ปัจจัยที่มีผลต่อการสกัดและการตรึงรูปสารออกฤทธิ์ทางชีวภาพ และกลิ่นในใบเตยหอม *Pandanus amaryllifolius*

นางสาวปรรัตน์ เซ็นกลาง

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FACTORS AFFECTING EXTRACTION AND ENCAPSULATION OF BIOACTIVE COMPOUNDS AND AROMA IN PANDAN *Pandanus amaryllifolius* LEAVES

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ใบเตยเป็นแหล่งที่อุดมไปด้วยคลอโรฟิลล์ แต่คลอโรฟิลล์ที่พบตามธรรมชาติเป็นรูปที่ไม่คงตัวต่อกรดและความร้อน ทำให้เสื่อมสภาพเป็นอนุพันธ์ที่มีสีเขียวมะกอกจนถึงสีน้ำตาล ซึ่ง Zn-chlorophyll เป็นรูปที่ทำให้คลอโรฟิลล์มีความคงตัว โดยทำปฏิกิริยาระหว่างคลอโรฟิลล์ในใบเตยกับซิงค์คลอไรด์ที่ความเข้มข้น 300 ppm ที่ pH 5 ควบคุมอุณหภูมิที่ 110 °C เป็นเวลา 15 นาที อนุพันธ์ที่เกิดขึ้นคือ Zn-pheophytin *a, b* และ Zn-pyropheophytin *a, b* ซึ่งอนุพันธ์ที่ได้นี้มีสีเขียวเหมือนกับคลอโรฟิลล์ที่พบตามธรรมชาติ แต่มีความคงตัวในภาวะที่เป็นกรดและความร้อนสูง นอกจากนี้อนุพันธ์ดังกล่าวยังมีประสิทธิภาพในการเป็นสารแอนติออกซิเดนท์ที่สูงขึ้นกว่าคลอโรฟิลล์ที่พบในธรรมชาติ จากนั้นสกัดอนุพันธ์ที่ได้ด้วยเอนไซม์เพคติเนส (Pectinex® Ultra SP-L, 10292 PGU/mL) วางแผนการทดลองแบบ response surface methodology (RSM) เพื่อหาภาวะการสกัดสูงสุด พบว่า ภาวะที่ให้ผลผลิตสูงสุดอยู่ที่เอนไซม์ความเข้มข้น 2.3-2.5% (v/w) เวลาในการสกัด 240-260 นาที และสกัดซ้ำสองครั้ง ภายใต้ภาวะดังกล่าวทำให้ได้ปริมาณคลอโรฟิลล์และสารแอนติออกซิแดนท์สูงสุด โดยเพิ่มขึ้นเป็น 17.3 และ 1.9 เท่า ตามลำดับเมื่อเทียบกับภาวะที่ไม่ได้ใช้เอนไซม์ในการสกัด จากนั้นนำสารสกัดที่ได้มาเตรียมให้เป็นผงโดยใช้วิธีการทำแห้งแบบพ่นฝอย โดยเปรียบเทียบชนิดของตัวพยุง ระหว่างกัมอะราบิก(GA) มอลโตเด็กซ์ทรินท์ (MD) และแป้งดัดแปร(MS) ต่อสมบัติทางเคมีกายภาพและความคงตัวของผงที่ได้ พบว่าการใช้แป้งดัดแปรที่ระดับ 30% (w/w) เป็นภาวะที่เหมาะสมเพราะผงซิงค์คลอโรฟิลล์ที่ได้มีสีเขียว ปริมาณคลอโรฟิลล์และสารแอนติออกซิเดนท์สูงสุด การเสื่อมสลายของผงซิงค์คลอโรฟิลล์ภายใต้ตัวพยุงต่างๆ พบว่าเป็นปฏิกิริยาอันดับหนึ่ง (first order kinetic) ที่มีค่าคงที่ปฏิกิริยา (rate constant, *k*) ของตัวพยุง GA , MD และ MS เท่ากับ 2.1×10^{-3},1.8×10^{-3} และ 1.5×10^{-3} วัน$^{-1}$ ตามลำดับ และค่าครึ่งชีวิต (half-life, $t_{1/2}$) เท่ากับ 330, 385 และ 462 วัน ตามลำดับ ผงซิงค์คลอโรฟิลล์ที่ได้มีความคงตัว โดยไม่เกิดการเปลี่ยนแปลงสีในภาวะกรดและทนความร้อนสูง จึงสามารถนำไปใช้เป็นสารแต่งสีเขียวในอาหาร นอกจากนี้ผงซิงค์คลอโรฟิลล์ที่ได้มีกลิ่นเฉพาะที่เป็นเอกลักษณ์ของใบเตยจากสาร 2-acetyl-1-pyrroline มีปริมาณซิงค์ตกค้างในผลิตภัณฑ์ไม่เกินข้อกำหนดขององค์การอาหารและยาสหรัฐอเมริกา การศึกษานี้สามารถใช้เป็นแนวทางในการสกัดสารแต่งสีเขียวจากธรรมชาติเพื่อใช้ในอุตสาหกรรมอาหาร

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PORRARUD SENKLANG: FACTORS AFFECTING EXTRACTION AND ENCAPSULATION OF BIOACTIVE COMPOUNDS AND AROMA IN PANDAN *Pandanus amaryllifolius* LEAVES. THESIS ADVISOR, ASSOC. PROF. PRANEE ANPRUNG, Ph.D., 134 pp.

The pandan leaf is a rich source of chlorophyll. In its natural state chlorophyll is unstable. It becomes extremely labile on exposure to acid or heat, breaking down into derivatives of olive brown coloration. Chlorophyll analogues, in which Zn is substituted for Mg, are more stable. This study optimized conditions for Zn-chlorophyll complex formation were 300 ppm zinc chloride at pH 5 and 110 ^{o}C for 15 min. The chlorophyll derivatives obtained included Zn-pheophytin *a, b* and Zn-pyropheophytin *a, b*. These analogues are green in color, like native chlorophyll, but more stable to acid and high temperature. The Zn-chlorophyll derivatives also show greater antioxidant properties than native chlorophyll. Subsequently, a hydrolytic enzyme was used to extract the derivatives. The commercial enzyme pectinase (Pectinex® Ultra SP-L, activity 10292 PGU/mL) assisted in the extraction process and an experimental design using response surface methodology (RSM) was utilized to further optimize the yield. The optimum conditions were 2.3–2.5% (v/w) Pectinex® Ultra SP-L, 240–260 min incubation time and extraction repeated twice. Under the optimum conditions, the extraction yields of green chlorophyll and antioxidant activity were increased 17.3 and 1.9 fold, respectively, over non-enzymatic treatment. From Zn-chlorophyll powder production by spray drying method, three different wall material types, gum arabic (GA), maltodextrin (MD) and *OSA*-modified starch (MS), were studied based on their physicochemical properties and the stability of encapsulated powder. It was found that the process with 30% (w/w) *OSA*-modified starch proved best for chlorophyll powder production. Under this optimum condition was obtained the highest of green coloration, chlorophyll content and antioxidant activity of the product. Decay of Zn-chlorophyll derivatives GA, MD and MS displayed first-order kinetics with rate constants (k) of 2.1×10^{-3}, 1.8×10^{-3} and 1.5×10^{-3} day^{-1}, respectively and the half-life value ($t_{1/2}$) of 330, 385 and 462 days, respectively. Chlorophyll powders proved to remain unchanged over a wide range of pH and temperature, validating their potential use as coloring additive in food product. The obtained chlorophyll powder contained 2-acetyl-1-pyrroline, the characteristic flavor of pandan leaves. The zinc content of the product was found to be within regulation limit set by the FDA. Collectively, the results support the potential for using natural green colorants in the food industry.

Department :…….Food Technology….. Student's Signature……...

Field of Study : …Food Technology…. Advisor's Signature……..

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CHAPTER I

INTRODUCTION

1.1 Background and significance

Pandan plant (*Pandanus amaryllifolius*) grow widely in tropical countries including Thailand. On account of the leaves' high chlorophylls content, pandan is becoming popular to use as green colorant in food. Pandan leaves also contain the aromatic compound 2-acetyl-1-pyrroline (Jiang, 1990; Laksanalamai and Ilangantileke, 1993) which is related to aromatic compounds found in Basmati and Jasmine rice. Pandan leaves display antioxidant properties due to the presence of quercetin (Miean and Mohamed, 2001), carotenoids, chlorophyll derivatives (Ferruzzi *et al*., 2002), tocopherols, tocotrienols (Lee, Su and Ong, 2004), and polyphenols. Nor *et al*. (2008) reported that polyphenols from the pandan leaf are excellent heat-stable antioxidants. From the information obtained, it indicates that pandan leaf is an interesting plant because of its various bioactive compounds and a rich source of diverse natural products (Wissgott and Bortlik, 1996).

Chlorophyll exists as a stable pigment in nature but when it is extracted from plant tissue, it is rapidly degraded by enzymatic reaction or other factors such as acid, oxygen, light and heat that convert it to derivatives such as pheophytin, pheophorbide, pyropheophytin or pyropheophorbide (Koca, Karadeniz and Burdurlu, 2007).

There are many methods to maintain the green color of chlorophyll during heat treatment. For example, gentle pre-heating is effective because it degrades destructive chlorophyllase enzymes (Tan and Fancis, 1962). Addition of alkali and brine salts such as sodium bicarbonate, hexametaphosphate, disodium glutamate, sodium hydroxide and magnesium hydroxide were including controlling pH (Blair and Ayres,

1943; Gupth, Elbisi and Francis, 1964). However, the studies have shown that under such controlling conditions, the finished product still exhibits brown coloration after storage.

Formation of stable chlorophyll analogues can be achieved by replacing the magnesium ion in the porphyrin ring with other divalent cations such as Zn^{2+} or Cu^{2+} (Humphrey, 1980). The resulting metallochlorophyll complexes are already extensively used in the maintenance of green color in canned fruits and vegetables (Fischbach, 1943; Fischbach and Newburger, 1943). Hence, the replacement of cations in chlorophyll is termed re-greening. The formation of metallochlorophyll derivatives produced depends on metallic salt concentration, chlorophyll concentration, pH and temperature. These derivatives are green in color like native chlorophyll but are more stable to acid and heat and behave more effectively as antioxidants (Tonucci and von Elbe, 1992). Ngo and Zhao (2007) found that in order to maintain the green color of canned pears for more than 19 weeks, the fruit had to be pre-blanched at 94 ^{o}C in 1300 ppm zinc chloride. Canjura, Watkins and Schwartz (1999) suggested that the aseptic process of a green pea, when blanched with zinc chloride a concentration of 300 mg/L at 83 ^{o}C for 5 min as pretreatment, the green color could be retained. Leunda, Guerrero and Alzamora (2000) similarly intensified the green color of kiwi fruit by blanching and adding zinc chloride. Although formation of copper chlorophyll complexes is achieved more readily than for the zinc analogue (Schanderl, Marh and Chichester, 1965; Jones *et al*., 1977), there is more interest in developing use of the zinc derivative due to toxicity of the copper analogue (LaBorde and von Elbe, 1994 a; Humphrey, 2004).

In plant tissue, chlorophyll is tied up in the form of chlorophyll-protein complexes from which it can be extracted by organic solvents such as acetone,

methanol, ethanol, dichloromethane (Humphrey, 2004), dioxane/water (Khalyfa, Kermasha and Alli, 1992) and *N,N*-dimethylformamide (*N,N*-DMF). These solvents disrupt linkages between the pigment and protein, thereby releasing the chlorophyll (Holden, 1976). However, disruption of chlorophyll-protein binding aggregates leads to water insolubility of the pigment, rapid oxidation (Çinar, 2005 a), browning color and toxic by-products.

Enzymatic treatment offers an alternative method for extracting pigments and aromatic compounds such as anthocyanin, lycopene, and carotenoid (Delgado-Vargas and Paredes-Lopez, 1997; Stoll *et al*., 2003; Muñoz, Sepulveda and Schwartz, 2004; Çinar, 2005 a,b ; Landbo *et al*., 2007; Choudhari and Ananthanarayan, 2007) from plant tissue. Enzymatic extraction produces no toxic residues, and the pigment stays in its native form i.e. linked with protein, ensuring in color remains intact (Fenema, 1985; Bassi *et al*., 1993; Çinar, 2004; Çinar, 2005 a). However, some problems occur with enzyme use in chlorophyll extractions. For example, Sun, Power and Tang (2007) used an enzyme in the commercial pectolytic enzyme to extract rutin, total antioxidant and chlorophyll from asparagus. Although this approach resulted in higher yields, significant browning still occurred. Consequently, this study sought to substitute metal-chlorophyll compounds for Zn-chlorophyll complexes prior to enzymatic extraction.

Spray drying is a convenient technique for changing liquids into solid powder form. This makes the compound easies to handle and improves shelf life and stability of the product. The initial step in drying a colorant involves the selection of a suitable wall material, known as the carrier or encapsulating agent. The ideal wall material should have good emulsifying properties, a good film formation, low viscosity at highs solid levels (<300 cps at > 35% solids levels), low hygroscopicity, low cost, and

afford good protection to the encapsulated material. Gum arabic (GA), hydrolyzed starches, and modified starches are the three most important classes of wall materials that are widespread used.

GA is the traditional standard encapsulating agent, widely used because it displays many of the desirable traits described above and shows good retention of active compounds (Thevenet, 1988). GA is a polysaccharide exudate from *Acacia senegal* and *Acacia seyal* trees. Typically each tree produces 300 g/year. GA is expensive to use and its quality depends on the climate conditions under which it was produced. These disadvantages have prompted many manufactures to look for gum arabic to use as a wall material for encapsulation. Starch and products derived from it, such as maltodextrin and n-octenyl succinic anhydride-treated starch (*OSA*-starch) have proved to be popular choices. Maltodextrin is a good compromise between cost and effectiveness, as it is bland in flavor, has low viscosity at high solid ratios and is available in a variety of molecular weights (Apintanapong and Noomhorm, 2003). *OSA*-starch contains both hydrophobic and hydrophilic groups. Both starches exhibit excellent volatile retention during spray drying.

Most researchers seeking optimum conditions for enzyme treatment focus on one variable, whist hold others constant. This so-called single-variable optimization can be successful, but many overlook synergistic interactions between parameters (Rai *et al.*, 2004). Experimental design using response surface methodology (RSM), allow the effects of several process parameters and their interactions on response variables to be evaluated. RSM is a collection of statistical and mathematical techniques for analysis and solving multivariable equations (Roy, Daoudi and Azaola, 2002) and involves contour plotting to find response surface and locate optimal parameters which help to assess optimizing conditions clearly. The technique has been

successfully used for developing, improving and optimizing biochemical processes including those related to enzyme systems (Rai *et al.,* 2004; Sun *et al.,* 2006; Sin *et al.,* 2006; Landbo, Kaack and Meyer, 2007; Liew Abdullah *et al*., 2007; Schweiggert *et al*., 2008; Fan *et al.,* 2008).

As discussed above, pandan appears to be suitable to be evaluated as potential source of natural green colorants. Besides its high chlorophyll content, pandan also exhibits antioxidant activity which can prolong the stability of food. However, native chlorophyll had to change to chlorophyll analogues which more stable derivative form prior to enzymatic extraction. This extraction process was designed with the aid of RSM to evaluate all of involved variables together. The solid Zn-chlorophyll derivative was substituted for the liquid form by using spray drying technique. The suitable wall materials were studied for the maximum encapsulating efficiency. Finally, the stability of Zn-chlorophyll powder was determined. Results of this research proved useful in the development of Zn-chlorophyll derivative powders to use as colorants in food processing.

1.2 Research objectives

1. To determine the factors affecting the formation of Zn-chlorophyll complexes in the pandan leaf.

2. To evaluate the optimal conditions for enzymatic extraction of Zn-chlorophyll complexes by using Response Surface Methodology.

3. To investigate and select a suitable wall material for Zn-chlorophyll powder prepared by spray drying.

1.3 Benefit of this research

There is demand for natural colorant in food industry especially green color from chlorophyll, but chlorophyll in natural source is in the unsuitable form for use as colorant because of its rapid degradation. Thus, the extraction procedure should start with formation of the stable chlorophyll derivatives by reacting zinc chloride under suitable conditions to form the zinc chlorophyll which is stable in green color and have higher antioxidant activity. Therefore, hydrolytic enzyme can be used to extract the derivatives and produce the Zn-chlorophyll derivatives to the powder form. The product is green in color like the natural form but has higher stability, is easy to handle. Hence, the process offers an alternative for production of natural green colorant in the food industry.

CHAPTER II

LITERATURE REVIEW

2.1 Pandan

Pandanus amaryllifolius Roxb. is a member of the screw pine family Pandanaceae. *P. amaryllifolius* possess uniquely fragrant leaves, and are widely cultivated in Southeastern Asian counties, such as Thailand, Malaysia, and Indonesia. Pandan leaf extracts have been widely used in various foodstuffs such as rice, chicken, cake and confectionery. Pandan leaves are sometimes added to iced drinks prepared from the juice of young coconuts and also to sweet puddings and custards prepared from sticky, glutinous rice (Bhattacharjee, Kshirsagar and Singhal, 2005). Moreover, the leaves are immersed in cooking oils to impart flavor to fried foods.

Pandan leaves are also used medicinally in South East Asia to treat fevers, and relieve indigestion and flatulence (Cheeptham and Towers, 2002). Oils contained in the leaf have been proven to act as stimulants and antispasmodics and are effective against headaches, sore throats, rheumatism, and epilepsy (Quisumbing, 1951). The pandan leaf contains quercetin (Miean and Mohamed, 2001), carotenoids, chlorophyll derivatives (Ferruzzi *et al*., 2002), tocopherols, tocotrienols (Lee *et al*., 2004) and polyphenols which are heat-stable antioxidants (Nor *et al*., 2008). Nor *et al.* (2008) reported that pandan leaf extract contained polyphenols that exhibited excellent heat-stable antioxidant properties suggesting its potential as a natural alternative to existing synthetic antioxidants in the food industry. Moreover, the pandan leaf also yields vitamins, minerals and other nutrients (Table 2.1).

Table 2.1 Nutrient composition of pandan leaf (per 100 g of edible component)

	Nutrient composition	
Proximate	Energy	35 Kcal
	Moisture	85.3 g
	Protein	1.9 g
	Fat	0.8 g
	Carbohydrate	4.9 g
	Crude fiber	5.2 g
	Ash	1.9 g
Minerals	Calcium	124 mg
	Phosphorus	27 mg
	Iron	0.1 mg
Vitamins	Beta-carotene	2987 µg
	Total A (RE)	498 µg
	Thiamin	Trace
	Riboflavin	0.20 mg
	Niacin	1.2 mg
	Vitamin C	8 mg

Source: Department of Health, Thailand (1992)

The flavor components of pandan leaves were not very well known until Teng, Shen and Goh (1979) isolated many of the constituent sweet-smelling compounds by chloroform–methanol extraction. One pandan leaf flavor component, 2-acetyl-1-pyrroline (2-AP) is responsible for a popcorn-like scent, often described by oriental people as a pandan-like aroma (Fig 2.1). Spectroscopic analysis of their extracts revealed that much of the leaf's characteristic flavor comes from oxidative degradation products of a yellow carotenoid pigment that develop only when the plant withers. The 2-AP compound is virtually absent in fresh intact plants of minimal aroma.

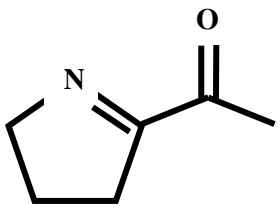


Fig. 2.1 The structure of 2-Acetyl-1-pyroline

(Source: Buttery, Ling and Mon, 1986)

2-AP has been identified as a major component of the volatile oil of freeze-dried pandan leaves (Buttery *et al*. 1986; Paule and Powers, 1989; Adam and Kimpe, 2006). This compound also gives rise to the pleasant smell of also aromatic rice varieties such as Basmati and Jasmine. However, the concentration of 2-AP in pandan leaves is higher than in aromatic rice (Buttery, Juliano and Ling, 1983; Buttery, Turnbaugh and Ling, 1988).

2.2 Chlorophyll

1) Structure

Chlorophyll is the green pigment found in grasses, trees and all higher plants. Chemically, chlorophyll ($C_{55}H_{70}MgN_4O_6$) is classified within the porphyrin group. The structural unit common to this series is porphin, comprising four units of pyrrole, whose α-positions are linked by methine bridges, forming a macrocyclic aromatic system that is very stable (Fig. 2.2).

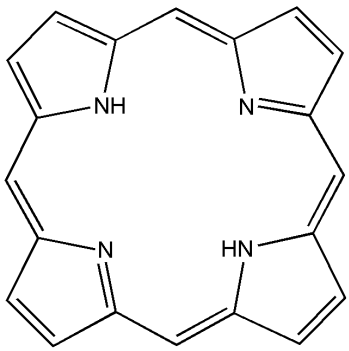


Fig. 2.2 The structure of porphin.

(Source: Minguez-Mosquera *et al*., 2002)

Porphin forms non-covalent bonds with some of metal ions, leaving the metal firmly bound by the four nitrogen atoms of the planar ring. Porphin's structure comprises 11 conjugated double bonds comprising a chromophore group that absorbs

visible light (Fig. 2.3). In consequence, porphyrins generally appear brightly colored. The uncomplexed porphin system is orange, and the color of porpyrin metal complexes depends both on the nature of the ring substituents and the central metal cation.

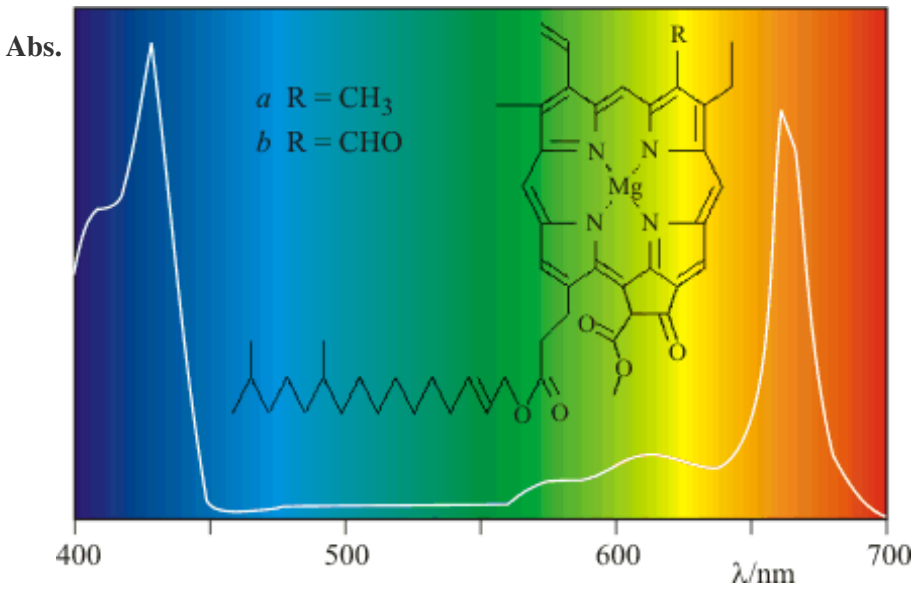


Fig. 2.3 Absorption spectrum of chlorophyll

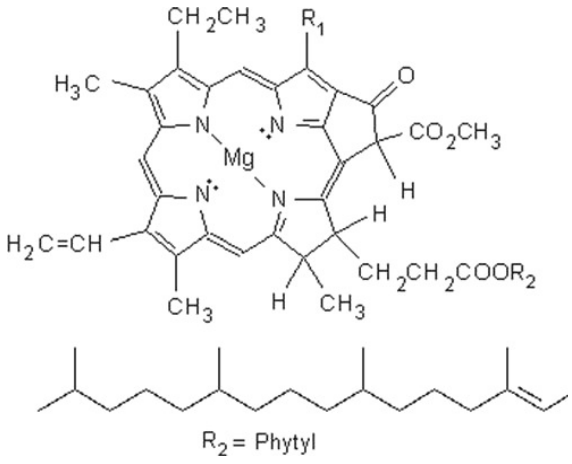
(Source: Genalic, 1997)

Chlorophyll contains a modified propionic acid chain in the form of a cyclic β-ketoester (isocyclic ring) and, on C−17, a chain of propionic acid esterified with a C−20 diterpene alcohol (phytol). This phytol group makes the molecule liposoluble. In higher plants, only chlorophylls *a* and *b* are present. The *a:b* ratio normally varies between 3 and 1, depending on a multitude of both genetic factors (e.g. species, variety) and environmental factors (luminosity, water stress, mineral nutrition, etc.).

2) Chemical properties

In nature, chlorophylls are quite stable when inside the chloroplast, but when this organelle is damaged and loses its physiological element, the chlorophyll complex becomes extremely labile, and susceptible to a wide range of structural modifications.

Chlorophylls are readily transformed or degraded into a series of derivatives with olive-brown and green colors or non-fluorescent colorless substances due to chlorophyll bleaching or photobleaching. The olive-brown degradation products of chlorophyll are magnesium-free derivatives that include the pheophytins, pheophorbide, pyropheophorbides, and pyropheophytins (Fig. 2.4 and 2.5).



Derivative	Metal	R_1	R_2
Chlorophyll a	Mg	CH_3	Phytyl
Chlorophyll b	Mg	CHO	Phytyl
Pheophytin a		CH_3	Phytyl
Pheophytin b		CHO	Phytyl
Pheophorbide a		CH_3	H
Pheophorbide b		CHO	H

Fig. 2.4 The structure of chlorophyll and chlorophyllin derivatives.

(Source: Lanfer-Marquez, Barros and Sinnecker, 2005)

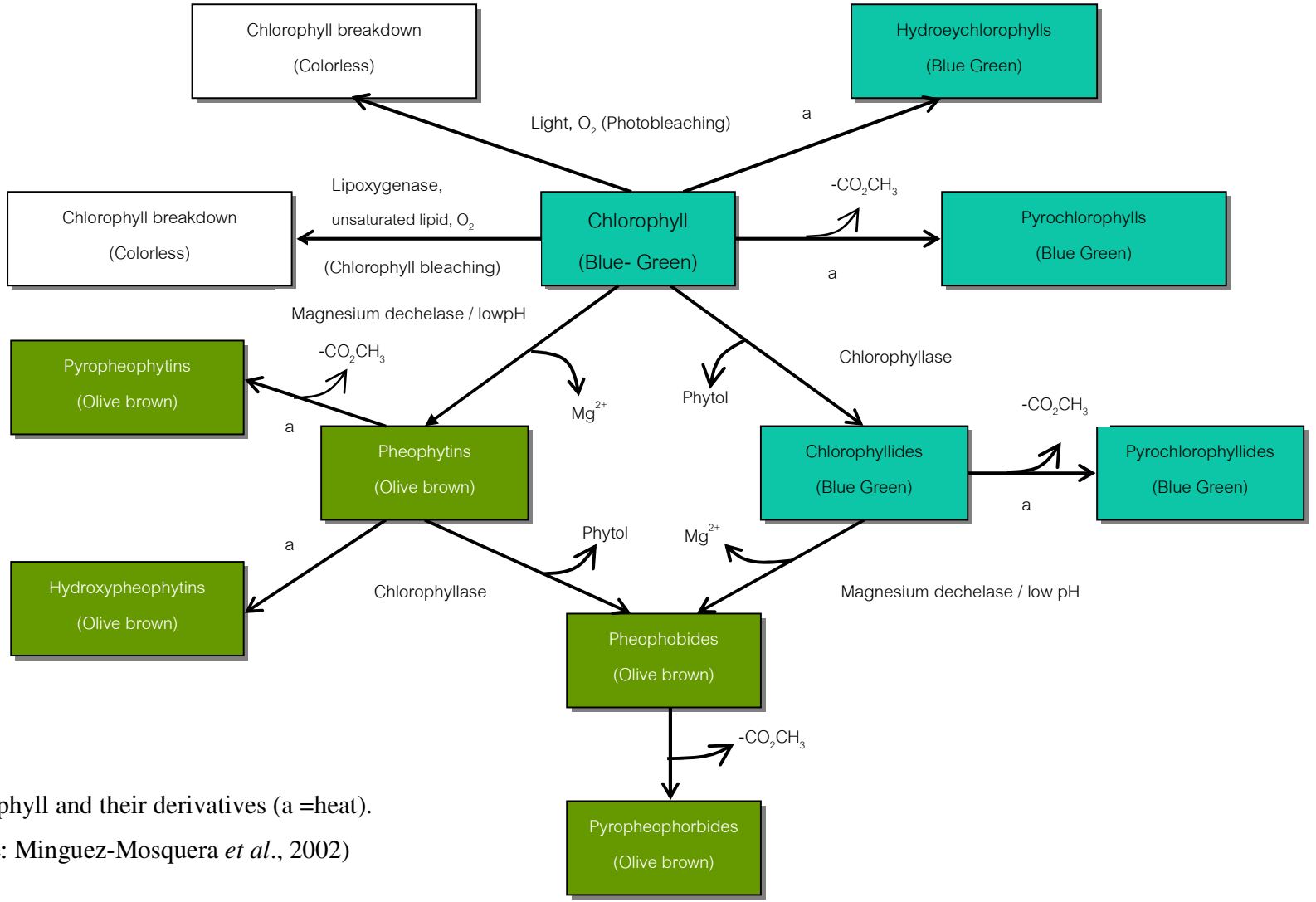


Fig. 2.5 Chlorophyll and their derivatives (a =heat).
(Source: Minguez-Mosquera *et al*., 2002)

3) Spectroscopic properties

The absorption of visible light by chlorophylls arises because of extensive conjugation in the porphyrin structure. Both chlorophylls *a* and *b* exhibit an absorption maximum in the blue-violet region, with λ_{max} at 428 and 454 nm, respectively. Smaller peaks are seen at 410 and 430 nm, respectively. Displacement of these absorptions toward the red region of the spectrum changes the greenish-blue color of chlorophyll *a* to yellowish-green in chlorophyll *b*.

The substitution of Mg by H in chlorophyll *a* produces a marked blue shift in the *Soret band*, from 428 to 408 nm. Displacement of the secondary band (*Q band*) is less marked (Fig. 2.6), shifting from 662 to 666 nm, with an increase in the peak ratio. Similar changes are seen in the absorption spectrum of chlorophyll *b* following the pheophytinization reaction. The Soret band shifts from 454 to 430 nm, and the secondary displaced from 646 to 656 nm (Fig. 2.7). Insertion of other divalent cations such as Cu^{2+} or Zn^{2+} shifts the absorption bands towards the red. However, the shapes of the spectral peaks, and the profile of absorptions appear similar to those observed for the original Mg complex.

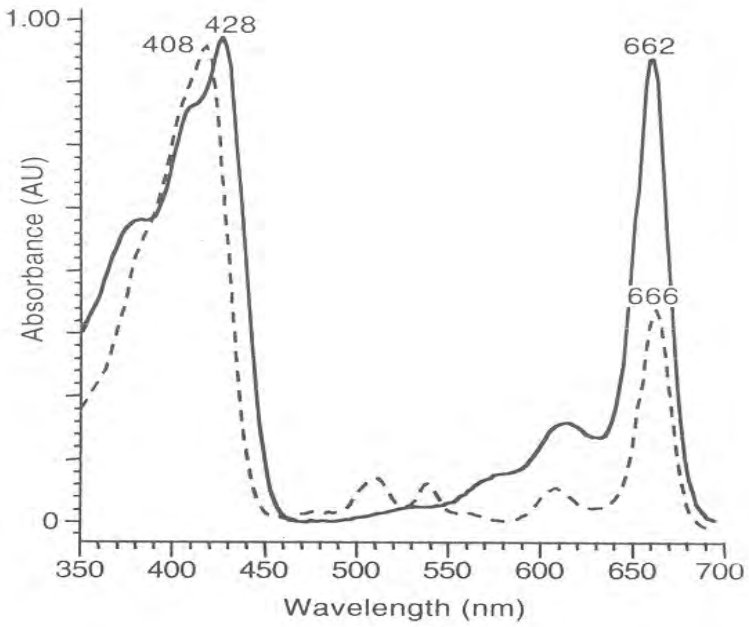


Fig. 2.6 Electronic absorption spectra of chlorophyll *a* (‾) and pheophytin *a* (- - -)

(Source: Minguez-Mosquera *et al*., 2002)

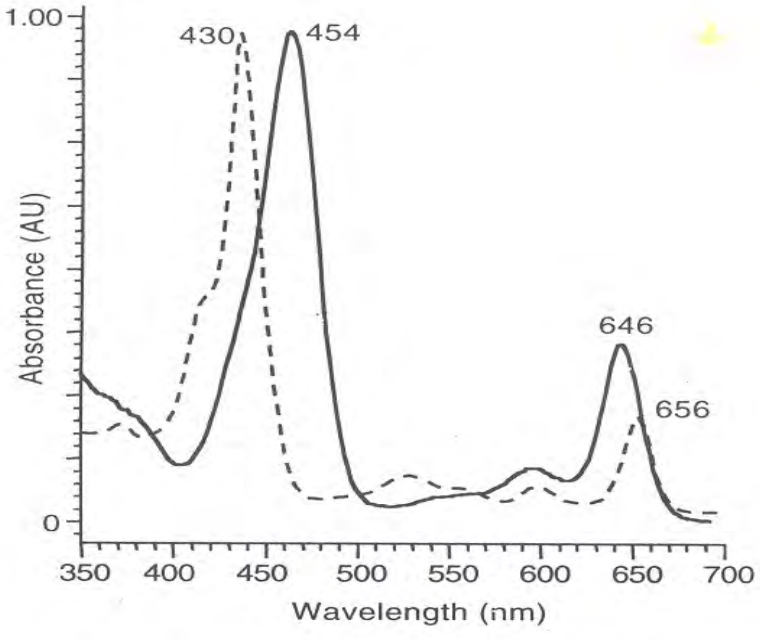


Fig. 2.7 Electronic absorption spectra of chlorophyll *b* (‾) and pheophytin *b* (- - -)

(Source: Minguez-Mosquera *et al*., 2002)

4) Chlorophyll stability during food processing

Since consumers perceive green color as an indicator of the "freshness" in many fruits and vegetables, many studies have been carried out on chlorophyll, seeking ultimately to limit the degradation of prized compound (Gauthier-Jaques *et al.,* 2001). Chlorophyll retention has been used as a measure of quality in green vegetables (Sweeney and Martin, 1961). The degradation of chlorophyll during the industrial processing of fruits and vegetables remains problematic. When fragile cellular structures are disrupted during processing, pigments become susceptible to various enzymatic and non-enzymatic degradation reactions, as discussed in the following section.

A. Effect of heating

Chlorophyll *a* and *b* are partially or totally broken down to pheophytins and pyropheophytins during high temperature processing of vegetables (Haisman and Clarke, 1975: Schwartz and von Elbe, 1983). Schwartz and von Elbe (1983) suggested this decomposition occurs as a two-step process: Chlorophyll → Pheophytin→ Pyropheophytin (Fig 2.8). The conversion of chlorophylls to pheophytins begins only above 60 ^{o}C (Haisman and Clark, 1975; Weemaes *et al*., 1999) as a result of increased permeability of hydrogen ions across cell membranes (Haisman and Clark, 1975). Several investigations have found that such thermal chlorophyll degradation follows first-order kinetics, with the degradation rate rising steadily with temperature (Haisman and Clarke, 1975; Schwartz and von Elbe, 1983; Steet and Tong, 1996).

