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Bioremediation of Herbicide Atrazine by Fungal sp. *Aspergillus alliaceus* Strain JAV1 Isolated from Paddy Field Soil in Vellore

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Abstract: The herbicide atrazine (2-Chloro-4-ethylamino-6-isopropylamino-s-triazine) is widely used on crops such as maize, sugarcane, corn, pineapple and sorghum. Although atrazine is an effective herbicide, the persistence of this herbicide leads to contamination of soil, groundwater and surface water. This work presents the laboratory studies on the degradation of atrazine. Atrazine degrading fungal strain JAV1 was isolated from paddy field. The molecular characterization based on 18S rRNA sequence revealed the strain JAV1 as *Aspergillus alliaceus.* The strain was able to degrade atrazine at the concentration of 1500 mgL^{-1} and their complete degradation was seen on sixth day of incubation by strain JAV1. The results were confirmed with the help of sophisticated instruments such as high performance liquid chromatography (HPLC), gas chromatography mass spectroscopy (GC-MS) and fourier transform infrared spectroscopy (FTIR).

Key words: Atrazine, mycoremediation, *Aspergilus alliaceus* strain JAV1, growth pattern.

Introduction

Atrazine was the second most highly consumed herbicide in India. Annually, 304 tons of technical grade atrazine is being consumed (Kadian et al., 2008). For the past five decades atrazine has been extensively used in many parts of the world for controlling many varieties of weeds (Tomlin, 1994). It is one of the selective herbicides belonging to the group of the s-triazines (Feria-Reyes et al., 2011). However, it has greater potential; problems emerge because of its long half-life (ranges from 21 days to one year) and low degradability leading to elevation of atrazine concentration above the permissible limit resulting in the contamination of soil (Khan and Saidak, 1981; Swain, 1981; Schiavon, 1988), surface water (Thurman et al., 1992; Richards and Baker, 1993), rain water (Nations and Hallberg, 1992) and ground water (Belluck et al., 1991). Toxicity of atrazine affects both human beings and animals by entering in their food chain (Zhang et al., 2009). It has the ability to induce mammary gland tumours in Sprague-Dawley (SD) female rats (Stevens et al., 1994) and attributed as a potential disruptor in male frogs during sexual development as well as immune responses in some other animals (Christin et al., 2004; Murphy et al., 2006).

Atrazine has been proposed as possible carcinogen and teratogen (Fazlurrahman et al., 2009; Shenoy, 2012). Its structural activity is highly affected by several potent environmental factors including pH,

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temperature, humidity and microbial activity (Feria-Reyes et al., 2011). The use of atrazine has been banned in many European countries but in USA and China till now in practice (Chen et al., 2011). Biodegradation is the economical, effective and residues-free clean up approach for removal of toxic compounds present in the contaminated environment. Till date, many researchers have reported that there are various microbes that have the ability to utilize atrazine as their sole carbon and nitrogen source in fungus genera *Fusarium, Aspergillus*, *Trichoderma, Penicillium*, *Rhizopus*, *Aspergillus*, *Trichoderma*, *Phanerochaete* and *Fusarium.* In bacteria genera *Arthrobacter* (Wang and Xie, 2012)*, Rhodococcus opacus* (Umar et al., 2012) sp., *Acinetobacter* sp., *Norcardioides* sp., *Streptomyces* sp., *Rastonia* sp., *Pseudomonas aeruginosa, Clavibacter michiganense* (Popov et al., 2005), *Chelatobacter heintzii* (Rousseaux et al., 2001), *Enterobacter cloacae* (Shapir et al., 2006), *Bacillus megatherium*, *Alcaligenes faecalis*, *Agrobacterium tumefaciens* and *Klebsiella ornithinolytica* (Siripattanakul et al., 2009). And the fungus *Phanerochaete chrysosporium* (Khan and Saidak, 1981; Hickey et al., 1994), *Fusarium, Aspergillus*, *Trichoderma, Penicillium*, *Rhizopus*, *Aspergillus*, *Trichoderma* and *Pleurotus pulmonarius*. These microbes degrade atrazine through biological pathways such as N-dealkylation, dechlorination, dehalogenation, ring-cleavage and deamination (Struthers et al., 1998; Gebendinger and Radosevich, 1999).

