M.Tech (Industrial Biotechnology)

Adithya Nair

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‘ENZYME ASSISTED AQUEOUS EXTRACTION OF VEGETABLE OIL AND ENZYMATIC TREATMENT OF VEGETABLE OIL MILL EFFLUENT’

A DISSERTATION

SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE

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IN

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Submitted By:

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CANDIDATE'S DECLARATION

I, Adithya Nair, Roll no 2K16/IBT/02 of M. Tech (Industrial Biotechnology), hereby declare that the project Dissertation titled "Enzyme Assisted Aqueous Extraction of Vegetable Oil and Enzymatic Treatment of Vegetable Oil Mill Effluent" 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 ADITHYA NAIR

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CERTIFICATE

I hereby certify that the project dissertation titled "Enzyme Assisted Aqueous Extraction of Vegetable Oil and Enzymatic Treatment of Vegetable Oil Mill Effluent" which is submitted by Adithya Nair, 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 by my permission and supervision. 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.

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Date:

Prof. JAI GOPAL SHARMA

(SUPERVISOR)

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Dear Adithya,

We are happy to inform you that the research work "*Thesis title: '**Aqueous enzymatic vegetable oil extraction and enzymatic treatment of vegetable oil mill effluent**' 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,

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ABSTRACT

Vegetable oil represents a major share in the global consumable fats and oil produced. The major types of vegetable oil produced are Palm oil, Soybean oil, Canola oil etc. with palm accounting for more than 30% of the entire production. Conventional oil extraction steps include mechanical and solvent extraction. An alternative for these conventional steps of oil extraction is the aqueous enzymatic extraction, here the solvent used for extracting the oil is water and thus it minimises the many environmental hazards posed by solvent extraction. The enzymatic extraction involves use of a combination of hydrolytic enzymes to degrade the plant cell wall components and make the oil accessible for aqueous extraction. The major classes of enzymes include, cellulases, xylanases, proteases etc.

This research work focuses on enzymatic aqueous extraction and its various parameter optimisation which include, dosage studies, oil loss analysis, and down streaming techniques like centrifugation. The dosage analysis shows the optimum dosage for effective enzymatic activity on the substrate. The enzymatic aqueous extraction is not as efficient as the solvent extraction hence oil loss studies are important to calculate the enzymatic efficiency, and this is done using Soxhlet analysis. Microscopic analysis of the substrate treated with enzyme is useful for understanding the enzymatic activity. The downstreaming of the extracted oil is also a vital step in getting higher oil yields and thus optimisation studies for centrifugation steps was carried out. These studies include, rotor type analysis and rcf value analysis for optimum oil yield.

The effluent coming from the mills have high BOD and COD. The effluent is also rich in a lot of complex sugar polymers. Biogas production from effluent has an enormous potential in tackling various environmental problems including lowering greenhouse gas emission. The anaerobic digestion of effluent can be enhanced using the enzymes to break down complex polymers to soluble monomers that can be readily metabolized by methanogenic bacteria. The use of enzyme on effluent resulted in increase in the concentration of glucose and this was confirmed using DNS assay and HPLC. This further supports the idea of using enzymes in effluent treatment and suggests immense potential of enzymatic treatment of the effluent. Further research must be carried out for making this viable on an industrial scale.

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1. INTRODUCTION

Vegetable oils represent a major share in the total consumable oils produced. The global demand for the product is increasing and has reached an all-time high, the demand is met from several types of vegetable oils produced. An estimated 213.5 million tons of oil and fats were produced in the year 2016-2017, out of which vegetable oil produced was estimated at 186 million tons. The major produce includes that of soybean, olive oil, corn oil, canola oil, palm oil etc. The share of palm oil being the highest in the lot at an estimated 59 million ton followed by soybean oil at 53 million tons and canola oil at 29 million tons. [1] The global increase in demand has led to increased production and this in turn has put the conventional practices for oil production under pressure. Oil palm (*Elaeis guineensis Jacq.*) is the highest yielding edible oil crop in the world and is cultivated in 42 countries on 11 million ha worldwide [2]. The global demand is also responsible for several environmental issues caused due to the side effects of increased production which relies heavily on conventional practices that aren't environment friendly, this includes oil extraction using solvents etc. The crops for the same are produced by cutting down natural vegetation covers and replacing them with plantation, this has turned out to be a disaster for biodiversity in certain regions. The idea of more from less is a major solution for this looming problem and it essentially helps us in tackling the problem without reducing our demand. Aqueous extraction of oil is a very well-established method for obtaining several oil products, this has proven to be very effective in combination with advanced mechanical press systems (expellers and screw pressers), yet it relies on the bulk of the raw material and a lot of waste produced from the process still has potential for oil extraction. This oil which is still present in the raw material that cannot be accessed by conventional methods is the answer to meeting the global demand without causing further environmental issues. The solvent extraction process is efficient but not environment friendly, we should employ a process or tool that is least hazardous to the environment at the same time be as potent as the solvent extraction. Enzymes are the answer to this problem and this was established in the 1950's, these biomolecules are the catalysts of biological origin and are the driving force behind all the biological processes happening within a living system. The very efficiency of a biological system in carrying out complex biochemical reactions can be credited to the enzymes that are part of the system. Greater than 90% oil extraction efficiency has been

achieved for various vegetable oils (e.g., canola, soybean, peanut and coconut oils) with this approach and the enzymatic extraction of olive oil has been reported. [3] In vegetable oil industry the main substrate is either the fruit or the seed of a specific plant. The oil molecules are either present in the seed as a reserve for the nourishment of the embryo or present in the mesocarp or present in both. This is structurally within the cell and to access it the structural components must be degraded first. To extract the lipid reserves stored in cells, it is necessary to be able to cross several barriers first the extra cellular walls (or secondary cell walls), then the cell wall, and finally oleosomes. Each cell wall has its own constituents, sometimes organized in a complex structure, and synthesized and degraded in a natural manner by specific enzymes. [4] For example, the secondary cell walls of rapeseeds are constituted of 39% pectins, 29% hemicelluloses, 22% cellulose, 8% of arabinogalactans seed. The primary cell wall contains 10% of glycoproteins which are very rich in hydroxyproline, at first called extensins but called today HRGP (HydroxyProline Rich Glycoprotein). Majority of plant material is cellulosic in nature and has variation depending on the type of the plant, this may include lignocellulosic as well as other complex structural biopolymers. Cellulases, a class of enzyme that degrades the polymer cellulose is one of the vital component that can be used for effective cell wall degradation. There are several types of cellulases depending on the site of action on the polymer, this can include endo-cellulase and exo-cellulase. Xylanases (EC 3.2.1.8) hydrolyse β-1,4 linked chains of xyloses, producing small xylooligomers. Lateral chains of glucuronic acids or of arabinoses fixed to this xylosidic skeleton can hide the action of these enzymes. β-Mannanases (EC 3.2.1.78) hydrolyse hemicelluloses composed of mannoses and liberate β-1,4-manno-oligomers, which can be then hydrolysed in mannose by β-mannosidase (EC 3.2.1.25). α-L-Arabinofuranosidases (EC 3.2.1.55) and α-L-arabinanases (EC 3.2.1.99) hydrolyse hemicellulose constituted of arabinose. Proteases would allow the proteins of cell wall structures to break, as well as oleosins, which stabilize oleosomes. [5] The enzymes for oil aqueous enzymatic extraction that are most frequently reported in the literature are protease, a-amylase, cellulase and pectinase other than cellulases, lignin degrading enzymes, cellobiose degrading enzymes, xylanases and proteases can also be employed. The enzymes to be used in industrial conditions must be tolerant to the harsh physical parameters present in the extraction mills, this includes elevated temperature, pH, presence of salts etc. The enzymes are produced from fungal strains that are modified genetically for higher enzyme production. The enzyme formulation also includes stabilizer chemicals for better storage and longevity. The final

