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Phosphatase Activities of Root-nodule Bacteria and Nutritional Factors Affecting Production of Phosphatases by Representative Bacteria from Three Different Genera

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Abstract

Fifty six strains of root-nodule bacteria isolated from 3 medicinal legumes including *Indigofera tinctoria* L., *Derris elliptica* Benth. and *Pueraria mirifica* Airy Shaw & Suvat. were measured for their acid-, neutral- and alkaline phosphatase activities. All strains produced extracellular phosphatases, while almost no cell-bound phosphatase activities were observed. The unidentified strain DASA 68062 and *Bradyrhizobium* sp. DASA 64011 produced the highest activity of acid phosphatase, the unidentified strain DASA 68032 and *Bradyrhizobium* sp. DASA 68056 produced the highest activity of neutral phosphatase and the strain DASA 68056 also produced the highest activity of alkaline phosphatase which differed significantly from other strains. Effects of 14 nutritional factors on production of phosphatases by the strain DASA 57020 in *Ralstonia/Cupriavidus* group, *Bradyrhizobium* sp. DASA 68056 and *Rhizobium* sp. DASA 68066 were determined. The stimulation of extracellular acid-, neutral- and alkaline phosphatase activities of the strain DASA 57020 occurred when the strain utilized D-fructose, D-xylose and D-mannitol as sole carbon sources and urea as a sole nitrogen source. The significant increase of extracellular acid-, neutral- and alkaline phosphatase activities of the strain DASA 68056 was detected in the presence of D-fructose and D-xylose. The factors including D-fructose, sucrose and KNO_3 could increase extracellular acid-, neutral- and alkaline phosphatase activities of the strain DASA 68066. The carbon source, D-fructose, was the only factor that induced production of acid-, neutral- and alkaline phosphatases by all strains tested.

Keywords: acid phosphatase, alkaline phosphatase, neutral phosphatase, root-nodule bacteria

1. Introduction

Phosphorus (P) is the major essential macronutrient for biological growth and development [1]. Inorganic phosphate and its organic esters and anhydrides are the predominant forms of phosphorus found in biological systems. These compounds are required metabolites in all living organisms [2]. Phosphorus may exist in 2 forms: soluble and insoluble forms. Phosphorus in insoluble forms, such as tricalcium phosphate [$Ca_3(PO_4)_2$], iron phosphate ($FePO_4$) and aluminium phosphate ($AlPO_4$), was unavailable to living organisms including microorganisms and plants.

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However, some microorganisms play the important role in the P solubilization by converting insoluble forms of P to accessible forms. These specific groups of microorganisms termed as "phosphate-solubilizing microorganisms" have been reported in different genera and species. Phosphorus solubilization has been reported in bacteria such as *Agrobacterium* sp. [3], *Arthrobacter ureafaciens* [4], *Bacillus firmus* [5], *Bacillus* sp. [3-4], *Chryseobacterium* [4], *Delftia* sp. [4], *Enterobacter agglomeran* [6], *Enterobacter* sp. [7], *Flavobacterium* sp. [3], *Gordonia* sp. [4], *Klebsiella* sp. [7], *Paenibacillus* sp. [1], *Pantoea* sp. [7], *Phyllobacterium myrsina* [4], *Pseudomonas* sp. [3], *Rhizobium* sp. [3], *Rhodococcus erythropolis* [4], and *Serratia marcescens* [4]. Fungi such as *Aspergillus niger* [8], *Penicillium italicum* [8], and *Trichoderma* spp. [9] have also been reported for their activity to solubilize phosphate.

P-solubilizing activity of microorganisms was strongly associated with either the secretion of low molecular weight organic acids [4] or enzyme activity [10-13]. Phosphatase is one of enzymes that releases phosphorus from organic compounds by dephosphorylation of phospho-ester or phosphoanhydride bonds in organic matter [14]. Acid-, neutral- and alkaline phosphatases catalyze such a reaction at acid, neutral and alkaline conditions, respectively. For examples, the optimal pHs 4.8 or 5.8 have been reported for acid phosphatase and the optimal pH 9.0 has been reported for alkaline phosphatase of *Bordetella bronchiseptica* isolates [15]. The optimal pH 6.4 has been reported for neutral phosphatase of *Treponema denticola* [16].

In this study, 56 strains of root-nodule bacteria isolated from 3 medicinal legumes *Indigofera tinctoria* L. (true indigo), *Derris elliptica* Benth. (derris, tubaroot) and *Pueraria mirifica* (white Kwao Krua) were measured for their acid-, neutral- and alkaline phosphatase activities. Effects of 14 factors on production of phosphatases and growth of 3 selected strains were compared. The obtained data should be useful for the application of these nitrogen-fixing microsymbionts to increase P availability.

2. Materials and Methods

2.1 Bacteria and culture conditions

Fifty-six strains of root-nodule bacteria used in this study are listed in Table 1. Bacteria were isolated from root nodules of *I. tinctoria* L., *D. elliptica* Benth. and *P. mirifica* Airy Shaw & Suwat. grown in Thailand. Nodulation tests with their original hosts were examined previously. The strains generated different profiles in a randomly amplified polymorphic DNA (RAPD), indicating that they are individual strains [17]. These bacteria were identified by the use of 16S rRNA gene sequence analysis in the previous studies [18-20]. Yeast-Mannitol (YM) medium [21] was used for growth and maintenance, unless indicated otherwise.

