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THE ENANTIOSELECTIVE OXIDATION OF HETEROCYCLIC PROCHIRAL SULFIDES TO CHIRAL SULFOXIDES USING THE FUNGAL BIOCATALYSTS

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ABSTRACT

Bioconversion of sulfoxidation of sulfides is gaining importance in synthetic chemistry as an alternative tool for the synthesis of enantiomeric sulfoxides.

Different micro organisms belonging to the class of fungi were selected for the study. The selected microorganisms include *Rhyzopus stolonifer* MTCC 2198, *Rhizopus stolonifer* MTCC 2591, *Rhizopus stolonifer* MTCC 162, *Aspergillus niger, Aspergillus ochraceous, Aspergillus flavus, Saccharomyces cerevisiae* (Soil isolates) and *Baker's yeast* (Locally purchased).

The substrate chosen for biosulfoxidation was 5-Methoxy-2-[[(4-methoxy–3, 5- dimethyl-2-pyridinyl) methyl]-sulphinyl]-1H benzimidazole.

Among the selected microorganisms used for biosulfoxidation of the above mentioned substrate, *Rhizopus stolonifer* 2591 showed maximum conversion. *Rhizopus stolonifer* 2198 and *Aspergillus ochraceous* showed less conversion while no conversion was observed with *Aspergillus niger, Aspergillus flavus, Baker's yeast and Rhizopus stolonifer* 162. Hence, *Rhizopus stolonifer* 2591 was

considerd for optimization. The parameters evaluated during optimization include pH, temperature, substrate concentration, biomass concentration, incubation time and effect of surfactants.

The optimized conditions at which maximum bioconversion was achieved were at pH 7.6, temperature 30˚C, incubation time 48hrs, substrate concentration 2mg and biomass concentration 10g for *Rhizopus stolonifer* 2591. Various surfactants were used in the study, among them anionic (SLS) and cationic (CTAB) surfactants were found to be toxic while very low conversion was observed in case of nonionic surfactant (Tween 80). The hydrophilic carrier (β-Cyclodextrin) did not have any significant effect on the yield of the product.

Keywords: ***Rhizopus stolonifer, Aspergillus niger, Aspergillus ochraceous, Aspergillus flavus, Saccharomyces cerevisiae, Baker's yeast,*** **Biosulfoxidation, Optimisation**

INTRODUCTION

Enantiopure drugs are of great importance in modern pharmaceutical industry because the racemates of some pharmaceuticals have shown unwanted effects in clinical practice. A worldwide sale of chiral drugs in single enantiomer dosage forms continue to grow at more than 13% annual rate and have reached $200 billion market, as of today.

The manufacture of single enantiomers of optically active compounds has become critical since chirality is a crucial element in the efficacy of many medicinal products. As a result, the industry is adopting the practice of producing the drug's active form. Despite significant scientific and technological advancements, several chiral synthesis methods remain challenging and costly **[1-3]**. The manufacture of the active form of the drug is consequently becoming a custom in the industry. Despite of tremendous scientific and technological advances, chiral synthesis still remains complicated and expensive.

Biocatalysis has often emerged as a viable alternative. It is well known that microorganisms are useful biocatalysts for asymmetric synthesis. These biocatalysts catalyze the reactions under mild and economically viable conditions in an eco-friendly environment when compared to chemical reactions **[4]**. Among the microorganisms most commonly employed for sulfoxidation are the fungi *Mortierella isabellina* NRRL 1757 (ATCC 42613), and *Helminthosporium* species NRRL 4671; the former organism has also been extensively examined for its ability to perform the benzylic hydroxylation reaction **[5]**.

Hence, the present work is to focus on identifying potential microorganisms, which can be used as alternative for chemical oxidizing agents, to get an enantiopure

product to meet the industrial demand at low cost. The present work relates to microbial sulfoxidation of 5- Methoxy -2- [(4- methoxy –3, 5-dimethyl-2-pyridinyl) methyl] - sulphinyl]-1H benzimidazole, which is a drug intermediate in the synthesis of Omeprazole, a proton pump inhibitor **[6]**. Several fungi were screened for their capacity to bring about the sulfoxidation **[7]**.

