PRODUCTION OF THE MYCOTOXIN PATULIN IN NATURE

by

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To my parents,

my wife, Ebtisam

my sons Mushari, Nawwaf and Mushaal,

and

my family

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CONTENTS

		Page
	SUMMARY	i
1.	INTRODUCTION	1
1. 1.	Secondary metabolites.	1
1. 2.	Mycotoxins as secondary metabolites.	2
1. 3.	Patulin as a secondary metabolite.	3
1. 4.	Patulin producing fungi.	4
1. 5.	Patulin production in nature.	7
1. 6.	The biosynthesis of patulin.	10
1. 7.	Physical and chemical properties of patulin.	11
1. 8.	Toxicity of patulin.	12
1. 8. 1.	Effect of patulin on bacteria.	13
1. 8. 2.	Effects of patulin on fungi.	14
1. 8. 3.	Effect of patulin on viruses.	15
1. 8. 4.	Effect of patulin on cells and tissue cultures.	16
1. 8. 5.	Effect of patulin on protozoa.	16
1. 8. 6.	Effect of patulin on human and animals.	17
1. 8. 7.	Effect of patulin on higher plants.	17
1. 9.	Factors which influence patulin production.	18
1. 9. 1.	Physical factors.	18
i.	Moisture.	18
ii.	Temperature.	18
iii.	pH	19
1. 9. 2.	Biological factors.	19
i.	Fungal strain.	19
ii.	Patulin production in relation to interaction with other microorganisms.	20

1. 9. 3.	Nutritional factors influencing patulin production.	20
1. 9. 4.	Effect of incubation period on patulin production.	21
1. 9. 5.	Cytological factors influencing patulin production.	21
1. 9. 6.	Some properties of patulin.	22
2.	PRODUCTION OF PATULIN IN APPLES.	23
2. 1.	Introduction.	23
2. 2.	Materials and Methods.	24
2. 2. 1.	Occurrence of fungi in fruits.	24
i.	Procurement of fruits.	24
ii.	Mould isolation.	24
2. 2. 2.	Isolation of bacteria from fruits.	25
2. 2. 3.	Natural occurrence of patulin on fruits.	26
2. 2. 4.	Evaluation of potential patulin toxicity.	26
2. 2. 5.	Ability of the isolated fungi to produce patulin.	26
i. a.	Laboratory medium.	27
i. b.	Evaluation of the potential patulin toxicity produced of extracts from fungi grown in Czapek Dox and the patulin standard.	27
ii.	Patulin production on apples.	27
ii. a.	Patulin extraction.	28
ii. b.	Preparation of patulin standard solution.	28
ii. c.	Sample clean-up.	29
ii. d.	Preparation and development of TLC plates.	29
ii. e.	Detection, identification and confirmation of the presence of patulin on TLC plates.	30
ii. f.	Quantitative analysis of patulin.	30
ii. g.	Two-dimensional TLC.	31
2. 3.	Results and Discussion.	31
2. 3. 1.	Occurrence of fungi on fruits.	31

2. 3. 2. Occurrence of bacteria in fruits. 32

2. 3. 3. Patulin production on fruits. 33

2. 3. 4. Evaluation of potential patulin toxicity. 35

i. a. Ability of the fungal isolates to produce patulin in Czapek Dox liquid medium. 35

i. b. Evaluation of the toxicity of patulin produced by fungi growing in Czapek Dox liquid medium. 36

ii. Ability of the isolated fungi to produce patulin on apple. 38

2. 4. Conclusions. 40

3. PATULIN PRODUCTION ON SUGAR BEET. 42

3. 1. Introduction. 42

3. 2. Materials and Methods. 43

3. 2. 1. Source of sugar beet. 43

3. 2. 2. Isolation of indigenous moulds. 43

3. 2. 3. Determination of natural occurrence of patulin in the mouldy sugar beet. 43

3. 2. 4. Determination of the ability of the isolated indigenous fungi to produce patulin in various culture media. 44

i. Czapek Dox liquid medium (CD). 44

ii. Inoculation of sugar beet slices. 44

iii. Patulin production on sugar beet extract amended with range of nitrate concentrations. 45

3. 2. 5. Patulin extraction. 45

3. 2. 6. Clean-up procedure. 45

3. 2. 7. Patulin assay. 46

3. 2. 8. Quantitative analysis. 46

3. 3. Results and Discussion. 46

3. 3. 1. Description of moulds isolates. 46

3. 3. 2. Ability of the isolated indigenous fungi to produce patulin in Czapek Dox liquid medium. 46
3. 3. 3. Ability of the isolated indigenous fungi to produce patulin when growing on sugar beet slices. 47
3. 3. 4. Patulin production by fungi growing on sugar beet extract amended with nitrate. 49
3. 4. Conclusions. 49
4. DO MICROORGANISMS PRODUCE PATULIN WHEN GROWING ON WHEAT STRAW? 52
4. 1. Introduction. 52
4. 2. Materials and Methods. 53
4. 2. 1. Substrate preparation. 53
4. 2. 2. Organisms and inoculum. 53
a. Patulin production in Czapek Dox liquid medium (CD). 53
b. Patulin production on wheat straw. 54
4. 2. 3. Extraction of patulin. 54
4. 2. 4. Clean-up, chromatography and quantitative analysis. 55
4. 3. Results and Discussion. 55
4. 4. Conclusions. 57
5. STUDIES ON THE INTER-RELATIONSHIP BETWEEN NITRIFICATION AND PATULIN PRODUCTION BY FUNGI. 59
5. 1. Introduction. 59
i. Ammonification. 59
ii. Nitrification. 59
iii. Denitrification. 60
iv. Conversion of nitrogen and patulin production. 61
5. 2. Materials and Methods. 61
5. 2. 1. Media preparation. 61

5. 2. 2. Inoculum and incubation. 62

5. 2. 3. Determination of fungal biomass. 62

5. 2. 4. Determination of ammonium nitrogen. 63

i. Reagents used. 63

i. a. Ethylenediaminetetraacetic acid (EDTA). 63

i. b. Phenolate reagent. 63

i. c. Phenol solution. 63

i. d. Caustic soda solution. 64

i. e. Sodium hypochlorite solution. 64

5. 2. 5. Determination of nitrate nitrogen. 64

i. Reagents used. 65

i. a. Reagent (1) 65

i. b. Reagent (2) 65

i. c. Reagent (3) 65

i. d. Reagent (4) 65

5. 2. 6. Determination of patulin. 65

5. 3. Results and Discussion. 65

5. 3. 1. Determination of fungal biomass. 65

5. 3. 2. Nitrification of ammonium. 66

5. 3. 3. Urea utilization. 66

5. 3. 4. Patulin production. 66

5. 4. Conclusion. 67

6. EFFECT OF CARBON AND NITROGEN ON GROWTH OF FUNGI AND PATULIN PRODUCTION. 68

6. 1. Introduction. 68

i. Relationship of fungal growth to carbon and nitrogen. 68

ii. Relationship between patulin production and levels of carbon and nitrogen present in the medium. 69

6. 2. Materials and Methods. 70
6. 2. 1. Ability of the isolated fungi to grow oligotrophically. 70
i. Preparation of silica gel. 70
ii. Fungal inoculation. 71
iii. Preparation of samples for scanning electron microscopy (SEM). 71
6. 2. 2. Effect of addition of various levels of carbon and/or nitrogen on fungal growth and patulin production. 72
i. Preparation of liquid medium. 72
ii. Fungal inoculation. 72
iii. Determination of the biomass of oligotrophically grown fungi. 73
iv. Extraction of patulin. 73
6. 2. 3. Effect of various amounts of carbon and/or nitrogen on fungal growth and patulin production. 73
i. Preparation of liquid medium. 73
ii. Fungal inoculation. 74
iii. Determination of the biomass of oligotrophically grown fungi. 74
iv. Patulin determination. 74
6. 2. 4. Effect of nitrogen source on fungal growth and patulin production by *Penicillium* sp (1). 74
i. Fungal inoculation. 74
ii. Patulin detection. 75
6. 3. Results and Discussion. 75
6. 3. 1. Oligotrophic of fungi grown on silica gel. 75
6. 3. 2. Growth of fungi in liquid medium under oligotrophic conditions. 76
6. 3. 3. Patulin production at various amounts of carbon and nitrogen. 77
6. 4. Conclusion. 78
7. EFFECT OF NATURAL ISOLATES OF BACTERIA AND FUNGI ON PATULIN. 80

7. 1.	Introduction.	80
7. 2.	Materials and Methods.	81
7. 2. 1.	Patulin synthesis by the isolated fungal species in dual cultures or in association with the isolated contaminant bacteria (I, II and III) and *Phanerochaete chrysosporium.*	81
i.	Effect of *Penicillium expansum*, *Penicillium* sp (1), *Penicillium* sp (2) and *Penicillium* sp (3) in dual culture on patulin production.	81
a.	Czapek Dox liquid medium (CD).	81
b.	Patulin production on apple.	82
ii.	Effect on patulin production of *Penicillium expansum* in dual culture with *Phanerochaete chrysosporium.*	82
iii.	Effect on patulin production of *Penicillium expansum* and other isolates of *Penicillium* species in dual culture with the isolated contaminant bacterial species I, II and III.	83
7. 2. 2.	Effect of patulin on *Penicillium expansum*, *Phanerochaete chrysosporium* and the isolated contaminant bacteria (I, II and III).	84
i.	Influence of patulin addition on *Penicillium expansum.*	84
i. a.	Evaluation of patulin toxicity.	85
ii.	Influence of patulin on *Phanerochaete chrysosporium.*	85
iii.	Influence of patulin on the isolated contaminant bacterial species I, II and III.	85
7. 2. 3.	Effect of patulin and glucose addition on the growth and patulin production by *Penicillium expansum.*	86
7. 2. 4.	The effect of patulin on the growth of *P.expansum.*	87
7. 2. 5.	Patulin detection.	87
7. 3.	Results and Discussion.	88
7. 3. 1.	Patulin production by *Penicillium expansum*, *Penicillium* sp (1), *Penicillium* sp (2) and *Penicillium* sp (3) in dual culture when	

growing in Czapek Dox liquid medium. 88

7. 3. 2. Effect of *Penicillium expansum*, *Penicillium* sp (1) and *Penicillium* sp (3) in separate or in dual culture on patulin production in apple fruits. 89

i. Effect of fungal competition on lesion development on apple fruits. 91

7. 3. 3. Effect of *Penicillium expansum* in dual culture with *Phanerochaete chrysosporium* on patulin production. 91

7. 3. 4. Effect of *Penicillium expansum* in dual culture, with the isolated contaminant bacterial species No. I, II and III, on patulin production. 91

7. 3. 5. Influence of patulin addition on *Penicillium expansum*, *Phanerochaete chrysosporium* and the isolated contaminant bacteria (I, II and III). 92

i. Effcet of *P. expansum* on patulin degradation in C-free Czapek Dox medium. 92

ii. Influence of patulin addition on *Phanerochaete chrysosporium*. 93

7. 3. 6. Effect of patulin and glucose addition on the growth and patulin production of *Penicillium expansum*. 94

7. 3. 7. Effect of patulin on the growth of *Penicillium expansum*. 94

7. 4. Conclusions. 95

8. GENERAL DISCUSSION. 97

9. APPENDIX. 100

9. 1. Solid media used for isolation and growth of fungi and bacteria. 100

9. 2. Liquid media used for isolation and growth of fungi and bacteria. 102

9. 3. Method for preparation of fungal sample for scanning electron microscopy (SEM). 106

9. 4. Analysis of inorganic N-ion. 107

9. 5. Glucose estimation by di-nitrosalicynic acid method. 109

9. 6. Detection of patulin. 109

10. REFERENCES. 113

SUMMARY

A study was made of the factors governing the production of the mycotoxin patulin in nature, including biological and physiological factors. The objective of the research described in this thesis was to study the ability of the indigenous fungi of fruits and the apple rot fungus, *Penicillium expansum*, to grow and to produce patulin in different substrates, both natural (apples, sugar beet, wheat straw) and laboratory media. The effect of carbon and nitrogen sources and the relationship between the production of the toxin and nitrification and its action with the natural isolated bacteria and fungi was also investigated.

Common members of the genus *Penicillium* were found to represent a high percentage of the indigenous fungal flora isolated from both apples and sugar beet. Most of these isolates were able to produce patulin in Czapek Dox liquid medium.

Although both apple fruit and sugar beet were naturally highly contaminated with moulds, only apples were contaminated with patulin (7598 $\mu g\ kg^{-1}$).

Confirmatory tests showed patulin production of 8.3% and 50% (after 7 days) to 99.2% (after 30 days) by the indigenous fungi in apple and sugar beet, respectively.

The indigenous fungal flora of wheat straw failed to produce patulin when growing naturally. Patulin was produced only by *Penicillium expansum* alone and not when growing in association with the white rot fungus *Phanerochaete chrysosporium*.

The accumulation of ammonium and nitrate during urea hydrolysis and ammonium nitrification by *Penicillium* sp (1), *Penicillium* sp (3) and *Penicillim expansum* was achieved with varying degree of efficiency. Urea hydrolysis, but not ammonium nitrification was associated with patulin production.

Growth of *P. expansum* and *Penicillium* species (1 and 3) occurred under oligotrophic conditions. Both carbon and nitrogen are required for patulin production but it is the depletion of nitrogen which is important for production of the toxin.

A wide range of Gram-negative bacteria naturally isolated from apple fruits showed growth stimulation even in the presence of high concentrations of patulin (100 μg patulin ml^{-1}). Growth stimulation by patulin was also observed for *Penicillium expansum.*

Patulin degradation using natural (apples) and artificial (Czapek Dox C-free liquid medium with added patulin with/without glucose) substrates, was demonstrated in 7 to 14 day old by dual cultures of either (a) patulin producing fungi grown together or (b) with *Phanerochaete chrysosporium* or (c) the isolated patulin producing fungi in dual culture with the indigenous bacteria.

Finally the data obtained demonstrated that natural substrates are generally better than artificial media for patulin production.

1. INTRODUCTION

1. INTRODUCTION

Microorganisms require major growth substrates, such as carbohydrates (as an energy source), organic or inorganic source of nitrogen, as well as trace elements and growth factors. Although, most of these required substrates are available in the environment, they are often present in complexed forms and their concentration is seldom constant, but instead continually changes due to competition from other organisms. Microorganisms secrete enzymes which break down the substrate allowing the substrate to be assimilated. Natural interactions between the various components, some of which can be regarded as pioneer organisms, of a mixed population can lead to the release of food products required for growth of other microorganisms. On the other hand, competition between organisms more often leads to a reduction in the substrate availability between competing microorganisms.

1. 1. Secondary metabolites

A number of common metabolic pathways are distributed in green plants, fungi, and bacteria. These pathways lead to products which can be termed "primary" and "secondary" metabolites. Generally, primary metabolites are produced in association with growth during tropophase (i.e. log phases of growth), whereas secondary metabolites are produced after active growth has ceased, during the so-called idiophase. Thus secondary metabolites may originate from a few simple precursors of primary metabolism (Bu'Lock *et al.*, 1965; Bu'Lock, 1967; Drew and Demain, 1977). Numerous filamentous fungi can grow on feed and foodstuffs, and many of them posses the ability to produce secondary metabolites. Secondary metabolites may be produced by the following systems:

(1) Fully growth associated system, where the product arises directly from as essential growth process, e.g., alcohol production during the anaerobic growth of yeast.

(2) Overproduction of primary metabolic products occurs when one of the enzymes in central metabolism is blocked or eliminated, e.g. in the production of citric acid.

(3) Secondary metabolic products are evolved by special enzymatic reactions, forming substances which are apparently unrelated to central metabolism, and which may appear to have no value to the organism concerned (Bu`Lock *et al.*, 1965; Bu`Lock, 1967; Bu'Lock, 1975). Moreover, the secondary metabolites often start to be produced following a decrease in growth rate (Luckner *et al.*, 1977).

Bu`Lock (1975) pointed out that secondary metabolites possess the following characteristics:

(1) Secondary metabolite biosynthesis is mainly reliant upon only a few basic pathways.

(2) Secondary metabolites productivity is generally restricted to a single species, or a single strain of a species.

(3) Secondary metabolites do not play an obvious role in the growth and metabolism of the producer microorganisms .

(4) Microorganisms start to produce secondary metabolites when growth is restricted, e.g. during the idiophase.

1. 2. Mycotoxins as secondary metabolites

Many filamentous fungi have the ability to produce secondary metabolites. These products include pigments, antibiotics, alkaloids, gibberellins and toxic metabolites for both plants and animals. These toxic metabolites are called mycotoxins, a term which is derived from the Greek "mykos" meaning fungus, and the Latin "toxicum" meaning poison. This term was introduced into microbial biochemistry after being borrowed from plant physiologists (Bu' Lock, 1961). Fungi that elaborate mycotoxins from feed and foodstuffs are usually in the category referred to as moulds. However, the great majority of moulds do not produce poisonous substances. Toxins may also be

elaborated by fungi which produce large macroscopic fruiting bodies - e. g., certain poisonous species of *Amanita* (Smith and Moss, 1985).

Many moulds regularly grow in feed and foodstuffs, so not surprisingly these species are often refered to as storage fungi. Of particular importance in this respect are species of the genera *Aspergillus* and *Penicillium* (Hesseltine, 1968).

There is a clear relationship between mycotoxins, as a final products of secondary metabolism, and primary metabolites. Thus, primary metabolites are linked to production of secondary metabolites by some common intermediates. For example, several workers (e. g. Steyn, 1980; Turner and Aldridge, 1983) have reported that the reaction of acetyl coenzyme A, which is the key branch point for catabolic and anabolic systems (Packter, 1973), with molecules of malonyl coenzyme A during fatty acids production is one of the most important routes for secondary metabolites production. The pathway of the synthesis of polyketides derived mycotoxins (Turner and Aldridge, 1983) is shown in Fig. 1. 1.

1. 3. Patulin as a secondary metabolite

Patulin is a fungal secondary metabolite which was evaluated for use as an antibiotic in the early 1940's for the treatment of various human infectious diseases, including the common cold (Chain *et al.*, 1942; Birkinshaw *et al.*, 1943). Studies on patulin production have largely been devoted to submerged laboratory culture. Patulin production was originally associated with two strains of *Aspergillus clavatus* (Wiesner, 1942). This mycotoxin has been given a variety of names. Chain *et al.* (1942) found that *Penicillium claviforme* (NCTC 1718) was able to produce a crystalline substance which was called claviformin, while under the name clavacin and clavatin a number of workers described its production by several strains of *A. clavatus* (Waksman and Horning, 1943; Bergel *et al.*, 1943; Raistrick *et al.*, 1943; Waksman *et al.*, 1943). Further studies on crystallised products of penicillia, discovered in 1942, were named

penicidin. Atkinson *et al.*, 1944 demonstrated that the same products were produced by *Penicillium patulum*, but named them patulin (Raistrick *et al.*,1943). Finally, the crystalline antibiotic, expansine was isolated and identified from six different strains of *Penicillium expansum*, and subsequently re-named patulin. Patulin is now regarded as a fairly common fungal toxin, being produced by numerous fungal species (Anslow *et al.*, 1943; Hooper *et al.*, 1944; Oosterhuis, 1946). Other names given to patulin include:- mycosin C_3, penicidin, leucopin and tercinin (Brian *et al.*, 1956; Scott, 1974 a; Wyllie and Morehouse, 1977).

1. 4. Patulin producing fungi

The principal moulds involved in patulin production are various members of the genera *Aspergillus*, *Penicillium*, *Paecilomyces* and *Byssochlamys* (Scott, 1974 a; Lovett and Thompson, 1978; Palmgren and Ciegler, 1983). Steiman *et al.* (1989) stated that patulin is produced by approximately 60 species of moulds belonging to over 30 genera. Most of these fungi are common contaminants of foods. Thus, *P. expansum*, the common storage rot of fruit; and *Byssochlamys nivea*, the heat-resistant fruit juice contaminant, have been identified as contaminant food fungi which can produce patulin on a variety of substrates (Kuehn, 1958). *Gymnoascus* sp. was reported as a patulin producing fungus by Karow and Foster (1944). Most of the above isolates were confirmed as patulin biosynthesis fungi by Abraham and Florey (1949) and Efimenko and Yakimov (1960). Some of the fungi which have been reported to produce patulin on various media are listed in Table 1. 1.

Table 1. 1. Patulin producing fungi.

Fungal species	Reference
Penicillium expansum	Van Luijk (1938); Anslow *et al.* (1943)
P. rivolii	Berestetskii *et al.* (1975)
Penicillium sp.	Atkinson (1942)
P. claviforme	Chain *et al.* (1942)
P. patulum	Raistrick *et al.* (1943)
P. melinii	Karow and Foster (1944)
P. urticae	Kent and Heatley (1945)
Penicillium spp.	Lochhead *et al.* (1946)
P. equinum and *P. novae-zeelandiae*	Burton (1949)
P. leucopus and *Penicillium* spp.	Umezawa *et al.*, (1947)
P. lapidosum	Myrchink (1967)
P. griseofulvum	Torres *et al.* (1987); Simonart and de Lathouwer (1956-1957)
P. divergens	Barta and Mecir (1948)
P. cyclopium	Efimenko and Yakimov (1960)
P. cyaneofulvum	Berestetskii *et al.* (1974)
Aspergillus clavatus	Wiesner (1942); Bergel *et al.* (1943); Waksman *et al.* (1943)
A. giganteus	Florey *et al.* (1944)
A. terreus	Kent and Heatley (1945)
Gymnoascus sp.	Karow and Foster (1944)
Byssochlamys fulva	Escoula (1975)

Tsai *et al.*, (1988) also reported that *P. roqueforti*, *P. cyclopium*, *P. viridicatum*, *P. crustosum* can produce patulin *in vitro*. Recently, a new strain of the species of *Byssochlamys fulva* described by Tokatli and Ozilgen (1991) has been recorded as a patulin producing fungus. Furthermore, some species of fungi which had not been reported as patulin producers have been found to produce patulin in liquid medium. These fungi belong to the order *Micromycetes* and include species of Mucorales and Fungi Imperfecti (Steiman *et al.*, 1989). The list of fungi capable of producing patulin has been further extend to include *Cunninghamella bainieri*, *Mortierella bainieri*, *Mucor hiemalis* (b), *Mucor racemosus* var. *globosus*, *Aspergillus amstelodami* (a), *A. echinulatus*, *A. fumigatus* (b), *A. manginii*, *A. parasiticus* (b), *A. repens* (b), *A. variecolor*, *A. versicolor* (b), *A. versicolor* (c), *Eupenicillium brefeldianum* (b), *Penicillium aurantiogriseum* (a), *P. aurantiogriseum* (b), *P. canescens*, *P. chrysogenum* (c), *P. chrysogenum* (f), *P. citreonigrum* (a), *P. funiculosum* (a), *P. griseofulvum*, *P. roqueforti* (b), *P. roqueforti* (c), *P. variabile* (b), *Acremonium zeae*, *Alternaria alternata* (d), *A. papaveris*, *Ascochyta imperfecta*, *Aureobasidium pullulans* var. *pullulans*, *Botrytis allii* (b), *Calcarisporium arbuscula* (c), *Chrysosporium pannorum* (b), *C. pannorum* (c), *Cladobotryum varium* (b), *C. verticillatum*, *Cladorrhinum* sp. *Colletotrichum musae*, *Curvularia lunata* (b), *Fusarium culmorum* (b), *F. oxysporum* (a), *F. proliferatum* (a) var. *proliferatum*, *F. proliferatum* (b) var. *proliferatum*, *Oidiodendron echinulatum* (b), *O. tenuissimum* (b), *Paecilomyces lilacinus* (a), *Pseudodiplodia sp.*, *Rhinocladiella atrovirens* (a), *Scopulariopsis* sp., *Sporothrix schenckii* (a), *Trichoderma pseudokoningii* (a), *T. polysporum* (a), *Trichophyton mentagrophytes* (c), *Gymnoascus reesii*, *Sporormiella minimoides*, *Basidio* 2 and *Trametes squalens* (Steiman *et al.*, 1989).

Several new fungal species have also been reported as patulin producers; including *Eupenicillium javanicum*, *Penicillium cyaneum*, *Penicillium diversum*, *Penicillium duclauxii*, *Penicillium echinulatum*, *Penicillium glabrum*, *Penicillium lignorum*,

Alternaria tenuissima, *Chaetomium atrobunneum*, *Scopulariopsis flava*, and a *Stemphylium* sp. (Okeke *et al.*, 1993).

Aspergillus sulphureus, which has previously been found in grain meals, is also considered to be a patulin- producing mould (Papa *et al.*, 1992). Jimenez *et al.* (1990) reported patulin production by an apparently new specie of *Penicillium* (which was subsequently identified as *P. griseofulvum*). Early studies by Bassett and Tanenbaum (1958) showed that a mutant strain of *P. urticae* (syn. *P. patulum* (NRRL 2159 A). also produced patulin.

1. 5. Patulin production in nature

Patulin is frequently produced where feeds and foodstuffs are infected with one or more fungi. However, the presence of potentially patulin producing fungi on agricultural products does not necessarily indicate patulin contamination. As was mentioned above, patulin producing fungi are widely distributed in nature, with the result that patulin is produced on diverse substrates including various foods and animal feeds. It is particularly commonly found in apples, as well as other fruits and their juices (Stoloff, 1975). In 1972, Scott *et al.*, appear to have been the first to have reported the presence of patulin in apple juice (produced by *Penicillium expansum*). The quantity of patulin detected was 1 mg l^{-1}. Later on several workers recorded the presence of patulin in both naturally rotted apples (Brian *et al.*, 1956; Walker, 1969; Harwig *et al.*, 1973 a), and pears. About 50% of apples and pears suffering from brown rot were found to be contaminated with patulin (Frank, 1977). Even under good storage conditions, patulin is able to attack apples and pears within 3 days after removal from cold storage (Gimeno, 1979). The detected amounts of patulin vary depending on the fruit type suffering contamination. For instance, Burdaspal and Pinilla (1979) found that patulin levels ranged from between 1 to 250 mg kg^{-1} in 53% of the naturally rotted apples. But levels found were less in the naturally rotted pears, where 33% of the

samples contained amounts of patulin ranging between 0.9 to 10 mg kg^{-1}. A concentration of 136 mg kg^{-1} of patulin was detected in naturally rotted apples by Harwig *et al.* (1973 a), while values of patulin from 0.8 to 100 mg kg^{-1} were detected in 66.7% of naturally rotted apples and pears (Gimeno and Martins, 1983). Reported patulin concentrations in apple juices vary from low concentrations (Scott, 1974 b) from Canadian apple juice, when quantities of patulin ranged from 20 to 120 µg l^{-1} was detected from 5 out of 11 samples. Eight to 13 samples of juice contained 49 to 309 µg patulin l^{-1} (Ware *et al.*, 1974), with concentrations ranging between 40 to 440 µg l^{-1} by Stoloff (1976). During a survey of some apple juice Wilson (1981) reported detecting patulin in 10 out of 24 samples at levels up to 56 µg l^{-1}.

Frank (1977) demonstrated that 61% of contaminated commercial apple juices contained 300 µg l^{-1} of patulin. The level of patulin was a little higher in sweet apple cider. Pasteurized apple cider produced in Georgia contained patulin levels varying between 244 to 3993 µg patulin l^{-1} of cider (Wheeler *et al.*, 1987).

Fruit and vegetables naturally contaminated by patulin producing fungi were also found to contain varying concentrations of patulin. Dvali *et al.* (1985) found that in 8 out of 160 samples of fruit and vegetables tested the highest concentration of patulin was found in the berries of sea buckthorn where up to 54,000 µg kg^{-1} was detected. They also recorded patulin in 28 out of 185 samples of foods manufactured from fruit including juices, purees and jams. The concentration of patulin in juices did not however, exceed the average allowable concentrations. Patulin has also been detected in apple, sea buckthorn and plum juices. Burda (1992) reported finding patulin when he assayed 328 samples of apple, pear, and mixed fruit products, including juices, sauces, purees, jellies, diced apples; as well apple pulps collected from 38 Australian producers. Patulin was detected in 75 of 258 juice and juice concentrate samples ranging from 5 to 50 µg l^{-1} and in 73 samples ranging from 51 to 1130 µg l^{-1}. Of 70 samples other than fruit juices, patulin was detected in 18 samples at levels below 50 µg kg^{-1} of patulin. The detection limits for patulin in juice (l^{-1}) and other products (kg^{-1})

were 5 μg. Furthermore, in Germany Frank *et al.* (1977) reported detectable amounts of patulin in bananas, pineapples, grapes and peach.

Apricots are also a source for naturally occurring patulin (Frank *et al.*, 1977); levels ranging between 2 to 13 mg kg^{-1} were found in 12 mouldy samples (50%) by Burdaspal and Pinilla (1979).

The natural occurrence of patulin in silage was reported by Escoula (1974), who found that 50% of the contaminated samples tested contained around 40 mg kg^{-1}. Silage has also been reported to contain patulin at concentrations between 1 and 5 mg kg^{-1} (Escoula, 1992). Wheat straw has also been reported to contain levels of patulin of 75 and 40 mg kg^{-1} (Norstadt and McCalla, 1969).

It is likely that patulin is produced wherever patulin-producing fungi are found. As has been mentioned, the list of potentially contaminated products is a long one including apples and apple products (juice, juice concentrate, jam, compote, confectionery and cider). Furthermore, patulin has occasionally been found in silage, cereals, flour, bread, cakes and cheese and in other fruits such as apricots, pears, peaches, plums, grapes as well as there products (Harwig *et al.*, 1973 a; Buchanan *et al.*, 1974; Scott *et al.*, 1977; Harrison, 1989); patulin has also been identified in surplus cheese (Tsai *et al.*, 1988). Indigenous fungi grown on nuts including almonds, peanuts, hazelnuts and pistachio nuts and sunflower seeds excreted patulin to their substrates (Jimenez *et al.*, 1991). Dried foods, mainly beans and macaroni, are also favourable substrates for patulin production (Trucksess *et al.*, 1987). Moreover, a detectable amount of patulin has been found in cereals including maize (Leming *et al.*, 1993) and grain meals (Papa *et al.*, 1990 and 1992). In contrast, although patulin producing fungi were present, the toxin has not been identified in fig, apricot, plum and raisin (Zohri and Abdel-Gawad, 1993).

Finally, soil samples were found to contain patulin at a concentration of 1.5 mg kg^{-1} (Norstadt and McCalla, 1969).

1. 6. The biosynthesis of patulin

It is well known that the polyketide pathway proceeds from acetyl Co A, which is the key branch point for catabolic and anabolic reactions of primary metabolism including fatty acids, as well as being the mainstay for secondary metabolites production (Bassett and Tanenbaum, 1960; Packter, 1973). While polyketides are derived from a polyketide chain consisting of acetate unit, the polyacetate pathway is an acetate polymalonate pathway where enzyme-bound acetyl CoA is condensed with malonyl CoA, and loss of CO_2 (Bennett, 1981; Turner and Aldridge, 1983) (Fig. 1. 1). Murphy and Lynen (1975) observed, during studies on cultures of *Penicillium patulum*, that the biosynthesis of patulin goes from *m*-hydroxybenzyl alcohol through gentisyl alcohol forming gentisaldehyde, ending in patulin. Bassett and Tanenbaum (1958) showed that the production of gentisaldehyde is a connecting link for patulin production. In 1960, they also reported the transformation of labeled 6-methyl-salicylate from 2-^{14}C-acetate into patulin. Bu`Lock and Ryan (1958) independently noted excreted radioactive 6-methylsalicylate during enzymatic rearrangement of acetate-1-^{14}C.

The excretion of 6-methylsalicylic acid by *Penicillium patulum* has been reported to occur only after aerial mycelium has begun forming, although its biosynthesis has not been confirmed (Bu`Lock *et al.*, 1969; Grootwassink and Gaucher, 1980; Peace *et al.*, 1981). Studies on intermediate substances in cultures of *Penicillium urticae* Bainier (Birkinshaw *et al.*, 1943) have led to the identification of 16 metabolites, involved in patulin biosynthesis from 6-methylsalicylic acid, including *m*-hydroxybenzyl alcohol, *m*-cresol, m-hydroxybenzaldehyde, *m*-hydroxybenzoic acid, and toluquinone (Bu`Lock *et al.*, 1965 and 1969). Scott and Yalpini (1967) proposed a major route for patulin biosynthesis by *P. urticae* via *m*-cresol, toluquinol, and gentisyl alcohol. They pointed out that *P. urticae* could convert deuterium-labeled m-cresol into toluquinol, gentisyl alcohol, gentisaldehyde, and patulin.

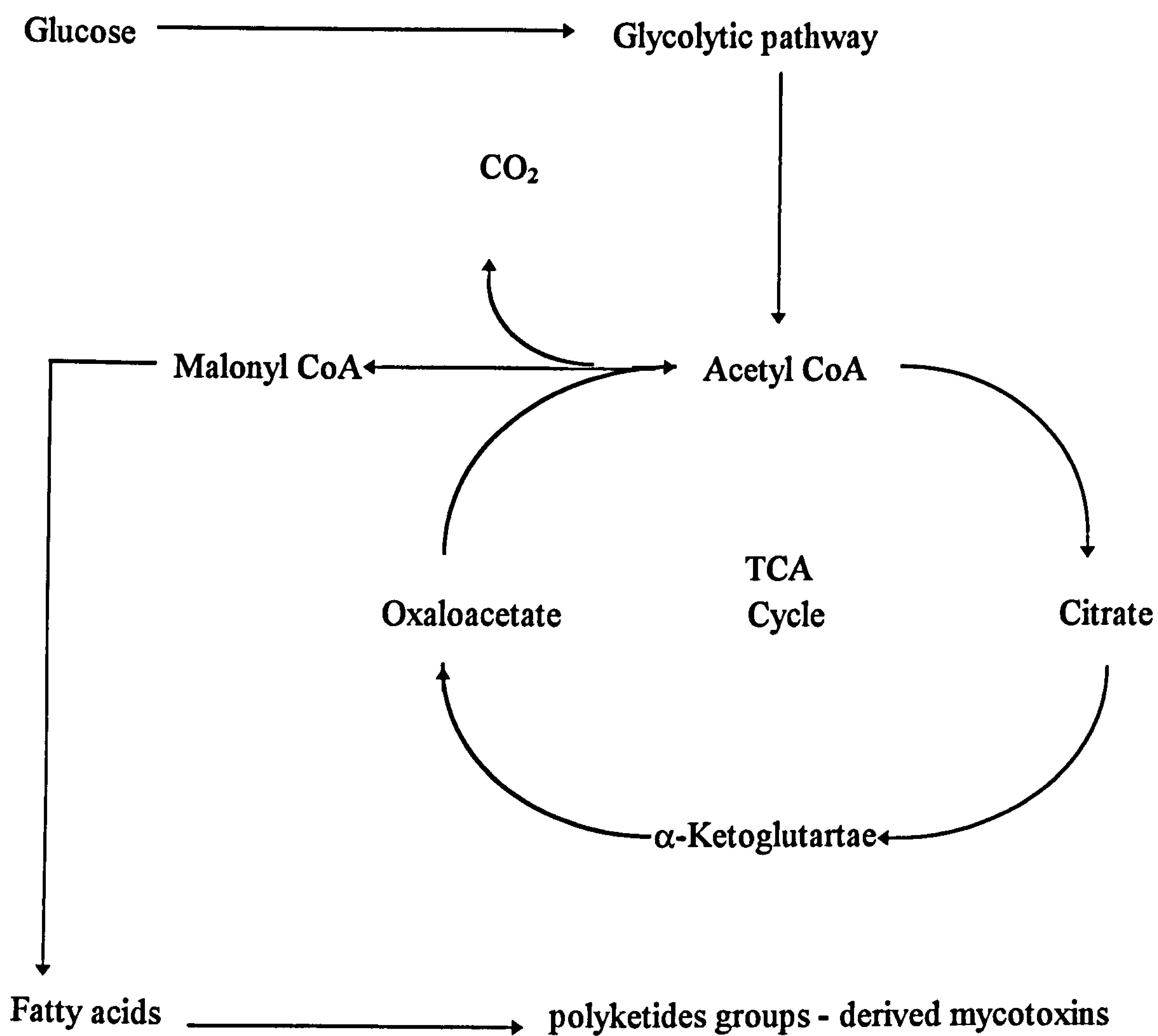


Figure 1. 1. A simplified diagram of the relationship between primary and secondary metabolites biosynthesis.

Forrester and Gaucher (1972) divided *P. urticae* secondary metabolites into three groups. Firstly, they isolated 6-methylsalicylic acid, *m*-cresol, *m*-hydroxybenzyl alcohol, gentisyl alcohol and patulin in sufficient amounts to allow their identification by comparison of their physical properties to those of authentic samples. Secondly, they identified toluquinol and *m*-hydroxybenzoic acid solely by their chromatographic characteristics. Thirdly, production in small amounts of the aldehydes, *m*-hydroxybenzaldehyde and gentisaldehyde was reported. Recently the six steps from 3-hydroxyanthranilic acid to the epoxyquinol LL-C10037-alpha, 1, produced by *Streptomyces* LL-C10037 have been determined by whole-cell labeling with deuterated substrates as well as using cell-free extracts. Gould *et al.* (1989) reported the conversion of 3-hydroxyanthranilic acid, 2 to 2-hydroxyaniline,11 after decarboxylation, and then oxidization to 2,5-dihydroxyaniline,8. In the presence of O_2 and either NaDH or NADPH the epoxyquinone 16 results from epoxidize 4. The steps beyond 3-hydroxyanthranilic acid and its relationship to production of 6-methylsalicylic acid have been listed by Gould *et al.* (1989). On the other hand, a study in the late fermented cultures of *P. patulum* indicated that epoxidation of toluquinol and gentisyl alcohol produce quinone epoxide phyllostine, by microsomal enzyme(s) and retention of one of the carbinol protons (Iijima *et al.*, 1986), which is an intermediate products in patulin biosynthesis (Priest and Light, 1989) (Fig. 1. 2).

1. 7. Physical and chemical properties of patulin

Patulin is Q,B-unsaturated lactone metabolite (Steiman *et al.* 1989). Its chemical name is 4- hydroxy-4*H*-furo[3,2-*c*] pyran-2 (6*H*)-one and it has the empirical formula $C_7H_6O_4$ and a molecular weight of 154. Patulin forms colourless crystals and has a melting point (m.p.) of 109 to 110.5°C (Lindroth and Wright, 1990). The structure of patulin was elucidated by Birkinshaw *et al.*,(1943). On treatment with hot dilute acid patulin hydrolyses to tetrahdro-γ-pyrone-2-carboxylic acid and formic acid. Patulin is a

Malonyl-CoA

$3CO_2$

3CoASH

Triketide chain

Acetylphloroglucinol

Orsellinic acid

4,6-Dihydroxy-2,3-dimethylbenzoic acid

6-Methylsalicylic acid

Orcinol

3,5-Dihydroxy-1,2-dimethylbenzene

Flavipin

m-Cresol

Amudol

Aurantiogliocladin

m-Hydroxybenzyl alcohol

Gentisaldehyde

Patulin

Figure. 1. 2. Biosynthesis of patulin. (from Garraway and Evans, 1984).

pyrone derivative. Patulin loses its biological activity under alkali conditions (Atkinson, 1942; Chain *et al.*, 1942), but it is stable under acidic conditions (Chain *et al.*, 1942). Furthermore, patulin is soluble in water, acetone, ethanol, diethyl ether, ethyl acetate and chloroform, but insoluble in benzene and petroleum. The ultraviolet absorption spectrum was reported at 275 nm and 276 nm (log $\varepsilon = 4.22$) in ethanol and 278 nm on TLC silica gel plates described the IR spectra of patulin in potassium bromide.

The structure of patulin was elucidated by Woodward and Singh (1949, 1950) (Fig. 1. 3).

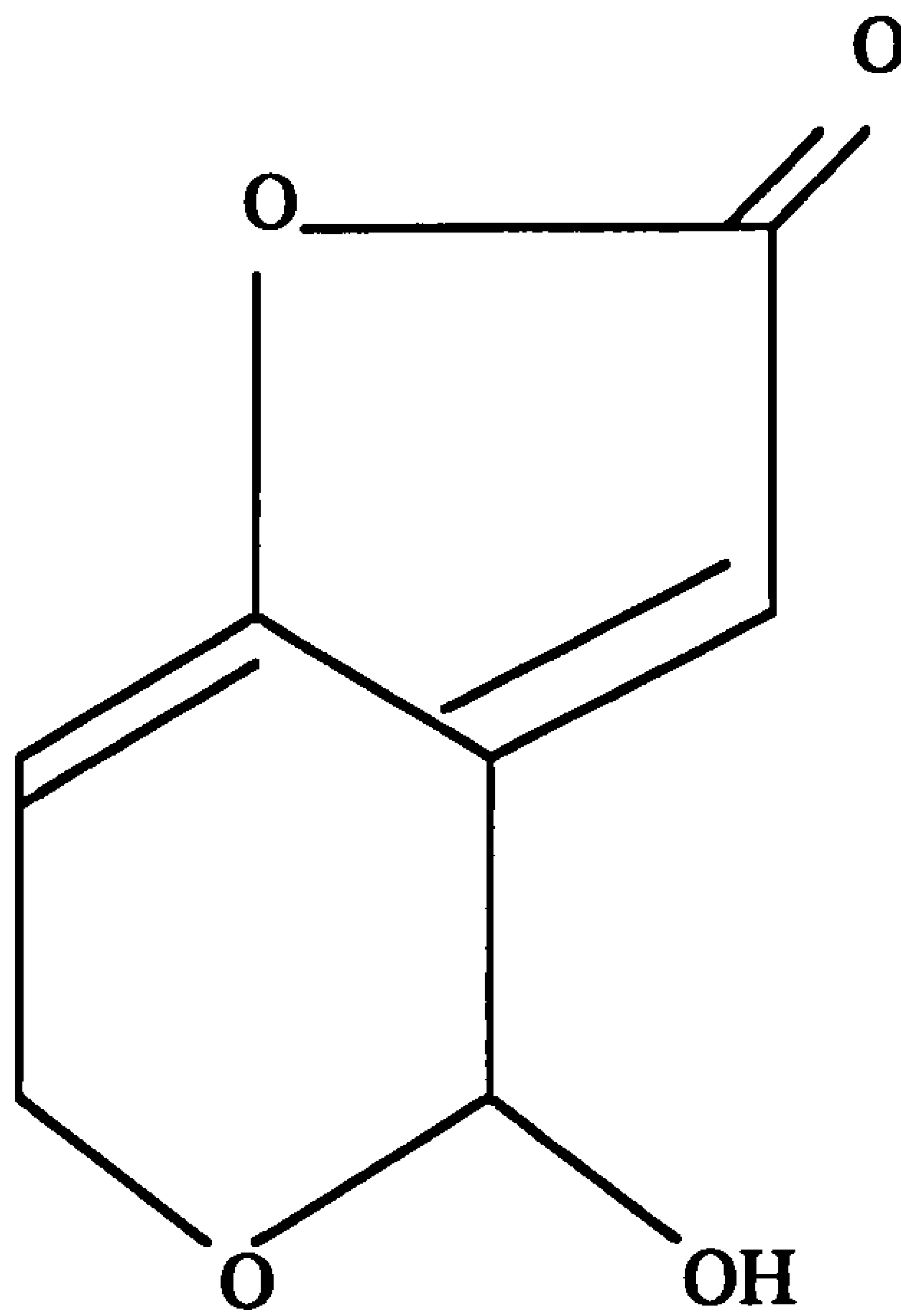


Figure 1. 3. The structure of patulin.

1. 8. Toxicity of patulin

Although patulin was first isolated in the early 1940's as an antibiotic (Chain *et al.*, 1942), it was classified as a mycotoxin due to its toxic activity on a wide range of plants, animals and cells (Ciegler *et al.*, 1971).

1. 8. 1. Effect of patulin on bacteria

Patulin has been reported as a generalised antibacterial toxin inhibiting the growth of more than 75 species of Gram-negative and Gram-positive bacteria (Nauta *et al.*, 1945; Ciegler *et al.*, 1971) including *Mycobacterium tuberculosis* (Betina, 1984). Of all the tested bacteria, none showed complete resistance to the effects of patulin. It acts not only as bacteriostatic agent against gram-negative bacteria, but also possesses marked bactericidal properties. Furthermore, it has been noticed that most bacterial species are inhibited by patulin at a concentration of less than 20 μg ml^{-1}, although some required toxic concentrations of as much as 500 μg patulin ml^{-1} (Singh, 1967). DeRosnay *et al.* (1952) reported that 6 - 10 μg patulin ml^{-1} inhibited the growth of *Escherichia coli*, while Borecka *et al.* (1984) reported the sensitivity of *Agrobacterium tumefaciens* to this mycotoxin as did Klemmer *et al.* (1955). On the other hand, Oosterhuis (1945/1947) observed resistance of some gram-negative bacteria to levels of less than 20 μg patulin ml^{-1}.

The ability of patulin to inhibit the growth of gram-positive bacteria has been reported by several workers. Kavanagh (1947) recorded that a concentration of 4 μg patulin ml^{-1} inhibited the growth of *Bacillus subtilis.* Lembke and Frahm (1947) and Lembke *et al.* (1950) reported growth inhibition of both *Bacillus subtilis* and *Micrococcus pyogenes* by patulin levels ranging from 12.5 to 100 μg ml^{-1}. Similarly, Wiesner (1942), reported that *Staphylococcus aureus* and some other organisms that are not attacked by penicillin were killed by patulin, Kavanagh (1947) recorded the inhibition of this bacterium by 8 μg patulin ml^{-1} and 12.5 to 30 μg ml^{-1} (Raistrick *et al.*, 1943). Lee and Roeschenthaler (1986) reported that *Bacillus brevis* appeared to be sensitive for patulin, where a concentration of 1 μg per disc caused zones of inhibition. *Actinomyces scabies* was inhibited by patulin at a concentration as low as 6.25 μg ml^{-1} (Gillivein, 1946).

Patulin was reported to inhibit sulfhydryl enzymes, a process which is reversed by thiols. Furthermore, it is believed that antibacterial action depends upon some reaction, perhaps addition across the reactive double bond, with sulfhydryl groups, although patulin does not inhibit urease, or sulfhydryl enzymes (Hoffmann-Ostenhof and Lee, 1946).

The effect of patulin on bacteria could result from mutation due to its effect on DNA, where a concentration of 10 μg ml^{-1} of patulin can cause breakage of single-stranded DNA in *Escherichia coli* (Lee and Roeschenthaler, 1986). This toxin completely inhibits oxygen uptake in *Staphylococcus* and *Bact. coli* in 10 minutes, even at a dilution of 1 in 1,000. Furthermore, it inhibits enzyme respiratory systems such as glucose dehydrogenase, succinoxidase, maltic acid dehydrogenase, glycerophosphate dehydrogenase, and tryptophanase (Waksman, 1947). On the other hand, patulin induces a weak response in genotoxic activity agent of SOS (increased repair and mutagenic activities) in *Escherichia coli* K 12 (Auffray and Boutibonnes, 1987).

1. 8. 2. Effects of patulin on fungi

While a wide range of bacteria are highly sensitive to patulin, not all fungal species react in this way sensitive. Those that are slightly affected include *Aspergillus niger*, *A. oryzae*, *Fusarium culmorum*, some species of *Rhizopus* and *Trichoderma* (Geiger and conn, 1945). A concentration of 1 mg patulin ml^{-1} did not affect the growth of *A. clavatus* (Katzman *et al.*, 1944). The first investigation on the antagonism of patulin to fungi was undertaken by Van Luijk (1938), who demonstrated that a culture fluid of *Penicillium expansum* diluted 1,280 times exerted a strongly antagonistic action on various species of *Pythium* and *Trichophyton rosaceum* Sab. In 1943, Anslow *et al.* pointed out that patulin completely inhibited the growth of *Pythium ultimum*, *P. aphanidermatum*, *P. mammilatum* and *Pythium* sp. in dilution's of 1:400,000 to 1:1,000,000, while *P. de-Baryanum* was inhibited at from 1 in 300,000 to 1 in 500,000.

Van Luijk (1938) showed that the culture filtrate of *Penicillium expansum* completely inhibited the growth of *Pythium debaryanum* Hesse at a dilution of 1 in 1,280, and it was antagonistic to certain species of *Fusarium*. Waksman and Bugie (1943) stated that patulin showed some limited fungistatic action and was fungicidal to *Ceratostomella ulmi*; while Anslow *et al.* (1943) and Gilliver (1946) reported that patulin inhibited the growth of *Gloeosporium musarum*, *Penicillium digitatum*, *Rizoctonia crocorum*, *Sclerotinia sclerotiorum* and *Verticillium dahliae* Patulin inhibited fungi as follows: at dilutions of 1 in 5,000, and 1 in 20,000 for *Rhizoctonia solani* and *Stereum purpureum*, 1 in 80,000 for *Claviceps purpurea*, 1 in 320,000 for *Phytophthora erythrosepitca* and 1 in 800 for *Pythium ultimum*. It also inhibits the growth of *Rhizopus nigricans*, *Monilia albicans*, *Saccharomyces cerevisiae*, and *Sporotrichium schenkii* (Katzman *et al.*, 1944). Growth of *Monilia albicans* and *Oidium asteroides* was inhibited at patulin dilutions ranging from 1 in 5,000 and 1 in 10,000 to 1 in 100,000 respectively. At the latter dilution of patulin the growth of *Trichophyton gypseum* was totally inhibited (Herrick, 1945). On the other hand, a specie of *Ustilago tritici* was inhibited by patulin at the higher dilution of patulin (1 in 100,000). Timonin (1946) found that the concentration 62.5 $\mu g\ ml^{-1}$ inhibited the growth of *Penicillium nigricans* and 10 $\mu g\ ml^{-1}$ inhibited *Ustilago tritici* (Wright, 1955). Reiss (1973) reported growth reduction and inhibition of spore germination and formation in both *Rhizopus nigricans* and *Thamnidium elegans*. Moreover, patulin inhibited the growth of more than 24 isolates of the genera *Allomyces*, *Achyla*, *Dicthyuchus*, *Pythium* and *Phytophthora* (Burghardt *et al.*, 1992).

Candida albicans is inhibited by a patulin concentration of 300 $\mu g\ ml^{-1}$ (Reilly *et al.*, 1945). Non inactivation for CO_2 production was seen, even at concentrations up to 100 $\mu g\ ml^{-1}$ (Reiss, 1973).

1. 8. 3. Effect of patulin on viruses

Jones (1945) and Hall *et al.* (1951) pointed out that patulin in high concentrations was able to inactivate *Escherichia coli* phages and more than 11 other bacterial phages. On the other hand, it did not inactivate *Pseudomonas pyocyanea* phage at low concentration (Dickinson, 1948), nor influenza virus when inoculated in mice (Rubin and Giarman, 1947).

1. 8. 4. Effect of patulin on cells and tissue cultures

The effect of patulin on the growth of tissue cultures was evaluated by Burghardt *et al.* (1992), and Vollmar (1947). The last named author found that low concentrations of patulin (20 - 40 μg ml^{-1}) stimulated mouse leukocytes and normal epithelium cultures from rabbit cornea but inhibited them at concentrations ranging from 100 - 200 μg ml^{-1}. Patulin was able to reduce the multiplication of mouse fibroblasts in suspension cultures to 50% (Perlman *et al.*, 1959), and also to inhibit aerobic respiration of cells in which the following enzymes were active: co-carboxylase, dehydrogenase, succinate oxidase and succinate dehydrogenase. Patulin may also inhibit cellular membrane permeability leading to leakage of vital metabolites from out of the cell, or else inhibit nutrient transport into the cell, e.g. the transport of K^+ ions in erythrocytes and glucose in fungal mycelium (Kahn, 1957; Singh, 1967). Recent studies have demonstrated that protein prenylation is also inhibited by patulin (Miura, *et al.* 1993).

Patulin can have a toxic effect on the bacterial chromosomes leading to the formation of giant forms (Babudieri, 1948), binucleate cells (Wang, 1948; Keilova-Rodova, 1949) and fragmentation (Sentein, 1955). Furthermore, Steineger and Leupi (1956) reported deleterious effects of patulin on the nucleus and chromosomes of *Allium cepa* and *Lepidium sativum.*

1. 8. 5. Effect of patulin on protozoa

Patulin has been found to be toxic to several species of protozoa. While a level of 200 µg ml^{-1} was found to kill *Glaucoma pyriformis* within an hour, *Strigomonas* needed a larger amount, and longer length of exposure time for complete killing. On the other hand, *Glaucoma pyriformis* was more resistant to a concentration of 2 µg patulin ml^{-1} and required 24 hours to be completely killed (Jirovec, 1949).

1. 8. 6. Effect of patulin on human and animals

In humans, the toxic antibiotic, patulin, produces nausea and stomach irritation (Freerksen and Bonicke, 1951) and also causes edema when applied to the skin in the form of a 1% ointment (Abraham and Florey, 1949; Anonymous, 1974).

In animals, patulin is known as a carcinogenic, sarcomagenic (Dickens and Jones, 1961; Orth, 1973; Ciegler, 1977; Palmgren and Ciegler, 1983), mutagenic (Mayer and Legator, 1969), tetratogenic (Ciegler *et al.*, 1976) and a mitotic poison (Dustin, 1963).

1. 8. 7. Effect of patulin on higher plants

In vivo studies have shown that patulin causes decreased growth and abnormal morphology in crops when taken up from stubble-mulch (Barnum, 1924; Bustinza and Lopez, 1949; McCalla *et al.*, 1963; Norstadt and McCalla, 1963). In laboratory studies, patulin was shown to reduce seed germination, (Timonin, 1946; Wallen and Skolko, 1951; Gattani, 1957; Borecka *et al.*, 1984), and cause plant wilting, stunting, and leaf necrosis (Gaumann and Jaag, 1947; Iyengar and Starkey, 1953; Nickell and Finlay, 1954; Klemmer *et al.*, 1955). Patulin has been found to inhibit the germination of *Pseudoperonospora cubensis* (Ark and Thompson, 1957) and *Elodea canadensis* plasma streaming (Gaumann *et al.*, 1947). It also releases scopoletin from oat roots

(Martin, 1958) prevents *Spirogyra* plasmolysis and arrests the motility of *Chlamydomonas* (Gaumann and v.Arx, 1947; Meyer *et al.*, 1952).

1. 9. Factors which influence patulin production

1. 9. 1. Physical factors

1. 9. 1. i. Moisture

One of the most important factors affecting patulin production is the moisture content of the substrate. For instance, *P. urticae* Bainier consistently produces detectable amounts of patulin on wheat, barley, oats and maize grains, but the highest amounts obtained from grains were where these were stored in the humidity range between 15 to 30% (Obrazhei and Pogrebnyak, 1984).

1. 9. 1. ii. Temperature

It is well known that many common fungi can grow over the temperature range from slightly below 0°C to as high as 60°C (Hesseltine, 1968). Escoula (1992) reported that *P. granulatum* was able to produced patulin on liquid Czapek medium over a temperature range from 20°C to 32°C. At medium temperature (26°C), patulin production was maximal, while at lower or higher temperature (20°C and 32°C) less patulin production was detected. Generally, most moulds grow and produce patulin in the temperature range from 20 to 30°C. Thus, Sanchis *et al.* (1992) recorded patulin production in large amounts by *P. griseofulvum* at 20 to 30°C. Furthermore, patulin production may be stimulated by the imposition of a variable temperature regime. For example, Criseo *et al.* (1991) observed higher rates of patulin production by *P. expansum* in the pre-incubated cultures than in constant 4° and 24°C incubated

cultures. Bullerman, (1985), during studies on the production of patulin by *P. patulum* at different temperatures (5, 12 and 25°C), reported that best growth and patulin production occurred at 25°C. On the other hand, Podgorska (1993) reported that *P. expansum* strains grown on Czapek-Dox liquid medium excreted maximum amounts of patulin at 25°C, although mycotoxin production was also observed at 5°C. *Penicillium urticae* Bainier produced patulin at a variety of temperatures (10°, 20°, 25°, and 30°C). The level of toxin formed increased at 20° and 25°C, and decreased at 10° and 30°C (Obrazhei and Pogrebnyak, 1984).