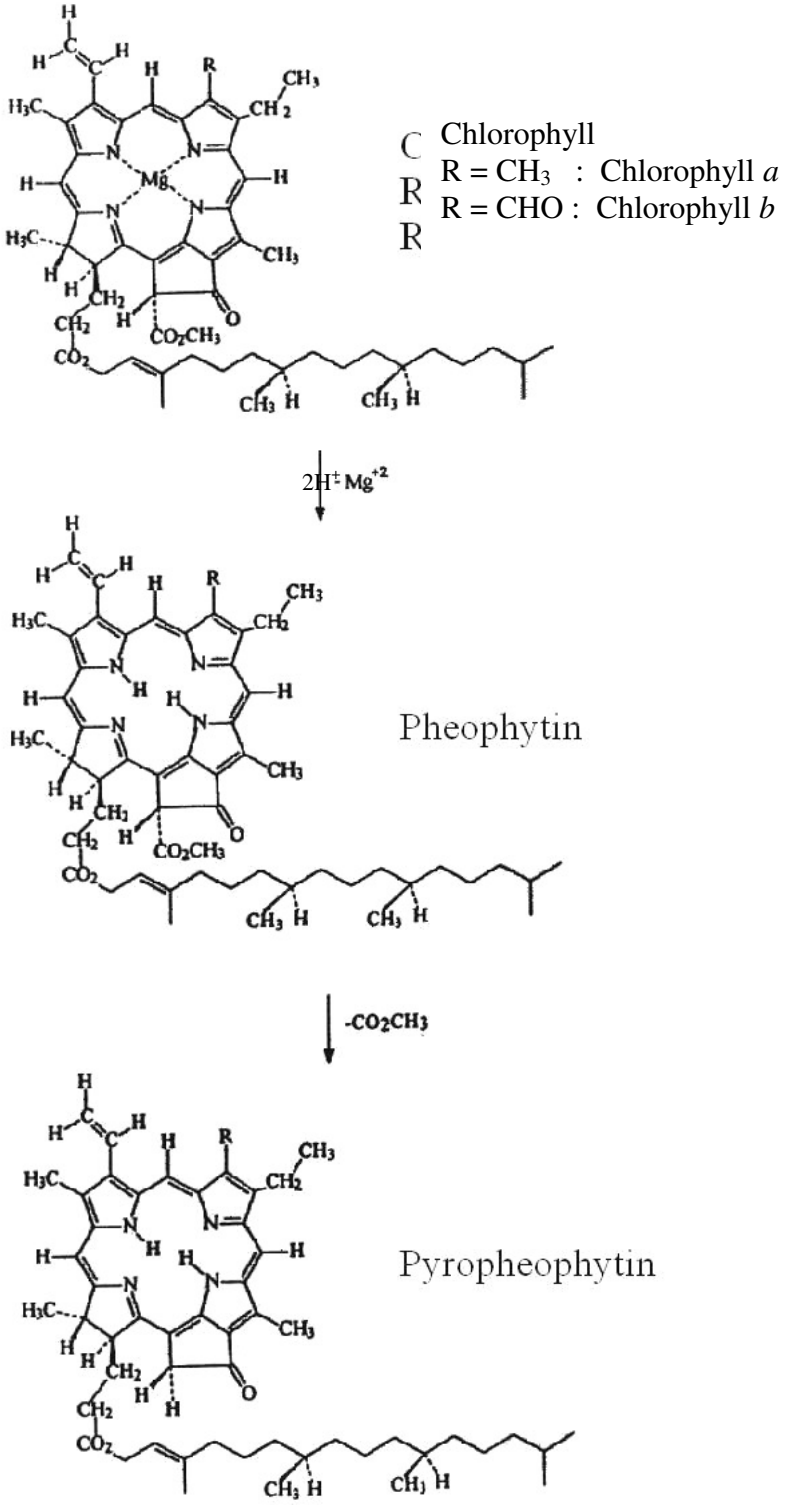


Fig. 2.8 Consecutive degradation of chlorophylls by heat.

(Source: von Elbe and Schwartz, 1996)

B. Effect of pH

During heating process and storage of fruits and vegetables, there is a release of intercellular acids and enzymes, which further facilitates chlorophyll degradation. Like heat exposure, these agents also promote the formation of pheophytin, chlorophyllide, pheophorbide, fluorescent compound and olive green pigment.

The removal of Mg^{2+} from chlorophyll occurs in acidic media, forming pheophytin (Leunda *et al.*, 2000) and resulting in a change from bright green to dull olive-green or olive-yellow (Gupte *et al.*, 1964). In dilute alkali solution chlorophylls can become saponified into chlorophyllin carboxylate salts, phytol and methanol which, deceptively, retain a vivid green color.

C. Improving the stability of green color

There are many methods to preserve the intensity of chlorophyll's green coloration. For example, gentle preheating to degrade destructive chlorophyllases (Tan and Francis, 1962), adding alkali and brine salt such as sodium bicarbonate, hexametaphosphate, disodium glutamate, sodium hydroxide and magnesium hydroxide including controlling pH (Blair and Ayres, 1943; Gupth and Francis, 1964). Clydesdale and Francis (1968) created derivatives of phytol-free pigments to improve the color of spinach. However, formation of chlorophyllide proved to be a limiting factor. Many studies have shown that even under conditions discuss above, the products still turn brown during storage.

D. Metallochlorophyll complexes

By contrast with natural chlorophylls, metal-porphyrin complexes featuring zinc and copper are relatively resistant to acid and heat (Humphrey, 1980). The patented Veri-Green procedure in which blanching of green vegetables is performed in the presence of zinc (II) salts, results in the formation of zinc complexes of pheophytin

and pyropheophytin. Zinc complexes are favored over to copper preserve green color because zinc is more beneficial for human health.

These metallocomplexes can be synthesized and yields depend on metallic salt concentration, chlorophyll concentration, pH value, and temperature. Derivatives were of chlorophyll *a* form metallocomplexes more rapidly than those of chlorophyll *b* (Berezin and Koifman, 1970; John *et al*., 1977). John *et al*. (1977) suggested that the synthesis of zinc chlorophyll complexes doesn't occur form native chlorophylls because the steric hindrance of their molecular structure. Pheophytin forms metallocomplexes more slowly than pyropheophytin and pheophorbide because of the presence of interfering substituent groups (Berezin and Koifman, 1970; Tonucci and von Elbe, 1992). The rate of zinc complex formation increases with increasing pH and Zn^{2+} ion and pigment concentrations (LaBorde and von Elbe, 1990). However, high pH conditions may result in retention of native chlorophyll form after heating and a reduction in the amount of chlorophyll derivatives available for complex formation (LaBorde and von Elbe, 1994 a). von Elbe *et al*. (1986) reported that the major pigments in Zn-chlorophyll complexes are zinc pheophytin *a*, and zinc pyropheophytin *a*.

The formation of Zn-chlorophyll complexes has been used in food processes. For example, Ngo and Zhao (2007) reported that in the production of canned pears when blanching at 94^{o}C with 1300 ppm zinc chloride the product still had green color. Canjura *et al*., (1999) suggested that in production of green peas by aseptic process (blanching with zinc chloride (300 mg/L) at 83^{o}C for 5 min as pretreatment) the green color could be retained. Leunda *et al.* (2000) improved the green color of kiwi fruit by blanching and adding zinc chloride. Luke and von Elbe (1990) reported that the

formation of zinc complex increased when sodium dodecyl sulfate was added because it improves diffusion efficiency of zinc within the chloroplast.

5) Antioxidant activity of chlorophyll

Endo, Usuki and Kaneda (1985) first suggested that chlorophyll derivatives can act as hydrogen donors in chain-breaking antioxidant activity. Derivatives of chlorophyll *a* have been found to be more effective radical quenchers than chlorophyll *b*. Cahyana, Shuto and Kinoshita (1993) demonstrated a structural relationship within related porphyrins for the inhibition of lipid hydroperoxide formation from ferric thiocyanate and ferric nitrilotriacetate.

Hoshina, Tomita and Shioi (1998) found that metal chlorophylls were better antioxidants than their metal-free derivatives and confirmed the importance of the porphyrin ring on the inhibition of lipid autoxidation. Metal-free derivatives such as chlorins, pheophytins, and pyropheophytins exhibited significantly lower antiradical capacity than complexes such as Mg-chlorophylls, Zn-pheophytins, Zn-pyropheophytins, Cu-pheophytin *a*, and Cu chlorophyllins (Ferruzzi *et al*., 2002).

6) Chlorophyll as a food colorant

Natural chlorophyll is not in a suitable form for use as food additive due to its poor stability and water insolubility (Humphrey, 1980). Metallochlorophyll complexes continue to gain interest as a source green colorant on account of their increased stability and antioxidant activity.

2.3 Chlorophyll extraction

1) Solvent extraction

In plant tissue, chlorophyll is present in the form of chlorophyll-protein complexes which can be extracted by organic solvents such as acetone, methanol, ethanol, dichloromethane (Humphrey, 2004), dioxane/water (Khalyfa *et al*., 1992) and

N,N-dimethylformamide. These solvents disrupt linkages between pigment and protein, releasing unbound chlorophyll (Holden, 1976). However, disruption of these aggregates causes water insolubility of the pigment, rapid oxidation (Çinar, 2005b), color change. Residues of toxic solvent remain in chlorophyll extracts.

2) Enzymatic extraction

Plant materials are rich in natural colorants and flavor compounds. Polysaccharides such as pectin and cellulose are mainly responsible for their sequestration, hindering their extraction by solvent. Enzymatic hydrolysis of plant cell walls increases the extraction yield, and recovery of reducing sugars, soluble dry matter, galacturonic acid and titrable acidity of the products (Joshi, Chauhan and Lal, 1991; Drilleau, 1994).

Enzymes which split the glycosidic bond of polygalacturonic acid (pectic acid), polymethylgalacturonic acid (pectin), and cellulolytic substances are generally classified based on substrate specificity and whether the substrate is split at a random (endo) or terminal (exo) residue. Enzymic preparations disintegrate the plant tissues allowing improved the pigment product.

Pectinex Ultra® SP-L (Novozyme company) is a highly active enzyme preparation which is specially designed for mashing. It is produced from a selected strain of *Aspergillus niger*. These preparations have both pectolytic activities, including polygalacturonase, pectinlyase, pectinesterase and others including hemicellulase, cellulase, protease and amylase. Polygalacturonase depolymerises the polygalactronic acid chain via lysis of α-1,4 glycosidic bound. While pectinlyase depolymerises the high esterified pectin, pectin esterase hydrolyses the methoxyl group of the pectin chain. Therefore Pectinex® Ultra SP-L has the ability to disintegrate plant cell walls, also to degrade haze provoking polysaccharides and

pectin. When Van den Broek *et al.* (1997) investigated the macerating effects of Pectinex® Ultra SP-L enzyme preparation they found endopectinlyase (EC 4, 2, 2, 10) to be the main maceration enzyme.

Cellulase randomly splits cellulose chains into glucose monomers whereas commercial pectinase preparations from *Aspergillus niger* have pectinesterase (PE), polygalacturonase (PG) and pectinlyase (PL) activity. Celluclast®1.5L (Novozyme company) is made by submerged fermentation of a selected strain of the fungus *Trichoderma reesei* which catalyzes the breakdown of cellulose into glucose, cellobiose and higher glucose polymers.

The use of pectinase and cellulase enzymes disrupts the cell wall of orange peel, sweet potato and carrot and frees the carotenoids from the chloroplasts, allowing their diffusion in to the cytoplasm. These released pigments remain in their natural state, bound to proteins. These aggregates prevent pigment oxidation, thereby stabilizing the complex and hence the green coloration (Fenema, 1985). Solvent extraction, by contrast, dissociates the pigments from the proteins and causes water insolubility and susceptibility to oxidation

Similar enzyme treatment in the extraction of other plant pigments such as anthocyanins, lycopenes, carotenoids, and betacarotenes (Delgado-Vargas and Paredes-Lopez, 1997; Santamaria *et al*., 2000; Stoll *et al*., 2003; Muñoz *et al*., 2004; Çinar, 2005 a; Sun *et al*., 2006; Landbo *et al*., 2007; Choudhari and Ananthanarayan, 2007).

2.4 Encapsulation

There has been increased interest in the development of food colorants from natural sources as alternatives to synthetic dyes because of both legislative action and consumer concern (Giusti *et al*., 1998). Complete microencapsulation aims to totally entrap the pigment particles in a protective network, which isolates and stabilizes the pigment from factors that can cause color loss or change, such as oxidation or acid (Wissgott and Bortlik, 1996).

Microencapsulation by using spray drier offers an economical approach for preservation of natural colorants by entrapping dyes within a coating material (Cai and Corke, 2000). Microcapsules range widely in millimeters in size (0.2–5000 μM) and can be prepared in a variety of shapes, depending on the materials and methods used (Balassa and Fanger, 1971).

The encapsulation efficiency of active ingredients is largely dependent on the performance of the wall material. Among the several encapsulating agents, emulsifying starches and gums are the most common wall materials (Reineccius, 1989). GA has now become a traditional standard (Thevenet, 1988). Being a tree exudate, its supply and quality are inconsistent, depending on the climatic conditions in which the source plant is grown. Furthermore its high cost is also a disadvantage. As a consequence many manufactures are seeking for substitutes for GA for use as a wall material for the encapsulation of sensitive food constituents. Starch and its derivatives, such as maltodextrin and OSA-starch are popular choices of colorant encapsulation.

1) Gum arabic

GA largely constitutes of cross-linked residues of a variety of different sugars including galactose, arabinose, rhamnose and glucoronic acid (Anderson and Stoddart, 1966; Williams and Phillips, 2000). However, approximately 2% (w/w) of GA is protein, which plays a crucial role in encapsulation (Fig. 2.9) (Randall, Phillips and Williams, 1988). Gum arabic is most often used as wall material. Its solubility, low viscosity, emulsification characteristics and its reliable retention of active compounds make it very versatile for most encapsulation procedures. In addition, the GA is ideally suited to the encapsulation of lipid droplets as it fulfils the roles of both surface active agent and drying matrix, thus preventing the loss of volatiles during contact with the atmosphere.

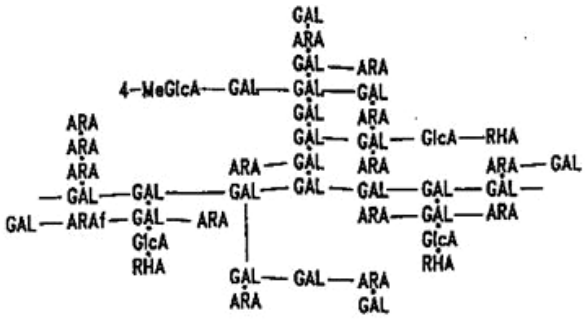


Fig. 2.9 Structure of gum arabic (GAL = Galactose; ARA = Arabinose; GlcA = Glucuronic acid; RHA= Rhamnose; 4-MeGlcA = 4-*O*-methylglucuronic acid)

Some studies have demonstrated the use of GA in microencapsulation for the retention of piperine or volatile compounds extracted from black pepper oleoresin (Shaikh, Bhosale, and Singhal, 2006). Krishnan, Bhosale and Singhal (2005) used GA in encapsulation of 1,8-cineole and α-terpinyl acetate extracted from cardamom oleoresin.

2) Maltodextrins (MD)

MD is formed during partial hydrolysis of flour with acids or enzymes. It is typically supplied as dextrose equivalents (DEs), the value of which reflects the extent of starch polymer hydrolysis. MD has been investigated as a replacement for GA in spray dried emulsions (Anandaraman and Reineccius, 1986). MD has the ability to form matrices that are important in forming wall systems (Shahidi and Han, 1993; Kenyon, 1995).

In selecting wall materials for encapsulation, maltodextrin offers a good compromise between cost and effectiveness. It is bland in flavor, has low viscosity at a high solid ratio and is available in a wide variety of average molecular weights, (4, 10, 15, 20, 25, and 42 DE) allowing selection of the appropriate matrix for the desired result (Apintanapong and Noomhorm, 2003). Studies indicated that higher DE starches form a tighter and more gas-impermeable matrix resulting in a more extended shelf-life of product. However, major shortcomings of these products include a total lack of emulsifying capacity and marginal retention of lipophilic volatiles.

Some research has recommended maltodextrin in retaining volatiles such as those from orange oil, orange peel oil, lemon oil, cheese volatile compounds and 2-AP (Anandaraman and Reineccius, 1986; Change, Scire and Jacobs, 1988; Bang and Reineccius, 1999; Mongenot, Charrier and Chalier, 2000; Apintanapong and Noomhorm, 2003) in addition to effectively encapsulating pigments such as betacyanins, lycopenes, β-carotenes, anthocyanins and carotenes (Wagner and Warthesen, 1995; Cai and Corke, 2000; Quek, Chok and Swedlund, 2007; Ersus and Yurdagel, 2007). Loh *et al*. (2005) studied the production of spray-dried pandan (*Pandanus amaryllifolius*) powder using MD as a wall material. Goubet, Quere and Voilley (1998) reported that MD had chemical bonds related with encapsulation that

included hydrophobic, van der Waals, hydrogen bonding and electrostatic force. Moreover, MD had low viscosity at high concentration, film forming property and low price (Anandaraman and Reineccius, 1986; Wang and Wang, 2000; Apintanapong and Noomhorm, 2003).

3) OSA-Starch

Native starch and its hydrolysis by-products are hydrophilic, and have no affinity for hydrophobic substances such as oils. However, it is possible to modify starch with fatty acids to increase its hydrophobicity. Esterification with oleic acid has been carried out for the preparation of starch oleates. The hydrophilic nature of modified starch can similarly be diminished by esterifying the polymer with *n*-octenyl succinic anhydride (*n*-OSA). The hydrophobic octenyl side chains impart an emulsifying capability to the starches. Starch alkenyl esters contain both hydrophobic and hydrophilic groups (Fig 2.10). Most *n*-OSA starches used for encapsulation are depolymerized to lower the viscosity (Qi and Xu, 1999). These starches display excellent volatile retention during spray drying and superior emulsification properties.

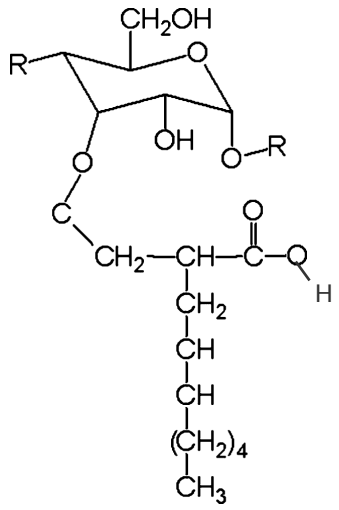


Fig. 2.10 Structure of starch octenyl succinate

(Source: Drusch and Schwarz, 2006)

The use of starch octenyl succinicate (OSA) is permitted in food applications, though the US FDA has restricted modified OSA content to 3% (w/w). Unlike typical surfactants, OSA starch forms strong films at the oil-water interface giving emulsions that are resistant to re-agglomeration. As a result, aqueous solutions of OSA starches have been used to stabilize flavor-rich emulsions in beverages and the oils in salad dressings and to encapsulate flavor and fragrances (Trubiano and Lacourse, 1988).

Many studies have used OSA-starch as a wall material (Ascheri, Marquez and Martucci, 2003) including those investigating *D*-limonene from orange oil, *L*-menthol (Sootitantawat *et al*., 2005), fish oil featuring long-chain polyunsaturated fatty acids (Drusch and Schwarz, 2006), vanillin (Chattopadhyaya, Singhal and Kulkarni, 1998) and carotenoids (Partanen *et al.*, 2002). Furthermore, Tesch, Gerhards and Schubert (2002) reported that OSA-starch can be used to prevent oxidation as efficiently as GA.

2.5 RESPONSE SURFACE METHODOLOGY (RSM)

RSM can be divided into three stages. The first stage is the preliminary work in which the determination of the independent parameters and their levels is carried out. The second stage is the selection of the experimental design and the prediction and verification of the model equation. The last step involves obtaining the response surface plot and contour plot of the response as a function of the independent parameters and the determination of optimum points.

CHAPTER III

METHODOLOGY

3.1 MATERIALS

Pectinex® Ultra SP-L enzyme was obtained from Novozyme (Denmark), manufactured from *Aspergillus aculeatus* which had activity of 10292 PGU/mL. Celluclast®1.5L, obtained from Novozyme (Denmark), is a liquid cellulase preparation produced by submerge of a selected strain of fungus *Trichoderma reesei*. The activity was 700 EGU/g (EGU = Endo-Glucanase Units).

Gum arabic was purchased from S.R. Lab, Ltd. (Thailand). Maltodextrin was obtained from Burly Jucker Specialties, Ltd. (Bangkok, Thailand). *OSA*-modified starch was a kindly provided by Sayuan Wong company, Ltd. (Nakhon Ratchasima, Thailand). All chemicals used in this study were analytical grade. Pandan leaves were purchased from a reputable local market in Bangkok, Thailand.

3.2 METHODOLOGY

Studies on pandan leaf extract powder were divided into six experiments. The first of these analyzed the pandan leaf compositions, second studying the factors effecting on the color change of pandan leaf extract, third studying the optimum condition for formation of Zn-chlorophyll complexes in the stabilization of chlorophyll derivatives. Fourth determined the most effective enzymatic extraction, fifth scrutinized the production of Zn-chlorophyll derivatives encapsulated powders, and finally determining the physicochemical properties and stability of Zn-chlorophyll derivatives powder.

1) Pandan leaf composition

The physical and chemical properties of pandan leaves were studies color values (L^*, a^*, b^*, C, and h^o). Measured, levels of chlorophyll contents, antioxidant activity, pH, total dietary fiber**,** zinc content, and aroma compounds also (Appendix A).

2) Factors affecting on the color change of pandan leaf extract

Fresh pandan leaves were washed, chopped into small pieces and homogenized by blending with the warring blender for 2 min. The ratio between pandan leaf and water was 1:4 by weight.

The color degradation was investigated in pandan puree as a function of pH, temperature and combination of pH and temperature. The pH of pandan puree was adjusted to 3-8 by citric acid or NaOH. Second were studied the effect of heating temperatures and times on the stability of color in the range 70-100 ^oC, and 0-30 min, respectively. Finally, the combination factors were studied in the pH range 3-8 at 121^oC for 15 min. The color change of pandan puree was determined by measuring color values (L^*, a^*, b^*, C, and h^o) at 0, 7, 14 and 21 days after storage at 4 ^oC.

3) Formation of Zn-chlorophyll complexes

Pandan pulp (500 g) was used in each case and adjusted as described above. Mixtures were then reacted with zinc chloride (300 ppm), heated (121 ^oC for 15 min). Color changes in the pandan pulp were recorded. Measurement of zinc chloride concentration at a pH level that resulted in the highest greenness intensity was further analyzed by varying the concentration of zinc chloride in the range of 0-600 ppm and heating at 121 ^oC for 15 min. Determination of the optimum temperature and reaction time were done by varying range of heat between 80-120 ^oC at the optimum conditions noted above.

Zn-chlorophyll derivatives were identified by absorption spectral and high performance liquid chromatography (HPLC). Separation of chlorophyll derivatives was accomplished using the method of Guzmán *et al.* (2002): pandan leaves (10 g) were extracted with acetone (20 mL, 80%), filtered into a separation funnel, and added diethyl ether and dried with anhydrous sodium sulfate. Separation of the pigments was then done by using preparative thin layer chromatography (TLC) using Kiesegel $60F_{254}$ plate 0.5 mm thick (Merck) (Germany) and hexane/diethyl ether/acetone (6:2:3 v/v/v). Identification of chlorophyll and its derivatives was determined by using RF measurement (distance of component migration/distance of solvent migration). Absorption spectra were recorded from 400 to 800 nm.

Separation of chlorophyll derivatives was achieved by HPLC with a Phenomenex 5 μm particles 4.6 mm i.d. × 250 mm (VARIAN, Prostar, USA). The solvent system used was ethyl acetate/methanol/water in the following respective proportions: solvent A (15:65:20 v/v/v), and solvent B (60:30:10 v/v/v). Initial conditions consisted of 100% solvent A at a flow rate of 1.3 mL/min. A gradient was applied after 6 min under isocratic conditions as shown in Fig. 3.1. Final conditions consisted of 100% solvent B at a flow rate of 1.5 mL/min. After 10 min, the solvent composition was returned to the initial proportions.

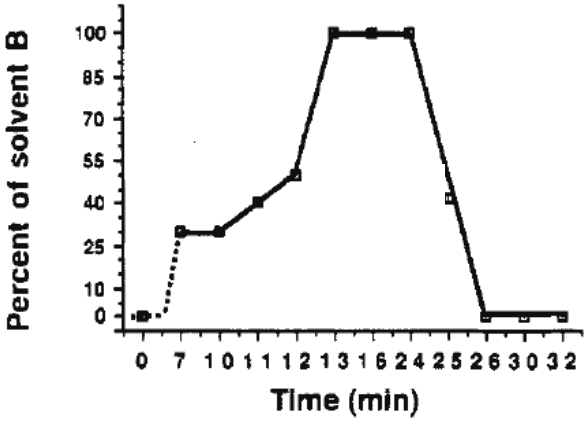


Fig. 3.1 Reversed phase HPLC gradient elution profile

4) Enzymatic extraction

4.1 Preliminary study

The preliminary step for finding the optimum condition involved screening of the dependent variables and their levels. The effect of varying the enzyme, and concentration of enzyme the pH, optimum temperature were then studied. Two types of commercial enzyme, Pectinex® Ultra SP-L and Celluclast®1.5L, were compared for capacity to extract Zn-chlorophyll derivatives from pandan leaves. The choice of enzyme for each treatment depended on which provided the higher reducing sugar level. The optimum condition of selected enzyme was examined in the ranges of enzyme on the amount of reducing sugar concentration 0-5%, pH 3.0-6.0, and incubation temperature 35-55^{o}C.

The relative activity of the enzyme in the pandan puree under set of conditions was determined by the reduction of the viscosity of pectin solution in Mcvalline buffer (at pH 3.5, 35°C). The pectin solution was heat-treated in order to obtain the gel form. Pectin solution (100 mL) was reacted with pandan sample (1 mL) in a water bath (35 °C for 30 min). Then the reaction was quenched by heating (90 °C for 10 min) and then immediately cooling in an ice bath. The reduction of viscosity was observed using a Brookfield Viscometer (Model DV-I).

4.2 Optimizing enzymatic extraction of Zn-chlorophyll derivatives

Response surface methodology was employed to determine the optimal conditions. The experiments were based on a central composite design with the quadratic model employed to study the combined effects of independent variables. According to preliminary studies, the enzymatic extraction parameters were studied on the concentration at enzyme concentration in the range of 1–3%, reaction times of 90–270 min, and 1–3 rounds of re-extraction. These three independent variables were

coded as X_1, X_2, and X_3, respectively. Each independent variable had three levels of parameters: low level (-1), medium level (0), and high level (+1). A total of 19 combinations including five replicates of the center point were carried out in random order. The levels of the variables for (*x*) and (*X*) are shown in Table 3.1.

Table 3.1 Matrix of experimental central composite design (CCD) for enzymatic extraction of pandan leaf

Treatment No.	Level		
	enzyme code, (%)	time code, (min)	re-extract code, (times)
1	−1, (1)	0, (180)	0, (2)
2	+1, (3)	0, (180)	0, (2)
3	0, (2)	−1, (90)	0, (2)
4	0, (2)	+1, (270)	0, (2)
5	0, (2)	0, (180)	−1, (1)
6	0, (2)	0, (180)	+1, (3)
7	+1, (3)	+1, (270)	+1, (3)
8	−1, (1)	+1, (270)	+1, (3)
9	+1, (3)	−1, (90)	+1, (3)
10	−1, (1)	−1, (90)	+1, (3)
11	+1, (3)	+1, (270)	−1, (1)
12	−1, (1)	+1, (270)	−1, (1)
13	+1, (3)	−1, (90)	−1, (1)
14	−1, (1)	−1, (90)	−1, (1)
15−19	0, (2)	0, (180)	0, (2)

The dependent variables measured were color values (L^*, $-a^*$, b^*, C, h^o), total chlorophyll content, and antioxidant activity of the pandan leaf extract. The obtained results were used to create quadratic equations to determine the correlation of data by SPSS program version 13 (SPSS Inc. Chicago, USA) and to make response surface curves and a contour plot by STATISTICA program version 5 (Stat Soft Inc., Tulsa, USA).

5) Zn-chlorophyll derivatives encapsulated powders

For the preparation of 10, 20 and 30% solution of each wall material (GA, MD and *OS*A-MS) by dispersion in water the final volume was made to 100 mL. 10 grams (10% based on wall material used) of Zn-chlorophyll derivatives were added to the mixture. The mixture was homogenized (3 min at 3000 rpm) until dispersion was complete. The Tween 80 was added as an emulsifying agent.

The resulting slurry was spray dried in a Eyela spray dryer equipped with a 0.5 mm diameter nozzle. The atomizer pressure and blower controls were adjusted to 50 kPa and 0.70m^3/min, respectively. The inlet and outlet temperatures were maintained at 150 ± 5 ^oC and 90 ± 5 ^oC, respectively, and the feed rate was set at 300 mL/h. The powder obtained was collected, and stored in airtight, self-sealable polyethylene pouches, in a dessicator.

The physicochemical properties of pandan powder including SEM micrograph, particle size distribution, bulk density, total chlorophyll content, color of the powder, antioxidant activity, water activity, and % yield were examined.

The decay of chlorophyll powder in different types of wall material were explored in 0-120 days after storage in clear glass bottle at room temperature. Pigment retention (%) was calculated by the formula:

$$Pigment\ retention(\%) = \frac{Chlorophyll\ content\ after\ storage\ time \times 100}{Chlorophyll\ content\ at\ initial\ content}$$

Rate constant (k) and half-life time ($t_{½}$) were calculated by the method of Cai *et al.* (1998) using the regression analysis of *ln* (pigment retention) against storage time when plotted on a natural logarithmic scale. Half life ($t_{1/2}$) for the retention of Zn-chlorophyll derivatives was calculated from the rate constant as 0.693/k.

6) Characteristic of Zn-chlorophyll derivatives powder

The physical and chemical properties of the Zn-chlorophyll derivatives powders were evaluated as for the pandan powder described above.

The color stability of obtained Zn-chlorophyll derivatives powder was studied as a function of pH and temperature. Preparation of Zn-chlorophyll solution by Zn-chlorophyll powder (10g) were dissolved in water (90 mL) and pH were adjusted in range of 3-8 by adding citric acid or NaOH and the temperature at 70-100 ^{o}C for 0-30 min. The color change of pandan purees were determined in color values (L^*, a^*, b^*, C, and h^o)

Volatile compound in the powder were analyzed by using method of solid phase microextraction (SPME) fiber assembly (Supelco, Bellefonte, PA). SPME was performed with fibers of Divinyl Benzene-Caroson-Polydimethyl Silosane. The fiber was exposed the headspace of flavors for 20 min at ambient temperature. Subsequently, the fiber was withdrawn into the housing, the SPME device was removed from the sample vial, and the fiber was desorbed into the GC-MS injector.

CHAPTER IV

RESULTS AND DISCUSSION

PART 1: Pandan leaves compositions

The composition analysis of fresh pandan leaves showed chlorophyll *a, b* and total chlorophyll contents of 66.15, 42.01 and 142.22 mg/100g fw, respectively. The antioxidant activity was 319.87 ± 4.23 μM TE/g fresh mass, and *L*, a*, b*, C* and h^0 values were 36.11, -11.65, 15.14, 22.80 and 127.26, respectively. The pH value was 5.4, total dietary fiber content was 8.09 g/100 g fw, and zinc content was 2.70 mg/kg.

The result of the volatile compounds by using SPME-GC-MS (Fig 4.1 and Table 4.1) showed that 2-AP was the characteristic flavor of pandan leaf. This finding is similar to results from previous investigations (Buttery *et al*., 1983; Paule and Power, 1989; Laksanalamai and Ilangantileke, 1993). Furthermore, pandan leave yielded other volatile compounds including three alcohols (2-penten-1-ol, 3-hexen-1-ol and 1-hexanol), seven types aldehyde (hexanal, (*E*)-2-pentenal, heptanal, 2-hexenal, 4-heptenal, (*E,Z*) 2,6-nonadienal, 2-nonenal and 2-octenal), a ketone (1-penten-3-one), and a furanone (2-pentyl-furan). The main flavor that was more than 40% in pandan scent was hexanal displayed an aroma of freshly cut grass. It was found that many of these volatile compounds for example, 2-acetyl-1-pyrroline, hexanal, and 2-nonenal are similar to those found in Khao Dawk Mali 105 rice (Mahatheeranont, Promdang and Chiampiriyakul, 1995; Grimm *et al*., 2001).

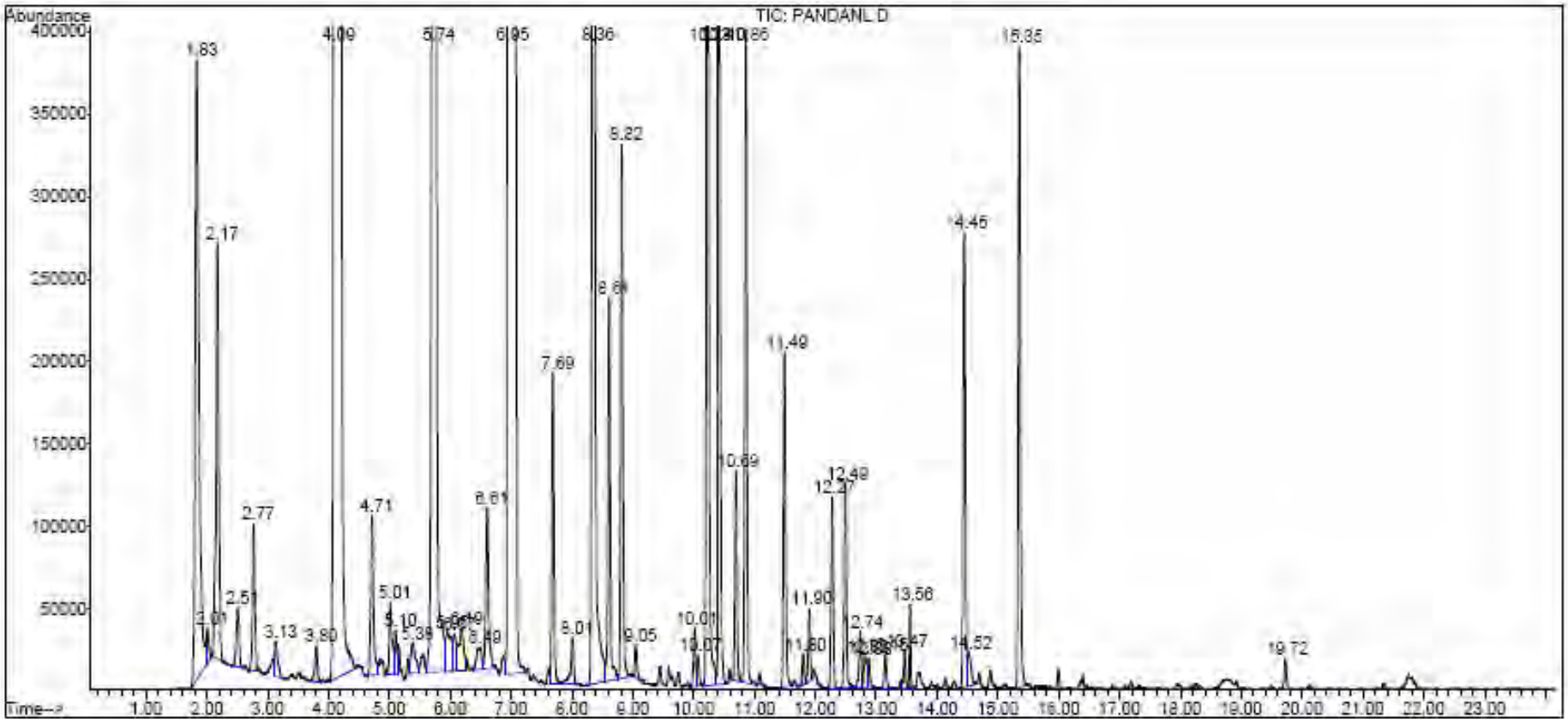
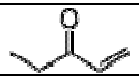
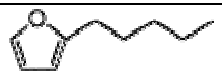
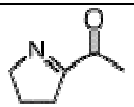


Fig. 4.1 The SPME-GC-MS chromatogram of pandan leaf compounds.

