Chapter 3 : Enzymatic Methods

The term enzymatic analysis is generally understood to mean analysis with the aid of enzymes. The major advantages of enzymes in analysis lie in their ability to react specifically with individual components of a mixture. This avoids lengthy separations of the components and reduces the time needed for an analysis.

1. Nitrate Reductase

1.1. Molybdopterin enzymes

Molybdenum is the only second row transition element required by the majority of living organisms, being widespread in the environment because of the water solubility of its high-valent oxides. Mo has been incorporated in a diverse range of biological systems complexed to a pyranopterin organic compound constituting the molybdenum cofactor (Moco).

1.1. Definition of nitrate reductase

Nitrate reductases ara enzymes responsible for the initial reductive steps of the nitrogen cycle, according to the reaction:

$$NO_3^- + 2H^+ + 2e^- \rightarrow NO_2^- + H_2O$$

1.2. Taxonomy of nitrate reductases

Nitrate reductases belong to the DMSOR family of Mo containing enzymes, and have been divided into four major groups: eukNR, Nas, Nap, and Nar.76 Nitrate reductases have been classified according to physiological function, subcellular location (i.e., cytoplasmic, periplasmic, or membrane) of the enzyme, structure of the molybdenum active site (e.g., dioxo-Mo(VI) and monooxo-Mo(VI)). In plants and fungi, eukNR functions in nitrate assimilation, while the three prokaryote nitrate reductases can function in both assimilatory and dissimilatory nitrate reduction: assimilatory nitrate reductase (Nas) or dissimilatory nitrate reductases i.e., Nar and Nap.

1.3. Assimilatory and dissimilatory nitrate reduction

The assimilatory and dissimilatory nitrate reductase enzymes were designated based on the ultimate fate of the nitrogen. Assimilatory nitrate reduction, a form of anabolism, incorporates nitrogen from nitrate into the organism's biomass. Nitrate is first reduced by nitrate reductases to nitrite, and then nitrite is transformed into ammonia via assimilatory nitrite reductases. Dissimilatory nitrate reduction, a catabolic pathway, does not incorporate nitrogen into the biomass; instead the end products of dissimilatory nitrate reduction are excreted from the cell. Although dissimilatory NR also forms nitrite from nitrate, nitrite can be furthermetabolized into dinitrogen or ammonia as part of denitrification or dissimilatory nitrate reduction to ammonia (DNRA), respectively. DNRA is an important part of the global nitrogen cycle because ammonia can be stored in the soil. Denitrification generates volatile compounds, which accumulate in the atmosphere. In some cases, dissimilatory nitrate reduction is an energy yielding process; therefore, the term ‘‘nitrate respiration’’ is used to specify nitrate reduction coupled to ATP generation.

