Downstreaming Of Bioactive Peptides Using Nano Crystalline Molecular Sieves

A Thesis

submitted in the partial fulfillment of the requirement for the award of the degree of

MASTER OF SCIENCE

IN

MICROBIOLOGY

Under the guidance of

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CANDIDATE'S DECLARATION

I hereby declare that the work presented in this thesis entitled, **"Downstreaming bioactive peptides using nano crystalline molecular sieves"** in partial fulfillment in the requirement for the award of Degree of **Master of Science in Microbiology**, submitted in the **Department of Biotechnology, Thapar University, Patiala**, is an authentic record of my own work carried out under the supervision and guidance of **Dr. Moushumi Ghosh and Dr. sanghamitra Barman**, Thapar University, Patiala and refers other researcher's work which are duly listed in the reference section.

The matter embodied in this thesis has not formed the basis for the award of any other degree of this or any other university.

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This is to certify that the above statement made by the student concerned is correct and true to the best of my knowledge.

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CERTIFICATE

This is to certify that dissertation entitled, **"Downstreaming of Bioactive Peptides by using Nano Crystalline Molecular Sieves"** submitted by Mr, Manish Kumar Sharma in partial fulfilment of the requirements for the award of M.Sc in Microbiology at Thapar University, Patiala is an authentic work carried out by her under our supervision and guidance. To the best of our knowledge, the matter embodied in this dissertation has not been submitted to any other university/ institute for award of any Degree or Diploma.

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CONTENTS

ABSTRACT

The potential applications of bacteriocins as food preservatives and their ability to inhibit the growth of pathogens has drawn the attention of researchers around the world. Due to its importance in various industrial applications, an innovative approach for their purification is required. In the present study, ability of zeolite for bacteriocin recovery is exploited. For this, two types of zeolites i.e., zeolite A and Na-X were synthesized from coal fly ash as cheap raw material. Crystallanity and microporous structure of zeolite was confirmed by SEM & XRD analysis. These zeolites were used to study the recovery of bacteriocin from lactic acid bacteria, LAB 4 and *L. acidophilus* ATCC 43121. Zeolite Na-X showed maximum recovery efficiency of 98% for LAB 4 and 97 % for ATCC 43121 with in a time span of 60 and 90 minutes respectively while with zeolite A maximum recovery efficiency of 95% with LAB 4 and 91% for *L. acidophilus* was observed at 60 and 90 minutes respectively. The results considered the application of zeolite in bacteriocin recovery & purification irrespective of bacteriocin concentration.

Abbreviations

$Cacl_2$	Calcium chloride
CFA	Coal fly ash
et al.	Et alteri/et alii (and others)
NaOH	Sodium hydroxide
SiO_2	Silica oxide
MRS	De Man Regosa Sharpe
Al_2O_3	Aluminium Oxide
XRD	Xay Ray Diffraction
SEM	Scanning Electron Microscopy
O.D.	Optical Density

LIST OF SYMBOLS

%	Percentage
µl	Micro litre
µm	Micrometre (1×10^{-6}m)
^{0}C	degree(s) Celsius
G	Gram
Mg	Miligram
mg/ml	Milligram per millilitre
Min	Minutes
Ml	Millitre
Rpm	Revolutions per minute

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CHAPTER 1 INTRODUCTION

Bio active peptides are the specific fragments of the proteins which have a positive impact on body and they play important role in human health (Kitts and Weiler, 2003). They are synthesized in the cell in the form of large prepropeptides, which are then cleaved and modified to give active products. They play important role in physiological functions and are generally present in milk proteins but also found in plant and muscle protein.

Bacteriocins are bioactive peptides produced by variety of bacteria which inhibits the growth of similar or closely related bacteria (Beshkova and frengova, 2012). Though produced by a variety of bacterial species, antimicrobial peptides produced by Lactic Acid Bacteria (LAB) have gained a lot of interest and attention due to their potential applications in the food industries as food preservatives and their ability to for the biological control of organisms causing food borne diseases and spoilage (Cleveland *et al*, 2001). Various techniques have been employed by these sectors for downstreaming these peptides. Among the available methods chromatographic techniques such as ion exchange and affinity have been used but these results into significant loss of product and also the cost of purification by these techniques are very high and time consuming. The alternatative method is by specific and reversible binding by zeolites as adsorbants (Faisal, 2009).

Zeolites are nanocrystalline moleculer sieves of aluminosilicates having uniform and precise size. They have wide applications in environmental protection, medicines, agriculture, downstream processing etc. These are already been applied for the purification and downstream processing of protein and receptors. As the current purification methods are very sensitive and time consuming, so there is a need for alternative processes. Accordingly for the application of

zeolite in downstream processing of bacteriocin, various objectives were framed for present study.

Objectives:

- Synthesis of nano zeolite-X, A from Fly ash
- Characterization of synthesized nano zeolite
- Application of synthesized zeolite for purification of bacteriocins.

CHAPTER 2 LITERATURE REVIEW

Bioactive peptides positively impacts the different functions of the body (Kitts and Weiler, 2003). Most of the bioactive peptides are derived from milk and milk proteins. Some plant and animal proteins serve as potential sources of bioactive peptides. They exhibit activities like antimicrobial, antioxidative, antihypersensitivity, antithrombic and immunomodulatory which results in beneficial effects on health (FitzGerald and Meisel, 2003; Korhonen and Pihlanto, 2003; Shimizu, 2004). Lactic acid bacteria (LAB) are known to produce physiologically active peptides from a wide variety of food proteins during gastrointestinal digestion and food material fermentation. The activity of these peptides depends on the composition and sequence of the inherent amino acids. The active sequences varies in size from two to twenty amino acid residues and many peptides are known for their multifunctional properties (FitzGerald and Meisel, 2003). Bcteriocins are bioactive peptides showing antimicrobial activity, have been synthesized in response to bacterial infections and act as first chemical barriers. They have applications in food preservation, biomedical devices and food processing equipments. For the preservation of food, they can be used for creating antimicrobial packaging (Appendini and Hotchkiss, 2002). They plays important role for the maintainance of food quality and safety as they reduces bacterial growth on the surface of the food and increases their shelf life.

2.1 Bacteriocin

Bacteriocin are bioactive peptides produced by a variety of gram positive and gram negative microoraganisms during growth. They exhibits antimicrobial activity against the relative species or species with similar nutritive requirements. Antimicrobial peptides from Lactic acid bacteria have gained lots of attention in past decades as they are considered as GRAS (Generally

Recognized As Safe). They have a very low molecular weight and their degradation can be done easily by the use of proteolytic enzymes making them a lot safer for the consumption of human. The consumption of these peptides by humans has been continued for a long time as they are isolated from dairy and meat products. In human and animals, these bacteriocin producing lactic acid bacteria have been shown strengthening barrier function of the gut micro flora and promoting the improvement of immune system (Tome *et al*, 2008).

Bacteriocins or potential antimicrobial peptides from lactic acid bacteria are ribosomally synthesized and are cationic in nature and they contain less than 100 amino acid residues (Marcus *et al*, 1999; Jenssen *et al*, 2006). Most of them showed a narrow spectrum of antimicrobial activity i.e., they only affect the growth of only those species which are related phylogenetically to the producer strain (Caplice and Fitzgerald, 1999) while some are found to be showing much broader spectrum of antimicrobial activity and inhibits the growth of protozoa, yeast fungi, viruses and protozoa (Reddy *et al*, 2004). These peptides are cytotoxic and exhibit antimicrobial properties against sperm and tumour cells also.

Bacteriocins are secreted by many lactic acid bacteria which includes members of *Lactococcus*, *Lactobacillus*, *Carnobacterium*, *Enterococcus* and *Pediococcus* sp. Various type of bacteriocins from LAB have been isolated and characterized. Nisin, diplococcin, acidophilin, bulgarican, helveticins, lactacins, and plantaricins are the most important ones (Nettles and Barefoot, 1993) . Nisin produced by *L. lactis* species has been characterized extensively (Buchman *et al*, 1988; Liu and Hansen, 1990) and is more effective against spore forming gram positive bacteria (Delves-Broughton, 1990)

2.2 Production conditions of bacteriocin

The production of bacteriocins depends on various physiochemical factors such as pH, temperature and source of nutrients (Todorov and Dicks 2004). Bacteriocins have been produced in pH ranging from 5-10 . Agitation supposed to have no effect during the production and optimum temperature for bacteriocin production is averaged at 30°C.

2.3 Classification of bacteriocin

The categorization of bacteriocins is done on the basis of name of the producing strain, their mechanism of killing and also on the common resistance mechanisms. A large number of bacteriocins are only phenomenologically connected including gram positive bacteria, colicins, microcins and archaea bacteriocins. Bacteriocins are divided into four classes i.e., class I, class II, class III and class IV bacteriocins.

2.3.1 Class I Bacteriocins

Class I Bacteriocins includes small peptide inhibitors and antibiotics. They have generated lots of interest for their use as food preservatives in industries due to its resistant properties. The bacteriocins of this class have a very low molecular weight of less than 5 KDa. Lantibiotics are divided into two classes according to their chemical structure and their antimicrobial activities i.e., type A lantibiotics and type B lantibiotics. Type A lantibiotics are cationic peptides which are upto 34 residues in length. Examples of these class of lantibiotics are nisin, epidermin, Pep 5 and Lactocon 5. Among these, nisin is the most important and industrially utilized bacteriocin. Type B lantibiotics are low positive charge carrying globular peptides which are upto 19 residues in length. Examples of Type B are mersacidin, actagardin and cinnamycin.

