A PROTOTYPE MODEL FOR INDOLE-3-ACETIC ACID (IAA) PRODUCTION BY *AZOSPIRILLUM BRASILENSE* SP245

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Abstract: Given the vital role of nitrogen for plant growth on the one hand, versus the severe impact on the environment of extensive use of inorganic nitrogen fertilizers on the other hand, the need for alternative fertilization becomes apparent. In this respect, the nitrogen fixing bacteria of the genus *Azospirillum* offer interesting perspectives since inoculation of the plant roots with these bacteria positively affects plant growth. Studies have shown that the growth promoting power of the bacteria can probably be attributed more to the production of the auxin indole-3-acetic acid (IAA) than to the nitrogen fixation qualities of the genus. The aim of this study is therefore to characterize and quantify the IAA production of *Azospirillum brasilense* Sp245 on a macroscopic level as to exploit the resulting model in optimal experiment design studies. The data obtained from these optimal experiments should be rich enough to aid in further unravelling the studied IAA production mechanism.

Keywords: *Azospirillum brasilense*, IAA production, bioreactor model identification

1. INTRODUCTION

Given the fact that nitrogen is undoubtedly one of the most important plant nutrients (together with inorganic phosphate), the production of inorganic nitrogen fertilizers has been of immense benefit for the agricultural sector. Unfortunately, the intensive application of fertilizers gives rise to severe ecological problems (Wright and Black, 1979). Alternative means have been sought to increase crop production without compromising the environment. In this context, the nitrogen fixing bacteria of the genus *Azospirillum* present a challenging alternative for, or a supplement to chemical fertilization (Baldani *et al.*, 1983; Dobbelaere *et al.*, 2001) since they belong to the class of *plant growth promoting rhizobacteria*. Indeed, after inoculation of *Azospirillum* spp., a significant change in root morphology can be detected. The key factor in this root number and root surface increase after *Azospirillum* inoculation is generally believed to be the auxin indole-3-acetic acid (IAA) (Okon and Vanderleyden, 1997). 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; Dobbelaere *et al.*, 2002). In addition, it has been shown that *Azospirillum* inoculants neither cause any environmental hazards (Fages, 1992) nor present any health prob-

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lems in plants (Okon and Vanderleyden, 1985). Unfortunately, not all field results are consistent.

Since the (factors that influence the) IAA production mechanisms by *Azospirillum* are not fully understood yet, the aim of this paper is to quantitatively assess the IAA production by *Azospirillum brasilense* Sp245 based on a set of strictly controlled bioreactor experiments. The resulting model can then be used to design future, *information rich* experiments to rule out certain hypotheses and to extend as such our knowledge on this production mechanism. The structure of the paper is as follows. First the material and methods are introduced after which the growth of the biomass on the carbon source is modelled (Section 3). In a second stage (Section 4), the IAA production process is quantified. Afterwards, it is outlined how the obtained preliminary model can be exploited to design future experiments (Section 5). Finally, Section 6 summarizes the conclusions.

2. MATERIALS AND METHODS

Bacterial strain. *Azospirillum brasilense* Sp245 was collected from the stock held in the CMPG laboratory at the K.U.Leuven. The preculture used to inoculate the bioreactor was initiated by inoculating a 250 mL Erlenmeyer flask containing 150 mL of a minimal malate medium (MMAB) (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 mm) of about 1.8 was reached.

Experimental conditions. Four batch cultures were conducted in the computer controlled BioFlo 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 the fermentor vessel containing 3.0 L MMAB medium. PID cascade controllers ensured that the fermentation temperature was kept at 30°C and the dissolved oxygen (DO) at 3% (micro-aerobic range) by changing the agitation rate while keeping the aeration rate at 0.1 L/min. L-malate is provided as carbon source. To test whether pH has a significant effect on growth and/or production, in the first and third experiment the pH is kept at 6.3 while in the second and fourth experiment the pH is kept at 6.8. Exogenous tryptophane (Trp) is only added at the beginning of the batch culture (5 μg/mL) of the first and the second experiment.

