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The Effect of pH on Indole-3-Acetic Acid (IAA) Biosynthesis of *Azospirillum brasilense* Sp7

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Abstract

This study reports on qualitative and quantitative aspects of indole-3-acetic acid (IAA) production of *Azospirillum brasilense* Sp7. Hereto, micro-aerobic batch cultures of *Azospirillum* were conducted in minimal medium with malate as the sole carbon source at different pH values (in the range of common soil pH values) in a controlled bioreactor. Biomass, malate, IAA concentrations as well as tryptophan (Trp) and anthranilate (AA) concentrations were measured. Irrespective of the imposed pH conditions, the kinetics of malate consumption and biomass accumulation are similar and can be modeled with a system of mass balance equations in which Monod type kinetics are assumed. Significant amounts of IAA are secreted only after a lag of several hours when malate gets depleted. The experiments illustrate that, while pH has no effect on this lag time, it has a significant

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effect on the amount of IAA produced. Furthermore, at the onset of IAA production, tryptophan also starts to accumulate in the medium while anthranilate is at its minimum value.

Keywords: *Azospirillum brasilense*, pH, indole-3-acetic acid, bioreactor

1. Introduction

Nitrogen is undoubtedly the most important plant nutrient. In this respect, the production of inorganic nitrogen fertilizers has been of immense benefit to the agricultural sector. Unfortunately, the application of fertilizers gives rise to ecological problems (Wright and Black, 1979). Alternative means have been sought for increasing crop production without generating adverse effects on the environment. The nitrogen fixing bacteria of the genus *Azospirillum* present an alternative for, or a supplement to chemical fertilization (Baldani et al., 1983; Dobbelaere et al., 2001). These free-living diazotrophic bacteria have a loose association with various grasses and other plants in both temperate and tropical regions (Döbereiner and Day, 1978; Patriquin et al., 1983). They have been referred to as plant growth promoting rhizobacteria (PGPR) (Okon, 1994) because after inoculation of *Azospirillum*, the number and length of the root hairs, the number of lateral roots and adventitious roots and the total root surface are all enhanced. Three types of plant growth promoting substances namely, auxins, cytokinins and gibberellines can be detected in supernatants of *Azospirillum* cultures (Tien et al., 1979; Reynders and Vlassak, 1979; Bottini et al., 1989). The culprit in the drastic changes in root morphology after *Azospirillum* inoculation is generally believed to be the auxin indole-3-acetic acid (Okon and Vanderleyden, 1997). Inoculation of wheat with a low IAA producing mutant showed a reduced ability to promote root development (Barbieri et al., 1991). Inoculated plants with moderate fertilizer level are as productive as heavily fertilized un-inoculated ones (Barbieri and Galli, 1993). Using *Azospirillum* mutants unable to fix nitrogen or drastically reduced in IAA synthesis, Barbieri et al. (1986) confirmed that the morphological plant root changes observed after inoculation with *Azospirillum* are attributed to bacterial IAA secretion rather than nitrogen fixation. The contribution of biological nitrogen fixation in the observed plant response has indeed often been questioned (Okon et al., 1983; Giller and Day, 1985). An increased number of lateral roots and root hairs expand the root surface area available for nutrient absorption. This results in a higher nutrient uptake by inoculated roots and an improved water status of the plant, which in turn could be the main factor enhancing plant growth. Several recent studies have shown significant

enhancement in crop yields under glasshouse and field conditions in response to inoculation with *Azospirillum* spp. (Dobbelaere et al., 2001; 2002). It has been shown that *Azospirillum* inoculants neither cause any environmental hazards (Fages, 1992) nor present any health problems in plants (Okon, 1985). Unfortunately, the yields have been somewhat inconsistent. Some portions of a farmer's land may respond very well to inoculation with *Azospirillum* while other fields may not respond at all. These inconsistencies in response to *Azospirillum* inoculation are due to the wide variations in soil, plant and microfloral (other microorganisms in the soil) components. Factors such as temperature, pH, and the availability of nutrients can affect the synthesis of IAA by bacteria associated with plant roots. Some of these factors are growth limiting and are known to either directly or indirectly restrict plant growth through interference with processes such as chemotaxis and the development and functioning of symbiotic associations between soil microbes and plants roots. Soil pH is a crucial factor that regulates plant/microbe interaction. The work presented here represents an attempt to qualitatively and quantitatively evaluate the effect pH might have on the biosynthesis of indole-3-acetic acid by *Azospirillum brasilense* under micro-aerobic conditions.

2. Material and Methods

Bacterial strains

Azospirillum brasilense Sp7 used for this study was collected from the stock held in our laboratory (Wild type: ATCC 29145) (Tarrand et al., 1987). A preculture of 100 ml used to inoculate the Bioreactor was initiated by inoculating a 250 ml Erlenmeyer flask containing 150 ml of MMAB medium (Vanstockem et al., 1987) with a loop of cells taken from an overnight plate culture. The culture was incubated in an incubator rotary shaker (New Brunswick Scientific; USA) at 200 rpm and 30°C until an optical density (at 600 nm) of about 1.8 was reached.

