Biochemical and nanoparticle characterization of *Strychnos potatorum* L. seed matrices and their applications

Thesis submitted for the Degree of

Doctor of Philosophy

by

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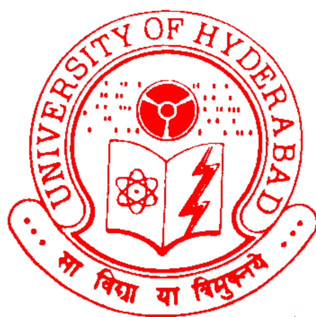

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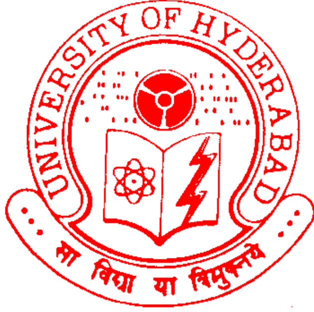

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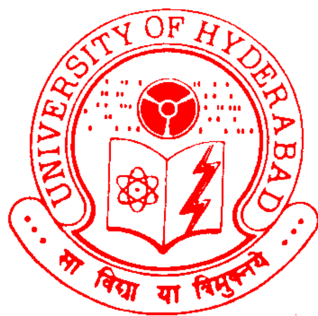
DECLARATION

I hereby declare that the work presented in my thesis is entirely original and was carried out by me in the department of Biochemistry, School of Life Sciences, University of Hyderabad, under the supervision of Prof. N. Siva Kumar and Prof. M. N. V. Prasad. I further declare that this work has not been submitted earlier for the award of degree or diploma from any other University or Institution.

Mohammad Mansour Saleh Saif

Date:



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CERTIFICATE

This is to certify that this thesis entitled **"Biochemical and nanoparticle characterization of *Strychnos potatorum* L. seed matrices and their applications"** submitted to the University of Hyderabad by Mr. **MOHAMMAD MANSOUR SALEH SAIF** for the degree of Doctor of Philosophy is based on the studies carried out by him under my supervision. I declare to the best of my knowledge that this work has not been submitted earlier for the award of degree or diploma from any other university or institution.

Prof. N. Siva Kumar
supervisor

Prof. M.N.V.Prasad
Co- supervisor

Head
Department of Biochemistry

I/c Dean
School of Life Science

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IN THE NAME OF ALLAH, MOST GRACIOUS, MOST MERCIFUL

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Mohammad Mansour

To the memory of my mother, mother in law

To the memory of my brother Abdul-Hakeem

To all of my beloved parents

To my wife and my sons Jamal and Tameem

This work is dedicated

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Abberiviations

AFM	Atomic Force Microscope
APS	Ammoniumperoxodisulfate
AS	Atomic Spectrophotometer
b	is a parameter related to the energy of adsorption
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
Cd (II)	Cadmium
C_e	equilibrium concentration
C_0	initial concentration of the metal ions
^oC	Degree centigrade
Con- A	Concanavalin A
DDSs	Drug delivery systems
DE-52	DiEthylAminoEthyl Cellulose
DLL-I	Glucose- mannose- specific lectin from seeds of *Dolichos lablab*
DLL-II	Galactose-specific lectin from seeds of *Dolichos lablab*
DTT	Dithiothreitol
DVS	Divinyl sulfone
EC	Enzyme Commission
EDTA	Ethylene diamine tetra acetic acid
et al	et alii (Latin: and others)
EtBr	Ethidium bromide
Gal	Galactose
GHs	Glycoside hydrolases
Glu/Glc	Glucose

Gp%	Glycoprotein %
h	Hour
HEPES	N-(2-Hydroxyethyl) piperazine-N'-2-ethane sulfonic acid
HMs	Heavy metals
kDa	Kilo Dalton
L	Liter
Lac	Lactose
MALDI	Matrix assited laser desorption ionization
mg	Milligram
min	Minute
mL	Milli Litre
MS	Mass Spectrometry
MW	Molecular Weight
NC	Nitrocellulose
nm	Nanometer
NP	Nanoparticles
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
Pb	Lead
PBS	Phosphate-buffered saline
pH	-log (H+) concentration
pNP	*para* nitro phenol
*p*NPG	*p*-nitrophenyl-α-D-Galactopyranoside.
*p*NPM	*p*-nitrophenyl-α-D-Mannopyranoside.
PMSF	Phenyl methyl sulfonyl fluoride

PVDF	Polyvinyl difluoride
q_e	Amount of metal ion adsorbed at equilibrium.
q_t	Amount of metal ion adsorbed at any time
rpm	Rotations per minute
RT	Room temperature
SDS	Sodium dodecyl sulfate
SEM	Scanning Electron Microscope
SPS	*Strychnos potatorum* seeds
SPSP	*Strychnos potatorum* seed powder
t	Time
TB	Tris-buffer
TBS	Tris-buffered saline
TCA	Trichloroacetic acid
TEM	Transmission Electron Microscope
TEMED	N, N, N', N'-Tetramethylendiamine
Tris	Tris-(Hydroxymethyl) aminoethane
UV	Ultraviolet
β-M.E	β -mercaptoethanol

Chapter 1

Introduction

1.1 *Strychnos potatorum* L.

Strychnos is the largest genus in *Loganiaceae*. It is a common tree of medicinal importance in India popularly used to purify drinking water. Past traditions in India reported the use of *S. potatorum* seeds for cleaning the turbid water [Cooke, 1871; Gupta et al., 1992]. *S. potatorum* is non-toxic plant and its seeds (popularly known as cleaning nuts) have high economic importance. Its seeds are collected by locals from different forest divisions of Andhra Pradesh. Its seeds are one of the most important minor forest products collected by the members of the Girijan Co-operative Corporation, Andhra Pradesh (the predominant participating tribal groups are Bagatas, Valmikis, Nookadoras, Malis, and Kutias) along with other minor forest produce such as *Strychnos nux-vomica* (nux-vomica), *Sterculia urens* (Gum karaya), *Terminalia chebula* (myrobalan), *Tamarindus indica (*Tamarind*), Phyllanthus emblica* (Amla), *Madhuca longifolia* (Mohwa). A commercial product by name "NATFLOC" a "natural polyelectrolyte" has been developed with the seeds of *S. potatorum* by the GCC. NATFLOC is recommended for water turbidity removal upto 3000 NTU (Nephelometric Turbidity Unit). Additionally, there are many traditional and medicinal applications of the *S. potatorum,* in Ayurveda, Siddha, Unani, Folk, Modern, Tibetan and Homeopathy Systems of Medicine (Rao 2008).

Figure 1: *Strychnos potatorum*

a	Habit
b	Flowers
c, d	Fruits
e	Seeds
f	Seed powder

1.1.1 Properties

S. potatorum Linn. (Family: *Loganiaceae*) commonly known as Katakam in Ayurveda is a moderate-sized tree found in southern and central parts of India, Srilanka and Burma [Kirtikar and Basu, 1933]. *S. potatorum* contains many monomeric and dimeric indole alkaloids, the root bark being especially rich. *S. potatorum* L. is the only *Strychnos* species that grows in Africa and in Asia [Leeuwenberg; 1969]. Twenty-four compounds have been isolated and identified in the root bark of *S. potatorum*. They are: harmane carboxamide, cantleyine, l&19-dihydrousambarensine, polyneuridine, norharmane, akuammidine, nor- C-flurorocurarine, ochrolifuanine A, bisnordihydrotoxiferine, ochrolifuanine E, normacusine B, normavacurine, henningsamine, 1 1-methoxyhenningsamine, dihydrolongicaudatine, dihydrolongicaudatin Y, antirhine, (20R)- and (20S)-dihydroantirhine, 1 1-methoxy-lZhydroxydiaboline, diaboline, 1 1 methoxydiaboline, desacetylretuline and diaboline N-oxide [Georges et al 1992]. The ripe seeds are used for clearing muddy water. They are reported to be very effective as coagulant aids. Alum aided by the seeds has been found to be very effective in removing the suspended impurities from coal-washery wastes. The clarification is due to the combined action of colloids and alkaloids in the seeds [Anonymous, 1976]. Presence of diaboline (major alkaloid) and its acetate [Singh et al., 1975]; sitosterol, stigmasterol, oleanolic acid and its 3β acetate, saponins containing oleanic acid, galactose and mannose [Singh and Dhar, 1977]; triterpenes [Singh and Kapoor, 1975]; mannogalactans (polysaccharides) [Venkata Rao et al., 1991; Adinolfi et al., 1994] etc; have been reported. Seeds are rich source of polysaccharide gum suitable for use in paper and textile industries. The wood is very hard and termite resistant, but splits easily and is therefore not suitable for carving.

1.1.2 Previous phytochemical and biochemical studies on *S. potatorum*

Selected phytochemical and biochemical investigations carriedout on *S. potatorum* are shown in Table 1.

Table 1: Selected phytochemical and biochemical studies on *S. potatorum*

Research findings	Reference
Mechanism of the clarification of muddy water by seeds.	Subbaramiah and Sanjiva Rao,1937
A pilot study on the use of nirmali seed as a coagulant.	Bulusu and Sharma 1965
Diaboline, the major alkaloid in root, stem, leaves and seeds.	Singh, 1974; Rao *et al.,*1990
Nirmali seed as an efficient naturally occurring coagulant.	Tripathi *et al.,* 1976
Reported the presence of isomotiol, sitosterol, stigmasterol and campesterol from the leaves and the bark mixtures.	Singh *et al.,*1978
Reported the chemical constituents of the seeds, and seed oil and as well as pharmacological studies on its alkaloid	Singh *et al.,* 1973; 1976; 1977; 1980; Kapoor,1988
Reported the occreence of mannogalactan (composed of mannose and galactose) and its antihypercholesterolemic activity (inhibition on cholesterol and triglycerides) .in rats	Rao *et al.,*1990
24 phytochemcials have been isolated and identified in the root bark.	Massiot *et al.,*1992
Report on a coagulant polysaccharide fraction from seeds and found that this fraction comprises a 1:1.7 mixture of a galactomannan and a galactan.	Adinolfi *et al.,* 1994
Seeds were found to be useful for nuclear waste treatment including heavy metals.	Jayaram,1993 ; Puvvada and Chandrasekhar 1997
The anti-diarrhoeal activity of the methanol extract of the dried seeds has been evaluated in rats using different models	Biswas *et al.,* 2002
Posses strong anti diabetic activity.	Mandal, *et al.,* 2002
The methanolic seed extract exhibited strong anti diuretic activity in Wistar albino rats and concluded that this evidence further supports the use of the seeds as a diuretic in folk remedies.	Biswas *et al.,* 2001
Seeds are used in the Indian traditional system of medicine for the treatment of hepatopathy, nephropathy, gonorrhea, leucorrhoea, gastropathy, bronchitis, chronic diarrhoea, strangury, renal and vesicle calculi, diabetes and eye diseases. Seeds are reported to exhibit the hepatoprotective and antioxidant activities.	Sanmugapriya *et al.,* (2006)
Toxicological investigations on *Strychnos potatorum* Linn seeds in experimental animal models	Ekambaram and Subramanian; 2006
Biosorption of lead from aqueous solution by its seed powder	Jayaram et al.,(2009)
Binding of cadmium to *S. potatorum* seed proteins in aqueous solution: Adsorption kinetics and relevance to water purification	Mohammad et al., (2012)

As The ripe seeds are used traditionally for clearing muddy water, this attracted us to study the biochemistry of the seeds as there are no reports till the date on that, the first deal with this seeds was to isolate and purify the protein components of the seeds and study their ability to bind the heavy metal from the aqueous solutions to be used in the future for water purifications and removal of heavy metals from the water.

1.2 Heavy metals

Heavy metals toxicity and the danger of their bioaccumulation in the food chain represent one of the major environmental and health problems of our modern society. Metal ions are probably the oldest toxins known to mankind and since they are not subjected to biodegradation and for all practical purposes they have infinite residence time in different environment compartments [Horwarth; 1996]. The degree of toxicity varies greatly depending on the metal itself and its mode of action on the specific organism [Sarkar et al 2007]. There are 35 metals that concern us because of occupational or residential exposure; 23 of these are the heavy elements or "heavy metals": antimony, arsenic, bismuth, cadmium, cerium, chromium, cobalt, copper, gallium, gold, iron, lead, manganese, mercury, nickel, platinum, silver, tellurium, thallium, tin, uranium, vanadium, and zinc [Glanze; 1996]. A term “heavy metal” is commonly used to describe the toxic metals. However, the term is rather used in causal language and has never been defined by any authority i.e., IUPAC [Duffus; 2002]. There are numerous definitions of “heavy metals” classifying according to certain physical, chemical or biological properties, including density (specific gravity), atomic weight and number, other chemical properties, definitions without a clear basis other than toxicity or nonchemical definitions [Duffus; 2002]. Another definition of heavy metals was defined them as chemical elements with a specific gravity that is at least 5 times the specific gravity of water. The specific gravity of water is 1 at 4 °C (39 °F). Simply stated, specific gravity is a measure of density of a given amount of a solid substance when it is compared to

an equal amount of water. Some well-known toxic metallic elements with a specific gravity that is 5 or more times that of water are arsenic, 5.7; cadmium, 8.65; iron, 7.9; lead, 11.34; and mercury, 13.546 [Lide 1992]. The majority of definitions have no relation with toxicity of these metals to living organisms since in fact no relation between density and atomic / molecular weight and toxicity has been observed [Duffus; 2002]. Toxic metals are understood to be elements (not only metals) commonly used in industry and generally toxic to living organisms even at low concentrations [Volesky and Naja; 2005], including As, Cd, Cr, Cu, Pb, Hg, Ni, Se, Zn [Scott and Smith; 1981]. Se and As are frequently named with a term “heavy metal” although these elements are not metallic [Wang and Chen; 2009]. Three kinds of heavy metals are of concern, including toxic metals (such as Hg, Cr, Pb, Zn, Cu, Ni, Cd, As, Co, Sn, etc.), precious metals (such as Pd, Pt, Ag, Au, Ru etc.) and radionuclides (such as U, Th, Ra, Am, etc.) [Wang and Chen, 2006., 2009]. However, when considering toxic properties, they are classified to the same group, similarly as the remaining toxic elements, and are named toxic metals [Kabata-Pendias, 2002]. It is interesting to observe that the small amounts of these elements are common in our environment and diet and are actually necessary for good health, in low quantities, but simultaneously at elevated levels are toxic [Conti and Cecchetti 2001]. To follow the fate of metallic species after they enter the ecosystem becomes very difficult and they start to inflict the damages as they move through from one ecological trophic layer into another. They accumulate in living tissues throughout the food chain which has humans at its top. With increasing amounts of metals on earth the amounts of toxic heavy metals entering the environment increase. They threaten humans as they become pre-concentrated throughout the food chain. The danger multiplies and humans eventually tend to receive the problems associated with the toxicity of heavy metals pre-concentrated and from many different directions. The resulting health problems demonstrate themselves on the acute as well as chronic levels and are reflected in the well-being of

individuals and in society's spiraling health care costs [Volesky; 2001]. Large amounts of any of them may cause acute or chronic toxicity (poisoning) figure 2. Shows the some toxically effects of heavy metals on humans. There are metals that play only harmful role in living organisms, through enzyme inhibition or activation, damage of subcellular organelles, carcinogenicity, and effects on kidneys, nervous system, endocrine system, reproduction, and respiratory system [Hodgson; 2004].

Figure 2 : Heavy metal toxicity. Drawn from the source, (International Occupational Safety and Health Information Centre 1999).

They do not play any positive function in living organisms. This is so-called *toxic trio* that includes Hg, Pb and Cd – the mostly toxic elements used by the industry [Hui et al; 2006]. Heavy metal pollution has become a severe public health concern and one of the most important environmental problems worldwide [Lin, et al; 2011]. The discharge of heavy metals to the environment is increasing continuously as results of industrial activities and technological developments [Jayaram and Prasad; 2009]. Mining, agriculture, smelting of metalliferous ores, metallurgy, surface finishing industry, energy and fuel production, combustion of fossil fuels, faulty waste disposal, fertilizer and pesticide industry and application, metallurgy, iron and steel, electroplating, electrolysis, electro-osmosis, leatherworking, photography, electric appliance manufacturing, metal surface treating, aerospace and atomic energy installation etc and military operations have released enormous amounts of toxic heavy metals and metalloids into the environment [Wernick and Themelis, 1998; Wijnhoven et al, 2007; Wang and Chen 2009]. Among these, the following four appear as the main priority targets, particularly in the industrialized world [Volesky; 2007] i) acid mine drainage (AMD)—associated with mining operations, ii) electroplating industry waste solutions (growth industry), iii) coal-based power generation (throughput of enormous quantities of coal), iv) nuclear power generation (uranium mining/processing and special waste generation), Since the presence of toxic metals in the environment is not desired, their emission by the industry is regulated by law. It is difficult to employ conventional methods to remove metal ions below the level of “ppms” since these methods if applied at such low concentrations cause that the methods become expensive and highly energy consuming [Diniz and Volesky; 2005]. These disadvantages of conventional technologies created the need to elaborate a new generation of efficient methods of environmental prevention (intervening at the impact source, in advance of pollutant event), protection (elimination of

The effects of pollutant actions or minimization of these effects) and restoration (removing damages caused by previous actions) [Beolchini; 2006], as well as monitoring [Kabata-Pendias; 2002]. Due to their persistence in nature, these pollutants could be dispersed in water, accumulated in plants and animals, and finally in human beings through the food chain or by consumption of contaminated water, causing serious health hazards [Li et al., 2009)]. Metal removal from waste streams and provides brief overview of the potential of biosorbents and biosorption technology. Considered are various aspects of utilization of microbial and plant derived biomass in connection with biosorption and the possibility of exploiting such material for heavy metal removal form solutions.

1..2.1 Necessity of water treatment (removal of heavy metals) and recycle

The quality of human life depends on the chemical composition of food and of the surroundings. The improvement in quality of life and growth in urban centers, and manufacturing activities have led to increased consumption of water. Currently, most of the used water in urban centers ends up at wastewater treatment facilities from where it is disposed of into bays or large water bodies (rivers, lakes, seas or oceans). In order to improve the quality of life and water resources, regulatory agencies have imposed high quality wastewater disposal standards. In some parts of the developed world, used water from secondary treatment facilities is recycled for human consumption and other uses including groundwater recharge and irrigation. At the industrial scale, however, only part of the water (boiler feed water, cooling tower water, and chilled water) is recycled. Water reuse and recycling should be part and parcel of an efficient water resources management scheme. Water resources management schemes should certainly include the large volumes of industrial wastewaters that mostly end up at disposal sites. To realize the goal of recycling used water, it is imperative to develop a rapid and efficient technology to treat secondary wastewater-treatment effluents. A combination of rapid mass exchangers (low-efficiency

filtration, high-efficiency molecular filtration, followed by or combined with bio-treatment) followed by disinfection via irradiation of the treated water with UV light can be optimized to treat secondary wastewater effluents to produce recycled water for human consumption, industrial use, irrigation, and/or artificial recharge. The performance of mass exchangers should be optimized in terms of particulate-separation efficiency, microbial presence detection due to bio-fouling and efficiency in reducing the biological oxygen demand (BOD) and certainly, efficiency at removing metals. Treated water can then be analyzed to detect BOD and microbial populations and these data should be compared to natural groundwater. More work is needed to understand nanoparticles and copper interactions and how they affect treatment of wastewater for reuse. Markets in water rights, based on regional and ethical trading practices, are likely to evolve as a rising population leads to shortages and climate change causes drought and famine. Trading water is unethical or even a breach of human rights, but water rights are already bought and sold in arid areas of the globe. Heavy metal contamination of both surface and ground water has become a common universal phenomenon. Heavy metals are directly or indirectly discharged into the environment by industries such as metal plating facilities, mining, fertilizer, tanneries, batteries, paper and pesticides etc. besides anthropogenic and geogenic sources.

Figure 3: Water security [Current Opinion in Environmental Sustainability 2011, 3:497–505 Oswald Spring,Ursula].

As shown in figure 4. Many technologies have been employed to eliminate heavy metal from aqueous solution, such as ion-exchange [Maheswari and Subramanian; 2004], coprecipitation [Soylak and Tuzen; 2008., Soylak, et al; 2005], membrane filtration [Ihara, et al; 2008., Feng, et al; 2011], electrolysis [Feng et al; 2007] and extraction [Li et al; 2011., Hidalgo et al 2006]. However, these processes have some disadvantages, including low treatment efficiency for trace amount of heavy metal ion, high operational cost and difficult further treatment due to generation of toxic sludge [Chakravarty et al; 2010].

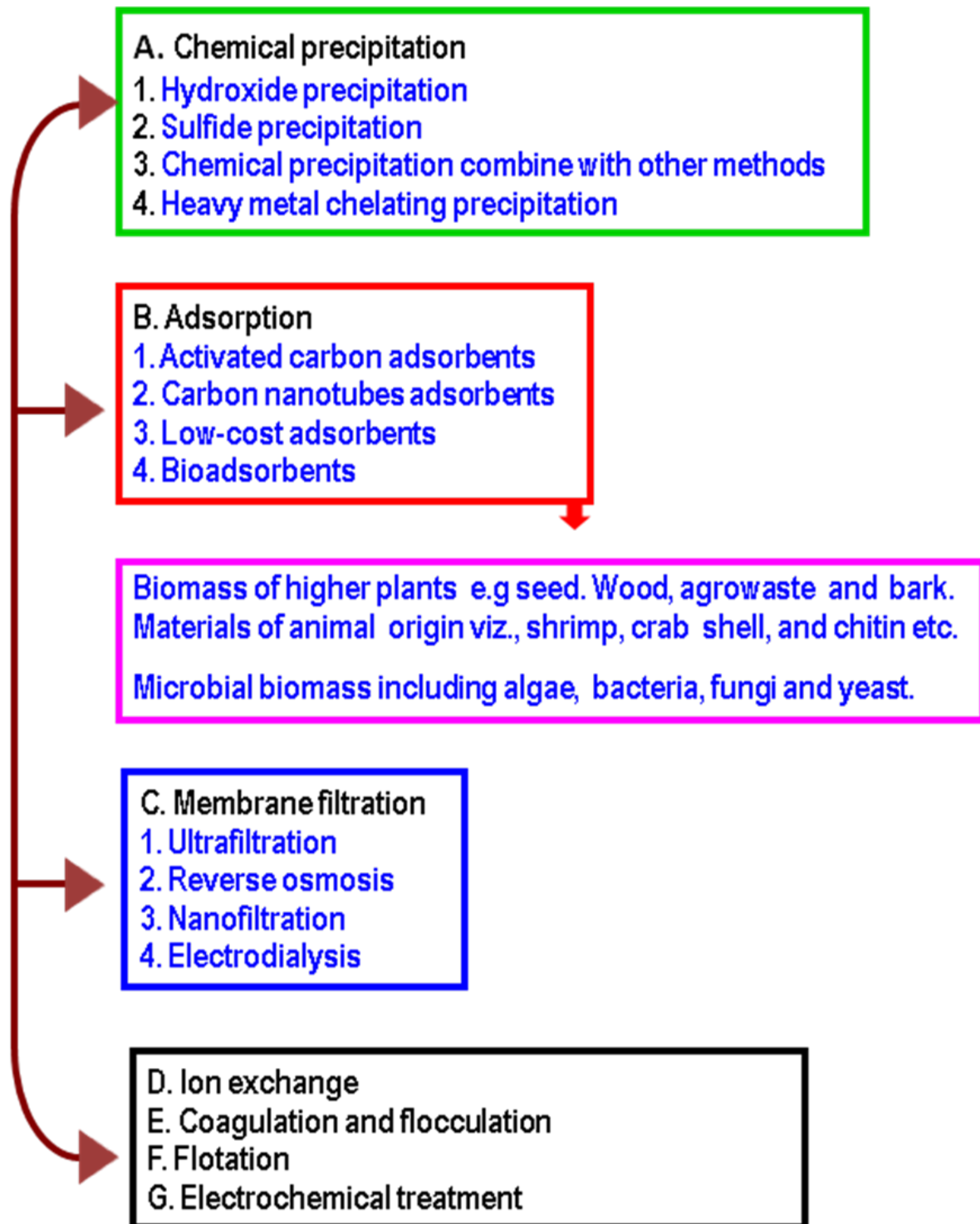


Figure 4: Important strategies for removal of heavy metals in wastewater [for details see Fu and Wang 2011].

In contrast, adsorption could be the most cost-effective for heavy metal removal due to a variety of advantages such as easy operation, high efficiency over a wide concentration range and low secondary pollution with suitable regeneration operation [Rafatullah et al, 2009., Semerjian, 2010., Chen et al, 2011., and Boyac et al, 2010].

1.3 Biosorption

Natural and controlled processes of biosorption and bioaccumulation may be efficiently used in the assessment of environmental pollution as well as in pollution prevention and cleaning the polluted environment. The property that is used in such pollution control processes is the ability of all types of biomasses to bind toxic metals, in different extent though, depending on morphology and physiology of an organism. If the processes are performed at controlled conditions, the efficiency can be greatly improved. By biosorption process, metals can be transferred from e.g., aquatic environment and become concentrated in bottom sediments. Biosorption is significant stage of toxic elements cycles in the environment. This phenomenon have been used for decades in conventional biological wastewater treatment plants in which scattered soluble impurities (nutrients, toxic metals and organic compounds) are transferred from dilute soluble form into concentrated, condensed several fold in the biomass of activated sludge with the simultaneous use of processes of biosorption, bioaccumulation and biodegradation. While organic pollutants can be processed by the biomass with the use of all three processes, metal ions can only be utilized with biosorption and bioaccumulation – biodegradation is not possible. The advantage of activated sludge method is that the impurities become concentrated and transferred into solid state that facilitates their utilization. Capacity of the biomass to bind and concentrate toxic metals from solutions may create the fundamentals for a cost-effective technology for detoxification of industrial effluents [Davis et al 2003] mainly from mining and electroplating industry or to recover precious metals from processing solutions [Williams et al; 2000]. Biosorption by

different materials of plant (microalgal biomass, aquatic plants, plant leaves, straw, and grass) and animal origin (eggshells, bones) can be applied in industrial wastewater treatment processes [Volesky and Naja, 2005]. It is probably true that most biosorption studies have been and continue to be carried out on microbial systems, chiefly bacteria, microalgae and fungi, and with toxic metals and radio nuclides, particularly actinides and lanthanides [Texier et al; 1999., Gadd and White; 1989., Gadd and White; 1992., Tsezos and Volesky; 1981]. However, practically all biological material has an affinity for metal species and a depth of other research exists with macro algae (seaweeds) as well as plant and animal biomass and derived products (e.g. chitosan). While most biosorption research concerns metals and related substances, unsurprising in view of the nature of adsorption and ion exchange mechanisms, the term is now applied to particulates and all kinds of organic substances. However, despite continuing dramatic increase in published research on biosorption (Figs 5) there has been gradual rise in publications on "Biosorption" using a variety of materials and applications. A total of 21,745 articles with only 472 in 1999 have been indexed in the Scopus web of science, (www.sciencedirect.com), in between 1999 and 2011, there has been little or no exploitation in an industrial context.

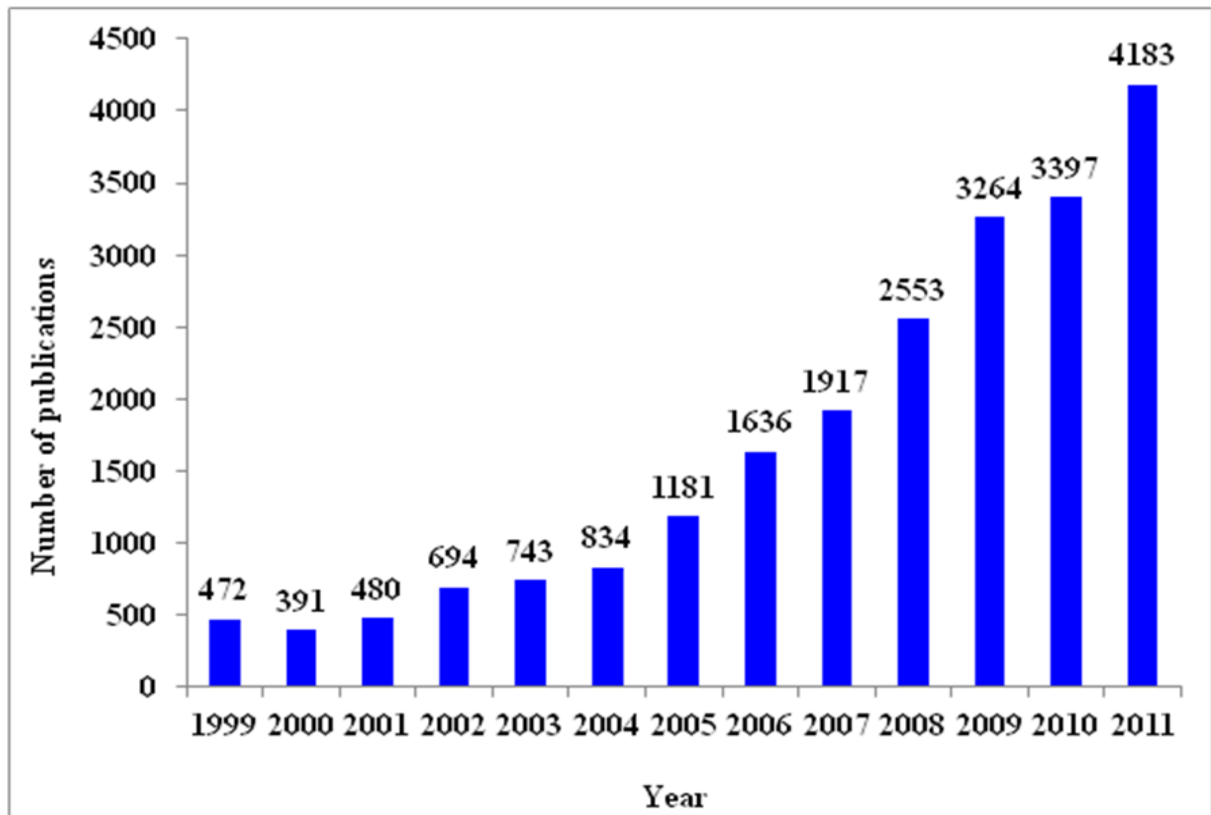


Figure 5 : Number of papers as we can also observe gradual rise in publications on "Biosorption" using a variety of materials and applications. A total of 21,745 articles have been indexed in Scopus web of science starting with only 472 in 1999. (Source: www.sciencedirect.com).

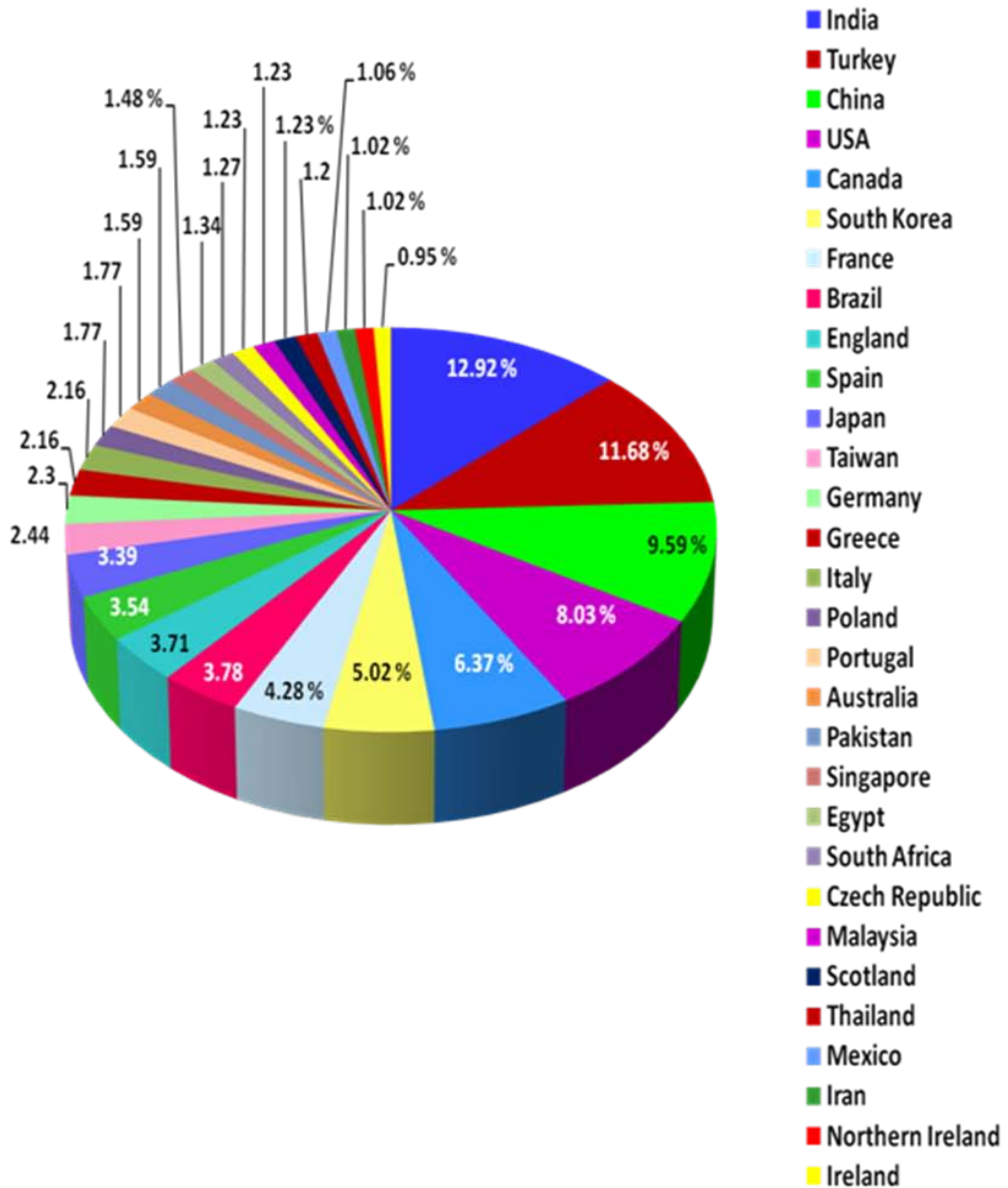


Figure 6: Top 30 countries publishing papers with 'biosorption' in the topic as listed in the ISI web of science database for 'All Years' (1970–2008) (out of a total of 2824 articles appearing : database searched 7.4.08: percentage values listed to two decimal places only).

Recently attention has been drawn towards the development of alternative methods like biosorption which uses organic materials as biosorbent. Since the last decade, biosorption or sorption of contaminants by sorbents of natural origin has gained important credibility due to its good performance and low cost of these complexing materials [Jayaram and Prasad; 2009].

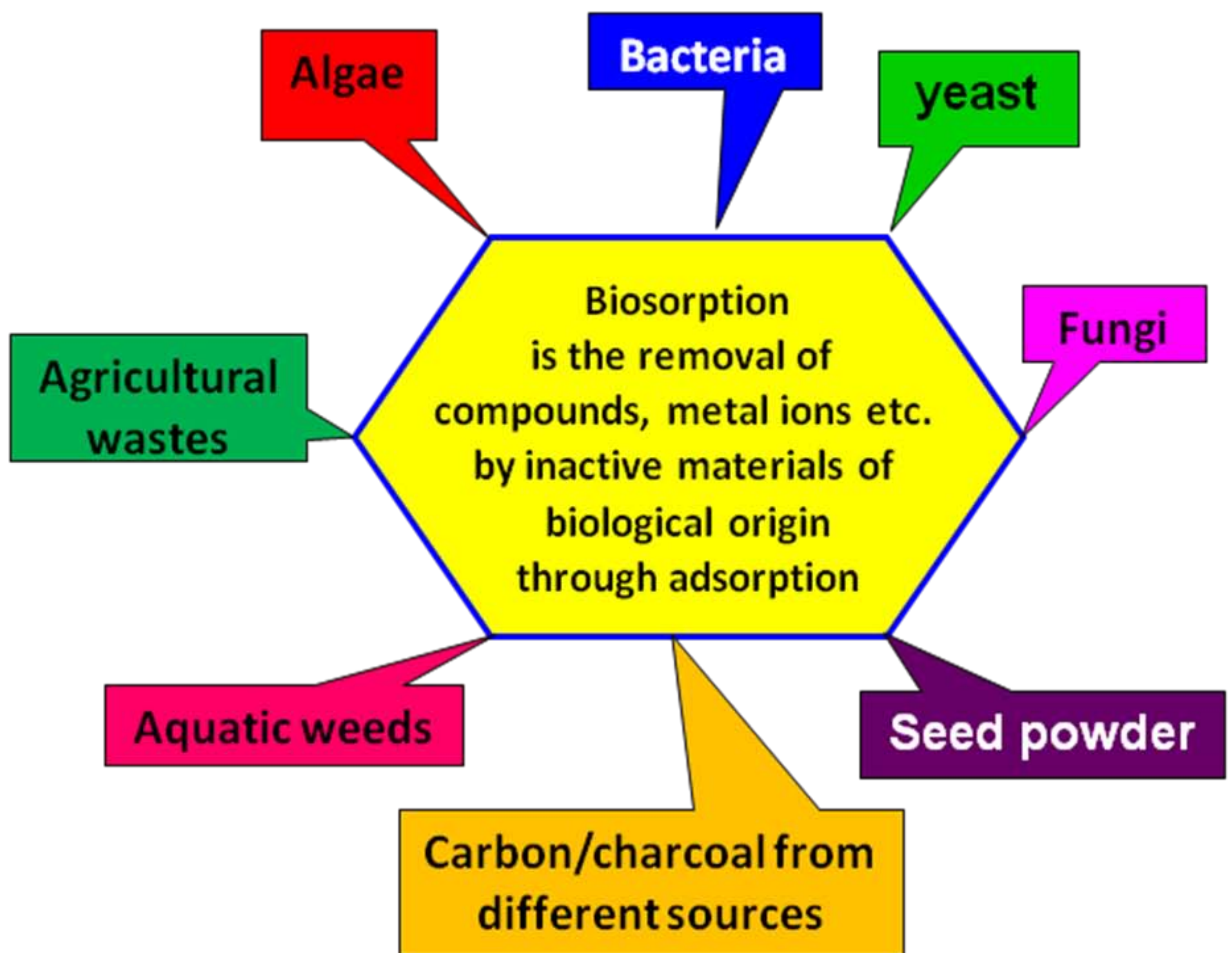


Figure 7: Wide variety of bioresources that are used for biosorption of toxic heavy metals [Volesky, B. and Holan, Z.R. 1995. Biosorption of heavy metals. Biotechnol. Progress 11, 235-250].

Biosorption is a general property of living and dead biomass to rapidly bind and abiotically concentrate inorganic or organic compounds from even much diluted aqueous solutions. Biosorption can be defined as the microbial adsorption of soluble/insoluble organic/inorganic metals by a metabolism independent, passive, or by a metabolism dependent, active process [Kaushik and Malik; 2009., Ma et al, 2011a]. Biosorption may be simply defined as the removal of substances from solution by biological material. Such substances can be organic and inorganic, and in gaseous, soluble or insoluble forms. Biosorption is a physico-chemical process and includes such mechanisms as absorption, adsorption, ion exchange, surface complexation and precipitation. Biosorption is a property of both living and dead organisms (and their components) and has been heralded as a promising biotechnology for pollutant removal from solution, and/or pollutant recovery, for a number of years, because of its efficiency, simplicity, analogous operation to conventional ion exchange technology, and availability of biomass [Geoffrey; 2009]. As a specific term, biosorption is used to depict a method that utilizes materials of biological origin biosorbents formulated from non-living biomass for the removal of target substances from aqueous solutions. Biosorption "traditionally" covers sequestration of heavy metals as well as rare earth elements and radio nuclides or metalloids, but the research and applications extended to the removal of organics, namely dyes [Kaushik and Malik 2009]. Due to high uptake capacity and very cost effective source of raw materials, biosorption is a progression towards a perspective method. Biosorption is being proposed for the recovery of high-value proteins, steroids, pharmaceuticals and drugs [Volesky; 2007].

13.1 The principle of biosorption science, and future thrusts

The underlying principles of biosorption for the removal of metal ions, the kinetics of mass transfer during the process of biosorption of metal ions, the theory and models that can be used to describe the mass transfer process and the thermodynamics of biosorption of heavy

metals onto biomass and the models, which can be used to quantify metal– biomass interactions at equilibrium, all are key knowledge areas in biosorption science [Rajkumar et al; 2012., Tan and Khan; 1988]. Compared with conventional or some biological methods for removing metal ions from industrial effluents, the biosorption process offers the advantages of low operating cost, minimization of the use of chemicals, no requirements for nutrients or disposal of biological or inorganic sludge, high efficiency at low metal concentrations, and no metal toxicity issues. Future efforts to improve selectivity and shelf life of bio-sorbents, further information on biosorption mechanisms and reliability and performance of biosorption models as well as more pilot scale demonstrations should bring convincing marketing arguments for large-scale applications. Biosorption also has the potential to find an industrial application in the future separation technologies with renewable bio-sorbents complementing conventional methods in hybrid or integrated installations.

1.3.2 Factors affecting biosorption

Biosorption is affected by various physical and chemical factors such as pH of the solution, temperature, biomass loading, pretreatment, ionic strength, biosorbent dosage, biosorbent size, initial solute concentration and agitation rate [Vijayaraghavan and Yun 2008]. pH usually plays a major role in biosorption and seems to affect the solution chemistry of metals/dyes and the activity of the functional groups of the biomass. Temperature seems to affect biosorption only to a lesser extent within the range from 20 to 35 ^{o}C. Higher temperatures usually enhance sorption due to the increased surface activity and kinetic energy of the solute [Vijayaraghavan and Yun 2008]. The ionic strength is a very important parameter that effect in biosorption, which influences the adsorption of solute to the biomass surface. The effect of ionic strength may be ascribed to the competition between ions, changes in the metal activity, or in the properties of the electrical double layer. In many instances, lower biosorbent dosages yield higher uptakes and lower percentage removal

efficiencies. An increase in the biomass concentration generally increases the amount of solute biosorbed, due to the increased surface area of the biosorbent, which in turn increases the number of binding sites [Ackmez et al; 2012].

