

VIT-CMJ2: Endophyte of *Agaricus bisporus* in Production of Bioactive Compounds

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Received: July 08, 2015; Revised: December 05, 2015; Accepted: February 15, 2016

Background: *Agaricus bisporus* is an edible basidiomycete fungus. Both the body and the mycelium contain compounds comprising a wide range of antimicrobial molecules, contributing in improvement of immunity and tumor-retardation.

Objectives: The presence of endophytes capable of producing bioactive compounds was investigated in *Agaricus bisporus*.

Materials and Methods: Endophytes from *Agaricus bisporus* was isolated on LB agar. The obtained isolates were characterized morphologically and biochemically. Further 16S rRNA sequencing was implemented for molecular analysis of isolates. The isolate was mass produced and the bioactive compounds were extracted using ethyl acetate, chloroform and hexane. Agar well diffusion method was carried out to seek the potential of any antimicrobial activity of the crude bioactive compounds against known pathogens. GC-MS and FT-IR analysis were performed for the identification of bioactive compounds.

Results: VIT-CMJ2 was identified as *Enterobacter* sp. as revealed by 16S rRNA sequencing. Chloroform extract of VIT-CMJ2 showed a maximum zone of inhibition of 19 mm against *Salmonella typhi* followed by hexane and ethyl acetate extracts. The GC-MS analysis revealed the presence of several bioactive compounds having effective antimicrobial activity like butyl ester, Behenic alcohol, S, S-dioxide derivatives and some others which were later confirmed by FT-IR spectral stretches.

Conclusions: The present study shows the insight on the way endophytes interact with *Agaricus bisporus*; thereby improving the nutritional profile.

Keywords: Antibacterial activity; Behenic alcohol; Button mushroom; Butyl ester; Endophytes; FT-IR; GC-MS; 16S rRNA

1. Background

Mushrooms are used as a common edible food and as a traditional Asian medicinal system across the globe (1). About 2000 varieties of edible mushrooms have been reported amongst which less than 100 varieties are cultivated, the rest are obtained from the wild varieties (2). Among the cultivated varieties of mushroom, the button mushroom (*Agaricus bisporus*) dominates the global market (3). Mushrooms are rich in protein, minerals, vitamin D, unsaturated fatty acids, immunologically active polysaccharides and sterols (4-6). Further, low fat and presence of dietary fibers make it a good choice for calorie conscious people (3). Like plants, mushroom is also associated with some (mainly Mushroom Growth Promoting Bacteria) microbes known as endophytes, which help in its growth.

Endophytes are mainly bacteria or fungi living inside plants, animals or other living system. They generally show endosymbiosis with the host, resulting in mutual benefit. Fungal endophytes in plant help the

roots in nutrients absorption, preventing infection and supports in growth (7, 9). Twenty three strains of mushroom growth promoting bacteria have been identified from 14 different mushroom farms (7, 8).

The most widely consumed mushroom; *Agaricus* have also been investigated for its association with some beneficial bacteria. *Agaricus blazei* have been found to be associated with actinobacteria that help to reduce the harvest time and at the same time increases the total polysaccharide-protein complex content (9). So far, association studies were used to analyze interaction between the microbes and mushroom substrate or mycelium. Only a meagre work has been reported on the endophytes isolated from edible mushroom, its identification and metabolite profiling.

The bioactive metabolites are either produced by mushroom itself or by some endophytes. Recent studies focuses on identification of numerous bioactive compounds with immuno-active and anticancer properties, optimization of substrate, strain improvement,

improvement of culture conditions and microbial ecology of the mushroom (10).

2. Objectives

The microbial association of *Agaricus bisporus* was investigated. The metabolites of the endophytic microbes from this mushroom were analyzed to identify the bioactive molecules and were tested against known pathogens for the antibacterial activity. The metabolites of these endophytes may be responsible for some of the bioactive compounds in button mushroom.

