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0014-4754/83/111221-11$1.50+0.20/0

Use of specialized microbial strains in the treatment of industrial waste and in soil decontamination

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A natural tendency among most waste treatment specialists is to regard any use of tailor-made cultures as useless in the effort to abate pollution. Such skepticism is deeply founded and is expressed, for example, by engineers whose training and experience lie not so much in microbiology as in the workaday world of processing enormous quantities of dilute municipal sewage containing traces of every imaginable substance. Traditionally, and to some extent even today, the practice has been to dilute the organic matter and then to disperse it broadly into the environment. Ecologists, by virtue of to their training, are usually primarily concerned with observing and sampling natural environments. Again, the systems they study are complex and disparate. The general criterion for assessing the stability and 'health' of any microcosm has to do with the diversity of species – both flora and fauna, including a broad array of microorganisms – of which that microcosm is comprised. Consequently, one hears such objection as, 'Your cannot run a waste-treating facility like an antibiotics plant' or 'Any open system will seek its own equilibrium and will arrive at the same steady state no matter what the initial conditions imposed from outside', or 'Monocultures are unstable'.

There is a truth in such skepticism. One cannot afford to be sanguine in the face of many failures by overzealous advocates of a controlled environment. One of the earliest proponents was Charles Darwin himself. Having established the beneficial effects of common earthworms on soil structure and fertility, he pictured their widespread use as inoculants in poor soils. His suggestions did not work in practice. Earthworms are indeed abundant in nature and when conditions are favorable they will multiply rapidly, but in poor soils they will die out. In more recent times there have been similarly unsuccessful attempts to establish free-living *Azotobacter* in soils so as to fix atmospheric

nitrogen and thereby reducing the need for commercial fertilizers. In the process of waste treatment itself the idea of adding enzymes or bacterial concoctions has also been largely discredited because attempts to adapt the techniques for commercial use have been premature since adequate scientific and technical controls were lacking. As a consequence of such failures, the view has often been expressed that 'if they're not there already, then they probably can't survive the competition'.

Reasoning by analogy alone is not very productive because counter examples can always be cited. It has been known, for example, since early biblical times that adding of leaven or starter to bread is far better than just waiting around for something to happen or for 'nature to take her course'. Listed in table 1 are various commercial processes that exemplify the maintenance of quasi-pure cultures under relatively clean, but not sterile, conditions. Furthermore, many of these processes are started from either pure or well-defined mixed cultures.

Of considerable interest for the future use of special bacteria in waste treatment is the current success of seed inoculation to promote the nodulation of some legumes[3,28]. Early efforts in this direction often failed to show any beneficial effect. Lack of knowledge on how to maintain culture viability and how to match the bacteria to the particular plant species caused such failures. Premature introduction of an innovative technology will almost certainly make its subsequent adoption more difficult.

An important feature of most 'directed fermentations', such as those in table 1, is that the starting material is rather well-defined and fermentation conditions are often so special that they are highly selective for a narrow range of microorganisms. Therefore new methods are likely to capitalize on the special properties that distinguish industrial wastewater from domestic sewage: its high strength, better-defined composition and often unusual pH, temperature or mineral content. It is similarly important to recognize that segregation and intensive pre-treatment are especially suited to the strong and sometimes toxic or refractory wastes from the chemical or food industries. To imagine the advantages that could accrue, one should consider the treatment of effluents from the manufacture of the herbicide, 2,4-dichlorophenoxyacetic acid (2,4-D). Raw waste contains 300–500 mg/l of chlorinated compounds, including both 2,4-D and 2,4-dichlorophenol (2,4-DCP). The latter is more toxic for microorganisms than 2,4-D itself. When such a mixture is added to domestic sewage, as practiced near Jacksonville, Arkansas, in the U.S.A., the use of retention ponds with several weeks holdup is a necessary after-treatment before the effluent can be discharged into natural waterways[11]. Such treatment is expensive, especially in situations where land costs are high; it is predicated on the assumption that the biodegradation is inherently slow. However, if one uses a relatively pure culture of a *Pseudomonas* strain, both 2,4-D and 2,4-DCP can be completely degraded at a rate almost $\frac{1}{3}$ as fast as that for glucose[37]. In a well-mixed, aerated chemostat it should be possible to reduce the concentrations to less than 10 mg/l in 14 h instead of 14 days. At such low concentrations it might then be possible to blend the pretreated waste into domestic sewage without the need for aftertreatment, although further development work would have to be done on any such process. Temperature and pH are more critical variables than is the richness of the growing media. Also special engineering problems arise because of time lags in the adaptation of the culture to shock loads[37]. Nevertheless, it would seem advantageous to avoid the binding of chlorophenolic residues onto the humus compounds of sewage[10]. The relatively small volumes that are encountered nearer the source of waste production will permit the economic use of closely controlled temperature and pH.

