Academic Sciences International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 4, Suppl 5, 2012

Research Article

PRODUCTION OF CYCLOSPORIN- A BY SAPROPHYTIC FILAMENTOUS FUNGUS FUSARIUM OXYSPORUM

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Received: 29 May 2012, Revised and Accepted: 12 July 2012

ABSTRACT

In the present study, wheat bran was evaluated as an inert supports for the production of Cyclosporin A (CyA) using *Fusarium oxysporum* by solid state fermentation (SSF). Initially fungus *F. oxysporum* was isolated from sediments of mangrove forest. It was screened for production of CyA with four different locally available materials like wheat bran, rice bran, groundnut cake and coconut cake. Among them wheat bran gave maximum CyA (176.83 mg /100g) and fungal biomass (26.4 g/100g) production. Further different parameters such as fermentation time and different solvents were optimized in wheat bran for maximum production of CyA. Column chromatography, FT-IR, ^{1}H and ^{13}C NMR spectroscopy was employed for purification and structural elucidation of CyA. Results conclude that maximum CyA production at 14th day of fermentation. Among the different solvents tested, ethyl acetate offered maximum production of CyA (163.28mg/100g). The above studies indicated that it is possible to obtain significantly higher yields of CyA through SSF by optimizing various parameters and to produce it at commercially viable levels.

Keywords: Cyclosporin A; *Fusarium oxysporum*; Saprophytic fungi; Peptides; Solid State Fermentation

INTRODUCTION

Modern drugs cure several diseases through precise mechanisms. However the toxicities associated with these drugs limit their use on a long term therapy. Although many drugs have originated from natural sources[1]. Natural compounds isolated from marine organisms have been

found to be a very rich source of bioactive molecules. Reported biological effects of these compounds include anti-tumor, anti-inflammatory and anti-viral activities as well as immunomodulatory and analgesic properties[2]. Immunosuppressants are class of drugs which are capable of inhibiting the body's immune system. Many of the agents included in this category are also cytotoxic (cell poisons) and are used in the treatment of cancer. These drugs are used in organ transplant patients to prevent rejection of the organ by the body and are also useful in the treatment of autoimmune diseases including AIDS owing to its superior T-Cell specificity and low levels of myelotoxicity. Immunosuppressants have gained considerable importance in the world market such as cyclosporin A (CyA), tacrolimus, rapamycin and mycophenolate mofetil. Among these immunosuppressants CyA is the first new generation of drugs with a specific site of action on the immune system. It is a family of neutral, highly lipophilic, cyclic undecapeptides containing unusual 11 amino acids (Fig.1). In addition to the immunosuppressive property, it has anti- inflammatory, antifungal and anti-parasitic properties and reversing multidrug resistance in several types of cancers[3].

A number of fungi from the marine environments have been reported to be important sources of bioactive compounds[4,5,6,7]. Fungi, when cultivated on a solid substrate under the conditions that are similar to their natural habitat could produce metabolites more efficiently than in liquid fermentation[8]. SSF has been used for the fermentation of several species of fungi including *Aspergillus, Rhizopus, Alternaria, Fusarium, Monilia, Mucor, Trichoderma* and *Penicillium*. Among these genus, *Fusarium* has great industrial and pharmaceutical applications for its richness in proteins and peptides[9,10,11]. Cyclosporins are generally produced by *Tolypocladium inflatum* in solid state fermentation process. But there is no report on the production of CyA from mangrove derived fungus, especially *F. oxysporum*. Hence, an attempt has been made to study the production, optimization, purification and chemical characterization of CyA from *F. oxysporum* using different solid substrates.

