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Methods to Increase Phenothiazine Conversion in *Beauveria bassiana*

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Authors' contributions

This work was carried out in collaboration between both authors. Author FN designed the study, performed the analysis, and wrote the first draft of the manuscript. Both authors read and approved the final manuscript.

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ABSTRACT

Aims: The degradation of phenothiazine with the filamentous fungus *Beauveria bassiana* was studied under different fermentation conditions. The objective was to demonstrate methods to increase substrate conversion in wild type *B. bassiana* using phenothiazine as a screening substrate. A bio catalytic system was optimized to increase the conversion of phenothiazine by resting cell reactions.
Methodology and Results: Reactions were carried out with cells grown in different concentrations of glucose, 5 - 40 g/L, and different resting cell densities, 0.78-6.2 mg cdw/mL. The conversion was monitored with gas chromatography and was characterized by mass spectroscopy, chiral HPLC, and NMR. The highest conversion, 74%±1, was achieved with 0.78 mg cdw/mL. Glucose didn't have an effect over the conversion of phenothiazine. This fungus oxidized phenothiazine into its sulfoxide and hydroxyl metabolites.
Conclusion: *B. bassiana* degrades more phenothiazine at lower cell densities than higher densities. The success of this project helps us expand the oxidation capacity of *B. bassiana* as a fungal biocatalyst while improving its utility for industrial purposes.

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1. INTRODUCTION

Catalysts are utilized in 80 percent [1,2] of all chemical synthesis operations. The industrial catalysts primarily used in oxidation reactions are precious and base metal types, organometallics, and zeolites. Enzymes and whole cell biocatalysts are used to a lesser extent [1,2]. As global manufacturing trends move toward increasing quality, safety, health, and environmental requirements of industrial chemical transformations, interest in advancing biocatalyst use in industrial processes is increasing. However, these applications are limited by the challenge of developing economically competitive biologically based systems [3]. The key for adopting these sustainable advancements is the development of novel process designs, which assure robustness, simplicity, and sustainability operations compatible with the current development of chemical reactions [4]. A potential strategy for more sustainable chemicals production is the use of entomopathogenic fungi, such as *Beauveria bassiana*. *B. bassiana* catalyzes hydroxylations [5-11], epoxidations [12], glycosylation [7,13], reductions [12] and sulfoxidations [5,14,15].

The synthesis of sulfoxides is important in the commercial production of flavors, antibacterial, and pharmaceuticals [11]. Sulfoxides are produced industrially by the Modena oxidation with the modification of the Sharpless catalyst [16,17]. Other industrial methods include reactions catalyzed by isolated enzymes and whole-cells, although they are used on a small scale. Enzymes used in sulfoxide production include peroxidases, flavin-dependent monooxygenases, cytochrome P450 and semisynthetic metalloenzymes [12,18]. Whole cell sulfoxidation has been catalyzed by *Cunnighamella elegans*, *Aspergillus niger, Penicillium verticillium, Mortierella isabellina, Helminthosporium sp.,* and *Pseudomonas putida* [19-24]. Current sulfoxidation processes based on organometallic catalysts are more efficient (89-98 % yields) and faster [12,25-27] than biocatalytic processes; the wide industrial application demonstrates that sustainability needs are surpassed by profitability targets. In addition, major progresses in fungal catalysis are needed in order to overcome low substrate conversions [5,14], low enzymatic expression [28,29], gaining control over cell type, and handling mycelia [2,28]. The following work demonstrates methods to increase substrate conversion in *B. bassiana* using phenothiazine, as a model substrate, under conditions that are milder than chemical catalysis and where the use of pollutants are reduced. Phenothiazine is the parent molecule of antipsychotics, antiemetics, antihistamines (H1-receptor antagonists) drugs, and azo dyes such as methylene blue [30]. The phenothiazine molecule induces Flavin-containing monooxygenases and several cytochrome P450 in mammals [31]; its metabolism has been thoroughly studied in mammals [30]. Phenothiazine [19] and substituted phenothiazine drugs [22,32,33] oxidation had been studied in the lower fungi *C. elegans* and endophytic fungi. The success of this project helps us expand the capacity of *B. bassiana* as a fungal biocatalyst while improving its utility for industrial purposes.

2. METHODS

2.1 *B. bassiana* Strain of Interest

B. bassiana, ATCC 7159, was purchased from the American Type Culture Collection (Manassas, VA). It was grown on potato dextrose agar (PDA) slants which were incubated at 26°C for 10-15 days and aerial conidia were harvested.

2.2 Preparation of Media

B. bassiana cultures were grown on Sabouraud Dextrose Emmons Broth (SDB); the medium was composed of glucose (20 g/L), yeast extract (5 g/L), and peptone (10 g/L). The pH was adjusted to 7 with HCl or NaOH. The biotransformation was done in a medium comprised of phosphate buffer amended with glucose as a carbon source. The medium included the following components: KH_2PO_4 (5.2 g/L), K_2HPO_4 (10.7 g/L), and glucose (5 g/L) for 0.5% (w/v) glucose potassium phosphate buffer solution (GPPB). Prior to inoculation, the medium was sterilized by autoclaving (121°C, 15 psi) for 20 minutes. Phenothiazine oxidations with *B. bassiana* were conducted with either growing cells or resting cells.

2.3 Phenothiazine Biooxidation with Growing Cells

B. bassiana conidia were harvested by flooding PDA slants with sterile detergent solution, 0.02%

Tween 80, and were transferred into a 250 mL baffled flask with 50 mL of Sabouraud Emmons Broth (SDB). The seeding stock of *B. bassiana* was incubated for 72 h at 26°C and 180 rpm. To a 1 L baffled flask, 150 mL of SDB was added along with a 10% inoculum (15 mL of seeding stock). The culture was grown for 72 h at 26°C and 180 rpm. After 3 days, 1.5 mL of a phenothiazine solution in acetone (50 mg/mL) was added into the flask. The reaction was monitored by thin layer chromatography. To evaluate the effect of glucose on the conversion, 1 mL of 72 h cells were harvested and suspended into 10 mL SDB with different glucose concentrations 5-40 g/L. After addition of cells, 100 µL of a phenothiazine solution (5 mg) in acetone (50 mg/mL) was added into each reaction mixture. The reaction was run at 26°C and 180 rpm for 7 days.

