9. Venteurello, C. and D'Aloisio, R., *J. Org. Chem.*, 1988, **53**, 1553.
10. Hirotoshi, I. and Tsutomu, K., *Tetrahedron Lett.*, 1997, **38**, 251.
11. Sandrine, V. D., Phillippe, M. and Michel, M., *J. Mol. Catal.*, 1996, **A113**, 201.
12. Michel Ange, G., Alain, M. and Pierre, B., *Synlett.*, 1996, **11**, 1049.
13. Yao, X., Chen, H., Lu, W., Pan, G., Hu, X. and Zheng, Z., *Tetrahedron Lett.*, 2000, **41**, 10267.
14. Karjalainen, J. K., Hormi, O. E. O. and Sherrington, D. C., *Tetrahedron: Assymetry*, 1998, **9**, 1563.
15. Karjalainen, J. K., Hormi, O. E. O. and Sherrington, D. C., *Tetrahedron: Assymetry*, 1998, **9**, 3895.
16. Canali, L., Sherrington, D. C. and Deleuze, H., *React. Funct. Polym.*, 1999, **40**, 155.
17. Wing-Chi, C., Wai-Hong, F. and Chi-Ming, Che, *J. Mol. Catal.*, 1996, **A113**, 311.
18. Gelbard, G., Breton, F., Ouenard, M. and Sherrington, D. C., *J. Mol. Catal.*, 2000, **A153**, 7.

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Presence of stable active oxygen scavenging enzymes superoxide dismutase, ascorbate peroxidase and catalase in a desiccation-tolerant cyanobacterium *Lyngbya arboricola* under dry state

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Freshly collected and one- and two-year-old dry mats of a desiccation-tolerant cyanobacterium *Lyngbya arboricola*, inhabiting the bark-surface of *Mangifera indica* exhibited enhancement in the activity of active oxygen scavenging enzymes superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase in their cell-free extracts on increasing the incubation period. Comparatively, the dry mats showed considerable activity of all these enzymes in the cell-free extracts, but lower than that of freshly collected mats. Five, two and three isoforms of SOD, APX and catalase enzymes respectively, were observed in the crude extracts of the freshly collected mats on native PAGE. SOD comprised of one Mn, one Fe–Mn and three Fe-containing isoforms. Excepting the disappearance of Mn and Fe–Mn SODs and a minor loss in the intensity of the isoforms of APX and catalase on native gels, the AOS enzymes were found stable on storage of the mats for one and two years respectively, under dry state.

CYANOBACTERIA inhabiting the surfaces of barks of trees, besides frequent drying and wetting during rainy season (growing season), are found invariably exposed to extremes of desiccation from the months of October to June with variable temperatures of cold winter and hot summer seasons[1]. Recovery of photochemical reactions, ^{14}C-fixation and C_2H_2 reduction on rehydration have reflected their capacity to survive such extremes of conditions–desiccation, in particular[2–4]. Certain metabolic activities of these cyanobacteria recovered even after their treatment in dry heat at 100°C for 1 h (ref. 4). Generation of highly reactive active oxygen species (AOS), which need to be scavenged in order to maintain normal plant growth, is almost inevitable on imposition of almost all types of stresses, particularly, in photosynthetic organisms[5,6]. Scavenging of AOS is reported to follow the Halliwell–Asada cycle[6,7]. Activity of some of the enzymes of the cycle has been found absent in cyanobacteria[8]. Also, there is no information about purification and characterization of ascorbate peroxidase (APX) in cyanobacteria[8]. Effect of salinity on superoxide dismutase (SOD) and APX activity from *Microcoleus chthonoplastes* Strain SC7B9002–1 obtained from tidal channel have been studied[9]. Nevertheless, there is no information about behaviour of AOS systems in the desiccation-tolerant cyanobacteria subjected to variations in the levels of their hydration and especially under dry state. The main purpose of the present study was to find out any roles of AOS enzymes such as catalase, SOD and APX in survival of subaerial cyanobacterium *Lyngbya arboricola* under the extreme oxidative conditions and drought in its natural habitats.

