Novel degradation pathway of 2-chloro-4-aminophenol in *Arthrobacter* sp. SPG

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Abstract

A novel degradation pathway of 2-chloro-4-aminophenol (2C4AP) was studied in an *Arthrobacter* sp. SPG that utilized 2C4AP as a sole carbon and energy source. The 2C4AP degradation was initiated by a 2C4AP-deaminase that catalyzed the conversion of 2C4AP into chlorohydroquinone (CHQ) with removal of ammonium ion. In the next step, a CHQ-dehalogenase catalyzed the dehalogenation of CHQ to hydroquinone (HQ). The further degradation of HQ was proceeded with ring cleavage and the formation of γ-hydroxymuconic semialdehyde. This is the first report of degradation of 2C4AP by any bacteria.

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Introduction

Chloroaminophenols (CAPs) are used as hair dyes and have been identified as toxic substances because of their carcinogenicity [1]. Examples are 2-chloro-4-aminophenol (2C4AP) and 4-chloro-2-aminophenol (4C2AP).

CAPs are released into the environment because of anthropogenic activities. They have been released into soil and water as by-products during the synthesis of cosmetic dyes and chemicals. They may also release into soil because of microbial degradation of various chemicals [2,3]. The 4C2AP may release into the soil due to degradation of 3-chloronitrobenzene and 4-chloro-2-nitrophenol [2,3].

Few bacteria capable of utilizing CAPs as the sole carbon and energy source have been identified and characterized [1, 4]. Examples are *Exiguobacterium* sp. PMA [4] and *Burholderia* sp. RKJ 800 [1]. Both of the strains utilized 4C2AP as the sole source of carbon end energy. The complete mineralizatio of 4C2AP was studied in *Burkholderia* sp. RKJ 800 that degraded it with release of ammonium and chloride ions [1]. The 4C2AP degradation was initiated with the formation of 4-chlorocatechol that was further degraded via ring cleavage [1].

The bacterial degradation of CAPs may be initiated by one of the following mechanisms: (i) the removal of ammonium ion from a CAP by a deaminase [1]; (ii) the ring cleavage of a CAP by an aminophenol dioxygenase [5]; (ii) dehalogenation of a CAP by a dehalogenase [4] (iii) acetylation of a CAP [2,3,.6]

In this communication, we have reported degradation of 2C4AP by *Arthrobacter* sp. SPG. Strain SPG was previously isolated from the soil collected from a pesticide contaminated site, India by an enrichment method using 4-nitrophenol [7]. Strain SPG utilized 4-nitrophenol, 2-chloro-4-

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nitrophenol (2C4NP), 2-nitrobenzoate, 3-methyl-4-nitrophenol and nitrocatechol as the sole carbon and energy sources [7]. In addition, *Arthrobacter* sp. SPG is also capable of utilizing 2C4AP as a sole carbon and energy source. The aim of this study is to study of the degradation pathway of 2C4AP by strain SPG.

Material and Methods

Chemicals

2C4AP, Hydroquinone and Chlorohydroquinone were purchased from Sigma-Aldrich. All other chemicals used were of high quality graded.

Growth and Degradation studies

Strain SPG was grown on 500 ml Erlenmeyer flask containing 100 ml minimal medium and 0.3 mM 2C4AP as a carbon and energy source. The composition of minimal media was exactly same as described previously [1]. For the growth studies, samples were collected at regular intervals and the growth was measured taking the absorbance at 600 nm. For degradation studies, samples were centrifuged and extracted with ethyl acetate and the extracted samples were dissolved in the methanol and analyzed by the high performance liquid chromatography by previously described method [1].

Release of Chloride and ammonia

Chloride and ammonia ions were analyzed by the previously described method [1].

Identification of metabolites

Strain SPG was grown on 500 ml Erlenmeyer flask containing 200 ml minimal media and 0.3 mM 2C4AP as a carbon and energy source. Samples (50 ml) were collected at regular intervals

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(0h, 8h, 16h, 24h) and centrifuged. The supernatant was extracted with ethyl acetate and the extracted samples were analyzed using the high performance liquid chromatography (HPLC) and gas chromatography-mass spectrometry (GC-MS) by the previously described method [1]. GC-MS analysis was carried out using a GC-MS-QP5000 instrument (Shimadzu, Tokyo, Japan) equipped with quadrupole mass filter and DB-1 capillary column with ionization of 70 eV, scan interval 1.5 s and mass range of 50–300 m/z. The column temperature was initially increased from 50°C to 190°C at the rate of 5°C/min and then from 190°C to 280°C at the rate of 10°C/min. The carrier gas (nitrogen) flow rate was 15 ml/min [1].