2.4 Phenothiazine Biooxidation with Resting Cells

For resting cell conversions, 72-h-grown cells were harvested and suspended in different volumes of GPPB to have different cell concentrations (0.78-6.2 mg cdw/mL). The dilutions were prepared as serial dilutions (1-, 2-, 4- and 8-fold) of the original cell stock. A 10 mL cell suspension was washed and was dried at 65°C for 24 h for cell dry weight determination. After the concentration was adjusted, 100µL of a phenothiazine solution (5 mg) in acetone (50 mg/mL) was added into each 10 mL cell suspension. For the determination of the optimal substrate concentration, 1.5 mg cdw/mL cells were suspended in 10 mL of GPPB and 50-2000 ppm phenothiazine were added into each reaction mixture, respectively. The reactions were run at 26°C and 180 rpm for 7 days.

2.5 Extraction and Characterization

The cell broth was centrifuged at 5,000 rpm for 20 min. The supernatant was extracted with three volumes of 50 mL ethyl acetate. The cell pellet was washed with two volumes of 15 mL ethyl acetate and incorporated into the total organic phase. The organic phase was dried over anhydrous Na_2SO_4 and vacuum distilled into a brown solid. The metabolites were purified by silica gel flash column chromatography (2 cm x 12 cm) eluting with a petroleum ether-ethyl acetate gradient (80%-100%) and then eluting with 10% methanol in ethyl acetate.

Phenothiazine conversion was followed by GC-MS. Samples (1 µL) were injected into a SPB-5ms (60 m x 0.25 mm ID, 0.25 um film thickness). Analyses were performed in a Thermo Voyager single quadrupole mass spectrometer interfaced with a Trace 2000 GC, equipped with an AS3000 auto sampler. Full scans were performed from 50-600 amu. Conversion was monitored via single ion monitoring (SIM) scans at [M+.]199 with m/z reference peaks [M+.–32] 167 and [M+.– 45] 154. Carbazole was used as internal standard. Data were processed using Xcalibur 1.4 software. Ionization was performed using electron ionization (EI) with electron energy at 70 eV. The temperature ramp was 100-250°C at 20°C/min ramp and 250°C for 10 min [19,34]. The injector and interface were at 250°C.

The isolated metabolites were analyzed by LC-MS-TOF using both electrospray ionization (ESI) and in-source collision induced dissociation (CID) modes. Samples (15 µL) were injected into an Acquity BEH C18 (100 mm x 2.1 mm ID, 5 µ m). Analysis was performed in an Acquity equipped with a Waters Q-TOF Premier detector. Full scans were performed from 120-1,000 m/z in gradient mode, 20 min gradient at a 0.2 mL/min flow rate [19], with ACN:H_2O with 3mM NH_4OAc (20:80) to (80:20). Ionization was performed using positive electron spray ionization (ESI) at 100 V for accurate mass determination and low energy (20 eV) MS/MS collision energy for fragmentation pattern validation by in source CID. The enantiomeric excess for 3-hydroxy phenothiazine sulfoxide was analyzed by chiral HPLC. Samples (5 µL) were injected into a CHIRALPAK ®-MA+ (50 mm x 4.6 mm ID, 3 µm). Analysis was performed in a Shimazdu 1200 equipped with a photodiode detector. The compounds were monitored at 254 nm in an isocratic mode with 2 mM $CuSO_4$ solution for 15 min at a flow rate of 0.5 mL/min. 1H-NMR spectra were recorded on a 300 HZ Bücher NMR Spectrometer. Tetramethylsilane was used as internal standard. Samples were dissolved in deuterated chloroform.

2.6 Phenothiazine sulfoxide (II)

Rf 0.28 (1:9 petroleum ether–ethyl acetate); 1H NMR (300 MHz, $CDCl_3$) 10 (NH broad s) 7.89 (2H, dd, J=7.7,1.5 Hz), 7.56(2H,ddd, J= 8.2, 7.7,1.5 Hz) 7.38(2H, dd, J=8.2, 1.1 Hz) 7.21(2H,ddd, J=7.7, 7.7, 1.1 Hz); HRMS (MALDI-TOF) calc. from $C_{12}H_9NSO+H$ $[M+H]^+$ calc m/z 215.0556, found 215.0478.

2.7 3-hydroxy Phenothiazine Sulfoxide (III)

Rf 0.15 (1:9 petroleum ether–ethyl acetate); 1H NMR (300 MHz, $CDCl_3$) 9.69 (1NH s), 7.84(1H J=8,1.5), 7.52 (1H, J=8.4,1.5), 7.33(1H, J=2.8),7.31 (1H J=8.4, 1.1), 7.28 (1H J= 8.8), 7.14 (1H, J=8, 1.1), 7.13(1H, J=8.8,2.8) HRMS (MALDI-TOF) calc. from $C_{12}H_9NSO_2$+H $[M+H]^+$ calc m/z 231.055, found 231.0391.

2.8 Statistical Analyses

Statistical evaluations of substrate conversion were conducted using GraphPad Prism 6 (GraphPad Software, La Jolla CA). The analyses were run in triplicates and were repeated twice for reproducibility purposes. The threshold for statistical significance was set at $P \leq 0.05$. The data were tested by one way analysis of variance (ANOVA); whenever a significant effect was indicated, a Dunnett multiple comparisons test was carried out. Time course experiments involved sampling three replicates at 4 distinct time points, at intervals of 48 hrs., after the addition of phenothiazine. Substrate conversion was plotted against time using GraphPad Prism, and the rate was determined after curve-fitting to a one-phase association function. All data were expressed as mean ± standard deviation.