2.2 Measurement of phosphatase activity

The strains were cultured in YM broth and used as inoculums. The total cell counts in inoculums were examined by the standard plate count method. Cell cultures were precipitated by centrifugation, washed, resuspended in sterile water and inoculated into Pikovskaya's broth containing 5 g/l of $Ca_3(PO_4)_2$ as a sole P source [22]. The initial cell density of each strain was 5.00×10^6 colony forming unit (CFU)/ml. After inoculation for 3-5 days depending on the growth rate of each strain, the cultures were centrifuged at 8,000 rpm for 10 min. Cell pellets were sonicated for 10 min. and resuspended in distilled water. The sonicated cell pellets were used to measure cell-bound phosphatase activity and the supernatants were used to measure extracellular phosphatase activity. Acid-, neutral- and alkaline phosphatase activities were assayed as described

Table 1 Root-nodule bacteria used in this study and their closest genera.

Host plants	The closest genera of root-nodule bacteria[a]	Strains
Indigodera tinctoria L.	*Ralstonia/Cupriavidus*	DASA 57009, DASA 57020 and DASA 57038
	Rhizobium	DASA 57010, DASA 57027, DASA 57053, DASA 57065 and DASA 57076
	Sinorhizobium	DASA 57015
	Bradyrhizobium	DASA 57019
	n.d.	DASA 57003, DASA 57004, DASA 57005, DASA 57024, DASA 57034, DASA 57050, DASA 57057, DASA 57066, DASA 57075 and DASA 57098
Pueraria mirifica Airy Shaw & Suwat.	*Rhizobium*	DASA 64006, DASA 64012, DASA 64016, DASA 64021, DASA 64023, DASA 64026, DASA 64027, DASA 64038 and DASA 64040
	Bradyrhizobium	DASA 64008, DASA 64011 and DASA 64042
	n.d.	DASA 64010, DASA 64014, DASA 64020, DASA 64022, DASA 64031 and DASA 64034
Derris elliptica Benth.	*Rhizobium*	DASA 68006, DASA 68020, DASA 68025, DASA 68053, DASA 68061, DASA 68066, DASA 68069 and DASA 68070
	Sinorhizobium	DASA 68012
	Bradyrhizobium	DASA 68056
	n.d.	DASA 68003, DASA 68010, DASA 68030, DASA 68032, DASA 68055, DASA 68058, DASA 68062 and DASA 68071

n.d.: not determined

[a]The closest genera based on 16S rRNA gene partial sequence (approx. 500 bp)

previously [23]. One ml of reaction buffer (citric acid/sodium citrate buffer, pH 5.0 for acid phosphatase estimation, sodium phosphate buffer, pH 7.0 for neutral phosphatase estimation or Tris-HCl buffer, pH 9.0 for alkaline phosphatase estimation) was mixed with 100 µl of p-nitrophenyl phosphate solution (0.6 mg/ml in diethanolamine), then 3 ml of supernatant or sonicated cell suspension was added. Reaction was incubated at 37°C for 20 min and terminated by adding 1 ml of 3M NaOH. Distilled water was used in the reaction instead of the supernatants to prepare blanks. Hydrolysis of p-nitrophenyl phosphate was estimated by measuring the concentration of p-nitrophenol with a spectrophotometer at wavelength 405 nm. The concentration of p-nitrophenol was determined by comparison with a standard curve. One unit of enzyme activity was defined as the amount of enzyme (mg) required to liberate 1 µmole of p-nitrophenol per min. [24].

2.3 Effects of factors on production of phosphatases and growth of the selected bacteria

Three strains belonging to different genus groups producing the highest phosphatase activity were selected. Effects of 14 factors on production of phosphatases and growth of the selected bacteria were determined. The tested factors included 1) monosaccharide (D-fructose and D-xylose); 2) disaccharide (maltose and sucrose); 3) sugar alcohol (D-mannitol and D-sorbitol); 4) polysaccharide (soluble starch); 5) inorganic nitrogen (NH_4Cl and KNO_3); 6) organic nitrogen (urea); 7) amino acid (L-alanine and L-lysine); and 8) phosphate compound ($AlPO_4$ and $NaPO_4$). For the carbon sources, D-fructose, D-xylose, maltose, sucrose, D-mannitol, D-sorbitol or soluble starch were separately added in Pikovskaya's broth instead of glucose with an equal amount (10 g/l). For inorganic and organic nitrogen, NH_4Cl, KNO_3 or urea was separately added in Pikovskaya's broth instead of yeast extract with an equal amount (0.5 g/l). For amino acid, L-alanine or L-lysine (1.0% w/v) was separately added into Pikovskaya's broth. For phosphate compounds, $AlPO_4$ or Na_3PO_4 was separately added in Pikovskaya's broth instead of $Ca_3(PO_4)_2$ with an equal amount (5 g/l). The inoculum was prepared as described above. The initial cell density of each strain was 5.00×10^6 colony forming unit (CFU)/ml. The cultures were grown for 3-5 days depending on the growth rate of each strain. The phosphatase activity was measured as described above. The cell numbers was measured by the standard plate count method.

2.4 Statistical Analyses

Experimental data were compared by using the SPSS program version 13.0 (SPSS Inc., Chicago, IL).

3. Results and Discussion

3. 1 Measurement of phosphatase activity

Phosphatase activities were measured in cell-free supernatants (extracellular enzyme) and disrupted cell pellets (cell-bound enzyme). Extracellular phosphatase activity was calculated as mU/ml supernatant and cell-bound phosphatase activity was calculated as mU/mg cell. Phosphatase activities of strains are shown in Figure 1-6. All strains were observed to produce extracellular phosphatases, while almost no cell-bound phosphatases were detected. The strains produced extracellular acid phosphatase ranged between 2.81 ± 0.17 to 7.86 ± 0.26 mU/ml. The extracellular neutral phosphatase ranged between 2.46 ± 0.14 to 5.88 ± 0.01 mU/ml. The extracellular alkaline phosphatase ranged between 2.96 ± 0.28 to 12.65 ± 0.13 mU/ml. The unidentified strain DASA 68062 and *Bradyrhizobium* sp. DASA 64011 produced the highest activity of acid phosphatase. The unidentified strain DASA 68032 and *Bradyrhizobium* sp. DASA 68056 produced the highest activity of neutral phosphatase. *Bradyrhizobium* sp. DASA 68056 also produced the highest activity of alkaline phosphatase which differed significantly from other strains.

