ORIGINAL ARTICLE

Effecf of pH and some cations on activity of acid phosphatase secreted from *Ustilago* sp. isolated from acid sulphate soil

Jumpen Onthong[1], Sayjai Gimsanguan[2], Ashara Pengnoo[3], Chairatana Nilnond[4] and Mitsuru Osaki[5]

Abstract

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Effecf of pH and some cations on activity of acid phosphatase secreted from *Ustilago* sp. isolated from acid sulphate soil
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Acid phosphatase secreted from *Ustilago* sp. is able to hydrolyze organic phosphorus. These soil yeast microorganisms were isolated from rice roots grown in acid sulphate soil that generally contains high amount of aluminum (Al), iron (Fe) and manganese (Mn) ions. Therefore, the objectives of this study were to examine the effect of pH and some cations on acid phosphatase activity. Two isolates of *Ustilago* sp., AR101 and AR102, were cultured in 100 mL of modified Pikovskaya's broth containing Na-phytate, pH 4, and acid phosphatase activity was determined at pH 2.0-7.0. Effect of Al, Fe, and Mn, including calcium (Ca) ions, on growth of AR101 and AR102, secreted acid phosphatase activity, and the ability of acid phosphatase on the phosphorus release from Na-phytate by *Ustilago* sp. were investigated. It was found that the optimum

[1]Ph.D. (Agricultural Chemistry) Asst. Prof., [2]M.Sc. student in Soil Resource Management, [3]Ph.D. (Bioresources and Product Science) Asst. Prof., [4]Ph.D. (Soil Science) Assoc. Prof., Department of Earth Science, Faculty of Natural Resources, Prince of Songkla University, Hat Yai, Songkhla, 90112 Thailand, [5]Ph.D. (Plant Nutrition) Prof., Department of Bioscience and Chemistry, Faculty of Agriculture, Graduate School of Agriculture, Hokkaido University, Kita 9, Nishi 9, Kita-Ku, Sapporo, 060-8589, Japan.
Corresponding e-mail : jumpen.o@.psu.ac.th
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pH for acid phosphatase activity was 3.5-4.5. The activity of acid phosphatase secreted from AR101 (3,690 nmol min^{-1} mL^{-1}) was remarkably higher than that from AR102 (956 nmol min^{-1} mL^{-1}). Aluminum, iron, manganese and calcium ions in the medium did not affect the growth of either isolate. The activity of secreted acid phosphatase of AR101 was inhibited by Al and Ca ion, and synthesis of acid phosphatase of *Ustilago* sp. AR102 was possibly stimulated by Fe ion. Both AR101 and AR102 solubilized Na-phytate, resulting in the release of P. However, some amount of released P was then precipitated with Al and Fe ions as the highly insoluble Fe- or Al- phosphate.

Key words : acid phosphatase, acid sulphate soil, *Ustilago* sp., aluminum, iron, manganese, calcium

บทคัดย่อ

จำเป็น อ่อนทอง[1] สายใจ กิ้มสงวน[1] อัจฉรา เพ็งหนู[1] ชัยรัตน์ นิลนนท์[1] และ มิตซูรุ โอซากิ[2]

ผลของพีเอชและแคตไอออนบางชนิดต่อกิจกรรมของแอซิดฟอสฟาเทส จากเชื้อ *Ustilago* sp. ที่แยกจากดินกรดจัด

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เชื้อ *Ustilago* sp. เป็นยีสต์ชนิดหนึ่งที่คัดแยกมาจากบริเวณรอบรากข้าวที่ปลูกในดินกรดจัด มีความสามารถในการปลดปล่อยเอนไซม์แอซิดฟอสฟาเทส ซึ่งมีบทบาทสำคัญในการแปรสภาพอินทรีย์ฟอสฟอรัสให้กลายเป็นอนินทรีย์ฟอสฟอรัสที่เป็นประโยชน์กับพืช แต่ในสภาพของดินกรดจัดมักมีอะลูมินัม เหล็ก และแมงกานีสไอออนละลายอยู่มาก ซึ่งอาจส่งผลต่อกิจกรรมของเอนไซม์แอซิดฟอสฟาเทส จึงทำการศึกษาผลของพีเอชและแคตไอออนบางชนิดต่อกิจกรรมของเอนไซม์แอซิดฟอสฟาเทส โดยเลี้ยงเชื้อ *Ustilago* sp. สายพันธุ์ AR101 และ AR102 ในอาหารเหลวสูตร modified Pikovskaya's medium ซึ่งมีอินทรีย์ฟอสฟอรัสรูปโซเดียมไฟเทต พีเอช 4 และนำมาทดสอบกิจกรรมของเอนไซม์แอซิดฟอสฟาเทสที่พีเอช 2-7 รวมทั้งทดสอบผลของอะลูมินัม เหล็ก แมงกานีส และแคลเซียมไอออน ต่อการเจริญเติบโต การปลดปล่อยเอนไซม์แอซิดฟอสฟาเทสของเชื้อทั้งสองสายพันธุ์ และทดสอบผลของแคตไอออนดังกล่าวต่อความสามารถของเอนไซม์แอซิดฟอสฟาเทสในการปลดปล่อยฟอสฟอรัสจากสารละลายโซเดียมไฟเทต พบว่า พีเอชที่เหมาะสมต่อกิจกรรมของเอนไซม์ที่ปลดปล่อยจาก *Ustilago* sp. ทั้งสองสายพันธุ์อยู่ในช่วง 3.5-4.5 โดยเอนไซม์จากสายพันธุ์ AR 101 มีกิจกรรม (3,690 นาโนโมล/นาที/มล.) สูงกว่าสายพันธุ์ AR102 (956 นาโนโมล/นาที/มล.) อย่างชัดเจน ในขณะที่อะลูมินัม เหล็ก แมงกานีส และแคลเซียมไอออนในอาหารเลี้ยงเชื้อไม่ได้มีผลต่อการเจริญเติบโตของเชื้อทั้งสองสายพันธุ์ แต่อะลูมินัมและแคลเซียมไอออนยับยั้งกิจกรรมของเอนไซม์ที่ปลดปล่อยจากเชื้อสายพันธุ์ AR101 ในขณะที่เฟอรัสไอออนอาจส่งเสริมให้เชื้อสายพันธุ์ AR102 สร้างเอนไซม์ได้เพิ่มขึ้น อย่างไรก็ตาม เชื้อทั้งสองสายพันธุ์สามารถปลดปล่อยฟอสฟอรัสจากสารละลายโซเดียมไฟเทตให้ออกมาได้ แต่ฟอสฟอรัสที่ถูกปลดปล่อยออกมาในสารละลายที่มีอะลูมินัม และเหล็กละลายอยู่มากจะเข้าทำปฏิกิริยากับอะลูมินัมและเหล็กทำให้ความเป็นประโยชน์ของฟอสฟอรัสลดลง

