Indian Journal of Marine Sciences
Vol. 38(1) March 2009, pp. 38-44

Batch culture fermentation of *Penicillium chrysogenum* and a report on the isolation, purification, identification and antibiotic activity of citrinin

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Received 7 Jan 2007, revised 29 October 2007

Batch fermentation of *Penicillium chrysogenum*, MTCC 5108 was carried out using potato dextrose broth medium prepared in seawater: distilled water (1:1). Biomass as dry weight was determined by gravimetric analysis. Citrinin, the main secondary metabolite, is produced in large quantities during the stationary phase of growth. The yield amounted to approximately 530 mg l^{-1}. After optimization of culture conditions, *P. chrysogenum* was mass cultured and citrinin was isolated and purified from the medium using a combination of chromatographic techniques (Thin layer and column chromatography). Citrinin, thus obtained was characterized on the basis of its spectral data (UV, Proton Nuclear Magnetic Resonance and Electrospray Ionization Mass spectra). The present study consists report on the effect of the concentration of citrinin on the growth of the culture and antibiotic activities assayed by disc diffusion method using clinical pathogens.

[**Key words**: Citrinin, secondary metabolite production, isolation, spectral identification, antibiotic activity]

Introduction

Microorganisms produce primary metabolites (amino acids, proteins, carbohydrates, vitamins, acetone, ethanol, organic acids etc.) and secondary metabolites (antibiotics, toxins, alkaloids, gibberellins etc.) during active cell growth or near the onset of stationary phase. Marine fungi are potentially prolific sources of highly bioactive secondary metabolites[1,2]. Genus *Penicillium* is known world wide for the production of secondary metabolites and extracellular enzymes of commercial value[3]. To mention a few, isochroman toxin produced by *P. steckii*[4], tanzawaic acids from *P. citrinum*[5,6], compactins from the same species[7-9], penigequinolone A and B from *P. scabrosum*[10], patulin, citrinin chaetoglobosins, communesins and roquefortine from *P. expansum*[11] etc.

Penicillium chrysogenum is a common mould, found, both, from terrestrial and marine sources. Since, Penicillin was first identified in isolates of this species, its secondary metabolism has been the subject of intensive study for decades. Secalonic acid D is the toxin generated by this species[12]. Roquefortine C, omega (w)-hydroxy emodine, pyrovoylaminobenzamide, chrysogine, meleagrin and xanthocillin X are also produced by *P. chrysogenum*[13]. Recently, a diketopiperazine derivative has been reported from *P. chrysogenum*[14].

Citrinin is a mycotoxin initially isolated as a pure compound from a culture medium of *Penicillium citrinum*[15]. Since then, a number of species of *Penicillium* including *P. miczynskii, P. westlingi, P. expansum, P. hirsutum, P. verrucosum, P. steckii, P. corylophilum*[16,17] species of *Aspergillus* viz. *A. niveus, A. terreus*[18,19] and species of *Monascus rubber* and *M. purpureus*[20] have been reported to produce citrinin.

Many species of *Penicillium* produce citrinin, however, till date, no information on the production and isolation of citrinin from *Penicillium chrysogenum* has been described earlier. Citrinin is reported to be a broad spectrum antibiotic especially against Gram positive-bacteria. The present study consists report on Gram-positive bacteria, its activity against Gram-negative bacteria and on its antifungal properties. Primarily, citrinin is a quinone methine. It has a conjugated planar structure and the intramolecular hydrogen bonds linking the phenol and keto functional groups to the carbonyl group of the citrinin nucleus gives it a natural fluorescence ability, hence can be easily detected using UV-VIS spectrophotometer.