Chiral sulfoxides have been asymmetric auxillary group that assist various stereoselective reactions. The sulfoxide functional group activates adjacent C-H bonds toward attack by a base & the resulting anions can be alkylated or acylated with high diasteroselectivity. Thermal elimination & reduction of keto sulfoxides can proceed with transfer of chirality from sulfur to carbon. Inspite of this with rare exceptions, no general method is available for the synthesis of sulfoxides with high enantiomeric purity **[8, 9]**.

In this present work, we will edge on discussing the bioconversion methods for oxidation of sulfides over synthetic methods; usuage of biocatalysts is undoubtedly gentle method and eco friendly for the synthesis of sulfoxides **[10]**.

MATERIALS AND METHODS

Microorganisms:

1. *Saccharomyces cerevisiae MTCC 174:*

Saccharomyces cerevisiae MTCC 174 was obtained from MTCC, Chandigarh. The organism was maintained on Yeast extract peptone dextrose growth medium (YEPD) media containing.

Yeast Extract	**3.0 g**
Peptone	**10.0 g**
Dextrose	**20.0 g**
Agar	**20.0 g**
Distilled water	**1000 ml**

***2. Aspergillus niger*, *Aspergillus flavus*, *Aspergillus ochraceous* (soil isolate) [11]:**

The organisms were isolated from soil and maintained in microbiology lab. These organisms were maintained on MRBA media containing:

Dextrose	**10.0 g**
Peptone	**5.0 g**
Potassium dehydrogenate phosphate	**1.0 g**
Magnesium sulphate	**0.5 g**
Rose Bengal	**0.0035g**
Agar	**20.0 g**
Distilled water	**1000**
Streptomycin	**0.03**

3. Baker's yeast:

The organism was obtained from local sources.

***4. Rhizopus stolonifer* [12] MTCC 2198**:

The organism was obtained from MTCC, Chandigarh and maintained on YEPD medium.

***5. Rhizopus stolonifer* MTCC 162**:

The organism was obtained from MTCC, Chandigarh and maintained on YEPD medium.

***6. Rhizopus stolonifer* MTCC 2591:**

The organism was obtained from MTCC, Chandigarh and maintained on YEPD medium.

Cultivation of Microorganisms:

Cultivation of *Saccharomyces cerevisiae* [13]:

The organism from the slant culture was subcultured into 100ml YEPD medium and was adjusted to pH7.0 and was sterilized at 121ºC for 15 min. The cultures were grown at 30ºC, 160 rev min^{-1} for 24h. 5ml of the inoculum was used to inoculate 100x20 ml of the YEPD medium. The inoculated medium was incubated at 30ºC, 160-rev min^{-1} for 48 h. After 48 h of growth, the cells were separated by filtration using buchner funnel and the biomass obtained was washed with phosphate buffer twice.

The spore from the maintenance culture was inoculated onto 100x20 ml of potato dextrose medium (PDB) containing potato 200.0 g dextrose 5.0 g and distilled water 1000ml. The pH of the medium was adjusted to 6.0. The medium was sterilized at 121 ºC for 15 min. The inoculated medium was later incubated at 25ºC for 7 days to get sufficient biomass. The mycelial biomass was separated by filtration and washed with phosphate buffer twice.

EXPERIMENTAL PROCEDURE

Biosulfoxidation with **Aspergillus *niger*, *Aspergillus flavus*, *Aspergillus ochraceous*** are isolated from soil.

Baker's yeast (locally purchased**).**

***Rhizopus stolanifer* MTCC 2198, *Rhizopus stolanifer* MTCC162, *Rhizopus stolanifer* MTCC 2591** (maintained on YEPD medium), ***Saccharomyces cerevisiae* MTCC 174**.