[1]ภาควิชาธรณีศาสตร์ คณะทรัพยากรธรรมชาติ มหาวิทยาลัยสงขลานครินทร์ อำเภอหาดใหญ่ จังหวัดสงขลา 90112 [2]Department of Bioscience and Chemistry, Faculty of Agriculture, Graduate School of Agriculture, Hokkaido University, Kita 9, Nishi 9, Kita-Ku, Sapporo, 060-8589, Japan.

Acid phosphatase is a group of enzymes which catalyse hydrolytic cleavage of the C-O-P ester bond organic phosphorus in soil and release phosphorus as plant available inorganic form ($H_2PO_4^-$, HPO_4^{2-}) (Marschner, 1991; Hysek and Sarapatka, 1998; Yadav and Tarafdar, 2001;

Magboul and McSweeney, 1998; Hayes *et al.*, 2000). Several types of acid phosphatase, such as phosphomonoesterase and phosphodiesterase are able to hydrolyze organic phosphorus e.g. phospholipids and nucleic acid, whereas, phytase is able to hydrolyze myo- inositol hexaphosphatate (phytic acid) to inositol and ortho-phosphoric acid (Sarapatka, 2003; Sylvia *et al.*, 2005).

The acid phosphatase exuded by plant roots and microorganisms hydrolyzes organic phosphorus and liberates orthophosphates which are released into the rhizosphere to be available to plants. Therefore, it plays an important role in the increase of soil P availability, especially that secreted by soil microorganisms which are commonly found in soil and have a short life cycle (Sarapatka, 2003).

Ustilago sp., isolates AR101 and AR102, are yeasts which have been isolated from the rhizosphere of rice in acid sulphate soil. Both isolate AR 101 and AR 102 were able to grow in medium containing $AlPO_4$, $FePO_4$ and Na-phytate (Pengnoo, 2005), indicating that these isolates can solubilize insoluble P in the medium. It was reported that the pH of medium decreased from the initial pH 4.0 to become even more acidic (pH = 2.3), resulting in the solubilization of $AlPO_4$ and $FePO_4$. However, the release of P from organic P, Na-phytate, was due to the activity of acid phosphatase secreted from 2 isolates. Since these 2 isolates tolerate an acidic environment, it is possible to use them for promoting P availability from organic P in acid and acid sulphate soils which are widely found in the tropic.

Organic phosphorus may constitute 20-80% of the total phosphorus in soil, especially in tropical soils, in which this fraction is generaly as high as 80 % (Foth and Ellis,1997). It was reported that organic P in Kohong and Hat Yai soil series, which are highly weathered soils in the south of Thailand, were 54.71% and 48.62% respectively (Onthong *et al.*, 1999). Consequently, organic phosphorus may play an important role as P sources for plants. Phosphorus availability is limited in acid and acid sulphate soil. Both soils contain high amounts of Al, Fe and Mn ions, leading to plant toxicity. Moreover, these cations can precipitate with available P, resulting in a reduction of available soil P. This serious problem can be alleviated by liming in order to decrease Al, Mn and Fe toxicity, but soil pH and Ca ion are increased. The condition of high Al, Fe, Mn and Ca ions will possibly affect the growth of both isolates and acid phosphatase activity. The objectives of this study were to investigate the effect of pH and Al, Fe, Mn and Ca ions on growth and acid phosphatase activity of *Ustilago* sp. AR101 and AR102 , as well as the release of P from Na-phytate by these isolates.

