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Antifungal activity of violacein purified from a novel strain of *Chromobacterium* sp. NIIST (MTCC 5522)

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A novel strain of *Chromobacterium* sp. NIIST (MTCC 5522) producing high level of purple blue bioactive compound violacein was isolated from clay mine acidic sediment. During 24 h aerobic incubation in modified Luria Bertani medium, around 0.6 g crude violacein was produced per gram of dry weight biomass. An inexpensive method for preparing crystalline, pure violacein from crude pigment was developed (12.8 mg violacein/L) and the pure compound was characterized by different spectrometric methods. The violacein prepared was found effective against a number of plant and human pathogenic fungi and yeast species such as *Cryptococcus gastricus*, *Trichophyton rubrum*, *Fusarium oxysporum*, *Rhizoctonia solani*, *Aspergillus flavus*, *Penicillium expansum*, and *Candida albicans*. The best activity was recorded against *Trichophyton rubrum* (2 μg/ml), a human pathogen responsible for causing athlete's foot infection. This is the first report of antifungal activity of purified violacein against pathogenic fungi and yeast.

Keywords: antimycotic, *Chromobacterium*, chromatography, purification, violacein

Introduction

The microbial bioactive compounds, violacein have great commercial interest due to their application in many industrial sectors (Brown, 1987; Durán and Menck, 2001). Violacein [3-(1, 2 dihydro-5-(5-hydroxy-1H-indole-3-yl)-2-oxo-3H-pyrrol-3-ylidene)-1, 3-dihydro-2H-indole-2-one] is a purple blue, high value bioactive compound first reported in *Chromobacterium violaceum* (Boisbaudran, 1882). Subsequently a number of other bacteria were also reported producing violacein including, *Chromobacterium fluviatile* (Moss *et al*., 1978), *Janthinobacterium lividum* (Shirata *et al*., 1997), *Alteromonas luteoviolacea* (McCarthy *et al*., 1985), *Pseudoalteromonas luteoviolacea* (Yang *et al*., 2007), *Duganella* sp. B2 (Wang *et al*., 2009) etc. The high value and wide application potential of violacein makes bacterial strains producing excess of the compound relevant to fermentation industry. In this scenario novel strains physiologically adapted to different environments have been reported in the past (Yada *et al*., 2008; Lu *et al*., 2009; Wang *et al*., 2009).

Violacein is reported to have important activities such as antibacterial (Nakamura *et al*., 2002), antioxidant (Konzen *et al*., 2006), antiviral (May *et al*., 1991), antiprotozoal (Lopes *et al*., 2009), antitumor (Durán *et al*., 2007). The antifungal activity of pure violacein is not yet reported. However, violacein containing crude pigmented extract reported to have antifungal activity against phytopathogenic fungi *Rosellinia necatrix* (Shirata *et al*., 1997). Moreover, violacein producing bacteria such as *C. violaceium* and *J. lividium* were reported to have biocontrol potential against fungi pathogenic to plants and amphibian (Barreto, 2008; Brucker, 2008; Becker, 2009).

As per literature, the recovery of pure violacein from broth after solvent extraction is laborious and the yield is very low (Table 1). Therefore, more efficient and economic method to recover pure violacein will be attractive. With this background the present study focuses on developing an efficient method to prepare pure violacein from an isolated *Chromobacterium* sp. NIIST (MTCC 5522) producing high level of the compound and evaluating antifungal activity of pure violacein produced against human and plant pathogenic fungi including yeast.

Materials and Methods

Isolation and characterization of violacein producing bacterium

The bacterial strain was isolated from sediment sample collected from a clay mine acid lake (pH 4.5) in Thiruvananthapuram district in Kerala, India (Lat_long=8°55′53″ N 76° 85′77″E). Sediment sample (1–3 cm deep) was collected in sterile plastic bottles and brought to laboratory. Sub samples after serial dilution were spread plated on Luria-Bertani (LB) agar media and incubated at 32°C. After 24 h, the purple pigmented colonies grown on plates were subcultured on LB agar under similar conditions for pure culture preparations. The morphological features of the bacterium were observed with scanning electron microscopy (JEOL JSM-5600LV Scanning Electron Microscopy).

