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ORIGINAL RESEARCH PAPER

Synthesis of Nanostructure Molecularly Imprinted Copolymer for Separation of Antifungal Bioactive Di-(2-Ethylhexyl) Phthalate from Biocontrol Fungi Metabolites

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ABSTRACT

Among biocontrol fungi, *Trichoderma* species produce a wide range of bioactive compounds with antifungal activities. In this study, Di-(2-Ethylhexyl) Phthalate (DEHP) is identified ***via*** gas chromatography-mass spectrometry (GC-MS) device in *Trichoderma atroviridae* (1-3) secondary metabolites and its antifungal effectiveness is confirmed. An eco-friendly approach for the extraction of DEHP is carried out by a nanoporous molecularly imprinted methacrylic acid-based network copolymer as a solid sorbent. Molecularly imprinted polymers (MIPs) are synthesized by precipitation polymerization using DEHP as a template, methacrylic acid (MAA) as a functional monomer, and trimethylolpropane trimethacrylate (TRIM) as a cross-linker with molecular ratio (1: 4: 8). After the removal of DEHP, the nanoporous polymer could recognize and rebind specifically the same or structurally very similar molecules. The synthesized MIPs exhibit a suitable tendency to absorb the template with the highest binding capacity of 300 mg/g for DEHP in n-Hexane solvent as a solid phase extraction (SPE) system. The measured particle size of the MIPs with dynamic light scattering (DLS) is reported at 75.38 nm. In addition, the porosity of the MIPs is evaluated by nitrogen gas adsorption/desorption using Brouneur Emmet Teller (BET) analysis. Results indicate that nanoporous MIPs with an average pore diameter of 2.70 nm and a specific surface area of 309 (cm^3/g) is achieved. According to the above-mentioned results, nanoporous MIPs could be considered as an acceptable candidate for the separation of antifungal bioactive compounds (natural fungicide) such as DEHP as an eco-friendly method in order to replace chemical pesticides.

Keywords: *Antifungal, Molecularly imprinted polymers, Nanostructure, DEHP, Separation*

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INTRODUCTION

Molecularly imprinted polymers as an adsorbent are used in order to recognize target molecules. They have a memory of the size, shape, and functionalities complementary to the template molecules [1]. The MIPs are introduced as a good alternative for a variety of applications in solid-phase extraction, drug delivery, sensing applications, etc. [2]. Applications of the MIPs, such as protein recognition, solid-phase extraction, sensors, drug delivery systems, environmental matters, and antibody substitutes have been expanded due to the facility and low cost of preparation [3-6]. The template-functional monomer complex could be stabilized by different interactions including van

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der Waals force or hydrogen bond in the non-covalent imprinting approach. MAA contains a carboxyl group that could act as both hydrogen bond donor and acceptor in order to bind to the carboxylate group in DEHP. Meanwhile, it has a methyl group which could be effective to increase the van der Waals force with DEHP. Subsequently, MAA was selected as the best monomer in this study [7]. Some polymerization approaches such as bulk polymerization, precipitation polymerization, suspension polymerization, and polymerization on a solid surface have been developed in order to synthesize three-dimensional network polymers. Since in bulk polymerization methods, some cavities would be lost during the grinding process and polymer mass will be decreased and according to a few advantages in precipitation polymerization, this technique is preferred over other techniques in this research [8-9]. Nowadays, due to environmental pollution, biocontrol fungi are assumed a significant role in agriculture. *Trichoderma* species are the most studied fungal biocontrol agents. They are successfully used as bio fungicides and biofertilizers in greenhouse and field plant production. Their high potential in crop protection and promoting vegetative growth are related to their secondary metabolites with antimicrobial and antifungal activity. These chemical bioactive compounds are very important to the biological control of different phytopathogens as natural fungicides especially with environment-friendly properties [10].

