Amino Acids (1995) 8: 15–21

Amino
Acids
© Springer-Verlag 1995
Printed in Austria

Production of amino acids by free-living heterotrophic nitrogen-fixing bacteria

Minireview Article

J. González-López, M. V. Martínez-Toledo, B. Rodelas, C. Pozo, and **V. Salmerón**

Group of Nitrogen Fixation, Department of Microbiology, Faculty of Pharmacy and Institute of Water Research, University of Granada, Granada, Spain

Accepted November 20, 1993

Summary. Interest of microbial production of amino acids has been increased greatly since development of biotechnological methods. These methods represent a perspective way applied in a future large-scale manufacture of inexpensive amino acids. In this context, the isolation of producing organisms that may be exploited in the desing of alternative methods for the production of amino acids could be of primary importance.

In this review we will describe the liberation of amino acids (methionine, lysine, arginine, tryptophane and glutamic acid) by *Azotobacter* and *Azospirillum* during growth in culture media with different carbon sources under diazotrophic and adiazotrophic conditions. These organisms may be useful in developing new methods for the industrial production of amino acids.

Keywords: Amino acids – *Azotobacter* – *Azospirillum* – Biotechnology

Introduction

Azotobacter are free-living nitrogen-fixing microorganisms widely distributed in soil and the rhizosphere (Martínez-Toledo et al., 1985; Kennedy and Tchan, 1992). The ability of *Azotobacter* to fix dinitrogen was definitively established by Beijerinck (1901) and has served as the distinguishing characteristic of these bacteria since that time. However, it has been shown that they fix dinitrogen only in the absence or under critically low quantities of nitrogenous compounds, such as ammonia, nitrate and urea (Salmerón et al., 1989).

Members of the genus *Azotobacter* have the ability to oxidize many different carbon sources such as glucose, mannitol, sucrose, alcohols and aromatic compounds (Wu et al., 1987). Phenolic acids are energy rich substances metabolized only by a few members of soil microorganisms, *Azotobacter* being prominent among these (Moreno et al., 1988).

Azotobacter is able to synthetize biologically active substances such as vitamins and phytohormones (Martínez-Toledo et al, 1991). However, the production of these substances is influenced by the growth conditions and duration of incubation.

Azospirillum spp. are nitrogen-fixing organisms, frequent inhabitants of the rhizosphere of a wide variety of plants in diverse regions of the world (Zimmer et al., 1988). *Azospirillum* proliferates under aerobic and anaerobic conditions but it's preferentially microaerobic in both the presence or ausence of combined nitrogen in the medium. *Azospirillum* is capable of using many organic compounds as carbon and energy sources in the presence of ammonia or dinitrogen, mainly organic acids such as malate and succinate (Okon and Itzigsohn, 1992).

The plant growth-promoting influence of *Azospirillum* has been extensively examined (Janzen et al., 1992), and some of the recent research concerning the *Azospirillum*-plant interaction has focused on phytohormones and vitamins (Kucey, 1988; Dahm et al., 1993; Rodelas et al., 1993). However, practically nothing is known about the production of amino acids by *Azospirillum*.

The liberation of amino acids from soil microorganisms into the medium was demonstrated by Payne et al. (1957). Diazotrophic bacteria, such as *Azotobacter* and *Azospirillum*, produce amino acids in chemically-defined media. We will discuss the influence of growth media and their nutrient content on the liberation of amino acids by these organisms.

Production of amino acids by *Azotobacter*

Most studies on production of amino acids by *Azotobacter* have been carried out in chemically-defined media, specially N-free media. Thus, it has been

Table 1. Production of amino acids by *Azotobacter vinelandii* under diazotrophic (a) and adiazotrophic (b) conditions (González-López et al., 1983)

	Incubation time (d)					
	1		2		7	
	a	b	a	b	a	b
Glutamic acid	0*	0.05	0.40	0.60	0.40	4.00
Lysine	0.60	0.05	500.0	500.0	200.0	200.0
Arginine	0	0	0.01	0	0.02	0
Tryptophane	0	0	0	0	0	0.09
Methionine	0	0.05	0	0.05	0	0.09

* Values are given as ng/ml. Liberation of amino acids was studied in chemically-defined nitrogen-free media with 0.5% glucose (a) and chemically-defined media with 0.5% glucose and 0.3% NH_4Cl (b). Cultures were incubated at 28° C on a shaker (100 rpm) to maintain aerobic conditions and throughout mixing.

reported the liberation of methionine, lysine, arginine, tryptophane and glutamic acid by *A.vinelandii* and *A.chroococcum* during growth in culture media amended with different carbon sources under diazotrophic and adiazotrophic conditions (Table 1).

