CHARACTERIZATION OF FREEZE DRIED CRUDE XYLANASE ON ITS STABILITY DURING STORAGE AT DIFFERENT TEMPERATURES

MURUGAN VISHNUPURAM KRISHNAMOORTHY

This thesis has fulfilled the requirement in terms of scope and quality for the award of the degree of Master of Engineering

Faculty of Chemical and Energy Engineering

Universiti Teknologi Malaysia

JULY 2017

ACKNOWLEDGEMENT

I would like to express my gratitude to all those who provide assistance in completing this thesis. First and foremost, I offer my sincere thanks to my supervisor, Dr. Syed Anuar Faua'ad bin Syed Muhammad, for his support, guidances and encouragement to carry out successfully my research work. In addition, my sincere thanks to my co-supervisor En Nik Azmi bin Nik Mahmood and all staff in the Department of Bioprocess and Polymer Engineering, Faculty of Chemical and Energy Engineering, Universiti Teknologi Malaysia, UTM Skudai Johor, Malaysia, for their support and I am indebted to all lab technicians, who deserve special thanks for their assistance and help with the instrumentation during the research.

ABSTRACT

Most of the enzymes are commercially available in powder form after undergoing freeze-drying process. However, such form is unstable if exposed to humidity at relatively higher temperature for a long-period of time that could affect the stability of the enzyme. Recently, crude xylanase enzyme has been produced through solid-state fermentation (SSF) and subjected to freeze-drying. The freeze-dried form of crude xylanase was characterised in terms of its stability after storage at different temperatures. Oil palm frond (OPF) is used as a substrate in a SSF to produce crude xylanase after fermentation for 18 days, then the samples of crude xylanase enzyme were extracted and analysed. Prior to centrifugation, crude xylanase solution was subjected to freeze-dry (Lyophilisation approach). In this research, storage test was conducted at various temperature points (-20°C, 4°C and 25°C), which were the common storage temperature for biological products. In addition, each sample taken was analysed in terms of xylanase activities using a standard xylanase assay. Meanwhile, qualitative structural changes of the crude xylanase were analysed using the Fourier Transform Infrared Spectroscopy (FTIR). No significant changes were observed in the peak profile in the FTIR analysis for all samples as compared to commercial xylanase. However, there were slight modifications of peak profile in the secondary structure pattern. Preliminary results showed that the freeze-dried crude xylanase was stable within the storage period of 91 days. Reduction of xylanase activities was observed after 120 days for the samples stored at 25°C. However, at temperature of -20°C and 4°C, results show that freeze-dried xylanase was stable or can be stored in a long-period of time. In conclusion, the crude xylanase can be stored in a long-period of time at -20°C, 4°C and 25°C for at least more than 3 months after freeze-drying process.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS

A_{cmc}	-	Cellulase activity
A_{xyl}	-	Xylanase activity
mg	-	Amount of cellulose or xylose produced
Stdev	-	Standard deviation
dil. Factor	-	Dilution factor
delOD	-	Amount of sample minus enzyme
delblank	-	Buffer solution
%	-	Percent
g	-	Gramme
°C	-	Degree celcius
ml	-	milliliter
nm	-	nanometer
Kcat	-	Kinetics catalyst

LIST OF ABBREVIATIONS

DOE	-	Design of Experiments
CCD	-	Central Composite Design
OPT	-	Oil palm trunk
EFB	-	Empty fruit bunch
OPF	-	Oil palm frond
Wt	-	Weight
IR	-	Infrared
UV	-	Ultraviolet spectrophotometer
pH	-	Potential hydrogen
RPM	-	Rotation per minutes
WL	-	Wavelength
SSF	-	Semi solid fermentation
SSC	-	Solid state culture
SDS	-	Sodium dodecyl sulphate
PAGE	-	Polyacrylamide gel electrophoresis
RSM	-	Response Surface methods
RSTW	-	Rice straw waste

LIST OF APPENDICES

CHAPTER 1

INTRODUCTION

1.1 Background of Study

In Malaysia, oil palm is first introduced as an ornamental plant and covered 4.48 million hectares of planted area through combination of processing capacity of 92.49 million tons through 406 mills and 52 refineries. It has generated 17.73 million tons of palm oil annually (Malaysia Palm Oil Board 2009).

