

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

Sandeepa Inamdar^{*,†}, Ravi U. Kanitkar^{*} and Milind G. Watve[#]

^{*}Krishi Tantra Sanstha, 12, Ganeshwadi, Off F.C. Road, Pune 411 004, India

[#]Abasaheb Garware College, Department of Microbiology, Karve Road, Pune 411 004, India

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, anti-fungal metabolites and phosphate solubilization¹⁻⁵. The conventional *Azotobacter* bioinoculants consist of vegetative cells of *Azotobacter* in peat, vermiculite, compost, lignite, etc. as carriers⁶. 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 time⁵⁻¹⁴. The cysts have a long life and viable cysts have been isolated from sun-dried mud bricks of the 4th to 7th century BC¹⁵. 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 deficiencies¹⁶⁻¹⁹. The cyst-based liquid bioinoculants were introduced in the mid-1990s (ref. 20) and were found to benefit a wide variety of crops²¹. 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 appli-

cations of *Azotobacter* have been shown to increase yields in paddy, wheat and sorghum²². 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 pH⁶. 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 $100 \times 10^8 \text{ ml}^{-1}$, 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 Ciocalteu method²³ and residual sugar by the phenol sulphuric acid method²⁴. 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 protocol⁶. 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

[†]For correspondence. (e-mail: ravikan@vsnl.com)

on a shaker at a speed of 180 rpm, after which the contents of the flask were heated in an evaporating dish at 80°C. 0.25 g of dry biomass was used for nitrogen estimation by Kjeldahl's method⁶.

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). 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* bioinoculants²⁵. 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 10⁹ cysts/ml would continue to give viable counts above 10⁸ 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-

