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Production of penitrem A by *Penicillium crustosum* isolated from foodstuffs

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Sixty one isolates of *Penicillium crustosum* originating from various foodstuffs were screened for penitrem A production by thin-layer chromatography The highest producers of penitrem A (4 isolates) were grown in various liquid media. Skimmed milk (2%)/potato extract (2%)/sucrose (2%) (SPS) medium supported the highest toxin production and *P crustosum* Sp 1552 was selected as the best producer of penitrem A Optimal conditions for the production of penitrem A were to grow the isolate Sp 1552 (stationary) in 100 ml of SPS medium (with 4% sucrose) in 500-ml flasks for 3 weeks at 25 °C with initial pH 5 7 The isolation and purification of penitrem A is described Approximately 246 mg of pure penitrem A was obtained from 200 flasks each containing 100 ml of SPS medium

Key words Penitrem A, Mycotoxin production, *Penicillium crustosum*

Introduction

Penitrem A, ($C_{37}H_{44}O_6NCl$) is a member of a group of tremorgenic fungal metabolites, now known as penitrems (but initially called tremortins) (Wilson et al., 1968; Steyn and Vleggaar, 1985). This mycotoxin causes tremor, limb weakness, ataxia and convulsion in mice. Its LD_{50} for mice dosed intraperitoneally is 1.05 mg/kg. Other animals that are susceptible to neurotoxic effects of penitrem A are calf, rat, chicken, rabbit, guinea pig, hamster, dog and sheep (Hayes et al., 1976; Penny et al., 1979; Cole and Cox, 1981).

Penitrem A has been detected in nature on mouldy cream cheese. The fungi which are capable of producing tremorgenic mycotoxins can be found on several important agricultural commodities. Also, the toxin has been produced by several species of fungi when grown on maize, rice, wheat, oat, rye, barley and sorghum (Hou et al., 1971a; Richard and Arp, 1979; Steyn and Vleggaar, 1985).

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Penitrem A has been reported to be produced by *Penicillium crustosum, P. cyclopium, P. granulatum, P palitans, P. puberulum* (Wilson et al., 1968; Ciegler, 1969, Ciegler and Pitt, 1970, Hou et al., 1971b). However, Pitt (1979) concluded that all isolates involved in the production of penitrem A belong to *P crustosum*. El-Banna et al (1987) confirmed the production of penitrem A by *P crustosum*. Penitrem A has also been isolated from *P. commune* (Wagener et al., 1980), *P. canescens* and *P janczewskii* (Di Menna et al., 1986).

Penitrem A has been produced at incubation temperatures from 4 to 30 °C, and after 7 to 120 days of incubation. A wide variety of media have been used at an initial pH from 3.9 to 6.8 for penitrem A production (Ciegler, 1969; Hou et al., 1971c, Yoshizawa et al., 1976, Wagener et al., 1980).

In this contribution we record some effects of medium, pH, incubation temperature and time, as well as medium volume per flask on the production of penitrem A by *P crustosum* The isolation and purification of penitrem A are also reported.

Materials and Methods

Fungi

1420 isolates of the genus *Penicillium* from the culture collection of the Federal Centre for Meat Research, Kulmbach; have been re-identified in 1985 by Dr. John I. Pitt (CSIRO, Australia). According to Pitt (personal communication) 61 (4 3%) of these isolates represented *P. crustosum*. These 61 isolates were drawn from several culture collections (9) or isolated from foods· meat and meat products (30), fruits (6), cereal and cereal products (5), cheese (4), spices (2), vegetables (2), fat (1), pudding (1), pistachio nuts (1). Conidia from 7-day-old subcultures, started from lyophilized stock-cultures and grown on malt extract agar at 25 °C, served as inocula

Screening the isolates for penitrem A production

The isolates were inoculated on malt extract agar (Merck) with three points per Petri dish (90 mm diameter). From each isolate, the mould colonies of 5 Petri dishes were cut out and extracted with acetone (100 ml) in a stomacher for 4 min. The acetone extract of each isolate was filtered through sodium sulphate. Then the extract was evaporated to dryness by using a rotary evaporator at 40 °C. The residue was redissolved in acetone and transferred to a test tube. The extract was concentrated to 1 ml by using a stream of nitrogen. The concentrated extract was used for penitrem A determination

Media and cultivation for penitrem A production

P crustosum was grown (stationary) in 500-ml Erlenmeyer flasks in 100 ml of 7 different media (Table II). The media were incubated at 25 °C for 1, 2, 3 and 4 weeks. SPS medium (containing 2% of each skimmed milk, potato extract and

sucrose) was used to study the effect of some nutrients, initial pH of the medium, the temperature of incubation and the medium volume per 500-ml flasks on the yield of penitrem A. In these experiments, static cultures in 500-ml flasks were incubated for 2 and 3 weeks. All media were autoclaved at 121°C, cooled, and inoculated with 1 ml of a spore suspension, containing approx. 10^4 spores per ml.

