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Maintenance of biodegradation capacities of aerobic bacteria during long-term preservation

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Abstract

Six strains of aerobic Gram negative bacteria degrading toluene, 2,4-dichlorophenoxyacetate, 2,2-dichloropropionate or 3-chlorobenzoate were freeze-dried and liquid-dried in the presence or absence of a protective agent. Survival and maintenance of the biodegradation capability was checked before and after drying, and after storage of the ampoules for one year at 4 ° or 25 °C. In many cases, stability of the degradation potential was low although viability was high. Survival and stability of all strains was always highest after preservation by liquid drying in the presence of *myo*-inositol and activated charcoal as protective agents. Losses of biodegradation abilities were highest after freeze-drying using no protective agents. Cells grown on complex medium were less sensitive to drying than cells grown under selective pressure (on mineral medium with a special compound as the sole carbon source). A choice of the most appropriate preservation method and the use of an effective protectant is recommended to avoid genetic alterations, and to maintain biodegradation capacities during long-term preservation.

Introduction

There is a growing interest in biotransformation of environmental pollutants and in degradative strains because biodegradation provides the possibility to remove toxic compounds from contaminated sites or waters by biological reclamation techniques. The aerobic bacterial metabolism of many pollutant compounds was investigated intensively (Smith 1990; Hardman 1991; Bedard 1992; Cerniglia 1992) but studies on the stability of the degradation capacities during subcultivation or preservation are rare. In Gram negative bacteria, the decomposition of xenobiotics is often connected with the existence of one or more plasmids in the bacterial cells (Sayler et al. 1990). As plasmid- encoded activities are generally less stable than chromosomally encoded activities (Sherratt 1982), the probability to lose the useful degradative properties of bacterial cultures during inadequate maintenance or preservation appears to be high.

Published data on the stability of plasmid-encoded properties like antibiotic resistance suggest a high stability of the plasmid during growth even under non-selective conditions (Wouters & van Andel 1983; Miwa et al. 1984; Keshavarz et al. 1985). Despite of this, every scientist working with strains with special properties – especially plasmid-encoded properties – is aware of the risk to lose the properties or the plasmids during subcultivation. Due to this risk and to problems during subcultivation under selective conditions, the latter is not desirable for maintenance of a strain over a long time and should be replaced by a long-term preservation method.

The vital problem during preservation is to ensure genetic stability since the viability of cells after preservation may not necessarily correlate with the full maintenance of all properties. As changes in various properties have been reported particularly during inadequate lyophilization (Ashwood-Smith & Grant 1976; Malik 1976, 1988a; Sakane et al. 1983; Simione 1992) and as the plasmid DNA is not stably maintained due to replication and segregation control, plasmid-encoded degradation activites may be lost at high frequencies

during drying of a culture although little loss of viability occurs during preservation.

The aim of this study was to show the influence of several drying techniques on the maintenance of special degradation activities and to select the technique retaining them best. Six strains belonging to the alpha-, beta- and gamma-subclasses of the *Proteobacteria* were tested. The transformation capabilities of three of the strains have been described to be plasmid coded. Before drying, the cultures were grown under non-selective (on complex medium) or under selective conditions (on mineral medium with a xenobiotic compound as sole source of carbon and energy).

Material and methods

Organisms and growth conditions

All strains were from the DSM – Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany: *Alcaligenes eutrophus* DSM 4058 (strain JMP 134) harbouring pJP4, *Alcaligenes xylosoxidans* DSM 6758 (strain AB IV) harbouring pFL40, *Pseudomonas alcaligenes* DSM 6284 (strain C-O), *Pseudomonas putida* DSM 3931 (strain mt-2) harbouring the natural TOL-plasmid pWWO, *Pseudomonas putida*, DSM 6899 (strain F1) and an unidentified strain DSM 6986.