Table 4.1 The flavor compounds found in the pandan leaf by using SPME-GC-MS.

no	name	class	RI	CAS NO.	structure	aroma description	relative peak area (%)	retention time(min)
1	1-Penten-3-one	ketone	245.20	1629-58-9		fish, pungent	1.26	4.71
2	hexanal	aldehyde	1083.65	66-25-1		grass, green, woody, green	41.39	5.74
3	(*E*)-2-pentenal	aldehyde	1131.82	1576-87-0		strawberry, fruit, tomato	1.39	6.61
4	heptanal	aldehyde	1187.98	111-71-7		green, fresh green, sweet, herbal, rancid	2.37	7.69
5	2-hexenal	aldehyde	1221.54	6728-26-3		fresh green, fruity , apple	19.10	8.35
6	2-penty-furan	furanone	1234.60	3777-69-3		green bean, butter	2.78	8.61
7	4-heptenal	aldehyde	1245.15	6728-31-0		biscuit, cream	3.71	8.82
8	2-penten-1-ol	alcohols	131.32	1576-95-0		green, plastic, rubber	10.15	10.23
9	2-acetyl-1-pyrrolline	nitrogeneous compounds	1339.93	85213-22-5		nut, roast, pandan	1.64	10.69
10	1-hexanol	alcohols	1348.66	111-27-3		resin,flower,green	4.32	10.86
11	3-hexen-1-ol	alcohols	1381.51	928-96-1		fresh green, cut grass	2.38	11.49
12	2-octenal	aldehyde	1433.69	2363-89-5		green	1.49	12.49
13	2-nonenal	aldehyde	1540.38	18829-56-6		cucumber, fat, green	3.49	14.44
14	(*E,Z*) 2,6-nonadienal	aldehyde	1590.85	557-48-2		cucumber, wax, green	4.47	15.35

PART 2: Factors effecting on color change of pandan leaf extracts

Measurement of color values in the CIE system is a simple and rapid method which also relate with chlorophyll content and consumer acceptance (Ryan-Stoneham and Tong, 2000; Guzman *et al*., 2002; Ngo and Zhao, 2005; Turkmen *et al*., 2006; Koca *et al*., 2007). Determination of the *-a** values which indicate the greenness can be done to assess the chlorophyll degradation and indicate the kinetic parameters (Steet and Tong, 1996; Weemaes *et al*., 1999). Hue angle has been shown to be a reasonable predictor of sensory perception of green color (Gnanasekharan, Shewfelt and Chinnan, 1992; Lau, Tang and Swanson, 2000). An angle of 90^{o} represents a yellow hue. The higher the hue angle, the greener the sample the less yellow it appears (Little, 1975; Maharaj and Sankat, 1996; Lau *et al*., 2000; Turkmen, 2006). Several studies have reported that increase or decrease of green color and h° value can be related with the chlorophyll content (Little, 1975; Albanese *et al*., 2007). As Koca *et al*. (2007) have found, visual color parameters (*-a*, -a*/b** and h°) and chlorophyll contents have the correlation coefficients varying from 0.91– 0.99.

Therefore, this experiment used *–a** and h° values to monitor the color change of pandan puree whilst considering the effect of pH, heat and pH-heat interaction as shown in results that follow.

1) Effect of pH

Acidic conditions (pH 3–6) can affect the color change of pandan leaf puree significantly ($p \leq 0.05$). Both *–a** and h^{0} values tending to decrease (Fig. 4.2 A, B) which means the green color tends to lessen resulting in browning. Storage at 4°C for 0–21 days showed that brown color increased over time. Moreover, fresh pandan leaf puree at pH 5.4 showed a marked color change from green to olive green, but in neutral to alkali conditions (pH 7–8) did not noticeable lose its original green color.

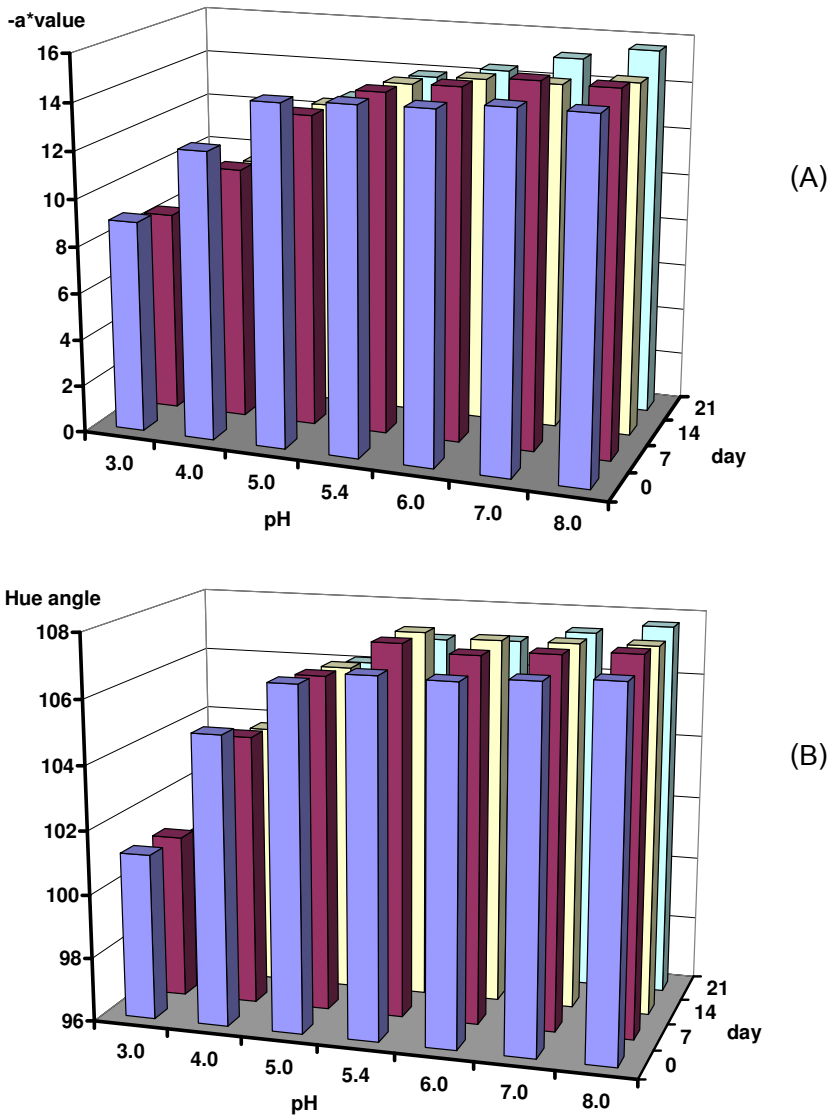


Fig. 4.2 The color values of pandan puree at pH 3-8 when A) $-a^*$ value (B) h^o value at the storage periods ■ 0 day, ■ 7 days, ■ 14 days, and ■ 21 days.

Changing the pH can alter the degradation reaction, as shown in Fig. 4.3 Magnesium in chlorophyll compounds can be replaced by two hydrogen atoms in acidic condition and hence the absorption spectra shifted in the different wavelength. This caused changing from bluish-green to grey color of chlorophyll *a* and from yellow-green to brown color of chlorophyll *b*, and this derivative was called as pheophytin (Mahanta and Hazarika, 1985; Koca *et al*., 2007). Minguez-Mosquera, Garrido-Fernándaz and Gandul-Rojas (1989) also observed a similar increase in the

concentration of pheophytin at pH < 5. In strongly acidic conditions (pH < 3), the pandan leaf puree in lowest green and highest brown color because hydrogen ions can replace with both magnesium atom and phytol group, subsequently became a derivative called pheophorbide with dark brown color.

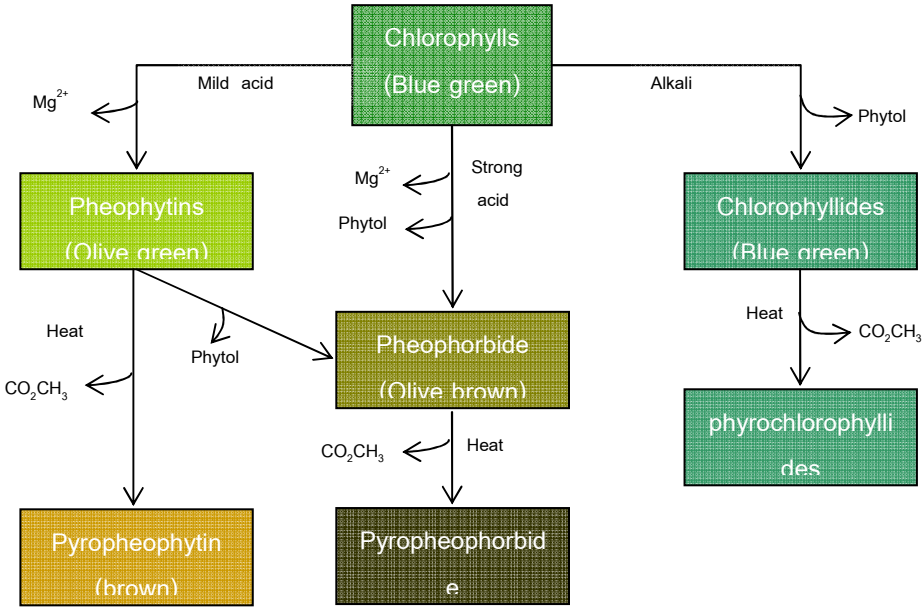


Fig. 4.3 Schematic of chlorophyll derivative in acidic and alkali conditions

Chlorophyll is more stable in alkali conditions due to formation of stable porphyrin structure (Gold and Weckel, 1959; Sweeney and Martin, 1961; Schwartz and Lorenzo, 1991). Thus, the reactions at pH 7 and 8 resulted in a green color. At pH 8 can brought the most intense greenness because the hydrophobic molecules in phytol chains were removed. The chlorophyllin derivatives which had bright green color and more water soluble characteristic were formed. However, it is not desirable to retain the green color by using alkaline condition because the texture of vegetables often becomes very soft at high pH and vitamin B can is unstable.

3) Effect of heating temperature

Heating the pandan leaf puree at 70 – 100 ^{o}C for 0 – 30 min brought about the reduction of greenness and hue values differences significantly (Fig. 4.4). The reduction rate of greenness was most rapid at high temperature. Heating for 5 min at 70, 80, 90 and 100 ^{o}C were saw reductions of greenness of 53.3%, 62.5%, 71.25%, and 75.73%, respectively. When heating for 30 min, the reductions were 71.54 %, 72.72%, 77.86%, and 79.55%, respectively.

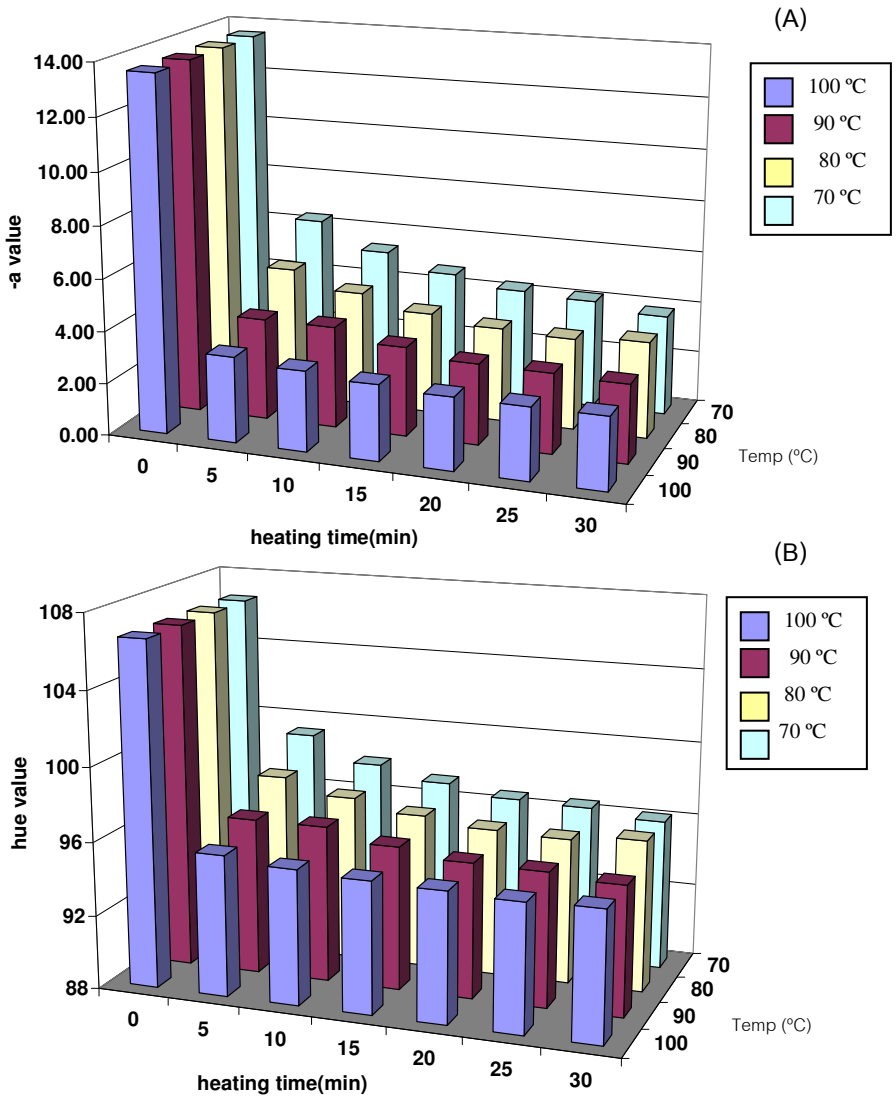


Fig. 4.4 The color values of pandan puree at heating temperature 70 –100 ^{o}C for 0 – 30 min A) The greenness value (B) The h^{o} value at the storage periods of 0 day, 7 days, 14 days and 21 days.

Irreversible degradation of chlorophyll can occur during heating process. The reduction of greenness correlated with that of chlorophyll content. Prolong heating of the puree can degrade chlorophyll compound. Heat caused some changes in cell wall structure in plant tissue, which was the organic acid in cell sap can contact to chloroplast and react with chlorophyll to form pheophytin. Prolonged heating brought about decarbo-methoxylation of pheophytin, converting it to pyropheophytin. Schwartz and von Elbe (1983) found that pheophytin *a* and pheophytin *b* were in turn to pyropheophytin *a* and pyropheophytin *b*, major thermal chlorophyll degradation products found in commercially canned spinach puree heated (116 to 126 °C). Consequently, the major pigments in green plant after heating were pheophytin and pyropheophytin (Ngo and Zhao, 2005).

4) Combined effect of heating and pH

Heating the pandan leaf extract in different acid or alkali conditions causes some changes as shown in Fig. 4.5. In strong acid (pH 3), the extract had olive green color prior to getting heat, while heating at 70–90 ^{o}C for 1 min caused brown color rapidly occurred. For neutral and alkali conditions (pH 7 and 9), the extract had green color, and obvious precipitation of chlorophyll was observed. This occurred because of chlorophyll-protein complex (Smith and Pickel, 1941). Therefore, increasing temperature until the protein was denatured brought about the sedimentation (Fig. 4.5).

In the commercial sterilization process (121 ^{o}C, 15 min) greenness and hue values decrease significantly ($p \leq 0.05$) and the pandan leaf extract have changed from green to brown totally (Fig 4.6). It can be concluded that this temperature let to complete chlorophyll degradation.

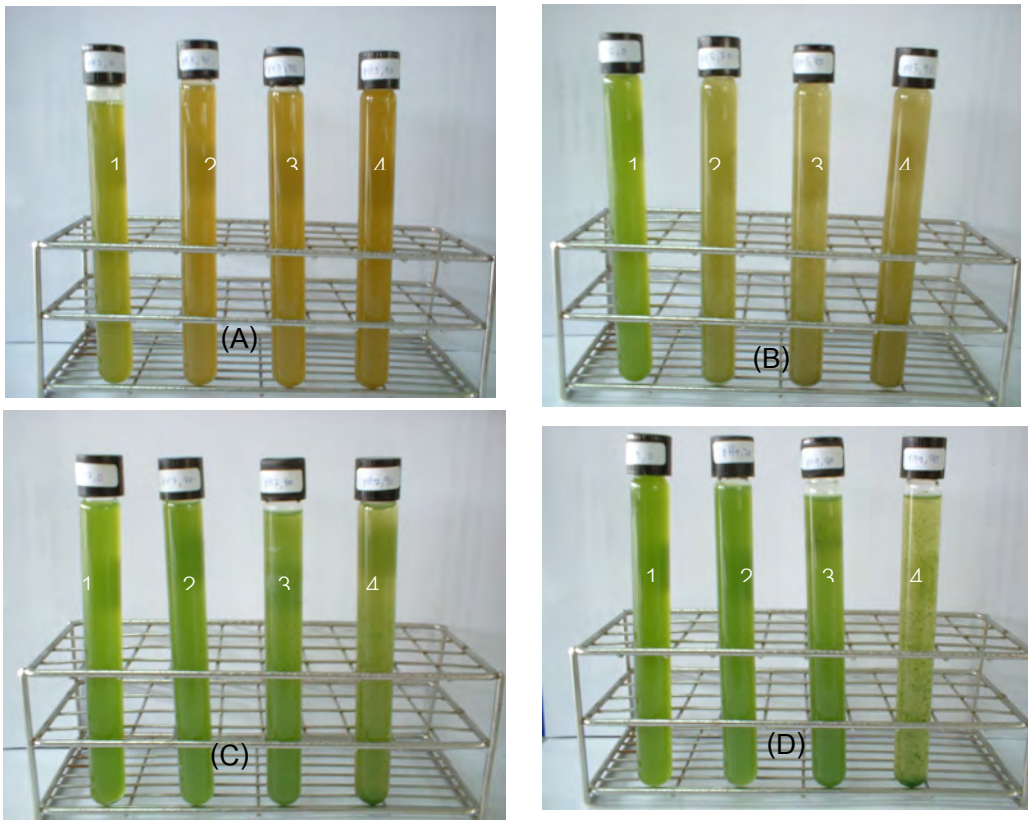


Fig. 4.5 The effect of pH and temperature on change of color of pandan puree (A) pH 3 (B) pH 5 (C) pH 7 and (D) pH 9 when (1) control, (2) heating 70^{o}C for 1 min, (3) heating 80 ^{o}C for 1 min, and (4) heating 90 ^{o}C for 1 min.

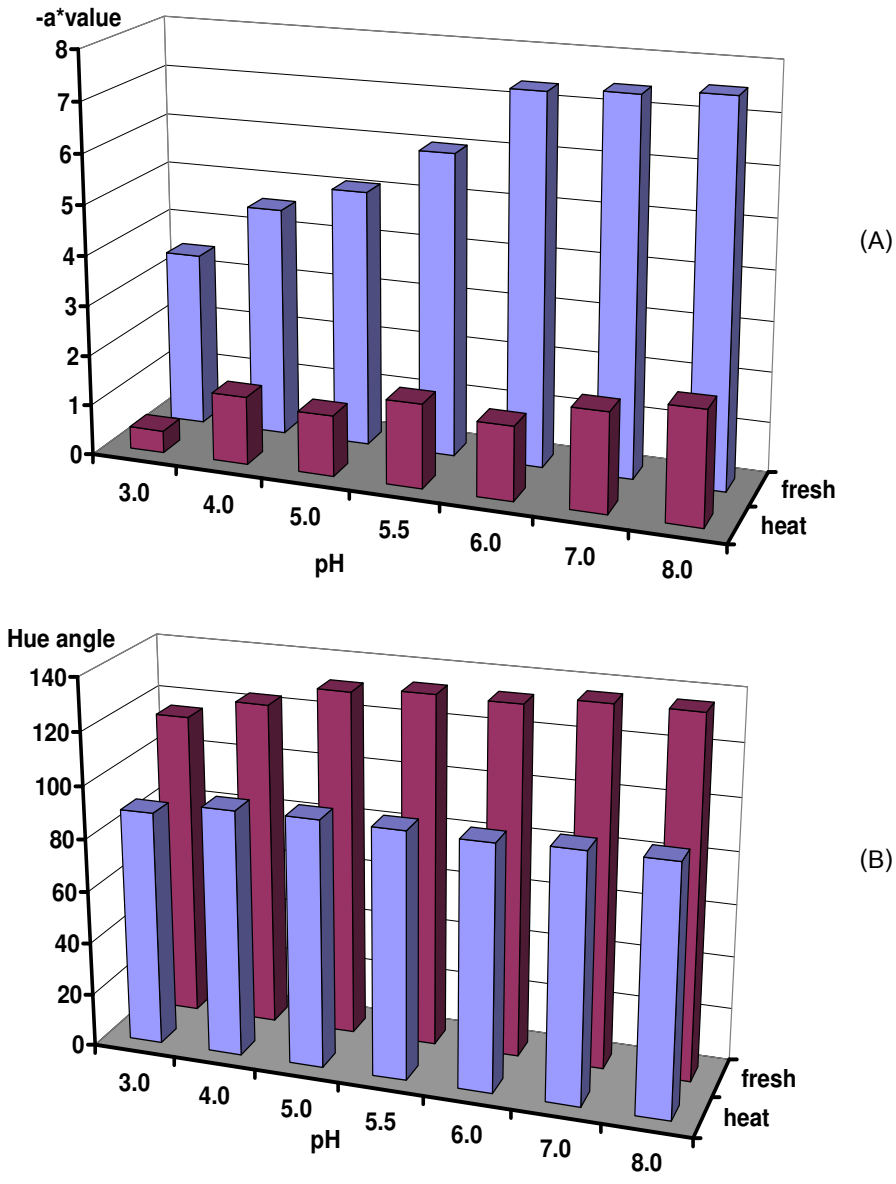


Fig. 4.6 The color change of pandan puree at pH 3.0 –8.0 when ■ fresh pandan puree and ■ heating at 121^{o}C for 15 min.

This experiment showed the instability of pandan leaf extract at high temperature which caused alteration of color from green to olives green to brown color. From FDA limitation, it was necessary to form the stable derivative before the application in food processed.

PART 3: Formation of metallochlorophyll complexes

The formation of a stable chlorophyll molecule can be managed by replacing the magnesium ion in the porphyrin ring with other divalent cations such as Zn^{2+} or Cu^{2+} to change the native form to a more stable porphyrin structure (Humphrey, 1980). These derivatives are green in color like native chlorophyll but are more stable in acid and heated conditions. The factors effecting on green color formation of Zn-chlorophyll derivatives in the pandan leaf were pH, concentration of zinc chloride, and heat treatment as described below.

1) Effects of pH

The pH had a significant ($p \leq 0.05$) effect on the formation of Zn-chlorophyll complex. The results showed that formation of Zn-chlorophyll complex was slow at pH 3.0 and pH 7.0–8.0. However, green color was detected at pH 4.0–6.0 and peaked in intensity at pH 5.0 (Fig. 4.7). In weakly acidic conditions, the Mg^{2+} molecule in the porphyrin ring may be removed and replaced by the two hydrogen ions and pheophytin were formed. With Zn^{2+} ion at a suitable concentration and temperature, it can in turn replace hydrogen resulting in a Zn-chlorophyll complex. Procedures for Zn-chlorophyll complexes formation are outlined in Fig.4.8. In alkaline conditions, the solubility of metallic salt may decrease and cause formation of insoluble $Zn(OH)_2$ (LaBorde and von Elbe, 1990). Moreover, alkaline conditions resulted in high retention of chlorophyll after heating and a reduction in the amount of chlorophyll derivatives available for the formation of zinc complexes (LaBorde and von Elbe, 1994a). LaBorde and von Elbe (1994 b) reported that zinc chlorophyll complexes can be formed at pH 4.0–6.0 and less formation occurs at pH > 8.0. This research found that pandan leaf extract had the most greenness when the reaction occurred at pH 5.0.

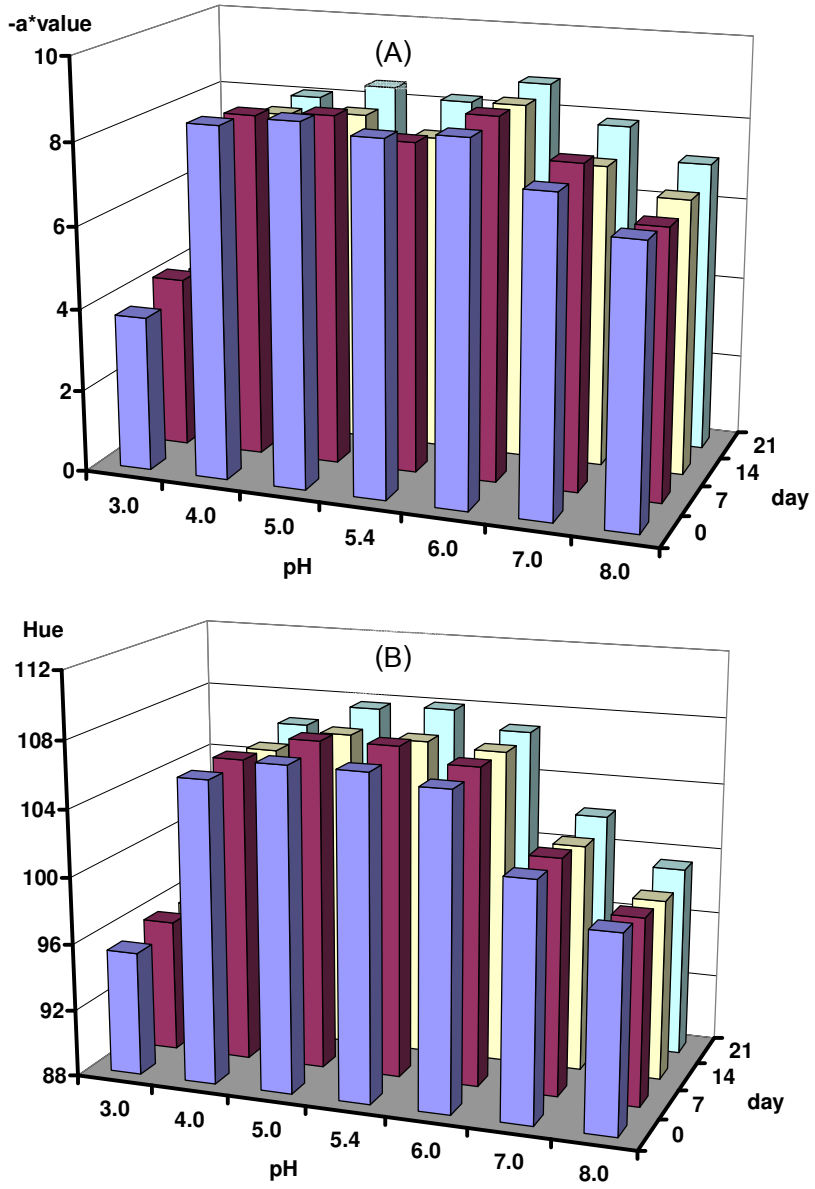


Fig. 4.7 Change in the (A) -*a** value and (B) hue value of pandan leaf extract containing of 300 ppm of Zn^{2+} ion at pH 3-8 and heating at 121°C for 15 min when storage at ■ 0 day, ■ 7 days, ■ 14 days and ■ 21 days

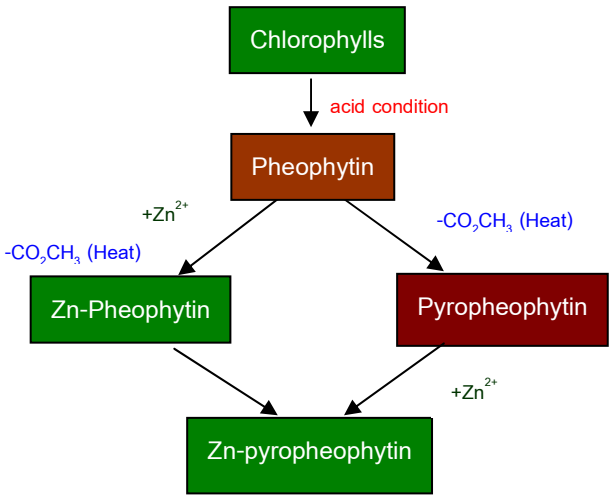


Fig. 4.8 Zn-chlorophyll complexes formation

(Source: Adapted from LaBorde and von Elbe, 1994)

2) Effect of zinc chloride concentration

The results show that higher $ZnCl_2$ concentration causes increased greenness and hue value. As seen in Fig. 4.9, 100 ppm $ZnCl_2$ increased the green color of the pandan leaf extract significantly when compared the control (0 ppm $ZnCl_2$). At 300 ppm $ZnCl_2$, the extract had green color, and at a concentrations above 300 ppm green color values were not significantly different ($p>0.05$). These finding imply that 300 ppm $ZnCl_2$ allows in complete formation of Zn-chlorophyll complexes in the pandan leaf extract. Zinc ions can replace two hydrogen ion in chlorophyll structure and form metallo-chlorophyll complexes which are a new green color form of chlorophyll derivatives (LaBorde and von Elbe, 1994 a,b ; Canjura *et al*., 1999; Ngo and Zhao, 2007).

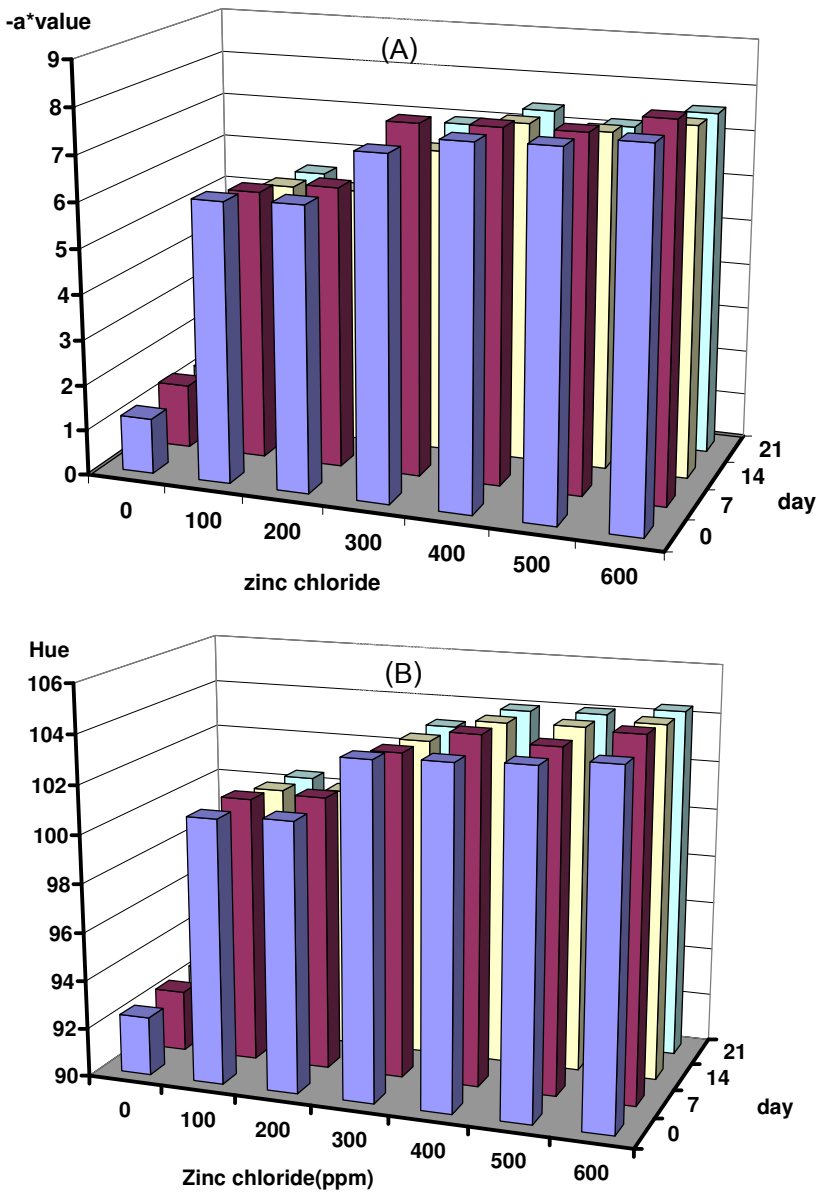


Fig. 4.9 The concentration of $ZnCl_2$ on the formation of green color at pH 5.0 and heating at 121°C for 15 min, when the storage time 0 day, 7 days, 14 days, and 21 days

3. Effect of heating The heating temperature and time had significant ($p \leq 0.05$) effect on the greenness and hue value (Fig. 4.10). Higher temperatures can decrease the formation period of Zn-chlorophyll complexes. As illustrated in Fig. 4.11, at temperatures of 80, 90, 100, 110 and 120 ^{o}C, pandan leaf extracts turned green in color at 90, 50, 20, 15 and 10 min, respectively. The most intense of green color occurred after at 110 ^{o}C for 15 min (Fig 4.12). Formation of metallo-chlorophyll complexes cannot occur with native chlorophyll, but does so with certain chlorophyll derivatives (LaBorde and von Elbe, 1994b). Heat was attributed to the removal of carbomethoxyl at the C10 carbon position on the isocyclic ring which acted like a steric hindrance. After the pyrrole nucleus became a cation and was suitable for Zn^{2+} or other cations to react with chlorophyll derivative (Tonucci and von Elbe, 1992). In addition, treatment by heating in acidic conditions resulted in a modification of the chlorophyll to pheophytin, pyropheophytin and pheophorbide. These derivatives can react with Zn^{2+} and resulted in Zn-pheophytin complexes (Cajura *et al*., 1999). The optimal formation of Zn-chlorophyll complexes occurred when the pandan leaf extract was added with 300 ppm $ZnCl_2$ at a pH of 5.0 and heating was applied at 110 ^{o}C for 15 min.