product being manufactured for industrial use must undergo several tests before being projected for sales, these tests include stability tests, dosage tests, process parameter tests etc. Dosage test is one of the important tests and is one of the focus of this research work, the effective dosage is calculated on various scale of the substrate for understanding the activity of the enzyme. Other than this the work also focuses on oil losses, the highest amount of oil that can be extracted from the substrate is obtained using organic solvent extraction usually done with hexane as solvent. The soxhelt/soxtherm analysis of the sludge which is a by-product of the oil extraction is done to determine the remaining quantity of oil present in the sludge. Other process parameters like effective centrifugation setup, this includes assessment of optimum g-force, choice of rotor etc. these steps come in the downstreaming of the oil produced. In the industrial scale the centrifuge is replaced with the decanter centrifuge. At the lab scale centrifuge configuration can play a vital role in obtaining the appropriate result for oil yield and this is also dependent on the scale of substrate. Simple fixed angle rotor can give optimum results while dealing with substrate as low as 50 g at the same time for a substrate size of 1 kg a swinging bucket configuration is preferred. Microscopic analysis of the sample also provides an insight into the enzyme activity and how it helps in increasing the efficiency of the process. Using selective staining can help us in understanding the oleosome size and location within the cell and how it is affected by enzyme treatment. The enzymes are also capable of acting on the effluent that is being generated from these mills. The vegetable oil mill effluent (VOME) is a rich source of carbohydrates, both monomers and polymers. The effluent from these mills are directly drained to water bodies and this causes a lot of environmental issues. An example of vegetable oil mill effluent causing environmental problems can be seen in Malaysia, where the palm oil mill effluent (POME) are causing wide range of environmental issues such as increased greenhouse gas (GHG) emission and eutrophication of water bodies etc. The effluent has high BOD and COD, and this can be a grave issue if not treated effectively. The studies have found that the VOME still has a considerable amount of oil left in it that can be extracted using enzymes at the same time this effluent can be used in the production of renewable energy source like Biogas. The conventional effluent treatment plants can do this, yet their efficiency is hampered due to the complex nature of the polymers present in the effluent. These polymers can be broken down with the help of enzymes so that the sugar monomers that act as a food source for the methanogenic bacteria is accessible to them readily, thus increasing the efficiency of the production of the Biogas. Anaerobic

digestion is the most commonly used method for primary POME treatment. Anaerobic digestion is divided into four processes i.e. hydrolysis, acidogenesis, acetogenesis and methanogenesis respectively. In hydrolysis stage, the hydrolytic bacteria degrade organic polymers (polysaccharides, proteins and lipids) into monomers, such as long chain volatile fatty acids (VFA), alcohols, hydrogen (H) and carbon dioxide (CO2). In the acidogenesis stage, fermentative or acidogenic bacteria transform hydrolytic products into acetic acid and intermediate compounds, such as ethanol, lactic acid, propionic acid, formic acid, butyric acid, H and CO2. The acetogenic bacteria transform these products to acetate, H and CO2. Finally, the methanogenic bacteria produce methane from acetate, H. [6] The research work carried out suggests stark difference in the concentration of glucose present in VOME with and without enzyme treatment, the enzyme treatment of the VOME/POME can help in hydrolysis of the polymers at a faster rate. Thus, more efficient breakdown of carbohydrate polymers into monomers like glucose can help in better biogas production. The research hence suggests the potential for the use of enzyme in effluent treatment. Further research work has to be carried out before making this an industrially applicable option, yet the initial findings suggest a significant potential.

1.1 OBJECTIVE

1. Enzyme enhanced vegetable oil extraction process parameter optimisation with respect to enzyme dosage
2. Calculation of oil loss in aqueous enzyme oil extraction in sludge and substrate fibre.
3. Microscopic analysis of substrate treated with enzyme.
4. Downstream process parameter optimisation with respect to centrifugation
5. Enzyme treatment of Vegetable oil mill effluent and analysis with respect to conc. of soluble sugar present in the effluent.

2. REVIEW OF LITERATURE

2.1 Vegetable oil and its significance

The global production of fats and oils have reached an all time high and was calculated at 213.5 million tons. Out of the 213.5 million tons the majority of share comes from vegetable oil and it is estimated at 186 million tons, making vegetable oil the most important constituent in all the oil and fat produced for consumption and other uses. [7] Vegetable oil production is under pressure of the rising demand and the conventional processes involved in the production and processing are proving to be less efficient to meet the rising demands. The major types of vegetable oils include, Palm oil, soybean oil, canola oil, olive oil etc. Amongst these the largest share comes from Palm and soybean accounting for almost 30% and 25% respectively of the total vegetable oil produced. [8]