The purpose of this study was to isolate and identify atrazine degrading fungus from the selected fields by enrichment technique and to evaluate the potential fungus for atrazine biodegradation.

Materials and Methods

Chemicals

Certified analytical grade atrazine (99% purity) was purchased from Sigma-Aldrich. The technical grade atrazine, a 55% wettable powder used in this study was purchased from Vellore, Tamil Nadu, India. All other reagents used in this study were of high purity and analytical grade.

Soil Sample

Soil sample was collected from the top layer (0-20 cm) of the paddy field which had been exposed to atrazine herbicide in Vellore district, India. The soil sample was dried at the room temperature in the laboratory and sieved.

Enrichment Procedure and Isolation of Fungal Strain

Atrazine degrading fungal strains were obtained by enrichment culture in the Czapek Dox broth containing (g L^{-1}) yeast extract, 3; peptone 10; dextrose 2; and atrazine 100 mg L^{-1}. Approximately 5 g of soil sample was inoculated in 50 ml of Czapek Dox broth containing atrazine and the flask was kept on a rotary shaker at 120 rpm, at room temperature. Following this it was incubated in orbital shaker for about 7 d, 10 fold dilutions of cultures were prepared and 100 µl of sample was spread on Czapek Dox agar medium containing 100 mg L^{-1} of atrazine. Isolated fungal strains were maintained on agar slopes of the same medium containing atrazine.

Gradient Plate

The enrichment experiment which resulted in four isolates was then further screened for atrazine tolerance capacity following gradient plate method. The atrazine concentration gradient was prepared by adding base layer of 20 ml of Czapek Dox agar without herbicide to the Petriplate tilted at 30° angle. The agar was allowed to solidify at room temperature and the other half of the Petriplate, 20 ml of same media containing atrazine (1000 mg L^{-1}) was poured to give atrazine gradient across the plate surface. Then, the isolated fungal strains were picked using a sterile cotton swab and were inoculated at the middle of the gradient plate agar. The inoculated plates were incubated at 30 ± 2 °C for 8 d. After that the length of the fungal growth along the gradient was recorded (Peter et al., 2014).

Minimum Inhibitory Concentration

Minimum Inhibitory Concentration (MIC) and tolerance to atrazine were determined for the efficient fungal strain which was screened from gradient plate technique using broth assay. Erlenmeyer flasks (250 ml) containing 100 ml of M1 medium composed (g L^{-1}) of $NaNO_3$ 2; KCl 0.5; $MgSO_4.7H_2O$ 0.5; glucose 10; $FeCl_3$ 10 mg; $BaCl_2$ 0.2; and $CaCl_2$ 0.5; per litre at pH 6.8 were taken with increasing concentration of atrazine. The flasks were then inoculated with fungal spore suspension and incubated at 30 ± 2 °C on a rotary shaker at 120 rpm. Mycelial growth was obtained in the flask after 7 d of incubation and Whatman no.1 filter paper was used for the filtration of these mycelial mass in order to separate. Then the mycelial mass was washed with deionised water and the dry weight was checked by drying at constant weight for 80 °C in preweighed aluminium

foil cups. The MIC was noted as the concentration of atrazine resulting in the complete inhibition of mycelial growth in the flasks.

Growth Kinetics

The determination of growth pattern of fungal strain, 1 ml of spore suspension was inoculated in a series of flask containing Czapek Dox broth with and without atrazine (1500 mg L^{-1}). The flasks were maintained in a rotary shaker at 120 rpm at 30 ± 2 °C of duration. After incubation at regular time intervals, the mycelial mass from each series was separated by filtration using Whatman no. 1 filter paper and washed with deionised water. Biomass determination was done by drying the fungal biomass for a constant weight at 80 °C in preweighed aluminium foil cups.

