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The International Biodeterioration Bulletin is published four times per year (Spring, Summer, Autumn and Winter). Typescript contributions should be sent to the Editors, at the above address.

The Bulletin acts as a vehicle for the publication of original works, including reviews, on all aspects of biodeterioration, i.e., deterioration of materials, artefacts or facilities, of economic importance by living organisms, which include microorganisms, insects, rodents, birds, higher plants, etc. Articles on biodegradation, that is conversion of materials to less objectionable, more easily disposable, or higher value products by living organisms, are also published.

Contributions are published only in English. Each article must be accompanied by a summary in 50 - 150 words which will be translated into French, German and Spanish. Native speakers of these languages are invited to submit their summaries in their own language; in certain circumstances complete articles may be submitted in French, German or Spanish and will be translated into English for publication.

Illustrations must be very clearly drawn, normally larger than the size finally desired. The suggested final size should be clearly indicated but the Editors reserve the right to vary this in the interest of economy and clarity.

As far as possible diagrams will be reduced to single column width (80 mm) or to half page (170 mm). In any event, neither these nor half tone photographs can exceed full page (260 by 170 mm). Authors should bear in mind that it is generally more convenient for readers if legends which accompany diagrams or photographs appear with them on the same page and should proportion their illustrations accordingly. Lettering on diagrams will normally be inserted by the printer; authors are therefore asked to insert lettering or symbols in pencil on the originals or preferably, in ink on a photocopy.

All articles are submitted by the Editors to one or more independent referees for advice on their clarity, originality, and general suitability for publication, but the final decision whether or not to publish an article rests with the Editors. If articles are rejected the substance of the referee's report will usually be communicated to the author and in suitable cases the Editors will be pleased to help authors to improve their papers with a view to possible publication.

Bibliographic references are indicated in the text by author names (no initials) and year only, viz: Reese and Levinson (1952); or: Darby et al. (1968) and in the bibliography in strict alphabetical order of first author's names, thus:

Reese E T and Levinson H G (1952)
Comparative study of the breakdown of cellulose by microorganisms
Physiologica Plantarum 5: 354-366

or:
Darby R T, Simmons E G and Wiley B J (1968)
A survey of fungi in a military aircraft fuel supply system.

International Biodeterioration Bulletin 39-41

References to books, conference proceedings, etc. should quote first the author(s) or editor(s), then the year of publication and title followed by the city in which it is published and the name of the publisher. As far as possible titles of journals should be given in full except for such abbreviations as "Journ.", "Proc.", "Trans." etc. 20 reprints will be sent free of charge to the first named author unless otherwise instructed. Any number (normally not more than 50) of additional reprints may be purchased if ordered sufficiently in advance. An order form and price will be sent giving about one month's notice.
Forthcoming Meetings in Great Britain

Following is the latest list of meetings which have been arranged to take place in Britain:

7th - 8th July 1983
‘Post-Harvest Biodeterioration’
Wolfson College, Cambridge.

13th December 1983
‘Packaging in relation to Biodeterioration’
Shoreditch College (Brunel University)
Egham Green, Surrey.

17th - 18th April 1984
‘Developments in the Study of Microbial Growth’
Hatfield Polytechnic

5th - 6th July 1984
‘Microbial Corrosion’
University of Surrey, Guildford.

Bunker Memorial Prize - Amended regulations

The Council of the Biodeterioration Society has agreed the following changed arrangements for the annual Bunker Memorial Prize competition.

The award of the Bunker Prize will in future be on the basis of an essay competition. The winner will be invited to present his/her entry as a short paper at a suitable future meeting of the Biodeterioration Society, but willingness to do this is not a condition for award of the prize. The conditions will be as follows:

1. Eligibility. All persons under the age of 25 and not holding a higher degree at the time of the closing date of the competition will be eligible.

2. Entry. Competitors will be invited to submit an essay or short paper based on any aspect of biodeterioration.

3. The entry must be based upon the candidate’s own work; in the case of essays based on published literature the candidate will be expected to demonstrate an original approach to the material.

4. The competition will be decided by a panel of three judges who will be appointed by the Council.

5. The winning entry will be announced at the December meeting of the Society and the winner awarded a prize of £50 plus one year’s membership of the Society.

6. Entries must be received by the first Monday in November by the secretary to the Society, Mrs. Joan Maw, Hatfield Polytechnic, School of Natural Sciences, P.O. Box 109, Hatfield, Herts.

Dr K J Seal - Biodeterioration at Cranfield

Dr K J Seal, whom many will know as the manager of contract research at the Biodeterioration Centre, University of Aston, has recently left to join the Cranfield Institute of Technology, Cranfield, Bedford. There he is establishing a Biodeterioration laboratory in the Biotechnology Centre. We wish him every success in this venture and welcome the accession of Cranfield to the list of Institutions which are entering the study of Biodeterioration.

Abstracts of papers presented at a Symposium
on
The Biodegradation of Solid Wastes
held at the Sheffield City Polytechnic
on
14th December 1982

Title: The use of Biodegradable Wastes in the Third World

Author: J.A. Vogler

Address: Interwaste, 40, The Avenue, Roundhay, Leeds LS8 1JG

Abstract

Given that the Third World is characterized by shortages of fertile soil, of energy and of feedstuffs for animals and humans, and that weaker legislation and lack of capital result in severe urban and industrial pollution, many people have seen the use of biodegradable wastes as offering tremendous opportunities to Developing Countries. The success of attempts to exploit these, particularly in the fields of composting of municipal refuse and of domestic scale biogas production, will be discussed. The contrast between highly successful programmes in China and widespread failures elsewhere will be discussed and suggestions made to improve the prospects of success in future projects. A number of other imaginative uses of organic wastes will also be discussed.
Title: The Biodegradation of Agricultural Wastes

Author: K.J. Seal

Address: Biodeterioration Centre, University of Aston St. Peter's College, Saltley, Birmingham B8 3TE

Abstract

Agricultural wastes in the U.K. represent an ever increasing problem particularly as available land for disposal is limited and legislation introduced in recent years is now being implemented. This paper discusses the types and quantities of wastes arising in the U.K. and shows that they may be considered as resources. Research over the last 15 years has demonstrated that micro-organisms can be harnessed to treat wastes for either recycling as a feedstuff or for safer disposal. Examples of this and the author's own work are given.

Title: Fungal Protoplasts as Tools in Biodeterioration Studies

Author: K. Thomas, B. Davis and J. Mills

Address: Department of Biological Sciences, Sheffield City Polytechnic, Pond Street, Sheffield S1 1WB

Abstract

Fungal protoplasts have a range of uses in biodeterioration studies. They may aid isolation of organelles, localization of metabolites or provide a system to investigate the effect of various agents directly on the fungal cytoplasm. Other uses include the ability to incorporate DNA and to fuse with protoplasts of different strains or species.

Lytic digestion of the hyphal wall and survival of protoplasts are affected by temperature, pH, lytic enzyme type and concentration, osmotica, co-factors and prior cultural conditions.

Protoplasts released from Aspergillus fumigatus regenerate in media containing carboxymethyl-cellulose and release CMCase while intact hyphae on this substrate retain the enzyme within the cytoplasm. Regenerating protoplasts thus offer an opportunity to investigate the details of enzyme secretion and the effect of the cell wall on this.

Title: The Biodegradation of Plastics Waste

Author: B.S. Brown

Address: Biochemistry Department, Medical School, Stopford Building, Oxford Road, Manchester M13 9PT

Abstract

Polyethylene has been converted to single-cell protein by oxidation or pyrolysis followed by fermentation. Oxidation of 100g polyethylene (3h reflux with conc. HNO₃) yielded 77g soluble dicarboxylic acids (C-6 to C-12) and 23g insoluble wax. Batch fermentation (5-litres, 2d, 30°C) of the soluble fatty acids yielded 41g biomass (38% crude protein), 95% of the dicarboxylic acids being utilised by the mesophilic bacteria employed. Pyrolysis of 100g polyethylene (60min, 560°C) yielded 90g insoluble wax comprising homologous series of n-alkanes, n-alkenes and α,ω-alkadienes (C-8 to C-40). Batch fermentation (8-litres, 5d, 30°C) of pyrolysed polyethylene dispersed in pristane yielded 45g biomass (35% crude protein), 50% of the pyrolysate being utilised by the Candida cells employed. Continuous fermentation of 46g pyrolysate (obtained from 46g polyethylene) yielded 5.2g biomass, 33% of the pyrolysate being utilised. Polypropylene and polystyrene can also be converted to biomass by these processes, but polyvinyl chloride resists both oxidation and pyrolysis.
Energy Recovery and Effluent Treatment by Anaerobic Fixed Films

L. Cassell and A. Wheatley

Pollution Research Unit, UMIST, Sackville Street, P.O. Box 88, Manchester M60 1QD

Abstract

Laboratory research, on a range of effluents, has established the feasibility of using anaerobic filtration for effluent treatment and gas recovery, from warm, strong food, drinks and fermentation wastes. This type of effluent is not easily treated by conventional techniques because air for bio-oxidation and sludge disposal are now very expensive. pH control was found to be critical and difficult to control, even by recycle with some wastes. Experiments have been extended to a 10m³ test plant treating the waste from a local sweet factory. The plant has been running for 3 months and demonstrated the potential to save 70% of the effluent treatment costs and make a 5% contribution to fuel costs. COD removals of up to 80% can be achieved at residence times of 24-30 hours and loads of 5-15 kgs COD per m³ per day. Gas yields are between 0.5-0.7m³ per kg of COD removed at 50-70% methane. The best gas production is at neutral pH. A full-scale plant has now been built.

The Biotechnology of Waste Treatment

A. Wheatley

Pollution Research Unit, UMIST, Sackville Street, P.O. Box 88, Manchester M60 1QD

Abstract

Waste treatment is one of the oldest and largest applications of biological organisms in the manufacturing and service industries. It is assumed that the current advances in genetic engineering and other biotechnological techniques will improve the efficiency of waste treatment and by-product recovery.

Biological Limits to Ethanol Production

S.G. Oliver

Department of Biochemistry & Applied Molecular Biology, UMIST Sackville Street, P.O. Box 88, Manchester M60 1QD

Abstract

The production of industrial alcohol by fermentation is often quoted as an end product of the treatment of waste materials. The efficiency of ethanol production, in both energetic and economic terms, is limited by the ethanol tolerance of yeast. The inhibitory action of ethanol on yeast is complex, there are different and separate effects on growth rate, fermentation rate, and viability. This complexity means that ethanol tolerance will be modified by a wide range of environmental and genetic factors. For instance, growth temperatures just below the optimum increase the ethanol tolerance of yeast growth, whereas fermentation is more resistant to elevated temperatures. Both nuclear and cytoplasmic mutations which increase ethanol sensitivity have been discovered. However, it has been possible to obtain ethanol tolerant mutants by a continuous selection system in which the intensity of selection is determined by the culture itself via a feedback control circuit and these should prove useful in increasing product concentration.