Mats of the cyanobacterium, freshly collected as well as stored for one and two years respectively, over silica gel at 25 ± 1°C in dark just after the rainy season under dry state from their natural habitats, were used during the study. The freshly collected mats were placed over filter papers soaked with double distilled water (0 MPa, osmotic water potential) and were incubated under light intensity of 35 µE m^{-2} s^{-1} at 25 ± 1°C for 72 h and thereafter dried over conc H_2SO_4 overnight. The freshly collected mats and those stored for one and two years respectively, after treatment with dry and wet heat at 100°C for 1 h as described by Talpasayi and Tripathi[4], were incubated under growing condition at 0 MPa at different time periods. Thereafter, at each time period of incubation, ^{14}C-fixation and activity of AOS enzymes in cell-free extract as well as on native polyacrylamide gels were recorded. ^{14}C-fixation was measured by employing the Conway dish method[10].

For preparing cell-free extract, nearly 1 g algal mats were homogenized at 4°C with 100 mM phosphate buffer (pH 7.8) containing 2 mM EDTA, 1% polyvinylpolypyrrolidone and 1 mM phenyl-methane sulphonyl fluoride by using mortar and pestle, and thereafter, sonicating them for

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15 min at 150 mA. The homogenates were filtered through four layers of cheesecloth and centrifuged at 17,000 *g* for 30 min. The supernatants were concentrated with millipore 10 kDa cut-off filters to a final protein concentration in the range of 35–160 μg ml^{-1}, according to requirements. The cell-free extract prepared for measurement of APX activity was made by adding ascorbate (5 mM) and sorbitol (20% w/v).

Catalase (EC 1.11.1.6) activity was determined by monitoring the consumption of H_2O_2 as described by Rao *et al.*[11]. SOD (EC 1.15.1.1) activity was determined by measuring the inhibition in the photochemical reduction of nitroblue tetrazolium (NBT) at 560 nm. Also, the value of one unit of the enzyme was adopted as described by Donahue *et al.*[12]. APX activity (EC 1.11.1.11) was determined by following the decrease in A_{290}, due to H_2O_2-dependent oxidation of ascorbate as described by Rao *et al.*[11]. The amount of ascorbate was determined by using an extinction coefficient of 2.8 mM cm^{-1} as mentioned by Nakano and Asada[13]. The reactions of all the enzymes were initiated by transferring the reaction mixtures prepared at 4°C to a water bath maintained at 25 ± 1°C.

Assays of catalase, SOD and APX were also performed on algal samples separated by non-denaturing 8% and 10% PAGE respectively, at 4°C for 4–6 h with a constant current of 30 mA and using algal samples containing 110 to 160 μg protein in each lane, following Davis[14]. In addition, gels were supported by 10% glycerol[15]. In case of SOD assays, the method described by Donahue *et al.*[12] was followed. For identification of individual isoforms, gels were treated with either 2 mM KCN or 2 mM H_2O_2 in the pre-equilibrated buffer[16]. For detection of APX activity on gels the method of Chen and Asada[17] was used. Activity of catalase on gels was also detected following the same procedure described for APX, except that ascorbate was omitted from the reaction mixture[18]. Protein concentrations were determined colorimetrically as described by Lowry *et al.*[19], with lysozyme as the standard.

The freshly collected 72 h grown but dry mats and those stored for one and for two years respectively, showed almost similar pattern of ^{14}C-fixation and activities of AOS enzymes on incubation for different time periods at 0 MPa osmotic water potential under growing conditions (Figures 1–4). During the first 10–20 h of incubation, a sharp increase in the ^{14}C incorporation and activity of AOS enzymes was recorded, which gradually reached a steady state on further increasing the incubation period. In general, the values of ^{14}C-fixation and activity of AOS enzymes in the one and two year stored dry mats, after complete recovery on rehydration were not so low compared to the freshly collected mats. Such observations are expected either due to synthesis of enzymes or recovery in the activity of the enzymes or both, during the period of rehydration. Synthesis of not any novel proteins, but ^{35}S incorporation into protein for 90 min has been recorded during short periods of rewetting of immobilized cells of *Nostoc commune*

