

LB/DON/76/2015

RICE BRAN BASED NANOFIBERS FOR STABILIZATION OF PHYTASE ENZYME

Upendra Amal Rathnayake

(138046L)

Degree of Master of Science

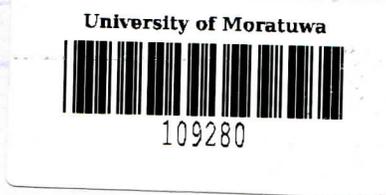
Department of Chemical and Process Engineering

University of Moratuwa

Sri Lanka

66"15"

66(043)



109280
+
CD-ROM
TH2929

109280

**RICE BRAN BASED NANOFIBERS FOR
STABILIZATION OF PHYTASE ENZYME**

Upendra Amal Rathnayake

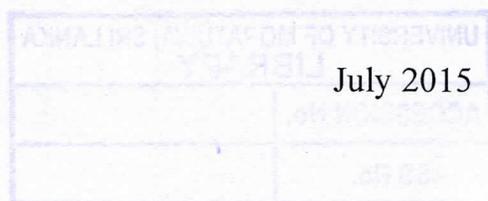
(138046L)

Thesis/ Dissertation submitted in partial fulfillment of the requirements for the
degree Master of Science

Department of Chemical and Process Engineering

University of Moratuwa

Sri Lanka



DECLARATION OF THE CANDIDATE & SUPERVISOR

I declare that this is my own work and this thesis does not incorporate without acknowledgement any material previously submitted for a Degree or Diploma in any University or other institute of higher learning and to the best of my knowledge and belief it does not contain any material previously published or written by another person except where the acknowledgement is made in the text.

Further, I hereby grant the University of Moratuwa the right to archive and to make available my thesis or dissertation in whole or part in the University Libraries in all forms of media, subject to the provision of the current copyright act of Sri Lanka. I retain all proprietary rights, such as patent rights. I also retain the right to use in the future works (such as articles or books) all or part of this thesis or dissertation.


Signature:

27/07/2015
Date:

I have supervised and accepted this thesis for the award of the degree

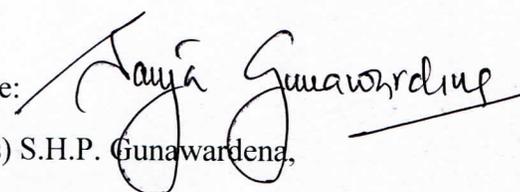

Signature:

27/07/15
Date:

Dr. Nilwala Kottegoda

Senior Lecturer, Dept. of Chemistry, University of Sri Jayewardenepura

Senior Research Scientist/Sri Lanka Institute of Nanotechnology (SLINTEC)

Signature: 
Dr. (Mrs) S.H.P. Gunawardena,

Date: 27/07/15

Senior Lecturer,

Dept. of Chemical and Process Engineering,

University of Moratuwa

ACKNOWLEDGEMENT

I acknowledge with gratitude the Sri Lanka Institute of Nanotechnology (SLINTEC) for giving me the opportunity to conduct this project with full financial support along with National Science Foundation while providing the access for its valuable high end equipment facilities.

I am grateful to Dr. Nilwala Kottegoda and Dr. (Mrs.) S. H. P. Gunawardena, my supervisors who very generously spent their precious time to provide necessary guidance and assistance to carry out this task.

I also extend my gratitude and thank to Dr. Asitha Siriwardena at SLINTEC, providing me with all necessary guidance in carrying out this project.

Further I wish to sincerely thank Mr. Dileepa Premathunga and Mr. Sunanda Gunasekara at SLINTEC who helped me in numerous ways to complete my project successfully.

I am also thankful to all my colleagues at SLINTEC for encouragement.

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 nanofibers. 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 .

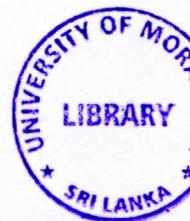
The presence of nanofibers was essential to offer a large surface area for large amount of enzyme encapsulation. The morphology and the size of the fibers were studied using scanning electron microscopic technique and the elemental composition was determined using energy dispersive X-ray analysis. It 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.