The publication of these abstracts by the Biodeterioration Society does not constitute publication in the usual sense. No reprints are available. Those wishing to pursue these subjects further should write directly to the authors at the addresses shown and not to the Society.
A CONTRIBUTION TO THE EXAMINATION OF THE ROT RESISTANCE OF TEXTILES

Paul Raschle

Summary

Results from laboratory soil burial tests can provide good forecasts of behaviour in practice. These tests can act as simple screening tests for manufacturers of biocides or can serve as a method for quality control based on the user's specific requirements.

It is important to standardize some main parameters for a soil burial test: the water content in the test system should be near 60% of maximum moisture holding capacity, acclimatization of the test soil is recommended, the system should be aerobic, test vessels should be incubated in an aerated atmosphere of more than 95% relative humidity, and a temperature of about 29°C is optimal for standardizing a rot resistance test.

Contribution concernant l'examen de la solidité de textiles à la putréfaction

Des résultats d'essais par enfouissement dans le sol donnent de valables informations quant au comportement en pratique. Cet essai représente une méthode simple pour le screening par le fabricant de biocides ou une méthode à contrôler les exigences spécifiques par le client.

La grande importance de quelques facteurs majeurs doit être observée pour l'enfouissement dans le sol: l'humidité du sol devrait être à 60% de la capacité maximale de rétention d'eau, la terre devrait être acclimatée avant d'effectuer l'essai d'enfouissement, un système aérobie est propé et l'incubation des récipients à plus de 95% d'humidité relative de l'air circulant et environ 29°C sont considérés optimaux pour la normalisation des essais d'enfouissement dans le sol.

Introduction

Examining the rot resistance is interesting and meaningful for the various parties concerned. The manufacturer of biocides is interested, among others, in an economic reproducible laboratory method for screening, and the buyer or user is interested mainly in the rot resistance in practice. The person who finishes the textile so as to protect it against rot will take into consideration the results from laboratory tests and the flow of results from practical usage.

This article deals mainly with the parameters which must be fulfilled in order to achieve a laboratory test which effectively forecasts the efficiency of a product against rot.

Standardization of a laboratory soil burial test

Hausam (1967) has dealt critically with the effectiveness of laboratory tests in forecasting practical experience. Objections have been raised against the soil burial test concerning its reproducibility and its relevance to practical experience. These two objections are lessened, however, by reference to the wide scatter of results especially with a treatment of minimum effectiveness. This shows that these two aspects, the erratic results of interpretation of results from soil burial tests. Turner (1972) describes the influence of many factors in rot resistance tests by soil burial methods, e.g. different results depending on the operator, re-use of soil, degree of compaction of the soil etc.

Bemerkungen zur Prüfung der Verrottungsbeständigkeit von Textilien

Resultate einer Laborvergräbungsprüfung geben gute Hinweise für das Praxisverhalten. Die Erdvergräbung ist eine einfache Prüfmöglichkeit für das Screening beim Biocidehersteller oder für Abnahmekontrollen aufgrund spezifischer Anforderungen durch den Verbraucher.

Es ist wichtig, einige Hauptparameter der Erdvergräbungsprüfung zu beachten: Der Wassergehalt im Testsystem sollte etwa 60% des max. Wasserrückhaltevermögens sein, eine Voraklimatisierung der Prüferde vor dem Test ist empfohlen, das System sollte aerob sein und die Inkubation der Prüfproben in einer belüfteten Atmosphäre von über 95% rel. Luftfeuchtigkeit und etwa 29°C werden als optimal betrachtet, eine Verrottungsprüfung zu normieren.

Contribución suiza al examen de resistencia de textiles a la putrefacción

Los resultados de las pruebas de enterramiento en suelos en laboratorio pueden dar buenas previsiones del comportamiento en la práctica. Estos métodos pueden servir como comprobación rutinaria para los fabricantes de biocidas o como un método para el control de calidad según las necesidades específicas del usuario.

Es importante normalizar algunos de los principales parámetros en este tipo de pruebas: el contenido de agua debe estar cerca del 60% del total admisible en el sistema, deben controlarse las condiciones ambientales, el sistema debe ser aeróbico, los recipientes deben airearse con aire que contenga más del 95% de humedad relativa y la temperatura de 29 grados es la óptima para esta clase de pruebas.

Similar projections were also done by many other authors: Siu (1951) mentions the "high degree of variability of results from different soils". For Lloyd (1968) "the chief problem is the lack of agreement which may be experienced, both, between different laboratories and on different occasions in the same laboratory" and he remarks the difficulty in interpreting results from soil burial tests. Turner (1972) describes the influence of many factors in rot resistance tests by soil burial methods, e.g. different results depending on the operator, re-use of soil, degree of compaction of the soil etc.
Therefore it should be postulated that a good laboratory soil-burial method shall optimally decay any material which is not protected. However, it shall also identify as good, during a defined testing time, any treated homogenous material which has been proved in practice under wet conditions. A minimal requirement for a test system is required, in respect of the microbial decay of textiles in order to make sure that the test is carried out under conditions which are optimal to the greatest possible extent. On the other hand, it would be desirable to set a limit value for a given method which classifies any material going beyond that value as "insufficiently protected". However, as this point is related to practical experience, such practical experience is needed to establish it. The method and the test material, compared with unprotected control material, determine the fixed points for the "rot resistance" and "regular test procedure" (Lloyd (1968): validity of the test).

It is an indispensable condition, however, that the upper limit values are only meaningful if there is a real danger of the material rotting in practice. No limit values on an evaluation criteria should be defined if it is known, for any material, that its liability to rot in practice is very low, as rot does not occur. Anything which rots in the laboratory under optimal conditions cannot be rot resistant in practice.


As rot-resistance tests already exist, or are under discussion, for various industries, some important parameters of that laboratory test are now reviewed:

Important Parameters of the Laboratory Soil Burial Test

a. Moisture content of the test soil.

Walchli (1978) found the optimum for the cellulolytic activity of microorganisms to be at about 60% of the maximum moisture holding capacity of the soil. Similar results are known from Siu (1951), who mentions the abundance of cellulolytic bacteria in the range of 50-80% moisture holding capacity and good decomposition of cellulose by cellulolytic fungi also between 50-80% of moisture holding capacity.

Khaziev (1976) also found that the largest spectrum of aerobic microorganisms occurs at the same proportion of water holding capacity in a soil specimen; he also found the maximum enzyme activity of aerobes at this moisture level.

El-Shinnawi et al., (1981) mentions the highest activity of both urease and phosphatase in a soil at 60% of water holding capacity. Rubidge (1977) found for John Innes No.1 soil best cellulolytic activity at 53% maximum moisture holding capacity.

It seems therefore that for many different soils a water content of about 60% max. MHC may favor different groups of cellulolytic microorganisms.

Moisture level must, therefore, not be determined as an absolute value but must be related to the moisture holding capacity of the soil. For soils with different moisture holding capacities, the optimum moisture contents are different. Our own test with different soils and different fractions of water holding capacity confirm this "optimal" level of about 60% of MHC.

b. Type of test soil

Microbially active, and less active, soils are known from nature and from eco-biological studies. Different levels of microbial activity are to be expected as different populations of microorganisms predominate, depending on the substrate, e.g. C/N ratio, pH and temperature. The test soils defined in the different standards are therefore of great importance for the understanding of soil burial tests. Depending on the soil, and also on the test material, different results are to be expected (see Turner (1972)). The standardized soil "Einheitserde Typ ED 73", of which we have good experience, may be mentioned as an example of a commercially available soil. (Einheitserde- und Torfwerke, Gebr. Patzer KG, D-6491 Simtal-Jossa; Agencies also in Austria, Italy, Switzerland and France). It contains about 66% of ashable substances, a nitrogen content of 0.24% and the pH-value of the 10% suspension is 5.2. This soil has a maximum moisture holding capacity of about 185% (Graf, 1979).

c. Pretreatment

As a laboratory rot test should not take too long a time, the initial activity of the soil is decisive for the results. This implies that a high enzymic activity in the soil is required. Kaplan (1978) has emphasized the importance of this pretreatment of a test soil prior to the test. That such "acclimatization" is necessary to aid the laboratory test is generally accepted. It is known that, for example, during maturation of a compost, different phases of the mineralization occur whereby the structure of the organisms present and responsible is changing completely with time. Without a deliberate, controlled, acclimatization there is no guarantee of a constant microbial activity in a commercial soil. In Table I are results which show that stability in a test soil may be obtained by acclimatization. The method commonly used at EMPA consists of a preliminary incubation for at least one month at 97 ± 2% relative humidity and about 20°C for a freshly bought soil, and a controlled maturation of an active soil by adding acclimatized fresh soil.
d. Aeration of the test soil

The pore volume, the compaction of the soil bed (Turner (1972)) and the moisture content of the soil, as well as the choice of test vessels are important allied parameters which may promote or prevent aerobic microbial activity. Wälchli (1981) has dealt with the choice of the test vessels and the factors of aeration in connection with the EMPA method. Khaziev (1976) has, furthermore, done some microbial counts of soils having differing moisture contents and has shown that anaerobic organisms and their type of metabolism, predominate if there is "too much moisture".

e. Test climate

A material comes into balance (water activity) with a definite environmental climate. Therefore, the climate of the incubator, which results neither in drying nor addition of water to the test soil during a test time of some weeks and which, of course, does not prevent ventilation of the test soil, is considered to be ideal.

Table 1.

Change in tensile strength of cotton strips 20 mm wide, by a laboratory soil burial test of one week duration. Comparison between soil test vessels exposed simultaneously.

<table>
<thead>
<tr>
<th>Test</th>
<th>Test vessel</th>
<th>n</th>
<th>Change in tensile strength percent. (Mean of n strips)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>4</td>
<td>-82.0</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>4</td>
<td>-78.3</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>4</td>
<td>-78.9</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>4</td>
<td>-72.7</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>4</td>
<td>-74.0</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>4</td>
<td>-72.7</td>
</tr>
<tr>
<td>3</td>
<td>4</td>
<td>4</td>
<td>-75.8</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>4</td>
<td>-71.2</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>4</td>
<td>-73.2</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>4</td>
<td>-74.0</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>4</td>
<td>-72.7</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>4</td>
<td>-74.3</td>
</tr>
</tbody>
</table>

Soil "Einheitserde Typ ED 73"; moisture content 60% of max. moisture holding capacity, incubation 97 ± 2% relative humidity, temperature 29 ± 1°C (viz. Raschle, 1981).

