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Selectivity of Plant and Microbial Mediated Reduction of Ketones

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Original Research Article

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Abstract: Bioreduction of ketones is an alternative tool for the synthesis of enantiomeric alcohols. Biocatalytic reduction of acetophenone and its derivatives have been widely reported. Some of the microorganisms obtained from culture collection centre as well as those isolated in our microbiology lab and few plant tissues obtained from local market were screened for their potential and performance in reduction of p-chloroacetophenone and dimethoxy indanone. The parameters such as pH, temperature, substrate concentration and incubation time were evaluated during optimization. Aspergillus flavus, Aspergillus niger, Aspergillus ocraceae showed significant bioconversion. Aspergillus flavus showed maximum ability of bioreduction, it was considered for optimization. Among the screened plant tissues, used for the reduction p-chloroacetophenone, radish showed significant bioconversion. The maximum reduction was observed with Onion and the least with Beetroot.

Keywords: Bioreduction, p-chloroacetophenone, Radish, Onion.

INTRODUCTION

Enantiopure drugs are in great demand and hence, developing new techniques to obtain the same has become a necessity in organic synthesis [1]. Enantioselective reduction of ketones represents a straightforward and an atom-economical approach towards production of optically active alcohols which are important building blocks of pharmaceuticals [2].

Bioreduction of acetophenone and its derivatives have been extensively reported in literature. In the present work, we have made an attempt to screen some of the fungi and vegetables for the bioreduction, which have not been reported. Totally six different fungi were selected for the study. They were: Aspergillus niger, Aspergillus flavus, Aspergillus ochraceous (soil isolate), Rhizopus stolanifer, Baker's yeast, Mushroom. Also, seven different vegetables Daucus carota (Carrot), Raphanus sativus (Radish), Malus Domestica (Apple), Cucumis sativus (Cucumber), Beta vulgaris (Beetroot), Solanum tuberosum (Potato), Allium cepa (Onion) were selected for the study [3-4].

The bioreduction process was carried out in two stages. In the first stage, screening of different micro-organisms and plant tissues were carried out and in the next stage, the optimization of different parameters was carried out using Onion and Radish.

The objective of the present study was to screen potential microorganisms and plant tissues for the reduction of p-chloroacetophenone[5-8].

A varied range of fungi, obtained both from collection centre and locally isolated species were employed for bioreduction: Aspergillus niger, Aspergillus flavus, Aspergillus ochraceous (soil isolate), Rhizopus stolanifer, Baker's yeast, Mushroom. Varied plant tissues, obtained from local market were employed for bioreduction: Carrot, Potato, Radish, Beetroot, Cucumber, Onion, and Apple. The substrate for bioreduction chosen was p-chloro acetophenone Biotransformation of pro-chiral ketone to corresponding alcohol. Product recovery, Spectral characterization and quantification of the product, Optimization of biotransformation parameters like temperature, pH, Incubation time, Substrate concentration, Biomass concentration and effect of different surfactants have been evaluated.

MATERIALS AND METHODS [9, 10]

Aspergillus niger, Aspergillus flavus, Aspergillus ochraceous, Rhizopus stolanifer (soil isolate) were isolated from soil and maintained in microbiology lab. This organism was 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 (0.0035 g), Agar(20.0 g), Distilled water (1000 ml), Streptomycin

(0.03 g), Baker's yeast and Mushrooms were obtained from local sources.

Cultivation of Aspergillus niger, Aspergillus flavus, Aspergillus ochraceous, Rhizopus stolanifer (soil isolate)

The spore from the maintenance culture was inoculated onto 100x20 mL 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 ^{0}C for 5 days to get sufficient biomass. The mycelial biomass was separated by filtration and washed with phosphate buffer twice.

The following plant tissues were isolated from the species obtained from the local market Daucus carota (Carrot), Raphanus sativus (Radish), Malus Domestica (Apple). Cucumis sativus (Cucumber), Beta vulgaris (Beetroot), Solanum tuberosum (Potato), Allium cepa (Onion).

Chemical reduction of p-chloroacetophenone was done by treating p-chlorophenone with sodium borohydride to yield p-chlorophenylethanol. 1 g of p-chloroacetophenone and 10 g of the wet biomass was taken in a 250mL conical flask, 20 mL of phosphate buffer of pH 7.0 and reaction mixture was incubated at 30^{0}C, 160-rev min^{-1} for 48 h. The cells were separated by filtration. The filtrate was extracted thrice with 20mL of dichloromethane. The combined extracts were then washed with 20mL of brine solution twice, dried over anhydrous sodium sulphate and evaporated to get the residue. The reaction was monitored by TLC using ethyl acetate: hexane: acetic acid (6:4:0.5) solvent system. The product formation was confirmed and quantified by HPLC.

