Biotransformation of Meloxicam by *Cunninghamella blakesleeana*: Significance of Carbon and Nitrogen Source

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Abstract Influence of carbon and nitrogen source, on biotransformation of meloxicam was studied by employing *Cunninghamella blakesleeana* NCIM 687 with an aim to achieve maximum transformation of meloxicam and in search of new metabolites. The transformation was confirmed by HPLC and based on LC–MS–MS data and previous reports the metabolites were predicted as 5-hydroxymethyl meloxicam, 5-carboxy meloxicam and a novel metabolite. The quantification of metabolites was performed using HPLC peak areas. The results obtained indicate that glucose as carbon source, ammonium nitrate as nitrogen source, were found to be optimum for maximum transformation of meloxicam. The study suggests the significance of these factors in biotransformation of meloxicam using microbial cultures. The fermentation was scaled up to 1 l level.

Keywords Meloxicam · *Cunninghamella blakesleeana* · 5-Hydroxymethyl meloxicam · 5-Carboxymeloxicam · Fermentor · Carbon · Nitrogen

Microorganisms can transform a huge variety of organic compounds and this ability to carry out the key steps for the production of useful compounds has been recognized for many years. Many compounds of therapeutic and/or industrial interest are obtained by microbial transformations. These reactions are important routes for introducing

chemical functions into inaccessible sites of molecules and thereby produce rare structures. The biotransformation reactions can involve high degree of regio- and stereo-specificity and require mild reaction conditions. They can yield new drugs and existing drugs can be improved as to increased activity and decreased toxicity. Side-effects can be reduced and the stability can be increased by modification of the parent drug. In addition to this, microorganisms are able to perform a large variety of reactions, including some nearly inaccessible by chemical processes. The interest in bioconversion is mainly because the product of the process is more useful or valuable than the precursor used. The transformation of organic compounds using biocatalysts, cell organelles or whole cells are important processes in organic synthesis and have been widely used in the production of steroids, antibiotics, vitamins and other high valuable products [1]. Use of biocatalyst also minimizes the problems of isomerization, racemisation, epimerization and rearrangement that are common in chemical processes [2]. Biotransformation encompasses practically every type of chemical reaction possible and is extremely useful since some of these reactions can be carried out more economically.

Meloxicam is a non steroidal anti-inflammatory drug (NSAIDS) with a selective inhibition of cyclooxygenase-2 (Cox-2) [3]. It is effective in the treatment of rheumatoid arthritis [4] and osteoarthritis [5] and appears to be well tolerated due to its preferential inhibition of cyclooxygenase (Cox-2) [6]. Some of the reported side effects of the meloxicam include signs of bleeding, allergic reactions, blurred vision, difficulty in swallowing, severe heart burning, pain in throat, pain or difficulty passing urine, stomach pain, swelling of feet or ankles, unexplained weight gain or edema, yellowing of eyes, of skin, diarrhea, dizziness, gas or heart burn, nausea or vomiting [7].

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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 [8].

In our previous work we reported the transformation of meloxicam by the filamentous fungus *Cunninghamella blakesleeana* NCIM 687 into three metabolites viz., 5-hydroxymethyl meloxicam, 5-carboxy meloxicam and a novel metabolite, and the effect of various parameters like pH, temperature, media, incubation period, influence of solvents and glucose concentration [9].

In the present study, effect of carbon and nitrogen source on biotransformation of meloxicam were studied for maximum transformation of meloxicam and to achieve novel metabolites. The fermentation was also scaled up to 1 l fermentor level.

