Biotransformation of albendazole by *Cunninghamella blakesleeana*: influence of incubation time, media, vitamins and solvents

Shyam Prasad Gurram*, Narasimha Rao Kollu, Girisham Sivadevuni, Madhusudan Reddy Solipuram

Department of Microbiology, Kakatiya University, Warangal 506009, AP, India

Abstract
The present investigation was aimed at studying the effect of incubation period, media, vitamins and solvents on biotransformation of albendazole by *Cunninghamella blakesleeana*. The transformation was evaluated and identified by high performance liquid chromatography (HPLC) and the structures of the transformed products were assigned by liquid chromatography-tandem mass spectrometry (LC/MS/MS) analysis. The fungus was found to metabolize albendazole into albendazole sulfoxide (M_1), albendazole sulfone (M_2) and the N-methyl metabolite of albendazole sulfoxide (M_3). Incubation period was found to influence the biotransformation significantly; 4 days was found to be optimum, but the effect was neither linear nor progressive. There was a significant effect of medium on the extent of biotransformation, with the highest substrate depletion produced by the glucose broth. The media also influenced qualitative metabolite formation. Presence of thiamine in the glucose media produced the maximum extent of transformation when compared to other vitamins studied. Dimethylformamide produced a higher extent of biotransformation. The fermentor was found to produce an increased level of biotransformation as compared to that obtained in shake flasks.
Keywords: *Albendazole; Fungal biotransformation; Fermentor; Media; Solvents; Vitamins*

INTRODUCTION

Albendazole is a benzimidazole carbamate with a broad anti-parasitic spectrum (Theodorides, *et al*., 1976). In general, most ascariasis, trichuriasis, enterobiasis and hookworm infections can be successfully treated with a single dose of albendazole and strongyloidiasis with multiple doses of albendazole. Albendazole is also used in the treatment of capillariasis, gnathostomiasis and trichostrongyliasis, the cestode infections hydatidosis, taeniasis and neurocysticercosis and the tissue nematode's cutaneous larva migrans, toxicariasis, trichinisis and filariasis (in combination with other anthelmintics) (Menon, 2002). 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, also has anthelmintic activity and albendazole efficacy is attributed to this metabolite (Hussar, 1997; Marriner *et al*., 1986; Bogan and Marriner, 1984). 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. After subcutaneous injection, albendazole sulfoxide is widely distributed from blood to other tissues, especially the gastrointestinal tract (Lanusse *et al*., 1998), so that plasma concentrations closely reflect those in fluids and tissues where the target parasite may be located (Lanusse and Prichard, 1993).

Biotransformation is a process involving the use of biological agents as catalysts to perform transformations of chemical compounds. Biotransformation processes that involve enzymatic or microbial biocatalysts, when compared to their chemical counterparts, offer the advantages of high regioselectivity, stereospecificity and mild operating conditions. Microbial

Correspondence to:* **Shyam Prasad Gurram, Ph.D.
Tel: +91 970 503 7655; Fax: +91 870 245 5144
E-mail: shyamprasad1919@yahoo.com

transformation is one of the most attractive approaches for introducing functional groups into various positions of organic compounds (Smith, 1984). A previous reports indicated that the filamentous fungus *C. blakesleeana* transformed albendazole into three metabolites *viz*., albendazole sulfoxide, albendazole sulfone and the N-methyl metabolite of albendazole sulfoxide, and the effects of various parameters like pH, temperature, substrate concentration, carbon source, nitrogen source and glucose concentration were significant (Prasad *et al*., 2008a; Prasad *et al*., 2008b). The objective of the present investigation is to study the effect of incubation period, various media, vitamins and solvents on the transformation of albendazole by *C. blakesleeana*. The biotransformation was also attempted in the fermentor in order to study the scale-up capability of the system.

MATERIALS AND METHODS

Chemicals and microorganism: Albendazole was gifted by GlaxoSmithKline, Mumbai, India. Acetonitrile was of high performance liquid chromatography (HPLC) grade, obtained from Ranbaxy Laboratories Ltd., New Delhi, India. Peptone, yeast extract, potato dextrose broth, glucose and all other chemicals of highest available purity were obtained from Himedia Labs, Mumbai, India. *C. blakesleeana* NCIM 687 was procured from the National Collection of Industrial Microorganisms (NCIM), Pune, India. Stock cultures were maintained on potato dextrose agar slants (PDA) at 4°C and subcultured every 3 months.

Biotransformation in different media: The present study was performed to determine a suitable medium for maximum transformation of albendazole by *C. blakesleeana* NCIM 687. Biotransformation was performed using a two-stage fermentation protocol while running suitable controls. In the first stage, the fermentation was initiated by inoculating 50 ml of liquid broth in a 250 ml culture flask. Glucose media used in the earlier studies (Prasad *et al*., 2008a; Prasad *et al*., 2008b) was taken as a control. The compositions (per liter) of the media used in the study are given below:

Asthana and Hawker's A broth (glucose, 10 g; KNO_3, 3.5 g; KH_2PO_4, 1.75 g and $MgSO_4.7H_2O$, 0.75 g), Czapek-Dox broth ($NaNO_3$, 2 g; KH_2PO_4, 1 g; $MgSO_4.7H_2O$, 0.5 g; KCl, 0.5 g, $FeSO_4$, 0.01 g and sucrose, 30 g), glucose broth (glucose, 20 g; yeast extract, 5 g; peptone, 5 g; NaCl, 5 g and K_2HPO_4, 5 g), Glucose asparagine medium (glucose, 20 g; asparagine, 5 g; KH_2PO_4, 3.4 g; $MgSO_4.7H_2O$, 1.9 g and NaCl, 0.01 g), malt extract, glucose, peptone (MGP) broth (glucose, 20 g; malt extract, 20 g and peptone, 1 g), nutrient broth (peptone, 5 g; beef extract, 3 g and NaCl, 5 g), rice flour broth (rice flour, 40 g; sucrose 30 g and yeast extract, 1 g), Richard's broth (KNO_3, 10 g; KH_2PO_4, 5 g; $MgSO_4.7H_20$, 2.5 g; sucrose, 30 g and $FeCl_2$, 0.02 g), Singh and Wood medium (glucose, 5 g; asparagine, 4 g; KH_2PO_4, 1 g; $MgSO_4.7H_2O$, 0.75 g and pectin, 10 g), sucrose magnesium sulfate, potassium nitrate, yeast extract (SMKY) broth (sucrose, 20 g; $MgSO_4.7H_2O$, 0.5 g; KNO_3, 3 g and yeast extract, 7 g) and yeast extract, sucrose (YES) broth (yeast extract, 20 g and sucrose, 40 g).