Experimental conditions

A batch culture was conducted in the BioFlow 3000 benchtop fermentor (New Brunswick Scientific; USA) with an autoclavable vessel of 1.25 to 5 l working volume. 100 ml of the preculture was transferred to a 6.0 l fermentor vessel (total volume) containing 3.0 l of a previously sterilised MMAB medium. The fermentation temperature was set at 30°C, dissolved oxygen (DO) at 3%, and aeration rate 0.1 l/min. Three different pH values, 6.0, 6.3 and 6.8 were implemented. An additional fermentation was also carried out without pH

control. Sulphuric acid solution (1N) was used as the pH control solution. All fermentations were carried out without added tryptophan. Two sets of fermentations were performed in each case. The parameters of fermentation (pH, temperature, dissolved oxygen and air flow) were maintained at set points by the Proportional Integral Derivative (PID) cascade controller of the fermentor. The speed of agitation was automatically regulated to keep the DO level at the set point. In addition to the fermentor's internal controller, a bioprocess software (AFS-BioCommand, New Brunswick Scientific, USA) was routinely used to supervise the process. All data from the fermentor were transmitted to a computer loaded with the 'AFS-BioCommand®' software. Dissolved oxygen level was monitored by an autoclavable Ingold polarographic probe.

Analytical procedures

Culture media samples were taken at regular intervals and each sample divided into subsamples. Optical density at 600 nm was measured with a Perking Elmer Lamda 2 UV/VIS spectrophotometer. Biomass concentration (defined as cell dry weight per ml of culture broth) was determined by weighing dry cells with a micro balance (Mettler, Switzerland) as described by Wang and Lee (1997). A calibration curve between OD_{600} and cell dry weight was established. L-malate concentrations were determined using test kits from Roche (R-Biopharm, Germany). Indole-3-acetic acid, anthranilate and tryptophan were purified from 5 ml fermentation medium by solid phase extraction using [phenyl-$^{13}C_6$]-indole-3-acetic acid (100 ng, CIL, Andover, MA, USA), [indole-$^{2}H_5$]-L-tryptophan (100 ng, CDN-Isotopes, Quebec, Canada), and $^{15}N_1$-AA (500 ng, CIL) as internal tracers and analysed by gas chromatography/mass spectrometry (GC/MS) (Prinsen et al., 2000) as pentafluorobenzyl (PFB) ester (Epstein and Cohen, 1981). All data shown are the average of at least two replicates.

3. Results

Qualitative results

Table 1 gives a generalized comparison of the experiments carried out at various pH values. Generally, irrespective of the imposed pH conditions, the growth profiles were similar and no significant differences in biomass accumulation were observed. Malate in the fermentation medium became depleted after 8–10 hours. Fig. 1A show that the onset of IAA accumulation coincides with the time at which tryptophan is released in the medium and

