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SOURCE AND MAINTENANCE OF MICROORGANISMS USED FOR TESTING PLASTICS

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ABSTRACT

In some countries legislation on the use of biodegradable plastics has outpaced the development of reliable standard methods of measuring degradation. There are numerous methods designed to assess soluble and water-miscible products, but standards designed specifically for plastic materials are few. This paper discusses some current techniques and those under development with particular reference to the organisms employed. The need to use defined test strains, mixed organisms from the environment, or both is discussed along with the problems that can arise when attempting to maintain the relevant enzyme activities in test strains in the laboratory. The speed with which some isolates can lose activity and vigor during maintenance is assessed.

INTRODUCTION

Plastics are heterogeneous materials. They may contain impurities such as residual oligomers, monomers, reagents, and products of side reactions. They may also contain compounds included intentionally such as plasticizers, fillers, pigments, mold release agents, and starch added to enhance degradability. These must be borne in mind when selecting suitable organisms or sources of inocula to test their resistance or susceptibility to attack.

The eventual use and/or likely method of disposal is also of significance. The UK disposed of 85% of its domestic waste by landfill in 1990 while Denmark, for example, disposed of only 10% by this method [1]. This means that the bulk of the

UK plastic waste would experience anaerobic conditions with high levels of bacterial activity, while in countries where composting is of more significance, mixed populations of aerobic microorganisms will be the major degraders. Plastic waste which never finds its way into a disposal system and finishes its life as litter in soil contact will be heavily reliant on fungal species to bring about biodegradation as will biodegradable plastic mulches and other agricultural materials.

Wastes in fresh water or marine environments are more likely to encounter aerobic bacterial activity. Susceptible materials which have been stored badly at high humidity are likely to be attacked by fungi while those in water contact may be damaged by bacterial activity. All this must be taken into account when designing test regimes.

DEFINITIONS

Biodeterioration and biodegradation are terms which are often considered to be interchangeable. However, Heuck [2] defined biodeterioration as "any undesirable change in the properties of a material of economic importance caused by the vital activities of organisms." Biodegradation, on the other hand, has been defined as "the harnessing by man of the decay abilities of organisms to render waste material more useful or acceptable" [3]. This definition is inadequate for current requirements as it implies "the hand of man" must be present, but materials in a natural uncontrolled environment will obviously biodegrade. It does, however, indicate the desirable nature of biodegradation as opposed to the undesirable biodeterioration. An acceptable definition of the term "biodegradable" is yet to be agreed upon internationally. Is complete mineralization and removal from the environment necessary before a material is considered to be "biodegradable," or is breakdown to a smaller molecule acceptable? If so, how simple must the molecule be, must it be nontoxic (certainly) and biologically inert (in which case it is not biodegradable), and so the argument continues.

A number of bodies are working toward acceptable definitions, and there are drafts in various stages of preparation and completion. All extremes are covered with DIN requiring "naturally occurring metabolic endproducts" while the Biodegradable Plastics Society (BPS) in Japan suggests a biodegradable polymer is one capable of "being decomposed into low molecular weight components."

TESTS FOR BIODETERIORATION AND BIODEGRADATION

"A testing protocol should include an environment that fairly represents that to which the substrate polymer will be exposed" [4]. The above statement holds true when we know exactly what this final environment will be, and such factors must be taken into account when designing a test method. However, problems can arise on the numerous occasions when the end use/disposal is not known or the material may be destined for multiple fates as is more often the case. Simulated environment tests, however, only form one part of the total testing protocol which is required.

There have always been conflicting opinions regarding the source of inocula for testing. Many workers feel that only organisms freshly isolated from relevant

substrates should be employed or that inocula based on soil or sewage with or without acclimatization are the sole answer. Many of the standard biodeterioration tests require the use of defined species (but not always strains).

There is justification for all points of view. In a research and development environment where specific conditions of use and disposal are known, then every effort should be made to obtain the relevant, active organisms that are likely to be encountered, either in isolation or as an acclimatized inoculum. However, rapid screening methods are often required in the early stages of a development program when the use of a standard test set can be helpful. The use of defined organisms to assess degradability or otherwise of additives (plasticizers, etc.) is also recommended. Standard sets of organisms are also useful to provide final "bench mark" testing which aids comparisons between products. Lee et al. [5] used pure fungal culture systems to allow the distinction between chemical and biological degradation of novel materials. Yakabe et al. [6] concluded that although the biodegradability of plastics in simulated natural environments is desirable, the reproducibility of these methods is not high, while the use of specific enzymes [7] or microorganisms [7, 8] can estimate biodegradability quickly and reproducibly.