2.3.2 Class II Bacteriocin

The bacteriocins of class II are most abundant among all classes and are small peptides of molecular weight less than 10 KDa. They are heat stable. They are further divided into 5 subclasses. Class IIa are the largest subgroup of this class and have strong antilisterial activity, and broad range of activity due to which they have huge potential applications in food preservation as well as medical applications (Heng *et al*, 2007). Class IIb are two peptide bacteriocins which require both peptides for their activity. Class IIc peptides are thiol activated peptides and they require a reduced cystiene for their activity. Class IId consists of single peptide bacteriocins and example of this group of bacteriocin is aureocin A53 which exhibit stability under highly acidic environment. Class IIe is most recently proposed class which consists of bacteriocin having 3 to 4 non pediocin like peptides and aureocin A70, a four peptide bacteriocin is the best example of these class which is highly active against *L. monocytogenes.*(Netz *et al*, 2001)

2.3.3 Class III Bacteriocin

These are large heat labile bacteriocin which have a molecular weight more than 10 KDa. They are further divided into 2 subclasses i.e., subclass IIIa or bacteriolysins and subclass IIIb. The bacteriocin of subclass IIIa consists of peptides which kills bacteria by degrading cell wall causing cell lysis. Subclass IIIb consist of peptides which instead of lysis, disrupts the membrane potential causing ATP efflux and kills the target cells.

2.3.4 Class IV Bacteriocin

These are complex bacteriocins which are composed of lipid and carbohydrate moieties (Klaenhammer, 1993).

Table 2.1 Classification of bacteriocins (Cotter *et al*, 2006)

Classification	Characteristics	Example
Class I Lanthionine-containing Bacteriocins or lantibiotics	Ribosomally produced peptides Includes both single- and two-peptide lantibiotics	Single-peptide: nisin, mersacidin, lacticin 481 two-peptide: lacticin 3147, cytolysin
Class II Non-lanthionine-containing bacteriocins	Low molecular weight, heat stable peptides. Formed exclusively by unmodified amino acids Heterogeneous class of small peptides; includes pediocin-like (subclass a bacteriocins), two-peptide (subclass b bacteriocins), cyclic (subclass c; formerly class V), non-pediocin single linear peptides (subclass d)	Class IIa: pediocin PA1, leucocin A class IIb: lactacin F; class IIc: enterocin AS48, reuterin 6; class IId: lactococcin A, divergicin A
Class III Bacteriolysins Non-bacteriocin lytic proteins.	Large, heat-labile proteins, often murein hydrolases	Lysostaphin, enterolysin A

2.4 Mode of bacteriocin action

The members of class Ia lantibiotics of lactic acid bacteriocins have been studied for their mode of action and among them the Nisin has generated maximum interest due to its industrial applications. Nisin exhibits dual mode of action. They prevent the synthesis of cell wall by binding to lipid II on surface of the cell wall. The insertion of peptide into phospholipid bilayer can lead to change the permeability of cell membrane drastically and thus may be resulted in cell death (Wiedemann *et al*. 2001). Nisin disrupts the proton motive force (PMF) and kills the

sensitive organisms (Abee *et al*, 1995; Chung and Hancock 2000; Kraaij *et al*, 1999; Ruhr and Sahl, 1985). Nisin due to their amphiphilic nature interacts with hydrophobic region and hydrophilic heads of plasma membrane causing an efflux of ions, solute and small particles and forces biosynthetic processes to stop (Sahl, 1998). Also they can initiate a membrane insertion and pore formation process by using lipid II as a docking molecule which can cause rapid cell death. Class Ib lantibiotics have different mode of action as they inhibits the activities of essential enzymes (McAuliffe *et al*, 2001).

Class II bacteriocins forms voltage free pores or channels in plasma membrane of sensitive cells by recognizing specific protein receptors (Hechard and Sahl, 2002). pediocin PA-1, lactococcin, and sakacin A and B exhibits this mode of action (Chikindas *et al*, 1993) . Class II bacteriocin with the help of their amphiphilic helical structure insert into target cell's membrane for deplorization and causing death.

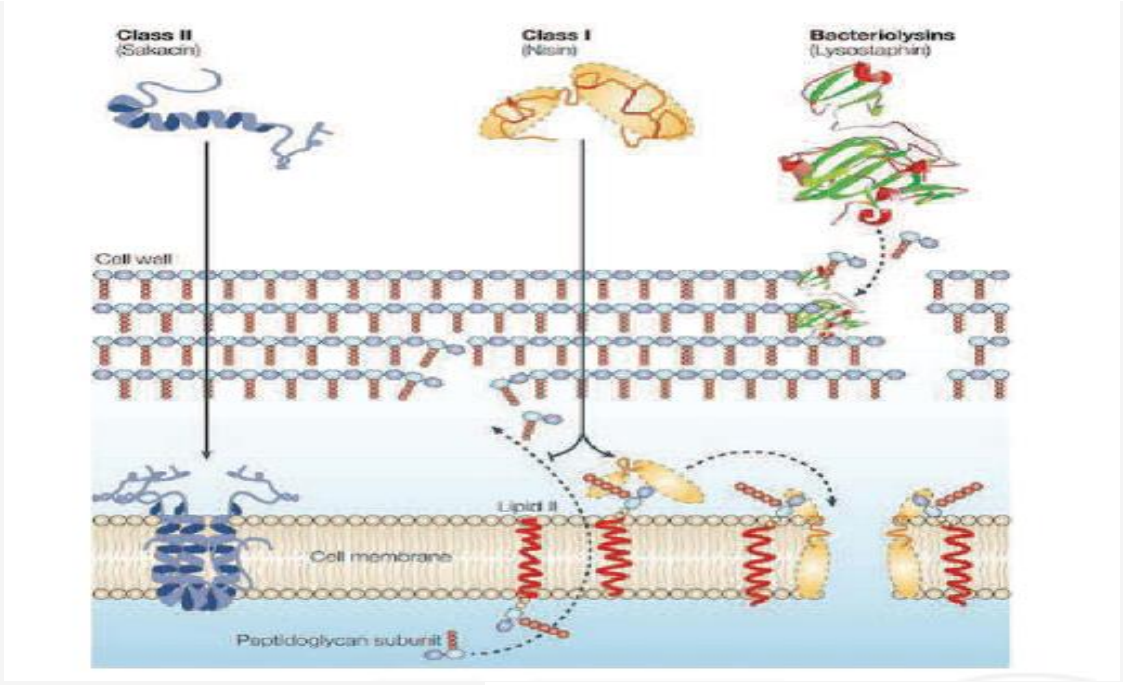


Figure 2.1 Mode of action of bacteriocin

Bacteriolysin which are previously included in class III are large size nacteriolytic proteins which directly acts on the gram positive cell wall resulting in the lysis of target cell (Cotter et al, 2005)

2.5 Applications of bacteriocins

Bacteriocins have several potential applications in health and food industries. There is an increased awareness in the consumers regarding the harmful effects of chemicals used in preservation of food products. Also a rapid increase in food borne illness raise concerns about the safety of the food. The bacteriocin from lactic acid bacteria gained a lot of attention in recent years due to their application as food preservatives from food borne disease and they are generally recognized as safe. Bacteriocin are also being studied as an alternative for antibiotics as antibiotics kills the beneficial bacteria present in the humans along with the pathogens.

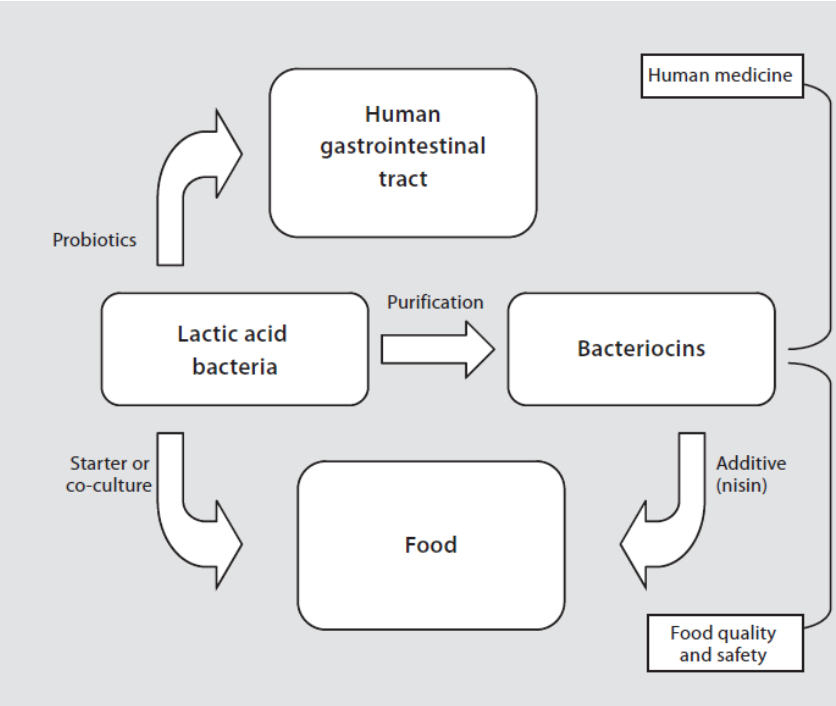


Figure 2.2 An overview of applications from bacteriocin of lactic acid bacteria.

