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Abstract
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Sargent, M. L. and H D. Braymer. Selection of intramural-enzyme mutants.

Selection for mutants with altered enzymes in Neurospora is a formidable task if the enzyme is normally located outside the cell membrane, whether within the wall (intramural) or outside of it (extracellular). Thir difficulty is due to cross-feeding of hydrolytic products from wild-type conidio to mutant conidio. Our efforts to isolate mutants damaged in the carbohydrases, invertase and trehalase, hove yielded a combination of techniques that offers some promise for the selection of altered, intramural enzymes.

One assumption is basic to our strategy: "the products of hydrolysis ore not completely equilibrated with the external milieu, but may be preferentially taken up by the cell that performs the hydrolysis," (Marzluf and Metzenberg 1967 Arch. Biochem. Biophyr. 120:487), i.e., Thot release is cytotropic. Marzluf ond Metzenberg were unable to detect cytotropic release of glucose from invertore-hydrolyzed sucrose. However, our reconstitution experiments with the one available invertase mutant (spontaneous) demonstrated that wild-type conidio suffered inositol-less death IO-100 timer faster than the mutant conidio in mixed populations. This result coupled with our ability to select invertase and trehalase mutants suggests that the assumption is valid in certain situations.

We have attacked the problem of crossfeeding in five ways. First, parent strains with a minimum amount of enzyme were chosen. From these, partial mutants were obtained, and were used as parents for subsequent mutant hunts. Second, conidia were dialyzed in water to deplete endogenous reserves which might allow initial growth. Third, enzyme on the surfaces of conidia was inactivated by treatment with acid (Metzenberg 1963 Biochem. Biophys. Acta 77:455). Fourth, inositol-less death selection on plates (Lester and Gross 1959 Science 120: 572) was chosen over filtration selection, since cell death occurs more promptly after germination with the inositol-less death technique. Fifth, selection was conducted, where practicable, on a substrate which is hydrolyzed rapidly enough to support growth and inositol-less death, but slowly enough to prevent accumulation and crossfeeding of reaction products (e.g., glucose). We used raffinose as a carbon source for invertase mutant hunts, and low levels of trehalose in the trehalase mutant hunts.

Cultures of inositol-requiring strains (allele 89601) were grown for 7 days at 20°C on Vogel's salts, 1.5% sucrose, 20 µg/ml inositol, and 2% agar. Conidia were harvested in water, filtered through cheesecloth and glasswool, and dialyzed against three changer of distilled water (once overnight). The washed conidio were collected by centrifugation, and resuspended in water (107 conidia/ml). An equal volume of 0.2 N HCl was added and the suspension was shaken at 37°C (8 min. treatment wholly inactivated invertase and treholore with a 40-60% survival of cells). The acid treatment was terminated by bringing the suspension to pH 5.5 with 1.O M sodium citrate. The acid-treated conidio were collected by centrifugation (60-80% recovery), resuspended in 0.1 M sodium citrate, pH 5.0 (I-2 x 10^7 conidia/ml), and either irradiated with UV light (to 10% survival) or shaken at 25°C with on equal volume of 50 µg/ml N-methyl-N'-nitro-N-nitrcaoguanidine (MNNG) made up in the same pH 5.0 buffer (15 min. treatment, 40-60% survival). MNNG was more efficient in producing the desired mutations, but it increased both the reversion rate at the inositol locus, and the number of secondary mutations in the selected strains. The mutagenized conidio were collected by centrifugation, washed, resuspended (I-2 x 10^6 conidia/ml) in Vogel's salts and a carbon source (trehalose or raffinose, 0. 1%), and incubated for 6 hours at 25°C. After this incubation, the conidia were filtered through cheesecloth and glasswool, collected, washed twice with water, and resuspended in water (10^6 conidia/ml). One ml aliquots were spread on prepared plates (Vogel's salts, 0.2% carbon source and 1.5% agar). After 3-5 days incubation at 259, the plates were overplated with Vogel's salts, 150 µg/ml inositol, 1.5% fructose, 3.0% sorbose, and 1.5% agar, and incubated at 25°C.

The colonies which appeared were either isolated into separate slants for subsequent analysis (trehalase mutant hunts), or scored directly on the plates with a liquid, in vivo, invertase assay. The assay solution is prepared by dissolving 500 mg glucostat enzyme reagent (Worthington Biochemical Co.) in 45 ml of 44% ethanol, adding 42 mg tartrazine and 100 mg o-tolidine, filtering and adjusting the pH to 5.0 with 5% formic acid. Immediately before use the above solution is diluted 1:1 with 1.0 M sucrose. After adding 3-4 ml of the combined solution to each plate, and allowing it to soak into the agar far 20-30 min., the residue is poured off. Within 30-60 min. all colonies possessing invertase activity are surrounded by a bright green halo, reprerenting the liberated glucose, while mutant colonies remain white with no halo. This reaction is light-sensitive, and is best done in subdued light.

While none of there techniques ore particularly novel, their combined use has allowed us to isolate previously unobtainable mutants. Over 200 trehalase mutants (many with no detectable activity) hove been isolated at the end of three cycles of experiments, in which the best partial mutants recovered in one were used as parents in the next. In addition, two cycles of selection hove yielded 5 partial invertase mutants (having less than 10% of the invertase activity of the original parent). We hope additional cycles will produce complete invertase mutants similar to the one known spontaneous mutant (Sargent and Woodward 1969 J. Bact., in press). The genetics and biochemistry of the treholore mutants will be reported elsewhere. We thank D.O.Woodward, A.S.Sussman, and R. H. Davis for advice in the course of this work. - - - Department of Botany, University of Illinois, Urbana, Illinois 61801, and Deportment of Microbiology, Louisiana State University, Baton Rouge, Louisiana 70803.