The present study reports on the biomass built-up of *P. chrysogenum* when grown in potato dextrose medium. It also provides a comparative data on the

biomass built-up and citrinin production with time. A process is also described on the mass culturing of *P. chrysogenum* for the production of citrinin, followed by its isolation from the fermentation medium. This study further describes its purification using a combination of chromatographic techniques and identification using spectroscopic data like UV-VIS spectrophotometer, ^{1}HNMR (proton nuclear magnetic resonance) and ESI-MS/MS (Electrospray ionization tandem mass spectrometer). The present study consists the influence of citrinin concentration on the overall growth of the culture in the experimental flasks and evaluates its antibiotic activity against clinical pathogens.

Materials and Methods

Microorganism

The fungal strain, *Penicillium chrysogenum,* isolated from the leaves of a mangrove plant *Porteresia coarctata* (Roxb.) is deposited at the Institute of Microbial Technology (IMTEC), Chandigarh, India, bearing the accession no. MTCC 5108. The leaves of the mangrove plant was collected from Chorao Island, along the Mandovi estuary of Goa, India, in sterile polythene bags and transported to the laboratory. On reaching the laboratory, the leaves were rinsed with sterile seawater to remove adherent particles and detritus material. The leaves were then kept in a sterile moist chamber for 2 weeks to allow the fungus to grow and sporulate. Fungal hyphae were picked and repeatedly subcultured on potato dextrose agar (PDA, HiMedia) plates to obtain pure isolate.

Media

Basically, four different media were used in the study.

- Potato dextrose medium (PDM, HiMedia) comprised of 200 g of potato starch and 20 g of dextrose sugar prepared in 1 liter of seawater:distilled water (1:1, V/V). The medium had a salinity of 16 ppt and its pH was adjusted to 6.5.
- Potato dextrose agar (PDA, HiMedia) had the same composition as PDM with additional 15 g of agar in 1 liter of seawater:distilled water (1:1, V/V).
- Nutrient broth (NB, HiMedia), comprised of 5 g of peptic digest of animal tissue, 5 g of NaCI, 1.5 g of Beef extract and 1.5 g of Yeast extract prepared in 1 liter of seawater:distilled water (1:1, V/V).
- Nutrient agar (NA, HiMedia) had the same composition as NB with additional 20 g of agar prepared in 1 liter of seawater:distilled water (1:1, V/V).

Preparation of inoculum

Fungal spores were collected from 8-day-old culture grown on Potato dextrose agar (PDA) slants with sterile seawater (5 ml). The spore suspension containing about 5×10^4 spores ml^{-1} was used as inoculum.

Fungal biomass and citrinin production

Fifty, 100 ml Erlenmeyer flasks containing 25 ml of Potato dextrose medium (PDM), were inoculated with 1 ml of inoculum. The flasks were incubated at 27±2°C on a rotary shaker at 200 rpm. Fungal biomass of the culture was measured by gravimetric analysis. One ml of the growth medium on days 0 - 45 at regular intervals was filtered through preweighed GF/C filters (45 mm dia. & 0.45 µm pore size) and the fungal cell mass with the filter paper was freeze dried to a constant weight in a lyophilizer (Heto DRYWINNER). The difference in weight of the filter paper before and after filtration gave the biomass of the culture for different time interval. Presence of citrinin in the medium was initially detected on thin layer chromatography (TLC) plates. Later, the filtrate was used for Optical Density (OD) measurements using UV-VIS spectrophotometer for determining the peak intensity from which the concentration of citrinin in the fermentation medium was estimated (as described below).

A pilot experiment was conducted in replicates of 4, wherein, serial dilutions of citrinin from a known stock solution (3 µg. μl^{-1}) was added to sterile potato dextrose medium (known volume) and scanned using spectrophotometer. Absorbance maxima were evident at 315 nm in potato dextrose medium. The graph plotted (concentration versus absorbance) of the standard, helped to determine the concentration of citrinin in the experimental fermentation medium.

Mass culture

Ten ml of inoculum, each, was added to four nos. of 5-liter flasks, each containing 1 liter potato dextrose medium. The flasks were incubated at room temperature (27±2°C) for 17 days on a shaker at 200 rpm.

Extraction of fungal toxin

All solvents used for the extraction, isolation, and purification were of HPLC grade.