Applications in Food Industry

In food industry, bacteriocins have been widely used as food preservatives and extensive investigations for their use in preservation of dairy products, eggs and meat in past years have been carried out (Zacharof and lovitt, 2012). They can be added to the food or may be directly produced in food products as starter culture. Nisin, a bacteriocin from lactic acid bacteria is the most important example of industrially utilized bacteriocin and is worldwide approved for their use as food additives. Nisin and its natural variant Nisin A are known to be very effective against pathogens causing spoilage and food poisoning (Deegan *et al*, 2006). Bacteriocins are preferred over other successful methods of preservation due to increase in demand for food products which are natural, safe from microbial contamination and provide high health benefits to consumers. They can also be used for improving food quality.

Bioactive packaging, a process used to protect the food from external microbial flora is another application in which bacteriocins are used (Zacharof and lovitt, 2012).

Bacteriocins in human health

Bacteriocins have a narrow spectrum of killing and are produced by non pathogenic bacteria colonizing the human body which make them ideal alternative for antibiotics. The use of antibiotics which have a very broad spectrum of killing results in elimination of pathogens as well as some beneficial microflora. These absence of this bacteria increases the risk of invasion of human body by harmful pathogens. Bacteriocins can be considered as designer drugs due to their capability of killing only specific pathogens. They are also investigated for their potential for treatment of cancer. They are promising cancer diagnostic agents. According to some studies, a lantibiotic Mersacidin is produced by a strain of Bacillus species (Sass *et al*, 2008), and

can be potentially used in the treatment of acne. (Jung 1991a, b; Kellner *et al*, 1988; Niu and Neu, 1991). Lantibiotics Epidermin and gallidermin produced by *Staphylococcus gallinarum* and *Staphylococcus epidermidis* proved very effective in the treatment of skin infections (Kellner et al, 1988).

Some studies suggested the use of bacteriocins in prevention of tooth decay and gingivitis (Blackburn and Goldstein, 1995; Howell *et al*, 1993; McConville, 1995; Peek *et al*, 1995). Nisin is being used in mouth washes due to its antimicrobial activity against bacteria responsible for causing plaque and gingivitis (Kraaij *et al*, 1999). Bacteriocin which are activated against vaginal infection have been reported to show spermicidal activities. Due to this feature, they can be utilized in the manufacture of health care and contraceptives products for females.

2.6 Technologies employed for downstream processing and their limitations

Various techniques have been used for the purification of bacteriocins. The widely used techniques for the purification and recovery of are chromatographic methods such as ion exchange chromatography, affinity chromatogrhy and hydrophobic interactions. Other methods of purification are ultafiltration, precipitation, centrifugation etc,

Chromatographic techniques are the most important method used for the purification and recovery of the protein and peptides. The product purity is very high by the chromatography methods like ion exchange chromatography.

Main limitation of above mentioned techniques are that they are very expensive methods and they require high maintainance. Also physical and chemical factors such as acid ,alkali and temperature can affect the purification process. These methods are also very time consuming and very small quantity of compound is handled.

An alternative method for the downstream processing of bacteriocin can be adsoption method by using zeolites which is a non economical method (Faisal, 2009).

2.7. Zeolites as adsorbants

Zeolites are inorganic crystalline solids having a very small pore size. They are hydrated, micro-porous, aluminosilicates that are built from an infinitely extending three dimensional network of $[AlO_4]^{4-}$ and $[SiO_4]^{4-}$ tetrahedral linked to each other by the sharing of oxygen atom.

2.7.1 Structure of Zeolite:

The primary building unit for zeolites is the tetrahedron and the secondary building units (SBUs) are the geometric arrangements of tetrahedra. The SBUs may be simple polyhedra such as cubes, hexagonal prisms, or cubo-octahedra. The structures can be formed by repeating SBUs.

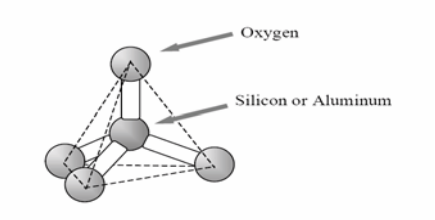


Figure 2.3 Primary building unit of zeolite structure.

Types of zeolite:

Table 2.2 Typical oxide formula of some synthetic zeolites

Zeolites	Typical oxide formula
Zeolites A	$Na_2O.Al_2O_3.2SiO_2.4.5H_2O$
Zeolites X	$Na_2O.Al_2O_3.2.5SiO_2.6H_2O$

2.7.2 Zeolites by CFA

Raw material used for the synthesis of zeolites are alumina and silica which are most abundantly found element on earth . They can be synthesized by kaolin, rice husk, other than coal fly ash.

Many recent investigations have shown the use of coal fly ash as a raw material for the synthesis of various types of zeolites. Due to intensive research on zeolite growth in geological materials such as clay minerals and volcanic rock, the conversion of zeolites from fly ash, which is a byproduct of thermal power plants, have gained a lot of interest. Fly ash containing aluminosilicate makes it interesting starting material for the synthesis of zeolite with a wide range of applications. Various methods of synthesis of a wide variety of zeolites from fly ash have been invented and patented. The important techniques are alkali fusion followed by slurry method (Grutzeck and Siemer, 1997), hydrothermal treatment (Shigemoto *et al*, 1993), molten salt method (*Park et al*, 2000a, 2000b). Among all these methods, alkali fusion method and hydrothermal treatment method are most effective and general method of synthesis of X and A type of zeolites.

Lu *et al*. [2010] synthesized zeolite NaPI by a hydrothermal method from coal fly ash. Modified zeolite NaPI was used as a material for removing fluorine from drinking water. Sutarno *et al*. [2007] synthesized faujasite from fly ash by hydrothermal reaction in alkaline solution via combination of reflux treatment of fly ash with HCl and fusion with NaOH. The synthesis of nano sized zeolite crystals has generated a considerable interest due to their potential of serving as a model system for fundamental studies of zeolite crystal growth. Fathizadeh *et al*. [2010] synthesized the nano particles of Na-X zeolite via hydrothermal method by controlling heat and mixing rate during crystallization. The particle sizes of NaX zeolite crystals ranged from 40 nm to 150 nm. The characterization of physical properties of Na-X zeolite was done by X-Ray

Diffraction(XRD), Field-Emission Scanning Electron Microscope (FESEM), and X-ray Fluorescence (XRF). Results showed that the nano X zeolite have average crystal size 105 nm, Si/Al ratio 1.25 and Na^{+1} active site. Larsen *et al*. [2007] synthesized nano crystal zeolites such as ZSM-5 and zeolite Y, the nano crystal were also used as building blocks to form larger hollow zeolite structure with encapsulated metal and organic species. Application of nano crystalline zeolite in the selective reduction of NO_X and the photo reduction of Cr (IV) to Cr (III) in aqueous solution was investigated. Khalil *et al*. [2007] synthesized MCM-41 nano composite material via a direct non hydrothermal method at room temperature from tetra-ethoxysilane, n-hexadecyl trimethyl ammonium bromide, and ammonia solution and cerium ammonium nitrate precursor. Composite material containing 5-10% (w/w) was targeted. The obtained material was investigated by TGA, DSC,FTITR, XRD. Wang *et al*. [2004] reported a novel strategy of using thermo reversible polymer hydrogel to control zeolite growth rate and produced size controllable zeolite nano crystal from template free precursors. Ojha *et al*. [2004] synthesized X-type zeolite by alkali fusion followed by hydrothermal treatment.

The characterization of synthesized zeolite was carried out by using various techniques such as X-ray diffraction, scanning electron microscopy, Fourier transform infrared spectroscopy. The simultaneous sequestration of ammonium and phosphate from ADSW using nano zeolite synthesized from fly ash was investigated by Yan *et al*. [2003]. . The nanometer scaled zeolite crystal increased the level of specific surface area. Schmidt *et al*. [2000] synthesized zeolite by confined space synthesis. It involved crystallization of the zeolite inside the pore system of an inert mesoporous matrix. Here, confined space synthesis was adopted to prepare nanosized ZSM-5, zeolite Beta, zeolite X, and zeolite A with tailored crystal size distributions using mesoporous carbon blacks as inert matrices. All zeolites were characterized by X-ray powder diffraction,

transmission electron microscopy, and nitrogen adsorption/desorption prior to and after removal of the carbon matrix. ZSM-5 with Si/Al ratios of 50, 100, were synthesized with controlled average crystal sizes in the range 20-75 nm. Nanosized zeolite Beta (7-30 nm), zeolite X (22-60 nm), and zeolite A (25-37 nm) were prepared similarly. Removal of the carbon matrix by controlled combustion proved to be a convenient method for isolation of the pure and highly crystalline zeolites.

2.7.3 Applications Of zeolites

Zeolites are known to have a wide range of commercial uses mainly as catalyst and sorbents (Vjunov *et al*, 2014).

(A) Petrochemical Industries- In petrochemical industries zeolites are widely used as catalysts in processes like fluid catalytic cracking and hydrocracking. The hydrogen form of zeolites which are prepared by ion exchange act as powerful solid acids which have ability to facilitate a host of acid catalyzed reactions like isomerization, alkylation and cracking.

(B) Nuclear Industry- Zeolites have been used in advanced reprocessing systems where they help in removal of various fission products from nuclear waste and permanently trapping them. The mineral properties of zeolites are also very important. Their aluminosilicate framework shows extreme durability and ressistance even if they are in porous form thus are also very useful in managing the leaks of radioactive materials.

(C) Construction- They are added to Portland cement to reduce chloride permeability and increase workability. Synthetic zeolites can act as pozzolanic materials and water reservoir

simultaneously when added to lime mortar (Andrejkovicova *et al*, 2012). They are used as an additive in production of warm mix asphalt concrete.