Preparation of crude extracts for enzyme assays

For, the preparation of the cell extacts, strain SPG was grown in 500 ml minimal media containing 0.3 mM 2C4AP and 20 mM sodium suncinnate. Bacterial cells were harvested by centrifugation at the end of the exponential growth phase, washed twice with phosphate buffer (50 mM, pH 7.5), and finally suspended in the same buffer. The cells were broken by sonication and the extract was centrifuged (12,000 x g for 120 min), and the resulting supernatant was used for enzyme assays. All procedures were carried out at 4°C.

Enzyme assays for a 2C4AP-deaminase

The 2C4AP activity was determined by measuring ammonia released from 2C4AP upon incubation with cell-free lysate. The standard reaction mixture contained 50 mM phosphate buffer (pH 7.5), 0.2 mM NADH, 30 mg of cell-free lysate, and 300 µM 2C4AP in a total reaction volume of 2 ml. After, the 5 min, the reaction mixture were centrifuged and 0.5 ml supernatant was subjected to ammonia release and the remaining 1.5 ml was extracted with the equal volume of ethyl acetate and analyzed by the GC-MS.

Enzyme assay for a CHQ-dehalogenase

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CHQ dehalogenase activity was determined as the total chloride released at 30°C in a reaction contained 50 mM phosphate buffer (pH 7.5), 0.2 mM NADPH, 30 mg of cell-free lysate and 200 µM of CHQ. The final volume of the reaction mixture was 5 ml. Samples were collected at regular intervals and assayed for chloride ions as described above. Samples were also extracted with equal volume of ethylacetate and extracted samples were analyzed by GC-MS to identify the product of reaction.

Enzyme assay for a HQ-dioxygenase

The HQ dioxygenase activity was determined spectrophotometry by monitoring the formation of γ-hydroxymuconic semialdehyde at 320 nm. The reaction mixture contained (in a final volume of 1 ml) 50 mM phosphate buffer, 0.1 mM hydroquinone, 0.1 mM ferrous sulphate and 30 mg crude extracts of the protein. Samples were taken at 0 and 5 min and the UV spectra were recorded.

Results

Growth and degradation studies

The degradation and growth studies showed that *Arthrobacter* sp. SPG utilized 0.3 mM 2C4AP as a sole carbon and energy source and degraded 2C4AP within 48 h (Fig. 1a). The stoichiometric releases of chloride and ammonia ions were detected during the degradation of 2C4AP. Ammonia release occurred before the chloride release that suggested the initiation of the 2C4AP degradation via ammonia release (Fig. 1b).

Identification of metabolites

HPLC analysis confirmed the degradation of 2C4AP within 48h (Fig. 2). In the sample of 0 and12 h, only peak of 2C4AP was detected with the retention time of 13.36 min. However, in the

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sample of 24 h, a peak of metabolite 1 was detected along with a peak of 2C4AP. The retention time of metabolite 1 (6.89 min) was exactly match with the authentic standard of chlorohydroquinone. In the sample of 36 h, the peaks of metabolite I and II were detected along with the peak of 2C4AP. The retention time of metabolite 11 (5.09 min) was exactly match with the authentic standard of hydroquinone. In the sample of 48 h, neither the peak of 2C4AP nor the peak of any metabolite was detected that suggested the complete degradation of 2C4AP by *Arthrobacter* sp. SPG.

GC-MS analysis showed that the mass fragment of the metabolite I and II were observed at 144 m/z and 110 m/z, respectively that were exactly match with authentic standards of CHQ and HQ (Fig. 3). On the basis of the GC-MS, metabolite I and II were identified as CHQ and HQ.

Enzyme assays

We have detected enzyme activities of a 2C4AP-deaminase, a CHQ-dehalogenase and HQ-dioxygenase in the crude extracts of 2C4AP induced cells of strain SPG. The 2C4AP deaminase activity was confirmed by the stoichiometric release of ammonium ions and identification of the product chlorohydroquinone by the GC-MS. The mass fragment of product was observed at 144 m/z equivalent to CHQ. The CHQ-dehalogenase activity was confirmed by the stoichiometric release of choride ions and detection of the product, HQ by the GC-MS. The mass fragment of product was observed at 110 m/z equivalent to HQ. The HQ dioxygenase catalyzed the conversion of HQ to γ-hydroxymuconic semialdehyde via ring cleavage. The spectrophotometric analysis of HQ dioxygenase assay showed that peak of the HQ at 289 nm was disappeared and peak of γ-hydroxymuconic semialdehyde (HMS) around 320 nm was appeared.

