M.Tech (Industrial Biotechnology)

Parul Sharma

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ENZYME ASSISTED AQUEOUS EXTRACTION OF VEGETABLE OIL AND EVALUATION OF ITS PHYSICOCHEMICAL AND NUTRITIONAL PROPERTIES

A DISSERTATION

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Submitted By:

PARUL SHARMA

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Under the supervision of

NAVNEETA BHARADVAJA

DEPARTMENT OF BIOTECHNOLOGY

DELHI TECHNOLOGICAL UNIVERSITY

(Formerly Delhi College of Engineering)

Bawana Road, Delhi-110042

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DELHI TECHNOLOGICAL UNIVERSITY

(Formerly Delhi College of Engineering)

Bawana Road, Delhi – 110042

CANDIDATE'S DECLARATION

I, Parul Sharma, Roll no 2K16/IBT/06 of M.Tech (Industrial Biotechnology), hereby declare that the project Dissertation titled "Enzyme assisted aqueous extraction of vegetable oil and evaluation of its physicochemical and nutritional properties" which is submitted by me to the Department of Biotechnology, Delhi Technological University, Delhi in partial fulfillment of the requirement for the award of the degree of Master of Technology, is original and not copied from any source without proper citation. This work has not previously formed the basis for the award of any Degree, Diploma Associateship, Fellowship or other similar title or recognition.

Place: Delhi PARUL SHARMA

Date:

DELHI TECHNOLOGICAL UNIVERSITY

(Formerly Delhi College of Engineering)

Bawana Road, Delhi – 110042

CERTIFICATE

I hereby certify that the project dissertation titled "Enzyme assisted aqueous extraction of vegetable oil and evaluation of its physicochemical and nutritional properties" which is submitted by Parul Sharma, 2K16/IBT/06 (Department of Biotechnology), Delhi Technological University, Delhi in partial fulfillment of the requirements for the award of the degree of Master of Technology, is a record of the project work carried out by the student under my supervision at Novozymes South Asia Pvt. Ltd. To the best of my knowledge this work has not been submitted in part or in full for any Degree or Diploma to this University or elsewhere.

Place: Delhi

Date:

Dr. NAVNEETA BHARDVAJA

(SUPERVISOR)

CERTIFICATE

Dear Parul,

We are happy to inform you that the research work *"Thesis title:'Enzyme assisted aqueous extraction of vegetable oil and evaluation of its physicochemical and nutritional properties' at Novozymes South Asia Pvt. Ltd*" which you have initiated on 19th Feb'18 during your internship is granted for your submission.

You can use this mail for your submission work and the experience certificate will be given as per the company protocol on the final day of your internship.

Wish you good luck!

Sincerely

Sibabrata

Best regards,

Sibabrata Mukherjee

Scientist II, Project lead.

Novozymes South Asia Pvt. Ltd.

Plot No. 32, 47-50

EPIP Area

Whitefield

560066 Bangalore India

Phone: +91 8030593500

Mobile: +91 9513371537

E-mail: smu@novozymes.com

ABSTRACT

Edible oils are among the most abundant cooking ingredients in the world and form a big sector of the food industry. Soybean, sunflower, rape and palm oil account for more than 70% of vegetable oils. Solvent method of extraction is the most common method employed at the industrial scale for extraction of vegetable oil. This method, however, has a lot of disadvantages from health hazard to environmental pollution being the primary. Aqueous enzymatic extraction of vegetable oils has recently gained popularity. This method is environmentally clean and poses no health hazard. The study was done to optimize the process parameters of aqueous enzymatic extraction to enhance the oil yield and identify the change in physicochemical properties of the oil before and after treatment with oil.

Process parameters like enzyme dosage, G-force, and rotor type were optimized to obtain maximum oil yield. Soxhlet was run using hexane as solvent to identify losses in the waste stream i.e. sludge and fiber. The oil obtained after the enzymatic treatment was assessed for its free fatty acid content and deterioration of bleachability index. Other properties like carotene content and total moisture content was also analyzed. Effect of heat on oil quality was also analyzed by quantifying free fatty acids after subjecting oil to 90^{o}C for five hours. Role of exogenous lipases in deteriorating oil quality was assessed by adding a lipase at 100ppm, 50ppm and 5ppm and then determining the FFA generated.

Enzyme A_1 (higher dose), 9600 G-force, and swing out rotor were identified as the best fit giving maximum oil yield. Soxhlet run showed that after enzyme addition, oil losses were significantly reduced in the waste stream leading to more oil yield in the product. Microscopic analysis of the sample revealed cell wall degradation and release of the oil droplet in the surrounding environment. The FFA was relatively same in oil derived from enzymatic and non-enzymatic method. FFA value was seen to rise slightly and then become stable after subjecting the oil to 90^{o}C for five hours. The DOBI value, however, showed a sharp decline. 100ppm concentration of lipase upon addition showed a sharp increase in FFA content while 50ppm and 5ppm dosages had FFA content similar to the control sample.

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CHAPTER 1 – INTRODUCTION

Oils are one of the major components of the food industry and are an essential part of human diet. With an increasing population, demand for edible vegetable oils is at its highest. Vegetable oils can be extracted from various parts of the plants like nuts e.g. walnut oil, almond oil, cashew oil, hazelnut oil or seeds e.g. sunflower oil, sesame oil, rapeseed oil or fruits e.g. coconut oil, olive oil, palm oil etc. 70 percent of the vegetable oils are Soybean, sunflower, rape and palm (Domínguez and Lema, 1994). These oils can be used for various cooking (frying, baking, dressing) or non-cooking (cosmetics, bio-fuel) purposes. Oil can be extracted with the help of either mechanical pressing methods like hydraulic press or solvent extraction. Various enzymatic methods have also been employed in order to increase the oil yield.

Solvents like hexane are generally employed to extract oil which extracts 99% oil leaving behind as little as 0.5% to 0.7% oil residue in the raw material. Organic solvent extraction is dependent on the nature of the solvent, reaction time, size of seeds/mesocarp, process temperature and the solid/solvent ratio (Ali and Watson, 2014). Oil from raw materials with low oil content or pre-pressed oil cakes can easily be extracted using this method. Because of these obvious advantages and very high yield of oil, this has become the most commonly used oil recovery method (Ali and Watson, 2014). While the method is very efficient in terms of oil yield, it is not environmentally clean and is associated with various health hazards due to constant exposure to solvents (Ricochon and Muniglia, 2010). Volatile Organic compounds produces from the process is harmful and is responsible for pollution of the atmosphere. These react with the other pollutants of the atmosphere to create a toxic environment. It is also carcinogenic and contributes towards global warming as it is a greenhouse gas. Because of its toxic nature, it also damages crops. (Rosenthal et.al, 1996).

Direct mechanical pressing of fruit for oil extraction gives yield lower in comparison to solvent based methods with residual oil in the range of 6% to 14%. During mechanical screw pressing, the efficacy of the process is dependent on the moisture content of the substrate. Increasing the moisture content softens the seeds while lowering the moisture content increases friction (Ali and Watson, 2014).

Although use organic solvents and mechanical pressing have high yield, its environmental impact is worrisome and therefore an alternate method of extraction

using enzyme was developed. Enzyme assisted aqueous extraction process has been developed in the last 50 years to provide an alternative for traditional methods (Ricochon and Muniglia, 2010). The choice of enzyme used in the process is dependent on the target macromolecules. A combination of hydrolytic enzymes like cellulases, hemicellulases, pectinases etc. are used to break down the cell wall of the oil-bearing cells. The enzymes target the structural polysaccharides of the cell wall or proteins of the lipid body membrane. This leads to higher oil yield and because enzymatic reactions are temperature controlled, mild extraction conditions lead to energy saving, making the process cost effective. On industrial scale factors like moisture content, size of the particle, enzyme/substrate ratio, temperature etc. play direct role extraction efficiency (Mariano et.al, 2009). Along with high oil yield, the process is economical since the solvent recovery step is eliminated and is safer because of lower risk of fire due to an explosion. However, there are certain limitations associated with the process. Because water is used in the process, emulsions are formed which needs to be broken down to recover oil. Large oil mills which process tons of oil everyday produce a very high quantity of oil mill effluent which has high amount of suspended solids and a high biological oxygen demand. For example, Palm oil mill effluent (POME), a highly polluting material needs to be processed prior to its release in the environment. This increases the cost of the process (Rosenthal et.al, 1996).

While fats are an important dietary constituent, consumption of healthier oil with the balanced intake is of utmost importance. Based on the type of fatty acids present in the oil, it can be classified as healthy or non-healthy. Unsaturated fatty acids are generally regarded as healthier fats. Unsaturated fats can be monounsaturated with a single double (e.g. olive oil, peanut oil, sesame oil) and polyunsaturated with multiple double bonds. Saturated fats are fatty acids with single bonds in the carbon chain (e.g. coconut oil, palm kernel oil). Palm oil has been widely used in parts of Brazil, South-east Asia and Africa mainly due to lower cost and high stability at high temperatures for longer duration of time. The oil palm is a monocotyledonous plant which hails from the genus *Elaeis*. The oil palm tree is perennial and produces very large quantities of oil of about 3.7 tonnes of oil per hectare annually making Malaysia a big player in the oil industry. The crop is unique on its own in the sense that it produces two types of oil: the palm oil from its thick mesocarp and the palm kernel oil from its kernel. The palm oil from the

mesocarp is used for edible purposes while palm kernel oil has other uses like in the cosmetic industry and the oleo-chemical industries.

The genus *Elaeis* is part of two species, namely *E. Guineensis* and *E. Oleifera.*(Rees,1965). *E. Guineensishas* its origins in West Africa and the commercially grown plants are mainly of this species. *E. Oleifera,* bearing a South American origin, is a stumpy plant and its oil is high oleic acid. South-east Asia countries like Malaysia and Indonesia are clearing out acres of land everyday to for cultivation of oil palm trees. A very high amount of oil is exported out of these two countries. Malaysia has become the biggest supplier of crude palm oil after its oil export increased by 2.9 million between 1998 and 2002. It is the second most important oil after soyabean and it contributes to 13% of worldwide oil and fats production. Oil palm initially grown as ornamental became commercial only after its cultivation in 1917 where it was planted on a commercial scale.

Elaies guineensis which originated in West Africa, was initially introduced to Brazil as early as the 15th Century by the Portuguese (Corley, 1976). However, its application and use did not start until the 19th Century when the Dutch brought with them, the seeds from West Africa all the way to Indonesia. The palms were *dura,* and their offsprings were planted as ornamentals in Deli , hence the name Deli *Dura*. Finally, it was brought to present west Malaysia in 1878.

The South American species *Elaeis oleifera* has a lower content of palmitic acid and higher amount of oleic and linoleic acid with its iodine values ranging from 78-80. The fruit is a drupe that forms in a tight bunch. The pericarp is made up of three layers, exocarp or skin; mesocarp or the outer pulp rich in palm oil; and endocarp or the hard shell covering the kernel or the endosperm which contains oil As well as carbohydrate which act as stored supplies for the embryo. Its fruit begin developing approximately 2 weeks after anthesis (WAA). The deposition of oil in the endosperm initiates about 12 WAA and is 95% completed within 16 WAA (Oo Kc, 1984).This is the period when the endosperm and endocarp slowly start hardening and by 16 WAA, the endocarp becomes a hard shell enveloping a white hard endosperm which is the kernel. It takes about 15WAA for the oil deposition to start in the mesocarp and it continues till the fruit is completely mature at about 20 WAA. All the fruits on a bunch have different ripening times due to variations in the pollination time. Fruits at the base ripe last and the end of

each spikelet ripen first. Fruits on the outside of the bunch turn large and deep orange in colour after ripening while the fruits on the inner side are smaller and much paler.

Chemistry

Tri Glycerides (TGs) form the major composition of palm oil. More than 95% of palm oils are made of combination of different fatty acids making esters with the glycerol molecules. Three fatty acids are associated with a single glycerol molecule forming a triglyceride. During extraction of oil from its mesocarp, the triacylglycerols attract other hydrophobic components with it and the final product has minor components like Vitamin E (tocopherols, tocotrienols), phosphatides, sterols, and pigments like beta carotene. Other than these major and minor component are present the metabolic by products of biosynthesis of triacyglycerols. Monoglycerides, diglycerides and free fatty acids are present in various proportions.The fatty acids are members of a class of aliphatic acids, namely: palmitic acid (16:0), stearic acid (18:0) and oleic acid (18:1) in animal and vegetable fats and oils respectively. Myristic acid (14:0), palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1) and linoleic acid (18:2) are the major fatty acids present in palm oil (Siewet al. 2000). Palm oil also contains saturated and unsaturated fatty acids in almost equal amounts. Most of the fatty acids are present in the form of TGs. Tripalmitin is the majorly present TG of the 7% to 10% of saturated TGs (Kifli 1981) and unsaturated TGs comprise 6 to 12%.

Minor constituents of palm oil

The minor constituents of palm oil can be segregated into two groups. The first consists of fatty acid derivatives like partial glycerides (MGs, DGs), esters, phosphatides, and sterols. The second group is made up of classes of compounds which are not related to fatty acids chemically. Hydrocarbons, esters aliphatic alcohols, x tocopherols, trace metals and pigments make up this group (Sambanthamurthi et al., 2000). A large fraction of the m*i*nor components in the unsaponifiable portion of palm oil are composed of sterols, higher aliphatic alcohols, pigments and hydrocarbons. Partial glycerides and phosphatides, are saponifiable by alkaline hydroxide.

Vitamin E, a fat soluble vitamin has isomers like Tocopherols and tocotrienols is present in oil palm. These give the oil its characteristic antioxidant property and is also responsible for the high stability of the oil. Tocopherols have two main categories:

tocols and tocotrienols. The tocotrienols are unsaturated in nature while the side chain present in tocols is starurated. In case of vegetable oils, Tocotrienols are rarely seen except in palm and rice bran oils. Tocols and tocotrienols are present in 600 to 1000 ppm in crude palm oil. Refining process however, reduces their levels as low as to 350-630 ppm. Because of its antioxidant property, they serve as radical scavengers in vivo and thus are beneficial.

The palm fruit pigmentation is related to their maturity stage. Carotenoids and chlorophylls are the two classes of natural pigments found in crude palm oil. The palm oil procured from young fruits is made up of more chlorophyll and less carotenoids as compared to mature or ripe fruits. These pigments are involved in autoxidation, photooxidation and antioxidation within the plant (Tan et al., 1986). Carotenoids are identified as highly unsaturated tetraterpenes which are formed by the bio-synthesized of eight isoprene units. There are two main classes of Carotenoids, namely; carotenes (polyene hydrocarbons) and xanthophylls (contain oxygen). This oxygen could be of hydroxy like zeaxanthin and lutein, keto, epoxy or carboxyl groups. Lycopene is the simplest carotene. The rich orange-red colour of crude palm oil is due to its high carotene content 700–800 ppm. The α and β –carotene account for 90% of the total carotenoids (Yap et al., 1997). Palm oil has about 11 different types of carotenoids.

