รายงานวิจัยฉบับสมบูรณ์

การสกัดวิตามิน และสารออกฤทธิ์ทางชีวภาพจากผลปาล์มหลังการเก็บเกี่ยว และการประยุกต์ใช้กากผลปาล์มสำหรับผลิตพอลิไฮดรอกซีอัลคาโนเอต

Extraction of Vitamins and Bioactive Compounds from Oil Palm Fruit after Harvested and The Application of Oil Palm Fruit Residue for Polyhydroxyalkanoates Production

ผู้ช่วยศาสตราจารย์ ดร.กนกพร สังขรักษ์

สนับสนุนโดย งบประมาณเงินแผ่นดิน
ประจำปีงบประมาณ พ.ศ. 2558
มหาวิทยาลัยทักษิณ

คำรับรองคุณภาพ

รายงานวิจัยเรื่อง สารสกัดวิตามินและสารออกฤทธิ์ทางชีวภาพจากผลปาล์มหลังการเก็บเกี่ยวและการประยุกต์ใช้กากเยื่อใยปาล์มสำหรับผลิตพอลิไฮดรอกซีอัลคาโนเอต

ผู้วิจัย กนกพร สังขรักษ์

สถาบันวิจัยและพัฒนา มหาวิทยาลัยทักษิณ ขอรับรองว่ารายงานวิจัยฉบับนี้ได้ผ่านการประเมินจากผู้ทรงคุณวุฒิแล้ว มีความเห็นว่าผลงานวิจัยฉบับนี้มีคุณภาพอยู่ในเกณฑ์

- ☐ ดีมาก
- ☑ ดี
- ☐ ปานกลาง
- ☐ พอใช้
- ☐ ควรปรับปรุง

.....

(รองศาสตราจารย์ ดร. พรพันธุ์ เขมคุณาศัย)

ผู้อำนวยการสถาบันวิจัยและพัฒนา

23 ธันวาคม 2558

กิตติกรรมประกาศ

ขอขอบพระคุณมหาวิทยาลัยทักษิณ และสถาบันวิจัยและพัฒนา ม.ทักษิณ สำหรับ
เงินสนับสนุนตลอดโครงการในการทำวิจัย เรื่องการสกัดวิตามิน และสารออกฤทธิ์ทาง
ชีวภาพจากผลปาล์มหลังการเก็บเกี่ยว และการประยุกต์ใช้กากเยื่อใยปาล์มสำหรับผลิตพอ
ลิไฮดรอกซีอัลคาโนเอตจนกระทั่งงานวิจัยนี้สำเร็จลุล่วงไปได้ด้วยดี

ขอขอบพระคุณอาจารย์ และบุคลากรประจำสาขาวิชาเคมี คณะวิทยาศาสตร์
มหาวิทยาลัยทักษิณ ที่ได้ให้การสนับสนุน อำนวยความสะดวกในการทำวิจัยในครั้งนี้

ขอขอบพระคุณ รองศาสตราจารย์ เกษม อัศวตรีรัตนกุล อาจารย์ ดร. ปิยาภรณ์
ภาษิตกุล นางนิษา ไพจิตร นายเทวัญ หยู่หนู นางสาวศศิธร สอนนำและนางสาวภัทรวดี
กิ้มตั้น อาจารย์และบุคลากรประจำหน่วยวิจัยเคมีเพื่อการใช้ประโยชน์จากวัสดุเศษเหลือ
สาขาวิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยทักษิณ ที่ให้คำแนะนำที่เป็นประโยชน์ตลอด
โครงการวิจัย จนงานวิจัยสำเร็จลุล่วงไปได้ด้วยดี

งานวิจัยนี้ได้รับงบประมาณสนับสนุนจาก งบประมาณแผ่นดิน ประจำปี
งบประมาณ พ.ศ.2558

ผู้ช่วยศาสตราจารย์ ดร.กนกพร สังขรักษ์
หัวหน้าโครงการวิจัย

บทคัดย่อ

ชื่อโครงการวิจัย การสกัดวิตามิน และสารออกฤทธิ์ทางชีวภาพจากผลปาล์มหลังการเก็บเกี่ยว และการประยุกต์ใช้กากเยื่อใยปาล์มสำหรับผลิตพอลิไฮดรอกซีอัลคาโนเอต

ชื่อผู้วิจัย ผู้ช่วยศาสตราจารย์ ดร.กนกพร สังขรักษ์
หน่วยวิจัยเคมีเพื่อการใช้ประโยชน์จากวัสดุเศษเหลือ
สาขาวิชาเคมี คณะวิทยาศาสตร์
มหาวิทยาลัยทักษิณ
อำเภอป่าพะยอม จังหวัดพัทลุง 93110
โทรศัพท์ 0 7460 9600 ต่อ 2355

ได้รับงบประมาณเงินแผ่นดิน ทุนอุดหนุนการวิจัยประเภท การวิจัยประยุกต์
ประจำปี พ.ศ. 2558 จำนวน 500,000 บาท
ระยะเวลาทำการวิจัย 1 ปี ตั้งแต่วันที่ 1 ตุลาคม 2557 ถึงวันที่ 30 กันยายน 2558

งานวิจัยนี้มีวัตถุประสงค์เพื่อสกัดวิตามิน และสารออกฤทธิ์ทางชีวภาพจากผลปาล์มหลังการเก็บเกี่ยว (0-240 ชั่วโมง) และยังทำการศึกษากิจกรรมของไลเปสจากผลปาล์มด้วย กากผลปาล์มที่ผ่านการสกัดสารออกฤทธิ์ทางชีวภาพแล้วจะถูกนำมาใช้สำหรับผลิตพอลิไฮดรอกซีบิวทิเรต การทดลองแบ่งออกเป็น 2 ส่วนคือการสกัด และศึกษาสารออกฤทธิ์ทางชีวภาพจากผลปาล์มหลังการเก็บเกี่ยวที่ 0-240 ชั่วโมง และการนำผลปาล์มหลังการสกัดหรือกากผลปาล์มมาใช้เป็นสารตั้งต้นในการผลิตพอลิไฮดรอกซีบิวทิเรต

ผลปาล์มน้ำมันหลังการเก็บเกี่ยวที่ 0-240 ชั่วโมงจะถูกนำมาสกัดด้วยตัวทำละลาย 6 ชนิด ได้แก่ อะซิโตน เอทานอล สารละลายทริส-เอชซีแอล (พีเอช 8) เมทานอล

คลอโรฟอร์ม และเฮกเซน ผลการทดลองพบว่าการสกัดด้วยเมทานอลจะได้ผลผลิตสาร ออกฤทธิ์ทางชีวภาพสูงสุด เท่ากับ 10.82 กรัม (5.41 เปอร์เซ็นต์) ผลปาล์มน้ำมันที่ 0 ชั่วโมง จะมีความชื้น 30.24 เปอร์เซ็นต์ โปรตีน 5.12 เปอร์เซ็นต์ ลิปิด 52.94 กรัมน้ำมันต่อกรัมผล ปาล์ม และเถ้า 1.84 เปอร์เซ็นต์ นอกจากนี้ปริมาณสารออกฤทธิ์ทางชีวภาพจะมีปริมาณ สูงสุดที่เวลา 0 ชั่วโมงหลังการเก็บเกี่ยวเช่นกัน โดยที่เวลา 0 ชั่วโมงจะมีอัลคาลอยด์ (1.6 มิลลิกรัมต่อกรัมเซลล์แห้ง) ซาโปนิน (3.5 มิลลิกรัมต่อกรัมเซลล์แห้ง) ฟลาโวนอยด์ (0.3 มิลลิกรัมต่อกรัมเซลล์แห้ง) ฟีนอลิก (0.3 มิลลิกรัมต่อกรัมเซลล์แห้ง) แทนนิน (3.5 มิลลิกรัมต่อกรัมเซลล์แห้ง) คาร์โบไฮเดรต (470 มิลลิกรัมต่อกรัมเซลล์แห้ง) โปรตีน (2.4 มิลลิกรัมต่อกรัมเซลล์แห้ง) โทโคฟีรอล (850 มิลลิกรัมต่อกิโลกรัม) โทโคไตรอีนอล (800 มิลลิกรัมต่อกิโลกรัม) และแคโรทีน (790 มิลลิกรัมต่อกิโลกรัม) สำหรับกิจกรรมของ เอนไซม์ไลเปสสูงสุดจะสกัดได้จากสารละลายทริส-เอชซีแอลบัฟเฟอร์ (พีเอช 8) ในสาร สกัดจะมีกิจกรรมของเอนไซม์ไลเปสเท่ากับ 0.4 ยูนิตต่อมิลลิลิตร และมีค่ากิจกรรมของ เอนไซม์ทั้งหมด และกิจกรรมจำเพาะของเอนไซม์เท่ากับ 0.1 ยูนิต และ 1.38 ยูนิตต่อ มิลลิกรัมโปรตีน ตามลำดับ

หลังจากสกัดสารออกฤทธิ์ทางชีวภาพออกจากผลปาล์ม กากผลปาล์มจะถูกนำมาใช้เป็นสารตั้งต้นในการผลิตพอลิไฮดรอกซีบิวทิเรต จากผลการทดลองพบว่าเมื่อใช้กากผลปาล์มเป็นแหล่งคาร์บอนในการผลิตพอลิไฮดรอกซีบิวทิเรตโดยใช้จุลินทรีย์ *Cupriavidus necator* จะให้ผลผลิตพอลิไฮดรอกซีบิวทิเรตเท่ากับ 50.4 เปอร์เซ็นต์ต่อน้ำหนักเซลล์แห้ง หลังการเลี้ยงเชื้อ 60 ชั่วโมง พอลิเมอร์ที่ได้เมื่อทำการศึกษาชนิดของโมโนเมอร์ และศึกษาหมู่ฟังก์ชันด้วยเครื่อง Gas chromatography และ ATR-FTIR พบว่ามีลักษณะที่บ่งชี้ได้ว่าเป็นพอลิเมอร์ชนิด พอลิไฮดรอกซีบิวทิเรต เมื่อเปรียบเทียบกับพอลิไฮดรอกซีบิวทิเรตทางการค้า

คำสำคัญ : กากผลปาล์ม พอลิไฮดรอกซีอัลคาโนเอต วิตามินเอ วิตามินอี สารออกฤทธิ์ทางชีวภาพ

ABSTARCT

This study aimed to extract bioactive compound from oil palm fruit after 0-240 h of harvested. In addition, lipase from oil palm was also partitioned and analyzed. Oil palm fruits after 0-240 h of harvested were firstly extracted by six different solvent including acetone, ethanol, Tris-HCl buffer (pH 8), methanol, chloroform and hexane. The highest bioactive compounds (10.82 g, 5.41% yield) were obtained from methanol extraction. Oil palm fruit after 0 h of harvested contained moisture (30.24%), protein (5.12%), lipid (52.94 g oil/g oil palm fruit and ash (1.84%). In addition, other phytochemical content were yielded the highest content after 0 h of harvested including alkaloid (1.6 mg/g DW), saponin (3.5 mg/g DW), flavonoid (0.3 mg/g DW), phenolic (0.3 mg/g DW), tannin (3.5 mg/g DW), carbohydrate (470 mg/g DW) and protein (2.4 mg/g DW). Tocopherol, tocotrienol and carotene from oil palm fruit after 0-240 h of harvested were also evaluated. The highest tocopherol (850 mg/kg), tocotrienol (800 mg/kg) and carotene (790 mg/kg) was obtained after 0 h of harvested. The highest lipase activity were obtained from Tris-HCl buffer (pH 8.0). The highest lipase activity (0.4 U/mL) with 0.1 U and 1.38 U/mg protein was achieved from oil fruit after 120 h of harvested.

After extraction of bioactive compound, the oil palm fruit (OPF) residue was used as substrate for production of polyhydroxybutyrate. Results indicated that OPF residue a s carbon source for PHB production by Cupriavidus necator gave the maximum PHB with the yield of 50.4% of dry cell weight after 60 h of cultivation. The polymer was identified by gas chromatography and ATR-FTIR to be polyhydroxybutyrate compared with commercial polyhydroxybutyrate.

Keywords: Oil palm fruit residue, Polyhydroxyalkanoates, Vitamin A, Vitamin E, Bioactive compounds

CONTENTS

LIST OF TABLES

List of Table (cont.)

LIST OF FIGURES

1. INTRODUCTION

Oil palm (*Elaeis guineensis* Jacq.) is the highest yielding edible oil crop in the world and is cultivated in 42 countries on 11 million ha worldwide (Abdul Khalil et al., 2008). Oil palm and its fractions are used in the manufacturing of cooking oil, magarines, spreads, icecreams and dairy products (Kok et al., 2011). Palm oil contains palmitic, monosaturated oleic, polyunsaturated linoleic and stearic acids (Kok et al., 2011; Mortensen, 2005; Sampaio et al., 2011) and also minor constituents with nutritional and beneficial health properties, including tocopherols, tocotrienols, carotenoids, phytosteropls, phenolic compounds and other phytonutrients (Edem, 2002; Sambanthamurthi et al., 2000). Palm oil possesses 1% minor components which amongst them are the carotenoids, vitamin E (tocopherols and tocotrienols) and sterols. The orange colour of palm oil is due to the presence of these carotenes. Its concentration normally ranges between 400 and 3,500 ppm and it contains about 15 times more retinol equivalents (vitamin A) than carrots and 300 times more than tomatoes (Sundram et al., 2003; Ahmad et al., 2008). In addition, palm oil contains very little amount of phytosterols (300-620 ppm), Squalene (250-540 ppm), phospholipids (20-100 ppm), co-enzyme Q10 (20-80 ppm) and polyphenolics (40-70 ppm).

Intense development of oil palm industry in Thailand contributes to the country's economy growth mainly through the trading of crude palm oil and its products. However, there are abundant of illegal entry of palm from Malaysia and Indonesia which make palm price in Thailand volatility. In addition, the other problem of palm oil industry is habitat degradation. Once

the oil palms have begun to produce, the fruit must be harvested at the right moment. Palm fruit becomes too ripe suddenly after harvest, many clusters will drop and the quality of the fruit will be less good. Therefore, the study of the alternative utilization of oil palm fruit is the interested issue. To our knowledge, there have been no reports documenting the chemical composition, phenolic acids, flavonoids, fatty acid and lipase profile of the palm fruit. Thus we decided to determine the chemical composition, bioactive compounds and antioxidant activity of oil palm fruit cultivated in southern Thailand. This study aimed to promote oil palm, increase oil palm price and prevent excess supply problem.

2. LITERATURE REVIEW

The oil palm gives two different types of oils which is distinct from each other in terms of its fatty acid composition. Palm oil and palm kernel oil are used for identify oil from the mesocarp and kernel, respectively. The bulk of palm oil that is produced goes into various applications. However, food applications is most well known, hence its nutritional properties have been extensively studied. Nutrition research related to palm oil has focused primarily on two major areas-the fatty acid composition and the micronutrient content of palm oil (Khosla, 2006).

1. Palm oil composition

1.1 Physicochemical characterization of palm oil

The Mongana report of 1955 was one of the earliest comprehensive research works on palm oil characterization. It dealt with palm oil milling and CPO quality in Africa. Then, CPO quality was mainly defined in terms of total percentage of FFA, moisture and impurities. After the Second World War, a more complex quality grading system was introduced to regulate oil production by cottage industries. A five point grading system was introduced. 'Grade 1' oils must have FFA 1% while 'grade 5' oils have FFA 436%. 'Grades 2, 3 and 4' oils have FFA ranges of 9–18%, 18–27%, and 27–36%, respectively. This grading system stimulated the small scale producers to improve their oil quality. Later, the specification 'Special Grade' palm oil with maximum FFA level of 4.5% at the point of sale was introduced. Further adjustment put the maximum FFA at 3.5%. Trade reports showed that by 1965 more than 80% of CPO export from Africa was of the 'Special Grade' quality

(Berger and Martin, 2000; Iwuchukwu, 1965). Since the 1990s, some countries such as Malaysia have the set limits of FFA to 5% and a maximum of 0.25% for moisture and impurities for locally produced CPO (Chong, 2012).

The physicochemical properties of palm oil and its fractions were extensively studied during the 1980s and 1990s. Brilliant works on the physicochemical properties of palm oil and its fractions have been published (Tan and Oh, 1981; Tan and Man, 2000; Tan and Nehdi, 2012). CPO is classified as saturated oil with iodine value (IV) range of 51–58 g/100 g oil. Palm oils with a wider IV range of 46–63 g/100 g oil have been reported. These types of palm oil may be mixtures of oils from different species of oil palm tree or oil mixed with various proportions of palm stearin (Edem, 2002; Elias and Pantzaris, 1997; O'Brien, 2010; Tavares and Barberion, 1995). The major physicochemical characteristics of palm oil are presented in Table 2.1.

Table 2.1 Physicochemical properties of palm oil.