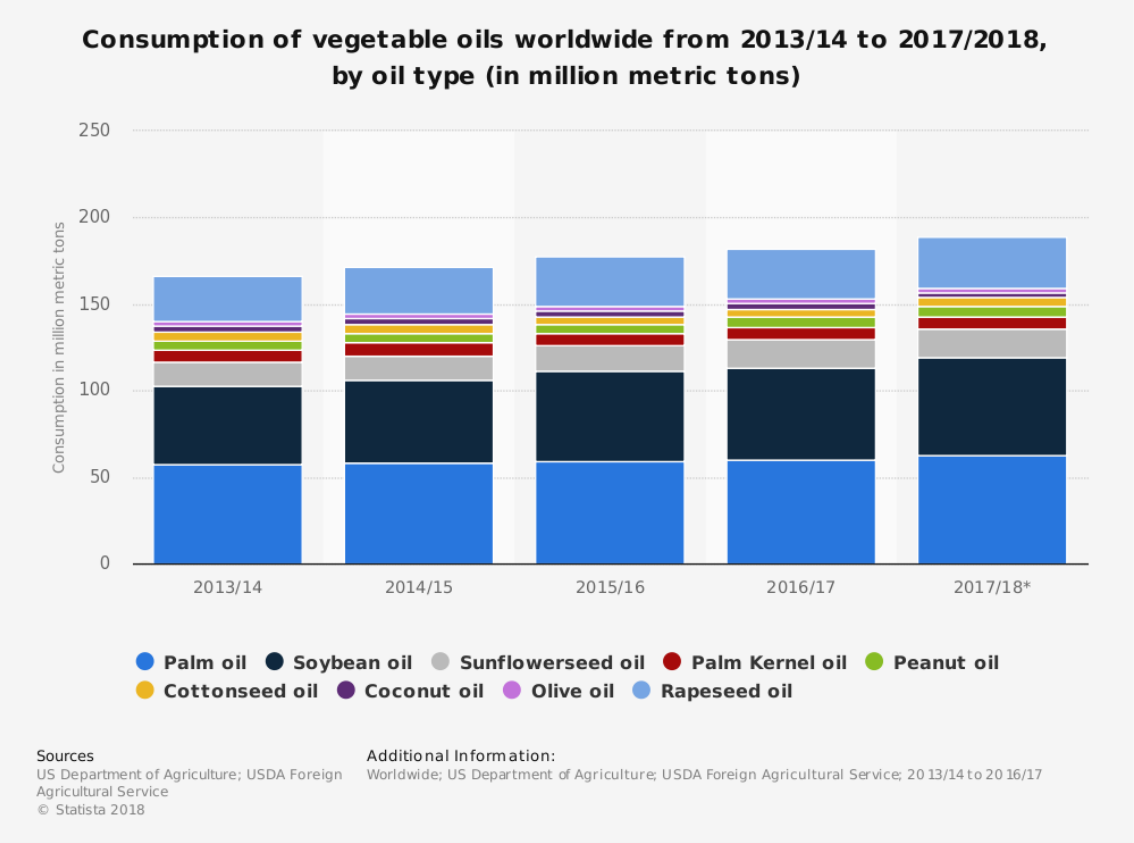


Fig 1. Consumption of vegetable oils worldwide from 2013-2018 (source: USFDA foreign agricultural service)

The major product in the vegetable oil being produced in terms of quantity is Palm oil and hence the further studies will be done with respect to palm and its derived oil. The global share of palm oil within the total vegetable oil produced is almost 60 million tons as estimated for the year 2016-2017, which is 30% of the total vegetable oil share. This huge production is mainly met by two important countries for Palm oil production namely Indonesia and Malaysia. They contribute to 30 million tons and 17 million tons of total palm oil production respectively. The area harvested under palm production has exponentially increased throughout the years and both Indonesia and Malaysia are the major contributors. [9]

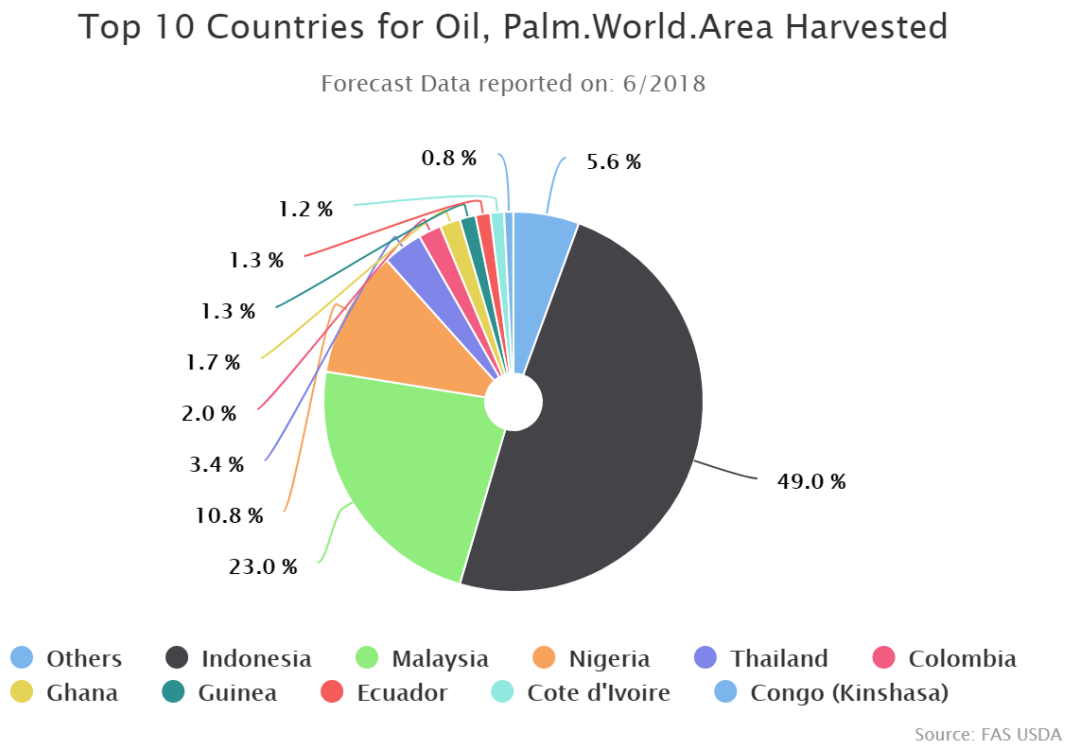


Fig 2. Area harvested for oil palm worldwide (source: USFDA foreign agricultural service)

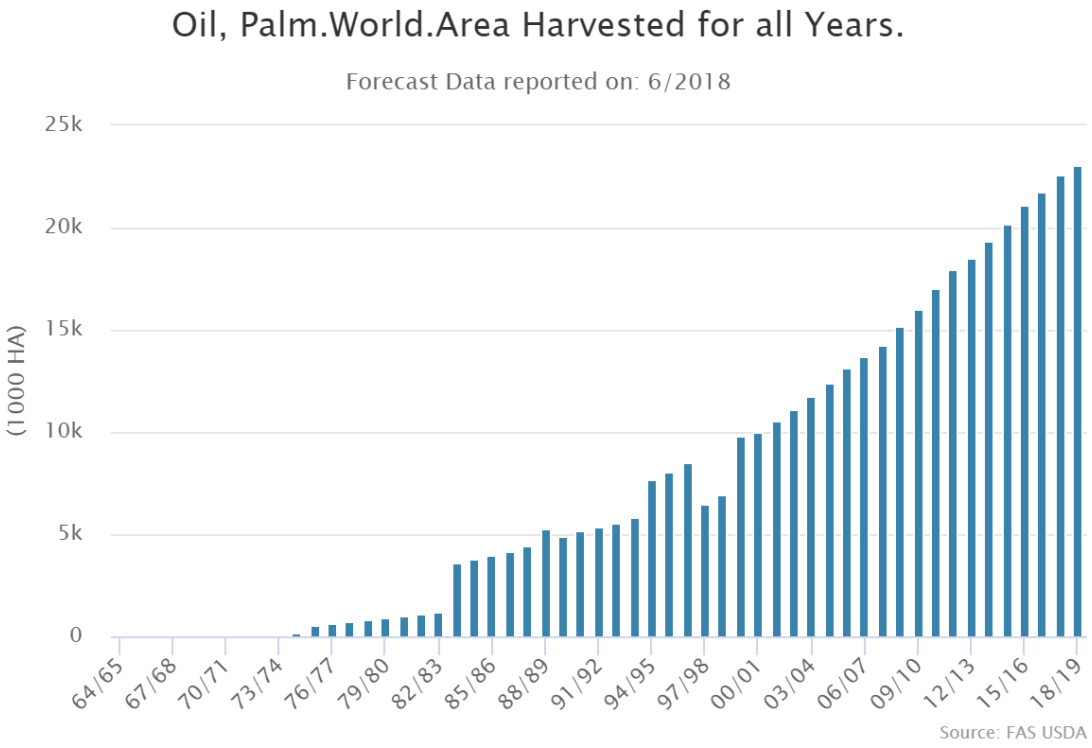


Fig 3. Area harvested for palm oil production from 1964-2018 (source: USFDA foreign agricultural service)

