Synthesis and characterization of molecularly imprinted polymers for solid phase extraction of biomolecules from fermentation broth.

Project report Submitted To

Faculty of Science, Savitribai Phule Pune University

By

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(IBB-2014-26)

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Certificate of Guide

This is to certify that the project entitled "Synthesis and characterization of molecularly imprinted polymers for solid phase extraction of biomolecules from fermentation broth", submitted by Mr. Sukrut Digambar Shishupal student of 3rd year integrated MSc. Biotechnology, Institute of Bioinformatics and Biotechnology (IBB), Savitribai Phule Pune University (SPPU), was carried out under my guidance successfully.

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Sukrut Shishupal
IBB-2014-26

Abstract

Title of Project	: Synthesis and characterization of molecularly imprinted polymers for solid phase extraction of biomolecules from fermentation broth.
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Name of the guide	: Prof. Smita Zinjarde
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Extraction of Biomolecules from various biological samples has been carried out on a large scale and is a tedious procedure. It takes a lot of time, requires huge amount of money and electric power. Hence to reduce the time taken for extraction and making it cost effective, Molecularly Imprinted Polymers (MIPs) can be used as an effective tool for extraction. MIPs are easy to synthesize, are cost efficient and provide a one-step extraction method. In this study, we deal with two biomolecules namely ectoine and streptomycin. MIPs were synthesized using methacrylic acid (MAA) as monomer, EGDMA as cross-linker and AIBN as initiator and acetonitrile as porogen in acetonitrile: toluene (3:1) solvent system. The imprinting was confirmed by TEM imaging, Thermogravimetric analysis (TGA). In-order-to elute the biomolecules from the MIP, solvent extraction method was used. Out of the solvent used, water and acetonitrile, ectoine was eluted efficiently using water. In case of streptomycin, the elution was successful with 1% cyclohexanol in water as compared to other solvents used (Water, Acetonitrile, acetic acid, toluene, heptane, methanol). To check the amount of molecule bounded to the MIP, we should carry out a rebinding experiment after elution is completed.

Key words: NPs, Molecularly imprinted polymers, extraction, specificity, template, recovery

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List of Abbreviations

MIPs	Molecularly Imprinted Polymers
NIPs	Non- Imprinted polymers
MAA	Methacrylic Acid
TEOS	Tetraethyl Orth silicate
EGDMA	Ethylene Glycol Dimethacrylate
SiO_2	Silicon Dioxide
TLC	Thin Layer Chromatography
TGA	Thermal Gravimetric Analysis
VSM	Vibrating Scanning Magnetometer
TEM	Transmission Electron Microscopy
NPs	NPs
DLS	Dynamic Light Scattering
MIT	Molecular Imprinting Technique
SNP	Sodium Nitroprusside

Chapter One

Literature Review

1.1 History of Molecular imprinting technique (MIT):

Molecular imprinting technique was first reported by Polyakov in which the polymerization of sodium silicate with ammonium carbonate was studied (Polyakov,1931). Later, in 1949, the polymerization of sodium silicate in the presence of four different dyes. The dyes were successfully eluted and during the rebinding experiment, it was found that silica prepared in the presence of any of the molecules would bind to the initial molecule in presence of other dyes (Dickey, 1949). Nicotine filter, consisting of nicotine imprinted silica, was synthesized which could adsorb 10.7% more nicotine than non-imprinted silica. The method was patented by Merck. The material was then used in cigarettes, cigars and pipes filters (Erlenmeyer H.,1965). After this discovery, many research groups started working on this. From 1931 to 2003, almost 1500 research articles, reviews were published (Alexander et. al, 2006) while the number reached to almost 4000 in the year between 2004 and 2011 (Whitcombe et. al, 2014). This method shows to be effective against small molecules of molecular weight less than 1000 and hence can be used for selective extraction of various proteins (Turner et. al, 2006). Initially, it was thought that their work is limited only to detection but now, this field is expanding fast and is now helpful in various other sides such as drug delivery, sensing of the molecules, solid phase extraction, etc. Although the term was first introduced in 1931, but the real interest was shown in 1973 when the synthesis of organic polymers was done to predestined binding of the ligand molecule (Wulff et. al. 1972).

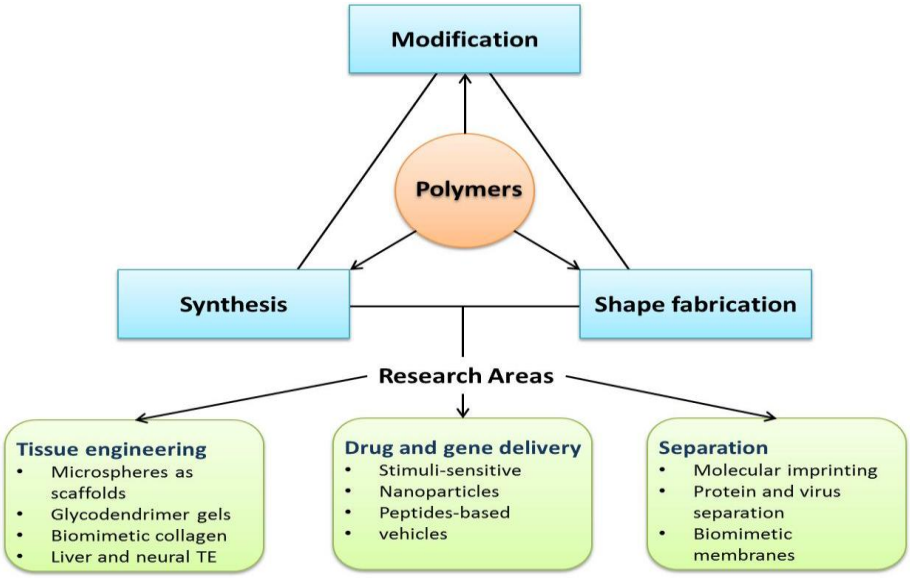


Fig 1.1.1 Application of MIPs in various fields.

1.2 Molecularly imprinted polymers (MIPs):

Molecular imprinting started a way back in 1931 and now it is a rapidly increasing field (Mosbach K. & Ramström O.,1996). MIP is a polymer which is processed using the MIT which forms a cavity or pore in the polymer and it is highly specific for the template molecule. Polymers is made by using monomer, cross-linking agent, initiator and a template molecule. The template is a biomolecule of our interest. Monomer binds to the template molecule at specific sites and forms a mesh, entrapping the biomolecule in it. The cross-linking agent binds the monomer together and initiator starts the polymerization reaction forming the polymer. Nanoparticle is in between the MIP as it provides a support to the polymerized material. Now, we must remove the template molecule so that we can get a porous structure or cavity formation.