Characteristics	Typical	Range
Apparent density at 50°C (g/mL)	-	0.892-0.899
AOM stability (h)	54.0	53.0-60.0
Melting point (°C)	37.5	33.0-45.0
Oxidative stability index at 110°C (h)	16.9	16.6-19.0
Refractive index at 50°C	-	1.449-1.456
Smoke point (°C)	-	230.0-235.0
Solidification point (°C)	-	35.0-42.0
Solid fat content		
10°C	34.5	30.0-39.0
21.1°C	14.0	11.5-17.0
26.7°C	11.0	8.0-14.0
33.3°C	7.4	4.0-11.0
37.8°C	5.6	2.5-9.0
40.0°C	4.7	2.0-7.0
Specific gravity at 50°C	-	0.888-0.889
Viscosity (cP)	45.0	45.0-49.0
Iodine value (g/100 g)	53.0	46.0-56.0
Free fatty acid (%FFA as palmitic)	-	3.17-5.0
Peroxide value (meqO_2/kg)	-	0.1-10.0
Anisidine value (mg KOH/g)	196.0	190.0-209.0
Unsaponification matter (%)	0.5	0.15-0.99

Table 2.1 (cont.)

Characteristics	Typical	Range
Total polar comounds (%)	13.5	9.47-19.50
Total polymer materials (%)	0.5	0.4-15.0
Saturated fatty acid, SFA (%)	-	49.9-54.7
Mono-unsatturated fatty acids MUFA (%)	-	37.1-39.2
Poly-unsatturated fatty acids PUFA (%)	-	8.1-10.58
Cystal habit	β	-

Source: Mba et al. (2015)

1.2 Fatty acid composition

Crude palm oil contains fatty acid ester of glycerol commonly referred to as triglycerides. Palm oil contains approximately an equal amount of saturated and unsaturated fatty acids. Palmitic, oleic, linoleic and stearic acid account for 45, 40, 10 and 5 percent of total fatty acids, respectively. Fractionation can give rise to a stearin (higher palmitic content) and an olein product (higher oleic content). Numerous dietary intervention studies designed to evaluate the ability of palm oil to influence blood cholesterol have been reported. The results from these have often been conflicting, primarily because some of the studies were designed to evaluate the effects of individual saturated fatty acid (SFA) and used palm oil as a source of palmitic acid, while others, compared palm oil with other dietary oils or fats. Palm oil samples collected from the local markets were analyzed using gas chromatograph for

their fatty acid (FA) compositions. The fatty acid percent compositions are shown in Table 2.2 (Khosla, 2006; Chowdhury *et al.*, 2007).

Table 2.2 Fatty acid composition of palm oil compare to the other edible oils

Fatty acids (%)	Sunflower oil (n=5)	Soybean oil (n=5)	Mustard oil (n=5)	Palm oil (n=3)	Coconut oil (n=6)
Caprylic (C8:0)	-	-	-	-	6.21±0.34
Capric (C10:0)	-	-	-	-	6.15±0.21
Lauric (C12:0)	-	-	-	-	51.02±0.71
Myristic (C14:0)	-	-	-	1.23±0.28	18.94±0.63
Palmitic (C16:0)	6.52±1.75	14.04±0.62	4.51±3.83	41.78±1.27	8.62±0.50
Stearic (C18:0)	1.98±1.44	4.07±0.29	2.78±0.59	3.39±0.65	1.94±0.17
Oleic (C18:1)	45.39±18.77	23.27±2.43	38.21±21.88	41.90±1.20	5.84±0.50
Linoleic (C18:2)	46.02±16.75	52.18±2.64	25.31±5.74	11.03±0.02	1.28±0.18
Linolenic (C18:3)	-	-	11.30±6.09	-	-
Arachidic (C20:0)	-	-	10.86±3.29	-	-
Erucic (C22:1)	-	-	11.35±13.83	-	-

Source : Chowdhury *et al.* (2007)

The mean of total SFA, monounsaturated fatty acid (MUFA) and polyunsaturated fatty acid (PUFA) percent are shown in Table 2.3.

Table 2.3 Percentage of saturated (SFA), monounsaturated (MUFA), polyunsaturated (PUFA) and total unsaturated fatty acid of various edible oils.

Fatty acids (%)	Sunflower oil (n=5)	Soybean oil (n=3)	Mustard oil (n=5)	Palm oil (n=3)	Coconut oil (n=6)
SFA	8.51±1.91	18.26±0.67	15.94±2.58	46.34±0.34	92.92±0.56
MUFA	45.5±16.89	23.28±1.99	49.57±8.56	41.16±0.56	5.84±0.46
PUFA	46.10±14.92	57.86±1.20	36.62±6.42	11.84±0.92	1.28±0.17
MUFA+PUFA	91.49±1.91	81.14±1.49	86.18±3.07	53.30±0.36	7.12±0.51

Source: Chowdhury *et al.* (2007)

1.3 Palm oil minor components

In addition to the fatty acid composition, minor components were also studied (Manorama *et al.*, 1997; van Stuijvenberg *et al.*, 2001; Sivan *et al.*, 2001; 2002; Hedren *et al.*, 2002; Zagré *et al.*, 2003; Canfield *et al.*, 2001; Radhika *et al.*, 2003; Lietz *et al.*, 2001). Crude palm oil is one of the richest known sources of biologically active carotenoids (500 - 700 parts per million), of which contain ~ 35 percent (α-carotene) and 56 percent (β-carotene). However, these are destroyed during conventional refining. A refined palm oil is available which retains in excess of 80 percent of the carotenoids that were originally present in the crude oil. Several reports have shown that red palm oil can be used as an effective medium for delivering Vitamin A without the need for resorting to synthetic Vitamin A (Manorama *et al.*, 1997). This, red palm oil, has been exploited by nutrition researchers for its pro-vitamin A activity. Besides the carotenoids, palm oil contains appreciable amounts of vitamin E -both the tocopherols and tocotrienols (Khosla, 2006). The

distinctive colour of the oil is due to the fat soluble carotenoids (pigment) which are also responsible for its vitamins E (tocopherols and tocotrienols) content.

There are several stages of processing the extract of palm oil from fresh fruit bunches. These include sterilization, bunch stripping, digestion, oil extraction and finally clarification and purifications; each process with its own various unit operations (Usoro, 1973). This palm oil production process is shown on Fig. 2.1.

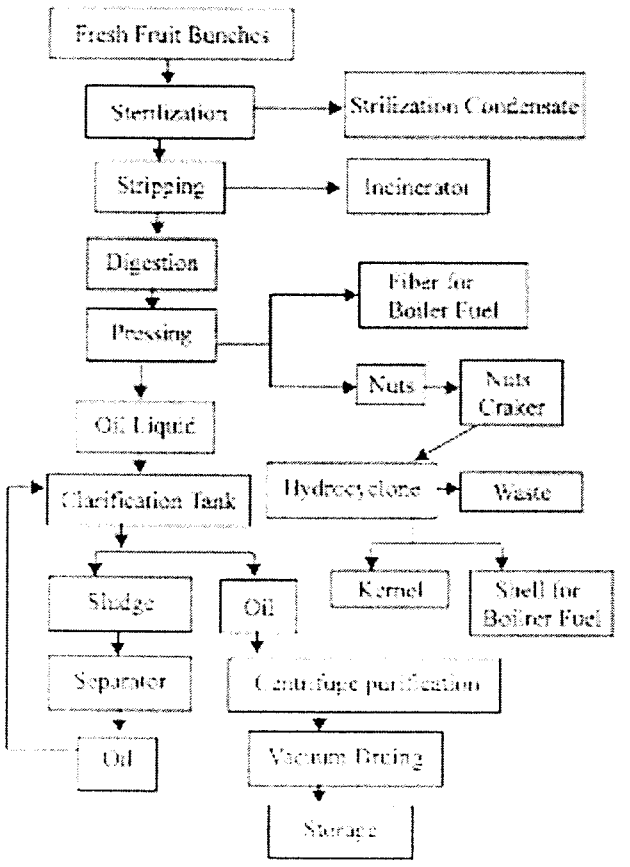

Fig. 2.1 A block flow diagram of the palm oil mill process.

Source: Hui (1992)

The first stage is sterilization, which involves subjecting freshly harvested fruit bunches to the mill and a high pressure steam (120 to 140°C at 40 psi) with a minimal delay. The objective of sterilization is inactivating the lipolytic enzymes that causes oil hydrolysis and fruit deterioration. Afterwards, the sterilized sample was subjected to next step call bunch stripping. This offers a means of separating the fruits from the bunch stalks by mechanical stripping. The separated and sterilized fruits thereafter were achieved by reheating the fruits using steam to a temperature of 80-90°C by a process of digestion to prepare the fruits for oil extraction. Palm oil was extracted by rupturing the oil bearing cells in the mesocarp and loosening the mesocarp from the nuts. Oil extraction followed by clarification and purification are the last processes of oil extraction. The crude oil is extracted from the digested fruit mash by the use of the screw press without kernel breakage (Hui, 1992). The extracted liquid and nuts are discharged from the screw press. However, the extracted oil contains varying amounts of water, solids and dissolved impurities that must be removed. The fiber particles from the pressed crude oil are first removed by passing the oil over a vibrating screen; sand and dirt are allowed to settle. Water is removed by settling or centrifuging and finally by vacuum drying. About 0.1-0.25% of moisture was obtained from the clarified crude oil after vacuum dry (Hui, 1992). The relatively low moisture content helps in maintaining oxidative stability and also prevents the deposition of small amounts of soluble solids known as gums. The final product is consumed locally as crude palm oil or can further be refined (Igwe and Onyegbado, 2007).

Palm oil contains minor components that demonstrate major nutritional and health benefits. The micronutrients are listed in Table 2.4.

Table 2.4 Micronutrients and other minor components of palm oil.

Micronutrient/component	**Range (ppm)**
Carotenoids	
α-carotene	30.0-35.16
β-carotene	50.0-56.02
Lycopene	1.0-1.3
Total carotenoids	500-700
Tocopherol	
α- tocopherol	129-215
β- tocopherol	22-37
γ- tocopherol	19-32
δ- tocopherol	10-16
Total tocopherol	500-600
Tocotrienol	
α- tocotrienols	44-73
β- tocotrienols	44-73
γ- tocotrienols	262-437
δ- tocotrienols	70-117
Total tocotrienols	1000-2000
Phytosterols	326-527
Phospholipids	5-130

Table 2.4 (cont.)

Micronutrient/component	Range (ppm)
Squalene	200-500
Ubiquinones	10-80
Aliphatic alcohols	100-200
Triterpene alcohols	40-80
Methyl sterols	40-80
Aliphatic hydrocarbons	50

Source: Mba et al. (2015)

These micronutrients include carotenoids, tocopherols, tocotrienols, sterols, phospholipids, glycerolipids and squalene (O'Brien, 2010). The carotenoids, tocopherols and tocotrienols maintain the stability and quality of palm oil and also act as biological antioxidants (Wu and Ng, 2007). The tocopherols and tocotrienols act as anti-cancer, antiinflammatory agents (Wu et al., 2008), control atherosclerosis, and decrease cholesterol (Das, Nesaretnam and Das, 2007). The growing interest in the bioactivities of these micronutrients has led to the development of functional foods or nutraceuticals incorporated with phytosterols, tocopherols, and tocotrienols (Zou et al., 2012). Carotenoids are responsible for the diversity of color in nature. Alpha-carotene, β-carotene, and cryptoxanthin have demonstrated provitamin A activity. Beta-carotene is the most potent provitamin A carotenoid. Vitamin A is necessary for vision, growth, cellular differentiation and other physiologic functions (Hendler and Rorvik, 2008). CPO contains 500– 700 ppm of carotenoids and is thus the natural richest source of carotenoids. CPO contains

33% α-carotene, 65% β-carotene and 2% other carotenoids such as γ-carotene and lycopene (Ng et al., 2012). The carotenes are responsible for the rich orange-red color of CPO. They act as antioxidants by trapping free radicals, neutralize thiyl radicals, chelate peroxy radicals and quench singlet oxygen in lipids. Stated simply, carotenoids protect the oil against oxidation by themselves being first oxidized before the oxidative attack on the triacylglycerols (Edem, 2002; Gunstone, 2011; Hendler and Rorvik, 2008). In 1992, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) accepted and included palm oil carotenoids as a permissible food colorant (Zou et al., 2012). CPO has been proposed as an alternative treatment for vitamin A deficiency. The digestibility of α- and β-carotene found in CPO is high and this enhances their bioavailability (Benadé, 2003; Edem et al., 2002). Rice and Burns (2010) reviewed a series of key intervention studies designed to investigate the impact of using red palm oil to improve the status of vitamin A. The review's focus was related to the use of palm oil in dietary supplementation and food fortification studies. The conclusion stated that red palm oil increased dietary intake of provitamin A carotenoids especially β-carotenes which are more abundant and better converted than α-carotenes. Palm oil is highly effective in improving vitamin A status amongst populations at risk of vitamin A deficiency. Tocopherols and tocotrienols (termed tocochromanols) are usually called vitamin E. They are fat soluble. They have a chromanol head, formed by phenolic and heterocyclic rings, and a phytyl tail. The number and position of methyl substitutions on the chromanol nucleus give rise to the subfamily of α-, β-, γ-, and δ-tocopherols/tocotrienols.

Alpha tocopherol is the most abundant. The difference in the structure of tocopherols and tocotrienols is only in the phytyl tail. The tocopherols have a saturated tail, while the tocotrienols have an unsaturated chain with three isolated double bonds (Rossi et al., 2007; Zou et al., 2012). The tocopherols and tocotrienols are present at different concentrations depending on the type of vegetable oil and its origin (Gunstone, 2011). Palm oil is one of the richest sources of vitamin E in nature. The vitamin E in palm oil is unique since it is composed of both tocopherols and tocotrienols. CPO contains 600–1200 ppm vitamin E. Tocopherols account for 18–22% while tocotrienols account for 78–82%. Amongst the tocotrienols, the major ones are γ-tocotrienol, α-tocotrienol and δ-tocotrienol (O'Brien, 2010; Ping and May, 2000; Zou et al., 2012). Some vitamins E in CPO are lost during processing and refining. During fractionation, vitamin E tends to partition preferentially into the olein fraction (Obahiagbon, 2012; Sambanthamurthi et al., 2000; Sundram et al., 2003). Recent findings showed that palm oil's tocotrienols significantly diminish the synthesis of pro-collagen 1 and 3; and inhibit the transforming growth factor-β1. These are responsible for the type of inflammatory bowel disease known as Crohn syndrome (Luna et al., 2011). The stability of the different tocopherols and tocotrienols present in the refined vegetable oils basically depend on the fatty acid composition of the oil, and the type of tocopherol and tocotrienol homologs present. The homolog, γ-tocotrienol in palm super olein proved to be the least stable during the deep-fat frying, thus preserving the other homologs (Rossi et al., 2007). As antioxidants, tocopherols and tocotrienols act as free radical quenchers which contribute to

the stability of palm oil. Tocopherols can interrupt lipid oxidation by inhibiting peroxide formation in the chain propagation step, or the decomposition process by inhibiting aldehyde formation. Alpha tocopherol is reported to be highly reactive towards singlet oxygen and protects the oil against photooxidation (Sundram et al., 2003). The tocotrienols and the isometric position of its fatty acids are credited as being responsible for palm oil's nutritional benefits (O'Brien, 2010). Carotenoids, along with vitamin E, protect the oil from thermal oxidation. During thermal oxidation carotene radicals are formed which are converted back to active carotene in the presence of tocotrienols. Schroeder et al. (2006) reported that this synergistic relationship decreased the oxidation of oil during frying of potato slices at 163 1C. Other minor components of palm oil such as the sterols, higher aliphatic alcohols and hydrocarbons are found in the unsaponifiable fraction. Similar to all other edible oils of vegetable origin, the cholesterol content of palm oil is negligible. Refining decreases the phytosterols, ketones, wax and methyl esters present (Edem, 2002; Sambanthamurthi et al., 2000). Ping and May (2000) reported that, generally the minor components act as antioxidants, boost energy, enhance the immune system and provide benefits in the prevention and treatment of coronary heart diseases (CHD). Palm oil also contains low levels (100 mg/L) of phenolic compounds. The phenolic compounds are responsible for the initial darkening of palm oil during frying (Berger, 2005; Sundram et al., 2003). Like all vegetable oils, a mixture of sterols is found in CPO, palm olein and their refined products. The sterols found in palm oil include β-sitosterol, campesterol, stigmasterol and

avenesterols. Amongst them, avenesterols exhibit antioxidant activity (Berger, 2005; Gunstone, 2011). The sterols of plant origin are referred to as phytosterols. Phytosterols are susceptible to oxidative degradation during food processing operations such as frying. During frying, phytosterols degradation occurs due to auto-oxidation. The products of this degradation are termed phytosterols oxidation products (POP). The POP can be found in both frying oil and the fried products (Dutta et al., 2007). Tabee et al. (2009) reported that the POP found in palm olein used in frying French fries at 180 1C for 5 h increased from 1.9 mg/g to 5.3 mg/g in the final batch. The degradation of phytosterols depends on the type of oil and polyunsaturated fatty acids present. Phytosterols appear to degrade faster in oils with high content of linoleic and linolenic acids. The rate of POP formation is also influenced by the type of sterol and the tocopherols content of the oil (Przybylski et al., 1999). Generally there is no clear evidence of a negative effect of palmitic acid on health (Fattore and Fanelli, 2013). CPO is a complex alimentary medium, in which palmitic acid is just one of its many components. The presence of oleic acid and many antioxidant compounds in palm oil provide some form of nutritional balance. While POP are absorbed and found in human serum, they do not directly affect the absorption of cholesterol (O'Callaghan et al., 2014).

