ANTIBIOTIC PURIFICATION BY USING IMA ADSORBENTS

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ANTIBIOTIC PURIFICATION BY USING IMA ADSORBENTS

ABDUL RAHIM BIN MOHD YUSOFF

Thesis submitted to the Faculty of Chemical and Natural Resources Engineering in
Partial Fulfillment of the requirement for the
Degree of Bachelor Engineering in Chemical Engineering

Faculty of Chemical and Natural Resources Engineering
University Malaysia Pahang

APRIL 2009

I declare that this thesis entitle "*Antibiotic Purification by Using IMA Adsorbents"* is the result of my own research except as cited in the references. The thesis has not been accepted for any degree and not concurrently submitted in candidature of any degree

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Name : ABDUL RAHIM BIN MOHD YUSOFF

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Special Dedication of This Grateful Feeling to My…

Beloved father and mother;
Mr. Mohd Yusoff bin Hussin and Mrs. Rabainah binti Hashim

Loving brothers and sisters;
Shahiful, Shahrizal, Asrol, Zaidi, Zurianie,
Azmir, Huda, Azizah and Shalehuddin.

Supportive friends

For Their Love, Supports and Best Wishes

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ABSTRACT

The intensity to achieve highly efficient and economical separation process can be seen in developing of various methods in the recent year. While in purification of antibiotic there are many methods use such as using High performance liquid chromatography (HPLC) and Counter-current chromatography (CCC). The purpose of this research is to develop immobilized metal ion affinity zeolite by using solid state ion exchange method to investigate the effect of pH, types of adsorbent using different metal into rifampicin adsorption capacity. Adsorption of rifampicin using zeolite has a greatly potential due to ability to scale up easily, and highly selective. It was found that the highest adsorption capacity for rifampicin occur at pH 8 with Zr-HBeta as adsorbent. H-beta zeolite give highest adsorption capacity because it has higher diameter size, surface area and pore volume compare to Y zeolite. Increasing the surface area and pore volume will give better chances of rifampicin to adsorb into adsorbent. Meanwhile, pH 8 gives the highest adsorption capacity because it is closer with the pKa_2 value of rifampicin which is 7.9. While zirconium is the only transition metal containing both acidic and basic surface sites. So this will make it gives better adsorption capacity of rifampicin compare with ferum and nickel. The adsorption isotherm data of rifampicin was well correlated by the Langmuir model.

ABSTRAK

Keinginan yang tinggi untuk mencapai proses pemisahan yang ekonomi dan berkecekapan tinggi dapat dilihat melalui penghasilan pelbagai cara sejak kebelakangan ini. Terdapat pelbagai cara dalam penyulingan antibiotic seperti HPLC and CCC. Tujuan kajian ini adalah untuk menyediakan ion logam tarikan dimasukkan ke dalam zeolite menggunakan kaedah penukar ion berkeadaan pepejal untuk melihat kesan pH, jenis penjerap daripada jenis logam yang berlainan terhadap kapasiti penjerapan rifampicin. Penjerapan rifampicin menggunakan zeolite mempunyai potensi yang besar kerana mudah dioperasikan pada skala yang lebih besar, beroperasi secara berterusan dan mempunyai kememilihan yang tinggi. Kapasiti penjerapan tertinggi untuk rifampicin adalah pada pH 8 dengan mengunakan penjerap logam zirkonium. Zeolite H-beta memberikan kapasiti penjerapan tertinggi kerana ianya mempunyai saiz diameter, luas permukaan and isipadu pori yang lebih besar berbanding dengan zeolite Y. Pertambahan luas permukaan serta isipadu pori akan memberikan peluang yang lebih kepada rifampicin untuk menyerap ke dalam penjerap. Dalam pada itu, pH 8 memberikan kapasiti penjerapan tertinggi kerana ianya dekat dengan nilai pK_{a2} bagi rifampicin iaitu 7.9. Sementara itu, hanya zirkonium sahaja logam peralihan yang mengandungi sifat asid dan alkali bagi kedua-dua belah permukaan. Ini menjadikan zirkonium memberikan kapasiti penjerapan rifampicin lebih baik berbanding dengan ferum dan nikel. Data penjerapan rifampicin menunjukkan ianya menghampiri model Langmuir.

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LIST OF SYMBOLS

Al	Aluminium
C	Concentration mM
Fe	Ferum
Kd	Langmuir adsorption parameter
pH	Negative logarithmic molar concentration of hydrogen ion, -log[H+]
pKa	Acid dissociation constant
n	Freundlich constant
Na	Sodium
Ni	Nickel
q	Solute concentration in adsorbent mmol/g
qm	Langmuir isotherm parameter mmol/g
Si	Silica
Zr	Zirconium

LIST OF ABBREVIATIONS

CASMAC	Cascade-mode multi-affinity chromatography
CCC	Counter-current Chromatography
CEC	Cation Exchange Capacity
CNS	Central Nervous System
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
FTIR	Fourer Transform Infrared
H_3PO_4	Phosphoric acid
HSCCC	High Speed Counter-current Chromatography
IDA	Iminodiacetic acid
IMA	Immobilized metal ion affinity
IMAC	Immobilized metal ion affinity chromatography
IUPAC	International Union of Pure and Applied Chemistry
FDA	Food and Drug Administration
HPLC	High Performance Liquid Chromatography
K_2CO_3	Potassium carbonate
K_2HPO_4	Potassium Hydrogen Phosphate
KH_2PO_4	Potassium Dihydrogen Phosphate
$KHCO_3$	Potassium hydrogen carbonate
LEC	Ligand Exchange Chromatography
NTA	Nitrilotriacetic acid

RNA	Ribonucleic acid
UV-VIS	Ultra Violet Visible

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CHAPTER 1

INTRODUCTION

1.1 Background of Study

Antibiotics are organic substances produced by special microorganisms or other living systems. Generally, antibiotic are produced on an industrial scale using a fermentation process and capable at low concentration of inhibiting the growth of, or destroying another microorganism. Antibiotics have been isolated from numerous sources but mainly from bacteria (tetracyclines, bacitracin, polymyxin, chloramphenicol, and streptomycin) and fungi (cephalosporins, penicillins). Penicillin was the first antibiotic discovered by Sir Alexander Flemming in 1928. It is derive from the Penicillium mold and acts by destroying the cell wall of bacteria. The name penicillium was taken from the Latin penicillum meaning a painter's brush because the fronds of the fungus were thought to look like a painter's brush.

Antibiotics are the most important bioactive and chemotherapeutic compounds made by microbiological synthesis. They also include antimicrobial compounds present in higher plants and animals. They have proven their significance in varied fields like medicinal chemistry, agriculture and food industry.

Up to now about 40 000 antibiotics have been found and about 80 of them are in therapeutic use. They are isolated primarily from metabolic products of living cells. Various penicillins, cephalosporins and several other antibiotics are semi-synthetic ones, which mean one part of the molecule, i.e. 6-amino penicillanic acid is

prepared from say penicillin G or penicillin V, followed by synthetic introduction of an appropriate side chain.

Zeolites are crystalline, hydrated aluminosilicates of alkali and alkaline earth cations characterized by an ability to hydrate/dehydrate reversibly and to exchange some of their constituent cations with dissolved cations in solution, both without a major change in structure. The ion exchange property of these minerals has generated worldwide interest for use in diverse applications such as the treatment of nuclear, municipal, and industrial waste water. Although commercial applications of ion exchange processes have used mainly synthetic zeolites, the earliest studies of ion exchange phenomena were based on observations of natural materials, including natural zeolites.

Commercial adsorbents that display ultra porosity include activated carbons, activated clays, inorganic gels, such as silica gel and activated alumina, and the crystalline aluminosilicate zeolite. Activated carbons, activated alumina, and silica gel do not possess an ordered crystal structure and consequently their pores are non-uniform. The distribution of the pore diameters within the adsorbent particles may vary widely from 20 to several thousand Angstroms, as is the case for some activated carbons. Hence, all molecular species, with the possible exception of high molecular weight polymers, may enter the pores. Zeolite molecular sieves, on the other hand, have pores of uniform size (3–10Å) which are uniquely determined by the unit structure of the crystal. These pores will completely exclude molecules that are larger than their diameter. J. W. McBain (1932) originated the term “molecular sieves” to define porous solid materials that exhibit the property of acting as sieves on a molecular scale.

Synthetic adsorbents are widely used as polymeric media for recovery and separation of antibiotic or their intermediates, foods, etc. For example, they are used for separation of antibiotics such as penicillin, cephalosporin and their derivatives, because of their high adsorption capacity, mechanical strength and chemical stability suitable for industrial operations.

Column operations are commonly adopted for those applications. In this sense, the synthetic adsorbents are used as chromatographic separation media. Therefore, both pore and chemical characteristics of the synthetic adsorbents will affect the separation and adsorption capacity of target compounds.

1.2 Problem Statement

The development of an antibiotic is a long and costly proposal. It begins with basic research designed to identify organisms, which produce antibiotic compounds. During this phase, thousands of species are screened for any sign of antibacterial action. When one is found, the species is tested against a variety of known infectious bacteria. This is a complex procedure because thousands of antibiotic materials have already been discovered. Repeatedly, scientists find that their new antibiotics are not unique. If the material passes this phase, further testing can be done. This typically involves clinical testing to prove that the antibiotic works in animals and humans and is not harmful. If these tests are passed, the government agencies like the Food and Drug Administration (FDA) must then approve the antibiotic as a new drug. This whole process can take many years.

Normally production of an antibiotic depends on a fermentation process. During fermentation, amounts of the antibiotic-producing organism are grown and the organisms produce the antibiotic material, which can then be isolated for use as a drug. Development of antibiotics necessitates isolation and purification of a desired compound from a complicated matrix such as fermentation broth and crude extract. Analysis of antibiotics in formulated and unformulated samples demand a highly specific and rapid method as many antibiotics (e.g. β-lactams) also have serious stability problems.

There are many methods in separation of antibiotic. HPLC technology using sophisticated equipments and refined adsorbents highly facilitate the isolation of antibiotics; there are some drawbacks due to various complications arising from the

use of a solid support. Other method is Counter-current chromatography (CCC) is a unique form of liquid partition chromatography which utilizes a separation column free of solid support matrix. Because of this support-free system, the method provides an important advantage over other chromatographic methods by eliminating various complications including an adsorptive loss and deactivation of samples, contamination, etc.

Immobilized metal ion affinity chromatography (IMAC) is one of the most powerful separation methods available for protein fractionation. For antibiotic separation, this method is use wisely yet. Other thing is traditional stationary phase for IMAC are based on soft gel. But for this research, we will use some inorganic material adsorbent.

1.3 Objective of Study

The purpose of this research is to use zeolite (H-beta, Y) as an immobilized metal ion affinity stationary phase by using three different metal (zirconium, ferum and nickel) and rifampicin as an antibiotic solution for antibiotic separation.

1.4 Scopes of Study

In order to achieve objectives, the scopes for this research are:

1. To study the effect of different metal use in IMA
2. To study type of zeolite
3. To study the effect of pH
4. To study the effect of antibiotic concentration

CHAPTER 2

LITERATURE REVIEW

2.1 Antibiotics

Antibiotics are chemical compounds used to kill or inhibit the growth of infectious organisms. The antibiotic terms originally referred only to the organic compounds that produced by bacteria or molds that are toxic to other microorganisms. The term is now used freely to include synthetic and semi synthetic organic compounds. Antibiotic refers generally to antibacterial, however, because the term is loosely defined, it is preferable to specify compounds as being anti malarial, anti viral, or anti protozoa's. All antibiotics share the property of selective toxicity: They are more toxic to an invading organism than they are to an animal or human host. Penicillin is the most well-known antibiotic and has been used to fight many infectious diseases, including syphilis, gonorrhea, tetanus, and scarlet fever. Another antibiotic, streptomycin, has been used to combat tuberculosis.

Rifampicin (Figure 2.1) is the most important compound of rifamycin group inhibits the growth of most gram-positive and some -negative microorganisms by inhibiting their RNA synthesis. Rifampicin is a bactericidal antibiotic drug of the rifamycin group. It is a semi synthetic compound derived from *Amycolatopsis rifamycinica* (formerly known as *Amycolatopsis mediterranei* and *Streptomyces mediterranei*). It is an antibiotic used to treat infections, including tuberculosis (also known as TB). It can also be used to prevent infections in those who have been in contact with serious infections. Rifampicin has 2 pKa since it is a Zwitterion, pKa 1.7 related to 4-hydroxy and pKa 7.9 related to 3-piperazine nitrogen.

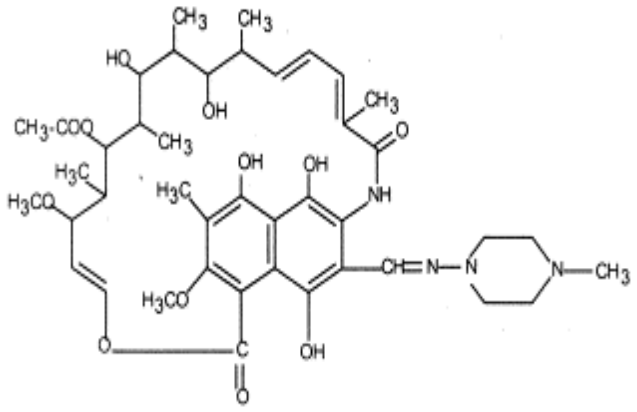


Figure 2.1: Chemical structure of rifampicin.

Penicillins can be divided into two groups, namely, (Albarellos et al., 2004) the natural penicillins, including benzyl penicillin (penicillin G) and its salts (sodium, potassium, benzathine and procaine), and penicillin V and (Albarellos et al., 2005) the semi-synthetic penicillins. This second group can be further divided into three sub-groups: (a) staphylococcal beta-lactamase-resistant penicillins or isoxazolilpenicillins (oxacillin, cloxacillin and dicloxacillin); (b) broad spectrum or aminobenzyl penicillins (ampicillin and amoxicillin), and (c) anti-pseudomonal penicillins (carbenicillin, ticarcillin, piperacillin).

Penicillin was the first microbial metabolite to distinguish between toxicity to the bacterial cell and toxicity to the mammalian host to permit its use in the systemic treatment of infections caused by gram-positive and -negative organisms in humans and animals. The basic structure of penicillin nucleus includes a β-lactam ring fused through nitrogen and adjacent tetrahedral carbon to a second heterocycle, which in natural penicillin is a five-membered thiazolidine ring that shown in figure 2.2. Semi-synthetic penicillins are produced starting from 6-aminopenicillanic acid, which are obtained from culture of *Penicillium chrysogenum*.

These molecule are more resistant to β-lactamase e.g. ampicillin, oxacillin etc.Penicillin and other β-lactams (cephalosporins) inhibit the synthesis of essential structural components of bacterial cell wall i.e. peptidoglycan which are absent in mammalian cells. Thus host cell metabolism remains unaffected and penicillins are regarded as one of the safest and most effective class of antibiotics being used for

bacterial infections. The analysis of degradation products in commercial penicillins has two-fold importance; firstly in pharmacokinetic studies it is desirable to distinguish between the drug and any degradation products, secondly allergic reactions attributed to penicillin may frequently because by such compounds. Accordingly it is essential to be able to detect the presence of these compounds in the pharmaceutical compounds.