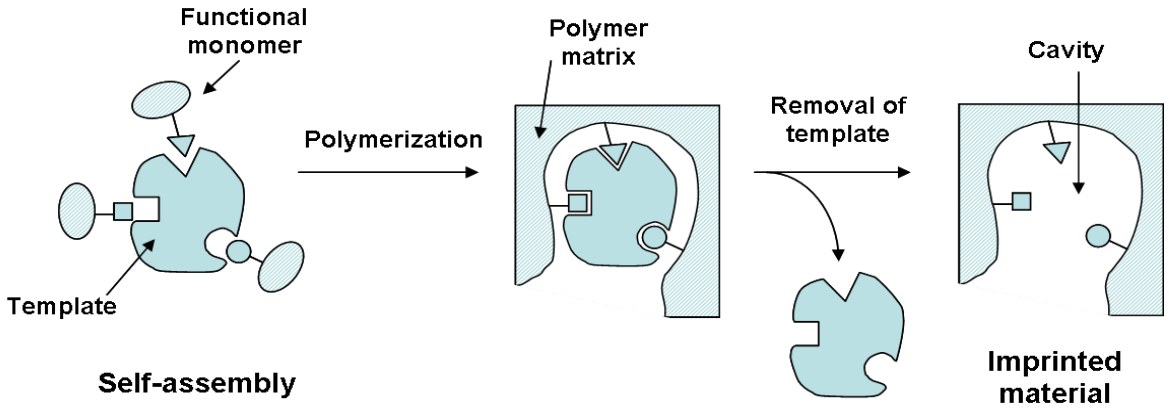


Fig 1.2.1 Molecularly Imprinted Polymer (MIP) basic synthesis mechanism (Satanaka, 2014)

Now once the cavity is formed, we can now react it with any sample containing the specific template molecule. Once the molecule binds to the MIPs, we can further extract them and quantify the amount of molecule contained in the sample.

MIPs can be synthesized by two methods namely self-assembly method, which involves the formation of polymer by combining all elements of the MIP such as monomer, initiator and cross-linker and allowing the molecular interactions to form the cross-linked polymer with the template molecule bound to it. The second method of formation of MIPs involves covalently linking the imprint molecule to the monomer (Sum Bui T, Bernadette, 2010). The polymerization product depends on the amount of cross-linker and the template molecule used. The selectivity also depends on the type of bond formation between monomer and template.

1.2.1 Removal of Template molecule:

Removing the template molecule from the MIP is a very tedious job and needs to be done with care (Ellwanger et. al, 2001, Lorenzo, 2011). There are several methods used to remove the template molecule, they are mainly classified into three methods which can be used for extraction of template molecule.

1) **Solvent extraction:**

   This method involves two ways to extract template from the MIPs. The first method is soxhlet extraction in which MIP particles are placed on a cartridge inside the extraction chamber and the washes are given of the solvent which is in a flask connected to the extractor chamber. This technique gives repeated washes to the MIP particles with fresh solvent which also favors solubilization because it uses hot solvent (Castro and Priego-Capote, 2010). The main disadvantage of this method is that it is very time consuming and large amount of solvent is also required (Castro, 2010) The second method involves incubation of MIPs with the elution solvent that can induce swelling of the polymer and hence favors the extraction of template molecule. This method also takes a lot of time for extraction (Hillberg et. al, 2009).

2) **Physically assisted extraction:**

   There are two methods namely ultrasound-assisted extraction (UAE) and microwave-assisted extraction (MAE). In UAE, ultrasound is used having a frequency of 20kHz. This process involves formation of small bubbles and erosion of solid particles. This causes an increase in temperature and pressure due to which solubility increases and helps in extraction of the template molecules (Cintas and Luche, 1999). In MAE, direct interaction between microwave and the molecule causes ionic condensation and dipole rotation. Excessive heating may cause heat sensitive polymer to melt and hence the structure may collapse (Ellwanger, A. et. al., 2001).

3) **Pressurized hot water extraction (PHWE):**

   This method involves use of water which is under high temperature (100-3740C) and pressure (10-60 bars). Due to these parameters, there is a change in viscosity and surface tension and helps in the elution process. The high thermal energy also helps to break the bond between template molecule and matrix (Batlokwa et. al., 2011).

1.2.2 Monomer selection:

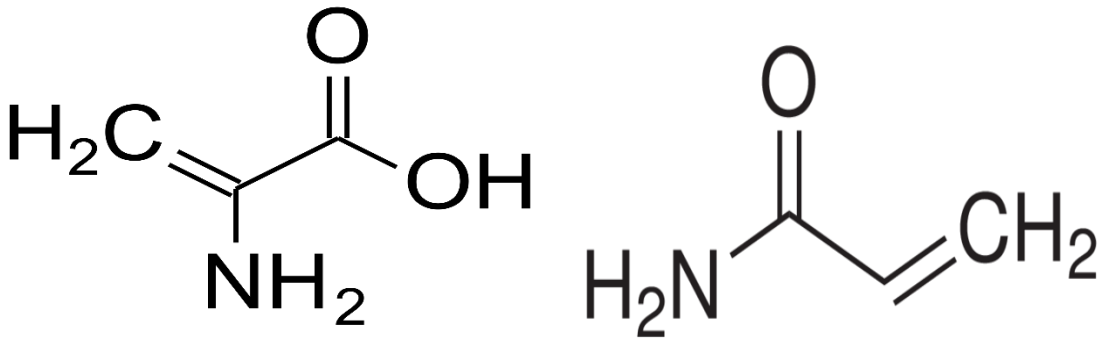
The choice of template also matters during the formation of MIPs. The type of bond formation during the template, monomer interaction matters because if the bond formed is covalent bond, it will be hard to break and hence we cannot extract the template molecule. In this study, we have used two important monomers MAA and Acrylamide.

1) **Methacrylic acid (MAA):**

   It is miscible with most of the organic solvents. It does not cause any toxicity and hence is widely used as a monomer to cause polymerization. It can form bonds with template molecules by hydrogen bond or electrostatic bond (Katoul S. Et. al., 2013)

2) **Acrylamide:**

   Acrylamide decomposes in the presence of acids, bases, oxidizing agent. It is used extensively in making Poly Acrylamide Gel Electrophoresis (PAGE). It forms a polymer once reacted initiator and cross-linker. It is carcinogenic, causes tumor it has also been found to have neurotoxic effects (Dotson, GS, 2011).



(a) (b)

Fig 1.2.2.1 Structure of (a) Methacrylic acid and (b) Acrylamide

1.3 NPs and their properties:

A nanoparticle is a microscopic particle with one dimension less than 100nm. they have different property as compared to their bulky counterpart. They have a wide variety of applications in biomedical, optical, and electronic fields which has made them an area of intense scientific research. They have a high surface area to volume ratio and hence can be used for the MIT. They can also provide support to the structure of MIP. In this study, we have focused on two important NPs, silica and magnetic NPs which have different properties and can be used for the extraction of biomolecules.

1.3.1 Silica NPs:

These NPs were first introduced (Chiola, V., et. al. 1971) and patented in 1970. They were later produced by Mobile Corporation laboratories (Beck, J. S., et. al. 1992) which suggested that silica NPs can be produced in large amount. In 1998, university of California, Santa Barbara had successfully synthesized silica NPs which have pore size from 4.6 to 30 nanometers (Zhao et.al., 1998). The researchers who invented these NPs thought of using these particles as molecular sieves, but today these NPs are used in bio sensing, imaging, synthesizing MIP and drug delivery. Many industries have started selling these NPs (www.sigmaaldrich.com).

