}	2.2×10^{3}	0.12
3.0	8.2×10^{2}	6.1×10^{2}	0.12
4.5	1.2×10^{2}	6.7×10	0.25

*lethality (Δ) = log 10 N_o/N_t, where
N_o = initial cfu g^{-1} and
N_t = final cfu g^{-1} (Mossel, D. A. A., 1982)

On Table 2, it was observed that with the extra heat treatment of 100℃ for 60 minutes the number of survived D. nigrificans spore were increased at the irradiation doses of 0 kGy, 0.75 kGy and 1.5 kGy and started to decrease from 2.25 kGy. This might be due to heat activation which various authers have observed (Russell, 1982) : some heat-shock increases the germination. Present work as well confirmed that lower than effective heat treatment activate the germination of spores instead of killing.

This preliminary experiment showed anyhow that 100℃ was not high enough to reduce the number of spores to see sensitizing effect of heat treatment and irradiation. Therefore, various higher temperatures were tried to obtain proper temperature and time in addition to 80℃ for 10 minutes which was applied to kill vegetative cells. The results on D. nigrificans (in hobby infusion) and C. thermosaccharolyticum (in hobby infusion) were shown on Table 3.

Table 3. Survival of D. nigrificans and C. thermosaccharolyticum with various heat treatment in addition to 80℃ for 10minutes

heat treatment	c. f. u ml^{-1}	
	D. nigrificans	C. thermosaccharolyticum
0℃	3.9×10^{5}	2.0×10^{5}
110℃, 10min.	5.0×10^{5}	1.3×10^{4}
110℃, 20min.	1.5×10^{5}	7.7×10^{2}
115℃, 10min.	1.8×10^{4}	<5
115℃, 20min.	6.8×10^{2}	<5

As shown on Table 3, the most promising temperature to be applied for further experiment to see combined effect of irradiation and heat treatment seems to be 115℃ for 10 minutes and 110℃ for 10 minutes for D. nigrificans and C. thermosaccharolyticum respectively, since both temperature could let the survival count of one log cycle lowered from 10^5 to 10^4. The results are shown on Table 4 and 5.

Table 4. Survival of D. nigrificans (in commercial compost, new) with combined treatment of irradiation and heat.

Dose (kGy)	c. f. u ml^{-1}		lethality
	80℃ for 10min.	80℃ for 10min. +115℃ for 10min.	(Δ=log N80℃ - log N 115℃)
0	5.8×10^5	1.3×10^5	0.65
0.75	2.4×10^5	3.9×10^4	0.79
1.5	7.2×10^4	1.5×10^4	0.69
2.25	5.1×10^4	6.1×10^3	0.92
3.0	2.1×10^4	3.1×10^3	0.83
4.5	4.8×10^3	3.9×10^2	1.09

Table 5. Survival of C. thermosaccharolyticum (in hobby infusion) with combined treatment of irradiation and heat.

Dose (kGy)	c. f. u ml^{-1}		lethality
	80℃ for 10min.	80℃ for 10min. +110℃ for 10min.	(Δ=log N80℃ - log N 110℃)
0	1.9×10^5	9.3×10^3	1.31
0.75	6.0×10^4	3.0×10^3	1.30
1.5	3.2×10^4	1.3×10^3	1.40
2.25	1.6×10^4	2.6×10^2	1.79
3.0	1.1×10^4	1.1×10^2	2.00
4.5	2.6×10^3	2.3×10	2.05

Table 3. also shows that C. thermosaccharolyticum spore is more heat sensitive than D. nigrificans spore. D values of radiation with the heat treatment of 80℃ for 10 minutes were 2.20 and 2.54 with D. nigrificans and C. thermosaccharolyticum respectively. This clearly indicates that D. nigrificans is more heat resistant than C. thermosaccharolyticum while in D value of radiation, C. thermosaccharolyticum is higher than D.

nigrificans. These D. values are close to the range of other spore formers such as Bacillus subtilis NCTC 8236(2.2 kGy), Clostridium botulinum type D(2.2 kGy) or Type F(2.5 kGy), Clostridium botulinum Type D(2.2 kGy) or Type F(2.5 kGy), (Russell, 1982).