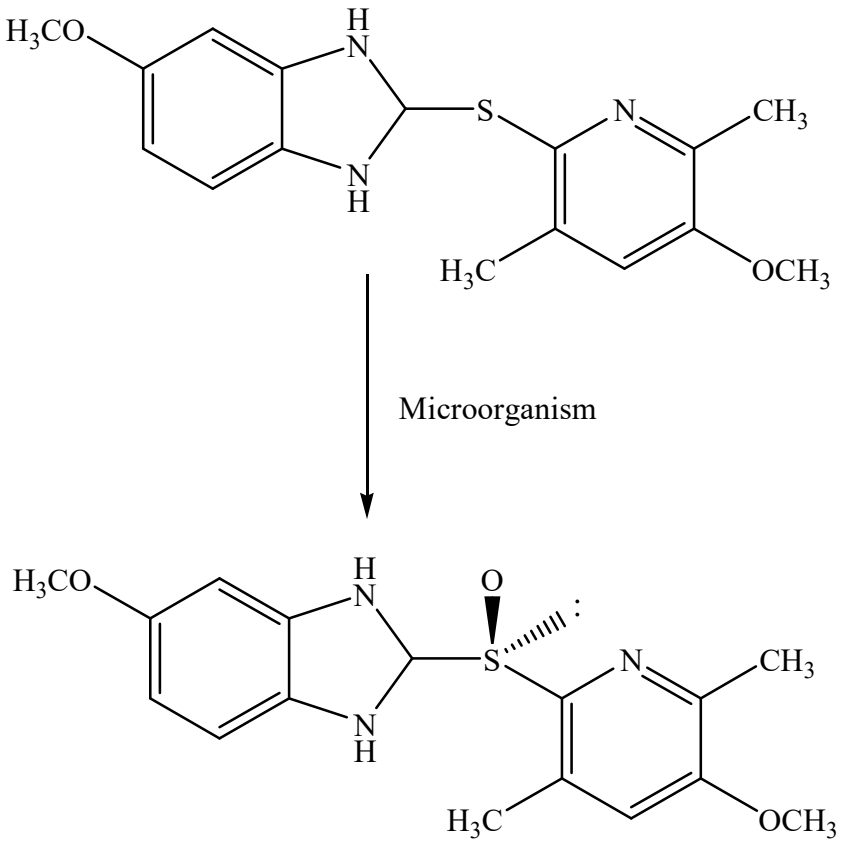

Figure 1: Sulfoxidation of 5- Methoxy-2-[[(4-methoxy –3, 5-dimethyl-2-pyridinyl) methyl]-sulphinyl]- 1H benzimidazole catalyzed by *Rhizopus stolonifer* 2591

Experimental procedure:

1ml of 5- Methoxy-2-[[(4-methoxy –3, 5-dimethyl-2-pyridinyl) methyl]-sulphinyl]- 1H benzimidazole & 5 g of the wet biomass was taken in a 250ml conical flask containing 20 ml of phosphate buffer of pH 7.6. The resulting reaction mixture was incubated at 30 ºC, 160-rev min^{-1} for 48 h. The cells were separated by filtration. The filtrate was extracted thrice with 20ml of alkaline dichloromethane. The combined extracts was then washed with 20ml of brine solution twice, dried over anhydrous sodium sulphate and evaporated to get the residue. The product formation was confirmed by TLC using benzene: ethyl acetate: methanol (50:30:10) solvent system.

HPLC ANALYSIS:

The sulfoxidised product of 5- Methoxy-2-[[(4-methoxy –3, 5-dimethyl-2-pyridinyl) methyl]-sulphinyl]-1H benzimidazole was quantified by HPLC.

Methodology: The Mobile phase consisted of Phosphate buffer pH 7.6 and methanol (75:25 ratio). The mobile phase was filtered through 0.45 µ membrane filter **[14]**.

Chromatographic condition:

Column	C18 phenomenex 250x4.6mm, 5µm

Flow rate	0.8ml/min
Wave length	280nm
Injection volume	20µl

Sample preparation: The sample obtained was lyophilized and diluted to 10 ml with methanol and injected.

