Expression of Assimilatory Nitrate and Nitrite Reductase of *Rhizobium meliloti*

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ABSTRACT

Purpose: *To investigate the expression of assimilatory nitrate- and nitrite reductase activity of Rhizobium meliloti SU 47.*
Methods: *Rhizobium cell were grown in medium, after 20 hrs cells were washes with potassium phosphate buffer and assayed the enzyme activity through nitrite estimation.*
Result: *Expression of assimilatory nitrate- and nitrite reductase was observed in GTS/glutamate medium. The enzymes were inducible in nature since it expressed only in presence of nitrate or nitrite. Ammonium inhibited the enzyme activity. Cyanate acted as a gratituous inducer of both the enzymes. Molybdenum acts as modulator whereas tungstate as an inhibitor of the enzyme.*
Conclusion: *Both the enzymes, assimilatory nitrate- and nitrite reductases expressed in GTS/glutamate medium in presence of nitrate.*

Key Words: *Assimilatory nitrate and nitrite reductase, Rhizobium meliloti, Cyanate*

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INTRODUCTION

Besides nitrogen fixation nitrate assimilation is another biological process by which inorganic nitrogen is converted to ammonia and ultimately to organic nitrogen. Root-nodule bacteria have in their possession this alternative route of nitrogen assimilation for the biosysthesis of proteins through the assimilatory reduction of nitrate to nitrite and then to ammonia. Assimilatory nitrate reductase activity is observed only in bacteria grown with nitrate as sole of nitrogen source and is, thus inducible. The assimilatory nitrate reducing system catalyzing the step wise reduction of nitrate to nitrite and then to ammonia and contains two metalloproteins, nitrate reductase (NR) and nitrite reductase (NiR).

Dissimilatory nitrate reduction is a mechanism of generating energy during the absence of oxygen in a variety of bacteria including certain strains of *Rhizobium* and *Bradyrhizobium*[1,2]. This process causes reduction of nitrate nitrogen usually no further than to nitrite. The enzymes, NR and NiR are synthesized constitutively without the presence of nitrate or nitrite.

Nitrate assimilation is a tightly regulated process which precedes slowly at the rate that ammonia is required for growth causing rarely accumulation of nitrite during nitrate assimilation.

MATERIALS AND METHODS

Bacterial Strain: The strain *Rhizobium meliloti* SU 47 used in the present investigation was procured CSIRO, Division of Plant Industry, Canberra City, Australia. The strain was maintained in yeast extract mannitol (YEM) agar medium[3] by routine transfer between experiments.

Preparation of Inoculum: The strain *R. meliloti* SU 47 was grown in YEM medium at 28^0C under shaking condition (120 r. p. m.). The culture was left to grow till their early stationary phase and was used as inoculum.

Media: Media used for the present investigation are as follows:

1. YM Medium[4] contained the following (g/l): Mannitol, 10; K_2HPO_4, 0.5; $MgSO_4.7H_2O$, 0.2; Yeast extract, 1; NaCl, 0.1; $CaSO_4$, 0.1.
2. GTS/Glutamate medium[5] contained the following (g/l) : Glucose, 10; Sodium succinate, 2.7; Sodium glutamate, 1; Tris, 3; NaCl, 1; K_2HPO_4, 0.1; Yeast extract, 0.01; $MgSO_4.7H_2O$, 0.246; $CaCl_2$, 0.011; and supplemented with the following (mg/l): $FeCl_3.6H_2O$, 0.27; $Na_2MoO_4.7H_2O$, 0.242; H_3BO_3, 3; $MnSO_4.4H_2O$, 2.23; $ZnSO_4.7H_2O$, 0.287; $CuSO_4.5H_2O$, 0.125; $CoCl_2$, 0.065; Biotin, 2.0. pH was adjusted to 7.5 before autoclaving.

The pH of the medium was adjusted to 7.0 before autoclaving at 15 lb pressure/sq. inch. for 15 mins. Stock solutions of KNO_3, NH_4Cl, $(NH_4)_2SO_4$, NH_4NO_3, glutamine, potassium cyanate, ammonium molybdate and sodium tungstate were autoclaved separately and added to the medium as and when necessary.