3.2 Effects of factors on production of phosphatases and growth of the selected bacteria

Among 56 strains tested, 22, 5, 3 and 2 strains were identified as *Rhizobium*, *Bradyrhizobium*, *Ralstonia/Cupriavidus* and *Sinorhizobium*, respectively. These genera have been reported as predominant symbionts that nodulate and fix nitrogen symbiotically with leguminous plants. Both *Rhizobium* and *Sinorhizobium* belong to the same family Rhizobiaceae. *Bradyrhizobium* is a genus of the family Bradyrhizobiaceae. *Ralstonia* and *Cupriavidus* belong to the family

Burkholderiaceae. One representative strain of each genus, *Rhizobium*, *Bradyrhizobium* and *Ralstonia/Cupriavidus*, were selected to examine effects of factors on production of phosphatases so that results could be compared among these distinct genera. The strains DASA 57020 in *Ralstonia/Cupriavidus* group, *Bradyrhizobium* sp. DASA 68056 and *Rhizobiuum* sp. 68066 were selected based on their highest activity compared to other strains of the same genus. Effects of 14 factors including D-fructose, D-xylose, maltose, sucrose, D-mannitol, D-sorbitol, soluble starch, NH_4Cl, KNO_3, urea, L-alanine, L-lysine, $AlPO_4$ and Na_3PO_4, on production of phosphatases and growth of the selected bacteria were studied. The growths of the strains in the presence of each factor are compared in Figure 7. Phosphatase activities of the strains DASA 57020, DASA 68056 and DASA 68066 in the presence of each factor are compared in Figure 8, 9 and 10, respectively. The results present that some factors stimulated production of phosphatases especially extracellular activities while almost no cell-bound phosphatase activities were observed in all treatments. However, production of phosphatases and growth of bacteria were not directly correlated. The stimulation of extracellular acid-, neutral- and alkaline phosphatase activities of the strain DASA 57020 was observed in medium containing D-fructose, D-xylose and D-mannitol as sole carbon sources and urea as a sole nitrogen source. The increasing of alkaline phosphatase activity up to 4.49 fold over control was found with the strain DASA 57020 grown in the presence of D-xylose. The significant increase of extracellular acid-, neutral- and alkaline phosphatase activities of the strain DASA 68056 was detected in the presence of D-fructose and D-xylose. The increase acid phosphatase acitivity of the strain DASA 68056 up to 4.01 fold over control was found with D-xylose. The factors including D-fructose, sucrose and KNO_3 could increase extracellular acid-, neutral- and alkaline phosphatase activities of the strain DASA 68066. D-xylose was found to stimulate the highest enhancement of neutral phosphatase from the strain DASA 68066 up to 4.13 fold over control. Among 14 factors tested, D-fructose was the only factor that could induce the production of acid-, neutral- and alkaline phosphatases of all strains tested. However, the most enhancement in all strains tested was found with D-xylose on different kinds of phosphatases depending on the strains. The influence of some environmental factors on production of phosphatase has been studied. In *Bacillus subtilis*, alkaline phosphatase was synthesized in media at a level of phosphate concentration lower than 0.37 mM and it was repressed at a level of phosphate concentration 3.7 mM. The enzyme activity can be increased by adding Zn^{2+} ion [25]. The influence of different carbon sources on the expression of alkaline phosphatase of *Vibrio vulnificus* has been reported. Their result is consistent with our study, the alkaline phosphatase activity of *V. vulnificus* was derepressed most profoundly in medium containing fructose as a sole carbon source. It was also found that the highest levels of the enzyme were observed in medium containing fructose and maltose as sole carbon sources. The difference in derepression and patterns of alkaline phosphatase expression might be suggestive of the existence of multiple phosphatases under different regulation in the bacteria and an additional regulatory system which performs a function in enzyme expression separate from the phosphate system [26]. The influence of carbon and nitrogen sources on alkaline phosphatase synthesis of *Streptomyces fradiae* has been reported in the previous study. Arabinose induced alkaline phosphatase synthesis, whereas glucose inhibited the synthesis of the enzyme. Asparagine was a very good inducer, with lysine and alanine having lower stimulatory effects. Glucosamine, NH_4NO_3 and NH_4Cl were to some extent inhibitory to the synthesis of the enzyme. The appearance of the enzyme was found to be due to protein synthesis [27]. Among bacterial species, the type of phosphatases made was different. Different phosphatases have efficiency for different substrates. Several phosphatases play a role on carbohydrate metabolisms. For instances, fructose-1, 6-diphosphatase catalyzes the dephosphorylation of fructose-1,6-diphosphate to yield fructose-6-phosphate and inorganic phosphate. The conversion of fructose-1,6-diphosphate to fructose-6-phosphate (fructose-1,6-diphosphatase activity) is essential for growth of *Escherichia coli* on glycerol, acetate, or succinate, but is unnecessary for growth on hexoses or pentoses [28].

Fructose-1,6-bisphosphatase and glucose-6-phosphatase involved in gluconeogenesis pathway that result in the generation of glucose from non-carbohydrate carbon substrates and glucogenic amino acids such as alanine [29]. In this study, D-fructose induced the synthesis of acid-, neutral- and alkaline phosphatases in all strains tested, suggesting the presence of phosphatases involved in fructose metabolisms. While some factors had different effects on different bacterial strains. D-mannitol induced the synthesis of acid-, neutral- and alkaline phosphatases in the strain DASA 57020 of *Ralstonia/Cupriavidus* group, but not in *Bradyrhizobium* sp. DASA 68056. The variation in effect of these nutritional factors on different strains is possibly due to particular metabolisms unique to each genus or species.