Oil palm frond can be used as substrates for the cultivation of various microorganisms for the production of different products which are important for industrial application through solid state fermentation (SSF), particularly in this research, for the production of xylanase enzyme. Enzymes are distinct biological polymers that catalyse the chemical reactions and convert substrates to particular products. They are specific in function and speed up reactions by providing alternative pathways of lower activation energy without being consumed. SSF is defined as the growth of the microorganisms on solid material in the absence or near absence of free water. The depolymerisation action of xylanase results in the conversion of polymeric substances into xylo-oligosaccharides and xylose (Omar *et al.*, 2008).

The agricultural wastes from oil palm tree are known to contribute to the agro-waste from oil palm trees which are rich in cellulose and hemicellulose. It was reported that approximately 51 million tons of oil palm frond (OPF) were generated in Malaysia (Goh 2010). OPF consists of polysaccharides and lignocellulosic

components such as celluloses, hemicelluloses and lignin which are an indication of high organic matter content.

The agricultural waste materials if manipulated properly can play a significant role in the economic uplift. There is an increasing gist to utilize such neglected materials in the production of enzymes which can be employed further such as in producing downstream products (Mohammadi *et al*., 2006; Okafor *et al*., 2007).

Xylans; the substrates for xylanases are polysaccharides composed of β-1,4 linked xylopyranose units. They are highly branched and in firm association with other polymers. Acid hydrolysis of xylans is a rapid process for the production of xylose but a number of toxic and undesirable substances are also produced (Jackson and Hoseney 1986). Nowadays, the demand for chemical free food products is increasing rapidly over the globe and biotechnology can play a significant role to meet the purpose. Accordingly, the fermentation industry has stepped forward and grown well in the recent past (Pandey *et al.,* 2000; Ahuja *et al.,* 2004).

There are two main methods for the production of cellulolytic enzymes which are SSF and submerged fermentation (SmF). In SSF, wastes of agro-industrial have been used as a substrate-support for enzyme production. SSF is different from submerged fermentation and is more favourable because there is no free-flowing water in the SSF, however the moisture present is enough to support cell growth.

The major factors that affect the production of cellulolytic enzymes in SSF systems are temperature, pH, type of substrate used, humidity of substrate bed, particle size of substrate and moistening agent. Different microorganisms require different conditions in producing cellulolytic enzymes, while temperature and pH have influence on the growth and the enzyme production. It has been reported that the success of SSF depended on the consumption of glucose, controlled temperature and pH, with the process closely monitored. (Frederic Leroy and Luc de Vuyst, 1999).

Freeze drying is a complex operation where the solvent (usually water) is removed from the product by sublimation. Sublimation occurs when a frozen liquid goes directly to the gaseous state without passing through the liquid phase. This direct phase transfer “ice - water vapor” is a function of pressure and ice temperature which can be delineated by the phase diagram of water.

These studies are designed as per centre composite design and choosing an ideal storage time and the temperature of freeze drying of crude enzyme materials. The response for this study is enzyme activities. Enzyme stabilization has notable importance due to the increasing number of enzyme applications. Stabilization of enzymes is used to realize their full potential as catalysts. There are different approaches to enzyme stabilization. It can be studied from the point of view of the various denaturation reactions that occur, the kinetics involved therein and with respect to intended industrial use and storage stability.

1.2 Statement of Problem

Enzymes are in demand to replace traditional chemical processes with advanced biotechnological fermentation process involving microorganisms. Enzymes are rapidly used in several different industrial products (David J.C. 2011). In terms of fungal based fermentation, several alternative methods are available, but reports emphasized the feasibility of solid-state fermentation (SSF) as the best.

Most of enzymes products face problems on thermal stability, during storage and transportation. Enzyme products are normally stored in liquid form, however it has issues on the activity reduction and stability of the products (shelf life). Therefore, the mixed crude liquid enzymes are converted to powder formed by freeze drying process, in order to extend the product shelf life of the enzyme.

However, the primary concern of prolonging storage will influence most enzymes regardless of how the enzyme is prepared. Moreover, the knowledge of

freeze-dried protein and prolonged storage relationship so far has not been extensively discussed.

1.3 Objective of Study

1.3.1 To optimise the factors of stability storage time and temperature on the activity of freeze dried crude xylanase enzyme.

1.3.2 To produce crude xylanase enzyme from oil palm frond (OPF) using solid-state fermentation (SSF) process.

1.4 Scope of Study

1.4.1 To produce crude xylanase enzyme using microorganism of thermophilic fungi (Aspergillus Niger).

1.4.2 To determine crude xylanase activity using standard enzymatic assay method.

1.4.3 To characterise crude xylanase enzyme using Fourier Transformed Infrared (FTIR) Spectroscopy.

1.4.4 To evaluate the factors of storage time and temperature on the activity of crude xylanase enzyme using design expert 7 crack (D-optimal factor) experiment.

