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RICE BRAN BASED NANOFIBERS FOR STABILIZATION OF PHYTASE ENZYME

Upendra Amal Rathnayake

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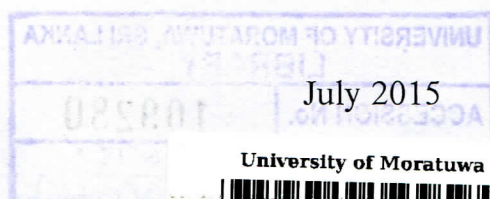
Degree of Master of Science

Department of Chemical and Process Engineering

University of Moratuwa

Sri Lanka

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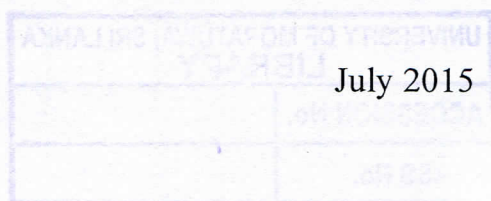
Thesis/ Dissertation submitted in partial fulfillment of the requirements for the
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Department of Chemical and Process Engineering

University of Moratuwa

Sri Lanka

July 2015



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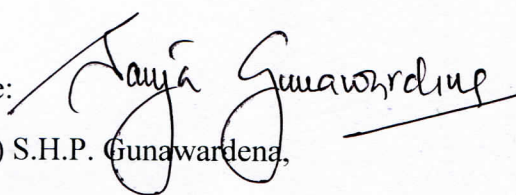

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U.A.Rathnayake

Abstract

Phytases is an enzyme belonging to the histidine acid phosphatase family which has the capability to initiate the stepwise dephosphorylation of phytate, the primary storage form of phosphorus in most seeds and cereal grains. Phytase enzyme has become the most widely used enzyme in the world, particularly in poultry and swine industries as a source for the dephosphorylation of phytic acid, the primary storage form of phosphorus in most seeds and cereal grains. The problem involved with the phytase applications in animal feed is its thermal instability. Hence, a substantial thermal stabilization of the enzyme is required to increase its practical applications.

In this research, phytase enzyme was stabilized in to agro waste based nanaofibers. Nanofibers with a fiber diameter ranging from 30 – 50 nm were produced successfully using electrospinning techniques. For the best of our knowledge only few researches have reported on the nanofiber synthesis from agro waste and probably this could be the first study that reports the use of rice bran for this purpose.

First, rice bran received from a local mill was characterized and the soluble fiber fraction without any fat was isolated. This fiber solution was used for electrospinning. The apparatus containing the high voltage supply, electrodes, solution input compartment and the collector plates were fabricated in-house and the instrument was automated prior to electrospinning of fibers. The initial polymer solution parameters such as pH, viscosity, and conductivity were adjusted in order to facilitate the spinning process. The solution viscosity was modified using a food grade bio-polymer, polyvinyl alcohol (PVA). Then, the spinning parameters such as accelerating voltage, distance between the two electrodes, PVA content were optimized to result in nanofibers with uniform diameter.

After achieving the best conditions for electrospinning, two different techniques were investigated to encapsulate phytase enzyme in to nanofibers synthesized from rice bran. First, attempts were made to in-situ encapsulate the phytase enzyme into nanofibers. Here, electrospinning process was carried out in the presence of phytase enzyme in the initial polymer solution followed by cross-linking of the fibers using

boric acid. Secondly, phytase enzyme was immobilized into the nanofibers as a post modification after spinning followed by cross linking with sodium tripolyphosphate. The activity of the phytase enzyme in the encapsulated product was established at the gutter pH. 2.5 and at different temperatures up to 170 °C .

The presence of nanofibers was essential to offer a large surface area for large amount of enzyme encapsulation. The morphology and the size of the fibers were studied using scanning electron microscopic technique and the elemental composition was determined using energy dispersive X-ray analysis. It was possible to produce nanofibers with uniform diameter between 30 – 50 nm by this technique. However, when the phytase enzyme was encapsulated a beaded-string like morphology was observed. The bonding and extent of encapsulation was studied using Fourier transform infra-red spectroscopy (FT-IR). Any changes in the peak positions or shape allowed predicting about the nature of bonding. Based on the FT-IR data it was confirmed that the enzyme was H-bonded to the nanofiber surface. Differential scanning calorimetric studies further confirmed the successful encapsulation of the phytase enzyme. Melting point of the enzyme has increased by 40 °C due to the encapsulation. Thermal stability of the final product was investigated using thermogravimetric analysis (TGA) technique. It was observed that the decomposition temperature of the enzyme has increased from 238 °C to 348 °C due to the encapsulation.

Interestingly, as expected it was found that the thermal stability of the enzyme has increased almost by 100% after encapsulated into the nanofibers followed by crosslinking. It was found that the pure enzyme loses its activity at 70 °C while after encapsulation into nanofibers based on rice bran, it is active up to 170 °C.

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LIST OF ABBREVIATIONS AND SYMBOLS

%	Percentage
°C	Degree Celsius
Å	Angstrom
cm	Centimeter
DI	Deionized
DSC	Differential Scanning Calorimetry
DTG	Derivative Thermogravimetric Analysis
EDX	Energy Dispersive X-ray analysis
FT-IR	Fourier Transform Infrared Spectroscopy
g	Gram
Hz	Hertz
kV	Kilo Volts
M	Molarity
mg	Milligram
ml	Milliliter
mm	Millimeter
Mt	Metric Tons
nm	Nanometer
PDF	Portable Document Format
PXRD	Powder X-ray Diffraction
rpm	Revolutions per minute
SEM	Scanning Electron Microscope
SLINTEC	Sri Lanka Institute of Nanotechnology
STPP	Sodium tripolyphosphate
TGA	Thermo Gravimetric Analysis/ Analyzer
USA	United State of America
UV-VIS	UltraViolet-Visible
wt%	Weight percentage
λ	Wavelength

μm

Micrometer

CHAPTER 1

INTRODUCTION

Nanotechnology involves engineering of functional systems at the nano scale (1 – 100 nm) to create materials and devices with new or different properties, thus attracting great scientific interest, in many fields.

Inspiringly, nanotechnology has greater applications in various industries, including food and bioactives. In recent years there has been a tendency to employ smart nano-encapsulation systems (nanocarriers) to deliver bioactive materials into biological systems, thus providing better stability with improved bioavailability.

Among nanomaterials, nanofibers are known as the revolutionary material in the new millennium due to variety of applications that they can be incorporated in to, and due to the extraordinary physical properties they possess, such as greater strength and reactivity. High surface area to volume ratio of nanofibers compared to monolithic materials alone is responsible for these improved physical properties (Huang et al., 2003). Biomedical field is one of the key areas where nanofibers are effectively utilized and the number of growing publications over the years by many researchers exhibits its extensive uses in drug release, wound dressing, tissue engineering and enzyme immobilization (Agarwal et al., 2008).

Phytase is an enzyme which belongs to the histidine acid phosphatase family which has the capability to initiate the stepwise dephosphorylation of phytic acid, the primary storage form of phosphorus in most seeds and cereal grains. Phytic acid contributes to about 1-7% of the total dry weight (Cheryan et al., 1980) and 50% - 80% of the total phosphorous content in cereals. The ruminants digest phytic acid with the help of phytases produced by their anaerobic ruminal micro flora. However, monogastric animals such as pig, poultry and fish are deficient in gastrointestinal tract phytases. Hence, their food is supplemented with inorganic phosphorus to meet the phosphorous requirement. This is extremely expensive and also contributes for the environmental pollution with excessive manure phosphorus runoffs, because of

the fecal phosphate excretion by the animals. To overcome these challenges phytase enzyme has been largely used as an animal feed additive mainly for swine and poultry, and for fish to some extent, in order to improve the utilization of phosphate from phytic acid. This has opened a market volume over 150 million euro (Greiner & Konietzny, 2006).

In recent years, phytase has also been found increasingly useful in processing and manufacturing of food for human consumption, due to its ability to decline food phytic acid, results in an enhancement of mineral bioavailability. Phytic acid rich diet can reduce the absorption of dietary mineral significantly, by making complexes with them. A phytase supplement diet can catalyzes the release of phosphate from phytic acid and hydrolyses the complex formed by phytate and dietary minerals, rendering them more soluble. This improves the intestinal uptake of dietary minerals (Greiner et al., 2006). However until now no phytase product has found its way to the market, thus the market demand is urging over billions of euros. The problem involved with the phytase use is its thermal instability. However, application of high temperature during feed stock preparation is unavoidable, since the feed pelleting process requires higher temperature in the range of 60 - 70 °C. Denaturation of phytase enzyme during the pelletization process at temperatures around 80 °C is reported (Kirkpinar et al., 2006; Silversides et al., 1999). Hence, a substantial thermal stabilization of the enzyme is required to overcome these challenges and to increase its practical applications.

Conformational stability of an enzyme is responsible for the extended thermal stability and prolonged activity. Conformational stability of an enzyme can be increased by rigidifying the structure by the meanings of immobilization or crosslinking thus is being tested for phytase enzyme in this research. Phytase enzyme is encapsulated in to agro waste based nanofibers by different techniques and tested for the thermal stability.

Agricultural waste is a great source of dietary fiber, which includes cellulose, hemicelluloses, lignin, pectin, gums, and other polysaccharides (Sharoba et al., 2013). These bio polymers will be used to synthesize nanofibers with suitable



amendments using a biocompatible synthetic polymer for the stabilization of Phytase enzyme in this research. The objectives of this research are.

- (1) Encapsulation of phytase into agricultural waste (rice bran) based nanofibers
- (2) Study the activity and stability of synthesized phytase encapsulated nanofibers
- (3) Identify structural properties of the nanocomposite with best/optimum activity and stability

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction to nanotechnology

A nanometer is one billionth of a meter (10^{-9} m) which is in equivalent to 10 hydrogen or 5 silicon atoms aligned in a line. Along with this extremely small dimensions they tend to exhibit physical properties distinctively different from the bulk thus it is not just miniaturization from the micrometer scale in where the physical properties are intact with the bulk. It is not only the fact that the nano scale permits more functionality in a given space but also some remarkable specific properties such as reduced melting point and lattice constants in crystals, loss of ferroelectricity and ferromagnetism in materials, loss of conductivity in semiconductors and turning in to insulators and inert materials such as gold converting in to excellent low temperature catalysts. "In general, nanotechnology can be understood as a technology of design, fabrication and applications of nanostructures and nanomaterials" (Cao, 2004).

2.2 Nanofabrication Techniques

Due to rapid growth of nanotechnology in the past decade advance nanofabrication techniques have been developed to fabricate nano-devices. These nanofabrication methods can be divided in to two main catagories. Bottom up method and top down method (Cao, 2004). Bottom up method mainly focuses on chemical synthesis or self-assembly and colloidal dispersion while the top down method focuses on attrition or milling and etching techniques. Both techniques are really important, do a significant impact on modern industry and most likely in nanotechnology, while there are advantages and disadvantages in both methods (Rogers et al., 2014). For example in top-down method, significant imperfection of surface structures can happen. Especially in a technique such as lithography, the possibility getting crystallographic damages and defects are very high. This phenomenon can be observed during the process of making nanowires through lithography and the resulting surfaces contain a lot of surface defects and impurities thus directly impact

on physical and surface chemistry. The conductivity of these nanowires would be reduced significantly due to inelastic surface scattering which can lead to generation of excessive heat (Cao, 2004). Though these challenges are there to be addressed in top-down method, their industrial impacts are still substantial.

So far, the most promising strategy to exploit nanomaterial and devices is offered by the bottom-up approach. As well as the name implies bottom up method refers to build the material, atom by atom, molecule by molecule or cluster by cluster. This method facilitates the possibility of getting materials with more homogeneity, chemical composition, better ordering and less defects.

2.3 Nanomaterials

2.3.1 Zero-Dimensional Nanostructures: Nanoparticles

Nanoparticles can be metals, semiconductors or insulators and be made from a single crystal, an aggregate crystals or be completely non-crystalline. Depending on the size, the physical and chemical properties of particles can significantly vary thus the particles those are too small to be considered as bulk and larger than to be considered as typical molecules or nanoparticles can show hybrid properties (Rogers et al., 2014). When the size of the particles decreases the surface area increases. For example in a bulk material, typically less than 1% of the atoms are on the surface while the particles are reaching to nanoscale and beyond over 90% of its atoms are on the surface. A particle with 50 nm has 6% surface atoms though a particle with 10 nm diameter has more than 15% surface atoms (Rogers et al., 2014). More surface atoms facilitates more reactivity for a given quantity thus industrially very important as catalysts. For example even though bulk gold is relatively an inert metal, gold nanoparticles perfectly are useful catalysts in numerous chemical reactions thus is used in the production of agrochemicals, pharmaceuticals and many more.

Both bottom and top-down methods can be employed to synthesize nanoparticles. Among them bottom-up approach has been the most utilized due to the uniformity and the defects freeness of the synthesized particles (Cao, 2004).

2.3.2 Two-Dimensional Nanostructures: Thin Films

Depositions of thin films is an area where intensive research has been focused on. Physical interaction that works between particles are mainly surface interactions.

Therefore deposition of a thin layer of material onto an object can make it more efficient, less susceptible to friction, more resistant wear, modify and improve the product performance (Rogers et al., 2014). Many techniques have been developed and used in industries to achieve this. These can be divided in to two groups; first, vapor-phase deposition which includes evaporation, molecular beam epitaxy (MBE), sputtering, chemical vapor deposition (CVD) and atomic layer deposition (ALD) and the second, liquid-based growth which includes are electrochemical deposition, chemical solution deposition (CSD), Langmuir-Blodgett films and self-assembled monolayers (SAMs) (Cao, 2004).

Among these techniques SAMs are one of the most popular thin film deposition methods. In the forming of this layer, usually molecules or particles are assembled themselves and form a densely packed layer on the top of a substrate usually less than 1 μm thick. These thin films can significantly enhance the surface area of the object and boost the available interaction sites (Cao, 2004).

2.3.3 One-Dimensional Nanostructures: Nanowires and Nanorods

Nanowires and nanorods are important one dimensional nanomaterial with very high specific surface area due to their small diameters and in addition they have high porous membrane with excellent pore interconnectivity. As same as nanoparticles and thin films many techniques have been developed to synthesis nanowires or fibers. Depending on the synthesizing technique of nanowires the aspect ratio, surface conditions, geometrical configuration and crystal conditions can vary. According to literature many techniques have been developed to synthesize nanowires.

2.4 Nanofiber synthesis methods.

Nano fibers are the widely applied nano materials compared to others, mainly due to the ease in production process, industrially. There are different methods available to synthesize nanofibers such as drawing, phase separation, self-assembly, template synthesis and electrospinning (Huang et al., 2003). Each of these techniques has their own advantages and disadvantages. A few of vastly utilized methods are discussed below.

2.4.1 Drawing

In the drawing process suspended fibers are fabricated by contacting a previously deposited polymer solution droplet by a sharp tip and drawing them as a liquid fiber (Ramakrishna et al., 2010). This drawn fiber is connected to another previously deposited polymer solution droplet and then to another as such until the desired length is obtained. Very sharp probe such as a scanning tunneling microscope nanoprobe is ideal for this purpose. Immediately after the fabrication / drawing the liquid fibers get dried up and solidify due to the high surface area to volume ratio at nano scale, which intensify the evaporation of the solvent. There are several limitations in this process, predeposition of the relevant polymer solution droplet is one factor this limits the fabrication of three dimensional configurations and spatial geometries. Solvent evaporation of the predeposited droplets is another limitation. Due to that the viscosity of the polymer solutions continuously increases and affects the diameter of the drawn fibers, hence there is a limited time within which the fibers can be pulled out (Nain et al., 2006).

Nain et al., (2006) reported a method to overcome some of those limitations in the drawing process. In this method, suspended fibers are drawn continuously using a glass micropipette (Figure 2. 1). Polymer solution is pumped continuously through the micropipette which has enabled continuous drawing at any configuration. They have used polystyrene dissolved in xylene has been used as the polymer solution and by varying the drawing parameters suspended fibers having amorphous internal structures with uniform diameters from micrometers down to sub-50-nm has been achieved (Figure 2. 2).

Key advantages in this reported method is the possibility of synthesizing/drawing fibers with different diameter from the same process. By varying the drawing speed and the time spent on the polymer droplet (step B in FIG 1) the researchers had been able to obtain fibers which are around 100 nm and 1000 nm in diameter (Nain et al., 2006).

However due to the above discussed limitation drawing was not used in this research to synthesis nanofibers.

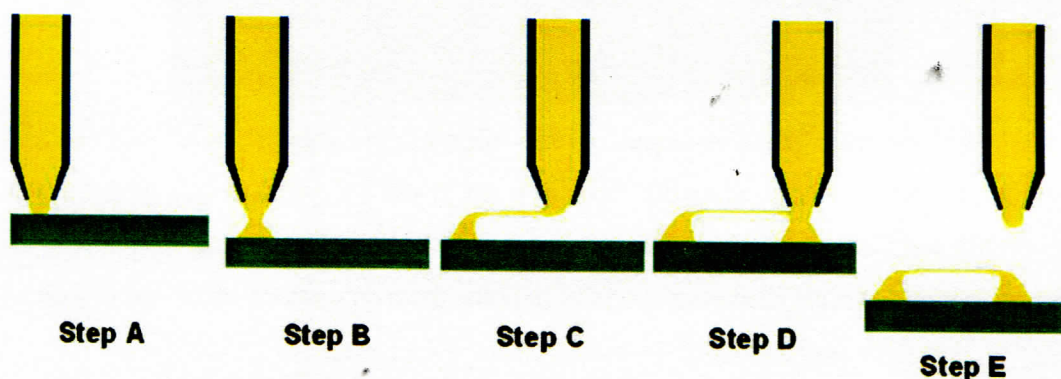


Figure 2.1: Schematic steps of the micropipette based suspended polymer fiber drawing method for a single fiber case: Step A: Raise the substrate until it comes into contact with the polymer droplet. Step B: Move the micropipette vertically with a constant speed and stop at a constant height. Step C: Move the stage along a predetermined trajectory with a constant speed while forming the solid polymer fiber by the evaporation of the solvent. Step D: Contact the substrate with the pipette to suspend the fiber. Step E: Break the fiber or continue to draw subsequent ones (Nain et al., 2006).

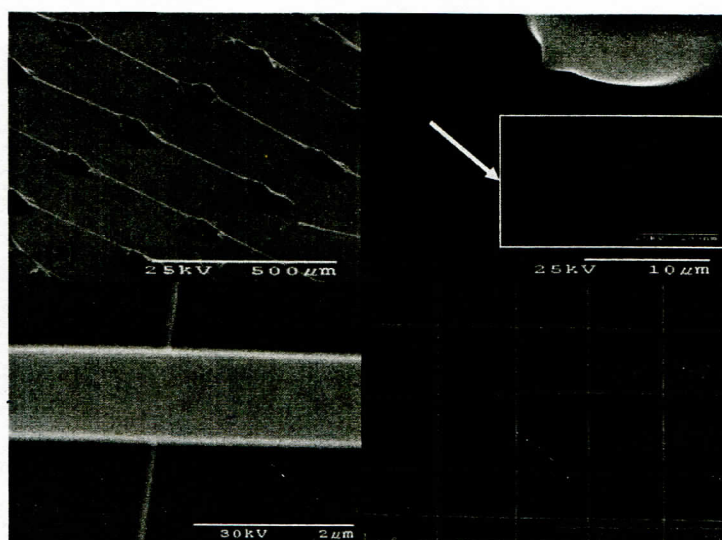


Figure 2.2: SEM images of sample drawn suspended polystyrene fibers: (a) continuously drawn array of fibers on a silicon substrate, (b) a 37 nm nanofiber ($M_w=650\,000$ g/mol), (c) a cross pattern of two fibers showing the fiber diameter control in the same drawing process, and (d) 2D fiber network (Nain et al., 2006).

2.4.2 Phase separation.

Ma et al., (1999) have published a method to engineer synthetic nano-scale fibrous which has the potential to use as an extracellular matrix in tissue engineering. The phase separation process involves five major steps.

1. Thermally induced gelation
2. Solvent exchange
3. Freeze drying

First, a suitable polymer is dissolved in an organic solvent in an elevated temperature. Then the solution is rapidly frozen into a gel and subsequently immersed into distilled water for solvent exchange. After that the gel is blotted to remove the excess water and transferred into a freezer. Then the gel is freeze dried under vacuum until a porous nano fibrous matrix is obtained. By employing phase separation method, Ma et al., (1999) have been able to synthesize three-dimensional interconnected fibrous network with a fiber diameter ranging from 50 to 500 nm and with the porosity over 98.5% from bio degradable aliphatic polyesters (Figure 2.3).

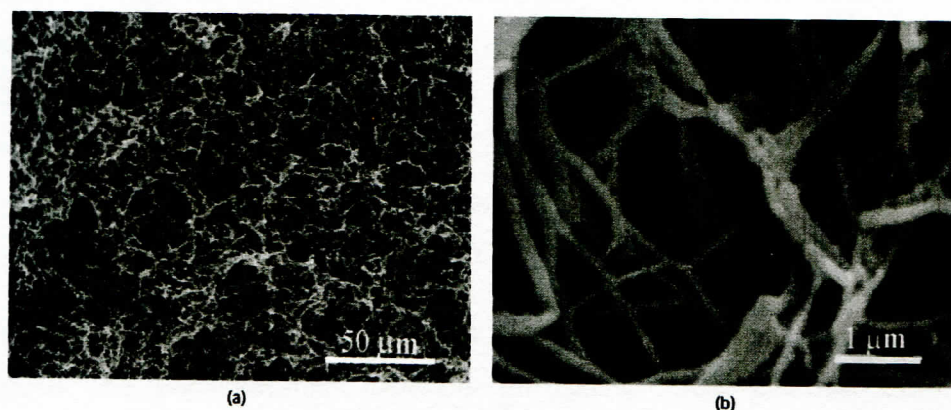


Figure 2.3: SEM micrographs of a Poly(L-lactic acid) fibrous matrix prepared from 2.5% (wt/v) Poly(L-lactic acid) / Tetrahydrofuran solution at a gelation temperature of 8°C: (a) $\times 500$; (b) $\times 20K$ (Ma et al., 1999).

Ma et al., (1999) have also studied the effect of polymer concentration, thermal annealing, solvent exchange, and freezing temperature before freeze-drying of the nano-scale structures. A platelet-like structure had been formed at a high gelation temperature and, nano-fibrous structure have been formed at lower temperatures. The polymer concentration has not affected much on the average fiber diameter, though the porosity has been decreased with the increased concentration. However, the mechanical properties had shown to increase with the concentration.

2.4.3 Template synthesis

Template based synthesis is a highly used method to synthesis nanorods, nanowires and nanotubules of polymers, metals, semiconductors and oxides. Material is grown in various nano sized templates and channels. There are three main template-based synthesis techniques.

1. Electroplating and electrophoretic deposition
2. Colloid dispersion, melt, or solution filling
3. Conversion with chemical reaction

Electro chemical deposition is basically a deposition of solid material on an electrode as a result of electrolysis. In this process, charged ions of the desired species are

diffused through a solution under the influence of an external electrical field. Then these charged ions, generally cations are reduced at the growth or deposition surface (Cao, 2004). This technique can be only be applied for conductive material such as alloys, metals semiconductors and conductive polymers and is method is widely used in the process of preparing metallic coatings in which the deposition is confined only to the inside of the pores of template membrane (Wu et al., 1994).

The electrophoretic deposition technique has been widely used in film deposition of ceramic and organoceremic materials on cathode from colloidal dispersions (Zhitomirsky, 2002). This method is different from electroplating or electrochemical deposition by many aspects, though the major difference is the deposit by electrophoretic deposition does not need to be electrically conductive. An electrical charge can be developed on to the surface of the deposited material via preferential dissolution, deposition of charges or charged species and adsorption of charged species such as polymers (Cao, 2004).

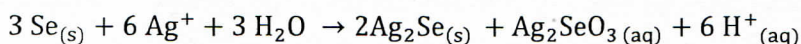
In this technique is, it uses the oriental motion of the charged particles is used to grow films by enriching the solid particles from a colloidal dispersion on to the surface of an electrode. When the colloid or the particles in the dispersion are positively charged they will abstract electrons from the anode and deposit on it accordingly.

In melt and solution filling method to synthesize metallic nanowires, a template is by filled with molten metal. One such example is the preparation of bismuth nanowires by injecting molten bismuth into the nanochannels of an anodic alumina template under pressure (Cao, 2004). In this method alumina template is degassed and immersed in molten bismuth under the application of high pressure argon gas which facilitates the injection of bismuth in to nanochannels of the alumina template. Wolfe and Coskren., (1965) reported the synthesis of nanowires of other metals such as In, Sn and Al, Se, Te, GaSb and Bi₂Te₃ following the same method.

Nanorods, nanotubes and nanowires can also be fabricated by conversion with chemical reactions. In this technique first nanowires of the constituent element is

prepared, then the prepared nanowires are reacted with chemicals containing the desired element.

One such example is reacting single crystalline selenium nanowires with aqueous AgNO_3 (aq) solutions at room temperature to make single crystalline nanowires of Ag_2Se (s) (Cao, 2004).



Another way to synthesize nanorods from the same technique is between the reaction of volatile metal halides or oxide species with carbon nanotubes to form solid carbide nanorods.

2.4.4 Electrospinning

The word electrospinning is a decedent of the word electrostatic spinning, a technique which uses electrical potential to draw fibers from a polymer solution. Electrospinning is a continuous, versatile and a convenient method to synthesize nanofibers with diameters ranging from tens nanometers to several micrometers (Huang et al., 2003). Eventhough this technique is discussed more recently, the original idea dates back more than 60 year. Formalas pioneering work on nanofiber synthesis during the period of 1934 to 1944 has resulted a series of patents revealing the possibility of fiber synthesis using an experimental setup using electrostatic force (Huang et al., 2003). In this work a polymer solution has been introduced into an electric field. One of the electrode was connected to the polymer solution which is injected out of a metal spinnerette with a small hole and the resulting solution charged jets are travelled to the collector where the opposite electrode is connected. During the time of the migration the solution is evaporated and the dried fibers are collected in the collector.

Many modification and development have been done for the process after wards, until an apparatus from which non-woven ultra-thin and ultra-light fabrics could be synthesized with different patterns has been patented. In this electrical spinning apparatus positive electrode was connected to a polymer solution and the negative

terminal was connected to a belt where non-woven fibers were connected (Simons, 1966).

Taylor, (1969) studied the shape of the polymer droplet produced at the tip of the needle of the electrospinning apparatus. He observed that the droplet arrange in to a cone when an electric field is applied. This conical shape at the edge of the needle is now known as the "Taylor Cone". Taylor was able to come up with very important conclusion, in electrospinning process to draw a fiber, viscosity and electrostatic potential are needed to be maintained so that Taylor cone has an angle of 49.3 degrees to balance the surface tension and electrostatic forces.

Early synthesis of fibers in sub-micron dimensions of 0.05–1.1 microns range has been reported by Baumgarten, 1971. In this work the droplet that came out from the stainless steel capillary tube has been maintained by an infusion pump. As done by scientists other (Huang et al., 2003), a high voltage DC current has been connected to the capillary tube and the fibers have been collected to a grounded metal screen.

After 1970s the technique and the apparatus did take much of different shapes but the application. From there onwards upto today publications reporting the use of electrospinning technique is significantly increase, probably due to its affiliation with nano-technology (Huang et al., 2007; Wang et al., 2006). Up to date literature reports successful synthesis of fine and nano fibers based on more than hundreds of synthetic and bio-polymers (Huang et al., 2003).

The most remarkable advantage of fibers produce by electrospinning process is the ability to produce fibers with a very smooth surfaces. It is reported that these fibers have very high surface area and small pore sizes and can be used to produce three dimensional structures effectively (Deitzel et al., 1999).

2.4.4.1 Electrospinning theory

It is reported that there are three major requirements in the electrospinning process (Huang et al., 2003).

- 1 High voltage supplier

- 2 Capillary tube with a pipette or needle of small diameter
- 3 Metal collecting screen

The importance of high voltage (DC) in the electrospinning process is to create an electrically charged jet of polymer from the needle. One electrode is connected to the needle or to the spinning solution directly and the other is to the collector or in most of cases collector is just grounded (Huang et al., 2003).

The electrode connected to the polymer solution exerts an electric field at the end of the capillary tube which contains the solution fluid which is held by the surface tension. This results an induction of the respective charges on the surface of the liquid. The repulsions between the mutual charges and the contractions of the surface charges to the opposite electrode, cause a force directly opposite to the surface tension (Fang et al., 1997). When the intensity of the electric field is increased, this force is also increased hence the hemispherical shape of the liquid surface at the end of the capillary tube elongates to form a conical shape known as the Taylor cone. With further increase of the electric field, this repulsive electrostatic force becomes a critical value which overcomes the surface tension of the fluid surface and a charged jet of the fluid is ejected from the tip of the Taylor cone. This jetting mode is known as electrohydrodynamic cone-jet (Yarin et al., 2001). During its motion to the collector this discharged fluid jet will undergo an instability and elongation process which results the jet to become very thin and long and to bend from the original trajectory resulting formation of webs like polymer structures. This bending instability causes splitting of the jet and results fibers with small diameters and this is known as splaying (Doshi et al., 1995). The solution jet evaporates before it reaches the collector screen leaving behind a polymer as an interconnected web of small fibers (Huang et al., 2003).

2.4.4.2 Parameters that affect electrospinning

Effect of process and fiber solution parameters on electrospinning and the fiber morphology has been studied by many scientists (Shen et al., 2014). They are mainly applied voltage, distance between two electrodes, flow rate, spinning environment,

solution viscosity or concentration, conductivity of the solution and solvent volatility.

(a) Applied voltage

In the electrospinning process an electric potential is used to generate nanofibers. Applied voltage, which should be from a DC source, determines the jet initiation and splaying, which determine the fiber morphology and diameter. Deitzel et al. 2001 showed that the increase of applied voltage is related to instability mode and if the applied voltage is not controlled properly fibers with beaded structures are resulted. Working with polyetheleneoxide/ water system, it was further reported that defect free fibers were obtained at 5.5 kV while beaded structures were resulted at 9.0 kV. These beaded structures have a reduced surface area and hence, some desired properties of nanofibers are lost. Bumagarten et al., (1971) report increased fiber lengths with increasing applied voltage, and further reported that the diameter of the jet reached a minimum value after an increase in the applied field and then getting larger diameters because polymer droplet saturated with charge (Zhang et al., 2005).

(b) Distance between two electrodes

Distance between the two electrodes (nozzle to collector distance) also, determine the fiber morphology. Deposition time, evaporation rate, and instability region (Huang et al., 2003). Buchko et al., (1999) studied the dependence of electrode distance on the above parameters and showed that if the nozzle collector distance was not set correctly (too large or too small) beaded structures were resulted even without changing the viscosity of the fiber solution. They further reported that fiber solution prepared using organic solvents need lesser distances than fiber solutions prepared using water as the solvent.

(c) Flow rate

Polymer solution flow rate determines the fiber diameter (Megelski et al., 2002). It is important to set a constant flow rate, to avoid beaded structures or fibers with

variable diameters can form. Megelski et al., (2002) showed that fiber diameters increased with increasing flow rate using poly styrene fibers.

(d) Spinning environment

Fiber morphology is determined by environmental conditions like type of environment (oxygen %, inert), relative humidity (RH), and pressure of the environment (vacuum conditions). It has been shown that RH greater than 60% has a significant effect on fiber drying and fibers do not properly dry before reaching the collector (Baumgarten, 1971).

(e) Solution viscosity or concentration

Solution viscosity is directly related to the surface tension. In electrospinning process a polymer solution is introduced through a small capillary or a needle in the presence of an electric field. Initially, a hemispherical meniscus of liquid will be equilibrate at the end of the capillary due to the surface tension of the solution. At a critical voltage the contact angle of the solution droplet must be suitable to release the polymer solution as fibers. Low viscous polymer solutions form droplets while high viscous polymer solutions are inhibiting fiber formation. It has been proven that with increasing solution viscosity, fiber diameter increases (Deitzel et al., 2001).

(f) Conductivity of the solution

Most polymers are conductive, with few exceptions, with those behave as dielectric materials. Charge induction in polymer solution significantly effect on electrospinning especially in jet formation. Baumgarten et al., (1971) showed that jet radius is inversely proportional to solution conductivity. It is reported that adding salts help to produce bead-less fibers with smaller diameters (Zong et al., 2002).

(g) Solvent volatility

In electrospinning process, it is very important to dry the fiber (evaporate solvent) before it reaches the collector. Therefore, solvent volatility plays a major role in electrospinning process (Huang et al., 2003). Due to phase separation when the jet

gets thinner within the traveling period solvents tend to evaporate rapidly. Megleski et al., (2002), studied the relationship between solvent properties and fiber morphology and showed that fiber diameters can be reduced with use of highly volatile solvents (Megelski et al., 2002)

2.5 Applications of nanofibres

Among nanomaterials, nanofibers are known as the revolutionary material for the new millennium with the fastest emerging market within the next decade. Owing to its structure nanofibers possess improved properties such as greater strength, reactivity, electrical and optical quality thus, they are used in applications such as filtration, barrier, wipes, personal care, material reinforcements, garments, insulation, medical, energy storage and many more, making them perfect solution applicable across all industries (Huang et al., 2003).

Biomedical field is one of the key areas where nanofibers are effectively utilized and the number of growing publications over the years by many researchers exhibits its extensive uses in drug release, wound dressing, tissue engineering and enzyme immobilization (Agarwal et al., 2008).

Nanofibers are also, used in drug delivery applications. This has been studied using tetracycline as the drug and poly ethylene-co-vinyl acetate as the fiber. These nanofibers have shown good drug releasing properties than films with the same material (Kenawy et al., 2002) . This observation highlights the importance of nanofibers in drug delivery applications specially drugs that are very unstable.

Apart from those applications research interests are increasing in the use of nanofibers incorporated nanocomposites in material engineering, since these composites can provide superior structural properties such as stiffness strength and reinforcement (Huang et al., 2003). The improvement of the inter laminar toughness and delamination strength of the high performance composite laminate by incorporating Polybenzimidazole in between plies of the laminate without a substantial reduction of in in-plane properties, weight or ply thickness has been reported (Huang et al., 2003). Further they have revealed the applications of

nanofibers in applying to manufacture military cloths expected to help maximize the survivability, sustainability, and combat effectiveness against extreme weather conditions, ballistics, and NBC (nuclear, biological, and chemical) warfare. Due to high porosity and less pore sizes of electrospun fibers are extremely resistant against the penetration of harmful gases in aerosol form. The high surface area of nano fibers facilitates more neutralization of chemical agents and without impedance of the air and water vapor permeability to the clothing which are more effective against current existing charcoal absorbents over chemical warfare agents such as sarin, soman, tabun and mustard gas from inhalation and absorption through the skin (Huang et al., 2003).

The potential use of nanofibers in Electrical and optical applications is reported (Madhavan et al., 2012). High surface areas of nano fibers has been taken as an advantage in developing porous electrodes for high performance batteries since the electrochemical reactions are proportional to the surface area of the electrode (Huang et al., 2003).

Due to high surface area of nanofibers they can be used in applications where high surface area within a low volume is needed to enhance the reactivity, like in catalysts. Fibrous catalysts provide many advantages over conventional catalysts as they can be formed to any geometry, and process nano-level webs with very small pores. Therefore, reactants cannot flow through without having contact with the catalyst. For example, carbon nanofibers loaded with iron particles have shown high conversion of hydrocarbons compared to those of active carbon and alumina (Rodriguez et al., 1994)

Nanofibers have been used in filtration processes for more than a decade. Nanowebs that are formed during synthesis of nanofibers have tiny pores and therefore, they can be used as a nanofilter in chemical masks, protective clothing and water filters (Qin et al., 2006).

Use of nanofibers for filtration gives some desired properties like high moisture vapour transfer, increased fabric breathability, and enhancing toxic chemical resistance (Qin et al., 2006).