Measurement of bacterial growth

Bacterial growth was measured turbido-metrically using a Balanced Cell Colorimeter 102 equipped with a red filter. Cell number was estimated by dilution plating.

REAGENTS

Phosphate buffer: 0.2 M stock solution of each of monobasic and dibasic potassium phosphate was prepared separately. 50 mM Potassium phosphate buffer, pH 7.5, was prepared by mixing 16 ml of monobasic with 84 ml of dibasic potassium phosphate stock solution[6] and diluted to 400 ml.

1% Sulphanilamide solution: 1.0 g of sulphanilamide was dissolved in 100 ml acid solution prepared by mixing 75 ml of distilled water and 25 ml of concentrated hydrochloric acid. The reagent was stored in an amber bottle.

0.02% N-(1-napthyl) ethylene di-amine dihydrochloride: 20 mg of N-(1-napthyl) ethylene di-amine dihydrochloride was dissolved in 100 ml of distilled water and stored in an amber bottle.

Estimation of nitrite: Cells were pelleted by centrifugation at 10,000 x g for 10 mins. at 4^0C and NO_2^- was estimated in the supernatant by the method of Nicholas and Nason[7]. The absorbance was measured at 540 nm in Shimadzu UV 240 spectrometer.

RESULT

Expression of assimilatory nitrate and nitrite reductase of *R. meliloti* SU 47

Expression of assimilatory NR and NiR by *R. meliloti* SU 47 in GTS/Glutamate medium was investigated. For the purpose, cells were grown till the cell number reached 10^8/ml when the culture was distributed equally in 6 flasks. In a set of 3 flasks KNO_3 to a concentration of 6 mM was added, one of these 3 flasks also received chloramphenicol (200μg/ml) and another flask received NH_4Cl (6 mM). The flasks containing chloramphenicol or NH_4Cl did not exhibit any NR activity at least for 6 hours while the cells in the control flask containing KNO_3 alone produced nitrite by the activity of NR (Figure 1A). After an initial lag of one and a half hours level of nitrite in this culture increased linearly throughout the entire period of incubation. The results indicated that the presence of NO_3^- was required for the *de novo* synthesis of assimilatory NR and the expression of the enzyme was inhibited by the presence of ammonium.

To the other set of 3 flasks $NaNO_2$ was added to a concentration of 1 mM. One of these flasks also received chloramphenicol (200 g/ml) and to the other NH_4Cl was added. Nitrite estimation was carried out in the flasks at regular time intervals. Nitrite content remains unchanged in the flasks containing chloramphenicol or NH_4Cl. However, the concentration of nitrite started declining linearly after 4 hours of incubation in the control flask containing $NaNO_2$ alone (Figure 1B) revealing the presence NiR for assimilatory reduction of nitrite.

Role of cyanate on the activity of NR of *R.* meliloti SU 47

Cyanate has been reported to act as a gratuitous inducer of assimilatory nitrate uptake in *Azotobacter*[8]. To study the effect of cyanate on the expression of NR in *R.* meliloti SU 47, the cells were grown in either YM medium or YM medium supplemented with 6 mM potassium cyanate, 6 mM KNO_3 plus 6 mM potassium cyanate. The NR activity of the cells was observed to be lower when grown in presence of cyanate alone. However, the activity was better in cells grown in medium supplemented with nitrate plus cyanate than when nitrate was present alone in the growth medium (Figure 2).

When potassium cyanate at different concentrations ranging from 1 to 6 mM was added to the assay medium along with 6 mM KNO_3, NR activity increased significantly. Data from Table 1 show that with increasing concentrations of cyanate in the assay medium the level of nitrite increased and at 6 mM cyanate the level of nitrite was much higher (294 nmole/ml after incubation for an hour) as compared to that in the control (190 nmole/ml).

