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Potential of Thermophilic Fungus *Rhizomucor pusillus* NRRL 28626 in Biotransformation of Antihelmintic Drug Albendazole

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Abstract In the present investigation, thermophilic fungus *Rhizomucor pusillus* was used to study biotransformation of antihelmintic drug albendazole to produce its active metabolite, albendazole sulfoxide and novel metabolites of commercial interest. A two-stage fermentation procedure was followed for biotransformation of albendazole. The transformation was identified and structures were confirmed by high-performance liquid chromatography and liquid chromatography-tandem mass spectrometry analysis. Four metabolites albendazole sulfoxide, the active metabolite, albendazole sulfone, *N*-methyl metabolite of albendazole sulfoxide, and a novel metabolite were produced. The study demonstrates the biotransformation ability of thermophilic fungus *R. pusillus* NRRL28626 in the production of, the active metabolite of albendazole which has industrial and economic importance, other metabolites and a novel metabolite in an ecofriendly way.

Keywords Biotransformation · ABZ · ABSO · LC-MS/MS · *Rhizomucor pusillus* NRRL28626

Introduction

A large group of wide spectrum, high-efficiency anthelmintics, such as the benzimidazole 2-carbamates, is marketed worldwide for the control of helminthiasis. It has been reported that benzimidazole anthelmintics with a sulfide group are the most active against intestinal nematodes in humans, as well as in animals [1–3]. Albendazole is a benzimidazole carbamate with a broad antiparasitic spectrum [4], used in treatment of ascariasis; trichuriasis; enterobiasis; and hookworm infections, capillariasis, gnathostomiasis, and trichostrongyliasis; the cestode infections hydatidosis, taeniasis, and neurocysticercosis; and the tissue nematode's cutaneous larval migrans, toxicariasis, trichinisis, and filariasis (in combination with other anthelmintics). The drug is thought to act by binding to ß-tubulin, thereby inhibiting its polymerization and thus blocking nuclear division and other MTs

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function of the pathogen. Absorption of albendazole by the gastrointestinal tract is poor which results in reduced systemic availability and efficacy after oral administration. The primary metabolite, albendazole sulfoxide is active metabolite and albendazole efficacy is attributed to this metabolite [5]. Albendazole sulfoxide undergoes bioconversion to albendazole sulfone, which is pharmacologically inactive.

Metabolites of albendazole are commercially available but not affordable, although there are reports in the pertinent literature for the synthesis of albendazole sulfoxide, albendazole sulfone in addition to the general methods of *S*-oxidation, these are not easy to carry out, or fail, due to insolubility problems in albendazole which often leads to mixtures of sulfoxides and sulfones that are difficult to separate [6].

Thermophilic fungi are a small assemblage in eukaryotes having a unique mechanism of growing at elevated temperature extending up to 60–62 °C. They are the chief components of the microflora that develops in heaped masses of plant material, piles of agricultural and forestry products, and other accumulations of organic matter wherein the warm, humid, and aerobic environment provides the basic conditions for their development [7, 8]. They constitute a heterogeneous physiological group of various genera in the Phycomycetes, Ascomycetes, Fungi imperfecti, and Mycelia sterilia [9], as the only representative of eukaryotic organisms that can grow at temperatures above 45 °C. They are also valuable experimental systems for investigations of mechanisms that allow growth at moderately high temperature.

Biocatalysis is well established as a key technology in the production of chiral intermediates and active pharmaceutical ingredients. Extremophilic organisms are often proposed as sources of industrially relevant enzymes, not only because of their innate properties but also the diversity of the Kingdom Archaea to which many belong [10]. However, thermophilic fungi are potential sources of enzymes with scientific and commercial interests. They offer robust catalyst alternatives, able to withstand the often relatively harsh conditions of industrial processing. The main advantages of performing biocatalytic processes at higher temperatures are reduced risk of microbial contamination, lower viscosity of medium, improved transfer rates, and improved solubility of substrates [11, 12]. Apart from high temperature, they are also known to withstand denaturants of extremely acidic and alkaline conditions. These enzymes are highly specific and thus have considerable potential for many industrial applications. The use of such enzymes in maximizing reactions accomplished in the food and paper industry, detergents, drugs, toxic wastes removal, and drilling for oil is being studied extensively [13].