Beta carotene present in palm oil has a very high provitamin A activity since caretenoids are known precursors of Vitamin A. Palm oil has 15 times more Retinol Equivalents in comparison to carrot and 300 times more in comparison to tomato. They are also oxygen and light sensitive. In presence of light, oxidation leads to formation of peroxides because of lipid peroxidation leading to bleaching and discoloration of the oil. This refined, bleached, deodorized palm oil is sold in the market. Refining crude palm oil first produces carotenoids which are removed partially by adsorption on activated earth followed by high temperature steam deodorization that destroys the chromogenic properties of the carotenoids that are remaining and results in light yellow coloured oil. The fatty acid composition of palm oil is1:1 saturated to unsaturated fatty acids which keeps the oil in a semi-solid state at room temperature. This property of palm oil and its melting range permit its use as a key ingredient in margarine (Nor et al., 2000). Hence, palm oil does not need hydrogenation for most practical purposes.

The biotechnology company Novozymes, headquartered in Copenhagen, Denmark develop and manufactures industrial enzymes that enhance oil extraction efficiency, and biopharmaceutical ingredients using genetically engineered microorganisms (Novozymes, Wikipedia). It was founded in 2000 when the original Novo was split into three companies namely: Novo A/S, Novo Nordisk A/S, and Novozymes A/S (Novozymes Global). The inception of the company goes back to 1925 when two brothers Harald and Thorvald Pedersen with the aim of producing insuln, founded Novo Terapeutisk Laboratorium and Nordisk Insulin Laboratorium (Novozymes A/S).Since then the company expanded to countries like China, India, Brazil, Argentina, United Kingdom, the United States, and Canada (Novozymes, Wikipedia). Novozymes South Asia covers India, Sri Lanka, Bangladesh, Nepal and Bhutan and is the largest manufacturer of microorganisms and industrial enzymes (Novozymes India).

A big part of the company is involved in production of enzymes that assist detergents for cleaning of fabric. Novozymes was the first company to introduce a fat-splitting enzyme for detergents that was manufactured from genetically engineered microbes. Novozymes started its R&D activities in India in 2006 in Bangalore. The centre was created to play an important role towards discovering new applications and technologies both globally and locally. Since then, the state-of-the-art centre has grown to four departments, namely Strain and Process Development, Protein Engineering, Protein Assay and Technology and Application Technology. The centre also serves as an application technology excellence centre for Novozymes' global juice and wine industry requirements (Novozymes, India).

Some of the key business areas for Novozymes India are (Novozymes, India):

- Household Care
- Textiles
- Food & Beverages
- **Oils & Fats**
- Baking and
- Beverage/ Alcohol

Because palm oil is the second highest utilized and consumed worldwide, production of enzymes for extraction of oils and fats are therefore one of the top priority projects at

Novozymes, India. As mentioned above, the aqueous enzymatic extraction of oil is environmentally safer; the company aims to produce enzymes that will help develop an efficient extraction system in order to eliminate the traditional methods of extraction.

OBJECTIVES

1. Optimization of process parameters for enzymatic extraction of vegetable oil from the fruit.
2. Assessing the physicochemical properties of oil before and after enzymatic treatment.
3. Effect of heat on oil stability
4. Effect of exogenous lipase on oil quality

CHAPTER 2 – REVIEW OF LITERATURE

The advantage of aqueous enzymatic oil extraction over conventional extraction has made it an emerging technology in the fats and oil industry. An example would be its ability to eliminate solvent consumption which, consequently, may lower investment costs (Lusas et al, 1982) and energy requirements (Barrios et al, 1990). Along with the lower cost of operation, it also facilitates concurrent recovery of oil and high standards of proteins which comply with the Codex specifications. Degumming operations can be eliminated and allows for the elimination of some toxins or antinutritional compounds from certain oilseeds (Caragay and Pacing, 1983). While monetary returns plays a major role in the oil sector, other facts like health and environment safety, cost cutting solutions, and nutritional needs have to be considered and can be achieved by enzyme based extraction process. The last four decades has seen several studies on aqueous processing of oilseeds (Rosenthal, 1996). Even though the concept might appear potentially attractive as compared to the conventional hexane-based process, the lower yield of oil and relatively high content of oil in the residue have discouraged its commercial application.

Some studies (Lanzani 1975, Fullbrook 1983, Sosulski 1988) have shown that hydrolytic enzymes can be employed to break down cell structure and extract out oil in order to combat the problem of low oil yield and less extraction efficiency of aqueous enzymatic processes. Apart from this, the problems associated with solvent based extraction like emission of volatile organic compounds that react with the gases in the environment forming low level ozone and causing smog has also led to the shift in focus towards aqueous methods of extraction. A review (Dominguez, 1994) discussed about the enzymatic pretreatments applied to various oil seeds and fruits. Its main motive was to discuss the essential advantages and disadvantages of aqueous enzymatic processes as compared to conventional solvent-based processes more specifically with respect to the environmental and economic aspects and the resulting products quality; to analyze published information on processes that employ aqueous extraction media which may or may not use enzymes; to discuss the structure of oilseeds and the mechanisms of enzyme action on the oilseed which would lead to the accurate enzyme selection for the aqueous or conventional process; to describe the function of operational parameters on process optimization; and finally to review the formation and stability of oil-in-water emulsions and alternative strategies for the downstream processing for oil recovery.

The main cost management techniques employed by the food industries focus on high yield of the product, reducing the number of by products or products that require secondary treatment before disposal, and finally use of mild operational conditions to save energy. These can be achieved by using enzymes as part of the production cycle. (Christensen 1991, Dominguez 1994, Rosenthal 1996) reviewed the use of enzymes in the extraction of oil or protein or both simultaneously from fruits or seeds rich in these components. The main idea in these operations is to use hydrolytic enzymes and cellular components like the cell wall or the oleosome to extract out oil or proteins under mild temperature and pH conditions without the use of explosive solvents and minimum waste generation (Christensen 1991, Dominguez 1994, Rosenthal 1996).

This technology has been developed in order to extract oil from many sources: avocado (Buenrostro, 1986), coconut (McGlone et al 1986, Che Man et al 1996, Tano-Debrah et al 1997), corn germ (Karlovic et al 1994), rapeseed (Sarker et al 1998), soybean (Dominguez et al 1993, Kashyap et al 1997), and sunflower (Dominguez,1993,1995, Singh et al, 1999).

In a recent study (Hanmoungjai et al 2001), enzymatic extraction of oil from rice bran was carried out. This investigation was carried out using Alcalase, a commercial protease followed by evaluation using response surface methodology. It concluded that the enzyme concentration has a significant role to play in the oil yield. On the other hand, other parameters such as incubation time and temperature did not have a notable effect. The maximal yield of oil was about 79%. In terms of free fatty acid, iodine value, and saponification value, the quality of oil recovered was comparable to that recovered using the solvent based method. However the peroxide level was higher in the oil extracted by the enzymatic method.

A similar study was carried out (Najafian et al, 2008) on virgin olive oil extraction. Three varities of olives were used, namely, Kroneiki, Iranian Native Oleaginous and Mission. These were treated with Pectinex Ultra SP-L and Pectinase 1.6021 at zero, low and high concentration to check the effect on yield, total polyphenols, turbidity, colour, acidity, peroxide value and iodine value. The yield, colour, turbidity and total polyphenol level of oil was affected by the change in concentration of the enzymes. But, there were no effects on acidity, peroxide value and iodine value. The results of this study concluded that Pectinex Ultra SP-L was more efficient than Pectinase 1.06021.

The production of corn oil from corn germ has also been shown to produce 80% yield when it is recovered using three different commercial cellulases (Robert et al 2004). In the control study, yields as low as 27% were observed in the absence of enzymes. The chemical compositions however, were very similar for hexane-extracted and aqueous-enzymatic-extracted corn oils.

In the case of coconut oil extraction, various techniques are available. The wet process of extraction that recovers only about 30-40% oil relies on the grating of the coconut. The oil is then separated from the milk extracted by cooking. This process, however, is not very efficient and leads to low oil yield (Thieme et al 1968). The oil is surrounded by different proteins and cellular material making the separation hard thereby reducing the extraction efficiency (Gonzales et al 1990). Also, the quality of the oil obtained by this process is poor as a result of the moisture content being quiet high and short shelf life (Thieme et al 1968). Mechanical methods like grating and pressing are also employed to extract oil. The mechanical force generated breaks down the cell walls of the plant (Hagenmaier et al 1980). This breakdown of plant cell walls could also be hydrolyzed and degraded with the help of various enzymes, releasing the oil (McGlone *et al 1986*). A study of coconut oil extraction (McGlone *et al, 1986.)* was done using polygalacturonase, a-amylase, and protease. These enzymes were added on diluted coconut paste, the results confirmed 80% of yield, even without further purification steps as per Official Mexican Standards.

A more detailed study of this application was done using cellulase, α-amylase, polygalacturonase, and protease at varying concentrations, pH conditions,as well as temperatures to investigate their effects on extraction yield and quality of the coconut oil (Che Man et al, 1996). After oil release by enzyme treatment, centrifugation was carried out. The findings of this study showed that 1% (w/w) enzyme mixture, each of cellulase, a-amylase, polygalacturonase, and protease at a pH of 7.0 and 60^{o}C of extraction temperature characterized the most efficient extraction conditions giving an oil yield of 73.8%. The resulting oil had a moisture content of 0.11%; iodine value of 8.3; free fatty acid, 0.051%; saponification value of 260; peroxide value of 0.016 meq oxygen/kg; anisidine value of 0.026; and color of 0.6 (Y + 5R). This technique was a significant improvement in both oil yield and quality over the traditional wet process.

A comparative study in the enzymatic extraction of *Moringa oleifera* seed oil was undertaken by Abdulkaram et al. which proved that a combination of enzymes produces better results over enzymes used individually (Abdulkarim et al., 2005). In this venture, the enzymes Neutrase 0.8L which is a neutral protease, Termamyl 120L, an type L α-amylase, Pectinex Ultra SP-L, a pectinase and Celluclast 1.5L FG, a cellulose were used on their own as well as in combination. Neutrase showed the best results followed by Termamyl, Celluclast and lastly, Pectinex. However, in combination, their yields were higher than the yields obtained with the use of Neutrase. The percentage of oil recovery for individual enzymes under optimum conditions and pH adjustment to the individual enzyme's optimum pH was, 71.9%, 64.8%, 62.6% and 56.5% for Neutrase, Termamyl, Celluclast and Pectinex, respectively. Neutrase, Pectinex and the combination of all the four enzymes at 2% (v/w) gave the best resullts at 45C, while Termamyl and Celluclast most efficient at 60C. The physic-chemical properties of the oils extracted such as iodine value was 66.0–67.2 g iodine/100 g of oil, free fatty acid (FFA) content of 1.13–1.25 as % oleic acid, complete melting points of 18.6–19.1^{o}C and viscosities of 83.1–85.0 cP.

This study was followed by another investigation by the same team to establish the physic-chemical properties of the oil obtained from the *Moringa oleifera* oil seed. The enzyme Neutrase 0.8L was selected for its performace in the aforementioned study. The same seeds were extracted using solvents as well and a comparative analysis was carried out. The fatty acid compositions of the oil extracted by solvent and enzyme-method from *M. oleifera* seed were determined. The inferences concluded that the solvent-extracted oil has 67.9% oleic acid as compared to enzyme-extracted oil which had an oleic acid content of 70.0%. Analysis results of the extracted oil showed that the oil is highly unsaturated due to the high percentage of oleic acid. Other than oleic acid, other prominent fatty acids present were palmitic acid (7.8% and 6.8%), stearic acid (7.6% and 6.5%), and behenic acid (6.2% and 5.8%) for solvent and enzyme-extracted processes respectively. The oil was liquid at room temperature having a pale-yellow colour. The values were 0.7R + 5.9Y for solvent extracted oils and 0.7R + 3.0Y for enzyme-extracted oils. The oil was found to have a flavour similar to peanut oil using Electronic nose analysis. Differential scanning calorimetry was used to find out the melting points. The melting temperature for the solvent extracted oils was 19.0^{o}C and 18.9 ^{o}C for the enzyme extracted oils. Triolein was the main triacylglycerol at 36.7%. It

was observed that extraction methods slightly influence the relative amounts of the fatty acids present in the oil. The percentage composition of fatty acids in the oils extracted using these two methods were found to differ. Enzyme extracted oils however had better quality attributes such as percentage of unsaturated fatty acids, relative percentage of oleic acid, iodine value, unsaponifiable matter contents, free fatty acid and colour (Abdulkarim et al., 2005).

Another investigation was carried out by Response Surface Methodology in the extraction of Soybean oil. The enzymes used were, protease and cellulase. These enzymes were selected after preliminary experiments which showed their increments in oil yield as compared to hemicellulase and pectinase. The quantitative parameters which gave the best results were as follows: enzyme concentration: 0.1, 0.45, 2 w/w %; liquid-to-solid ratio: 0.05, 0.125, 0.2; mean particle size: 212.5, 449.5, 855 mm; and time of hydrolysis: 30; 60; 120 min (Rosenthal, 2001).

Grapeseed oil extraction was also optimized using a cocktail of cell wall degrading enzymes (Cláudia, 2008). The difference in this venture was the duration of enzyme treatment. The previous studies that were carried out (Dominguez et al.1994; Rosenthalet al., 1996; Sineiro et al, 1998) involved enzyme treatments for short durations like 15-120 mins. This study however, was done with longer duration of enzyme treatment. When treated for 24hrs at a pH of 4 with temperatures between 30–40^{o}C, particle diameters between 1.0–1.4 mm, and cocktail concentration of cellulase, protease, xylanase, and pectinase (29, 1191, 21, 569 respectively) U/g of seed sample, the extraction yield was 13.7%, which represents an increment of 106% over non-treated samples. The yield was 17.5% and the increase reached to 163% when the enzyme treatment was carried out for 120 hrs. Thus, the results illustrate that enzymatic treatment for a long period of time can certainly be used for the extraction of oil.

A similar study was conducted for the extraction of rice bran oil using a mixture of Protizyme^{TM} (protease; Jaysons Agritech Pvt. Ltd., Mysore, India), Palkodex^{TM} (α-amylase; Maps India Ltd., Ahmedabad, India), and cellulose (crude cellulase; Central Drug House, Delhi, India).A recovery of 77 % was achieved when 10 g of rice bran in 40 mL of distilled water was treated with the mixture of enzymes at 65^{o}C for 18 h of constant shaking at 80 rpm followed by centrifugation for 20 min at 10,000xg. The pH was maintained at 7.0 throughout process (Aparna et al., 2001).