TABLE OF CONTENTS

ACKNOWLEDGEMENT	ii
Abstract.....	iii
TABLE OF CONTENTS	v
LIST OF FIGURES.....	x
LIST OF TABLES.....	xiv
LIST OF ABBREVIATIONS AND SYMBOLS.....	xv
CHAPTER 1	1
INTRODUCTION	1
CHAPTER 2	4
LITERATURE REVIEW	4
2.1 Introduction to nanotechnology.....	4
2.2 Nanofabrication Techniques.....	4
2.3 Nanomaterials.....	5
2.3.1 Zero-Dimensional Nanostructures: Nanoparticles	5
2.3.2 Two-Dimensional Nanostructures: Thin Films	6
2.3.3 One-Dimensional Nanostructures: Nanowires and Nanorods.....	6
2.4 Nanofiber synthesis methods.	7
2.4.1 Drawing	7
2.4.2 Phase separation.	9
2.4.3 Template synthesis	10
2.4.4 Electrospinning.....	12
2.4.4.1 Electrospinning theory.....	13
2.4.4.2 Parameters that affect electrospinning	14
2.5 Applications of nanofibres	17
2.6 Agro wastes as a source for nanofiber synthesis.....	19
2.6.1 Rice bran	20

2.6.1.1 Dietary fiber.....	21
2.6.1.2 Physiological effect of dietary fiber	21
2.7 Enzymes	22
2.7.1 Enzyme Immobilization Methods	22
2.7.1.1 Covalent Binding:.....	23
2.7.1.2 Entrapment.....	23
2.7.1.3 Adsorption:	23
2.7.1.4 Ionic Binding	24
2.7.1.5 Affinity Binding	24
2.7.1.6 Metal Linked immobilization	24
2.8 Phytase.....	25
2.9 Phytic acid (<i>Myo</i> -inositol (1,2,3,4,5,6) hexakisphosphate)	26
2.9.1 Phytic acid as an antinutritional factor	26
CHAPTER 3	28
MATERIALS AND METHOD.....	28
3.1 Electrospinning apparatus (built in house).....	28
3.2 Characterization Techniques	29
3.2.1 Fourier Transform Infrared Spectroscopy (FT-IR).....	29
3.2.2 Thermo Gravimetric Analysis (TGA)	30
3.2.3 Differential scanning calorimetry (DSC)	30
3.2.4 Scanning Electron Microscopy (SEM).....	30
3.2.5 Energy Dispersive X-ray Spectroscopy.....	31
3.2.6 UV-VIS Spectroscopy	31
3.3 Experimental Method	31
3.3.1 Characterization of Crude Phytase enzyme, Rice bran and poly vinyl alcohol (PVA).....	31
3.3.2 Extraction of phytase enzyme from the crude phytase source	31
3.3.3 Condition optimization for Phytase enzymatic assay	31
3.3.3.1 Determination of required substrate concentration for enzymatic assay	31



3.3.3.2 Determination of required the enzyme concentration for enzymatic assay	32
3.3.3.3 Determination of the incubation time for the enzymatic assay.	32
3.3.4 Determination of the activity of phytase enzyme.	33
3.3.5 Preparation and processing of rice bran for fiber synthesis.	33
3.3.6 Pretreatment of rice bran	33
3.3.6.1 Defatting	33
3.3.6.2 Alkali treatment	33
3.3.7 Condition optimization for electrospinning	34
3.3.7.1 Solution viscosity	34
3.3.8.2 Applied voltage.	34
3.3.8.3 Distance between two electrodes.	34
3.3.9 Production of nanofibers using electrospinning	34
3.3.9.1 Characterization of nanofibers and phytase enzyme encapsulated nanofibers.	35
3.3.10 Production of phytase enzyme encapsulated nanofibers	35
3.3.11 Production of phytase enzyme encapsulated nanofibers	35
3.3.11.1 Production of cross-linked Phytase enzyme encapsulated nanofibers.	35
3.3.11.2 Production of cross-linked nanofibers with boric acid.	36
3.3.11.3 Determination of the activity of cross-linked Phytase enzyme encapsulated nanofibers.	36
3.3.12 Production of cross-linked surface modified nanofibers with phytase enzyme.	36
3.3.12.1 Production of cross-linked nanofibers with STPP.	36
3.3.12.2 Determination of the activity of cross-linked surface modified nanofibers with phytase enzyme	37
3.2.12.3 Determination of the thermal stability of cross-linked surface modified nanofibers with phytase enzyme	37
CHAPTER 4	38
RESULTS AND DISCUSSION	38
4.1 Characterization of phytase enzyme.	38