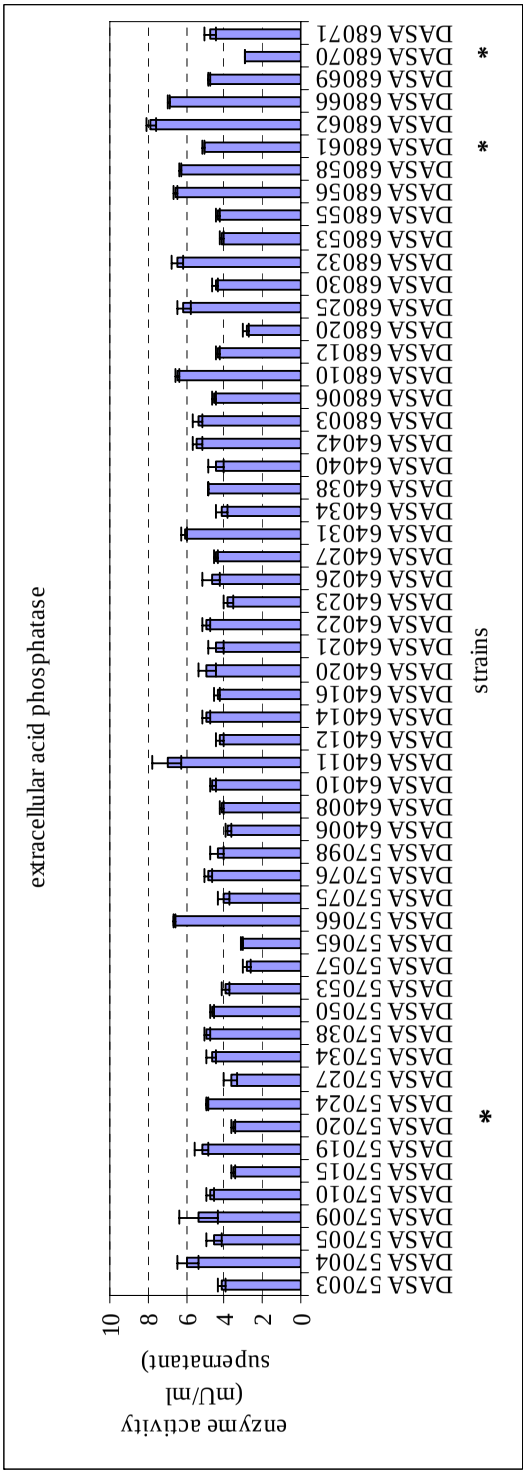

Figure 1 Extracellular acid phosphatase activity of root-nodule bacteria in Pikovskaya's broth.The values shown are the mean values of triplicates. Error bars indicate ± standard deviations. Asterisks indicate strains that were selected for further study: DASA 57020 in *Ralstonia/Cupriavidus* group, *Bradyrhizobium* sp. DASA 68056 and *Rhizobium* sp. DASA 68066.

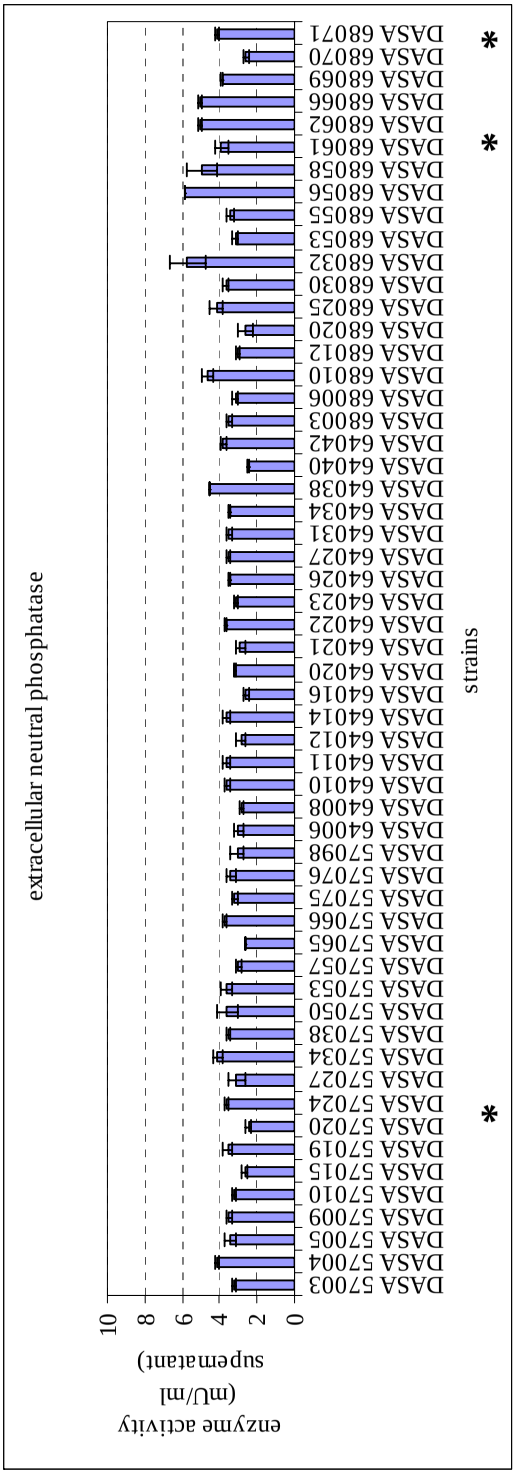

Figure 2 Extracellular neutral phosphatase activity of root-nodule bacteria in Pikovskaya's broth. The values shown are the mean values of triplicates. Error bars indicate ± standard deviations. Asterisks indicate strains that were selected for further study: DASA 57020 in *Ralstonia/Cupriavidus* group, *Bradyrhizobium* sp. DASA 68056 and *Rhizobium* sp. DASA 68066.

