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SCREENING AND OPTIMISATION OF BIOCONVERSION PARAMETERS FOR THE REDUCTION OF 3-[5-[(4-FLUROPHENYL)-1,5, DI-OXOPENTOL]-YL] –4-(S) PHENYL OXAZOLIDIN-2-ONE.

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ABSTRACT: The reduction of ketones is one of the most important and practical reaction for producing non racemic alcohols, which are needed to synthesize industrially important chemicals such as pharmaceuticals, agrochemicals and natural products. Biocatalysis has turned out to be a highly competitive technology for asymmetric ketone reduction. In the present work, an attempt was made to identify a potential microorganism for the reduction of 3-[5-[(4-flurophenyl)-1,5, di-oxopentol]-yl] –4-(S) phenyl oxazolidin-2-one. Some of the fungi screened were *Saccharomyces cerevisiae, Aspergillus niger(2 strains), Pichia farinosa, Candida vishwanathii, Rhizopus stolonifer and Penicillin species.* The experimental results showed that *S. cerevisiae*, *Aspergillus niger* and *C. viswanathii* strains were able to bring about the conversion of selected ketone to alcohol. As *Saccharomyces cerevisiae* was found to be more effective in bringing about reduction, it was selected for further experiment. In order to improve the yield certain bioconversion parameters like pH of reaction medium, time of incubation, incubation temperature and biomass to substrate ratio were studied. The results showed that the bioreduction of the above mentioned substrate was maximum in pH 7.6 at 30º C when incubated for 48 h. The conversion increased with increase in biomass, however it reached saturation at the ratio of 300:1

KEY WORDS: Bioreduction, *Saccharomyces cerevisiae*, Optimisation.

INTRODUCTION

Synthesis of chiral drugs within the pharmaceutical industry has become very important. Chirality is a key factor in the efficiency of many drug products and the production of single enantiomer of optically active molecules has become vital[1,2,3]. The manufacture of the active form of the drug is consequently becoming a norm in the industry. Despite tremendous scientific and technical advances, however, several chiral syntheses still remain difficult and/or expensive. Asymmetric biocatalysis employing either whole microorganisms or isolated enzymes has often emerged as a viable alternative[4,5]. It is well known that microorganisms are useful biocatalysts for asymmetric reduction of ketones[6]. These biocatalysts catalyze the reactions under mild and economically viable conditions in an eco-friendly environment when compared to chemical reactions. Biocatalysis may be performed using whole cells or isolated enzymes[7,8]. There are advantages in using whole cells, as enzymes require the presence of expensive cofactors. Whole cells are an economic and continuous source of enzyme as well as co- factors, thus simplifying these reactions significantly. Hence, the present work is to focus on identifying potential microorganisms, which can be used in place of reducing agents to get an enantiopure product to meet the industrial demand at low cost.

3-[5-[(4-flurophenyl)-1,5-dioxopentol]-4-(S)phenyl oxazolidin-2-one is an important prochiral intermediate in the synthesis of Ezetimibe, an antihyperlipedimic agent and it's S isomer is the preferred biologically active molecule. The present work relates to microbiological reduction of carbonyl group of the above mentioned intermediate. Several fungi were screened for their capacity to bring about the reduction. Among the seven strains of fungi which were examined, *S. cerevisiae*, s

howed considerable reduction. Therefore it was selected for optimization experiments.

EXPERIMENTAL

MATERIALS AND METHODS

MICROORGANISMS:

1.*Saccharomyces cerevisiae:* MTCC 174 was obtained from MTCC, Chandigarh.The organism was maintained on YEPD media containing, yeast extract 3.0 g, peptone 10.0 g, dextrose 20.0 g, agar 20.0 g and distilled water 1000ml.

2. *Pichia farinosa* MTCC 246 was obtained from MTCC, Chandigarh.The organism was maintained on MYA media containing, malt extract 3.0 g, yeast extract 3.0 g, peptone 5.0 g, dextrose 10.0 g and agar 20.0 g and distilled water 1000ml.

3. *Aspergillus niger* (soil isolate) and *Aspergillus niger* MTCC 961. The organismwas maintained on MRBA media containing, dextrose 10.0 g, peptone 5.0g, potassium dihydrogen phosphate 1.0g, Magnesium sulphate 0.5 g, Rose Bengal 0.0035 g, Agar 20.0 g, distilled water 1000 ml and streptomycin 0.03 g.

4. *Candida viswanathii MTCC 1629:* The organism was obtained from MTCC, Chandigarh and maintained on YEPD medium.

5. *Pencillium species* (soil isolate): The organism was maintained on MRBA medium.

6.*Rhizopus stolonifer* MTCC 2198: The organism was maintained on MRBA Medium.