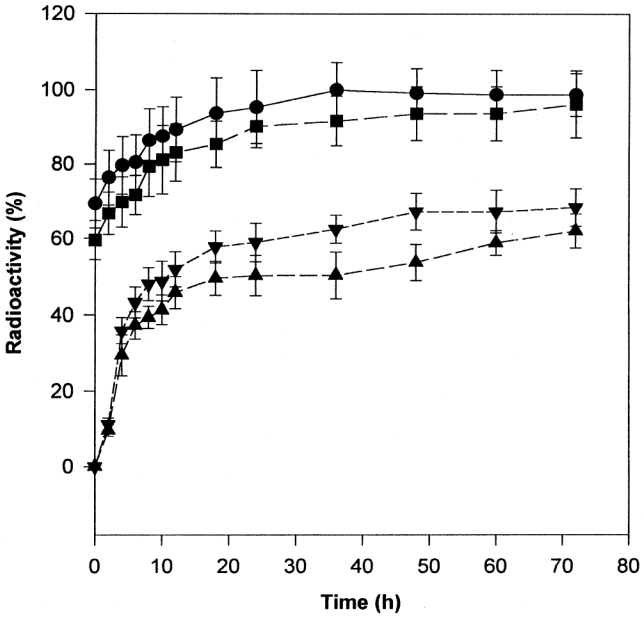

Figure 1. ^{14}C-fixation by the freshly collected 72-h grown but dry mats (●) and these mats dry heated (■), dry mats stored for one year (▼) and these mats dry heated (▲) on incubation for different time periods at 0 MPa under growing conditions ($n = 3$).

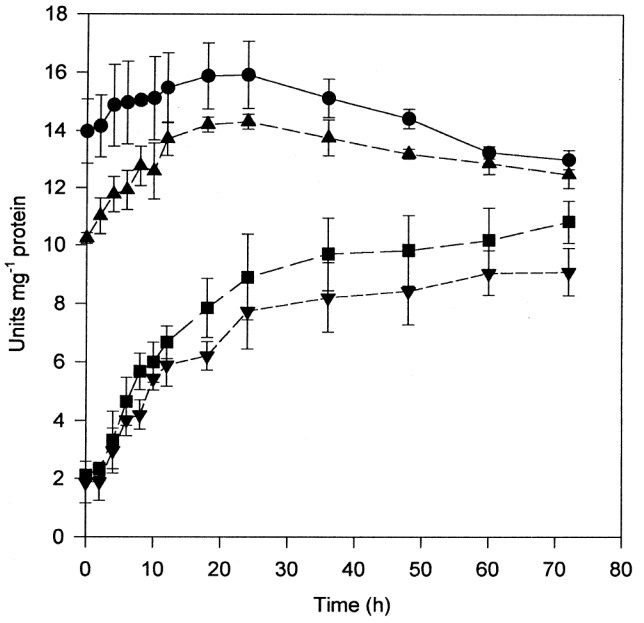

Figure 2. Activity of SOD in the freshly collected 72-h grown but dry mats (●) and these mats dry heated (▲), dry mats stored for one year (■) and these mats dry heated (▼) on incubation for different time periods at 0 MPa under growing conditions ($n = 3$).

UTEX 584 (ref. 20). On the other hand, osmolytes such as sucrose, sorbitol, trehalose, glycine-betaine, etc. are proposed for preserving the integrity of proteins by forming hydrogen bonds with the proteins in the place of water molecules which are removed during the process of drying[1,21]. It is difficult to propose whether the sharp recovery

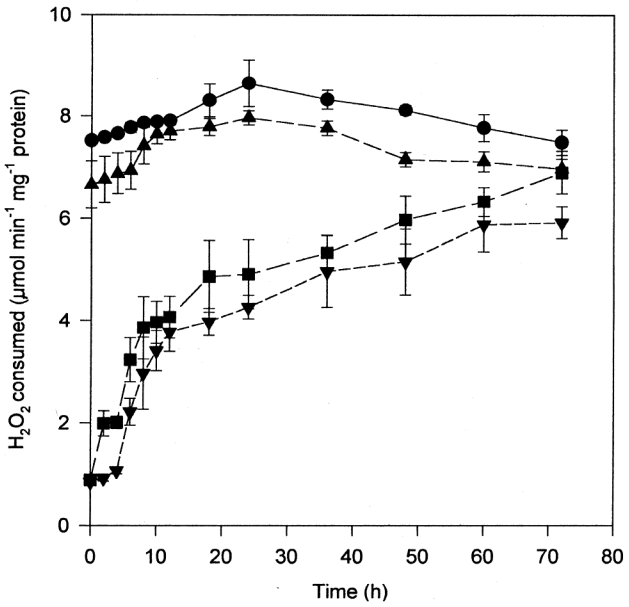

Figure 3. Activity of catalase in the freshly collected 72-h grown but dry mats (●) and these mats dry heated (▲), dry mats stored for one year (■) and these mats dry heated (▼) on incubation for different time periods at 0 MPa under growing conditions (n = 3).

