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# Microcalorimetric measurements of the microbial activities of single- and mixed-species with trivalent iron in soil

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**Abstract**

A microcalorimetric technique was applied to a series of experiments to follow the toxic effect caused by the trivalent iron on the single and mixed microbes in sterilized soil that was inoculated with the single *Bacillus subtilis* (*B. subtilis*) (prokaryotic bacterium), single *Candida humicola* (*C. humicola*) (eukaryotic fungus) and the mixed-species. The microbial activity was stimulated by the addition of 5.0 mg glucose and 5.0 mg ammonium sulfate under a 35% controlled humidity in the studied soil samples of 1.2 g. The power–time curves from every experiment were analyzed, and from these analyses characteristic parameters, such as growth rate constant ($k$) and total thermal effect ($Q$) which can reflect the biochemical reactions were determined. The mixed-species have moderate tolerance to the iron overload, comparing with single species, and exhibit synergistic interaction in exponential growth phase (0–400.0 $\mu g\,mL^{-1}$). Meanwhile, there is no much difference in the thermal effect ($Q$) per gram soil sample for the single and mixed culture. This also validates that the nutrient substances in natural environment determine the organisms' metabolic activities. Ultraviolet–visible spectrophotometry and dissolved oxygen sensor also were successfully applied to reflect the activities of *B. subtilis* and *C. humicola* in the pure culture. The investigation could provide insight into the microbial ecology of bacteria and fungi in ecological niches.


*Keywords:* *Bacillus subtilis*; *Candida humicola*; Bacterial–fungal interaction; Calorimetric method; Biochemical processes; Toxic effect; Trivalent iron

## 1. Introduction

Soil is the most important region of the geosphere, in which soil microorganisms play an essential role in the environment due to their role in cycling mineral compounds (Critter et al., 2004). However, iron and its compounds as a natural component of soil (Gurzau et al., 2003) are ubiquitous in soil, which also have been a recognized physiological requirement for life; its role extends beyond that of a nutritional necessity (Weber and Achenbach, 2006). In addition, iron and its compounds present as pollutants in the environment can cause deleterious effects to human being, animals, plants and microorganisms in soil.

Though, the low solubility product of iron minerals makes the inorganic form of iron unavailable to plants and forms the most common widespread nutritional disorder world over (Chatterjee et al., 2006), iron is readily chelated by a variety of organic molecules including haem, oxalate, citrate, EDTA, humic acids, tannins and siderophores (or siderochromes as they are also callded). In natural environments, chelation reactions may stabilize iron (either in the ferrous or ferric state) as soluble chelates thereby

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increasing the amount of iron in solution far above that expected from thermodynamic calculation (Nealson, 1983). Ethylenediaminetetraacetic acid (EDTA) is a chelator and combines stoichiometrically with iron. Ferric iron forms a strong complex with EDTA and has a high stability constant (Nayak and Nair, 2003). Some researches have reported that iron chelated by EDTA has bioavailability which can be absorbed by living organism (Candela et al., 1989). And Fe-EDTA supplementation will result in better expansion for the effect of the ferric iron on the plants (Chatterjee et al., 2006).

The presence of high metal concentrations have significant adverse effects on whole soil microbial biomass and activity (Khan and Scullion, 1999; Preston et al., 2000) and soil hydrolase activities (Renella et al., 2003, 2004, 2005). The major scientific and medical interest in iron is based on the essential bio-element, but toxicological considerations are also important in terms of accidental acute exposures by sensitive and accurate methods to assess the microbial activities in vitro.

Biochemical processes of living organisms results in a final exothermic effect in nature (Prado and Airoldi, 1999). Microcalorimetry is just one of the methods that can be applied to study the whole biochemical processes of microorganisms under the effect of different given laboratory conditions in soils (Critter et al., 2001, 2002; Raubuch and Beese, 1999). It is useful for evaluating the metabolism of microbial biomass in soils, because the heat released in the various biochemical processes depends solely on the initial and final energy states of the system, and is independent of the type of microorganisms and their form of evolution (Núñez-Regueira et al., 2006). Furthermore, the method has been confirmed to permit the continuous monitoring of the activity of a living process in situ for a prolonged period without disturbing the system (Wadsö, 1980; Núñez-Regueira et al., 2006; Barros et al., 1999).

