



Development of Enzymatic Microtoxicity Test for the Toxicity Assay of Chlorophenolic Compounds

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Author's contribution

The sole author designed, analysed, interpreted and prepared the manuscript.

Article Information

DOI: 10.9734/AJOB/2020/v9i230083

Editor(s):

(1) Dr. Angelo Mark P. Walag, University of Science and Technology of Southern Philippines, Philippines.

Reviewers:

(1) Anna Rafaela Cavalcante Braga, Universidade Federal de São Paulo (UNIFESP), Brazil.

(2) José Alfredo Hernández Maldonado, UPIIG-IPN, Mexico.

Complete Peer review History: <http://www.sdiarticle4.com/review-history/57625>

Original Research Article

Received 22 March 2020

Accepted 27 May 2020

Published 01 June 2020

ABSTRACT

Enzymes are proteins that serve as catalysts of biological reactions in the animal, plant, and microbial cells. They are quite specific concerning with regard to their substrates. Enzymes lower the activation energy and increase the rate of biochemical reactions. According to the type of reaction catalyzed, enzymes are currently subdivided into six classes, namely oxidoreductases, hydrolases, transferases, lyases, isomerases, and ligases.

The effects of toxic chemicals and chlorophenolic compounds on enzyme activity are determined by fast, useful, and simple enzymatic methods. These methods can be carried out in low volumes and automatically using equipment such as a spectrophotometer, fluorometer and scintillation counters.

In this study, a new microtoxicity test was developed with the use of the fungal and bacterial β -galactosidase enzyme. In the toxicity experiments, the effects of 2,4,6-trichlorophenol (2,4,6 TCP), 2,4-dichlorophenol (2,4 DCP) and 4-chlorophenol (4 CP) over the β -galactosidase enzyme activity were investigated.

In this study, the bacterial and fungal β -galactosidase enzyme was found to be effective in determining the detoxification of some chlorinated phenolic compounds, 2,4,6-TCP, 2,4-DCP, and 4-CP, through the treatment with laccase enzyme. The inhibitor effect of toxic compounds on the activity of the β -galactosidase enzyme was examined. As a result of the studies, 61% inhibition for 2,4,6-TCP, 58% inhibition for 2,4-DCP and 47% inhibition for 4-CP was observed.

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Mikrotoxicity test system developed in our laboratory which works principally based on inactivation of β -galactosidase activity by toxic chlorinated phenolics, was used for toxicity detection. Data obtained from microtoxicity tests have shown that the dechlorination of chlorinated phenolics with laccase has resulted in the loss of toxic potentiality of these chemicals to a substantial extent. We thought that this study is to be an important contribution to bioremediation science and environmental technology with a new biotechnological approach.

Keywords: *Microtoxicity test; β -galactosidase enzyme; chlorophenolic compounds; Bacillus sp.; Trichoderma viride.*

1. INTRODUCTION

The chemical industry has a wide range of usage area of chlorophenolic compounds. However, biodegradation of these compounds in natural environments is a process that is quite slow. Chlorinated aromatics cause significant environmental health problems due to their xenobiotic properties due to their toxic and recalcitrant properties. Dechlorination of these group compounds is an important stage in their detoxifications and biodegradation [1]. In the developing world, biological processes, which are now an alternative to chemical processes in the protection of environmental health controlled by international agreements, are gaining increasing potential. The dissemination of the use of low cost and environmentally friendly biological processes within the scope of "Clean Technology-Clean Environment" is one of the leading goals of Biotechnology Science.

Since microorganisms are fast-paced tests due to short life cycles, it is possible to observe the effects of toxic substances on numerous

generations over a short period of time of time and also toxic compounds are also applied to these organisms at high concentrations (Table 1). Therefore they are known as highly productive and reliable tests [2].

Microbial toxicity assays are of great importance today, especially in treatment facilities. In these facilities, the application areas of toxicity assays are examined in three groups [3].

1. Microbial toxicity tests are used to examine the toxicity of wastewater coming to the plant. In such facilities, it is aimed to protect the biological treatment system from the toxicant effect. The source of the toxicant entering the system can be determined.
2. Microbial toxicity tests are used to assess the possibility of pretreatment for detoxification of industrial wastes.
3. Short-term microbial and enzymatic methods are used to study toxic inhibition in biological processes used during the treatment of wastewater and mud.

