International Journal of Current Advanced Research
ISSN: O: 2319-6475, ISSN: P: 2319 - 6505, Impact Factor: SJIF: 5.995
Available Online at www.journalijcar.org
Volume 6; Issue 7; July 2017; Page No. 4594-4597
DOI: http://dx.doi.org/10.24327/ijcar.2017.4597.0541

Research Article

BIOTRANSFORMATION OF DICLOFENAC BY THERMOPHILIC COPROPHILOUS FUNGUS *SCYTALIDIUM THERMOPHILUM* ISOLATED FROM SHEEP DUNG

Ajmera ShanthiPriya[1]*., Girisham S[1]., Shyam Prasad G[1] and Chandra Sekhar Rao D[2]

[1]* Department of Microbiology, Palamuru University, Mhabubnagar-509001
[1]Department of Microbiology, Kakatiya University, Warangal-506009, Telangana State, India
[2]Department of Chemistry, Kakatiya University, Warangal-506009, Telangana State, India

A R T I C L E I N F O

Article History:

Received 13th April, 2017
Received in revised form 7th May, 2017
Accepted 19th June, 2017
Published online 28th July, 2017

Key words:

Diclofenac, Scytalidium thermophilum, HPLC, LC-MS, coprophilous fungi.

A B S T R A C T

Biotransformation of Diclofenac was performed by *Scytalidium thermophilum* isolated from sheep dung at pH 6.0, a temperature of 45±°C, in 4 day incubation period. By morphological features and 28S rDNA sequencing, the strain was identified as *Scytalidium thermophilum*. The metabolites were confirmed by HPLC analysis of the extract of experimental flasks. The transformation of Diclofenac was identified by LC-MS. The results prove that thermophilic coprophilic fungus S. thermophilum is a promising fungus for diclofenac biotransformation. Hence, this fungus can be explored for the synthesis of biotransformation products.

INTRODUCTION

The coprophilous mycota is a distinct community with morphologically and physiologically specialized and play an important role in the decomposition and recycling of organic wastes (Richardson, 2008). These are a subgroup of saprophytic fungi that can inhabit feces or herbivorous animal dung most commonly herbivore (Webster *et al.,* 2007). Coprophilous fungi have not only interesting life cycles, but are often aesthetically very attractive. These fungi spend their entire life the droppings of herbivorous animals. Animals while grazing on vegetation ingest many spores of these fungal along with their food and pass through the gut and excreted in the dung (Bell, 2003). These fungi produce thick-walled, pigmented spores that require passage through the gut of an animal to germinate (Bell, 2003), breaking their dormancy. There have also selecting advantages of giving resistantance to temperature and digestive enzymes. The forceful discharge of these spores will facilitate their reaching distant places and other animal during grazing. Microorganisms have the ability to transform a variety of organic compounds to a distinct product with structural similarity in an eco-friendly and economical way. The Microbial process can make reactions feasible that are not likely to be carried out in other methods.

Corresponding author:* **Ajmera ShanthiPriya
Department of Microbiology, Palamuru University, Mhabubnagar-509001

Currently a wide range of enzymatic reactions was adapted for synthesis of pure intermediates for pharmaceutical purposes, for the synthesis of different chemical alcohols, carboxylic acids, diamines, terminal olefins, and other important chemicals, conversion of biogenic amino acids into platform chemicals such as styrene and acrylamide Prasad *et al*.,.The use of coprophilous fungi for biotransformation of different substances into the desired products remains unexplored. Owing to the importance of reaction, the present investigation is directed to isolate coprophilous fungi for their potential to perform diclofenac biotransformation.

MATERIALS

Diclofenac was gifted by Unichem labs, Mumbai, India. Methanol, Acetonitrile was of HPLC grade obtained from Ranbaxy Laboratories Ltd., New Delhi, India. Yeast extract, starch, $MgSO_4$, K_2HPO_4, Agar agar and other chemicals were obtained from Himedia Labs, Mumbai, India.

METHODS

Isolation and screening of fungi

Different source of sheep dung were collected from Warangal district of Telangana, India in sterilized polyethylene bags and were brought to the laboratory and analyzed for the presence of fungi capable of diclofenac transformation using paired plate method (Cooney and Emerson 1964). In this method, two sterile Petri plate lids were taken and top plate inner side was fixed with sterile filter paper and the fungal source was

serially diluted by taking 1g in 250ml of Erlenmeyer conical flask containing 100 ml of sterilized distilled water and shaken thoroughly. From this solution, serial dilutions were prepared and later plated on yeast extract starch agar (YESA) (Starch, 30g; yeast extract, 5.0g; $MgSO_4$. $7H_2O$, 0.5 g; KH_2PO4, 1.0g; agar -agar, 20g, and distilled water 1000ml). The paired plate is thus sealed with cellophane tape to prevent moisture. The plates, thus prepared were incubated in an inverted position at 45± 2˚C for 4 days. The fungal colonies developed on Petri plates were isolated and transferred to YESA slants, allowed to grow and stored at 4°C for further use.