Materials and methods

1. Effect of pH on acid phosphatase activity

Ustilago sp., isolates AR101 and AR102, were cultured in 100 mL of modified Pikovskaya's broth which contained the following ingredients (per liter); glucose 10 g, $(NH_4)_2SO_4$ 0.5 g, NaCl 0.2 g, KCl 0.2 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, $MnSO_4 \cdot H_2O$ 0.005 g and P 5 mg in the form of Na-phytate. The pH of broth was adjusted to 4.0. The flasks were shaken at 150 rpm for 12 days at room temperature (28-32^{o}C). After that the acid phosphatase activity was determined with 3 replicates by incubation of 0.1 mL of supernatant with 0.1 mL of 10 mM ρ-nitrophenyl phosphate (ρ-NPP) in 200 mM sodium acetate buffer pH 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5 and 7 at 37^{o}C for 10 min. The reaction of nitrophenol release was stopped by the addition of 3.6 mL saturated sodium carbonate. The sample was set aside at room temperature (28-32^{o}C) for 20 min, and then centrifuged at 10,000 x g for 10 min. The nitrophenol standard solution at concentrations of 0, 100, 200, 400, 800, 1,600 and 3,200 mM was conducted as the samples. The absorbance of supernatant was measured using visible spectrophotometer at 410 nm. The acid phosphatase activity corresponds to the amount of released nitrophenol per min per mL (nmol min^{-1} mL^{-1}).

2. Effect of Al, Fe, Mn and Ca ion on *Ustilago* sp. growth and acid phosphatase activity

Ustilago sp. AR101 and AR102 were cultured in 100 mL of modified Pikovskaya's broth containing Na-phytate (P 5 mg L^{-1}, pH 4). Al, Fe, Mn and Ca ions were separately added until the medium reached at the concentrations of 0, 5 and 10 mg L^{-1} with 3 replicates. The pH of broth and acid phosphatase activity were measured every 4 days after inoculation. The viable cell of *Ustilago* sp. AR101 and AR102 after culturing in the medium containing different concentrations of cation were also examined using PDA plate count technique.

3. Effect of Al, Fe, Mn and Ca ions on phosphate solubilization from Na-phytate by *Ustilago* sp.

Ustilago sp. AR101 and AR102 were cultured in 100 ml of modified Pikovskaya's broth containing Na-phytate (P 5 mg L^{-1}, pH 4) for 12 days. Three mL of supernatant was added into 100 mL of Na-phytate solution (P 5 mg L^{-1}). Al, Fe, Mn and Ca ions were separately added with 3 replicates to reach the concentrations of 0, 5 and 10 mg L^{-1} and incubated at 37°C for 24 hours. The released phosphorus was measured by molybdenum blue method and the acid phosphatase activity was measured at the start and the end of incubation.

Results

1. Effect of pH on acid phosphatase activity

The optimum pH for acid phosphatase activity was approximately 3.5-4.5, and the activity of acid phosphatase secreted from AR101 was higher than that from AR102. The highest activity of acid phosphatase secreted from AR101 and AR102 was observed at pH 3.5 (3,690 and 956 nmol min^{-1} mL^{-1} respectively). The acid phosphatase activity of both isolates was very low at pH 2.0-3.0. It increased rapidly as the pH increased from 3.0 to 3.5, and then decreased sharply when the pH increased from 3.5 to 4.5. The activity of acid phosphatase was not found at pH higher than 5.5 (Figure 1).

2. Effect of Al, Fe, Mn and Ca ion on *Ustilago* sp. growth and acid phosphatase activity

The pH of culture medium in all treatments rapidly decreased from initial pH 4 to about 2.5 after 4-days of culturing, and then was stable throughout the test. Addition of Fe, Mn and Ca ions did not significantly affect pH, compared to the control treatment (without these ions) (Figure 2c-2h). However, the pH of the culture medium containing 5 and 10 mg L^{-1} of Al slightly decreased, and was higher than that in the control treatment (Figure 2a and 2b). Addition of Al, Fe, Mn and Ca ions up to 10 mg L^{-1} did not affect the growth of

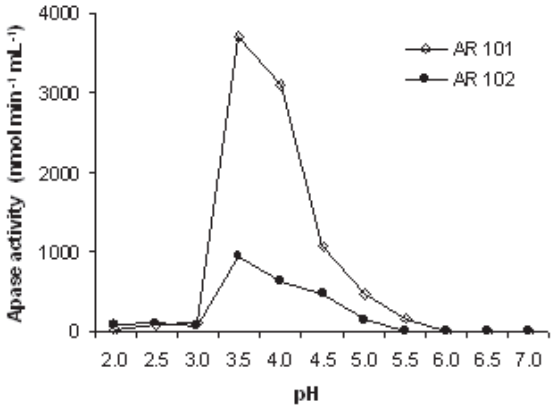

Figure 1. Effect of pH on the activity of acid phosphatase secreted from *Ustilago* sp. AR101 and AR102 (3 replications)