Table 1. Important features of microbial toxicity tests [3]

Feature	Description
Economical	The cost of the test varies depending on the using method
They are usually simple methods	Microbial methods are much more effortless compared to bio-tests on fish
They provide a large amount of sample	This is the case if automation technology is applied
Cultures are easily obtained	Cultures obtain easier with the application of the lyophilization method
Laboratory and incubation area needs are simple	Microbial methods are more easily than fish bio-tests
Pre-experiment preparations are very low	Disposable plastic materials used in large experimental environments are not included in these methods
The response absolutely and quickly to toxic substances	Microorganisms give results in a few minutes or hours
Their response to toxic substances is exact and precise	Since there is a large number of organisms in the experimental medium, there is no margin of error.

More than 50,000 chemicals, most of which are xenobiotics, are in regular use and new ones are continually and regularly added to the inventory. Serious concern has been raised over the release of these xenobiotics or their metabolites [4] into the environment. Their deleterious effect on the environment can be assessed via acute and chronic toxicity tests, using mostly fish and invertebrate bioassays [5]. However, due to the large number of chemicals to be tested, ecotoxicologists and environmental scientists and engineers are now using short-term toxicity assays which are mostly based on inhibition of the activity of enzymes.

Soil scientists pioneered studies on the relationship between enzyme activity and microbial activity in soils and examined the possibility of using enzymes for indicating the adverse effect of toxic chemicals on microbial populations in soil [6]. The ecological role of enzymes was later examined in aquatic environments and some of them were found (e.g., dehydrogenases) to correlate well with microbial activity in water [7].

2. MATERIALS AND METHODS

In this study, a toxicity testing system was selected in order to monitor the presence of the substances (such as pesticides and heavy metals) that may have toxic effects in terrestrial and aquatic environments, and this microtoxicity test was aimed to be developed. Toxic effect assessment was performed by evaluating the effect on the β -galactosidase activity synthesized by *Bacillus sp.*

The effects of 2,4,6-trichlorophenol, 2,4-dichlorophenol and 4-chlorophenol organic toxic substance on β -galactosidase activity were examined for toxicity experiments.

2.1 Bacterial β -galactosidase Production

In the study method was used for the production of a β -galactosidase enzyme [8]. Nutrient Broth (Difco) liquid medium containing 1% lactose was prepared for the production of enzymes to be transferred to the production medium from Nutrient Agar (Difco) slanted-agar media and transferred to the medium where the enzyme is produced, stored in the refrigerator at +4°C.

Media were prepared to be 50 mL into 100 mL flasks, and their pH was adjusted to pH: 7.5 in the same way as enzyme-containing media.

They were sterilized in an autoclave at 100°C under 1.5 atmospheric pressure for 25 min. Following sterilization, *Bacillus sp.* culture media were incubated for 72 h in a shaking incubator at 30°C.

2.2 Fungal β -galactosidase Production

In the study Fiedurek's method was used for production of β -galactosidase enzyme [9].

Media were prepared to be 50 mL into 100 mL flask, and their pH was adjusted to pH: 5.0 in the same way as enzyme-containing media. They were sterilized in an autoclave at 121°C under 1.5 atmospheric pressure for 15 minutes. Following sterilization, *Trichoderma viride* ATCC 32098 culture media were incubated for 8 days in a shaking incubator at 30°C.

2.3 β -galactosidase Activity Assay

The activity of the galactosidase enzyme was measured using the method as suggested by [8]. Measurements, reaction mixtures consisting of 3 ml were prepared by placing 1 ml of enzyme supply and 2 ml and 2 mM ONPG (ICN Biomedicals Inc.) solutions into reaction tubes.

The enzyme source used in reaction mixtures was created by solving 2 mM ONPG in the potassium phosphate buffer (0.1 M; pH:7), which is caused by centrifuge of culture medium for 10 minutes at 7200 rpm.

Reaction mixtures were incubated in the bath for 30 minutes at a temperature of 50°C and 30 minutes within the optimum conditions detected for the enzyme. After incubation, the reaction was stopped by adding 2 ml from 1M Na₂CO₃ solution to the reaction tubes. Enzyme activity was detected by 420 nm wavelength optical density in a spectrophotometer.

In the study, 1 unit enzyme activity was defined as enzyme activity that formed 1 μ mol o-nitrophenol in 1 minute [10].

2.4 Assay of the Effect of Chlorophenolic Compounds on β -Galactosidase Activity

Reaction mixtures prepared to measure the effect of 4-chlorophenol, 2,4-dichlorophenol and 2,4,6-trichlorophenol toxic compounds on β -galactosidase activity, 4mM ONPG solution in 1 ml phosphate buffer (0.1M, pH 7.0), 1ml

phosphate buffer (0.1M, pH 7.0) prepared in 3 mL volume to contain chlorophenol solution dissolved in ethyl alcohol and 1ml enzyme source.

