UNIVERSITI PUTRA MALAYSIA

EXTRACTION, PURIFICATION AND CHARACTERIZATION OF AMYLASE ENZYME FROM WHITE PITAYA (Hylocereus undatus (Haworth) Britton & Rose) PEEL USING AQUEOUS TWO- PHASE SYSTEM

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By

ZAHRA SHAD

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for theDegree of Master of Science

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DEDICATION

To my parents for their uncontional love and prayers

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the Degree of Master of Science

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Chair : Mohd Yazid Manap, PhD
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Amylase is one of the most important enzymes largely used in biotechnological and industrial applications. Thirty percent of the World's enzyme production is accounted by Amylase. Malaysia has around 927.4 ha (363.2 ha production areas) pitaya fruit-growing areas produces about 2,534.2 tons worth around US$3.5 million. Peels are one of the byproducts that are obtained from the processing of pitaya. Pitaya peel is mostly a waste material from fruit and beverage industries. Although it consists about 33% of whole fruit by weight and possesses valuable enzymes such as amylase, it is not being presently used commercially and is considered as a waste. It could have been efficiently used for commercial and economical production of natural enzymes.

Therefore, this research studied the extraction, purification and characterization of amylase from white pitaya (*Hylocereusundatus*) peel. Extraction of amylase from peel of white pitaya was optimized using full factorial design (FFD) with three variables, sodium phosphate buffer (pH 4.5-7.5), mixing time (1- 3min) and buffer to sample ratio (1:3-1:5).

The purification was carried out using aqueous two phase system (ATPS). The effectiveness of different parameters on purification and selective separation, such as polyethylene glycol (PEG) molecular weight (4000 to 8000), PEG concentration (10 to 18%), sodium citrate concentration (12-20%) and NaCl (2-8%) were optimized using response surface methodology (RSM). The purified amylase enzyme was characterized based on pH, temperature and metal ions, surfactants and oxidizing agents.

It was found that optimum condition for amylase extraction was with sodium phosphate buffer pH 6 and buffer to sample ratio of (1:4) for 2 min which yielded the enzyme with specific activity of 5.89 U/mg. The purification of amylase was studied with ATPS method polyethylene glycol/sodium citrate. The optimum purification

factor and yield were obtained when 14% (w/w) of polyethylene glycol 6000 g/mol, 16 % (w/w) sodium citrate buffer and 5% of NaCl were used. Amylase purification factor and yield using ATPS were 4.43 and 89.12%, respectively. The optimum temperature and pH activity of amylase were 55 °C and 6, respectively. This enzyme was also stable in the presence of surfactants and oxidizing agents. Moreover, it was found that the activity of amylase was increased in the presence of calcium ions. The unique characteristics of amylase from white pitaya peel indicate the great potential application of the enzyme in food and biotechnology industries.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
Sebagai memenuhi keperluan untuk Ijazah Master Sains

PENGEKSTRAKAN, PENULENAN DAN PENCIRIAN ENZIM AMILASE DARI KULIT PITAYA PUTIH (*Hylocereus undatus* (Haworth) Britton & Rose) MENGGUNAKAN SISTEM AKUAS DUA- FASA

Oleh

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Amilase adalah salah satu enzim terpenting yg digunakan secara meluas dalam aplikasi bioteknologi dan industry.Tiga puluh peratus pengeluaran enzim dunia diambilkira oleh amylase. Malaysia mempunyai kira-kira 927.4 hektar (363.2 hektar kawasan pengeluaran) kawasan penanaman buah pita yang menghasilkan kira-kira 2,534.2 tan bernilai kira-kira US$3.5 juta. Kulit merupakan salah satu hasil sampingan yang diperolehi dari pemprosesan buah pitaya.Kulit pitaya kebanyakannya adalah bahan buangan dari buah dan industry minuman.Walaupun ia terdiri kira-kira 33% dari keseluruhan berat buah dan mempunyai enzim yang bernilai seperti amilase, ia tidak digunakan pada masa kini secara komersil dan dianggap sebagai bahan buangan. Ia boleh digunakan secara efisien untuk tujuan komersil dan pengeluaran economi bagi enzim semulajadi.

Oleh itu, penyelidikan ini mengkaji pengekstrakan, penulenan dan pencirian bagi amylase dari kulit pitaya putih (*Hylocereusundatus*). Pengekstrakan amylase dari kulit pitaya putih telah dioptimumkan melalui rekabentuk factor ia l penuh (FFD) dengan 3 pembolehubah, penampan natrium fosfat (pH 4.5-7.5), masa pencampuran (1-3min) dan nisbah penampan kepada sampel (1:3-1:5).

