

SOIL DEHYDROGENASE ACTIVITY: A COMPARISON BETWEEN THE TTC AND INT METHOD. A REVIEW

Susan Marlein Mambu¹⁾

¹⁾Jurusan Biologi FMIPA Universitas Sam Ratulangi, Manado
e-mail: susan.mambu@gmail.com

ABSTRACT

Soil enzyme activities are very sensitive to both natural and anthropogenic disturbances and show a quick response to the induced change. Soil dehydrogenase enzyme are one of the main components of soil enzymatic activities participating in and assuring the correct sequence of all the biochemical routes in soil biogeochemical cycles. Dehydrogenase activity is measured by two methods using the TTC and INT substrate. Different biotic and abiotic factors such as incubation time and temperature, pre-incubation, soil aeration and moisture content have significant effect on dehydrogenase activity in soil. Dehydrogenase enzyme is often used as a measure of any disruption caused by pesticides, trace elements or management practices to the soil, as well as a direct measure of soil microbial activity. This review describes the role of intracellular enzyme-dehydrogenase in the soil environment, and the most common laboratory procedure used for measure dehydrogenase activity by two methods using the TCC and INT substrate.

Keywords: Dehydrogenase Activities, Flooded Soils, Soil Quality

AKTIVITAS DEHIDROGENASE TANAH: KAJIAN PERBANDINGAN ANTARA TTC DAN METODE INT

ABSTRAK

Aktivitas enzim tanah sangat sensitif terhadap gangguan yang disebabkan oleh alam dan antropogenik dan menunjukkan respon yang cepat terhadap perubahan yang terjadi. Enzim tanah dehidrogenase adalah salah satu komponen utama dari aktivitas enzim tanah yang berpartisipasi dalam siklus biogeokimia tanah. Aktivitas dehidrogenase dapat diukur dengan menggunakan dua metode TTC dan INT substrat. Perbedaan faktor biotik dan abiotik seperti waktu inkubasi dan suhu, pre-inkubasi, aerasi tanah dan kadar air memiliki pengaruh yang signifikan pada aktivitas dehidrogenase dalam tanah. Enzim dehidrogenase sering digunakan sebagai ukuran dari gangguan yang disebabkan oleh pestisida, elemen atau manajemen pengelolaan tanah, serta dapat mengukur langsung aktivitas mikroba tanah. Ulasan ini menjelaskan peran enzim dehidrogenase intraseluler dalam lingkungan tanah, dan prosedur laboratorium yang paling umum digunakan untuk mengukur kegiatan dehidrogenase, yaitu dengan menggunakan metode TTC dan INT substrat.

Kata Kunci: Aktivitas Dehidrogenase Tanah, TTC, INT

INTRODUCTION

Interesting in evaluating the quality and health of our soil resources has been stimulated by increasing awareness that soil is a critically important component of the earth's biosphere, functioning not only in the production of food and fibre but also in the maintenance of local, regional, and global environmental quality. The thin layer of soil

covering the surface of the earth represents the different between survival and extinction for most land-based life (Gliessman 1984). Soil quality is the phenomenon that has been developed to evaluate the factors effecting soil functionality. It is mainly concerned with sustainable use of soil resources in terms of enhanced agricultural productivity, environmental quality and human health. Basic soil quality indicators is expected to

integrate the combined effects of the soil's physical, chemical and biological properties that together: (i) provide a medium for plant growth and biological activity, (ii) regulate and partition water flow and storage in the environment, and (iii) serve as an environmental buffer in the formation and destruction of environmentally hazardous compounds, (Dexter 2004 ; Larson and Pierce 1994).

Soil physical quality is manifest in various ways. Examples of poor physical quality are when soils exhibit one or more of the following symptoms: poor water infiltration, run-off of water from the surface, hard-setting, poor aeration, poor rootability, and poor workability. Good soil physical quality occurs when soils exhibit the opposite or the absence of the conditions listed above (Dexter 2004). Chemical indicators include pH, salinity, organic matter content, phosphorus availability, cation exchange capacity, nutrient cycling, and the presence of contaminants such as heavy metals, organic compounds, radioactive substances, etc. These indicators determine the presence of soil-plant-related organisms, nutrient availability, water for plants and other organisms, and mobility of contaminants (Anderson 2003).