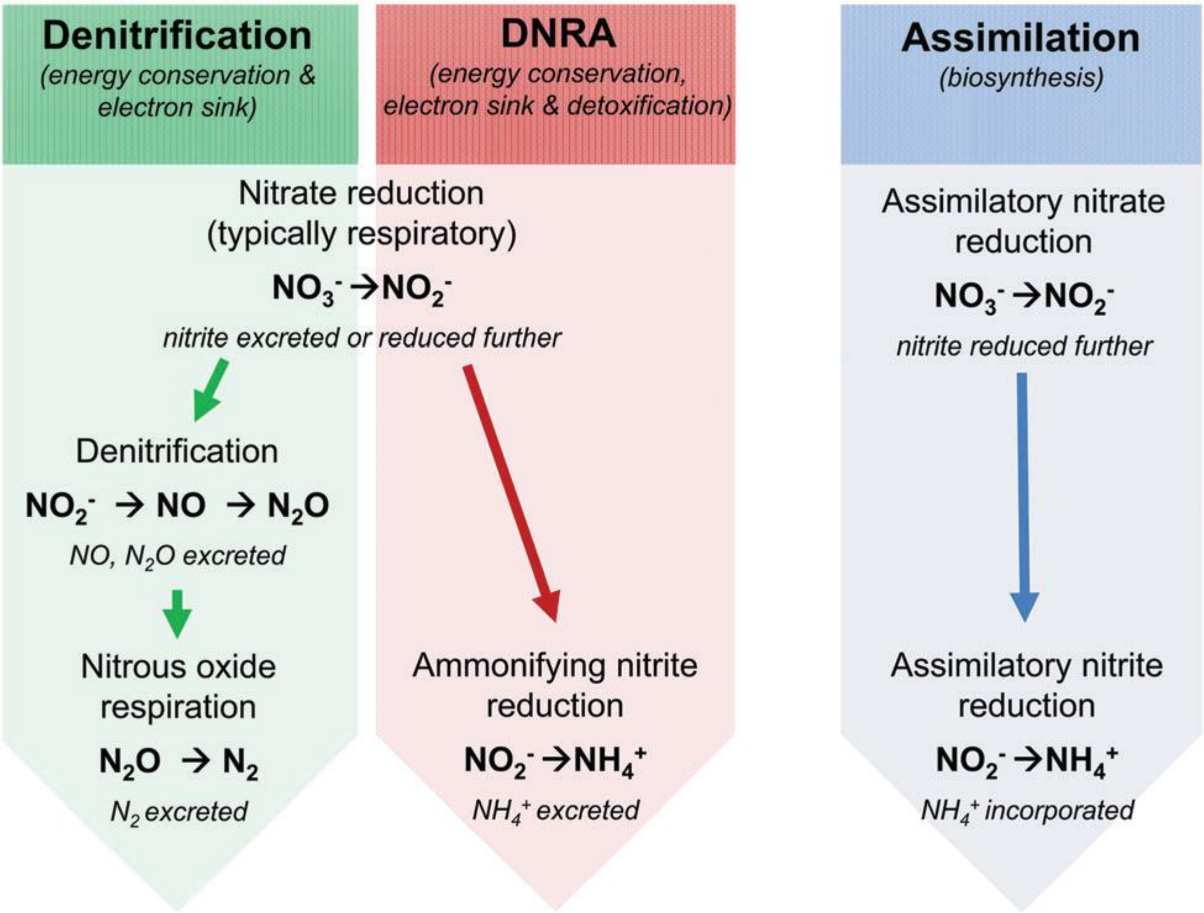

Figure 1 : Schematic representation of dissimilatory and assimilatory nitrate reduction.

1.4. Nitrate Reductase Assay (NRA)

Nitrate Reductase Assay (NRA) is a simple colorimetric method. It is simple, inexpensive, reliable and reproducible test.

Nitrate reducing bacteria (NRB) might be expected to be found where nitrate is plentiful (i.e., vegetables) and/or where nitrate-reducing bacteria might be found (intestinal tract, rumen).

1.4.1. The Nitrate Reduction Test (in Broth)

The nitrate reduction test is a qualitative procedure used to determine the ability of bacteria to reduce nitrate into nitrite. Bacteria are cultured overnight in nitrate broth (e.g., from HiMedia Laboratories) at 37 °C and then tested for the presence of nitrite.

M17 broth (e.g., from BD Difco), supplemented with 0.1% potassium nitrate (e.g., from Fisher Scientific), can also be used when Gram-positive cultures do not grow well in commercial nitrate broth. M17 broth supports the growth of lactic acid bacteria (LAB), and the absence of Tween 80 (a component of MRS broth) allows more non-LAB organisms to grow than would grow in MRS broth.

Nitrite detection is a two-step process. Nitrate reagent A (sulfanilic acid) is added first, followed by reagent B (alpha-naphthylamine). A red color indicates the presence of nitrite.

If no color develops, zinc powder is added. Zinc reduces nitrate to nitrite, producing a red color if unreduced nitrate is present. If there is still no color change after adding zinc, this indicates that nitrate was reduced to nitrite and then further reduced to other nitrogen compounds.