Taxonomic Identification of Fungal Strain

The isolated fungal strain was identified by 18S rRNA sequence analysis. The fungal genomic DNA was isolated by using AMpurE Fungal gDNA Mini kit. In this kit detergent and other non-corrosive chemicals are used to break open the cellulosic cell wall and plasma membrane to extract DNA from fungal cells. The 18S rRNA gene was amplified by polymerase chain reaction (PCR) using the universal primers CGWCGRAANCCTTGTNACGASTTTTAC TN and AWGCTACSTGGTTGATCCTSCCAGN. PCR reaction mix of 50 µl final volume contained: 50 ng sample gDNA, 100 ng forward primer, 100 ng reverse primer, 2 µl dNTP's mixture (10 mm), 5 µl 10X Taq polymerase buffer, 3 U Taq polymerase enzyme and PCR grade water to make up the volume. Amplified PCR product was sequenced by using ABI3730xl genetic analyzer (Amnion Biosciences Pvt. Ltd. Bangalore, India). The sequencing result was submitted to the Genbank National Centre for Biotechnology Information (NCBI) database.

Biodegradation of Atrazine in Mineral Medium

To study the degradation of atrazine in liquid medium, Erlenmeyer flask containing 100 ml of M1 medium spiked with 1500 mg L^{-1} of atrazine as the sole carbon source and inoculated with 1 ml of fungal spore suspension of JAV1 strain. The flasks were incubated at 30 °C on a rotary shaker at 120 rpm and samples were taken at regular time intervals. The removal of atrazine was determined by High Performance Liquid Chromatography (HPLC).

Analytical Methods

The liquid samples from atrazine degradation flask were recovered and centrifuged at 8000 rpm for 15 min. Atrazine residues were extracted from supernatant with equal volume of chloroform. Organic layer was evaporated and then the residues were dissolved in HPLC grade methanol. 0.22 µm filter membrane was used to filter the samples before injecting in HPLC. The isocratic mobile phase was composed of methanol:water (70:30, V:V), which was pumped through the column at a flow rate of 0.8 ml/min and run time of 25 min. Atrazine and its metabolite were detected at 230 nm (Wang et al., 2014).

Metabolite produced during atrazine degradation under aerobic condition was monitored by GC-MS. The sample from chloroform extraction of M1 medium was injected in GC-MS. Perkin Elmer Clarus 680 gas chromatographic instrument equipped with a mass spectrometer detector (Clarus 600 model) and an Elite-5MS (30.0 m, 0.25 mmID, 250 µm df) column was used. The carrier gas used was helium at a flow rate of 1 ml min^{-1}. The following temperature programme was used: initially the oven temperature was held at 60 °C for 2 min and then ramped from 10°C/min to 300 °C with hold time for 4 min, total run time 30 min. The temperature of the injector was maintained at 300 °C. The ion trap was operated at 70 eV with a scan range of m/z from 50 to 600. A sample of 1 µl was injected in split mode (10:1). The intermediate and end product was identified based on the Wiley registry of mass spectral data.

Infrared spectra of the atrazine parent compound and sample after fungal degradation were recorded at room temperature at a frequency range of 4000-400 cm^{-1} with FTIR. Spectrophotometer (8400 Shimadza, Japan with Hyper IR-1.7 Software for windows) with helium neon laser lamp as a source of IR radiations. Pressed pellet were prepared by grinding the extract samples with potassium bromide in motor with 1:100 ratio and immediately analyzed in the region of 4000-400 cm^{-1} at a resolution of 4 cm^{-1}.