Owing to immense potential of thermophilic biocatalysts and very limited reports on thermophilic biotransformations, an attempt was made to study biotransformation of albendazole to produce the active metabolite albendazole sulfoxide and novel metabolites of commercial interest if any in an ecofriendly and economical way.

Materials and Methods

Microorganism and Media

The thermophilic fungus *Rhizomucor pusillus* NRRL28626 was collected from culture deposit of Microbiology Research Laboratory, Kakatiya University. The organisms were maintained on yeast extract starch agar (starch, 30 g; yeast extract, 5 g; $MgSO_4.7H_2O$, 0.5 g; KH_2PO4, 1 g; agar–agar, 20 g and distilled water 1,000 ml). The pH of medium was adjusted to 6.0 with 0.1 N HCl or 0.1 N NaOH and stored at 4 °C and subcultured for every 3 months.

Chemicals

Albendazole was gifted by GlaxoSmithKline, Mumbai, India. Methanol and acetonitrile were of high-performance liquid chromatography (HPLC) grade obtained from Ranbaxy, New Delhi, India. Peptone, yeast extract, glucose, and all other chemicals were obtained from Himedia, Mumbai, India.

Fermentation Procedure

Microbial transformation was performed using a two-stage fermentation process. The first-stage preculturing was initiated by inoculating a 250-ml culture flask consisting of 50 ml of sterile liquid broth with a loopful of spores (approximately 20 spores) obtained from a freshly growing agar slant. The liquid broth used contained (per liter) glucose (20 g), peptone (5 g), yeast extract (5 g), K_2HPO_4 (5 g), and sodium chloride (5 g). The pH of the broth was adjusted to 6.0 with 0.1 N HCl or 0.1 N NaOH. The flasks were incubated at 120 rpm and 45 °C for 48 h. After incubation for 48 h second-stage cultures were initiated in the same media using an inoculum of 1 ml of first-stage culture per 50 ml of medium in a 250 ml culture flask. The cultures were incubated for 24 h and albendazole (2 mg) was dissolved in 200 μl dimethyl sulfoxide and added. The flasks were incubated under similar conditions for 4 days. Two kinds of controls were maintained simultaneously with the biotransformation procedure. Culture controls consisted of a fermentation blank in which the microorganism was grown under identical conditions and no substrate was added. The substrate controls were prepared by adding albendazole to sterile medium and incubated without microorganism to determine chemical decomposition or spontaneous transformation of albendazole under experimental conditions.

Isolation of Metabolites

After incubation period, the contents of the flasks were transferred to separating flask and extracted with three volumes of ethyl acetate; the combined organic extracts were evaporated using a rotary vacuum evaporator and dried over a bed of sodium sulfate. The resultant residues were reconstituted with 1 ml of methanol and analyzed by HPLC and liquid chromatography-tandem mass spectrometry (LC-MS/MS) for the presence and identification of metabolites.

Analytical Methods

HPLC was performed as described by Prasad et al. [14] using Water's PDA 2595 system by injecting 100 μl of sample into the syringe-loading sample injector. The column used was Water's, Symmetry shield, C18, 4.6×250 mm, 5 μm. The metabolites were eluted isocratically with a mixture of acetonitrile–water (pH adjusted to 3.0 with orthophosphoric acid) in the ratio of 1:9 at a flow rate of 1 ml/min with 290 nm using a photodiode array detector. The metabolite peaks were identified based on the similarity in the UV spectra of albendazole with that of metabolites.

LC-MS/MS analysis was carried out using system MDS SCIEX API-4000, Q-TRAP, Canada with MS/MS API-4000, Q-Trap detector. Chromatographic separation was achieved

by Waters column C18, 25×0.46 cm, 5 μm, and a mobile phase consisting of acetonitrile and water (pH adjusted to 3.0 with formic acid) in a 1/9 ratio. The ESI detection was set to positive mode. A temperature of 300 °C and scan range of 50–600 was set for the analysis. The data was acquired and processed by means of Analyst 1.4.2 software. The transformed compounds were identified from the masses of the fragmentation products obtained.