Following incubation, culture filtrate was concentrated under vacuum to reduce the volume to approximately 800 ml. This concentrated filtrate was extracted using chloroform (5 × 200 ml). Any emulsion formed during separation was overcome by the use of celite (545, LR). The extraction procedure resulted in approximately 1000 ml of chloroform extract. The solvent was next evaporated from the resulting mixture with the aid of a vacuum rotary evaporator, leaving only the dry crude extract behind (1.1 g. L^{-1}).

Isolation and purification of citrinin

Around 100 mg of crude extract was taken in a 100 ml beaker, 2 ml chloroform was added to prepare a slurry using silica gel (20 mg). The silica gel slurry was evaporated to dryness under a stream of nitrogen. This dry slurry was loaded onto a silica gel (60-120 µm mesh size) column prepared in petroleum ether. The column was eluted initially with 200 ml of petroleum ether to remove the low polar fractions. This was followed by elution with 500 ml petroleum ether: ethyl acetate (95:5 V/V) and finally elution with petroleum ether: ethyl acetate (90:10, V/V) yielded the fluorescent yellow compound. Sub fractions (10 ml) were collected in tubes, which were later centrifuged under vacuum (Jouah RC 1022) and combined after studying the spots on thin layer chromatography (TLC) plates. Repeated chromatographic separation (column and TLC) yielded pure citrinin.

Analytical methods

Thin Layer Chromatography (TLC)

TLC was performed on aluminium sheets precoated with silica gel 60 F254 (Merk KgaA, Damstadt, Germany, Cat No. 1.05554). The compound was spotted using a fine capillary tube (2-5µl). The toxin was detected as a fluorescent spot in a UV-chamber at 366 nm UV light. The plate was developed using chloroform:acetic acid (99:1%) and visualized after keeping the plates in iodine chamber for 2 minutes.

UV-VIS Spectrophotometer

UV absorbance of citrinin was studied using a UV-VIS Spectrophotometer (Shimadzu, UV 2401 PC) in methanol.

Proton Nuclear Magnetic Resonance (^{1}HNMR)

^{1}H NMR spectra were generated in deuterated chloroform ($CDCl_3$) at 300 MHz, using a Bruker Avance 300 instrument. Tetramethyl silane (TMS) was used as an internal standard.

Electrospray Ionization Mass spectrometry (ESI-MS/MS)

Mass data was obtained using Electrospray Ionization tandem Mass Spectrometer (ESI-MS/MS, QSTAR XL System) using spray voltage of 5.5 kv. Typically about 10 µg of sample was dissolved in methanol containing 1% formic acid. About 10 scans were averaged to get a spectrum.

Antibiotic assay

Antibacterial activity was determined against *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Salmonella typhi*, *Shigella flexineri*, *Klebsiella* sp. and *Vibrio cholerae* using the paper disc assay method as described by Rodrigues *et al*., (2004)[21]. Briefly, Whatman No.1 filter paper (GF/C) discs of 6 mm diameter were sterilized at 15 Ib pressure for 15 min. The sterile discs were impregnated with 250 µg.$disc^{-1}$ of citrinin. Nutrient agar (NA) plates were surface inoculated and uniformly spread with the test organism consisting of approximately 1.2 × 10^8 CFU/ml. The impregnated discs were placed on the nutrient agar (NA) plates along with standard streptomycin discs (100 µg.$disc^{-1}$) which served as a positive control while discs containing only solvent (chloroform), served as negative control. NA plates were incubated at 37°C for 24 h. The zone of growth-inhibition around each disc was measured in millimeters. All the assays were carried out in replicates of three.