Materials and Methods

Chemicals and Microorganism

Meloxicam was gifted by Unichem Laboratories Mumbai, India. Methanol and acetonitrile were of HPLC grade obtained from Ranbaxy, New Delhi, India. Peptone, yeast extract, potato dextrose agar, glucose and all other chemicals of highest available purity were obtained from Himedia, Mumbai, India. The culture *Cunninghamella blakesleeana* NCIM 687 was procured from National Collection of Industrial Microorganisms (NCIM), Pune, India. Stock cultures were maintained on potato dextrose agar slants at 4°C and subcultured for 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 consists of 50 ml of liquid broth. The liquid broth used contain (per litre) 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 prepared media was autoclaved and cooled to room temperature. The media was inoculated with a loopful of culture obtained from freshly grown potato dextrose agar slants. The flasks were incubated at 120 rev/min 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 second stage cultures were incubated for 24 h and the substrate meloxicam (2 mg) in dimethyl formamide (200 μ l) 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 of meloxicam added to the sterile medium were incubated under similar conditions. Each culture was studied in triplicate. The cultures were extracted with three volumes of ethyl acetate and 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 LC–MS–MS for identification of metabolites.

Influence of Carbon and Nitrogen Sources

Influence of carbon source was studied by replacing the glucose (20 g) of the medium with different carbon sources viz., citric acid, D-fructose, D-galactose, D-mannose, glycerol, lactose, L-sorbose, maltose, manitol, sorbitol, starch, sucrose and xylose separately so as to get equal number of carbon atoms.

The influence of nitrogen source was studied by replacing the peptone of the medium with different nitrogen sources viz., ammonium nitrate, ammonium chloride, ammonium dihydrogen ortho phosphate, ammonium molybdate, ammonium nitrate, ammonium oxalate, ammonium sulfate, barium nitrate, bismuth nitrate, calcium nitrate, peptone, potassium nitrate, sodium nitrate, thiourea and urea. These nitrogen sources were added so as to provide same number of nitrogen atoms when compared with 3.5 g of potassium nitrate.

Biotransformation of Meloxicam in Laboratory Fermentor

Biotransformation was carried out in a 1 l stirred jar bench top fermentor 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 into the media and the fermentor was run for 24 h. 0.02% of meloxicam was added as a solution in dimethyl formamide and the fermentor was run for further 7 days. The fermentor contents were extracted with three volumes of ethyl acetate and analyzed for the presence of metabolites.

Analysis

HPLC analysis was performed according to the method described by Elbary et al. [10] with a slight modification. The samples were analysed using an LC-10AT system (Shimadzu, Japan) by injecting 20 μ l of 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 methanol and water (pH adjusted to 3.0 with orthophosphoric acid) in the ratio of 60:40. 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). 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 60:40 ratios. 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.

Results and Discussion

In our previous work [9] we reported that the fungus *Cunninghamella blakesleeana* was found to transform meloxicam into three metabolites: 5'-hydroxy methyl meloxicam (M2), 5'-carboxy meloxicam (M1) and a new metabolite (M3). In the present investigation the fungus was found to produce similar metabolites in presence of all the parameters studied. The pathway containing the structures of the metabolites was shown in Fig. 1.

Influence of Carbon Source

Carbon is the major structural and functional component of microbial cells and plays an important role in the nutrition of fungi. A wide variety of carbon sources including carbohydrates, organic acids and amino acids, along with their derivatives and some polycyclic compounds and alkaloids are used by fungi as source of carbon. Previous reports demonstrated that the quantitative effect of glucose, dextrose, sucrose and fructose on fungal transformation of

aromatic and heterocyclic substrates viz., 4-ethylphenol, sclareol, cinobufagin, 3-hydroxy flavone, 10-deoxoartemisinin and *O*-nitro styrene was significant [11–16].