Gram staining and biochemical tests such as oxidase, cata-

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lase, urease, TSI, indole, voges-proskauer test, methyl red and citrate tests were done as per standard protocols (Brown, 2010). The carbon utilization profile of the strain was analyzed by inoculating to BIOLOG 96 well plate (GN2) specific to Gram-negative bacteria. The genomic DNA was extracted through CTAB method (Wilson, 1987) and 16S rRNA gene was amplified with EUB 341f (5′-CCT ACG GGA GGC AGC AG-3′) and EUB 907r (5′-CCG TCA ATT CMT TTG AGT TT-3′) primers (IDT) under standard PCR conditions. The amplified PCR product was purified and sequenced. The gene sequence obtained (~550 bp) was compared with corresponding sequences of related organisms retrieved from GenBank database using BLAST algorithm. The sequence was deposited in GenBank database with accession number FJ982784. A neighbor joining phylogenetic tree was constructed with the sequence of the present strain and previously reported strains producing violacein using MEGA (Molecular Evolutionary Genetic Analysis, MEGA 6) software (Kumar *et al*., 2008). The culture was deposited at Microbial Type Culture Collection (MTCC-India).

Growth and violacein production by the bacterium

The growth and violacein production of the present strain was studied in modified LB broth in which the sodium chloride is lacking (Peptone 10 g/L, Yeast Extract 5 g/L). A 24 h old culture suspended in sterile saline was used as the inoculum at 5% (v/v) level (initial OD at 600 = 0.32). The experiment was conducted in a 250 ml Erlenmeyer flask, incubated under room temperature (33±2°C) in an environmental shaker at 150 rpm (New Brunswick). To assess the increase in biomass and violacein, 1 ml culture broth was taken and centrifuged at 9,660 × *g* for 10 min, 1 ml distilled ethanol was added to the pellet-pigment mixture and mixed well to extract the pigment to ethanol phase. The mixture was centrifuged again and the supernatant and pellet were separated. The OD of ethanolic supernatant was taken directly at 575 nm (Thermoscientific) to quantify violacein. The pellet was resuspended in 1 ml saline and OD was taken at 600 nm to quantify biomass. Growth and violacein production by the culture was studied further under different pH (4–10), temperatures (20–40°C) and salinity (0–3%). All batch experiments were repeated with triplicates and the average values accounted with standard deviation.

Extraction, purification, and characterization of violacein

30 ml LB broth containing violacein and biomass was centrifuged first at 9,660 × *g* for 10 min. The supernatant was discarded and the pellet with violacein was suspended in 10 ml ethanol to extract crude violacein. It was centrifuged again to separate pellet from ethanol-violacein portion. To the pellet again added 10 ml ethanol, mixed well and centrifuged as above for complete extraction of crude violacein. The ethanol-extracted pigment was pooled and dried in a rotavapour (Buchi R-200) at 40°C under complete vacuum; it was then kept overnight in a desiccator and finally weighed.

A novel, inexpensive column chromatographic method was developed for purifying violacein in this study. The crude violacein was initially subjected to silica gel open column chromatography to remove low polar compounds. Ethyl acetate at different polarities (up to 40% ethyl acetate was prepared by mixing with hexane) was used for this. The semipurified pigment was eluted from the column using 100% ethyl acetate. The dried mass of partially purified violacein was then adsorbed to alumina to perform succeeding column chromatography using alumina as the stationary phase and methanol-chloroform mixture prepared in different polarities as the mobile phase (up to 10% methanol). On spotting the individual fractions with different polarities on a TLC plate, it was found that in 10% methanol pure violacein eluted. 10% methanol fraction collected was concentrated in rotavapour to a small volume and was allowed to stand overnight. Crystals of purple blue violacein precipitated was harvested through centrifugation at 9,660 × *g*. The overall methodology for preparing pure crystalline violacein is presented in Fig. 1.