3. Materials and Methods

3.1. Sample Collection and Chemicals

Fresh button mushrooms (*Agaricus bisporus*) were procured from a local vegetable market in Vellore, Tamil Nadu, India and were processed immediately. Luria Bertani (LB) agar media and broth were purchased from the HiMedia Laboratories, Mumbai.

3.2. Isolation of Endophytic Bacteria

To isolate endophytic bacteria, mushroom were surface sterilized with three washes of dH₂O followed by 4% NaOCl solution and 70% ethanol (11). Blocks of mushroom (1 cm³) were impregnated on LB agar plates and were incubated at 28±2°C for 24 h. The endophytic isolates were selected based on colony morphology and were purified and maintained on LB agar slant. Biochemical and morphological characterization was performed based on Bergey's manual. One distinct isolate was selected and named as VIT-CMJ2 (11, 12).

3.3. Endophytic Bacterial Culture Inoculum

VIT-CMJ2 was cultured in LB and incubated at 28±2°C till the optical density reached 0.5 (1.5×10⁸ cfu.mL⁻¹). Inoculum (2% v/v) from this pre-grown culture was used to inoculate 350 mL of LB at 28±2°C for 5 days on a rotary shaker at 120 rpm (13).

3.4. Solvent Extraction

The broth of VIT-CMJ2 culture was extracted using chloroform, n-hexane and ethyl acetate. The extracted metabolites were dried over anhydrous Na₂SO₄, dissolved in HPLC grade solvents and were further analytically analyzed using GC-MS and FT-IR (14).

3.5. Antimicrobial Activity

Antimicrobial activity of crude extracts of endo-

phytic bacteria VIT-CMJ2 was assessed against known pathogens such as *Salmonella typhi* (MTCC No. 8587), *Escherichia coli* (MTCC No. 9721), *Bacillus subtilis* (MTCC No. 2547) and *Staphylococcus aureus* (MTCC No. 3160). Seed culture (O.D = 0.5) of all the bacterial pathogens were prepared and swabbed evenly onto Muller hinton agar plate; wells were prepared using cork borer and 100 µL (10⁸ cfu mL⁻¹) of crude VIT-CMJ2 extracts were added at a concentration of 300 mg.L⁻¹ to each well. The well containing only solvent was considered as negative control and Ciprofloxacin and Rifampin (100 mg.L⁻¹) as positive control. The plates were incubated at 37°C and zone of inhibition were measured in mm after overnight incubation (15).

3.6. GC-MS Analysis

Gas chromatography coupled to mass spectrometer was used to identify the metabolites produced by the endophytic microbes (16, 17). For the detection of various metabolites obtained in the solvent extracts of effective isolate VIT-CMJ2, the samples were given for GC-MS analysis along with the controls *i.e.* (LB). GC-MS analysis was performed using Perkin Elmer GC model (30 m×0.25 mm×0.25 µm) and Clarus 680 (Mass spectrometer Clarus 600 EI) apparatus with an injection volume of 1 µL, oven temperature was programmed from 60°C to 300°C for 2 min at the rate of 10°C/min and isothermally held for 6 min until the analysis was completed.

3.7. FT-IR Analysis

FT-IR analysis was performed in order to investigate the different functional groups present in crude VIT-CMJ2 extract. FT-IR analysis was performed on Nicolet FT-IR (Avatar-330) in 400-4000 cm⁻¹ with 16 scan speed (18).

3.8. 16S rRNA Sequencing

The isolated bacteria VITCMJ-2 were sequenced using 16S rRNA technique using universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') The bacterial DNA was extracted from the cells and 16S rRNA sequence was determined using fluorescent dye terminator method with the help of the sequencing kit (ABI Prism Big dye terminator cycle sequencing ready reaction kit v.3.1). Products were run on an ABI13730XL capillary DNA sequencer (ABI Prism 310 genetic analyzer, Tokyo, Japan). The ClustalW software was used to compute the aligned sequences and an evolutionary

distance matrix was created through determination of sequence homologies using BLASTn search (31).