A unique investigation was undertaken in the aqueous extraction of peanut oil wherein Protizyme^{TM}, which comprises of acid neutral and alkaline proteases, was used (Aparna et al, 2002). This study diverges from previous studies that focussed on a combination of different enzymes like α-1,4-galacturonide glucanohydrolase, cellulose and protease. Employing the same enzyme with different pH optima 3-4, 5-7, 7-10 resulted in a recovery of 86-92%. The optimal parameters for this recovery percentage were pH 4.0 at 40^{o}C, incubation period of 18hrs and enzyme concentration of 2.5% (w/w) in 10g of peanut seeds. A study was then done which compared aqueous enzymatic extraction using Protizyme^{TM} in one and enzymes like chymotrypsin, trypsin and papain in the other. Papain showed better results in comparison to trypsin or chymotrypsin which backed previous studies relating to higher yield when applying aqueous enzymatic oil extraction methodology over simple water based extraction without use of enzyme. Minute amount of lipases, amylases and cellulases were also present in enzyme prepearation which might have helped the extraction and recovery of oil. Because the proteases had a wide range of optimum pH, it gave a unique advantage of choosing the optimum pH depending on what extraction conditions are feasible and the isolelectric point of the protein that is present in majority. The study showed that proteins like papain which are non specific in nature can also give high oil yield in comparison to well defined and characterized proteases like trypsin and chymotrypsin.

The *Jatropha* wood and fruit can be utilised for various purposes including fuel. Its seeds contain viscous oil, which has been harnessed in the manufacturing of candles and soap, in the cosmetics industry sector, as a diesel or paraffin substitute or extender. This latter application has notable implications in order to meet the demands for the rural energy services and also in the case of exploring practical replacements for fossil fuels to counter the effect of greenhouse gas accumulation in the atmosphere. These traits along with its versatility make it of essence to the developing countries (Foidl and Kashyap, 1999).Being a drought-resistant shrub or tree, and also having a wide distribution in the wild or semi-cultivated areas in places like South East Asia, Central and South America, India, and Africa (Cano-Asseleih, 1986; Cano-Asseleih et al., 1989). Its commercial applications debuted from Lisbon, where the oil was imported from Cape Verde and was used for the production of soap and lamps. Other than just being a source of oil, *Jatropha* also served as a highly nutritious and cost saving protein supplement in animal feed, only if the toxins were eliminated (Becker and Makkar,

1998). The plant could also be used to help prevent soil erosion, land reclamation. It could be grown into a living fence, specifically to keep away farm animals and also planted as a commercial crop (Heller, 1996). Most of the parts of the plant were shown to have medicinal value, for instance, its bark contains tannin, the flowers help attract bees and thus it has a potential for honey production. Fuel can be obtained from its wood and fruit along with other numerous uses. The most advantageous fact about this plant is its relatively quick growth time.

In order to enhance the process of aqueous enzymatic oil extraction from the seeds of *Jatropha curcas* L, ultrasonication was performed as a pre-treatment before aqueous as well as aqueous enzymatic oil extraction (Shweta et al., 2004). The study concluded that 10 min exposure to ultrasonication at pH 9.0 followed by aqueous oil extraction yields 67% oil. On the other hand, a maximum yield of 74% was attained by ultrasonication for just 5 min before aqueous enzymatic oil extraction with the help of an alkaline protease at a pH of 9.0. Ultrasonication also reduced the process time from 18 to 6 h.

The oils extracted also need to be analysed for Free Fatty acids (FFA) and yield. Both are somewhat co-related. It is desirable to obtain the final product with a low FFA and high yield. For this purpose, various parameters must be changed and optimised. A study was done for optimising this process by Chu et al and studying the physiochemical properties of the palm oil extracted. Their findings were verified and optimised with the help of response surface methodology. The inferences they came up with were the conditions for maximum oil yield with the lowest FFA percentage. They confirmed that the optimum condition of the drying process in order to yield minimum FFA and maximum oil recovery were 12.8 h at 66.8^{o}C, respectively. The application of these pre-extraction parameters predicted responses of <1% FFA and 31.5% oil yield. Another viable option was increasing the drying at 70.2^{o}C for 17.9 h which resulted in an FFA of <1% and 33.6% oil yield. The former method was preferred over the latter even though the latter condition may slightly increase the oil yield because the reaction time was longer (Choon et al., 2009).

When oil quality is talked about, free fatty acids play a major role as it talks about the degree of oil deterioration. The palm mills in Malaysia allow 5% of FFA in their extracted oil. This value generally increases because of the inherent lipases present in the fruit which start breaking down the triglycerides into free fatty acids leading to oil

deterioration (Corley & Tinker, 2003). Another factor responsible for FFA increase is the moisture content. If the moisture content is reduces by drying, FFA generation can be inhibited. The FFA of the extracted oil was <1% in the study mentioned above due to less moisture content. This lipase can be found in the oil body fraction with an optimal activity at pH 7.5 (Sambanthamurthi et al., 2000). In the study, due to the process of drying, water activity was reduced leading to the inhibition of lipase activity which required an optimum pH of 7.5. This led to reduction in the FFA content in the oil. Even in the case of extra virgin olive oil the FFA is <1% as a result of the extraction technique which involves physical processes only at temperatures greater than 30^{o}C (Torres & Maestri, 2006). This extraction technique retains the heat sensitive natural antioxidants in the olive.

Flax (*Linum usitatissimum L.*) is an important oilseed crop worldwide. Apart from its industrial applications, investigation have been carried out for its medicinal potential (Zhang et al., 2008; Oomah and Sitter, 2009) and nutritional value (Pradhan et al., 2010; Özkal, 2009). The high costs of enzymes make it challenging for its use at an industrial scale. Immobilization of enzymes is a very old technique employed to enhance the efficacy of the enzyme. The process also reduces the product separation process as immobilized enzymes are separated easily from the product. The enzyme can further be reused making the process very economical (Wan et al., 2008). A recent study focussed on identifying the change in oil yield in case of free and immobilized enzymes when used in aqueous enzymatic extraction. Different parameters like enzyme dosage, pH, incubation time and temperature, were optimized. It was concluded that the highest oil recovery of 68.1% was achieved when the ground flaxseed treated with 130 U/g of cellulase, pectinase, and hemicellulase for a period of 12 h, at 45^{o}C and a pH 5.0. The aqueous enzymatic process aided by ultrasound extraction (AEP-UE) derived oil had a 1.5% higher content of unsaturated fatty acids than the Organic solvent extraction derived oil. The AEP-UE is convincingly a promising and environmentally friendly method for the large-scale preparation of flaxseed oil at minimum costs (Jing-jing et al., 2011).

The oil which was recovered from the AEP-UE had a lower peroxide value of 1.0 ± 0.3 m mol/kg as compared to the organic solvent extracted oil which was 1.2 ± 0.2 m mol/kg. This implied that the rancidity under the same storage conditions for the AEP-UE oil was lesser. The lower acid value of 1.1 ± 0.1 mg KOH/g of the oil recovered

from AEP-UE indicated that this oil comprised of lesser number of free fatty acids ($P < 0.05$). The oil also had a higher iodine value (161.2 ± 0.4) when compared to the organic solvent extracted oil which was 140.8 ± 0.1.The inference from this observation was that AEP-UE oil had more number of unsaturated fatty acids ($P < 0.05$). These results were verified by Gas chromatography-Mass Spectrophotometry analysis. Five fatty acids were identified through the comparison of mass spectra of the unknown compound with the mass spectra available in the spectral libraries. Poly unsaturated fatty acids are made up of of two crucial fatty acids, omega-6-fatty acid and omega-3-fatty acid, which are the key constituents and are known for the synthesis of vitamins in biological processes. The enzyme extracted oil was composed of 15.4 ± 0.1% omega-6-fatty acid and 53.7 ± 0.3% omega-3-fatty acid. The amounts of both mono unsaturated fatty acid and poly unsaturated fatty acid were higher in case of aqueous enzymatic extract when compared to the conventional solvent methods ($P < 0.05$). Further, performing the least significant difference test confirmed that the differences in the composition of the two methods were statistically significant, the exception being Palmitic acid. Thus, AEP-UE oil proved to be a functional food resource with a high polyunsaturated fatty acid concentration of 69.1%. As unsaturated fatty acids have desirable nutritional properties, AEP-UE oil could be preferred over organic solvent extracted oil.

Traditionally, palm oil has been extracted from the highly perishable oil palm fruit with the help of wet processing. The steps involved for the conventional extraction of palm oil have been broken down to: Harvesting the fresh fruit bunches (FFB) of mature fruits and transportation without causing any damage to the fruit.

Sterilization of the FFB at 3 bars of steam pressure for 1 hour in a horizontal sterilizer is the next step. This is followed by stripping the sterilized FFB in a rotary drum stripper in order to separate the fruits from the bunch. Digestion of the loose fruits is then carried out where they are converted to semisolid pulp. The next step involves pressing this digested mash by mechanical method. Hydraulic press is used for this step in small mills which has an efficiency of 1 tone FFB/hr. In case of larger mills, a continuous screw press is for pressing 5 tone and higher amounts of FFB. The oil: water mixture obtained after the pressing step is then separated into oil and water in a clarifier. This step is called Clarification. The oil then needs to purified using a high speed centrifuge to remove all the solid impurities from the oil.

Vacuum Drying is the final step of this process where moisture is removed from the oil by vacuum drying. All these steps combined produce a high quality edible grade red palm oil. An additional step is the nut recovery from the press cake in a seed recovery unit.

This conventional method of palm oil extraction has high energy consumption as a result of the sterilization and milling processes before the pressing step. The oil yield obtained by this conventional method is about 17 to 18 % which is low compared to 24% of oil content in the FFB. In addition, the palm oil industry also produces a large quantity of waste water during the clarification step which contains a considerably high volume of organic substances that affect the environment negatively. There lies a need for special treatment or biogas production. The disintegration of cellular material which an essential step prior to extraction or pressing steps is often performed by a thermal treatment or mechanical grinding and recently, enzymatic maceration.

It has been shown previously that aqueous enzymatic oil extraction from plant material has the capability of increasing the oil yield in rapeseed, soybean, peanut and coconut oils (Sarker et al., 1998; Barrios et al., 1990; Subrahmanyan et al., 1959) with the help of enzymes like Alkalase, pectinase and cellulase. A study was thus carried out to investigate the possibility of increasing oil yield from the palm mesocarp

Through the application of enzymes like cellulase, pectinase, hemicellulase and natural enzymes obtained from waste of pineapple (Mohammad et al., 2015). The results of this study showed that enzymatic treatment of the fruit mesocarp is a suitable technique for oil extraction. The enzyme concentrationas well as the type of enzyme applied was important for the digestion of cells of palm mesocarp and decreasing the pressed pulp which remains after the digestion step. Increase in the cellulsae concentration from 0.05% to 0.15%, treated for 4h at 50 ^{o}C decreased the remaining press pulp by 5% from 18 % to about 13%.It was also interestingly discovered that the enzyme from pine waste was effective in decreasing the overall weight of pressed pulp up to about 15% and had a better efficieny when compared to the pectinase enzyme treated samples. The evaluation of the oil extracted was carried out on the basis of total extractable oil and the results confirmed the affectivity of the cellulase enzyme for increasing the oil yield. On the other hand, the oil extracted from untreated samples were less than 75% as compared to the extracted oil from the enzyme treated samples which was 88, 89, and

95 % for 0.05, 0.1 and 0.15% of cellulase enzyme concentration respectively). Pectinase was less effective in this regard compared to cellulase. The use of pineapple waste as an enzyme source surprisingly raised the extracted oil percentage up to 86% which was distinctively higher than the oil extracted from untreated samples (75% only). The analysis of the final oil obtained inferred that the peroxide value of the enzyme treated sample as well as the free fatty acid, were minutely higher than the thermally pretreated and untreated samples. However, these values were well within the range of acceptable value for crude palm oil.

A deep red coloured palm oil is obtained from the mesocarp of the oil palm fruit which contains about 700-800ppm of carotenoids. The properties of these carotenoids and advances in technology aimed towards the production of red palm oil and palm carotene concentrate. The activity of provitamin A in carotenoids has been well recorded (Kalyana et al., 2003). ß-carotene is an important precursor of vitamin A but not all carotenoids act as precursors. The activity of vitamin A formation also depends on the bioavailability of the provitamin precursors. As far as human nutrition is concerned, ß-carotene acts as the most important source of vitamin A.

A study done by Doll and Peto has identified dietary components and the role it plays in the development of cancer (Doll et al., 1981). Diet plays a major role in protection against cancer causing agents. Epidemiological cancer investigations have shown evidence that chemo-preventive substances naturally form a part of our diets. Daily consumption of fruits and vegetables have shown an inverse relationship with the development of cancer specially lung and colon. This property may be attributed to the various antioxidants present in these fruits and vegetables which effectively scavenge free radicals and prevent cancer development. Out of the multitude of these chemo-protective agents, carotenoids and retinoids play an important role. Studies show the inhibitory effect of these compounds on cancer when taken in via diet. These compounds are also believed to have an anti carcinogenic effect. Studies have shown different sites in the cell that could be influenced by carotenoids in order to prevent cancer growth. Tan Chu (Tan, 1991) reported inhibition of chemical carcinogenesis with respect to Benzo(a) pyrene metabolites by carotenoids derived from oil palm. Both in vivo and in vitro inhibition has been explained in details in rat hepatic (Tan, 1991). Similar inhibitory effects of carotenoids derived from palm have been documented in different human cancer cells like Neuroblastoma, pancreatic cancer, GOTO, PANC-1,

glio-blastoma A172 and gastric cancer HGC-27 (Murakoshi et al., 1989). In this study a comparative analysis of alpha carotene and carotene concentrate from palm with synthetic beta carotene was done. The former showed protective effect against cancer while the latter promoted cancer growth. Murakoshi also isolated the α-carotene and a carotene concentrate derived from palm and demonstrated its capability to inhibit liver, lung and skin tumours in mice. Unfortunately, β-carotene did not show similar results. All these results combined, concluded that the natural array of carotenoids present in palm oil have effective anti-cancer properties.

Effect of tocotrienols was also investigated on breast cancer cells of human origin. The palm TRF at 180ug/ml inhibited the insertion of thymidine in human breast cancer cells by 50%. In comparison, α-tocopherol at 500ug/ml did not result in growth inhibition of the same (Nesaretnam et al., 1995). Different tocotrienol concentrations were tested on human breast cancer cells of two types: one with osterogen-receptor and the other without. The experiments showed greater inhibitory effects on these cells at concentration lower than TRF. There also appears to be an Association in the inhibition of human cancer cells amongst palm tocotrienols and flavonoids. Combining tocotrienols, flavonoids and tamoxifen proved to show better results than the individual components (Guthrie et al., 1997). Palm tocotrienols have been seen to have a positive effect against transplantable mice tumours.