As shown on both Table 4, and 5, in both strains, the combined treatments of radiation and heat give synergistic effect. Lethalities at various doses and sensitzing factor (S. F=/\4.5 kGy//\0 kGy) of heat treatment were calculated between 80℃, 10 minutes and 115℃, 10 minutes for D. nigrificans and between 80℃, 10 minutes and 110℃, 10 minutes for C. thermosaccharolyticum. Lethalities with the temperature of 115℃ for 10 minutes for D. nigrificans were generally much lower than with the temperature of 110℃ for 10 minutes for C. thermosaccharolyticum. However, sensitizing factor of D. nigrificans was slightly higher than C. thermosaccharolyticum, 1.67 and 1.56 respectively.

Microbiological qualtity evaluation of cultivated mushrooms

Prior to the application of ionizing radiation, overall microbiological quality of cultivated mushroom was evaluted with the counts such as total mesophilic cell, thermophilic aerobic spore, thermophilic anaerobic gas-forming spore, and thermophilic anaerobic H_2S-producing spore.

Total mesophilic aerobic cell count was done to check the total number of bacteria grown on the mushroom. Counting of both aerobic and anaerobic spore was to see if mushroom contains any of those thermophilic spores which will survive normal heat treatment of 100℃ to 120℃ and give problems of various spoilage after processing in canning industry.

After 4 days of incubation, total mesophilic aerobic cell count was 5.6×10^7 per gram mushroom. No growth of either thermophilic aerobic spore or gas-forming anaerobic spore was observed while 5 out of 10 tubes showed the growth of H_2S-producing thermophilic anaerobic spores.

Comparison of microbiological quality of unirradiated and irradiated mushrooms

Table 6. Microbiological qualities of unirradiated and irradiated mushrooms.

Count	c. f. u g^{-1}	
	Unirradiated	Irradiated (2.5kGy)
mesophilic aerobic cell	3.4×10^8	$<1.3\times10^2$
mesophilic anaerobic cell	4.8×10^3	1.3
mesophilic aerobic spore	$<1.3\times10^2$	<4
mesophilic anaerobic spore	<1.3	<1.3
thermophilic anaerobic spore	1	<0.2

Total mesophilic aerobic cell count in fresh unirradiated mushroom was 3.4×10^8 per gram while irradiated at 2.5 kGy mushroom gave no count at the 10^{-2} level. Hence, lethality at 2.5

kGy and apparent D value of mesophilic aerobic cell count could be calculated as follows.

Δ(lethality)=log N_0-log Ni

Δ2.5kGy>8.53-2.11

Δ2.5kGy>6.42

$$D\,\text{apparent} = \frac{2.5\text{kGy}}{>6.42}$$

<0.39 kGy

<~0.4 kGy

Mesophilic anaerobic cell count as well showed marked difference between the unirradiated and irradiated mushrooms. No mesophilic aerobic and anaerobic spores could be counted either in unirradiated or irradiated mushroom at 10^{0} to 10^{-2} level.

In thermophilic anaerobic spore count, only 1 tube out of 10 unirradiated sulphite agar gave five colonies while no positive tube was detected in irradiated mushroom sample.

In general, the quality of fresh mushrooms used were in so good sanitary condition that artificial contamination using pure culture strain was inevitable to see the effect of irradiation.

Effect of irradiation on heat resistance of thermophilic bacterial spores in mushrooms

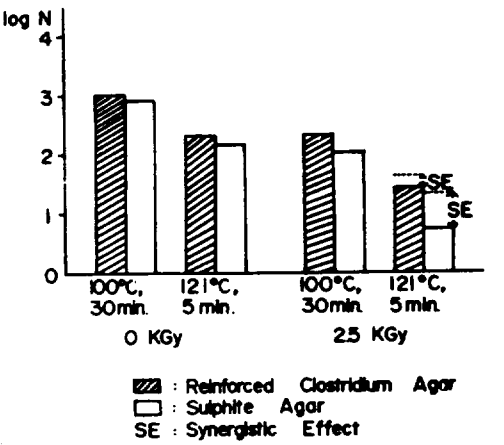

Fig. 1. Spore counts and synergistic effect of irradiation and heat treatment on Desulfotomaculum nigrificans.