The purified violacein was characterized through different spectrometric methods. The UV-VIS spectrum of the violacein was taken using Spectrophotometer (Thermo scientific). A saturated solution of violacein (1 mg/500 µl) in methanol was used for the liquid chromatography mass spectroscopy (Thermo scientific exactive mass spectrometer, Thermoscientific USA). The proton NMR was also taken for the pure violacein (Bruker AVANCE^{II} 500MHz).

Antimycotic activity of violacein by minimum inhibitory concentration (MIC)

The antimycotic activity of purified violacein was tested for medically important fungi/yeast such as *Aspergillus flavus*

Table 1. Violacein producing bacterial strains, crude pigment yield, purification steps adopted and pure violacein recovery reported

Bacterial strain	Crude violacein (g)	Purification method adopted	Pure violacein	Reference
C. violaceium CCT 3496	≈ 0.750 g	Soxhlet purification, crystallization & HPLC	1 mg	Rettori and Durán (1998)
Pseudoalteromonas sp. strain DSM 13623	2.10 g	Silica gel column chromatography with different solvents	2.9 mg	Tan *et al.* (2011)
Pseudoalteromonas sp.520P1 and 710P1	Data not available	Silica gel column chromatography	Data not available	Yada *et al.* (2008)
C. violaceium JCM 1249	Data not available	Amberlite XAD 2 adsorption, solvent washing & column chromatography	Data not available	Hoshino *et al.* (1987)
Alteromonas uteoviolacea	Data not available	Sephadex LH-20 column chromatography	Data not available	McCarthy *et al.* (1985)
Duganella sp. B2	1.62 g	Not specified	Data not available	Wang *et al.* (2009)
***Chromobacterium* sp. NIIST (MTCC 5522)**	**1.6 g**	**Successive silica gel & alumina column chromatography with different solvents**	**12.8 mg**	**Present study**

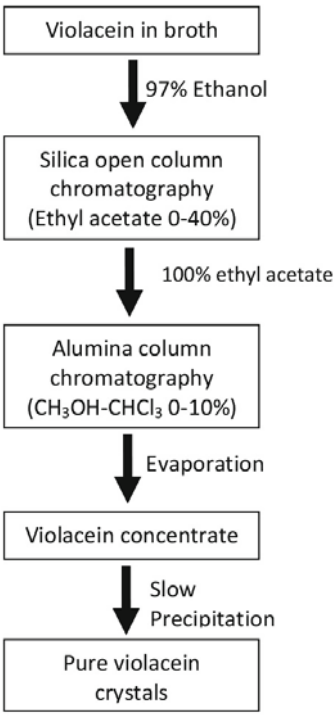

Fig. 1. Diagrammatic representation of violacein purification.

MTCC 183, *Candida tropicalis MTCC* 184, *Candida albicans* MTCC 277, *Cryptococcus gastricus* MTCC 1715, and *Trichophyton rubrum MTCC* 296 as well as agriculturally important fungi such as *Fusarium oxysporum* MTCC 284, *Rhizoctonia solani* MTCC 4634, and *Penicillium expansum* MTCC 2006. All the test microorganisms were procured from Microbial Type Culture Collection Centre (IMTECH), Chandigarh, India. In antimycotic study, MIC was determined using potato dextrose agar media against the standard fungicide bavistin by the poisoned food technique (Rollas *et al.*, 1993). The MIC was defined as the lowest antibiotic concentration showing no growth. A stock solution of 2,000 µg/ml of the violacein was prepared, which was further diluted with methanol to give the required concentrations 1000 to 1 µg/ml. Same dilution in sterile distilled water was done for bavistin and amphotericin B (commercial antimycotics) used for comparison in this study. To check the response of methanol, a solvent control was maintained without the antimycotic compounds. For *C. albicans*, the broth dilution method (CLSI, 2008) was adopted using potato dextrose broth against the standard fungicide amphotericin B. All experiments were done in triplicate for each treatment against each fungus.