1.4 Palm based oils sources for vitamin E

Tocopherols and tocotrienols can be directly extracted from the oil palm fruits, or can be simultaneously obtained from crude palm oil (CPO) extraction process which being carried out in the palm oil mill. In terms of raw material costand availability, it would be more favourable to get the tocopherols and

tocotrienols from CPO or its derivative products in comparison to direct extraction from oil palm fruits. It is because the CPO is the main commodity of oil palm fruits, thus it will be difficult to obtain the raw material supplies for Vitamin E production. By-products and residues from palm oil mills and palm oil refineries also can be used for the source of vitamin E, since they have similar characteristics and components with the CPO. The composition of Vitamin E (tocopherols and tocotrienols) from several palm-based oil materials is shown in Table 2.5.

Table 2.5 Vitamin E content in the several palm-based oil materials

Raw material	Range composition (%)					Total vitamin E
	α-T	γ-T	α-T3	γ-T3	δ-T3	(ppm)
Crude palm oil (CPO)	22-25	1-3	20-25	36-45	7-10	600-1000
Palm pressed fiber oil (PFO)	55-60	0-3.5	13-20	18-23	8-10	2000-4000
Refined bleaching deodorized palm oil (RBDPO)	21-25	-	23-29	36-50	6-10	500-1000
Palm fatty acid distillate (PFAD)	23-24	-	23-24	36-38	13-15	4000-5000
Palm olein	21-24	-	26-28	25-40	6-10	500-1000
Palm phytonutrient concentration (PPC)	8-10	0-4	17-21	55-60	6-7	14000-15000

Source: Maarasyid et al. (2014).

Crude palm oil (CPO), refined bleaching deodorized palm oil (RBDPO) and palm olein are the major products in palm oil milling and refinery, whereas palm-pressed fibre oil (PFO) and palm fatty acid distillate (PFAD) are the by-products of palm oil milling and palm oil refinery, respectively. Palm-pressed fibre is the fibrous waste material from fruit mesocarp after

extracted and separated from kernel in the palm oil mills. It is usually burned in the boiler as fuel to provide the energy in the palm oil mills. May et al. (2003) reported about 5-7% of oil residue in palm-pressed fibre which can be recovered. Although the relative residue oil is lower, higher concentration of Vitamin E isomers and β-carotene content is obtained from PFO (Sanagi et al., 2015).

PFAD is a by-product resulted by deodorizing unit in palm refinery. The aim of this unit process is to remove the free fatty acid components in refined palm oil. However, thermal utilization in deodorizer unit also indirectly led to the elution of Vitamin E content, resulting 0.5% wt. of Vitamin E content in PFAD (Posada et al., 2007). Similarly with PFAD, PPC is also a by-product of vacuum distillation which is obtained in refinery of biodiesel. Since it is derived from CPO, the occurrence of individual vitamin E components in CPO and the phytonutrient concentrate would be identical.

The refined product of palm oil mill and palm oil refineries contain only small amount of tocopherols and tocotrienols. It also tends to have a reduction in total vitamin E from the palm oil mill to product of palm refineries, as shown in Table 1. Wei et al. (2007) also observed that there is degradation of tocopherol concentration from CPO to deodorizing product during the physical refining.

On the contrary, there is an accumulation of vitamin E in the by-product of each stage. It also seems an enhancement of total Vitamin E in the by-product from palm oil mills to palm oil refineries. The utilization of these products is also limited. Therefore, PFO, PFAD and PPC as the by-products

and the residues from the palm oil mills can be effective sources as the raw material of Vitamin E.

1.4.1 Vitamin E extraction from palm based oil

Extraction of Vitamin E can be achieved by several methods as shown in Table 2.6. Solvent-based extraction is a conventional method and widely applied to extract the natural product. It usually uses organic solvents such as hexane, chloroform and short chain alcohols. For the food industries, short chain alcohols (ethanol and isopropanol) are preferred to be used than hexane and chloroform, due to the health hazard issues.

Table 2.6 Several extraction methods the separation of vitamin E

Method	Information
1. Solvent Extraction	
a. Direct Extraction	Compare three extraction methods to determine vitamin E in peanuts and peanut butters. The extraction methods involve direct solvent extraction with hexane:ethyl acetate, saponification, and soxhlet extraction with hexane (Lee et al., 1998)
b. Soxhlet Extraction	Extraction of tocopherols and phytosterols from soybean oil deodorizer distillate by using modified soxhlet extraction with severals organic solvent (Kasim et al., 2010)
c. Pressure Liquid Extraction (PLE)	Extraction of vitamin E and carotene in residue oil from palm pressed fiber using through pressurized liquid extraction with n-hexane (Sanagi et al., 2005)
d. Supercritcal Fluid Extraction (SFE)	Recovery the high value substances such as FFA, tocopherols, sterols and squalene from soybean oil deodorizer distillate by using the SC-CO_2 distillation-extraction (Chang et al., 2000)
2. Enzymatic Process	Extraction of sterols and tocopherols from severals oil deodorizer distillate through lipase-catalyzed modification (Ramamurthi and McCurdy, 1993)
3. Chemical Modified	
a. Saponification	Optimize the parameters of saponification process on the extraction of vitamin E from tomato and broccoli (Lee et al., 2000)

Table 2.6 (cont.)

b. Esterification	Recovery tocopherols from rapeseed oil deodorizer distillate though a process involving acid-catalysed methyl esterification and followed by molecular distillation (Jiang et al., 2006)
4. Adsorption	Determination of tocopherols and tocotrienols from palm fatty acid distillate using hydrolysis, neutralization, adsorption and chromatography methods (Chu et al., 2003)
5. Molecular Distillation	Extraction of tocopherols using molecular distillation with the comparison of with and without preparation (saponification) (Martins et al., 2006)
6. Microwave-assisted Extraction	Evaluate the fffect of temperatures on vitamin E extraction from rice brain using microwave-assisted method with solvent (isopropanol and hexane) (Zigoneanu et al., 2008)
7. Membrane Technology	Determine the membrane selectivity for tocopherol using hexane dilution from esterified and crude soy oil deodorizer distillate (Nagesha et al., 2003)

Source: Maarasyid et al. (2014).

Also, ethanol and isopropanol tend to extract more non-glyceride material than hexane due to their greater polarity (Hu et al., 1996). Lee et al. (1998) have found that direct solvent was the best method for extracting Vitamin E from peanuts and peanut butter, in order of quantify of yield. Unfortunately, direct solvent is usually a laborious procedure which requires high purity and high cost of solvent. Its procedures also need a long time period (Jiang et al., 2006 Sahena et al., 2005); . Another report from Lim et al.

(2007) claimed that soxhlet extraction was the best method to extract Vitamin E which obtained yield of 13% higher than direct solvent. Later, Kasim et al. (2010) used the modification in soxhlet extraction and could recover up to 83% of Vitamin E from soybean deodorizer distillate.

There are several processes to extract the vitamin E from palm-based oil. However, the weakness of each method has not completely recovered the problems faced during the process of extraction. Rapid process and high recovery of vitamin E obtained are the desired achievement of the process. This achievement must be balanced against the cost for the process that involving the investment cost and the operational cost for the process (amount of solvent and energy used for the high temperature and pressure). Recently, environmental and health aspects have also been considered. Reduction in the use of organic solvents, combinations of various extraction processes, and prospecting environmental friendly method are developed. Therefore, process design and selection for the chosen technology are crucial in order to determine the recovery of vitamin E obtained, time efficiency, cost effectiveness, safety of products and environmentally friendly impacts. A green technology approach could be further diversified and manifested for sustainable process of vitamin E.

1.5 Lipase production from palm oil

Lipase are special kind of esterases belong to sub class 1 of hydrolytic enzymes class 3 and have been assigned sub-sub-class 3.1 due to their specificity for carboxylic acid ester bonds. They have thus been called carboxylic acid esterases and numbered as E.C.3.1.1 according to the

classification recommended by enzyme commission of the International Union of Biochemists. Glycerol-ester hydrolases or triglycerides acyl hydrolases (E.C.3.1.1.3) are generally known as lipases and may not have positional specificity for the primary ester bonds. Usually lipase hydrolyze triacylglycerols stepwise to diacylglycerols, monoacylglycerol and free fatty acids. The most prominent member of the group is pancreatic lipase along with monoglyceride lipase and galactolipase. The sequential breakdown of triacylglycerol by lipase shown in Fig 2.2, lipase do not attack the secondary ester bond of triacylglycerols and it has been proposed that 1 or 2-diacylglycerol intermediate isomerized to 1 or 3-isomer form and then primary esters are hydrolyzed. However, in subsequent studies demonstrated the sequence of reaction as under: Triacylglycerol+1,2-diacylglycerol+FFA → 1- or 2-monoacylglycerol+FFA+glycerol+FFA.

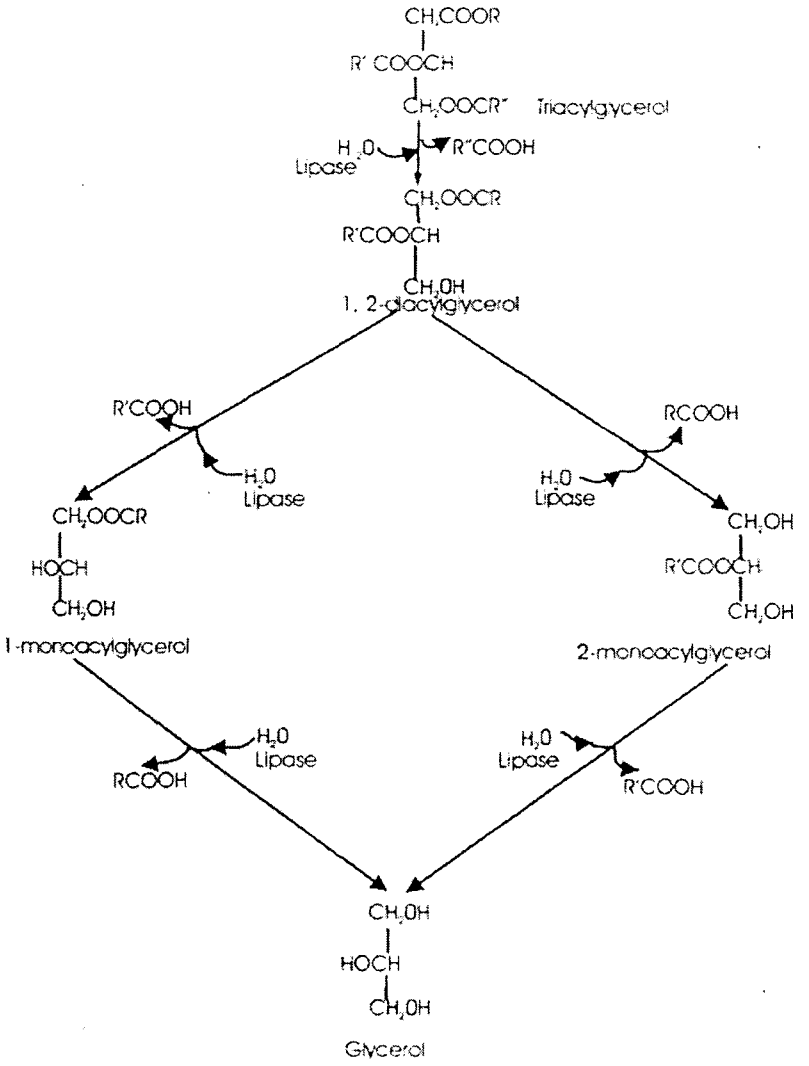

Fig. 2.2 Reaction mechanism of lipase

Source: Pahoja and Sethar (2002)

The presence of 1,3-diacylglycerol during the hydrolysis was wxamined by an acyl transfer reaction. Lipase catalyzed not only the hydrolysis of triacylglycerols but also synthesis of ester bonds by transesterification. 1-monoacylglycerol+Fatty acid → 1,3-diacyglycerol. The primary ester group is attached preferentially with 1,2- or 1,3-diacylglycerols and are hydrolyzed at similar rates. The overall reaction is not specific for the position on the glycerol molecules. The enzymatic reaction of lipolysis present in biological

system is an example of heterogeneous catalysis. The lipases are perfectly soluble in water but the substrate are not and these reactions usually carried out by making emulsion of the substrate in order to provide a oil-water interfacial area to which the enzyme binds with substrate. The individual catalytic steps occur essentially at the lipid/water interfaces and this unique property is known as interfacial activation. The interfacial activation is associated with conformational change in the lipase molecule. Generally, glycerol trioleate (olive oil which contains 70% oleic acid) is used as substrat for the detection or determination of lipase activity and it is acceptable as universal and low cost substrate.

Lipases are widely distributed in plants, animals and microorganisms. In plants mostly lipases are present in food reserve tissues of growing seedling and especially in those which contains large amount of triacylglycerols. The storage triacylglycerols are localized in the organelles called lipid bodies (oleosomes, oil bodies, spherosomes) which are surrounded by a membrane. Lipase is the first enzyme in the gluconeogenic pathway, and is associated with the membrane of the lipid bodies where the intracellular lipid is localized. Lipase activity in plant seeds increases rapidly after germination of the seeds. In seeds containing oil (mainly triacylglycerols) as storage material, it is obvious that lipase action is essential in germination when reserve (insoluble) triacylglycerols are converted to soluble sugars which can be transported to the growing tissues to supply structural carbon and energy. Lipase hydrolyze triacylglycerol to glycerol and fatty acids which are converted to sugar and support the growth of young plants. Fatty acids released by lipase act as

precursors for the phospholipid synthesis necessary to support proliferation of cellular endomembrane systems which may be their primary role in the germination process. The growing interest in this area arises from the potential applications of lipase. Among the enzymes so far investigated, lipase is one of the most advantageous because it is stable, inexpensive and widely used in the development of various applications. Lipases are produced from different sources such as plants, animals and microorganisms.

The amount of enzyme is not determined on the basis of its catalytic activity but it is estimated by measuring the decrease in substrate concentration or measuring the product concentration with the passage of time under the use of appropriate method of assay. Enzymologist have developed a number of assay methods in an attempt to throw some light on the complex enzymatic interactions involved in the breakdown of triacylglycerols and other lipids. For both detection and assay of lipases, it is essential that substrate be chosen and reaction conditions arranged so that the definition of a lipase be met i.e. hydrolysis of long-chain acylglycerols at an oil-water interface. Generally lipase is present in low amount in crude subcellular fractions, therefore the assay method should be sensitive and rapid. Mostly assay methods are based on the release of free fatty acids or labeled fatty acids from natural and radioactive substrates. Various methods have been developed to measure free fatty acids such as titration, calorimetric, fluorometric, thin layer chromatography and gas chromatography.

1.5.1 Lipase applications

Lipolytic enzymes are widely used in the manufacturing process throughout the world in varied and interesting applications. In recent years the growing demand of lipolytic enzymes has been increased due to its potentials use in the various manufacturing process of industrial goods such as detergent industry, food industry and medicine, which inspired to search new sources for enzyme isolation. Lipases are incorporated with the products of pharmaceuticals, cosmetics, leather, detergents and perfumery. They are also used in the medical diagnostics and other organic synthetic materials. In medical and therapeutic applications the manipulation of lypolytic activities will probably play a part in future methods for treating malfunctions of fat metabolism and thus control cardiovascular disease. The pancreatic lipase is necessary for the absorption of fat. Hormone-sensitive lipase is used for the mobilization of fat from adipose tissue. The assay of serum lipase is a clinical tool of some importance in the diagnosis and to check the severity of pancreatitis. Microbial lipases are used in combination with other enzymes to help in the degradation of sewage. An acid lipase has been added to bread dough for the uniform production of monoglycerides which greatly improve the resistance of the bread to staling. It is observed that uncontrolled lipolysis during milk processing can cause flavor defect and accumulation of free fatty acids in the extraction of cotton seed oil due to undesirable action of lipase requiring extra operation for their removal prior to marketing. They are many report of the use of lipase in a variety of industrial process such as removing

of fat strains from fabrics, accelerating maturation in cheese and degrading fats in waste products.

1.5.2 Plant lipases

Lipases activity has been identified in the various tissues of plants but relatively high concentration is found in seeds. Seeds are generally rich in triacylglycerols, which serve as compact source of energy for the newly emerging plant. During germination of the seed, the reserved triacylglycerols are disappeared, since the fatty acids cannot be oxidized to provide energy until they are released from the triacyglycerol. Lipases are probably rate controlling during germination and the activity of the lipase is high during germination.

