

## Production of amino acids by *Azotobacter vinelandii* and *Azotobacter chroococcum* with phenolic compounds as sole carbon source under diazotrophic and adiazotrophic conditions

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**Summary.** *Azotobacter vinelandii* strain ATCC 12837 and *Azotobacter chroococcum* strain H23 (CECT4435) were tested to grow in N-free or NH<sub>4</sub>Cl amended chemically defined media, with protocatechuic acid or sodium p-hydroxybenzoate as sole carbon (C) sources at a concentration of 2 mmol/L. Both substrates supported growth at similar rates than bacteria grown in control media amended with 2 mmol/L sodium succinate as C source. The two strains produced aspartic acid, serine, glutamic acid, glycine, histidine, threonine, arginine, alanine, proline, cysteine, tyrosine, valine, methionine, lysine, isoleucine, leucine and phenylalanine after 72 h of growth in chemically defined media with 2 mmol/L of phenolic compounds or sodium succinate as sole C source amended or unamended with 0.1% (w/v) NH<sub>4</sub>Cl. Qualitative and quantitative production of all amino acids was not affected by the use of different C and N substrates.

**Keywords:** Amino acids – *Azotobacter* – Phenolic compounds – Soil microbiota

### Introduction

Microorganisms of the genus *Azotobacter* are common inhabitants of soils of neutral pH in cold and temperate regions, where they constitute a major fraction of the free-living N<sub>2</sub>-fixing population that keeps nitrogen cycling in the biosphere (Tchan and New, 1984). *Azotobacter* is able to fix at least 10 mgN per gram of carbohydrate (Becking, 1992). It has been shown that the soil characteristics and climate conditions affect the distribution of this microorganisms (González-López, 1992); it includes organic matter content and C/N relation. In addition, strains of this genus are often able to colonize the rhizosphere and root surfaces of many agrochemical important plants and establish, under natural conditions, non-endosymbiotic root association that lead to plant growth promotion (Revillas et al., 2000).

Agronomical exploitation of *Azotobacter*-plant interactions has been successfully tested under laboratory and field conditions (Becking, 1992), and the positive effect of inoculation of plants with these bacteria has been attributed to the cession of fixed N to the plant, enhancement of soluble P availability, antibiosis to pathogenic microorganisms and production of soluble substances such as hormones, vitamins and amino acids (Okon and Itzigsohn, 1995; González-López et al., 1999). The influence on *Azotobacter* root colonization of biologically-active substances produced and released by these microorganisms, such as vitamins (Revillas et al., 2000), on success of the establishment of root surface colonization has been proposed but not clarified.

One of the main factors that influences the biological activities of *Azotobacter* spp in soil and rhizosphere is the availability of carbon compounds. In this way, sugars and organic acids supplied in plant root exudates are readily incorporated by these microorganisms (Tchan and New, 1984). However, survival and persistence of *Azotobacter* in natural habitat is believed to be highly dependent on their ability to metabolize simple phenolic compounds, which are commonly present in soils at concentrations of 1–2 mmol/L (Wu et al., 1987). Phenolic acids in the soil come mainly from plant litter decomposition or soil contamination by agrochemical and are toxic for most microorganisms, but a few genera of bacteria and fungi are able to degrade and use them as carbon sources. In this sense, growth and N<sub>2</sub>-fixation supported by some phenolic

compounds, such as p-hydroxybenzoic acid or protocatechuic acid, at concentrations well over the range commonly found in natural soils, have been reported for *A. vinelandii* (Moreno et al., 1999).

Production of some amino acids (methionine, lysine, arginine, tryptophane and glutamic acid) has been reported by *Azotobacter* spp during growth in culture media amended with glucose as sole carbon source under diazotrophic and adiazotrophic conditions (González-López et al., 1995). As far as is known, there are no previous data on amino acids production by *Azotobacter* spp, or any other soil bacteria when phenolic compounds are the only carbon source available at the concentrations commonly found in soils in nature.

In this paper, the production of aspartic acid, serine, glutamic acid, glycine, histidine, threonine, arginine, alanine, proline, cysteine, tyrosine, valine, methionine, lysine, isoleucine, leucine and phenylalanine by *Azotobacter vinelandii* strain ATCC 12837 and *Azotobacter chroococcum* H23 (CECT 4435) growing on chemically-defined media amended with low concentration of two phenolic compounds as sole carbon source, under diazotrophic and adiazotrophic conditions, is reported.