Scanning Electron Microscopy (SEM)

Scanning electron microscopy was used to investigate the morphological changes in the surface of fungal mycelia before and after degradation of atrazine. Strain JAV1 treated with 1500 mg L^{-1} concentration of atrazine after six days of incubation were mounted on specimen stub with double-sided adhesive tape and coated with gold in a coater and examined under SEM (HITACHI, Japan).

Results and Discussion

Many researchers have reported enrichment techniques with carbon sources as a standard approach for isolating microbes for pesticide degradation (Umar et al., 2012; Solomon et al., 2013). In the present study, a selective enrichment method was used to isolate atrazine degrading fungal strains from paddy field and three distinct strains were obtained. Solomon et al. (2013) have achieved enrichment technique for isolating nine bacterial strains which have the greater ability to degrade atrazine. Gradient plate assay was applied to screen the potential strain for highest tolerance to atrazine and the growth performance was recorded as the length of fungal growth (in cm) across the atrazine gradient. Among the three isolates, strain JAV1 shows growth of >5 cm on gradient plate which was further assessed using broth assay. The minimum inhibitory concentration of atrazine was determined for the JAV1 strain in accordance with gradient plate assay. The isolate JAV1 showed luxuriant growth up to 1600 mg L^{-1} of atrazine, and hence it was selected for further biodegradation.

The molecular characterization based on 18S rRNA sequence analysis was used to identify JAV1 strain. BLAST results of the 18S rRNA gene sequence of JAV1 strain exhibiting close relationship with 99% similarity to that of the 18S rRNA gene of *Aspergillus alliaceus*. Multiple sequence alignments and phylogenetic tree (Figure 1) revealed that the strain JAV1 cluster with *Aspergillus* sp. Therefore, the JAV1 isolate was designated as *Aspergillus alliaceus* JAV1 and the sequence result was submitted to GenBank NCBI database and accession number KT148626 was obtained.

Growth kinetics of *Aspergillus alliaceus* JAV1 in the presence and absence of 1500 mg L^{-1} atrazine is depicted in Figure 2. A visible increase of mycelial mass was observed with time which indicated the metabolism of atrazine *Aspergillus alliaceus* JAV1. Initially, the growth was found to be suppressed in the presence of atrazine but after the acclimation to atrazine, the fungus showed a higher growth rate. Moreover, the amount of biomass produced in the medium containing atrazine was much higher as compared to the growth in the absence of atrazine. This could be due to the availability of an additional carbon source upon degradation of atrazine in the medium.

The JAV1 strain in the liquid medium containing atrazine as the sole organic compound revealed that fungal strain was capable of utilizing applied