Effect of molybdate and tungstate on NR activity of *R.* meliloti SU 47

As NR is reported to be a metalloprotein containing molybdenum, the effect of molybdate and tungstate on NR activity of *R.* meliloti SU 47 was studied. The bacteria were grown for 20 hours in YM medium with Na-molybdate or Na-tungstate at a concentration of 0.5 mM. Cells were collected and assayed in fresh YM medium containing 6 mM KNO_3 as substrate. Measurement of nitrite of different hours of incubation shows that the NR activity in molybdate grown cells was almost twice in comparison to that in control (Figure 3). On the other hand, tungstate grown cells exhibited only negligible activity.

Table 1: **Effect of added cyanate to the assay medium on nitrate reductase activity of *Rhizobium meliloti* SU47**

Potassium Cyanate (mM)	NO_2^- (n mole/ml)	
	1 Hour	2 Hours
Control	190	349
1	233	425
3	256	505
6	294	515

Cells were grown in YM medium `for 20 hours, resuspended in fresh YM medium containing 6 mM of KNO_3 to a cell density of 2 x 10^9 cells/ml. Potassium cyanate was added at various concentrations to the assay mixture. Nitrite was measured after one and two hours of incubation respectively.

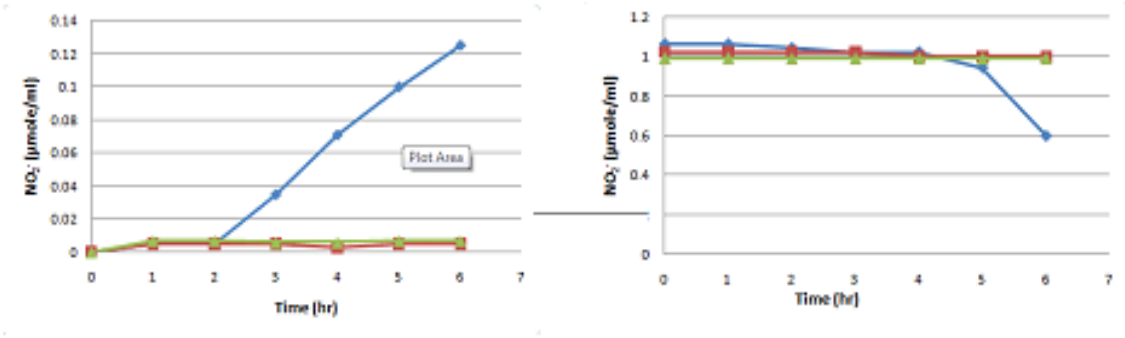

Figure 1A

Figure 1B

Figure 1: **A.** Detection of assimilatory nitrate reduction in *Rhizobium meliloti* SU47.
Cells were grown in GTS/glutamate medium to 10^8 cells/ml and divided into three subcultures and incubated further with the following compounds: 6 mM KNO_3 (♦----♦); 6 mM KNO_3 + 6 mM NH_4Cl (■----■) ; 6 mM KNO_3 + 200 □g cloramphenicol/ml (▲----▲)
B. Detection of assimilatory nitrite reduction in *Rhizobium meliloti* SU 47.
Cells were grown in GTS/glutamate medium to 10^8 cells/ml and divided into three subcultures and incubated further with the following compounds: 1 mM $NaNO_2$ (♦----♦); 1 mM $NaNO_2$ + 6 mM NH_4Cl (■----■) ; 1 mM $NaNO_2$ + 200 □g cloramphenicol/ml (▲----▲)

