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A Study on the Effect of Various Physicochemical Parameters on Biosulphoxidation of Omeprazole Intermediate

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ABSTRACT

Biosulphoxidation of 5-Methoxy-2-[[(4-methoxy –3, 5-dimethyl-2-pyridinyl) methyl]- sulphinyl]-1H benzimidazole, an intermediate in the synthesis of block buster drug omeprazole, was carried out using different microorganisms belonging to the class of fungi. Totally eight fungi were considered for the study. . The selected microorganisms include *Rhyzopus stolanifer* MTCC 2198*, Rhizopus stolanifer* MTCC 2591*, Rhizopus stolanifer* MTCC 162*, Aspergillus niger, Aspergillus ochraceous, Aspergillus flavus, Saccharomyces cerevisiae* (Soil isolates) and *Baker's yeast* (Locally purchased). Among the selected microorganisms, *Rhizopus stolanifer*-2591 showed maximum conversion. *Rhizopus stolanifer*-2198 and *Aspergillus ochraceous* showed conversion in negligible amounts while no conversion was observed with other microorganisms. Hence, *Rhizopus stolanifer*-2591 was considered for the study of effect of various physicochemical parameters that affect the biotransformation process. The parameters varied in the study include pH of the reaction mixture, incubation temperature and time, concentration of substrate and concentration of biomass. The results obtained indicated that the optimum biosulphoxidation of the above mentioned substrate with *Rhizopus stolanifer*-2591 was observed at pH 7.6, temperature 30˚C, incubation time being 48h at substrate concentration 2mg and biomass concentration at 10g.

Keywords: Biosulphoxidation, screening, *Rhizopus stolanifer*-2591, Physicochemical parameters.

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INTRODUCTION

Sulphoxide group often occurs in various pharmacologically active agents. Enantioselective synthesis of sulphoxides have elicited great interest in the production of many therapeutically effective chemical entities. Several methods are available for the synthesis of enantiopure sulphoxides [1]. A potential source for obtaining chiral sulphoxides is by making use of regio and stereoselective property of enzymes in microorganisms [2]. Enantiomerically pure sulphoxides are useful building blocks for the synthesis of many pharmaceuticals and biologically active compounds [3]. Conventional synthetic methods involve an additional step of resoluting the racemic mixture limiting the enantiopure yield to 50%. This problem is taken care of in asymmetric synthesis. Among them, chemical asymmetric oxidation using a chiral metal catalyst is however expensive and contributes to environmental pollution where as biooxidation is highly regio- and stereo specific and environment friendly.

Biocatalysis using isolated enzymes such as peroxidases and monooxygenases is expensive and laborious owing to the purification and stability of the isolated enzymes. Use of whole cells as biocatalyst is more preferred as it is simple and economical. Many such bioconversions are reported in the literature [4, 5, 6]. If the enzyme involved in the reactionis a constitutive enzyme, use of resting cells is more appropriate in biotransformation of toxic xenobiotic substrates. Screening and identification of the right microorganism is critical towards achieving maximum conversion and hence good yields. In this context, several fungi were screened for biosulphoxidation of 5-methoxy-2-[[(4-methoxy 3,5-dimethyl-2-pyridinyl)methyl] sulphinyl]1H-benzimidazole, which is an intermediate in the synthesis of omeprazole, a proton pump inhibitor. Among the selected microorganisms, *Rhizopus stolanifer*-2591 alone exhibited considerable conversion. In order to further improve the yield, manipulation of various physicochemical parameters that affect the biotransformation was thought of. The study included variations in pH of the reaction medium, incubation time and temperature, substrate concentration and biomass concentration.

MATERIALS AND METHODS

Chemicals

All chemicals and solvents were obtained from local suppliers and are of analytical grade.

5 – methoxy - 2 -[[(4 –methoxy –3, 5- dimethyl -2- pyridinyl)methyl] - sulphinyl] -1 H benzimidazole, the sulphide and omeprazole standard were kind gift samples from Cipla Pvt Ltd, Bangalore.

Microorganisms

***Saccharomyces cerevisiae* MTCC 174:** The organism was obtained from MTCC, Chandigarh and was maintained on YEPD media containing Yeast Extract 3.0g, Peptone 10.0g, Dextrose 20.0g, Agar 20.0g and Distilled water to 1000 ml.

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***Aspergillus niger*, *Aspergillus flavus*, and *Aspergillus ochraceous* (soil isolates):** The organisms were isolated from the soil in our microbiology lab and identified in Bangalore University. These organisms were maintained on MRBA media containing Dextrose 10.0g, Peptone 5.0g, Potassium dihydrogen phosphate 1.0g, Magnesium sulphate 0.5g, Rose Bengal 0.0035g, Agar 20.0g, Distilled water 1000 ml and Streptomycin 0.03g.