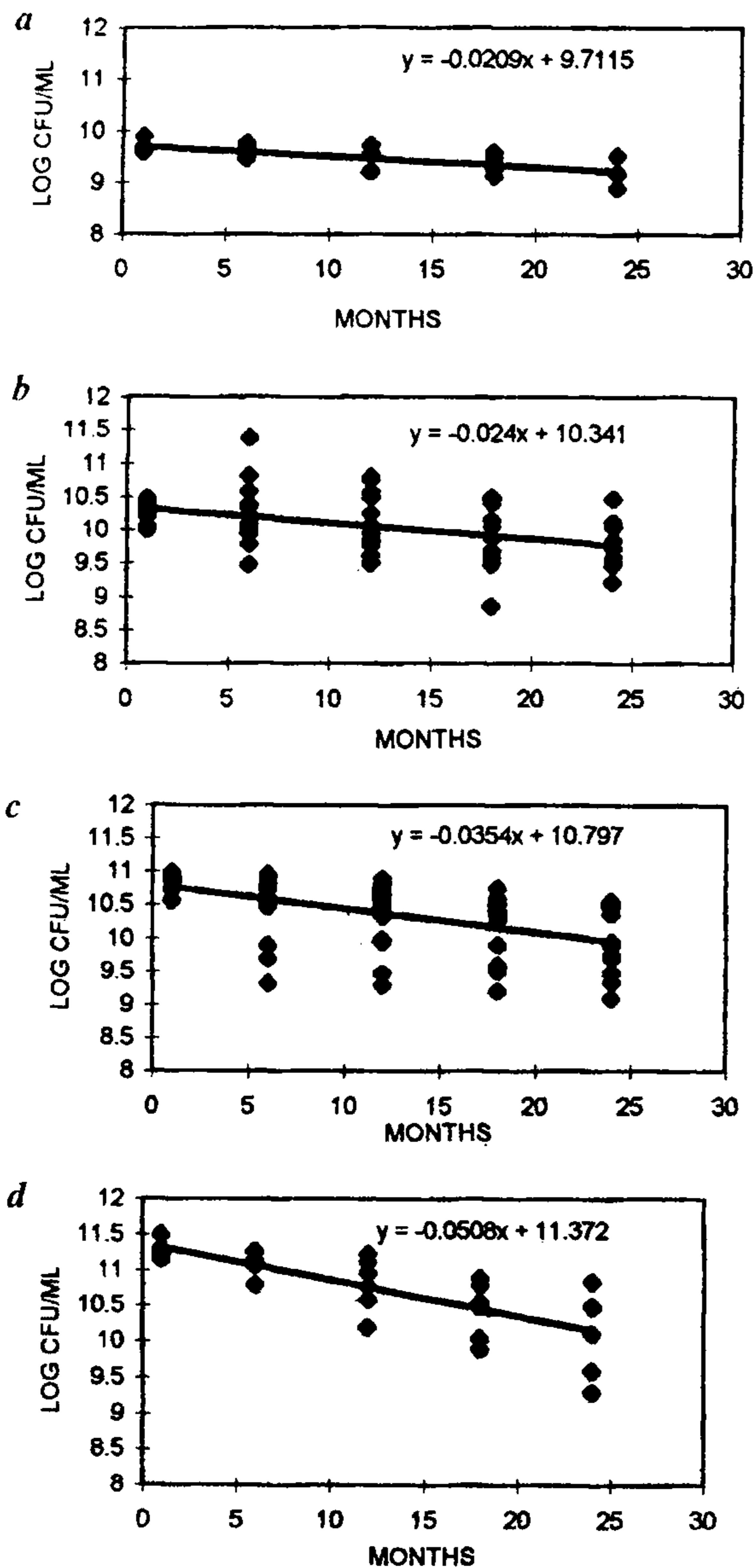


Figure 1. Death rate of cysts as a function of initial cyst density. The initial cyst density/ml is between a, 10^{9.5} and 10¹⁰; b, 10¹⁰ and 10^{10.5}; c, 10^{10.5} and 10¹¹; and d, 10¹¹ and 10^{11.5}.

