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TITLE

Improvement of antifungal and antibacterial antibiotic producing strain of Bacillus subtilis AFCI-69 by radiation and chemical mutagens, (part of a coordinated programme on radiation biology)

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FINAL REPORT

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Improvement of antifungal and antibacterial antibiotic producing strain of *Bacillus subtilis* AECL69 by radiations and chemical mutagens.

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Introduction

A bacterial strain from the soil samples of Lahore District was isolated, that had antagonistic activity against Aspergillus niger on Czapek's agar petri plate. The strain was identified; and it belonged to Bacillus subtilis and it was designated AECL69. Also it was found out that it could antagonise the growth of Staphylococcus aureus 6571(16)N.C.T.C. Antibiotic substance active against A.niger, and S.aureus was obtained from the culture fluid grown with B.subtilis AECL69[1].

As the quantity of antibiotic produced in the culture fluid was very small, therefore; it could be advantageous to obtain higher antibiotic yield mutants of B.subtilis AECL69. X-ray, ultraviolet light and nitrogen mustard were used to mutate wild type Penicillium chrysogenum and a mutant strain Q 176 was obtained, that produced far higher yields of penicillin[2]. Streptomyces strains were improved by irradiation methods for higher antibiotic yield and reduced pigment[3,4]. Gamma radiations were used to produce higher tubercidin yield strains of Streptomyces tubercidicus[5].

In the present work gamma radiations were used to select higher antibiotic yield mutants of B.subtilis AECL69, and also search of fermentation, purification and characterization of antibiotics of parent strain and its mutants was carried out.

Materials and Methods

Strains

The antibiotic producing strain Bacillus subtilis AECL69 and its mutants were maintained on MPMS agar slants. The test organisms, Aspergillus niger RAGENI 70 and Staphylococcus aureus 6571(16)N.C.T.C. were maintained on nutrient agar slants.

Culture media

For antibiotic production, cell populations irradiation and LD90 determination the culture media were:- (1) MPMS broth: cane

molasses, 10g; Bacto-peptone, 10g; Bacto-malt extract, 10g; sucrose, 10g; and water to 1000 ml; pH 6.3.(2) BPS broth: bacto-beef extract, 3g; Bacto-peptone, 10g; sodium chloride, 5g; and water to 1000ml; pH 6.5 (3) GMSG broth; l-glutamic acid, 5g; KH_2PO_4, 0.5g; K_2HPO_4, 0.5; $MgSO_4 \cdot 7H_2O$; 0.2g; $MnSO_4 \cdot 4H_2O$, 0.01g; $FeSO_4 \cdot 7H_2O$, 0.01g; $CuSO_4 \cdot 5H_2O$, 0.01g; NaCl, 0.01g; $CaH_4(PO_4)_2$, 2ml saturated solution; water to 1000ml, pH 6.8; and Glucose, 1.0%; sterilized and added seperately. Sterilization was at 15 lb for 15 minutes. For solid media, Bacto-agar was added at 2.0% concentration.

Antibiotic Production

B.subtilis AECL69 and its mutants, for antibiotic production, were grown in 100 ml media contained in 250 ml Erlenmeyer flasks. A loopful of inoculum from an overnight grown slant was transferred to each flask. The incubation was at 37°C. Mat formation was visible after 24 hour incubation. Small portions of media were taken at different growth intervals and passed through millipore fiters and the filtrates assayed for antibiotic activity.

Antibiotic Assay

The antibiotic activity was tested against A.niger and S.aureus. A loopful of test organism inoculum was suspended in 10 ml of melted nutrient agar kept at 45°C, and the suspension seeded in a petri plate containing 20 ml of base layer of nutrient agar. Stainless steel cylinder(10.0mm diameter) were used to hold and diffuse the respective antibiotic filtrate. The S.aureus assay plates were incubated at 37°C for 16 hours and A.niger assay plates were incubated at 30°C for 48 hours, and inhibition zones that appeared were measured.

Irradiation and determination of LD90

A loopful of cells from an overnight grown slant of B.subtilis AECL69 was inoculated in 100 ml medium contained in 250 ml Erlenmeyer flask. The culture was incubated at 37°C with shaking at 60-80 strokes

per minute. After 18 hours of growth, the cells were centrifuged at 500xg for 3 minutes, and then the first cell pellet discarded; the supernatant again centrifuged at 5000xg for 5 minutes and the second cell pellet saved. The cells were washed three times with the cold water and suspended in water to an O.D.1.25-1.30 at 650nm. The cell suspension was distributed in various aliquots for irradiation. After irradiation at different doses the cell populations diluted suitably with phosphate buffer(Na_2HPO_4, 7.0g; KH_2PO_4, 3.0g; NaCl, 4.0g; $MgSO_4 \cdot 7H_2O$, 0.2; and water to 1000ml, pH 7.2), and plated out in the medium of choice for cell survival counts and the cell colonies were counted after 48 hours of growth and the counts were rechecked after 72 hours of growth. The LD90 was determined graphically.