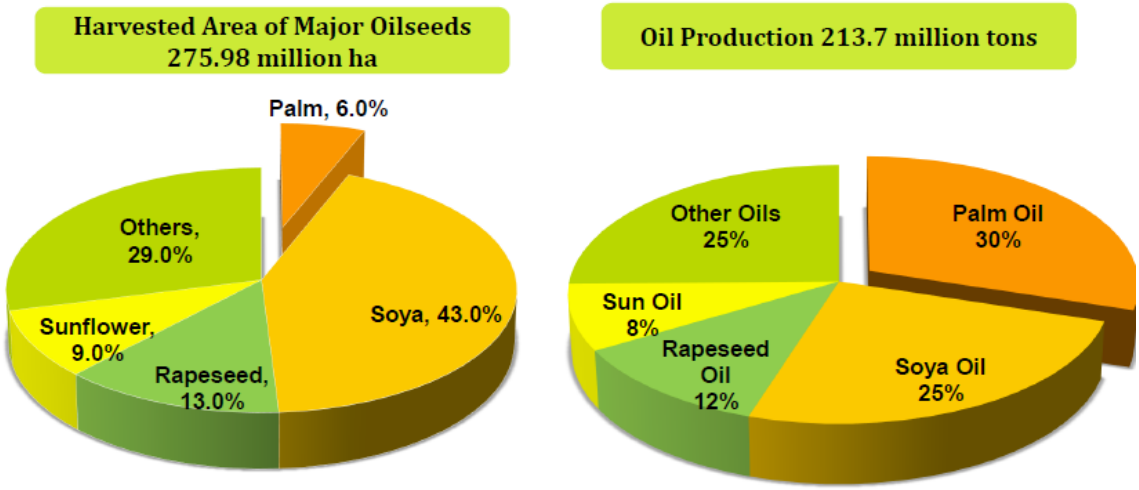


Fig 4. Harvested area under vegetable oil production and its correlation with quantity of oil produced (source Presentation by Thomas Mielke (Oil World) in POC 2017)

2.2 Palm oil

Palm oil is obtained from the fruit flesh (mesocarp) of *Elaeis guineensis* and the Palm kernel oil is derived from the palm kernel. The plant is native to west Africa. The fruit of the oil palm grows in bunches which weigh from 4 to 20 kg and contain 20&2000 individual fruits. Each is a sessile drupe, which varies in shape from nearly spherical to ovoid or elongated and bulging somewhat at the distal end, and from 3 g to over 30 g in weight. Oil palms can be classified into three main fruit forms

(1) dura, in which the fruits have a thick shell between the mesocarp and the kernel;

(2) tenera, in which the fruits have a thinner shell than dura and contain in the

mesocarp a characteristic ring of fibres encircling the nut which may be clearly seen in cross-section; and

(3) pisifera, in which the fruits contain no shell.

It was introduced to south east Asia, planted at the Bogor Botanical Garden, Indonesia, in 1848. The plant was introduced in Malaysia in the 1870s by the Royal botanic gardens in Kews, England. Initially it was used as an ornamental plant and later the industrial revolution in Europe laid the foundation of large scale palm plantation in Malaysia. [10] The major developments were seen in early 1900s and after independence the Malaysian government redistributed the land amongst the population for combating poverty in rural regions of the country through Federal Land Development Authority (Felda). From an initial 55000 ha of harvested land area in 1960 to 5.74 million ha in 2016, the area of palm plantation has increased exponentially. This increase in land under harvest directly correlates to the increase in palm oil production from 100 000 t in 1960 to about 17.32 million tonnes in 2016. [11]

Malaysia's palm oil production increased after the introduction of weevils. The national average fresh fruit bunches (FFB) before 1981 in Malaysia was low *i.e.* less than 19 t ha/ per year. Hand-assisted pollination was necessary to increase the yield, but this was arduous and expensive. The introduction of *Elaeidobius kamerunicus* weevil, which is a pollinating insect from Cameroon, at the Mamor Estate in Kluang, Johor in 1981 was a turning point for the Malaysian oil palm industry. [12]

With respect to the production the export of palm oil also increased, with Indonesia and Malaysia being the largest and second largest exporters in the world. In both the countries the palm oil industry is a major factor in the national GDP and thus the industry is vital for the countries economy. The global market consists of almost 200 counties of which the major markets include Europe, India, China, Japan etc. with India and China being the major importers. [13] The industry in Malaysia accounts for almost 5%-6% of the GDP. Palm crop helps in feeding more than 3 billion people on the planet, which is set to increase in the following years and estimated to feed 2 billion more by 2050. This estimate is a clear indication on why there is extensive research and development with respect to breeding of better variety of plant crops and development of advanced oil extraction and processing techniques. Compared to other oil bearing crops, palm has the highest production per land used, hence comparatively less land can be used for more production of the oil. An estimated 24% of global palm oil was produced in Malaysia using less than 0.1% of global agricultural land. The final product is highly sought after for various applications in food and non-food industries, making it a true global product. [14]

2.2.1 Palm oil in Malaysia

Malaysia being a major producer can explain the various parameters of palm oil industry and its functioning. The palm oil industry saw its growth in Malaysia from 1960s and ever since has been showing exponential growth. The country is second only to Indonesia in terms of production. The major area under cultivation was peninsular Malaysia but due to increase in demand of the product and decrease in suitable land, the area of production now includes regions of Sabah and Sarawak. The estimates include 47% in peninsular Malaysia, 27% in Sabah and 26% in Sarawak region which are under palm cultivation. The ownership of palm plantation is divided amongst various private plantations and few government bodies. The majority being under private ownership. [15]

Category	ha	%
Private estates	3 508 554	61.2
Government schemes:		
Felda	706 588	12.3
Felcra	173 032	3.0
RISDA	71 549	1.2
State schemes	344 314	6.0
Independent smallholders	933 948	16.3
Malaysia	5 737 985	100

Fig 5. Palm Cultivation land distribution (source MPOB 2017)

In 1960, Malaysia produced about 92 000 t of CPO. However, after six years, Malaysia's crude palm oil (CPO) production increased significantly to become the world's largest exporter of palm oil, overtaking Indonesia, Nigeria and the Belgian Congo. [16]

The production of CPO is dependent on the quality and quantity of the fresh fruit bunch (FFB) which is harvested from the palm tree. The FFB quality and quantity directly affects the yield. The oil palm starts producing FFB after three years of planting and reaching its maximum yield at the age of 12 to 15 years after which the yield, starts decreasing after the age of 15. Good agricultural practices will tend to give better quality FFB and this will result in higher yield of CPO. The palm cultivation is very sensitive to climate and unusual climatic conditions can affect the quality of the FFB as well as decrease the FFB production. [17] The floods in 2011 had a major impact in the FFB production in Malaysia and this resulted in less CPO production in comparison with previous year of 2009-2010. Regular well-distributed rainfall as well as the generally high amount of sunshine is necessary for a good yield. [18] The modem tenera fruit grown commercially in Malaysia is derived from high yielding Deli dura and pisifira palms of Sumatran and African origins. Good commercial tenera fruit now has 75-80% mesocarp and about 17% shell. The fresh fruit bunches of such palms will contain some 20-30% of oil and 1.34.1% of palm kernels. [19]