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Discussion

Arthrobacter sp. SPG utilized 2C4AP as a sole carbon and energy source and degraded it with release of stoichiometric amounts of chloride and ammonium ions. The CHQ was identified as an intermediate of the degradation pathway of 2C4AP. The enzyme 2C4AP-deaminase was involved in the conversion of 2C4AP into CHQ. Literature studies showed that the CHQ is a common metabolite in the degradation pathway of several chlorinated compounds [8, 9, 10, 11, 12]. Reddy et al. [10] reported that the CHQ was degraded either via HQ or via 2-chlorotrihydroxybenzene in degradation pathway of 2,4,6-trichlorophenol in *Phanerochaete chrysosporium.* Miyauchi et al. [11, 12] showed that CHQ cleaved to maleylacetate or dechlorinated to HQ in the degradation pathway of gamma-hexachlorocyclohexane in *Sphingomonas paucimibilis* UT260. *Arthrobacter* sp. SJCon degraded 2-chloro-4-nitrophenol via a CHQ pathway in which CHQ was cleaved to maleylacetate by a CHQ-dioxygenase. *Burkholderia* sp. RKJ 800 [8] and *Rhodococcus imtechensis* RKJ 300 [9] degraded 2-chloro-4-nitrophenol via a CHQ pathway in which CHQ dehalogenated to HQ. In this study, we have also detected HQ as a metabolite of degradation of 2C4AP by strain SPG. Furthermore, we have also detected the activity of the CHQ dehalogenase in the crude extracts of the 2C4AP-induced cells of strain SPG that confirmed the formation of the HQ from CHQ with release of chloride ion. The HQ was also detected as a metabolite of degradation pathway of 4-nitrophenol in a various Gram-positive bacteria [7, 13]. In the degradation pathway of 4-nitrophenol, HQ was cleaved into γ-hydroxymuconic semialdehyde by a HQ-dioxygenase [7, 13]. We have also detected the HQ-dioxygenase activity in the crude extracts of the 2C4AP-induced cells of strain SPG that suggested the cleavage of HQ into γ-hydroxymuconic semialdehyde.

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On the basis of the identified metabolites and the enzyme assays, we have proposed a pathway of degradation of 2C4AP for *Arthrobacter* sp. SPG (Fig. 4) The 2C4AP degradation was initiated with release of ammonium ion and the formation of CHQ that dehalgenated to HQ with release of chloride. The further degradation of HQ was proceeded via ring cleavage.

The 2C4AP degradation pathway identified in *Arthrobacter* sp. SPG was compared with the degradation pathway of 4C2AP in *Burkholderia* sp. RKJ 800 [1]. It was observed that the initial mechanism of degradation of 2C4AP in strain SPG was similar with that of the 4C2AP degradation in strain RKJ 800 [1]. Both stains initiated the degradation of an isomer of CAPs with removal of ammonium ion by a deaminase [1]. The difference in the degradation pathways of 2C4AP and 4C2AP is that the chloride release was occurred before the ring cleavage in the degradation pathway of 2C4AP whereas in the degradation of 4C2AP, chloride was released after the ring cleavage [1].

Conclusion

Arthrobacter sp. SPG degraded 2C4AP via a novel pathway in which CHQ and HQ were formed.

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Figure legends:

Fig. 1. Utilization and degradation of 2-chloro-4-aminophenol by *Arthtobacter* sp. SPG.

Fig 2. High performance liquid chromatography eluction profiles of the samples of the degrdation of 2-chloro-4-aminophnol by *Arthrobacter* sp. SPG.

Fig. 3. Mass spectrum of metabolite I(a), 11(b) and authentic standards chlorohydroquinone (c) and hydroquinone(d).

Fig. 4. Proposed pathway of degradation of 2-chloro-4-aminophenol for *Arthtobacter* sp. SPG.

