

## ISOLATION AND CHARACTERIZATION OF IMMUNOSUPPRESSANTS FROM FUNGI IN SOIL

S.ANBUSELVI, D.SHARMILA

Department of Industrial Biotechnology, Bharath University, Chennai 73. Email: anbuselvichennai@yahoo.com

Received: 13 Dec 2012, Revised and Accepted: 05 Feb 2013

## ABSTRACT

The discovery of immunosuppressant drugs, in recent years, brought astonishing impact on clinical therapy as a potent composite to perform successful organ transplantation. The isolation and characterization of immunosuppressants from non pathogenic fungi which was isolated from different soil samples of Chennai were collected. The fungal growth of isolate 1 was confirmed by mycelium with white colony morphology in PDA agar plates and appearance of a small leaflet with septa using lactophenol staining was observed microscopically. Antifungal activity of isolated fungal strain was analyzed by Paper Disc Agar Diffusion Method via the production of secondary metabolites. The presence of secreted protein (WCP) was confirmed by SDS-PAGE and found the components of WCP were in molecular weight range of 14 to 80 KDa. The immunosuppressant activity of culture supernatants were emulsified with Freund's adjuvant and injected into rabbit along with known antigen (Goat serum) by subcutaneous mode of injection. The immunization schedule was maintained for 28 days. Marginal ear vein bleeding was done after every dose of immunization and the antigen-antibody titer was analyzed by single radial immunodiffusion and Ouchterlony double diffusion method.

**Keywords:** Immunosuppressants, Fungi, Immunodiffusion and Antigen, Antibody

## INTRODUCTION

In recent years, many immunosuppressive drugs have been discovered and developed for clinical use in transplantation, to induce immunosuppression. Immunosuppressants may either be exogenous, as immunosuppressive drugs, or endogenous, as testosterone<sup>1</sup>. They are used in immunosuppressive therapy to prevent the rejection of transplanted organs and tissues (e.g., bone marrow, heart, kidney, and liver). It is also used to treat autoimmune diseases or diseases that are most likely of autoimmune origin (e.g., rheumatoid arthritis, multiple sclerosis, myasthenia gravis, systemic lupus erythematosus, Crohn's disease, pemphigus, and ulcerative colitis) and some other non-autoimmune inflammatory diseases (e.g., long term allergic asthma control). The majority of these immunosuppressive drugs act non-selectively, the immune system is less able to resist infections and the spread of malignant cells. There are also other side-effects, such as hypertension, dyslipidemia, hyperglycemia, peptic ulcers, liver, and kidney injury<sup>2</sup>. Immunosuppressants are actually natural products derived from bacteria or fungi with potent immunosuppressive activity. Ovalicin, a sesquiterpene, was the first immunosuppressive compound of fungal origin, and was isolated from *Pseudotorium ovalis*<sup>3</sup>. Further development of this drug, however, had to be abandoned due to its severe side effects. Activity of Cyclosporin A inhibits T-cell activation and prevents cytolytic T-cells from organ attack<sup>4</sup>. Its use in organ transplantation was first reported by Calne et al<sup>5</sup>. The immunosuppressive drugs also interact with other medicines and affect their metabolism and action<sup>6</sup>. Recently, several synthetic as well as bioactive compounds from several microorganisms were identified to possess immunosuppressant potentials. Among the microorganisms, fungal species are found to be at top hierarchy in terms of presence of bioactive principles<sup>7</sup>. In view of this information and clinical demands, the present study was designed to explore the soil fungal flora for the presence of clinically potential bioactive principles.

## MATERIALS AND METHODS

Soil samples were obtained from four different polluted areas of Chennai. The samples were immediately added with distilled water (1:5) and centrifuged at 5000 rpm for 10 min. The supernatants were collected. To the PDA agar medium, 0.1 ml of sample supernatant was added and spread by L-rod. It was allowed to incubate at 32°C for 3-4 days or until the appearance of growth. The growth morphology of each colony was observed with naked eye and the colonies with distinct morphology were noted. Microscopic examination of colonies were confirmed by Lactophenol cotton blue (Loba Chemie, India) method. Isolated fungal culture (isolate 1) was maintained using MCP medium and Sabouraud Dextrose medium and

incubated at 32°C for 3 days with constant shaking at 110 rpm. After the overnight growth a loopful of overnight culture was streaked on to PDA agar medium and incubated at 32°C for 3 days<sup>8</sup>.