Cultures were grown under two different conditions before preservation. For growth on complex nutrients, the medium contained peptone 5.0 g, meat extract 3.0 g, H_2O 1000 ml. For selective biodegradation conditions, the mineral medium (MM) after Brunner et al. (1980) supplemented with a special substrate as sole source of carbon was used. For DSM 3931 and DSM 6899, 20 μl toluene was applied to a cotton stopper fixed onto the gas tight screw cap of the flasks. 2,4-dichlorophenoxyacetate (1 g/l; for DSM 4058), 3-chlorobenzoate (1 g/l; for DSM 6284), 2,2-dichloropropionate (2.8 g/l; for DSM 6758) and naphthalene-2-sulfonate (0.5 g/l; for DSM 6986) were added to the medium after autoclaving from filter sterilized stock solutions. Flasks were inoculated with 2 ml of a well growing culture in the same medium and incubated on a rotary shaker at 28 °C for 20–44 hours.

Preservation studies

Before preservation, each strain was cultivated on complex medium or on special medium with the compound to be transformed as sole source of carbon. After harvesting, each culture was divided into three parts. The first part was liquid-dried, the second part was freeze-dried using skim milk as a protective agent, and the third part was lyophilized without a protectant. For prolonged storage test, the dried cultures were kept at 25 °C or 4 °C for one year.

Assay of viability and testing of stability

Viability and biodegradative capacity were checked before drying and after reactivation of the dried cultures (i)immediately after preservation and (ii) after one year of storage at 4 °C or 25 °C. For determination of viability, serial decimal dilutions were plated in duplicate on complex medium. Maintenance of the degradation capabilities was assessed by plating the dilutions on mineral agar supplemented with 100 mg/l yeast extract and bromophenolblue (to detect pH changes resulting from degradation of chlorinated organic compounds). Substrates except toluene were added to the agar from stock solutions (see growth conditions). For growth with toluene, plates were incubated in a toluene-enriched atmosphere in desiccators. Cultures were also lysed and the lysates checked for occurrence of plasmids.

Freeze-drying procedure

Freeze-dried skim milk vials were prepared as described previously (Malik 1988a). Cells were harvested by centrifugation at 4000 *g* and resuspended in 2 ml medium (drying with protectant) or water (unprotected drying), respectively. A drop (30 μl) of this thick cell suspension was added to empty glass vials (in case of lyophilization without a protectant) or to the freeze-dried skim milk vials. The vials were frozen at −30 °C and transferred quickly to the freeze-drying chamber. Primary freeze-drying was continued at 0.05 mbar for 12–16 hours to achieve maximum desiccation. Vials were transferred into soft glass tubes, constricted, subjected to secondary drying for 3–4 hours at 0.001 mbar, and sealed under vacuum (Malik 1988a).

Preparation of carrier material for liquid-drying

Small glass vials were filled with 0.1 ml of 20% (w/v) skim milk (Bacto, Difco 0032) containing 1.0% (w/v) neutral activated charcoal (medical grade) and 5% (w/v) *myo*-inositol (Malik 1990). The vials were sterilized at 115 °C for 13 minutes, frozen at about

−40 °C for a few hours and then freeze-dried for 8–24 hours using a standard freeze-drying technique (Malik 1988a). This resulted in a disc of freeze-dried carrier material.

Liquid-drying procedure

A solution of *myo*-inositol (5% w/v) and neutral activated charcoal (1.0% w/v) was prepared in distilled water and autoclaved at 115 °C for 13 minutes (Malik 1990). About 30 μl of cell suspension in this *myo*-inositol charcoal-solution was added to each vial with a freeze-dried disc of carrier material and the vials were subjected to liquid-drying (L-drying; cell suspension are dehydrated in liquid and not in frozen state (lyophilization)) under vacuum in a metallic jar maintained at 20 °C. The outline of the simplified L-drying procedure and the major steps involved have been reported in detail (Malik 1990). First step drying was continued for about two hours at 15–40 mbar and second step drying under 0.1 to 0.01 mbar vacuum for about one hour while maintaining the temperature at about 20 °C. Then vacuum was replaced by sterile nitrogen or argon gas. The ampoules were transferred to soft glass tubes and sealed under vacuum.