Penulenan dijalankan menggunakan system dua fasa akueus (ATPS). Keefektifan oleh parameter yang berlainan ke atas purifikasi dan pemisahan terpilih seperti polietilenaglicol (PEG) jisim molecular (4000 ke 8000), kepekatan PEG (10 ke 18%), kepekatan natrium sitrat (12-20%) dan natrium klorida (2-8%) telah dioptimumkan menggunakan metodologi kesan permukaan (RSM). Enzim amilase yang ditulenkan telah dicirikan berdasarkan kepada pH,suhu dan ion logam, surfaktan dan agen pengoksidaan.

Di dapati bahawa kondisi optima bagi pengekstrakan amylase adalah dengan pH 6 penampan sodium fosfat dan nisbah penampan kepada sampel (1:4) bagi 2 minit telah menghasilan enzim dengan 5.89 U/mg aktiviti spesifik.Penulenan amylase dikaji dengan kaedah ATPS polietilenaglikol/natriumsitrat. Faktor purifikasi optima dan hasil diperolehi apabila 14% polietilenaglikol 6000 g/mol, 16 % (berat/berat)

penampan natrium sitratand 5% of natrium kloridadigunakan. Faktor purifikasi amilase and hasilan menggunakan ATPS adalah masing-masing 4.43 dan 89.12%. Suhu dan aktiviti pH bagi amilase masing-masing adalah 55°C dan 6. Enzim ini turut stabil dengan kehadiran surfaktan dan agen pengoksidaan. Lebih-lebih lagi, di dapati bahawa aktiviti amylase meningkat dengan kehadiran ion potassium. Ciriunikamilase dari kulit pitaya putih menunjukkan potensi besar aplikasi enzim berkenaan dalam industry makanan dan bioteknologi.

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I certify that a Thesis Examination Committee has met on 27 November 2015 to conduct the final examination of Zahra Shad on her thesis entitled "Extraction, Purification and Characterization of Amylase Enzyme from White Pitaya (*Hylocereus undatus* (Haworth) Britton & Rose) Peel Using Aqueous Two-Phase System" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

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This is to confirm that:

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LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
APS	Ammonium Persulfate
ATPS	Aqueous Two Phase System
BSA	Bovine Serum Albumin
CCD	Central Composite Design
cm	Centimeter
DEAE	Diethylaminoethyl
DNS	3, 5-dinitrosalicylic acid
DOE	Design of Experiments
FFD	Full Factorial Design
g	Gram
Ha	Hectare
kDa	Kilodalton
MW	Molecular Weight
OD	Optical Dnesity
PEG	Polyethylene Glycol
RSM	Response Surface Methodology
SD	Standard Devation
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gelelectrophoresis
TEMED	TetramethylethyleneDiamine

LIST OF NOMENCLATURES

A_b → Activity of enzyme in bottom phase U/ml

A_t → Activity of enzyme in top phase U/ml

C_i → Concentration of proteinininitial extract mg/ml

C_t → Concentration of proteinin the top phase mg/ml

K_e → Partition coefficient of enzyme

P_F → Purification factor of enzyme

S → Specific activity of enzyme U/mg

T_A → Total activity of enzyme U

T_p → Total protein of enzyme mg

Y → Yield of enzyme %

CHAPTER I

INTRODUCTION

Pitaya or dragon fruit is a valuable tropical fruit, popularity of which has spread rapidly worldwide especially in countries like Vietnam, Taiwan, Malaysia, and the Philippines (Lim et al., 2010; Mizrahi et al., 1997). It has been universally recognized as an ornamental plant due to its large, scented flowers that bloom uniquely in the night. Dragon fruit has also gained popularity for its health properties (Kim et al., 2011; Li et al., 2003). These vine cacti of the genera *Hylocereus* and *Selenicereus,* indigenous to northern South America, Central America, and Mexico are currently being cultivated as a novel exotic fruit crop. The genus *Hylocereus* and *Selenicereus* comprises 18 and 28 species, respectively (Anderson, 2001; Nobel & La Barrera, 2004).

At present, three species are commercially cultivated and these include H. *undatus*, H. *polyrhizus* and S. *megalanthus* in Colombia, Israel, Vietnam and Nicaragua (Tel-Zur et al., 2005). Vietnam is the biggest producer of the *Hylocereus* spp. However, only H. *undatus* and H. *polyrhizus* are cultivated in Malaysia. In the market, these two fruits are commonly differentiated based on the peel and pulp color. The first large scale cultivation of dragon fruit was at Sungai Wangi Estate in the state of Perak by Golden Hope Company at the end of 1990. As at 2006, Malaysia had about 927.4 ha (363.2 ha production areas) dragon fruit-growing areas with total production about 2,534.2 tons (production value around US$3.5 million) and Johor has the highest pitaya plantation area of 326.7 ha in Malaysia (Cheah & Zulkarnain, 2008). A significant advantage is the 20-year lifespan of the plant, with an average of 800 plants to the hectare (Gunasena et al., 2006) .