1.3.3 Biosorption current studies

Recently the research on biosorption as it is shown in (Figure 8). focuses mainly on removal of toxic metals commonly used in industrial processes, such as Pb [Naja et al; 2006., Lodeiro et al; 2005., Kiran et al; 2005., Vilar et al; 2006., Moon et al; 2006., Tunali et al; 2006., Ruelas-Inzunza et al; 2005], Cu [Lodeiro et al; 2005., Kara; 2004., Aksu and Dönmez; 2000, Al-Rub et al; 2006., Chen et al; 2005., Kiran et al; 2005., Vilar and Botelho; 2005., Beolchini et al; 2006] Cr [Loukidouet al; 2004., Sun et al; 2008., Ozdemir and Baysal; 2004., Srinath et al; 2002], Cd [Naja et al; 2006., Lodeiro et al; 2005., Kiran et al; 2005., Vilar et al; 2006., Akar and Tunali; 2005., Lodeiro et al; 2005], Zn [Kargi and Cikla; 2006., Chen et al; 2005], Ni [Hawari and Mulligan; 2006] There are few studies devoted Hg [Chen et al; 2005], Al [Ozdemir and Baysal; 2004] and Co, As, Th. Currently studied sorbents include Microorganisms: aerobic [Hui et al; 2006., Liu et al 2003] and anaerobic [Hawari and Mulligan; 2006] granular biomass, bacteria (*Sphaerotilus natans* [Beolchini et al; 2006., Beolchini et al; 2006., Kamer et al; 2003], *Pseudomonas putida* [Chen et al; 2005]), microalgae (*Chlorella vulgaris* [Al-Rub et al; 2006], *Microcystis aeruginosa* [Chen et al; 2005]), macroalgae (*Gelidium*) and algal waste [Vilar and Botelho; 2005., Vilar et al; 2006], *Sargassum muticum* [Lodeiro et al; 2005], fungi (*Cephalosporium aphidicola* [Tunali et al; 2006], *Rhizopus arrhizus* [Naja et al; 2005., Zhou; 1999], *Neurospora crassa* [Kiran et al; 2005], biological products (extracellular polysaccharide (Pestan) produced by fungus *Pestalotiopsis* [Moon et al; 2006] and other fungal by-products (*Botrytis cinerea*) [Akar and Tunali; 2005], cellulose/chitin bed [Zhou et al; 2005], chaff [Hassler et al; 2005]. Various plant materials viz.waste tea leaves [Sun et al; 2008], *Quercus ilex* leaf, stemand

rootphytomass[Prasad and Freitas; 2000], sphagnum moss peat [Ho et al; 1996], *Medicago sativa* [Gardea-Torresdey et al; 1998], sawdust [Yu et al; 2001], peat [Ho and McKay; 1999], rice polish [Singh et al; 2003], *Caladium bicolor* biomass [Jnr and Spiff; 2005], maize leaf [Adesola et al; 2006, *Moringa oleifera* seed powder and whole seed kernel [Sajidu et al; 2005., Mataka et al; 2006], maize bran [Singh et al; 2006], sago waste [Quek et al; 1998], *Oryza sativa* husk [Zulkali et al; 2006], palm shell activated carbon [Issabayeva et al; 2006], *Azadirachta indica* leaf powder [Bhattacharyya and Sharma; 2004], olive pomace [Pagnanelli et al; 2006], *M. stenopetala* [Mataka et al; 2006], sawdust [Abdel et al; 2007], *Saraca indica* leaf powder [Goyal et al; 2008], *S. potatorum* seed powder [Jayaram et al; 2009], coconut and seed hull [Gueu et al; 2007], have been studied for heavy metals removal from aqueous system. Trends in current research include studies on kinetics [Liu et al; 2003., Kargi and Cikla; 2006., Tunali et al; 2006] and equilibrium [Beolchini et al; 2006., Vilar et al; 2005., Loukidouet al; 2004., Beolchini et al; 2006., Chen et al; 2005., Tunali et al; 2006], influence of pH [Hui et al; 2006., López et al; 2000., Vilar and Botelho; 2005], ionic strength [Beolchini et al; 2006., Vilar and Botelho; 2005., Beolchini et al; 2006] and temperature [Vilar and Botelho; 2005], including modelling [Beolchini et al; 2006., Schiewer and Volesky; 1996., Vilar et al; 2005., Beolchini et al; 2006., Vilar et al; 2006]. Reports contain investigation of biosorption in single- and multi-metal systems [Al-Rub et al; 2006]. A particular attention is paid to identification of the mechanism of the process [Zhou et al; 2005], including i.e., metal-based potentiometric titration [Naja et al; 2005., Naja et al; 2005]. Also, some modifications (pretreatments) of the biomass are tested [Kaduková and Virčíková; 2005], i.e., protonation [Lodeiro et al; 2005]. The new process configurations are also studied extensively, including carrying out the process in membrane bioreactors [Beolchini et al; 2006].

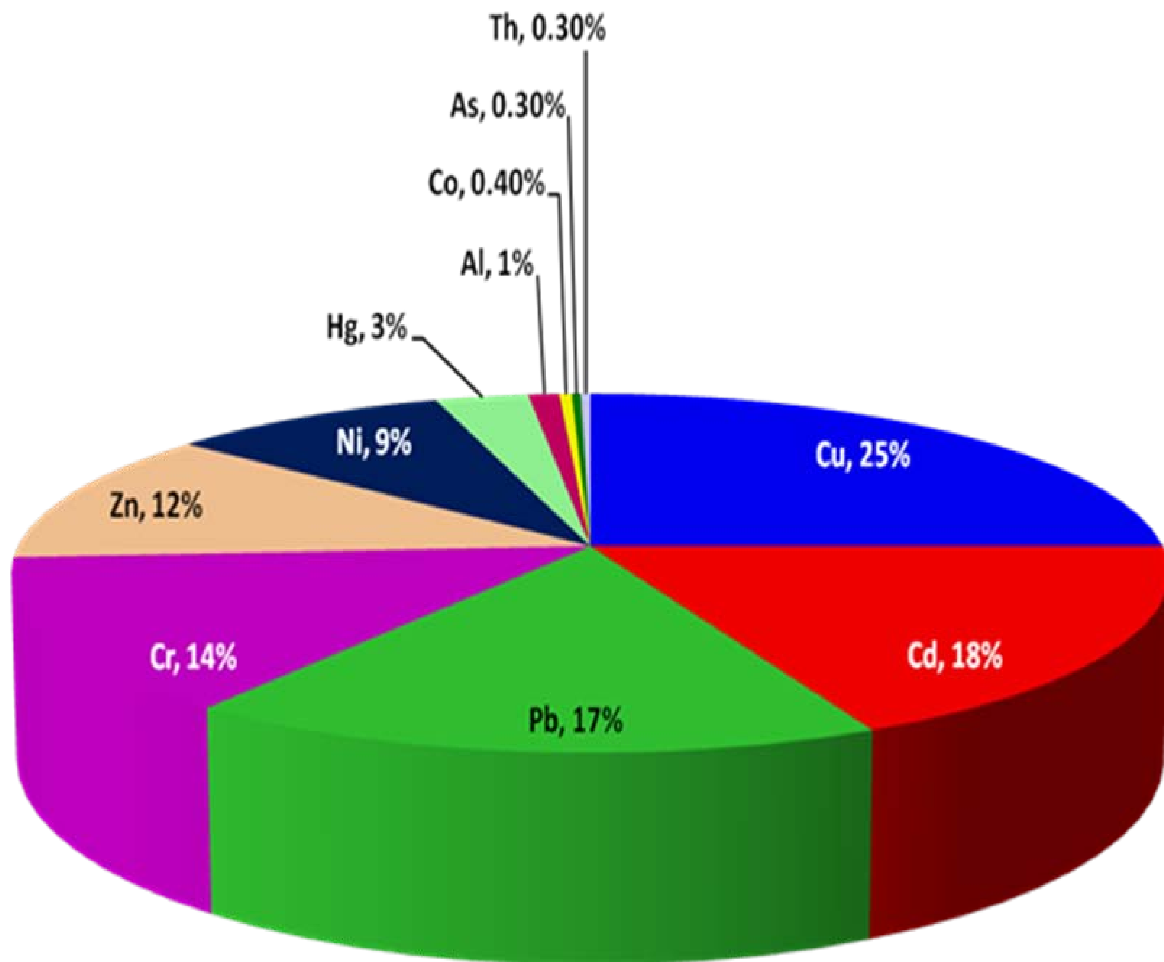


Figure 8: Current trends in research in biosorption

1.4 Polysaccharides

There is a growing interest in the use of polysaccharides due to their biocompatible, biodegradable and environmentally benign characteristics. Polysaccharides are high molecular weight polymers constituted with simple sugar monomers such as glucose, fructose and mannose. They are abundant in nature, universally found in almost all living organisms. They are present in various tissues of seeds, stems and leaves of plants, body fluids of animals, shells of crustaceans and insects. They are also found in the cell walls and extra cellular fluids of bacteria, yeast and fungi and are thus renewable reservoirs for synthesizing high performance materials [Davidson, 1980., BeMiller and Whistler, 1992]. Polysaccharides have complex structures and they are the most abundant of many natural products and the source of most of the biological energy. They consist of monosaccharides linked together by O-glycosidic linkages, and diversification of their monosaccharides yields a variety of properties. Although they are made up of single type of building blocks, their enormous diversity has led to a bewildering variety of species, structures and properties all performing a large variety of functions of great significance [Linhardt, 1986]. They can also easily undergo chemical [Miyata et al, 2002] and biochemical modification [Donnelly, 1999] to generate novel products with unique rheological and applicative properties. They are found in abundance, widely available, inexpensive, and able to select some properties according to their monosaccharides [Hovgaard and Brondsted1996]. The interactions of some of the polysaccharides with other synthetic and biopolymers have further increased their range of applicability [Doublier, 1994., Perissutti et al, 2002., Alexandridis and Tsianou, 1999., Akiyoshi et al 1998]. The versatility in the structure and properties of the polysaccharides and their derivatives, along with their modifications find widespread applications including food [Sanderson, 1991], cosmetics [Glasser, 2000], petroleum [Best and Baird, 1989] and pharmaceutical [Franz et al, 1995] industries. Moreover polysaccharides are hydrophilic,

biodegradable, non toxic, stable and safe to use, which suggests their use in targeted drug delivery systems, can profoundly affect the immune system and therefore have the potential as immunomodulators with wide clinical applications [Sinha and Kumria, 2001., Coviello et al, 2007., Tzianabos, 2000]. Many of polysaccharides have specific receptors on the cells so they can be easily taken by the cells, it was reported that hyaluronic acid (HA) was taken by HA specific receptor-mediated endocytosis, and HA was suitable for the targeted drug delivery systems via their specific receptor [Coradini et al, 1999., Luo et al, 2002]. Some glucosides, such as asialoglycoproteins and galactosides, were known to be suitable for the receptor-mediated drug delivery systems. It was reported that the mannosylated, fucosylated, and galactosylated liposomes showed high accumulation in the liver via each specific receptor [Kawakami et al, 2000]. Polysaccharides have been successfully used as drug carriers due to their superior properties and biocompatibility [Mao et al, 2010., Liu et al, 2008., Huh et al, 2010., Mok et al, 2007., Na et al, 2007., Mochizuki et al, 2009]. The polysaccharides can have linear, branched or cyclic structures containing residues of only one type of monosaccharides or of different types of monosaccharides. The polymers of one type of monosaccharide units are called homopolysaccharides (e.g. cellulose, starch, etc) and those containing different monosaccharide units are known as heteropolysaccharides (e.g., glucomannans: polymers of glucose and mannose, galactomannans: polymers of galactose and mannose sugars, cyclodextrins(CD) are cyclic polysaccharide with six or more glucose units arranged on a doughnut shaped ring [D'Souza and Lipkowitz, 1998]. Depending on their structure, monomer composition and conformations, the polysaccharides show different physical and chemical properties. Although large varieties of polysaccharides have a multitude of industrial uses, in many cases they need to be further modified to improve their applicability across the wide spectrum of end-uses. In the last few decades, wide ranges of chemical derivatives of these polysaccharides were developed [Srivastava, 1983]. The

chemical modifications improved the rheological properties of their solutions and hydrogels, while still maintaining their biodegradability and biocompatibility. Storage polysaccharides in seeds are mostly starches or galactomannans, in which the mannan back bone is built of *β* (l→4) linked mannose residues and single unit galactose side chains are attached α (l→6) to all or some of the mannose residues [Baker and Whistler, 1976]. The extent and pattern of galactosidation on the mannan backbone varies among plant varieties [Dey, 1978].

1.4.1 Galactomannan

Galactomannans are heteropolysaccharides containing residues of galactose and mannose monosaccharide units. They are an important group of polysaccharides produced in plants as cell wall and storage polysaccharides [Reid, 1985]. Galactomannan polysaccharides serve as water retainers for the seeds due to their swelling property, in addition to being food reserve for the germinating seeds [Appukuttan and Basu, 1987]. A large group of galactomannans are produced from seeds of *Leguminosae* family. Seed galactomannans are heterogeneous polysaccharides consisting mainly of the monosaccharides mannose and galactose units. The mannose-elements form a linear chain consisting of (1→4)-β-D-mannopyranosyl residues, with (1→6) linked α-D galactopyranosyl residues as side chain at varying distances, dependent on the plant of origin [Manjoosha and Kapoor, 2005], according to Aspinall [Aspinall, 1959], galactomannansare those mannans that contain more than 5% of D-galactose. The major difference in galactomannans from different seed species is in the ratio of D- galactose to D- mannose (M/G) ratio; however, there is also some variation in molecular weight of the polysaccharides. As these gums and their derivatives have different chemical properties which make them versatile materials used in for many different applications, including food, drug delivery, and health care products because of their natural abundance and their low cost and other desirable functionalities[Cheng et al, 2002., Mahammad et al, 2006]. In the open literature there is only little information available about

the possibility of using guar gum based nanosized materials as a drug carrier. Nanoparticles of galactomannan (Guar gum) from the seeds of *Cyamopsis tetragonoloba*, was prepared and characterised [Rema et al, 2010].

1.4.2 Occurrence

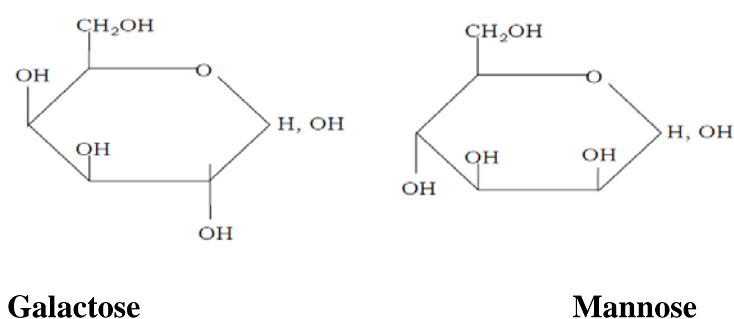
The majority of galactomannans originate from the endosperm of dicotyledonous seeds of numerous plants, particularly the *Leguminosae* family. Out of 163 legume- seeds examined, 119 seed endosperm mucilage contained galactomannans as the major, reserve polysaccharide constituent [Vandana and Mathur, 2005], some of them are presented in (Table 2). The endosperm has several functions: it serves as food reserve for germinating seeds and it retains water, preventing the complete drying of the seeds [Gidley and Reid, 2006]. The two main groups of galactomannan polysaccharides are those derived from (a) the endosperm of plant seeds (the vast majority of which originate in the *Leguminosae*) and (b) microbial sources, in particular yeasts and other fungi. In these two groups the commercially preferable sources for the gum is the seed endosperm. Galactomannans also have been found in few species which are non- leguminous seeds. It has been extracted from members of *Annonaceae*, the *Convolvulaceae* and the *Palmae*, *Ebenaceae* and *Loganiaceae* such as *Annona muricata, Convolvulus tricolor, Ipomoea muricata, Diospyros virginiana, Strychnos nux-vomica, Cocos nucifera, Elaies guinensis, Coffea arabica, Phoenix dactylifera, Phyetelephas macrocarpa,* in few exceptional cases the gum occurs in the hull, the inner side of the seed coat, and kernel, such as in *Glycine max, Gymnocladus diocia,* and *Mucuna*, respectively

Table 2 Galactomannans of Leguminosae species these differ from each other on mannose / galactose (M/G) ratio

Family	Species	References
CAESALPINIACAE	*Cassia absus*	[Kapoor and Mukherjee, 1971., Kapoor and Mukherjee, 1969., Kapoor and Mukherjee, 1971]
	C. marginata	[Tookey et al 1962]
	C. leptocarpa	[Anderson, 1949]
	C. grandis	[Bose and Srivastava, 1978]
	C. occidentalis	[Gupta and Mukherjee]
	C. tora	[Gupta and Mukherjee]
	Ceratonia siliqua	[Anderson, 1949]
	Caesalpinia cacalaco	[Anderson, 1949]
	C. spinosa	[Anderson, 1949]
	Delonix regia (flame tree)	[Anderson, 1949]
	G. triacanthos	[Leschziner and Cerezo, 1970]
	Gymnocladus dioica	[Anderson, 1949]
	Parkinsonia aculeata	[Anderson, 1942]
MINNOSACEAE	*Leucaena leucocephala*	[Morimoto et al 1962]
	Leucaena galauca	[**Kapoor** and Farooqi, 1993., Morimoto and Unrar, 1962., Unrau, 1961]
	Genista raetan	[Dea and Morrison, 1975]
	G. scoparia	[Dea and Morrison, 1975]
	G. tanditica	[Anulov and Shcherbukhin, 1996]
	G. patula	[Anulov et al, 1998]
	Desmanthus illinoensis	[Anderson, 1949]
	Chamaecytisus ruthenicus	[Anulov and Shcherbukhin, 1996]
	Lotus corniculatus	[Somme, 1966]
	L. pedunculatus	[Dea and Morrison, 1975]
	L. scoparius	[Tookey et al, 1962]
	Lembotropis nigricans	[Anulov et al, 1998]
	Desmodium pulchellum	[Sinha and Tiwari, 1970]

1.4.3 Chemical structure and properties of galactomannan

The first report on galactomannan structure was probably the one made by Nadelman in the last century [Nadelmann, 1890]. He reported the presence of galactose and mannose in several legume species and noticed the property of galactomannans as viscous substances. With development of linkage determination by methylation analysis techniques, mainly during the second half of 20th century, the structures of galactomannans from several sources were determined. It is now clear that the majority of the molecules found in plants are composed of a linear backbone of β-(l- 4)-linked D-mannose residues to which single units of D-galactose residues are attached by α-(l- 6)-linkages. To understand the galactomannan chemical structure we have to know the structure of the monosaccharide units of the galactomannan



Galactose **Mannose**

Figure 9 the chemical structure for galactose and mannose polysaccharides

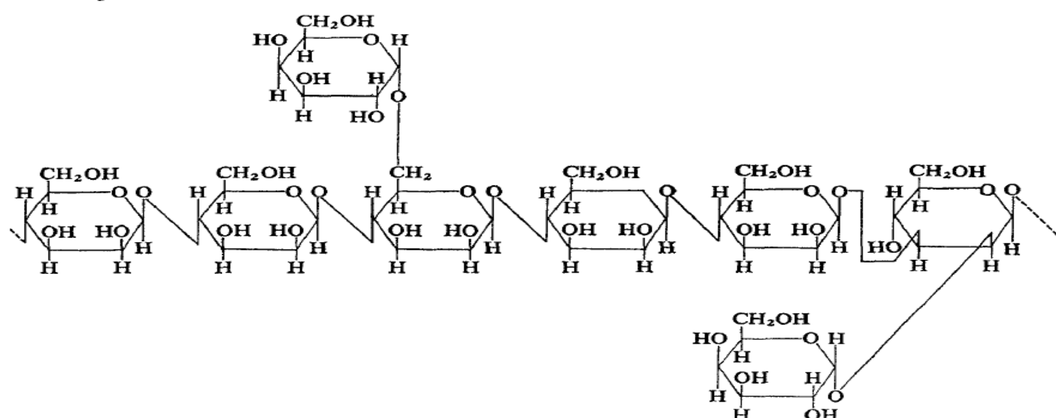


Figure 10 Probable structure of *Cassia absus* seed galactomannan

It is obvious that mannose is provided with cis-OH groups in the galactomannan polymer. Therefore, an enhanced deposition to form hydrogen bonds between the polymannan chains is expected as long as neighboring groups like galactose do not develop steric hindrance to prevent the galactomannan chains from coming too closer together and prevent the mannose cis-OH groups in forming the hydrogen bonds. Generally seed galactomannans, known as seed gums, are reserve heterogeneous polysaccharides consisting mainly of the monosaccharides, β 1-4 – linked linear mannose backbone, to which galactose linked by α, 1-6 glycosidic bounds. The mannose-elements form a linear chain consisting of (1→4)-β-D-mannopyranosyl residues, with (1→6) linked α-D-galactopyranosyl residues as side chain at varying distances, dependent on the plant of origin. Galactomannans from different legume seeds differ from each other in M: G ratio, for example the fenugreek gum has the highest galactose (~ 48%) in its molecule and its linear mannan backbone, has α, 1- 6 linked single galactose on each the mannose group of the main chain, where as other galactomannans from other seeds are having less galactose residues as it is shown in Table 3.

Table 3 Chemical structure of galactomannans from different sources

Galactomannan type	Source	Chemical structure
Galactomannan-1, 1	Fenugreek Gum	-M-M-M-M-M-M-M-M-M-M-M- each M substituted with G (11 G)
Galactomannan-1, 2	(Guaran) from the endosperm of *Cyamopsis tetragonoloba*	-M-M-M-M-M-M-M-M-M-M-M- every second M substituted with G (6 G)
Galactomannan-1, 3	(Tara gum) from the endosperm of *Cesalpinia spinosa*	-M-M-M-M-M-M-M-M-M-M-M- G substitution on 4 M units
Galactomannan-1, 4	(Carubin) from the endosperm of *Ceratonia siliqua*	-M-M-M-M-M-M-M-M-M-M-M- G substitution on 3 M units
Galactomannan-1, 5	(*Cassia* gum) from the endosperm of *Cassia tora/obtusifolia*	-M-M-M-M-M-M-M-M-M-M-M- G substitution on 3 M units

From the chemical structure of different galactomannans, showed in Table 3 and by the comparison of different galactomannans it is obvious that since additional cross linking via hydrogen bonds goes hand in hand with less solubility, an increase in substitution leads to higher solubility. The best solubility is found with galactomannan-1, 1 the viscosity of galactomannan-1 at a given concentration is lower than other galactomannans due to lower molecular weight. Because of a fully substituted backbone by galactose obviously establishes so much steric hindrance that it dissolves easily in cold water and prevents attack for some time for degrading enzymes. However, galactomannan-1, 2 where on average each second mannose unit is blocked by galactose, shows so much steric hindrance and so little hydrogen bonding between the molecular chains that it hydrates instantly. Galactomannan-1, 5 shows only partial solubility in cold water resulting in a low viscosity. After boiling, the same

suspension yields a thick, colloidal solution of high viscosity. This is caused by the thermal breakdown of the inter-molecular hydrogen bonds so that water can penetrate in between the chains leading to hydration of the molecules. Galactomannan-1, 4 does not dissolve in cold water but its solubility in boiling water is already better than that of galactomannan-1, 5 due to slightly higher degree of substitution by galactose side groups compared to galactomannan-1, 5. From the foregone discussion, it can be concluded that galactomannans, are rich in hydroxyl groups; this enables them to bind and take up water. Rich in cis-OH groups, which allow aggregation from chain to chain via hydrogen bonding so that hydration becomes more complicated if interchange cross linking can take place. By substitution with galactose, nature establishes steric hindrance between the molecules and thus enhances water solubility.

1.4.4 Applications and medicinal importance of galactomannans

Due to the unique rheological properties of galactomannans, a large of industrial sectors uses galactomannan. Galactomannans can often be used in different forms for human consumption. Featuring different physicochemical properties, galactomannans are a versatile material used for many applications: they are excellent stiffeners and stabilizers of emulsions, and the absence of toxicity allows their use in the textile, pharmaceutical, biomedical, cosmetics and food industries [Baveja et al, 1991., Krishnaiah et al, 2002., Varshosaz et al, 2006., Vendruscolo et al, 2009., Vieira et al, 2007]. Galactomannans have been reported to be useful for industrial applications and are usually classified as seed gums. In foods, they are normally used as thickeners, emulsifiers and stabilisers and also because they interact with other polysaccharides, changing the rheological features of gels such as xanthan, carragennan and agar [Klose and Glicksman, 1968]. If the small amount of galactomannan is ingested, it is capable to form an unstirred water layer within the large intestine, which has been suggested to inhibit absorption of cholesterol and glucose by humans [Cerda et al 1987].

Other reports has come to show if the diet complement withed with galactomannan the cholesterol absorption can be decreased by 10- 15%, and on this basis propose that galactomannan could be used therapeutically in patients who are not dependent on insulin [Torsdottir et al, 1989]. Galactomannans are also used a part from food, in cosmetic, paper, textile, explosives, oil prospection to name but a few. In the textile and paper industry, galactomannans are used mainly for its capacity to increase color printing quality. In this case, guar polymer is mixed with cellulose, to which it is thought to be adsorbed [Scherbukhin and Anulov, 1999]. The three major galactomannans of commercial importance in food and non-food industries are guar gum (GG, *Cyamopsis tetragonolobo*, M/G ratio: 2:1), tara gum (TG, *Caesalpinia spinosa*, M/G ratio: 3:1) and locust bean gum (LBG, *Ceratonia siliqua*, M/G ratio: 3.5:1) [Dakia et al, 2008]

Table 4 commercial and alternative sources of galactomannan and their possible applications [Cerqueira et al, 2011]

Species	Applications	references
Adenanthera pavonina *Prosopis pallida (*mesquite gum*)* *Mimosa scabrella* *Trigonella foenum graecum* (fenugreek gum)	Stabilizer, thickener,	[Cerqueira, Pinheiro et al., 2009] [Chaires-Martınez, Salazar-Montoya & Ramos-Ramırez, 2008] [Vendruscolo et al., 2009] [Prado et al., 2005]
*Ceratonia siliqua (*locust bean gum*)*	Thixotropic, binder, stabilizer, lubricator	[Prado, Kim, €Ozen & Mauer, 2005]
Cesalpinia spinosum (tara gum) *Dimorphandra gardneriana Tul* *Cyamopsis tetragonolobus* (guar gum)	Stabilizer, thickener, emulsifier, firming agent	[Prado et al., 2005] [Cunha et al., 2009] [Prado et al., 2005]
Caesalpinia pulcherrima *Gleditsia triacanthos*	Stabilizer, thickener, emulsifier	[Cerqueira, Pinheiro et al., 2009] [Bourbon et al., 2010; Cerqueira, Pinheiro et al., 2009; Sciarini, Maldonado, Ribotta, P_erez & Le_on, 2009]

Within the past decade detection of the *aspergillus* antigen galactomannan has become an important and reliable tool for the early diagnosis of invasive aspergillosis [Paule et al 2006]. The detection of circulating galactomannan (GM) has become an important tool in the early diagnosis of invasive aspergillosis (IA). GM is part of the outer layer of the *aspergillus* cell wall, and is released during growth of the fungus at the tips of the hyphae [Latge et al, 1994., Mennink-Kersten et al 2004]. The antigen can be detected using a commercially available sandwich ELISA (Platelia *Aspergillus*, BioRad, France)(PA-ELISA), which employs a monoclonal antibody (EB-A2) that binds to the galactofuran epitope of the GM antigen [Stynen et al 1995., Stynen et al, 1992]. The assay has been extensively studied and is now commonly used to monitor patients at high risk for invasive aspergillosis [Mennink-Kersten et al 2004., Verweij et al, 2006., Denning, 2000., Hope et al, 2005., Maertens et al, 2002]. Medicinal properties of the seed are due to soluble dietary fiber. Being non- metabolized in human system, the galactomannans act as dietary fibers and promote the beneficial pre-biotic coclon bacteria. Fenugreek gum, compared to other dietary fibers, shows maximum efficiency in lowering blood glucose and lipids including LDL and cholesterol. Clinical studies have shown that 2-3 g/day of fenugreek gum is effective in controlling blood sugar [Vandana and Mathur, 2005].

1.5 Nanotechnology and nanoparticles

Almost every day we read the "nano" words (nanoscience, nanotechnology, nanoscale, nanometer, nanosecond, nano-device, nano-electronics, nano-optics, and so on) from television commercials, research journal papers, magazine articles, newspaper headlines, and conferences. Such excitement comes from the undisputed fact that scientists all over the world have begun to control materials at a nanometer scale resulting in a great many new techniques and skills which can be used to make new materials with unique and useful properties [Nalwa, 2008]. Within the last decade, nanoscience and nanotechnology [Drexler, 1997., Drexler, 1992., Wilson, 2002., Regis a-nd Chimsky, 1996., Crandall and Lewis, 1992., Krummenacker and Lewis, 1994., Timp, 1999] have reached the status of leading sciences with fundamental and applied research in all basic physical, life, and earth sciences as well as engineering and materials science [Klabunde, 2001., Bard, 1994., Kawata, 2002., Grundmann, 2002., Nalwa, 1999., Mitura, 2000., Nalwa, 2001., Edelstein and R Cammarata, 1998., Hofmann et al 2002., Koch, 2002]. It shall lead to a tremendous amount of in-depth understanding as well as to the fabrication of novel high technological devices in many fields of applications from electronics to medicine [Reed and Lee, 2003., Freitas, 1999., Robinson, 1996., Hoch et al, 1996]. Therefore, it should improve tremendously the level of technological advance to a much greater rate than human history has ever experienced. Obviously, these extensive studies in nanoscience and nanotechnology have led to a range of commercial applications in electronics, photonics, spintronics, sensing, military uses, medicine, and so on. Certainly, the best and most exciting days of obtaining novel properties or other kinds of discovery in the nanoscience and nanotechnology are still ahead as some of these applications are represented in table 5 [Nagarajan et al 2008].

Table 5 Examples of present and potential applications of nanoparticles

Biomedicine	• Antibacterial creams and powders (Ag) • Gene delivery (CNT) • Biocompatible coatings for implants • Dental composites • Biolabeling and detection (Au, Ag, Quantum dots) • Biosensors (metal oxide, polymer nanoparticles, CNT) • Bone growth promoters (hypoxyapatite ceramics) • Cancer diagnostics and targeted drug delivery (magnetic nanoparticles) • Cell, receptor, antigen, enzyme imaging (Quantum dots) • Fungicides (ZnO, Cu20) • MRI contrast agents (Fe203, Fe304)
Consumer Goods and Personal Care Products	• Anti-bleaching, scratch resistance additives in paints • Anti-scratch coated tiles (alumina) • Barrier packaging (silicates) • Glass coatings for anti-glare, anti-misting mirrors (Ti02) • Skin creams with antioxidant vitamins (nanocapsules) • Sunscreens (ZnO and Ti02) • Water- and stain-repellent textiles • Tennis balls, rackets (nanoclays, carbon nanotubes))
Electronics and Computers	• Chemical mechanical planarization (alumina, silica, ceria) • Coatings and joining materials for optical fibers (Si) • Conductive coatings/fabrics (rare-earth-doped ceramics) • Display technologies (conducting oxides) • Ferro-fluids (Fe, FeCo, Fe304) • EMI shielding using conducting and magnetic materials • Electronic circuits (Cu, Al) • Magnetic particles for high-density data storage (Fe) • Optoelectronics devices (Gd203, Y 2 0 3 doped with rareearth metals)
Engineering Materials	• Anti-scattering layers in photographic film • Molecular sieves • Conducting/magnetic inks (metal powders) • Cutting tool bits (A1203, Zr02,WC, TaC)

	• Chemical sensors • Thermal spray coating techniques (Ti02 , TiC-Co) • Flame retardant polymer formulations (nanoclay) • Lubricants and sealants/hydraulic additives (Cu MoS2) • Pigments (metals and metal oxides) • Polymer composites (nanoclays, Ti02 , Si02) • Spark plugs (metal and ceramic powders) • Wear/abrasion-resistant coatings (alumina, Y-Zr203)
Environmental	• Controlled delivery of herbicides and pesticides • Self-cleaning glass (Ti02 based coatings) • Soil remediation (Fe) • Water treatment (photo-catalyst treatments, Ti02)
Food	• Flavors and colors in food and beverages (nanocapsules) • Frying oil refining catalysis (ceramics) • Food pathogen sensing • Food packaging materials (nanoclays, Si02, Ti02, Ag) • Neutraceutical delivery (liposomes, block copolymer micelles)
Power and Energy	• Anode and cathode materials for solid oxide fuel cells (nanoclays, CNT) • Catalysts for various fuel technologies (metals and metal oxides) • Conducting polymers for bipolar plates in fuel cells • Dye-sensitized solar cells (Ti02 , ZnO, Au) • Thermal control fluids (Cu) • Environmental catalysts (Ti02 , Ce02 as diesel additive) • Fuel cell catalysts (Pt in PEM cells) • Hydrogen storage (metal hydrides) • Improved electrodes in batteries and supercapacitors
Transportation	• Automated highways • Battery technology • High strength, light weight composites for increasing fuel efficiency • High temperature sensors • Improved displays • Thermal barrier and wear resistant coatings • Wear-resistant tires

1.5.1 Nanoparticles

The term nanoparticle is a collective name for any colloidal carrier of submicrometer dimension and includes nanospheres, nanocapsules, and liposomes. According to the national institutes of health (US), nanoparticles (NP) are defined as being in the 10-100 nm diameters. Biocompatibility and toxicity are major issues and vary between different forms of NP. They are generally polymeric in nature (synthetic or natural) and can be biodegradable or nonbiodegradable in character. As the sizes of the particle become smaller, the ratio of the surface atoms to those in the interior increases, thereafter leading those kinds of particles to play an important role in the properties of novel functional material. The unique properties and utility of nanoparticles arise from a variety of attributes, including the similar size of nanoparticles and biomolecules such as proteins and polynucleic acids. Additionally, nanoparticles can be fashioned with a wide range of metal and semiconductor core materials that impart useful properties such as fluorescence and magnetic behavior. Those significant properties, such as chemical, electronic, mechanical, and optical properties, of nanoparticles obviously distinguish them from those of the corresponding “bulk” material [Ferrari, 2005].

1.5.2 Nanobiotechnology

Bionanotechnology is an emerging field with great promise in molecular science. The field is so new that it has yet to be formally defined. However, it can be characterized as a primitive technology that takes advantage of the properties of highly evolved natural products like nucleic acids and proteins polysaccharides by attempting to harness them to achieve new and useful functionalities on the nanoscale. Nanobiotechnology can be defined as an interdisciplinary field of research integrating engineering, physical sciences and biology through the development of very small physical and biological devices using biomimetically inspired nano-fabrication techniques. The application of nanotechnology principles and methodology to problems in biotechnology is providing an exciting discipline that will

generate a revolution of novel applications in several arenas. Applications of nanotechnology range from novel nanosensors, to novel methods for sorting and delivering bioactive molecules, to novel drug-delivery systems. Nanomaterials hold great promise for biological applications in magnetic resonance imaging, drug delivery, fluorescent probes, and cancer treatment "nanovectors" which can be tailored to penetrate cells to simultaneously deliver therapeutic agents with response to external stimuli [Ferrari, 2005]. Modern bionanotechnologists use knowledge of chemistry, biochemistry, and molecular biology to identify components and processes for the construction of self-assembling materials and devices. Like other forms of nanotechnology, bionanotechnology seeks to define approaches to the fabrication of useful materials and devices. However, the construction principles utilized in the field often originate in biology and the goals are often biomemetic (e.g., the construction of biosensors [Yun et al 2002]) or aimed at the solution of long-standing research problems (e.g., protein crystallization [Seeman, 1991]. Nonbiological problems have also been approached in attempts at the construction of electronic circuitry using biomolecules [Gil, 2002] and the construction of a fuelled nanomechanical oscillator [Yurke et al, 2000] and a nanomechanical switch [Mao et al, 1999]).

Figure 11: Applications of nanomaterials to biology and or medicine

1.5.3 Nanotechnology in pharmaceutical applications

The delivering of a pharmaceutically active molecule to a specific site in the body was adream and it has been a long-held aspiration with beginnings that may be traced back to Paul Erhlich, who in the early 20th century coined the phrase “magic bullet” to describe such an entity [Sykes, 2000]. Drug delivery systems (DDSs) can improve several crucial properties of ‘‘free’’ drugs, such as solubility, in vivo stability, [Allen and Cullis, 2004] pharmacokinetics, and biodistribution, enhancing their efficacy. The extensive pharmaceutical research today has led to the development of drug delivery systems and strategies, which go some way to fulfilling this idea, but few which could be described as “magic bullets.” Side-effects and toxicities still afflict these approaches and, hence, erhlich’s visionary thinking has not yet been fully realized. This is especially relevant in tumour chemotherapy, where selective delivery to neoplastic cells in comparison to surrounding normal cells is an important principle [Gupta, 1990].

There are many challenges to be faced while delivering the drug from the point of administration to the specific intended destination site in the body, in site-specific delivery of drugs is immense due to the numerous obstacles barricading the drug along its desired route. Cellular structures and indeed the very components of the cell itself will either prevent or act in some selective manner to hinder to the migration of drug from its point of administration to the intended destination site. It is obvious that modern medicine still faces many challenges, till today as we are moving forward into the 21st century. Nanotechnology is the area of research that may offer scientific advances in the future, which could lead to significant progress in the improvement of therapeutic outcomes. Instead of relying on the physicochemical properties of the drug to dictate its biodistribution, the drug is incorporated as a payload into a particle resulting in a different transit mechanism for the drug after administration [Damge et al, 1987].

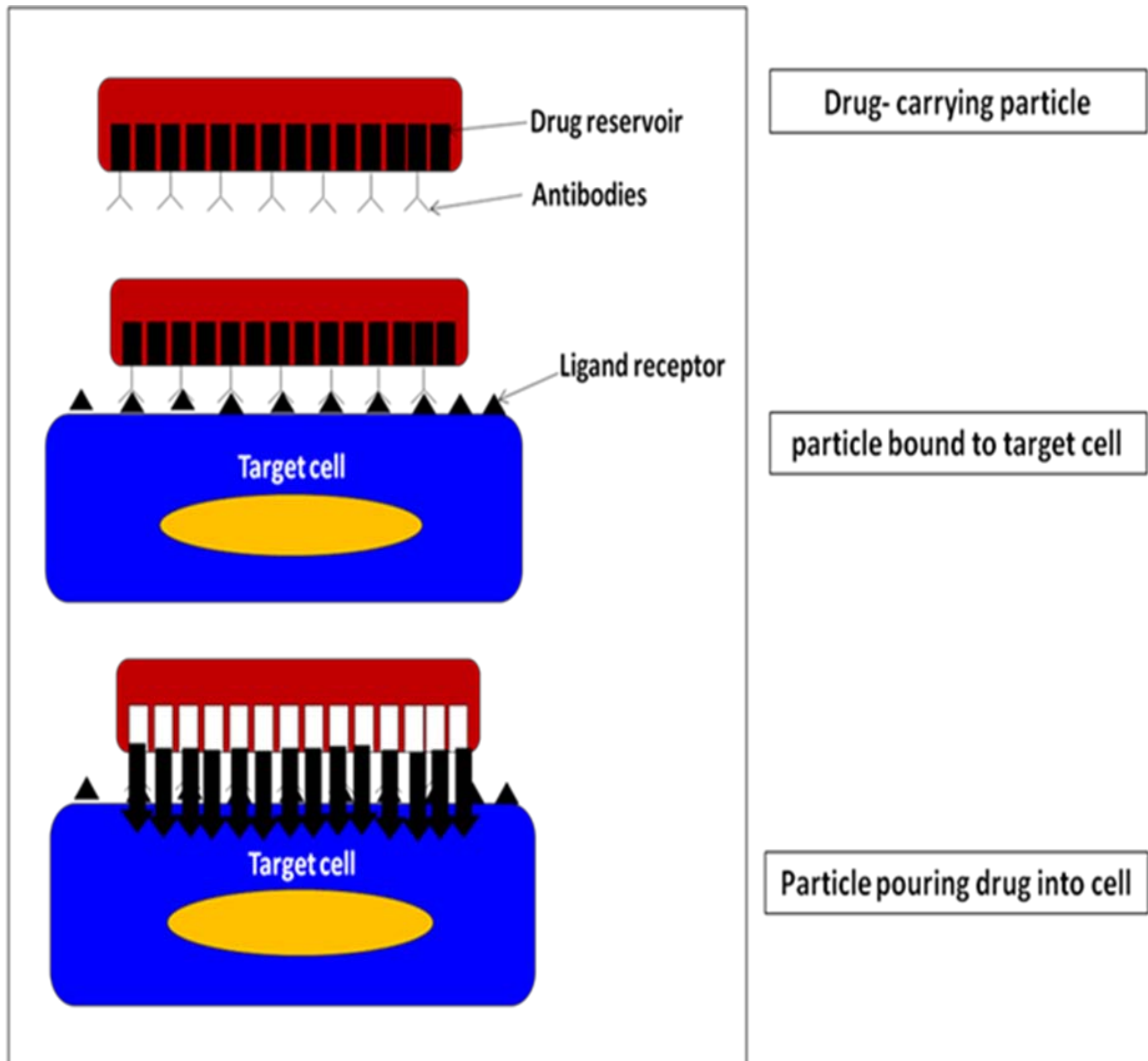


Figure 12 : Nanocarriers for drugs, mode of action.

In particular, the development of nanoparticulate drug delivery systems may enhance the probability of getting a drug to its target site [Lockman et al, 2002]. The nanoparticle should have flexible nature and several properties which are incorporated onto the particle, mostly by covalent bonding to surface groups. A targeting system, such as a monoclonal antibody, will recognize binding sites that are unique to the target cell and allow the particle to dock onto the exposed surface [Mrinmoy et al, 2008]. For successful delivery, carriers must: (i) form condensed complexes with biomolecules, (ii) facilitate penetration of the cell membrane after complexation, and (iii) unload their payloads inside of cells [Illum et al, 1983]. The current research in the fields of nanoparticles and drug delivery systems are focusing into developing strategies for targeting nanoparticles to the site of drug action [Davis et al, 1993., Kreuter, 1994., Moghimi, 1995]. A fusion protein will instigate the process of merging with the target cell, thereby bringing the particle into the cytoplasm [Silhol et al, 2002]. As polymeric nanoparticles are recognized as foreign by the body's immune system, they are removed quite effectively by phagocytosis on exposure to the endoreticular system [Muller et al, 1992]. This will prevent the particle from reaching the target site and must be prevented. Steps toward this goal have already been taken with the production of so-called "stealth"nanoparticles. These are nanoparticles which incorporate a biomimetic polymer, usually polyethylene glycol, into their structure to avoid elicitation of an immune response [Peracchia et al, 1999., Bazile et al, 1995., Li et al, 2001]. Presently, such an idealized nanoparticle with these three important properties has yet to be realized, but attempts have been made to attach some of the subsystems described.

1.6 Glycoside Hydrolases

Glycoside hydrolases (GHs) (also called glycosidases or glycosyl hydrolases), are enzymes that catalyze the hydrolysis of the glycosidic bond between two carbohydrate residues or a carbohydrate unit linked to a non-carbohydrate aglycon unit. Glycosidic linkages have been shown to be remarkably stable, with an estimated half-life of approximately 5 million years for a single glycosidic linkage in cellulose [Wolfenden et al; 1998]. Glycoside hydrolases catalyze the hydrolytic cleavage of these linkages, making them some of the most proficient enzymes, with rate enhancements on the order of 1017 [Wolfenden et al; 1998]. They are ubiquitous in Nature. About 1% of the genome of any organism encodes for GHs [Vocadlo et al; 2008], and they are involved in several processes that are essential for life on earth.

1.6.1 Occurrence importance of glycoside hydrolases

Glycoside hydrolases (EC 3.2.1.-) are a widespread group of enzymes which hydrolyse the glycosidic bond between two or more carbohydrates or between a carbohydrate and a non-carbohydrate moiety. Glycoside hydrolases are widespread group of enzymes present in almost all living organisms and found in essentially all domains of life, in prokaryotes, they are found both as intracellular and extracellular enzymes that are largely involved in nutrient acquisition. In higher organisms glycoside hydrolases are found within the endoplasmic reticulum and golgi apparatus where they are involved in processing of *N*-linked glycoproteins, and in the lysozome as enzymes involved in the degradation of carbohydrate structures. GHs are important in industrial settings. Glycoside hydrolases are also involved in several processes that are essential for life on earth. For example: they participate in cellular recognition processes; carbohydrate moieties are covalently linked to proteins and lipids, where they interact with other molecules to mediate recognition in and among cells. Post-processing of the attached carbohydrates, via addition and trimming of individual carbohydrate units, is crucial for this process, and GHs and glycosyltransferases are

employed in this step. In addition, the hydrolysis of carbohydrate polymers, such as cellulose and starch, into smaller sugar units provides some of the required building blocks to construct biopolymers and obtain energy in organisms. Some of the GHs were identified as drug targets for number of diseases including cancer and influenza as these enzymes plays variety of roles in the human body[Jacob et al; 1995., von Itzstein et al; 1996]. GHs found in other organisms have been employed to break down starch, cellulose, and hemicellulose to produce fermentable sugars that can be converted into biofuels [Bayer et al; 2007., Himmel et al; 2007].