REFERENCES

A.E. Segneanu., C. Macarie., M. Ungureanu., I. Balcu., V. Gherman & I. Grozescu. (2013). Comparative Study on Enzymatic Hydrolysis of Cellulose. *Digest Journal of Nanomaterials and Biostructures.* Vol. 8, p. 1061 – 1068.

Andreas Jabs. (2005). The jena library of biological macromolecules, Fourier Transform Infrared spectroscopy to determine the structure of biological macromolecules.

Ashok Pandey., P.Selvakumar., Carlos R.Soccol & Poonam Nigam. (1999). Solid state fermentation for the production of industrial enzymes. School of Applied Biological and Chemical Sciences, University of Ulster, Coleraine BT2 IAS, N.Ireland,UK.

Amid Mehrnoush., Shuhaimi Mustafa & Abdul Manap Mohd Yazid. (2012). Optimization of Freeze Drying Conditions for Purified Pectinase from Mango.

Arief Widjaja., Evi Lestari., Akbar Tanjung., Widiawan & Alfian Hiroyasu Ogino. (2009). Optimized production of xylanase from fungal strains and its purification strategies. Journal of Applied Sciences in Environmental Sanitation.

Brijwani & Vadlani, P. V. (2011). Cellulolytic Enzymes Production via Solid-State Fermentation: Effect of Pretreatment Methods on Physicochemical Characteristics of Substrate.

B.V.Kilikan., L.C.Afonso., T.F.C.Souza., R.G.Ferreira & I.R.Pinheiro. (2013). Filamentous fungi and media for cellulase production in solid state cultures. Brazilian Journal of Microbiology.

Claudio Henrique Cerri e Silva., Jurgen Puls., Marcelo Valle de Sousa & Edivaldo Ximenes Ferreira Filho. (1998). Purification and characterization of a low molecular weight xylanase from solid-state cultures of Aspergillus fumigatus Fresenius.

Cristiane S. Farinas1., Marcel Moitas Loyo1., Anderson Baraldo., Paulo W. Tardioli., Victor Bertucci Neto1 & Sonia Couri. (2010). Finding stable cellulose and xylanase: evaluation of the synergistic effect of pH and temperature.

Cristica, M., Barbaneagra, T., Ciornea, E., & Manoliu A. (2012). Influence of pH on beta-xylanase activity in the filamentous fungi, Trichoderma Reesei, Trichorderma Viride, and Phanerochaete Chrysoporium. Lucrări Ştiinţifice. 55 (2): 321 – 325.

Concepcion Jimenez-Gonzalez & David J.C.Constable. (2011). Green Chemistry and Engineering. ISBN:978-0-470-17087-8.

Dayana Amira R., Roshanida A.R & Rosli M.I. (2012). Effects of Xylanase and Cellulase Production during Composting of EFB and POME using Fungi. World Academy of Science, Engineering and Technology International Journal of Biological, Biomolecular, Agricultural, Food and Biotechnological Engineering Vol:6, No:8, 2012

D.K.Buslov., F.N.Kaputskin & N.I.Sushko. (2009). Infrared spectroscopic analysis

of the structure of xylans.

Frederic Leroy & Luc De Vuyst. (1999). Temperature and pH conditions that prevail during fermentation of sausages are optimal for production of the antillisterial bacteriocin sakacin K. Applied and Environmental Microbiology. Volume 65 no.3 974-981.

Gary W. Oehlert. (2000). A first Course in Design and Analysis of Experiments. ISBN 0-7167-3510-5.

Gaurav kanojia., Geert-jan Willems & Henderik W.Frijilink. (2016). Institute for Translational Vaccinology. AL Bilthoven, The Netherlands. International journal of pharmaceutics, 511(2), 1098-1111.

Gloria Lopez & Pilar Estrada. (2014). Effect of Temperature on Xylanase II from Trichoderma reesei QM:9414: A Calorimetric, Catalytic and Conformational Study. Facultad de Biologia, Universidad Complutense, Madrid, Spain.

Gupteshwar Gupta., Vikram Sahai & Rajinder K.Gupta. (2013). Thermal Stability and Thermodynamics of Xylanase from Melanocarpus albomyces in Presence of Polyols and Salts.

Heinz Fabian & Werner Mantele. (2002). Infrared Spectroscopy of Proteins. Johann Wolfgang Goethe-University Frankfurt am Main, Frankfurt am Main, Germany.