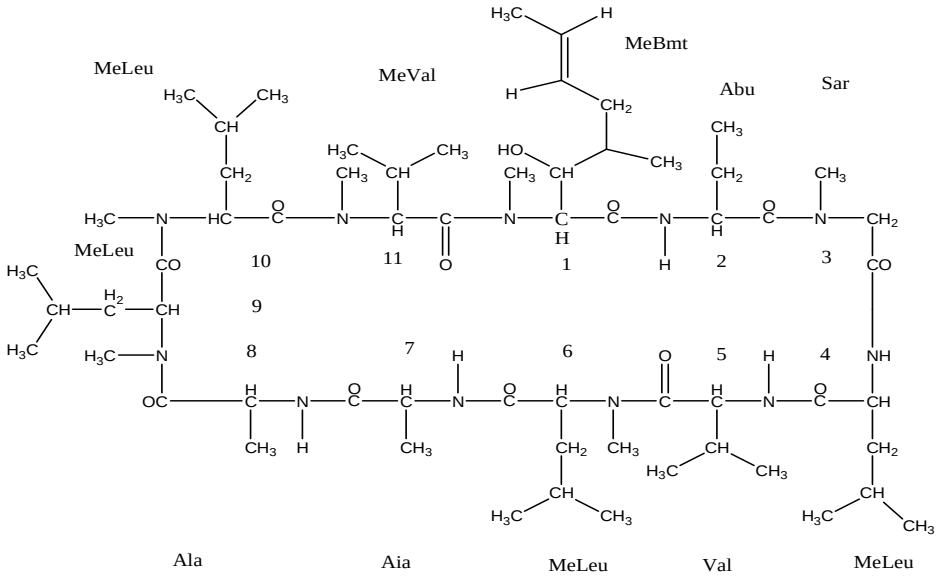

Fig. 1: Chemical structure of CyA comprising 11 amino acids

MATERIALS AND METHODS

Chemicals

All analytical reagents and media components were purchased from Hi-Media (Mumbai, India) and authentic CyA was provided by Sigma company (St. Louis, USA). The solvents used are HPLC grade. Wheat barn, rice bran, groundnut cake and coconut cake were obtained from a local flour mill.

Microorganisms

The fungus used in this study was *F. oxysporum,* isolated from the Rhizosphere soil of *Rhizopora annamalayana* Kathir, endemic species to India. It was sub-cultured in Malt and yeast extract agar medium containing malt extract 20 g/l, yeast extract 4 g/l, agar 20 g/l, at pH 5.3.

Preparation of inoculums

The 5ml of sterile saline containing 0.1% tween 20 was added to the spores (approp. 2.5× 10^7 spores/ ml) were, scrapped from 14 days slant, and mixed thoroughly and then was added to 10 ml of medium (glucose 2%, casein acid hydrolysate 1%, peptone 1%, malt extract 1%, pH 5.4) taken in a 50 ml flask and incubated in a rotary shaker at 150 rpm for 24 ˚C for 48 hrs. This was then inoculated to 200ml of medium in 1000 ml flask at 10% level (v/v) and incubated for 2 days under same condition. This was used as the inoculum for solid state fermentation process.

Preparation of solid substrate

Wheat bran, rice bran, groundnut cake, and coconut cake were used as the solid substrate for the production of CyA. 100 grams of each substrate were taken in a 1000 ml flask and 70 ml of 0. 2 N HCl was added and mixed well and was autoclaved for 60 min at 121 °C/ 15 psi. The flasks were then cool to room temperature and then 40% (v/w) inoculum was added, mixed thoroughly and incubated at 24 °C for16 days in a slanting position.

Analytical Methods

Extraction of CyA

On every alternate day, in each substrate, 300ml (1: 3 w/v ratio) of ethyl acetate was added and the flasks were kept in shaker at room temperature for overnight extraction. This was then filtered through a muslin cloth and extracted once more with methanol. The combined extracts were filtered through a Whatman 1 filter paper to obtained dark brown color extract.

Optimization of incubation period.

Different incubation time was optimized for maximum CyA production from wheat bran. The inoculated flasks were kept 6 days for initial growth. From 6th day of incubation CyA was measured every alternative day up to 16 days.

Monitoring of pH during SSF.

From 6th day of incubation, about 1g of fermented wheat bran was removed under sterile condition and it was mixed with 10 ml of distilled water, pH of the water was measured every alternative day up to 16th day of incubation.

Optimization of extraction with different solvents

In order to extract maximum amount of CyA from wheat bran fermentation, four different solvents like methanol, ethyl acetate, chloroform and butyl acetate were used in the ratio of 1:3 volumes of the solvent and the extraction was done overnight in a rotary shaker at 200 rpm at room temperature.

Qualitative and quantitative determination of CyA:

CyA content was qualitatively identified by the TLC techniques. Silica gel plate was prepared and the test sample was resolved by the solvent system (chloroform: n-hexane, 1:1 v/v). The developed plates were subjected to iodine vapor as color reagent; wherein in the CyA acquired a faint brown color as compared with authentic[12]. The CyA was purified by silica gel column chromatography and its chemical structure was confirmed by IR, ^{1}H and ^{13}C NMR spectroscopy.

