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RESEARCH PAPER

Synthesis of a Nanoporous Molecularly Imprinted Polymers for Dibutyl Phthalate Extracted from *Trichoderma Harzianum*

Maede Shahiri Tabarestani[1,2], Kamran Rahnama[1], Mohsen Jahanshahi[3], Saeed Nasrollanejad[1] and Mohammad Hossein Fatemi[4]

[1]*Department of Plant Protection, Faculty of Crop Production, Gorgan University of Agricultural Sciences and Natural Resources, Gorgan,Iran*

[2] *Department of agriculture, mohammad reza Hariri, Science fundation, Babol, Iran*

[3]*Nanotechnology Research Institute, Babol Noshirvani University of Technology, Babol, Mazandaran, Iran*

[4]*Department of Chemistry, Mazandaran University , Babolsar, Iran*

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ABSTRACT

In this study, molecularly imprinted polymers were synthesized for dibutyl phthalate as a bioactive chemical compound with antifungal activity which produced by *Trichoderma Harzianum* (JX1738521). The molecularly imprinted polymers were synthesized via precipitation polymerization method from methacrylic acid, dibutyl phthalate and trimetylolpropantrimethacrylate as a functional monomer, template and cross-linker, respectively. After removal of the template by the eluent from the MIPs, the leached nanoparticles of the MIPs had a good binding capacity as equal 830 mg/g. The polymer particles have been evaluated by field emission scan electron microscopy and Brunauer–Emmett–Teller techniques. The excellent specific surface area in the molecularly imprinted polymers as equal to 690.301 m^2/g comparatively to non-imprinted polymers (ca. 89.894 m^2/g), confirms that the nanoporous MIPs were synthesized, successfully. The results indicated that the nanoporous MIPs can be used in solid phase extraction. This is a novel method for separation of the bioactive compounds from fungi secondary metabolites in biological control.

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INTRODUCTION

Phthalates are naturally found as the bioactive components in plants and fungi. They are known as curative drugs with antifungal, antitumor, anticancer, antiretroviral, antidiabetic and antimalarial effects [1-6]. Trichoderma species are saprophytic fungi with minimal nutritional needs, which secrete a wide range of secondary metabolites that confer major ecological benefits to their host plants. The secondary metabolites are explored for their antimicrobial, antioxidant, insecticidal, nematicidal, plant growth promoting and plant strengthening bioactivities which render their hosts resistant to environmental stresses [7-8]. Since a little amount of the bioactive compounds exist in the secondary metabolites, researchers are interested to find a suitable method to extract them from natural products. The MIPs technology is one of the best techniques in this regard.

The MIPs are introduced as a good alternative for a variety of applications in solid phase extraction, synthetic binding assays, biomimetic catalysts, drug delivery and sensing applications [9-13]. Recently the MIPs technique was used to extract the bioactive compounds from herbal

* *Corresponding Author Email: Maedeshahiri@yahoo.com*

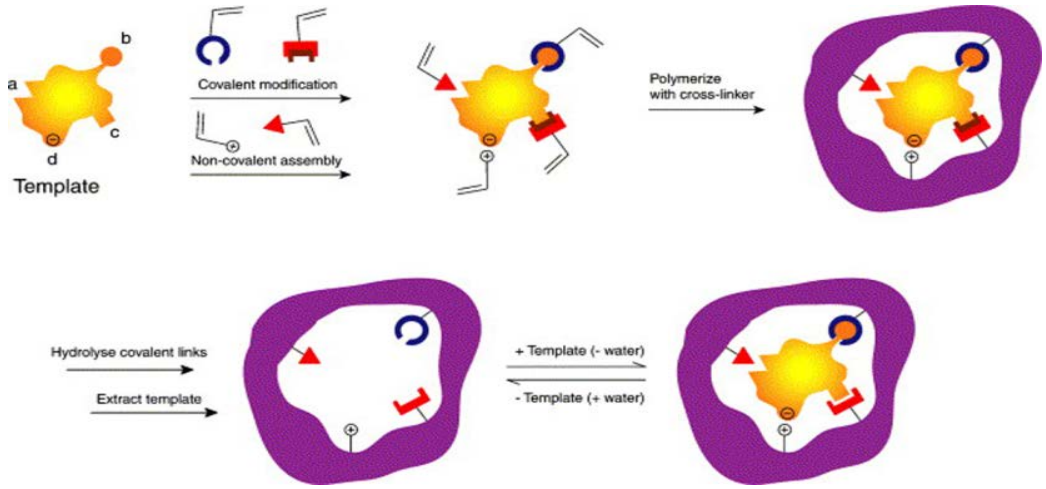

Fig. 1. The planning of the MIPs.