Measurements. Culture media samples were removed 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. L-malate concentrations were determined using test kits from Roche. Indole-3-acetic acid and tryptophane concentrations in the fermentation medium were determined by Gas Chromatography/Mass spectrophotometry (GC/MS) (Prinsen *et al.*, 2000). All data shown are the average value of at least two replicates.

Mathematical tools. The implemented identification routine for model parameter estimation is the `e04UCF` routine from the NAG library (Numerical Algorithms Group) in Fortran. Apart from Fortran, Matlab 6.1 (The Mathworks Inc., Natick) was used as simulation software. It must be mentioned that the absolute and relative tolerances of the integration routines had to be set to a very small value (i.e., 10^{-8}) to ensure correct simulations.

3. GROWTH MODELLING

Since there is no evidence that malate consumption or biomass growth is influenced by the presence of Trp or IAA in the medium, the growth process is first tackled as a separate phenomenon. When focussing on (*macroscopic*) mass balance equations for batch reactors, the only term that needs to be specified is the reaction term since no transport has to be taken into account.

For the modelling of the specific growth rate μ, a simple monotonically increasing function, i.e., the Monod equation is proposed. The link between the specific growth rate and the substrate consumption is the so-called *linear law* in which, for the time being, the maintenance term is neglected. All substrate consumed is therefore assumed to be built in as new biomass with a certain efficiency or yield factor $Y_{X/S}$ [OD L /g]. The evolution in time of the substrate concentration (i.e., malate) C_S [g/L] and the biomass concentration C_X [OD] is then described by following system of mass balance equations:

$$\begin{aligned} \frac{dC_S}{dt} &= -\frac{\mu}{Y_{X/S}} \cdot C_X \\ \frac{dC_X}{dt} &= \mu \cdot C_X \end{aligned} \tag{1}$$

in which

$$\mu = \mu_{max} \frac{C_S}{C_S + K_M}. \tag{2}$$

In this specific growth rate expression, μ_{max} [1/h] is the maximum specific growth rate and K_M [g/L] the half saturation constant. As can be seen, only the influence of the carbon source is taken into account at this stage.

Inspection of Equations (1) and (2), reveals that 5 parameters have to be identified, i.e., the yield factor $Y_{X/S}$, the Monod growth constants μ_{max} and K_S and the initial values of the substrate and biomass concentration $C_S(0)$ and $C_X(0)$ (to account for measurement errors these latter initial conditions are also considered as degrees of freedom during parameter identifcation). Hereto, following sum of squared errors (SSE) between the model predicted and experimentally obtained values for substrate and biomass concentration is minimized:

$$\begin{aligned} SSE_G = & \sum_{k=1}^{n_{C_S}} (C_S(k) - C_{S,exp}(k))^2 \\ & + \sum_{k=1}^{n_{C_X}} (C_X(k) - C_{X,exp}(k))^2 \end{aligned} \tag{3}$$

Growth parameters					
	Initial[a]	pH6.3+	pH6.8+	pH6.3-	pH6.8-
$C_S(0)$	exp[b]	2.426	2.477	2.427	2.595
$C_X(0)$	exp	0.079	0.099	0.088	0.195
μ_{max}	1.000	0.421	0.612	0.414	0.253
K_S	1.000	0.439	1.425	0.473	$1{\cdot}10^{-7}$
$Y_{X/S}$	0.800	0.777	0.756	0.745	0.636
SSE_G		0.090	0.088	0.045	0.036

[a]the initial values to start the identification procedure
[b]the first experimental data point

Table 1. Growth parameter estimates for the 4 experiments. The + and - sign denote addition of exogenous Trp or not.