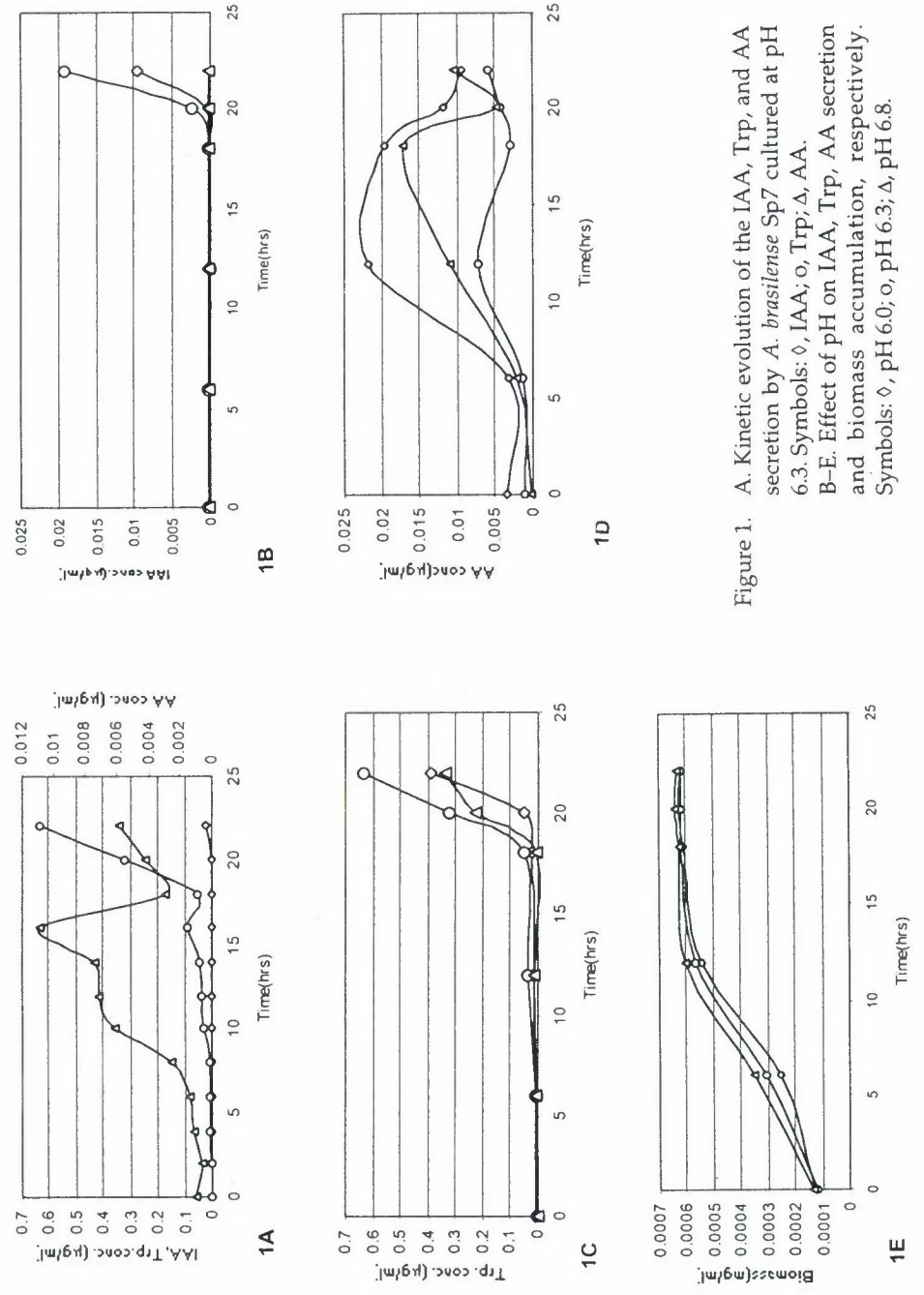

Figure 1. A. Kinetic evolution of the IAA, Trp, and AA secretion by *A. brasilense* Sp7 cultured at pH 6.3. Symbols: ◊, IAA; o, Trp; Δ, AA. B–E. Effect of pH on IAA, Trp, AA secretion and biomass accumulation, respectively. Symbols: ◊, pH 6.0; o, pH 6.3; Δ, pH 6.8.

Table 1. Data collection for malate fermentation by *A. brasilense* Sp7 with and without pH control

Parameters	Non-controlled	pH 6.8	pH 6.3	pH 6.0
Elapsed fermentation time (EFT)* (hr)	30	28	22	22
Initial malate concentration (g/l)	2.464	2.601	2.573	2.464
Maximum biomass (mg/ml)	6.41×10^{-4}	6.05×10^{-4}	6.22×10^{-4}	6.24×10^{-4}
Final pH	8.74	6.8	6.3	6.0
Maximum IAA concentration (μg/ml)	5.1×10^{-4}	6.4×10^{-4}	1.9×10^{-2}	9.1×10^{-3}

*Beyond this EFT, it became difficult to keep the culture at its set points.

anthranilate was at its lowest value. IAA was significantly secreted only after a lag of several hours (i.e., after about 20 hours) (Fig. 1B). The highest amount of IAA in the medium was detected at pH 6.3. Below and above this pH value, a reduction in IAA levels was observed (Table 1 and Fig. 1B). This reduction in IAA values was more drastic at higher pH values than at lower pH values. Figs. 1C, 1D, and 1E show the respective kinetics of Trp, AA and biomass at different pH values.

Quantitative data

The microbial growth as well as the substrate consumption during the batch experiments can be mathematically described by means of the following system of mass balance equations:

$$\frac{dC_S}{dt} = -\frac{\mu}{Y_{X/S}} C_X \qquad \text{(eq. 1)}$$

$$\frac{dC_X}{dt} = \mu C_X \qquad \text{(eq. 2)}$$

The left hand side terms denote the change in time of the malate concentration C_S [g/l] and biomass concentration C_X [OD], respectively, while the right hand side terms reflect the consumption of malate (with $Y_{X/S}$ [OD/g] being the yield coefficient of biomass on malate) and the growth of biomass, respectively. μ is the specific growth rate according to the Monod equation which is a black box equivalent of the well-known Michaëlis-Menten enzyme kinetics. The kinetic constants are the maximum specific growth rate μ_{max} and

the half saturation constant K_M. While other factors such as dissolved oxygen concentration, pH and temperature can influence the specific growth rate, in a first approach only the impact of the substrate concentration is taken into account (eq. 3).

$$\mu = \mu_{max} \frac{C_S}{C_S + K_M} \qquad \text{(eq. 3)}$$

Based on the experimental data of the batch culture conducted at pH 6, the 3 parameters were identified with the E04JAF optimization routine in MATLAB (The MathWorks Inc., Natick).