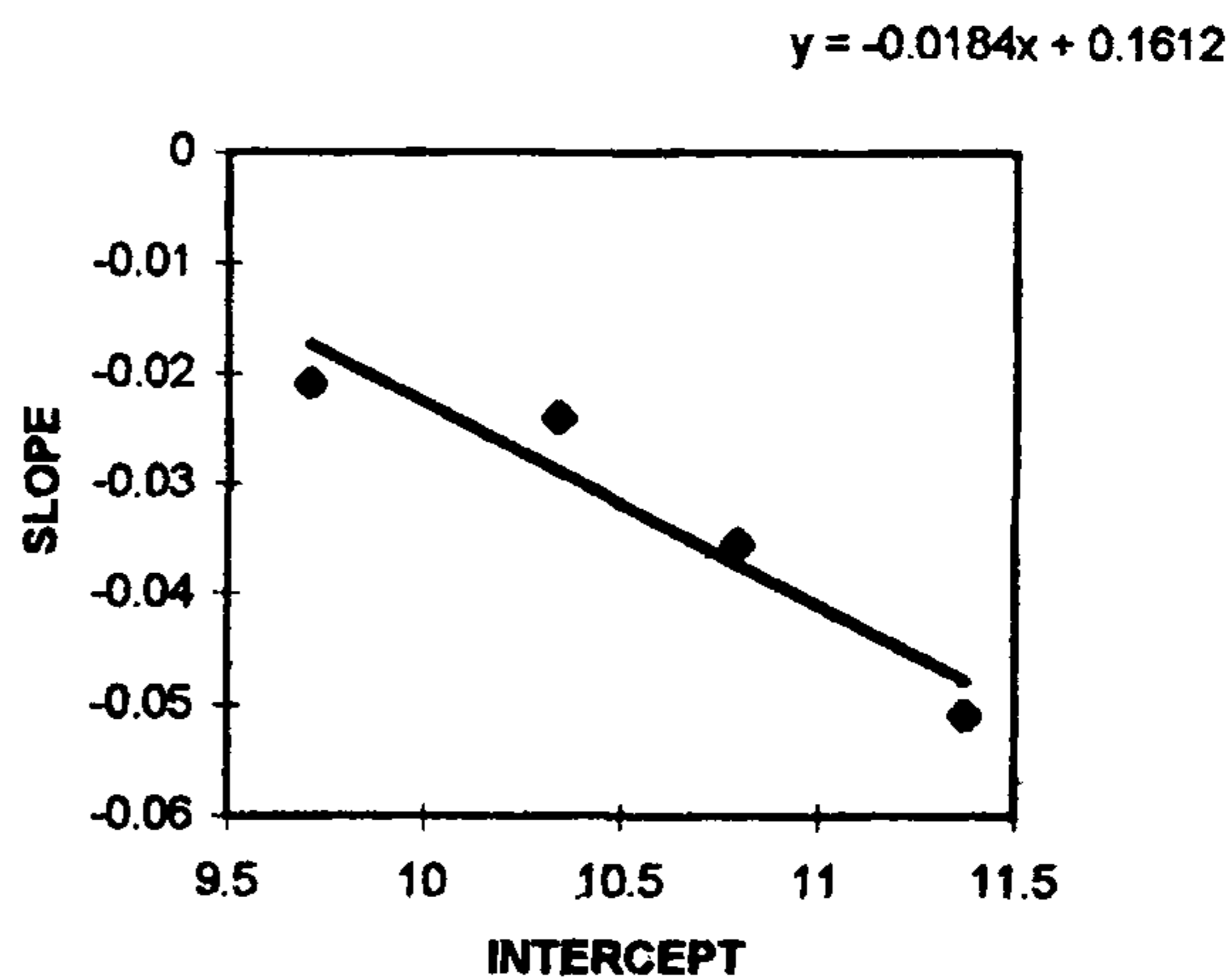


Figure 2. Relationship between death rate and mean cyst densities from Figure 1. Estimated K and C in eq. (4) are 0.1613 and 0.0184, respectively.