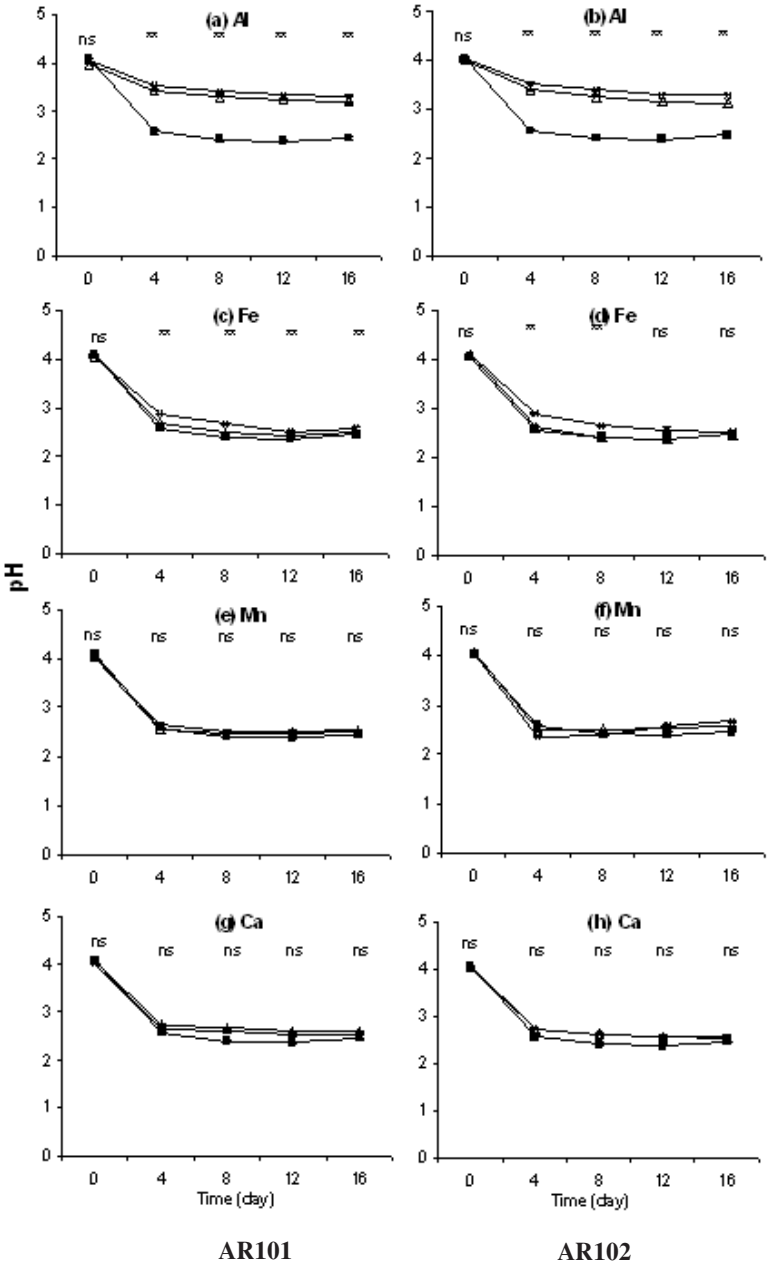

Figure 2. Effect of Al, Fe, Mn and Ca ions (■ = 0 mg L^{-1}, △ = 5 mg L^{-1}, X = 10 mg L^{-1}) on the pH of modified Pikovskaya's medium containing Na-phytate which cultured *Ustilago* sp. AR101 and AR102 (3 replications) (ns = not significant and ** = significant at 0.01)

Ustilago sp. AR101 and AR102. The number of viable cell of these isolates in the Al, Fe, Mn and Ca treatments was similar to the control treatment (Figure 3a-3h).

The activity of acid phosphatase secreted from AR101 was inhibited by Al ion, but activity was not found in AR102 (Figure 4a-4b). In contrast, the activity of acid phosphatase secreted