Plasmid testing

A miniaturized plasmid isolation procedure of Crosa & Falkow (1981) was used. Two milliliter of a late log phase cell suspension was centrifuged, washed in TE buffer (50 mM Tris, pH 8.0, 10 mM EDTA), resuspended in 40μl TE buffer and lysed by adding 0.6 ml of lysis buffer (TE containing 4% SDS, pH 12.45). This procedure gave crude lysates which were sufficiently pure for qualitative plasmid analysis. The plasmid DNA and the chromosomal residues were separated by horizontal gel electrophoresis. Agarose mini gels (0.8%) were run in a TRIS-borate-EDTA buffer system at 50 mA for 4 hours. For visualizing of bands, ethidium bromide was used.

Results and discussion

During preservation, microorganisms are exposed to severe stress and potentially selective conditions. Although cryopreservation generally results in good viability and stability of properties, especially of antibiotic resistance (Nierman & Feldblym 1980; Miwa et al. 1984; Snell 1991; Sidyakina & Golimbet 1991), loss of viability and of various capabilities (particularly plasmid-bound ones) during inadequate drying have been reported. Degradation of dibenzothiophene and other thiophenes was encoded by plasmids in several bacterial strains used to study the microbial desulfurization of fossil fuels. They lost their degradation abilities during conventional lyophilization without protectants (Malik & Claus 1976; Malik 1978 and 1979). Similarly, many strains lost their ability to nodulate plant roots, to grow chemolithotrophically (where mostly a plasmid is the carrier of hydrogen autotrophy), to degrade special substrates or to produce antibiotics and alkaloids after conventional (unprotected) lyophilization (Ashwood-Smith & Grant 1976; Malik 1976 and 1988a). For reactivation of dried microorganisms, rich media and favourable growth conditions showed higher recoveries and were recommended previously (Malik 1990).

At the DSM, we found a loss of degradation capabilities with several strains which had been lyophilized using routine procedures. Out of 55 unusual catabolic activities described in the literature we could reproduce only 42 activities in cultures obtained from such lyophilized cultures (data not shown). We presume that in the other cases, capabilities were probably lost during subcultivation or preservation. Instability of metabolic plasmids was also found by Salkinoja-Salonen et al. (1979). Strains isolated by these authors pleiotropically lost their plasmid-encoded abilities to utilize methoxylated aromatic acids.

Maintenance of degradative capabilities in surviving cells

In the present study, we determined survival and maintenance of the biodegradative capacities of six strains after dry-preservation with three different methods. For most strains, there was a clear difference between viability and biodegradative capacity after all three preservation procedures. Considerable losses of degradative capabilities after drying were observed in 3 out of 6 strains. The transformation abilities were maintained totally in surviving cells of strains DSM 3931 and 6986 if they had been dried with a protectant. Stability of degradation in DSM strain 6986 was reduced to 63% if no protective agent had been added prior to drying (Fig. 1). In cells of *Burkholderia* sp. DSM 6284, degradative capabilities were uniformly reduced to 6% with all drying methods. Losses of special activities in *A. eutrophus* were even higher: only one out of 10^4 viable cells utilized 2,4-dichlorophenoxyacetate as substrate