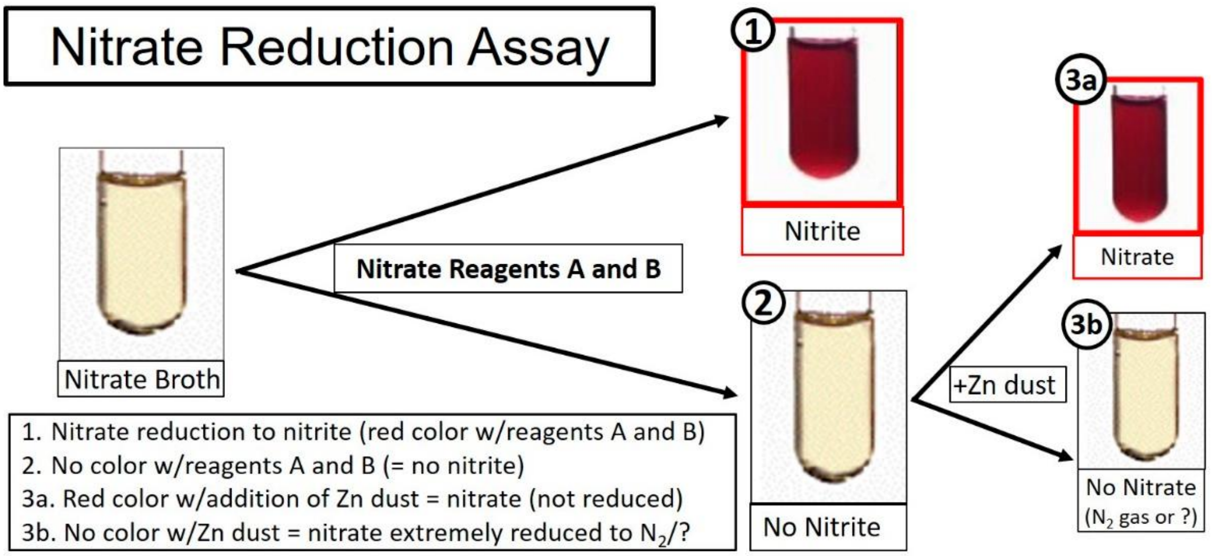

Figure 2 : Representation of the nitrate reduction test assay performed in liquid with nitrate broth culture samples by addition of reagents A and B.

1.4.2. The Nitrate Reduction Assay (on Agar)

The nitrate reduction assay using individual broth cultures was slow and laborious for screening potential NRB from food/animal samples. The nitrate reduction colony assay method for use on agar plates was developed in order to facilitate the screening of colonies from various food and animal samples. The agar plate nitrate reduction assay uses a similar principle of the culture broth (tube) nitrate reduction test but is performed directly on colonies on petri plates. A dilution series of test samples were surface plated on pre-poured M17 agar plates, allowed to dry (adsorb), and then overlaid with 10–12 mL nitrate agar (HiMedia) to entrap the plated colonies in a sandwich overlay technique. The overlaid plates were allowed to incubate overnight at 37° C, and then plates with a range of 25–250 colonies were selected to be overlaid with reagent agar layers tempered at 46–48° C before use. A plain agar layer containing addition of nitrate reagent A (2 mL reagent A is added in 50 mL 0.5% soft agar) was mixed and ~6–8 mL is overlaid onto the colony-sandwiched plate. After 5–10 min, another 6–8 mL plain agar layer containing nitrate reagent B (2 mL reagent B is added in 50 mL 0.5% soft agar) was overlaid on top of the nitrate reagent A layer and then tilted to facilitate the soft agar in running to the other side of the plate. The chemicals in the two separate overlays diffuse to the lower levels, reacting with nitrite in the order of addition as in the liquid nitrite test, and zones of red color observed around colonies indicated the presence of nitrite and nitrate-reducing bacteria. The various overlays are similar to those used for other purposes including sandwich overlays for detection of bacteriocin-producing colonies and thin agar overlays for recovery of injured bacteria.