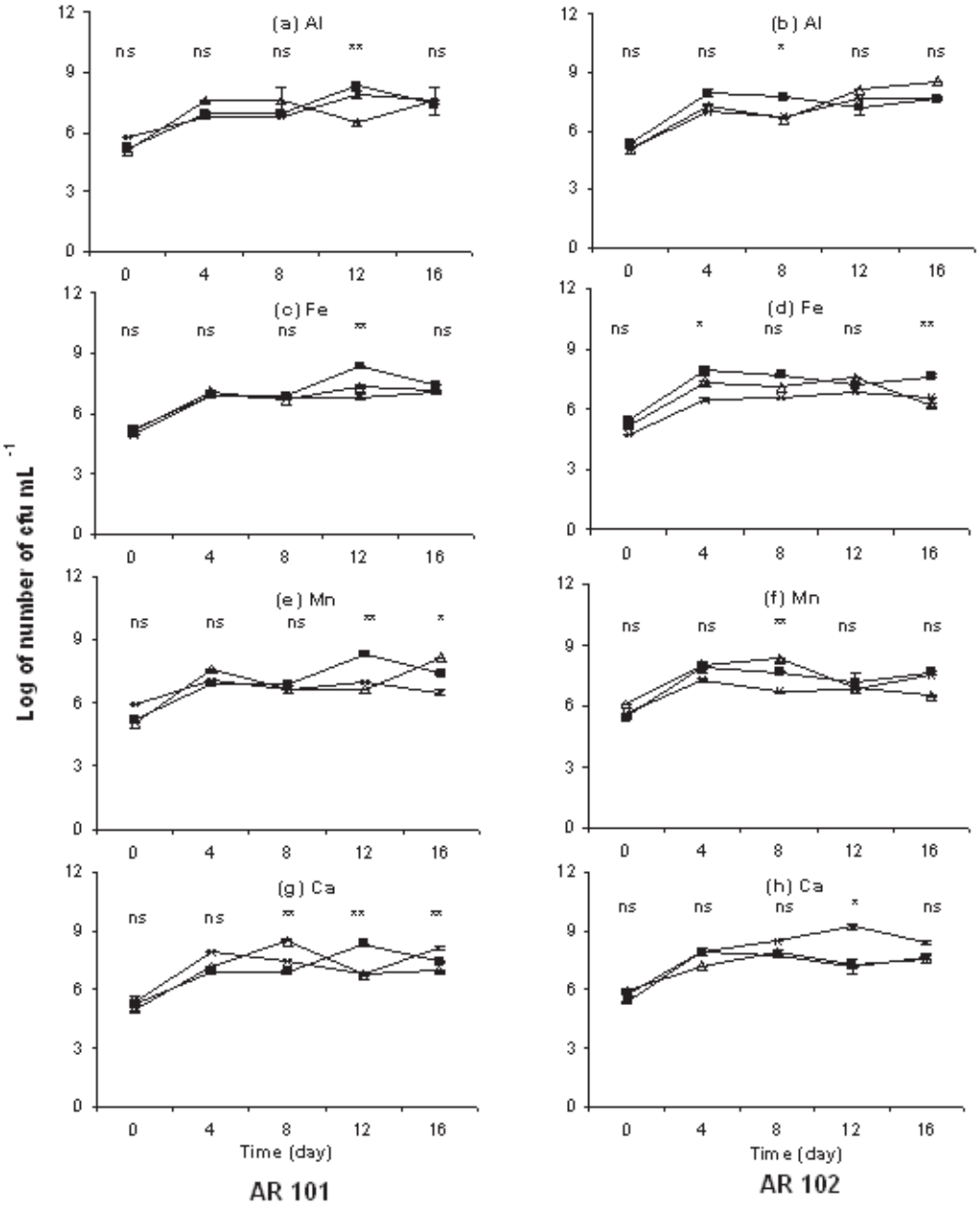

Figure 3. Effect of Al, Fe, Mn and Ca ions (■ = 0 mg L^{-1}, △ = 5 mg L^{-1}, X = 10 mg L^{-1}) on the growth of *Ustilago* sp. AR101 and AR102 cultured in modified Pikovskaya's medium containing Na-phytate (3 replications) (ns = not significant, * and ** = significant at P=0.05 and 0.01 respectively) (I = standard error)

from AR102 was stimulated by Fe ion, but was not found in AR101 (Figure 4c-4d). Addition of Mn and Ca ions did not affect the activity of acid phosphatase secreted from AR102 but Mn and Ca stimulated the activity of acid phosphatase secreted from AR101 at 4 days after culturing. However, after 4-days of culturing Ca tended to inhibit enzyme activity (Figure 4e-4h).