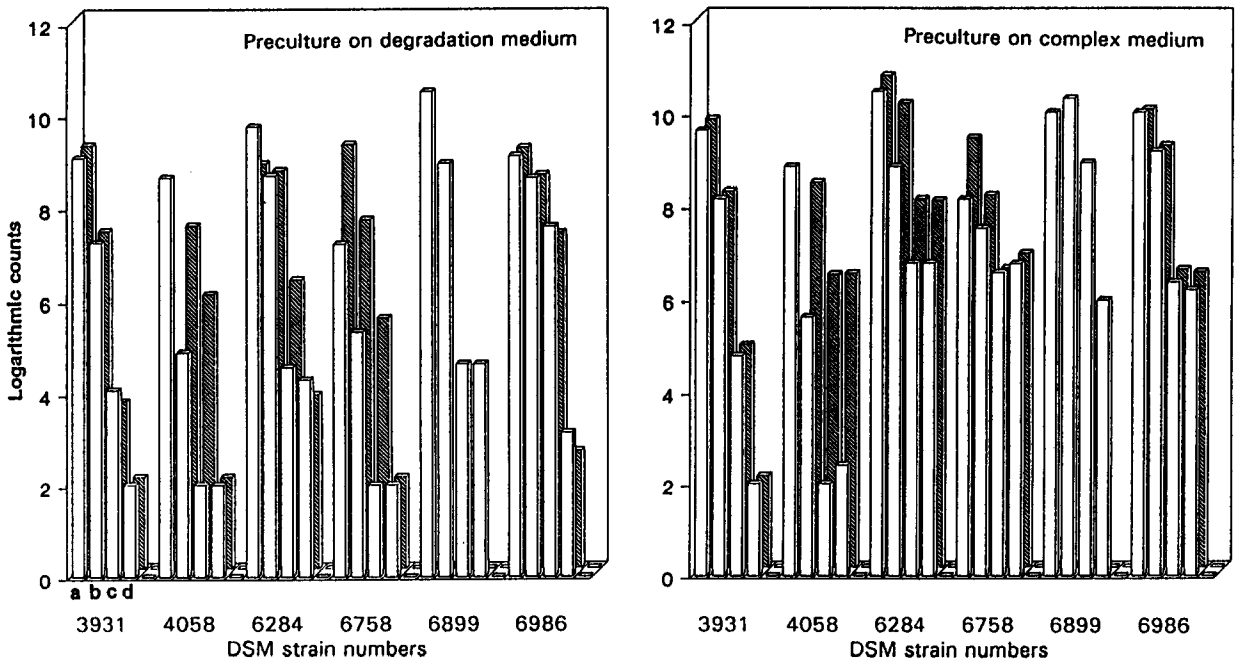

Fig. 1. Comparison of biodegradation capacities of *Alcaligenes eutrophus* DSM 4058, *Alcaligenes xylosoxidans* DSM 6758, *Pseudomonas alcaligenes* DSM 6248, *Pseudomonas putida* DSM 3931 and DSM 6899, and unidentified DSM 6989 after dry preservation.
Vacant columns (front): colony counts on special medium (selective conditions) showing biodegradation abilities; hatched columns (rear): colony counts on complex medium showing survival.
Log counts of average colony forming units per sample (detection limit log 2) a: before drying, b: after liquid drying, C: after freeze drying with skim milk, d: after freeze drying without protectant.

after lyophilization. The biodegradation activities were protected best during liquid-drying with charcoal and *myo*-inositol which also resulted in maximum viability.

Effect of drying method

Survival and stability of all strains was uniformly highest after preservation by liquid-drying in the presence of appropriate protective agents (Fig. 1, columns b) whereas lyophilization resulted in 10 to 1000 fold lower counts.

Freeze-drying is generally conducted after suspending cells in skim milk. This commonly used method was tested and provided some protection as compared to freeze-drying without protective agent. Addition of a protectant was especially important for cells pregrown on mineral medium, and for the maintenance of biodegradation as compared to viability. The difference between viability and biodegradative activity increased in the order: L-drying < lyophilization with skim milk < lyophilization without protectant.

Effect of precultivation

During growth on complex medium for about 16 hours before drying, degradation capabilities were retained fully in 3 out of 4 strains tested. Only *A. xylosoxidans* DSM 6758 lost the capability to grow on dichloropropionate at high rates during non-selective subcultivation but the rate of activity loss in this strain was even higher during cultivation under selective conditions than during growth on complex medium.