## (i) Antifungal assay (Paper Disc Diffusion Method)

The *Aspergillus niger* was obtained from Microbial collection center and was subcultured and maintained in Potato Dextrose medium (Himedia, India). The fungal isolates were inoculated into 25 ml of MCP medium and incubated at 32°C for 4 to 5 days with constant shaking at 110 rpm. About 100 µl of *Aspergillus* stock culture was poured over CM agar medium and incubated at 26 °C for 48hrs. The *Aspergillus* spores were scraped by adding 0.9% Sodium chloride and the mycelium was harvested by filtering through sterile Whatman filter paper no.1. The filtrate containing *Aspergillus* conidial spores were stored at -20°C.

To 100 ml of PDA medium (at hand bearable warmth), 0.5 ml of *Aspergillus* conidial suspension was added and mixed uniformly. About 25 ml of media was poured on to a sterile petridish and allowed to solidify. Sterile paper disks were placed over the PDA plate containing *Aspergillus* conidial spores and pressed to fix properly. The paper discs were labeled from 1 to 7 along with a control. To the discs labeled from 1 to 7, increasing volume of isolate 1 culture supernatant was spotted, whereas in control disc, 10 µl of Cycloheximide was added. The plates were incubated for 16 hours at 32°C<sup>9</sup>.

## (ii) Extraction of whole cell protein

About 2 ml of overnight culture of Isolate 1 was taken and centrifuged at 5000 rpm for 10 minutes. The supernatant was discarded and the pellet was suspended in 250 µl of TEP buffer. The suspension was incubated at 37°C for 4 to 5 hours with intermittent vortexing. The homogenous suspension hence obtained was centrifuged at 10000 rpm for 10 minutes. The supernatant was transferred to a fresh tube and the pellet was again treated with TEP buffer and the subsequent steps were carried out as mentioned above. The pellet was repeatedly lysed with TEB buffer, at least for 5 times, in order to obtain proper lysis of cell wall and high yield of intracellular proteins. Each supernatant was quantified for protein using Lowry's method and the proteins were separated by 10% SDS-PAGE<sup>10</sup>. The resultant supernatants were pooled and used for evaluation of immunosuppressant potential using Rabbit model.

## (iii) Evaluation of immunosuppressant potential of whole cell protein (wcp) from isolate 1

Rabbits were categorized under three groups, and each group with three animals; 1) Group 1 forms a control group, which was given

immunization with Goat serum (GS) as antigen. 2) Group 2 forms a Test group, which was given immunization with GS and WCP, 3) Group 3 forms a Test Control, which was immunized with WCP.

The emulsification of antigen and adjuvant was achieved by the simple syringe method, where the appropriate dose of antigen to be used was vigorously added to either FCA or FIA in a clean, dry, sterile, wide mouth beaker. To 0.5 ml of FCA or FIA, about 0.5 ml of Protein Antigen, which contains 1 mg of protein (WCP), was forcefully added.

About 0.5 ml of Goat Serum was emulsified with 0.5 ml of FCA or FIA for the preparation of control antigen. About 0.25 ml of Goat Serum and 0.25 ml of WCFP was emulsified with 0.5 ml of FCA or FIA for the preparation of test antigen. For preparation of Test Control Antigen: About 0.5 ml of WCFP was emulsified with 0.5 ml of FCA or FIA. Primary dose of antigen was given subcutaneously (SC), whereas the subsequent doses were given intradermally[11].

#### (iv) Immunization schedule

Rabbits were given a primary dose which lasts for 10 days and followed by 3 secondary doses with the time interval of 5-7 days between each doses. A final booster dose was given on the 28<sup>th</sup> day, and after seven days of final dose, the rabbits were bled at regular interval after injecting the next dose. The serum samples were collected and determination of immune response in rabbits against antigen and PCEP by Single Radial Immuno Diffusion(SRID) and Ouchterlony double diffusion methods[12].

#### RESULTS AND DISCUSSION

In order to obtain fungal strains for the evaluation of immunosuppressant potential, polluted soil samples from four different locations of Chennai. Soil samples were mixed with water and the supernatants were obtained. The supernatant was plated on PDA agar medium. After incubation at 32°C for 3 days, the fungal colonies with different morphology, pigmentation, and sporulation were appeared, were observed. All the colonies with different characteristics were observed by microscopy. Most of the colonies showed fungus with cotton wooly growth morphology, showed thick, dense mycelium with terminal spores.

