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Environmental Parameters Affecting Urease Production and Ammonification in *Phaseolus vulgaris*-Nodulating Rhizobia and *Vigna radiata*-Nodulating Rhizobia

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Abstract: Twenty three isolates of symbiotic nitrogen-fixing bacteria that nodulate common bean (*Phaseolus vulgaris*) or mungbean (*Vigna radiata*) were examined for their urease activity and ammonification. All isolates were observed to produce extracellular urease that ranged between 5.458 ± 1.021 to 28.326 ± 5.618 unit/ml supernatant. The ammonia produced from ammonification of peptone ranged between 70 ± 15.564 to 266.354 ± 23.833 µM. A different trend between urease production of common bean-nodulating rhizobia and mungbean-nodulating rhizobia was observed. In the case of 2 isolates of common bean-nodulating rhizobia, hydrolysis of urea slightly increased during 2 days of culture and the most increase was found on the third day. After the third day, urease activity was markedly decreased. Maximum production of urease was detected during the mid-log to late-log phases of growth. The production of urease by 2 isolates of mungbean-nodulating rhizobia was not significantly different during 2 days of culture, then it was continuously increased until the last day of cultivation in which cells were in their stationary phases. Similar profiles were observed from ammonification of 3 isolates of common bean-nodulating rhizobia and 3 isolates of mungbean-nodulating rhizobia. Ammonification, as measured by the amount of ammonia produced, increased along with growth until cells reached the stationary phases. Effects of factors on production of urease and ammonifcation of the selected isolates were studied. Effects of these factors varied in different isolates. In summary, Ni^{2+}, Co^{2+} and $NaNO_3$ decreased urease production in common bean-nodulating rhizobia. While, yeast extract and NH_4Cl decreased urease production in mungbean-nodulating rhizobia. Two heavy metals, Co^{2+} and Zn^{2+}, had negative effects on ammonification in almost all isolates tested.

Key words: Ammonification · Rhizobia · urease

INTRODUCTION

The specific groups of bacteria, collectively known as rhizobia, induce the formation of root or stem nodules of leguminous plants and establish a nitrogen-fixing symbiosis [1, 2]. Nitrogen fixation that is catalyzed by nitrogenase enzyme, is a reduction of nitrogen gas into ammonia [3]. This process can provide a source of assimilable nitrogen for bacteria and plants. Besides nitrogen fixation, ammonia can be obtained from the other metabolic processes such as urease production and ammonification. Urease is a nickel-containing, multi subunit enzyme that catalyzes the hydrolysis of urea to ammonia and carbonic acid [4]. Urease expression in bacteria is often regulated in response to environmental parameters such as nitrogen availability and pH [5]. Bacteria perform ammonification with the production of ammonia from nitrogen compounds such as nitrate [6], casamino acids and trypticase peptone [7].

Several factors were found to affect urease production in bacteria. Addition of nickel to a nickel-free culture led to recovery of urease activity of the cyanobacterium *Anabaena cylindrica* [8]. Supplementation of nickel in medium could result in up to 3.5-fold-increased expression of the urease subunit proteins UreA and UreB of *Helicobacter pylori* [9]. Acetohydroxamic acid (100 µg/ml), hydroxyurea (85 µg/ml), flurofamide (0.05 µg/ml) and EDTA (8 mM) inhibit enzyme activity of *Campylobacter pylori* by 50% [10]. Temperature and urea were found to affect urease gene expression in *Proteus mirabilis* [11]. The effects of some factors on ammonification by bacteria have been investigated; trichloroethylene (TCE) concentration has no effect on arginine ammonification potential of soil microbial populations [12]. Also, it was found that the effects of aldrin on ammonification of peptone are minor and irregular [13].

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Table 1: Urease activity and ammonification of rhizobia used in this study

Host plants	Rhizobial isolates	Urease activity: Extracellular urease (U/ml supernatant)	Urease activity: Intracellular urease (U/mg cell)	Amount of ammonia (μM) produced from ammonification
Phaseolus vulgaris (common bean)	PV001	10.634±1.574	0.365±0.093	119.219±23.202
	PV002	14.869±2.367	0.544±0.006	139.688±27.194
	PV006	12.799±0.454	0.996±0.034	147.083±32.991
	PV008	26.327±0.100	0.775±0.063	169.688±72.138
	PV009	17.504±4.908	0.554±0.070	187.396±31.961
	PV010	8.846±0.918	0.302±0.046	161.146±6.5120
	PV011	19.762±1.835	0.224±0.036	82.083±10.723
	PV012	19.763±1.956	0.291±0.036	113.229±16.514
	PV013	28.326±5.618	0.376±0.206	162.084±56.047
	PV014	27.949±2.834	0.426±0.029	70.104±15.564
	PV015	11.528±6.057	0.255±0.017	115.105±26.475
	PV016	22.515±3.693	0.412±0.077	187.500±65.188
	PV017	21.221±1.200	0.598±0.102	163.230±23.396
	PV018	23.809±1.765	0.567±0.055	113.229±80.931
	DASA 06008	12.846±1.230	0.440±0.036	99.167±63.987
	DASA 06006	14.822±1.474	0.529±0.017	173.959±22.418
Vigna radiata (mungbean)	DASA 02007	6.775±2.473	0.351±0.042	106.979±35.842
	DASA 02008	7.482±1.156	0.468±0.083	199.167±36.406
	DASA 02009	11.010±1.717	0.481±0.205	252.709±29.359
	DASA 02010	17.269±5.364	0.357±0.087	223.750±64.939
	DASA 02011	5.458±1.021	0.199±0.009	218.230±21.940
	DASA 02068	12.704±2.136	0.617±0.021	233.750±59.722
	DASA 02077	6.214±1.114	0.349±0.022	266.354±23.833