Biological indicators represent different aspects of soil quality in different ecosystems. These indicators strive to monitor or measure three basic functions or parameters:

1. soil structure development;
2. nutrient storage;
- and 3. biological activity (Gregorich *et al.* 1994).

Many biological indicators of soil quality measure the processes or components of soil organic matter accumulation and mineralization. Biological indicators often recommended include: nitrogen mineralization, microbial biomass, microbial biomass to total carbon ratios, soil respiration, respiration to microbial biomass ratios, faunal populations and rates of litter decomposition. Respiration, used to measure microbial activity related to decomposition of organic matter in soil, and a commonly used index: the metabolic quotient (qCO_2), defined as the respiration to microbial biomass ratio, which is associated to mineralization of organic substrate per unit of microbial biomass (Bastida *et al.* 2008).

Soil microbial activity leads to the liberation of nutrients available for plants but also to the mineralization and mobilization of pollutants and xenobiotics. Thus microbial activity is of crucial importance in biogeochemical cycling. Microbial activities are regulated by nutritional conditions, temperature and water availability. Other important factors affecting microbial activities are proton concentrations and oxygen supply. Microbial activity measurements include enzymatic assays that catalyze substrate-specific transformations and may be helpful to ascertain effects of soil management, land use and specific environmental conditions (Burns 1978).

Enzymes are biologically produced proteinic substances, having specific activation in which they combine with their substrates in such a stereoscopic position that they cause changes in the electronic configuration around certain susceptible bonds. Their significance in all spheres including soil is worth tested and reported. In plant nutrition, their role cannot be substituted by any other substance and their function is quite pragmatic in solubilizing and dissolving the much needed food in ionic forms for the very survival of the animal and plant kingdom. Enzymes are the key to understanding below-ground biochemistry and the role of soil in the global carbon cycle. These biological mediators of change are active both within living soil organisms and independently as extracellular proteins that are actively secreted into the soil by roots and fungi, or released as prokaryotic and eukaryotic cells that die and decompose. These enzymes can persist in the soil for weeks while maintaining a ghostly after life activity. Soil enzymes are a group of enzymes whose usual inhabitants are the soil and are continuously playing an important role in maintaining soil ecology, physical and chemical properties, fertility, and soil health. Soil enzymes play key biochemical functions in the overall process of organic matter decomposition in the soil system. They are important in catalyzing several important reactions necessary for the life processes of microorganisms in soils and the stabilization of soil structure, the decomposition of organic wastes, organic matter formation, and nutrient cycling, hence playing an important

role in agriculture (Dick *et al.* 1994 ; Sinsabaugh *et al.* 1991).

The enzyme levels in soil systems vary in amounts primarily due to the fact that each soil type has different amounts of organic matter content, composition, and activity of its living organisms and intensity of biological processes. In practice, the biochemical reactions are brought about largely through the catalytic contribution of enzymes and variable substrates that serve as energy sources for microorganisms (Kiss *et al.* 1978). These enzymes may include amylase, arylsulphatases, β -glucosidase, cellulase, chitinase, dehydrogenase, phosphatase, protease, and urease. Soil dehydrogenases are the major representatives of the Oxidoreductase enzymes class. Among all enzymes in the soil environment, dehydrogenases are one of the most important, and are used as an indicator of overall soil microbial activity, because they occur intracellularly in all living microbial cells (Gu *et al.* 2009).

The main purpose of the chapter is to clarify the description of the role of intracellular enzyme- dehydrogenase in the soil environment, and in the current chapter will concentrate on the precise description of the most common laboratory procedure used for measuring Dehydrogenase activity by two methods using the TTC and INT substrate.

Soil Dehydrogenase Enzyme Activity

The dehydrogenase enzyme activity is commonly used as an indicator of biological activity in soils (Burns 1978). This enzyme is considered to exist as an integral part of intact cells but does not accumulate extracellularly in the soil. Dehydrogenase enzyme is known to oxidize soil organic matter by transferring protons and electrons from substrates to acceptors. These processes are part of respiration pathways of soil microorganisms and are closely related to the type of soil and soil air-water conditions. Studies on the activity of dehydrogenase enzyme in the soil are very important as it may give indications of the potential of the soil to support biochemical processes which are essential for maintaining soil fertility. A study by (Brzezinska *et al.* 1998) suggested that soil water content and temperature influence dehydrogenase activity indirectly

by affecting the soil redox status. The relationship between dehydrogenase activity and redox potential (Eh) as well as Fe^{2+} content may also be used to illustrate the reactions of soil microorganisms to the changes in soil environment.