## Material and methods

### Microorganisms

The strains used in this study were *Azotobacter vinelandii* strain ATCC 12837 and *Azotobacter chroococcum* H23 (Spanish Type Culture Collection, CECT 4435). Both strains are amino acids produced in media amended with glucose, and have shown growth-promoting ability on plants (Rodelas et al., 1999; Pozo et al., 2000).

### Growth conditions

*Azotobacter* spp were maintained in Burk's N-free agar slants amended with 0.5% glucose as carbon source (Pozo et al., 2003). *Azotobacter* strains were transferred from fresh agar slants to Erlenmeyer flasks with 50 ml Burk's N-free or Burk's  $\text{NH}_4^+$  liquid medium containing 0.3%  $\text{NH}_4\text{Cl}$  amended with sodium p-hydroxybenzoate (PB), protocatechuic acid (PA) or sodium succinate (S) at concentrations of 2.0 mmol/L. Succinate was selected as control substrate as it is readily incorporated into the tricarboxylic acid cycle (TCA), as well as being the main product of both PA and PB degradation by *Azotobacter* spp (Hardison et al., 1969). All chemicals used as carbon sources were purchased from Sigma. PA, PB and S was added to culture media, after autoclaving and cooling down, from a freshly-made, filter-sterilized, 10 fold concentrated stock solution. After 72 h incubation at 28°C in a shaker (Gallekamp, INR-200, UK) with gentle agitation (110 rpm), total cell numbers were determined using a Petroff-Hausser counting chamber (C.A. Hausser and Son, Philadelphia, PA, USA).

### Amino acids productions assay

Samples from Erlenmeyer flasks containing 50 ml Burk's N-free or Burk's  $\text{NH}_4^+$  medium amended with 2 mmol/L of the corresponding carbon source were taken after 72 h of incubation at 28°C. Aliquots were cen-

trifuged at 5000 g for 30 min in a Sorvall RC-5B centrifuge (Dupont Instruments, Wilmington, DE, USA) at 4°C, and the supernatant fluid were passed through 0.22  $\mu\text{m}$  of Millipore filter membranes. Aliquots (100  $\mu\text{l}$ ) of filtrate supernatants were added to Eppendorf tubes containing 200  $\mu\text{l}$  of acetic acid (2 mol/L). The amino acids fraction was isolated with pre-prepared Dowex column (AG 50WX8 in the  $\text{H}^+$  form) according to Stoll et al. (1999), and the purified amino acids were used for HPLC analysis (Water ACQO.TAG) with 6-amino quinolyl-N-hydroxysuccinimide carbamate derivative. The solution was analysed for amino acids by injecting 5  $\mu\text{l}$  into a HPLC 2690 (Water) with a water column of 150 mm. Standard curve for amino acids was calculated from 3 concentrations (20, 50 and 100 pmol/ml of amino acids) and sensitivity of the method was in the range of 2 pmol of amino acid per ml.

### Statistical analysis

Data obtained from this study were analyzed by multifactorial analysis of variance (ANOVA) with all interactions, using the software package *Statgraphics* version 5.0 (STC Inc. Rockville, MD, USA). Least significant differences (LSD) were calculated at the 95% level of significance ( $p < 0.05$ ).

## Results and discussion

Total cell numbers of *A. chroococcum* H23 and *A. vinelandii* ATCC12837 after growing for 72 h on chemically-defined media amended with 2 mmol/L of PA, PB or S as sole carbon source is shown in Table 1. No significant differences in the growth of the two *Azotobacter* strains were found on media amended with all the substrates

**Table 1.** Total cell numbers per ml of *Azotobacter vinelandii* ATCC 12837 and *A. chroococcum* H23 after 72 h of incubation in Burk's N-free and Burk's  $\text{NH}_4^+$  medium amended with 2 mmol/L of protocatechuic acid (PA), sodium p-hydroxybenzoate (PB) or succinate (S) as sole carbon source

