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Full Length Research Paper

Isolation of *Acremonium* species producing cephalosporine C (CPC) from forest soil in Gilan province, Iran

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Cephalosporin C (CPC) is a major precursor of cephalosporin antibiotics which is produced by a group of deuteromycets. After biosynthesis in optimized fermentation media, CPC is recovered and converted to a variety of potent cephalosporin antibiotic drugs. This study was performed to determine the mycoflora producing CPC in various parts of Iran soil. Soil samples were collected and cultured on selective media and *Acremonium* sp. were isolated. The presence of CPC antibiotic in the fermentation broths of these species was determined by a combination of biological, HPLC and mass spectrometry methods. From 350 fungi isolated, 21 (6%) were *Acremonium* spp, which one strain was able to produce CPC. This species was recognized as A*cremonium persicinum* according to its macroscopic and microscopic criteria. It is possible to apply different characteristics of the isolated species in strain optimization processes such as protoplast fusion.

Key words: *Acremonium persicinum*, Soil, Cephalosporin C, Iran.

INTRODUCTION

Cephalosporin C (CPC) is a major precursor of semi-synthetic cephalosporin antibiotics, which is naturally produced in a secondary metabolite form in *Acremoniun* sp. (a group of deuteromycets). After biosynthesis in optimized fermentation media, CPC is recovered and converted to a variety of potent cephalosporin antibiotic drugs (Velasco et al., 2001; Demain and Zhang, 1988; Domsch, 1980).

CPC antibiotic have been believed to be exclusively fungal metabolite and microorganisms with the ability of CPC production include *Acremonium chrysogenum, Acremonium strictum, Emericellopsis minima, Acremonium persicinum, and Emericellopsis salmosynnemata* (*Cephalosporium solmosynnemata telemorph*) (Gams, 1997; Domsch, 1980). Among them, a well recognized mould is *A. chrysogenum* which has been genetically modified, optimized and used as an industrial strain available for biotechnological/pharmaceutical industries in the recent decades (Velasco et al., 2000).

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Today, many screening studies are trying to discover additional fungi with the high production rate and better biological characteristics. The present study aims to isolate organisms in soil native able to produce CPC that could be used in biotechnological processes.

MATERIAL AND METHODS

Top soil samples to a depth of 10 cm were obtained from dead plants, wastage material and dead woods of 67 separate sites in various provinces of Iran. The samples were brought to laboratory in sterilized plastic bags. For the soil culture, dilution and plating technique was used which 5 g of each sample was dissolved in 500 ml of sterilized DW and suspended. Subsequently 5 ml of this mixture was delivered to a sterile screw-capped tube containing 0.5 ml of 200 IU/ml of penicillin and incubated for 24 h. The tube was then centrifuged at 1200 g for 10 min using sterile pipettes the precipitate was cultured on selective culture media (Rose- Bengal, Chapex, SDA and malt extract agar) and incubated at 25 – 27°C for 5 - 10 days (Moo-Young, 1985).

Macroscopic colony characteristics (shape, size, texture, and color and growth rate) as well as microscopic criteria on slide culture were studied for strain identification and taxonomy (El Nagdy and Hafez, 1990). Final recognition of *Acremonium* sp. was done by comparison of above criteria with those given by Gams (Gams,

Table1. Fungi genera and occurrence frequencies in soil samples of 67 localities of investigated areas.

Fungi genus	Frequency (%)
Aspergilus sp.	31%
Penicillium sp.	28%
Alternaria sp.	11%
Acremonium sp.	6%
Mucor sp.	4%
Others	20%

1975; Gams, 1997). The strain with ability to produce CPC was transferred to CBS (Central bureau Voor Schimmelcaltures, Baarn, and the Netherland) in order to obtain complete taxonomy.

For confirmation of CPC production in the isolated *Acremonium* spp., the fungi conidia/mycelia were inoculated into a 250 ml baffled shake-flask containing 40 ml of seed medium (Paul et al., 1997). This flask was then incubated at 27°C on an orbital shaker at 180 rev/min which after 72 h, transferred into a 2 liter shake-flask containing 400 ml of two different self prepared complex production media including: Medium A contains sucrose (80 g), soy bean meal (60 g), DL-Methionine (1.5 g), distilled water (up to 1 L), pH = 6.8 and Medium B contains sucrose (20 g), CSL (12 g), ammonium acetate (4.6 g), $Na_2S0_4.2H_2O$ (0.5 g), $MgSO_4.7H_2O$ (0.5 g), distilled water (up to 1 L), pH = 7. These media were incubated under the same conditions as for seed medium. The culture was harvested and centrifuged after 3 - 5 days (Paul et al., 1997; Zhou et al., 1992).