Cereal seeds contain significant amount of triacylglycerols normally concentrated in germ (embryo) and outer layers of the grains, although oats (5-9% oil) are unusual in having most of the oil in the endosperm. Because lipolytic activity in these seeds causes major problems in their commercial use, the responsible enzymes have received attention, particularly in wheat, rice and oats. Lipase plays a central role in the release of fatty acids from the reserve triacylglycerols of lipid bodies in the aleurone and scutellum of germinating cereals. Lipases are synthesized de novo on free ribosomes in the scutellum of germinating maize and are translocated to the membrane of the lipid bodies where they remain bound. Lipase synthesis in the aleurone is more complicated. Significant lipase activity is detected in isolated barley aleurone layers before gibberellic acid application, but preliminary imbibitions of half grains may induce its synthesis. Lipase activity in rice is concentrated

in the outer layer of the grain and causes problem in the storage. The enzymatic properties of rice bran lipase that maximum activity was observed at pH 7.5 and 8.0, but stable over the pH range from 7.0 to 9.0. The optimum temperature was 37°C and the enzyme was stable up to 40°C. The enzyme was activated by calcium ion (<0.01M) but inhibited with 0.1M. Lipase activity was also strongly inhibited by EDTA. It was more specific for glycerol than alkyl alcohol. Moreover, the enzyme is capable of hydrolyzing the olive oil, rice bran oil, and coconut oil, in addition to synthetic triacylglycerol and trioelin. The hydrolysis rate was maximum at C_4 and decreases until C_{18}, among these triolein was hydrolyzed at the lowest rate. The enzyme catalyzes the hydrolysis of short chain ester bond faster than in longer ones. The hydrolysis of 2-oleo -1,3-disterin resulted in the formation of monoacylglycerol, 1,2-diacylglycerol; 1,3-diacylglycerol and fatty acids. Rice bran lipase preferentially splits fatty acid at the 1,3-position of substrate. The enzymatic properties of a galactolipase from rice bran that its molecular weight was about $4x10^4$ and the Km value 0.34 mM for monogalactosyl diacylglycerol. The enzyme exhibited maximal activity at pH 7.5 in a Tris-HCl buffer and its optimum temperature was 35°C. It lost 95% of its activity at pH 7.5 at 50°C. The enzyme activity was inhibited by EDTA, Triton X-100, acetyltrimethylammonium chloride, NaCl and sodium dodecyl sulfate. Whereas, the activity increased in the presence of Ca^{2+} and sodium deoxycholate. Maximum hydrolytic activity was found with digalactosyldiacylglycerol. Monogalactosyldiacylglycerol and its lysolipids were hydrolyzed at about two-third of the rate of digalactosyldiacylglycerol.

Cystein, mercaptoethanol, and diisopropyl flourophosphate inactivated the purified galactosidase. It catalyzes the hydrolysis of the fatty acid ester at C_1 and 2-positions of the galactolipid. The purification sequence showed a 480 fold increase in specific activity. The purified enzyme was electrophoretically homogeneous by polyacrylamide disc gel electrophoresis and ultracentrifugation. The isoelectric point of the enzyme was found 8.56 by ampholine electrophoresis and the sedimentation coefficient was 2.97s with maximum and minimum absorption at 278 mμ (nm) and 250 mμ (nm), respectively. The molecular weight of lipase was estimated 40,000 and the value of extrapolation to zero time was 0.800 and 0.835. The optical rotator dispersion (ORD) constant values of a_o and b_o at 239 mμ (nm) were -164 and -123, respectively. The chemical properties of rice bran lipase that the enzyme was comprise of disulfide linked sub units with molecular weight of 32000. Rice bran lipase hydrolyze long chain triacylglycerols and rice bran oil less readily than triacylglycerol of short chain (C_2-C_4) fatty acids.

The lipolytic enzyme activity of buckwheat was 880 pmol/min/mg which was 7 times higher than wheat flour. The water activity above 0.28 did not depress the lipolytic enzyme activity during 1 month storage at 25°C. The activity was inhibited at 120°C within 7 sec. and increases free fatty acids. The accumulation of free fatty acids in buckwheat during storage are mainly caused by lipase. The induction of lipase activity in the germinating wheat grain yield the maximum lipase activity upto 6 days of germination in the starchy endosperm. Lipase activity in the tissue of endosperm halves incubated with 1 mM hydroxylamine was optimum at 4^{th} day. In endosperm

halves, lipase activity can be induced in the starchy endosperm by glutamine and hydrolamine. The activity in the bran of endosperm halves is induced by indol acetic acid in the presence of nitrogen sources. Both of these induction processes are inhibited by energy metabolism inhibitors, RNA and protein synthesis. The bran pre-incubated with cytokinin, hydroxylamine and indol acetic acid were able to induce lipase activity slowly.

Oat contain a true lipase that hydrolyzes triolein and tributyrin. This is an alkaline lipase (optimum pH 7.4). The lipase activity of oats was found mainly in the epidermal layers. It is important to know that it is essentially a property of the seeds, and does not arise from subepidermal fungal mycelium, which is known to occur in some specimens of grain. This lipase has been purified some 2000 fold on a dry matter basis compared with the activity in the original oat meal. The purified preparation has an optimum temperature of 37-38°C, and a Michealis-Menten constant (Km) of 0.006 M when tributyrin is the substrate. The purified lipase splits off one butyric acid radical only from tributyrin and does not hydrolyze the various mono- and di-tributyrins at pH 7.4 and 37°C. Lipase activity of *Cajanus cajan* L. (matri) was found maximum upto 1 hr and then declined. The rate of reaction was increased upto 20% enzyme solution with 10% substrate concentration. The maximum activity was found at pH 5.5 and at temperature of 30°C. The enzyme was activated in the presence of Ca^{2+} and Zn^{+2} but it was inhibited with EDTA, Mn^{2+}, Co^{2+} and mercaptoethanol. The enzyme was highly stable upto 40°C. The activity was thermostable and retains 3% activity at 90°C within 10 min. The lipase activity of *Cajanus cajan* was highly specific towards tristearin.

Dialysed sample of *Cajanus cajan* seeds was purified on Sephadex G-100 column and it was separated into four fractions (I, II, III, IV) which were subjected to SDS-disc gel electrophoresis. Fraction II was found homogeneous and the molecular weight was estimated 19000 by SDS-polyacrylamide disc gel electrophoresis. The optimum temperature of *Cajanus cajan* seed lipase II was found 20°C and pH 5.0. The enzyme was found fairly stable upto 40°C but more than 20% activity remained active after 10 min incubation at 98°C. Since the lipase is intimately linked to the lipid both physically and metabolically, a different in the genetic control of the gluconeogenic enzymes and a co-selection for high lipid content and high lipase activity through breeding. The lipase of lipid bodies and the enzymes of the glyoxysome are under separate genetic control. A noncoordinate expression of their activities occur in the high and low lipid maize lines. Selection for high lipid in maize lines apparently also selects for high lipase activity but not the subsequent gluconeogenic enzymes. The mechanism of coordinate selection for both high lipid and high lipase activity is unknown. There are about 50 genes for the expression of high lipid in illinosis high oil and they are only expressed only in kernel formation and not in seedling growth. Yet, lipase activity is absent in the forming and ungerminated kernel and appears only after germination (post germinative growth). It is unlikely that the genes for high lipid content are tightly linked to the lipase genes. It is possible that the high lipid genes and the lipase genes are both expressed in kernel formation but the later expression results in the production of pro-lipase protein or mRNA, which is processed to active lipase in post germinative growth. Alternatively, the lipases synthesized

or degraded in post germinative growth in proportion to the availability of substrate and thus metabolic need.

1.5.3 Lipase activity in oil seeds

During germination of oil seeds the utilization of the storage fats is initiated by the stepwise hydrolysis of the triacylglycerides to free fatty acids and glycerol. Lipolyti activity in a range of germinating oil seeds was evaluated in castor bean, none contained acid lipase activity, but all had an alkaline lipase activity in their storage tissues. During the germination of peanuts, the lipase activity was increased rapidly and it was highest at pH 9.0. The subcellular localization of alkaline lipase was studied by using sucrose gradient centrifugation of peanut cotyledon homogenate of 3-days old seedling. About 60% of enzyme was found to be associated with glyoxysomes, 15% with the mitochondria, and 25% with a membrane fraction. The glyoxysomal lipase hydrolyzed only monopalmitin, where as the enzyme associated with the mitochondria and the membrane fraction hydrolyzed equal amount of tri-, di- and monoacylglycerol. The intracellular localization of lipase activity in *Brassica napus* L. (rape seed) cotyledons. The growth of the seedlings was observed in the light and in the dark. The higher lipase activity was found at 4^{th} day of seedling growth when crude homogenates of the cotyledons produced about 5 μmol of fatty acids/min/pair of cotyledons with sunflower oil as a substrate at 37°C with pH 9.0. The maximum activity was observed at pH 8.5 with N-methylindoxyl-myristate as a substrate. Significant effect of lipase was not observed on the development of lipase activity from the cotyledons after fat utilization but it may be dependent on nitrogen nutition

of seedlings. Crude homogenates by sucrose density gradient centrifugation yields two major and one minor visible protein bands in the gradients. About 90% of the lipase activity was associated with a microsomal membrane fraction by sucrose solution. One major protein bands was recovered from the gradients at a density of 1.085 kg/L and lipase activity was maximum in this protein band. Some properties of a membrane bound triacylglycerol lipase of rape seed cotyledons. The apparent Km of the lipase reaction was 6.5 mmol/L with sunflower oil. About 25% of the total radioactivity was lost from the triacylglycerol within 60 min and 23.5% were recovered from the fractions representing 1,2- and 1,3-diacylglycerol, monoacylglycerol and free fatty acids. In addition, increasing amount of free glycerol accumulated in the text mixture, indicating that the lipolytic membranes of rape seed cotyledons are capable of splitting triacylglycerol into glycerol and free fatty acids. The rate of hydrolysis was highest with sunflower oil followed by linseed oil. In the presence of linoleic acid or oleic acid in sunflower oil, lipase activity was completely inhibited. Erucic acid, a major component of rape seed oil blocks the lipase activity. The microsomal lipase of rape seed cotyledons is also affected by the presence of inorganic ions and detergents in the assay mixture. Lipase activity was increased in the presence of 0-0.1mol/L NaCl and above this concentration the system was obviously saturated with the respect to the salt. Pre-incubation of the lypolytic membrane fraction in 0.15 mol/L NaCl solution increased lipase activity while sodium deoxycholate and $CaCl_2$ stimulated the activity; and EDTA, triton X-100 and Tween-85 also inhibited the activity. The lipase activity was present in the soluble cell fraction and the

oil body fraction of cotyledons of 4-day-old rape seed. Optimum activity was obtained with triolein emulsion as substrate at pH 9.0. Addition of deoxycholate to the triolein substrate emulsion improves the stabilization of the surface of the lipid globules and shifts pH optimum to pH 8.0. In contrast, rape seed oil emulsified in gum Arabic was a poor substrate for the soluble lipase. The apparent activity of lipase was enhanced with the addition of deoxycholate to the emulsified rape seed oil and fatty acids. 1,2 or 2,3-diacylglycerols, and monoacylglycerols were produced at alkaline pH values. The autolysis of a crude oil body fraction demonstating the lipolytic activity associated with these subcellular compartments by the liberation of free fatty acids from the native neutral lipids with maximum activity at pH 5.0. Lipase prepared from 4-day old rape seed seedling hydrolyzed the triacylglycerols of the native oil bodies. Native oil bodies purified from cotyledon of rape seed started to show autolysis between day 1 and 2 of germination, with optimum activity at pH 5.0. While optimum activity of the cytoplasmic (soluble) lipase was detected at pH 7.0 with native oil bodies as substrate. However, such lipase catalyzed degradation was found only with oil bodies isolated from seedlings at least 2 days old, but not with oil bodies obtained from dry seeds or 1-day old seedling. The inhibition of the action of lipase is caused by the chaff unit membrane, surrounding such oil bodies. These data cast doubt on the role of the prominent oil body proteins (oleosins) in anchoring lipase. It is proposed that the interaction of lipase with the surface of the oil bodies relies on negatively charged constituents of the oil body coat, which are different from the oleosins. In early germination the activity of acylester acyl-hydrolase

rise prior to lipase activity, it should be assumed due to the initial enzyme in storage oil mobilization in germinating rape seeds.

The free fatty acid level of palm oil is a major determinant of quality and commercial value. It is therefore important to know the reasons why fatty acids appear, and lipase of various kinds have been implicated. Oil palm mesocarp contains a very active endogenous lipase. As much as 40% of the triglyceride present in mesocarp could split in 15 min. Purified fungal lipases split fatty acids from triacylglycerides at the rate of the order of 2000 μmol min^{-1} mg^{-1}. If the specific activity of the enzyme in mesocarp was similar to that of fungal lipases, the remarkably high rate of fatty acid production would have needed about 1% lipase in mesocarp tissue. The mesocarp contains only about 1% protein and it seems unlikely that the whole of this would be lipase. Most fruit contain only a few percent fatty acids and the level is variable. This has been attributed to variable activation of supposed endogenous lipase by for example, bruising of the fruit. This is hardly conclusive evidence for an endogenous lipase, since heating to 100°C would eliminate lipase from any source, as well as minimizing the microbial population. It is generally accepted that fungi can indeed bring about lipolysis of oil palm mesocarp. Lipase activity in ripening and mature fruit of the oil palm extract was inactivated *in vitro* above 0°C but *in vivo* the enzyme activity increase as ripening proceeds. The highest activity was present in the ripest fruit coincident with the abscission of the fruit base from the pedicel. Activity rose from background values in the unripe fruit to just detectable in fruit that were first synthesizing carotene and lipid (Ca 125-130 days after synthesis).

Lipase activity of this aqueous layer was always lower than that of the equivalent of frozen mesocarp powder. The enzyme activity of both aqeous extracts and mesocarp powder decreased after 30 min incubation with the increase of assay temperature from 18 to 35oC and this was assumed to be associated with a greater instability of the lipid micelles at these temperatures. Tween 20, Tween 80 or Triton X-100 in the extraction medium and cocktail increase stability of enzyme activity. Tween 20 was found to be superior for the retaining activity. To improve extractability and stability of the enzyme by increasing ionic strength were unsuccessful. At 0.2 M NaCl, enzyme activity was reduced by 30% in the lipid layer and by 50% by 200mM in the aqueous layer. Addition of Ca^{2+}, papain or PMSF also failed to enhance or prolong the lipase activity in mesocarp powder or in lipid or aqueous layer of the homogenates. Detection of enzyme activity from mesocarp powder [^{14}C] oleat hydrolysis product increase with time. The reaction is linear for at least 20 min at 18oC and the rate of reaction increases with increasing concentration of the glycerol tri [1-^{14}C] oleat substrate in the medium. Maximum product release per 30 min assay occurred at 6 mg from mesocarp powder but inhibition was not observed with increasing sample weight. The lipase activity of ripe fruit is sensitive to chilling, which inactivate enzyme activity at 8°C without restoration of activity at 25°C. However, enzyme activity is stable at least 8 days in fruit held continuously at 20°C or for short period (30 min) at 45°C. The addition of lipid present in the tissue of lipase inactivated ripe fruit neither enhances activity from unripe mesocarp or reduces the activity from ripe mesocarp. An active lipase enzyme is indeed absent from unripe fruit. The

presence of lipase activity in the *Elaeis guineensis* (palm oil) seedlings. An active lipase were found to be present in shoot extracts during germination and reached highest after 18 to 21 days. The optimum pH for lipase activity was found to be 6.2 and enzyme was more active with monopalmitin in comparison to other substrates.