2.6 Agro wastes as a source for nanofiber synthesis

Agriculture is the most important part of the human civilization and agricultural wastes are becoming more and more problematic with the population growth. By products or agricultural residues that are not been utilized are generally known as agro wastes. Disposing some of these agro wastes are problematic because they can cause environmental pollution. Hence, researches based up on agriculture are attracting a significant interest. Some of these agro wastes such as rice bran contain very valuable components like cellulose, hemicellulose and lignin and can be effectively utilized for the synthesis of valuable end products.

Initial attempt to use rice bran as a dietary fiber source for the synthesis of nanofibers was investigated by Senapathi, (2013). He has attempted the synthesis of dietary nanofibers from agro waste as a nutraceutical encapsulant. In his work he had been able to synthesize a network structure of fibers with the diameter varying from 200 nm to 10 μ m (Senapathi, 2013).

Shankar et al., (2013) revealed the possibility of synthesizing soy bean protein by blending soy protein with other spinnable polymers; cellulose, polyethylene oxide (PEO), PVA and chitosan by electrospinning process and also showed that these fibers have the potential of wound-healing.

The potential of soluble dietary fibers from okara which is an agro waste from soya bean solid waste, oil palm frond and oil palm trunk has been reported (Fung et al., 2011). Soluble dietary fibers had been extracted from alkali treatment of agro wastes. Poly(vinyl alcohol) (PVA) had been amended with soluble dietary fibers prior to use in the electrospinning process and the authors have obtained fine fibers (Figure 2.5) (Fung et al., 2011).

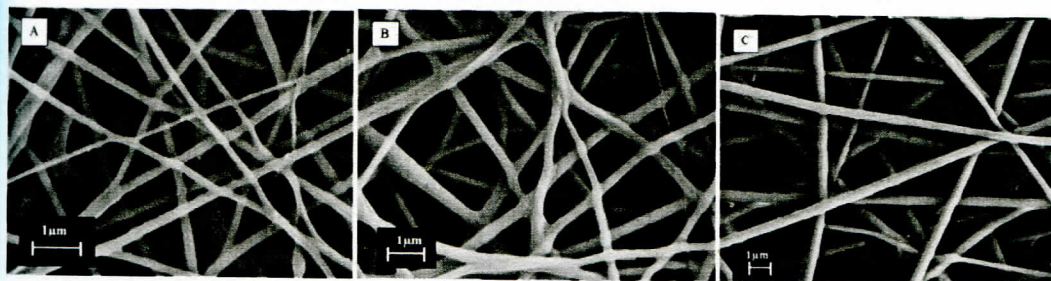


Figure 2.4: SEM micrographs of electrospun nanofibers from the soluble dietary fiber fraction of (A) okara, (B) oil palm trunk, and (C) oil palm frond (Fung et al., 2011).

2.6.1 Rice bran

According to the Department of Agriculture, Sri Lanka, rice is the single most important crop occupying nearly 0.77 million ha, which is 34% of total cultivation area of Sri Lanka. Sri Lanka currently produces 2.7 million tons of rice annually which satisfies around 95% of the domestic requirement (Sharma, 2010).

Rice bran is the layer between the inner white rice grain and the outer hull and has around 8% of total weight. The annual rice bran production in Sri Lanka is around 200000 tons. Which is mainly used as animal feed (Senapathi, 2013).

Abdul et al., (2000), studied the constituents of rice barn and has been concluded that dietary fiber content is 27% of rice bran and the analysis data is shown in the table 1. 1. In addition, it is also an excellent source of protein, minerals, unsaturated fat and vitamins. Therefore, more researches have to be conducted to minimize this wastage.

Table 1.1: Constituents of rice bran (Abdul-Hamid et al., 2000)

Components	Percentage (%)
Fat	16.62 ± 0.05
Protein	14.6 ± 0.21
Moisture	11.19 ± 0.03
Ash	7.41 ± 0.01
Total dietary fiber	27.04 ± 0.30



Insoluble dietary fiber	24.99 ± 0.43
Soluble dietary fiber	2.25 ± 0.13
Digestible carbohydrates	23.16

2.6.1.1 Dietary fiber

Dietary fiber consists of carbohydrate polymers and non-starch polysaccharides that are primary components of plant cell walls. These include cellulose, hemicelluloses, hemiglucans and pectins that do not digest utilizing the available human enzymes. However, these non-digestible poly saccharides pass through small intestine facilitating the digestion (Senapathi, 2013).

Dietary fibers are commonly classified as soluble and insoluble fibers. Soluble fiber is the type of fiber that dissolves in water to form a gel-like material and helps to lower blood cholesterol and glucose levels (Senapathi, 2013). Insoluble fiber is the type of fiber that promotes the movement of food through the digestive system and increases stool bulk, so it can be of benefit to those who struggle with constipation or irregular stools. Whole-wheat flour, wheat bran, rice bran, nuts, beans and vegetables, such as cauliflower, green beans and potatoes, are good sources of insoluble fibers (Senapathi, 2013).

2.6.1.2 Physiological effect of dietary fiber

Dietary fibers are complex compounds and the composition varies with the source and the area of cultivation. Each of these components is having a unique chemical structure and physical properties. Normally dietary fibers present in plant cell walls (as structural polymers) combined with other organic compounds such as vitamins flavonoids (Senapathi, 2013).

Since human cannot digest dietary fiber, they reach the large intestine without undergoing any changes in structure and are fermented by the colonic microflora with the production of short chain fatty acids, H₂, CO₂, CH₄ and biomass (Senapathi, 2013). This fermentation process is very important for the activity of the large intestine as energy needed for the functioning is gained by absorption of short chain

fatty acids and not by glucose oxidation (Roberfroid, 1993). Therefore intake of dietary is very important.

2.7 Enzymes

Enzymes are the most remarkable and highly specialized proteins, with extraordinary catalytic power, often far greater than that of synthetic or inorganic catalysts. Enzymes are highly substrate specific, and function tremendously under very mild conditions of temperature and pH and especially in aqueous solutions. In biological systems they are central to every biochemical reaction, catalyze hundreds of metabolic reactions which degrade nutrient molecules, transform chemical energy and synthesize macromolecules from simple precursors.

The main draw back with enzymes in industrial application is that the biotechnological production of enzymes are very expensive. Somehow, in affiliation with the recombinant DNA technology, in principle it has become possible to produce most of enzymes for a commercially viable price. Apart from that enzymes have many other limitations such as lack of long-term operational stability, high sensitive to process conditions, difficult recovery and re-use of enzymes. To overcome these challenges in the industrial utilization of enzymes immobilization techniques have been implemented (Nisha et al., 2012; Sheldon, 2007).

2.7.1 Enzyme Immobilization Methods

As a technique immobilization of enzymes have been tested and used since 1916, when Nelson and Griffin observed that the hydrolysis of sucrose is possible with invertase even after the enzyme is absorbed to charcoal (Nelson et al., 1916). Late in 1953 Grubhofer and Schelth have been able to report the covalent immobilization of several enzymes, their reuse and stability which reduce the production of enzymes over and over again. In addition to cost effectiveness, it gives more convenient handling to the enzyme, more facile separation from the product thus eliminate the enzyme contamination of the product, efficient recovery and reuse of the costly enzymes, enables the use in continuous, fixed-bed operation and most importantly it

provides enhanced stability under both storage and operational conditions towards denaturation by heat or organic solvents(Sheldon, 2007).

With the development of the nanotechnology, immobilization of enzymes on various nanomaterials is being developed.

2.7.1.1 Covalent Binding:

Covalent binding is the most traditional enzyme immobilization technique. This is gained by a direct attachment of the enzyme with the material via a covalent linkage. This covalent linkage is strong and stable. This technique is mainly used in an industrial process where it does not need the enzyme to be in the product. Covalent binding involves the activation of the supporting material with the addition of a reactive compound, which produces electrophilic groups on the supporting materials and the coupling those groups with strong nucleophiles on the proteins. This attachment in between the enzyme and the supporting material can be achieved via a direct linkage or a spacer arm. The advantage of using a spacer arm is that it provides a greater mobility to the enzymes when compared to the direct attachment.

2.7.1.2 Entrapment

This is rather a convenient and a traditional method of immobilizing enzymes. In this technique what really happens is that the enzymes are entrapped or occluded in a synthetic or a natural polymeric matrix while allowing the substrate and products to pass through. This is achieved by microencapsulation, fiber entrapping or by a gel. The advantages of this method is fast, cheap, the requirement of mild condition for the reaction and the protection of enzyme by microbial contamination via the supporting matrix. The disadvantage of this method is the limited mass transfer and the inactivation of enzyme during encapsulation(Bernfeld et al., 1963; Rosevear et al., 1987).

2.7.1.3 Adsorption:

This is the oldest and the easiest immobilization technique. Matrixes used for the adsorption are activated charcoal, Alumina and Ion exchange resins. This

immobilization is believed to be reversible. Enzyme molecules are adsorbed to the respective surface of the matrix through weak forces by salt linkage, hydrogen bonds, hydrophobic bonds, ionic bonds and van der Waals forces. Strength of these bonds could vary due to the charges of the matrix and the protein arrangement. The advantage of this technique is that additional activation steps are not required or minimally required thus chemical consumption is minimal, hence was used for this research

2.7.1.4 Ionic Binding

The enzyme and the matrix is bound through a salt linkage. This is a reversible immobilization technique and the immobilization process will be reversed by changing the polarity, temperature and the ionic strength. This method was also tested in this research.

2.7.1.5 Affinity Binding

In this method either enzyme or the substrate is activated in such a way that each will have bond like attraction to the other. There are two methods in affinity binding. First is the activation of the supporting material, in which the coupled affinity ligand had been added. To this enzyme is added, so that the enzyme will not be exposed to harsh chemical conditions. Second method is the modification of the enzyme with an affinity ligand so that it will have an affinity towards the supporting matrix.

2.7.1.6 Metal Linked immobilization

In this method metal salts are precipitated over the surface of the matrix so that the nucleophilic groups in the enzymes can bind with it. This is relatively a simple method and the activity of the immobilized enzyme is relatively high over 30-80%. This is a reversible process and the enzyme can be separated from the matrix by lowering the pH.

2.8 Phytase

Phytases [*myo*-inositol(1,2,3,4,5,6)hexakisphosphate phosphohydrolases] is an enzyme belongs to the histidine acid phosphatase family which have the capability to initiate the stepwise dephosphorylation of phytic acid [*myo*-inositol(1,2,3,4,5,6)hexakisphosphate] (Greiner et al., 2006; Liu et al., 2004). Generally, phytase behaves like a monomeric enzyme with molecular masses between 40 and 70 kDa (Konietzny et al., 2002).

Phytase enzyme was first discovered in rice bran by Suzuki et al. in 1907 and for the last fifty years scientists have been able to identify the existence many sources of the nature such as in plants, certain animal tissues and in microorganisms like bacteria, fungi and yeast.

Among them filamentous fungi has become one of the best sources of phytase for the use in feed industry due to the easy cultivation and high production of extracellular enzymes(de Antioquia et al., 2012). De Antioquia, M. A. *et al.* has reported the use of fungi in genera *Aspergillus*, *Penicillium*, *Mucor* and *Rhizopus* for the enzyme production. Other strains including *Cladosporium species*, *Myceliophthora thermophila*, *Emericella nidulans*, *Talaromyces thermophilus* and *Thermomyces lanuginosus* also have been reported for their potential for the phytase production.

Among these, *Aspergillus strains* have been reported to be the best for the extracellular phytase production as a feed additive. Generally, these strains are reported as health wise safe with the highest secretory potential for enzymes, particularly *Aspergillus niger*. Therefore, phytase enzyme produced from *Aspergillus niger* was used for this reaseach.

Phytase plays a major role during seed germination. It hydrolysis phytic acid in seeds and release phosphate and metallic cations bound to phytic acid required for the metabolism of the seed during the germination period. This natural phenomenon has influenced to use Phytase enzyme as a feed additive to utilize phosphate from phytic acid (Bohn et al., 2008).

2.9 Phytic acid (*Myo*-inositol (1,2,3,4,5,6) hexakisphosphate)

The structure of the inositol phosphates consist of an inositol ring and at least one phosphate group. In the name the prefix “*myo*” refers to the conformation of the hydroxyl groups on the inositol ring (Figure 2.6) and it is the major nutritionally relevant form of inositol. *Myo*-inositol (1,2,3,4,5,6) hexakisphosphate has 6 groups of phosphates which is esterified to the inositol ring (Figure 2.6) (Bohn et al., 2008).

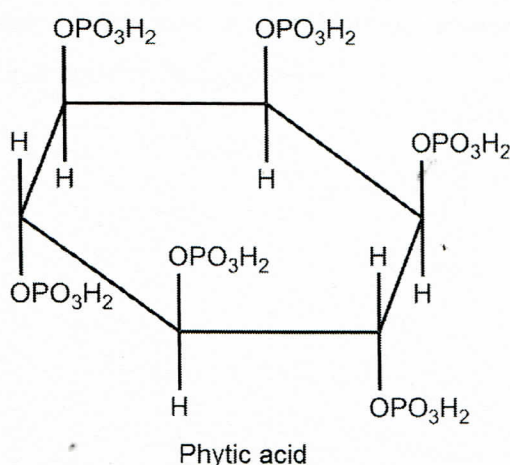


Figure 2.5: Phytic acid

Phytic acid is the primary storage form of phosphorus in most seeds and cereal grains, which contributes about 1-7% of the total dry weight (Cheryan et al., 1980) and 50%-80% of the total phosphorous content. Phytic acid amount in different grains, nuts legumes and seeds are highly varying due to the growth condition, harvesting condition and the age of the food that are being tested. In monocotyledonous seeds like wheat, rice, millet and barley phytic acid is concentrated in aleurone layer, whereas in corn more than 80% is concentrated in germ (Coulibaly et al., 2011). It is also found in roots and tubers in significant quantities in some plants (Omoruyi et al., 2013).

2.9.1 Phytic acid as an antinutritional factor

Phytic acid is generally known as an antinutrient due to its ability to chelate divalent metal ions such as Fe, Ca, Cu and Zn, precipitate and decrease its availability by

forming very insoluble salts at the pH of the gastrointestinal tract and exhibit very poor absorptivity at the gut (Ekholm et al., 2003). Apart from the multivalent metal ions the ability of phytic acid to complex with proteins and decrease its availability at the digestive tract has also been proven (Coelho et al., 2002). At lower and high pH proteins can make ions and salt bridges accordingly, that are able to bind directly with phytic acid through electrostatic charges, hence reduce the digestibility via two mechanisms; either by reducing the solubility of proteins or changing its structure to a form that makes steric hindrance to the binding mechanism of endogenous proteases thus reduce their activity in digestion.

CHAPTER 3

MATERIALS AND METHOD

All chemicals and reagents used in this study were analytical grade and purchased from Sigma-Aldrich, USA and used without further purification.

Crude phytase enzyme was purchased from Boading Faithful Industries Co. Ltd, China. The enzyme was originated from a fungal source *Aspergillus niger*.

All the characterizations were carried out in the laboratory of Sri Lanka Institute of Nanotechnology (SLINTEC), Sri Lanka.

3.1 Electrospinning apparatus (built in house)

Electrospinning apparatus consists of high voltage source (DC), electrodes, a syringe and a collector (Figure 3.1).

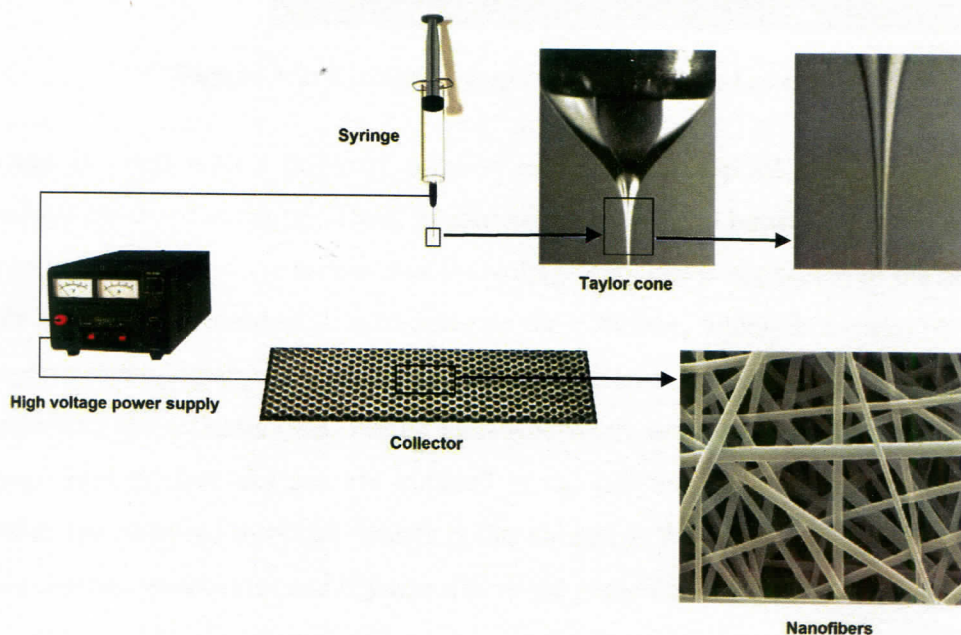


Figure 3.1: Major components of the electrospinning apparatus.

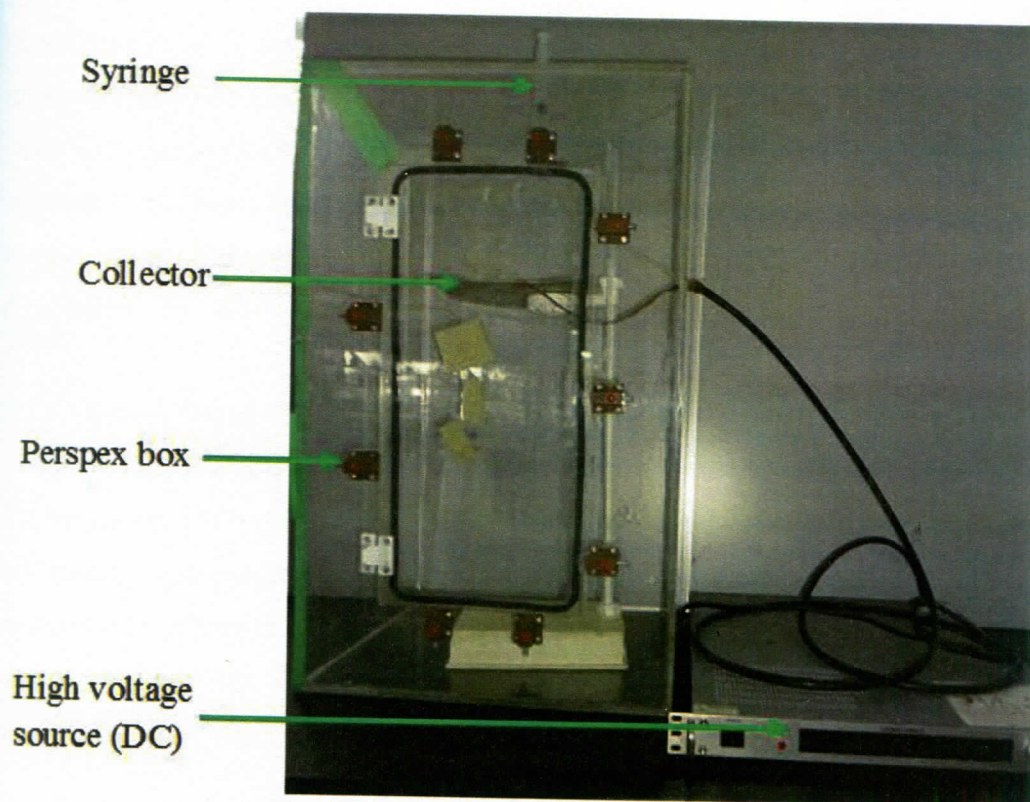


Figure 3.2: Electrospinning machine built in house

Syringe is filled with a polymer solution and force is applied manually, until a droplet is observed at the tip. Then, a high voltage is applied between the needle and the collector. The positive terminal of the voltage supplier is connected to the needle while the negative terminal is connected to the collector, which is a metal plate in this electrospinning apparatus (Figure 3.2). The distance between the two electrodes (needle and the collector) was varied until fine fibers are obtained. Due to the high voltage applied, free charges are induced in the polymer solution and they travel towards the negative terminal, which is the collector. While travelling through the space solvent evaporates and dry nanofibers are gathered on the collector.

3.2 Characterization Techniques

3.2.1 Fourier Transform Infrared Spectroscopy (FT-IR)

FT-IR characterization was carried out to determine the chemical structure and the bonding nature of the products. Bruker Vertex 80 coupled with Ram-FT module

(RAM II) Fourier Transform Infrared Spectrophotometer at SLINTEC was used for measurements. The spectra was obtained in a range of 600 to 4000 cm^{-1} . Attenuated Total Reflectance (ATR) FT-IR technique was used to obtain the spectra. Potassium bromide (KBr) beam splitter and RT-DTGS detector were used with a SiC globar sample source. Data were acquired and analyzed by instrumental software; OPUS-6.5 version.

3.2.2 Thermo Gravimetric Analysis (TGA)

The TGA thermograms in this study were obtained under nitrogen atmosphere using TA Instruments SDTQ600, at SLINTEC. Nitrogen gas purge flow rate was set as 100 ml/ minute. The sample (5-15 mg) was filled into an alumina pan and heated from ambient temperature to 1000 °C at a rate of 10 °C/min. Data were acquired by TA Q series, version 5.4.0. Thermograms were analyzed using universal analysis 2000, version 4.5.

3.2.3 Differential scanning calorimetry (DSC)

The DSC measurements were obtained under nitrogen atmosphere using TA Instruments SDTQ200, Nitrogen gas purge flow rate was set as 50 ml/minute. The sample (5-10 mg) was filled into a zero aluminium pan and heated from 0 °C to 1000 °C at a rate of 10 °C/minute. Data were acquired by TA Q series, version 5.4.0. Thermograms were analyzed using universal analysis 2000, version 4.5.

3.2.4 Scanning Electron Microscopy (SEM)

The particle size and the morphology of the samples were analyzed using HITACHI SU 6600 Scanning Electron Microscope at an accelerating voltage between 20-30 kV. The sample was mounted on to an aluminium stub using double-sided carbon tape. Then, to overcome the charging effect, the sample was sputtered with gold using HITACHI, E-1020 Ion Sputter. Then, the sample was introduced into the vacuum chamber of the SEM. The sample was subjected to SEM imaging using the scanning electron mode.

3.2.5 Energy Dispersive X-ray Spectroscopy

All the elemental analysis was done using Energy Dispersive X-ray (EDX) mode attached to HITACHI SU 6600 Scanning Electron Microscope. Sample preparation was done in a similar way as described in the section 3.1.4 and secondary electron mode was used for measurements.

3.2.6 UV-VIS Spectroscopy

UV-Vis spectrophotometer analysis were done using UV-Vis-NIR spectrophotometer (UV 3600, Shimadzu, Japan) in the absorbance mode. Absorption spectras were analyzed using UV-Prob 2.33.

3.3 Experimental Method

3.3.1 Characterization of Crude Phytase enzyme, Rice bran and poly vinyl alcohol (PVA).

Crude Phytase enzyme, rice bran and PVA was characterized using SEM, EDX, FT-IR, DSC and TGA techniques.

3.3.2 Extraction of phytase enzyme from the crude phytase source

Exactly 5.0 g of the crude phytase was taken and dissolved in 500.0 ml of deionized water in beaker. The solution was magnetically stirred (100 rpm) for 1 hour until a homogenous suspension was obtained. The solution was filtered using a filter paper, covered with an aluminium foil and stored at 4 °C (stock phytase enzyme solution).

3.3.3 Condition optimization for Phytase enzymatic assay

The best substrate concentration, enzyme concentration and incubation time required for the Phytase enzymatic assay were established.

3.3.3.1 Determination of required substrate concentration for enzymatic assay

Two phytic acid solutions were prepared to determine the required substrate concentration for the enzymatic assay. Exactly, 0.05 mmol dm⁻³ and 0.5478 mmol dm⁻³ phytic acid solutions were prepared using glycine buffer (pH 2.5). From each of

the solution 7.5 and 15 ml portions were taken in a reaction vial and kept in a water bath at 37 °C for 15 min. Separately, hundred fold diluted phytase enzyme solution was prepared from the stock solution (3.3.2) and four 0.5 ml aliquots were taken from the diluted solution and kept in a water bath at 37 °C for 15 min. After 15 min, phytase enzyme solutions were mixed with each of the phytic acid solutions incubated at 37 °C. Mixtures were incubated again at 37 °C and the reactions were terminated at 30 min and at 60 min, by adding 12.5 ml of vanadomolybdate reagent. The amount of generated from the enzymatic reaction was determined using the standard vanadomolybdate spectrophotometric method (Heinonen et al., 1981).

3.3.3.2 Determination of required the enzyme concentration for enzymatic assay

A hundred fold and a thousand fold diluted solutions of phytase enzyme were prepared from the stock solution (3.3.2) and three 0.5 ml aliquots from each of the solutions were kept in a water bath at 37 °C for 15 min. 0.5478 mmol dm⁻³ phytic acid solution was prepared using glycine buffer (pH 2.5) and six 15 ml aliquots from this solution were kept in a water bath at 37 °C for 15 min in a reaction vials. Incubated 0.5 ml phytase enzyme solutions and 15 ml of 0.5478 mmol dm⁻³ phytic acid solutions were mixed together and further incubated at 37 °C, keeping in a water bath. The reactions were terminated at 15 min, 30 min and 60 min by adding 12.5 ml of vanadomolybdate reagent. Next, the amount of inorganic phosphate content was determined (Heinonen et al., 1981).

3.3.3.3 Determination of the incubation time for the enzymatic assay.

A thousand fold diluted phytase enzyme solution (3.2.3.2) was prepared from the stock phosphate solution and 0.5478 mmol dm⁻³ phytic acid solution was prepared as in (3.2.3.1). Pre incubated 0.50 ml of the prepared phytase enzyme solution was mixed with 15 ml of 0.5478 mmol dm⁻³ phytic acid solution (pre incubated at 37 °C for 15 min) and the reaction system solution was again incubated at 37 °C, keeping in a water bath. Thirteen reaction vials prepared and incubated in this manner were terminated at 5 min intervals between 5 min and 60 min of the reaction time and then

after 90 min of the reaction by adding 12.5 ml of vanadomolybdate reagent and the amount of inorganic phosphate resulted was quantified (Heinonen et al., 1981).

3.3.4 Determination of the activity of phytase enzyme.

A thousand fold diluted phytase enzyme solution and a 0.5478 mmol dm⁻³ phytic acid solution were prepared using glycine buffer (pH 2.5) and kept in a water bath at 37 °C for 15 min. Exactly, 0.5 ml of phytase enzyme solution was mixed with 15 ml of 0.5478 mmol dm⁻³ phytic acid solution and again incubated at 37 °C for 20 min. The reaction was terminated by adding 12.5 ml of vanadomolybdate reagent. Next, the amount of inorganic phosphate content was determined (Heinonen et al., 1981).

3.3.5 Preparation and processing of rice bran for fiber synthesis

Rice bran was obtained from a local rice mill. Then, rice bran was oven dried at 60 °C until a constant weight is obtained to minimize the moisture present. The oven dried rice bran was sieved through a 500 µm mesh screen (STIEVE-TRONIC model AO59-12 with 50 Hz vibration) to remove any remaining rice husks, seeds and other contaminants. Sieved rice bran (fiber material) was packed under vacuum (ITECO glove box, model sgs 20) for further use.

3.3.6 Pretreatment of rice bran

3.3.6.1 Defatting

Defatting was done using diethyl ether at a ratio of 1:10 (w/v). Diethyl ether was added to rice bran in a beaker and the solution was magnetically stirred for 15 min. Then, the mixture was allowed to settle and the liquid fraction containing fat was carefully removed. This process was repeated two times to ensure complete defatting and the residue was allowed to stand for few minutes to remove any remaining diethyl ether.

3.3.6.2 Alkali treatment

Defatted fiber material was suspended in water at a ratio of 1:10 (w/v) (Fung et al., 2010). Then, pH was adjusted to 9 with 1 mol dm⁻³ NaOH (aq) using a pH meter

(OHAUS STARTER 3000 bench pH meter). The solution was heated at 60 °C with stirring for 60 min and allowed to cool to room temperature (Fung et al., 2010). Solution pH, conductivity (OHAUS STARTER 3000C Conductivity meter), and viscosity (Fungi lab ALPHA SERIES rotational viscometer version 1.2) were measured.

To separate the base soluble dietary fiber fraction, the solution was centrifuged for 1 h at 9000 rpm rotation. After centrifugation, fiber supernatant was decanted carefully to a container, sealed under nitrogen environment and kept at 4 °C until further use.

3.3.7 Condition optimization for electrospinning

3.3.7.1 Solution viscosity

The viscosity of soluble dietary fiber fraction was not sufficient for electrospinning. Therefore, solutions with various viscosities were made by amending with various PVA percentages (6, 7 and 8% by weight) and the best composition (weight to volume percentage) was identified.

3.3.8.2 Applied voltage

Applied voltage was optimized to find the lowest voltage needed to form a Taylor cone. Voltage was increased from 15 kV at 1 kV intervals until the Taylor cone was formed at the end of the needle tip.

3.3.8.3 Distance between two electrodes

Distance between the two electrodes was set to 10 cm, 15 cm and 20 cm and fibers were made accordingly. Fiber morphology was observed with the use of scanning electron microscope and the suitable electrode distance was identified.

3.3.9 Production of nanofibers using electrospinning

The supernatant containing soluble fibers were electrospun to nanofibers. For this, fiber supernatant was amended with PVA at a concentration of 8 % (w/v) to gain the required viscosity. The temperature was increased to 100 °C to completely dissolve

PVA with stirring until a homogeneous solution was obtained. Then, the solution was left to cool to 30 °C. The viscosity and pH were measured. The solution was filled in to a syringe and fixed in to electrospinner, built in house (3.1).

Ground electrode (collector plate) was covered with an aluminum foil and electrospinning parameters were set as follows: tip-collector distance, 15 cm and voltage 20 kV. Flow rate was set manually until a Taylor cone was observed. After electrospining, fibers were collected carefully and packed under vacuum.

3.3.9.1 Characterization of nanofibers and phytase enzyme encapsulated nanofibers.

Nanofibers and all types of phytase enzyme encapsulated nanofibers were characterized using SEM, EDX, FT-IR, DSC and TGA techniques.

3.3.10 Production of phytase enzyme encapsulated nanofibers

Two different techniques were investigated to encapsulate phytase enzyme in to the nanofibers synthesized from rice bran and tested for thermal stability in this research.

1. Cross-linked Phytase enzyme encapsulated nanofibers.
2. Cross-linked, surface modified nanofibers with phytase enzyme.

3.3.11 Production of phytase enzyme encapsulated nanofibers

The supernatant containing soluble fibers was prepared following the procedure (3.3.6). Then, 40 µl of phytase enzyme from the stock phytase enzyme solution was added to 10 ml of fiber solution in a beaker and stirred well until a homogeneous suspension was obtained. Then the solution was filled to the syringe and fibers were drawn using the electrospinning apparatus. After electrospining, fibers were collected carefully and packed under vacuum.

3.3.11.1 Production of cross-linked Phytase enzyme encapsulated nanofibers.

Synthesized nanofibers in 3.2.11 were dipped in a 4% boric acid solution for 30 s and sequentially in three deionized water baths for 1 min in each, until all the



remaining boric acid was removed from the film. The resulted fibers were dried in a vacuum oven for 12 h at 30 °C and stored at 4 °C.

3.3.11.2 Production of cross-linked nanofibers with boric acid.

Nanofibers synthesized in 3.3.9 were cross-linked following the method mentioned in 3.3.11.1 (Cross-linked nanofibers with boric acid were produced as the control fibers (control experiment) for cross-linked Phytase enzyme encapsulated nanofibers).

3.3.11.3 Determination of the activity of cross-linked Phytase enzyme encapsulated nanofibers.

10.0 mg, of the synthesized cross-linked Phytase enzyme encapsulated nanofibers in a reaction vial was kept in a water bath at 37 °C for 15 min. 0.5478 m mol/dm³ phytic acid solution prepared using glycine buffer (pH 2.5) was also kept in a water bath at 37 °C for 15 min. 15 ml of this phytic acid solution was added to dietary fibers and incubated at 37 °C for 20 min. The reaction was terminated by adding 12.5 ml of vanadomolybdate reagent and the amount of inorganic phosphate resulted was quantified (Heinonen et al., 1981).

3.3.12 Production of cross-linked surface modified nanofibers with phytase enzyme

About 350 mg of nanofibers (3.3.9) were dipped in a phytase enzyme solution (10 fold diluted) and kept in a shaker (100 rpm) for 1 h. Then the, excess enzyme was drained off. In order to cross-link, sodium tripolyphosphate (STPP) solution (1.5 w/v %) was added to the fibers, drop wise for 5 min. The excess STPP was washed off thoroughly by dipping in three deionized water baths for 1 min in each. The resulted fibers were dried in a vacuum oven for 12 hours at 25 °C and stored at 4 °C.

3.3.12.1 Production of cross-linked nanofibers with STPP

Nanofibers synthesized in 3.3.9 were cross-linked by adding STTP solution (1.5 w/v %) drop wise for 5 min. Excess STPP was washed dipping in three deionized water

baths for 1 min in each (cross-linked nanofibers with STPP were produced as the control fibers (control experiment) for cross-linked surface modified nanofibers with phytase enzyme).

3.3.12.2 Determination of the activity of cross-linked surface modified nanofibers with phytase enzyme

5.0 mg from the synthesized cross-linked surface modified nanofibers with phytase enzyme was weighed in a vial and kept in a water bath at 37 °C for 15 min. 0.5478 mmol dm⁻³ phytic acid solution prepared using glycine buffer (pH 2.5) was kept in a water bath at 37 °C for 15 min. 15 ml of 0.5478 mmol dm⁻³ phytic acid solution was mixed with cross-linked surface modified nanofibers with phytase enzyme and incubated at 37 °C for 20 min. The reaction was terminated by adding 12.5 ml of vanadomolybdate reagent. The amount of inorganic phosphate resulted from the enzymatic reaction was determined using the standard vanadomolybdate spectrophotometric method (Heinonen et al., 1981).

3.2.12.3 Determination of the thermal stability of cross-linked surface modified nanofibers with phytase enzyme

The synthesized cross-linked surface modified nanofibers with phytase enzyme (5.0 mg) and equal amount of free enzyme were subjected to 50 to 170 °C temperatures in 10 °C intervals, keeping in a water bath (up to 100 °C) a mineral oil bath (above 100 °C) respectively, for 5 min. After 5 min, the samples were cooled to room temperature and the activity was measured as mentioned in 3.3.12.2.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Characterization of phytase enzyme.

Crude enzyme used in this work is in the powder form and a photograph of that is shown in Figure 4.1

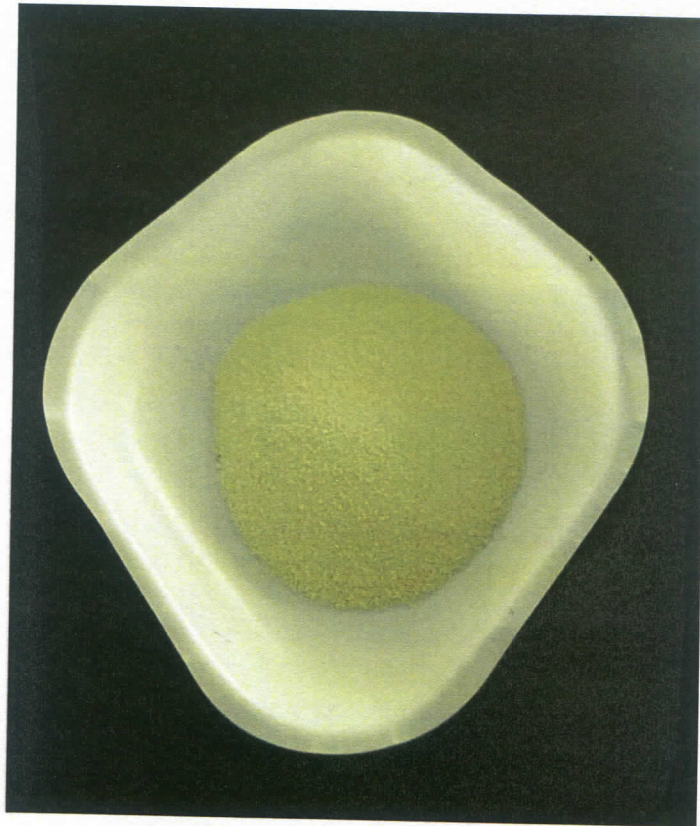


Figure 4.1: Crude phytase

(a) Scanning electron microscopy (SEM)

SEM micrograph of the crude phytase enzyme shows irregular shape particles in the range of 30 – 50 μm . Uniformity between particle size and shape was not seen in the sample (Figure 4.2.).