Antifungal activity was determined against *Aspergillus fumigatus*, *Fusarium* sp., *Cryptococcus neoformans*, *Aspergillus niger*, *Rhodotorula* sp. *Nocardia* sp., *Candida albicans*, *Fusarium semitectum*, *Aspergillus ochraceous*, *Fusarium* sp., F. *nivale*, *Aspergillus niveus*, *Trichoderma* sp., and *Curvularia lunata*, using the paper disc assay method as described above for antibacterial assay. The sterile discs were impregnated with 300 µg.$disc^{-1}$ of citrinin. Potato dextrose agar (PDA) plates were inoculated with test organisms having inoculum concentration of 0.5-2.8 × 10^3 CFU/ml. Nystatin was used as positive control at concentrations of 100 µg.$disc^{-1}$. The plates were incubated at 37°C for 48 h. The growth inhibition around each disc was measured in millimeter to determine the activity of each disc and compared with standard discs. All the assays were carried out in replicates of three.

In order to study the influence of citrinin on the growth of the culture producing it, discs loaded with varying concentrations of citrinin i.e. 50, 100, 250 and 500 µg.$disc^{-1}$ were placed on *P. chrysogenum* seeded

Potato dextrose agar (PDA) plates. Inhibition zone developed around the discs were evaluated.

Results and Discussion

Industrial enhancement using filamentous fungi lies in their production of secondary metabolites. Thus, optimization of growth conditions accompanied by optimum secondary metabolite production go hand in hand.

A time course of biomass accumulation and citrinin production during growth of *Penicillium chrysogenum* is shown in Fig. 1. When grown in batch fermentation medium the biomass production and metabolite concentration generally change constantly as a result of the metabolism of the cells. After inoculation of sterile potato dextrose medium with *P. chrysogenum,* four typical phases of growth are observed as indicated in Fig. 1. The lag phase (0-7 days), wherein, physiochemical equilibrium exists between micro-organisms and the environment, showed very little growth (0.1-0.24 mg.ml^{-1}). The log phase in which there is doubling of biomass per unit time was seen from day 8 to 15 with biomass values ranging from 0.2 to 1.04 mg.ml^{-1}. Maximum biomass buildup was seen on day 15. The rapidly growing cells alter the medium through uptake of substrates and excretion of metabolic products. From day 16 to 18, there was no remarkable change in biomass values (1.03-1.04 mg.ml^{-1}) indicating the stationary phase of growth. This phase is followed by decrease in biomass of the culture in the medium from day 19 till the end of incubation indicating cell death (0.95-0.08 mg.ml^{-1}).

The presence of citrinin in the culture medium was initially detected by spotting on thin layer chromatography (TLC) plates. The concentration of citrinin in the experimental flasks was next determined spectrophotometrically which is also plotted in the same figure (Fig. 1). Production of citrinin was noticeable from day 3, (3.83 µg.ml^{-1}) High concentration of 530 µg.ml^{-1}was evident during the stationary growth phase (day 17). This indicated that citrinin biosynthesis is associated with secondary metabolism[19]. Thus, mass culturing for extraction of secondary metabolites need to be done only after evaluating the growth pattern of microorganisms. No citrinin was detected in the fermentation flasks from 30 days of incubation.

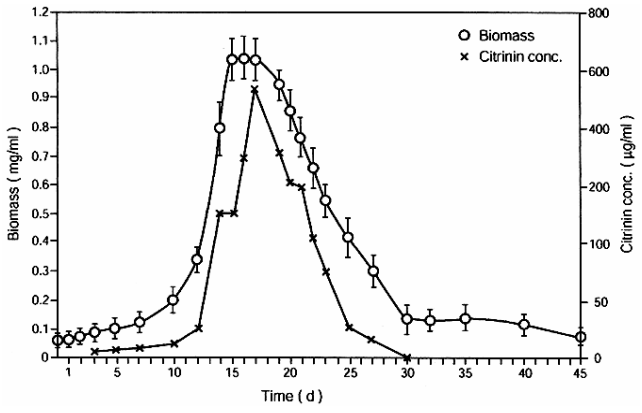

Fig. 1—Time course of biomass accumulation and citrinin production during growth of *Penicillium chrysogenum* in PDB medium.