The values shown are the mean values of triplicates±standard deviations

In this study, we investigated urease production and ammonification by symbiotic nitrogen-fixing bacteria that nodulate common bean (*Phaseolus vulgaris*) or mungbean (*Vigna radiata*). Sixteen isolates of common bean-nodulating rhizobia and 7 isolates of mungbean-nodulating rhizobia were screened for their urease activity and ammonification.

MATERIALS AND METHODS

Rhizobia and Culture Conditions: The 23 rhizobial isolates used in this study and their host plants are listed in Table 1. Nodulation tests and random amplified polymorphic DNA (RAPD) analysis of mungbean-nodulating rhizobia were carried out in the previous study [14]. These strains nodulated mungbean and generated particular RAPD profiles, suggesting that they were individual strains. Nodulation tests of common bean-nodulating rhizobia were carried out in this study. Yeast-Mannitol (YM) medium [15] was used for growth and maintenance unless otherwise indicated.

Measurement of Urease Activity: Bacteria were grown in Christensen's urea broth [16]. Bacterial cultures were incubated at 28°C with shaking at 150 rpm for 3-5 days until cultures reached the mid-log phases. Then the cultures were centrifuged at 10,000 rpm for 10 min. Cell pellets were sonicated for 10 min and resuspended in distilled water. The sonicated cell pellets were used to measure intracellular and/or surfaced-associated urease activity and the supernatants were used to measure extracellular urease activity. Urease activity was measured as described by Askin and Kizilkaya [17] with some modifications. A 3.0 ml of citrate buffer (pH 6.7) and 5.0 ml of 10% urea substrate solution were added to 1.0 ml of supernatant or sonicated cell suspension and the sample was then incubated for 3 h at 37°C. Then 5.0 ml of sodium phenolate (12.5% (w/v) phenol + 5.4% (w/v) NaOH) and 1.0 ml of 0.9% sodium hypochloride were added to the sample. The absorbance at 578 nm of the reaction mixture was measured. Controls without addition of urea were also included. Ammonia concentrations without and with addition of urea were used to determine the released ammonia resulting from urease activity.

The concentration of ammonia was determined by comparison with a standard curve. A standard curve was constructed by preparing reaction mixtures in which ammonium chloride at varied concentration (equivalent to 0-12 mg/ml ammonia) was used in instead of the supernatant or sonicated cell suspension. One unit of urease activity is equal to the amount of urease that hydrolyzes 0.5 μmol of urea and liberates 1.0 μmol of ammonia in 1 min at 37°C [18]. Based on the maximum activity, 2 isolates specific to each host plant were selected for further studies.

Measurement of Ammonification: Bacteria were propagated in peptone water and incubated at 28°C with shaking at 150 rpm for 3-5 days until cultures reached the mid-log phases. A 0.1 ml of Nessler's reagent was added into 2 ml of the culture [19]. The absorbance at 395 nm was immediately measured [20]. Ammonium chloride in the range of 0-500 μM was used in the reaction to prepare a standard curve. The concentration of ammonia produced was determined by comparison with a standard curve. Based on the maximum amount of ammonia produced, 3 isolates specific to each host plant were selected for further studies.

Urease Production and Ammonification During Growth of the Selected Rhizobia: To examine urease production and ammonification during growth of the selected rhizobia, cells were grown in YM broth at 28°C with shaking at 150 rpm for 3-5 days and used as inocula. The inocula were added into Christensen's urea broth (for measurement of urease activity) and peptone water (for measurement of ammonification) to obtain the initial cell number 1.00 x 10^5 Colony Forming Unit (CFU)/ml. Cultures were grown at 28°C with shaking at 150 rpm for 5 days. At a 1-day interval, the cell numbers in media were measured by the standard plate count method on YM agar containing 25 μg/ml congo red, urease acitivity and ammonification were determined as described above.

Effects of Environmental Parameters on Urease Production, Ammonification and Growth of the Selected Rhizobia: Factors tested for urease production were inoculated into Christensen's urea broth including yeast extract 1 g/l; peptone 5 g/l; NH_4Cl 10 mM; $NaNO_3$ 10 mM; $NiSO_4$ 0.1 mM and $CoCl_2$ 0.1 mM. Factors tested for ammonification were added to peptone water including $CuSO_4$ 0.2 mM; $CuSO_4$ 2.0 mM; $ZnSO_4$ 0.2 mM and $ZnSO_4$ 2.0 mM. The inocula were prepared and the cultures were grown as described above. The cell numbers, urease activity and ammonification were measured on the day that each isolate had maximum urease activity or ammonification.

Statistical Analysis: The standard deviation was calculated using Microsoft Excel 2003 software.