Antifungal assay by disc diffusion technique

Violacein was screened for their antifungal activity against test fungi by disc diffusion method (CLSI, 2009, 2010). The fungal cultures were grown on potato dextrose broth. The mycelia mat of fungi of 7-day old culture was suspended in normal saline solution and test inoculum was adjusted to 5 $\times 10^5$ CFU/ml. Inocula (0.1 ml) were applied on the surface of the potato dextrose agar plate and spread by using a cotton swab. Subsequently, filter paper discs (6 mm in diameter, Himedia) containing MIC concentration of test compounds for each test fungi were placed on the agar plates after drying and incubated at 35°C for 24–48 h. Afterwards, the diameter of the inhibition zone was measured.

Statistical analysis

All batch experiments were repeated with triplicate. The mean value was accounted with standard deviation. The significance level for all tests was set at $P < 0.05$. MS Excel was used for the calculations.

Result and Discussion

Characteristics of the violacein producing strain

The present strain of *Chromobacterium* sp. NIIST (MTCC 5522) produced purple blue pigmented circular colonies (0.5–3 mm diameter) on LB medium in 24 h. The SEM images showed the rod shaped cells in a size range of 0.4–0.6 × 1.8–2.2 µm. Cells exist as single, chain or as cluster (Fig. 2).

Cells were Gram-negative. Biochemical tests revealed the strain was positive for oxidase, methyl red test and citrate utilization and negative for catalase, urease, indole production and voges-proskauer test, TSI test was positive for glucose and no sulphide production. Analysis of substrates utilization profile (BIOLOG) indicated the strain is able to metabolize a number of naturally occurring aliphatic and aromatic compounds such as trehalose, thymidine, inosine, uridine, cellobiose, hydroxybutyric acid, alaninamide, organic acids, amino acids, esters and sugars (mannose, fructose). The potential of this strain to metabolize different substrates offer advantage for the bacterium to survive in natural conditions with limited carbon or nutrient sources. *Chromobacterium* sp. reported from diverse natural ecosystems exhibits more or less uniform phenotypic and biochemical characteristics, meanwhile diverse physiological features to adapt specific environment (Hungria *et al.*, 2004). The neighbor joining phylogenetic tree of the strain and other related strains is presented in Fig. 3. The strain is deposited in MTCC under the accession number 5522.

Growth and violacein production by the isolated strain

The growth and violacein production by the present strain was observed over a wide pH, temperature, and salinity and aerobic to micro-aerophilic conditions. The growth and violacein production by the new strain was observed over a range of pH 5–9, with optimum value around seven. At pH 4.0 and 10 growth was very poor without any violacein pro-

Fig. 2. SEM image of *Chromobacterium* sp. NIIST (MTCC 5522) cells in cluster.

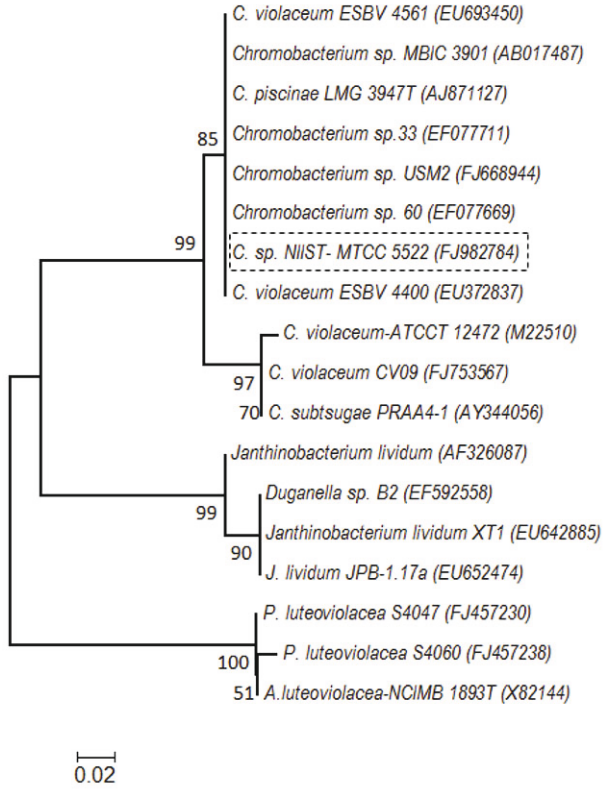

Fig. 3. Neighbour-joining phylogram based on 16S rRNA gene sequences showing the phylogenetic position of *Chromobacterium* sp. NIIST (MTCC 5522) with other violacein producing organisms retrieved from the GenBank. Scale bar represents 0.02 substitutions per nucleotide position.