Preparation of standard graph of 5-Methoxy-2-[[(4-methoxy –3, 5-dimethyl-2-pyridinyl) methyl]-sulphinyl]-1H benzimidazole:

Procedure:

Preparation of standard stock solution1:

10mg of standard product (obtained from company) was accurately weighed and transferred into a clean, dry 10ml volumetric flask and dissolved in small quantity of methanol. The volume was made upto 10ml mark, with HPLC methanol, which gave a concentration of 1000µg/ml.

Preparation of standard second stock solution 2:

From the above standard stock solution, 1ml was pipetted into 10ml volumetric flask and the volume was made up to 10ml mark with methanol, which gave concentration of 100µg/ml & this solution was kept as standard stock solution 2.

Preparation of working standard solution:

From the standard stock solution 2, aliquots of 100µl, 200µl, 300µl, 400µl, 500µl were pipetted into 10ml volumetric flasks. The volume was made up with methanol upto the mark. This gave the working standard solutions with a concentration of 10µg, 20µg, 30µg, 40µg and 50µg of Standard product respectively.

Optimization of Biosulfoxidation [15] of 5-Methoxy-2-[[(4-methoxy –3, 5-dimethyl-2-pyridinyl) methyl]-sulphinyl]-1H benzimidazole by *Rhizopus stolonifer* MTCC 2591 (soil isolate):

Optimization of pH:

The bioconversion was carried out at different pH values like 7.6, 8.0, 8.4, & 8.8 at 30 ºC.

Experimental Procedure:

1ml of 5-Methoxy-2-[[(4-methoxy –3, 5-dimethyl-2-pyridinyl) methyl]-sulphinyl]-1H benzimidazole & 5.0 g of the wet mycelium was taken into five different 250 ml conical flask containing 20 ml of pH 7.6, 8.0, 8.4 & 8.8 phosphate buffer. The reaction mixture was incubated at 30 ºC, 160-rev min^{-1} for 48 h. The biomass was separated by filtration and the filtrate was extracted with 20 ml of alkaline dichloromethane thrice. The collected organic extracts was washed twice with 20 ml brine and dried over anhydrous sodium sulphate. The dried extract was then evaporated to get the residue. The product formation was confirmed by TLC using

benzene: ethyl acetate: methanol (50:30:10) solvent system.

Optimization of Temperature:

Bioconversion was carried out at different temperature 25 ºC, 30 ºC, 35 ºC, & 40 ºC keeping pH constant at 7.6

Experimental Procedure:

1ml of 5- Methoxy-2-[[(4-methoxy –3, 5-dimethyl-2-pyridinyl) methyl]-sulphinyl]-1H benzimidazole & 5.0 g of the wet mycelium was taken in four different 250 ml conical flasks containing 20 ml of pH 7.6 phosphate buffer. The reaction mixture was incubated at different temperature like 25 ºC, 30 ºC, 35 ºC & 40 ºC at 160-rev min^{-1} for 48 h. The biomass was separated by filtration and the filtrate was extracted with 20 ml of alkaline dichloromethane thrice. The collected organic extracts was washed twice with 20 ml brine and dried over anhydrous sodium sulphate. The dried extract was then evaporated to get the residue.The product formation was confirmed by TLC using benzene: ethyl acetate:methanol (50:30:10) solvent system.

Optimization of Substrate concentration:

The bioconversion was carried out at different Substrate concentration like 0.2ml (2mg), 0.4ml (4mg), 0.6ml (6mg), 0.8ml (8mg), & 1.0ml (10mg) in pH 7.6 at 30 ºC.

Experimental Procedure:

0.2ml (2mg), 0.4ml (4mg), 0.6ml (6mg), 0.8ml, (8mg), &1.0ml (10mg) of 5-Methoxy-2-[(4- methoxy –3, 5-dimethyl-2-pyridinyl) methyl]-sulphinyl]-1H benzimidazole & 5.0 g of the wet mycelium were taken in five different 250 ml conical flask containing 20 ml of pH 7.6 phosphate buffer. The reaction mixture was incubated at 30 ºC, 160-rev min^{-1} for 48 h. The biomass was separated by filtration and the filtrate was extracted with 20 ml of alkaline dichloromethane thrice. The collected organic extracts was washed twice with 20 ml brine and dried over anhydrous sodium sulphate. The dried extract was then evaporated to get the residue. The product formation was confirmed by TLC using benzene: ethylacetate: hexane: methanol (50:30:10) solvent system.