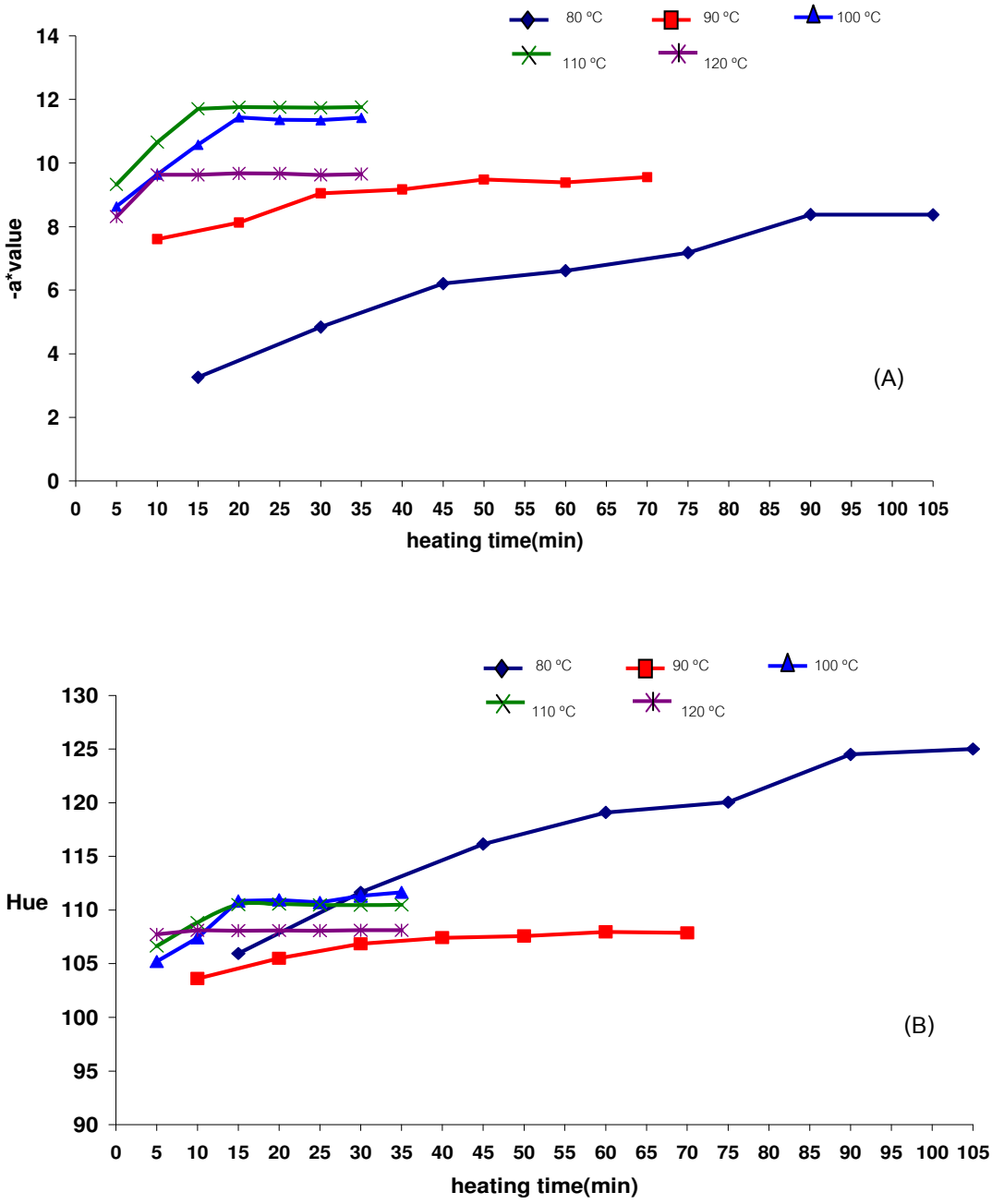


Fig. 4.10 The effect of heating temperature (80 −120 °C) and time (0 −105 min) on the (A) greenness, and (B) hue value when added 300 ppm $ZnCl_2$ at pH 5.0.

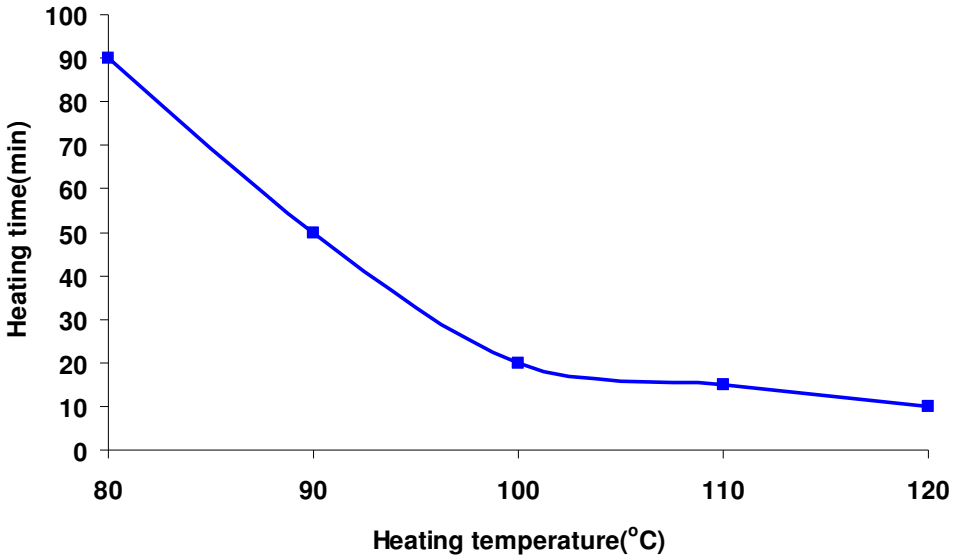


Fig. 4.11 The heating temperature and heating time required for greenness color when pandan leaf extract at pH 5.0 and 300 ppm $ZnCl_2$

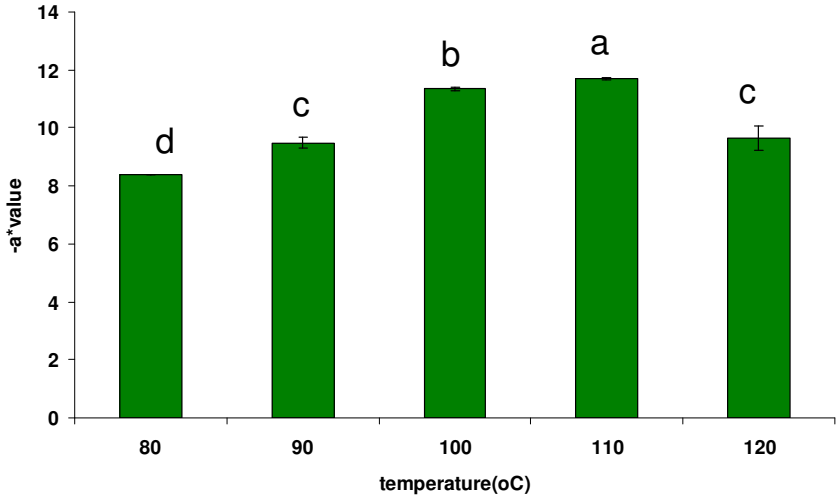


Fig. 4.12 The greenness value of pandan leaf extract at 300 ppm $ZnCl_2$, pH 5.0 when heated at 80 °C, 90 °C, 100 °C, 110 °C and 120 °C for 90, 50, 20, 15 and 10 min, respectively.

5) Evidence of Zinc-chlorophyll complex formation

4.1 Thin layer chromatography (TLC)

The present investigation detected the presence of complex Zn-chlorophyll derivatives by thin layer chromatography (Fig 4.13). The pigments of samples are shown in Table 4.2; RF values and spectra detect them.

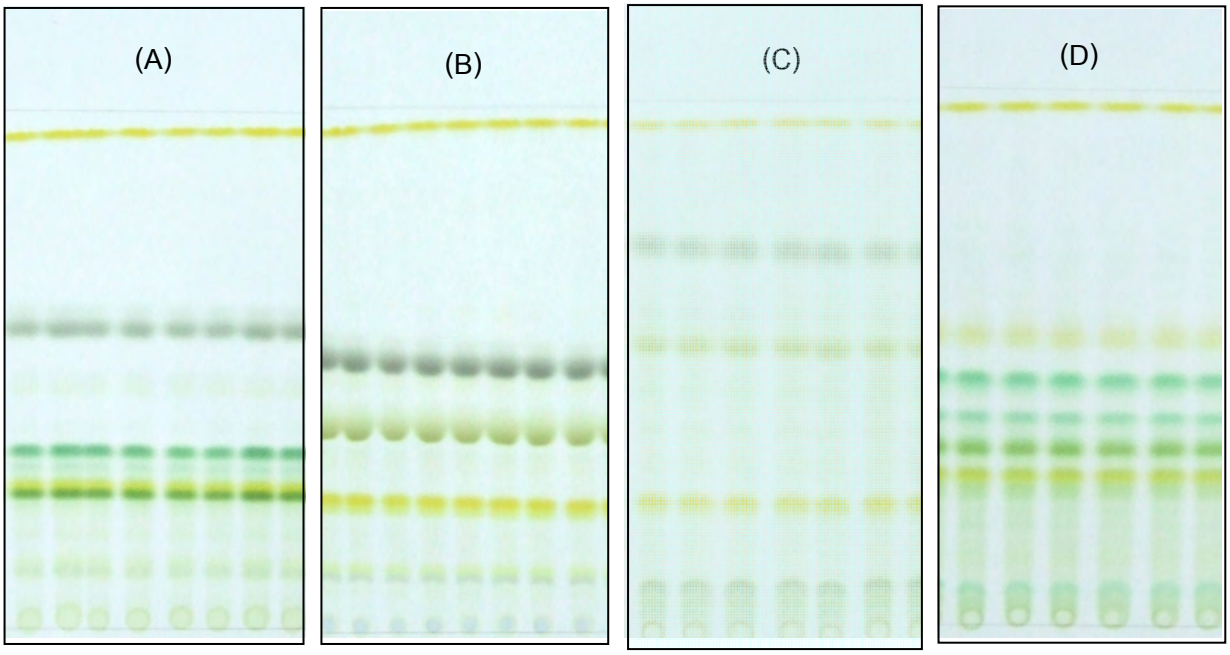


Fig. 4.13 TLC of fresh pandan leaf extract (A), acid pandan leaf extract (B), heated pandan leaf extract (C), and Zn-chlorophyll pandan leaf extract (D).

Table 4.2 Pigments detected from pandan leaf extract of fresh, acid, heated, and zinc-chlorophyll complex (diethyl ether)

no	Spectral absorption max (nm) (diethyl ether)	RF value	Pigment	Color	Fresh Pandan extract	Acid Pandan extract	Heated pandan extract	Zn-chlorophyll pandan extract
1	430, 450	0.97	β- carotene	yellow	√	√	√	√
2	408, 471, 534, 609, 668	0.72	Pyropheophytin a	Black			√	
3	434, 525, 555, 600, 655	0.54	Pyropheophytin b	brown			√	
4	663, 471, 413	0.77	Pheophytin a	grey	√	√		
5	435.655	0.37	Pheophytin b	brown		√		
6	425, 522, 564, 606, 655, 730	0.54	Zn-pheophytin a	green				√
7	445, 637, 730	0.45	Zn-pheophytin b	green				√
8	425, 522, 564, 606, 656, 684, 730	0.37	Zn-pyropheophytin a	green				√
9	445, 637, 730	0.29	Zn-pyropheophytin b	green				√
10	410,503, 533, 615, 662, 730	0.44	Chlorophyll a	Blue green	√			
11	430, 456, 644, 730	0.35	Chlorophyll b	Yellow green	√			

4.2 Absorption spectra of chlorophyll derivatives

Distinct visible absorption spectra were observed for each chlorophyll derivative. These spectra were used subsequently to identify the different derivatives (Jones *et al*., 1968; Guzman *et al*., 2002) (Table 4.2, Fig 4.14) different derivatives of chlorophyll formed under different conditions as follows:

1) ***Fresh pandan leaf extracts*** (A), yielded chlorophyll *a* and *b* which had bluish-green and yellowish-green colors, respectively. Furthermore, they were found beta carotene, pheophytin *a* and xanthophylls which caused yellowish-green color.

2) ***Pandan leaf extract in acidic condition*** (B), yielded pheophytin *a* and *b* which had grey-black and olive-green colors, respectively. Other pigments were found beta-carotene and xanthophylls which caused olive-drab green to brown color of this extract.

3) ***Pandan leaf extract in commercial sterilize heating condition*** (C), yield pigments including pyropheophytin *a* and *b* which had black and dark brown colors, respectively. This level of heating temperature can convert all chlorophyll molecules to their derivatives, so the band of chlorophyll *a* and *b* can not be determined like that found in fresh pandan leaf, this brought about dark brown color of pandan leaf extract.

4) ***Pandan leaf extract derived after zinc-chlorophyll complex formation*** (D), afforded pigments the Zn-pheophytin *a*, Zn-pheophytin *b*, Zn-pyropheophytin *a* and Zn- pyropheophytin *b* which had green color. This brought about new green color of chlorophyll extract (Guzman *et al*., 2002; Minguez-Mosquera *et al*., 2002; Tonucci and von Elbe, 1992). This occurred green color was stable in heat and acidic conditions more than the natural form.

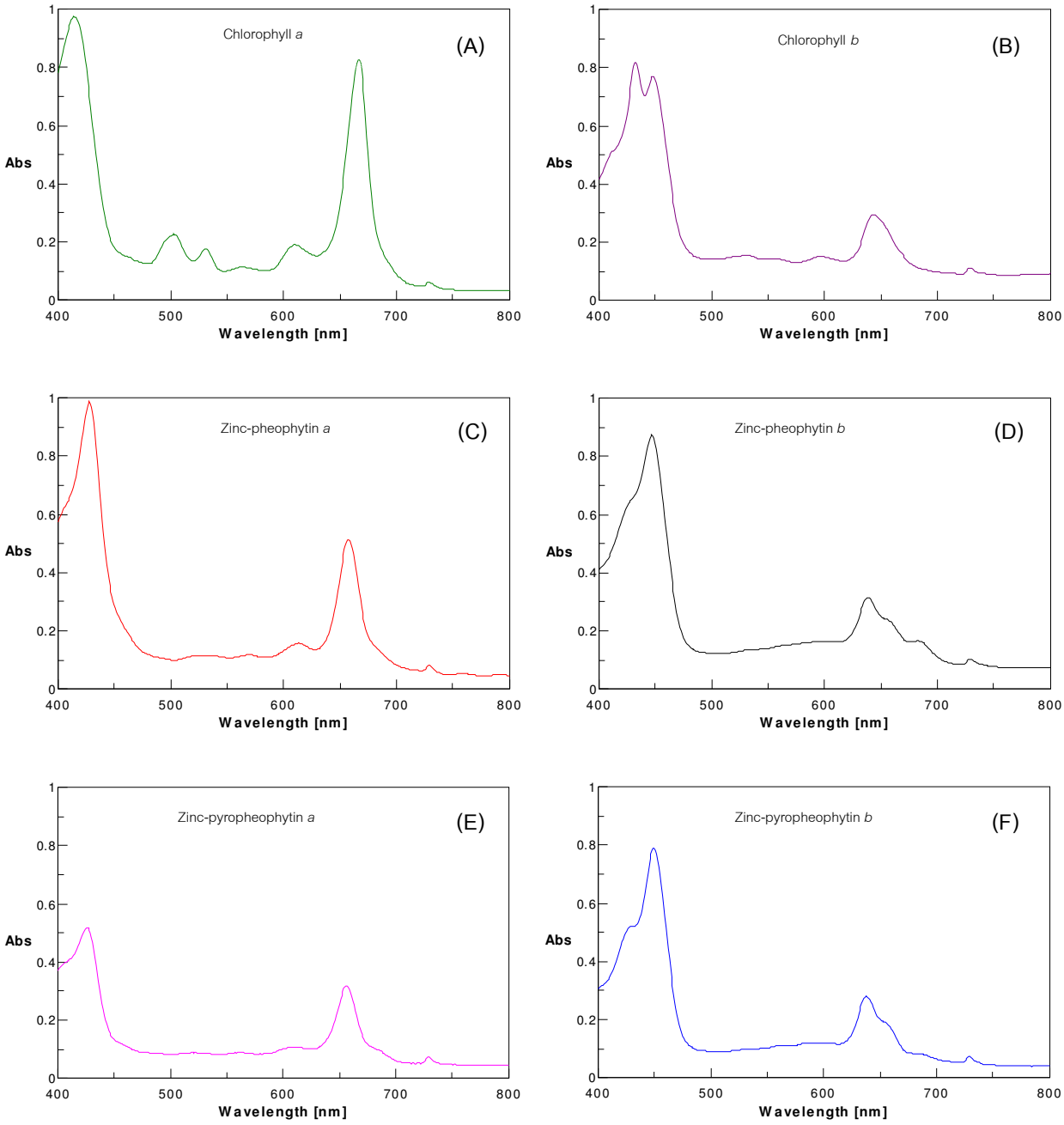


Fig. 4.14 Absorption spectra of chlorophyll derivatives (A) chlorophyll *a* (B) chlorophyll *b* (C) Zn-pheophytin *a* (D) Zn-pheophytin *b* (E) Zn-pyopheophytin *a*, and (F) Zn-pyopheophytin *b*.

4.3 High Performance Liquid Chromatography (HPLC)

Separation the chlorophyll derivative by prepararive HPLC, using reverse phase C18 column, the running conditions as in method of Canjura *et al*. (1991) which there were methanol, ethyl acetate and water as solvents, the chromatogram was shown in Fig 4.15.

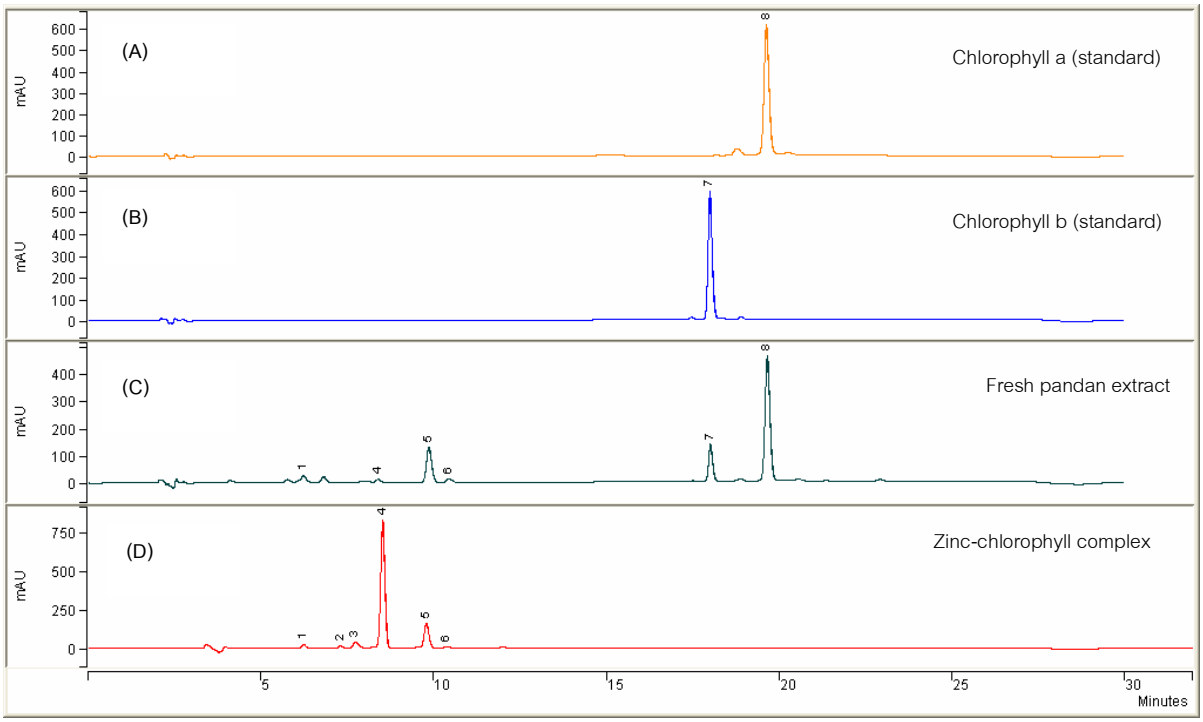


Fig. 4.15 High Performance Liquid Chromatography of chlorophyll in pandan leaf extract (A), standard chlorophyll *a* (B) , standard chlorophyll *b* (C) , fresh pandan leaf extract, and (D) zinc-chlorophyll complexes in pandan leaf extract.

It was found that fresh pandan leaf extract had derivatives of chlorophyll *a* and *b*, while pandan leaf extract derived after zinc treatment of Zn-chlorophyll which also showed new green color as presented in band 4.

The HPLC traces suggest that the main derivatives from the zinc treatment reaction were Zn-pheophytin *a*, Zn-pheophytin *b*, Zn-pyropheophytin *a*, and Zn-

pyropheophytin *b* which brought about new green color of chlorophyll extract, found by other researchers (Guzmán *et al*., 2002; Minguez-Mosquera *et al*., 2002; and Tonucci and von Elbe, 1992). However, these derivatives were attached to pandan leaf tissue. Therefore it was decided to investigation an extraction method for these derivatives, as detailed below.

PART 4: Optimum conditions in enzymatic extraction process

Enzyme extraction of pigments allows the pigments to remain in their natural form, linked with protein, and so the color remains unaffected (Çinar, 2005 *a*; Bassi *et al*., 1993). Enzyme treatment of plants is an alternative method developed to extract pigments such as anthocyanin, lycopene, beta-carotene, and carotenoid (Stoll *et al*., 2003; Muñoz *et al*., 2004; Çinar, 2005 *a,b* ; Choudhari and Ananthanarayan, 2007).

1) Seeking suitable conditions for an enzymatic extraction

A preliminary study was conducted in which independent variables and their levels were determined. In extraction of Zn-chlorophyll derivatives by type of enzyme, there should be screening of the enzyme type, pH range, heating temperature and enzyme concentration as follows:

1.1 Screening enzyme types

The comparison between the enzyme activities whilst employing pectinase (Pectinex® Ultra SP-L) and cellulase (Celluclast 1.5L) at concentrations of 0–3% (v/w) were determined. The results showed that both enzyme-treated samples tended to higher reducing sugar level than did the control ($p \leq 0.05$) (Fig 4.16). Furthermore, Pectinex® Ultra SP-L enzyme contributed to greater reducing sugar content than Celluclast 1.5 L enzyme at all concentrations evaluated. The activity in degradation of

plant cell wall by enzyme can be determined from the increase of reducing sugar content. As a result of disruption the glycosidic linkage, the monosaccharide resulted. The fast rates of hydrolysis bring about high levels of reducing sugar.

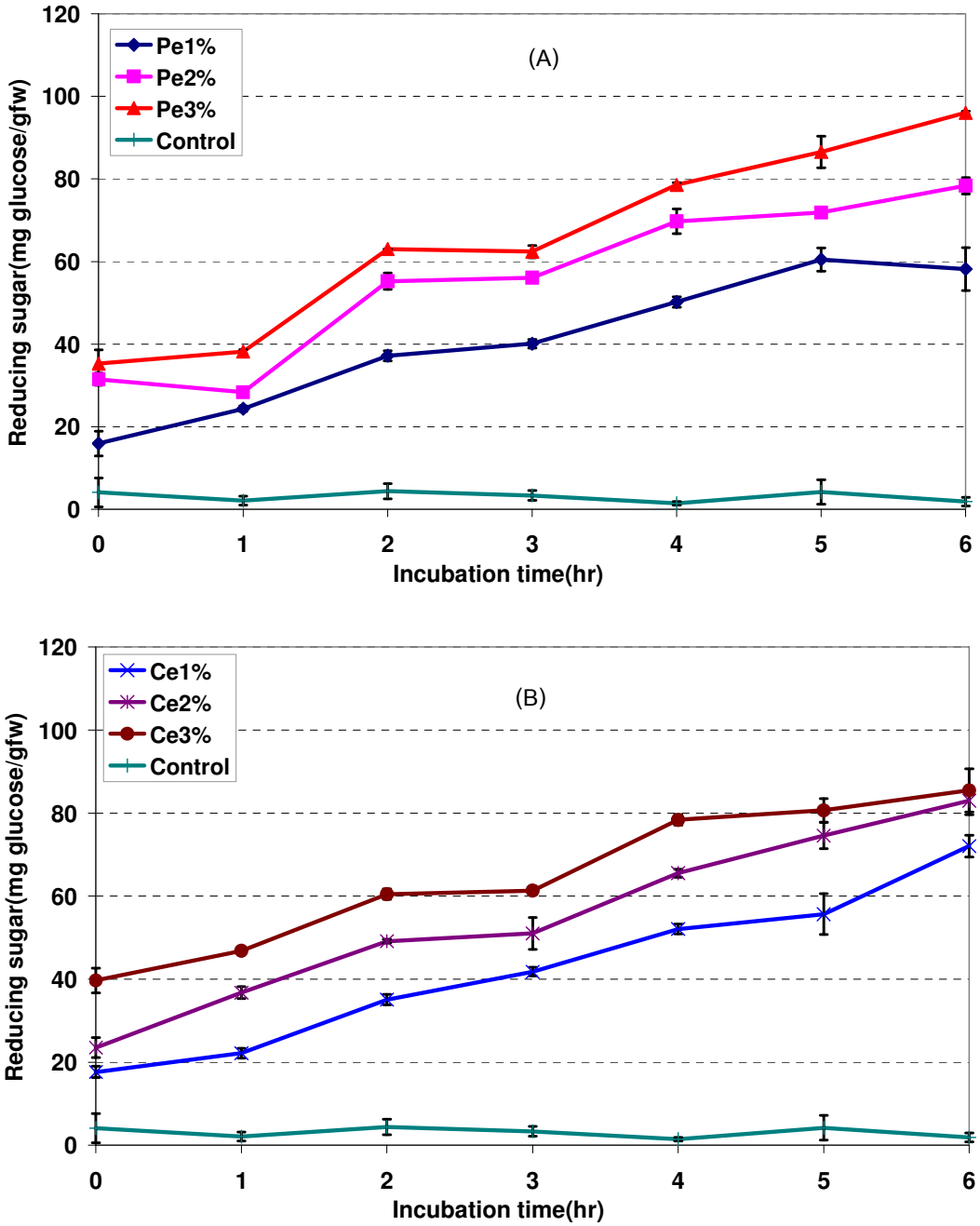


Fig. 4.16 The amount of reducing sugar when the enzyme applied at concentration of 1–3% (v/w) (A) Pectinex® Ultra SP-L, and (B) Celluclast® 1.5 L.

Thus, enzyme treatment conditions can yield significantly greater amounts of Zn-chlorophyll derivatives than control group ($p \leq 0.05$) (Fig 4.17). The Pectinex® Ultra SP-L enzyme could extract more green color and Zn-chlorophyll derivatives than Celluclast 1.5L enzyme at every concentration. In particular 2% Pectinex® Ultra SP-L can be used to extract more Zn-chlorophyll derivatives increase above control 30.61 to 47.31 mg/100g fw. In addition, the function of enzyme in disrupting at cell wall interaction releases pigments inside the cell (Choudhari and Ananthanarayan, 2007). Cellulase specifically hydrolyses cellulose, which is present in the primary wall beneath the first layer of middle lamella of plant cell walls. Primary wall consists of a rigid skeleton of cellulose embedded in a gel-like matrix composed of pectins, hemi-cellulose and glycoproteins. The cellulase enzyme catalyzes the breakdown of cellulose into glucose, cellobiose, and higher glucose polymers (Choudhari and Ananthanarayan, 2007). Pectinase, being pectolytic and hemicellulolytic, has the ability to disintegrate pectic compounds and pectin, the latter a polymer of 100–200 galacturonic acids, found in the middle lamella and primary wall. Pectinase and cellulase hydrolyses pectin and cellulose, liberate chlorophylls from cell walls and hence increase extraction efficiency.

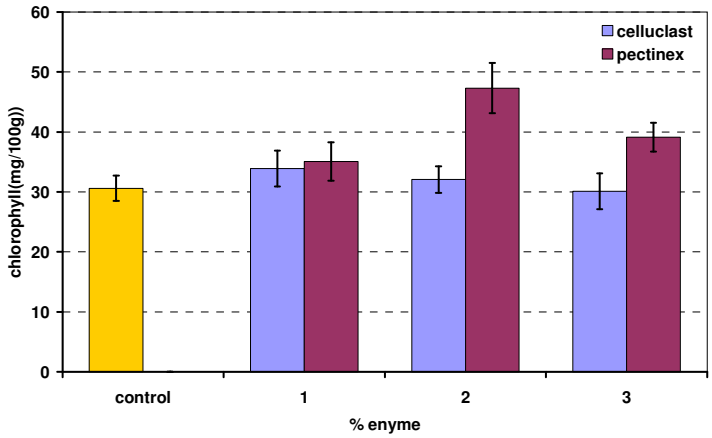


Fig. 4.17 Comparison of Pectinex® Ultra SP-L and Celluclast 1.5L at concentration of 1-3%(v/w) on the extracted chlorophyll.

The efficiency in chlorophylls derivative extraction of pectinase was greater than that of cellulase across all concentration values (Fig 4.17). This finding agrees with a previous study by Wilkins *et al.* (2007) which performed a similar comparison study on polysaccharide extraction from grape skin. Moreover, Grohmann and Baldwin (1992) found that pectinase can degrade cell wall polysaccharides of citrus peel, while cellulase had lower efficiency. Choudhari and Ananthanarayan (2007) reported that pectinase proved to be more effective than cellulase for lycopene extraction from tomatoes.

Pectinex® Ultra SP-L infact comprises a mixture of enzymes including polygalacturonase, pectatelyase, pectinesterase, hemicellulase, cellulase, protease and amylase. These enzymes can disrupt the glycosidic bond of pectin, cellulose and hemicellulose of cell wall in plant tissue and induce the increase of reducing sugar content (Whitaker, 1994; Sreenath, Sudarshana and Santhanam, 1995; Anprung, 2004). From our study was found that the Pectinex® Ultra SP-L enzyme would be an alternative method for Zn-chlorophyll derivative extraction.

1.2 Screening for optimum pH

The effect of pH on the ionization of prototropic groups at active site of the enzyme has been shown to cause changes in the conformation of the active site (Anprung, 2004). The activities of Pectinex® Ultra SP-L enzyme at pH 3.0–6.0 brought about the significantly different amount of reducing sugar ($p \leq 0.05$) (Fig 4.18). pH 4.0 showed the highest amount of reducing sugar ($p \leq 0.05$). At pH 6.0, the reducing sugar content was lowest for every incubation period. Therefore, pH 4 was chosen as the optimum condition for activity of Pectinex® Ultra SP-L.

Furthermore, pH affects to enzyme stability because enzyme is protein which can have loss of its activity at inappropriate pH. From this experiment, it can be

concluded that Pectinex® Ultra SP-L enzyme had the optimum activity at pH 4. This result is consistent with a previous study by Landbo and Meyer (2004) which reported that an optimum pH of 3.5.

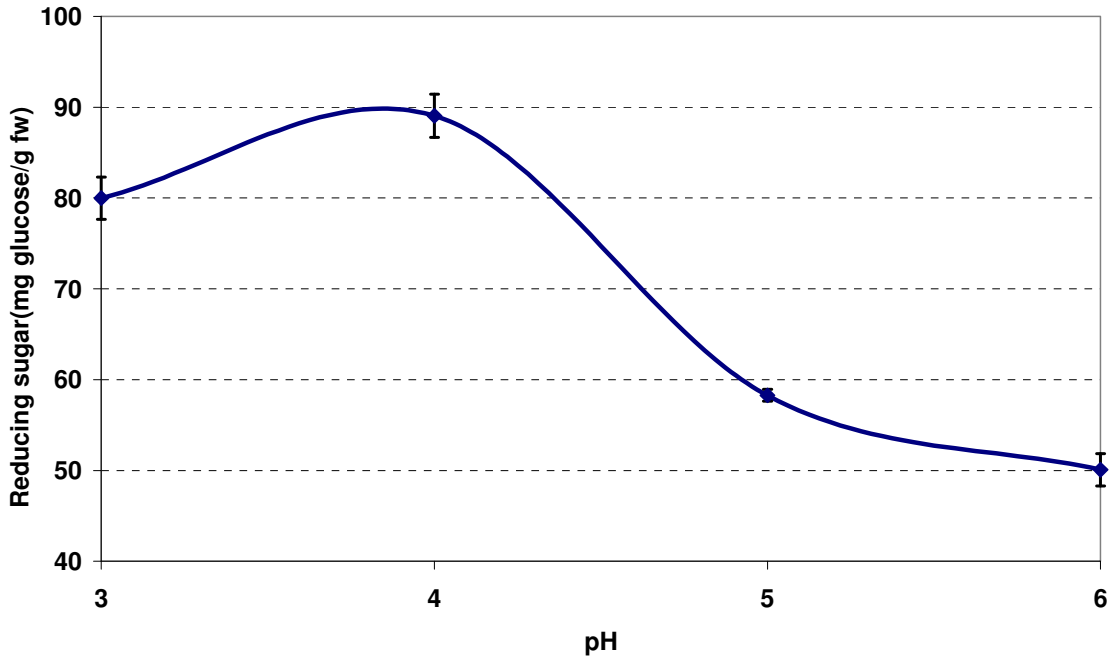


Fig. 4.18 The reducing sugar yield of pandan leaf extract after addition of Pectinex® Ultra SP-L at various pH 3.0–6.0 for 2 hours at 35 °C.

1.3 Screening for optimum temperature

The effect of changing incubation temperature from 25 to 55°C on reducing sugar content is presented in Fig 4.19. The reducing sugar content was lowest in at 25 °C highest at 35 °C.

Temperature can affect to enzyme activity because higher temperature caused higher kinetic energy of molecule which subsequently induced more frequency of the particles colliding with each other per time unit. Moreover, temperature also affects activation energy (E_A). The optimum temperature should show the highest activity of enzyme throughout the analysis period. From this experiment, the amount of reducing

sugar was lowest at 25 °C because it was low kinetic energy. At 55 °C, the amount of reducing sugar was lower than 35 and 45 °C. This implies that enzyme had reduced of its activity. Therefore, this study selected temperature at 35 °C to be used for enzyme activity. This finding that the optimum temperature of Pectinex® Ultra SP-L at 35 °C is similar to results reported by Landbo and Meyer (2004).