1. 9. 1. iii. pH

Patulin production in media can be detected at a pH of less than 6 (Sanchis *et al.*, 1992). Furthermore, pH values ranging from 2 to 4 are sufficient to yield high concentrations of patulin, either on natural or on artificial substrates. For example, *P. patulum* was found to produce the greatest amount of patulin at 25°C at pH values of 3.5, 5.0 and 6.5 (Bullerman, 1985). On the other hand, Sanchis *et al.* (1992) detected the production of large quantities of patulin by *P. griseofulvum* at a pH value near 3.5. *Penicillium expansum* strains excreted patulin into the medium after 12 days at pH 3 and pH 6 (Podgorska, 1993). Patulin production by strains of *P. griseofulvum* and *P. expansum* was found to vary over the pH range pH 5.5 to near neutrality (Sanchis *et al.*, 1991). Escoula (1992) reported maximum patulin production by *P. granulatum* when the pH of the liquid Czapek medium was about 4.

1. 9. 2. Biological factors

1. 9. 2. i. Fungal strain

While a wide range of moulds, representing several genera of different species, have been reported to produce patulin only a few strains within each specie have the ability to produce the toxin. Within the genus *Penicillium*, some groups of species such as *P. expansum* readily produce patulin on both artificial and natural substrates. Vinas-Almenar *et al.* (1993) found that out of 122 *P. expansum* strains isolated from different sources (environmental contamination, boxes and decayed apples) only 10 were able to produce patulin on media and on artificially inoculated apples. The results in liquid media were even less impressive, with only 4 strains producing patulin in the minimal medium and 1 strain in yeast extract sucrose (YES) medium.

1. 9. 2. ii. Patulin production in relation to interaction with other microorganisms

Microbial interactions extensively influences the amount of patulin produced by a given patulin producing fungus (Hesseltine, 1968). Antagonists can influence sporulation (Sadoff, 1971) or produce some inhibitory metabolites, which can lead to disappearance, diminution or stimulation of patulin production in the medium (Harwig *et al.*, 1973 b). Furthermore, interaction may occur in the competition between microorganisms for nutrients, especially during storage (Cuero *et al.*, 1987; Nout, 1989). For instance, yeasts have been found to prevent more than 90% of patulin production by patulin producing fungi (Burroughs, 1977; Stinson *et al.* (1978).

1. 9. 3. Nutritional factors influencing patulin production

It is well known that secondary metabolites are produced in the phase where nutrients uptake and utilization is in the maximal rate. This phase is termed the idiophase (Bu`Lock, 1965; Nover and Luckner, 1974). Thus, the onset of growth limitation and secondary metabolites production could be obscured by cultural

conditions. Pytel and Borecka (1982), during studies on the effect of medium conditions on patulin production, found that all the fourteen *P. expansum* isolates obtained from apples, pears and the air in Poland were able to produce from 268 to 2225 μg patulin ml^{-1} into the liquid medium. The productivity of these isolates depended on the source of carbon in the medium, with less patulin being produced when the fungus was grown on apple tissue in comparison to Czapek liquid medium. Furthermore, Podgorska, (1993) recorded increasing amounts of produced patulin by *P. expansum* strains when fructose rather than sucrose was used as the carbon source. Grootwassink and Gaucher (1980) pointed out that glucose-nitrate or glucose-yeast extract medium are both required for maximal patulin production. They noticed that it was the depletion of nitrogen which actually triggers patulin production. Other nutrient factors such as oxygen (Grootwassink and Gaucher, 1980; Damoglou *et al.*, 1985), ammonium ions (Rollins and Gaucher, 1994) and iron concentrations also affect patulin production. Similarly, *Penicillium urticae* in a low-zinc medium forms predominantly 6-methylsalicylic acid, but at higher concentrations of zinc this compound is replaced by a mixture of gentisyl alcohol, patulin, and toluquinone (Cochrane, 1958).

1. 9. 4. Effect of incubation period on patulin production

Penicillium expansum produced largest amounts of patulin when grown in Czapek Dox agar after 12 days of incubation (Podgorska, 1993). Escoula (1992) reported that the maximum patulin production by *P. granulatum* on liquid Czapek medium (plus glucose), was obtained during the 16th day of culture at 26°C (2.9 g l^{-1}), after 24 days at 32°C and after 32 days at 20°C.

1. 9. 5. Cytological factors influencing patulin production

The main compounds affecting patulin production are those containing sulfhydryl (SH) groups including glutathione, cysteine, thioglycollate and dimercaptopropanol. These group of compounds react with patulin forming adducts (Ciegler *et al.*, 1976) diminishing its toxicity to bacteria and animals (Singh, 1967) or interact with patulin preventing its mitostatic and general toxic effects (Geiger and Conn, 1945; Rondanelli *et al.*, 1957). In higher plants, Afridi (1962) reported augmentation of the inhibitory effect of patulin on the formation of nitrate reductase due to presence of glutathione, cysteine and tryptophan.

Patulin toxicity was, on the other hand, found to be diminished by peptone, glycine, methionine, asparagine, p-aminobenzoic acid (Waksman and Bugie, 1943; DeRosnay *et al.*, 1952), sodium thiosulphate, sodium sulphate, sodium pyrosulphate (Miescher, 1950). However, some other compounds such as tryptophan, urea and thiourea are able to increase patulin toxicity (DeRosnay *et al.*, 1952).

1. 9. 6. Some properties of patulin

Patulin is hydrolysed at pH of >6 and its degradation increases at high pH (Brackett and Marth, 1979). Timonin (1946) reported that a dilute patulin solution lost its antibiotic activity in the presence of whole wheat flour or vitamin B_1. Jefferys (1952) found patulin to be stable for several weeks, as an antibiotic, over the pH range 3.3 to 6.3, but that it was slowly inactivated at 6.8. It retains its activity after 15 minutes at 100°C and pH 2 (Jefferys *et al.*, 1953). Patulin does not survive this treatment at pH 9.5 (Heatley and Philpot, 1947; Wheeler *et al.*, 1987).

2. PRODUCTION OF PATULIN IN APPLES

2. PRODUCTION OF PATULIN IN APPLES

2. 1. Introduction

Patulin is produced by a range of filamentous fungi growing on a wide range of substrates. These may be waste residues, as in the case of straw, or food products such as fruits and fruit juices.

Culture conditions under which the patulin-producing fungus grows are critical in relation to maximal patulin production. Such factors include the nature of the isolate (Pytel and Borecka, 1984), temperature, incubation time, inoculum potential, fungal species or strains (Hesseltine, 1976). The growth of patulin-producing isolates and the rate of production of patulin on natural substrates have been reported to be generally much less than that seen under laboratory conditions (Pytel and Borecka, 1982). Interestingly, the original substrate from which the producing mould was isolated, appears to be more suitable for the production of patulin than other natural substrates (Tsai *et al.*, 1988). The natural microflora of a food or beverages consists of two components, the first is associated with the raw materials used and the second is acquired during processing, some of which survive preservation and storage conditions. The occurrence of patulin in apples and apple products has been widely investigated throughout the world. The natural occurrence of patulin has been particularly associated with *Penicillium expansum*, as well as some of other fungal species (Brian *et al.*, 1956). Scott *et al.* (1972) was the first reported the presence of patulin in apple juice and showed that it was produced by *Penicillium expansum.* Isolates of *Penicillium griseofulvum* were also found to produce patulin naturally in apple juice (Torres *et al.*, 1987). The level of patulin found in apple juice from a variety of countries was published by Wilson (1981). He found that between 1972 and 1980 the minimum value of patulin was 5 $\mu g\ l^{-1}$ and the maximum was 1000 $\mu g\ l^{-1}$. Patulin has mainly been found in apples and apple products including juice, juice concentrate, jam,

compote, confectionery and cider (Harrison, 1989). Some fifty per cent of apple, pear, and mixed fruit products including sauces, purees, jellies, diced apples and apple pulps were also found to be contaminated with patulin, with concentrations ranging from 5 to 1130 $\mu g\ l^{-1}$ (Burda, 1992). A recent survey of patulin present in apple juice collected at stores indicated that 82% of samples contained patulin at a variety of concentrations (Prieta *et al.*, 1994). Mortimer *et al.* (1985) found that 41.7% of retail samples of apple juice were contaminated with patulin at a level ranged from 5 to 56 $\mu g\ l^{-1}$.

The patulin content of apple juice is therefore a good indicator of the quality of the apples used in the process (Forbito and Babskey, 1985) as well as in the processing leading to the end-product food and drink (Prieta *et al.*, 1994).

The aim of the work described in this chapter was to:

i) obtain data on the occurrence of fungi on fruits;

ii) obtain data on the occurrence of bacteria on fruits;

iii) obtain data on the occurrence of patulin producing fungi and levels of the toxin on fruits;

iv) evaluate the potential toxicity of patulin to bacteria, an approach which might be used as a biological indicator of patulin production..

2. 2. Materials and Methods

2. 2. 1. Occurrence of fungi in fruits

2. 2. 1. i. Procurement of fruits

Mouldy fruits were collected from local retail outlets. Fruits examined included: apples, grape-fruits, lemons, oranges and pears.

2. 2. 1. ii. Mould isolation

Indigenous fungi were isolated from all varieties of the mouldy fruits. Either skin (2x 2 mm) or heavily sporulating colonies were picked off and maintained on Czapek Dox agar (Oxoid) and incubated at 25°C for 7 to 10 days (Plate 2. 1). Mycelial growth was taken from the leading edge of the isolate and subcultured on Czapek Dox agar (Oxoid) until a pure culture was obtained. Subcultures of all isolates were then stored at 4°C. The fungi were identified with the aid of various keys including those published by Raper and Thom (1968); Barnett and Hunter (1972); and Ellis (1971 and 1976).

Penicillium expansum Link was obtained from the CAB, International Mycological Institute, Surrey.

A heavy loopful of spores of each isolate grown on Czapek Dox agar (Oxoid) Petri dishes were spread in triplicate on 10 ml slopes of Czapek Dox agar (Oxoid) in Universal tubes. The stock cultures were incubated at 25°C for 7 days then stored in the fridge at 4°C.

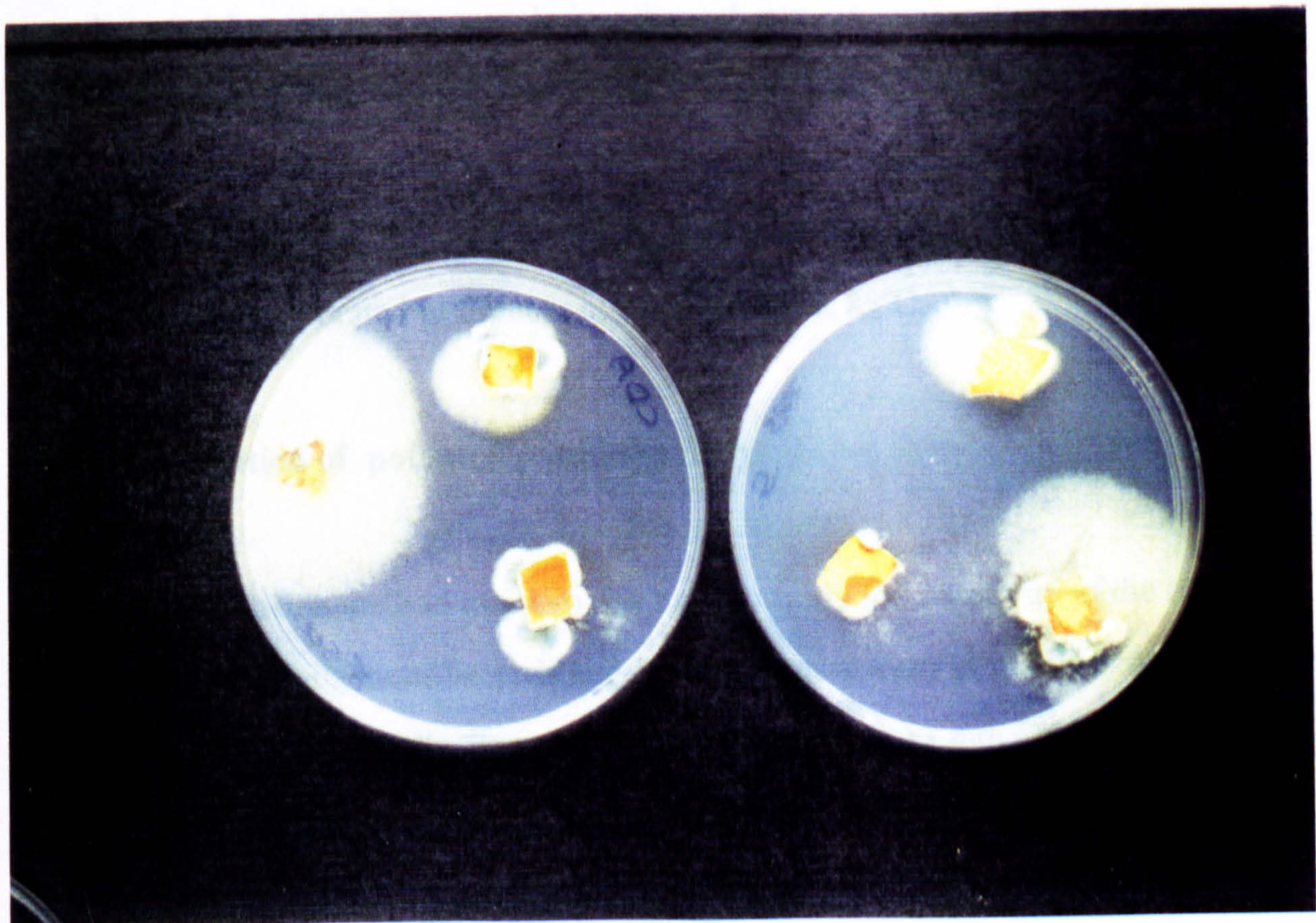
2. 2. 2. Isolation of bacteria from fruits

Indigenous bacteria were isolated from fruits, and their numbers determined by serial dilution. Skin (0.5 g) was removed and transferred into 20 ml 1/4 strength Ringer's solution in 250 ml bottles and shaken at 250 rpm, 25°C for 30 minutes. The suspension was then serially diluted using 9 ml of 1/4 strength Ringer's. Serial dilutions were made to 10^{-3} for the control suspensions and to 10^{-4} for the mouldy- fruit suspensions. Each dilution (0.1 ml) was then spread onto plate count agar. The plates were incubated at 37°C overnight, and the colonies which developed were then counted.

Each obviously different bacterial colony type was picked off and subcultured by streaking onto Nutrient Agar (Oxoid). Plates were incubated at 37°C overnight. Pure cultures were obtained by subculturing and these were then transferred to 10 ml slopes of Nutrient Agar (Oxoid), incubated overnight at 37°C and then stored at 4°C.

Plate 2. 1

Isolation of indigenous apple fungi on Czapek Dox agar after 7 days of incubation at 25°C.



2. 2. 3. Natural occurrence of patulin on fruits

Fruit pulp was prepared by homogenizing mouldy fruits in a kitchen blender with 50 ml sterile distilled water. Portions (40 g) were centrifuged in sterile 30 ml Universal bottles at 4500 rpm for 10 mins. The supernatants were then filtered through sterile Whatman filter paper No.1. Each extract (2 x 1 ml) was applied to Nutrient agar (Oxoid) for subsequent growth and application to the patulin bioassay, the remainder was then saved at -18°C. The sample was thawed at room temperature prior to assay. The collected fruit pulp was adjusted to pH 2.0 by adding 6 N HCl, then extracted twice with equal volume of ethyl acetate. The extracts were combined via filtering through Whatman No. 2 filter paper containing 1 g of anhydrous sodium sulphate, then the filtrate was evaporated to dryness in a water bath at 85°C.

2. 2. 4. Evaluation of potential patulin toxicity

The agar diffusion method of assay was used to study the sensitivity of the isolated contaminant and indigenous bacteria and *Serracia marcescens* to mould extracts prepared as in 2. 2. 3. Wells were cut into the agar using a 13 mm diameter sterile cork borer. Nutrient agar (Oxoid) was prepared in flat-bottomed plates (Nunc bioassay). The slope on which each bacterial isolate was maintained was washed with 2 ml sterile distilled water which was transferred to 150 ml of Nutrient agar (Oxoid) at 50°C. This was mixed and poured into Nunc bioassay plates. Then 100 µl of each fruit extract under test were added to the wells. The plates were incubated at 37°C overnight and any resulting zones of inhibition were measured.

2. 2. 5. Ability of the isolated fungi to produce patulin

All of the mould isolates and *Penicillium expansum* Link maintained on slopes were plated onto Czapek Dox agar (Oxoid) and incubated for 7 days at 25°C. The following media were used to determine their ability to produce patulin:

2. 2. 5. i. a. Laboratory medium

The fungi were grown in Czapek Dox liquid medium (Oxoid), pH 6.8 for the patulin production studies. The medium was dispensed into Erlenmeyer flasks (100 ml in 250 ml flasks) and sterilized by autoclaving at 121°C for 20 minutes. On cooling, the medium was inoculated with a single mycelial disc (13 mm diameter) of each isolates cut from the leading edge of a 7 day culture grown on Czapek Dox agar (Oxoid). All flasks were set up in triplicate and incubated at 25°C on a reciprocal shaker (150 rpm) for 7 days.

2. 2. 5. i. b. Evaluation of the potential patulin toxicity produced of extracts from fungi grown in Czapek Dox and the patulin standard

The Czapek Dox liquid medium extracts and patulin standard solution (Patulin standard solution was prepared by dissolving pure crystalline patulin (Aldrich) in sterile distilled water at concentrations of 0, 0.5, 1, 10, 15, 20, 25, 30, 35, 40, 50 and 60 μg 100 μl^{-1}) were tested using the agar diffusion technique. The assays were applied using the isolated contaminant and indigenous bacteria and *Serracia marcescens* following the procedure stated in 2. 2. 4. At the end of the incubation period, the zones of inhibition were measured.

2. 2. 5. ii. Patulin production on apples

All the patulin producing strains on Czapek Dox liquid medium were tested for there ability to produce patulin when growing on sound apples fruit. Golden Delicious apples were wiped with alcohol and divided into three sets. A set of 48 fruits (six apples for each isolate, in duplicate) was spiked with spores applied from 7 days apple paste cultures using a sterilized glass rod. The other two sets were treated as controls, where one set was left unwounded and the second set was wounded, but not inoculated. All groups were placed in a propagator which was partially sterilized by washing and wiped with alcohol (Plate 2. 2 and 2. 3). The base of the propagators was covered with four layers of Kimwipe lab roll. The apples were watered with 50 ml sterile distilled water and incubated at 25°C for 7 days. At the end of the incubation period ,decayed parts of the apples were impressed into Nutrient Agar (Oxoid) for bacterial isolation and patulin bioassay (As stated in 2. 2. 4). The decayed apples tissues were assayed for the presence of patulin.

2. 2. 5. ii. a. Patulin extraction

At the end of the incubation period, the Czapek Dox liquid medium culture flasks, set up in triplicate, were removed and the contents were filtered through Whatman No. 1 filter paper. The decayed tissue from the apple fruit were homogenized in a kitchen blender. Portions of 40 g were centrifuged in 30 ml Universal at 4500 rpm for 10 minutes, then the extract was collected and measured. All apple extracts were adjusted to pH 2.0 by adding 6 N HCl, then extracted with two equal volume of ethyl acetate. The extracts were combined, dried on 1 g of anhydrous sodium sulphate and evaporated to dryness.

2. 2. 5. ii. b. Preparation of patulin standard solution

Patulin standard solution was prepared by dissolving pure crystalline patulin (Aldrich) in chloroform ($CHCl_3$) to produce a concentration of 100 μg ml^{-1}. (Appendix 9. 6. a) (Gimeno and Martins, 1983).

2. 2. 5. ii. c. Sample clean-up

The dried residue obtained from 2. 2. 3. and 2. 2. 5. ii. a. was re-dissolved in 20 ml of chloroform plus 0.5 ml of water and dried for 1 h over approximately 1 g of anhydrous sodium sulphate. Five mls of the chloroform solution were then introduced into a silica Sep-Pak cartridge (No.51900, Water, Milipore Corp.) previously conditioned with 5 ml chloroform. The cartridge was washed with 1 ml of chloroform, 1 ml of chloroform-ethyl acetate (8 + 2), and 1 ml of chloroform-ethyl acetate (5 + 5). All of these fractions were then discarded. Then 2 ml of chloroform-ethyl acetate (2 + 8) were passed through the cartridge. The eluent was collected, and the solution was evaporated . The dried residue was dissolved in 1 ml of benzene for thin layer chromatography (TLC) (Rovira *et al.*, 1993).

2. 2. 5. ii. d. Preparation and development of TLC plates

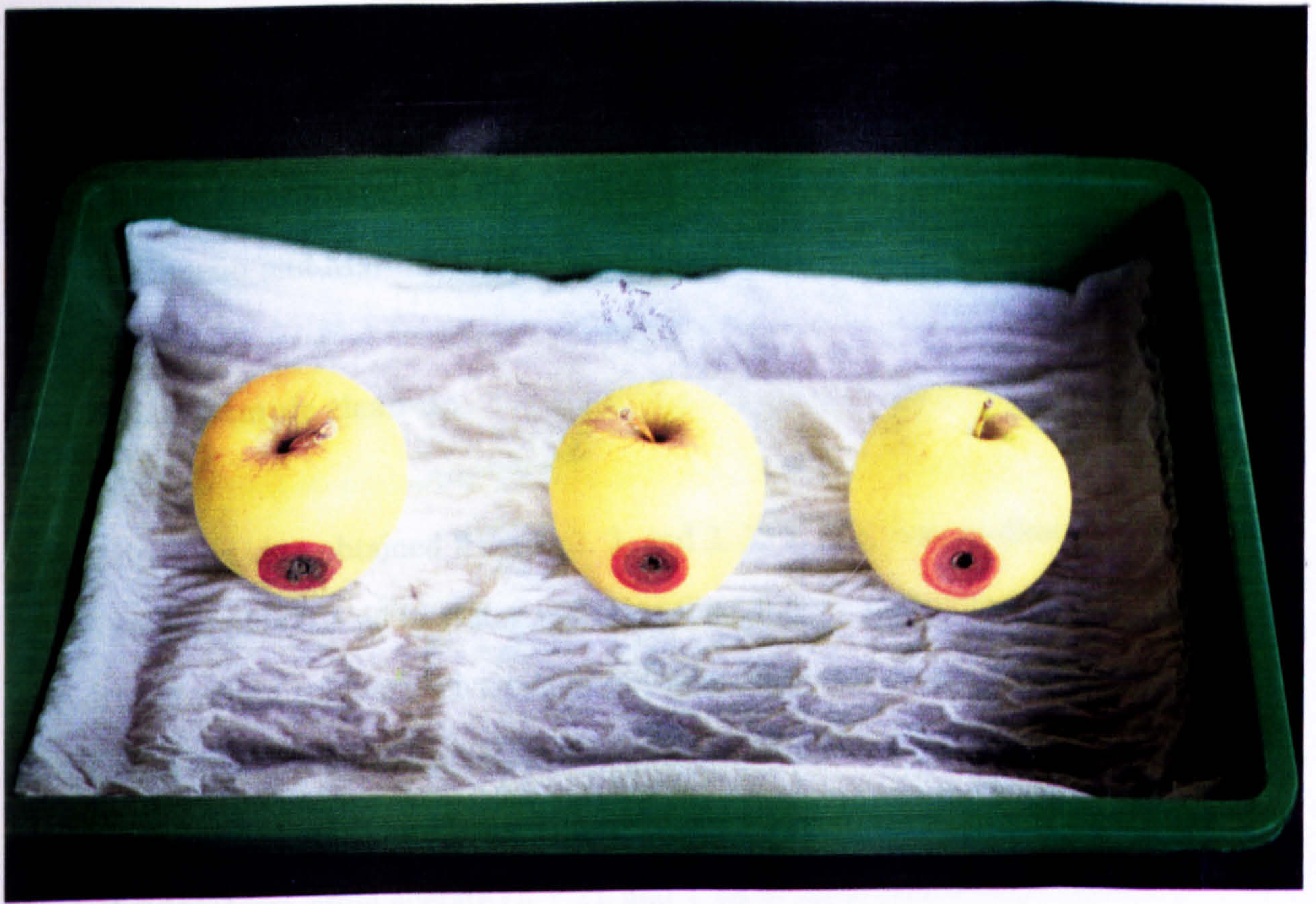
For both qualitative and quantitative studies on the extracts, E. Merck pre-coated silica gel, aluminium-backed Kieselgel 60 plates without fluorescence indicator (Cat. No. 5554) as supplied (20 x 20 cm), were used after activating by drying for 2 hr at 120°C. Plates were spotted along a line 3 cm from the bottom with two 10 μl aliquots of extracts and different concentrations of patulin standard (external standard). On one 10 μl sample extract, 5 μl of patulin standard were spotted as a reference standard solution (internal standard). The plates were developed in one dimension for about 90 min (15 cm distance) in benzene : methanol : acetic acid - BMA (90 : 5 : 5 v/v/v) equilibrated, in unlined Shandon TLC chromatanks, at room temperature until the

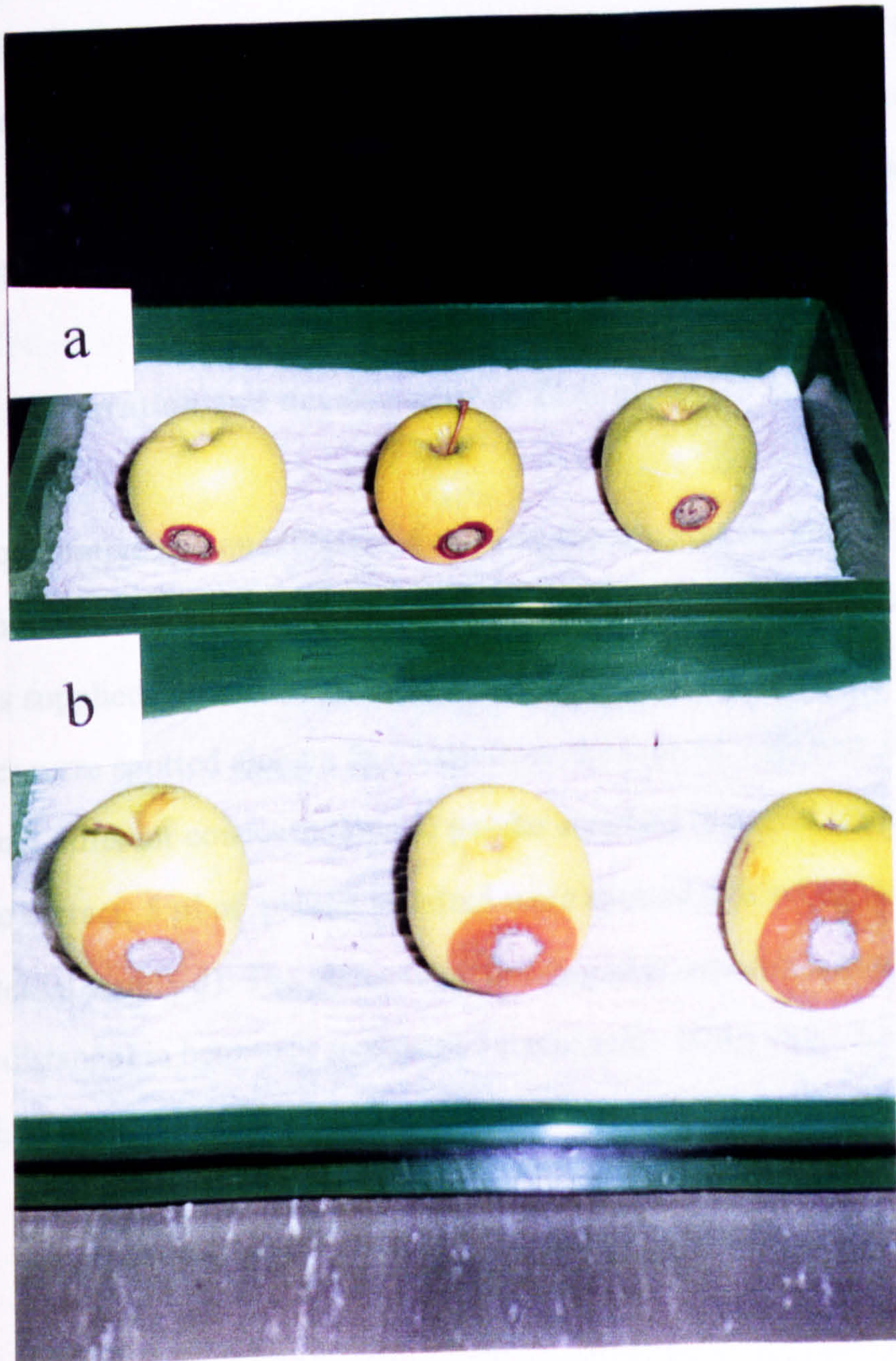
Plate 2. 2

Golden delicious apples, placed in a propagator, inoculated with the indigenous isolate *Penicillium* sp (1) after 7 days of incubation at 25°C.

Plate 2. 3

Golden delicious apples placed in a propagator; (a) inoculated with the indigenous isolate *Penicillium* sp (2); (b) inoculated with the indigenous isolate *Penicillium* sp (3) after 7 days of incubation at 25°C.




a
b

solvent front had breached a line marked 2 cm from the top of the plate. The plates were then removed and air-dried in a fume cabinet and sprayed with a freshly prepared mixture of 0.5 ml ρ-anisaldehyde (in 85 ml of methanol containing 10 ml of glacial acetic acid and 5 ml of concentrated sulfuric acid) and then heated at 110° in a hot-air oven for 10 minutes. Plates were then examined under visible light, short wavelength UV light (254 nm) and long wavelength (366 nm) UV light (Gimeno, 1979; Gimeno and Martins, 1983).

2. 2. 5. ii. e. Detection, identification and confirmation of the presence of patulin on TLC plates

After being reagent-sprayed, the developed chromatograms were examined under visible and UV light for the presence of fluorescent spots. The anisaldehyde spray allows detection of patulin. After spraying, it detects patulin as faint brown spots under the visible light and which appear yellow-orange under longwave UV light. Replacement of methanol in the spray reagent by ethanol improves the detection of patulin which then forms a reddish spot at a detection limit of 0.1 µg (Scott and Somers, 1968; Scott *et al.*, 1970).

2. 2. 5. ii. f. Quantitative analysis of patulin

Once patulin was identified on the TLC plate by comparison of extract spots with internal and external standards, quantitative analysis for positive samples was conducted.

A series of dilutions from the sample extract were chromatographed to determine the lowest dilution at which patulin can still be detected. From this lower limit of detection and the relevant dilution factor the quantity of patulin can be detected (Gimeno and Martins, 1983).

μg patulin kg^{-1} or l^{-1} product = (S x Y x V) / (X x W)

where S = µl ml of patulin standard equal to unknown

Y = concentration of patulin standard, $\mu g\ ml^{-1}$ = 100 $\mu g\ ml^{-1}$

V = µl of final dilution of sample extract = 1000 µl

X = µl sample extract spotted given fluorescent intensity equal to S (patulin standard) = 10 µl

$$W = \frac{\text{Sample weight x filtrate volume}}{\text{Total solution used for extraction}}$$

W = weight of sample, g, or volume of sample, ml (AOAC, 1984).

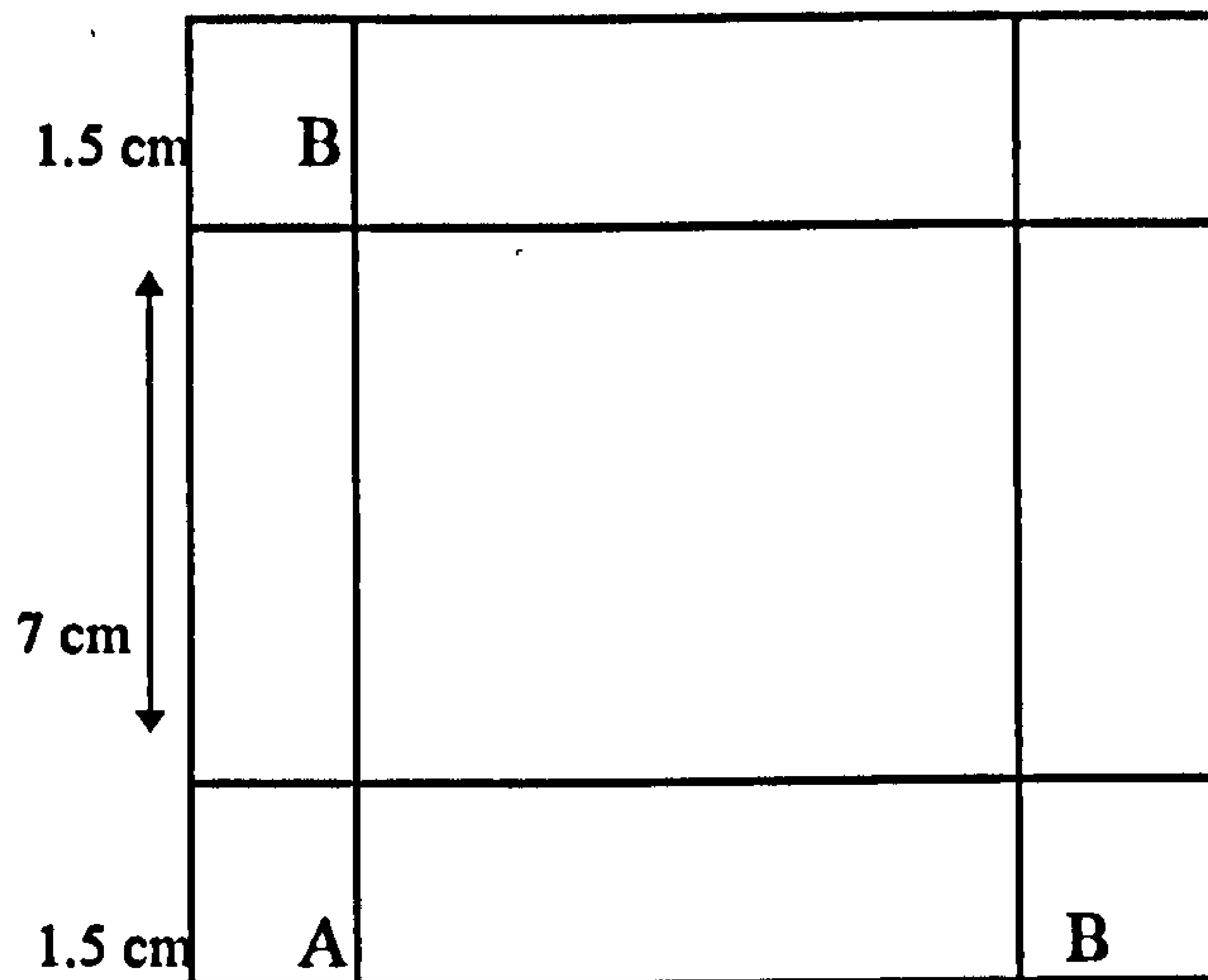
2. 2. 5. ii. g. Two-dimensional TLC

Two-dimensional (bi-directional) chromatography was conducted as described by Stoloff *et al.*(1971). The extract was spotted on 10 x 10 cm TLC plate together with the standard as shown in Fig. 2. 1. The plate was then developed in a mini glass tank in direction 1 with the solvent CA (chloroform : acetate, 93 : 7) to the score line, dried and turned at right-angles in direction 2 with the solvent BMA to score the line and then dried as before. The plate was sprayed with a freshly prepared *p*-anisaldehyde, heated for 10 minutes at 110°C, then observed under visible and UV light. The standard was scanned as before, the position of sample spot in relation to reference standard was noted and checked by their characteristic fluorescent colour under UV lights. If the sample spot of sample extract was too intense to match the standard, the sample was diluted and re-chromatographed.

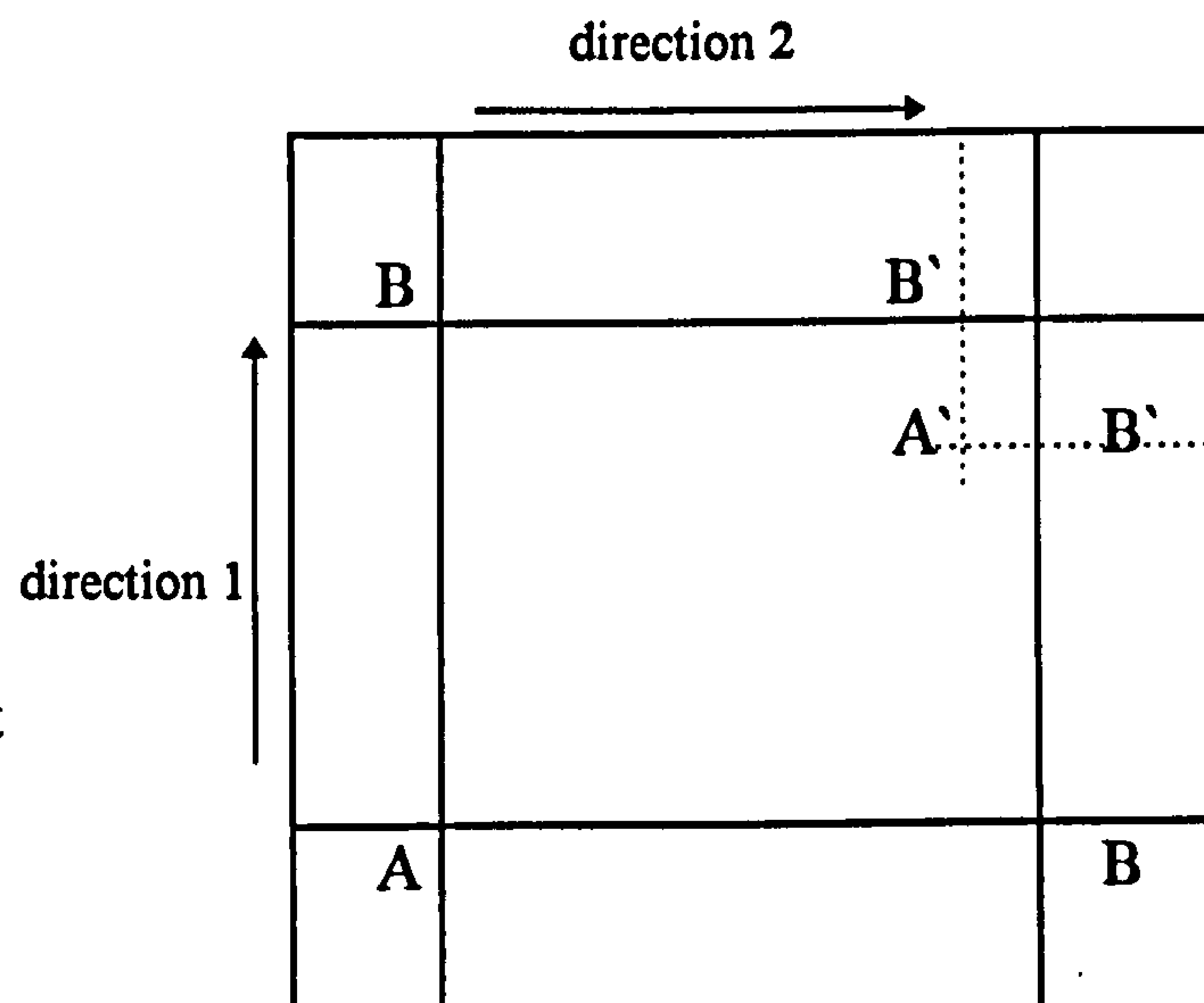
2. 3. Results and Discussion

2. 3. 1. Occurrence of fungi on fruits

a. TLC plate before development



b. TLC plate after development



A = Sample extract spotting place.

B = Patulin standard spotting place.

A` = Patulin place of sample.

B` = Patulin place of standard.

Direction 1, chloroform : acetate (93 : 7).

Direction 2, benzene : methanol : acetic acid (90 : 5 : 5)

<u>Figure 2. 1</u>. Spotting and developing pattern for two-dimensional TLC plate.

Twenty four mould isolates, representing four different genera, and an unknown fungus were isolated from apples fruit samples (Table 2.1). These isolates belonged to the genera- *Penicillium*, *Fusarium*, *Alternaria* and *Mucor*. It was clear that the most frequently isolated genus was *Penicillium* , which accounted for 66.7% of total isolates, followed by *Fusarium* (12.5%) (Table 2.1). Most of these isolates have been reported as common naturally contaminant fungi on fruits as well as being the major pathogenic fungi responsible for toxin production in apples (Wojtas-Koziel and Borecka, 1988).

Table 2. 1. Fungal genera isolated from mouldy apples.

Isolated fungi	Total No. of isolates	Frequency %
Penicillium	16	66.7
Fusarium	3	12.5
Alternaria	2	8.3
Mucor	2	8.3
Unknown	1	4.2

2. 3. 2. Occurrence of bacteria in fruits

Table 2. 2. a. shows the number of bacterial cells isolated from mouldy apples. Mouldy apples were clearly highly contaminated with bacteria in comparison with the control (i. e. non-mouldy apples). On the other hand, no bacteria were isolated from other mouldy fruits.

Six obviously morphologically different types of bacteria were isolated from the mouldy apples and Gram-stained. Bacteria species were combined, two isolates from mouldy apples as well as sound apples including a Gram-positive rods (bipolar) and another Gram-negative rod. Three isolates of Gram-negative rods and one isolate of a Gram-negative cocco-bacilli were identified from the mouldy apples (Table 2. 2. b).

2. 3. 3. Patulin production on fruits

Patulin was detected on mouldy apples. The quantity of patulin detected was 7598 $\mu g\ kg^{-1}$. On the other hand, no detectable amount of patulin was found in other mouldy fruits (Table 2. 3). Although, pear and grape-fruit are well known to contain patulin (Buchanan *et al.*, 1974), this current finding is consistent with the observations of Mortimer *et al.* (1985). Indeed, patulin is a mycotoxin produced naturally by relatively

Table 2. 2. a. Bacterial cell count from mouldy fruits.

Sample	count (dilution 10^{-4})
Apple	169
Grape-fruit	0
Lemon	0
Orange	0
Pear	0
Control	1

Control = sound apples

Values - means of triplicates

Table 2. 2. b. Gram-stain for the isolated indigenous bacteria from both mould and sound apples.

Isolated bacteria	Gram-stain
I	Gram-negative bacilli
II	Gram-negative bacilli
III	Gram-negative cocco-bacilli
IV	Gram-negative bacilli
V	Gram-positive bacilli (bipolarity)
VI	Gram negative bacilli (*Proteus* sp., suspected)

V and VI isolates from mouldy apples as well as from sound apples (Control)

Table 2. 3. Concentration of patulin detected from mouldy fruits.

Sample	Concentration of patulin detected (μg kg^{-1})
Apple	7598
Grape-fruit	ND
Lemon	ND
Orange	ND
Pear	ND
Control	ND

Control = sound apples

ND = non detected

few strains of fungi. The amount of patulin produced appeared not to be directly dependent on the degree of moulding.

Furthermore, patulin production on fruit has been shown not to be essential for fungal growth (Calam, 1979). Natural substrate composition is another important factor governing patulin production (Hesseltine, 1976); the presence of sulphydryl (HS) group in citrus fruits for example, appears to inhibit patulin production (Geiger and Conn, 1945) by inactivating (Timonin, 1946).

2. 3. 4. Evaluation of potential patulin toxicity

Extracts of the natural effected apples produced zones of inhibition of *Serratia marcescens* and 42.9% of the isolated indigenous bacterial growth (Plate 2.4).

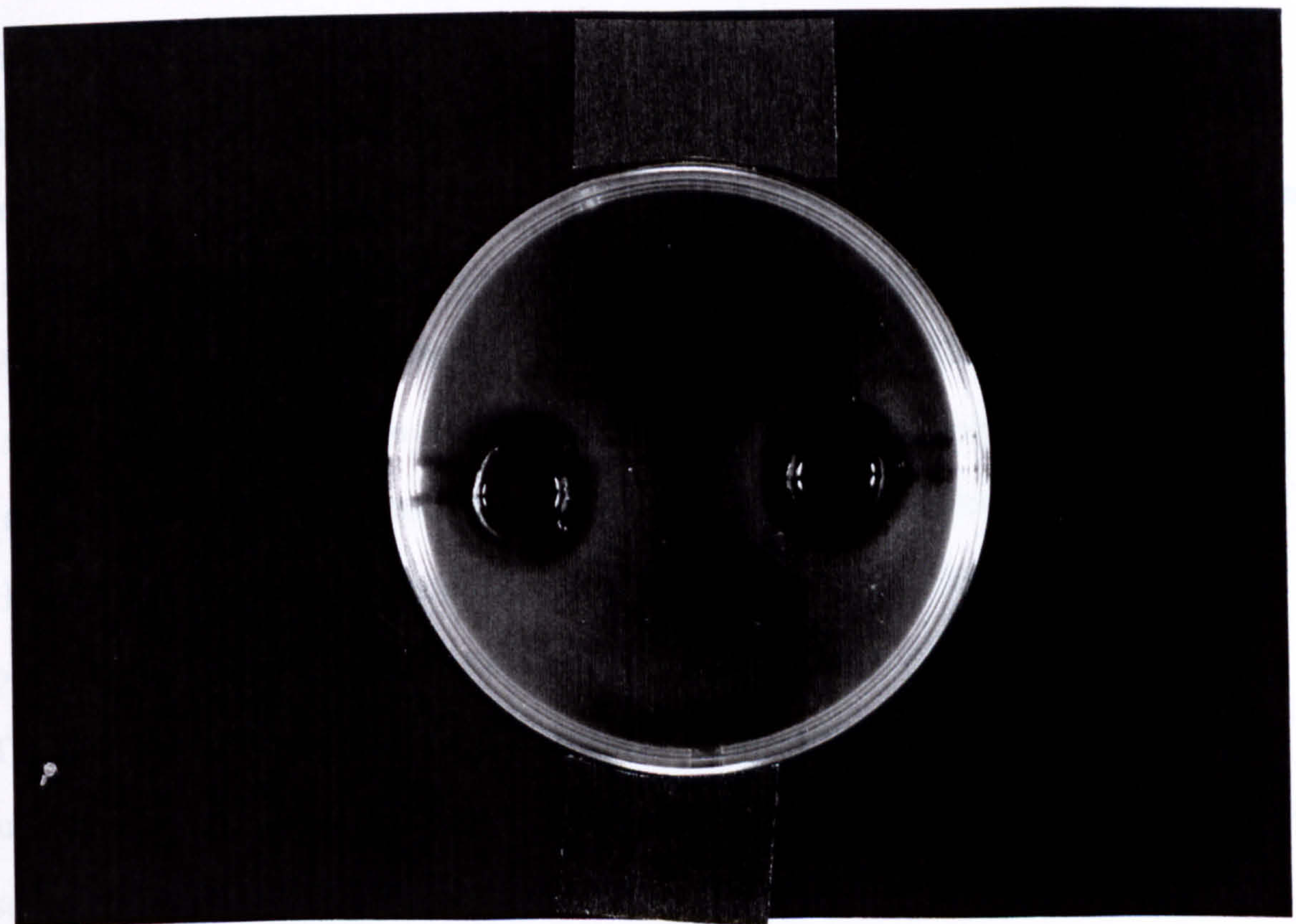
The size of the zones of inhibition varied from 5.1 to 21.3 mm in diameter (Table 2. 4). This result shows that there is considerable variation in the sensitivity shown by different bacteria to patulin. Borecka *et al.* (1984) reported that the action of the metabolite on some bacterial species is specific. One indigenous isolate (IV) proved to be particularly sensitive to patulin. Indigenous bacteria from non-mouldy fruits and from one of the mouldy apples were not susceptible to patulin. *S. marcescens* proved sensitive, but not as sensitive as isolate IV. Singh (1967) reported that pure patulin is toxic to a variety of bacteria at a wide range of concentrations; in some cases however, as much 500 $\mu g\ ml^{-1}$ was needed to achieve inhibition.

2. 3. 5. i. a. Ability of the fungal isolates to produce patulin in Czapek Dox liquid medium

Out of the twenty four isolates and *Penicillium expansum* (Link), 16.7% were found to produce patulin on Czapek Dox liquid medium. Concentrations detected ranged from 450 to 1750 $\mu g\ l^{-1}$ patulin in Czapek Dox liquid medium (Table 2. 5). It is well

Plate 2. 4

An assay plate showing zones of inhibition for the isolated indigenous bacteria I caused by the crude extract of mouldy apples after 24 hours of incubation at 37°C.



known that the productivity of the isolates in regard to patulin production depends on the culture conditions as well as fungal species or strain. For example, the presence of fructose in medium supports patulin production by some strains of the fungus *Penicillium expansum* (Pytel and Brorecka, 1982; Podgorska, 1993).

2. 3. 5. i. b. Evaluation of the toxicity of patulin produced by fungi growing in Czapek Dox liquid medium

The bioassay test for the fungal culture metabolites produced in the Czapek Dox liquid medium showed that the sensitivity of the isolated indigenous bacteria was low, with inhibition zones ranging from 0.3 to 2.8 mm. Table 2. 6 shows that metabolites excreted in Czapek Dox medium from *Penicillium* sp (2) were particularly inhibitory to the isolated indigenous bacteria (I - VI) than the other two isolates (Plate 2.5).

With the exception of two isolates, most of the isolated bacteria (I - VI) were found not to be sensitive to the pure patulin standard solution, at the levels tested. Moreover, the nonsensitive bacteria (I and II) demonstrated growth stimulation in the presence of the lowest concentration of patulin (Plates 2. 6. a and 2. 6. b). The sensitivity of their response to patulin was found to be linear between 25 and 60 μg (Table 2. 7).

The response of bacteria to the Czapek Dox liquid medium extracts, but not to the patulin standard solutions (Table 2. 6 and Table 2. 7), pointed to the possible presence of some other factors (possibly even the presence of other mycotoxins) which might have affected the growth of the bacterial isolates.

Bacillus megaterium (Kellen K) proved remarkably sensitive to patulin even at a concentration as low as 0.5 μg (Table 2. 7). Stott and Bullerman (1975) similarly reported that patulin at a concentration of 1.7 μg inhibited the growth of *B. megaterium.*

Table 2. 4. Effect of crude patulin in apple extracts on *Serratia marcescens* and indigenous bacteria isolated from mouldy apples*.

Bacteria	Zone of inhibition (mm)
Serratia marcescens	5.10
Isolation I	10.0
II	0.0
III	0.0
IV	21.3
V	0.0
VI	0.0

* Concentration of crude patulin detected was 7598 $\mu g\ kg^{-1}$

V and VI isolates from the control apples

Table 2. 5. Concentration of patulin detected in Czapek Dox liquid medium supporting the fungal isolates or *Penicillium expansum* (7 days of incubation at 25°C).

Fungal isolates	Concentration of patulin detected ($\mu g\ l^{-1}$)
Penicillium sp (1)	1250
Penicillium sp (2)	1200
Penicillium sp (3)	1750
Penicillium expansum	450.0

Table 2. 6. Microbiological assay for crude patulin for the indigenous bacteria isolated from mouldy apples and *Serratia marcescens.*

Isolated bacteria	Zone of inhibition (mm) Fermentation of isolated fungal species on CD medium		
	Penicillium sp (1)	*Penicillium* sp (2)	*Penicillium* sp (3)
Serratia marcescens	0.0	0.0	0.0
I	0.3	0.5	1.0
II	0.5	2.8	0.0
III	0.0	2.0	0.0
IV	0.0	0.0	2.5
V	0.0	0.0	0.0
VI	0.0	0.0	0.0

2. 3. 5. ii. Ability of the isolated fungi to produce patulin on apples

The various patulin-producing fungi, found to be capable of producing patulin in Czapek Dox liquid medium, showed varying abilities to colonise apples. The growth rate of the indigenous *Penicillium* sp (2) was low when compared to *Penicillium* sp (3) and *Penicillium* sp (1) (Plates 2. 2 and 2. 3). These two isolates produced patulin on apple in amounts ranging from 2750 to 4000 µg kg^{-1}, respectively (Table 2. 8). This yield is higher than that seen when the same fungus was grown in Czapek Dox; a result which shows that apples are more favorable substrate for patulin production than is a synthetic medium like Czapek Dox.

Plate 2. 5

An assay plate showing a zone of inhibition for the isolated indigenous bacteria III caused by the ferment of *Penicillium* sp (2) in 100 ml Czapek Dox liquid medium. No zones of inhibition caused by the ferment of *Penicillium* sp (1) and *Penicillium* sp (3). The plate was incubated at 37°C overnight.

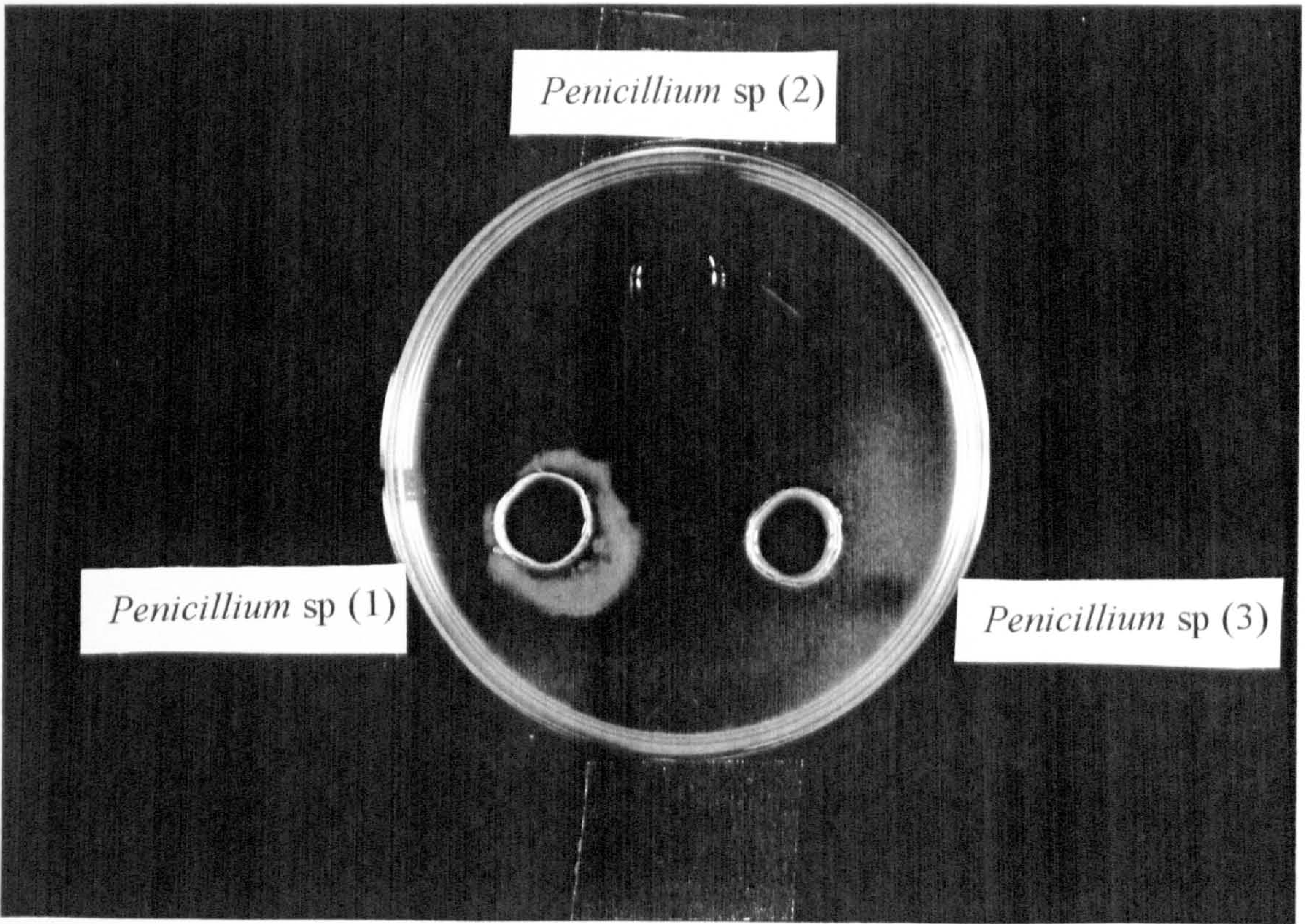

Penicillium sp (2)
Penicillium sp (1)
Penicillium sp (3)

Plate 2.6.a

An assay plate for the sensitivity of the isolated indigenous bacteria I to different concentrations of patulin (0 - 60 μg) after 24 hours of incubation at 37°C. Stimulation zones appeared with the low concentrations of patulin (0 - 20 μg).

