

## Evaluating the Growth Performance of Two *Azotobacter* spp. in Liquid Glucose Broth and Tryptic Peptone Broth as Inoculum for the Production of Bio-Fertilizers

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**ABSTRACT.** Biofertilizers with *Azotobacter* spp. releases available nitrogen to the plant rhizosphere. A media that yield a high viable inoculum cell count in a shorter incubation period is important for commercial production of biofertilizers. Current study evaluates the growth performances of *Azotobacter chroococcum* and *Azotobacter vinelandii*, two spp. commonly found in sugarcane rhizosphere, in two broth cultures: tryptic peptone broth (TPB) and liquid glucose broth (LGB) using optical density (OD) measurements at a wavelength of 620 nm over an incubation period of 12 - 15 hours. The analysis of variance revealed significant ( $p < 0.001$ ) species, broth and species  $\times$  broth interaction on OD. *Azotobacter* spp. had a higher growth rate in the protein enriched TPB compared to LGB. *Azotobacter chroococcum* had a higher growth rate than *A. vinelandii* particularly in TPB. Despite the cost, *A. chroococcum* inoculum grown in TPB can be recommended for commercial biofertilizer production, due to yield and time advantage.

**Keywords:** *Azotobacter*, Bio-fertilizers, Inoculation media, Liquid glucose broth, Tryptic peptone broth

### INTRODUCTION

Bio-fertilizers are looked upon as an eco-friendly and sustainable mode of supplementing the soil with nutrients compared to the use of synthetic fertilizers (Singh *et al.*, 2011). Previous studies have shown that nitrogen fixing bacteria such as *Azospirillum* spp. (Yasari & Patwardhan, 2007) and *Azotobacter* spp. (Kizilkaya, 2008) can potentially be used in bio-fertilizers to enhance crop production.

*Azotobacter* is a soil borne, free-living nitrogen fixing bacterial genus, abundantly found in sugarcane plantations (Tejera *et al.*, 2005). The spent-wash from sugarcane processing plants, which is rich in P, S, Fe, Mn, Zn and Cu and low in N is an ideal substrate for a biofertilizer plant. Thus, the potential of using *Azotobacter* spp. as a biological agent to produce fertilizer from sugarcane spent-wash is currently being looked at globally (Dawood *et al.*, 2005; Patil *et al.*, 2013). The spent-wash from sugarcane processing

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plants, which otherwise considered as a pollutant can be used as a culture medium for the production of bio-fertilizer. For such task, mass culturing of initial inoculum will be an utmost necessity.

A higher yield of the initial inoculum will improve the efficiency of production in terms of yield and time. For culturing *Azotobacter* spp. bacterial broths including nitrogen-free liquid glucose medium (Lipman, 1904), Burk's medium (Wilson & Knight, 1952), *Azotobacter* agar modified II (Atlas, 2010) and tryptic soy broth (TSB; as used by Aquilanti *et al.*, 2004) are used in laboratories. It is noted that the protein enriched broth cultures generally result in higher bacterial growth compared to conventional media without an added protein source (Kihara & Snell, 1960). However, the major constraint of using a protein enriched broth is the added cost of the protein supplement.

This study is aimed to compare the growth of *Azotobacter* spp. in culture media with or without an added protein source. Nevertheless, if there is a significant growth advantage, the added cost may give a better return in terms of time-efficiency and inoculum yield in biofertilizer production.

## METHODOLOGY

### Preparation and inoculation of broth media

To compare the growth performance of the *Azotobacter* spp. between the two inoculation media, *A. chroococcum* and *A. vinelandii*, two common *Azotobacter* spp. associated with sugarcane rhizosphere were selected. The two broths, tryptic peptone broth (TPB) and liquid glucose broth (LGB) were selected for the study as media with and without a protein supplement, respectively. From a pure culture of *A. chroococcum* and *A. vinelandii* 100  $\mu$ l were streaked on a modified liquid glucose agar medium [described by Lipman (1904) with the modifications made by Turner & Gibson (1980)]. The petri dishes were incubated at 30 °C for three days and a loop-full of bacteria from a single colony forming unit (CFU) was inoculated into a 150 ml Pyrex<sup>®</sup> conical flask containing 30 ml of TPB [composition of 1 liter of TPB: enzymatic digest of casein (Pancreatic digest of casein) 17.0g, peptone bacteriological 3.0g, NaCl 5.0g, K<sub>2</sub>HPO<sub>4</sub> 2.5g and dextrose (anhydrous) 2.5g] modified from Aquilanti *et al.* (2004).