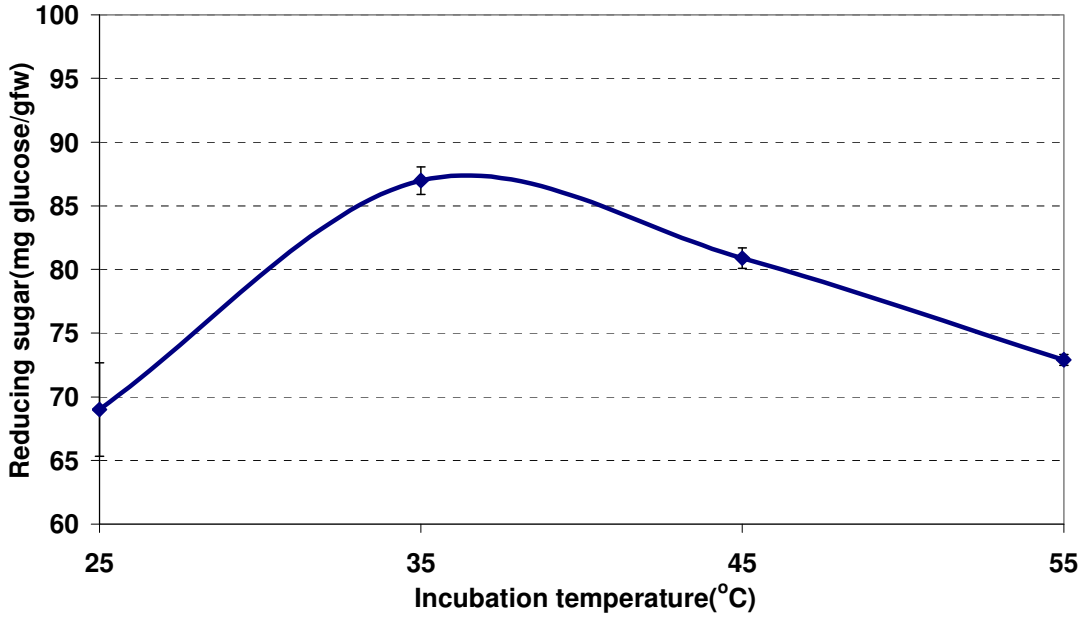


Fig. 4.19 The amount of reducing sugar of 2% (v/w) Pectinex® Ultra SP-L incubation at temperature 25–55 °C for 2 h.

1.4 Screening the optimum enzyme concentration

After varying the pectinase enzyme concentration from 0 to 5%, it was found that the enzyme-treated sample tended to significantly produce more reducing sugar than the control group ($p \leq 0.05$) (Fig. 4.20). When enzyme concentration was increased, the reducing sugar content also increased. At 1% enzyme concentration, the reducing sugar content increased and then leveled off after 2 h. At enzyme concentration of 2% and 3%, the reducing sugar content leveled off after 4 h. At 4%

and 5% enzyme concentration, the reducing sugar content was highest after 4 h and leveled off thereafter. At 4-5% enzyme concentration, the amount of reducing sugar was not statistically different ($p>0.5$) from the 3% enzyme concentration. From the statistical point of view, the result of 3% enzyme concentration showed the highest amount of reducing sugar. The major advantages of high enzyme loading are faster rate of hydrolysis and increased sugar yields (Çinar, 2005 *b*).

Enzyme concentration affected to the increase of reducing sugar content because the higher amount of substrate and enzyme brought about more greater binding of enzyme-substrate complexes and hence the product yield was higher. In contrast, when substrate and enzyme decreased, binding was also diminished and the product level reduced. Thus, reaction rate was depended on proportional of enzyme–substrate complexes.

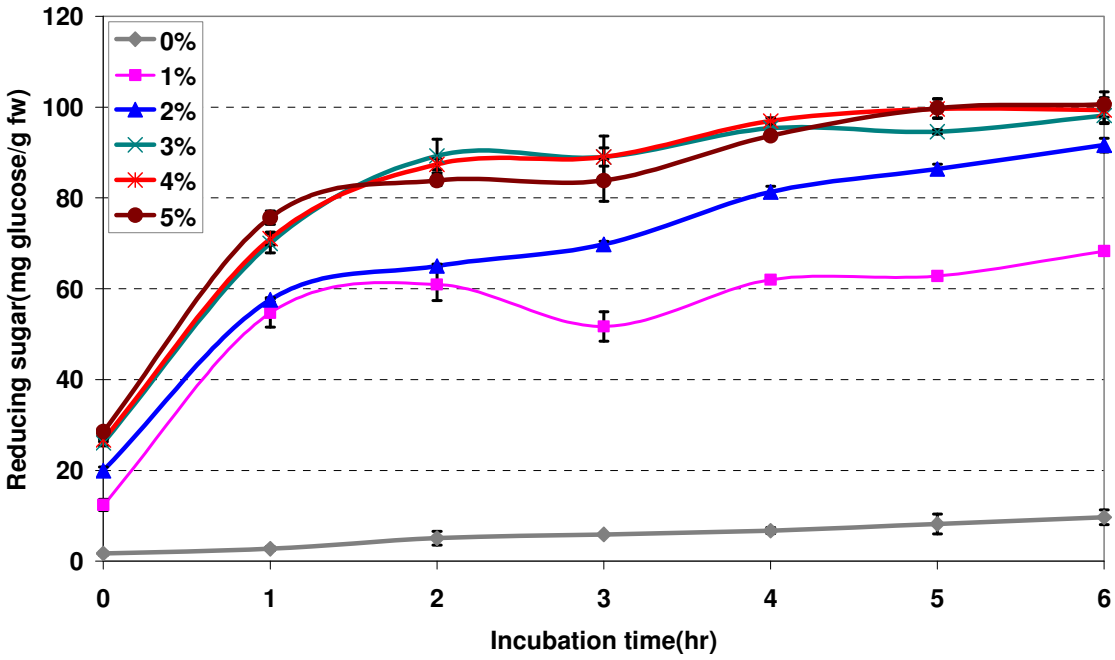


Fig. 4.20 The reducing sugar yield after incubation at 35°C with 0–5%(v/w) Pectinex® Ultra SP-L.

Although there was a high amount of substrate, the reaction rate did not increase because the enzyme content was not great enough to react with the excess substrate. But providing more incubation time until some enzymes were released from product and the reaction of other substrate molecules was activated, the reaction rate can be increased (Whitaker, 1991; Anprung, 2004). More enzyme contents also not related with the higher reaction rate because there was not enough substrate for the reaction. Furthermore, Çinar (2005 a) studied about carotenoid extraction and found that applying the enzyme in too a high concentration can reduce the yield because hydrolysis reaction occurs rapidly and brings about the end product inhibitor. Thus, to compromise the amount of enzyme substrate and time needed to obtain acceptable product yield, rate is necessary because the main drawback is the high cost of enzyme.

1.5 Determine the relative activity

From the enzymatic extraction of the chlorophyll derivatives results show that at longer incubation times contributed to the constant rate of extracted amount of chlorophyll derivatives compounds. For example, using Pectinex® Ultra SP-L enzyme at 2% (v/w) concentration, the chlorophyll content started to be constant level after 4 h. The reasons of extraction yield not increasing after this point can be determined by measuring the relative activity of enzyme at each incubation time. The experiment had to collect the extract sample in each extraction period to monitor the enzyme activity by measuring relative activity (Fig. 4.21).

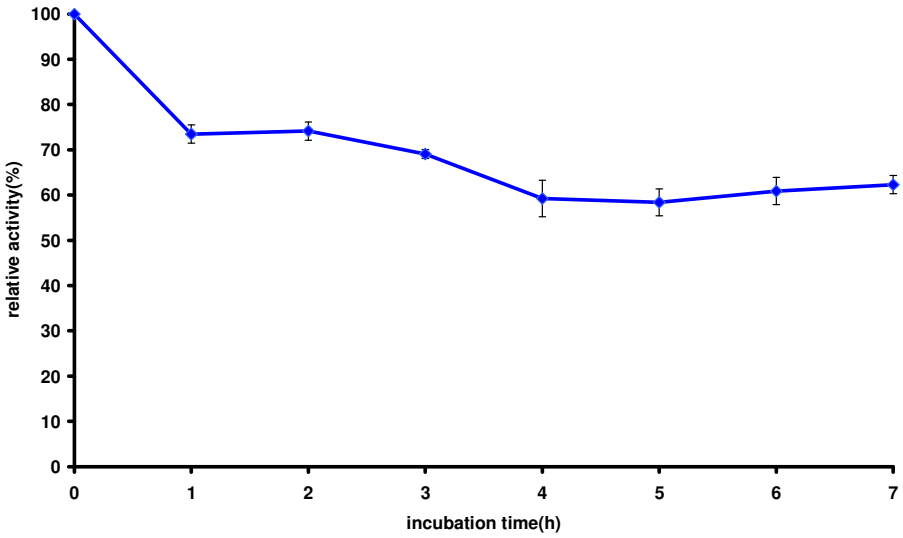


Fig. 4.21 The relative activity of 2% (v/w) Pectinex Ultra SP®-L in the pandan extract with the incubation in 0-7 h.

Initially, relative activity equaled 100% and after incubation time of 1–3 h, the relative activity decreased to 73.5–69.07 % and after 4–7 h, it reduced to 62.3–58.4%. The reduction of relative activity may come from zinc-chlorophyll derivatives product which composed of zinc ion that can inhibit activity of pectinase enzyme (Moyo *et al.*, 2003).

Reduction of enzyme activity caused a slower rate for the chlorophyll derivatives extraction after 4 h and some chlorophyll derivatives were still left in the pandan residue. Therefore, this study tested the addition of enzyme in the same concentration with the first round of extraction to extract the remaining chlorophyll (Fig. 4.22). The result showed that the higher amount of chlorophyll derivatives compound can be extracted.

From our preliminary study, there was a comparison between using 4% (v/w) enzyme for extraction and 2% (v/w) enzyme for twice re-extractions with its residue. It was found that the former procedure caused lower efficiency in extraction of colorant, Zn-chlorophyll derivatives, and antioxidant than the latter one. The reason

may be due to its product after extraction, Zn-chlorophyll derivatives, which can inhibit the activity of the pectinase enzyme (Moyo *et al*., 2003). Therefore, removal of the Zn-chlorophyll derivatives product after the first round of extraction and then re-extraction can enhance quantity and quality (enzymic activities) levels of the product.

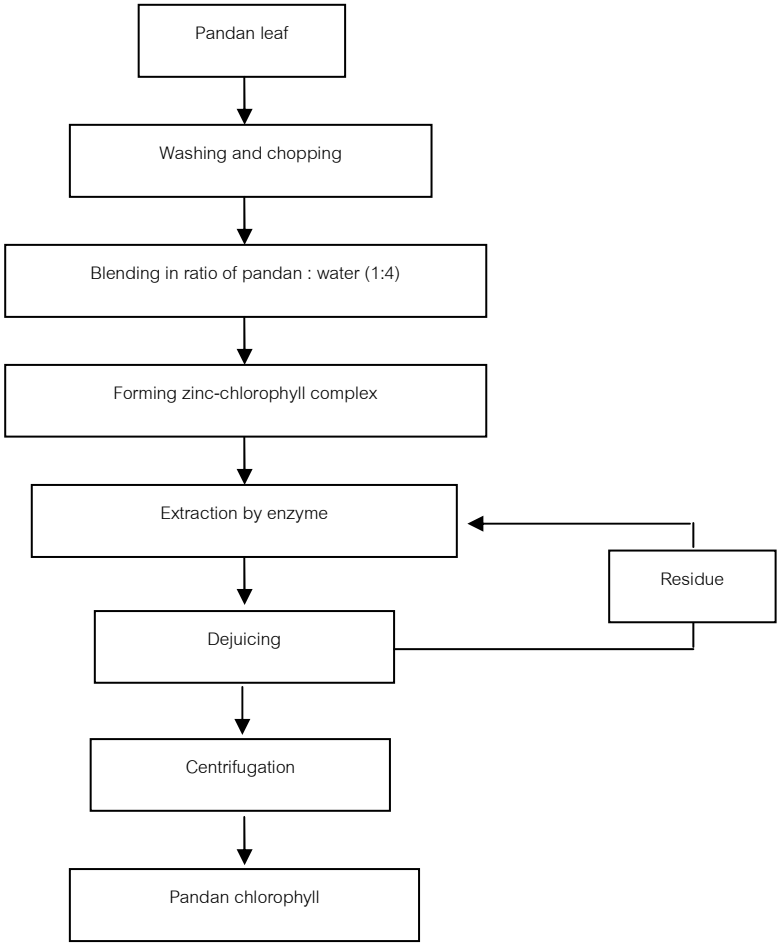


Fig. 4.22 Process flow chart for zinc chlorophyll formation and extraction

According to preliminary studies, the important enzymatic extraction parameters for determining the RSM included the concentration of enzyme, reaction time and number of re-extractions. The studies varied the concentration of enzyme Pectinex® Ultra SP-L in the range of 1–3%, reaction time by 90–270 min, and 1–3

rounds of re-extraction whilst holding the incubation at the optimal temperature of 35 ^{o}C and optimal pH of 4.0.

2) Optimization of enzymatic extraction of zinc-chlorophyll complex by Response Surface Methodology (RSM)

2.1 Comparison of enzyme treated and control

From table 4.3, results of control No.1 (no formation of zinc-chlorophyll derivatives and no enzyme treatment) showed that hue value was 96.57 representing the dark brown color of pandan leaf extract, greenness (-2.38), total chlorophyll content (1.23 mg/gfw) and antioxidant activity (300.85 μMTE/gfw) were very low. For control No.2 (with formation of Zn-chlorophyll complexes but no enzyme treatment), the extraction yield showed that hue value was 105.67 representing green color of pandan leaf extract, greenness (-3.46), total chlorophyll content (1.03 mg/gfw) and antioxidant activity (314.88 μMTE/gfw) were low.

From comparison of chlorophyll contents between control 2 and extract treated with lowest enzyme level (treatment 14; 1% Pectinex Ultra SP®-L, 90 min incubation time and one round of extraction), the result showed that using enzyme can increase extracted chlorophyll content 5.84 times including increase the antioxidant activity 1.91 times. When compared with medium enzyme level (treatment 15-19; 2% Pectinex® Ultra SP-L, 180 min incubation time and two rounds of extraction), the result showed that using enzyme could increase extracted chlorophyll yield 16.58 times and increase the antioxidant activity 1.95 times. When compared with high enzyme level (treatment 7; 3% Pectinex® Ultra SP-L, 270 min incubation time and 3 rounds of extraction), the result showed that using enzyme can increase extracted chlorophyll content 19.07 times and increase the antioxidant activity 1.95 times.

Table 4.3 The effect of enzyme extraction on the color value, chlorophyll content and antioxidant activity of pandan leaf.

No	Level			Response variable						
	Enzyme (%)	Time (min)	Re-extract (round)	*L**	*a**	*b**	*Chroma*	*Hue*	Chlorophyll (mg/g fw)	Antioxidant (μM TEAC)
1	1	180	2	23.05 ± 0.02 ^{m}	-11.58 ± 0.08 ^{hi}	33.07 ±0.03 ^{h}	35.04 ± 0.03 ^{h}	109.23 ± 0.06 ^{i}	18.39 ± 0.22 ^{i}	607.44 ± 3.53 ^{ab}
2	3	180	2	21.66 ± 0.01 ^{o}	-11.56 ± 0.03 ^{h}	31.59 ± 0.03 ^{l}	33.61 ± 0.06 ^{j}	109.93 ± 0.12 ^{g}	15.11 ± 0.02 ^{l}	615.63 ± 1.46 ^{a}
3	2	90	2	31.20 ± 0.01 ^{h}	-11.45 ± 0.05 ^{g}	36.41 ±0.02 ^{b}	38.28 ± 0.05 ^{b}	107.37 ± 0.06 ^{m}	14.24 ± 0.01 ^{k}	606.04 ± 7.83 ^{ab}
4	2	270	2	28.94 ± 0.01 ^{i}	-11.59 ± 0.04 ^{hi}	36.59 ±0.03 ^{a}	38.43 ± 0.01 ^{a}	107.43 ± 0.06 ^{lm}	17.76 ± 0.03 ^{c}	612.82 ± 3.17 ^{ab}
5	2	180	1	34.32 ± 0.02 ^{e}	-11.21 ± 0.01 ^{e}	35.74 ±0.01 ^{d}	37.49 ± 0.02 ^{d}	107.40 ± 0.00 ^{lm}	11.08 ± 0.11 ^{n}	612.59 ± 1.86 ^{ab}
6	2	180	3	18.19 ± 0.01 ^{t}	-11.65 ± 0.05 ^{ij}	27.71 ±0.02 ^{n}	30.03 ± 0.03 ^{n}	112.60 ± 0.10 ^{b}	18.40 ± 0.08 ^{m}	613.99 ± 0.70 ^{a}
7	3	270	3	16.68 ± 0.01 ^{u}	-11.36 ± 0.05 ^{f}	25.79 ±0.05 ^{o}	28.23 ± 0.04 ^{o}	113.70 ± 0.10 ^{a}	19.58 ± 0.20 ^{a}	612.82 ± 1.72 ^{ab}
8	1	270	3	20.26 ± 0.01 ^{s}	-11.75 ± 0.03 ^{k}	30.32 ±0.01 ^{l}	32.53 ± 0.09 ^{l}	111.07 ± 0.06 ^{c}	16.36 ± 0.09 ^{gh}	614.69 ± 11.86 ^{a}
9	3	90	3	21.03 ± 0.02 ^{r}	-11.77 ± 0.09 ^{k}	31.17 ±0.03 ^{k}	33.39 ± 0.06 ^{k}	110.53 ± 0.15 ^{d}	16.26 ± 0.03 ^{h}	615.86 ± 1.46 ^{a}
10	1	90	3	24.07 ± 0.01 ^{k}	-11.57 ± 0.05 ^{h}	33.81 ±0.05 ^{f}	35.79 ± 0.07 ^{f}	108.87 ± 0.06 ^{j}	14.61 ± 0.12 ^{j}	608.61 ± 7.88 ^{ab}
11	3	270	1	31.23 ± 0.01 ^{g}	-11.35 ± 0.04 ^{f}	36.46 ±0.02 ^{b}	38.24 ± 0.02 ^{b}	107.40 ± 0.00 ^{lm}	12.37 ± 0.23 ^{m}	613.05 ± 5.84 ^{ab}
12	1	270	1	32.38 ± 0.01 ^{f}	-11.42 ± 0.04 ^{fg}	36.12 ±0.03 ^{c}	37.94 ± 0.02 ^{c}	107.53 ± 0.06 ^{l}	12.97 ± 0.11 ^{l}	609.78 ± 2.53 ^{ab}
13	3	90	1	42.11 ± 0.01 ^{d}	-10.43 ± 0.01 ^{d}	34.74 ±0.03 ^{e}	36.37 ± 0.02 ^{e}	106.60 ± 0.00 ^{n}	9.06 ± 0.10 ^{o}	613.99 ± 0.70 ^{a}
14	1	90	1	54.24 ± 0.02 ^{c}	-8.95 ± 0.02 ^{c}	30.22 ±0.08 ^{m}	31.80 ± 0.03 ^{m}	106.37 ± 0.06 ^{o}	6.00 ± 0.08 ^{p}	602.76 ± 1.86 ^{b}
15	2	180	2	23.53 ± 0.01 ^{l}	-11.76 ± 0.02 ^{k}	33.53 ±0.04 ^{g}	35.51 ± 0.06 ^{g}	109.17 ± 0.12 ^{i}	17.81 ± 0.08 ^{c}	613.99 ± 0.78 ^{a}
16	2	180	2	21.58 ± 0.01 ^{p}	-11.68 ± 0.04 ^{j}	31.63 ±0.03 ^{i}	33.76 ± 0.05 ^{i}	110.17 ± 0.06 ^{f}	17.03 ± 0.10 ^{e}	613.52 ± 1.07 ^{a}
17	2	180	2	22.88 ± 0.01 ^{n}	-11.87 ± 0.04 ^{l}	33.04 ±0.06 ^{h}	35.10 ± 0.04 ^{h}	109.60 ± 0.00 ^{h}	16.61 ± 0.02 ^{f}	611.42 ± 5.08 ^{ab}
18	2	180	2	21.36 ± 0.01 ^{q}	-11.80 ± 0.03 ^{k}	31.51 ±0.01 ^{j}	33.73 ± 0.05 ^{i}	110.40 ± 0.10 ^{e}	17.47 ± 0.12 ^{d}	613.75 ± 2.26 ^{a}
19	2	180	2	24.31 ± 0.01 ^{j}	-11.45 ± 0.02 ^{g}	33.82 ±0.04 ^{f}	35.75 ± 0.01 ^{f}	108.67 ± 0.06 ^{k}	16.51 ± 0.21 ^{fg}	615.16 ± 1.07 ^{a}
Control 1 (no pretreatment, no enzyme)				73.24 ± 0.01^{b}	-2.38 ± 0.02 ^{a}	20.37± 0.02 ^{p}	20.58 ± 0.02 ^{p}	96.57 ± 0.06 ^{q}	1.23 ± 0.03 ^{q}	300.85 ± 16.16 ^{d}
Control 2 (no enzyme)				85.36 ± 0.03 ^{a}	-3.46 ± 0.02 ^{b}	12.12 ± 0.04 ^{q}	12.79 ± 0.07 ^{q}	105.67 ± 0.12 ^{p}	1.03 ± 0.11 ^{r}	314.88 ± 9.86 ^{c}

Note; *Control 1; no zinc-chlorophyll formation and no added enzyme

**Control 2; no added enzyme.

It can be concluded that all levels of enzyme concentration increase efficiency in chlorophyll derivative and antioxidant activity significantly ($p \leq 0.05$). Comparisons of enzymatic and non-enzymatic extraction of Zn-chlorophyll derivatives from pandan leaves found that enzymatic extraction resulted in higher green color values, increased amounts chlorophyll derivatives, and increased antioxidant activity. The range of

green color values, chlorophyll contents, and the antioxidant activity were increased 2.58-3.43, 5.82-19.00 and 1.91-1.95 fold, respectively, over non-enzymatic extraction (Fig 4.23). Since the plant cell wall comprises cellulose and pectins, cellulase, and pectinase have been used for this purpose. Enzymatic cell wall lysis employing hydrolytic enzymes, that can degrade the cell wall constituents, thus enzyme is assisting in the release of intercellular content (Schmitt, 1988).

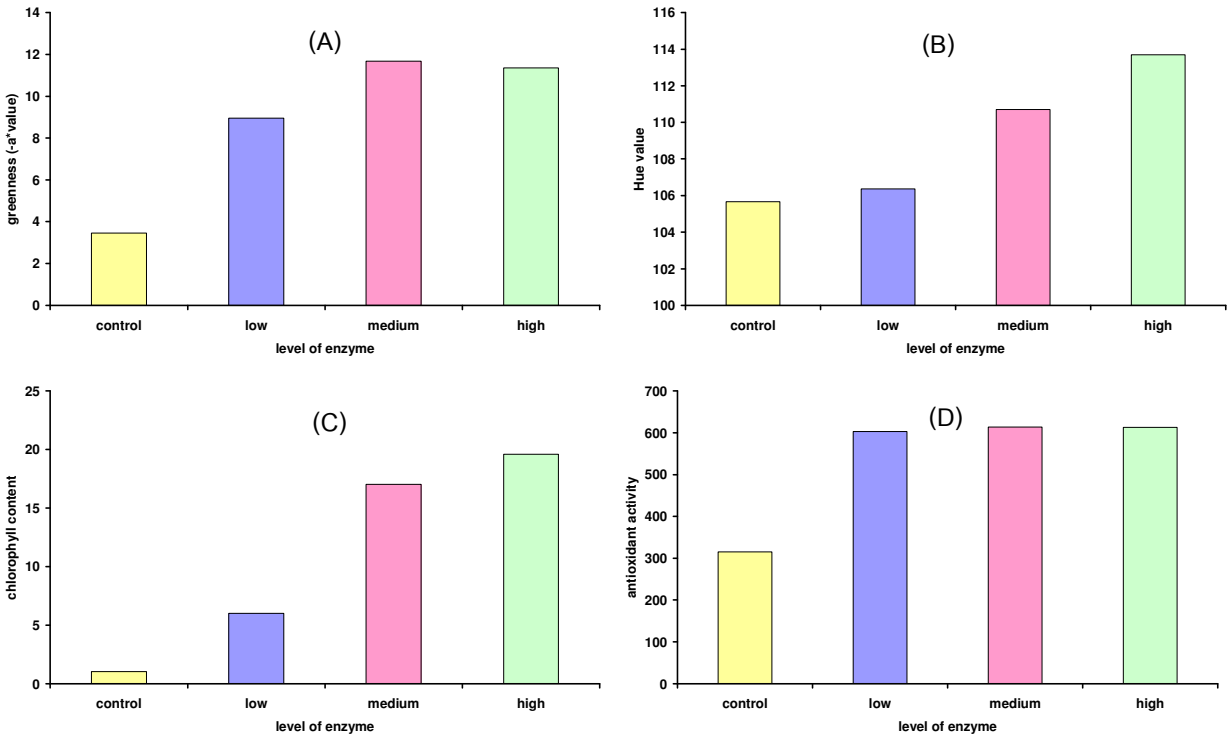


Fig. 4.23 The extraction of Zn-chlorophyll complexes in pandan leaf at the low level (-1, -1, -1) , the medium level (0, 0, 0), and the high level (+1, +1, +1) compare with the control No.2 when (A) greenness values, (B) Hue values, (C) chlorophyll content (mg/g fw), and (D) antioxidant activity (μM TEAC/gfw)

Color values and chlorophyll derivatives increased when extracted by Pectinex® Ultra SP-L because this enzyme had activities including polygalacturonase, pectinlyase, pectin esterase, β-galactosidase, chitinase, and transgalactosidase (Mutlu

et al., 1999; Liew Abdullah *et al*., 2007). These enzymes were used to disintegrate the cell wall of the chloroplast which contain chlorophyll and then the pigment was extracted (Essa, 2002; Çinar, 2005 a,b; Choudhari and Anathanarayan, 2007).

Furthermore, higher amounts of chlorophyll derivatives increased antioxidant activity because chlorophyll derivatives were able to break down the radical chains due to chlorophyll being an electron donor (Endo *et al*., 1985). Hoshina *et al*. (1998) found that the porphyrin structure in chlorophyll molecules was capable of inhibiting formation of lipid hydroperoxide from thiocyanate and ferric nitrolotriacetate. Ferruzi *et al*.(2002) reported that metal-free derivatives such as chlorins, pheophytins, and pyropheophytins exhibited significantly lower antiradical capacity than metallo-derivatives such as Mg-chlorophylls, Zn-chlorophylls and Zn-pyropheophytins. Metal chelation would result in concentration of electron density toward the centrally bound metal and away from the porphyrin backbone of the chlorophyll molecule, resulting in an increased ability to donate electrons from the conjugated porphyrin system (Ferruzi *et al*., 2002).

Consequently, the formation of metallochlorophyll complexes by changing chlorophyll to zinc-chlorophyll derivatives not only made the color of chlorophyll stable due to the porphyrin's stability, but also brought about greater antioxidant activity than native chlorophyll (Ferruzzi *et al*., 2002).

2.2 Model fitting and response surface plotting

The regression coefficient equation of color values (L^*,-a^*, b^*, C, h^o), and the amount of chlorophyll derivatives and antioxidant activity, showed that the coefficient values of determination (R^2) were more than 0.9 ($p \leq 0.05$) in all parameter except antioxidant activity (table 4.4). Results indicate that these equations had a model fit with experimental data (Rastogi and Rashmi, 1999) which may be used to estimate

greenness value and chlorophyll derivatives content after varying enzyme concentration (x_1), incubation time (x_2), and number of re-extractions (x_3). The relationship of greenness (-*a** value) and chlorophyll derivatives content can be predicted using equation 1 and 2, respectively.

Table 4.4 Regression coefficient and R^2 for color values, chlorophyll derivatives content, and antioxidant activity response of enzymatic extraction.

coefficient	*L** value	*a** value	b* value	chroma	hue	total chlorophyll	antioxidant activity(TEAC)
β_0	23.223**	-11.717**	33.170**	35.202**	109.398**	16.814**	612.855**
β_1	-2.128**	-0.120**	-0.378**	-0.328*	0.510**	0.720**	2.807**
β_2	-4.317**	-0.332**	-0.106	-0.026	0.740**	1.887**	1.591**
β_3	-9.406**	-0.473**	-2.447**	-2.187**	2.147**	3.373**	1.380**
$\beta_1 \beta_2$	1.305**	0.268**	-0.759**	-0.771**	0.075**	-0.262	-2.135
$\beta_1\beta_3$	0.834*	0.200**	-1.505**	-1.447**	0.525**	0.300**	-1.140**
$\beta_2\beta_3$	3.073**	0.454**	-2.061**	-2.055**	0.425**	-0.652**	-0.380**
β^2_1	-1.479**	0.149*	-1.425**	-1.414**	0.438**	-1.294**	-0.427**
β^2_2	6.238**	0.199**	2.750**	2.612**	-1.745**	-0.471**	-2.531**
β^2_3	2.421**	0.293**	-2.023**	-1.979**	0.855**	-1.731**	1.326**
R^2	0.959	0.924	0.907	0.906	0.933	0.963	0.509

Note: Subscripts; β_0 =constant, β_1 = enzyme concentration (x_1), β_2= incubation time (x_2) β_3 =number of re-extract (x_3).

*Significant at 0.05 level, **Significant at 0.01 level

$$\text{Greenness (-a*values)} = -11.717 - 0.120\,x_1 - 0.332\,x_2 - 0.473\,x_3 + 0.268 x_1.x_2 + 0.200 x_1.x_3 + 0.454\,x_2.x_3 + 0.149\,x_1^2 + 0.199\,x_2^2 + 0.293 x_3^2$$

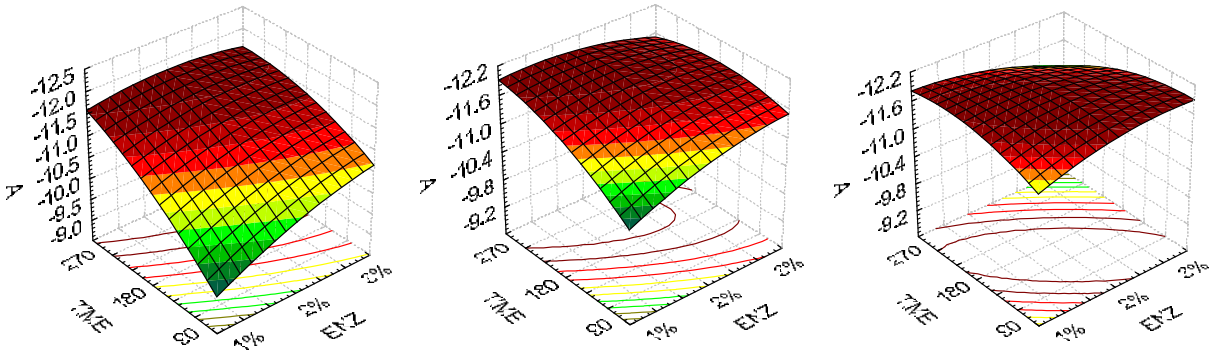
$$(R^2 = 0.924,\ p \leq 0.01) \quad \text{Equation 1}$$

$$\text{Chlorophyll content} = 16.814 + 0.720\,x_1 + 1.887 x_2 + 3.373 x_3 + 0.300 x_1.x_3 - 0.652\,x_2.x_3 - 1.294\,x_1^2 - 0.471\,x_2^2 - 1.731 x_3^2$$

$$(R^2 = 0.963,\ p \leq 0.01) \quad \text{Equation 2}$$

From these equations, the relationship between enzyme concentration, incubation time, and number of re-extractions was shown in terms of linear, quadratic and interaction, respectively. Increases in greenness and chlorophyll derivatives corresponded to a rise in the concentration of the enzyme, incubation time, and number of re-extractions until a critical point is reached, after which point all three factors decrease quadratically. Thus, it can be explained that higher concentrations of enzyme lead to more efficient enzyme hydrolysis. Moreover, when the concentration of the pectinase enzyme involved a high rate of hydrolysis, Zn-chlorophyll derivatives end-product inhibition might have occurred (Çinar, 2005 a,b). As processing time and number of re-extractions increased, a gradual increase in yield of chlorophyll derivatives was observed until the point after which no more chlorophyll derivatives would be formed.

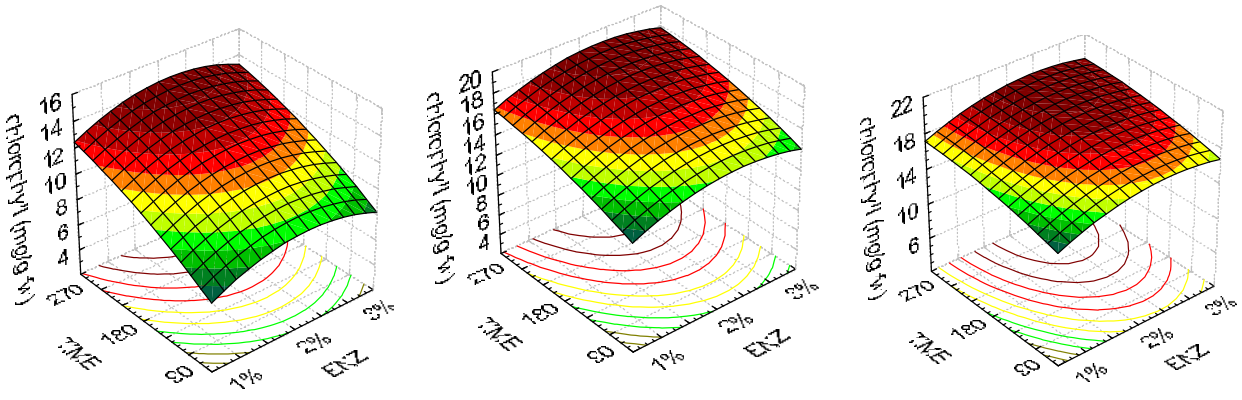
Furthermore, equations showed a correlation among enzyme concentration, incubation time, and number of re-extractions. An increase or decrease one of these parameters could affect the others. When considering coefficient values, increasing the number of extractions resulted in more green color extract and the highest yield of chlorophyll derivatives. The number of extractions applied was the most important factor affecting the yield of chlorophyll derivatives.



(A) **(B)** **(C)**

Fig. 4.24 Response surface showing effects of enzyme concentration and incubation time on the greenness value at (A) first extraction, (B) second extraction, and (C) third extraction.

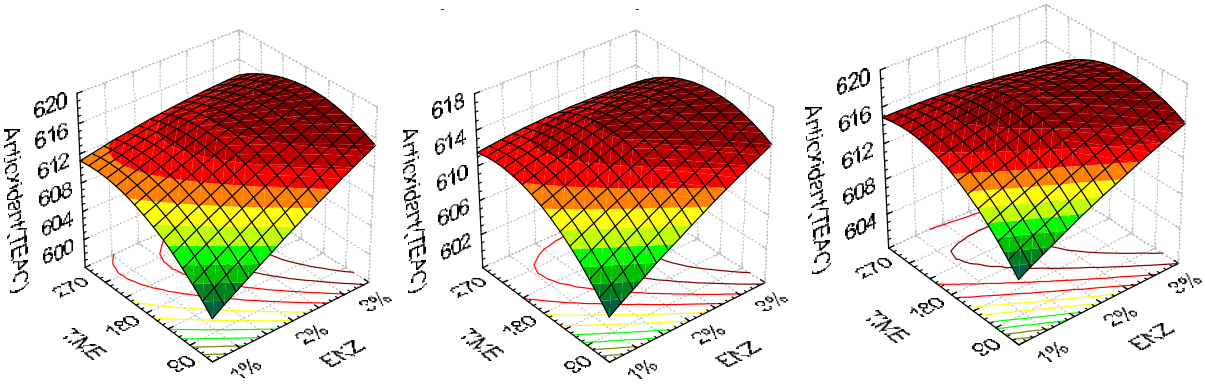
Fig. 4.24 shows the response surface curves of the relationship between enzyme concentration (1–3%v/w) and incubation time (90–270 min) on green color pigment extracted. It can be interpreted that for the first extraction (A), the greatest amount intensity of green color extracted was reached at 3% (v/w) pectinase for 270 min of reaction time. For the second extraction (B), after treatment of the residue with the same enzyme concentration to re-extract, conditions for the highest yield were 1) treatment with 2% pectinase and 180-270 min or 2) to decrease enzyme content to 1% pectinase and increase reaction time to 270 min. At re-extraction third time (C), many conditions could be chosen for the highest yield by modification of the incubation time or concentration of enzyme (i.e. 1% pectinase for 180–250 min, 2% enzyme for 90–200 min, or 3% pectinase for 90 min).