1.3.2 Magnetic NPs:

This type of NPs can be manipulated by providing a magnetic field. Such NPs consist of magnetic material such as iron, cobalt or nickel. In this study, we have used iron magnetic material and synthesized the nanoparticle. The magnetic property of these NPs depends on the method followed for the synthesis. It has been found that, particle size from 1 to 100nm may show superparamagnetic properties (A-H. Lu; E. L. Salabas, F. Schüth 2007). Ferrite NPs are mostly studied and their surface can be modified by using silica (Tadic et. al., 2014), silicone which increases the stability of NPs in the solution. In the extraction process, we can apply magnetic field to the solution and the NPs will agglutinate and extraction process can be easily done.

1.4 Synthesis of NPs:

There are mainly two approaches to synthesize NPs bottom-up and top-down approach.

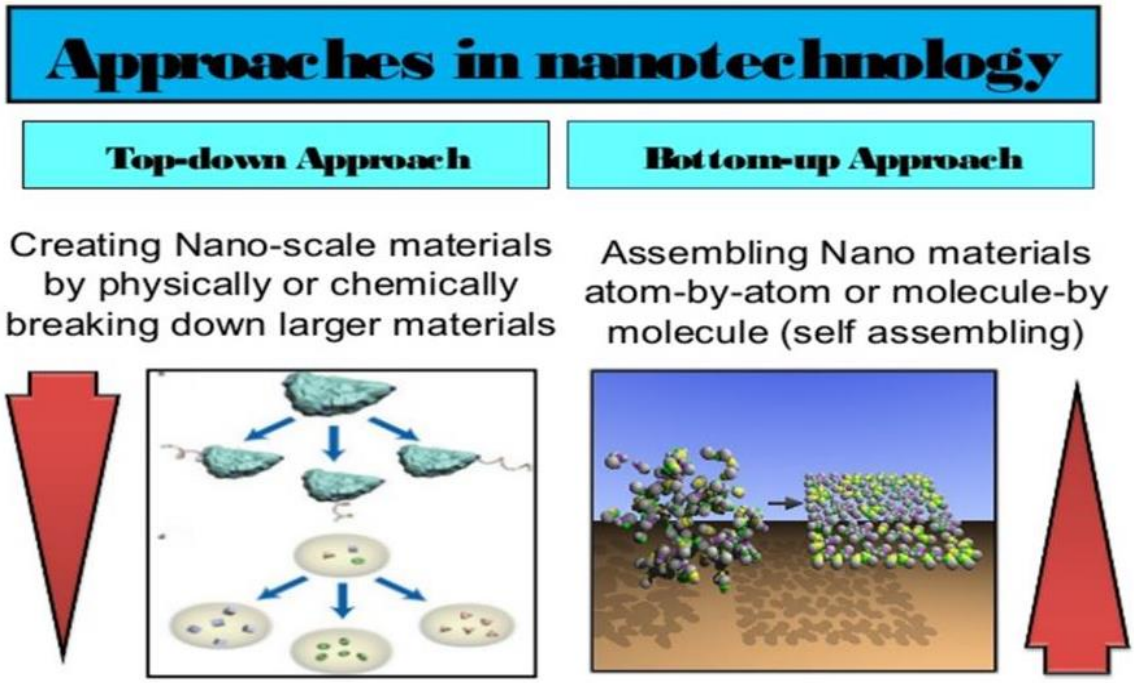


Fig 1.4.1 Approaches used for the synthesis of NPs (www.slideshare.net)

1.4.1 Bottom-up approach:

This approach uses smaller components that are built into complex assemblies. It uses the chemical properties of molecules to cause the components to assemble and self-organize to form a useful conformation. During the production of MIP, we have used this approach.

1.4.2 Top-down approach:

This approach is based on breaking large particles into small particles by using various methods. The other parameters are externally controlled and the process is carried out. These methods basically cut, mill and shape the material into desired shape and can be used further.

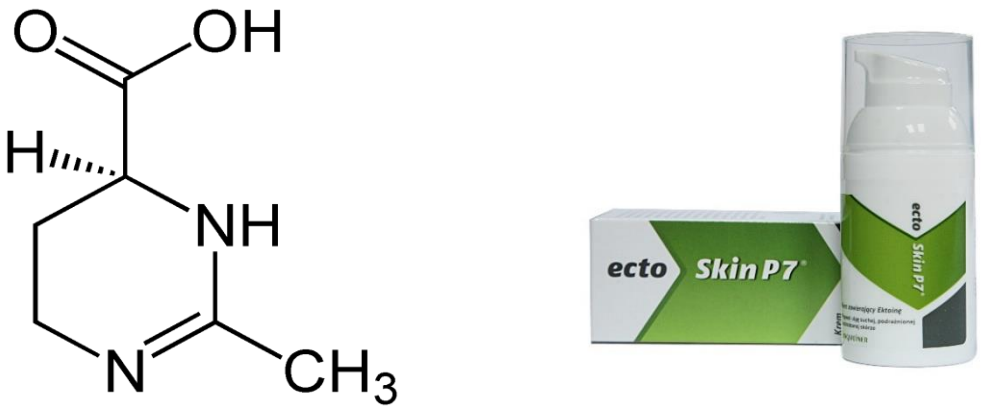
In this study, we have used bottom-up approach so that from small molecules, we get the big MIP molecules.

1.5 Template molecule:

The choice of template molecule depends on us. The cavity formed on the MIP structure depends on the template of choice. In this study, we have used two different biomolecules as template molecules ectoine and streptomycin.

1.5.1 Ectoine:

Ectoine also known as 1,4,5,6-tetrahydro-2-methyl-4-pyrimidinecarboxylic acid is a natural compound found in several species of bacteria. It is a solvent which acts as an osmolyte and helps organism during osmotic stress (Pastor M. J. et.al, 2010). Ectoine is found in high concentration in halophilic microorganisms and shows resistance towards salts and temperature stress. It is a very small molecule. Ectoine is used as an active ingredient in skin care and sun protection products. It stabilizes proteins and other cellular structures and protects skin from stress like UV irradiation and dryness (Stöveken, N. Et. Al. 2011). In sun protection creams, it is found in very low concentration and hence it becomes very important to extract ectoine from these compounds. This ectoine can be eluted out from a bacterial culture by giving osmotic shock and hence, we can also characterize the total amount of ectoine present inside the bacteria.