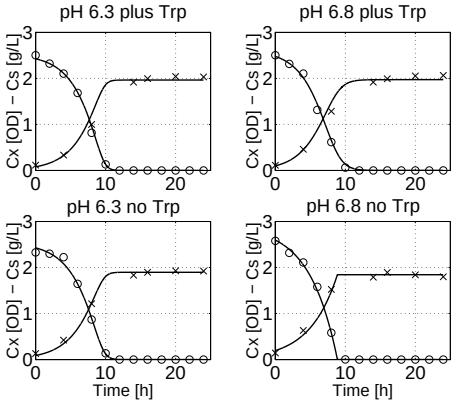

Fig. 1. Biomass and malate concentration profiles at different pH values and with or without extra Trp: experimental values (x,o) and model predictions (–) (Equations (1) and (2) and Table 1).

with n_{C_S} and n_{C_X} the number of experimental data points for the substrate and biomass concentration, respectively. Since the measurements are in the same order of magnitude, no rescaling of the individual errors is performed.

The obtained growth parameter sets for each experiment are listed in Table 1 together with the corresponding SSE_G value. As can be seen from Figure 1, these parameter values give rise to excellent model fits.

However, correct identification of the parameters is not a trivial task since (*i*) the experimental data points are scarce and (*ii*) batch experiments are known as not the most optimal setup for estimation of both Monod constants at once (Holmberg and Ranta, 1982). When plotting the value of the SSE_G for a whole range of μ_{max} and K_S values as is done in the contour plot of Figure 2 for the first experiment, it is apparent that their is a whole *valley* in which several local minima can appear. Indeed, the final values of the parameter estimation routine are largely affected by (*i*) the initialization of the parameters and (*ii*) the quality and positioning of the data points. Since there are relatively few data points at the zones of significant transition in dynamics, a small difference in the measurements for C_S and C_X will

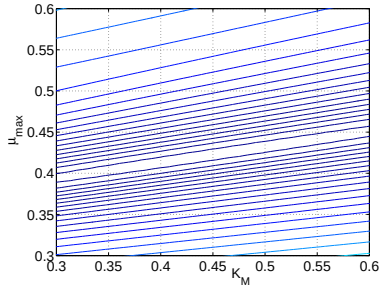

Fig. 2. Contour plot for the SSE_G value obtained for a wide range of μ_{max} and K_S values.

have a large influence on the final value, probably explaining the difference in the K_S values for both pH setpoints. Nevertheless, the estimated parameter sets from one experiment can be used to describe another data set, i.e., at another pH value (results not shown due to space limitation) and a significant pH effect on growth cannot be inferred from the data at hand.

Section 5 discusses how the identifiability problem related to the Monod kinetics constants can be overcome.

4. IAA PRODUCTION MODELLING

While for the modelling of the biomass growth and substrate consumption one could rely on intuitive microbiological knowledge, the quantification by a mass balance type model of the IAA production by *Azospirillum* has, to the authors knowledge, not been published yet. Evidently, some hypotheses have been formulated with respect to the influencing factors of this production process. One of these hypotheses, namely, the need for additional, exogenous tryptophan as a precursor (Zimmer *et al.*, 1991), is explored in this paper. The experimental results for IAA in the four data sets are illustrated in Figure 3. When no exogenous Trp is added (upper plots), very limited (in fact hardly detectible) amounts of IAA are produced. If, however, an initial amount of 5 μg/mL of Trp is added to the batch bioreactor, the IAA production soon becomes significant, i.e., already during the exponential growth phase (lower plots).

Remains the question whether the production of IAA is growth related or not, i.e., whether the growth limiting substrate (or the energy derived from it) is directly needed in the IAA production pathway or not. Two model types for the IAA production are therefore proposed. In the first model hypothesis, IAA is formed due to the presence of Trp only but the biomass is needed as (bio)catalyst. In the second hypothesis, production of IAA is also growth related. Following sets of additional mass balance equations are proposed for the evolution in time of the IAA concentration [μg/mL] and Trp concentration [μg/mL].