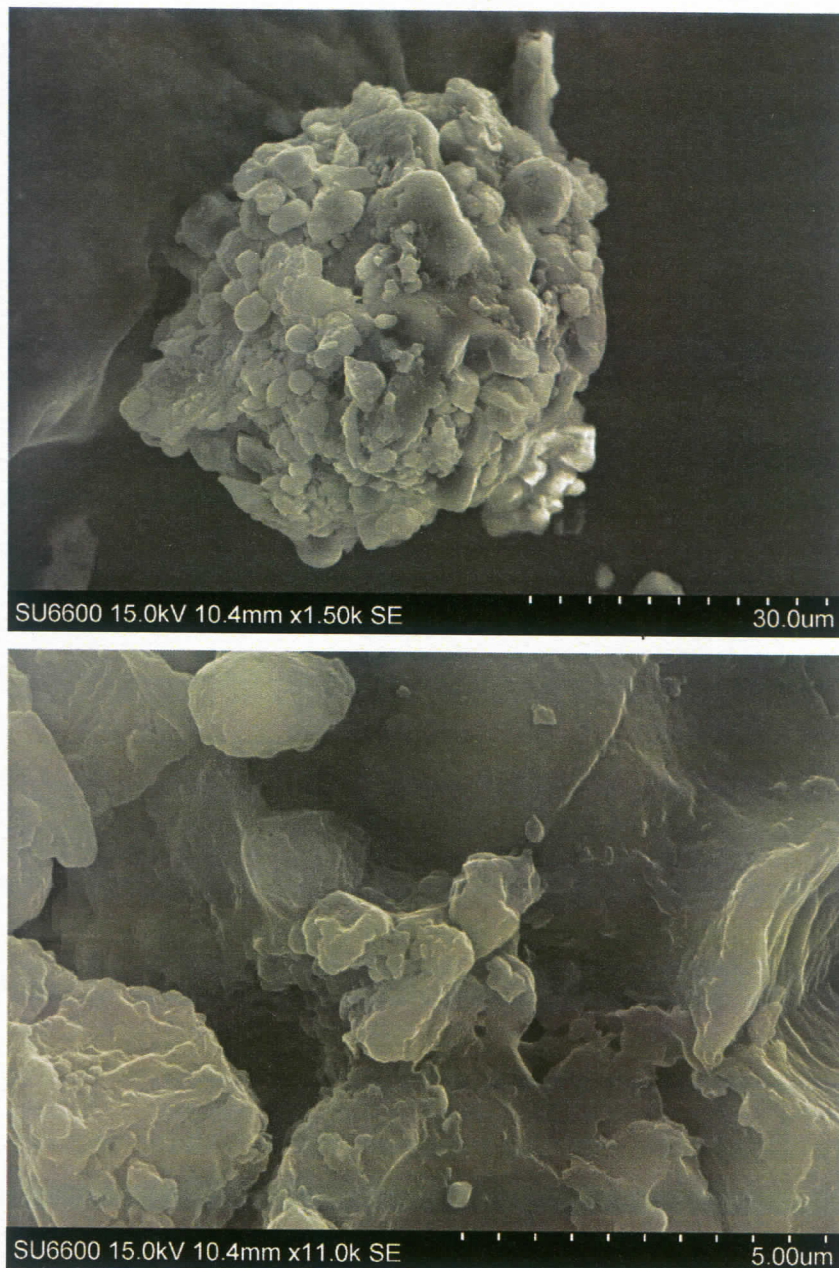


Figure 4.2: Scanning Electron Microscopic image of crude phytase enzyme

(b) Energy-dispersive X-ray spectroscopy (EDX).

In a very pure enzyme C, H, N and O are the major components. However, depending on the type of enzyme, some metal ions can also be expected which are working as cofactors.

Table 4.1: Elemental composition of crude phytase enzyme

Element Line	Weight %	Atom %
C	27.29 ± 0.22	31.84
N	46.34 ± 0.57	46.36
O	23.37 ± 0.25	20.47
Mg	0.63 ± 0.01	0.36
Al	0.02 ± 0.01	0.01
Si	0.07 ± 0.01	0.03
P	1.15 ± 0.02	0.52
K	1.12 ± 0.02	0.40

The elemental composition and weight percentage of the crude enzyme are listed in Table 4.1.

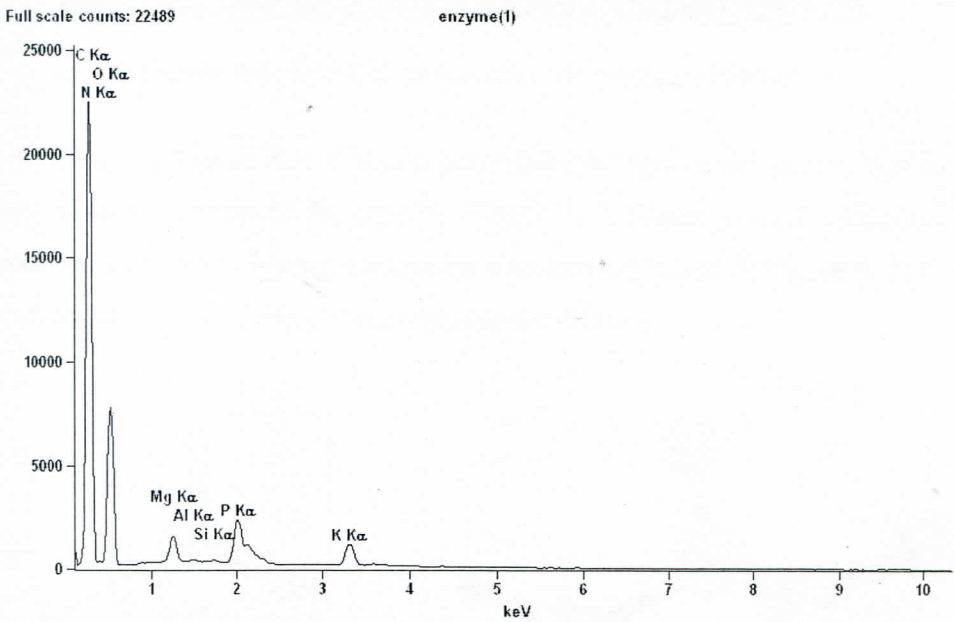


Figure 4.3: EDX Spectrometric data of crude phytase enzyme

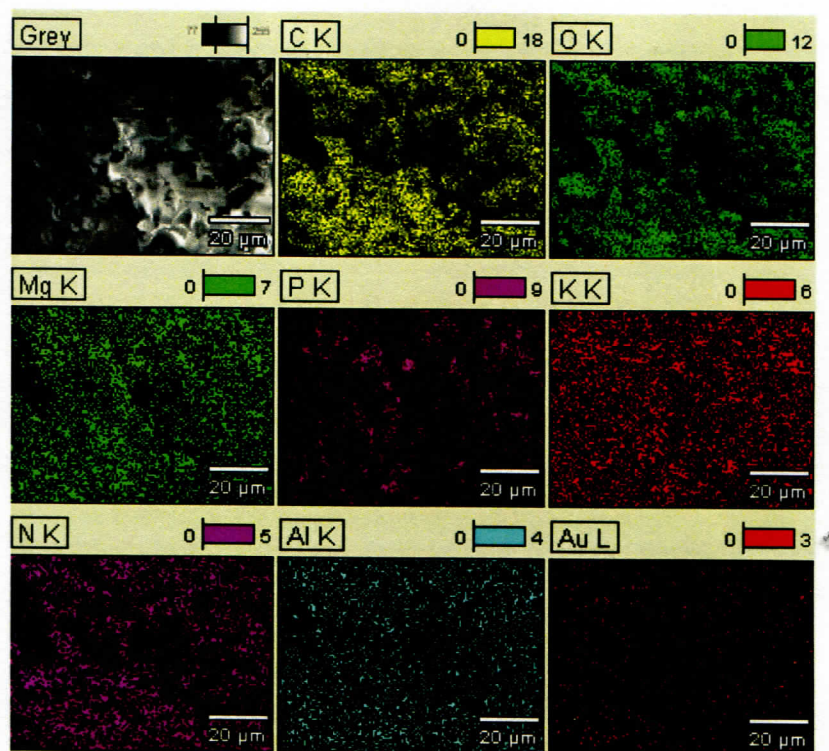


Figure 4.4: Spectral image of crude phytase enzyme

As can be seen in Figure 4.3, nitrogen percentage is high in the sample due to the presence of amino groups in the enzyme (Figure 4. 3, Figure 4. 4). An enzyme is a sequence of amino acids having a common structure explained in Figure 4. 5, thus a high carbon, nitrogen and oxygen percentages are obvious.

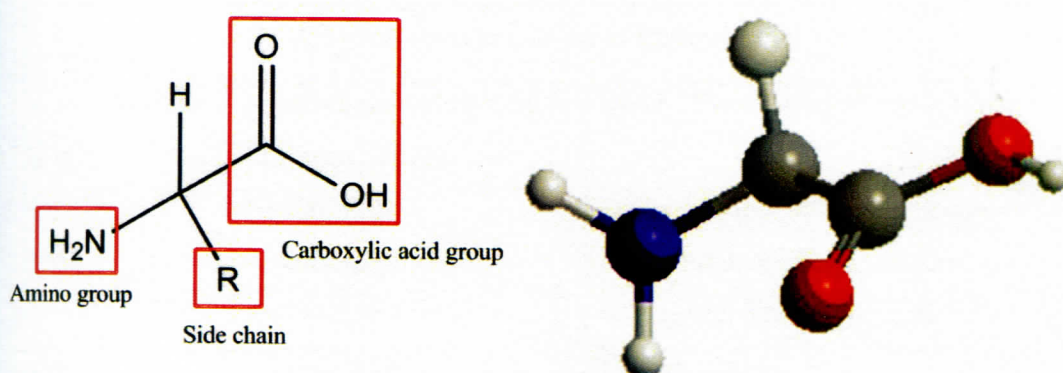


Figure 4.5: Common structure of an amino acid

Apart from N, C and O the EDX analysis shows a considerable amount of Mg, Al, Si, P and K in the crude phytase enzyme. This may be mainly due to presence of impurities in the crude enzyme used in this work.

(c) Fourier Transform Infrared Spectroscopy (FT-IR)

The FT-IR spectrum obtained for crude enzyme is shown in Figure 4.6 and Table 4.2 presents the FT-IR peak interpretation of phytase enzyme.

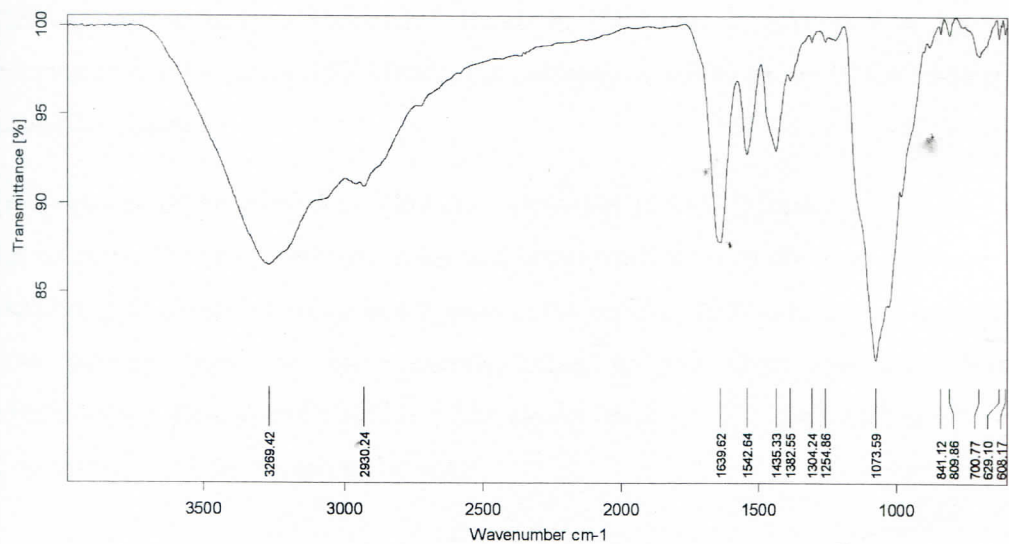


Figure 4.6: FT-IR Spectrum of phytase enzyme

Table 4.2: FT-IR peak interpretation of phytase enzyme

FT-IR peak interpretation of Phytase ensyme		
Wave number (cm ⁻¹)	Reference values (cm ⁻¹) (Yadav, 2013)	Bond / Functional group vibration
3269	3500-3200	O-H stretching (alcohols, phenols)
	3400-3250	N-H stretch amines or amides
	3300-2500	O-H stretch carboxylic acid
2930	3000-2850	C-H stretching
1639	1690–1600	Amide C=O Stretch
1542	1575 - 1480	NH Bending of amides

1435	1500–1400	C–C stretch (in–ring) aromatics
1304	1320–1000	C–N stretching of amine groups
1073	1320–1000	C–O stretch alcohols, carboxylic acids

The peptide and protein repeat units in enzymes give rise several characteristic IR absorption bands. The most prominent of them in the protein secondary structural component is the C=O stretch vibrations of the peptide linkages, which can be attributed to the band at 1639 cm^{-1} . Bands at 1542 cm^{-1} is attributed to the N-H Bending of amides (table 4.2). Hence, the presence of amino group in the enzyme is further confirmed.

The presence of broadband at 3269 cm^{-1} attributes to O-H stretching. This may be due to both –O-H in carboxylic acids and water (moisture) in the crude enzyme. In addition N–H stretching of amines appear in the region $3300\text{--}3000\text{ cm}^{-1}$. They might have overlap with the band corresponding to the O-H stretching, hence differentiating them is not possible. The strong band at 1073 cm^{-1} attributes to the C–O stretch alcohols or carboxylic acids.

(d) Differential scanning calorimetry (DSC)

DSC thermogram of the phytase enzyme shows an endotherm at $120\text{ }^{\circ}\text{C}$ (217.3 J/g) which most probably corresponds to the melting of the crude enzyme (Figure 4.7). In DSC thermograms sharp narrow melting endotherms correspond to the purity of the sample. The broadness of the peak corresponds to the presence of substantial amount of impurities and that is acceptable since the enzyme was crude, which can have unfermented substrate left behind.

Deactivation of an enzyme happens in two steps. First step is denaturation in which breaking of H bonds in the secondary and tertiary structure of an enzyme. This is principally a reversible and an endothermic process. The small endotherm at $59\text{ }^{\circ}\text{C}$ (0.17 J/g), correspond to this denaturation (De Maria et al., 2011). Second step is degradation and the endotherm at $229\text{ }^{\circ}\text{C}$ (64 J/g) most probably corresponds to the

degradation of phytase enzyme, since after that the heat capacity of the sample had increased to a great extent.

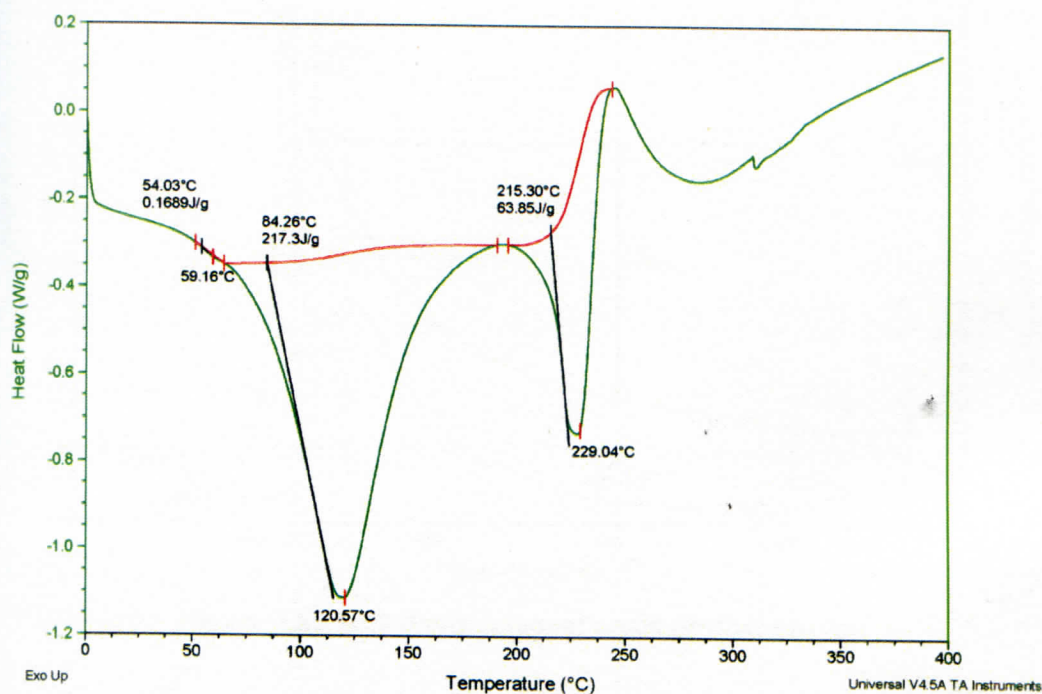


Figure 4.7: DSC thermogram of crude phytase enzyme

(e) Thermal gravimetric analysis (TGA)

Phytase enzyme shows thermal decomposition in the temperature ranges of 34-154 °C, 166-271 °C, 271-609 °C and 609-1000 °C (Figure 4.8).

Weight loss (11%) corresponding to the temperature range of 34-154 °C might be due to loss of moisture and other volatile components. From 166 to 609 °C there is a rapid weight loss (53%) that could correspond to the degradation of organic components. This could not be due to oxidation; because oxidation is not possible under nitrogen environment. Hence, it is possible to assume that the enzyme is physically stable up to 165 °C. At 1000 °C, there was about 26 % weight which could be char and other inorganic substances under nitrogen environment.

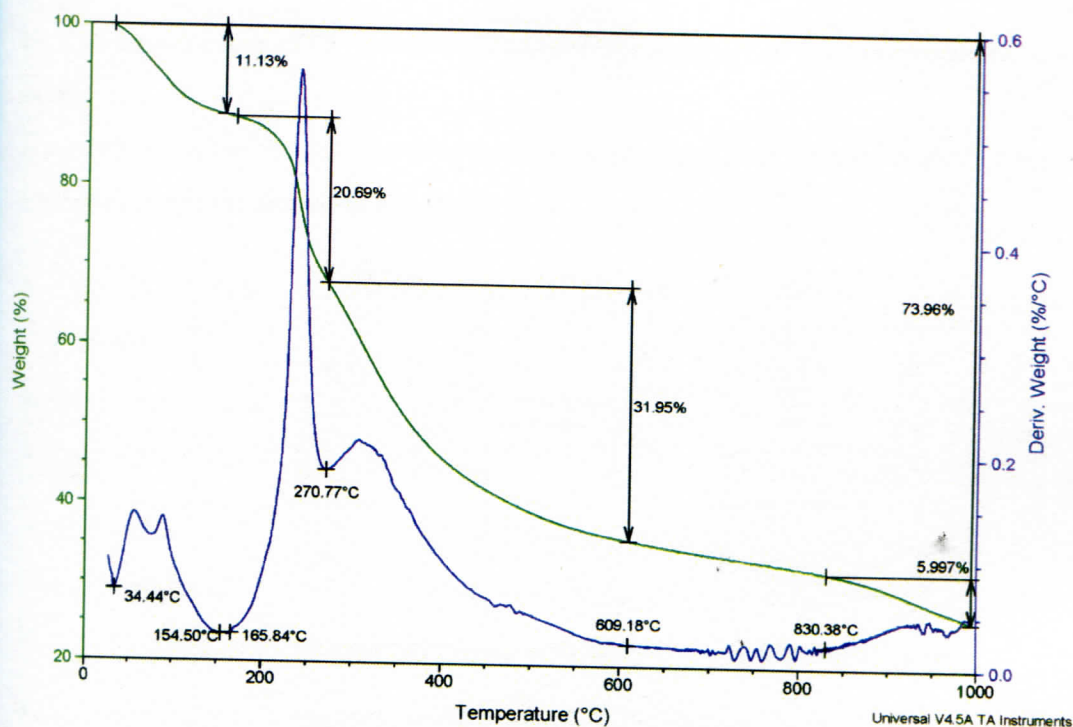


Figure 4.8: TGA thermogram of crude phytase enzyme

4.1.1 Extraction of phytase enzyme from a crude phytase source

Determination of the activity of the enzyme is the most important step in this research. Since any change of the activity will assess the effectiveness of the immobilizing technique. Any decrease of the activity under the influence of temperature will access the thermal stability of the enzyme.

Deionized water was used in sample preparation considering the possibility of enzyme inhibition by some metal ions. One hour stirring was required to ensure the complete dissolution of the enzyme. The stirring speed was limited only to 100 rpm to avoid any heat generation which can cause the denaturation of the enzyme. The solution was filtered to remove the unfermented particles, which can interfere the fiber formation process as well as the activity establishment.

4.1.2 Determination of the substrate concentration required for the enzymatic assay

The mechanism for the enzymatic hydrolysis of phytic acid by phytase enzyme from *Aspergillus niger* is shown in Figure 4.9.

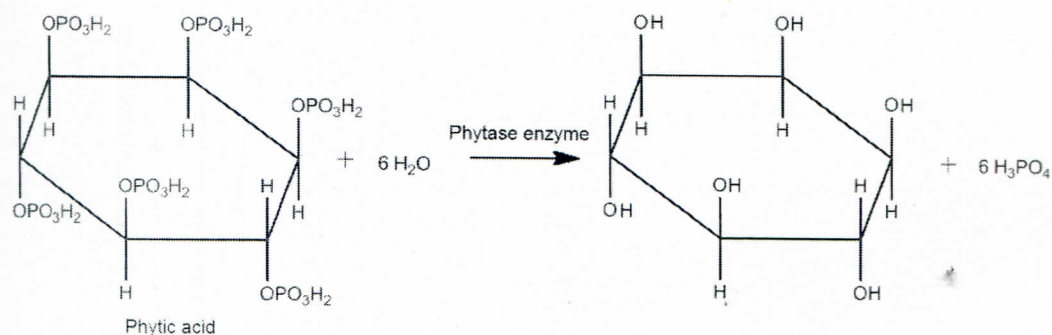


Figure 4.9: Mechanism of phytic acid hydrolysis by phytase enzyme

When phytic acid is enzymatically reacted with phytase enzyme six inorganic phosphate molecules are released. This inorganic phosphate is determined (3.2.3.1). If the substrate concentration is very low, phosphate content will be very low to be detected by the spectrophotometer and if the substrate concentration is very high the time required for a particular amount of enzyme to complete the reaction will be considerably high, and the absorbance values will be beyond the calibration level. Therefore, determination of the correct amount of substrate concentration is very important for the enzymatic reaction.

In this work, phytic acid was prepared in glycine buffer at pH-2.5 and the enzymatic assay was carried out at the same pH. That is because the ultimate objective is to synthesize Phytase enzyme stabilized nanofibers with a dietary value and hence the enzyme should be stable at stomach pH which is around 2.5.

According to Figure 4.10, the enzymatic reaction conducted using 7.5 ml and 15 ml, 0.05 m mol/dm³ phytic acid has not resulted considerable amount of phosphate. Further, there is no significant increase of the phosphate release even at 60 min indicating that the reaction had already been completed by 30 min (Figure 4.10).

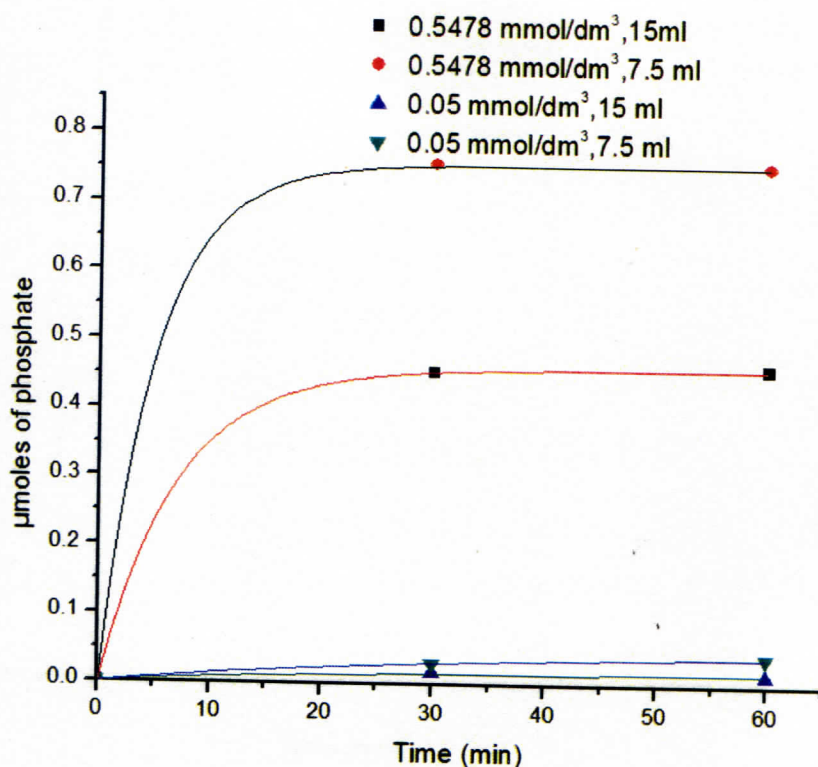


Figure 4.10: Amount of phosphate released at different phytic acid initial concentrations

Both 7.5 ml and 15 ml, 0.5478 mmol dm⁻³ phytic acid solutions gave a substantial amount of phosphate at 30 and 60 min. From the two samples with two different volumes, 15 ml sample gave a highest amount of phosphate. Hence 15 ml and 0.5478 mmol dm⁻³ were selected as the best sample size and the concentration of the phytic acid solution for the enzymatic assay.

4.1.3 Determination of required enzyme concentration required for the enzymatic assay

Inorganic phosphate liberation as a result of enzymatic hydrolysis of phytic acid (0.5478 mmol dm⁻³), corresponding to several initial phytase enzyme concentrations (100 fold and 1000 fold diluted from the stock solution) is shown in Figure 4.11.

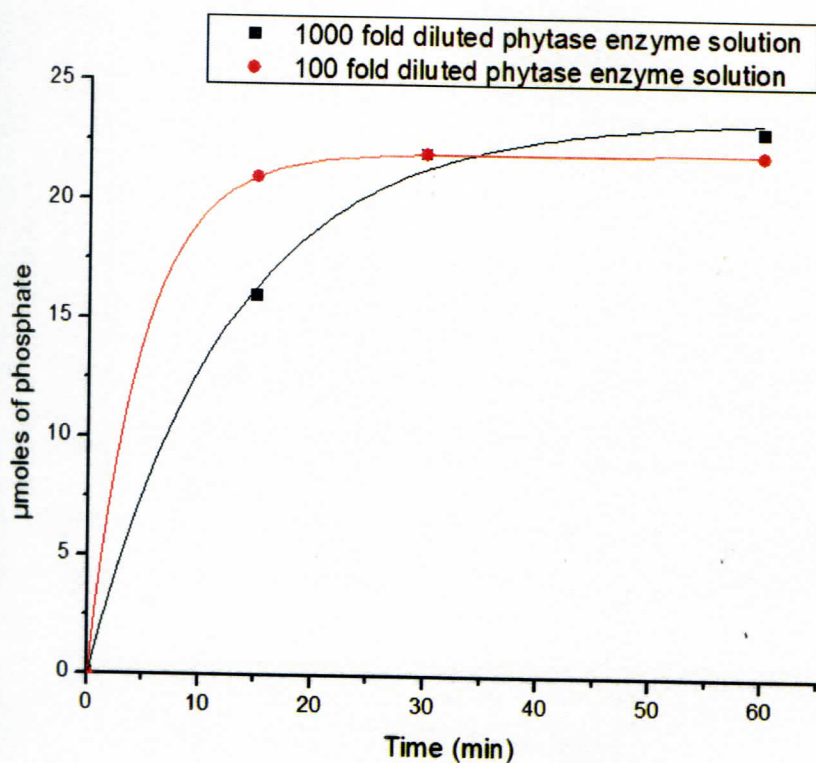


Figure 4.11: Amount of phosphate released at different initial phytase enzyme concentrations

According to Figure 4.11 a hundred fold diluted phytase enzyme sample has completed the reaction even before 20 min. While thousand time diluted sample completed the reaction after 45 min.

For an enzymatic assay, a measurable time is required to minimize experimental and measuring errors that can happen. Therefore a thousand fold diluted enzyme solution was selected as the best concentration for the enzymatic assay.

4.1.4 Determination of the incubation time for the enzymatic assay.

The absorbance corresponding to the amount of inorganic phosphate liberated as a consequence of the enzymatic hydrolysis of phytic acid ($0.5478 \text{ mmol dm}^{-3}$) at different time intervals, is shown in Figure 4.12.

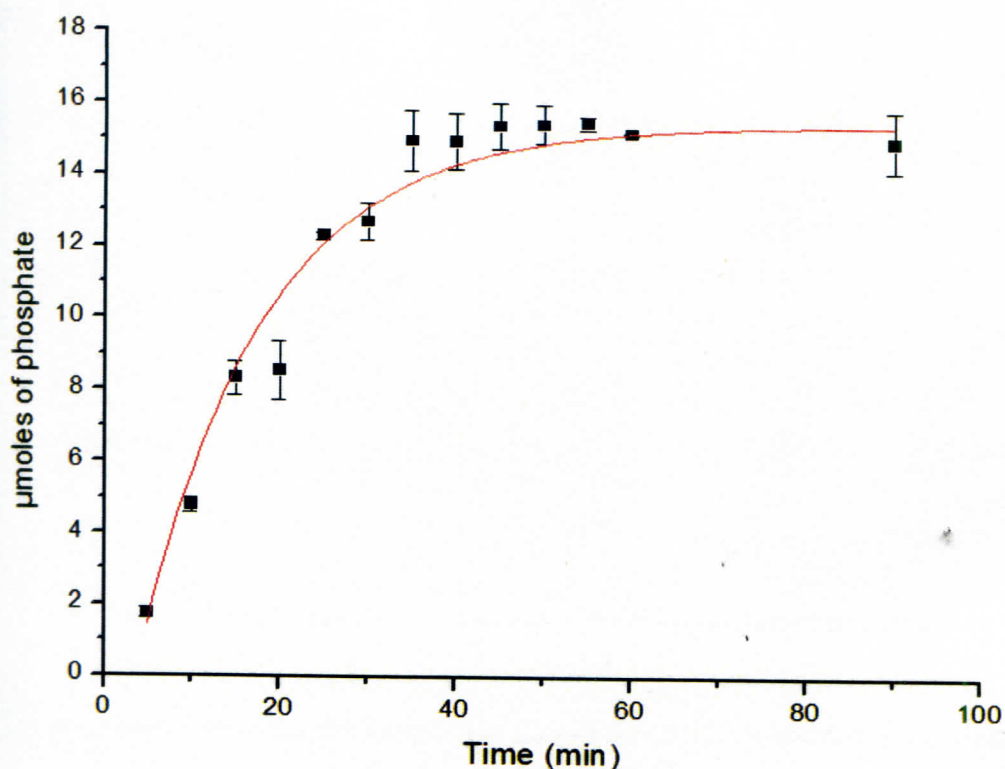


Figure 4.12: Phytic acid hydrolysis at different incubation times

As can be seen, the absorbance becomes constant after 50 min indicating phytic acid hydrolysis has completed after 50 min. After 25 min the reaction rate decelerated due to decrease in substrate concentration. Hence the time required for the enzymatic reaction was decided as 20 min (Figure 4.12).

4.1.5 Determination of the activity of phytase enzyme

The activity of the phytase enzyme was calculated using the following formula (Applegate, 2004).

$$\text{Units/ml enzyme} = \frac{(\text{Phosphate released} / \mu\text{moles}) (\text{Dilution factor})}{(\text{Time} / \text{min})(\text{Volume of enzyme used} / \text{ml})}$$

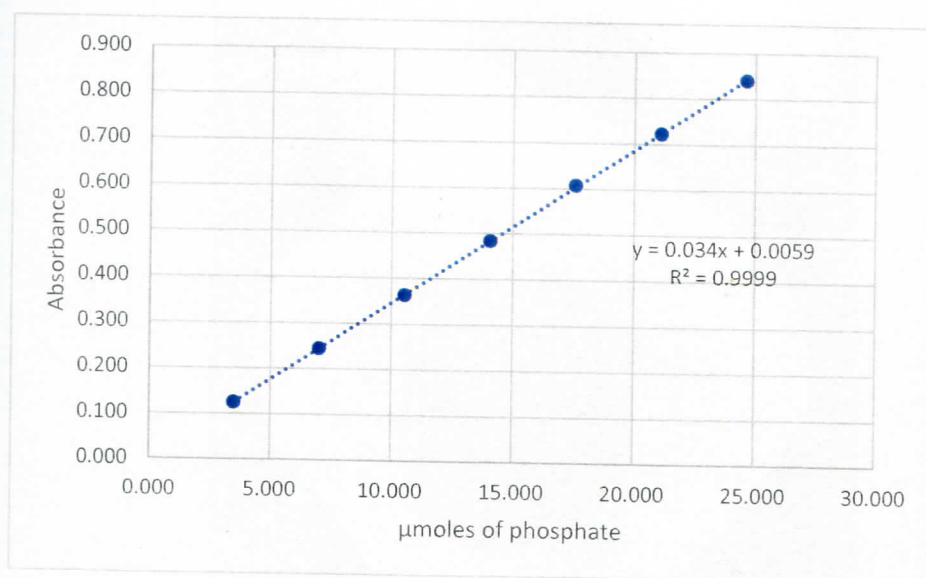


Figure 4.13: Calibration plot of absorbance vs amount of phosphate

The absorbance value for the experiment (3.3.4) was 0.390. Therefore the activity of the stock enzyme solution was 1129.7 units/ml.

Similarly the activity of the crude enzyme was calculated using the following formula.

$$\text{Units/mg solid} = \frac{\text{units/ml enzyme}}{\text{mg solid/ml enzyme}}$$

and was found as 1.1297 units/mg.

4.2 Preparation of dietary fiber using rice bran

4.2.1 Preparation and characterization of rice bran

Rice bran purchased from a local rice mill is shown in Figure 4.14. It is important to dry rice bran since moisture can make the rice bran susceptible for fungal and mold attacks during storage. Sieving of rice bran removes any seeds, husks and other contaminants which can disturb the formation of fibers and also enhances the efficiency of alkaline treatment.



Figure 4.14: Sieved dried rice bran

(a) Scanning electron microscopy (SEM)

SEM images of rice bran is shown in Figure 4.15 and it is composed of particles with an average diameter of 100-250 μm .