Biodeterioration Testing

Table 1 shows some of the standard tests which are available for use in biodeterioration and biodegradation testing of plastics. The problems arising out of current biodeterioration tests and the organisms employed have been discussed elsewhere [9]. These standards are mentioned here because they contain techniques which are valid for both biodeterioration and biodegradation testing, e.g., soil burial methods. A number of workers have looked at the role of soil burial methods in biodegradation testing. Yakabe et al. [6] studied the factors affecting the biodegradability of polyester in soil.

Biodegradation Testing

The definition and measurement of biodegradation is hampered by the lack of recognized standards. There are three major questions which require an answer to predict the likely behavior of a plastic in the environment.

TABLE 1. Some Standard Tests Designed for Use on Plastics

ISO 846	Plastics—determination of behavior under the action of fungi and bacteria
ASTM G21-90	Standard practice for determining resistance of synthetic polymeric materials to fungi
ASTM D 5209-92	Standard test method for determining the aerobic biodegradation of plastic material in the presence of municipal sewage sludge
ASTM D 5210-92	Standard test method for determining the anaerobic biodegradation of plastic materials in the presence of municipal sewage sludge

1. Is the material readily biodegradable? For this a simple stringent test is often used employing organisms and inocula with limited time for acclimatization.
2. Is the material inherently biodegradable? Current tests usually provide more favourable conditions using larger inocula and acclimatized organisms. Limits may be set on all tests, for example, 60% degradation after 28 days may be considered to indicate biodegradability in the Sturm test.
3. Is the material going to degrade under field and waste disposal conditions? The tests here tend to be simulated environment techniques.

The terminology and early biodegradability tests were mainly devised to cover liquid and sparingly-soluble or limited water-immiscible liquids such as detergents, surfactants, oils, etc. Testing of water-soluble polymers is consequently relative easy; methods include biological oxygen demand (BOD), carbon dioxide evolution (e.g., Sturm test [10]) and semicontinuous activated sludge tests (SCAS) [11]. Water-insoluble plastics, however, are more difficult. Two relatively new ASTM tests [12, 13] (Table 1) have begun to address the problem. Degradation of a known standard is employed as an organism control and 70% degradation of this material is required to confirm a valid test. These, in a program together with the established ASTM Biodeterioration tests [14–16], can give the experimenter a great deal of information. The International Biodeterioration Research Group (IBRG) is currently ring testing a modified Sturm test for use with biodegradable plastics. There are a number of techniques employed to simulate environmental situations, e.g., soil burial [16], simulated landfill, and simulated composts. Biodegradation may be assessed by weight loss, gas evolution, etc.

Many naturally occurring polymers can take a number of years to degrade, e.g., some timbers, pine needles. There are examples of landfill sites where newspapers are still legible after 40 years; this supposedly readily degradable polymer did not degrade in this field situation. We must ensure that we are not demanding more from biodegradable plastics than we expect from naturally occurring polymers.

EXPERIMENTAL WORK

There is a case for the use of defined cultures within the testing protocols of plastics materials. However, if test organisms are to be employed, it becomes imperative that the correct strains are selected and that these strains are well maintained. The International Mycological Institute (IMI) has had a rolling program of monitoring fungal test strains and screening new isolates with a view to improving test methods. This work has fallen into three stages: investigations of the variation in enzyme activities within species, looking at the variation between test strains held in different culture collections, and assessing the effect of maintenance regimes on enzyme activities. Data resulting from the first two studies have been published [17]. The work reported here completes one aspect of part three above.

METHODS

A series of semiquantitative screening tests has been developed to investigate enzyme activities in fungal cultures and has been described previously [17]. These methods were used to investigate the effect of subculturing strains as a means of culture maintenance.

There are a number of recognized test strains held by the IMI Genetic Resource Collection which are cited in standard test methods. Eighteen of these strains (Table 2) were put through six of the enzyme screens to investigate amylase, cellulase, protease, polycaprolactone degradation (as an indicator of potential to degrade polyurethanes, plasticizers, etc.), lipase, and pectinase activities. The tests were selected to monitor the activities of enzymes which could be of significance in the breakdown of commercial plastic formulations and plastics designed or amended to be biodegradable.

All results were rated on a 0–5 scale from zero to very high activity. Growth was also assessed and designated A–E, indicating very good growth to no growth, so that A5 would be the rating of a strain giving good growth and high enzyme activity.

The 18 isolates were then subcultured weekly for 24 weeks onto their recommended growth media, i.e., no special precautions were taken to ensure enzyme activities were retained by challenging with the relevant inducing substrate. The subcultures were grown for 7 days and then put through the screens described above. After 24 weeks the cultures were subbed onto media containing relevant inducing substrates for a further 10 weeks. Once more the cultures were screened after each subculture to assess recovery of enzyme activities.