Bioreduction with Aspergillus niger, Aspergillus flavus, Aspergillus ochraceous, Rhizopus stolanifer (soil isolate)

1g of p-chloroacetophenone and 10 g of the wet biomass was taken in a 250mL conical flask, 20 mL of phosphate buffer of pH 7.0 and reaction mixture was incubated at 30^{0}C, 160-rev min $^{-1}$ for 48 h. The cells were separated by filtration. The filtrate was extracted thrice with 20mL of dichloromethane. The combined extracts were then washed with 20mL of brine solution twice, dried over anhydrous sodium sulphate and evaporated to get the residue. The reaction was monitored by TLC using ethyl acetate: hexane: acetic acid (6:4:0.5) solvent system. The product formation was confirmed and quantified by HPLC.

Bioreduction with Baker's yeast

10 mg (8.3 µl) of p-chloroacetophenone and 5.0 g of the biomass was taken in a 250mL conical flask, 20 mL of phosphate buffer pH 7.0 and the resulting reaction mixture was incubated at 30^{0}C, 160-rev min^{-1} for 48 h. The cells were separated by filtration using Buchner funnel and the biomass was washed with phosphate buffer twice. The filtrate was extracted thrice with dichloromethane. The combined extracts were combined and washed with 20mL of brine solution twice, dried over anhydrous sodium sulphate and evaporated to get the residue. The reaction was monitored by TLC using ethyl acetate: hexane: acetic acid (6:4:0.5) solvent system. The product formation was confirmed and quantified by HPLC.

Bioreduction with Mushroom, Carrot, Radish, Apple, Cucumber, Beetroot, Potato and Onion

10 mg (8.3 µl) of p-chloroacetophenone and 30.0 g of the grated tissue was taken in a 250 mL conical flask, 20 mL of phosphate buffer pH 7.0 and the resulting reaction mixture was incubated at 30 ^{0}C, 160-rev min^{-1} for 48 h. The tissues were separated by filtration using Buchner funnel and they were washed with phosphate buffer twice. The filtrate was extracted thrice with dichloromethane. The combined extracts were combined and washed with 20mL of brine solution twice, dried over anhydrous sodium sulphate and evaporated to get the residue. The reaction was monitored by TLC using ethyl acetate: hexane: acetic acid (6:4:0.5) solvent system. The product formation was confirmed and quantified by HPLC.

HPLC ANALYSIS

The reduced product p-chlorophenylethanol was quantified by HPLC. The Mobile phase consisted of acetonitrile and water (1:1 ratio). The mobile phase was filtered through 0.45 µms membrane filter. The conditions for the chromatographic technique performed was as follows C18 phenomenex column (250 x 4.6 mm, 0.5 µm) was used with the flow rate of 1ml/min, Wavelength of 215nm, and Injection volume: 20 µl. The sample obtained was diluted to 10 mL with methanol and injected. 1mg/mL of the standard stock solution was prepared by adding chemically reduced product in methanol. From the standard stock solution, working solutions with a concentration of 20µg, 40µg, 60µg, 80µg and 100µg were prepared. The standard graph of p-chlorophenyletanol was plotted using substrate concentration vs. AUC (Table 1).

Table-1: Standard graph of p-chlorophenylethanol

Product Concentration (µg/ml)	AUC
20 µg/ml	2023458
40 µg/ml	3881488
60 µg/ml	4886937
80 µg/ml	6195991
100 µg/ml	7452847

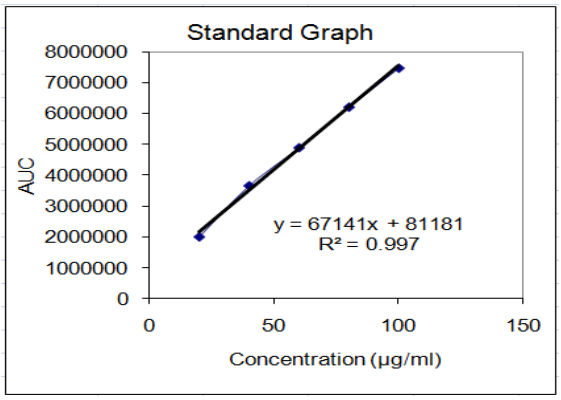

Fig-1: Standard graph of p-chlorophenylethanol

Optimization of Bioreduction of p-chloroacetophenone by Onion

Optimization of pH, substrate concentration, incubation time, biomass concentration was carried out and analysed for the bioconversion.