Screening of higher yield antibiotic mutants

For mutant isolates the cultures were established by taking colonies grown from cells exposed to different radiation doses and were marked for irradiation dose and numbered as these colonies were picked up at random from plates grown with irradiated cell populations. The isolates were tested for higher yield antibiotic mutants by growing in MPMS medium and assaying antibiotic yield.

Estimation of cell growth

Dry weight of cells for any culture was estimated by centrifuging the cells at 5000xg. The cells were washed with water three times and finally pelleted in preweighed centrifuge tubes. The cells were dried under vacuum over calcium chloride in a desiccator to a constant weight, for growth estimation.

Chromatographic separation of antibacterial and antifungal antibiotics

About 100 ml cell free antibiotic impregnant culture fluid was passed through a celite-charcoal bed(2gm celita+2gm charcoal) layered over celite bed(2gms). Then the chromatographic bed was washed with 50 ml of water. The antibacterial antibiotic was

adsorbed by the charcoal bed and antifungal antibiotic came out in the run out fluid.

The adsorbed antibacterial antibiotic from charcoal bed was eluted with 100 ml of acetone-water-con. ammonia(70:30:0.2) solution. The ammonia was evaporated on Evaporator and the antibiotic was passed through Amberlite CG-50 bed(1.2 x 10cm) equilibrated at pH 5.5 with phosphate buffer. The antibiotic was retained by the resin and then elution was made with acetone-water-con. ammonia(70:30:1). The antibiotic was concentrated on Evaporator and further characterized by thin layer chromatography.

The run out fluid from celite-charcoal bed that had antifungal activity was passed through Dowex-50 x 8 bed(1.2 x10 cm) equilibrated with pyridine-acetate buffer, pH 5.5; the antibiotic was retained by the resin. The elution was made with 100 ml 0.1M ammonia. The ammonia was evaporated on Evaporator. The antifungal antibiotic was further characterized by thin layer chromatography.

Concentration and desalting of antibiotics by ultrafiltration

The concentration and desalting of antibacterial antibiotics by Diaflo untrafiltration using UM2 membrane(Amicon, 21 Hartwell Avenue, Lexington, Massachusetts 023173 USA) was obtained. The contaminents with molecular weight below 1000 were eliminated. Antifungal antibiotic could pass through Diaflow UM05 membrane and it could not be desalted by ultrafiltration.

Gel filtration on Sephadex G-50

Gel filtration was carried out using a Sephadex column (15x900mm). The concentrated antibiotic was applied to the column and elution was made with water. Fractions, 5 ml, were collected and their activity assayed. The active fractions were pooled and further characterised by thin layer chromatograpy.

Characterization of antibiotics by thin layer chromatography and bioautography.

The antibiotics were characterized by thin layer chromatography. The solvant system, n-butanol-acetic acid-water(40:10:20) and another solvent system ethanol-water(77:23) were used. The antibiotics were spotted on POLYGRAM PRECOATED CEL 300 SHEET(MACHEREY-NAGEL + CO., D-516 DUREN, GERMANY) and were allowed to develop when the solution had moved a distance of at least 10 cm. The sheet was dried and cut out into strips for bioautography. For bioautography the 15 cm petri plates were seeded with test organisms and the cut out strips with cellulose layer facing the seeded agar were placed in the petri plates. The antibiotic was allowed to diffuse for 3 hours and then the strips removed. The plates were incubated and antibiotic inhibition zones were used to mark the antibiotic position on the chromatograms and for Rf value determination.

Results

Taxonomic characters of strain AECL69

The vegetative cells are gram-positive rods, with rounded ends. The cells occur in BPS medium singly or in pairs. The cells are motile with peritrichous flagella. Sporangia are not swollen and spores are cylindrical to oval, central to subterminal. Young colonies on nutrient agar are circular, but with age become undulate, effuse, motile, and spread on the agar surface. Yellow-brown-black pigments are produced in nutrient broth and GMSG broth. The cells are aerobic and pellicle is formed on the surface of liquid stationary cultures. The bacterial cells grow well at 8% NaCl in BPS broth, and these can grow at 10% NaCl concentration with reduced growth. The optimum temperature for growth is 37°C. There is acid but no gas formation in glucose medium. The marked liberation of ammonia in peptone-carbohydrate media leads to

complete neutralization of acids from carbohydrates and so results in a false negative reaction for acid production for sugars. The bacterium hydrolyses starch, peoptonizes milk, liquefies gelatin, produces nitrite from nitrate, forms acctylmeltycarbinol from glucose-peptone-water. Indole production is negative. Citrate is utilized as sole source of carbon. It contains catalase, urease but no oxidase or lecithinase. These observations indicate that the strain AECL69 is of Bacillus subtilis.

