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Estimation of *Frankia* growth using Bradford protein and INT reduction activity estimations: application to inoculum standardization

Y. Prin, M. Neyra [1] and H.G. Diem

BSSFT (CTFT/Orstom) Nogent sur Marne Cedex, France

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1. SUMMARY

The growth of *Frankia* spp. strain ORS 020607 in BAP medium was studied by using two methods simultaneously: determination of Bradford protein content and INT (2-(*p*-iodophenyl-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride) reduction activity (IRA). With the latter test, red formazan crystals formed intracellularly were extracted with methanol. Colouration intensity was estimated by absorbance spectrophotometry at 490 nm. The protein content and IRA of the culture were monitored for 96 days. IRA appeared to reflect the 'metabolically active' biomass of *Frankia* more accurately than the Bradford protein estimations.

Correspondence to: Y. Prin, BSSFT (CTFT/Orstom), 45 bis, Av. de la Belle Gabrielle, 94736 Nogent sur Marne Cedex, France.

2. INTRODUCTION

Frankia is a soil nitrogen-fixing actinomycete whose growth characteristics have been increasingly studied during the last decade. However, because of its filamentous form and very low biomass production in vitro, estimating growth has been difficult. Growth in a liquid medium has been measured through determination of cell densities, packed-cell volumes and dry weight. More accurate methods of growth estimation are based on total protein content or total organic carbon [1]. All these methods reviewed by Nittayarjarn and Baker [2] provide an estimate of the total cell mass but do not bring out differences between active and inactive *Frankia* cells. More attractive is the estimation of adenosine triphosphate [3], but the method is too time-consuming and costly to be used routinely.

Amongst microscopy techniques used either to identify or enumerate metabolically active microorganisms or both, the technique based on in-

tracellular reduction of tetrazolium salts to red formazan crystals by dehydrogenase enzymes is of great interest because it is simple, rapid and sensitive [4]. The technique has been already used by Akkermans [5] to estimate the metabolic activity of *Frankia* vesicles in relation to nitrogen fixation. To estimate the viability of different structures of *Frankia*, Faure-Raynaud et al. [6] visualized the intracellular formation of INTFormazan resulting from the reduction of 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride (INT) by dehydrogenase enzyme. Extraction of intracellular INTFormazan (INTF) from microbial cells followed by spectrophotometric measurement has also been proposed to quantitatively estimate the viability of microbial populations [7,8]. Prior to this work, INT Reduction Activity (IRA) has never been used to estimate the changes of metabolic activity during *Frankia* growth.

Since in preliminary experiments, IRA occurring in *Frankia* hyphae seemed to reflect differences in hyphal metabolic activity, we attempted to determine whether these variations in INT reduction by metabolically active cells could be used as an indicator of *Frankia* growth. The approach used was to compare IRA with growth as determined by the conventional method of Bradford protein evaluation. Values from IRA and from the Bradford protein method were also compared as a means of standardizing the inoculum used in growth studies.

3. MATERIALS AND METHODS

3.1. Frankia isolate and inoculum preparation

The *Frankia* strain used in this study was strain ORS 020607 isolated from *Casuarina equisetifolia* [9].

Preparation of 8–10 day old inoculum. The actinomycete was first successively cultured three times for 8–10 days. This was necessary to provide an homogenous culture of *Frankia* only composed of young vegetative hyphae. Inocula were obtained by concentrating *Frankia* cultures by centrifugation in sterile 10 ml tubes (10 000 × *g* for 10 min at 4° C) using a J2 21 ME Beckman centrifuge (rotor JA-20). The pellets were then washed three times in sterile BAP medium [10]. Finally, colonies were disrupted into small hyphal fragments by forced passages through a 0.8 mm diameter needle. Each inoculum was assayed for its protein content and its IRA.

Preparation of 10, 27, 76 day old inocula. Inocula were obtained by collecting culture samples from a long term culture of *Frankia* at day 10, 27 and 76. Preparation of the inocula was as described above.

3.2. Inoculation, growth conditions and biomass estimations

The suspension of fragmented hyphae was used to inoculate stationary glass tubes (25 × 150 mm) containing 10 ml of BAP medium. After inoculation, the concentration of *Frankia* in the fresh medium was 1.3 μg protein per ml of medium. According to P. Benoist (personal communication) this concentration induced optimal subsequent growth of Frankia. This value was equivalent to 1.3 nmol IRA per ml of medium. Inoculated tubes were incubated at 28° C for 96 days. Throughout this period, estimations of protein and IRA were made at regular intervals from three different tubes.