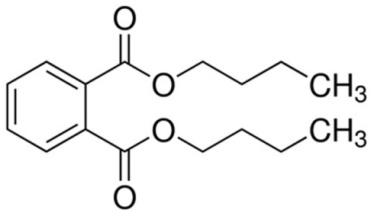

Fig. 2. DBP structure as a template.

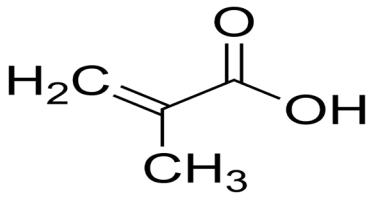

Fig. 3. MAA as a monomer.

plants [14]. During the non-covalent approach, the template molecules are surrounded by the monomers via H-bonding [13]. The cross-linkers enter to polymerization reactions and then the steady space around the templates, are created. After template removal, the leached MIPs with nanoporous cavities are produced. These MIPs can absorb the same templates or very similar molecules based on their shape and 3D structure (Fig. 1). Since, DBP is one of the bioactive chemical compounds in secondary metabolite of *T. harzianum* (JX173852.1) with antifungal effect; it was used as a case study for synthesis of the MIPs.

MAA, TRIM and DBP have hydroxyl and carbonyl functional groups, so there are some interactions between MAA and TRIM with DBP through H-bonding. These interactions enable the MIPs to absorb DBP from the solutions. Figs. 2 and 3 illustrate DBP and MAA structures.

The MIPs have been synthesized by other researchers for dimethyl phthalate [15], dibutyl phthalate [16] and other phthalates through different monomers, cross-linkers and ratios. In this research, synthesis of the MIPs was carried out via TRIM as a cross-linker with a new ratio of template: monomer: cross-linker.

MATERIALS AND METHODS

Dibutyl phthalate, 2, 2´ azobisisobutyronitrile (AIBN), methacrylic acid (MAA), trimethylolpropane trimethacrylate (TRIM) were prepared from Sigma-Aldrich. Methanol, acetic acid, toluene and n-hexan were prepared in analytical grade. Jenway 6305 UV-spectrometer was used to determine the templates amount in loading process. The porosity was evaluated by nitrogen gas adsorption/desorption analysis using BET analysis (PHS1020-China). *T. harzianum* (JX173852.1) was collected from the Mycology Laboratory, Department of Plant Protection, Gorgan University of Agricultural Sciences & Natural Resources, Gorgan [17]. It was cultured in PDB (potato dextrose broth) and its secondary metabolites were extracted according to defined procedure [18]. DBP was identified in its secondary metabolite via GC/MS device. Meanwhile, its antifungal effectiveness was confirmed.

Synthesis of MIPs and NIPs

Dibutyl phthalate dissolved in 50 ml toluene as a porogen in a round-bottom flask. After that, the functional monomer, cross-linker and AIBN (as an initiator) were added in a molecular ratio of 1:4:8

Table 1. The absorbance value of the filtered solution in 280 nm.

Process No.	Absorbance value
1	3.000
2	3.000
3	2.508
4	2.200
5	1.980
6	0.090
7	0.060
8	0.040
9	0.002
10	0.001

Table 2. Loading results of polymers.

Polymers	C_i (ppm)	C_f (ppm)	Q (mg/g)	IF
MIPs	125	93	64	3.5
MIPs	200	165	70	2.9
MIPs	250	205	90	3.4
MIPs	400	166	468	2.3
MIPs	500	85	830	2.7
MIPs	600	250	700	3.5
MIPs	750	675	150	3.7
NIPs	125	116	18	
NIPs	200	188	24	
NIPs	250	237	26	
NIPs	400	300	200	
NIPs	500	350	300	
NIPs	600	500	200	
NIPs	750	730	40	

Table 3. BET analysis for MIPs and NIPs.