Lysine is the amino acid that *Azotobacter* produces in larger amounts. The production of this amino acid is not affected by the presence or limitation of nitrogen. However, it has been reported that the liberation of arginine, tryptophane and methionine is influenced by the amount of nitrogen added into the media (González-López et al., 1983).

The production of lysine in chemically-defined media by *Azotobacter* is significantly affected by the concentration of carbohydrate (Fig. 1). Thus, lysine is produced in large amounts in culture medium containing 5.0 g/l of glucose, incubated for three days at 30° C. However, the liberation of this amino acid is decreased after 7 days.

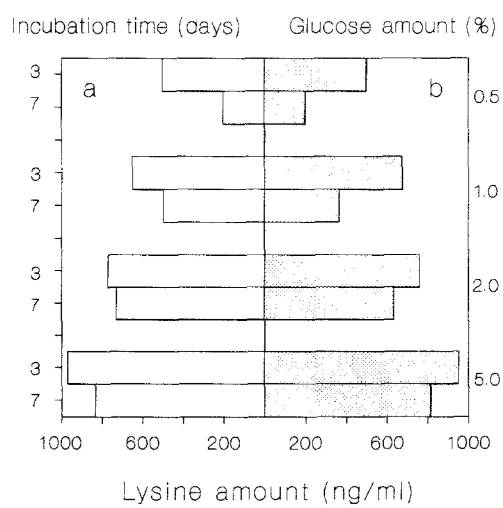

Fig. 1. Production of lysine by *Azotobacter vinelandii* in chemically-defined nitrogen-free media with glucose (**a**), and chemically-defined media with glucose and 0.3% NH_4Cl (**b**) (From González-López et al., 1983)

Production of amino acids by *Azospirillum*

The liberation of amino acids by *Azospirillum* in chemically-defined media with different carbon sources is shown in Fig. 2. Data show that the production of those substances is significantly influenced by the growth conditions. Methionine, lysine and arginine are produced in large amounts in culture medium containing fructose for 24 h at 30° C. However, the liberation of those amino acids drecreases strongly after 48 h. Therefore, the production of amino acids by *Azospirillum* is influenced by the carbon source added into the culture media. When malate or gluconate are present in the culture medium, the liberation of methionine, lysine and arginine by *Azospirillum* is drastically modified, compared with culture medium amended with fructose.