duction (Fig. 4A). The tolerance of *Chromobacterium* sp. NIIST (MTCC 5522) to wide pH ranges could be a physiological adaptation to natural environments. This view is supported by the fact that the present strain was isolated from an acid mine lake (pH 4.5). The growth and pigment production by *Chromobacterium* sp. isolated from different geographical and environmental conditions have been reported in the past (Hungria *et al.*, 2005).

Temperature considerably affected pigment production, but showed little effect on growth of the present strain. At 30°C, violacein production started in 10 h incubation, whereas at 35°C pigment production started at 6 h. The maximum violacein production was found at 30°C in 24 h incubation period (Fig. 4B). Hungria *et al.* (2005) have studied about 22 isolates of *C. violaceum* from Brazilian Amazon and reported that all isolates were able to grow *in vitro* at 44°C, but were adversely affected by temperatures below 15°C, and unable to survive at 4°C. In a different study, *C. violaceum* (strain BB-78) isolated from Brazil showed maximum violacein production at 28°C compared to 30°C or 37°C (Riveros *et al.*, 1989). The strain *Alteromonas luteoviolacea* produced maximum violacein at 18–22°C, while more biomass was formed at 25–30°C (McCarthy *et al.*, 1985). Similarly the strain RT102 produced maximum violacein at 20°C; under pH 6 and 1 mg/L of dissolved oxygen level (Nakamura *et al.*, 2003).

Moreover, the present strain exhibited violacein production under static (confined to top medium) to agitated conditions (Fig. 4C). In static culture, violacein production only at top layer could be due to availability of oxygen at this point. McCarthy *et al.* (1985) have also recorded a similar observation for *A. luteoviolacea.*

Chromobacterium sp. NIIST (MTCC 5522) showed growth and violacein production under different initial culture salinity (Fig. 4D). The experiment showed that the bacterial strain is saline tolerant and this can be an added advantage of bacterium to widely spread in tropical and subtropical regions of the world.

The isolated bacterium is an improved strain under the species *C. violaceum* as the *Chromobacterium* sp. NIIST (MTCC