The fungal cultures which appeared on Petri plates were isolated and screened individually for of diclofenac. First stage fermentation was carried out in 100 ml flask with 20 ml of broth containing (per liter) glucose (20 g), peptone (5 g), yeast extract (5 g), K_2HPO_4 (5 g), and NaCl (5 g), pH 6.0. The sterilized medium was inoculated with fungi from 7d old culture. At the end of incubation, the substrate diclofenac was added aseptically and incubated under similar conditions for 4 days.

Extraction and analysis of diclofenac metabolite

After the incubation period, the culture flasks were extracted with ethyl acetate and evaporated to dryness. The residues were analyzed by HPLC for confirmation of diclofenac biotransformation and the metabolite was identified by LC-MS.

HPLC and LC-MS analysis

HPLC analysis was performed by injecting 20µl of the sample into a syringe loading sample injector. The mobile phase consists of a mixture of acetonitrile, methanol, water (40:40:20 ratio).Column used was Wakosil II C18, 5 µ, and 250×4.6-mm i.d. (SGE, USA). The analysis was performed at a flow rate of 1 ml/min, and diclofenac and its metabolite was detected by SPD-M10Avp, Shimadzu at a wavelength of 274 nm. The calculations were performed with respect to the total area of drug and metabolite. LC-MS analysis was performed using SCIEX API-4000, Q-TRAP, Canada system with MS/MS API-4000, Q-Trap detector. Waters C18, 4.6×250 mm, 5µm, was the column used with the same mobile phase as HPLC (pH adjusted to 3.0). The instrument was operated in electrospray ionization (ESI) mode. The scan range of 120-440 and a temperature of 300^{o}C were set for the analysis. Analyst 1.4.2 software was used for acquiring and processing the data. The structure of the transformed compound was elucidated by interpreting the m/z value of the metabolite peaks obtained in LC-MS.

Morphological identification of fungi

Microscopic features of isolated promising strains such as hyphae, conidiophores, branching conidia, pigmantation etc. were determined using a microscope (X40).

Identification of isolated fungal culture by Partial 28S rDNA gene sequence

For identification of isolated fungal culture, total genomic DNA was isolated from the fungal mycelial mat as described (Vonarx *et al*., 975). DNA concentrations were determined by measuring the absorbance at 260 nm. Further, D2 region of LSU (large subunit 28S rDNA) gene fragment was amplified using primers: DF: 5'-ACCCCGCTGAACTTAAGC-3' and 'DR: 5'-GGTCCGTGTTTCAAGACGG-3' respectively (Matsuda *et al*., 1999; Gillespie, 2005). The amplified products were purified with a Quiagen quick PCR purification kit (Qiagen Inc., Chatsworth, USA) and sequenced by methods as described earlier. Chimeric sequences were discarded using Gene Tool version 2 (www.biotools.com) and the sequences were then subjected to BLAST (Jarvis *et al*., 2006) to identify the nearest taxa and aligned with sequences, belonging to the nearest taxa using CLUSTALW (Sarkar, 2013). Phylogenetic trees were constructed using the Neighbour Joining method using the Kimura 2-parameters (Altschul *et al*., 1990) using MEGA4 software.

RESULTS AND DISCUSSION

In the present study, thermophilic coprophilous fungi capable of diclofenac transformation were isolated. Four fungal cultures were found to grow on YES culture plates. Among the fungi screened, only one fungus could transform of diclofenac while, rest of the fungal culture could not perform any transformation. Based on the morphological and molecular features, the promising fungus was identified as *Scytalidium thermophilum.*

Morphological Identification of fungal strains

Colonies of *S.thermophilum* at 45±2 ˚C were very fast growing. Inhibition of growth of fungus was observed at 25°C and a slight growth was seen at 30°C. Colonies were white later turns to grayish, to jet-black. Hyphae are colorless branched, septate. Conidiogenous cells are small, smooth walled, generally globose, oval produced basipatally in chains on hyphal branches or developed intercalarily. By comparing above morphological features with standard manual (Cooney and Emerson, 1964) the fungus identified as *Scytalidium thermophilum*

In the study, molecular identification of the fungus was done by extracting DNA and tested using a molecular assay. A PCR amplified product of the expected size (700 base pairs) has been sequenced. The Blast analysis (NCBI) confirmed its identity as *S.thermophilum* with 100 % similarity at the species level. Along with molecular studies, the morphological characteristics of the culture isolate (KT253886.1) also confirmed its identity as *S.thermophilum.*
The fungal culture *S.thermophilum* biotransformed diclofenac. Biotransformation reaction employing microorganisms (biocatalysts) is increasingly being used industrially. Many researchers have reported commercially valuable products by biotransformation reactions employing microbial cultures. Devi *et al*. (2006) reported biotransformation of citrinin, using Moraxella sp. Yoshida *et al*. (1984) reported biotransformation of Gallic acid. Similarly, Pyruvate decarboxylase, enzyme is capable of acyloin-type condensation reactions leading to the formation of chiral α-hydroxy ketones, which are versatile building blocks in the pharmaceutical and chemical industries. Gabmeyer *et al*. (2016) reported microbial biotransformation of arylmalonates to important pharmaceutical ingredient flurbiprofen.

