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Studies on Microbial Transformation of Meloxicam by Fungi

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Screening-scale studies were performed with 26 fungal cultures for their ability to transform the anti-inflammatory drug meloxicam. Among the different fungi screened, a filamentous fungus, *Cunninghamella blakesleeana* NCIM 687, transformed meloxicam to three metabolites in significant quantities. The transformation of meloxicam was confirmed by high-performance liquid chromatography (HPLC). Based on the liquid chromatography–tandem mass spectrometry (LC–MS/MS) data, two metabolites were predicted to be 5-hydroxymethyl meloxicam and 5-carboxy meloxicam, the major mammalian metabolites reported previously. A new metabolite was produced, which is not detected in mammalian systems. Glucose medium, pH of 6.0, temperature of 27°C, 5-day incubation period, dimethylformamide as solvent, and glucose concentration of 2.0% were found to be suitable for maximum transformation of meloxicam when studied separately. It is concluded that *C. blakesleeana* can be employed for biotransformation of drugs for production of novel metabolites.

Keywords: Biotransformation, fungi, meloxicam, 5-hydroxymethyl meloxicam, 5-carboxy meloxicam, HPLC, LC–MS/MS

Meloxicam (MLX), 4-hydroxy-2-methyl-*N*-(5-methyl-2-thiazolyl)-2H-1,2 benzothiazine-3-carboxamide-1,1-dioxide), is a nonsteroidal anti-inflammatory drug (NSAID) and a selective cyclooxygenase-2 (COX-2) inhibitor used in the treatment of rheumatoid arthritis, osteoarthritis, and other joint diseases. Meloxicam preferentially inhibits cyclooxygenase-2, which is induced by inflammatory stimuli in pathophysiological conditions, rather than cyclooxygenase-1, which is responsible for normal physiological processes in mammals [9, 12, 28]. The pharmacokinetics of meloxicam has been investigated in a number of animal species including mice, rats, dogs, mini-pigs, and baboons, to provide comprehensive profiles and to determine which animal species exhibits a profile most closely resembling the pharmacokinetic profile in humans [5]. The main metabolites of meloxicam in humans, rats, mice, and mini-pigs were a 5-hydroxymethyl derivative and a 5-carboxy derivative. Other metabolites have also been detected in rats and humans [34, 35]. The occurrence of hydroxylation pathway suggests the involvement of a cytochrome p450 (CYP) [8].

Some of the reported side effects of the meloxicam include signs of bleeding, signs of an allergic reaction, blurred vision, change in the amount of urine passed, difficulty in swallowing, severe heart burning, pain in throat, pain or difficulty in passing urine, stomach pain or cramps, swelling of feet or ankles, unexplained weight gain or edema, yellowing of eyes, diarrhea, dizziness, and nausea or vomiting [3]. Meloxicam is practically insoluble in water. The poor solubility and wettability of meloxicam leads to poor dissolution and thereby variation in bioavailability. Thus, increasing the aqueous solubility and dissolution of meloxicam is of paramount therapeutic importance [16].

Microorganisms are able to perform a large variety of reactions, including some nearly inaccessible by chemical process. From a pharmaceutical point of view, hydroxylations and glycosylations [1, 2] are considered to be particularly useful bioconversions. They can yield new drugs, and existing drugs can be improved so as to increase activity and/or stability and decrease toxicity.

In light of the above facts, the present work was aimed to produce novel metabolites (i.e., new analogs of meloxicam) by transformations using filamentous fungi. The effect of media, temperature, pH, solvents, incubation period, and glucose concentration on transformation of meloxicam were studied in detail.

Materials and Methods

Chemicals

Meloxicam was gifted by Unichem Laboratories, Mumbai, India. Methanol was of high-performance liquid chromatography (HPLC) grade, obtained from Ranbaxy, New Delhi, India. Peptone, yeast

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extract, glucose, potato dextrose agar, and all other chemicals of highest available purity were obtained from Himedia, Mumbai, India.

Microorganisms

Twenty-six fungal cultures were selected based on the previous reports [2, 38]. The cultures were procured from Microbial Type Culture Collection and Gene Bank (MTCC), Chandigarh, India or National Collection of Industrial Microorganisms (NCIM), Pune, India. The procured fungal cultures included *Aspergillus flavipes* NCIM 1209, *A. niger* NCIM 1006, *A. niger* NCIM 589, *A. niger* NCIM 620, *A. ochraceous* NCIM 1140, *A. flavus* NCIM 557, *Cunninghamella elegans* NCIM 690, *C. elegans* NCIM 689, *C. blakesleeana* NCIM 687, *C. echinulata* NCIM 691, *Curvularia lunata* NCIM 716, *Fusarium equiseti* MTCC 3731, *F. graminearum* MTCC 1893, *F. lateritium* MTCC 3320, *F. moniliforme* NCIM 1099, *F. oxysporum* NCIM 1008, *F. pallidoroseum* MTCC 2083, *Mucor plumbeus* NCIM 984, *M. rouxii* MTCC 386, *Penicillium chrysogenum* NCIM 733, *P. chrysogenum* NCIM 738, *Pestalotiopsis* spp. MTCC 3689, *Rhizopus arrhizus* NCIM 997, *R. stolonifer* NCIM 880, *Saccharomyces cerevisiae* NCIM 3090, and *Trichothecium roseum* NCIM 1147. Stock cultures were maintained on potato dextrose agar slants at 4°C and subcultured every 3 months.