(D) Medical Uses- Medical grade oxygen production has been done using zeolite based oxygen concentrator systems. They are used as molecular sieves to purify oxygen from air due to their properties of trapping impurities resulting in highly purified oxygen. Zeolites have been used in haemostatic agents which stop severe bleeding (Rhee *et al*, 2008)

(E) Agriculture- They are used for treatment of soil and for removal of odor from soil. They can also be used as water moderators i.e., they absorb 55% water and then release it when plant require water. They increase the nitrogen level in manure thus providing high fertilizer value. They limit the moisture levels in organic wastes and diminish toxicity of ammonia.

(F) Aquarium Keeping- Zeolites are used as filter additives in aquariums and are used to absorb ammonia and other nitrogenous compounds (Virta, 2009). Some marine aquarium used zeolite filteration for keeping the concentration of nutrients at low levels for benefits of corals.

CHAPTER 3 MATERIALS AND METHODS

3.1 Reagents, chemicals and materials used

Coal fly ash (CFA) was used for the synthesis of nano-sized zeolites and it was obtained from Guru Gobind Singh thermal power plant of Ropar distt. Punjab (India). All the chemicals used for zeolite synthesis were purchased from Himedia (India) and were of high purity of analytical grade.

3.2 Zeolite synthesis

Before using CFA, calcination i.e., giving thermal treatment at a high temperature of around 800 K, was done for the removal of iron oxide and other impurities. In this study, two different zeolites were synthesized namely zeolite Na-X and zeolite A .

3.2.1 Synthesis of zeolite Na-X

Synthesis of zeolite Na-X was carried out by alkaline hydrothermal process (Querol *et al* 2002, Murayama *et al*, 2003). 30 g of CFA and 180 mL of 2.0 mol/ L NaOH solution were added into a 500 mL flask at a solid liquid ratio of 1: 6. This mixture was then heated at 95°C for 48 hours in a water bath. The mixture was then rinsed with deionized water over three times until no NaOH was detected, further washed with 0.5 mol/L $Cacl_2$ solution and again rinsed with distilled water to remove chlorine ions. It was then dried in an oven for 24 hours at 90°C.

3.2.2 Synthesis of zeolite A

For the synthesis of zeolite A, 30 g of CFA and 300 mL of 2 M NaOH solution was mixed in a sealed polypropylene bottle and was kept in water bath at 100°C for 3 hours at agitation speed of 150 rpm. Then, the solution formed was separated from the mixture by a filtration process. The molar ratio of SiO2/Al2O3:Na2O/SiO2:H2O/Na2O in the solution was adjusted to

1.64:8.09:56.51 by adding 100 mL of aluminum solution. The solution was then agitated at 500 rpm for 30 min at room temperature (30° C) and kept at its first reaction temperature of 90 °C for 1.5 hour and subsequently at the second reaction temperature of 95 °C for 2.5 hour. The precipitated sample was separated from the mixture by a filtration process and washed with deionized water until the pH reaches 10. The sample was kept in an oven and dried at 100 °C for 12 hour.

3.3 Characterization Of Zeolites

Characterization of synthesized zeolites were done by two techniques (1) XRD (X-Ray Diffraction) and SEM (Scanning Electron Microscopy)

3.3.1 XRD- Powdered X-ray diffraction analysis of zeolite was carried out with X-ray diffractometer. Copper Kα radiation was used with a wavelength of 1.54 A°. The scan rate was measured between 5° and 80°. Zeolite materials being crystalline solids have characteristic diffraction patterns that can be used to identify their exact structure and to determine their degree of crystallinity.

3.3.2 SEM- SEM analysis was carried out to obtain surface morphology and EDX was performed to evaluate the contents present in coal fly ash sample. Samples of zeolite an coal fly ash were coated with a conductive layer of gold and then analyzed by SEM(JSM 541-V, JOEL, Japan) at an accelerating voltage of 20.0 kV complemented with an energy dispersive X-ray micro-analyzer (EDX).

3.4 Antimicrobial bioactive peptide production by lactic acid bacteria (LAB)

3.4.1 Bacterial strains & cultural conditions

de Man Rogosa Sharpe (MRS) media was used for the production of bioactive peptide (bacteriocin) from different strains. The strains used were Lactic Acid Bacteria (LAB) i.e., LAB

1, LAB 2, LAB 3, LAB 4 and Lactobacillus *acidophilus*. The media components were purchased from Himedia Laboratories, Mumbai (annexure I). The bacterial strain used for the production of bacteriocin has been previously isolated from fermented foods were screened for their ability to produce bacteriocin (Singh , 2012).. These isolates were inoculated in 100 mL flask containing MRS broth and then incubated at 37°C for 48 hours.

3.4.2 Screening of antimicrobial peptide producing LAB

Total of five isolates were screened for their ability to produce bioactive peptides. Cell free supernatant of all five isolates were obtained by centrifuging the cultures at 10000 rpm for 10 minutes. The pH of supernatant was adjusted to 6.5 using 4 N sterile NaOH. Hydrogen peroxide present in the supernatant was removed by heating the supernatant at 60°C or by adding catalase. The antimicrobial activity of peptide produced by the supernatant was determined by agar well diffusion method (Soumya *et al* , 2012).

3.4.3 Measurement of peptide content and its antimicrobial activity

Peptide content from supernatant was measured by method of Church *et al*, 1983. To the 50 µL supernatant, 2 mL OPA reagent was added (annexure I). The reaction mixture was incubated for 2 minutes at room temperature and absorbance was measured at 340 nm with spectrophotometer (Hitachi Japan).

Antimicrobial activity was determined by agar well diffusion method using *B. subtilis* as indicator strain. 30 µL aliquots of culture supernatant was added to well in agar plates spreaded with *B. subtilis.* Plates were incubated at 37°C for 24 hours and zone of inhibition was measured.

3.4.4 Batch fermentation for antimicrobial peptide production (Bacteriocin)

The isolated strains were used for the bacteriocin production. Batch fermentation was carried out using MRS media under static conditions and at incubation temperature of 37°C for 48 hours. The peptide content was monitored after every six hours of growth.

3.4.5 Recovery of bacteriocin by synthesized zeolite

For determination of zeolite applications in recovery of bacteriocin, binding of bacteriocin with zeolite A and Na-X was carried out. To study the effect of synthesized zeolite dose on adsorption, different concentration of zeolites ranging from 10 mg to 150 mg was used. Zeolite was used to form a bed on spin column. A working solution of bacteriocin diluted 10 to 100 folds was added to zeolite and incubated for 30 minutes.

Column was spin for 30 seconds. The filterate obtained were used to analyze the residual peptide present in the sample. Each experiment was performed in triplicate. To analyse the effect of contact time on the peptide binding by zeolite, columns containing optimum zeolite and peptide concentration was added and incubated for time interval ranging from 0 to 180 minutes and filterate was analyzed for peptide estimation by the method described above.

Percentage recovery of peptide by zeolite was calculated by the following formula

$$\text{Percentage recovery} = \frac{(P_I - P_f)}{P_I} * 100$$

P_I= initial peptide concentration

P_F= final peptide concentration

CHAPTER 4 RESULT AND DISCUSSION

Bacteriocins from Lactic acid bacteria offers potential as biopreservatives and exhibits broad spectrum of antimicrobial activity. The main hurdles concerning the applications of bacteriocins is expensive purification methods which are suitable at laboratory scale but cannot be used at industrial scale. So, in this study, zeolites were synthesized with cheap and economic raw material coal fly ash (CFA) and determining their efficiency in the downstream processing of bacteriocin peptides.

4.1 Zeolite-: Synthesis and Characterization

Zeolites were synthesized by CFA which is a byproduct of thermal power plants. The zeolite synthesized are zeolite Na-X and zeolite A. zeolite Na-X was synthesized by hydrothermal process by treating CFA with NaOH at 95 °C for 4 days. The yield of zeolite obtained from this method is 15 gms. Zeolite A is obtained by treating CFA with NaOH and subjecting this solution to temperature variations and ammonium solution. The total time required was 12 hours to produce 14 gm of pure zeolite A powder.

Table 4.1 Yield of zeolites

Zeolite	Time	Yield
A	12 hours	14 gms
Na-X	96 hours	15 gms

Factors affecting the quality and yield of the zeolites are temperature. reaction time and agitation. With the increase in temperature and agitation along with the time, the pore size of the zeolite decreases which results in production of small or nano-sized zeolites.

The chemical composition of the coal fly ash sample was determined by using EDX. The CFA sample used for the synthesis of nano-zeolites composed of aluminium (40.85%) and silica (31.34%) which are the major components. Potassium, platinum and iron serve as minor components.