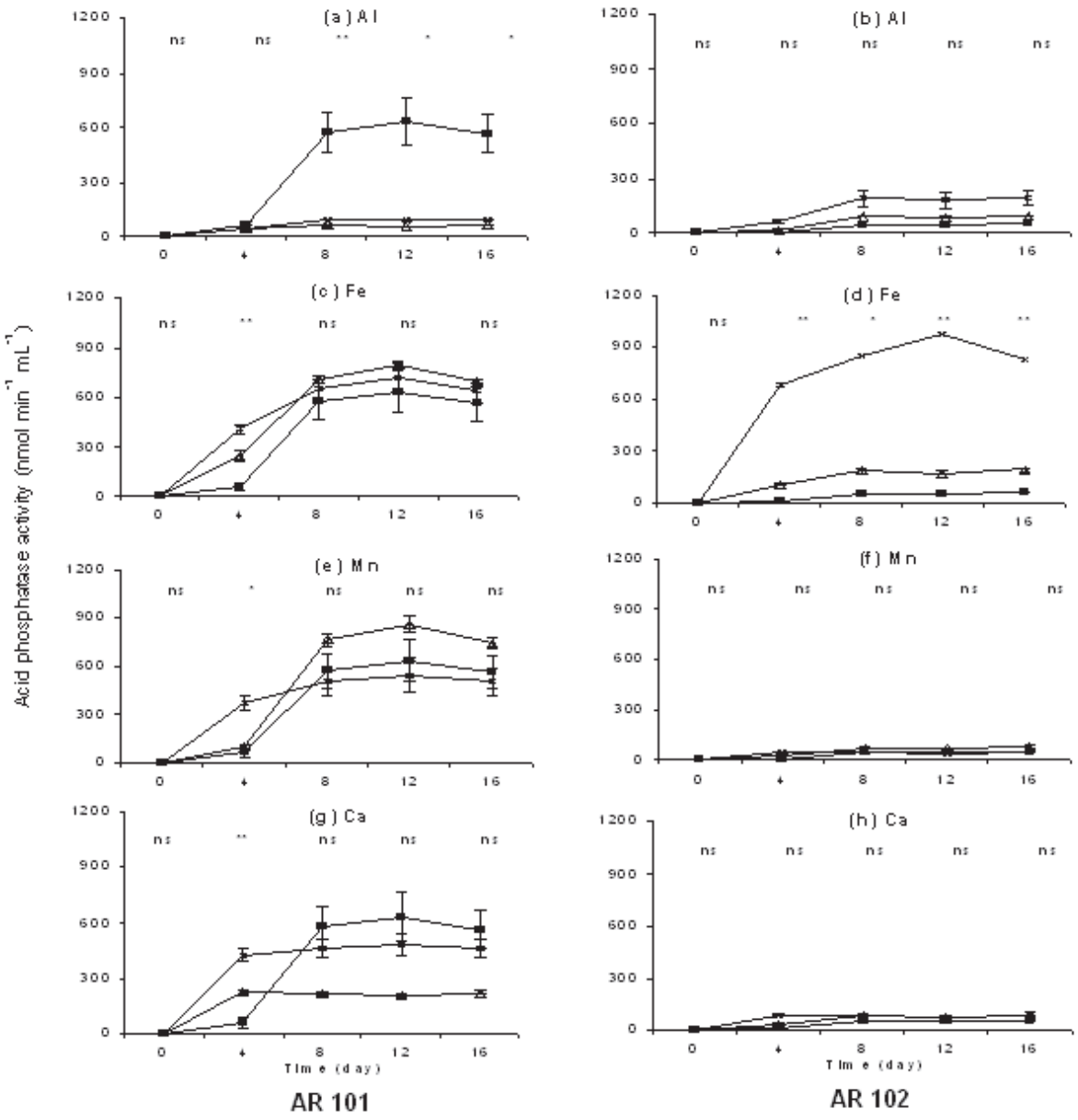

Figure 4. Effect of Al, Fe, Mn and Ca ions (■ = 0 mg L^{-1}, △ = 5 mg L^{-1}, X = 10 mg L^{-1}) on the activity of acid phosphatase secreted from *Ustilago* sp. AR101 and AR102 (3 replications) (ns = not significant, * and ** = significant at P=0.05 and 0.01 respectively) (I = standard error)

3. Effect of Al, Fe, Mn and Ca ions on phosphate solubilization from Na-phytate by *Ustilago* sp.

At 24-hour-incubation of the Na-phytate solution with AR101 and AR102, the released P was higher than that in the control (without isolate), but it was decreased in the Al treatment. The released P in the 0, 5 and 10 mg L^{-1} Al treatments by AR101 was 2.55, 1.06 and 0.96 mg L^{-1} respectively, and by AR102 was 1.91, 1.10 and

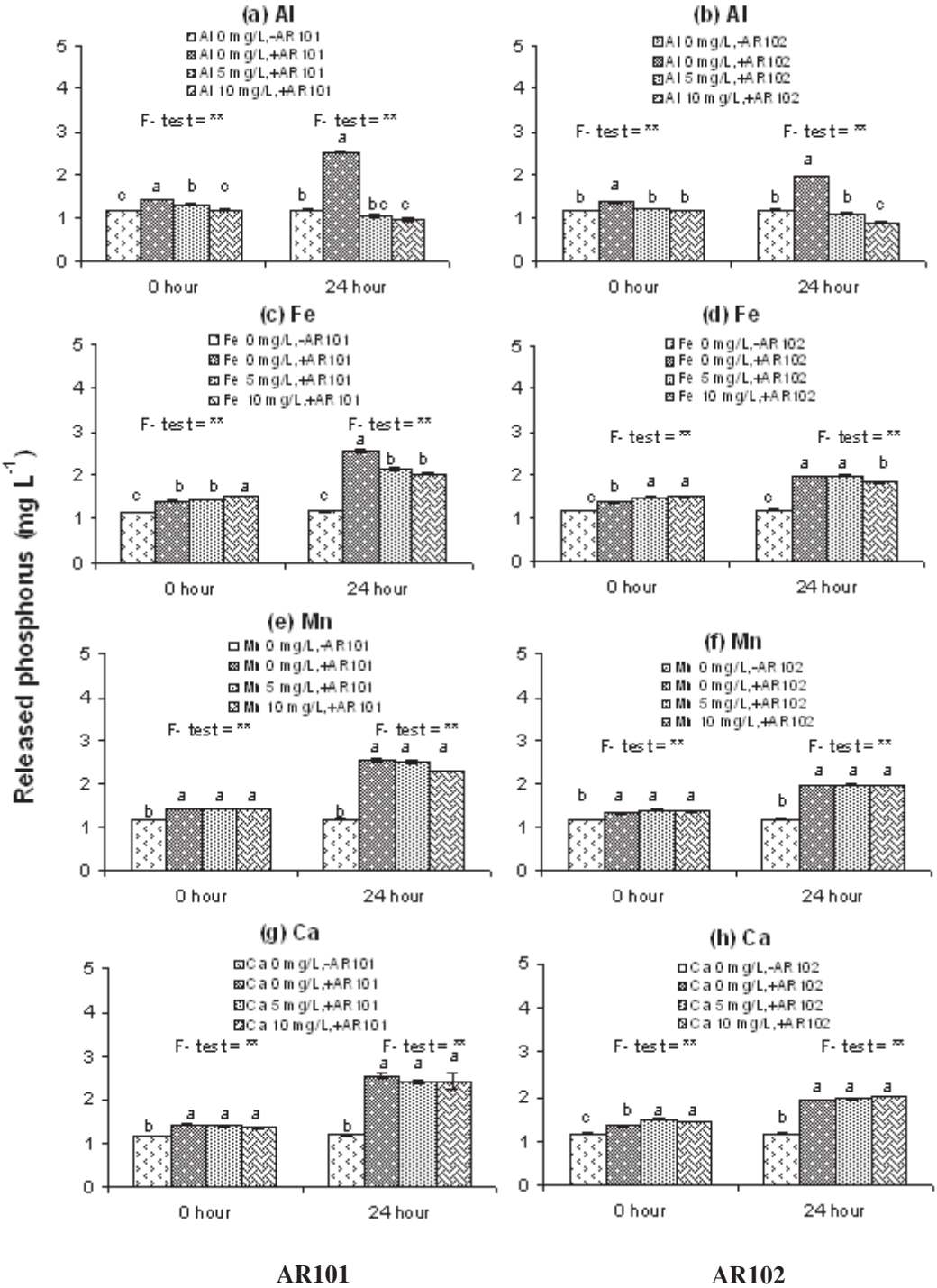

Figure 5. Effect of Al, Fe, Mn and Ca ions on the phosphorus release from Na-phytate by *Ustilago* sp. AR101 and AR102 (3 replications) (= significant at 0.01) (I = standard error)**