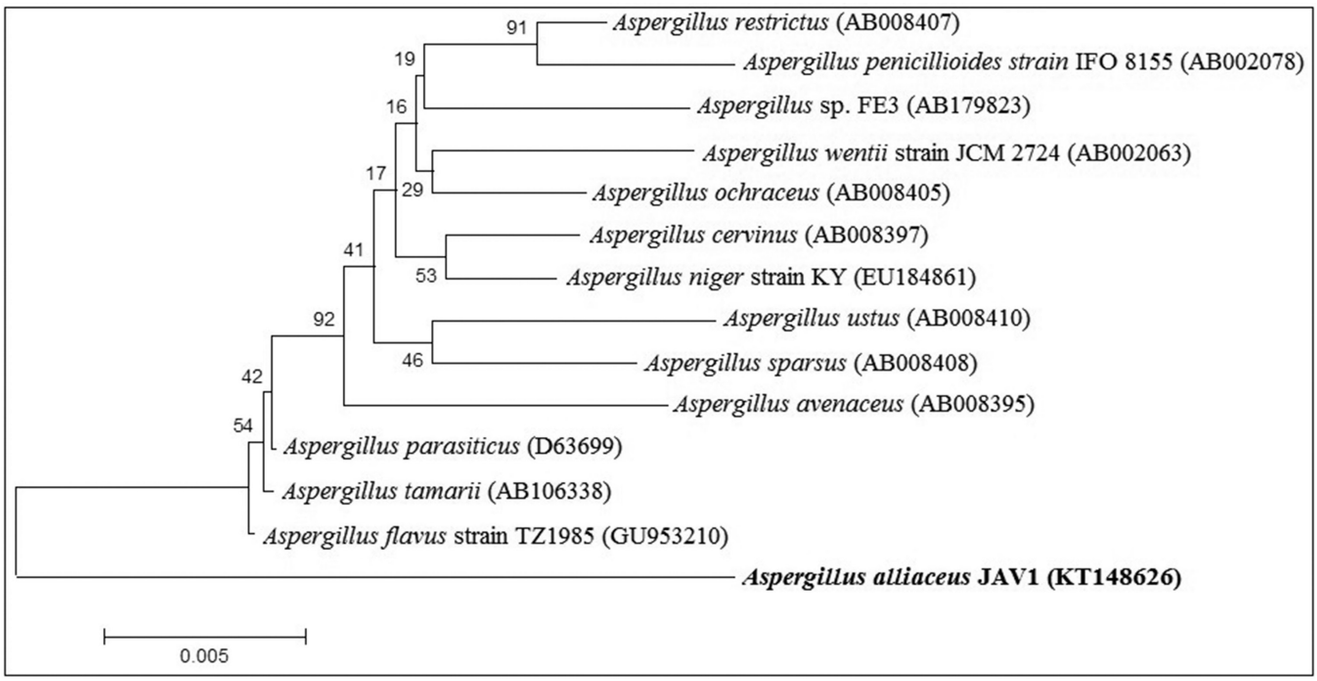

Figure 1: The neighbour-joining tree was constructed using MEGA5 and a bootstrap analysis was performed with 1000 replicates. Phylogenetic analysis of 18S rRNA sequence of *Aspergillus alliaceus* JAV1 (KT148626).

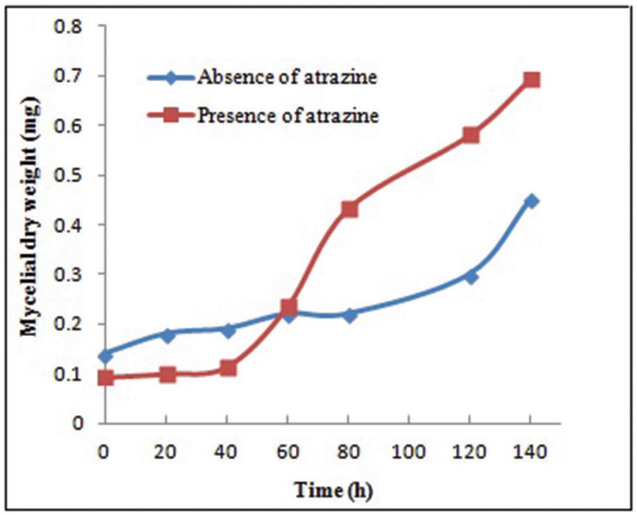

Figure 2: Growth pattern of strain JAV1 in the presence and absence of 1500 mgL^{-1} concentration of atrazine.

herbicide as a source of carbon and energy to grow, and confirmed the degradation of atrazine. Standard HPLC chromatogram of atrazine is shown in Figure 3. *Aspergillus alliaceus* JAV1 degraded 1500 mg L^{-1} of atrazine in the aqueous medium in six days of incubation (Figure 4). This was confirmed with the standard HPLC peaks of atrazine. Our findings are supported by Azizullah et al. (2014) who observed biodegradation of atrazine by both in bacteria and fungus; out of 12 fungal strains, *Aspergillus fumigates* (55.24%) showed highest degradation rate and in bacterial isolates, *Streptococcus* sp. showed 62.77% rate of degradation. Solomon et al. (2013) have reported the strain *Enterobacter cloacae* strain JS08.Deg01 degraded 90% of atrazine within three days of incubation. Topp (2001) reported biodegradation ability of *Pseudomonas* sp. Fungus *Phanerochaete chyrososporium* ME446 degraded 74%