Polymer	Total specific surface area (m^2/gr)	The micropore specific surface area (m^2/gr)	Micropore Volume (cm^3)	Average pore diameter of MP model (nm)
MIPs	690.301	501.925	0.1192	0.303
NIPs	427.578	89.894	0.0517	0.521

(template: functional monomer: cross-linker) [19]. The pre-polymerization solution was prepared in an ice-bath and dispersed by ultrasound irradiation for three times [20]. The solution was purged with nitrogen gas for 5 minutes to remove dissolved oxygen. The polymerization procedure was thermally initiated at 60 °C in a water bath and stirred by magnetic stirrer for 24 hrs to complete the polymerization reaction [21]. The related process was repeated to synthesize non-imprinted polymer (NIPs) exactly by the same synthetic route of MIPs (without DBP). After polymerization, the polymeric particles were separated using centrifuging and then washed by the solvent.

Eluting the template from MIPs

For removing of the template from the MIPs, the polymers were put in a round-bottom flask containing 200 ml of the eluent (methanol/acetic acid 9:1 V/V) and the solution was continuously stirred with a magnetic stirrer during the extraction. After 3 hrs, the mixture was centrifuged and the supernatant was checked by UV spectrometer to measure the concentration of DBP. This procedure was carried out for 10 times till the absorbance value of the filtered solution in 280 nm, reach to about zero (Table 1).

It means that the entire template molecules were removed from the MIPs. The MIPs were separated from the eluent by centrifuge 11000 rpm and washed two times with the pure methanol and acetone. The leached MIPs were dried at 60 °C over night for further use.

RESULTS AND DISCUSSION

Binding studies

The amounts of DBP in the solutions (before and after loading process) were measured according to the beer-lambert law and absorbance value at 280 nm via UV- spectrometer. The experiments were conducted at 25°C for three times and were investigated in static adsorption mode. 10 mg of the leached MIPs and NIPs was loaded by 20 ml of the solution with different concentrations of DBP in n-hexane solvent. The solution was stirred by magnetic stirrer for 2 hrs at room temperature. In each process, after loading time, the solution was placed in centrifuge tubes and the solid materials were spin down by centrifuge 11000 rpm. The supernatant solution was withdrawn by the sampler and diluted 500 -1000 times for determination of unloaded concentration of DBP by UV- spectrometer. Concentration of DBP was calculated according to the equation which has been already earned by standard absorbance curve of DBP. The results were listed in Table 2.

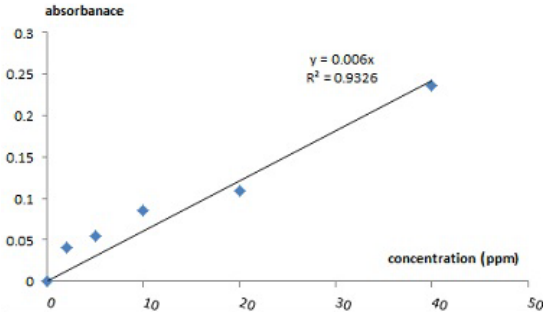

Fig. 4. Standard curve for DBP.

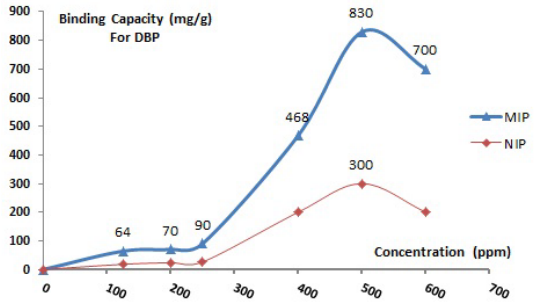

Fig. 5. The binding capacity Vs concentration of DBP.