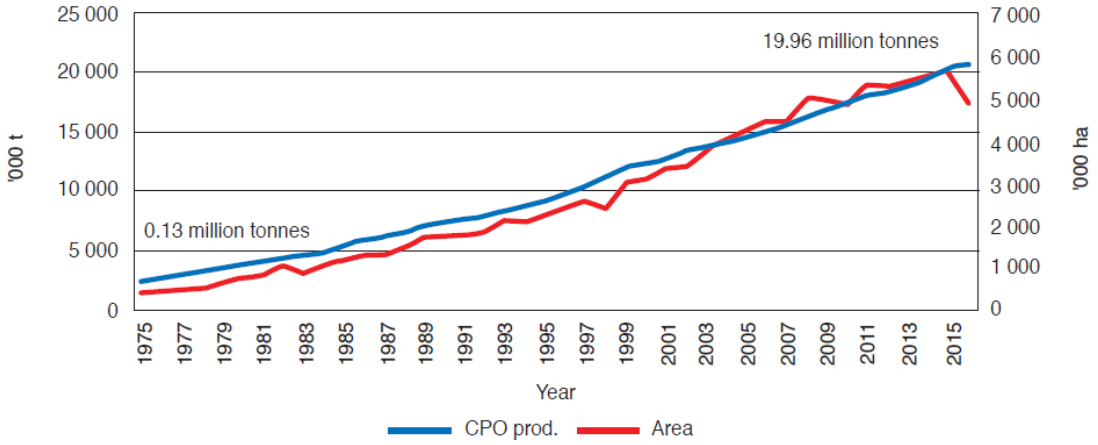


Fig 6. Malaysian oil palm planted area and crude palm oil (CPO) production (1975-2016). (Source: MPOB 2017)

2.2.2 Conventional Palm oil extraction

Like many oil crop, the palm oil is conventionally extracted using high pressure expellers/screw presses or solvent extraction.

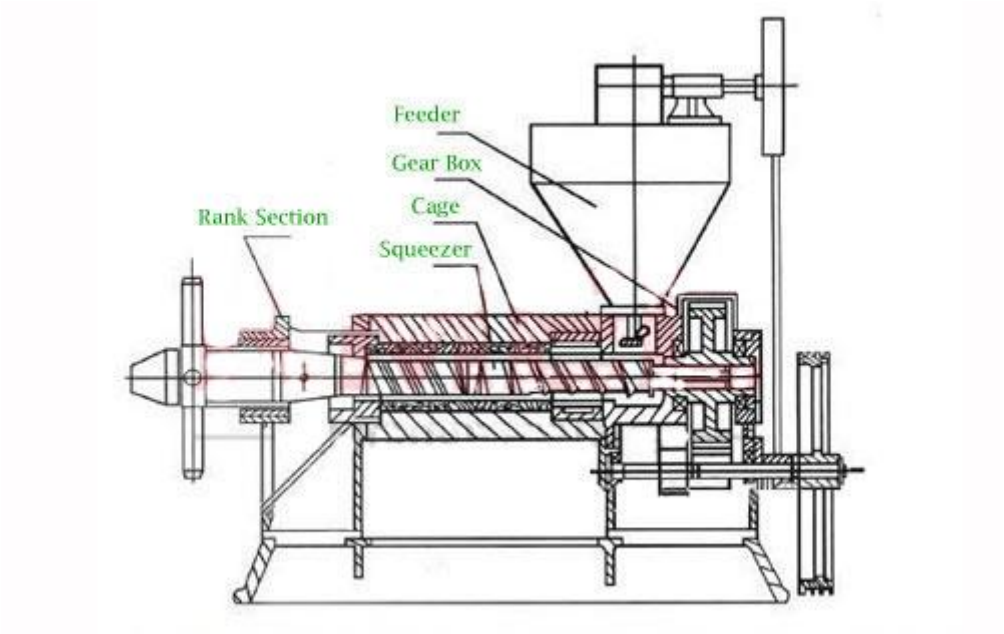


Fig 7. Basic design of an Industrial screw press. (source MPOB)

Solvent extraction employs the use of an organic solvent, usually a hydrocarbon like hexane. This is one of the main methods employed due to its very high efficiency. Every other extraction step is compared with solvent extraction step for determining the efficiency of the extraction process. The organic solvent is chemically more suitable to extract the hydrophobic oil droplets within the fruit. Even though the efficiency is very high the solvent extraction process isn't the ideal procedure for oil extraction due to its wide ranging economic and environmental drawbacks. Usage of solvents has come under a lot of scrutiny in the past years due to its ill effects on the environment.

An alternative to solvent extraction is aqueous extraction, where the solvent used is water. Usually high temperature water. This method is used along with mechanical pressing like screw press for better results. The method is better than solvent extraction in terms of being environment friendly, but it lags in efficiency and oil yield. [20]

An improvement to aqueous extraction is the enzyme assisted aqueous extraction, where hydrolytic enzymes are used to degrade cell components and make the oil within the cells more accessible. To completely understand Enzyme assisted oil extraction, proper knowledge of enzymes and the nature of the composition of the fruit is necessary.

2.2.3 Biochemical nature of palm fruit

Just like all the plant materials, most of composition is consisting of Cellulose. Cellulose is a polymer of glucose. Cellulose is a linear chain of several hundred to over nine thousand β-(1→4)-linked D-glucose units. In the cell wall, the molecules of cellulose are assembled in parallel rows: microfibrils with a diameter from 5 to 12 nm. They are constituted from 36 to 1,200 molecules of cellulose maintained together by hydrogen bonds between the hydroxyl groups of the nearby glucose residues. They are between 5 and 15 nm wide and separated from one to another by 20-40 nm. Crystalline and amorphous domains can be observed. Other constituents of a plant cell include pectin, hemicellulose, protein moieties, lignin etc. Hemicelluloses are linear or branched out polysaccharides bound to the celluloses microfibrils by hydrogen bonds or connected to the lignin by covalent bonds, so they form a complex and solid structure around plant cells. [21]

Structural Characteristics	%
Total lipid content	56.68
Soluble sugars	2.89
Total structural carbohydrate	13.76
Glucan	8.27
Xylan	4.52
Arabinan	0.97
Soluble lignin	3.58
Insoluble lignin	0.038
Water extractives	17.16
Ethanol extractives	17.98

Fig 8. Composition of palm material (source MPOB)

2.3 Enzymes

Enzymes are catalysts of biological origin and are mainly proteinaceous in nature. Enzymes can be credited for the high efficiency of biological systems in carrying out complex biochemical reactions.