1.6.2 Classification of glycoside hydrolases

Classification of glycoside hydrolases, or glycosidases, based on amino acid sequences was first performed in the early 1990's [Henrissat; 1991]. It was determined that the 301 known glycoside hydrolase sequences could be grouped into 35 families. The enzyme classification system of the "International Union of Biochemistry and Molecular Biology" is based on substrate specificity, the substrate specificities and reaction mechanisms of the classified enzymes corresponded to their family placement. And the "Enzyme Commission" (EC) number corresponding to glycoside hydrolases (GHs) is EC 3.2.1.x. However, the structural relationship (if any) between specific enzymes is not revealed by this classification system. Currently, the carbohydrate-active enzyme database (CAZy; www.cazy.org) has classified thousands of glycoside hydrolases into more than one hundred families [Coutinho and Henrissat; 1999]. The member enzymes of a given family come from a wide variety of organisms, but retain similarity in the types of reactions catalyzed, and the mechanism employed. This indicates that, despite the increase in sequence data, the classification scheme remains robust, as it has been validated by biochemical means. In addition, numerous structural studies of glycoside hydrolases from a variety of species have shown that this enzyme class encompasses a large part of the genome, and contains a wide diversity of

protein folds [Coutinho and Henrissat; 1999]. Glycoside hydrolases can also be classified according to the stereochemical outcome of the hydrolysis reaction: thus they can be classified as either retaining or inverting enzymes. Glycoside hydrolases can also be classified as exo or endo acting, dependent upon whether they act at the (usually non-reducing) end or in the middle, respectively, of an oligo/polysaccharide chain.

In this work we focused only on alpha-manosidase

1.6.3 Mannosidase

It is an enzyme which can remove mannose residues from the glycosidic linkage of the glycoproteins and oligosaccharides.

There are two types of mannosidase:

- α-mannosidase
- β-mannosidase

β-Mannosidase (MANB, β-D-mannosidase mannohydrolase, EC 3.2.1.25) is lysosomal enzyme and it is one of the exoglycosidases that cleave the single β-linked mannose residue from the nonreducing end of all N-linked glycoproteins. It is essential for the complete hydrolysis of polysaccharides such as galactoglucomannan and mannooligosaccharides, which are produced by β-mannanase activity [Zahoor and Muhammad, 2011]. (MANB) enzyme is involved in removing mannose residue from the nonreducing end, and its impaired activity leads to β-mannosidosis. MANB enzyme has been purifi ed from fungi [Ademarket al 1999., Kurakake and Komaki, 2001], hyperthermophilic microbes [Duff aud et al, 1997., Bauer et al, 1996], plants [Mo and Bewley, 2002., Ishimizu et al, 2004], goats [Pearce et al, 1990], bovines [Sopher et al, 1983], and humans [Guadalupi et al, 1996].

1.6.4 Alpha mannosidase

α-Mannosidases are widely distributed in nature. Purified from various vegetable and animal species, the enzymes can serve as a structural reagent for those glycoproteins having D-

mannose as a common constitutent [Andree et al, 1980]. α-D mannosidases are assigned five EC numbers by the "Nomenclature Committee of the International Union of Biochemistry and Molecular Biology" [NC-IUBMB, 1992] as following: EC 3.2.1.24 to those that do not have strict linkage specificity, EC 3.2.1.77 to those acting on (1-2) - and (1-3)-linkages, EC 3.2.1.113 to those acting on (1-F2) - only, EC 3.2.1.114 to those acting on (1-3) - and (1-6) - linkages and EC 3.2.1.137 to those acting on (1-2) - and (1-6)-linkages. There are no (1-6) - or (1-3) - linkage specific mannosidases included yet [Vasileios, 2005]. There has, however, been one (1-6) - specific enzyme reported in the literature [Wong-Madden, 1995] and is commercially available, but it has not been fully characterized. A (1-3) - linkage specific exo-mannosidase has not been discovered to date.

1.6.5 Classification of α-mannosidase

Class I α-mannosidase:

Class I α-mannosidases comprise a homologous and functionally diverse of glycoside hydrolases. Class I enzymes are inverting glycosidases [Park et al, 2005., Dorling et al, 1980], that specifically cleave α-1,2- linked mannosyl subunits and form glycosyl hydrolase family 47 localized in the ER. [Bischoff et al, 1990], they are inhibited by pyranose monosaccharide analogs such as 1- deoxymannojirimycin but not by swainsonine, and they required Ca^{+} for their activity.

Class II α-mannosidase:

These are belongs to GH38 along with other α-mannosidases [Cantarel et al, 2009]. Removal of α- 1,3- and α-1,6- linked mannosidases is essential for the synthesis of complex *N*-glycans, a task performed specifically by class II mannosidase in the golgi apparatus. They are more heterogeneous group of the lysosomal mannosidases. They are also retaining [Akama et al, 2006], and they can cleave α-1,2-, α-1,3-, α-1,6- linked mannosyle residues, they are inhibited by furanose analogs such as swainsonine, and compris glycosyl hydrolase

family 38. GH38 members hydrolyze mannosyl glycosidic bonds using a retaining mechanism, GH38 members contains a zinc ion that interacts directly with the nucleophile (Asp204) and two hydroxyl groups (C2'–OH and C3'–OH) of the substrate[van den Elsen et al, 2001].GH38 is the first known GH family whose members employ a catalytic Zn ion[Vasella et al, 2002].

Lysosomal α-mannosidase

Lysosomal α-mannosidase (LM) is a GMII-related enzyme that catalyzes the catabolic breakdown of oligosaccharides in the lysosome [Park et al, 2005]. It too employs a retaining mechanism, but has much broader substrate specificity than GMII, cleaving all α-linked mannoses from high mannose oligosaccharides. In addition, due to its subcellular localization in the lysosome, it is more highly active at lower pH values [Park et al, 2005]. Inhibition of lysosomal α-mannosidase produces a phenocopy of α-mannosidosis, a lysosomal storage disorder [Dorling et al, 1980].

Cytosolic α-mannosidase

There is a cytosolic α-mannosidase that does not seem to contain a transmembrane helix and has a unique metal dependency for this class of enzyme. It seems to be Co2+ activated, and likely functions to trim mannoses from proteins that have been incompletely glycosylated yet transported to the cytosol [Bischoff et al, 1990].

Golgi α-mannosidase IIx

Golgi α-mannosidase IIx (GMIIx) is a Golgi-resident enzyme that is similar to GMII. A recent GMII/GMIIx double knockout mouse has been shown to lack complex-type *N*-glycans [Akama et al, 2006]. Recombinant mouse GMII and GMIIx enzymes showed identical substrate specificities toward N-glycan substrates (conversion of GnMan5Gn2 to GnMan3Gn2), suggesting that GMIIx is an isozyme of GMII [Akama et al, 2006].

Golgi α-mannosidase III

Golgi α-mannosidase III (GMIII) is a class II cobalt-dependent α-mannosidase from insect (Sf9) cells with amino acid sequence and biochemical similarities to mammalian Golgi α-mannosidase II [Kawar et al, 2001]. GMIII catalyzes the cleavage of two mannosyl linkages, converting Man5Gn2 to Man3Gn2 but it cannot act on GnMan5Gn2, making it quite distinct from GMII. GMIII appears to provide an alternate pathway to complex oligosaccharide synthesis that does not rely on GnT I or GMII.

1.6.6 Occurrence and importance of α-mannosidase.

α-Mannosidases (α -D-mannoside mannohydrolase, E.C. 3.2.1.24) were found in all organisms from bacteria to human [Yamamoto and Nagasaki, 1975., Bagiyan et al, 1997., Yoshida et al, 1993., Jelinek-Kelly et al, 1985., Oeltmann et al, 1994., Curdel and Petek, 1980., Kumano et al, 1996., Hamagashira et al, 1996., Schatze et al, 1992]. Some α -mannosidases have a high specificity towards terminal mannosidic residues of glycans [Schweden et al,1986., Moore and Spiro, 1990] and participate in mannose trimming reactions (endoplasmic reticulum mannosidase, Golgi mannosidases IA, IB and IC). The enzyme α-mannosidase (EC. 3.2.1.24) is ubiquitous in nature. It has been purified and well characterized from various plants, microbial and animal sources. The enzyme is an abundant constituent of plant hydrolytic system [Forsee et al, 1989., Howard et al, 1998., Snaith, 1975]. It accumulates in vacuoles and is thought to be involved in catabolism and turnover of *N*-linked glycoproteins [Pastuszak et al, 1990., Woo and Kimura, 2005]. A deficiency of α-mannosidase in degradatory system of higher eucario tic cells causes the lethal decease, the mannosidosis [Hocking et al, 1972., Burditt et al, 1980., Pohlmann et al 1983]. 1,2-α-Mannosidases which cleave side chains of yeast mannan were isolated from *Bacillus* sp. [Maruyama et al, 1994], *Aspergil lus orizae* [Tanimoto et al 1989], *Aspergillus saitoi* [Ichishima et al,1981]. Secreted 1,2-1,6- - α -mannosidase from *Cellulomonas sp.* [Takegawa

et al,1989] and 1,2-1,3- - α -mannosidase from *Arthrobacter* [Jones and Ballou, 1969] were found to hydrolyse α-mannan. The distinction of the *Trichoderma* α -mannosidase is that it hydrolyses the yeast mannan at very low rate. Another role played by a-mannosidases is the processing of secreted glycoproteins like those from *A. awamori* [Neustroev et al, 1993]. The most enzymes of *T. reesei* are mannose-containing glycoproteins [Gum and Brown, 1976., Maras et al, 1997] and may be affected by own mannosidase after secretion.

Chapter 2

Isolation and characterization of total proteins from *S. potatorum* seed powder

2.1. Introduction

Proteins are important in plants as they are involved in plant structure and metabolism, defence, environmental interaction and metabolic regulators and mony other activities and functions. Plants are emerging as a viable alternative for the production of high-value proteins such as therapeutic antibodies, as they offer the advantage of practically unlimited scalability in the field, without the requirement of high-cost fermentation facilities needed for mammalian cell cultures, and freedom from potential contamination with zoonotic pathogens [Saskia et al, 2008]. Several plant-derived biopharmaceutical proteins have successfully been expressed and produced in plants and plant cell cultures, some of which have reached clinical trials. In contrast to microbial production systems, plants offer the full potential of protein folding, secretion, as well as *N*-glycosylation [Twyman et al, 2005].

Seed proteins

In plants the amino acids formed as products of nitrate assimilation are stored as proteins. These are mostly special storage proteins, which have no enzymatic activity and are often deposited in the cell within protein bodies. Protein bodies are enclosed by a single membrane that derived from the endo-membrane system of the endoplasmic reticulum and the Golgi apparatus or the vacuoles [Robinson et al, 2005]. Storage proteins can be deposited in various plant organs, such as leaves, stems, and roots. They are stored in seeds and tubers and also in the cambium of tree trunks during winter to enable the rapid formation of leaves during seed germination and sprouting. Storage proteins are located in the endosperm in cereal seeds and in the cotyledons of most legume seeds [Hans-Walter et al, 2011]. In cereals the protein content amounts to 10% to 15% of the dry weight, in some legumes (e.g., soybean) it is as high as 40% to 50%. About 85% of these proteins are storage proteins. Globally, about 70% of the human demand for protein is met by the consumption of seeds, either directly or indirectly by feeding them to animals for meat production [Shewry et al, 1995]. Therefore

plant storage proteins are the important basis for human nutrition. However, in many plant storage proteins the content of nutritionally essential amino acids is too low. In cereals, for example, the storage proteins are limited in threonine, tryptophan, and particularly in lysine, whereas in legumes there is a limitation of methionine. Since these essential amino acids cannot be synthesized by the humans they have to be obtained through their diets. In humans with an entirely vegetarian diet, the deficiency of essential amino acids can lead to irreparable physical and mental damage, especially in children. A research goal in plant genetic engineering is to improve the amino acid composition of the storage proteins of harvest products. Scientists have long been interested in plant proteins [Jacopo Beccari, 1745]. In Italy proteins were isolated from wheat [Thomas Osborne, 1924]. Connecticut Agricultural Experimental Station, classified plant proteins according to their solubility properties. Plant proteins are classified into albumins (soluble in pure water), globulins (soluble in diluted salt solutions), glutelins (soluble in diluted solutions of alkali and acids), and prolamins (soluble in aqueous ethanol) [Shutov et al, 2003]. Later, when the structures of these proteins were determined, it turned out that glutelins and prolamins were closely related. Therefore, in more recent literature, glutelins are regarded as members of the group of prolamins. Storage globulins occur in varying amounts in practically all plants. The most important globulins are legumin and vicilin, both of which are encoded by a multigene family. Legumin is the main storage protein of leguminous seeds. In broad bean, for instance, 75% of the total storage protein consists of legumin. Prolamins are only present in grasses, such as cereals. 2S-proteins are present in seeds of dicot plants. The protein bodies of some seeds contain additional proteins, which, although also acting as storage proteins, protect the seeds from being eaten [Haq et al, 2004]. The seeds of some legumes contain lectins, which bind to sugar residues, irrespective of whether these are free sugars or constituents of glycolipids or glycoproteins. When these seeds are consumed by animals, the lectins bind to glycoproteins

in the intestine and thus interfere with the absorption of nutrients [Peumans and van Damme, 1995]. The seeds of some legumes and other plants also contain proteinase inhibitors, which block the digestion of proteins by inhibiting proteinases in the animal digestive tract. Because of their content of lectins and proteinase inhibitors, many beans and other plant products are suitable for human consumption only after being denatured by cooking. Castor beans contain the extremely toxic protein ricin. Beans also contain amylase inhibitors, which specifically inhibit the hydrolysis of starch by amylases in the digestive tract of certain insects. Seed storage proteins are formed by ribosomes at the rough endoplasmic reticulum (ER). The newly synthesized proteins occur in the lumen of the ER, and the storage proteins are finally deposited in the protein bodies. The proteins isolated in this part of the study from the seeds of *S. potatorum* seems to be storage proteins as they are not exhibiting hemagglutination activity and are not showing any glycoprotein property or enzymatic activity.

Metalloproteins

Metal ions serve a variety of functions in proteins. They have been implicated in enchancing the structural stability of the protein in the conformation required for biological function and/or to take part in the catalytic processes of enzymes. Metal ions can activate chemical bonds and make them more amenable to reaction. They can take part in trigger and control mechanisms by specifically altering or stabilizing a macromolecular conformation on binding [Rasmussen, 1990]. Certain metals can also undergo redox reactions. The presence of metal ions in the active site may be crucial to the activity of an enzyme. Sometimes only one specific metal ion in a specific oxidation state can be employed to aid in catalysis, while in other cases a variety of metal ions of similar sizes can be used. On the other hand, the role of the metal ion may be to bind various domains of the protein together. In this way the metal acts as a template, bringing reacting groups into the correct relative orientation for reaction [Hughes, 1981]. The atoms or groups of atoms that surround a metal ion and which are close

enough to be chemically bonded are termed ligands [Kauffman et al, 1983]. Ligands donate an electron pair to the bond and are generally negatively charged or neutral. The number of such liganding atoms surrounding a central metal ion is termed the coordination number of the metal ion. Important in a study of metal-ligand interactions are the polarizabilities of both of the metal ion and the ligand, the number of the ligands around each metal ion, and the stereochemistry of the resulting arrangement [Williams, 1959, 1970].

Metal –binding Sites in protein

The major metal-binding amino acid side chains in proteins [Gurd and Wilcox, 1956., Voet and Voet, 1990] are side chains of carboxyl (aspartic acid and glutamic acid), imidazole (histidine), indole (tryptophan), thiol (cysteine), thioether (methionine), hydroxyl (serine, threonine, and tyrosine), and possibly amide groups (asparagine and glutamine, although generally via their side-chain carbonyl, rather than amino, groups). About 65% of the various types of amino acid side chains are potential metal-binding groups. In addition protein main-chain carbonyl and amino groups bind metal ions. The binding of a metal ion to a ligand can be considered in terms of lewis acid-base theory [Lewis, 1923., Allred and Rochow, 1958., Brown and Skowron, 1990] because in accepting an electronic pair the metal ion acts as a lewis acid. When a metal ion coordinates a ligand, it can affect the electron distribution of the ligand and therefore its reactivity. Metals can perform catalytic functions that are difficult or impossible to carry out by amino acid functional groups or organic cofactors. These include activation of water molecules, reactions involving gases and, to some extent, redox processes. Although recent results have shed considerable light on this field, some caution should be exerted when interpreting active site structures. On the one hand, many metal sites seem to be rather promiscuous, being able to bind different ions, sometimes even keeping the catalytic activity. On the other hand, photoreduction by X-ray photons may be a very common phenomenon. In our laboratory some work has been carried out on the Bioprospecting

Strychnos L. (*Strychnaceae*) Ph.D thesis accepted by the Univ. Hyderabad, he studied *S. potatorum, S. nux-vomica and S. wallichiana* [Dr Pasupuleti Sreenivasa Rao (2008)]. The following topics were covered 1) in vitro differentiation for multiplication, 2) Preparation of alkaloids extract, 3) Anti-proliferative and cytotoxic properties of plant extract on myeloma cell lines. But to our knowledge till date there are no studies carried out on the proteins of the *S. potatorum*.

In this chapter we isolated and separated the total proteins from the seeds of *S. potatorum* and we studied some characterstics of these proteins. We then immobilized these proteins separately on affigel matrix and used the gels for metal binding studies from the aqueous medium.

2.2 Methods and materials

All the chemicals and reagents used in the present study were of analytical grade. Seralose 4B was purchased from Sisco Research Laboratory, India (equivalent to Sepharose 4B supplied by Sigma, USA). Standard proteins used were purchased from sigma chemical company USA. pH of the solutions was adjusted with 1M NaOH or 1M HCl. Affigel-10 was procured from Bio-Rad Laboratories, USA.

2.2.1. Plant material

S. potatorum L. seeds were obtained from the divisional forest, flying squad division, Rajahmundry, East Godavari district, Andhra Pradesh India. Seeds were dried at 40 ^{o}C for 2 days in hot air oven. Seeds were made into powder in Clotech 1093 sample mill. 10 g of the seed powder was used for every batch of protein extraction.

2.2.2 Extraction and isolation of total proteins

S. potatorum seeds were ground to a fine powder and defatted with acetone and hexane and the powder air dried. From the dried seed powder (10 g) total proteins were extracted overnight at 4 ^{o}C using 25 mM Tris- HCl buffer pH.7.4 containing 150 mM NaCl (TBS), 1 mM phenylmethylsulfonyl fluoride (PMSF) was added as a protease inhibitor. The suspension was clarified by centrifugation at 10000 rpm for 20 minutes. The clear supernatant containing the soluble proteins was subjected to precipitation by addition of solid ammonium sulfate to 0- 30 and 30- 70 % saturations. 0- 30% ammonium sulfate precipitated fraction was very viscous and contained negligible amount of proteins. The 30- 70% fraction was containing high concentrations of proteins. 30- 70 % ammonium sulfate precipitated proteins were separated by centrifugation at 12000 rpm for 30 minutes, the pellet was dissolved in the minimal amount of Tris buffer saline (TBS), and the protein solution was dialysed through cellulose dialysis tubing membrane with a molecular weight cut-off of 12-

14 kDa (Himedia). The proteins remained inside the dialysis tube and were collected to be used for further studies.

Protein concentration in the extracts as well as the ammonium sulphate precipitated proteins was determined using Lowry's method [Lowry et al, 1951], Bradford method [Bradford, 1976] and bicinchoninic acid (BCA) method [Smith et al, 1985] employing Bovine Serum Albumin (BSA) as standard. Protein estimation was done using BCA reagent with BSA as standard following manufacturer's instructions. BCA reagent: 10 mL of BCA and 0.5 of 4% copper sulfate. The volume of the protein sample was made upto 500 µL with distilled water and mixed with 1.0 mL of BCA reagent and incubated for 30 minutes at 37°C. After incubation the absorbance was measured at 562 nm.

2.2.3. Hemagglutination assay

This was carried out according to [Siva Kumar and Rajagopal Rao, 1986]. Rabbit blood was collected by ear vein puncture, into Alsevier's solution. It was centrifuged at 3000 rpm for 10 minutes at 4 ^{o}C. The sedimented erythrocyte pellet was washed thrice with 0.9% saline and the pellet was made to 4% suspension with saline. Processed erythrocytes were treated with 0.1% trypsin and incubated at 37 ^{o}C for one hour. Erythrocytes were centrifuged at 3000 rpm for 10 minutes at 4 ^{o}C and then made upto their original volume. To 200 µL of sample serially diluted in 200 µL of saline, 200 µL of the trypsin treated erythrocytes were added separately in a plexiplate and incubated at 37 ^{o}C for one hour and the hemagglutination was visually observed.

2.2.4. Gel filtration

Seralose 4B matrix was used for separation of the 30-70% ammonium sulfate precipitated protein. The column (75 ml gel) was equilibrated with 25 mM TBS. The precipitated protein was dissolved and dialyzed against TBS and applied onto the gel in batches to separate the proteins. Protein in column fractions was monitored by measuring the absorbance at 280 nm.

2.2.5. Molecular weight and sub units composition of the proteins: sodium dodecyl sulphate-polyacrylamide gel electrophoresis

The electrophoresis separation of proteins was done by discontinuous SDS-PAGE according to Laemmli (1978). (Tank buffer): 3 g Tris, 14g glcine and 1g SDS dissolved in 1 litre of double distilled water., (2X sample buffer "Reducing") : 0.5 M Tris HCl pH 6.8, 2.5 mL 10% SDS, 4.0 mL 100% glycerol, 2.0 mL β- mercaptoethanol, 1.0 mL bromophenol blue 0.05% and made up the volume to 10 mL,. (Ressolving gel buffer) 1.5 M Tris- HCl pH 8.8., (stacking gel buffer) 0.5 M Tris- HCl pH 6.8., (Acrylamide): 30% (w/v) acrylamide, 0.8% (w/v) N N' methylenebisacrylamide, (ammonium peroxide sulfate) (APS): 10% (w/v) in water. Resolving gel and stacking gels are made as shown in the tables respectively. APS and TEMED were added at the end to resolving gel and poured in to the sealed glass plates. After polymerization the gel was rinsed with water. Stacking gel solution was poured, a comb with required number of wells was inserted and allowed to polymerize for 30 minutes. Wells were rinsed with water. The samples were cooked at 95°C for 5 minutes with sample buffer mixed in 1:1 ratio, centrifuged briefly and the supernatant loaded into the wells. These were overlaid with tank buffer and placed in the electrophoretic chamber. Samples were subjected to electrophoresis for 2-3 h at 50 100 V at room temperature. Details are given in the Table below.

% of PAA gel	7.5%	10%	12.5%
H_2O (mL)	3.650	3.025	2.400
Resolving gel buffer (mL)	1.875	1.875	1.875
Acrylamide (mL)	1.875	2.500	3.125
10% SDS (µL)	75	75	75
APS (µL)	40	40	40
TEMED (µL)	10	10	10

Table 6 Reagents used for the preparation of resolving gel with different polyacrylamide concentrations

H_2O (mL)	1.875
Resolving gel buffer (mL)	0.375
Acrylamide (mL)	0.3125
10% SDS (µL)	25
APS (µL)	40
TEMED (µL)	8

Table 7 reagents used for the stacking gel.

The protein bands were visualized with coomassie and silver staining.

2.2.7. Carbohydrate estimation

Carbohydrate content in the extracts was determined by the phenol- sulphuric acid method [Dubois et al., 1956] using glucose as the standard.

2.2.8. Immobilization of proteins on affigel

The proteins eluted from the gel filtration column as two distinct peaks (peak A), (peak B) were separately pooled and concentrated. These and the 30-70% ammonium sulfate precipitated proteins (gel 3) were separately immobilized to affigel-10 (Bio-Rad laboratories) following manufacturer's instructions. Coupling the proteins to affigel was carried out at $4^{o}C$. For each protein sample 2 ml of affigel-10 was packed in a sintered syringe and washed successively with ice cold isopropanol, water and then with 0.1 M HEPES buffer pH 7.4. To this the protein sample was added, column was closed and coupling was allowed to proceed overnight at 4 ^{o}C by rotating the column end over end for 24 hours. At the end of this period, the unbound fraction was collected and saved (to determine the extent of coupling), and the gel was incubated with 0.1 M HEPES buffer containing 400 uL of 0.1 M ethanolamine hydrochloride to block the unreacted sites and allowed to rotate for 1 hour in cold. The gel was finally washed with PBS and stored in cold until use.

2.2.9. Silver staining of the gel

This was carried out according to [Blum et al., 1978].

Reagents: Methanol, Ethanol, $AgNO_3$, Hypo, Na_2CO_3, Formaldehyde and acetic acid.

All the following steps were carried out at room temperature by placing the gel on a rocking platform, as follows

1. Fixative: 50% methanol, 12% glacial acetic acid, 50 µL formaldehyde (HCHO)was taken in a flask and the volume made up to 50 mL with double distilled water. The gel was soaked in fixative solution for 45 minutes to overnight (fixative solution can be stored in a brown bottle and can be reused 4-5 times).
2. The gel was transferred to 50% ethanol and incubated for 1 hour.
3. The gel was rinsed 3 times with double distilled water.

4. 10 mg of Hypo was dissolved in 50 mL double distilled water. The gel was soaked exactly for 1 minute in the above solution with constant shaking.
5. The gel was rinsed 3 times with double distilled water.
6. 100 mg of $AgNO_3$ was dissolved in 50 mL of double distilled water. To this 18 µL of formaldehyde was added. The gel was soaked in the above solution for 20 minutes with constant shaking.
7. The gel was rinsed 3 times with double distilled water.
8. Developer: 3 g of Na_2CO_3 was dissolved in 50 mL of double distilled water. To this 26 µL of formaldehyde was added. The gel was soaked in the developer solution to visualize the protein bands.
9. The gel was rinsed with double distilled water.
10. The developing reaction was stopped by the addition of 12% glacial acetic acid. The gel was washed with double distilled water.

2.2.10. Determination of glycoprotein nature

To determine the carbohydrate nature of the protein qualitatively, periodic acid- Schiff's staining was carried out following the method of [Zacharius et al., 1969]. Schiff's reagent was prepared as follows: 1 g of Basic Fuschin was added to 200 ml of water at 70 ^{o}C. This was boiled for few minutes, cooled and filtered. The temperature was adjusted to 50 ^{o}C. To this 5 ml of HCl and 2 g of potassium metabisulphite were added and incubated overnight. The solution turns colorless or pale straw yellow. To decolourise completely 0.25 g to 0.5 g of activated charcoal was added and filtered. This stain solution has to be tightly closed and stored in brown bottle at 4 ^{o}C. SDS-PAGE gel was stained in 1% periodic acid in 3% acetic acid for one hour. The gel was washed for one hour with water and stained in schiffs reagent for 30 minutes in dark. It was then destined with 10% acetic acid and finally stored in 3% acetic acid.

2.3. Results

2.3.1 Extraction of total proteins

The total proteins from the seeds of *S. potatorum* were extracted using Tris buffer saline (TBS) containing 0.001 M PMSF as protease inhibitor. Due to the abundant polysaccharides and alkaloids in the seeds the extract appeared very viscous and jelly. To precipitate the total proteins the extract was subjected to ammonium sulfate fractionation. Solid ammonium sulfate was added to the crude extract to a saturation of 30 % and stirred at 4 ^{o}C for six hours, centrifuged at 10000 rpm for 20 minutes. The pellet contained negligible amount of protein and therefore discarded. To the supernatant solid ammonium sulfate was added to 70 % saturation and then stirred overnight at 4 ^{o}C. Bulk of the protein precipitated in this fraction and was pelleted by centrifugation at 12000 rpm for 30 minutes. The pellet was dissolved in a minimal amount of TBS and dialyzed through cellulose dialysis tubing membrane with a molecular weight cut-off of 12-14 kDa (Himedia). This was treated as the source of seed protein.

2.3.2. Gel filtration

The seed proteins obtained above after dialysis was subjected to gel filtration, using seralose 4B matrix, to separate the proteins. Fractions 3 ml were collected and the proteins in column fractions were monitored by measuring the absorbance at 280 nm. Figure 13 shows the separated proteins on Seralose 4B column. Two distinct peaks A and B were obtained.

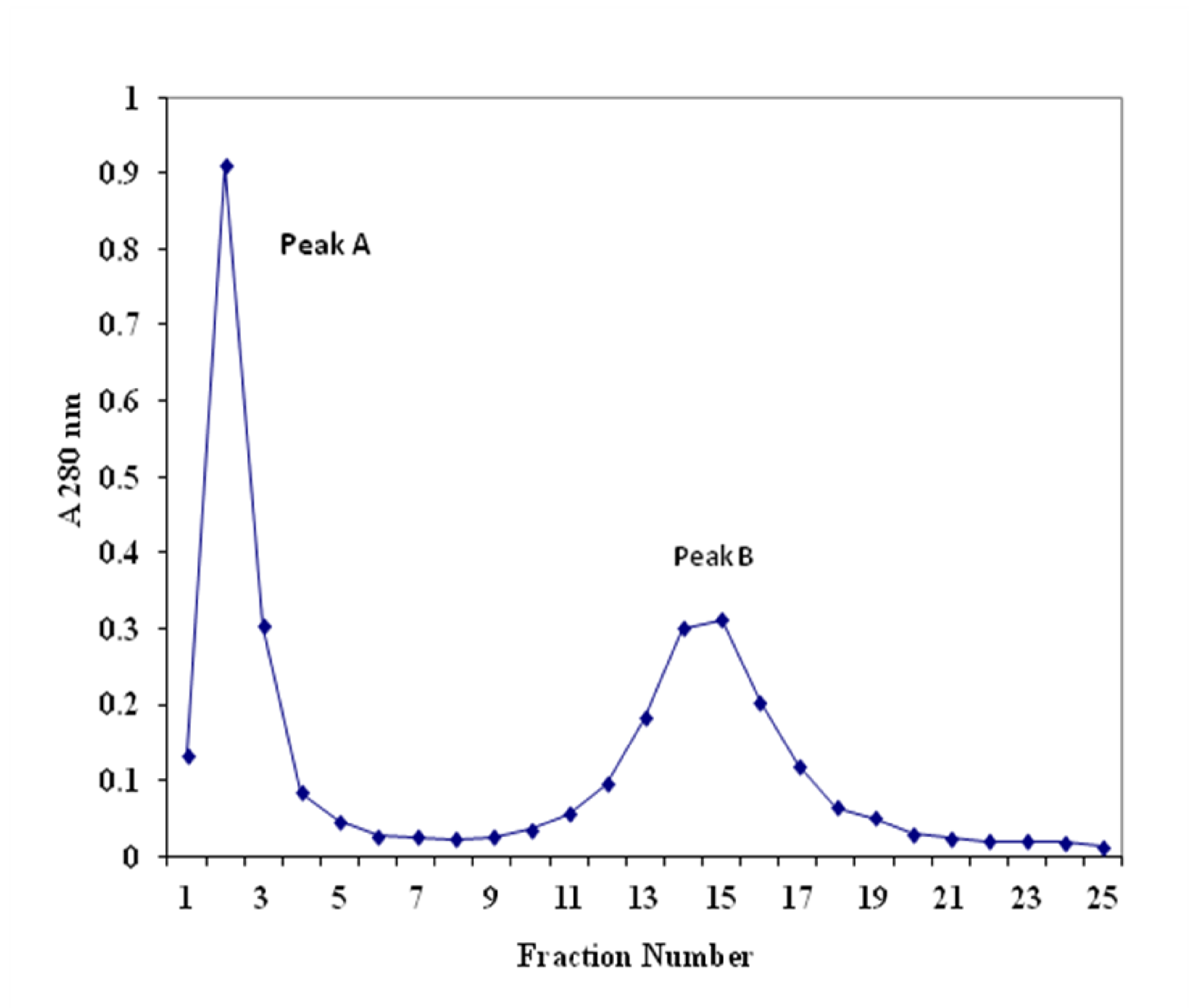


Figure 13: Gel filtration of the 30-70% ammonium sulphate precipitated proteins on Seralose 4B. The column was equilibrated with 25 mM TBS. After collecting the avoid volume fraction in a measuring cylinder (25ml). Fractions of 3mL were collected and the absorbance monitored at 280 nm.

2.3.3. Molecular weight and sub unit composition of the proteins

The total proteins (30- 70 % fraction) as well as the peak A and B protein fractions were analyzed using 12.5% SDS- PAGE. Fermentas unstained markers served as standards. The protein bands were visualized by silver staining. Figure 14 shows the 12.5% SDS-PAGE analysis obtained. From the gel it is apparent that the proteins identified in the total protein fractions could be separated on a gel filtration column in to two distinct peaks A and B. Protein bands are marked by arrows.

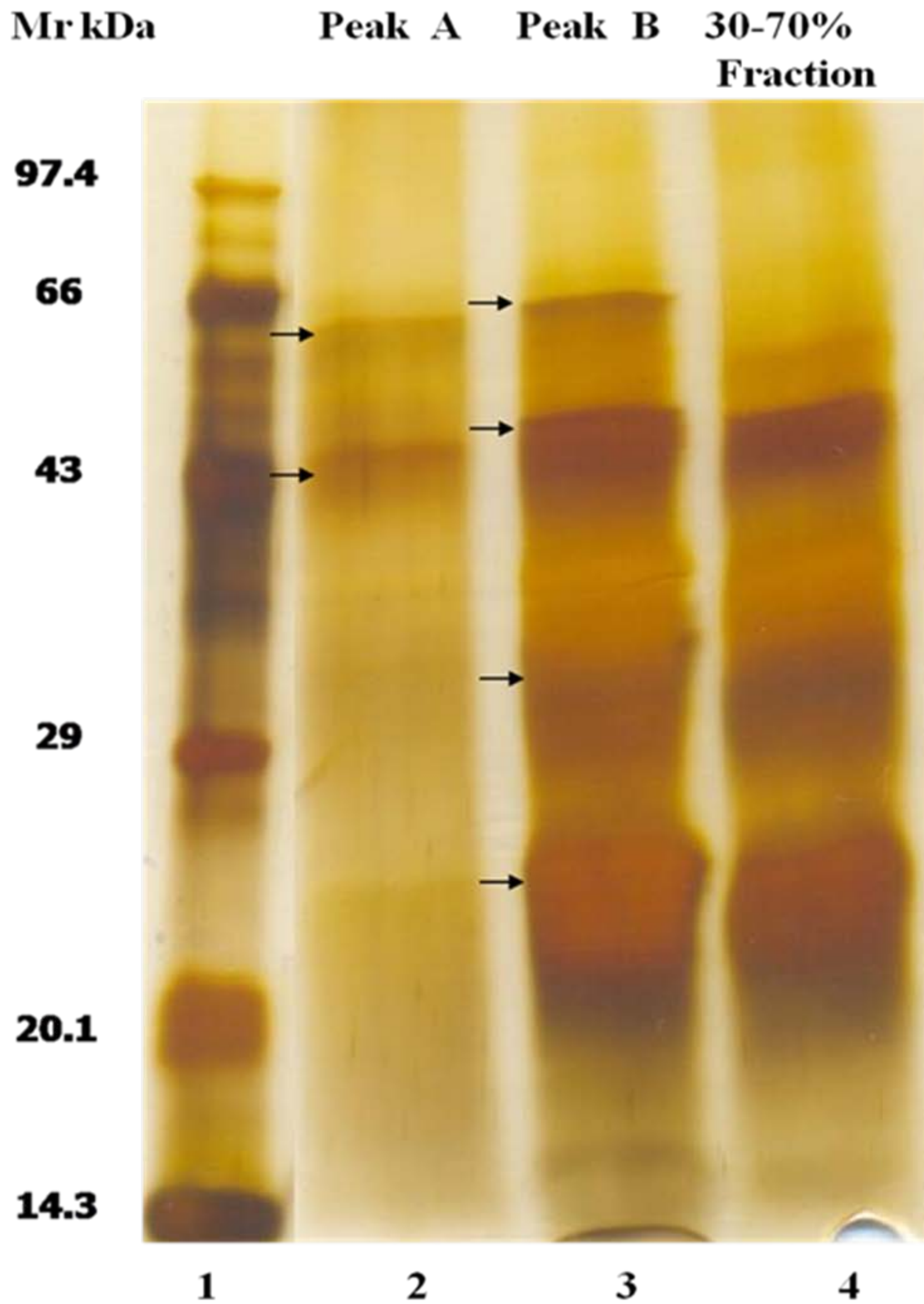


Figure 14: 12.5% SDS-PAGE analysis obtained from the seed extracts. After electrophoresis gel was stained using silver staining method. Lane 1 is showing the standard molecular weight marker, lane 2 is showing the proteins from peak A (gel filtration sample), lane 3 is showing the proteins from peak B (gel filtration sample) and lane 4 is showing 30-70% ammonium sulfate fractionated proteins.

2.3.4. Hemagglutination assay

To determine if the proteins extracted from the seeds of *S. potatorum* exhibit any lectin activity, hemagglutination assay was carried out. When the total proteins were analyzed for hemagglutination activity using trypsin treated rabbit erythrocyte, no agglutinating activity was detectable. Under the same conditions control (lectin sample) showed agglutination. Figure 15 shows the results of hemagglutination assay for the proteins isolated from *S. potatorum* seed powder, using plant lectin as appositive control and also running the same experiment steps except the addition of the protein extracts to be used as negative control and each experiment was done twice for results conformation as we can observe from the picture the proteins extracted from the seeds of *S. potatorum* do not exhibit any hemagglutination activity. The periodic- schiffs staining was carried out as described under methods to determine the carbohydrate nature of the proteins which also shows negative results and no pink color bands were observed.

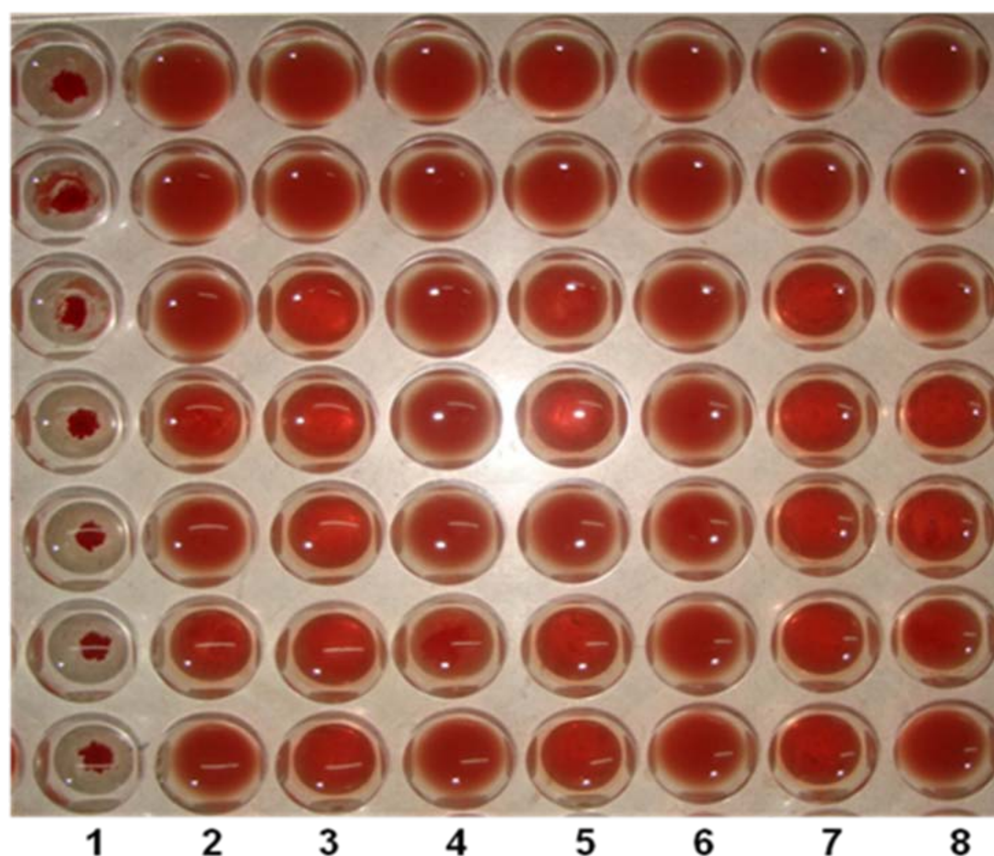


Figure 15: Hemagglutination assay for the proteins isolated from *S. potatorum* seed powder. Lane1 is showing positive control for the test, lane 2, 3 are showing proteins from peak A (gel filtration), lane 4, 5 are showing proteins from peak B (gel filtration), lane 6,7 are showing 30-70% ammonium sulfate fractionated proteins and lane 8 is showing negative control for the test.

2.4. Discussion

Seed proteins are of different kinds and some exhibit distinct biological activity. The total proteins isolated from the seeds of *S. potatorum* did not exhibit any hemaggltinating activity and were also not glycoproteins. Our idea was to analyze these proteins for their metal binding properties in detail as these could be one of the potential metal binders. To identify the possible components in the seed matrix that is responsible for metal binding and removal the present study was undertaken. By virtue of the charged side chain amino acids in proteins, it has been already postulated the role of proteins in specific binding of metals (these aspects have been discussed in the earlier pages of this chapter). Earlier studies have revealed that the total seeed matrices when used for removing metals from aqueous solutions, the matrix was effective in removing the metals. To our knowledge there has been no study to look at the role of specific proteins in metal binding from the *S. potatorum* seeds. Therefore as the first step towards achieving our goals, we isolated the total seed proteins which could be separated into two peaks by gel filtration. When the proteins in both peaks were analyzed by SDS PAGE analysis, a number of protein bands were visualized. The total proteins as well as the proteins separated in the gel filtration were separately coupled to affigel-10, in order to analyze their binding abilities to metals. The total protein and proteins in peak A, B were coupled at a concentration of 2.84 mg/ ml, 2.97 mg/ml and 4.1 mg/ ml respectively.

Chapter 3

Binding of cadmium to *S. potatorum* seed proteins in aqueous solution: adsorption kinetics and relevance to water purification

3.1 Introduction

Heavy metal contamination of both surface and ground water has become a common universal phenomenon [Mahvi et al, 2008]. Cadmium is known to be a harmful heavy metal. Cadmium is No 7 in the priority list of hazardous substances in USA [ATSDR, 2001]. Cadmium is among the heavy metals, which is regarded as a non-essential element for metabolic processes and one of the most toxic metals [IARC, 1994]. Cadmium is attracting the attention of environmentalists as one of the most toxic heavy metals. It is identified as a soft, blue-white malleable, lustrous metal or a greyish white powder that is insoluble in water and reacts readily with dilute nitric acid. Cadmium intentional applications include: pigments, coatings and stabilizers, It is abundantly widely used in electroplating, smelting, alloys and electronic components Figures 16, 17 and 18.