The macroscopic observation of isolate 1 showed diffused white growth colony morphology in PDA agar plates, whereas in microscopic observation, it appeared as a small leaflet with septa after lacto phenol staining. Isolate 2 showed a bulky white mass with yellow spores in the centre of the colony. The microscopic observation revealed thin mycelium with elongated spores at the terminus of the mycelium. Isolate 3 showed a white circular colony with an elevated mucoidal disc at the centre of the colony. However, no discrete structure was observed in microscopical observation. Isolate 4 showed a dispersed growth with deep yellow to orange pigmentation(Figure1).

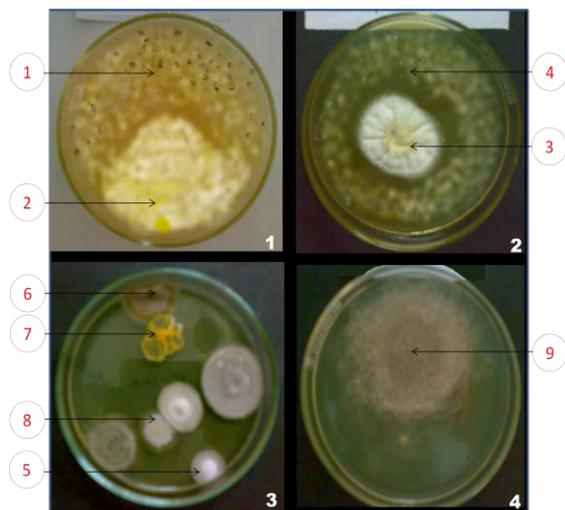


Fig. 1: Culture morphology of soil fungus on PDA agar plates

The microscopic observation revealed clumps with spherical buddings or spores upon lacto phenol cotton blue staining. Based on the macroscopic and microscopic characterization, the fungal isolate 1 was selected for the evaluation of immunosuppressant potential. The distinct one between these two organisms might be due to the inhibition of growth of one or both of the organisms. and also reported to contain antifungal property using paper disc diffusion method(Figure2).

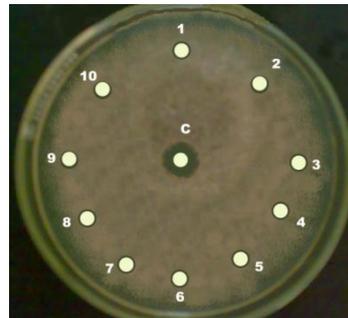


Fig. 2: Antifungal activity for isolate 1 against *Aspergillus niger*

SDS-PAGE was carried out to analyze the presence of proteins in the supernatant. The analysis revealed the presence of different protein bands with different molecular weight ranging from 14 to 80 kDa (Figure 3). Grouped rabbit animals were immunized with known antigen; goat serum and WCP to check the antigenicity of WCP and total WBC count(Table 1).

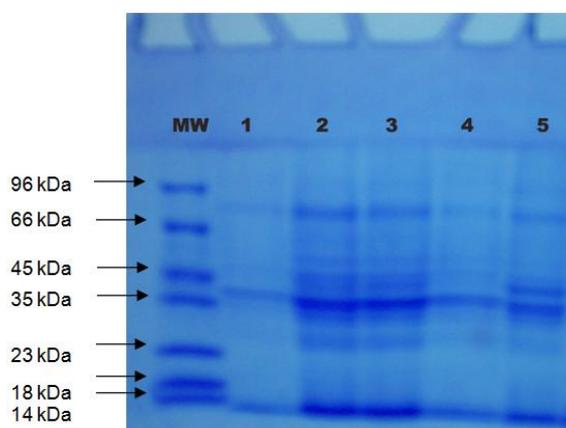
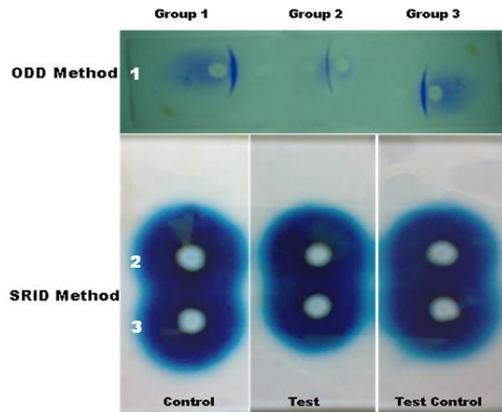