Table 1. Microbial processes. A listing in order of decreasing culture purity during the process itself

Antibiotics, vitamins, amino acids (pure culture)
*Bakers' yeast
*Beer, wine
*Cheese
Sauerkraut, pickles
Pulche (palm wine)
Silage
*Legume nodules, tree mycorrhizia
Activated sludge from domestic waste (impure culture)

* Process is normally started with a pure or well-defined microbial culture.

Pure culture studies provide guidelines

A very large amount of knowledge is rapidly accumulating not only on metabolic pathways for catabolism of xenobiotics, but also on the regulatory mechanisms including genetic transfer[26]. Studies of mixed culture fermentations[4] are also highly relevant for improving the performance of existing waste treatment plants. One simple study could be based on the likelihood that a sludge adapted to biodegrade 2,4-D would also be pre-adapted to degrade 2,4-dichlorophenol since the latter lies on the biochemical pathway. Haller[16] has described several similar examples for mono-substituted benzoates and phenols.

It is especially interesting when organisms isolated from soil or sewage display behavior similar to that shown by the more complex ecosystem. For example, the degradation of p-nitrobenzoate (p-NBA) by fully adapted sewage is inhibited by the presence of benzoate in the waste. Moreover this result can be reproduced in an axenic culture of a pseudomonad isolated from soil[15]. Kinetic data as well as proposed mechanisms for the competitive inhibition could, in

this instance, be more clearly delineated in the bacterial isolate than in sewage itself. The modelling of sewage behavior as if it were a single culture[14] is the reverse approach, but it is also useful.

Further guidelines stem from the studies by Knackmuss and co-workers[24] and from Williams[39] on the interrelated routes of breakdown of chloroaromatic compounds by pure and mixed cultures of pseudomonads. Their studies included plasmid-modified strains. The extension of such fundamental work to sewage itself is an important middle step toward facilitating technology transfer and needs to be emphasized. In this vein, we recently compared the behavior of 2 bacterial isolates with the behavior of sewage in a study on the accumulation of unwanted chlorocatechols[17,23]. One isolate, H-1, and the B-13 organism of Knackmuss mimicked the behavior of sewage. As shown in table 2, black color due to polymerized chlorocatechols will be formed from a mixture of benzoate and 3-chlorobenzoate (3-CBA) if both substrates are presented together or if pre-adaptation to benzoate has been made. Neither chemical alone causes accumulation of an unwanted intermediate, and pre-adaptation to 3-CBA also directs metabolism into the desired *ortho* pathway. As shown in table 2, however, another sewage isolate, designated H-2, was insensitive to the mode of pre-adaptation. Apparently H-2 is not the natural dominant organism in sewage but might perhaps be established in a less complex waste stream.

Other researchers have emphasized the behavior of mixed cultures, pointing out for example that it is a common mistake to ignore the interactions among populations[18,20,23]. Such knowledge is especially helpful when laboratory studies attempt to simulate actual environments.