Optimisation of pH

The bioconversion was carried out at different pH 5.8, 6.4, 7.0, 7.6, 8.2 at 30 ^{0}C for which 10 mg (8.3 µl) of p-chloroacetophenone and 30.0 g of the grated onion was taken into five different 250 mL conical flask containing 20 mL of pH 5.8, 6.4, 7.0, 7.6, 8.2 phosphate buffer. The reaction mixture was incubated at 30 ^{0}C, 160-rev min^{-1} for 5 days. The biomass was separated by filtration and the filtrate was extracted with 20 mL of dichloromethane thrice. The collected organic extracts were combined and washed twice with 20 mL brine and dried over sodium sulphate. The dried extract was then evaporated to get the residue. The reaction was monitored by TLC using ethyl acetate: hexane: acetic acid (6:4:0.5) solvent system. The product formation was confirmed and quantified by HPLC.

Optimisation of substrate concentration was done using various substrate concentrations like 4 mg (3.32 µl), 8 mg (6.64 µl), 10 mg (8.38 µl), 14 mg (11.62 µl), 18 mg (14.94 µl) and pH 7.0 at 30 ^{0}C. P-chloroacetophenone of the above-mentioned concentrations and 30.0 g of the grated onion were taken in five different 250 mL conical flask containing 20 mL of pH 7.0 phosphate buffer. The reaction mixture was incubated at 30 ^{0}C, 160-rev min^{-1} for 5 days. The biomass was separated by filtration and the filtrate was extracted with 20 mL of dichloromethane thrice. The collected organic extracts were combined and washed twice with 20 mL brine and dried over sodium sulphate. The dried extract was then evaporated to get the residue. The reaction was monitored by TLC using ethyl acetate: hexane: acetic acid (6:4:0.5) solvent system. The product formation was confirmed and quantified by HPLC.

Optimization of Incubation time for the bioconversion was carried out at different incubation time like 24 h, 48 h, 72 h, 96 h and 120 hat pH 7.0 at 30 ^{0}C. Here 10 mg (8.3 µl) of p-chloroacetophenone and 30.0 g of the grated onion was taken into five different 250 mL conical flask containing 20 mL of pH 7.0 phosphate buffer. The reaction mixture was incubated at 30 ^{0}C, 160-rev min^{-1} for 24 h, 48 h, 72 h, 96 h and 120 h. The biomass was separated by filtration and the filtrate was extracted with 20 mL of dichloromethane thrice. The collected organic extracts were combined and washed twice with 20 mL brine and dried over sodium sulphate. The dried extract was then evaporated to get the residue. The reaction was monitored by TLC using ethyl acetate: hexane: acetic acid (6:4:0.5) solvent system. The product formation was confirmed and quantified by HPLC.

Optimization of Biomass Concentration was done by taking different biomass concentration like 5 g, 10 g, 15 g, 20 g and 30 g at pH 7.0 at 30 ^{0}C. Here10 mg (8.3 µl) of p-chloroacetophenone and 5 g, 10 g, 15 g, 20 g, 25 g and 30 g of the grated onion was taken into five different 250 mL conical flask containing 20 mL of pH 7.0 phosphate buffer. The reaction mixture was incubated at 30 ^{0}C, 160-rev min^{-1} for 5 days. The biomass was separated by filtration and the filtrate was extracted with 20 mL of dichloromethane thrice. The collected organic extracts were combined and washed twice with 20 mL brine and dried over sodium sulphate. The dried extract was then evaporated to get the residue. The reaction was monitored by TLC using

ethyl acetate: hexane: acetic acid (6:4:0.5) solvent system. The product formation was confirmed and quantified by HPLC.

Optimization of Bioreduction of p-chloroacetophenone by Radish

Optimization of Bioreduction of p-chloroacetophenone by Radish was also done for varying pH, temperature, Substrate concentration, Incubation time, Biomass Concentration

Optimization of pH for the bioconversion was carried out at different pH 6.2, 6.6, 7.0, 7.4, 7.8 at 30 ^{0}C. Here 10 mg (8.3 µl) of p-chloroacetophenone and 30.0 g of the grated radish was taken into five different 250 mL conical flask containing 20 mL of pH 6.2, 6.6, 7.0, 7.4, 7.8 phosphate buffer. The reaction mixture was incubated at 30 ^{0}C, 160-rev min^{-1} for 5 days. The biomass was separated by filtration and the filtrate was extracted with 20 mL of dichloromethane thrice. The collected organic extracts were combined and washed twice with 20 mL brine and dried over sodium sulphate. The dried extract was then evaporated to get the residue. The reaction was monitored by TLC using ethyl acetate: hexane: acetic acid (6:4:0.5) solvent system. The product formation was confirmed and quantified by HPLC.