Biotransformation

Biotransformation was performed using a two-stage fermentation protocol. In the first stage, fermentation was initiated by inoculating a 250-ml culture flask consisting of 50 ml of liquid broth. 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 with 0.1 N HCl or 0.1 N NaOH. The prepared media were autoclaved and cooled to room temperature. The medium was inoculated with a loopful of culture obtained from freshly grown potato dextrose agar slants. The flasks were incubated at 120 rpm and 28°C for 48 h. Second-stage cultures were initiated in the same media using an inoculum of 1 ml of first-stage culture per 20 ml of medium in a 100-ml culture flask. The cultures were incubated for 24 h and the substrate meloxicam (2 mg) in dimethyl formamide was added to give a final concentration of 100 mg/l. The flasks were incubated under similar conditions for 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 comprised meloxicam added to the sterile medium and incubated under similar conditions. Each culture was studied in triplicate. The cultures were 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 analyzed by HPLC and liquid chromatography–tandem mass spectrometry (LC–MS/MS) for the presence and identification of metabolites.

Analysis

HPLC analysis was performed according to the method described by Elbary *et al.* [11] with a slight modification. The samples were analyzed using an LC-10AT system (Shimadzu, Japan) by injecting 20 µl of sample into the syringe-loading sample injector (Model 7725i; Rheodyne, U.S.A.). The column used was Wakosil II, C18, 250×4.6 mm, and 5 µm (SGE, Australia). The mobile phase consisted of a mixture of methanol-water (pH adjusted to 3.0 with orthophosphoric acid) in the ratio of 6:4. The analysis was performed isocratically at a flow rate of 1 ml/min and the analytes were detected at 360 nm using a photodiode array detector (Model SPD M10Avp; Shimadzu, Japan). The metabolite peaks were identified based on the similarity in the UV spectra of meloxicam with that of metabolites.

LC–MS/MS analysis was carried out using a Waters system, column X Terra C18, 25×0.46 cm, 5 µm, and a mobile phase consisting of methanol and water (pH adjusted to 3.0 with formic acid) in a 6:4 ratio. The ESI detection was set to positive mode. A temperature of 300°C and scan range of 50–500 were set for the analysis. The transformed compounds were identified from the masses of the fragmentation products obtained.

Effect of Media

To find a suitable media for maximum transformation of meloxicam and to produce novel metabolites by employing *C. blakesleeana* NCIM 687, different media were screened. The compositions (per liter) of media used are given below:

Asthana and Hawkers A broth (glucose, 10 g; KNO_3, 3.5 g; KH_2PO_4, 1.75 g; and $MgSO_4 \cdot 7H_2O$, 0.75 g), Czapeck's broth ($NaNO_3$, 2 g; KH_2PO_4, 1 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; KCl, 0.5 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 aspargine (glucose, 20 g; aspargine, 5 g; KH_2PO_4, 3.4 g; $MgSO_4 \cdot 7H_2O$, 1.9 g; and NaCl, 0.01 g), 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; $MgSO_4 \cdot 7H_20$, 2.5 g; sucrose, 30 g; and $FeCl_2$, 0.02 g), Singh and Wood medium (glucose, 5 g; aspargine, 4 g; KH_2PO_4, 1 g; $MgSO_4 \cdot 7H_2O$, 0.75 g; and pectin, 10 g), SMKY broth (sucrose, 20 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; KNO_3, 3 g; and yeast extract, 7 g), and YES broth (yeast extract, 20 g and sucrose, 40 g)

Specified quantities of the media ingredients were dissolved in distilled water, the pH was adjusted to 6.0 with either 0.1 N HCl or 0.1 N NaOH, and the media were sterilized by autoclaving.

Effects of Temperature and pH

Studies were performed to determine the suitable temperature and pH for higher transformation of meloxicam by *C. blakesleeana* NCIM 687. The transformation was studied at the temperatures 20, 25,27, 30, 35, and 40°C and at various media pH of 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, and 10.

Influence of Incubation Period

The effect of incubation period on transformation of meloxicam was studied by analyzing the culture broth at the end of 1, 2, 4, 5, 6, 8, 10, 12, 14, 18, and 20 days.

Influence of Different Solvents

Influence of different solvents (*viz.*, acetone, dimethylformamide, dimethylsulfoxide, ethanol methanol, and propanol) on the biotransformation of meloxicam was studied. The drug was dissolved in each solvent and added to the culture. The addition of respective solvent without drug served as the control.

Effect of Glucose Concentration

Influence of glucose concentration on growth and biotransformation of meloxicam was studied by adding different glucose concentrations of 0%, 0.5%, 1%, 1.5%, 2%, 2.5%, 3%, and 3.5%.