However, in most complex natural environments, microbial populations consist of a multitude of species. Interaction between prokaryotes and eukaryotes are ubiquitous. Bacteria and unicellular eukaryote, such as yeast and filamentous fungi, are found together in a myriad of environments and exhibit both synergistic and antagonistic interactions, which may play an ecological role within microbial communities (Hogan and Kolter, 2002). So far, yet most laboratory studies of bacterial physiology have focused on the artificial setting of single-species cultures. Relatively little is known about how bacteria of different species interact with each other when cocultured (Straight et al., 2006; Weaver and Kolter, 2004).

This investigation aims at evaluating the toxic effect of Fe(III)-EDTA on the individual and the mixed species (*Bacillus subtilis* and *Candida humicola*, two ubiquitous soil microorganisms) inoculated into the sterilized orchard soil by microcalorimetric technique, and studying of bacteria–fungi interactions in order to provide an insight into microbial ecology. At the same time, the biochemical oxygen demand (BOD) and the absorbency (reflecting the turbidity) in controlled experimental conditions at different intervals based on the power–time curves were measured by dissolved oxygen sensor and ultraviolet–visible spectrophotometry, respectively. The combination of these methods can better elucidate the microbial activities. Meanwhile, the results also will be useful to understand the tolerance of prokaryotes and eukaryotes to iron overload.

## 2. Materials and methods

### 2.1. Materials

*B. subtilis* (prokaryotic bacterium) and *C. humicola* (eukaryotic fungus) were provided by the National Key Laboratory of Agromicrobiology, Huazhong Agricultural University (Wuhan, PR China).

Ferric chloride (Tianjin Shuangchuan Chemical Factory, PR China) was combined with EDTA (Tianjin Shuangchuan Chemical Factory, PR China) in molar ratio of Fe/EDTA of 1:1 (Nayak and Nair, 2003) to prepare concentration ($Fe^{3+}$) of 10 000.0 μg $mL^{-1}$.

Potato sucrose medium (PSM) was used to incubate *C. humicola*. PSM was prepared as following process: massive potatoes without peel (200.0 g) were boiled in 1.0 L distilled water for 30 min. Then the solution was filtered with gauzes. Filtrate was added 20.0 mg glucose (Sinopharm Group Chemical Reagent Co., Ltd.), adjusted to consistent volume (1.0 L) with distilled water, finally sterilized at 112 °C for 30 min.

Peptone culture medium that incubated the *B. subtilis* was prepared by dissolving 5.0 g peptone (Beijing Shuangxuan Microbe Culture Medium Products Factory, China), 10.0 g beef extract (Beijing Shuangxuan Microbe Culture Medium Products Factory, China) and 5.0 g NaCl (Sinopharm Group Chemical Reagent Co., Ltd.) in 1.0 L of deionized water at pH 7.2. It was then sterilized in high-pressure steam at 120 °C for 30 min.

### 2.2. Soil sampling and analysis

The soil sample was collected from orchard soil in Wuhan, Hubei province, central China, where is the typical subtropical zone, at a depth of 5–10 cm. After removal of the surface layer, it was air-dried and sieved (mesh size $2 \times 2\,mm^2$) to remove root fragments and large particles. The soil was stored in polyethylene bags at 25 ± 0.3 °C (Triegel, 1988).

Microbial activity is related to the main characteristic of the soil. Thus, the inherent physical and chemical properties such as pH, organic matter content and nutritional elemental content are important features to be considered. Soil pH value was determined with a pH-meter (Beckman Φ690). The measurement was performed by introducing the electrode in the supernatant solution prepared, 10.0 g of soil and 25.0 mL of distilled water. Organic matter (OM) of each sample was determined by titrating the samples in an acidic medium, with the end point followed by a redox reaction. The elements of C, H, N were analyzed by element analyzer (VARIO EL3, Germany), using a previously obtained calibration curve.

The determination of Na, K, Mg, Ca and P was performed by extracting a percolated fraction of 5.0 g of soil with 50.0 ml Mehlich 3 solution (0.2 mol $L^{-1}$ acetic acid, 0.25 mol $L^{-1}$ ammonium nitrate, 0.015 mol $L^{-1}$ ammonium fluoride, 0.013 mol $L^{-1}$ nitric acid and 0.001 mol $L^{-1}$ EDTA, pH 2.5) (Mehlich, 1984). Na and K were determined by flame photometry and P by photometry, using a previously obtained calibration curve. Mg and Ca were obtained by atomic absorption spectrometry (WFX-1F2B, Beijing Ruili) (Triegel, 1988). The results were shown in Table 1.

### 2.3. Incubation procedure

The soil was sterilized at 120 °C for 30 min, and the processes were repeated three times at interval of three days in order to assure the soil