Specified quantities of the media ingredients were dissolved in distilled water, pH was adjusted to 6 with either 0.1N HCl or 0.1N NaOH and sterilized by autoclaving. The sterilized media were inoculated with a loop of spores (approximately 20 spores) obtained from freshly grown PDA slants. Spore concentration was evaluated by using a Neubauer's chamber (haemocytometer) following classical procedure.The flasks were incubated at 120 rpm and 28°C for 48 h. Second stage cultures were initiated in the same media using inocula (of 1 ml) from the first stage culture per 20 ml of medium in a 100 ml culture flask. The second stage cultures were incubated for 24 h under similar conditions. Two mg of albendazole (in 100 µl of dimethylformamide) was added to the culture flasks and the incubation was continued for another 5 days. Culture controls consisted of a fermentation blank in which the microorganism was grown under identical conditions and no substrate was added. Substrate controls were comprised of albendazole added to the sterile medium and incubated under similar conditions. Each group was studied in triplicate. At the end of the incubation period the generated fungal mat was separated from the culture broth and the latter was 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 mat was dried and weighed to find out the dry cell weight of the fungus. The resultant residues were analyzed by HPLC and LC/MS/MS for the presence and identification of metabolites.

Analysis: HPLC and LC/MS/MS analyses were performed according to the method described by Prasad *et*

al. (2008b). In the HPLC procedure, the samples were analyzed using an LC-10AT system (Shimadzu, Japan) by injecting 20 µl of the sample into the syringe-loading sample injector (Model 7725i, Rheodyne, USA). The column used was Wakosil II, C18, 250×4.6 mm and 5 µm (SGE, Australia). The mobile phase consisted of a mixture of acetonitrile-water (pH adjusted to 3.0 with orthophosphoric acid) at a ratio of 15:85. The analysis was performed isocratically at a flow rate of 1 ml/min and the analytes were detected at 290 nm using a photodiode array detector (Model SPD M10Avp, Shimadzu, Japan). LC/MS/MS analysis was carried out using a Waters system, column XTerra C18, 250×4.6 mm, 5 µm and a mobile phase consisting of acetonitrile and water (pH adjusted to 3.0 with formic acid) at a 15:85 ratio. The ESI detection was set to positive mode. A temperature of 300°C and scan range of 50-500 was set for the analysis. The transformed compounds were identified from the masses of the fragmentation products obtained.

Effect of vitamins: Influence of the presence of different vitamins *viz*., riboflavin, niacin, choline, folic acid, inositol, pantothenic acid, thiamine, pyridoxine and ascorbic acid added to the glucose broth before inoculation of the organism was studied to observe their effects on the extent of biotransformation. Each vitamin was studied at a concentration of 10 µg per culture flask (20 ml media). The pH of the media was adjusted to 6 with either 0.1 N NaOH or 0.1 N HCl and sterilized by autoclaving. The resultant media were used for biotransformation of albendazole by the fungus *C. blakesleeana* NCIM 687.

Effect of solvents and incubation period: Influence of dimethylformamide and dimethyl sulfoxide on bio-

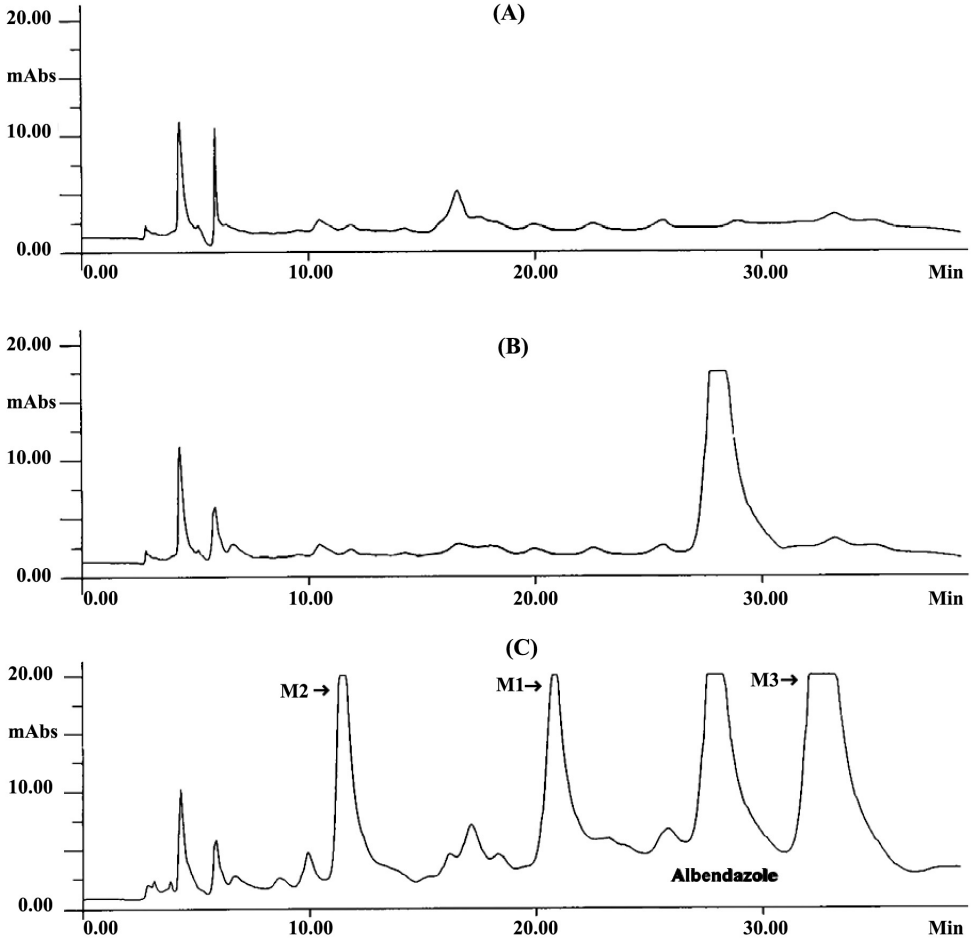

Figure 1. High performance liquid chromatography **(**HPLC) chromatograms showing A: Culture control B: Culture medium with standard albendazole C: albendazole fed culture of C. *blakesleeana*. (X-axis: time in minutes,Y-axis: millivolts).

transformation of albendazole was studied by dissolving 2 mg of the substrate in 100 µl of the solvent and adding to 20 ml of culture grown in glucose broth. The effect of incubation period on transformation of albendazole was also studied by analyzing the culture broth at the end of 2, 4, 6, 8, 10, 12, 14, 18 and 20 days.