Test 1. Fresh soil acclimatized in the tropical chamber by adding additional substrate in the form of oatmeal to each vessel.

Test 2. Acclimatized soil without any further treatment.

Test 3. Acclimatized soil which was inoculated at the beginning of the test with a mixed spore suspension of *Chaetomium globosum*, *Stachybotrys chartarum* and *Trichoderma viride*.

Test 4. Acclimatized soil which was inoculated at the beginning of the test with a mixed spore suspension of fungi for testing plastics (ISO 846, 1978).

A traversable chamber with admission of fresh air, and air ventilation controlled at 97 ± 2% relative humidity and 29 ± 1°C has proved capable of maintaining, throughout 32 weeks, a moisture content of 60% of the moisture holding capacity. This chamber has also proved able to guarantee the requirement of the microbial activity of the test soil, viz: at least 70% loss of the strength of a cotton strip (cotton cloth 250 g/m2) within one week.

Preliminary treatment and duration of burial test

Kaplan (1978) refers to an earlier publication (Kempton et al., 1963) that "good treatments pass soil burial tests; poor treatments fail; and marginal treatments, as expected, give results befitting marginal treatments, i.e. equivocal results". This fact is discussed in the two following examples.

1st example: Table 2 shows results of a performance test of preservative finishes of canvas cloth (800 g/m2) for which a customer required a quality test on taking delivery.

Based on profound practical experience by that customer, all materials (A, B and C) show a good rot behaviour in practice. For this reason a pre-delivery test could be decided to confirm acceptable quality by means of a 4 weeks' laboratory soil burial.

However, prolonged tests make it possible to gain a deeper knowledge concerning the status of the preservative finishes and also help to evaluate new products. Obviously, the material with preservative B shows lower rot resistance compared with A or C.

Table 2.

Initial and residual tensile strength of canvas cloth strips 20 mm wide as affected by duration of test and protective treatment, compared with an outdoor test in forest soil.

<table>
<thead>
<tr>
<th>Duration of soil burial</th>
<th>Tensile strength of differently protected canvas material (kN)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Material A</td>
</tr>
<tr>
<td>Initial tensile strength</td>
<td></td>
</tr>
<tr>
<td>nil</td>
<td>1.11 ± 0.021</td>
</tr>
<tr>
<td>Laboratory soil</td>
<td></td>
</tr>
<tr>
<td>2 weeks</td>
<td>1.10 ± 0.063</td>
</tr>
<tr>
<td>4 weeks</td>
<td>1.06 ± 0.080</td>
</tr>
<tr>
<td>8 weeks</td>
<td>1.08 ± 0.048</td>
</tr>
<tr>
<td>12 weeks</td>
<td>0.65 ± 0.318</td>
</tr>
<tr>
<td>Burial in forest soil</td>
<td></td>
</tr>
<tr>
<td>6 months</td>
<td>1.09 ± 0.026</td>
</tr>
</tbody>
</table>
2nd example:
Graf et al., (1980) have given an example of an artificial leather (polyester, coated with plasticised PVC) which lost about 9% of its weight in a 4 weeks' laboratory burial test, under the action of soil microorganisms. When comparing the damages caused by cold bending it becomes obvious that this weight loss of approximate 9% largely reduced the resistance against cold-bending.

Outdoor weathering (1.5 m above ground, exposed to the south, 45°) gave much smaller reduction in resistance against cold bending as well as weight loss of that material through weathering (Fig. 1). The amount of damage caused by cold bending of a coating, following a laboratory test, correlates with the loss of weight given by the burial test.

Conclusions

Results from laboratory soil burial tests provide very good prognosis of performance if environmental conditions which encourage rot, such as contact with the soil and high humidity, are expected. The necessary condition to ensure the prognostic value of these results is, however, a good standardization and supervision of the test method because numerous factors determine its results. Good experience has been obtained with acclimatized soils (with preliminary incubation), having a moisture activity adjusted to 60% of the maximum water holding capacity, in an aerobic test system.

By adjusting the test period, at continuously optimal conditions for rot, this test can be made to answer different questions. It can be a screening method for manufacturers of biocides, or it can serve as a quality assurance test for the user, based on specific requirements. In the latter connection it is particularly useful to determine, by reference to behaviour of products in practice, the limit values for the different physical assessments of a perfect material in order to obtain performance-relevant information from short-time laboratory burial tests.

The above parameters should be given attention in order to standardize a soil burial test for general application. Anaerobic systems or tests altered in other ways, may be helpful for dealing with biodeterioration outside the usual rotting.

By such standardization, with optimal conditions for fungi and bacteria, the laboratory soil burial test may not only serve the rot resistance test on protected cellulosic textiles but may also be useful to test man-made fibres and coatings.

Figure 1 Influence of outdoor weathering and soil burial testing on the mass constancy and on the cold bending resistance of a coated textile.

C = Control
SB = after soil burial test (4 weeks in soil type ED73) (according to Graf (1977)).

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BIOLOGICAL FACTORS OF FOXING IN POSTAGE STAMP PAPER

Lea Nol, Y. Henis and R.G. Kennet

Summary

Extracts of naturally and artificially blotched postage stamps reacted positively to phenol reagents in three different tests. Microscopic examination of blotches revealed sparse living and dead hyaline hyphae. Six species of Aspergillus and one each of Penicillium and Gliocladium, isolated from foxed stamps, were capable of producing pigments in agar media and filter paper, sometimes more intense when grown in pairs than alone. All fungal isolates showed some degree of cellulolytic ability; all but A. fumigatus and G. roseum were capable of germinating, growing, sporulating and producing pigments at high osmotic pressures, and all at low. Of three species inoculated on new stamp paper, one, A. terreus var. aureus, developed hyphae and fluorescent yellow orange -brown pigments and produced pigments at constant RH 32.5% over an eight month period. Ferrous and ferric salts appeared to be unnecessary for production of pigments diffused into agar media, liquid medium or filter paper, but in some isolates they intensified the colour. Stamps inoculated with A. terreus var. aureus conidia and irradiated with a dose of 2.5 Mrad remained free of blotches throughout the two-year experimental period.

Introduction

Although foxing of paper (irregular yellowish-brown patches) is a common phenomenon, and can be a problem in such expensive items as old manuscripts and postage stamps, Meynell and Newsam (1979) considered it "amazing that we know almost nothing of their cause" for they are so common. These authors summarized the various possible causes as cited in the literature, as abiotic, or purely chemical, such as accumulation of visible iron salts in the lesions, dampness affecting impurities in the paper, etc., and biotic, such as fungi. Baynes-Cope (1976) and Press (1976) suggested that what they considered to be true foxing was of biological origin, although they were unable to indicate the causative organism(s). Meynell and Newsam (1978, 1979) appear to be the first to have concluded, through observation of hyphae and spores only within foxed areas of paper, that the lesions arise from fungal growth.

Biologische Faktoren bei der Bildung von Stockflecken auf Briefmarkenpapier


Facteurs biologiques de piqûres dans le papier des timbres postaux

Les pigments dans des timbres postaux naturellement tachés se comportent comme des phénoliques. Un examen microscopique des taches a révélé des hyphes hyalins et des conidies mortes. Six espèces d’Aspergillus et une de Penicillium et de Gliocladium isolées de timbres piqûrés ont pu produire des pigments phénoliques sur agar et papier filtre, d’une manière quelquefois plus intense quand ils poussaient par paires plutôt que seuls. Tous les isolats fongiques ont montré quelque action cellulolytique; tous sauf A. fumigatus et G. roseum ont été capables de germer, de croître, de sporuler et de produire du pigment à des pressions osmotiques élevées et tous à des bases pression. Une des trois espèces inoculées à du papier à timbre neuf, le A. terreus, var. aureus a développé des hyphes et produit du pigment en huit mois à un RH constant de 32.5%. Il apparaît que des sels ferreux ou ferriques ne sont pas nécessaires pour produire des pigments diffusant dans le milieu ager, le milieu liquide ou le papier filtre, mais que dans quelques cas ils intensifient la couleur.

Des timbres inoculés avec A. terreus var. aureus sous la forme de conidies puis irradiés à la dose de 2.5 Mrad sont restés non tachés pendant la période expérimentale de deux ans.
As the value of postage stamps to the philatelist may drop considerably due to foxing, we investigated this phenomenon with the object of ascertaining whether fungi could be shown to be responsible, and if so, to identify the kinds involved. Furthermore, an attempt was made to pinpoint conditions which favour, or at least allow, development of blotches on stamps.

Below we recount experiments which tend to corroborate Meynall and Newsm's findings on paper items other than stamps, mostly by other means, and to deepen our understanding of the factors connected with foxing.

**Materials and Methods**

Although foxing of stamps is a world-wide problem, we used Israeli stamps in our investigations simply in order to have a more detailed knowledge of time of issue and conditions under which they had been held until the start of the experiments. All stamps and stamp paper were obtained from the Israel Philatelist Service, Israel Ministry of Communications. All were gummed and perforated and none were franked. Four types were used, of which the first three had been held at the archives of the Service, Jaffa-Tei Aviv, since fabrication: Old (1952-1957) blotted individual stamps of different issues; Sheets of relatively new "Gan Hashlosha (1972)", unblotted stamps of one issue; sheets of old (1957) unblotted, unprinted stamp paper; sheets of newly produced, unblotted, unprinted stamp paper. In the new sheets, which were used for inoculation experiments, the glue consisted of polyvinyl acetate obtained from Harrison, High Wycombe, England.

For identification of phenols in agar or liquid media or blotched paper, we added a freshly-made 1:1 mixture of 1% solution (on anhydrous salt basis) of FeCl₃·6H₂O and K₃Fe(CN)₆·3H₂O to the material (Barton, Evans and Gardner, 1952; Sundman and Nåse, 1972). The appearance of a blue to green colour indicated the presence of phenols. For agar media, the fungus colony was first removed before testing.

In some experiments, phenols were tested according to Swain and Hillis (1959) with the Folin-phenol reagent, originally used for tannins (AOAC, 1980) which is based on sodium tungstate and phosphomolybdic acid. Water extracts of uninfected stamps were used as a control and dihydroxy-phenylalanine (DOPA) was originally used for tannins (AOAC, 1960). For each treatment, 10 sheets were used.