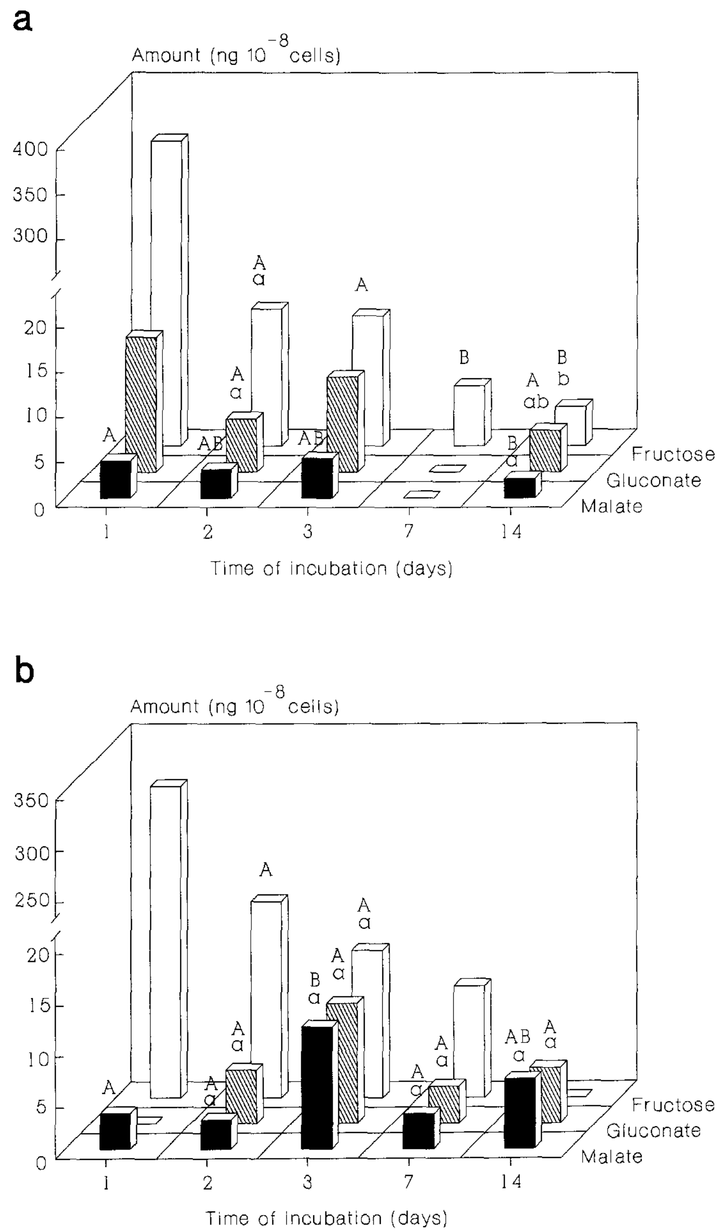

Fig. 2a, b. Production of lysine (**a**) and arginine (**b**) by *Azospirillum brasilense* in chemically-defined media. Influence of the carbon source and incubation time (B. Rodelas, M.sc. thesis). Values with a common letter are not significantly different (Student's t-test, $P = 0.05$). Lower case letters refer to analysis of the influence of the carbon source between cultures with the same incubation time, and capital letters refer to analysis of the influence of the time of incubation between cultures amended with the same carbon source

Discussion

Amino acids are used in medicine, in the food and the fodder industry and in the chemical and pharmaceutical industries too. Amino acids such as tryptophan and aspartic acid can be prepared by a precursory method or by an enzymatic one, but the predominant way of preparation is a fermentation. Lysine,

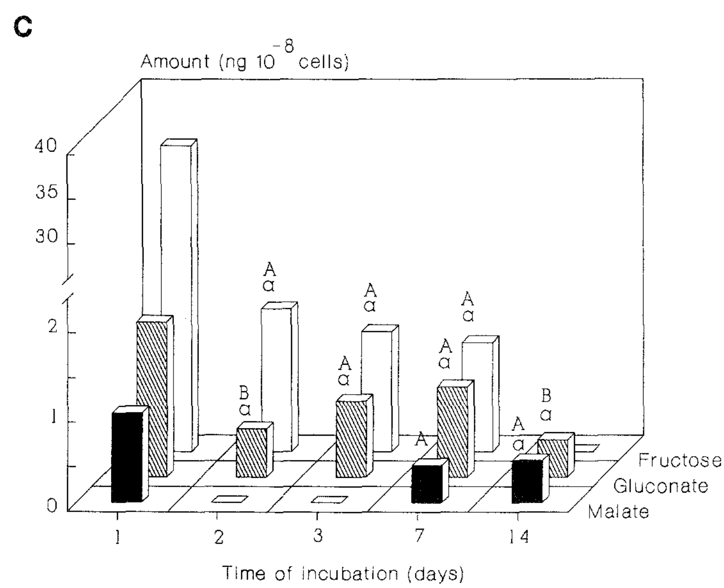

Fig. 2c, d. Production of methionine (**c**) and glutamic acid (**d**) by *Azospirillum brasilense* in chemically-defined media. Influence of the carbon source and incubation time (B. Rodelas, M.sc. thesis). Values with a common letter are not significantly different (Student's t-test, $P = 0.05$). Lower case letters refer to analysis of the influence of the carbon source between cultures with the same incubation time, and capital letters refer to analysis of the influence of the time of incubation between cultures amended with the same carbon source

threonine, methionine, tryptophan and glutamic acid are the amino acids most often produced microbiologically in the largest amounts. Thus, with *Corynebacterium glutamicum* 180,000 tons of L-lysine are produced annually.

The most significant factor in developing microbial processes for amino acids has been the use of auxotrophic strains. However, due to their nutritional requeriments, nitrogen-fixing bacteria (*Azotobacter* and *Azospirillum*) could be applied in a future for the production of amino acids (i.e. lysine) in batch cultures.

The first step for batch production of amino acids using *Azotobacter* and *Azospirillum* in a fermentor is the optimization of medium and culture conditions. Rather surprisingly, few studies have been devoted to the physiology of *Azotobacter* and *Azospirillum* growth in fermentor for biomass and amino acids production. Most of these studies were in fact related with nitrogen-fixation activity (Dalton and Postgate, 1969; Cacciari et al., 1989). For amino acid production, growth cannot be limited by nitrogen and combined nitrogen salts must be added to the medium. A strict control of sterility is required since the optimal temperature (28–30° C) and pH (7.0–7.2) of the culture allow development of all kinds of potential contaminants.

