Biodegradation of n-cyclohexyl benzothiazole-2-sulfenamide by *Pseudomonas desmolyticum* NCIM 2112

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**ABSTRACT**

Additives are generally added during insecticide formulation to compensate the time required before the action of active ingredient commence. N-cyclohexyl benzothiazole-2-sulfenamide is used as supplementary additive during insecticide formulation. Overexposure of insecticide results into deposition of additives in the soil region, results into soil infertility. This investigation describes the biodegradation of N-cyclohexyl benzothiazole-2-sulfenamide by *Pseudomonas desmolyticum* NCIM 2112. *Pseudomonas desmolyticum* NCIM 2112 degrades N-cyclohexyl benzothiazole-2-sulfenamide into nontoxic metabolites like benzothiazole and cyclohexane.

**Keywords:** Insecticide, N-cyclohexyl benzothiazole-2-sulfenamide, *Pseudomonas*, biodegradation

**INTRODUCTION**

Insecticides are used to control the damages caused by insects in the agriculture field. To compensate the time required before the main action of insecticide commence additives are generally added in the insecticide formulation [1]. Benzothiazole and its derivatives are generally used in pesticide formulation and are of concern for potential ecotoxicity [2]. A (dihalopropenyl) phenyl alkyl substituted bezoxazole and benzothiazole derivatives shows significant insecticidal and acaricidal activities [3]. Insecticide additives show a marked effect on the growth of soil microorganisms, they reduce their number significantly [4]. 2-Mercapobenzothiazole interfered with the bacterial cell membrane and affects bacterial cell respiration [5]. Benzothiazole and its derivatives are considered as nitrification inhibitors of majority of crop plants [6]. Nodulation and nitrogen fixation processes are reduced and sometime abolished by recurring use of insecticide and its additives [7]. As far as the aquatic toxicity of benzothiazole is concerned it is known to induce tumor and found to be toxic in the concentration of 600mmol L\(^{-1}\) for the aquatic life [8]. It also hampers waste water treatment [9]. The 2-(Thiocyanomethylthio) benzothiazole shows an half life in natural waters varying from 4 months in river water to 30 days in sea water indicating a potential contaminant in aquatic system as visualized on fishes like coho salmon, *Oncorhynchus kisutch* where adverse effect on blood lactate level has been reported [10].

Biodegradation is the only way to lower the toxicity of xenobiotic and recalcitrant compounds [11]. By investigating physicochemical properties of soil with a particular strategy biodegradation of recalcitrant compound can be achieved [12,13] Bacteria possess the capability to utilize virtually all naturally and synthetically occurring compounds as their sole carbon and energy source therefore by studying effect of different physico chemical parameters on the degradation effective biodegradation strategy can be employed [14,15]. This investigation deals with biodegradation of N-cyclohexyl benzothiazole-2-sulfenamide by *Pseudomonas desmolyticum* NCIM 2112.
MATERIALS AND METHODS

Insecticide additive
The commercial grade formulated insecticide Dentok (350g kg\(^{-1}\)) was used for extraction of N-cyclohexyl benzothiazole-2-sulfenamide.

Method of extraction
The chemical extraction of N-cyclohexyl benzothiazole-2-sulfenamide was performed as per the method described [16]. The product sample (50g) was suspended in 100ml distilled water and centrifuged at 8,000 \(x\) g for 20min. The residue was redissolved in 100ml dichloromethane and again centrifuged like wise. The supernatant was air dried at 22\(^{\circ}\)C and redissolved in 50 ml dichloromethane, and was centrifuged by the method as described above. The process was repeated till there was no visible residue left in the cuvettes. The supernatant was analyzed by UV-Vis spectrophotometer (Cyberlab UV 100).

Growth of bacteria
\(P.\) \(desmolyticum\) NCIM 2112 was grown in a mineral based medium containing 0.3\% NaNO\(_3\), 0.1\% K\(_2\)HPO\(_4\), 0.05\% MgSO\(_4\), 0.05\% KCl, 0.0001\% FeSO\(_4\), 0.05\% yeast extract, glucose 1.0 \% and pH 7.00 at 28\(^{\circ}\)C under aerobic conditions on rotary shaker at 120 rpm. Growth of organism was monitored at 660nm by using spectrophotometer.