**Experimental and Results**

**Microscopical Examination**

Stamps of the first three categories - under Materials and Methods - were cut into small squares, each square chosen having one or more blotches, were placed on glass microscope slides and were given the following treatments: (a) Drops of lactophenol containing cotton-blue stain were applied to the upper surface so as to cover the entire square, a cover slip was added and the preparation heated until bubbles appeared. Observations were made under a microscope with brightfield and phase contrast illumination at 200-500x magnification. (b) Freshly prepared trypan-blue in distilled water was added as drops, and a cover slip was placed. Preparations were immediately observed under the microscope, trypan-blue acting as a vital dye staining living cytoplasm within hyphae. Other preparations were made by adding distilled water to the squares, placing a cover slip, heating three times till bubbles appeared so as to be certain that any hyphae present would not remain alive; trypan-blue in distilled water was then run under the cover slip and the excess absorbed by filter paper.

We found several kinds of hyphae within the same square. All hyphae were septate and irregular, 2-4 μm wide. Cytoplasm, but not cell walls, was stained by cotton-blue and the latter were hyaline. The hyphae extended in all directions, running along, under and sometimes spirally around the fibres. They were particularly noticeable at edges of cut squares, as fibres and hyphae became exposed to a greater degree. Hyphae were more numerous, as a rule, within a lesion than outside of it, but were often sparser in the centre of the blotch than near the periphery. Old stamps that

**Production of spots by artificial inoculation**

Sheets of unused stamps (50/sheet) were inoculated by smearing with a brush an area of ca. 9 x 9 cm in the center of the glued side of each sheet (10-12 stamps) with a suspension of Aspergillus terreus var. aureus spores (10⁷/ml, 0.5 ml/sheet). Controls were smeared with an equal amount of sterile water. The sheets were then sealed separately in polyethylene bags and incubated for 12 months at 28°C. Controls were run with sterile distilled water. For each treatment, 10 sheets were used.

Constant relative humidities were obtained in hermetically sealed containers with stamps in one experiment, by employing a saturated solution method (Winston and Bates, 1960) at 25-30°C: RH 93.5-96%, -CaH₂(PO₄)₂·H₂O; RH 75.5%, -NaCl; RH 55%, - glucose; RH 32.5%, - MgCl₂·6H₂O.

γ - irradiation: stamps placed in hermetically sealed polyethylene bags were irradiated with a dose of 2.5 Mrad from a cobalt source of γ rays at the Soreq Nuclear Research Centre.
appeared to the naked eye to have few blotches nevertheless sometimes displayed more hyphae than did stamps of different issues with many blotches. Globose and oval, unicellular, echinulate conidia, ca. 4μm diam, occasionally in short chains, were found on the surface of some blotches, near to but not attached to hyphae; they resembled conidia of some species of *Aspergillus*. Many individual typically flask-shaped phialides were observed (Fig. 1).

No hyphae could be discerned in new stamps, nor in old stamps without lesions. In old foxed stamps, hyphae were observed both in blotches and in areas between neighbouring blotches, and to a smaller extent some distance from blotches.

Drops of water with trypan-blue were absorbed quickly only by the paper of stamps in which lesions were present, and rapidly only in the blotched areas themselves. No hyphae could be discovered in any of the material which had been heated before staining, whereas many short hyphae (with stained cytoplasm and hyaline cell-walls and septa) were observed in, and adjacent to, blotches when the dye was applied without heating. Phialidic cells attached to hyphae were sometimes discerned.

In all the preparations the mycelium, which was hyaline, was never found to be dense in foxed areas and was always even sparser in areas beyond the periphery of blotches. When these foxed stamps were tested for presence of phenolics by the ferric chloride-ferric cyanide test, the results were positive for blotched areas, but negative for unblotched ones. As the old stamps were 25-30 years old, and it was not known exactly when blotches began to appear, it was not possible to gauge the age of the mycelium, even when shown, through trypan-blue vital staining, to be living.

**Isolation of Fungi**

Old, foxed and new, unblotched stamps were tested. Two methods were employed: (a) Homogenate of stamps and (b) Small squares plated in Petri dishes on agar media. The stamp preparations were plated simultaneously on PDA, nutrient agar, Czapek agar and malt-salt agar. For homogenization, pieces of stamps in a small amount of sterile distilled water were ground in a sterile mortar with pestle until a thick slurry was obtained, which was diluted in distilled water to a tenth, with nine further dilutions, poured into Petri dishes containing 15 ml agar medium, and spread with a sterile glass rod. All dishes were sealed with parafilm and held in an incubator in darkness at 28°C. The dishes were examined after 7 days. For (a), four dishes and for (b) ten dishes were employed at each of 10 dilutions for new and for old stamps.

With the rare exception of some bacilli, neither fungi nor bacteria were found on nutrient agar. Bacteria, therefore, apparently were not associated with these blotches. Fungi very often grew out of the paper of both old and new stamps into the three other media; there were no great differences between the number of colonies obtained from the two types of stamps. Among the fungi occasionally isolated from each, on all but malt-salt agar, were some species of *Alternaria*, *Cladosporium* and *Bipolaris*. As the dematiaceous fungi showed no cellulolytic activity nor osmotolerance (on malt-salt agar), and as their hyphae are usually dark-walled, unlike those in our stamps, they were eliminated from consideration, as simply adventitious fungi, probably isolated on the media from conidia as surface contaminants on the stamps. The rest of the fungi also did not differ much (Table 1). Of particular interest was the finding of very common "ordinary" mould species, rather than specialized kinds that might have been expected to find a particularly favourable ecological niche on paper held under relatively non-humid conditions, such as obligate osmophiles. None of the latter were isolated on malt-salt agar (osmotic pressure ca. 31 atmospheres), a substrate which did not prevent, however, the isolation of six species (Table 1).

All the above isolates secreted pigments which diffused into the agar. Colours were yellow, orange, brown or reddish-brown, differing according to isolate. In all cases, the pigments behaved as phenolics, as tested by the methods of Sundman and Näse (1972) and Swain and Hillis (1959).

In order to ascertain which fungal species are likely to be encountered within the centre of a lesion, within a lesion at its periphery and at a distance of 1 mm from the edge of a blotch, we scratched fibre aggregates from stamps (1953) with a needle, plated them on Czapek agar in Petri dishes and incubated the cultures at 28°C (5 lesions x 10 replications for each of three areas = 150 samples). After 10 days, only 20% of

Figure 1. Direct microscopic observation of fungal elements in a naturally foxed postage stamp.

The cytoplasm of the hyaline hypha (ca. 2μm wide) within a blotch is stained with cotton-blue in lactophenol, on the background of teased cellulose fibres. Arrow points to flask-shaped phialide. White bar = 10μm.
lesions yielded colonies, which were all fungal; in the rest, any fungi that might have been present were no longer viable. A single lesion often yielded two species, one or three occasionally, four rarely. Fungi sometimes arose from one area of a lesion and not from others. Most common were colonies from the lesion periphery and least common from 1 mm from the lesion. The most common species isolated was A. niger, from all lesions from which isolations had succeeded. This was the only species found outside the lesion proper. Others, in order of frequency, were A. flavus, A. terreus var. aureus and P. funiculosum. Repetition of the experiment with stamps of the same series and source yielded similar results.

Production of Spots by Artificial Inoculation and Their Chemical Nature

Unused stamps were artificially inoculated with A. terreus var. aureus. After two weeks of incubation at 28°C, they developed brown and yellow spots on the glued side and heavy yellow-brown sporulation on the printed side. When examined under a long wave UV lamp, the yellow spots fluoresced strongly, whereas the brown spots appeared darker, orange-brown. It should be noted that the area of the sterile uninoculated control smeared with sterile water also showed some pale-blue fluorescence. However, the fluorescence appeared to be weaker and different in range from that of the inoculated treatment. Individual stamps were taken from the treated area and examined for the presence of phenol-like compounds by the ferric chloride potassium ferric cyanide test (Sundman and Näsä 1972), by the Folin reagent (Swain & Hillis, 1959) and by their UV spectrum (Scott, 1964). All these tests were positive for the treatments and negative for the controls. Extracts of some stamps showed colour intensity equivalent to ~ 50 μm/ml/stamp, when examined by the Folin-phenol reagent and using DOPA as a standard. Extracts of all the tested infected stamps showed a maximum absorbance peak at the range of 270-280 nm, typical for phenolic compounds. Furthermore, a 20-30 unit shift to the longer UV range was observed in extracts to which 0.1 ml of 1 N NaOH was added, and this shift was reversed by reneutralizing the solution by HCl, indicating the presence of typical-OH phenolic groups (Scott 1964).

Nature of the Coloration of the Lesions

Table 1.

<table>
<thead>
<tr>
<th>Fungal Species</th>
<th>Old, Foxed</th>
<th>New, Unfoxed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspergillus carneus van Tieghem</td>
<td>++**</td>
<td>-</td>
</tr>
<tr>
<td>A. flavus Link (with sclerotia)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>A. flavus Link (lacking sclerotia)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>A. fumigatus Thom*</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>A. niger van Tieghem</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>A. terreus var. aureus Thom &amp; Raper</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>A. tamarii Kita</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Gloeocadium roseum (Link) Thom*</td>
<td>++**</td>
<td>-</td>
</tr>
<tr>
<td>Penicillium funiculosum Thom</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* Did not grow on malt-salt agar.
** Infrequent

The same treatments were made with single and paired agar discs on 1 cm² sterile filter paper (Whatman No.1) placed in Petri dishes (4 squares/dish), mycelium side in contact with the paper. For each treatment, four replications were made.

At the meeting point of two fungal species on Czapek agar, the colour (light yellow to reddish brown according to species) was always different and stronger than that of each constituent species. On filter paper, one fungus sometimes overgrew the other, and the colour of the paper was that of the pigment of the stronger of the two, or both grew and formed a meeting line at which a stronger colour was noticeable, or both species which produced the same colour (light yellow e.g. A. niger, A. flavus) continued to do so when grown in pairs. The pigments, both in agar media and filter paper, were tested for phenols, with positive results.

(b) The Effect of Addition of Iron Ions on Pigment Production by the Fungi.

1. Intensity of colour in blotches on paper

On Czapek agar in which the carbon source was absent, four sterile squares (1 cm²) of filter paper were placed in each Petri dish and these were inoculated with various isolates. The following treatments were employed: medium without iron; medium enriched with Fe ++ (FeSO₄ 0.1 g/l); medium enriched with Fe +++ (FeCl₃ 0.1 g/l). The Petri dishes were incubated at 28°C for 6 weeks in darkness. There were 8 replicated Petri dishes per treatment.

Addition of iron, as Fe ++ and Fe ++++, changed the colour in some cases. Of the five species tested, A. terreus and A. carneus reacted to both Fe ++ and Fe ++++, the former by change from brown to reddish, the
latter by a deepening of the yellow-brown colour. *P. funiculosum* reacted only to Fe **++**, by giving red rather than reddish-brown spots. *A. niger* and *A. flavus*, however, were indifferent to the addition of iron to the medium.