Pitaya peels are usually disposed during various pitaya-based products processing, particularly in the production of beverages (Bakar et al., 2011). Pitaya peel contains several bioactive compounds, dietary fiber, and many enzymes (such as pectinase, pectin methylesterase, polygalacturonase, amylase and invertase) (Awang et al., 2013; Lim, 2012). In the present study, *Hylocereus undatus* [(Haworth) Britton & Rose] with white-fleshed and red-colored pericarp (Mizrahi & Nerd, 1999) was selected for extraction and purification of amylase from the peel, which is the most widely cultivated *Hylocereus* species in the world and even it is cheaper than H. *polyrhizus*.

Amylases are a group of amylolytic enzymes that hydrolyse the glycosidic bond in the amylose and amylopectin large molecules to small molecules such as maltose, dextrin and/or glucose. They play an important role in seed germination and maturation. They are also active in starch digestion in animals for the formation of sugars used for various metabolic activities (Godon & Boudreau, 1994; Muralikrishna & Nirmala, 2005). Amylases comprise 30% of the world's enzyme market. They are used in many applications in industry including starch liquefaction, textile, paper, brewing, baking, detergents, distilling industries, the manufacturing of

digestive aids, fruit juices, cakes, starch syrups, and pharmaceutical products (Sivaramakrishnan et al., 2006).

Amylase from different sources has different properties such as kinetic parameters, pH optimum, temperature optimum and substrate specifically (Terashima et al., 1197). However, thermo-stability, pH response, and specificity of amylase are important properties for its applications in industry (Souza, 2010). Amylases with optimum activity in acidic pH, are primarily used in glucose syrup and baking industries, whereas those showing activities at alkaline pH have been applied in laundry detergent formulations (Ghorbel et al., 2009). Thus, amylase characteristics are key factors for its applications in manufacturing of glucose syrup, bread making, warp sizing of textile fibers, clarifying of haze in beer or in fruit juice, and also in the production of detergents (Janeček & Baláž, 1992; Van Der Maarel et al., 2002)..

Amylases from different sources have been studied in great depth. They are the products of animals, plants, and microorganisms. Now a large number of bacterial α-amylases especially from genus Bacillus are commercially available and they are the most used in industry. However, the production of such enzymes does not meet industry requirements in the world, because the demand for this enzyme has increased in the last two years. The use of bacterial α-amylase has caused allergies affecting 15% of global population (Gomez et al., 2013). As such, there is a necessity to discover novel sources to reduce the valuable enzyme.

Another important sources of amylase are plant and fungi, which have higher productivity than bacteria and also have formed the center of amylase studies due to their ubiquitous nature in developing countries (Afiukwa et al., 2009).. It is well documented that plants are abundant source of amylases (Sivaramakrishnan et al., 2006), so using plants as an alternative source of the enzyme have great advantages over microbial sources due to their cost-effective production, easy scale-up, and available natural storage organs (Stanley et al., 2005).

Generally, there are two processes used as conventional methods of protein extraction and purification: (i) obtaining crude extract through sample pretreatment to free the intracellular material; and (ii) purifying proteins using traditional chromatography process. These methods involve cell wall disintegration, ammonium sulfate-aided precipitation, centrifugation, dialysis of the samples to get a crude extract, and then applying one or more chromatographic processes (Ramos et al., 2010). The common procedures for purification of amylase from plants include homogenization, centrifugation, filtration, dialysis, ion exchange chromatography on DEAE-cellulose and CM-cellulose (Noman, Hoque, Sen, & Karim, 2006; Rahman & Absar, 2001). Kanwal et al. (2004) reported that purification of amylase from apple includes homogenization, centrifugation, filtration, ammonium sulfate with 60% precipitation, dialysis and gel filtration chromatography on sephadex G-150. However, a fast and desirable purification method is required to minimize deactivation and modification of the product (Bierau et al., 2001).

Aqueous two-phase extraction (ATPE) has been widely used for purification of protein, enzyme, biopharmaceutical and extractive fermentation (Albertsson, 1986; Hatti-Kaul, 2000). The formation of the ATPS involves mixing two incompatible

polymers (polyethylene glycol, dextran, etc.) or a polymer and a salt (phosphate, citrate, sulfate, etc.) in aqueous condition (Albertsson, 1986; Yücekan & Önal, 2011). Successful separation occurs as a result of the different distribution between the two phases of target compound and contaminants. Because of high water content of the two phases and low interfacial tension, they produce mild conditions particularly appropriate for separating the biological macromolecules (Marcos, Fonseca, Ramalho, & Cabral, 1999).