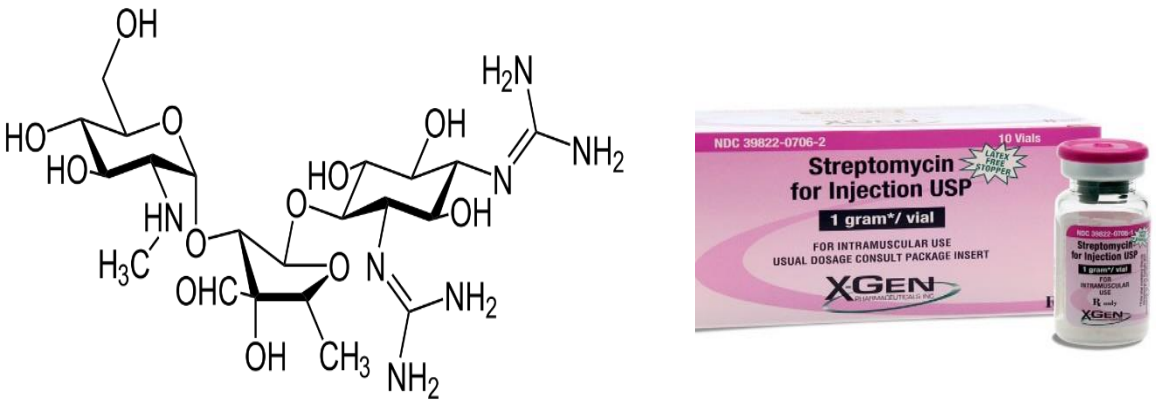


(a) (b)

Fig. 1.5.1.1 (a)Structure of ectoine molecule (b)Skin product containing ectoine. (www.bestmedicalbrands.com)

1.5.2 Streptomycin:

It is used as an antibiotic used to treat many bacterial infections and is bigger in size. Streptomycin is in the aminoglycoside class of medication. It works by blocking the ability of 30S ribosomal subunits in bacteria to make proteins which results in bacterial death and preventing the organism (www.drugs.com, 2016). It was first discovered in 1934 in *Streptomyces griseus* (Torok, et. al., 2009) and is on the World Health Organization's list of essential medicines and is the most effective and safe medicine in the health system (WHO Model List of Essential Medicines (19th List), 2016). There is a recommended dosage, if intake is excess, it may lead to side effects such as nephrotoxicity and ototoxicity (Prayle A. et. al, 2010). Hence, it is necessary to keep a track on the amount of streptomycin used. Hence, extraction of streptomycin is necessary from the broth used.



(a) (b)

Fig. 1.5.2.1 (a)Structure of streptomycin molecule and (b) product containing streptomycin. (www.x-gen.us)

1.6 Applications of MIPs:

In the recent years, MIPs has been extensively used in many fields of sciences such as sensing (Akiba and Anzai, 2016), drug delivery (Lulińsk1i P, 2017), solid phase extraction (Yoshimatsu et. al., 2007), selective extraction and many other applications. There are many other fields on which MIPs are being used such as TV technology, in which they are replacing the light emitting diodes (LEDs) and replacing them with organic light emitting diodes (OLEDs) in which, a dielectric layer was inserted which are proving to be more efficient in terms of power consumption and brighter light (Mann and Rastogi, 2016).

1.6.1 Application in drug delivery:

Recent progresses are made in this topic, MIPs can be used for drug delivery very efficiently and prove to be effective. The template used for the synthesis of MIPs is based on the drug required for the treatment of disease. Once this molecule goes in, it can directly target the affected area without nay side effects (Ansari and Karimi, 2017). This technique can also be used for the drug release method which can directly affect the cancer cell, the change in pH, enzyme activity is sensed and hence the drug is released accordingly (An et. al, 2016).

1.6.2 Application in extraction of molecules:

There are many methods used for the selective extraction of molecules but they are very costly, time consuming and power consumption is also high. Hence, MIPs can be used as an easy method and one step extraction of molecules technique. Various NPs are used based on the ease with which they can be eluted. In the table below (**Table 1.6.2.I**), it can be see that the application of MIP for selective recognition has reached new heights.

Template molecule	Nanoparticle	Detection	Reference
Bacteriophage *MS2*	silica	bacteria	Altintas Z. et.al 2015
Sulfonamide	Ferric	Sulfonamide in poultry feed	Kong et. al 2012
cholic	Quartz	Cholic acid level in body	Gültekin et.al 2014
caffeic acid	Quartz	caffeic acid in plant materials	Gültekin A et. al 2013
diazinon	silica	Diazinon in pesticides	Motaharian A. et. al 2016
Helicobacter pylori	silica	*Helicobacter pylori* eradication	Han et.al 2015
Pyrraline	magnetic	Pyrraline in milk sample	Liu et. al. 2017

Table 1.6.2.I Application of MIPs for selective extraction of molecules.

Chapter Two

Genesis of hypothesis and objectives

2.1 Genesis of hypothesis:

MIPs are molecules which are highly specific and can be used to elute the required biomolecule form the solution. Various NPs such as silica NPs, magnetic NPs are found to be effective in the drug delivery, selective extraction, and many more applications. Ectoine and Streptomycin during the down-stream processing need a lot of power and hence, MIPs can be used for selectively extraction of these molecules. Silica and magnetic NPs are used in the production of MIPs and magnetic NPs can easily be extracted out just by applying the magnetic field to these NPs. While centrifugation is required to extract the silica NPs.

Highlights of MIPs:

1. Highly specific molecules.
2. Elution can be carried out in one step.
3. Easily produced, only 3 steps required for the production.
4. Lower cost of production these molecules.
5. Reduces the downstream process and makes it cost efficient.

In this study, we focus on the extraction of ectoine and streptomycin. Synthesis of these MIPs imprinted with these molecules is carried out. The non-imprinted polymers (NIPs) are molecules which do not have a template molecule imprint on them so that they cannot form cavities and hence binding will not take place.

2.2 Objectives:

1) Standardization and characterization of biomolecules.
    - Ectoine and streptomycin needed to be standardized using various methods such as TLC, spectrophotometry.
    - The obtained MIPs are then characterized using various instruments in order to confirm the binding of template molecules and if cavity formation has taken place.

2) Synthesis of silica and magnetic NPs.
    - These NPs can be used to provide strength as well as can provide easy way to elute them from the solution.
    - Different methods used for the synthesis of NPs.

3) Synthesis of MIPs:
    - Ectoine and streptomycin used as template molecule to produce the MIPs.
    - There are various parameters which need to be standardized in order to synthesize the MIPs.

4) Elution of the template molecule.
    - The template molecule should be removed in order to create cavity or pore.
    - Elution of the template molecule can be completed by various methods. One such method is solvent extraction which was used for the extraction.

Chapter Three

Methods and materials

3.1 Chemicals:

Ectoine and streptomycin were obtained from HiMedia which were used as the template molecules. Methacrylic acid (MAA) which acts as a cross-linking agent was purchased from HiMedia and Ethylene Glycol Dimethacrylate (EGDMA) was purchased from Sigma Aldrich. AIBN was purchased from HiMedia which acts as an initiator. Different solvents were used such as acetonitrile, toluene, acetic acid were brought from SRL labs. methanol, acetic acid, acetone, heptane and cyclohexane were obtained from Merck. Potassium hexacyano ferrate was obtained from Merck, sodium nitroprusside was obtained from Merck and sodium hydroxide pellets were obtained from HiMedia which were used for the preparation of SNP solution. Cyclohexanol was obtained from SRL labs.