Production and purification of penitrem A

A selected isolate of *P crustosum* was grown in stationary culture at 25°C in Erlenmeyer flasks (200 × 500) containing SPS medium (100 ml) After 3 weeks the culture was filtered and the mycelium was macerated with acetone in a Waring blender. The homogenate was filtered and the acetone evaporated. The aqueous residue was extracted with dichloromethane. After evaporation of the solvent, the residue was partitioned between 90% methanol and hexane (1 : 1, v/v). The methanol layer was evaporated and the aqueous residue extracted with dichloromethane. The crude extract, after evaporation of the solvent was percolated through a silica gel column with dichloromethane-acetone (75 : 25, v/v) (De Jesus et al., 1983). The eluent was treated with activated charcoal, concentrated and purified by column chromatography on silica gel (Merck, 0.065–0.200 mm) using benzene-acetone (85 · 15, v/v). Fractions (2 ml) were evaluated by thin-layer chromatography (TLC), fractions containing penitrem A were combined and concentrated. The concentrate was further purified by preparative TLC on silica gel F_{254} plates (Merck, type 60, 2 mm thick) with hexane-acetone (70 : 30, v/v). The chromatogram was developed twice in this solvent system. The toxin-containing band was scraped off and the toxin was eluted with hexane-acetone (70 : 30, v/v). The penitrem A solution was permitted to evaporate slowly at room temperature and dried in vacuo at 40°C.

Penitrem A quantitation

TLC was used for quantitation of penitrem A. The toxin was chromatographed on glass plates precoated with silica gel F_{254} (Merck, type 60, 0.25 mm thick) and developed in benzene-acetone (85 : 15, v/v). The developed plates were examined under ultraviolet light and sprayed with two spray reagents: 2% $FeCl_3$ in butanol and 1% $Ce(SO_4)_2$ in 6 N H_2SO_4. The characteristic colours of penitrem A were. light purple at 254 nm, green after heating at 110°C for 5 min and purple after heating at 110°C for 5 min, respectively (Gorst-Allman and Steyn, 1979; Maes et al., 1982). The estimation of the penitrem A concentration relied on visual comparison with standards. Two standards of penitrem A were kindly supplied by Dr. A. Ciegler, Northern Regional Research Laboratory, USDA, Peoria, U.S.A. and Dr. P.S. Steyn, Council for Scientific and Industrial Research, Pretoria, South Africa.

Results and Discussion

The amounts of penitrem A produced by *P. crustosum* were widely variable among the isolates tested (Table I). The highest amount of penitrem A (100

TABLE I

Screening of 61 *P crustosum* isolates for penitrem A production [a]

Amount of penitrem A (μg/Petri dish)	Number of isolates which produced the corresponding amounts of the toxin
No detectable amount [b]	6
up to 10	7
20–45	21
50–60	18
70–80	5
100	4

[a] Isolates were cultured on malt extract agar medium for 7 days at 25 °C
[b] Detection limit of the method used is 10^{-7} g penitrem A

TABLE II

Effect of different liquid media on penitrem A production in mycelium of selected *P crustosum* isolates

Isolate No	Incubation time at 25 °C (weeks)	Amounts of penitrem A produced using the following media [a b]						
		CD	CDY	CDP	CDT	MEB	YES	SPS
Sp 1444	1	ND [c]	ND	ND	ND	0 03	ND	0 75
	2	0 02	ND	0 04	0 03	0 05	ND	1.42
	3	0 15	ND	0 08	0 13	0 10	ND	1 50
	4	0 10	0 28	0 33	0 13	0 08	ND	2 06
Sp 1552	1	0 02	2 50	4 38	0 13	0 05	0 04	2 56
	2	1 38	3 13	4 75	1 88	0 18	0 02	5 94
	3	3 88	4 75	5 00	4 06	0 50	0 10	6 87
	4	5 00	3 75	6 25	3 94	0 25	0 05	6 56
Sp 1891	1	0 05	0 03	0 05	0 01	0 08	ND	2 06
	2	0 04	0 05	0 50	0 08	0 25	ND	4 37
	3	0 38	0 13	1 50	0 38	0 25	ND	4 50
	4	0.06	0 04	1 25	0 23	0 20	ND	4 50
Sp 2300	1	0 04	0.30	0 30	0 03	0 15	ND	3 44
	2	ND	0 73	0 85	0 15	0 40	ND	4 16
	3	0 75	0 70	1 25	0 20	0 15	0 02	5 00
	4	1 42	0 81	1 15	0 28	0 20	0.01	4 81