3. RESULTS AND DISCUSSION

Throughout the course of experimentation, only two metabolites were isolated from the *B. bassiana* mediated conversion of phenothiazine. The isolated metabolites were phenothiazine sulfoxide (II) and 3-hydroxy phenothiazine (III) (See Fig. 1). Phenothiazine sulfoxide positive ESI-MS with CID had significant ions at m=z 216$[M + H]^+$, 199 (MH^+ - 17), 167 (MH^+- 49) and 166 (MH^+ -50), which were consistent to loses of oxygen and sulfur. 3-Hydroxyphenothiazine sulfoxide positive ESI-MS with CID had significant ions at m=z 232 $[M + H]^+$, 215 ($[MH^+$- 17), 186 (MH^+- 46), 183 (MH^+- 49), 182 (MH^+- 50) and 154, which were consistent to losses of oxygen, sulfur and CO fragments. The extrusion of oxygen from the molecular ion gave the base peak on both cases. The enantiomeric excess for 3-hydroxy phenothiazine sulfoxide was 63±4% (R)-3-hydroxy phenothiazine sulfoxide (See Appendix). These results are similar to bioconversions of phenothiazine with the yeast *C. elegans* [19] and the functional group conversions identified in the oxidation of phenothiazine drugs [22,32,33,35].

Whole-cell sulfoxidation reactions were performed with different cell concentrations in triplicate experiments for each concentration. Values of the cell concentrations tested ranged from 0.78 to 6.2 mg cdw /mL (See Fig. 2). The highest conversion, 74±1% at a rate of 1.56E-01±1.7E-02 h^{-1}, was achieved at the lowest cell concentration, 0.78 mg cdw /mL (See Table 1). This value was demonstrated to be statistically distinct from the conversion experiments at other concentrations. Higher concentrations, 1.5-6.2 mg cdw /mL, suffered slight variations that were not statistically significant throughout the 7 days. Since sulfoxides are metabolic intermediates which can enter different reactions, variation in phenothiazine conversion is common; the phenothiazine can be either further oxidized to alternative metabolites or reduced to the parent molecule [30]. One possible explanation in the low cell density phenomena, is the differential regulation of enzyme activities in response to increased cell population [36,37]. This might be verified through evaluation of proteomics or gene expression, but was beyond the scope of the current study. The consistent data from these current experiments indicates that reduction of phenothiazine sulfoxide into phenothiazine has a lesser impact on the conversion in the more diluted cells, 74±1%, in comparison to more concentrated cells, 62%±2. In addition to potential metabolic phenomena at higher cell densities, physical variations, diffusion, and viscosity [2] play a larger role in conversion sometimes being factors which impose greater limitations than low catalytic power or low enzymatic expression.

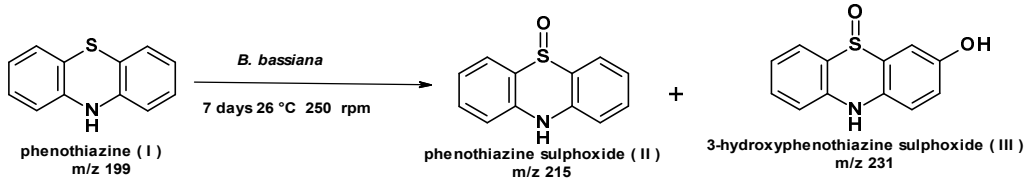

Fig. 1. Isolated and characterized metabolites from the degradation of phenothiazine by *B. bassiana*

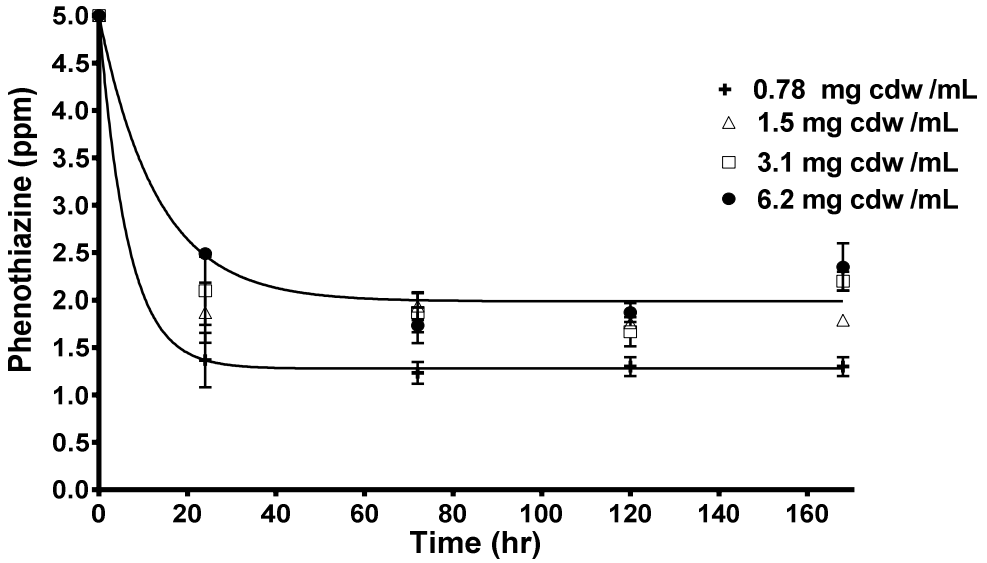

Fig. 2. Phenothiazine time course reaction with different cell concentrations in GPPB at 26°C and 180 rpm, fitted to a one phase –exponential decay; bars represent standard deviations of 3 replicates

Table 1. Kinetic parameters derived from biotransformation of phenothiazine with different cell concentrations in glucose buffer solution after 7 days

Initial Cell concentration (mg cdw/ mL)	0.78	1.55 #	3.1	6.2
Rate (h^{-1})	1.56E-01±1.7E-02	1.07E-01±9.6E-02	1.17E-01±5.5E-02	7.63E-02±2.6E-02
Plateau (ppm)	1.28±1.8E-02	1.8±4.5E-02	1.91±1.2E-01	1.99±1.6E-01
Max conversion	74%±1*	64%±1	62%±2	60%±7
R^2	0.99	0.99	0.98	0.97

**p<0.05 significant difference, ±SD, # control group*

These results further demonstrate the value of exploring the concentration space experimentally rather than assuming higher cell density will result in higher conversion.