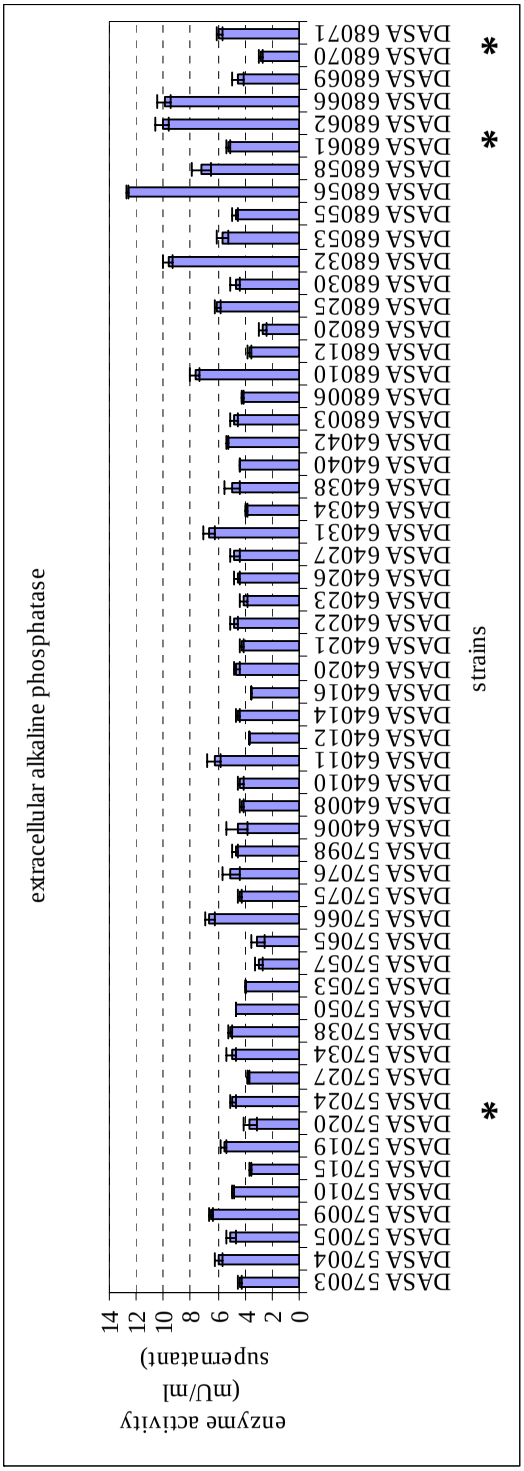

Figure 3 Extracellular alkaline phosphatase activity of root-nodule bacteria in Pikovskaya's broth. The values shown are the mean values of triplicates. Error bars indicate ± standard deviations. Asterisks indicate strains that were selected for further study: DASA 57020 in *Ralstonia*/*Cupriavidus* group, *Bradyrhizobium* sp. DASA 68056 and *Rhizobium* sp. DASA 68066.

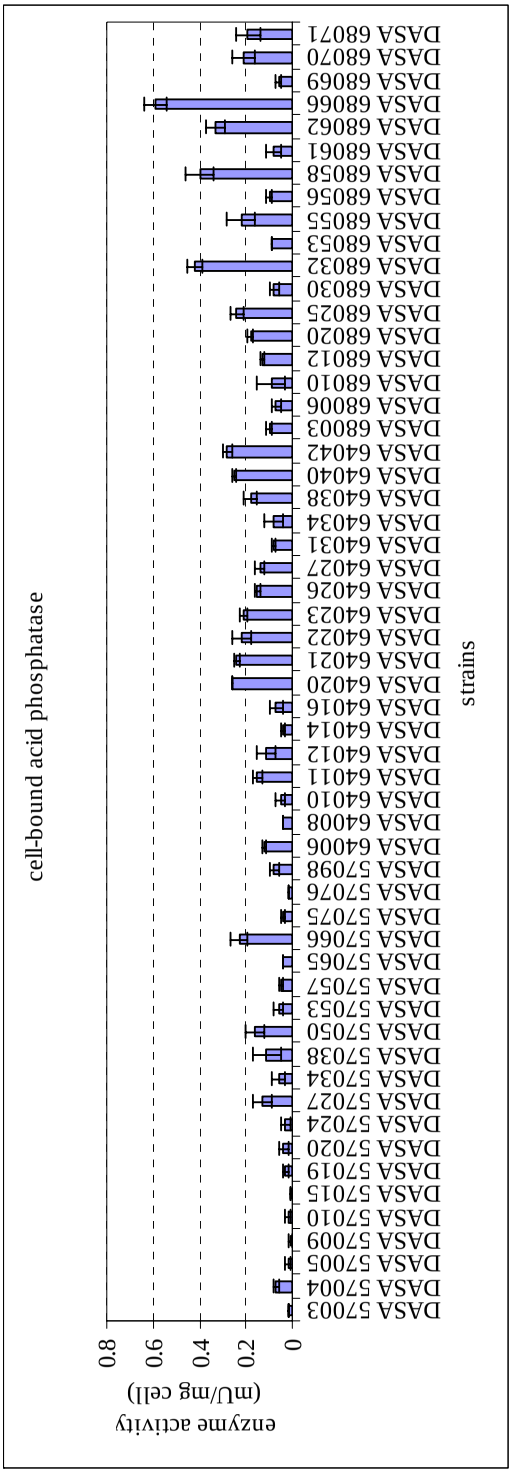

Figure 4 Cell-bound acid phosphatase activity of root-nodule bacteria in Pikovskaya's broth. The values shown are the mean values of triplicates. Error bars indicate ± standard deviations.