(A) **(B)** **(C)**

Fig. 4.25 Response surface showing effects of enzyme concentration and incubation time on the chlorophyll content at (A) first extraction, (B) second extraction, and (C) third extraction

Fig. 4.25, represents the relationship between enzyme concentration (1-3% v/w) and incubation time (90-270 min) on chlorophyll derivatives content. It was found that after extraction 1, 2 & 3, the chlorophyll derivatives obtained were 14.23, 19.54 and 20.12 mg/g fresh weight, respectively (2%v/w enzyme, 270 min). While the second extraction yielded chlorophyll derivatives content significantly different from that first extraction, the second extraction resulted in almost complete removal of the chlorophyll derivatives, and the third extraction was deemed unnecessary. It could be concluded that nearly all of chlorophyll derivatives contents were collected from the leaf by the second extraction, so the third extraction did not significantly increase the final amount of chlorophyll derivatives removed. So two times extraction was deemed most favorable, as three times extraction resulted in both loss of enzyme and time. The response surface graphs of antioxidant activity values are illustrated in Fig. 4.26. The results showed that the higher the enzyme concentration, the longer the incubation time, and the higher the number of extractions, led to higher antioxidant activity.



(A) **(B)** **(C)**

Fig. 4.26 Response surface showing effects of enzyme concentration and incubation time on antioxidant activity at (A) first extraction (B) second extraction, and (C) third extraction

2.3 Optimization

Using the central point (of the second extraction) from the response surface plots of 1) greenness values, 2) chlorophyll derivatives contents, and 3) antioxidant activities, a superimposed contour plot was made and is shown in Fig. 4.27. The optimal conditions for Pectinex® Ultra SP-L in the extraction of chlorophyll derivative from the pandan leaf (shown at a position drawn in the circle) were a 2.3-2.5% enzyme concentration, 240-260 min of incubation time, and two times of extraction at 35 ^{o}C. At these optimum conditions, the obtained extract had a green color value of -10.36, chlorophyll derivatives content of 17.82 mg/g fw, and antioxidant activity of 612.89 μMTE/g fw. This was different from non-enzymatic extraction that resulted in a -3.46 green color value, 1.03 mg/g fw of chlorophyll derivatives content, and 314.88 μMTE/g fw of antioxidant activity. Therefore, enzyme treatment yielded greenness, Zn-chlorophyll derivatives content, and antioxidant activity of 3.00, 17.31 and 1.916 fold more than control, respectively.

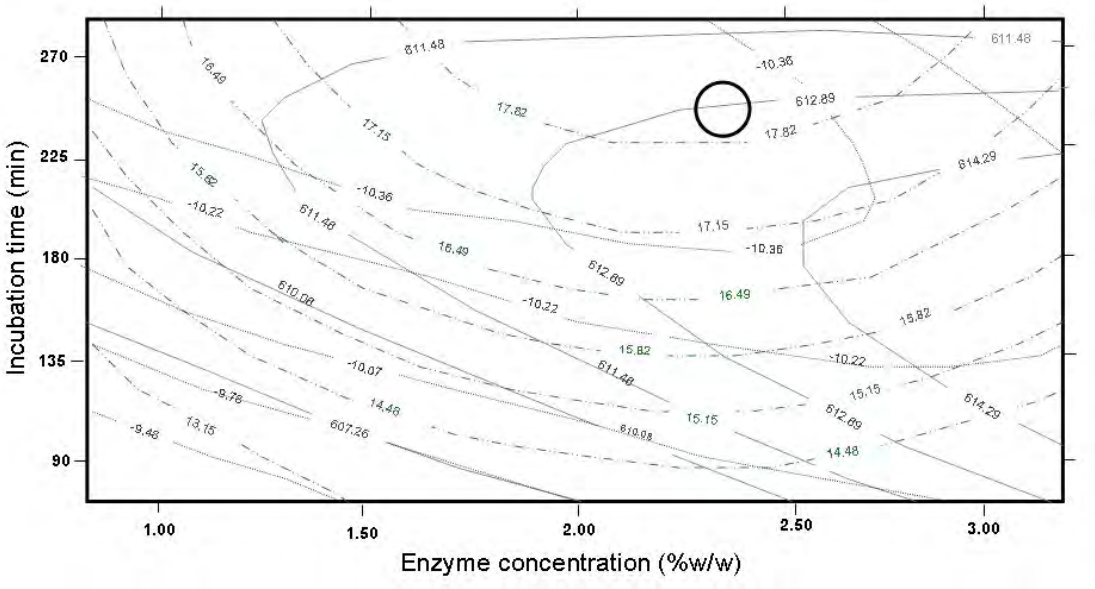


Fig. 4.27 Superimposed contour plots for optimization of greenness, chlorophyll content, and antioxidant activity when re-extraction was kept constant at central point.

The optimal conditions for the formation of Zn-chlorophyll derivatives to form a stable in green color and improve antioxidant activity in the pandan leaf occurred when a 300 ppm concentration of $ZnCl_2$ was used at a pH of 5.0 and was heated at 110 ^{o}C for 15 min. This should be followed by extraction of the derivatives by Pectinex® Ultra SP-L at pH 4.0 with 2.3-2.5% (v/w) enzyme concentration, 240-260 min of extraction time, and two times of extraction at 35 ^{o}C. This treatment resulted in a stable green colorant, more chlorophyll content and increased antioxidant activities.

PART 5: Zn-Chlorophyll derivatives encapsulated powders

Spray drying method was used to prepare chlorophyll powder form liquid extract bring about stability of product and also more convenient in application. Selection of suitable encapsulating agent at the appropriate ratio can improve the

quality of product by preventing the chemical reaction with environmental factors like heat, acidity-alkalinity and oxidation.

Production of Zn-chlorophyll derivatives powder can be done by drying the chlorophyll derivatives extract using spray drying method. From preliminary study, it was found that the optimum conditions for Zn-chlorophyll derivatives powder production process by spray dryer were the inlet and outlet temperatures as 150 ± 5 ^{o}C and 90 ± 5 ^{o}C, respectively, atomizer pressure control at 50 kPa, flow rate at 300 mL/h and blower control at 0.70 m^3/min. Therefore, this experiment was set followed these conditions to evaluate the suitable types of carriers by comparison between GA, MD and *n*-OSA-starch and vary the suitable total solid content of 10, 20 and 30% (w/w).

1) Physicochemical property

The physicochemical properties of chlorophyll powder monitored were: Scanning Electron Microscope (SEM) micrograph, average particle size distribution, bulk density, greenness of powders, total chlorophyll contents, antioxidant activity, a_w, and yield.

1.1 SEM micrograph

Fig. 4.28 illustrates the shape of spray-dried particles as results of wall materials of GA, MD and MS. The GA and MD had the spherical particles with dented surface, while MS had the spherical particle with smooth surface. These related to Rosenberg, Kopelman and Talman (1990) and Kim and Morr (1996) who reported that GA encapsulated powder had high dense on surface and also high release rate of orange oil flavor which brought to short term storage. Furthermore, Desobry, Netto and Labuza (1997) suggested that the spray dried particle in spherical shape had high surface area/volume ratio which was the appropriate character for spray-dried product. Reineccius (2004) recommended that particle in spherical shape can retain the highest

amount of flavoring agent. In this study, it can be concluded from considering the shape of Zn-chlorophyll derivatives powder that MS was a proper wall material.

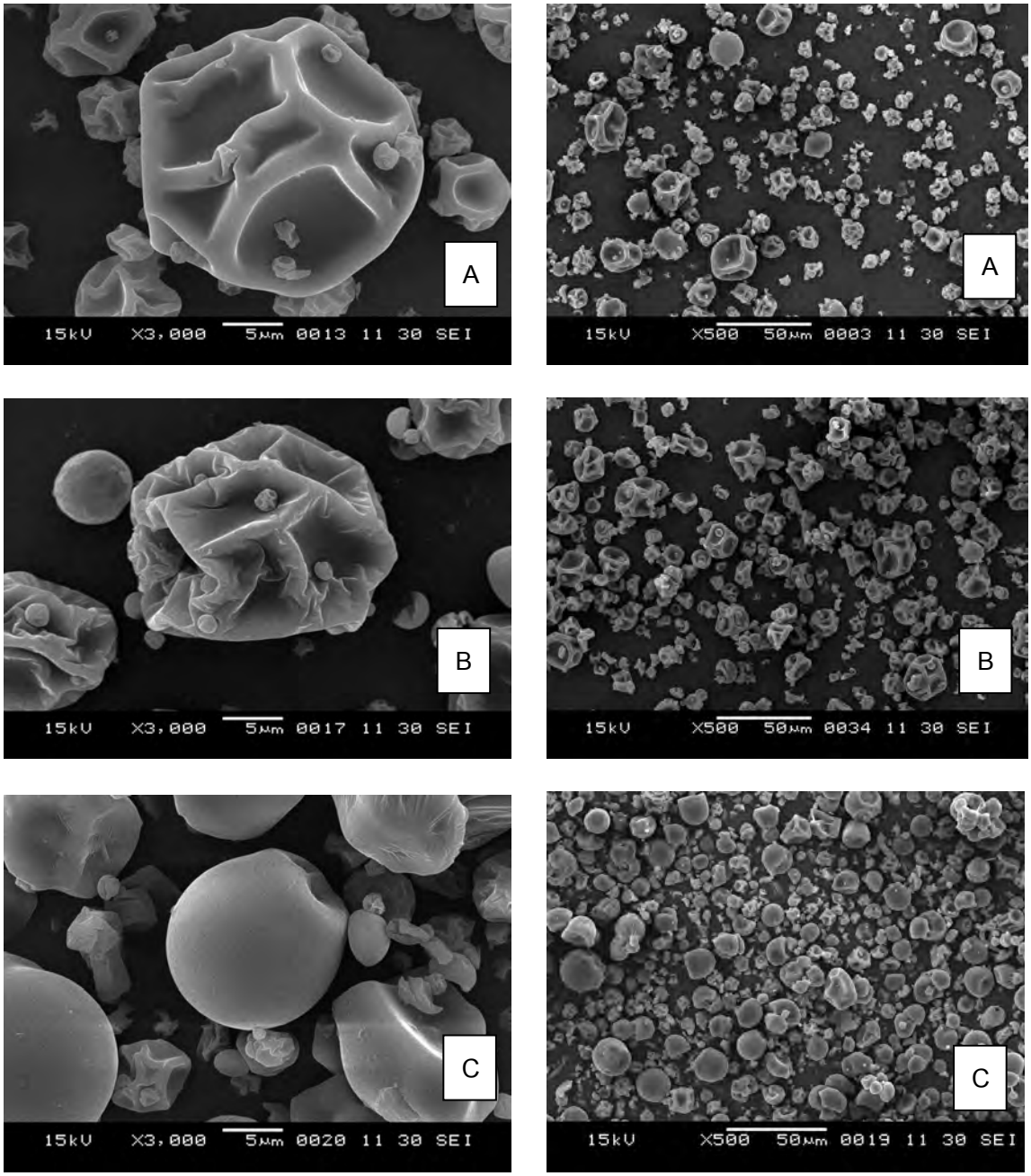


Fig. 4.28 SEM micrographs of spray-dried chlorophyll encapsulated powders. Wall material system: (A) Gum arabic, (B) Maltodextrin, and (C) Modified starch.

1.2 Particle size distributions

Average particle sizes of GA, MD and MS were 34.46, 30.11 and 16.13 μm, respectively (Fig. 4.29). The average particle sizes of GA and MD were about 2 times greater than that of MS. These large particle sizes of GA and MD caused high dense on surface when they were processed by spray drying as shown in SEM micrographs.

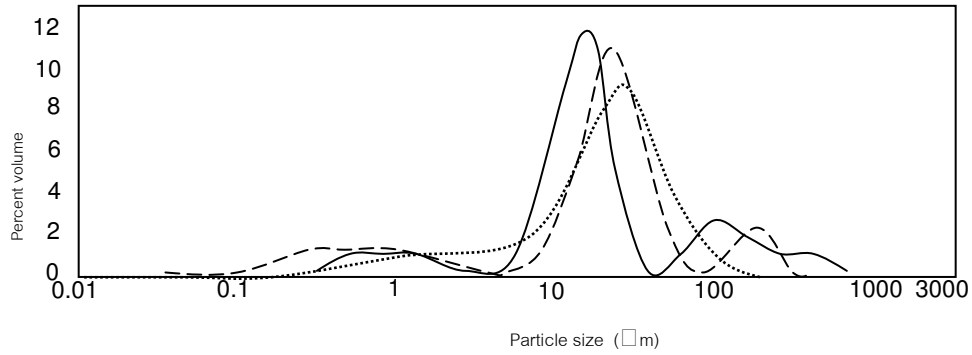


Fig. 4.29 Particle size distribution of pandan powder with different carrier: (A) Gum arabic (·····), (B) Maltodextrin (- - - -), and (C) Modified starch (——) wall material.

1.3 Bulk density

Bulk density value correlated with the particle size as found that powders derived from GA and MD had large sizes, while that from MS had the smallest size and hence MS can be contained most tightly and represented the highest bulk density value. Reineccius (2004) found that particle in spherical shape had the highest bulk density value, best packing and best flowing ability. Moreover, Buffo and Reineccius (2000) have concluded that powder in spherical shape can be compacted. The high bulk density had an advantage because its can decrease the oxygen permeability which caused oxidation, so the shelf life of product can be extended. Therefore, MS in the smallest particle with smooth surface and high bulk density was the suitable characteristic of wall materials.

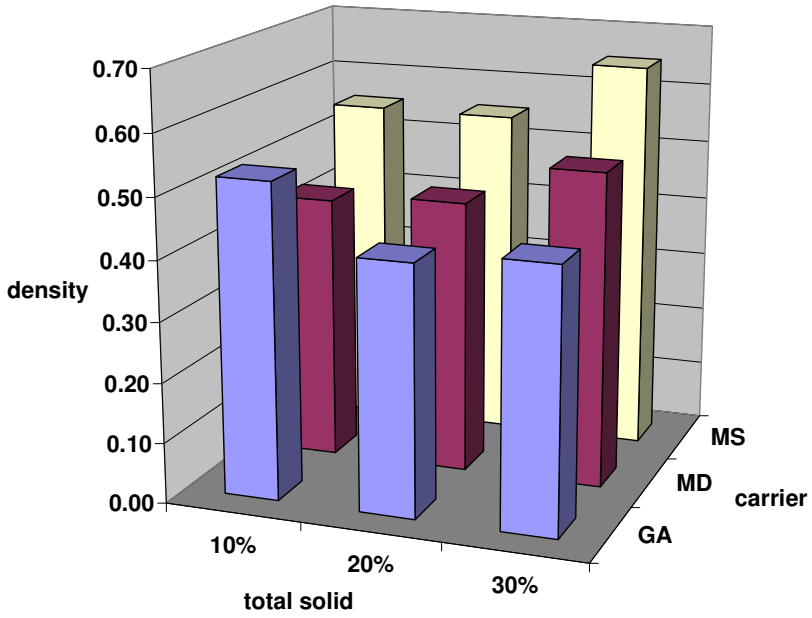


Fig. 4.30 Bulk density of the GA, MD, and MS powder at total solid content at 10-30%.

1.4 Chlorophyll content

Results of chlorophyll content derived from different types of wall material as presented in Fig. 4.31 showed that MS had the highest amount of Zn-chlorophyll derivatives, the second was GA and the lowest was from MD due to the difference in chemical structure as follows:

GA comprised subunits of oligosaccharides, polysaccharides, and glycoproteins, especially glycoproteins which had property of emulsion, thus they can bind with Zn-chlorophyll derivatives molecules that had both hydrophobic part of phytol group and hydrophilic part of porphyrin ring.

For MD, it was derived from the modified starch with acid or enzyme which subsequently brought about monosaccharide or short chain polymer that it form thin film cover the flavoring and coloring agents to prevent the loss of them during drying. The involved chemical bonds were hydrophobic, van der Waals, hydrogen bond and

electrostatic (Goubet *et al.*, 1998). Nevertheless, some disadvantages of MD were having no property of emulsification.

Native starch and starch hydrolysis products are hydrophilic, and have no affinity for hydrophobic substances such as oils. However, modifying the starch with fatty acids can introduce hydrophobic groups. For OSA-MS, it has been modified by octenyl succinic anhydride to add hydrophobic groups to the starch molecules that comprised amphiphilic character (Viswanathan, 1999; Kshirsagar and Singhal, 2007). Subsequently, it can bind with chlorophyll derivatives molecule or this meant OSA-MS has emulsifying property. Furthermore, it had character of strong film at the oil-water interface area, so this can be resistant to re-agglomeration. Hence OSA-MS was deemed suitable to be a wall material (Bhosale and Singhal, 2006).

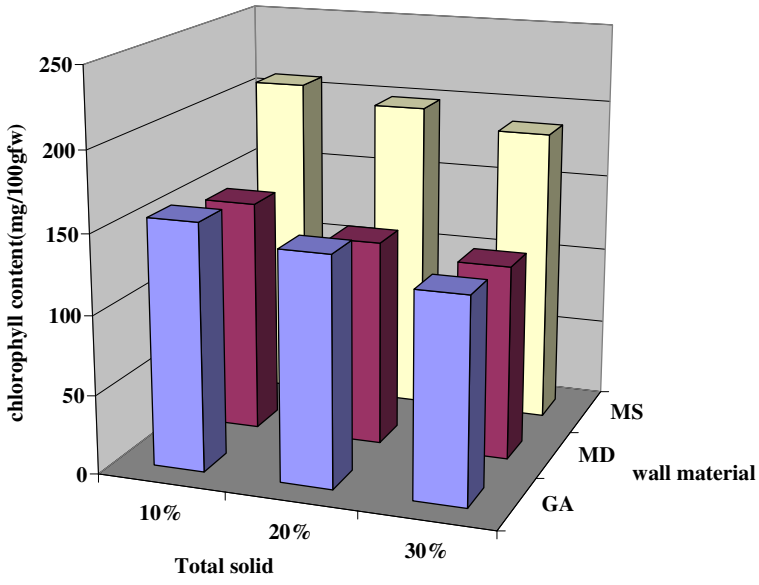


Fig. 4.31 The chlorophyll content of GA, MD, and MS powder at total solid range 10–30% (w/w)

1.5 Greenness of the powder

For greenness of the Zn-chlorophyll derivatives powder derived from different wall materials as shown in Fig. 4.32 can be compared by considering at the same amount of total solid of wall material. It was found that using MS as wall material gave the highest greenness, the second was MD and GA had the lowest one. The greenness decreased when total solid content increased and it was the same trend with the result of chlorophyll content.

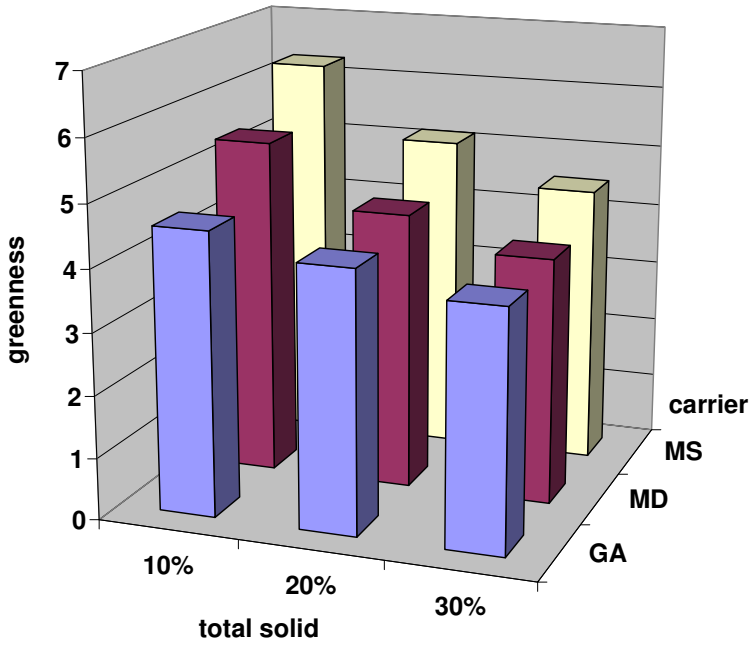


Fig. 4.32 The greenness power of GA, MD, MS powder at total solid range 10-30% (w/w)

1.6 Antioxidant activity

The result of antioxidant activity value was represented in Fig. 4.33. The result showed that the highest antioxidant activities were the MS powder and the lower levels were GA, and MD, respectively. The antioxidant value related with chlorophyll derivatives content, thus the higher chlorophyll derivatives due to higher antioxidant activity (Endo *et al.*, 1985; Cahyana *et al.*, 1993; Hoshina *et al.*,1998 ; Ferruzzi *et al.*,

2002). According to MS and GA had abilities to bind with both hydrophilic and hydrophobic groups of chlorophyll derivatives molecules. Consequently, their antioxidant values were higher than that of the MD which had no emulsifying property, then can bind with low amount of chlorophyll derivatives, leading to low antioxidant activity.

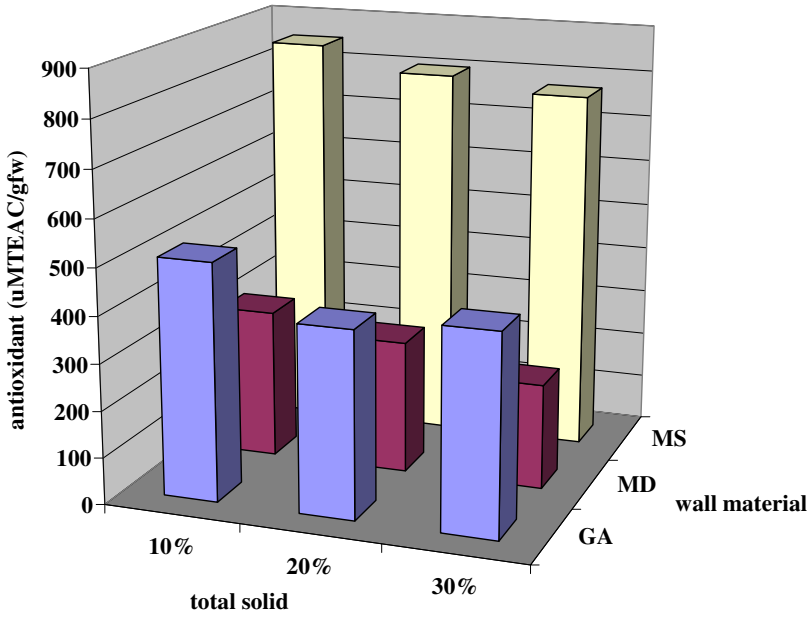


Fig. 4.33 The antioxidant activity of GA, MD, MS powder at total solid range 10–30% (w/w)

1.7 Water activity (a_w)

The a_w values resulted from GA, MD and MS (presented in Fig. 4.34) were in range of 0.19-0.28. At higher total solid contents were leading to more rapid drying and lower a_w value of the encapsulated powder. The suitable a_w values were 0.2-0.3 because many chemical reactions such as oxidation, Maillard reaction and chlorophyll degradation can occur slowly, and no microbial proliferate in this condition (Fenema, 1985). Moreover, the a_w value at less than 0.3 can be considered as ensure of product stability (Drusch and Schwarz, 2006).

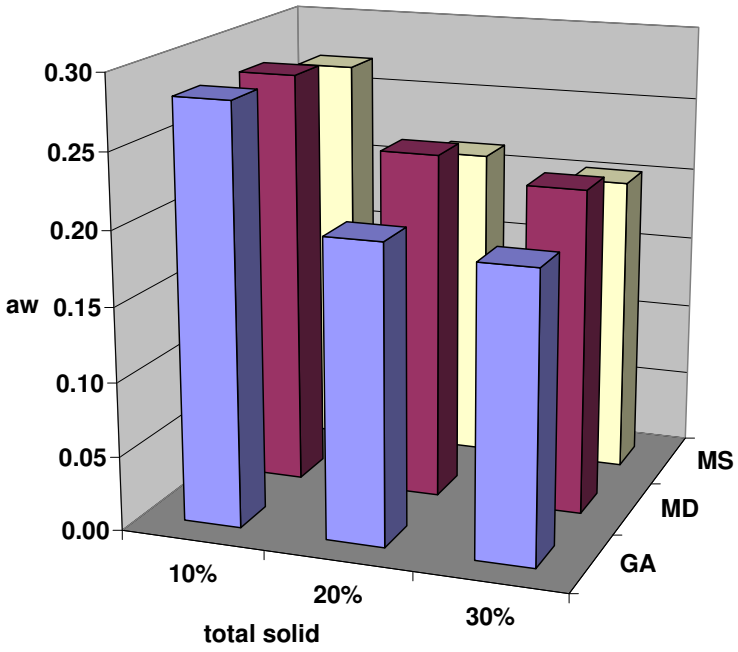


Fig. 4.34 The a_w of GA, MD, MS powder at total solid range 10-30% (w/w)

1.8 % yield

The result of percent yield in Fig. 4.35 indicated that using MD at total solid content of 10-20 % brought about the highest % yield, while GA yielded the lowest. But at 30% total solid content, MS had the lowest viscosity when it was compare between GA and MD. Therefore, this solution can be dried into powder due to the higher yield. This result agrees with Pegg and Shahidi (1999) who reported that *OSA*-MS can be used at higher feed solid level than GA.

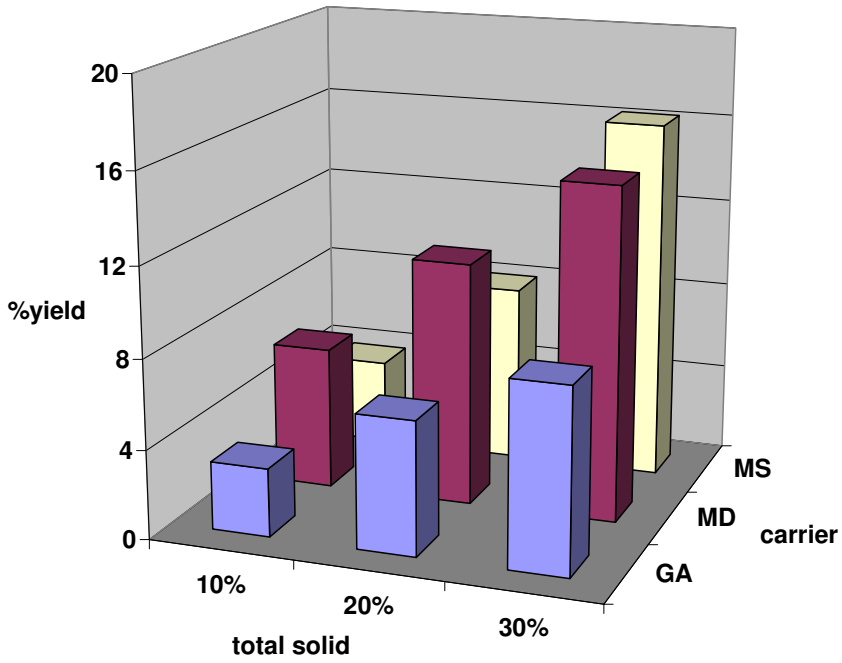


Fig. 4.35 The % yield of GA, MD, MS powder at total solid range 10–30% (w/w)

2) Decay of Zn-chlorophyll derivatives

Decay of Zn-chlorophyll derivatives derived from different wall materials and kept in clear glass bottles at room temperature for 120 days (Fig. 4.36). It was found that Zn-chlorophyll contents of GA, MD and MS wall material were remained as 77.73, 80.62 and 82.73 %, respectively. Regression equation of *ln* (% chlorophyll derivatives retention) against storage time showed the linear relation with negative slope when plotted on a natural logarithmic scale. This decrease of the Zn-chlorophyll derivatives can be concluded as first-order kinetics with rate constant (k) of 2.1×10^{-3}, 1.8×10^{-3} and 1.5×10^{-3} day^{-1} for the GA, MD and MS, respectively. Moreover, the half-life values were 330, 385 and 462 days for GA, MD and MS, respectively. From this experiment results, it was clear that MS was the appropriate wall material which can retain most of Zn-chlorophyll derivatives as shown the longest shelf life.

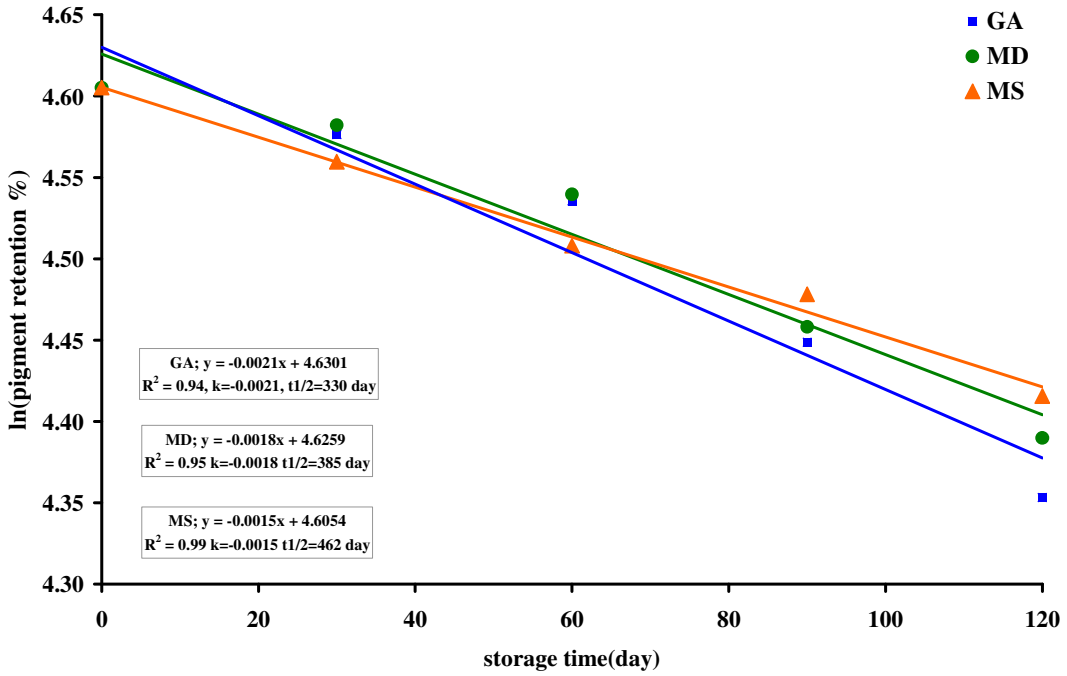


Fig. 4.36 The decay of Zn-chlorophyll derivatives content in different type of wall materials.

3) The suitable wall material for spray dried Zn-chlorophyll powder.

From the above results, comparison the encapsulation properties between GA, MD and MS in Zn-chlorophyll derivatives powder production from pandan leaf. It can be concluded that using MS as wall material brought about the highest greenness and chlorophyll derivatives content. The MS molecules had both hydrophobic and hydrophilic parts; amphiphilic character (Baranauskiene *et al*., 2007; Viswanathan, 1999) which can bind with chlorophyll derivatives molecules in both porphyrin (hydrophilic) and phytol (hydrophobic). According to MS had high chlorophyll content, it had also high antioxidant activity. The a_w values of chlorophyll derivatives powder from MS, GA and MD wall materials were in range of 0.28-0.30 which was the suitable range that many chemical reactions such as oxidation and chlorophyll degradation and no microbial proliferation. For considering of particle size, it was found that MS had the smallest size which affected to high bulk density

because they can be packed more densely (Reineccius, 2001), leading to reduction of oxygen transmission and oxidation (Buffo and Reineccius, 2000). Moreover, SEM micrograph can confirm the result of MS that it was a suitable wall material for conservation the green color of chlorophyll derivatives. Due to its spherical shape with smooth surface, the ratio between surface and volume was the highest (Desobry, Netto and Labuza, 1997) and this shape can retain chlorophyll derivatives mostly. Furthermore, MS had low viscosity at high concentration which was good property that can use it to apply in high total solid content (Baranauskiene *et al*., 2007). Therefore, using MS as encapsulating agent at 30% total solid was suitable from Zn-chlorophyll derivatives powder production process.

PART 6: Characteristic of Zn-chlorophyll derivatives powder

1) Analysis of Zn-chlorophyll derivatives powder properties

The chemical and physical properties of Zn-chlorophyll derivatives powder showed in Table 4.5. Zn-chlorophyll derivatives powder had greenness color, chlorophyll derivatives content of 187.34 mg/100g fw and antioxidant activity of 772.50 μMTE/gfw. When compared with fresh pandan leaf, extract powder had higher in both chlorophyll derivatives and antioxidant activity. Moreover, zinc content in pandan leaf powder was 13.12 mg/kg which was not excess limitation of FDA that allowed having zinc in product not more than 75 mg/kg.

Table 4.5 Physicochemical properties of pandan powder at 30% (w/w) of wall material

Physicochemical properties	OSA-MS
Color value	
Lightness(L*value)	52.90 ± 4.13
Greenness(-a*value)	-14.64 ± 1.54
Yellowness (b*value)	20.16 ± 2.16
Chroma(*C*)	23.50 ± 1.11
Hue (h^o)	130.17 ± 1.94
Chlorophyll content(mg/100gfw)	187.34 ± 2.19
pH	7.04 ± 0.03
a_w	0.28 ± 0.02
Moisture content	9.27 ± 0.74
Bulk density	0.55 ± 0.01
Antioxidant activity(μMTE/gfw)	772.50 ± 4.32
D(0.5)	16.13 ± 1.36
Zinc content (mg/kg)	13.12 ± 2.76

2) Flavor analysis in pandan leaf powder

Chlorophyll powder comprises 21 types of flavoring agents (Fig. 4.37, Table 4.5): two furan (2-ethyl furan and 2-pentyl furan), three ketones (1-penten-3-one, 2-heptanone and 4-(2,6,6-trimethyl-2-cyclohexan-1-ol), seven aldehydes (hexanal, (*E*)-2-pentenal, heptanal, 2-Hexenal ,4-heptenal, nonanal ,2-octenal,2-nonenal and (*E,Z*) 2,6-nonadienal), six alcohol(2-penten-1-ol, 1-hexanol, 3-hexen-1-ol ,2-hexenol, 1-octen-3-ol, and 2-ethyl hexanol), and a nitrogeneous (2-acetyl-1-pyrroline).

Comparisons between fresh pandan leaf and Zn-chlorophyll derivative powders showed that Zn-chlorophyll derivative powder had seven flavoring compounds more higher than the fresh pandan leaf. These compounds were (1) 2-Ethyl furan (sweet and burnt flavor), (2) 2-heptanone (soap flavor), (3) nonanal (fat and green flavor), (4) 2-hexenol (green leaf flavor), (5) 1-octen-3-ol (green flavor), (6) 2-ethyl hexanol (green flavor) and (7) 4-(2,6,6-trimethyl-2-cyclohexan-1-ol)-3buten-2-one (wood flavor). These flavoring agents may derive from heat process like

metallo-chlorophyll formation and spray drying which can cause some flavors like burnt, fat, soap and wood. When considered flavor of 2-AP, it was found that Zn-chlorophyll derivative powder from pandan leaf had this flavor a little bit less than fresh leaf because 2-AP compound was not stable and can be degraded rapidly, subsequently its concentration decreased after process.