The inoculated broth was incubated at 30 °C for 15 hours. From a well-mixed cultured broth, 100  $\mu$ l was used to make a ten-fold dilution series and 100  $\mu$ l of the diluted samples was inoculated into a modified liquid glucose agar medium using sterilized beads to obtain even distribution of the inoculum on the plate. After two days of incubation colony counts were made and CFU in the initial inoculum was calculated. From the broth cultures of two *Azotobacter* species in TPB, 1.5 ml (which contained approximately  $6.6 \times 10^8$  CFU) was inoculated separately into 150 ml of freshly prepared TPB and LGB in 250 ml Pyrex<sup>®</sup> conical flasks in ten replicates for each species [Composition of LGB: K<sub>2</sub>HPO<sub>4</sub> 0.29 mM, KH<sub>2</sub>PO<sub>4</sub> 1.1 mM, CaCl<sub>2</sub>·2H<sub>2</sub>O 0.137mM, MgSO<sub>4</sub>·7H<sub>2</sub>O 0.811 mM, Na<sub>2</sub>MoO<sub>4</sub>·2H<sub>2</sub>O trace, Na<sub>2</sub>MoO<sub>4</sub> trace, FeCl<sub>3</sub>·6H<sub>2</sub>O trace and Sucrose 58.4 mM, pH of the medium adjusted to 7 (Atlas, 2010)]. The broth cultures were incubated at 30 °C. After three hour incubation period the optical density (OD) of

sub-samples drawn from the broth cultures were measured using a spectrophotometer (Model: Optizen POP, Mecasys Co. Ltd., Korea) at 620 nm wavelength. For each sample, three repeated OD measurements were taken. The measurements were repeated every 90 minutes until the OD values of the broth cultures started to drop. Un-inoculated TPB and LGB samples were used as the blank samples in OD measurements.

### Data analysis

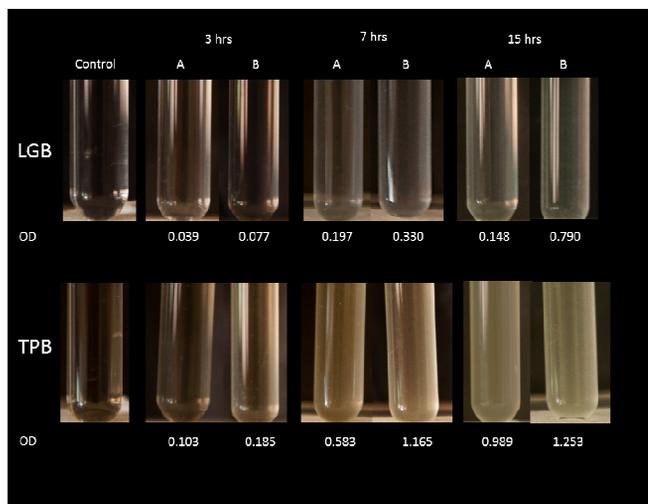
The significance of the broth type for the growth of *Azotobacter* species (OD values) was statistically analyzed using analysis of variance (ANOVA). Species, broth types, and species  $\times$  broth type interaction were used as the main effects in ANOVA. As the species  $\times$  broth type interaction was significant, regression analysis was performed for each species under each broth type separately. A non-linear regression with a second order polynomial (Delignette-Muller, 1998) was performed to explain the variability of OD values with time (i.e. with the highest  $R^2$ ). Data were analyzed using Statistical Analysis System (SAS) v9.13 (SAS Institute Inc, USA) and the interpretations were made at  $P < 0.05$ . A cost analysis was done for the preparation of 1 L of TPB and LGB based on the ingredients used. The prices were based on Fisher Scientific International Inc. USA as of 19<sup>th</sup> October, 2015.

## RESULTS AND DISCUSSION

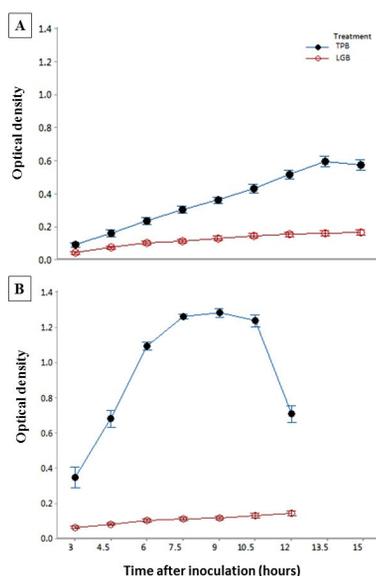
For a cost-effective production of bio-fertilizer an inoculation medium that facilitates rapid growth of *Azotobacter* spp. is essential. However, to our knowledge growth performance comparison of *Azotobacter* spp. in different media has not been reported to-date. Thus, here we report the evaluation of the growth performance of two commonly used broth media, LGB and TPB, suitable for culturing *Azotobacter* spp. as an inoculum for biofertilizer production.

The standard TSB medium, which is commonly used to culture *Azotobacter* spp., consists of enzymatic digest of soybean meal and enzymatic digest of casein as sources of nitrogen. In the modified TPB medium used in the present study, nitrogen is supplied *via* peptone (peptone and tryptophan from animal tissue digest (Oxoid Ltd., Thermo Fisher Scientific Inc., UK)) and enzymatic digest of casein (pancreatic digest of casein by Sigma-Aldrich Co., USA). The OD value directly relates to the turbidity of the medium, which is a resultant of the cell density in the broth. Turbidity of the medium increased with time irrespective of the culture medium (Fig. 1). Throughout the experiment growth rates of the two *Azotobacter* species in TPB were higher compared to that in LGB (Fig. 2A and 2B). The maximum growth of *A. vinelandii* and *A. chroococcum* were observed at 9 and 13.5 hours, respectively, after inoculation in TPB medium and continued to drop thereafter. However, in the LGB neither species reached the peak during the experimental period of 15 hours (Fig. 2 A and 2B). The observed growth patterns in the present study are in agreement with the sigmoidal growth curves for microorganisms reported in Monod (1949).