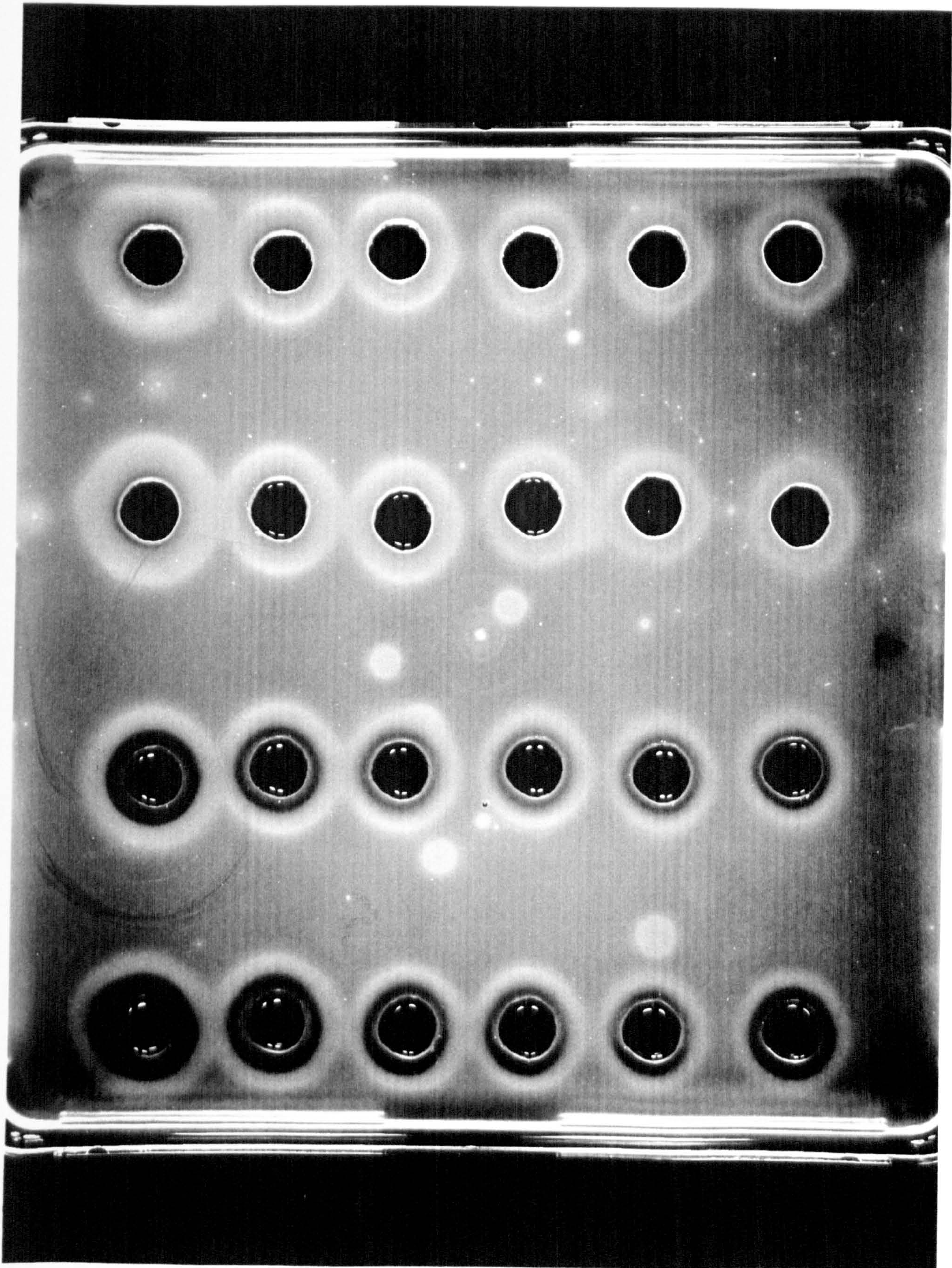


Plate 2.6.b

An assay plate for the sensitivity of the isolated indigenous bacteria II to different concentrations of patulin (0 - 60 μg) after 24 hours of incubation at 37°C. Stimulation zones appeared with the low concentrations of patulin (0 - 20 μg).

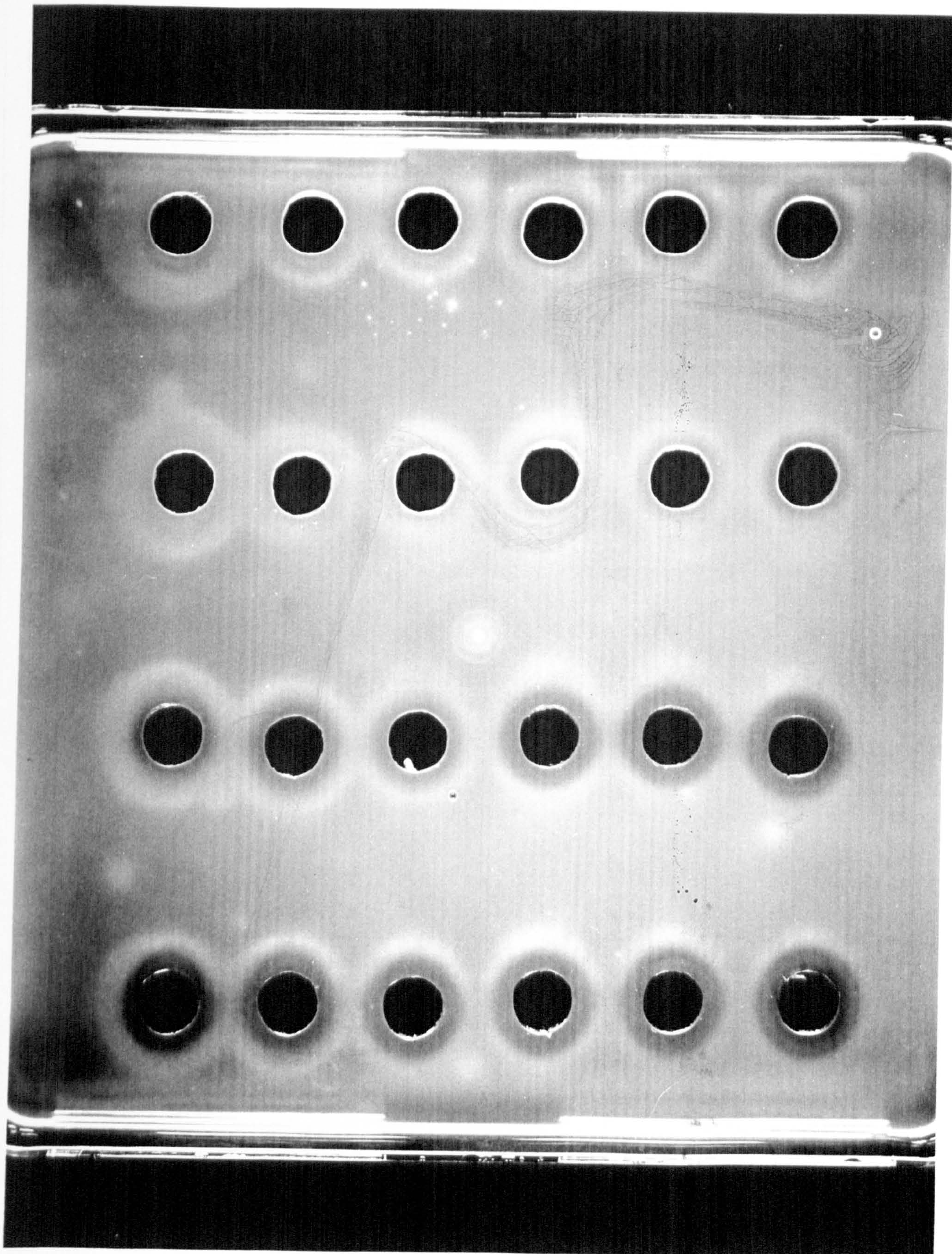


Table 2. 7. Patulin bioassay for the isolated bacteria and *Bacillus megaterium*.

Patulin concentration	Zone of inhibition (mm)		
(μg 100 μl^{-1})	*B. megaterium*	I	II
0.0	0.0	0.0	0.0
0.5	1.0	0.0	0.0
1.0	1.5	0.0	0.0
5.0	1.8	0.0	0.0
10	2.0	0.0	0.0
15	2.5	0.0	0.0
20	3.0	0.0	0.0
25	3.2	4.3	3.0
30	3.5	5.0	3.9
35	3.6	6.1	4.3
40	3.8	6.2	4.6
50	4.0	6.6	4.7
60	4.2	6.8	5.0

Table 2. 8. Patulin production in inoculated apples *in vitro* by fungi capable of producing patulin in Czapek Dox liquid medium (7 days of incubation at 25°C).

Isolated fungi	Concentration of patulin detected (μg kg^{-1})
Penicillium sp (1)	4000
Penicillium sp (2)	ND
Penicillium sp (3)	2750

ND = non detected

By impressing the decayed parts of the inoculated apples onto bacterial growth medium confirmed that the isolated bacteria (I - VI) could grow in the presence of patulin.

2. 4. Conclusions

Amongst the filamentous fungi there are numbers of species adapted for growth over a wide range of substrates. Some of which are commonly found in fruits and cause spoilage. The fungal species isolated in this investigation are clearly associated with the spoilage of fruits and are commonly found growing on the fruit surface. Most of the moulds associated with apple and other fruit decay belong to the genus *Penicillium* (Hesseltine, 1976; Stinson *et al.*, 1981). However, Schiewe and Mendgen (1992) noted the presence of more than 700 types of microorganisms on apple fruits and trees.

The occurrence of patulin in apples, but not in other fruits, indicated the suitability of this substrate for patulin production. The presence of fungi capable of producing patulin on a substrate does not however, necessarily mean that patulin is present (Bullerman, 1974). Since 1972 reports have been made concerning the production of patulin in nature in food and foodstuff, with the exception that is natural substrates containing sulphydryl group, such as citrus fruits (Scott *et al.*, 1972; Geiger and Conn, 1945), a fact which explains why no detectable amounts of patulin were found in this study to occur in lemons and oranges, even where contaminated with patulin-producing fungi.

It is well known that the production of particular mycotoxins is generally restricted to certain species or to single specific strains. Out of 24 isolates examined here on 12.5% produced patulin in Czapek Dox medium (excluding *P. expansum*). The rate of production was distinctly lower when apples were used as substrate, where only two out of the 24 isolates (8.3%) were able to produce patulin. This can be explained on the

basis that the carbon source was different, or possibly apples contain some factor which is inhibitory to patulin production.

The quantity of patulin produced by the indigenous fungi in Czapek Dox liquid medium (Table 2. 5) and in apple fruits (Table 2. 8) reflects the fact that natural substrates appear better suited for patulin production than artificial media. Similar results were reported by Vinas-Almenar *et al.* (1993). They found that in liquid medium (yeast extract sucrose), only 0.8% of the fungal isolates were able to produce patulin, but the percentage was higher when the isolates were grown on apples (8.2%).

An important point to note is that during inhibition studies, the zone of inhibition was not always directly related to the concentration of patulin measured in Czapek Dox media in which fungi grew, a fact which suggests that perhaps more than one antibiotic\mycotoxin was being produced (Table 2. 6).

Patulin is generally highly toxic to a wide range of both Gram-positive and Gram-negative bacteria (Wiesner, 1942; Waksman *et al.*, 1943; Waksman, 1947). However, the isolated indigenous bacteria I to VI were insensitive to even high concentrations of patulin, whereas isolates I and II proved sensitive and exhibited linear inhibition responses over the concentration range of 25 and 60 µg patulin. This result is in agreement with the findings of Madhyastha *et al.* (1994) who demonstrated the lack of sensitivity to patulin amongst certain bacteria, and even that low concentrations of patulin stimulate bacterial growth. However, it should be borne in mind when discussing the production of antibacterial agents, like patulin, that fungi are capable of producing more than one antibiotic\mycotoxic agent at the same time. *Penicillium expansum* for example, is known to be able to produce patulin and traces of citrinin when growing on a single substrate *in vitro* (Harwig *et al.*, 1973 a; Ciegler *et al.*, 1977). Moreover, an atypical strain of *Penicillium requeforti* was confirmed to produce both patulin and penicillic acid (Olivigni and Bullerman, 1978).

3. PATULIN PRODUCTION ON SUGAR BEET

3. PATULIN PRODUCTION ON SUGAR BEET

3. 1. Introduction

The previous studies showing that patulin was produced by natural isolates in larger concentrations on apples than in liquid culture led to an investigation into the ability of fungi to produce the mycotoxin in other agricultural products. Agricultural products sustain considerable physical damage during harvesting, transportation, storage, processing and production, all processes which allow for rapid and extensive mould colonization. Such contamination leads to the obvious possibility of *in vivo* patulin production.

The most common storage fungi are members of the genera *Aspergillus* and *Penicillium* (Scott, 1974 a), fungi which, as we have seen, are noteworthy for their ability to produce patulin. Again, as has already been noted, patulin production is associated with *Penicillium expansum*, as well as some other commonly occurring spoilage fungi (Scott, 1974 b).

Although a variety of agricultural substrates have been reported in the literature to be ideal for patulin production, sugar beet has been largely neglected. However, fungal growth and antibiotic production on this substrate has been demonstrated naturally by Grossbard (1954), who found that dried sugar beet pulp supported patulin production in a variety of different types of soils. Geiger and Conn (1945) also reported the production of patulin on brown sugar in place of glucose.

Grossbard (1948) showed that both glucose and sugar beet waste supported patulin production by *P. patulum*. The ability of fungi to grow and produce antibiotics on organic materials and in autoclaved soil has also been reported. Falih (1995), for example, reported the production of an antibiotic substance (penicillin) by two strains (1255 and 1951) of *Penicillium chrysogenum* when grown on Czapek Dox C-free

liquid medium amended with autoclaved sugar beet extract (sucrose concentration in excess of 16% w/w).

The purpose of the work described in this Chapter was:

i) to isolate and identify the predominant fungal species occurring as contaminants of mouldy sugar beet; and

ii) to determine the ability of the isolated indigenous fungi to produce patulin using sugar beet as a source of carbohydrate.

3. 2. Materials and Methods

3. 2. 1. Source of sugar beet

Sugar beet tubers were collected from a sugar beet storage pile situated at Worksop, Nottingham, UK. The sugar beet were stored at 2°C until required.

3. 2. 2. Isolation of indigenous moulds

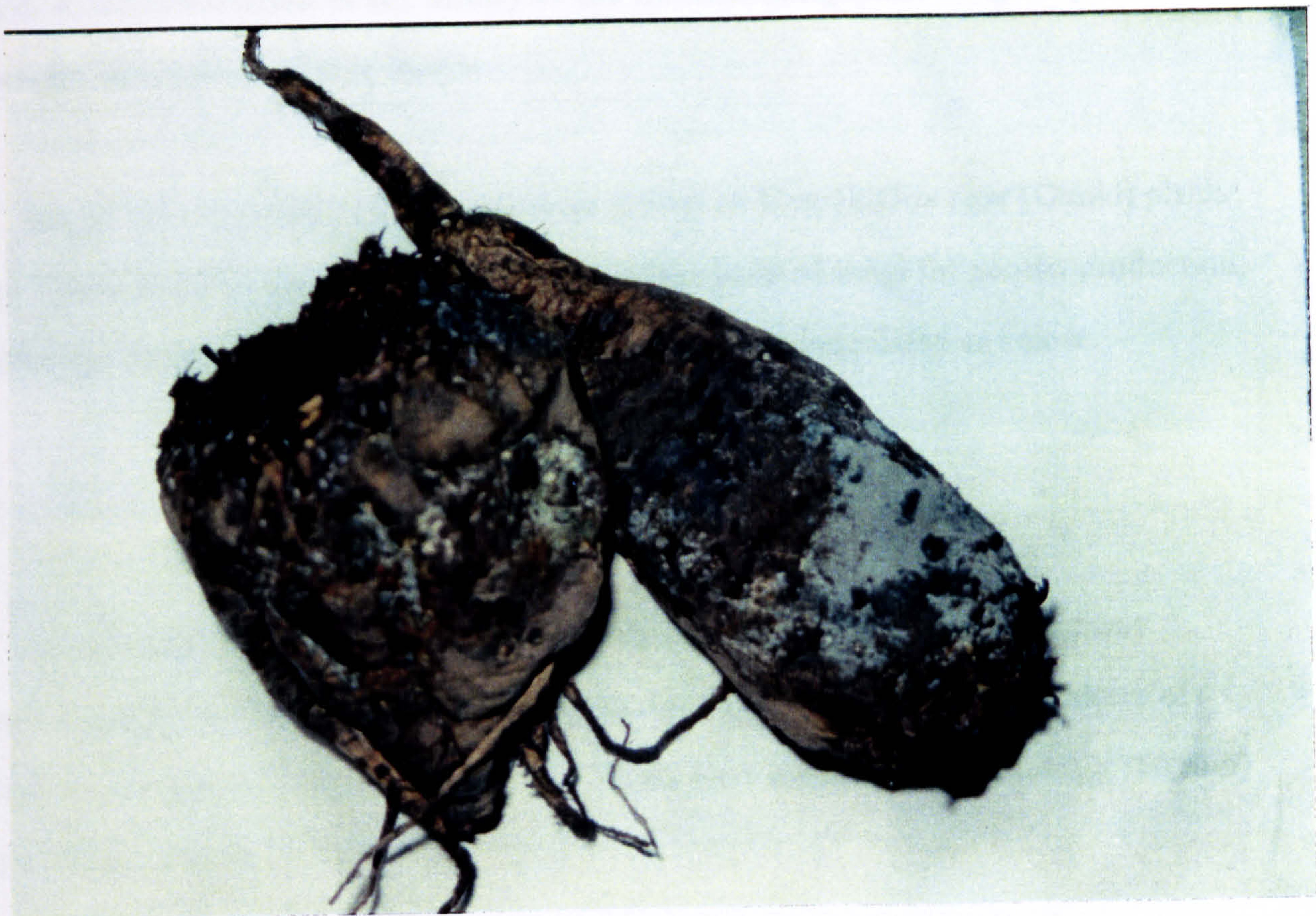
Naturally contaminated sugar beet tubers were removed from cold storage and sampled for the presence of indigenous fungi (Plate 3. 1). Spore colonies were picked off and transferred to Czapek Dox Agar (Oxoid). Plates were incubated at 25°C for 7 to 10 days. The isolated fungi were maintained in triplicate on slope Czapek Dox agar (Oxoid) for 7 days at 25°C then stored at below 4°C.

3. 2. 3. Determination of natural occurrence of patulin in the mouldy sugar beet

Sugar beet tubers were cut into small pieces, placed in a kitchen blender and homogenized with 50 ml sterile distilled water. Portions (40 g) were centrifuged in sterile 30 ml Universal tube at 4500 rpm for 10 minutes. The supernatants were then

Plate 3.1

Spoiled sugar beet tubers, naturally contaminated with moulds. Stored at 2°C for 3 months.



filtered through Whatman filter paper No.1. Sugar beet juice (30 ml) was collected and adjusted to pH 2.0 by the addition of 6 N HCl, then placed in 250 ml separating funnel. The filtrate was extracted with two 30 ml portions of ethyl acetate. The upper layers of ethyl acetate (organic compounds) were combined after filtration through Whatman No. 2 filter paper containing 1g of anhydrous sodium sulphate and evaporated to dryness (Scott and Kennedy, 1973; Wilson and Nuovo, 1973).

3. 2. 4. Determination of the ability of the isolated indigenous fungi to produce patulin in various culture media

Fungal colonies used as inoculants were grown on Czapek Dox agar (Oxoid) plates for 7 days at 25°C. To determine the ability of the isolated fungi for patulin production, (after the desired incubation period) culture media were inoculated as below:

3. 2. 4. i. Czapek Dox liquid medium (CD)

Autoclaved Czapek Dox liquid medium (Oxoid, 100 ml) in 250 ml flasks was inoculated in triplicate with a hyphal growth disc (13 mm) of a 7 day old culture of each isolate grown on Czapek Dox agar. Flasks were incubated with shaking(150 rpm) at 25°C for 7 days.

3. 2. 4. ii. Inoculation of sugar beet slices

Fresh sugar beet tubers were cleaned, and, after the skin was removed, cut into similar sized (about 0.5 cm in thick) slices. Each slice was inoculated with a heavy loopful of spores of each isolate and *Penicillium expansum* grown on Czapek Dox agar 25°C for 7 days. The inoculated slices were placed in sterile propagators which had previously been washed and wiped with alcohol. The base of the propagators were

covered with four layers of Kimwipe laboratory tissue. The sugar beet slices were then wetted with 50 ml sterile distilled water and incubated at 25°C for 7 days. Those isolates which did not produce patulin after 7 days of incubation were incubated for 30 days. The propagators were moistened manually once a week.

3. 2. 4. iii. Patulin production on sugar beet extract amended with range of nitrate concentrations

A range of nitrate concentrations (NO_3) (0.0, 0.1, 0.5, 1.0, 1.5 and 2.0 mg ml^{-1}) were added to 40 g of sugar beet paste and 100 ml of distilled water in 250 ml flasks. The flasks were autoclaved at 120°C for 20 minutes. The medium was inoculated with spores of fungi which are incapable of producing patulin on sugar beet slices which had been washed with sterile, distilled water; 1 ml of spore suspension, was added and the flasks were incubated with shaking for 7 days at 25°C.

3. 2. 5. Patulin extraction

The contents of all growth flasks were filtered through Whatman No. 1 filter paper. The decayed tissues of the sugar beet slices were homogenized in a kitchen blender with 50 ml sterile of distilled water. Portions (40 g) were centrifuged (in 30 ml Universals) at 4500 rpm for 10 minutes then filtered through Whatman No. 1 filter paper. All extracts were then adjusted to pH 2 using 6 N HCl. The solutions were then extracted with two equal volumes of ethyl acetate. Organic compounds were dried over anhydrous $NaSO_4$ and evaporated to dryness (Appendix 9. 6. b).

3. 2. 6. Clean-up procedure

The dried residues obtained from 3. 2. 3 and 3. 2. 4. iv were dissolved in 20 ml of chloroform with 0.5 ml of water then dried for 1 h over approximately 1 g of anhydrous sodium sulphate. Chloroform solution (5 ml) was next added to a pre-conditioned silica Sep-Pak cartridge (No.51900, Water). The cartridge was washed (1 ml, chloroform; 1 ml chloroform-ethyl acetate (8 + 2) Chloroform-ethyl acetate (1 ml. 5 + 5) then 2 ml of chloroform-ethyl acetate (2+8) was passed through the cartridge. The final eluent was collected, evaporated and redissolved in 1 ml of benzene for thin layer chromatography (TLC) (Rovira *et al.*, 1993) (Appendix 9. 6. c).

3. 2. 7. Patulin assay

Chromatography for patulin determination was assayed according to the methods described by Scott *et al.* (1970) and Stoloff *et al.* (1971) (Appendices 9. 6. a and 9. 6. g).

3. 2. 8. Quantitative analysis

Quantity of patulin detected was calculated as:-
μg patulin kg^{-1} or l^{-1} = (S x Y x V) / (X x W) according to the methods described by Gimeno and Martins (1983) and AOAC (1984) (Appendix 9. 6. g).

3. 3. Results and Discussion

3. 3. 1. Description of moulds isolates

Six species of filamentous fungi belonging to the genus *Penicillium* were isolated from the mouldy sugar beet, these were common fungi , frequently isolated from food and foodstuffs and are refer to as storage fungi (Hesseltine, 1968).

3. 3. 2. Ability of the isolated indigenous fungi to produce patulin in Czapek Dox liquid medium

Growth in Czapek Dox liquid medium was initially used to study the production of patulin by the isolates. Table 3. 1 shows that the isolates produced substantial concentrations of patulin, ranging from 800 to 8000 μg l^{-1}. The fact that 33.3% of the isolates were incapable of producing patulin seems to indicate that this is not an optimal medium for patulin production by all of the isolates.

Table 3. 1. Patulin production by the isolates in CD liquid medium after 7 days incubation at 25°C.

Isolated fungi	Concentration of patulin detected (μg l^{-1})
Penicillium sp (1)	ND
Penicillium sp (2)	2933.3
Penicillium sp (3)	8000.0
Penicillium sp (4)	800.00
Penicillium sp (5)	ND
Penicillium sp (6)	2666.7

ND = not detected

3. 3. 3. Ability of the isolated indigenous fungi to produce patulin when growing on sugar beet slices

All of the patulin and the non-patulin producing moulds in Czapek Dox liquid medium, together with *Penicillium expansum* Link, were tested for the ability to produce patulin while growing on sugar beet slices (Plate 3. 2 and 3. 3). The results (after 7 days incubation) showed that 50% of the isolates produced patulin (Table 3. 2). After 30 days incubation however, two of the fungi previously found to be incapable of patulin production after 7 days, began to produce detectable quantities of the toxin. Of those fungi assayed, only one isolate, *Penicillium* sp (1), was unable to produce patulin on sugar beet slices even after 30 days of incubation. This fungus grew well on this substrate so the fact that it failed to produce patulin suggests either the presence of some inhibitor or the absence of an intermediary substrate (Plate 3. 2).

Table 3. 2. Determination of naturally occurring patulin in inoculated sugar beet slices *in vitro* after 7 days and 30 days of incubation at 25°C.

Fungal Isolate	Concentration of patulin detected (µg kg^{-1})	
	7 days	30 days
Penicillium sp (1)	ND	ND
Penicillium sp (2)	2500	-
Penicillium sp (3)	2500	-
Penicillium sp (4)	ND	510.2
Penicillium sp (5)	ND	952.4
Penicillium sp (6)	3250	-
Penicillium expansum	3250	-

- = not assayed

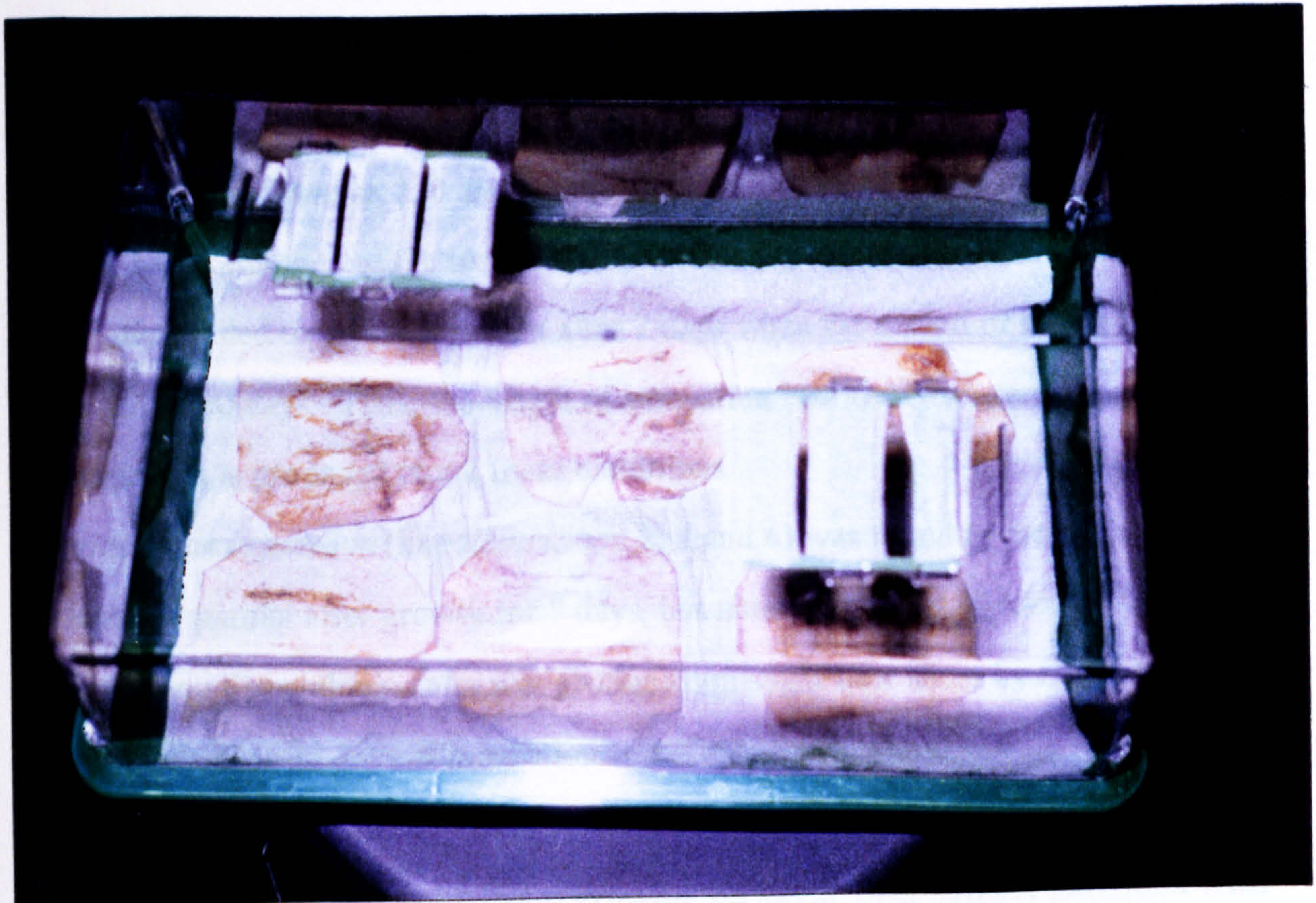
ND = not detected

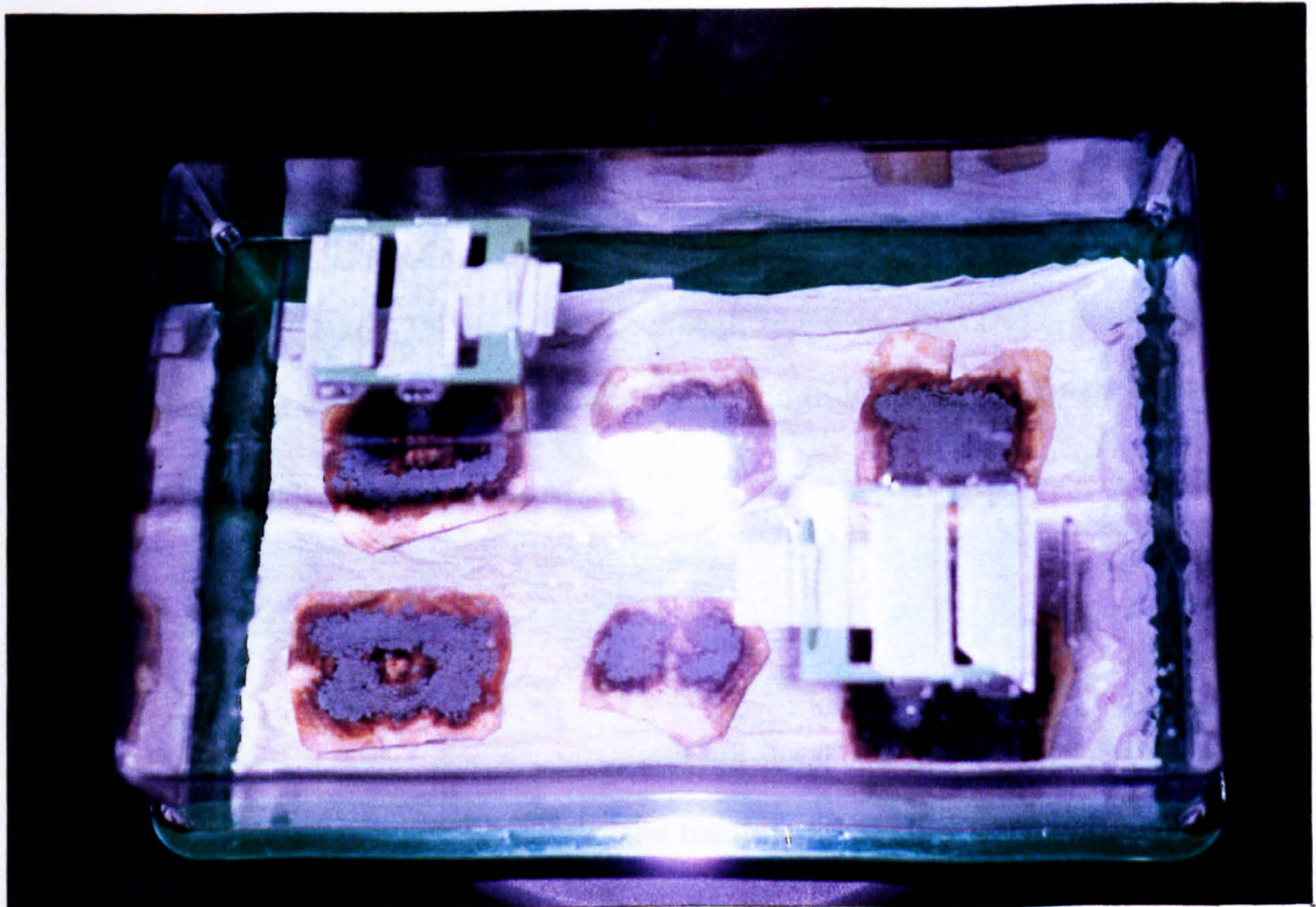
Plate 3. 2

Inoculated sugar beet slices with the isolated indigenous fungus *Penicillium* sp (1).

Plate 3. 3

Inoculated sugar beet slices showing high growth rate of *Penicillium expansum*.





High yields of patulin of 2500 to 3250 μg kg^{-1} were detected after 7 days in cultures containing *Penicillium* sp 2, 3 and 6, whereas *Penicillium* sp 4 and 5 did not produce patulin. Small amounts of the toxin (510.2 - 952.4 μg kg^{-1}) were produced by those fungi which failed to produce patulin after 7 days when the period of incubation was increased to 30 days. Thus, the length of incubation is critical in determining whether or not patulin is produced under these conditions.

Penicillium expansum (like *Penicillium* 2, 3 and 6) was found to produce large amounts of patulin after growth for 7 days, but none was detected after 30 days, while in contrast no detectable patulin was produced on sugar beet slices by *Penicillium* sp (1) after 7 days or 30 days of incubation.

3. 3. 4. Patulin production by fungi growing on sugar beet extract amended with nitrate

Garraway and Evans (1984) pointed out that the biosynthesis of patulin is influenced mainly by carbon but that nitrogen is also a crucial nutrient for its production. As a result, the effect of nitrate on the production of patulin by the fungus *Penicillium* sp (1), which failed to produce patulin when grown on non-autoclaved sugar beet slices even after 30 days of incubation, was determined following amendment with nitrate (0.0, 0.1, 0.5, 1.0, 1.5 and 2.0 mg ml^{-1}). The result suggested that this isolated fungus was either inhibited by some factors present or period of incubation did not allowed patulin production.

3. 4. Conclusions

As agricultural products are easily infected with fungi during storage, contamination with patulin is frequently likely to occur. The results presented in this Chapter show

that fungi (six species of *Penicillium*) can grow on sugar beet tubers . Hesseltine (1968) pointed out that members of this genus are the most common group of storage fungi causing mycotoxin contamination of stored agricultural products. However, patulin was not detected when naturally contaminated sugar beet was assayed. One explanation for this might be that there may have been competition between the different isolates for limiting concentrations of available nutrients in the sugar beet.

When the natural fungal isolates were grown on CD medium all were found to be able to produce patulin in Czapek Dox liquid medium with the exception of *Penicillium* sp (1) and *Penicillium* sp (5). Sucrose is generally regarded as an ideal carbon source for patulin production (Wiesner, 1942; Chain *et al.*, 1942), so the ability of these fungi to produce patulin under these conditions is unlikely to have been due to the nature of the C-source.

Sugar beet was used to study the effect of a natural substrate on patulin production. The results show that this substratum was a favorable source for carbon. While within 7 days of incubation 50% of the isolates were able to produce patulin in sugar beet slices. After 30 days of incubation, *Penicillium* sp (4) and *Penicillium* sp (5) were able to produce patulin in sugar beet slices at either sampling time. Only *Penicillium* sp (1) was found not to produce patulin in this substrate and Czapek Dox liquid medium as well. It is surprising that the concentration of patulin produced in Czapek Dox liquid medium was greater than that detected from sugar beet slices. This is possibly due to presence of some inhibitory factors. *Penicillium* sp (5) produced patulin in sugar beet slices after 30 days of incubation, while it failed to produce the substance in the liquid medium after 7 days of incubation. Geiger and Conn (1945) obtained large amounts of patulin in medium containing glucose as a source of carbon. Grossbard (1954) reported detectable amounts of patulin by six strains of *Penicillim patulum* isolated from soil amended with sugar beet. The length of incubation could be another significant factor effecting patulin production. By growing the common storage rotting fungus of fruit, *Penicillium expansum*, on sugar beet slices it was readily demonstrated that fungus

could produce patulin on this substrate. Only *Penicillium* sp (1) was unable to produce patulin in both liquid medium and sugar beet slices.

As nitrogen is recommended for patulin production (Grootwassink and Gaucher, 1980). *Penicillium* sp (1) was applied to autoclaved sugar beet extract amended with nitrate (0 - 2 mg ml^{-1}). The results showed that this isolate is possibly not a patulin producing fungus as it's growth was poor (Plate 3. 2), although a species of *Penicillium*, *Penicillium patulum*, formed a detectable amount of patulin in sugar beet waste (Grossbard, 1948).

4. DO MICROORGANISMS PRODUCE PATULIN WHEN GROWING ON WHEAT STRAW?

4. DO MICROORGANISMS PRODUCE PATULIN WHEN GROWING ON WHEAT STRAW?

4. 1. Introduction

El-Kady (1986) demonstrated the presence of 160 isolates of *Aspergillus* and *Penicillium* from 50 samples of wheat straw and 25 samples of each of wheat, barley, maize and sorghum collected from different locations in Egypt. Mycotoxins, including patulin, were found to be produced by 44.4% of these isolates.

The ability of fungi to use wheat straw as a source of carbohydrate for patulin production by fungi was investigated by Grossbard (1948) who also reported patulin production by *Penicillium patulum* on sterilized wheat straw. The concentration of patulin produced was enhanced by the addition of 3.5% glucose. Patulin was also found to be produced by this fungus in soil containing 5% of fresh wheat straw. Patulin was detected in more than 83% of soil samples amended with 2.5 to 5% wheat straw following inoculation with *Penicillium patulum* (Grossbard, 1954). Moreover, sterilization of soil allowed for the production of antibiotics where straw was added (Grossbard, 1949).

The occurrence of patulin in 800 samples of agricultural commodities including cereals, feeds, hay and silage was investigated. Results demonstrate that patulin was the most common mycotoxin next to aflatoxin B_1 (Dutton and Westlake, 1985).

Mycological studies of wheat straw and silage demonstrated contamination of such foodstuffs with patulin producing fungi. Escoula (1977) reported that silage spoils naturally in less than a month. The indigenous fungi isolated were *Penicillium granulatum* and *Byssochlamys nivea.* The concentration of patulin produced by these isolates was 40 ppm (Escoula, 1974).

In this Chapter, an investigation was made of the ability of either the indigenous fungi or *Penicillium expansum* to produce patulin when growing naturally on wheat straw.

4. 2. Materials and Methods

4. 2. 1. Substrate preparation

Wheat straw, obtained from a local pet shop, was air dried, cut into short lengths (approx. 5 cm). Samples (25g) were then placed in glass cylinders (15 cm length and 20 cm width) sealed with cotton gauze. The straw was washed with running tap water then with 1500 ml of sterile distilled water, followed by 1500 ml of boiling hot, sterile distilled water, both ends of the glass cylinder were then covered with glass Petri dishes (Plate 4. 1). The samples were sterilized by autoclaving at 120°C for 20 minutes on 3 successive occasions then divided into two sets. One set was inoculated with fungi, while the other served as the control.

4. 2. 2. Organisms and inoculum

Penicillium expansum was obtained from the CAB International Mycological Institute, Surrey. *Phanerochaete chrysosporium* was obtained from Dr. Wainwright, Department of Molecular Biology and Biotechnology, University of Sheffield.

The fungus *Penicillium expansum* was grown on Czapek Dox agar (Oxoid) slope at 25°C for 7 days, while *Phanerochaete chrysosporium* was grown on potato dextrose agar (Oxoid) slopes at 37°C for 5 days. Spore suspensions of both cultures were harvested in sterile distilled water using sterile loop after gentle agitation.

4. 2. 2. a. Patulin production in Czapek Dox liquid medium (CD)

Plate 4. 1

Apparatus for straw incubation studies.

Autoclaved CD liquid medium (100 ml), dispensed in 250 ml flasks, was inoculated with 1 ml of *P. expansum* suspension alone or in association with 1 ml of *Phanerochaete chrysosporium* suspension. The flasks were then incubated at 25°C on a reciprocal shaker (150 rpm) for 7 to 14 days.

4. 2. 2. b. Patulin production on wheat straw

Sterile wheat straw (25 g) was used as the natural nutrient source. One ml of the suspension of each isolates alone or in association with each other were used as the inoculant. The inoculated substrate was incubated at 25°C for 7, 14, 21 and 30 days. Non inoculated, sterilized wheat straw was used as the control. In order to study the ability of the indigenous fungal flora to produce patulin, non sterile and non washed wheat straw was moistened with sterile distilled water and incubated at 25°C for 7, 14, 21 and 30 days in triplicate. All samples were moistened weekly with sterile distilled water to a water content equal to the sample weight at beginning of the experiment.

4. 2. 3. Extraction of patulin

Patulin was extracted from CD by filtering the medium through Whatman No. 1 filter paper. The filtrate was then adjusted to pH 2.0 by addition of 6 N HCl. Two equal volumes of ethyl acetate were used for the extraction. The extracts were combined, dried on 1 g of anhydrous sodium sulphate and evaporated to dryness.

Wheat straw (25 g) was extracted for patulin determination as follows. At the end of the incubation period the contents of the glass cylinder were transferred to a kitchen blender and then extracted with 125 ml of ethyl acetate. The samples were shaken vigorously for 5 minutes and the contents was filtered through Whatman No.1 filter paper containing 5 g of anhydrous sodium sulphate. The

filtrate was then separated using a 250 ml separating funnel. The filtrate was then filtered through a No. 2 filter paper containing 1 g anhydrous sodium sulphate and then evaporated to dryness (Appendix 9. 6. b).

4. 2. 4. Clean-up, chromatography and quantitative analysis

Clean-up, chromatography and quantitative analysis was performed as described above (Appendixes 9. 6. c to 9. 6. g).

4. 3. Results and Discussion

Table 4. 1 shows that *Penicillium expansum* was able to produce patulin in nutrient-rich medium (Czapek Dox). When CD liquid culture was inoculated with both *P. expansum* and *Phanerochaete chrysosporium*, the amount of patulin produced was 34.2% less than when *P. expansum* alone was inoculated. A larger amount of patulin was produced by the culture of *P. expansum* incubated for a week then inoculated with *Phanerochaete chrysosporium* and then re-incubated for a further week. *Phanerochaete chrysosporium* was incapable of producing patulin in the nutrient-rich medium (Table 4. 1).

Patulin was produced by *P. expansum* growing on sterile wheat straw after 14 days of incubation (Table 4. 2). The maximum level of patulin produced appeared after 21 days when 3730.3 μg kg^{-1} was detected. Thus, *P. expansum* was able to produce patulin on wheat straw when growing on a realistically, if sterile, natural substrate. The quantity of patulin produced increased with increasing length of incubation, but declined after 30 days of incubation, when 2048.3 μg kg^{-1} was detected.

Penicillium expansum failed to produce patulin, at any point in the incubation period, when growing together with *Phanerochaete chrysosporium*. Patulin was not produced by the indigenous microflora, nor in sterile controls.

Table 4.1. Production of patulin by *Penicillium expansum* alone or in association with *Phanerochaete chrysosporium* when growing in CD liquid medium (7 days incubation at 25°C).

Treatments	Concentration of patulin detected ($\mu g\ l^{-1}$)
Penicillium expansum	423.0
P. expansum + P. chrysosporium	296.0
*P. expansum + P. chrysosporium**	669.8
Phanerochaete chrysosporium	ND

*Incubation of *P. expansum* for 7 days then inoculation the culture with *Phanerochaete chrysosporium* then re-incubation for 7 days.

ND = not detected

When wheat straw was used without being washed or sterilized, in order to study the ability of the indigenous fungal flora to produce patulin, extensive fungal growth and natural colonization of the straw occurred (Plate 4. 2) but patulin was not detected even after 30 days of incubation (Table 4. 2). Presumably either the indigenous fungi present are not patulin producers on wheat straw or wheat straw alone is not a suitable substrate for patulin production by the indigenous flora.

The white rot filamentous fungus *Phanerochaete chrysosporium* is renowned for its ability to release carbon from lignin substrates (Tien and Kirk, 1983). This fungus was applied in dual culture with straw in the hope that it would release sufficient carbon from the straw to meet both its own needs and to provide carbon to support the growth and patulin production by *P. expansum.* Table 4. 2 shows that this expectation was not

Plate 4. 2

Non-autoclaved and non-washed raw wheat straw after incubation for 21 days at 25°C.

S57
S57

met, as no patulin was produced when the two fungi were grown together, even though sufficient carbon was released by straw degradation to support luxurious growth of *P. expansum* (Plates 4. 3 and 4. 4).

Table 4. 2. Patulin production on wheat straw by the indigenous mycoflora, *Penicillium expansum* and *Phanerochaete chrysosporium* in 7, 14, 21, and 30 days of incubation at 25°C.

	Concentration of patulin detected (µg kg^{-1})			
Treatment	7 days	14 days	21 days	30 days
P. expansum	ND	827.5	3730.3	2048.3
P. expansum + *P. chrysosporium*	ND	ND	ND	ND
Indigenous mycoflora*	ND	ND	ND	ND
Control	ND	ND	ND	ND

* non sterilized and non washed wheat straw

Control = sterilized and non inoculated wheat straw

ND = not detected

4. 4. Conclusions

The ability of *Penicillium expansum* and the indigenous fungal flora to produce patulin in the presence of the white rot fungus *Phanerochaete chrysosporium* in CD liquid medium and wheat straw was studied. The lowest amount of patulin produced in CD medium by the combination of *P. expansum* and *Phanerochaete chrysosporium*

Plate 4. 3

Wheat straw inoculated with *Penicillium expansum* after 14 days of incubation at 25°C.

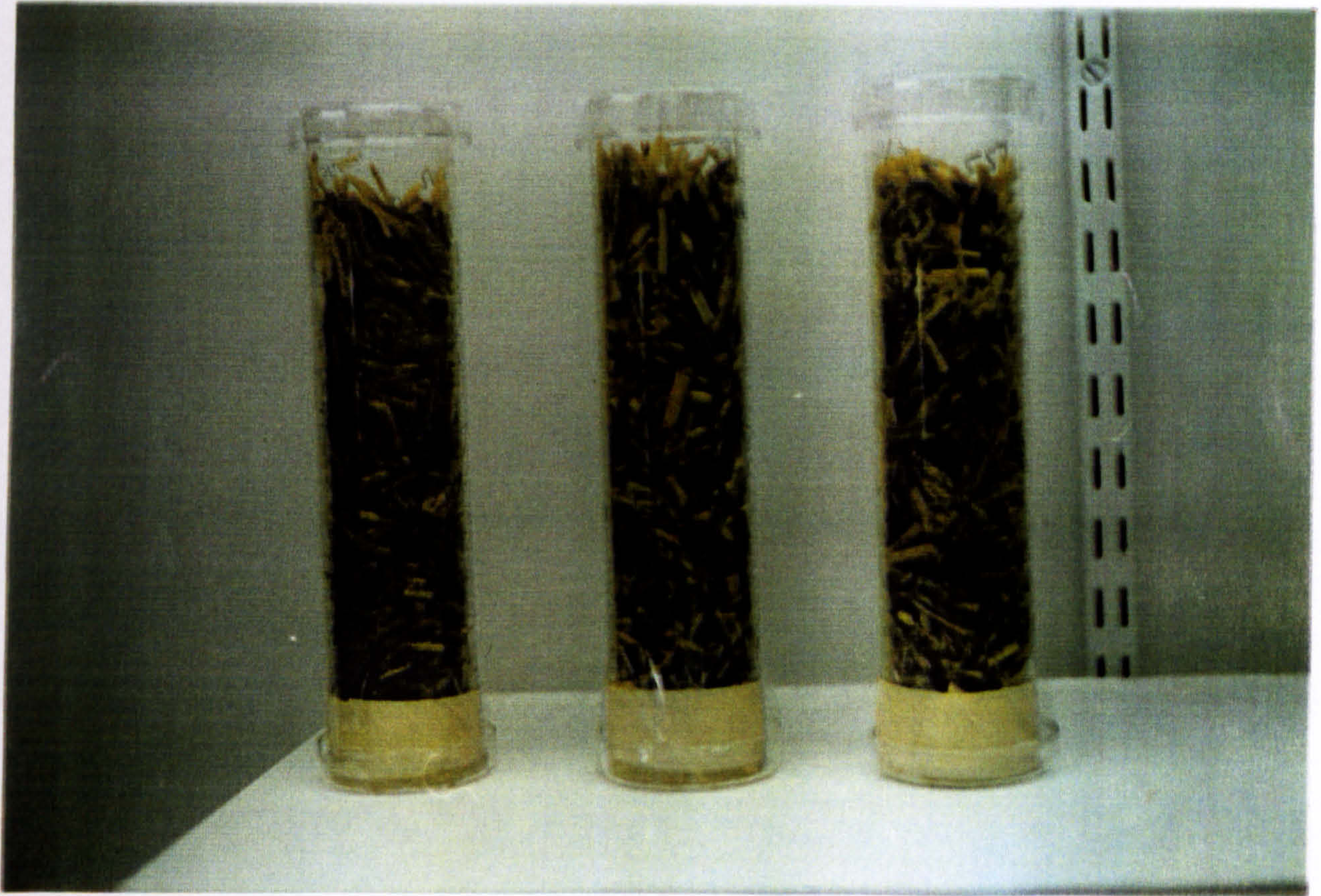


Plate 4. 4

Wheat straw inoculated with both *P. expansum* and *P. chrysosporium* after 7 days of incubation at 25°C.

indicated that both fungi were in competition for nutrients available in the medium or produced some natural inhibitor. Furthermore, inoculation of *P. expansum* culture, after 7 days of incubation, with the white-rot fungus *Phanerochaete chrysosporium* did not effect the productivity of patulin with production of the toxin increasing at the final period of incubation.

Although patulin has been detected in raw wheat straw (El-Kady, 1986), the raw straw sample used here did not contain patulin at the beginning of the experiment.

Indigenous straw fungi are unable to produce patulin on wheat straw under non-sterile conditions. On the other hand, inoculation of wheat straw with *P. expansum* alone showed that sterilized wheat straw can support patulin production by this fungus after 14 days of incubation. It was also clear that 21 days of incubation is an optimum time for patulin production on by *P. expansum* growing on wheat straw. Patulin formation on wheat straw has previously been reported by Grossbard (1948) who concluded that *P. patulum* was able to produce patulin when grown on sterilized wheat straw.

To demonstrate the effect of co-culture on patulin production on a natural substrate both *P. expansum* and *Phanerochaete chrysosporium* were grown together. It is perhaps surprising that *P. expansum* was incapable of producing patulin when growing in association with *Phanerochaete chrysosporium* on straw. Possibly the white rot fungi require available metabolizable carbon cosubstrate in order to degrade lignin (Kirk *et al.*, 1976) and organic nitrogen to stimulate lignin degradation (Barder and Crawford, 1981).

5. STUDIES ON THE INTER-RELATIONSHIP BETWEEN NITRIFICATION AND PATULIN PRODUCTION BY FUNGI

5. STUDIES ON THE INTER-RELATIONSHIP BETWEEN NITRIFICATION AND PATULIN PRODUCTION BY FUNGI

5. 1. Introduction

Nitrogen plays a crucial role in the metabolism of animals, plants and microbes. Microorganisms play a major part in nitrogen transformations and are largely responsible for mediating the transformation of organic, inorganic, and volatile N-compounds.

5. 1. i. Ammonification

The process of ammonification involves the production of ammonia or ammonium ions through the mineralization of organic nitrogen (Atlas and Bartha, 1987).

Microorganisms including species of *Bacillus*, *Pseudomonas*, *Micrococcus*, *Arthrobacter*, *Achromobacter*, *Clostridium* and *Coryneformbacterium* and numerous fungal species of for example, the genera *Aspergillus* and *Penicillium* are capable of hydrolysing proteins to ammonia (Nicholas, 1965; Roberge and Knowles, 1967; Hawker and Linton, 1979). Others such as *Aspergillus nidulans* and *Dictyostelium discoideum* can convert nitrite from a simple nitrogenous organic compound to ammonium and ammonium ions (Pateman and Kinghorn, 1976). Among the bacterial genera *Bacillus*, *Pseudomonas* and *Mycobacterium* and the fungal genera *Aspergillus*, *Penicillium*, *Fusarium*, *Mucor*, *Rhizopus* and *Cephalosporium* there are several species responsible for breaking down proteinaceous material (i.e., nucleic acids, purines and pyrimidines or their derivatives) and liberating ammonia (Alexander, 1977; Hawker and Linton, 1979).

5. 1. ii. Nitrification

As described above that the final product of the mineralization of organic and inorganic nitrogen is ammonia or ammonium ions. A number of autotrophic bacteria including members of the genera *Nitrosomonas*, *Nitrosospira*, *Nitrosococcus*, *Nitrosovibrio* and *Nitrosolobus* can oxidize ammonia to nitrite (equation 1); while members of the genera of *Nitrobacter*, *Nitrospira* and *Nitrococcus* oxidize nitrite to nitrate (equation 2) (Campbell, 1977).

(1) $2NH_4^+ + 3O_2 \longrightarrow 2NO_2^- + 4H^+ + 2H_2O$

(2) $2NO_2^- + O_2 \longrightarrow 2NO_3^-$

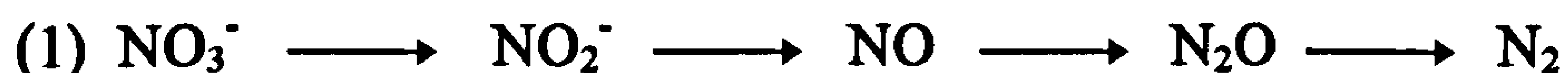
Similarly, heterotrophic bacteria and fungi can also nitrify (Odu and Adeoye, 1970); some, including species of *Aspergillus*, *Fusarium*, and *Penicillium* are capable of hydrolyzing urea and nitrifying ammonium (Killham, 1986). *Aspergillus flavus* has been observed to produce significant amounts of nitrate from both organic and inorganic nitrogen sources (Schimel *et al.*, 1984). Moreover, a species of *Penicillium* isolated from an acid environment can nitrify at low pH (pH 5) (Remacle, 1977; Johnsrud, 1978).

Fungal nitrification probably involves intermediates such as hydroxylamine, the nitroxyl radicle, hyponitrite and nitrohydroxylamine, although the exact pathways involved have yet to be determined. Oxidation of these products leads to nitrite then to nitrate (Campbell, 1977).

5. 1. iii. Denitrification

The process whereby nitrate is converted, via reduction processes, through nitrite to molecular nitrogen or to nitrous oxide is termed denitrification. Several members of the genera *Pseudomonus*, *Achromobacter* and *Bacillus* are active denitrifiers (Hawker and

Linton, 1979). Focht and Verstraete (1977) reported that bacteria like *Paracoccus denitrificans* reduce nitrate through nitrite to nitric oxide (NO), nitrous oxide (N_2O) and then molecular nitrogen (equation 1). Other denitrifying bacteria include species of *Alcaligenes*, *Rhizobium*, *Propionibacterium*, *Azospirillum* and *Rhodopseudomonas*.



