Longevity of *Azotobacter* cysts and a model for optimization of cyst density in liquid bioinoculants

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Species of *Azotobacter* are known to form heat and desiccation-resistant cysts that have a long life span. Recently this property has been used to prepare nitrogen-fixing liquid bioinoculants useful for a variety of crops. We determined the survival of *Azotobacter* cysts in a liquid medium in order to estimate the shelf-life of a liquid cyst bioinoculant. A negative exponential model was fitted to the survivorship curve. The death rate was found to increase with the initial density of cysts and ranged from 0.02 to 0.05 per month. The nitrogen-fixing ability, on the other hand, dropped after two years. The shelf-life of cyst bioinoculants therefore should be decided by the nitrogen-fixing ability rather than cyst survival. Based on the derived kinetics a model for optimization of cyst density in *Azotobacter* cyst-based liquid bioinoculants is proposed.

THE use of non-symbiotic nitrogen fixer, *Azotobacter* sp., as a bioinoculant is known to benefit a wide variety of crops, due to its properties like nitrogen fixation, secretion of growth promoting substances, vitamins, antifungal metabolites and phosphate solubilization[1–5]. The conventional *Azotobacter* bioinoculants consist of vegetative cells of *Azotobacter* in peat, vermiculite, compost, lignite, etc. as carriers[6]. The carrier-based bioinoculants have a short shelf-life of about 6 months and have to be stored at temperatures not exceeding 30°C (ref. 6). The ability of *Azotobacter* to form resistant cysts has been known for a long time[5–14]. The cysts have a long life and viable cysts have been isolated from sun-dried mud bricks of the 4th to 7th century BC[15]. It is surprising, however, that for a long time cysts of *Azotobacter* were not used in bioinoculants, although methods of induction of cyst formation have been described by several workers[10,16–19]. Encystation in *Azotobacter* can be induced by *n*-butanol, beta-hydroxy butyrate or mineral deficiencies[16–19]. The cyst-based liquid bioinoculants were introduced in the mid-1990s (ref. 20) and were found to benefit a wide variety of crops[21]. Cyst bioinoculants are now available commercially which do not require a carrier and therefore can be distributed in liquid form. Liquid bioinoculants are more convenient to use as seed dip as well as foliar sprays. Foliar applications of *Azotobacter* have been shown to increase yields in paddy, wheat and sorghum[22]. The cyst-based liquid bioinoculants are claimed to have a longer shelf-life than the carrier-based ones. We studied the survivorship curve of *Azotobacter* cysts in liquid bioinoculants and developed a model for the optimization of the initial cyst density in the liquid bioinoculants. Bioplin, a cyst-based liquid bioinoculant manufactured by M/s Kumar Krishi Mitra Bioproducts (I) Pvt. Ltd. was used for this study. The product consisted of cysts of two strains of *Azotobacter*, namely KTS 11 and KTS 23 in a proportion 9:1. KTS 11 had *in vitro* nitrogen-fixing ability ranging between 15 and 17 mg/g of sugar consumed. KTS 23 had poor nitrogen-fixing ability (< 10 mg/g of sugar consumed) but showed other desirable properties including enhancement of seed germination, soil aggregation, antifungal activity, tolerance to pesticides and acidic pH[6]. The two strains had distinctive colony characters and were recognizable in plate counts. KTS 11 showed dark brownish-black pigmented, smooth colonies, whereas KTS 23 showed cream-coloured colonies with abundant slime.

The manufacturing process for Bioplin involved growing the two strains separately in 30 l fermenters in modified Jensen's medium, to get cell densities of 100×10^8 ml^{-1}, mixing them in an encystation vessel containing 0.2% calcium carbonate and 0.3% butanol for 72 h (ref. 20). This medium was observed to support growth for 5–6 generations culminating into encystation in almost 100% cells as checked by phase contrast microscopy. The final product densities were between 10^9 and 10^{11} ml^{-1}. The product bottles were stored at room temperature which fluctuated from 7 to 42°C.

One batch of the product was sampled every month up to 46 months for viable counts, packed cell volume, pH, total protein by Folin Ciocalteau method[23] and residual sugar by the phenol sulphuric acid method[24]. A total of 82 batches was monitored for cyst density with a 6-month interval for a period of 24 months.

For viable counts the sample bottles were homogenized on a rotary shaker for 10 min, and serially diluted to 10^{-8}. Vigorous shaking for 3 min before every dilution was done to overcome the tendency of cysts to form clumps. Plate counts were taken in Jensen's medium after incubation at 30°C for 6 days. No significant difference was noted in the relative proportion of the two strains throughout the monitoring period. Therefore we have used the total counts in the statistical analysis presented in the article.

Nitrogen-fixing ability was determined using an ISI recommended protocol[6]. Three to five colonies from the above, showing characteristic morphology of KTS 11 and diameter >3 mm were subcultured on Jensen's slant. The slant after incubation at 30°C for 4 days was used to seed 650 ml of Jensen's medium in a 1000 ml Erlenmeyer flask. The flask was incubated at 30°C for 4 days on a shaker at a speed of 180 rpm, after which the contents of the flask were heated in

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an evaporating dish at 80°C. 0.25 g of dry biomass was used for nitrogen estimation by Kjeldahl's method[6].