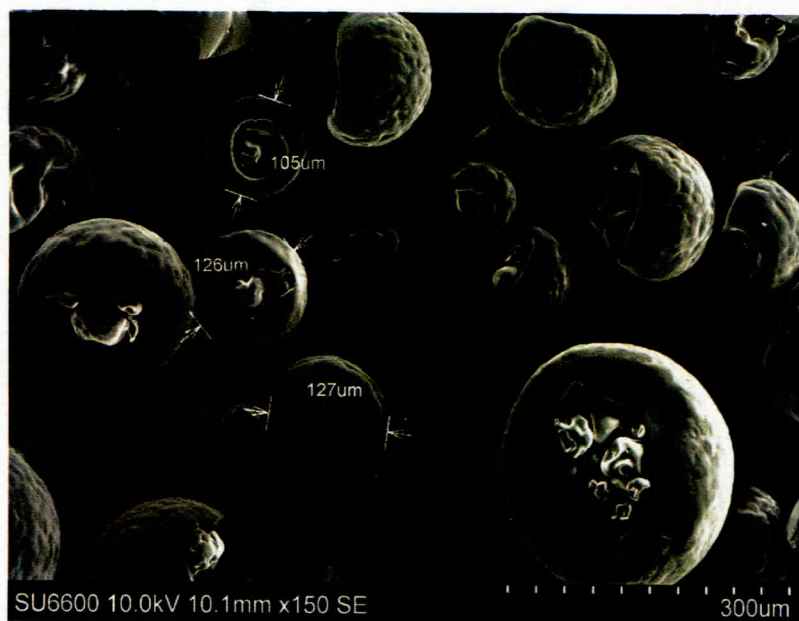




Figure 4.15: Scanning Electron Microscopic images of rice bran

(b) Energy-dispersive X-ray spectroscopy (EDX).

From the EDX data the elemental composition of rice bran can be found, hence the nutritional composition of the rice bran can be estimated.

Table 4.3: Elemental composition of rice bran

Element Line	Weight %	Atom %
C	40.28 ± 0.64	49.37
O	49.84 ± 0.36	45.86
Mg	2.03 ± 0.05	1.23
Al	0.19 ± 0.02	0.11
P	2.65 ± 0.08	1.26
S	3.54 ± 0.08	1.62
K	1.47 ± 0.03	0.55

The elemental composition and the weight percentage of rice bran is listed in Table 4.3.

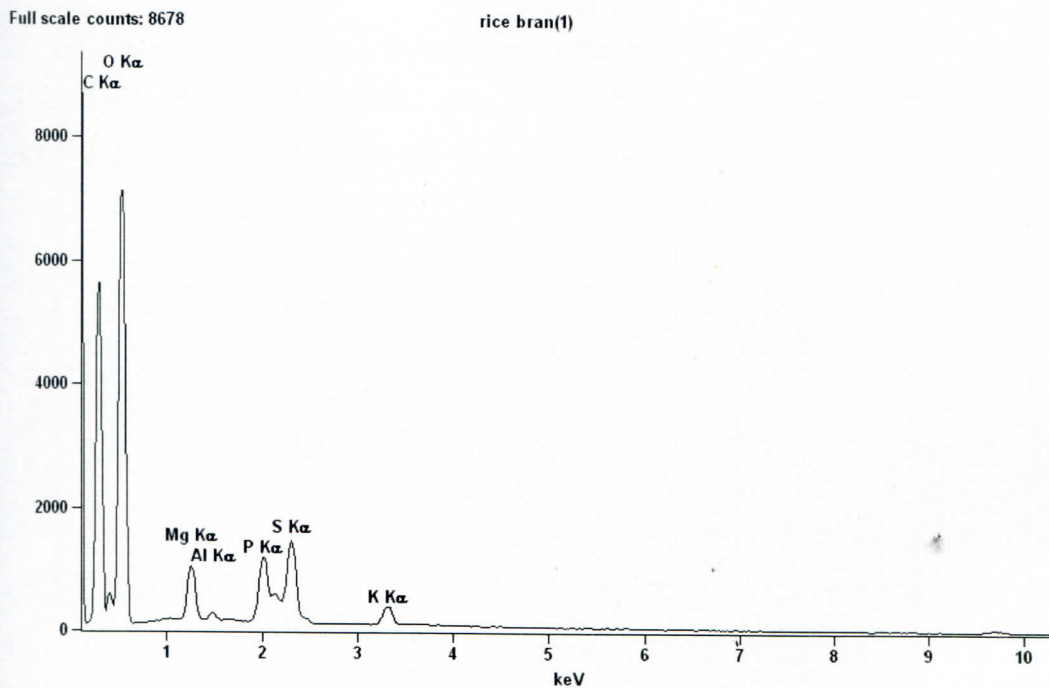


Figure 4.16: EDX Spectrometric data of rice bran

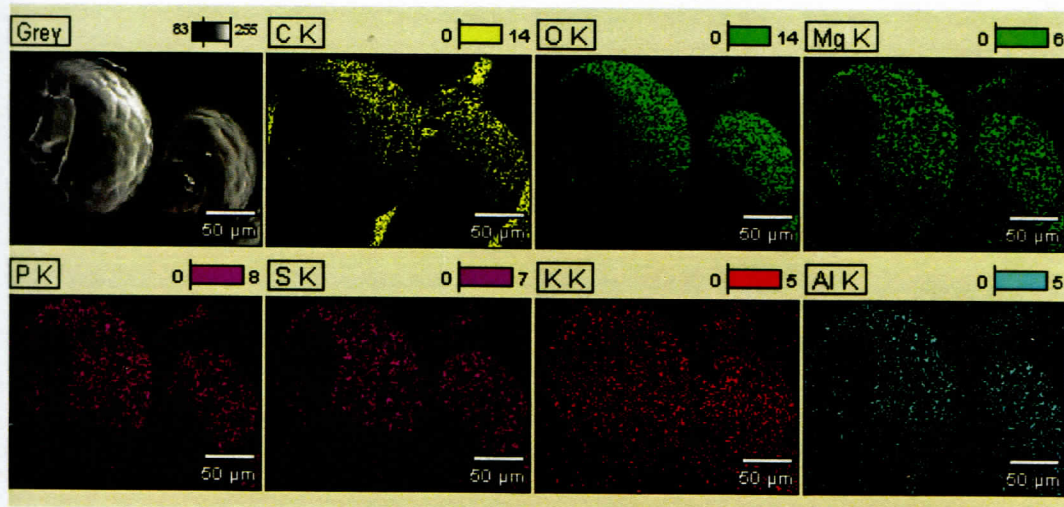


Figure 4.17: Spectral image of rice bran

According to the EDX analysis (table 4.3 and Figure 4.16) rice bran is a good source of Mg, Al, S, P, K thus a suitable dietary mineral source.

(c) Fourier Transform Infrared Spectroscopy (FT-IR)

The FT-IR spectrum obtained for rice bran is shown in Figure 4.18 and Table 4.4 presents the FT-IR peak interpretation of rice bran.

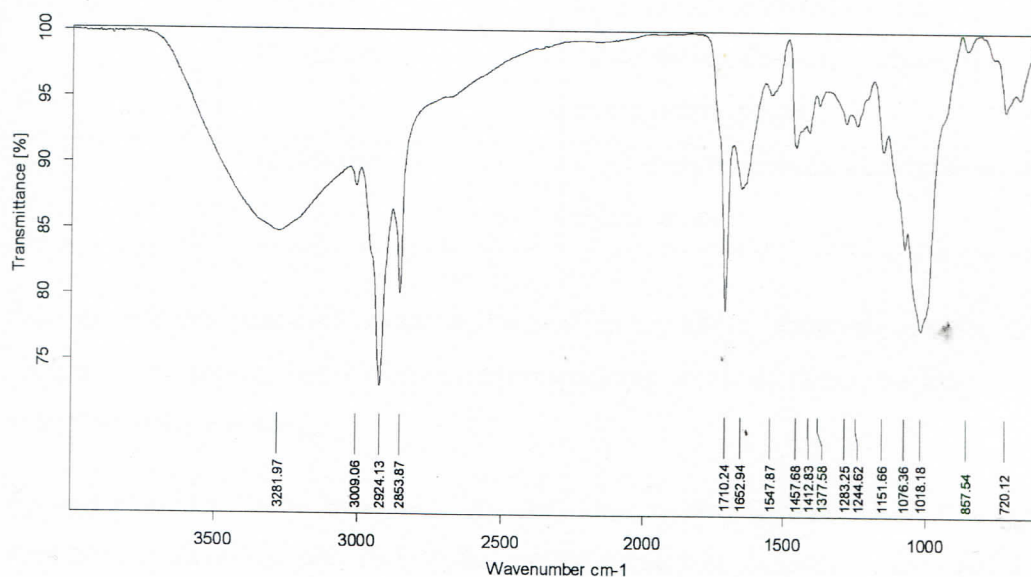


Figure 4.18: FT-IR Spectrum of rice bran

Table 4.4: FT-IR peak interpretation of rice bran

FT-IR peak interpretation of rice bran		
Wave number (cm ⁻¹)	Reference values (cm ⁻¹) (Yadav, 2013)	Bond / Functional group vibration
3281	3500–3200 3400–3250	O–H stretch, H–bonded alcohols, phenols, N–H stretch 1°, 2° amines, amides
3009	3100–3000 3100–3000	C–H stretch aromatics =C–H stretch alkenes
2924	3000–2850	C–H stretch alkanes
2853	3000–2850	C–H stretch alkanes
1710	1710–1665	C=O stretch α, β unsaturated aldehydes, ketones
1652	1680–1640	–C=C– stretch alkenes

	1650–1580	N–H bend 1° amines
1457	1500–1400 1470–1450	C–C stretch (in–ring) aromatics –C–H bend (alkane)
1283	1335–1250 1320–1000	C–N stretch aromatic amines C–O stretch alcohols, carboxylic acids, esters, ethers
1018	1320–1000	C–O stretch alcohols, carboxylic acids, esters, ethers

Rice bran is composed of cellulose, hemicellulose, lignin, vitamins, protein, fats, minerals, etc. Hence, bond vibration corresponding to all of these compounds are available in the spectrum.

As expected the strong band at 3281 cm^{-1} can be attributed to O–H stretching vibration of alcoholic and carboxylic groups present in cellulose, hemicellulose, lignin and vitamins in rice bran while the band at 1087 cm^{-1} represents the C–O stretch in C–OH groups. Bands at 2924 cm^{-1} and 2853 cm^{-1} can be attributed to C–H stretching of the bio polymers.

(d) Differential scanning calorimetry (DSC)

In DSC thermograms sharp narrow endotherms attribute to the purity of the sample. Rice bran is not a pure material, it does not results in a sharp melting point in the DSC (Figure 4.19). Since it is a complex material a broad peak extending through a temperature range $43\text{ }^{\circ}\text{C}$ to $206\text{ }^{\circ}\text{C}$ is observed and it is due to the complex nature of rice bran. A small endothermic peak can also be seen at $27\text{ }^{\circ}\text{C}$ which is again due to the melting of a constituent in rice bran. In the thermogram $280\text{ }^{\circ}\text{C}$ the decomposition of rice bran has taken place.

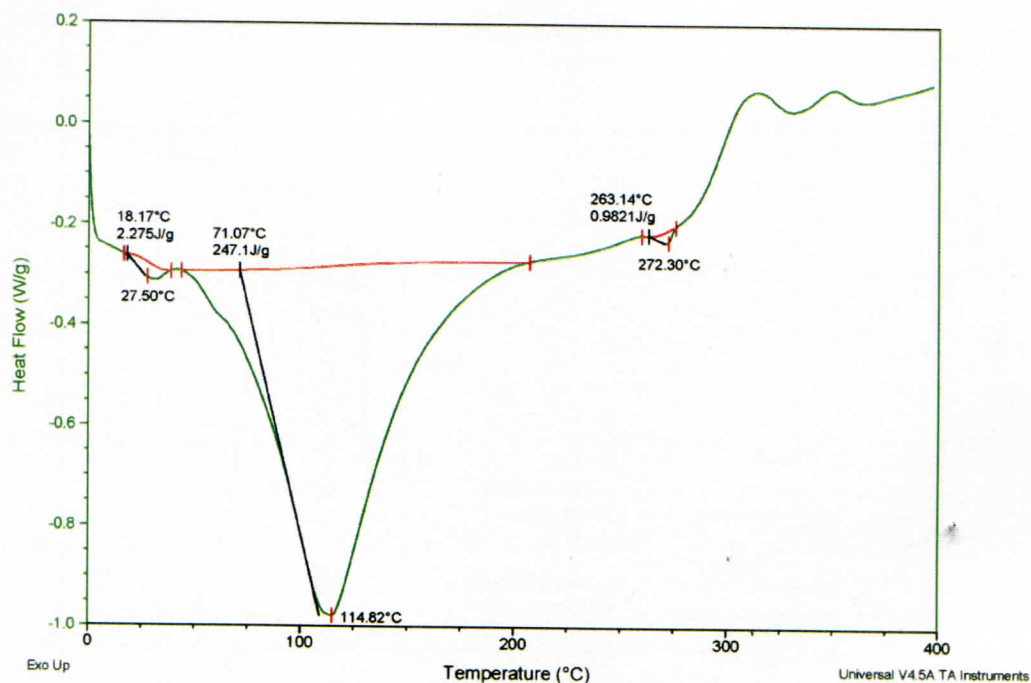


Figure 4.19: DSC thermogram of rice bran

(e) Thermal gravimetric analysis (TGA)

Raw rice bran shows thermal decomposition in temperature ranges of 43-126 °C, 126-251 °C, 251-387 °C, 387-483 °C and 483-1000 °C (Figure 4.20).

Weight loss (5.4 %) corresponding to the temperature range of 43-126 °C is due to the loss of moisture and other volatile components. There is a rapid weight loss (68%) from 126 to 483 °C which could correspond to the pyrolysis of organic components. There is also a possibility to evaporate some of the components at this temperature. At 1000 °C, about 7 % weight of char under nitrogen environment corresponding to the remaining inorganic materials (Mg, Al, K).

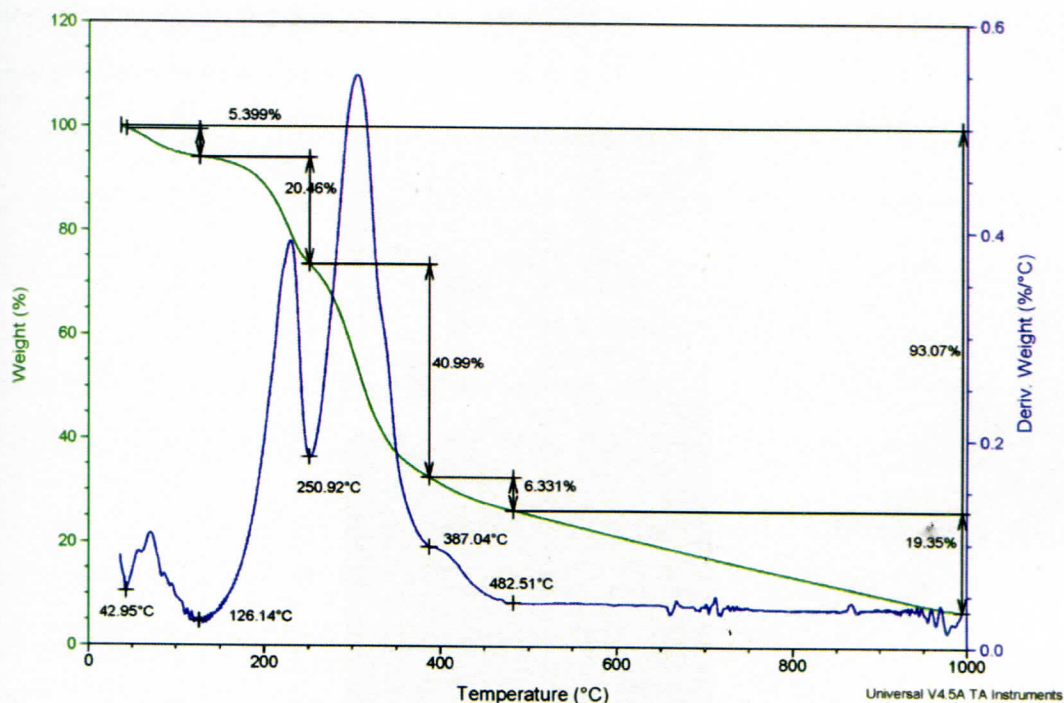


Figure 4.20: TGA thermogram of rice bran

4.3 Pretreatment of rice bran

Rice bran should be defatted to remove the fat and other encrusting lipids that is over 17 % by weight in their native state (Abdul-Hamid et al., 2000). This pretreatment step is very important in electrospinning process since fat can results in emulsions affecting the physical parameters of the polymer solution. Such polymer solutions can impact morphologies of fiber produced. Fat molecules are long chain carboxylic acids thus soluble in organic solvents. Diethyl ether the organic solvent used in this work is non polar making it miscible with fat molecules easily. Further it is considerably volatile and easily removed from the fiber material.

Upon alkaline treatment and heating, the solubility of dietary fiber increases. This process helps to dissolve cellulose, hemicellulose, pectin, lignin, proteins and carbohydrate (Fung et al., 2010). Reaction between cellulose, which is one of the major components of the fibers and NaOH is represented bellow.



Reaction increases the polarity of dietary fibers and hence increases water solubility which results in fiber rich supernatant (Figure 4.21).



Figure 4.21: Dietary fiber supernatant

Table 4.5: Fiber supernatant characterization

pH	7.30
Conductivity/ (mS cm ⁻¹)	8.90
Viscosity/ (cP)	30

Even though the pH of the solution was set to 9 initially for the extraction of dietary fibers, upon heating and reacting with NaOH the pH value decreases to 7.30. Solution pH is a very important factor in the process of electrospinning and for the stability of the enzyme employed. Conductivity of the solution is strongly dependent on the pH of the solution hence low and high pH values leads to high conductive solutions (Keun Son et al., 2005). These parameters have a direct effect on the fiber morphology. Depending on these parameters fiber-like, bead-like, sheet-like morphologies can be resulted. For example, it has been found that high conductivity or charge density results in more uniform fibers with fewer beads present (Pham et

al., 2006). This effect is attributed to high charge density on the surface of the ejected jet during spinning, thus stronger elongation forces are imposed to the ejected jet under the electrical field employed (Keun Son et al., 2005; Zong et al., 2002).

Beaded electrospun fibers are usually considered as "poor" quality fibers, since they can reduce the total surface area significantly. This can directly reduce the enzyme loading capacity of the fibers and therefore, industrially undesirable.

It is reported that high alkaline are favourable over high acidic conditions for electrospinning process (Keun Son et al., 2005). However, in this study high alkaline conditions are not desirable since it will influence on the denaturation of the enzyme and also the consumability of the final product. Although phytase enzyme is stable under acidic conditions, according to the literature when the medium is highly acidic electrospun fibers are not continuous leading to beads-on-string structures (Keun Son et al., 2005). This observation has been explained referring to the protonation of PVA molecules under acidic conditions and the induced local variation in charge density due to its very lower mobility under an external electric field leading to an uneven elongational forces on the ejected jets. Apart from that it has been assumed that under high electrical field repulsive forces between protonated PVA backbone could have partially inhibit the formation of continuous fibers especially in the process of jet stretching by whipping and bending. Considering all these factors electrospinning was carried out at pH 7.

4.4 Viscosity modification of the polymer solution

Dietary fibers were extracted from rice bran under alkaline condition to synthesize nanofibers. The viscosity of this extract was not enough to electrospin into nanofibers. Therefore, poly vinyl alcohol (MW- 60,000 - 125,000) was used as a viscosity modifier.

4.4.1 Characterization of PVA

(a) Fourier transform infrared spectroscopy (FT-IR)

The FT-IR spectrum obtained for PVA is shown in Figure 4.22 and Table 4.6 presents the FT-IR peak interpretation of PVA.

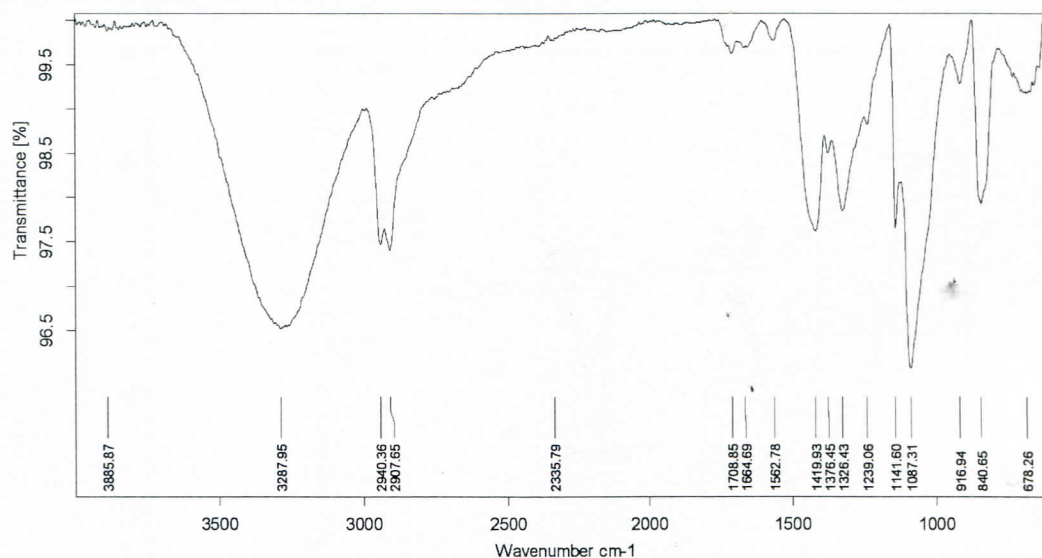


Figure 4.22: FT-IR spectrum of PVA

Table 4.6: FT-IR peak interpretation of PVA

FT-IR peak interpretation of PVA		
Wave number (cm ⁻¹)	Reference values (cm ⁻¹) (Yadav, 2013)	Bond / Functional group vibration
3287	3000–2850 (m)	O–H stretch, H–bonded alcohols
2940	3000–2850 (m)	C–H stretch alkanes
2907	1320–1000 (s)	C–H stretch alkanes
1087	1320–1000 (s)	C–O stretch alcohols

Polyvinyl alcohol is a polymer produced by the reaction of polyvinyl acetate with methanol.

As expected the strong band at 3287 cm⁻¹ can be attributed to O–H stretching vibrations of alcohols present in the back bone of PVA molecules and the band at

2940 cm^{-1} and 2907 cm^{-1} can be attributed to C–H stretching of alkanes, while band at 1087 cm^{-1} represents the C–O stretching vibrations in alcoholic C–OH groups.

(b) Differential scanning calorimetry (DSC)

DSC thermogram of PVA is shown in Figure 4.23.

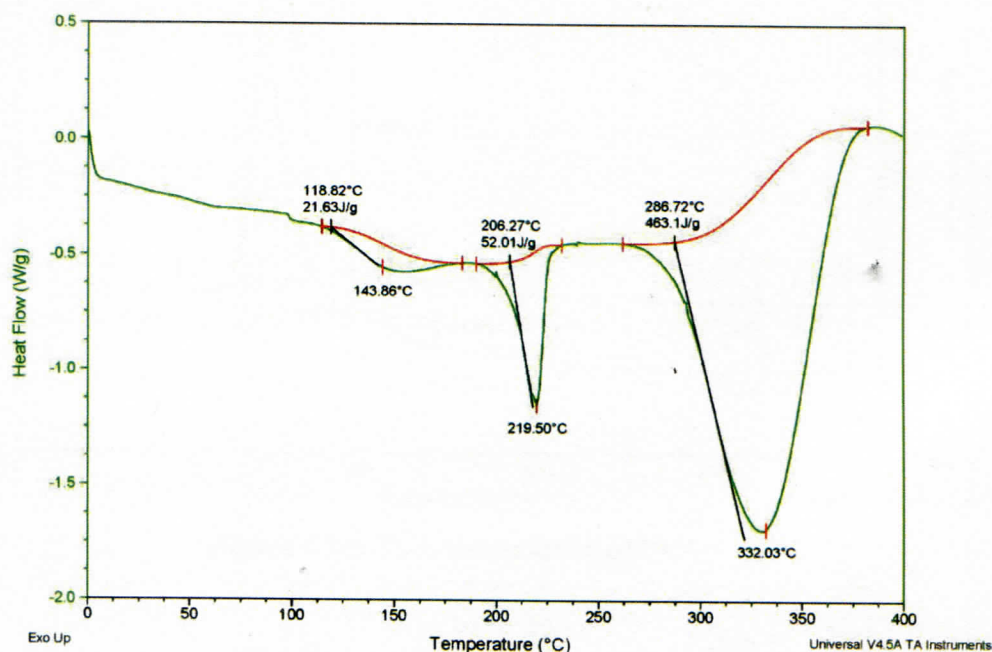


Figure 4.23: DSC thermogram of PVA

The small peak present at 144 °C (23 J/g) in the DSC thermogram, suggests the presence of impurities. The relatively sharp endothermic peak at 219 °C (52 J/g) corresponds to melting of PVA while the endotherm at 332 °C (463 J/g) corresponds to degradation or evaporation of PVA (Figure 4.23).

(c) Characterization by Thermal gravimetric analysis (TGA)

PVA shows thermal decomposition in temperature ranges of 230 to 482 °C. The rapid decomposition from 230 °C to 386 °C, corresponds to 80 % weight loss and a slow and a small thermal degradation from 405 °C to 482 °C corresponds to 7 % weight loss (Figure 4.24). These weight losses are corresponding to the degradation and evaporation of organic components in PVA under nitrogen environment. At 1000 °C PVA shows a 100 % weight loss.

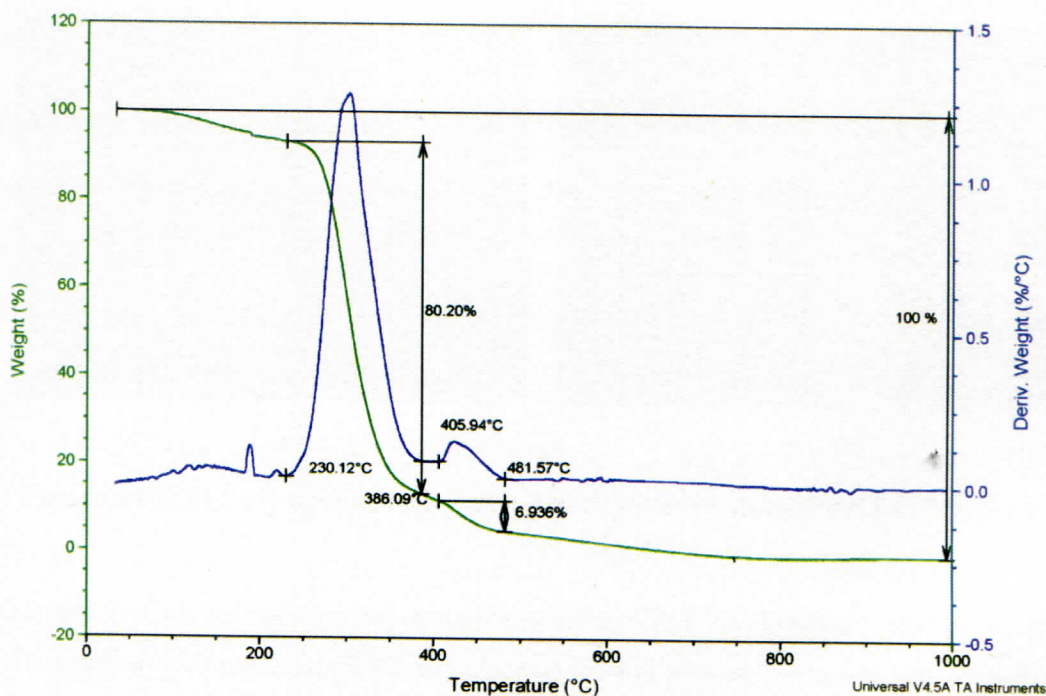


Figure 4.24: TGA thermogram of PVA

4.5 Condition optimization for electrospinning

4.5.1 Solution viscosity

Viscosity, resulting from the polymer concentration of the electrospinnig solution is one of the factors affecting the size and morphology of electrospun fibers (Pham et al., 2006). If the size of the fibers is large the surface area decreases and the amount of enzyme that can load on the fiber surface is also less. This is undesirable.

Viscosity, resulting from the polymer concentration of the electrospinnig solution is one of the factors affecting the fiber size and morphology of electro-spun fibers (Pham et al., 2006). If the size of the fibers is large the surface area decreases, hence the amount of enzyme that can load on the fiber surface is also less. This is undesirable.

Therefore, correct amendment of the solution viscosity is really important.

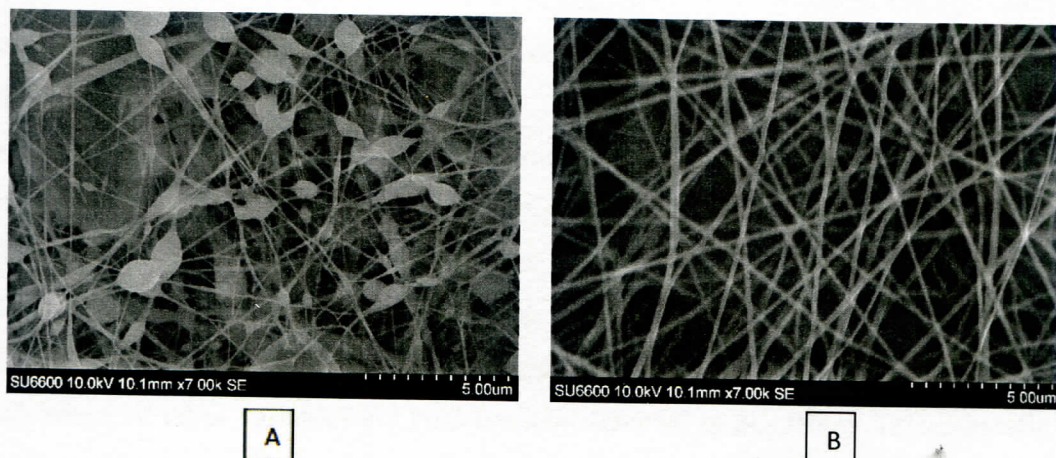


Figure 4.25: SEM images of fibers after electrospinning using PVA (A) 7% (B) 8%

Viscosity of the solution varied using 6% to 8% (w/v) PVA, however viscosity of the 6% solution was not enough for the electrospinning process. When it was loaded to the syringe droplets were resulted without drawing in to fibers. The same observation was reported by Son et al., (2005) and they were able to obtain uniform fibers from 7% (w/v) polymer solution.

However, in this experiment a considerable amount of beads-on-string structures were resulted with 7% (w/v) PVA. Subsequently the weight percentage of PVA was increased to 8% (w/v) and then it resulted less amount of beads. Therefore 8% (w/v) was chosen as the minimum concentration that gives reasonably uniform fiber morphology with low diameters (Figure 4.25 (B)). Since further increase of the polymer concentration might not be cost effective and industrially viable.

Table 4.7: Fiber supernatant characterization after adding PVA

pH	7.00
Conductivity/ (mS/cm)	17.00
Viscosity/ (cP)	800

In addition to viscosity amendment in the electrospinning solution, -OH groups in PVA makes hydrogen bonds with cellulose and other biopolymers in dietary fibers increasing the integrity of the synthesizing nanofibers.

4.6.2 Applied voltage

Applied voltage is another important parameter in deciding the fiber morphology. It has been observed that the shape of the initial drop changes with the applied voltage. The mechanism of charge transfer from the tip/needle to the collector is through the flow of polymer that is resulting due to the voltage difference generated. Therefore, increase in the electrospinning current coincide with the mass flow rate away from the tip, when all other variables are held constant (Deitzel et al., 1999). This generally means, at higher applied voltage more is ejected fluid in a jet, resulting in a larger fiber diameter. Therefore, the applied voltage was increased from 15 kV until a Taylor cone was observed. Taylor cone was initially observed at 20 kV but in the meantime substantial amount of beads-on-string structures were observed (Figure 4.26). The voltage was further increased to 27 kV, at which smooth fibers were resulted.

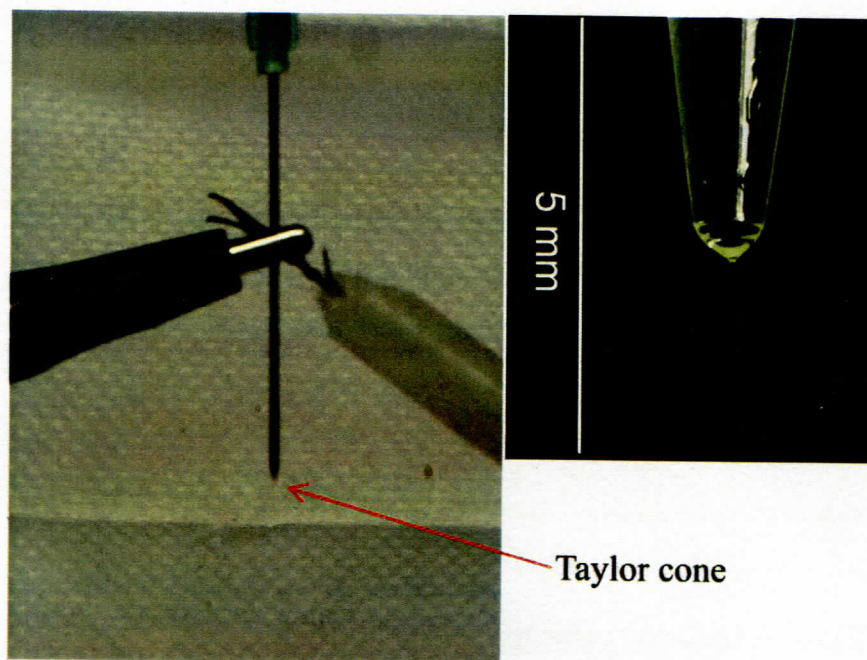


Figure 4.26: Taylor cone observed at 20 kV

Further increase of voltage is not viable in scaling up and commercialization. In addition high voltages increase the defect density of electrospun fibers (Deitzel et al., 1999).

4.5.3 Distance between two electrodes

The distance between the needle and the collector was another parameter that determined the morphology and the diameter of the electrospun fibers. The distance had to be maintained in such a way that the fibers would get sufficient time to dry before it reached the collector. Solvent of the fiber solution evaporated while traveling to the collector. Distances that were too short produced bead structures. If the distance was too long fibers gathered at the edges of the collector making it difficult to collect. Distances were set at 20 cm, 15 cm and 10 cm during the work. When using 10 cm, droplet formation was observed. When the distance was adjusted to 20 cm, very small amount of fibers were formed at the edge of the collector indicating that the distance is too high. At 15 cm, reasonable amount of fibers were collected at the collector (Figure 4.27). Senapathi, (2013) reports the same observation as he had been able to synthesize nanofibers from agrowaste by keeping the distance between electrodes around 15 cm.

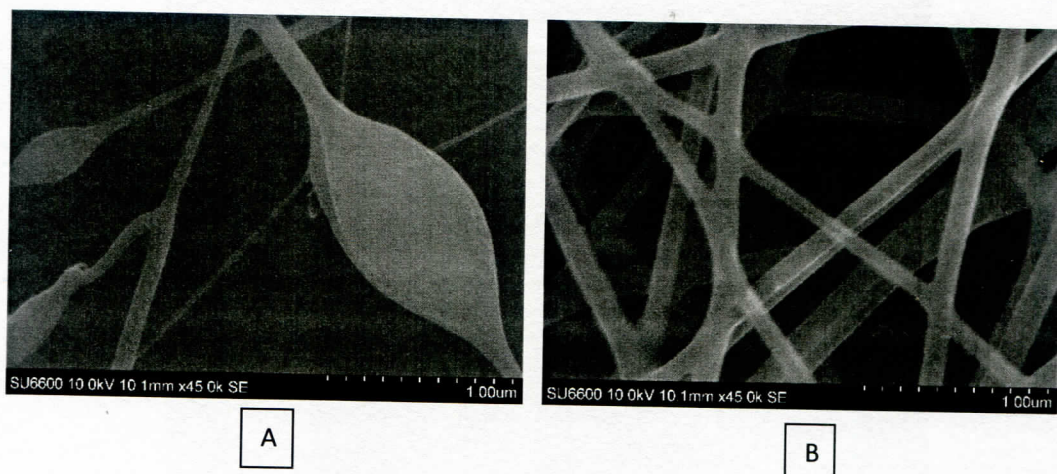


Figure 4.27: SEM images of fibers after electrospinning using (A) 10 cm (B) 15 cm distances between the two electrodes.

4.6 Synthesis of nanofibers

Using the optimized conditions; viscosity of the solution around 850 cP, the distance between electrodes 15 cm and the applied voltage 27 kV, nanofibers were synthesized.