After drying, the yield of surviving cells was uniformly 1–2 orders of magnitude higher after precultivation on complex medium than after precultivation under selective pressure (Tab. 1, line A versus B, counts in brackets). Cells grown under selective conditions appeared to be much more sensitive towards the drying stress than cells grown on complex medium. Cells precultivated on complex medium (with the exception of one strain) survived lyophilization without protectant in the range of 10^6–10^8 cells per ml whereas selectively grown cells did not survive with acceptable results (10^4 cells per ml or less). The maintenance of

Table 1. Maintenance of biodegradation capacities and survival of various strains during dry-preservation and after storage.

Species and strain no.		Logarithmic values of cell counts[a]									
		Before drying	After drying with drying method[b]			After storage at 4 °C			After storage at 25 °C		
			I	II	III	I	II	III	I	II	III
Alcaligenes eutrophus											
DSM 4058	A[c]	8.9	5.6(8.3)	<2(6.4)	2.4(6.4)	5.4(8.3)	<2(6.2)	<2(<5)	<3 (7.9)	<2(<6)	<2(<5)
	B[d]	8.6	4.9(7.4)	<2(<6)	<2(<2)	4.9(7.6)	<2(3.9)	<2(<2)	4.8(<7)	<2(<3)	<2(<2)
Alcaligenes xylosoxydans											
DSM 6758	A	8.2(9.3)	7.5(8.1)	6.6(6.5)	6.8(6.8)	7.5(8.4)	5.8(6.5)	6.6(6.6)	5.7(7.7)	<4(5.9)	4.3(4.7)
	B	7.2(9.2)	5.3(7.6)	<2 (5.5)	<2 (<2)	4.8(7.3)	<2 (5.5)	<2 (3.6)	4.3(7.3)	<2(5.4)	<2 (<2)
Pseudomonas alcaligenes											
DSM 6284	A	10.5(10.7)	8.9(10)	6.8(8)	6.8(>8)	8.9(9.8)	6.8(7.3)	6.5	9(9.2)	4.9(6.1)	<3(<6)
	B	9.8 (8.8)	8.7(8.6)	4.6(6.3)	4.3(<3.8)	7.9(7.6)	4.6(5)	4.1(2.6]	8.2(7.9)	<2(4.1)	<2(<2)
Pseudomonas putida											
DSM 3931	A	9.6(9.7)	8.2(8.2)	4.8(4.9)	<2(<2)	8.5(8.5)	5.1(5.1)	–	8.5(8.5)	4.6(4.8)	–
	B	9.1(9.2)	7.2(7.3)	4.1(3.7)	<2(<2)	7.4(7.5)	3.6(3.5)	–	7.1(7)	2.4(2.6)	–
Pseudomonas putida											
DSM 6899	A	10	10.3	9	6	9.4	6.8	6.6	8.7	6.8	<4
	B	10.5	9	4.7	<4.7	9	4.7	3	8.1	4.9	<2
Unidentified strain											
DSM 6986	A	10 (9.9)	9.2(9.1)	6.4(6.5)	6.2(6.4)	<7(9.1)	<4(6.8)	<4(6.7)	<7(8.9)	<4(6.5)	<4(5.2)
	B	9.1(9.1)	8.6(8.5)	7.6(7.3)	3.2(2.6)	>9	7.8	<2(<2)	>9	7.2	<2(<2)

[a] The numbers indicate the average logarithmic counts of cells showing the capacity of biodegradation (growth on selective medium). The total viable cell population able to grow on complex medium is given in brackets.
[b] Drying methds: I liquid-drying with meso-inositol; II lyophilization with skim milk; III lyophilization without protective agent.
[c] Before drying, the cultures were grown on complex media.
[d] Before drying, the cultures were grown on mineral media with selective compounds for biodegradation

metabolic activities showed the same tendency. With all strains and all drying methods, precultivation on nutrient media resulted in higher yields of degrading cells after drying as compared to precultivating in mineral medium. This effect was more pronounced for drying without protectant than for drying in the presence of a protectant: After drying without protectant, recoveries of degrading cells were up to 5 orders of magnitude higher in bacteria which had been precultivated on complex medium than in bacteria which had been cultivated under selective conditions.