Results and Discussion

Of the 26 fungal species representing 10 genera, 6 species could exhibit the ability to transform meloxicam. The degree of transformation, however, varied with the species. Even the same species differed in their ability to transform. The results of this study are shown in Table 1. From the table, it can be observed that the six cultures altogether produced three metabolites, evidenced from HPLC analysis. Of the different species of *Aspergillus* studied, *A. niger* NCIM 589 could transform meloxicam to an extent of 1.67%, producing two metabolites, M_1 and M_2, which were identified as 5-hydroxymethyl meloxicam (M_2) and 5-carboxy meloxicam (M_1) based on LC–MS/MS analysis, whereas *A. ochraceous* NCIM 1140 could transform meloxicam to nearly 8%, producing M_1 and M_2. Similarly, out of three species of *Cunninghamella*, *C. blakesleeana* NCIM 687 could transform meloxicam to nearly 93% of M_2, a trace amount of M_1, and to nearly 4% of a new metabolite (M_3). M_3 was not detected in any of the other fungi that transformed meloxicam. *C. echinulata* NCIM 691 could also transform meloxicam to form M_2 and M_1. Interestingly *C. elegans* NCIM 690 could not transform meloxicam to M_1 and M_3, in which case only M_2 was produced. Among the three *Cunninghamella* species, *C. blakesleeana* transformed the highest amount of meloxicam (nearly 97%). When the transformation was calculated with respect to the dry cell weight (DCW), an almost similar pattern was observed, where *C. blakesleeana* transformed the highest amount of meloxicam (Table 1). *S. cerevisiae* NCIM 3090 could also transform meloxicam, but only to produce M_2. Among all the fungi screened, the complete range of transformation of meloxicam was performed by *C. blakesleeana* NCIM 687, hence this fungi was selected for further investigation.

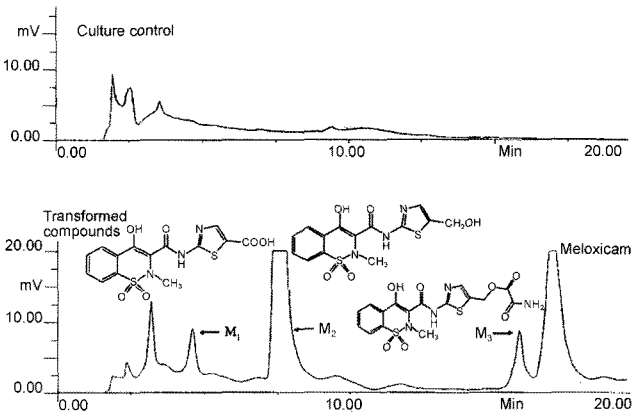

Fig. 1. HPLC chromatogram showing culture control and transformed compounds obtained in *C. blakesleeana*.

The metabolites formed were identified basing on observation of new peaks in HPLC (Fig. 1) and characterized with the help of the mass values of fragmentation ions obtained in LC–MS/MS analysis (Fig. 2).

Mass spectrometric analysis of the metabolite M_2 showed a molecular ion at *m/z*=367 (an increase of 16 units), indicating addition of a single oxygen atom, which results in formation of 5-hydroxymethyl meloxicam. Another molecular ion was found at *m/z*=381 (an increase of 14 units), indicating further addition of an oxygen atom and removal of two hydrogens to M_2, which results in the formation of 5-carboxy meloxicam (M_1). A third metabolite was observed with *m/z* of 437, indicating the addition of 86 units to meloxicam. This might be arising from an oxamic acid analog of meloxicam. An oxamic metabolite of meloxicam was reported in rat previously [35].

These analyses indicated that the metabolites were 5-hydroxymethyl meloxicam (M_2, eluting at 7.0 min), 5-carboxy

Table 1. Transformation products of meloxicam by some fungi.

Name of the fungi	Metabolites in % (% per mg DCW)				Final pH		Dry weight (mg/20 ml)	
	M_1	M_2	M_3	M	C	T	C	T
Aspergillus niger NCIM589	1.30 (0.28)	0.37 (0.08)	0.00 (0.00)	98.32 (21.01)	6.05	6.25	4.84	4.68
A.ochraceous NCIM 1140	0.27 (0.03)	7.97 (0.92)	0.00 (0.00)	91.74 (10.61)	8.41	8.36	9.04	8.65
Cunninghamella blakesleeana NCIM 687	0.75 (0.06)	92.61 (7.87)	4.04 (0.34)	2.37 (0.20)	7.07	6.82	11.60	11.77
C.echinulata NCIM 691	0.01 (0.00)	1.94 (0.32)	0.00 (0.00)	96.04 (15.74)	7.27	7.17	6.37	6.10
C.elegans NCIM 690	0.00 (0.00)	3.05 (0.33)	0.00 (0.00)	96.94 (10.42)	7.16	6.72	12.51	9.30
Saccharomyces cerevisiae NCIM 3090	0.00	14.35	0.00	85.64	8.20	8.01	-	-

Reaction conditions: Meloxicam concentration 2.0 mg/20 ml reaction medium; incubation period 5 days.
M1=5-carboxy meloxicam. C=culture control.
M2=5-hydroxymethyl meloxicam. T=meloxicam-fed culture.
M3=New metabolite.
M=Meloxicam.