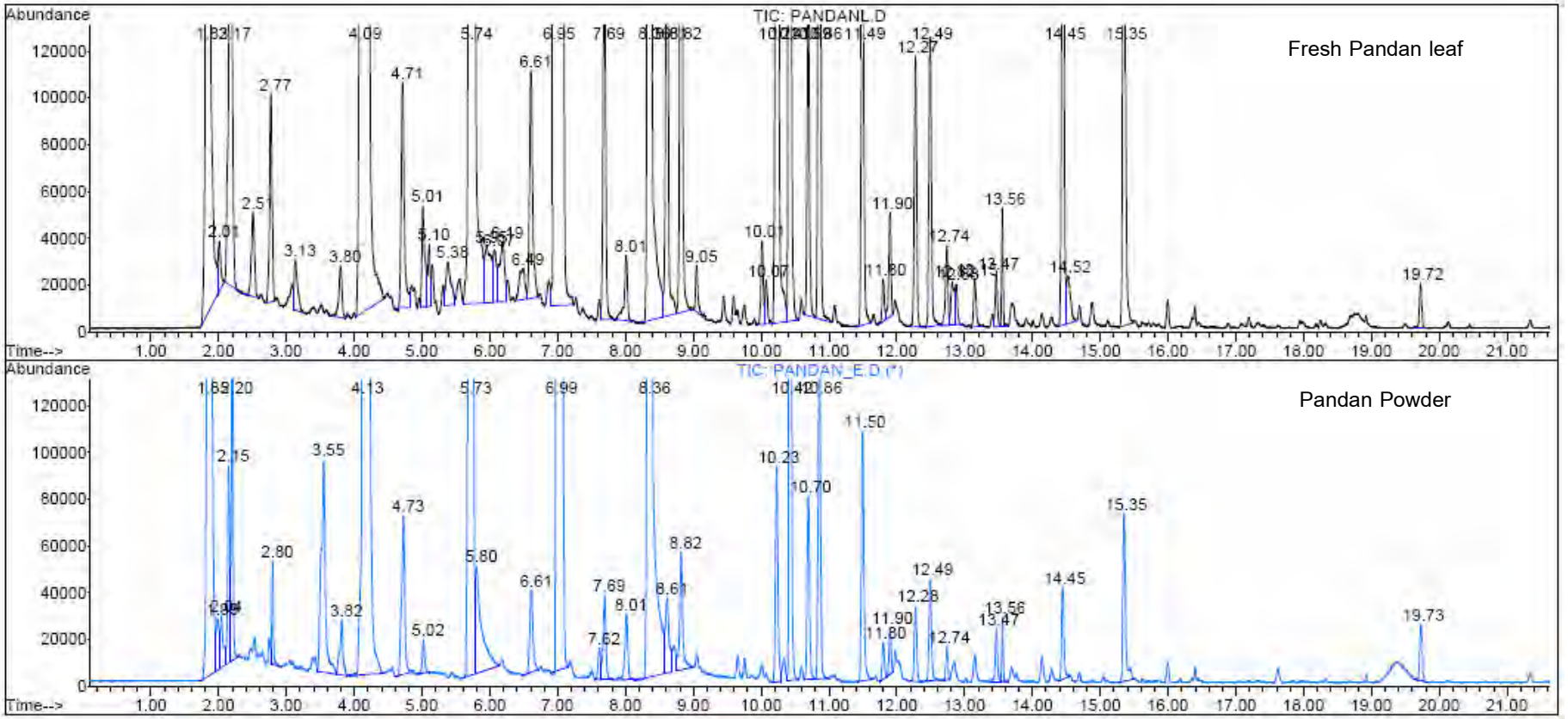


Fig. 4.37 The GC-MS chromatograms of (A) fresh pandan leaf and (B) chlorophyll pandan powder.

Table 4.6 Flavor compounds detected in the Zn-chlorophyll powder by SPME-GC-MS.

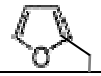
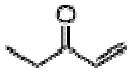
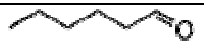
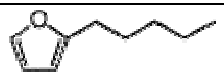
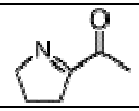
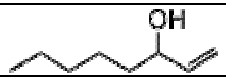
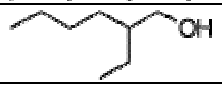
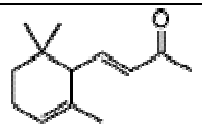
no	structure	group	RI	CAS	flavor	description	retention time	fresh (%)	powder (%)
1	2-ethyl furan	furan	133.41	3208-16-0		sweet, burnt, earthy, malts	3.81	-	0.52
2	1-penten-3-one	ketone	245.20	1629-58-9		fish, pungent	4.73	1.26	1.37
3	hexanal	aldehyde	1083.65	66-25-1		grass, green, woody, green	5.73	41.39	48.27
4	(*E*)-2-pentenal	aldehyde	1131.82	1576-87-0		strawberry, fruit, tomato	6.61	1.39	0.74
5	2-heptanone	ketone	1184.34	110-43-0		soap	7.62	-	0.24
6	heptanal	aldehyde	1187.98	111-71-7		green, fresh green, sweet, herbal, rancid	7.69	2.37	0.69
7	2-hexenal	aldehyde	1221.54	6728-26-3		fresh green, fruity , apple	8.01	19.10	34.46
8	2-penty-furan	furan	1234.60	3777-69-3		green bean, butter	8.61	2.78	0.94
9	4-heptenal	aldehyde	1245.15	6728-31-0		biscuit, cream	8.82	3.71	1.09

Table 4.6 Flavor compounds detected in the Zn-chlorophyll powder by SPME-GC-MS. (continue)

no	structure	group	RI	CAS	flavor	description	Retention time	Fresh (%)	Powder (%)
10	2-penten-1-ol	alcohols	131.32	1576-95-0		green, plastic, rubber	10.23	10.15	1.62
11	2-acetyl-1-pyrrolline	nitrogeneous compounds	1339.93	85213-22-5		nut, roast, pandan	10.42	1.64	1.33
12	1-hexanol	alcohols	1348.66	111-27-3		resin,flower,green	10.86	4.32	2.52
13	3-hexen-1-ol	alcohols	1381.51	928-96-1		fresh green, cut grass	11.50	2.38	1.84
14	nonanal	aldehyde	1396.92	124-19-6		fat, citrus, green	11.80	-	0.19
15	2-hexenol	alcohols	1402.14	928-95-0		green, leaf, walnut	11.90	-	0.28
16	2-octenal	aldehyde	1433.69	2363-89-5		green	12.49	1.49	0.74
17	1-octen-3-ol	alcohols	1447.06	53907-72-5		mushroom, green coffee	12.74	-	0.20
18	2-ethyl hexanol	alcohols	1486.09	104-76-7		rose, green	13.47	-	0.36
19	2-nonenal	aldehyde	1540.38	18829-56-6		cucumber, fat, green	14.45	3.49	0.75
20	(E,Z) 2,6-nonadienal	aldehyde	1590.85	557-48-2		cucumber, wax, green	15.35	4.47	1.36
21	4-(2,6,6-trimethyl-2-cyclohexan-1-ol)-3buten-2-one	ketone	1840.22	127-41-3		wood, violet	19.73	-	0.43

3) The stability of as affected by pH condition

Zn-chlorophyll derivatives powder made from pandan leaf was objected to be stable in heat and acidic condition which was advantageous to apply as natural green colorant. Consequently, this research included study on product characteristics and effects of pH and temperature on stability compared to fresh pandan leaf extract.

The stabilities of fresh pandan leaf extract and Zn-chlorophyll derivative powder derived from this experiment were affected under acidic/basic conditions as shown in Fig. 4.38. Fresh leaf extract had brown to olive green color, while still was green in alkali condition. For Zn-chlorophyll derivative powder, pH not had affect color change. Therefore, it can be concluded that Zn-chlorophyll derivative powder was stable in pH range wider than fresh extract. This Zn-chlorophyll derivative powder was advantageous for application as colorant in acid containing food without color change.

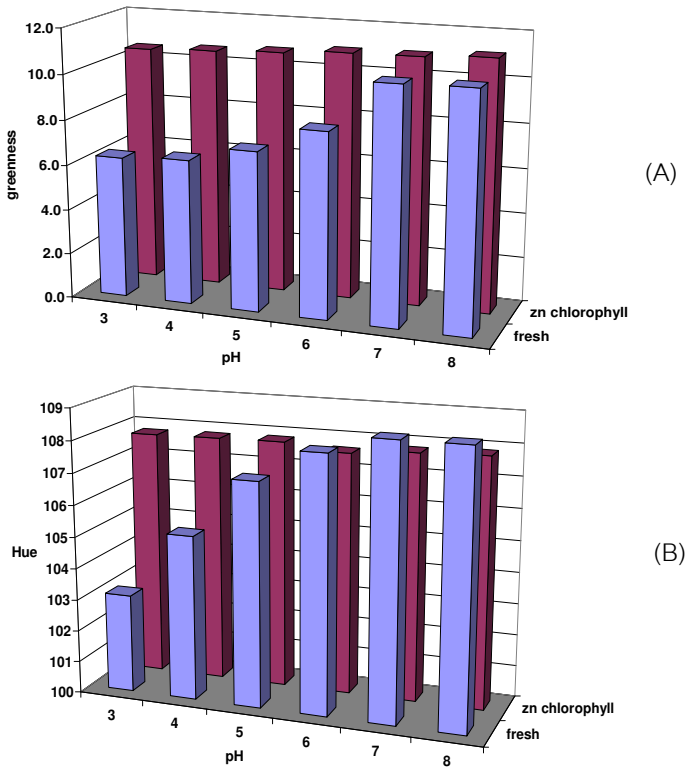


Figure 4.38 Comparison of green color (A) and Hue (B) of the fresh pandan leaf extract ( ), and Zn-chlorophyll powder ( ) at pH 3-8.

4) Comparison of product stability in heating temperature

Comparison of product stability at heating temperature range of 70–100 ^{o}C and incubation period for 0–30 min (Fig 4.39), it was found that fresh pandan leaf extract have been changed its green color to brownish green within 5 min of heating and turned to more brown when heated in longer time. For the result of Zn-chlorophyll derivative extract derived from this experiment at 70–100 ^{o}C for 0–30 min, the green color was still not changed. It can be implied that the obtained powder can be resisted in high temperature condition.

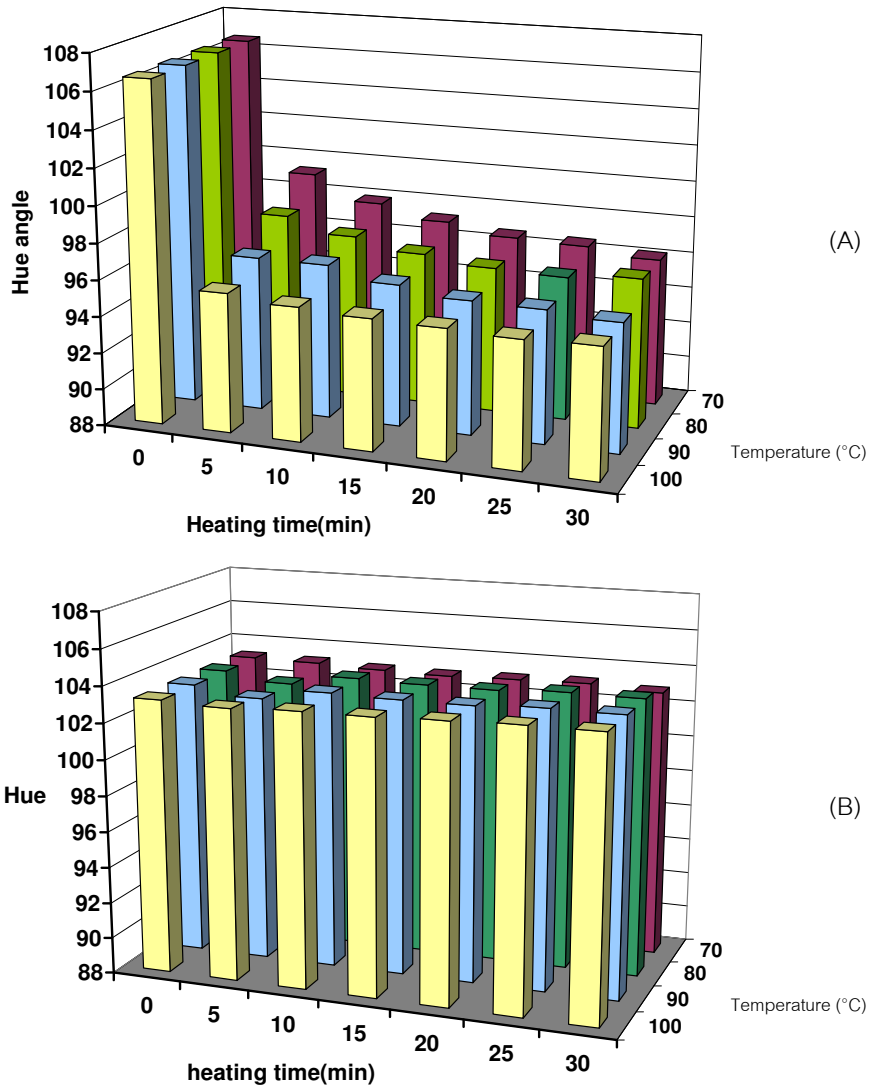


Figure 4.39 Comparison of hue of the fresh pandan leaf extract (A) and Zn-chlorophyll powder (B) when heating temperature 70 –100 ^{o}C for 0 –30 min.

5) Conclusion

From the data obtained it was found that pandan leaf is a good source of the chlorophyll. However, chlorophyll in the natural form is not stable. It becomes extremely labile by factor such as pH and temperature. Chlorophyll degraded into the derivatives with olive brown degradation product. More stable form of chlorophyll produced though metallo-chlorophyll complexes by the reaction of chlorophyll compounds in fresh pandan leaves with the divalent cations. This study found that the optimal conditions for Zn-chlorophyll complexes formation were 300 ppm zinc chloride at pH 5 and 110 ^{o}C for 15 min. The obtained chlorophyll derivatives were Zn-pheophytin *a, b* and Zn-pyropheophytin *a, b.* This product has a more stable green color and has higher amounts of antioxidants. Subsequently, hydrolytic enzyme is used to extract the derivatives. The commercial enzyme pectinase (Pectinex® Ultra SP-L) assisted in the extraction process and an experimental design using response surface methodology (RSM) was further optimized for the highest yield. The optimum conditions were 2.3–2.5% v/w pectinase, 240–260 min incubation time, and extraction repeated twice. Under the optimum conditions, the extraction yields of green chlorophyll and antioxidant values were increased 17.31 and 1.92 fold, respectively, over non-enzymatic sample. From Zn-chlorophyll derivative powder production by spray drying method it was found that the process with 30% (w/w) OSA-modified starch was suitable for chlorophyll powder production. Under this optimum condition was obtained the highest of greenness color, Zn-chlorophyll derivative content, and antioxidant activity in the powder. The outcome has a natural green color with higher stability and is also safe because there are no solvents used during extraction which can bring about toxic residues. The process studied could be an alternative for the production of natural green colorant in the food industry.

CHAPTER V

CONCLUSION

PART 1: Pandan leaf compositions

The composition analysis of pandan leaves, this plant had the chlorophyll *a, b* and total chlorophyll contents as 66.15, 42.01 and 142.22 mg/100g fw, respectively. The radical scavenging activity was 319.87 ± 4.23 μM TE/g fresh mass, and *L*, a*, b*, C* and h^0 values were 36.11, -11.65, 15.14, 22.80 and 127.26, respectively. The pH value was 5.4±0.5, total dietary fiber content was 8.09 g/100g fw, and zinc content was 2.70 mg/kg. The hexanal was the main flavor compound found in pandan leaf which had smell like greeny grass. The odor characteristic found in the pandan leaf had the smell like greeny, fresh greeny or freshly cut green grass. Moreover, the pandan leaf was composed the flavor component of 2-acetyl-1-pyrrolline responsibility for pandan-like aroma.

PART 2: Factors effecting the color change of pandan leaf extract

The pH and temperature had significantly effect on color value. The non-stability of pandan leaf extract at acid condition and high temperature which caused alteration of color from green to olives green to brown rapidly. From this limitation, it was necessary to form the stable derivative before the application in food processed with heating and acidic conditions.

PART 3: Formation of metallochlorophyll complexes

The formation of a stable chlorophyll molecule can be managed by replacing the magnesium ion in the porphyrin ring with divalent cations such as zinc. The optimal conditions for the formation of Zn-chlorophyll derivatives to form a stable color and improve antioxidant activity in the pandan leaf occurred when a 300 ppm

concentration of $ZnCl_2$ was used at a pH of 5 and was heated at 110 °C for 15 min. At these conditions green color derivatives of Zn-pheophytin *a,* Zn-pheophytin *b*, Zn-pyopheophytin *a*, and Zn-pyopheophytin *b* could be detected. These derivatives were stable in green color and were suitable to be extracted for use as a green colorant.

PART 4: Optimum conditions in enzymatic extraction process

Comparisons of enzymatic and non-enzymatic extraction of Zn-chlorophyll from pandan leaf found that enzymatic extraction resulted in higher green color values, increased amounts chlorophyll pigment, and increased the antioxidant activity values. The range of green color values, amount of chlorophyll pigment, and the amount of antioxidant increased 2.58-3.43, 5.82-19.00, and 1.91-1.95 fold, respectively, over non-enzymatic extraction.

The optimal conditions for Pectinex® Ultra SP-L in the extraction of chlorophyll derivative from the pandan leaf were a 2.3-2.5% enzyme concentration, 240-260 min of incubation time, and two times of extraction at 35 °C. At these optimum conditions, the obtained extract had a green color value of -10.36, chlorophyll content of 17.82 mg/g fw, and antioxidant activity of 612.89 μMTE/g fw. This was different from non-enzymatic extraction sample that resulted in a -3.46 green color value, 1.03 mg/g fw of chlorophyll content, and 314.88 μMTE/g fw of antioxidant activity. Therefore, enzyme treatment yielded greenness, chlorophyll content, and antioxidant of 3.00, 17.31 and 1.916 fold more than control, respectively.

PART 5: Zn-chlorophyll derivative encapsulated powders

Zn-chlorophyll powder production by spray drying method, the effects of gum arabic (GA), maltodextrin (MD) and OSA-modified starch (MS) on physicochemical properties and the stability of Zn-chlorophyll derivative powder showed that using MS as wall material can bring about spherical particle shape with smooth surface, while

GA and MS caused particles with densely surface. Average particle sizes of Zn-chlorophyll derivatives powder products when used GA, MD and MS as carriers were 34.46, 30.11 and 16.13 μm, respectively. MS had small particle size which can be compacted tightly and hence had high bulk density value. This reduced the oxygen permeability which contributed to decrease in the oxidation reaction. The a_w values of all chlorophyll powder products were in range of 0.19-0.23 which was the value that had low oxidation. The process with 30% (w/w) MS was suitable for chlorophyll powder production. Decay of Zn-chlorophyll derivative powders derived from different wall materials were first-order kinetics with rate constant (k) of 2.1×10^{-3}, 1.8×10^{-3} and 1.5×10^{-3} day^{-1} for the GA, MD and MS, respectively and the half-life value of 330, 385 and 462 days for GA, MD and MS, respectively.

PART 6: Characteristic of Zn-chlorophyll derivative powder

Zn-chlorophyll derivative powder had greenness color, chlorophyll content of 187.34 mg/100g fw and antioxidant activity 772.50 μMTE/gfw. When compared with fresh pandan leaf, extract powder had higher in both chlorophyll and antioxidant content. Moreover, zinc content in pandan leaf powder was 13.12 mg/kg which was not excess limitation of FDA that allowed having zinc in product not more than 75 mg/kg. The obtained chlorophyll powder had 2-AP compound which was characteristic flavor of pandan leaf. The stability test of chlorophyll powder showed that it can be unchanged under wide range of pH and heating process, thus it can be applied as green colorant in food product.

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APPENDICES

APPENDIX A

MATERIALS

1. Enzyme source

Pectinex Ultra® SP-L, obtained from East Asiatic (Novozymes, Thailand), was used for enzymatic treatment for pandan leaf extract and stored at 4 °C. Pectinex Ultra SP®-L is a commercial enzyme preparation from *Aspergillus aculeatus* used in the food industry for fruit juice processing to reduce viscosity. It contains different pectinolytic and cellulolytic enzymes [endo-polygalacturonase (EC 3.2.1.15; C.A.S. No. 9032-75-1), endopectinylase (EC 4.2.2.10; C.A.S. No. 9033-35-6) and pectin esterase (EC 3.1.1.11; C.A.S. No. 9025-98-3)], and other activities, such as beta-galactosidase, chitinase and transgalactosidase (Iraj *et al*., 2005). The activity of pectinex ultra SP-L enzyme is 10292 PGU per mL (polygalacturonase activity per mL). The optimum enzyme reaction conditions are at pH 3.5–6.0 and temperature range below 50 °C (Kashyap, *et al*., 2001).

Celluclast®1.5L, obtained from East Asiatic (Novozymes; Thailand), is a liquid cellulase preparation produced by submerge of a selected strain of fungus *Trichoderma reesei*. The enzyme catalyzes the breakdown of cellulose into glucose, cellobiose, and higher glucose polymers. The activity is 700 EGU/g (EGU=Endo-Glucanase Units). The optimum conditions are about 50-60 °C and a pH of 4.5-6.0.

METHODS

1) Determination of chlorophylls

Measurement of chlorophyll contents by Vernon method (1960): 5 g of pandan leaves were accurately weighed, then extracted with 20 mL of 80% acetone, mixed by homogenizer, dejuiced, centrifuged at 8,000 rpm for 15 min, filtered through *Whatman* no. 1 and 42 filter papers, adjusted to 25 mL in a volumetric flask and absorbance values were measured at 663nm and 645 nm.

$$Chlorophyll\ total\ (mg/g\ fw) = 20.2\ A_{663} + 8.02\ A_{645} \times Dilution\ factor / 1000$$

2) Assessment of Trolox equivalent antioxidant capacity (TEAC)

Evaluation of scavenging properties using long-lived $ABTS^{\cdot+}$ radical was performal by using the method of Thaipong *et al.* (2006). A 7.4 mM of $ABTS^{\cdot+}$ solution was used to react with 2.6 mM of potassium persulfate solution in a ratio 1:1, then allowing the solutions to react and form free radicals for 12 h at room temperature in the dark (the output chemical should be used within 4 h). Then 1 mL of $ABTS^{\cdot+}$ solution was mixed with 60 mL of methanol to obtain an absorbance of 1.1± 0.2 units at 734 nm ($ABTS^{\cdot+}$ should be prepared everyday). Subsequently, pandan leaf extracts (150 μL) were allowed to react with 2850 μL of the $ABTS^{\cdot+}$ solution for 2 h in a dark condition and absorbance value measured at 734 nm. The standard curve was prepared by using Trolox as a standard substance in a concentration range of 25 – 600 μM and results were expressed in μM Trolox equivalent (TE)/g fresh mass.

3) Determination of color value

Minolta colorimeter (Minolta Spectrophotometer CR300 and CT310) was used to determine *L*, a*, b**, chroma (*C*), and Hue (h^o) values in which *L** is the lightness of color (100 = white, 0 = black), *a** value (+*a** = red, -*a** = green), *b** value (+*b** = yellow, -*b** = blue) , $C= ((-a^*)^2 + (b^*)^2)^{1/2}$ and h^o $(180+ \tan^{-1}(b^*/-a^*))$.

4) Thin layer chromatography (TLC)

Separation of chlorophyll derivatives by means of Guzmán *et al*. (2002): 10 g of pandan leaves were extracted with 20 mL of 80% acetone, filtered into a separation funnel, and added with ethyl ether and dried with anhydrous sodium sulphate. Then separation of the pigments were done by using thin layer chromatography (TLC) using Kiesegel $60F_{254}$ plate 0.5 mm thick (Merck) and hexane/ethyl ether/acetone (6:2:3 v/v/v) as solvent. Identification of chlorophyll and its derivatives was processed by

using RF measurement (distance of component migration/distance of solvent migration) and detection of absorption spectra was recorded from 400 to 700 nm.

5) High performance Liquid Chromatography (HPLC)

Pigment Extraction. The following a modified method described by Schwartz and von Elbe (1983). The chlorophylls were extracted by adding 20 mL of acetone to 5 g of puree and blending with a homogenizer for 2 min. The mixture was then filtered under vacuum through *Whatman No. 42* filter paper (Fisher Scientific, Springfield, NJ). An additional 5 mL of acetone was used to wash the filter paper. The filtrate was transferred to a 50 mL volumetric flask and brought to volume with acetone. Prior to injection into the HPLC column, the sample was filtered through 0.22 μm filter paper (Fisher Scientific, Springfield, NJ).

Separation of chlorophyll derivatives was achieved by the Phenomenex, 5 μm particles 4.6 mm i.d. × 250 mm (VARIAN, Prostar). The solvent system used was ethyl acetate/methanol/water with the following proportions: solvent A, 15/65/20, and solvent B, 60/30/10 (v/v/v). Initial conditions consisted of 100% solvent A at a flow rate of 1.3 mL/minute. The gradient was applied after 6 minute under isocratic conditions. Final conditions consisted of 100% solvent B at a flow rate of 1.5 mL/ minute. After 10 min, the solvent composition was returned to the initial proportions. The absorption spectra were measured at 658 nm.

6) SEM micrograph

The outer structural features of the dry powder were studied by scanning electron microscopy (SEM). A JSM-5410 LV scanning electron microscope (JSM-5410, Jeol., Ltd., Tokyo, Japan) was used to investigate the outer microstructural properties of spray-dried microencapsulated products. Microencapsulated specimens were loaded onto a specimen stub, coated with gold with a ion sputter coater (Balzers

Union SCD 040, Balzers, Liechtehstein). Examinations were made at 500x, and 3000x magnifications.

7) Determine the reducing sugar by DNSA method (Miller, 1959)

The pandan pulp in each condition were collected and filtrated through Whatman No 1. For assay of pandan pulp extracted (1 mL) were react with DNSA reagent. DNSA reagent (1 mL) was added to stop the reaction, the mixture was heated for 10 min in a boiling water bath, distilled water (9 mL) was added and Ab_{530} read in a UV160A spectrophotometer. Standard curves were prepared from glucose ranged of 0-1000 mg/mL and results were expressed in g glucose/g fresh weight.

8) Determine the relative activity

Standard activity of the enzyme was determined by the reduction of the viscosity of pectin solution at pH 3.5 and 35 $^{\circ}$C. The pectin solution had 40% sucrose solution and Mcllvaine buffer at pH 3.5. The pectin solution was heat treated in order to obtain the gel form. 1% of pandan pulp in each time was added to pectin solution and incubate for 30 min at 35°C and stop reaction by heating for 10 min in a boiling water bath. Then, viscosity reduce were determined. The viscosity measurements were carried out using a Brookfield Viscometer (Model DV-I). 100 rpm was chosen as the working spindle speed.

9) Determine the particle size distribution

A laser diffraction-based Malvern particle size analyzer Mastersizer 2000 (Malvern Instruments Inc., UK) was used for determination of powder particle diameter. The Malvern Mastersizer S, which is considered a spatial sampling device, utilizes the phenomena that a powder particle when it falls through the laser beam causes laser light to scatter through an angle dependent on the diameter of the particle. Particle characteristics were computed automatically from a compressed range.

Particle size measurement tests were replicated four times. The area-weighted mean diameter d_{32} used as the particle size distribution parameter. Specific surface area (m^2/g) was taken into account as well.

10) Flavor analysis

10.1 Headspace solid phase microextraction: Volatile flavors in headspace of pandan leaf extract and encapsulated powder removed were extracted using solid phase microextraction (SPME) fiber assembly (Supelco, Bellefonte,PA). SPME was performed with fibers of Divinyl benzene-caroson-polydimethyl silosane. The fiber was exposed the headspace of flavors for 20 min at ambient temperature. Afterwards the fiber was withdrawn into the housing, the SPME device was removed from the sample vial, and the fiber was desorbed into the GC–MS injector.

10.2 Gas chromatography–mass spectrometry: Gas chromatography/ mass spectrometry condition: An Agilent 6890 GC equipped with an Agilent 5973 mass-selective detector (Agilent Technologies) was used, with the injector and detector maintained at 200 and 250 °C, respectively. The column (HP-Innowax) dimensions were 0.25 mm i.d. × 30 m × 0.25 μm film thickness. The carrier gas (Helium) had a flow rate of 5.0 mL/min. The temperature program was isothermal at 50 °C for 10 min, increase to 170 °C at 15°C/ min, then held for 10 min. The mass spectrometer was operated in the electron impact (EI) mode with an electron energy of 70 eV; ion source and quadrupole maintained at 230 and 150 °C, respectively; mass range m/z 10-350. Compounds were identified by matching mass spectra (quality match >80%) and retention indices with the Chemstation Wiley Spectral Library of standard compounds. Moreover, the standard Alkane C1-C19 was using for determined the retention indices (R.I).

APPENDIX B

The statistical analyze data

Table 1 The color values of fresh pandan leaf extract at pH 3.0–8.0

Day	pH	L*value	a*value	b* value	Chroma	Hue
0	3.0	40.86 ± 0.03^{c}	-9.00 ± 0.05 ^{a}	45.47 ± 0.02 ^{e}	46.35 ±0.21^{e}	101.19 ± 0.06 ^{f}
	4.0	37.69 ± 0.07^{e}	-12.21 ± 0.07 ^{b}	45.51 ± 0.15 ^{e}	47.12 ± 0.32^{e}	105.04 ± 0.07 ^{e}
	5.0	39.22 ± 0.65^{d}	-14.41 ± 0.03 ^{c}	48.05 ± 0.22 ^{ba}	50.16 ± 0.42^{b}	106.69 ± 0.06 ^{d}
	5.4	35.33 ± 0.03^{f}	-14.55 ± 0.02 ^{d}	47.42 ± 0.03 ^{d}	49.60 ± 0.80^{d}	107.06 ± 0.01 ^{c}
	6.0	37.68 ± 0.09^{e}	-14.62 ± 0.02 ^{e}	47.77 ± 0.13 ^{c}	49.96 ± 0.41^{c}	107.01 ± 0.07 ^{c}
	7.0	42.27 ± 0.02^{b}	-14.88 ± 0.01 ^{f}	48.22 ± 0.01 ^{a}	50.46 ± 0.77^{a}	107.15 ± 0.01 ^{b}
	8.0	54.23 ± 0.05^{a}	-14.90 ± 0.03 ^{f}	47.91 ± 0.06 ^{bc}	50.17 ± 0.53^{b}	107.27 ± 0.03 ^{a}
7	3.0	42.29 ± 0.05 ^{c}	-8.53 ± 0.01 ^{a}	43.35 ± 0.03 ^{f}	44.18 ±0.23^{f}	101.13 ± 0.06 ^{f}
	4.0	38.45 ± 0.02 ^{e}	-10.74 ± 0.01 ^{b}	41.21 ± 0.04 ^{g}	42.59 ±0.35^{g}	104.48 ± 0.03 ^{e}
	5.0	39.84 ± 0.01 ^{d}	-13.28 ± 0.01 ^{c}	44.67 ± 0.02 ^{e}	46.60 ±0.53^{e}	106.50 ± 0.00 ^{d}
	5.4	34.46 ± 0.13 ^{g}	-14.45 ± 0.01 ^{d}	45.62 ± 0.23 ^{d}	47.85 ±0.34^{d}	107.61 ± 0.11 ^{c}
	6.0	37.58 ± 0.01 ^{f}	-14.89 ± 0.01 ^{e}	47.34 ± 0.02 ^{c}	49.63 ±0.84^{c}	107.37 ± 0.06 ^{ba}
	7.0	43.16 ± 0.01 ^{b}	-15.33 ± 0.02 ^{g}	48.48 ± 0.02 ^{a}	50.85 ±0.65^{a}	107.51 ± 0.02 ^{b}
	8.0	56.60 ± 0.06 ^{a}	-15.25 ± 0.02 ^{f}	47.75 ± 0.02 ^{b}	50.13 ±0.07^{b}	107.64 ± 0.10 ^{a}
14	3.0	42.45 ± 0.01 ^{c}	-7.88 ± 0.01 ^{a}	42.02 ± 0.06 ^{f}	42.75 ±0.34^{f}	100.57 ± 0.06 ^{f}
	4.0	37.58 ± 0.01 ^{e}	-10.36 ± 0.01 ^{b}	40.56 ± 0.05 ^{g}	41.86 ±0.94^{g}	104.23 ± 0.06 ^{e}
	5.0	40.69 ± 0.02 ^{d}	-13.08 ± 0.02 ^{c}	44.36 ± 0.06 ^{e}	46.25 ±0.23^{e}	106.35 ± 0.05 ^{d}
	5.4	34.16 ± 0.01 ^{f}	-14.21 ± 0.03 ^{d}	44.85 ± 0.01 ^{c}	47.05 ±0.52^{c}	107.57 ± 0.06 ^{b}
	6.0	31.56 ± 0.01 ^{g}	-14.61 ± 0.01 ^{e}	44.73 ± 0.05 ^{d}	47.06 ±0.23^{d}	108.02 ± 0.02 ^{a}
	7.0	44.79 ± 0.04 ^{b}	-14.61 ± 0.02 ^{e}	46.43 ± 0.02 ^{b}	48.67 ±0.36^{b}	107.45 ± 0.05 ^{c}
	8.0	55.56 ± 0.01 ^{a}	-14.88 ± 0.02 ^{f}	47.11 ± 0.03 ^{a}	49.40 ±0.45^{a}	107.49 ± 0.04 ^{ba}
21	3.0	40.11 ± 0.01 ^{c}	-8.35 ± 0.02 ^{a}	43.32 ± 0.09 ^{f}	44.12 ±0.22^{f}	100.73 ± 0.06 ^{f}
	4.0	40.08 ± 0.03 ^{d}	-10.42 ± 0.03 ^{b}	40.74 ± 0.06 ^{g}	42.05 ±0.32^{g}	104.23 ± 0.06 ^{e}
	5.0	39.98 ± 0.01 ^{e}	-12.78 ± 0.01 ^{c}	44.29 ± 0.04 ^{e}	46.10 ±0.54^{e}	106.10 ± 0.00 ^{d}
	5.4	36.46 ± 0.02 ^{f}	-13.93 ± 0.03 ^{d}	45.25 ± 0.02 ^{d}	47.35 ±0.34^{d}	106.97 ± 0.06 ^{c}
	6.0	42.57 ± 0.02 ^{b}	-14.40 ± 0.02 ^{e}	46.78 ± 0.03 ^{c}	48.95 ± 0.66^{c}	107.03 ± 0.06 ^{c}
	7.0	42.57 ± 0.01 ^{b}	-15.11 ± 0.02 ^{f}	47.85 ± 0.01 ^{b}	50.18 ± 0.45^{b}	107.40 ± 0.00 ^{b}
	8.0	54.44 ± 0.01 ^{a}	-15.64 ± 0.01 ^{g}	48.75 ± 0.02^{a}	51.20 ±0.55^{a}	107.70 ± 0.00 ^{a}

Table 2 The color values of fresh pandan leaf extract when heating temperature 70 –100 °C for 0 –30 min.