2. Intensity of colour in standing liquid culture

We employed Czapek medium (50 ml in 100 ml Erlenmeyer flasks), with either no iron, 0.1 g FeSO₄/1, or 0.1 g FeCl₃/1. There were three replicate flasks for each treatment. Each flask was inoculated with 0.2 ml conidial suspension at a conc. of 40-50 x 10⁷/ml., and incubated in darkness at 28°C for 25 days, after which samples of the supernatant fluid were checked for absorbance (γ = 400 nm). The medium was dried on filter paper at 100°C for 24 h and weighed. The results are summarized in Table 2.

<table>
<thead>
<tr>
<th>Species</th>
<th>Without Iron</th>
<th>With Fe ++</th>
<th>With Fe +++</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. carneus</em></td>
<td>1.70</td>
<td>0.195</td>
<td>0.238</td>
</tr>
<tr>
<td><em>A. terrest</em></td>
<td>0.80</td>
<td>0.350</td>
<td>0.65</td>
</tr>
<tr>
<td><em>A. funicul</em>i</td>
<td>0.75</td>
<td>0.290</td>
<td>0.135</td>
</tr>
</tbody>
</table>

*A. carneus* allowed much greater pigment intensity with addition of Fe ++ and particularly Fe +++ than in the controls. The other two were definitely affected by Fe +++; as the amount of mycelium containing FeSO₄ was much less than for controls, Fe ++ actually yielded greater colour intensity for equal weight of mycelium.

**Additional Characterisation of Isolates of Fungal Species on Stamp Paper**

(a) Cellulolytic activity

As we could often see hyphae coiled around fibres in stamp paper, it seemed possible that there might be some degree of cellulolytic activity. Furthermore, the paper within blotches appeared to be weaker than outside, for, upon scratching the surface of dry stamps with a fine needle, 'crumbs' were raised only where lesions were present. However, as no holes had appeared in the paper, we could not be certain that weakness in the paper necessarily indicated degradation by fungi of cellulose rather than of size.

We employed two methods for assessing cellulolytic activity:

1. Placing sterile filter paper (1 cm²) in petri dishes on Czapek agar leaching a source of carbon, and holding at 28°C in darkness, after having placed agar discs with growing mycelium of the fungus in question on the paper. Change toward translucency in the paper and the inability to remove the paper from the agar without tearing in relation to time indicated cellulolytic ability. The dishes were examined bi-weekly for as long as 6 weeks (3 dishes x 4 pieces). At the same time, the degree of discoloration in the stamps by phenol was recorded. Four types were found:

Strong cellulolytic activity + blotches within 6 days. *A. terreus*, *G. roseum* and *P. funiculosum*.

Weak to moderate cellulolytic activity + blotches within 6 days. *A. carneus*.

Strong cellulolytic activity, but blotches only after a number of weeks. *A. funigatus*.

Weak to moderate cellulolytic activity, but blotches only after a number of weeks. *A. flavus*, *A. niger* and *A. tamarii*.

(b) Osmotolerance

As the macroclimatic conditions for holding postage stamps seldom are very humid, it is possible that fungi present on and in stamps would need to be tolerant of dryness and to be able to continue some degree of growth under such conditions. Osmophilic and osmotolerant fungi are known for seed coats (Christensen and Kaufmann, 1969) and other dry substrates (Griffin, 1972). Three methods were used to ascertain osmotolerance: (1) Malt-salt agar containing 7.5 or 20% NaCl. All the species except *A. funigatus* and *G. roseum* grew and sporulated on the 7.5% medium (31 atm.), and grew as well as on Czapek medium without salt. On the medium with 20% salt, only *A. terreus* and *A. tamarii* grew and sporulated, but their colonies were more restricted than in the medium lacking salt. (2) Czapek agar at 10-50% sucrose vs. controls at 3% sucrose were tested at 28°C in darkness, with four replicates. *A. flavus*, *A. niger* and *A. tamarii* displayed no differences between treatments and controls in growth, sporulation and coloration of the medium even at 50% sucrose (which would give 35 atmospheres (Scott, 1957)). *A. terreus* and *A. carneus*...
grew more poorly above 20% sucrose than in the controls and color intensity was reduced. *P. funiculorum* grew more poorly at above 20%, but coloration was stronger. (3) On liquid standing Czapek medium, with addition of 10-50% sucrose compared with 3% sucrose controls, *P. funiculorum* showed after 3 wk. growth a slight rise in dry weight of mycelium with rise in sucrose concentration, but a steep continuous drop in absorbance at 400 nm. *A. terreus* showed a greater rise in dry weight but high and almost unchanging absorbance at all concentrations (ca. 1.0-1.3).

(c) Capacity to Produce Hyphae on Paper at Different Relative Humidities.

1. Twelve 1-litre glass jars with hermetic closure were used. Within, R.H. of 32.5, 55, 75.5 and 93.5-96% were attained by use of saturated salt solutions according to the method described by Winston & Bates (1960). New unprinted stamps, with and without glue*, were inoculated with a great number of dry conidia of *A. niger*. *A. terreus* and *A. fumigatus*, held in the jars on fine plastic screening on glass upright rods above the solutions that determined R.H. within the jars. Six pieces of stamp paper with, and six without glue had been introduced into each jar. The jars were held in darkness at 28°C for 3 months, followed by 5 months on a laboratory bench.

The stamp paper with all three species displayed a weft of mycelium and sporulation at 55% to 93.5-96% R.H., irrespective of whether glue was present or not. Those with *A. fumigatus* and *A. terreus* did so even at 32.5% R.H. Foxing, however, occurred only in those with *A. terreus*, and at all humidities tested.

2. The same stamp paper was heavily inoculated, as above (from the glue side when glue was present), with the same species and then inserted by sterile tweezers in cellophane strips on new stamp album pages. Stamps with and without glue were placed in alternate rows. Two pages were assigned to each species and faced each other, being sealed all around with cello tape. All pages were kept in a laboratory desk drawer for 9 months (15-22°C).

All stamps showed presence of mycelium on both sides of each stamp, particularly near the edges. Foxing, however, appeared only on those inoculated with *A. terreus*, and regardless of whether glue was present or not.

Effect of γ Irradiation on Blotching

Fifteen sheets of stamps of 1972 issue (with 50 stamps in each) were inoculated with spores of *A. terreus* var. *aureus*, placed in hermetically sealed polyethylene bags and irradiated with γ rays at a dose of 2.5 Mrad. Five inoculated control sheets in sealed bags were left unirradiated. No change in colour could be observed in the irradiated stamps. Three inoculated and irradiated sheets and three inoculated control sheets were wetted by injecting 1 ml sterile water into the bags to allow for adequately moist conditions for fungal development. All the bags were incubated at 28°C for two weeks. No blotches were discerned in non-inoculated, non-irradiated stamps, and in the inoculated irradiated ones, whereas strong blotching developed in the inoculated, non-irradiated treatments.

Discussion

Although it has lately been claimed (Meynell and Newsam, 1978) that the causal agents of foxing of paper can be fungi, this has been by microscopic observations alone and little has been done experimentally. Furthermore, no such evidence has so far been presented for postage stamps. Our results obtained by microscopic examination confirmed their findings in postage stamps. We found, by examination of and isolation from naturally blotched paper of postage stamps, that hyphae could be discerned not only in the blotches themselves, but in areas which seemed to be devoid of spots. The centre of blotches often no longer showed evidence of viable hyphae, and the periphery of the blotch often showed the greatest amount of visible hyphae. The low density of fungal hyphae in the paper, as well as their hyalinity, made it obvious that hyphae per se could not be the actual cause of discoloration. We found, instead, that they produced pigments, which reacted positively to phenol reagents such as ferric chloride potassium ferric cyanide (Sudman and Næse, 1972) and Folin (Swain and Hillis, 1959). Many fungi, especially those involved in the humification of soil organic matter, are capable of synthesizing phenolic compounds (Feldbeck, 1971). A number of fungal species may be present in a single blotch, and our experiments on agar media and filter paper show, that in some combinations, a stronger colour may be produced than by each alone. As already indicated by other authors, (Press, 1976, Baynes-Cope, 1976), we found no clear relationship between blotching and iron. Most of the fungal species we encountered were indifferent to both Fe++ and Fe ++ for causing intensity of colour in agar media and paper. However, *Aspergillus carneus*, *A. terreus* var. *aureus* and *Penicillium funiculorum* produced stronger colour in the presence of iron salts.

It was surprising that the isolated causal agents of the blotching were very common, "ordinary" fungi, rather than any specialized types. They may be characterized, however, by their relative osmotolerance, a character which should allow them to exist in the paper under rather dry conditions. *A. terreus* produced hyphae and conidia and yielded blotches on stamp paper even at constant 32.5% R.H. and grew on agar media with 50% sucrose or 20% NaCl, and thus seemed to meet the qualifications for a blotch-inducing species more than did the others. No obligate osmophiles were found.
Although all these species can utilize cellulose to one degree or another, it is not at all certain that strong cellulolytic capacity is necessary for the fungi to exist in paper and cause blotching. Dematiaceous hyphomycetes, such as Alternaria, Cladosporium, Bipolaris, etc. occasionally isolated from new and old stamps, did not yield blotches nor prove to have cellulolytic capacity, and were considered to simply be adventitious, by surface contamination by conidia. It is likely that such common species of Aspergillus, capable of inducing blotches on stamp paper, colonize the paper by way of air-borne conidia.

In this work, the principles of Koch postulates were applied for the first time to demonstrate the relationship between the fungus Aspergillus terreus and blotching of stamp paper. Only stamp sheets inoculated with A. terreus spores and inoculated under suitable conditions developed brown and yellow blotching. The A. terreus-produced brown spots seemed to fit the definition of Baynes-Cope (1976) of foxing, i.e. "a brown area which fluoresces an orange-brown colour and is surrounded by a pale-blue zone". However, in addition to the brown pigmentation, A. terreus produced yellow spots which showed strong bright fluorescence. Water extracts of the blotched stamps reacted positively to phenol reagents. According to Swain (1969), the ferric chloride-potassium ferric cyanide reagent used in this work is fairly specific for phenolic compounds. Other tests used (Folin phenol reagent and UV spectrum) were also positive. At present, there is no data available regarding the precise chemical nature of the blotching involved in "natural" foxing processes. One cannot say, therefore, whether or not the A. terreus-produced spots are identical with the natural foxing phenomenon and more information is required regarding its chemical nature.