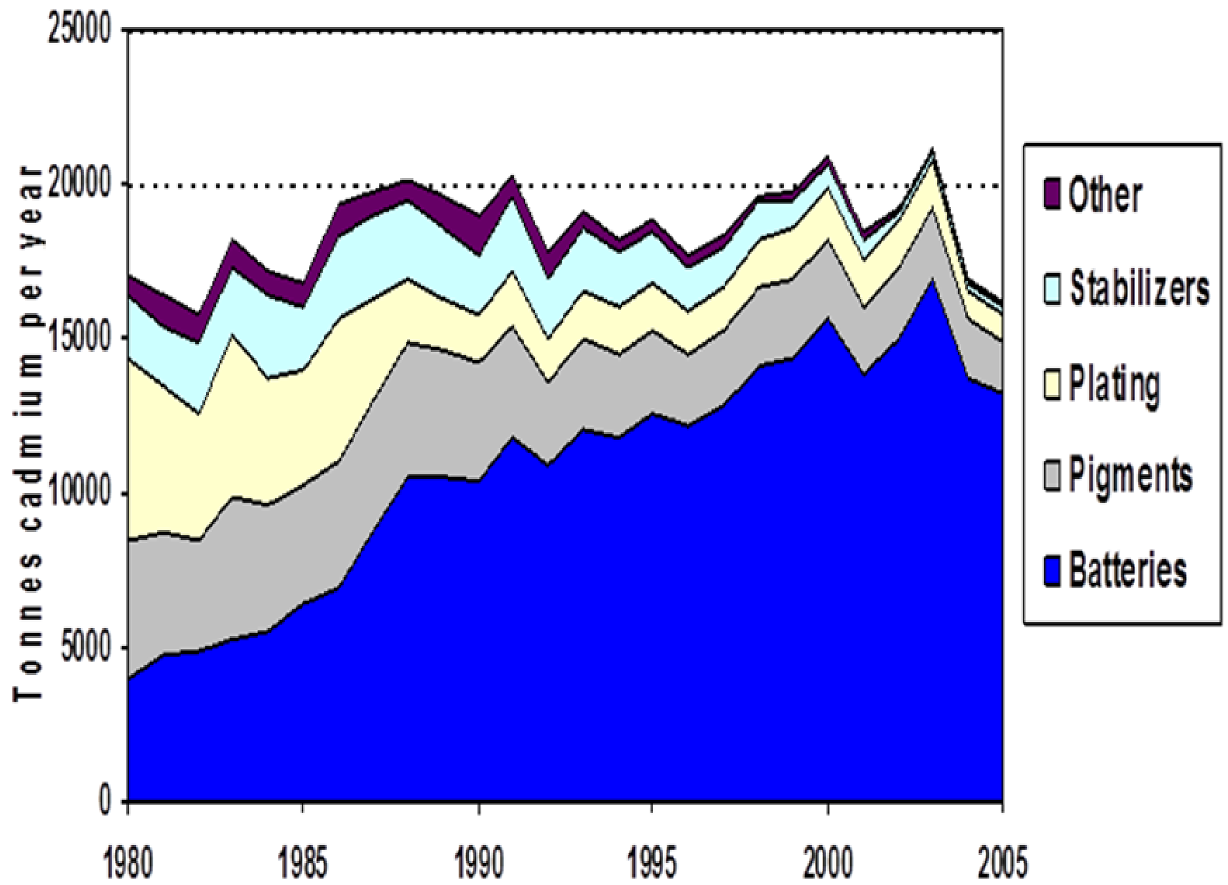
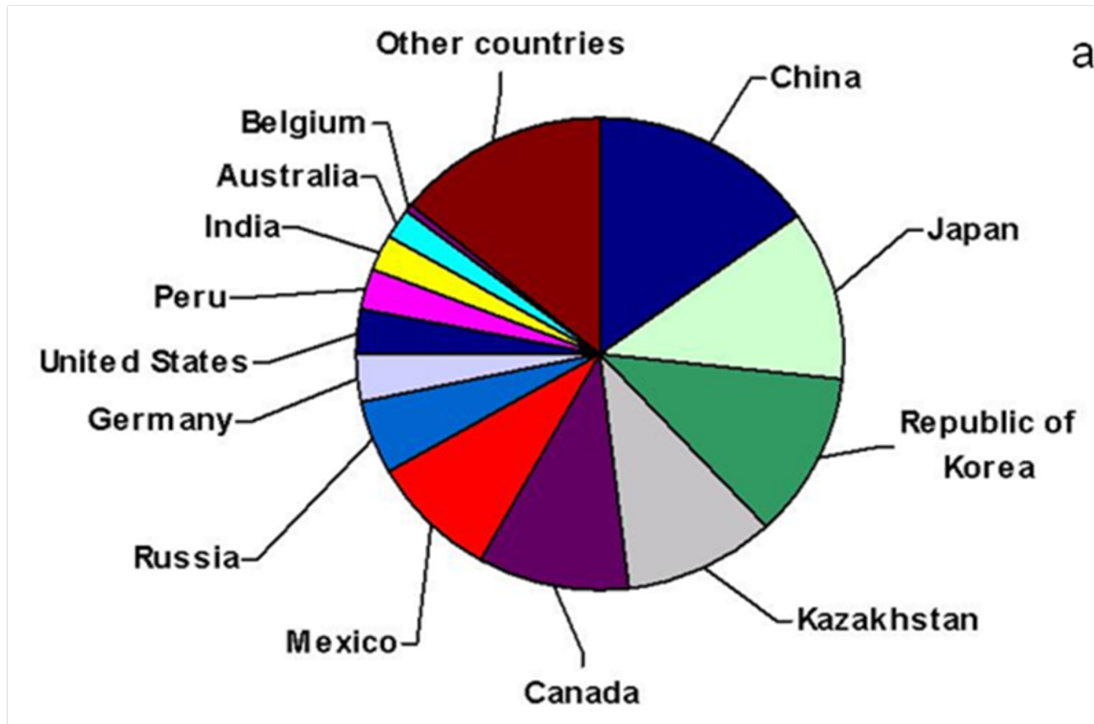


Figure 16: Global primary cadmium consumption by end-use, 1980-2005. Data compiled by the International Cadmium Association [ICdA, 2006].



b

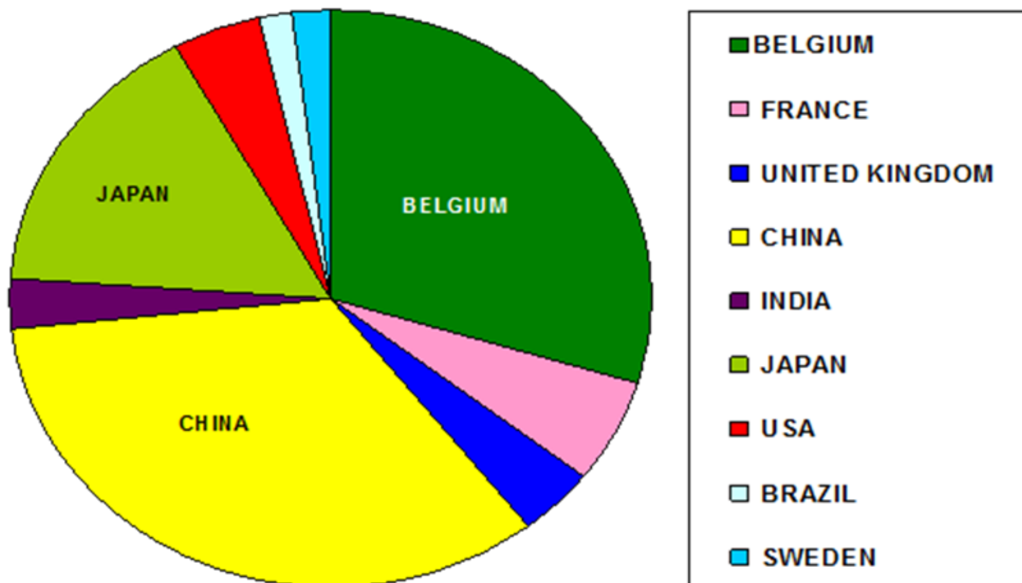


Figure 17 : Consumption of cadmium in (a) 2004 and (b) 2006 [data source: International Cadmium Association, Brussels, Belgium 2005].

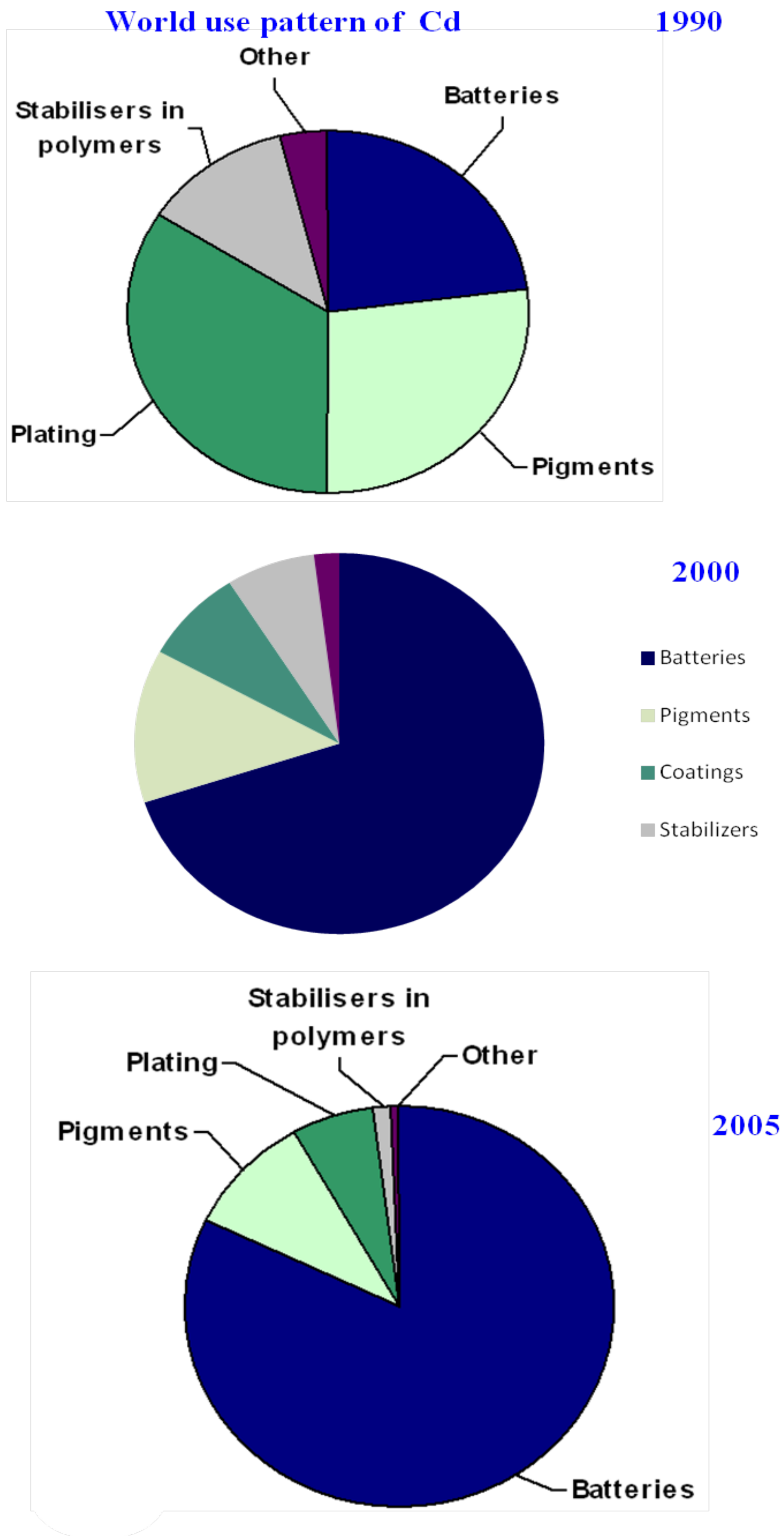


Figure 18: World use pattern of Cd 1990,2000 and 2005.

Cadmium is also released into the environment through non-ferrous (zinc, lead and copper) metal smelters, cement, iron, and steel manufacturing industries, as an emission from fossil fuels and as a contaminant of phosphate fertilizers [Cook and Morrow, 1995]. It is also released through natural and geogenic processes such as volcanic activities [Hutton, 1983]. It has an extremely long biological half-life (>20 years) and is listed by the U.S. Environmental Protection Agency (US-EPA) as one of the 126 priority contaminants and as a known carcinogen by the International Agency for Research on Cancer [Wanna et al, 2009]. Cadmium safety level in drinking water has been set at 0.01 mg L^{-1} as per USA-EPA [Volesky, 1990]. The permissible limits of Cadmium in waste water and drinking water are 0.1 and 0.05 mg L^{-1}, respectively [Gupta and Rastogi, (2008]. Cadmium contamination and toxicity in human beings was first reported in Japan in the 1950s where the municipal sewage sludge was used as a fertilizer for the rice crop. [Nasrin et al, 2011., Kaneta et al, 1986]. It is non-biodegradable and tends to accumulate in living organisms, causing significant threats to both the environment and public health [Xiao et al, 2010].The cadmium exposure leads to adverse health effects to human, adverse health and toxic effects due to cadmium exposure were well documented, cadmium induced genomic instability through induction of DNA damage, inhibition of DNA repair process, cell cycle progression control and apoptosis, stimulation of cell proliferation and epigenetic modulation of gene expression Figure 20, [Filipic, 2012]. Diseases such as renal dysfunction and damage [Nakagawa et al, 1990], liver damage, bone degradation and hypertension [Nordberg et al., 1993], hypertension [Schroede, 1965], anaemia [Jacobs et al, 1969], and itai-itai [Bui et al, 1975] are associated with excess bioavailability of cadmium to humans. Due to this cadmium has been included in red list of priority pollutants by Department of Environment, UK [UK Red List Substances, 1991] and in List I (the ‘‘black list”) of Directive 76/464/EEC [Council directive, 1976]. USEPA has also classified cadmium as group B1 carcinogen [USEPA, 1999].

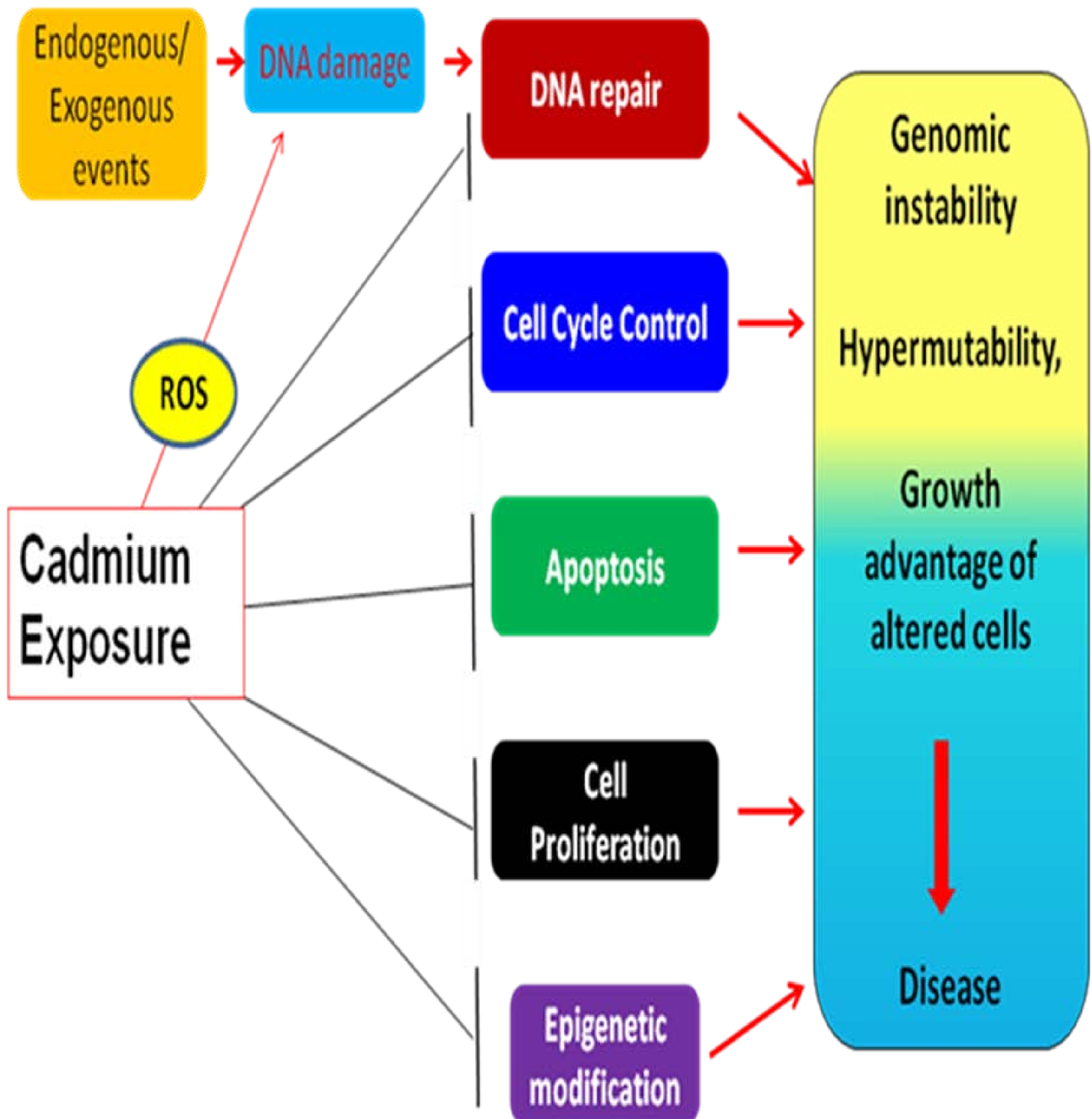


Figure 19: Mechanisms involved in cadmium induced genomic instability through induction of DNA damage, inhibition of DNA repair process, cell cycle progression control and apoptosis, stimulation of cell proliferation and epigenetic modulation of gene expression.

The removal and recovery of heavy metals is very important with respect to environmental and economical considerations [Nourbakhsh et al, 2002]. A number of methods such as the coagulation, chemical precipitation, ion exchange, evaporation, membrane processing, electrolytic and adsorption technologies are used for removal of toxic metals from industrial waste waters and other effluents [Fenglian and Wang, 2011]. However, these methods have some disadvantages and other limitations which include: low efficiency, intense energy requirement and often are not feasible to reduce the cadmium concentration to the level required by environmental legislation. Furthermore, production of toxic chemical sludge as a secondary contaminant is an additional problem that needs further treatment. Thus, there is a need for the development of economic, effective and safe, method for removal of cadmium from aqueous solutions [Rathinam, et al, 2010]. Biosorption is an efficient, low cost method, operates in short time, and does not produce toxic secondary wastes [Mungasavalli et al, 2007]. Figure 20 shows the Number of published papers as we can also observe gradual rise in publications on "Biosorption" and "Cadmium Biosorption" using a variety of materials and applications. A total of 21,745 among them 9985 Cadmium articles have been indexed in Scopus web of science starting with only 472 in 1999. (Source: http://www.hub.sciverse.com).

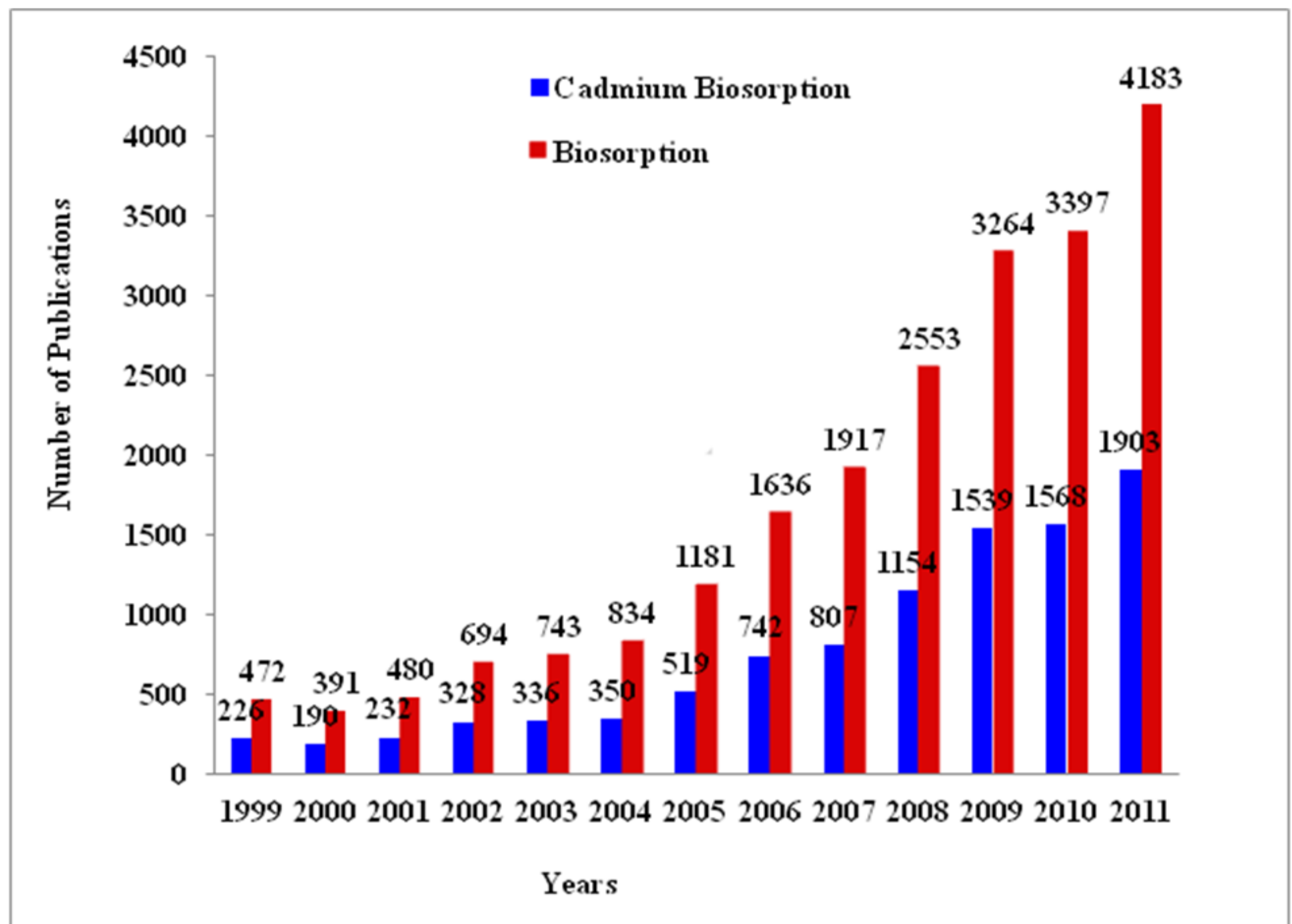


Figure 20: Number of published papers as we can also observe gradual rise in publications on "Biosorption" and "Cadmium Biosorption" using a variety of materials and applications. A total of 21,745 among them 9985 Cadmium articles have been indexed in Scopus web of science starting with only 472 in 1999. (Source: http://www.hub.sciverse.com).

A wide variety of agro-forestry and biological materials are proposed as adsorbents such as wheat shell [Basci et al, 2004], brown algae [Liu et al, 2009], olive stone [Blazquez et al, 2005], orange peel [Li et al, 2008], red mud [Nadaroglu et al, 2010], brown seaweed [Basha et al, 2009], coconut copra meal [Ho and Ofomaja, 2006], olive pomace [Pegnanelli et al, 2003], macrofungus [Sari and Tuzen, 2009], mushrooms [Vimala and Das, 2009], tree fern [Ho and Wang, 2004], saw dust and neem bark [Naiya et al, 2009], eucalyptus bark [Ghodbane et al, 2008], activated sludge [Kumar et al, 2009], *Rosa gruss an teplitz* [Bhattia et al, 2009], *Hydrilla verticillata* biomass [Bunluesin et al, 2007], *Cupressus sempervirens*, *Eucalyptus longifolia* and *Pinus halepensis* [Al-Subu, 2002], *Carpobrotus edulis, Euphorbia echinus*, *Senecio anthophorbium*. *Launea arborescens* leaves [Benhima et al, 2008] and papaya wood [Asma et al, 2005] for removal of toxic metals from aqueous solutions. Usage of phytocoagulants for water and waste water treatment has gained considerable importance recently [Yin, 2010]. The tribals in the forest areas of Visakhapatnam and Vizainagaram, Srikakulam districts of Andhra Pradesh are actively involved in collecting minor forest produce by forming “Girijan Co-operative Corporation (GCC)” and “Vana Samrakhshana Samithis” (Forest Protection Organisations). *S. potatorum* seeds (popularly known as cleaning nuts) are one of the most important minor forest products collected by the members of the GCC along with *Strychnos nux-vomica* (nux-vomica), *Sterculia urens* (Gum karaya), *Terminalia chebula* (myrobalan), *Tamarindus indica* (Tamarind), *Phyllanthus emblica* (Amla), *Madhuca longifolia* (Mohwa). A commercial product by name “NATFLOC” a “natural polyelectrolyte” has been developed with the seeds of *S. potatorum* by the GCC. NATFLOC is recommended by the GCC for turbidity removal of water in a wide range of turbidity levels up to 3000 NTU (Nephelometric Turbidity Unit). It is being used as a secondary flocculent in conjunction with alum for cost reduction of turbid natural water treatment. The raw seed powder is by indigenous tribals for cleaning the turbid and metal

contaminated natural waters. However, the biochemical characterization of the coagulant seed proteins for removal of heavy metals in water has not been scientifically investigated. Therefore, in this study *S. potatorum* seed proteins were isolated to study their ability for the removal of cadmium from aqueous solution. *S. potatorum* seeds are also reported to exhibit antidiarrheal active principles [Biswas et al, 2002] and possesses diuretic activities [Biswas et al, 2001]. The seeds constitute unique coagulant polysaccharides [Corsaro et al, 1995., Adinolfi et al,1994], indole alkaloids [Massiot et al, 1992], sitosterol, stigmasterol and campesterol [Singh et al, 1978], diaboline [Singh et al, 1975] .

3.2 Materials and Methods

All the chemicals and reagents used in the present study were of analytical. All the glassware used were washed with 10 % (v/v) HNO_3 and subsequently rinsed several times with deionized distilled water to remove any possible interference by other materials. Cd(II) stock solution (1000 mg L^{-1}) was prepared by dissolving 1.6306 gm of $CdCl_2$ (qualigens fine chemicals, Mumbai; minimum assay 99%) in 200 ml of (Millipore) Milli-Q water and the final volume made up to 1000 ml with Milli-Q water. Different concentrations of Cd (II) were prepared after diluting the stock solution appropriately. Standard solution of Cd (II) (1000 mg L^{-1}) for atomic spectrophotometer was procured from Sisco Research Laboratories (India). pH of the solutions was adjusted with 1 M NaOH or 1 M HCl. Affigel-10 was procured from Bio-Rad laboratories, USA.

3.2.1. Plant material

S. potatorum L. seeds were obtained from the divisional forest, flying squad division, Rajahmundry, East Godavari district, Andhra Pradesh India. Seeds were dried at 40°C for 2 days in hot air oven. Seeds were made into powder in Clotech 1093 sample mill. 10 g of the seed powder was used for every batch of protein extraction.

3.2.2 Extraction and isolation of total proteins

S. potatorum seeds were ground to a fine powder and defatted with acetone and hexane. From the dried seed powder, total proteins were extracted overnight at 4°C using 25 mM Tris- HCl buffer pH.7.4 containing 150 mM NaCl (TBS), 1 mM phenylmethylsulfonyl fluoride (PMSF) was added as a protease inhibitor. The suspension was clarified by centrifugation at 10000 rpm for 20 minutes. The clear supernatant containing the soluble proteins was subjected to 0-30, 30- 70 % ammonium sulfate fractionation. Protein concentration in the extracts as well as the ammonium sulphate precipitated proteins was determined using Lowry's Method [Lowry

et al, 1951], Bradford method [Bradford, 1976] and bicinchoninic acid (BCA) method [Smith et al, 1985] employing Bovine Serum Albumin (BSA) as standard.

3.2.3. Gel filtration

Seralose 4B (Sisco Research Laboratory, India: equivalent to Sepharose 4B supplied by Sigma, USA) matrix was used for separation of the 30-70% ammonium sulfate precipitated protein. The column (75 ml gel) was equilibrated with 25 mM TBS. The precipitated protein was dissolved and dialyzed against TBS and applied onto the gel in batches to separate the proteins. Protein in column fractions was monitored by measuring the absorbance at 280 nm.

3.2.4. SDS- PAGE analysis of the proteins

SDS-PAGE analysis was carried out according to Laemmli [Laemmli, 1970]. The protein bands were visualized by silver staining as described by Blum et al., [Blum et al, 1978].

3.2.5. Immobilization of proteins to Affigel-10 (Bio-Rad laboratories, N-hydroxysuccinamide esters of a derivatized cross-linked agarose bead support)

The proteins eluted from the gel above as two distinct peaks (peak A) and (peak B) were separately pooled and concentrated. These and the 30-70% ammonium sulfate precipitated proteins were separately immobilized to 2 ml of affigel-10 each following the manufacturer's instructions. Coupling the proteins to affigel was carried out at $4^{o}C$. For each protein sample 2 ml of affigel-10 was packed in a sintered syringe and washed successively with ice cold isopropanol, water and then with 0.1 M HEPES buffer pH 7.4. To this the [protein sample was added, column was closed and coupling was allowed to proceed overnight at $4^{o}C$ by rotating the column end over end for 24 hours. At the end of this period, the unbound fraction was collected and saved (to determine the extent of coupling), and the gel was incubated with 0.1 M HEPES buffer containing 400 uL of 0.1 M ethanolamine Hydrochloride to block unreacted sites and allowed to rotate for one hour in cold. The gel was finally washed with PBS and stored in cold until used.

3.2.6. Interaction of proteins with metal ions (adsorption and desorption cycle)

To evaluate if the proteins from the seed extracts isolated and immobilized above can specifically interact with metal ions such as cadmium, the following experiments were done. The three gels prepared above were separately packed into columns (2 ml of each gel) and equilibrated with 25 mM phosphate buffer pH 7.4. The columns were washed with de-ionized Milli-Q (Millipore) water. To each of the gels, 3 ml of (80 mg L^{-1}) cadmium metal ion (pH 5.0) was added and rotated for six hours at $4^{o}C$ to ensure optimal binding. The effect of different pH solutions on the equilibrium adsorption of Cd(II) ions was investigated (pH 2.0, 3.0, 4.0, 5.0, 6.0, 7.0). Since pH 5.0 was found to be optimal for binding, the other three separate experiments viz., i) to analyze the binding efficiency at different time intervals (5, 10, 20, 30, 60, 180, 360, 480 and 600 minutes). ii) to study the effect of different concentrations of the metal ions (80, 90, 100 and 110 mg L^{-1}) and iii) to study the efficiency of binding at different temperatures (4, 24 and 40 ^{o}C) were conducted at pH 5.0. In all these experiments the gels were processed in the same way. After loading the sample, the unbound solution was collected; the gel was washed with de-ionized Milli-Q water for removing the excess metal ions. The bound metal ions were eluted using 0.15 M HCl. The metal concentrations were measured using flame atomic absorption spectrometer (GBC 932 plus, Australia). The wavelength used for analysis of the metal in this study was 228.8 nm. The instrument was calibrated within the linear range of analysis and a correlation coefficient (R^2) of 0.996-1.000 was obtained for the calibration curve. To check the reproducibility, all the experiments were repeated thrice and each experiment in turn was carried out in triplicates. The instrument was periodically checked throughout the analysis with known standards.

The equilibrium metal uptake q_e (mg g^{-1}) and the sorption efficiency (%) can be calculated according to the mass balance equations [Bhatti et al, 2007].

Amount of adsorption: Q_e (mg g^{-1}) = (C_o - C_e) V / M (1)

Sorption efficiency $R = \frac{Co-Ce}{Co}100$ (2)

Where C_o and C_e are the initial and equilibrium concentrations (mg L^{-1}), V the volume of solution (L), M is the weight of the protein used (g).

3.2.7. Desorption of the adsorbed Cd(ll)

Cd(II) was first bound on the affigels. The gels were washed to remove unbound metal ion. After extensive washing with deionized distilled (milli-Q) water, the Cd (II) bound on the gel was eluted using 0.15 M HCl. In all cases 3 ml fractions were collected. The Cd(II) concentration in the solutions was measured using flame atomic absorption spectrometer (GBC 932 plus, Australia).

3.3 Results

3.3.1 Extraction and isolation of total proteins

In the present study a detailed analysis of the protein profiling of the extracts from the seeds of *S. potatorum*, was carried out in an attempt to understand and identify the components in the seeds that impart the property for metal binding. Toward achieving these goals, the seed extracts were subjected to ammonium sulfate fractionation 0–30 and 30–70%. The 30–70% fraction that contained most proteins was further processed by gel filtration. Figure 21 shows that the proteins applied on the gel separated into two distinct peaks A and B. When these were analyzed by 12.5% SDSPAGE and silver staining, protein bands could be seen in both A and B samples (Figure 21, inset). The proteins isolated might represent storage proteins in the seed material and so we further investigated if these proteins have the ability to bind metal ions. For this purpose the 30–70% ammonium sulfate precipitated fraction, the peak A and peak B separated on gel filtration, were all separately immobilized to affigel at a concentration of 2.97 mg mL^{-1}, 4.1 mg mL^{-1} and 2.84 mg mL^{-1}, respectively. In all figures solid squares: cadmium adsorption by protein of the peak A in gel filtration. Solid circle: cadmium adsorption by proteins from the peak B in gel filtration. Solid triangle: cadmium adsorption by 30–70% ammonium sulfate precipitated proteins, the Figure 21 Gel filtration of the 30–70% ammonium sulfate precipitated proteins on Seralose 4B. After collecting the avoid volume fraction in a measuring cylinder (25 mL). Fractions of 3 mL were collected and the absorbance monitored at 280 nm. Inset shows SDS-PAGE pattern. Error bars show the standard deviation for the data of triplicate experiments done.

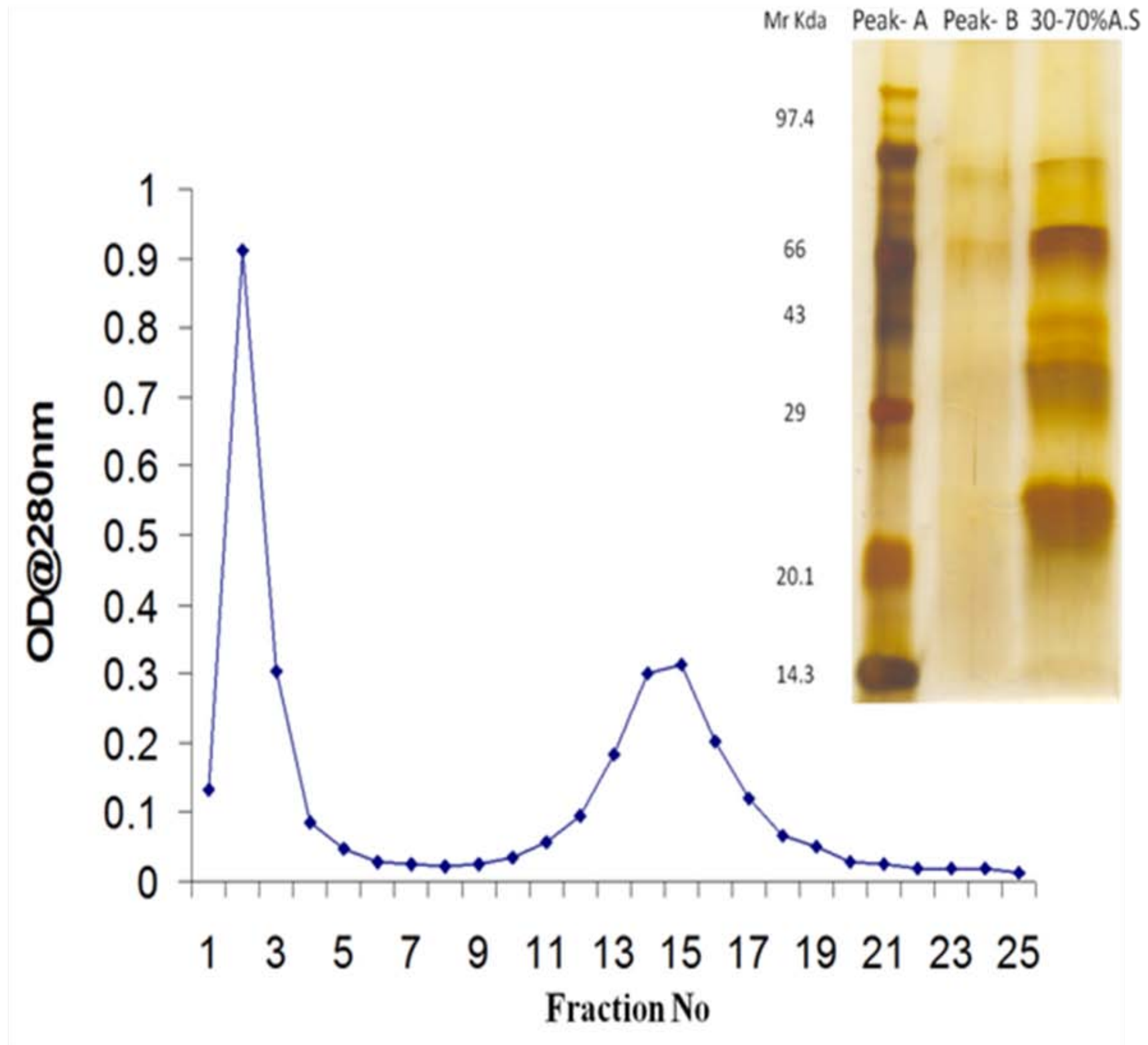


Figure 21 : Gel filtration of the 30-70% ammonium sulfate precipitated proteins on seralose 4B. After collecting the avoid volume fraction in a measuring cylinder (25 ml). Fractions of 3 mL were collected and the absorbance monitored at 280 nm.

Inset shows 12.5% SDS- PAGE analysis of the proteins obtained from the seed extracts. After electrophoresis gel was stained using silver staining method.

3.3.2. Effect of pH on metal biosorption

The pH of the aqueous solution is an important controlling parameter in the biosorption process [Yu and Kaewsarn, 2002]. In our study the effect of pH solution on the biosorption of cadmium ions using proteins isolated from *S. potatorum* seed powder with different pH solutions was studied and the results are shown in the Figure 22 There was a gradual increase in cadmium ion adsorption with the increase of the pH from 2.0 to 7.0. The maximum cadmium uptake was obtained at pH 5.0 for all proteins used in this study; the increase in the biosorption of cadmium with increase in pH can be explained by the fact that at low pH, the biosorbent surface became more positively charged thus reducing attraction between the biomass and metal ions. These bonded active sites thereafter become saturated and therefore are inaccessible to other cations [Yu et al, 2006]. At higher pH, the biosorbent surface is more negatively charged, thus attracting more cadmium ions. However, with further increase in pH the formation of anionic hydroxide complexes decreases the concentration of free cadmium ions; thereby the biosorption capacity of cadmium ions also decreases [Stephen and Sulochana, 2004]. The results obtained in the present study can be correlated with the cited references above.

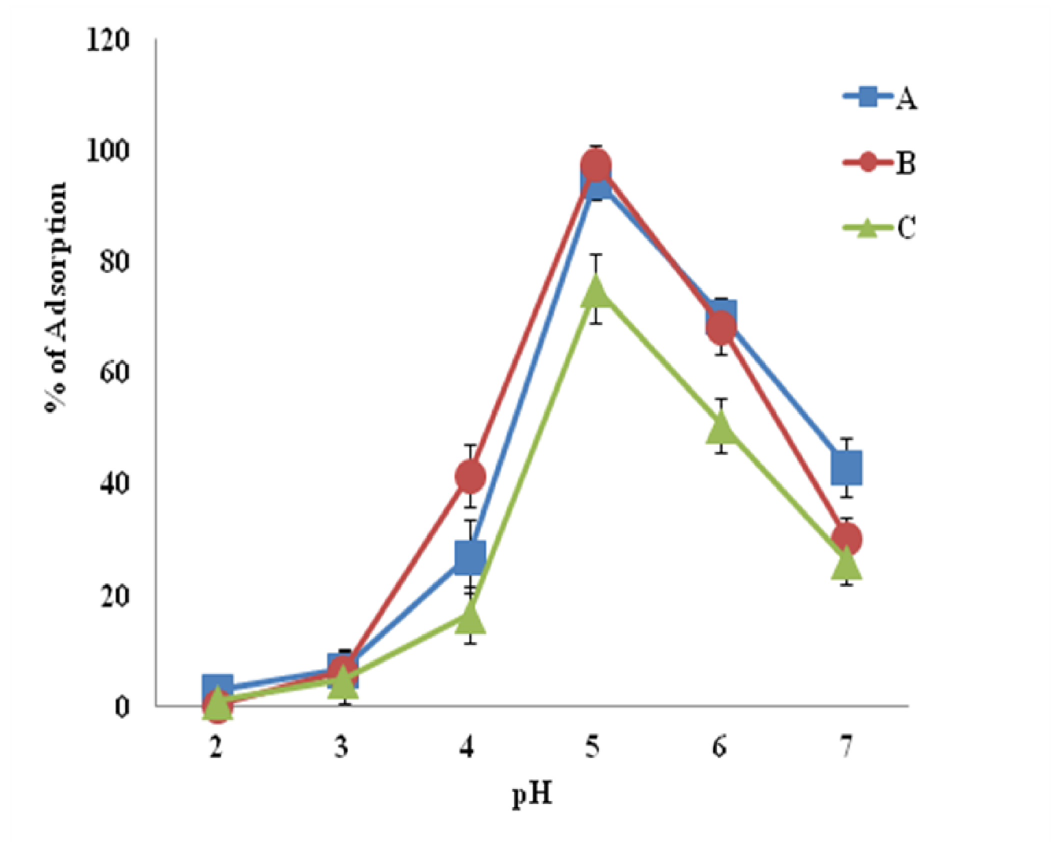


Figure 22: Effect of pH on Cd(II) adsorption by proteins isolated from the seeds of *S. potatorum* . Initial metal concentration = 80 mg / L, temperature 4 °C, contact time = 360 minutes. (Solid squares: Cadmium adsorption by protein from peak A in gel filtration. Solid circle: Cadmium adsorption by proteins from the peak B in gel filtration. Solid triangle: Cadmium adsorption by 30-70% ammonium sulfate precipitated proteins). Error bars show standard deviation, n= 3.

3.3.3. The effect of time

The effect of contact time on the adsorption of Cd(II) at 80 mg L^{-1} and pH 5, is shown in the Figure 23 The cadmium adsorption increased with increasing the contact time, the maximum removal of cadmium occurred at 180 min, after which there were no significant changes. The equilibrium was reached at 360 min for the Cd(II) adsorption by proteins isolated from the seeds of *S. potatorum* as we can observe from the figure that the adsorption started fast and increased rapidly till 180 min. Following this, the adsorption rate was uniform as there was no significant change in adsorption with the increasing time. The initial fast adsorption is due to the availability of more active sites and more functional groups which participate in the cadmium uptake till equilibrium is attained and thereafter, there was no further adsorption. Therefore, there is no significant change in the cadmium concentration in the solution.