Chlorophenol solutions were added to the reaction mixtures in amounts of 21.5 M for 4-CP, 17.8 M for 2,4-DCP and 16.5 M for 2,4,6-TCP. The reaction mixture prepared as the 1st control was prepared only from the enzyme source (1 ml) and the substrate (ONPG) solution (2 ml). 96% ethyl alcohol (Birpa) used in dissolving chlorophenols in volumes equivalent to test tubes was added to the reaction mixes prepared as the 2nd control.

The reaction mixtures were incubated for 30 minutes in a water bath set at 50°C. Enzyme activities were read as absorbance (OD_{420 nm}) value in spectrophotometer set to 420 nm and unit enzyme activities were calculated according to the o-nitrophenol standard curve (10). The change of the enzyme activity was calculated using the following formula (1).

$$(C-A)-(C-B)=D$$

- A. Enzyme activity value calculated in the test tube (U/mL)
- B. Enzyme activity value calculated in 2. control tube (U/mL)
- C. Enzyme activity value calculated in 1. control tube (U/mL)
- D. The change caused by chlorophenol on enzyme activity (U/mL)

3. RESULTS AND DISCUSSION

In this study Bacterial β -galactosidase enzyme activity was assayed as 0.01 U/mL. The measured activity value for the fungal β -galactosidase enzyme was calculated as 0.011 U/mL.

In the measurements of β -galactosidase activity with samples of chlorinated toxic solution that is chlorinated toxic substance-free medium are

accepted 100 percent. Activity percentages were found 18.81% for 2,4,6-TCP, 6.97% for 2,4-DCP, 21.79% for 4-CP. In β -Galactosidase enzyme activity, 81.19%, 93.03%, and 78.21% inhibition were observed, respectively (Table 2).

In the measurements of β -galactosidase activity with samples of chlorinated toxic solution that is chlorinated toxic substance-free medium are accepted 100 percent. Activity percentages were found 39% for 2,4,6-TCP, 42% for 2,4-DCP, 53% for 4-CP. In β -Galactosidase enzyme activity, 61%, 58%, and 47% inhibition were observed, respectively (Table 2).

For confirmation of detoxification, Laccase enzyme was used in this study. It was observed that the laccase enzyme tested for detoxification eliminates the inhibitor effect of toxic substances on the β -galactosidase enzyme activity.

In this study, microtoxicity tests and determination of detoxification due to enzymatic dechlorination were carried out.

Recently, many researches have been carried out on the toxicity, genotoxicity and mutagenicity of environmental pollutants in many laboratories. Microbial tests are simple, fast and economical applications [11]. Recalcitrant substances, which are compounds resistant to biological destruction, are given to the environment in which we live through humans or rarely occur within nature itself. Acute toxicity tests using different organisms are used to demonstrate the toxic effects of these substances. The pollution in the environmental area as a result of industrial and agricultural activities has increased the importance of toxicity tests and led researchers to focus on the subject. Fungi, bacteria, algae, and protozoans, which have a short life cycle use reproduction, enzyme activities, respiratory, and nitrification activities in toxicity tests.

In our microtoxicity test studies, bacterial β -galactosidase enzyme obtained from *Bacillus sp.* and fungal β -galactosidase enzyme obtained from *Trichoderma viride* were used.

Table 2. Effect of chlorophenolic compounds on bacterial β -galactosidase and fungal β -galactosidase activity

Chlorophenolic Compounds	Bacterial β -galactosidase (Inhibition %)	Fungal β -galactosidase (Inhibition %)
2,4,6-TCP	81.19	61
2,4-DCP	93.03	58
4-CP	78.21	47

As a result of our microtoxicity studies, it was found that bacterial β -galactosidase enzyme and fungal β -galactosidase enzyme were effective in determining detoxification related enzymatic dechlorination of chlorophenolic compounds (2,4,6-TCP, 2,4-DCP, 4-CP).

4. CONCLUSION

Mikrotoxicity test system developed in our laboratory which works principally based on inactivation of β -galactosidase activity by toxic chlorinated phenolics, was used for toxicity detection. Data obtained from microtoxicity tests have shown that the dechlorination of chlorinated phenolics with laccase has resulted in the loss of toxic potentiality of these chemicals to a substantial extent.

Our method is an economic, an easy, rapid for determining the toxicity assay of chlorophenolic compounds.

COMPETING INTERESTS

Author has declared that no competing interests exist.

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Peer-review history:

The peer review history for this paper can be accessed here:
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