Optimization of temperature was carried out at different temperature 25 ^{0}C, 30 ^{0}C, 35 ^{0}C, and 40 ^{0}C keeping pH constant at 7.0 for this 10 mg (8.3 µl) of p-chloroacetophenone and 30.0 g of the grated radish was taken in to four different 250 mL conical flask containing 20 mL of pH 7.0, phosphate buffer. The reaction mixture was incubated at different temperature like 25 ^{0}C, 30 ^{0}C, 35 ^{0}C, 40 ^{0}C and 160-rev min^{-1} for 5 days. The biomass was separated by filtration and the filtrate was extracted with 20 mL of dichloromethane thrice. The collected organic extracts were combined and washed twice with 20 mL brine and dried over sodium sulphate. The dried extract was then evaporated to get the residue. The reaction was monitored by TLC using ethyl acetate: hexane: acetic acid (6:4:0.5) solvent system. The product formation was confirmed and quantified by HPLC.

Optimisation of Substrate concentration

The bioconversion was carried out at different Substrate concentration (p-chloroacetophenone) 4 mg (3.32 µl), 8 mg (6.64 µl), 10 mg (8.38 µl), 14 mg (11.62 µl), 18 mg (14.94 µl) at pH 7.0 and 30.0 g of the grated radish were taken in five different 250 mL conical flask containing 20 mL of pH 7.0 phosphate buffer. The reaction mixture was incubated at 30 ^{0}C, 160-rev min^{-1} for 5 days. The biomass was separated by filtration and the filtrate was extracted with 20 mL of dichloromethane thrice. The collected organic extracts were combined and washed twice with 20 mL brine and dried over sodium sulphate. The dried extract was then evaporated to get the residue. The reaction was monitored by TLC using ethyl acetate: hexane: acetic acid (6:4:0.5) solvent system. The product formation was confirmed and quantified by HPLC.

Optimisation of Incubation time

The bioconversion was carried out at different incubation time was carried out by taking 10 mg (8.3 µl) of p-chloroacetophenone and 30.0 g of the grated radish into five different 250 mL conical flask containing 20 mL of pH 7.0 phosphate buffer. The reaction mixture was incubated at 30 ^{0}C, 160-rev min^{-1} for 24 h, 48 h, 72 h, 96 h and 120 h. The biomass was separated by filtration and the filtrate was extracted with 20 mL of dichloromethane thrice. The collected organic extracts were combined and washed twice with 20 mL brine and dried over sodium sulphate. The dried extract was then evaporated to get the residue. The reaction was monitored by TLC using ethyl acetate: hexane: acetic acid (6:4:0.5) solvent system. The product formation was confirmed and quantified by HPLC.

Optimisation of Biomass concentration

The bioconversion was carried out with different biomass concentration was done by 10 mg (8.3 µl) of p-chloroacetophenone and 5 g, 10g, 15g, 20g and 30g of the grated radish into five different 250 mL conical flask containing 20 mL of pH 7.0 phosphate buffer. The reaction mixture was incubated at 30 ^{0}C, 160-rev min^{-1} for 5 days. The biomass was separated by filtration and the filtrate was extracted with 20 mL of dichloromethane thrice. The collected organic extracts were combined and washed twice with 20 mL brine and dried over sodium sulphate. The dried extract was then evaporated to get the residue. The reaction was monitored by TLC using ethyl acetate: hexane: acetic acid (6:4:0.5) solvent system. The product formation was confirmed and quantified by HPLC.

Optimisation of Surfactant Concentration

The bioconversion was also carried out with different surfactants, CTAB and SLS with concentrations of 10 mg, 20 mg and 30 mg. The procedure used was as follows 10 mg (8.3 µl) of p-chloroacetophenone and 10 mg, 20 mg and 30 mg of CTAB, 10mg, 20mg and 30mg of SLS and was stirred vigorously for 10 mins and 30g of grated radish was taken into six different 250 mL conical flask containing 20 mL of pH 7.0 phosphate buffer. The reaction mixture was incubated at 30 ^{0}C, 160-rev min^{-1} for 5 days. The biomass was separated by filtration and the filtrate was extracted with 20 mL of dichloromethane thrice. The collected organic extracts were combined and washed twice with 20 mL brine and dried over sodium sulphate. The dried extract was then evaporated to get the residue. The reaction was monitored by TLC using ethyl acetate: hexane: acetic acid (6:4:0.5) solvent system. The product formation was confirmed and quantified by HPLC.