Results and Discussion

In the present study, thermophilic fungus *R. pusillus* NRRL 28626 was employed to biotransform antihelmintic drug albendazole. HPLC analysis of the extract showed that the fungus was able to transform albendazole into four metabolites viz. albendazole sulfoxide (M1) the active metabolite, albendazole sulfone (M2), *N*-methyl metabolite of albendazole sulfoxide (M3), and a novel metabolite (M4). The production of metabolites by the fungus may be due to induction of enzymes by substrate albendazole for its transformation or may be due to natural production of enzymes.

Metabolites of albendazole were identified based on formation of new peaks in HPLC analysis (Fig. 1) and absence of these peaks in drug as well as culture controls. The structure elucidation of the metabolites was carried out from the *m*/*z* values of the fragmentation ions in LCMS/MS analysis (Fig. 2) and HPLC retention times. The LCMS/MS data of albendazole and the metabolites synthesized by thermophilic fungus *R. pusillus* NRRL 28626 was presented in Table 1.

Mass spectrometric analysis of the metabolite M1 eluted at 8.4 min in LCMS/MS analysis showed a molecular ion peak at *m*/*z* 282, that is, 16 units higher to the parent compound, albendazole indicating addition of single oxygen atom to sulfur group of albendazole, resulted in formation of M1. This is an active metabolite having antihelmintic activity, and albendazole efficacy is attributed to this metabolite [5]. Similar type of sulfoxidation reactions employing mesophilic fungi was reported by many researchers [15,

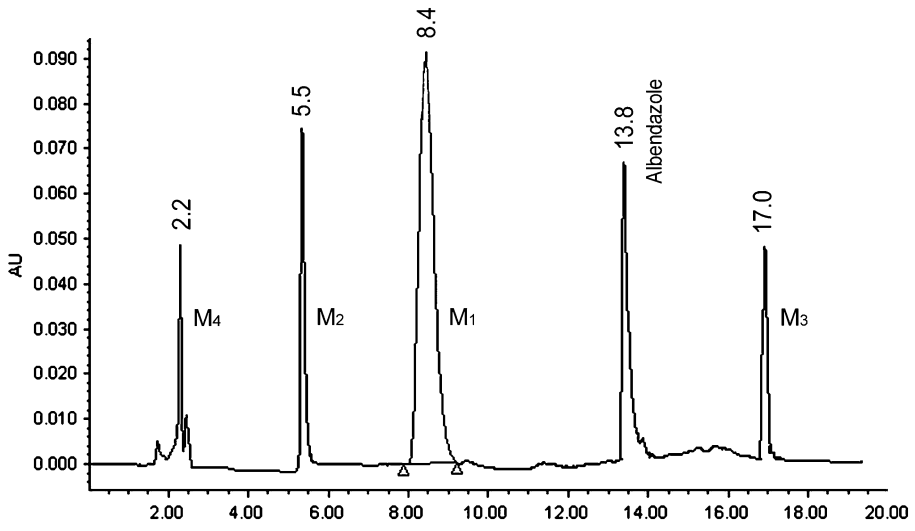


Fig. 1 HPLC chromatogram showing transformed compounds of albendazole obtained in culture medium of *R. pusillus* NRRL 28626