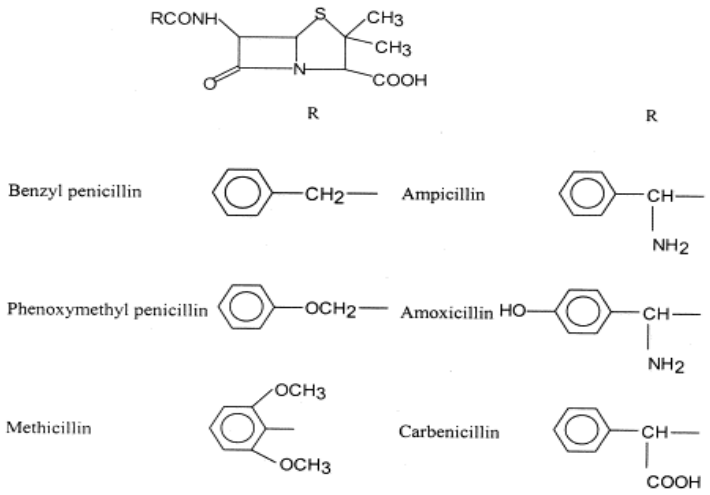


Figure 2.2: Chemical structure of some important penicillins.

Cephalosporins are β-lactam antibiotics, with the same fundamental structural requirements as penicillin that shown in figure 2.3. Heterocyclic ring fused to β-lactam ring is six membered (dihydrothiazine) in cephalosporins. The fused rings in β-lactams are not coplanar but folded along the C-N bond common to both rings; less markedly in cephalosporins than in penicillins. The first generation of cephalosporins are administered parenterally (cephalothin, cefazolin) or orally (cephalexin, cefadroxil). The second generation includes cefoxitin, cefotetan, cefamandole and cefuroxime. The third generation includes cefotaxime, ceftazidime, ceftizoxime, ceftriaxone and ceftiofur. Cefotaxime, ceftazidime, ceftizoxime and ceftriaxone consistently reach effective antibacterial concentrations in the central nervous system (CNS) in humans.(Albarellos et al., 2007).

Cephalosporins are used for the treatment of infections caused by most gram-positive and -negative bacteria, especially *Escherichia coli*, *Proteus mirabilis* and klebsiella. As discussed in penicillin only cephalosporin C is found in nature isolated

from cultures of fungi other i.e. semi-synthetic cephalosporins are derived from 7-aminocephalosporanic acid, product obtained from cephalosporin C hydrolysis. Literature suggests use of C18 column for chromatographic analysis of this class of antibiotics. Carbanepem, a newly synthesized β-lactam antibiotic, was analysed for its degradation products by multistage liquid chromatography-electrospray mass spectrometry.

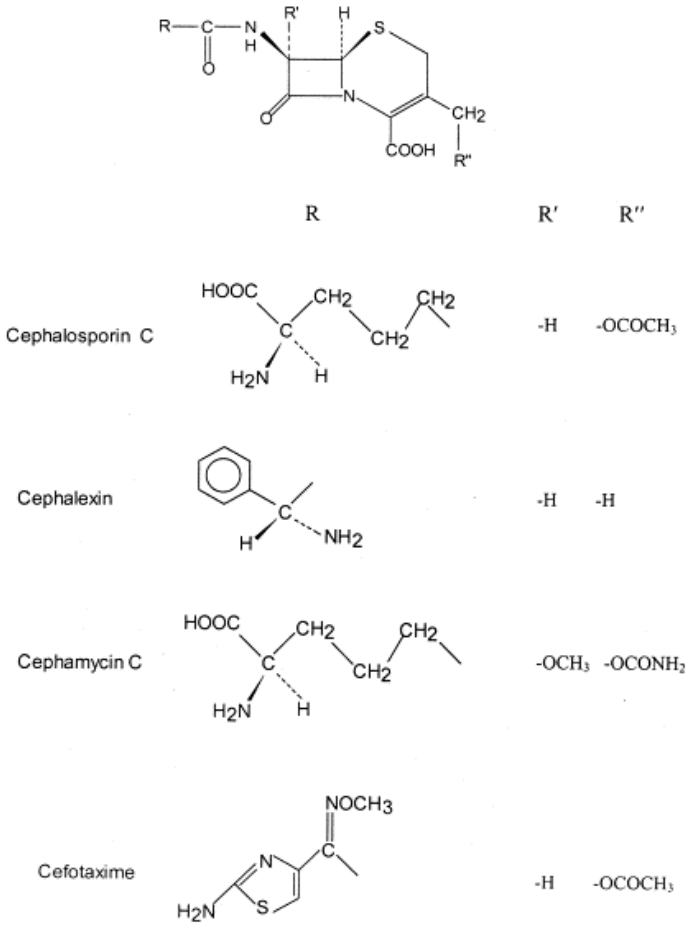


Figure 2.3: Chemical structure of cephalosporins.

2.1.1 Major principle and definition

While our scientific knowledge of antibiotics has only recently been developed, the practical application of antibiotics has existed for centuries. The first known use was by the Chinese about 2,500 years ago. During this time, they discovered that applying the moldy curd of soybeans to infections had certain therapeutic benefits. It was so effective that it became a standard treatment. Evidence

suggests that other cultures used antibiotic-type substances as therapeutic agents. The Sudanese-Nubian civilization used a type of tetracycline antibiotic as early as 350 A.D. In Europe during the Middle Ages, crude plant extracts and cheese curds were also used to fight infection. Although these cultures used antibiotics, the general principles of antibiotic action were not understood until the twentieth century.

The behavior of antibiotics and heavy metals in soil greatly depend on their adsorption–desorption characteristics, and the knowledge of these processes are important to predict their bioavailability, fate and transport mechanism through soil column into groundwater or surface water. Many studies have been conducted to investigate the adsorption and desorption of heavy metals (Morillo, 2000, Zhou, 2004 and Wang, 2006)

The development of modern antibiotics depended on a few key individuals who demonstrated to the world that materials derived from microorganisms could be used to cure infectious diseases. One of the first pioneers in this field was Louis Pasteur. In 1877, he and an associate discovered that the growth of disease-causing anthrax bacteria could be inhibited by a saprophytic bacterium. They showed that large amounts of anthrax bacilli could be given to animals with no adverse affects as long as the saprophytic bacilli were also given. Over the next few years, other observations supported the fact that some bacterially derived materials could prevent the growth of disease-causing bacteria.

Commercial antibiotic are produced by microbial fermentation and the process involve three stages, fermentation, purification and chemical modification. Fermenters in the pharmaceutical industry are low pressure vessels designed for high horse-power agitation to facilitate dissolution of oxygen in the growth medium. Although many of the production fermenters in the industry are well over 20 years old, the continual refinement of these vessels has kept them quite serviceable. The relatively short commercial period of patent protection for healthcare products combined with the high capital cost of building new fermenters has led the industry toward multiple use facilities rather than construction of new, dedicated factories. The continual drive toward higher volumetric productivity has led to high oxygen

demand. Most of the larger fermenters were designed on the basis of oxygen transfer, modification of existing fermenters maybe facing new constraints. Higher volumetric productivity has often meant increased broth viscosity (with increased cell mass) and increased that load (increased power transfer from higher horsepower agitators and increased metabolic rates). Fluid bulk mixing and heat transfer are becoming increasingly more important parameters for achieving high fermenter productivity, particularly in older units.

Characteristics of antibiotic such purity and form are controlled by purification and final isolation procedures. Antibiotic products are generally of high purity (usually greater than 95% and often over 98% purity). Crystalline products possibly are sought as a means of achieving high purity and desirable form (color, stability, dissolution rate). The ultimate use of these products contraindicates use of toxic separation agents in the final steps of product isolation. Fermentation products often have limited chemical and thermal stability. Generally, the larger the molecular weight of the product, the gentler must be the recovery.

The manufacturers of antibiotics maintain pilot plant facilities whose purpose is to upgrade yields and to bring about improvements in processing procedures. Studies are continually being made on strain improvement, inoculum conditions, fermentation conditions, and various combinations of these factors. For example, improved mutant strains almost always require adjustments in fermentation conditions in order to achieve the high yields in fermenters that are obtainable in shaken flasks.

In 1928, Alexander Fleming made one of the most important contributions to the field of antibiotics. In an experiment, he found that a strain of green *Penicillium* mold inhibited the growth of bacteria on an agar plate. This led to the development of the first modern era antibiotic, penicillin. A few years later in 1932, a paper was published which suggested a method for treating infected wounds using a penicillin preparation. Although these early samples of penicillin were functional, they were not reliable and further refinements were needed. These improvements came in the early 1940s when Howard Florey and associates discovered a new strain of

Penicillium, which produced high yields of penicillin. This allowed large-scale production of penicillin, which helped launch the modern antibiotics industry.

After the discovery of penicillin, other antibiotics were sought. In 1939, work began on the isolation of potential antibiotic products from the soil bacteria streptomyces. It was around this time that the term antibiotic was introduced. Selman Waxman and associates discovered streptomycin in 1944. Subsequent studies resulted in the discovery of a host of new, different antibiotics including actinomycin, streptothricin, and neomycin all produced by *Streptomyces.* Other antibiotics that have been discovered since include bacitracin, polymyxin, viomycin, chloramphenicol and tetracyclines. Since the 1970s, most new antibiotics have been synthetic modifications of naturally occurring antibiotics.

2.1.2 Antibiotic resistance

Antibiotic resistance is the ability of a microorganism to withstand the effects of antibiotics. It is a specific type of drug resistance. Antibiotic resistance evolves naturally via natural selection acting upon random mutation, but it could also be engineered by applying an evolutionary stress on a population. Once such a gene is generated, bacteria can then transfer the genetic information in a horizontal fashion (between individuals) by plasmid exchange. If a bacterium carries several resistance genes, it is called multi resistant or, informally, a superbug. The term antimicrobial resistance is sometimes using to explicitly encompass organisms other than bacteria.

Antibiotic resistance can be a result of horizontal gene transfer, and also of unlinked point mutations in the pathogen genome and a rate of about 1 in 10^8 per chromosomal replication. The antibiotic action against the pathogen can be seen as an environmental pressure; those bacteria which have a mutation allowing them to survive will live on to reproduce. They will then pass this trait to their offspring, which will result in a fully resistant colony.

Several studies have demonstrated that patterns of antibiotic usage greatly affect the number of resistant an organism which develop overuse of broad-spectrum antibiotics, such as second- and third-generation cephalosporins, greatly hastens the development of methicilin resistance. Other factors contributing towards resistance include incorrect diagnosis, unnecessary prescriptions, improper use of antibiotics by patients, the impregnation of household items and children's toys with low levels of antibiotics, and the administration of antibiotics by mouth in livestock for growth promotion.

Antibiotic resistance can also be introduced artificially into a microorganism through transformation protocols. This can aid in implanting artificial genes into the microorganism. If the resistance gene is linked with the gene to be implanted, the antibiotic can be used kill off organisms that lack the new gene.

Rational use of antibiotics may reduce the chances of development of opportunistic infection by antibiotic-resistant bacteria due to dysbacteriosis. In one study the use of fluoroquinolones are clearly associated with *Clostridium difficile* infection, which is a leading cause of nonosocomial diarrhea in the United States, and a major cause of death, worldwide. Vaccines do not suffer the problem of resistance because a vaccine enhances the body's natural defenses, while an antibiotic operates separately from the body's normal defenses. Nevertheless, new strains may evolve that escape immunity induced by vaccines.

While theoretically promising, anti-staphylococcal vaccines have shown limited efficacy, because of immunological variation between *Staphylococcus* species, and the limited duration of effectiveness of the antibodies produced. Development and testing of more effective vaccines is under way.

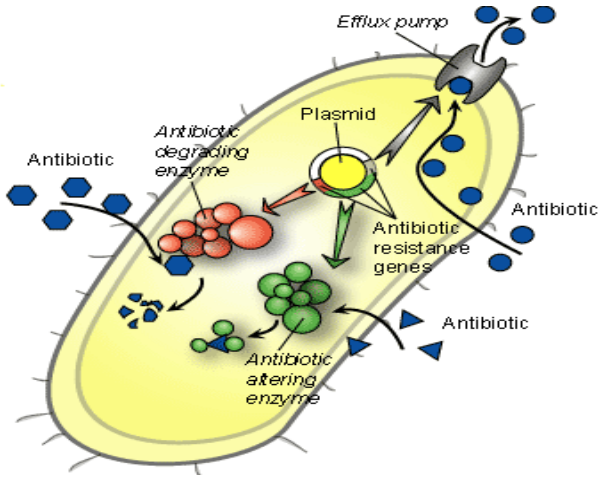


Figure 2.4: The bacterial mechanisms of antibiotic resistance

Several mechanisms have evolved in bacteria which confer them with antibiotic resistance. These mechanisms can chemically modify the antibiotic, render it inactive through physical removal from the cell, or modify target site so that it is not recognized by the antibiotic. The bacterial mechanisms of antibiotic resistance can be shown in figure 2.4.

The most common mode is enzymatic inactivation of the antibiotic. An existing cellular enzyme is modified to react with the antibiotic in such a way that it no longer affects the microorganism. An alternative strategy utilized by many bacteria is the alteration of the antibiotic target site. These and other mechanisms are shown in the figure and accompanying table 2.1 below.

Table 2.1: Mechanisms of preventing antibiotic resistance

Antibiotic	Method of resistance
Chloramphenicol	reduced uptake into cell
Tetracycline	active efflux from the cell
β-lactams, Erythromycin, Lincomycin	eliminates or reduces binding of antibiotic to cell target
β-lactams, Aminoglycosides, Chloramphenicol	enzymatic cleavage or modification to inactivate antibiotic molecule
Sulfonamides, Trimethoprim	metabolic bypass of inhibited reaction
Sulfonamides, Trimethoprim	overproduction of antibiotic target (titration)

2.1.3 Categories of antibiotic

Antibiotics are categorized as bactericidal if they kill the susceptible bacteria or bacteriostatic if they reversibly inhibit the growth of bacteria. In general the use of bactericidal antibiotics is preferred but many factors may dictate the use of a bacteriostatic antibiotic. When a bacteriostatic antibiotic is used the duration of therapy must be sufficient to allow cellular and humoral defense mechanisms to eradicate the bacteria. If possible, bactericidal antibiotics should be used to treat infections of the endocardium or the meninges. Host defenses are relatively ineffective at these sites and the dangers imposed by such infections require prompt eradication of the organisms. Today the most classification of antibiotics is unambiguously based on the chemical structures of the active compounds as shown in Table 2.2.

Table 2.2: Classification of antibiotic by their structures

Class	Example of antibiotics	Common uses
Aminoglycosides	Amikacin Gentamin Kanamycin Neomycin Streptomycin	Infection caused by Gram-negative bacteria, such as Escherichia coli and Klebsiella
Macrolides	Azithromycin Dirithromycin Erythromycin Troleandomycin	Streptococcal infections, Syphilis, respiratory infections, Mycoplasmal infections and Lyme disease
Penicilin	Ampicillin Azlocillin Dicloxacillin Nafcillin	Wide range of infections, Penicillins used for streptococcal Infections, syphilis and Lyme disease
Polypeptides	Bacitracin Colistin Polymyxin B	Eye, ear or bladder infections; Usually applied directly to the eye or inhaled into the lungs, rarely given by injection.
Quinolones	Ciprofloxacin Enoxacin Levofloacin Ofloxacin	Urinary tract infections, bacterial prostatics, bacterial diarrhea and gonorrhea
Tetracyclines	Doxycycline Oxytetracycline Tetracycline	Syphilis, chlamydial infections, Lyme disease, and acne rickettsial infections
Ansamycins	Rifampicin	Curing tuberculosis and other infections caused by Gram-positive bacteria

2.1.4 Antibiotics and chemotherapeutic agent

The term antibiotic strictly refers to substances that are of biological origin whereas the term chemotherapeutic agent refers to a synthetic chemical. The distinction between these terms has been blurred because many of our newer "antibiotics" are actually chemically modified biological products or even chemically synthesized biological products.

Antibiotics are effective against bacteria, usually by breaking down the cell wall. These are given to ward off infection after surgery or injury or for diseases like TB that are caused by bacteria. While chemotherapeutic agents are poisons used to fight cancer. Cancer cells are "young" because they are rapidly dividing. "Young" cells are more vulnerable to poisons, and also radiation in radiation therapy.