4.6.1 Characterization of nanofibers

(a) Scanning electron microscopy (SEM)

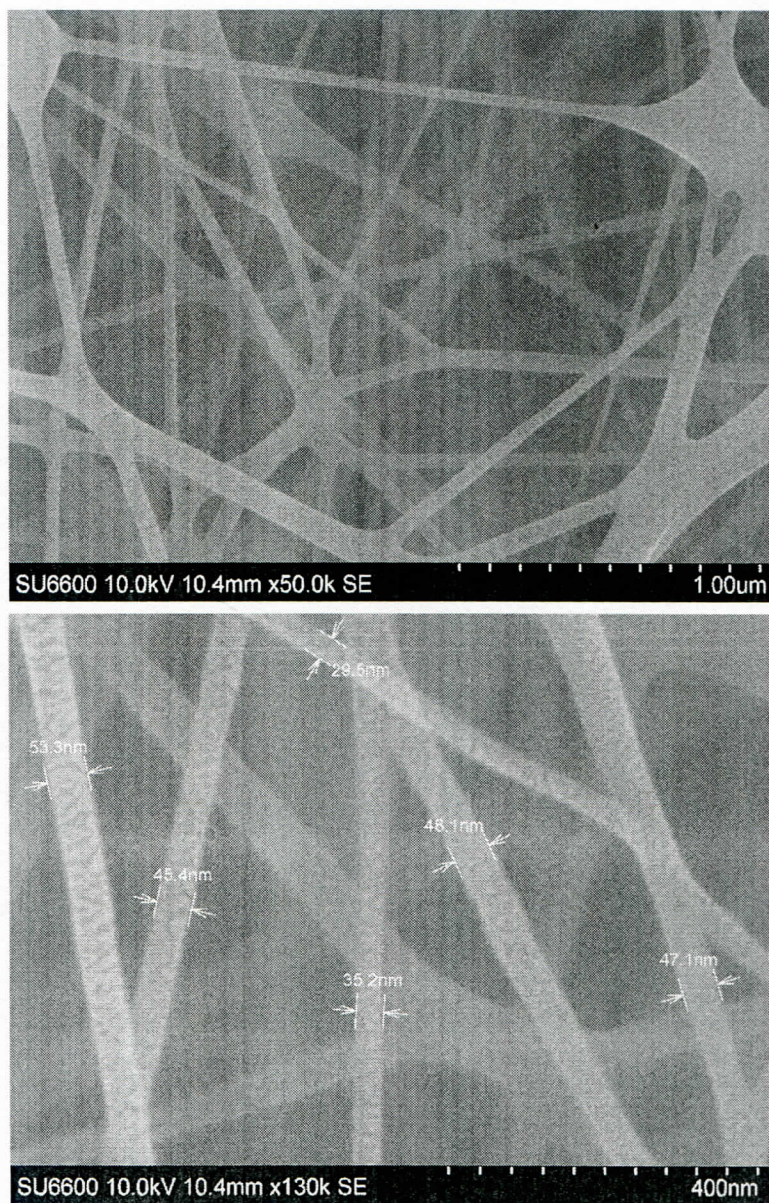


Figure 4.28: Scanning Electron Microscopic images of nanofibers

The dietary fiber solution was successfully spun in to nanofibers and a network structure having a fiber diameter of 30-50 nm with uniform size was obtained (figure 4.28). The fiber surface was smooth and less number of bead structures were observed. Wu et.al., (2005) produced nanofibers with diameter around 200nm using PVA alone where as in this work fibers synthesized using dietary fibers amended with PVA have smaller diameters proving that the composite solution is better for electronspin than PVA alone. The diameter of the fibers produced are well within the nano range (1 – 1000 nm).

(b) Energy-dispersive X-ray spectroscopy (EDX).

From the EDX data, elemental composition of the synthesized fibers can be established hence the nutritional composition of fibers can be established.

Table 4.8: Elemental composition of the nanofibers

Element Line	Weight %	Atom %
C K	82.99 ± 0.45	88.67
O K	11.20 ± 0.56	8.98
Na K	1.80 ± 0.10	1.00
Si K	1.08 ± 0.04	0.49
K K	1.80 ± 0.06	0.59
Fe K	1.14 ± 0.17	0.26

The elemental composition and the weight percentage of nanofibers are given in Table 4.8.

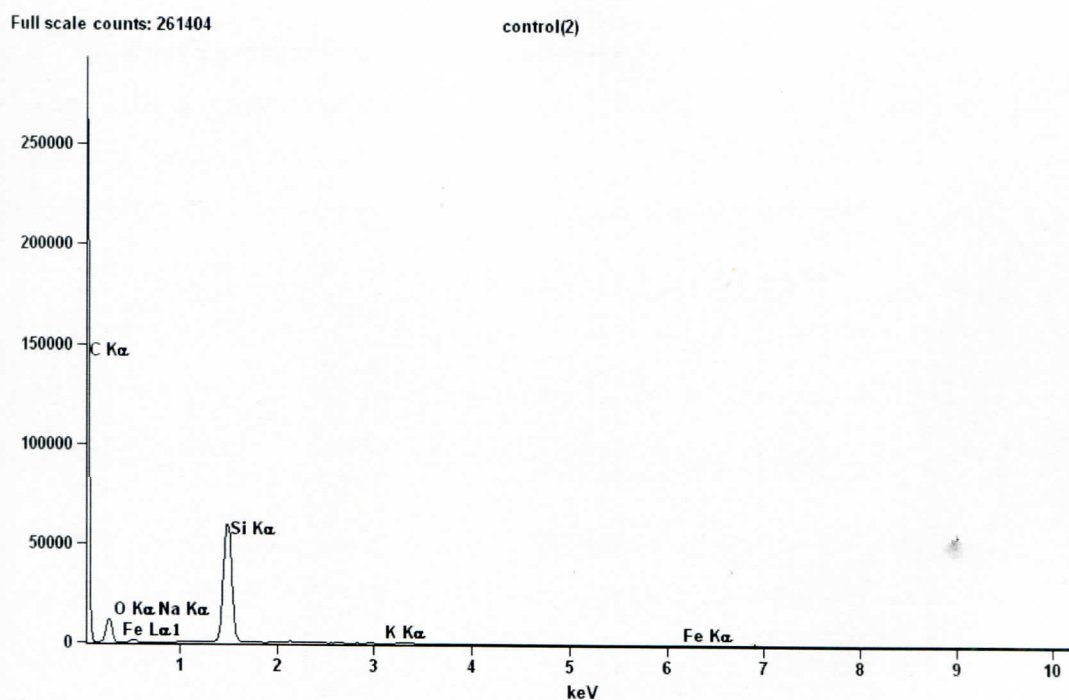


Figure 4.29: EDX Spectrometric data of nanofibers

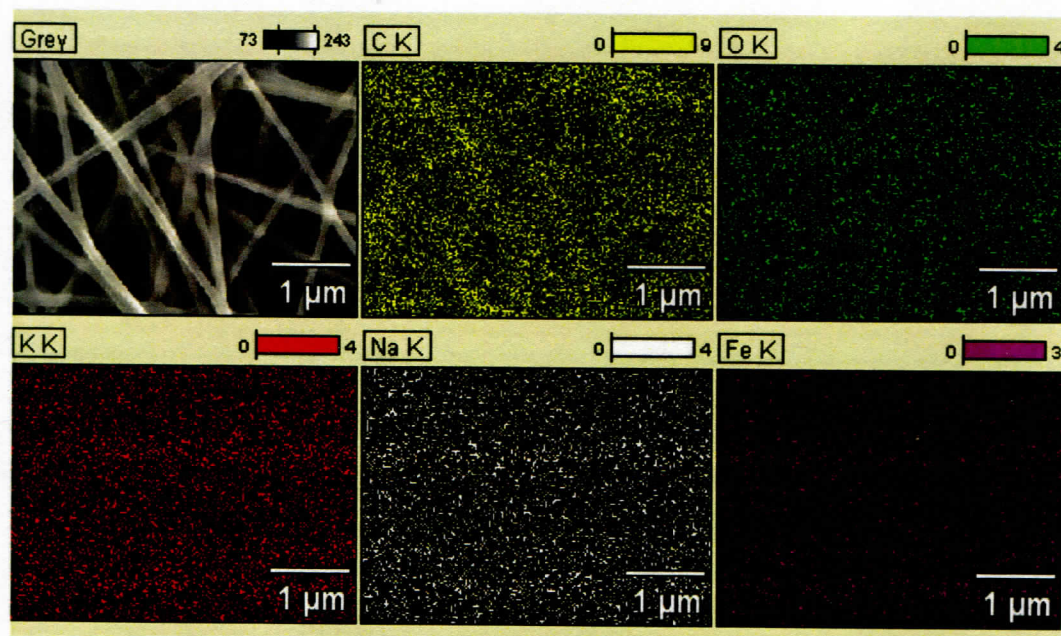


Figure 4.30: Spectral image of nanofibers

As can be seen in the graph the synthesized nanofibers are a good source of Fe and K along with Na.

(c) Fourier transform infrared spectroscopy (FT-IR)

The FT-IR spectrum obtained for nanofibers is shown in Figure 4.31 and Table 4.9 presents the FT-IR peak interpretation.

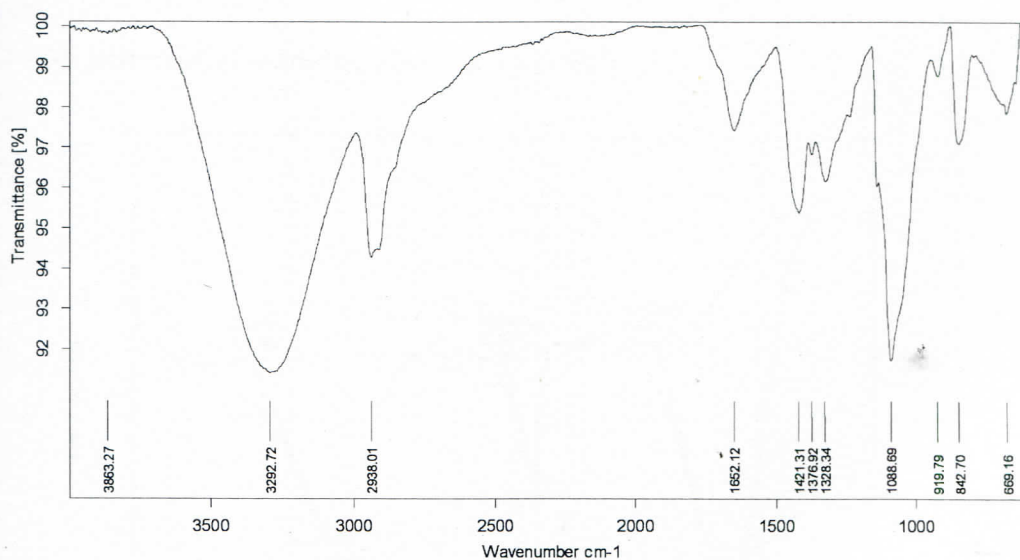


Figure 4.31: FT-IR spectrum of nanofibers

Table 4.9: FT-IR peak interpretation of nanofibers

FT-IR peak interpretation of nanofibers		
Wave number (cm ⁻¹)	Reference values (cm ⁻¹) (Yadav, 2013)	Functional group interpretation
3292	3500-3200	OH stretching (alcohols)
2938	3000-2850	C-H stretching
1421	1350-1480	C-H bending
1088	1320-1000	C-O stretching of alcohol
842	895-856	skeletal vibrations of syndiotactic and isotactic PVA stereosequences

In Figure 4.31, the presence of broad band at 3292 cm⁻¹ attributes to the O-H stretching. This may be due to -O-H groups in both PVA and rice bran. The strong band at 1088 cm⁻¹ can be attributes to the C-O stretch in alcohols. While the band at 2938 cm⁻¹ corresponds to C-H stretching in PVA molecules and rice bran. Band at

1421 cm^{-1} is attribute to the C-H bending while the band at 842 cm^{-1} is due to the skeletal vibrations of syndiotactic and isotactic PVA stereosequences (Labidi et al., 2008; Wu et al., 2012)

(d) Differential scanning calorimetry (DSC)

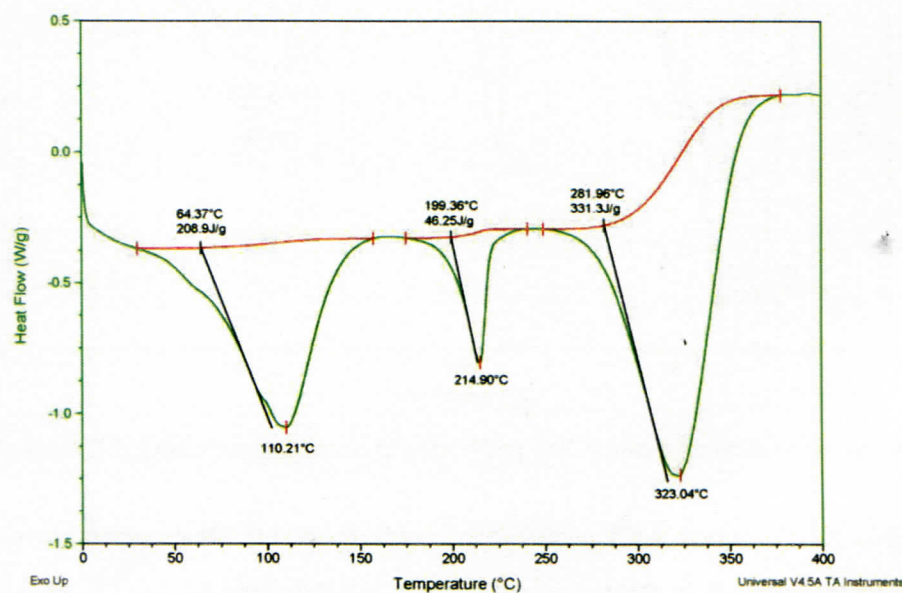


Figure 4.32: DSC thermogram of nanofibers

The DSC thermogram of the synthesized nanofibers basically shows three main endotherms (Figure 4.32). First endotherm at 110 °C corresponds to the melting of the dietary fibers and other components came from rice bran in the composite. The second endotherm at 215 °C represents melting of PVA and the endotherm at 323 °C is related to subsequent decomposition of the composite under nitrogen environment.

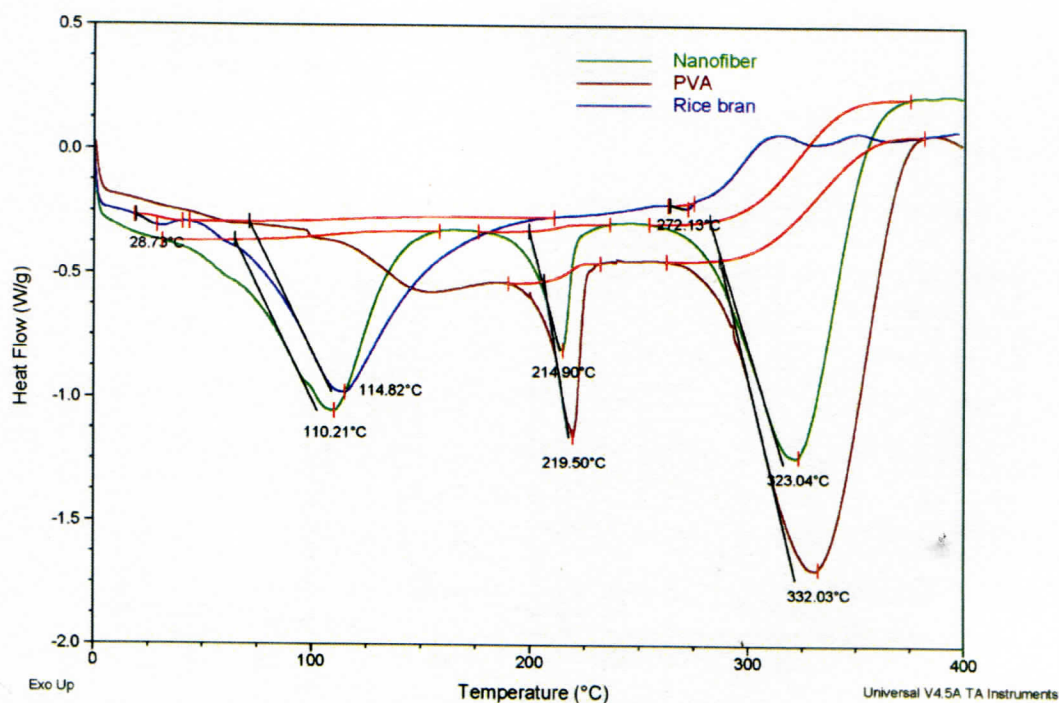


Figure 4.33: DSC thermogram of nanofiber, PVA and rice bran (comparison)

Comparison between the thermograms of nanofibers, PVA and rice bran is given in Figure 4.33. As can be seen in the graph the melting point of the rice bran and PVA have not been changed significantly. However, the broadness of the endotherms corresponding to the melting of dietary fibers fraction has become much narrower. This may be due to better alignment of the polymer chains with in individual nanofibers (Peresin et al., 2010).

(e) Thermal gravimetric analysis (TGA)

Nanofibers show thermal decomposition in the temperature ranges of 39-99 °C, 99-212 °C, 212-376 °C, 376-510 °C and 510-1000 °C (Figure 4.34)

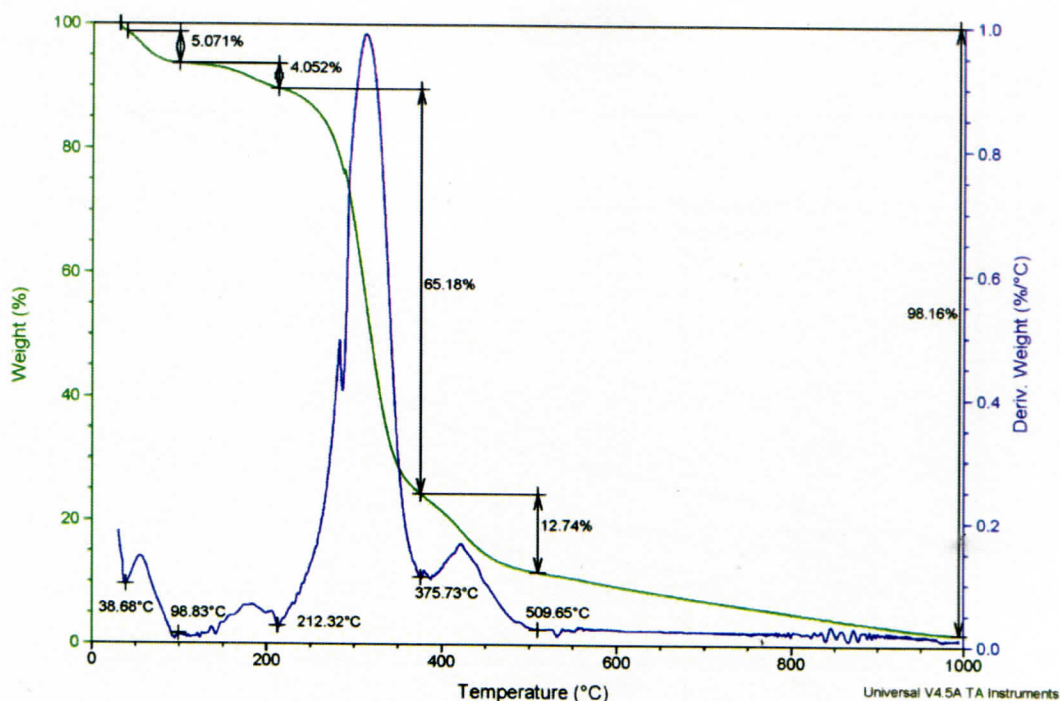


Figure 4.34: TGA thermogram of nanofibers

Weight loss (5 %) corresponding to the temperature range of 39-99 °C might be due to the loss of moisture and other volatile components. From 99-212 °C there is a small weight loss (4%) which could be due to the loss of volatile components in nanofibers. From 212-509 °C there is a rapid weight loss (78%) correspond to the decomposition of nanofibers. At 1000 °C, nanofibers left about 2 % of char and other inorganic compounds under nitrogen environment.

TGA Comparison between the thermograms of the synthesized fibers and its constituents; PVA and rice bran are given in Figure 4.35. As seen from the graph rice bran shows a weight loss around 93 % while PVA show a complete weight loss 100 %. Synthesized nanofibers shows a weight loss in-between which is 98 %, confirming the presence of both components in the composite. By 1000 °C all the PVA fraction in the compost have decomposed and the remainder is the fraction that is coming from the rice bran.

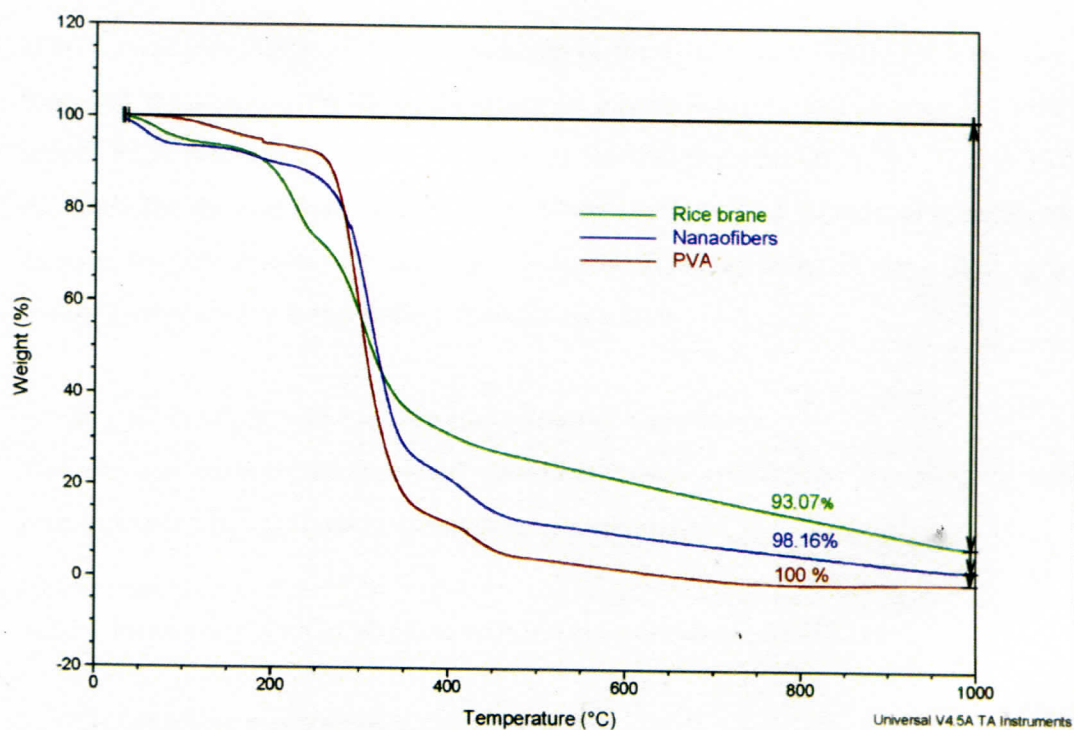


Figure 4.35: Comparison between TGA thermograms of nanofiber, PVA and rice bran.

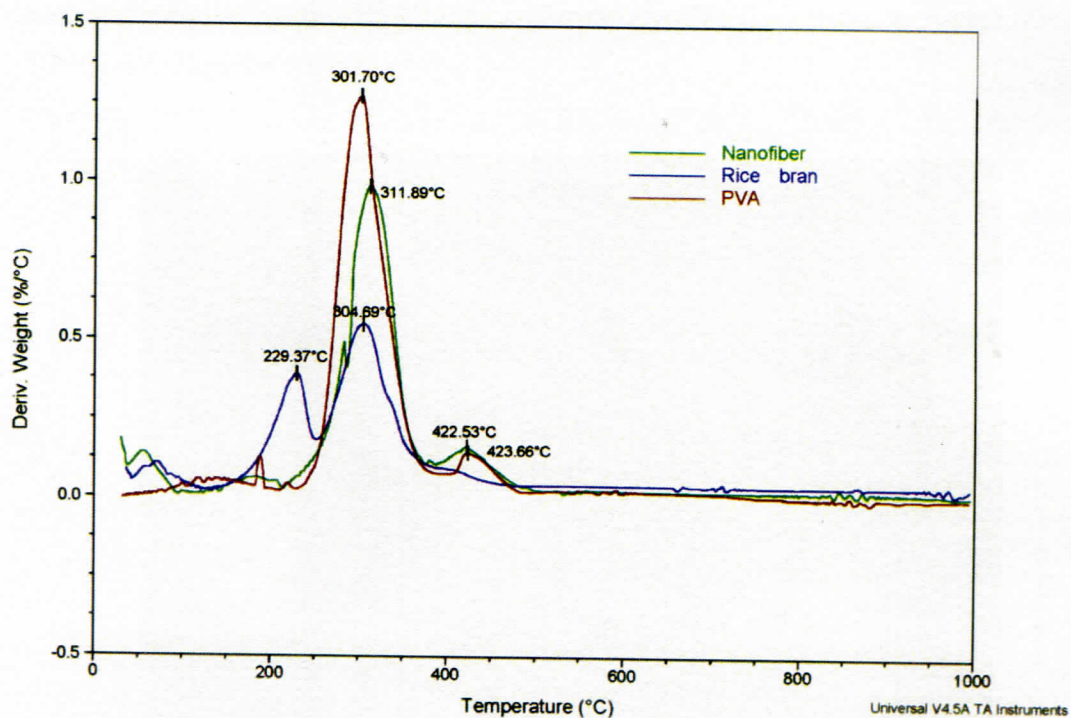


Figure 4.36: DTG analysis of nanofibers, PVA and rice brans (comparison).

DTG Comparison between the components of the synthesized fibers; PVA and rice bran and the synthesized fibers are given in Figure 4.36. As can be seen from the graph, PVA and rice bran have the greatest thermal degradation at 302 °C and 305 °C, while the thermal degradation of nanofibers is around 312 °C indicating a gain of an extra thermal stability by spinning into fibers. This may be again due to hydrogen bonding between PVA and dietary fibers in rice bran.

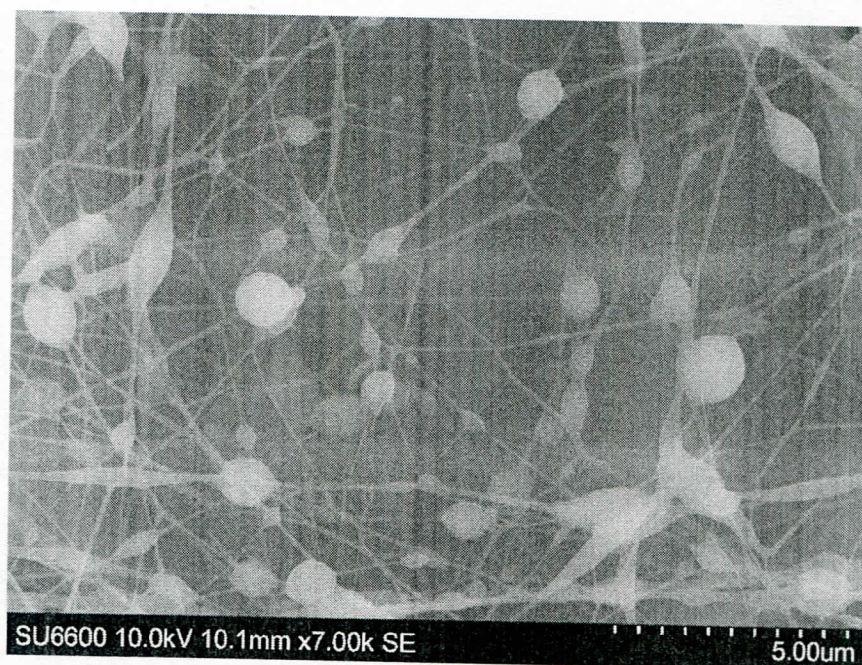
4.7 Synthesis of phytase enzyme encapsulated nanofibers

The phytase enzyme encapsulated nanofibers were synthesized according to the protocol stated in 3.2.10 and characterize accordingly.

4.7.1 Characterization of phytase enzyme encapsulated nanofibers

(a) Scanning electron microscopy (SEM)

The physical morphology and the elemental composition of phytase encapsulated electrospun fibers were examined using the scanning electron microscope and the energy dispersive X-ray (SEM/EDX) spectroscopy. The fiber diameter varied from 50-100 nm (Figure 4.37)



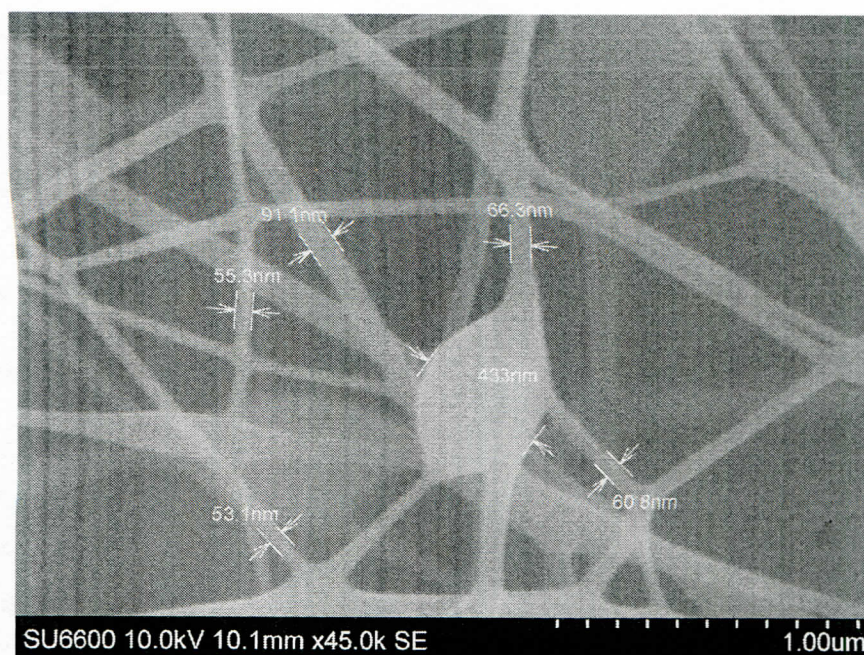


Figure 4.37: Scanning Electron Microscopic images of Phytase enzyme encapsulated nanofibers

Compared to the electrospun pure nanofibers (Figure 4.28), phytase enzyme encapsulated nanofibers (Figure 4.37) are much less uniform. As can be seen in figures with the addition of phytase enzyme, fibers become more irregular in shape and interspersed with shuttle-shaped beads. The same observation has been reported by Ren et al., (2006) and Wu et al., (2005) with their work on immobilization of enzymes on PVA membranes.

Phytase enzymes alone cannot be electrospun in to nanofibers due to complex conformational structures it possess. Hence, amending with a suitable polymer is required. Polymers in the electrospinning solution can form secondary bonding with protein, interrupt the complex structures and destroy molecular interactions making it possible to electrospin into nanofibers. At the same time this process reduces the stability of the solution thus beads-on-string structures have been resulted. With the increasement of the enzyme concentration the instability increase hence the beads structures increases.

(b) Energy-dispersive X-ray spectroscopy (EDX).

From EDX data the elemental composition of Phytase enzyme encapsulated nanofibers can be established. Hence, the nutritional composition of fibers can be established.

Table 4.10: Elemental composition of phytase enzyme encapsulated nanofibers

Element Line	Weight %	Atom %
C	86.58 ± 0.38	91.44
O	7.72 ± 0.36	6.12
Na	1.93 ± 0.06	1.07
Mg	0.25 ± 0.02	0.13
Si	0.25 ± 0.02	0.11
P	0.86 ± 0.06	0.35
K	2.42 ± 0.04	0.78

The elemental composition and the weight percentage of the Phytase enzyme encapsulated nanofibers are listed in Table 4. 10.

As can be seen from Figure 4.38 and table 4.10 phytase enzyme encapsulated nanofibers can be a good source of Mg, P and K along with Na. High amount of C was expected since both PVA and dietary fibers have long C chains. Both components have -OH groups in the structure; hence, high amount of O is also possible.

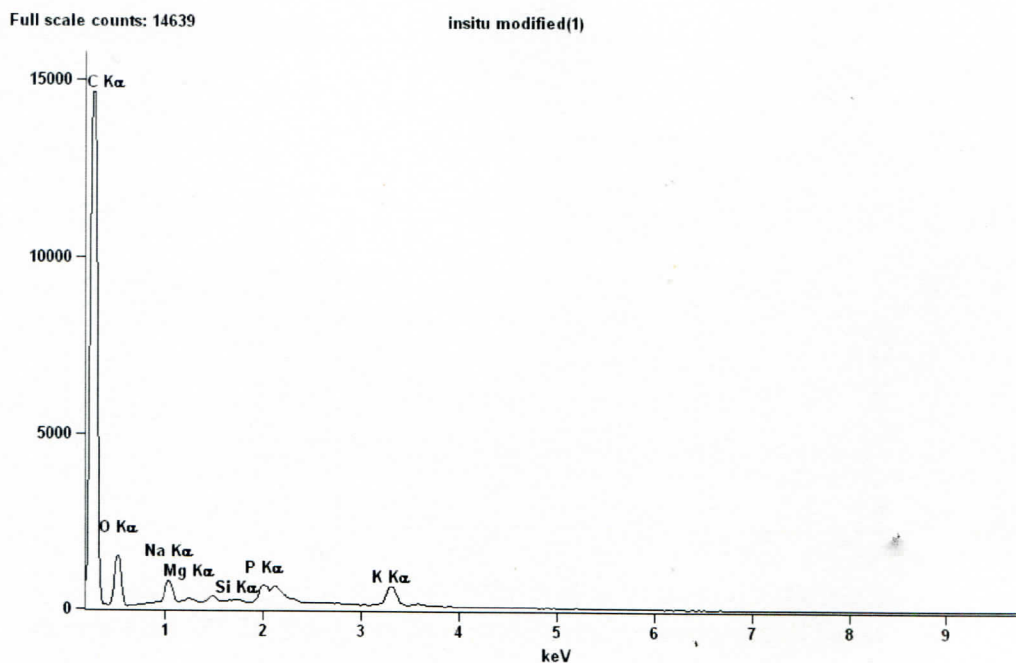


Figure 4.38: EDX Spectrometric data of phytase enzyme encapsulated nanofibers

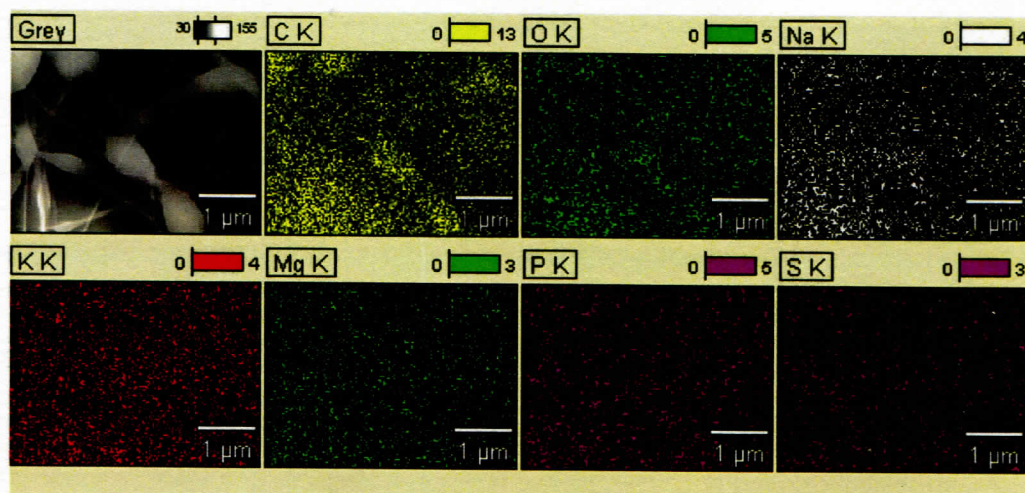


Figure 4.39: Spectral image of phytase enzyme encapsulated nanofibers

(c) Fourier transform infrared spectroscopy (FT-IR)

The FT-IR spectrum of phytase enzyme encapsulated nanofibers is shown Figure 4.40 and Table 4.11 presents the FT-IR peak interpretation.