RESULTS AND DISCUSSION

In the screening, it was found that all the selected fungi and vegetables were capable of bringing out the reduction of p-chloroacetophenone. This indicated that the selected fungi and plant tissues had the required oxidoreductase enzyme which accepted the xenobiotic substrate. Out of the six fungi, Aspergillus species were found to be more efficient in reduction. Among the Aspergillus species, Aspergillus flavus showed maximum conversion. Out of the seven vegetables, Onion and Radish were found to be more efficient in reduction whereas Cucumber and Potato showed considerable activity. To our surprise, we found out that the Baker's yeast and Carrot, which were reported for maximum activity, exhibited the least conversion.

Optimization of bioreduction parameters

As Onion and Radish showed maximum conversion they were selected for optimization of bioreduction process. The parameters evaluated were: pH, temperature, substrate concentration, biomass concentration, incubation time, effect of surfactants.

Optimization of pH

The bioreduction was carried out at pH values, 5.8, 6.4, 7.0, 7.6 and 8.2 for onion and 6.2, 6.6, 7.0, 7.4 and 7.8 for radish as it is one of the important parameters for enzymatic activity as most of the enzymes possess optimum activity at a particular pH. Enzymes receptor sites are active at particular pH depending on the nature of the enzyme and thus maximum yield will be obtained at optimum pH (Table No. 02, Figure No. 2, Figure No. 03). The study indicated that pH 7.0 is optimum for the reduction of p-chloroacetophenone with both biocatalysts.

Table-02: Effect of pH on Bioreduction

pH	Product concentration (µg/ml)
5.8	1.04 µg/ml
6.4	14.78 µg/ml
7.0	24.98 µg/ml
7.6	16.58 µg/ml
8.2	0.96 µg/ml

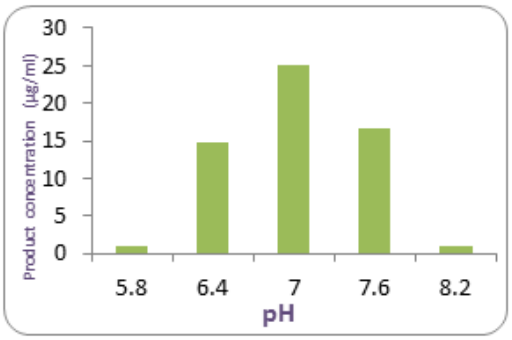

Fig-02: Effect of pH on Bioreduction

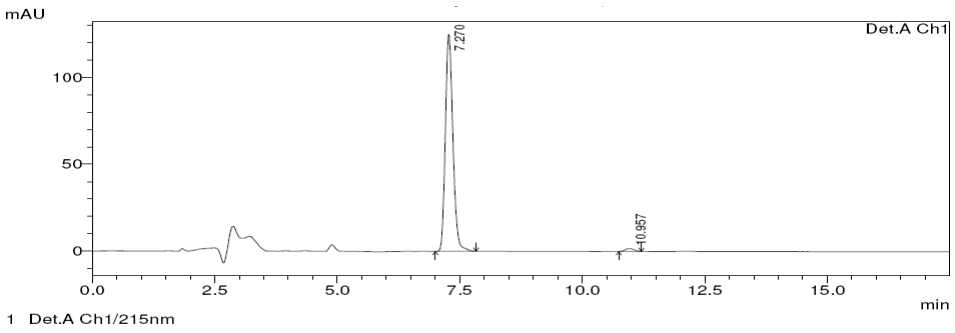

Fig-03: Chromatogram of reaction carried out at pH 7.0

Optimisation of temperature

Four different temperatures were selected for the study 25 ^{0}C, 30 ^{0}C, 35 ^{0}C, and 40 ^{0}C respectively. The optimum temperature was found to be 30 ^{0}C, for the reduction of p- chloroacetophenone with Radish (Table No. 3, Figure No. 04, Figure No.05).

Table-03: Effect of Temperature on Bioreduction

Temperature	Product concentration (µg/ml)
25 ^{o}C	3.07 µg/ml
30 ^{o}C	8.10 µg/ml
35 ^{o}C	0.62 µg/ml
40 ^{o}C	0.41 µg/ml