Fermentation and antibiotic production

B.subtilis produced in BPS medium an antibiotic that was active against S,aureus alone. The antibiotic could be detected after the bacterium had passed the rapid growth phase; and then it disappeared within a few hours(Figure 1 and table 1). Likewise antibacterial antibiotic alone was observed in GMSG medium and the formation of antibiotic paralleled the active growth; thereafter the antibiotic disappeared rapidly as the growth entered into stationary phase(Figure 1 and table 1). Both antibacterial and antifungal antibiotics were produced in MPMS medium. The formation of antibacterial antibiotic paralleled the active growth phase, while formation of antifungal antibiotic could be detected at the beginning of stationary growth phase by 4th day of growth when antibacterial antibiotic was at peak productioh(Figure 1, figure 2 and table 1). In MPMS medium the antibiotics remained at peak production level for some days, then started slowly decreasing and disappeared by 17th day (Figure 1 and figure 2).

Lethal effect of gamma irradiation on Bacillus subtilis AECL69 population grown in different media.

B .subtilis AECL69 populations were submitted to gamma radiation with lethal effects. The media GMSG, BPS, MPMS were used for growth of cell populations and survivors before and after exposure to radiations. The LD90 doses for cell populations grown

in GMSG, BPS and MPMS were 10 Kr, 13.5 Kr, 15 Kr respectively (Figure 3).

Mutagenesis of strain AECL69

The antibiotic productivity of natural isolates of strain AECL69 and cell populations that survived 10 Kr and 15 Kr doses were studied. The medium MPMS was used because it was found to be quite suitable for antibiotics production. The productivity distribution of various natural isolates for both antibacterial and antifungal antibiotics had a narrow range. The productivity distribution in antibacterial antibiotic for 10 Kr and 15 Kr survivors was somewhat broader than that observed for natural isolates(Figure 4). The productivity distribution for the antifungal antibiotic for 10 Kr survivors was much broader and therewere more higher yield variants, however; the higher yield productive variants sharply reduced at 15 Kr(Figure 5).

The best higher yield survivors were collected from population irradiated at 15 Kr and these produced more than 18% of antibacterial and antifungal antibiotics produced by the parent. When higher yield survivors were re-tested, many of them reverted to level of productivity of the parent. However some survivors retained their productivity, and these were preserved for antibiotic isolation and characterization studies.

Comparison of parent and higher yield selected survivor strains.

i) Antibiotic production

The higher yield strains: M 315, M 715, M 1815, M 3115, like the parent strain AECL69, produced both antibacterial and antifungal antibiotics in MPMS medium, and peak production titers for both the antibiotics were higher than the parent strain(Table 2). These higher yield strains visibly produced more cell mat that was also more wrinkled. The strain M 315 was examined for dry weight and antibiotic yield; and it showed pattern of antibiotics formation similar to that of parent

strain that antibacterial antibiotic could alone be detected by 3rd day of growth and antifungal antibiotic titer reached maximum by 5th day of growth(Table 3).

ii) Antibiotic isolation and characterization.

Separation of antibacterial and antifungal antibiotics from each other was achieved by charcoal adsorption process. The antibiotic culture fluid, free from cells, was fed to the celite-charcoal bed. Antibacterial antibiotic was adsorbed to charcoal and antifungal antibiotic was not adsorbed and thus was recovered in the effluent. The antibacterial antibiotic was eluted from the charcoal bed by acetone-ammonia-water mixture.

Further purification of antibacterial antibiotics was carried out by Amberlite CG-50 ion-exhcange chromatography and ultrafiltration. The antibiotic was retained by Diaflo UM2 membrane and could pass out of UM 10 membrane; and that implicated the molecular weight of the substance between 1,000-10,000. The gel filtration was carried with sephadex G50 column(1.5x90 cm), using water as eluant. The antibiotic eluted after 60 ml blank portion as a symetrical peak. Thin layer chromatography using cellulose as supporting medium, showed a single spot upon bioautography for antibacterial substance. Also silica gel thin layer plates showed one spot. Solvents: n-butanol-acetic acid-water(40:10:20), Rf Ca 0.76; ethanol-water(77:23), Rf Ca 0.96.

The antifungal antibiotic was further purified by chromatography on Dowex-50x8 column. Antifungal antibiotic could pass through Diaflo UM05 membrane, that implicated its molecular weight below 500. The antifungal substance showed a single spot on TLC using cellulose plates with solvents:n-butanol-acetic acid-water(40:10:20), Rf Ca 0.20; ethanol-water(77:23), Rf Ca 0.60. The spots were visualized with bioautography, ninhydrin.

Antibiotics produced by parent strain AECL69 and higher yield strain M315 could be isolated and separated using similar procedures and these were identical in chromatographic behaviour.

Conclusion

1. Composition of fermentation media was very important for antibiotic production. In GMSG and BPS media, antibacterial antibiotic alone was produced by B.subtilis AECL69. In MPMS medium the bacterium produced both the antibiotics; antibacterial antibiotic as well antifungal antibiotic.

2. Antibiotic assay system based upon inhibition zone of filtered culture fluid was tedious and it was more so when two test organisms, S.aureus and A.niger were used. No other suitable assay method could be devised for these antibiotic for mass screening purpose.