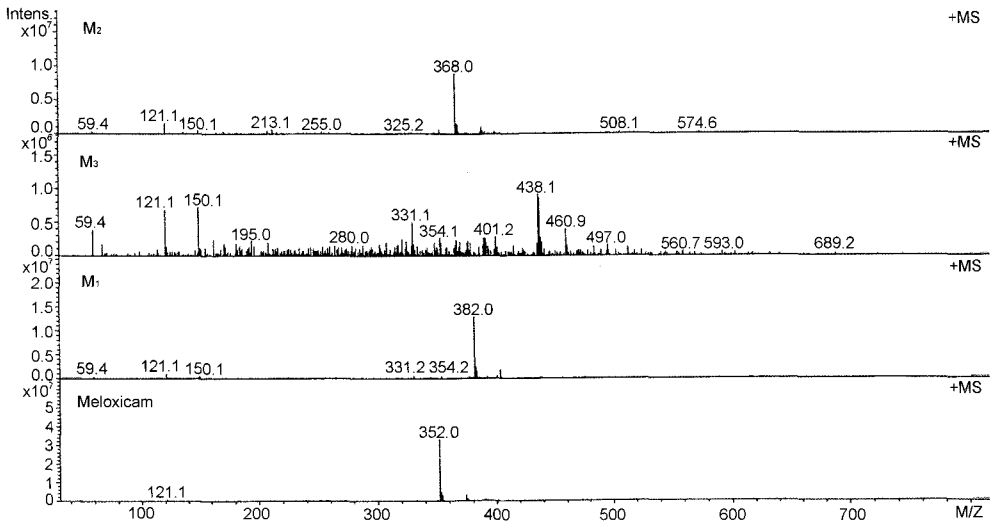

Fig. 2. LC–MS/MS spectra of metabolites detected in meloxicam-fed culture broth of *C. blakesleeana*.

meloxicam (M_1, eluting at 5.0 min), and a new metabolite (M_3, eluting at 17 min), where the substrate meloxicam was eluted at 19.0 min. The pathway of the metabolite formation is shown in Fig. 3. The metabolites were quantified based on the peak areas obtained in HPLC analysis taking the drug and metabolites' peak areas together as 100%.

Addition of meloxicam to the growing cultures of fungi (Table 1) showed marginal influence on vegetative growth. Vegetative growth in all the controls was slightly high when compared with meloxicam-treated cultures. Interestingly, vegetative growth of *C. blakesleeana* NCIM 687 was more in meloxicam-treated culture flasks when compared with the control. The final pH recorded in the control and meloxicam-fed culture media of different fungi was near neutral. However, the pH recorded in *A. ochraceous* NCIM 1140 and *S. cerevisiae* NCIM 3090 culture flasks was slightly alkaline.

Metabolites obtained in transformation of meloxicam by *C. blakesleeana* NCIM 687 were further studied for optimizing cultural conditions for transformation to a maximum extent and to produce novel metabolites.

Effect of Different Media

The effect of different synthetic media on transformation of meloxicam by *C. blakesleeana* was tested and the results are depicted in Table 2. The transformation ability of *C. blakesleeana* varied significantly with the medium, where glucose medium could induce maximum transformation of meloxicam. Nearly 97% of the meloxicam could be transformed to M_1, M_2, and M_3. Malt extract medium was also found to be a good medium; however, it could induce transformation of most of the meloxicam into M_2 only. Czapeck's medium followed by SMKY medium could induce about 50% of the meloxicam transformation. Only M_2 was seen in Czapeck's medium. In SMKY medium, besides forming a major amount of M_2, a limited amount of M_1 was also detected. Nutrient broth was the most unfavorable for the transformation of meloxicam, as only a

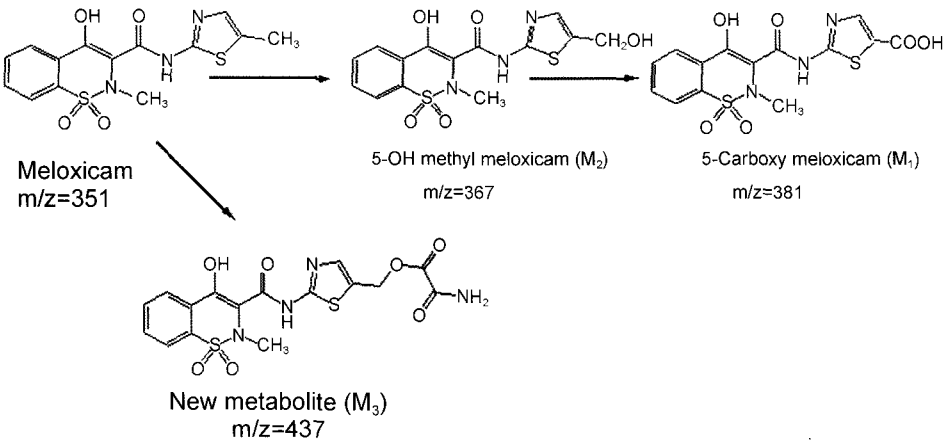

Fig. 3. Proposed pathway of meloxicam metabolism in culture broth of *C. blakesleeana*.