Yap *et al* did various compositional analysis in oil extracted from different palm species and drew comparison in their carotenoid content. Thirteen different kinds of carotenoids were identified out of which alpha and beta carotene accounted for 54% to 60% and 24% to 60% respectively of the total. There was no significant difference in the types of carotenoids found in the oils of *E. oleifera* and *E. guineensis*, As well their hybrids and backcrosses to *E. guineensis.* This investigation also concluded that a higher level of lycopene is present in *E. guineensis* as compared to *E. oleifera* and the hybrid it forms with *E. guineensis.* When consumed in a low-fat diet (<30%energy), palm oil has been observed to be effective in maintaining healthy plasma cholesterol and lipoprotein cholesterol levels (Sundram, 1997). The healthiest of the edible fats in the human diet now considered are monounsaturated oils rich in oleic acid. As compared to 48% of this monounsaturated fatty acid in palm oil, olive, rapeseed and canola have in excess of 60% of their fatty acid composition as cis-oleic acid. A series of human trials were conducted to answer the question of whether this oleic acid level

of palm olein is enough to result in a lipoprotein cholesterol profile which can be protective against coronary heart diseases (CHD). The team exchanged palm olein (rich in 16:0) and olive oil (rich in 18:1) and this resulted in similar plasma and lipoprotein cholesterol values like total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C) as well as high density lipoprotein cholesterol (HDL-C). This inferred that in a healthy normocholesterolaemic individual, palm olein could be switched for olive, canola or rapeseed oils without any adverse effects on serum lipids and lipoprotein levels (Sundram, 1997). This team also fed 23 healthy normocholesterolaemic volunteering males meticulously designed whole food diets containing canola oil (18:1-rich), palm olein (16:0-rich) or an American Heart Association Step 1 diet (AHA), which made up 31% energy as fat and less than 200mg of dietary cholesterol every day. The AHA oil blend was prepared by blending 50% of soyabean oil, 40% of palm oil and 10% of canola oil resulting in a 1:1:1 ratio of the saturates, monounsaturates and polyunsaturates. TC and LDL-C from serum were not significantly affected by these three diets even though manipulations of the key fatty acids were done. The high canola and high palm olein resulted in an almost identical plasma and lipoprotein cholesterol profile. Only HDL-C after the AHA diet was found to be raised significantly when compared with the other two diets. When the habitual Dutch diet, which is primarily a typical Western diet, was maximally swapped with palm oil, TC and LDL-C remained unaffected. On the other hand, in the palm diet, significant improvements in the HDL2-C and the apolipoprotein A1/B ratio signalling some cardiovascular benefits were observed rather than the opposite (Sundram et al., 1992). These aforementioned research activities focussed on the oleic acid content in the oils tested, namely; palm olein, canola, rapeseed and olive, for their property of modulating cholesterol. Oleic acid has undoubtedly been proven to have cholesterol-lowering properties, which are considered to be equal if not better the polyunsaturates. However, the optimal amount of oleic acid required in order to result in beneficial lipoprotein profiles has not been determined .

In an investigation conducted by Khor et al., commercial lipase from *Candida rugosa* was used to study the hydrolysis of palm oil, palm olein and palm stearin, soybean oil, corn oil and peanut oil was studied. Lipases hydrolyze fats and oils to their simplest components, fatty acids and glycerol. There are a large number of lipases available commercially; and its enzymology is well recorded (Brockerhoff et al., 1974;

Werdelmann et al., 1982). Lipase have been used for the extraction of fatty acids from triacylglycerols, olive oil, coconut oil, soybean oil, etc and has been documented in earlier studies (Tomizuka et al., 1966; Shinota et al., 1971; Benzonana and Eposito, 1971; Linfield et al., 1984). This team described the optimal conditions for palm oil hydrolysis employing lipase from *Candida rugosa.* According to their study, lipase from *Candida rugosa* was faster in hydrolyzing palm oil as compared to the lipase obtained from porcine pancreas and wheat germ under similar conditions. The lipase exhibited an optimal activity at 37 C and a pH of 7.5. The rate of hydrolysis of palm oil by the lipase was found to be linear on a logarithmic scale (Khor et al., 1986).

An investigation was also carried out to determine the kinetics of enzymatic hydrolysis of palm oil with the help of lipase in a batch reactor (Sulaiman et al., 2003). The lipase enzyme which was used was not selective of the ester bond position and it displayed higher activity at the interface compared to that in the bulk. The mathematical model took into account the mechanism of the hydrolysis reaction and also catered to the effect of interfacial area between the oil phase and the aqueous phase which contained the enzyme. A correlation was established experimentally between interfacial area and operating conditions which included agitation speed as well as oil volume fraction. The kinetic parameters were estimated by placing the data obtained into the model and the results were compared to the values reported previously. The study concluded by demonstrating that the kinetic model represented the experimental data accurately.

A recent study was conducted to analyze the effect of different combinations of enzymes on the palm oil recovery and the degree of digestibility its correlation. It also catered to the investigation of the structural carbohydrate composition of the oil palm fruit mesocarp. A combination of Cellic CTec2 (X1), Cellic HTec2 (X2) and Pectinex Ultra SP-L (X3) for Aqueous Enzymatic Oil Extraction Process (AEOEP) was found to be the optimal composition and this was determined by Simplex Lattice mixture design under fixed parameters. It resulted in 88% of oil recovery. The enzymes were used in 0.46: 0.34: 0.2 (X1:X2:X3) ratios, and loaded at 30 mg of protein /10 gm of substrate, the substrate was loaded at 50 %w/v, pH and temperature were 4.8 and 50ºC respectively and the incubation period was 2 hours. The effectiveness of the combination in fruit cell wall degredation for the releasing of trapped oil was measured by the conversion of reducing sugar at corresponding condition. Apart from this, hexane based solvent extraction was also performed on the fresh palm fruit resulting in 49.77%

oil yield. It was also revealed that, cellulose in the form of glucan and hemicellulose in the form of xylan & arabinan contribute to the cell wall polysaccharides constituents to a large extent. This was followed by soluble lignin. Lignin coating on the mesocarp fibre is believed to have acted as a physical barrier preventing cellulose and hemicelluloses accessibility (Glauet., 1980). Other than this, arabinan, was present in the cell-wall pectic-substances and were responsible for the integrity and coherence of the plant tissue (Perez et al., 2013). Hence, in order for the oil located in the vacuoles and cytoplasmic membranes to be released, the cellular wall of fruit mesocarp needs to be degraded and ruptured. This was why, the Cellic CTec2, a cocktail of aggressive cellulases, high level of beta-glucosidases and hemicellulase, Cellic HTec2, which is an endoxylanase having a high specificity towards soluble hemicellulose and Pectinex Ultra SP-L which is rich in pectolytic activities were used in the aqueous enzyme reaction. The team also found Simplex lattice mixture design as a wiser alternative over the conventional process For the purpose of formulation and optimisation of dosage ratios because of its fewer experiment requirements and shorter time hence, proving to be a more economic technique (Hemavathi et al., 2015).

The current study deals with the extraction of edible vegetable oil using enzymes and the physio-chemical properties of the oil obtained. The previous studies act as a background to this investigation and optimisation of the process for the extraction of oil is documented further. The oil quality parameters after enzymatic treatment and the effect of heat on oil stability were also analysed as part of this thesis.

CHAPTER 3 – MATERIALS AND METHODS

3.1 Materials

Raw Materials: The raw fruit was obtained from Malaysia and was immediately transported to the laboratory. Part of the sample underwent sample preparation while extra sample was stored in a cold room at 4˚C.

Sample preparation: The received fruit was peeled and the mesocarp was separated from the kernel. This mesocarp forms the substrate on which the enzyme will act.

Enzymes: Proprietary Enzyme A – A combination of hydrolytic enzymes like cellulases, hemicellulases, pectinases, Proprietary Enzyme B – a lipase.

Solvent: Hexane was used for Soxhlet extraction method

Instrument: Mash bath, water bath, hot air oven, Soxhlet apparatus (round bottom flask, extracter, and condenser, chiller), fume hood, analytical balance, centrifuge, centrifuge tubes, beakers, aliquot vessels, spatula, centrifuge tube, micropipette, tips, light microscope, gun pipette, petri plates, incubator shaker, DOBI tubes, conical flasks, digital burette, and spectrophotometer.

3.2 Methods

3.2.1 Optimization of process parameters for enzyme assisted extraction

For this study, enzyme dosage, type of centrifuge rotor and G-force were optimized for maximum yield. The sludge and fibre of the process obtained after extraction was checked for loss of oil and the fibre structure was analyzed under a light microscope. For comparison, oil was also extracted by Soxhlet extraction method using solvent. The whole procedure was done once to set enzyme dosage, repeated again to set optimum rotor speed and finally done for selecting the type of rotor.

a. Dose Response Study and identification of the rotor type.

Sample collection and preparation - Fresh fruit was collected and the good fruit was separated from the trash (calyx, twigs, dirt, and pebbles). It was then sterilized at 121°C for 30min in a pressure cooker. The softened fruit was then peeled and the mesocarp was separated from the nut. The mesocarp was mashed in a mash bath and then

aliquoted into containers depending on the scale of trial. The aliquoted sample was spread evenly on the base of the container so that maximum substrate is exposed to enzyme.

Pre-incubation – Prior to the addition of the enzyme, the substrate temperature was brought to an optimum value. This step is known as pre-incubation. The sample was kept at 90°C for 10mins and 70°C for 5mins to achieve desired temperature. Enzyme dilution Enzyme A_1 (higher dose in ppm) and Enzyme A_2 (lower dose in ppm) were prepared using Novozymes proprietary Enzyme A.

Inoculation and Incubation – Dilutions of the hydrolytic enzyme A_1 and A_2 were added to the substrate and was mixed well. The substrate was then kept for incubation at 70°C for 30 mins. Water was added in place of enzyme for the control sample. After the incubation was complete, water was added in order to assist the oil to leach out and the enzyme was heat inactivated by keeping the substrate at 90°C for 15mins.

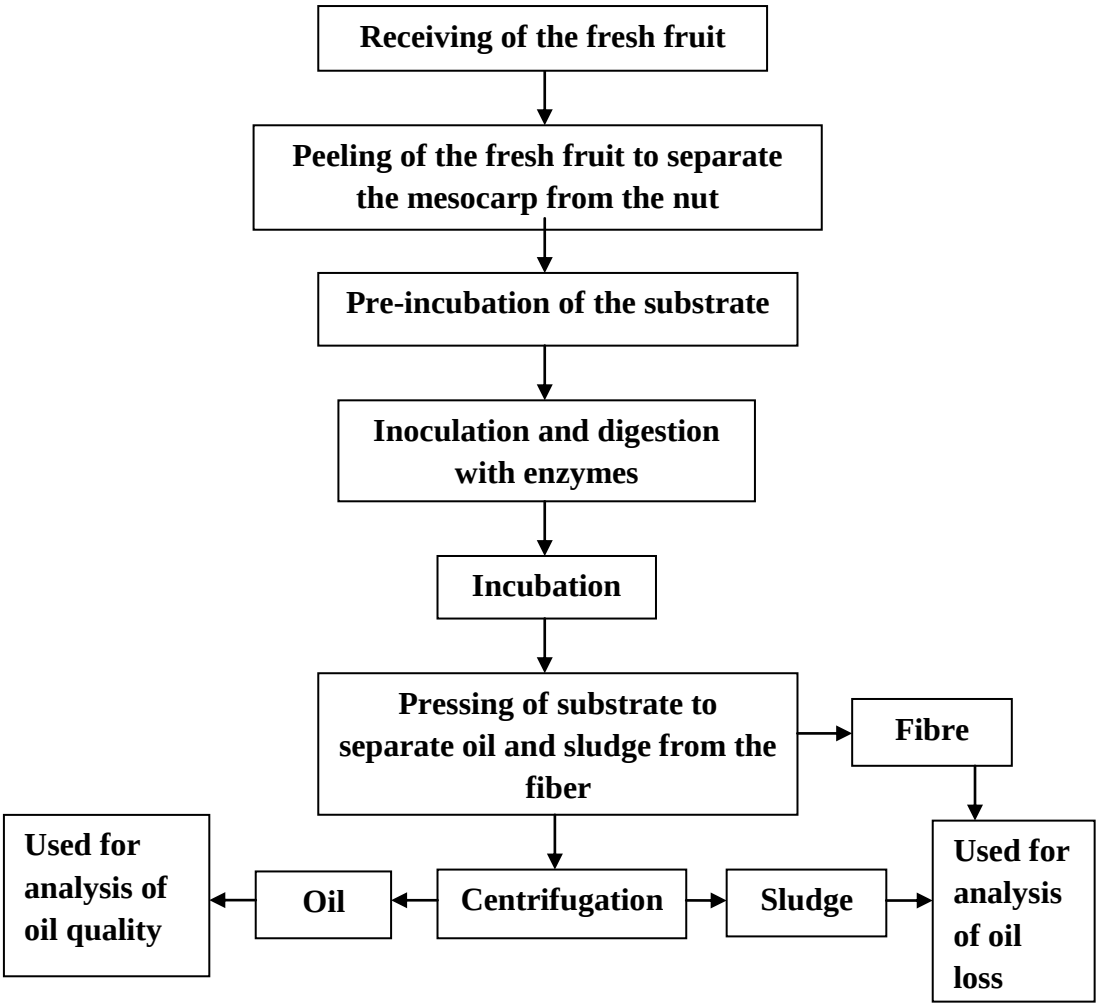
Para-pressing – The substrate was transferred to a para-press and pressure of four bars was applied to extract - a combination of oil and sludge from the fruit fibre. The process was done twice to ensure maximum removal of oil from the fiber. The extract was transferred to centrifuge tubes and the pressed fibre was weighed.

Clarification and Centrifugation – The centrifuge tubes were transferred to a clarifying water bath at 90^{o}C for 30mins. In this step some amount of oil-water-sludge separation takes place. After the initial sedimentation, the clarified extract was subjected to centrifugation at 5000rpm (Beckmann Coultier – Avanti J-E).

Oil recovery – After centrifugation, the topmost oil layer was carefully retrieved in a petri plate using gun pipette and the weight of oil was recorded. The fibre and the sludge were further tested for oil loss using Soxhlet.

The oil which was recovered was further analyzed for its physicochemical properties like free fatty acid value, deterioration of bleachability index, carotene content and moisture content. The whole procedure was also done using two different sets of rotor: fixed angle and swing bucket rotor at different g-force – 3200G, 6300G and 9600G. The yield of oil was measured for different enzyme dilutions A1 and A2 for different rotors at different speeds. The combination that provided maximum yield was selected and used for further analysis.

A flow chart of the process is given below:



b. Analysis of loss of oil in sludge and fruit fibre

After the best combination of the aforementioned parameters was determined, Soxhlet was done to identify the amount of oil lost in the waste streams during the process. Both the sludge and the fibre were analysed for oil content. The extractor, heating mantle, round bottom flask and the condenser were set up as given in the picture below. The round bottom flasks were pre-weighed prior to the start of the process. It was then filled with the extraction solvent hexane. The fibre and sludge samples were dried in a hot air oven at 65^{o}C. Weight of the samples kept in dry oven was taken after every two hours until the weight became constant. 8-10g of sample was weighed and then thimbles were prepared. The thimble was put in the extractor and the extraction was started. The condenser was connected to regular supply of chilled water. The extraction process continued for four hours. After the extraction, the hexane was recovered and the oil was retained in the round bottom flasks. The final weight of the flask with oil was recorded.