Antibiotics are fairly safe, although their misuse or overuse can be disastrous. They also kill "friendly" bacteria that inhabit every inch of your body. These friendly colonies help keep malicious bacteria from getting a foothold. Chemotherapy can be a very difficult experience. Although the target is cancer cells, the whole body is poisoned. This and radiation is why cancer patients so often lose hair, are nauseated, etc.

Most chemotherapy agents and medications work by interfering with DNA synthesis or function. Each chemotherapy drug works during different phases of the cell cycle. Based on their action, chemotherapy agents can be classified as cell-cycle specific agents (effective during certain phases of cell cycle) and cell-cycle nonspecific agents (effective during all phases of cell cycle).

Depending on their characteristics and nature of treatment, chemotherapy agents can be categorized as alkylating agents, antimetabolites, anthracyclines, antitumor antibiotics, monoclonal antibodies, platinums, or plant alkaloids. The generic terms to refer to either antibiotics or chemotherapeutic agents are

antimicrobic or antimicrobial agent. However, the term antibiotic is often used to refer to all types of antimicrobial agents

2.2 Zeolite

Zeolites are hydrated aluminosilicate minerals and have a micro-porous structure. The term was originally coined in the 18th century by a Swedish mineralogist named Axel Fredrik Cronstedt who observed, upon rapidly heating a natural mineral that the stones began to dance about as the water evaporated. Using the Greek words which mean "stone that boils," he called this material zeolite. More than 150 zeolite types have been synthesized and 48 naturally occurring zeolites are known. Zeolites have an "open" structure that can accommodate a wide variety of cations, such as Na^{+}, K^{+}, Ca^{2+}, Mg^{2+} and others. These positive ions are rather loosely held and can readily be exchanged for others in a contact solution. Some of the more common mineral zeolites are: analcime, chabazite, heulandite, natrolite, philipsite, and stilbite. An example mineral formula is: $Na_2Al_2Si_3O_{10}$-$2H_2O$, the formula for natrolite. The type of ZSM zeolite is shown in table 2.3.

Table 2.3: ZSM-Type zeolite

ZSM-Type Zeolite			
Zeolite	Cations	Si/Al ratio	Type
ZSM-4	TMA, Na	1.5-10	
ZSM-5	TPA or precursors Na	6-50	Pentasil family
ZSM-11	TBP,BTPP,TBA	10-45	Pentasil family
ZSM-21	ED, P, C, Na	4-25	Ferririte
ZSM-35	ED, P, Na	4-25	Ferririte
ZSM-38	C(Chloride),Na	4-25	Ferririte
ZSM-20	TEA, Na	3.5-5	Faujasite
ZSM-34	C or TMA, Na, K	4-10	Offeretite / erionite

Zeolite was discovered exactly 250 years ago. Axel A. F. Cronstedt (1722-1765), the famous Swedish mineralogist, was the first scientist who described the distinctive property of this class of minerals, i.e., its peculiar frothing characteristics when heated before the blowpipe. Cronstedt examined two samples, one which was said to come generically from Iceland, the other coming from Svappavaara in northern Sweden. From Cronstedt's indications, the Swedish site has been located near Kiruna, where the first zeolite, a stilbite, was collected. This work describes the structure and microstructure of the first discovered zeolite, the stilbite, from Svappavaara (northern Sweden).

The discovery and description of zeolite minerals during the late 18th century depended on new concepts concurrently developed in crystallography, mineralogy, and chemistry. Of particular importance were the discoveries in chemistry and crystallography. Axel F. Cronstedt's invention of the blowpipe provided the means for melting a mineral sample and thereby providing a qualitative test for water within

the sample. This was also the time when many chemical elements were discovered and the techniques of analytical chemistry were developed. Two notable chemists, Martin H. Klaproth and Louis N. Vauquelin, provided many useful analyses of minerals, but in some cases, especially with zeolites, missed some important elements. The only method of determining the quantitative proportion of individual components was by gravimetric analysis, meaning each component had to be chemically separated from the sample and weighed. If an element, such as sodium, could not be precipitated, there was no way to determine its abundance.

Several fundamental concepts of crystallography were developed during these years. Using the new Carangeot contact goniometer, Romé de l'Isle (1772) proposed his general law of constancy of interfacial angles and thereby established the science of crystallography. RenéJust Haüy (1801) proposed a geometrical law of crystallization that gave crystallography a mathematical footing.

Using the available chemical analyses and crystallographic observations, Haüy (1801) organized information for eight minerals thought to be zeolites. The names he used are harmotome, stilbite, chabasie, analcime, mesotype, prehnite, lapis lazuli (lazurite), and zeolithe efflorescente (laumontite). During the next few decades, improvements in analytical methods and refined observations on crystals caused the number of known zeolite minerals to more than double. Heulandite was separated from stilbite, and mesotype was divided into natrolite, mesolite, and scolecite. Other zeolites discovered in the early 19th century are brewsterite, epistilbite, phillipsite, gismondine, gmelinite, levyne, and edingtonite.

The emergence of the polarizing microscope as a research tool early in the 19th century was a valuable tool in refining crystallographic observations on zeolites. It was (and remains) most useful in documenting the ubiquitous twinning of minerals, like stilbite and phillipsite.

Near the end of the 19th century Dana (1899) listed twenty two minerals in the zeolite group, of which all but two, ptilolite and laubanite, remain valid today.

Members of the zeolite group were considered to be hydrated silicates of aluminum with sodium, potassium, calcium, and rarely, barium and strontium. All occurred in cavities of volcanic rocks, and less commonly in veins cutting granite, and all had the property of expelling water when heated.

2.2.1 Natural and synthesis zeolite

Synthetic and natural zeolites are hydrated aluminosilicates with symmetrically stacked alumina and silica tetrahedra which result in an open and stable three-dimensional honeycomb structure with a negative charge. The negative charge within the pores is neutralized by positively charged ions (cations) such as sodium. Over 150 zeolite structural types have been identified

Natural zeolites form where volcanic rocks and ash layers react with alkaline groundwater. Zeolites also crystallized in post-depositional environments over periods ranging from thousands to millions of years in shallow marine basins. Naturally occurring zeolites are rarely pure and are contaminated to varying degrees by other minerals, metals, quartz or other zeolites. For this reason, naturally occurring zeolites are excluded from many important commercial applications where uniformity and purity are essential.

There are nearly 50 different types of natural zeolites (clinoptilolite, chabazite, phillipsite, mordenite, etc.) with varying physical and chemical properties. For example, clinoptilolite, the most common natural zeolite, has 16% more void volume and pores as much as 0.2 nm larger than analcime, another common zeolite. It is important to know the specific type of zeolite one is using in order to assure that it is appropriate for one's needs. The image of clinoptilolite zeolite is shown at figure 2.5

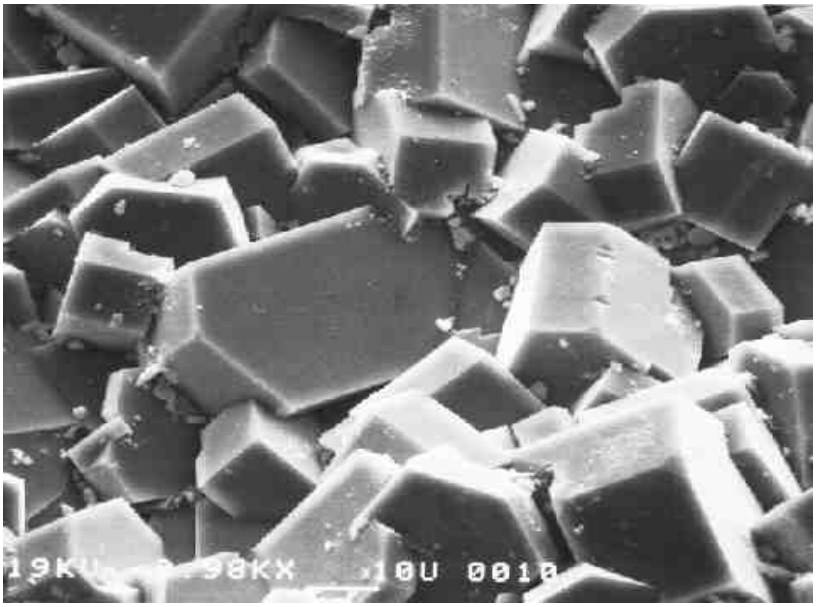


Figure 2.5: Image of clinoptilolite zeolite

In common with other zeolites, clinoptilolite has a cage-like structure consisting of SiO4 and AlO4 tetrahedra joined by shared oxygen atoms. The negative charges of the AlO4 units are balanced by the presence of exchangeable cations - notably calcium, magnesium, sodium, potassium and iron. These ions can be readily displaced by other substances, for example heavy metals and ammonium ions. This phenomenon is known as cation exchange, and it is the very high cation exchange capacity of clinoptilolite which provides many of its very useful properties. Clinoptilolite is also known to be a powerful adsorbent of certain gases, such as hydrogen sulphide and sulphur dioxide.

Synthetic zeolites are high-purity specialty chemicals that serve a wide range of applications. Most synthetic zeolites are thermodynamically meta-stable products formed kinetically under special synthesis conditions. Therefore, they are prepared not only under closely controlled conditions of temperature, pressure and time but with specific reactants and physical reaction environments. These restrictions on their production make their reproducibility an issue.

Typically, many zeolite syntheses yield two or more crystalline phases with unreacted gel components. Much development work is, therefore, aimed at the

process and compositional control needed to produce a reaction window, where a single-phase fully-crystallized product may be isolated.

The simplest synthetic zeolite is the zeolite A with a molecular ration of one silica to one alumina to one sodium cation. The zeolite A synthesis produces precisely duplicated sodalite units which have 47% open space, ion exchangeable sodium, water of hydration and electronically charged pores. These properties lead to the various uses of natural and synthetic zeolites.

Zeolite beta is one of the most important high silica zeolites because its interconnected large pore network and strong acidity give it special catalytic properties. The contributions on zeolite beta came after the framework structure was successfully solved by Newsam and Treacy. Marler confirmed the topology of the basic layer as the building unit by refining the superposition structure of zeolite B-beta about 200 μm in size. The B-beta has been synthesized from aqueous solutions containing silica, boric acid and 4,4′- trimethylene dipiperidine as templates and only a few zeolite beta crystals were obtained after 5 months of thermal treatment. All of these previous investigations illustrated that the structure of the zeolite beta consists of an intergrowth hybrid of two distinct polytypic series of layers, namely, polymorphs A and B. A hypothetical polymorph C, closely related to A and B, was first described by Newsam in 1988. Recently, using germanium to stabilize the structure, Corma synthesized the pure polymorph C structure either in the presence of fluoride anions or in a fluoride-free system under alkaline conditions. In addition, materials denoted ITQ-16 containing different proportions of polymorph C have also been obtained. Polymorph C has also been found as an impurity in all-silica zeolite Beta synthesized in the F- system. Figure 2.6 provides the structural characteristics of the three polymorphs of zeolite beta.

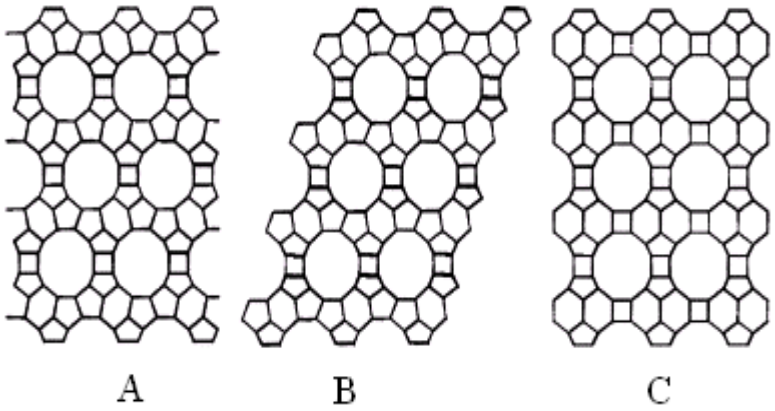


Figure 2.6: Beta polytypes A(tetragonal, $P4_122$,), B(monoclinic C2/c,) and C($P4_2/mmc$)

In zeolite Y cations are located in their supercage, sodalite and double hexagonal prisms. Supercages are able to accommodate many cations, even those with high hydrated radii, due to their aperture (7.4Å) and diameter (12.5Å). On the other hand, sodalite and hexagonal prisms have an aperture of 2.2 Å, being less favorable for ion exchanging, because of steric factors (Breck, 1984).

High charges and small radii cations are preferred by zeolites of lower Si/Al ratio such as zeolite Y (Townsend, 1991). Few studies concerning heavy metal exchange isotherms in zeolite Y have been reported (Chen *et al*., 1990; Keane, 1996). In all cases, it has been emphasized that the entering ions are preferably located in large cages such as supercages and sodalites. Exchange degree in such cages depends on the nature of each entering ion (Keane, 1994). Figure 2.7 shows the structure of Y zeolite as seen by red light.

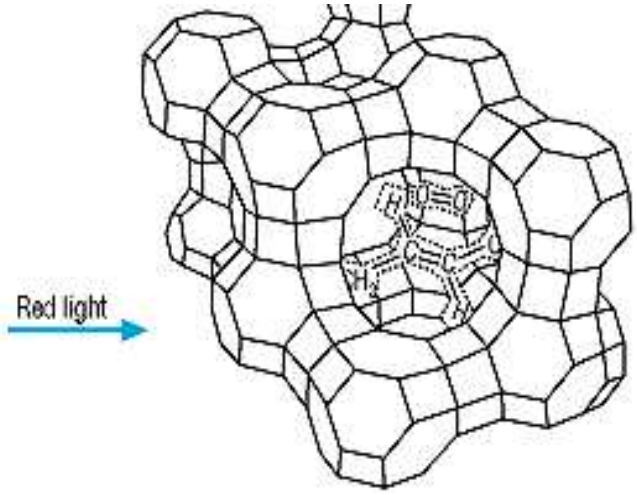


Figure 2.7: Y Zeolite crystal structure

2.2.2 Characteristic of natural and synthesis zeolite

The characteristics of a zeolite deposit are decided in its genesis. Small natural differences such as temperature, geographic location and ash/water properties impart a slightly different composition and therefore some unique properties to a few of the deposits. These small differences present during the formation of a zeolite deposit are the reason that each natural zeolite property has distinctly unique properties.

The alumina and silica from the ash stack into a stable, open and three three-dimensional honeycombs structure–there are over forty other natural zeolite structures. For example, clinoptilolite (clino) has silica to alumina ratio of 5 to 1, while chabazite has a ratio of 2 to 1.In the natural zeolite structure, the net negative charge within the symmetrical voids hold the cations for the cation exchange capacity (CEC). Ion exchangeable ions, such as potassium, calcium, magnesium and sodium, the major cations, are held electronically within the open structure (pore space)—up to 38% void space.

Zeolites are inorganic materials which have a highly ordered structure and can be synthesized with a nanocrystallline size. As inorganic support materials to

immobilize biological species, zeolites offer interesting characteristics, such as mechanical and chemical resistance as well as high surface area (Breck et al., 1974) and (Szostak et al., 1978). They have the advantage that the basic/acidic nature of the material can be modified by varying the Si/Al ratio or by introducing different metals into the crystalline framework and by changing the Si/Me ratio (Tavolaro et al., 2005). Furthermore, zeolite acidity can be modified by exchanging extra-framework metal cations with H^+. Finally, zeolites are known to be stable both in wet and dry conditions and well-tolerated by microorganisms, and therefore normally compatible with biochemical analyses. These materials are receiving increased interest as biomaterials. In fact, zeolite containing antibiotic toothpaste and antimicrobial orthopedic agents were reported in recent patents (Barry et al., 2000).