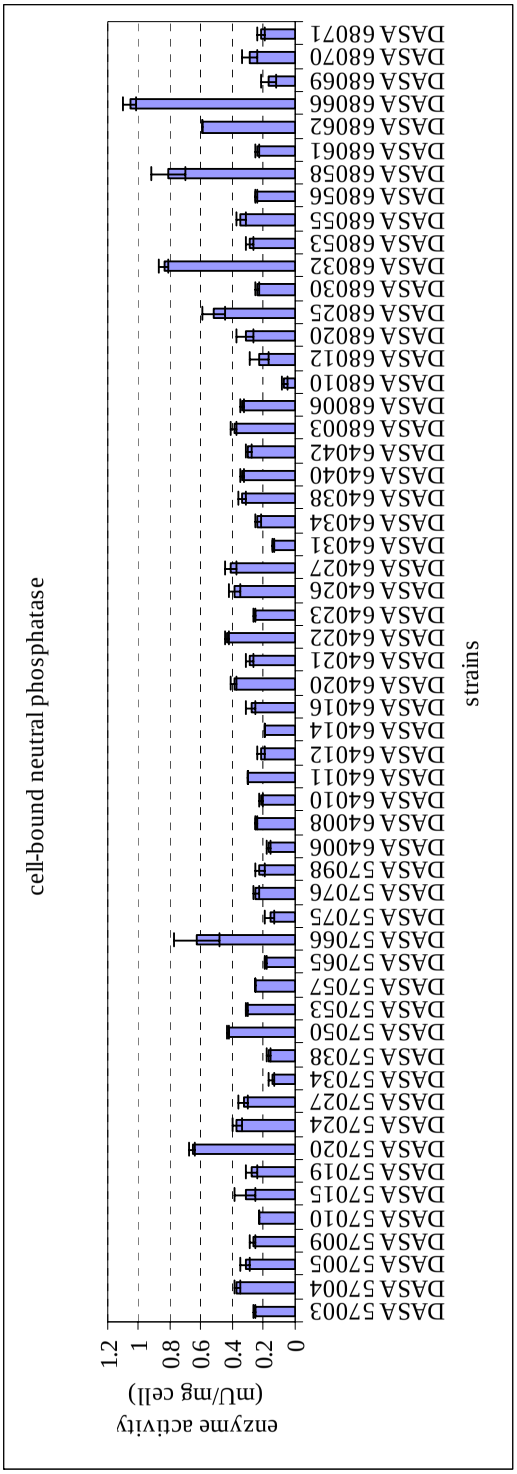

Figure 5 Cell-bound neutral phosphatase activity of root-nodule bacteria in Pikovskaya's broth. The values shown are the mean values of triplicates. Error bars indicate ± standard deviations.

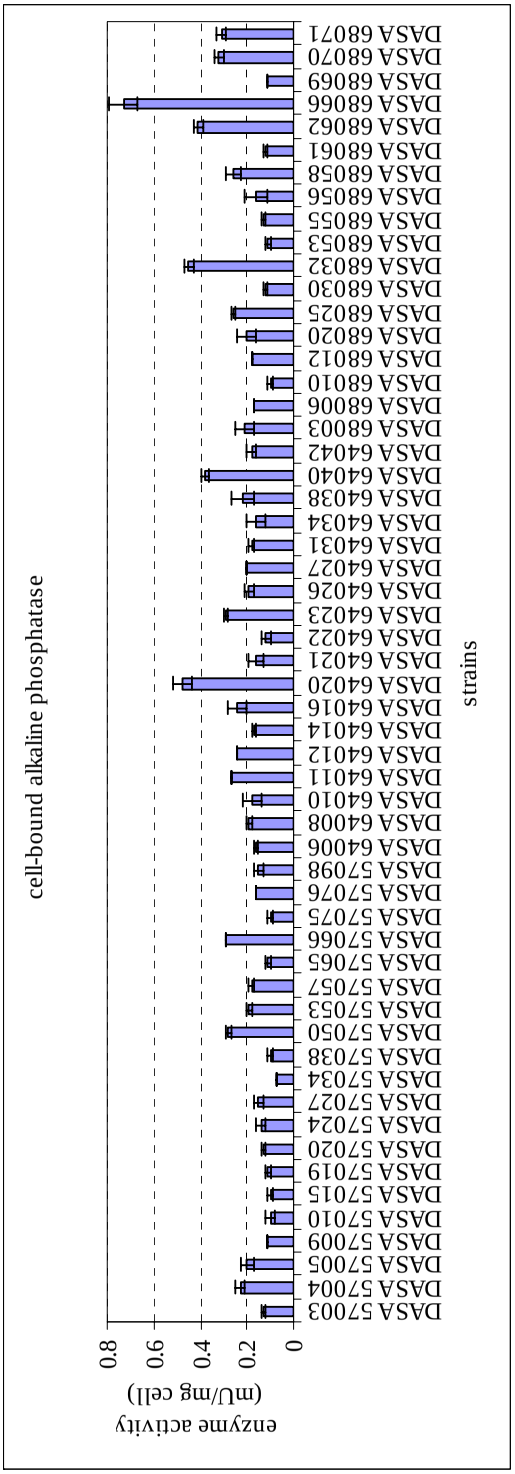

Figure 6 Cell-bound alkaline phosphatase activity of root-nodule bacteria in Pikovskaya's broth. The values shown are the mean values of triplicates. Error bars indicate ± standard deviations.