[a] Amounts of penitrem A produced are expressed in mg per flask, containing 100 ml of the medium (average of 2 flasks, difference between single values of duplicate determinations ranged from 0 0 to 15 0%)
[b] CD = Czapek-Dox broth, CDY = Czapek-Dox broth + 2% yeast extract, CDP = Czapek-Dox broth + 2% peptone, CDT = Czapek-Dox broth + 0 1% tryptophan, MEB = malt extract broth, YES = 2% yeast extract + 4% sucrose SPS = 2% skimmed milk + 2% potato extract + 2% sucrose Initial pH of MEB, YES and SPS was 5 5, 6 5 and 5 7, respectively The initial pH of the other media was 6 8
[c] ND = no detectable amounts (detection limit of the method used is 10^{-7} g penitrem A)

TABLE III

Effect of some nutrients on penitrem A production of *P crustosum* Sp 1552

No of cultivation medium	Composition of cultivation medium [a b]	Amounts of penitrem A produced after incubation at 25 °C for [c]	
		2 weeks	3 weeks
1	SPS	5 01	6 78
2	SPS + 2% yeast extract	4 96	6 96
3	SPS + 0 5% peptone	5 22	7 07
4	SPS + 2% peptone	5 22	7 05
5	SPS + 0 1% tryptophan	4 95	7 07
6	2% skimmed milk + 2% sucrose	2 61	3 61
7	2% potato extract + 2% sucrose	4 44	5 99
8	2% skimmed milk + 2% potato extract	4 89	6 66
9	SP + 4% sucrose	6 03	8 16
10	SP + 8% sucrose	5 19	7 35
11	SP + 16% sucrose	2 83	3 26
12	SP + 2% lactose	5 92	7 98
13	SP + 2% maltose	4 90	6 77
14	SP + 2% galactose	5 50	6 77
15	SP + 2% fructose	5 30	6 66
16	SP + 2% glucose	5 30	6 70
17	SP + 2% xylose	5 30	6 75

[a] Initial pH of the media ranged from 5 0 to 6 5

[b] SPS = 2% skimmed milk + 2% potato extract + 2% sucrose, SP = 2% skimmed milk + 2% potato extract

[c] Amounts of penitrem A produced are expressed in mg per 500-ml flask containing 100 ml of the corresponding medium (average of 2 flasks, difference between single values of duplicate determinations ranged from 0 0 to 15 0%)

μg/Petri dish) was produced by four isolates. These isolates were selected for studying the production of penitrem A on seven liquid media in stationary culture. Shaken cultures have been reported to be unsuitable for the production of penitrem A (Wagener, 1980; Di Menna et al., 1986). The results are presented in Table II. SPS medium supported higher toxin production than the other liquid media tested. Isolate Sp 1552 produced the highest amounts of the toxin in comparison with the other 3 isolates investigated. The highest amount of penitrem A (6.87 mg/100 ml medium) was produced by Sp 1552 in SPS medium after 3 weeks incubation at 25 °C. Czapek-Dox medium containing yeast extract which was used by some other investigators for the production of penitrem A by *P crustosum* (De Jesus et al., 1981, 1983; Maes et al., 1982) is inferior to SPS.

The effect of some nutrients on penitrem A production of *P crustosum* Sp 1552 is shown in Table III. Supplementation of SPS medium with yeast extract, peptone or tryptophan did not affect penitrem A production by *P crustosum* Sp 1552 (media 1 to 5). Removal of potato extract from SPS medium (medium 6) resulted in a remarkable decrease of the amount of penitrem A produced, while the removal of skimmed milk or sucrose (media 7, 8) from SPS media only slightly decreased the amount of penitrem A produced. However, an increase in the amount of sucrose to

TABLE IV

Effect of pH of the SPS medium on penitrem A production of *P crustosum* Sp 1552 [a]

Initial pH of SPS medium	Amounts of penitrem A produced after incubation at 25 °C for [b]	
	2 weeks	3 weeks
3	4 00	5 00
4	4 00	5 00
5	4 00	5 00
5 7	4 06	6 88
6	4 00	6 50
7	4 00	5 00
8	3 88	5 00
9	3 81	5 00