RESULTS

Effect of solid substrates on the CyA production.

In this study wheat bran, rice bran, groundnut cake and coconut cake were used as solid substrate. Among the four solid substrates, wheat bran was found to be best supportive in terms of biomass (24.4 g/ 100g) and drug production (175.83 mg/ 100g). In case of rice bran showed good fungal growth (20.6 g/100g) as well as drug production (126.63 mg/100g), but it was comparatively less than the wheat bran. Whereas groundnut cake and coconut cake enhanced only the fungal biomass about 17.81g/100g and 15.58 g / 100g respectively, but not enhanced the drug production (fig.2).

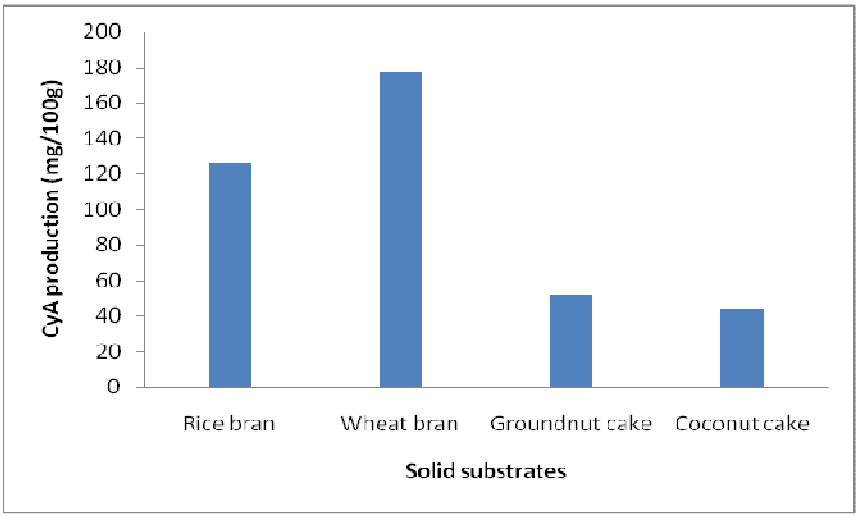

Fig. 2: Effect of different solid substrates on the production of CyA.

pH and CyA levels

Fungal growth was initiated at 6th day of incubation. After that CyA yield as well as pH was checked every alternative days. The yield of CyA was increased from 6th day (78.12 mg/100g) of fermentation and reaches maximum production (176.83 mg/100g) at 14th day of incubation. Above this incubation period affect the CyA production. In case of pH of wheat bran was in increased from 6.2 to 8.4. The impact of pH and incubation periods on the production of CyA was given table.1.

Table 1: Effect of pH and incubation periods on the production of CyA

Days	pH	CyA(mg/ 100g)
6	6.2	78.12
8	6.9	108.59
10	7.3	134.90
12	7.6	162.68
14	8.0	176.83
16	8.4	158.02

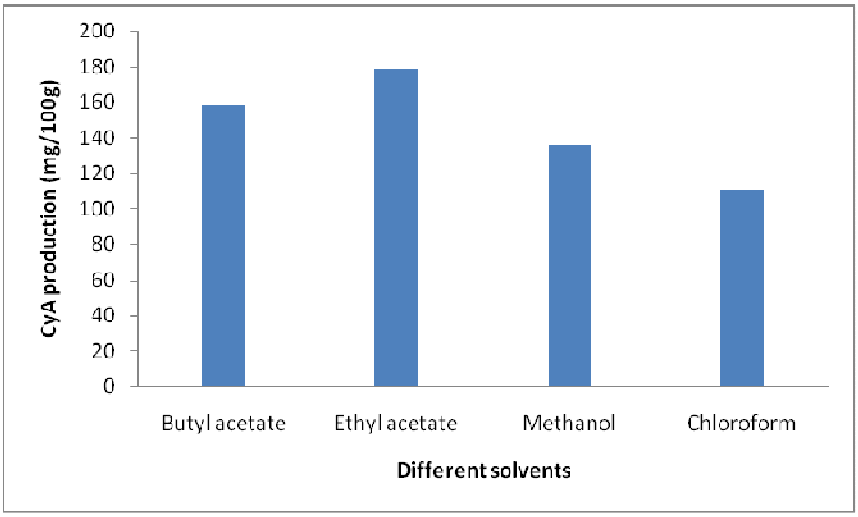

Fig. 3: Effect of different solvents on the production of CyA.