3. Gamma radiations increased variability and the largest number of higher producing variants were observed for 10 Kr irradiated population. At 15 Kr doses the antibiotic production variability decreased, however, the best high yielding strains were obtained at 15 Kr exposures.

4. The high antibiotic producing strains yielded more biomass that could be related with antibiotic production.

5. Procedure has been devised to separate the two antibiotics.

6. Antibiotics have been characterized by chromatographic methods.

References

1. Malik, M.A., Shaukat, G.A., Ahmad, M.S., and Muhammad, A. The Nucleus, 9, (1972) 111.
2. Backus, M.P., and Stauffer, J.F., Mycologia, 47(1955)429.
3. Alikhanian, S.I., Advances in Appl. Microbiol., 4(1962)1.
4. Vezina, C., Bolduc, C., Kudelski, A., and Sehgal, S.N. J.Antibiotics, 29(1976)248.
5.

Legends to figures

Figure 1 Antibiotic production of antibacterial antibiotic in GMSG, BPS and MPMS media at different days of fermentation. Fermentation was at 37°C in stationary culture flask.

Figure 2 Antibiotic production of antifungal antibiotic in MPMS medium at different days of fermentation. Incubation was at 37°C in stationary culture flask.

Figure 3 Lethal effects of gamma radiations on Bacillus subtilis AECL69 populations grown in GMSG, BPS and MPMS medium.

Figure 4 Distribution of productivity variants of antibacterial antibiotic as dependent on gamma radiation doses in Bacillus subtilis AECL69.

Figure 5 Distribution of productivity variants of antifungal antibiotic as dependent on gamma radiation doses in Bacillus subtilis AECL69.

Details of project expenditure

Year 1973-74

IAEA funds made available — US $ 3,000.00

Expenditure

a)	Amicon ultrafiltration apparatus	US $ 840.47	
b)	Antibiotic zone reader	334.13	
c)	Incubator, cooled, Gallenkamp	875.00	
	Total	2049.60	
Balance			+950.40

Year 1974-75

IAEA funds made available — 3,200.00

Expenditure

a)	Millipore filtration apparatus	2317.30	
b)	Sephadex columns, gels, peristaltic pump etc.	1803.00	
c)	Pipettes serological, 0.1 ml	305.40	
d)	Siphon stand and siphons for LKB fraction collector	165.00	
	Total	4590.70	
Balance			-1390.70

Year 1976-77

IAEA funds made available

Ist instalment	US $(U.S.Currency)	1050.00
	+ US $(Pak.Currency)	450.00
2nd instalment	US $(U.S.Currency)	1050.00
	+ US $(Pak.Currency)	450.00
	Total	3000.00

Expenditure

a)	Ist instalment remitted to PAEC was used in purchase of bench top agar sterilizer.	US $(US currency)	1050.00
		+ US $(Pakcurrency)	450.00
b)	Precoated thin layer plates.	U.S. $	575.00
	Total		2075.00
Balnce		US $(U.S.Currency)	475.00
		US $(Pak.Currency)	450.00
			+925.00

Accounts Officer,
Nuclear Institute for Agric. & Biology,
Pakistan Atomic Energy Commission,
P.O.

Table 1

Relationship between growth and antibiotic production in different media from B.subtilis AECL69

Medium	BPS			GMSG			MPMS		
Growth period (day)	Dry weight (mg)	S.aureus inhibition zone(mm)	A.niger inhibition zone(mm)	Dry weight (mg)	S.aureus inhibition zone(mm)	A.niger inhibition zone(mm)	Dry weight (mg)	S.aureus inhibition zone (mm)	A.niger inhibition Zone (mm)
1	20	0	0	40	13	0	30	10	0
2	32	0	0	80	18	0	50	13	0
3	50	11	10	108	24	70	70	18	0
4	50	0	0	110	0	0	100	24	14
5	50	0	0	110	0	0	100	20	18

Table 2

Antibiotic yields of various strains of *B.subtilis*

Strains	Antibiotic production on 3rd day of fermentation		Antibiotic production on 5th day of fermentation	
	S.aureus inhibition zone (mm)	*A.niger* inhibition zone (mm)	*S.aureus* inhibition zone (mm)	*A.niger* inhibition zone (mm)
AECL69	22-23	0	21-22	17-20
M315	36-38	0	31-22	30-32
M715	33-35	0	28-29	33-35
M1815	34-35	0	28-30	28-32
M3115	28-30	0	28-30	29-30