RESULTS AND DISCUSSION

Measurement of Urease Activity: Urease activity was measured in cell-free supernatants (for extracellular urease) and disrupted cell pellets (for intracellular and/or surfaced-associated urease) (Table 1). Among the 23 rhizobia tested, all of them produced extracellular but almost no intracellular urease. Extracellular urease activity ranged between 5.458 ± 1.021 to 28.326 ± 5.618 U/ml supernatant, while intracellular urease activity ranged between 0.199 ± 0.009 to 0.996 ± 0.034 U/mg cell. Some other bacteria such as *Lactobacillus* sp. and *Helicobacter pylori* were found to produce extracellular urease [21, 22]. Five isolates of common bean-nodulating rhizobia (PV008, PV013, PV014, PV016 and PV018) produced the highest urease activity, which was not significantly different. Two isolates specific to each host plant were selected based on the maximum unit of extracellular urease/ml supernatant. Common bean-nodulating rhizobia (PV013 and PV014) as well as mungbean-nodulating rhizobia (DASA 02010 and DASA 02068) were selected for further studies.

Measurement of Ammonification: Ammonification was measured from the amount of ammonia produced from peptone. The results are shown in Table 1. The ammonia produced from rhizobial isolates ranged between 70 ± 15.564 to 266.354 ± 23.833 μM. One isolate of common bean-nodulating rhizobia (PV016) and 4 isolates of mungbean-nodulating rhizobia (DASA 02009, DASA 02010, DASA 02068 and DASA 02077) produced the highest amount of ammonia, which was not significantly different. Common bean-nodulating rhizobia (PV009, PV016 and DASA 06006) as well as mungbean-nodulating rhizobia (DASA 02009, DASA 02068 and DASA 02077) were selected for further studies.

The values shown are the mean values of 3 replicates. Error bars indicate standard deviations