Azospirillum with a nitrogen source like an ammonium salt is often described as a complete aerobe organism. However, recent study has show that high dissolved oxygen tensions (DO) are detrimental while low DO ($<30\%$ saturation) are favourable. It is likely that most nitrogen-fixing organisms behave in the same way since such sensitivity is also reported with *Azotobacter* (Senior et al., 1972).

The development of optimized fermentation strategies (medium composition, temperature, pH and O_2 supply) might lead to improved amino acid production by diazotrophic bacteria. Fed-batch cultures where nutrients are added in the medium during the culture can help in reaching the desired physiological state while increasing amino acid productivity, and such studies probably deserve more attention.

References

Beijerinck MV (1901) Ueber oligonitrophile Microben. In: Rubenchick I (ed) *Azotobacter* and its use in agriculture. Washington 1963, National Science Foundation, p 1

Cacciari I, Lippi D, Ippoliti T, Pietrosanti T, Pietrosanti W (1989) Response to oxigen of diazotrophic *Azospirillum brasilense-Arthrobacter giacomelloi* mixed batch culture. Arch Microbiol 152: 111–114

Dahm H, Rozycki H, Strzelczyk E, Li CY (1993) Production of B-group vitamins by *Azospirillum* spp. grown in media of different pH at different temperatures. Zentralbl Mikrobiol 148: 195–203

Dalton H, Postgate JR (1969) Growth and physiology of *Azotobacter chroococcum* in continuous culture. J Gen Microbiol 56: 307–319

González-López J, Salmerón V, Moreno J, Ramos-Cormenzana A (1983) Amino acids and vitamins produced by *Azotobacter vinelandii* ATCC 12837 in chemically-defined media and dialysed soil media. Soil Biol Biochem 15: 711–713

Janzen RA, Rood SB, Dormaar JF, Mc Gill WB (1992) *Azospirillum brasilense* produces gibberellin in pure culture on chemically-defined medium and in co-culture on straw. Soil Biol Biochem 24: 1061–1064

Kennedy IR, Tchan Y-T (1992) Biological nitrogen fixation in non-leguminous field crops: recent advances. Plant Soil 141: 93–118

Kucey RMN (1988) Alteration of size of wheat root systems and nitrogen fixation by associative nitrogen-fixing bacteria measured in field conditions. Can J Microbiol 34: 735–739

Martinez-Toledo MV, González-López J, Ramos-Cormenzana A (1985) Isolation and characterization of *Azotobacter chroococcum* from the roots of *Zea mays*. FEMS Microbiol Ecol 31: 197–203

Martínez-Toledo MV, Salmerón V, González-López J (1991) Biological characteristics of *Azotobacter* spp. in natural environments. Trends Soil Sci 1: 15–23

Moreno J, González-López J, Martinez-Toledo MV, de la Rubia T, Ramos-Cormenzana A, Vela GR (1988) Growth and nitrogenase activity of *Azotobacter chroococcum* in the presence of several phenolic acids. Arch Microbiol 150: 113–116

Okon Y, Itzighson R (1992) Poly-ß-hydroxybutyrate metabolism in *Azospirillum brasilense* and the ecological role of PHB in the rhizosphere. FEMS Microbiol Ecol 103: 131–140

Payne TMB, Ronatt JW, Lochhead AG (1987) The relationship between soil bacteria with simple nutritional requirements and those requiring amino acids. Can J Microbiol 3: 73–82

Rodelas B, Salmerón V, Martínez-Toledo MV, González-López J (1993) Production of vitamins by *Azospirillum brasilense* in chemically-defined media. Plan Soil 153: 97–101

Salmerón V, Martínez-Toledo MV, González-López J (1989) Effect of the nitrogen source on the adenine nucleotide content and energy charge of *Azotobacter chroococcum.* Chemosphere 19: 1779–1786

Senior PJ, Beech GA, Ritchie GAF, Dawes EA (1972) The role of oxygen limitation in the formation of poly-ß-hydroxybutyrate during batch and continuous culture of *Azotobacter beijerinckii.* Biochem J 128: 1193–1201

Wu FJ, Moreno J, Vela GR (1987) Growth of *Azotobacter vinelandii* on soil nutrients. Appl Environ Microbiol 53: 489–494

Zimmer W, Roeben K, Bothe H (1988) An alternative explanation for plant growth promotion by bacteria of the genus *Azospirillum.* Planta 176: 333–342

Authors' address: Prof. J. González-López, Department of Microbiology, Faculty of Pharmacy, University of Granada, E-18071 Granada, Spain.

Received May 25, 1993