2.3.1 Cell wall degrading enzymes

Cell-wall degrading enzymes can be used to extract oil by solubilizing the structural cell wall components of the oilseed or mesocarp. Proteolytic enzymes are also found to improve yields of oil and protein by hydrolysing the structural fibrous protein in which fat globules are embedded. Major class of cell wall degrading enzymes for plant cells are that of cellulases, hemicellulases/xylanase etc.

a) Cellulases

Cellulases (EC 3.2.1.4 and EC 3.2.1.91) and a β-glucosidase (EC 3.2.1.21), the first cellulase, called 1,4-β-cellobiosidase, is able to hydrolyse the intermolecular β-(1→4)-glucosidic bonds. The second cellulase can hydrolyse the cellulose from the extremities of glucosidic chains; they consequently form either glucose or cellobiose. Finally, β-glucosidases can hydrolyse the cellobiose molecules, forming some glucose, to eliminate its inhibition on the other activities. [22]

b) Xylanase

Xylanases (EC 3.2.1.8) hydrolyse β-1,4 linked chains of xyloses, producing small xylooligomers.

c) β-Mannanases

(EC 3.2.1.78) hydrolyse hemicelluloses composed of mannoses and liberate β-1,4-manno-oligomers, which can be then hydrolysed in mannose by β-mannosidase (EC 3.2.1.25).

d) α-L-Arabinofuranosidases (EC 3.2.1.55) and α-L-arabinanases (EC 3.2.1.99) Hydrolyse hemicellulose constituted of arabinose.

e) β-xylosidases (EC 3.2.1.37)

are exoglycosidases which hydrolyse short oligomers in simple units of xyloses.

f) Pectins

are a linear chain of β-(1→4)-linked D-galacturonic acid? Into this backbone, regions where galacturonic acid is replaced by (1-2)-linked L-rhamnose can be found. Lateral chains of neutral sugar can be attached to the rhamnose. The neutral sugars are mainly D-galactose, L-arabinose and D-xylose; the types and the proportions of neutral sugars vary with the origin of pectin.

g) Proteases

Proteases correspond to any enzyme which conducts proteolysis. Any enzyme which begins protein catabolism by hydrolysis of the peptide bonds linking amino acids together in the polypeptide chain. There are 5 types of proteases, classified according to their mechanism of action and amino acid (serine, threonine, aspartate, cysteine) or a metallic atom directly implicated in the mechanism. [22]

2.3.2 Aqueous Enzyme oil extraction

Aqueous extraction processing (AEP) has found increasing interest due to the need for environmentally cleaner alternative technologies for oil extraction. Low oil recovery is one of the major challenges for Aqueous extraction process due to poor solubility of oil in water. This drawback can be mitigated to a very large extent with the help of hydrolytic enzymes. These hydrolytic enzymes, few of which have been mentioned before helps in breaking down the cell wall components and make the oil more accessible. This is combination with mechanical press systems have seen efficiency above 90% in several oilseed substrate. The extraction efficiency and quality of the oils depend on the combination of the applied enzymes. The enzyme assisted oil recovery is done by digesting the substrate with enzymes for a certain period under set parameters. The mash after digestion is subjected to the press and this obtains better results than that of simple aqueous extraction. Other than degrading the cell wall components the enzyme assistance in oil recovery has also proved to reduce the viscosity of the extract. This is particularly useful while the Undiluted crude palm(UDCO) oil is obtained after extraction from the digested mash. The main factors that affect the hydrolytic process are particle size, moisture, hydrolysis time and the enzyme/substrate mass ratio. Other advantages of the aqueous process compared with solvent-based processes include: (i) simultaneous production of edible oil and protein isolate or concentration in the same process, (ii) lower protein damage during extraction, and (iii) improved process safety due to the lower risk of fire and explosion. It is also reported that aqueous extraction processes may be more cost effective since the solvent recovery step is eliminated. The main limitations of this process appear to be: (i) lower efficiency of oil extraction as evident in earlier studies, (ii) demulsification requirements to recover oil when emulsions are formed, and (iii) treatment of the resulting aqueous effluent [23]

2.3.3 Factors affecting enzyme function

a) Most of the early studies did not consider the particle size of the oil-bearing material as a key factor influencing extraction efficiency. Theoretically, the lower the particle size, the higher the oil yield for a given set of extraction conditions which is attributable to higher cell wall disruption during size reduction as well as the lower diffusion path

b) Enzyme/substrate ratio higher enzyme concentration leads to greater interaction between the enzyme and substrate, thus promoting cell wall degradation and rupturing more peptide. However, too high enzyme concentration may result in bitterness and off flavours, as reported possibly due to the extraction of undesirable components.

c) Ratio of water to oil-bearing material the water used in AEE not only serves as an extraction medium but also enters the oil-bearing material and modifies its water activity. The resulting moisture content of the oil-bearing material can assist hydrolytic reaction, diffusion, and mobility of the enzymes and products.

d) pH of extraction medium the pH at which enzymes attain maximum activity varies with the enzyme. In most earlier studies, the pH value of the solution, be it for soaking pre-treatment or extraction itself, was set at a value corresponding to maximum enzyme activity. However, the optimum pH of several enzymes is in the range of the isoelectric pH of proteins which depends on the nature of the oilseeds.

e) Incubation temperature besides being active over a narrow range of pH, enzymes also active over a narrow temperature interval.

f) Incubation time degradation of cell wall components can be enhanced by prolonging the incubation time.

g) Agitation assists in mixing and additional rupture of the cell wall, and agitation rate is one of the factors affecting the disruption of cell wall.

h) Pre-treatment (grinding) of oleaginous materials it is necessary to reduce the size of oleaginous materials (seeds/fruits) either by grinding or flaking to gain much access by enzymes.

Enzyme assisted aqueous extraction has better efficiency than normal aqueous extraction and since the enzyme is biological in nature it does not pose a threat to the environment. There are additional steps as described above which must be taken into consideration that makes enzyme assisted extraction more complex, but the end result is both economically and environmentally viable.

2.4 Environmental hazards due to Oil processing

As the production of the oil has seen an exponential rise, it should be no surprise that in order meet the global demands there has been excessive production where care for the environment has not been considered. Conventional methods have proven to be effective in just getting the yield and they have not taken into account further environmental issues caused due to increase in the production. Vegetable oil mill effluent is one of the major contributors in this issue. VOME discharged into water bodies without proper treatment can cause an array of problems starting with increased GHG emission and eutrophication of water bodies.

2.4.1 Palm oil mill effluent

POME the waste generated after palm oil production is characterized with high BOD and COD, if the effluent is not treated and discharged without decreasing the BOD and COD, it can cause severe damage to the environment. It is characterized with brownish colour and excessive amounts of suspended solids. The POME still has very high amount of sugars both monomers and polymers. It also has adequate quantities of oil that cannot be recovered in the conventional process. This POME if treated properly can be a solution to many problems, one of them being production of Biogas. POME as already discussed has excessive amounts of organic matter, mainly sugars. These are complex in nature and hence cannot be readily be utilized by the microbes in the effluent treatment plants. If these complex sugars, mainly cellulose and its derivatives can be broken down to its monomers of glucose it can be readily be utilized by the microorganisms. This in combination with an anaerobic digestor in an effluent treatment plant can be the ideal source for Biogas production. The methanogens can do complex conversion on their own, but the process efficiency is not high, if the source of nutrition for these bacteria are simple in nature like reducing sugars, then the process of methanogenesis can be rapid and the final product of CH_4 produced can be collected in larger quantity which can be utilized. The use of enzyme in treating POME has shown stark difference in the sugar profile of POME treated with enzyme and POME not treated with enzyme. The final product of biogas can be utilized for electricity generation and this in turn can reduce the energy demands of the mill, making the entire system sustainable. A proper understanding of the POME constituents and from it the choice of enzyme to be used is the most important criteria in making this idea effective. Appropriate effluent tanks and digestors

also play a crucial role. Understanding the microbial consortium responsible for the biogas production is another challenge and this can be utilized to increase the overall production of the biogas. It can be done by learning the optimum growth conditions required by these microbes to produce the Biogas. The overall power generation potential from effluent treatment can be estimated based on the calculated methane yield from anaerobic POME treatment. According to Malaysia Palm Oil Board (MPOB), 0.65 m3 POME is generated from every processed ton of fresh fruit bunch. [24]