Chemicals: All chemicals and solvents were from local suppliers and of Analytical grade.

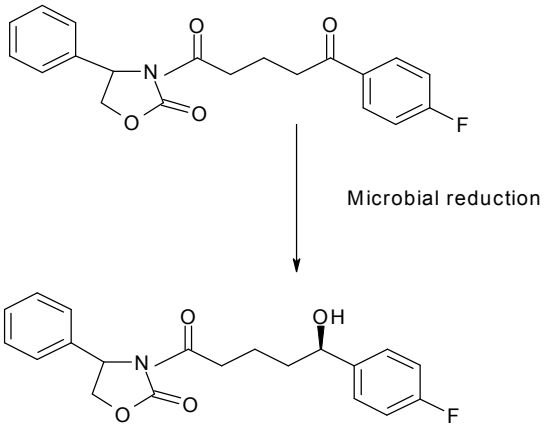
Cultivation of *Saccharomyces cerevisiae, Pichia farinosa and Candida viswanathii:* The organisms from the slant culture was subcultured into 300ml YEPD medium containing Yeast extract 0.3g, peptone 1.0 g, dextrose 2.0 g and distilled water 100ml, pH was adjusted to 7.0 and was sterilized at 121ºC for 15 min. The cultures were grown at 30ºC, 160 rev min^{-1} for 24 h.

10% volume of the fermentation medium was used for inoculation of 2.5 L of fermentation 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 was washed with phosphate buffer twice.

Cultivation of *Aspergillus niger, Rhizopus stolonifer and Pencillium* The spores from the maintenance culture was inoculated onto 100 X 20 of potato dextrose medium containing potato 200.0 g, dextrose 5.0 g and distilled water 1000 ml.

The pH of the medium was adjusted to 6.0. The medium was sterilized at 121 ºC for 15 min. The inoculated medium was incubated at 25 ºC for 5 days to get sufficient biomass. The mycelial biomass was separated by filtration and washed with phosphate buffer twice.

BIOREDUCTION OF 3-[5-[(4-FLUROPHENYL)-1,5 – DI - OXOPENTOL]-YL] –4-(S) PHENYL OXAZOLIDIN-2-ONE.



Screening methodology

5 g of the wet biomass was taken into 20ml of phosphate buffer (pH7.0) containing 2.5g of glucose. 20 mg of the substrate was dissolved in 1ml of DMSO and charged into the buffer and incubated at 30ºC, 160-rev min^{-1} for 48h.

The cells were separated by filtration.

The filtrate was extracted thrice with methylene dichloride (20 ml each). The combined extract was then washed with 20ml of brine solution twice, dried over anhydrous sodium sulphate and evaporated to get the residue.

The reaction was monitored by using TLC (ethyl acetate: hexane: HAC: 6:4:0.1). The standard for analysis was procured from industry. Confirmation of the reduced product and quantification was done by HPLC and LCMS.

HPLC ANALYSIS

The reduced product of 3-[5-[(4-flurophenyl)-1,5,- di-oxopentol] y –4-(S) phenyl oxazolidin-2-one was quantified by HPLC.

Methodology- The Mobile phase consisted of 250 ml of buffer (0.05M Ammonium acetate adjusted to pH 5.0 with acetic acid), 500 ml methanol The mobile phase was filtered through 0.45 µ using membrane filter.

Chromatographic condition:

Column:	C18 phenomenex 250x4.6mm, .5µm,
Flow rate:	1.5ml/min.
Wave length:	215nm
Injection volume:	20µl.

Standard Preparation: 25 mg of the Standard was dissolved in 25 ml of methanol and diluted to get concentration of 80-120 µg /ml.

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

1. Optimization of pH:
5 g of the wet biomass was taken into 20ml of buffer solution of pH ranging from 5.2 – 8.8 containing 2.5 g of glucose. 20mg of the substrate was dissolved in 1ml of DMSO and charged into the buffer which was incubated at 30˚C, 160-rev min^{-1} for 48h.
The biomass was separated by filtration and the filtrate was extracted with 20ml of MDC thrice. The combined extract was washed with 20ml of brine solution twice , dried over anhydrous sodium sulphate and evaporated to get the product.

2. Optimization of reaction time:
The reaction was carried out as described above at different time intervals 24, 48, 72 and 96 h, maintaining the pH at 7.6 which was found to be optimum for the bio-reduction.

3. Optimization of biomass:substrate ratio
20mg of substrate was added to varied concentrations of biomass (1 -10 g) in buffer solution of pH 7.6 and incubated at 30˚C, 160-rev min^{-1} for 48h. The extraction was carried out in the same way as mentioned above.