The 82 replicates from the experiment were put into 5 groups according to the initial cyst densities. The log of survivors was plotted against time for each group separately and a linear regression was fitted to each.

The cyst survivorship had a negative exponential and the log of survivors showed a linear decrease with time

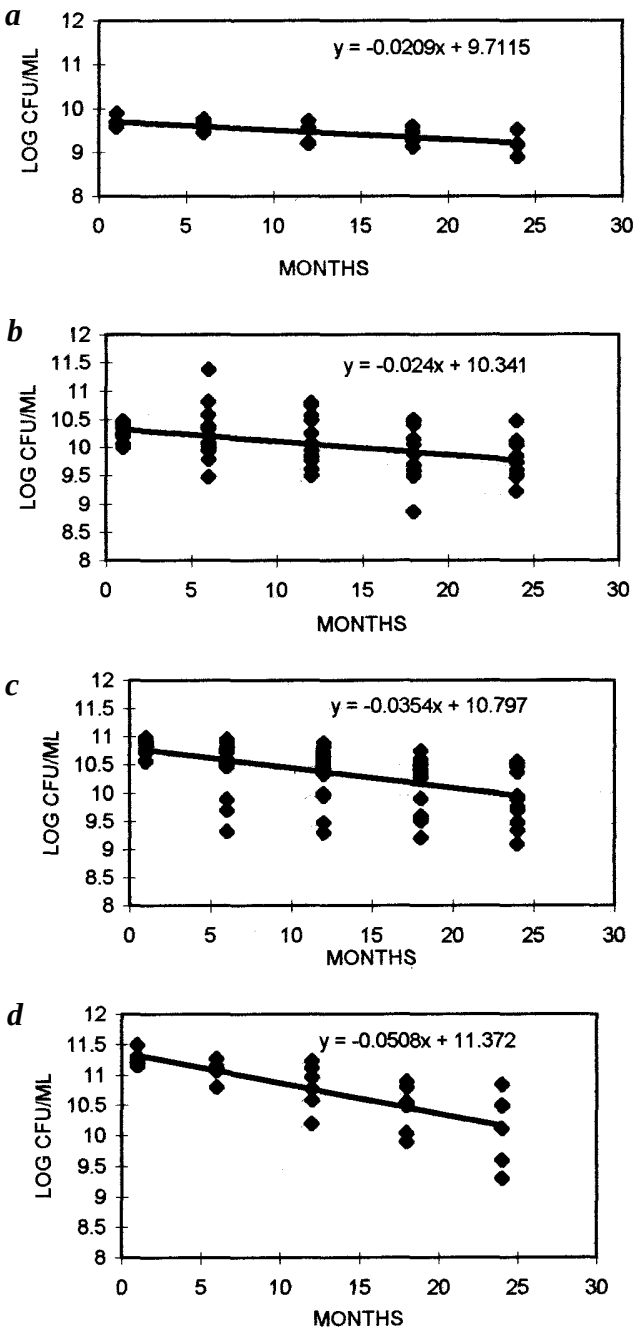

Figure 1. Death rate of cysts as a function of initial cyst density. The initial cyst density/ml is between ***a***, $10^{9.5}$ and 10^{10}; ***b***, 10^{10} and $10^{10.5}$; ***c***, $10^{10.5}$ and 10^{11}; and ***d***, 10^{11} and $10^{11.5}$.

(Figure 1). The negative slopes of the regression lines increased with increasing initial density of cysts (Figure 2). The death rates, however, were considerably slower than those reported for carrier-based *Azotobacter* bioinoculants[25]. A cyst-based liquid bioinoculant, therefore, can enjoy a much prolonged shelf-life. The ambient temperatures ranged between 7 and 42°C during the experimental period. Evidently the cyst-based liquid bioinoculant was stable at wider temperature fluctuations, unlike the vegetative cells-based carrier bioinoculants. The death curves predict that formulations having 10^9 cysts/ml would continue to give viable counts above 10^8 cysts/ml for more than four years.

The other parameters monitored namely pH, packed cell volume by centrifugation at 5000 *g*, total protein and residual sugar did not show any statistically significant trend with time (data not shown). The nitrogen-fixing abilities of the surviving population however, declined after two years. Unlike the survivorship curve, this decline was convex (Figure 3). The reason for the decline in the nitrogen-fixing ability is difficult to explain. Since the nitrogen-fixing ability was checked after two subcultures, it is unlikely to be a simple physiological consequence of starvation. It would either indicate an irreversible damage to the nitrogen-fixing system or may reflect natural selection during storage, if there is a negative relationship between survival and nitrogen-fixing ability and mutants arise during growth or storage. In any case the shelf-life of the cyst-based bioinoculants is limited by the decline in the nitrogen-fixing ability than by the survival of cysts.