Table 2. Effect of different media on biotransformation of meloxicam by *C.blakesleeana.*

Name of media	Metabolites in %				Final pH		Dry weight (mg/20 ml)	
	M_1	M_2	M_3	M	C	T	C	T
Asthana and Hawkers media A	0.00	24.14	0.00	75.85	5.62	5.92	1.45	1.76
Czapek's	0.00	55.18	0.00	44.81	5.60	5.72	8.96	9.78
Glucose	0.75	92.61	4.04	2.37	7.07	6.82	11.60	11.77
Glucose asparagine	0.00	21.14	0.00	78.85	7.64	7.85	7.98	7.67
Malt extract	0.00	84.71	0.00	15.28	7.98	8.14	3.83	3.77
Nutrient broth	0.00	0.39	0.00	99.60	7.10	6.91	1.00	0.84
Rice flour	0.00	27.18	0.00	72.81	4.42	4.27	7.01	7.10
Richard's	0.00	38.55	0.00	61.44	5.82	6.14	3.40	3.86
Singh and Wood	0.00	21.47	0.00	78.52	7.73	8.05	2.91	3.10
SMKY	1.59	44.93	0.00	53.42	7.56	7.45	10.28	10.44
YES	0.00	35.74	0.00	64.25	7.98	8.14	16.22	15.59

Reaction conditions: Meloxicam concentration 2.0 mg/20 ml reaction medium; incubation period 5 days.
M1=5-carboxy meloxicam. C=culture control.
M2=5-hydroxymethyl meloxicam. T=meloxicam-fed culture.
M3=New metabolite.
M=Meloxicam remaining.

trace amount of M_2 was detected. The rest of the media under investigation supported varying degrees of transformation of meloxicam to M_2 only. Glucose medium was found to be suitable for maximum transformation of meloxicam. Hence, this medium is selected for further investigation.

Table 2 reveals that the efficiency of medium in induction of biomass production varied significantly. YES medium followed by SMKY medium and glucose medium supported good mycelial growth of *C. blakesleeana.* On the other hand, nutrient broth and Asthana and Hawkers medium A supported poor growth of *C. blakesleeana.* Richards medium, malt extract medium, and Singh and Wood medium were also found to be poor supports. The rest of the media produced intermediate extent of mycelial growth.

In general, the vegetative growth was marginally inhibited by the addition of meloxicam, as biomass production was not much different. This may be attributed to addition of meloxicam during the growth that has resulted in increase in the carbon source, or it may be the temporary adverse effect of the compound on the fungus. More detailed studies are required in order to know the exact mechanism in temporary growth inhibition of the fungus.

A significant change in pH was recorded owing to the growth of fungus, which, however, varied with the medium. pH in rice flour medium, Czapeck's medium, Richards medium, and Asthana and Hawkers medium A was found to be strongly acidic. In the rest of the media, the final pH recorded was either neutral or slightly alkaline.

Influence of Temperature

Incubation temperature exerted a profound impact on transformation of meloxicam (Table 3). A temperature of 27°C was the most favorable for transformation by *C. blakesleeana,* as most of the meloxicam was transformed to different compounds. Similarly, maximum transformation of naproxen by *Cunninghamella* species was obtained at 28°C by Zhong *et al.* [40]. Sun *et al.* [37] have reported maximum transformation of verapamil by *C. blakesleeana* at 28°C. In the present study, at 20°C, nearly 60% of the meloxicam was transformed to M_2 and M_1. The transformant formed in higher concentration was M_2. At 25°C, the transformation of meloxicam to M_2 was nearly 88%, whereas M_1 was formed only in trace amounts. At 30°C, 60% of the meloxicam was transformed to M_2. These results indicate that the degree of transformation was decreased with increase in the incubation temperature. Temperatures above 35°C did not favor transformation of meloxicam to M_3. Inhibition of M_3 formation at higher and low temperatures suggests its specific temperature requirement. Temperature of 27°C was found to be optimum as most of the meloxicam was transformed.

Biomass production was found to be maximum at 30°C, which decreased with increase in temperature. At all temperatures, the pH drift was towards the alkaline side with the growth of the organism, and the final pH recorded was near neutral.