RESULTS AND DISCUSSION

Among the seven strains of microorganisms screened for bioreduction of the selected substrate, only three strains *S. cerevisae*, *C. vishwanathii* and soil isolated *A. niger* exhibited reduction of Oxazolidine 2-one. The results are tabulated in Table-1.
Of the three microorganisms, which showed ability to bring about ketone reduction, *A.niger* and *Candida vishwanathii* exhibited considerably less bioreduction compared to *S. cerevisae*. Hence *S. cerevisae* was taken up for optimization experiments.
While optimizing pH parameter, it was found that pH 7.6 was optimum for the bio-reduction of the substrate indicating the specific dehydrogenase is more active in alkaline medium.
The optimum temperature for the reduction of the selected substrate with the selected organism was found to be 30ºC
The optimum reaction time for the reduction of the selected substrate was identified as 48 h. Increase in reaction time showed decreased product concentration suggesting probable degradation of the reduced product.
While studying the effect of biomass:substrate ratio, it was found that the conversion of the substrate increased with increase in concentration of biomass up to 6g and reached saturation at higher concentrations

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Table: 1 Bioreduction with different organisms at pH 7.0 and temp. 30ºC in duplicates

MICROORGANISM	% YIELD
Saccharomyces cerevisiae	2.05
Candida vishwanathii	0.34
Pichia farinose	0.00
Aspergillus niger (soil isolate)	0.43
Aspergillus niger MTCC	0.00
Rhizopus stolonifer	0.00
Pencillium (soil isolate)	0.00

Figure 1

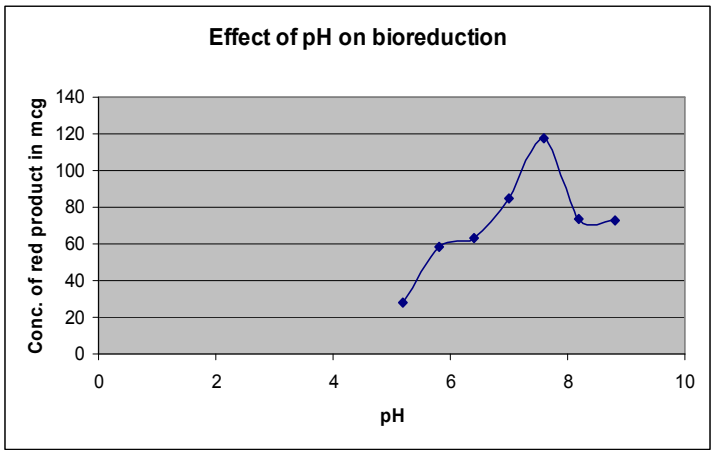


Figure 2

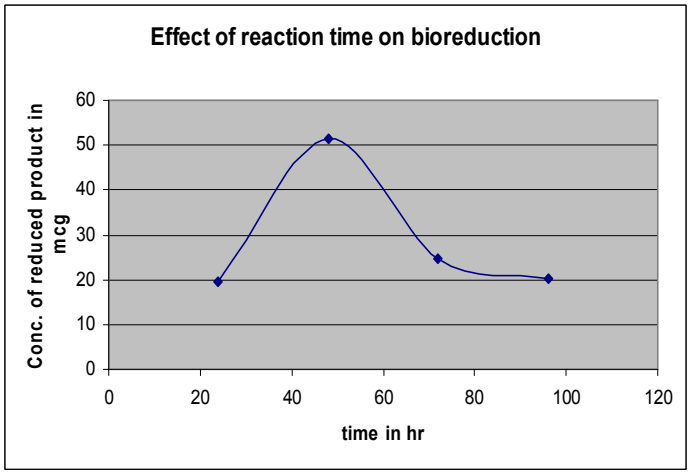
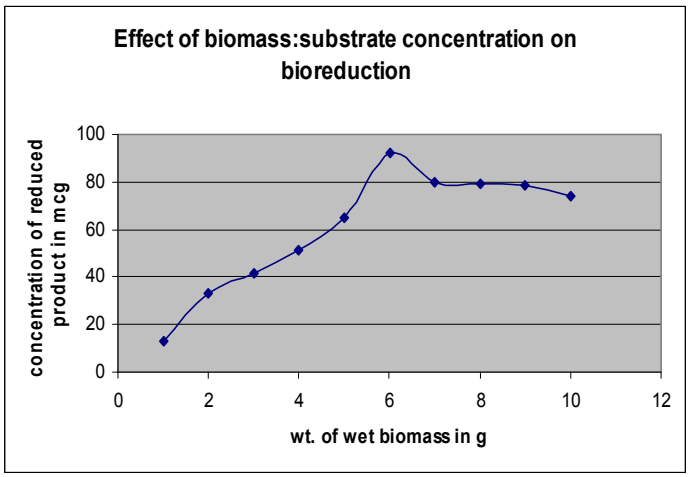


Figure 3



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