3.2 Standardization of TLC technique for detection of ectoine:

Solvent system was standardized for detection of ectoine using thin layer chromatography (TLC) technique. Solvents such as butanol, formic acid, acetic acid, water were prepared in different ratios to get the separation between ectoine and its derivative hydroxyectoine (Hisayo et. al, 1998). Such solvent system can detect both ectoine and hydroxyectoine (convertible forms) from the fermentation broth. Different proportions of butanol, water and acetic acid or formic acid were examined as mentioned in **Table 3.2.I**

Number	Butanol (ml)	Acid (ml)	Water (ml)	Butanol:Acid:Water
1	0.60	0.15 (Acetic acid)	0.25	4:1:1.6
2	0.60	0.20 (Acetic acid)	0.20	3:1:1
3	0.75	0.15 (Formic acid)	0.10	7.5:1.5:1

Table 3.2.I: Different ratios of solvent system used to compare acetic acid and formic acid.

It can be seen that, formic acid gives us much more clear separation between hydroxyectoine and ectoine than acetic acid. Hence, formic acid was finalized for further analysis. Further, the ratio of butanol: formic acid: water was standardized to give constant R_f value for ectoine.

Table 3.2.II summarizes the proportions of the solvents that were analyzed.

Number	Butanol (ml)	Formic acid (ml)	Water (ml)
1	7.5	1.5	1
2	8.5	1	0.5
3	4	3	1

Table 3.2.II Different ratios of butanol: formic acid: water used for TLC.

The R_f values for the solvent system were obtained and are given below in the **Table 3.2.III**.

Number	R_f value
1	0.288
2	0.394
3	0.527

Table 3.2.III R_f value of the TLC obtained by different ratios of butanol: formic acid: water

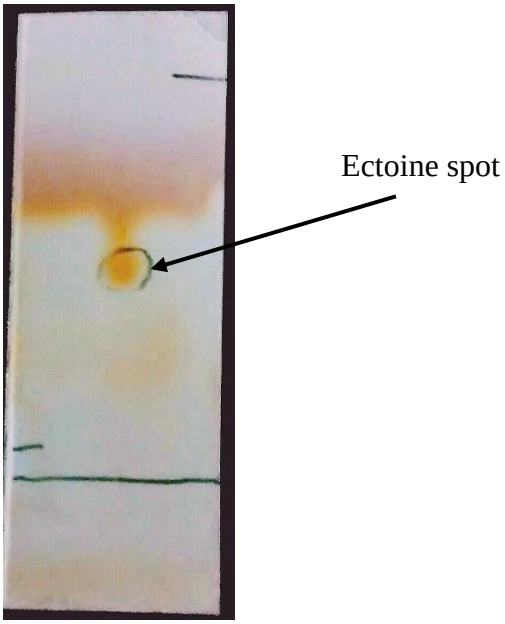


Fig. 3.2.1 Standardization of TLC for detection of ectoine.

Hence, the solvent system of butanol: formic acid: water in the ratio 4:3:1 was finalized.

3.3 Synthesis of MIPs:

Two molecules were selected as template namely ectoine and streptomycin. These two molecules are used because they require a huge amount of energy during the downstream processing and the concentration of these molecules produced is also low. Ectoine synthesis was carried out using two monomers, acrylamide and MAA.

3.3.1 Synthesis of ectoine MIPs using MAA:

The synthesis of ectoine MIPs was carried out (Scorrano S, 2011). In the synthesis of MIPs, 30 µl of ectoine (0.5mM) was added along with 30 µl of MAA (1.7mM) which is a monomer and dissolved in 3ml of solvent acetonitrile: toluene (3:1) (v/v). This mixture was stirred at 4^0C overnight. After this step, 10mg of SiO_2 NPs were added along with 30 µl of EGDMA and 4mg of AIBN. Another 2ml of acetonitrile: toluene (3:1) (v/v) was added to dissolve all the other added components and to make the complete solution to 5ml. After the addition, the vial was placed at 50^0C for 6 h with constant stirring. The polymerization takes place at this step and it can be noticed by change in turbidity. The vial is then placed at 60^0C for aging for 24 h. The unreacted template molecules were removed by giving water washes. The bound ectoine molecules were eluted by using acetonitrile as eluent. Non-imprinted polymers (NIPs) were synthesized in similar way without adding the template molecule.

3.3.2 Synthesis of ectoine MIPs using acrylamide:

In this method, ectoine (0.5mM) was added along with acrylamide (1mM) and they were dissolved in 3ml of solvent system toluene: 1-octanol: methanol (1:2:2). The mixture was then sonicated for 20 min at room temperature. After the sonication, 10mg of silica NPs were added along with 30µl of EGDMA and 4mg of AIBN respectively. The mixture was again sonicated for 20 min and polymerization was noticed by the colour change. The above mixture was kept at 60^0C for 24h for aging. Non-imprinted polymers (NIPs) were produced in the similar manner, but template was not added.

3.3.3 Removing the unreacted template molecules:

Ectoine was not detected in acetonitrile fractions. However, it was later observed that it was eluted during water washes as confirmed by TLC analysis. Water washes were given to remove the unreacted template molecule from the MIP. Hence, three water washes were given to remove the unreacted template molecule.

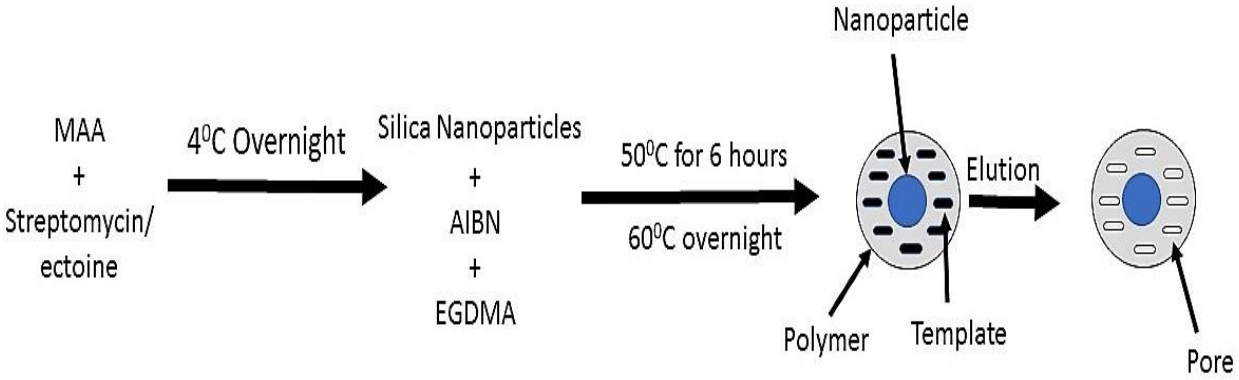


Fig 3.3.1 Standard protocol for the synthesis of MIPs.

3.4 Streptomycin quantitation:

The quantitation was done by reacting streptomycin with a solution containing sodium nitroprusside (SNP) reagent (Li and Gao, 2008). This reagent includes potassium ferricyanide, sodium nitroprusside and sodium hydroxide each weighing 100mg which were dissolved in 30ml of distilled water. A change in colour from yellow to red is observed read at 495 nm and the intensity changed linearly depending upon amount of streptomycin. Streptomycin solutions (0- 200 mM) were made and reacted with SNP reagent. The following standard dosage response graph was obtained which is shown in **Fig 3.4.1** and the concentration are given in **Table 3.4.I** respectively.