In addition to the above experiment, mass culturing of *P. chrysogenum* was also initiated in order to isolate pure citrinin in larger quantities for further spectroscopic studies. Four liters of fermentation medium yielded a little over 2 g of citrinin which was used for all the bioassay as well as spectroscopic studies.

We report here the antibacterial (Table 1) as well as antifungal activity of citrinin (Table 2). Previous

Table 1—Antibacterial activity of citrinin

Bacterial pathogens	Gram stain	Streptomycin (100µg.disc^{-1})	Citrinin (250µg.disc^{-1})
E. coli	Gram –ve	-	-
Pseudomonas aeruginosa	Gram –ve	5 mm	5 mm
Staphylococcus aureus	Gram +e	4 mm	3 mm
Salmonella typhi	Gram –ve	-	-
Shigella flexineri	Gram –ve	5 mm	3 mm
Klebsiella sp.	Gram –ve	-	-
Vibrio cholerae	Gram –ve	5 mm	6 mm

- = zone of growth inhibition <1mm

Table 2—Antifungal activity of citrinin

Fungal pathogens	Nystatin (100µg.disc^{-1})	Citrinin (300 µg.disc^{-1})
Aspergillus fumigatus	-	-
Fusarium sp.	-	13 mm
Cryptococcus neoformis	-	-
Aspergillus niger	-	-
Rhodotorula sp.	-	-
Nocardia sp.	-	-
Candida albicans	-	-
Fusarium semitectum	-	-
Aspergillus ochraceous	-	-
Fusarium sp.	-	-
F. nivale	-	12 mm
Aspergillus niveus	-	6 mm
Trichoderma sp.	-	-
Curvularia lunata	-	-

- = zone of growth inhibition <1mm

report showed citrinin to be antibacterial especially against Gram-positive bacteria[20]. We observed strong antibacterial activity of citrinin against *P. aeruginosa* and *V. cholerae* while weak antibacterial activity was observed against *S. aureus* and *S. flexineri.* Citrinin is also fungicidal against *Fusarium* sp., *F. nivale* and *Aspergillus niveus.*

Earlier studies are available to show that secondary metabolites produced by a culture had inhibitory effect on the culture itself. For e.g. lovastatin inhibits its own synthesis in the fungus *Aspergillus terreus*[23]. However, this was not the case for the present study using *P. chrysogenum.* A disc assay experiment, wherein varying concentrations of citrinin loaded onto discs were placed on plates grown with *P. chrysogenum,* had no deleterious effect on the growth of the culture. Since, no zone of growth inhibition was observed around the citrinin-loaded discs (Table 3).

Structural information

The aim of this experiment was to study the secondary metabolite of *P. chrysogenum,* with special reference to citrinin. Various analytical tools were used to confirm the presence of citrinin in the experimental flasks. UV spectrum of pure citrinin in methanol showed absorption at λmax 213, 253 and 321 nm (Fig. 2).

Table 3—Effect of varying concentration of citrinin on the growth of *P. chrysogenum*

Citrinin conc. (μg.disc^{-1})	Zone of growth inhibition
50	-
100	-
250	-
500	-

^{1}H NMR (300 MHz) spectrum of citrinin in $CDCl_3$ is well in agreement with the reported values[24]. Two doublets at δ1.173 (J=7.2Hz) and δ1.291 (J=6.7Hz) are attributed to two methyl (-CH_3) protons. The resonance at δ2.925 is assigned to methine (-CH) proton. The three singlets at δ1.964, 8.181 and 15.860 correspond to the methyl (-CH_3) protons attached to a double bond, olefenic -CH and carboxylic (-COOH) protons respectively (Fig. 3).