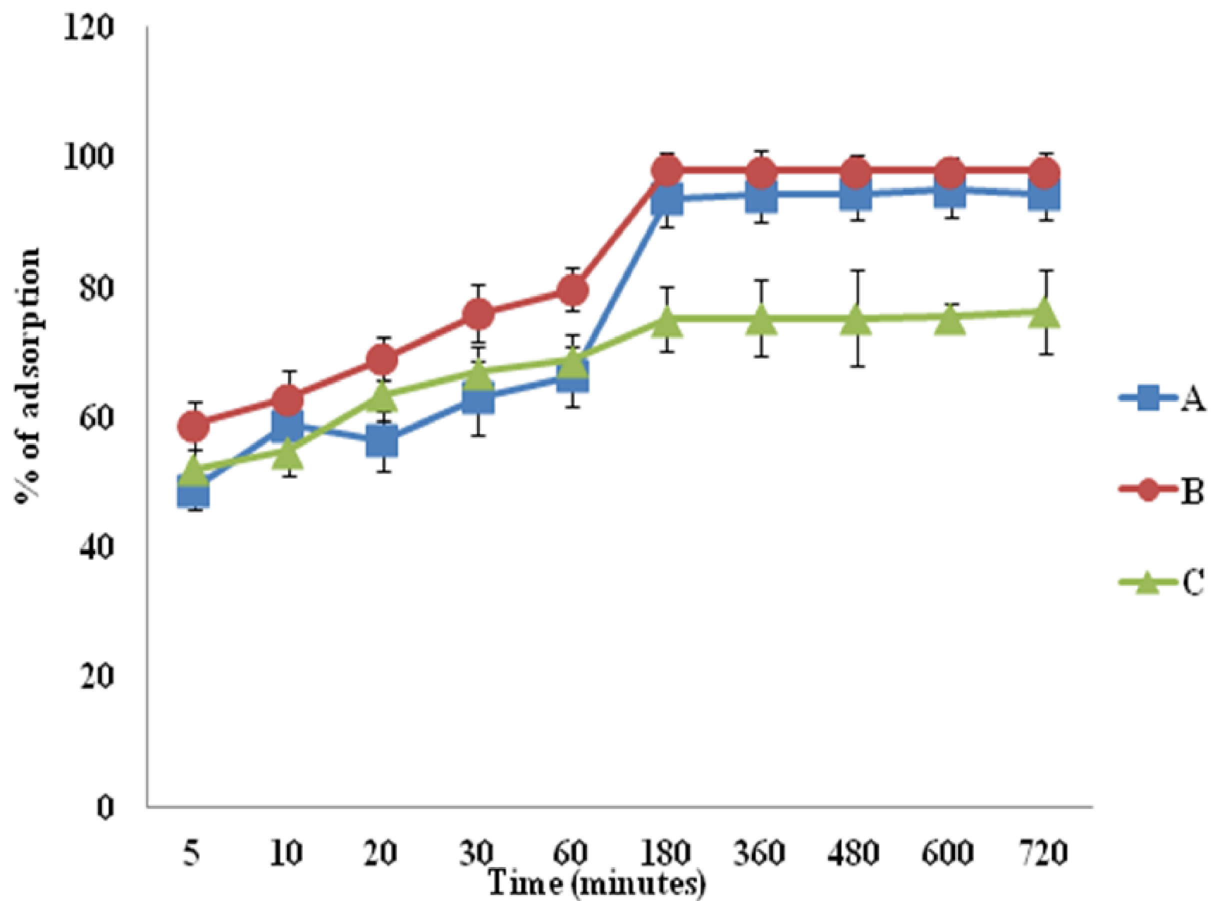


Figure 23: Effect of Time on Cd(II) adsorption by proteins isolated from the seeds of *S. potatorum*. Initial metal concentration = 80 mg/L, temperature = 4 ^{0}C, pH 5.0, contact time 5, 10, 20, 30, 60, 120, 180, 240, 360 and 600 (Solid squares: Cadmium adsorption by protein from peak A in gel filtration. Solid circle: Cadmium adsorption by proteins from the peak B in gel filtration. Solid triangle: Cadmium adsorption by 30-70% ammonium sulfate precipitated proteins). Error bars show standard deviation, n=3.

3.3.4. Adsorption kinetics

The information on the kinetics of solute uptake is necessary to select the optimum operating conditions for full-scale batch process. A number of models have been developed to describe the kinetics of the sorption process. In this study we used two different models to investigate the mechanism of Cd(II) biosorption to the *S. potatorum* seed powder proteins. These kinetic models included pseudo first-order Lagergren model and pseudo second order model. The pseudo first order model derived by Lagergren [Lagergren, 1898] is one of the most widely used models for the biosorption of solutes from a liquid solution [Ho and McKay, 1999., Yu et al, 2007]. And it can be expressed with the following Eq. (3).

$$\text{Log } (q_e - q_t) = \log (q_e) - Ks_1 / 2.303 \times t \qquad (3)$$

Where qe and qt are the amount of metal ions adsorbed on the adsorbent at equilibrium and at any time t (mg g^{-1}) respectively. Ks_1 (min^{-1}) is the Lagergren constant of the pseudo first order biosorption. As shown in Figure 24 the rate constant Ks_1 for the pseudo first order and also the value of q_e were calculated from the plot of ($q_e - q_t$) versus the time. As they are presented in Table 8 The constants determined from the model indicate that the kinetic of adsorption does not fit with this model since the correlation coefficient is low and the experimental q_e value differs from the estimated q_e, for the total incubation time, whereas the pseudo first order could be applicable for the short time less than 100 min only after that linearity of the graph missed, the possibility of this kinetic change may due to the structural change of the proteins exposed to cadmium for long time.

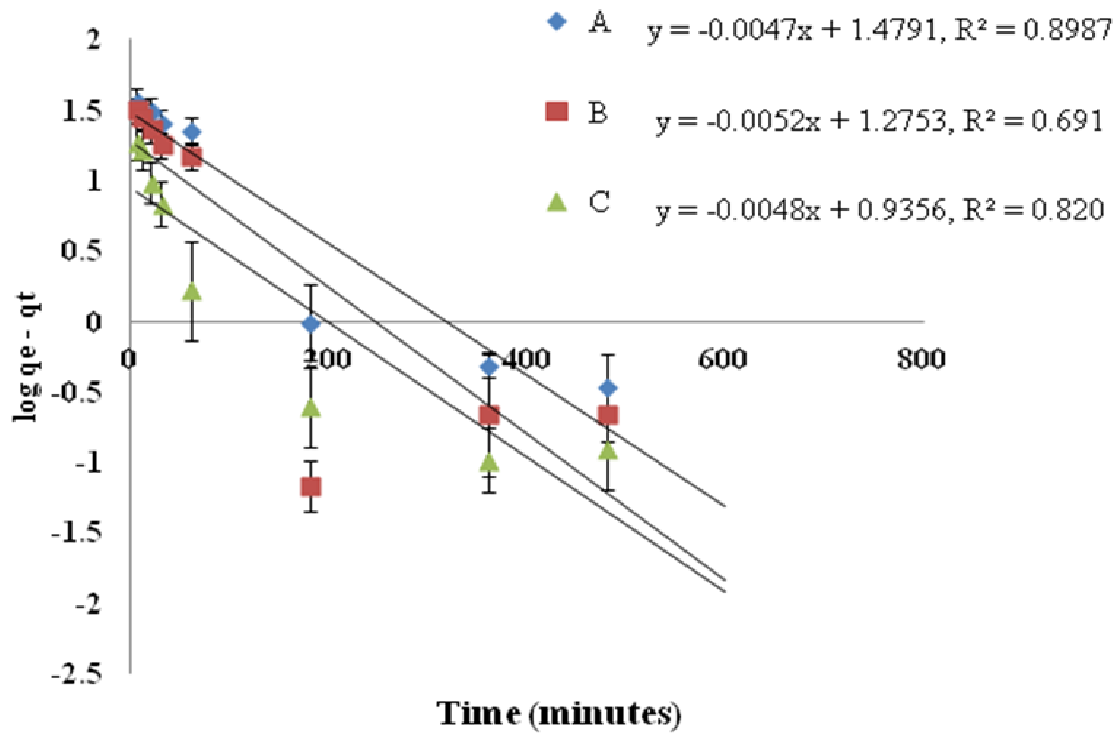


Figure 24: Pseudo first order kinetic model for adsorption of Cd(II) by *S. potatorum* seed proteins (Solid squares: Cadmium adsorption by protein from peak A in gel filtration. Solid circle: Cadmium adsorption by proteins from the peak B in gel filtration. Solid triangle: Cadmium adsorption by 30-70% ammonium sulfate precipitated proteins). Error bars show standard deviation, n= 3.

Table 8: The Pseudo first order model Kinetic constants, slope, intercept, correlation coefficient, q_e (mg g^{-1}) (which is the amount of cadmium adsorbed by the proteins at the equilibrium) and K_1 (min^{-1}) is the Lagergren constant of the pseudo first order biosorption. For Cd(II) uptake by *S. potatorum* seed proteins.

	peak - A protein	peak - B protein	30- 70% proteins
Slope	**0.004**	**0.005**	**0.004**
Intercept	**1.479**	**1.275**	**0.935**
R^2	**0.898**	**0.691**	**0.820**
qe (mg g^{-1})	**30.130**	**18.837**	**8.61**
K_1(min^{-1})	**$9.212x10^{-3}$**	**0.0115**	**$9.212x10^{-3}$**

Pseudo second order model

The pseudo second order model [Ho and McKay, 1999] assumes that biosorption follows a second order mechanism, so that the rate of occupation of biosorption sites is proportional to the square of the number of occupied sites. The pseudo second order model expressed as following.

$$t/q_t = t/q_e + 1/K_2 q_e^2 \qquad (4)$$

Where K_2 is the equilibrium rate constant of the second order biosorption (g mg^{-1} min^{-1}). The pseudo-second order rate constant K_2 and the value of q_e were calculated from the plot of t/q_e versus t Figure 25 and presented in Table 9. The value of correlation coefficient (R^2) of Cd(II) for the pseudo second-order kinetic model on to *S. potatorum* seed powder are very high (0.999), (0.999), (1) for Peak-A, Peak-B and 30–70% ammonium sulfate precipitated proteins respectively. The value of theoretical q_e is closer to the experimental q_e value. On the other hand and if we compare the first and second order correlation coefficients (R^2) and q_e values we can observe that in the first order the correlation coefficients are lower than (R^2) in the second order model, also the theoretical q_e values in the second order are closer to the experimental q_e values than in the first order model. From all that we can conclude that the Cd(II) biosorption onto *S. potatorum* seed proteins follows the pseudo second order kinetic model.

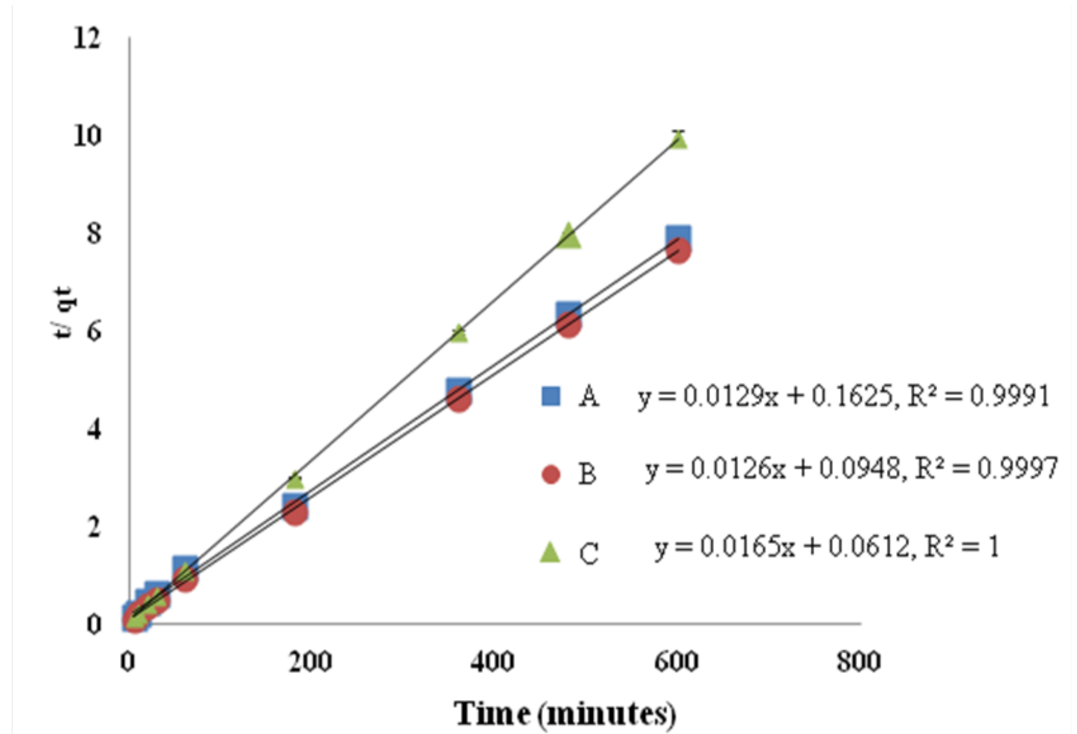


Figure 25: Pseudo second order kinetic model for adsorption of Cd(II) by *S. potatorum* seed proteins (Solid squares: Cadmium adsorption by protein from peak A in gel filtration. Solid circle: Cadmium adsorption by proteins from the peak B in gel filtration. Solid triangle: Cadmium adsorption by 30-70% ammonium sulfate precipitated proteins). Error bars show standard deviation, n= 3.

Table 9: The Pseudo second order model Kinetic constants, slope, intercept, correlation coefficient, q_e (mg g^{-1}) (which is the amount of cadmium adsorbed by the proteins at the equilibrium) and K_2 is the equilibrium rate constant of the second order biosorption (g mg^{-1} min^{-1}). For Cd(II) uptake by *S. potatorum* seed proteins.

	peak- A proteins	peak- B protein	30-70% proteins
Slope	0.0129	0.012	0.016
Intercept	0.162	0.094	0.061
R^2	0.999	0.999	1
q_e (mg g^{-1})	77.52	83.33	62.5
K_2 (g mg^{-1} min^{-1})	$1.02x10^{-3}$	$1.532x10^{-3}$	$4.196x10^{-3}$

3.3.5. Effect of initial Cd(ll) concentration

The rate of adsorption is a function of the initial concentration of metal ions, which makes it an important factor to be considered for effective biosorption [Ahalya et al, 2005]. The effect of metal concentration is shown in Figure 26 The percentage of cadmium ions adsorption at different metal concentrations using proteins isolated from the seeds of *S. potatorum* decreased with increase in metal ion concentration and showed little decrease in percentage (%) of adsorption at higher concentration. This may be due to saturation of active adsorption sites on proteins.

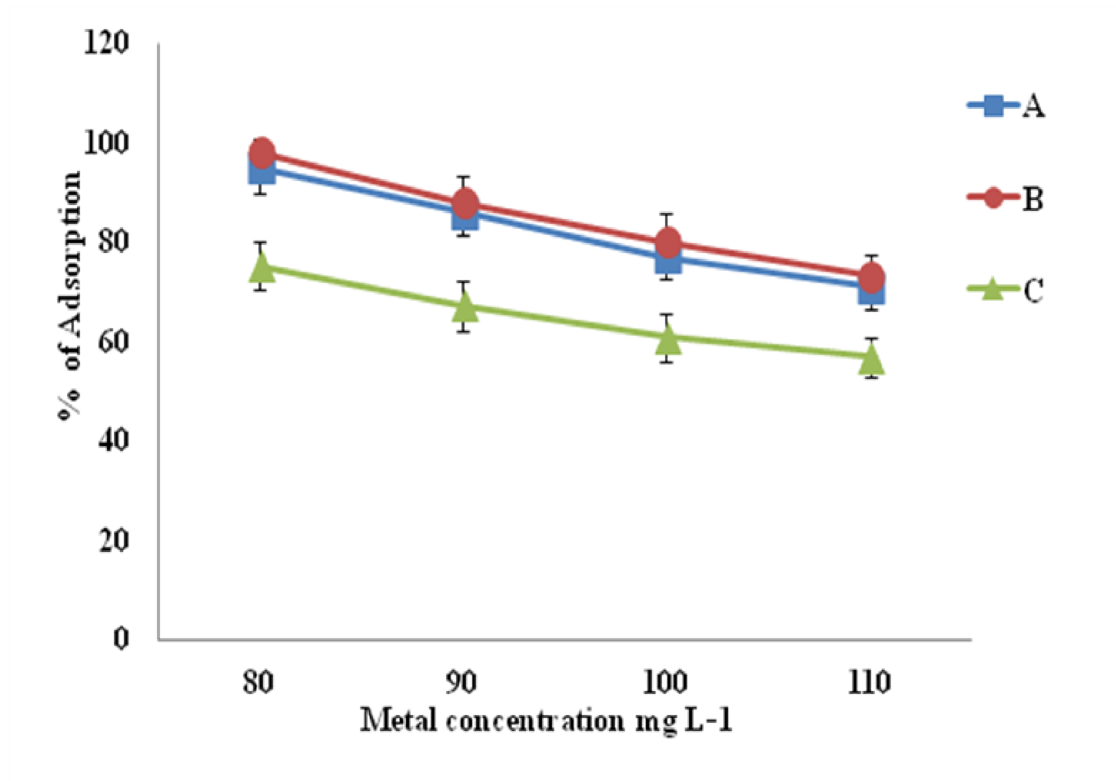


Figure 26: Effect of initial metal (Cd(II)) concentrations on adsorption by proteins isolated from the seeds of *S. potatorum*. Cd(II) concentrations = 80, 90, 100 and 110, at temperature = 4^0C, contact time = 360 minutes, pH 5.0. (Solid squares: Cadmium adsorption by protein from peak A in gel filtration. Solid circle: Cadmium adsorption by proteins from the peak B in gel filtration. Solid triangle: Cadmium adsorption by 30-70% ammonium sulfate precipitated proteins). Error bars show standard deviation, n=3.

3.3.6. Adsorption isotherm

The cadmium adsorption capacity by *S. potatorum* seed proteins at different concentrations of cadmium on a fixed amount of adsorbents was evaluated using the Freundlich and Langmuir adsorption isotherms [Freundlich, 1907]. The Freundlich isotherm represented by the following

$$\text{Log } q_e = \log K_f + (1/n) \log C_e \qquad (5)$$

Where Ce (mg L^{-1}) is the equilibrium concentration; q_e (mg g^{-1}) is the amounts adsorbed per specific amount of adsorbent at equilibrium, K_f (mg g^{-1}) and n are constants which are adsorption capacity and intensity of adsorption, respectively. A plot of log q_e versus log C_e in Figure 27 represents a measure of non-linearity involved. The values of the constants are given in Table 10 which suggests that the cadmium adsorption by *S. potatorum* seed proteins is not following the Freundlich isotherm model.

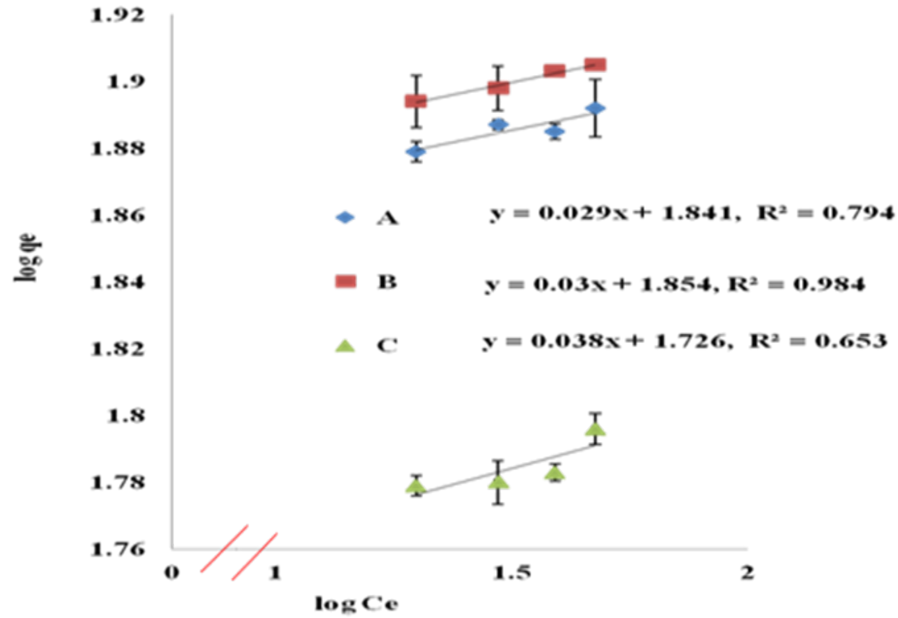


Figure 27: Linearized Freundlich isotherm plot for adsorption of Cd(II) by *S. potatorum* seed proteins, (Solid squares: Cadmium adsorption by protein from peak A in gel filtration. Solid circle: Cadmium adsorption by proteins from the peak B in gel filtration. Solid triangle: Cadmium adsorption by 30-70% ammonium sulfate precipitated proteins). Error bars show standard deviation, n= 3.

Table 10: The Linearized Freundlich isotherm model constants, slope, intercept, correlation coefficient, Kf (mg g^{-1}) and n are constants which are adsorption capacity and intensity of adsorption, respectively. For Cd(II) uptake by *S. potatorum* seed proteins.

	peak- A proteins	peak- B protein	30-70 % proteins
Slope	**0.029**	**0.03**	**0.038**
Intercept	**1.841**	**1.854**	**1.726**
R^2	**0.794**	**0.984**	**0.653**
k_f(mg g^{-1})	**69.34**	**71.45**	**57.81**
n	**34.483**	**33.33**	**26.316**

Langmuir isotherm model

The Langmuir isotherm model [Langmuir, 1916] assumes monolayer coverage and constant binding energy between surface and adsorbate suggesting that the adsorbate surface has fixed number of binding sites and each can hold only one metal ion at a time. The monolayer forms when the equilibrium is attained. The Langmuir linear form is as in the Eq. (6).

$$C_e / q_e = 1 / q_{max} b + C_e / q_{max} \quad (6)$$

Where C_e (mg L^{-1}) is the concentration of the metal ions in solution at the equilibrium, q_{max} (mg solute/g adsorbent) is the maximum adsorption capacity corresponding to complete monolayer coverage, and b (L mg^{-1}) is a parameter related to the energy of adsorption. A plot of C_e/q_e versus C_e gives straight line as shown in Figure 28. The values of q_{max} and b and the other parameters are given in Table 11 The q_{max} was calculated from the slope of the plot, and also these calculated q_{max} is matching with the theoretical q_e of Cd(II) with all of the three protein samples that were used in this study. According to the correlation coefficient it can be observed that the Cd(II) adsorption experimental data was better fitted to the Langmuir model than that of the Freundlich isotherm model.

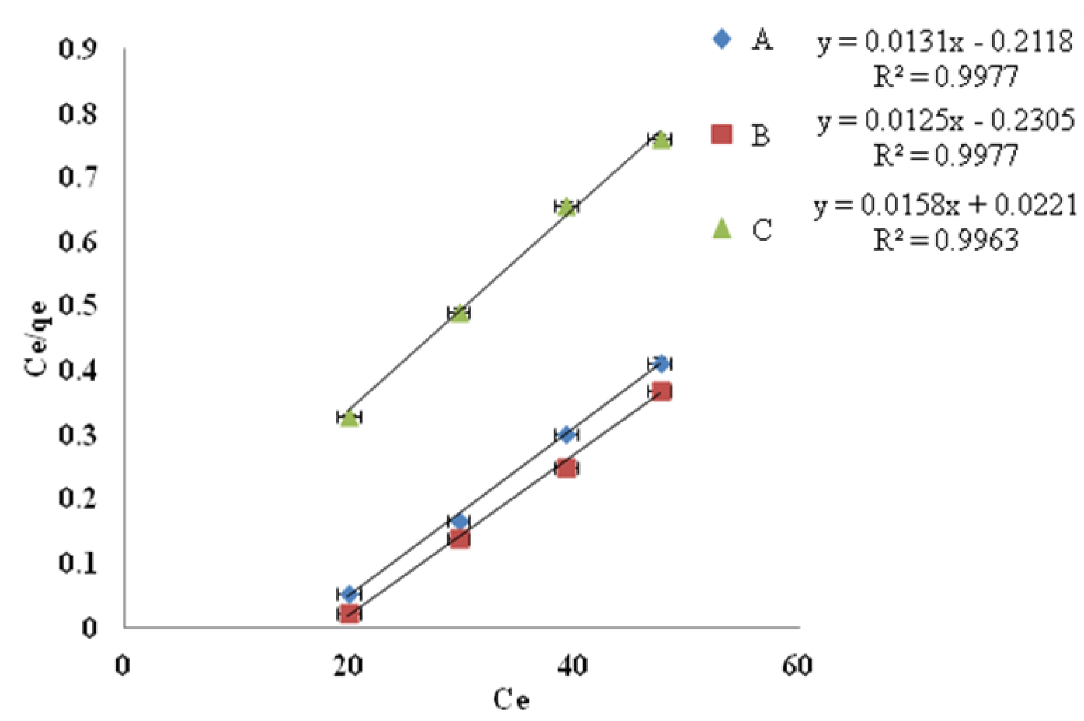


Figure 28: Linearized Langmuir isotherm plot for adsorption of Cd(II) by *S. potatorum* seed proteins, (Solid squares: Cadmium adsorption by protein from peak A in gel filtration. Solid circle: Cadmium adsorption by proteins from the peak B in gel filtration. Solid triangle: Cadmium adsorption by 30-70% ammonium sulfate precipitated proteins). Error bars show standard deviation, n= 3.

Table 11: The Linearized Langmuir isotherm model constants slope, intercept, correlation coefficient, qmax (mg solute / g adsorbent) is the maximum adsorption capacity corresponding to complete monolayer coverage, and b (L mg^{-1}) is a parameter related to the energy of adsorption. For Cd(II) uptake by *S. potatorum* seed proteins.

	peak- A proteins	peak- B protein	30-70 % proteins
Slope	0.013	0.012	0.015
Intercept	0.211	0.230	0.022
R^2	0.997	0.997	0.996
q_{max} (mg g^{-1})	76.92	83.33	66.67
b (lmg^{-1})	61.61x10^{-3}	52.2x10^{-3}	0.682

3.3.7. The effect of temperature

The effect of temperature on cadmium uptake by proteins isolated from *S. potatorum* was studied using constant initial cadmium ion concentration of 80 mg L^{-1}, pH 5.0 for 6 h, at various temperatures and the results are shown in Figure 29 The maximum cadmium uptake by the proteins of *S. potatorum* seeds occurred at 24 ◦C. At 40 ◦C decrease of q_{max} was observed. This decrease at higher temperature may be due to the limitation of the accessibility of metal ions to metal binding sites, as a consequence of biomass denaturation [Michalak et al, 2007., Jayaramet al, 2009]. It is known from literature that some plant proteins can efficiently bind divalent metal ions. Apparently the binding of the positively charged metal ions to the proteins in general is attributed to their specific interaction with acidic amino acids such as the aspartic acid and glutamic acid present in the proteins [Rogers et al, 2000., Clemens, 2006]. Our long-term goal is also to identify the specific amino acids in the proteins we isolated that are responsible for the metal binding which is beyond the scope of this present investigation.

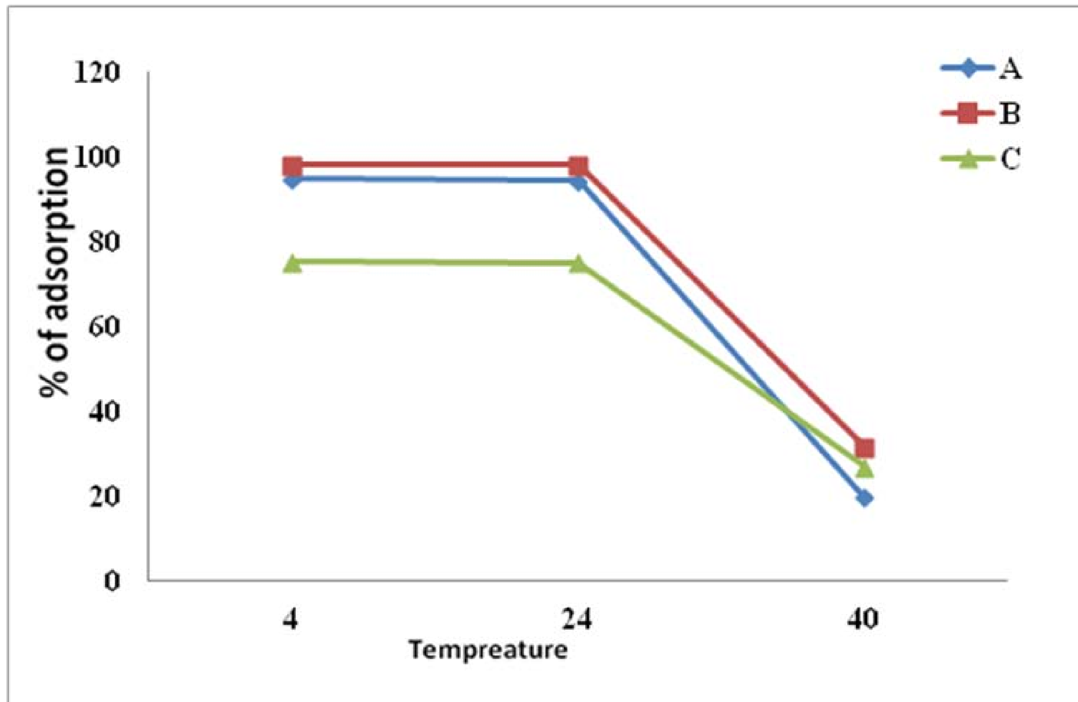


Figure 29 : Effect of temperature on Cd(II) adsorption by proteins isolated from the seeds of *S. potatorum*. Initial metal concentration = 80 mg/l. pH 5.0, contact time = 360 minutes. (Solid squares: Cadmium adsorption by protein from peak A in gel filtration. Solid circle: Cadmium adsorption by proteins from the peak B in gel filtration. Solid triangle: Cadmium adsorption by 30-70% ammonium sulfate precipitated proteins). Error bars show standard deviation, n= 3.

3.4. Discussion

S. potatorum seeds (popularly known as cleaning nuts) are traditionally used in India by tibals to purify turbid water. *S. Potatorum* seeds are also one of the most important minor forest products collected by the members of the GCC along with other products. A commercial product by name "NATFLOC" a "natural polyelectrolyte" has been developed with the seeds of *S. potatorum* by the GCC. NATFLOC is recommended by the GCC for turbidity removal of water in a wide range of turbidity levels up to 3000 NTU (Nephelometric Turbidity Unit**).** It is being used as a secondary flocculent in conjunction with alum for cost reduction of turbid natural water treatment. The raw seed powder is by indigenous tribals for cleaning the turbid and metal contaminated natural waters. However, the biochemical characterization of the coagulant seed proteins for removal of heavy metals in water has not been scientifically investigated. In the present study the proteins isolated from this seed powder were used in an attempt to understand the role of proteins in Cd(II) adsorption from the aqueous media. The present study provided evidence that the immobilized proteins are effective biosorbents for adsorption of Cd(II) from aqueous solution. The Cd(II) adsorption was dependent on pH, contact time, initial metal concentration and temperature. The optimal Cd(II) adsorption conditions were almost same for all three proteins used in this study. The maximum cadmium adsorption by *S. potatorum* proteins were pH dependant and it was occurred at pH 5.0. That can be explained as, the increase in the biosorption of cadmium with increase in pH due to the fact that at low pH, the biosorbent surface became more positively charged thus reducing attraction between the biomass and metal ions. These bonded active sites thereafter become saturated and therefore are inaccessible to other cations [Yu et al, 2006]. At higher pH, the biosorbent proteins surfaces become more negatively charged, thus attracting more cadmium ions. However, with further increase in pH the formation of anionic hydroxide complexes decreases the

concentration of free cadmium ions; thereby the biosorption capacity of cadmium ions also decreases [Stephen and Sulochana, 2004]. The results obtained in the present study can be correlated with the cited references above.

The equilibrium was attained at 360 min for the Cd(II) adsorption by proteins isolated from the seeds of *S. potatorum,* as it was observed there are rapid increase of adsorption rate in the beginning and after 180 minutes the rate of adsorption becomes uniform, till the equilibrium occurred then there was no significant increase in the adsorption, that can be discussed as The initial fast adsorption is due to the availability of more active sites and more functional groups which participate in the cadmium uptake till equilibrium is attained and thereafter, there was no further adsorption. Therefore, there is no significant change in the cadmium concentration in the solution the rate decreased in with time.

To select the optimal condition for the adsorption in full- scale many of kinetics models have been developed to describe the kinetics of the sorption process. In this study we used two different models to investigate the mechanism of Cd(II) biosorption to the *S. potatorum* seed powder proteins. These kinetic models included pseudo first-order Lagergren model and pseudo second order model. From the experimental data we found that the Cd(II) adsorption could be fitted with the pseudo first order model since the correlation coefficient is low and the experimental q_e value differs from the estimated q_e, for the total incubation time, whereas the pseudo first order could be applicable for the short time less than 100 min only after that linearity of the graph missed, the possibility of this kinetic change may due to the structural change of the proteins exposed to cadmium for long time. The pseudo second order model provides the best correlations of the experimental data as the correlation coefficients are high and the experimental data are fitting with the calculated data. Whereas the pseudo first order model fits the experimental data well for an initial period only.

The adsorption was also is metal concentration dependant also as the rate of adsorption increase in the beginning with increasing the metal concentration wher as at high metal concentration the percentage of adsorption shows decrease that may due This may be due to saturation of active adsorption sites on proteins. The cadmium adsorption capacity by *S. potatorum* seed proteins at different concentrations of cadmium on a fixed amount of adsorbents was evaluated using the Freundlich and Langmuir adsorption isotherms. From the experimental data it was found that the correlation coefficients are low and the constants calculated from the experimental data and calculated data are incompatible which indicates that the cadmium adsorption by these proteins are not fitted with Freundlich isotherm model, whereas according to the correlation coefficient it can be observed that the Cd(II) adsorption experimental data was better fitted to the Langmuir model than that of the Freundlich isotherm model. The maximum cadmium uptake by the proteins of *S. potatorum* seeds occurred at 24 ◦C. At 40 ◦C decrease of q_{max} was observed. This decrease at higher temperature may be due to the limitation of the accessibility of metal ions to metal binding sites, as a consequence of biomass denaturation.

Chapter 4

Isolation and purification of polysaccharides, (galactan and galactomannan) from *S. potatorum* seeds

4.1. Introduction

Polysaccharides play an important role in various physiological and structural functions in plants, such as in growth, source of carbon and energy, defense against pathogen attack, signaling, mechanical resistance, and interactions with the environment, [Zagrobelnyet *et al.,* 2004., Thompson, 2005., Lloyd *et al.,* 2005., Parre and Geitmann, 2005., Wingler *et al.,*2006]. Plant polysaccharides can be divided into two main groups with respect to their localization: plant cell wall polysaccharides and storage polysaccharides. Plant cell wall polysaccharides can divided into three major groups: cellulose, hemicellulose and pectin [Pedro et al, 2009]. Cellulose is a linear polymer of β-1, 4-linked D-glucose residues. The polymeric molecules are organized in bundles (microfibrils) which provide the main strength for the plant cell wall [Kolpak and Blackwell, 1976]. These microfibrils are crosslinked by different hemicellulose polymers (xylan, galactomannan and xyloglucan). Xylan is the most abundant hemicellulose structure and consists of a backbone of β-1,4-linked D-xylose residues that contains several side groups such as L-arabinose, D-galactose, D-(4-Omethyl)-glucuronic acid and acetyl and ferulic acid [Ebringerová and Heinze, 2000]. That provides links to the cell-wall strengthening aromatic polymer lignin. Xyloglucan consists of a β-1,4-linked D-glucose backbone to which D-xylose is connected through an α-1,4-linkage [Vincken et al., 1997]. D-galactose, L arabinose and L-fucose can be attached to the D-xylose residues. Galacto(gluco)mannan consists of a β-1,4-linked D-mannose backbone that can be interrupted by D-glucose and can contain side groups like acetyl and a-1,6-linked D-galactose [Dea and Morrison, 1975., Dey, 1978., Timell, 1967]. Pectin is a highly diverse polymer that contains several specific polysaccharides [Perez et al., 2000]. Homogalacturonan consists of an α-1,4-linked D-galacturonic acid backbone that can be methylated or acetylated. Xylogalacturonan is a homogalacturonan that contains β-1,4-linked D-xylose residues linked to the galacturonic acid main chain. In rhamnogalacturonan I (RG I), the backbone is

interrupted by α-1,2-linked L-rhamnose residues to which side chains consisting of L-arabinose (arabinan), D-galactose (galactan) or both (arabinogalactan) can be attached [Guillon and Thibault, 1989]. Ferulic acid attached to pectin or xylan plays an important role in the structure of the plant cell wall due to its ability to form covalent crosslinks between xylan, pectin and the aromatic polymer lignin [Ishii, 1997., Lam et al., 1994], providing additional rigidity to the plant cell wall, that is believed to play a role in limiting cell growth [Wakabayashi et al., 1997] and defense against enzymatic hydrolysis by microorganisms [Eraso and Hartley, 1990]. Starch and inulin are the most common storage polysaccharides in plants. Starch consists of amylose and amylopectin that both have a main chain of α-1,4-linked D-glucose polymer, but amylopectin has α-1,6-linked D-glucose residues resulting in side chains [Peters, 2006]. Inulin is a β-1, 2-linked D-fructose polymer with terminal D-glucose residues [Vijn and Smeekens, 1999]. Polysaccharides and their derivatives have gained increasing attention in the biomedical and drug delivery fields and other applications, Figures 30 Shows the number of publications employing of polysaccharides as potential tools for drug delivery has remarkably increased over the last few years over the last few decades.

Among these polysaccharides Galactomannan, Galactan and their derivatives have become very attractive polysaccharides due to their biological and physicochemical properties that include their structural organizations, viscosity, solubility, gelling properties, molecular weights, interaction with other hydrophilic polymers and degradation behaviors. The importance of galactan and galactomannan as potential tools having varied applications is evident from the large number of research publications that appeared [Figures 32 and 33]. These and other properties of polysaccharides and their derivatives suitable to be used in pharmaceutical formulations successfully as solubilizers or adhesives in the last few years [Alonso- Sande et al, 2009].

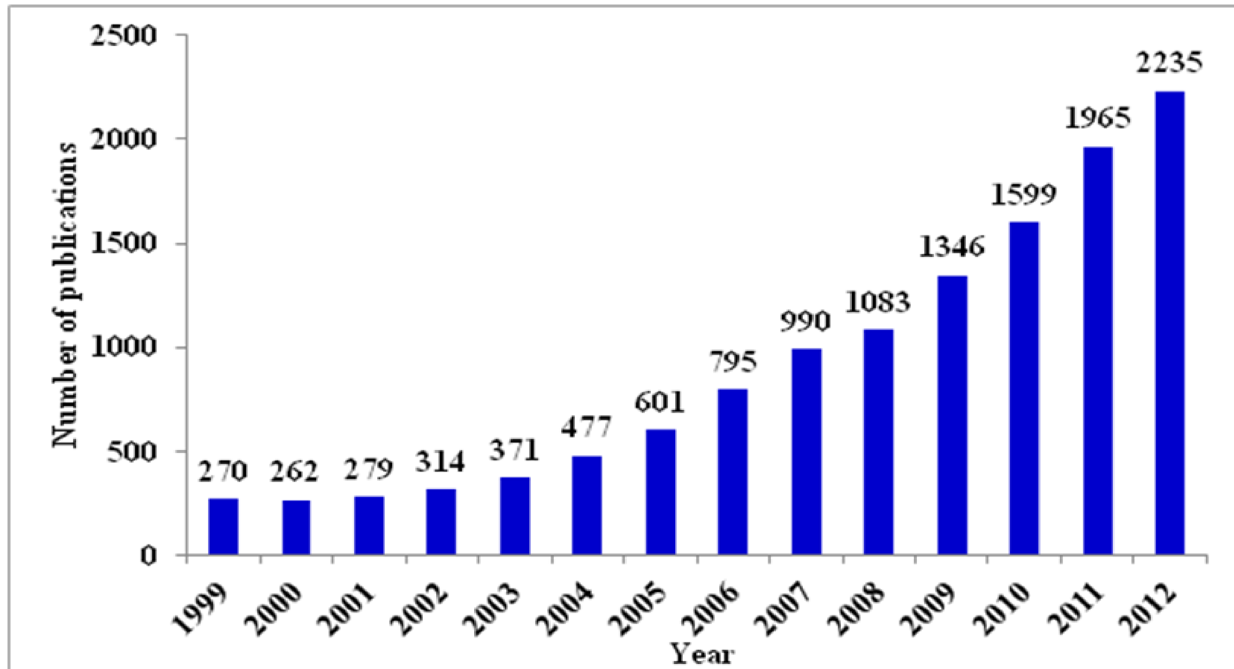


Figure 30 : Number of scientific publications on the topic of polysaccharides and drug delivery shows the gradual increasing of publication as function of the publication year, which indicates the importance of polysaccharides in different fields of research; these were taken from scopus scientific web site.

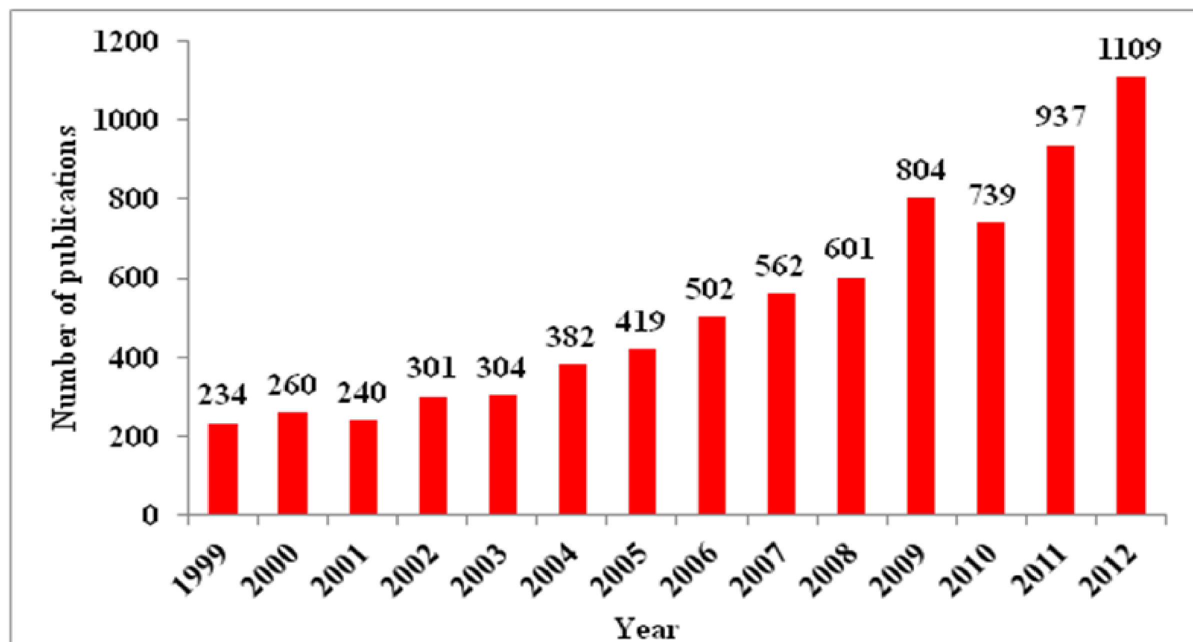


Figure 31 : Number of scientific publications on the topic of galactomannan shows the gradual increasing of publication as function of the publication year, which indicates the importance of galactomannan in different fields of research; these were taken from scopus scientific web site.