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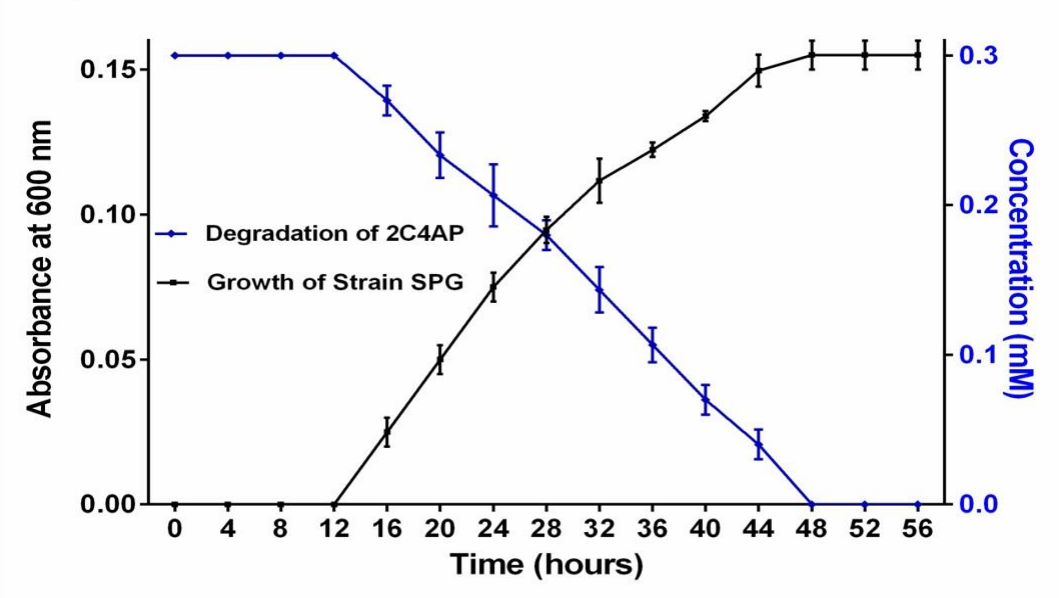
(a)



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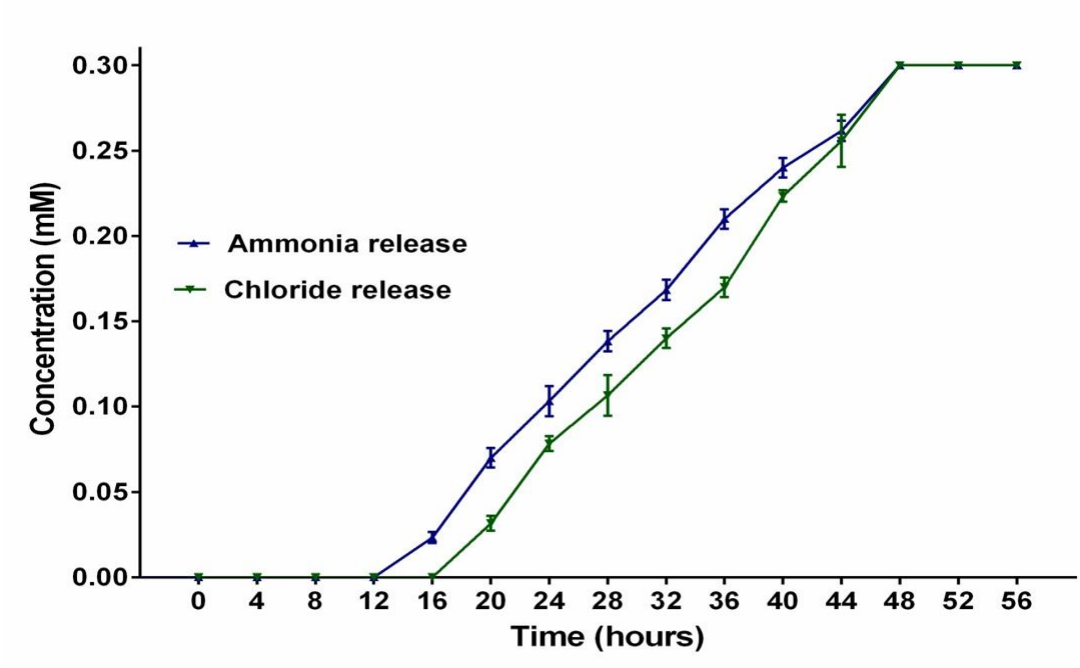