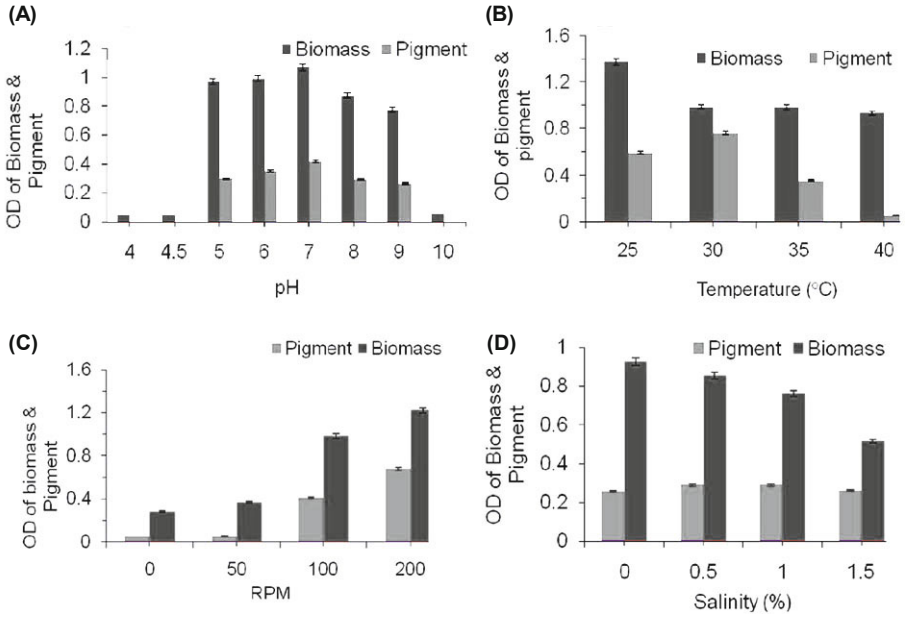

Fig. 4. Effect of different parameters on growth and pigment production by *Chromobacterium* sp. NIIST (MTCC 5522) in LB broth. (A) pH levels, (B) Temperature, (C) Agitation, and (D) Culture salinity. Data represent value after 24 h incubation under specific RPM. Biomass and pigment OD were measured at 600 and 575 nm respectively.

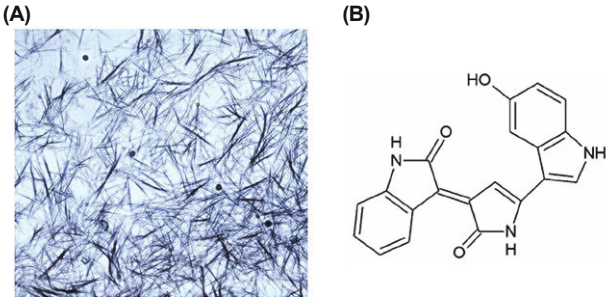

Fig. 5. (A) Microscopic image of pure violacein crystals precipitated and (B) Chemical structure of violacein.

5522) used in this study produced substantial amount of violacein under simple growth conditions. Significant genetic variability within and between populations of *Chromobacterium* sp. have been reported earlier (Dall'Agnol *et al.*, 2008).

Extraction, purification, and characterization of violacein

Under optimum conditions of pH (7.0) and temperature (30°C), in modified LB medium, around 1.6 g crude violacein/L of LB broth in 24 h was produced through extraction with ethanol. TLC of the crude violacein at this stage revealed a mixture of co-occurring contaminanants with violacein. The new column chromatographic method was found very effective to recover pure violacein in crystalline needle form (Fig. 5). The previously reported purification methods are more laborious and expensive, that include multiple steps using specific columns (Table 1). However, the present method is simple and the recovery of pure violacein was 12.8 mg/L culture, that is many fold higher than reported values. The TLC of pure violacein recovered was co-spotted with pure violacein purchased (Sigma Aldrich) had the same Rf values (1.2 cm in 1: 6 methanol-dichloromethane mixture) and the chromatogram was devoid of any other impurities. The proton NMR spectra (Fig. 6A) also confirmed the purity of violacein obtained. In the absorption spectrum the pigment showed a maximum absorbance at 571 nm in methanol. It was reported that the UV-VIS spectrum shows