***Rhizopus stolanifer* MTCC 2198, *Rhizopus stolanifer* MTCC 162 and *Rhizopus stolanifer* MTCC 2591:** The organisms were obtained from MTCC Chandigarh and maintained on MRBA media.

Baker's yeast: Was obtained from local market and maintained on YEPD medium.

Experimental

Cultivation of Microorganisms

Cultivation of *Saccharomyces cerevisiae*

The organism from the slant culture was subcultured into 100ml YEPD medium, adjusted to pH 7.0 and was sterilized at 121ºC for 15 min. The cultures were grown at 30 ºC, 160rpm for 24h. 5ml of the inoculum was used to inoculate 100x20 ml of the YEPD medium. The inoculated medium was incubated at 30 ºC, 160-rpm 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.

Cultivation of *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus ochraceous, Rhizopusm stolanifer* MTCC 2198, Rhizopus *stolanifer* MTCC 162 and *Rhizopus stolanifer* MTCC 2591

The spores from the maintenance culture was inoculated onto 100x20 ml of potato dextrose medium (PDB) containing potato 200.0g, dextrose 5.0g, 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 25 °C for 7 days to get sufficient biomass. The mycelial biomass was separated by filtration and washed with phosphate buffer twice.

Procedure for Bio-oxidation

10 g of the biomass was taken in 20 ml phosphate buffer of pH 7.6, 10 mg of omeprazole intermediate dissolved in 2 ml of alcohol was added to the above suspension and incubated at 30 ºC, 160 rpm for 48 h. The reaction mixture was filtered to remove the biomass. Filtrate was extracted with alkaline methylene dichloride (20 ml x 3), washed with 20ml of brine twice and dried over anhydrous sodium sulphate. The organic extract was then concentrated by evaporation. The product formation was monitored by TLC using benzene: ethyl acetate: methanol (50:30:10) solvent system.

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

Chromatographic condition

Mobile phase: phosphate buffer (pH 7.6) : methanol (25:75)
Column: C18 phenomenex 250 × 4.6 mm, .5 µm,
Flow rate: 0.8 ml/min.
Wave length: 280 nm
Injection volume: 20µl

Effect of various physicochemical parameters on Biosulphoxidation of omeprazole intermediate by *Rhizopus stolanifer* MTCC 2591:

Effect of pH: 10mg of 5-Methoxy-2-[[(4-methoxy –3, 5-dimethyl-2-pyridinyl) methyl]- sulphinyl]-1H benzimidazole dissolved in 1ml alcohol & 5.0 g of the wet mycelium were taken into four 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, 160rpm for 48h. The biomass was separated by filtration and the filtrate was extracted with 20 ml of alkaline dichloromethane thrice. The combined organic extract was washed twice with 20 ml brine and dried over anhydrous sodium sulphate. The dried extract was then evaporated to get the residue.

Effect of Temperature: Bioconversion was carried out as mentioned above at different temperatures like 25 ºC, 30 ºC, 35 ºC & 40 ºC keeping pH constant at 7.6.

Effect of incubation time: The bioconversion was carried out as described above at different incubation time intervals like 24h, 48h, 72h & 96h in pH 7.6 at 30 ºC with a biomass concentration of 5g and substrate concentration of 10 mg.

Effect of Substrate Concentration: The bioconversion was carried out as mentioned above at different substrate concentrations like 2mg, 4mg, 6mg, 8mg &10mg in pH7.6 at 30 ºC.

Effect of biomass concentration: The bioconversion was carried out as described above by using different quantities of biomass like 2g, 4g, 6g, 8g & 10g in pH 7.6 at 30 ºC and a substrate concentration of 10mg.

RESULTS AND DISCUSSION

Screening of microorganisms

In the screening, it was found that only some of the selected fungi were capable of bringing about the sulphoxidation of 5-Methoxy-2-[[(4-methoxy –3, 5-dimethyl-2-pyridinyl) methyl]-sulphinyl]-1H benzimidazole. This indicated that, only some fungi possess the required monooxygenase enzyme which accepted the selected xenobiotic substrate. Out of the eight

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fungi selected for the study, *Rhizopus* species were found to be more efficient in bringing about sulphoxidation of the selected substrate. Among the three *Rhizopus species* screened, *Rhizopus stolanifer* 2591 showed maximum conversion (table. no.1).