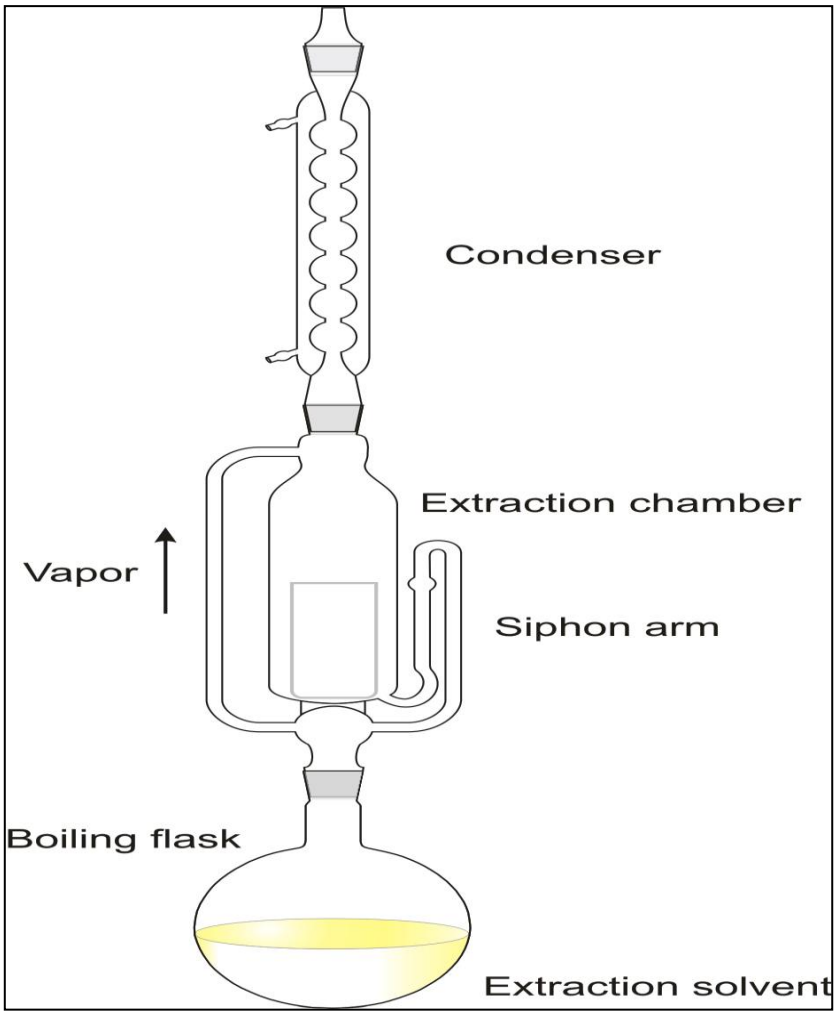


Fig 1: Soxhlet apparatus (Generalic, Eni. *Croatian-English Chemistry Dictionary & Glossary*. 29 Aug. 2017)

Calculation

% Yield of crude oil = $\frac{W1}{W2} \times 100$

where: W1- Weight of oil (g). [Final wt. of round bottom flask – initial wt. of round bottom flask], W2- Weight of sample (g).

c. Microscopic analysis of the substrate

The substrate was subjected to microscopic analysis before and after the enzymatic treatment. The equipment used was a light microscope (OLYMPUS CX41). 10µL of the sludge sample was taken on a slide and viewed directly under the light microscope. No stains were used for sludge samples.

3.2.2 Analysis of oil quality parameters before and after enzymatic treatment

The enzyme concentration that gave more oil yield was used in the following tests. For the purpose of this study, biological duplicates (Control 1 and 2, Enzyme A1(a) and A1(b)) and process triplicates were used. Oil was extracted as described in section 2.2.1(a). The oil obtained from was subjected to the following quality tests. Free fatty acids analysis was done to check the relative stability of oil when enzyme is added in the extraction process. The crude oil is bright red in color due to the presence of high amount of carotenoids. The oil loses color with time which was calculated by its bleachability index from which carotene concentration was determined. Moisture content is an important parameter and is responsible for conversion of triglycerides to free fatty acids. Moisture content was also analyzed for both control and enzyme treated sample in order to identify effect of moisture on oil.

a. Free fatty acid analysis

FFA was done according to the protocol by The American Oil Chemists' Society (AOCS). 0.05N sodium hydroxide was prepared by weighing 4g of NaOH and making the volume to 2000ml in a volumetric flask using RO water. The solution was then standardized using potassium phthalate. Potassium phthalate was dried at 105^{o}C for two hours in an oven till a constant weight was observed. It was then cooled in a desiccator and 0.4g of it was weighed and kept in a conical flask. 50ml of RO water was added and the solution was heated up to 50^{o}C for the phthalate to dissolve completely. 2-3 drops of phenolphthalein indicator was added and it was titrated against 0.05N sodium hydroxide until a pale pink color appeared. The volume of NaOH used was noted and normality was calculated using the following formula:

$$\text{Normality} = \frac{W \times 1000}{V \times 204.2}$$

Where: W- weight of potassium phthalate, V-volume of 0.05N NaOH, 204.2 – equivalent weight of potassium phthalate.

After the titrant (NaOH) was standardized, 4g of crude oil (liquid state) was weighed and kept in a conical flask. Iso-propanol was neutralized by adding 2-3 drops of phenolphthalein indicator and titrated against 0.05N NaOH till a plae pink color persisted. 40ml of neutralized iso-propanol and 2-3 drops of phenolphthalein indicator were then added to the crude oil. This solution was then titrated against 0.05N NaOH until a brick red color was observed. Volume of NaOH used was noted and %FFA as palmitic acid was calculated using the following formula:

$$\%FFA = \frac{25.6 \times Normality\ of\ NaOH \times Volume\ of\ NaOH}{Weight\ of\ sample\ taken}$$

b. Determination of Bleachability Index (DOBI) and Carotene content

DOBI was done according to the protocol by The American Oil Chemists' Society (AOCS). 0.1g of the oil obtained from the trials was weighed in the DOBI tubes. 20ml of iso-octane was added to the tubes and the oil was thoroughly dissolved in it. A quartz cuvette was first rinsed and then filled with iso-octane and this was set as reference in the spectrophotometer at wavelengths 269nm and 446nm. The cuvette was then rinsed with the test solution and the absorbance of test solution was taken on the same wavelengths. This was done with oil obtained from both control and enzyme trial. DOBI and carotene content was calculated based on the following formula:

DOBI = $\frac{A446}{A269}$ where: A_{446} is the absorbance at 446nm and A_{269} is the absorbance at 269nm.

Carotene (C) = $\frac{383\ A446}{l\rho}$ where: C – is the total carotene content of oil as β-carotene in milligrams per kilogram, l – path length, ρ – is the concentration in grams per 100ml, used for absorption measurement.

c. Variation in moisture content

Moisture analyzer (Mettler Toledo) was used to check the moisture content in oil sample of both control and enzyme. 1g of sample was placed on soft aluminium plates and kept inside the moisture analyser at a drying temperature of 105^{o}C. The process was done in replicates of three. The moisture content was calculated as %MC (moisture content) of the total oil.

3.2.3 Effect of heat on oil quality

Oil is very commonly used for frying purposes and is thus subjected high temperatures for long duration of time. If often results in foul smell and off putting flavour. To identify oil stability at high temperatures condition, following protocol was used. Control and enzyme oil samples were obtained by method described in section 2.2.1(a). The samples were aliquoted in tarson tubes and kept in a water bath at 90^{o}C for 5 hours. The samples were assessed for FFA and DOBI as described in section 2.2.2 (a,b) at every hour for five hours.

3.2.4 Effect of lipase on oil quality

Oil derived from fruits generally contains inherent lipases which are responsible for degradation of triglycerides to free fatty acids which are responsible for rancidity. These inherent lipases were deactivated by the process of sterilization. However, exogenous lipases excreted by certain type of fungi growing on the skin of the fruit might affect oil quality. Therefore, for the purpose of this study, exogenous lipases were used to study the generation of free fatty acids. Control and enzyme oil samples were obtained by method described in section 2.2.1(a). Three dilutions 100ppm, 50ppm, 5ppm of Enzyme B, a lipase, were prepared. Initial stock solution 100ml (1mg/ml) was prepared. From the stock, 1ml was added for 100ppm, 0.5ml stock + 0.5ml R.O water was added for 50ppm and 0.05ml + 0.95ml R.O water was added for 5ppm to three separate tarson tubes along with 15ml oil in each. Thus five samples – Enzyme, Control, Control+100ppm lipase, Control + 50 ppm lipase, and Control + 5ppm lipase were prepared and kept in an incubator-shaker at 50^{o}C for 8 days. FFA and DOBI were done as described in section 2.2.2 (a,b) on Day 0, Day 2, Day 3, Day 6 and Day 8.

CHAPTER 4 – RESULTS AND DISCUSSION

4.1 Optimization of process parameters

a. Dose Response Study and identification of the rotor type and G-force

Table 1: Dose Response

Sr. No.	Treatment	Tube+ Extract weight (g)	Fibre Weight (g)	Empty pan weight (g)	Pan + Oil weight (g)	Final weight of oil (g)
1	Control	144.26	11.57	11.23	31.16	19.93
2	Enzyme A_1	144.61	10.61	11.24	32.12	20.88
3	Control	146.26	11.28	11.26	31.86	20.6
4	Enzyme A_1	142.22	11.67	11.26	32.45	21.19
5	Control	143.64	11.93	11.25	31.19	19.94
6	Enzyme A_1	146.2	11.22	11.25	32.21	20.96
7	Control	142.95	11.7	11.25	30.36	19.11
8	Enzyme A_2	145.9	10.89	11.25	32.71	21.46
9	Control	145.74	9.96	11.26	31.4	20.14
10	Enzyme A_2	145.16	10.9	11.25	31.5	20.25
11	Control	144.54	10.75	11.24	30.16	18.92
12	Enzyme A_2	144.62	11.12	11.25	31.16	19.91

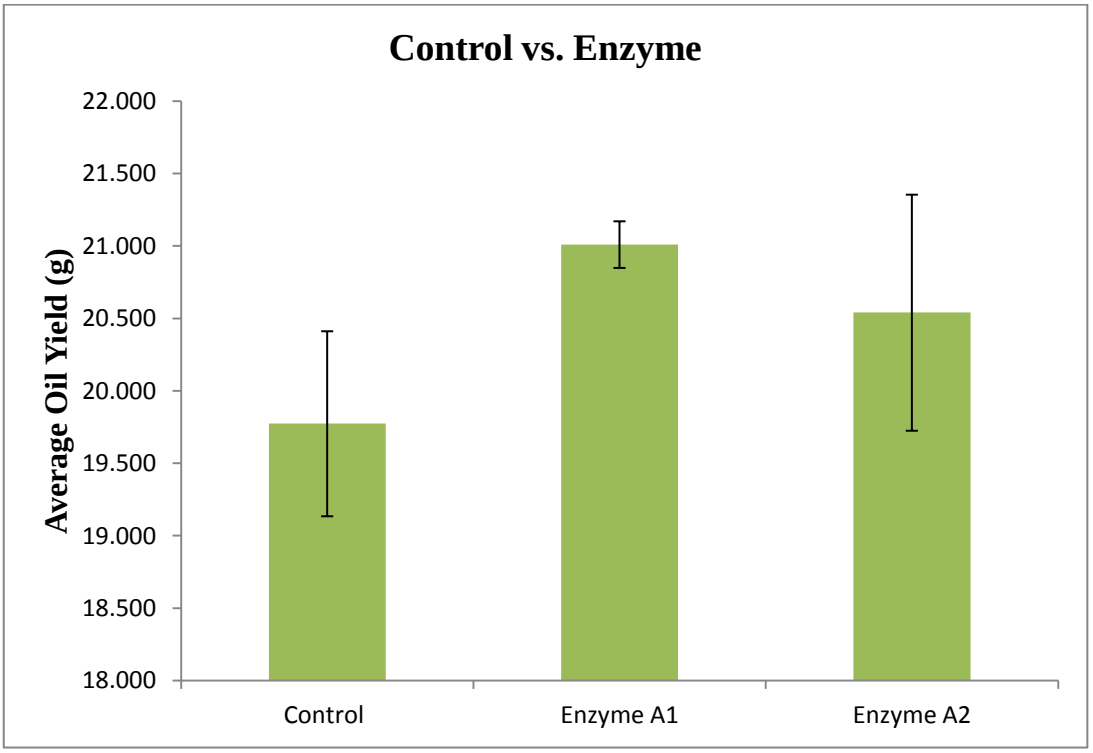
Table 2: Control vs. Enzyme A_1 vs. Enzyme A_2 dose response

	Control	Enzyme A_1	Enzyme A_2
Average	19.77	21.01	20.54
Standard deviation	0.638	0.16	0.815
CV	3.23%	0.77%	3.97%
Delta		1.237	0.767

Table 1 shows the dose response study done by proprietary enzyme A. A_1 is higher enzyme dosage while A_2 is lower. A direct correlation between treatment and oil yield is established. Pan is the container in which the oil was collected and tube + extract is

the final extract that was obtained after para-pressing. Table 2 shows average value of oil yield, its standard deviation, coefficient of variation and increase in oil yield in control, enzyme A_1 and enzyme A_2. From the two tables it is observed that enzyme A_1 (higher dose) gives higher oil yield in comparison to enzyme A_2 (lower dose). Lower coefficient of variation in enzyme A_1 shows higher precision of estimated values. Graphical depiction of the result is given below.

Graph 1: Average Oil Yield in Control vs. Enzyme A_1 vs. Enzyme A_2



Graph 1 depicts oil yield in substrate treated with enzyme A_1 is more in comparison to the control. The error bars do not overlap showing the yield is significantly higher than the control sample. It also shows oil yield in substrate treated with enzyme A_2 is more in comparison to the control. However, the overlapping of error bar shows that the oil yield in enzyme treated substrate is not significantly higher than the control. Therefore it can be concluded that higher dose of enzyme extracts out more oil and therefore was used for further analysis.