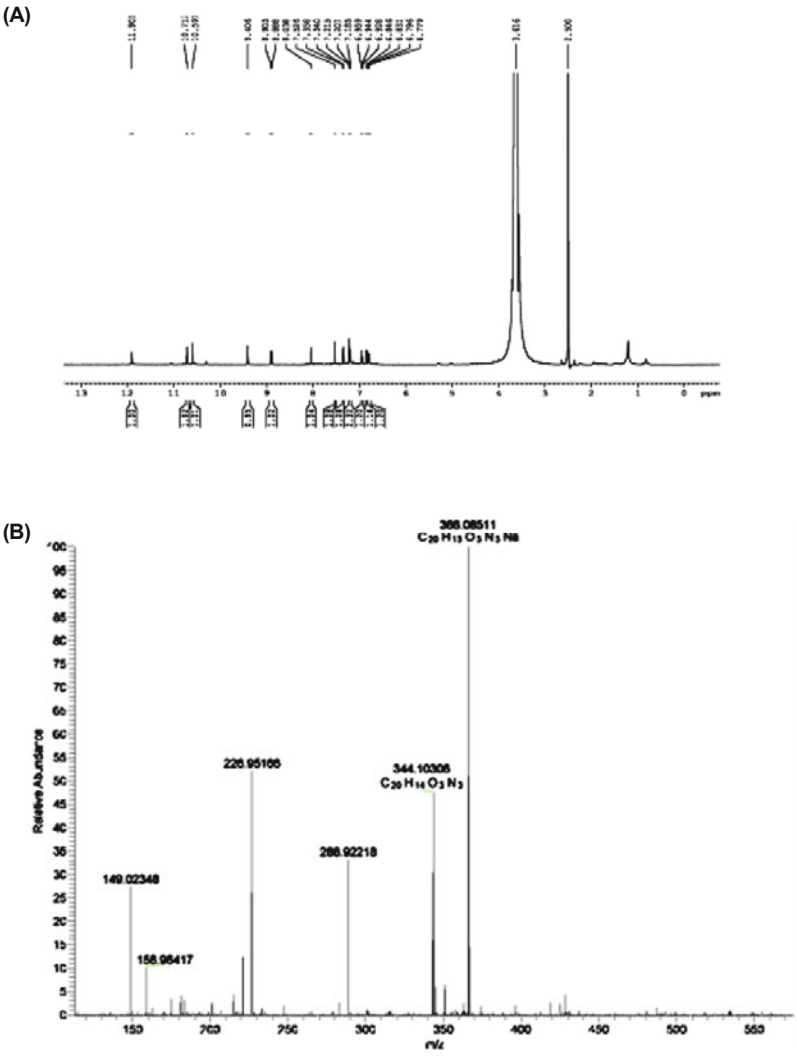

Fig. 6. (A) ^{1}H NMR spectrum of purified violacein dissolved in DMSO-d_6. (B) Liquid chromatography mass spectrum of purified violacein

Table 2. Antifungal activity of violacein

Test fungi	MIC (µg/ml)			Disc diffusion (zone of inhibition in mm)		
	Violacein	Bavistin	Amphotericin B	Violacein	Bavistin	Amphotericin B
A. flavus	32	64	-	20±0.52	22±0	-
C. albicans	8	-	2	22±1	-	26±1
C. tropicalis	16	-	2	19±0	-	30±0
C. gastricus	32	-	8	19±1.12	-	26±1.12
T. rubrum	2	-	4	32±2.12	-	33±1.72
R. solani	4	8	-	20±1.17	25±0.72	-
F. oxysporum	4	16	-	21±0.52	24±1.15	-
P. expansum	2	32	-	31±0.52	27±0.72	-

Values represent mean of three replications;
- not tested

strong absorption at the visible region due to resonance of violacein (Rettori and Duran, 1998). The FTMS {1, 1} + p ESI Full lock ms [100.00–2000.00] was taken and got the major peak in 366.085 (Fig. 6B) which corresponds to $C_{20}H_{13}N_3O_3Na$. The MS/MS spectrum of the ion at m/z 342.5 showed the fragment ions at m/z 298.4, m/z 209.3, and m/z157.3. The characterization data supported recovery of highly purified violacein through the new column chromatographic method.

Antimycotic activity of the violacein

Pure violacein recovered from the present isolate was highly effective against all human and plant pathogenic fungi/yeast tested like *Candida albicans* and *Aspergillus flavus* and phytopathogenic *Fusarium oxysporum*, *Rhizoctonia solani*, and *Penicillium expansum*. Compared to common antifungal agents such as bavistin and amphotericin B available in the market, violacein was more effective against four out of eight fungal strains tested (Table 2). The zone of inhibition created by the minimum inhibitory concentration of violacein against the fungi tested was presented in Fig. 7 and Table 2. Methanol used for dissolving violacein has no effect on the antifungal activity. The antifungal activity of pure violacein against medically important fungi was not reported yet.