Additionally, dehydrogenase enzyme is often used as a measure of any disruption caused by pesticides, trace elements, or management practices to the soil (Wilke 1991), as well as a direct measure of soil microbial activity. It can also indicate the type and significance of pollution in soils (Garcia and Hernandez 1997). It has been found that dehydrogenase enzyme is high in soils polluted with pulp and paper mill effluents but low in soils polluted with fly ash. Similarly, higher activities of dehydrogenases have been reported at low doses of pesticides and, lower activities of the enzyme at higher doses of pesticides. As most areas of the world are often polluted by different industrial bio-chemical products, better understanding of the role of this enzyme in environmental science will open greater possibilities of using it as a diagnostic tool for better ecosystem assessment and amelioration (Baruah *et al.* 1986 ; Leiros *et al.* 2000).

Dehydrogenases are generally present in every upper layer of soils. Soil microflora is responsible for the decomposition and conversion of organic substances, aggregation stability and the carbon, nitrogen, sulphur and phosphorus cycles. Dehydrogenases, as respiratory chain enzymes, play the major role in the energy production of organisms. They oxidize organic compounds by transferring two hydrogen atoms. Dehydrogenases play a significant role in the biological oxidation of soil organic matter (OM) by transferring hydrogen from organic substrates to inorganic acceptors (Zhang *et al.* 2010). Many specific dehydrogenases transfer hydrogen to either nicotinamide adenine dinucleotide or nicotinamide adenine dinucleotide phosphate. Throughout mentioned co-enzymes hydrogen atoms are involved in the reductive processes of biosynthesis. Due to this fact, the overall DHA of a soil depends on the activities of various dehydrogenases, which are fundamental parts of the enzyme system of all living microorganisms, like enzymes of the

respiratory metabolism, the citrate cycle, and N metabolism (Subhani *et al.* 2001). Thus, DHA serves as an indicator of the microbiological redox-systems and could be considered a good and adequate measure of microbial oxidative activities in soil. Brzezińska *et al.* (2001) found that active dehydrogenases can utilize both O₂ and other compounds as terminal electron acceptors, although anaerobic microorganisms produce most dehydrogenases. Therefore, DHA reflects metabolic ability of the soil and its activity is considered to be proportional to the biomass of the microorganisms in soil. However, the relationships between an individual biochemical property of soil DHA and the total microbial activity is not always obvious, especially in the case of complex systems like soils, where the microorganisms and processes involved in the degradation of the organic compounds are highly diverse (Salazar *et al.* 2011).

Different Method Used for Measurement of Dehydrogenase Activity

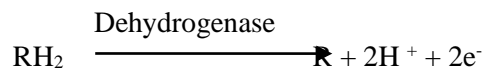
Dehydrogenase activity can be considered to be a good measure of microbial oxidative activity in soils. It is usually determined by measuring the amount of an artificial electron acceptor reduced by microbial activity, such as a soluble tetrazolium salt with a red colored reduced form (a formazan) that can be determined colorimetrically following extraction with a suitable solvent. Concentration of soil dehydrogenases depends on conditions and intensity of biological conversion of organic compounds. Addition of suitable chemical (triphenyltetrazolium chloride) enhances bioavailability of endogenous soil organic compounds to microflora. At the same time chloride is converted by hydrolytic reaction to formazan which can be extracted by organic solvents (methanol, acetone). Formazan concentration can be determined spectrophotometrically at 485 nm. Addition of organic substrate, e.g. compost, induces maximum DHA. H can be transferred to soluble tetrazolium salts (e.g., TTC, INT) with the formation of red formazans, which can be determined calorimetrically after extraction with a solvent (Tabatabai 1982).

Use of Tetrazolium Salts:

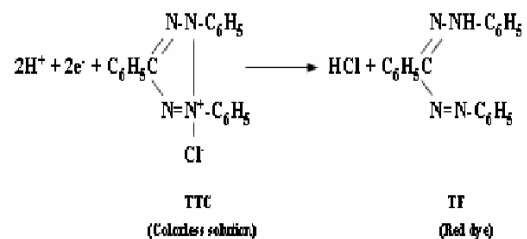
Dehydrogenase assays based on the reduction of 2, 3, 5- triphenyltetrazolium chloride (TTC) to the creaming red-colored formazan (TPF), have been used to determine microbial activity in soil. The triphenyltetrazolium chloride (TTC) is a stable, water-soluble heterocyclic organic salt that can be easily reduced to form a highly colored insoluble product (red formazan), which can be quantified colorimetrically by visible light absorption (Ghaly and Mahmoud 2006).