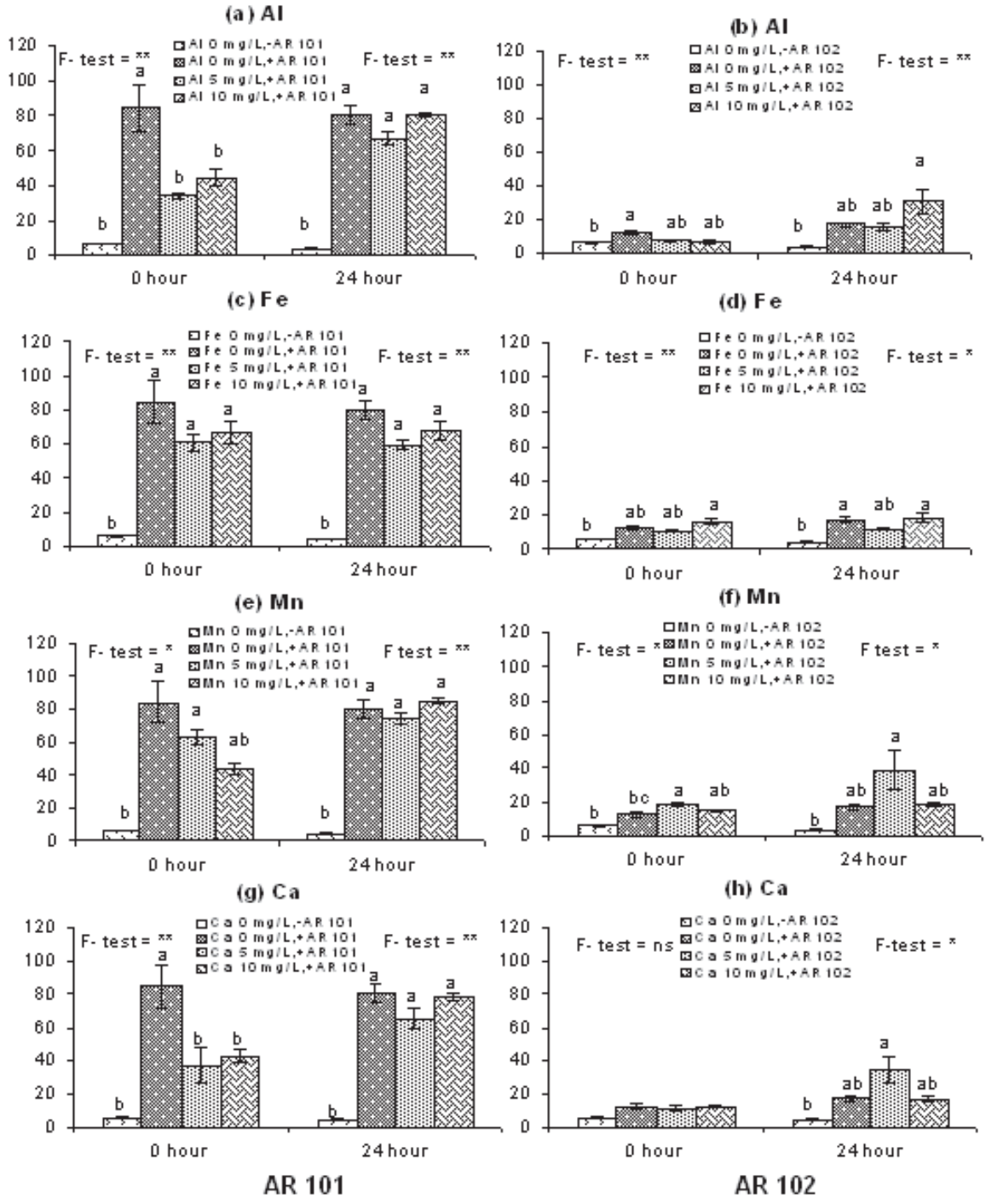

Figure 6. Effect of Al, Fe, Mn and Ca ions on the activity of acid phosphatase at the start and the end of incubation. (3 replications) (ns = not significant, * and ** = significant at P=0.05 and 0.01 respectively) (I = standard error)

0.88 mg L^{-1} respectively. Addition of Fe also gave the similar results as Al. However, the addition of Mn and Ca showed no effect on the ability of phosphate solubilization from Na-phytate by *Ustilago* sp. (Figure 5a-5h).

The activity of acid phosphatase secreted from AR101 in the Fe treatments was similar to the control (without Fe), and the activity in 0- and 24-hour-incubation was not significantly different. However, the initial activity (0-hour-incubation) in the Al, Mn and Ca treatments was significantly lower than in the treatments without these cations. The activity of acid phosphatase secreted from AR102 with and without Al, Fe, Mn and Ca addition at the start and the end of incubation was not significantly different (Figure 6a-6h).

Discussion

1. Effect of pH on acid phosphatase activity

The optimum pH for the activity of acid phosphatase secreted from AR101 and AR102 was approximately 3.5-4.5 (Figure 1). It is consistent with the acid phosphatase secreted from other microorganisms such as *Aspergillus niger* (pH = 2.0-3.5) (Gargovar and Sariyska, 2003) and *Lactobacillus plantarum* DPC2739 (pH = 3.5-5.0) (Magboul and McSweeney, 1999). *Ustilago* sp. AR101 and AR102 were isolated from rhizosphere and rhizoplane of rice grown in acid sulphate soil which had low pH (pH = 4), implying that the natural pH of acid sulphate soil is optimum for activity of acid phosphatase secreted from *Ustilago* sp. The adjustment of soil pH to greater than 4.5 may reduce the activity of acid phosphatase (Figure 1), consequently, it is not necessary to lime when using *Ustilago* sp. for enhancing organic phosphorus availability in acid sulphate soil. However, liming reduces the harmful effects of low pH and aluminum toxicity, and increases calcium and magnesium in soil.