ESI-MS (in positive ion mode) of the compound yielded molecular ion $(M+H)^+$ peak at m/z 251.0367, which corresponds to the molecular formula $C_{13}H_{14}0_5$. ESI-MS/MS of the molecular ion m/z 251.0367, yielded fragment ion at 223.0247 (M^+-18), due to the loss of water molecule. Another fragment ion is evident at 205.0419 (M^+-46,) which is attributed to the loss of -CO in addition to water, yet another ion at

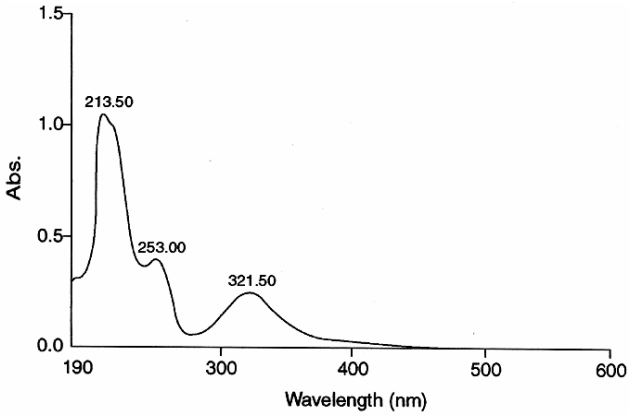

Fig. 2—UV-VIS spectrum of citrinin in methanol.

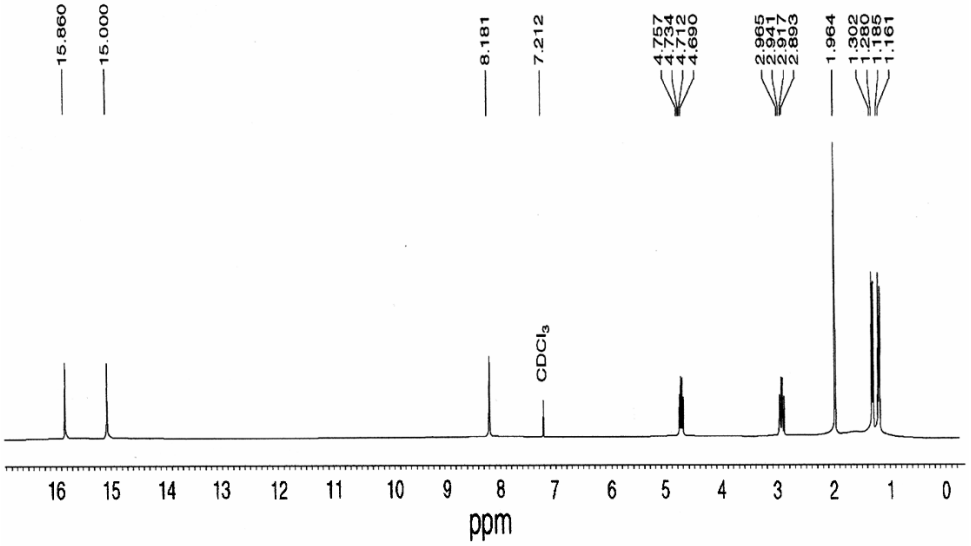

Fig. 3—^{1}H NMR spectrum of citrinin.