1.1.Problem statements

- The increasing demand for amylase to be used in different industries such as brewing, baking, sugar, starch syrups, fruit juices, detergents, leather, textile, paper, digestive juices, pharmaceutical and distilling industries
- There is a huge volume of pitaya peel waste in Malaysian Food industry
- Unsuitable enzyme extraction method or any changes in extraction condition results in denaturation and deactivation of it
- Conventional methods are multi-step, time-consuming, hardly reproducible, discontinuous, and need costly equipment at every step
- Conventional methods may also result in a decreased yield and purity of enzyme

The purpose of the current research is to develop efficient processes for extraction of amylase from white pitaya peel and development of a fast and convenient purification method of amylase using ATPS, which can enhance the overall yield, purity and activity of the enzyme. The novelty of this study is the extraction and purification of amylase as an important enzyme from waste (white pitaya peel) at a high level of purification factor and yield with easy scale-up and fast processing at low material cost.

1.2.Objectives

The general objective of this research is to study the impact of extraction and purification conditions on yield and enzyme activity of amylase from white pitaya (*Hylocere usundatus*) peel. The specific objectives of this study are as follow:

1. To optimize the extraction conditions of amylase from white pitaya peel
2. To develop the applicable aqueous two-phase system for purification of the crude amylase
3. To determine the pH, temperature, effect of metal ions and surfactant of amylase from white pitaya peel

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APPENDICIES

Appendix 1

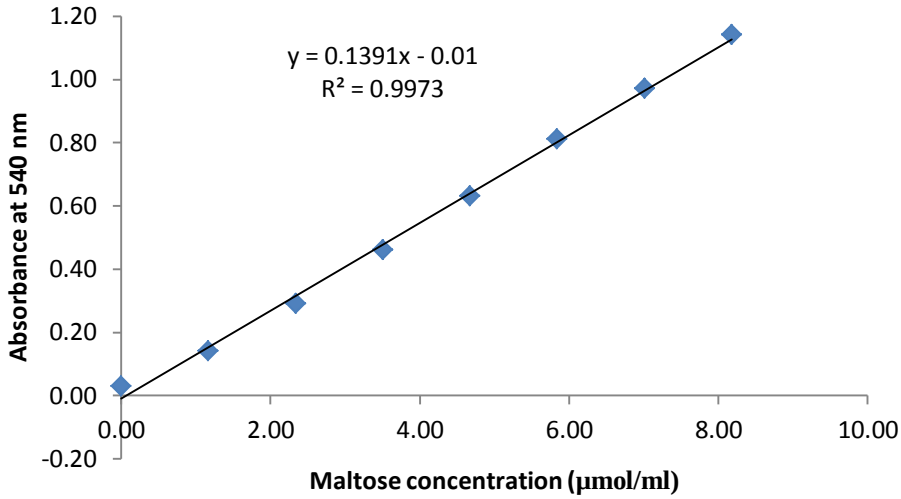

Figure 1. The standard curve of Maltose

Appendix 2

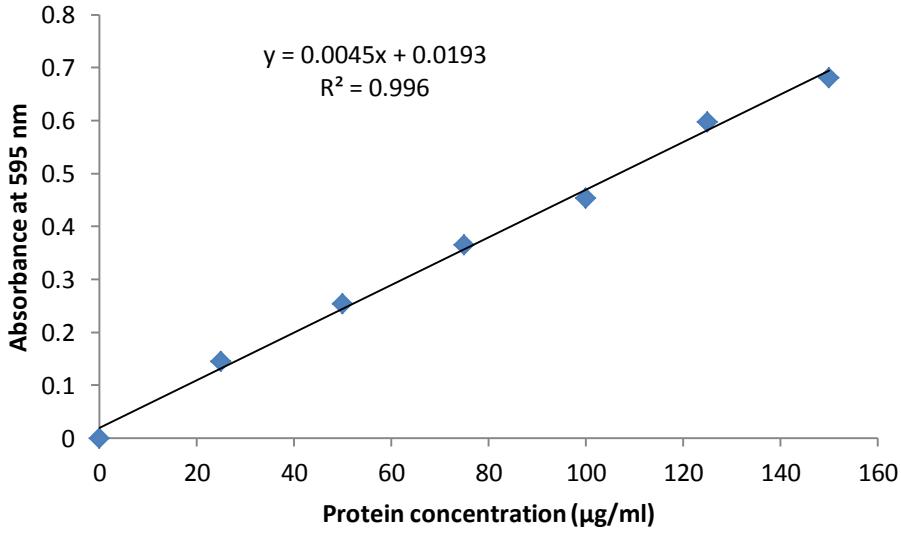

Figure 2. The standard curve of Coomassie protein analysis

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