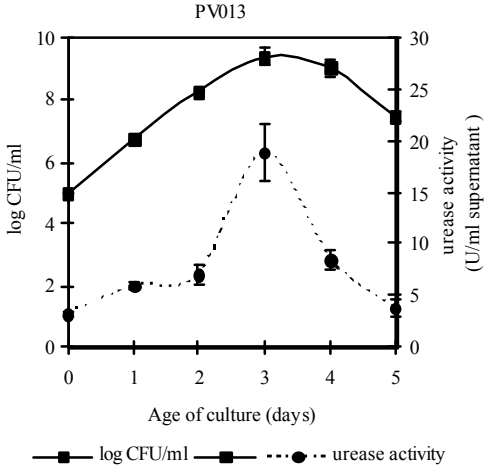

Fig. 1: Growth curve and urease production of the isolate PV013

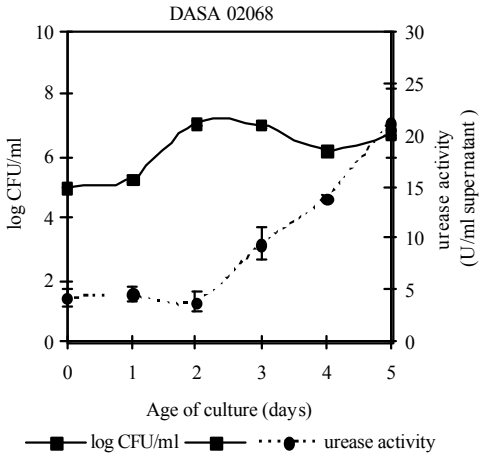

Fig. 4: Growth curve and urease production of the isolate DASA 02068

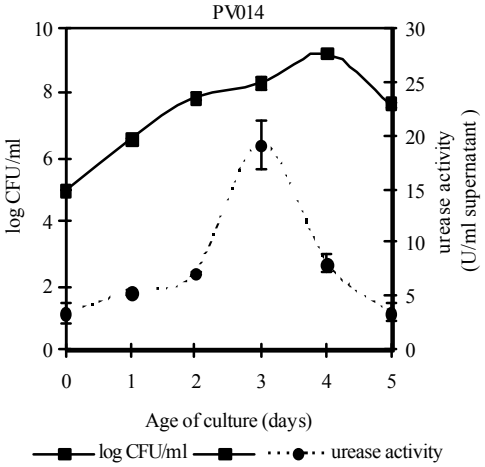

Fig. 2: Growth curve and urease production of the isolate PV014

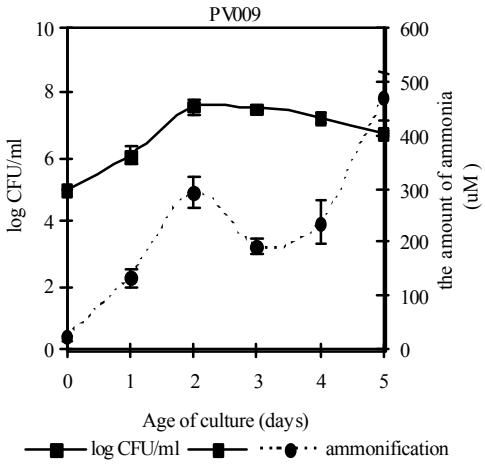

Fig. 5: Growth curve and ammonification of the isolate PV009

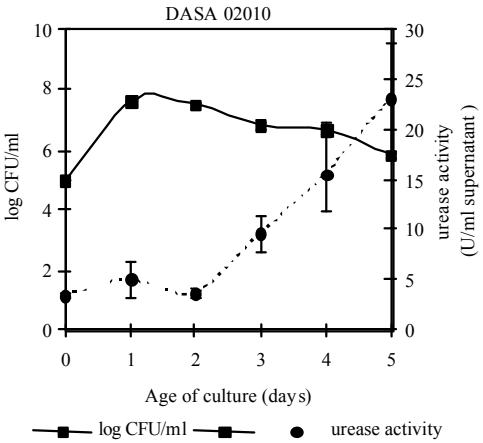

Fig. 3: Growth curve and urease production of the isolate DASA 02010

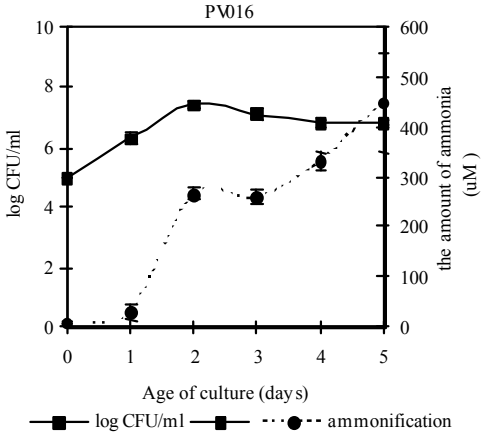

Fig. 6: Growth curve and ammonification of the isolate PV016

The values shown are the mean values of 3 replicates. Error bars indicate standard deviations