Optimization of Incubation time:

The bioconversion was carried out at different incubation time intervals like 24h, 48h, 72h & 96h in pH 7.6 at 30 ºC.

Experimental Procedure:

1ml of 5- Methoxy-2-[[(4-methoxy –3, 5-dimethyl-2-pyridinyl) methyl]-sulphinyl]- 1H benzimidazole & 5.0 g of the wet mycelium was taken in to five different 250 ml conical flask containing 20 ml of pH 7.6 phosphate buffer. The reaction mixture was incubated at

30 ºC, 160-rev min^{-1} for 24h, 48h, 72h & 96h. The biomass was separated by filtration and the filtrate was extracted with 20 ml of alkaline dichloromethane thrice. The collected organic extracts was washed twice with 20 ml brine solution and dried over anhydrous sodium sulphate. The dried extract was then evaporated to get the residue. The product formation was confirmed by TLC using benzene: ethyl acetate: methanol (50:30:10) solvent system.

Optimization of biomass concentration:

The bioconversion was carried out by using different quantities of biomass like 2g, 4g, 6g, 8g & 10g in pH 7.6 at 30 ºC.

Experimental procedure:

1ml of 5- Methoxy-2-[(4-methoxy –3, 5-dimethyl-2-pyridinyl) methyl]-sulphinyl]-1H benzimidazole & 2g, 4g, 6g, 8g, 10g of the wet mycelium were taken in five different 250 ml conical flask containing 20 ml of pH 7.6 phosphate buffer. The reaction mixture was incubated at 30 ºC, 160-rev min^{-1} for 48 h. The biomass was separated by filtration and the filtrate was extracted with 20 ml of alkaline dichloromethane thrice. The collected organic extracts was washed twice with 20 ml brine and dried over anhydrous sodium sulphate. The dried extract was then evaporated to get the residue. The product formation was confirmed by TLC using benzene: ethyl acetate: methanol (50:30:10) solvent system.

Effect of surfactants:

4 mg of substrate dissolved in alcohol was mixed with 12 mg of sodium lauryl sulphate (SLS) and stirred for 10 min. The solvent was then removed under reduced pressure and the solid obtained was added to 20 ml of pH 7.6 buffer with 5 g of wet biomass. The reaction mixture was incubated at 30 ˚C, 160-rev min^{-1} for 48 h. The reaction mixture was filtered and the filtrate was extracted with 20 ml alkaline MDC thrice and washed twice with brine soln and dried over anhydrous sodium sulphate. The experiment was repeated using tween 80 and cetyltrimethyl ammonium bromide (CTAB) in the same manner as described above. The product formation was confirmed by TLC using benzene: ethyl acetate: methanol (50:30:10) solvent system.

Effect of Hydrophilic carrier:

4 mg of substrate dissolved in alcohol was mixed with 12 mg of β-cyclodextrin and stirred for 10 min. The solvent was then removed under reduced pressure and the solid obtained was added to 20 ml of pH 7.6 buffer with 5 g of wet biomass. The reaction mixture was incubated at 30˚C, 160-rev min^{-1} for 48 h. The reaction mixture was filtered and the filtrate was extracted with 20 ml

alkaline MDC thrice, washed with brine solution twice and dried over anhydrous sodium sulphate. The product formation was confirmed by TLC using benzene: ethyl acetate: methanol (50:30:10) solvent system.

RESULTS AND DISCUSSIONS

Enantiopure drugs are in great demand in pharmaceutical industry as desired therapeutic activity is characteristic of a particular isomer. Hence, developing new techniques to obtain the same has become a necessity in synthetic chemistry.