CyA recovery using different solvents

CyA was extracted with different solvents like, methanol, chloroform, ethyl acetate and butyl acetate and the results are given in fig.3. Maximum production of CyA (178.28mg/100g) was obtained by ethyl acetate extraction followed by butyl acetate (158.27mg/100g), methanol (135.89mg/100g) and chloroform (110.43mg/100g). Hence, the ethyl acetate was the best choice of solvent for the optimum extraction of CyA.

CyA purification by Column Chromatography

The crude ethyl acetate extract was dried and residue was suspended in methanol and extracted twice with petroleum ether. The methanol layer containing CyA was dried and loaded into a silica gel column, eluted with mixture of hexane: chloroform: methanol in the ratio of 10:9:1. The TLC positive fraction containing CyA were pooled, evaporated to dryness.

FTIR and NMR spectroscopy

Infrared (IR) spectrum of this compound exhibited absorption at 3421 cm^{-1} indicating the presence of N-H group. The adsorption at 2926 and 2855 cm^{-1} are characteristics of aliphatic C-H stretch indicating the presence of methyl group. The adsorption at 1689 cm^{-1} stretching vibration was indicating the presence of amide carbonyl carbon.

^{1}H NMR (CoCl3): 1- MeBmt: δ 4.70(H-2), 4.15(H-3), 1.30(H-4), 2.31, 2.04(H-5), 5.36(H6, 7), 1.30(H-8), 0.85(H-9), and 2.18(OH); 2-Abu: 4.53(H-2), 2.04(H-3), 0.85(H-4), and 8.10(N-H); 3- San: 4.19, 4.14(H-2), 2.77(N-CH3).4, 6, 9 & 10- MeLeu: 4.30(H-2), 1.25(H-3), 1.63(H-4), 0.85(H-5), 2.77(N-CH3); 5- Val; 4.30(H-2), 2.68(H-3), 1.01(H-4), 8.10(N-H), 7, 8- Ala; 4.30(H-2), 1.30(H-3), 8.10(N-H), 11-Me Val; 4.30(H-2), 2.31(H-3), 0.85(H-4), 2.77(N-CH3). Peak values of ^{1}H NMR has showed in (fig.4).

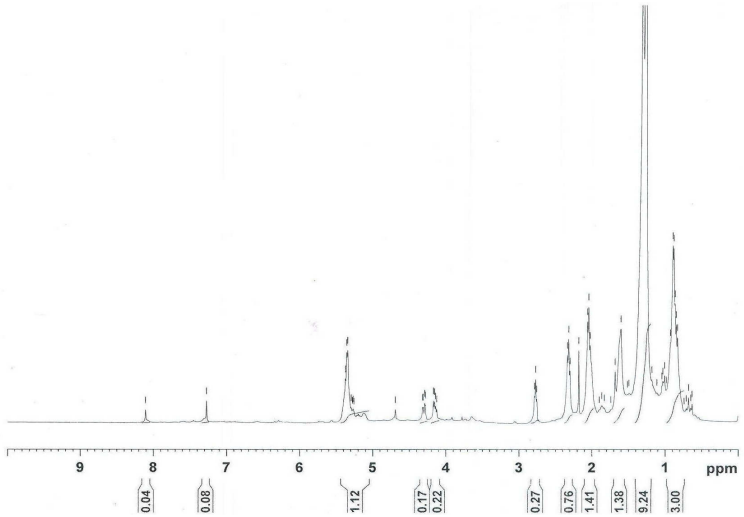

Fig. 4: ^{1}H NMR spectra of purified CyA

^{13}C NMR(CoCl3); 1- MeBmt; δ 172.92(C-O), 62.77(C-2), 68.90(C-3), 36.17(C-4), 26.42(C-5), 130.24(C-6), 127.91(C-7), 14.20(C-8), 14.10(C-9), 2-Abu; δ173.38(C ≈ O), 62.77(C-2), 25.65(C-3), 12.60(C-4), 3-San; δ 172.92(C ≈ O),, 42.84(C-2), 32.22(C-3), 4, 6,9 & 10- Me Leu; δ172.92(C ≈ O), 62.70(C-2), 34.21(C-3), 25.65(C-4), 29.70(C-5), 32.22(C-6), 5-Val; δ 173.34(C ≈ O), 62.70(C-2), 31.95(C-3), 27. 20(C-4). 7&8- Ala; δ 172.92(C ≈ O), 62.77(C-2), 23.40(C-3), 11- MeVal; 173.34(C ≈ O), 62.77(C-2), 31.95(C-3), 27.20(C-4), 32.22(C-5). Peak values of ^{13}C NMR has showed in (fig.5).