Table 1: Screening of Microorganisms for Biosulphoxidation

SL. No.	Microorganisms	Product concentration (mg/L)
1.	*Rhizopus stolonifer* MTCC 2591	5.2
2.	*Rhizopus stolonifer* MTCC 2198	3.1
3.	*Aspergillus ochraceous*(from soil)	1.84
4.	*Aspergillus flavus*(from soil)	0
5.	*Aspergillus niger*(from soil)	0
6.	*Rhizopus stolonifer* MTCC 162	0
7.	*Saccharomyces cerevisiae* MTCC174	0
8.	*Baker's yeast*	0

Effect of pH:

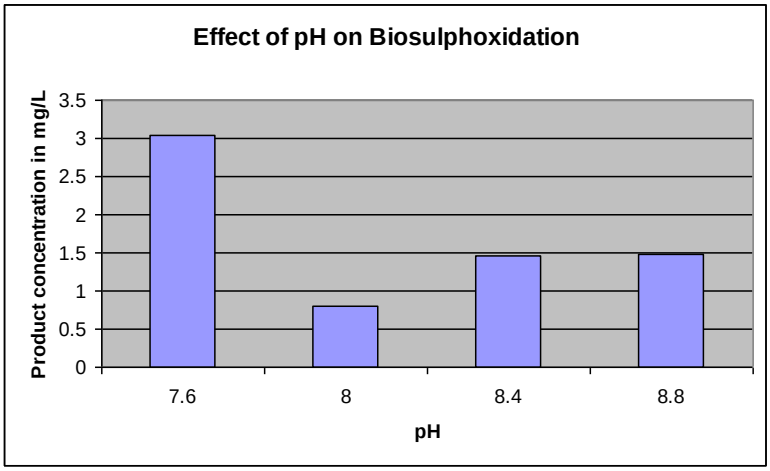

Fig: 1

pH of the reaction medium plays a crucial role in biotransformation. Enzymes possess optimum activity at a particular pH. Variations in pH alter 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 biosulphoxidation was studied by varying pH values at 7.6, 8.0, 8.4 and 8.8 as it is one of the important parameter for enzymatic activity. It is evident from figure no.2 that the yield of omeprazole depends on the pH value. The highest yield was achieved when the pH was 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. stolonifer* 2591.

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Effect of temperature

Incubation temperature plays an important role in determining the extent of enzyme activity. It affects the catalytic characteristics of a biocatalyst. Low temperatures may deter while very high temperatures may decrease enzymatic activity owing to denaturation of enzymes. Hence, bioconversion was attempted at four different temperatures i.e. 25 °C, 30 °C, 35 °C, and 40 °C. When the temperature was increased from 25 °C to 30 °C, the yield of omeprazole increased. The highest yield of omeprazole was achieved at 30 °C. When the temperature was increased more than 30 °C, the yield decreased considerably. The decrease in bioconversion could be attributed to the loss of the enzymatic activity. Hence the optimum temperature was found to be 30 °C for the sulphoxidation of 5- Methoxy-2-[[(4-methoxy –3, 5-dimethyl-2-pyridinyl) methyl]- sulphinyl]-1H benzimidazole, using *R..Stolanifer* 2591 (fig.no.2).

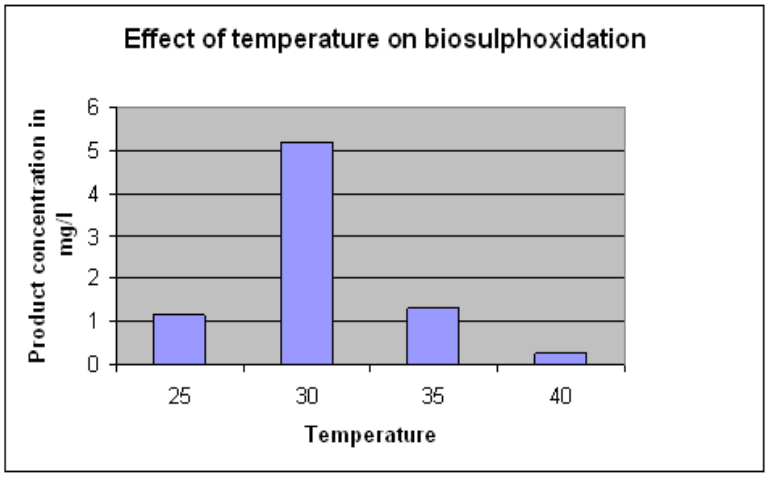

Fig : 2

Effect of incubation time

Reaction time plays an important role in the enzymatic reactions. The effect of incubation time in the production of omeprazole was carried out by varying the incubation time from 24 - 96h. When the reaction time increased from 24h to 48h, the yield of omeprazole increased. It was found that the maximum conversion was observed at 48h (Fig no. 3). Beyond 48h, the concentration of the sulphoxidised product decreased by increasing the incubation time, probably due to degradation of the sulphoxidized product by other cellular constituents of the organism.