| Strain     | Carbon source          | Log CFU/ml |
|------------|------------------------|------------|
| ATCC 12837 | PA (N-free)*           | 6.56       |
|            | PA ( $\text{NH}_4^+$ ) | 7.10       |
|            | LDS ( $p < 0.05$ )     | 0.16       |
|            | PB (N-free)            | 6.89       |
|            | PB ( $\text{NH}_4^+$ ) | 7.03       |
|            | LDS ( $p < 0.05$ )     | 0.15       |
|            | S (N-free)             | 6.60       |
|            | S ( $\text{NH}_4^+$ )  | 6.21       |
|            | LDS ( $p < 0.05$ )     | 0.32       |
| H23        | PA (N-free)            | 7.58       |
|            | PA ( $\text{NH}_4^+$ ) | 7.76       |
|            | LDS ( $p < 0.05$ )     | 0.24       |
|            | PB (N-free)            | 6.23       |
|            | PB ( $\text{NH}_4^+$ ) | 7.00       |
|            | LDS ( $p < 0.05$ )     | 0.27       |
|            | S (N-free)             | 6.99       |
|            | S ( $\text{NH}_4^+$ )  | 7.23       |
|            | LDS ( $p < 0.05$ )     | 0.18       |

\* All media were inoculated with an initial cell number around  $10^5$  cells/ml

tested. The positive effect of the addition of 0.3%  $\text{NH}_4\text{Cl}$  to the media on growth of *Azotobacter* strains was slight.

Our experiments show that *A. chroococcum* H23 and *A. vinelandii* ATCC12837 are able to degrade phenolic structures on chemically-defined media. Thus, it is well known that *Azotobacter* spp can grow and biotransform a wide range of phenolic substances (Moreno et al., 1999) and complex wastes with a high phenolic compound content (Pozo et al., 2002). However, it has been reported that higher concentrations (up to 5 mmol/L) of phenolic acids may be toxic for some *Azotobacter* strains (Revillas et al., 2000) or at least induce a poor utilization of these substances (Moreno et al., 1999). Obviously, our data show that the natural levels of these compounds present in soils (2 mmol/L) should not affect *Azotobacter* strains negatively, either with  $\text{NH}_4\text{Cl}$  (diazotrophic condition) or atmospheric  $\text{N}_2$  (diazotrophic condition) as the nitrogen source.

The production of aspartic acid, serine, glutamic acid, glycine, histidine, threonine, arginine, alanine, proline, cysteine, tyrosine, valine, methionine, lysine, isoleucine, leucine and phenylalanine by strains ATCC 12837 and H23, in both Burk's N-free and Burk's medium amended with 0.3%  $\text{NH}_4\text{Cl}$ , is shown in Tables 2 and 3. The amounts of each amino acid detected varied largely within strain; however the production of those substances was not significantly influenced by the nature of the available C and

N sources. *A. vinelandii* ATCC 12837 released higher amounts (up to 30 pmol/ml) of serine, glycine, alanine and proline, whereas *A. chroococcum* H23 produced large amounts of serine, glycine, alanine, valine and leucine.

*A. chroococcum* H23 and *A. vinelandii* ATCC 12887 produced all the amino acids tested showed that those microorganisms are able to produce and liberate these biologically active substances under diazotrophic and adiazotrophic conditions. Succinate was used as control substrate as it is the main product of protocatechuic and p-hydroxybenzoic acids degradation by *Azotobacter* spp (Hardisson et al., 1969). Our results (Tables 2 and 3) show that the presence of succinate did not significantly affect amino acids production by these bacterial strains when compared with culture media amended with phenolic compounds as sole C source. Moreover, the liberation to the media of the amino acids was independent of the phenolic compound (protocatechuic acid or p-hydroxybenzoate) available, suggested that at a concentration of 2 mmol/L (natural levels in soils) the response to the presence of different phenolic acids on amino acids production followed a similar pattern for each particular strain and amino acid.

The presence of  $\text{NH}_4\text{Cl}$  did not significantly affect amino acids production by any of the strains when the carbon source was protocatechuic acid, p-hydroxybenzoic acid or succinate. Thus, the nature of the available N source did not affect the quantitative production and