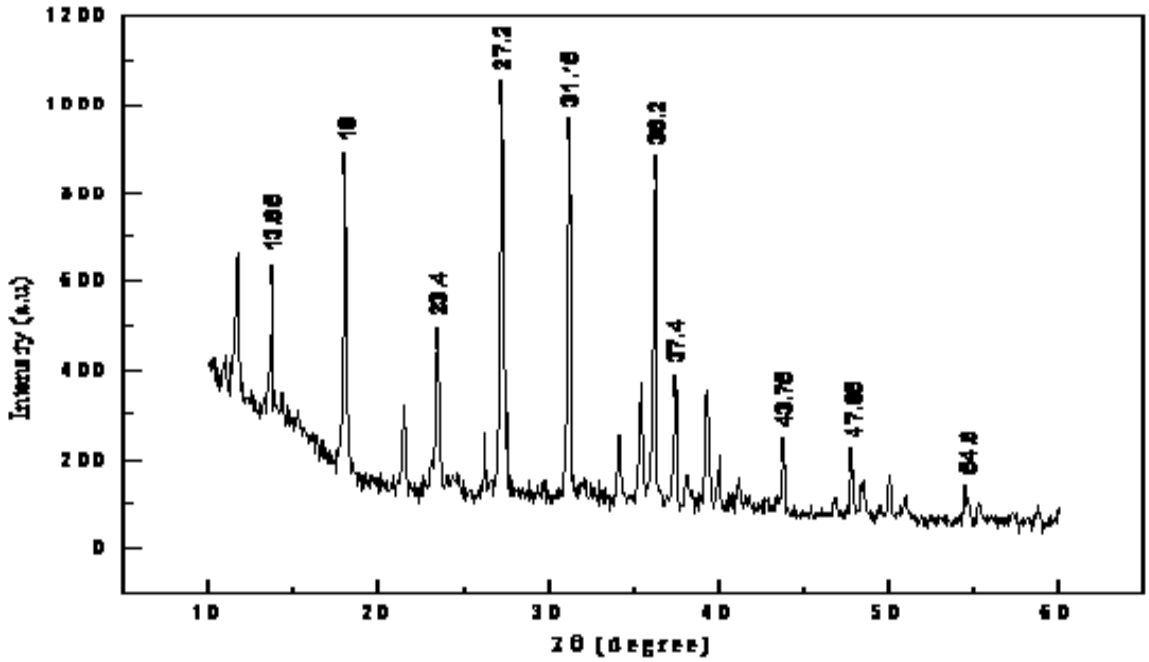
Table 4.2 Composition of coal fly ash

Elements	Weight %	Atomic %
Al	31.34	40.91
Si	40.85	51.22
K	0.99	0.89
Pt	22.32	4.03
Zr	-	-
Fe	3.42	2.15
Ti	1.09	0.80

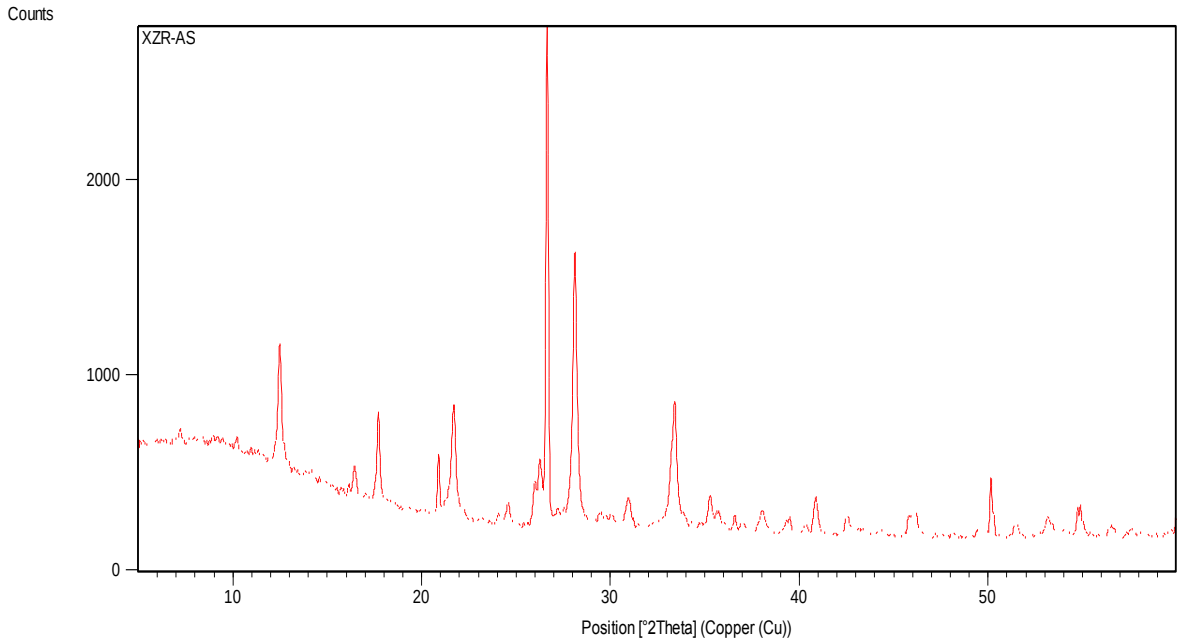
4.2 Characterization of zeolites

Synthesized zeolite were characterized for their crystalline behavior by X-ray diffraction (XRD) and morphology of the synthesized zeolite was observed by SEM analysis.

The XRD patterns of nano-zeolites Na-X and A were compared with commercially used Zeolites X (Barman 2006) and A (Hui *et al* , 2009) respectively . The XRD patterns of zeolite Na-X synthesized from coal fly ash suggested that the newly synthesized nano zeolite was more crystalline when compared to industrially synthesized zeolite X. Similarly XRD patterns of Zeolite A showed they were more crystalline than commercially obtained microsized zeolite . The XRD analysis revealed sharp and intense peaks (figure 4.1)

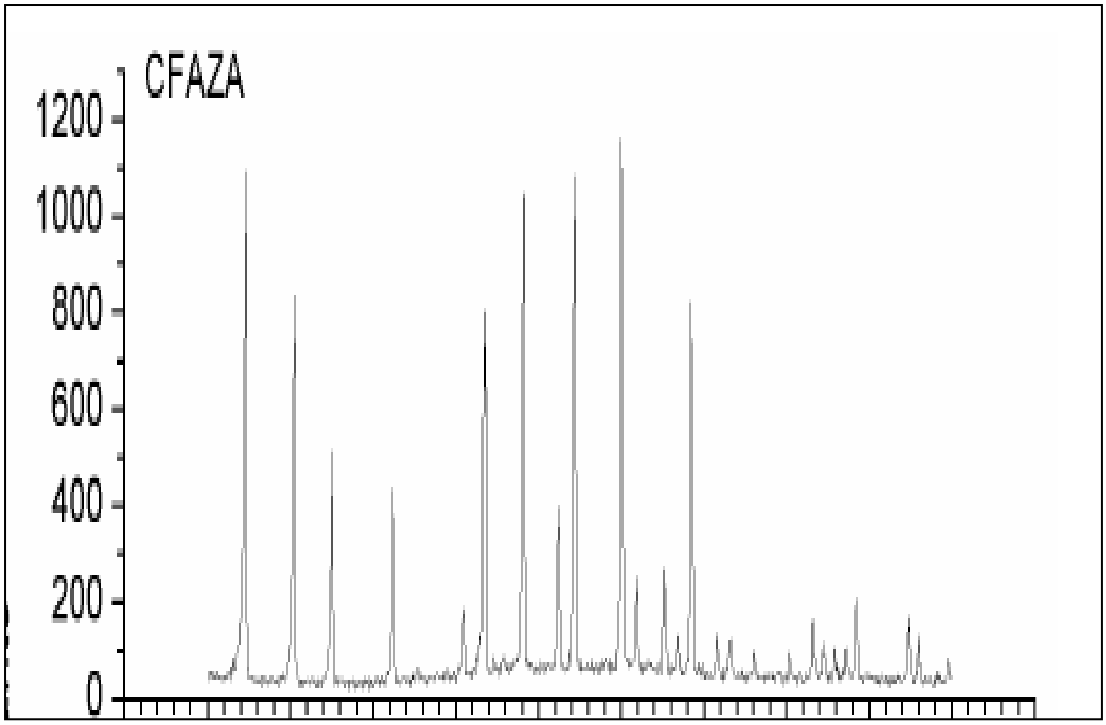


(a)



(b)

Figure 4.1 XRD pattern of (a) commercial zeolite X (b) CFA synthesized zeolite Na-X