Figure 1

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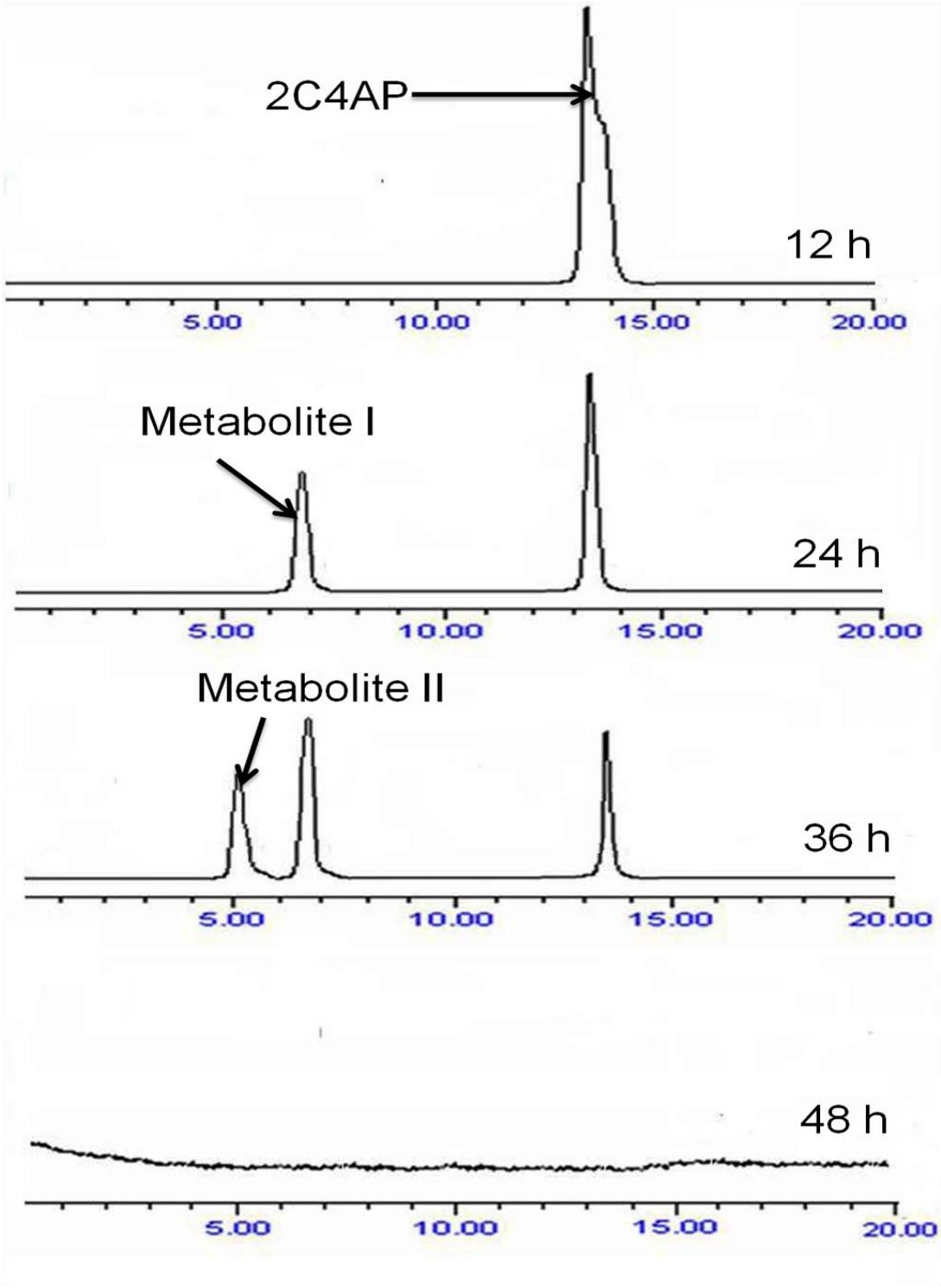