**Table 2.** Production (pmol/ml) of amino acids by *Azotobacter chroococcum* H23 in Burk's N-free medium and Burk's medium amended with 0.3%  $\text{NH}_4\text{Cl}$  containing 2 mmol/L of protocatechuic acid (PA), sodium p-hydroxybenzoate (PB) or succinate (S) as sole carbon source after 72 h of growth under aerobic conditions

| Amino acid    | PA                 |                          | PB      |                          | S       |                          |
|---------------|--------------------|--------------------------|---------|--------------------------|---------|--------------------------|
|               | N-free             | $\text{NH}_4^+\text{Cl}$ | N-free  | $\text{NH}_4^+\text{Cl}$ | N-free  | $\text{NH}_4^+\text{Cl}$ |
| Aspartic acid | 4 ± 1 <sup>a</sup> | 4 ± 1                    | 4 ± 1   | 5 ± 1                    | 2 ± 1   | 2 ± 1                    |
| Serine        | 37 ± 12            | 46 ± 1                   | 42 ± 7  | 54 ± 14                  | 61 ± 19 | 45 ± 19                  |
| Glutamic acid | 14 ± 6             | 9 ± 4                    | 13 ± 4  | 13 ± 3                   | 11 ± 4  | 8 ± 4                    |
| Glycine       | 100 ± 15           | 98 ± 16                  | 76 ± 12 | 86 ± 16                  | 78 ± 10 | 75 ± 13                  |
| Histidine     | 5 ± 1              | 6 ± 3                    | 4 ± 2   | 8 ± 3                    | 2 ± 1   | 9 ± 3                    |
| Threonine     | 16 ± 7             | 18 ± 5                   | 16 ± 4  | 23 ± 5                   | 23 ± 4  | 14 ± 4                   |
| Arginine      | 8 ± 3              | 5 ± 2                    | 10 ± 3  | 20 ± 9                   | 8 ± 3   | 8 ± 3                    |
| Alanine       | 40 ± 9             | 44 ± 12                  | 37 ± 10 | 63 ± 13                  | 27 ± 11 | 36 ± 11                  |
| Proline       | 12 ± 4             | 20 ± 4                   | 16 ± 3  | 20 ± 3                   | 23 ± 3  | 20 ± 2                   |
| Cysteine      | 11 ± 6             | 10 ± 7                   | 17 ± 6  | 24 ± 7                   | 15 ± 5  | 10 ± 6                   |
| Tyrosine      | 15 ± 3             | 15 ± 4                   | 19 ± 2  | 14 ± 2                   | 18 ± 2  | 19 ± 2                   |
| Valine        | 36 ± 8             | 41 ± 9                   | 39 ± 4  | 51 ± 12                  | 32 ± 6  | 40 ± 15                  |
| Methionine    | 16 ± 6             | 16 ± 4                   | 12 ± 4  | 13 ± 4                   | 7 ± 3   | 8 ± 2                    |
| Lysine        | 14 ± 2             | 18 ± 7                   | 20 ± 5  | 23 ± 8                   | 19 ± 3  | 16 ± 5                   |
| Isoleucine    | 9 ± 4              | 13 ± 5                   | 10 ± 2  | 24 ± 9                   | 7 ± 2   | 8 ± 2                    |
| Leucine       | 31 ± 10            | 37 ± 8                   | 34 ± 7  | 43 ± 11                  | 38 ± 4  | 48 ± 7                   |
| Phenylalanine | 5 ± 1              | 5 ± 1                    | 4 ± 1   | 8 ± 1                    | 3 ± 1   | 7 ± 1                    |

<sup>a</sup> Values are mean of three replicates (± standard error)

**Table 3.** Production (pmol/ml) of amino acids by *Azotobacter vinelandii* ATCC 12837 in Burk's N-free medium and Burk's medium amended with 0.3% NH<sub>4</sub>Cl containing 2 mmol/L of protocatechuic acid (PA), sodium p-hydroxybenzoate (PB) or succinate (S) as sole carbon source after 72 h of growth under aerobic conditions