Eight seed oils from *Vernonla galamensis*, *Ximenia kaffra*, castor, corn, soybean, palm kernel, sunflower and olive as a substrate for the action of lipase (acetobe powder) followed by methylation using diazomethane in ethyl ether. The *V. galamensis* lipase (acetone powder) is probably nonspecific in the hydrolysis of triacylglycerols. The reaction products were analyzed by gas chromatography and mass spectrometry and the presence of different fatty acids, confirm the lipolytic hydrolysis of the lipase (acetone powder). The lipolytic hydrolysis of triacylglycerol probably does not result in isomerization because the resulting fatty acids are unaffected during hydrolysis. The fatty acids obtained are representative of the fatty acid spectrum of the parent oil. In the scutella of corn, lipase activity is present in the lipid bodies of germinated but not ungerminated seeds. It has an optimal activity at neutral pH values, and is highly specific for the native corn triacylglycerols. The lipase activity in lipid bodies increased during seedling growth, parallel to the increase of catalase activity. At 6^{th} day, the lipase activity releases 47 nmol fatty acid/min/scutellum at 34°C. The lipid bodies were isolated from ungerminated seeds and the maximal autolytic activity was found at pH 7.5. About 50% of the lipase activity was recovered in the lipid bodies, and the rest was distributed among other subcellular fractions. The optimum activity was found

with trilinolein, or N-methylindoxylmyristate at pH 7.5. Lipase activity was slightly enhanced in the presence of 0.05 M NaCl and was reduced by $CaCl_2$, EDTA and phosphate. The enzyme activity was completely lost by Triton-X and SDS. The lipase was found active on trilinolein and triolein but it was totally inactive on tristearin, tripalmitin, trierucin, triarachidin and tribehenin. The corn lipase was purified by DE-52 ion exchange chromatography and sucrose density gradient centrifugation from isolated lipid bodies. Only one protein band was detected by SDS PAG electrophoresis. The molecular weight was 65,000 by electrophoresis but 270,000 by sucrose density gradient centrifugation. The lipase was purified about 272 fold. Total proteins are associated with the membrane of the isolated lipid bodies but the lipase contained a substantial amount of neutral amino acids. Glyoxysomal lipase was purified from castor bean glyoxysomal membrane by PEG fractionation, CM-cellulose and hydroxylapatite chromatography. Only one band was visible after SDS-polyacrylamide gel electrophoresis and the molecular weight was estimated to be 62,000. The reaction was stimulated by Na^+ and K^+ salts. Deoxycholate, octylglucoside and Triton-X 100 inhibited the reaction. Triricinolein (the endogeneous lipid) was hydrolyzed by the purified enzyme. However, when intact glyoxysomes in 0.5 M sucrose were incubated in trypsin the lipase activity was rapidly inhibited, but the isocitrate was scarcely affected. An antibody preparation raised in a rabbit against the purified enzyme, inhibited the purified enzyme in glyoxysomal membranes. The lipase activity was completely inhibited by mercuric ion and PCMB, but the inhibition was reserved by cysteine and EDTA. The dry seeds of castor bean

showed a little or no activity of lipase. But highest activity was found at pH 9.0 during the seed germination. Castor bean contains two types of lipase activities such as acidic and alkaline in nature. In dry castor bean, an acidic active lipase was present but it was disappeared completely after 6^{th} day of germination. In contrast alkaline lipase was absent in the dry seeds but its activity increased markedly after 6^{th} days of germination. Total lipid of the castor bean endosperm remains unchanged until the beginning of day 3, and completely disappeared at day 7. Simple lipid comprised essentially the entire lipid until day 5. The enzyme in the fat layers shows optimal activity at pH 5.0. The Km values of acid and alkaline lipase for *N*-methylindoxylmyristate were 1.67 and 0.23 mM, respectively. The fatty acyl-ester hydrolase from post germinated sunflower seeds. Fatty acyl ester hydrolase was not detectable in dry seeds of sunflower using various *p*-nitrophenyl caprylate or emulsified sunflower oil as substrate. After imbibitions of seeds enzyme activity slowly developed in cotyledon and was maximal after 5^{th} day. The triacylglycerol level was began to decrease after one day and showed a continuous decrease throughout the post germinating period. One main activity peak was eluted upon performing Macro-preparative anion chromatography and one band was visible under SDS-PAGE analysis. The molecular mass was 44 kDa and purification factor was close to 615 with a yield of 6.7% and was found homogeneous by SDS-PAGE electrophoresis. The total number of amino acids was 459 for molecular mass of 45 kDa. The glycine and serine content were high. The maximum absorbance of the protein was at 277.5 nm at pH 8.0. The purified acyl-ester hydrolase was very stable at $30^{o}C$ and maximum

activity was obtained at pH 8.0 using p-nitrophenyl caprylate as substrate. o-phenanthroline, Mg^{2+}, Ca^{2+}, or Mn^{2+} had no effect on the enzyme activity but EDTA had only a very slight inhibitory effect at a concentration of 10 mM. The DTT, DTNB and PMSF efficiently inhibited lipase activity but the activity was enhanced with 1% Triton X-100. The enzyme was capable to hydrolyze various p-nitrophenyl esters and highest rate was obtained with p-nitrophenyl caproate.

Five bacterial lipolytic enzymes throught to constitute a different group from the classical lipases and esterases. The occurrence of fatty acyl-ester hydrolase activity in post-germinated sunflower is due to a de novo synthesis of the protein. The influence of the enzyme and the substrate concentrations (sunflower oil). The interfacial area was modified by changing the colume fraction of oil, while keeping other variable constant. A phase inversion is indicated, which influenced the reaction kinetics by the possible formation of enzyme multilayers, due to stearic hindrance. Lipophilization of soy protein isolates, containing high amount of lysine residues was achived by biocatalysis. Reactions have been carried out at 60°C, when the acylation was carried out by lipozyme without solvent. A level of 60, 33 and 42% of lysine residues were covalently attached by capric, lauric and oleic groups, respectively. The lipophilization was achieved by *Rhizopus arrhizus* lipase in the presence of tertiobutanol, a level of 50% of lysine residues were acylated by a mixture of palmitic and stearic acids.

2. Polyhydroxyalkanoate

2.1 Introduction

The broad utility of petroleum-based plastics in terms of mechanical properties and durability has been manipulated by mankind to enhance quality of life without realizing they have become increasingly ubiquitous (Kunasundari and Sudesh, 2011). The world's plastics production was estimated to be 260 million tones in 2007 (Lazarevic et al., 2010). It is clear from this figure that the long term deleterious environmental impacts caused by plastics were entirely overlooked and this in turn poses greater difficulties for plastic waste disposal. Therefore, the development and use of biodegradable plastics is gaining more serious attention (Kunasundari and Sudesh, 2011). The most extensively studied thermoplastic biopolymer is the polyhydroxyalkanoate (PHA) and its derivative (Chen, 2009). PHA is attractive because of its biodegradability and physical properties that similar to those of chemically-synthesized polymers such as polypropylene (PP) and low-density polyethylene (LDPE) (Steinbüchel and Fuchtenbusch, 1998; Sudesh et al., 2000) and can be broken down in natural environments including soil, sea water and lake water (Mergaert et al., 1992; Khanna and Srivastava, 2005). PHA is naturally-occuring biopolymer, produced by a diverse group of Gram-negative and Gram-positive bacteria (Madison and Huiman, 1999; Sudesh et al., 2000). In general, microbial biosynthesis of PHA results from the limitation of nutrients, such as nitrogen, oxygen or phosphate (Anderson and Dawes, 1990), in the presence of abundant carbon. These polymers serve as storage for carbon or reducing equivalents and in some

strains of bacteria, PHA content accounts for more than 80% of the dry cell weight (DCW) (Khanna and Srivastava, 2005). The characteristics of PHA differ, depending on the type of monomer incorporated and particularly on the monomer chain length (Doi et al., 1995; Jendrossek and Handrick, 2002). The various PHA monomers can be classified based on the number of carbon atoms as short-chain length PHA (scl-PHA), medium-chain length PHA (mcl-PHA) and long-chain PHA (lcl-PHA). Scl-PHA refers to PHA comprised of monomers having 5 or less carbon atoms. These include 3-hydroxybutyrate and 3-hydroxyvakerate. The mcl-PHA is comprised of monomers having 6 to 14 carbon atoms. These include 3-hydroxyhexanoate, 3-octanoate and 3-hydroxydecanoate. The lcl-PHA, which is uncommon and least studied, consists of monomers with more than 14 carbon atoms (Kunasundari and Sudesh, 2011). Recently, it has also been made possible to synthesize a new type of PHA containing lactide as a co-monomer (Taguchi et al., 2008; Yamada et al., 2009; Shozui et al., 2009). All these developments indicate that PHA may become the preferred next generation bioplastic. However, PHA has not been successfully commercialized due to its high costs as compared to conventional plastics. As an intracellular product, the recovery of PHA contributes significantly to the overall economics (Mohammadi et al., 2012). Therefore developing an inexpensive and less-polluting PHA recovery method to achieve low-cost production is an attractive proposition (Ghatnekar et al., 2002). This article examines the effectiveness of several methods in recovering PHA. Some of the large-scale production of PHA and the strategies employed to reduce the production cost are also discussed.

2.2 PHA production

Among several microorganisms that are known to synthesize PHA, only a few bacteria have been utilized for the PHA production. These include *Curpiavidus necator* (former *Ralstonia eutropha*) (Madison and Huisman, 1999; Kobayashi et al., 2000; Song et al., 2001; Tohyama et al., 2002), *Alcaligenes latus* (Grothe and Chistri, 2000), *Azotobacter vinelandii*, several strains of *Methylotrophs*, *Pseudomonas* species (Chen et al., 2001; Ashby et al., 2002) and recombinant of *C. necator*, *Klebsilla aerogenes* and *Escherichia coli* (Ramachander et al., 2002; Yu et al., 2002). Aforementioned bacteria have been selected because they can be cultivated efficiently to high cell densities with a high PHA content (defined as the ratio of PHA concentration to dry cell concentration expressed as a percentage) in a relatively short period of time, resulting in high PHA productivity (defined as g PHA produced/liter/hour) (Lee, 1996).

2.3 The PHA biosynthetic pathway

Many studies have been made on the pathway of metabolic synthesis of PHA in various microorganisms. The best understood biochemical pathway for PHA and copolymer P(HB-co-HV) production is found in the bacterium *C. necator* and probably the most widely distributed, and has been confirmed by experiments using ^{13}C-labelled substrates and ^{13}C nuclear magnetic resonance (NMR) spectral analysis of the isolated PHA (Doi et al., 1987). The cyclic nature of biosynthesis of PHA is demonstrated in Figure 2.3.

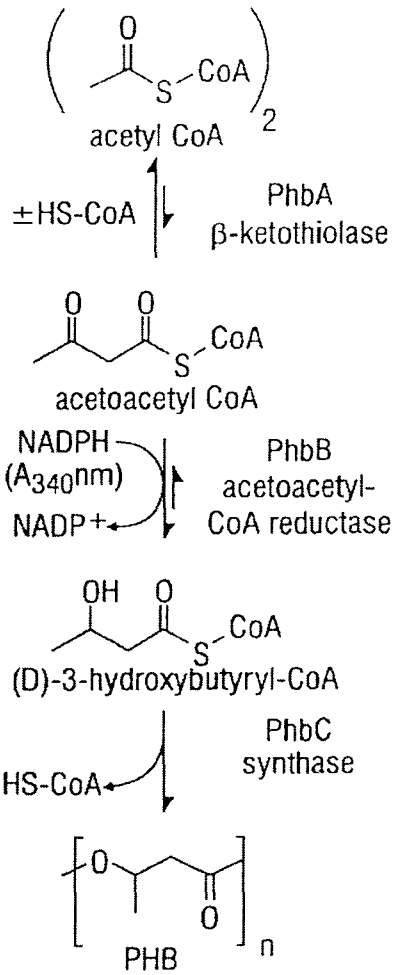

Figure 2.3 The cyclic nature of biosynthesis of PHA.

Source: Gruys et al. (2003)

The biosynthesis pathway of PHA consists of three steps and three enzymatic reactions catalyzed by three distinct enzymes (Figure 2.4). The first reaction, Acetyl-CoA is the starting substrate employed in the biosynthetic pathway, produced from feedstocks such as glucose, fructose, sucrose, methanol or acetic acid. A β-ketothiolase enzyme condenses two acetyl-CoA moieties to form acetoacetyl-CoA. The second reaction is the reduction of acetoacetyl-CoA to *R*-β-hydroxybutyryl-CoA by an NADPH-dependent stereoselective acetoacetyl-CoA reductase. The third and last step of the

biosynthesis is the action of the PHA synthase enzyme, which takes the *R*-β-hydroxybutyryl moiety and binds the β-hydroxyl group to the carboxyl end of a pre-existing PHA molecule to form an ester bond, increasing the chain length by one (Reddy *et al.*, 2003).

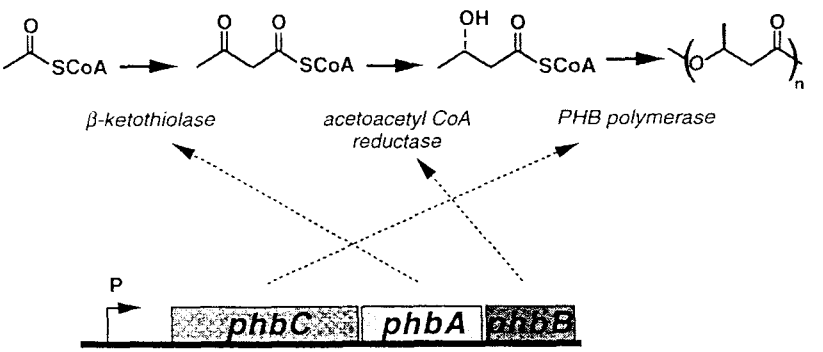

Figure 2.4 Biosynthetic pathway of poly(3-hydroxybutyrate), P(3-HB).

Source: Madison and Huisman (1999)

In *C. necator*, PHA inclusions can typically accumulate to 80–85% of DCW when bacteria are grown in media containing excess carbon, such as glucose, but limited in one essential nutrient, such as nitrogen or phosphate (Poirier, 2002). Under these conditions, PHA synthesis acts as a carbon reserve and an electron sink. Under autotrophic conditions, *C. necator* can also utilize carbon dioxide for the production of PHA. When growth limiting conditions are alleviated (by addition of phosphate or nitrogen), PHA is depolymerized by the action of an intracellular PHA depolymerase to give acetoacetate, which is then catabolized further to acetyl-CoA (Poirier, 2002). Several carbon sources have been reported that can be utilized by *C. necator* for PHA production. *C. necator* is also capable of accumulating P(3HB-*co*-3HV) in the culture medium contains glucose and propionic acid or valeric acid as carbon sources. The mechanism of co-polyester biosynthesis is shown

in Figure 2.5. Sudesh and his co-workers (2000) reported that *C. necator* can produce copolymer of 4-HB monomers along with 3-HB, P(4HB-*co*-3HB) from specialized carbon sources such as 4-hydroxybutyric acid, γ-butyrolactone and 1,4 butanediol.

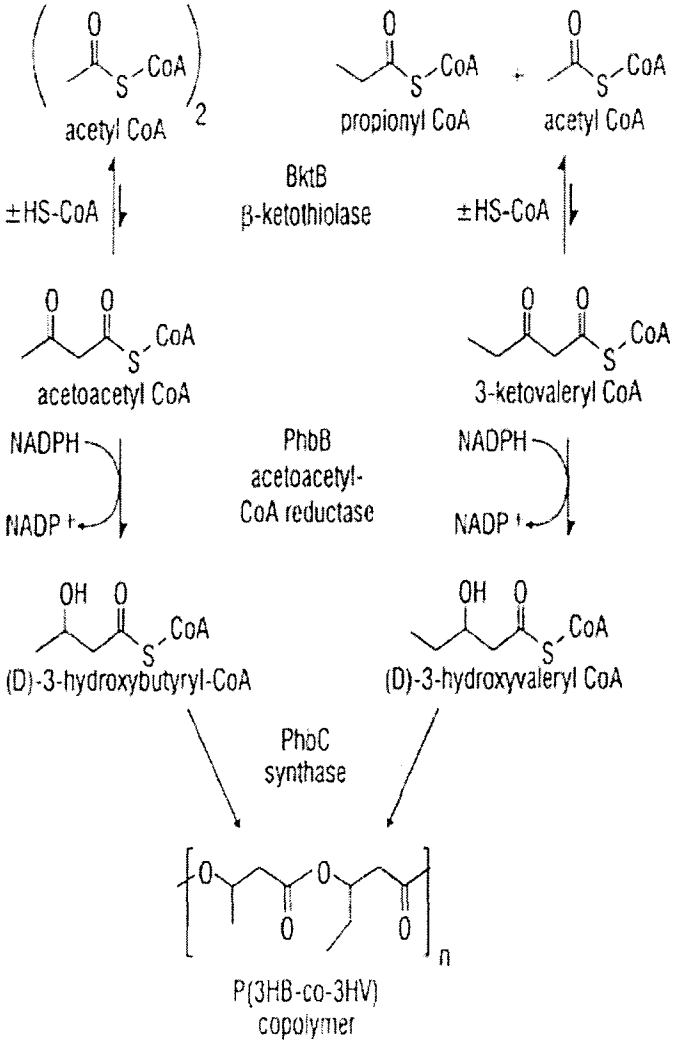

Figure 2.5 Biosynthesis pathway of P(3HB-*co*-3HV) copolymer by *C. necator*.

Source: Gruys et al. (2003)

3. MATERIALS AND METHODS

3.1 Plant collection

Oil palm fruit (*E. guineensis*) were collected from Faculty of Science, Thaksin University (Thailand) and used as the sample for this study. Oil fruit were picked randomly from different parts of bunch. Fruits of uniform size (15.29±3.0 g) and with no visible defects were collected and left for 0-240 h after harvested under room temperature. Afterward, the sample were separated under specific time, washed thoroughly under running tap water, cut into smaller pieces and blended under suitable solvent.

3.2 Solvent extraction

Organic solvents such as acetone, ethanol, 0.05 mM Tris-HCl buffer (pH 8), methanol, chloroform and hexane were used for extraction. Sample (50 g) was dissolved in 500 mL of respective solvents by sonication for 10 minutes. The extract was litered through Whatman No.1 liter paper using vacuum pump literation and the extraction process was repeated for two times. The extract was concentrated under reduced pressure at 40°C, 90 rpm using a rotary evaporator. The yield s obtained were kept at -20°C for further analysis. Extraction yields were weighted and calculated the formula as followed:

$$\text{Yield (\%)} = \frac{\text{Dry weight of extract}}{\text{Dry weight of plant powder}} \text{x100}$$

3.3 Phytochemical study (qualitative analysis)

Phytochemical studies were conducted qualitatively and quantitatively to identify the presence of bioactive chemical constituents such as alkaloids, lavonoids, terpenoids and steroids, saponins, tannins, phenolic compounds,

coumarins and carbohydrates. Qualitative phytochemical analysis was done according to the standard protocols as described by Karthishwaran et al. (2010), while quaitiative phytochemical analysis was done based on standard proximate measurement as described by Konate et al. (2012) and Chanput et al. (2009).