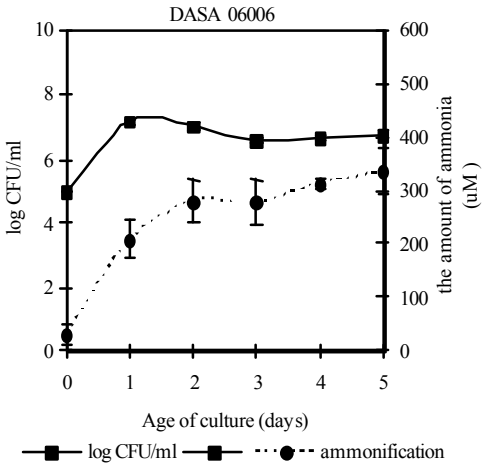

Fig. 7: Growth curve and ammonification of the isolate DASA 06006

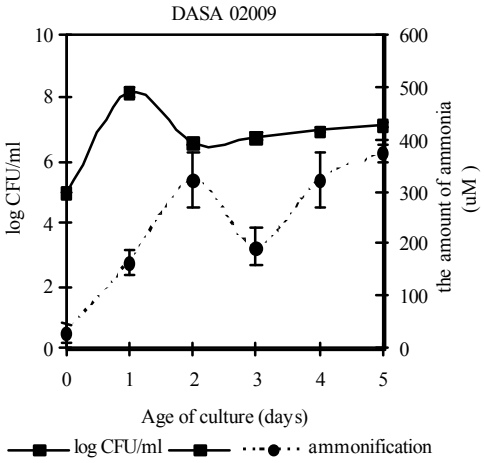

Fig. 8: Growth curve and ammonification of the isolate DASA 02009

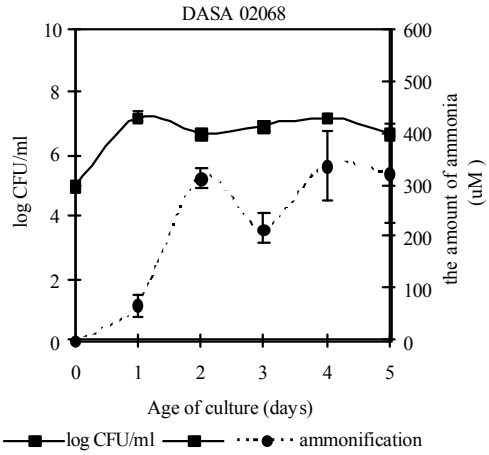

Fig. 9: Growth curve and ammonification of the isolate DASA 02068

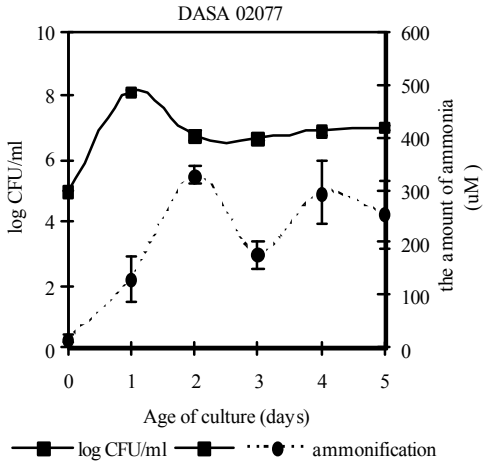

Fig. 10: Growth curve and ammonification of the isolate DASA 02077

Urease Production and Ammonification During Growth of the Selected Rhizobia: As only extracellular urease was detected in all rhizobia tested, it was measured during growth of the selected rhizobia (Figure 1-4). In case of common bean-nodulating rhizobia (PV013 and PV014), hydrolysis of urea slightly increased during 2 days of culture and the maximum increase was found on the third day. After the third day, urease activity decreased markedly. Maximum production of urease was detected during the mid-log to late-log phases of growth. The production of urease by mungbean-nodulating rhizobia was not significantly different, during the first 2 days of culture, (then it continuously increased until the last day of cultivation in which cells were in their stationary phases). In the previous study, a parallel difference between the two strains with regard to the onset of ureolysis was observed, hydrolysis beginning at the onset of growth in the case of *Actinobacillus* and only at the end of the exponential growth phase in the case of *Lactobacillus* [21]. Figure 5-10 present the growth curves and ammonification of the isolates PV009, PV016, DASA 06006, DASA 02009, DASA 02068 and DASA 02077, respectively. Similar profiles were observed from ammonification of common bean-nodulating rhizobia and mungbean-nodulating rhizobia. The amount of ammonia increased along with growth until cells reached the stationary phases, after that it still maintained or decreased during the stationary phases, then increased again at the end of the stationary phases.

The values shown are the mean values of 3 replicates. Error bars indicate standard deviations

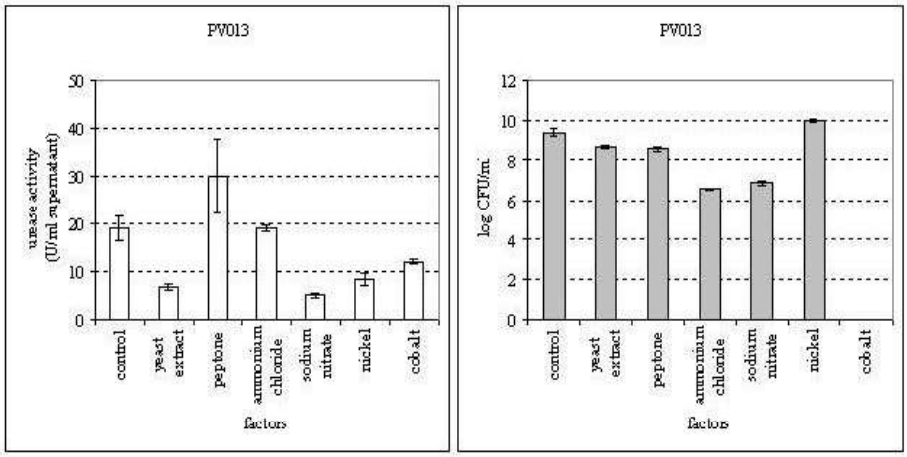

Fig. 11: The influence of environmental factors on urease activity and growth of the isolate PV013

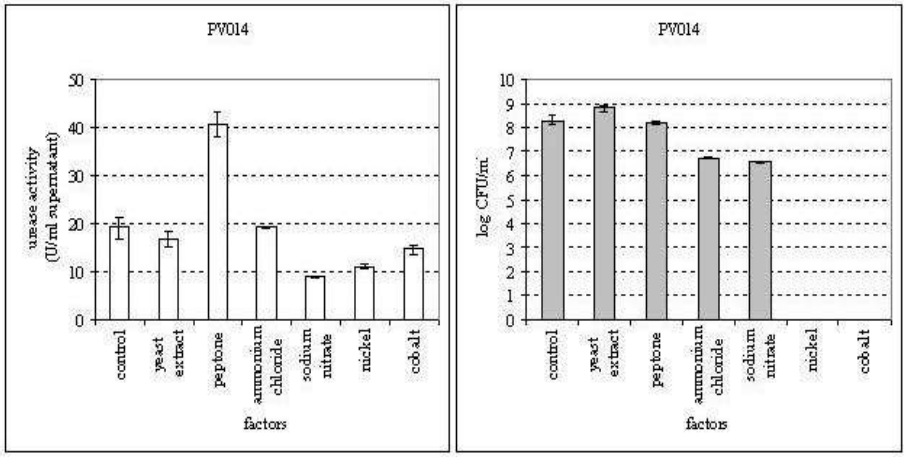

Fig. 12: The influence of environmental factors on urease activity and growth of the isolate PV014

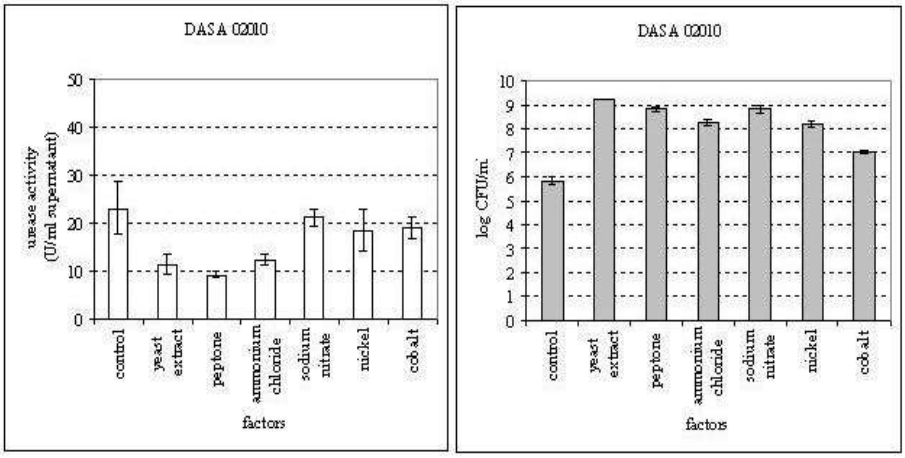

Fig. 13: The influence of environmental factors on urease activity and growth of the isolate DASA 02010