Finally, we demonstrated here that γ irradiation can be successfully used for inactivation of biological blotching agents in stamps as well as other paper items. This can turn out to be a relatively cheap and safe method for high value paper items.

Acknowledgements

This work was supported by a grant from the Israel Ministry of Communications. The authors are grateful to Mr. M. Krupp, Mr. G. Menchel, Mr. M. Cohen and Mr. I. Granott of the Ministry for their encouragement and valuable assistance.

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Saturation solutions for the control of humidity in biology research.
CELL WALL DEGRADING ENZYMES ASSOCIATED WITH DETERIORATION OF COCOA BEANS BY PENICILLIUM STECKII

P.O. Olutola

Summary

During the deterioration of cocoa beans caused by Penicillium steckii, a complex of proteins which possessed cellulase, polygalacturonase, pectin methyl-esterase and pectin transeliminase activities were produced. These enzymes, except for the occasional traces of pectin methyl-esterase, were not detectable in healthy cocoa beans. The enzyme complex was partially purified by a combination of ammonium sulphate precipitation, gel filtration and ion-exchange chromatography, and possessed a molecular weight of approximately 91400 Daltons. Maximum activity of the cellulase, polygalacturonase, pectin methyl-esterase and pectin transeliminase occurred at pH 4.5, 5.0, 8.0 and 8.5 respectively. Optimum temperature for cellulase was 30°C, and 40°C for polygalacturonase, pectin methyl-esterase or pectin transeliminase. The enzymes were stimulated by low concentrations of Ca++ and Mg++ but inhibited by ethyldiamine tetraacetic acid.

Enzymes dégradant la paroi cellulaire en association avec la détérioration des fèves de cacao par Penicillium steckii.

Au cours de la détérioration des fèves de cacao par Penicillium steckii, il s'est produit un complex de protéines à activité cellulase, polygalacturonase, pectine méthyl-ésterase et pectine trans­liminase. Ces enzymes, sauf pour des traces occasionnelles de pectine méthylésterase, n' étaient pas détectables dans les fèves de cacao saines. On a partiellement purifié le complexe d' enzymes par une combinaison de précipitation au sulfate, filtration sur gel et chromatographie d' échange d' ions; leur masse molaire était d' environ 91400 Daltons. L' activité maximum de la cellulase, polygalacturonase, pectine méthylésterase et pectine transliminase s'est produite respectivement aux pH de 4,5, 5,0, 8,0 et 8,5. La température optimum pour la cellulase fut de 30°C et de 40°C pour les polygalacturonase, pectine méthylésterase et transliminase. Les enzymes furent stimulées par de basses concentration en Ca++ et Mg++ mais inhibées par l'acide éthylené tetraacétique.

Introduction

Cocoa beans have been found to be liable to serious microbial deterioration during storage (Riley, 1968; Broadbent, 1968; Broadbent and Oyeniran, 1968). One of the important fungi associated with this deterioration is Penicillium steckii Zaleski (Oyeniran, 1970).

Microbiological attack on stored products usually results in economic loss either by imparting unpleasant flavour, odour and colour or by causing structural changes which render the material unusable for its original function. The major cell-wall constituents are pectic and celluloses substances (Alexander, 1961; Joslyn, 1962; McClendon, 1964).

Therefore the ability of a phytopathogen to produce enzymes necessary for the degradation of these cell wall components will be an advantage in infection (Wood, 1960; Hunter and Elkan, 1975). Hence it was thought necessary to examine production of pectic and cellulolytic enzymes during infection of cocoa beans by P. steckii. Attempts were made to partially purify and characterise the enzymes.

Methods and Materials

Inoculation of cocoa beans

The isolate (NSPRI. 111) of Penicillium steckii Zaleski used was from the culture collection of the Nigerian Stored Products Research Institute, Ibadan. It was

1 Department of Microbiology, University of Ilé-Ifé, Nigeria. Present address: Department of Botany, Obafemi Awolowo University, P.M.B. 5363, Ado-Ekiti, Ondo State, Nigeria.

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isolated from mouldy cocoa beans. Stock cultures were maintained on 1% malt extract agar slants. Seventy-two-h old agar slant cultures were employed as inoculum.

Healthy cocoa beans were surface-sterilized, aseptically sliced into approximately 2 mm sections and placed in sterile 250 ml Erlenmeyer flasks. Each flask contained 20 g of cocoa bean tissue plus 1 ml spore suspension (diluted to give approximately 10⁷ spores/ml). All experimental flasks were incubated at 30°C. Control cocoa beans sliced and similarly treated, but without inoculation, were incubated under the same conditions.

*Extraction of enzymes from tissues*

Within fourteen days of incubation, the inoculated cocoa slices had become covered with dense mats of mycelia and conidia. The contents of each flask were removed, chilled and macerated in a homogeniser (MSE, England) as previously described (Olutiola and Akintunde, 1979). The extractant was 0.1 M citrate phosphate buffer (pH 7.0) containing 0.2 N NaCl. The homogenate was filtered through glass fibre paper and clarified by centrifugation at 15,000 x g for 15 min at 4°C. The supernatant was employed as crude enzyme preparation.

*Preparation of enzymes for column chromatography*

The crude enzyme preparation was dialysed using acetylated cellophane tubing (Olutiola and Cole, 1977). The dialysis was for 18 h against several changes of 0.1 M citrate phosphate buffer (pH 7.0) at 4°C. The dialysate was coloured and somehow viscous. Effective colour removal was carried out by a batch adsorption step ('A guide to ion exchange chromatography', Pharmacia Fine Chemicals, Sweden). Dry DEAE Sephadex A-25 was added to the dialysate (15 mg/ml, w/v) and stirred continuously at 4°C for 24 h. The resulting slurry was layered on top of a DEAE Sephadex A-25 column (250 mm x 25 mm) equilibrated with 0.1 M citrate phosphate buffer (pH 7.0) and eluted as previously described (Olutiola and Cole, 1977). In this way an approximately 5-fold concentrate of the enzyme preparation was obtained and the slight viscosity was eliminated. Proteins in the preparation were precipitated with ammonium sulphate (analytical grade) between the limits of 40-95% saturation (Olutiola and Akintunde, 1979). The precipitates were redissolved in 0.1 M citrate phosphate buffer (pH 7.0) to give a 10-fold concentration of the original enzyme preparation. The filtrate was then dialysed as mentioned above. The dialysate was further clarified by centrifugation (15,000 x g at 4°C for 15 min) and sterilized by membrane filtration (Oxoid, 0.45 μm) before separation on Sephadex G - 100 column.

*Fractionation on Sephadex G - 100*

A column (640 x 25 mm) of Sephadex G-100 was prepared as previously described (Andrews, 1964; Olutiola, 1972), and was contained in a water jacket at 4°C. The column was equilibrated with 0.1 M citrate phosphate buffer (pH 7.0) containing 0.1 M NaCl and 5 mM NaN₃. Ten ml of the sterile enzyme concentrate was applied to the column and eluted with the same buffer. Measurement of protein content of the eluted fractions (5 ml/tube) and the calibration of the column with proteins of known molecular wt were as previously described (Olutiola, 1976). Each of the fractions was analysed for cellulase, polygalacturonase, pectin methylesterase and pectin transeliminase activities.

*Fractionation by ion-exchange chromatography*

Fractions (16-26) from Sephadex G-100 column which exhibited appreciable enzymic activity were pooled, and the proteins were precipitated with ammonium sulphate as described above. The ammonium sulphate precipitate was treated as before and applied to a column (340 x 25 mm) of CM Sephadex C-50. Preparation of the column and monitoring of column effluents were as mentioned above. Each fraction was also analysed for cellulase and pectinase enzymes.

*Enzyme assays*

**Cellulase**: Cellulase activity was determined by the percentage reduction in the viscosity of CM-cellulose and by the release of reducing sugars from this substrate as described by Olutiola (1976). Activity towards insoluble celluloses (cellulose powder, viscose cellulose and Whatman No.1 filter paper) was performed as previously described (Olutiola, 1976).

**Polygalacturonase**: Polygalacturonase activity was determined as mentioned above for cellulase activity except that the substrate was 1% (w/v) pectin in citrate phosphate buffer (pH 5.0) unless otherwise stated. D-galacturonic acid was used as standard for the reducing sugars.

**Pectin methylesterase and pectin transeliminase activities**: Pectin methylesterase and pectin transeliminase activities were analysed as previously described (Olutiola and Akintunde, 1979).

**Tissue maceration and cellular death**

The ability of the enzyme preparation to macerate potato tissue disks was determined by the technique of Olutiola and Akintunde (1979). The experiments were incubated for 60 min at 35°C, employing 0.02 M citrate phosphate (pH 5.0) and 0.02 M Tris - HCl (pH 8.0) as buffers respectively.

Cellular death was estimated by the technique of Tribe (1955). Potato disks were placed in enzyme buffered at pH 5.0 (0.02 M citrate phosphate) and pH 8.0 (0.02 M tris-HCl) respectively. After incubation for 60 min at 35°C, disks were transferred to a plasmozizing solution containing Neutral Red and 1 ml of either 0.05 M citrate phosphate buffer (pH 5.0) or 0.05 M Tris-HCl buffer (pH 8.0). After 20 min the disks were washed in 1 M KNO₃ solution. Cells which retained the Neutral Red were scored as dead.
### Table 1.
Partial purification of cellulase from cocoa beans infected by *Penicillium steckii.*
Each value is the mean of three replicates with standard error.

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total activity (U)</th>
<th>Total protein (mg)</th>
<th>Specific activity (U/mg)</th>
<th>Yield (%)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>1075 ± 3.7</td>
<td>415 ± 1.4</td>
<td>2.6 ± 0.02</td>
<td>100</td>
<td>1.0</td>
</tr>
<tr>
<td>Dialysed extract</td>
<td>977 ± 2.8</td>
<td>284 ± 2.5</td>
<td>3.4 ± 0.03</td>
<td>90.9</td>
<td>1.3</td>
</tr>
<tr>
<td>Batch adsorption (DEAE-Sephadex)</td>
<td>898 ± 1.9</td>
<td>179 ± 2.5</td>
<td>5.0 ± 0.07</td>
<td>83.5</td>
<td>1.9</td>
</tr>
<tr>
<td>1st ammonium Sulphate precipitation</td>
<td>863 ± 2.1</td>
<td>30.9 ± 0.3</td>
<td>27.9 ± 0.25</td>
<td>80.3</td>
<td>10.7</td>
</tr>
<tr>
<td>Sephadex G-100</td>
<td>785 ± 1.9</td>
<td>6.7 ± 0.4</td>
<td>117.2 ± 7.2</td>
<td>70.0</td>
<td>45.1</td>
</tr>
<tr>
<td>Peak A</td>
<td>660 ± 2.5</td>
<td>3.4 ± 0.07</td>
<td>194.1 ± 3.3</td>
<td>61.3</td>
<td>74.7</td>
</tr>
<tr>
<td>2nd ammonium sulphate precipitation</td>
<td>384 ± 2.8</td>
<td>1.20 ± 0.08</td>
<td>320.0 ± 3.9</td>
<td>35.7</td>
<td>123.1</td>
</tr>
<tr>
<td>Sephadex C-50</td>
<td>270 ± 3.1</td>
<td>0.70 ± 3.1</td>
<td>385.7 ± 4.1</td>
<td>25.1</td>
<td>148.3</td>
</tr>
</tbody>
</table>

### Table 2.
Partial purification of polygalacturonase from cocoa beans infected by *Penicillium steckii.*
Each value is the mean of three replicates, with standard error.