Biotransformation of albendazole in a laboratory fermentor: Biotransformation was carried out in a 1 l stirred-jar benchtop Fermentor Murhopye Scientific co: Mysore, India with an operating volume of 800 ml. The biotransformation was performed at 30°C, 1.2 lpm aeration and a stirring speed of 200 rpm. The media used contained (per litre), glucose, 20 g; KNO_3, 3.5 g; yeast extract, 5.0 g and NaCl, 5 g. Ten percent of the inoculum was added in to the fermentation medium and incubated for 24 h. A 0.02% (w/v) sample of albendazole was added as a solution in dimethylformamide and incubated for a further 7 days. The contents were extracted with three volumes of ethyl acetate and analyzed for the presence of metabolites.

RESULTS

Identification of metabolites: Based on the HPLC and LC/MS/MS analyses, no additional metabolites were produced with any of the parameters studied. The transformation was identified based on observation of new peaks by HPLC analysis of samples from the test culture flasks (albendazole-fed flask) as compared to those of the control culture flasks (without addition of albendazole) (Fig. 1).The metabolites were identified based on LC/MS/MS analysis and previous reports (Guyrik *et al*.,1981). Mass spectrometric analysis of the metabolite M_1 showed a molecular ion at m/z 281 (an increase of 16 units with respect to the parent drug) indicating the addition of a single oxygen atom to albendazole, which results in the formation of albendazole sulfoxide (M_1). Another molecular ion was found at m/z 298 (an increase of 32 units) indicating the addition of two oxygen atoms to albendazole, which results in the formation of albendazole sulfone (M_2) (Fig. 2). The proposed metabolic pathway of albendazole in the culture broth of *C. blakesleana* is shown in Figure 3.

Effect of media: *C. blakesleeana* was specific in its choice of medium for the transformation of albendazole (Table 1). The glucose asparagine and glucose media followed by the rice flour and YES media supported the growth of the fungus to the maximum extent. In contrast, SMKY media followed by Sing and Wood and Richard's media produced minimal growth of the fungus. The growth of the fungus was estimated on a dry weight basis. Glucose broth was responsible for highest extent of biotransformation in

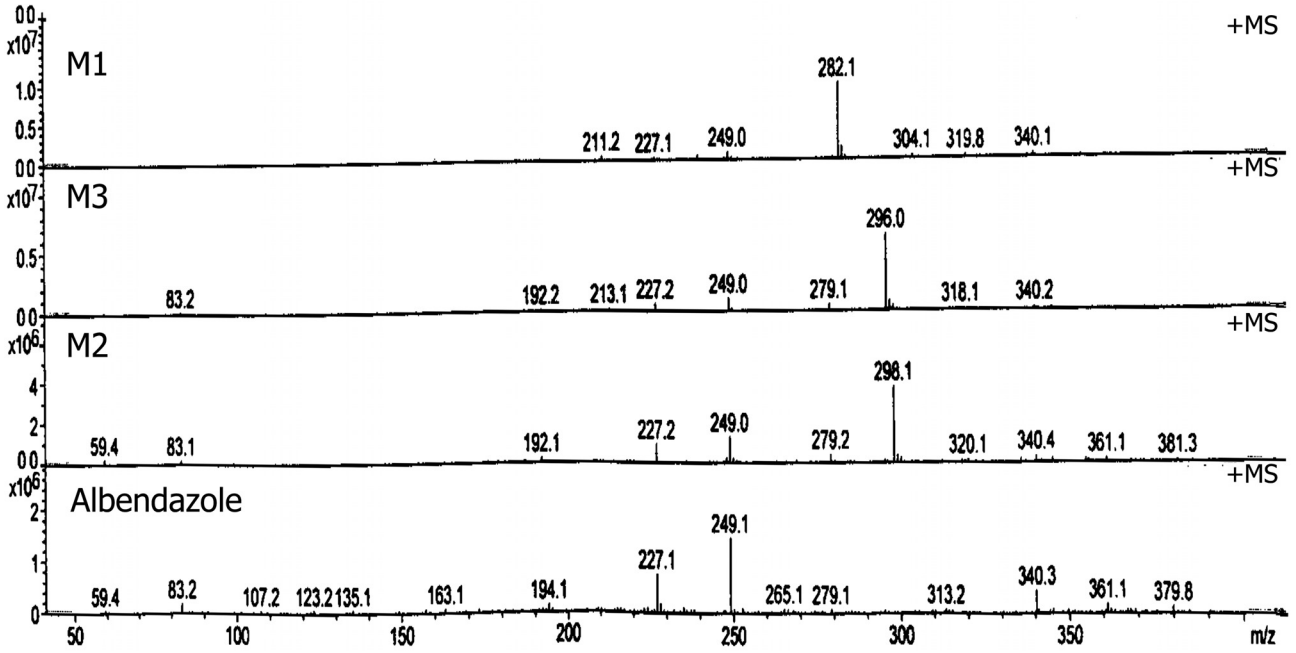

Figure 2. LC/MS/MS spectra of metabolites detected in albendazole fed culture broth of *C.blakesleeana* (X-axis: mass, Y-axis: intensity,LC-MS/MS:liquid chromatography-mass spectrometry/mass spectrometry).