Generally, adsorption on zeolites is mainly determined by two main factors, structure and physicochemical properties of adsorbent. The pore size which depends on the structure of zeolite, determines the accessibility of molecules into the zeolite framework. However, in certain cases it can adapt the opening upon adsorption because of the flexibility of the framework.

The degree of flexibility is a function of the framework structures in the presence of extra-framework cations and molecules. The availability of internal space volume is another interesting characteristic of zeolites for separation/purification applications. Effect of physical properties such as the type of framework structure, pore volume, pore size distribution as well as chemical properties of adsorbent (electrostatic force of cations, hydrophobicity and acidity) are both important in tailoring the effective adsorbent. The effects of zeolite structural parameters namely type of framework, surface area, average pore size and pore volume which were obtained by zeolite structural synthesis and modifications.

The biggest differences between natural and synthetic zeolite:

- Synthetics are manufactured from energy consuming chemical and naturals are processed from natural ore bodies.
- Synthetic zeolite have a silica to alumina ratio of 1 to 1 and clinoptilolite (clino) zeolite have a 5 to 1 ratio.
- Clino natural zeolites do not break down in a mildly acid environment, where synthetic zeolite do. The natural zeolite structure has more acid resistant silica to hold its structure together. The clino natural zeolite is broadly accepted for use in the agricultural industry as a soil amendment and as a feed additive.

Table 2.4: Properties of zeolite (natural & synthetic)

Zeolite	Si/Al Range	Structure	Application
Mordenite	4.4-5.5	Orthorhombic blocky crystal system with 6.0-7.0 a pore size, crystal shape tends to be equate, 'kidney-like' in shape	NH_3 and CO_2 adsorbent, mineral specimen, hydro-isomerization catalyst, chemical filter
Ferrierite	3.2-6.2	Elliptical 10-ring channels of dimension 5.4 x 4.2 Å and 8-ring channels (4.7 x 3.4 Å) parallel to the c-axis	Benzene and CO sorption, ionexchangers, hydrocracking catalyst
Clinoptilolite	2.7-5.3	Sheet like structure. Sheet contains open rings of 8 to 10 sides. The rings stack together to form channels	Gas adsorber, molecular sieve, food additive, odor control agent, and catalyst
Zeolite Y	1.5-3.0	Faujastic structure, three dimension pore structure formed by 12-member oxygen rings, large cavity of 13 Å and surrounded by 10 sodalite cages.	Petroleum cracking catalyst, hydrophobic molecules adsorbent, NOx reduction and gas separation
Beta	5.0-100	Tetra crystal structure with straight 12-memberd rings channels (7.6 x 6.4 Å) with crossed 10-membered ring channel (5.5 x 6.5 Å)	Aromatic catalyst, hydrocarbon absorbent, isomerization of waxes, NOx reduction
ZSM-5	10-50	Zig-zag pattern interesting two-dimension pore structure formed by 10-membered oxygen rings.	Hydrocarbon conversion catayst

2.2.3 Uses of zeolite

Zeolites are widely used as ion-exchange beds in domestic and commercial water purification, softening, and other applications. In chemistry, zeolites are used to separate molecules (only molecules of certain sizes and shapes can pass through), as traps for molecules so they can be analyzed. Zeolites have the potential of providing precise and specific separation of gases including the removal of H_2O, CO_2 and SO_2 from low-grade natural gas streams. Other separations include: noble gases, N_2, O_2, freon and formaldehyde.

However at present, the true potential to improve the handling of such gases in this manner remains unknown. Zeolite-based oxygen concentrator systems are widely used to produce medical grade oxygen. The zeolite is used as a molecular sieve to create purified oxygen from air using its ability to trap impurities, in a process involving the absorption of undesired gases and other atmospheric components, leaving highly purified oxygen and up to 5% argon. QuikClot® brand hemostatic agent, which continues to be used successfully to save lives by stopping severe bleeding, contains a calcium loaded form of zeolite. Synthetic zeolites are widely used as catalysts in the petrochemical industry, for instance in fluid catalytic craking and hydro-cracking.

Zeolites confine molecules in small spaces, which cause changes in their structure and reactivity. The hydrogen form of zeolites (prepared by ion-exchange) is powerful solid-state acids, and can facilitate a host of acid-catalyzed reactions, such as isomerisation, alkylatin, and cracking. The specific activation modality of most zeolitic catalysts used in petrochemical applications involves quantum-chemical Lewis acid site reactions.

Zeolites are marketed by pet stores for use as a filter additive in aquariums. In aquariums, zeolites can be used to absorb ammonia and other nitrogenous compounds. However, due to the high affinity of some zeolites for calcium, they may be less effective in hard water and may deplete calcium. Zeolite filtration is

used in some marine aquaria to keep nutrient concentrations low for the benefit of corals adapted to nutrient-depleted waters. Where and how the zeolite was formed is an important consideration for aquariums. Northern hemisphere natural zeolites were formed when molten lava came in contact with sea water, thereby 'loading' the zeolite with Na (sodium) sacrificial ions. These sodium ions will spec ate with other ions in solution, thus the take-up of nitrogen in ammonia, with the release of the sodium. In southern hemisphere zeolites, such as found in Australia, which were formed with fresh water, thus the calcium uptake on formation. Zeolite is an effective ammonia filter, but must be used with some care, especially with delicate tropical corals that are sensitive to water chemistry and temperature.

2.3 Metal Ion Affinity Chromatography (IMAC) Adsorbents

Immobilized Metal Ion Affinity Chromatography (IMAC) was introduced by Porath and coworkers in 1975 under the name of Metal Chelate Affinity Chromatography. In this short publication, the authors described the use of immobilized zinc and copper metal ions for the fractionation of proteins from human serum. IMAC is regarded as a special case of Ligand Exchange Chromatography (LEC), which was introduced by Helfferich in 1961 for the separation of small molecules. LEC has been reviewed by Davankov and Semechkin.

In their first paper, Porath and coworkers introduced the means to fractionate proteins on solid supports based on their differential affinity towards immobilized metal ions. This paper, as well as a significant number of papers that followed, was devoted to the development of this method as a group-specific affinity separation principle. **(**Chaga et al, 2001)

2.3.1 Principle of IMAC

Immobilized metal ion affinity chromatography (IMAC) represents a relatively new separation technique that is primarily appropriate for the purification of bioproducts. For proteins purifications, IMAC utilizes the differential affinity of proteins for immobilized metal ions to effect their separation. This differential affinity derives from the coordination bonds formed between metal ions and certain amino acid side chains exposed on the surface of the protein molecules. Since the interaction between the immobilized metal ions and the side chains of amino acids has a readily reversible character, it can be utilized for adsorption and then be disrupted using mild (i.e., non-denaturing) conditions (Chaga, 2001)

IMAC was based on the formation of weak coordination bonds between metal ions immobilized on a chromatographic support and the exposed residues of target proteins, mainly His residues. The additional His tag confers the fusion protein specific binding strength to divalent metal ions immobilized on the adsorption column. Being of small size and uncharged, the His tag tail does not generally interfere with the secretion or folding of expressed foreign proteins.

The most commonly used metal ions in IMAC are Ni2+, Zn2+, Cu2+, Co2+ and Ca2+, which are classified as transition metal ions. These metal ions are chelated to a multidentate ligand immobilized onto a support material. The most commonly used ultidentate ligand is iminodiacetic acid (IDA) and nitrilotriacetic acid (NTA). Nickel ions have been chosen to be immobilized to the adsorbent of this study because nickel ions are most often used in IMAC and have the higher affinity to the His residues of target proteins.

Following the interaction between proteins and metal ions, the target protein can be eluted by three methods: by lowering the pH of the system, addition of competing agents such as imidazole or histidine or by the addition of a strongly chelating agent such as ethylenediaminetetraacetic acid (EDTA). Nevertheless, the use of EDTA can lead to the contamination of eluted protein fractions because

EDTA tends to strip the metal ions off the column. Therefore, addition of competing agents such as imidazole was preferred.

Furthermore, imidazole is a common and inexpensive chemical, which has little or no complexity on the further purification steps. The advantages of IMAC include the stability of the metal chelates over a wide range of solvent conditions and temperatures, the high metal loadings that result in high protein loading capacities and the ease of product elution and ligand regeneration. (B.T. Tey et al., 2005)

2.3.2 Flexibility of IMAC

Since IMAC is applicable under a wide range of conditions, one would expect it to be incorporated in a significant number of purification procedures, either as a capture step and/or as a final polishing step in the purification of wild-type and recombinant proteins. The wide range of conditions will be discussed in more detail below, but one could point out here that IMAC, with intermediate and hard metal ions, covers almost completely the biologically relevant pH range (i.e., pH 4–10).

Furthermore, IMAC can be utilized in the presence or absence of chaotropic salts as well as a number of structure-forming reagents. It is compatible with strong denaturing reagents, such as urea and guanidinium–HCl, as well as a large number of non-ionic detergents, making it extremely useful in the initial steps of purification immediately after the extraction/isolation procedure.

IMAC has also found application in the purification of DNA and oligonucleotide derivatives tagged with histidine residues. The major application so far is in the enrichment of phosphorylated proteins and peptides by immobilized Fe^{3+} and Ga^{3+}, but there is no doubt that is use will broaden to includes intermediate metal ions.

2.3.3 IMAC Adsorbents

IMAC adsorbents can also be utilized as ion exchangers. While this feature was undesired during the initial development of the technique, it can deliver additional selectivity or capacity for solving particular separation problems. For example, modulation of binding/elution of proteins to intermediate and hard metal ions can be obtained by simultaneous change of salt and pH (unpublished data). IMAC adsorbents can also be used for negative adsorption as exemplified by the purification of horseradish peroxidase on a tandem column of Cu^{2+}-IDA and thiophilic adsorbent. The broad range of conditions under which IMAC can be carried out have also proved useful in the isolation of proteins from organisms that live in extreme natural environments. For example, immobilized Fe^{3+} ions have been used at high salt concentrations (1.5–3 M NaCl) for the purification of proteins from halophilic microorganisms.

Such a broad environmental compatibility enables the incorporation of IMAC steps before or after any other adsorption step or Size Exclusion Chromatography (SEC), or, for that matter, even as a part of such a step. Porath et al. were among the first to suggest the use of IMAC columns in such cascade-mode multi-affinity chromatography experiments (CASMAC).

IMAC adsorbents have a number of advantages including high ligand stability, high protein throughput and low cost (Gaberc-Porekar, 2001). They can be easily sanitized and regenerated, making them suitable for large scale applications. Another distinct advantage of IMAC is its applicability under denaturing conditions in the presence of strong denaturing agent, which is often necessary for solubilisation when recombinant proteins are expressed in the form of inclusion bodies.

2.4 Adsorption

Adsorption is a process that occurs when a gas or liquid solute accumulates on the surface of a solid or a liquid (adsorbent), forming a film of molecules or atoms (the adsorbate). It is different from absorption, in which a substance diffuses into a liquid or solid to form a solution. The term sorption encompasses both processes, while desorption is the reverse process.

2.4.1 Introduction

Adsorption is present in many natural physical, biological, and chemical systems, and is widely used in industrial applications such as activated charcoal, synthetic resins, and water purification. Adsorption, ion-exchange, and chromatography are sorption processes in which certain adsorbents are selectively transferred from the fluid phase to the surface of insoluble, rigid particles suspended in a vessel or packed in a column. Similar to surface tension, adsorption is a consequence of surface energy.

In a bulk material, all the bonding requirements (be they ionic covalent, or metallic) of the constituent atoms of the material are filled by other atoms in the material. However, atoms on the surface of the adsorbent are not wholly surrounded by other adsorbent atoms and therefore can attract adsorbents. The exact nature of the bonding depends on the details of the species involved, but the adsorption process is generally classified as physisorption (characteristic of weak van der Waal forces) or chemisorptions (characteristic of covalent bonding).

2.4.2 Adsorbent

Adsorbents are natural or synthetic materials of amorphous or microcrystalline structure. Those used on a large scale, in order of sales volume, are activated carbon, molecular sieves, silica gel, and activated alumina (Keller, 1983).

Ion exchange usually occurs throughout a polymeric solid, the solid being of gel-type, which dissolves some fluid-phase solvent, or truly porous. In ion exchange, ions of positive charge in some cases (cations) and negative charge in others (anions) from the fluid (usually an aqueous solution) replace dissimilar ions of the same charge initially in the solid. The ion exchanger contains permanently bound functional groups of opposite charge-type (or, in special cases, notably weak-base exchangers act as if they do). Cation-exchange resins generally contain bound sulfonic acid groups; less commonly, these groups are carboxylic, phosphonic, phosphinic, and so on. Anionic resins involve quaternary ammonium groups (strongly basic) or other amino groups (weakly basic).

Most ion exchangers in large-scale use are based on synthetic resins—either preformed and then chemically reacted, as for polystyrene, or formed from active monomers (olefinic acids, amines, or phenols). Natural zeolites were the first ion exchangers, and both natural and synthetic zeolites are in use today.

Ion exchange may be thought of as a reversible reaction involving chemically equivalent quantities. A common example for cation exchange is the familiar water-softening reaction

$$Ca^{++} + 2NaR \leftrightarrow CaR_2 + 2Na^{+}$$

Where *R* represents a stationary univalent anionic site in the polyelectrolyte network of the exchanger phase.

Table 2.5 classifies common adsorbents by structure type and water adsorption characteristics. Structured adsorbents take advantage of their crystalline structure (zeolites and silicalite) and/or their molecular sieving properties. The hydrophobic (nonpolar surface) or hydrophilic (polar surface) character may vary

depending on the competing adsorbate. A large number of zeolites have been identified, and these include both synthetic and naturally occurring (e.g., mordenite and chabazite) varieties.

Table 2.5: Classification of common adsorbents

	Amorphous	Structured
Hydrophobic	Activated carbon Polymers	Carbon molecular sieves Silicalite
Hydrophilic	Silica gel Activated alumina	Common zeolites: 3A (KA), 4A (NaA), 5A (CaA), 13X (NaX), Mordenite, Chabazite, etc.

The classifications in Table 2.5 are intended only as a rough guide. For example, a carbon molecular sieve is truly amorphous but has been manufactured to have certain structural, rate-selective properties. Similarly, the extent of hydrophobicity of an activated carbon will depend on its ash content and its level of surface oxidation. Zeolites are crystalline aluminosilicates. Zeolitic adsorbents have had their water of hydration removed by calcination to create a structure with well-defined openings into crystalline cages. The molecular sieving properties of zeolites are based on the size of these openings.

Two crystal types are common: type A (with openings formed by 4 sodalite cages) and type X or Y (with openings formed by 6 sodalite cages). Cations balancing charge and their locations determine the size of opening into a crystal unit cell. Nominal openings sizes for the most common synthetic zeolites are 0.3 nm for KA, 0.4 nm for NaA, 0.5 nm for CaA, and 1.0 nm for NaX. Further details, including effective molecular diameters, are widely available (Breck, 2001)

Commercial adsorbents that exhibit ultra porosity include activated carbons, activated clays, inorganic gels, such as silica gel and activated alumina, and the crystalline aluminosilicate zeolite. Activated carbons, activated alumina, and silica gel do not possess an ordered crystal structure and consequently their pores are nonuniform.

The distribution of the pore diameters within the adsorbent particles may vary widely from 20 to several thousand Angstroms, as is the case for some activated carbons. Hence, all molecular species, with the possible exception of high molecular weight polymers, may enter the pores. Zeolite molecular sieves, on the other hand, have pores of uniform size (3–10Å) which are uniquely determined by the unit structure of the crystal. These pores will completely exclude molecules that are larger than their diameter. J. W. McBain (1932) originated the term “molecular sieves” to define porous solid materials that exhibit the property of acting as sieves on a molecular scale.

