Instructor's Information **Project one**

Identification of unknown mutants in the arginine biosynthetic pathway of *Neurospora crassa* through growth tests and measurements of levels of intermediates

Overview: The goal of this project is to identify a series of unknowns as either being wild type or one of several possible mutations in the arginine biosynthetic pathway. More specifically, the students will receive strains known only by a letter and will have to identify them as being either wild type, *arg 1*, *arg6, arg10, arg12* or *arg14* by doing two types of experiments: growth tests in the presence of various intermediates and measurement of level of intermediates. In order to measure the levels of intermediates, it is necessary to break open the *N. crassa* and collect metabolites. Since the amino acids interfere in the colorimetric tests for each other, it is necessary to separate them by cation exchange chromatography and this requires that the students calibrate the columns beforehand. This is done by passing known amounts of the three amino acids over the column, following a set elution schedule and determining both which fraction contains which amino acid as well as the % recovery.

Procedure: This lab consists of two types of experiments—growth tests and measurement of the level of intermediates. As indicated in the lab, these experiments can proceed simultaneously. The measurement for the level of intermediates requires that the columns are calibrated. There really is no preparation on the instructor's part for the measurement of the level of intermediates and thus, instructions for the two components of the lab that require preparation by the instructor are described below: growth tests and calibration of columns.

Growth tests

1. It is necessary to obtain stock cultures of the mutants as well as the wild type. These can be obtained from the Fungal Genetics Stock Center. There are several versions of the wild type, *arg-1, arg-6, arg-10*, and *arg-12* mutants and only one version of *arg-14*. The mating type does not matter. The wild type most commonly used is called 74A where 74 designates the allele and A is the mating type.
2. The wild type and mutants need to be transferred from the stock cultures to slants. In order to make the slants, a solution that contains 1 X Vogel's salts (made from 50 X), 1.5% sucrose, 2% agar and 1 mg/ml L-Arginine is prepared and heated on a hot plate stirrer. When dissolved, 2 ml aliquots are transferred to 13 x 100 mm test tubes which are covered with Bacto caps and placed in an autoclave. When removed from the autoclave, the tubes are allowed to cool to room temperature at an angle and thus form the slants. The tubes are inoculated using sterile technique with the arg mutants as well as wild type and grown for 2 days at 30 °C and at least 2 days in the light at room temperature.
3. These slants can then be used to inoculate VM+1 mg/ml Arginine slants for the students using sterile lab technique. These are grown as described above and labels are removed. Each student should receive a set of slants labeled as A through F which contain 74A, *arg-1, arg-5, arg-6, arg-10, arg-12* and *arg-14*.

Calibration of cation exchange columns

1. The cation exchange columns need to be prepared. The cation exchange columns contain Dowex 50W, 200 x 400 mesh and the resin should be prepared following manufacturers instructions. The columns need to be packed with resin. Columns are available from a variety of vendors and need to be packed so that they contain 1.15 ml of the resin.
2. The solutions required for the elution schedule should be prepared as well as the standards. There are two solutions for elution: 0. 2 M NaOH and 0.116 M Na_3 citrate pH 5.3. In order to prepare the citrate buffer, an appropriate amount of Na_3 citrate is dissolved in distilled water and the pH is adjusted to 5.3 using 4 M HCl. The standards contain 10 mM arginine, ornithine or citrulline.
3. Prior to use, the columns should be washed by following the standard elution schedule. This is listed in the project and is repeated here. The schedule is as follows
    a. sample plus citrate buffer with 2.0 ml total volume
    b. 2.0 ml citrate buffer (citrulline should emerge here)
    c. 2.0 ml citrate buffer
    d. 2.0 ml citrate buffer(ornithine emerges here)
    e. 2.0 ml citrate buffer (ornithine emerges here)
    f. 2.0 ml of 0.2 M NaOH(arginine emerges here)
    g. 2.0 ml of 0.2 M NaOH (arginine emerges here)
    h. approximately 4.0 ml of water to wash the column

It is not necessary to collect the last fraction.

4. The students will take over from here by preparing mixtures using the 10 mM solutions of the amino acids and passing them over the column. Once the columns are calibrated, the students will grow the strains in liquid medium, collect and collect metabolites by boiling water extraction. The metabolites are then passed over the cation exchange column and only those fractions which contain argnine, ornithine and citrulline need to be collected.

Reference:

1. A complete description of the contents of Vogel's minimal medium (50X) as well as how to grow Neurospora is contained in
   Rowland H. Davis and Frederick DeSerres. 1970. Genetic and Microbiological Research Techniques for *Neurospora crassa Meth. Enzymol*. **17A**: 70-143.
2. A description of how the cation exchange columns can be used to separate arginine, ornithine and citrulline is described in
   Kelly A. Keenan, et al. 1998. Isolation and characterization of strains defective in vacuolar ornithine permease of *Neurospora crassa Fungal Gen. and Biol*. **23**: 237-247.