Table 3

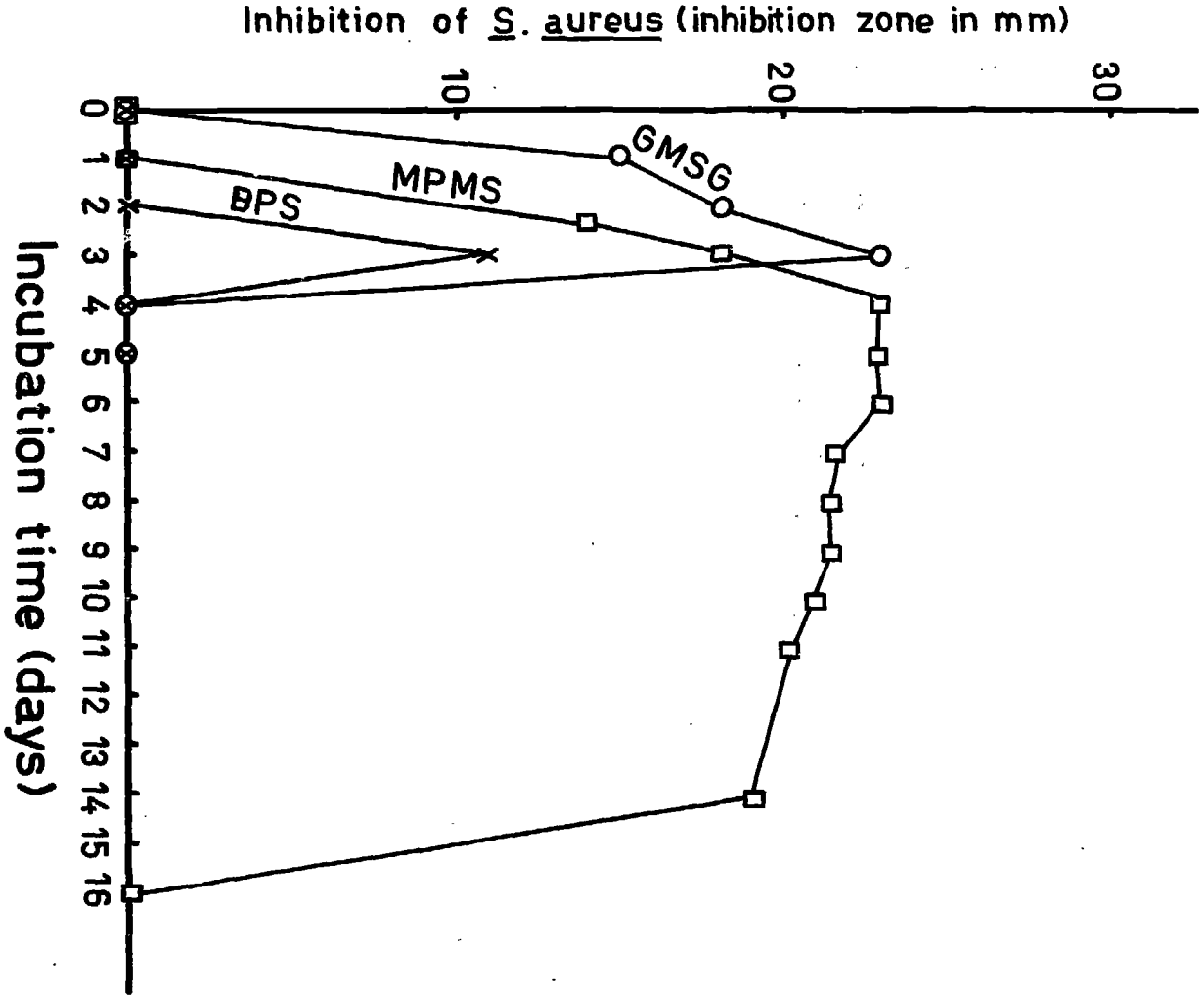
Antibiotic production during growth by *Bacillus subtilis* AECL69 and higher producing strains M315

Growth period (day)	*Bacillus subtilis* AECL69			*Bacillus subtilis* M315		
	Dry weight (mg)	*S. aureus* inhibition zone (mm)	*A. niger* inhibition zone (mm)	Dry weight (mg)	*S. aureus* inhibition zone (mm)	*A. niger* inhibition zone (mm)
1	28	10	0	70	15	0
2	50	13	0	150	25	0
3	78	16	0	180	38	0
4	103	22	14	194	37	29
5	97	21	17	200	35	30
6	99	20	19	199	33	28