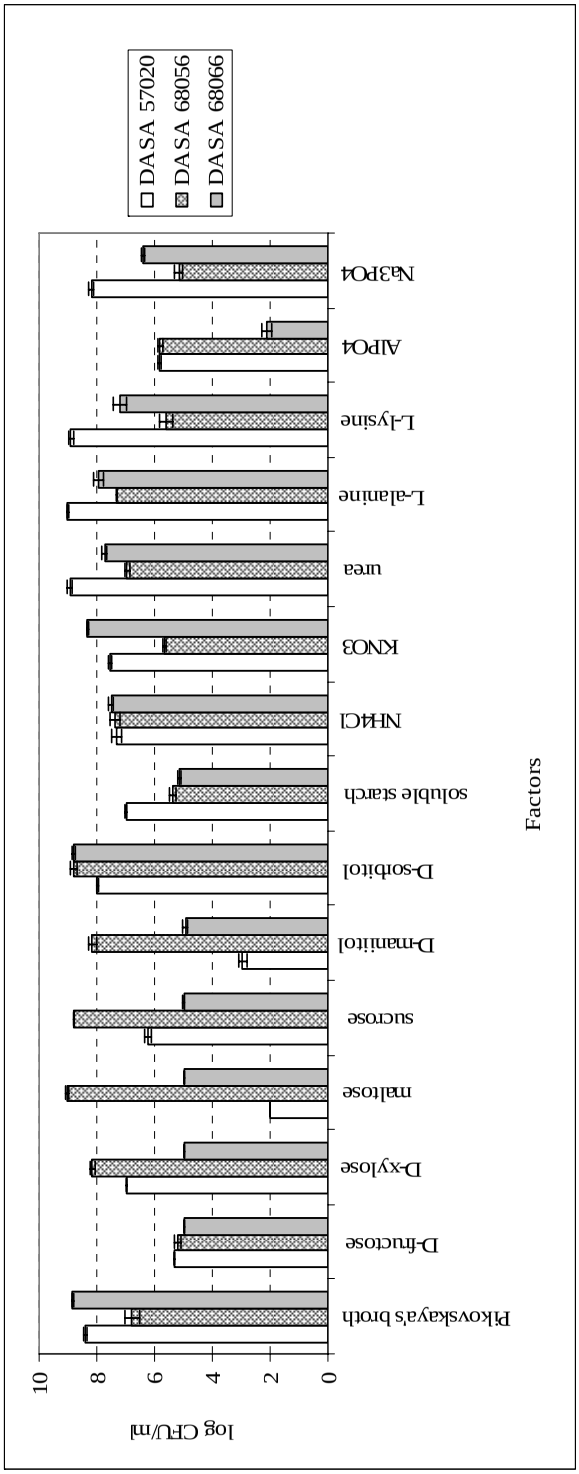

Figure 7 Growths of the selected strains in the presence of each factor. The values shown are the mean values of triplicates. Error bars indicate ± standard deviations.

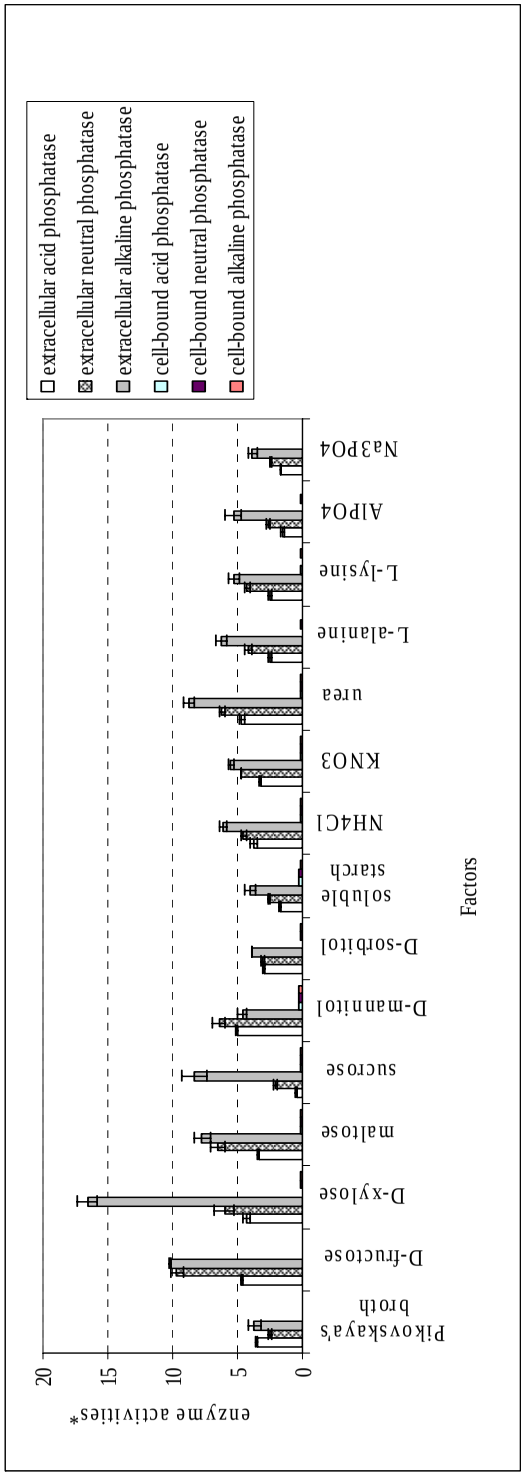

Figure 8 Phosphatase activties of *Cupriavidus* sp. DASA 57020 in the presence of each factor. The values shown are the mean values of triplicates. Error bars indicate ± standard deviations.