3.4 Antioxidant assay using DPPH method

Methanol solution of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical (0.004 %w/v) was prepared and stored at 10°C in the dark. Sample in methanol solution with different concentration of 0.0625 to 4 mg/mL were prepared. Aliquot (0.05 mL) of the sample solution was then added to 5.0 mL of methanolic DPPH solution. The mixture was shaken vigorously and left to stand for 30 min in the dark. Absorbance measurements were recorded immediately at 517 nm. The absorbance of the DPPH radical without the crude extract samples (control) and the reference compound DL-α-tocopherol and gallic acid were measured. All the determinations were performed in three replicates and average was counted. The percentage of inhibition (PI) of the DPPH radical was calculated according to the formula as followed:

$$PI\ (\%) = \frac{AC - AT}{AC} x100$$

Where AC = Absorbance of the control and AT = absorbance of the sample.

3.5 Analysis of total lipid content

The total amount of oil palm was determined by solvent extraction (Teixeira et al., 2013).

3.6 Determination of total carotenes, tocopherols and tocotrienols

The analyses of carotenes, tocopherols and tocotrienols were carried out simultaneously by chromatographi analyses, as described previously by Silva et al. (2011) with a Shimadzu HPLC (Japan) equipped with a quaternary pump, auto sampler, degasser, SPD-M20A spectrophotometric detector that was set at 292 and 455 nm and RF-10AXL fluorescence detector that was set at 290 nm for excitation and 330 nm for emission. Chromatographic separation of the compounds was achieved at 30°C with a normal-phase column (250 x 4.6 mm id; 5 μm particle size) with a guard column (10 x 4.6 mm) purchased from Merck (Germany). The concentration gradient used was as follows: 0-7 min 99.5% hexane and 0.5% isopropanol; 7-9 min linear gradient of 0.5-1% isopropanol; 9-20 min 99.0% hexane and 1.0% isopropanol; 20-25 min reconditioning of the column with 0.5% isopropanol isocratic for 10 min. The chromatographic run time for each analysis was 35 min. Samples were dissolved in hexane, and aliquots of 20 μL were injected into the HPLC system.

3.7 Analysis of fatty acid composition

The fatty acid composition was determined by conversion to fatty acid methyl esters (FAMEs) based on the method of Rodrigues et al. (2010) and the esters were detected by a gas chromatograph equipped with a flam ionization detector and a capillary column (length 60 m, internal diameter 0.25 mm, film thickness 0.25 μm). The operating conditions were as follows: helium as the carrier gas, a flow rate of 0.9 mL/min, FID detector temperature of 250°C, an injector temperature (split ratio 1:100) at 245°C and an injector volume of 1

μL. The temperature program for the column was 4 min at 80°C and a subsequent increase to 220°C at 4°C/min. The individual fatty acid peaks were identified by comparison of the retention times with those of known mixtures of standard fatty acids run under the same operating conditions. The retention time and the area of each peak were computed. The results were expressed as relative percentages of total fatty acids (Teixeira et al., 2013)

3.8 Determination of the total phenolic content

The total phenolic content was quatified using Folin-Ciocalteu reagent, with minor modifications. A 300-μL aliquot of extract solution was mixed with 5 mL of Folin-Ciocalteu reagent (10% in distilled water). After 5 min, 4 mL of sodium carbonate (7.5% in distilled water) was added. The samples were incubated for 2 h at room temperature in the dark. The absorbance was measured at 765 nm. A standard curve was prepared with gallic acid solutions of know concentrations. The results were expressed as mg gallic acid equivalents (GAE) per kg of extract.

3.9 Measurement of oxidative stability

The oxidative stability of the sample was evaluated with a Metrohm 743 Rancimat instrument which measures the rate of oxidation under accelerated conditions. The tests were performed with 3 g samples at a temperature of 130°C and flow of air 20 L/h. The oxidative stability was expressed as the induction time for oxidation of the oils.

3.10 The production of polyhydroxybutyrate (PHB) from oil palm fruit (OPF) residue

3.10.1 PHB-accumulating bacteria

Cupriavidus necator TISTR1095, PHB-producing strains, was obtained from culture collection, Thailand Institute of Scientific and Technological Research, Thailand and employed. Bacterial strains were maintained at 37°C in PHA-producing medium containing glucose 30 g/L, [$(NH_4)_2SO_4$ 2 g/L, KH_2PO_4 13.3 g/L, $MgSO_4.7H_2O$ 1.2 g/L, citric acid 1.7 g/L, trace element solution 10 mL/L]. The medium was adjusted to pH 7.0 using 5 mol/L NaOH.

3.10.2 The preparation of oil palm wastes

Oil palm fruit (OPF) was obtained from The Krabi Oil Palm Farmers Cooperatives Federation Limited (Krabi, Thailand). OPF was firstly cut and homogenized in suitable solvent for phytochemical analysis as described above. Therefore, OPF after homogenized were indicated as "palm pressed fiber, PPF". PPF was pretreated before use. PPF were cut into small piece and sun dried for 2 days, then stored in a plastic bag and kept at room temperature until used. Afterward, PPF was soaked overnight in a commercial dish washing detergent before they was washed with tap water to remove oil and dust. Then, the washed materials were dried in an oven at 60°C for 24 h (Ibrahim *et al.*, 2015). 10 g of PPF was soaked again in distilled water for 4 h followed by autoclaving at 121°C for 60 min.

3.10.3 Hydrolysis of PPF

Cellulase from *Aspergillus niger* (Sigma Chemical Co., USA) was utilized for the saccharification of pretreated PPF into fermentable sugar. The cellulase stock was diluted in 0.1 M of phosphate buffer, pH 5.5 to give an initial β-glucosidase activity of 5.0 U/ mL before it was filtered through 0.45

μm of membrane filter using vacuum pump to remove the remaining debris from the cellulase solution (Ibrahim *et al.*, 2015).

3.10.4 The production of polyhydroxyalkanoate

10 g of PPF with β-glucosidase solution (5.0 U/mL) was firstly added and incubated. After 24 h of incubation, the supernatant was recovered and utilized as substrate for polyhydroxybutyrate production.

Starter culture of *C. necator* TISTR1335 was prepared by cultivating aerobically with shaking (150 rpm) in PHA-producing medium. The culture was cultivated in medium at 37°C for 24 h. Afterward aliquots were removed to determine cell growth by measurement of optical density at 660 nm.

The starter culture (5%) was added into the 200 mL medium and cultivated on a rotary shaker (150 rpm) at 37°C for 96 h. Samples were taken at the first 6 h and then every 12 h to measure for pH, growth (OD 660 nm), dry cell weight, the concentrations of polyhydroxybutyrate.

3.11 Analytical method

The cellulose and lignin amounts from oil palm wastes were estimated by the A.O.A.C method (A.O.A.C, 1990). The reducing sugar amounts were estimated by dinitrosalicylic acid (DNS) method (Miller, 1959) using a glucose standard calibration curve. Cellulase activity was determined by the method of Wood and Bhat (1988). One unit (U) of cellulase is defined as the amount of enzyme the release 1 mole of glucose equivalent per min under the assay conditions.

The PHA content and composition were determined by gas chromatography-mass spectrometry (GC-MS) and benzoic acid was used as the internal standard [9]. PHA concentration is defined as the percentage of DCW, i.e., 100 x (g PHA / g DCW). Molecular weight was estimated by gel permeation chromatography (GPC) with polystyrene and $CHCl_3$ as a standards and solvent, respectively. All data were calculated with the mean and standard derivation of three parallel studies.

4. RESULTS AND DISCUSSION

4.1 Extraction yield

Different solvents have different resolving strength towards the plant constituents which resulted in different yield as shown in Table 4.1. The extraction of methanol resulted with the highest amount of yield. Oil palm fruit (100 g) used in methanol-polar solvent extraction obtained the highest yield (5.41%) while the other yielded only 1.20-3.58%.

Table 4.1 Yield of oil palm fruit (200 g) extracted using different types of solvents.

Solvents	**Yield**	
	gram	**%**
Acetone	4.84	2.42
Ethanol	7.16	3.58
0.05 mM Tris-HCl buffer (pH 8)	7.16	3.58
Methanol	10.82	5.41
Chloroform	2.40	1.20
Hexane	2.90	1.45

The extraction yield of palm oil fruit (this study) showed the similar pattern with the result from Yin et al. (2013). The extraction yield of leaves from *Elaeis guineensis* gave the highest value when oil palm leaves were extracted in methanol-polar solvent extraction obtained the highest yield (8.28%).

4.2 Chemical composition

The moisture, crude proteins and ash contents of oil palm fruit are shown in Table 4.2. The moisture content was 30.24%, the crude protein content was 5.12% on a dry matter (DM) basis. The lipid content was 52.94 g oil/100 g oil palm fruit.

Table 4.2 Chemical characterization of oil palm fruits cultivates in Southern Thailand

Content	**Unit**	
Moisture	%	30.24
Protein	%	5.12
Lipid	g oil/100g oil palm fruit	52.94
Ash	%	1.84

4.3 Phytochemical study (Qualitative analysis)

Based on the highest yield obtained from Table 4.1, methanol was chosen for further used for extraction and phytochemical screening. The quantitative analysis of the phytochemicals in oil palm fruit revealed the presence of phenolic compounds, lavonoids, tannins, coumarins, alkaloids, saponins, terpenoids and steroids, and carbohydrates (Table 4.3).

Table 4.3 Qualitative analysis of the phytochemical constituents of oil palm fruit extracted with methanol.

Group of phytochemicals	Test	Results
Alkaloids	Dragendorff's test	++
	Mayer's test	++
Coumarins	NaOH test	++
Phenolic compounds	Gelatin test	++
Saponins	Frothing test	++
Tannins	FeCl3 test	++
Terpenoids and Steroids	Salkowski test	++
Flavonoids	Shinoda test	++
Carbohydrate	Benedict test	++

+ indicates presence; number of (+) indicates number of replication

4.4 Proximate analysis of phytochemical in oil palm fruit

The qualitative analysis results in Table 2 showed promising phytochemical properties presence in oil palm fruit. Therefore, a proximate analysis of phytochemicals using methanolic extract of oil palm fruit was performed to further quantify the amount of these properties and results are shown in Table 4.4. The result suggests that oil palm fruit are rich in saponin, tannin, carbohydrate and protein. The total alkaloids content was 1.6±0.1 mg/g dry weight (DW), contributed the least amount of phytochemicals in this study. The flavonoid content of the extract was 0.3±0.1 mg QE/g DW. The flavonoid content was

calculated in terms of quercerin equivalent. The content of total phenolic was 0.3±0.01 mg GAE/g DW, which was measured in terms of gallic acid equivalent. The content of tannin and saponin in extract contained 3.5±0.1 mg TAE/g DW, which is the highest amount among the phytochemical group in this study.

Table 4.4 Mean values of phytochemical content and relative percentage of mean in oil palm fruit.

Group of phytochemical	**Quantity (mg/g dry weight, DW)**
Alkaloids	1.6±0.1
Saponin	3.5±0.2
Flavonoid	0.3±0.1
Phenolic	0.3±0.01
Tannin	3.5±0.1
Carbohydrate	470±1.4
Protein	2.4±0.01

In addition, the amount of primary metabolites such as carbohydrates and protein content were also calculated. The carbohydrate content was determined from the glucose standard curve, showed a total amount of 470±1.4 mg glucose/g DW of oil palm fruit. In protein content determination, bovine serum albumin (BSA) was used as standard, showed regression equation of calibration curve. The protein content obtained from oil palm fruit was 2.4±0.01 mg BSA/g DW.

4.5 The phytochemical content of oil palm fruit at various time of harvesting

The DPPH radical scavenging activity of the methanolic extract from oil palm fruit after 0-240 h harvested as shown in Table 4.5. The scavenging activity for methanolic extract was up to 88.45±0.07 % after 0 h harvested.

Table 4.5 DPPH radical scavenging activity of the methanolic extract from oil palm fruit after 0-240 h of harvested. Gallic acid and α-tocopherol were used as reference antioxidant.

Hour after harvested (h)	DPPH radical scavenging activity (%)	% Reduction (%)
0	88.45±0.07	0.00
24	85.57±0.08	3.26
48	79.93±0.05	9.63
72	76.51±0.08	13.50
96	72.04±0.08	18.55
120	68.32±0.07	22.76
144	64.96±0.06	26.56
168	61.87±0.02	30.05
192	51.17±0.06	42.15
216	49.88±0.03	43.61
240	39.10±0.03	55.79

The results for the DPPH assays are presented on Table 4.5. These results represent the antioxidant capacities of the lipophilic and hydrophilic fractions and show relatively large standard derivations as is common natural samples (Hanson et al., 2004; Ou et al., 2002).

In addition, phenolic contents were also determined from oil palm fruit after 0-240 h harvested as shown in Table 4.6. The highest phenolic content for methanolic extract was 0.3±0.01 mg GAE/g DW after 0 h harvested.

Table 4.6 Phenolic content of the methanolic extract from oil palm fruit after 0-240 h of harvested. Gallic acid was used as reference.

Hour after harvested (h)	**Phenolic content (mg GAE/g DW)**
0	0.3±0.01
24	0.2±0.01
48	0.2±0.01
72	0.2±0.01
96	0.2±0.01
120	0.2±0.01
144	0.2±0.01
168	0.1±0.01
192	0.1±0.01
216	0.1±0.01
240	0.1±0.01

Vitamin E contents were also determined from oil palm fruit after 0-240 h harvested as shown in Table 4.7. The highest vitamin E content for methanolic extract was 0.1±0.01 mg/g DW after 0 h harvested.

Table 4.7 Vitamin E content of the methanolic extract from oil palm fruit after 0-240 h of harvested. Tocopherol was used as reference.

Hour after harvested (h)	**Vitamin E content (mg/g DW)**
0	0.1±0.01
24	0.09±0.01
48	0.09±0.01
72	0.08±0.01
96	0.08±0.01
120	0.07±0.01
144	0.07±0.01
168	0.06±0.01
192	0.05±0.01
216	0.03±0.01
240	0.03±0.01

Protein contents were also determined from oil palm fruit after 0-240 h harvested as shown in Table 4.8. The highest protein content for methanolic extract was 2.4±0.01 mg/g DW after 0 h harvested.

Table 4.8 Protein content of the methanolic extract from oil palm fruit after 0-240 h of harvested.

Hour after harvested (h)	**Protein content (mg/g DW)**
0	2.4±0.01
24	1.0±0.01
48	0.9±0.01
72	0.8±0.01
96	0.8±0.01
120	0.7±0.01
144	0.7±0.01
168	0.6±0.01
192	0.6±0.01
216	0.50±0.01
240	0.5±0.01

In addition, lipase activity was also determined from oil palm fruit after 0-240 h harvested as shown in Table 4.9. The highest lipase for Tris-HCl extract was 0.4 U/mL after 120 h harvested.

Table 4.9 Lipase activity of the Tris-HCl extract from oil palm fruit after 0-240 h of harvested.

Hour after harvested (h)	Lipase activity (U/mL)	Total activity (U)	Specific Activity[a] (U/mg protein)
0	0.2±0.01	0.59	0.30
24	0.2±0.01	0.61	0.63
48	0.3±0.01	0.64	0.75
72	0.3±0.01	0.77	0.95
96	0.3±0.01	0.88	1.15
120	0.4±0.01	0.10	1.38
144	0.3±0.01	0.66	1.06
168	0.2±0.01	0.63	1.03
192	0.2±0.01	0.58	1.03
216	0.1±0.01	0.49	0.96
240	0.01±0.01	0.35	0.77

4.6 Oil composition

Table 4.10 shows the values for tocopherol, tocotrenol and carotene in oil palm fruit during various times after harvested. Puah et al. (2007) studied the tocopherol concentration of palm oil during physical refining and observed a mean value of 1273±18 ppm in crude palm oil, 1134±20 ppm after degumming. 1095±18 ppm after bleaching and 1029±18 ppm after deodorization.

Table 4.10 Tocopherol, tocotrienol and carotene in oil palm fruit after 0-240 h of harvested.

Hour after harvested (h)	Total tocopherol (mg/kg)	Total tocotrienol (mg/kg)	Total carotene (mg/kg)
0	850±1.2	800±2.4	790±7.0
24	845±2.1	789±2.1	720±1.3
48	820±2.0	750±1.9	680±2.0
72	780±1.5	751±2.4	650±4.0
96	700±2.1	700±2.4	580±8.0
120	650±1.8	680±2.1	530±5.0
144	650±1.8	650±1.5	450±7.0
168	600±1.0	600±2.1	340±2.0
192	600±1.5	600±1.9	260±2.0
216	600±1.0	600±1.8	200±3.0
240	580±1.5	589±1.9	130±1.0

Szydlowska-Czernial et al. (2011) evaluated the total phenolic, antioxidant capacity and total carotenoid content of oil palm samples after non-enzymatic refining. The total phenolic compounds range from about 41 to 124 mg GA/kg and carotenoids from 1.8 to 458 mg/kg oil samples.