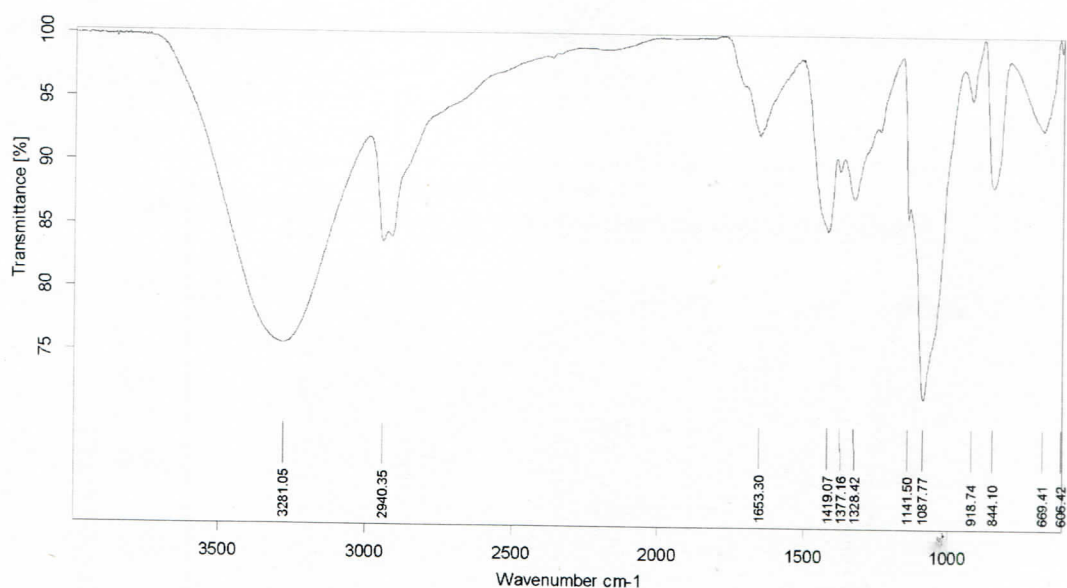


Figure 4.40: FT-IR Spectrum of phytase enzyme encapsulated nanofibers

Table 4.11: FT-IR peak interpretation of phytase enzyme encapsulated nanofibers.

FT-IR peak interpretation of phytase enzyme encapsulated nanofibers		
Wave number (cm ⁻¹)	Reference values (cm ⁻¹) (Yadav, 2013)	Bond / Functional group vibration
3281	3500-3200 3400-3250	OH stretching (alcohols,phenols) N-H stretch amines or amides
2940	3000-2850	C-H stretching
1653	1690–1600	Amide C=O Stretch
1328	1320–1000 (s)	C-N stretching of amine groups
1088	1320–1000 (s)	C–O stretch alcohols

In the FT-IR Spectrum of phytase enzyme encapsulated nanofibers the presence of PVA, dietary fibers from rice bran and phytase enzyme is expected.

The peptide and protein repeat units in enzymes give rise to several characteristic IR absorption bands. In the protein secondary structure C=O stretch vibrations of the peptide linkages can be attributed to the band at 1653 cm⁻¹. Hence, the presence of enzyme in the Phytase enzyme encapsulated nanofibers is further confirmed (Figure

4.41). Also, the band at 1328 cm^{-1} corresponds to the C-N stretching of amine groups in the enzyme.

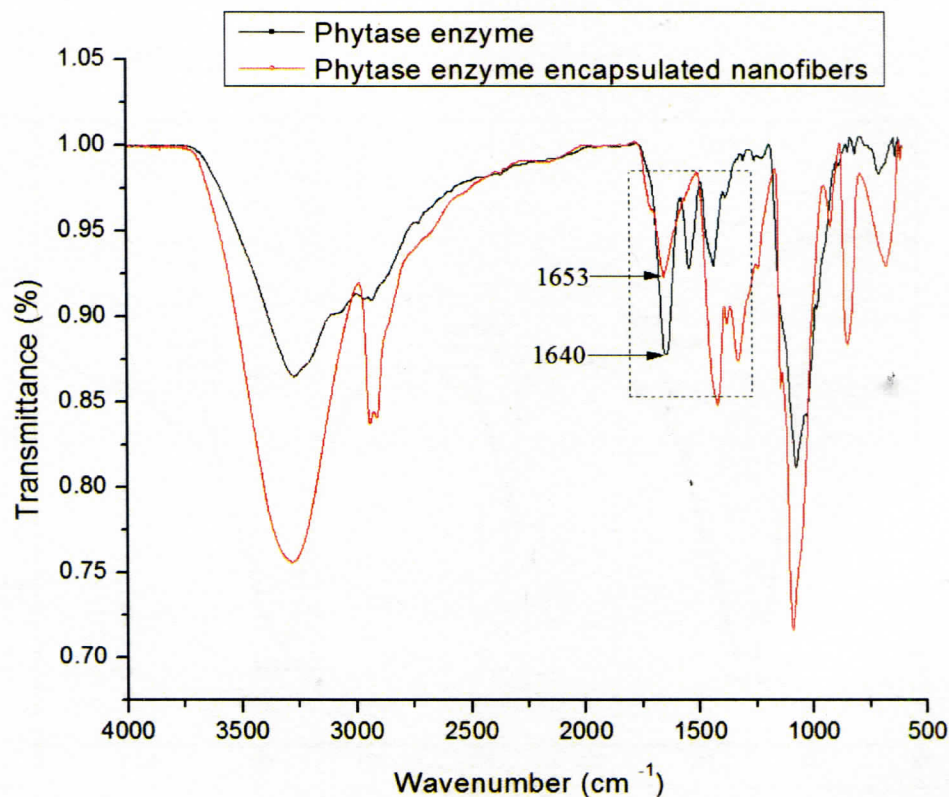


Figure 4.41: FT-IR Spectrum of phytase enzyme encapsulated nanofibers and phytase enzyme (comparison)

The presence of broad band at 3281 cm^{-1} attributes to the O-H stretching. This may be due to both -O-H bond vibration in the PVA molecules as well as in the enzyme. In addition N-H stretches of amines appears in the region $3300\text{--}3000\text{ cm}^{-1}$ (Figure 4.40). They might have overlapped with the band corresponding to the O-H stretching, hence differentiating them is not possible. The strong band at 1088 cm^{-1} attributes to the C-O stretch alcohols or carboxylic acids (Figure 4.40).

(d) Differential scanning calorimetry (DSC)

DSC thermogram of phytase enzyme encapsulated nanofibers is shown in figure 4. 42.

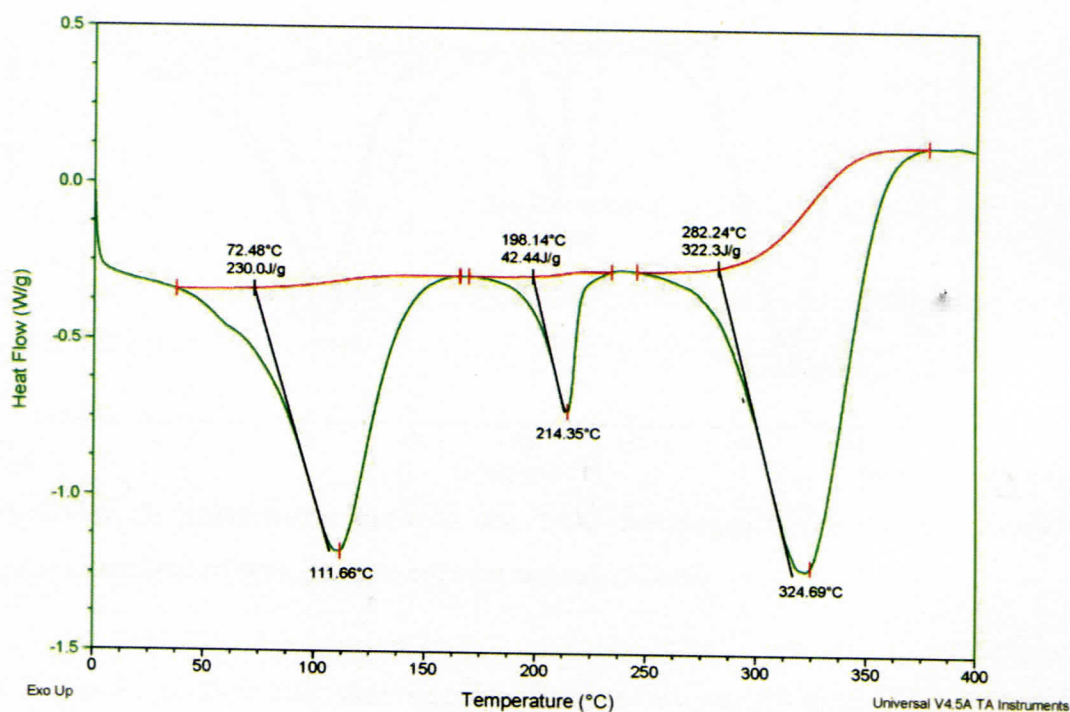


Figure 4.42: DSC thermogram of phytase enzyme encapsulated nanofibers

The DSC thermogram of the synthesized insitu enzyme encapsulated fibers basically shows three main endotherms. First endotherm at 112 °C corresponds to melting of the rice bran in the composite. The second endotherm at 214 °C represents melting of PVA and the endotherm at 325 °C is related to subsequent decomposition of the composite.

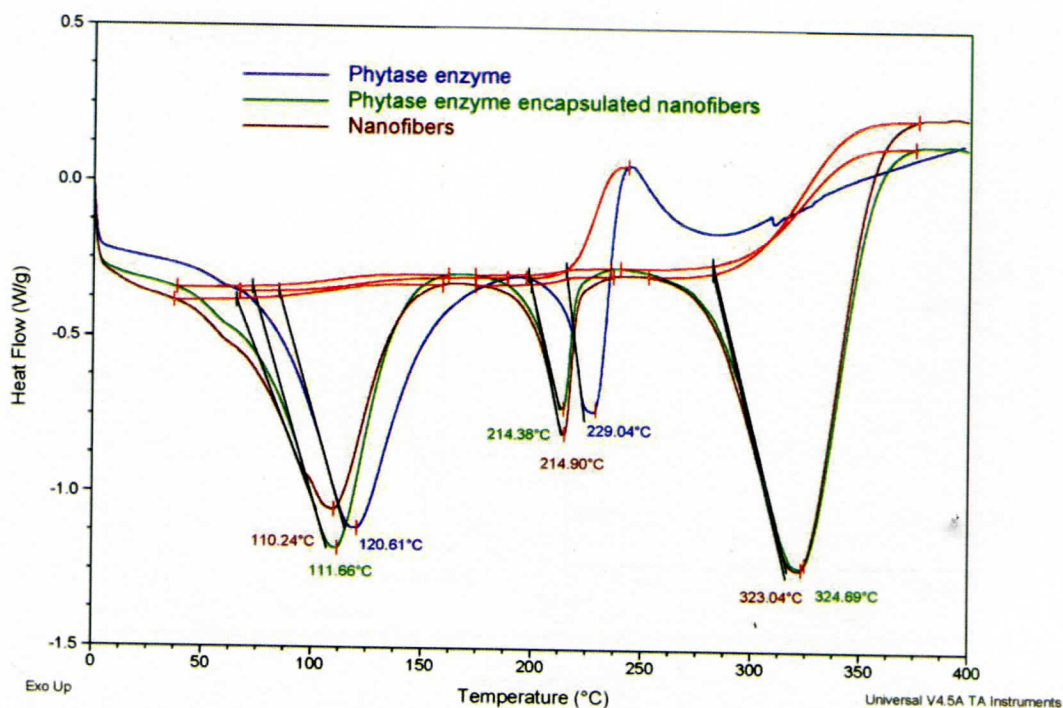


Figure 4.43: Comparison between the DSC thermograms of phytase enzyme encapsulated nanofibers, phytase enzyme and nanofibers.

Comparison between DSC thermograms of phytase enzyme encapsulated nanofibers, phytase enzyme and nanofibers are given in Figure 4.43. As can be seen, the melting point at 112 °C in phytase enzyme encapsulated nanofibers, corresponding to the melting of dietary fiber fraction has not been changed due to the presence of phytase enzyme and also the endotherm corresponding to the melting of PVA has not been changed. It shows a very similar thermogram to that of nanofibers.

(e) Thermal gravimetric analysis (TGA)

Phytase enzyme encapsulated nanofibers fibers show thermal decomposition in temperature ranges of 43-119 °C, 119-213 °C, 213-384 °C, 384-526 °C and 526-1000 °C (Figure 4.44)

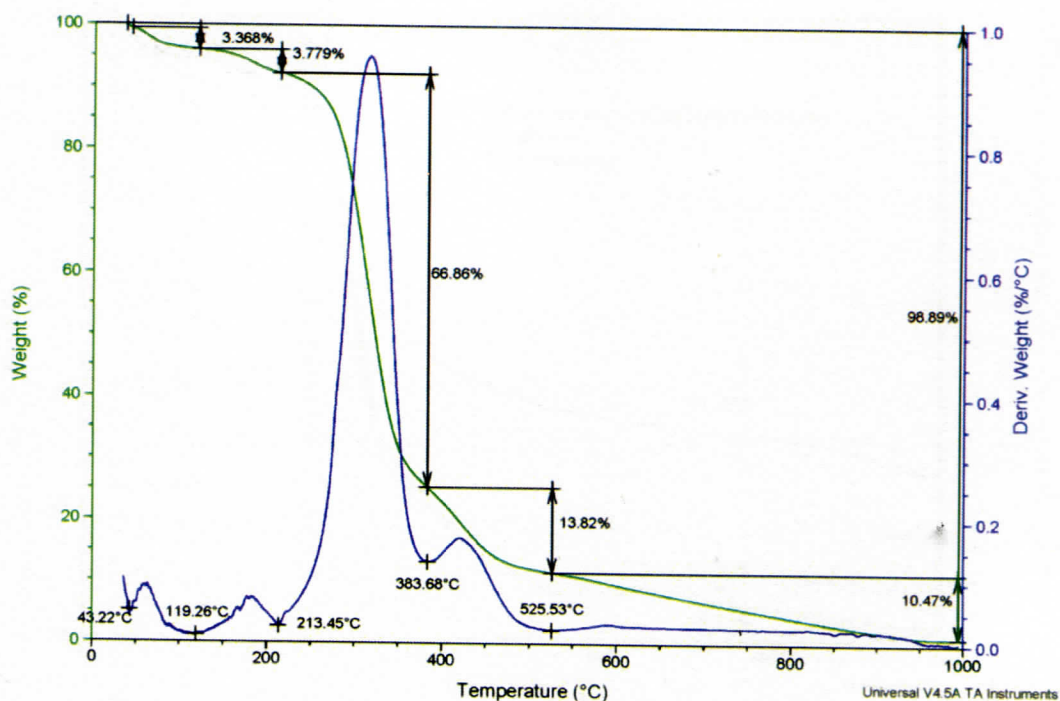


Figure 4.44: TGA thermogram of Phytase enzyme encapsulated nanofibers

Weight loss (3 %) corresponding to the temperature range of 43-119 °C might be due to the loss of moisture and the weight loss from 119-213 °C corresponds to the loss of other volatile components. From 213-526 °C there is a rapid weight loss (81%) that could be corresponding to the pyrolysis of organic components. At 1000 °C, phytase enzyme encapsulated nanofibers left only about 1 % weight that could be char under nitrogen environment.

TGA thermograms of nanofibers, phytase enzyme and phytase enzyme encapsulated nanofibers are shown in Figure 4.45. Crude enzyme has left 26 % of weight from the initial as can be seen in the graph. However, there is no much difference between the residual weight of nanofibers and phytase enzyme encapsulated nanofibers at 1000 °C under nitrogen environment. There is only 2 % of weight from the initial weight is remaining in both sample. Comparatively this might be due to the lower amount of phytase present in phytase enzyme encapsulated nanofibers.

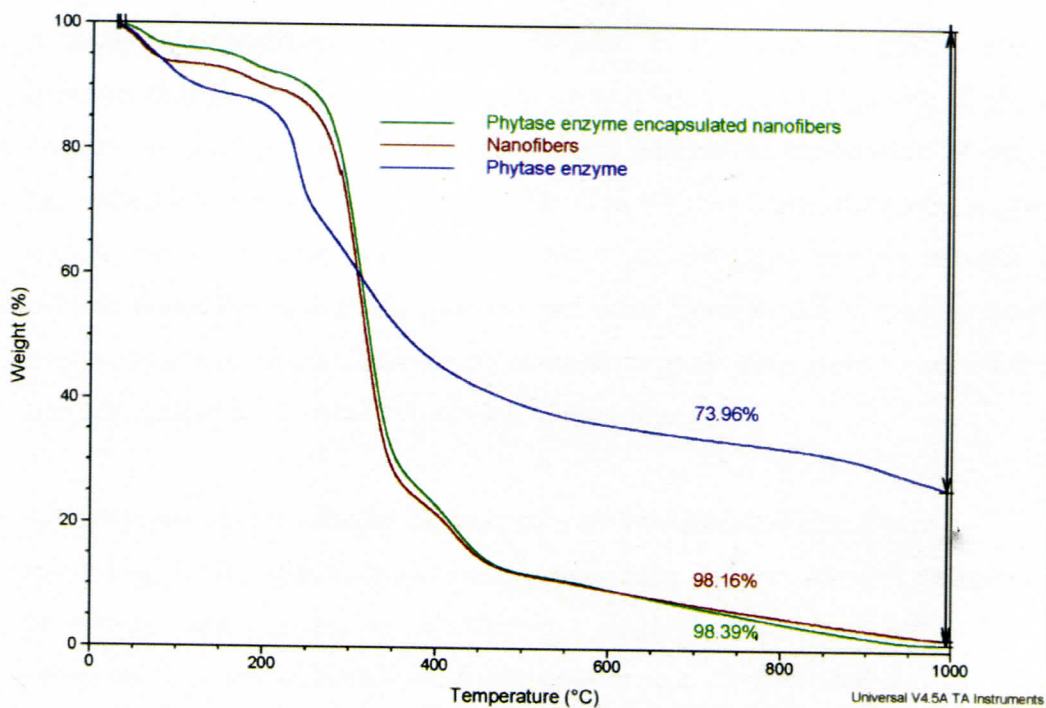


Figure 4.45: TGA thermogram of Phytase enzyme encapsulated nanofibers with its components.

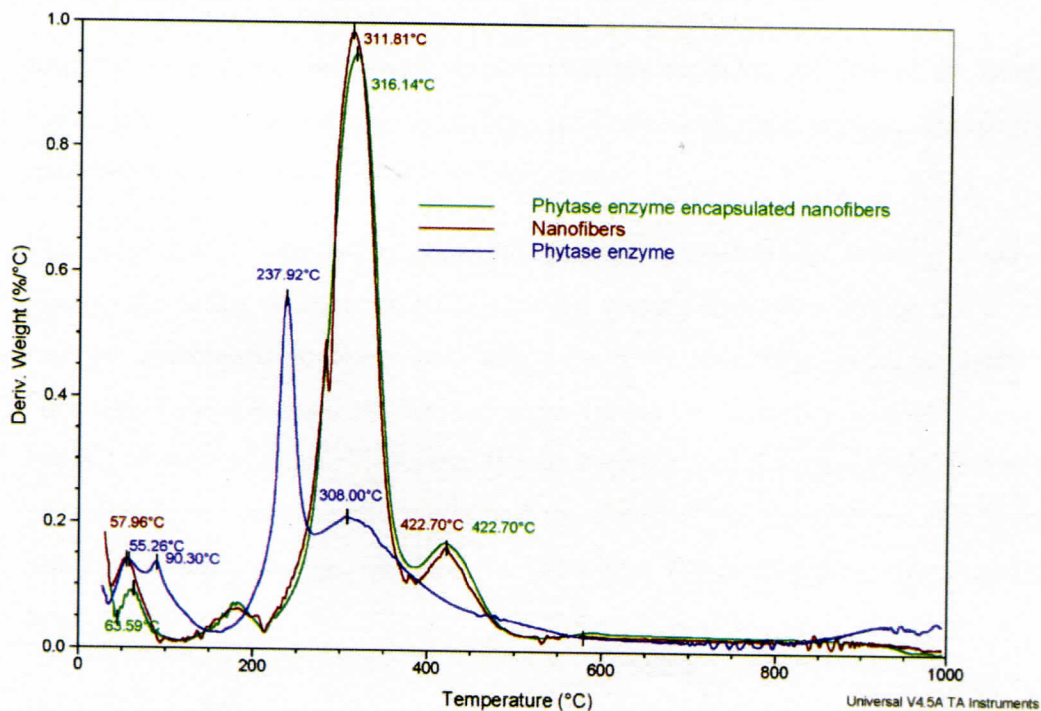


Figure 4.46: DTG thermograms of phytase enzyme encapsulated nanofibers, nanofibers and Phytase enzyme (comparison).

A separate degradation step cannot be seen in the range of 230 – 240 °C corresponding to the degradation of phytase enzyme, in the DTG profile of phytase enzyme encapsulated nanofibers. The thermal degradation temperature of enzyme has shifted into a higher value (From 238 °C to $\approx$ 316 °C) give the range as can be seen in the graph. This can be mainly due to the hydrogen bonding between the enzyme molecules with PVA, cellulose and other biomolecules in phytase enzyme encapsulated nanofibers. These strong interactions might have given an extra thermal stability against the thermal degradation of the enzymes.

4.8 Synthesis of cross-linked Phytase enzyme encapsulated nanofibers

According to the objective of the research these phytase enzyme encapsulated nanofibers were expected to be stable in a range of temperatures and in low pH values as they are ultimately be formulated in to a chicken feed and for human consumption. But once the fibers were tested for their activity it was found that they started to get dissolved in lower pH (2.5) values. This challenging observation had to be addressed since the human as well as chicken stomach pH is very close to 2.5.

Improvements had to be brought to overcome the solubility problem of the fibers at lower pH. To decrease the solubility, synthesized phytase enzyme encapsulated nanofibers were crossed linked with boric acid.

The solubility of phytase enzyme encapsulated nanofibers in lower pH may be mainly due to the presence of PVA. Oxygen atoms present in – OH groups in PVA can get protonated in lower pHs and as a result solubility could be increased. Although PVA has good mechanical properties in the dry state, its applications are limited in wet medium. Therefore, it was hypothesized that, by, cross-linking the phytase enzyme encapsulated nanofibers the chemical and the mechanical properties would drastically change, specially the solubility. The cross-linking agent used was boric acid.

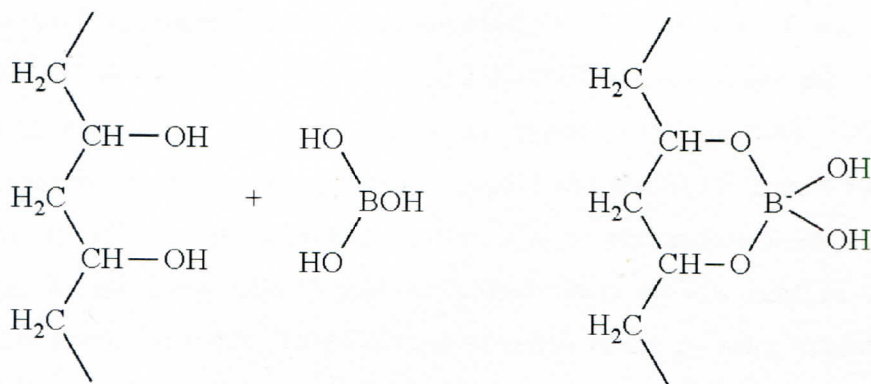


Figure 4.47: Cross-linking mechanism of boric acid with PVA

Boric acid reacts with an hydroxide ion to produce tetrahydroxy borate anion (B(OH)_4^-), which will react with two alcohol groups in the PVA backbone to make two covalent bonds linking the tetraborate ion with one polymer chain (Figure 4.47). The two remaining hydroxyl groups attach to the boron then react with two additional alcohol groups within an adjacent polymer chain. This reaction completes the borax cross-link and creates a covalent bond that links these polymer chains together.

Therefore, the synthesized phytase enzyme encapsulated nanofibers were dipped in a 4% boric acid solution for 30 seconds and sequentially in three deionized water baths for 1 min. in each, until all the remaining boric acid was removed from the film (Figure 4.48).



Figure 4.48: cross-linking of phytase enzyme encapsulated nanofibers with boric acid.

The resulted fibers were dried in a vacuum oven for 12 hours at 30 °C and stored at 4 °C. Due to the cross links phytase enzyme encapsulated nanofibers get covalently bonded to each other via boron atoms as shown in Figure 4.49. Finally, a nanofibrous film with good mechanical properties was resulted (Figure 4.50). When there are nanofibers they offer high surface area to encapsulate large amount of enzymes. At the cross linked stage individual fibers do not exist as they are covalently bonded together. This net-work structure offers an extra stability to the enzyme since it give a better protection from external conditions.

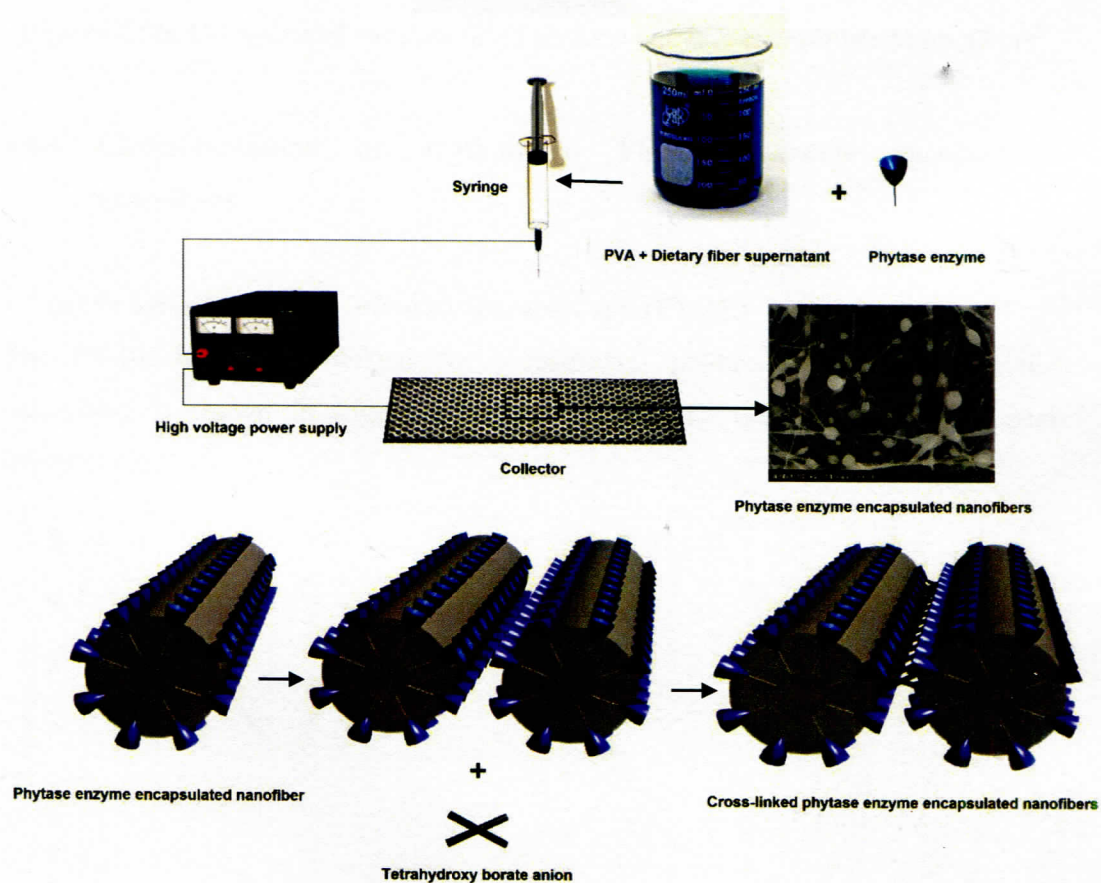


Figure 4.49: Mechanism of the synthesis of cross-linked Phytase enzyme encapsulated nanofibers



Figure 4.50: Cross-linked vacuum dried phytase enzyme encapsulated nanofibers

4.8.1 Characterization of cross-linked Phytase enzyme encapsulated nanofibers

(a) Fourier transform infrared spectroscopy (FT-IR)

The FT-IR spectrum obtained for cross-linked phytase enzyme encapsulated nanofibers is shown in Figure 4.51 and Table 4.12 presents the FT-IR peak interpretation.

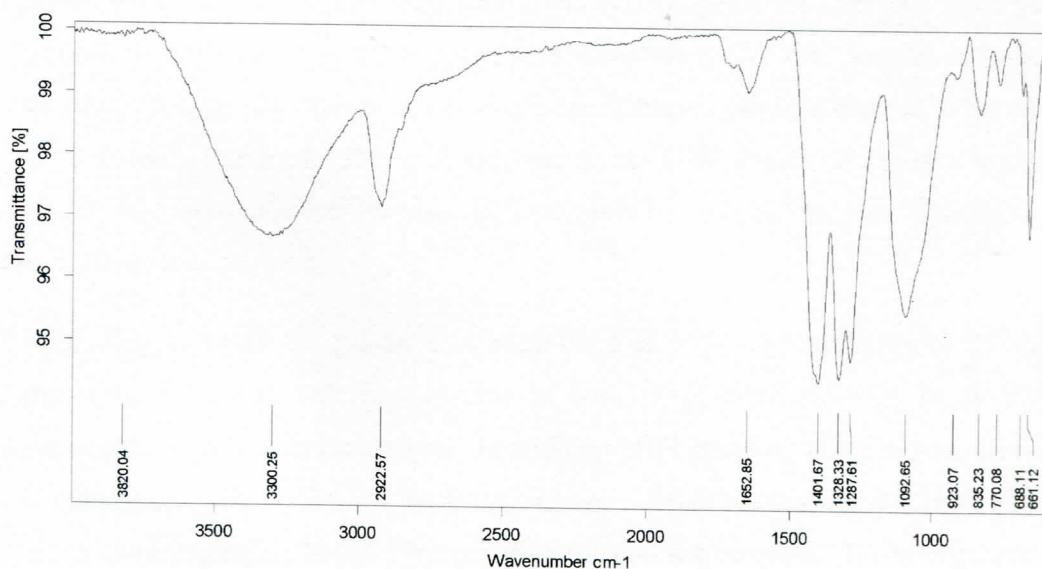


Figure 4.51: FT-IR Spectrum of cross-linked phytase enzyme encapsulated nanofibers.

Table 4.12: FT-IR peak interpretation of cross-linked Phytase enzyme encapsulated nanofibers.

FT-IR peak interpretation of cross-linked Phytase enzyme encapsulated nanofibers.		
Wave number (cm ⁻¹)	Reference values (cm ⁻¹) (Yadav, 2013)	Bond / Functional group vibration
3300	3500-3200	OH stretching alcohols, carboxylic acid
2922	3000-2850	C-H stretching
1652	1690-1600	Amide C=O Stretch
1402		asymmetric B-O stretching
1328	1320-1000	C-O stretch carboxylic acids
1287	1335-1250	C-N stretch
1092	1320-1000	C-O stretch alcohols
688	690-670	O-B-O bending

In the FT-IR spectrum of cross-linked phytase enzyme encapsulated nanofibers functional groups present in both nanofibers and enzymes are expected to be very similar to Phytase enzyme encapsulated nanofibers. In the protein secondary structure C=O stretch vibrations of the peptide linkages, can be attributed to the band at 1652 cm⁻¹. Bands at 1287 cm⁻¹ attribute to the C-N stretch of aliphatic amines. Hence, the presence of enzyme in cross-linked phytase enzyme encapsulated nanofibers is confirmed.

According to Table the presence of broadband at 3300 cm⁻¹ attributes to the O-H stretching vibration. This may be due to both -O-H bond vibration in the PVA molecules as well as in the enzyme. In addition N-H stretches of amines appears are in the region 3300-3000 cm⁻¹. They might have overlap with the band corresponding to the O-H stretching, hence differentiating them is not possible. The strong band at 1092 cm⁻¹ attributes to the C-O stretch alcohols.

The peak at 688 cm^{-1} in the FT-IR spectrum of cross-linked phytase enzyme encapsulated nanofibers is due to the O-B-O bending of cross links while the band at 1402 cm^{-1} is due to the asymmetric B-O stretching in the cross links. Therefore the presence of boron cross-links is also confirmed (Uslu et al., 2007).

(b) Differential scanning calorimetry (DSC)

DSC data indicates a broad endotherm in cross-linked nanofibers with boric acid (Figure 4.52) and the cross-linked phytase enzyme encapsulated nanofibers (Figure 4.53). This may be due to the chemical and physical changes that takes place due to the strong cross linking of the nanofibers. In DSC thermograms sharp narrow endotherms correspond to the purity of the sample. Therefore the broadness of the melting peaks relative to the phytase enzyme encapsulated nanofibers indicates the presence of impurities in cross-linked nanofibers with boric acid (Figure 4.52) and the cross-linked phytase enzyme encapsulated nanofibers (Figure 4.53). In this situation boron is working as an impurity.