RESULTS

Table 3 summarizes the overall results after 24 weeks subculturing. A number of activities were very rapidly lost or much reduced. *Chaetomium globosum* 16203 lost enzyme activities allowing polycaprolactone degradation after one subculture; *Stachybotrys atra* 82021 lost this ability after two subcultures. Other strains lost this activity after five and six subcultures. Amylase activity, important in the degradation of starch-based and amended plastics, was lost by *Scopulariopsis brevicaulis*

TABLE 2. Organisms Tested

Fungal species	IMI strain numbers
Aspergillus amstelodami	17455
A. flavus	91856
A. niger	17454 and 91855
A. terreus	45543
A. versicolor	45554
Aureobasidium pullulans	45533
Chaetomium globosum	45550 and 16203
Paecilomyces vaiotii	108007
Penicillium cyclopium	19759
P. funiculosum	14933, 211742, and 87160
P. ochrochloron	61271
Scopulariopsis brevicaulis	49528
Stachybotrys atra	82021
Trichoderma viride	45553

TABLE 3. Enzyme Activity Levels in Fungal Test Strains after Twenty-four Subcultures

Enzyme activity	Activity level maintained	Activity level reduced	Activity level not detectable	No initial activity
Amylase	5	5	7	1
Cellulase	5	7	3	3
Protease	2	4	8	4
Polycaprolactone degradation	6	4	8	0
Lipase	0	3	13	2
Pectinase	4	12	2	0

49528 after four subcultures and by *Stachybotrys atra* 82021 after seven subcultures. Cellulase activity in two *Chaetomium globosum* strains (45550 and 16203) was reduced from Level 5 to Level 1 after six and seven subcultures respectively.

In general, lipase activity seemed to be the most readily lost with no strains retaining maximum activity, 13 losing it completely. The ability to break down polycaprolactone was retained by the highest number of strains but also had the second largest number of total losses together with protease activity. Only two strains lost pectinase activity completely but only four retained full activity and 12 showed much reduced activity. The ability to degrade polycaprolactone and pectinase activity was found in all organisms tested.

After 24 weeks, 10 of the 18 strains still showed some ability to degrade polycaprolactone (six fully maintained) and again 10 strains still showed amylase activity (five fully maintained).

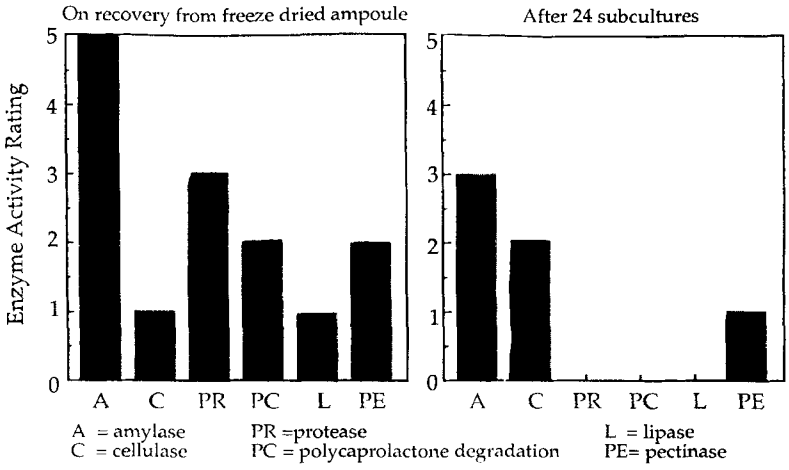

FIG. 1. Enzyme activity ratings before and after subculturing *P. cyclopium* 19759.

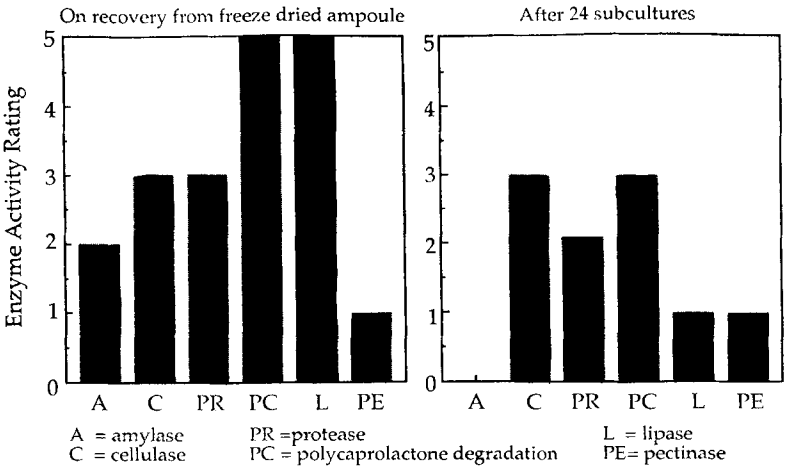

FIG. 2. Enzyme activity ratings before and after subculturing *A. niger* 91855.