LD90 determination the culture media were:- (1) NFMS protein cane

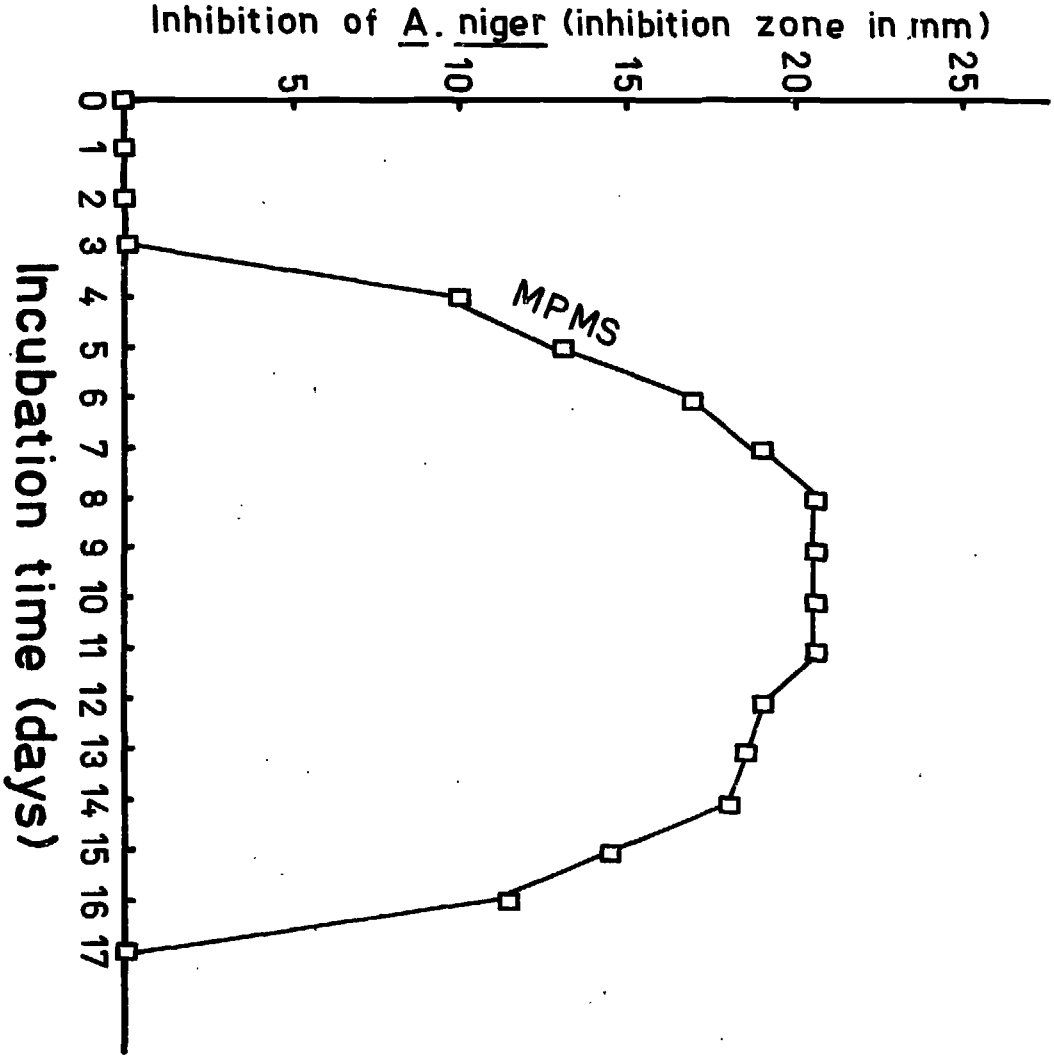
flask. The culture was incubated at 37 C with shaking at 80-90 strokes