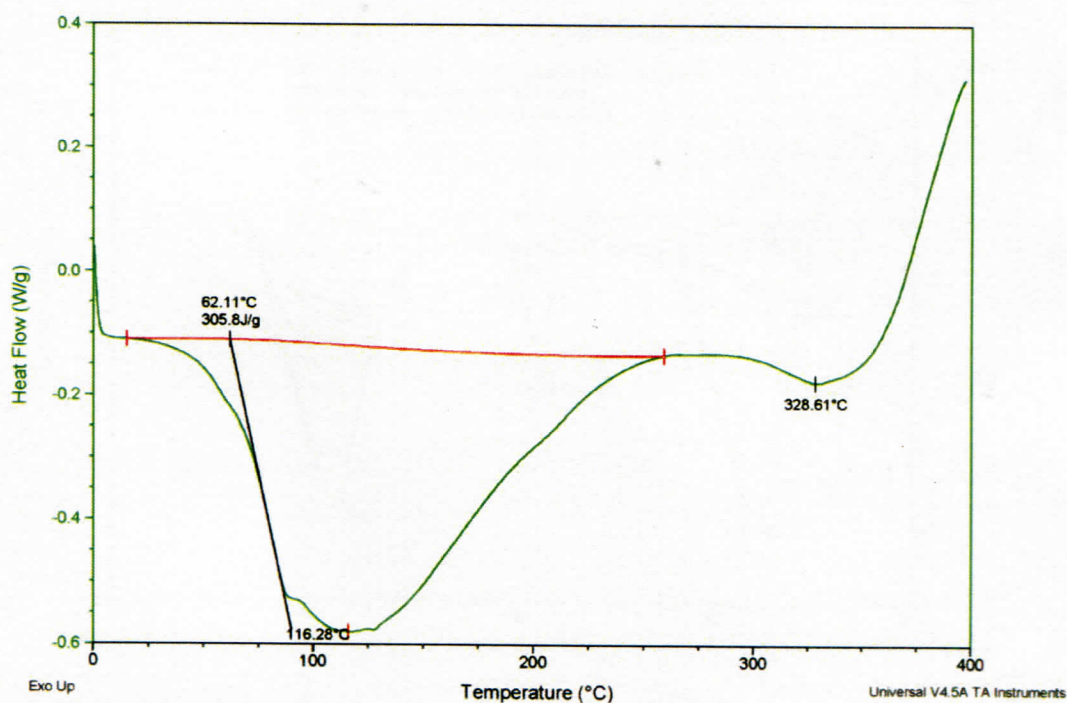


Figure 4.52: DSC thermogram of cross-linked nanofibers with boric acid

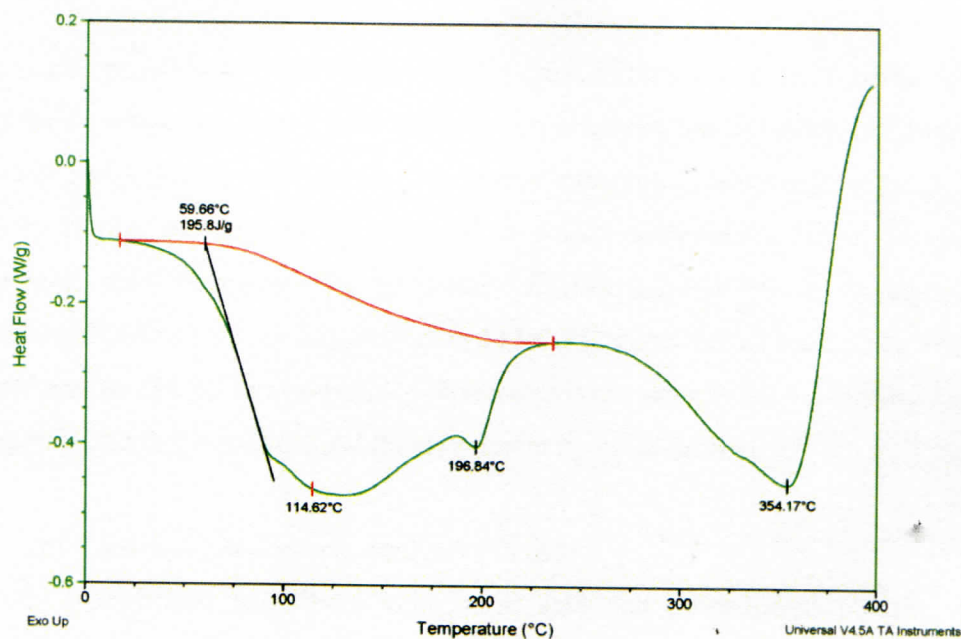


Figure 4.53: DSC thermogram of cross-linked phytase enzyme encapsulated nanofibers

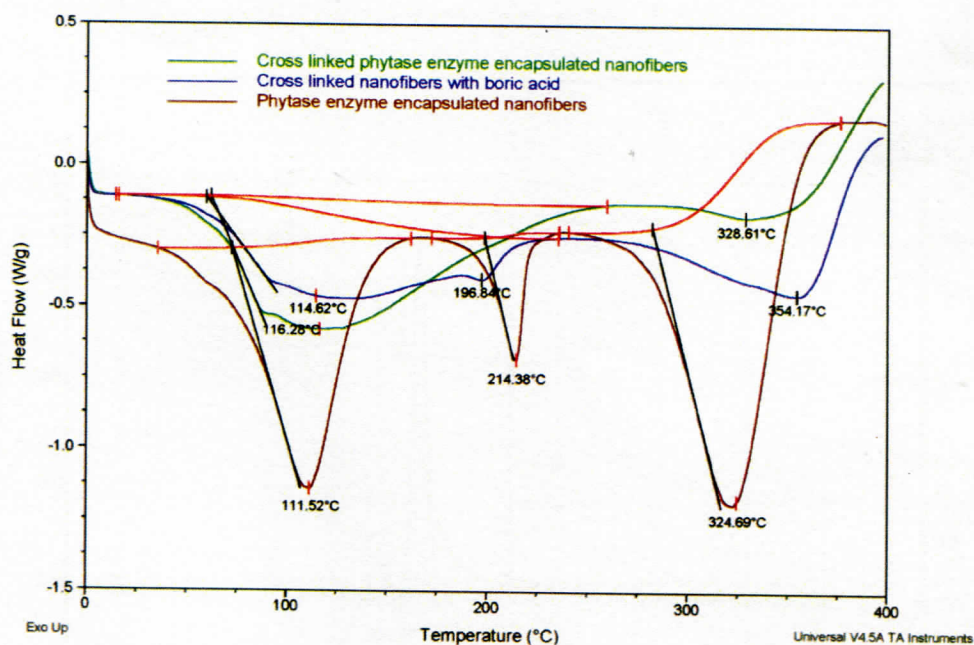


Figure 4.54: DSC thermogram of cross-linked phytase enzyme encapsulated nanofibers, phytase enzyme encapsulated nanofibers and cross-linked nanofibers with boric acid.

It is also possible to expect a variation in cross linking density in separate locations of the nanofibrous mat. Areas where the crosslinking has not taken place melts in a lower temperature, while areas with higher crosslink density requires much heat to melt. Hence, melting can be expected in a wide temperature range. Similarly the melting point corresponding to dietary fraction at 111 °C in phytase enzyme encapsulated nanofibers has shifted to 115 °C in cross-linked nanofibers with boric acid and to 116 °C in cross-linked phytase enzyme encapsulated nanofibers and this again is due to the thermal stabilization attained by cross linking.

(c) Thermal gravimetric analysis (TGA)

Both cross-linked nanofibers with boric acid and cross-linked phytase enzyme encapsulated nanofibers show a similar thermal profiles in shape. Cross-linked nanofibers show the thermal decomposition in the temperature range 39 – 200 °C and 259-540 °C (Figure 4.55). Cross-linked phytase enzyme encapsulated nanofibers decomposes in the temperature range 39 – 200 °C and 240-520 °C (Figure 4.56).

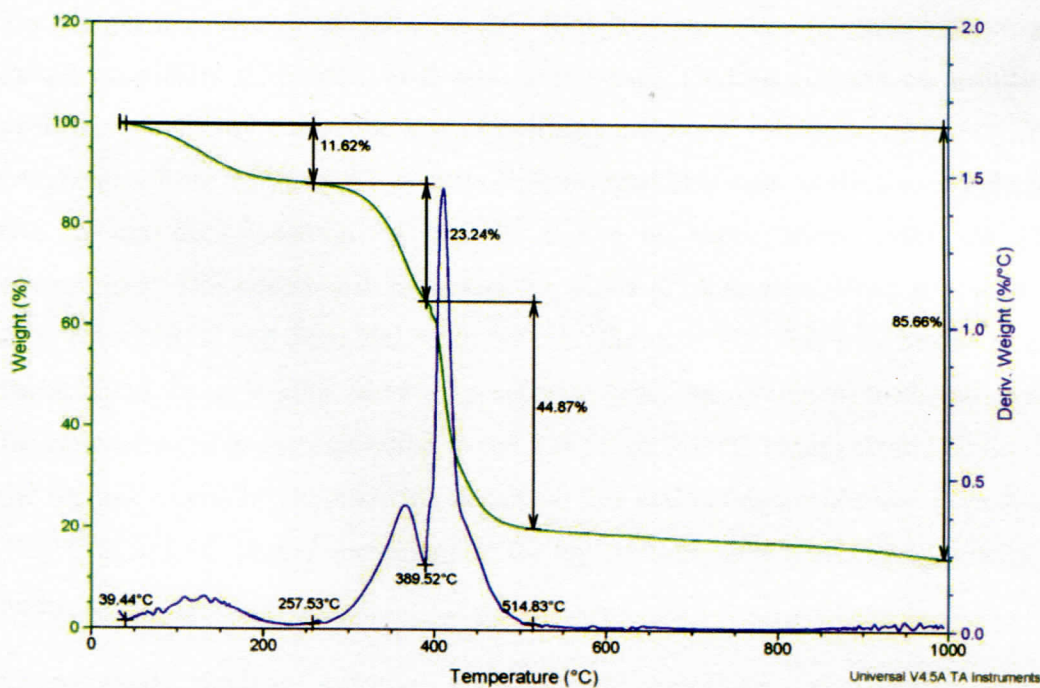


Figure 4.55: TGA thermogram of cross-linked nanofibers with boric acid

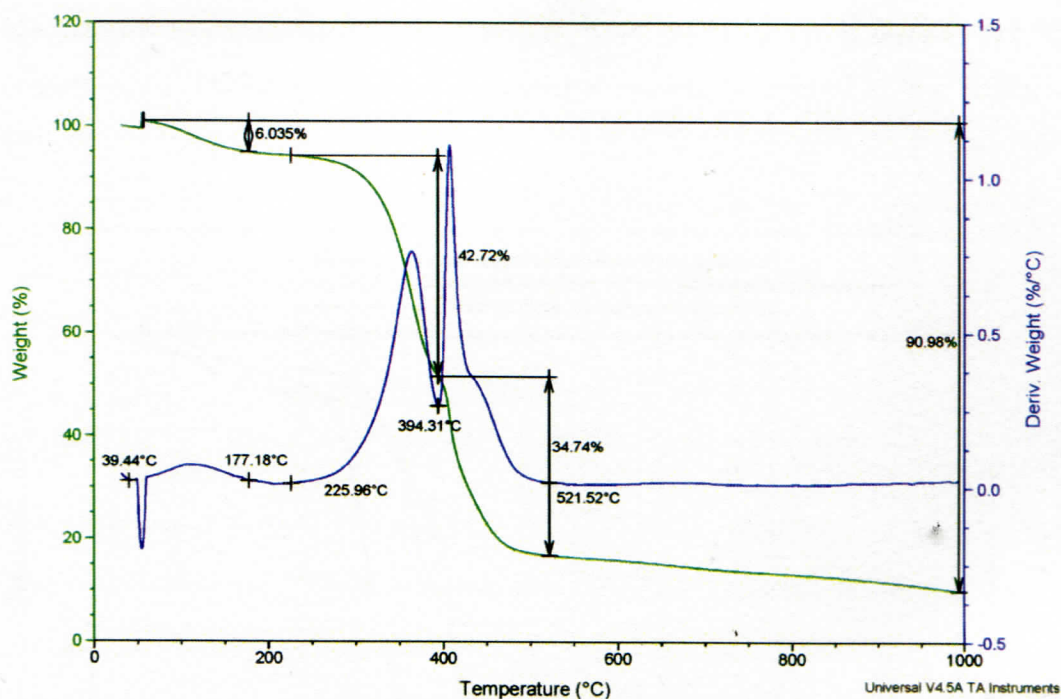


Figure 4.56: TGA thermogram of cross-linked phytase enzyme encapsulated nanofibers

The weight loss takes place between 39 – 200 °C temperature range in both cross-linked nanofibers with boric acid and cross-linked phytase enzyme encapsulated nanofibers is mainly due to the loss of moisture and other volatile components. The weight loss from 258-515 °C in cross-linked nanofibers with boric acid should be due to the decomposition of organic matter or vaporization under nitrogen environment. This decomposition associates with two close steps. They appear from 258 °C to 390 °C and from 390 °C to 540 °C (Figure 4.55). This may be due to the variation in cross linking density in separate locations of the nanofibrous mat. Decomposition step corresponding to the 258 °C to 390 °C region should be due to the regions where the cross linking density is less and the decomposition steps from 390 °C to 515 °C should correspond to the regions where the crosslinking density is high.

Cross-linked phytase enzyme encapsulated nanofibers also exhibits the decomposition of organic matter in the temperature range from 226-522 °C. It also

associates two decomposition steps from 226 °C to 394 °C and from there to 522 °C (Figure 4.56). The same argument discussed above can be applied to this situation also.

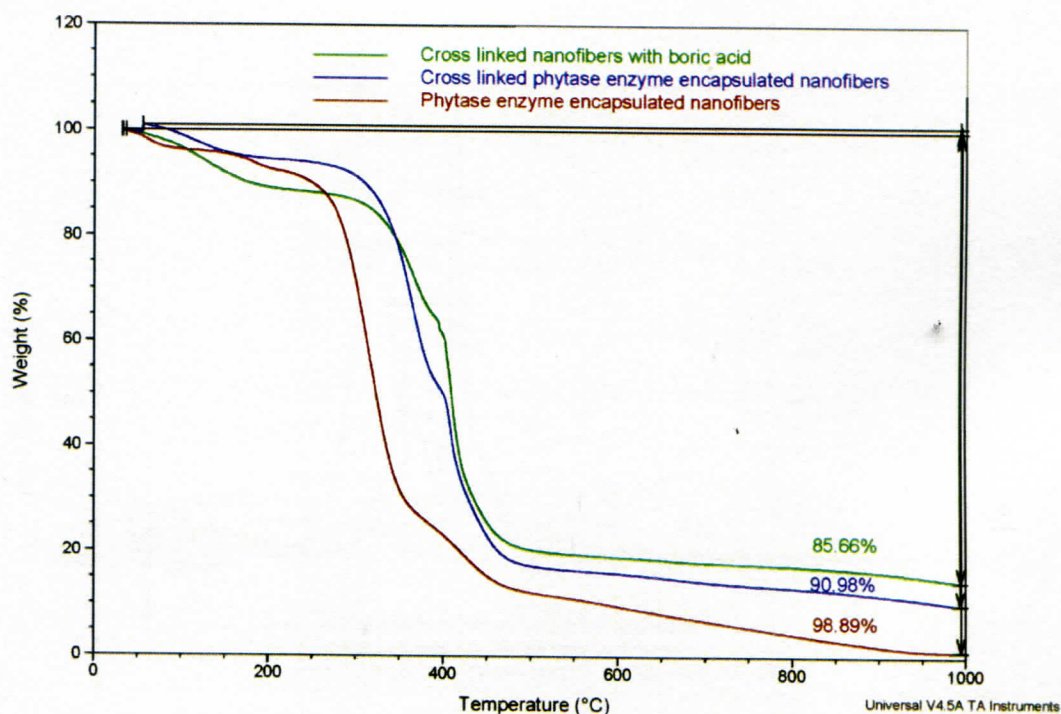


Figure 4.57: TGA thermogram of cross-linked phytase enzyme encapsulated nanofibers, cross-linked nanofibers with boric acid and phytase enzyme encapsulated nanofiber.

Phytase enzyme encapsulated nanofibers left around 2% of char under nitrogen environment while cross-linked phytase enzyme encapsulated nanofibers left around 10%. Cross-linked nanofibers with boric acid left around 14% of char under nitrogen environment. Higher char percentage in cross-linked nanofibers with boric acid is due to the presence of boron in cross links. Phytase enzyme encapsulated nanofibers are composed only with PVA and dietary fibers rice bran, thus small char percentage is expected. In cross-linked phytase enzyme encapsulated nanofibers the char percentage is around 10%, 4% lesser than in the cross-linked nanofibers with boric acid and this might be due to the reason explained bellow. Cross linking takes place between the surface - OH groups of the nanofibers and boric acid molecules as

explained in (4.9). In phytase enzyme encapsulated nanofibers, enzyme molecules have replaced some of the surface – OH groups, hence the number of cross links that can take place also is less. Therefore less number of boron atoms can be expected. Hence, lesser the char percentage (Figure 4. 57).

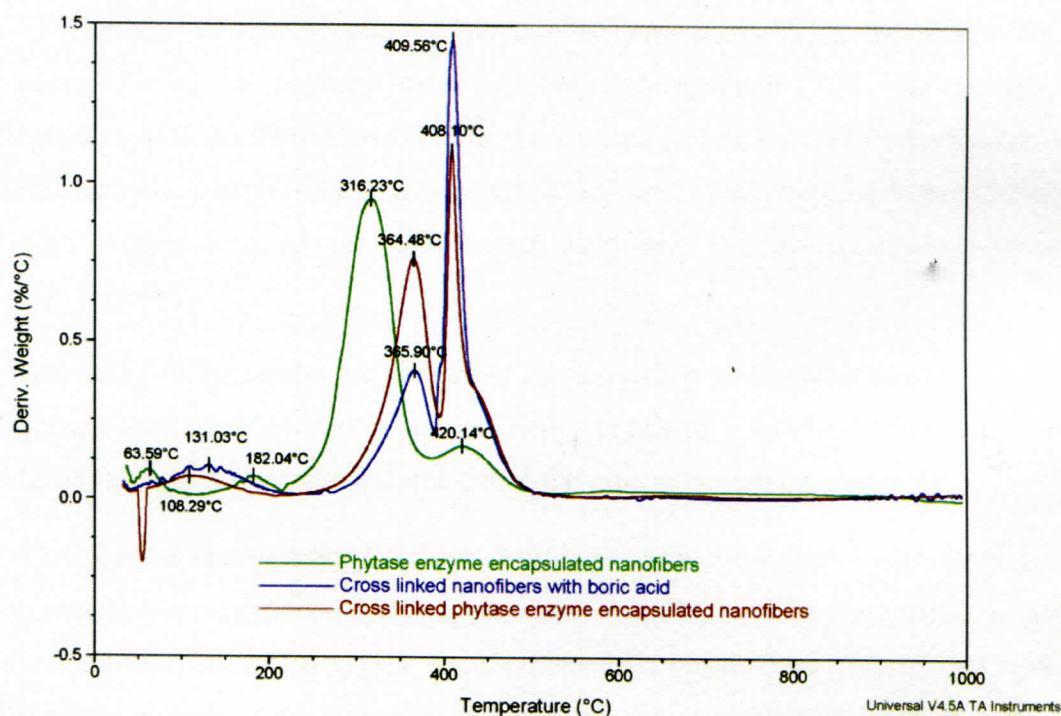


Figure 4.58: DTG thermograms of cross-linked phytase enzyme encapsulated nanofibers, cross-linked nanofibers with boric acid and phytase enzyme encapsulated nanofiber (comparison).

DTG data indicates the thermal decomposition temperatures of phytase enzyme encapsulated nanofiber is around 316 °C and in the cross-linked nanofibers with boric acid, show the thermal decomposition with two distinctive maximums in 366 °C and 410 °C and in cross-linked phytase enzyme encapsulated nanofibers again with two maximums in 364 °C and 408 °C (Figure 4.58). The decomposition temperature of cross-linked nanofibers with boric acid and cross-linked phytase enzyme encapsulated nanofibers has increased significantly relative to the phytase enzyme encapsulated nanofibers. This again is due to the cross links that holds the

nanofibers tightly together and make it difficult to decompose. Therefore an elevation of the decomposition temperature was expected.

4.9.1 Determination of the activity of the cross-linked phytase enzyme encapsulated nanofibers

The activity of the cross-linked phytase enzyme encapsulated nanofibers were accessed using the enzymatic assay protocol established in 3.2.10.6. The hypothesis had been that the thermal stability of the enzyme will be resulted by multipoint or multisubunit immobilization (El-Aassar, 2013) and, along with the extraordinarily high surface area of the synthesized nanofibers this effect should increase significantly.

According to the results the activity of the enzyme in cross-linked phytase enzyme encapsulated nanofibers have lost completely indicating a significant denaturation or inactivation of the phytase enzyme due to the process conditions employed.

This may be due to several reasons. In this research, cross-linked phytase enzyme encapsulated nanofibers were synthesized using the insitu encapsulation and entrapment method. During the insitu encapsulation method the enzyme was mixed with the polymer solution and subsequently electrospun in to fibers. Hence, there is no particular mechanism to control the amount of enzymes coming to the surface of the fibers and that is trapped inside, rather than controlling the fiber diameter. It is possible to expect that a significant amount of enzymes might be trapped inside the nanofibers rather than on the surface. Fibers with much smaller diameter could have given a higher fraction of enzymes on the surface, due to the increased surface area. Also, due to the initial mixing of the polymer with the enzyme could have covered and blocked the active sites of the enzymes making, it inactive.

Bernfeld et al., (1963) and Rosevear et al., (1987) also have reported, the limited mass transfer and inactivation of enzyme as a disadvantage in the insitu encapsulation technique as a method to immobilize enzymes.

In this research, to decrease the solubility of synthesized fibers in the gastric pH (pH-2.5), they were cross-linked using boric acid. This resulted cross-linked fibrous mats and it could have further masked the active sites of the enzymes and reduced the surface area.

All together these reasons could have hindered the arrival of the substrate to the active sites of the enzymes and exit of the products thus the effective activity might have gone down.

4.10 Synthesis of cross-linked surface modified nanofibers with phytase enzyme

Phytase enzyme carrying surface modified nanofibers were synthesized according to the procedure stated in the 3.2.10. Synthesized fiber mats were dipped in 10 fold diluted enzyme solutions for one hour under mechanical shaking (100 rpm) to make sure the complete adsorption of enzymes to the nanofibers. In a different study carried out by wang et al., (2006) have found that complete adsorption of lipase enzyme in to polysulfone nanofibers happen in one hour.

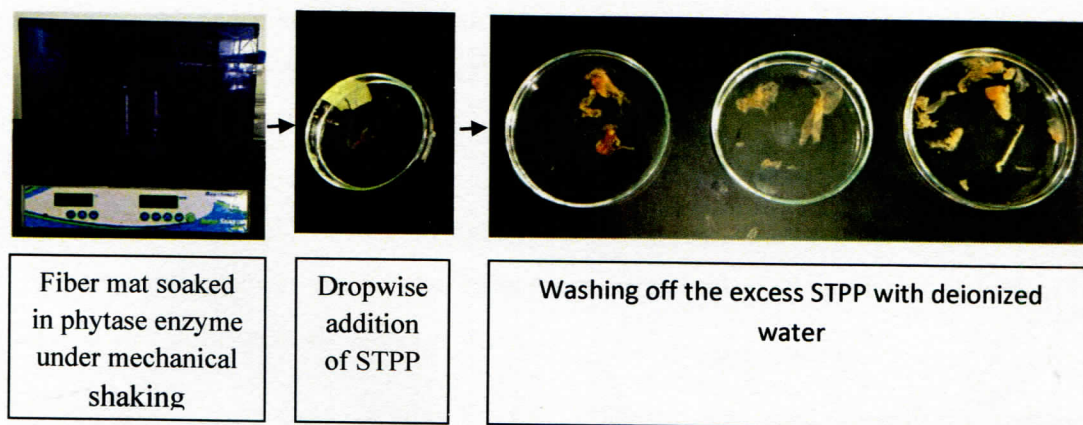


Figure 4.59: surface modification of nanofibers with Phytase enzyme and STPP.

Strong hydrogen bondings between surface $-OH$ groups present in nanofibers and free $C=O$ and NH groups present in phytase enzymes are expected in this step thus immobilization of enzyme in to nanofibers takes place.

After that excess enzyme was poured off and sodium tripolyphosphate (STPP) was added to the fibers drop wise. Sodium tripolyphosphate is an FDA approved chemical

widely used in the synthesis of chitosan nanoparticles. In chitosan there are lot of amino groups, hence they possess positive charge, thus it can self-assemble with tripolyphosphate groups possessing negative charges via ionic gelation (Yang et al., 2009). Similar mechanism is expected in between the enzyme molecules and sodium tripolyphosphate, since amino acids composed of phytase enzyme have amino groups which can undergo protonation and similar type of self-assembly can be expected with tripolyphosphate groups. Lot of researchers prefer to cross link enzyme with glutaraldehyde which reacts with enzymes and make permanent covalent bonds. Gluteraldehyde is a toxic chemical and is not recommended for dietary applications thus not been used in this research. Also glutaraldehyde make covalent bonds with enzymes while cross linking which make them extra rigid and lose the flexibility. This also can limit the activity of enzymes. Sodium tripolyphosphate can also make hydrogen bonds with surface -OH groups of nanofibers hence cross-links can also be expected in between them which provides more resistant towards dissolution in gastric pH.

Synthesized nanofibers were within the range of 30-50 nm, which are extremely small compared to the other nanofibers synthesized using agro wastes by various researchers. Fung et al. reports the synthesis of nanofibers using agrowastes generated from palm oil and soy industries and their diameters were within 100-500nm (Fung et al., 2010). Generally, nanofibers are defined in the U.S textile industry and Japanese and Korean strategic research initiatives as fibers of less than 1 μm in size (Gibson et al., 2007).

Once they are soaked in the enzyme solution and subsequently in sodium tripolyphosphate they have been formed into nanofibrous films. Hence, cross-linked surface modified nanofibers with phytase enzyme was resulted (Figure 4.60).

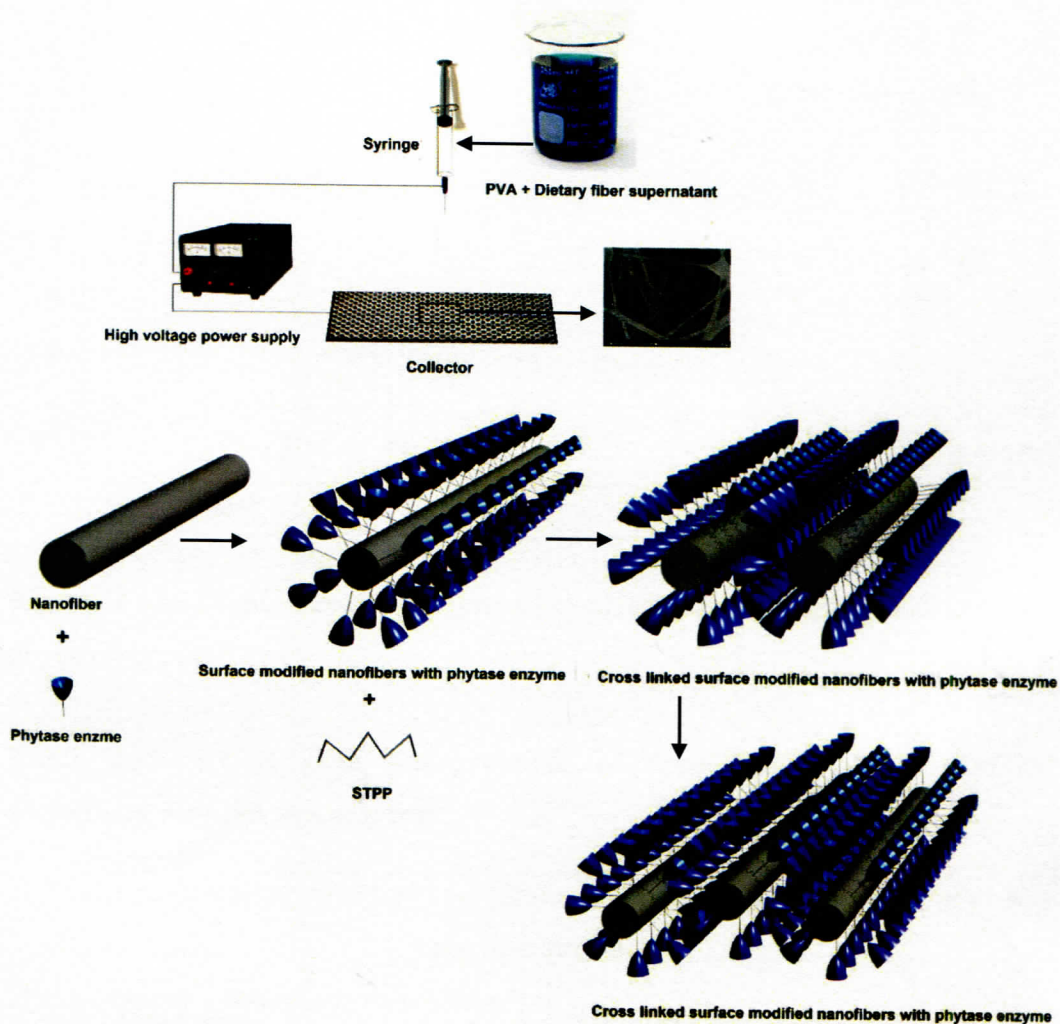


Figure 4.60: Mechanism for the surface modification of nanofibers with Phytase enzyme and STPP.

4.10.1 Characterization of surface modified nanofibers with phytase enzyme.

(a) Fourier transform infrared spectroscopy (FT-IR)

The FT-IR spectrum obtained for cross-linked surface modified nanofibers with phytase enzyme is shown in Figure 4.61 and Table 4.13 presents the FT-IR peak interpretation.

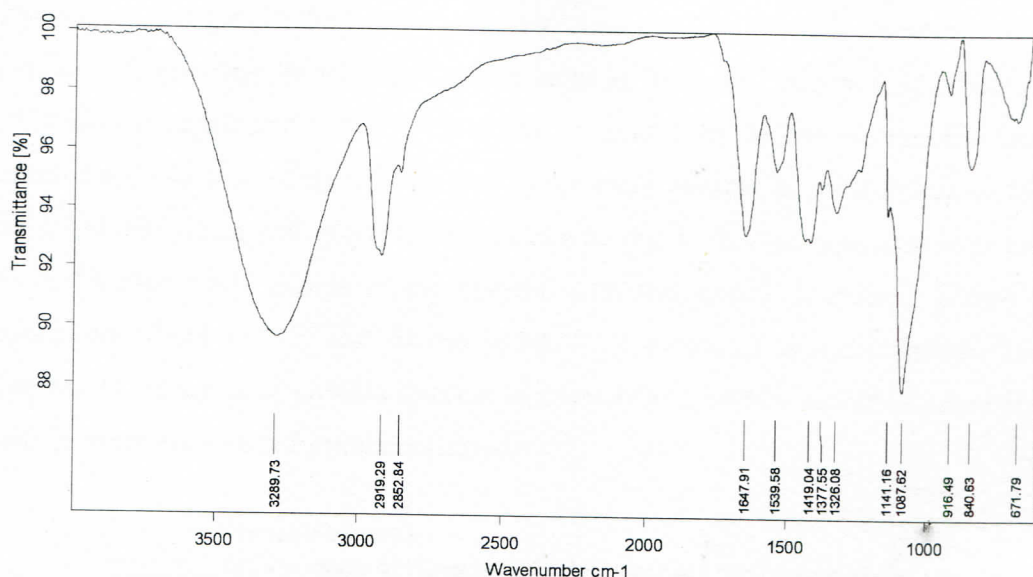


Figure 4.61: FT-IR spectrum of cross-linked surface modified nanofibers with phytase enzyme.

Table 4.13: FT-IR peak interpretation of cross-linked surface modified nanofibers with phytase enzyme.

FT-IR peak interpretation of cross-linked surface modified nanofibers with phytase enzyme		
Wave number (cm ⁻¹)	Reference values (cm ⁻¹) (Yadav, 2013)	Bond / Functional group vibration
3289	3200-3500	O-H , stretch(H bonded) in alcohols
2919	3000-2850	C-H stretch (alkanes)
2852	3000-2850	C-H stretch (alkanes)
1647	1690-1600	Amide C=O Stretch
1539	1575 - 1480	NH Bending of amides
1326	1320-1000	C-N stretch of aliphatic amines
1087	1320-1000	C-O stretch alcohols

In the FT-IR spectrum of cross-linked surface modified nanofibers with phytase enzyme gave several characteristic IR absorption bands corresponding to the

presence of enzyme. In the protein secondary structure C=O stretch vibrations of the peptide linkages, can be attributed to the band at 1647 cm^{-1} (figure 4.62) Bands at 1539 cm^{-1} are attributed to the NH Bending of amides. In the free enzyme this band appears at 1542 cm^{-1} . This shifting to a lower wave number in cross-linked surface modified nanofibers with phytase enzyme can be due to the hydrogen bondings take place between -NH groups of the enzyme with the surface functional groups in nanofibers. Band at 1326 cm^{-1} is due to the C-N stretch of aliphatic amines. That Hence, the presence of phytase enzyme in cross-linked surface modified nanofibers with phytase enzyme is further confirmed.

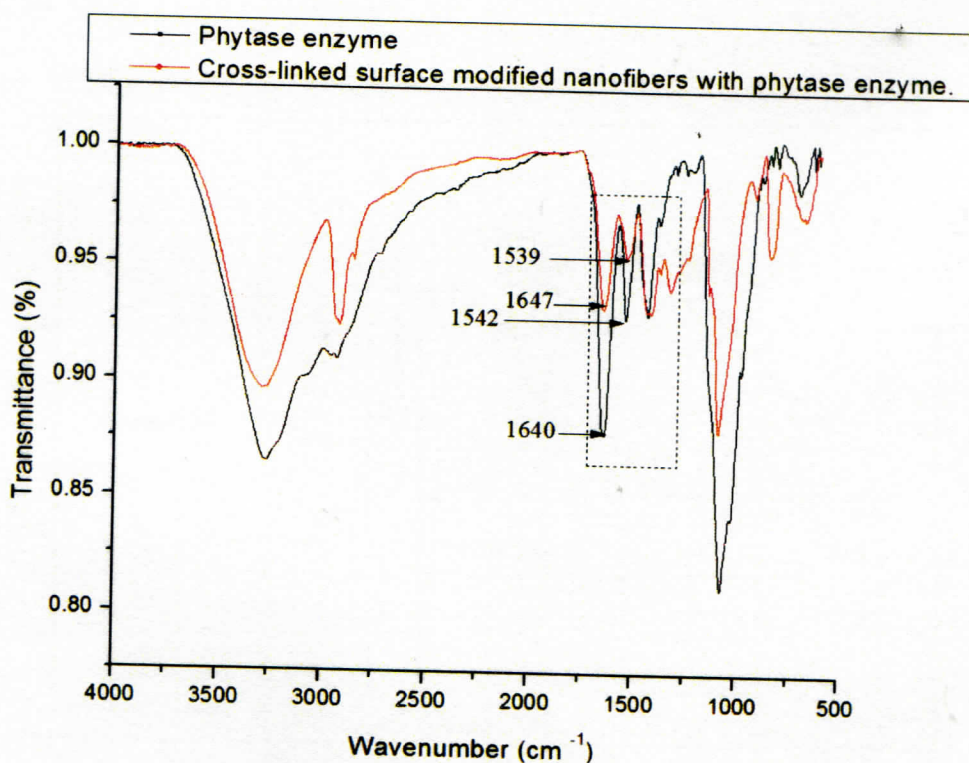


Figure 4.62: FT-IR spectrum of cross-linked surface modified nanofibers with phytase enzyme and phytase enzyme.

In Figure 4.62, the presence of broad band at 3289 cm^{-1} attributes to the O-H stretching. This may be due to both -O-H bond vibration in the PVA molecules as well as in the enzyme. In addition N-H stretches of amines appears are in the region $3300\text{-}3000\text{ cm}^{-1}$, usually to the higher region. They might have overlap with the band

corresponding to the O-H stretching, hence differentiating them is not possible. The strong band at 1092 cm^{-1} attributes to the C–O stretch alcohols or carboxylic acids.

(b) Differential scanning calorimetry (DSC)

The interaction between cross-linked nanofibers with STPP and cross-linked surface modified nanofibers with phytase enzyme were examined by their thermal behavior. The cross-linked nanofibers with STPP exhibited four endotherms. The endotherm corresponding to the melting of rice bran is at $167\text{ }^{\circ}\text{C}$ and the melting of PVA is at $220\text{ }^{\circ}\text{C}$. The endothermic peak at $346\text{ }^{\circ}\text{C}$ is due to the decomposition of the fiber (Figure 4.63).

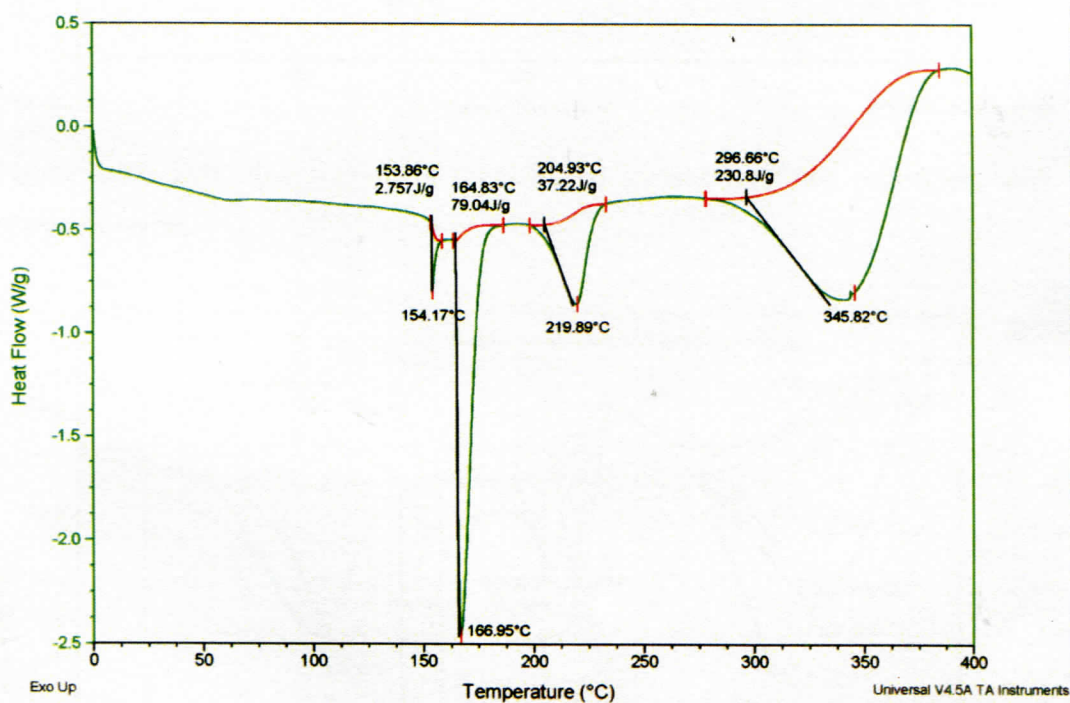


Figure 4.63: DSC thermogram of cross-linked nanofibers with STPP.