Enantioselective oxidation of sulfides represents a straightforward and an atom economical approach towards production of optically active sulfoxides which are important building blocks of pharmaceuticals.

Biosulfoxidation is employed as an alternative tool for the production of sulfoxides, as enzymes produce enantiopure products by virtue of their stereoselective property. Biosulfoxidation of 5-Methoxy-2-[[(4-methoxy –3, 5-dimethyl-2-pyridinyl) methyl]- sulphinyl]-1H benzimidazole has been extensively reported in the literature. As there are still many microorganisms whose sulfoxidation ability has not been realized, in our present work, we have made an attempt to screen some of the fungi for the biosulfoxidation, which have not been reported yet.

As whole cells are easy to handle and cheaper compared to isolated enzymes, we have opted for resting cell bioconversion.

Totally eight different fungi were selected for the study. They were:

1. *Saccharomyces cerevisiae* MTCC174
2. *Baker's yeast* (locally purchased)
3. *Rhizopus stolonifer* MTCC 2591
4. *Rhizopus stolonifer* MTCC 2198
5. *Rhizopus stolonifer* MTCC 162
6. *Aspergillus niger* (soil isolate)
7. *Aspergillus flavus* (soil isolate)
8. *Aspergillus ochraceous* (soil isolate)

The Biotransformation process

The whole bioprocess was carried out in two stages.

1. Culturing the selected microorganism in suitable media to obtain sufficient active biomass to serve as catalyst.

2. Biosulfoxidation of the substrate mentioned earlier using the biomass from stage-1.

In the first stage, microorganisms were cultivated in the specified culture media to obtain the biomass. In the next stage the biosulfoxidation of the sulfide was carried out as defined in methodology.

Screening of microorganisms

In the screening, it was found that only some

of the selected fungi were capable of bringing out the sulfoxidation of 5-Methoxy-2-[[(4-methoxy –3, 5-dimethyl-2- pyridinyl) methyl]-sulphinyl]-1H benzimidazole. This indicated that, some fungi only possess the required monooxygenase enzyme which accepted the xenobiotic substrate. Out of the eight fungi selected for the study, *Rhizopus* species were found to be more efficient in bringing about sulfoxidation. Among the three *Rhizopus species* screened **(Figures 1-3)**, *Rhizopus stolonifer* 2591 showed maximum conversion.

Screening of some selected fungi for the biosulfoxidation:

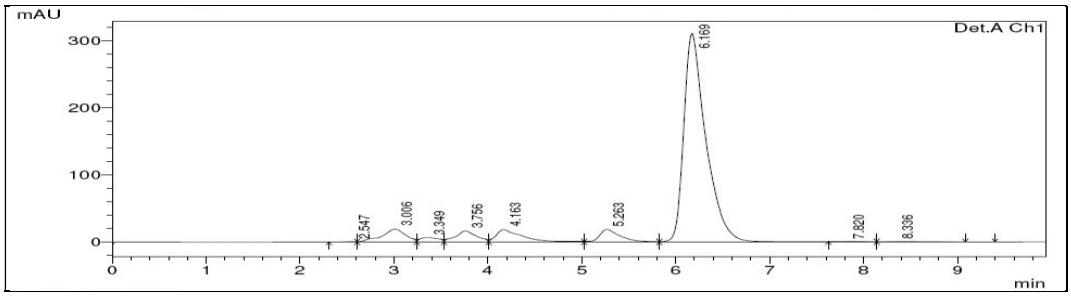

Figure 2: Chromatogram with *Rhyzopus stolanifer* 2591 for biosulfoxidation

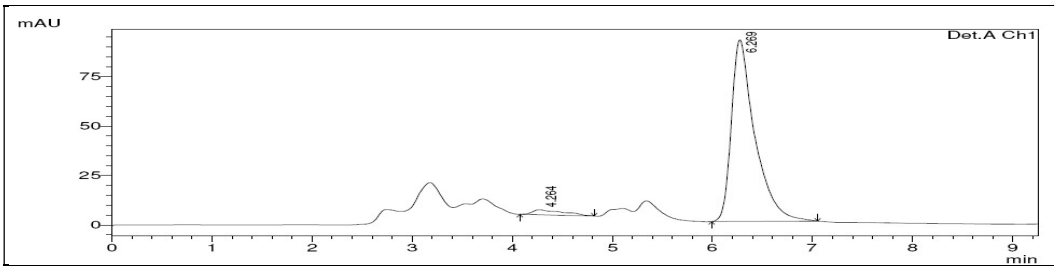

Figure 3: Chromatogram with Rhyzopus *stolanifer* 2198 for biosulfoxidation

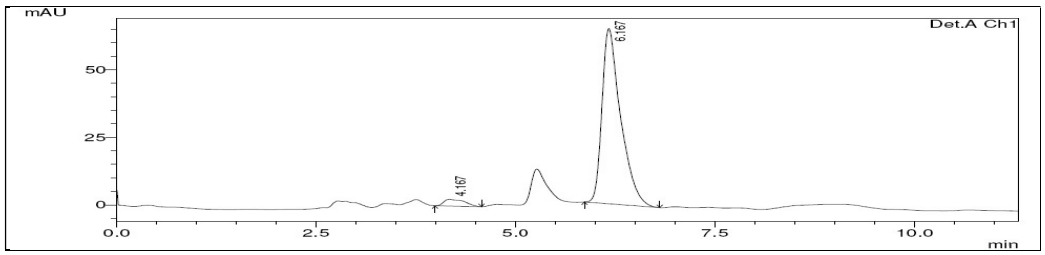

Figure 4: Chromatogram with *Aspergillus ochraceae* for the biosulfoxidation

Rhizopus stolonifer species, *Rhizopus stolonifer* 2591 showed maximum conversion. Hence it was selected for optimization of biosulfoxidation process. Some of the parameters evaluated were:

1. pH
2. Temperature

3. Substrate concentration
4. Incubation time
5. Biomass concentration
6. Presence of surfactant
7. Hydrophilic carrier

Effect of pH

The pH plays a crucial role in biotransformation. Enzymes possess optimum activity at a particular pH. Variation in pH alters the ionic state of substrate and enzymes involved in the reaction, so pH influences enzymatic reactions. The effect of the pH value of the reaction medium on the asymmetric biosuloxidation was studied by varying pH values at 7.6, 8.0, 8.4 and 8.8. The highest yield was achieved when the pH was at 7.6. The study indicated that pH 7.6 is optimum for the sulfoxidation of 5-Methoxy-2-[[(4-methoxy- 3, 5-dimethyl-2-pyridinyl) methyl]-sulphinyl]-1H benzimidazole, by *R. Stolanifer* 2591.

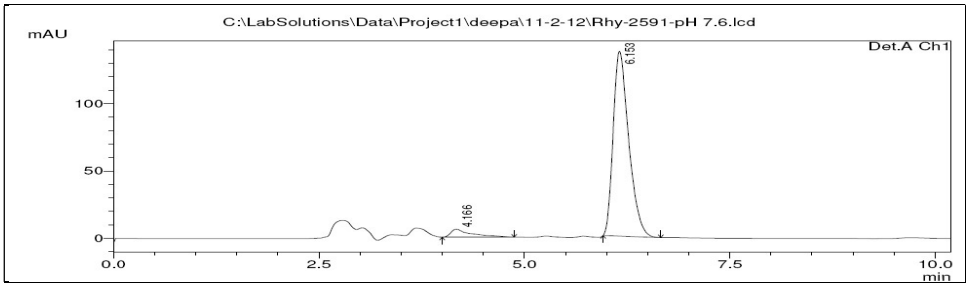

Figure 4: Effect of pH on bioconversion of substrate by *Rhizopus stolonifer* 2591

Optimization of Temperature:

Bioconversion was carried out at different temperature 25^{o} C, 30^{o} C, 35^{o} C, & 40^{o} C keeping pH constant at 7.6. The highest sulfoxidation of 5-Methoxy-2-[[(4-methoxy- 3, 5-dimethyl-2-pyridinyl) methyl]-sulphinyl]-1H benzimidazole, by *R. Stolanifer* 2591 was achieved at the temperature of 30^{o} C.