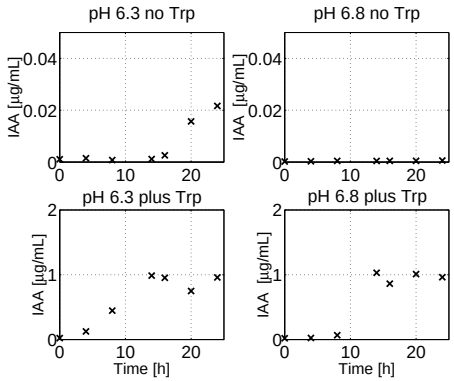

Fig. 3. Experimental IAA profiles at different pH values and with or without extra Trp.

Production model hypothesis 1 (H1)

$$\begin{aligned} \frac{dTrp}{dt} &= -A_T \frac{Trp}{Trp + K_T} \cdot C_X \\ \frac{dIAA}{dt} &= Y_{P/X} \cdot \frac{Trp}{Trp + K_T} \cdot C_X \end{aligned} \tag{4}$$

Production model hypothesis 2 (H2)

$$\begin{aligned} \frac{dTrp}{dt} &= -A_T \frac{Trp}{Trp + K_T} \cdot C_X \\ \frac{dIAA}{dt} &= Y_{P/X} \cdot \mu \cdot \frac{Trp}{Trp + K_T} \cdot C_X \end{aligned} \tag{5}$$

with μ [1/h] the Monod expression of Equation (2), A_T [μg/h] and K_T [μg/mL] Monod kinetics constants for the Trp consumption and $Y_{P/X}$ [μg/h] for H1 and [μg] for H2 a yield like coefficient of IAA on biomass.

In the first model hypothesis, the left hand side of the balance equation for IAA reflects the need for Trp via a Monod like *switching* function: if the amount of Trp is very low, no IAA will be produced but once Trp is present (to some extent) IAA production can occur. In the second model hypothesis this Trp dependent term is multiplied with the specific growth rate μ to reflect the presumed dependency on the growth.

The global identification cost SSE_T is equal to the sum of the SSE for growth (Equation (3)) and an SSE term for the production related concentrations:

$$SSE_T = SSE_G + SSE_P \tag{6}$$

with

$$\begin{aligned} SSE_P = & \sum_{k=1}^{n_{Trp}} (Trp(k) - Trp_{exp}(k))^2 \\ & + \sum_{k=1}^{n_{IAA}} (IAA(k) - IAA_{exp}(k))^2 \end{aligned} \tag{7}$$

Production parameters					
		Hypothesis 1		Hypothesis 2	
	Initial	pH6.3+	pH6.8+	pH6.3+	pH6.8+
$Trp(0)$	exp	4.024	4.082	4.022	4.080
$IAA(0)$	exp	0.109	$1{\cdot}10^{-4}$	$1{\cdot}10^{-4}$	$1{\cdot}10^{-4}$
A_T	1.000	0.276	0.246	0.275	0.245
K_T	1.000	$1{\cdot}10^{-4}$	$1{\cdot}10^{-4}$	$1{\cdot}10^{-4}$	$1{\cdot}10^{-4}$
$Y_{P/X}$	1.000	0.056	0.058	0.476	0.457
SSE_T		0.197	0.542	0.176	0.878

Table 2. The identified production parameter sets for the experiments with exogenous Trp (with the identified growth parameters of Table 1) for both hypothesis.

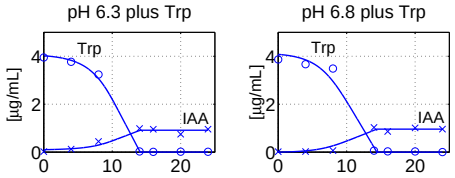

Fig. 4. IAA and Trp profiles at different pH values and with exogenous Trp: experimental values (x,o) and model predictions (–) following H1, i.e., Equations (1), (2) and (4).

with n_{Trp} and n_{IAA} the number of experimental data points for the Trp and IAA concentration, respectively.