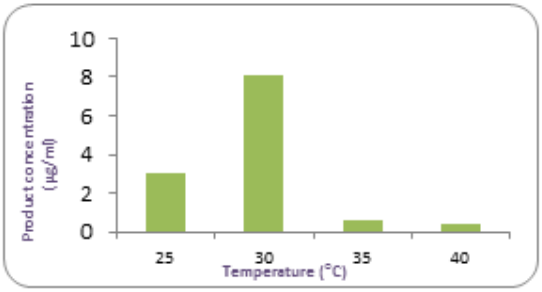

Fig-04: Effect of Temperature on Bioreduction

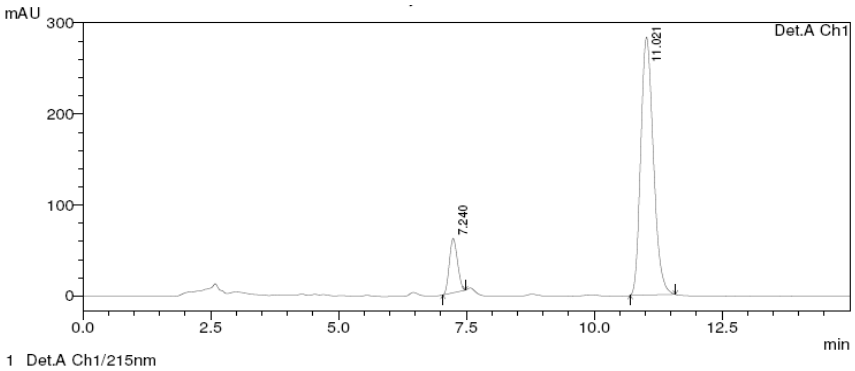

Fig-05: Chromatogram of reaction carried out at 30 ^{o}C

Optimisation of incubation time

The incubation time was varied from 24 - 120 hrs, the maximum conversion was observed at 96 hrs with Radish as biocatalyst. Beyond 96 hrs, the concentration of the reduced product decreased probably due to degradation of the product by other cellular constituents. In case of Onion, percentage conversion increased with increase in incubation time and maximum activity was found at 120 hrs (Table No. 04, Figure No. 06, Figure No.07).

Table-04: Effect of Time on Bioreduction

Incubation time	Product concentration (µg/ml)
24 h	1.98 µg/ml
48 h	3.27 µg/ml
72 h	3.79 µg/ml
96 h	13.11 µg/ml
120 h	8.10 µg/ml

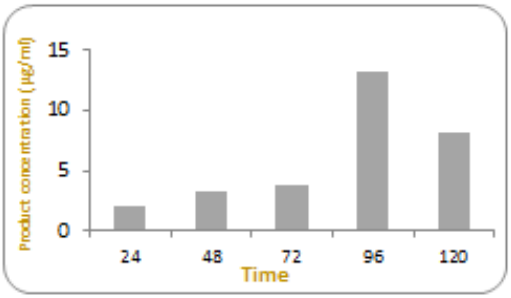

Fig-06: Effect of Time on Bioreduction

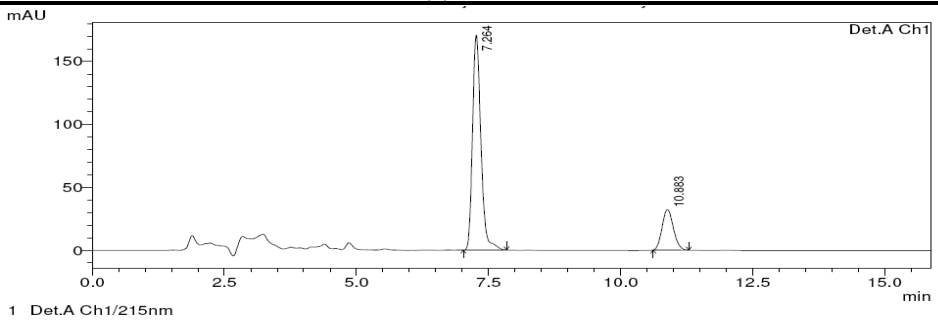

Fig-07: Chromatogram of reaction carried out for incubation time 120 h

Optimisation of substrate concentration

The reductase activity was evaluated by varying substrate concentration in the range of 4 mg, 8 mg, 10 mg, 14 mg and 18 mg. The enzyme activity was highest at 10 mg with Onion and at 14 mg substrate concentration with Radish and further decreased with increased concentration (Table No. 05, Figure No. 08, Figure No. 09). This infers that the increased substrate concentration inhibits the enzymatic activity may be due to the toxic effect of the substrate on the enzyme.