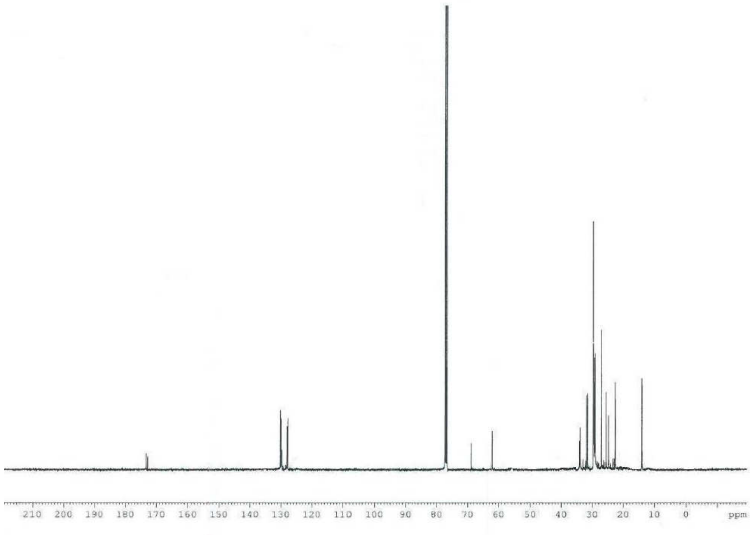

Fig. 5: ^{13}C NMR spectra of purified CyA

The proton spectra at 400 MH 2 and proton decoupled 13 C NMR spectra at 100 MH 2 were recorded at room temperature on DRX 400 NMR spectrometer of CyA in CDCl3-d6. The signals in the ^{1}H NMR spectrum were assigned based on their positions, intervals and multiplicities. The sharp signals at 8.10 ppm (interval corresponds to three protons) is assigned to amide proton (N-H). Low frequency signals centered at 2.77 ppm is assigned to methyl proton attached to the nitrogen atoms (N-CH3 in Me Leu 4, 6, 9 &10 Me Val group). The up field singlet 2.18 ppm is assigned to OH proton appears around 1.25- 4.15 ppm, γ and δ protons appears in the range 0.85-2.31ppm respectively. In Me Bmt group H-6 and H-7 protons resonate at 5.36 ppm (integral corresponds to two protons).

The ^{13}C NMR spectrum downfield signals at 172.92 173.34 and 173.38 ppm are assigned to carbonyl carbons in the CyA moiety. The frequency signal at 32.22 ppm is assigned to methyl carbon attached to nitrogen atom (N-CH3) in MeLeu 4, 6, 9 & 10 and MeVal groups. The α carbons appears around at 23.40- 68.90 ppm, γ and δ carbon observed in the range 12.60-36.17 ppm respectively. In MeBmt group C-6 and C-7 carbons resonate at 130.24 and 127.91 ppm. The ^{1}H and ^{13}C chemical shifts obtained in this manner are listed in table. 2.

Table 2: ^{1}H and ^{13}C-NMR spectral data of CyA

Functional groups	Position	^{1}H chemical shifts (ppm)	^{13}C chemical shifts (ppm)
MeBmt	C=O	_	172.92
	α(CH)	4.70	62.77
	β(CH)	4.15	68.90
	γ (CH)	1.36	36.17
	δ (CH2)	2.31, 2.04	26.42
	6(CH)	5.36	130.24
	7(CH)	5.36	127.91
	8(CH3)	1.30	14.20
	9(CH3)	0.85	14.10
	V (OH)	2.18	_
Abu	C=O	_	173.38
	α(CH)	4.53	62.77
	β(CH2)	2.04	25.65
	γ (CH3)	0.85	12.60
	V-H	8.10	_
San	C=O	_	172.92
	α(CH2)	4.19, 4.14	42.84
	N-CH3	2.77	32.22
MeLeu	C=O	_	172.92
4,6,9& 10	α(CH)	4.30	62.70
	β(CH2)	1.25	34.21
	γ (CH)	1.63	25.65
	δ,δ2(CH3)	0.85	29.70
	N-CH3	2.77	32.22
Val	C=O	_	173.34
	α(CH)	4.30	62.70
	β(CH)	2.68	31.95
	γ1,γ2 (CH3)	1.01	27.20
	N-H	8.10	_