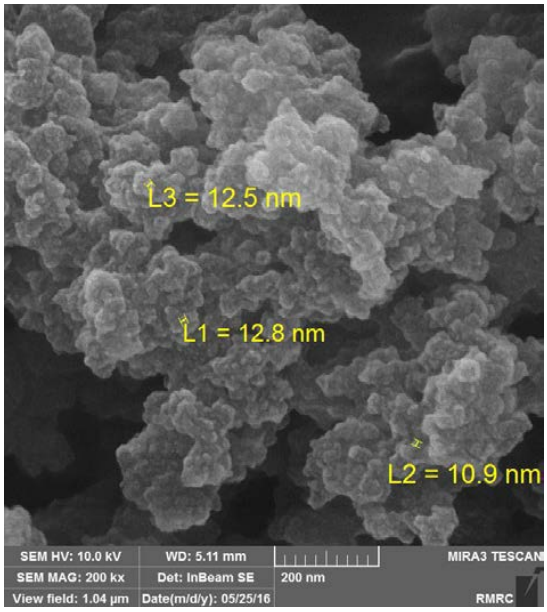

Fig. 6. FESEM imaging for the MIPs.

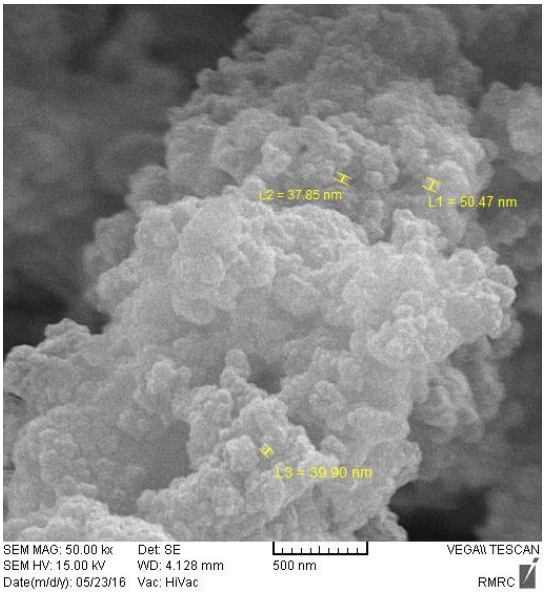

Fig. 7. SEM imaging for the MIPs

Standard curve for DBP

Standard curve for DBP was earned based on measuring of the absorbance in 280 nm Vs for different concentrations of DBP (Fig. 4).

Binding capacity (Q)

The binding capacity is defined as the amount of the absorbed template per one gram of the polymer as the following equation:

$$Q= (C_i – C_f) * V / W \quad (1)$$

Where, C_i is the initial concentration of DBP, C_f is the concentration of DBP after loading time, V is the volume of the feed with initial concentration and W represents mass of the polymers. Fig. 5 shows the variations of the binding capacity Vs different concentrations of DBP.

Porosity Studying

Nanopores in the MIPs were created resulting in the strong hydrogen bonding between the functional groups in monomers and the template. The inner structure and the porosity of the MIPs have been detected by BET technique (Table 3).

Referring to the IUPAC definition, these kinds of the polymers mainly possessed micropores (pore size < 2 nm) [22].

Imprinting Factor (IF)

The imprinting factor can be calculated according to equation 2:

$$IF = Q_{(MIPs)} / Q_{(NIPs)} \quad (2)$$

The optimized IF is 2.7 which pertain to 500ppm of DBP concentration (Table 2).

Morphological studies

Field emission scanning electron microscopy (FESEM) model MIRA3 TESCAN was used to characterize MIPs, morphologically. As shown in Fig. 6, the particles of the MIPs had spherical shapes as agglomerated. The diameter of the spherical MIPs was specified by FESEM imaging around 12nm.

CONCLUSION

The best binding capacity and specific surface area of this research were 830.00 mg/gr and 690.301 m^2/g which it appertained to MIPs.

The results indicated that the nanoporous MIPs are good candidate for extraction of the special bioactive compound from *Trichoderma* species in solid phase extraction. This is a new technique to separate and concentrate an effective antifungal compounds among many chemical compounds in secondary metabolites which are unused for biological control in plants. Synthesis of the MIPs for extraction of the other bioactive components from secondary metabolites is our future schedule.

ACKNOWLEDGMENT

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest regarding the publication of this manuscript.

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