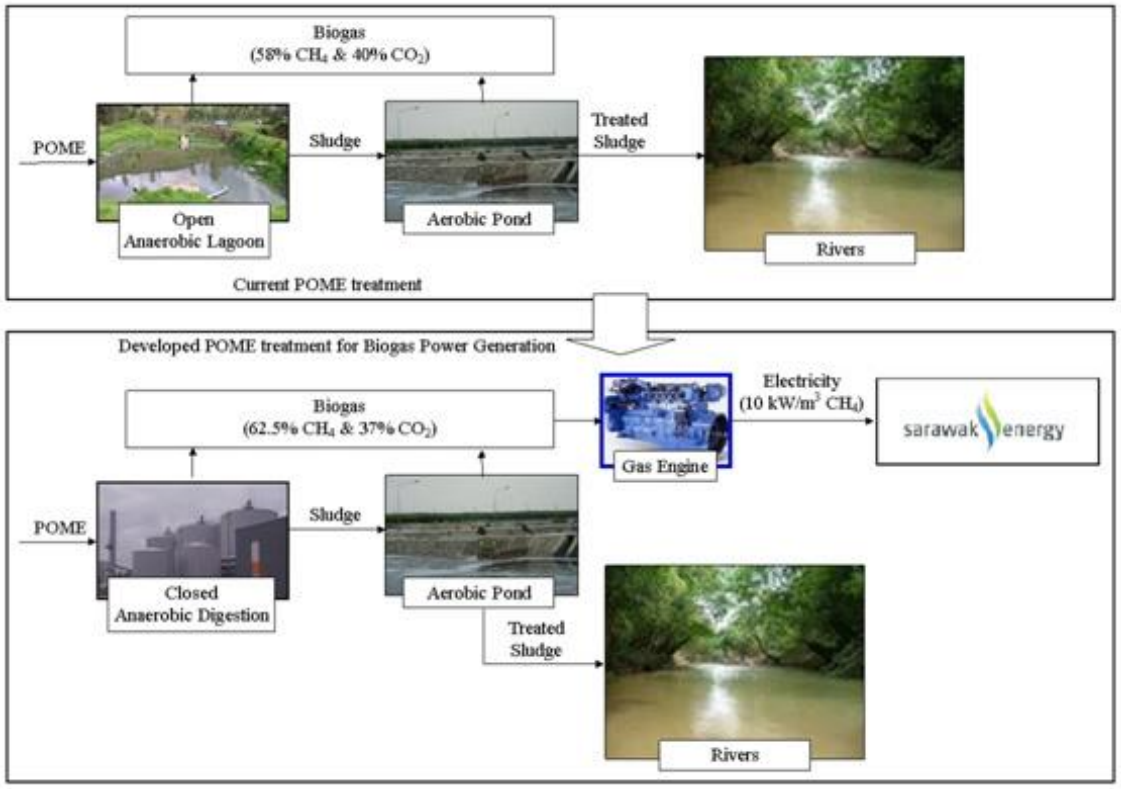


Fig 9. Conversion of conventional effluent treatment to Biogas powered electricity generation systems (source Sarawak energy).

Sarawak energy case study

Based on annual production of 9,288,000 tons of FFB process in Sarawak; resulting in an annual effluent generation of 6,037,200 m3 and therefore 150,930,000 m3 of biogas could be harnessed. If the effluent is treated properly under anaerobic conditions, the total methane production amounted to 94,000,000 m3. The calorific value of methane is stated as 10kWh/m3. The annual energy content of the generated methane gas can be calculated to 940 GWh (~108 MW). Based on a conversion efficiency of 38 % (gas engine), the potential annual electrical power generation would be 360 GWh. Assuming 100 %

availability of the conversion system shall result in an installed power generation capacity of 41 MW from POME derived methane gas. [25]

Parameters	Value	Unit
FFB	9,288,000	ton/year
POME yield	6,037,200	m3/ton-FFB
Biogas yield from POME	25	m3-biogas/m3-POME
CH4 gas fraction in biogas	0.625	m3-CH4/m3-biogas
CH4 emitted	0.94E+08	m3
Electricity equivalent (38 % eff)	3.6E+08 or (41)	kWh or (MW)

Fig. 10 Parameter for Methane production (source Sarawak energy)

POME has emerged as an alternative option as a chemical remediation to grow microalgae for biomass production and simultaneously act as part of wastewater treatment process.

Utilizing POME as nutrients source to culture microalgae is not a new scenario, especially in Malaysia. Most palm oil millers favour the culture of microalgae as a tertiary treatment before POME is discharged due to practically low cost and high efficiency. Therefore, most of the nutrients such as nitrate and ortho-phosphate that are not removed during anaerobic digestion will be further treated in a microalgae pond. Consequently, the cultured microalgae will be used as a diet supplement for live feed culture.

The microalgae thus grown find multiple uses in phytoremediation, growth of single cell protein and in third generation biodiesel production (which employs the use of microalgae).

The analysis on POME with respect to reducing sugar concentration can give us an idea about the amount of substrate that is available for the microbes to synthesis methane, which in turn is the measure of Biogas produced. The reducing sugar concentration can be done on POME sample using the DNS assay and this can be further confirmed with HPLC of the sample for sugar molecules.

3. MATERIALS AND METHOD

Raw Materials

The substrate is obtained from Malaysia and is stored in refrigeration at 4^{o}C.

Sample preparation

The received fruit is peeled and the mesocarp is separated from the kernel. This mesocarp forms the substrate on which the enzyme will act.

Enzymes

Cellulases, Xylanases, Pectinases etc. all produced by Novozymes.

Solvent

Hexane was used for Soxhlet extraction method

Instrument used Mash bath, water bath, hot air oven, Soxhlet apparatus (round bottom flask, extractor, condenser), analytical balance, centrifuge, centrifuge tubes, beakers, aliquot vessels, spatula, centrifuge tube, micropipette, tips, microscope, gun pipette, petri plates, Methods – the process flow chart is given below

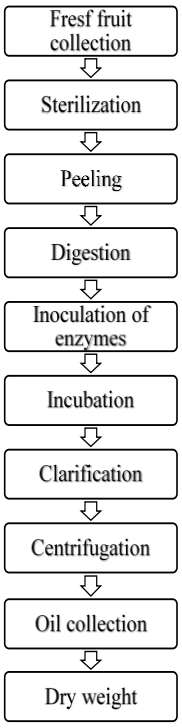


Fig 11. Flow chart for the extraction process.

1. **Collection of fruits:**

The bunch of fruits were collected from specific region. The trashes were sorted out from the collected fruits. The good fruits were separated and sterilized at 121°C for 30min in pressure cooker. The cooked fruits were peeled to remove the nuts separately from the pulp (mesocarp). The weight of the nuts and pulp were measured for calculating the mass balance.

2. **Preparation of substrate:**

The required amount of mesocarp were taken as substrate for the extraction. The peeled mesocarp of the fruits was mashed homogenously for 3mins at 50°C using masher. The homogenized substrate was taken out from the masher. 50g of substrate were aliquoted in each vessel.

3. **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 and 70°C to achieve optimum enzyme action.

4. **Enzyme preparation**

Various dilutions of different proprietary Enzyme A&B were prepared.

5. **Inoculation and Incubation**

Dilutions of hydrolytic proprietary enzymes A, B, from Novozymes was added to the substrate and was mixed well. The substrate was then kept for incubation at 70°C. No enzyme was added to 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.