Temp (°C)	Time (min)	L*value	a* value	b*value	chroma	Hue
70	0	61.49 ± 0.40^{a}	-13.60 ± 0.06^{a}	45.29 ±0.19^{a}	47.46 ±0.24^{a}	106.63 ± 0.15^{a}
	5	58.49 ± 0.20^{c}	-6.35 ± 0.04^{b}	38.54 ±0.03^{b}	39.27 ±0.04^{b}	99.25 ± 0.06^{b}
	10	58.34 ± 0.17^{c}	-5.34 ± 0.03^{c}	37.68 ±0.04^{c}	38.13 ±0.03^{c}	97.90 ± 0.08^{c}
	15	58.51 ± 0.58^{c}	-4.71 ± 0.02^{d}	36.94 ±0.11^{d}	37.25 ±0.06^{d}	97.20 ± 0.00^{d}
	20	59.24 ± 0.25^{b}	-4.32 ± 0.05^{e}	36.42 ±0.06^{e}	36.70 ±0.01^{e}	96.65 ± 0.13^{e}
	25	58.45 ± 0.09^{c}	-4.19 ± 0.02^{f}	36.80 ±0.07^{d}	36.80 ±0.08^{e}	96.55 ± 0.17^{e}
	30	59.75 ± 0.62^{b}	-3.87 ± 0.01^{g}	35.79 ±0.18^{f}	36.08 ±0.23^{f}	96.18 ± 0.10^{f}
80	0	61.49 ±0.40^{a}	-13.60 ±0.06^{a}	45.29 ±0.19^{a}	47.46 ± 0.24^{a}	106.63 ±0.15^{a}
	5	58.11 ±0.44^{b}	-5.10 ±0.02^{b}	36.56 ±0.08^{b}	37.01 ± 0.06^{b}	97.80 ±0.08^{b}
	10	59.10 ±0.10^{b}	-4.43 ±0.05^{c}	35.55 ±0.03^{c}	35.88 ± 0.09^{c}	97.00 ±0.12^{c}
	15	58.79 ±0.09^{b}	-3.91 ±0.07^{d}	34.72 ±0.23^{d}	34.85 ± 0.17^{d}	96.40 ±0.18^{d}
	20	58.75 ±0.46^{b}	-3.62 ±0.02^{e}	34.11 ±0.05^{e}	34.31 ± 0.04^{e}	96.00 ±0.00^{e}
	25	58.78 ±0.20^{b}	-3.53 ±0.04^{e}	33.73 ±0.03^{f}	33.96 ± 0.03^{f}	95.90 ±0.00^{e}
	30	58.73 ±1.86^{b}	-3.71 ±0.42^{de}	33.56 ±0.08^{f}	33.77 ± 0.05^{f}	96.23 ±0.67^{de}
90	0	61.49 ±0.40^{a}	-13.60 ± 0.06^{a}	45.29 ±0.19^{a}	47.46 ±0.24^{a}	106.63 ±0.15^{a}
	5	57.93 ±0.63^{b}	-3.91 ± 0.05^{b}	34.41 ±0.06^{b}	34.41 ±0.06^{b}	96.48 ±0.13^{b}
	10	57.93 ±0.06^{b}	-3.91 ± 0.42^{b}	34.41 ±0.33^{c}	34.41 ±0.33^{c}	96.48 ±0.72^{b}
	15	58.24 ±0.10^{b}	-3.44 ± 0.10^{c}	33.56 ±0.32^{d}	33.56 ±0.32^{d}	95.80 ±0.12^{c}
	20	58.50 ±0.81^{b}	-3.13 ± 0.06^{d}	33.17 ±0.08^{e}	33.17 ±0.08^{e}	95.35 ±0.06^{d}
	25	58.37 ±0.22^{b}	-3.09 ± 0.15^{d}	33.01 ±0.08^{e}	33.01 ±0.08^{e}	95.30 ±0.23^{d}
	30	58.45 ±0.44^{b}	-3.01 ± 0.01^{d}	32.39 ±0.05^{f}	32.60 ±0.12^{f}	95.05 ±0.33^{d}
100	0	61.49 ±0.40^{a}	-13.60 ± 0.06^{a}	45.29 ±0.19^{a}	47.46 ±0.24^{a}	106.63 ±0.15^{a}
	5	57.67 ±0.18^{c}	-3.30 ± 0.03^{b}	33.30 ±0.11^{b}	33.47 ±0.08^{b}	95.65 ±0.06^{b}
	10	58.03 ±0.11^{b}	-3.10 ± 0.12^{c}	32.54 ±0.12^{c}	32.76 ±0.13^{c}	95.33 ±0.21^{c}
	15	58.05 ±0.06^{b}	-2.91 ± 0.10^{d}	31.99 ±0.04^{d}	32.12 ±0.07^{d}	95.15 ±0.17^{cd}
	20	58.05 ±0.09^{b}	-2.79 ± 0.11^{e}	31.54 ±0.14^{e}	31.54 ±0.14^{e}	95.08 ±0.21^{d}
	25	57.96 ±0.18^{bc}	-2.77 ± 0.10^{e}	31.30 ±0.16^{f}	31.03 ±0.09^{f}	94.95 ±0.06^{d}
	30	57.89 ±0.17^{bc}	-2.78 ± 0.06^{e}	30.76 ±0.07^{g}	30.91 ±0.09^{f}	95.05 ±0.06^{d}

Table 3 The color value of fresh pandan leaf extract when heating at 121°C for 15 min at the pH range 3.0 – 8.0

Treatment	pH	L*value	a* value	b*value	chroma	Hue
Control	3.0	4.37 ± 0.01	-3.47 ± 0.06	7.08 ± 0.03	7.75 ± 0.03	115.80 ± 0.3
	4.0	4.36 ± 0.02	-4.58 ± 0.11	6.85 ± 0.04	8.23 ± 0.04	123.50 ± 0.7
	5.0	2.47 ± 0.01	-3.24 ± 0.10	3.71 ± 0.05	5.09 ± 0.05	131.40 ± 0.0
	5.9	3.89 ± 0.03	-6.02 ± 0.12	6.27 ± 0.02	8.50 ± 0.02	133.40 ± 0.5
	6.0	4.81 ± 0.04	-7.35 ± 0.06	7.71 ± 0.04	10.57 ± 0.04	132.97 ± 0.0
	7.0	2.20 ± 0.01	-3.53 ± 0.06	3.40 ± 0.02	2.20 ± 0.02	136.13 ± 1.2
	8.0	2.66 ± 0.00	-4.28 ± 0.11	4.17 ± 0.07	5.94 ± 0.07	136.17 ± 0.2
Heating	3.0	31.57 ± 0.01	0.44 ± 0.02	33.24 ± 0.04	33.25 ± 0.04	89.20 ± 0.17
121°C	4.0	53.93 ± 0.12	-1.36 ± 0.01	20.31 ± 0.02	20.23 ± 0.02	93.70 ± 0.00
15 min.	5.0	50.62 ± 0.33	-1.21 ± 0.02	23.70 ± 0.10	22.73 ± 0.10	94.07 ± 0.06
	5.9	50.06 ± 0.02	-1.67 ± 0.02	25.01 ± 0.00	25.01 ± 0.00	93.73 ± 0.06
	6.0	48.46 ± 0.05	-1.47 ± 0.02	25.88 ± 0.03	25.92 ± 0.03	93.10 ± 0.00
	7.0	49.75 ± 0.05	-1.98 ± 0.02	26.48 ± 0.03	26.59 ± 0.03	94.17 ± 0.06
	8.0	43.31 ± 0.04	-2.25 ± 0.03	30.68 ± 0.02	30.83 ± 0.02	94.17 ± 0.06

Table 4 The formation of Zn-chlorophyll complexes at pH 3.0–8.0 on the color values

day	pH	L*value	a*value	b* value	Hue
0	3.0	45.53 ± 0.02^{a}	-3.76 ± 0.04^{a}	39.77 ± 0.08^{a}	95.40 ± 0.06^{f}
	4.0	41.47 ± 0.04^{b}	-8.51 ± 0.03^{d}	29.57 ± 0.04^{d}	106.06 ± 0.06^{c}
	5.0	38.37 ± 0.03^{e}	-8.74 ± 0.02^{e}	28.12 ± 0.06^{e}	107.27 ± 0.07^{a}
	5.4	39.19 ± 0.05^{d}	-8.51 ± 0.04^{d}	27.40 ± 0.01^{f}	107.24 ± 0.07^{a}
	6.0	31.28 ± 0.02^{f}	-9.67 ± 0.02^{f}	32.31 ± 0.03^{c}	106.67 ± 0.05^{b}
	7.0	41.48 ± 0.04^{b}	-7.63 ± 0.02^{c}	35.55 ± 0.05^{b}	102.11 ± 0.04^{d}
	8.0	39.82 ± 0.02^{c}	-6.73 ± 0.01^{b}	39.71 ± 0.07^{a}	99.62 ± 0.03^{e}
7	3.0	47.11 ± 0.01^{a}	-4.16 ± 0.01^{a}	39.33 ± 0.01^{b}	95.90 ± 0.01^{f}
	4.0	44.04 ± 0.04^{b}	-8.36 ± 0.02^{e}	28.76 ± 0.05^{e}	106.21 ± 0.02^{c}
	5.0	39.92 ± 0.07^{e}	-8.50 ± 0.01^{f}	26.54 ± 0.01^{f}	107.67 ± 0.06^{a}
	5.4	43.16 ± 0.12^{c}	-8.01 ± 0.01^{d}	25.09 ± 0.01^{g}	107.72 ± 0.01^{a}
	6.0	31.90 ± 0.01^{f}	-9.76 ± 0.04^{g}	32.06 ± 0.02^{d}	106.90 ± 0.05^{b}
	7.0	40.11 ± 0.02^{d}	-7.83 ± 0.01^{c}	36.41 ± 0.05^{c}	102.08 ± 0.06^{d}
	8.0	31.03 ± 0.16^{g}	-6.54 ± 0.01^{b}	41.07 ± 0.04^{a}	99.11 ± 0.01^{e}
14	3.0	48.31 ± 0.03^{a}	-3.85 ± 0.01^{a}	38.55 ± 0.02^{b}	95.67 ± 0.06^{f}
	4.0	44.92 ± 0.04^{b}	-8.00 ± 0.01^{e}	28.05 ± 0.00^{d}	105.80 ± 0.00^{c}
	5.0	41.48 ± 0.05^{e}	-8.12 ± 0.02^{f}	26.42 ± 0.03^{f}	107.08 ± 0.03^{a}
	5.4	44.06 ± 0.18^{c}	-7.69 ± 0.02^{d}	25.23 ± 0.04^{g}	107.03 ± 0.06^{a}
	6.0	28.25 ± 0.03^{g}	-9.63 ± 0.03^{g}	31.89 ± 0.02^{c}	106.77 ± 0.06^{b}
	7.0	42.86 ± 0.05^{d}	-7.33 ± 0.01^{c}	35.72 ± 0.02^{b}	101.57 ± 0.06^{d}
	8.0	39.32 ± 0.13^{f}	-6.69 ± 0.02^{b}	42.90 ± 0.01^{a}	98.77 ± 0.06^{e}
21	3.0	46.85 ± 0.01^{a}	-4.22 ± 0.02^{a}	39.61 ± 0.03^{b}	95.97 ± 0.06^{g}
	4.0	46.82 ± 0.01^{a}	-8.05 ± 0.02^{d}	27.09 ± 0.04e	106.43 ± 0.06^{d}
	5.0	40.48 ± 0.06^{d}	-8.43 ± 0.01^{f}	26.02 ± 0.05^{f}	107.77 ± 0.06^{b}
	5.4	40.25 ± 0.18^{e}	-8.20 ± 0.04^{e}	24.84 ± 0.06^{g}	108.03 ± 0.06^{a}
	6.0	31.27 ± 0.01^{f}	-9.78 ± 0.02^{g}	31.89 ± 0.04^{d}	107.00 ± 0.00^{c}
	7.0	40.72 ± 0.02^{c}	-7.87 ± 0.03^{c}	36.23 ± 0.03^{c}	102.20 ± 0.00^{e}
	8.0	41.22 ± 0.01^{b}	-7.10 ± 0.04^{b}	42.30 ± 0.02^{a}	99.40 ± 0.10^{f}

Table 5 The formation of Zn-chlorophyll complexes at the concentration of $ZnCl_2$ 0–600 ppm on the color values

Day	$ZnCl_2$	L*value	a*value	b*value	Hue
0	0	37.14 ± 0.02 [b]	-1.22 ± 0.02 [f]	33.03 ± 0.07 [a]	92.40 ± 0.52 [d]
	100	32.82 ± 0.02 [d]	-6.14 ± 0.03 [e]	32.01 ± 0.01 [b]	100.87 ± 0.04 [c]
	200	40.61 ± 0.01 [a]	-6.22 ± 0.02 [d]	31.86 ± 0.02 [c]	101.05 ± 0.02 [c]
	300	35.42 ± 0.01 [c]	-7.42 ± 0.01 [c]	31.72 ± 0.06 [d]	103.68 ± 0.03 [b]
	400	29.58 ± 0.02 [f]	-7.79 ± 0.03 [b]	31.72 ± 0.06 [d]	103.80 ± 0.06 [ab]
	500	27.46 ± 0.02 [c]	-7.83 ± 0.03 [b]	31.60 ± 0.02 [e]	103.93 ± 0.03 [ab]
	600	32.42 ± 0.02 [e]	-8.03 ± 0.07 [a]	31.78 ± 0.05 [d]	104.20 ± 0.11 [a]
7	0	36.14 ±0.01 [b]	-1.41 ± 0.04 [a]	32.37 ± 0.03 [a]	92.49 ± 0.06 [f]
	100	30.71 ± 0.01 [d]	-5.94 ± 0.03 [b]	30.73 ± 0.02 [c]	100.94 ± 0.04 [e]
	200	39.53 ± 0.01 [a]	-6.18 ± 0.02 [c]	31.07 ± 0.06 [b]	101.26 ± 0.04 [d]
	300	29.25 ± 0.04 [e]	-7.68 ± 0.03 [d]	30.16 ± 0.03 [e]	103.31 ± 0.05 [b]
	400	28.53 ± 0.02 [f]	-7.73 ± 0.03 [de]	30.45 ± 0.03 [d]	104.25 ± 0.04 [b]
	500	27.12 ± 0.08 [g]	-7.75 ± 0.02 [e]	31.10 ± 0.08 [b]	104.00 ± 0.05 [c]
	600	30.88 ± 0.01 [c]	-8.15 ± 0.03 [f]	30.66 ± 0.03 [c]	104.69 ± 0.04 [a]
14	0	37.56 ± 0.08 [b]	-1.26 ± 0.02 [a]	28.32 ± 0.04 [e]	92.55 ± 0.03 [g]
	100	31.68 ± 0.05 [d]	-5.66 ± 0.01 [b]	30.16 ± 0.08 [a]	100.62 ± 0.03 [f]
	200	39.95 ± 0. 04 [a]	-5.69 ± 0.02 [b]	29.80 ± 0.01 [b]	100.81 ± 0.03 [e]
	300	33.68 ± 0.11 [c]	-6.72 ± 0.04 [c]	28.76 ± 0.10 [d]	103.15 ± 0.03 [d]
	400	27.28 ± 0.01 [f]	-7.46 ± 0.02 [e]	29.69 ± 0.05 [b]	104.10 ± 0. 02 [c]
	500	25.48 ± 0.02 [g]	-7.40 ± 0.01 [d]	29.38 ± 0.03 [c]	104.15 ± 0.02 [b]
	600	29.98 ± 0.18 [e]	-7.67 ± 0.02 [f]	29.73 ± 0.04 [b]	104.47 ± 0.02 [a]
21	0	32.57 ± 0.04 [b]	-1.16 ± 0.02 [a]	29.03 ± 0.01 [c]	92.27 ± 0.06 [f]
	100	29.61 ± 0.01 [d]	-5.56 ± 0.03 [b]	29.81 ± 0.03 [a]	100.47 ± 0.06 [e]
	200	38.16 ± 0.04 [a]	-5.85 ± 0.02 [c]	29.40 ± 0.32 [b]	101.30 ± 0.00 [d]
	300	30.54 ± 0.04 [c]	-6.96 ± 0.02 [d]	29.57 ± 0.02 [b]	103.13 ± 0.06 [c]
	400	25.20 ± 0.01 [f]	-7.39 ± 0.04 [f]	29.47 ± 0.04 [b]	104.00 ± 0.10 [b]
	500	24.04 ± 0.06 [g]	-7.16 ± 0.04 [e]	28.48 ± 0.03 [b]	104.07 ± 0.12 [b]
	600	27.97 ± 0.01 [e]	-7.58 ± 0.01 [g]	29.50 ± 0.02 [a]	104.40 ± 0.01 [a]

Table 6 The effect of heating temperature on the formation of Zn-chlorophyll derivative complexes at 80 °C for 15–105 min.

Heating Time (min)	Color				
	L*value	-a*value	b* value	Chroma	Hue
15	7.16 ± 0.00^{e}	-3.26 ± 0.07^{a}	11.51 ± 0.00^{e}	12.01 ±0.04^{e}	105.95 ± 0.64^{e}
30	7.41 ± 0.00^{d}	-4.84 ± 0.07^{b}	11.91 ± 0.00^{d}	12.86 ±0.00^{d}	111.65 ± 0.07^{d}
45	7.46 ± 0.01^{cd}	-6.21 ± 0.06^{c}	12.96 ± 0.03^{b}	14.51 ±0.02^{a}	116.15 ± 0.35^{c}
60	7.50 ± 0.00^{c}	-6.61 ± 0.14^{d}	11.93 ± 0.00^{d}	13.67 ±0.01^{c}	119.10 ± 0.28^{b}
75	7.90 ± 0.00^{b}	-7.18 ± 0.07^{e}	12.63 ± 0.00^{c}	14.62 ±0.10^{a}	120.05 ± 0.49^{b}
90	8.55 ± 0.07^{a}	-8.38 ± 0.01^{f}	13.23 ± 0.16^{a}	14.05 ±0.07^{b}	124.50 ± 0.71^{a}
105	8.58 ± 0.03^{a}	-8.38 ± 0.02^{f}	13.02 ± 0.04^{b}	14.15 ±0.07^{b}	125.00 ± 1.41^{a}

Table 7 The effect of heating temperature on the formation of Zn-chlorophyll derivative complexes at 90 °C for 10–70 min.

Heating Time(min)	Color				
	L*value	-a*value	b* value	Chroma	Hue
10	29.53 ± 0.04^{cd}	-7.60 ± 0.22^{a}	31.24 ± 0.16^{a}	32.15 ± 0.26^{a}	103.61 ± 0.31^{f}
20	33.84 ± 1.86^{a}	-8.13 ± 0.12^{b}	29.23 ± 0.39^{cde}	30.26 ± 0.37^{d}	105.50 ± 0.40^{e}
30	28.12 ± 1.09^{d}	-9.05 ± 0.18^{c}	29.66 ± 0.04^{bc}	30.90 ± 0.06b^{c}	106.85 ± 0.32^{d}
40	32.05 ± 2.48^{ab}	-9.17 ± 0.29^{cd}	29.19 ± 0.48^{de}	30.58 ± 0.49^{cd}	107.42 ± 0.28^{c}
50	31.08 ± 1.41^{bc}	-9.48 ± 0.19^{e}	29.84 ± 0.04^{b}	31.14 ± 0.25^{b}	107.58 ± 0.28^{abc}
60	29.61 ± 1.29^{cd}	-9.38 ± 0.08^{de}	28.84 ± 0.04^{d}	30.33 ± 0.08^{d}	107.97 ± 0.12^{a}
70	30.36 ± 1.97^{bc}	-9.55 ± 0.19^{e}	29.49 ± 0.70^{bcd}	30.98 ± 0.73^{bc}	107.88 ± 0.10^{ab}

Table 8 The effect of heating temperature on the formation of Zn-chlorophyll derivative complexes at 100 °C for 5–35 min.

Heating time	Color				
	L*value	-a*value	b* value	Chroma	Hue
5 min	24.04 ± 3.76 ^{a}	-8.64 ± 0.25 ^{a}	31.61 ± 2.18 ^{ab}	32.83 ± 2.20 ^{ab}	105.22 ± 0.52 ^{d}
10 min	24.11 ± 0.37 ^{a}	-9.64 ± 0.06 ^{b}	30.60 ± 0.31 ^{bc}	32.15 ± 0.26 ^{ab}	107.42 ± 0.12 ^{c}
15 min	19.97 ± 1.11 ^{b}	-10.58 ± 0.08 ^{c}	27.80 ± 1.01 ^{d}	29.79 ± 0.97 ^{c}	110.83 ± 0.56 ^{b}
20 min	22.93 ± 0.88 ^{a}	-11.43 ± 0.07 ^{d}	30.21 ± 0.52 ^{c}	32.30 ± 0.54 ^{ab}	110.95 ± 0.63 ^{b}
25 min	22.47 ± 0.42 ^{a}	-11.36 ± 0.05 ^{d}	30.13 ± 0.51 ^{c}	31.90 ± 0.42 ^{b}	110.71 ± 0.33 ^{b}
30 min	22.98 ± 0.83 ^{a}	-11.34 ± 0.06 ^{d}	30.72 ± 1.42 ^{bc}	32.82 ± 0.78 ^{ab}	111.31 ± 0.83 ^{ab}
35 min	24.33 ± 0.10 ^{a}	-11.42 ± 0.05 ^{d}	32.29 ± 0.06 ^{a}	33.35 ± 0.02 ^{a}	111.66 ±0.36 ^{a}

Table 9 The effect of heating temperature on the formation of Zn-chlorophyll derivative complexes at 110 °C for 5-35 min.

Heating time	Color				
	L*value	-a*value	b* value	Chroma	Hue
5 min	32.28 ± 2.44 [a]	-9.33 ± 0.06 [a]	31.08 ± 0.13 [ns]	32.48 ± 0.13 [d]	106.63 ± 0.10 [c]
10 min	31.91 ± 0.29 [ab]	-10.65 ± 0.02 [b]	31.02 ± 0.07 [ns]	32.80 ± 0.09 [c]	108.83 ± 0.05 [b]
15 min	29.23 ± 2.40 [cd]	-11.71 ± 0.05 [c]	31.25 ± 0.39 [ns]	33.16 ± 0.40 [b]	110.51 ± 0.22 [a]
20 min	28.39 ± 1.71 [d]	-11.75 ± 0.07 [c]	31.15 ± 0.31 [ns]	33.34 ± 0.30 [ab]	110.57 ± 0.08 [a]
25 min	30.33 ± 0.02 [bc]	-11.75 ± 0.06 [c]	31.13 ± 0.29 [ns]	33.38 ± 0.07 [ab]	110.48 ± 0.04 [a]
30 min	30.21 ± 0.06 [bcd]	-11.74 ± 0.04 [c]	31.11 ± 0.28 [ns]	33.37 ± 0.06 [ab]	110.46 ± 0.03 [a]
35 min	30.12 ± 0.23 [bcd]	-11.76 ± 0.04 [c]	31.10 ± 0.26 [ns]	33.42 ± 0.05 [a]	110.49 ± 0.05 [a]

Table 10 The effect of heating temperature on the formation of Zn-chlorophyll derivative complexes at 120 °C for 5-35 min.

Heating time	Color				
	L*value	-a*value	b* value	Chroma	Hue
5 min	41.29 ± 0.40 [a]	-8.31 ± 0.15 [a]	26.37 ± 0.25 [g]	27.47 ± 0.21 [f]	107.73 ± 0.06 [b]
10 min	34.94 ± 0.61 [d]	-9.63 ± 0.43 [d]	27.61 ± 0.06 [d]	29.13 ± 0.32 [c]	108.11 ± 0.01 [a]
15 min	30.72 ± 0.97 [g]	-9.63 ± 0.19 [g]	29.52 ± 0.04 [a]	31.05 ± 0.23 [a]	108.08 ± 0.04 [a]
20 min	36.54 ± 0.86 [b]	-9.67 ± 0.04 [b]	26.75 ± 0.26 [f]	28.10 ± 0.17 [e]	108.10 ± 0.04 [a]
25 min	35.96 ± 0.26 [c]	-9.67 ± 0.21 [c]	27.44 ± 0.58 [e]	28.44 ± 0.10 [d]	108.08 ± 0.01 [a]
30 min	34.70 ± 0.12 [e]	-9.62 ± 0.06 [e]	28.02 ± 0.17 [c]	29.03 ± 0.23 [c]	108.11 ± 0.01 [a]
35 min	33.32 ± 0.15 [f]	-9.65 ± 0.10 [f]	29.13 ± 0.21 [b]	30.11 ± 0.10 [b]	108.11 ± 0.02 [a]

Table 11 The heating temperature and heating time require for greenness color of Zn-chlorophyll derivative.

Temp(°C)	Time (min)	Color value				
		L*value	-a*value	b* value	Chroma	Hue
80	90	8.55 ± 0.07 [d]	-8.38 ± 0.01 [a]	13.23 ± 0.16 [d]	14.05 ±0.07 [e]	124.50 ± 0.71 [a]
90	50	31.08 ± 1.41 [b]	-9.48 ± 0.19 [b]	29.84 ± 0.04 [b]	31.14 ± 0.25 [c]	107.58 ± 0.28 [c]
100	20	24.11 ± 0.37 [c]	-9.64 ± 0.06 [c]	30.60 ± 0.31 [b]	32.15 ± 0.26 [b]	107.42 ± 0.12 [b]
110	15	29.23 ± 2.40 [b]	-11.71 ± 0.05 [c]	31.25 ± 0.39 [a]	33.16 ± 0.40 [a]	110.51 ± 0.22 [b]
120	10	34.94 ± 0.61 [a]	-9.63 ± 0.43 [b]	27.61 ± 0.06 [c]	29.13 ± 0.32 [d]	108.11 ± 0.01 [c]

Table 12 The effect of Celluclast®1.5 L and Pectinex® Ultra SP-L using for extract Zn-chlorophyll derivative in pandan on the color, chlorophyll and antioxidant activity.

Enzyme	Conc. (%)	*L**	*a**	*b**	*C*
control	0	53.75 ± 0.21[a]	-7.67 ±0.20[a]	30.01 ± 0.39[e]	30.98 ± 0.30[e]
Celluclast	1	50.48 ±0.64[b]	-7.98 ±0.05[ab]	30.98 ± 0.03[cd]	31.89 ± 0.15[cd]
	2	49.66 ±3.49[b]	-8.07±0.52[b]	31.00 ± 1.27[c]	32.08 ± 1.49[c]
	3	53.20 ±0.85[a]	-7.72 ±0.26[a]	31.12 ± 0.56[ed]	31.12 ± 0.56[e]
Pectinex	1	48.63 ±1.12[b]	-8.42 ±0.06[c]	32.97 ± 0.13[b]	34.00 ± 0.22[b]
Ultra SP-L	2	40.72 ±0.90[c]	-9.02 ±0.29[d]	34.80 ± 0.35[a]	35.92 ± 0.48[a]
	3	45.29 ±2.74[d]	-8.66 ±0.04[c]	34.05 ± 0.50[a]	35.19 ± 0.41[a]

Table 12 The effect of Celluclast®1.5 L and Pectinex® Ultra SP-L using for extract Zn-chlorophyll derivative in pandan on the color, chlorophyll and antioxidant activity (continuous)

Enzyme	Conc. (%)	$^{\circ}h$	Chlorophyll	Antioxidant
control	0	104.20 ±0.23[ab]	0.31 ± 0.02[e]	592.24 ±1.23[c]
Celluclast	1	104.37 ±0.12[abc]	0.34 ± 0.03[cd]	614.34 ±1.33[a]
	2	104.52 ±0.35[a]	0.32 ± 0.02[de]	617.85 ± 2.20[a]
	3	104.30 ±0.22[abc]	0.30 ± 0.00[e]	613.99 ± 1.43[a]
Pectinex	1	104.23 ±0.19[abc]	0.35 ± 0.04[c]	606.62 ± 2.35[b]
Ultra SP-L	2	104.50 ±0.33[ab]	0.47 ± 0.10[a]	605.57 ± 1.34[b]
	3	104.17 ±0.10[c]	0.39 ±0.21[b]	606.97 ± 1.76[b]

Table 13 The pH profile of 2% (v/w) Pectinex® Ultra SP-L incubation at 35°C for 2 h.

pH	Reducing sugar (mg glucose/g fw)
3	80.45 ± 1.04 [b]
4	89.73 ± 1.89 [a]
5	58.88 ± 0.32 [c]
6	50.09 ± 1.43 [d]

Table 14 The temperature profile of 2% (v/w) Pectinex® Ultra SP-L at pH 4.0 and incubated for 2 h.

Temperature(°C)	Reducing sugar (mg glucose/gfw)
25	68.93 ± 2.03 [d]
35	87.11 ± 0.46 [a]
45	80.32 ± 0.62 [b]
55	73.44 ± 0.03 [c]

Table 15 The effect of enzyme Pectinex® Ultra SP-L concentration on the reducing sugar (mg glucose/g fw) when incubated 35°C at pH 4.0

Conc.	Incubation time (hour)						
(%)	0	1	2	3	4	5	6
0	1.68 ±0.39 [e]	2.73 ±0.42 [e]	5.04 ±1.53 [f]	5.88 ±0.00 [e]	6.72 ±0.69 [e]	8.19 ±2.21 [e]	9.66 ±1.61 [d]
1	12.39 ± 1.26 [d]	54.62 ±3.07 [d]	60.92 ±3.53 [e]	51.68 ±3.22 [d]	61.97 ±0.42 [d]	62.82 ±0.42 [d]	68.28 ±0.42 [c]
2	19.96 ±0.80 [c]	57.56 ±0.49 [c]	65.00 ±0.41 [d]	69.75 ±0.69 [c]	81.30 ±1.26 [c]	86.34 ±1.06 [c]	91.60 ±1.53 [b]
3	26.05 ±0.69 [b]	69.96 ±2.10 [b]	89.23 ±3.72 [a]	88.98 ±2.00 [a]	95.34 ±1.20 [a]	94.54 ±0.49 [b]	98.11 ±1.73 [a]
4	26.68 ±0.42 [b]	71.01 ±1.46 [b]	87.39 ±1.19 [b]	89.00 ±4.62 [a]	96.90 ±0.72 [a]	99.58 ±2.11 [a]	99.32 ± 2.78 [a]
5	28.57 ±2.47 [a]	75.63 ±1.94 [a]	83.82 ±0.80 [c]	83.82 ±0.80 [b]	93.70 ±0.49 [b]	99.79 ±0.80 [a]	99.54± 1.73 [a]

Table 16 The relative activity of pandan contain of 2% Pectinex Ultra® SP-L at 0-7 h.

Incubation time(h)	Relative activity (%)
0	100
1	73.50
2	74.16
3	69.07
4	59.24
5	58.40
6	60.90
7	62.30

Table 17 Physicochemical properties of pandan powder at 30% of wall materials

Physicochemical properties	Gum arabic	Maltodextrin	Modified starch
Color value			
Lightness(L*value)	86.14 ± 3.29 a	86.71 ± 2.84 a	82.90 ± 4.13 b
Greenness(-a*value)	-8.75 ± 1.20 c	-9.47 ± 1.31 b	-11.84 ± 0.94 a
Yellowness (b*value)	15.71 ± 2.12 b	15.73 ± 1.34 b	20.16 ± 2.16 a
Chroma(*C*)	17.78 ± 1.25 c	18.22 ± 1.71 b	23.50 ± 1.11 a
Hue (°*H*)	119.10 ± 2.29 b	120.84 ± 1.98 a	120.17 ± 1.94 a
Chlorophyll content(mg/100gfw)	128.65 ± 1.45 b	122.79 ± 1.03 b	187.34± 2.19 a
pH	6.53 ± 0.34 c	7.35 ± 0.63 a	7.04 ± 0.03 b
aw	0.30 ± 0.01 a	0.28 ± 0.01 b	0.28 ± 0.02 b
Moisture content	9.41 ± 0.95 a	8.39 ± 0.81 b	9.27 ± 0.74 a
Bulk density	0.48 ± 0.03 b	0.50 ± 0.01 b	0.55 ± 0.01 a
Antioxidant activity(μMTEAC)	428.00 ± 6.02 b	225.00± 4.63 c	772.50 ± 5.32 a
Specific surface area(m^2/g)	0.12 ± 0.03 c	0.37 ± 0.04 b	0.70 ± 0.03 a
D(0.5)	34.46 ± 1.23 a	30.11 ± 1.39 b	16.13 ± 1.36 c
%yields	8.25 ± 0.43 c	14.94 ± 0.68 b	16.22 ± 1.62 a

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Patent and International papers:

1. Porrarud Senklang and Pranee Anprung. 2009. “Production of green chlorophylls concentrate extract from pandan leaf”. Thailand Patent.
2. Senklang, P. and Anprung P. 2009. “Optimizing enzymatic extraction of Zn-chlorophyll derivatives from pandan leaf using response surface methodology”. Journal of Food Processing and Preservation. Article in press.

3. Senklang, P. and Anprung P. 2009. “Selection of wall material for microencapsulation of Zn-chlorophyll derivatives by spray drier”. Journal of Food Processing and Preservation. Article on submitting: scoring process.

Oral presentation:

1. งานนวัตกรรมจากการบูรณาการผลงานวิจัยด้านอาหาร จัดโดย สำนักงานเทคโนโลยี SMEs มหาวิทยาลัยเทคโนโลยีพระจอมเกล้าธนบุรี สนับสนุนโดย สำนักงานส่งเสริมวิสาหกิจขนาดกลางและขนาดย่อม **วันที่** 28 **พฤศจิกายน** 2550 ห้องราชเทวี 2 โรงแรมเอเชีย
2. โครงการนวัตกรรมเพื่อยกระดับคุณภาพและความปลอดภัยทางอาหารสู่โครงสร้างเศรษฐกิจยุคใหม่ (ครั้งที่ 1) จัดโดย จุฬาลงกรณ์มหาวิทยาลัย งบประมาณแผ่นดินปี 2549 ตึกมหามกุฎ คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย **วันที่** 30 **พฤศจิกายน** 2550
3. การประชุมวิชาการและเสนอผลงานวิจัยพืชเขตร้อนและกึ่งร้อน ครั้งที่ 2 นำเสนอผลการวิจัยเรื่อง ผลของชนิดตัวพยุงต่อสมบัติทางเคมีกายภาพและความคงตัวของผงคลอโรฟิลล์จากใบเตยด้วยวิธีทำแห้งแบบพ่นฝอย ณ โรงแรมเดอะทวินทาวเวอร์ กรุงเทพมหานคร **วันที่** 21-22 **สิงหาคม** 2551
4. โครงการนวัตกรรมเพื่อยกระดับคุณภาพและความปลอดภัยทางอาหารสู่โครงสร้างเศรษฐกิจยุคใหม่ (ครั้งที่ 2) จัดโดย จุฬาลงกรณ์มหาวิทยาลัย งบประมาณแผ่นดินปี 2550 ตึกมหามกุฎ คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย **วันที่** 17-18 **ธันวาคม** 2551