Equations (1) and (2) describe the two step reaction of TTC (Lenhard *et al.* 1964):

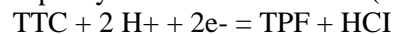
- a) Biological oxidation of organic compounds:



- b) Chemical reduction of tetrazolium salts:



Lenhard (1956) appears to have been the first to use 2, 3, 5-triphenyltetrazolium chloride (TTC) in studies of microbial activity in soil. The method is based on the assumption that in the absence of O₂ TIC acts quantitatively as the terminal H acceptor for dehydrogenase systems, with the formation of red triphenyltetrazoliumformazan (TPF):



When this test is applied to soil, the conditions are made anaerobic, and the aerobic dehydrogenases as well as the anaerobic dehydrogenases are assumed to use the TIC as H acceptor. If no substrate is added, the preferential stimulation of any group of organisms is avoided. If TTC does in fact act quantitatively as the terminal H acceptor for dehydrogenase systems, it replaces the respiratory enzyme chain which normally transfers the H and, in aerobic conditions, brings about combination of the H with O₂. When comparing O₂ uptake and CO₂ release, measurements have shown that

only 4-5% of the H developed was recorded by the TTC reduction.

Various possible reasons have been proposed for these poor results (Von Mersi and Schinner 1991):

1. TTC inhibits the transformation by its own toxicity.
2. Not all the H is transferred to the TTC, when TTC functions as an H acceptor it is competitively inhibited, and reduction occurs only after other H acceptors are exhausted.
3. A cell can only take in a limited amount of TTC, so that the potential for TTC reduction is limited (2,3,5,-triphenyl formazan, TPF may cause the death of the cell).

Subsequent work produced similar results which led us to conclude that a measurement of TTC reduction is only a qualitative, not a quantitative, determination of biological activity, and suspect that the TTC method may not be very efficient.

TTC standard curve: Water-soluble tetrazolium salts are the preferred oxidants because they form water-insoluble colored formazans which can be measured spectrophotometrically. Soil was Prepared with CaCO₃ and dispensed in three tubes as 6g each. The total volume of fluids added to the soil was 3.5 ml; this included any fluids added during preincubation of the soil. Most substrate solution concentrations were 1% dextrose solutions. All of these were sterilized by autoclaving and 1 ml was added to the soil at the time of TTC addition. However, the substrates were added as 0.5 ml of a double-strength solution if there was danger of exceeding the 3.5-ml fluid volume limit. TTC (Calbiochem, San Diego, Calif.) was prepared as a 3% aqueous solution and was sterilized by passage through a 0.30-, μ m membrane filter (Millipore Corp., Bedford, Mass.). Each tube received 1 ml of TTC, except for the soil blank, which received water instead. After addition, the water, substrate and TTC were simultaneously mixed through the soil with a sterile glass rod; then rubber stoppers were inserted and the tubes were incubated at 37°C at different incubation period of 6 hours, 12 hours and 24 hours. Upon completion of incubation, the tubes could be extracted immediately. Each soil sample was transferred with methanol to a funnel containing Whatman no. 5 filter

paper (W. and R. Balston, Ltd.) placed on a 100-ml graduated cylinder. Additional portions of methanol were passed through the soil until 50 ml of methanol, containing the formazan, had been collected in the graduated cylinder. If the filtrate passing from the funnel still had a red color, additional methanol was passed through the soil until all formazan had been extracted and corrections in calculations were made for the additional methanol. The red methanolic solutions of the formazan were read at 485 nm against the extract from the non-TTC soil blank by using a Spectronic 20 colorimeter (Bausch and Lomb, Rochester, N.Y.). The values obtained were compared against a formazan (Calbiochem) standard curve prepared with methanol and they are reported as milligrams of formazan per gram of soil (Kumar *et al.* 2013).