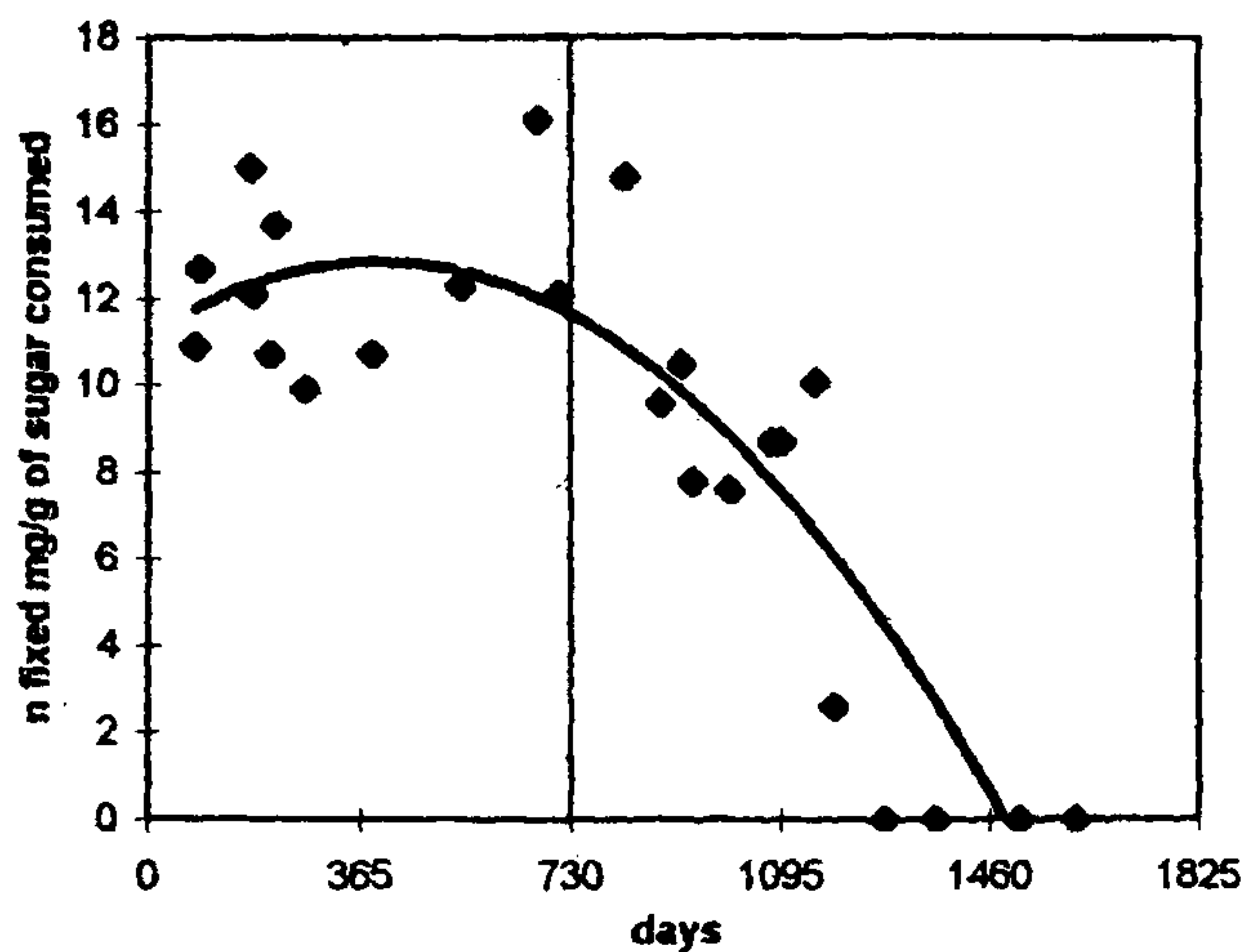


Figure 3. Decline in the nitrogen-fixing ability with time of storage of cysts in liquid formulation. The decline is sharp after two years.

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:

$$X = X_0 e^{-D't} \text{ or } \log X = \log X_0 - D't, \quad (1)$$

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' through the expiration period T , we can rewrite eq. (1) keeping in mind the errors in the cyst-counting methods as follows:

$$\log(X' + 2s.e.) = \log X_0 - DT. \quad (2)$$

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

$$\log(X' + 2 s.e.) = \log X_0 - T(K - c \log X_0), \quad (3)$$

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,

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

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, $X' = 10^9$ cysts/ml, the initial cyst density should be 2.45×10^9 cysts/ml and for $X' = 10^{10}$ 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 *Azoto-*

bacter 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.

1. Pandey, A. and Kumar Sushil, *J. Sci. Ind. Res.*, 1989, **48**, 134-144.
2. Shende, S. T. and Apte, R., Proceedings of the National Symposium held at IARI, New Delhi, 1982, pp. 25-27.
3. Brown, M. E. and Burlingham, S. K., *J. Gen. Microbiol.*, 1968, **53**, 135-144.
4. Chakre, D. S., Patil, P. L. and Patil, B. C., *Indian J. Microbiol.*, 1982, **22**, 3.
5. Mishustin, E. N. and Shilkinova, V. K., *Biological Nitrogen Fixation of Atmospheric Nitrogen*, Macmillan Press Ltd., London, 1971.
6. *Indian Standard Specification for Azotobacter chroococcum inoculants*, IS: 9138-1979, Indian Standards Institution, New Delhi.
7. Goldschmidt, M. C. and Wyss, O., *J. Bacteriol.*, 1966, **91**, 120-124.
8. Goldschmidt, M. C. and Wyss, O., *Appl. Microbiol.*, 1968, **16**, 871-876.
9. Hitchins, V. M. and Sadoff, H. L., *J. Bacteriol.*, 1970, **104**, 492-498.
10. Layne, J. S. and Johnson, E. J., *J. Bacteriol.*, 1964, **87**, 684-689.
11. Page, W. J., *Can. J. Microbiol.*, 1983, **29**, 1110-1117.
12. Socolofsky, M. D. and Wyss, O., *J. Bacteriol.*, 1961, **81**, 946-954.
13. Socolofsky, M. D. and Wyss, O., *J. Bacteriol.*, 1962, **84**, 119-124.
14. Sadoff, H. L., *Bacteriol. Rev.*, 1975, **39**, 516-539.
15. Abd-al Malek and Ishac, *Plant Soil*, 1996, **XXIV**, 325-327.
16. Hitchins, V. M. and Sadoff, H. L., *J. Bacteriol.*, 1973, **113**, 1273-1279.
17. Lin, L. P. and Sadoff, H. L., *J. Bacteriol.*, 1968, **95**, 2336-2343.
18. Page, W. J., *Can. J. Microbiol.*, 1983, **29**, 1110-1117.
19. Sadoff, H. L. and Loperfido, B., *J. Bacteriol.*, 1971, **105**, 185-189.
20. Kanitkar, R. and Inamdar, S., Indian Patent No. 178503, 1993

21. Sawant, D. M. *et al.*, *Ann. Agric. Res. Rev. Rep.*, Department of Plant Pathology and Agricultural Microbiology, Mahatma Phule Krishi Vidyapeeth, Rahuri, 1997-1998.
22. Wani, S. P. and Rai, P. V., *Indian J. Microbiol.*, 1980, **20**, 1319-1320.
23. Lowry, O. H., Rosenbrough, N. J., Farr, A. L. and Randall, R. J., *J. Biol. Chem.*, 1951, **193**, 265-275.
24. Dubois, M., Gilles, K. A., Hamilton, J. K., Rebres, P. A. and Smith, F., *Anal. Chem.*, 1956, **26**, 350.
25. Patil, A. S. and Hapse, D. G., Deccan Sugar Institute (DSTA), 36th Convention, 1986, A353-A361.