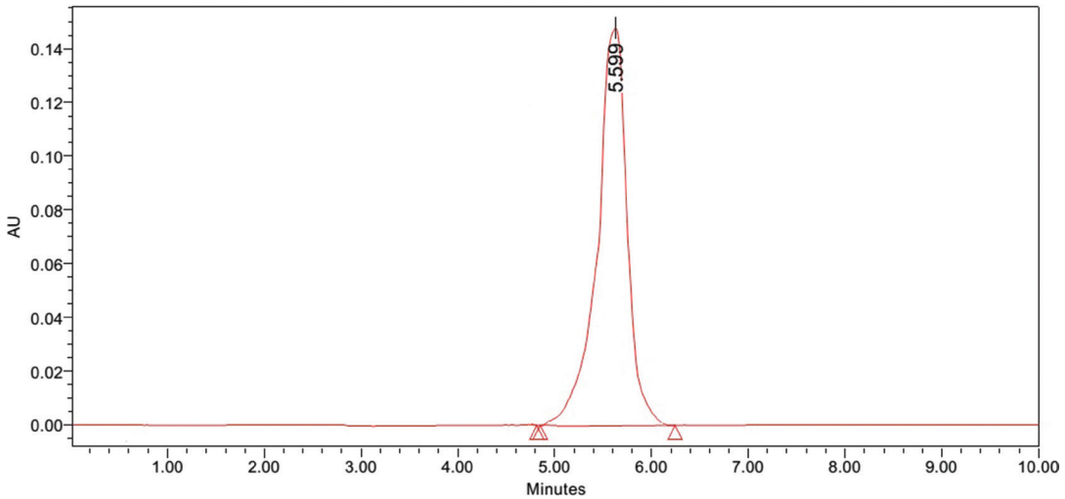

Figure 3: HPLC chromatogram of atrazine at standard condition.

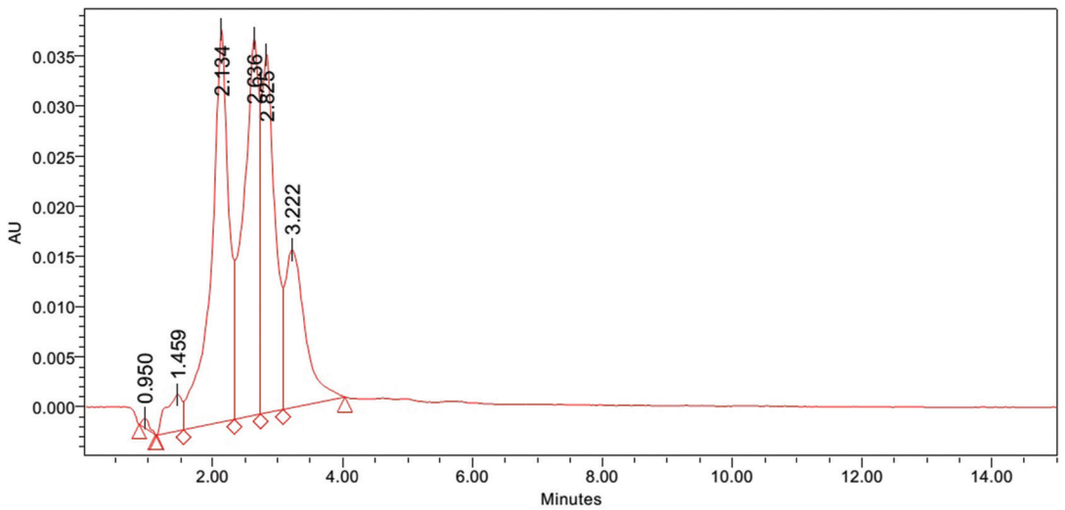

Figure 4: HPLC chromatogram of degradation of atrazine in aqueous medium by *Aspergillus alliaceus* JAV1.