[a] SPS = 2% skimmed milk + 2% potato extract + 2% sucrose

[b] Amounts of penitrem A produced are expressed in mg per 500-ml flask containing 100 ml of SPS medium (average of 2 flasks, difference between single values of duplicate determinations ranged from 0 0 to 15 0%)

4% in SPS medium (medium 9) appeared to increase the production of the toxin On the other hand, an increase in the amount of sucrose to 16% decreased the amount of the toxin by more than 50% Replacement of sucrose by lactose in SPS medium (medium 12) appeared to increase the amount of the toxin, while the replacement of sucrose by maltose, galactose, fructose, glucose or xylose in SPS medium (media 13–17) did not affect the production of penitrem A by *P crustosum* Sp 1552.

A change of the initial pH of SPS medium to 5 or lower and to 7 or higher resulted in a remarkable decrease of the amount of penitrem A produced by *P crustosum* Sp 1552 after 3 weeks incubation (Table IV)

TABLE V

Effect of incubation temperature on penitrem A production by *P crustosum* Sp 1552

Incubation temperature (°C)	Amounts of penitrem A produced after incubation at the corresponding temperature for [a]	
	2 weeks	3 weeks
5	0 08	0 15
10	1 50	2 00
15	4 06	4 50
20	5 00	5 00
25	4 38	6 50
30	2 50	2 75

[a] Amounts of penitrem A produced are expressed in mg per 500-ml flask containing 100 ml of SPS medium (average of 2 flasks, difference between single values of duplicate determinations ranged from 0 0 to 15 0%) SPS = 2% skimmed milk + 2% potato extract + 2% sucrose (initial pH = 5 7)

TABLE VI

Effect of medium volume per 500-ml flask on penitrem A production by *P crustosum* Sp 1552

Volume of SPS medium in 500-ml flasks [a] (ml)	Amounts of penitrem A produced after incubation at 25 °C for [b]	
	2 weeks	3 weeks
50	2 50	2 75
100	4 50	6 86
200	8 00	9 00
300	9 00	10 00

[a] The initial pH of SPS medium was 5 7 SPS = 2% skimmed milk + 2% potato extract + 2% sucrose

[b] Amounts of penitrem A produced are expressed in mg per flask containing the corresponding medium volume (average of 2 flasks, difference between single values of duplicate determinations ranged from 0 0 to 15 0%)

The effect of incubation temperature on penitrem A production by *P crustosum* Sp 1552 is presented in Table V. Maximum toxin yield (6.5 mg) was observed after 3 weeks at 25 °C.

The yield of penitrem A in 500-ml flasks containing different media volumes is shown in Table VI. The mycelia of *P crustosum* spread more rapidly in flasks

TABLE VII

Recovery of penitrem A, produced by *P crustosum* Sp 1552, during purification steps [a b]

Treatment	Total amount of penitrem A (mg)	Recovery (%)
Acetone extract	1362 0	100 0
Partition chromatography		
dichloromethane - aqueous residue after evaporation of acetone	1296 0	95 2
aqueous methanol 90% - hexane	949 0	69 7
dichloromethane - aqueous residue after evaporation of methanol	815 0	59 8
Percolation through short silica gel column with dichloromethane-acetone (75 25)	740 0	54.3
Filtration through activated charcoal	666 8	49 0
Separation on silica gel column with benzene-acetone (85 15)	633 5	46 5
Preparative thin layer chromatography on silica gel F_{254} (hexane-acetone, 70 30)	246 0	18 1

[a] Total amount of culture medium used was 20 liters (200 × 100 ml in 500-ml Erlenmeyer flasks)

[b] The fungus was grown in stationary culture at 25 °C for 3 weeks in Erlenmeyer flasks containing SPS medium SPS = 2% skimmed milk + 2% potato extract + 2% sucrose (initial pH 5 7)

containing small amounts of SPS medium than in flasks with more medium; consequently, the initial growth rate and initial toxin production were higher at the lower volumes. After 3 weeks of incubation at 25 °C, the highest toxin level (10 mg) was encountered with 300 ml of SPS medium per 500-ml flask. Similar results have been observed by Wagener et al. (1980) during their study with penitrem A and roquefortine production by *P commune*

The recovery of penitrem A, produced by *P crustosum* Sp 1552, during purification is shown in Table VII. Penitrem A was extracted only from the mycelium of *P crustosum* Sp 1552 (the toxin is an intracellular metabolite and is formed only within the mycelial mat· Wilson et al., 1968; Ciegler, 1969; Hou et al., 1971b), and acetone proved suitable for extraction. Chloroform has been used by other investigators for extraction of the toxin (Ciegler, 1969, Hou et al., 1971b; Wagener et al., 1980); however, penitrem A is very unstable in chloroform when directly exposed to light, presumably as a result of acid formation in the solvent (Hou et al., 1971b; Steyn and Vleggaar, 1985). Therefore, any contact of penitrem A with chloroform or mineral acids was avoided throughout our investigation. Approximately 246 mg pure penitrem A were recovered from 200 flasks, each containing 100 ml SPS medium.