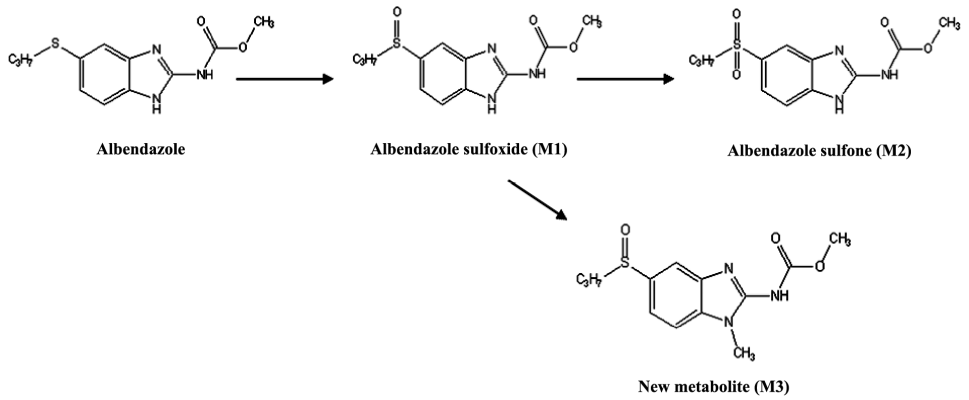

Figure 3. Proposed metabolic pathway of albendazole in *C. blakesleeana.*

terms of substrate depletion, while the MGP broth produced minimal biotransformation. SMKY, Singh and Wood and Richards broths produced intermittent quantities of biotransformation.

Among the media studied, SMKY and glucose broths supported the transformation of albendazole to all the three known metabolites *viz*., albendazole sulfoxide (M_1), albendazole sulfone (M_2) and the N-methyl metabolite of albendazole sulfoxide (M_3). Glucose medium induced transformation of albendazole to significant levels of M_3. However, in the present study, SMKY and Singh and Wood media were responsible for maximum production of M_1. M_2 was found to be high in Richard's broth whereas a maximum amount of M_3 was found to be produced in Glucose broth. The extent of biotransformation, in terms of substrate depletion, produced by various media is depicted in Figure 4.

Effect of vitamins: The effect of B-complex vitamins on the biotransformation of albendazole by *C. blakesleeana* was studied and the results obtained are presented in Table 2. Vitamins exerted significant influences on both transformation as well as biomass production. None of the vitamins supported the formation of M_3. Ascorbic acid, choline, folic acid, inositol and pantothenic acid suppressed the formation of M_2 while niacin, pyridoxine, riboflavin and thiamine pro-

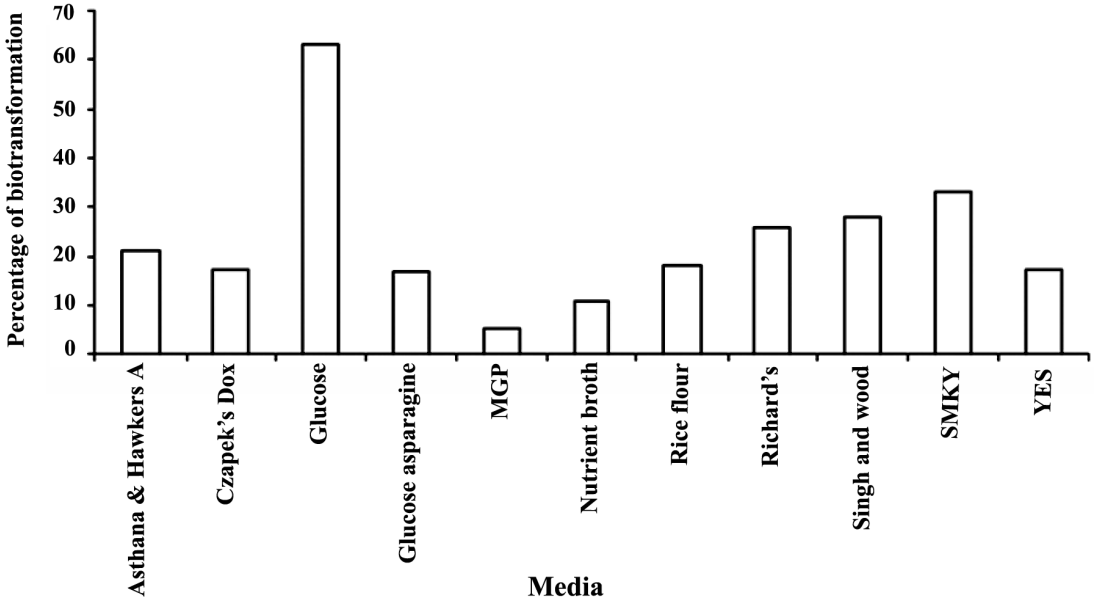

Figure 4. Biotransformation of albendazole by *C.blakesleeana* NCIM 687 in different media.

Table 1. Biotransformation of albendazole by C. blakesleeana NCIM 687 in different media.

Media	% of metabolites			Dry weight of cells (mg/ml)
	M_1	M_2	M_3	
Asthana and Hawkers A	17.39	3.77	0.00	8.96
Czapek's Dox	12.42	4.99	0.00	7.01
Glucose	16.40	9.67	37.23	10.99
Glucose asparagine	13.33	3.67	0.00	16.22
MGP	3.07	2.30	0.00	3.83
Nutrient	8.57	2.52	0.00	7.98
Rice flour	10.62	7.63	0.00	10.28
Richard's	10.33	15.59	0.00	2.91
Singh and wood	24.55	3.45	0.00	1.45
SMKY	22.92	6.85	3.15	1.00
YES	17.09	0.06	0.00	10.18

M_1: Albendazole sulfoxide M_2: Albendazole sulfone M_3: New metabolite.

Table 2. Biotransformation of albendazole by *C. blakesleeana* NCIM 687 in presence of different vitamins.

Vitamin	% of metabolites			Dry weight of cells (mg/ml)
	M_1	M_2	M_3	
Ascorbic acid	39.75	0.00	0.00	10.17
Choline	28.50	0.00	0.00	9.13
Folic acid	19.51	0.00	0.00	9.75
Inositol	21.80	0.00	0.00	9.52
Niacin	13.52	9.04	0.00	9.14
Pantothenic acid	31.59	0.00	0.00	6.07
Pyridoxine	22.32	0.00	0.00	6.56
Riboflavin	21.49	12.18	0.00	8.40
Thiamine	44.32	1.44	0.00	8.80
Control	16.40	9.67	37.23	10.99

M_1: Albendazole sulfoxide. M_2: Albendazole sulfone. M_3: New metabolite.

moted the formation of both M_1 and M_2. M_1 formed, was significantly high when compared to M_2 in all the vitamin added cultures. The amount of albendazole transformed was at a maximum in the glucose broth containing thiamine than other vitamins (Fig. 5). Interestingly most of the vitamins produced M_1 that was higher than that of the control (glucose broth without a vitamin addition). When compared to the control, there was no significant change in vegetative growth of the fungus in the presence of vitamins. The ascorbic acid treated culture produced maximum vegetative growth of the fungus, which was slightly lower than that of the control.