Rotor and Relative Centrifugal Force Test

Table 3: Experimental replicates to show effect of Relative Centrifugal Force on Oil Yield

No. of Experimental Replicates	R.C.F / Treatment	3200G	6300G	9600G
		Oil yield (g)	Oil yield (g)	Oil yield (g)
1	Control	3.124	3.551	4.137
	Enzyme A_1	3.238	3.601	4.062
	Enzyme A_2	3.305	3.554	3.893
2	Control	3.144	3.634	3.881
	Enzyme A_1	3.198	3.542	4.035
	Enzyme A_2	3.178	3.557	3.683
3	Control	3.432	3.663	4.152
	Enzyme A_1	3.518	3.392	4.143
	Enzyme A_2	3.391	3.421	3.947
4	Control	3.352	3.445	3.739
	Enzyme A_1	3.397	3.496	3.851
	Enzyme A_2	3.364	3.501	3.826

Graph 2: Effect of Relative centrifugal force on Oil Yield

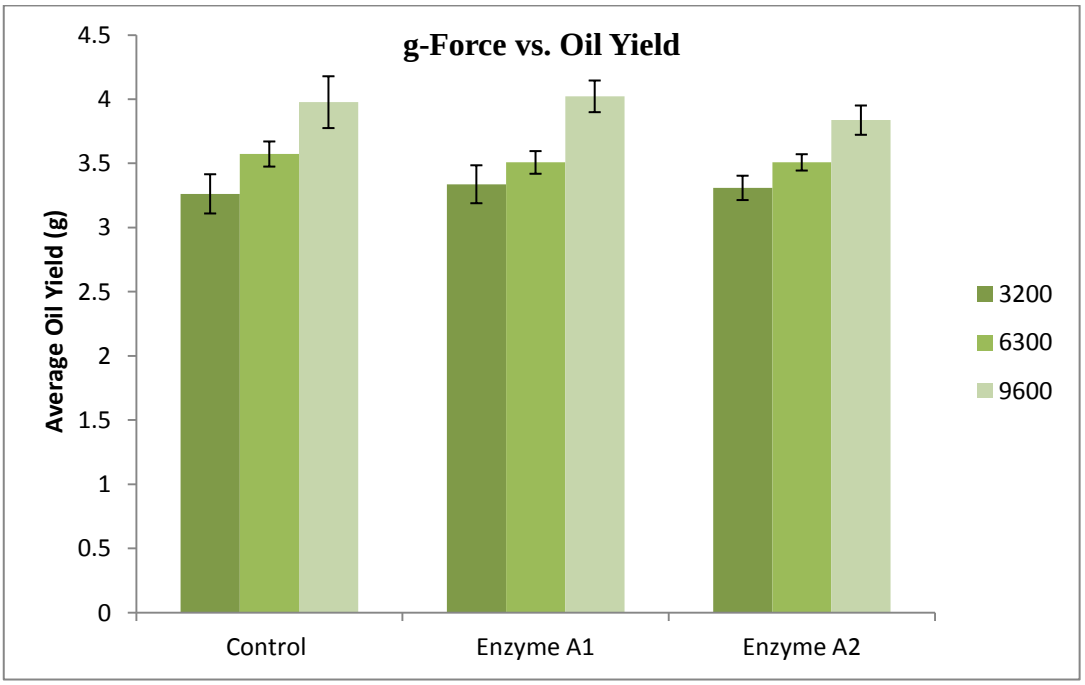


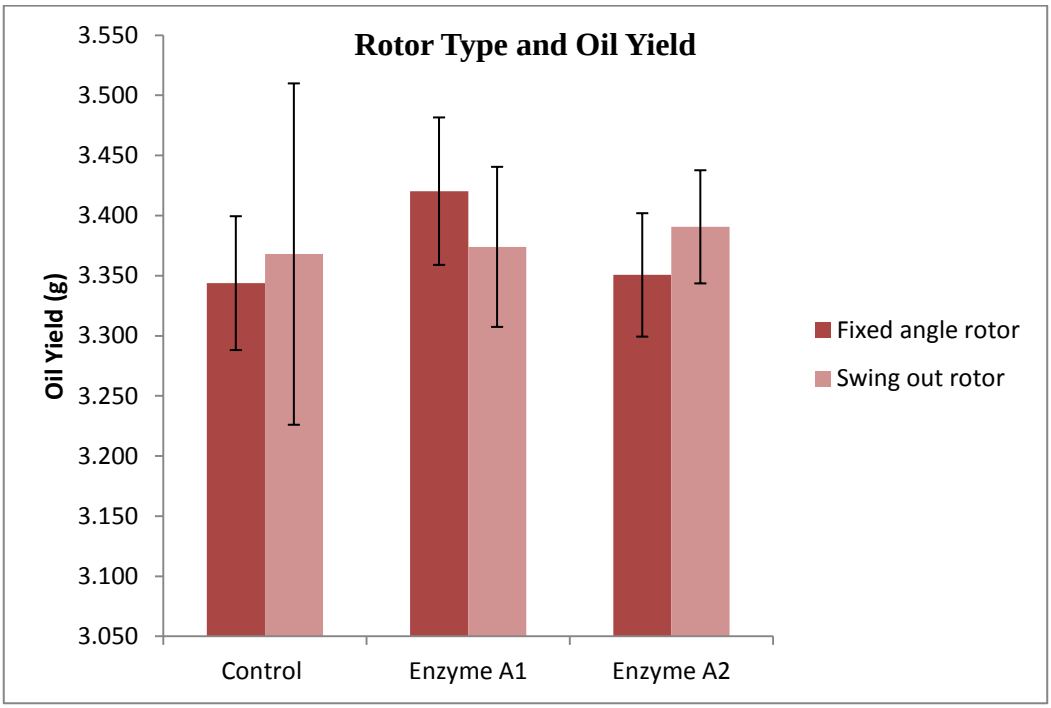
Table 3 shows four experimental replicates to test the effect of relative centrifugal force at 3200G, 6300G and 9600G on oil yield. The average value of Control, Enzyme A_1 and Enzyme A_2 was taken and Graph 2 was plotted. The graph indicates that with the increase in G-force, oil yield increases irrespective of whether the sample is with or without enzyme. 9600G showed maximum oil yield in all three cases. The non-overlapping error bars in case of 9600G depict that the increase in oil yield is significantly higher. Therefore, it can be concluded that oil yield is dependent on the R.C.F.

Table 4: Effect of Rotor Type on Oil Yield

No. of Experimental replicates	Rotor type / Treatment	Swing out rotor	Fixed angle rotor
		Oil yield (g)	Oil yield (g)
1	Control	3.241	3.308
	Enzyme A_1	3.277	3.543
	Enzyme A_2	3.331	3.4
2	Control	3.362	3.436
	Enzyme A_1	3.43	3.387
	Enzyme A_2	3.456	3.398
3	Control	3.412	3.274
	Enzyme A_1	3.325	3.417
	Enzyme A^2	3.367	3.312
4	Control	3.235	3.337
	Enzyme A_1	3.393	3.383
	Enzyme A_2	3.438	3.39
5	Control	3.337	3.337
	Enzyme A_1	3.363	3.391
	Enzyme A_2	3.378	3.321
6	Control	3.621	3.371
	Enzyme A_1	3.456	3.401
	Enzyme A_2	3.374	3.283

Table 4 shows six experimental replicates used to assess the effect of rotor on oil yield. The two types of rotors that were used were swing bucket and fixed angle rotor using centrifuge (Beckmann Coulter) and oil yield was analysed. The average value of Control, Enzyme A_1 and Enzyme A_2 was taken and graph 4 was plotted.

Graph 3: Effect of rotor type on oil yield



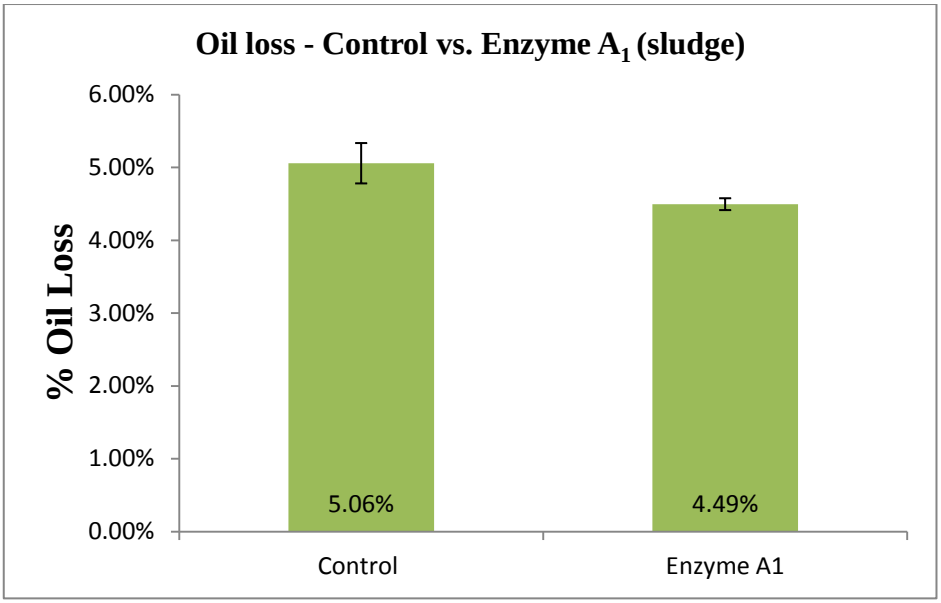
Graph 3 shows similar oil yield in case of swing out rotor, however, Enzyme A_1 shower slightly higher yield in comparison to control and Enzyme A_2. The overlapping error bars show the difference in oil yield is not significant and hence it can be concluded that oil yield is independent of rotor type. For the purpose of this study, swing bucket was used for future analysis because of ease of oil extraction and quantification.

b. Analysis of loss of oil in sludge and fruit fibre

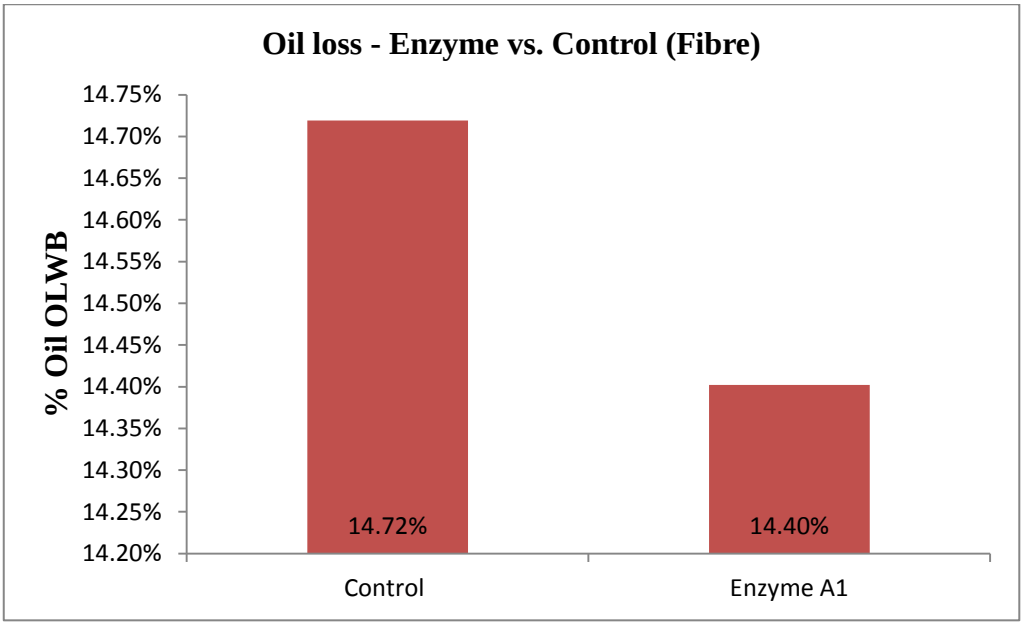
After selection of the optimum dose of enzyme, loss of oil was evaluated by using hexane as solvent for the soxhlet extraction. The sludge and fibre collected after processing oil from Enzyme A_1 was tested. The sludge sample was run twice while the fibre sample was run thrice for maximum removal of oil. The table 5 and 6 given below gives the experimental value of the oil loss from the sample. The total oil obtained was calculated based on which the average % of oil loss on wet basis (OLWB) was calculated by final weight of oil obtained divided by initial wet weight of the substrate multiplied by hundred.

From the result it can be said that oil loss in sludge was reduced in sample where enzyme was used. The non overlapping error bars show significant difference in yield. This can imply the increase in oil yield as depicted in graph 1 can be due to loss of oil in the waste stream. Based on table 5 the following graph 4 was plotted.

Graph 4: Oil loss in sludge Control vs. Enzyme A_1



Graph 5: Oil loss in fibre Control vs. Enzyme A_1



Graph 5 was plotted based on table 6. The pressed fibre sample was run twice to remove maximum oil. The oil loss was calculated in a manner similar to the sludge sample. The graph shows a difference in oil loss. Control has more oil loss in comparison to enzyme.

Thus from the soxhlet analysis we can conclude that the increase in oil yield in case of samples treated with enzyme might be partly because of the reduction in oil losses in the waste streams.

Table 5: Soxhlet for sludge sample

							1st Run			2nd Run					
Sr. no.	Treatment	Petri dish weight (g)	Petri dish+Sample wt. (wet) (g)	Sample wt. (wet) (g)	Petri dish+Sample wt. (dry) (g)	Sample wt. (dry) (g)	Flask wt. (g)	Flask+oil wt. (g)	Oil wt. (g)	Flask wt. (g)	Flask+oil wt. (g)	Oil wt. (g)	Total wt. (g)	% Oil (OLWB)	Average % OLWB
1	Control	23.7	61.52	37.82	27.054	3.354	124.497	126.3	1.803	103.01	103.018	0.008	1.811	4.79%	
2	Control	22.215	55.345	33.13	25.168	2.953	102.246	103.911	1.665	129.004	129.008	0.004	1.669	5.04%	5.06%
3	Control	22.48	50.917	28.437	25.193	2.713	103.605	105.121	1.516	96.011	96.014	0.003	1.519	5.34%	
4	Enzyme A_1	21.527	56.354	34.827	24.445	2.918	123.763	125.351	1.588	102.128	102.13	0.002	1.59	4.57%	
5	Enzyme A_1	22.423	51.817	29.394	24.776	2.353	116.041	117.331	1.29	103.895	103.9	0.005	1.295	4.41%	4.49%
6	Enzyme A_1	22.663	60.345	37.682	25.818	3.155	124.013	125.7	1.687	102.027	102.04	0.013	1.7	4.51%	

Table 6: Soxhlet for fibre sample

							1st Run			2nd Run			3rd Run				
Sr. no.	Treatment	Petri dish weight (g)	Petri dish+Sample wt. (wet) (g)	Sample wt. (wet) (g)	Petri dish+Sample wt. (dry) (g)	Sample wt. (dry) (g)	Flask wt. (g)	Flask+oil wt. (g)	Oil wt. (g)	Flask wt. (g)	Flask+oil wt. (g)	Oil wt. (g)	Flask wt. (g)	Flask+oil wt. (g)	Oil wt. (g)	Total wt. (g)	% Oil (OLWB)
1	Control	45.506	54.963	9.457	50.366	4.86	107.491	108.871	1.38	101.603	101.61	0.007	104.517	104.522	0.005	1.392	14.72%
2	Enzyme A_1	41.276	51.094	9.818	46.4	5.124	100.512	101.91	1.398	106.124	106.13	0.006	124.45	124.46	0.01	1.414	14.40%

c. Microscopic analysis of the substrate

Microscopic examination of substrate before and after enzyme treatment was done. Fig1. which is a control sample shows a presence of an intact cell wall and the oil droplet bound inside the cell wall. Fig2. which is an enzyme treated sample shows absence of cell wall and free oil droplets. Microscopic examination showed the enzymes work on the disintegration of cell wall to release the oil droplet. This released oil droplet can be extracted easily in comparison to bound droplet when mechanical force from para-press in laboratory scale or screw press in industrial scale is applied on it.