Many adsorbents, particularly the amorphous adsorbents, are characterized by their pore size distribution. The distribution of small pores is usually determined by analysis, using one of several available methods, of a cryogenic nitrogen adsorption isotherm, although other probe molecules are also used. Russell and LeVan compare popular methods using a single nitrogen isotherm measured on activated carbon and provide numerous references. The distribution of large pores is usually determined by mercury porisimetry (Gregg, 2003).

Table 2.6 shows the IUPAC classification of pores by size. Micropores are small enough that a molecule is attracted to both of the opposing walls forming the pore. The potential energy functions for these walls superimpose to create a deep well, and strong adsorption results. Hysteresis is generally not observed. (However, water vapor adsorbed in the micropores of activated carbon shows a large hysteresis loop, and the desorption branch is sometimes used with the Kelvin equation to determine the pore size distribution.) Capillary condensation occurs in mesopores and a hysteresis loop is typically found. Macropores form important paths for

molecules to diffuse into a particle; for gas-phase adsorption, they do not fill with adsorbate until the gas phase becomes saturated.

Table 2.6: Classification of pore sizes

Type	Slit Width* (w)	Characteristic
Micropore	$w < 2$ nm	Superimposed wall potentials
Mesopore	2 nm $< w <$ 50 nm	Capillary condensation
Macropore	$w > 50$ nm	Effectively flat walled until $p \rightarrow Ps$

2.4.3 Adsorption process

In general, there are two classes in adsorption process. The first is the bulk separation and the other is the purification. In the adsorption process, molecules are concentrated on the surface of the activated carbon. Adsorption is caused by a type of Van der Waals Force which exists between molecules. The force acts in a similar way to gravitational forces between planets. These Sources are extremely short ranged and therefore sensitive to the distance between the carbon surface and the adsorbate molecule they are also additive, meaning the adsorption force is the sum of all interactions between all the atoms.

The short range and additive nature of these forces results in activated carbon having the strongest physical adsorption forces of any known material. In general, the absorbability of a compound increases with Increasing molecular weight, a higher number of functional groups such as double bonds or halogen compounds and increasing polarisability of the molecule. This is related to electron clouds of the molecule.

Adsorption from solution into a solid thus can occur as a result of one or both of two characteristic properties for a given solvent-adsorbent-adsorbate system. Adsorption affected by a number of parameter specific that is concentration,

molecular weight, molecular size, molecular structure, molecular polarity, steric form or congulation and the nature of background or competitive of equilibrium (Cheng, 2005).

The performance characteristics of adsorbents relate in large measure to their intraparticle properties. Surface area and the distribution of area with respect to pore size generally are primary determinants of adsorption capacity. The nature of the intraparticle surface area markedly affects the types of adsorption interactions that will be operative for an adsorbent, and is a major distinguishing factor between activated carbons and synthetic adsorbents.

Recently, there has been rapidly increasing interest in the use of functionalized, selective solid-phase adsorbents for treatment of aqueous multicomponent protein solutions for the sorptive removal of one or several members of a common class of protein. In this process, the solution is contacted with the adsorbent until the solid is virtually saturated with the adsorbate. The solid phase flushed free from unadsorbed solutes, and then treated with a desorbing solution which displaces the adsorbed species in concentrated form, and regenerates the adsorbents.

The solid phase may be functionalized in a number of ways: (1) attachment of ionogenic functions renders it an ion exchanger, which can differentiate between proteins of different isoelectric points; (2) attachments of generic affinity ligands such as triazine dyestuffs can render it selectively sorptive for certain classes of enzyme; (3) attachment of cell surface immunoproteins such as protein A can be render it selectively sorptive for classes of immunoglobin such as IgG; or (4) attachment of specific monoclonal antibodies can render it uniquely sorptive for one antigen protein (Ismail, 2005).

2.4.4 Adsorption theory

Adsorption at various interfaces has concerned scientists since the beginning of this century. This phenomenon underlies a number of extremely important processes of utilitarian significance. The technological, environmental and biological importance of adsorption can never be in doubt. Its practical applications in industry and environmental protection are of paramount importance. The adsorption of substrates is the first stage in many catalytic processes.

The methods for separation of mixtures on a laboratory and on an industrial scale are increasingly based on utilizing the change in concentration of components at the interface. Moreover, such vital problems as purification of water, sewages, air and soil are involved here too. On the other hand, many areas in which technological innovation has covered adsorption phenomena have been expanded more through art and craft than through science. A basic understanding of the scientific principles is far behind; in part because the study of interfaces requires extremely careful experimentation if meaningful and reproducible results are to be obtained. In recent years, however, considerable effort has been increasingly directed toward closing the gap between theory and practice.

Crucial progress in theoretical description of the adsorption has been achieved, mainly through the development of new theoretical approaches formulated on a molecular level, by means of computer simulation methods and owing to new techniques which examine surface layers or interfacial regions. Moreover, during the last 15 years new classes of solid adsorbents have been developed, such as activated carbon fibers and carbon molecular sieves, fullerenes and heterofullerenes, micro porous glasses and nonporous--both carbonaceous and inorganic--materials. Nanostructures solids are very popular in science and technology and have gained extreme interest due to their sorption, catalytic, magnetic, optical and thermal properties.

Although the development of adsorption up to the 1918s has been following rather a zigzags path, this arm of surface science is now generally considered to have become a well-defined branch of physical science representing an intrinsically interdisciplinary area between chemistry, physics, biology and engineering. This review presents in brief the history of adsorption and highlights the progress in theoretical description of the phenomenon under consideration. The paper deals with the above problems critically, showing the development of adsorption, presenting some of the latest important results and giving a source of up-to-date literature on it. Moreover, in this paper the most important aspects are overviewed referring to today's trends and visions in application of adsorption science in industry, environmental protection and in environmental analysis.

The relationship between development of adsorption theory and adsorption practice is pointed out. Current understanding and perspectives pertaining to applications of adsorption phenomena on laboratory and on industrial scale as well as environmental protection are discussed and illustrated by means of a few spectacular examples.

2.4.5 Adsorption theorem

There are basically two well established types of adsorption isotherm:

- the Langmuir adsorption isotherm
- the Freundlich adsorption isotherm

2.4.5.1 Langmuir equation

Langmuir (1918) was the first to propose a coherent theory of adsorption onto a flat surface based on a kinetic viewpoint, that is there is a continual process of

bombardment of molecules onto the surface and a corresponding evaporation (desorption) of molecules from the surface to maintain zero rate of accumulation at the surface at equilibrium.

The assumptions of the Langmuir model are:

1. Surface is homogeneous, that is adsorption energy is constant over all site.
2. Adsorption on surface is localized, that is adsorbed atoms or molecules are adsorbed at definite.
3. Each site can accommodate only one molecule or atom

The Langmuir theory is based on a kinetic principle, that is the rate of adsorption (which is the striking rate at the surface multiplied by a sticking coefficient, sometimes called the accommodation coefficient) is equal to the rate of desorption from the surface The Langmuir equation or Langmuir isotherm or Langmuir adsorption equation relates the coverage or adsorption of molecules on a solid surface to gas pressure or concentration of a medium above the solid surface at a fixed temperature. The equation was developed by Irving Langmuir in 1916. The equation is stated as:

$$\theta = \frac{\alpha \cdot P}{1 + \alpha \cdot P}$$

Θ or theta is the percentage coverage of the surface, P is the gas pressure or concentration, α alpha is a constant.

The constant α is the Langmuir adsorption constant and increases with an increase in the strength of adsorption and with a decrease in temperature.

It has been widely used to describe many real sorption processes. A basic assumption of the Langmuir theory is that sorption takes place at specific homogeneous sites. Theoretically, a saturation value is reached beyond which no further sorption can take place. The curve is represented by the expression:

$$q = \frac{q_0 KC}{1 + KC}$$

Where q is the amount adsorbed (mmol·g^{-1}), q_0 and K are related to monolayer adsorption capacity and energy of adsorption, respectively, and C is the equilibrium solution concentration of solute. In the analysis of the results, q_0 is assumed as the total number of binding sites, N_T. Example graph for Langmuir isotherm is shown in Figure 2.8.

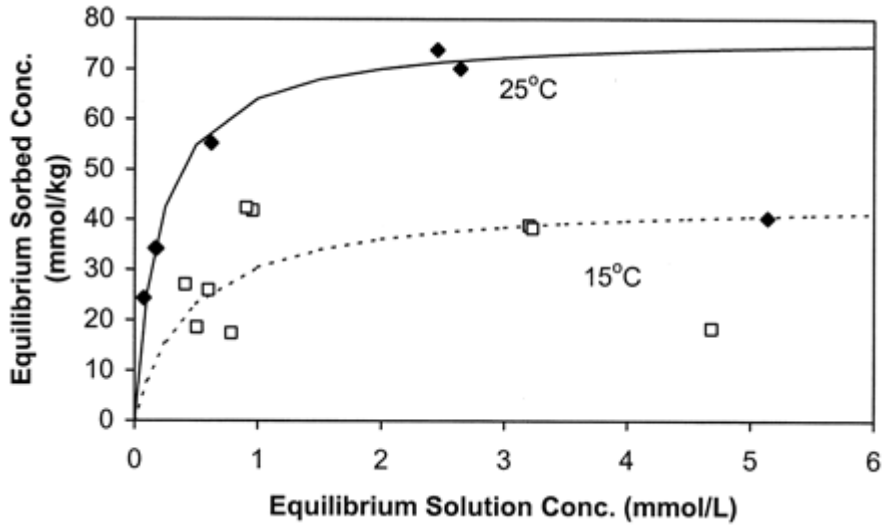


Figure 2.8: Example graph for Langmuir isotherm

2.4.5.2 Freundlich equation

The freundlich equation is one of the earliest empirical equations used to describe equilibria data. The name of this isotherm is due to the fact that is used extensively by Freundlich (1932) although it was used by many other researchers. This equation takes the following term:

$$C_\mu = KP^{1/n}$$

Where C_μ is the concentration of the adsorbed species, and K and n are generally temperature dependent. The parameter n is usually greater than unity. The larger is this value; the adsorption isotherm becomes more nonlinear as its behavior deviates further away from the linear isotherm.

The Freundlich Adsorption Isotherm is an adsorption isotherm, which a curve is relating the concentration of a solute on the surface of an adsorbent, to the concentration of the solute in the liquid with which it is in contact.

The Freundlich Adsorption Isotherm is mathematically expressed as

$$x/m = Kp^{1/n}$$

Or

$$x/m = Kc^{1/n}$$

Where

x = mass of adsorbate

m = mass of adsorbent

p = Equilibrium pressure of adsorbate

c = Equilibrium concentration of adsorbate in solution.

K and 1/n are constants for a given adsorbate and adsorbent at a particular temperature

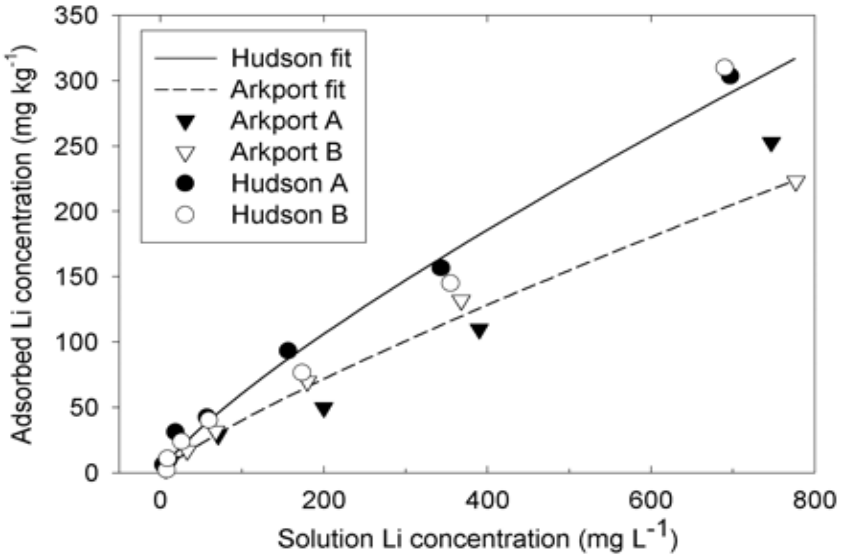


Figure 2.9: Example graph for Freundlich isotherm

The Langmuir adsorption isotherm describes quantitatively the buildup of a layer of molecules on an adsorbent surface as a function of the concentration of the adsorbed material in the liquid in which it is in contact. In a modified form it can also describe a bi-layer deposition. The shape of the isotherm (assuming the (x) axis represents the concentration of adsorbing material in the contacting liquid) is a gradual positive curve that flattens to a constant value. It often represents an initial surface adsorption followed by a condensation effect resulting from extremely strong solute-solute interaction. In chromatography the Freundlich isotherm is not common; most adsorption processes are best described by the Langmuir isotherm. Example graph for Freundlich isotherm is shown in Figure 2.9.

CHAPTER 3

METHODOLOGY

3.1. Material

3.1.1 General Chemical and Material

The deionized water that having water resistivity ~ 15-16 MΩcm is use to prepare rifampicin and buffer solution. The rifampicin solutions were mixed with buffered solution at the desired pH and used immediately to minimize the decomposition. Buffer solutions were prepared using phosphoric acid (H_3PO_4), potassium dihydrogen orthophosphate (KH_2PO_4), dipotassium hydrogen phosphate anhydrous (K_2HPO_4), potassium hydrogen carbonate ($KHCO_3$), and potassium carbonate anhydrous (K_2CO_3).

3.1.2Adsorbent

Zeolite was used as an adsorbent in this research. To study the effect of adsorbent on the adsorbent capacity, various zeolite were used, which are H-Beta, and Y.

H-beta zeolite was form in white powder and mostly odorless. As an high-silica zeolite (Si/Al from ca 10-100), it has large pore saiz (12 membered ring

apertures). Y zeolite was form in white and odorless powder. It is noncombustible but can cause respiratory, eye and skin irritation.

3.1.2.1 Adsorbents selection criteria

Guidelines for adsorbent selection are different for regenerative and nonregenerative systems. For a nonregenerative system, generally wants a high capacity and a strongly favorable isotherm for a purification and additionally high selectivity for a separation. For a regenerative system, high overall capacity and selectivity are again desired, but needs for cost-effective regeneration leading to a reasonable working capacity influence what is sought after in terms of isotherm shape.

For separations by pressure swing adsorption (or vacuum pressure swing adsorption), generally a linear to slightly favorable isotherm was needed (although purifications can operate economically with more strongly favorable isotherms). Temperature swing adsorption usually operates with moderately to strongly favorable isotherms, in part because one is typically dealing with heavier solutes and these are adsorbed fairly strongly (e.g., organic solvents on activated carbon and water vapor on zeolites). Exceptions exist, however; for example, water is absorbed on silica gel and activated alumina only moderately favorably, with some isotherms showing unfavorable sections. Equilibrium for ion exchange separations generally vary from moderately favorable to moderately unfavorable; depending on feed concentrations, the alternates often exist for the different steps of a regenerative cycle. Other factors in sorbent selection are mechanical and chemical stability, mass transfer characteristics, and cost.

Zeolite was be chosen as an absorbent because it has molecular sieves that potentially ideally suited for this type of separation as a number of them have pores of similar diameter to the kinetic diameters of the molecules to be separated. Zeolite are crystalline aluminosilicate inorganic materials with unique natural properties

such as high surface area, excellent thermal/hydrothermal stability, high shape selectivity and superior ion-exchange ability, which form the basis for their traditional application in catalysis and separation of small molecules. However, it is not obvious which zeolite in which particular cation-exchanged form would be successful in the separation of rifampicin.