The above facts influenced the author to study the effect of carbon source on transformation of meloxicam by *C. blakesleeana* by substituting glucose of the basal medium with different carbon substances so as to supply equal amount of carbon and the results are summarized in Table 1. Citric acid, mannitol, D-mannose, L-sorbose and sucrose were responsible for total inhibition of transformation of meloxicam. Parshikov et al. [17] by using sucrose and glucose as carbon source reported maximum transformation of artemisinin by *C. elegans*. D-glucose followed by D-galactose favoured transformation of most of the meloxicam added to the medium, while rest of the carbon sources supported intermediate degree of transformation. Glycerol induced nearly 30% of transformation of meloxicam. Glucose, galactose and maltose were most favourable carbon source for transformation of meloxicam to 5'-OH methyl meloxicam (M2). Higher hydroxylation product of mutilin by *S. griseus* and *C. echinulata* was also achieved with glucose as carbon source compared to glycerol [18]. *O*-Demethylation and sulfation of 7-methoxylated flavanones by *C. elegans* was achieved when dextrose and glycerol used as carbon source [19]. Choudary et al. [20] have reported maximum transformation of mestronol by *C. elegans* when glucose was used as carbon source. Lactose could facilitate only marginal transformation of meloxicam into 5'-OH methyl meloxicam (M2). Starch favoured for nearly 60% transformation of meloxicam. Xylose and sorbitol were responsible for transformation of meloxicam to 5'-OH methyl meloxicam (M2) only.

Influence of Nitrogen Source

Nitrogen is also used for functional and structural development by the fungi. Thus the source of nitrogen could play a profound influence on the biotransformation by

Fig. 1 Proposed metabolic pathway of meloxicam in *Cunninghamella blakesleeana*

Table 1 Effect of carbon source on transformation of meloxicam by *C. blakesleeana*

Carbon source	Metabolites in %			
	M1	M2	M3	M
Citric acid	0.00	0.00	0.00	100
D-Fructose	4.85	53.84	1.75	40.05
D-Galactose	6.78	64.55	1.54	27.12
D-Glucose	4.68	66.73	4.30	24.28
D-Mannose	0.00	0.00	0.00	100
Glycerol	0.45	19.80	0.25	79.41
Lactose	0.00	0.50	0.00	99.49
L-Sorbose	0.00	0.00	0.00	100
Maltose	0.23	60.09	1.14	38.53
Mannitol	0.00	0.00	0.00	100
Sorbitol	0.00	20.06	0.00	79.00
Starch	3.84	52.23	3.37	40.55
Sucrose	0.00	0.00	0.00	100
Xylose	0.00	18.17	0.00	81.82

M1 5-carboxy meloxicam, M2 5-hydroxymethyl meloxicam, M3 new metabolite, M meloxicam

fungi. Literature is depleted with conflicting claims regarding the comparative superiority of a particular form or source of nitrogen over the other. Farooq et al. [21] found that sodium nitrate as nitrogen source produced transformation of (–) α -pinene to maximum extent by *Botrytis cinerea*. Diez et al. [12] by using ammonium nitrate as nitrogen source, reported maximum transformation of sclareol by *Rhizopus stolonifer*.

Basing on the above reports on the effect of nitrogen source, it was felt worthwhile to study the effect of nitrogen source on the biotransformation of meloxicam by *C. blakesleeana*. Influence of nitrogen source on transformation of meloxicam by *C. blakesleeana* was investigated by substituting peptone of the basal medium with different nitrogen source so as to supply equal amount of nitrogen (equivalent to 3.5 g of potassium nitrate) and the results are depicted in Table 2. Nitrogen source present in the medium exerted significant influence on transformation of meloxicam by *C. blakesleeana*. Ammonium nitrate followed by ammonium chloride was the most favoured nitrogen source for transformation of major amount of meloxicam. Incidentally in all the nitrogen sources, 5'-OH methyl meloxicam (M2) was formed in large amount except with *p*-nitro benzoic acid. In the medium containing *p*-nitro benzoic acid, higher amount of 5' carboxy meloxicam (M1) was detected. The new metabolite (M3) could not be recorded in medium containing ammonium acetate, ammonium dihydrogen ortho-phosphate, ammonium molybdate, ammonium nitrate, ammonium oxalate, calcium nitrate, *para*-nitro benzoic acid and sodium nitrate. Sodium nitrate

Table 2 Effect of nitrogen source on transformation of meloxicam by *C. blakesleeana*