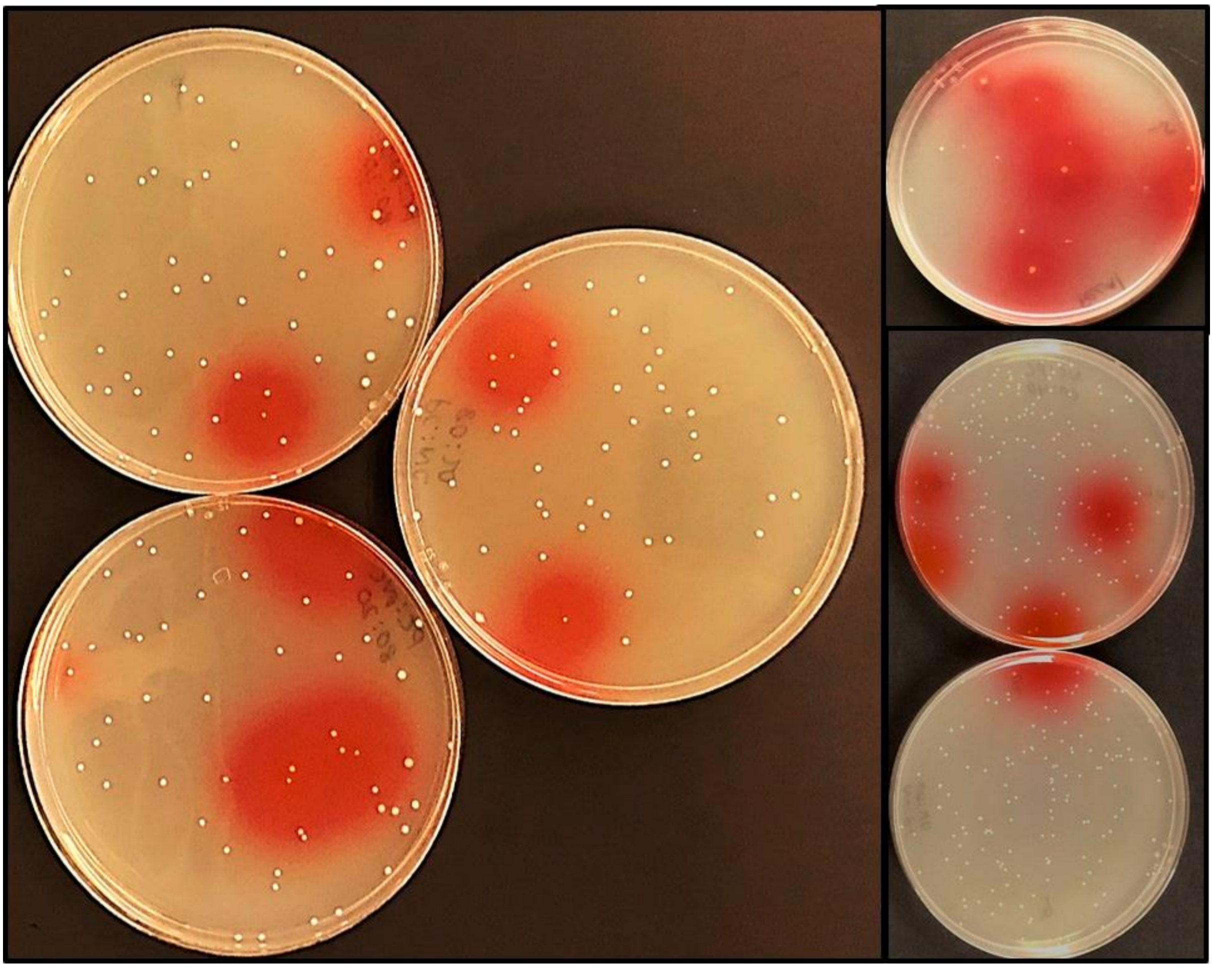

Figure 3 : Nitrate reducing bacterial colonies showing red color zones after sandwich overlays with soft agar containing nitrate reagents A and B.

2. ELISA Test

The quantity of the antigen depends on the antibody - antigen reaction in immunoassays, which are a type of bio-analytical approach. The antigen binds to the antibody to produce an immunological complex when the immunoanalytical reagents (antigen and antibody) are combined and incubated. Enzyme-linked immunosorbent assay (ELISA) is one of the most popular types of assays used as diagnostic instruments in medicine, as quality control measures in various sectors, and for the detection of specific antigens or antibodies in a collected sample.

2.1. Terminology

-ELISA plate: a container for antigen-antibody collection, which generally contains 96 test wells.

-Antigen: A protein to be assessed for levels, derived from the sample to be tested.

-Antibody: A protein that binds to an antigen.

2.2. Definition

ELISA (enzyme-linked immunosorbent assay) is an immunological biochemical assay used to detect and measure antibodies, antigens, peptides, proteins, glycoproteins, and hormones in biological samples.

2.3. Principle

ELISA test is carried out in 96-well polystyrene plates. The principle behind ELISA test is that specific antibodies bind to the target antigen and detect the presence of antigens and amount of antigenic load present in the given sample. In order to increase the sensitivity and accuracy of the test, antibodies with high affinity should be coated on the plate. This test also provides the information regarding the concentration of antigen and antibody in the given sample.