Fig I



washed with 50 ml of water. The antibacterial antibiotic was

Fig. 2



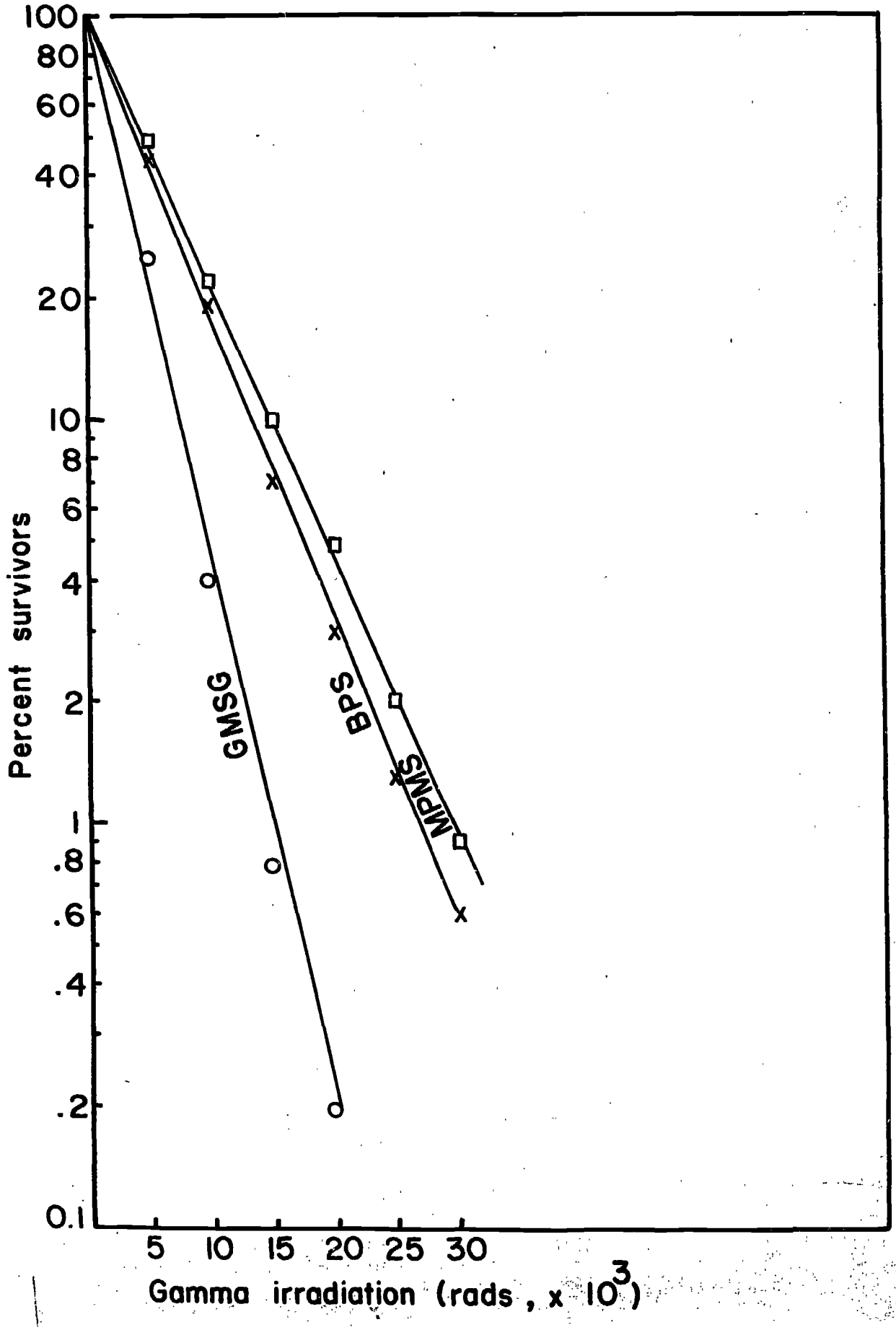
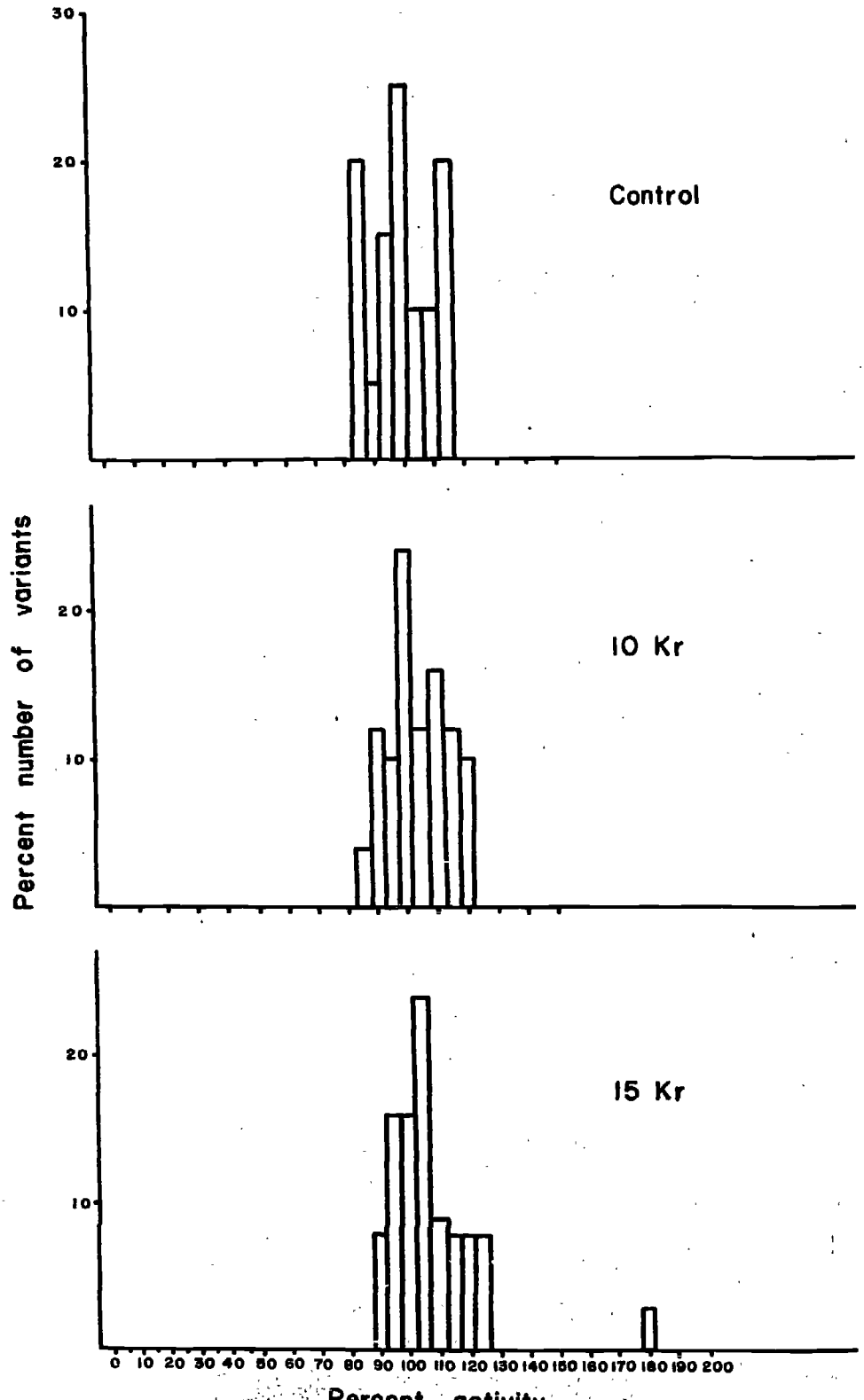