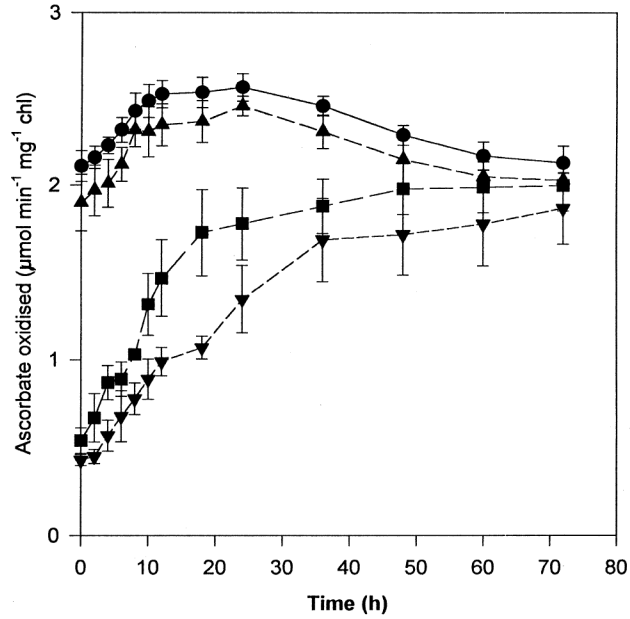

Figure 4. Activity of APX in the freshly collected 72-h grown but dry mats (●) and these mats dry heated (▲), dry mats stored for one year (■) and these mats dry heated (▼) on incubation for different time periods at 0 MPa under growing conditions (n = 3).

in the values of all the parameters during the early 10–20 h of wetting of the dry mats of *L. arboricola* is either due to synthesis of enzymes or a gradual replacement of osmolytes bound to preserved enzymes by water molecules or both. Nevertheless, the native polyacrylamide gel-activity assays of the cell-free extracts clearly reflected presence of all the three studied AOS enzymes in the one and two year stored dry, besides freshly grown but dry mats of the cyanobacterium (Figure 5). Plausibly, while growing in its natural habitats, the cyanobacterium has been directly exposed to sunlight together with frequent drying and wetting, resulting in generation of high amounts of AOS enzymes and during subsequent dry phase the enzymes remained preserved and stable in their cells. On incubation of the mats in laboratory conditions, enhancement in activity of the AOS enzymes was apparent only for certain periods (up to 80–100 h) and thereafter showed a decline, whereas there was no decline in ^{14}C-fixation even on more than 100 h incubation of the mats in growing conditions (Figure 1). Hence, the synthesis of proteins may not be the main cause for the sharp recovery in the values of all the metabolic activities at early hours of rewetting, but the enzymes are stabilized during the course of dryness, at least up to two years, may be by involvement of the osmolytes. Such a phenomenon of stability of enzymes under dry state has also been reported in other organisms[1,21]. Even, over a long period of millions of years, catalase was active in free-dried permafrost samples[22].