4. Results

4.1. Isolation and Identification of Effective Endophytic Bacteria

Based on different cellular and morphological characteristics VIT-CMJ2 was selected and maintained on LB agar plates. Morphological and biochemical analysis revealed VIT-CMJ2 to be gram negative rod and positive for nitrate reduction, glucose, citrate, ornithine, arabinose, and sorbitol utilization and negative for urease, phenylalanine deamination, H₂S production and lysine, adonitol and lactose utilization test. 16S rRNA gene sequencing revealed VITCMJ2 to be a close neighbor of *Enterobacter ludwigii* (Figure 1). The sequence was further submitted in NCBI Genbank (accession number KJ437474).

4.2. Antibacterial Activity

The hexane, ethyl acetate and chloroform extract of the VIT-CMJ2 strain exhibited antibacterial activity against the tested pathogens. The maximum activity was displayed by the chloroform extract having a zone of inhibition of 19 mm, followed by ethyl acetate (15 mm) and hexane (11 mm) extracts. All the extracts showed maximum activity against *Salmonella typhi* and minimum activity against *E. coli*.

4.3. GC-MS Analysis

The bioactive compound present in chloroform, hexane and ethyl acetate extracts of VIT-CMJ2 was detected by GC-MS. The peaks obtained were compared with the database available and the analysis revealed the presence of the following major group of compounds (Figure 2A, B and C). GC-MS analysis revealed the presence of compounds like butyl ester, behenic alcohol, 1-amino cyclopentane hydroxamic acid and methylphenidate among the various secondary metabolites produced by VIT-CMJ2 effective in possessing antimicrobial activities.

4.4. FT-IR Analysis

The FTIR analysis of the ethyl acetate, chloroform and hexane extract of VIT-CMJ2 revealed the major peaks in IR range of 3446 cm⁻¹, 2935 cm⁻¹, 1641 cm⁻¹, 1402 cm⁻¹, 1051 cm⁻¹ and 1219 cm⁻¹ corresponding to O-H stretch, H-bonded stretch, C-H stretch, C=C stretch, C-C stretch (in-ring) of aromatic compounds, C-N stretch of aliphatic amines respectively (Figure 3A, B and C).

5. Discussion

Mushrooms are valued throughout the world as both food and medicine for thousands of years and have been considered as a rich source for the production of effective bioactive metabolite and drugs since ancient times (19, 10). Endophytes are relatively