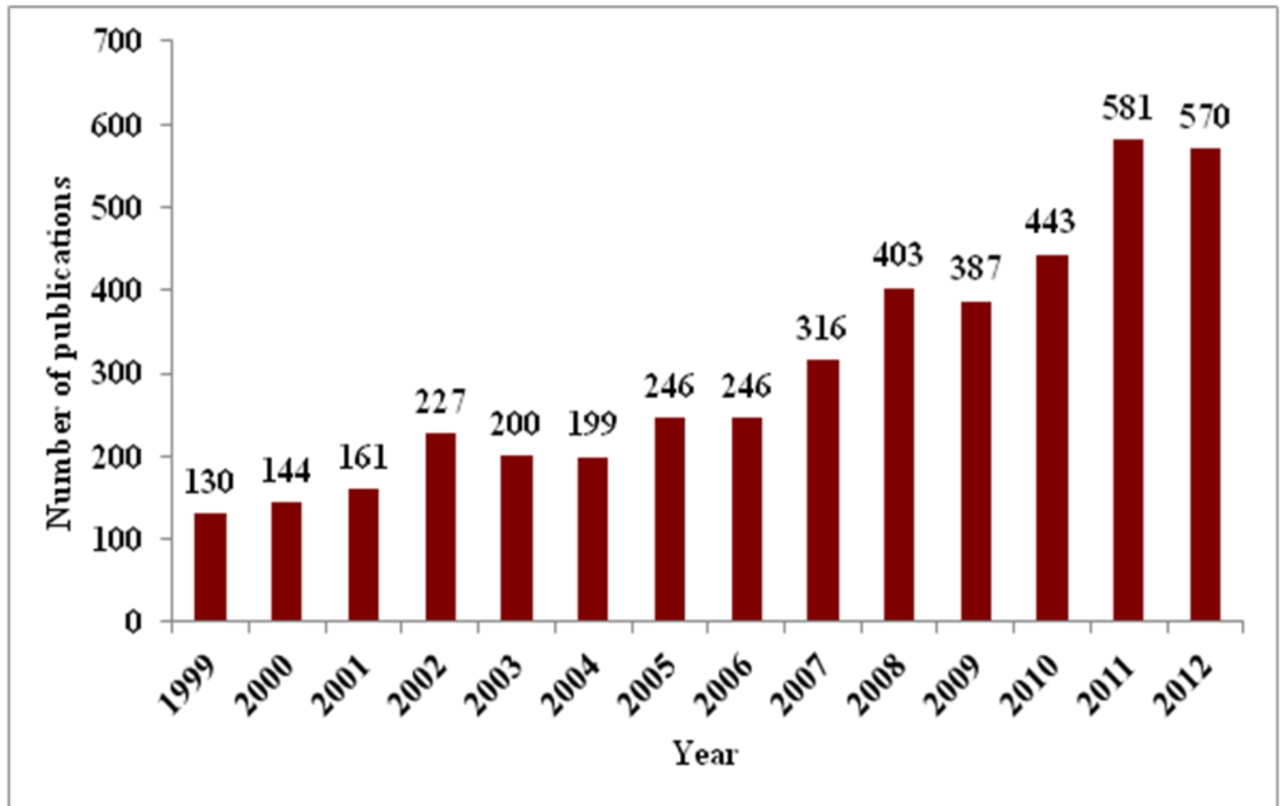


Figure 32 : Number of scientific publications on the topic of galactan shows the gradual increasing of publication as function of the publication year, which indicates the importance of galactan in different fields of research; these were taken from scopus scientific web site.

Few reports have been published on the polysaccharide composition of *Strychnos* seeds. [Andrews et al, 1954] described a galactan and also the presence of galactomannan from the seeds of *S. nux-vomica*. [Dea and Morrison, 1975], reported the occurrence of polymer containing galactose and mannose. [Rao et al (1979)., Corsaro et al, 1995], investigated the polysaccharide content of the seeds of *S. potatorum* and described the structure of a mannogalactan, which is made up of a backbone of β-(1 - 4) - linked D-galactopyranose residues that bear side chains with mannopyranosyl non-reducing ends [Adinolfi et al, 1994] described the composition of the coagulant polysaccharide fraction from the seed of *S. potatorum* comprises of mixture of galactan and galactomannan.

The polysaccharides composition of the seed of *S. potatorum* indicates presence of galactan and galactomannan. Their chemical composition was determined on the basis of acid hydrolysis and the chromatographic analysis of the acid hydrolysis products for both galactan and galactomannan. Additionally, it was also tested if these polysaccharides can bind galactose specific lectins. These data are presented in this chapter.

4.2. Materials and Methods

4.2.1. Materials

All the chemicals and reagents used in the present study were of analytical grade. Bio-Gel P-60 (Polyacrylamide Gel) was procured from Bio-Rad Laboratories, USA. All the glassware used were washed with 10% (v/v) HNO_3 and subsequently rinsed several times with de-ionized distilled water. diethyl ether, D-Galactose are from SRL, India , Seralose 6B, DVS from Sigma Aldrich, (USA) Equipments such as AFM-(SPA-400, USA), Probe sonicator, SEM-(PHILIPS FEI-XL ESEM, USA),Spin Coater, TEM - JOEL JEM 1011, USA, were available in the central facilities and center for nanotechnology of the University of Hyderabad.

4.2.2. Seed material

Seed material and their processing to obtain seed pwder are described in the earlier chapter. 5 g of the seed powder was used for every batch of polysaccharides extraction.

4.2.3. Extraction and purification of polysaccharides from *S. potatorum* seed powder

Seed powder 5g was added to 250 ml of double distilled water, stirred for 15 min, and then centrifuged at 10000 rpm for 10 minutes. The supernatant solution was filtered through Whatman GFC glass fibre disc (0.45 µm) followed by Whatman cellulose acetate filters. The filtrate was added to an equal volume of a 1: 1 mixture of 10 mM pH 7.5 Tris-saturated phenol and chloroform, and the emulsion was stirred at room temperature for 30 min followed by centrifugation at 10000 rpm for 10 min. The aqueous layer was removed, shaken with an equal volume of chloroform, centrifuged at 10000 rpm for 10 min, separated from the organic phase, and mixed with 2.5 volumes of ethanol. The polysaccharide fraction was precipitated overnight at -20 ^{o}C, washed in ethanol, and dried in a vacuum desiccator. From 1 g of seed powder 100 mg of polysaccharides were obtained.

4.2.4. Gel filtration

The polysaccharides extracted above were dissolved in a minimal amount of sodium acetate buffer pH 5.2 and chromatographed on a Bio-gel P-60 (Bio-Rad Laboratories), 75 ml gel in 100 ml column. The column was equilibrated with sodium acetate buffer pH 5.2, and equilibrated with the same buffer.

4.2.5. Acid hydrolysis

The peak (1) and peak (2) polysaccharides were separately subjected to acid hydrolysis using concentrated hydrochloric acid in closed test tubes and heated at 100 ^{o} C, the monosaccharide composition of each peak were monitored on paper chromatography, using solvent of *n*-butanol/ pyridine/ water 6: 4: 3 v/ v/ v. Stained with alkaline silver nitrate.

4.2.6. Phenol sulphuric acid test

The column fractions were assayed for carbohydrate using phenol sulphuric acid method [M. Dubois, et al 1956] using glucose as standard.

4.2.7. Preparation of Seralose-divinyl sulfone-galactomannan and galactan gels (affinity chromatography)

20 ml Seralose 6-B was washed thoroughly with double distilled water on a sintered glass funnel and the wet cake was suspended in 20 ml of 0.5 M sodium carbonate / bicarbonate buffer pH 11. To this 2 ml of Divinyl Sulfone (DVS) was added and the suspension was gently shaken at room temperature for 70 min and washed thoroughly with double distilled water on a sintered glass funnel.

4.2.8. Coupling of galactomannan and galactan to the activated gel

Galactomannan and Galactan were coupled to the activated sepharose 6B separately. The activated gel (2.5 ml) obtained above was washed with 0.5 M sodium carbonate / Bicarbonate buffer pH 10.0 and the wet cake was suspended in 3ml of carbonate buffer pH 10.0 containing 25 mg of Galactomannan and Galactan in separate experiments. Coupling was

allowed to proceed in cold (4° C) for 24 hours. At the end of the coupling reaction, the gel was passed through sintered glass funnel. The gel was washed with deionized water and suspended in 0.5 M sodium bicarbonate buffer pH 8.5 containing β-mercaptoethanol to block the unreactive sites and mixed at room temperature for three hours. The gel was finally washed with distilled water and was packed in a 10 ml column for further use.

4.2.9. Analysis of binding of galactose specific lectin (DLL-II) to galactomannnan and galactan gels prepared

100 g of *Dolichos lablab* seed powder which is known to contain a galactose lectin (DLL-II) was extracted overnight with 700 ml of 25 mM Tris buffered saline pH 7.4 (TBS) at 4 ^{o}C. The suspension was clarified by centrifugation (10,000 rpm at 4 ^{o}C) and the clear supernatant (crude extract) was subjected to 0- 60 % and 60- 80 % ammonium sulfate fractionation. The fractionation allowed the separation of the glucose/ mannose lectin (DLL-I) in the 0- 60 % fraction and the galactose lectin in the 60- 80 % fraction. The fraction containing the galactose lectin was dialyzed against TBS and passed through Seralose – mannose gel to deplete the fraction of any glucose/ mannose lectin. To the unbound protein from Seralose – mannose gel 1.5 M ammonium sulfate was added and applied on a fresh Seralose - Galactomannan and Galactan gels separately, that were equilibrated with TBS containing 1.5 M ammonium sulfate (column buffer). The gel was thoroughly washed with the column buffer until the A_{280} was 0.05 and the bound protein was eluted using 0.3 M galactose in column buffer and the protein was analyzed by coomassie staining as shown in the results.

4.3. Results

Total polysaccharides were extracted from the *S. potatorum* seed powder. The extracted polysaccharides were chromatographed on Bio-gel P-60 gel equilibrated with the column buffer, (50 mM sodium acetate pH 5.2) and the same buffer was also used for the polysaccharides elution. Fractions were monitored for carbohydrate using phenol sulphuric acid method. Figure 33 shows the separation of polysaccharides on Bio-gel P- 60 column into two distinct peaks (1) and (2). Fractions in both peaks were separately pooled and lyophilized for further analysis. The lyophilized powder obtained was hydrolyzed with acid and the hydrolysate was processed as described under methods. The galactomannan component was hydrolyzed yielding galactose and mannose sugars while the galactan component was hydrolyzed yielding galactose only as evidenced by the paper chromatography using *n*-butanol / pyridine / water 6: 4: 3 v / v / v, as solvent. Subsequent staining with alkaline silver nitrate, showed the sugar spots. Comparing the standards mobility to those in the experimental samples, it can be concluded that the isolated polysaccharides are galactomannan and galactan respectively (Figure 34).

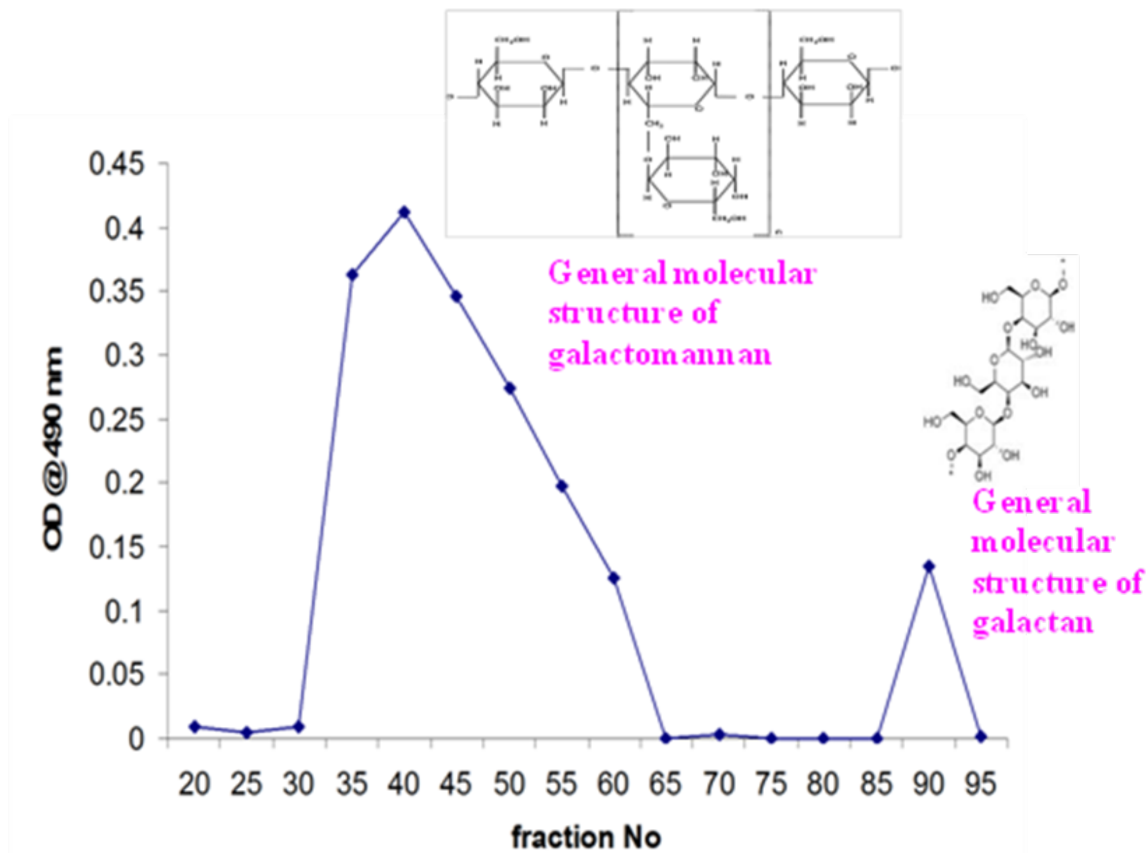


Figure 33 : Gel filtration of the polysaccharides isolated from the seeds of the *S. potatorum*. The polysaccharide fraction obtained above was applied to a Biogel P-60 column equilibrated with 50 mM sodium acetate buffer pH 5.2 at a flow rate of 30 ml / h. Fractions of 0.5 ml were collected. Carbohydrate in the column fractions were monitored using phenolsulphuric acid method. Two distinct peaks (1) and (2) could be obtained, which were pooled separately and lyophilized for further analysis.

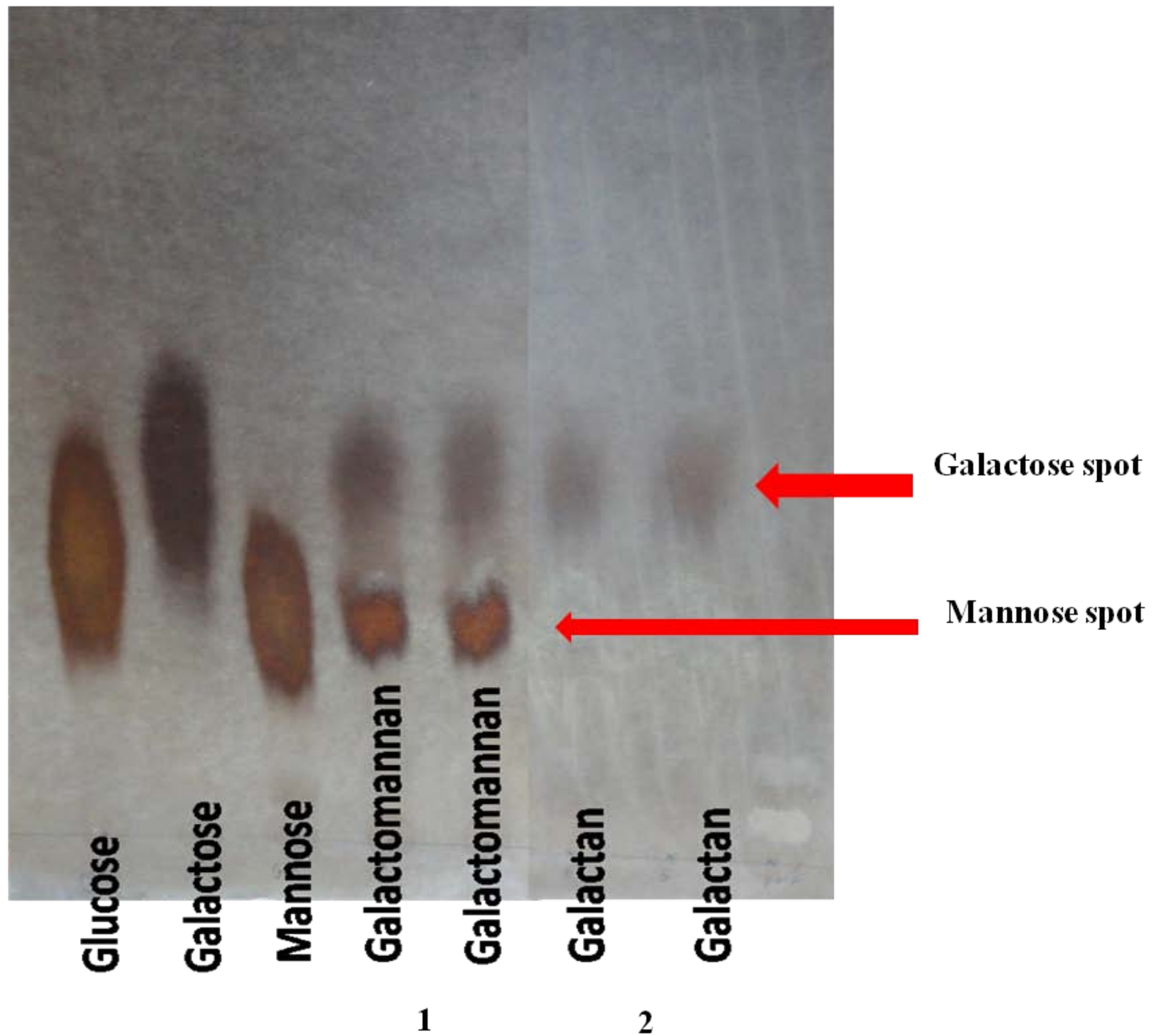


Figure 34 : Paper chromatogram showing reference compounds (glucose, mannose and galactose) and the products of acid hydrolysis, galactomannan (peak 1) and galactan (peak 2) isolated from *S. potatorum* seeds. The solvent used is n-butanol / pyridine / water 6: 4: 3 v / v / v. Stained with alkaline silver nitrate

Both peak (1) and peak (2) fractions were lyophilized and the polysaccharides were separately immobilized on Seralose - divinyl sulfone activated gel (Affinity gel) in order to explore their potential to bind sugar specific lectins. This study shows that the gels can bind galactose specific lectin that was isolated and partially purified in our laboratory from the seeds of *Dolichos lablab* (DLL-II) and can be eluted specifically with galactose as it is shown in Figure 35 A DLL-II lectin already characterized is known to contain two subunits of molecular weights 31 and 29 kDa respectively and this has also been observed in this study.

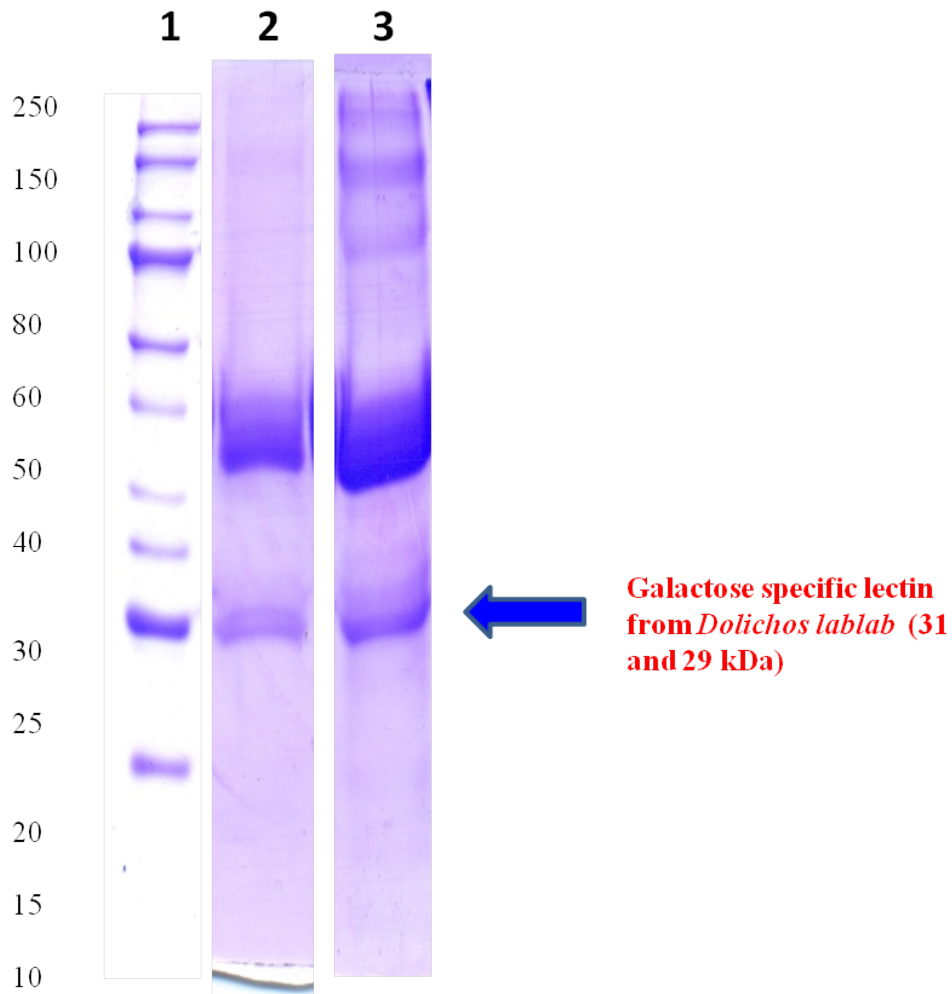


Figure 35

DLL-II containing sample applied on galactan / galactomannan polysaccharide gel separately: the columns were equilibrated with TBS containing 1.5 M ammonium sulfate (column buffer). The gel was thoroughly washed with the column buffer until the A280 was 0.05 and the bound protein was eluted using 0.3 M galactose eluate analyzed by 10% SDS-PAGE and stained by comassie staining, analysis of the eluted lectin after Affinity chromatography, lane 1 molecular weight markers. Result in lane 2 shown for galactan gel elution, and lane 3 shown for galactomannan gel elution, two subunits of 29 and 31 kDa of DLL-II are shown. Additional bands that were stained with coomassie gel could be seen from both elutions, which might represent other galactomannan/galactan binding proteins from the seed of *Dolichos lablab* which needs to be further evaluated.

4.4. Discussion

The present study was undertaken for the isolation and characterization of the polysaccharide components of *S. potatorum* seed matrices. The long term objective of the study was to understand the nature and structural properties of the polysaccharide component of the seeds and study their applications. Previous studies on the *S. potatorum* by [Tripathi P N et al 1976] mentioned that the seed extracts are anionic polyelectrolytes that destabilize particles in water by means of interparticle bridging [Sen AK et al 1962]. Tripathi P N et al [1976] have established that the seed extracts also contain lipids, carbohydrates and alkaloids containing the –COOH and free –OH surface groups which enhance the extracts coagulation capability. [Adinolfi et al 1994] reported that *S. potatorum* seeds contained a mixture of polysaccharides designated as galactomannan and galactan. The present study shows that the seed material contains two distinct polysaccharides that can be separated by gel filtration in support of the earlier study. That the isolated polysaccharides are indeed galactomannan and galactan was confirmed by acid hydrolysis of the separated polysaccharides and separation of the hydrolysate by paper chromatography into the constituent monosaccharides which identified the separated polysaccharides to be galactomannan and galactan respectively. Both these when separately immobilized to Seralose - divinyl sulfone gel, proved to efficiently bind galactose specific lectin from the seeds of *Dolichos lablab* that has already been well characterized in the lab. This protein was purified earlier in our lab on Seralose-galactose gel [Lavanya latha et al., 2006]. The protein binds on this gel only in presence of 1.5 M ammonium sulfate and can be eluted with 0.3 M galactose. Therefore a similar condition for binding and elution from the Galactomannan and Galactan gels was tested. The preliminary result suggests that these polysaccharides can be used as alternate ligands for the purification of galactose lectins. This can be assessed further using a battery of galactose lectins and

analyzing their ability to bind on these polysaccharide gels, which could suggest the high potential of these polysaccharides as tools for isolation of galactose lectins.

Chapter 5

Preparation and characterization of polysaccharides (Galactan and Galactomannan) nanoparticles

5.1. Introduction

The nanomaterials research is the most advanced at present, both in scientific knowledge and in commercial applications. It is expected that nanotechnology will be developed at several levels: materials, devices and systems. Nanotechnology is enabling technology that deals with nano-meter sized objects [Feynman, 1991]. A decade ago, nanoparticles were studied because of their size-dependent physical and chemical properties [Murray et al, 2000]. Now they have entered a commercial exploration period [Mazzola, 2003., Paullet al, 2003]. This simple size comparison gives an idea of using nanoparticles as very small probes that would allow us to understand at the cellular machinery without altering physiological conditions [Taton, 2002]. Over the past few decades, there has been considerable interest in developing biodegradable nanoparticles as effective drug delivery systems. Understanding of biological processes on the nanoscale level is a strong driving force behind development of nanotechnology [Whitesides, 2003]. Out of plethora of size-dependant physical properties available to someone who is interested in the practical side of nanomaterials, optical [Parak et al, 2003] and magnetic [Pankhurst et al, 2003] effects are the most used for biological applications. There are many applications of nanotechnology to biology and medicine such as in food and bio detection of pathogens [Edelstein et al, 2000], Fluorescent biological labels [Bruchez et al, 1998., Chan and Nie, 1998., Wang et al, 2002], Separation and purification of biological molecules and cells [Molday and MacKenzie, 1982], Drug and gene delivery [Mah et al,2000., Panatarotto et al, 2003], Phagokinetic studies [Parak et al, 2002], Detection of proteins [Nam et al, 2003], MRI contrast enhancement [Weissleder et al, 1990], Tumor destruction via heating (hyperthermia)[Yoshida and Kobayashi, 1999], Probing of DNA structure [Mahtab et al, 1995], Tissue engineering [Ma et al, 2003., de la Isla et al, 2003] because of their natural abundance and their low cost and other desirable functionalities [Cheng et al, 2002., Mahammad et al, 2006]. Nanoparticle delivery systems are essentially

nanosized carriers (10–1000 nm size) having different morphologies such as the nanocapsules, nanomicelles, nanospheres and dendrimers that can be used for specific delivery of drugs, biomolecules into cells. Nanoparticle drug delivery systems have outstanding advantages [Feynman, 1991]:

(1) They can pass through the smallest capillary vessels because of their ultra-tiny volume and avoid rapid clearance by phagocytes so that their duration in blood stream is greatly prolonged;

(2) They can penetrate cells and tissue gap to arrive at target organs such as liver, spleen, lung, spinal cord and lymph;

(3) They could show controlled release properties due to the biodegradability, pH, ion and/or temperature sensibility of materials;

(4) They can improve the utility of drugs and reduce toxic side effects; etc.

Nanoparticles (NPs) act as potential carries for several classes of drugs such as anticancer agents, antihypertensive agents, immunomodulators, hormones and macromolecules such as nucleic acids, proteins, peptides and antibodies [Akagi et al, 2006., Torchilin, 2007]. Nanoparticles can be prepared from a variety of materials such as proteins, polysaccharides and synthetic polymers. Figure 36 shows the gradual increasing of the publications on the polysaccharide nanoparticles that reflects the important of polysaccharides as potential tools for drug delivery. Nanoparticles that are biodegradable offer better advantages as they would be non-toxic to the cells. [Khan and Kumar, 2011].

Presently, nanoparticles have been widely used to deliver drugs, polypeptides, proteins, vaccines, nucleic acids, genes and so on. Over the years, nanoparticle drug delivery systems have shown huge potential in biological, medical and pharmaceutical applications**.** The current researches on nanoparticle drug delivery system focus on:

(1) The selectness and combination of carrier materials to obtain suitable drug release speed;

(2) The surface modification of nanoparticles to improve their targeting ability;

(3) The optimization of the preparation of nanoparticles to increase their drug delivery capability, their application in clinics and the possibility of industrial production;

(4) The investigation of in vivo dynamic process to disclose the interaction of nanoparticles with blood and targeting tissues and organs, etc.

Polymeric materials used for preparing nanoparticles for drug delivery must be biocompatible and should be biodegradable. To this aim, many polymeric materials have been applied, including poly (lactic acid), poly (glycolic acid), polycaprolactone, polysaccharides (particularly chitosan), poly (acrylic acid) family, proteins or polypeptides (such as gelatin), etc. Among them, polysaccharides are the most popular polymeric materials to prepare nanoparticles for drug delivery. The various methods used for the preparation of NPs include precipitation, sonochemical, dialysis, conservation, spray drying and a combination of these methods [Gaucher et al, 2005]. As the natural polysaccharides are nontoxic, biodegradable, safe and are available in abundance, so the natural polysaccharides, as well as their derivatives, have been classically used in pharmaceutical formulations as solubilizers or adhesives. Over the last few years, the evolution of these polysaccharides from the concept of ‘‘pharmaceutical excipient” to ‘‘bioactive material” has raised their potential use in the design of drug-delivery carriers. This conceptual change has, partially, been motivated by the recent emphasis in the design of biomimetic and intelligent drug delivery nanostructures, which can be recognized and assimilated in the body [de la Fuente and Penades, 2006., Alonso and Sanchez, 2004., Janes. K.-A, Calvo and Alonso, 2001., Challa et al, 2005]. The polysaccharides nanoparticles, due to their unique properties are promising carriers to deliver and protect the physiological properties of hydrophilic drugs and have been successfully applied as drug-delivery systems [Goncalves, Pereira and Gama, 2010., Hu et al, 2008., Liu, Jiao, Wang, Zhou and Zhang, 2008., Vauthier and Couvreur, 2000]. As natural biomaterials,

polysaccharides are stable, safe, non-toxic, hydrophilic and biodegradable. In addition, polysaccharides have abundant resources in nature and low cost in their processing. Recently, polysaccharides nanoparticles based on maltodextrin and gum arabic have been reported as a delivery system for catechins [Ferreira, Rocha and Coelho, 2007., Gomes et al, 2010]. In fact, polysaccharides can be used as ligands in order to facilitate the interaction of a nanostructure with a specific biological surface [Tomizawa et al, 1993., Cui et al, 2003]. As a consequence of these new potential applications, the number of publications dealing with the use of polysaccharides for drug delivery has remarkably increased over the last 10 years Figure 38.

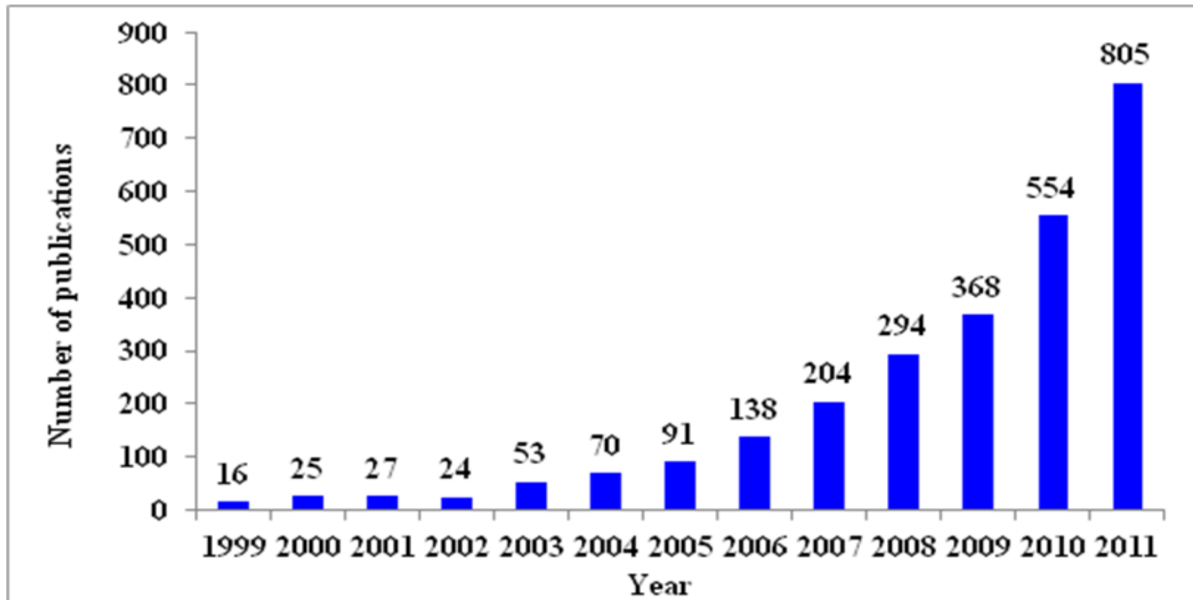


Figure 36 : Number of scientific publications published on the topic of polysaccharides nanoparticles, as function of the publication year, total of 2669 published paper, starting with only 16 papers in 1999, these data was taken from scopus scientific website. Keywords entered: polysaccharides nanoparticles.

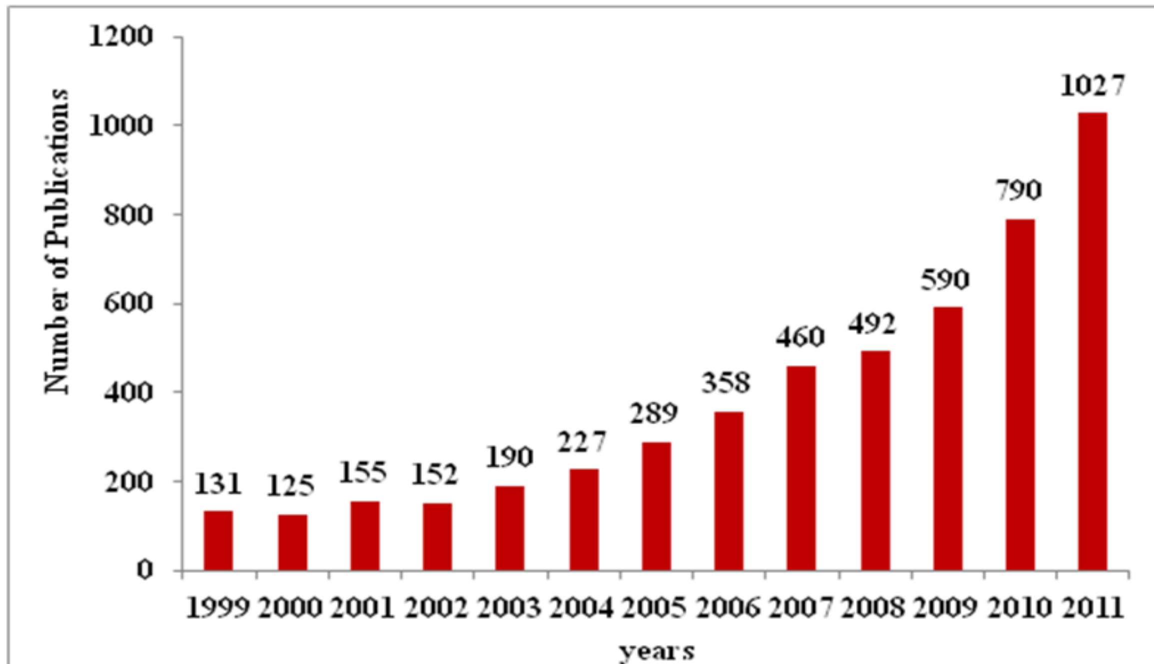


Figure 37 : Number of scientific publications published on the topic of polysaccharides and drug delivery field as function of the publication year. , total of 4986 published paper, starting with only 131 papers in 1999, these data was taken from scopus scientific website. Keywords entered: polysaccharides and drug delivery.

A very promising polysaccharide, which has been lately incorporated into the drug delivery field, is galactomannan (GM). Galactomannans and Galactan are very important polysaccharides for their industrial applications and for polysaccharides interaction studies. [Dea and Morrison, 1975., Turquoiset al 1993]. Galactomannans are found as reserve carbohydrates in the endosperm for the germinating seeds of numerous plants, particularly the Leguminosae. Galactomannans are neutral water-soluble polysaccharides extracted from the seeds of *S. potatorum* to be used in this study to prepare nanoparticles of both galactomannan and galactan. Galactomannan and its derivatives are extensively used in many applications including food, drug-delivery and health care products because of their natural abundance and their low cost and other desirable functionalities [Cheng et al, 2002., Mahammad et al, 2006].

In order to explore the polysaccharides isolated in the present study as potential tools for nanoparticle preparation and possible usage of these in the long run as alternate nanocarriers for drug delivery, the present study was undertaken and in this chapter we have prepared and characterized galactan and galactomannan nanoparticles.

5.2. Materials and methods

5.2.1. Materials

All the chemicals and reagents used in the present study were of analytical grade. Bio-Gel- P-60 (Polyacrylamide Gel) was procured from Bio-Rad Laboratories, USA. All the glassware used were washed with 10% (v/v) HNO3 and subsequently rinsed several times with de-ionized distilled water. diethyl ether, D-Galactose are from SRL, India , Seralose , DVS from Sigma Aldrich, (USA) Equipments such as AFM-(SPA-400, USA), Probe sonicator, SEM-(PHILIPS FEI-XL ESEM, USA),Spin Coater, TEM - JOEL JEM 1011, USA, were available in the central facilities and center for nanotechnology of the University of Hyderabad.

5.2.2. Seed material

Seeds were processed as described in the chapter 4. Seed powder 5 g was used for every batch of polysaccharides extraction.

5.2.3. Extraction and purification of polysaccharides from *S. potatorum* seed powder.

Extraction isolation and separation of the glactomannan and galactan polysaccharides from the seeds have been described in chapter 4.

5. 2. 4. Preparation of galactomannan and galactan nanoparticles

For the preparation of nanoparticles, the procedure described by Krishna et al., with slight modifications was adopted. 20 mg each of lyophilized galactomannan and galactan were separately dissolved in 500µl of 1XPBS buffer of pH 7.4 and kept on ice for 5 minutes. 15 ml olive oil was added drop wise with continuous stirring on a cyclomixer. It was sonicated 15 times, each time for 30 sec with 1 min gap. Immediately it was frozen in liquid nitrogen for 10 min. It was then kept at $4^{o}C$ for 4 hr and the nanoparticle pellet was collected by centrifugation at 8000 rpm for 10 min. The residual oil was removed by washing the pellet with chilled diethyl ether 3-4 times. The clear pellet was dissolved in PBS pH 7.4 and the nanoparticle sample was stored at $4^{o}C$.

5. 2. 5. Nanoparticles characterization

Structure and morphology of the nanoparticles were investigated using Scanning electron microscope (Philips FEI-XL 30 ESEM, USA - operated at 20 kV), Transmission electron microscope (JOEL JEM 1011, USA - operated at 100 kV), and Atomic force microscope (SPA 400, USA); manufacturers' instructions were followed for sample preparation, data collection, and analysis of particles as describe below.

5. 2. 6. Atomic force microscopy (AFM) [Erickson B et al 2008]

Nanoparticle sample was uniformly dispersed on a clean glass cover slip using a spin cater and dried in a dust free zoon. The unit containing the sample was kept in SPA-400 and size and morphology of particles was studied following manufacturer's instructions.

5. 2. 7. Scanning electron microscopy (SEM) [Lochmann D et al 2005]

Operated at 10 kV. Nanoparticle sample was uniformly dispersed on a clean glass cover slip using a spin coater and dried. Metal stubs were coated with double-sided adhesive tape, the cover slip with nanoparticle sample was kept on the sticky surface and sample was coated with gold in Sputter Coater. Specimens were stored in dry, dust free environment during the analysis.

5. 2. 8. Transmission electron microscopy (TEM) [Baalousha M et al 2005]

Galactomannan's and Galactan's unstained samples of the nanoparticles were prepared for electron microscopy measurements/observations, by air-drying on dust free zone, small drops of a sample solution onto carbon-coated copper electron microscopy grids, to obtain stained images of polysaccharides nanoparticles, the electron microscopy grids containing air-dried samples were incubated with a 2% (w/v) aqueous uranyl acetate solution for 10 min at room temperature and washed 3–4 times with distilled water. Polysaccharides nanoparticle images were examined using the JOEL JEM 1011 100 kV electron microscope. Electron diffraction patterns were recorded from a selected area that is well occupied with polysaccharides

nanoparticles in order to obtain high diffraction intensities. Particle size distributions were made by measuring diameters for polysaccharides nanoparticles.

5.3. Results

The galactomannan and galactan polysaccharides after separation were used for the nanoparticle preparation, using the procedure described by Krishna et al., with slight modifications was adopted. The atomic force microscopy characterization of the galactomannan nanoparticles is shown in the [Figure 38] Which reveals that the nanoparticles of galactomannan are spherical in shape. Analysis of galactomannan nanoparticle and characterization using scanning electron microscope (SEM) revealed that the particles are spherical in shape and the size varies from 45 nm to 110 nm [Figure 39]. These results were further confirmed by the characterization of the galactomannan nanoparticles using transmission electron microscope as shown in [Figure 40].