Despite the abundance of biochemical information on catabolism there remains a lack of data necessary to design and operate waste treatment facilities. As emphasized recently by Cook and Hütter[7] it is also important to have kinetic data on growth or degradation rates as well as the saturation constants, K_s. For toxic substrates, information about tolerance and metabolic lags is very necessary.

Use of starter cultures and treating spills

The time needed to start up a new process or to adjust it to new conditions can be shortened by proper inoculation procedures. Sometimes these procedures entail the simple transferring of an active culture from another well-operating plant. Increasingly though, specialized strains or defined microbial mixtures will be used to point treatment plants in the right biological direction. The handling of spills or upsets to a system can also be improved by inoculation, and commercial suppliers of bacterial mixtures exist in many countries. One hopes that the procedures will become less empirical in the future; to this end several successful procedures will be cited here.

Table 2. Formation of black color due to accumulated chlorocatechols

	Pseudomonads H-1, B-13, or sewage	Pseudomonad H-2
Adapted to grow on:		
Benzoate	−	−
3-CBA	−	−
Both substrates together	+	−
Benzoate, then both	+	−
3-CBA, then both	−	−

+, color formation; −, no color formation.

The slow response of methane digestors is notorious and therefore it is not surprising that they should be inoculated at start-up. In the Netherlands a rather special methane process has recently been developed[27]. It requires adaptation to form a sludge with large agglomerates or cell clusters, that settle rapidly. Careful start-up procedures must be followed which last 8–12 weeks. However, once established, the granular consortium of bacteria is rather stable. In starting up a full-scale reactor 200 m^3 in size, a seed of 1800 kg of sludge solids was used. After only 14 days of operation its capacity to digest sugar beet waste water was fully developed[27]. Similar but more sophisticated processes, designed to handle special effluents from the chemical industry, are under development[1,2]. These will no doubt also benefit from inoculation at start-up.

Even in aerobic processes the building up of microbial communities to form a stable association for treating industrial wastes takes time. The recent review by Harder[18] cites several examples including the work of Bull and his associates on biodegradation of the herbicide Dalapon (2,2-dichloropropionic acid) where a 6-membered stable community developed after many weeks in a continuous-flow chemostat[31,32]. Two types of adaptation are involved: the adaptation of existing catabolic systems to the degradation of novel compounds and the acquisition of altogether novel metabolic pathways through mutation/selection or by plasmid transfer[6,24,35]. Why such complex communities are formed, though, is not understood in detail[29,33].

Inoculation with pure cultures has merit in special cases. By using a repeated fill-and-draw technique it was possible to adapt domestic activated sludge to utilize 40 mg/l of pentachlorophenol (PCP) present in a simulated industrial waste. This procedure routinely took 6–7 days. Even though the degrading bacteria are fairly ubiquitous[36] they are probably present in small numbers. Therefore another procedure was used whereby PCP-degrading bacteria were inoculated directly into an operating activated sludge unit in the laboratory. In this procedure 10% of the mixed liquor was removed and replaced by an equivalent volume of a batch culture of *Arthrobacter* ATCC 33790. Flow was not interrupted. In 1–2 days

instead of 6 or 7, the level of PCP was reduced from 40 mg/l to less than 1 mg/l. The hydraulic retention time was 9–10 h and sludge age, 6.2 days (Edgehill and Finn, unpublished)). Once acclimated, the activated sludge was stable under laboratory condition, i.e. where the feed and temperature were closely regulated. Problems encountered in the dynamic behavior of such a system will be pointed out later. The relative ease with which a variety of chemical compounds can be degraded by adapted sewage has been measured by Pitter[30].