Subsequently, 3 sets of triplicate tubes containing 10 ml of BAP medium per tube were inoculated with the three inocula obtained at day 10, 27 and 76. Because protein contents varied greatly according to the age of *Frankia*, the amount of inoculum was adjusted so that the final concentration was 1.3 μg protein per ml of medium whatever the age of the culture. 3 additional set of triplicate tubes were inoculated with the same inocula but, as indicated above, the amount of each type of inoculum used was adjusted in such a way that, after inoculation, the final concentration of the inoculum was 1.3 nmol IRA per ml of medium. In each set of triplicate tubes, the time course of Bradford protein content and IRA was followed for 15 days.

3.3. Protein and IRA evaluations

The protein concentration of the homogenized culture was determined with the Bradford procedure [11] using the 'Bio-Rad Protein Assay Kit' (Bio-Rad. U.S.A.). Cell fractions for protein assay

were boiled for 10 min in 1 N NaOH to solubilize protein, cooled and neutralized with 1 N HCl, before adding the BioRad reagent dye.

The IRA of *Frankia* was determined using the following procedure: a 0.2% (w/v) solution of INT (Aldrich) in water was prepared and 10% of this solution was added to 1 ml of homogenized culture. This mixture was incubated at 28° C for 60 min in the dark. After centrifugation at 15 000 $\times g$ for 10 min, the supernatant was completely removed. INTF was extracted in methanol at 70° C for 2 h (until complete bleaching of the pellet). The extract was then centrifuged at 15 000 $\times g$ for 10 min to remove the cell fragments, and absorbance at 490 nm was read on a Bausch and Lomb Spectronic 601 spectrophotometer. The INTF equivalent of the absorbance value was determined from a standard assay curve with concentrations of INTF (Aldrich) ranging from 1 to 7×10^{-5} M in methanol.

4. RESULTS AND DISCUSSION

4.1. Time course of Bradford protein content and IRA during a 96-day growth cycle

Variations of Bradford protein content and IRA of *Frankia* strain ORS 020607 are shown in Fig. 1. Since the time course of *Frankia* biomass expressed in terms of Bradford protein is assumed to reflect the growth of *Frankia*, the following phases can be distinguished: (A) a lag phase up to day 5 after inoculation; (B) an active growth phase between days 5 and 15: the Bradford protein content rapidly increased during this period; (C) a decline phase after day 15: the Bradford protein content decreased from 50 to about 32 μg protein per ml of medium during this phase. When the values of protein content were plotted on a logarithmic scale, the protein decrease was not clearly visible (Fig. 1) but it was statistically significant and more visible on a linear scale (not shown). This suggests that autolysis occurred in the *Frankia* culture between day 15 and 35 as already reported elsewere [10]; (D) a stabilization phase between days 35 and 96; (There were no significant differences between the measured values of proteins during this period).

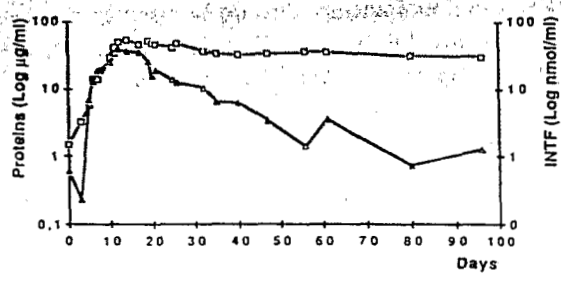

Fig. 1. Kinetics of Bradford protein content and IRA during the long-term cultivation of *Frankia* ORS 020607. Each value is the mean of three culture tubes. The standard errors are 1.86 and 1.49 for the kinetics of Bradford protein content and IRA repectively. △, IRA; □, proteins.

The IRA time course fitted relatively well with that of Bradford protein during the lag and the active phases of *Frankia* growth (day 0 to day 15). During the active growth phase, the higher the protein content, the greater was the IRA. In contrast, after day 15, IRA of *Frankia* declined much more abruptly than protein values. The sharp decline of IRA suggests that a marked reduction of metabolically active structures occurred after *Frankia* growth had reached the 15 day peak. These findings are reminiscent of those reported by Fliermans and Schmidt [12] when using autoradiography combined with immunofluorescence to detect and enumerate dead and active cells of *Nitrobacter*. They found that the proportion of active cells of *Nitrobacter* dropped off sharply while population density, as determined by direct count, remained stabilized at its peak level.