Bioactivity of the complex fermentation liquors were assayed by the method of cylinder-agar plate embedded with *Micrococcus luteus* ATCC 9341 and *Alcaligenes faecalis* ATCC 8750 as test strains (US Pharmacopieal convention, Rockville Md, USA, 1996). 20 µL of samples with bioactivity against the mentioned test strains were then analysed by HPLC (Knauer model) on a C18 cartridge column (4 mm (i.d) 300 mm) contained in a radial compression module, and eluted with phosphate buffer eluent (0.03 M, pH = 3) at a flow rate of 1 ml/min (Rieger et al., 1990). Subsequently, the results of samples and CPC standard analysis were compared.

Mass spectrometry (MS) was finally performed for exact identification of the produced CPC in the fermentation broths extracts. Fast-atom bombardment mass spectra were acquired using a Finning Mat TSQ 70 instrument (Varian co.) fitted with an antex Cs^+ ion gun and a post-acceleration detector. CPC standard spectrum was also acquired and compared.

RESULTS

Three hundred fifty filamentous fungi species were isolated and classified in 6 genera. The occurrence frequency of these fungal genera is given at Table 1. Twenty-one (6%) of the species belonged to genus *Acremonium* or *Cephalosporium*-like hyphomycets. All of these species were screened for their ability to excrete CPC. From these, 19 isolates were not able to produce CPC. The remaining (2 species) had simultaneous inhibitory effects on test strains (*M. luteus* and *A. faecalis*).

There was a peak with a retention time (RT) similar to CPC standard in HPLC of fermentation broth of only one isolated species which could produce antibiotic .The related chromatograms are presented in Figure 1.

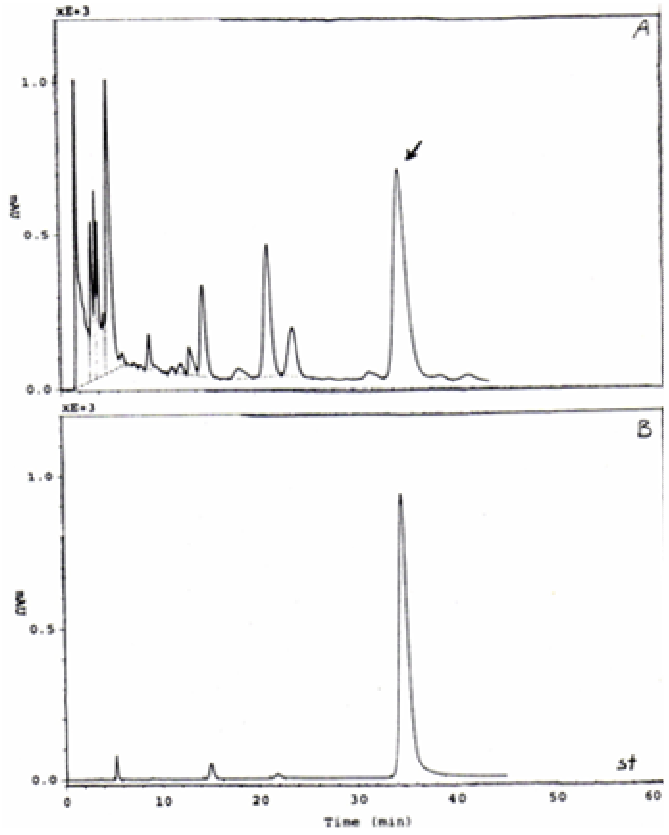

Figure 1. HPLC analysis of fermentation broths of the isolated *Acremonium* sp. and CPC standard (there are similar peaks at RT = 34 – 37 min).

Mass spectrum of the fermentation broth of the isolated strain indicated a molecular ion of about 415, similar to which determined by CPC standard and the calculated mass of CPC (free acid). Thus, the composition of the produced antibiotic is supported by these values (Figure 2).

The strain with ability to produce CPC which was priorly transferred to CBS, was recognized as *Acremonium persicinum*. Figures 3 and 4 show macroscopic and microscopic (slide culture) views of this species respectively.