Table 1: Effect of temperature on bioconversion of substrate by *Rhizopus stolonifer* 2591

Temperature	Product concentration (mg/L)
25^{o}C	1.165
30^{o} C	5.2
35^{o} C	1.30
40^{o} C	0.257

Effect of substrate concentration

It is a well known fact that, in enzymatic reaction, substrate concentration plays an important role, worthy of careful investigation. It influences either sufficient expression of the enzyme activity or may

results in the enzyme inhibition. Hence, the enzyme activity in production of omeprazole was evaluated by varying substrate concentration in the range of 2, 4, 6, 8, and 10 mg. The results showed that the enzyme activity was highest at 2 mg substrate concentration and when the substrate concentration was higher than 2 mg the yield decreased. From this, we can infer that the increased substrate concentration inhibits the enzymatic activity which may be due to the toxic effect of the substrate on the enzyme.

Table 2: Effect of substrate concentration on bioconversion of substrate by *Rhizopus stolonifer* 2591

Biomass	Product concentration (mg/L)
2g	0.25
4g	0.6
6g	0.75
8g	0.98
10g	2.08

Effect of surfactants:

The microbial transformation of non polar substrates is a limited process due to poor solubility of substrates in aqueous medium. Low solubility of substrates leads to low transport rates to and from the cells, resulting in lower product yields. An alternative approach to increase the penetration of a hydrophobic substrate into microbial cells is to use the surfactants. Hence, the surfactant effect was evaluated by using three different surfactants SLS (anionic), CTAB (cationic), Tween-80 (non-ionic). Among the surfactants used in the study, anionic and cationic surfactants were found to be toxic while very low conversion was observed in case of non ionic surfactant.

Table 3: Effect of surfactant concentration on bioconversion of substrate by *Rhizopus stolonifer* 2591

Surfactant	Concentration of omeprazole in mg/L
SLS	-
CTAB	-
Tween 80	2.88

Effect of hydrophilic carrier:

Cyclodextrins are cyclic oligosaccharides with a hydrophilic outer surface and a hydrophobic central cavity. The hydrophilic exterior renders the cyclodextrin water soluble and the hydrophobic interior provides a microenvironment for relatively non-polar molecules. In aqueous solutions, cyclodextrins can form inclusion complexes with hydrophobic compounds by entrapping either the entire molecule or

a nonpolar part of it inside the hydrophobic cavity**.** Based on this concept, physical mixture of the substrate and cyclodextrin was prepared and this mixture was used in the sulphoxidation reaction. The results indicated that the product concentration was lower than the yield, without cyclodextrin

Hydrophilic Centre	Concentration of omeprazole in mg/L
β-cyclodextrin	5.1
Absence of β-cyclodextrin	5.2

CONCLUSION

Biosulfoxidation of 5-Methoxy-2-[[(4-methoxy –3, 5-dimethyl-2-pyridinyl) methyl]- sulphinyl]-1H benzimidazole was carried out using eight different strains of fungi. Some of the strains exhibited considerable sulfoxidation capability. However, maximum sulfoxidation was observed with *Rhizopus stolonifer* 2591.

While, optimising the various biosulfoxidation parameters like pH, temperature, substrate concentration, incubation time, biomass variation, effect of surfactants and hydrophilic carrier. The following observations were made:

1. Optimum pH: 7.6
2. Optimum incubation temperature: 30 ^{o} C
3. Optimum substrate concentration: 0.2mg
4. Optimum incubation time: 48hrs
5. Optimum biomass: 10g
6. Surfactant: Non significant
7. Hydrophilic Carrier: Non significant

Further works can be pursued to improve the yield by altering the other biosulfoxidation parameters like:

1. Performing biosulfoxidation in organic solvents/biphasic systems.
2. Performing biosulphoxidation using disrupted cells/growing cells.

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