of atrazine in 14 days of incubation period (Doruk et al., 2012). Mougin et al. (1994) reported that *Phanerochaete chyrososporium* decreased 48% of initial concentration of 2 µM atrazine in four days on incubation. Zhang et al. (2009) used *Microbacterium* sp. and *Arthrobacter* sp. for the removal of atrazine with degradation rate of 77.7% and 65.5%, respectively. Ojo (2007) reported the atrazine degrading activity of the bacteria as well as fungi and found that in mineralization the hydrocarbon component of atrazine is utilized by the microbial consortium.

The degradation products of atrazine in the culture extracts were analyzed by GC-MS (Figure 5). After six days of incubation, final metabolites of atrazine was identified as Trans-2-Hydroxycinnamic Acid, Trimethylsilyl Ether, Trimethylsilyl. The FTIR studies indicate the various structural changes of atrazine. FTIR spectrum of extracted metabolites after complete degradation of atrazine by *Aspergillus alliaceus* JAV1 clearly confirmed the degradation of atrazine (Figure 6). The infrared spectrum of atrazine degraded sample showed a band at 3005.1 cm^{-1} corresponding to C=C-H asymmetric stretch. The two bands were presented at 1737.86 and 1535.34 cm^{-1}, which are the characteristics of C=O stretch, N-H bend. The peak positions at 1365.60 cm^{-1}, 1274.95 cm^{-1}, 1217.95 cm^{-1} and 748.38 cm^{-1} are N=O bend, C–O stretch, C=O stretch and C-H bending, respectively. In SEM analysis, Figure 7 depicts the rough and irregular surface of fungal spores after degradation of 1500 mg L^{-1} concentration of atrazine.