Influence of solvents: The effect of dimethylformamide and dimethyl sulfoxide on biotransformation of albendazole by *C. blakesleeana* was investigated. The substrate albendazole is soluble in these solvents and hence these were selected for the study. The results obtained in the study are shown in Table 3. Dimethylformamide supported the transformation of albendazole to a maximum extent followed by dimethyl sulfoxide with a slight decrease. Dimethylformamide and dimethyl sulfoxide produced 63.3 and 61.4% of substrate depletion, respectively. Both solvents could show significant extent of transformation of albendazole by the fungus. However,

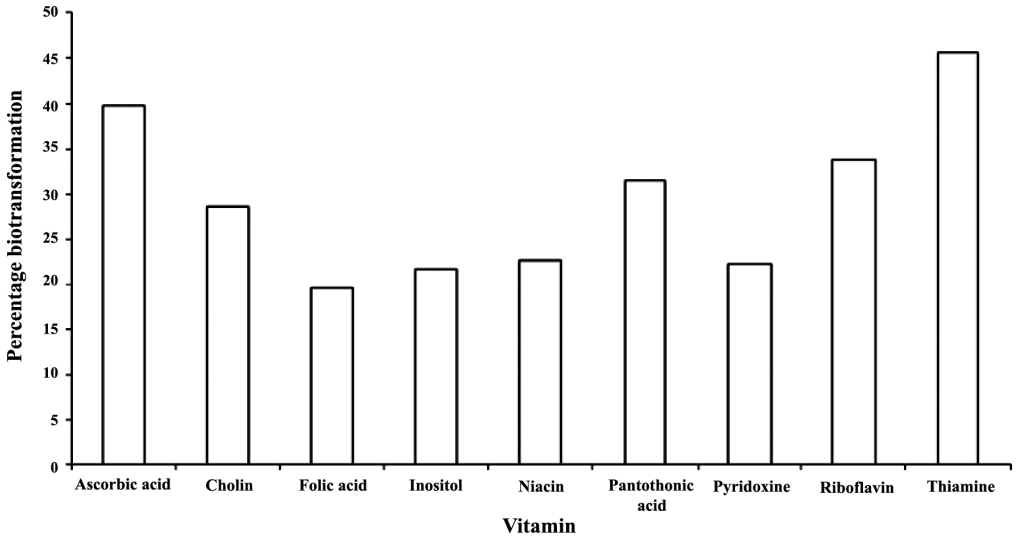

Figure 5. Biotransformation of albendazole by *C.blakesleeana* NCIM 687 in presence of vitamins.

dimethyl sulfoxide produced maximum amounts of M_1, which was almost 3 times that of dimethylformamide. M_2 was produced to maximum extent by dimethyl sulfoxide followed by a slight decrease with dimethylformamide. However, Dimethy lformamide produced maximum dry weight of the cells when compared to that of dimethyl sulfoxide.

Effect of incubation period: The influence of incubation period on the transformation of albendazole by *C. blakesleeana* was monitored by analyzing the medium periodically during the 20 days incubation period (Table 4). No transformation of albendazole by *C. blakesleeana* was recorded during the first 48 h of incubation. By the end of 4 days, *C. blakesleeana* could transform nearly 71% of albendazole to M_1, M_2 and to M_3. By the end of 6 days of the incubation period, nearly 70% of albendazole was transformed to M_1, M_2 and M_3. Amount of M_1 formed decreased to a minimum while the amount of M_2 increased significantly. By the end of 8 days of the incubation period, albendazole sulfoxide was formed in maximum amounts. After 10 days of incubation, all the three compounds, M_1, M_2 and M_3 could be detected in varying amounts. During 12 to 18 days incubation, M_1 and M_2 formation were almost the same. The 4 days incubation period was found to be the optimum, because a major amount of albendazole was transformed. The patterns of formation of M_1, M_2 and M_3 metabolites of albendazole are depicted in Figure 6.

Table 3. Biotransformation of albendazole by *C. blakesleeana* NCIM 687 in presence of dimethylformamide and dimethyl sulfoxide.

Solvent	% of metabolites			Dry weight of cells (mg/ml)
	M_1	M_2	M_3	
Dimethyl-formamide	16.40	9.67	37.23	10.99
Dimethyl-sulfoxide	50.82	10.58	0.00	7.51

M_1: Albendazole sulfoxide. M_2: Albendazole sulfone. M_3: New metabolite.

Table 4. Effect of Incubation period on transformation of albendazole by *C. blakesleeana.*

Incubation (in days)	metabolites %		
	M_1	M_2	M_3
2	0	0	0
4	4.61	11.46	55.70
6	0.50	28.14	36.99
8	16.26	10.64	38.00
10	8.44	14.76	46.05
12	5.16	4.23	46.21
14	5.98	6.09	41.00
16	4.13	4.50	45.15
18	4.06	4.10	53.57
20	3.82	6.66	44.13

M_1: Albendazole sulfoxide. M_2: Albendazole sulfone. M_3: New metabolite.

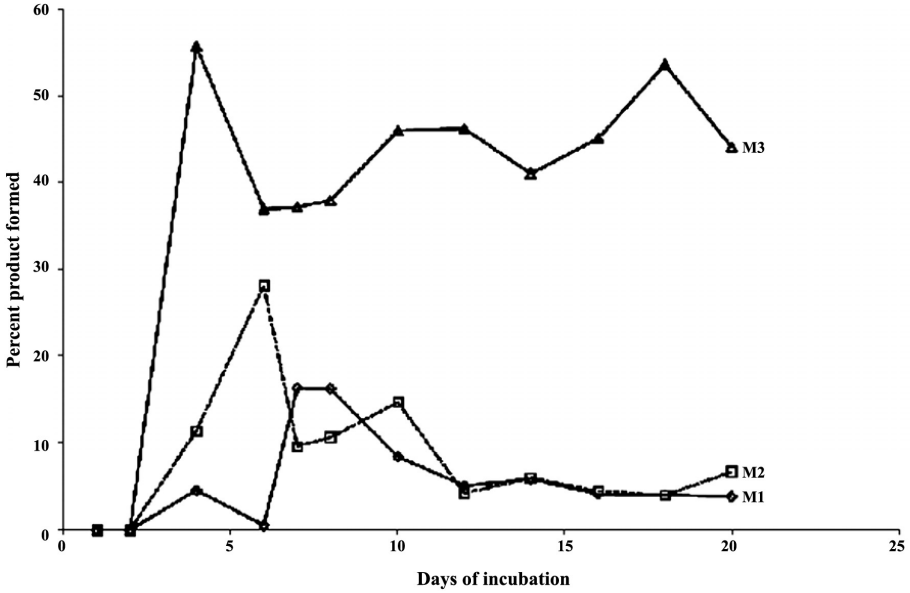

Figure 6. Effect of incubation period on albendazole transformation by *C. blakesleeana* National collection of industrial microorganisms (NCIM 687).