To avoid an overload of parameters to be identified, the growth related parameters were first fixed to their optimal values (as listed in Table 1). The identified values for the remaining parameters (for both hypotheses) can be found in Table 2. Re-identification of all 10 parameters afterwards revealed that the identified values hardly changed. The difference between the identified values for $Y_{P/X}$ in both hypotheses, being an order of a magnitude smaller for H1 can probably be attributed to the different units for this parameter.

Obviously, no production related parameters have been identified for the experiments to which no Trp is added, and in which accordingly hardly any IAA is produced. Both model hypotheses will predict zero IAA production which is in accordance with the experimental data since the recorded values, which are close to the detection limit, are questionable.

Figures 4 and 5 illustrate the model predictions following the first and second hypothesis for the experiments to which exogenous Trp was added. As can be seen, for H1, the IAA production ceases when the Trp concentration goes to zero, while for H2, the IAA production levels already when the malate concentration becomes depleted. However, the experimental data at hand do not enable discrimination between the proposed hypotheses, since both figures illustrate a positive match between the model predictions and the experimental data.

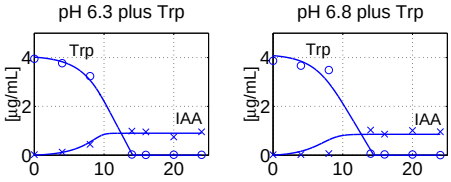

Fig. 5. IAA and Trp profiles at different pH values and with exogenous Trp: experimental values (x,o) and model predictions (–) following H2, i.e., Equations (1), (2) and (5).

	Set 1	Set 2	Set 3
$C_S(0)$	2.390	2.364	2.263
$C_X(0)$	0.056	0.011	0.010
μ_{max}	0.476	41.078	0.589
K_S	0.475	136.002	0.010
$Y_{X/S}$	0.802	0.828	0.875
$Trp(0)$	3.983	3.933	4.036
$IAA(0)$	0.005	0.013	0.022
A_T	0.281	0.295	416.975
K_T	$1{\cdot}10^{-4}$	$1{\cdot}10^{-4}$	2243.264
$Y_{P/X}$	0.467	0.453	330.529
SSE_T	0.161	0.237	0.598

Table 3. Different parameter sets for the first experiment, obtained by starting from different initial parameter estimates.

5. OPTIMAL EXPERIMENT DESIGN

To refine (and/or validate) the proposed model structure, following necessary steps are proposed. First of all, the uniqueness problem of the identified parameter values has to tackled. Afterwards, the alleged growth dependency of the production process must be investigated. Finally, it must be examined whether IAA production is persistent with prolonged availability of malate and tryptophan.

5.1 *Uniqueness of the parameter values*

If Monod type kinetics have to be identified from batch experiments, it is known that the parameters will be highly correlated and a unique identification will be hard since the cost surface is littered with local minima. As an illustration, Table 3 lists three sets of parameters that exhibit more or less the same identification performance (reflected by similar SSE_T values). The first two sets differ at the level of growth constants while the latter has different Trp consumption rate constants.
Intuitively, one could propose a *fed-batch* experiment with a very low feed rate in which malate is present. This more controlled feeding strategy enables to discriminate between the different parameter sets as illustrated in Figure 6. A more rigorous approach would be based on optimal experiment design. Through the optimization of a scalar function of the Fisher Information Matrix an optimal input (i.e., feeding profile) can be designed, that ensures high information content

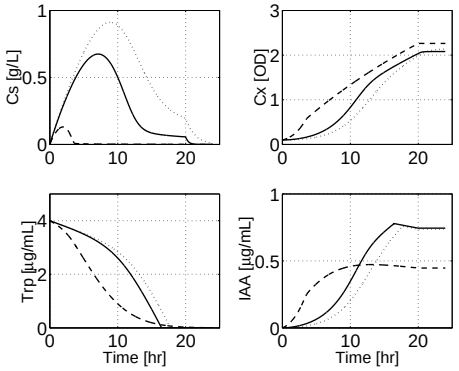

Fig. 6. Proposed fed-batch experiment to discriminate between different parameter sets. Set 1 (–), set 2 (...) and set 3 (--) as listed in Table 3. Fin = 0.0625 L/h, t_{feed}=20 h, $C_{S,in}$ = 10 g/L, $C_X(0)$ = 0.1 OD, $Trp(0)$ = 4 µg/mL, $C_S(0)$=$IAA(0)$=0 g/L.

of the data with respect to uncorrelated, unique parameter estimation (Versyck *et al.*, 1997). This is the subject of ongoing research.