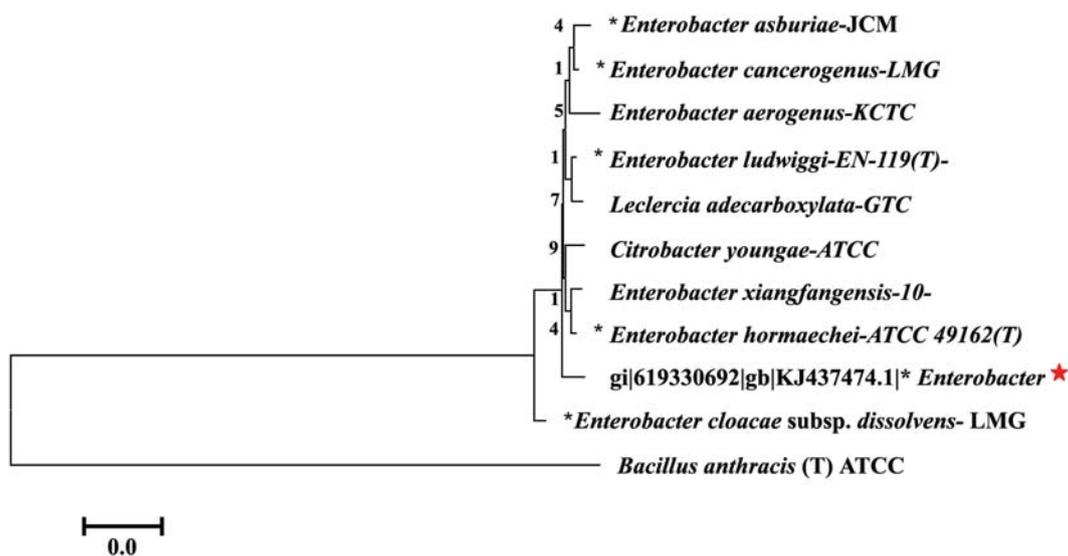


Figure 1. Phylogenetic tree of VIT CMJ 2(Creamy) colony based on the nucleotide sequences of 16S rRNA genes. ★Represents a taxonomic group that includes species/subspecies that are not distinguishable by 16S rRNA gene sequence