DEHP is one of the bioactive compounds which is found in the secondary metabolites of fungi with antimicrobial and antifungal effects [11]. In this research, DEHP is used as a template to synthesize MIPs. The reason for that is the feasibility of the separation of DEHP from secondary metabolites in the solid-phase extraction procedure. In order to synthesize MIPs, the first step is to solve the templates in toluene. By adding monomer after sonication, between the carbonyl group of DEHP and the -OH functional groups contained in MAA monomers, non-covalent interactions would be created via hydrogen bonds. The polymerization process between MAA monomers is carried out in the presence of 2, 2'-azobisisobutyronitrile (AIBN) as the initiator of the reaction, and the polymer matrix is completely formed *via* TRIM. The next step is to remove DEHP from the polymer in order to fabricate polymer matrix as imprinted polymers containing three-dimensional binding sites named MIPs. Schematic of the MIPs preparation and removal of DEHP to create the binding site is shown in Fig. 1.

DEHP compounds could interact again non-covalently with the carboxyl groups in the MIPs through hydrogen bonds due to the suitability of shape, size, and functional groups. Finally, the last step could be introduced as a solid-phase extraction. In this research, MIPs for DEHP as one of the antifungal varieties in the secondary metabolite of *Trichoderma* is synthesized by the precipitation polymerization and the polymer characteristic is evaluated. A good tendency to absorb DEHP with a binding capacity of 300 mg/g indicated that the nanoporous MIPs could be suggested as a good candidate for the separation of the antifungal bioactive compounds from the secondary metabolites. The afro-mentioned result is carried out for the first time as a novel eco-friendly separation of the antifungal bioactive compound from the secondary metabolite, and up to now, no researches on the synthesis of the MIPs

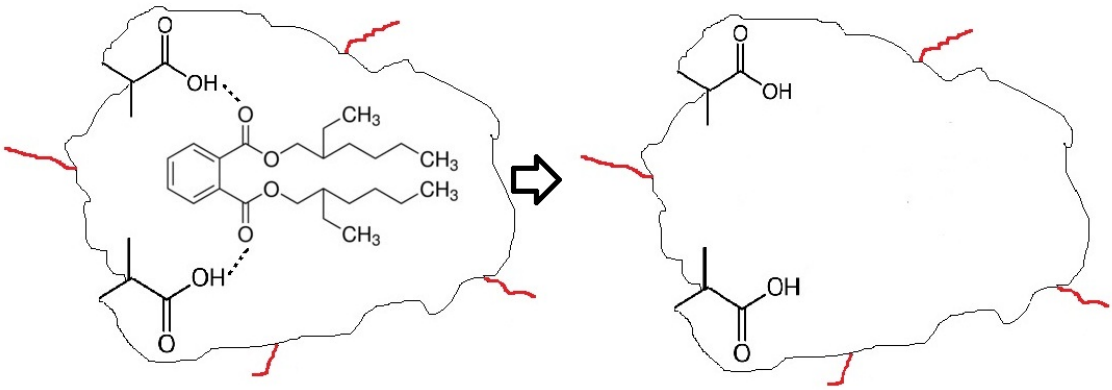

Fig. 1. Schematic representation of MIPs preparation and removing of DEHP to create Matrix polymer

through precipitation method with molecular ratio (1:4:8) DEHP/MAA/TRIM has been reported.

EXPERIMENTAL PROCEDURE

Material

High-purity di (2-Ethylhexyl) phthalate (DEHP), methacrylic acid, Trimethylolpropane trimethacrylate, and 2,2'-azobisisobutyronitrile, are prepared as Sigma-Aldrich products. All of the solvents such as toluene, acetone, and so on are used general pure but acetic acid with high purity and methanol with HPLC grade are purchased. Mycology Laboratory, Department of Plant Protection, Gorgan University of Agricultural Sciences & Natural Resources, Gorgan, was responsible to provide *T. atroviridae* (1-3), *Rhizoctonia solani*, and *Fusarium graminearum*.