Concentration (mM)	Average absorbance
0	0.160333
20	0.316667
40	0.489667
60	0.695333
80	0.832667
100	0.975333
120	1.119
140	1.263
160	1.396
180	1.543
200	1.647

Table 3.4.I Absorbance value of the standard dosage response.

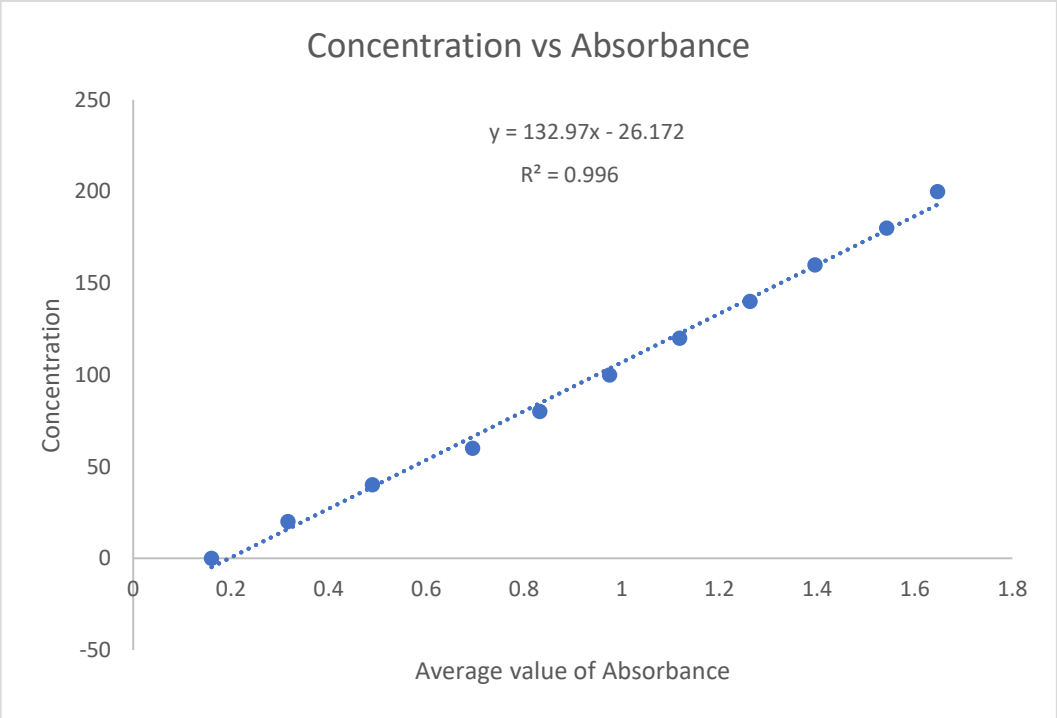


Fig. 3.4.1 Streptomycin standard dosage response curve.

Hence, SNP containing solution can be used to find the amount of streptomycin in the solution.

3.4.1 Synthesis of streptomycin MIPs:

The synthesis of MIPs using streptomycin was carried out using the same method as given for synthesis of ectoine MIPs using MAA as monomer. The concentration of streptomycin used was 10mg/ml.

3.5 Synthesis of magnetic NPs:

The synthesis of magnetic NPs was carried out using ferric ammonium sulphate ($NH_4Fe(SO_4)_2 \cdot 12\ H_2O$) and ferrous ammonium sulphate [$(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$] respectively. Ferric ammonium sulphate (0.2M) and ferrous ammonium sulphate (0.2M) were dissolved in 3ml of distilled water. In this solution, 10ml of ethanol and 2ml of sodium hydroxide was also added. The solution was sonicated for 10 min and while sonicating, TEOS was added drop by drop to generate silica coating on the magnetic NPs. After this process, the solution was allowed to sonicate further for 30 min.

3.5.1 Synthesis of ectoine MIPs using magnetic NPs:

The synthesis of ectoine MIPs was done using the same technique as reported earlier. Instead of silica NPs, magnetic NPs were used. Concentration of the materials required were taken the same as reported earlier.

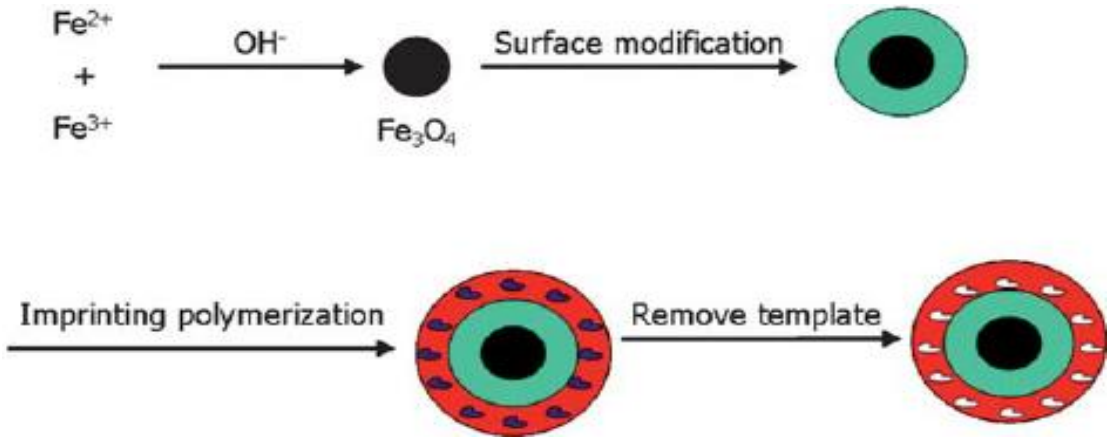


Fig. 3.5.1 General steps for the synthesis of magnetic NPs and then synthesizing MIP.

Chapter Four

Results and Discussion

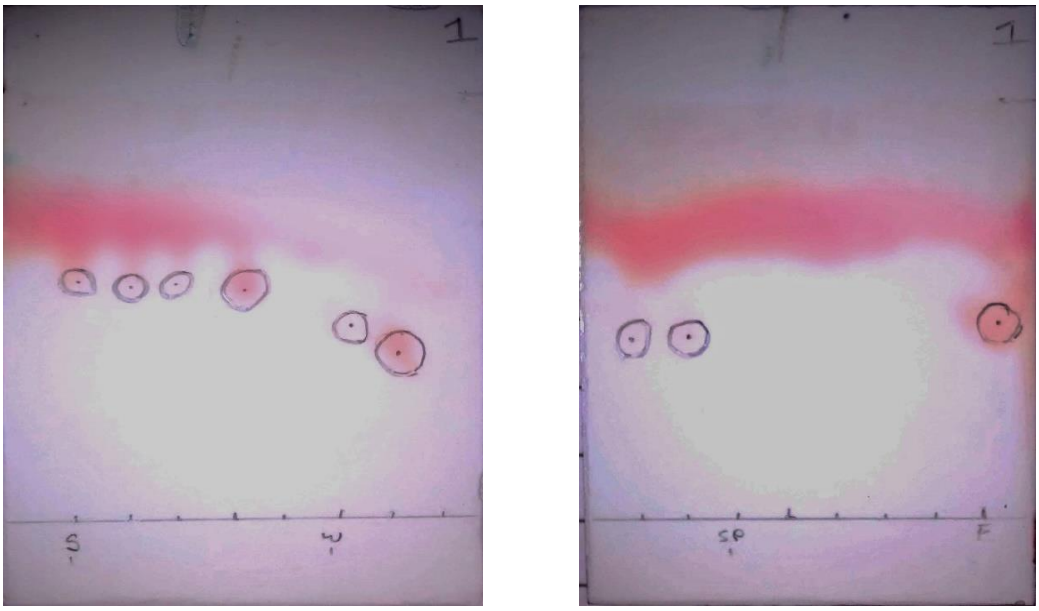
4.1 Standardization of important parameters for MIP synthesis:

The concentration of monomer and cross-linker also matters during the process of MIP synthesis. The cavity formed during the elution of template molecule depends on the amount of monomer and cross-linker concentration (Bunch and Wang, 2013). Hence, we have taken different concentrations as given below. (**Table 4.1.I)**

Number	MAA (mM)	EGDMA (mM)	AIBN (mg)
1	1	4	1
2	1.7	6.8	4
3	2.5	10	8
4	1	10	8
5	2.5	4	1

Table 4.1.I Different concentration of MAA, EGDMA and AIBN used for the synthesis of MIPs.

Using these varying concentrations of MAA and EGDMA, we synthesized the MIPs. They were given water washes to elute the ectoine molecule. TLC analysis was done to check which concentration can be used effectively to get the best MIPs. The TLC analysis photos are shown below in **Fig 4.1.1**. We tried to elute the ectoine molecule using various different solvents such as acetonitrile, acetic acid, octanol, pentanol but we didn't get any spot on the TLC to predict that elution has taken place by using these solvents. Hence, water was used for the elution process of the ectoine.



(a) (b)

Fig. 4.1.1 Standardization of important factors such as concentration of monomer and cross-linker. Here, (a) Sample (S) and water wash 1 (W1) is seen and (b) shows supernatant (SP) and ectoine standard (E). The TLC is done for the five different concentration of MAA, EGDMA and AIBN.

The R_f values obtained from these TLC are given below. (**Table 4.1.II**)

Number	Sample	Water wash	Supernatant	Ectoine
1	0.57	0.44	-	0.44
2	0.57	0.38	-	
3	0.57	-	-	
4	0.57	0.42	-	
5	-	0.42	-	

Table 4.1.II R_f values obtained from TLC due to different concentration of MAA, EGDMA and AIBN used to prepare the MIPs.

Hence, it is seen that, water is a good eluent and can be used for the elution of ectoine molecule.

4.2 Elution of streptomycin from MIPs

Various chemicals such as octanol, acetonitrile, acetic acid, were used for the elution of streptomycin from the MIPs. The elution was carried out using 1% cyclohexanol in distilled water (Hoffman, 1951). The elution was successfully done and comparison between the efficiency of MIPs and NIPs can also be predicted from the graph (**Fig. 4.2.1**). The mean absorbance reading is taken after subtracting the standard solution value (**Table 4.2.I**). Absorbance was taken at 495nm. Washes were given to elute the streptomycin molecule.

Wash Number	MIPs	NIPs
1	0.159	0.066
2	0.083	0.073
3	0.022	0.05

Table 4.2.I Absorbance value of streptomycin. Comparison between MIPs and NIPs.

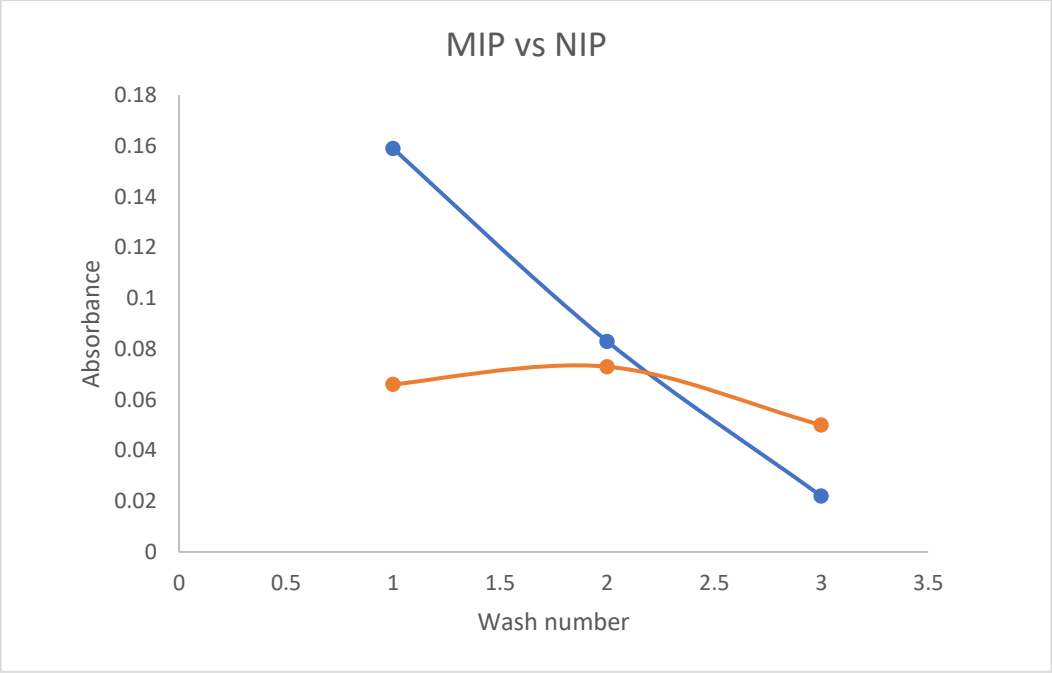


Fig 4.2.1 Comparison between MIPs (blue) and NIPs (orange).

Hence, it can be seen that MIPs are more selective towards streptomycin as compared to NIPs. Thus, MIPs were successfully synthesized and is specific to streptomycin.

4.3 TGA analysis of streptomycin MIPs:

TGA analysis is used to evaporate the organic material and only inorganic material will be left. In this method, the temperature in the furnace is increase till 1000^0C at which all the sample will evaporate. This evaporation will take place step by step in which, the first layer (template molecule) will be evaporated and then the second layer (polymer) and so on. The weight change during this cycle is measured. Hence, by plotting a graph of the weight change versus the temperature increase (**Fig 4.3.1**), we can quantify which material in the MIPs will evaporate first.