Cross-linked surface modified nanofibers with phytase enzyme exhibited similar endotherms at $150\text{ }^{\circ}\text{C}$, $222\text{ }^{\circ}\text{C}$ and $340\text{ }^{\circ}\text{C}$ with respect to similar thermal events (Figure 4.64).

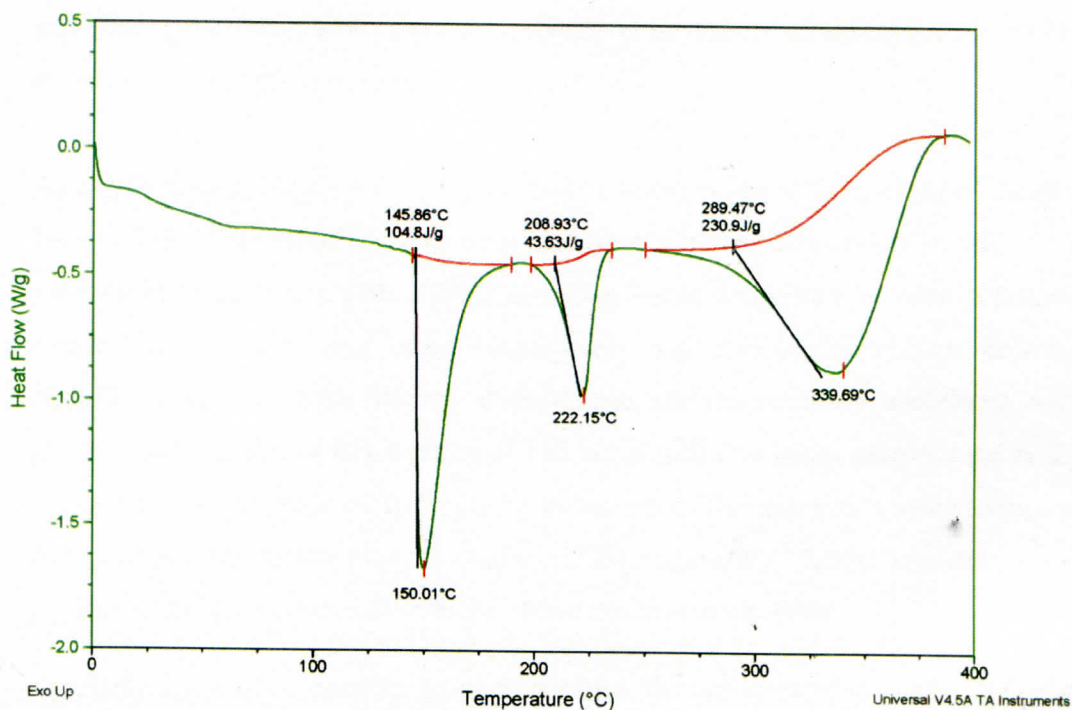


Figure 4.64: DSC thermogram of cross-linked surface modified nanofibers with phytase enzyme.

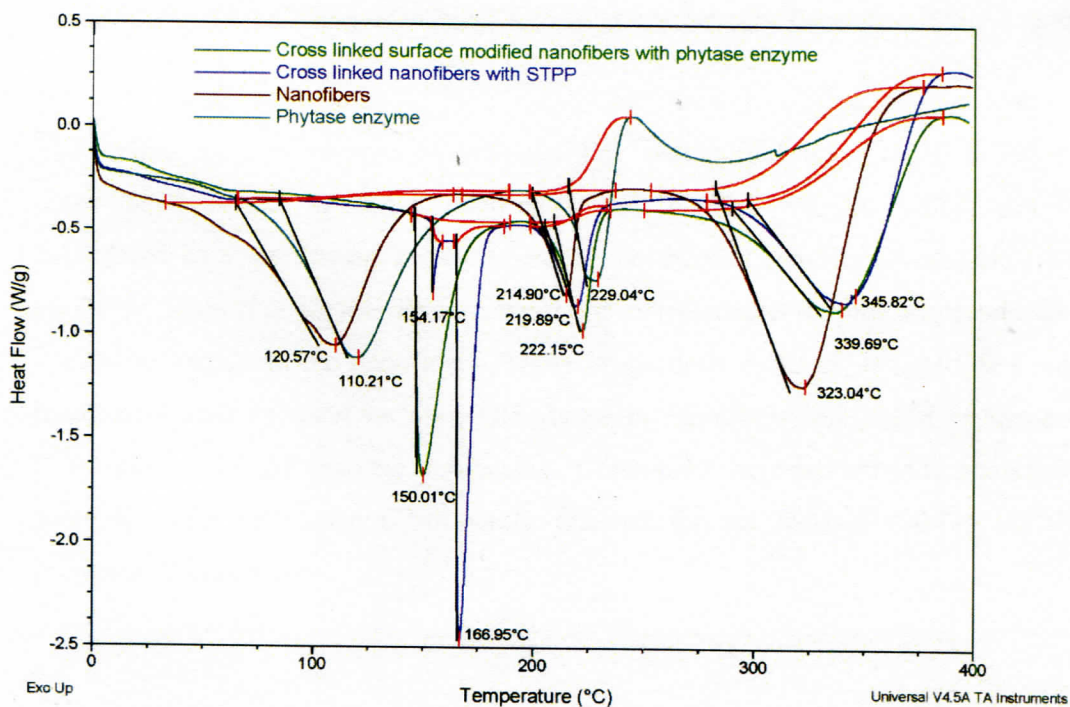


Figure 4.65: Comparison between DSC thermograms of cross-linked surface

modified nanofibers with phytase enzyme, cross-linked nanofibers with STPP, nanofibers and phytase enzyme.

As can be seen in Figure 4.65 the endotherm corresponding to the melting of the rice bran at 110 °C in nanofibers has been shifted to a higher temperature to 167 °C in cross-linked nanofibers with STPP, indicating better interaction between cellulose, hemicellulose, lignin and other biopolymers and stronger interaction between nanofibers due to cross linking. Cross-linked surface modified nanofibers with phytase enzyme shows this melting at 150 °C, a shift to a lower temperature value, suggesting the presence of an impurity in the fiber. The impurity corresponding to this phenomenon is the phytase enzyme. This argument is further assisted by the peak broadening corresponding to the above mentioned transition.

Similarly, the melting corresponding to the PVA has been shifted in to a higher value in both cross-linked surface modified nanofibers with phytase enzyme and cross-linked nanofibers with STPP compared to nanofibers which is from 215 °C to 222 °C and 220 °C. This is due to the same reason explained above. Decomposition temperatures of the fibers also have increased accordingly from 323 °C to 340 °C and 346 °C (Figure 4.65).

The melting point of phytase enzyme also has been shifted from 110 °C to 150 °C cross-linked surface modified nanofibers with phytase enzyme, which can be considered as a significant increasement of the thermal stability of enzyme. This could be again due to the strong interaction between the enzyme molecules and functional groups in the nanofibers. These interactions could be assigned as strong hydrogen bonds between surface -OH groups in nanofibers and -NH groups and C=O groups in the enzyme molecules. Cross-links between enzyme molecules through STPP also have significantly affected for the thermal stability of the enzymes (Figure 4.65).

(c) Thermal gravimetric analysis (TGA)

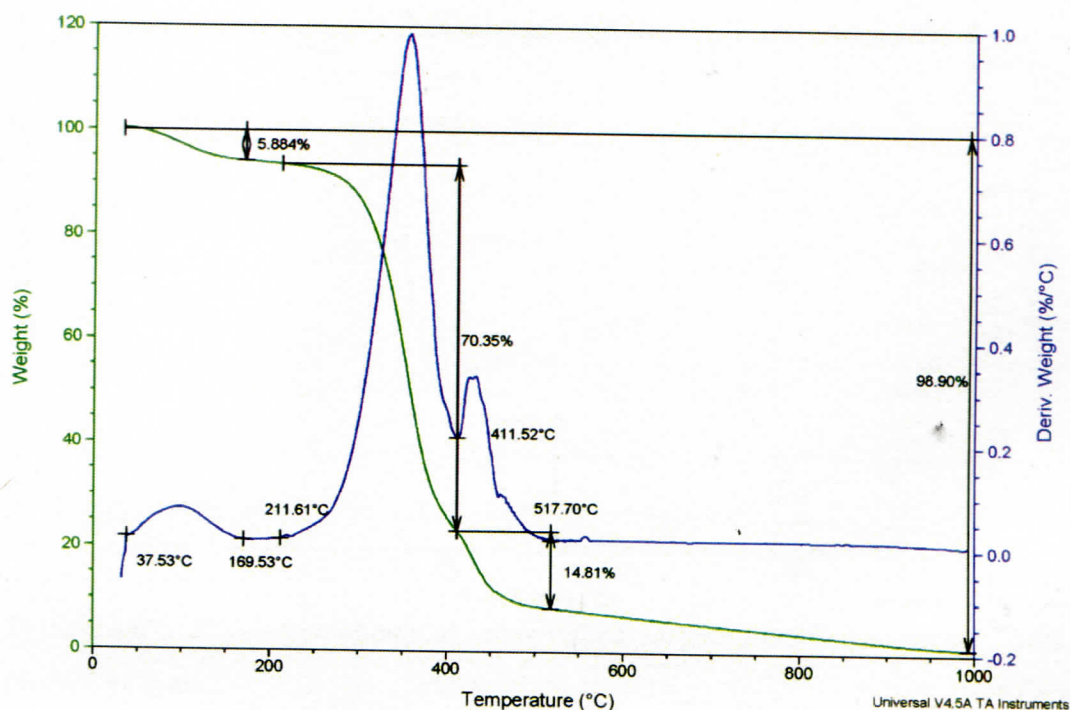


Figure 4.66: TGA thermogram of cross-linked nanofibers with STPP

Both cross-linked nanofibers with STPP and cross-linked surface modified nanofibers with phytase enzyme shows very similar thermal profiles in shapes. Both fibers shows the thermal decomposition in the temperature range 202-517 °C (Figure 4.66, Figure 4.67).

The weight losses corresponding to 37-170 °C temperature range in cross-linked nanofibers with STPP and 37-213 °C temperature range in cross-linked surface modified nanofibers with phytase enzyme, might be due to the loss of moisture and other volatile organic matter.

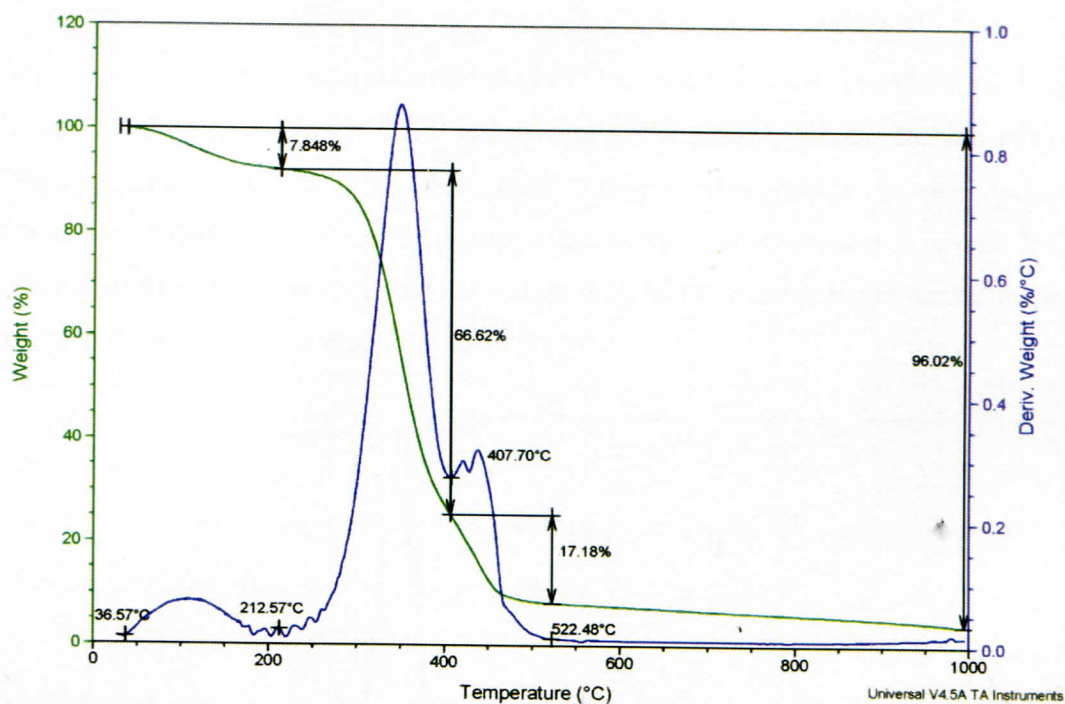


Figure 4.67: TGA thermogram of cross-linked surface modified nanofibers with phytase enzyme

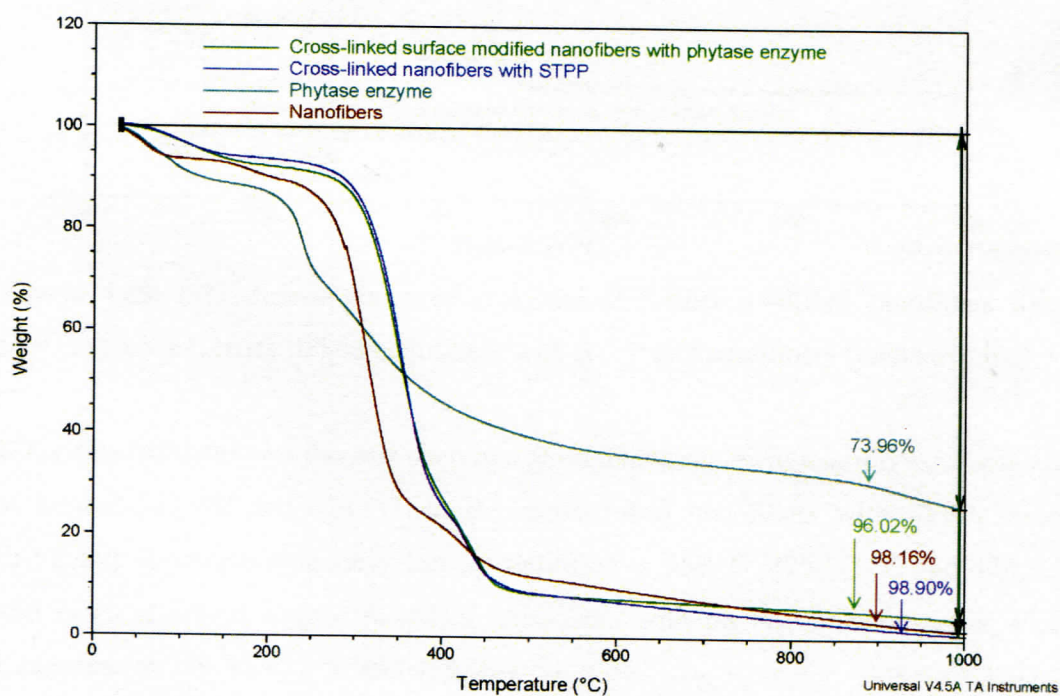


Figure 4.68: TGA thermogram of cross-linked surface modified nanofibers with phytase enzyme, cross-linked nanofibers with STPP, phytase enzyme and nanofibers.

When heated under nitrogen environment in TGA, nanofibers left around 2% of char, cross-linked nanofibers with STPP left around 1.5%, roughly similar to each other. Phytase enzyme left 26 % of char under nitrogen environment. In cross-linked surface modified nanofibers with phytase enzyme, the char percentage is around 4%, 2% higher than in the cross-linked nanofibers with STPP and this might be due to the presence of enzymes and high amount of STPP.

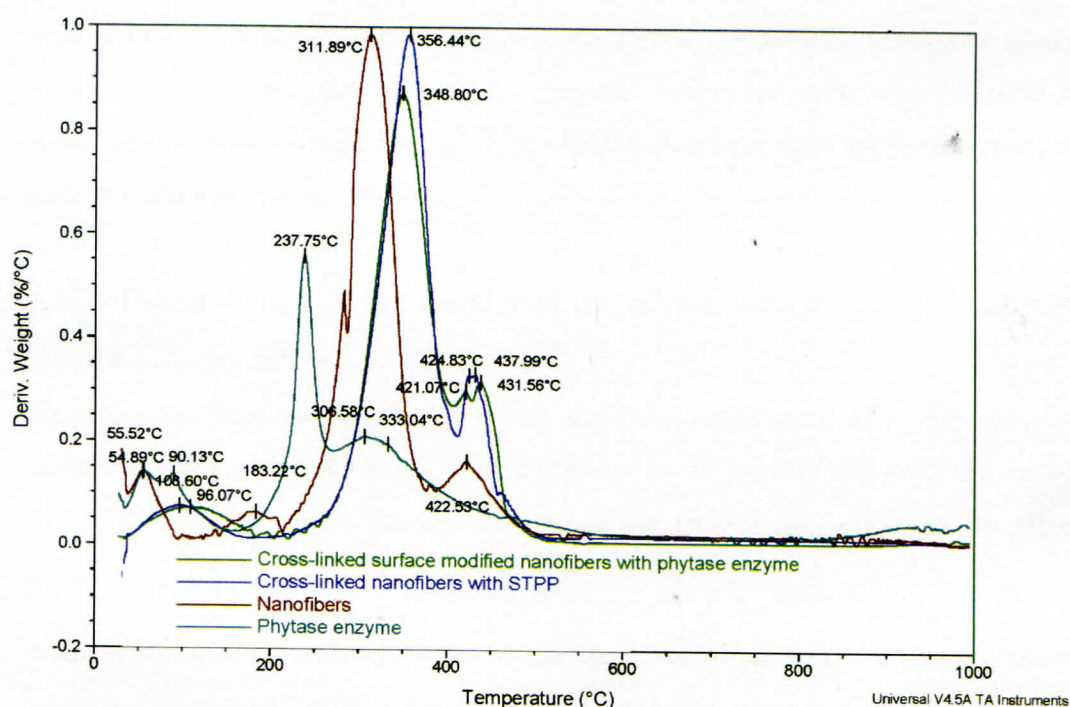


Figure 4.69: DTG thermograms of cross-linked surface modified nanofibers with phytase enzyme, cross-linked nanofibers with STPP and nanofibers (comparison).

DTG data indicates two thermal decomposition temperatures in nanofibers which are at around 312 °C and 423 °C. In the cross-linked nanofibers with STPP, three distinctive decomposition steps can be identified at 356 °C, 425 °C °C and 438 °C and in cross-linked surface modified nanofibers with phytase enzyme again with three steps at 349 °C, 421 °C and 438 °C .

The thermal decomposition step at 312 °C in nanofibers has been shifted to 356 °C in cross-linked nanofibers with STPP and to 348 °C in cross-linked surface modified

nanofibers with phytase enzyme. This is due to the thermal stability attained by the cross linking of fibers with STPP. Due to the cross linking fiber strands holds together strongly making it resistant to thermal decomposition.

The thermal decomposition step at 238 °C in phytase enzyme cannot be seen in cross-linked surface modified nanofibers with phytase enzyme. It has been shifted to 349 °C which is a significant thermal stabilization of the enzyme. This could be due to the strong hydrogen bonds between surface –OH groups in nanofibers and –NH groups and C=O groups in the enzyme molecules. Cross-links between enzyme molecules with enzyme molecules, enzyme molecules with nanofibers and nanofibers with nanofibers through STPP might also have affected for the thermal stability of the enzymes.

4.10.2 Determination of the activity of the phytase enzyme carrying surface modified dietary fibers.

Free and the fiber bound activity of the enzyme was assessed using the protocol established in 3.2.11.4. An extra thermal stability of the enzyme was expected due to the multi-point or multi-subunit immobilization (El-Aassar, 2013) to the fiber surface.

In this method, the surface modification of the synthesized nanofibers with phytase enzyme was carried out by submerging an appropriate amount of nanofibers in the tenfold diluted enzyme solution and crosslinking it with STTP, subsequently. Equal amount of free enzyme and fiber bound enzyme was taken and subjected to 50 to 170 °C temperatures in 10 °C intervals, keeping in a water bath and a mineral oil bath respectively, for 5 min. After wards the samples were brought back to room temperature and the activity was established (3.2.11.5)

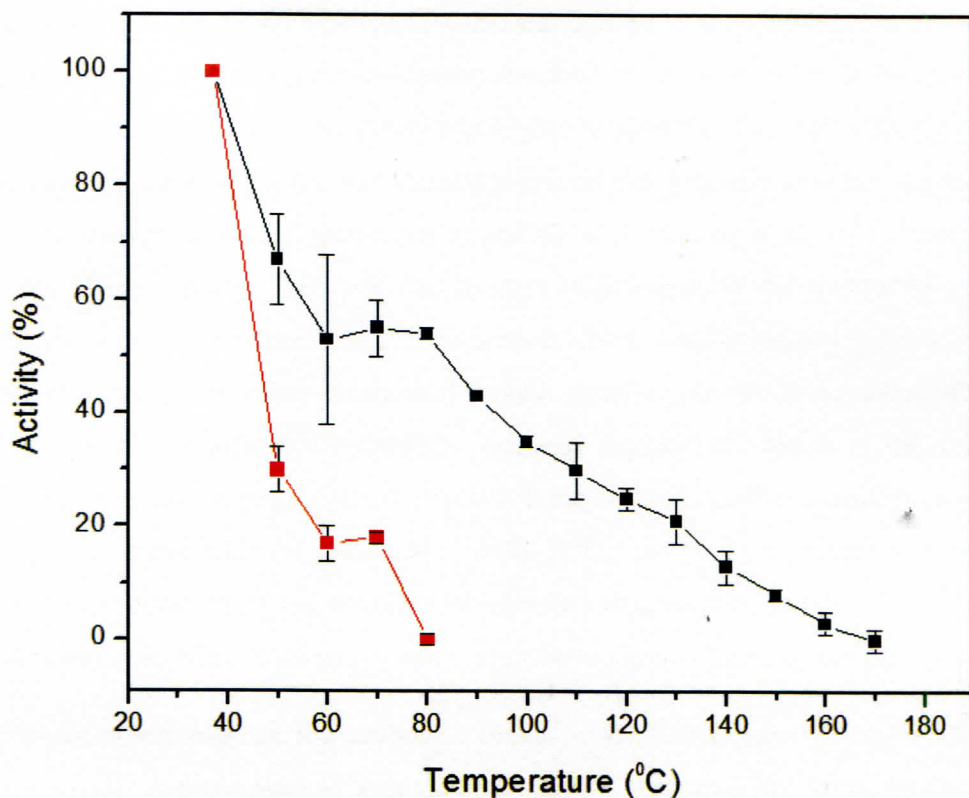


Figure 4.70: Activity (%) of cross-linked surface modified nanofibers with phytase enzyme and free phytase enzyme after being exposed to different temperatures

Each enzyme has an optimum temperature at which the enzyme works at its best. For phytase enzyme the optimum temperature is around 40 – 45 °C (Naves et al., 2012). As the temperature continues to rise above the optimum, the activity and the rate of the reaction decrease abruptly due to irreversible denaturation of enzymes and losing the biological activity.

This is clearly compatible with the results (Figure 4.70) as the free phytase enzyme lost its activity completely around 70 °C -80 °C. The same observation has been reported by Wyss et al., (1998). He has reported the deactivation of phytase enzyme at 70 °C.

Phytase enzyme has been widely used as animal feed additive mainly for swine and poultry, and for fish to some extent in order to improve the utilization of phosphate from phytic acid, main storage of phosphate in cereals and grains. This option though,

has been challenged a lot with the low thermal stability of phytase enzyme. However, application of high temperature during the feed stock preparation is unavoidable, since the feed pelleting process requires higher temperature i.e. $\sim 60 - 70^{\circ}\text{C}$. Lot of researchers have reported the denaturation of the phytase enzyme during the pelletization process at temperatures around 80°C (Kirkpinar et al., 2006; Silversides et al., 1999). Usually, there are two process responsible for the activity loss of an enzyme with temperature. First, denaturation which usually happen because of the loss of tertiary (and often secondary) protein structure not involving covalent bond cleavage, and principally reversible. Second, degradation which is the loss of primary structure with associated covalent bond cleavage and/ or formation and this process is irreversible (Daniel et al., 1996). Either one or both process can involve for the inactivation of the enzymes at elevated temperature, usually above 80°C . Inactivation by heat denaturation has a profound effect on the enzymes productivity.

Contrast to free enzyme, the surface modified enzyme carrying nanofibers have shown a significant improvement in their thermal stability (Figure 4.70). While free enzyme loses its activity around $70 - 80^{\circ}\text{C}$, surface modified enzyme carrying nanofibers continues to show the activity up to 170°C , an improvement of 100°C . Upper temperature limit for enzyme stability depends on the conformational integrity of the enzyme or protein. Greater the conformational integrity, greater the stability is. Greater thermal stability of the surface modified enzyme carrying nanofibers could have been due to the immobilization of enzymes on the nanofiber surface. Strong hydrogen bonding can be expected in between the surface - OH groups present in synthesized nanofibers and free $\text{C}=\text{O}$ and NH groups present in the phytase enzyme. PVA and the biopolymers which are present in rice bran extract are responsible for the surface - OH groups in the nanofibers. Subsequent cross linking with sodium tripolyphosphate may have further increased the thermal stability. Strong hydrogen bonding between $^-\text{O}-\text{P}$ groups in sodium tripolyphosphate and free NH groups in enzyme and - OH groups in the fiber surface is responsible for stable cross-links between enzyme molecules and enzyme molecules, enzyme molecules with fiber surface and fibers with fibers.

Similarly, greater thermal stability up to 140 °C has been obtained for different enzymes through immobilization and in the presence of stabilizing agents (Daniel et al., 1996).

CHAPTER 5

5. CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE WORKS

5.1 Conclusions

In this research dietary fibers extracted from rice bran were effectively used to synthesize nanofibers in the diameter range of 30 – 50 nm. Dietary fibers were extracted from rice bran under alkaline conditions (pH - 9) and amended with PVA (8% v/w) to adjust the viscosity. SEM micrographs indicate that the surface of the electrospun nanofibers are smooth, free from beads-on-string structures and uniform in diameter. Presence of both PVA and dietary fibers extracted from rice bran in nanofibers were confirmed by DSC analysis. TGA data confirmed the better thermal stability of nanofibers over PVA and dietary fibers alone, suggesting an extra stability gained by spinning into fibers. So far, only few reports can be found in the literature regarding synthesis of nanofibers using agrowaste and this could be the first to report a successful utilization of rice bran to this purpose.

In this research two different techniques were investigated encapsulate Phytase enzyme in to synthesize nanofibers.

First technique is insitu encapsulation, in which the phytase enzyme was mixed with dietary fibers and PVA solution initially and then electroapun in to nanofibers. This technique resulted phytase enzyme encapsulated nanofibers in the diameter range of 50 – 100 nm. Due to the addition of phytase enzyme the stability of the electrospinning solution decreased. Hence, the diameter of the fibers increased. However, that was well within the nano range. SEM micrographs indicate that the surface of the phytase enzyme encapsulated nanofibers are smooth, though associated with considerable amount of beads-on-string structures and this is due to the instability of the electrospinning solution. FT-IR data confirmed the presence of enzyme in the phytase enzyme encapsulated nanofibers and DSC data indicated the stabilization of phytase enzyme to the nanofibers possibly via hydrogen bonds. TGA

data indicated the thermal degradation temperature (238 °C) of the enzyme has been shifted to a higher value (316 °C) due to spinning into fibers.

However, phytase enzyme encapsulated nanofibers tend to dissolve in the stomach pH (pH - 9). To overcome this solubility problem they were cross-linked with boric acid. This resulted cross-linked fiber mats of phytase enzyme encapsulated nanofibers with better stability towards solubility. DSC and TGA data confirmed a significant stabilization of the cross-linked phytase enzyme encapsulated nanofibers against thermal degradation under nitrogen environment.

However, the enzymatic assays confirmed the phytase activity of the cross-linked phytase enzyme encapsulated nanofibers have been lost completely. This may be due to limited mass transfer and inactivation of enzyme by the technique and the conditions employed.

Second technique is the surface modification in which the phytase enzyme was immobilized in to nanofibers by adsorption and subsequently cross-linked with sodium tripolyphosphate. Strong hydrogen bondings between surface -OH groups present in nanofibers and free -C=O and -NH groups present in phytase enzymes were expected in this step. FT-IR analysis confirmed the presence of enzymes in the nanofibers.

Cross linking was done to increase the enzyme loading capacity of cross-linked surface modified nanofibers with phytase enzyme. Phytase enzyme possess amino groups hence can bare positive charge via protonation. These can self-assemble with tripolyphosphate groups possessing negative charges via ionic bonds. Hence, enzyme cross linking takes place. STPP can also make hydrogen bonds between surface -OH groups of nanofibers and cross-linking is also expected in between them thus better stability under stomach pH is observed. DSC analysis confirmed better interaction and better stability of cross-linked surface modified nanofibers with phytase enzyme as the melting point of the nanofibers (110 °C) has been shifted to a higher value (150 °C) with the surface modification. TGA data confirmed the degradation

temperature of the enzyme (238 °C) has been shifted to a higher value (349 °C) by encapsulation and cross linking in to nanofibers.

The enzymatic assays confirmed a significant increasement of the thermal stability of phytase enzyme in cross-linked surface modified nanofibers with phytase enzyme over free enzyme. While free enzyme loses its activity around 70 – 80 °C, surface modified enzyme carring nanofibers continues to show the activity up to 170 °C, an improvement of 100 °C.

5.2 Recommendations for future work

1. In this research phytase enzyme was stabilized in to rice bran based nanofibers. Similarly, the study can be extended for other enzyems, which have significant industrial importance.
2. The agro waste selected for this research was rice bran. The study can also be extended to other agro waste materials as well.
3. Insitu encapsulation technique was found to be ineffective for phytase enzyme in this research. Rather than electrospinning, co-electrospinning could be a better method to associate with insitu encapsulation. Therefore, that can be tested for phytase enzyme.
4. Better air pressure controlling system could be designed to improve the multi-syringe pump, since the operation of this pump is very cost effective.

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APPENDIX

Appendix 1:

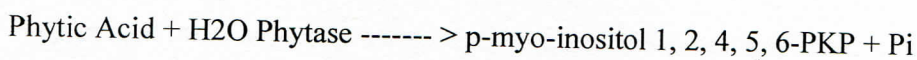
Enzymatic Assay of Phytase (Heinonen et al., 1981)

1. Objective

Determine the activity of phytase,

2. Scope

The scope of this procedure includes the following reaction:



3. Definitions

3.1. Purified Water – Water from a deionizing system

3.2. p-myo-inositol 1,2,4,5,6-PKP = p-myo-inositol 1,2,4,5,6-Pentakisphosphate

3.3. Phytic Acid = Myo-Inositol Hexakis (dihydrogen phosphate)

3.4. Pi = Inorganic Phosphate

4. Discussion

4.1. Phytic Acid + H₂O Phytase -----> p-myo-inositol 1, 2, 4, 5, 6-PKP + Pi

4.2. Method = Colorimetric Determination, Temperature (T) = 37°C, pH = 2.5,
A_{420nm}, Light path = 1cm

5. Procedure

5.1 Reagents

5.1.1 200 mM Glycine Buffer, pH 2.8 at 37°C (Buffer)

5.1.1.1. Prepare 500 ml in purified water using Glycine.

5.1.1.2. Adjust to pH 2.5 at 37°C with 1M NaOH or 1M HCl.

5.1.2. 0.5478 mM Phytic Acid Solution, pH 2.5 at 37°C (Phytic Acid)

5.1.2.1. Prepare 500 ml in Reagent 5.1.1 using Phytic Acid, Dipotassium.

5.1.2.2. Adjust to pH 2.5 at 37°C with 1M HCl.

5.1.3. 20% (w/v) Ammonium Molybdate Solution (Amm Moly)

Prepare 100 ml in hot water using Molybdic Acid, Ammonium Tetrahydrate Salt.

5.1.4 70% nitric acid.

5.1.5 1% (w/v) ammonium vanadate

Prepare 100 ml in hot water using Molybdic Acid, Ammonium Tetrahydrate Salt.

5.1.6 Color Reagent Solution (CRS)

5.1.6.1 Prepare 1000 ml by adding 100 ml of Reagent 5.1.3 to 140 ml of Reagent 5.1.4 and 100 ml of Reagent 5.1.5.

5.1.6.2 Mix and use immediately.

5.1.6.3 Prepare fresh.

5.1.7 2.8 mM Potassium Phosphate Solution (P Std.)

Prepare 250 ml in deionized water using Potassium Phosphate, Monobasic.

5.1.8 Phytase Enzyme Solution (Enzyme)

Immediately before use, prepare a solution containing 0.5 – 2.0 units/ml of Phytase in cold Reagent 5.1.1.

5.2. Procedure – Blank/Test Preparation

5.2.1. Pipette (in milliliters) the following reagents into a 4 dram vial:

	Test	Blank
Reagent 7.1.2 (Phytic Acid)	15	15

5.2.2. Equilibrate to 37°C, then add:

Reagent 5.1.8 (Enzyme)	0.5	----
Reagent 5.1.1 (Buffer)	----	0.5

5.2.3. Immediately mix by inversion and incubate at 37°C for exactly 20 minutes.

Then add:

Reagent 5.1.6 (CRS)	12.50	12.50
Purified Water	22	22

5.2.4. Mix by swirling, then transfer to suitable cuvettes and record the A_{420nm} for both the test and blank using a suitable spectrophotometer.

5.3. Procedure – Standard Curve

5.3.1 Prepare by pipetting (in milliliters) the follow reagents into suitable containers:

	Std	Std	Std	Std	Std	Std	Std	Std.
	1	2	3	4	5	6	7	Blk.
Purified Water	7.5	6.25	5	3.75	2.5	1.25	-----	8.75
Reagent 5.1.7 (P Std.)	1.25	2.5	3.75	5.00	6.25	7.5	8.75	-----
Reagent 5.1.2 (Phytic Acid)	15	15	15	15	15	15	15	15

5.3.2 Mix by swirling and incubate at 37°C for 20 minutes. Then add:

Reagent 5.1.6 (CRS)	12.5	12.5	12.5	12.5	12.5	12.5
Purified Water	13.75	13.75	13.75	13.75	13.75	13.75

5.3.3 Mix by swirling, then transfer to suitable cuvettes and record the $A_{420\text{nm}}$ for both the standards and the standard blank using a suitable spectrophotometer.

5.4. Calculations

5.4.1 Standard Curve

5.4.1.1 $\Delta A_{420\text{nm}} \text{ Standard} = A_{420\text{nm}} \text{ Standard} - A_{420\text{nm}} \text{ Standard Blank}$

5.4.1.2 Prepare a standard curve by plotting $\Delta A_{420\text{nm}}$ versus μmoles of phosphate.

5.4.2 Sample Determination

5.4.2.1 $\Delta A_{420\text{nm}} \text{ Test} = A_{420\text{nm}} \text{ Test} - A_{420\text{nm}} \text{ Test Blank}$

5.4.2.2 Determine the μmoles of phosphate liberated using the standard curve.

$$5.4.2.3 \quad \text{Units/ml enzyme} = \frac{(\mu\text{moles of phosphate released}) (df)}{(20)(0.5)}$$

df = dilution factor

20 = Time (in minutes) of assay per the Unit Definition

0.5 = volume (in milliliters) of enzyme used

$$5.4.2.4 \quad \text{Units/mg solid} = \frac{\text{units/ml enzyme}}{\text{Mg solid/ml enzyme}}$$