Based on these findings an optimization model can be constructed for the standardization of initial cyst density in the liquid bioinoculant preparation. Since the limit to the shelf-life is set by the declining nitrogen-fixing abil-

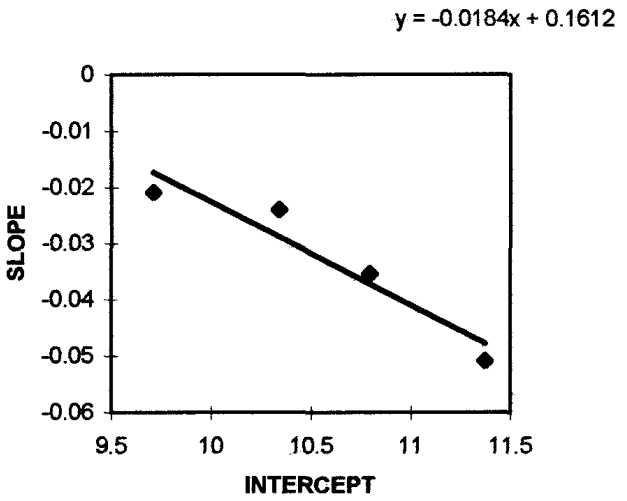

Figure 2. Relationship between death rate and mean cyst densities from Figure 1. Estimated *K* and *C* in eq. (4) are 0.1613 and 0.0184, respectively.

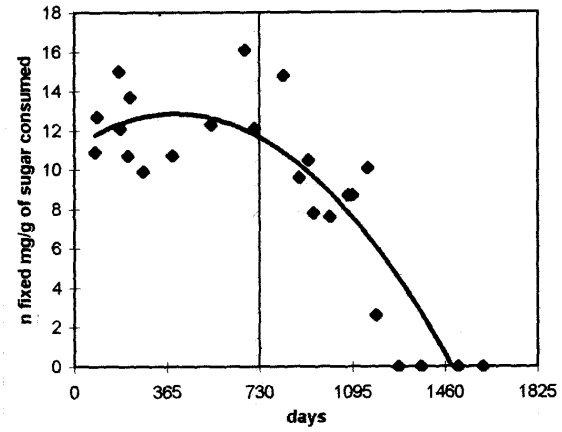

Figure 3. Decline in the nitrogen-fixing ability with time of storage of cysts in liquid formulation. The decline is sharp after two years.

ity, the target shelf-life period can be taken to be a constant, *T*. Since the cyst survival is a negative exponential, the population *X* after time *t* is given as:

$$X = X_0 e^{-D\heartsuit t} \text{ or } \log X = \log X_0 - D\heartsuit t, \quad (1)$$

where X_0 is the initial cyst density and *D* is the death rate of cysts. Since we aim at keeping a minimum viable population of cysts $X\heartsuit$ through the expiration period *T*, we can rewrite eq. (1) keeping in mind the errors in the cyst-counting methods as follows:

$$\log(X\heartsuit + 2\text{s.e.}) = \log X_0 - DT. \quad (2)$$

However, since *D* also increases with X_0 (Figure 2)

$$\log(X\heartsuit + 2\text{ s.e.}) = \log X_0 - T(K - c\log X_0), \quad (3)$$

where *K* is the intercept and *c* the slope of the line describing the relationship between initial cyst density and death rate (Figure 2).

Rearranging we get,

$$\log X_0 = [\log(X\heartsuit + 2\text{s.e.}) + TK]/(1 - Tc). \quad (4)$$

The methods of counting bacterial population generally used in quality control laboratories have a coefficient of variation between 15 and 20%. Using this expected error parameters for the strains studied above and taking $T = 24$ months, the optimum initial cyst density in the liquid bioinoculant could be calculated. For example, for the final cyst density after two years, $X\heartsuit = 10^9$ cysts/ml, the initial cyst density should be 2.45×10^9 cysts/ml and for $X\heartsuit = 10^{10}$ cysts/ml the initial cyst density should be 1.51×10^{11} cysts/ml. For different strains of *Azotobacter* the rate constants *D*, *K* and *c* are likely to differ. Apart from being useful for *Azotobacter* cysts the model might also be used for other carrier-based or liquid bioinoculants of *Azotobacter* if kinetic parameters of death on storage are estimated. At present prescribed standards exist for the minimum necessary counts of organisms needed in bioinoculants. Manufacturers usually keep a higher initial cell density so that the minimum required density is maintained until expiration. Our study points out that the rate of death could be density dependent. This model takes the possible density dependence into account which will help the manufacturer to optimize the initial density of organisms after estimating the necessary parameters required for the model.

Traditional nitrogen-fixing biofertilizers have suffered from problems of short shelf-life, instability to ambient temperatures and laborious large-scale application. The first two have been particularly important since the user has no way to know whether the product has been stored and transported under appropriate conditions before reaching him. The fact that cysts of *Azotobacter* in a liquid formulation survive and retain the nitrogen-fixing ability for over two years can boost the use of biofertilizers. A sturdy *Azotobacter* cyst-based liquid bioinoculant therefore can be more reliable and safely distributed to remote agricultural areas.

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