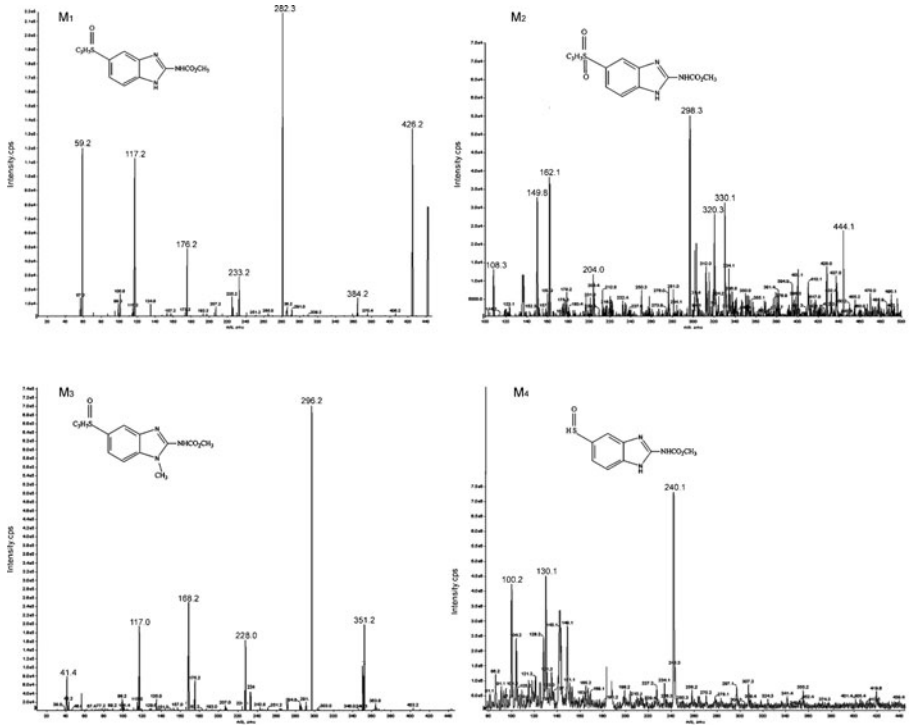


Fig. 2 LC-MS/MS spectra of metabolites detected in albendazole fed culture broth of *R. pusillus* NRRL28626

16, 17]. For metabolite M2, the molecular ion peak at *m/z* 298 (32 units higher to albendazole) was recorded, indicating addition of two oxygen atoms to sulfur group of albendazole which results in the formation of M2. This type of sequential sulfoxidation and sulfonation reactions was reported by Gai et al. [17] using *Sphingomonas* sp. The formation of metabolites M1and M2 was also recorded in animal tissues studied by Gyurik et al. [1]. The flavin-containing monooxygenases and cytochrome P-450 (CYP, mainly CYP3A in rat) appear to mediate the conversion of albendazole to albendazole sulfoxide, whereas the biotransformation of albendazole sulfoxide to albendazole sulfone is influenced by CYP only (CYP1A in rat) [14].

The metabolite M3, eluted at 17.0 min in HPLC has shown a molecular ion peak at *m/z* 296 indicating that *R. pusillus* has introduced a methyl group into structure of albendazole sulfoxide, that is, there is an addition of CH2 (14 units) to the metabolite M1 resulted in

Table 1 LCMS/MS data of albendazole and its metabolites produced by *R. pusillus* NRRL28626

Metabolites	Rt (Min)	*m/z* Values	Fragments *m/z* values	Predicted molecular formulae	Predicted reaction
Albendazole	13.8	265.09	234.1	$C_{12}H_{15}N_3O_2S$	–
M1	8.4	282.3	234.1,240.1	$C_{12}H_{15}N_3O_3S$	Sulfoxidation
M2	5.5	298.3	234.3,266.2	$C_{12}H_{15}N_3O_4S$	Sulfonation
M3	17.0	296.2	234.2,266.0	$C_{13}H_{17}N_3O_3S$	*N*-Methylation
M4	2.2	240.1	234.1	$C_9H_9N_3O3S$	Depropylation

Fig. 3 LC-MS/MS fragmentation pattern of metabolites M1 and M2 detected in albendazole fed culture broth of *R. pusillus* NRRL 28626

formation of *N*-methyl metabolite of M3. The fragmentation pattern of the metabolite M3 indicated that there was a methylation of N atom on the five-membered ring. The enzyme *S*-adenosyl methionine-dependent methyl transferases are well known in fungi, bacteria, and *Streptomyces* and are found to catalyze methylation reactions [18]. Methylation reactions employing mesophilic microbes viz. *Cunninghamella elegans*, *Streptomyces griseus*, and *Bruveria* was reported by Park et al. [19], Hosny et al. [20], and Costa et al. [21]. Biological activity studies of this metabolite are to be performed.

Formation of M1, M2, and *N*-methyl metabolite of M3 was also recorded in mesophilic fungi and bacteria by Prasad et al. [14, 22] which clearly states existence of similar type of enzyme system in mesophilic fungi, bacteria and thermophilic fungi studied.

Similarly, the metabolite M4, eluted at 2.2 min in HPLC analysis has shown a molecular ion peak at *m*/*z* 240. It was assumed that the metabolite M4 has produced from dealkylation of albendazole sulfoxide. The metabolite M1 has undergone depropylation to produce the metabolite M4. The fragmentation of M4 confirms this production path of the metabolite. Further investigations are needed to find the biological activity of this metabolite. This type of dealkylation reaction was also reported in fungi and mammals [23].