DISCUSSION

In this study a native *Acremonium* sp. with the ability of CPC production was isolated. Regarding to macroscopic and microscopic characteristics, this strain was finally recognized as *A. persicin*um. The producing species was isolated in the soil of an old woody forest (Saravaan) in the south-west of Gilan province (North of Iran). It is one of the 108 *Acremonium* spp. that has been classified by Gams according to his latest monograph (Gams, 1997). In spite of world-wide distribution of *A. persicinum*, it is unclear that the name of this species is related to

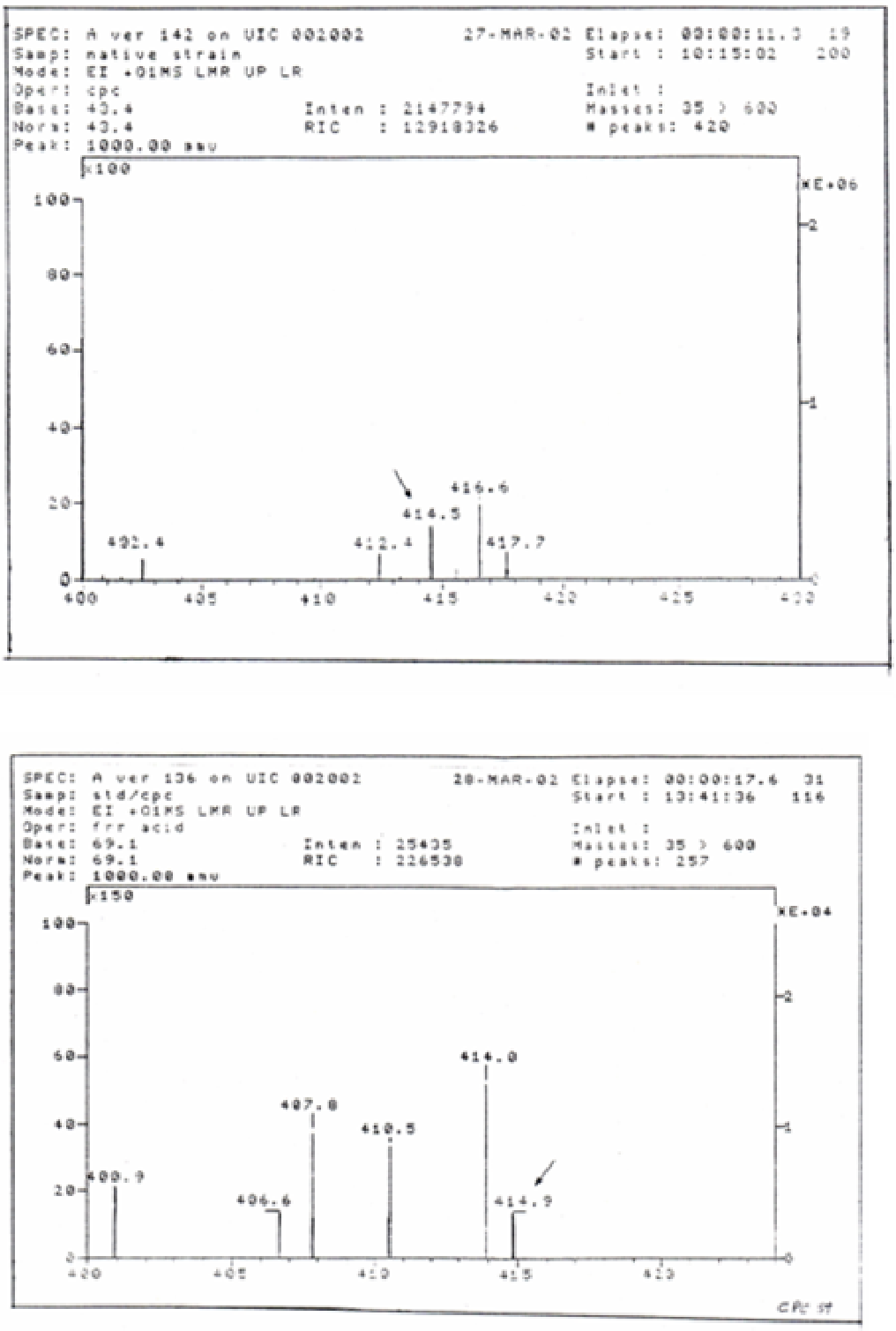

Figure 2. Mass spectra of the extract of fermentation broth of the isolated strain and that of CPC standard (the same molecular Ions with Mr~415 are revealed, some fragment ions are observed as well).

persica (Fars, IRAN) or not.

Cellulose hydrolysis is an out-standing characteristic of this fungus (Piston et al., 1996). So its isolation in the soil of an old forest is expectable due to suitable ecologic conditions of the investigated area.