Fermentor studies: The transformation of albendazole in a laboratory fermentor under conditions such as pH 6.0, temperature of 30ºC, aeration rate of 1.2 lpm and stirrer speed of 200 rpm was also investigated in the present study. The results revealed that approximately 80% of the substrate albendazole could be transformed in 7 days. All the three products were detected in the fermentor culture medium leaving about 20% of albendazole in the medium as a residue. The transformed products M_1, M_2 and M_3 were produced to the extents of 23.49, 11.12 and 45.23%, which were 16.4, 9.67 and 37.23% in the case of shake flasks, respectively.

DISCUSSION

In previous works (Prasad *et al*., 2008a; Prasad *et al*., 2008b), the fungus *C. blakesleeana* has been reported to transform albendazole into three metabolites: albendazole sulfoxide (M_1), albendazole sulfone (M_2) and an N-methyl metabolite of albendazole sulfoxide (M_3). In the present investigation, the fungus was found to produce similar metabolites in presence of all the parameters studied. The transformation of albendazole by *C. blakesleeana* was influenced by several physical and biological conditions. The present organism could transform albendazole readily when it was grown in glucose medium while limited transformation could be recorded in nutrient broth. Such specificity of the medium for transformation have also been reported by Sutherland *et al*. (1991) for maximum transformation of phenanthrene in malt extract glucose medium by employing *Phanerochaete chrysosporium*. Zhang *et al*. (1996), by using Sabouraud's dextrose broth have reported 95% of azatadine transformation by *Cunninghamella elegans*. Faster transformation of phenanthrene in Sabouraud's medium by *C. elegans* has been reported by Lisowska and Dlugonski (2003). Zhang *et al.* (2005) have also shown the increased hydroxylation of cinobufagin in potato dextrose medium by *Mucor spinosus*. Specific 12 β-hydroxylation of cinobufagin in potato dextrose medium by *Alternaria alternata* has also been demonstrated by Ye *et al*. (2004).

In the present investigation, SMKY and glucose broths supported the transformation of albendazole to all the three known metabolites *viz.,* albendazole sulfoxide (M_1), albendazole sulfone (M_2) and the N-methyl metabolite of albendazole sulfoxide (M_3). Glucose medium induced transformation of albendazole to a significant amount of M_3. SMKY and Singh and Wood media were responsible for maximum production of M_1 in the present study. M_2 was found to be high in Richard's broth whereas a maximum amount of M_3 was found to be produced in Glucose broth. The inconsistency in the production of metabolites in different media may be due to the suitability of

that medium in induction of enzymes of *C.blakesleeana* for the production of metabolites. The results also reveal that the less grown cultures produced maximum extents of biotransformation, except for the culture grown in glucose broth. This indicates that the uptake of nutrients is directed towards enzyme activity rather than primary metabolism, cell division and thereby growth of cells (biomass). Interestingly, glucose broth produced good biomass as well as biotransformation.

Vitamins are organic compounds which are necessary for the growth of some organisms. They function as coenzymes or constituent parts of coenzymes which catalyze special reactions. Vitamins are necessary for proper utilization of carbohydrates, fats and proteins. The capacity of different organisms to synthesize the vitamins varies. Fungi with regard to their vitamin requirements occupy a position in between the totally independent green plants and completely dependent animals (Bilgrami and Varma, 1978). In the present investigation, when compared to other vitamins, the amount of albendazole transformed was at a maximum in the glucose broth containing thiamine. Interestingly, most of the vitamins produced M_1, which was higher than that of the control (glucose broth without a vitamin addition). Ascorbic acid, choline, folic acid, inositol and pantothenic acid suppressed the formation of M_2. None of the vitamins supported the formation of M_3. Production of M_1 in large quantities by all the vitamins studied may be due to activation of the enzyme responsible for production of M_1. Supression of formation of M_3 and M_2 by vitamins may be by inhibiting the enzymes responsible for the production of these metabolites. When compared to the control, there was no significant change in the vegetative growth of the fungus in presence of vitamins. The ascorbic acid treated culture led to maximum vegetative growth of the fungus, which was slightly lower than that of the control.

The solvent used to dissolve the substrate has also been found to have an effect on the biotransformation ability of the culture. This is due to the fact that some solvents induce and some of them inhibit the enzymes required for biotransformation. In flunitrazepam metabolism using human liver mesosomes, dimethylformamide inhibited the Cytochrome P450 2C19 (CYP2C19) enzyme activity and caused a phenotypic change in the metabolism reaction (Coller *et al*., 1999). Chauret *et al*. (1998) have reported that dimethyl sulfoxide inhibits some metabolic reactions in liver microsomes. The use of solvents may also help for the better solubilization of the substrate in the media. Solvents like dimethyl sulfoxide promote substrate uptake and product release by increasing the permeability of the cells to the substrate. Hickman *et al*. (1998) have shown that dimethylformamide could produce maximum transformation of caffeine and tolbutamide in human liver microsomes. In the present investigation, the metabolite M_3 was not produced when dimethyl sulfoxide was used to dissolve the substrate, indicating that this solvent might have an inhibitory effect on the enzyme responsible for the production of this metabolite. The amounts of M_1 and M_3 produced with the two solvents also indicate that M_1 might be converting to M_3 in presence of dimethylformamide. The results indicate that the effect of solvents on transformation was significant both qualitatively and quantitatively.