Effect of pH

pH exerted significant influence on transformation of meloxicam (Table 4). At pH 3.0, meloxicam was transformed to M_2 to an extent of 40%. pH 6.0 was most favorable, as most of the meloxicam was transformed. pH 6.0 was also reported to be favorable for *O*-demethylation and sulfation of 7-methoxylated flavanones by *C. elegans* [22]. Zhong *et al.* [40] have reported maximum transformation of naproxen by *Cunninghamella* species at pH 6.0. At pH 4.0, meloxicam could be transformed to M_2, M_1, and M_3, with M_2 being the major metabolite. At pH 5.0, nearly 26% of M_2 was

Table 3. Effect of temperature on transformation of meloxicam by *C.blakesleeana.*

Temperature in °C	Metabolites in %				Final pH		Dry weight (mg/20 ml)	
	M_1	M_2	M_3	M	C	T	C	T
20	3.84	52.23	0.00	40.55	7.22	7.24	5.96	5.48
25	0.09	87.19	9.43	3.29	7.13	7.21	7.69	10.38
27	0.7	91.57	5.04	2.69	6.97	6.52	11.52	11.37
30	0.23	60.08	1.15	38.53	6.56	5.46	12.65	11.09
35	0.01	26.04	0.00	73.94	6.60	6.15	9.51	8.51
40	0.27	7.97	0.00	91.74	7.41	6.82	9.04	8.65

Reaction conditions: Meloxicam concentration 2.0 mg/20 ml reaction medium; Incubation period 5 days.
M1=5-carboxy meloxicam. C=culture control.
M2=5-hydroxymethyl meloxicam. T=meloxicam-fed culture.
M3=New metabolite.
M=Meloxicam remaining.

formed and only trace amounts of M_1 could be detected. In contrast to our findings, the maximum transformation of *N*-acetyl phenothiazine was achieved at pH 5.0 by *Aspergillus niger*, *Cunninghamella verticellata*, and *Penicillium simplicissimum* [29]. M_2 was the major compound formed at pH 6.0 and M_1 and M_3 were formed only in trace amounts. pH 3.0, 5.0, and 7.0 did not favor formation of M_3. At pH 7.0, only M_2 to an extent of nearly 31% could be detected. In contrast to our results, Hanson *et al.* [17] reported maximum transformation of mutilin by *S.griseus* and *C. echinulata* at pH 7.0. pH 8.0 and 9.0 favored nearly 50% transformation of meloxicam to all the three metabolites (*i.e.*, M_1, M_2, and M_3).

The biomass production was maximum at pH 5.0 in meloxicam-fed culture followed by at pH 4.0, which decreased with further increase of acidity or alkalinity. A marginal decrease in biomass production with addition of meloxicam was recorded. However, at pH 9.0, there was a significant increase in biomass production.

The pH of the medium was shifted towards the alkaline side because of the growth of *C. blakesleeana* and final pH recorded was near neutral. With a few exceptions, the final pH was low in the control. However, in medium containing meloxicam, the final pH recorded was near neutral.

Influence of Incubation Period

The incubation period influenced the transformation of meloxicam in an erroneous manner (Table 5). Five days of incubation was optimum, as the major amount of meloxicam was transformed. Present observations are in agreement with the report of Pothuluri *et al.* [31], who recorded that transformation of vinclozolin by *C. elegans* was dependent on incubation time. Similarly, Chatterjee and Bhattacharysa [7] have reported maximum transformation of limonene in a 5-day incubation period by employing *P. putida*. In contrast to our findings, a 36-h incubation period was optimum for transformation of *S*-naproxen by *A. niger* ATCC 9142 [20]. Transformation of meloxicam was also maximum in a 16-day incubation period. The increase of transformation of meloxicam after 16 days of incubation may be attributed to the activity of enzymes released as a result of the lysis of cells or due to reversible reactions. At the end of 16, 18, and 20 days of incubation, M_2 was formed in maximum amounts. Maximum transformation of trachyloban-19-oic

Table 4. Effect of pH on transformation of meloxicam by *C. blakesleeana.*

pH	Metabolites in %				Final pH		Dry weight (mg/20 ml)	
	M_1	M_2	M_3	M	C	T	C	T
3.0	0.00	40.52	0.00	59.47	5.25	6.58	9.09	5.94
4.0	3.29	39.56	3.73	53.4	6.78	5.75	12.61	11.31
5.0	0.01	26.04	0.00	73.94	6.69	6.15	11.12	11.31
6.0	0.62	93.33	4.34	1.71	6.91	6.78	10.38	9.29
7.0	0.00	30.79	0.00	68.23	7.11	6.01	10.36	7.68
8.0	2.94	33.86	4.8	58.38	7.01	6.50	9.44	9.15
9.0	0.86	54.98	0.96	43.17	7.07	7.20	8.90	9.79

Reaction conditions: Meloxicam concentration 2.0 mg/20 ml reaction medium; incubation period 5 days.
M1=5-carboxy meloxicam. C=culture control.
M2=5-hydroxymethyl meloxicam. T=meloxicam-fed culture.
M3=New metabolite.
M=Meloxicam remaining.