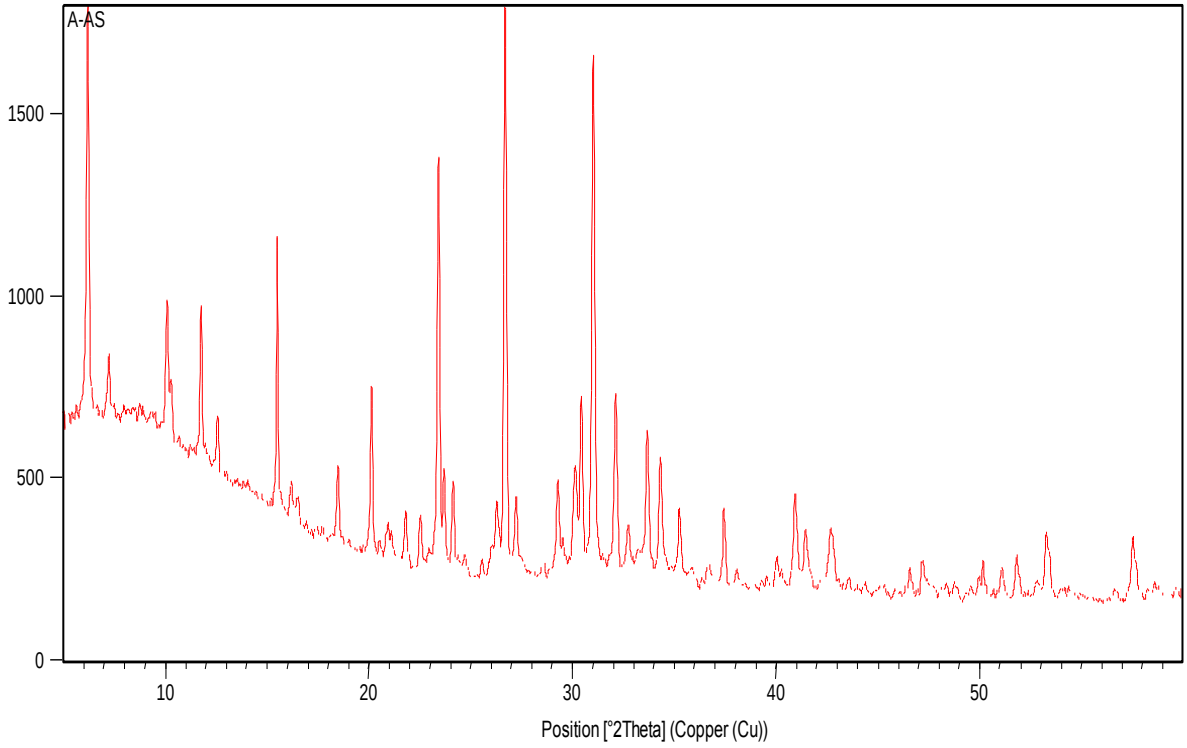
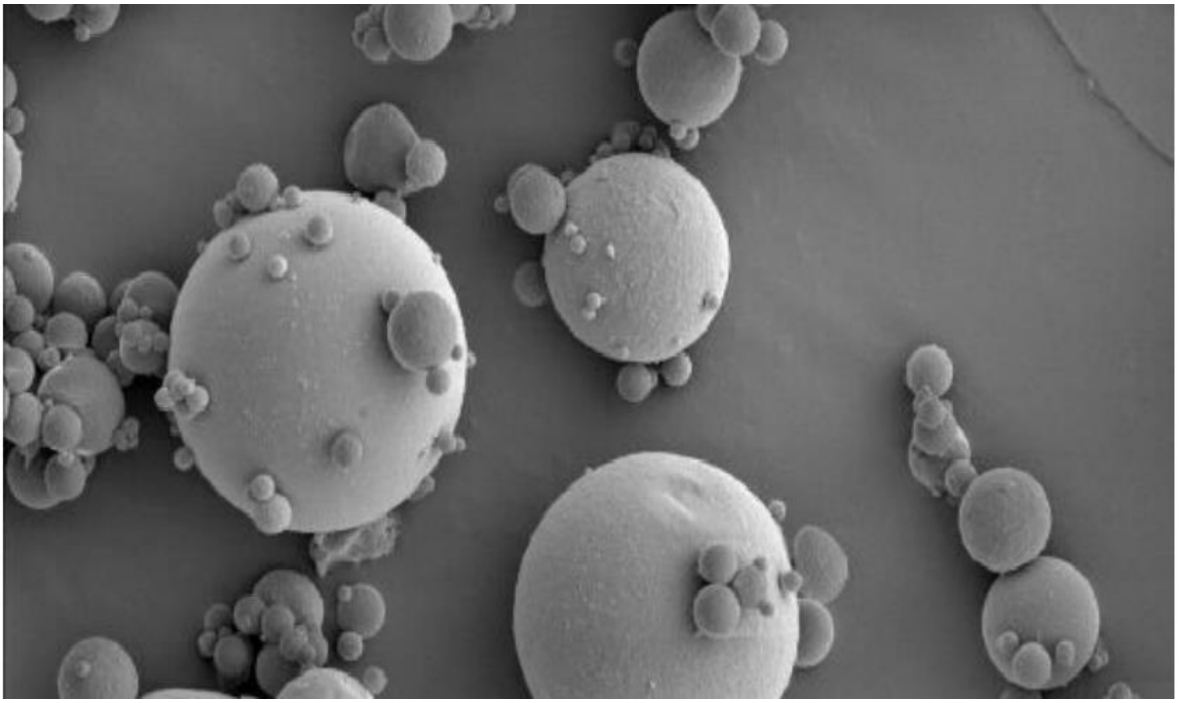


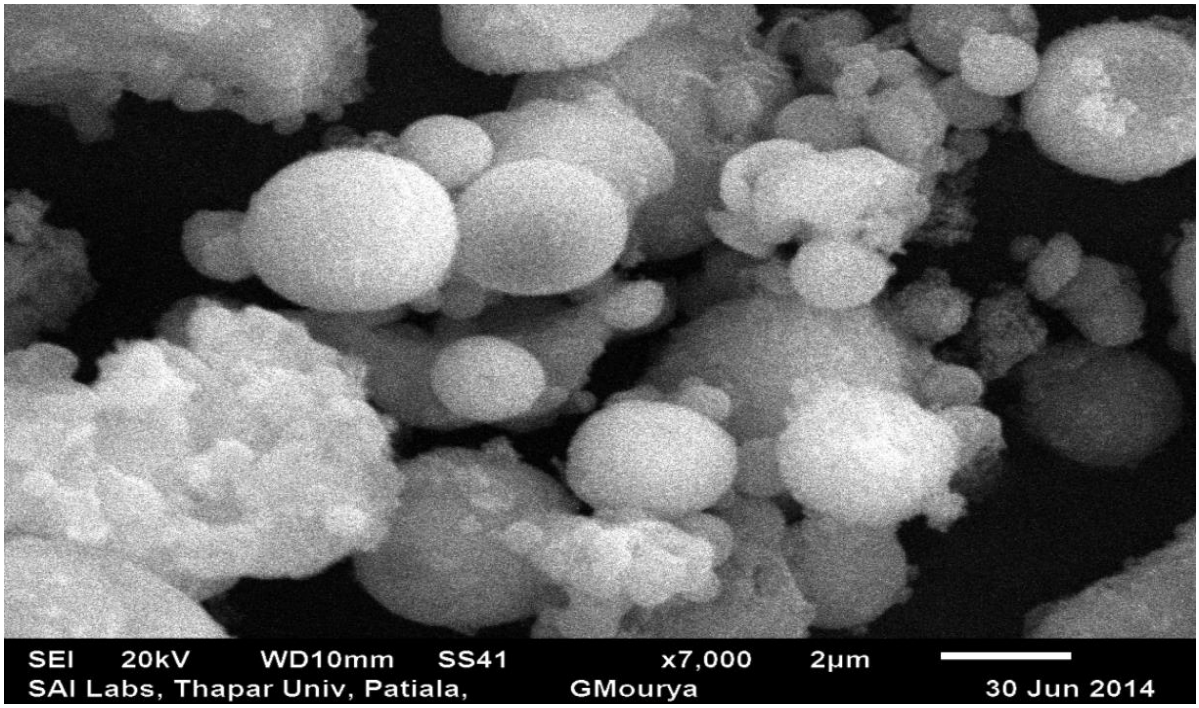
Figure 4.2 XRD pattern of (a) commercial zeolite A (b) zeolite by CFA

The diffraction of X-rays from zeolite crystallites produce a scattering pattern which is specific of periodic arrangements of regular arrays of atoms or ions located within the zeolite structure. XRD is important for inferring crystal size, crystalline phase purity and measurement of solid sample amorphous or crystalline structure (Kim *et al.,* 2001).

The SEM of coal fly ash and synthesized zeolite from fly ash are shown in figure (4.3 a,b) . The SEM of the synthesized zeolite sample shows the morphology of the crystal surface in which crystal size range are less than 2µm.



(a)



(b)

Figure 4.3 SEM micrograph of (a) Coal fly ash (b) zeolite X

4.3 Screening of bioactive peptide (bacteriocin) producing *Lactobacillus* strains

A total of five previously isolated strains obtained from fermented foods were evaluated for the production of bioactive peptides or bacteriocins (kumar,. 2012). From screening studies, it was observed that bacteriocin was found to be present in strains *L. acidophilus* and isolate LAB 4. All other isolates do not show any bacteriocin. The results are shown in Table 4.2. Antimicrobial activity of the crude peptide was estimated by agar well diffusion test and it was observed that peptide produced by these two strains show antimicrobial property and show strong zone of inihibition against *B.subtilis* (figure 4.7). Bacteriocins are the antimicrobial peptides produced by these strains and hence assay methods confirms the presence of bacteriocin peptides by these two strains.

Table 4.3 Peptide content with their relevant antimicrobial activity of isolates

Organism	Peptide activity	Antimicrobial activity
LAB 1	-	-
LAB 2	-	-
L. acidophilus ATCC 43121	+	++
LAB 3	-	-
LAB 4	+	++

(-) No peptide present, (+) Peptide present, (-) No microbial activity, (++) Presence of antimicrobial activity