T. atroviridae (1-3) was isolated from the soil of canola cucurbits farm in Gorgan and is cultured on PDB (potato dextrose broth) and is incubated at 28 ± 2°C (12 h darkness, 12 h light) for 30 days in *in-vitro* conditions [10]. Its secondary metabolites are extracted according to the defined procedure [12] and identified by the GC-MS device. Based on the NIST mass spectra library of the GC–MS analysis, in secondary metabolite of T. atroviridae (1-3), different categories of chemical materials with antifungal bioactivity are detected such as terpenes, phthalates (DEHP), alcohols, fatty acids, and their derivatives. For considering antifungal effectiveness of DEHP against two phytopathogenic fungi (*R. solani* and *F. graminearum*) in in-vitro conditions, concentrations of 1, 5 and 10 percent DEHP have been prepared and are compared with the Control (without DEHP). The obtained data are statistically analyzed at the level of one percent probability by SPSS software [13].

Tools

The equipment used in this study includes analytical balance, magnetic stirrer, water bath, sonicator, and oven. Jenway 6305 UV/Visible spectrometer is used to determine the amount of the templates in the loading process on the polymers at 280 nm wavelength. The porosity is evaluated by nitrogen gas adsorption/desorption analysis using Brouneur Emmet Teller (BET) analysis (PHS1020-China). The porosity measuring is based on the results of isothermal adsorption at 77 K. Surface morphological information of the MIPs is obtained by scanning electron microscope (SEM) model VEGA\\TESCAN-XMU (Canada). Particle size is measured by dynamic light scattering (DLS) model VASCO (Cordouan Tech- France).

MIPs & NIPs Synthesis

Synthesis and evaluation of the MIPs are carried out in three steps: (1) precipitation polymerization to prepare fine particles of the MIPs, (2) eluting of the template from particles of the polymers by eluent in order to achieve the blank MIPs, (3) loading on the blank MIPs by certain concentration to evaluate and measure the binding capacity of the MIPs [14]. Reactants are dissolved in a round bottom flask by 50 ml toluene as a porogen solvent in a molecular ratio of 1:4:8 for precipitation polymerization [12]. AIBN as an initiator for radical reactions is added and the solution is kept in an ice bath. The pre-polymerization solutions are sonicated by ultrasonic waves and purged with nitrogen gas to remove dissolved oxygen. The reaction is performed at 60 °C in a water bath for 24 h to achieve a solid polymer [15 -16]. After the polymerization reaction, the mixture of the container is centrifuged and then washed with acetone several times. According to the same synthetic routes in MIPs synthesis, the non-imprinted polymers (NIPs) as a control polymer are synthesized exactly by the similar procedure of MIPs without the template molecules.

Template removal from MIPs

The prepared polymers are eluted by methanol/acetic acid (9:1 V/V) with a magnetic stirrer. This procedure is allowed to the extent that the absorbance of the filtered solution in 280 nm reaches zero. It means that the entire template has been removed from the polymers. The MIPs are then centrifuged at 11000 rpm and washed two times with distilled water. Hence, nanoporous MIPs is prepared which is able to absorb DEHP again. The leached MIPs are dried at 60 °C overnight for further use.

Binding Capacity Measurement

Binding capacity is defined as mg of the absorbed template per 1 gram of the polymer. Binding affinity of the imprinted and non-imprinted polymers are evaluated using static adsorption experiment by separately mixing of 10 mg of the polymer particles with 20 ml of various concentrations of DEHP in the n-Hexane solvent. Due to the fact that n-Hexane has no hydrogen-bonding and limited ability to compete