(1) $NO_3^- \longrightarrow NO_2^- \longrightarrow NO \longrightarrow N_2O \longrightarrow N_2$

There have been reports suggesting that fungi can denitrify, the species involved being *Aspergillus nidulans*, *Neurospora crassa*, *Penicillium chrysogenum* and *Saccharomyces cerevisiae* (Pateman and Kinghorn, 1976). Although nitrite reductase to nitrous oxide has been associated with species of *Fusarium oxysporum* and *F. solani* and other fungi including species of *Aspergillus* and *Acremonium* (Bollag and Tung, 1972; Burth and Ottow, 1981; Malinowsky and Ottow, 1985).

5. 1. iv. Conversion of nitrogen and patulin production

A number of factors influence patulin production in fungi, including the type and quantity of N source. Patulin production is also sensitive to pH, being reduced at 3.2 to 3.8 (Damoglou and Campbell, 1986); a fact which is relevant to the observation that a fall in medium pH usually follows ammonium utilization by fungi (Garraway and Evans, 1984; Rollins and Gaucher, 1994).

The research in this chapter was aimed at studying the ability of patulin producing fungi to grow, nitrify and produce patulin in Czapek Dox N-free liquid medium to which was added either ammonium or urea as a nitrification substrate.

5. 2. Materials and Methods

5. 2. 1. Media preparation

The basal medium, free of nitrogen, was prepared by dissolving 0.5 g of potassium chloride (KCl), 0.5 g magnesium glycerophosphate, 0.01 g $FeSO_4.7H_2O$, 0.35 g potassium sulphate (K_2SO_4) and 30 g of sucrose in 1000 ml of distilled water. The pH was adjusted to 6.8 by 2N NaOH. The constituents of the basal medium were dispensed into Erlenmeyer flasks (50 ml in 250 ml flask), then autoclaved at 121°C for 20 minutes (Appendix 9. 2. c). The flasks were divided into two sets. One set was amended with membrane filter-sterilized (pore size 0.2 µm, Millipore filter) ammonium sulphate to achieve a concentration of 500 µg ml^{-1} while the other set received membrane filter-sterilized urea solution (500 µg ml^{-1}).

5. 2. 2. Inoculum and incubation

The patulin producing fungi, *Penicillium* sp (1) and *Penicillium* sp (3), and *Penicillium expansum* obtained from the CAB International Mycological Institute, Surrey, were grown on Czapek Dox agar (Oxoid) slopes at 25°C for 7 days. Spore suspensions of each culture were harvested by gentle agitation in sterile distilled water. Using a sterile loop, 1 ml of each fungus suspension (10^6 - 10^7) was inoculated in triplicate then incubated at 25°C on a reciprocal shaker (150 rev. min^{-1}) for 7, 14, 21 and 28 days. Non-inoculated flasks were included to account for any non-biological nitrification. At the end of period of incubation the biomass, ammonium ions, nitrate and patulin produced in the culture were determined as follows:-

5. 2. 3. Determination of fungal biomass

After each 7 days of incubation the flasks contents were filtered through pre-dried and pre-weighed Whatman No. 1 filter papers. The filter papers were dried at 50°C for 3 days to constant weight then the biomass was calculated as g 50^{-1} ml of the medium.

5. 2. 4. Determination of ammonium nitrogen

Ammonium nitrogen ions were determined colorimetrically by using the method described by Wainwright and Pugh (1973) (Appendix 9. 4. a). In this analysis 2 ml of the culture filtrate were mixed thoroughly with 1 ml of EDTA, (6% w/v), 7 ml of H_2O (distilled), 5 ml of phenolate reagent, and 3 ml of sodium hypochlorite solution (0.9% v/v active chlorine), were then incubated in the dark at 25°C for 20 minutes. The volume of was made up to 50 ml and mixed. The intensity of the indophenol-blue-ammonium complex was measured colorimetricaly at 630 nm. The concentration of NH_4^+-N was determined by reference to a standard curve (0 - 100 μg NH_4^+-N ml^{-1}) prepared from a standard solution of $(NH_4)_2SO_4$.

5. 2. 4. i. Reagents used:

5. 2. 4. i. a. Ethylenediaminetetraacetic acid (EDTA).

Ethylenediaminetetraacetic acid was prepared by dissolving 60 g of the powder in 900 ml distilled water, then the solution was diluted to 1 liter. This solution is stable for two months.

5. 2. 4. i. b. Phenolate reagent.

For the phenolate reagent 20 ml phenol solution (5. 2. 4. i. c) were mixed with 20 ml caustic soda solution (5. 2. 4. i. d) and diluting the solution to 100 ml with distilled water. The reagent was prepared freshly as required.

5. 2. 4. i. c. Phenol solution.

Phenol was prepared by dissolving 62.5 g phenol in a minimum amount of absolute ethanol (19 ml) and adding 18.5 ml acetone to give a final volume of 100 ml. The phenol solution was stored in the dark at 4°C.

5. 2. 4. i. d. Caustic soda solution.

Caustic soda solution was prepared by dissolving 27 g of sodium hydroxide (NaOH) in 100 ml distilled water.

5. 2. 4. i. e. Sodium hypochlorite solution.

Sodium hypochlorite solution was prepared from an active chlorine solution to give a final concentration of 0.9% v/v.

5. 2. 5. Determination of nitrate nitrogen

Nitrate nitrogen ions were determined by using Orange (1) method according to the procedure described by Middleton (1959) (Appendix 9. 4. b). In this method 2.5 ml of the culture filtrate were treated with 50 ml of reagent 1, 1 ml of reagent 2, and 0.2 g of reagent 3. The mixture was shaken for 5 minutes in closed flasks and then filtered immediately through Whatman No.1 filter paper. To 20 ml of the filtrate, 5 ml of reagent 4 were added then mixed thoroughly. The solution was filtered again using Whatman No. 1 filter paper and then left for 30 minutes until an orange colour appeared. This colour was then measured colorimetricaly at 475 nm and the concentration of NO_3^--N was determined by reference to a standard curve (0 - 1000 μg NO_3^--N ml^{-1}) prepared from a standard solution of sodium nitrate ($NaNO_3$).

5. 2. 5. i. Reagents used:

5. 2. 5. i. a. Reagent (1). Prepared by dissolving 12.5 g of calcium acetate in 350 ml Erlenmeyer flasks with 200 ml of distilled water and 5 ml of aqueous NH_3 (sp. gr. 0.8, 35% NH_3). Then 1 ml of reagent 2 (5. 2. 5. i. b) and 0.2 g of zinc dust (Zn) were added and the mixture was shaken thoroughly and left overnight in the dark. The flask contents was then boiled for 30 minutes and filtered through Whatman No. 1 filter paper. Aqueous NH_3 (100 ml) was then added to the filtrate and the volume was adjusted to 2500 ml with distilled water.

5. 2. 5. i. b. Reagent (2). Prepared by dissolving 1 g of manganese sulphate ($MnSO_4.4H_2O$) in 100 ml aqueous acetic acid (5% glacial acetic acid).

5. 2. 5. i. c. Reagent (3). Zinc dust powder.

5. 2. 5. i. d. Reagent (4). Prepared by dissolving 0.1 g of sulphanilic acid and 0.08 g of α-naphthol in 100 ml of 75% aqueous acetic acid (75 ml glacial acetic acid in 25 ml distilled H_2O).

5. 2. 6. Determination of patulin

Patulin was extracted and determined as described in Appendix 9. 6 a to g.

5. 3. Results and Discussion

5. 3. 1. Determination of fungal biomass

The biomass of all three fungi increased over the twenty eight days incubation period, with *Penicillium* sp (1) achieving the greatest biomass (Fig. 5. 1 and 5. 2). The concentration of added urea and ammonium did not therefore prevent fungal growth.

5. 3. 2. Nitrification of ammonium

Net nitrate production by the three fungi in media containing ammonium as nitrification substrate is shown in Figures 5. 3, 5. 5 and 5. 7. The only fungus which produced a net increase in nitrogen concentration was *Penicillium* sp 1 (Fig. 5. 5). Medium inoculated with the other two fungi showed a decrease in net nitrate concentration. Presumably in these media nitrate assimilation or possibly denitrification exceeded nitrification. The amount of nitrate produced by *Penicillium* sp (1) is small (approximately 13.5 $\mu g\ ml^{-1}$). Similar limited nitrification is achieved by other fungi when ammonium is used as N-substrate (Killham, 1986).

5. 3. 3. Urea utilization

Growth of all three fungi on medium containing urea as N-source led to a marked increase in ammonium concentration and a decrease in net nitrate concentration (Fig 5. 4 and 5. 6 and 5. 8). Clearly all of the fungi have the ability to liberate ammonium from urea (i. e. urea hydrolysis). However, none of the fungi nitrified the liberated ammonium. The decreased nitrate level is possibly due to either nitrate ammonification or denitrification. However, it is not clear why the fungi accumulated nitrate when large amounts of ammonium are being liberated from urea. It is possible however, that pH change in the medium influenced whether nitrate or ammonium was assimilated and utilized.

5. 3. 4. Patulin production

Figure 5. 1

Biomass of *Penicillium expansum*, *Penicillium* sp (1) and *Penicillium* sp (3) during ammonium (500 μg ml^{-1}) nitrification.

Values-means of triplicates ± standard deviation.
Significantly different from control ($P < 0.05$) except where marked *.

Figure 5. 2

Biomass of *Penicillium expansum*, *Penicillium* sp (1) and *Penicillium* sp (3) during urea (500 μg ml^{-1}) hydrolysis.

Values-means of triplicates ± standard deviation.
Significantly different from control ($P < 0.05$) except where marked *.

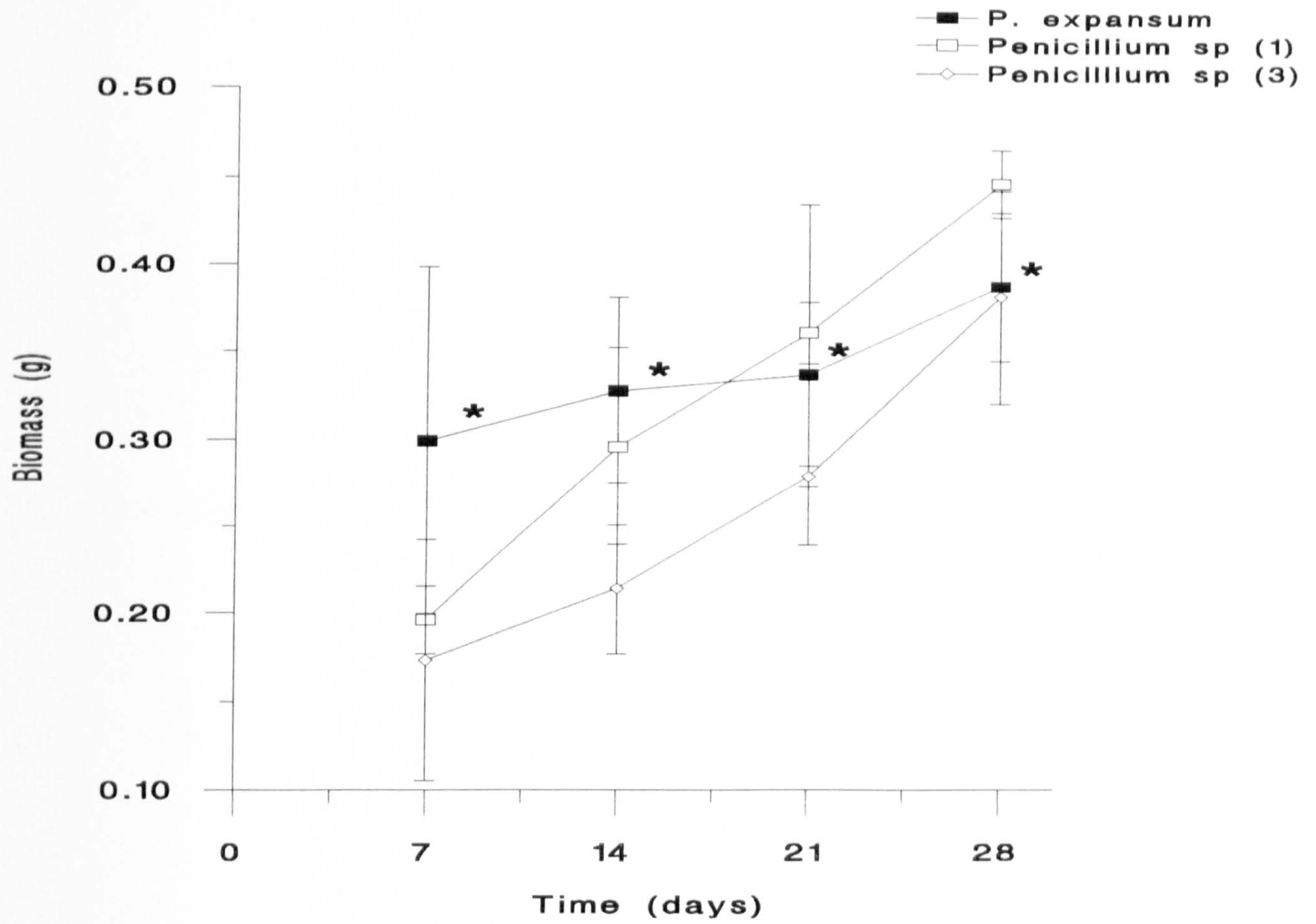


FIG. 5.1

FIG. 5.2

P. expansum
Penicillium sp (1)
Penicillium sp (3)

0.35
0.25
0.15
Biomass (g)

0
7
14
21
28
Time (days)

Figure 5. 3

Nitrate detected (μg ml^{-1}) during ammonium nitrification (500 μg ml^{-1}) by *Penicillium expansum*.

Values-means of triplicates ± standard deviation.

Significantly different from control ($P < 0.05$) except where marked *.

Figure 5. 4

Ammonium and nitrate detected (μg ml^{-1}) during urea hydrolysis (500 μg ml^{-1}) by *Penicillium expansum*.

Values-means of triplicates ± standard deviation.

FIG. 5.3

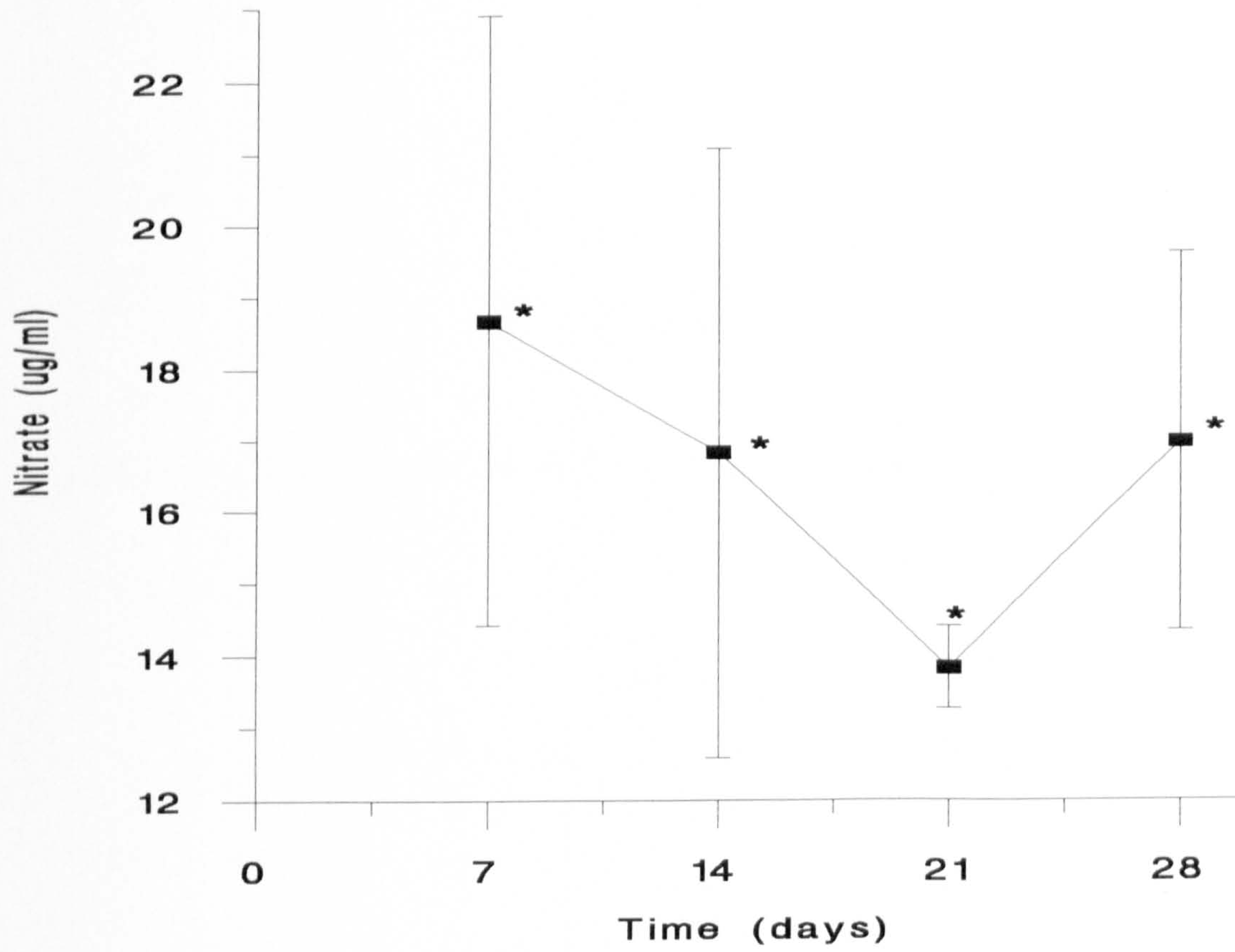


FIG. 5.4

Figure 5. 5

Nitrate detected (μg ml^{-1}) during ammonium nitrification (500 μg ml^{-1}) by *Penicillium* sp (1).

Values-means of triplicates ± standard deviation.

Significantly different from control ($P < 0.05$) except where marked *.

Figure 5. 6

Ammonium and nitrate detected (μg ml^{-1}) during urea hydrolysis (500 μg ml^{-1}) by *Penicillium* sp (1).

Values-means of triplicates ± standard deviation.

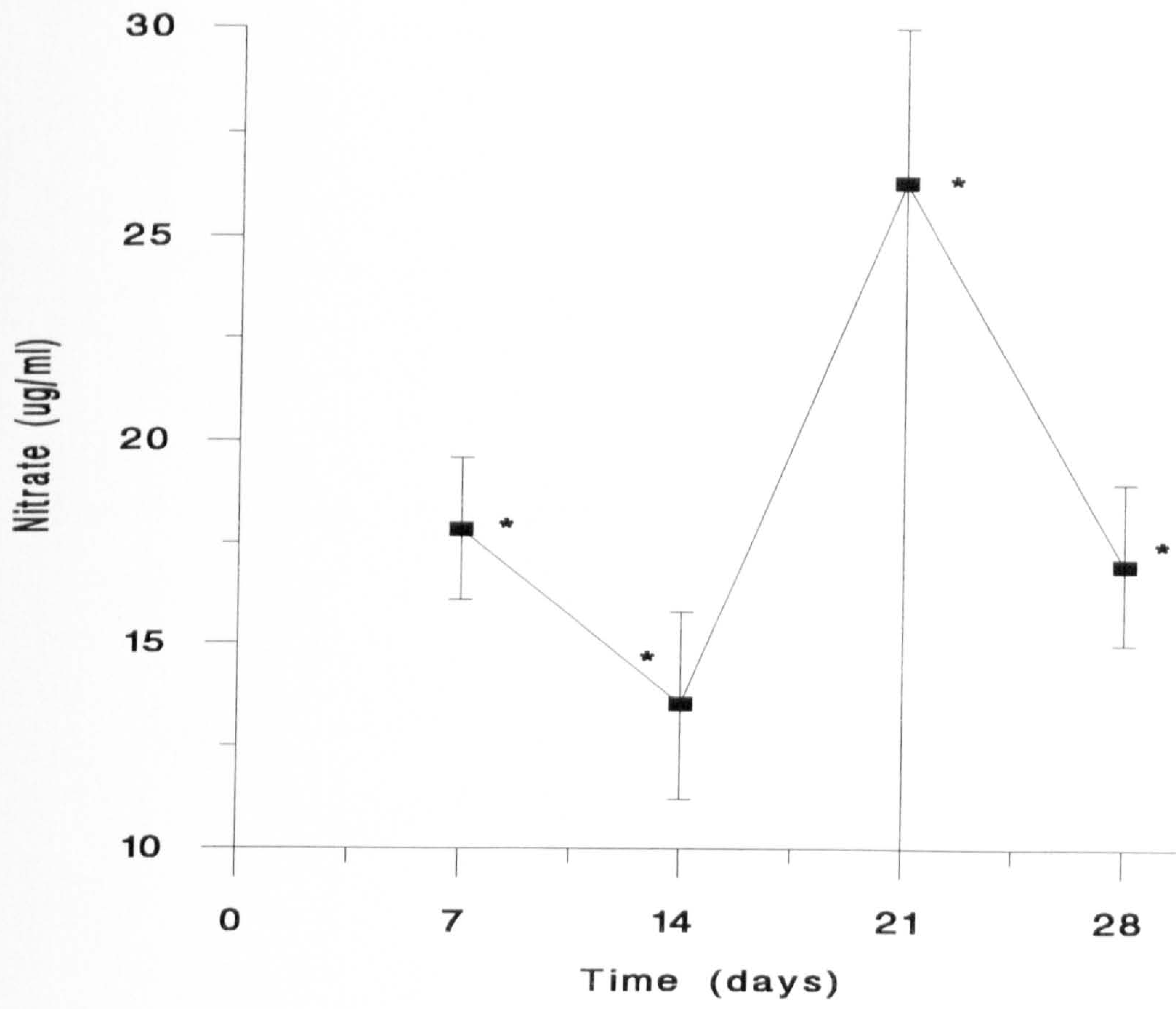


FIG. 5.6

Ammonium
Nitrate
3000
2500
2000
1500
1000
500
0
Ammonium (ug/ml)
20
15
10
5
0
Nitrate (ug/ml)
0
7
14
21
28
Time (days)

Figure 5. 7

Nitrate detected (μg ml^{-1}) during ammonium nitrification (500 μg ml^{-1}) by *Penicillium* sp (3).

Values-means of triplicates ± standard deviation.

Figure 5. 8

Ammonium and nitrate detected (μg ml^{-1}) during urea hydrolysis (500 μg ml^{-1}) by *Penicillium* sp (3).

Values-means of triplicates ± standard deviation.

FIG. 5.7

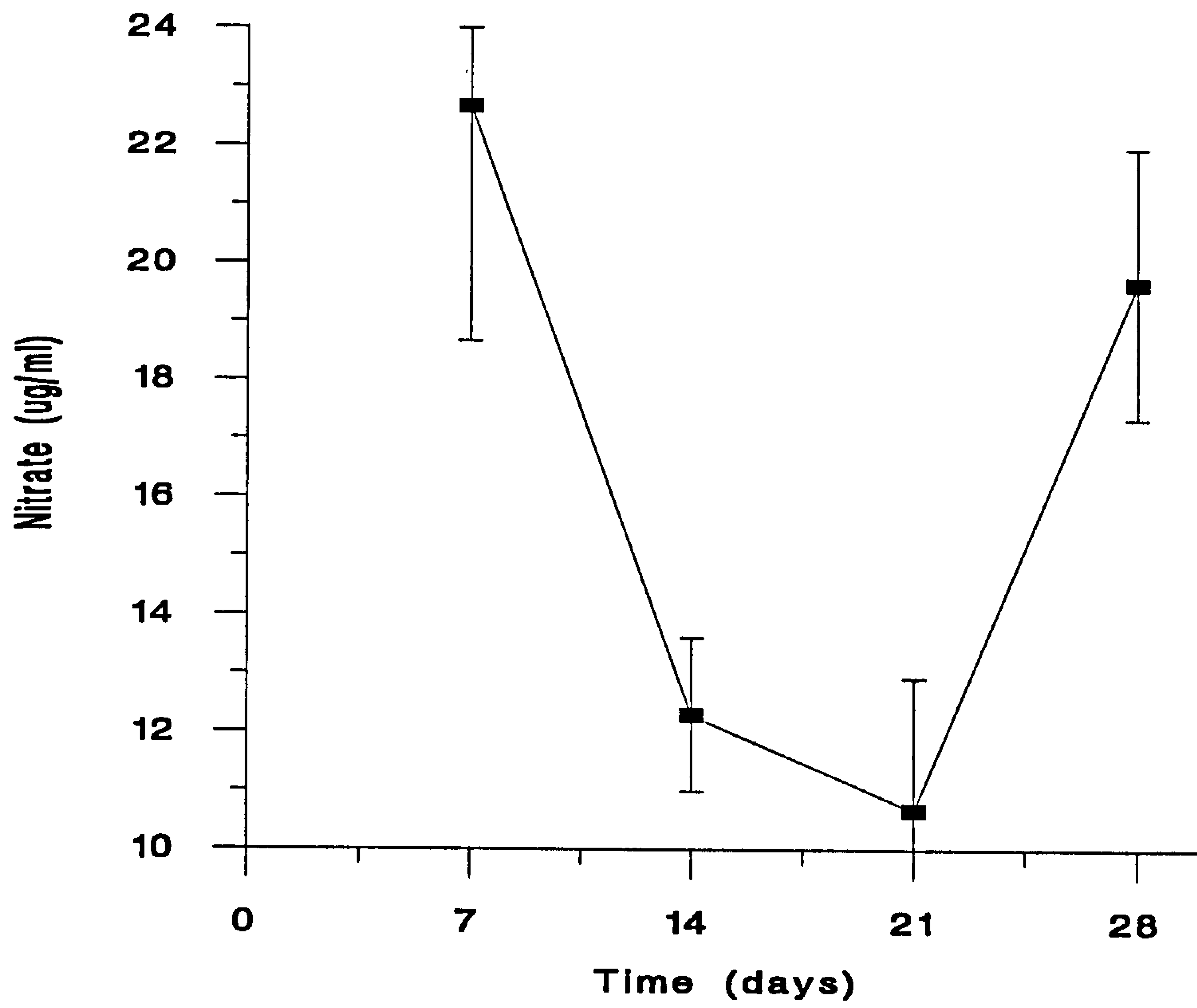


FIG. 5.8

Penicillium expansum produced patulin when ammonium was added at the 14 d sample. After this time, no patulin was detected (Fig 5. 9 and 5. 10). *Penicillium* sp (3) produced patulin after 7 days, but the amount detected decreased over the incubation period. *Penicillium* sp (1) on the other hand did not produce patulin at any period over the incubation period. Clearly the amount of patulin decreased, a fact which reflects both production and degradation. *Penicillium* sp (1) appears not to have produced patulin, while the other two fungi while initially producing the toxin, degrade patulin towards the end of the incubation period.

These was no obvious correlation between nitrification and patulin production in that the only fungus which nitrified was *Penicillium* sp (1), and this isolate failed to produce patulin.

The same pattern of patulin production was seen when urea was used in place of ammonium in the N-source. This shows that patulin production is related neither solely to urea hydrolysis or to nitrification.

5. 4. Conclusion

The three fungi used in this study were all capable of urea hydrolysis, but only one (*Penicillium* sp 1) was capable of nitrification, and this was very limited. No correlation could be found between the addition of urea or ammonium, nor with nitrification and patulin production. It is likely that pH is the main factor influencing patulin production in these experiments. Damoglou and Campbell, (1986) and Rice *et al.*, (1977) reported the production of patulin by a strain of *Penicillium expansum* over a pH range from 2.8 to 4.22 but the optimum pH was ranged from 3.2 to 3.8. On the other hand, patulin producing enzymes can be detected at a pH less than 6, whereas it is unstable at a pH near 6.0 (Sanchis *et al.* 1992).

Figure 5. 9

Concentration of patulin detected ($\mu g\ l^{-1}$) during ammonium nitrification (500 $\mu g\ ml^{-1}$) by *Penicillium expansum*, *Penicillium* sp (1) and *Penicillium* sp (3).

Values-means of triplicates ± standard deviation.

Significantly different from control ($P < 0.05$) except where marked *.

Figure 5. 10

Concentration of patulin detected ($\mu g\ l^{-1}$) during urea hydrolysis (500 $\mu g\ ml^{-1}$) by *Penicillium expansum*, *Penicillium* sp (1) and *Penicillium* sp (3).

Values-means of triplicates ± standard deviation.

Significantly different from control ($P < 0.05$) except where marked *.

FIG. 5.9

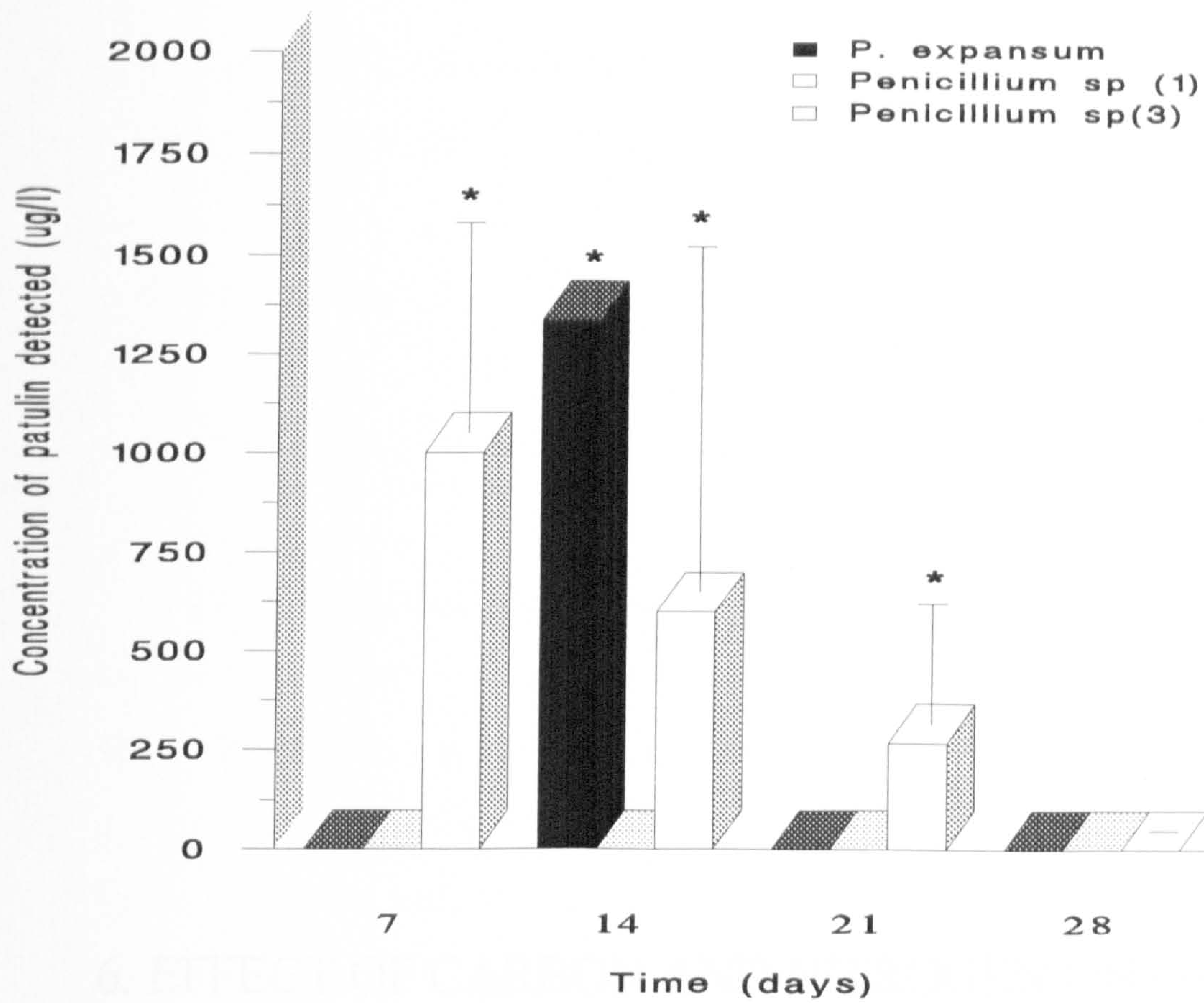
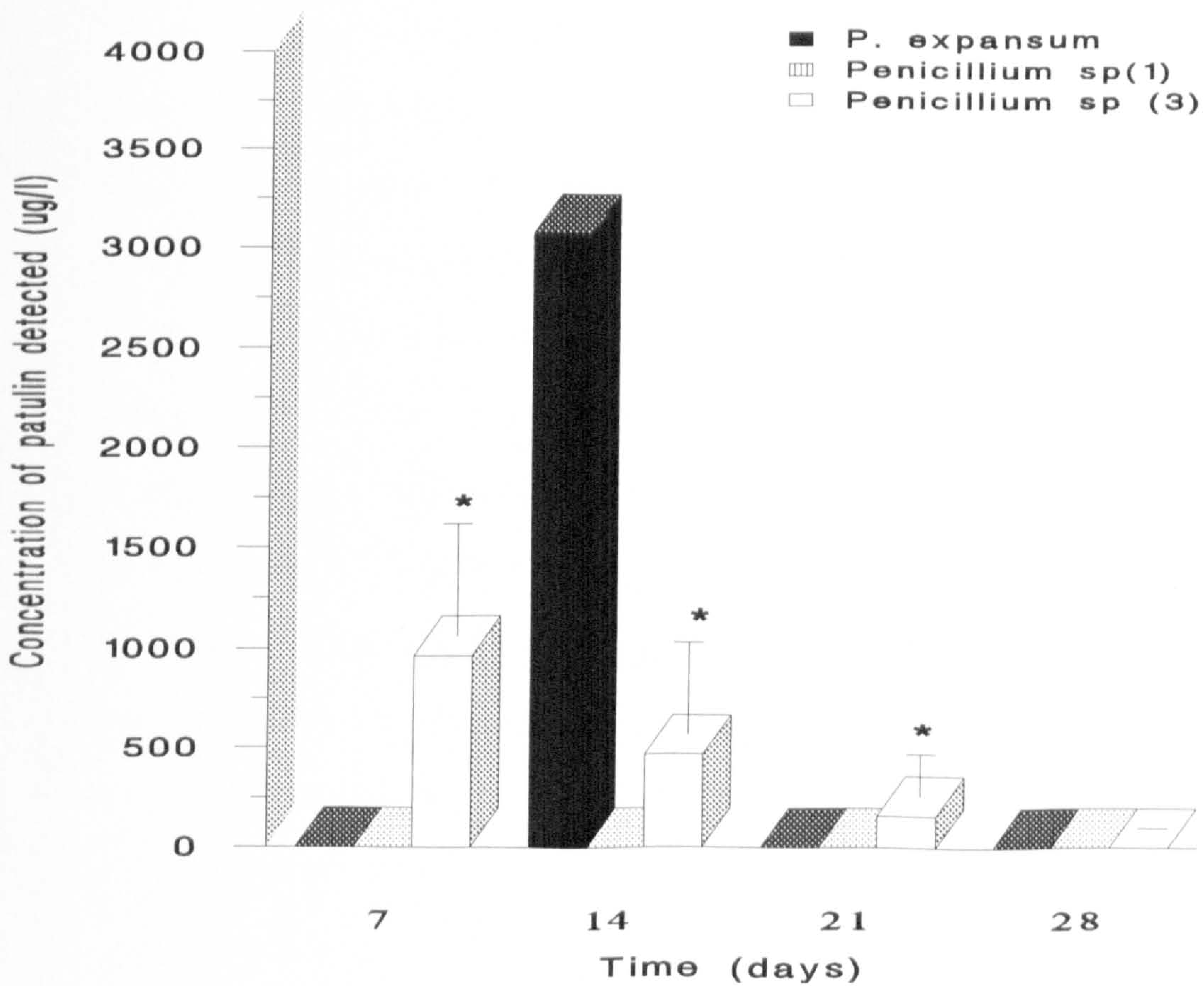


FIG. 5.10



6. EFFECT OF CARBON AND NITROGEN ON GROWTH OF FUNGI AND PATULIN PRODUCTION

6. EFFECT OF CARBON AND NITROGEN ON GROWTH OF FUNGI AND PATULIN PRODUCTION

6. 1. Introduction

6. 1. i. Relationship of carbon and nitrogen to fungal growth

Microorganisms require energy in order to grow, the habitat being the source of nutrients. The presence or absence of an essential element, in the environment obviously determines whether or not an organism can grow there. Conversely, the growth of an organism in an environment is of itself evidence that an essential nutrient is present. Carbon and nitrogen are the two most critical elements influencing fungal growth and activity. Fungi generally use simple carbon sources such as D-glucose to synthesize all their require substances. These type of fungi have catabolic and anabolic enzymes. On the other hand, some fungi lack such enzymes, which means they require several sources of carbon to grow (Hawker and Linton, 1979).

Earlier studies have shown that various microorganisms can grow in the apparent absence of added carbon (Castellani, 1939; Stern *et al.*, 1956). A few early studies suggested that a number of fungi including strains of *Actinomucor elegans*; *Fusarium culmorum*; *F. solani*; *Gliocladium roseum*; *Mucor hiemalis*; *Penicillium corymbiferum*; *Trichoderma viride* and *Zyorhynchus moelleri* can grow in media lacking carbon. These fungi are able to germinate and grow on silica gel, in the absence of added carbon (Tribe and Mabadeje, 1972). Moreover, Mirocha and Devay (1971) and Jones *et al.* (1991) showed that *Cephalosporium spp*, *Fusarium spp* and *F. oxysporum* can grow on carbon-free medium lacking all complex organic carbon. All these fungi appear to use organic compounds by scavenging trace amount of nutrients from the atmosphere as gases and volatiles, or otherwise from the substratum (Mirocha

and Devay, 1971; Postgate, 1988; Wainwright *et al.*, 1991; Wainwright and Barakah, 1993).

Nitrogen is required by fungi to synthesis proteins and nucleic acids. Ammonium salts and nitrate (but not nitrite) are the most important nitrogen sources used by fungi (Hawker and Linton, 1979). Numerous fungal species including actinomycetes are able to grow oligonitrotrophically where they can scavenge combined nitrogen from the atmosphere (Andriyuk, 1967). Parkinson *et al.* (1989) reported the ability of *Aspergillus flavus*, *Aspergillus niger*, *Mucor rouxii*, *Penicillium chrysogenum*, *Fusarium solani* and *F. spp* to grow on silica gel whereas no nitrogen was added.

6. 1. ii. Relationship between patulin production and levels of carbon and nitrogen present in the medium

Nutrition is an important key factor present concerning the relationship between secondary metabolites biosynthesis and environment. These metabolites usually depend on environmental factors affecting there products. Such factors are including carbon and nitrogen sources. Indeed, patulin biosynthesis becomes active in the presence of both carbon and nitrogen (Garraway and Evans, 1984). As carbon source is required for patulin production, Pytel and Borecka (1982) reported that the productivity of fourteen strains of *Penicillium expansum* to patulin was high on Czapek liquid medium than the quantity detected in apple tissues. Moreover, fructose was found to be a more favorable carbon source for patulin production than sucrose whereas some strains of *Penicillium expansum* excreted high levels of patulin on Czapek-Dox liquid medium containing fructose as a carbon source than the quantity detected from Czapek Dox medium (Podgorska, 1993). Grootwassink and Gaucher (1980) reported patulin production by several fungal species in medium containing either glucose-nitrate or glucose yeast extract medium.

The role of nitrogen on patulin production cannot be neglected because workers have shown nitrogen consumption during patulin production. Moreover, nitrogen leads to the accumulation of primary metabolic intermediate which then induce secondary metabolic producing enzymes (Bu`Lock, 1961). Acetylation of nitrogen and oxidation afford analogous intermediates from 6-methylsalicylic acid where patulin then produced (Gould *et al.*, 1989). Ammonium sulphate as a nitrogen source was found to stimulate the production of quinone epoxide phyllostine, a product of gentisyl alcohol epoxidation, by *Penicillium patulum* which is an intermediate in the biosynthesis of patulin (Priest and Light, 1989).

In general, patulin biosynthesis require a carbon source, as well as a high consumption of nitrogen (Grootwassink and Gaucher, 1980). Microorganisms growing oligotrophically never achieve large biomass yields, and are unlikely to produce primary and secondary metabolites (Dorofeev *et al.*, 1984; Wainwright *et al.*, 1993).

The research described in this Chapter was aimed at determining the following:

(i) The ability of the isolated fungi to produce patulin on Czapek Dox liquid medium and to grow as oligotrophs *in vitro*.

(ii) The effect of addition of various levels of carbon and/or nitrogen on fungal growth and patulin production.

6. 2. Materials and Methods

6. 2. 1. Ability of the isolated fungi to grow oligotrophically

6. 2. 1. i. Preparation of silica gel

Ultra carbon-free (UCF) silica gel medium free of carbon and nitrogen was prepared as described by Parkinson *et al.* (1989) using glassware which had been washed with chromic acid and then rinsed with purified distilled water (pdw). Glass Petri dishes

were acid washed and then heated to 150°C for 135 minutes in a muffle furnace to remove all traces of organic carbon. The pdw was obtained by passing distilled water through a `Mill Q` filtration system (Milipore Corp.) which the manufacturer claims produces water containing less than 50 p.p.b. of organic carbon. Silica gel was prepared by mixing together potassium silicate (8 g silicic acid plus 7 g KOH in 100 ml pdw), orthophosphoric acid (20% v/v) and sterile salts solution (1 g KH_2PO_4, 0.5 g KCl, 0.5 g $MgSO_4.7H_2O$, 0.01 g $FeSO_4.7H_2O$ in 1000 ml pdw, pH 6.8 by 1.5 N NaOH) (Appendix 9. 1. d). The contents were mixed and then rapidly poured into glass Petri dishes; the gel sets in about 15 min. The plates were then left overnight when any water of syneresis was poured off. All constituents were autoclaved at 120° for 20 min. Precautions were taken to avoid nutrient contamination.

6. 2. 1. ii. Fungal inoculation

Fungi isolated from mouldy fruits which were found to produce patulin on Czapek Dox liquid medium and *Penicillium expansum* Link were grown on Czapek Dox agar at 25°C for 7 days. Agar discs (13 mm) were cut from the edge of the colony and transferred to UCF gel. After two weeks of incubation at 25°C, a mycelial disc was transferred to new UCF gel. This process was repeated on four successive occasions. Six replicates were used throughout for each of the fungal isolates.

6. 2. 1. iii. Preparation of samples for scanning electron microscopy (SEM)

Penicillium expansum Link, *Penicillium* sp (1), *Penicillium* sp (2) and *Penicillium* sp (3) grown on Czapek Dox agar and UCF were prepared for SEM to study the effect of carbon and nitrogen lacking medium on the growth of fungi (Appendix 9. 3).

6. 2. 2. Effect of addition of various levels of carbon and/or nitrogen on fungal growth and patulin production

6. 2. 2. i. Preparation of liquid medium

The basal medium, free of carbon and nitrogen contained the following (g): K_2HPO_4, 1; KCl, 0.5; $MgSO_4.7H_2O$, 0.5; and $FeSO_4.7H_2O$, 0.01 in 1000 ml of purified distilled water (pdw) was adjusted to pH 6.8 by 1.5 N NaOH). The constituent of the basal medium was dispensed into Erlenmeyer flasks (100 ml in 250 ml flasks). Sucrose and/or ammonium sulphate were amended as carbon and/or nitrogen source as the following:-

1. Carbon concentrations in 0, 10, 50, 100, 250, 500, 750 and 1000 $\mu g\ ml^{-1}$ with added nitrogen in 100 $\mu g\ ml^{-1}$.
2. Carbon concentrations in 0, 10, 50, 100, 250, 500, 750 and 1000 $\mu g\ ml^{-1}$ lacking nitrogen.
3. Nitrogen concentrations in 0, 10, 50, 100, 250, 500, 750 and 1000 $\mu g\ ml^{-1}$ with added carbon in 500 $\mu g\ ml^{-1}$.
4. Nitrogen concentrations in 0, 10, 50, 100, 250, 500, 750 and 1000 $\mu g\ ml^{-1}$ lacking carbon. The Flasks contents were sterilized by autoclaving at 120°C for 20 min.

6. 2. 2. ii. Fungal inoculation

A hyphal disc (13 mm) of *Penicillium expansum* Link, *Penicillium* sp (1), *Penicillium* sp (2) and *Penicillium* sp (3) obtained from the four successive occasions on the UCF medium was transferred into the 100 ml basal medium (in 250 ml Erlenmeyer flasks) containing carbon and/or nitrogen. The cultures were incubated at 25°C for two weeks on a reciprocal shaker (150 rev. min^{-1}).

6. 2. 2. iii. Determination of the biomass of oligotrophically grown fungi

At two weeks intervals, the contents of the flasks were filtered through pre-dried and pre-weighed Whatman No.1 filter paper for 15 days. The filters plus mycelium were dried at 50°C for three days to constant weight.

6. 2. 2. iv. Extraction of patulin

Patulin was extracted and determined as described above (Appendix 9. 6. a - g).

6. 2. 3. Effect of various amounts of carbon and/or nitrogen on fungal growth and patulin production

Penicillium expansum, *Penicillium* sp (1), *Penicillium* sp (2) and *Penicillium* sp (3), all of which were found incapable of producing patulin at a low levels of carbon and/or nitrogen were next grown in medium containing high concentration of carbon and/or nitrogen.

6. 2. 3. i. Preparation of liquid medium

The basal carbon and nitrogen free medium (as on 6. 2. 2. i) was amended with sucrose and/or ammonium sulphate to produce the following concentrations:-

1. Carbon concentrations in 1500, 2000, 2500 and 3000 $\mu g\ ml^{-1}$ with added nitrogen in 100 $\mu g\ ml^{-1}$.
2. Carbon concentrations in 1500, 2000, 2500 and 3000 $\mu g\ ml^{-1}$ lacking nitrogen.
3. Nitrogen concentrations in 1500, 2000, 2500, 3000 $\mu g\ ml^{-1}$ with added carbon in 500 $\mu g\ ml^{-1}$.

4. Nitrogen concentrations in 1500, 2000, 2500, 3000 μg ml^{-1} lacking carbon. Flasks contents were sterilized by autoclaving at 120°C for 20 min.

6. 2. 3. ii. Fungal inoculation

The flasks were inoculated with the isolates under investigation (procedure as described in 6. 2. 2. ii).

6. 2. 3. iii. Determination of the biomass of oligotrophically grown fungi

The biomass was determined as the procedure described in 6. 2. 2. iii.

6. 2. 3. iv. Patulin determination

Patulin was extracted and determined as the procedure described in Appendix 9. 6 a to g.

6. 2. 4. Effect of nitrogen source on fungal growth and patulin production by *Penicillium* sp (1)

Penicillium sp (1), which was found to produce patulin and patulin production increased by low levels of nitrogen (10 to 250 μg ml^{-1}) amended as ammonium sulphate (in the presence of carbon, 500 μg ml^{-1}), was further investigated by replacement of sodium nitrate (0, 10, 50, 100, 250, 500, 750 and 1000 μg ml^{-1}) plus sucrose at the concentration of 500 μg ml^{-1}.

6. 2. 4. i. Fungal inoculation

The flasks were inoculated with the isolates on investigation (procedure as described in 6. 2. 2. ii).

6. 2. 4. ii. Patulin detection

Patulin was extracted and determined as the procedure described in Appendix 9. 6 a to g.

6. 3. Results and Discussion

6. 3. 1. Oligotrophic of fungi grown on silica gel

The growth of fungi on nutrient free silica gel was first described by Tribe and Mabadeje (1972) and confirmed by Wainwright and Graytson (1988) and Parkinson *et al.* (1989). A range of fungi obtained from mouldy fruits and having the ability to produce patulin on Czapek Dox liquid medium were found to be able to grow on Ultra carbon-free (UCF) silica gel medium to which no either carbon or nitrogen sources were added. Plates 6. 1, 6. 2. a, 6. 3 and 6. 4. a show that the fungi also grew on nutrient rich medium (Czapek Dox agar); while Plates 6. 2. b and 6. 4. b show their ability to grow as facultative oligotrophs. Oligotrophic growth was, not surprisingly, found to be less dense than when full strength Czapek Dox medium was used. The hyphae continued to grow when transferred on successive occasions to fresh silica gel without obvious signs of loss of viability. The term 'super oligotrophs' could be used to describe this type of fungi which are able to grow in the apparent absence of any added nutrients, including vitamins and trace elements (Parkinson *et al.*, 1989; and Wainwright *et al.*, 1991). Since these fungi grew on UCF silica gel to which neither carbon nor nitrogen sources had been added they can simultaneously grow both oligocarbotrophically and oligonitrotrophically. Under these conditions presumably

meet their nutrient requirements by scavenging them from the atmosphere. Mirocha and Devay (1971); and Barakah (1992) reported that some fungi grow on silica gel even when the air entering the growth chamber has been scrubbed free of CO_2 and organic carbon. Fungal growth on silica gel was invisible to the naked eye but clearly observed under the low magnification light microscope. Plates 6. 2. b and 6. 4. b show mycelium grown on nutrient free silica gel. The mycelium branches out from the surface of the solid substratum, often connected to form a mycelial network showing small hyphae and few spore structures. On the other hand, it is also worth noting that limited spore production was observed (Plate. 6. 2. b and 6. 4. b). Growth of *P. expansum* and the other isolates in the silica gel occurred in limit aerial mycelium network and spores (Plates 6. 5 to 6. 10). A small amount of hyphae was produced by *Penicillium* sp (2) (Plate 6. 9). When the hyphae of the fungi were transferred from the silica gel to Czapek Dox medium, growth occurred, showing that these fungi are facultative oligotrophs, a result which agrees with the findings of Parkinson *et al.*, (1989). The isolated fungus *Penicillium* sp (2) was found not to grow in silica gel after the third transfers. Oligotrophic growth was observed under the light microscope and by using SEM. Hyphae were seen to form a shallow groove in the silica gel (Plate 6. 11 to 6. 14). Similar observation were reported by Parkinson *et al.* (1989) and Barakah (1992). Similar groove-pitting was observed by Richards (1949) when fungi grow on glass.

6. 3. 2. Growth of fungi in liquid medium under oligotrophic conditions

As might be expected, in the absence of added carbon and nitrogen only a small amount of biomass was obtained. Figs. 6. 1 and 6. 2 show the biomass of *Penicillium* sp (1) grown oligotrophically. Fig. 6. 1 shows that *Penicillium* sp 1 can grow in the presence of relatively small amounts of carbon (10 - 1000 μg C ml^{-1}). It failed however, to grow in media lacking added carbon. The addition of nitrogen to the medium (100 μg ml^{-1}) generally had no significant effect on growth.

Plate 6.1

Penicillium expansum hyphal growth on Czapek Dox agar after 7 days of incubation at 25°C.

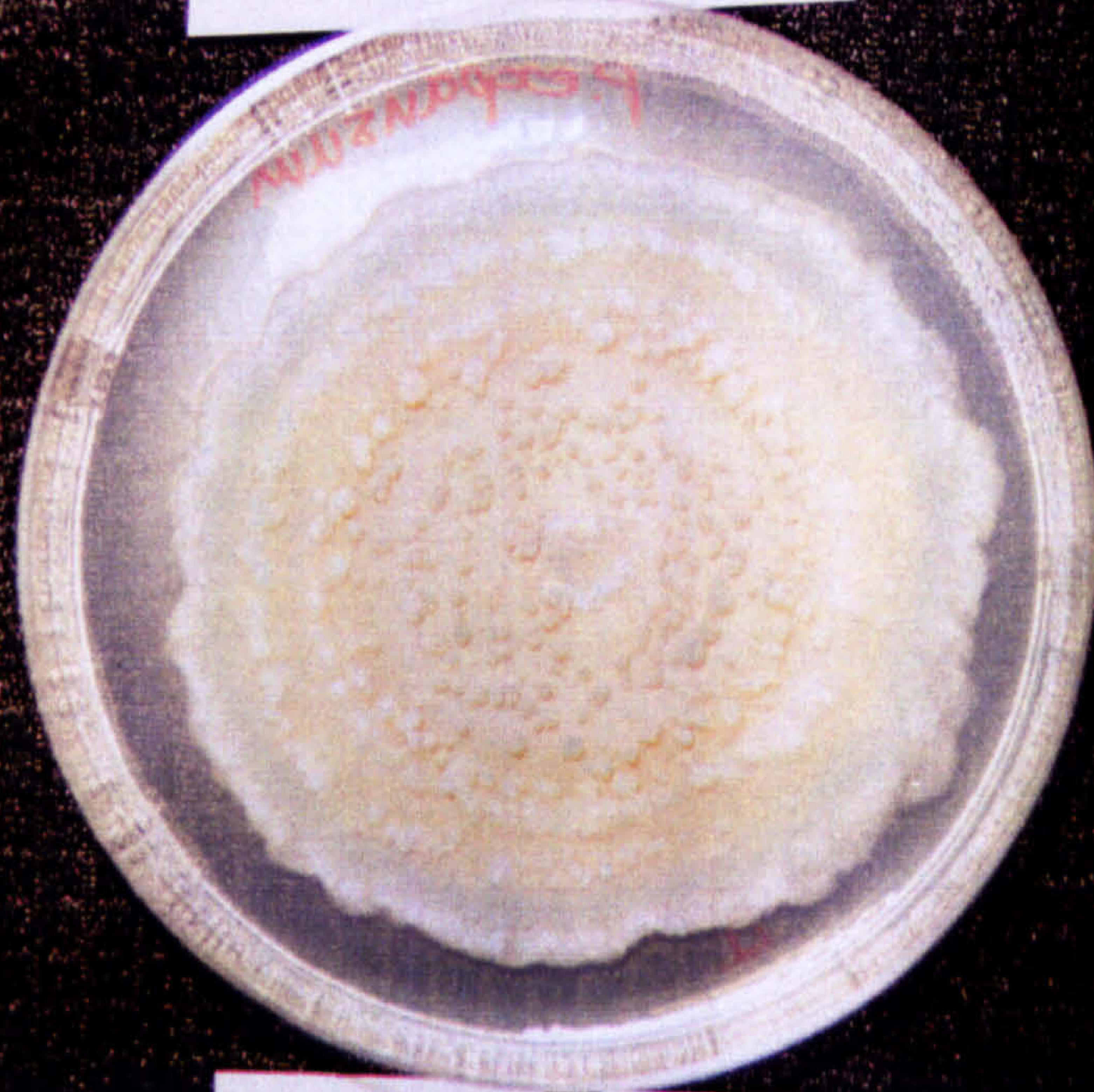

Penicillium expansum
CDA
25ºC

Plate 6.2.a

Penicillium sp (1) hyphal growth on Czapek Dox agar after 7 days of incubation at 25°C.

Plate 6.2.b

Light micrograph showing oligotrophically grown hyphae of *Penicillium* sp (1) (Final magnification x 400).