Identification of diclofenac metabolites

The metabolite of diclofenac was identified by HPLC (Fig. 1). Observation of new peak in experimental flasks confirms that

biotransformation of diclofenac was aided by thermophilic coprophilous fungal culture *S.thermophilum* with 46% yield.

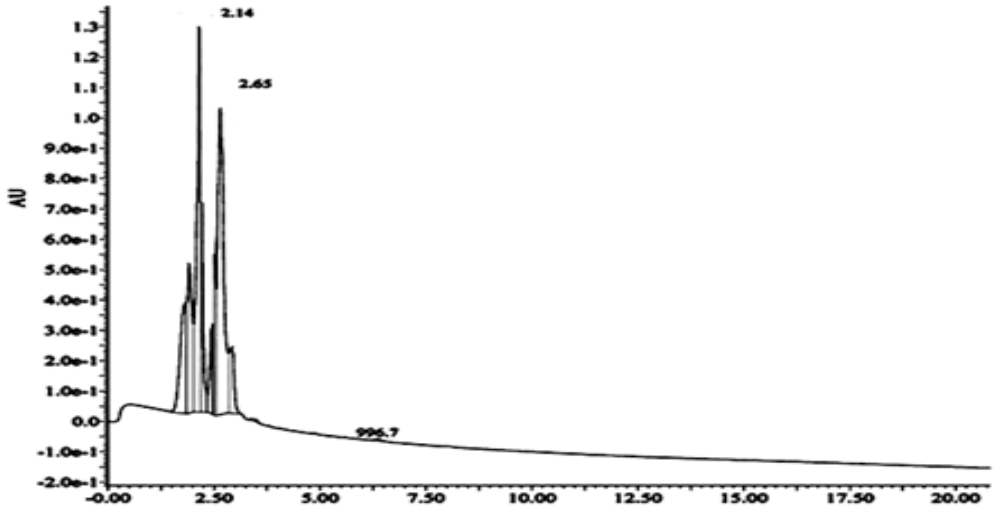

Fig 1 HPLC chromatogram showing diclofenac and its metabolite from *Scytalidium thermophilum*

The metabolite of diclofenac was characterized by mass value of obtained in LC-MS analysis. Mass spectrometric analysis showed a molecular ion at m/z =239 indicating the reaction to be biotransformation, removal resulting in formation of 2, 6-(Dichloro-phenyl)-phenyl l amino diclofenac (fig.2).