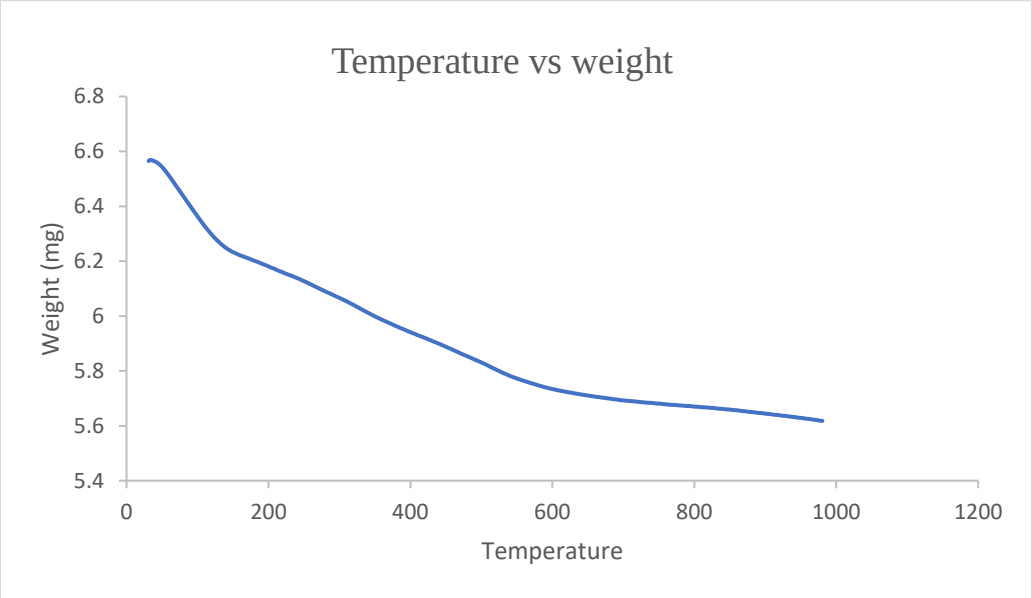


Fig 4.3.1 Temperature versus weight graph obtained by TGA analysis.

Hence, the first molecule to evaporate was streptomycin and then the polymer will get evaporated. Hence, we come to know that MIPs are created successfully in which streptomycin is on the top and polymer is bounded to it.

4.4 VSM analysis of magnetic NPs:

This analysis is used to find the strength of magnetic nanoparticles. If the nanoparticle is coated with other material, then its magnetic strength will decrease and hence we can confirm whether MIPs formation has taken place successfully or not. A magnetic field is applied to the sample and change in weight is noted. Magnetic NPs coated with silica and normal magnetic NPs were used for the comparison. Hence, a graph of mass vs magnetic field applied is plotted (**Fig 4.4.1**).

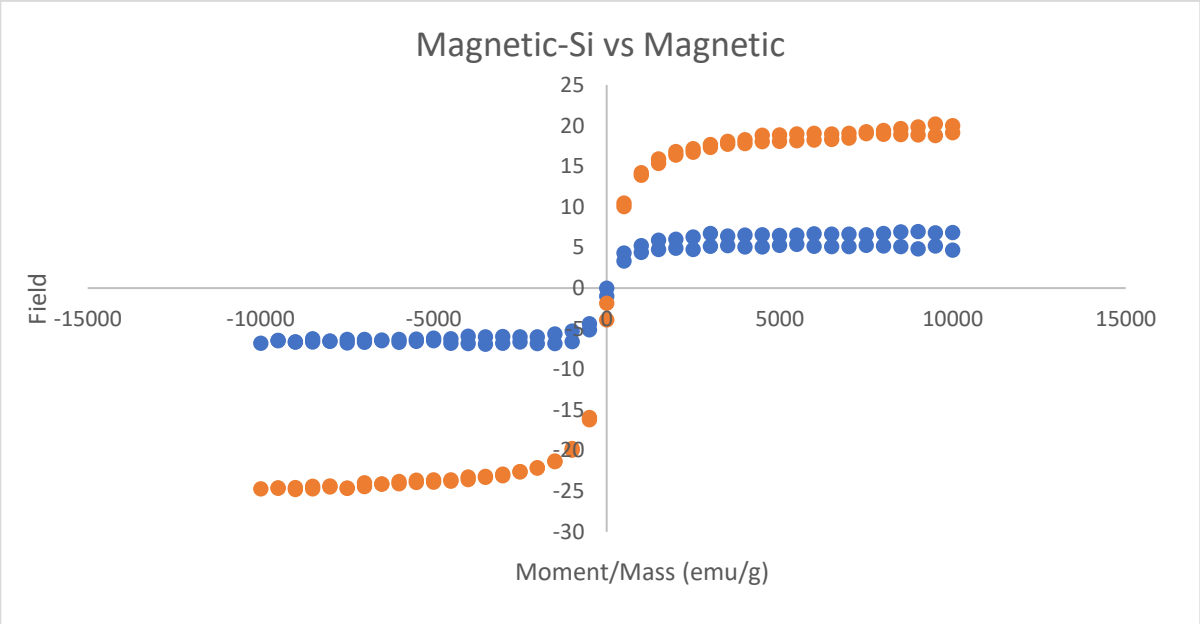


Fig. 4.4.1 VSM graph to compare coating on magnetic NPs. Mass vs magnetic field is compared. Magnetic NPs (orange) were compared with silica coated magnetic NPs (blue).

Hence, this graph can show the binding of silica on magnetic NPs and hence, the imprinting was done successfully.

4.5 Discussion:

MIPs have been used for sensing and solid phase extraction of various pesticides from natural environment. These MIPs are used for the solid phase extraction from various sources (Qiao, et. al., 2006). MIPs are used for the selective extraction using silica nanoparticles (Peng et. al, 2010; Attaran, 2013) as well as magnetic nanoparticles (Bagheri, 2016). Their use has been reported for selective extraction from fermentation broth on large scale (Javanbakht, 2012). Hence, MIPs are increasingly being used for selective extraction from fermentation broth. Their use has been reviewed earlier (Wackerlig and Lieberzeit, 2015; Bapat et al., 2016). The MIPs used in this study were synthesized using MAA, EGDMA and AIBN scheme and the template molecules - ectoine and streptomycin was successfully imprinted which is in agreement with other reports (Ji et al., 2013). The non-specific extraction of ectoine may be corrected using different scheme of monomer and cross-linker. As the MIPs can extract the molecules from the fermentation broths in just one step, the methods is going to be cost effective and can be used for large scale implement.

Chapter Five

Conclusion and future prospective

5.1 Conclusion:

NPs were synthesized as per the standardized protocols. MIPs synthesized using magnetic nanoparticles were not stable and aggregation upto 1 micrometer size was observed. Hence, these were not used for further studies. MAA mediated MIPs were non-aggregating and stable. These were used further for imprinting ectoine and streptomycin. Elution patterns in case of ectoine indicate that the elution is taking with water as eluent. However, when the MIPs were exposed to broth containing ectoine, the extraction was non-specific and other components of the broth were also getting extracted. This indicates that the scheme for synthesis of MIPs can be revised or different monomers can be used for checking suitability of ectoine extraction.

The MIPs were also synthesized using streptomycin as template. Out of various solvents used such as methanol, water, acetic acid, acetonitrile, heptane, cyclohexanol, only cyclohexanol (1% in water) eluted the streptomycin molecules. However, these trials need to be taken in fermentation broth and % recovery can be calculated to check the suitability of this technique for extraction of streptomycin on large scale.

5.2 Future prospective:

The activity and specificity of these MIPs has to be checked in fermentation broth to justify whether these synthesized MIPs can act as specific extraction tool.

TEM imaging of the cavity formed structures needs to done to confirm the imprinting as well as elution.

Chapter Six

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