The values shown are the mean values of 3 replicates. Error bars indicate standard deviations

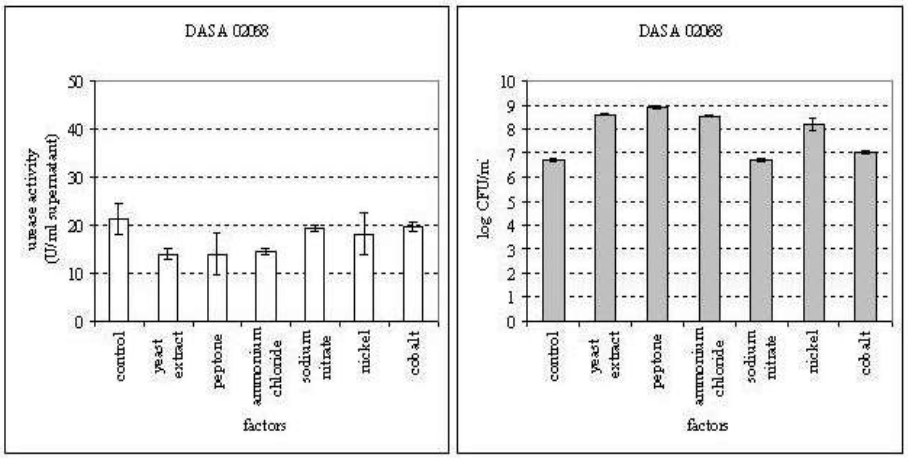

Fig. 14: The influence of environmental factors on urease activity and growth of the isolate DASA 02068

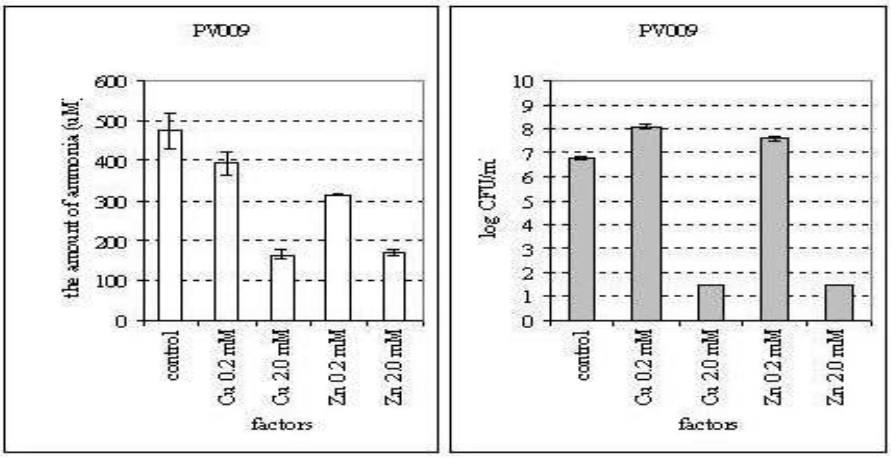

Fig. 15: The influence of environmental factors on ammonification and growth of the isolate PV009

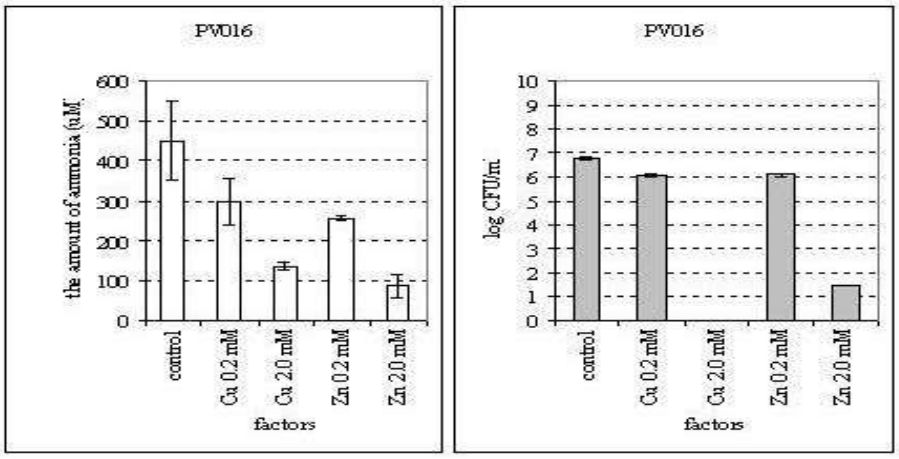

Fig. 16: The influence of environmental factors on ammonification and growth of the isolate PV016

The values shown are the mean values of 3 replicates. Error bars indicate standard deviations