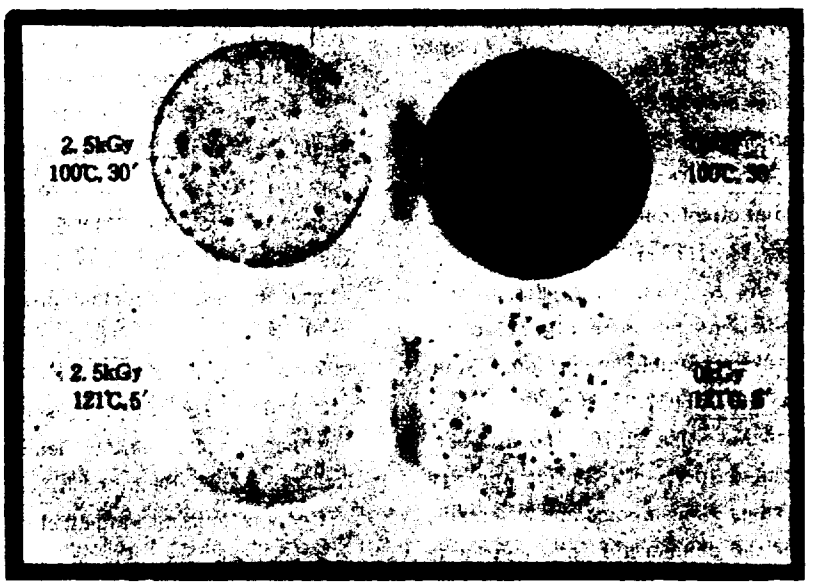

Plate I. Colonsies of D. nigrificans spore on sulphite agar after treatment of irradiation and heat

As illustrated on Fig 1. the number of D. nigrificans spore was decreased with the increase of temperature and irradiation on both sulphite agar and reinforced clostridium agar. With unirradiated sample the difference in log number between heat treatment of 100℃ for 30 minutes and 121℃ for 5 minutes was 0.7 on both media while with irradiated sample the differences were 1.3 and 0.9 on sulphite agar and reinforced clostridium agar respectively. On both media, certain synergistic effect could be detected. On sulphite agar the initial effect of elevating temperature from 100℃ for 30 minutes to 121℃ for 5 minutes was 0.7 in log number scale and irradiation at same temperature gave further 0.9 which will make the total of 1.6 if the temperature of heat treatment increased up to 121℃ for 5 minutes together with irradiation, but the difference actually turned out to be 2.2 which indicated the synergistic effect of 0.6 log cycle. Similarly the synergistic effect with heat treatment and irradiation shown on reinforced clostridium agar was 0.2 log cycle per gram mushroom.

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摘 要

Desulfotomaculum nigrificans 胞子의 種菌을 신선한 양송이에 인위적으로 첨가하여 放射線照射를 하고 두 가지의 熱處理를 한 후 sulphite agar와 reinforced clostridium agar 위에 나타난 胞子數를, 照射한 것(2.5kGy)과 照射하지 않은 양송이에 대해서 비교하였다. 照射한 양송이는 100℃에서 30분간 熱處理한 것에 있어서 reinforced clostridium agar에서 0.7 log cycle, sulphite agar에서 0.9 log cycle의 胞子數의 감소를 보인반면 121℃에서 10분간 熱處理한 것에 있어서는 reinforced clostridium agar에서 0.9 log cycle, sulphite agar에서 1.5 log cycle의 胞子數의 감소를 나타 내었다. 이것은 고온과 放射線照射의 二重處理에 의해서 reinforced clostridium agar에서는 0.2, sulphite agar에서는 0.6 log cycle의 상승효과가 나타났음을 의미한다.

또한 Desulfotomaculum nigrificans와 Clostridium thermosaccharolyticum의 D value와 heat sensitivity도 조사하였는데 80℃에서 10분간 열처리 한 것에 부가해서 D. nigrificans 에서는 115℃에서 10분간, C. thermosaccharolyticum 에서는 110℃에서 10분간의 熱處理가 가장 적절한 온도인 것으로 나타났다. D. nigrificans에 있어서는 80℃에서 10분간 熱處理한 것의 放射線照射의 D value는 2.20, 80℃에서 10분간 熱處理한 것에 부가해서 115℃에서 10분간 熱處理한 것은 1.78이었고 C. thermosaccharolyticum에 있어서는 80℃에서 10분간에서의 D value는 2.54, 80℃ 10분간에 부가해서 110℃에서 10분간 熱處理한 것에 있어서는 1.72로 나타났다. 두가지 균주가 다 加熱과 放射線照射의 二重處理에서 상당한 상승효과가 있음을 보여주었다. D. nigrifi cans와 C. thermosaccharolyticum의 sensitizing factor는 각각 1.69와 1.56이었다.