The OD values were affected by species, broth type and species × broth type interaction (Table 1). Thus, the yield of *Azotobacter* inoculum varied significantly with the different species and the broth type used ( $p < 0.05$ ).



**Fig. 1.** Comparison of media turbidity and optical density (OD) measurements over 3, 7 and 15 hours after inoculation in liquid glucose broth (LGB) and tryptic peptone broth (TPB) for (A) *A. vinelandii* and (B) *A. chroococcum*



**Fig. 2.** Variation in optical density of (A) *Azotobacter vinelandii* and (B) *Azotobacter chroococcum* when grown in liquid glucose broth (LGB – open symbol) or tryptic peptone broth (TPB – close symbol) with time, mean  $\pm$  s.e., n = 10

**Table 1. The F-statistics and probability values of ANOVA test of optical density values observed for two different *Azotobacter* species cultured in two growth media ( $p < 0.05$ )**

Source	Df	F value	P
Species	1	553.65	< 0.001
Broth type	1	1954.80	< 0.001
Species × Broth type	1	596.16	< 0.001

Changes in OD values with time could be best explained by a second order polynomial function for each species and broth type separately. The model equation is as follow (Eq1).

$$OD = m + (C_1 \times \text{time}) + (C_2 \times \text{time}^2) \text{ -----(Eq1)}$$

in which,  $m$ ,  $C_1$  and  $C_2$  are model constants.

Coefficient of determination values ranged from 76 - 95 % for the selected second order polynomial regression models (Table 2) indicating that a major proportion of the variability in OD values could be explained by the selected models using time as an independent variable. As indicted by model constant 'm', which is the intercept, the OD values in the TPB were higher compared to that in LGB even at three hours after inoculation irrespective of the *Azotobacter* spp. (Table 2). During the log phase of the growth curve, the OD values of the TPB increased at a rate of 0.19 OD increment per hour than that of LGB. Cell mass production depends on protein availability of the medium (Kihara & Snell, 1960). The reason for the slow growth rate of the *Azotobacter* spp. growing in the LGB could be the need to fix atmospheric N for catering own N demand in a N limiting medium, compared to TPB where nitrogen is supplied as peptone, tryptophan and casein proteins.

**Table 2. The coefficient of determination ( $R^2$ ), coefficient of variance (CV), mean optical density (OD) and the probability values (P) of the second order polynomial regression. The probability of the model fit is <0.001.**

Factors		$R^2$	CV	OD mean	Model constants		
<i>Azotobacter</i> spp.	Broth type *				m	C1	C2
<i>A. vinelandii</i>	LGB	0.85	14.1	0.119	-0.0205	0.0240	- 0.0008
	TPB	0.95	10.9	0.361	-0.0909	0.0599	- 0.0009
<i>A. chroococcum</i>	LGB	0.76	12.8	0.109	+0.0247	0.0154	- 0.0005
	TPB	0.93	9.4	0.946	-1.2083	0.6053	+ 0.0366

\*LGB – liquid glucose broth; TBP – tryptic peptone broth

Even though the species effect on the variation in OD values was significant irrespective of the broth type ( $p < 0.05$ ), the species *A. chroococcum* showed a higher growth performance compared to *A. vinelandii* (Fig. 2A and 2B). It is possible that *A.*

*chroococcum* might have vigorous growth capability and better nutrient assimilation ability compared to *A. vinelandii*.

The cost of ingredients per liter of LGB and TPB were USD 2.25 and 5.12, respectively [based on the prices listed in Thermo Fisher Scientific Inc., USA (<https://www.fishersci.com/us/en/home.html>) retrieved on the 19/05/2015; prices less than US \$ 0.01 were rounded to US \$ 0.01]. Use of a protein supplemented medium is 127% expensive but there is a significant advantage with respect to harvest. Further, within 9 hrs of incubation, an OD value of 1.28 can be achieved in TPB, while it remained at 0.12 in LGB. Therefore, rapid bacterial yield can be achieved in the protein-supplemented media. Even though the TPB is costly, considering the time and yield advantage, the use of a protein supplemented medium can still be considered as a feasible option for production of inoculum. To reduce the cost, we suggest to extend investigations on the use of non-purified, cost effective alternative protein sources such as crude soybean extracts in biofertilizer industry applications.

## CONCLUSIONS

In the TPB *Azotobacter* spp. showed a better growth performance than in the LGB. Thus, a growth medium supplying N such as TPB is ideal for preparing *Azotobacter* inoculum. Considering the differences in magnitude of response by *A. chroococcum* and *A. vinelandii* to TPB, it is recommended to test the response of the inoculum to selected growth medium and optimize growth conditions before using the medium in biofertilizer production.

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