4.1.1	Extraction of phytase enzyme from a crude phytase source.....	45
4.1.2	Determination of the substrate concentration required for the enzymatic assay	46
4.1.3	Determination of required enzyme concentration required for the enzymatic assay	47
4.1.4	Determination of the incubation time for the enzymatic assay.	48
4.1.5	Determination of the activity of phytase enzyme	49
4.2	Preparation of dietary fiber using rice bran	50
4.2.1	Preparation and characterization of rice bran	50
4.3	Pretreatment of rice bran	57
4.4	Viscosity modification of the polymer solution	59
4.4.1	Characterization of PVA	60
4.5	Condition optimization for electrospinning.....	62
4.5.1	Solution viscosity	62
4.6.2	Applied voltage.....	64
4.5.3	Distance between two electrodes.....	65
4.6	Synthesis of nanofibers.....	66
4.6.1	Characterization of nanofibers.....	66
4.7	Synthesis of phytase enzyme encapsulated nanofibers	74
4.7.1	Characterization of phytase enzyme encapsulated nanofibers	74
4.8	Synthesis of cross-linked Phytase enzyme encapsulated nanofibers.....	84
4.8.1	Characterization of cross-linked Phytase enzyme encapsulated nanofibers.....	87
4.9.1	Determination of the activity of the cross-linked phytase enzyme encapsulated nanofibers.....	95
4.10	Synthesis of cross-linked surface modified nanofibers with phytase enzyme	96
4.10.1	Characterization of surface modified nanofibers with phytase enzyme..	98
CHAPTER 5	111
5. CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE WORKS	111

5.1 Conclusions 111

5.2 Recommendations for future work..... 113

REFERENCES:..... 114

APPENDIX..... 118

Appendix 1:..... 118



LIST OF FIGURES

<p>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).</p> <p>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 ($M_w=650\ 000\ \text{g/mol}$), (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)....</p> <p>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).</p> <p>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).</p> <p>Figure 3.1: Major components of the electrospinning apparatus.....</p> <p>Figure 3.2: Electrospinning machine built in house</p> <p>Figure 4.1: Crude phytase</p> <p>Figure 4.2: Scanning Electron Microscopic image of crude phytase enzyme</p> <p>Figure 4.3: EDX Spectrometric data of crude phytase enzyme.....</p> <p>Figure 4.4: Spectral image of crude phytase enzyme</p> <p>Figure 4.5: Common structure of an amino acid</p> <p>Figure 4.6: FT-IR Spectrum of phytase enzyme.....</p> <p>Figure 4.7: DSC thermogram of crude phytase enzyme.....</p>	<p>8</p> <p>9</p> <p>10</p> <p>20</p> <p>28</p> <p>29</p> <p>38</p> <p>39</p> <p>40</p> <p>41</p> <p>41</p> <p>42</p> <p>44</p>
---	---

Figure 4.8: TGA thermogram of crude phytase enzyme	45
Figure 4.9: Mechanism of phytic acid hydrolysis by phytase enzyme	46
Figure 4.10: Amount of phosphate released at different phytic acid initial concentrations	47
Figure 4.11: Amount of phosphate released at different initial phytase enzyme concentrations	48
Figure 4.12: Phytic acid hydrolysis at different incubation times	49
Figure 4.13: Calibration plot of absorbance vs amount of phosphate	50
Figure 4.14: Sieved dried rice bran.....	51
Figure 4.15: Scanning Electron Microscopic images of rice bran.....	52
Figure 4.16: EDX Spectrometric data of rice bran	53
Figure 4.17: Spectral image of rice bran.....	53
Figure 4.18: FT-IR Spectrum of rice bran	54
Figure 4.19: DSC thermogram of rice bran	56
Figure 4.20: TGA thermogram of rice bran.....	57
Figure 4.21: Dietary fiber supernatant.....	58
Figure 4.22: FT-IR spectrum of PVA	60
Figure 4.23: DSC thermogram of PVA	61
Figure 4.24: TGA thermogram of PVA.....	62
Figure 4.25: SEM images of fibers after electrospinning using PVA (A) 7% (B) 8%	63
Figure 4.26: Taylor cone observed at 20 kV.....	64
Figure 4.27: SEM images of fibers after electrospinning using (A) 10 cm (B) 15 cm distances between the two electrodes.	65
Figure 4.28: Scanning Electron Microscopic images of nanofibers	66
Figure 4.29: EDX Spectrometric data of nanofibers.....	68
Figure 4.30: Spectral image of nanofibers	68
Figure 4.31: FT-IR spectrum of nanofibers	69
Figure 4.32: DSC thermogram of nanofibers	70
Figure 4.33: DSC thermogram of nanofiber, PVA and rice bran (comparison).....	71
Figure 4.34: TGA thermogram of nanofibers	72