Although measuring the dehydrogenase activity using the TTC test depends on the biochemical reduction of the TTC, in addition, the medium pH, incubation temperature, quantity of tetrazolium salt and incubation period are critical parameters that affect the accuracy of dehydrogenase activity measurements using tetrazolium salts (Mahmoud and Ghaly 2004).

Use of P-Iodonitrotetrazolium Violet or INT salts:

Various authors have used INT as a substrate to determine dehydrogenase activity. Benefield *et al.* (1977) and Griffiths (1989) used INT 2-(p-iodophenyl)-3- (p-nitrophenyl)- 5-phenyltetrazolium chloride (p-iodonitrotetrazolium violet, or INT) as a substrate to determine dehydrogenase activity; which act as an artificial electron acceptor as a substrate to determined DHA in soil. Later, Trevors *et al.* (1982) used INT for the determination of dehydrogenase activity in soils, finding it to be more suitable than TTC for this purpose. The INT is reduced to idonitrotetrazolium formazan (INT-formazan) by soil enzymes and microorganisms and it was easily extracted without interference from phenolic compounds normally present in soil. The INT-formazan can be easily extracted with methanol and quantified spectrophotometrically.

The production of formazan is affected by the conditions of reaction, for example, substrate concentration, temperature, duration, pH of any buffer added, degree of aeration and the addition of energy-yielding substrates (Trevors 1984). Formazan is usually extracted from soil with methanol (Trevors *et al.* 1982) or ethanol (Roberge 1978) but other solvents have been used, for example 1 : 1.5 tetrachlorethylene : acetone (Benefield *et al.*, 1977) and 1:9 acetone : trichloromethane (Benckiser *et al.* 1984). However, INT is less soluble in water, the INT reduction, like that of TTC, is strongly dependent on the soil reaction and gives values that are much too low compared to the TTC method when a soil with a high organic matter content is used. To alleviate the influence of soil reaction on dehydrogenase activity, optimal condition have been provided by using buffers with pH values between 7.0 and 8.0 (Von Mersi and Schinner 1991). The use of INT has been shown to give good reproducibility and differentiation of DHA in various soil types, where INT may actually be the superior substrate due to greater enzyme reduction, shorter incubation times and greater extractability of INTF from soils (Friedel *et al.* 1994; Rossel *et al.* 1997).

Griffiths (1989) and Von Mersi and Schinner (1991) have reported that, compared to TTC, INT is less toxic to microorganisms, INT affords a more accurate estimation of dehydrogenase activity than TTC, which they attributed to its higher electron affinity compared to TTC. As a consequence of this high electron affinity, INT competes more effectively with oxygen for free electrons and so it is not necessary to carry out the determinations under anaerobic conditions. Similar the results of Trevor (1984), comparison of TTC and INT, the INT method allows measurements in both aerobically and anaerobically incubated soils. They observed that the reduction from INT to INTF (iodonitrotetrazolium formazan) gives a more exact measurement of DHA in soil extracts, as O₂ does not interfere with the INT reduction. More formazan is produced from the INT reduction than TTC, both with anaerobic and aerobic incubation.

Benefield *et al.* (1977) and Trevors (1984) compared the INT and TTC assay and concluded that the INT assay offered a better

measure of DHA than the TTC assay. The INT method has the advantage of increased sensitivity over the TTC procedure or INT acceptor is more sensitive than TTC. INT is reduced more rapidly and easily than other tetrazolium compounds. This is ensured by the strong electron affinity of INT in comparison to the other tetrazolium compounds.

CONCLUSION

The following are the significant findings emerging from the present study:

Soil dehydrogenase enzymes are one of the main components of soil enzymatic activities participating in and assuring the correct sequence of all the biochemical routes in soil biogeochemical cycles. It has also found that measurement of changes in soil enzyme activities may provide a useful index of changes in soil quality.

Dehydrogenase enzymatic activities is measured by two methods, namely use TTC and INT as substrate, however, various reported the poor results of DHA when TTC is used as substrate. The report sets out the finding of a study that both the INT and TTC assay can be applied to measure the DHA of anaerobic activated sludge in hydrogen-producing system. Furthermore, the INT assay has the advantage of increased sensitivity over the TTC procedure under the optimal conditions. INT assay, compared to TTC assay, requires less sludge and shorter time and turns out to be a more sensitive determination. Therefore, INT assay is more suitable to measure DHA in biohydrogen production system.

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