3.1.3Metal selection

3.1.3.1Zirconium

Zirconium is a chemical element with the symbol Zr and atomic number 40. It is a lustrous, gray-white, strong transition metal that resembles titanium. Zirconium was first isolated in an impure form in 1824 by Jons Jakob Berzelius. Zirconium forms both inorganic and organometallic compounds such as zirconium dioxide and zirconocene dibromide, respectively. There are five naturally-occurring isotopes, three of which are stable. Short-term exposure to zirconium powder causes minor irritation, and inhalation of zirconium compounds can cause skin and lung granulomas.

In powder form, zirconium is highly flammable, but the solid form is far less prone to igniting. Zirconium is highly resistant to corrosion by alkalis, acids, salt water, and other agents. However, it will dissolve in hydrochloric and sulfuric acid, especially when fluorine is present. Alloys with zinc become magnetic below 35 K.The melting point of zirconium is at 1855°C, and the boiling point is at 4409°C. Zirconium has an electronegativity of 1.33 on the Pauling scale. Of the elements within d-block, zirconium has the fourth lowest electronegativity after yttrium, lutetium, and hafnium.

3.1.3.2 Ferum

Iron or ferum is a chemical element with the symbol Fe (Latin: *ferrum*) and atomic number 26. Ferum is a group 8 and period $ elements. Ferum is lustrous and silvery in color. It is soft, about 80 Brinell, relative to steel, which is about 140 Brinell. It is one of the few ferromagnetic elements.

Ferum is a metal extracted mainly from the iron ore hematic. It oxidizes readily in air and water to form Fe_2O_3 and is rarely found as a free element. In order to obtain elemental ferum, oxygen and other impurities must be removed by chemical reduction. The properties of ferum can be modified by alloying it with various other metals and some non-metals, notably carbon and silicon to form steels. Ferum (as Fe^{2+}, ferrous ion) is a necessary trace element used by almost all living organisms. The only exceptions are several organisms that live in iron-poor environments and have evolved to use different elements in their metabolic processes, such as manganese instead of iron for catalysis, or hemocyanin instead of hemoglobin. Ferum-containing enzymes, usually containing heme prosthetic groups, participate in catalysis of oxidation reactions in biology, and in transport of a number of soluble gases.

3.1.3.3 Nickel

Nickel is a chemical element, with the chemical symbol Ni and atomic number 28. It is a silvery-white lustrous metal with a slight golden tinge. It is one of the four ferromagnetic elements at about room temperature. Its use has been traced as far back as 3500 BC, but it was first isolated and classified as a chemical element in 1751 by Axel Fredrik Cronstedt.

Nickel is a silvery-white metal with a slight golden tinge that takes a high polish. It is one of only four elements that are magnetic at or near room temperature.

It belongs to the transition metals and is hard and ductile. It occurs most often in combination with sulfur and iron in pentlandite, with sulfur in millerite, with arsenic in the mineral nickeline, and with arsenic and sulfur in nickel galena. Nickel is commonly found in iron meteorites as the alloys kamacite and taenite.

Similar to the elements chromium, aluminium and titanium, nickel is a very reactive element, but is slow to react in air at normal temperatures and pressures. Due to its permanence in air and its slow rate of oxidation, it is used in coins, for plating metals such as iron and brass, for chemical apparatus, and in certain alloys such as German silver.

Nickel is chiefly valuable for the alloys it forms, especially many super alloys, and particularly stainless steel. Nickel is also a naturally magnetostrictive material, meaning that in the presence of a magnetic field, the material undergoes a small change in length. In the case of Nickel, this change in length is negative (contraction of the material), which is known as negative magnetostriction.

3.1.4 Rifampicin

Rifampicin (Figure 3.1) is a bacterial antibiotic drug of the rifamycin group. It is a semisynthetic compound derived from *Amycolatopsis rifamycinica* (formerly known as *Amycolatopsis mediterranei* and *Streptomyces mediterranei*). It has chemical structure C43H58N4O12 and a molecular weight of 823g/mol. The melting point is around 183^{O}C-188^{O}C. It also has two pKa values which is pKa 1.7 (anion forming) and pKa 7.9 (cation forming). Rifampicin appears in a form of red-orange platelet and basically odorless. It is soluble in chloroform and methanol but slightly soluble in water, acetone, ethanol (96%) and ether. There are various types of rifampicin from which this is derived, but this particular form, with a 4-methyl-1-piperazinaminyl group, is by far the most clinically effective.

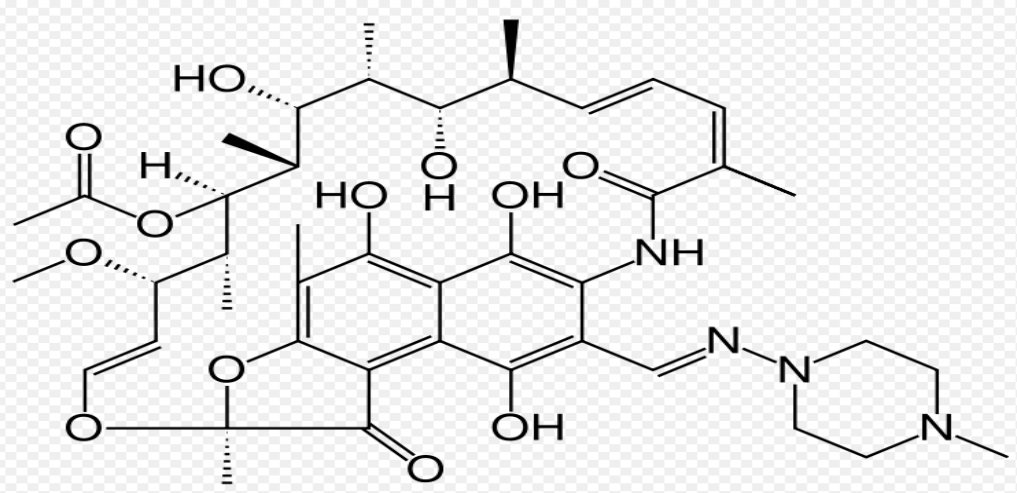


Figure 3.1: Structure of rifampicin

3.2 Preparation of Immobilized Metal Ion Affinity

Before used, H-Beta zeolite was calcined at 773.15K for 3 hours. Powdered metal oxide (Nickel, Ferum, and Zirconium) was mixed with zeolite with ratio 1: 9. Which mean 10% metal oxide with 90% zeolite. The mixture was mixed by hand using a mortar and pestle for about 10 minutes until it appeared to be uniformly mixed. The mixture was then calcined at 873.15K for 24 hours. Repeat this process with Y zeolite.

3.3 Solution Preparation

3.3.1Antibiotic solution

Rifampicin was dissolves in deionized water to get concentration 0.1 mM rifampicin solution. Other rifampicin solution was prepared by using dilution method to get concentration 0.8 mM, 0.6mM and 0.4 mM.

3.3.2 Buffer preparation

Five types of buffer solution was used for these experiments, which are Phosphoric acid (H_3PO_4), Potassium dihydrogen phosphate(KH_2PO_4), Potassium hydrogen phosphate (K_2HPO_4), Potassium hydrogen carbonate ($KHCO_3$), and Potassium carbonate (K_2CO_3).Phosphoric acid with phosphate (H_3PO_4, KH_2PO_4) was used for pH 3.0-4.0. Phosphates (KH_2PO_4, K_2HPO_4) for pH 4.0-8.0 and carbonates ($KHCO_3$, K_2CO_3) for pH 8.0-10.0.Preparation of buffer can be refer to Appendix A.

3.4 Experimental Procedures

All the experiment was conducted in room temperature.10 mg adsorbent was put in the test tube that containing 2ml of antibiotic solution and 1ml buffer solution at different pH. Sample was continuously shaken at room temperature for 20 minutes. Then sample was centrifuges for 10 minutes at 2000 rpm. After centrifuges, a sample supernatant was withdrawn and the rifampicin sample concentration was analyzed using UV-VIS at wavelength 257 nm. Experiment was repeated at different pH and zeolite.

3.5 Adsorption Isotherm Analysis

The analysis of rifampicin adsorption on adsorbent was interpreted from Langmuir isotherm shown in equation 3.2

$$q = \frac{qmC}{C+kd} \tag{3.2}$$

Where q is the amount of adsorbent at equilibrium, C is the equilibrium concentration of rifampicin, q_m and k_d are Langmuir constant related to the monolayer adsorption capacity energy of adsorption respectively.

CHAPTER 4

RESULTS AND DISCUSSIONS

4.1 Introduction

Purification of rifampicin was study by using separation method. The adsorption behavior of the separation process can be determined by using adsorption process. There are many parameters that can influence adsorption process such as electrostatic interaction, pH of solution, types of adsorbents, and antibiotic concentration. Types of absorbents, pH of solution, various types of metals and rifampicin concentration are the parameters that included in these studies. The Langmuir equation was used to investigated and examined of the adsorption isotherm data.

Firstly, the effect of equilibrium pH on the adsorption of rifampicin was examined. Then, the effect of various types of metal adsorbents was investigated. Finally the adsorption isotherm data was analyses using The Langmuir equation.

4.2 Effect of pH

Adsorption of rifampicin at various pHs was performed to study the effect of pH on adsorption capacity. From the literature, the highest adsorption occurs at pH 8. Figure 4.1 shows the effect of solution pHs on the adsorption of rifampicin onto six

different metal adsorbents which are Ni-Hbeta, Zr-Hbeta, Fe-Hbeta, Ni-Y, Zr-Y and Fe-Y.

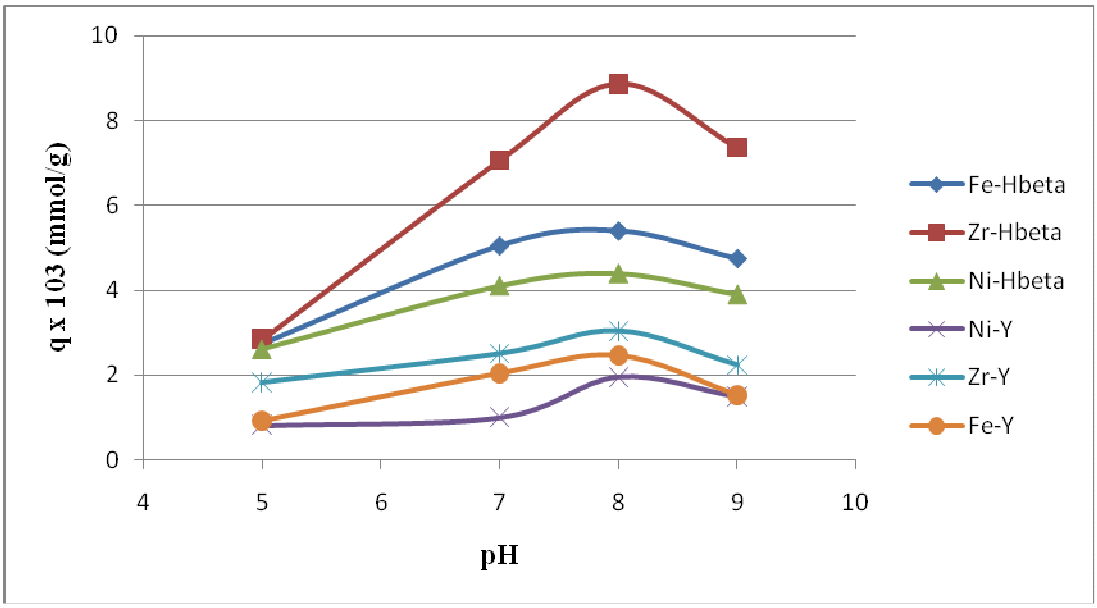


Figure 4.1: Effect of pH on the adsorption of rifampicin

From Figure 4.1, the adsorption capacity for rifampicin is highest at pH 8 at all six different adsorbents. The amount of adsorption capacity is increasing start from pH 5 to pH 8 and start decrease at pH 9.

The highest adsorption for rifampicin was found when Zr-Hbeta is used as adsorbent at pH 8 while the lowest adsorption was found at pH 5 with Ni-Y as an adsorbent. Zr-Hbeta has highest adsorption capacity in each pHs followed by Fe-Hbeta, Ni-Hbeta, Zr-Y, Fe-Y and Ni-Y.

The amount of rifampicin adsorbed on Zr-Hbeta at pH 8 is 0.0089 mmol/g and at pH 5 is 0.0028 mmol/g. While at pH 5 for Ni-Y, the lowest amount of rifampicin adsorbed is 0.00081 mmol/g and at pH 8 the value of rifampicin can adsorbed is 0.0019 mmol/g.

Rifampicin has two pKa values which are pKa_1 1.7 related to 4-hidroxy and pKa_2 7.9 that related to 3-piperizine nitrogen. So, the highest adsorption is pH 8, which is closer to the pKa_2 value.

4.3 Effect of Adsorbent

For this study, two adsorbents will be examine in batch adsorption experiments in order to get the best effect of metal adsorbent for rifampicin adsorption process. Result clearly shown that HBeta give best adsorption capacity compare to Y zeolite.

Figure 4.2 shown the comparison between Hbeta and Y zeolite at various pHs (pH 5, pH 7, pH 8 and pH 9) and concentration of rifampicin solution is 0.1 mM.

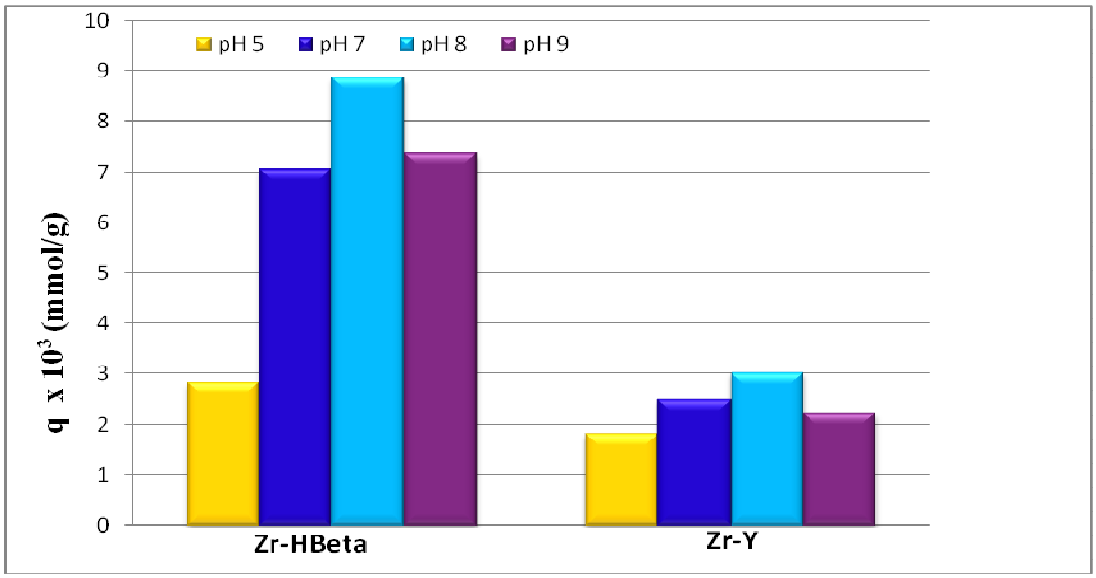


Figure 4.2: Effect of adsorbents on the adsorption to the zirconium ion.