Figures 1 and 2 show profiles of initial activity and that after 24 weeks for *Penicillium cyclopium* 19759 which had 50% of the activities tested no longer detectable and *Aspergillus niger* 91855 which retained all but amylase activity.

Table 4 summarizes the results at 34 weeks after transfer to inducing substrates. Amylase and pectinase activities were not regained by any strains which had lost detectable activities. Three strains regained cellulase and protease activities and one regained lipase. Many strains showed further reductions in activity, and a number lost detectable activities completely.

DISCUSSION

The results from part one of these studies [17] suggested that when developing test methods it is not sufficient to simply recommend species. The variations in activities between strains (Fig. 3) indicated that optimal activities must be screened

TABLE 4. Responses of Tests Strains after Transfer to Inducing Substrates between Twenty-four and Thirty-four Subcultures

Enzyme activity	Activity fully recovered	Activity maintained at reduced level	Activity further reduced	Activity lost
Amylase[a]	0	14	2	5
Cellulase[b]	3	7	1	3
Protease[b]	3	5	2	2
Lipase[b]	1	2	0	2
Pectinase[b]	0	9	4	0

[a]Extra strains were added to investigate amylase activity.
[b]Not all strains continued to be studied in this part of the work.

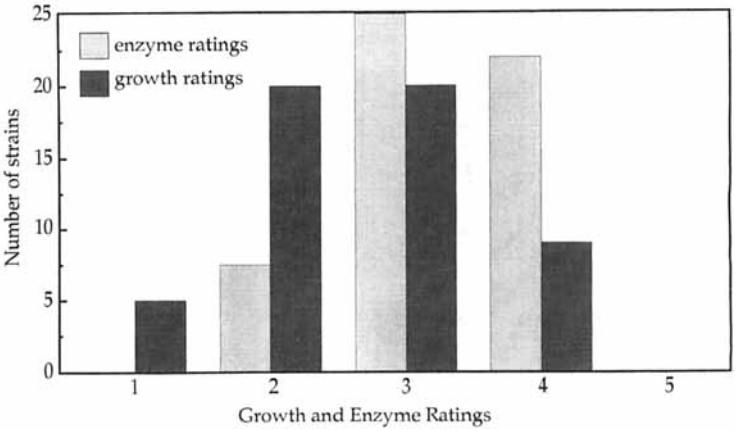

FIG. 3. Polycaprolactone degradation by *A. niger*. Reprinted from *International Biodeterioration, 24*, 289 (1988) with kind permission from Elsevier Science Ltd., The Boulevard, Langford Lane, Kidlington OX5 1GB, UK.

for, the best strains selected, and then referred to by culture collection number in the standard. Part two of the work showed that when cultures are well maintained by freeze-drying or in liquid nitrogen, optimal activities can be successfully retained as demonstrated by testing the same cultures held at IMI and at Centraalbureau voor Schimmelcultures (CBS), Baarn, the Netherlands.

The work described here demonstrates the importance of good maintenance techniques. While the tendency of organisms to become "laboratory strains" if maintained by subculturing is well recognized, the speed with which this occurred in some of these strains was surprising. It could be predicted that some strains having inducible activities would lose and then regain detectable activities when presented with the correct substrate. Some constitutive activity was retained throughout, but concern arises from the number of strains which lost activities rapidly and never regained them. This was presumably due to selection during subculture of conidia without the genetic information coding for these activities, and in this case the potential for change is probably even greater in fungi than in bacteria. Changes may also be due to heterokaryosis and the parasexual life cycle of fungi and to well documented intracellular DNA damaging processes which occur at a fairly constant low rate [18]. Maintaining fungi in an actively metabolizing state will inevitably introduce more of these problems when compared to freeze-drying or liquid nitrogen storage.

Maintaining "fed" cultures in fermenters etc. is not always the answer either, because while maintaining enzyme activities the ability to grow well and sporulate normally at air/substrate interfaces may be reduced with the organisms becoming "reactor" strains.

CONCLUSIONS

Testing protocols for plastics should always include environmental simulation tests and exposure to multiple organism inocula (sewage, soil), but there is an

argument for the use of defined test cultures of bacteria and fungi within the development program. When defined fungal cultures are employed, these should include test strains identified by culture collection number. The use of a defined "core" set should not preclude the inclusion of other additional organisms selected by the worker.

Test strains should not be maintained routinely by subculturing and should be monitored for required activities at regular intervals.

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