Figure 4.35: Comparison between TGA thermograms of nanofiber, PVA and rice bran	73
Figure 4.36: DTG analysis of nanofibers, PVA and rice brans (comparison).	73
Figure 4.37: Scanning Electron Microscopic images of Phytase enzyme encapsulated nanofibers.....	75
Figure 4.38: EDX Spectrometric data of phytase enzyme encapsulated nanofibers .	77
Figure 4.39: Spectral image of phytase enzyme encapsulated nanofibers.....	77
Figure 4.40: FT-IR Spectrum of phytase enzyme encapsulated nanofibers	78
Figure 4.41: FT-IR Spectrum of phytase enzyme encapsulated nanofibers and phytase enzyme (comparison).....	79
Figure 4.42: DSC thermogram of phytase enzyme encapsulated nanofibers	80
Figure 4.43: Comparison between the DSC thermograms of phytase enzyme encapsulated nanofibers, phytase enzyme and nanofibers.....	81
Figure 4.44: TGA thermogram of Phytase enzyme encapsulated nanofibers	82
Figure 4.45: TGA thermogram of Phytase enzyme encapsulated nanofibers with its components.	83
Figure 4.46: DTG thermograms of phytase enzyme encapsulated nanofibers, nanofibers and Phytase enzyme (comparison).....	83
Figure 4.47: Cross-linking mechanism of boric acid with PVA.....	85
Figure 4.48: cross-linking of phytase enzyme encapsulated nanofibers with boric acid.....	85
Figure 4.49: Mechanism of the synthesis of cross-linked Phytase enzyme encapsulated nanofibers	86
Figure 4.50: Cross-linked vacuum dried phytase enzyme encapsulated nanofibers..	87
Figure 4.51: FT-IR Spectrum of cross-linked phytase enzyme encapsulated nanofibers.....	87
Figure 4.52: DSC thermogram of cross-linked nanofibers with boric acid	89
Figure 4.53: DSC thermogram of cross-linked phytase enzyme encapsulated nanofibers.....	90
Figure 4.54: DSC thermogram of cross-linked phytase enzyme encapsulated nanofibers, phytase enzyme encapsulated nanofibers and cross-linked nanofibers with boric acid.....	90

Figure 4.55: TGA thermogram of cross-linked nanofibers with boric acid 91

Figure 4.56: TGA thermogram of cross-linked phytase enzyme encapsulated
nanofibers..... 92

Figure 4.57: TGA thermogram of cross-linked phytase enzyme encapsulated
nanofibers, cross-linked nanofibers with boric acid and phytase enzyme encapsulated
nanofiber. 93

Figure 4.58: DTG thermograms of cross-linked phytase enzyme encapsulated
nanofibers, cross-linked nanofibers with boric acid and phytase enzyme encapsulated
nanofiber (comparison)..... 94



LIST OF TABLES

Table 1.1: Constituents of rice bran (Abdul-Hamid et al., 2000)	20
Table 4.1: Elemental composition of crude phytase enzyme.....	40
Table 4.2: FT-IR peak interpretation of phytase enzyme	42
Table 4.3: Elemental composition of rice bran	52
Table 4.4: FT-IR peak interpretation of rice bran	54
Table 4.5: Fiber supernatant characterization	58
Table 4.6: FT-IR peak interpretation of rice bran	60
Table 4.7: Fiber supernatant characterization after adding PVA.....	63
Table 4.8: Elemental composition of the nanofibers	67
Table 4.9: FT-IR peak interpretation of nanofibers	69
Table 4.10: Elemental composition of phytase enzyme encapsulated nanofibers.....	76
Table 4.11: FT-IR peak interpretation of phytase enzyme encapsulated nanofibers.	78
Table 4.12: FT-IR peak interpretation of cross-linked phytase enzyme encapsulated nanofibers.....	88
Table 4.13: FT-IR peak interpretation of cross-linked surface modified nanofibers with phytase enzyme.....	99

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