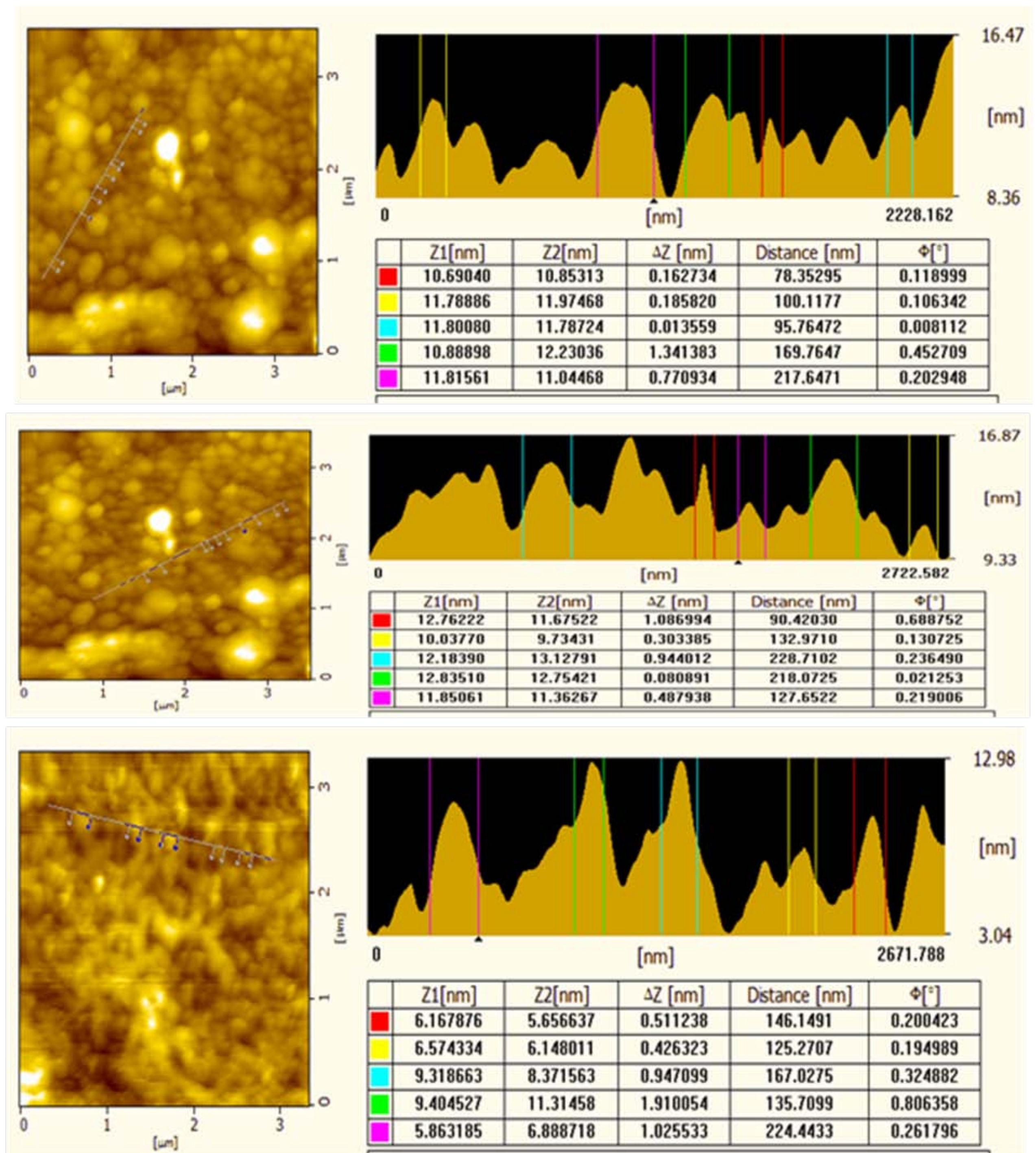


Figure 38 : Shows the atomic force microscope (AFM) image for galactomannan nanoparticles as we can observe the spherical shape of the nanoparticles.

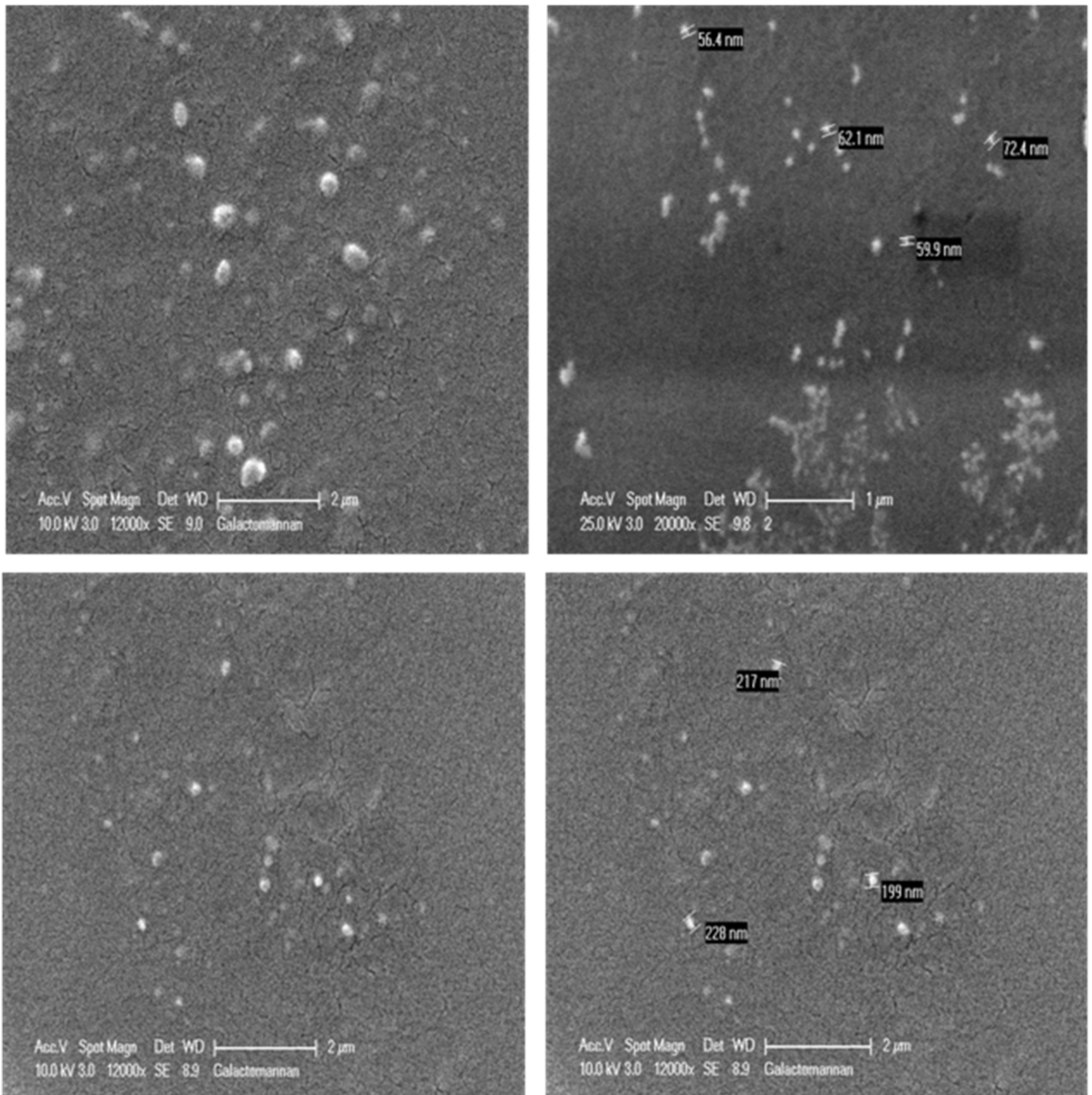


Figure 39 : Show the scanning electron microscope (SEM) images of the galactomannan nanoparticles which represent the shape and the size of the nanoparticles.

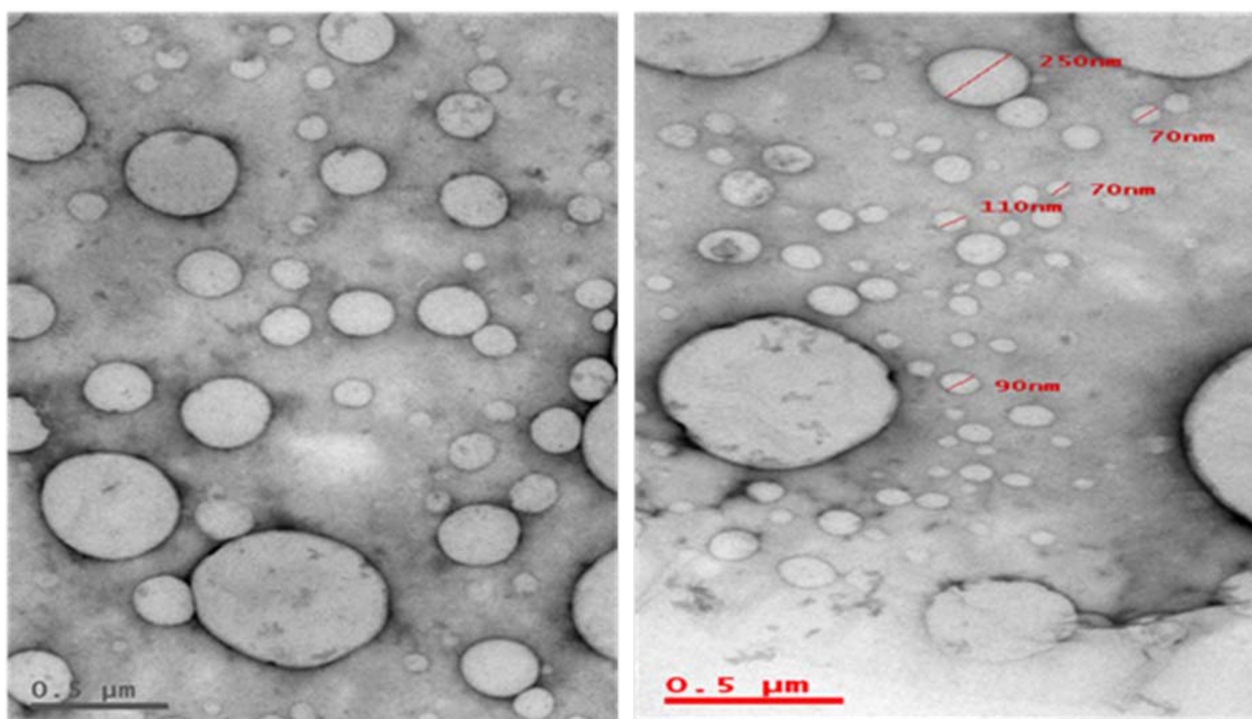


Figure 40 : Shows the transmission electron microscope (TEM) images for the galactomannan nanoparticles also represented the shape and size of the nanoparticles.

The preparation of galactan nanoparticles was done using the same method as done for galactomannan nanoparticles preparations. Atomic Force Microscopy analysis of the galactan nanoparticles are shown in [Figure 41 and Figure 42]. These nanoparticles of galactan are spherical in shape. Analysis of galactomannan nanoparticle and characterization using scanning electron microscope (SEM) revealed that the particles are spherical in shape and the size varies from 37 nm to 100 nm [Figure 43]. This was further confirmed using transmission electron microscope as shown in [Figure 44].

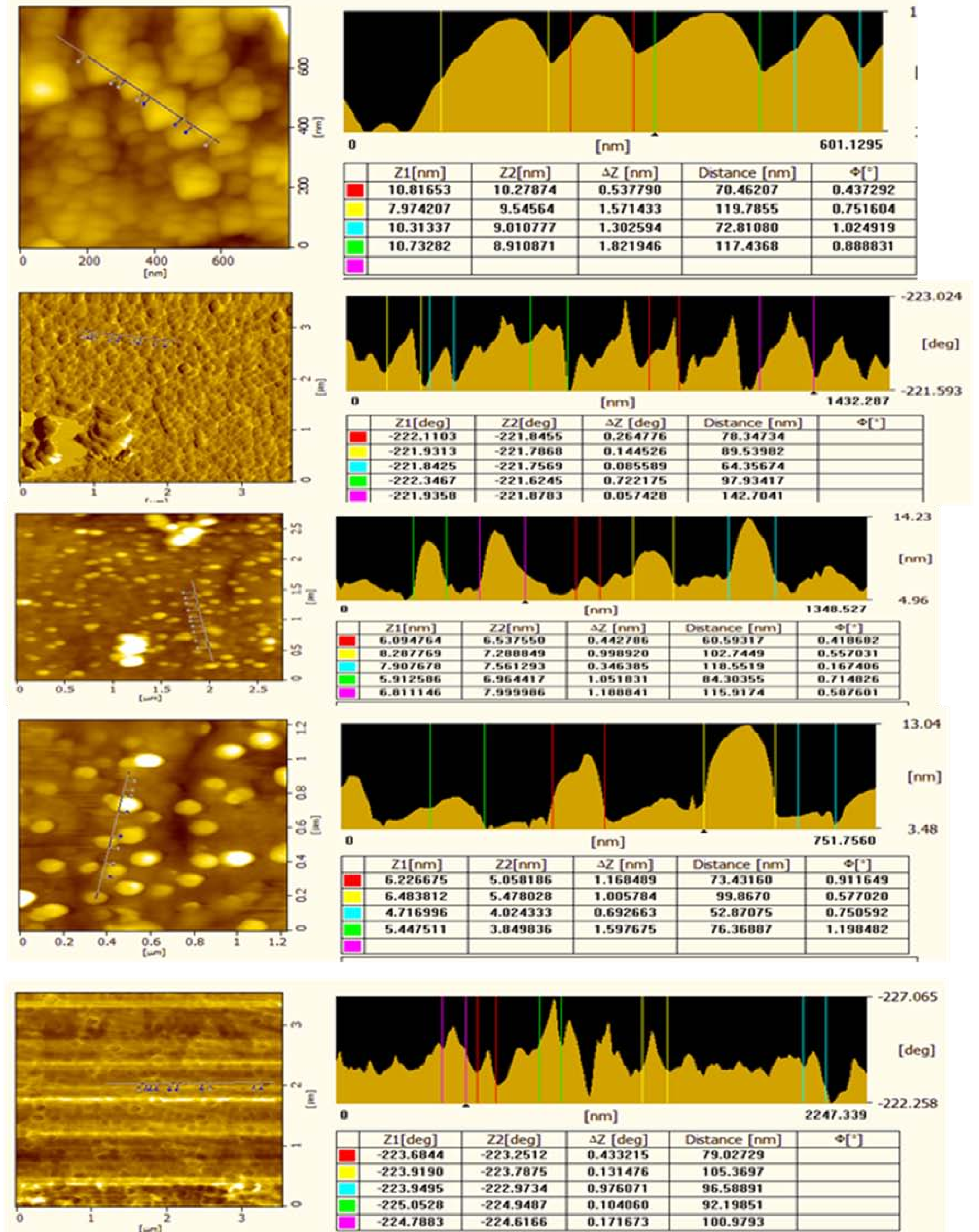
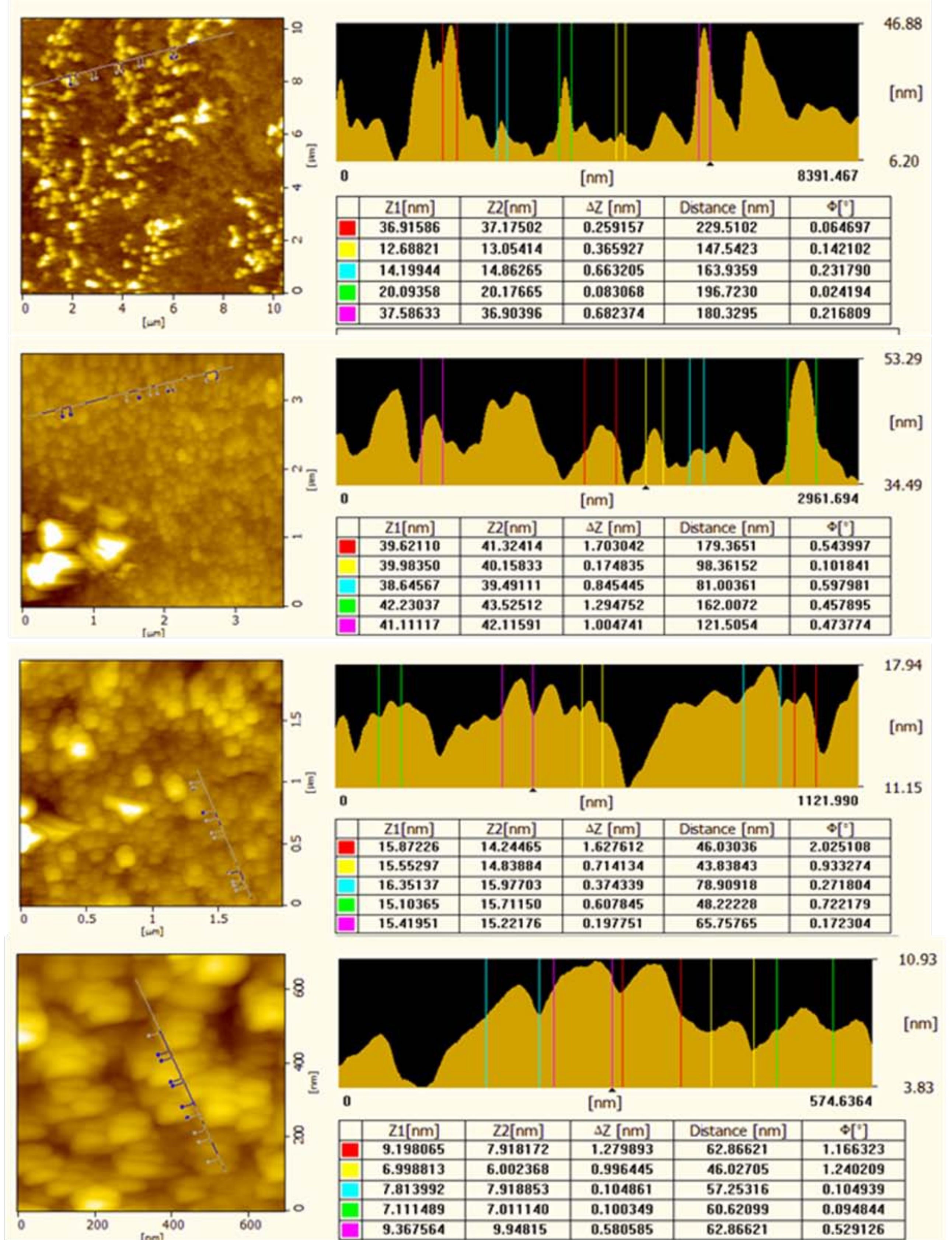


Figure 41 : Shows the atomic force microscope (AFM) image for galactan nanoparticles as we can observe the spherical shape of the nanoparticles.



	Z1[nm]	Z2[nm]	ΔZ [nm]	Distance [nm]	Φ[°]
	36.91586	37.17502	0.259157	229.5102	0.064697
	12.68821	13.05414	0.365927	147.5423	0.142102
	14.19944	14.86265	0.663205	163.9359	0.231790
	20.09358	20.17665	0.083068	196.7230	0.024194
	37.58633	36.90396	0.682374	180.3295	0.216809

	Z1[nm]	Z2[nm]	ΔZ [nm]	Distance [nm]	Φ[°]
	39.62110	41.32414	1.703042	179.3651	0.543997
	39.98350	40.15833	0.174835	98.36152	0.101841
	38.64567	39.49111	0.845445	81.00361	0.597981
	42.23037	43.52512	1.294752	162.0072	0.457895
	41.11117	42.11591	1.004741	121.5054	0.473774

	Z1[nm]	Z2[nm]	ΔZ [nm]	Distance [nm]	Φ[°]
	15.87226	14.24465	1.627612	46.03036	2.025108
	15.55297	14.83884	0.714134	43.83843	0.933274
	16.35137	15.97703	0.374339	78.90918	0.271804
	15.10365	15.71150	0.607845	48.22228	0.722179
	15.41951	15.22176	0.197751	65.75765	0.172304

	Z1[nm]	Z2[nm]	ΔZ [nm]	Distance [nm]	Φ[°]
	9.198065	7.918172	1.279893	62.86621	1.166323
	6.998813	6.002368	0.996445	46.02705	1.240209
	7.813992	7.918853	0.104861	57.25316	0.104939
	7.111489	7.011140	0.100349	60.62099	0.094844
	9.367564	9.94815	0.580585	62.86621	0.529126

Figure 42 : Shows the atomic force microscope (AFM) image for galactan nanoparticles as we can observe the spherical shape of the nanoparticles.

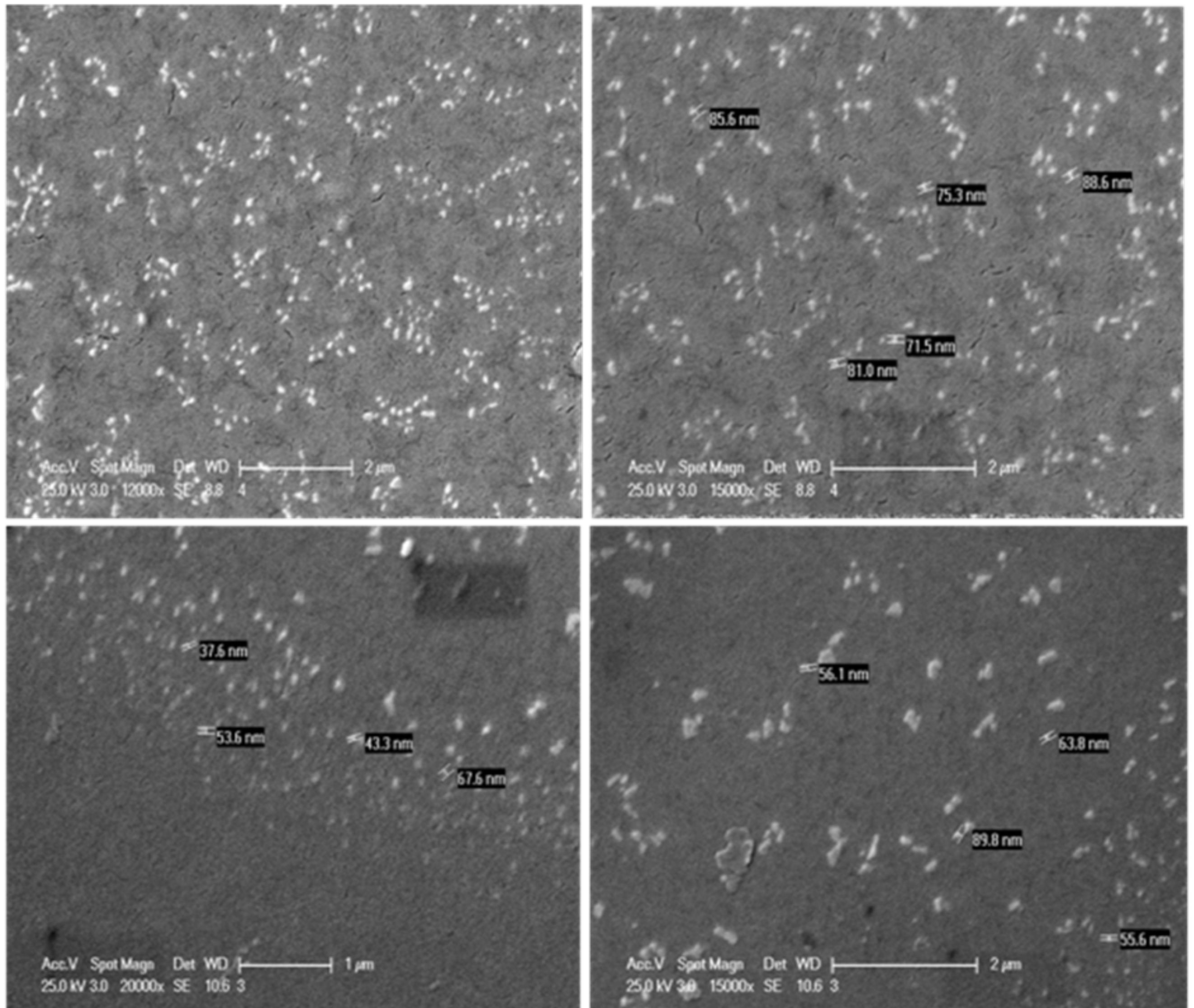


Figure 43 : Show the scanning electron microscope (SEM) images of the galactan nanoparticles which represent the shape and the size of the nanoparticles.

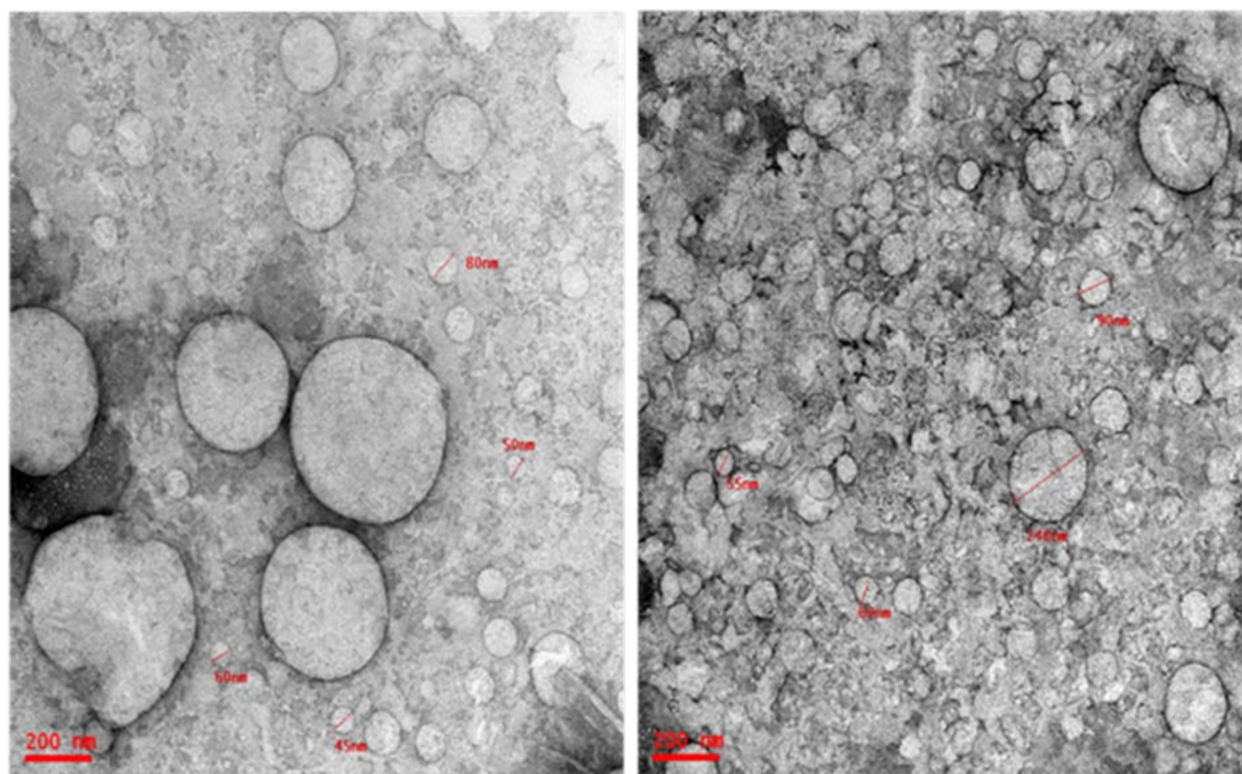


Figure 44 : Shows the transmission electron microscope (TEM) images for the galactan nanoparticles also represented the shape and size of the nanoparticles.

5.4 Discussion

The polysaccharides isolated from *S. potatorum* seeds were separately used for the nanoparticles preparation; Nanoparticles from both Galactan and Galactomannan were successfully prepared by Sol-oil chemistry method. From the AFM images it is shown that the nanoparticles are spherical in shape for both polysaccharides, and from the images of SEM and TEM we found the size of galactomannan nanoparticles are varied from 45 nm to110 nm and the size of Galactan nanoparticles also from 37 nm to 100 nm, these nanoparticle can be used for many applications such as Drug delivery. The seed material contains two distinct polysaccharides that can be separated by gel filtration. These have been identified as galactomannan and Galactan. As described in the earlier chapter both the polysaccharides when immobilized to Seralose were found to bind specifically galactsoe specific lectin. The present study further reveals that the polysaccharides can be used to prepare nanoparticles of varied sizes. Polysaccharides seem to be the most promising materials in the preparation of nanometeric carriers [Liu et al, 2008]. In literature, chitosan and its derivatives, alginate, dextran, pullulan, hyaluronic acid and chondroitin sulfate are prominent polymers investigated so far [Bodnar et al, 2005., Zahoor et al, 2005., Fuente et al, 2008]. Gum cordia is one such polymer, which has an excellent potential in designing of nanoparticulate delivery systems [Yadav and Ahuja, 2010], nanoparticles are widely used for drug delivery. The present study provided the basis to explore these polysaccaharide nanoparticles as potential tools for drug deliver and can be alternate sources of natural and cheap material for drug delivery as nano carriers. Furthermore, as these are polysaccharides in natural systems they would not be toxic as they are biodegradable. A detailed study on these aspects needs to be carried out which is beyond the scope of the present investigation.

Chapter 6

Isolation and purification of α-manosidase from *S. potatorum* seed powder

6.1. Introduction

The α-mannosidases (α-mannoside mannohydrolase) are a family of exoglycosidases which hydrolyse α-D-mannosyl residues from terminal non-reducing positions of oligosaccharides. α-mannosidase plays an important role in the processing of manno-oligosaccharides of mannoproteins *in vitro* [Sushama et al, 1995] and deficiency of this enzyme causes mannosidosis in humans [Ockerman, 1967] and cattle [Phillips et al, 1974., Burditt et al, 1978]. α-mannosidases have also been employed in the analysis of mannose containing glycans of manno-glycoproteins [Bischoff et al, 1986., Strube et al, 1988., Hase et al, 1987]. α-mannosidases (α-D-mannoside mannohydrolase, E.C. 3.2.1.24) were found in all organisms from bacteria to humans [Yamamoto and Nagasaki, 1975., Bagiyan et al, 1997., Yoshida et al, 1993., Jelinek-Kelly et al, 1985., Oeltmann et al, 1994., Curdel and Petek, 1980., Kumano et al, 1996., Hamagashira et al, 1996., Schatze et al, 1992]. Some α-mannosidases have a high specificity towards terminal mannosidic residues of glycans [Schweden et al, 1986., Moore et al, 1990] and participate in mannose trimming reactions (endoplasmatic reticulum mannosidase, Golgi mannosidases). 1,2-α-Mannosidases which cleave side chains of yeast mannan were isolated from *Bacillus* sp. [Maruyama et al, 1994], *Aspergillus orizae* [Tanimoto et al, 1989], *Aspergillus saitoi* [Ichishima et al, 1981]. Secreted 1, 2- 1, 6- α-mannosidase from *Cellulomonas* sp. [Takegawa et al, 1989] and 1, 2- 1, 3- α-mannosidase from *Arthrobacter* [Jones and Ballou, 1969] were found to hydrolyse α-mannan. α-mannosidase activity is involved in both biosynthesis and catabolism of N-linked and free oligomannosides. The extent of increased information available on α-mannosidase in the last 12 years is shown in Figure 40; data was taken from scopus scientific website http://www.hub.sciverse.com

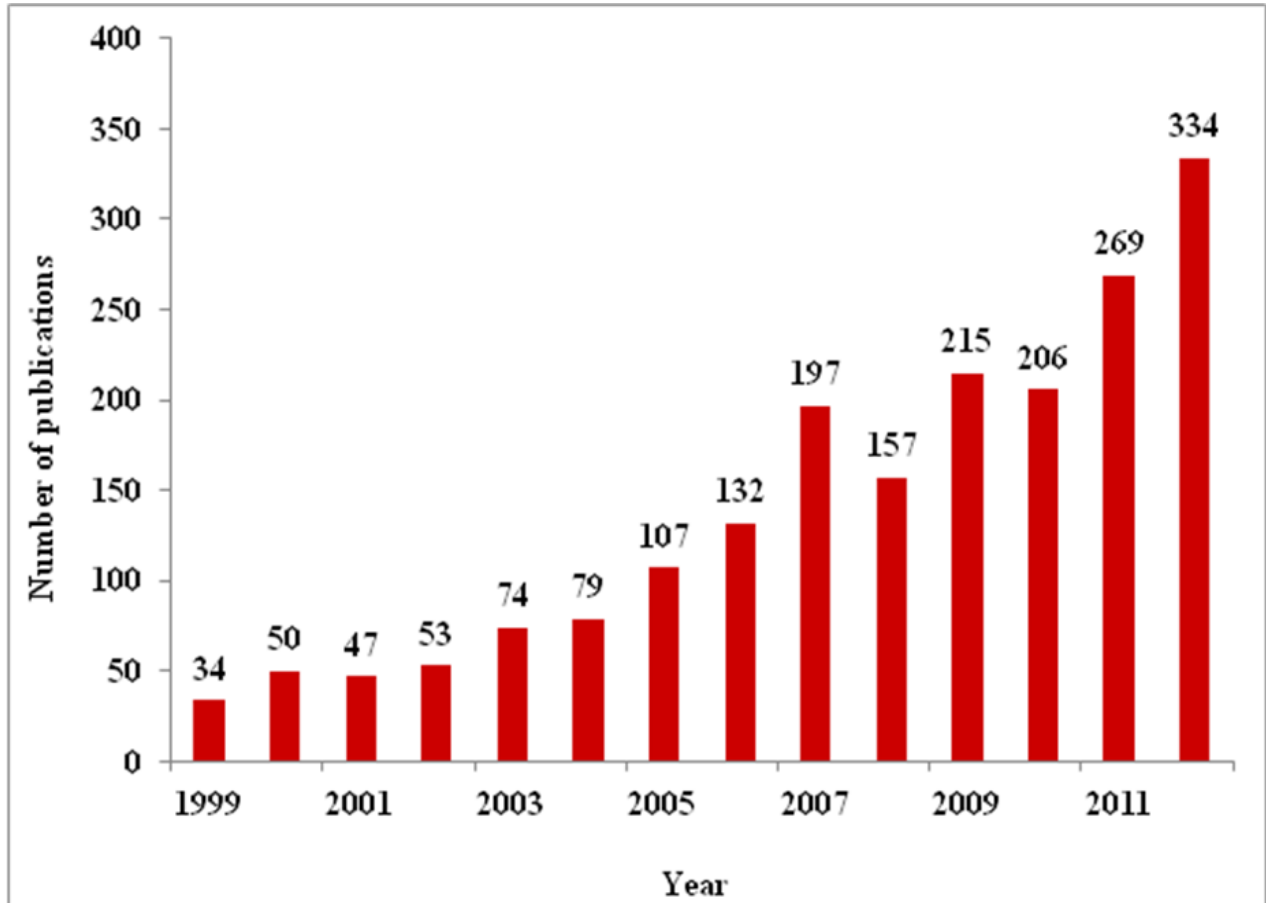


Figure 45 : Number of scientific publications published on the topic of α-mannosidase, as function of the publication year, total of 1954 published paper, starting with only 34 papers in 1999, these data was taken from scopus scientific website. Keywords entered: polysaccharides nanoparticles.

Initially cellular α-mannosidases were classified into three groups based on their properties and localization as acidic/lysosomal, intermediate/Golgi, and neutral/cytosolic [Winchester, 1984]. A more recent classification based on biochemical properties, catalytic mechanism and characteristic regions of conserved amino acid sequences, has shown that processing mammalian α-mannosidases fall into two distinct classes [Daniel et al, 1994]. Class I contains α 1,2 specific enzymes which are inhibited by DMM or kifunensine (KIF) (both pyranose analogues). The neutral/cytosolic α-mannosidase belongs to class II, retaining glycosidases found in glycosidase family 38 [Henrissat and Bairoch, 1996] and can cleave α1,2-, α1,3-, and α1,6-linked mannose residues and is inhibited by furanose analogues SWand DIM. α-mannosidase can be also classified into two types: one is involved in the biosynthesis of *N*-linked glycoproteins [Lerouge, 1998], the second one is involved in the degradation of *N*-glycans [Kimura *et al.,* 1999; Kishimoto *et al.,* 2001]. In plants α-mannosidase accumulates in vacuoles and is thought to be involved in catabolism and turnover of *N*-linked glycoproteins [Rakesh et al, 2007]. It is observed that the levels of α-mannosidase increase during seed germination and fruit ripening led to propose that the enzyme may be playing important role during these processes by removing mannose residues from mannoglycans and thereby exposing the core cell wall glycan structures to facilitate further hydrolytic attack [Kishimoto et al, 2001., Dey and Campillo, 1984., Gaikwad et al, 1995]. Several α-mannosidases have been purified and characterized from different legumes [Li, 1967; Paus, 1977; Tulasi and Nadimpalli, 1997] and non legume sources [Ohtani and Misaki, 1983; Priya Sethu and Prabha 1997; Kishimoto *et al.,* 2001; Mahadevi *et al.,* 2002]. The α-mannosidase from jack bean (*Canavalia ensiformis*) has generally been employed in determining the glycan structures [Kobata, 1979] because its properties such as pH optimum, kinetics, and substrate specificity [Li and Li, 1972., Li, 1967., Tomiya et al, 1991] have been extensively characterized. The glycan structure of the α-mannosidase purified from jack bean seedlings

has also been studied using lectins [Araki et al, 1994]. In addition to jack bean, α-mannosidases have been purified from almond [Schwartz et al, 1970], papaya [Ohtani and Misaki, 1983], kiwifruit [Ogawa et al, 1990], and watermelon [Nakagawa et al, 1988], and the properties of some of these enzymes have been characterized. Although almond α-mannosidase is commercially available and has been studied previously [Schwartz et al, 1970] several of its properties have not yet been elucidated. Plant α-mannosidases generally require metal ions for their activity, Zinc metal ion has been found to maintain and enhance the activity of preparations of α-mannosidase from many plant and animal tissues, other metal ions such as Co (II), Cd (II) were also found to enhance the enzyme activity [Nakajima *et al.,* 2003; Woo *et al.,* 2004].

Previously in our laboratory, we have purified and characterized α-mannosidase both from legume and non-legume sources (lablab beans and Triticale). This hydrolytic enzyme along with lectins is localized in protein bodies [Tulasi and Nadimpalli, 1997; Mahadevi *et al.,* 2002]. More recently the partial sequences and glycan structure analysis α-mannosidase from *Dolichos lablab* seeds has been reported [Gnanesh and Kumar, 2013].

Initial studies using the total proteins from *S. potatorum* seeds for glycosidase activities, showed α-mannosidase activity and therefore in the present work we describe the partial purification and characterization of α-mannosidase from the seeds of *Strychnos potatorum*.

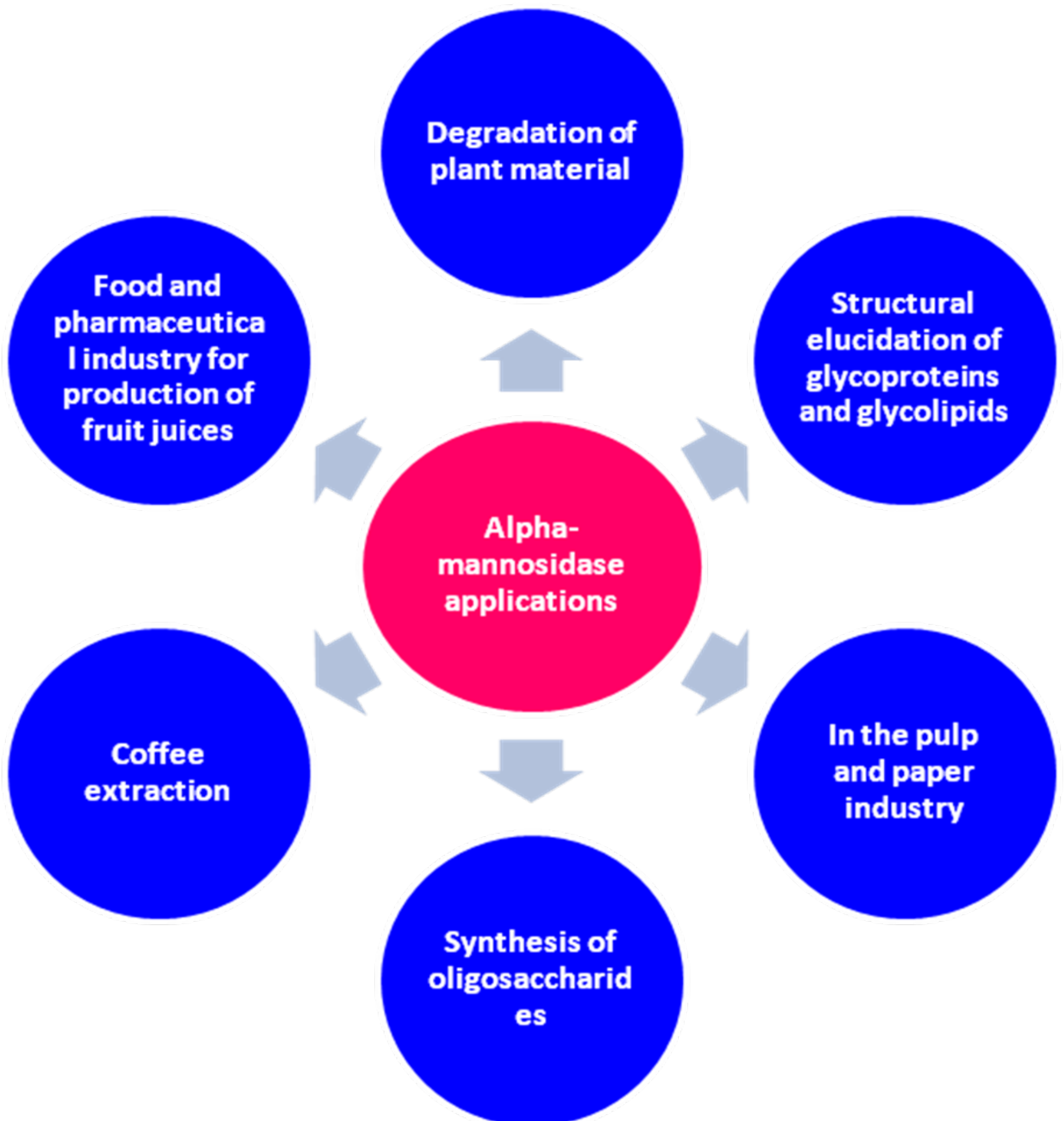


Figure 46: α-mannosidase applications

6.2 Materials and methods

6.2.1. Materials

All the chemicals and reagents used in the present study were of analytical grade. All the glassware used were washed with 10% (v/v) HNO3 and subsequently rinsed several times with de-ionized distilled water. *p*- Nitrophenyl-α-D-mannopyranoside, other *p*-nitrophenyl glycosides, α-methyl-D-mannopyranoside and phenyl-Sepharose CL-6B was obtained from Fluka (Sigma-Aldrich), DE-52 (DiEthyl cellulose) was obtained from Whattman, Ready to use standard protein molecular weight marker mixture for SDS-PAGE was obtained from Fermentas. Con-A Sepharose 4B gel supplied by Amersham (GE Healthcare), Uppsala, Sweden. All other chemicals and reagents were of analytical grade and procured from reputed firms.

6.2.2. Plant materials

S. potatorum L. seeds were obtained from the divisional forest, flying squad division, Rajahmundry, East Godavari district, Andhra Pradesh India. Seeds were dried at 40°C for 2 days in hot air oven. Seeds were made into powder in Cyclotech 1093 sample mill (Tecator AB, Höganäs, Sweden). 15 g of the seed powder was used for every batch of α-mannosidase extraction.

6.2.3. Extraction of α-mannosidase from *S. potatorum* seed powder

S. potatorum seeds were ground to a fine powder using Clotech 1093 sample mill instrument. Then the seed powder was defatted with chilled acetone and hexane. After removing the acetone and hexane by centrifugation the seed material was air dried at room temperature. From the air dried seed powder, 20 g of the seed powder was used in each batch of total protein extraction. The total protein was extracted using 25 mM Tris-HCl pH 7.4. At 4 ^{o}C, over night. The homogenate was centrifuged at 12,000 rpm for 30 min. The pellet was discarded and the supernatant which was designated as the crude extract of the enzyme

preparation was dialyzed against 25 mM Tris-HCl buffer. Protein, carbohydrate and periodic acid Schiff's staining procedures were also carried out as already described in chapter 2.

6.2.4. Enzyme assays

Under the standard conditions, α-mannosidase activity was measured by the release of *p*-nitrophenol from the chromogenic substrate *p*-nitrophenyl-α-D-mannopyranoside (5 mM stock of *p*NP-α-Man). The control contained all reactants except the enzyme. An assay mixture (500 µL) consisting of a 100 mM acetate buffer pH 5.0, 100 µL of a 5 mM *p*-nitrophenyl-α-D-mannopyranoside (1 mM final concentration) and the enzyme solution, was incubated at 37 °C for 30 min. Determination of other *p*-nitrophenylglycosidase activities was carried out under the same experimental conditions. The reaction was stopped after 30 min of incubation by addition of 1 M Na2CO3 buffer pH 10.0 to the reaction mixture. Liberated *p*-nitrophenol was measured spectrophotometerically at 405 nm. The activity of the enzyme (units/ml/min) was calculated according to the formula given below:

$$\text{Activity (units/mL/min)} = \frac{\text{Absorbance at 405nm}}{\text{Time of incubation x 18.5 x Volume of enzyme}}$$

Where 18.5 is the molar extinction coefficient of *p*-nitrophenol.