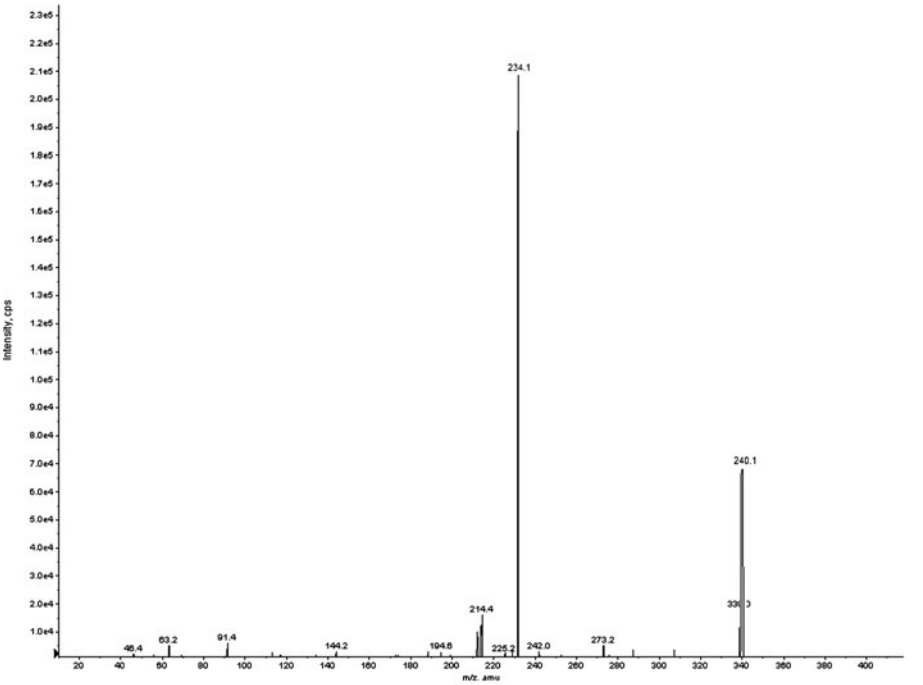
The fragments detected in all the four metabolites were found to be similar with the drug albendazole. The major fragments observed were 234.1, 266.2, and 240.1 (Fig. 3). Among all the metabolites produced, M1 was recorded in maximum quantity. The pathway of the metabolite formation is shown in Fig. 4.

Albendazole is a broad antihelmintic compound with a drawback of poor absorption from gastrointestinal tract which results in reduced systemic availability and efficacy after oral administration. Albendazole sulfoxide is an active metabolite. Hence, an injectable formulation of albendazole sulfoxide has been developed, exploiting its slightly greater solubility in water than that of other equally potent benzimidazole methyl carbamates. Interestingly in the present investigation, thermophilic fungus *R. pusillus* NRRL 28626 could produce the active metabolite (M1) in large quantities compared to all other metabolites. Hence, further investigations are needed to inhibit production of other metabolites for commercial exploitation of M1 which is in progress in our laboratory.

Most of the mesophilic fungi possess enzymes similar to mammals for detoxification of xenobiotics. Thus, they are useful as microbial models of mammalian drug metabolism and as a source for drug metabolites. The use of filamentous fungus belonging to genus *Cunninghamella*, as a model of mammalian metabolism has been well documented [24–26]. The use of microbial simulation of mammalian metabolism also gives an idea on the mechanism of action, toxicity, and pharmacological activity of the drugs and thus helps in discovery of new drug molecules [27]. In the present study, the thermophilic fungi *R. pusillus* NRRL 28626 biotransformed albendazole and produced two major metabolites M1 and M2 reported in mammals. Hence, this fungus is also suitable as microbial model.