| Amino acid    | PA                 |                                 | PB      |                                 | S       |                                 |
|---------------|--------------------|---------------------------------|---------|---------------------------------|---------|---------------------------------|
|               | N-free             | NH <sub>4</sub> <sup>+</sup> Cl | N-free  | NH <sub>4</sub> <sup>+</sup> Cl | N-free  | NH <sub>4</sub> <sup>+</sup> Cl |
| Aspartic acid | 2 ± 1 <sup>a</sup> | 5 ± 1                           | 3 ± 1   | 5 ± 1                           | 5 ± 1   | 3 ± 1                           |
| Serine        | 44 ± 5             | 67 ± 13                         | 28 ± 10 | 61 ± 16                         | 51 ± 13 | 47 ± 14                         |
| Glutamic acid | 2 ± 1              | 8 ± 3                           | 5 ± 2   | 10 ± 4                          | 12 ± 3  | 9 ± 3                           |
| Glycine       | 60 ± 6             | 64 ± 8                          | 77 ± 5  | 57 ± 10                         | 69 ± 8  | 67 ± 12                         |
| Histidine     | 4 ± 2              | 4 ± 2                           | 4 ± 1   | 2 ± 1                           | 9 ± 3   | 5 ± 3                           |
| Threonine     | 12 ± 4             | 20 ± 9                          | 17 ± 2  | 27 ± 7                          | 14 ± 7  | 16 ± 7                          |
| Arginine      | 19 ± 3             | 13 ± 3                          | 16 ± 2  | 13 ± 3                          | 17 ± 3  | 19 ± 4                          |
| Alanine       | 33 ± 10            | 44 ± 4                          | 42 ± 10 | 51 ± 16                         | 32 ± 11 | 36 ± 11                         |
| Proline       | 37 ± 10            | 46 ± 8                          | 33 ± 14 | 31 ± 8                          | 20 ± 5  | 23 ± 5                          |
| Cysteine      | 18 ± 3             | 13 ± 5                          | 16 ± 5  | 18 ± 6                          | 13 ± 2  | 14 ± 6                          |
| Tyrosine      | 20 ± 7             | 26 ± 7                          | 13 ± 6  | 19 ± 4                          | 19 ± 7  | 13 ± 6                          |
| Valine        | 13 ± 3             | 16 ± 6                          | 21 ± 6  | 22 ± 4                          | 22 ± 4  | 16 ± 7                          |
| Methionine    | 10 ± 3             | 15 ± 6                          | 14 ± 6  | 11 ± 4                          | 7 ± 3   | 6 ± 3                           |
| Lysine        | 18 ± 6             | 25 ± 8                          | 15 ± 4  | 16 ± 6                          | 21 ± 4  | 24 ± 7                          |
| Isoleucine    | 10 ± 4             | 13 ± 5                          | 10 ± 3  | 14 ± 3                          | 7 ± 2   | 8 ± 2                           |
| Leucine       | 22 ± 4             | 24 ± 6                          | 28 ± 9  | 25 ± 7                          | 19 ± 3  | 26 ± 7                          |
| Phenylalanine | 6 ± 2              | 6 ± 1                           | 9 ± 3   | 10 ± 3                          | 6 ± 2   | 4 ± 2                           |

<sup>a</sup> Values are mean of three replicates (± standard error)

liberation of amino acids in chemically-defined media amended with the phenolic compounds.

González-López et al. (1995) reported that the availability of NH<sub>4</sub>Cl affected the production of some amino acids by *Azotobacter* spp when the microorganisms were grown on glucose as sole carbon source suggested that the presence of combined N on amino acids production by *Azotobacter* spp seems to be related to the special pathways for the production of such compounds. However the results of our study show that the production of amino acids by *Azotobacter* spp grown in media amended with 2 mmol/L of organic compounds such as phenolic acids and succinate was not affected by the nature of the available N source. In this sense, aromatic compounds often support high rates of nitrogenase activity (D'Mello et al., 1997) in several free-living N-fixing bacteria including *Azotobacter*.

*Azotobacter* spp obtain the energy for N<sub>2</sub> fixation from soil and plant root exudates. The chemical composition and processes in the rizhosphere have recently gained much attention from student of soil-plant interactions but the main interest has focused on aspects other than amino acids (Broughton et al., 2003). However the exogenous application of biologically-active substances such as vitamins and amino acids, can have a direct effect on plant cells functions as well as indirect effects on plant growth (Oertli, 1987; Streit et al., 1996). Consequently,

the liberation of amino acids by *Azotobacter* spp could be one of the mechanisms that explain the positive effects of these plant growth promoting bacteria on plants and also their positive interactions with other microorganisms in the soil and rizhosphere.

In conclusion, the results of this study show that large amounts of amino acids are produced by two strains of *Azotobacter* with phenolic compounds as sole carbon sources supplied at the concentrations commonly found in the soil and rizhosphere. However, the amino acids production under these conditions followed pattern different from those reported when *Azotobacter* was grown in media amended with sugars both under adiazotrophic or N<sub>2</sub>-fixing conditions.

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