Table-05: Effect of Substrate on Bioreduction

Substrate concentration in (ml)	Product concentration (µg/ml)	% Yield
4 mg	0.56 µg/ml	1.4
8 mg	2.07 µg/ml	2.58
10 mg	8.10 µg/ml	8.1
14 mg	19.36 µg/ml	13.83
18 mg	6.57 µg/ml	3.65

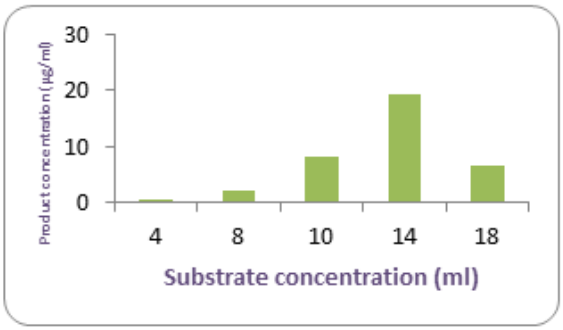

Fig-08: Effect of Substrate on Bioreduction

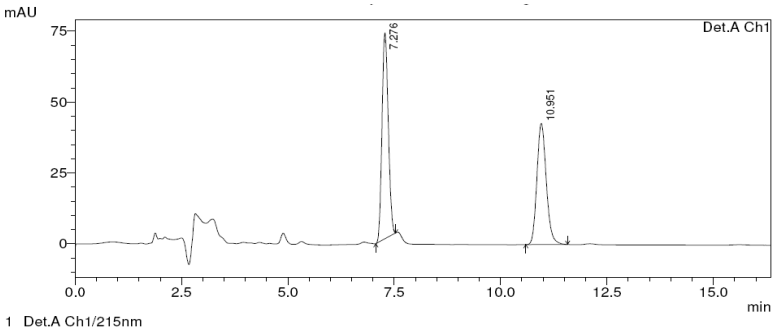

Fig-09: Chromatogram of reaction carried out for Substrate concentration of 14 mg

Optimisation of Biomass concentration

The effect of biomass on bioconversion was studied with biomass concentrations of 5 g, 10 g, 15 g, 20 g, 25 g and 30 g. The concentration of the product increased with increase in biomass concentration and it may further increase with increase in biomass concentration and the maximum rate of reaction was found with 30 g (Table No. 06, Figure No. 10, Figure No. 11).

Table-06: Effect of Biomass concentration

Biomass concentration	Product concentration (µg/ml)
5g	0.171 µg/ml
10g	0.174 µg/ml
15g	0.420 µg/ml
20g	6.52 µg/ml
25g	7.59 µg/ml
30g	8.10 µg/ml

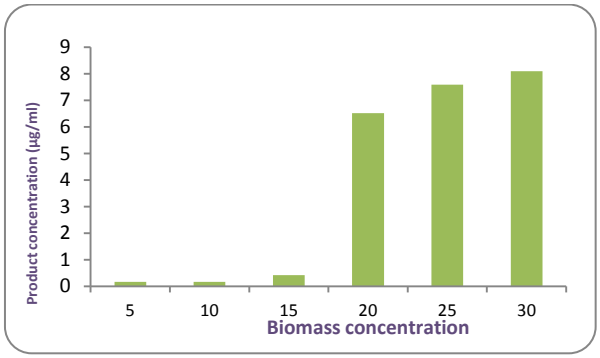

Fig-10: Effect of Biomass concentration

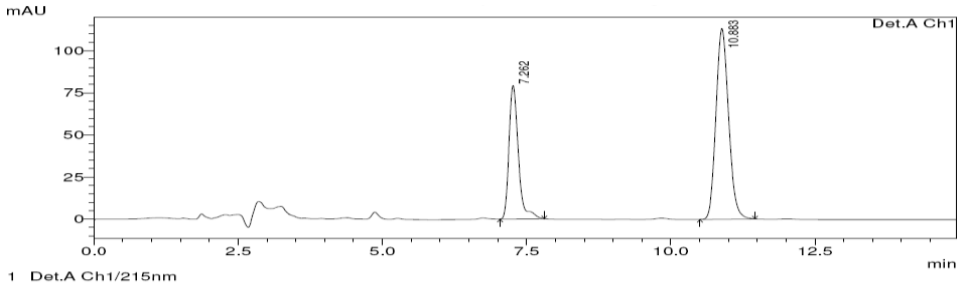

Fig-11: Chromatogram of reaction carried out with biomass 25g

Optimisation of Surfactants

The effect of both anionic (SLS) and cationic (CTAB) surfactants on bioreduction of ketone were studied. SLS was used in triple the quantity and CTAB was used in double the concentration of the substrate which showed maximum conversion of para-chloroacetophenone. Missiles were formed on stirring the substrate with surfactant which helps in better penetration of the substrate into the cell enzyme-substrate complex formation. Higher concentration of surfactant can damage the cells and can decrease the reduction of substrate.