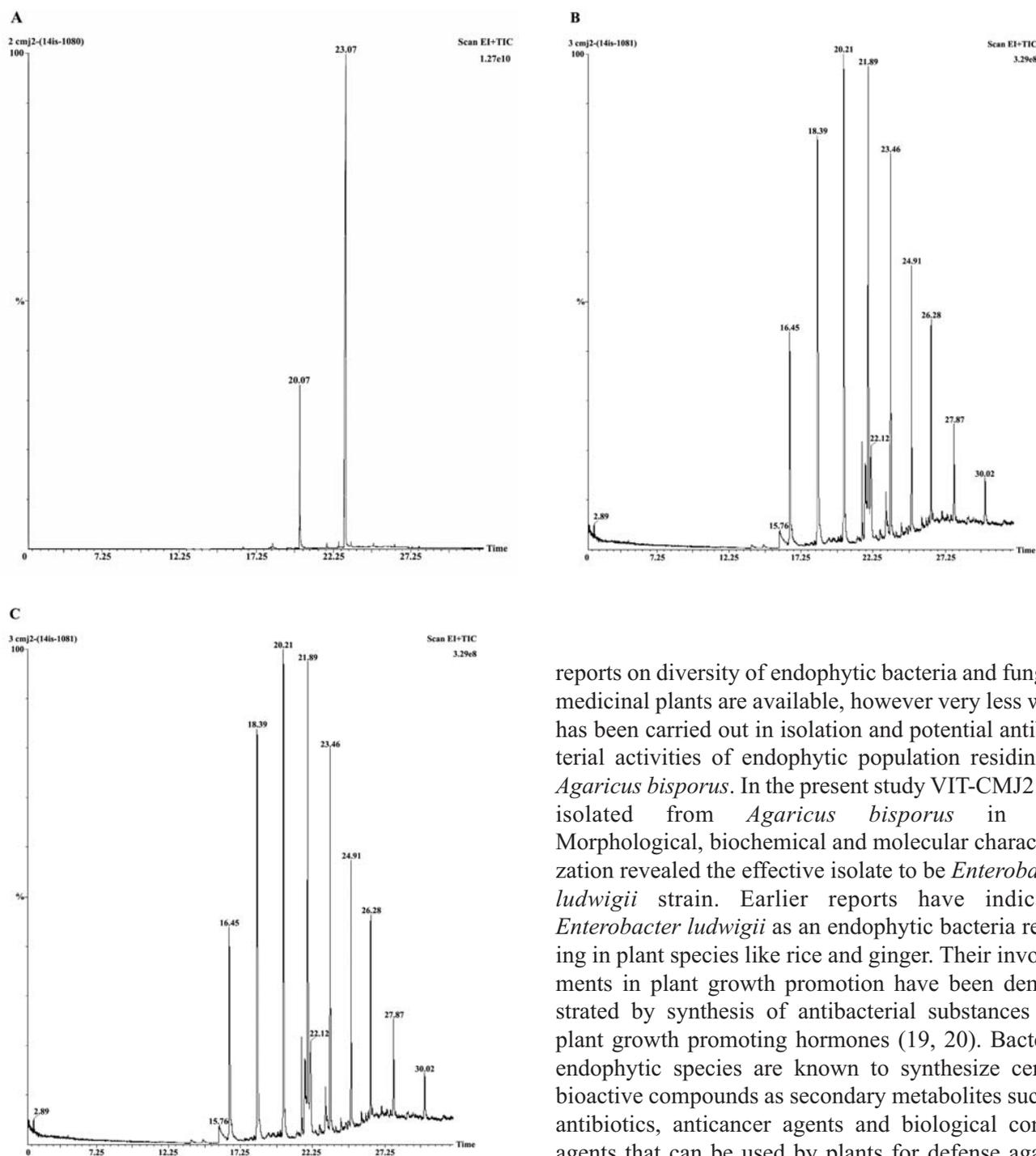


Figure 2. A: Chromatogram of *Enterobacter ludwigii* strain VIT-CMJ2 using hexane. B: Chromatogram of *Enterobacter ludwigii* strain VIT-CMJ2 using chloroform. C: Chromatogram of *Enterobacter ludwigii* strain VIT-CMJ2 using ethyl acetate

unstudied and can be of a great interest, especially in understanding their potential antibacterial activities and exploring their probabilities for the development of novel natural products. Countable numbers of

reports on diversity of endophytic bacteria and fungi in medicinal plants are available, however very less work has been carried out in isolation and potential antibacterial activities of endophytic population residing in *Agaricus bisporus*. In the present study VIT-CMJ2 was isolated from *Agaricus bisporus* in LB. Morphological, biochemical and molecular characterization revealed the effective isolate to be *Enterobacter ludwigii* strain. Earlier reports have indicated *Enterobacter ludwigii* as an endophytic bacteria residing in plant species like rice and ginger. Their involvements in plant growth promotion have been demonstrated by synthesis of antibacterial substances and plant growth promoting hormones (19, 20). Bacterial endophytic species are known to synthesize certain bioactive compounds as secondary metabolites such as antibiotics, anticancer agents and biological control agents that can be used by plants for defense against pathogens (21).

Secondary metabolites from effective isolate VIT-CMJ2 were extracted using hexane, chloroform and ethyl acetate as solvents and the obtained crude extract were assessed for its antimicrobial activity against known pathogens like *Salmonella typhi* (MTCC No. 8587), *E. coli* (MTCC No. 9721), *Bacillus subtilis* (MTCC No. 2547) and *Staphylococcus aureus* (MTCC No. 3160) by agar well diffusion method, where the zone of inhibition around the wells directly indicated