Penicillium sp (1)
CDA
25oC

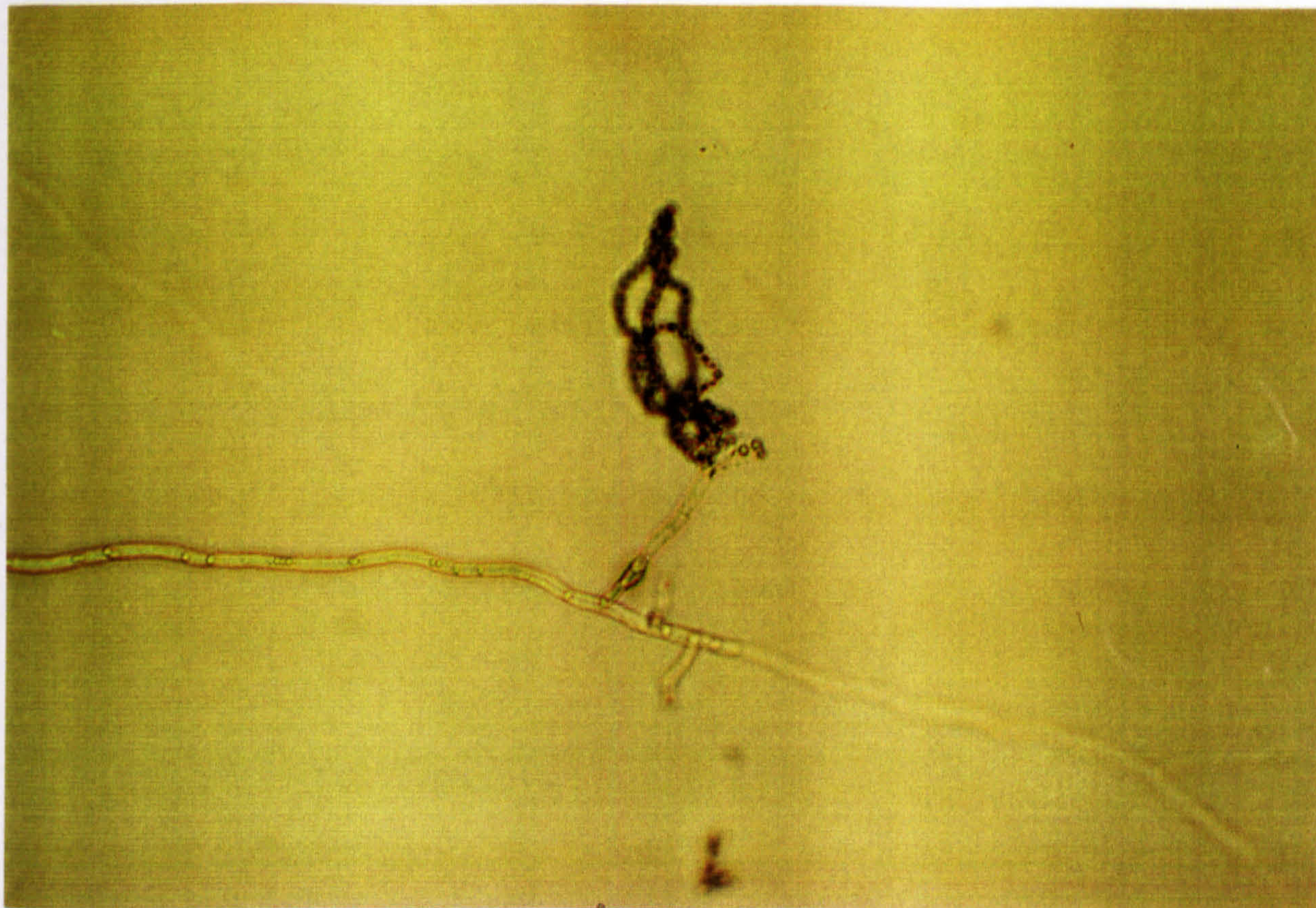


Plate 6.3

Penicillium sp (2) hyphal growth on Czapek Dox agar after 7 days of incubation at 25°C.

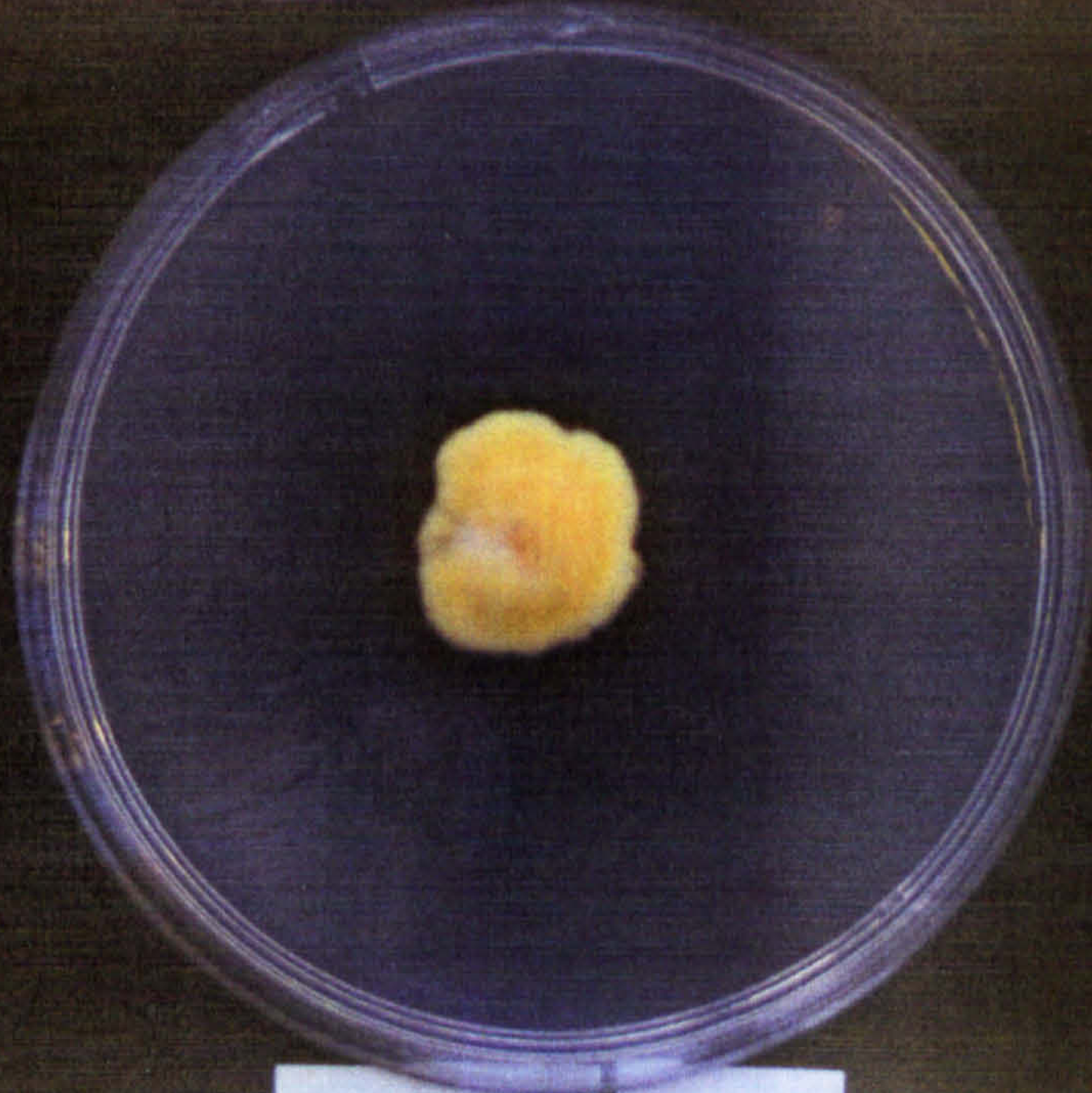

Penicillium sp (2)
CDA
25oC

Plate 6.4.a

Penicillium sp (3) hyphal growth on Czapek Dox agar after 7 days of incubation at 25°C.

Plate 6.4.b

Light micrograph showing oligotrophically grown hyphae of *Penicillium* sp (3) (Final magnification x 400).

Penicillium sp (3)
CDA
25oC

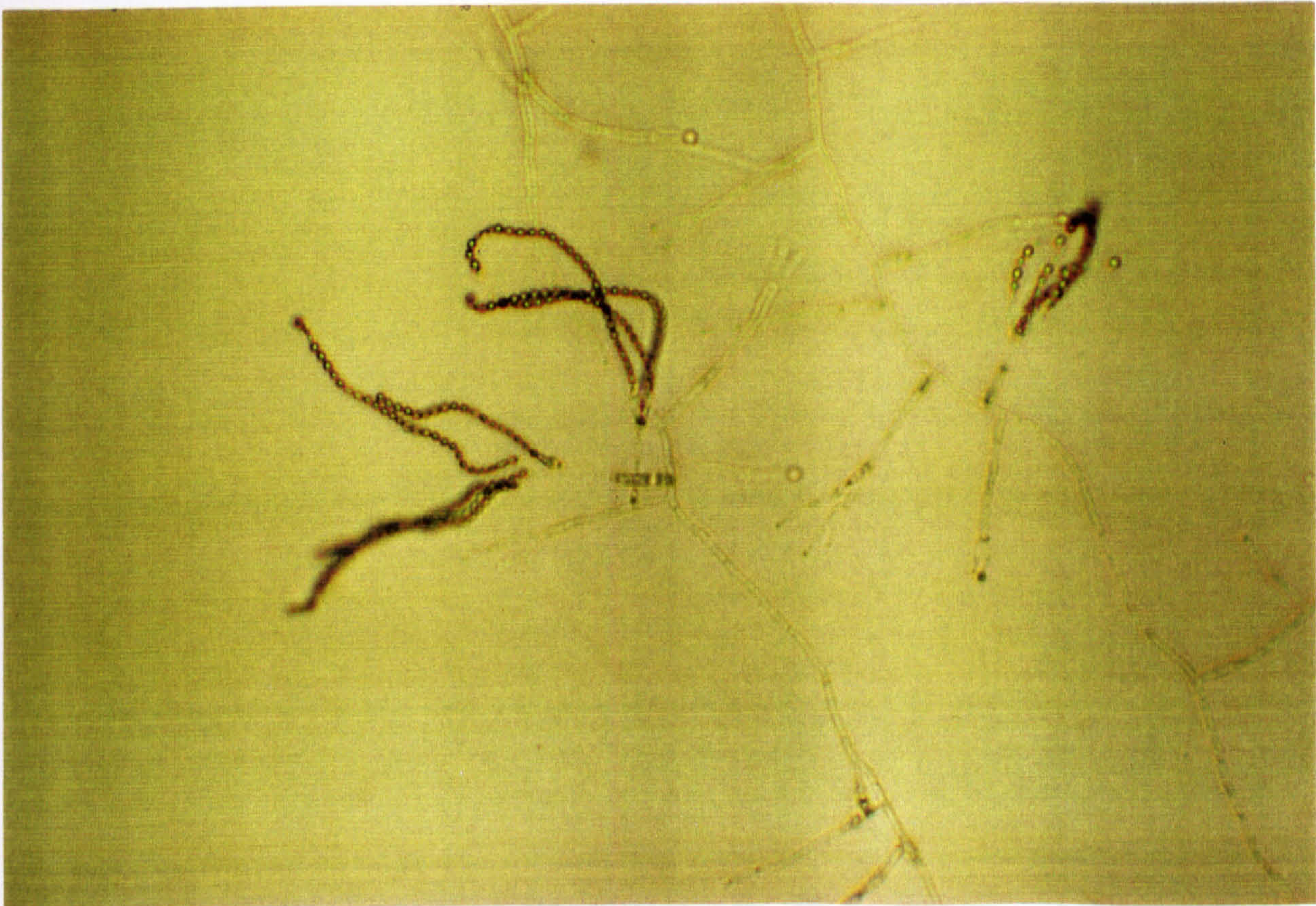


Plate 6. 5

Scanning electron micrograph showing oligotrophic growth of hyphae of *Penicillium expansum* (Magnification x 320).

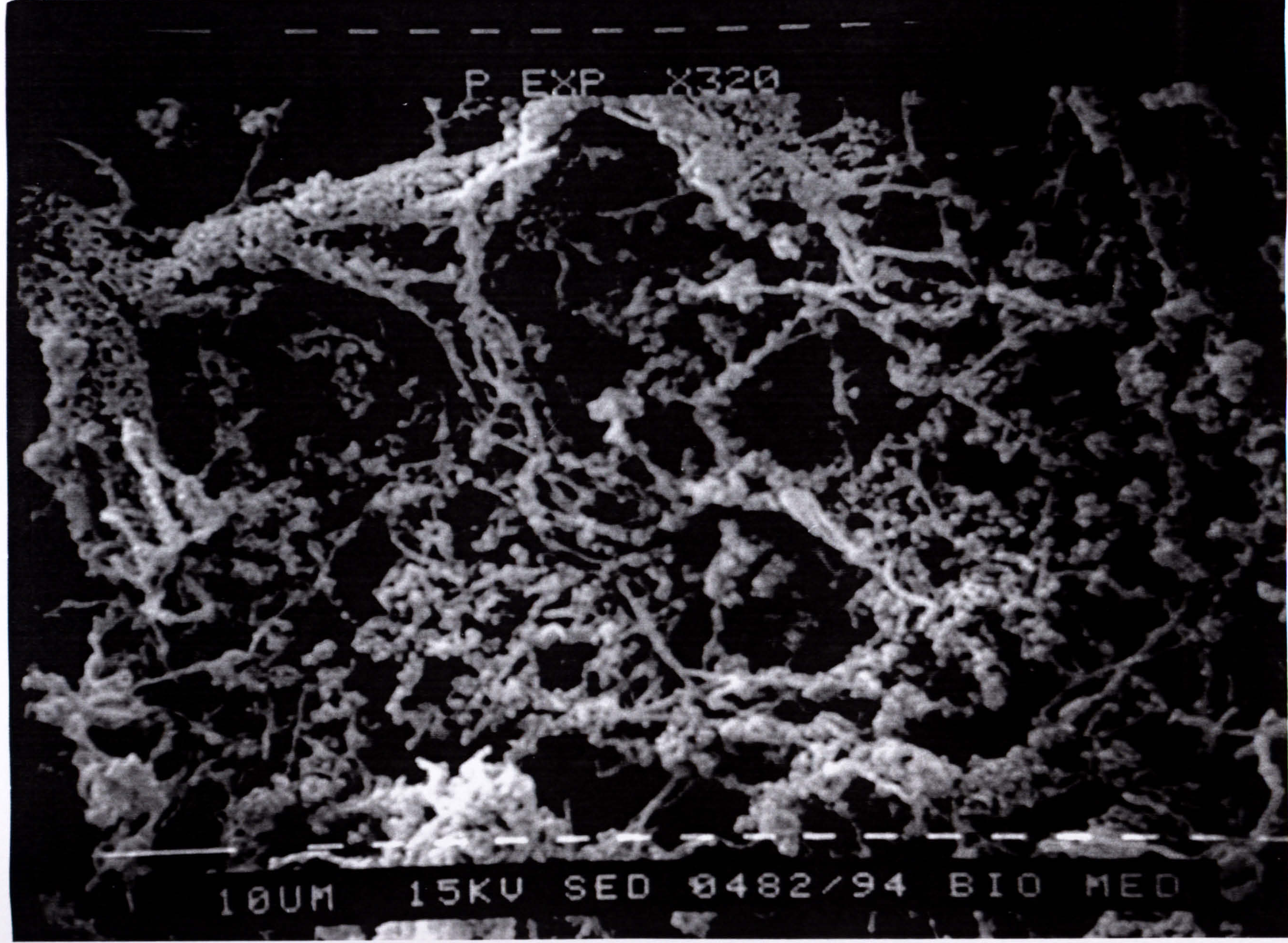

P EXP X320
10UM 15KV SED 0482/94 BIO MED

Plate 6. 6

Scanning electron micrograph showing oligotrophic growth and spore production by *Penicillium expansum* (Magnification x 1250).

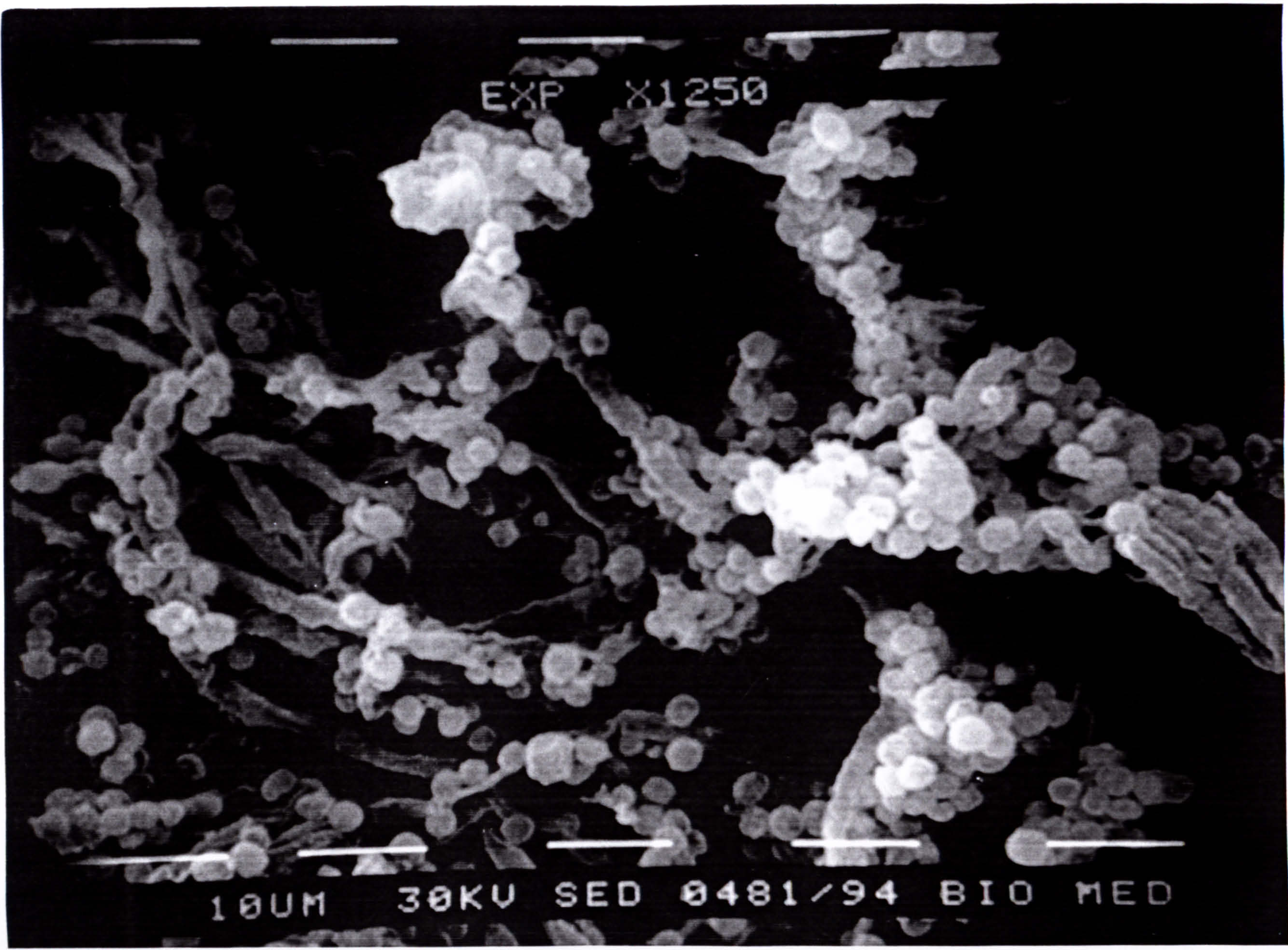

EXP X1250
10UM 30KV SED 0481/94 BIO MED

Plate 6. 7

Scanning electron micrograph showing oligotrophic growth and spore production by *Penicillium expansum* (Magnification x 5000).

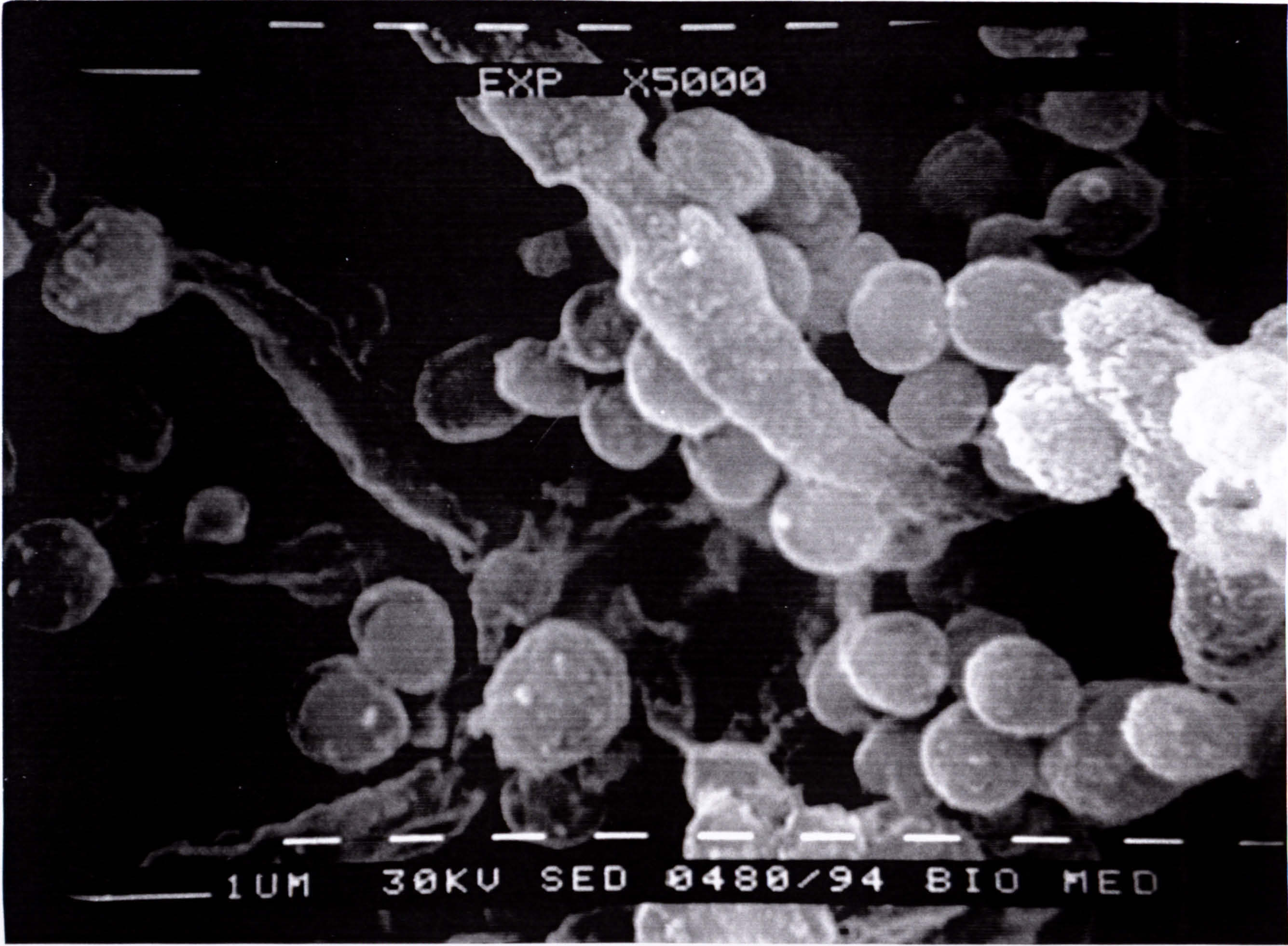

EXP X5000
1UM 30KV SED 0480/94 BIO MED

Plate 6. 9

Scanning electron micrograph showing oligotrophic hyphal growth of *Penicillium* sp (2) (Magnification x 320).

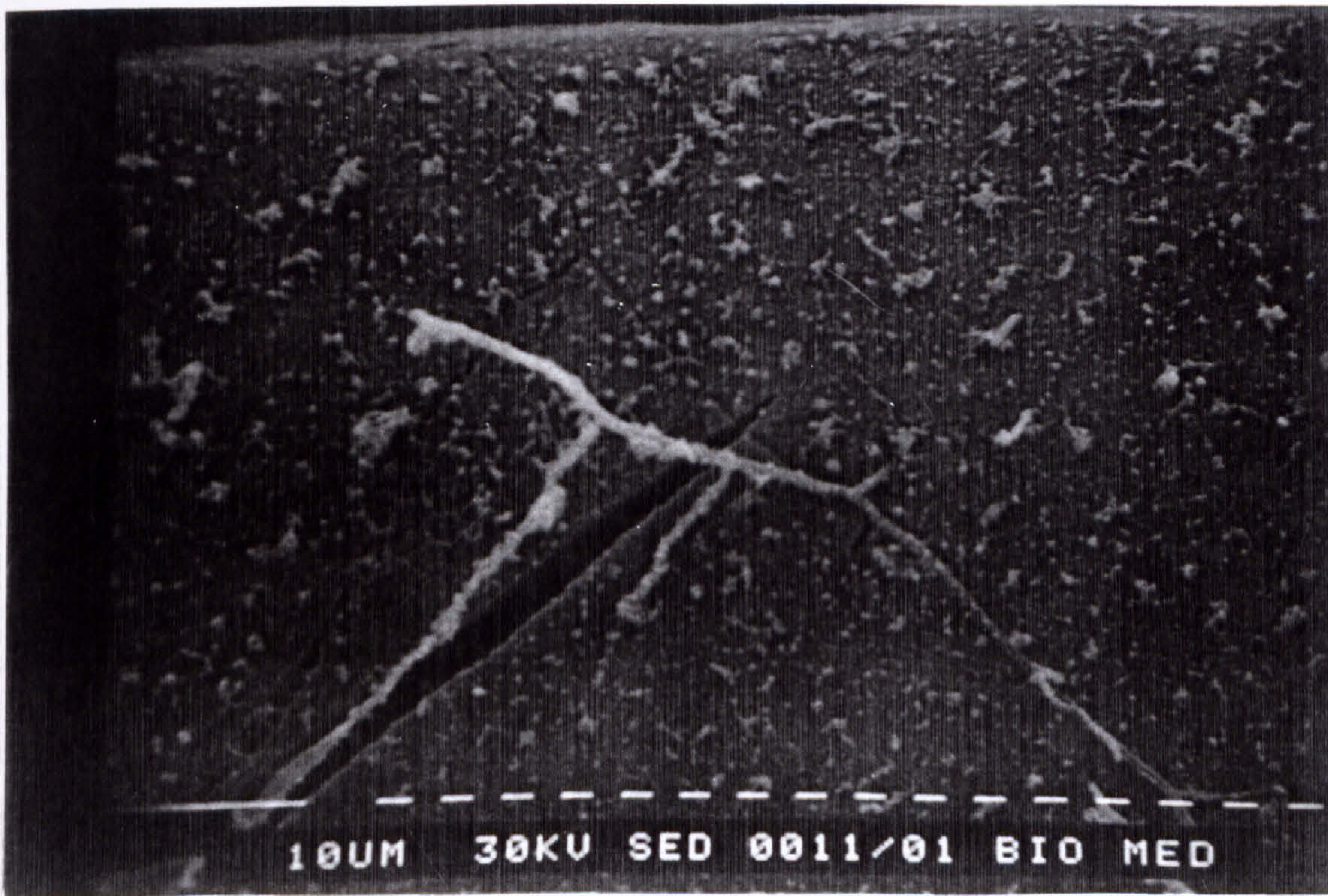

10UM 30KV SED 0011/01 BIO MED

Plate 6. 10

Scanning electron micrograph showing oligotrophic growth of hyphae of *Penicillium* sp (3) (Magnification x 2500).

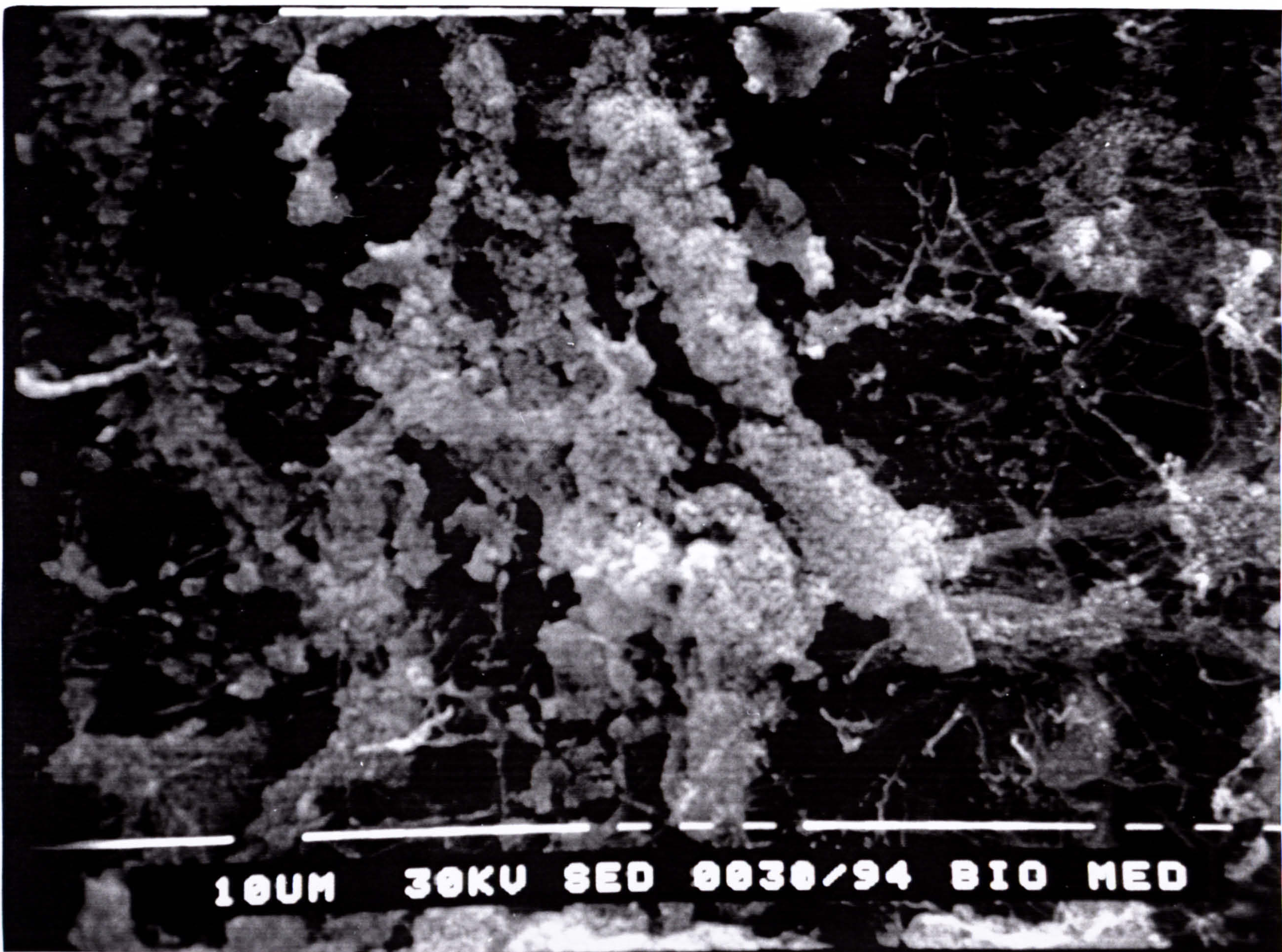

10UM 30KV SED 0030/94 BIO MED

Plate 6. 11

Scanning electron micrograph showing *Penicillium expansum* hyphae grown oligotrophically (Magnification x 320), a shallow groove is seen.

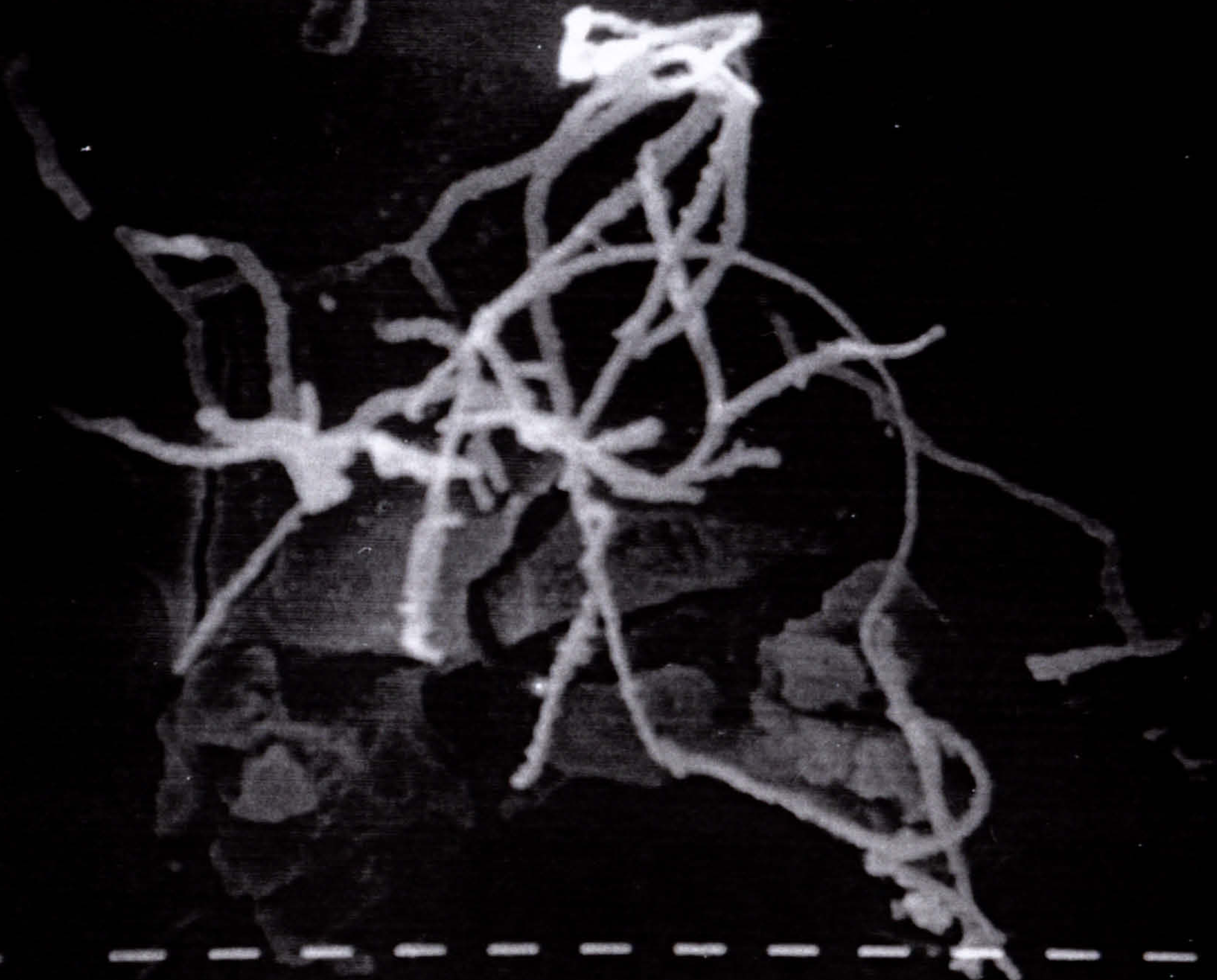

10UM 30KV SED 0026/94 BIO MED

Plate 6. 12

Scanning electron micrograph showing *Penicillium expansum* hyphae grown oligotrophically (Magnification x 1250), a shallow groove is seen.

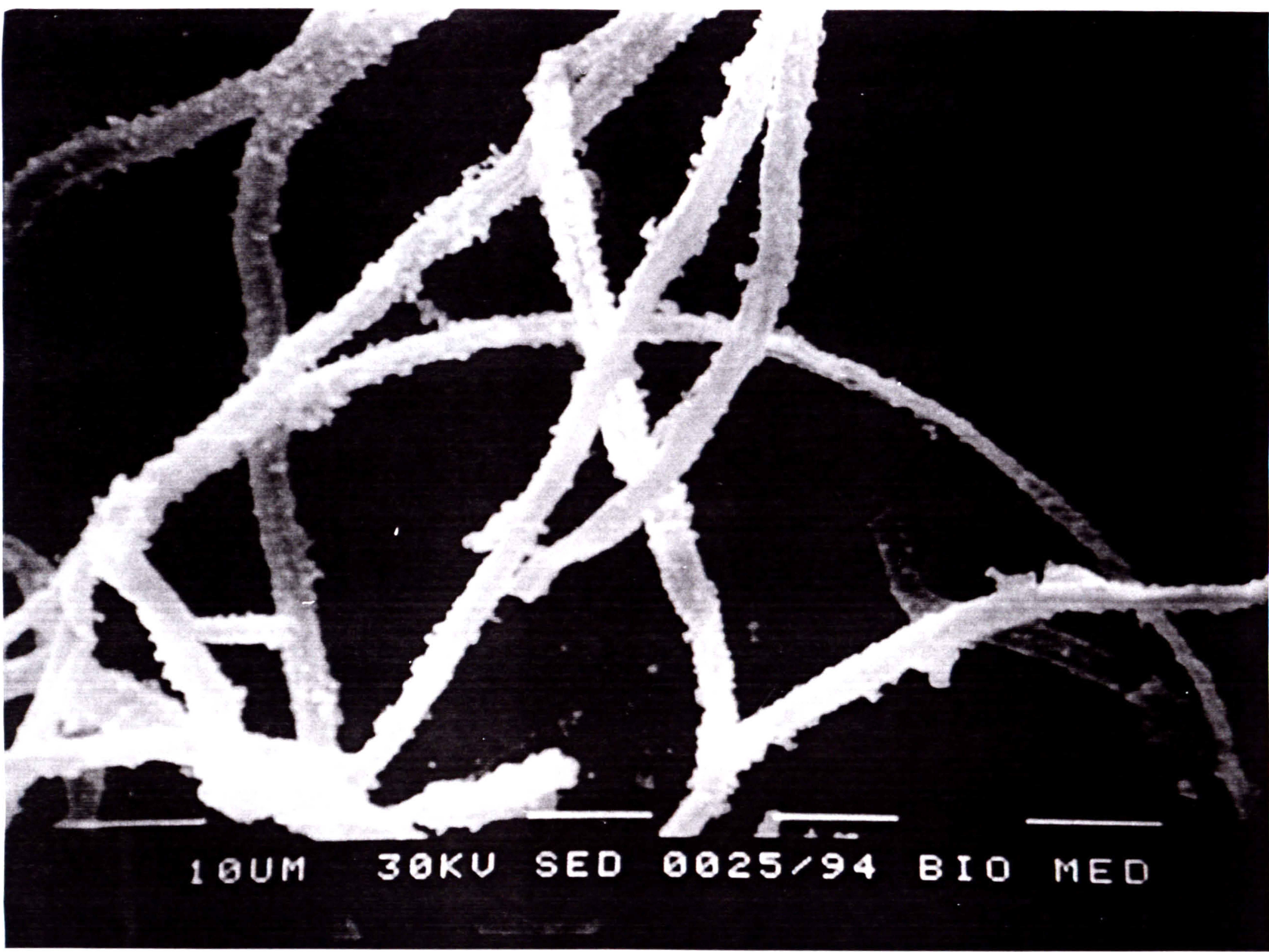
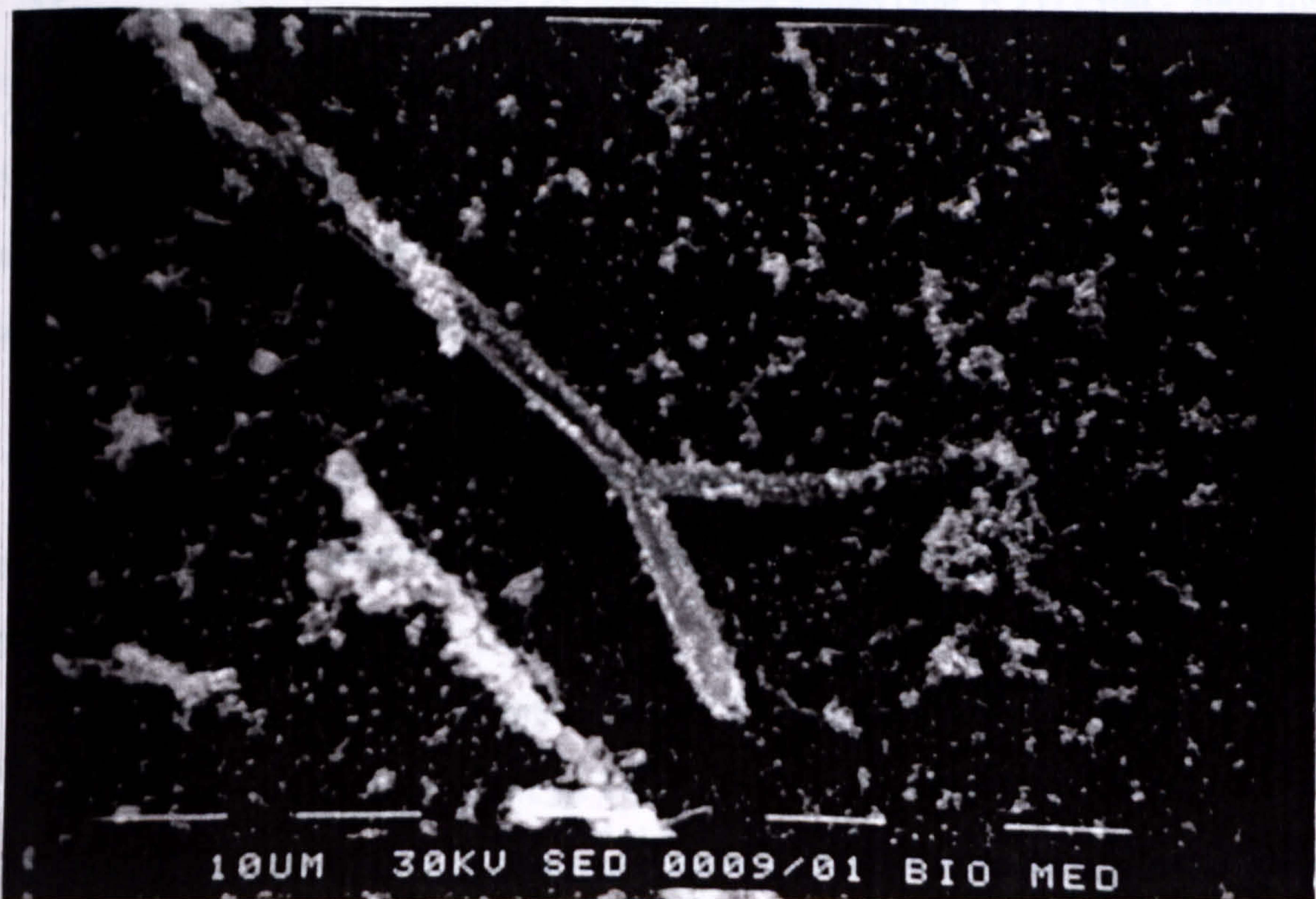

10UM 30KV SED 0025/94 BIO MED

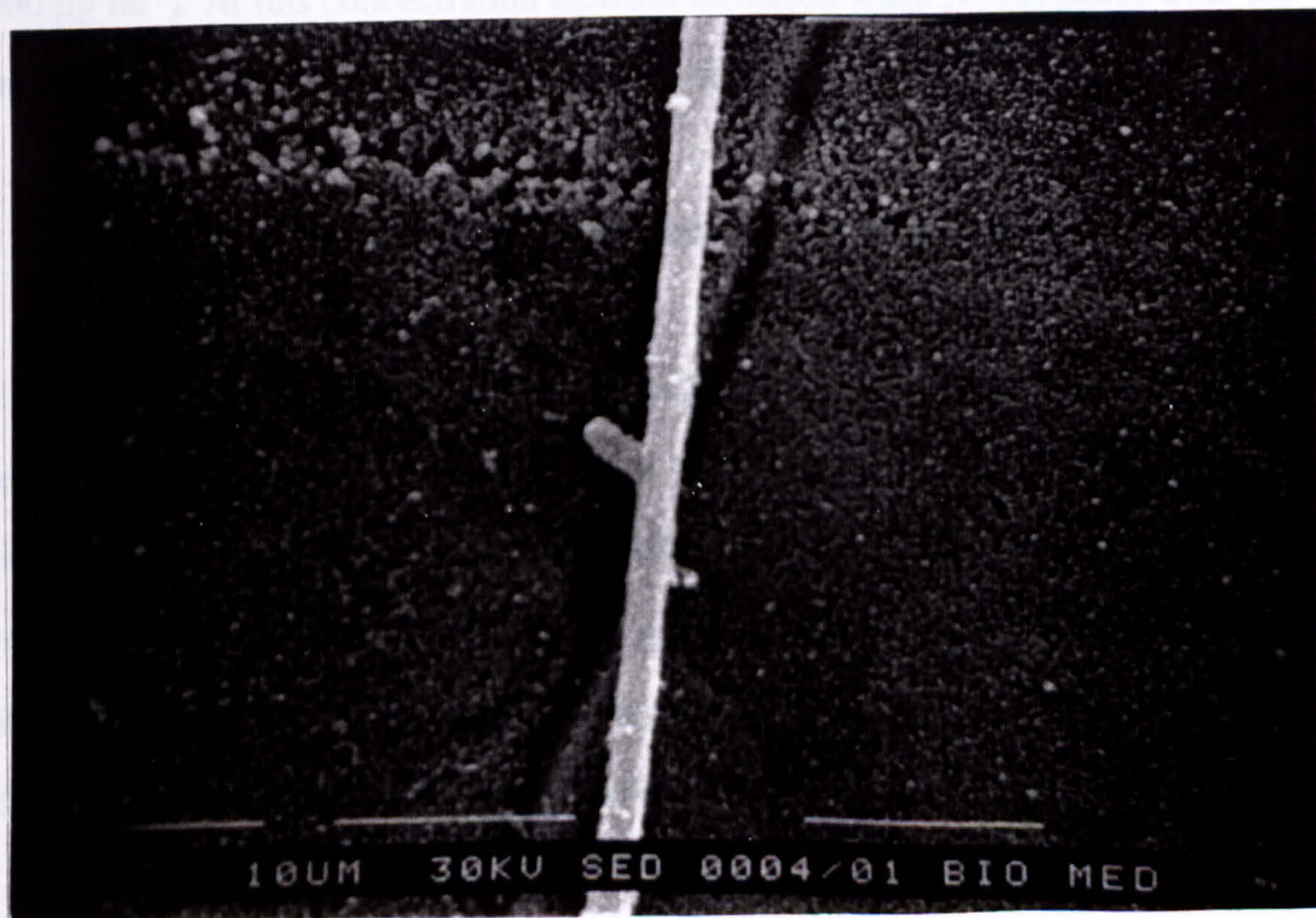
Plate 6. 13

Scanning electron micrograph showing *Penicillium* sp (2) hyphae grown oligotrophically (Magnification x 1250), a shallow groove is seen.

Plate 6. 14

Scanning electron micrograph showing *Penicillium* sp (2) hyphae grown oligotrophically (Magnification x 2500), a shallow groove is seen.


10UM 30KV SED 0009/01 BIO MED


10UM 30KV SED 0004/01 BIO MED

However, when large amounts of carbon were added (Fig. 6.2) (1500 - 3000 μg ml^{-1}) nitrogen addition stimulated growth (not statistically significant however). The effect of increasing the nitrogen content of the medium (10 - 1000 μg ml^{-1}) on fungal growth in the presence of 500 μg C ml^{-1} is shown in Fig. 6. 3. Here, increasing N addition led to an increase in fungal biomass.

Penicillium sp. 2 failed to grow at low carbon concentration (below 500 μg C ml^{-1}). Fig. 6. 4 shows that increasing the N concentration of the medium increased growth of *Penicillium* sp. 2 in the absence of added carbon and also when C (500 μg ml^{-1}) was added.

The results obtained when *Penicillium* sp. 3 was grown under the same medium nutrient conditions as *Penicillium* sp 1 (Figs 6. 1 - 6. 3.) are shown in Figs. 6. 5 - 6. 7. The only major difference was that *Penicillium* 3 generally produced a larger amount of biomass than did *Penicillium* 1. *Penicillium expansum* like *Penicillium* sp 2 failed to grow on carbon free medium and at carbon concentrations lower than 500 μg C ml^{-1} even in the presence of N.

As with *Penicillium* sp 2 however, this fungus grew at added C concentrations of (500 μg ml^{-1}). At this concentration biomass increased when N was added over the concentration range 10 - 100 μg N ml^{-1} (Fig. 6. 8).

6. 3. 3. Patulin production at various amounts of carbon and nitrogen

Patulin production by *Penicillium* sp 1 as influenced by high carbon and nitrogen concentrations is shown in Figs. 6. 9 and 6. 10 (Plates 6. 15 to 6. 17). Increasing the carbon concentration in the presence of 100 μg N ml^{-1} led to increased patulin production up to 2500 μg C ml^{-1} at 3000 μg C ml^{-1} however, patulin production declined dramatically, presumably became there was insufficient N available to support high levels of production at this high C concentration.

Figure 6. 1

Effect of carbon concentrations in medium containing added nitrogen (100 μg ml^{-1})/ or lacking nitrogen on the growth of *Penicillium* sp (1).

Values-means of triplicates ± standard deviation.

Significantly different from control ($P < 0.05$) except where marked *.

Figure 6. 2

Effect of high concentrations of carbon in medium containing added nitrogen (100 μg ml^{-1})/ or lacking nitrogen on the growth of *Penicillium* sp (1).

Values-means of triplicates ± standard deviation.

FIG. 6.1

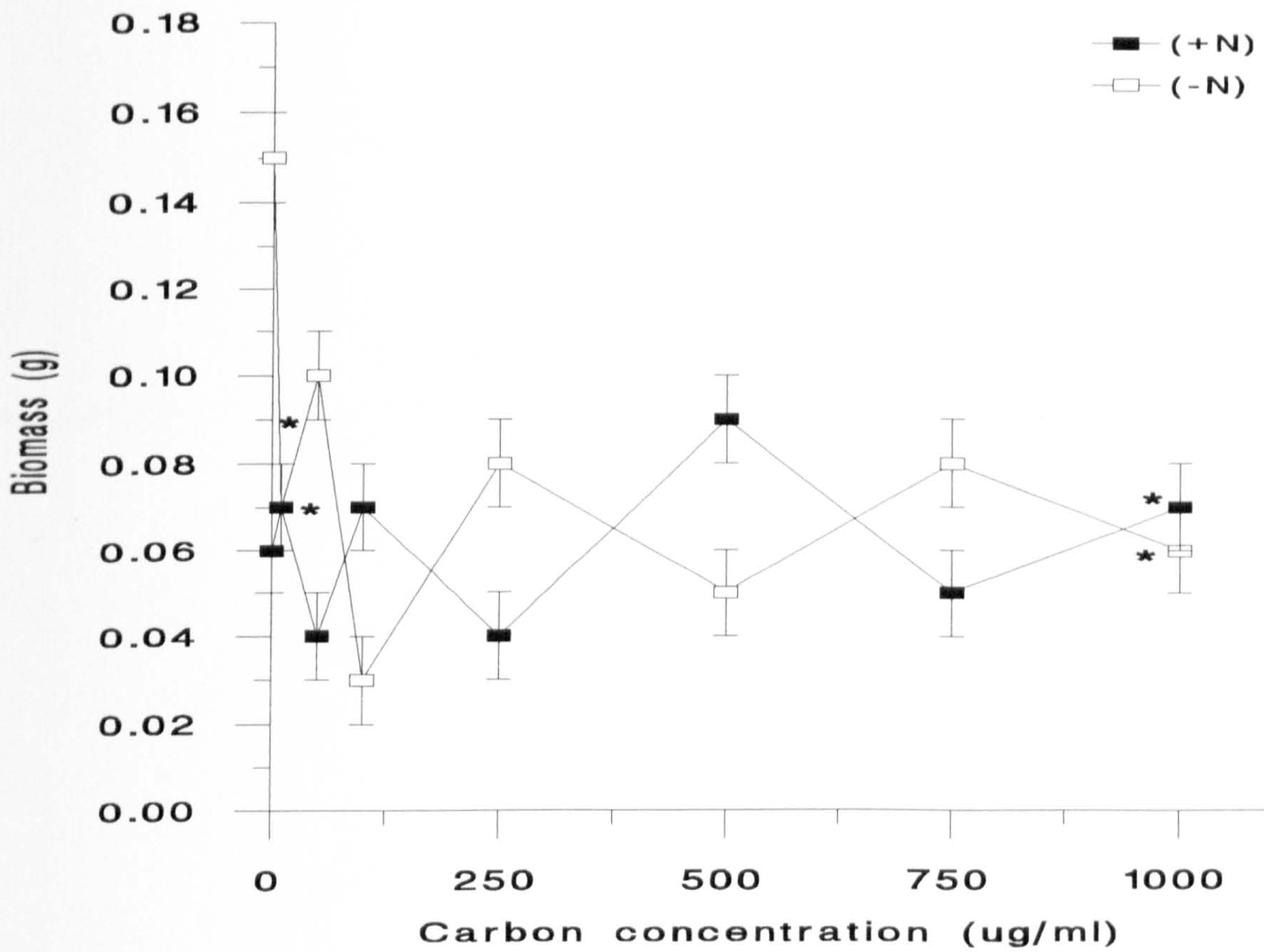


FIG. 6.2

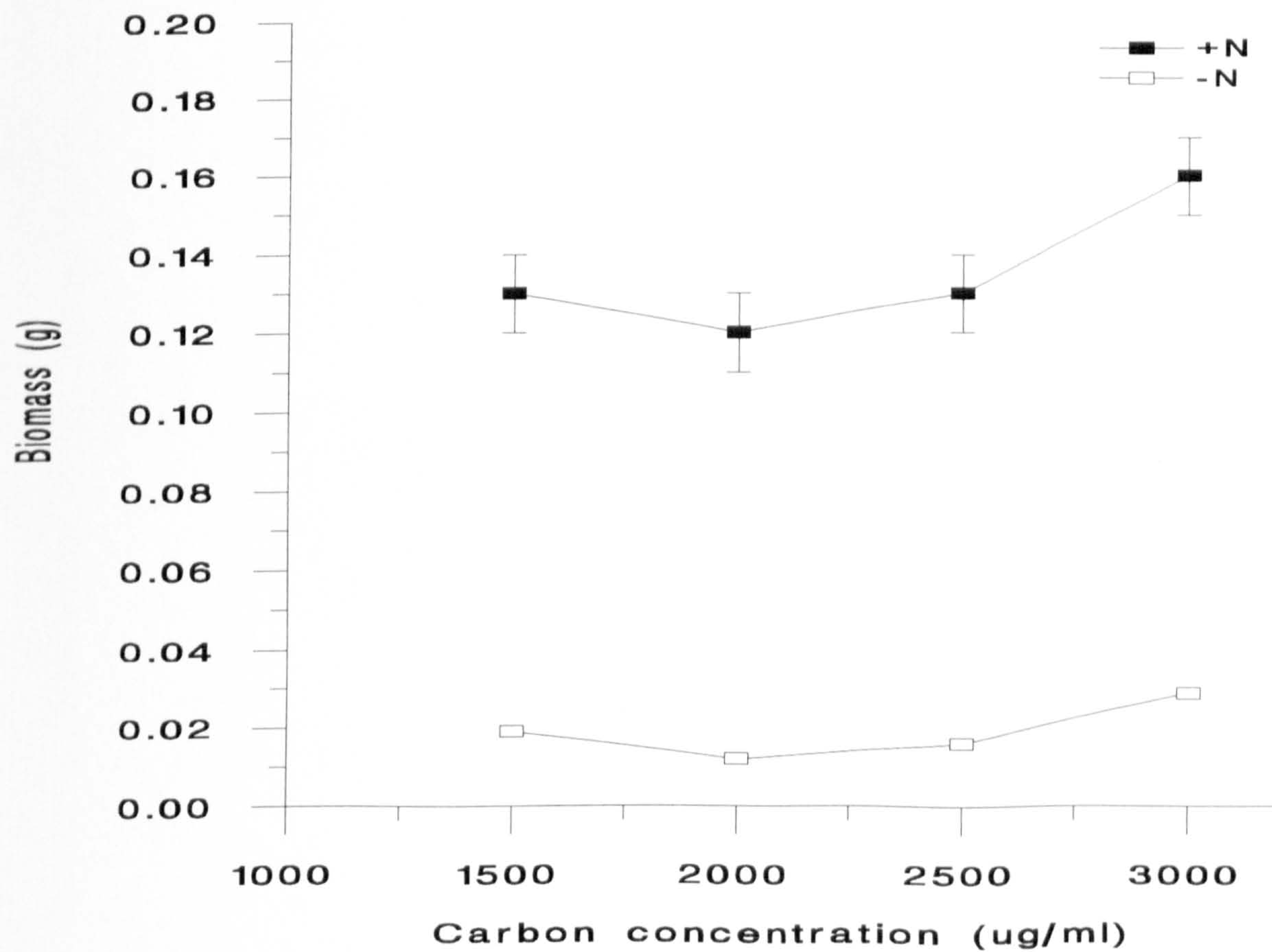


Figure 6. 3

Effect of nitrogen concentrations in medium containing added carbon (500 $\mu g\ ml^{-1}$) on the growth of *Penicillium* sp (1).