The effect of different phenothiazine concentrations, 50 – 2,000 ppm, was compared to the control group, 500 ppm. The optimal initial substrate concentration was selected for higher conversion. As starting substrate concentrations increased from 100 to 2000 ppm, a reduction in conversion was observed. Intitial concentrations of 100 -500 ppm did not result in differences that were statistically significant while higher starting values of 750 - 2,000 ppm produced a significant reduction in conversion (See Appendix). Phenothiazine concentration varied according to the substrate concentration, resulting in the highest conversion (65±1.4%) at a phenothiazine concentration of 500 ppm after 7 days (See Fig. 3).

The effect of initial glucose concentration, 5 – 40 g/L, was compared to the control group, 20 g/L. Glucose concentration effects on phenothiazine conversion were not seen over the time course of the experiments despite the impact that glucose levels have on cellular growth, affecting bioenergetics (See Table 2 and Appendix). Fungi have been reported to turn off a large number of genes in the presence of glucose as an energy saving response. This phenomenon primarily affects catabolic enzymes without affecting other cellular functions, such as xenobiotic metabolism or virulence of *B. bassiana* to aphids [38,39].

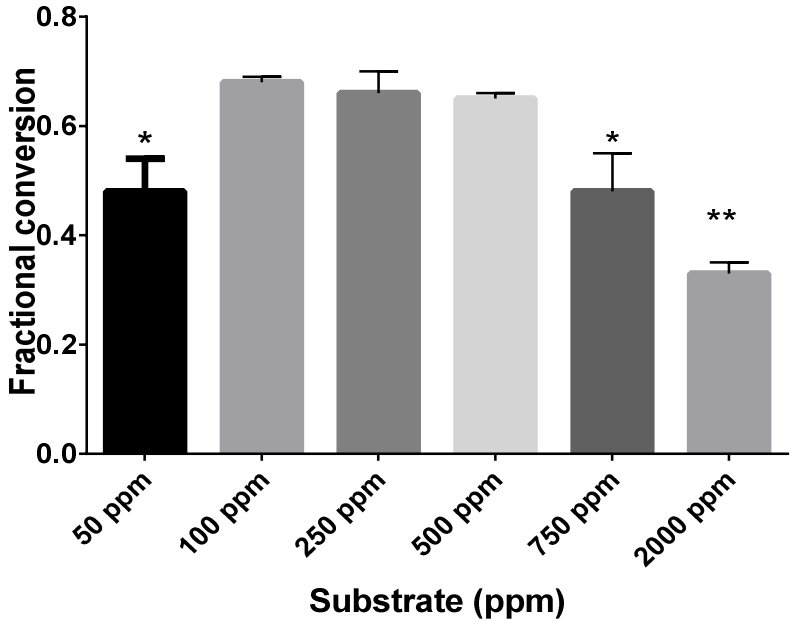

Fig. 3. Phenothiazine fractional conversion in 1.5 mg cdw/mL cells in GPPB after 7 days with different substrate concentrations, *statistical significant difference, bars represent standard deviations of 3 replicates

Table 2. Kinetic parameters of the biotransformation of phenothiazine in SDB with different glucose concentration

Glucose conc. (g/L)	5	10	20 #	30	40
Rate (h^{-1})	8 E-02±2 E-02	5E-02±1 E-03	7 E-02±2 E-02	6 E-02±1 E-02	6 E-02±8 E-03
Plateau (ppm)	1.6±0.09	1.6±0.01	1.6±0.13	1.5±0.14	1.6±0.08
Max Conversion	36%	34%	38%	39%	37%
R^2	0.99	0.99	0.98	0.98	0.99

No statistical significance, P<0.05 ±SD, # control group

Based on the experimental results from this present study, the oxidase expression involved in phenothiazine conversions is not as sensitive to glucose fluctuations. Quantitative effects of glucose in fungal biotransformation had been demonstrated over the conversion of meloxicam [40] and cyproheptadine hydrochloride [41] and with alternative carbon sources in the conversion of ferulic acid [42] and albendazole [43]. An increase in reversible reactions lowers phenothiazine conversion at higher glucose concentrations. More detailed experiments are required to assay the effects isolated enzymes have over conversion. Future work includes the analysis of physical and environmental stress effects over conversion; in addition to protein identification of enzymes involve on phenothiazine oxidation.

4. CONCLUSION

These results demonstrate that *B. bassiana* converts phenothiazine into phenothiazine sulfoxide and hydroxyl phenothiazine sulfoxide. This study highlights the effects of glucose and cell densities on phenothiazine conversion. The ability of *B. bassiana* to oxidize phenothiazine provides further foundation for a well-characterized biocatalyst of possible industrial interest.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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APPENDIX