As it was indicated, the production of CPC in *A. persi-*

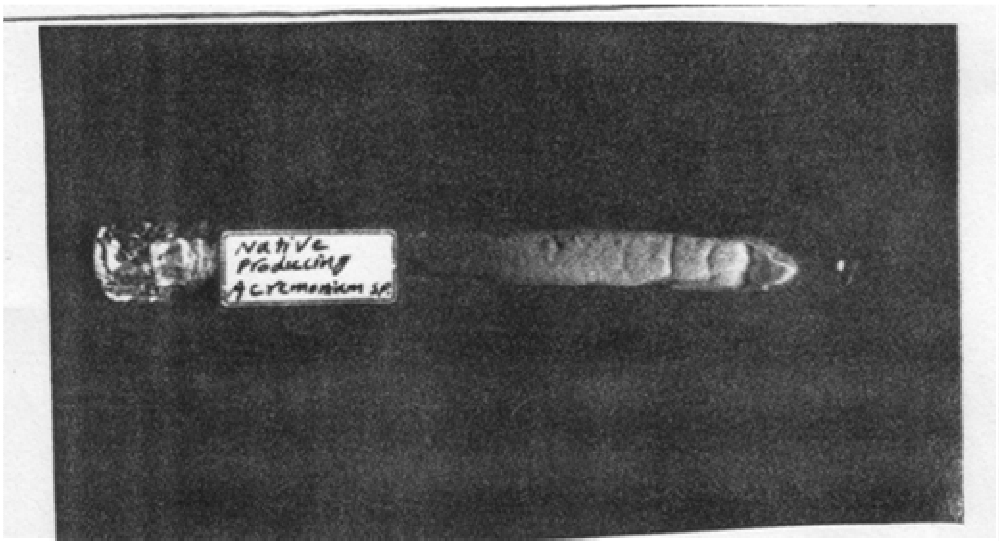

Figure 3. Macroscopic (colony) view of the isolated CPC-producing mould.

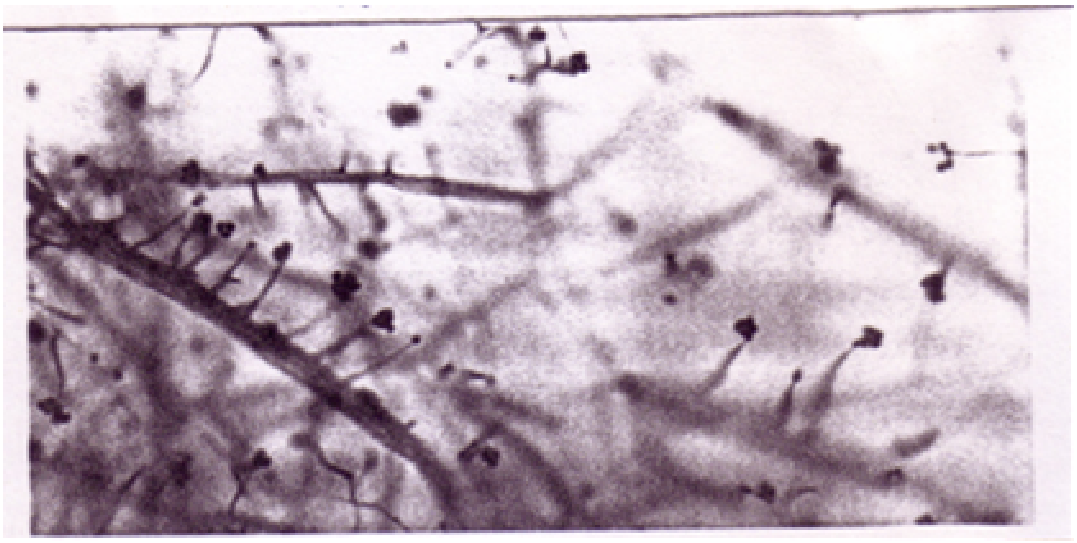

Figure 4. Microscopic (slide culture) view of the isolated CPC-producing mould.

cinum has already been proven. Pisano and Vellozzi, (1974) have shown the secretion of CPC in *P. persicinum* P-M1, the species with most similarities to the isolated native strain and Gams has classified the majority of the *Paecilomyces* sp. in *Acremonium* and *Cephaloporium* genera as well (W. Gams, personal communications).

The production of CPC in *A. persicinum* has shown to be 5 - 8 mg/dl by Eriquez and Malowitz studies (Eriquez and Pisano, 1979 and Malowitz, 1982), which was confirmed in this study to be 5 mg/dl (data not shown). Therefore, *A. persicinum* has not been applied for biotechnological processes due to its low CPC production.

Furthermore our studies have revealed that media supplementation of DL-methionine has no effect on CPC production of this strain which implies one of its advantage. Another advantage of the isolated strain is that its maximum peak of CPC production is lower than standard strain (3 days versus 5 days) as determined by our studies.

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