4.7 The production of polyhydroxybutyrate from palm pressed fiber

The characterization of PPF showed that PPF contained high cellulose (32.2%). After enzyme hydrolysis, the percentage of cellulose in PPF was increased to 64.8% (Table 4.11). Therefore, the pretreatment of palm biomass before used for fermentation seem to be important while their cellulose content increased significantly.

Table 4.11 Cellulose, hemicelluloses and lignin contents in palm wastes

Palm wastes	Dry weight (%)	Cellulose (%)		Hemicellulose (%)		Lignin (%)	
		Before[a]	After[b]	Before	After	Before	After
PPF	96.0±1.0	32.2±1.2	64.8±1.2	24.4±1.2	20.2±1.2	30.1±1.5	11.1±1.5

[a] = Before enzyme hydrolysis; [b] = After enzyme hydrolysis

After enzyme hydrolysis, the supernatant contained reducing sugar at 24.5 g/L. Therefore, the supernatant was suitable for PHB production. The maximum PHB accumulation in *C. necatoe* was reported to occur between 48-72 h of incubation. Hence, *C. necator* cells grown in supernatant were harvested between 0-96 h incubation. During cultivation, the pH increased to a slightly alkali pH (from 5.5 to 8.02) due to the depletion of carbon source and the generation of ammonia from nitrogen source consumption. Maximum growth was obtained after 60 h cultivation whereas PHB concentration and PHB content showed the same potential as cellular growth. The maximum PHB in the cells was 50.4% of dry cell weight (DCW) and gave the PHB concentration about 2.81±0.10 g/L. Afterward, the polymer was isolated and characterized. To specifically determine the

composition of the isolated polymer, the freeze dried cell material and the commercial polyhydroxybutyrate were subjected to esterification. The propyl esters formed were analyzed by GC and benzoic acid propyl ester was used as the internal standard. The peak corresponding to propyl ester of 3-hydroxybutyric acid was observed in the gas chromatogram (retention time 8.5 min) for PHB from *C. necator* (Fig 4.1). This indicated that the polymer accumulated by *C. necator* was PHB. The structure of PHB was confirmed by ATR-FTIR compared with commercial PHB.

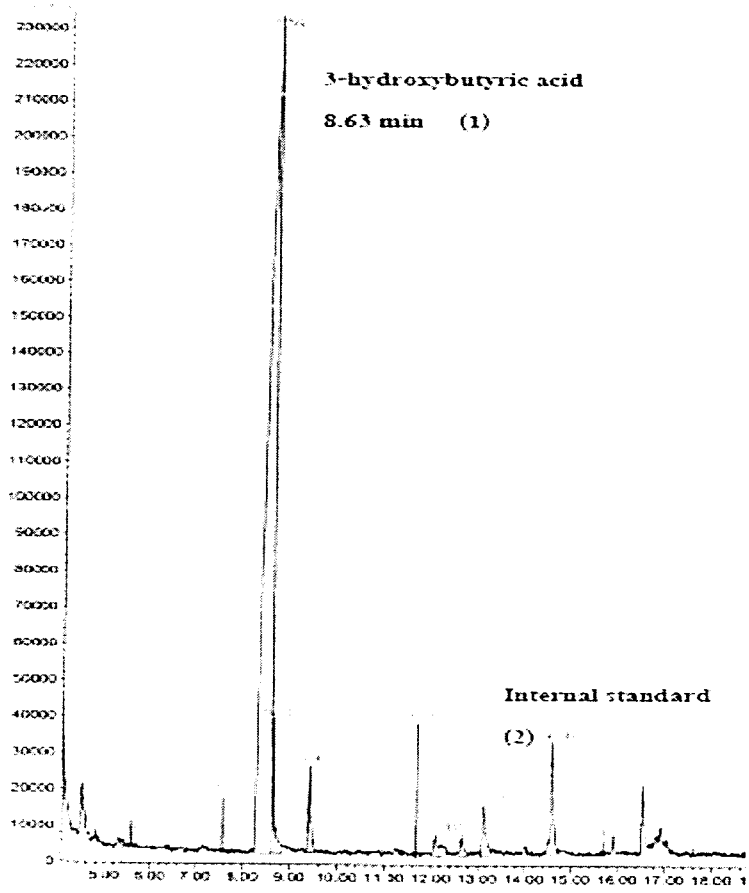

Figure 4.1 Chromatogram of extracted polyhydroxybutyrate (PHB) from PHB-producing bacteria, used in this study, cultivated in minimal medium analyzed by GC-MS.

ATR-FTIR spectrum of the PHB sample revealed 3 major peaks of C-O-C stretching at 1280 cm^{-1}, C-H banding at 2978-2934 cm^{-1} and C=O stretching at 1721 cm^{-1}. The structural analysis confirmed the presence of polyester group which consisted of polyhydroxybutyric acid (Fig 4.2).

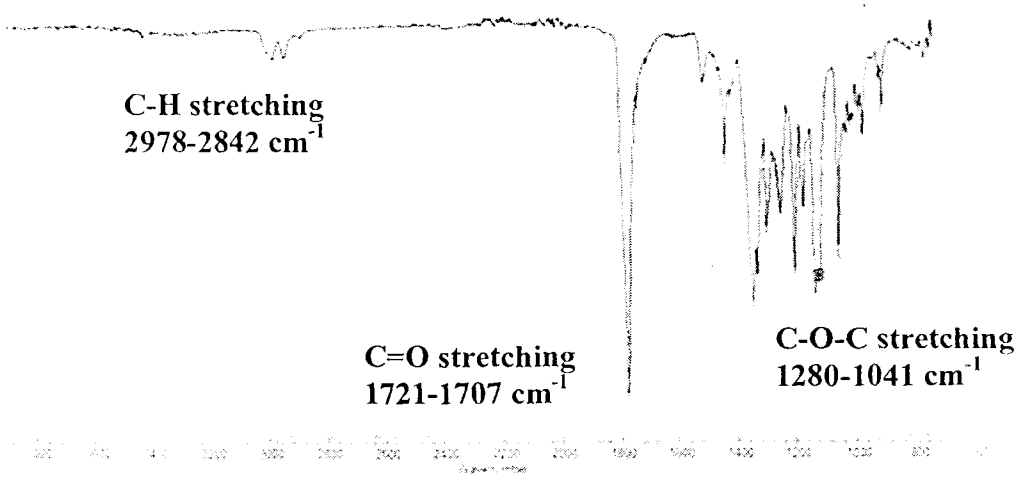

Figure 4.2 FTIR spectra of poly(3-hydroxybutyrate) [P(3HB)] from palm pressed fiber.

5. Conclusion

This study aimed to extract bioactive compound from oil palm fruit after 0-240 h of harvested. In addition, lipase from oil palm was also partitioned and analyzed. Oil palm fruits after 0-240 h of harvested were firstly extracted by six different solvent including acetone, ethanol, Tris-HCl buffer (pH 8), methanol, chloroform and hexane. The highest bioactive compounds (10.82 g, 5.41% yield) were obtained from methanol. Oil palm fruit after 0 h of harvested contained moisture (30.24%), protein (5.12%), lipid (52.94 g oil/g oil palm fruit and ash (1.84%). In addition, other phytochemical content were yielded the highest content after 0 h of harvested including alkaloid (1.6 mg/g DW), saponin (3.5 mg/g DW), flavonoid (0.3 mg/g DW), phenolic (0.3 mg/g DW), tannin (3.5 mg/g DW), carbohydrate (470 mg/g DW) and protein (2.4 mg/g DW). Tocopherol, tocotrienol and carotene from oil palm fruit after 0-240 h of harvested were also evaluated. The highest tocopherol (850 mg/kg), tocotrienol (800 mg/kg) and carotene (790 mg/kg) was obtained after 0 h of harvested. However, the highest lipase activity was obtained from Tris-HCl buffer (pH 8.0). The highest lipase activity (0.4 U/mL) with 0.1 U and 1.38 U/mg protein was achieved from oil fruit after 120 h of harvested.

Therefore, the supernatant was collected for PHB production by *Cupriavidus necator*. The maximum PHB (50.4% of dry cell weight, DCW) was obtained after 60 h of cultivation. The polymer was identified by gas chromatography and ATR-FTIR to be polyhydroxybutyrate compared with commercial polyhydroxybutyrate.

Acknowledgement

The authors would like to thank The Research and Development Institute Thaksin University for their financial support (National budget source Year 2015).

REFERENCES

Abdul Khalil, H.P.S., Siti Alwani, M., Ridzuan, R., Kamarudin, H. and Khairul, A. (2008). Chemical composition, morphological characteristics, and cell wall structure of Malaysian oil palm fibers. Polym. Plast. Technol. Eng. 47(3): 273-280.

Ahmad, A.L., Chan, C.Y., Abd Shukor, S.R. and Mashitah, M.D. (2008). Recovery of oil and carotenes from palm oil mill effluent (POME). Chemical Engineering Journal. 141: 383-386.

Anderson, A.J. and Dawes, E.A. (1990). Occurrence, metabolism, metabolic role, and industrial uses of bacterial polyhydroxyalkanoates. Microbiological Reviews, 54: 450-472.

Ashby, R.D., Solaiman, D.K. and Foglia, T.A. (2002). The synthesis of short- and medium-chain-length poly(hydroxyalkanoates) mixtures from glucose- or alkanoic acid-grown *Pseudomonas oleovorans*. Journal of Industrial Microbiology and Biotechnology, 28: 147-153.

Berger, K.G. (2005). The use of palm oil in frying. Frying oil series (pp. 1–73) Selangor, Malaysia: Malaysian Palm Oil Promotion Council.

Berger, K.G. and Martin, S.M. (2000). Palm oil. In K. F. Kiple, & K. C. Ornelas (Eds.), The Cambridge world history of food (pp. 397–411). Cambridge, England: Cambridge University Press.

Benade´, S.A.J. (2003). A place for palm fruit oil to eliminate vitamin A deficiency. Asia Pacific Journal of Clinical Nutrition, 12 (3), 369–372.

Canfield, L.M., Kaminsky, R.G., Taren, D.L., Shaw, E. and Sander, J.K. (2001). Red palm oil in the maternal diet increases provitamin A

carotenoids in breastmilk and serum of the mother-infant dyad. Eur J Nutr. 40(1): 30-38.

Chang, C.J., Chang, Y., Lee, H., Lin, J. and Yang, P. (2000). Supercritical Carbon Dioxide Extraction of High-value Substances from Soybean Oil Deodorizer Disti.

Chanput, W., Theerakulkait, C. and Bakai, S. (2009). Antioxidative properties of partially ouri ied barley hordein, rice bran protein fractions and their hydrolysates. J. Cereal Sci. 49: 422-428.

Chen, G.Q. (2009). A microbial polyhydroxyalkanoates (PHA) based bio- and materials industry. Chemical Society Reviews, 38: 2434-2446.

Chen, G.Q., Jun, X., Qiong, Q., Zengming, Z. and Kwok-Ping, H. (2001). Synthesis of copolyesters consisting of medium-chain-length beta-hydroxyalkanoates by *Pseudomonas stutzeri* 1317. Reactive and Functional Polymers, 48: 107-112.

Chowdhury, K., Banu, L. A., Khan, S. and Latif, A. (2007). Studies on the Fatty Acid Composition of Edible Oil. Bangladesh J. Sci. Ind. Res. 42(3): 311-316.

Chong, C.L. (2012). Measurement and maintenance of palm oil quality. In O.-M. Lai, C.-P. Tan, & C. C. Akoh (Eds.), Palm oil: Production, processing, characterization and uses (pp. 433–470). Urbana, Illinois, USA: AOCS Press.

Chu, B.S., Baharin, B.S., Quek, S.Y. and Che Man, Y.B. (2003). Separation of Tocopherols and Tocotrienols from Fatty Acid Distillate Using Hydrolysis-

neutralization-adsorption Chromatography Method. Journal of Food Lipids. 10: 141–152.

Das, S., Nesaretnam, K. and Das, D.K. (2007). Tocotrienols in cardioprotection. Vitamins & Hormones, 76, 419–433.

Doi, Y., Kitamura, S. and Abe, H. (1995). Microbial synthesis and characterization of poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate). Macromolecules, 28: 4822-4828.

Doi, Y., Tamaki, A., Kunioka, M. and Soga, K. (1987). Biosynthesis of terpolyester of 3-hydroxybutyrate, 3-hydroxyvalerate, and 5-hydroxyvalerate in *Alcaliges eutrophus* from 5-cholopentanoic and pentanoic acids. Die Makromolekulare Chemie, Rapid Communications, 8: 631-635.

Dutta, P.C., Przybylski, R., Eskin, N.A. and Appelqvist, L.A. (2007). Formation and analysis and health effects of oxidized sterols in frying fats. In M. D. Erickson (Ed.), Deep frying: Practices, chemistry and nutrition (pp. 125–178). Champaign, Illinois, USA: AOCS Press.

Edem, D.O. (2002). Palm oil: biochemical, physiological, nutritional, hematological and toxicological aspects: a review. Plant Foods Hum. Nutr. 57(3): 319-341.

Edem, D.O., Eka, O.U. and Umoh, I.B. (2002). Feeding of red palm oil-supplemented diets to rats may impact positively on vitamin A status. International Journal of Food Sciences and Nutrition, 53, 285–291.

Elias, B.A. and Pantzaris, T.P. (1997). Authenticity of palm oil-Assessment of a Brazilian survey. Palm Oil Technical Bulletin, 3 (2), 8–9.

Fattore, E. and Fanelli, R. (2013). Palm oil and palmitic acid: A review on cardiovascular effects and carcinogenicity. International Journal of Food Sciences and Nutrition, 64(5), 648–659.

Ghatnekar, M.S., Pai, J.S. and Ganesh, M. (2002). Production and recovery of poly-3-hydroxybutyrate from *Methylobacterium* sp. V49. Journal of Chemical Technology and Biotechnology, 77: 444-448.

Grothe, E. and Chistri, Y. (2000). Poly(β-hydroxybutyric acid) thermoplastic production by *Alcaligenes latus*: Behavior of fed-batch cultures. Bioprocess Engineering, 22: 441-449.

Gruys, K.J., Mitsky, T.A., Kishore, G.M., Slater, S.C., Padgette, S.R. and Stark, D.M. (2003). Methods of optimizing substrate pools and biosynthesis of poly-beta-hydroxybutyrate-*co*-poly-beta-hydroxyvalerate in bacteria and plants. U.S. Patent 20,030,028,917.

Gunstone, F.D. (2011). Vegetable oils in food technology: Composition, properties and uses (2nd ed.). West Sussex, UK: Blackwell Publishing Ltd. pp. 25–133.

Hedren, E., Mulokozi, G. and Syanberg, U. (2002). *In vitro* accessibility of carotenes from green leafy vegetables cooked with sunflower oil or red palm oil. Int J Food Sci Nutr. 53: 445-453.

Hendler, S.S. and Rorvik, D. (2008). PDR for nutritional supplements (2nd ed.). Montvale, NJ: Thomson Reuters, Physicians' Desk Reference.

Hu, W., Wells, J.H., Shin, T.S. and Godber, J.S. (1996). Comparison of Isopropanol and Hexane for Extraction of Vitamin E and Oryzanols

from Stabilized Rice Bran. Journal of American Oil Chemists' Society. 73: 1653–1656.

Hui, Y.H. (1992). Encyclopedia of Food science and Technology, John Wiley and sons Inc; Vol. 3, pp: 1992-1994.

Ibrahim, M.F., Abd-Aziz, S., Yusoff, M.E.M., Phang, L.Y. and Hassan, M.A. (2015). Simulraneous enzymatic saccharification and ABE fermentation using pretreated oil palm empty fruit bunch as substrate to produce butanol and hydrogen as biofuel. Renewable Energy. 77: 447-455.

Igwe, J.C. and Onyegbado, C.C. (2007). A review of palm oil mill effluent (POME) water treatment. Global Journal of Environmental Research. 1(2): 54-62.

Jendrossek, D. and Handrick, R. (2002). Microbial degradation of polyhydroxyalkanoates. Annual Review of Microbiology, 56: 403–32.

Jiang, S.T., Shao, P., Pan, L.J. and Zhao, Y.Y. (2006). Molecular Distillation for Recovering Tocopherol and Fatty Acid Methyl Esters from Rapeseed Oil Deodorizer Distillate. Biosystems Engineering. 93(4): 383–391.

Karthishwaran, K., Mirunalini, S., Dhamodharan, G., Krishnaveni, M. and Arulmozhi, V. (2010). Phytochemical investigation of methanolic extract of leaves of *Pergularia daemia*. J. Biological Sci. 10(3):242-246.

Kasim, N.S., Gunawan, S., Yuliana, M. and Ju, Y. (2010). A Simple twoStep Method for Simultaneous Isolation of Tocopherols and Free

Phytosterols from Soybean Oil Deodorizer Distillate with High Purity and Recovery. Separation Science and Technology. 45: 2437–2446.

Khanna, S. and Srivastava, A.K. (2005). Recent advances in microbial polyhydroxyalkanoates. Proocess Biochemistry, 40: 607-619.

Khosla, P. (2006). Palm Oil: a nutritional overview. J. Agro. Food Ind., 17: 21 – 23.