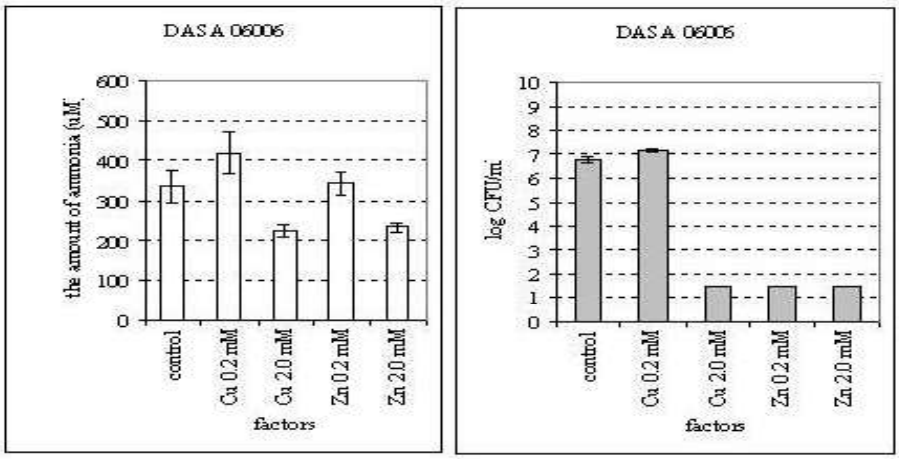

Fig. 17: The influence of environmental factors on ammonification and growth of the isolate DASA 06006

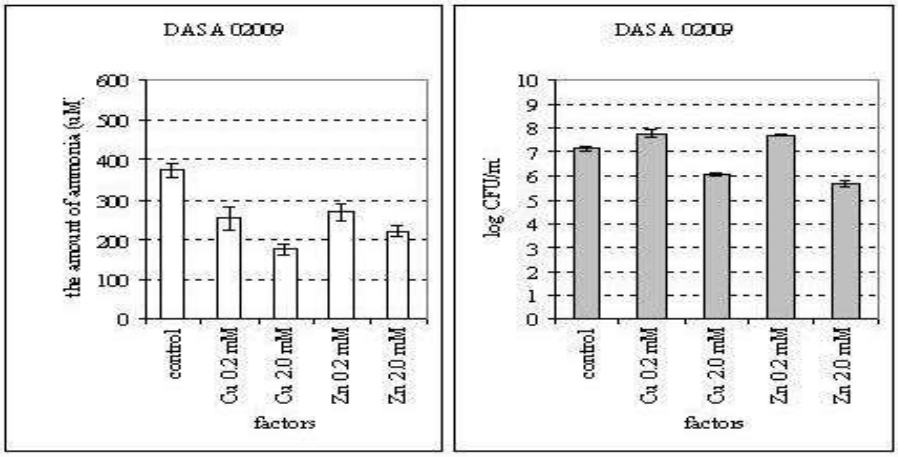

Fig. 18: The influence of environmental factors on ammonification and growth of the isolate DASA 02009.

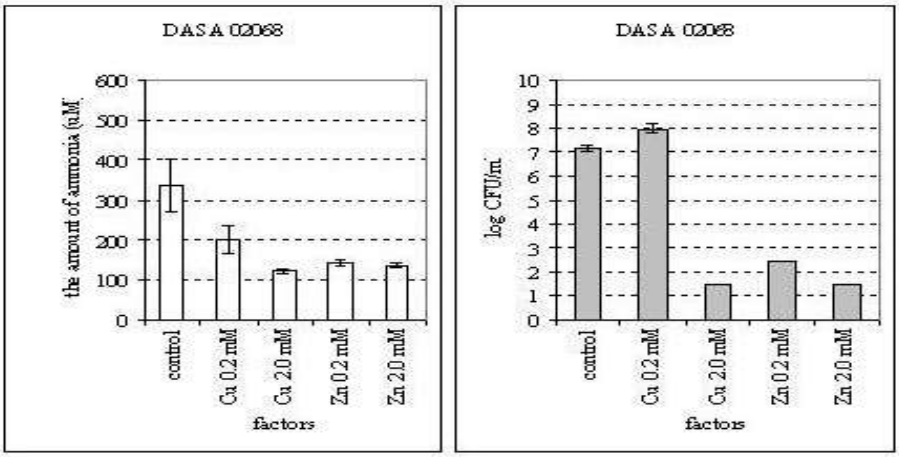

Fig. 19: The influence of environmental factors on ammonification and growth of the isolate DASA 02068

The values shown are the mean values of 3 replicates. Error bars indicate standard deviations