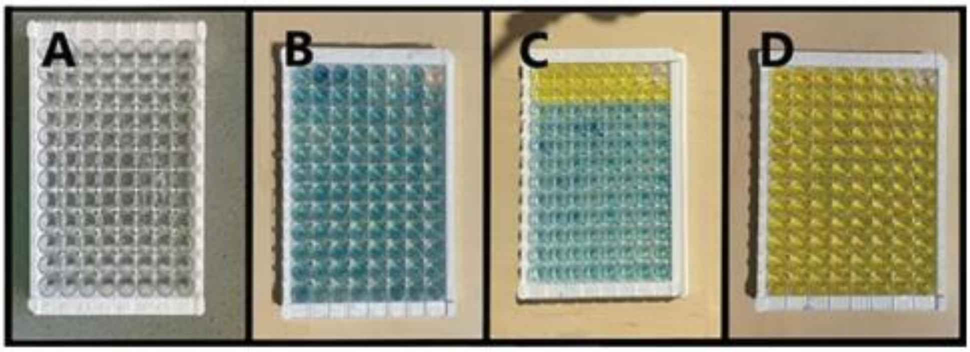

Figure 4 : ELISA kit contents. (a) Microplate; (b) Microplate loaded with analytes; (c) Yellow colour occurs when stopping solution is added and (d) Yellow colour occurs when stopping solution is added.

2.4. Types

2.4.1. Direct ELISA

In this type, the antigen is attached to the bottom of the plate, then the antigen will be detected through the antibody bound to the enzyme.

- **Advantages**

-Examination with this method is faster.

-Cross-reaction with secondary antibodies can be eliminated.

- **Disadvantages**

-The resulting signal amplification is weak.

-Lack of flexibility in selecting enzyme labeled primary antibodies.

-There may be reactions between primary antibodies and enzymes bound to these primary antibodies.

2.4.2. Indirect ELISA

In this method, the antigen is attached to the base of the plate, then, the primary antibody which is not labeled with the enzyme is inserted. Next, put back the enzyme labeled secondary antibody, which will bind to the primary antibody.

- **Advantages**

-The sensitivity of the test is increased with the use of primary and secondary antibodies.

- **Disadvantages**

-Cross-reactions can occur with secondary antibodies which will result in a non-specific signal.

-Longer incubation time is required.

-The cost required is greater than the direct method.

2.4.3. ELISA sandwich

In this method, the antibody is first attached to the base of the plate. Next, the test sample (antigen) is inserted into the well on the plate, then a secondary antibody bound to the enzyme is inserted into the well on the plate.

- **Advantages**

-Has high specificity.

-Suitable for use with less pure samples.

- **Disadvantages**

-The cost is quite large because it uses two antibodies.

2.4.4. Competitive ELISA

Competitive ELISA is an ELISA examination method where there is a competitive reaction between the sample antigen and antigen bond that is attached to the bottom of the plate well with the primary antibody. In this method, the non-sample antigen is attached to the bottom of the plate. Next, the sample antigen and primary antibody are inserted into the well. Then put secondary antibodies that are bound to the enzyme in the wells on the plate.

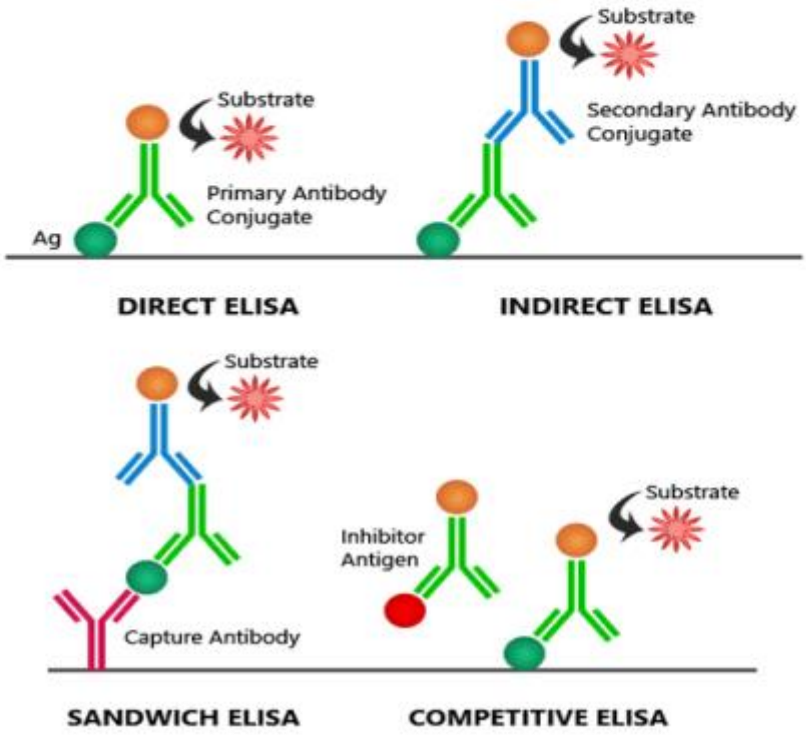

Figure 5 : Types of ELISA.