The advantage of precultivation in complex media can be attributed to the following two reasons. First, all selective carbon sources used in the mineral medium are toxic. Growth in mineral medium with such a compound as sole source of carbon and energy is a challenge even to the degrading strains adapted to grow on these substances. The substrate concentration during cultivation was obviously not high enough to kill the bacteria but nevertheless, cells could be sublethally damaged by the substrate or by metabolic intermediates. Further stress by the drying procedure seems to result in much higher losses of special properties and of viability in thereby predamaged cells than in cells which have not been exposed to such harmful conditions before. Second, this effect may be enhanced by lack of organic matter during drying in case of drying without addition of protective agents. Whereas some rests of peptones attached to the cells will protect the cells precultivated on complex medium, bacteria grown on mineral medium have no such support. These effects dominated clearly the possible but negligible losses of degradative capabilities during non-selective growth.

Similar observations were made with non-sulfur photosynthetic bacteria (Malik 1988b). The cultures were difficult to lyophilize after photoautotrophic growth, but successful drying was achieved if the strains had been cultured heterotrophically.

Effect of storage

The maintenance of viability and degradative capabilities during storage in ampoules for one year was strain dependent. As a rule, cell counts dropped 0 to 2 orders of magnitude during storage at 25 °C (Table 1), but losses up to 4 orders of magnitude also occurred. Losses of viability and of metabolizing activities were highest during storage after unprotected lyophilization and amounted to more than two orders of magnitude for all strains. After precultivation on complex medium, counts of degraders in lyophilized ampoules decreased about 10 times (mean) more than counts of viable cells. This result shows again the risk to lose selectively a property of interest while viability is maintained.

Decreasing the storage temperature from 25 °C to 4° C improved the recovery of degrading cells up to 10^3 times, especially with strains DSM 4058, DSM 6284, and DSM 6758 (Tab. 1). Low storage temperature was essential for cells preserved without protectant but was not so important for cells which had been dried in the presence of a protective agent.

Plasmid content and stability

Strains that were deposited as plasmid-bearing strains, *P. putida* DSM 3931, *A. eutrophus* DSM 4058 and *A. xylosoxidans* DSM 6758, were all confirmed to carry plasmids after precultivation under selective and under non-selective conditions. In two other strains, we also detected plasmids. The *Burkholderia*-like strain DSM 6284 haboured two plasmids and the unclassified strain DSM 6986 one plasmid. Only *P. putida* DSM 6899 proved to be plasmid free as expected.

We could not find an evident loss of any of the plasmids during subcultivation, preservation or storage of the ampoules. Even in cultures which had lost their degradative activity to a high extent during these steps the respective plasmid(s) could be shown still to be present. Nevertheless, the plasmids of the strains were all large plasmids in which deletions and other damages may occur. We can not exclude such injuries since only relatively large deletions can be detected by the technique applied (agarose gel electrophoresis of crude lysates).

Conclusions

Based on these results it is obvious that there is a high risk to lose degradative activities during unprotected drying. The use of effective protective agents and an optimized freezing and drying protocol is recommended in order to avoid the loss of important qualities of the cultures. In conclusion, the following measures should be taken for the protection of biodegradative activities during drying:

- Cultivation on complex medium directly before drying (irrespective of general cultivation under selective conditions);
- Protection of the cells by an appropriate agent such as skim milk or *myo*-inositol during drying;
- Storage of the ampoules with dried material at low temperatures;
- Reactivation of the dried cells in complex medium and thereafter transfer to selective media for biodegradation.

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