Fig 2: Control sample not treated with enzyme

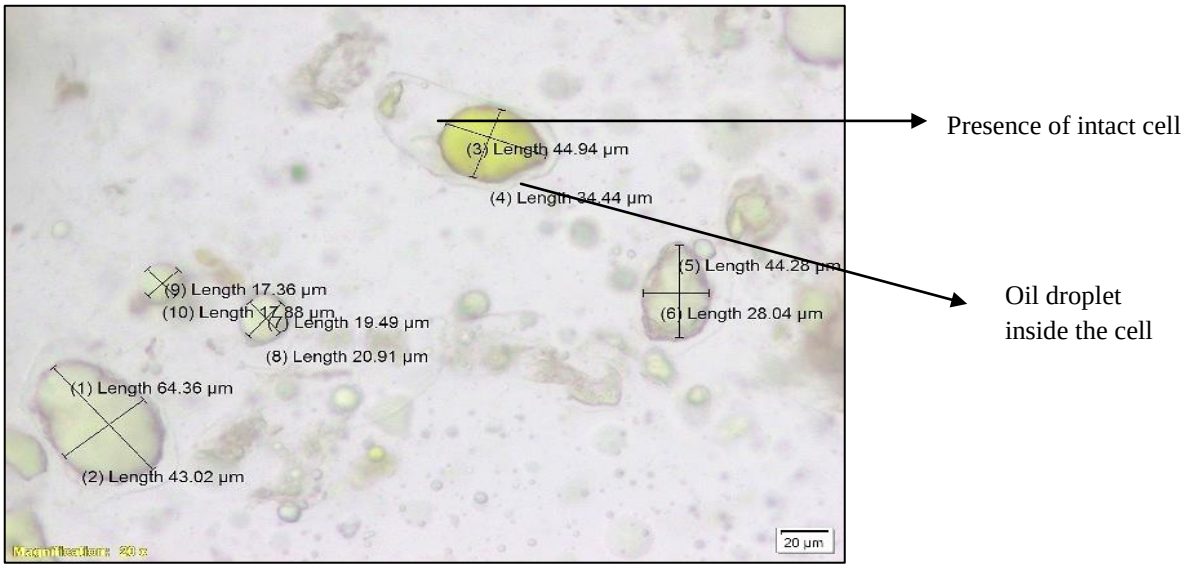
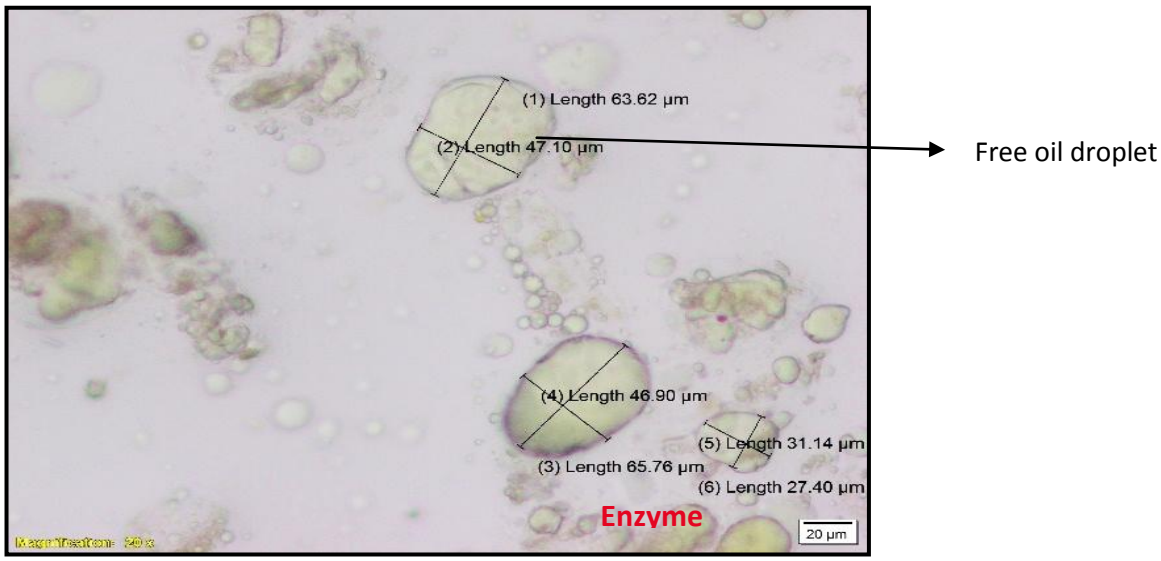


Fig 3: Enzyme treated sample



Enzyme A_1, swing bucket rotor and 9600G force were the final parameters that were identified as optimum and were thus used for the future experiments. Soxhlet analysis showed reduction in oil losses when treated with enzyme. Microscopic analysis showed cell wall degradation and extraction of oil droplet.

4.2 Analysis of oil quality parameters before and after enzymatic treatment

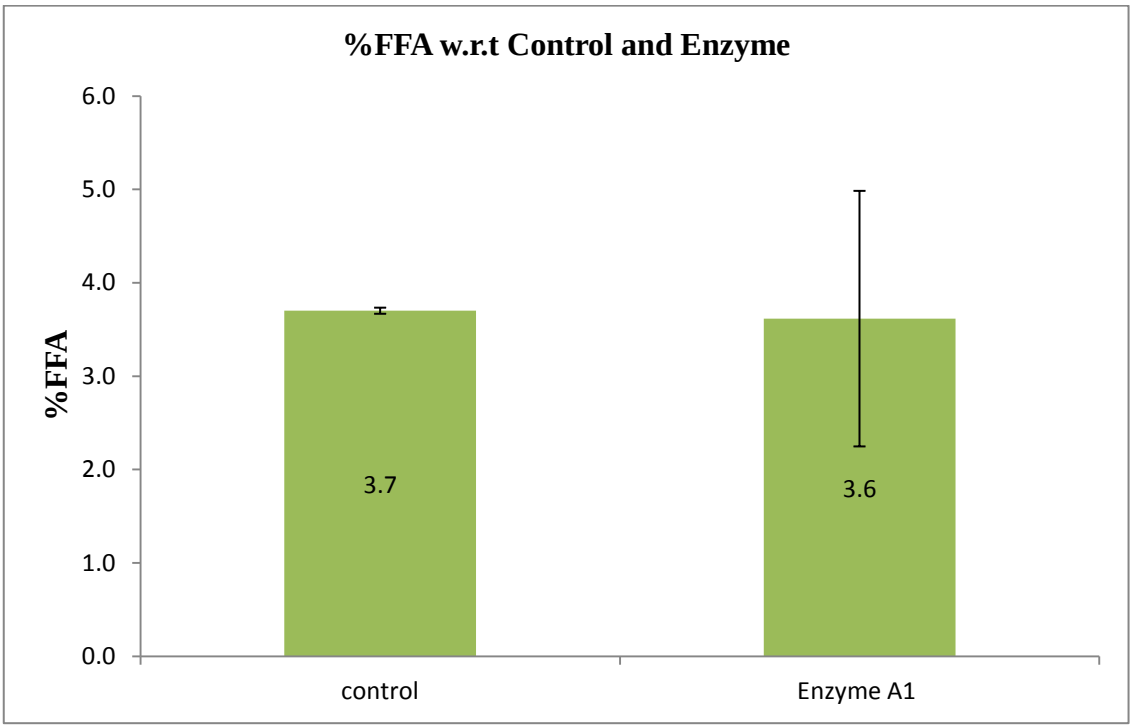
4.2.1 Free fatty acid analysis

Table 7: Free fatty acid analysis of Control and Enzyme

Free Fatty Acid Analysis			
Sample Name	**Weight of sample taken (g)**	**Volume of NaOH (ml)**	**%FFA**
Control 1			
a.	4.048	11.77	3.722
b.	3.987	11.38	3.653
c.	4.043	11.76	3.723
Control 2			
a.	4.009	11.59	3.700
b.	4.012	11.51	3.672
c.	3.983	11.63	3.737
Enzyme A_1			
a.	4.048	11.6	3.668
b.	4.044	11.51	3.643
c.	4.038	11.46	3.633
Enzyme A_1			
a.	4.028	11.56	3.673
b.	4.06	11.11	3.503
c.	3.988	11.15	3.579

Table 7 shows free fatty acids expressed in terms of %FFA as palmitic acid only. Two biological duplicates and three experimental triplicates were taken. The average value of experimental triplicates was used to plot graph 8. From the values given in the table it is seen that there is a slight reduction in %FFA in case of enzyme sample. Graphical depiction of the same is given below. Graph 6 shows slight decrease in %FFA for enzyme but the overlapping error bars indicate not significant difference. Thus it can be concluded that enzyme addition does not break down triglycerides to free fatty acids and the oil remains stable in the enzymatic extraction process.

Graph 6: Comparison of %FFA between control and enzyme samples



4.2.2 Deterioration of Bleachability Index

Table 8: Analysis of DOBI and Carotene in control and enzyme treated sample

Deterioration of Bleachability Index					
Sample name	**Weight of sample (g)**	**A_{269}**	**A_{446}**	**DOBI A446/ A269**	**Carotene (mg/kg)**
Control 1	0.104	0.35	0.864	2.469	661.82
Control 2	0.107	0.344	0.809	2.352	619.69
Enzyme A1(a)	0.107	0.331	0.775	2.341	593.65
Enzyme A1(b)	0.104	0.326	0.755	2.316	578.33

Table 8 shows DOBI remains relatively same in both enzyme and control sample. The color of oil is an inherent quality of oil and is seen to remain unaffected during the enzymatic extraction. Carotene content, however, is dependent on absorbance value at A_{446} and thus variation can be seen in it. Enzyme treated sample shows less carotene content in comparison to the control sample. We cannot, however, conclude that the reduction was due to the presence of enzyme. The starting concentration of carotene can vary with every fruit and the type of conditions the fruit grows in.

4.2.3 Moisture content analysis

Moisture content (%MC) in both control and enzyme treated sample was analyzed after the oil was extracted. Experimental triplicates were taken and average %MC was calculated. Table 10 shows the percentage moisture. No difference was seen in the moisture content in the samples.

Table9: Moisture content analysis in control and enzyme treated sample

Treatment	Weight of sample (g)	% MC	Average
Control			0.52
a.	1.104	0.3	
b	1.055	0.57	
c.	1.012	0.69	
Enzyme			0.583
a.	1.024	0.29	
b.	1.019	0.69	
c.	1.035	0.77	

4.3 Effect of heat on oil quality

Both biological duplicates were taken for this analysis. %FFA and DOBI was assessed every hour for five hours. %FFA determined is given below in Table 11.

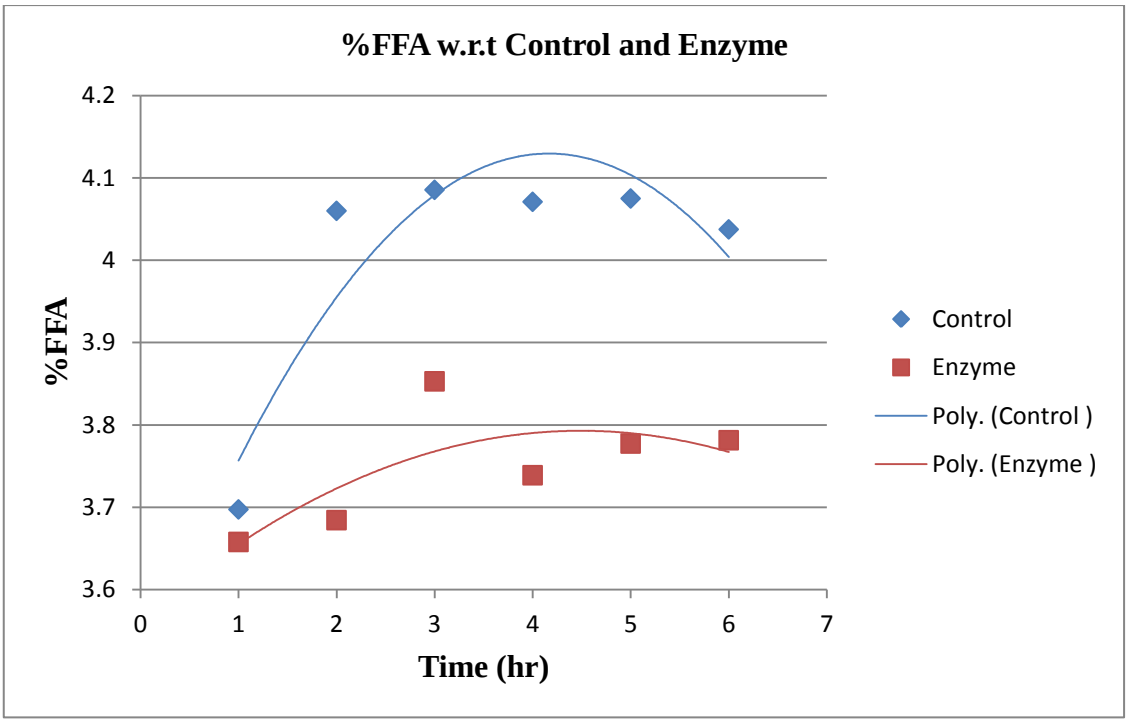
a. Free fatty acids

Table 10: %FFA for control and enzyme treated sample on an hourly basis

Treatment	Time (hr)	% FFA	Average %FFA
Control 1	0	3.723	3.6975
Control 2		3.672	
Enzyme A1a		3.643	3.658
Enzyme A1b		3.673	
Control 1	1	4.081	4.06
Control 2		4.039	
Enzyme A1a		3.737	3.6845
Enzyme A1b		3.632	
Control 1	2	4.194	4.0855
Control 2		3.977	
Enzyme A1a		3.921	3.853
Enzyme A1b		3.785	

Control 1	3	4.111	4.071
Control 2		4.031	
Enzyme A1a		3.777	3.739
Enzyme A1b		3.701	
Control 1	4	4.095	4.075
Control 2		4.055	
Enzyme A1a		3.737	3.7775
Enzyme A1b		3.818	
Control 1	5	4.069	4.0375
Control 2		4.006	
Enzyme A1a		3.798	3.7815
Enzyme A1b		3.765	

Graph 7: Analysis of variation in %FFA between control and enzyme on an hourly basis



Graph 7 shows that there was a slight increase in FFA from zero hour to one hour after which the FFA value remains relatively constant for control samples. The graph also shows that enzyme based samples had a lower initial value of FFA in comparison to control samples which is consistent with the previous results. Amongst enzyme treated sample, FFA value remained relatively stable over the course of five hours. From this analysis we can conclude that the oil extracted is stable when subjected to high

temperatures for long duration of time. This can also hint at high degree of saturated fats present in the oil.