Earlier, presence of only two forms, Fe-SOD and Mn-SOD, was demonstrated in *Plectonema boryanum*, *Anacystis nidulans*, *Anabaena variabilis* and *Anabaena cylindrica*[8]. However, later Canini *et al.*[23] reported presence of an additional type of SOD, i.e. hybrid-SOD (Hy-SOD or Fe-Mn-SOD) in *Anabaena azollae*, a symbiont to *Azolla filiculoides*. Five different isoforms of SOD were observed in the growing mats of *L. arboricola* (Lane 3, Figure 5 *c*). The lower bands are major bands as they were more intense compared to the top two bands. The topmost band was unaffected on addition of 2 mM H_2O_2 to the staining solution. The three lower-most bands disappeared in the presence of H_2O_2, but were unaffected by 2 mM cyanide. Intensity of the middle band was slightly lowered, but did not disappear on addition of H_2O_2 to the staining solution. Observations made on the basis of sensitivity and insensitivity of SOD forms to cyanide and H_2O_2, reflected that the topmost band belonged to Mn-SOD and the three bottom ones to Fe-SOD. The band next to Mn-SOD might be Fe-Mn-SOD (Hy-SOD) (Lane 3, Figure 5 *c*). This is in accordance with the observation made by Canini *et al.*[23] about the presence of Hy-SOD in *A. azollae*. Catalase activity has been shown to be associated with all cyanobacterial species[8]. Obinger *et al.*[8] have demonstrated one isoform of catalase in *A. nidulans* on native polyacrylamide gel, whereas there were three isoforms of catalase recorded in *L. arboricola* (Figure 5 *a*). Presence of three isoforms of catalase enzyme is more common in higher plants[11,17]. This may be due to availability of more oxidative atmosphere at its subaerial habitats and also to high activity of oxygenic photosynthesis[10]. The level of oxygen in the cavity of *Azolla*[23] and bubbling cultures of *A. cylindrica* with O_2-rich air[24] have been suggested for higher levels of AOS enzymes in these cyanobacteria. On the other hand, there are several reports that indicate the

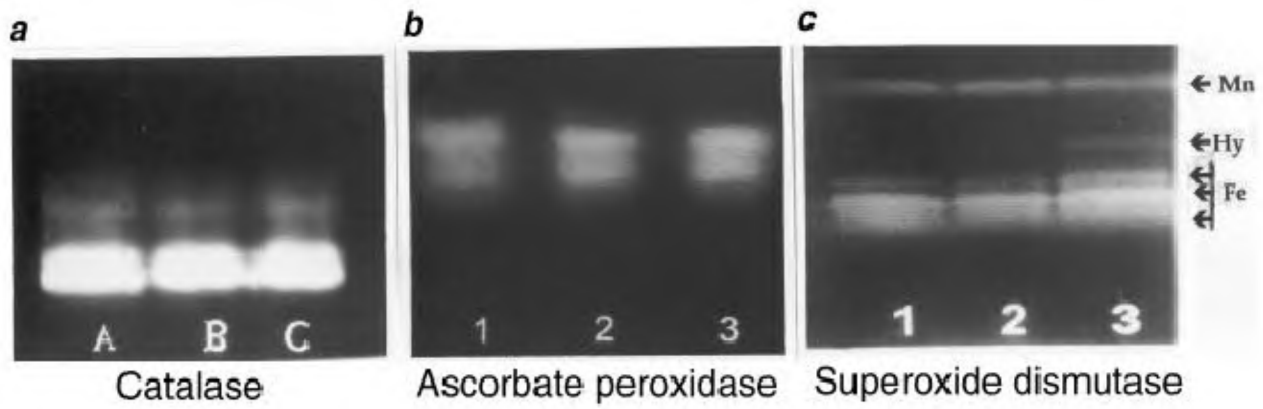

Figure 5. Native PAGE stained for (*a*), activity on catalase on one- (lane A) and two- (lane B) year-old dry and growing (lane C) mats; (*b*), activity of APX on growing (lane 1) and one- (lane 2) and two- (lane 3) year-old dry mats; and (*c*), activity of SOD on one- (lane 1) and two- (lane 2) year-old dry, and growing (lane 3) mats.

presence of APX, but only in crude extracts of some cyanobacteria[8]. However, two APX forms were recorded during the present study (Figure 5 *b*).

Dry heating at 100°C for 1 h of the dry as well as growing mats of the cyanobacterium resulted only in an insignificant lowering in the activity of the three studied AOS enzymes and in the amount of ^{14}C-fixation (Figures 1–4), whereas wet heating at 100°C for 1 h resulted in complete loss in ^{14}C-fixation and activity of AOS enzymes (data not shown). These observations suggest that dry cyanobacterium bears high ability to maintain stability of its enzymes under extremes of environmental conditions. Further, the cyanobacterium did not show complete stability of the enzymes on storage for longer duration. The top second (Hy-SOD) and third (Fe-SOD) bands were absent in the one (Lane A, Figure 5 *a*) and two (Lane B, Figure 5 *a*) year-old dry mats. In the case of APX and catalase, the number of bands was almost the same in the dry and growing mats, but intensity of the bands lowered in the one-year-old dry mats, which further lowered on increasing the storage period to two years (Figure 5 *b* and *c*). Proteolysis may be one of the reasons for such loss of enzymes as it was detected in *Nostoc commune* cells after its prolonged desiccation (17 days)[20]. Nevertheless, information about occurrence of stable AOS enzymes for longer duration under dry state in the cyanobacterium provides an interesting scope to understand the mechanism of survival of organisms in extremes of environmental factors and also their applications in biotechnology.