Kobayashi, G., Tanaka, T., Itoh, H., Tsuge, T., Sonomoto, K. and Ishizaki, A. (2000). Fermentative production of P(HB-*co*-HV) from propionic acid by *Alcaligenes eutrophus* in fed-batch culture with pH-stat continuous substrate feeding method. Biotechnology Letters, 22: 1067-1069.

Kok, S., Ong-Abdullah, M., Ee, G.C. and Namasivayam, P. (2011). Comparison of nutrient composition in kernel of tenera and clonal materials of oil palm (*Elaeis guineensis* Jacq.). Food Chem. 129(4): 1343-1347.

Konate, K., Souza, A., Therese, K.Y., Dibala, I.C., Barro, N., Millogo, R.J. and Nacoulma, O.G. (2012). Phytochemical composition, antioxidant and anti in lammatory potential of bioactive fractions from extracts of three medicinal plants traditionally used to treat liver diseases in Burkina Faso. Int. J. Phytomed. 3: 406-415.

Kunasundari, B. and Sudesh, K. (2011). Isolation and recovery of microbial polyhydroxyalkanoates. *eXPRESS* Polymer Letters, 5: 620-634.

Lazarevic, D., Aoustin, E., Buclet, N. and Brandt, N. (2010). Plastic waste management in the context of a European recycling society:

Comparing results and uncertainties in a life cycle perspective. Resource, Conservation and Recycling, 55: 246-259.

Lee, S.Y. (1996). Plastic bacteria? Progress and prospects for polyhydroxyalkanoate production in bacteria. TIBTECH. 14: 431-438.

Lee, J., Landen, W.O., Phillips, R.D. and Eitenmiller, R.R. (1998). Application of Direct Solvent Extraction to the LC Quantification of Vitamin E in Peanuts, Peanuts Butter, and Selected Nuts. Peanut Science. 25: 213–218.

Lee, J., Ye, L., Landen, W.O. and Eitenmiller, R.R. (2000). Optimization of an Extraction Procedure for Quantification of Vitamin E in Tomato and Broccoli Using Response Surface Methodology. Journal of Food Composition and Analysis. 13: 45–57.

Lietz, G., Henry, C.J.K., Mulokozi, G., Mugyabuso, J.K.L., Ballart, A., Ndossi, G.D., Lorri, W. and Tomkins, A. (2001). Comparison of the effects of supplemental red palm oil and sunflower oil on maternal vitamin A status. Food Nutr. Bull. 74(4): 501-509.

Lim, H., Woo, S., Kim, H.S., Jong, S. and Lee, J. (2007). Comparison of Extraction Methods for Determining Tocopherols in Soybeans. European Journal of Lipid Science and Technology. 109: 1124–1127.

Luna, J., Masamunt, M.C., Llach, J., Delgado, S. and Sans, M. (2011). Palm oil tocotrienol rich fraction reduces extracellular matrix production by inhibiting transforming growth factor β1 in human intestinal fibroblasts. Clinical Nutrition, 30(6), 858–864.

Maarasyid, C., Muhamad, I.I. and Supriyanto, E. (2014). Potential source and extraction of vitamin E from palm-based oils: a review. Jurnal Teknologi. 69(4): 43-50.

Madison, L.L. and Huisman, G.W. (1999). Metabolic engineering of poly(3-hydroxyalkanoates): from DNA to plastic. Microbiology and Molecular Biology Reviews, 63: 21-53.

Manorama, R., Sarita, M., Kavita, R. and Rukmini, C. (1997). Red palm oil for combating Vitamin A deficiency. Asia Pac J Clin Nutr. 6(1): 56-59.

Martin, P.F., Batistella, C.B., Maciel-Filho, R. and Wolf-Maciel, M.R. (2006). Comparison of Two Different Strategies for Tocopherols Enrichment Using a Molecular Distillation Process. Industrial Engineering Chemical Research. 45: 753–758.

May, C.Y., Harrison, L.L.N., Han, M.N.N., Ma, A. and Basiron, Y. (2003). Value-added Products from Palm-pressed Fibre. MPOB Information Series. 193.

Mba, O.I., Dumont, M.J. and Ngadi, M. (2015). Palm oil: processing, characterization and utilization in the food industry-a review. Food Bioscience. 10: 26-41.

Mergaert, J., Anderson, C., Wouters, A., Swings, J. and Kersters, K. (1992). Biodegradation of polyhydroxyalkanoates. FEMS Microbiology Reviews, 9: 317-321.

Miller, G.L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. Analyt. Chem. 31: 426-428.

Mohammadi, M., Hassan, M.A., Phang, L.Y., Ariffin, H., Shirai, Y. and Ando, Y. (2012). Recovery and purification of intrace+llular polyhydroxyalkanoates from recombinant *Cupriavidus necator* using water and ethanol. Biotechnology Letters, 34: 253-259.

Mortensen, A. (2005). Analysis of a complex mixture of carotenes from oil palm (*Elaeis guineensis*) fruit extract. Food Res. Int. 38(8-9): 847-853.

Nagesha, G.K., Manohar, B. and Sankar, K.U. (2003). Enrichment of Tocopherols in Modified Soy Deodorizer Distillate Using Supercritical Carbon Dioxide Extraction. European Food Research and Technology. 217(5): 427–433.

Ng, T.K.W., Low, C.X., Kong, J.P. and Cho, Y.L. (2012). Use of red palm oil in local snacks can increase intake of provitamin A carotenoids in young aborigines children: A Malaysian experience. Malaysian Journal of Nutrition, 18(3), 393–397.

Obahiagbon, F.I. (2012). A review: Aspects of the African oil palm and the implications of its bioactives in human health. American Journal of Biochemistry and Molecular Biology, 2(3), 106–119

O'Brien, R.D. (2010). Fats and oils: Formulating and processing for applications (3rd ed.). Boca Raton, Florida: CRC Press.

O'Callaghan, Y., McCarthy, F.O. and O'Brien, N.M. (2014). Recent advances in phytosterol oxidation products. Biochemical and Biophysical Research Communications, 446(3), 786–791.

Pahoja, V.M. and Sethar, M.A. (2002). A review of enzymatic properties of lipase in plants, animal and microorganisms. Pakistan Journal of Applied Sciences. 2(4): 474-484.

Ping, B. and May, C. (2000). Valiable phytonutrients in commercial red palm olein. Palm Oil Developments, 32, 20–25.

Przybylski, R., Zambiazi, R. and Li, W. (1999). Kinetics of sterols changes during storage and frying of canola oils. In Proceedings of the 10th international rapeseed congress. Canberra, Australia, 26–29 September

Poirier, Y. (2002). Polyhydroxyalknoate synthesis in plants as a tool for biotechnology and basic studies of lipid metabolism. Progress in Lipid Research, 41: 131–155.

Posada, L.R., Shi, J., Kakuda, Y. and Xue, S.J. (2007). Extraction of Tocotrienols from Fatty Acid Distillates Using Molecular Distillation. Separation and Purification Technology. 57: 220–229.

Puah, C.W., Choo, Y.M., Ma, A.N. and Chuah, C.H. (2007). The effect of physical refining on palm vitamin E (tocopherol, tocotrienol and tocomonoenol) AJAS. 4(6): 374-377.

Radhika, M.S., Bhaskaram, P., Balakrishna, N. and Ramalakshmi, B.A. (2003). Red palm oil supplementation: A feasible diet-based approach to improve the vitamin A status of pregnant women and their infants. Food and Nutrition Bulletin. 24(2): 208-217.

Ramachander, T.V.N., Rohini, D., Belhekar, A. and Rawal, S.K. (2002). Synthesis of PHB by recombinant *E. coli* harboring approximately 5

kb genomic DNA fragment from *Steptomyces aureofaciens* NRRL 2209. International Journal of Biological Macromolecules, 31: 63-69.

Ramamurthi, S. and McCurdy, A.R. (1993). Enzymatic Pretreatment of Deodorizer Distillate for Concentration of Sterols and Tocopherols. Journal of American Oil Chemists' Society. 70(3): 287–295.

Reddy, C.S.K., Ghai, R., Rasmi and Kalia, V.C. (2003). Polyhydroxyalkanoates: an overview. Bioresource Technology, 87: 137-146.

Rice, A.L. and Burns, J.B. (2010). Moving from efficacy to effectiveness: Red palm oil's role in preventing vitamin A deficiency. Journal of the American College of Nutrition, 29(3), 302–313.

Rodrigues, A.M.C., Darnet, S. and Silva, L.H.M. (2010). Fatty acid profiles and tocopherol contents of buriti (*Mauritia flexuosa*), patawa (*Oenocarpus bataus*), tacuma (*Astrocaryum vulgare*), mari (*Poraqueiba paraensis*) and inaja (*Maimiliana maripa*) fruits. J. Braz. Chem. Soc. 21(10): 2000-2004.

Rossi, M., Alamprese, C. and Ratti, S. (2007). Tocopherols and tocotrienols as free radical-scavengers in refined vegetable oils and their stability during deep-fat frying. Food Chemistry, 102(3), 812–817.

Sahena, F., Zaidul, I.S.M., Jinap, S., Karim, A.A., Abbas, K.A., Norulaini, N.A.N. and Omar, A.K.M. (2005). Application of Supercritical CO_2 in Lipid Extraction–A Review. Journal of Food Engineering. 95: 240–253.

Sambanthamurthi, R., Sundram, K. and Tan, Y. (2000). Chemistry and biochemistry of palm oil. Prog. Lipid Res. 39(6): 507-558.

Sampaio, K.A., Ceriani, R., Silva, S.M., Taham, T. and Meirelles. A.J.A. (2011). Steam deacidification of palm oil. Food Bioprod. Process. 89(4): 383-390.

Sanagi, M.M., See, H.H., Wan Ibrahim, W.A. and Naim, A.A. (2005). Determination of Carotene, Tocopherols and Tocotrienols in Residue Oil from Palm Pressed Fiber Using Pressurized Liquid Extraction-normal Phase Liquid Chromatography. Analytica Chimica Acta. 538: 71–76.

Schroeder, M.T., Becker, E.M. and Skibsted, L.H. (2006). Molecular mechanism of antioxidant synergism of tocotrienols and carotenoids in palm oil. Journal of Agricultural and Food Chemistry, 54(9), 3445–3453.

Shozui, F., Matsumoto, K., Nakai, T., Yamada, M. and Taguchi, S. (2009). Biosynthesis of novel terpolymers poly (lactate-*co*-3-hydroxybutyrate-*co*-3-hydroxyvalerate)s in lactate-overproducing mutant *Escherichia coli* JW0885 by feeding propionate as a precursor of 3-hydroxyvalerate. Applied Microbiology and Biotechnology, 85: 949-954.

Silva, M.G.P.C., Barretto, W.S. and Serodio, M.H. (2004). Comparacao nutrivional da polpa dos frutos de jucara e de acai. In: XVIII Congresso Brasileiro de Fruticultura. Florianopolis, Santa Catarina, 22 a 26 de novembro de 2004. Anais CD ROOM, Florianopolis, SC.

Song, C., Zhao, L., Ono, S., Shimasaki, C. and Inoue, M. (2001). Production of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) from cottonseed oil and valeric acid in batch culture of *Ralstonia* sp. strain JC-64. Applied Biochemistry Biotechnology, 94: 169-178.

Steinbüchel, A. and Fuchtenbusch, B. (1998). Bacteria and other biological systems for polyester production. TIBTECH. 16: 419-427.

Sudesh, K., Abe, H. and Doi, Y. (2000). Synthesis and properties of polyhydroxyalkanoates: biological polyesters. Progress in Polymer Science, 25: 1503-1555.

Sundram, K., Sambanthamurthi, R. and Tan, Y.A. (2003). Palm fruit chemistry and nutritional. Asia. Pac. J. Clin. Nutr. 12: 355-362.

Sivan, Y.S., Jayakumar, Y.A., Arumughan, C., Sundaresan, A., Balachandran, C., Job, J., Deepa, S.S., Shihina, S.L., Damodaran, M., Soman, C.R., Raman, K.V. and Sankara, S.P. (2001). Impact of beta-carotene supplementation through red palm oil. J. Tropical Pediatrics. 47: 67-72.

Sivan, Y.S., Alwin, J.Y., Arumughan, C., Sundaresan, A., Jayalekshny, A., Suja, K.P., Soban, K.D.R., Deepa, S.S., Damodaran, M., Soman, C.R., Raman K.V. and Sankara, S.P. (2002). Impact of vitamin A supplementation through different dosages of red palm oil and retinol palmitate on preschool children. J Trop Pediatr. 48: 24-28.

Szydlowska-Czerniak, A., Trokowski, K., Karlovits, G. and Szlyk, E. (2011). Effect of refining processes on antioxidant capacity total contents of

phenolics and carotenoids in palm oils, Food Chem. 129(3): 1187-1192.

Tabee, E., Ja¯gerstad, M. and Dutta, P.C. (2009). Frying quality characteristics of French fries prepared in refined olive oil and palm olein. Journal of American Oil Chemists Society, 86(9), 885–893

Taguchi, S., Yamada, M., Mutsumoto, K., Tajima, K., Satoh, Y., Munekata, M., Ohno, K., Kohda, K., Shimamura, T., Kambee, H. and Obata, S. (2008). A microbial factory for lactate-based polyesters using a lactate-polymerizing enzyme. Proceedings of the National Academy of Sciences of the United States of America, 105: 17323-17327.

Tan, B.K. and Oh, F.C. (1981). Malaysian palm oil: Chemical and physical charactristics. PORIM Technology, 3, 1–5.

Tan, C.P. and Nehdi, I.A. (2012). The physicochemical properties of palm oil and its components. In O.-M. Lai, C.-P. Tan, & C. C. Akoh (Eds.), Palm oil: Production, processing, characterization and uses (pp. 377–390). Urbana, Illinois, USA: AOCS Press.

Tan, C.P. and Man, Y.B.C. (2000). Differential scanning calorimetric analysis of edible oils: Comparison of thermal properties and chemical composition. Journal of American Oil Chemists Society, 77(2), 143–155.

Tavares, M. and Barberion, J.C. (1995). Fatty acid composition of Brazilian palm oil. In Paper presented at the PORIM international palm oil congress: Update and vision in chemistry and technologies. Kuala Lumpur.

Teixeira, C.B., Macedo, G.A., Macedo, J.A., da Silva, L.H.M., da C. Rodrigues, A.M. (2013). Simultaneous extraction of oil and antioxidant

compounds from oil palm fruit (*Elaeis guineensis*) by an aqueous enzymatic process. Bioresource Technology. 129: 575-581.

Usoro, E.J., (1974). The Nigerian oil palm Industry Ibadan University Press, pp: 1-3.

van Stuijvenberg, M.E., Dhansay, M.A., Lombard, C.J., Faber, M. and Benade, A.J. (2001). The effect of a biscuit with red palm oil as a source of beta-carotene on the vitamin A status of primary school children: a comparison with beta-carotene from a synthetic source in a randomised controlled trial. Eur J Clin Nutr. 55: 657–662.

Wei, P.C., May, C.Y., Ngan, M.A. and Hock, C.C. (2007). The Effect of Physical Refining on Palm Vitamin E (Tocopherol, Tocotrienol and Tocomonoenol). American Journal of Applied Science. 4(6): 374–377.

Wu, S.J., Liu, P.L. and Ng, L.T. (2008). Tocotrienol-rich fraction of palm oil exhibits anti-inflammatory property by suppressing the expression of inflammatory mediators in human monocytic cells. Molecular Nutrition and Food Research, 52, 921–929.

Wu, S.J. and Ng, L.T. (2007). Antioxidant and antihepatoma activities of palm oil extract. Journal of Food Lipids, 14, 122–137.

Yamada, M., Matsumoto, K., Nakai, T. and Taguchi, S. (2009). Microbial production of lactate-enriched poly[(R)-lactate-*co*-(R)-3-hydroxybutyrate] with novel thermal properties. Biomacromolecules, 10: 677-681.

Yin, N.S., Abdullah, S. and Phin, C.K. (2013). Phytochemical constituents from leaves pf *Elaeis guineensis* and their antioxidant and

antimicrobial activities. International Journal of Pharmacy and Pharmaceutical Sciences. 5(4): 137-140.

Yu, H.H., Shi, Y., Zhang, Y., Tang, S. and Shen, Z. (2002). Effect of *Vitreoscilla* homoglobin biosynthesis in *Escherichia coli* on production of poly(beta-hydroxybutyrate) and fermentative parameters. FEMS Microbiology Letters, 214: 223-227.

Zagré, N., Delpeuch, F., Traissac, P. and Delisle, H. (2003). Red palm oil as a source of vitamin A for mothers and children: impact of a pilot project in Burkina Faso. Public Health Nutrition. 6(8): 733–742.

Zigoneanu, I.G., Williams, L., Xu, Z. and Sabliov, C.M. (2008). Determination of Antioxidant Components in Rice Bran Oil Extracted by Microwave-assisted Method. Bioresource Technology. 99: 4910–4918.

Zou, Y., Jiang, Y., Yang, T., Hu, P. and Xu, X. (2012). Minor constituents of palm oil: Characterization, processing, and application. In O. -M. Lai, C.-P. Tan, & C. C. Akoh (Eds.), Palm oil: Production, processing, characterization and uses (pp. 471–524). Urbana, Illinois, USA: AOCS Press.