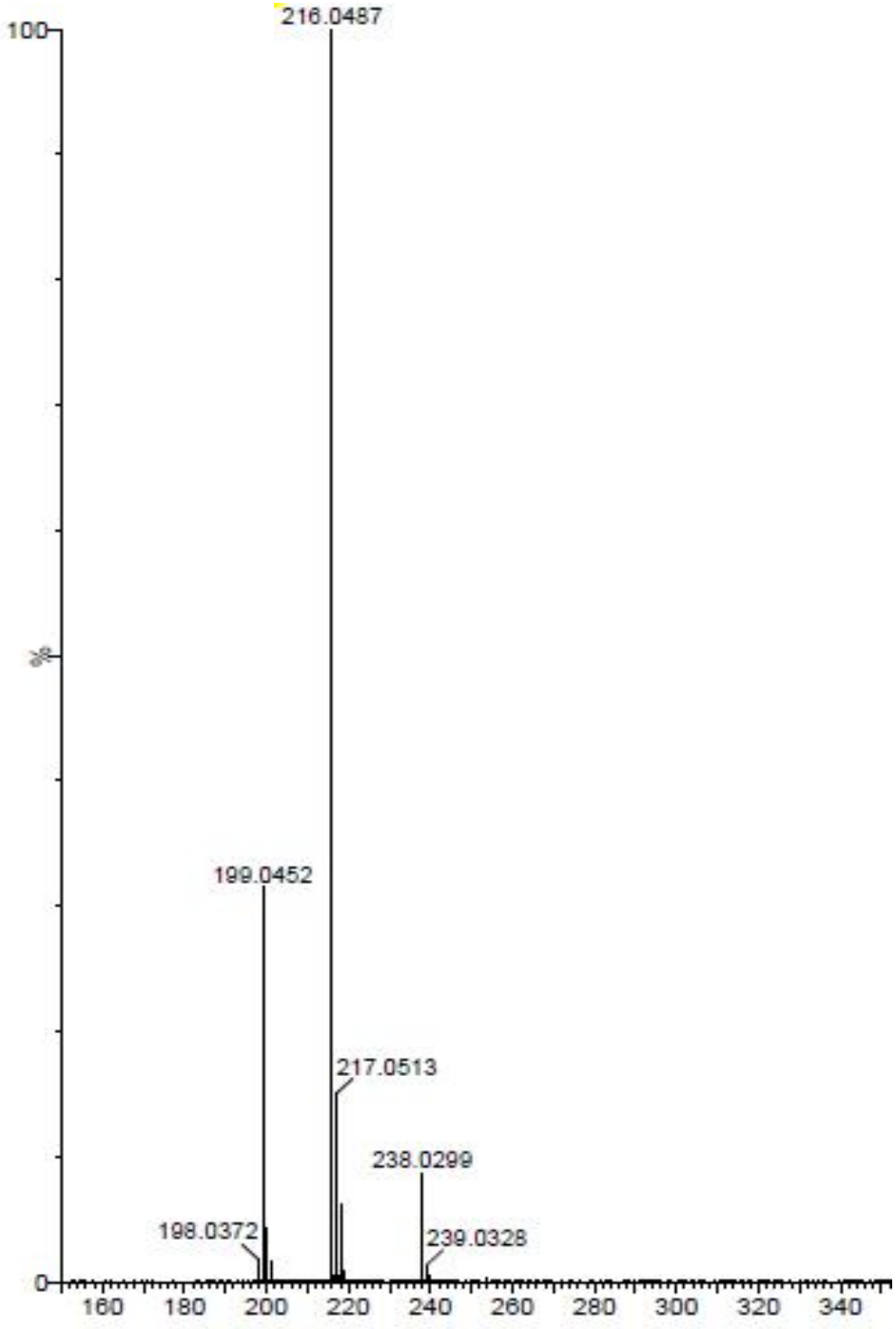

Fig. A-1. Phenothiazine sulfoxide positive ESI-MS at 100 V

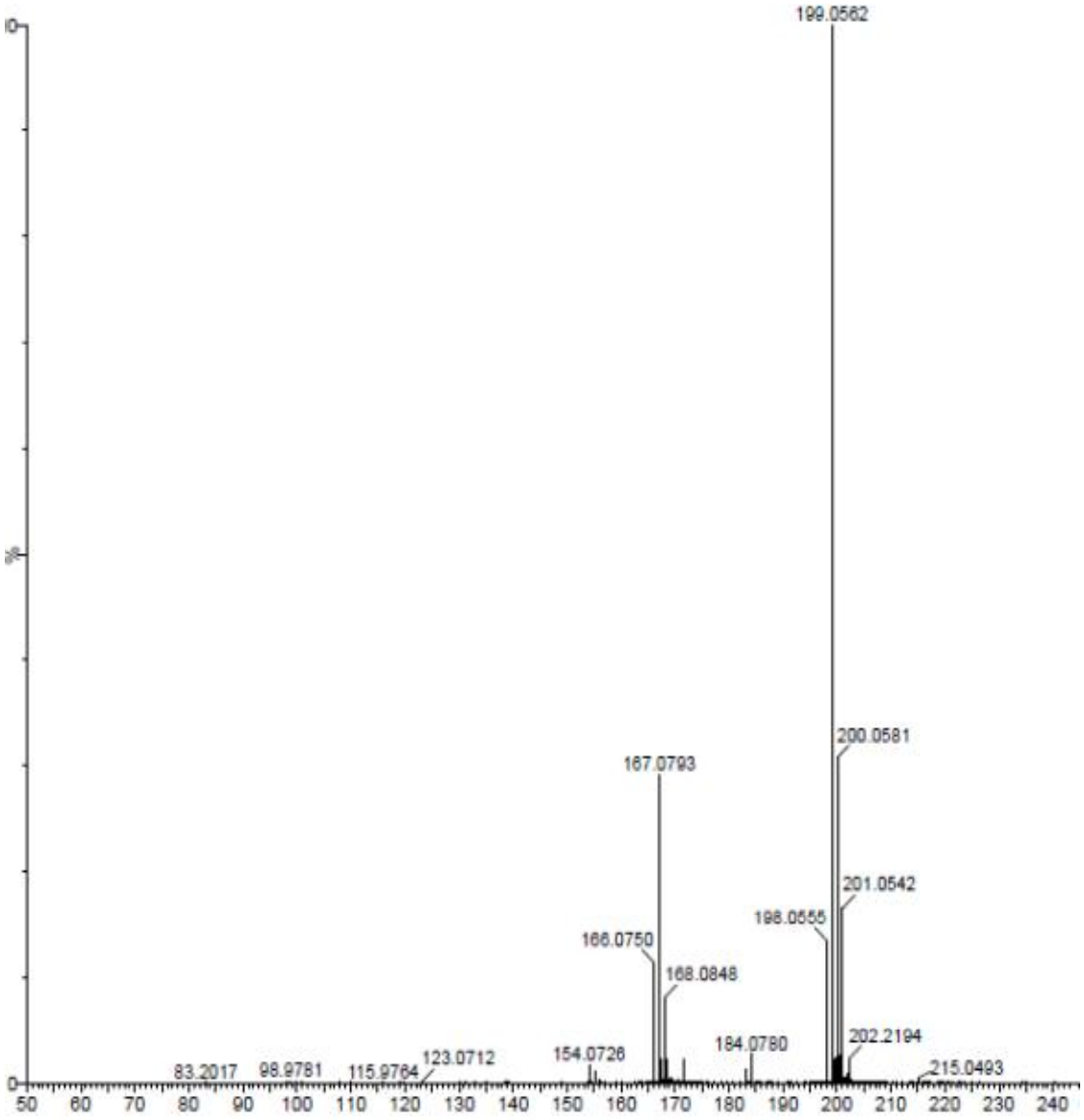

Fig. A-2. Phenothiazine sulfoxide positive ESI-MS with low energy in-source CID at 20 eV