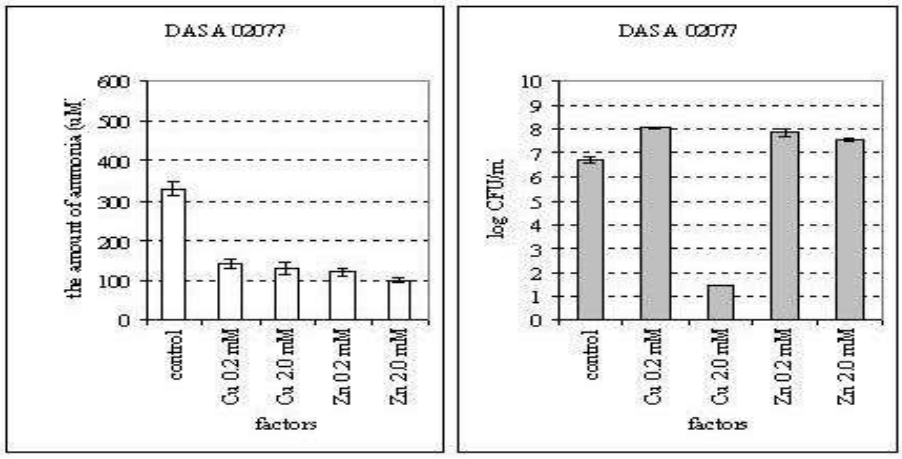

Fig. 20: The influence of environmental factors on ammonification and growth of the isolate DASA 02077

Effects of Environmental Parameters on Urease Production, Ammonification and Growth of the Selected Rhizobia: Previous studies showed that nitrogen availability is found as one of environmental parameters that regulate urease expression in bacteria [5] and urease is a nickel-containing enzyme [4] The urease activity of the cyanobacterium, *Anabaena cylindrica*, was also found to be nickel-dependent and the addition of nickel to a nickel-free culture led to recovery of urease activity [8]. The cobalt salt has been reported as a reversible inhibitor of intracellular urease produced by *Rhizopus oryzae* [23]. Figures 11-14 present the effects of these factors on urease production and growth of the isolates PV013, PV014, DASA 02010 and DASA 02068, respectively. It was found that effects of these factors varied in different isolates. As compared with controls, Ni^{2+}, Co^{2+} and $NaNO_3$ decreased urease production in both isolates of common bean-nodulating rhizobia (PV013 and PV014). The greatest decrease of urease production was found with $NaNO_3$. Peptone was found to stimulate urease production in both common bean-nodulating isolates. In contrast, it decreased urease production in mungbean-nodulating isolate DASA 02010 and no significant difference was found with mungbean-nodultating isolate DASA 02068. Yeast extract decreased urease production of all isolates except PV014. The variation of these factors might due to different nitrogen catabolic pathways in each isolate [24]. Heavy metals were found to inhibit growth of some isolates, leading to a decrease in urease production. Both common bean-nodulating isolates could not tolerate Co^{2+}. The growth of a common bean-nodulating isolate, PV014, was inhibited by Ni^{2+}. Although Ni^{2+} did not have a growth-inhibitory effect on another isolate (PV013), it had a negative effect on urease production. In contrary, Ni^{2+} and Co^{2+} did not have a growth-inhibitory effect on both mungbean-nodulating isolates (DASA 02010 and DASA 02068). The urease production of these isolates was not affected by Ni^{2+} and Co^{2+}. Regardless of a growth-inhibitory effect, it was found that 0.1 mM Ni^{2+} decreased urease production in the isolate PV013, but not in the other isolates (DASA 02010 and DASA 02068). Even though Ni^{2+} is the cofactor of the urease enzyme such as in *Helicobacter pylori* [25], it is toxic for some bacterial cells such as *Escherichia coli* [26]. Uncontrolled acquisition of transition metals like nickel may lead to toxicity, as they may participate in the generation of toxic oxygen radicals or block incorporation of cofactors into enzymes [27]. Bacterial survival in the presence of toxic compounds is dependent on resistance and tolerance mechanisms [26].

Factors tested for ammonification include 1) $CuSO_4$ 0.2 mM; 2) $CuSO_4$ 2.0 mM; 3) $ZnSO_4$ 0.2 mM; and 4) $ZnSO_4$ 2.0 mM. The effect of these heavy metals on ammonification of soil has been reported. Under aerobic incubation conditions, 100 ppm Cu^{2+} was found to increase ammonification of soil but 1,000 ppm Cu^{2+} had no effect. The 100 and 10,000 ppm Zn^{2+} levels reduced ammonification whilst 1,000 ppm Zn^{2+} did not affect ammonification [28]. Figure 15-20 present the effects of environmental factors on ammonification and growth of the isolates PV009, PV016, DASA 06006, DASA 02009, DASA 02068 and DASA 02077, respectively.

The Cu and Zn heavy metals, added as sulphate, at varied concentrations (0.2 mM and 2.0 mM) had a negative effect on ammonification as these heavy metals are toxic to cells. Regardless of a growth-inhibitory effect, 0.2 mM Cu^{2+} reduced ammonification of the isolates PV009, DASA 02009, DASA 02068 and DASA 02077 (by 17% to 57%). The 0.2 mM Zn^{2+} reduced ammonification of the isolates PV009, DASA 02009 and DASA 02077 (by 28% to 63%).

In conclusion, common bean-nodulating rhizobia and mungbean-nodulating rhizobia produced extracellular urease, with maximum production achieved during the mid-log phases to stationary phases of growth. Ammonification of peptone varied among these rhizobia. The effect of other nitrogen sources (yeast extract, peptone, NH_4Cl and $NaNO_3$) and heavy metals (Cu^{2+} and Zn^{2+}) on urease production and cell growth varied in different isolates whilst Co^{2+} and Zn^{2+} had negative effects on ammonification and cell growth of almost all isolates tested.

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