Ala 7& 8	C=O	_	172.92
	α(CH)	4.30	62.77
	β(CH3)	1.30	23.40
	N-H	8.10 _	
MeVal	C=O	_	173.34
	α(CH)	4.30	62.77
	β(CH)	2.31	31.95
	γ1,γ2 (CH3)	0.85	27.20
	N-CH	32.77	32.22

DISCUSSION

SSF has gained importance currently due to its several advantages over submerged fermentation. SSF is being observed as technique for producing higher yields of desired products, various workers have shown interest for the production of high value microbial metabolites including antibiotics through SSF route [13]. From this study, it is clear that SSF has a distinct advantage over submerged fermentation for the production of CyA because of its increased yield. The selection of substrates and optimization of its concentration plays an important role in yielding the higher growth rates of microbes. Though there are many publications for the production of CyA using submerged fermentation, but very few reports are available on SSF.

In the present study, wheat bran was found to be best supportive both in terms of biomass (24.66g/100g) and drug production (176.83 mg/100g) followed by rice bran (20.6 g/100g and 126.63mg/100g), groundnut cake (17.81g/100g and 52.35 mg/100g) and coconut cake (15.58 g/100g and 44.05mg/100g). These are more or less similar with the results of Nisha and Ramasamy[14] screened different indigenously available and cost effective solid substrates and to found that wheat bran support maximum CyA (179mg/100g) and biomass production (22g/100g).

This may due to that wheat bran is reported to contain high levels of polysaccharide but low levels of nitrogen. Since polysaccharides have much higher moisture absorption potential than lignin, wheat bran is able to retain higher moisture levels[15]. Among the various solid substrates tested, wheat bran was observed to be a complete solid medium for the fungal proliferation and cyclosporin production in the SSF process. Cyclosporin being an intracellular metabolite is released into the medium during the stationary phase after the autolysis of the fungal cell wall. The particles of wheat bran have the ability to attract and retain the moisture levels mainly because of the hydrophilic functional groups in its organic matter are able to form hydrogen bonds with water molecules.

In our results showed increasing of incubation period enhanced both the pH and CyA level of *F. oxysporum.* CyA yield of *F. oxysporum* was observed to reach the maximum on the 14th day of incubation period at pH 8.0 after it was declined (table.1). Sekar[16] evaluated CyA production by SSF using a local isolate of *Tolypocladium* sp on the 14th day of incubation periods and similarly the yield of CyA increases from 6-14th. This variation probably the organisms might have reached the death phase. He also pointed out pH of the bran gradually increased and reaches a maximum pH at 8.2. This was mainly due to the production of acids due to incomplete oxidation of the substrate or uptake of ammonium ions, which cause the pH to fall, while the release of ammonia by de-amination of urea, or other amines increase the pH [17].

In the present study, CyA (178.28mg/100g) yield was higher when it was extracted with ethyl acetate followed by butyl acetate, methanol and chloroform. Similar results were obtained by Ismaiel[18]. This may be due to ethyl acetate is very volatile and has a low boiling point of 77 °C. Due to these properties, it can be removed from a sample by heating in a hot water bath. The CyA was confirmed by comparison of its IR and NMR values with those reported earlier Jagtap[19].

The results we have obtained indicate that it is possible to obtain higher yields of CyA from *F. oxysporum* growing under SSF conditions are favorable for further industrial process. In earlier studies an attempt has been made to produce CyA mainly by *T. inflatum* using wheat bran as a substrate. The CyA obtained from *F. oxysporum* was considered to be a best strain next to *T. inflatum* from mangrove environment.

ACKNOWLEDGEMENT

Authors are thankful to Prof. T. Balasubramanian, Dean, Faculty of Marine Sciences and the authorities of Annamalai University for providing necessary facilities and University Grants Commission, Govt. of India, New Delhi for financial support (Grant no: 39-182/2010) to carry out this work.

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