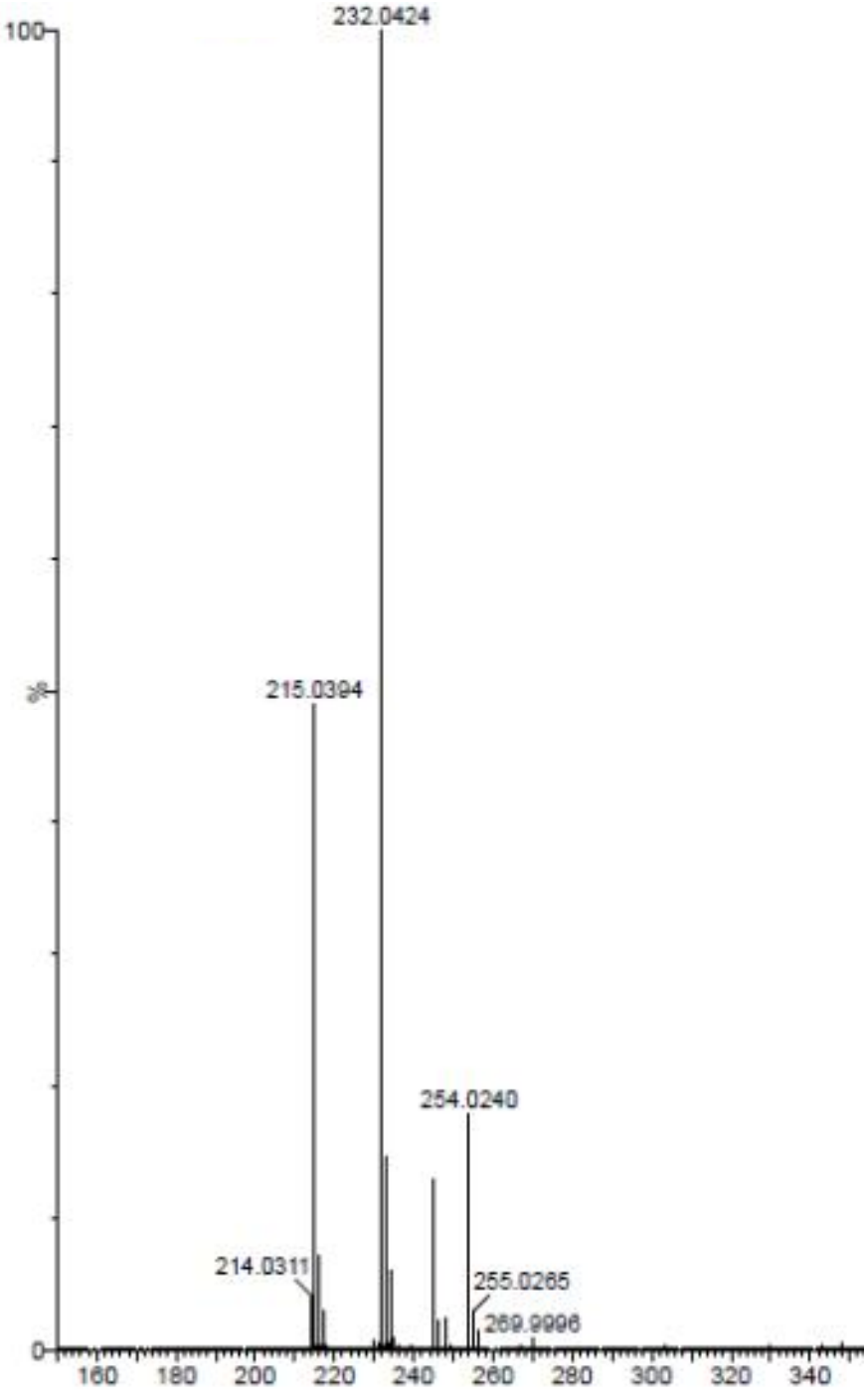

Fig. A-3. 3-hydroxy- phenothiazine sulfoxide positive ESI –MS at 100 V

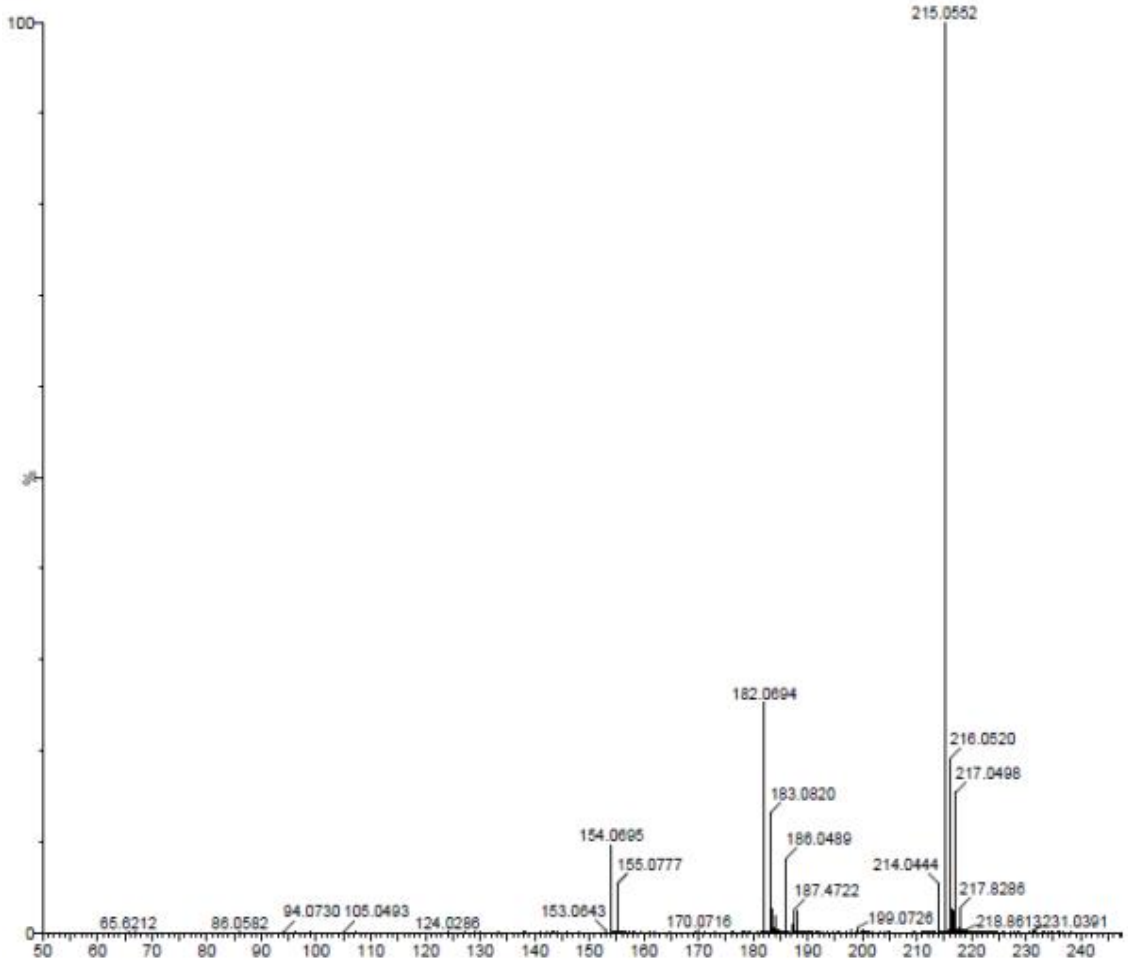

Fig. A-4. 3-hydroxy- phenothiazine sulfoxide positive ESI –MS with low energy in-source CID at 20 eV

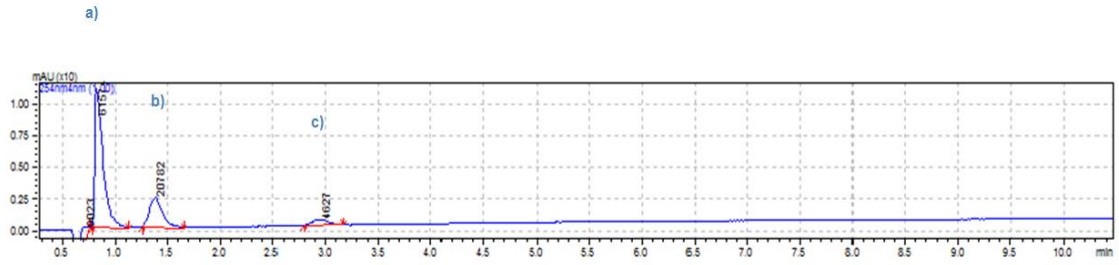

Fig. A-5. Chiral chromatogram of 3-hydroxy phenothiazine sulfoxide run in a CHIRALPAK ®-MA+ at a flow rate of 0.5 mL/min of 2mM $CuSO_4$ soln. a) phenothiazine sulfoxide, b) (R)-3-hydroxy phenothiazine sulfoxide and c) (S)-3-hydroxy phenothiazine sulfoxide

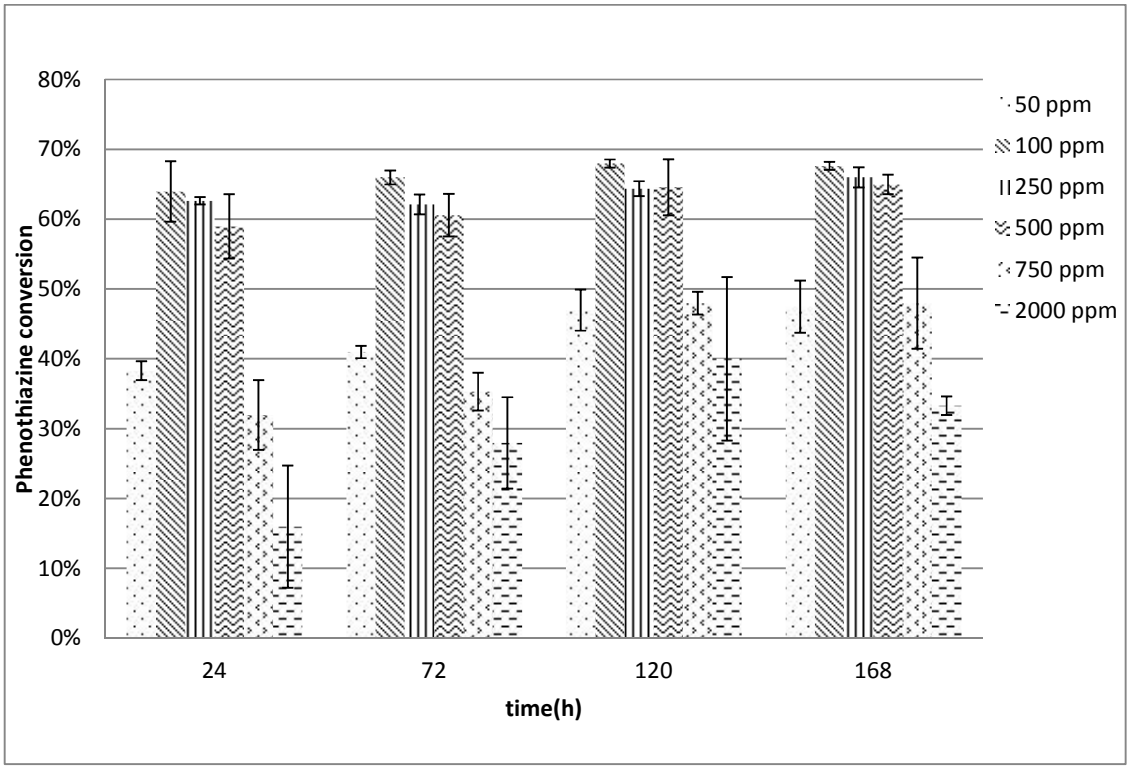

Fig. A-6. Time course conversion with different phenothiazine concentrations, with 1.5 mg cdw/mL suspended in GPPB at 26°C and 180 rpm, bars represent standard deviations