Rate and amount of product formed by the process of transformation have also been reported to be influenced by the incubation period. Miyazawa *et al*. (1995) have reported that the transformation of cedrol by *Glomerella cingulata* varies with the incubation period. Similarly transformation of limonene by *Pseudomonas putida* has been shown to be at a maximum after 5 days of incubation (Chatterjee and Bhattacharya, 2001). While an 8 days incubation period has been found to be the optimum for transformation of (L)-citronellal to (L)-citronellol by *Rhodotorula minuta* (Harshad and Mohan, 2003). In the present study, a 4 days incubation period was found to be optimum for biotransformation of albendazole employing *C. blakesleeana*. Contrary to present observations, *C. elegans* could transform azatadine to the extent of 95% within 72 h of incubation (Zhang *et al*., 1996). In support of the findings of this research, after 4 days of incubation, nearly 70% of fluorene was reported to be transformed by *C. elegans* (Pothuluri *et al*., 1993). In contrast to the results of this study, only 28% of doxepin has been reported to undergo transformation by *C. elegans* during the 4 days of incubation (Moody *et al*., 1999). The varying concentrations of albendazole and transformed products in the medium during different phases of the incubation period may be attributed to the reversible reactions of enzymes involved. This reversible reaction has even been observed in mammals by Formentini (2005), where the metabolites are converted back to the parent drug albendazole. No definite pattern could be observed in the transformation of albendazole to M_3. The patterns of formation of M_1, M_2 and M_3 metabolites of albendazole are depicted in Figure 6.

In industrial processes, screening of organisms for their potential to produce a product represents the primary task. Subsequently the conditions under which it can be carried out will be of the second step. Before going to large-scale production, the laboratory-scale workup in a fermentor and operation of the pilot plant will be the third step. After studying various parameters influencing the biotransformation of a substrate, the optimum conditions have to be provided in order to obtain the product in large quantities. Providing these conditions for large-scale production using fermentor will be the primary task to produce the product economically. Successful scale-up in biotransformations using the fermentor have been reported previously for protected carboxylic acids (Kreiner *et al*., 1996), mutilin (Hanson *et al*., 2002) and few other organic substrates. In the present study, there was a significant increase in the metabolite levels during the fermentor study. This could be due to a change in operation conditions, shaking/stirring patterns or both. The operating conditions might affect enzyme induction and thus the extent of biotransformation. The operating conditions in a fermentor might also influence metabolite production both qualitatively and quantitatively. Further scaling-up of the process is needed before recommendation to large-scale field production and commercialization.

In the present investigation, albendazole was transformed to albendazole sulfoxide, albendazole sulfone and a new metabolite. Albendazole sulfoxide and albendazole sulfone have been identified in plasma after oral administration of albendazole in several species *viz*., rat, human, porcine, ovine, bovine, caprine and chicken (Csiko *et al*., 1996; Moroni *et al*., 1995; Benchaoui *et al*., 1993; Lanusse *et al*., 1993; McKellar *et al*., 1993; El Amri *et al*., 1987; Penicaut *et al*., 1983). The flavin containing monooxygenases (FMO) and cytochromes P450 (CYP, mainly CYP3A in rat) appear to mediate the conversion of albendazole(ABZ) to albendazole sulfoxide(ABZSO), whereas the biotransformation of ABZSO to albendazole sulfone (ABZSO2) is influenced by CYP only (CYP1A in rat) (Moroni *et al*., 1995; El Amri *et al*., 1988; El Amri *et al*., 1987). This shows the presence of a similar type of enzyme system in *C. blakesleeana* which produces the same metabolites. Further investigations are needed to find out the enzyme system involved in biotransformation of albendazole by *C. blakesleeana*.

CONCLUSION

Previous studies indicate that albendazole can be transformed to albendazole sulfoxide, albendazole sulfone and the N-methyl metabolite of albendazole sulfoxide by *C. blakesleeana* in an eco-friendly way. The present investigation reveals the importance of incubation period, vitamins, media and solvents for optimization of biotransformation. Among the four factors studied, the use of suitable media was found to be critical for the development of a biotransformation system. The production of metabolite M_3 was found to be sensitive since a large variation was observed with all the four factors studied. Further investigations are needed to produce albendazole sulfoxide in large quantities by optimizing the fermentor conditions and by inhibiting the enzymes responsible for production of other metabolites.

Acknowledgements

The authors thank Ms. Akshaya for interpretation of LC/MS spectra and Head, Prof. M.A.Singara charya Department of Microbiology, Kakatiya University Warangal, Andhra Pradesh, India, for providing necessary facilities. The authors also thank the University Grants Commission, New Delhi, for providing financial assistance.

References

Bilgrami KS, Varma RN (1978). *Physiology of fungi*, 2nd Edition, Vikas Publishing House Private Limited, Ghaziabad, UP, India, PP. 274-297.

Bogan JA, Marriner SE (1984). Pharmacodynamic and toxicological aspects of albendazole in man and animals. In: *Albendazole in helminthiasis*, ed., M. Fock, Royal Society of Medicine International Congress and Symposium, Series 61, Royal Society of Medicine, London, PP. 13-21.

Benchaoui HA, Scott EW, McKellar QA (1993). Pharmacokinetics of albendazole, albendazole sulfoxide and netobimin in goats. *J Vet Pharmacol Ther* 16: 237-240.

Chatterjee T, Bhattacharyya DK (2001). Biotransformation of limonene by *Pseudomonas putida*. *Appl Microbiol Biotechnol*. 55: 541-546.

Chauret N, Gauthier A, Nicoll-Graffith DA (1998). Effect of common organic solvents on *in vitro* cytochrome P450-mediated metabolic activities in human liver microsomes. *Drug Metab Dispos*. 26: 1-4.

Coller JK, Somogyi AA, Bochner F (1999). Flunitrazepam oxidative metabolism in human liver microsomes: Involvement of CYP2C19 and CYP3A4. *Xenobiotica* 29: 973-986.

Csiko GY, Banhidi GY, Semjen G, Laczay P, Ványine Sándor G, Lehel J, Fekete J (1996). Metabolism and Pharmacokinetics of albendazole after oral administration to chickens. *J Vet Pharmacol Ther*. 19: 322-325.

El Amri HS, Fargetton X, Delatour P, Batt AM (1987). Sulphoxidation of albendazole by the FAD-containing and cytochrome P-450 dependent mono-oxygenases from pig

liver microsomes. *Xenobiotica* 10: 1159-1168.