Values-means of triplicates ± standard deviation.

FIG. 6.3

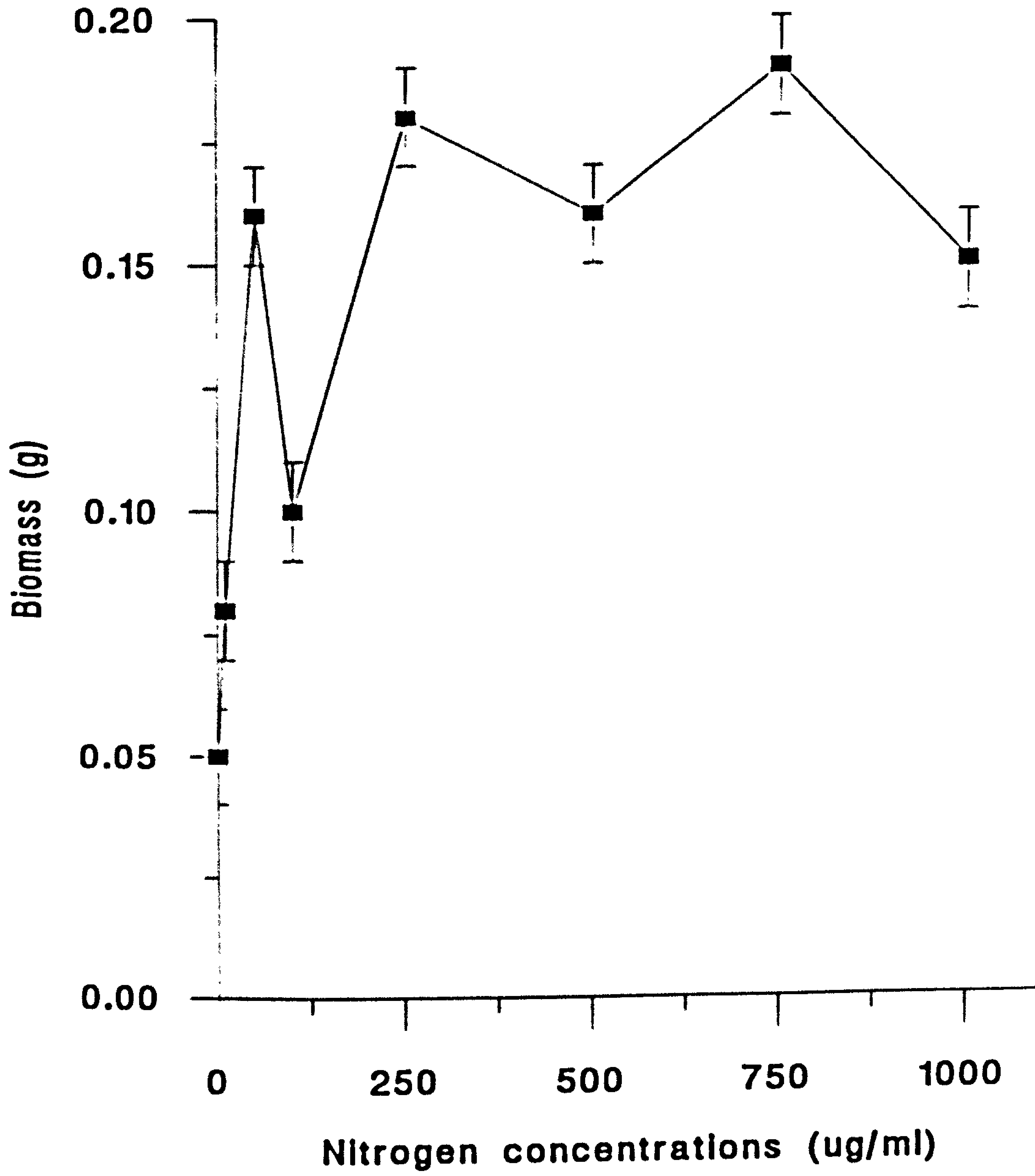


Figure 6. 4

Effect of nitrogen concentrations in medium containing added carbon (500 $\mu g\ ml^{-1}$)/ or lacking carbon on the growth of *Penicillium* sp (2).

Values-means of triplicates ± standard deviation.

Significantly different from control ($P < 0.05$) except where marked *.

FIG. 6.4

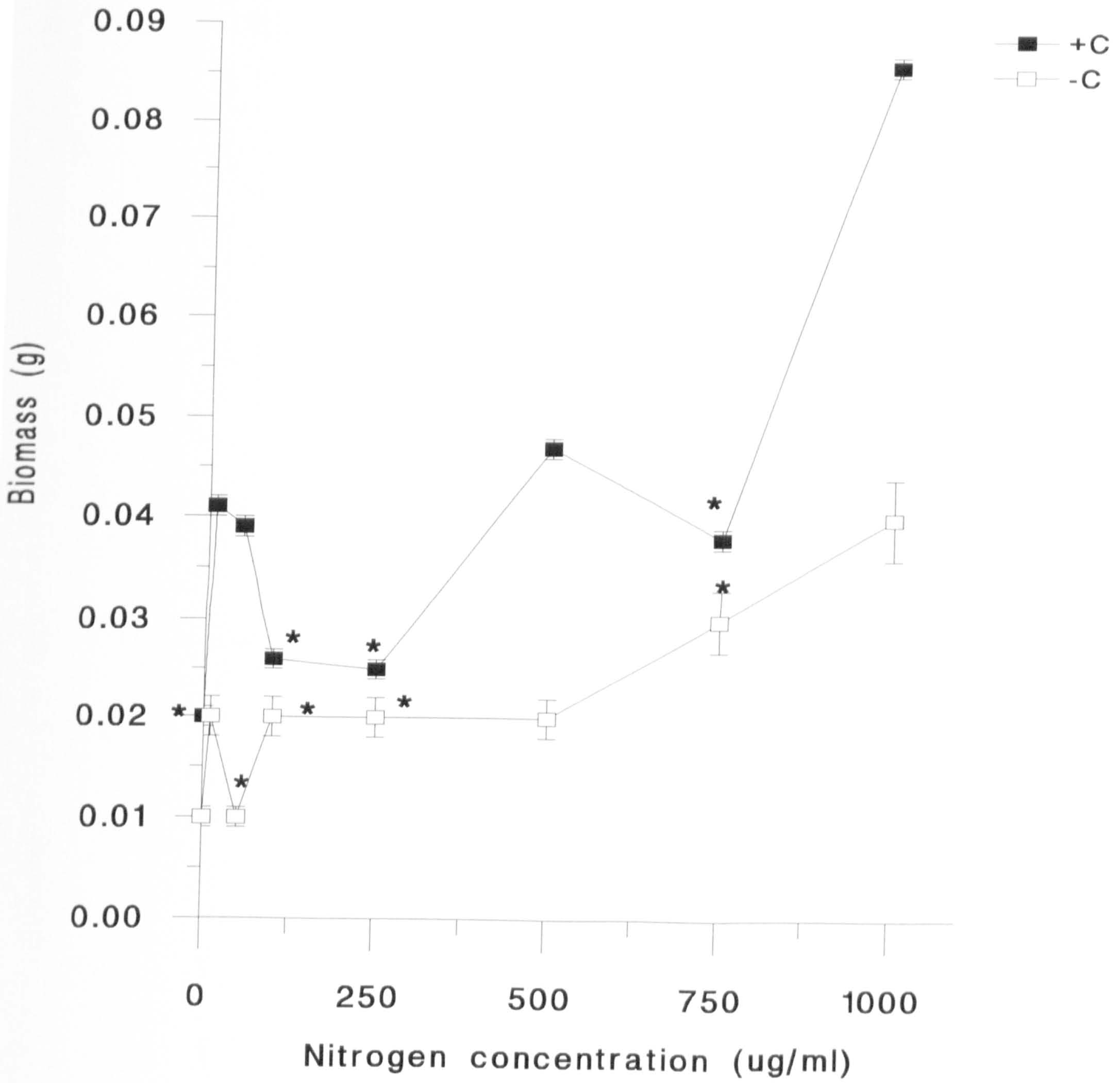


Figure 6. 5

Effect of carbon concentrations in medium containing added nitrogen (100 μg ml^{-1})/ or lacking nitrogen on the growth of *Penicillium* sp (3).

Values-means of triplicates ± standard deviation.

Figure 6. 6

Effect of high concentrations of carbon in medium containing added nitrogen (100 μg ml^{-1})/ or lacking nitrogen on the growth of *Penicillium* sp (3).

Values-means of triplicates ± standard deviation.

FIG. 6.5

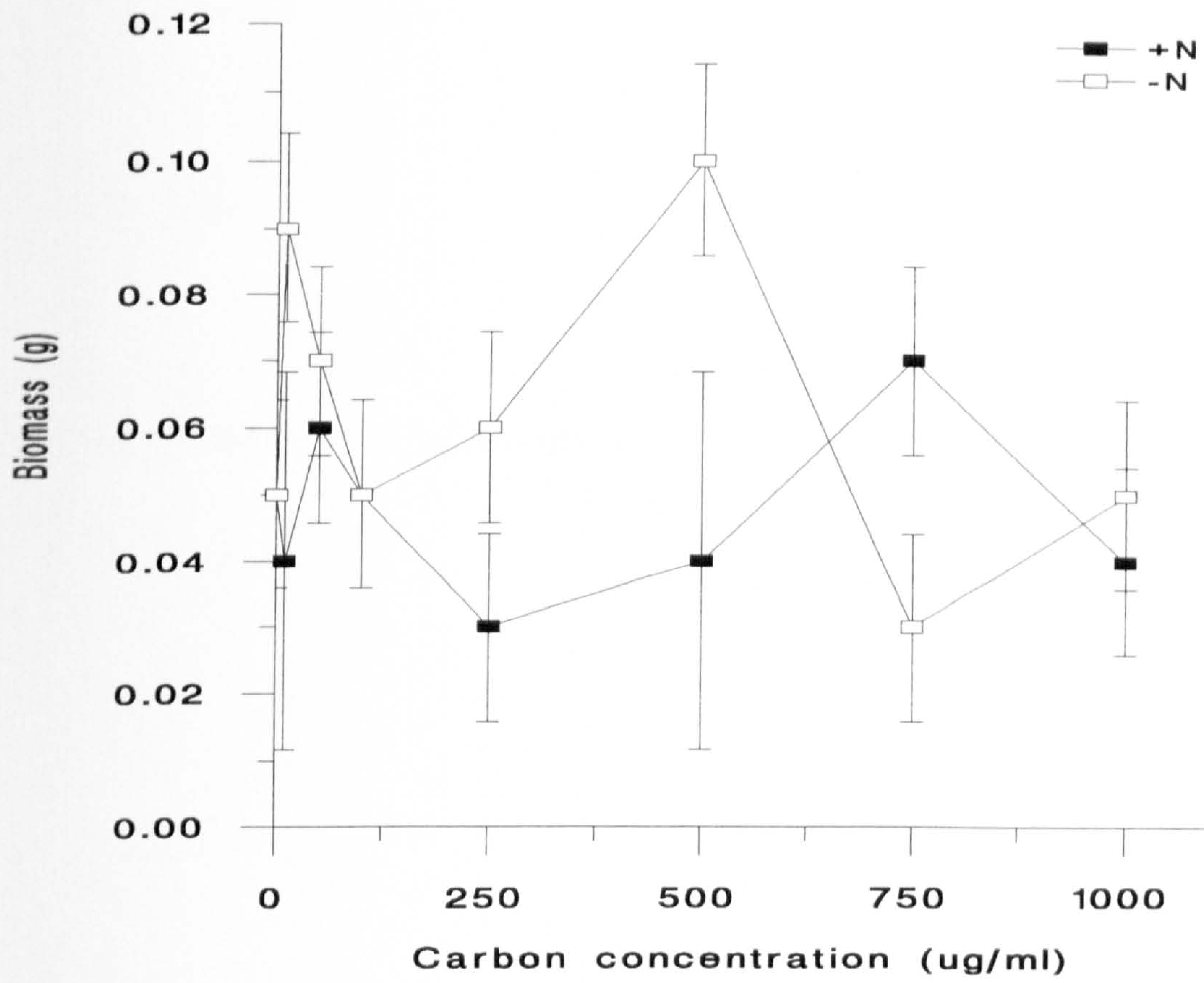


FIG. 6.6

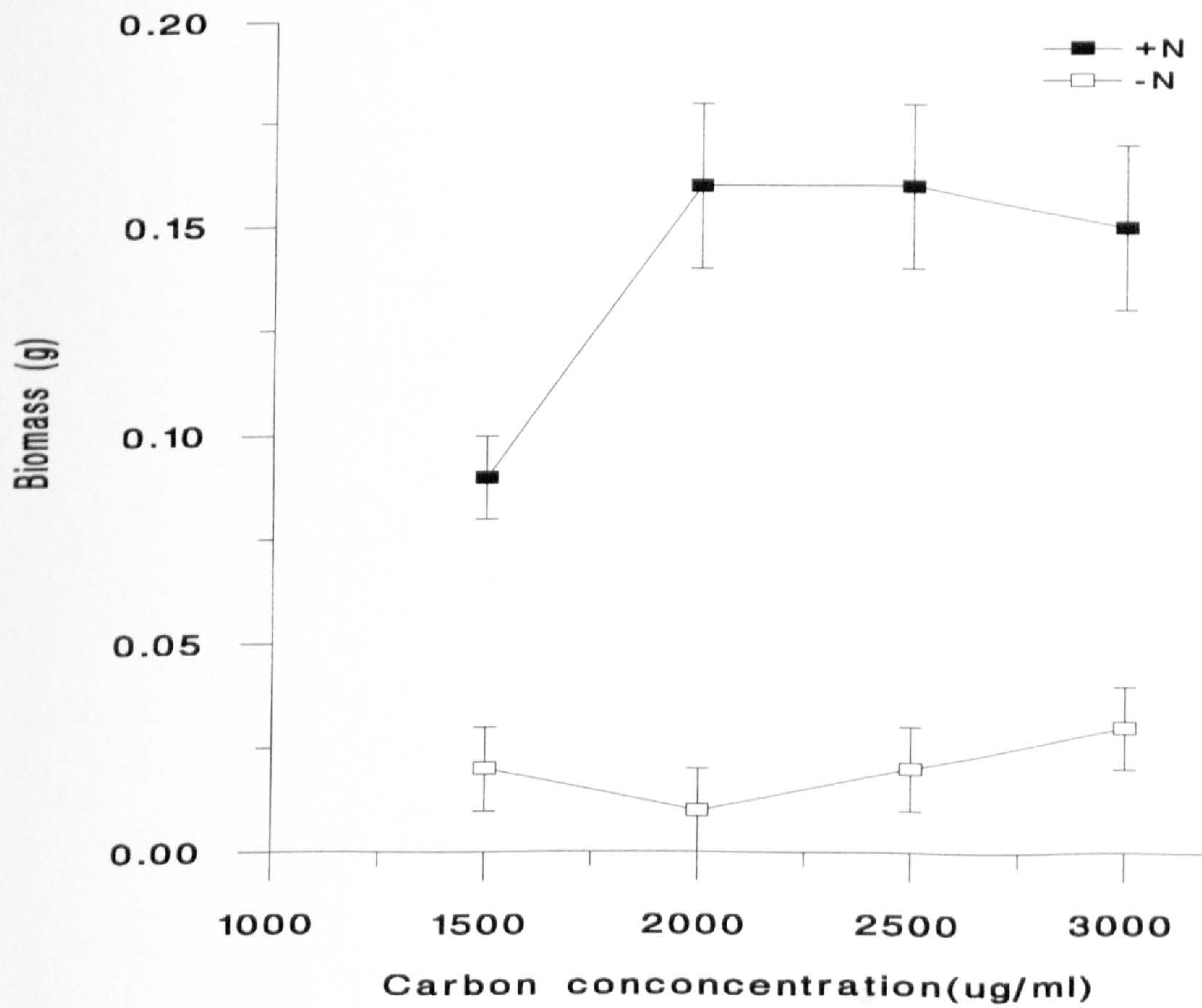


Figure 6. 7

Effect of nitrogen concentrations in medium containing added carbon (500 $\mu g\ ml^{-1}$) on the growth of *Penicillium* sp (3).

Values-means of triplicates ± standard deviation.

FIG. 6.7

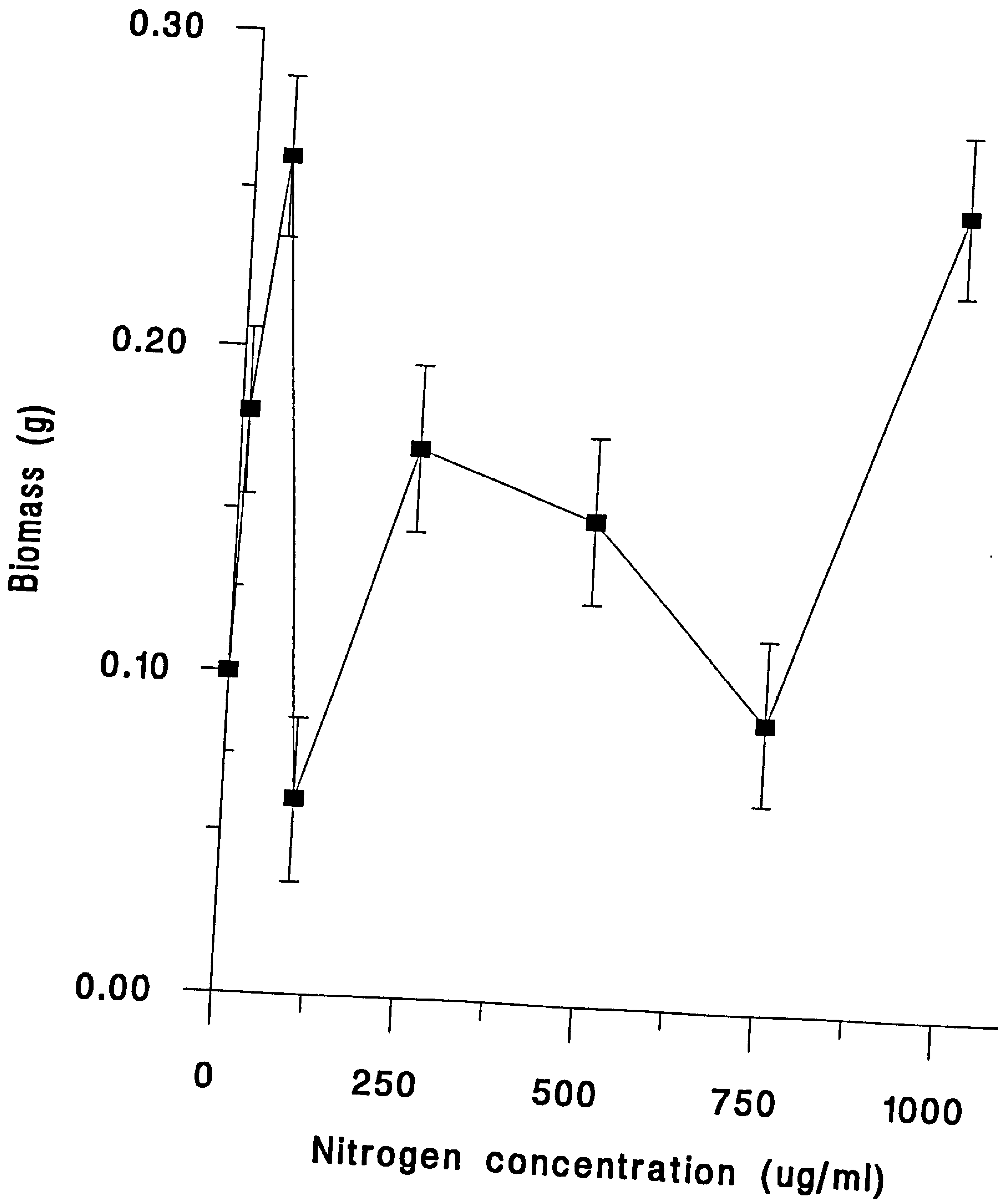


Figure 6. 8

Effect of nitrogen concentrations in medium containing added carbon (500 $\mu g\ ml^{-1}$) on the growth of *Penicillium expansum*.

Values-means of triplicates ± standard deviation.

FIG. 6.8

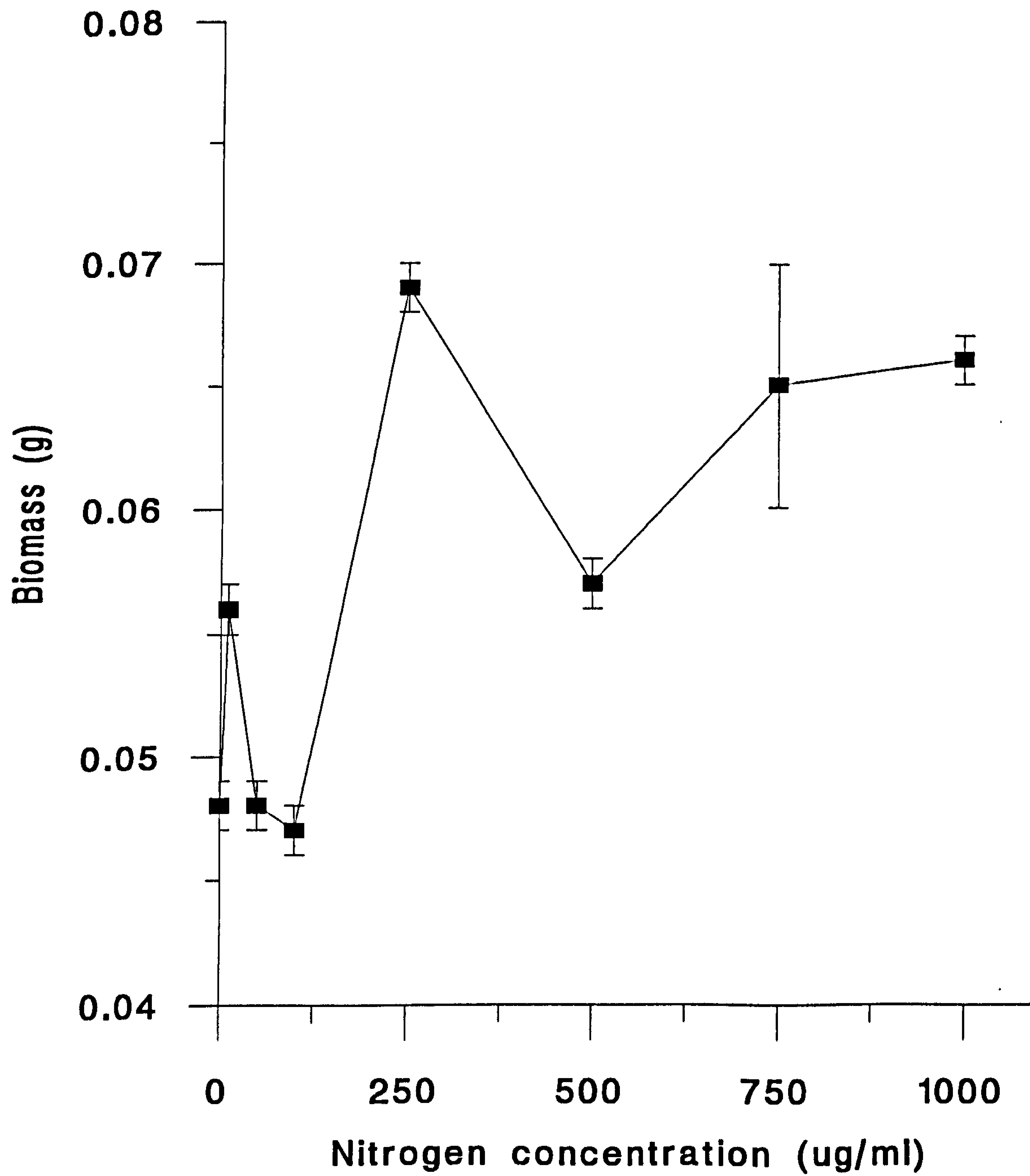


Figure 6. 9

Effect of high concentrations of carbon in medium containing added nitrogen (100 μg ml^{-1}) on the production of patulin by *Penicillium* sp (1).

Values-means of triplicates ± standard deviation.

FIG. 6.9

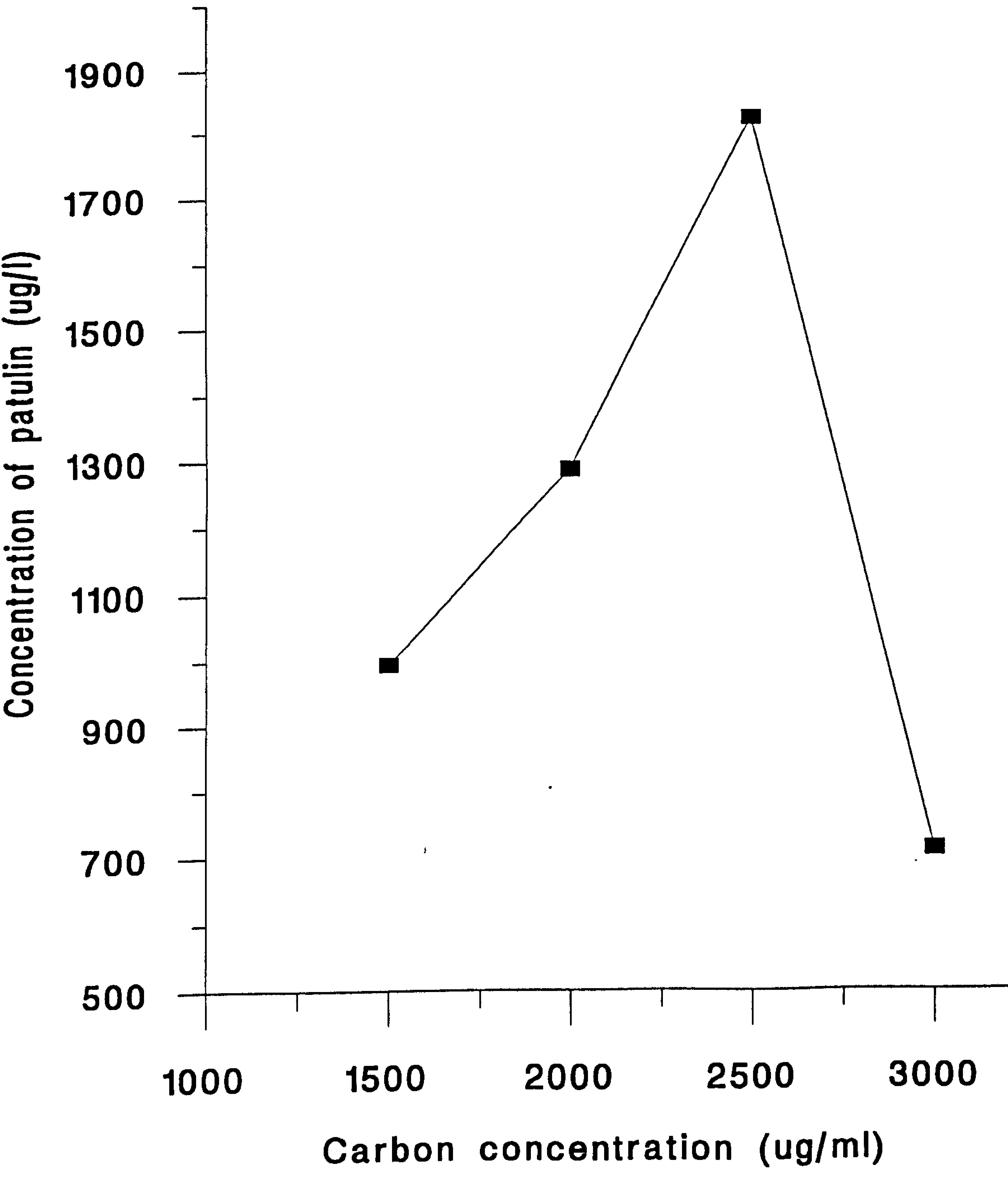


Figure 6. 10

Effect of nitrogen concentrations (amended as ammonium sulphate or sodium nitrate) in medium containing added carbon (500 $\mu g\ ml^{-1}$) on the production of patulin by *Penicillium* sp (1).

Values-means of triplicates ± standard deviation.

FIG. 6.10

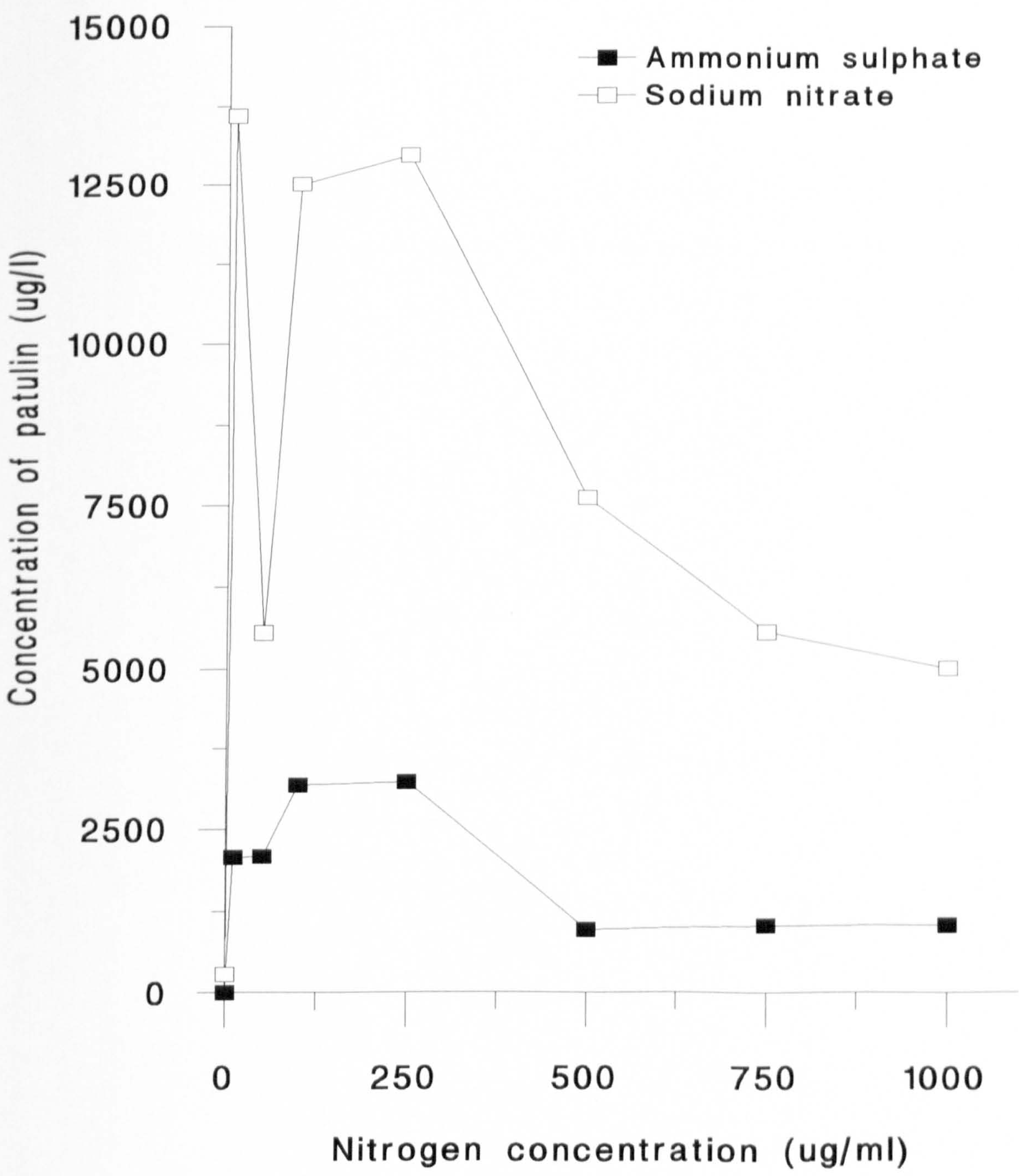


Plate 6. 15

Developed and sprayed chromatographic plate (20 x 20 cm).

Samples = different concentrations of spotted samples (20, 10 and 5 μl for each sample).

PAT std. = different amounts (1, 3, 5 and 7 μl) of spotted patulin standard (100 μg ml^{-1}) solution.

SAMPLES
PAT
std.

Plate 6. 16

Developed and sprayed chromatographic plate (20 x 20 cm).

1 - 6 = spotted sample extracts (20 μl for each sample).

PAT = different amounts (1, 3, 5, 7, 10, 15 and 20 μl) of spotted patulin standard (100 μg ml^{-1}) solution.

a - f = sample extracts superimposed with 5 μl patulin reference standard solution (internal standard).

Example.

Concentration of patulin detected from sample No. 4 when applying the equation:

S = 7 μl, Y = 100 μg ml^{-1}, V = 1000 μl, X = 20 μl, W = 22 ml

7 x 100 x 1000 / 20 x 22 = 1591 μg patulin l^{-1}.

1
2
3
4
5
6
PAT
a
b
c
d
e
f

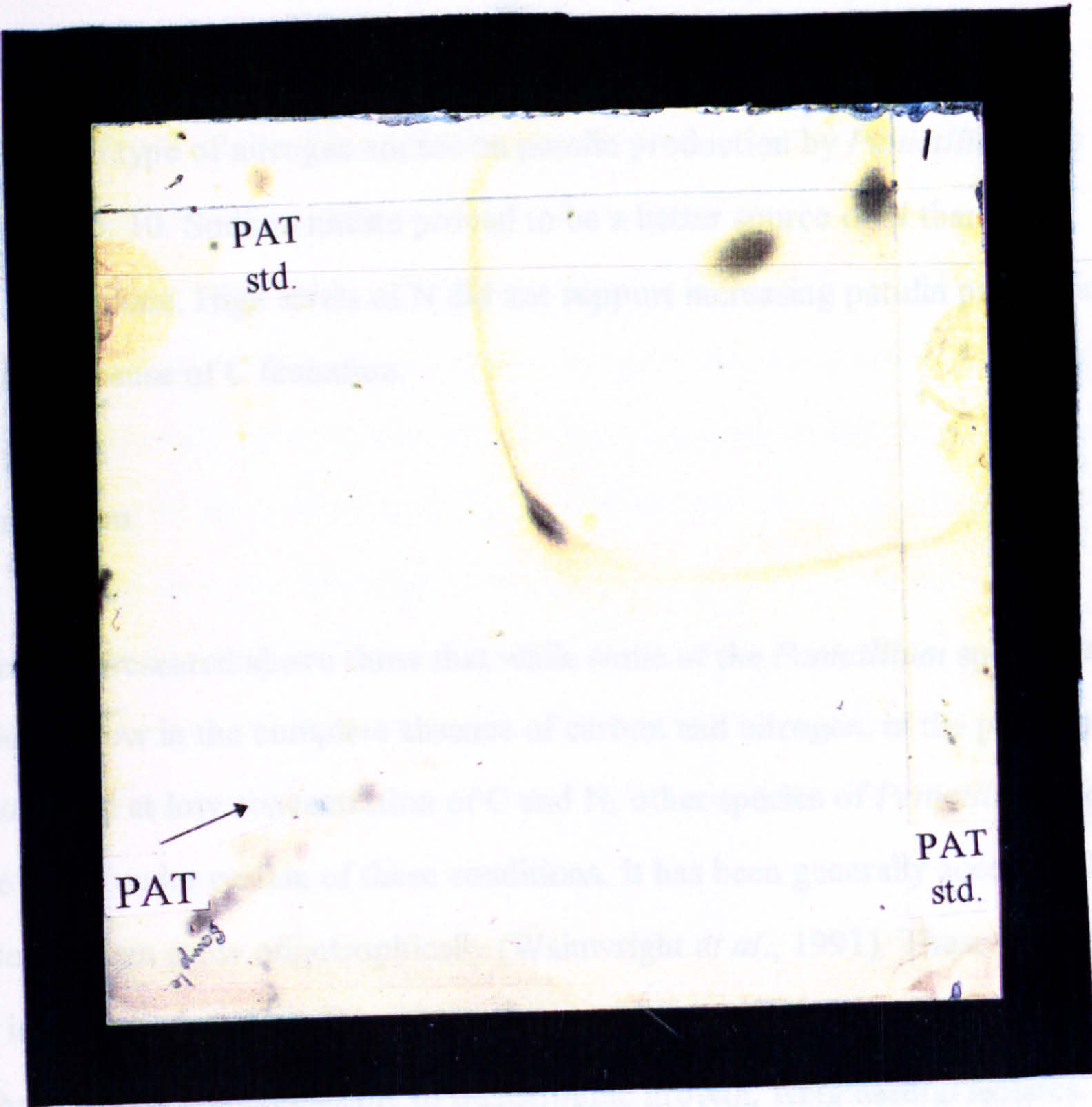
Plate 6. 17

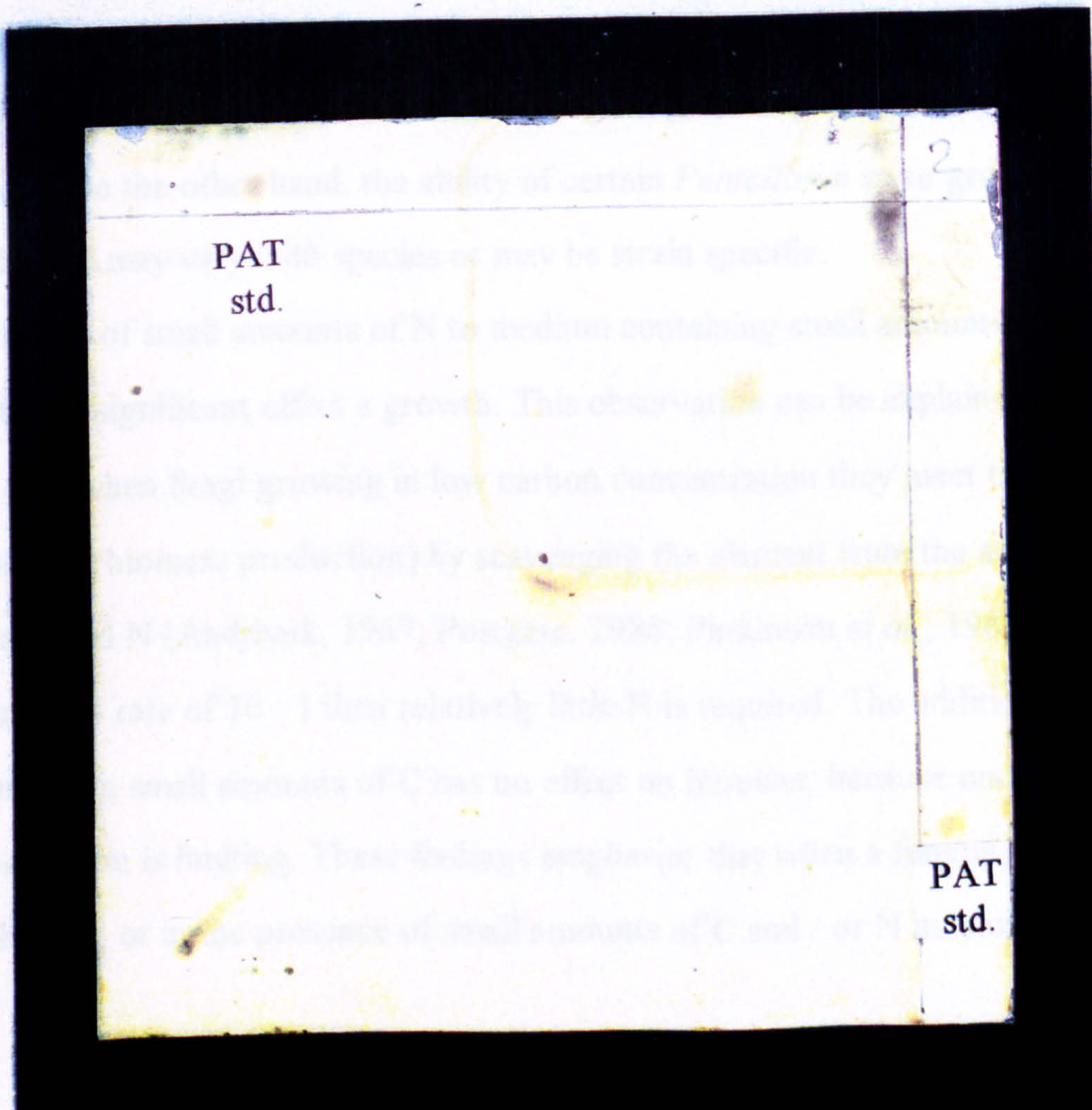
Developed and sprayed TLC plates (10 x 10 cm) in two-dimensional chromatography.

a) a positive sample for patulin.

b) a negative sample for patulin.

PAT std. = patulin standard (100 $\mu g\ ml^{-1}$) solution (5 μl).


1
PAT
std.
PAT
PAT
std.


2
PAT
std.
PAT
std.

The effect of type of nitrogen source on patulin production by *Penicillium* 1 is shown in Fig. 6. 10. Sodium nitrate proved to be a better source of N than did ammonium sulphate. High levels of N did not support increasing patulin production, presumably because of C limitation.

6. 4. Conclusion

The results presented above show that while some of the *Penicillium* species studied were able to grow in the complete absence of carbon and nitrogen, in the presence of N, but no C and at low concentration of C and N, other species of *Penicillium* were unable to grow under certain of these conditions. It has been generally accepted that *Penicillium* sp can grow oligotrophically (Wainwright *et al.*, 1991). These results show that this is not true for all species. This may be a function of the fact that the fungi studied here, which were incapable of oligotrophic growth, were natural isolates. *Penicillium expansum* grew under oligotrophic conditions, so this ability may be acquired during culturing. This possibility, if proved to be correct, would have a profound effect on our view of the ability of fungi to grow oligotrophically in the environment. On the other hand, the ability of certain *Penicillium* sp to grow oligotrophically may vary with species or may be strain specific.

The addition of small amounts of N to medium containing small amounts of C had no consistently significant effect a growth. This observation can be explained by assuming that when fungi growing in low carbon concentration they meet their N needs (to support low biomass production) by scavenging the element from the atmosphere in the form of fixed N (Andriyuk, 1967; Postgate, 1988; Parkinson *et al.*, 1989). If one assumes a C : N rate of 10 : 1 then relatively little N is required. The addition of N to media containing small amounts of C has no effect on biomass, because under these conditions carbon is limiting. These findings emphasise that when a fungus is growing oligotrophically, or in the presence of small amounts of C and / or N its biomass

production is exactly geared to the availability of individual nutrients. This is why it is misleading to talk about "starvation" under such conditions.

In the presence of small amounts of carbon (below 500 $\mu g\ ml^{-1}$) the fungi studied here did not produce patulin. This is perhaps not surprising since secondary metabolite production is generally considered to be nutrient demanding. It should be borne in mind however, that (as we have seen) the fungi studied here are able to produce patulin on natural substrates such as apples and straw (Chapter 2 and 3). These substrates will probably contain relatively small amounts of nutrients suggesting the possibility that the inability of the fungi to produce patulin under low nutrient conditions in laboratory media is due to the absence of certain growth factors which are present in natural substrates.

7. EFFECT OF NATURAL ISOLATES OF BACTERIA AND FUNGI ON PATULIN

7. EFFECT OF NATURAL ISOLATES OF BACTERIA AND FUNGI ON PATULIN

7. 1. Introduction

Early studies showed that secondary metabolites failed to be depleted in media containing small amounts of carbon, thereby suggesting that degradation was taking place (Foster, 1949). In 1973, Harwig *et al.* reported the effect of yeast on added patulin during *in vitro* studies. They noted the disappearance of the toxin from fermenting juice (after two weeks of fermentation) following inoculation with two strains of *Saccharomyces cerevisiae* (Y-99 and Mac # Y 2947) and *S. ellipsoideus* (Davis # 522). Sumbu *et al.* (1983) similarly noted decrease in patulin concentration during log-phase curve growth of *Saccharomyces cerevisiae.* The result of which demonstrated the relationship of protein synthesis and patulin degradation.

The disappearance of patulin from contaminated apple juice during alcoholic fermentation using different yeasts strains and methods of proceed has been highlighted by few workers. They reported patulin degradation within two weeks of fermentation (Drilleau and Bohoun, 1973; Harwig *et al.*, 1973 b; Burroughs, 1977). Stinson *et al.* (1978) for example reported a complete destruction of patulin in naturally contaminated apple juice during fermentation by eight wild strains of yeast.

The aim of the work described in this Chapter was to determine:

a) patulin production by *Penicillium expansum* in dual culture with *Penicillium* sp (1), *Penicillium* sp (2) and *Penicillium* sp (3) which were found to produce patulin in Czapek Dox liquid medium (Chapter 2).

b) patulin production by *Penicillium expansum* in dual culture with the white rot fungus *Phanerochaete chrysosporium.*

c) patulin production by *Penicillium expansum*, *Penicillium* sp (1) and *Penicillium* sp (3) in dual culture with the isolated contaminant bacterial species No. I, II and III (Chapter 2).
d) influence of patulin addition on *Penicillium expansum*, *Phanerochaete chrysosporium* and the isolated contaminant bacterial species No. I, II and III.
e) effect of patulin and glucose addition on the growth and patulin production of *Penicillium expansum*.

7. 2. Materials and Methods

7. 2. 1. Patulin synthesis by the isolated fungal species in dual cultures or in association with the isolated contaminant bacteria (I, II and III) and *Phanerochaete chrysosporium*

7. 2. 1. i. Effect of *Penicillium expansum*, *Penicillium* sp (1), *Penicillium* sp (2) and *Penicillium* sp (3) in dual culture on patulin production

In order to study the production of patulin by *Penicillium expansum*, *Penicillium* sp (1), *Penicillium* sp (2) and *Penicillium* sp (3) in dual cultures, Czapek Dox liquid medium and sound apples, were respectively used as a synthetic medium and as a natural substrate.

a. Czapek Dox liquid medium (CD)

Penicillium expansum Link (obtained from the CAB International Mycological Institute, Surrey), *Penicillium* sp (1), *Penicillium* sp (2) and *Penicillium* sp (3) (isolated as natural contaminants of samples of mouldy fruits and previously demonstrated to be patulin producers in Czapek Dox liquid medium, Chapter 2) were

grown on Czapek Dox agar (Oxoid) plates and slopes at 25° for 7 days. Spore suspensions of each isolate were prepared by gentle agitation using sterile distilled water.

Dual fungal cultures of *Penicillium* species were inoculated into Czapek Dox liquid medium (100 ml flask^{-1} in 250 ml Erlenmeyer flask) using 1 ml of the spore suspensions of each isolates. The inoculated flasks were incubated in triplicate (7 days) at 25°C on a reciprocal shaker (150 rev. min^{-1}).

b. Patulin production on apple

Sound Golden Delicious apples were wiped with alcohol and inoculated by spiking with each of the filamentous *Penicillium* fungal species spores, separately or in dual culture, at a distance of 1 cm from each other (Fig. 7. 1 and Plates 7. 1 to 7. 7). These isolates were applied from 7 days old cultures of *Penicillium expansum*, *Penicillium* sp. (1) and *Penicillium* sp.(3) grown on apple paste (20 ml autoclaved apple paste/plate). Each treated group of apples (six apples) was placed in duplicate in a propagator (12 apples each group, six apples each propagator) which was washed, wiped with alcohol and the base covered with four layers of Kimwipe laboratory roll. A group of apples was inoculated with each fungal species alone and used as control. The apples were moistened with 50 ml sterile distilled water and incubated at 25°C for 7, 14 and 21 days. The propagators were watered weekly and the fungal growth diameter was measured.

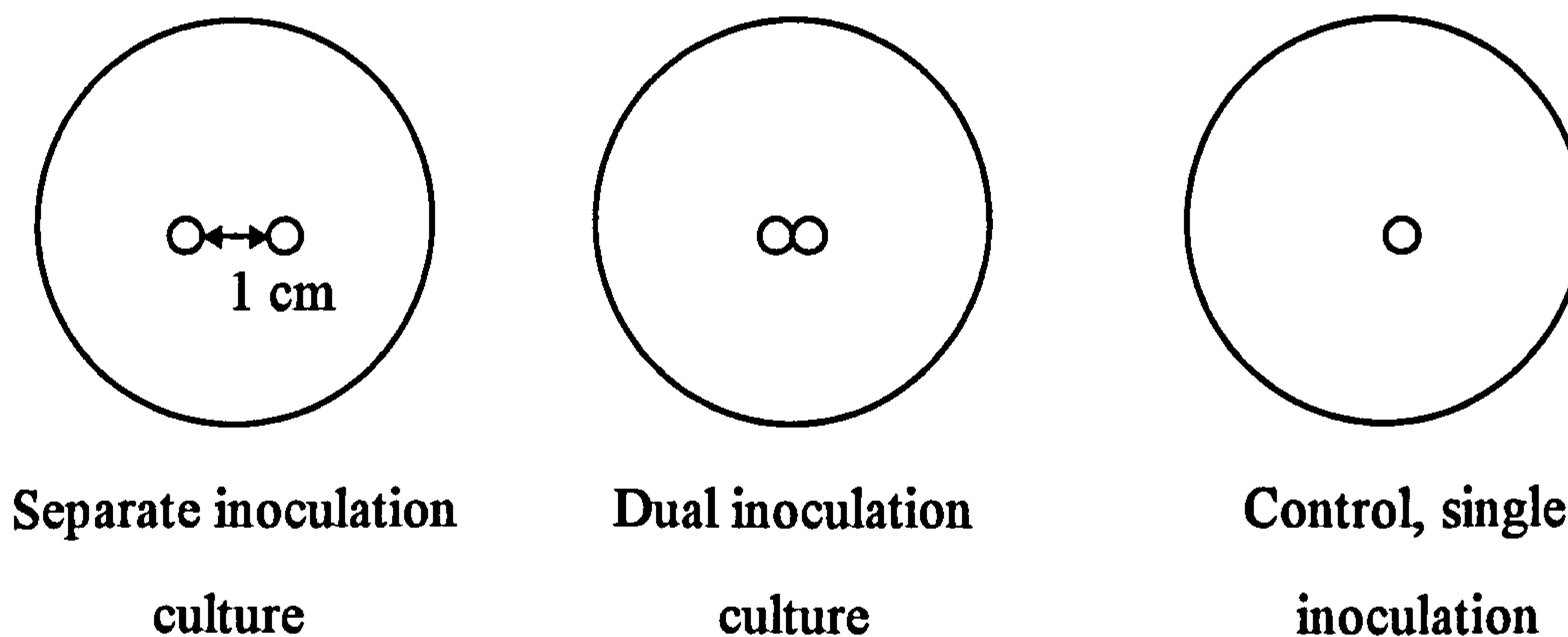
7. 2. 1. ii. Effect on patulin production of *Penicillium expansum* in dual culture with *Phanerochaete chrysosporium*

Phanerochaete chrysosporium (obtained from Department of Molecular Biology and Biotechnology, University of Sheffield) was grown on potato dextrose agar (Oxoid)

plates and slopes at 37°C for 5 days. Spore suspensions were prepared by gentle agitation using sterile, distilled water.

Dual microbial cultures of *Penicillium expansum* and *Phanerochaete chrysosporium* were inoculated in Czapek Dox liquid medium (100 ml in 250 ml Erlenmeyer flask) with 1 ml of each spore suspensions. The inoculated flasks were incubated in triplicate for 7 days at 25°C on a reciprocal shaker (150 rev. min^{-1}). Controls of both isolates were included.

Fig. 7. 1. A simplified diagram of the inoculation procedure on apples.



7. 2. 1. iii. Effect on patulin production of *Penicillium expansum* and other isolates of *Penicillium* species in dual culture with the isolated contaminant bacterial species I, II and III

Bacterial species No. I, II and III (isolated as natural contaminants of samples of mouldy fruit apples, Chapter 2) were grown on nutrient agar slopes and incubated at

25°C for 7 days. Suspensions of each culture were prepared by gentle agitation using sterile, distilled water.

Apple extract medium was used in order to study the ability of *Penicillium expansum*, *Penicillium* sp (1), and *Penicillium* sp (3) to produce patulin when grow in dual cultures with the bacterial species I, II and III.

The medium was prepared from fresh Golden Delicious apples which were blended using an electric mixer into a homogeneous paste. Apple paste (40g) was weighted into 250 ml Erlenmeyer flasks then 100 ml distilled water were added. The contents were sterilized by autoclaving at 121°C for 20 minutes.

After cooling, the medium were inoculated with 1 ml of the spore suspension of each fungal species in association with 1 ml of the cell suspension of each bacterial species. Inoculated flasks with each fungal species alone were also included as controls.

All flasks were set up in triplicate and incubated for 7, 14 and 21 days at 25°C, on a reciprocal shaker at 150 rpm.

7. 2. 2. Effect of patulin on *Penicillium expansum*, *Phanerochaete chrysosporium* and the isolated contaminant bacteria (I, II and III)

7. 2. 2. i. Influence of patulin addition on *Penicillium expansum*

The basal medium used in the following studies was Czapek Dox C-free liquid medium containing $NaNO_3$, 2 g; KH_2PO_4, 1 g; $MgSO_4.7H_2O$, 0.5 g; KCl, 0.5 g and $FeSO_4.7H_2O$, 0.01 g in 1000 ml of purified distilled water (pdw). The medium was adjusted to pH 6.8 with 1.5 N NaOH. The constituents of the basal medium were dispensed into Erlenmeyer flasks (50 ml or 100 ml in 250 ml flasks) and sterilized by autoclaving at 121°C for 20 minutes.

After cooling, the flasks were amended with different concentrations of patulin prepared by dissolving crystalline patulin standard in sterile purified distilled water

(pdw). This was then added to the CD, C-free medium (50 ml in 250 ml flasks) to give concentrations of 0.5, 1, 2.5, 5, 10, 15 and 20 μg ml^{-1}. The flasks were next inoculated with 1 ml of *Penicillium expansum* spore suspension (10^6 - 10^7). The inoculated flasks were incubated in triplicate at 25°C on a reciprocal shaker (150 rev min^{-1}) for 7 days. Flasks lacking patulin were also inoculated and used as controls.

7. 2. 2. i. a. Evaluation of patulin toxicity

The agar diffusion method of assay was used to study the sensitivity of *Bacillus megaterium* Kellen K to the extracts applied from 7. 2. 2. i. Wells were cut into Nutrient agar (Oxoid) prepared in flat-bottomed (Nunc bioassay) plates and seeded with the bacteria. Liquid medium (100 μl) was added to the wells then the plates were incubated at 37°C overnight and any resulting zones of inhibition were measured.

7. 2. 2. ii. Influence of patulin on *Phanerochaete chrysosporium*

The basal medium used in the following studies was Czapek Dox C-free liquid medium adjusted to pH 6.8 with 1.5 N NaOH, dispensed into Erlenmeyer flasks (100 ml in 250 ml flasks) and sterilized by autoclaving at 121°C for 20 minutes (Appendix 9. 2. e).

After cooling, the flasks were amended with 2 ml of patulin standard solution (1000 μg ml^{-1}) to give a final concentration of 20 μg ml^{-1}. The prepared medium was inoculated in triplicate with *Phanerochaete chrysosporium* (transferred from 5 days old culture of each) then incubated at 25°C, 150 rev min^{-1} for 7 days. Inoculated and non patulin amended flasks were added as controls.

7. 2. 2. iii. Influence of patulin on the isolated contaminant bacterial species I, II and III

The medium used in the following studies was apple extract prepared from fresh Golden Delicious (40 g of apple paste in 250 ml Erlenmeyer flasks mixed with 100 ml distilled water). The contents were sterilized by autoclaving at 121°C for 20 minutes.