Hooi Ling Ho & Jamila Said Hood. (2014). Optimisation of Medium Formulation and growth Conditions for Xylanase Production by Aspergillus brasiliensis. Faculty of Applied Science, UCSI University, Cheras, Kuala Lumpur.

Isil, S & Nilufer, A. (2005) Investigation of Factors Affecting Xylanase Activity from Trichoderma harzianum 1073 D3. Brazil. Arch. Biol. Technol. 48 (2): pp. 187-193.

Jilie kong & Shanoning YU. (2007). Fourier Transform Infrared Spectroscopic Analysis of Protein Secondary Structures. Department of Chemistry, Fudan University, Shanghai 200433, China.

John Bertie & John Wiley. (2001). Optical Contants in the Handbook of Vibrational Spectroscopy. 0-471-98847-2, Approx 3,572 pp.

Karim, A., Nawaz, M. A., Aman, A., Ali, S & Qader, U. (2014). Hyper production of cellulose degrading endo (1,4) b-D-glucanase from Bacillus licheniformis KIBGE-IB2.

Kamarul, A. A. H. (2008). Production of Cellulose Fiber from Oil Palm Frond Using Steam Explosion Method. Ph.D. Thesis, University Malaysia Pahang.

Keharom, S., Mahachai, R & Chanthai, S. (2016). The optimization study of α-amylase activity based on central composite design-response surface methodology by dinitrosalicylic method. International Food Research Journal 23(1): 10-17.

Kok Chang Lee., Takamitsu Arai., Darah Ibrahim., Panida Prawitwong., Deng Lan., Yoshinori Murata., Yutaka Mori & Akihiko Kosugi. (2015). Purification and Characterization of a Xylanase from the Newly isolated Penicillium rolfsii c3-2(1) IBRL.

Kunal A. Gaidhani., Mallinath Harwalkar., Deepak Bhambere., Pallavi S. & Nirgude. (2015). Lyophilization / Freeze drying. World Journal of Pharmaceutical Research 4(8):516-543.

Li, X. hua., Yang, H. jun., Roy, B., Park, E. Y., Jiang, L. jun., Wang, D & Miao, Y.

gen. (2010). Enhanced cellulase production of the Trichoderma viride mutated by microwave and ultraviolet. Microbiological Research.

Li Wan Yoon., Teck Nam Ang., Gek Cheng Ngoh & Adeline Seak May Chua. (2013). Fungal solid-state fermentation and various methods of enhancement in cellulase production. Department of Chemical Engineering, Faculty of Engineering, Univeristy of Malaya, Kuala Lumpur, Malaysia.

Marcia M.S.Moretti., Daniela A. Bocchini-Martins., Roberto Da Silva., Andre Rodrigues., Lara D. Sette & Eleni Gomes (2012). Selection of Thermophilic and Thermotolerant Fungi for the production of Cellulase and Xylanase under Solid State Fermentation. Brazilian journal of Microbiology.

Maria de Lourdes T.M.Polizeli., Simone C. Peixoto-Nogue., Tony M.da Silva & Alexandre Maller. (2010). Gel Electrophoresis for investigating Enzymes with Biotechnological Application. ISBN: 978-953-51-0457-5, In Tech.

Maciel, G.M., L.P.S. Vandenberghe., C.W.I. Haminiuk,R.C., Fendrich., B.E.D. Bianca., T.Q.S. Brandalize., A. Pandey & C.R. Soccol. (2008). Xylanase Production by Aspergillus niger LPB 326 Solid-State Fermentation Using Statistical Experimental Designs.Food Technology Biotechnology, 46: 183-189.

Md. Zahangir Alam., Nurdina Muhammad & Mohd Erman Mahma. (2005). Production of Cellulase from Oil Palm Biomass as Substrate by Solid State Bioconversion. American Journal of Applied Science 2 (2): 569-572, 2005.

M Kacurakova., N Wellner & A Ebringerova. (1999). Charaterisation fo xylan type polysaccharides and associated cell wall components by FTIR. Food Hydrocolloids, Volume:13.

Miller & G.L. (1959). Use of DinitrosaIicyIic Acid Reagent for Determination of Reducing Sugar.Analytical Chemistry, 31: 426-428.

Mohammad, N., Alam, M. Z., Kabbashi, N. A., & Ahsan, A. (2012). Effective composting of oil palm industrial waste by filamentous fungi: A review. Resources, Conservation and Recycling.

Mohamed Ahmed El-Sherbiny & Ghadir Aly El-Chaghaby. (2012). Storage temperature and stabilizers in relation to the activity of commercial liquid feed enzymes: a case study from Egypt. Reginal Center for Food and Feed, Agricultural Research Center, Egypt.