Table 5. Effect of incubation period on transformation of meloxicam by *C.blakesleeana.*

Incubation (in days)	Metabolites in %			
	M_1	M_2	M_3	M
1	0.43	15.11	0.05	84.38
2	3.37	47.35	3.11	46.15
4	2.92	61.57	5.21	30.28
5	0.75	92.61	4.04	2.37
6	1.18	59.43	4.98	33.38
8	1.27	53.14	5.57	40.00
10	1.04	53.91	5.60	39.43
12	0.36	35.96	3.12	60.53
14	0.50	32.50	0.11	66.87
16	0.64	68.94	5.26	25.14
18	0.64	69.94	5.26	24.14
20	1.63	73.18	0.07	25.10

Reaction conditions: Meloxicam concentration 2.0 mg/20 ml reaction medium.
M1=5-carboxy meloxicam.
M2=5-hydroxymethyl meloxicam.
M3=New metabolite.
M=Meloxicam remaining.

acid by *Rhizopus stolonifer* was after a 20-day incubation period [36]. At the end of 12 and 14 days of incubation period, the formation of M_2 was nearly 30%. Only marginal difference in the formation of M_1 and M_3 could be recorded with the advancement of incubation period. No consistency was observed in the formation of M_1.

Effect of Solvents

The effects of different solvents on transformation of meloxicam by *C. blakesleeana* was studied and the results are presented in Table 6. Dimethylformamide (DMF) was found to be most favorable for transformation of meloxicam. DMF was also reported to be favorable for transformation of amoxapine by *C.elegans* [27]. DMF was found to be

Table 6. Influence of solvents on transformation of meloxicam by *C.blakesleeana.*

Name of solvent	Metabolites in %				Final pH		Dry weight (mg/20 ml)	
	M_1	M_2	M_3	M	C	T	C	T
Acetone	0.00	28.42	0.00	71.57	7.67	7.67	10.81	11.43
DMF	0.71	91.01	4.34	4.94	7.01	6.91	11.20	11.56
DMSO	0.00	55.89	0.00	44.10	7.91	7.40	11.74	11.61
Ethanol	0.00	50.61	0.00	49.38	7.81	7.29	19.15	14.02
Methanol	0.00	46.69	0.00	53.30	7.74	6.09	9.80	7.75
Propanol	0.00	59.47	0.00	40.52	7.62	6.86	11.74	11.58

Reaction conditions: Meloxicam concentration 2.0 mg/20 ml reaction medium; incubation period 5 days.
M1=5-carboxy meloxicam. C=culture control.
M2=5-hydroxymethyl meloxicam. T=meloxicam-fed culture.
M3=New metabolite.
M=Meloxicam remaining.

suitable for fungal transformation of many organic compounds (*viz.*, malachite green [6], trachyloban-19-oic acid [36], 7-methoxylated flavanones [22]). In contrast to our findings, efficient transformation of artemisinin by *C. elegans* was obtained by dissolving in methanol [30]. Acetone produced transformation to the least extent. The rest of the solvents supported intermediate degree of transformation.

Mycelial growth of *C. blakesleeana* was maximum when the drug was dissolved in ethanol, followed by DMSO and propanol. The growth was moderate in the presence of methanol. The final pH recorded was near neutral with all the solvents studied.

Glucose Concentration

The effect of glucose concentration on transformation of meloxicam by *C. blakesleeana* was studied by steadily increasing the concentration of glucose in basal medium and the results are presented in Table 7. No transformation could be observed in medium lacking glucose. At 0.5%

Table 7. Effect of glucose concentration on transformation of meloxicam by *C. blakesleeana.*

Glucose conc. (in %)	Metabolites in %				Final pH		Dry weight (mg/20ml)	
	M_1	M_2	M_3	M	C	T	C	T
0.0	0.00	0.00	0.00	100	8.15	8.71	5.13	1.70
0.5	0.00	44.95	0.00	55.04	7.58	7.43	5.68	4.06
1.0	0.98	38.08	0.00	60.93	7.21	7.67	5.54	5.08
1.5	1.71	73.12	0.00	24.98	7.98	7.97	6.93	6.38
2.0	0.7	92.05	3.99	3.25	7.01	7.00	11.5	11.13
2.5	1.85	86.17	0.00	11.97	7.32	7.64	11.95	9.89
3.0	0.28	80.54	2.79	16.37	6.26	6.29	13.30	13.93
3.5	2.61	71.52	0.27	25.58	5.85	5.06	15.99	13.68

Reaction conditions: Meloxicam concentration 2.0 mg/20 ml reaction medium; incubation period 5 days.
M1=5-carboxy meloxicam. C=culture control.
M2=5-hydroxymethyl meloxicam. T=meloxicam-fed culture.
M3=New metabolite.
M=Meloxicam remaining.

glucose concentration, nearly 45% of meloxicam was transformed to M_2. Transformation of meloxicam was maximum at 2% glucose concentration, where maximum amount of M_2 was formed. This was followed by 2.5% and 3% glucose concentrations. Interestingly, higher concentrations of glucose also produced M_3.