Table-06: Effect of Surfactants on Bioreduction

Concentration of SLS	Product concentration (µg/ml)	Concentration of CTAB	Product concentration (µg/ml)
10mg	0.51 µg/ml	10mg	13.68 µg/ml
20mg	12.28 µg/ml	20mg	18.98 µg/ml
30mg	17.40 µg/ml	30mg	6.69 µg/ml

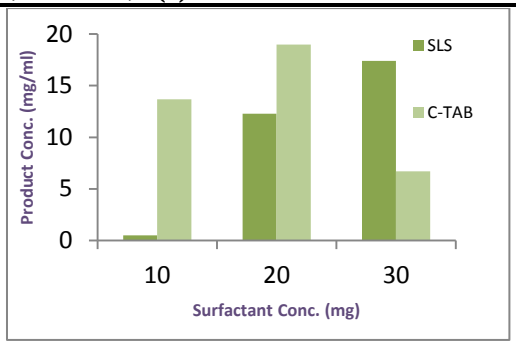

Fig-12: Effect of Surfactants on Bioreduction

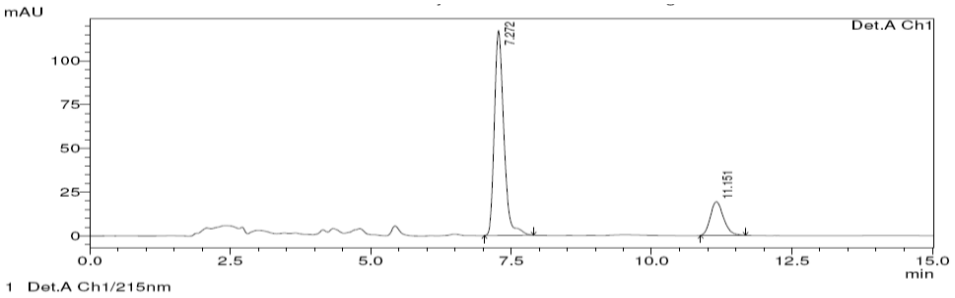

Fig-13: Chromatogram of reaction carried out with SLS 30mg

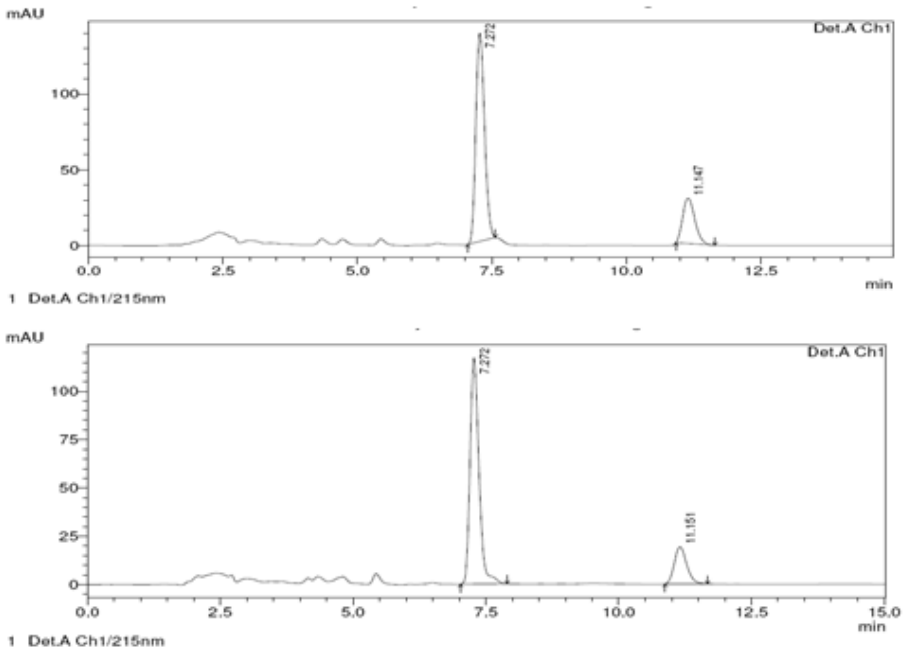

Fig-014: Chromatogram of reaction carried out with CTAB 20mg

CONCLUSION

The bioreduction of p-Chloroacetophenone was carried out using six different strains of fungi and seven plant tissues. All strains and plant tissues exhibited reduction capabilities. However, maximum reduction was observed with Onion and the least with Beetroot.

While, optimising the various bioreduction parameters like pH, temperature, substrate concentration and incubation time. The optimized conditions at which bioconversions was achieved was in pH 7.0. substrate concentration 10 mg (8.36 µL) biomass concentration of 30 g, and incubation time of 120 h for onion and pH 7.0, temperature 300 °C, Substrate concentration 14 mg, biomass concentration 30 g, incubation time 72 h and surfactants CTAB in 1:2 and SLS in 1:3 ratio for Radish.

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