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total activity (U)</th>
<th>Total protein (mg)</th>
<th>Specific activity (U/mg)</th>
<th>Yield (%)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>798 ± 5.7</td>
<td>415 ± 1.4</td>
<td>1.9 ± 0.01</td>
<td>100</td>
<td>1.0</td>
</tr>
<tr>
<td>Dialysed extract</td>
<td>609 ± 4.9</td>
<td>284 ± 2.5</td>
<td>2.1 ± 0.01</td>
<td>76.3</td>
<td>1.1</td>
</tr>
<tr>
<td>Batch adsorption (DEAE-Sephadex)</td>
<td>597 ± 4.4</td>
<td>179 ± 6.0</td>
<td>3.3 ± 0.09</td>
<td>74.8</td>
<td>1.7</td>
</tr>
<tr>
<td>1st ammonium sulphate precipitation</td>
<td>498 ± 6.5</td>
<td>30.9 ± 0.3</td>
<td>16.1 ± 0.04</td>
<td>62.4</td>
<td>8.5</td>
</tr>
<tr>
<td>Sephadex G-100</td>
<td>409 ± 3.2</td>
<td>6.7 ± 0.9</td>
<td>61.0 ± 4.1</td>
<td>51.3</td>
<td>32.1</td>
</tr>
<tr>
<td>Peak A</td>
<td>371 ± 3.5</td>
<td>3.4 ± 0.1</td>
<td>109.1 ± 1.2</td>
<td>46.5</td>
<td>57.4</td>
</tr>
<tr>
<td>2nd ammonium sulphate precipitation</td>
<td>180 ± 3.1</td>
<td>1.20 ± 0.2</td>
<td>150.0 ± 6.5</td>
<td>22.6</td>
<td>78.9</td>
</tr>
<tr>
<td>Peak Aa</td>
<td>83 ± 0.7</td>
<td>0.70 ± 0.02</td>
<td>118.6 ± 1.4</td>
<td>10.4</td>
<td>62.5</td>
</tr>
<tr>
<td>Peak Ab</td>
<td>105 ± 1.8</td>
<td>0.75 ± 0.03</td>
<td>140.0 ± 3.6</td>
<td>13.2</td>
<td>73.7</td>
</tr>
</tbody>
</table>
Table 3.

Partial purification of pectin methylesterase from cocoa beans infected by *Penicillium steckii*.
Each value is the mean of three replicates with standard error.

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total activity (U)</th>
<th>Total protein (mg)</th>
<th>Specific activity (U/mg)</th>
<th>Yield (%)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>1725 ± 2.1</td>
<td>415 ± 1.4</td>
<td>4.2 ± 0.01</td>
<td>100</td>
<td>1.0</td>
</tr>
<tr>
<td>Dialysed extract</td>
<td>1691 ± 5.7</td>
<td>284 ± 2.5</td>
<td>6.0 ± 0.04</td>
<td>98.0</td>
<td>1.4</td>
</tr>
<tr>
<td>Batch adsorption (DEAE-Sephadex)</td>
<td>1572 ± 7.4</td>
<td>179 ± 6.7</td>
<td>8.8 ± 0.3</td>
<td>91.1</td>
<td>2.1</td>
</tr>
<tr>
<td>1st Ammonium sulphate precipitation</td>
<td>1429 ± 3.1</td>
<td>30.9 ± 0.3</td>
<td>46.2 ± 0.4</td>
<td>82.8</td>
<td>11.0</td>
</tr>
<tr>
<td>Sephadex G-100</td>
<td>1327 ± 2.1</td>
<td>6.7 ± 0.4</td>
<td>198.1 ± 12.3</td>
<td>76.9</td>
<td>47.2</td>
</tr>
<tr>
<td>Peak A</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2nd Ammonium sulphate precipitation</td>
<td>1262 ± 3.7</td>
<td>3.4 ± 0.1</td>
<td>371.2 ± 6.7</td>
<td>73.2</td>
<td>88.4</td>
</tr>
<tr>
<td>Sephadex C-50</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peak Aa</td>
<td>771 ± 5.1</td>
<td>1.20 ± 0.04</td>
<td>642.5 ± 19.5</td>
<td>44.7</td>
<td>153.0</td>
</tr>
<tr>
<td>Peak Ab</td>
<td>487 ± 1.9</td>
<td>0.70 ± 0.01</td>
<td>695.7 ± 11.6</td>
<td>28.2</td>
<td>165.5</td>
</tr>
</tbody>
</table>

Table 4

Partial purification of pectin transeliminase from cocoa beans infected by *Penicillium steckii*.
Each value is the mean of three replicates with standard error.

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total activity (U)</th>
<th>Total protein (mg)</th>
<th>Specific activity (U/mg)</th>
<th>Yield (%)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>1495 ± 6.7</td>
<td>415 ± 1.4</td>
<td>3.6 ± 0.01</td>
<td>100</td>
<td>1.0</td>
</tr>
<tr>
<td>Dialysed extract</td>
<td>1299 ± 3.7</td>
<td>284 ± 2.5</td>
<td>4.6 ± 0.03</td>
<td>86.9</td>
<td>1.3</td>
</tr>
<tr>
<td>Batch adsorption (DEAE-Sephadex)</td>
<td>1157 ± 1.2</td>
<td>179 ± 2.7</td>
<td>6.5 ± 0.1</td>
<td>77.4</td>
<td>1.8</td>
</tr>
<tr>
<td>1st Ammonium sulphate precipitation</td>
<td>987 ± 1.9</td>
<td>30.9 ± 0.3</td>
<td>31.9 ± 0.5</td>
<td>66.0</td>
<td>8.9</td>
</tr>
<tr>
<td>Sephadex G-100</td>
<td>895 12.7</td>
<td>6.7 ± 0.3</td>
<td>133.6 ± 6.7</td>
<td>59.9</td>
<td>37.1</td>
</tr>
<tr>
<td>Peak A</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2nd Ammonium sulphate precipitation</td>
<td>814 ± 3.1</td>
<td>3.4 ± 0.1</td>
<td>239.4 ± 4.2</td>
<td>54.4</td>
<td>66.5</td>
</tr>
<tr>
<td>Sephadex C-50</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peak Ac</td>
<td>492 ± 1.9</td>
<td>0.75 ± 0.02</td>
<td>656.0 ± 14.2</td>
<td>32.9</td>
<td>182.2</td>
</tr>
<tr>
<td>Peak Ad</td>
<td>317 ± 4.9</td>
<td>0.58 ± 0.02</td>
<td>546.6 ± 10.2</td>
<td>21.2</td>
<td>151.8</td>
</tr>
</tbody>
</table>
Red colouration were assumed to be living while those which lost the dye were termed dead cells.

Results

Fractionation on Sephadex G-100.

Separation of the enzyme complex on Sephadex G-100 led to a single peak of absorption designated A (Fig. 1). The molecular wt of this component from its elution volume was approximately 91,400 Daltons. The component also exhibited cellulase, polygalacturonase, pectin methylesterase and pectin transeliminase activities.

Further fractionation on CM Sephadex C-50.

When the components of peak A (Fig. 1) were separated on Sephadex C-50, four peaks of absorption were obtained designated Aa, Ab, Ac and Ad respectively (Fig. 2). Components of peaks Aa and Ab exhibited cellulase, polygalacturonase and pectin methylesterase activities, but lacked pectin transeliminase activity, while components of peak Ac possessed Polygalacturonase and pectin transeliminase activities but lacked cellulase and pectin methylesterase activities. However components of peak Ad possessed only pectin transeliminase activity.

Properties of the partially purified enzymes.

The enzymes were partially purified by subjecting to ammonium sulphate fractionation followed by dialysis and were separated by molecular exclusion and ion-exchange chromatography (Table 1-4). The second ammonium sulphate extracts following gel filtration gave purification folds of approximately 75, 64 and 83 respectively.

These fractions were employed to examine some characteristics of the enzymes.

Effect of pH and temperature.

Maximum activity of the enzymes occurred at pH 4.5, 5.0, 80 and 8.5 for cellulase, polygalacturonase, pectinmethylesterase and pectintranseliminase activities respectively (Fig. 3). Optimum temperatures for their activities were 50°C for cellulase, and 40°C for each of the pectinase enzymes (Fig. 4).

Effect of Ca++, Mg++, and ethylenediamine tetraacetic acid (EDTA).

Activity of each enzyme was stimulated by Ca++ and Mg++ respectively (Fig. 5). Concentration of Mg++ exceeding 10 mM produced no further increase in pectin methylesterase activity and caused a slight inhibition of polygalacturonase activity. Also Ca++ concentration exceeding 15 mM was slightly inhibitory to polygalacturonase and pectin transeliminase activity. All the concentrations of EDTA (1 to 20 mM) employed were inhibitory to the activity of each of the enzymes (Fig. 5), and a concentration of 15 mM resulted in complete inhibition of enzyme activity.

Km values

Activities of cellulase, polygalacturonase, pectin methylesterase and pectin transeliminase increased gradually as the substrate concentration increased. From the Lineweaver-Burk plots of the rate of enzyme activity versus substrate concentration, the apparent Km of cellulase for the hydrolysis of CM-Cellulose was approximately 3.3 mg/ml (Fig. 6), and those for polygalacturonase, pectin methylesterase and pectin transeliminase for the hydrolysis of pectin were 16.7, 7.1, and 14.3 mg/ml respectively (Fig. 7).

Tissue maceration and cellular death

The partially purified enzyme complex caused maceration and cellular death of potato tissue, the effect being higher at pH 8.0 than at pH 5.0.