ACKNOWLEDGEMENTS. We thank the effort and co-operation rendered by our colleagues and associates at Krishi Tantra Sanstha.

Received 26 March 1999; revised accepted 3 January 2000

Territorial songs and calls of the oriental magpie robin *Copsychus saularis*

Dinesh Bhatt^{†, #}, Anil Kumar[†], Y. Singh* and Robert B. Payne**

[†]Department of Zoology and Environmental Science, and

*Department of Physics, Gurukul Kangri University, Haridwar 249 404, India

**Museum of Zoology, The University of Michigan, Ann Arbor, Michigan 48104, USA

There are very few studies on acoustic communication of Indian birds. A systematic study was made on the physical characteristics of territorial songs and calls of an oriental avian species *Copsychus saularis*, the magpie robin. Songs and calls of 14 male individuals were recorded in their territories from January 1995 to November 1997. The territorial song was delivered during territory formation and throughout the breeding season. Out of 360 song samples recorded, 84 types of song phrases were identified. These phrases were composed of 3 to 10 types of elements. Calls were uttered mainly during the non-breeding season to defend food resources in winter territory. Songs and calls differed from each other in several parameters. Typically, songs had lower frequencies and higher pulse rates, which may facilitate long-range transmission when compared to calls, increasing the conspicuousness of the male magpie robin and advertising his location to competitors and potential mates during the breeding season.

ALTHOUGH India has a large number of songbird species¹ and the Indian literature and folklore is filled with their descriptions, scientific studies on their acoustic communication are negligible. In fact, the study of

acoustic communication is a new field in India²⁻⁴. Our work in this area has generated some basic data on the vocal signals and their functional significance in the social life of a few songbirds of the Indian subcontinent viz. *Copsychus saularis*, *Sturnus pagodarum* and *Rhipidura albicollis*.

Song and call notes in birds perform a variety of functions. In many species of birds song is characteristic of the male and serves two important functions: territory establishment and maintenance through advertisement, and mate attraction⁵⁻¹⁰. Birds also use song for synchronization of reproductive behaviour between mates¹¹, mate guarding¹², mate recognition, parent-offspring recognition and neighbour-stranger discrimination¹³⁻¹⁵. Female song has also been reported in many passerine species^{16,17}. In some species the female duets with the male¹⁸⁻²². In the present study, an attempt has been made to investigate the physical characteristics and importance of territorial song and call-notes of a tropical bird, the oriental magpie robin *C. saularis*. The acoustic properties of songs and calls have been discussed in relation to territory size.

Three hundred and sixty samples of songs (18-20 song bouts per individual) and calls were recorded from 14 different males in their territories throughout the year periodically from January 1995 to November 1997 in and around Gurukul Kangri University Campus, Haridwar (29°55'N, 78°8'E) in a suburban area, using a JVC ZOOM Microphone MZ-500 and SONY CFS 1030S tape recorder. Most songs and call-notes were recorded at a distance of 2-10 m. Males were colour-banded for individual identification. The physical characteristics of the songs and call-notes were analysed with the help of Scientific 25 MHz Digital Storage Oscilloscope HM205-3 with interface HO 79-4, IEEE controller card HO 80 and signal analysis software SP 91 installed in a Pentium D × 2 100 MHz 16 MB RAM. The samples were analysed at the maximum sampling rate of 20 kHz/s, memory size 2 K × 8 bit per channel. The horizontal resolution was 200 points/cm and vertical resolution was 28 points/cm. Spectrograms were obtained from a Kay Elemetrics model 5500 signal analysis workstation (transform size 256 points (234 Hz); frequency range 16 kHz; buffer 19 s; time axis 100 ms; frequency division 2000 Hz).

A survey of the literature revealed that there was no standard terminology for the songs and calls of different species. In fact different authors used different terms for the same song categories of the same species¹⁷. We used the following criteria of classification to differentiate between song and calls on the basis of pre-existing information in the literature. A song is generally made up of a number of distinct sections called phrases. Each phrase consists of a series of similar or dissimilar units which occur together in a particular pattern. The units themselves are usually referred to as notes or elements.

[#]For correspondence. (e-mail: dd_bhatt@yahoo.com)