Spills into the environment represent another situation in which added inoculum can perhaps speed up the natural process of biodegradation. Such an approach will be effective only in situations where the lag in disappearance is not due to environmental factors such as moisture, temperature, or lack of oxygen or minerals. Thus attempts to speed up biodegradation of oils spills by inoculation have not generally been successful because the inoculum has not been the limiting factor[5,25]. It is often observed, however, that the initial application of a pesticide to soil is slow to disappear, whereas subsequent applications are degraded without so long a lag. Under such circumstances the initial lag could be shortened by judicious application of a soil inoculum, as shown for example by Daughton and Hsieh[9]. We have shown[9a] that soil sprayed with PCP at the level of 100 µg/ml of soil moisture was more rapidly cleared of the wood-treating chemical if inoculated with specialized *Arthrobacter* cells at the level of 10^4–10^6 per g of soil. In laboratory tests at 30 °C the half-life of residual PCP was reduced from about 2 weeks (uninoculated) to 15 h (inoculated). The degradation in soil plots in an outdoor shelter was slower because of lower temperatures but again inoculation was of considerable benefit. The tests with soil are complicated by adsorption and reaction of the phenol with organic matter or clays, as well as volatility losses. Practical considerations may limit the application of direct soil inoculation. In this instance, highly contaminated soil would have to be blended with uncontaminated soil or else be spread out over a large area so as to lower the aqueous concentration of PCP to less than the toxic level of 200 µg/ml of soilwater. In fact soil leaching and treatment of the leachate by more conventional methods might often be preferred.

Zitrides[40] recently described such a leaching method used to decontaminate a railroad bed after an entire tank car, 80 m^3, of 50% formaldehyde had been spilled. Contaminated liquid was contained, but residual formaldehyde on the stone ballast of the track bed and surrounding soil was a major problem. The total cost of physical removal, including interruption of train service, would have cost almost half a million dollars. The contractor together with a supplier of specialized microbial inoculum arranged to spray the area repeatedly using an aerated inoculated holding tank with recycle and sealed drainage ditches. Formaldehyde concentrations were reduced from 1400 mg/l to 1 mg/l within 18 days at a cost of less than $ 50,000.

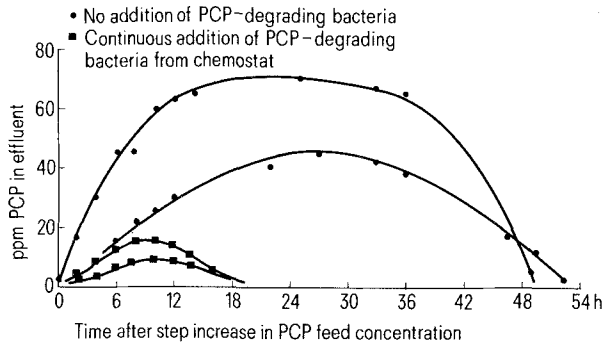
Stability of specialized cultures for long-term treatment

If industrial wastes are isolated close to the source of their production then their composition may be simple enough to allow specialized growth, on a sustained basis, of rather well-defined or even quasi-pure cultures. The concept of defined mixed cultures as mutually consuming all of the available carbon supply was developed already by the Shell Research group as a basis for producing single cell protein from methane[19], and has also been applied to treating waste with simple water-miscible compounds[38].

Even easier to maintain is a quasi-pure culture of *Azotobacter* cells, if the industrial waste happens to be nitrogen-deficient at the source. Because of their unique aerobic nitrogen-fixing capability these organisms can be maintained as the dominant culture in a chemostat under non-sterile conditions. Furthermore they have broad ability to attack even aromatic compounds like phenol. Upon aging, the cells leak other nitrogenous compounds, so cell recycling must be avoided. It has been pointed out[12] that the advantage of such an *Azotobacter* culture, as a form of waste pre-treatment, is the reduced yield of biomass. Complete oxidation of the waste to CO_2 and water occurs and with only a third as much cell mass accumulation as with activated sludge. (Organic matter is oxidized at a rapid, inefficient rate by these bacteria as a means of protecting their air-sensitive nitrogenase enzymes from damage). In pilot plants tests with *Azotobacter* a waste stream containing mostly ethanol (2000 mg/l COD) was treated in 5 h detention time to remove 80% of the organic matter even without cell separation. In this particular waste stream ethyl acetate was also present in small amounts, and the treatment would have been more complete if the *Azotobacter* could have metabolized it also. Despite their omnivorous capabilities, however, these bacteria are curiously unable to hydrolyze esters. Such simple esterases would nowadays be relatively easy to introduce, by genetic manipulation, into an otherwise useful organism.