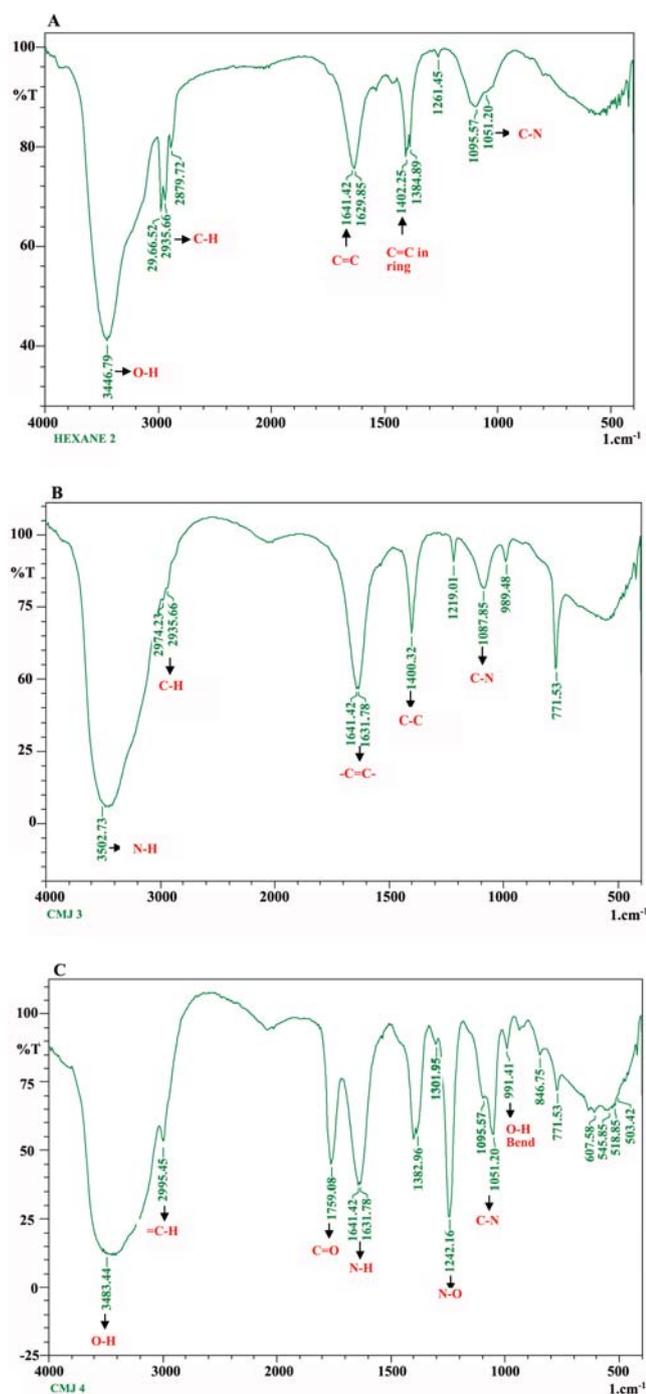


Figure 3. A: FT-IR spectra of VIT-CMJ2 using hexane. B: FT-IR spectra of VIT-CMJ2 using chloroform. C: FT-IR spectra of VIT-CMJ2 using ethyl acetate

the efficiency of the bioactive compound to act against the tested pathogens. The maximum activity was shown by the chloroform extract having the zone of inhibition (19 mm) against *Salmonella typhi* (15). The extract of VIT-CMJ2 on the basis of spectral data by GC-MS analysis was found to be a mixture of many compounds.

We have identified that the bacterial endophytes VIT-CMJ2 from button mushroom produces several bioactive molecules both aliphatic and aromatic. GC-MS analysis revealed the presence of compounds like butyl ester, behenic alcohol, 1-amino cyclopentane hydroxamic acid and methylphenidate effective in possessing antimicrobial activities. Recent reports have revealed the effectiveness of butyl alcohol to act as an antimicrobial agent against vast range of pathogens including *E. coli* DMF 7503, *B. cereus* DMF 2001, *Listeria monocytogenes* DMF 5776 and *Saccharomyces cerevisiae* DMF 1017 (20). Behenic alcohol, 1-aminocyclopentane hydroxamic acid, methylphenidate were reported to be effective against a wide range of microbial agents (21). GC-MS spectra also revealed the presence of compounds such as 1-heptacosenol in the chloroform extract which has multiple potential activities to act as a antimicrobial, nematicidal, anticancer and antioxidant agent. The ethyl acetate extract reported mainly the presence of chloro derivatives of hydrocarbons. It also showed the presence of methylphenidate, a substituted phenethylamine and psycho stimulant drug used for the treatment of the attention deficit hyperactivity disorders. The functional groups of the hexane, ethyl acetate and chloroform extract of VIT-CMJ2 were also analyzed using Fourier Transform Infra Red spectroscopy (FT-IR) and it revealed the presence of functional groups such as C-O, C=C (aromatic), C=O (amides), O-H (acid), C-H (alkyl) and C=O (amide) in the extracts of VIT-CMJ2, in agreement with GC-MS m/z ratio for the presence of same functional groups. Further this study proves that the endophytic bacterium residing in *Agaricus bisporus* is an endophyte that is capable of antibacterial activity. This study will help to enhance the knowledge of edible mushroom in production of novel bioactive compounds.

Acknowledgements

Authors are thankful to VIT University management, SIF, VIT University, Laboratory Assistants of School of Biosciences and Technology of VIT University, Vellore, India.

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