The vacuum-dried white amorphous solid of pure penitrem A showed typical physico-chemical characteristics and biological activities (in mice), and corresponds with the two standards we obtained from Dr. Steyn and Dr. Ciegler (data not included)

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References

Ciegler, A (1969) Tremorgenic toxin from *Penicillium palitans* Appl Microbiol 18, 128–129

Ciegler, A and Pitt, J I (1970) Survey of the genus *Penicillium* for tremorgenic toxin production Mycopathol Mycol Appl 42, 119–124

Cole, R J and Cox, R H (1981) Handbook of Toxic Fungal Metabolites, Academic Press, New York, 937 pp

De Jesus, A E, Steyn, P S, van Heerden, F R, Vleggaar, R, Wessels, P L and Hull W E, (1981) Structure and biosynthesis of the penitrems A–F six novel mycotoxins from *Penicillium crustosum* J Chem Soc Chem Commun, 289–291

De Jesus, A E, Steyn P S, van Heerden, F R, Vleggaar, R, Wessels, P L and Hull, W E (1983) Tremorgenic mycotoxins from *Penicillium crustosum* Isolation of penitrem A–F and the structure elucidation and absolute configuration of penitrem A J Chem Soc Perkin Trans I, 1847–1856

Di Menna, M, Lauren, D R and Wyatt, P A (1986) Effect of culture conditions on tremorgen production by some *Penicillium* species Appl Environ Microbiol 51, 821–824

El-Banna, A A , Pitt, J I and Leistner, L. (1987) Production of mycotoxins by *Penicillium* species System Appl Microbiol 10, 42–46.

Gorst-Allman, C P and Steyn, P S (1979) Screening methods for the detection of thirteen common mycotoxins J Chromatogr 175, 325–331

Hayes, A W , Presley, D B and Neville, J A (1976) Acute toxicity of penitrem A in dogs Toxicol Appl Pharmacol 35, 311–320

Hou, C T , Ciegler, A and Hesseltine, C W (1971a) Quantitative determination of tremortins in agricultural commodities J Assoc Off Anal Chem 54, 1037–1038

Hou, C T , Ciegler, A and Hesseltine, C W (1971b) Tremorgenic toxins from penicillia II A new tremorgenic toxin, tremortin B, from *Penicillium palitans* Can J Microbiol 17, 599–603

Hou, C T , Ciegler, A and Hesseltine, C W (1971c) Tremorgenic toxins from penicillia III Tremortin production by *Penicillium* species on various agricultural commodities Appl Microbiol 21, 1101–1103

Maes, C M Steyn, P S and van Heerden, F R (1982) High-performance liquid chromatography and thin-layer chromatography of penitrems A–F, tremorgenic mycotoxins from *Penicillium crustosum* J Chromatogr 234, 489–493

Penny, R H C , O'Sullivan, B M., Mantle, P G and Shaw, B I (1979) Clinical studies on tremorgenic mycotoxicoses in sheep Vet Rec 105, 392–393

Pitt, J I. (1979) *Penicillium crustosum* and *P simplicissimum*, the correct names of two common species producing tremorgenic mycotoxins Mycologia 71, 1166–1177

Richard, J L and Arp, L H (1979) Natural occurrence of the mycotoxin penitrem A in moldy cream cheese Mycopathology 67, 107–109

Steyn, P S and Vleggaar, R (1985) Tremorgenic mycotoxins Fortschr Chem Org Naturst (Prog Chem Org Nat Prod) 48, 1–80

Wagener, R E , Davis, N D and Diener, U L (1980) Penitrem A and roquefortine production by *Penicillium commune*, Appl Environ Microbiol 39, 882–887

Wilson, B J , Wilson, C H and Hayes, A W (1968) Tremorgenic toxin from *Penicillium cyclopium* grown on food materials Nature 220, 77–78

Yoshizawa, T , Morooka, N , Sawada, Y and Udagawa, S (1976) Tremorgenic mycotoxin from *Penicillium paraherquei* Appl Environ Microbiol 32, 441–442