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PHYSIOLOGY OF MUTANT STRAINS OF *ERWINIA CAROTOVORA* PRODUCING L-ASPARAGINASE

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SINCE the first demonstration[1] of L-asparaginase (L-asparagine amidohydrolase EC 3.5.1.1) as an anticancer enzyme, production of the enzyme by a variety of microbes has been investigated in many laboratories. The various sources reported, such as *Pseudomonas fluorescens*[2], *Serratia marcescens*[3], *Escherichia coli*[4], *Erwinia carotovora*[5] and *Proteus vulgaris*[6], provided active and immunologically different enzyme preparations. All these species were evaluated in the author's laboratory. As *E. carotovora* was shown to be a promising culture, it was mutated by UV and gamma radiation and by treatment with chemical mutagens as part of a strain improvement programme. The mutant strains derived by these traditional methods were further investigated by a classical method of genetic recombination reported by Barnes *et al.*[7]

The original wild-type strain *E. carotovora* was a gift from Prof. Wade of Microbiological Research Establishment (UK). It was mutated by the routine methods such as UV and gamma radiation and treatment with chemical mutagens. The mutants were screened and stock cultures were maintained. Working cultures were obtained by streaking the stock culture and transferring isolated colonies on tryptone yeast (TY) agar slants (in g/l: Tryptone, 5.0; yeast extract, 2.0; lactose, 5.0; K_2HPO_4, 1.0; NaCl, 5.0; agar, 15.0; pH 6.8-7.0). Purity was checked periodically.

Auxotrophic strains were isolated on minimal agar (in g/l: NaCl, 0.2; Na_2SO_4, 2.0; K_2HPO_4, 1.0; $MgSO_4{\cdot}7H_2O$, 0.2; lactose, 10; pH 7.0) supplemented with individual amino acids. To determine antibiotic resistance, the following antibiotics were added to minimal agar: penicillin, 200–2000 units/ml; ampicillin, 10–20 μg/ml; erythromycin, 5–10 μg/ml; chlortetracycline, 10–100 μg/ml; chloramphenicol, 250–500 μg/ml; and streptomycin, 10–20 μg/ml.

Complete medium (TGY) was used for the fermentation process (in g/l: Tryptone, 20.0; yeast exrtact, 5.0; monosodium glutamate, 15.0; lactose, 10.0; K_2HPO_4, 1.0; NaCl, 1.0; $CaCO_3$, 5.0; $MgSO_4{\cdot}7H_2O$, 0.2; pH 7.4).

The depleted mutant strains of *E. carotovora* were treated with a mixture of amino acids which included monosodium glutamate, L-asparagine, tryptophan, methionine, alanine, serine and threonine. After eliminating one of these amino acids from the mixture, their polyauxotrophic character was determined. They were screened to select producer and non-producer strains and tested for growth in presence of antibiotics. The producer strains were resistant particularly to streptomycin and chlortetracycline antibiotics at the higher concentration. They required specifically monosodium glutamate as carbon source. The non-producer strains lacked this requirement and their growth was inhibited at lower concentrations of streptomycin and chlortetracycline. All the strains—wild and polyauxotrophic—were however sensitive to penicillin and ampicillin.

The mutants were mated according to the procedure of Barnes *et al.*[7] Donor, recipient and recombinant cells were characterized by observing their growth on the third day. Only genetic recombinants grew and were picked up as isolated colonies. They were subcultured five times on minimal agar plates and stock cultures were prepared. After purification they were transferred to agar slants, incubated for 16 h, and inoculated into production flasks directly for fermentation studies. The fermentation was carried out in 100 ml medium in a 500 ml Erlenmeyer flask. The cultures were incubated for 24 h at 37°C on a rotary shaker (250 rpm). Changes in fermentation parameters in the recombinants were considered as evidence of successful application of the technique adopted. L-Asparaginase activity, being intracellular, was assayed by the routine Nesslerization method[8]. One IU represents 1 μmol of ammonia liberated per min at 37°C in 0.05 M borate buffer, pH 8.5.

In table 1, the polyauxotrophic and the streptomycin-sensitive (Sm^S) strains are shown as non-producers while the streptomycin-resistant (Sm^R)

Table 1 *L-Asparaginase production by mutant strains of E. carotovora*

Mutant strain	Phenotypic characters	PCV* (%)	Enzyme activity (IU/ml)
389	MSG⁺ Ala⁺ Sm^R	3	0.2
189	Asn* Gln⁺ Ala⁺ Sm^S	4	0.08
213	MSG⁺ Tr⁺ Met⁺ Sm^R	3	0.38
56	Asn⁺ Tr⁺ Met⁺ Sm^S	5	0.0
106	Asn⁺ Met⁺ Tr⁺ Sm^S	3	0.0
122	MSG⁺ Gln⁺ Sm^R	5	1.5

*Packed cell volume.

Table 2 *L-Asparaginase production by recombinants of E. carotovera obtained by genetic recombination*

Recombinant	Mutant strains in cross	PCV (%)	Enzyme activity (IU/ml)
R1	389 × 213	3.0	1.58
R2	189 × 56	3.0	1.43
R3	106 × 213	2.5	3.13
R4	106 × 122	2.5	3.13
R5	122 × 213	2.5	5.93
R6	389 × 122	2.0	6.10
R7	389 × 106	3.0	2.10
R8	56 × 109	3.0	1.30

strains are seen as poor producers. These strains, when hybridized, yielded, among others, the recombinants R5 and R6, which were at least four times more active than the original strains (table 2). It was further observed that the mutant strains requiring monosodium glutamate specifically and showing streptomycin resistance produced these high yielding recombinants. Hence Sm^R was considered a suitable selective marker. The prototrophic recombinants were also found to be induced when L-asparagine, at 0.1–0.3% was added to the culture medium; there was a 3.5-fold increase in enzyme activity. The parental strains were never capable of showing such induction.

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EFFECT OF *RHIZOBIUM* IN ASSOCIATION WITH GRANULAR INSECTICIDES ON NODULATION AND YIELD IN SOYBEAN

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INSECTICIDES containing mercury, copper or zinc, when used for seed protection, were found to be extremely toxic to *Rhizobium* sp.[1-4] Oblisami *et al.*[5], found in *in vitro* tests that endrin, carbofuran and disulfoton inhibited the growth of *Rhizobium* from red gram (*Cajanus cajan*). Seed inoculation with *Rhizobium* is advocated for achieving higher grain production in soybean. We therefore studied the effect of pesticides on nodulation *vis-a-vis* yield of soybean to evolve an effective combination of pesticide and rhizobial culture.

The experiment was conducted in randomized block design at the Research Farm of IARI, New Delhi. Soybean seeds, var. Harosoy-63, were coated with effective culture of *Rhizobium japonicum* (IARI strain, obtained from the Division of Microbiology, IARI) using 10% sucrose solution before sowing. The inoculated seeds were air-dried. Granular insecticides phorate 10, quinalphos 5, mephosfolan 5 and carbofuran 3 were drilled in soil furrows before sowing. All the insecticides except phorate were applied at 3 g/m row and phorate was applied at 1.5 g/m row. There were six treatments, each replicated four times. The size of each plot was 3.0 × 2.25 m. Row-to-row and plant-to-plant distances were 37.5 and 5 cm respectively.

The crop was given two light irrigations before uprooting, one at the beginning of flowering and the other at early pod formation stage, in addition to normal rainfall received during the crop season. Ten plants were uprooted from each plot each time.