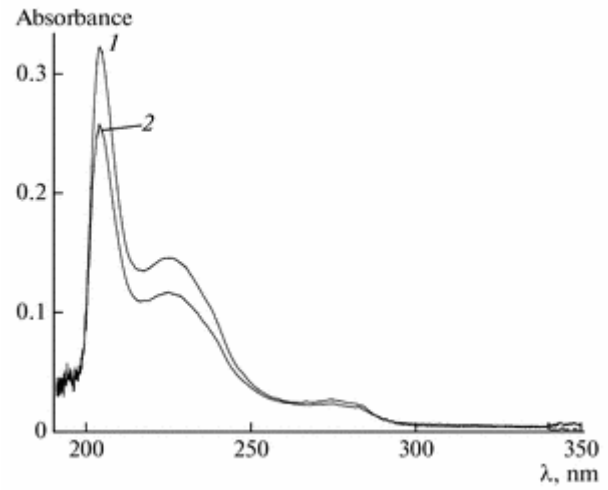

Fig. 2. UV-Visible Absorption Spectra of Di-Butyl Phthalate (1) and Di-(2-Ethyl hexyl) Phthalate (2) [17]

Table 1. Effect of different concentrations of DEHP on *R.solani* and *F. graminearum* , 96 hours after culturing. (Numbers followed by the same letter are not significantly different (P<0.01))

Concentration of DEHP in each plate (%)	Mean of colony diameter (mm) of *R.solani*	Mean of inhibition Percent of *R.solani*	Mean of colony diameter (mm) of *F. graminearum*	Mean of inhibition percent of *F. graminearum*
1	45 b	35 c	30 b	57c
5	30 c	57 b	20 c	71b
10	10 d	85 a	5 d	92a
(Control) 1	70 a	0 d	70 a	0 d
(Control) 5	70 a	0 d	70 a	0 d
(Control) 10	70 a	0 d	70 a	0 d

for the hydrogen-bonding sites on the template or the binding sites, this solvent is more suitable than other solvents in the loading processes. The mixture is then put in a conical flask and stirred by the magnetic stirrer for 2 hr. at room temperature. In each process, after loading time, the solution is placed in centrifuge tubes and the solid materials are spun down at 11000 rpm for 30 min. After that, the free concentration of DEHP is measured by a UV spectrometer at 280 nm wavelength based on Beer-Lambert law. According to Fig. 2, the UV spectrum of the DEHP shows that it absorbs UV light at different wavelengths, including 210 and 230, and 280 nm [17]. Since n-Hexane (Maximum wavelength absorbance at 195 nm) does not absorb light significantly at 280 nm, the binding analysis of the prepared MIPs is studied at 280 nm. The related concentration of DEHP is calculated according to the equation which has already been obtained by the standard absorbance curve of DEHP. The binding capacity can be calculated by the following Equation:

$$Q= (C_i - C_f) * V / W$$

Where, C_i, C_f, V, and w, are the initial concentration of DEHP in feed, the concentration of DEHP after loading procedure, the volume of the feed with an initial concentration of DEHP, and mass of the polymer in grams, respectively.

RESULTS AND DISCUSSION

Antifungal activity of DEHP

DEHP is one of the bioactive compounds in secondary metabolites of *T. atroviridae* (1-3) which in this study, its antifungal effects have been confirmed against *R.solani* and *F. graminearum* in *in-vitro* conditions. Statistically, there are significant differences in %1 level, between different concentrations of DEHP for control of the mycelial growth of *R. solani* and *F. graminearum* (Table 1).

Characterization of MIPs and NIPs

Imaging by SEM on the surface of the MIPs is shown in Fig. 3 which indicates that MIPs have a