Inoculum preparation
Biodegradation study of N-cyclohexyl benzothiazole-2-sulfenamide was performed using the inoculum as, \(P.\) \(desmolyticum\) NCIM 2112 was grown in the mineral based medium as mentioned above, but without glucose and with 10 mg L\(^{-1}\) of N-cyclohexyl benzothiazole-2-sulfenamide concentration. The flasks were incubated on rotary shaker at 120 rpm at 30\(^{\circ}\)C.

Biodegradation of N-cyclohexyl benzothiazole-2-sulfenamide
The flasks were incubated on rotary shaker at 120 rpm at 30\(^{\circ}\)C. The content of the flasks were checked by taking 5 ml of culture drawn from mineral based medium and centrifuged at 8000 rpm for 10 minutes. The pellet was discarded and the supernatant was collected filtered through 0.2 \(\mu\)m membrane filter and then the filtrate was scanned in the UV-Vis Spectrophotometer. The band width was set to 1 nm during scanning program. Control flask containing mineral based medium but without \(P.\) \(desmolyticum\) NCIM 2112 was run parallel along with the test flask. Degradation study was conducted for every 2 days interval up to 6 days. The percent degradation of compound was determined using the formula, Percent degradation = \(Ab – Aa / Ab \times 100\), where \(Ab\) is absorbance of compound before degradation and \(Aa\) is absorbance at same wavelength after degradation.

Extraction of metabolites
After 6 days of incubation the broth (100 ml) was centrifuged at 10000 x g for 15 min. The supernatant obtained was used to extract metabolites with dichloromethane (1:1). The extracts were dried over anhydrous Na\(_2\)SO\(_4\) and evaporated to dryness in rotary evaporator. The obtained residue was dissolved in small volume of HPLC grade methanol and used for HPLC and GCMS analysis.

HPLC analysis was carried out by using Waters chromatograph fitted with a reversed phase column (Interchrom Nucleosil C18; 5 mm, 250 9 4.6 mm) and a UV detector set at 240nm. The mobile phase consisted of acetonitrile (70\%) and water (30\%) with a flow rate of 0.6 ml min\(^{-1}\).

GCMS analysis were performed as per the method reported [16]. The extract was also analyzed by QP2010 gas chromatography coupled with mass spectroscopy (Shimadzu). Gas chromatography was conducted in temperature programming mode with a Resteck column (0.25 mm \(\times\) 30 mm; XTI-5) attached to a mass spectrophotometer. Samples were injected in splitless mode at 50\(^{\circ}\)C and column was maintained at 50\(^{\circ}\)C for an initial 1.5 minutes. This was followed by heating to 140\(^{\circ}\), then to 210\(^{\circ}\) and finally to 250\(^{\circ}\)C. Helium was used as carrier gas. The compounds were identified on the basis of mass spectra and were compared using National Institute of Standards and Technology (NIST) library.
Statistical analysis
All the experiments were carried out in triplicate. Analysis of the variants was carried out on all data at P< 0.05 using Graph Pad software. (Graph Pad Instat version 3.00, Graph Pad software, San Diego, CA, USA).

RESULTS AND DISCUSSION

UV-Vis analysis of N-cyclohexyl benzothiazole-2-sulfenamide
UV-visible spectral analysis of cell free broth at 200-400 nm wavelengths was carried out to confirm the degradation of N-cyclohexyl benzothiazole-2-sulfenamide. Figure 1 shows the change in the absorbance spectra of N-cyclohexyl benzothiazole-2-sulfenamide before and after degradation by *P.desmolyticum* NCIM 2112. Degradation of N-cyclohexyl benzothiazole-2-sulfenamide was found to be 40%.

![Figure 1. UV-visible spectra of N-cyclohexyl benzothiazole-2-sulfenamide and degraded metabolites after 6 days of incubation](image)

At every 2, 4, and 6 days of incubation the percent degradation was calculated, it was found to be goes on increasing with decrease in concentration of N-cyclohexyl benzothiazole-2-sulfenamide as summarized in Table 1.