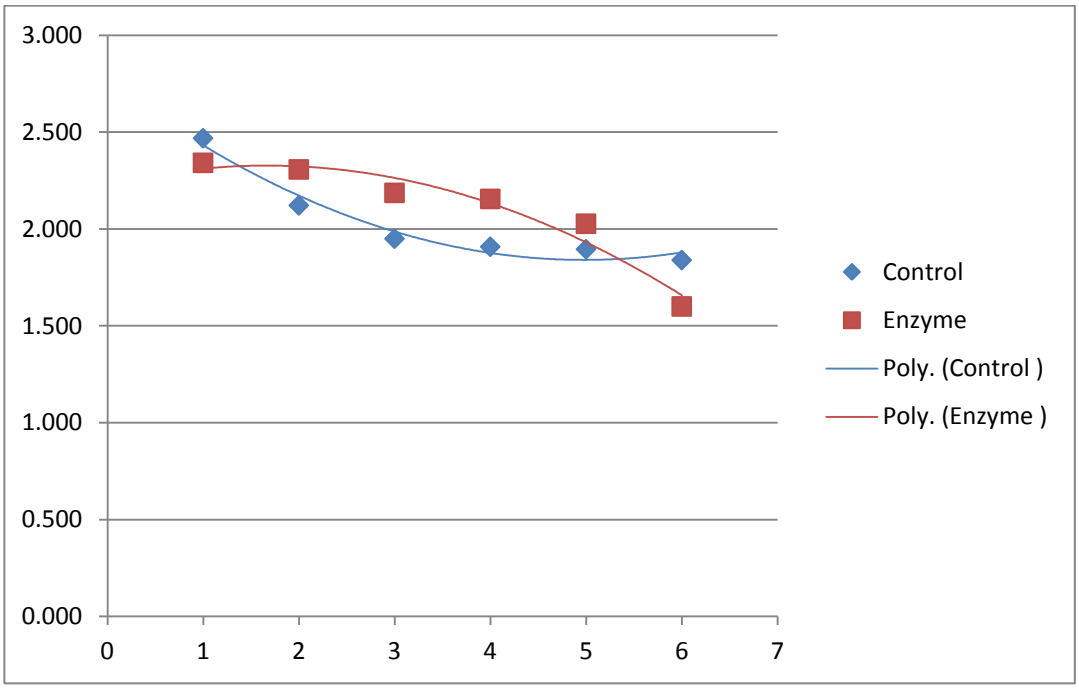
b. DOBI and Carotene analysis

Table 11: Analysis of bleachability and carotene content of oil on hourly basis

Treatment	Time (hr)	Weight of sample (g)	A_{269}	A_{446}	DOBI	Carotene (mg/kg)
Control	0	0.104	0.35	0.864	2.469	165.456
Enzyme	0	0.107	0.331	0.775	2.341	148.413
Control	1	0.126	0.443	0.94	2.122	180.01
Enzyme	1	0.111	0.348	0.803	2.307	153.775
Control	2	0.123	0.459	0.895	1.950	171.393
Enzyme	2	0.15	0.499	1.091	2.186	208.927
Control	3	0.106	0.459	0.876	1.908	167.754
Enzyme	3	0.106	0.348	0.75	2.155	143.625
Control	4	0.143	0.527	0.999	1.896	191.309
Enzyme	4	0.135	0.48	0.973	2.027	186.330
Control	5	0.156	0.536	0.986	1.840	188.819
Enzyme	5	0.133	0.598	0.957	1.600	183.266

DOBI is the ratio between absorbance at 446nm to absorbance at 269nm. The oil in question is bright red at the time of extraction due to high beta-carotenoids and is bleached and deodorized prior to its sale in the market. The oil loses its characteristic color with time which can be quantified by its bleachability. Table 11 shows values obtained on hourly basis for five hours at 90^{o}C. Graph 8 shows decrease in DOBI with time showing the loss of color in oil when subjected to high temperatures. While DOBI decreased with time, no significant change in carotene concentration (mg/kg) was observed.

Graph 8: Analysis of bleachability of oil with respect to time at high temperature



4.4 Effect of lipases on oil quality

a. Free fatty acids

The samples were treated with different concentrations of lipase to study the generation of free fatty acids over the course of 8 days when subjected to a temperature of 50^{o}C. Data for this is compiled in table 12. Sample treated with 100ppm of lipase shows very sharp increase in %FFA by the end of eight days. Lower doses of enzyme did not show a significant increase. 100ppm is a relatively high concentration and exogenous lipases might not be as potent since the enzyme used in this study of high purity. It can be concluded that exogenous lipases might not be a threat to oil quality. It will remain intact as long as the inherent lipases are deactivated by the process of sterilization.

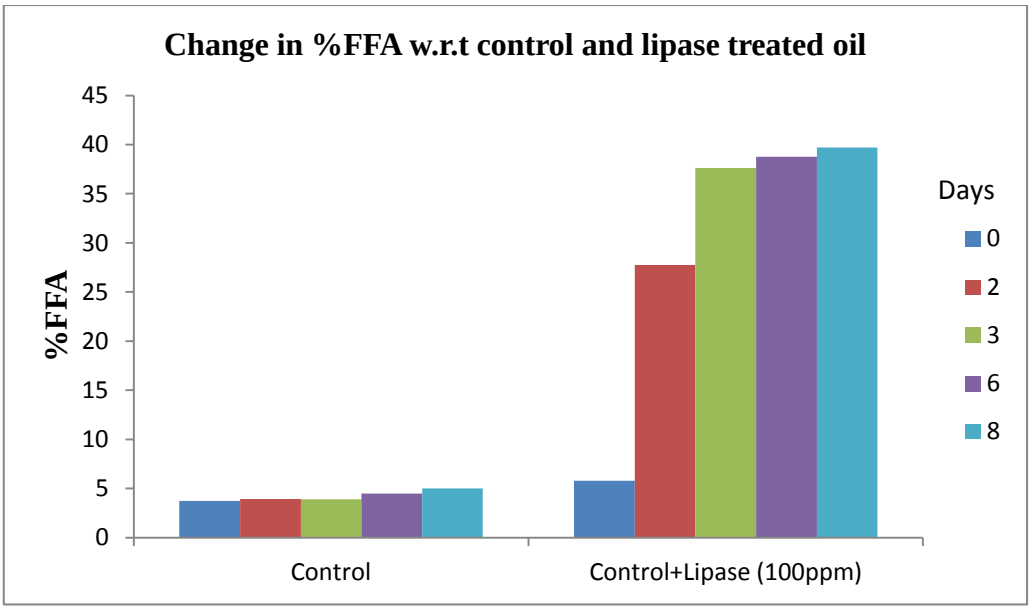
Table 12: Affect of lipases on free fatty acids

Treatment	Day	Weight of sample (g)	Vol. of NaOH (ml)	%FFA
Control	0	4.012	11.74	3.746
Enzyme		4.199	10.88	3.317
Control+Lipase (100ppm)		4.014	18.11	5.775
Control+Lipase (50ppm)		4.042	15.53	4.918
Control+Lipase (5ppm)		4	13.62	4.358

Control	2	4.006	12.32	3.936
Enzyme		4.04	11.87	3.761
Control+Lipase (100ppm)		4.001	86.74	27.75
Control+Lipase (50ppm)		4.02	15.36	4.891
Control+Lipase (5ppm)		4.015	12.58	4.011
Control	3	4.011	12.18	3.887
Enzyme		4.007	11.97	3.824
Control+Lipase (100ppm)		4.013	117.98	37.631
Control+Lipase (50ppm)		4.006	19.76	6.314
Control+Lipase (5ppm)		4.018	11.21	3.571
Control	6	4.024	14.09	4.482
Enzyme		4.016	12.34	3.933
Control+Lipase (100ppm)		2.022	61.22	38.755
Control+Lipase (50ppm)		4.003	19.86	6.35
Control+Lipase (5ppm)		4.003	14.76	4.72
Control	8	4.039	15.79	5.004
Enzyme		4.053	12.97	4.096
Control+Lipase (100ppm)		2.035	63.11	39.696
Control+Lipase (50ppm)		4.024	19.67	6.257
Control+Lipase (5ppm)		4.028	13.15	4.179

Graph 9 shows a very steep increase in FFA content for 100ppm while control sample FFA concentration grows gradually. From table 12 we can see that 5ppm and 50ppm showed FFA content similar to the control sample.

Graph 9: Variation of FFA observed with various enzyme treatments

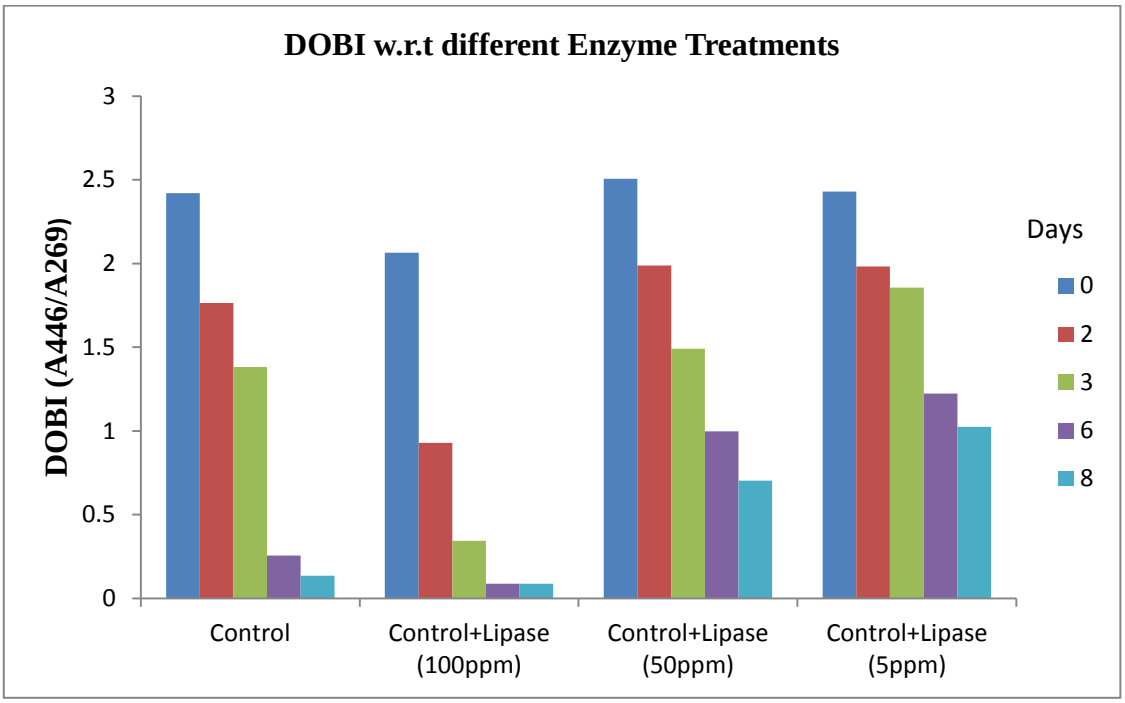


c. DOBI analysis

Table 13: Analysis of Bleachability index over the course of eight days with various enzymatic treatments.

Treatment	Day	Weight of sample (g)	A269	A446	DOBI
Control	0	0.114	0.338	0.818	2.42
Enzyme		0.117	0.352	0.879	2.497
Control+Lipase (100ppm)		0.122	0.277	0.572	2.065
Control+Lipase (50ppm)		0.12	0.414	1.037	2.505
Control+Lipase (5ppm)		0.119	0.329	0.799	2.429
Control	2	0.139	0.595	1.05	1.765
Enzyme		0.104	0.556	0.752	1.353
Control+Lipase (100ppm)		0.106	0.478	0.444	0.929
Control+Lipase (50ppm)		0.11	0.521	1.035	1.987
Control+Lipase (5ppm)		0.107	0.504	0.999	1.982
Control	3	0.11	0.367	0.507	1.381
Enzyme		0.107	0.387	0.358	0.925
Control+Lipase (100ppm)		0.118	0.385	0.132	0.343
Control+Lipase (50ppm)		0.106	0.361	0.538	1.49
Control+Lipase (5ppm)		0.135	0.399	0.74	1.855
Control	6	0.115	0.437	0.112	0.256
Enzyme		0.104	0.367	0.115	0.313
Control+Lipase (100ppm)		0.133	0.478	0.042	0.088
Control+Lipase (50ppm)		0.129	0.459	0.458	0.998
Control+Lipase (5ppm)		0.111	0.375	0.459	1.224
Control	8	0.121	0.475	0.064	0.135
Enzyme		0.101	0.391	0.062	0.159
Control+Lipase (100ppm)		0.12	0.449	0.039	0.087
Control+Lipase (50ppm)		0.12	0.435	0.306	0.703
Control+Lipase (5ppm)		0.114	0.376	0.385	1.024

Graph 10: Variation in Bleachability index with time



As mentioned earlier, the oil loses its color over a period of time. Graph 10 shows that oil is bleached in all samples irrespective of the treatment type. Although the rate of bleachability is varying, the bleaching property is independent of the presence of lipases since the control sample shows similar result. Therefore it can be concluded that oil is an intrinsic property of the oil and is independent of lipase. The oil gets bleached for all the samples indicating the independent of the type of treatment.

CHAPTER 5 – CONCLUSION

Global demand of vegetable oil has increased drastically over the past two decades. According to the Food and Agriculture Organisation of United Nations (FAOSTAT) report, there has been a 48% increase in the allocation of global edible vegetable oil for usage as food from the year 1995 to 2011. Strategies that increase oil yield without harming the environment needs to be formulated and implemented. Aqueous enzymatic extraction process is one such process which increases oil yield. This study was aimed to optimize this process and assess the oil quality parameters.

From the optimization process we can infer the following:

- Different enzyme dosages affect the oil yield during the extraction process. Higher enzyme dosage gave higher oil yield percentage. The entire enzyme mediated extraction process showed higher oil yield percentage compared to the control (with no enzyme). This proves that enzymes play a vital role in increasing the oil yield.
- Rotor types did not have an effect on oil yield but higher G-force showed more oil yield. Thus 9600g was identified as optimum for the process.
- Use of enzyme showed reduction in oil loss in the effluents i.e. sludge and fibre but the data was somewhat inconclusive.

The oil used in the study is stable at higher temperatures showing high degree of saturation. The study showed that the oil loses its characteristic color if kept outside for long durations or subjected to high temperatures for long duration of time which was seen by a significant decline in its Bleachability Index . FFA content was seen to be less in oil sample extracted with enzyme in comparison to control sample. The moisture content in both samples was almost similar. Lipases at higher concentration showed dramatic increase in FFA content, however at lower doses, the FFA content remained same. Carotene content remained same in enzyme treated oil and control sample.

The process needs further evaluation at higher scales so that it becomes industrially feasible and can help meet the global vegetable oil demand. The above mentioned parameters can be scaled up and used for mass extraction of oil.

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