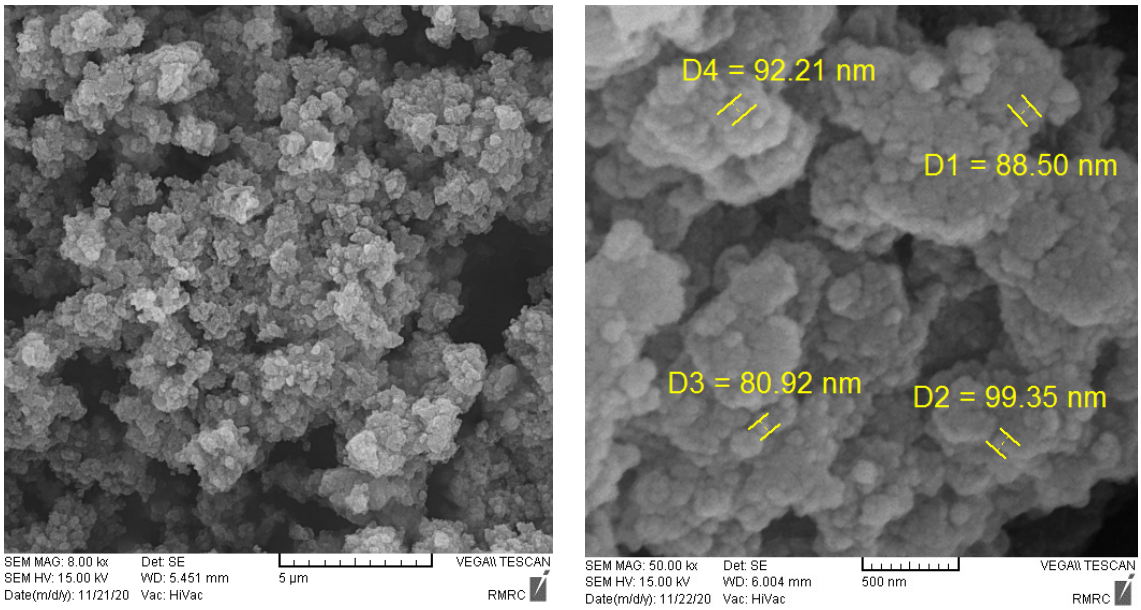

Fig. 3. SEM images of MIPs

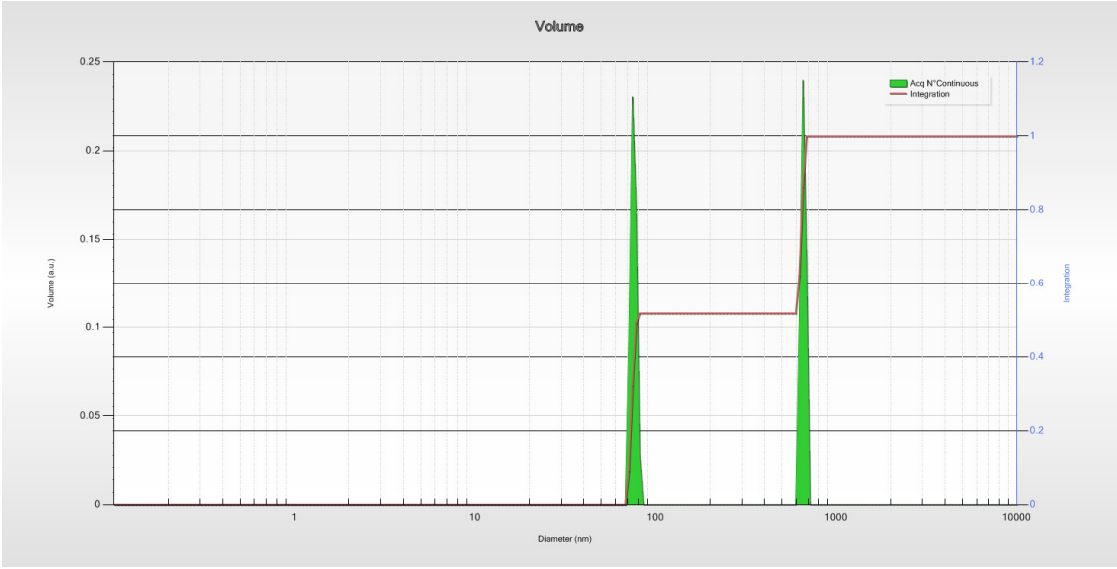

Fig. 4. Particle size distribution of MIPs measured by dynamic light scattering

spherical shape. The surface morphology of the polymers shows a collection of small granules with a diameter of about 80 nm.

Particle size distribution was measured with dynamic light scattering (DLS) analysis, too. Fig. 4 illustrates particle size distribution for the nanoparticles. This figure shows that the smallest particle size of the MIPs is 75.38 nm.