100
80
60
40
20
10
8
6
4
2
1
.8
.6
.4
.2
0.1
Percent survivors
GMSG
BPS
MPMS
5
10
15
20
25
30
Gamma irradiation (rads, x 10^3)

Fig. 3

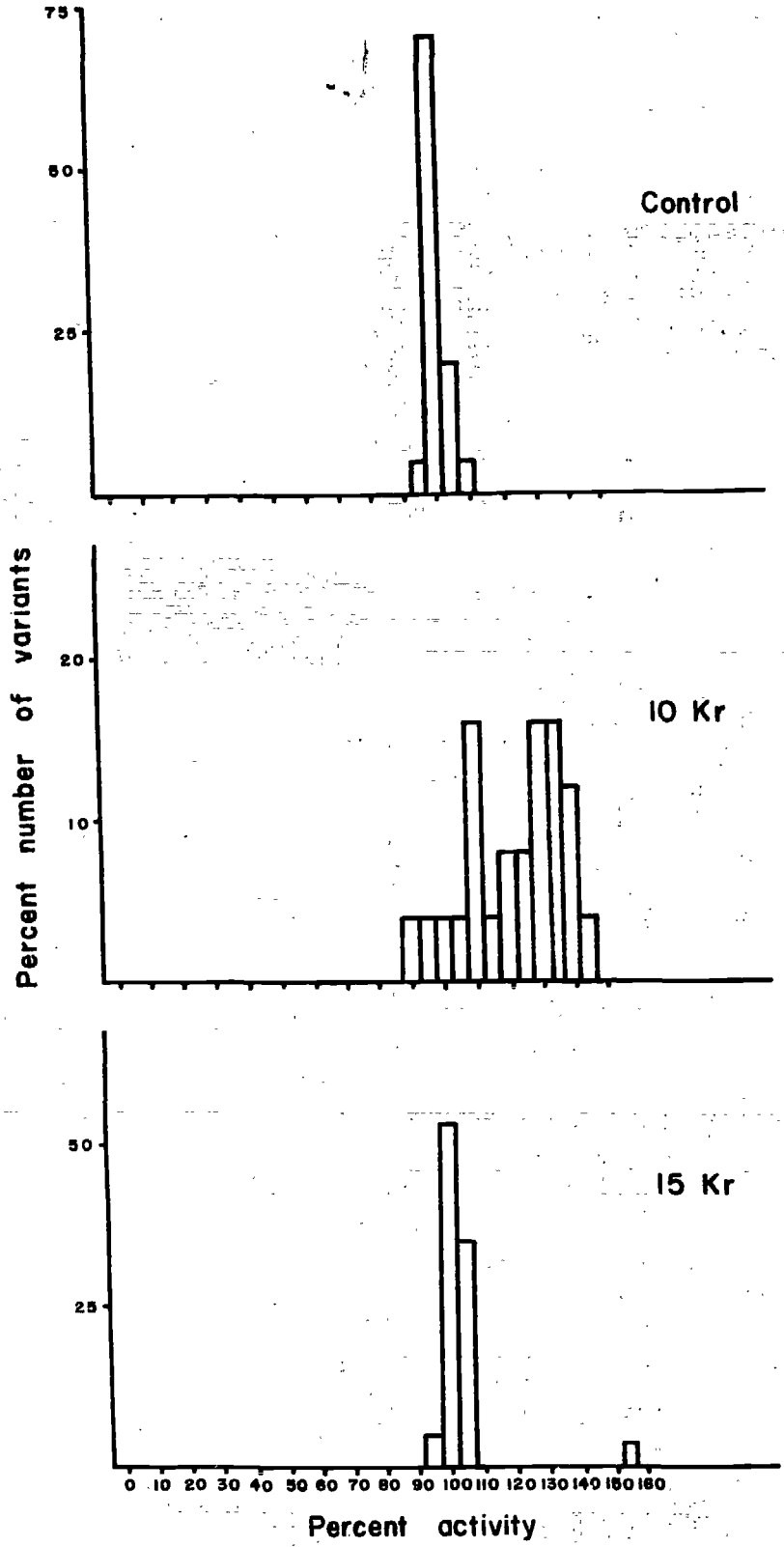
The marked liberation of ammonia in peptone-carbohydrate media leads to



Distribution of variation in antibacterial antibiotic production as dependent on gamma irradiation doses in *Bacillus subtilis* AECL69.

Fig. 4

exposure to radiations. The LD90 doses for cell populations grown



Distribution of variation in antifungal antibiotic production as dependent on gamma irradiation doses in *B. subtilis* AECL69.

Fig. 5