El Amri H, Mothe O, Totis M, Masson C, Batt AM, Delatour P, Siest G (1988). Albendazole sulfonation by rat liver cytochrome P-450c. *J Pharmacol Exp Ther*. 246: 758-764.

Formentini EA (2005). Pharmacokinetics behavior or ricobendazole in calves and sheep after its intravenous administration. *Rev Med Vet*. 156: 37-42.

Gyurik RJ, Chow AW, Zaber B, Brunner EL, Miller JA,Villani AJ, Petka LA, Parish RC (1981). Metabolism of albendazole in cattle, sheep, rats and mice. *Drug Metab Dispos* 9: 503-508.

Hanson RL, Matson JA, Brzozowski DB, Laporte TL, Springer DM, Patel RN (2002). Hydroxylation of mutilin by *Streptomyces griseus* and *Cunninghamella echinulata*. *Org Process Res Dev*. 6: 482-487.

Harshad RV, Mohan RH (2003). Biotransformation of (L)-citronellal to (L)-citronellol by free and immobilized *Rhodotorula minuta*. *Electron J Biotechnol*. 6: 90-103.

Hickman D, Wang J, Wang Y, Unadkat JD (1998). Evaluation of the selectivity of *in vitro* probes and suitability of organic solvents for the measurement of human cytochrome P450 monooxygenase activities. *Drug Metab Dispos*. 26: 207-215.

Hussar DA (1997). New drugs of 1996. *J Am Pharm Assoc*. 37: 192-228.

Kreiner M, Braunegg G, de Raadt A, Griengl H, Kopper I, Petsch M, Plachota P, Schoo N, Weber H, Zeiser A (1996). Stereospecific biohydroxylations of protected carboxylic acids with *Cunninghamella blakesleeana*. *Appl Environ Microbiol*. 62: 2603-2609.

Lanusse C, Virkel G, Sanchez S, Alvarez L, Lifschitz A, Imperiale F, Monfrinotlic A (1998). Ricobendazole kinetics and availability following subcutaneous administration of a novel injectable formulation to calves. *Res Vet Sci*. 65: 5-10.

Lanusse CE, Gascon LH, Prichard RK (1993). Gastrointestinal distribution of albendazole metabolites following netobimin administration to cattle: relationship with plasma disposition kinetics. *J Vet Pharmacol Ther*. 16: 38-47.

Lisowska K, Dlugonski J (2003). Concurrent corticosteroid and phenanthrene transformation by filamentous fungus *Cunninghamella elegans*. *J Steroid Biochem Mol Biol*. 85: 63-69.

Marriner SE, Morris DL, Dickson B, Bogan JA (1986). Pharmacokinetics of albendazole in man. *Eur J Clin Pharmacol*. 30: 705-708.

Menon K (2002). Assesment of the antiprotozoal activity of some tubulin inhibitors following cyclodextrin complexation, *PhD. Thesis*, Murdoch University, Perth, Western Australia.

Miyazawa M, Nankai H, Kameoka H (1995). Biotransformation of (+)-cedrol by plant pathogenic fungus, *Glomerella cingulata*. *Phytochemistry* 40: 69-72.

Moody JD, Freeman JP, Cerniglia CE (1999). Biotransformation of doxepin by *Cunninghamella elegans*. *Drug Metab Dispos*. 27: 1157-1164.

Moroni P, Buronfosse T, Sauvageon LC, Delatour P, Benoit E (1995). Chiral sulfoxidation of albendazole by the flavin adenine dinucleotide-containing and cytochrome P450-dependent monooxygenases from rat liver microsomes. *Drug Metab Dispos*. 23: 160-165.

McKellar QA, Jackson F, Coop RL, Baggot JD (1993). Plasma profiles of albendazole metabolites after administration of netobimin and albendazole in sheep: effects of parasitism and age. *Brit Vet J*. 149: 101-113.

Pothuluri JV, Freeman JP, Evans FE, Cerniglia CE (1993). Biotransformation of fluorene by the fungus *Cunninghamella elegans*. *Appl Environ Microbiol*. 59: 1977-1980.

Pothuluri JV, Sutherland JB, Freeman JP, Cerniglia CE (1998). Fungal biotransformation of 6-nitrochrysene. *Appl Environ Microbiol*. 64: 3106-3109.

Prasad GS, Girisham S, Reddy SM, Srisailam K (2008a). Biotransformation of albendazole by fungi. *World J Microbiol Biotechnol*. 24: 1565-1571.

Prasad GS, Girisham S, Reddy SM, Srisailam K (2008b). Biotransformation of albendazole by fungi: Effect of carbon and nitrogen sources. *World J Microbiol Biotechnol*. 24: 2055-2059.

Penicaut B, Maugein P, Maisonneuve H, Rossignol JF (1983). Pharmacokinetics and urinary metabolism of albendazole in man. *Bull Soc Pathol Exot Filiales*. 76: 698-708.

Smith LL (1984). Steroids. In: *Biotechnology*, ed., H.J. Rehm, G. Reed, Vol. 6a, Chrmie GmbH, Weinheim, PP. 31-78.

Sutherland JB, Selby AL, Freeman JP, Evans FE, Cerniglia CE (1991). Metabolism of phenanthrene by *Phanerochaete chrysosporium*. *Appl Environ Microbiol*. 57: 3310-3316.

Theodorides VJ, Gyerik RJ, Kingsbury WD, Parish RC (1976). Anthelmintic activity of albendazole against liver flukes, tapeworms, lung and gastrointestinal roundworms. *Experientia* 32: 702.

Ye M, Qu G, Guo H, Guo D (2004). Specific 12 beta-hydroxylation of cinobufagin by filamentous fungi. *Appl Environ Microbiol*. 70: 3521-3527.

Zhang D, Hansen EB, Deck J, Heinze TM, Sutherland JB, Cerniglia CE (1996). Fungal biotransformation of the antihistamine azatadine by *Cunninghamella elegans*. *Appl Environ Microbiol.* 62: 3477-3479.

Zhang W, Ye M, Qu GQ, Wu WY, Chen YJ, Guo DA (2005). Microbial hydroxylation of cinobufagin by *Mucor spinosus*. *J Asian Nat Prod Res*. 7: 225-229.