We agree with the authors who recognize that IRA is a reliable indicator of metabolic activities studied in other microorganisms [13–16], and suggest that IRA also reflects the amount of metabolically active structures in *Frankia*. The Bradford protein determination would be related to the total mass of *Frankia* cells, regardless of their metabolic state.

The rapid decrease of IRA in *Frankia* hyphae at the end of the growth phase (Fig. 1), indicates that many hyphae were probably subjected to senescence and death during the so-called stabilization phase. Microscopic observations of the strain ORS 020607, made at regular periods of time indicated that in BAP medium this strain

only formed few and small sporangia, and few or no vesicles, structures which are assumed to remain viable for longer periods than hyphae. This could explain the low level of IRA observed after day 15. After day 15 the hyphae became more and more senescent and the production of vesicles or spores was not significant enough to prevent the decrease of IRA. However, at the same time, the Bradford protein content decreased to a lesser extent. This result suggests that, under our experimental conditions, the autolysis of *Frankia* was not significant enough to induce a substantial loss of proteins by leakage of components from the dead cells.

4.2. Relationship between the method of inoculum standardization and the growth curve pattern of Frankia

When the inoculum standardization was based on IRA (1.3 nmol IRA per ml of medium) (Fig. 2), the growth curves derived from the 10, 27 and 76-day old inocula did not significantly differ from each other. This suggests that their inocula contained the same amount of live structures. With the 76-day old inoculum standardized with IRA, *Frankia* growth increased markedly only after day 5, suggesting that the active cells of the inoculum were not hyphae but mainly spores and sporangia which had to germinate before initiating new growth.

When the inoculum standardization was based on Bradford protein (1.3 μg protein per ml of medium), a marked decrease of biomass was observed prior to the active growth phase in the culture inoculated with 76-day old material. The growth was subsequently delayed (Fig. 3). The 10, 27 and 76-day old inocula standardized at 1.3 μg protein per ml of medium had IRA values of 1.3, 0.6 and 0.08 nmol per ml of medium respectively. The very low IRA value of the 76-day old inoculum may explain the behaviour of that culture, if it indicates that the inoculum contained mainly inactive or inviable biomass.

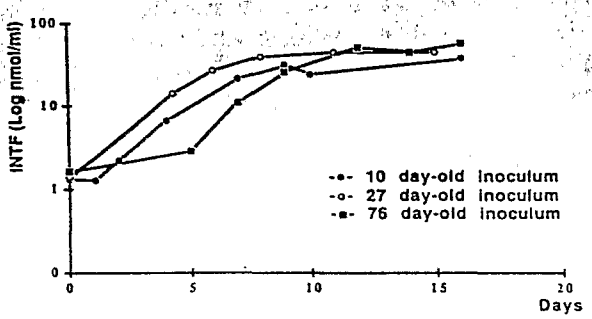

Fig. 2. Growth curves of *Frankia* ORS 020607 using inocula standardized in term of IRA. In the three cases, the inoculum contained the same amount of IRA (1.3 nmol INTF per ml of fresh medium). Each value is the mean of three culture tubes. The standard errors are 2.88, 3.6 and 4.84 for the curves obtained from the 10, 27 and 76-day old inocula respectively.

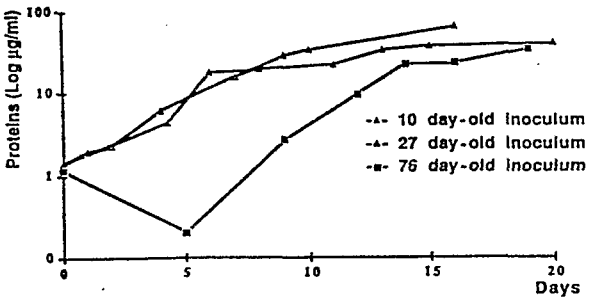

Fig. 3. Growth curves of *Frankia* ORS 020607 using inocula standardized in term of Bradford protein content. In the three cases, the inoculum contained the same amount of Bradford protein (1.3 μg of protein per ml of fresh medium). Each value is the mean of three culture tubes. The standard errors are 4.29, 3.08 and 2.76 for the curves obtained from the 10, 27 and 76-day old inocula respectively.

While protein content only indicates the total biomass, IRA estimation provides a complementary method to study accurately the changes of *Frankia* metabolic activity during its different growth phases. Moreover, standardization of the inocula based on IRA estimations rather than on total protein estimations should be used to ensure the reproducibility of *Frankia* experiments. Another advantage of the IRA technique is that it could be used in checking the viability of long-term preserved cultures and the effectiveness of commercial inoculants.

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