μ_{max}=0.34l/hr, K_M= 0.70g/l and $Y_{X/S}$=0.63OD/g

Fig. 2A illustrates the high quality fit of the model to the experimental data of the pH 6 batch experiment. The same model parameters were used to simulate the batch experiments at the other pH values (Figs. 2B, C and D).

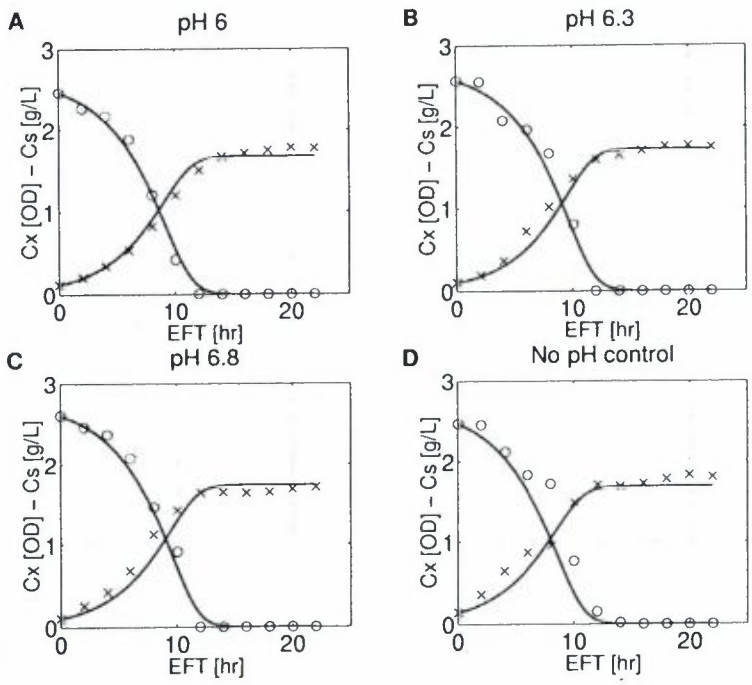

Figure 2. A–D. Biomass and malate concentration profiles at different pH values: experimental data (x,o) and model predictions (–) (eqs. 1–3).

4. Discussion and Conclusion

The results presented here indicate that pH affects the IAA production by *Azospirillum brasilense* Sp7 while the growth and substrate consumption are not significantly influenced. With respect to the latter, the proposed mathematical model based on mass balance equations with Monod type kinetics clearly explains the experimental data. As can be seen from Figs. 2B, C and D the model explains the experimental data very well without any re-identification of the model parameters for different pH values (only the initial values for the simulations are adjusted to the experimental initial values), confirming that the substrate consumption and microbial growth are not (significantly) affected by the pH value. As for the IAA production, variations in pH do not change the onset of production (i.e., in the stationary phase of growth) but do influence the total amount of IAA produced. There is a hundred fold increase in the quantity of IAA secreted at pH 6.3 when compared to values recorded at pH 6.8. pH 6.3 therefore seems to be optimal for IAA biosynthesis. The mechanism by which *Azospirillum* cells release tryptophan, anthranilate and IAA are not yet resolved. However, the observation that anthranilate accumulates in the medium during the fermentation and subsequently disappears followed by accumulation of tryptophan and IAA in the medium indicates indirectly that accumulation is not the result of cell lysis. Moreover, the tightly controlled growth conditions in the bioreactor argue against significant cell lysis. The hypothesis of Zimmer and Elmerich (1991) seems to be confirmed: tryptophan, assumed to be a precursor for IAA, attains high values when IAA is at its peak value while anthranilate (an alleged inhibitor) is at its lowest value. Since the culture medium was not supplemented with exogenous tryptophan, the quantity of IAA produced is significantly less than values normally reported in literature (Crozier et al., 1988; Zimmer et al., 1991). To refine the mathematical model such that it also includes IAA production, new (continuous culture) experiments need to be carried out, the design of which will be dictated by the growth related knowledge obtained from this study.

In conclusion, it is clearly demonstrated that pH has a drastic and specific effect on IAA production by *A. brasilense*. The reason for this is not clear. Presently genetic and biochemical experiments to decipher this mechanism are being done. Nevertheless, given the importance of IAA in plant growth promotion by *Azospirillum*, a clear understanding of the mechanism of IAA synthesis by *A. brasilense* should be regarded as a priority in research on plant growth promoting rhizobacteria. Moreover when conducting and reporting on field inoculation studies with *Azospirillum*, we consider it of utmost importance to specify the physico-chemical properties of the soil.

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