1. Tripathi, S. N., Tiwari, B. S. and Talpasayi, E. R. S., *Energy Build.*, 1990–91, **15–16**, 499–505.
2. Tripathi, S. N. and Tiwari, B. S., in *Research in Photosynthesis* (ed. Murata, N.), Kluwer Academic Publishers, The Netherlands, 1992, vol. IV, pp. 267–270.
3. Tiwari, B. S. and Tripathi, S. N., *Indian J. Biochem. Biophys.*, 1998, **35**, 172–178.
4. Talpasayi, E. R. S. and Tripathi, S. N., *Proc. Indian Natl. Sci. Acad. B.*, 1987, **53**, 527–529.
5. Potts, M., *Eur. J. Phycol.*, 1999, **34**, 319–328.
6. Halliwell, B. and Gutteridge, J. M. C., *Free Radicals in Biology and Medicine,* 2nd edn, Clarendon Press, Oxford, 1989, pp. 86–187.
7. Asada, K., in *Causes of Photooxidative Stress and Amelioration of Defence Systems in Plants* (eds Foyer, C. H. and Mullinaeaux, P. M.), CRC Press, Boca Raton, FL, 1994, pp. 77–104.
8. Obinger, C., Regelsberger, G., Pircher, A., Strasser, G. and Peschek, G. A., *Physiol. Plant*, 1998, **140**, 693–698.
9. Tovar, R., Gonzalez, A., Lopez-Cortes, A., Ascencio, V. and Ochoa, J. L., *Bull. Inst. Oceanogr., Monaco*, 1999, **19**, 197–202.
10. Tripathi, S. N. and Talpasayi, E. R. S., in Proceedings of National Symposium on Biological Nitrogen Fixation, IARI, New Delhi, 1982, pp. 138–149.
11. Rao, M. V., Paliyath, G. and Ormrod, D. P., *Plant Physiol.*, 1996, **110**, 125–136.
12. Donahue, Janet L., Okapodu, C. M., Cramer, C. L., Grabau, E. A. and A'lscher, R. G., *Plant Physiol.*, 1997, **113**, 249–257.
13. Nakano, Y. and Asada, K., *Plant Cell Physiol.*, 1981, **22**, 867–880.
14. Davis, B. J., *Ann. NY Acad. Sci.*, 1964, **121**, 404–427.
15. Mittler, R. and Zilinskas, B. A., *Anal. Biochem.,* 1993, **212**, 540–546.
16. Hassan, H. M. and Fridovich, I., *J. Bacteriol.*, 1977, **129**, 1574–1583.
17. Chen, G. X. and Asada, K., *Plant Cell Physiol.*, 1989, **30**, 987–998.
18. Woodbury, W., Spencer, A. K. and Stahmann, M. A., *Anal. Biochem.*, 1971, **44**, 301–305.
19. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J., *J. Biol. Chem.*, 1951, **193**, 265–275.
20. Potts, M., *J. Bacteriol.*, 1985, **164**, 1025–1031.
21. Potts, M., *Microbiol. Rev.*, 1994, **58**, 755–805.
22. Gilichinsky, D. A., Vorobyova, E. A., Erokhina, L. G., Fyodorov Dayvdov, D. G. and Chaikovskaya, N. R., *Adv. Space Res.*, 1992, **12**, 255–263.
23. Canini, A., Galiazzo, F., Rotilio, G. and Caiola Grilli, *Plant Physiol.*, 1991, **97**, 34–40.
24. Tel-Or, E., Huflejt, M. E. and Packer, L., *Arch. Biochem. Biophys.*, 1986, **246**, 396–402.

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