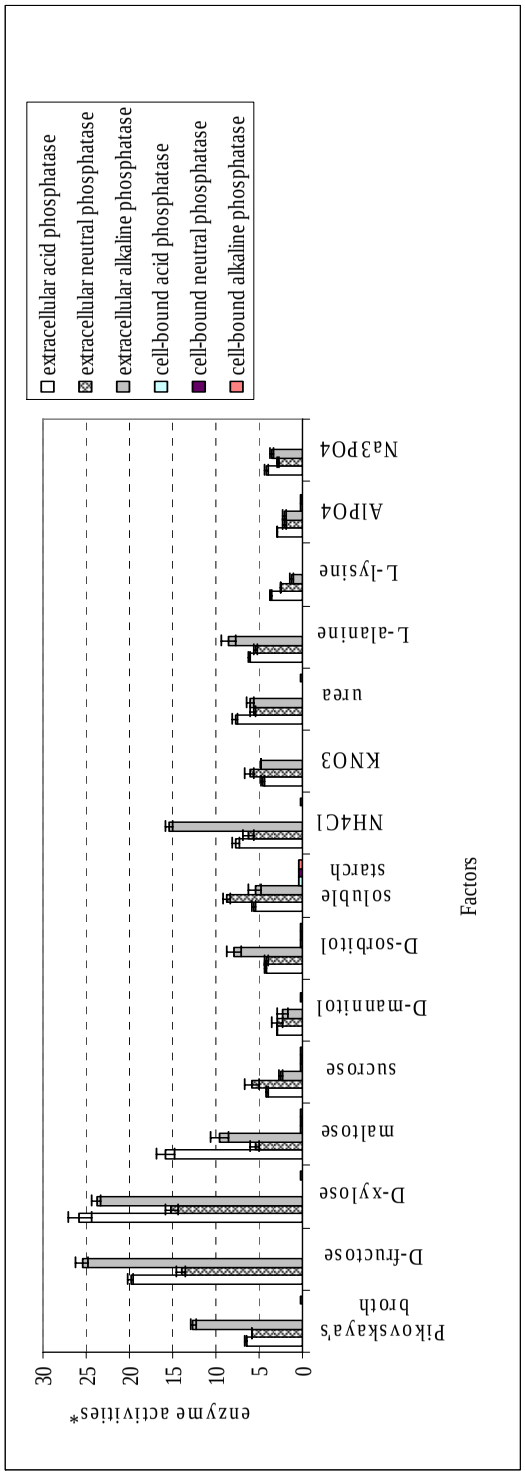

Figure 9 Phosphatase activities of *Bradyrhizobium* sp. DASA 68056 in the presence of each factor. The values shown are the mean values of triplicates. Error bars indicate ± standard deviations.

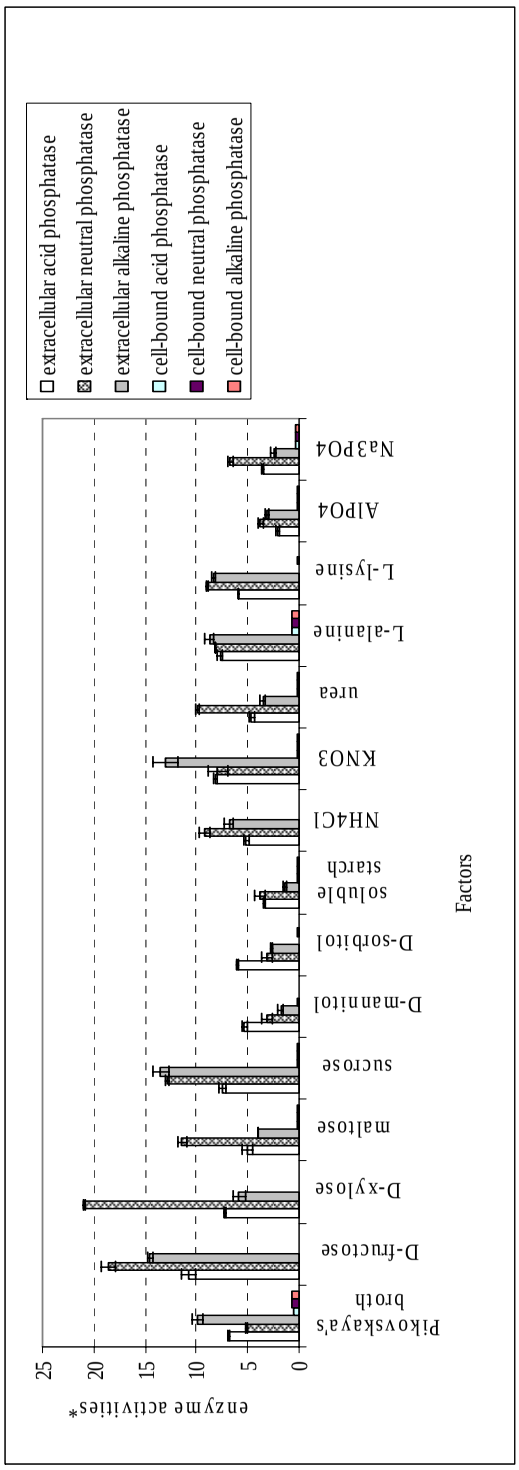

Figure 10 Phosphatase activties of *Rhizobium* sp. DASA 68066 in the presence of each factor. The values shown are the mean values of triplicates. Error bars indicate ± standard deviations.

4. Conclusions

Present study demonstrated production of acid-, neutral- and alkaline phosphatases in medium containing $Ca_3(PO_4)_2$ as a sole P source by 56 strains of root-nodule bacteria. Three strains in different genera utilized 14 nutritional factors differently, resulting in different total cell number. Some nutritional factors including D-fructose, D-xylose, maltose, NH_4Cl and urea could induce production of phosphatases by these bacteria. As these bacteria can fix nitrogen symbiotically with their host legumes, the obtained data should make useful in application of these strains to provide both N and P sources in agricultural system.

5. Acknowledgements

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