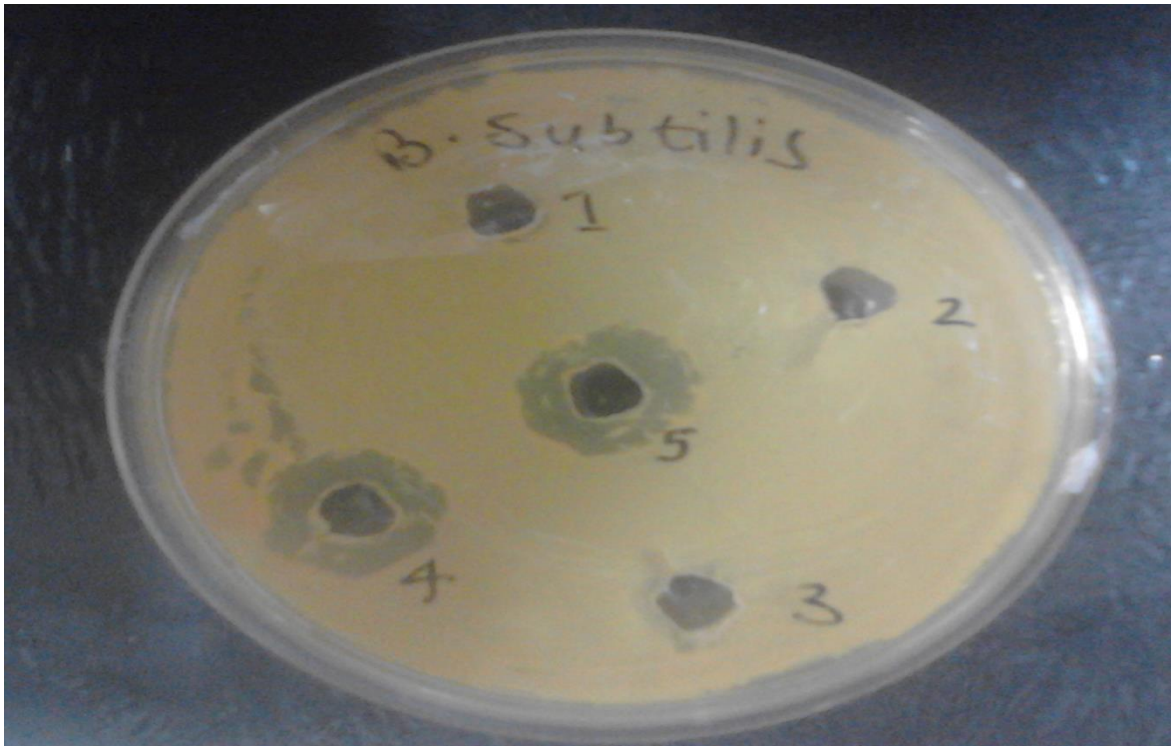


Figure 4.4 Zone of inhibition indicating presence of bacteriocin from LAB 4 and *L. acidophilus* ATCC 43121using *B. subtilis*. Well 1=LAB 1, well 2= LAB 2, well 3= LAB 3, well 4=LAB 4 and well 5= *L. acidophilus* ATCC 43121

4.4 Batch fermentation for production of bacteriocins by *L. acidophilus* ATCC 43121 and LAB 4

Production of bacteriocin by both strains i.e., *L. acidophilus* ATCC 43121 and LAB 4 was studied under shake flask conditions as well as under batch fermentation conditions. Batch fermentation was performed in 3 L media in 5 L fermenter.

It was observed that in case of *L. acidophilus* ATCC 43121, maximum bacteriocin activity was 2490 AU/mL at 36 hours of incubation and in case of LAB 4, bacteriocin activity was monitored to be maximum 1998 AU/mL at 24 hours of incubation . The results suggest that bacteriocin produced by both the strains are secondary metabolites as they were produced during late exponential and early stationary phase. The bacteriocin produced fermentatively was recovered by zeolites.

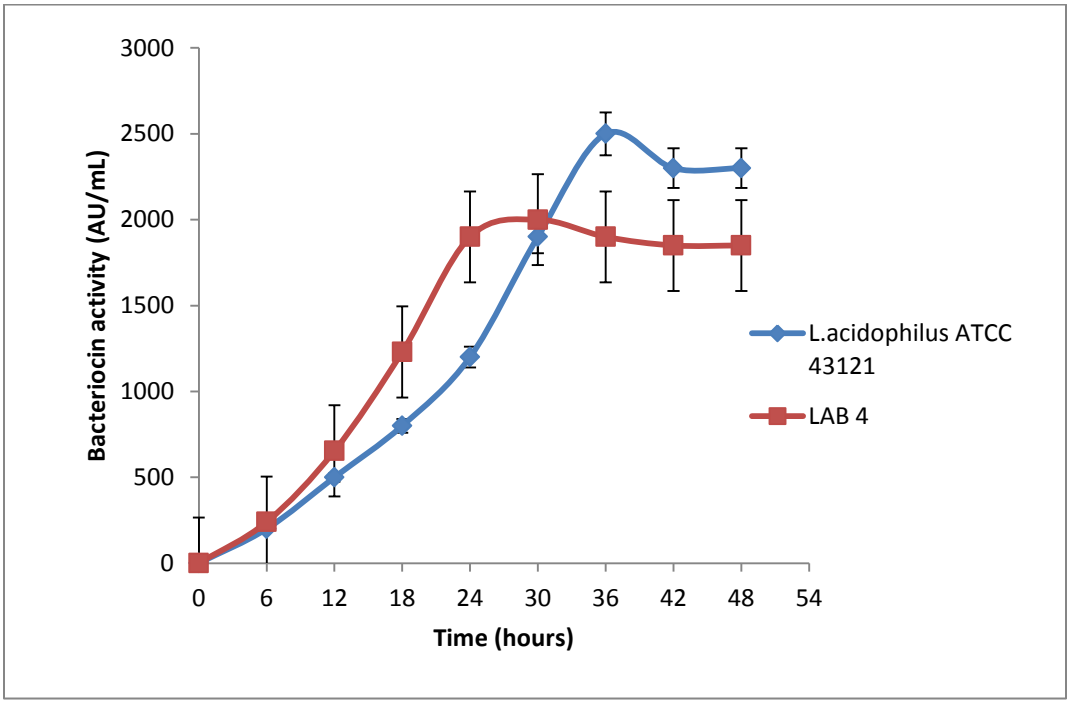


Figure 4.5 Bacteriocin activity of *L. acidophilus* ATCC 43121 and LAB 4 with time

4.5 Recovery studies of bacteriocin from fermentation broth

For purification and efficient recovery of bacteriocins, newly synthesized zeolites Na-X and A were used. Bacteriocin produced from fermentation of two bacterial isolates LAB 4 and *L.acidophilus* were analysed for their binding ability with zeolites. For their maximum and efficient binding, different parameters such as zeolite dose, bacteriocin concentration and time of contact between them were analysed.

4.5.1 Effect of zeolite and bacteriocin dose

One of the most important parameter used for determination of optimum conditions of recovery are zeolite and bacteriocin dose. In this study zeolites Na-X and A was used as adsorbants with bacteriocin *L. acidophilus* ATCC 43121 and LAB 4 as adsorbates. The zeolite dose used for the optimization was used in range of 10 mg to 150 mg.