The polydispersity index (PdI) was 0.24 for these particles. It means that all of the particles were agglomerated and were not dispersed well. Distribution statistics are reported in Table 2.

Binding Studies

The amounts of the extracted compounds are confirmed according to the beer-lambert law and measuring the absorbance at 280 nm by a UV-Visible spectrophotometer. In order to evaluate

Table 2. Distribution statistics and Mean diameters of the MIPs particles *via* DLS measuring technique

Mean particles size (Consist of both dispersed and agglomerated particles)	Mean diameter of the particles in Peak 1 (Intensity 51.81 %)	Mean diameter of the particles in Peak 2 (Intensity 48.19 %)
355.38 nm	75.38 nm	656.43 nm

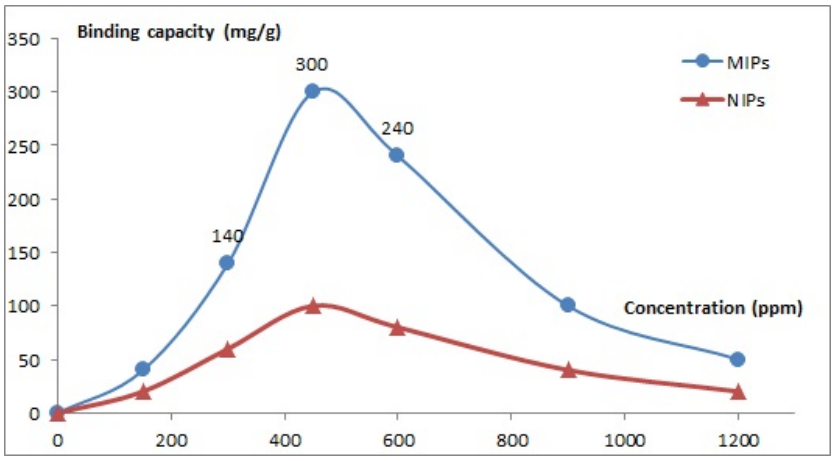

Fig. 5. The Binding Capacity Vs. Feed Concentration

Table 3. BET analyses of the MIPs and NIPs

Polymer	The BET specific surface area (m^2/g)	Pore volume (cm^3/g)	Mean pore diameter (nm)
MIPs	309.60	71.131	2.709
NIPs	236.37	54.307	2.797

the binding capacity of the MIPs and the NIP, experiments are conducted at 25°C for three times. Experiments as a function of the DEHP concentration are investigated in static adsorption mode.

Fig. 5 indicates the various amount of binding capacity of the MIPs and NIPs based on the initial concentration of DEHP. According to Fig. 3, the binding capacity of 300 mg/g is achieved in 450 ppm of the feed concentration.

Porosity Results

According to the amount of absorbed nitrogen gas by the sample, the pore diameter and pore volume of the MIPs are calculated. MIPs' and NIPs' BET analysis results are shown in Table 3.

Based on the classification of pore size by IUPAC[1], the pore diameter of the synthesized polymers is in the range of 2 to 50 nm, so they can be classified as mesopore nanostructured materials. This research shows that the nanoporous MIPs prepare the possibility for direct extraction of certain bioactive components from secondary metabolites with MIP technology.

CONCLUSIONS

Since water resources are limited, eco-friendly researches should be promoted to prevent water pollutions. In this research, the produced Nanoporous MIPs are able to separate the antifungal bioactive compounds (natural fungicide) such as DEHP. Instead of increasing the production line to produce effective chemical materials as antifungal agents which most of them are a hazard and poisonous for human health, the researchers should find and replace another alternative for this purpose. MIPs nanotechnology as an eco-friendly technique is suggested as a suitable solid sorbent for the separation of the effective bioactive compounds from secondary metabolites to use as a biocontrol system in agriculture.

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CONFLICTS OF INTEREST

There are no conflicts to declare.

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