The specific activity of the enzyme was expressed as units per mg protein (IU/mg). All assays were performed in triplicate and results were recorded as the mean of these experiments.

6.2.5. Anion exchange chromatography on DE-52 cellulose gel

The crude extract of the *S. potatorum* seed sample which exhibited the α-mannosidase activity after dialysis was loaded on to the DE-52 (25 ml of the gel), that had been previously equilibrated with 25 mM Tris-HCl (pH 7.4). The unbound proteins were removed from the gel by washing with five column volumes of the same equilibrating buffer. The bound proteins were then eluted using 0.2M NaCl buffer. Fractions (3 mL each) were collected at a

flow rate of 60 mL/h and assayed for the enzyme activity. The active fractions containing α-mannosidase were pooled and concentrated by 0-80% ammonium sulphate precipitation.

6.2.6. Hydrophobic interaction chromatography using phenyl-sepharose CL-6B

The active fractions precipitated above containing α-mannosidase pellet from the previous step were dissolved in a minimal amount of 25 mM Tris-HCl (pH 7.4) containing 1 M ammonium sulphate buffer and applied on a phenyl-Sepharose CL-6B column (1.5 x 5 cm) previously equilibrated with 25 mM Tris-HCl (pH 7.4) containing 1 M ammonium sulphate buffer. The column was washed with equilibration buffer and the bound proteins were then eluted using 25 mM Tris-HCl (pH 7.4). Fractions (1 mL each) were collected at a flow rate of 1mL/min and active fractions were pooled together. The pooled fractions were concentrated and were dialysed against 25 mM Tris-HCl (pH 7.4) buffer.

6.2.7. SDS- PAGE analysis of the proteins

SDS-PAGE analysis was carried out according to Laemmli [Laemmli, 1970]. Using Fermentas unstained markers as standards. The protein bands were visualized by Coomassie Brilliant Blue R-250, and also visualized by silver staining as described by Blum et al., [Blum et al, 1978].

6.2.8. Sephadex G-100 gel chromatography

Sephadex G-100 (75 ml gel) was packed into a glass column and equilibrated with TB. Enzyme containing fractions obtained above were concentrated and applied on this gel. Elution was performed using TB. 1.5 ml fractions were collected and the absorbance of the fractions monitored at 280 nm. Fractions were also analyzed for glycosidase activity and subjected to 12.5% SDS-PAGE analysis under reducing conditions and proteins detected using Coomassie Brilliant Blue R-250 staining method. Aliquot of the peak fraction eluted was also subjected to western blot analysis. The mannosidase bands were detectable by using an antibody to the jack bean α-mannosidase enzyme.

6.2.9. Affinity purification on Con-A sepharose 4B gel

The concentrated fractions from phenyl-Sepharose were applied on to a Con A-Sepharose 4B column (5 ml) previously equilibrated with 25 mM Tris-HCl pH 7.4 containing 0.5 M NaCl (Equilibration buffer). After washing the unbound protein bound α-mannosidase was eluted with 0.4 M α-methyl-D-mannopyranoside in the same equilibration buffer at 10 mL/h. The elution of protein is monitored by checking the absorbance at 280 nm as well as by checking the enzyme activity. The eluted protein was used in further studies.

6.2.10. Effect of pH and pH stability

To study the effect of pH on the stability of the enzyme activity, the enzyme activity was determined under the standard conditions at 37°C by incubation of the enzyme at different pH ranges varying from 2 to 8 for 12 hours, using 0.1 M Citrate buffer (pH 2-3), 0.1 M NaOAc (pH 4-5), 0.1 M Sodium phosphate (pH 6-7), 0.1 M Tris-HCl (pH 8). After incubation the residual enzyme activity was subsequently assayed under standard assay conditions.

6.2.11. Effect of temperature and thermal stability

To determine the optimum temperature for the *S. potatorum,* α-mannosidase, and the thermo stability of the enzyme, the assay was performed after incubating the enzyme at different temperatures in the range of of 30 °C to 90 °C with *p*-nitrophenyl-α-D-mannopyranoside (5 mM) in 100 mM NaOAc buffer pH 5.0 Thermal stability was determined by incubating the enzyme at 50 °C, 60 °C, 70 °C, 80 °C for 60 min, an aliquot was drawn at regular interval and immediately cooled. The residual enzyme activity was determined by standard assay method.

6.3. Results

6.3.1. Extraction of α-mannosidase

Among the various glycosidases tested, α-mannosidase activity was found to be higher in the seed extracts and therefore this enzyme was purified. After dialysis of the extract against Tris buffer it was loaded on to DE-52 (anion column) the column was calibrated with the same buffer before loading the sample. After washing the unbound proteins the α-mannosidase was eluted with 0.2 M NaCl, in Tris-HCl buffer pH 7.4. The protein content was monitored at 280 nm and α-mannosidase activity was estimated for the fractions [Figure 47 A and B]. The active fractions were pooled and concentrated by 0-80% saturation with solid ammonium sulphate, after the centrifugation at 12,000 rpm the pellet was dissolved in a minimal amount of Tris buffer containing 1 M ammonium sulfate.

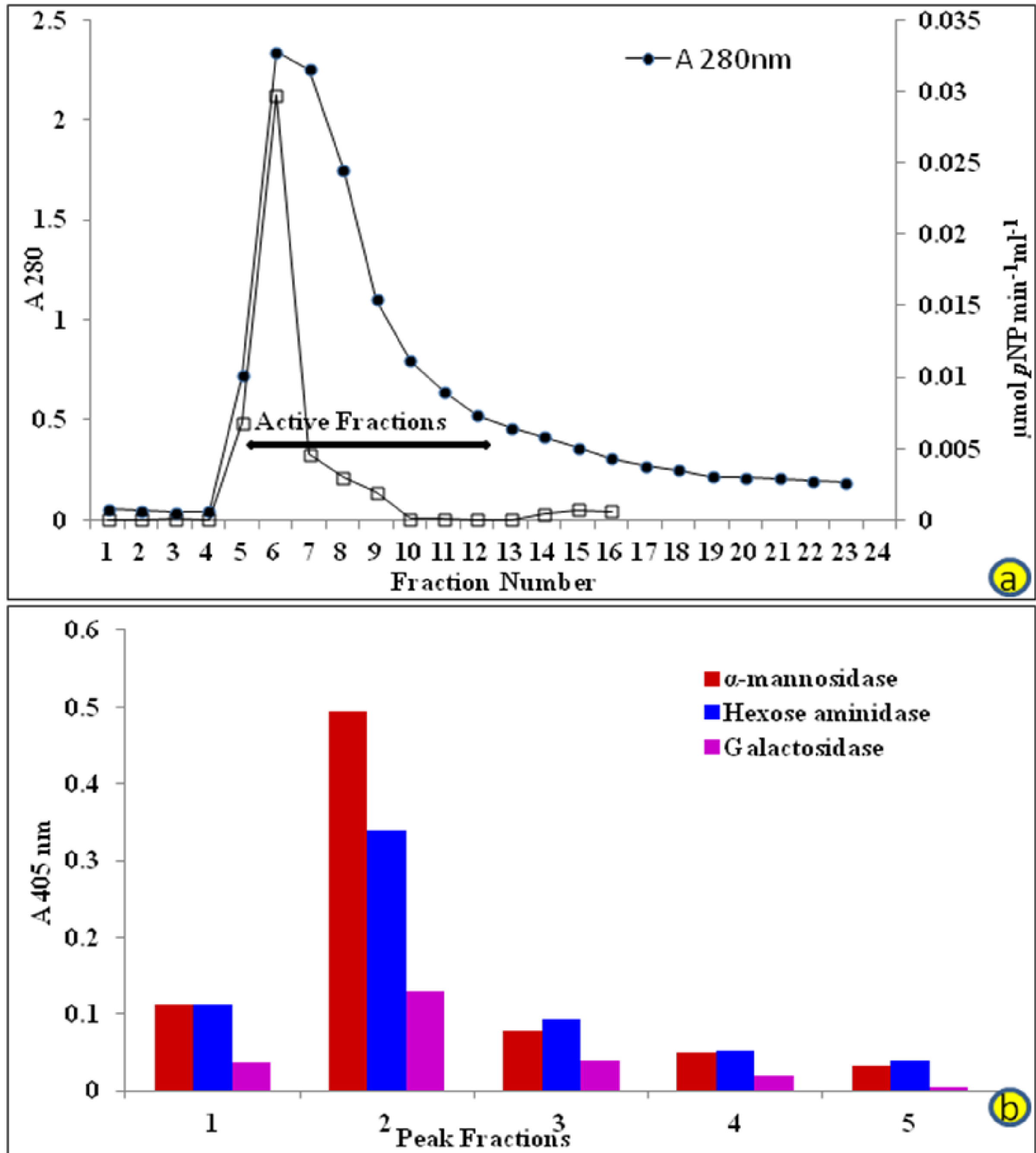


Figure 47: A- Ion exchange chromatography of the protein extracts on DE-52 cellulose gel. The fraction after dialysis was applied onto the gel (25 ml), and the unbound proteins washed with column buffer (TB). Bound proteins were eluted using 0.2M NaCl in column buffer.

B- Glycosidase activity after ion exchange chromatography for the peak fractions, among them the α-mannosidase activity is the highest.

6.3.2. Hydrophobic interaction chromatography using phenyl-sepharose CL-6B

The enzyme rich fractions from the previous step after ammonium sulphate concentration was dissolved in the minimal amount of Tris buffer containing 1 M ammonium sulphate and processed for hydriophobic chromatography as described under methods. The α-mannosidase enzyme retained on the gel at high concentration of ammonium sulphate and eluted using the Tris buffer in the absence of the ammonium sulphate [Figure 48 A]. The protein containing fractions were estimated for α- mannosidase activity [Figure 48 B]. The peak fractions with high α-mannosidase activity were pooled and concentrated using 0-80% ammonium sulphate precipitation, the pellet was collected and dissolved in a minimal amount of Tris buffer to be used in next steps. 10% SDS-PAGE analysis of the peak proteins after phenyl Sepharose chromatography as done to have an over view about the molecular weight of the protein components and the protein bands were visualized using Coomassie Brilliant Blue R-250 staining [Figure 49].

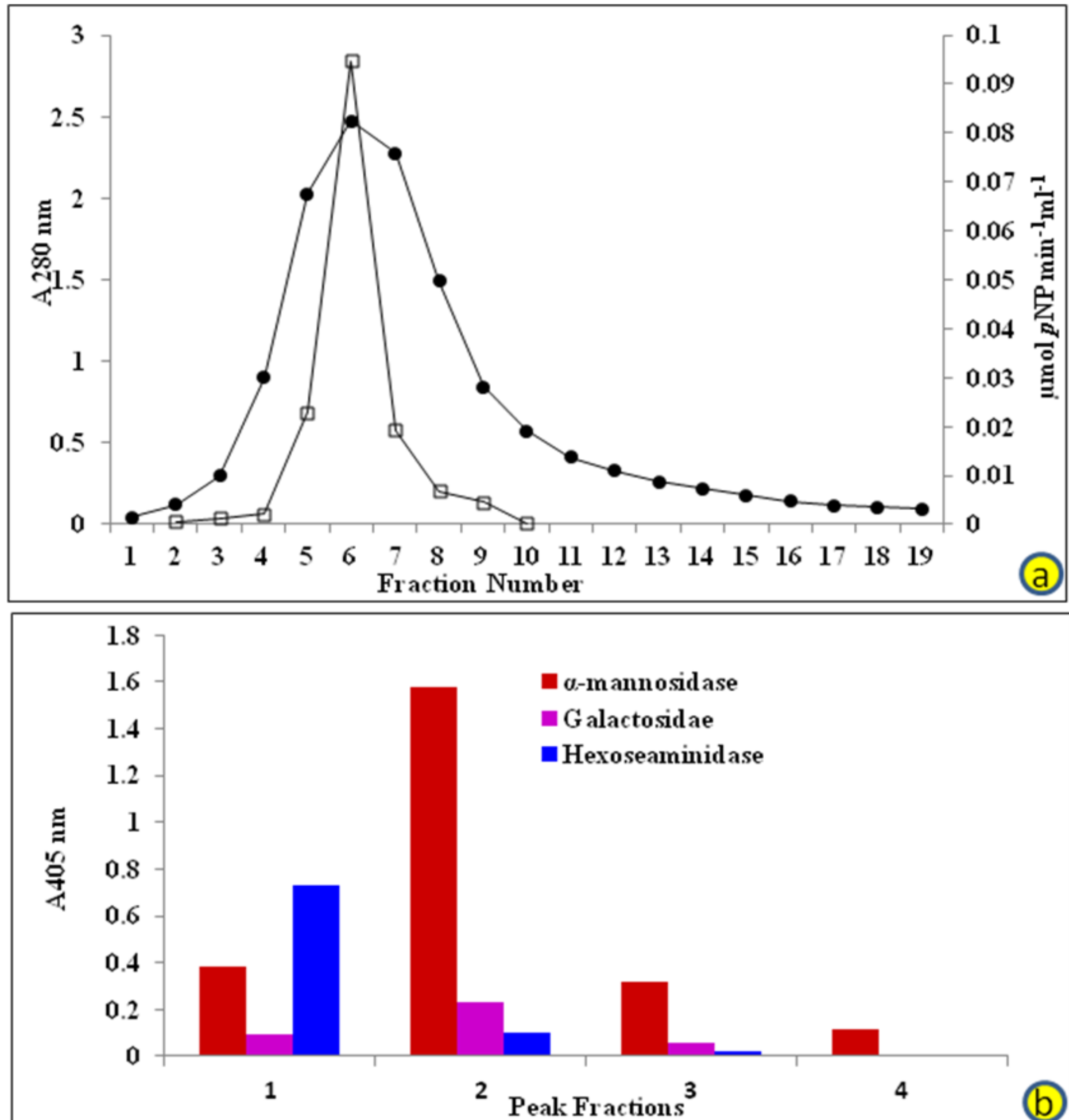


Figure 48 : A- Phenyl sepharose chromatography. The glycosidase active fractions obtained from the DE 52 cellulose gel were pooled, and solid ammonium sulfate was added to a final concentration of 1 M, and applied onto a phenyl sepharose gel (10 ml) equilibrated with 25 mM Tris-HCl buffer pH 7.5 containing 1 M ammonium sulfate. After washing the gel to remove unbound proteins with the column buffer, the bound proteins were eluted using 25 mM Tris HCl buffer pH 7.5 which is shown in the Figure.

B - Glycosidase activities for the fractions obtained after phenyl sepharose chromatography. Assays were performed as described under methods.

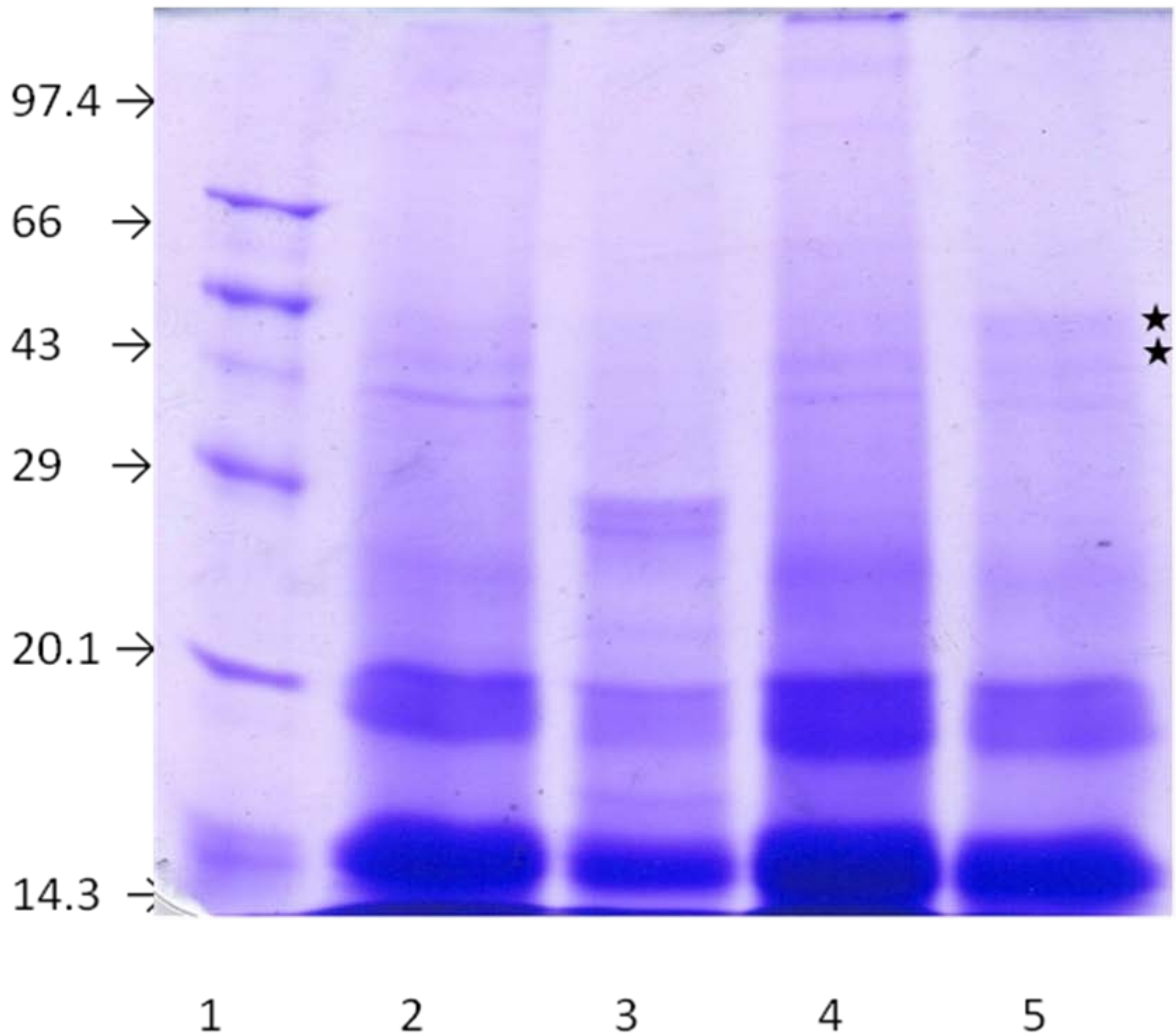


Figure 49 : 10% SDS-PAGE analysis of the proteins after phenyl sepharose chromatography. lane 1 molecular weight markers, lane 2, fraction 5, lane 3, fraction 6 (50 microlitres), lane 4, fraction 6 (100 microlitres), lane 5, fraction 10 (100 microlitres). * indicates possible enzyme bands

6.3.3. Sephadex G-100 gel chromatography and western blot analysis

For further purification, the enzyme rich fractions from the previous step after 0-80% ammonium sulphate precipitation was dissolved in the minimal amount of TB (25 mM Tris buffer) buffer this buffer also was used as column buffer, passed through sephadex G-100 (gel filtration) column, (75 ml gel) was packed into a glass column and equilibrated with TB. Elution was performed using TB. 1.5 ml fractions were collected and the absorbance of the fractions monitored at 280 nm [Figure 50 A]. The peak fractions were pooled and concentrated and also analyzed for glycosidase activity [Figure 50 B] and subjected to 12.5% SDS-PAGE analysis under reducing conditions and proteins detected using Coomassie Brilliant Blue R-250 [Figure 51 A]. Aliquot of the peak fraction eluted was also subjected to western blot analysis using an antibody to jackbean α-mannosidase. Some protein bands cross-reacting with the jack ben enzyme antibody were detectable [Figure 51 B].

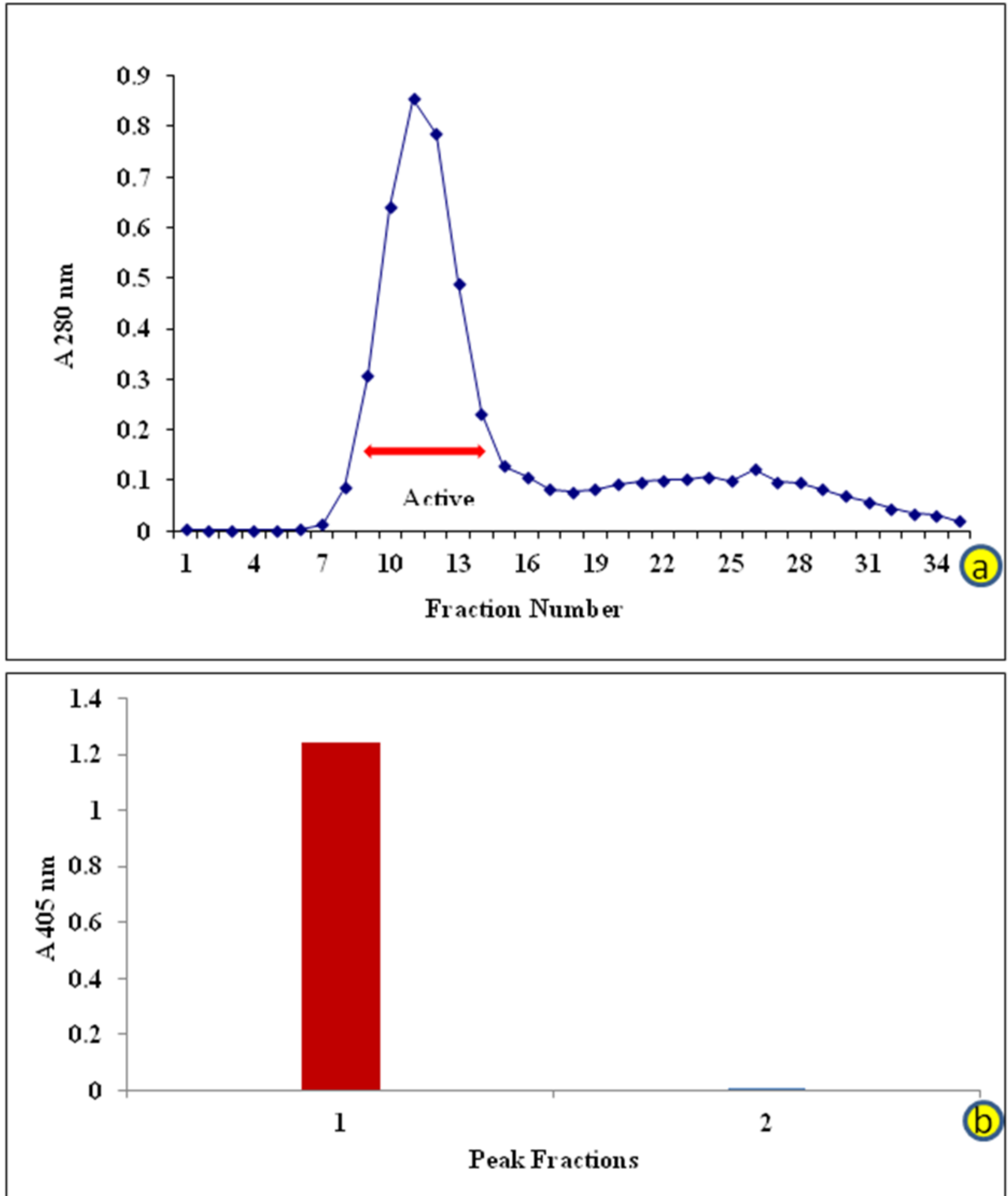


Figure 50 : A - Sephadex G-100, Gel filtration analysis of the glycosidase fractions obtained from sephadex G-100 fractions.

B - α-mannosidase activity after sephadex G-100 for the peak fractions.

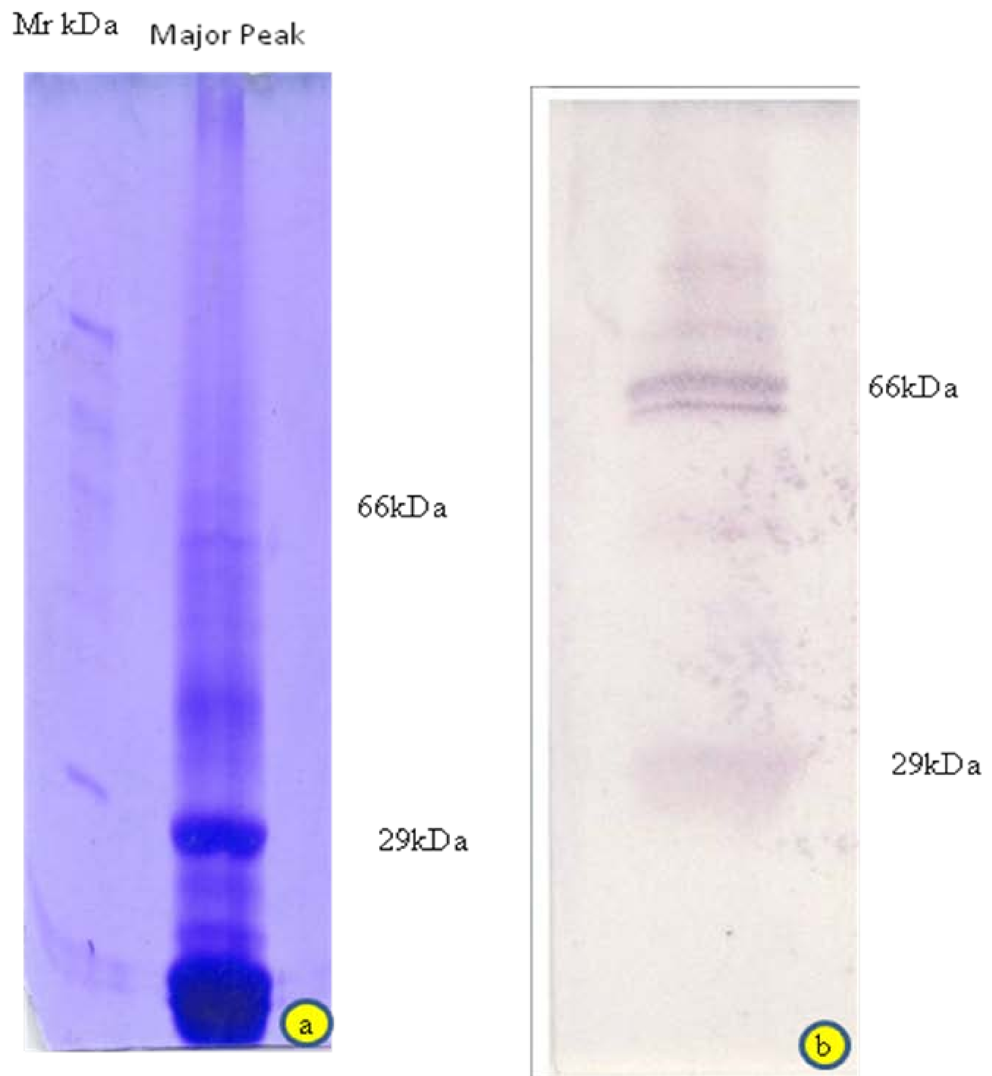


Figure 51 : A- 10% SDS-PAGE for the major peak of the sephadex G-100 elution protein.

B- Western blot analysis for the major peak of the protein eluted from the sephadex G-100 column, the α-mannosidase could be detected on the membrane using the jack bean antibody. It shows two closely migrating bands could be seen (~66 kDa).

6.3.4. Affinity purification on Con-A sepharose 4B column

Partially purified *S. potatorum* α-mannosidase was further analyzed for its binding to Con A sepharose gel as described under methods. When α-mannosidase rich fractions of the sephadex G-100 gel filtration was loaded on to the Con A sepharose *S. potatorum* α-mannosidase was strongly bound, which is later eluted specifically by using 0.4 M methyl-α-mannopyranoside in 25 mM Tris-HCl pH 7.4 containing 0.5 M NaCl [Figure: 52 A]. It was found to be a glycoprotein confirmed from its ability to bind to Con A-sepharose 4B gel. Con-A eluted enzyme rich fractions were pooled, concentrated and analyzed on 12% SDS-PAGE, [Figure 52 B]. By silver staining it shows two closely migrating bands (~66 kDa). These bands were also detectable in the previous step of purification.

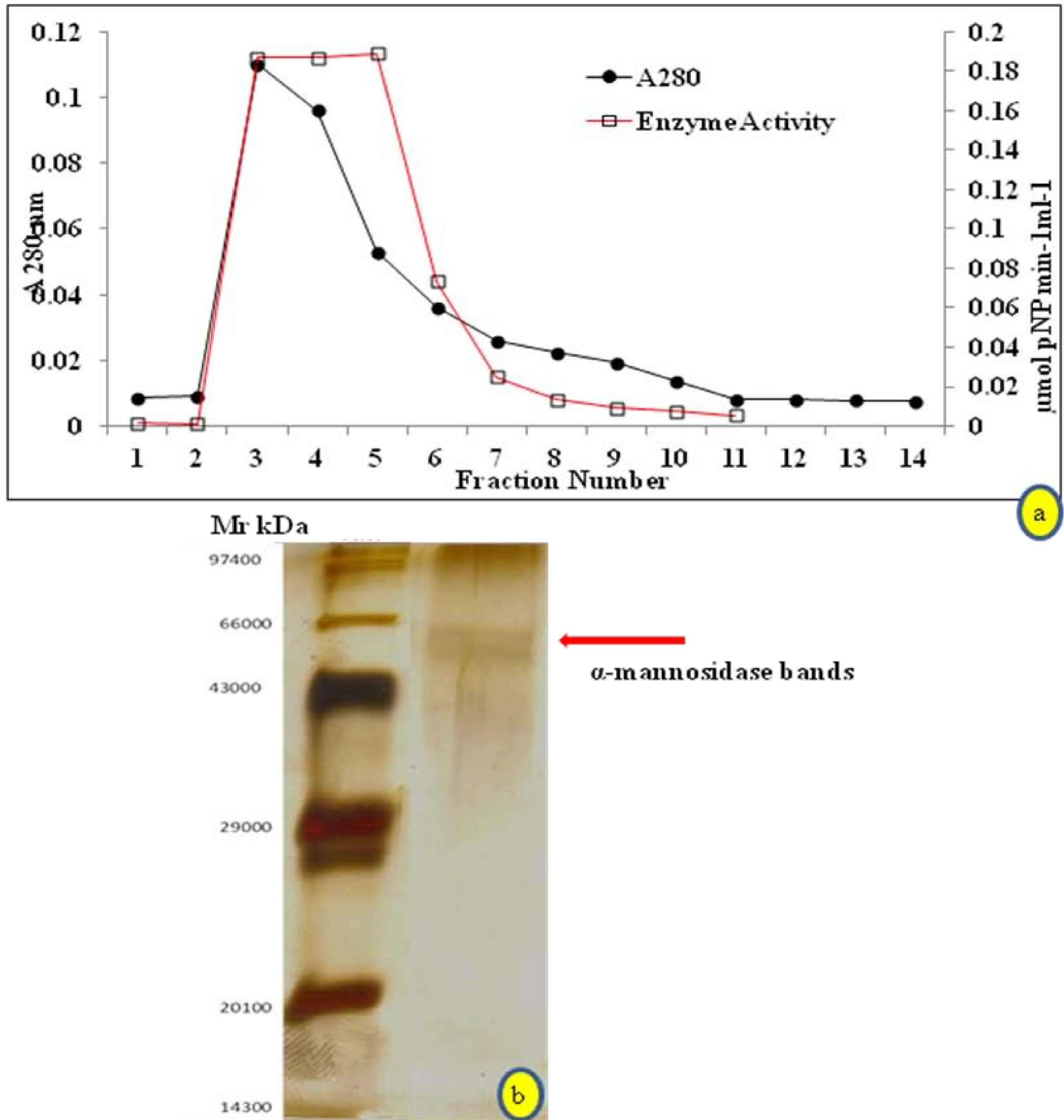


Figure 52 : A- Protein fractions eluted from Con A-sepharose 4B column (5 ml) previously equilibrated with 25 mM Tris-HCl pH 7.4 containing 0.5 M NaCl (Equilibration buffer). After washing the unbound protein, bound α-mannosidase was eluted with 0.4 M α-methyl-D-mannopyranoside in the same equilibration buffer at 10 mL/h, 0 .5 ml fractions were collected. The α-mannosidase activity for the fractions eluted from the Con-A affinity gel column was estimated.

B - 12.5% SDS-PAGE for the eluted fractions was done and silver stained. Two closely migrating bands were visualized in the region of 66 kDa.

6.3.5. Effect of pH, temperature and thermostability on the α-mannosidase activity

The effect of pH on the α-mannosidase activity of *S. potatorum* was determined at 37°C in buffers ranging from pH 2.0 to 8.0 [Figure 53 A]. The value obtained at pH 5.0 where α-mannosidase activity is the maximum that range was taken as 100%. The Effect of temperature on α-mannosidase activity was determined in 100 mM NaOAc buffer (pH 5.0) at 30–90°C [Figure 53 B]. The value obtained at 50°C was taken as 100%. The thermostability of α-mannosidase was determined by measuring α-mannosidase activity under standard assay conditions after pre-incubation of the enzyme in 100 mM NaOAc buffer (pH 5.0) at 50, 60, 70 and 80°C for various periods [Figure 53 C]. The activity of an unheated enzyme sample was taken as 100%. Data is the mean of triplicate experiments.

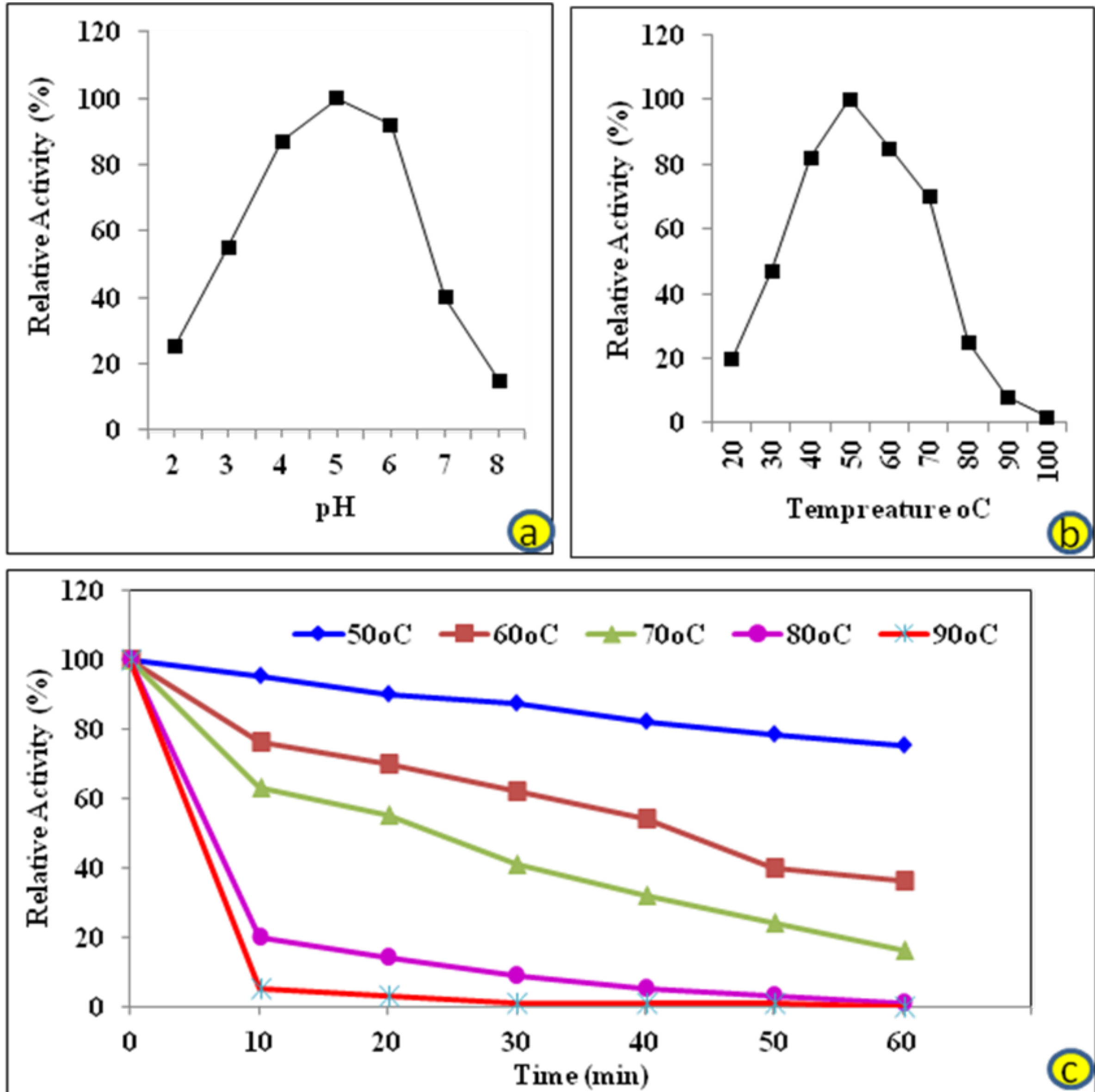


Figure 53 : A- Effect of pH on the α-mannosidase activity of *S. potatorum* was determined at 37 °C in buffers ranging from pH 2.0 to 8.0. The maximum activity was obtained at pH 5.0.

B- Effect of temperature on α-mannosidase activity was determined in 100 mM NaOAc buffer (pH 5.0) at 20–100 °C. The maximum activity was obtaind at 50 °C.

C- Thermostability of α-mannosidase was determined by measuring α-mannosidase activity under standard assay conditions after pre-incubation of the enzyme in 100 mM NaOAc buffer (pH 5.0) at 50, 60, 70, 80 and 90 °C for various periods. The activity of an unheated enzyme sample was taken as 100%. Data is the mean of triplicate experiments.

6.4 Discussion

In the frame work of our interest for new glycosyl hydrolases from the seeds of non-legumes we have successfully purified α-mannosidase from *S. potatorum* seed powder using conventional chromatographic techniques. As per our knowledge there have been no reports on purification of α-mannosidase from the seeds of *S. potatorum.* In the first steps of purification the extraction of α-mannosidase from the seeds of *S. potatorum* the crude extract was very viscous due to the abundant polysaccharides especially galactomannan and galactan in the seeds, so the proteins in the crude extract were ammonium sulfate precipitated as described in earlier chapter. When aliquots of the precipitated and dialyzed protein were analyzed for the glycosidases activity using different substrates such as *p*NP-α-D-glucopyranoside, *p*NP-β-D-glucopyranoside, *p*NP-α-D-galactopyranoside, *p*NP-β-D-galactopyranoside, it was observed that the α-mannosidase is the highest among all the glycosidases tested. Therefore, the protein was passed through the DE-52 ion exchange column, and the bound enzyme could be easily desorbed using 02 M NaCl. To further remove contaminating proteins, gel filteration was used. The enzyme eluted from the gel filteration column strongly binds to the Con-A gel indicating its glycoprotein nature which is further confirmed by its specific elution from the gel using the sugar methyl-α-mannopyranoside. In this preliminary study we obtained the following information.

For the purification we used the protocol that involved four chromatographic steps anion exchange chromatography, hydrophobic interaction chromatography, Con-A lectin affinity chromatography, and Sephadex G-100. SDS-PAGE for α-mannosidase shows that the enzyme dissociated into two closely migrating bands (~ 66 kDa), suggesting that this enzyme may be tetrameric. The α-mannosidase also bound to Con-A affinity gel suggesting its glycoprotein nature and by silver staining also two closely migrating bands could be seen (~66 kDa). In literature, a large number of mannosidases have been purified from different

plant sources and their native and subunit molecular masses vary in size [Gnanesh Kumar etal, 2013 in press]. Jack bean enzyme has two subunits of 66 kDa and 44 kDa, whereas, tomato enzyme has subunits of 70 kDa and 47 kDa respectively [Snaith, 1975, Hossain, *et al.,* 2009]. Monomeric forms of mannosidase were also observed in some plant species like *Artocarpus communis* seeds (Isoform I-75 kDa; II-61 kDa), *Erythrina indica* seeds (127 kDa) [Kestwal and Bhide, 2005; Ahi *et al.,* 2007]. However *Ginko biloba* seeds α-mannosidase is 340 kDa in the native form and subunit of 120 kDa [Woo *et al.,* 2004]. In tropical fruit babaco (brassicales) oligomeric form of α-mannosidase was observed [Blom *et al.,* 2008]. *Prunus serotina* Ehrh and *Medicago sativa* [Curdel and Petek, 1980; Waln and Poulton, 1987] also has the four-subunits with different molecular masses. The purified α-mannosidase was checked for other glycosidase activities which are present in the initial crude extract with *p*NP-substrates such as *p*NP-α-D glucopyranoside, *p*NP-β-D-glucopyranoside, *p*NP-α-D-galactopyranoside, *p*NP-β-D-galactopyranoside, This α-mannosidase from *S. potatorum* seeds did not show significant activity with these substrates, this indicates that the activity of this enzyme is restricted towards *p*NP-α-D-mannopyranoside only. The optimum pH of *S. Potatorum α-mannosidase enzymes was around pH 5.0.* The enzyme was active at acidic pH and activity decreased as the pH approached the alkaline range pH 8.0. The optimum temperature of this α-mannosidase was found to be 50 °C, above this temperature, enzyme activity declined rapidly as the temperature increased. Taken together the data we obtained on the *S. potatorum* enzyme describes the protein to be different in its maolecular weight and subnit properties. However, interestingly the two bands obtained for this enzyme cross-reacted with the jackbean mannosidase antibody suggesting some antigenic similarities among these two proteins. A detailed sequence analysis of the enzyme purified in this study using Mass spectrometry analysis would reveal

its structural relatedness to jack bean enzyme as well as to other known mannosidases which is beyond the scope of the present investigation.

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Annexe

Brief report on candidates academic highlights

a) Awarded the best poster prize from **The International Symposium on Environmental Risk Assessment 2011 (ISERA 2011)** October 17 – 19, 2011, Bharathiar University, Coimbatore.

b) Mohammad Mansour Saleh Saif, N. Siva Kumar, M.N.V. Prasad**,** (2012), **Binding of cadmium to *Strychnos potatorum* seed proteins in aqueous solution: Adsorption kinetics and relevance to water purification,** Colloids and Surfaces B: Biointerfaces (94) 73– 79.

c) Poster entitled "**Preparation and characterization of polysaccharide nanoparticles from the seeds of *S. potatorum*** "was presented in the International Conference on **Nano Science and Technology (ICONSAT 2012) January 20-23, 2012, held in Taj Krishna Hotel Hyderabad.**

d) University of Omar Al-Mukhtar, Al Bayda, Libya awarded travel grant for presenting a paper in **The first international conference on Water Resources of Al Jabal Al Akhdar: Reality & Prospective** 5 -7 June 2012 held at the Department of Natural Resources, Faculty of Natural Resources & Environmental Sciences. Omar Al-Mukhtar, Al Bayda, Libya.

e) Poster intiteled **"Preperation and characterization and applications of polysaccharides nanoparticles from the seeds of *S. potatorum*"** was presented in the **National carbohydrate conference / CARBO- XXVII prospects and prospective of Glycoscience and allied technologies (CARBO- XXVII, CSIR- CFTRI, Mysore), December 13- 15, 2012, held at the CSIR- Central Food Technological research institute, Mysore, Karnataka.**