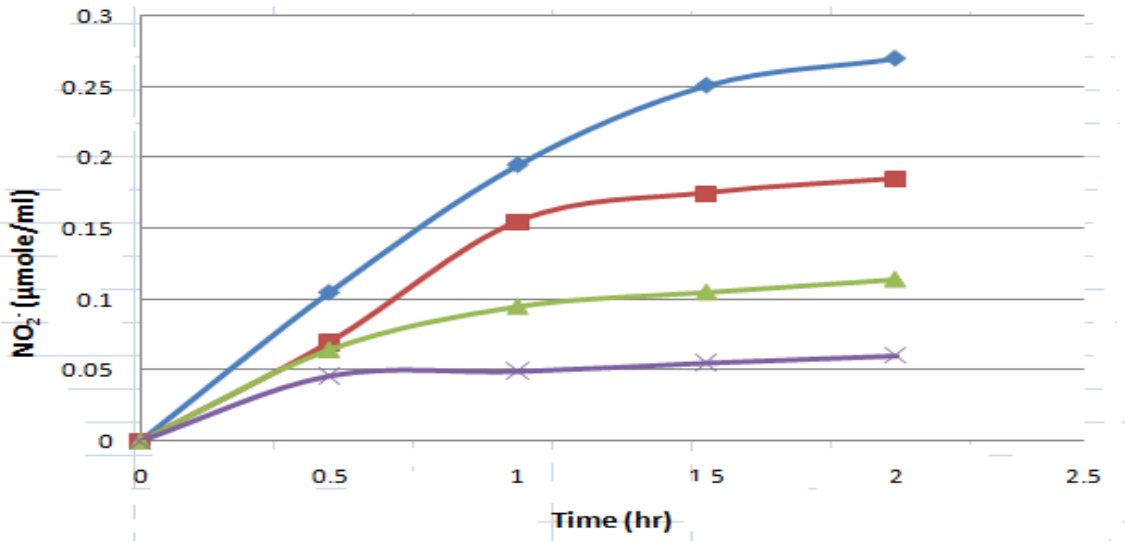

Figure 2: Nitrate reductase activity of *Rhizobium meliloti* SU 47 grown for 20 hours in YM medium alone (♦----♦) or in YM medium supplemented with 6 mM KNO_3 (▲----▲), 6 mM KOCN (×-----×) and KNO_3 and KOCN simultaneously (■----■), each at 6 mM.
Cells were resuspended at a density of 2 x 10^9/ml in fresh YM medium containing 6 mM KNO_3 as substrate and nitrite was measured at different time periods of incubation.