After cooling, the medium was inoculated with 1 ml of the cell suspension of the bacterial species No. I, II and III. Patulin solution (1 ml) was added to the medium to achieve a final concentration of 10 μg ml^{-1}. The inoculated medium was incubated at 25°C and 150 rpm for 7, 14 and 21 days.

7. 2. 3. Effect of patulin and glucose addition on the growth and patulin production by *Penicillium expansum*

The basal medium used in the following studies was Czapek Dox C-free liquid medium amended with 1000 μg ml^{-1} glucose then adjusted to pH 6.8 with 1.5 N NaOH (Appendix 9. 2. g). The constituents of the basal medium were dispensed into Erlenmeyer flasks (100 ml in 250 ml flasks) and sterilized by autoclaving at 121°C for 20 minutes. After cooling, 1 ml of patulin standard solution (1000 μg ml^{-1}) was added to the flask to achieve a concentration of 10 μg ml^{-1}. The flasks were then inoculated with 1 ml of a suspension of *Penicillium expansum* (10^6 - 10^7) on Czapek Dox agar, and grown for 7 days. The inoculated flasks were then incubated in triplicate at 25°C on a reciprocal shaker (150 rpm) for 0, 3, 7, 14, 21 and 28 days. Controls containing glucose, but lacking patulin were also included.

At the end of each incubation period, the contents of three flasks of each treatment were filtered through pre-dried (for 15 days at 50°C) and pre-weighed Whatman No. 1 filter paper. The filter papers were dried to constant weight for three days at 50°C then the biomass was calculated per 100 ml of the medium.

Glucose was estimated by using di-nitrosalicynic acid method according to the procedure described by Bernfield (1955). In this method 1 ml of the filtrate was diluted

with 1 ml distilled water and mixed with 2 ml of dinitrosalicylic acid (1 gm of 3, 5-dinitrosalicylic acid dissolved in 20 ml of 2 N NaOH and 50 ml H_2O, followed by the addition of 30 g of Rochelle salt, potassium sodium tartrate, and the volume was made up to 100 ml with distilled water) reagent. This mixture was heated for 5 minutes in boiling water and then cooled in running tap water. After the addition of 20 ml water, the optical density of the reaction mixture produced was determined photometrically, at 540 nm. A blank was similarly prepared. A calibration curve using glucose (0.2 to 2.0 mg in 2 ml of H_2O) was used to convert the colorimeter readings into milligrams of glucose.

7. 2. 4. The effect of patulin on the growth of *P.expansum*

The agar diffusion assay was used to determine the effect of patulin on the growth of *Penicillium expansum.* Czapek Dox agar (100ml, autoclaved and cooled in a water bath at 50°C) was seeded with *Penicillium expansum* obtained from a 7 days old culture grown on CD slopes, mixed then poured into Nunc bioassay plate. Wells were cut into the agar using a 13 mm diameter sterile cork borer. Portions (100 µl) of a range of concentrations of patulin, dissolved in sterile distilled water (0, 0.5, 1, 2.5, 5, 10, 15, 20, 25, 30, 35, 40, 50, 60, 80 and 100 µg ml^{-1}) were added to the wells in duplicate. Seeded plates with wells lacking patulin were also included as controls. All plates were incubated at 25°C for 5 to 10 days and any resulting zones of inhibition were measured.

7. 2. 5. Patulin detection

For patulin determination, the contents of both Czapek Dox and apple cultures were analyzed as described in Appendix 9. 6. a to g.

7. 3. Results and Discussion

7. 3. 1. Patulin production by *Penicillium expansum*, *Penicillium* sp (1), *Penicillium* sp (2) and *Penicillium* sp (3) in dual culture when growing in Czapek Dox liquid medium

Table 7. 1 shows that *Penicillium expansum* and the three isolates when growning alone in Czapek Dox medium produced large amounts of patulin. The natural isolates produced more patulin than did the laboratory culture of *P. expansum*. Patulin was also

Table 7. 1. Influence of *Penicillium expansum*, *Penicillium* sp (1), *Penicillium* (2) and *Penicillium* sp (3) in dual culture on patulin production using Czapek Dox liquid medium incubated at 25°C for 7 days.

Fungi	Quantity of patulin detected (μg l^{-1})
Penicillium expansum	395.0
Penicillium sp (1)	1175
Penicillium sp (2)	1089
Penicillium sp (3)	1813
P. expansum + *Penicillium* sp (1)	238.3
P. expansum + *Penicillium* sp (2)	158.4
P. expansum + *Penicillium* sp (3)	ND
Penicillium sp (1) + *Penicillium* sp (2)	ND
Penicillium sp (1) + *Penicillium* sp (3)	ND

produced when *P. expansum* and *Penicillium* sp 1 were grown in co-culture. However, the amount produced was relatively small. The other co-cultures failed to produce patulin. This finding agrees with the results from the apple inoculation experiments (see below). The results enphasise again that mixed cultures of fungi, whether growing in natural substrates or in synthetic medium produce less patulin than when growing in single cultures.

7. 3. 2. Effect of *Penicillium expansum*, *Penicillium* sp (1) and *Penicillium* sp (3) in separate or in dual culture on patulin production in apple fruits

While the isolate *Penicillium* sp (2) was able to produce patulin on Czapek Dox liquid medium it was unable to produce the mycotoxin when grown on apple (Chapter 2); as a result, this isolate was excluded from the following experiment.

Levels of patulin produced in apples inoculated with *Penicillium expansum* alone, and in dual culture with other selected fungi are shown in Figure 7.2 to 7. 4.

Figure 7. 2 shows patulin production in apples following fungal inoculation. The apples were inoculated with *P. expansum* and *Penicillium* species 1 initially (separate culture) separated by 1 cm. These fungi then grew together. *P. expansum* and *Penicillium* sp. 1 were also inoculated in the same place (dual culture). The controls consisted of the two fungi inoculated alone. Large quantities of patulin were produced after 7 days by *Penicillium* sp. 1 inoculated on its own. Small amounts were also produced over this period by *P. expansum.*

By 14 days, all fungi produced at least trace amounts of patulin. *Penicillium* sp. 1 produced the greatest amount at the time, but the quantity produced was much less than after 7 days. The amount of patulin produced by *Penicillium* sp 1 grown alone decreased from the high concentrations produced on day 7. Patulin degradation occurred over the 7 to 21 days incubation period.

At the 21 days sample all inoculated produced patulin. The amount produced in the separate culture exceeded that produced in dual culture by *P. expansum* and approached the amount produced by *Penicillium* sp 1.

The amount of patulin produced in the separate or dual cultures increased over the incubation period, but after 7 and 14 days never reached the levels seen in the controls where the fungi were grown alone.

A similar trend is seen in the case of *Penicillium* sp. 3 grown with *P. expansum* when grown on apples where the fungus, when growing alone, than when growing together with *P. expansum* in separate or dual culture (Fig. 7. 3).

However, the situation is not completely straightforward since *P. expansum* growing with *Penicillium* sp. 3 rarely produced less patulin than when growing alone than when growing after inoculation into apples in dual or separate culture.

Finaly Figure 7. 4 shows that *Penicillium* sp. 1 and 3 again produce more patulin when growing alone on apples than when growing after separate or dual inoculation.

In conclusion over the incubation period separate inoculation of the two fungi led to the production of more patulin than when the two fungi were inoculated together in dual culture, a trend which is particularly noticeable at day 21. No treatments ever produced patulin in excess of the amounts produced when *Penicillium* sp 1 was inoculated alone. And the exception of the 21 day sample the apple inoculated with *P. expansum* contained more patulin than when dual or separate co-cultures were used.

These findings point to the generalization that *Penicillium* sp 1 and *Penicillium expansum* produce large amounts of patulin when growing on their own than when growing in combination with other fungi.

This finding agrees with the results from the apple inoculation experiments. The results enphasise again that mixed cultures of fungi, whether growing in nature substrates or in synthetic medium produce less patulin than when growing in single cultures.

Figure 7. 2

Concentration of patulin detected from apple fruits inoculated with *Penicillium expansum* in separate or in dual places with *Penicillium* sp (1).

Values-means of triplicates ± standard deviation.

Significantly different from controls ($p < 0.05$) except where marked *.

FIG. 7.2

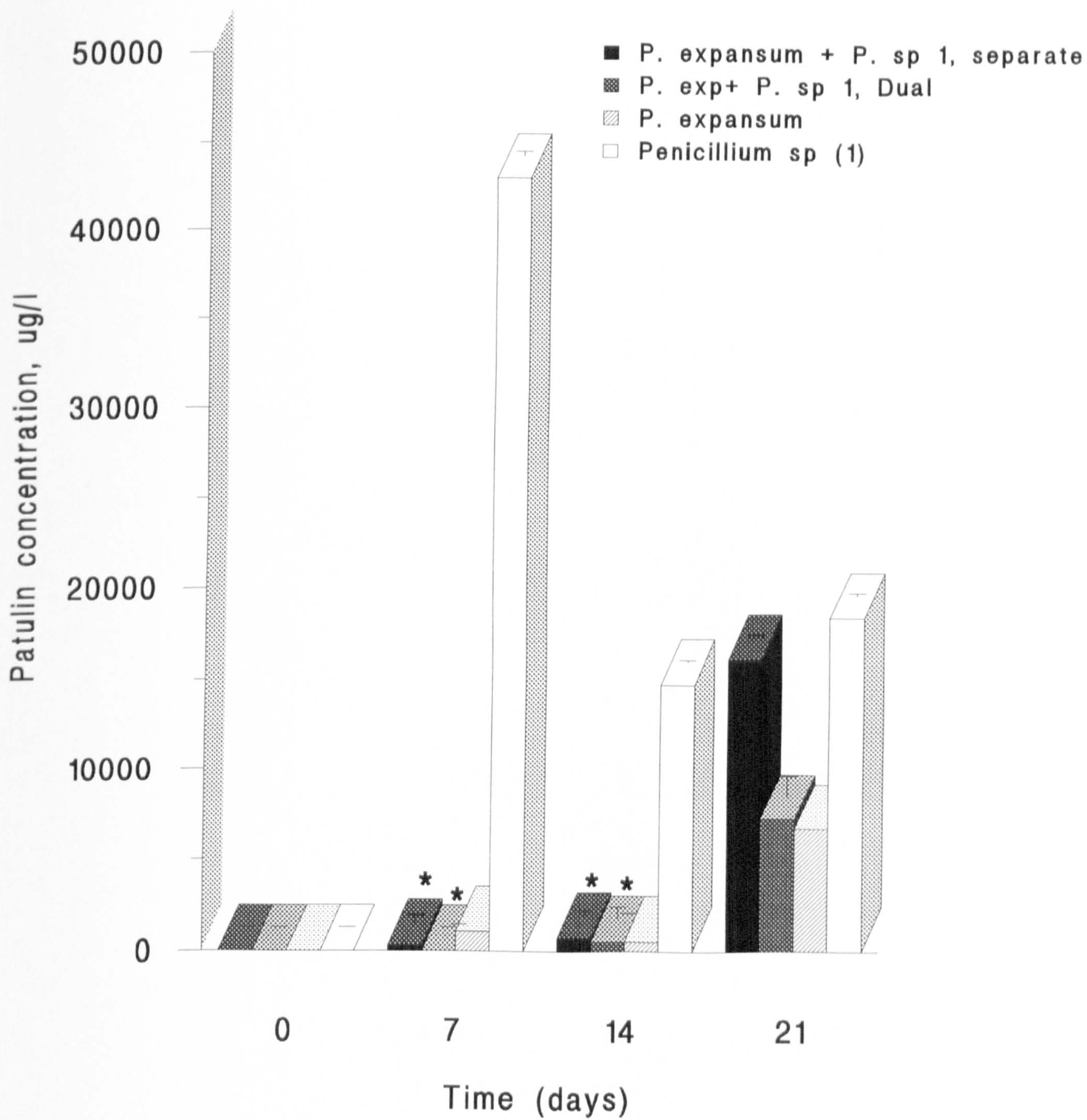


Figure 7. 3

Concentration of patulin detected from apple fruits inoculated with *Penicillium expansum* in separate or in dual places with *Penicillium* sp (3).

Values-means of triplicates ± standard deviation.

Significantly different from controls ($p < 0.05$) except where marked *.

FIG. 7.3

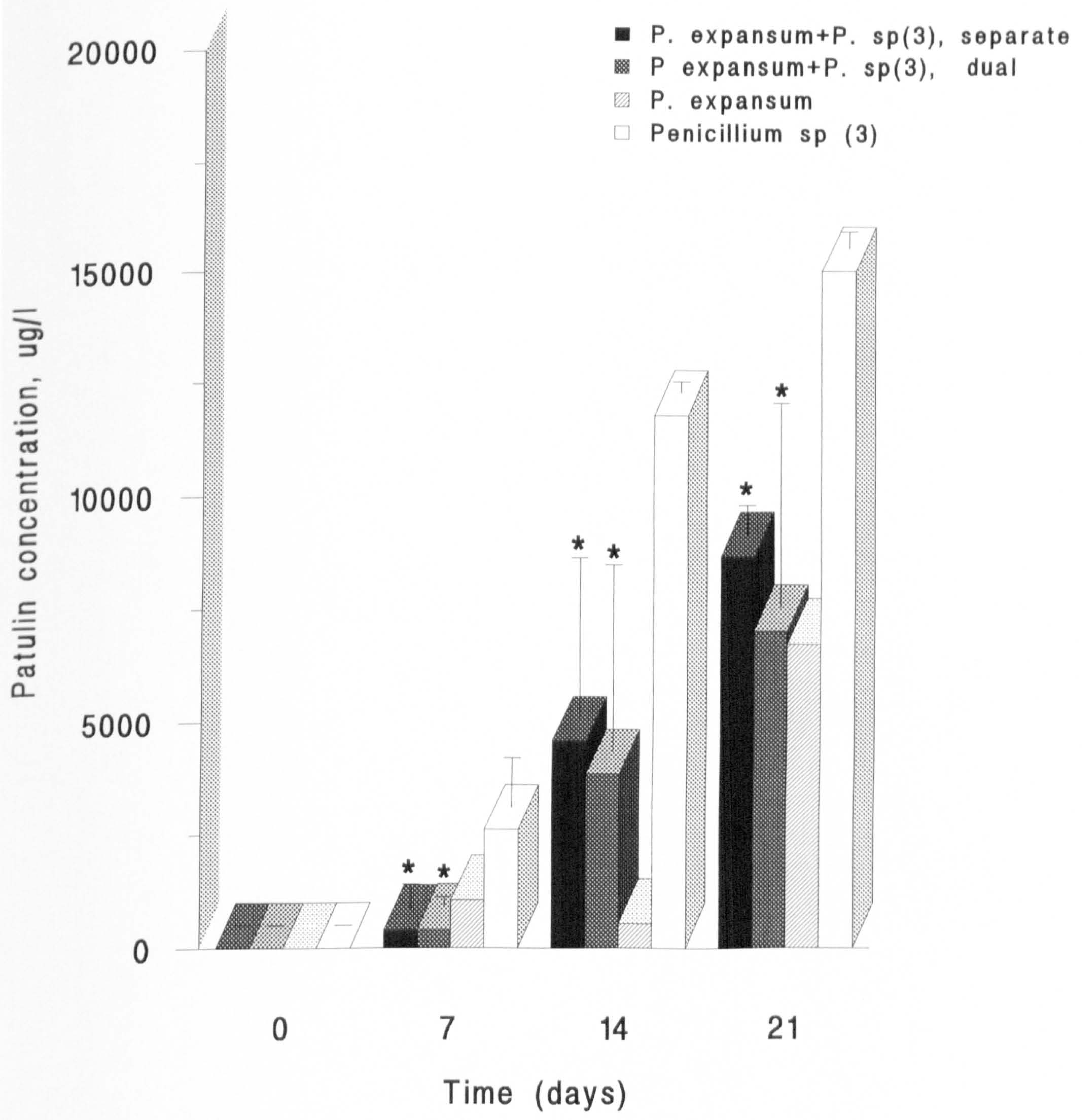


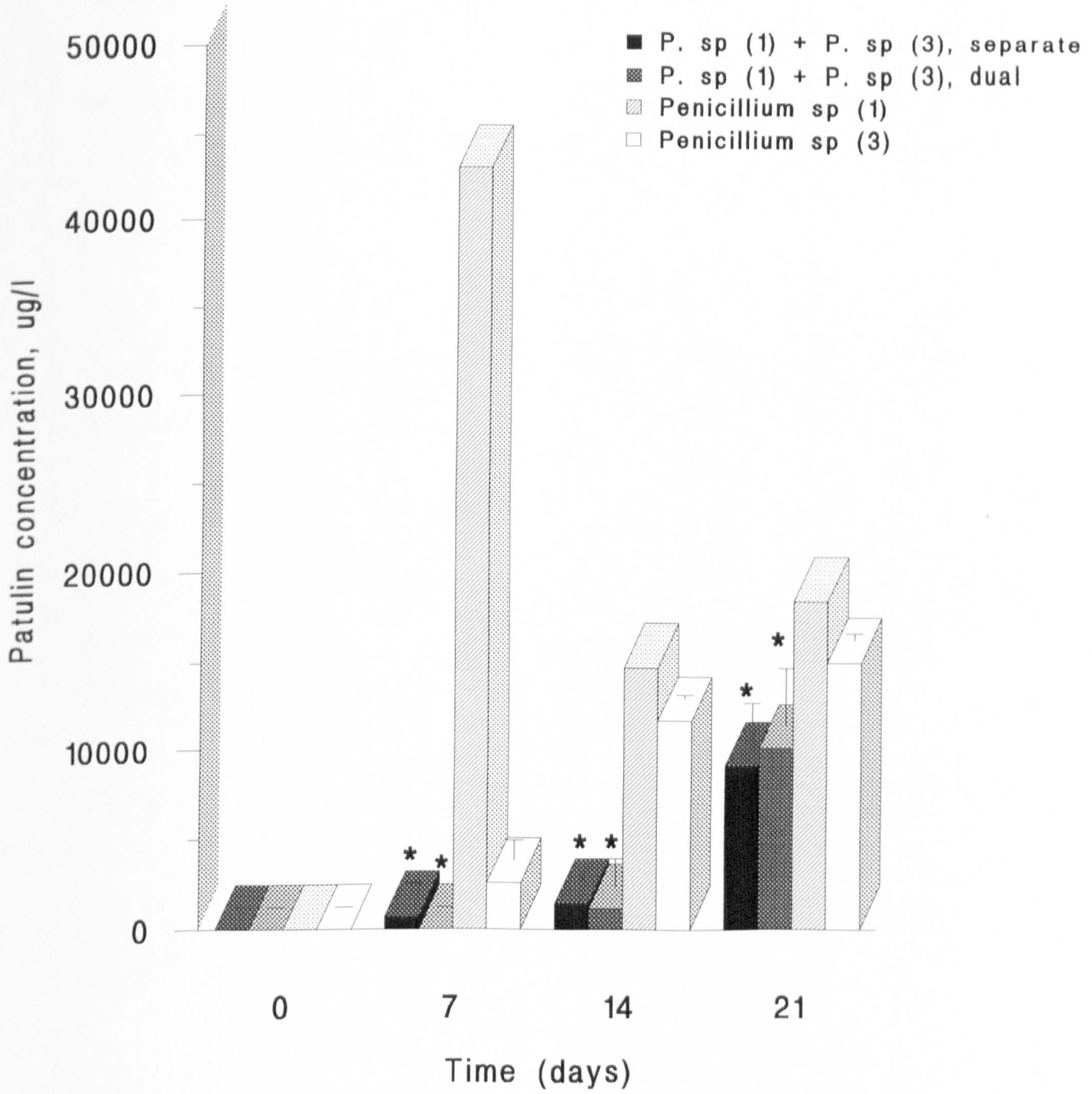
Figure 7. 4

Concentration of patulin detected from apple fruits inoculated with *Penicillium* sp (1) in separate or in dual places with *Penicillium* sp (3).

Values-means of triplicates ± standard deviation.

Significantly different from controls ($p < 0.05$) except where marked *.

FIG. 7.4



7. 3. 2. i. Effect of fungal competition on lesion development on apple fruits

In general, when spores of the following fungi were spiked into apple and incubated for 7 and 14 days after they inoculated in dual cultures with the spores smaller lesions were produced than when inoculated in separate cultures: *Penicillium expansum* and *Penicillium* sp (1), *Penicillium expansum* and *Penicillium* sp (3) or *Penicillium* sp (1) and *Penicillium* sp (3) (Plates 7.1 to 7. 5). The indigenously isolated fungi also tended to show more rigorous colonization of apple than did the laboratory culture of *P. expansum.*

7. 3. 3. Effect of *Penicillium expansum* in dual culture with *Phanerochaete chrysosporium* on patulin production

Table 7. 2. shows the effect on patulin production in Czapek Dox medium of co-culture with the wood-rotting fungus *Phanerochaete chrysosporium.* The amount of patulin in the medium by *P. expansum* was reduced by the presence of *Phanerochaete chrysosporium.* However, it is not clear whether *Phanerochaete chrysosporium* caused *P. expansum* to produce less patulin, or wether the wood-rotting fungus achively degraded patulin.

7. 3. 4. Effect of *Penicillium expansum* in dual culture, with the isolated contaminant bacterial species No. I, II and III, on patulin production

The above results illustrate the effect of patulin produce of growing fungi together in co-culture. In the natural environment fungus grow together with bacteria. It was therfore of interest to determine the effects of natural bacterial inoculated (from apple) on patulin production. Unfortunatly the bacterial isolated did not grow well in Czapek

Plates 7. 1. a

Penicillium sp (1) and *Penicillium* sp (3) when grown in separate places after 7 days of incubation at 25°C.

Plates 7. 1. b

Penicillium sp (1) and *Penicillium* sp (3) when grown in dual places after 7 days of incubation at 25°C.

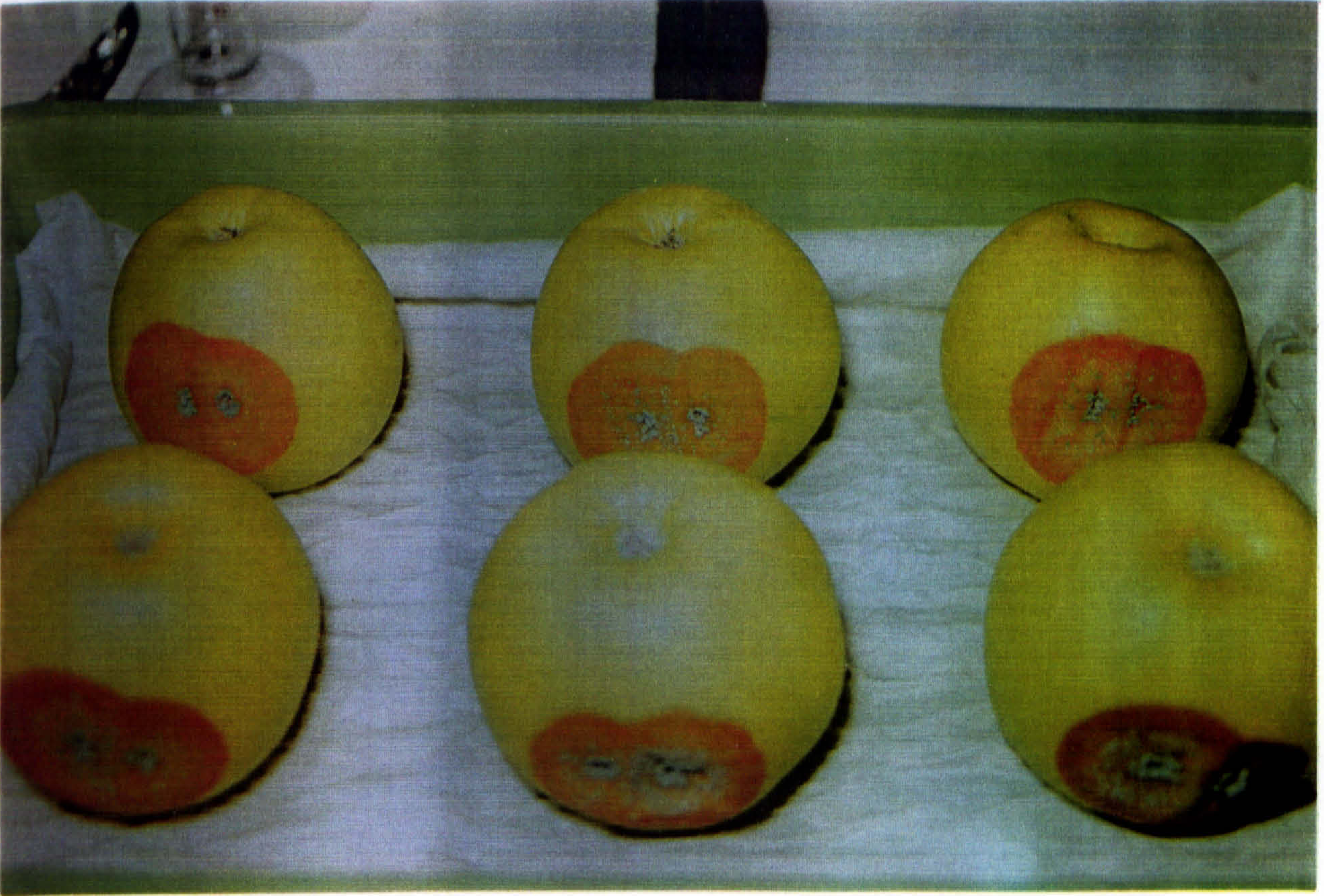


Plate 7. 2. a

Decayed apples showing lesion characteristic growth produced by *Penicillium expansum* inoculated in separate places with *Penicillium* sp (3) after 7 days of incubation at 25°C.

Plate 7. 2. b

Decayed apples showing lesion characteristic growth produced by *Penicillium expansum* inoculated in dual places with *Penicillium* sp (3) after 7 days of incubation at 25°C.

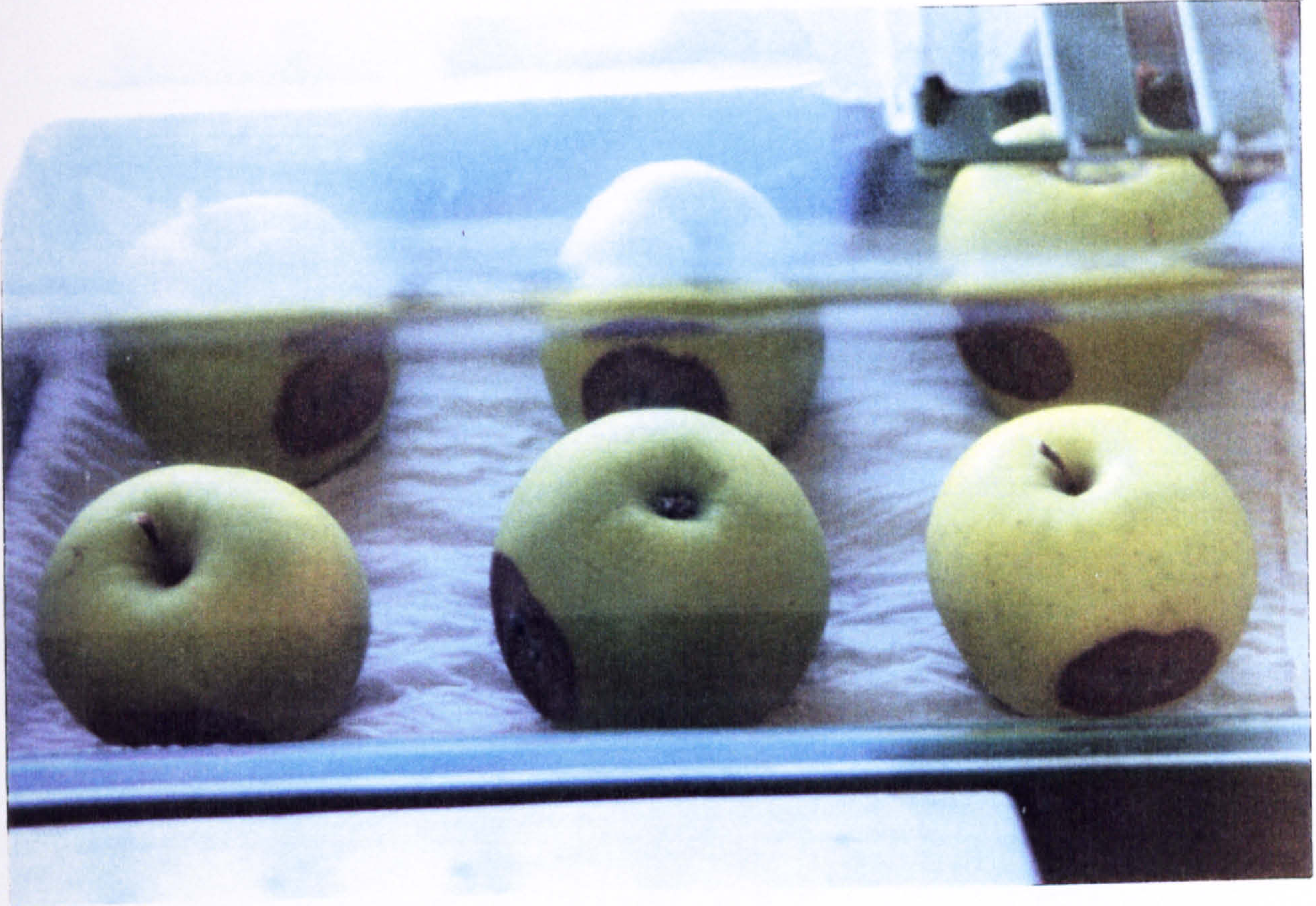


Plate 7. 3

Fungal growth and small diameter lesions produced by *Penicillium expansum* inoculated in dual places with *Penicillium* sp (3) after 14 days of incubation at 25°C.

Plate 7. 4

High rate of fungal contamination and zones of growth, as well as spore formation, produced by *Penicillium* sp (1) inoculated in separate places with *Penicillium* sp (3) after 14 days of incubation at 25°C.

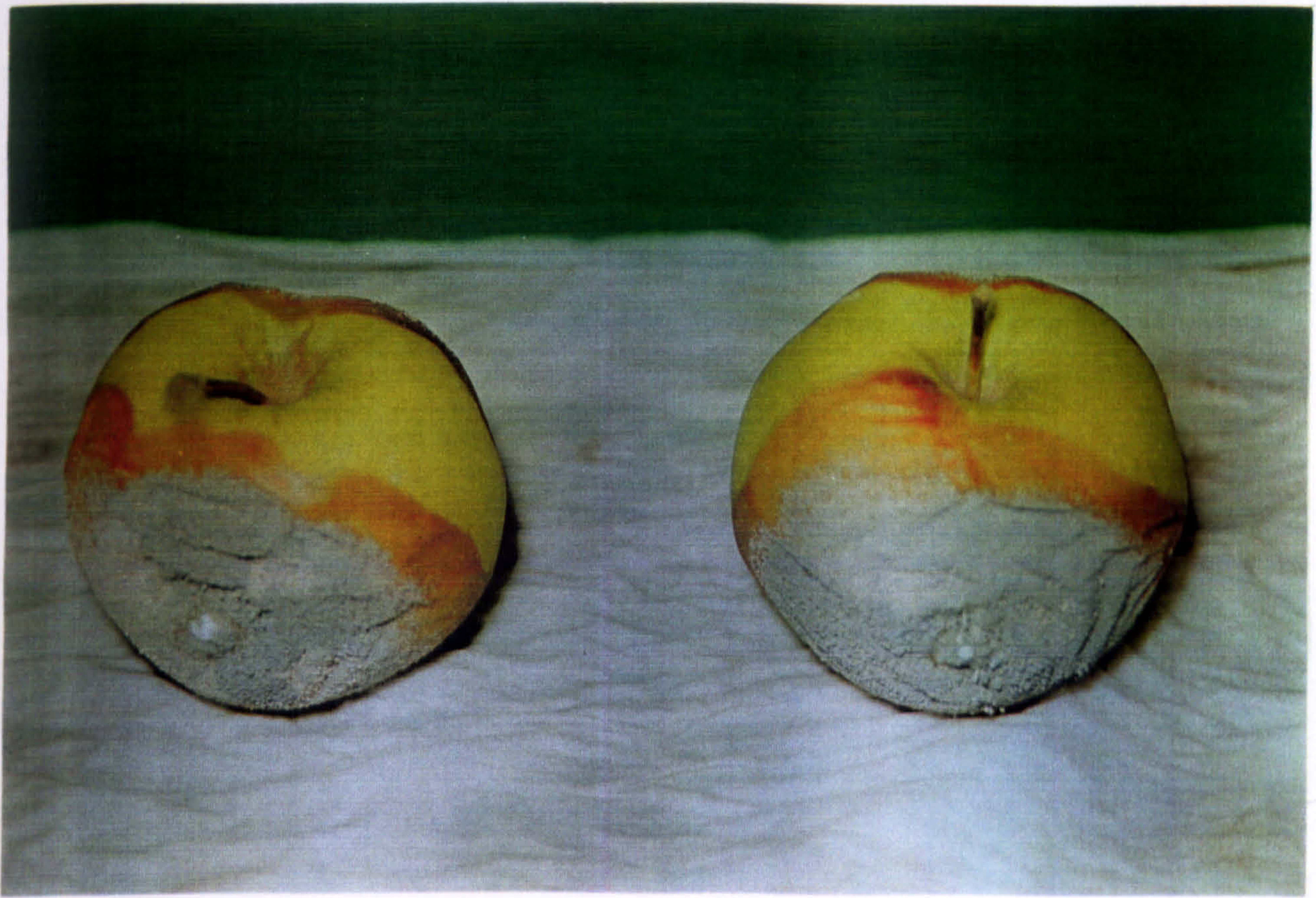


Plate 7. 5. a

Moderate fungal growth, as well as spore formation, produced by *Penicillium expansum* inoculated in separate places with *Penicillium* sp (1) after 14 days of incubation at 25°C.

Plate 7. 5. b

Moderate fungal growth, as well as spore formation, produced by *Penicillium expansum* inoculated in dual places with *Penicillium* sp (1) after 14 days of incubation at 25°C.

Plate 7. 6. a

Completely decayed apples, showing growth of each isolate and high rate of spore formation produced by *Penicillium* sp (1) inoculated in separate places with *Penicillium* sp (3) after 21 days of incubation at 25°C.

Plate 7. 6. b

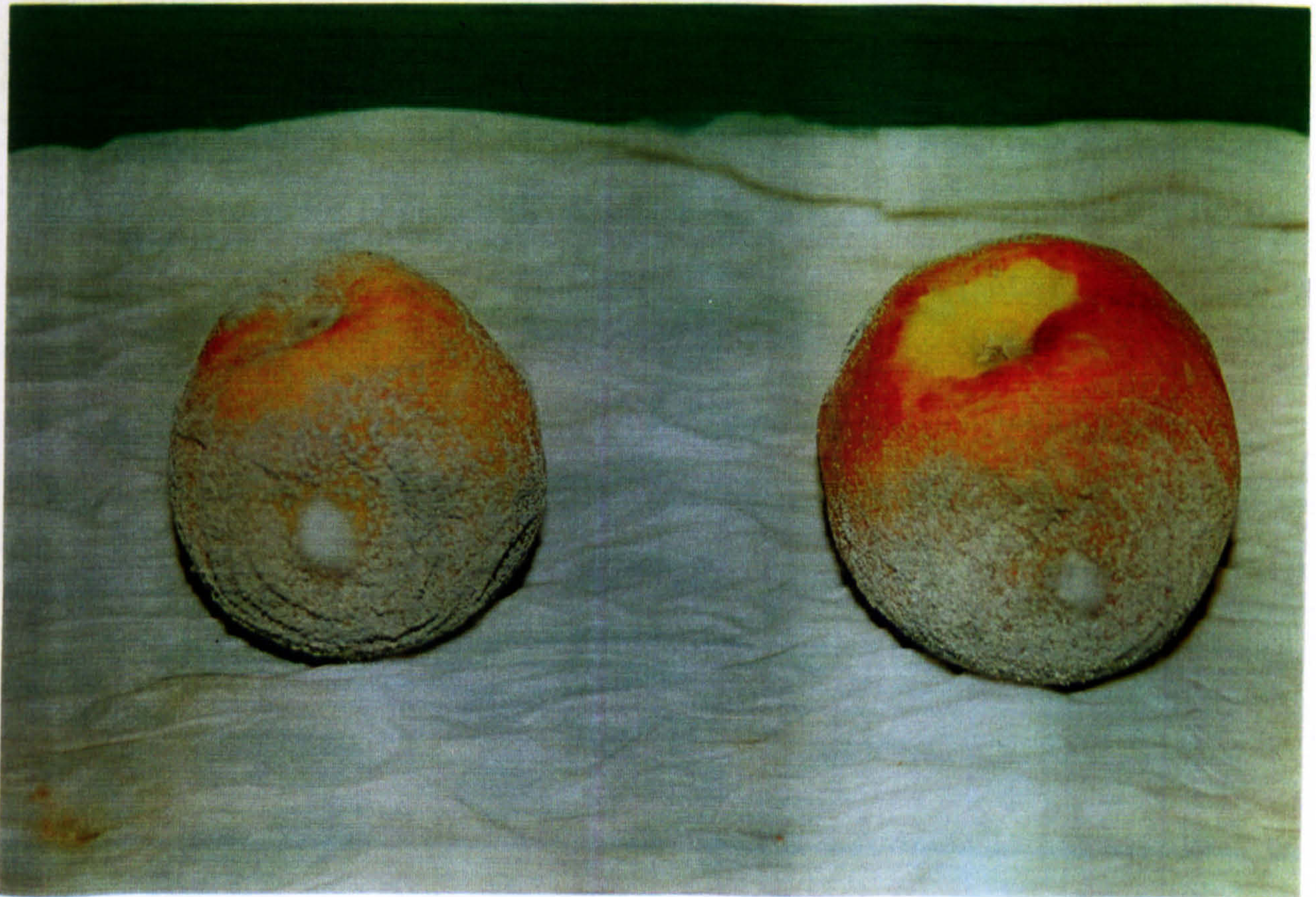
Completely decayed apples, showing high growth and high rate of spore formation produced by *Penicillium* sp (1) inoculated in dual places with *Penicillium* sp (3) after 21 days of incubation at 25°C. No dimidiate growth was formed.

Plate 7. 7. a

Almost completely decayed apples, showing moderate fungal growth and spores formation, produced by *Penicillium expansum* inoculated in separate places with *Penicillium* sp (1) after 21 days of incubation at 25°C.

Plate 7. 7. b

Almost completely decayed apples, showing moderate fungal growth and spores formation, produced by *Penicillium expansum* inoculated in dual places with *Penicillium* sp (1) after 21 days of incubation at 25°C.



Dox agar and nutrient agar, nor when inoculated into apple. For these experiments therfore, apple extract (40 g in 100 ml distilled water) was used as the substrate on which both fungus and bacteria were grown.

Table 7. 2. Effect of *Phanerochaete chrysosporium* on the production of patulin by *Penicillium expansum* after 7 days of incubation at 25°C in Czapek Dox liquid medium.

Isolates	Quantity of patulin detected ($\mu g\ l^{-1}$)
Penicillium expansum	435
P. expansum + *P. chrysosporium*	296
Phanerochaete chrysosporium	ND

ND = not detected

The results are shown in Figures 7. 5 - 7. 7. The general trend for all fungi and bacteria tested is analagous to that seen when fungi were grown in co-culture, is that patulin production by the patulin producing fungi was reduced when grown in co-culture with naturally isolated bacteria (An exception is seen in the case of *Penicillium* sp. 3 when grown for 14 days with bacteria isolate II and 21 days with bacterial isolate I).

7. 3. 5. Influence of patulin addition on *Penicillium expansum*, *Phanerochaete chrysosporium* and the isolated contaminant bacteria (I, II and III)

7. 3. 5. i. Effcet of *P. expansum* on patulin degradation in C-free Czapek Dox medium

Figure 7. 5

Concentration of patulin detected when *Penicillium expansum* was grown in CD in association with the isolated contaminant bacterial species I, II and III.

FIG. 7.5

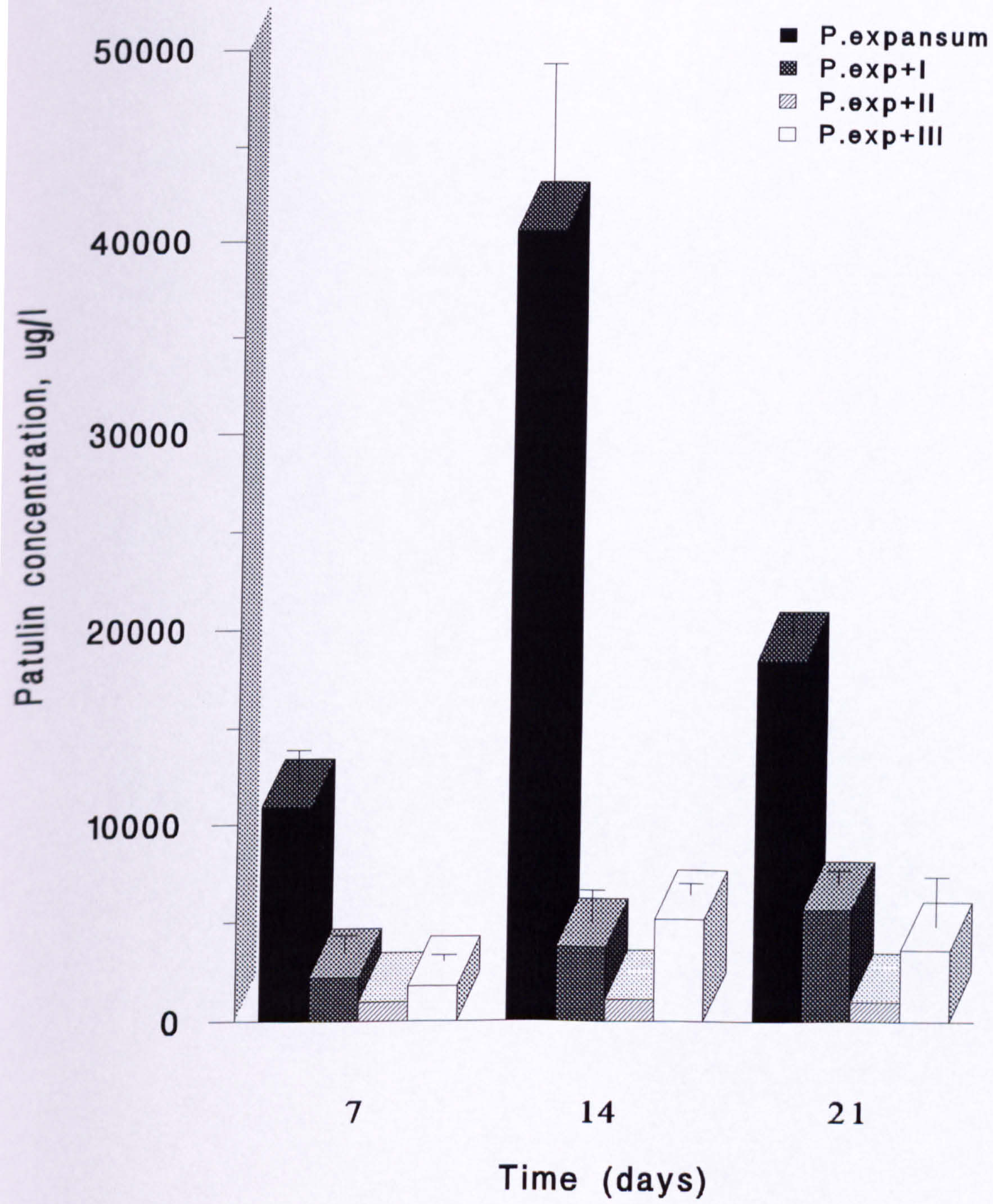


Figure 7. 6

Concentration of patulin detected when *Penicillium* sp (1) was grown in CD in association with the isolated contaminant bacterial species I, II and III.

FIG 7.6

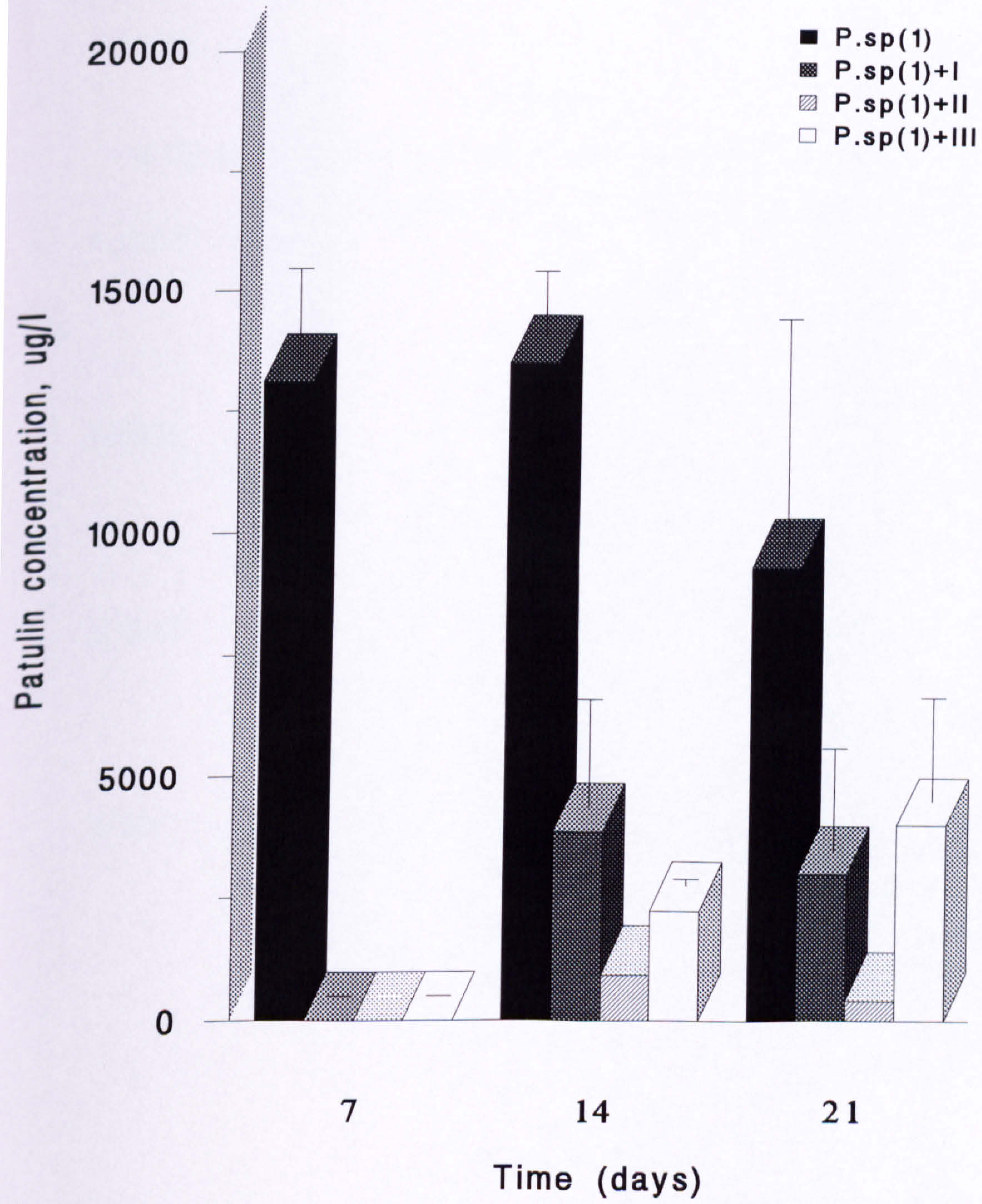
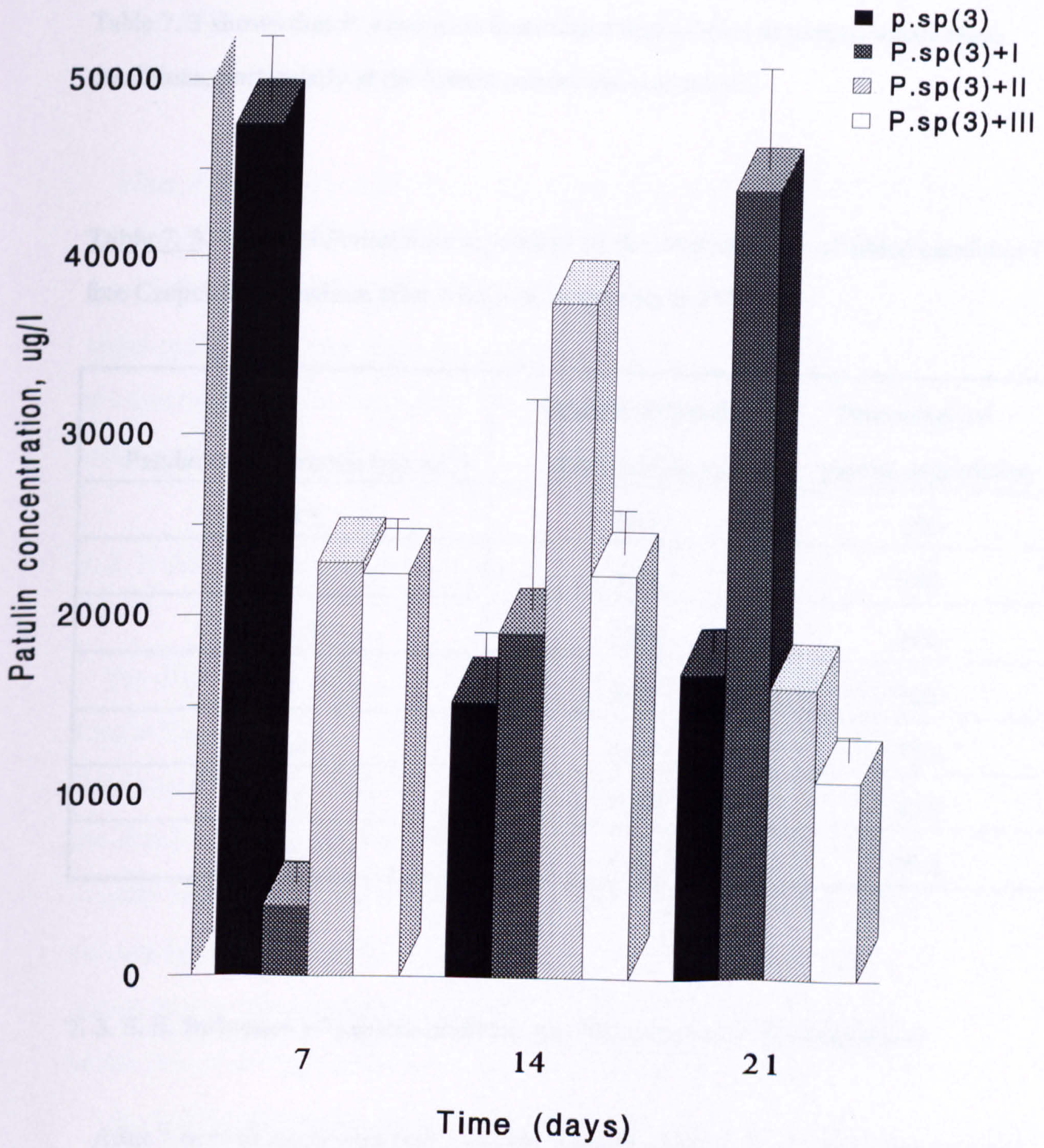


Figure 7. 7

Concentration of patulin detected when *Penicillium* sp (3) was grown in CD in association with the isolated contaminant bacterial species I, II and III.

FIG 7.7



The aim of these experiments was to determine whether *P. expansum* could degrade, as well as produce, patulin. For these experiments C-free Czapek Dox medium was used so that no carbon source for patulin production by the fungus was made available. Table 7. 3 shows that *P. expansum* is an active degradation of patulin where these conditions, particularly at the lowest patulin concentrations.

Table 7. 3. Effect of *Penicillium expansum* on the concentration of added patulin in C-free Czapek Dox medium after 7 days of incubation at 25°C.

Patulin concentration (μg ml^{-1})	Quantity of patulin detected (μg ml^{-1})	Percentage of patulin degradation
0.5	ND	100
1	ND	100
2.5	ND	100
5	ND	100
10	0.30	97.0
15	0.80	92.0
20	0.42	95.8

7. 3. 5. ii. Influence of patulin addition on *Phanerochaete chrysosporium*

After 7 days of incubation only a patulin concentration of 723.7 μg l^{-1} was detected out of 2000 μg l^{-1} of patulin added. As *Phanerochaete chrysosporium* has the ability to degrade a wide toxic, environmentally persistent compounds (Bumpus, 1993), so this

fungus may be able to degrade patulin as well. Moreover, since *Phanerochaete chrysosporium* is wide spread in nature, mainly soil (Ali, 1993), this might be a reason of insufficiency of patulin in soil.

7. 3. 6. Effect of patulin and glucose addition on the growth and patulin production of *Penicillium expansum*

When *P. expansum* was grown in the presence of patulin and represent small amount of carbon (1000 $\mu g\ ml^{-1}$) patulin degradation still occurred (Fig. 7. 8). Glucose was degraded under these conditions as well as patulin (Fig. 7. 9), the glucose acting as the major carbon source for biomass production (Fig. 7. 10) (the decrease in biomass seen towards the end of the incubation periods is presumably due to increase cellula lysis with time).

7. 3. 7. Effect of patulin on the growth of *Penicillium expansum*

The effect of increasing patulin concentration on the growth of *P. expansum* on Czapek Dox agar is shown in plates 7. 8 a - c. In the case of both duplicate treatments there was a stimulation of growth with increasing concentration of added patulin. (NB. the stimulation zones (shown in plate 7. 8) can, at first sight be confused with inhibition - clearly zones). Since this stimulation in growth occurred on Czapek Dox agar, which contains high levels of carbon, the effect is clearly not due to the patulin actions as a carbon source. The stimulatory effect seen is of interest because it suggests that the fungus was producing patulin as a means of stimulating growth and or metabolism.

Our results suggest that it could be production of some metabolites which altered the substrate to either enhance or inhibit growth of *P. expansum*, *Penicillium* sp (1), *Penicillium* sp (2), or *Penicillium* sp (3) or their ability to produce patulin or both. Harwig *et al.* (1973 b) stated that production of metabolites can be lead to

Figure 7. 8

Concentration of patulin detected during growth of *Penicillium expansum* on carbon free Czapek Dox liquid medium amended with 1000 μg ml^{-1} glucose and 10 μg ml^{-1} patulin.

Figure 7. 9

Hydrolysis of glucose (1000 μg ml^{-1}) by *Penicillium expansum* *in vitro*.

Figure 7. 10

Biomass of *Penicillium expansum* during glucose (1000 μg ml^{-1}) or glucose (1000 μg ml^{-1}) and patulin (10 μg ml^{-1}) hydrolysis *in vitro*.