Discussion

Cocoa beans infected by Penicillium steckii contained enzymes capable of degrading soluble and insoluble cellulasic substrates. Extracts from healthy cocoa beans lacked activity towards both forms of cellulose, indicating absence of cellulolytic activity. In this investigation, specific assays were not made to detect C enzyme. However hydrolysis of insoluble celluloses is believed to indicate presence of C activity (Selby and Maitland, 1967; Whitney et al., 1969; Olutiola, 1972). Production ofcellulases in plant tissues infected by pathogens has been reported (Bateman, 1964; Hancock and Millar, 1965). The Penicillium - infected tissues contained enzymes which degraded pectin hydrolytically and by a transeliminative mechanism, indicating polygalacturonase, pectin methylesterase and pectin transeliminase activities (Tellboy and Busch, 1970; Olutiola and Akintunde, 1979). Extracts from healthy tissues lacked such enzyme activity. Similar enzymes have been reported for other pathogen - infected plant tissues (Bateman, 1963, 1966; Reddy et al., 1969; Hagar and McIntyre, 1972). The absence of cellulase, polygalacturonase and pectin transeliminase activities from healthy tissues indicates that the enzymes are of fungal origin. However traces of pectin methylesterase occurred occasionally in uninfected cocoa beans although much more activity occurred in infected tissues. It has been suggested that perhaps host and pathogen contribute to the production of pectin methylesterase in infected tissues (Olutiola and Akintunde, 1979).

The occurrence of pectic and cellulolytic enzymes in the infected tissues coupled with their ability to cause tissue maceration and cellular death, suggest a role for these enzymes during the deterioration of cocoa beans by P. steckii. The role of pectinases and cellulases in pathogenicity has been discussed (Husain and Dimond), 1960; Bateman 1964; Hancock et al., 1964; Wood, 1967; Garibaldi and Bateman, 1971).
Cell Wall Degrading Enzymes Associated with Deterioration of Cocoa Beans by *Penicillium Steckii*  

P.O. Olutola

Figure 1  
Separation by gel filtration of proteins in cocoa beans infected by *Penicillium steckii*. Δ, protein (E<sub>260</sub>); ..., cellulase; ●, polygalacturonase; ---, pectin methylesterase; O, pectin transeliminase.

Figure 2  
Separation by ion-exchange chromatography (Sephadex C-50) of proteins (fractions 16-26 of Fig. 1) subjected to ammonium sulphate precipitation) and enzymic activity of the fractions towards cellulose and pectin. Δ, Protein (E<sub>260</sub>); ..., cellulase (CL); ●, polygalacturonase (PG); ---, pectin methylesterase (PME); O, pectin transeliminase (PTE).
Effect of pH on the activity of partially purified (fractions 16-26 of Fig. 1 subjected to ammonium sulphate precipitation) enzymes obtained from cocoa beans infected by *Penicillium steckii*. A, cellulase; B, polygalacturonase; C, pectin methylesterase; D, pectin transeliminase. Each point represents the mean of three replicates.

Effect of temperature on the activity of partially purified (fractions 16-26 of Fig. 1 subjected to ammonium sulphate precipitation) enzymes obtained from cocoa beans infected by *Penicillium steckii*. A, cellulase; B, polygalacturonase; C, pectin methylesterase; D, pectin transeliminase. Each point represents the mean of three replicates.
Cell Wall Degrading Enzymes Associated with Deterioration of Cocoa Beans by *Penicillium Steckii*  P.O. Olutiola

**Figure 5** Effects of Ca\(^{++}\) (○), Mg\(^{++}\) (△) and ethylenediamine tetraacetic acid (●) on the activity of partially purified (fractions 16-26 of Fig.1 subjected to ammonium sulphate precipitation) enzymes obtained from cocoa beans infected by *Penicillium steckii*. A, cellulase, B, polygalacturonase; C, pectin methylesterase; D, pectin transeliminase. Each point represents the mean of three replicates.

**Figure 6** Lineweaver - Burk plot for the hydrolysis of carboxymethylcellulose by the partially purified (fractions 16-26 of Fig.1 subjected to ammonium sulphate precipitation) cellulase obtained from cocoa beans infected by *Penicillium steckii*. X, experimental point; O, regression point.
Figure 7  Lineweaver - Burk plots for the hydrolysis of pectin by the partially purified (fractions 16-26 of Fig.1 subjected to ammonium sulphate precipitation) polygalacturonase (A), pectin, methyl esterase (B), and pectin transeliminase (C) obtained from cocoa beans infected by *Penicillium steckii*. X, experimental point; O, regression point.

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THE BIODETERIORATION OF THE PLASTICISER DIOCTYL PHthalate

G.R. Williams1, 2 and R. Dale1

Introduction

Plasticisers are added to plastic materials to increase flexibility. They work by separating the polymer chains so that the intermolecular forces between the chains are weakened. Chloroprene rubber also contains plasticisers to make the rubber suitable for low temperature use. The degree of susceptibility of plasticisers to microbial attack exercises a profound influence on the susceptibility of the whole material. Several surveys have been conducted on the resistance of plasticisers to mould growth (Brown, 1945; Lightbody et al., 1954; Stahl & Pessen, 1953; Turner, 1967). Phthalates, especially dioctyl phthalate (DOP), are generally considered to be resistant to mould growth, although traces of growth have been found on this compound (Berk et al., 1957). However, the bacterium Serratia marcescens has been found to utilise DOP as sole source of carbon and energy (Mathur & Rouatt, 1975), and DOP degradation occurred by constitutive esterases to phthalic acid, and further deterioration was catalysed by adaptive enzymes. This paper describes the breakdown of DOP by a Bacillus sp.

Experimental

Chemicals: All chemicals were obtained from Aldrich Ltd. and were of the purest grade available. Chloroprene rubber sheets (15cm x 7.5cm x 0.2cm) were supplied by the MQAD Rubber Mill Room, consisting of Neoprene: 100; DOP: 8; ZnO: 5; MgO: 4 (pph rubber).

Growth and harvesting of organisms: Organisms were grown in 500ml of Bushnell Haas broth (BH) (Busnell & Haas, 1941) supplemented with 5% DOP as the sole carbon source, contained in 1 litre conical flask on an orbital shaker (200 rev/min). Solid media were prepared by the addition of 1.5% Difco-Noble agar to the liquid medium. Organisms were harvested by centrifugation at 30,000g for 1 hour.

Cell free extracts: Cell free extracts were prepared by subjecting cells to ultrasonic disruption for 5 min and remaining whole cells removed by centrifugation.

Respirometric experiments: Respirometric experiments were performed at 25°C in a Warburg apparatus.

Thin layer chromatography (TLC): DOP and related compounds are separated on methylene chloride (system I) and on di-isopropyl either: formic acid: water (90:7:3) (system II) on silica gel G (UV 254) plates. RF values on system I and II respectively were, DOP (0.71, 0.95); dibutyl phthalate (0.57, 0.88); diethyl phthalate (0.43, 0.82); dimethyl phthalate (0.42, 0.74) and phthalic acid (0.07, 0.66). Spots were detected under UV light.

Gas liquid Chromatography (GLC): Chloroprene rubber samples were heated in a limited space at 130°C for 6 mins, in order to measure volatile compounds which were then examined by GLC using a 2 meter column packed with 8% OV-17 on chromosorb W-HQ, temperature programmed at 40°C min-1 up to 280°C, detector temperature 300°C.

Physical testing: Microhardness tests were performed according to British Standard 903 A26 method M (1969) and low temperature torsional modules for 70 mega pascals was performed according to British standard 903 A13 (1972).

Results

Organisms capable of growth on DOP were isolated on solid DOP media from unsterilized John Innes No.1 soil. Organisms were identified as large gram positive rods which formed spores predominately in the centre of the cell. Further tests on MR/VP media tentatively identified the organism as Bacillus megaterium. Growth of the organisms in liquid culture was complete in two weeks, however growth was accompanied with an emulsification of the medium. No accumulation of phthalates could be detected by TLC in the culture filtrates.

Organisms grown on DOP in liquid culture were harvested, washed and resuspended in 50 ml BH broth. These organisms could rapidly oxidise a range of phthalates without a lag period, as determined respirometrically. These phthalates include DOP (22.3), dibutyl phthalate (13.3), diethyl phthalate (16.6), dimethyl phthalate (53.3) and phthalic acid (103.9). Figures in brackets represent the QO2 values (in m moles/min/mg dry wt. cells) corrected for endogenous respiration. Two intermediates in aromatic ring degradation, catechol (1.8) and protocatecholic acid (27.3) were also tested, and the latter compound was readily oxidised without a lag period.

As no accumulated compounds could be detected in culture filtrates of DOP grown cells, cell free extracts of B. megaterium were incubated with NADH and DOP for 8 hours. After this period, an intermediate was found which co-chromatographed with dimethyl phthalate (0.42, 0.74 RF values for systems I and II respectively) as determined by TLC. Controls lacking NADH, DOP or cell free extract did not form dimethyl phthalate.

Ministry of Defence M.Q.A.D., Biodeterioration Laboratory, Royal Arsenal East, Woolwich, London SE18

Dr. Williams’ present address: Dairy Crest Creamery, Milk Marketing Board, Felinfach, Lampeter, Dyfed, Wales.

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Chloroprene rubber samples containing DOP were placed in sterile glass vessels (20cm x 10cm x 10cm) and inoculated with 100 ml of DOP grown B. megaterium in BH broth containing 3.0 x 10^5 cells per ml. One set of samples was maintained in air another sealed in cellophane sheets, and another set placed in vessels containing sterile BH broth to act as controls. Rubbers were incubated at 25°C for 28 days, after which time B. megaterium cells had sporulated. After this period, GLC examination of the rubbers indicated that control strips maintained both in air and in cellophane contained 6.5% (v/w) DOP, and chloroprene rubber placed in BH broth (sterile and infected with B. megaterium) contained 5.3 (v/w) DOP. Microhardness tests on these rubbers indicated that all four samples gave readings of 29 IRHD (initial rubber hardness degrees). Low temperature torsional modules for 70 mega pascals was -43°C for all rubbers.

Discussion

Evidence presented here suggests that DOP may be broken down to dimethyl phthalate in B. megaterium, with further conversion to phthalic acid, as suggested by respirometric and cell free extract experiments. This compound could then be converted into protocatechueic acid (as shown by respirometric tests), which is an intermediate in one branch of the β-ketodipate pathway used by aerobic bacteria in the catabolism of aromatic compounds (Mahler and Cordes, 1966). However, this study indicates that bacteria do not cause a measurable deterioration of chloroprene rubber containing DOP over a 28 day period, and no further deterioration would occur as cells responsible for DOP breakdown had sporulated. These organisms may grow on DOP which had leached out of the rubbers, as DOP content of the rubbers was found to decrease with immersion in water, as determined by GLC.

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