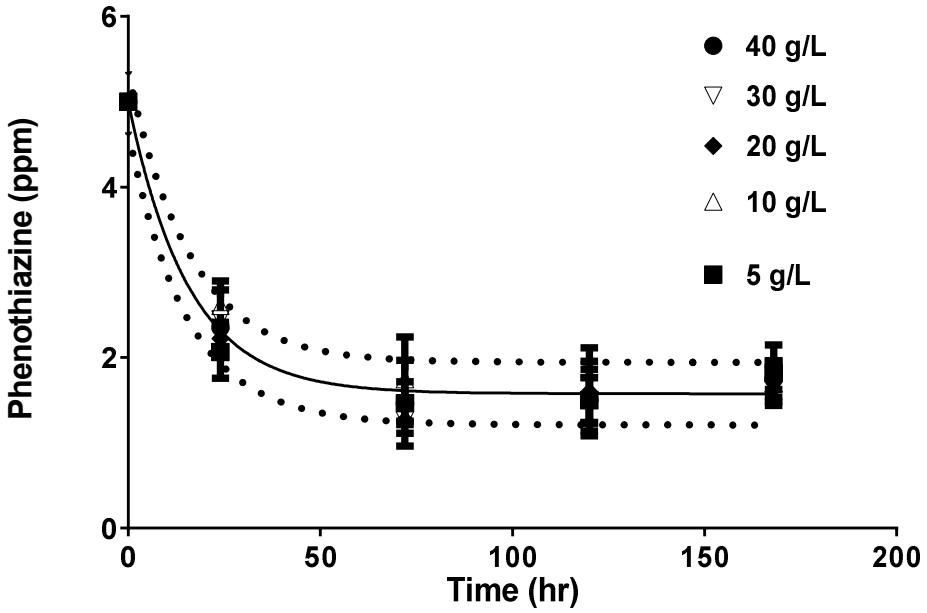

Fig. A-7. Effects of glucose concentration on the conversion of phenothiazine time course reaction plot fitted to a one phase exponential decay , dotted lines p<0.05 confidence intervals. Reaction ran for 7 days at 26°C and 180 rpm in SDB with different glucose concentrations

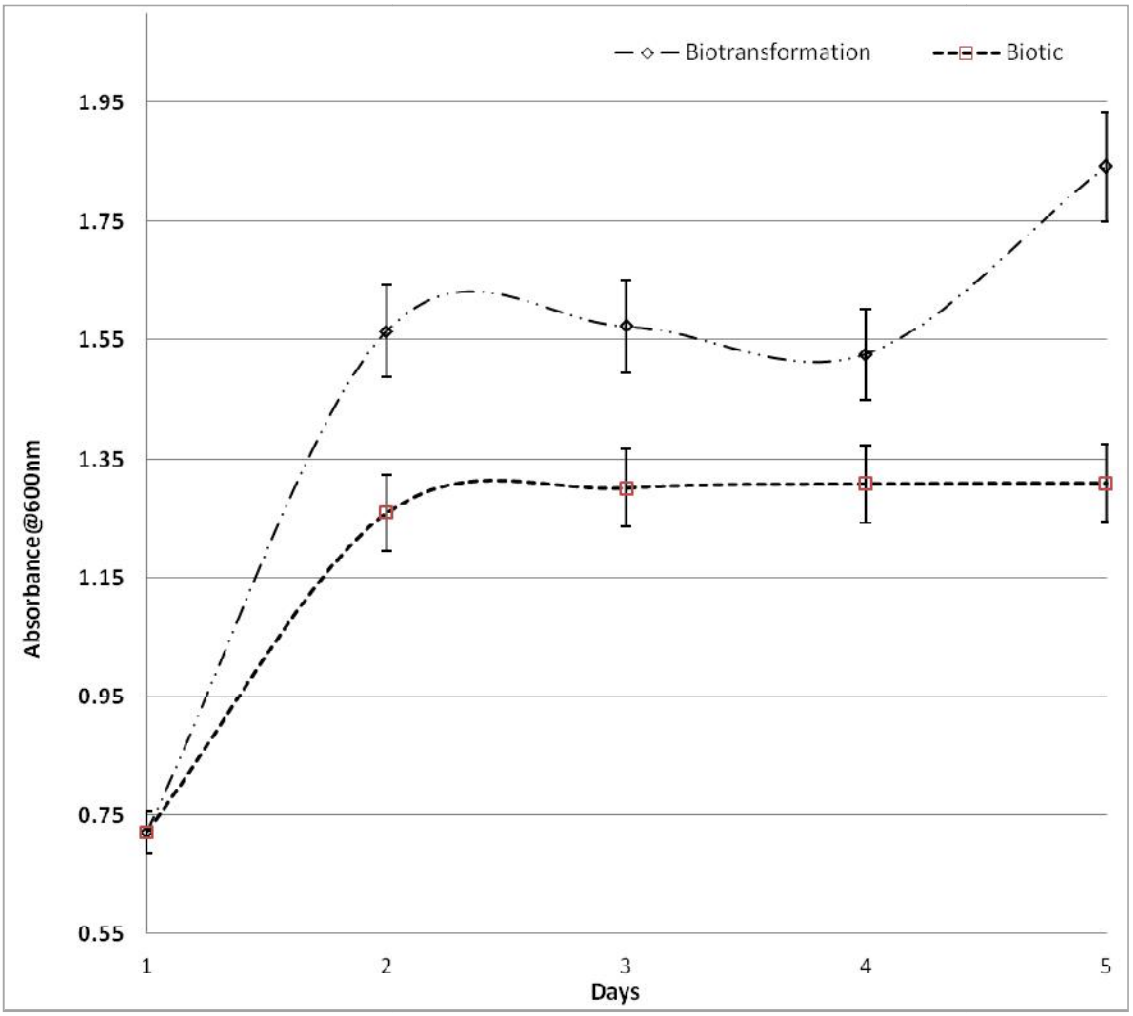

Fig. A-8. Phenothiazine effect over fungal growth

In presence of phenothiazine fungal growth was often higher than in pure cultures, although further analyses are needed to understand the metabolic effect played by different substrates on fungal growth. As a result, biomass and activity correlations are not defined since several factors may play single or combinatorial effects (i.e. primary or secondary metabolism, carbon catabolism, toxic substrate or product, stress response, transport & diffusion into mycelium bundles…) in substrate conversion.

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