Figure 2

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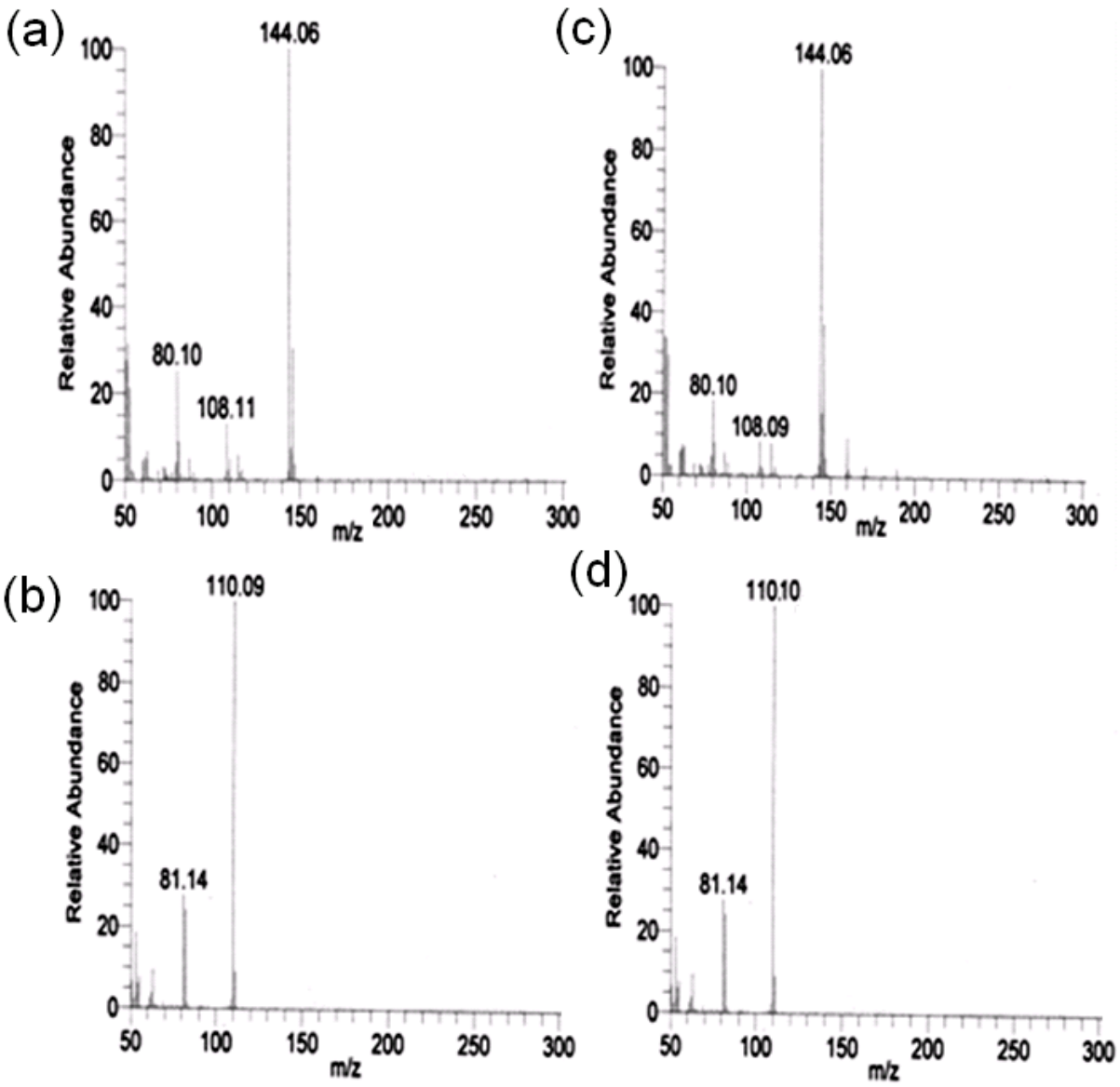

Figure 3

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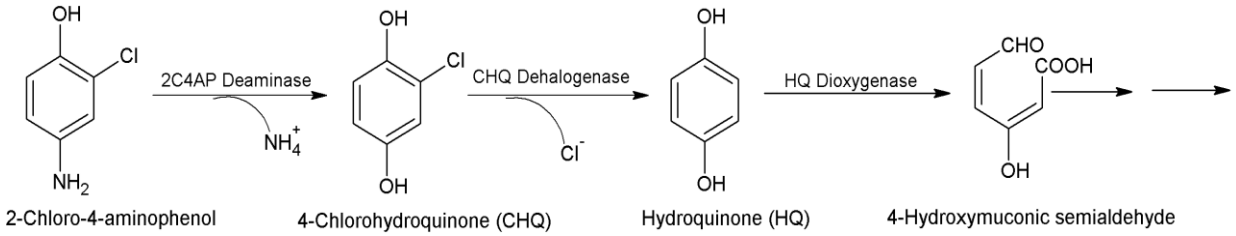

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