The mycelial growth was increased with the increase in the concentration of glucose in the medium. Addition of meloxicam to the medium that is devoid of glucose decreased the biomass production of *C. blakesleeana*. The final pH of the medium was slightly varied with glucose concentration and it was near neutral at all the glucose concentrations.

Studies on transformation of meloxicam by different microorganisms revealed that *A. niger* NCIM 589, *A. ochraceous* NCIM 1140, *C. blakesleeana* NCIM 687, *C. echinulata* NCIM 691, *C. elegans* NCIM 690, and *S. cerevisiae* NCIM 3090 could transform meloxicam into M_2, M_1, and M_3. The characterization of the transformants revealed that the organisms could perform oxidation reactions. Such oxidation of organic compounds has been carried out by a large number of microorganisms.

The extent of transformation was increased with time upto 5 days followed by a decrease and then an increase. The inconsistency of this transformation may be due to reversible reactions or release of enzymes from the cells upon cell lysis. More detailed investigations are desired in order to assess this pattern of transformation.

Some of the organisms under study could produce three compounds through different kinds of reactions, which suggest the involvement of more than one enzyme in these biotransformations.

As expected, the transformation of meloxicam by *C. blakesleeana* was influenced by several physical and biological conditions. The present organism could transform meloxicam readily when it was grown in glucose medium, whereas limited transformation could be recorded in nutrient broth. Such specificity to the medium for transformation was also reported by Medeiros *et al.* [24] for 10-deoxy artemisinin to its 7β-hydroxy derivative by *Mucor ramannianus*. Harshad and Mohan [18] have also reported specificity to the medium for biotransformation of (L)-citronellal to (L)-citronellol by *Rhodotorula minuta*.

The transformation of meloxicam was maximum at incubation temperature of 25–27°C and decreased both at lower and higher incubation temperatures, which indicates the requirement of an optimum temperature for the activity or induction of enzymes. Similarly, Chatterjee and Bhattacharysa [7] reported that transformation of limonene by *Pseudomonas putida* reached a maximum at 30°C. A temperature of 27°C was optimum for transformation of (L)-citronellal to (L)-citonellol by *Rhodotorula minuta* [18].

The pH of the medium is reported to be critical for metabolism by the organism, which may be attributed to its enzyme activity. The present study confirms this fact, as meloxicam transformation was maximum at pH 6.0, which decreased both with the increase of alkalinity or acidity.

The biochemical reactions are known to be affected by the solvent used to dissolve the substrate. Kreiner *et al.* [23] reported stereospecific biohydroxylations of protected carboxylic acids using ethanol as solvent and by employing *C. blakesleeana*. Solubilization of *N*-acetyl phenothiazine in DMF favored its transformation by using *Aspergillus niger*, *Cunninghamella verticillata*, and *Penicillium simplicissimum* [29]. Moody *et al.* [27] reported that DMF favored the transformation of amoxapine by *C. elegans*.

Glucose concentration was found to influence the extent of transformation of meloxicam by *C. blakesleeana*. The transformation of meloxicam was maximum at 2% concentration of glucose. Higher concentrations produced decreased transformations.

Generally, the transformation studies on organic compounds will be aimed either to widen the spectrum of activity [4, 10, 13, 14, 21, 25, 26] or to minimize the adverse effects [6, 15, 17, 19, 31–33, 39]. It may be aimed further to acquire additional physical properties to make an improved formulation from a drug molecule. Meloxicam is an established antiinflammatory agent with some side effects, such as signs of bleeding, signs of an allergic reaction, blurred vision, change in the amount of urine passed, difficulty of swallowing, severe heart burning, pain in throat, pain or difficulty in passing urine, stomach pain or cramps, swelling of feet or ankles, unexplained weight gain or edema, yellowing of eyes, diarrhea, dizziness, gas or heart burn, nausea, or vomiting. Moreover, there are no reports of its other biological activities so that it can be exploited in such fields. There are a number of new drugs of antiinflammatory activity that are safe and more effective. The testing for other activities of meloxicam is desired.

Present studies on transformation of meloxicam by different fungi revealed the possibilities of its giving rise to a variety of derivatives by employing different microorganisms. The screening studies revealed that meloxicam was transformed into a new metabolite along with the reported metabolites (*viz.*, 5-hydroxymethyl meloxicam and 5-carboxy meloxicam). Among all the fungi screened, *C. blakesleeana* could transform meloxicam to the maximum extent and has shown a strong reaction dependent on media, pH, temperature, incubation period, solvents, and concentration of glucose. Hence *C. blakesleeana* can be employed in biotransformation studies of meloxicam to produce metabolites in large quantities or to produce novel metabolites with improved biological activity.

The ability and parametric dependence of *Cunninghamella blakesleeana* to transform meloxicam into the known metabolites clearly indicates that, by this means, sufficient quantity of metabolites can be produced, isolated, and identified, and biological activity studies can be performed,

with the produts used as a standard for metabolite identification and to investigate the pharmacokinetic, pharmacological, and toxicological properties of meloxicam. The ability to produce a new metabolite in the study reveals the possibility of producing compounds with novel structures and biological activities.

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