2. Effect of Al, Fe, Mn and Ca ions on *Ustilago* sp. growth and acid phosphatase activity

The pH of medium rapidly decreased from the initial pH 4.0 to pH 2.3-3.6 after culturing. Pengnoo (2005) reported that these *Ustilago* sp. did not produce any organic acids in detectable amount, indicating that the reduction of the medium pH was not because of organic acid production but may be caused by H^+ released from the microbial cell due to the assimilation of ammonium and other cations (Paul and Clark, 1996).

Ustilago sp. AR101 and AR102 were isolated from rhizosphere and rhizoplane of rice grown in acid sulphate soil which contained highly soluble Al, Fe and Mn. However, these ions did not limit the growth of *Ustilago* sp., indicating that they can tolerate acidic condition even with Al concentration in the medium as high as 10 mg L^{-1} (Figure 3a-3f), whereas growth of *Psuedomonas* sp. and *Rhizobium trifilii* decreased when culturing in the medium containing Al higher than 1.1 mg L^{-1} (lllmer and Schinner, 1999) and 1.4 mg L^{-1} (Wood and Cooper, 1988), respectively. Because *Ustilago* sp. did not produce any organic acid, the mechanism of Al tolerance is possibly due to an internal mechanism. The Al taken up, including Fe and Mn, into the cell may be detoxified by binding proteins, tolerance enzymes, or the compartmentation of Al into the vacuole as explained by Jo *et al*. (1997). The increment of Ca ion did not affect the growth of *Ustilago* sp. (Figure 3g-3h) and *R. trifolii*. (Wood and Cooper, 1984), whereas the growth of *Bradyrhizobium* sp. SEMIA 6144 was stimulated by 204 mg L^{-1} of Ca (Maccio *et al*., 2002).

Aluminum inhibited activity of acid phosphatase secreted from AR101, but not that from AR102 (Figure 4a and 4b), implying that the two acid phosphatases are different. After addition of Al, it was found that activity was immediately lowered (Figure 6a and 6b). It is assumed that the released enzyme might form a complex with Al, resulting in the denaturation of acid phosphatase. The result seems consistent with the work of Minggang (1997) who reported that 22.0 mg L^{-1} of Al inhibited the activity of acid phosphatase secreted from lupin and tomato root.

The activity of acid phosphatase secreted from AR102 cultured in the medium containing Fe ion was remarkably high, compared to that in medium without Fe (Figure 4d), but the addition

of Fe did not affect enzyme activity (Figure 6d). These findings imply that Fe may be the component or involve in acid phosphatase synthesis. It was reported that the active form of the mammalian purple acid phosphatase contains a binuclear Fe^{3+} - Fe^{2+} metal center (Schenk *et al*., 1999).

3. Effect of Al, Fe, Mn and Ca ions on phosphate solubilization from Na-phytate by *Ustilago* sp.

The released P after 24-hour-incubation in the treatments of Al or Fe addition during incubation was lower than without Al and Fe treatment, except in the case of Fe addition for AR102, even though the activity of acid phosphates was not different (Figure 6d). Therefore, the reduction of released P was possibly caused by precipitation of the released P with Al or Fe ion as the highly insoluble Fe- or Al- phosphate. However, addition of Ca and Mn did not affect the released P from Na-phytate by *Ustilago* sp. AR101 and AR102.

4. Guideline for enhancing of organic phosphorus availability in acid sulphate soil using *Ustilago* sp.

The optimum pH for the activity of acid phosphatase secreted from *Ustilago* sp. AR101 and AR102 was 3.5-4.5 (Figure 1). Moreover, *Ustilago* sp. AR101 and AR102 can grow even if the pH dropped to 2-3 and contained high Al, Fe and Mn (Figures 2 and 3), indicating that the low pH of natural acid sulphate soil did not limit growth and activity. It was found that Al decreased activity of AR101 acid phosphatase (Figure 4a). In contrast, Fe seemed to promote the synthesis of AR102 acid phosphatase (Figure 4d). Because of precipitation of released P with Al and Fe ion, lowering these ions by liming should be done. However, care must be taken since Ca tended to reduce AR101 enzyme activity (Figure 4g). Further study of higher Ca on enzyme activity and release of P from Na-phytate, as well as Al- and Fe-phytate, should be carried out.

Conclusion

The activity of acid phosphatase secreted from AR101 was higher than that from AR102, and the optimum pH for enzyme activity was 3.5-4.5. Low pH (2-3) and high Al, Fe, Mn and Ca did not limit growth, but Al and Ca decreased activity of enzyme secreted from AR101, whereas Fe seemed to stimulate enzyme activity and synthesis of AR102. The AR101 and AR102 acid phosphatase stimulated the release of P from Na-phytate, but the released P was rapidly precipitated with Al and Fe ions as highly insoluble Fe- or Al-phosphate. Further investigation of lowering of these ions, increasing of solubilization of different kinds of organic P and application of *Ustilago* sp. AR101 and AR102 to increase soil P availability should be conducted.

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