<table>
<thead>
<tr>
<th>Wavelength maxima</th>
<th>Before incubation</th>
<th>After 2 days of incubation</th>
<th>After 4 days of incubation</th>
<th>After 6 days of incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percent Degradation</td>
<td>0%</td>
<td>18.740.333%</td>
<td>33.5240.333 %</td>
<td>40%40.333%</td>
</tr>
</tbody>
</table>

*Values are mean of ±SEM of three experiments*

HPLC analysis
HPLC analysis shows retention time of N-cyclohexyl benzothiazole-2-sulfenamide at 1.116 min whereas after biodegradation new peaks at 1.822 and 1.940 min were observed (Figure 2).
Figure 2. HPLC analysis of N-cyclohexyl benzothiazole-2-sulfenamide and its degraded metabolites

A) HPLC chromatogram of N-cyclohexyl benzothiazole-2-sulfenamide B) HPLC chromatogram of N-cyclohexyl benzothiazole-2-sulfenamide obtained after 7 days of incubation with *Pseudomonas desmolyticum* NCIM 2112.

**Proposed degradation pathway**

GCMS analysis of N-cyclohexyl benzothiazole-2-sulfenamide shows retention time of 9.558 minutes. The results obtained were matched with NIST library database where it shows retention time of N-cyclohexyl benzothiazole-2-sulfenamide. The results obtained were matched with respect to mass/charge ratio vs relative intensity. The results obtained from GCMS analysis clearly show the formation of benzothiazole and cyclohexane from N-cyclohexyl benzothiazole-2-sulfenamide degradation by *Pseudomonas desmolyticum* NCIM 2112 (Figure 3).

Figure 3. Proposed degradation pathway of N-cyclohexyl benzothiazole-2-sulfenamide by *Pseudomonas desmolyticum* NCIM 2112
As far as the adverse effect of insecticide additives are concerned, little is known about the environmental fate of additives on agricultural land [17]. Benzothiazole derivatives are commonly found in both surface water as well as in the industrial effluent and are responsible for considerable ecotoxicity [18]. Biodegradation by means of optimizing different soil parameters was reported and concluded that it can be achieved under favorable condition by means of *Pseudomonas desmolyticum* NCIM 2112 [19].

In case of bacterial degradation of insecticide additives, mechanism such as intracellular accumulation, oxidation or reductions are involved [20]. Pure bacterial cultures such as *Rhodococcus* strain PA and *Rhodococcus erythropolis* capable of degrading benzothiazole and use it as a sole carbon, nitrogen and energy source has been studied [21,22]. In aerobic degradation, thiocyanomethyl thiobenzothiazole was transformed to mercaptobenzothiazole and benzothiazole by unknown pathways [23]. Biodegradation of insecticide additive benzyl benzoate by means of *Pseudomonas desmolyticum* NCIM 2112 was previously reported and concluded that such additives can be degraded into non-toxic metabolites [24].

In this investigation, we found that the N-cyclohexyl benzothiazole-2-sulfenamide converted into benzothiazole and cyclohexane. The formed benzothiazole was not further cleaved into any other metabolite and are non-toxic in nature therefore it is assumed here that the formed metabolites is used by *Pseudomonas desmolyticum* NCIM 2112 for its metabolism.

**CONCLUSION**

Benzothiazole derivatives show considerable toxicity to microorganisms. Presence of N-cyclohexyl benzothiazole-2-sulfenamide in soil shows a strong inhibitory effect on the growth of germinating seeds as well as on the microorganisms. Biodegradation is the only way to minimize the toxicity of such compounds from the environment. In our studies, we tried to deduce a degradation strategy for N-cyclohexyl benzothiazole-2-sulfenamide by *Pseudomonas desmolyticum* NCIM 2112. *Pseudomonas desmolyticum* NCIM 2112 possess the capability to degrade such hazardous compound and convert it into non-toxic metabolites. By means of *Pseudomonas desmolyticum* NCIM 2112, effective strategy for degradation of xenobiotics compounds can be achieved.

**Acknowledgement**

The authors are very grateful to the Principal, Shri Vijaysintha Yadav Arts and Science College, Peth Vadgaon, Dist. Kolhapur and Bharati Vidyapeeth’s MBSK Kanya Mahavidyalaya, Kadegaon, Dist. Sangli (M.S.) for extending the laboratory facilities to complete the investigation.

**REFERENCES**