Nitrogen source	Metabolites in %			
	M1	M2	M3	M
Ammonium acetate	2.54	83.93	0.00	13.52
Ammonium chloride	0.37	88.81	2.35	8.45
Ammonium dihydrogen phosphate	0.10	84.16	0.00	17.85
Ammonium molybdate	0.72	29.33	0.00	69.94
Ammonium nitrate	3.11	89.42	0.00	7.46
Ammonium oxalate	0.09	67.03	0.00	32.86
Ammonium sulphate	0.45	83.97	2.06	13.50
Barium nitrate	0.30	66.86	1.56	31.26
Bismuth nitrate	0.02	45.44	0.33	54.20
Calcium nitrate	0.40	85.30	0.00	14.29
<i>Para</i> -nitro benzoic acid	57.49	27.31	0.00	15.18
Peptone	4.68	66.73	4.30	24.27
Potassium nitrate	0.07	85.43	1.23	13.27
Sodium nitrate	0.06	85.91	0.00	14.01
Thio urea	0.40	60.22	1.35	38.01
Urea	0.29	81.32	2.14	16.22

M1 5-carboxy meloxicam, M2 5-hydroxymethyl meloxicam, M3 new metabolite, M meloxicam

induced nearly 86% production of 5'-OH methyl meloxicam and traces of 5'-carboxy 22 meloxicam. By using sodium nitrate as nitrogen source Hu et al. [22] reported transformation of 2 α ,5 α ,10 β ,14 β tetra acetoxo-4(20), 11-taxadiene by employing *C. elegans* and *C. echinulata*. Ammonium sulfate favoured formation of 84% of 5'-OH methyl meloxicam, trace amounts of 5'-carboxy meloxicam (M1) and the new metabolite (M3) from meloxicam. By using ammonium sulphate as nitrogen source, Rosche et al. [23] reported the transformation of benzaldehyde into *R*-phenyl acetyl carbinol by *Rhizopus javanicus* and *Fusarium* sp. Yoshihara et al. [24] served ammonium sulphate as nitrogen source for bioconversion of D-psicose to *T*-tagatose and D-talitol by *Rhizopus oryzae*. Overall, 5'-carboxy meloxicam (M1) was formed in low amount when compared to M2 and M3.

Fermentor Studies

In the industrial processes, screening of organisms for their potential to produce a product is the primary task. Subsequently the conditions under which it can be carried out will be of second step. Before going to large scale production, the lab scale workup in a Fermentor and operation of 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 get

the product in large quantity. Providing these conditions for large scale production using fermentor will be the primary task to produce the product in an economical quantity. Successful scale-up in biotransformations using fermentor were reported previously for protected carboxylic acids [25], mutilin [18] and few other organic substrates.

The present investigation of transformation of meloxicam by *C. blakesleeana* revealed its potential to transform meloxicam into 5'-OH methyl meloxicam. The conclusions by these laboratory experiments have formed the basis for large scale production in a fermentor. Transformation of meloxicam by *C. blakesleeana* in a laboratory fermentor by providing optimal conditions (pH 6.0, temperature 30°C, glucose as carbon source and KNO₃ as nitrogen source) was tried, the medium was analysed for transformed products.

In the fermentor run, at the end of 7 days incubation period, it was revealed that about 30% of meloxicam remained unutilized by *C. blakesleeana* and only one transformed product, 5'-OH methyl meloxicam was detected in large quantity. About 70% of meloxicam was transformed to 5'-OH methyl meloxicam and no other compounds could be detected.

From the present investigation it is clear that *C. blakesleeana* can be employed as a biotransformant of meloxicam to produce 5'-OH methyl meloxicam in ecofriendly and economic manner.

The studies indicate that meloxicam could be transformed to 5-hydroxy methyl meloxicam, 5-carboxy meloxicam and a new metabolite using *C. blakesleeana* in an ecofriendly way. The present investigation reveals the importance of carbon source, nitrogen source, for optimum biotransformation of meloxicam. Among the factors studied, the use of suitable carbon source and nitrogen source were found to be critical for the development of a biotransformation system. Further investigations are needed to produce meloxicam metabolites in large quantities by optimizing the fermentor conditions.

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