Violacein containing crude extract produced by *Janthinobacterium lividum* was reported effective against the phytopathogenic fungi *Colletitrichum dematium* and *Rosellinia necatrix*, but ineffective against soil living yeast *Trichosporon cutaneum* (Shirata *et al*., 1997). In a different perspective, the biocontrol potential of violacein producing bacteria against pathogenic fungi was reported in two independent studies. In one of the studies, *C. violaceum* strains from the Brazilian Amazon were reported to have antagonistic activity against *Fusarium* sp., *Phomopsis* sp., *Cercospora kikuchi*, *Corynespora* sp., *Aspergillus* sp., and *Colletotrichum*, all pathogenic to soybean. It is speculated that the observed antifungal activity could be due to violacein toxicity, cyanide production and chitinolytic activity of the bacterium (Barreto, 2008). In a different study, *J. lividium* produced violacein containing metabolites on the skin surface of salamander which declined the growth of a fungi *Batrachochytrium dendrobatidis* pathogenic to this amphibian (Brucker, 2008).

Unlike these previous studies where either the crude violacein (metabolites including violacein and other compounds) or the source bacterium was used for fungal control, the antifungal potential of pure violacein is demonstrated first time in this study. Moreover, activity of violacein against pathogenic yeast is not reported yet. The novel information about

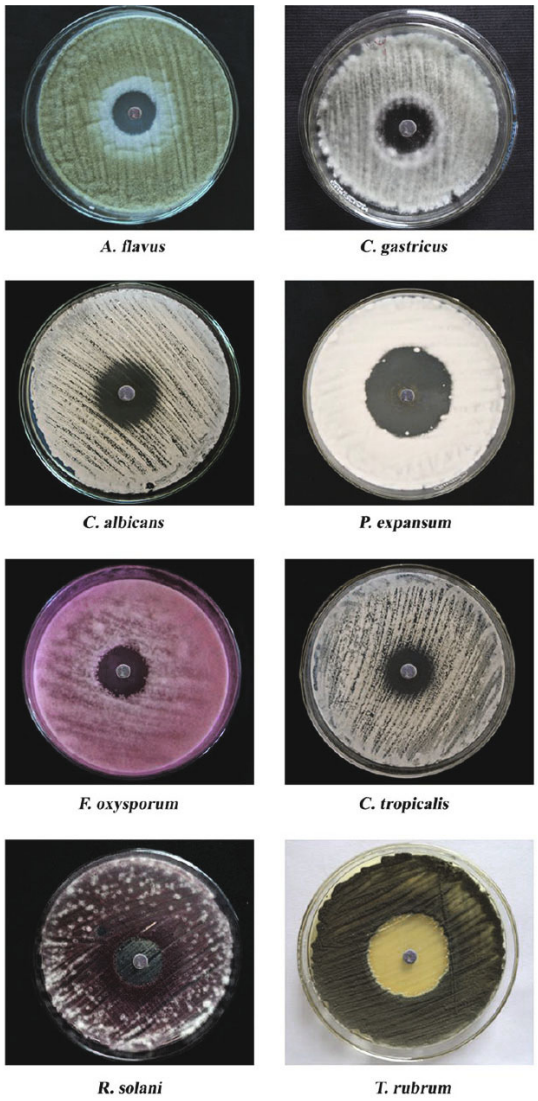

Fig. 7. Antifungal zone created by violacein against different test fungi.

antifungal activity of violacein against pathogenic fungi/yeast presented in this paper will have application potential. Infectious fungi/yeast can be controlled either by using antifungal formulations with violacein as active ingredient. A novel method for purification of violacein is also demonstrated and the recovery of 12.8 mg pure violacein/L culture broth is the highest value ever reported. In the current scenario of environmental contamination of highly persistent chemical pesticide, antifungal compounds of biological origin such as violacein will have more acceptances.

Conclusion

A novel *Chromobacterium* sp. NIIST (MTCC 5522) isolated from an acidic sediment found to produce the high value pigmented bioactive compound violacein under simple nutritional requirements and growth conditions. This study successfully established a novel and economic method for recovering pure crystalline violacein from the crude violacein that contains a number of co-occurring metabolites. The violacein prepared was tested for its bioactive potential and obtained a highly promising antifungal activity so far not reported.

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