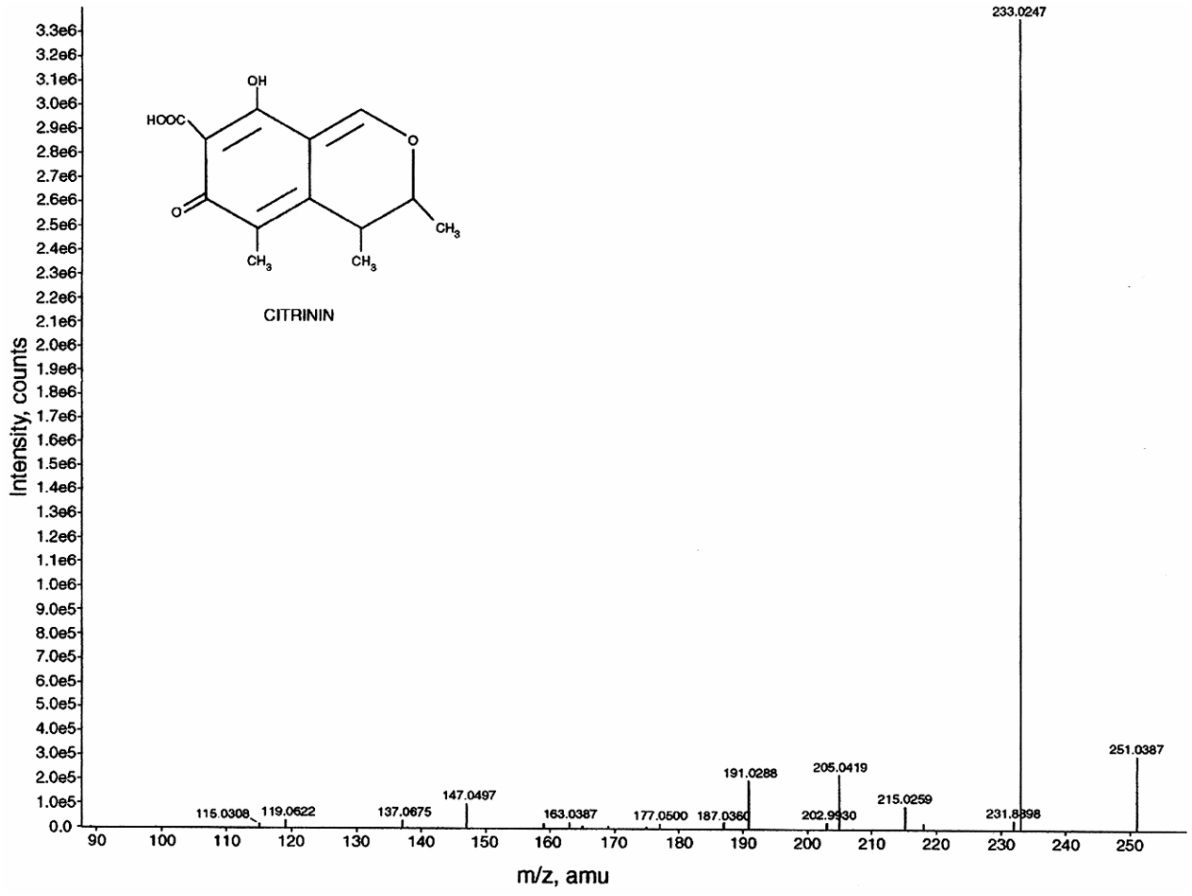

Fig. 4—MS/MS fragmentation pattern of citrinin.

191.0288 (M^+-60) etc, which further confirm the structure assigned to citrinin (Fig. 4.)

The present study report *Penicillium chrysogenum* to produce citrinin as a secondary metabolite under batch culture, in potato dextrose medium, at *p*H 6.5 and at temperature of 27±2°C. Optimum concentration was produced during the stationary period of growth phase. This could be expected since conditions favorable to growth of *Penicillium chrysogenum* are unfavorable to *secondary* metabolite production. During the death phase (from 19 day onwards), the concentration of citrinin dropped from 305 μg.ml^{-1} till it became undetected from day 30. It is essential to monitor the growth of the organism simultaneously with the metabolite production, for optimum yield, of secondary metabolite. The concentration of citrinin in the fermentation flask did not influence the growth of the culture. Citrinin was isolated from the experimental flask using a combination of chromatographic techniques (column chromatography and thin layer chromatography) and identified using various spectroscopic data. In addition to being antibacterial, citrinin also showed to be antifungal against a number of clinical pathogens.

Acknowledgements

Authors are grateful to Dr. S.R. Shetye, Director, NIO, for constant encouragement. And to the Dean, Goa Medical College for providing clinical pathogens. Authors thank Ministry of Earth Sciences (MoES) for funding the Project "Development of Potential Drugs from the Seas around India". This manuscript has NIO contribution No. 4145

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