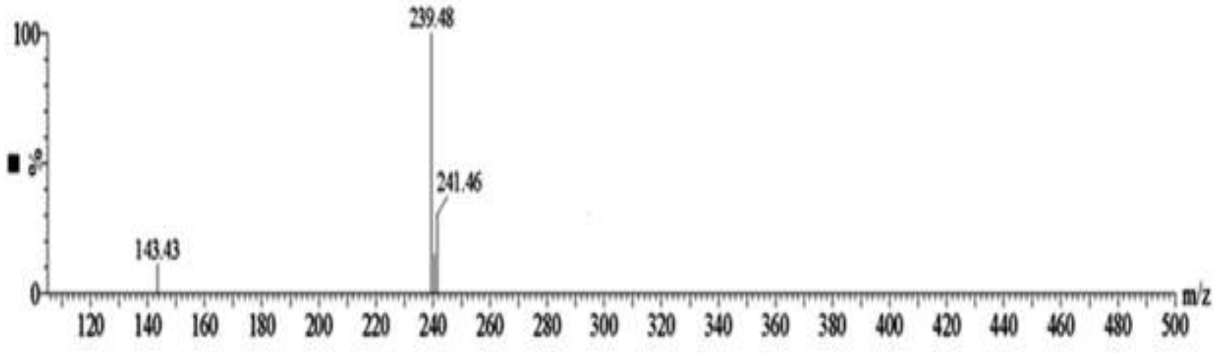

Fig 2 LC-MS spectra of Diclofenac and its metabolite

Production of chemicals and bioactive compounds using biological agents such as microorganisms or isolated enzymes is being considered as a superior alternate. Several yeasts and bacterial carboxylases have been evaluated for their potential in biotransformation for the synthesis of many products viz. Hydroxy ketones, cz-aryl acetic acid and D-amino acids, dopamine and organic acids. Several other decarboxylases from microbial sources like benzoyl format decarboxylase (*Pseudomonas putida, P. aeruginosa, Acinetobacter calcoaceticus*), pyruvate decarboxylase (*Saccharomyces cerevisiae, Kluyveromyces* marxianus, *Neurospora crassa, Zymomonas mobilis*), alpha acetolacetate decarboxylase (*Klebsiella aerogenes, Lactococcus lactis, Bacillus subtilis*), phenylpyruvate decarboxylase (*Achromobacter eurydice, Acinetobacter calcoaceticus, Thauera aromatica*) and arylmalonate decarboxylase (*Alcaligenes bronchisepticus*) used in chemoenzymatic synthesis have been compiled.

CONCLUSION

Microbial metabolism of diclofenac was reported previously employing mesophilic fungal cultures which were able to produce 4′-hydroxy metabolite of diclofenac 3′-hydroxy,4′-hydroxy, and 5′-hydroxy metabolites of diclofenac using the microbial cultures *Paecilomyces farinosus, Arthrinium phaeospermum, Mucor plumbeus, Scytalidium sp.,* *Pestalotiopsis sp., and* production of 4-OH diclofenac using *Epiccocum nigrum* was reported. In the present study, the thermophilic coprophilic fungus metabolic pattern is different in comparision to all other fungus.

The thermophilic coprophilic fungal culture *S.thermophilum* performed biotransformation reaction. Hence, the culture can also be explored for the synthesis of many biotransformed products in an ecofriendly method.

Acknowledgements

The authors are thankful to Head, Department of Microbiology, Kakatiya University, Warangal, Telangana, India for providing the necessary facilities.

References

1. Altschul SF, Gish W, Miller W, Myers E W, Lipman D J, Basic local alignment search tool; *J Mol Biol*; 1990; 215; 403-410.
2. Bell; Dung Fungi: An Illustrated Guide to Coprophilous Fungi in New Zealand; (Victoria University Press, Wellington).
3. Cooney DC, Emerson R; In: Thermophilic fungus: An account of their Biology, Activities and Classification (W.H. Freeman and Co., San Francisco); 1964; 1-188.

4. Devi P, Naik CG, Rodrigues C; Biotransformation of citrinin to decarboxycitrinin using an organic solvent tolerant marine bacterium,Moraxella sp.(MB1); *J Mar Biotechnol*;2006;8;129-138.
5. Gabmeyer SK, Wetzig J, Mugge C, Assmann M, Enoki J, Hilterhaus L, Zushe R, Miyamoto K, Liese A, Kourist R, Arylmalonate Decarboxylase-catalyzd Asymmetric synthesis of both enantiomers of optically pure flurbiprofen; 2016; 8;916-921.
6. Gillespie JJ, James B, Munro J B, Heraty J M, Yoder M J, Owen A K, Carmichael A E;A Secondary Structural Model of the 28S rRNA Expansion Segments D1/D2 and D3 for Chalcidoid Wasps (Hymenoptera: Chalcidoidea); *Mol Biol Evol*; 2005; 22;593-1608.
7. Jarvis BW, Wickes B L, Hoffman LM;direct genomic DNA sequencing for rapid fungal identification (ASM meeting Orlando); *J Clin Microbiol*; 2010; 48(3); 741-752.
8. Matsuda Y, Hijii N*;* Characterization and identification of *Strobilomyces confusus* ectomycorrhizas on Momi fir by RFLP analysis of the PCR-amplified ITS region of the rDNA*; J For Res*; 1999; 4; 145- 150.
9. Prasad GS, Girisham S, Reddy S M;Studies on microbial transformation of albendazole by soil fungi; *IJBT*;2009;8;425-429.
10. Richardson MJ; Coprophilous fungi. *Field Mycol*; 2003; 4(2); 41-43.
11. Sarkar S, Girisham S, Reddy S M;Identification of three fruit rot fungi of banana by 28S ribosomal DNA sequencing; *IJB*; 2013;2;422-429.
12. Webster J; *Fungal Ecology*; London: Chapman and Hall; 1995.
13. Yoshida H, Yamada H;Microbial production of pyrogallol through biotransformation of gallic acid; *Agric Biol Chem*;1984;49;659-663.

How to cite this article:

Ajmera ShanthiPriya *et al* (2017) 'Biotransformation of Diclofenac By Thermophilic Coprophilous Fungus Scytalidium Thermophilum Isolated From Sheep Dung', *International Journal of Current Advanced Research,* 06(07), pp. 4594-4597. DOI: http://dx.doi.org/10.24327/ijcar.2017.4597.0541

****