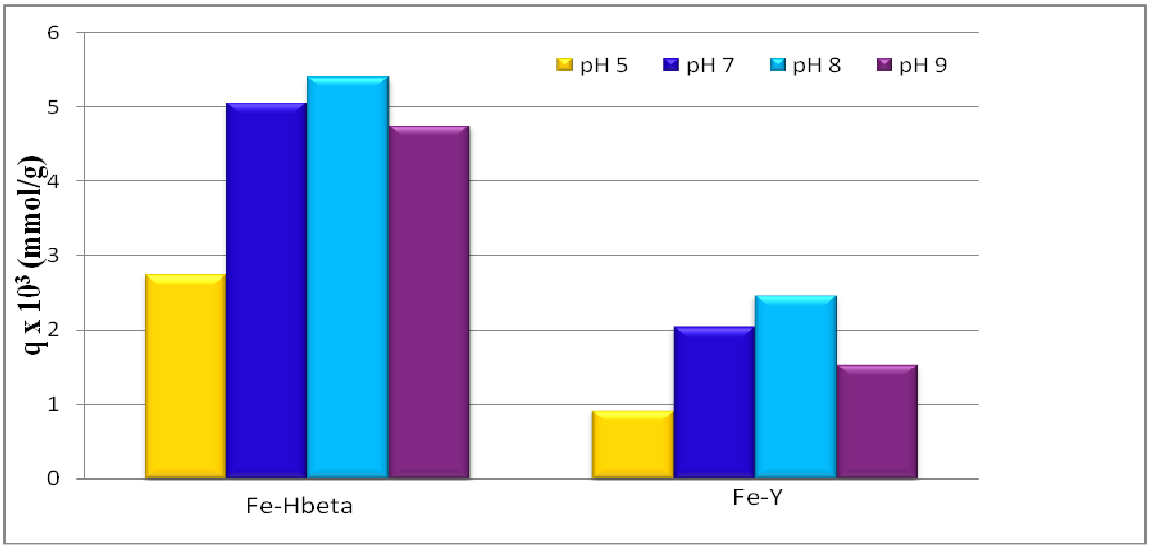


Figure 4.3: Effect of adsorbents on the adsorption to the ferum ion.

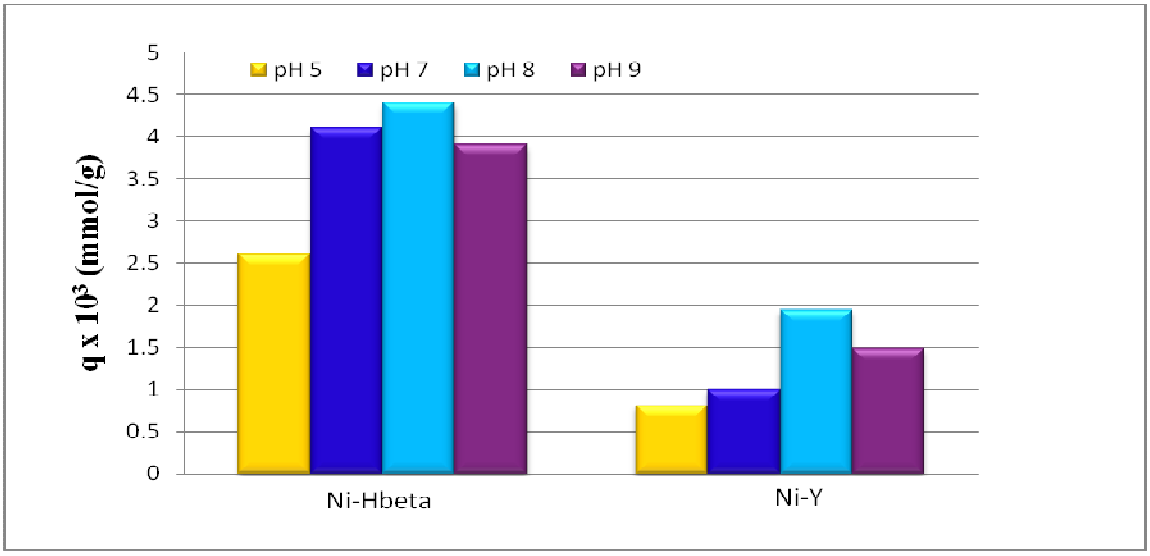


Figure 4.4: Effect of adsorbent on the adsorption to the nickel ion

Figure 4.2 shows that for metal zirconium, Hbeta give the highest adsorption for the rifampicin at pH 8 which is 0.008 mmol/g. While the amount of adsorption for Y zeolite is 0.003 mmol/g. This figure also shows that Hbeta has higher adsorption capacity than Y at all pHs.

Figure 4.3 and 4.4 shows the similar result for the ferum and nickel metal. Hbeta recorded 0.0054 mmol/g for ferum and 0.0044 mmol/g for nickel of rifampicin adsorption at pH 8 compare to Y at pH 8 just 0.0025 mmol/g and 0.0019 mmol/g for both metal.

Physical characteristic of adsorbents such as diameter size, surface area and pore volume are the several factors that can effluences of the rifampicin adsorption process. The diameter size of rifampicin should be smaller than diameter size of adsorbents. This is to enable rifampicin to go through into micropore of the adsorbent. The surface area and pore volume also important for the adsorption of the rifampicin. Increasing the surface area and pore volume will give better chances of rifampicin to adsorb into adsorbent.Highest capacity of HBeta zeolite can be explained by the higher diameter size, surface area and pore volume compare to Y zeolite.

4.4 Effect of Various Metal Ions

In order to study the effect of various metal ions in rifampicin adsorption, three different types of metals will be examine. Zirconium, ferum and nickel will be immobilized into zeolite (Hbeta and Y) using the solid state ion exchange method. Figure 4.3 illustrate the comparison of adsorption of rifampicin using different metal at pH 5, pH 7, pH 8 and pH 9. The result shown zirconium give highest adsorption compare with ferum and nickel.

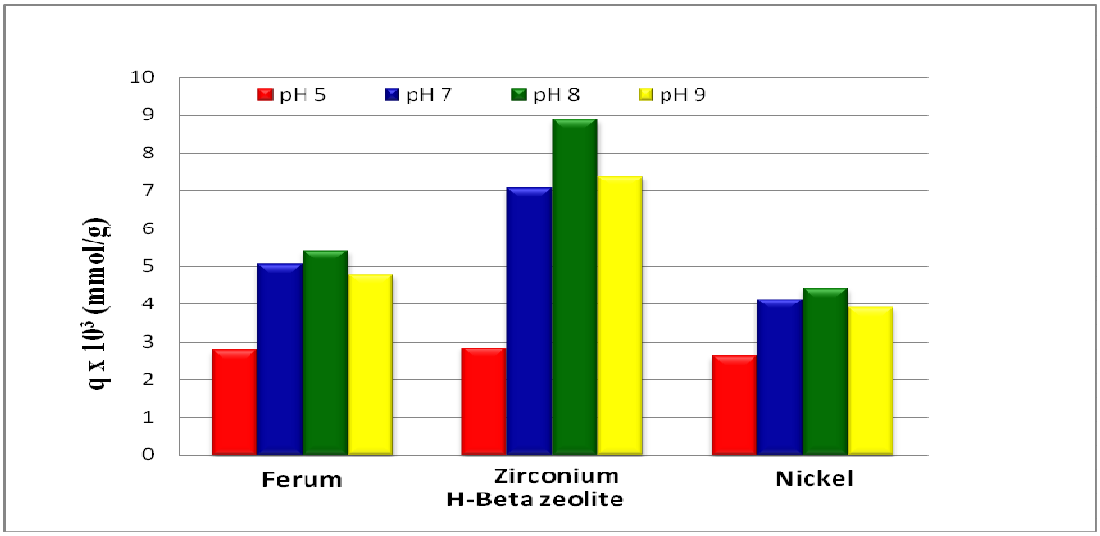


Figure 4.5: Effect of various metals on the adsorption onto Hbeta zeolite.

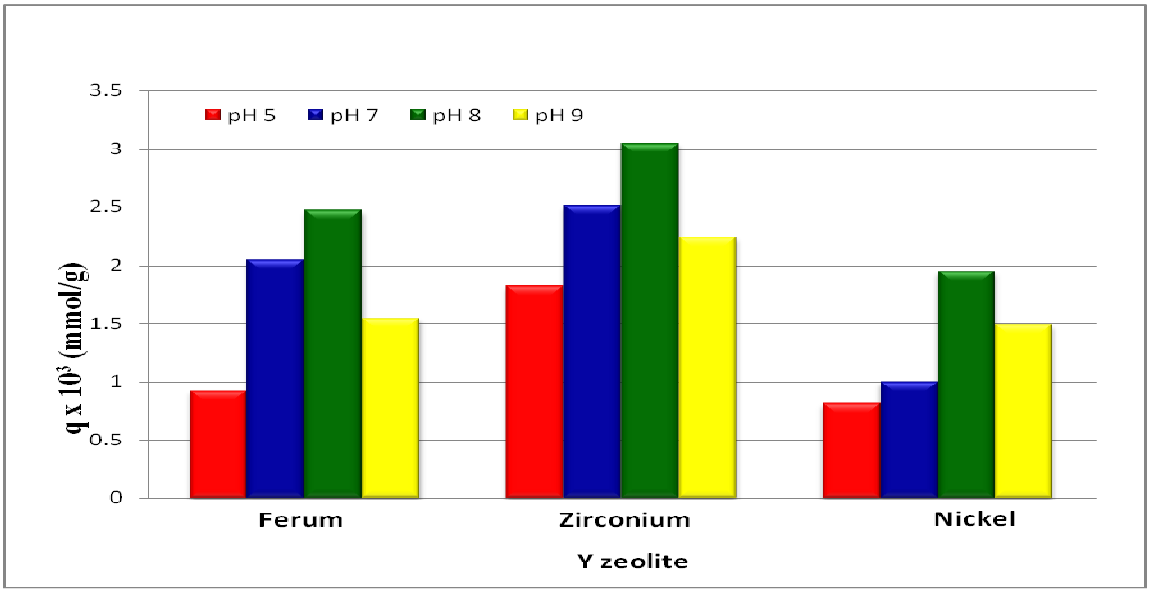


Figure 4.6: Effect of various metals on the adsorption onto Y zeolite.

Figure 4.5 and 4.6 shows even using different zeolite, zirconium still has highest adsorption capacity of the rifampicin. In Hbeta, adsorption capacity of rifampicin at pH 5, pH 7, pH 8 and pH 9 was recorded 0.0028mmol/g, 0.0071mmol/g, 0.0089mmol/g, and 0.0074mmol/g respectively. While rifampicin adsorption capacity in Y founded at pH 5 was 0.0018mmol/g, pH 7 was 0.0025mmol/g, pH 8 was 0.0030mmol/g and pH 9 was 0.0022mmol/g.

All these metals are transition metal but zirconium is the only transition metal containing both acidic and basic surface sites. So this is a major factor that why zirconium gives better adsorption capacity of rifampicin compare with ferum and nickel.

4.5 Adsorption Isotherm

The Langmuir model is used to examine the isotherm adsorption data. A linearized form of the Langmuir equation was used to find the dissociation coefficient. k_d and maximum rifampicin binding capacity, q_m as well as the

correlation coefficient. It is found that the Langmuir type expression fits the equilibrium isotherm data.

4.5.1 Effect of pH

The adsorption isotherms of rifampicin at various pHs are shows in Figure 4.7. The adsorption capacity of rifampicin is significantly high at pH 8 and the lowest at pH 5. The adsorption capacity shows a sharp initial rise and become constant with increasing the molar concentration.

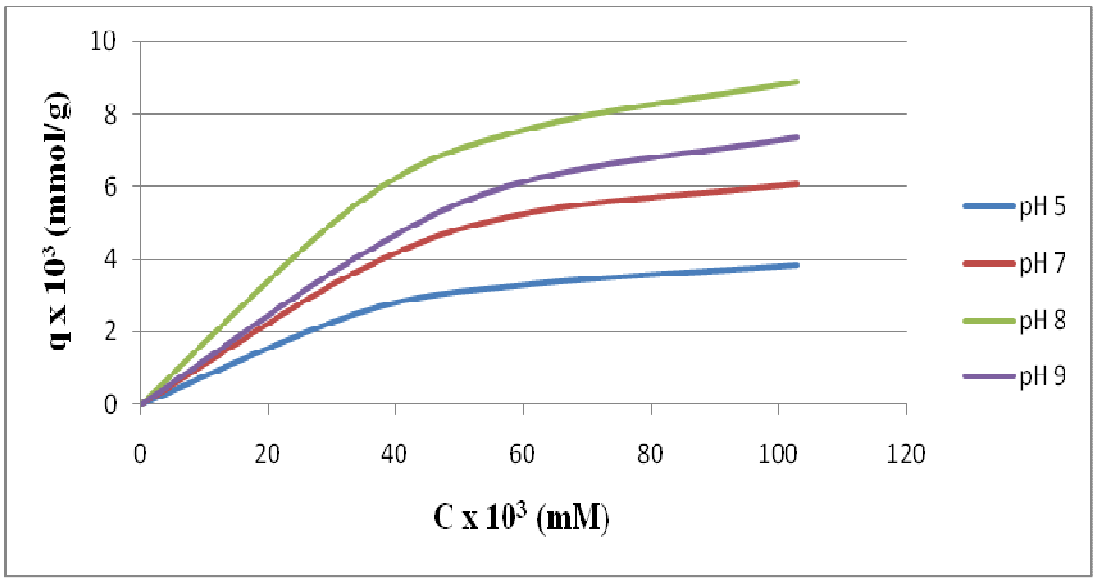


Figure 4.7: Effect of pH on adsorption isotherm of rifampicin onto Zr-Heta zeolite.

Data's was collected from Fe-Hbeta zeolite. The calculated data using Langmuir equation is obtained from Table 4.1

Table 4.1: Effect of pH on Langmuir constant for rifampicin

pH	q_m	K_d	R^2
5	3.5051	37.4990	0.8772
7	8.1699	18.2717	0.9799
8	4.1425	53.8870	0.9849
9	6.3735	34.7124	0.9951

The value of regression analysis should be to be greater than 0.99. This will show a strong indication that a suitable model has been chosen. When pH close to the pI value, the net charge of the rifampicin is low and the Coulombric repulsive force between the molecules are minimal.

4.5.2 Effect of adsorbent

From the Figure 4.8, it is clearly shown that immobilized zirconium metal ion with Hbeta zeolite give the highest adsorption capacity among the other modified zeolite. Figure also shown that adsorption isotherm of Y zeolite become constant is faster than Hbeta zeolite.

A sharp increase of the initial isotherm is seen for HBeta zeolite indicated that the favorable adsorbent for the adsorption is HBeta zeolite. A lower slope of Y zeolite shows that the materials have a lower affinity for rifampicin.

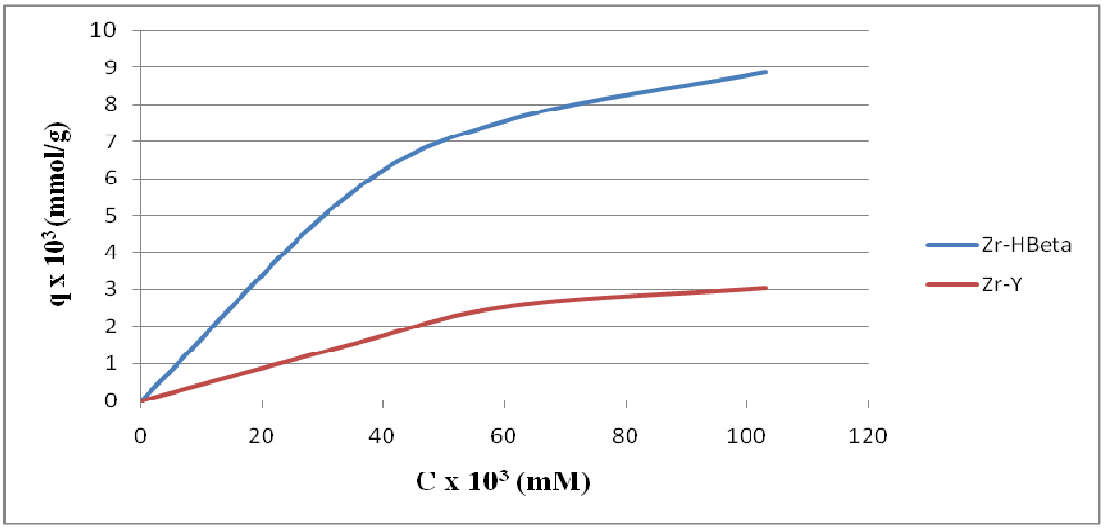


Figure 4.8: Effect of adsorbent on adsorption isotherm of rifampicin.