The use of chemostatic culture as a pretreatment for industrial wastes will probably come into more widespread use as various tailor-made cultures or mixed populations find special uses. Chemostats generally respond well to shock loads, especially if cell recycle is possible. Bacterial cells from such suspension culture would usually be removed in a subsequent activated sludge unit, using protozoa as discussed by Curds[8] and by methods presently under development in Japan.

If activated sludge units are used to treat unusual and sometimes toxic wastes, special problems in stability often arise due to relatively small changes in flow or feed composition. Under such circumstances continual or periodic addition of the critical bacterial types may be necessary. For the treatment of pentachlorophenol, as described earlier, an acclimated sludge could routinely treat a waste containing 40 mg/l of PCP, plus sugars and mineral salts. A step change in the concentration of PCP in the feed from 40 mg/l to just 120 mg/l caused a rapid rise in the amount passing into the effluent (see figure). After 2 days the system returned to a steady state. The intensity as well as the duration of the disturbance could be markedly reduced however, by continuous addition of PCP-degrading bacteria from a chemostat. The dosage rate of cells was 5–7% of the net sludge production rate (Edgehill and Finn, unpublished results). A cause for the unstable behavior is not yet understood in this case because in pure or quasi-pure culture the PCP-degrading *Arthrobacter* exhibit their maximum growth rate of 0.15 h^{-1} between concentrations of 10 and 135 mg/l of PCP. The tolerance of these bacteria is surprisingly high; the growth rate is still 0.1 h^{-1} at 300 mg/l of PCP (all values at pH 7.4, 30 °C).



Response of a laboratory activated sludge unit to an increase in the feed concentration of PCP from 40 mg/l to 120 mg/l. 2 replicates shown.

The above example illustrates a situation arising with a natural isolate. Highly bred organisms of the type developed by Chakrabarty and co-workers[21,22] for biodegradation of 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), are likely to perform even less well in an activated sludge unit. It now appears that the unusual degradative abilities of most specialized strains of microorganisms may be carried by plasmids[13,32]. The exchange of these genetic elements provides opportunities for the evolution of new degradative capacities, especially by selective challenges in the chemostat. The maintenance of such capabilities, however, will be especially difficult when, as in the case of PCP, the wastewater also contains considerable amounts of sugars leached from the wood being treated with the preservative. It will probably be easier in general to maintain degradative strains on wastes of more simple composition and to maintain them in chemostats rather than in treating units where flocculation must be accomplished at the same time as the degradation.

Acknowledgment. For assistance in preparing this manuscript, thanks are extended to the staff of the Institute of Biotechnology, Nuclear Research Center, Jülich (Federal Republic of Germany).

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0014-4754/83/111231-06$1.50 + 0.20/0

Detoxification of pesticides by microbial enzymes

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1. Introduction

The production and use of synthetic pesticidal chemicals increased dramatically after World War II. The need to know the fate and effect of these new chemicals in the environment has created tremendous research efforts. Early investigators were concerned with degradative processes and when it was realized that microorganisms could degrade xenobiotics, research projects were initiated to determine the basic principles of microbial metabolism. This led some scientists to be amazed at the ability of microbes to degrade chemicals, claiming that microbes were either infallible and could degrade any synthetic molecule, or, on the other hand, that certain molecules could not be metabolized and were refractory[14]. This debate led investigators toward a better understanding of the basic principles concerning the enzymology and biochemistry of pesticide-related metabolism. It also helped to establish that the persistence of xenobiotics in the environment was strongly affected by microbial activity.

In the 1970's the mood of the pesticide industry changed from being optimistic to recognizing the damages that agricultural chemicals could do to our