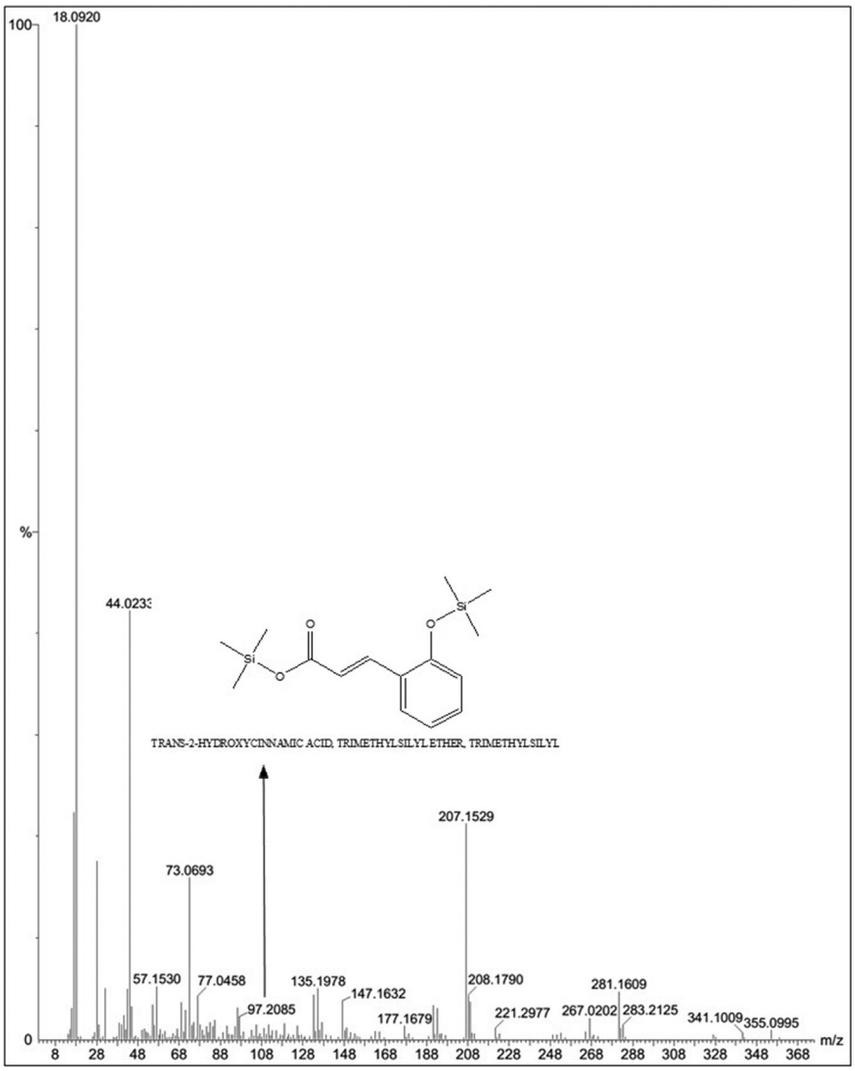

Figure 5: Mass spectrometry of trans-2-Hydroxycinnamic Acid, Trimethylsilyl Ether, Trimethylsilyl metabolite appeared during the degradation of atrazine.

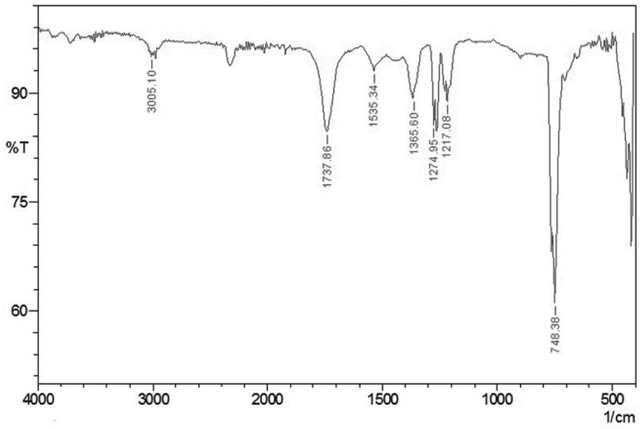

Figure 6. FTIR spectrum of biodegradation of atrazine by strain JAV1 in aqueous medium.

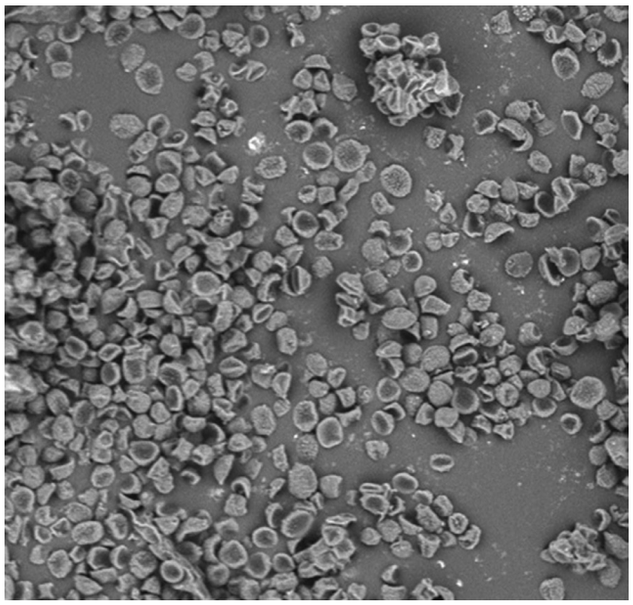

Figure 7: SEM micrograph of *Aspergillus alliaceus* JAV1 after degradation of 1500 mg L^{-1} of atrazine.

Conclusion

In the present investigation atrazine degrading strain *Aspergillus alliaceus* strain JAV1 was isolated and characterized. Strain JAV1 could utilize atrazine as a carbon source and it showed a very high efficiency of atrazine degradation over a short time period. On the basis of these results, *Aspergillus alliaceus* strain JAV1 has the potential to be used in the biodegradation of atrazine and can be used for environmental cleanup.

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