Table 4.2: Effect of adsorbent on Langmuir constant for rifampicin

System	q_m	K_d	R^2
Fe-Hbeta	3.5051	34.7122	0.8777
Fe-NaY	2.4582	181.197	0.6306

The adsorption isotherm for rifampicin onto several adsorbents is shown in Table 4.2. The effect of adsorbents on the adsorption is significant based on the isotherm for each adsorbent. Among the adsorbents study, Hbeta zeolite has the largest pore diameter size, making it possible for the rifampicin penetration to occur. While the pore diameter size of Y is smaller than the rifampicin. It will reduce the adsorption capacity of rifampicin. The adsorption might occur at surface of adsorbent only as the rifampicin cannot penetrate into the mesopore network.

4.5.3 Effect of various metal ions

Figure 4.9 is shown the effect of various metal ions on adsorption isotherm of rifampicin. The maximum adsorption is attained by Zr-HBeta. A sharp increase of initial isotherm is seen for Zr-HBeta zeolite.

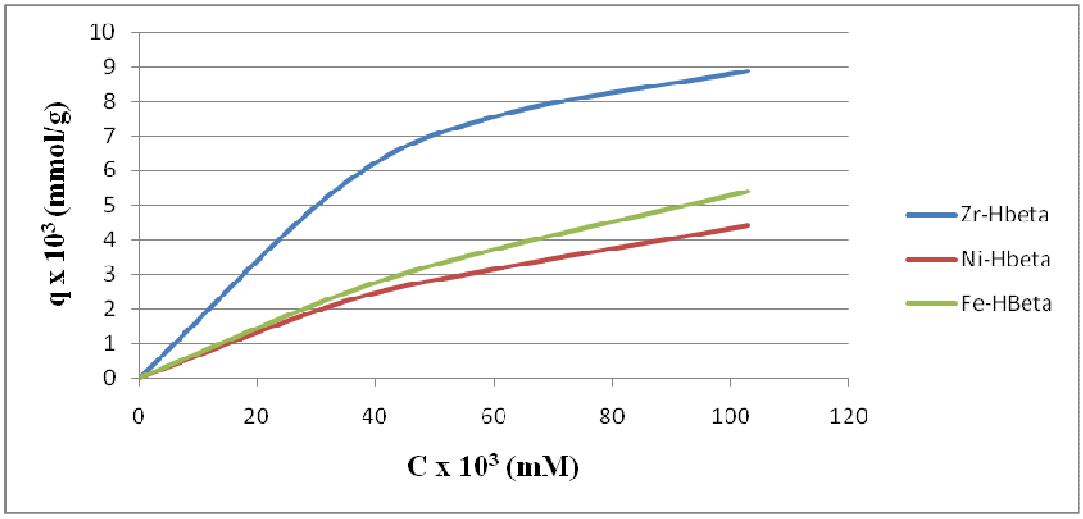


Figure 4.9: Effect of various metal ions on adsorption isotherm of rifampicin.

Table 4.3: Effect of various immobilized metal ion affinity adsorbents on Langmuir constant for rifampicin.

System	q_m	K_d	R^2
Ni-Hbeta	5.7142	37.4148	0.9544
Zr-Hbeta	9.5511	82.0220	0.9273
Fe-Hbeta	8.1699	53.8872	0.9799

The calculated Langmuir constants and regression values are given in Table 4.3. Rifampicin is highly adsorbed when Zr-Hbeta was used as an adsorbent. Higher values of equilibrium constant, q_m for Zr-Hbeta suggesting that the strength of the interaction with the molecular sieve is higher for this solute.

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 Conclusion

Antibiotic purification by using immobilized metal ion affinity (IMA) adsorbents has been developing to studies the effect on the rifampicin adsorption from aqueous solution in batch experiment. The parameters that that influence the adsorption process are pH, various types of adsorbents and various metal ions immobilized onto zeolites have been studies.

The maximum adsorption for rifampicin occurs at pH 8, which is near to the pKa_2 point of rifampicin. From the study, it clearly shows that the pH has a significant impact on the adsorption capacity.

This study also revealed that the adsorption capacity varies according to the types of adsorbent. The most effective zeolite that gives highest adsorption capacity was Hbeta. Among zirconium, ferum and nickel, zirconium possesses the suitable interaction with both the zeolite framework and the rifampicin, exhibited the best separation performance for rifampicin.

5.2 Recommendations

The adsorption characteristics of different antibiotic onto zeolite depend on part of experimental conditions used, antibiotic physical and chemical properties. The adsorption isotherm data of rifampicin is well fitted to the Langmuir model. FTIR analysis can be performed for both zeolite with and without adsorbed antibiotic to study the interaction between the antibiotic and zeolite surface.

It is recommended in further study other parameters such as other types of zeolite and metal, temperature ionic strength of buffer solution can be investigated for a better understanding on the adsorption process.

REFERENCES

A. Da Browski. 2001. Adsorption- from theory to practice. *Journel of Advance in Colloid and Interface Science 93: 135-224*

Adalgisa Tavolaro, PalmiraTavolaro and Enrica Drioli. 2007. Zeolite inorganic supports for BSA immobilization: Comparative study of several zeolite crystals and composite membrances. *Journal of Colloids and Surfaces B: Biointerfaces 55:67-76*

Barrer, R**.**M**.** (1938) The sorption of polar and nonpolar gases by zeolites. *Proceedings of the Royal Society of London, Series A, Mathematical and Physical Sciences*, **167,** 392–420.

Berdy, J. 1980. Recent advance in and prospects of antibiotic research. *Journal of Proc Boichem, 55:28-35*

Chase, H. A., and Clemmit, R. H. 2000. Immobilised metal affinity chromatography of β-galactosidase from unclarified *Escherichia coli* homogenates using expended bed adsorption. *Journal of Chromatography A, 874:27-43*

Cheng. L.L. 2005. *Adsorption characteristic of protein on silicaneous molecular sieve*. Universiti Teknologi Malaysia: B. Eng Thesis

Chunjuan Jia, Patricia beaunier, pascale Massiani. 1998. Comparison of conventional and solid-state ion exchange procedures for the incorporation of lanthanum in H-beta Zeolite. *Journal of Microporous and Mesoporous Materials 24: 69-82*

Daniela Todorova-Balvay, Olivier Pitiot, Mustapha Bourhim, Thamarapu Srikrishnan and mookambeswaran Vijayalakshmi. 2004. Immobilized metal-ion affinity chromatography of human antibodies and their proteolytic fragments. *Journal of Chromatography B 808: 57-62*

Duong D. Do. Adsorption Analysis: Equilibia and Kinetics. Imperial College Press. 1998

Gaberc-Porekar, V., and Menart V. 2001. Perspectives of immobilized-metal affinity chromatography. *Journal of Biochem, Biophys, Method., 49: 335-360*

Ganguly, A.K. 1978. Ansamycins. IN: Weinstein. M. J. and Wagman, G. H. Antibiotics: Isolation, separation and purification. *Journal of Chromatography Library, 15:39-68*

G. Kinger, A. Lugstein, R. Swagera, M. Ebel, A. Jentys and H. Vinek. 2000. Comparison of impregnation, liquid and solid-state ion exchange procedures for the incorporation of nickel in HMFI, HMOR and HBEA Activity and selectivity in *n*-nonane hydroconversion. *Journal of Microporous and Mesoporous Materials 39: 307-317*

GA Albarellos and M.F Landoni 2008. Current concepts on the use of antimicrobials in cats. *The veterinary Journel 80: 304-316*

Grigoriy S. Chaga. 2001. Twenty-five years of immobilized metal ion affinity chromatography: past, present and future. *Journal of Biochemical and Biophysical Methods 49: 313-334*

Hssao Oka, Ken-ichi Haradas, Yuko Itos, Yoichiro Ito. 1998. Separation of antibiotics by counter-current chromatography. *Journal of Chromatography A 812: 35-52*

Ismail, M. 2005. Adsorption characteristic of protein on zeolite adsorbent. Universiti Teknologi Malaysia: M. Eng thesis

Sadhana Sharmal and Gopal P.Agarwal. 2001. Interactions of proteins with immobilized metal ions-Role of ionic strength and pH. *Journal of Colloid and Interface Science 243: 61-72*

Shalini Joshi. 2002. HPLC separation of antibiotics present in formulated and unformulated samples. *Journal of Pharmaceutical and Biomedical Analysis 28: 795-809*

Thaer Abudiab, Robert R. Beitle Jr. 1998. Preparation of magnetic immobilized metal affinity separation media and its use in the isolation of proteins. *Journal of Chromatography A 795: 211-217*

Vesselina P. Mavrodinova. 1998. Solid-state ion exchange in beta zeolites. I alkaline chlorides/ NH_4-β. *Journal of Microporous and Mesoporous Materials 24: 1-8*

Vinu, A., Hartmann, M. 2004. Adsorption of Cytochrome C on MCM-41 and SBA-15: Influence of pH. *Proceeding of the 14th, International Conference on Zeolite: 2987-2994*

Vladka Gaberc-Porekar, Viktor Menart. 2001. Perspective of immobilized-metal affinity chromatography. *Journal of Biochemical and Biophysical Methods 49: 335-360*

Wang, D.J, Zhang, Y.H., Xu, F., Shan, W., Zhu, G.B., Yang, P.Y., and Tang, Y. 2004. The application of zeolite/FAC composites in protein separation. *Studies In Surface Science and Catalysis 154(2):2027-2033*

Zhang, C., Liu, Q., Xu, Z., and Wan, K., 2003. Synthesis and characterization of composite molecular sieve with mesoporous and microporous structure from ZSM-5 zeolite by heat treatment. *Journal of Micropores, Mesoporores,Material 62: 157-163*

APPENDICES

A.1 Preparation of antibiotic solution

Concentration of 0.1 mM rifampicin in 100 ml de-ionized water

Molecular weight of rifampicin = 823 g/mol

Rifampicin needed in gram to prepare the solution:

$$\text{Molarity} = \frac{\text{gram} - \text{moles solutes}}{\text{liter solution}}$$

$$0.1 \times 10^{-3}\ \text{mol} = \frac{\frac{\text{grams needede}}{0.1\ l}}{823\ \text{g/mol}}$$

Gram needed = 0.1 x 10-3 x 0.1 x 823

= 8.23 x 10-3

= 0.00823 g

To prepare the solution, 0.008 g rifampicin needed to disperse in 100 ml de-ionized water in 100 ml volumetric flask. Same procedure was applied for preparing other concentration of antibiotic in de-ionized water.

Dilution of Solution

Concentration 0f 0.08 mM rifampicin from 0.1 mM rifampicin

MaVa = MbVb

0.1 mM x Va = 0.08 mM x 100 ml

$$\text{Va} = \frac{0.08 \times 100}{0.1}$$

= 80 ml

To dilute 0.1 mM rifampicin into 0.08 mM rifampicin in 100 ml, 80 ml of 0.1 mM rifampicin is pipette and dilute with 20 ml deionized water in 100 ml volumetric flask. Same procedure was applied for other dilution of concentration

A.2 Preparation of Buffer Solution

Buffer (0.1M)	H_3PO_4	KH_2PO_4	K_2HPO_4	$KHCO_3$	K_2CO_3
JMR	98	136.09	174.18	100.12	138.21
Gram	0.6742 ml	1.3609	1.7418	1.0012	1.3821

Concentration of 0.1 M H_3PO_4 in 100 ml deionized water

Molecular weight of H_3PO_4 = 98 g/mol

H3PO4 = 85% wt

H2O = 15% wt

SG of H3PO4 = 1.71 g/ml

Grams in solution = molarity x liter solution

$= 0.1 \text{ mol/l} \times \frac{100 \text{ ml}}{1000 \text{ml/l}} \times 98 \text{ g/mol}$

= 0.98 g

volume needed $= \frac{\frac{\text{mass}}{\text{SG}}}{\text{purity}}$

$= \frac{\frac{0.98 \text{ g}}{1.71 \text{ g/ml}}}{0.85}$

= 0.6742 ml

To prepare this solution, 0.6742 ml H_3PO_4 needed to disperse in 100 ml de-ionized water in 100 ml volumetric flask

Concentration of 0.1 M KH2PO4 in 100 ml de-ionized water

Molecular weight of KH2PO4 = 136.09 g/mol

KH_2PO_4 needed in gram to prepare the solution

$$\text{Molarity} = \frac{Gram-moles\ solutes}{liter\ solution}$$

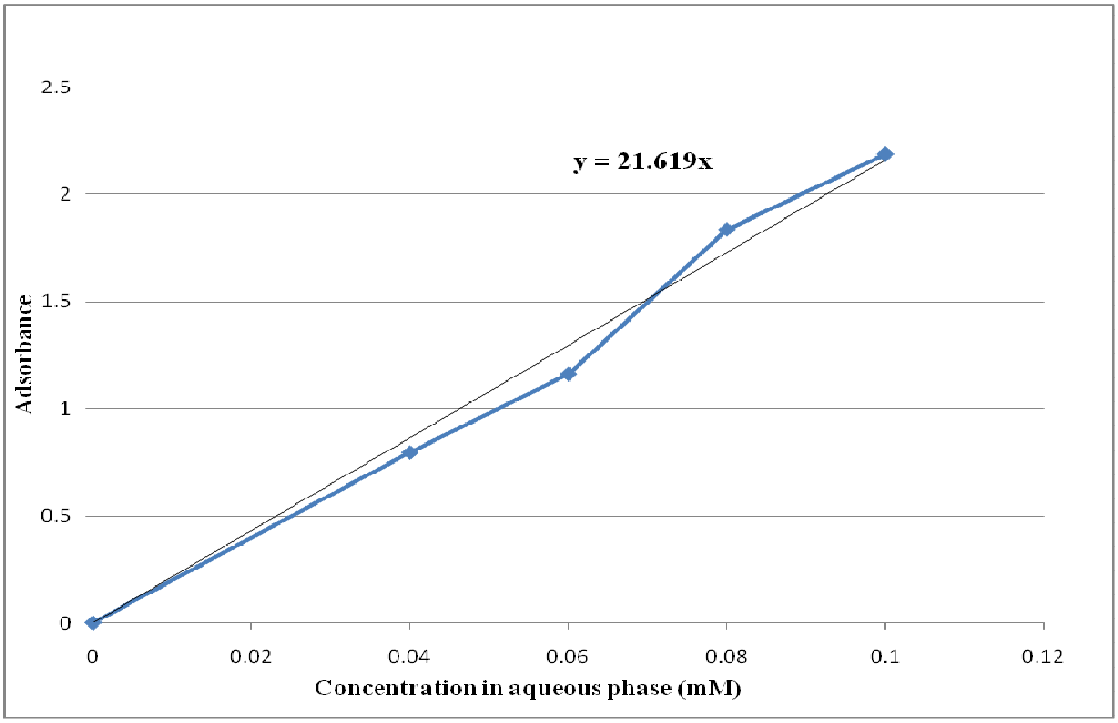
$$0.1 \text{ mol} = \frac{\frac{gram\ needed}{0.1\ l}}{136.09\ mol/g}$$

Gram needed = 0.1 x 0.1 x 136.09

= 1.3609 g

To prepare this solution, 1.3609 g KH_2PO_4 needed to disperse in 100 ml de-ionized water in 100 ml volumetric flask. Same procedure was applied for preparing other concentration of salt in de-ionized water

A.3 Calibration curve for rifampicin initial adsorbance



Experimental condition: pH 8, [Buffer] = 0.1 mM