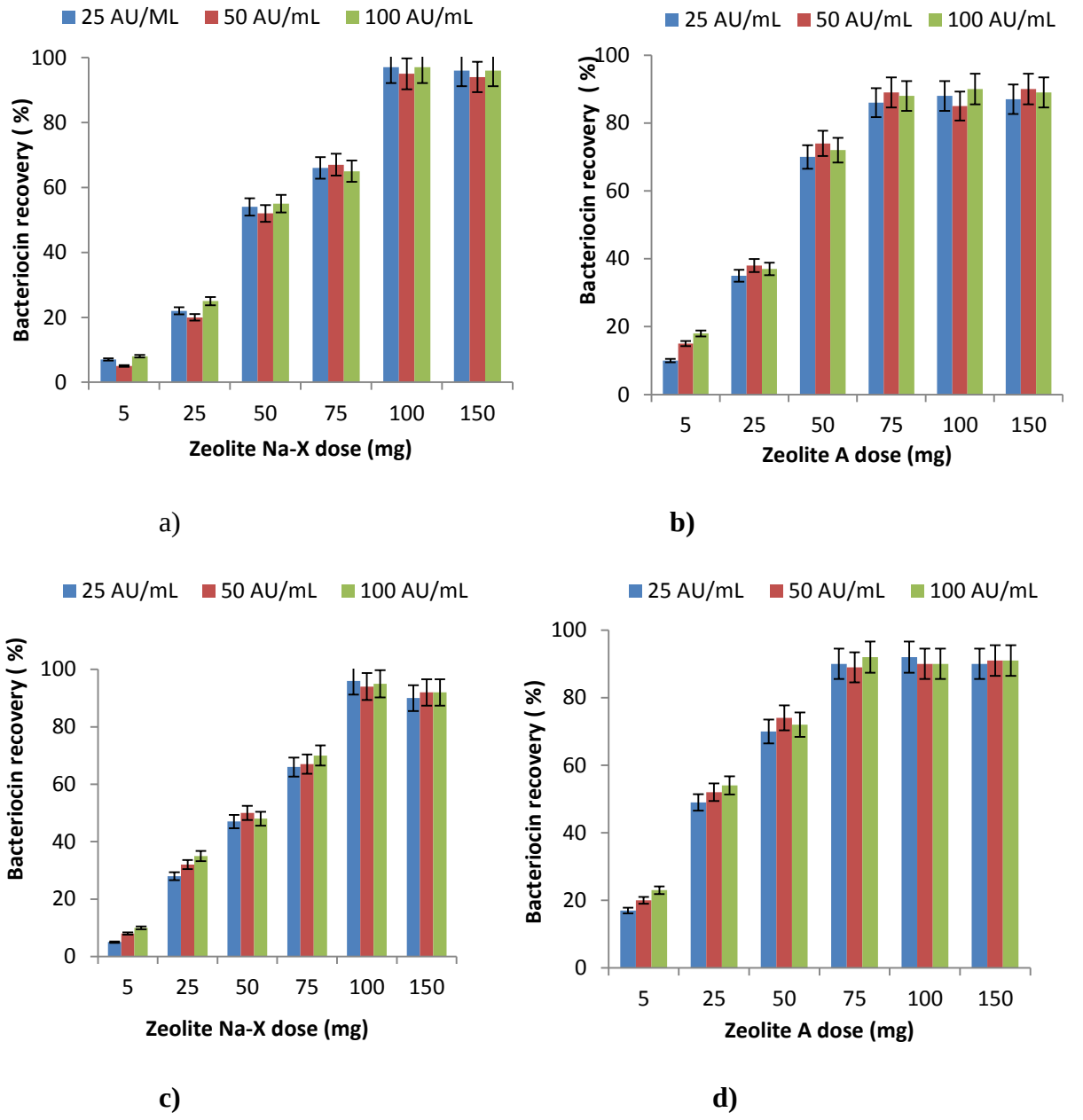


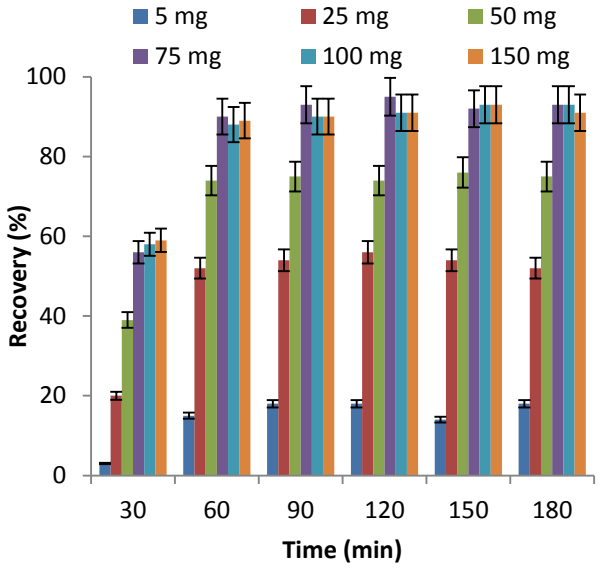
Figure 4.6 (a-d) Effect of zeolite and bacteriocin dose for recovery of bacteriocin by zeolites (a) zeolite Na-X with bacteriocin bacteriocin *L. acidophilus* ATCC 43121 (b) zeolite A with bacteriocin *L. acidophilus* ATCC 43121 (c) zeolite Na-X with bacteriocin LAB 4 (d) zeolite A with bacteriocin LAB 4. (Error bars represents the standard deviation from triplicate experiments).

In case of zeolite Na-X, the significant increase ($p<0.05$) in bacteriocin binding by increasing zeolite dose was observed. Maximum binding of 98 % of bacteriocin aws observed for LAB4 and 97% binding was observed for *L.acidophilus*. The initial increase was due to availability of active sites for the adsorption of bacteriocin and above 100 mg for both bacteriocin LAB 4 and *L. acidophilus* ATCC 43121, bacteriocin recovery was not significant ($p>0.05$) and similar results was observed in case of zeolite A, were maximum bacteriocin recovery was observed at the zeolite dose of 75 mg for LAB 4 and 91% for *L.acidophilus*. Above 75 mg of zeolite , no significant binding of bacteriocin was observed which was due to saturation of all active sites by the available bacteriocin (Figure 4.9).

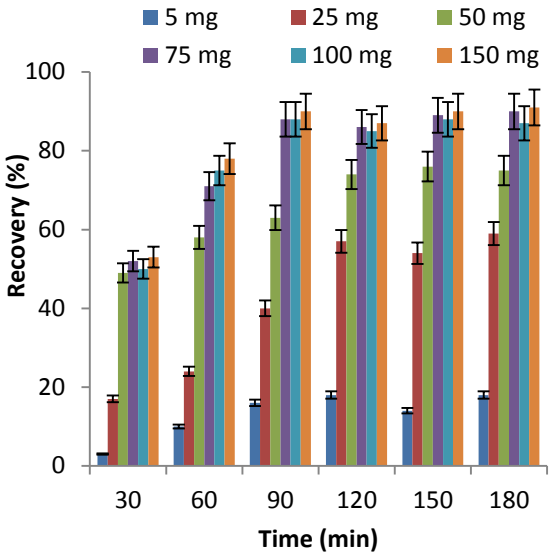
To study the effect of bacteriocin dose on its binding, extracted bacteriocin solution was diluted to the concentrations of 25, 50 and 100 AU/mL and the binding efficiency of bacteriocin by zeolites was observed. It was observed that , there was no significant effect ($p>0.05$) on bacteriocin binding when bacteriocin concentration was increased from 25 AU/mL to 100 AU/mL. So, The results describes the ability of zeolites to bind with bacteriocins without ch

4.5.2 Effect of contact time-

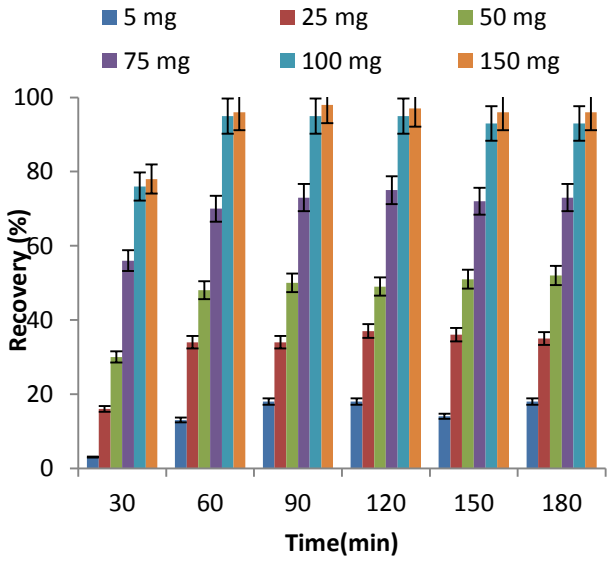
The time of contact was another important parameter for the bacteriocin recovery by zeolites. The recovery of bacteriocins was studied with each zeolite at various time intervals. In case of zeolite A, maximum binding of 95% was observed over time of 60 minutes for LAB 4 and 91% was observed for *L. acidophilus* ATCC 43121 at the time interval of 90 minutes. In case for zeolite Na-X maximum recovery of 98% and 97% of bacteriocin from LAB 4 and *L. acidophilus* ATCC 43121 was observed over a time span of 60 min and 90 min respectively (Figure 4.7).



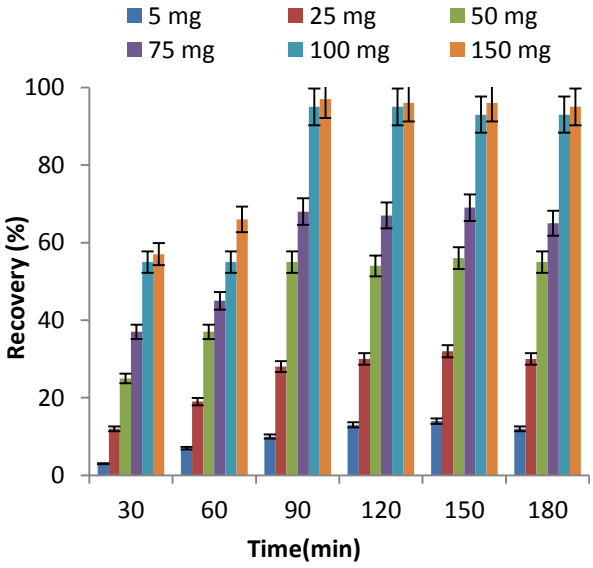
a)



(b)



c)



(d)

Figure 4.7 (a-d) Effect of contact time and zeolite dose for recovery of bacteriocin by zeolites. (a) zeolite A with LAB 4 (b) zeolite A with *L. acidophilus* ATCC 43121 (c) zeolite Na-X with LAB (d) zeolite Na-X with *L. acidophilus* ATCC 43121 by varying bacteriocin dose. (Error bars represents the standard deviation from triplicate experiments)

The results indicates the maximum bacteriocin recovery of 98% from LAB 4 bacteriocin by Zeolite Na-X and 95 % by zeolite A While Maximum recovery from *L.acidophilus* bacteriocin was 91 % by zeolite A and 97% by zeolite A. The more binding of zeolite NA-X was observed due to its large pore size which allowed the more recovery of bacteriocin LAB 4.

It can be concluded from the above results that zeolite Na-X was able to recover more bacteriocin LAB 4 at less contact time as compared to zeolite A.

CONCLUSION

1. Zeolites offer promising candidates for downstream process or recovery of products from fermentation media because of their inert and non reactive nature. Also they can cater to the requirement of desirable pore size.
2. Zeolite synthesized in the present study i.e., Zeolite Na-X and A were applied for the recovery and purification of an bioactive antimicrobial compound; bacteriocin obtained after the fermentation of *L. acidophilus* ATCC 43121 and LAB 4.
3. Zeolites synthesized were microporous in nature as evidenced by SEM and XRD analysis.
4. The product obtained was suitable in the recovery of bacteriocin. Percent recovery of bacteriocin LAB 4 obtained with zeolite Na-X (98 %) was maximum in comparison to zeolite A (95%) and for bacteriocin *L. acidophilus* ATCC 43121, it was maximum with zeolite Na-X (97%) compared to (91%) with zeolite A within the contact time of 90 minutes.
5. This study emphasizing the purification processes efficient for recovery of bacteriocin by zeolites. Zeolites have promising future in the downstream processing of various biotechnological products as they are cost effective and highly compatible.

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ANNEXURE 1

MRS Media Composition (for 250ml):

Peptone with casein –	2.5gm
Beef extract –	2.5gm
Yeast –	1.25gm
Dextrose –	5gm
Dipottassium hydrogen phosphate –	0.5gm
Tween 80 –	0.25gm
Triammonium citrate –	0.5gm
Sodium acetate –	1.25gm
Magnesium sulphate –	0.05gm
Magnese sulphate –	0.02 gm
Distilled water –	250 ml
pH _	7.0

O-pthaldialdehyde (OPA) Reagent

Borax	1.524 gm
Sodium Dodecyl Sulphate	2 gm
O-pthaldialdehyde	40 mg
Methanol	1 mL
B- mercaptoethanol	100 µL

Distilled water	50 mL