FIG. 7.8

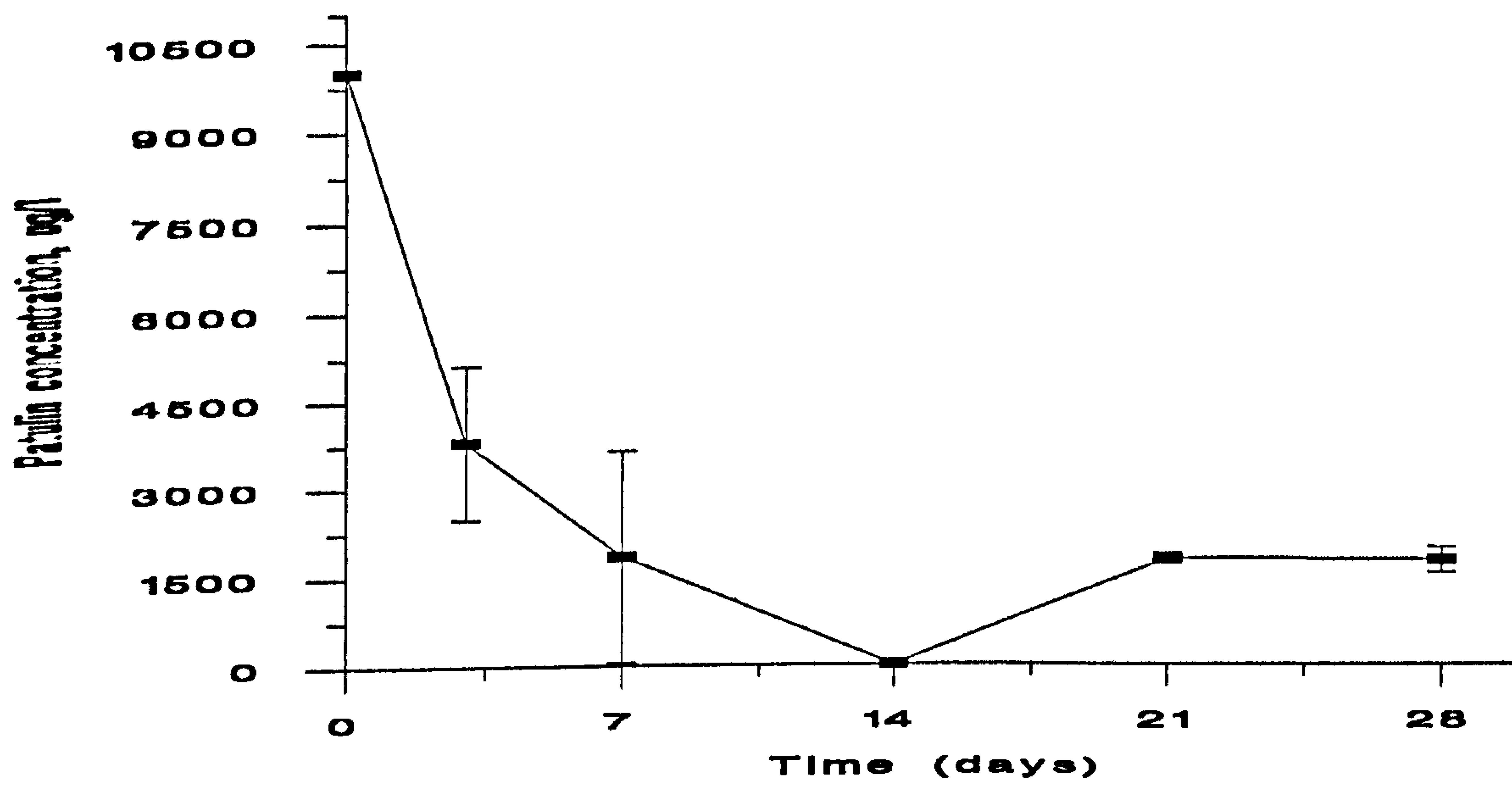


FIG. 7.9

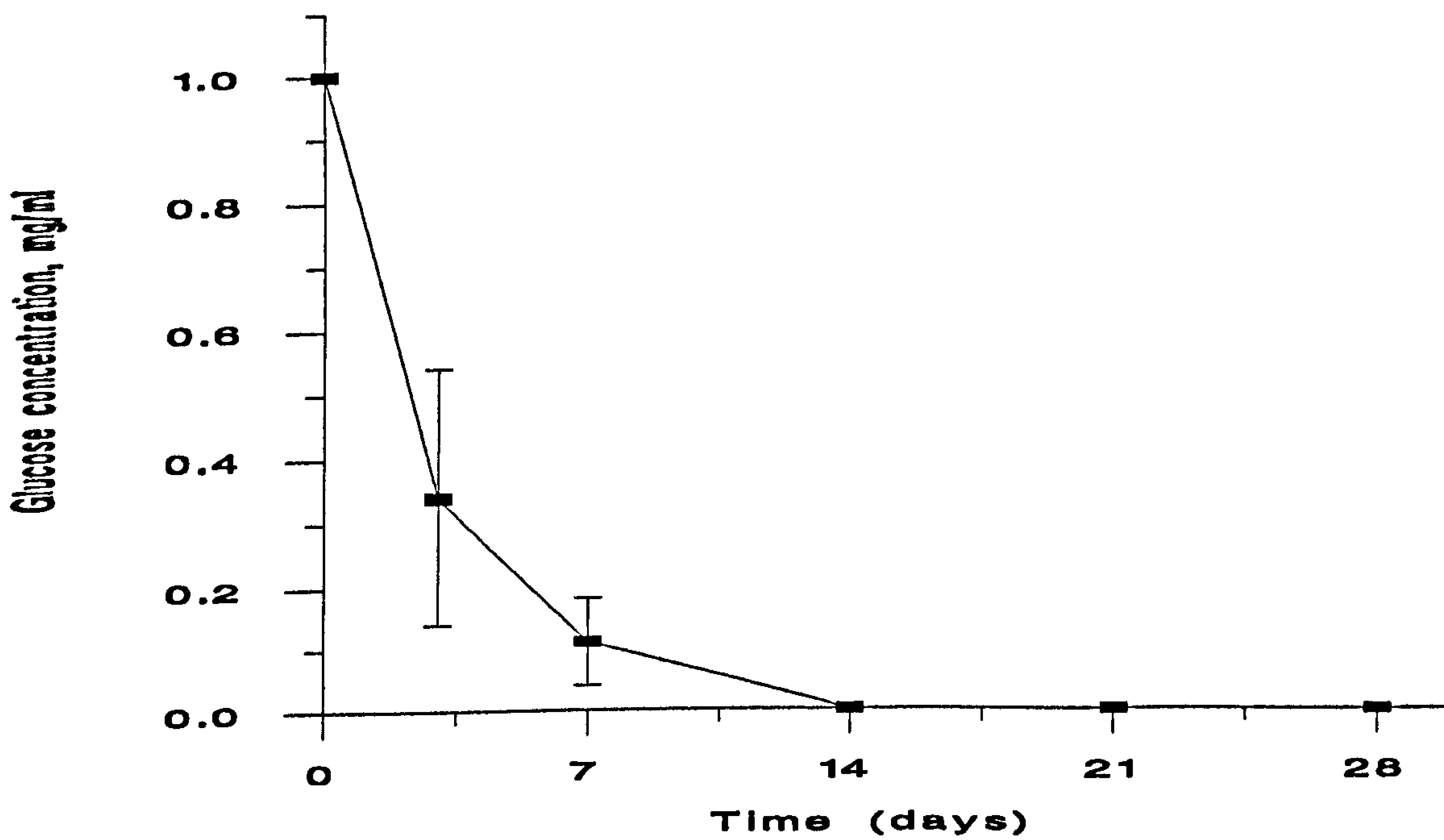


FIG. 7.10

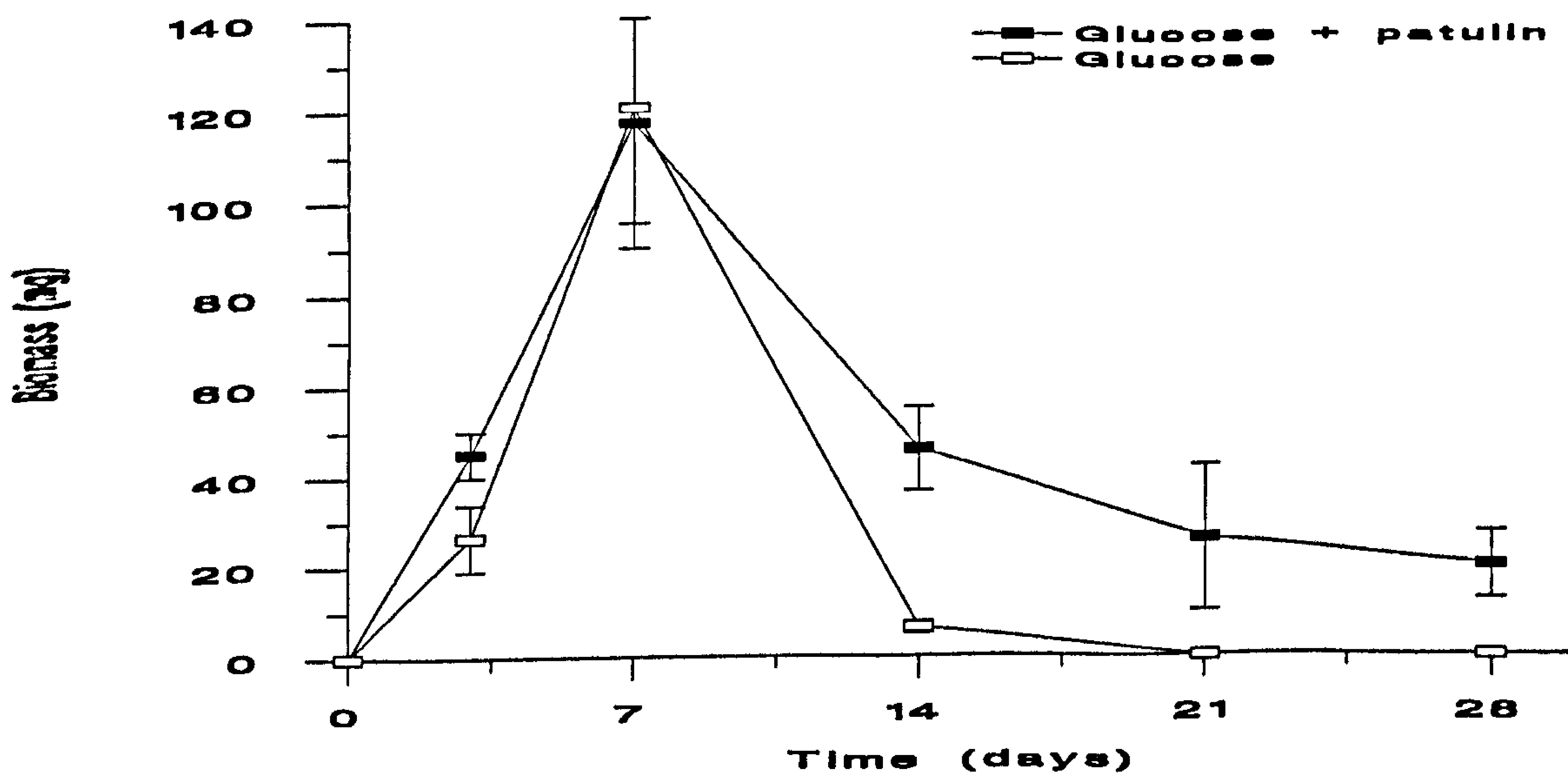


Plate 7. 8. a

Effect of patulin on the growth of *Penicillium expansum* (0, 0.5, 1, and 2.5 μg ml^{-1}).

Duplicates treatments are shown.

0 = Sterile distilled water lacking patulin.

Control = drilled wells without sterile distilled water or patulin addition.

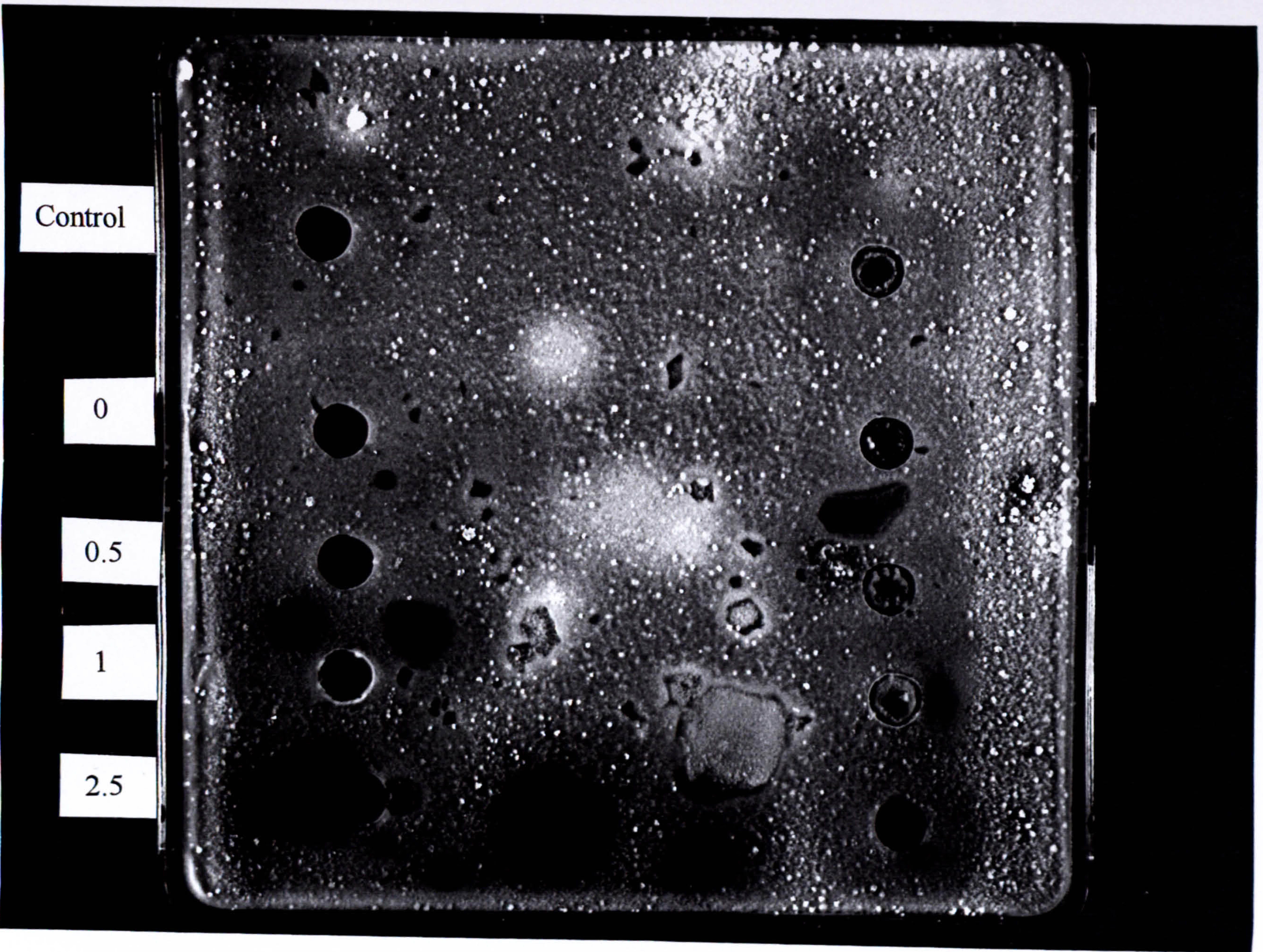
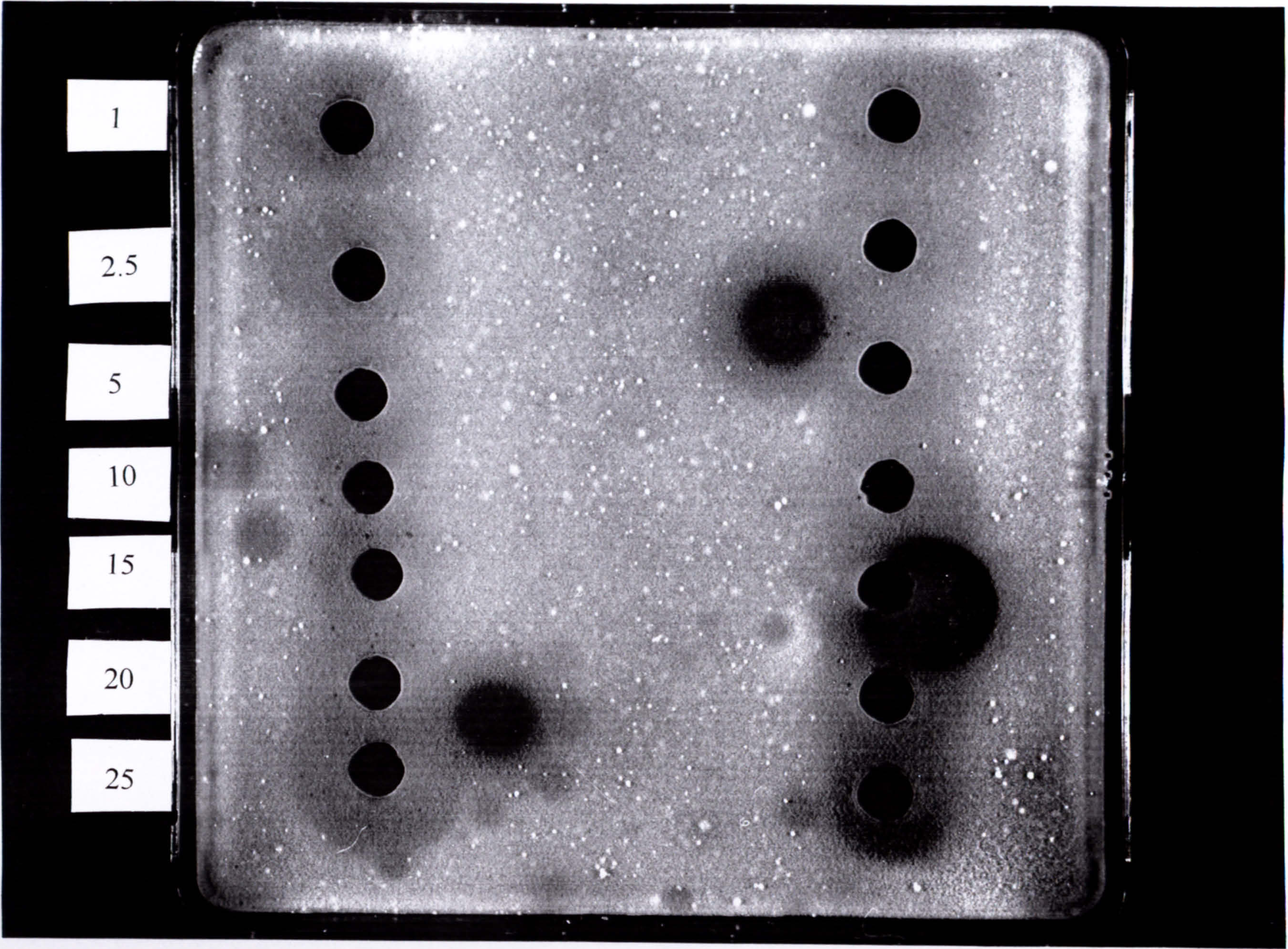

Control
0
0.5
1
2.5

Plate 7. 8. b

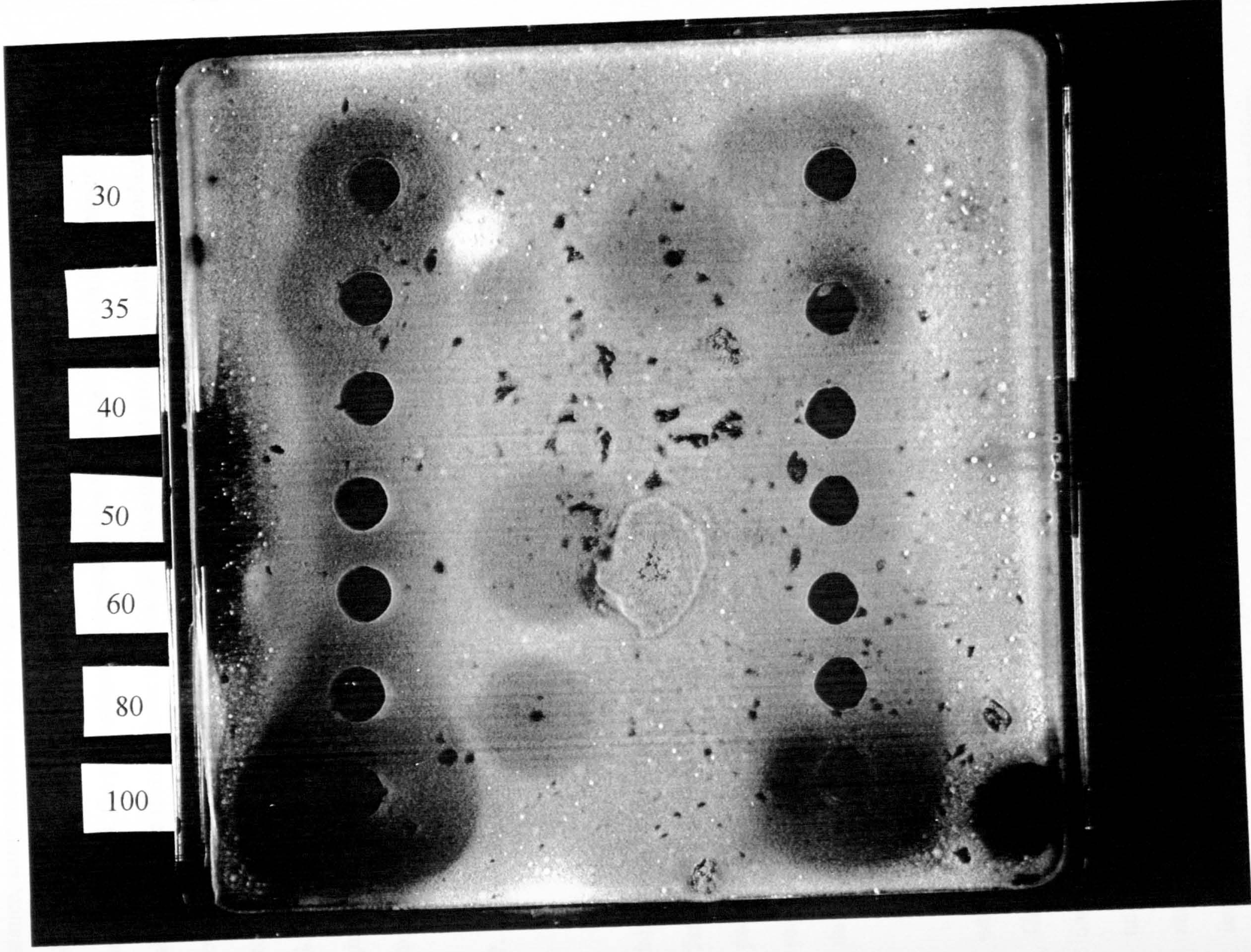
Stimulation the growth of *Penicillium expansum* by different concentrations of patulin (1 to 25 μg ml^{-1}).



1
2.5
5
10
15
20
25

Plate 7. 8. c

Stimulation the growth of *Penicillium expansum* by different concentrations of patulin (30 to 100 μg ml^{-1}).



30
35
40
50
60
80
100

disappearance, diminution or stimulation of patulin produced in the medium. For instance, some secondary metabolites act as hormones (Barksdale, 1969), controlling microbial sporulation (Sadoff, 1971) or detoxification processess (Luckner, 1972). On the other hand, the production of some enzymes for bacterial growth can change the substrate to be favourable or unfavourable to either the growth or patulin production or both by the selected fungi. Tautorus and Townsley (1983) reported mycelial growth increasing by *Agaricus bisporus* in association with bacterial cocultivation. During alcoholic fermentation of apple juice production of sulphydryl groups which containing glutathione compound or sulphite (SO_2) contributed to disappearance of patulin from apples (Pohland and Allen, 1970; Hofmann *et al.*, 1971).

7. 4. Conclusions

A wide range of microorganisms were found to produce secondary metabolites in laboratory or in nature or in both. Beside carbon and nitrogen to enhance the growth of fungi and influence their ability to produce mycotoxins or both growth and mycotoxins production. Although the effect of fungi in association with yeasts and bacteria was recorded, there are only a few references to patulin biosynthesis when fungi are grow in dual culture with fungi or bacteria (Harwig *et al.*, 1973 b; Stinson *et al.*, 1978).

The several trends from the results present above are as follows:

The fungi generally produced more patulin when growing alone on the apple than when growing together, following either separation or dual inoculation onto apples. The same trend was also observed in Czapek Dox medium. These results suggest that competition between the two fungi occurred. This competition is likely to have been for nutrients, or may have resulted from one fungus utilizing an intermediate produced by another, thereby reducing patulin production. It is also likely that antagonistic interactions occurred, such that growth of one or both fungi occurred, with the result that patulin production was inhibited. These results suggest that in natural conditions,

situations where the fungus is growing alone are likely to lead to larger patulin levels than where fungi grow as co-contaminants a separate inoculation led to higher quantities of patulin production than dual inoculation. Presumably patulin was produced under these conditions before negative interaction between the two fungi could occur. Some of which are altered the substrate due to metabolic activity led to enzymatic changes leading to enhanced or reduced patulin production. Burroughs (1977) reported inhibition of more than 90% of patulin during fermentation and more than 99% was demonstrated by Stinson *et al.* (1978). Cuero *et al.* (1987) found increased and decreased in growth or aflatoxin production or both by *Aspergillus flavus* which were refer to enzymatically changed in the media making them more suitable or unsuitable for the fungal growth and aflatoxin biosynthesis. Moreover, the production of more than one mycotoxin by a fungus could affect the growth and production of patulin by other fungus. Wicklow *et al.* (1980) reported the non-production of aflatoxin by *Aspergillus flavus* when grown paired with *Aspergillus niger* despite the fact that they are aflatoxin producer.

The experiment described shown patulin disappearance in all substrates applied. This suggests that patulin may act as a carbon source for both fungal growth and patulin production (Foster, 1949; Cuero *et al.*, 1987).

8. GENERAL DISCUSSION

8. GENERAL DISCUSSION

The production of mycotoxins is invariably studied from the viewpoint of their toxic effects on man. As a result, little thought has been given in the past to the question of why these compounds are produced in nature. Quite clearly, mycotoxins are not produced in order to act directly as toxins or carcinogens in relation to human health. It is possible that fungi produce mycotoxins as a way of conferring some growth advantage on the producer organism. However, it should be borne in mind that compounds can be produced by microorganisms as a result of their general metabolism and that the products of this metabolism need not necessarily serve a function. In this way mycotoxin production in nature may be regarded as unregulated or merely fortuitous.

Since mycotoxins, such as patulin, are also often antibiotics it is tempting to speculate that the producer organism gains a competitive advantage by producing them, so that fungi produce antibacterial compounds to prevent, or decrease, the effects of bacterial competition in the environment. This is analogous to the view, often put forward, generally without being well-evidenced, that antibiotics are produced by fungi in the environment in order to act as a deterrent to the growth of competing bacteria. In fact there is little evidence to suggest that medically useful antibiotics like penicillin are produced under natural conditions, rather than being merely laboratory artefacts (Falih, 1995).

In contrast, Grossbard (1948) has shown that patulin can be produced by fungi when growing on natural substrates. The major rider to this finding however, is that this mycotoxin was only produced on autoclaved substrates, such as straw. Quite clearly mycotoxins are produced in nature, since patulin for example, is known to contaminate fruit juices made from normally harvested apples and other fruits.

As has already been stated, and confirmed by results presented on this thesis, patulin is often produced in larger amounts when fungi grow on natural substrates, than when

grown on defined laboratory media such as Czapek Dox (Vinas-Almenar *et al.*, 1993). One must assume that necessary growth factors, absent from simple media, are found in complex natural substrates.

Although patulin is regarded as being toxic to bacteria (Waksman, 1947), the results present in Chapter 2 show that at least some of the bacteria found growing on apples are insensitive to even high concentrations of the toxin. This points to the view that patulin is unlikely to be effective if produced as a means of reducing bacterial competition. Low concentrations of patulin were even found to stimulate bacteria growth, a finding which could be used to suggest that fungi encourage the growth of bacteria, perhaps these organisms prove beneficial to their growth. It should be emphasised however, that while patulin was studied here, the possibility cannot be excluded that other mycotoxins/antibiotics are coincidentally produced when fungi grow in media or on natural substrates.

The results presented in Chapter 3 show that fungi can produce patulin when inoculated onto sugar beet slices. Only *Penicillium* sp1 was unable to produce patulin when growing on both liquid medium or sugar beet slices.

Fungus also produced patulin on autoclaved wheat straw. The wood decomposing fungus *Phanerochaete chrysosporium* was incapable of producing patulin on straw, and it did not stimulate patulin production by *P. expansum* when these fungi were grown together. Under these conditions, straw decomposition took place and, as a result, nutrients capable of supporting the growth of *P. expansum* were released. Again one must conclude that essential growth factors necessary for patulin production, were not liberated by the growth of the wood-decomposer.

In Chapter 5 it was shown that the three fungi used were all capable of hydrolysing urea, but that only one *Penicillium* sp1 was capable of nitrification. Again it is not clear what function nitrification plays in fungal metabolism, although it is clear that numerous species, including yeasts are capable of oxidising reduced N compounds, ultimately to nitrate (Killham, 1986; Wainwright and Grayston, 1987). No correlation

was found between nitrification and patulin production (Grootwassink and Gaucher, 1980).

White and Johnson (1982) concluded that aflatoxin production by *A. flavus* could be correlated with the ability of strains to nitrify; they suggested that the ability to nitrify might be used diagnostically to detect aflatoxin producers.

Oligotrophic growth of some of the *Penicillum* species used here was demonstrated, although same did not grow under these conditions. The addition of small amounts of N to C-free medium surprisingly did not increase growth of the fungi tested. It is assumed that fungi gain sufficient N by scavenging from the atmosphere under low C conditions. The further addition of N does not stimulate growth because carbon is limiting. This finding demonstrates that oligotrophic growth is "balanced growth" and that under these conditions the term "starvation" is inappropriate. The fungi did not produce patulin when the C-concentration was below 500 $\mu g\ ml^{-1}$. This finding is perhaps not surprising since secondary metabolite production is generally considered to be an energy demanding process. Since patulin production occurs on substrates such as straw, which are unlikely to contain large amounts of available carbon it is assumed that the lack of patulin production in low C media is probably due to the absence of unknown co-factors which are present in natural substrates.

When the fungi were grown directly on apples more patulin was produced when isolated cultures were set up than when co-cultures, either separate or dual inoculation, were used. The results suggest that competition between fungi occurred and that this led to a reduction in patulin production.

Finally patulin can also act as a carbon source for utilisation by fungi.

Although the results obtained during the course of this work extend our knowledge of patulin production on natural substrates, it is clear that far more research is needed on this area before we can gain a full understanding of why patulin is produced in nature and what factors influence its formation under non-laboratory conditions.

9. APPENDIX

9. APPENDIX.

9. 1. Solid media used for isolation and growth of fungi and bacteria.

(a). Czapek Dox agar (Oxoid).

composition:

$NaNO_3$	2.00 g
KCl	0.50 g
Magnesium glycerophosphate	0.50 g
$FeSO_4$	0.01 g
K_2SO_4	0.35 g
Sucrose	30.0 g
Agar (Oxoid No. 3)	12.0 g
Distilled water	1.0 liter

pH adjusted to 6.8

A proprietary formulation (Oxoid) of Czapek Dox agar was routinely used, prepared by suspending 45.4 g of the powder in 1 liter of distilled water. The medium was then dissolved and sterilized by autoclaving at 121°C for 15 minutes.

(b) Plate Count agar.

composition:

Tryptone	5.0 g
Yeast extract	2.5 g
Glucose	1.0 g
Agar (Oxoid No. 3)	15.0 g
Distilled water	1.0 liter

pH adjusted to 7.0

A proprietary formulation (Oxoid) of Plate Count agar was routinely used, prepared by suspending 17.5 g of the powder in 1 liter of distilled water. The medium was then dissolved and sterilized by autoclaving at 121°C for 15 minutes.

(c) Nutrient agar (Oxoid).

composition:

'Lab-lemco' Powder (Oxoid L 29)	1.0 g
Yeast extract (Oxoid L 20	2.0 g
Peptone (Oxoid L 37)	5.0 g
Sodium chloride	5.0 g
Agar (Oxoid No. 3)	15.0 g
Distilled water	1.0 liter

pH adjusted to 7.4 ± 0.2

A proprietary formulation (Oxoid) of Nutrient agar was routinely used, prepared by suspending 28 g of the powder in 1 liter of distilled water. The medium was then dissolved and sterilized by autoclaving at 121°C for 15 minutes.

(d) Silica gel medium (Parkinson *et al.*, 1989).

Mineral salt solution:

Composition:

KH_2PO_4	1.00 g
KCl	0.50 g
$MgSO_4.7H_2O$	0.50
$FeSO_4.7H_2O$	0.01 g
purified distilled water (pdw)*	1.0 liter

pH was adjusted to 5.2 ± 0.2

* Purified distilled water was obtained by passing distilled water through a "Mill Q" filtration system (Millipore Corp.).

Orthophosphoric acid:

20% orthophosphoric acid (20 ml orthophosphoric acid was added to 80 ml pdw)

Potassium silicate:

7 g of KOH were dissolved in 100 ml purified distilled water then added to 8 g of silicic acid. Mineral salts, orthophosphoric acid and potassium silicate were autoclaved at 121°C for 20 minutes, except $FeSO_4.7H_2O$ which was filter sterilized.

Procedure:

Potassium silicate (10 ml) was mixed with 10 ml of the mineral salts solution in a sterile glass Petri-dish then 2 ml of orthophosphoric acid were added and gently mixed thoroughly. The gel set in approximately 15 minutes. The plates were left overnight and used after discarding the moisture.

9. 2. Liquid media used for isolation and growth of fungi and bacteria.

(a) Czapek-Dox liquid medium.

composition:

$NaNO_3$	2.00 g
KCl	0.50 g
Magnesium glycerophosphate	0.50 g
$FeSO_4$	0.01 g
K_2SO_4	0.35 g
Sucrose	30.0 g
Distilled water	1.0 liter

pH adjusted to 6.8

A proprietary formulation (Oxoid) of Czapek Dox liquid medium was routinely used, prepared by suspending 33.4 g of the powder in 1 liter of distilled water. The medium was then dissolved and sterilized by autoclaving at 121°C for 15 minutes.

(b) Czapek-Dox C-free liquid medium.

composition:

$NaNO_3$	2.00 g
KCl	0.50 g
Magnesium glycerophosphate	0.50 g
$FeSO_4$	0.01 g
K_2SO_4	0.35 g
Distilled water	1.0 liter
pH adjusted to 6.8	

The medium was dissolved and sterilized by autoclaving at 121°C for 15 minutes.

(c) **Czapek-Dox N-free liquid medium.**

composition:

KCl	0.50 g
Magnesium glycerophosphate	0.50 g
$FeSO_4.7H_2O$	0.01 g
K_2SO_4	0.35 g
Sucrose	30.0 g
Distilled water	1.0 liter
pH adjusted to 6.8	

The medium was dissolved and sterilized by autoclaving at 121°C for 20 minutes.

(d) **Czapek-Dox C-free N-free liquid medium.**

composition:

K_2HPO_4	1.0 g
KCl	0.50 g
$MgSO_4.7H_2O$	0.5 g
$FeSO_4.7H_2O$	0.01 g
Purified distilled water	1.0 liter

pH was adjusted to 6.8

The constituent of the basal medium was dispensed into Erlenmeyer flasks (100 ml in 250 ml flasks) then amended with different concentrations of carbon (as sucrose) or nitrogen (as ammonium sulphate) (0, 10, 50, 100, 250, 500, 750, 1000, 1500, 2000, 2500 and 3000 $\mu g\ ml^{-1}$) with/without nitrogen (250 $\mu g\ ml^{-1}$)/carbon (500 $\mu g\ ml^{-1}$). The medium was dissolved and sterilized by autoclaving at 121°C for 20 minutes.

(e) Czapek-Dox C-free liquid medium.

composition:

$NaNO_3$	2.00 g
KH_2PO_4	1.00 g
$MgSO_4.7H_2O$	0.50 g
KCl	0.50 g
$FeSO_4.7H_2O$	0.01 gm
Distilled water	1.0 liter

pH adjusted to 6.8

The constituent of the basal medium was dispensed into Erlenmeyer flasks (50 ml or 100 ml in 250 ml flasks) and sterilized by autoclaving at 121°C for 20 minutes.

(f) Czapek-Dox C-free liquid medium for patulin degradation.

composition:

$NaNO_3$	2.00 g
KH_2PO_4	1.00 g
$MgSO_4.7H_2O$	0.50 g
KCl	0.50 g
$FeSO_4.7H_2O$	0.01 gm
Distilled water	1.0 liter

pH adjusted to 6.8

The constituent of the basal medium was dispensed into Erlenmeyer flasks (50 ml or 100 ml in 250 ml flasks) and sterilized by autoclaving at 121°C for 20 minutes.

After cooling the flasks were amended with patulin to give a final concentration of 10 μg ml^{-1} and 20 μg ml^{-1}or different concentrations (0.5, 1, 2.5, 5, 10, 15 and 20 μg ml^{-1}).

(g) Czapek-Dox C-free liquid medium amended with glucose and patulin for patulin degradation.

composition:

$NaNO_3$	2.00 g
KH_2PO_4	1.00 g
$MgSO_4.7H_2O$	0.50 g
KCl	0.50 g
$FeSO_4.7H_2O$	0.01 gm
Distilled water	1.0 liter
pH adjusted to 6.8	
Glucose	1.00 g (1000 μg ml^{-1})
Distilled water	1.0 liter
pH adjusted to 6.8	

The basal medium was dispensed into Erlenmeyer flasks (100 ml in 250 ml flasks) and sterilized by autoclaving at 121°C for 20 minutes.

After cooling 1 ml of patulin standard solution (1000 μg ml^{-1}) was added to each flask to achieve a concentration of 10 μg ml^{-1}.

(h) Nutrient broth (Oxoid).

composition:

'Lab-lemco' Powder (Oxoid L 29)	1.0 g
Yeast extract (Oxoid L 20)	2.0 g

Peptone (Oxoid L 37)	5.0 g
Sodium chloride	5.0 g
Distilled water	1.0 liter
pH adjusted to 7.4	

A proprietary formulation (Oxoid) of Nutrient broth was routinely used, prepared by suspending 13 g of the powder in 1 liter of distilled water. The medium was then dissolved and sterilized by autoclaving at 121°C for 15 minutes.

(i) **Apple extract medium.**

Apple extract medium was prepared from Golden Delicious fresh apple paste where 20 g or 40 g of apple paste was weighted into 250 ml Erlenmeyer flasks then mixed with 100 ml distilled water. The contents were sterilized by autoclaving at 121°C for 20 minutes.

(j) **Sugar beet extract amended with range amounts of nitrate (NO_3).**

Sugar beet extract medium was prepared from sugar beet tuber paste where 40 g of sugar beet paste were weighed into 250 ml Erlenmeyer flasks then mixed with 100 ml distilled water. Different concentrations of nitrate (0.0, 0.1, 0.5, 1.0, 1.5 and 2.0 mg ml^{-1}) amended as sodium nitrate were added to the flask contents, then autoclaved at 121°C for 20 minutes

9. 3. Method for preparation of fungal sample for scanning electron microscopy (SEM).

Mycelia of the fungi which were studied under the electron microscope were prepared by the following method. The mycelium was primarily fixed in a mixture of paraformaldehyde (2% w/v) and glutaraldehyde (2.5% w/v) in 0.1 M sodium cacodylate for 24 hours at 4°C. The fixed sample was then washed in buffer overnight, followed by three, half hour changes in sucrose (10% w/v) in 0.1 M sodium cacodylate

for 24 hours at 4°C. The sample was then subjected to secondary fixation in 2% aqueous osmium tetroxide for 1 hour at room temperature. This was followed by dehydration in acetone (15 minutes in steps of 30, 50, 75, 95 and 100% v/v acetone). The sample was then critical point dried with CO_2 then sputter coated with gold. The samples were all examined in a Cambridge S600 SEM.

9. 4. Analysis of inorganic N-ion.

(a) Indophenol blue method for determination of ammonium nitrogen (Wainwright and Pugh, 1973).

To 2 ml filtrate, EDTA (6% w/v, 1 ml), distilled water (7 ml), phenolate reagent (5 ml) and sodium hypochlorite solution (0.9% v/v active chloride, 5 ml) were added, mixed thoroughly, then incubated in the dark at 25°C for 20 minutes. The volume was made up to 50 ml and mixed properly, then the intensity of the indophenol-blue-ammonium complex was measured at 630 nm. The concentration of NH_4^+-N was determined by reference to a standard curve (0 - 100 µg NH_4^+-N ml^{-1}) prepared from a standard solution of $(NH_4)_2SO_4$.

Reagents:

Ethylenediaminetetraacetic acid (EDTA):

Prepared by dissolving EDTA (60 g) in distilled water (900 ml), then diluted to 1 liter. This solution is stable for two months.

Phenolate reagent:

Prepared by mixing 20 ml phenol solution (below) with 20 ml caustic soda solution (below) and diluting to 100 ml with distilled water. This reagent was prepared freshly when required.

Phenol solution:

Prepared by dissolving phenol (62.5 g) in a minimum amount of ethanol (19 ml) and addition of acetone (18.5 ml) to give a final volume of 100 ml. The phenol solution was stored in the dark at 4°C.

Caustic soda solution:

Prepared by dissolving 27 g of sodium hydroxide (NaOH) in 100 ml distilled water.

(b) Organic (1) method for determination of nitrate-N (Middleton, 1959).

To 2.5 ml of filtrate, reagent 1 (50 ml), reagent 2 (1 ml), and reagent 3 (0.2 g) were added, the flasks were closed with a bung and shaken for 5 minutes then filtered immediately through Whatman No.1 filter paper into a dry test tube. To 20 ml filtrate reagent 4 (5 ml) was added, mixed thoroughly and set for 30 minutes, for the development of the colour. The intensity of orange colour formed was measured at 475 nm. The concentration of NO_3^--N was determined by reference to a standard curve (0 - 100 μg NO_3^--N ml^{-1}) prepared from a standard solution of sodium nitrate.

Reagent (1): Calcium acetate (12.5 g) was dispensed in 350 ml flask with 200 ml of distilled water and 5 ml concentration aqueous NH_3 (sp. gr. 0.8, 35% NH_3). Reagent 2 (1 ml) and Zn (0.2 g) were added and the mixture were shaken thoroughly and set aside overnight. The mixture was boiled for 30 minutes and filtered using Whatman filter paper No. 1, concentration aqueous NH_3 (100 ml) was added and the volume was made up to 2.5 liters with distilled water.

Reagent (2): $MnSO_4.4H_2O$ (1% w/v) in aqueous acetic acid (5 ml of acetic acid in 95 ml of distilled water).

Reagent (3): Zinc dust.

Reagent (4): Sulphanilic acid (0.1% w/v) in 0.08% α-naphthol with (75% w/v) aqueous acetic acid (75 ml of glacial acetic acid in 25 ml of distilled water).

9. 5. Glucose estimation by di-nitrosalicylic acid method (Bernfield, 1955).

Di-nitrosalicylic acid reagent:

3, 5-dinitrosalicylic acid (1 g) was dissolved in 20 ml of 2 N NaOH and 50 ml distilled water. Then 30 g of potassium sodium tartrate (Rochelle salt) were added and the volume completed to 100 ml with distilled water. This solution was protected from CO_2.

Procedure:

Culture filtrate (1 ml) was added to distilled water (1 ml) and dinitrosalicylic acid (2 ml). The tube containing this mixture was heated for 5 minutes in boiling water, then cooled in running tap water. After addition of 20 ml distilled water, the optical density of the solution containing the brown reduction product was determined photometrically, at 540 nm. A blank was prepared in the same manner without sample. A calibration curve established with glucose (0.2 to 2.0 mg in 2 ml of distilled water) was used to convert the colorimeter readings into milligrams of glucose.

9. 6. Detection of patulin.

(a). Preparation of patulin standard solution (Gimeno and Martins, 1983).

Patulin standard solution was prepared by dissolving pure crystalline patulin (5 mg or 10 mg, obtained from Aldrich) in chloroform ($CHCl_3$) to give a concentration of 100 $\mu g\ ml^{-1}$. The solution was stored in well sealed, aluminium foil - wrapped containers at 4°C. The standard used at room temperature.

(b). Extraction of patulin.

Culture media were extracted by filtering the contents through Whatman No. 1 filter paper, then the extract was collected and measured. Each filtrate was adjusted to pH 2.0 by adding 6 N HCl, then extracted with two equal volume of ethyl acetate. The organic compound was filtered through Whatman No. 2 filter paper containing 1 g anhydrous sodium sulphate, combined, then evaporated to dryness in a water bath at 85°C (Scott and Somers, 1968; Scott and Kennedy, 1973; Wilson and Nuovo, 1973).

(c). Clean-up procedure (Rovira *et al.*, 1993).

The dried residue obtained was re-dissolved in 20 ml of chloroform plus 0.5 ml of water and dried for 1 h over approximately 1 g of anhydrous sodium sulphate. Five ml of the chloroform solution were then introduced into a silica Sep-Pak cartridge (No.51900, Water) previously conditioned with 5 ml chloroform. The cartridge was washed with 1 ml of chloroform, 1 ml of chloroform-ethyl acetate (8 + 2), and 1 ml of chloroform-ethyl acetate (5 + 5). All of these fractions were discarded. Then 2 ml of chloroform-ethyl acetate (2 + 8) were passed through the cartridge. The eluent was collected, and the solution was evaporated. The dried residue was dissolved in 1 ml of benzene for thin layer chromatograghy (TLC).

(d). Preparation and development of TLC plates (Gimeno, 1979; Gimeno and Martins, 1983).

For both qualitative and quantitative studies on the extracts, E. Merck or Whatman pre-coated silica gel, aluminium-backed Kieselgel 60 plates without fluorescence indicator (Cat. No. 5554, 4420 221 respectively) as supplied (20 x 20 cm), were used after activating by drying for 2 h at 120°C. Plates were spotted along a line 3 cm from the bottom with two 10 or 20 μliter spots of sample extracts and different concentrations of patulin standard (100 $\mu g\ ml^{-1}$) (external standard) at 1 cm intervals. On one 10 or 20 μl sample extract spot, superimpose 5 μl patulin reference standard

solution (internal standard). The plates were placed in Shandon TLC chromotanks for developing in one dimension for about 90 minutes (15 cm distance) in benzene : methanol : acetic acid - BMA (90 : 5 : 5 v/v/v) equilibrated at room temperature until the solvent front had reached a line marked 2 cm from the top of the plate, then removed and air-dried in a fume cabinet

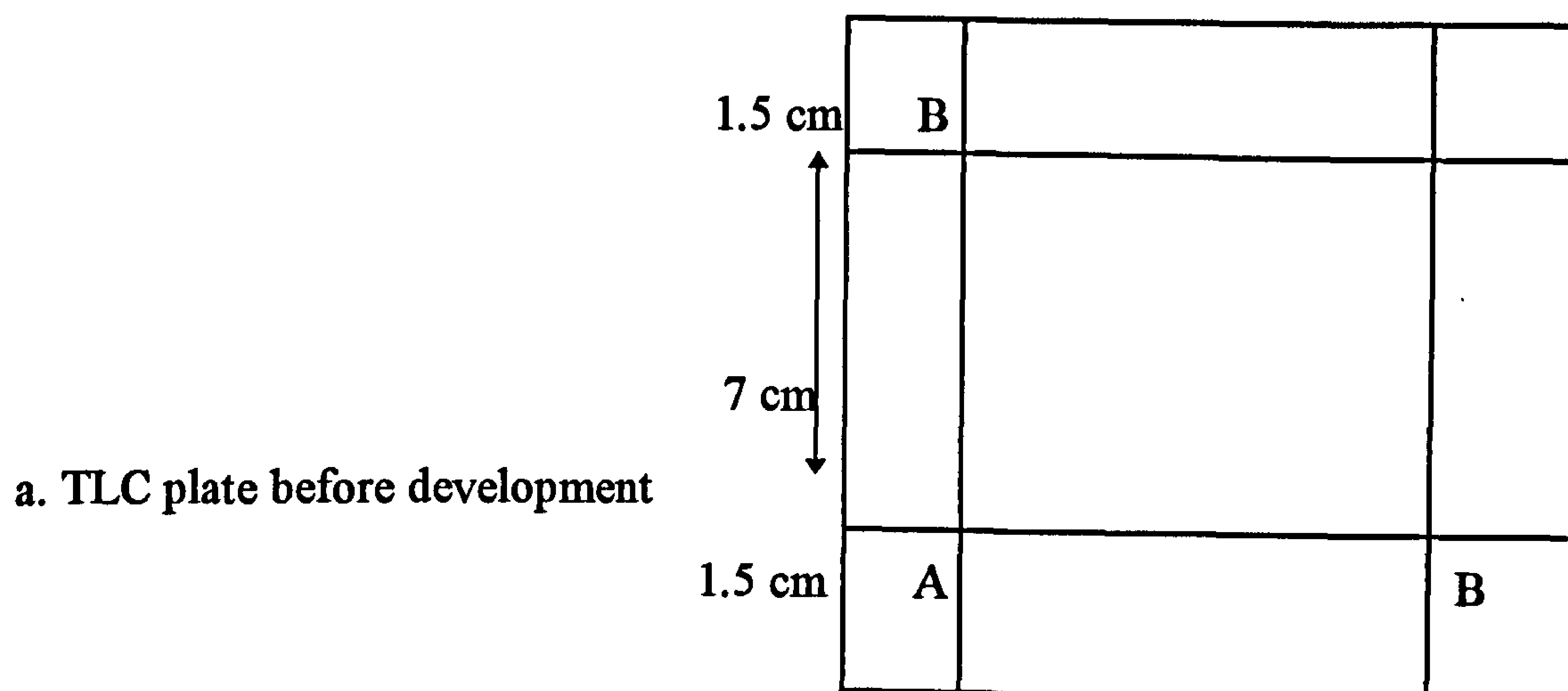
(e). Detection, identification and confirmation of patulin on TLC plates (Scott and Somers, 1968; Scott *et al.*, 1970).

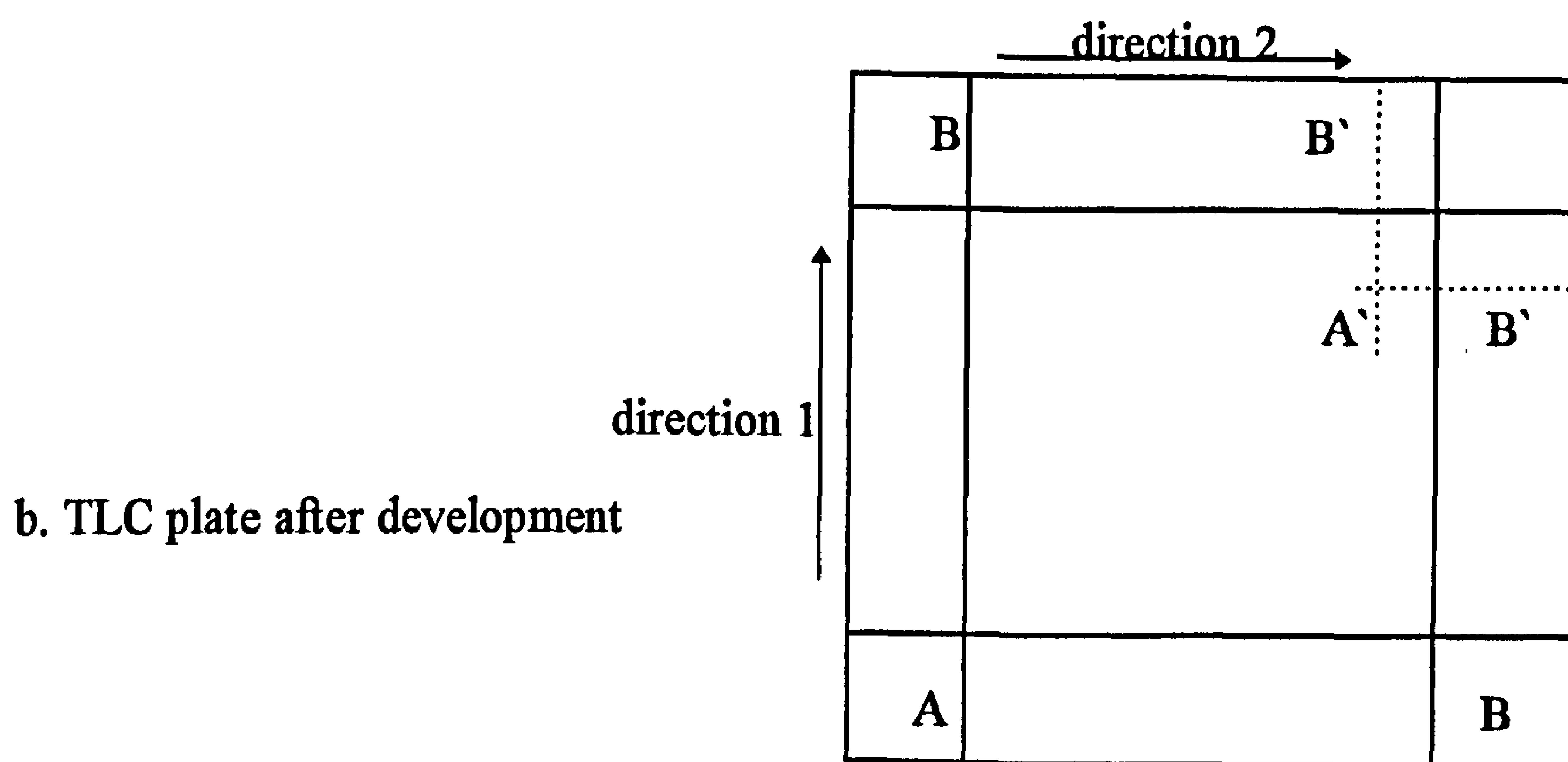
After drying, the developed plates were sprayed with a freshly preparation *ρ*-anisaldehyde* and then heated at 110° in a hot-air oven for 10 minutes. Plates were examined under visible light, short wavelength UV light (254 nm) and long wavelength (366 nm) UV light. The anisaldehyde spray allows detection of patulin where it appears faint brown under the visible light and yellow-orange colour under the longwave UV light. Replacement of methanol in the spray reagent by ethanol improves the detection of patulin which then forms a reddish spot of detection limit 0.1 μg.

*Prepared by dissolving 0.5 ml *ρ*-anisaldehyde in 85 ml of methanol containing 10 ml of glacial acetic acid and 5 ml of concentrated sulfuric acid

(f). Two-dimensional TLC (Stoloff *et al.*, 1971).

Two quarter plates (10x10 cm) of the TLC were used. On scribe lines according to pattern in Fig. 9. 1. On both plates at positions indicated in Fig. 9. 1, 5 μl spots of patulin standard solution and 10 μl spots of sample extract solution were placed. On one plate, 5 μl patulin reference standard solution was superimposed on sample extract spot. The plates were developed in mini glass tank in direction 1 with the solvent CA (chloroform : acetate, 93 : 7) to score line, dried and turned at right-angles in direction 2 with the solvent benzene-methanol acetic acid (95 : 5 : 5 v/v/v) to score line and dried as before. The plate was sprayed with a freshly prepared *ρ*-anisaldehyde, heated





A = Sample extract spotting place.

B = Patulin standard spotting place.

A` = Patulin place of sample.

B` = Patulin place of standard.

Direction 1, chloroform:acetate (93 : 7).

Direction 2, benzene : methanol : acetic acid (90 : 5 : 5)

Figure 9. 1. Scribing and spotting pattern for two-dimensional TLC plate.

for 10 minutes at 110°C, then observed under visible and UV light. The standard was scanned as before, the position of sample spot in relation to reference standard was noted and checked by their characteristic fluorescent colour under UV lights. If the sample spot of sample extract was too intense to match the standard, the sample was diluted and re-chromatographed.

(g). **Quantitative analysis (Gimeno and Martins, 1983; AOAC, 1984).**

Once patulin was identified on the TLC plate by comparison of extract spots with internal and external standards, quantitative analysis for positive samples was conducted.

A series of concentrations of patulin standards were spotted on the chromatography plates, together with a single concentration of the sample. The fluorescent intensity of both standards and sample were then compared and the patulin concentration determined as follows:-

μg patulin kg^{-1} or l^{-1} product = (S x Y x V) / (X x W)

where S = μl patulin standard equal to unknown

Y = concentration of patulin standard, ($\mu g\ ml^{-1}$) = 100 $\mu g\ ml^{-1}$

V = μl of final dilution of sample extract = 1000 μl

X = μl sample extract spotted given fluorescent intensity equal to S (patulin standard) = 10 or 20 μl

$$W = \frac{\text{Sample weight x Filtrate taken}}{\text{Total solution used for extraction}}$$

W = weight of sample, g, or volume of sample, ml.

10. REFERENCES

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