Fig. 3: SDS PAGE of whole cell protein

Table 1: Total WBC (Leucocyte content in mm)

	No. of Cells per mm <sup>3</sup>		
	Control	Test	Test Control
Rabbit 1	6290	6320	6280
Rabbit 2	6420	6480	6450
Rabbit 3	6380	6510	6430
<b>Mean</b>	<b>6363.33</b>	<b>6436.67</b>	<b>6386.67</b>
<b>SD</b>	<b>66.58</b>	<b>102.14</b>	<b>92.92</b>

About 1 ml of peripheral blood was collected from each animal from marginal ear vein incision on day 1, day 9, day 14, day 20, and day 27 after immunization. The final bleeding was done after the 10<sup>th</sup> day of booster dose. Serum was collected after incubation at room temperature and subsequent centrifugation at 10000 rpm for 10 minutes. The serum was added with 0.02% (w/v) sodium azide as preservative and stored at -20°C/ 4°C until use and to evaluate the immune response of animals against known antigen the Control animals were injected only with goat serum by Single Radial Immuno Diffusion (SRID) and ODD method. SRID gave precipitin lines when the known antigen and the serum obtained from the animals were allowed to react in the agar medium (Figure 4)



**Fig. 4: Immune response of animals against goat serum and wcp**

From the immunodiffusion assay results it is observed that the B-cells that are responsible for production of antibodies are not affected by the WCP, since all the three samples showed antigen-antibody reaction, which could be seen as precipitin line and ring. As there was presence of antibodies in the serum, it was assumed that the B-cells, which are responsible for antibody production, were not suffered or influenced by the components of WCP.

Increased risk of infection is a common side effect of all the immunosuppressant drugs. The immune system protects the body from infections and when the immune system is suppressed, infections are more likely. Immunosuppressant drugs are also associated with a slightly increased risk of cancer because the immune system also plays a role in protecting the body against some forms of cancer. Other side effects include loss of appetite, nausea or vomiting, increased hair growth, and trembling or shaking of the hands. The specific immunosuppressive therapy is needed to further

reduce the high morbidity due to infections, malignancies, and graft loss due to chronic rejection after kidney transplantation.

#### REFERENCES

1. Fimmel and Zouboulis CC. Influence of physiological androgen levels on wound healing and immune status in men. *Aging Male* 8: 166-174.
2. Davidson L. and Henry J. *Clinical diagnosis by laboratory methods*. 15<sup>th</sup> ed. Philadelphia, 1974.
3. Lazary S and Staehelin H. Immunosuppressive and specific antimetabolic effects of ovalicin. *Experientia*, 1968; 24: 1171 - 1173.
4. Borel JF, Feurer C., Gubler HU. *et al*. Biological effects of cyclosporin A: a new antilymphocytic agent. *Agents and Actions*, 1976; 6:468-475.
5. Calne RY, White DJH., Thiru S. *et al*. Cyclosporin A in patients receiving renal allografts from cadaver donors. *Lancet*, 1978; 2: 1323-1327
6. Halloran PF. Immunosuppressive drugs for kidney transplantation. *N Engl J Med*. 2004; 351:2715-29.
7. Hiroaki M, Fumie A., Satoko F. *et al*. FR235222, a fungal metabolite, is a novel immunosuppressant that inhibits mammalian histone deacetylase (HDAC): II. Biological activities in animal models. *J. Antibiotics* 2003; 56(2): 80-86
8. Warcup J H , Isolation of fungi from soil ,*Nature* 1950; 166,117-118.
9. Bauer AW, Kirby WM, Sherris JC, Turch M , Antibiotic susceptibility testing by a standardized single disk method ,*Am.J.Clin. Pathology*, 1966; 45(4):493-496.
10. Laemmli UK, *Nature* 1970; 227:680-685.
11. Barne, Bernard H) Quantitating immunoglobulins by immunodiffusion method, *Clinical chemistry* ,2012;20(1):60-69.
12. Manchini G, Vaerman JP, Carbonara AO, Heremans JF (2012) A single radial immunodiffusion and ODD for immunological quantitation of protein, Elsevier, Proceedings of 11<sup>th</sup> colloquium.