Conclusion

The present investigation states that thermophilic fungi *R. pusillus* NRRL 26826 can be employed in production of M1 which is of commercial interest in short time, in ecofriendly, and in economical way. As similar metabolites were produced by the fungus *R. pusillus* NRRL 28626 compared to mammals, this fungus can be used as a microbial model in


Intensity, cps
m/z, amu
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63.2
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273.2
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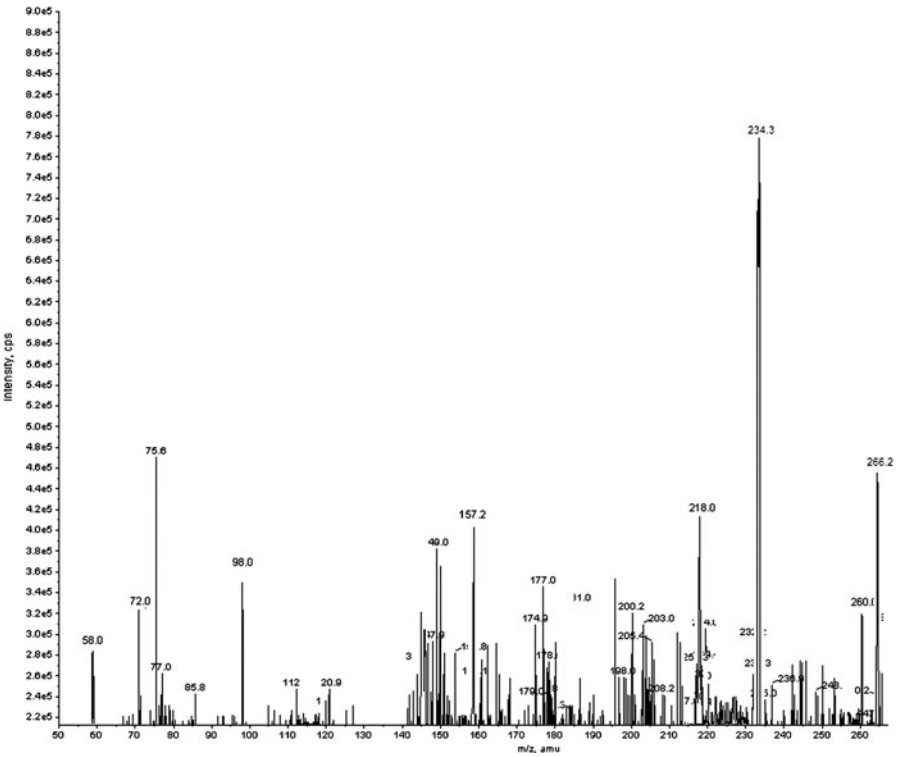
intensity, cps
m/z, amu
234.3
266.2
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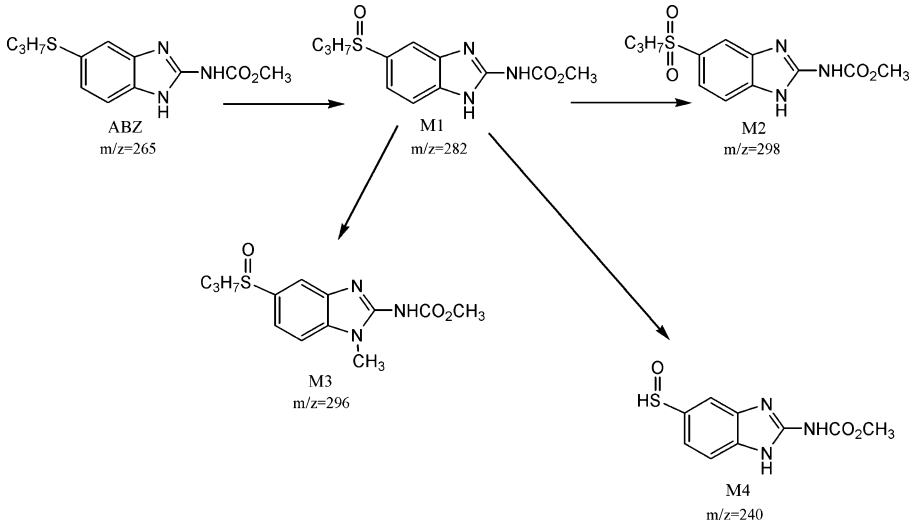

Fig. 4 Proposed pathway of albendazole in culture broth of *R. pusillus* NRRL28626

predicting mammalian drug metabolism. Further investigations are also needed to find the biological activity studies of the M4 produced.

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