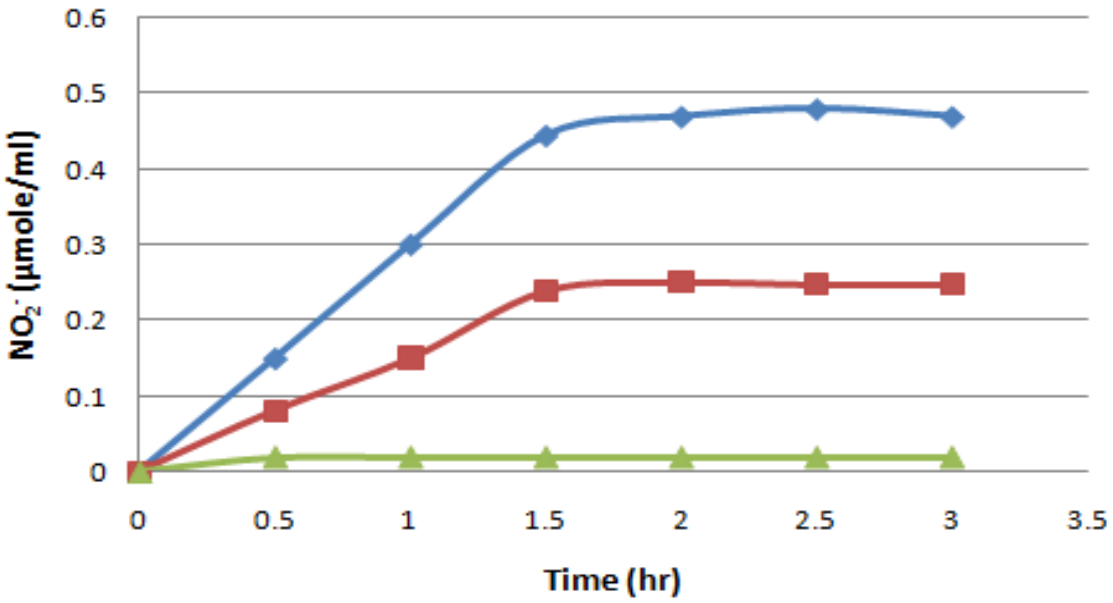

Figure 3: Effect of molybdate and tungstate on nitrate reductase activity of *Rhizbium meliloti* SU 47 grown in YM medium for 20 hours.
Cells were grown in YM medium alone (■----■) and also in presence of 0.5 mM sodium molybdate (♦----♦) or 0.5 mM sodium tungstate (▲----▲). Cells were resuspended in fresh YM medium containing 6 mM KNO_3 at a cell density of 2 x 10^9/ml. Nitrite was measured in the assay mixture at different time intervals during incubation.

DISCUSSION

The presence of assimilatory NR and NiR in *R. meliloti* SU47 was investigated in GTS/Glutamate medium (5) containing sodium succinate, glucose, sodium glutamate and traces of yeast extract as carbon and nitrogen sources. Results indicate that the presence of nitrate or nitrite is necessary for the induction of synthesis of functional assimilatory enzymes as observed by Kiss *et al*.[5] in *R. meliloti* SU 47. Earlier workers[9,10,11] postulated that the assimilatory nitrate- and nitrite reductases in *Rhizobium* and *Bradyrhizobium* are inducible in nature. Varinhos *et al*.[12] observed simultaneous assimilation and denitrification of nitrate by *Bradyrhizobium japonicum* when incubated under anaerobiosis. Addition of chloramphenicol or NH^{4+} to the suspending medium inhibited the expression of both the enzymes. Ammonium inhibition of expression of assimilatory nitrate reducing system in the stain *R. meliloti* SU 47 is similar to that observed in some N_2-fixing bacteria, e.g. *Azotobacter*, *Derxia*[8]. Also, no development of the nitrate reducing system took place when chloramphenicol, which acts at the level transcription, was present in the suspending medium with nitrate as inducer.

Cyanate inhibition of nitrate consumption in eukaryotic system is known[13]. Cyanate has been demonstrated to stimulate NR activity[14] and acts as a gratuitous inducer of assimilatory nitrate uptake in *Azotobacter chroococcum*[8]. In the present study the influence of the presence of cyanate in YM medium on the NR activity of *R. meliloti* SU 47 was tested. The results show that NR activity of the cells was only marginal in the presence of cyanate (6 mM) in the growth medium as compared to control. However, NR activity was enhanced when cyanate was supplemented to the growth medium along with nitrate (6 mM) as compared to nitrate alone. Remarkable as it is, cyanate when added in different concentrations to the assay mixture also enhanced NR activity considerably. Taken together, the results indicate that cyanate is possibly a modulator of nitrate reductase activity of *R. meliloti* SU 47.

The enzyme, NR, is reported to be a metalloprotein containing molybdate[15], as such the effect of molybdate and tungstate on its activity was studied. In nitrate assimilating organism's molybdenum is an essential trace element required for reduction of nitrate to nitrite[14]. Addition of sodium molybdate promoted NR-activity of *R. meliloti* SU47 grown in YM medium. Since NR activity seems to be synthesized constitutively in YM medium the role of Mo as an inducer in NR synthesized[16] cannot be supported.

Presence of tungstate in growth medium caused abolition of NR activity probably due to substitution of molybdenum of the enzyme with tungstate and forming an analogue of nitrate reductase. Tungtstate analogue of NR with NADH activity but no nitrate reducing activity has been reported[17,18]. Sorger *et al*.[19] also suggested that tungsten is inhibitory to NR activity, by being

incorporated in place of molybdenum of NR and making it non-functional. Guerrero *et al*.,[14] had made a similar observation on the inhibitory effect of tungnate which decreased the cellular level of NR activity in *Azotobacter chroococcum*. The growth of *R. meliloti* SU47 was not affected in the presence of tungstate in YM medium suggesting that the NR enzyme was not being used for assimilatory purposes.

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