5.2 *IAA production: growth related or not?*

To overrule or validate the hypothesis that the IAA production is growth related, the time instant at which the malate becomes depleted must be significantly different from the instant at which Trp becomes exhausted. Although chemostat cultures are often cited in this respect, as a first indicative experiment, a simple batch experiment with another initial malate and Trp concentration can rapidly elucidate the problem. As illustrated in Figure 7 (solid line for H1, dotted line for H2), it should then be possible to infer from the experimental data, whether IAA production stops when malate is depleted or continues until Trp is depleted (since both hypotheses are only different at the level of the IAA production mass balance, the predictions for C_S, C_X and Trp are the same). A high sampling rate during significant changes in the culture dynamics is recommended. Also for this type of *model structure discrimination*, techniques of optimal experiment design exist, some of which will be tested in future (Munack, 1992).

5.3 *Persistence of IAA production*

With the obtained model (and its parameters) a fed-batch experiment can be designed that maintains the substrate and Trp concentration in the reactor at a (low) constant level. As such, depletion of the growth/production limiting factors can be avoided. It is then possible to check whether *Azospirillum* IAA production is persistent or that other (limiting) factors or product degradation have to be taken into account.

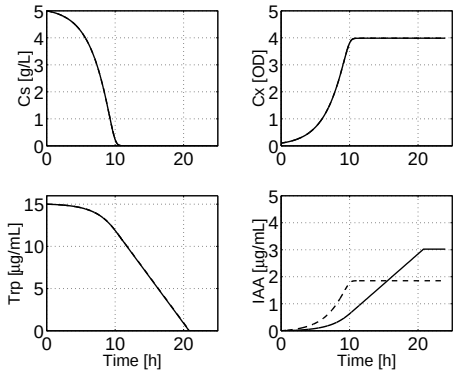

Fig. 7. Proposed batch experiment to discriminate between the two production hypotheses H1 (–) and H2 (- -). $C_S(0)$=5 g/L, $Trp(0)$ = 15 μ/mL, $C_X(0)$ = 0.1 OD, $IAA(0)$=0 μg/mL.

6. CONCLUSIONS

In this paper, a prototype model for the production of IAA by *Azospirillum brasilense* Sp245 is proposed. IAA is an auxine that could play an important role with respect to soil fertilization since it is shown that the inoculation of plants with the IAA producing *Azospirillum* species, has a positive effect on plant growth and crop yield. Based on the experimental batch data at hand, a simple, Monod law based model for the growth of the bacteria on malate is deduced. pH was not observed to have a significant effect on growth nor on production. The unique identification of the kinetic parameters μ_{max} and K_S asks however for additional fed-batch experiments. For the IAA production process, no distinction between the two hypotheses (only Trp or Trp and growth dependency) could be made on the basis of the available data. Future research will focus on accurate parameter estimation and model structure discrimination by means of optimal experiment design and the realization of the optimal proposed experiments.

ACKNOWLEDGMENTS

Ilse Smets and Kristel Bernaerts are postdoctoral fellows with the Fund for Scientific Research Flanders (FWO). Work supported in part by Projects FWO-G.0085.03, OT/99/24 and OT/03/30 of the Research Council of the Katholieke Universiteit Leuven and the Belgian Program on Interuniversity Poles of Attraction, initiated by the Belgian State, Prime Minister's Office for Science, Technology and Culture. The scientific responsibility is assumed by its authors.

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