Mohd, N. B. (2008). The Effects of Hydrothermal Treatment on the Physico Chemical Properties of Oil Palm Frond (OPF) Derived Hemicellulose.

Muhammad Suleman., Iftikhar Hussain Bukhari., Muhammad Ikram Aujla & Abu ul Hassan Faiz. (2016). Production and Characterization of Xylanase from Aspergillus Niger using wheat Brans, Corn cobs and Sugar cane bagasse as carbon sources with different concentrations. Department of Chemistry, Government College University Faisalabad-Pakistan.

N. Abdullah & F. Sulaiman. (2013). The Oil Palm Wastes in Malaysia. School of Physics, Universiti Sains Malaysia, 11800 Minden, Penang, Malaysia.

Neelam Gurung., Sumanta Ray., Supta Bose & Vivek Rat. (2013). Microbial enzymes and Their Relevance in Industries, Medicine and Beyond. Department of Earth Science, Indian Institute of Science, West Bengal.

Norazlina, K.H.Ku Halim & F.A.M. Shareena. (2014). Degradation of Oil Palm Leaf

by Aspergillusnigaer ATCC 16404. American-Eurasian J. Agric. & Environ. Sci., 14 (6): 495-501,2014. IDOSI Publications.

Petchluan, P., Pukahuta, C., & Chaikong, N. (2014) Characterization of Xylanase and Cellulase from Lentinus polychrous Lev. LP-PT-1. Chiang Mai J. Sci. 41(5.1): 1007-1019.

Polizeli, M. L. T. M., Rizzatti, A. C. S., Monti. R., Terenzi, H. F., Jorge, J. A. & Amorim, D. S. (2005). Xylanases from fungi: properties and industrial applications. Applied Microbiol Biotechnol 67: 577–591.

Prasad, P., Singh, T & Bedi, S. (2013). Characterization of the cellulolytic enzyme produced by Streptomyces griseorubens (Accession No. AB184139) isolated from Indian soil. Journal of King Saud University.

R. Agarwal., Biswanath Mahanty, V & Venkata Dasu. (2009). Modeling Growth of Cellulomonas cellulans under Substrate Inhibition during Cellulase Production. NRRL B 4567.

Reera Rani Singhania., Rajeev K., Sukumaran, Anu Pillai, P., Prema., George Szakacs & Ashok Pnadey. (2006). Solid-state fermentation of lignocellulosic substrates for cellulase production by Trichoderma reesei NRRL 11460. Vol 5(supply): pp 332-336.

Siti Sabrina., M. S. Roshanida & A. R, Norzita. (2013). Pretreatment of Oil Palm Fronds for Improving Hemicelluloses Content for Higher Recovery of Xylose.

Singh S., Reddy P., Haarhoff J., Biely P., Janse B & Pillay B. (2000). Relatedness of thermomyces lanuginosus strains producing a thermostable xylanase. Journal Biotechnol.

S.Kandil & M.El Soda. (2015). Influence of Freezing and Freeze Drying on Intracellular Enzymatic Activity and Autolytic Properties of Some Lactic Acid Bacterial Strains. Department of Dairy Science and Technology, Alexandria, Egypt.

Sohpal, V. K., Dey, A & Singh, A. (2010). Investigate of Process Parameters on Xylanase Enzyme Activity in Melanocarpus Albomyces Batch Culture. Proceedings of the World Congress on Engineering. Vol I WCE 2010, London, U.K.

Subramaniyan, S & Prema, P. (2000). Cellulase-free xylanases from Bacillus and other microorganisms. *FEMS* Microbiology Letters. Volume 183, Issue 1.

Tony, J.F., L. Bo-Chin & L. Shu-Chih. (2010). Enhanced production of xylanase by Aspergillus carneus M34 in solid-state fermentation with agricultural waste using statistical approach. N. Biotechnol., 27: 25-32.

Wan-Yi Chiu & Peter W.M. John. (1998). D-optimal fractional factorial designs. Department of Mathematics, the University of Texas at Austin, RLM 8.100, Austin, USA.

Wilson, D. B. (2011). Microbial diversity of cellulose hydrolysis. Current Opinion in Microbiology.

W.J.J.Van den Tweel., A.Harder & R.M.Buitelarr. (1992). Stability and Stabilization of Enzymes.

W.D Wanrosli., Z.Zainuddin., K.N.Law & R.Asro. (2007). Pulp from oil palm fronds by chemicals process. Industrial Crops and Products 25(1):89-94.