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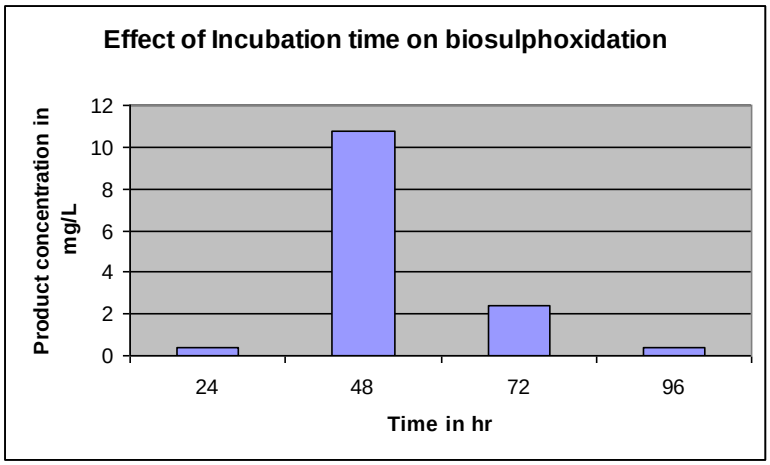

Fig: 3

Effect of substrate concentration

It is a well known fact that, in enzymatic reactions, substrate concentration plays an important role, worthy of careful investigation. It influences either sufficient expression of the enzyme activity or may results in 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 10mg. The results (fig no.4) showed that the enzyme activity was highest at 2mg substrate concentration and when the substrate concentration was higher than 2mg 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.

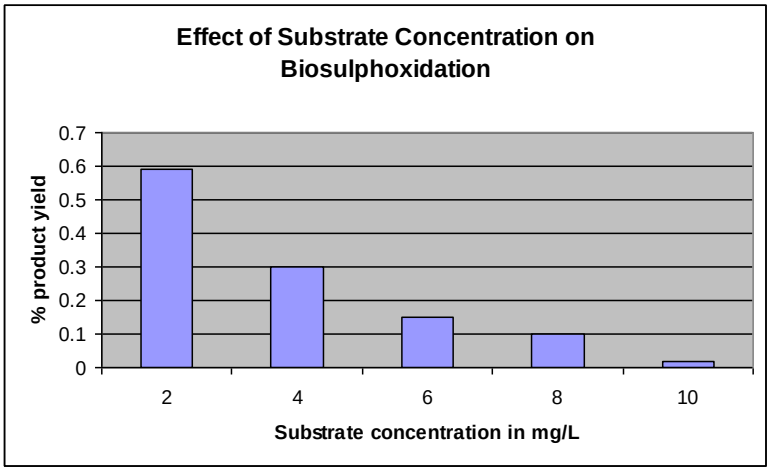

Fig: 4

Effect of biomass

It is a well known fact that, the enzyme activity depends on the concentration of biomass. The rate of reaction and amount of conversion depends on the quantity of enzymes available. Hence, the enzyme activity was evaluated by varying biomass concentration in the

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range of 2, 4, 6, 8, and 10g. When the biomass concentration was increased from 2 to 10g, the yield of omeprazole also increased (fig. no. 5). Maximum conversion was observed at 10g. From 2g to 10g the conversion increased with increase in biomass.

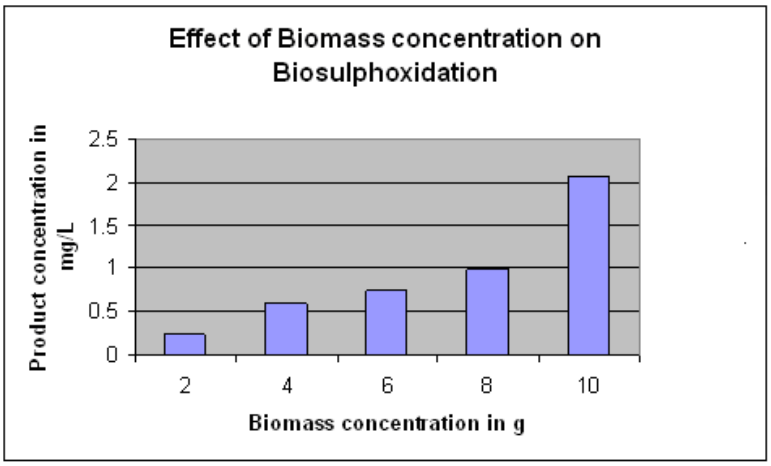

Fig: 5

CONCLUSIONS

The present work was aimed at identifying the right organism for biosulphoxidation of omeprazole intermediate. During screening, eight fungi were selected out of which *Rhizopus stolonifer* 2591 exhibited good bioconversion. Hence this organism was selected for further experiments where in which various physicochemical parameters affecting the bioconversion like pH, temperature, reaction time, substrate concentration and biomass concentration, were evaluated. During optimization it was found that the biosulphoxidation of omeprazole intermediate by *Rhizopus stolonifer* 2591 gave better yield at pH 7.6, temperature 30 °C, reaction time being 48h maximum conversion was achieved by using 2mg of substrate and the yield increased with increase in biomass concentration.

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