6. **Para-pressing**

The substrate was transferred to a para-press and a combination of oil and sludge was separated 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.

7. Clarification and Centrifugation

The centrifuge tubes were transferred to a clarifying water bath. 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.

8. Oil recovery

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

3.2. Laboratory trial (Rotor test)

The above given steps were repeated for this trial. In the centrifugation step, two different set of rotors were used: (i) swing bucket rotor and (ii) fixed angle rotor. The yield of oil was measured in both cases and the rotor which gave higher oil yield was identified. The optimum yield in case of varying g-force was also done.

The substrate amount per sample was measured at 50g

3.3 Oil loss in sludge and fruit fibre

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.

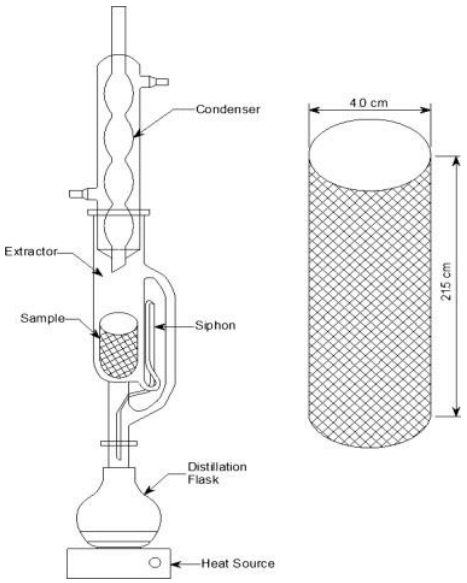


Fig12. Soxhlet Apparatus

The extractor, heating mantle, round bottom flask and the condenser were set up as given in the picture above. 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. Weight of the samples kept in dry oven was taken after every two hours until the weight became constant. Particular amount of sample in grams 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.

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)

3.4 Microscopy

The substrate was subjected to microscopic analysis before and after the enzymatic treatment. The equipment used was a light microscope. 10µL of the sludge sample was taken on a slide and viewed directly under the light microscope. No stains were for sludge samples. Sudan black was used for fibre samples. The dye binds specifically to fat droplets within the cell cytoplasm.

3.5 Vegetable oil mill effluent analysis for reducing sugar conc. and treatment with different proprietary enzyme conc.

Reducing sugar conc. can be used for estimating the amount of Biogas that is being produced from the effluent. The DNS assay is the basic test for quantifying the sugar concentration.

The VOME collected is subjected to treatment with different conc. of proprietary enzyme so that the effects may be observed and conc. of reducing sugar maybe analysed.

The different conc. of enzyme treatment includes – 0.01% w/w of sample, 0.1%w/w of sample and 1 % w/w of sample and a control sample.

The samples are subjected to DNS analysis in the first hour, second hour, fourth hour, sixth hour, eighth hour and twenty fourth hour after inoculation with enzyme in different conc.

The analysis is done while taking 3 replicates for each treatment. (set A, B and C).

The VOME is aliquoted in 50ml falcon centrifuge tubes, an equal amount of 40g is added to each tube.12 tubes in total with three replicates of each treatment included.

The sample is kept in for incubation at a temperature of 50^0c and agitation of 15 rpm.

At the end of every pre-set incubation period the samples are centrifuged, and the 1 ml of aqueous phase is pipetted for DNS and HPLC assay.

DNS assay is done with 100µL of sample and HPLC is done with 10µL of sample.

The DNS assay is done with spectrophotometer of Molecular devices (spectramax) and the HPLC is done using Agilent infinity series HPLC unit with RID detector.

The chromatography is isocratic, and the mobile phase used is 5mM H_2SO_4.

The flow rate being 0.6mL/min. The column temperature is set at 60^oc and the RID detector is set at 55^0c temperature. The run time is for 20 min.

Standard sugar solutions are first run so that the peaks in the sample can be identified.

4. RESULTS and DISCUSIONS

4.1 Dose response Studies

Table 1. Dose response study for proprietary enzyme A and B

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	143.26	10.07	11.25	31.16	19.91
2.	Enzyme A	142.61	10.56	11.26	32.12	20.86
3.	Control	146.26	11.27	11.26	31.86	20.6
4.	Enzyme A	144.22	10.56	11.26	32.45	21.19
5.	Control	143.22	11.54	11.26	31.19	19.93
6.	Enzyme A	146.24	10.67	11.26	32.21	20.95
1.	Control	142.95	10.71	11.25	30.76	19.51
2.	Enzyme B	143.9	10.55	11.25	33.21	21.96
3.	Control	145.55	10.96	11.25	30.4	19.15
4.	Enzyme B	144.43	9.98	11.25	31.65	20.4
5.	Control	144.76	11.75	11.23	30.1	18.87
6.	Enzyme B	144.62	11.12	11.25	32.2	19.91

The dose response study suggests that enzyme treatment of sample is leading to higher yield of oil in those specific samples. Both the proprietary Enzymes (A and B) show better oil yield than control sample, in comparison with each other the proprietary enzyme B is having higher value in terms of oil yield. The oil yield with enzyme A is 21g and the oil yield with enzyme B is 20.57

Average oil yield values for sample treated with Enzyme A and B

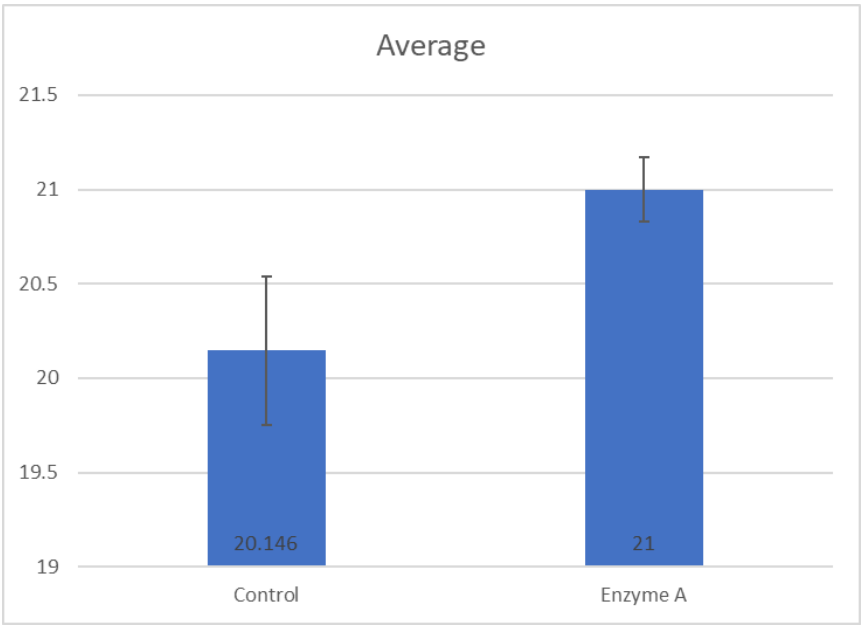
	Control	Enzyme A
Average	20.146	21
Standard Deviation	0.393	0.171
CV	2%	1%
Increase in Oil Yield		0.854

Average oil yield using proprietary Enzyme A.

	Control	Enzyme B
Average	19.177	20.757
Standard Deviation	0.321	1.071
CV	2%	5%
Increase in Oil Yield		1.580

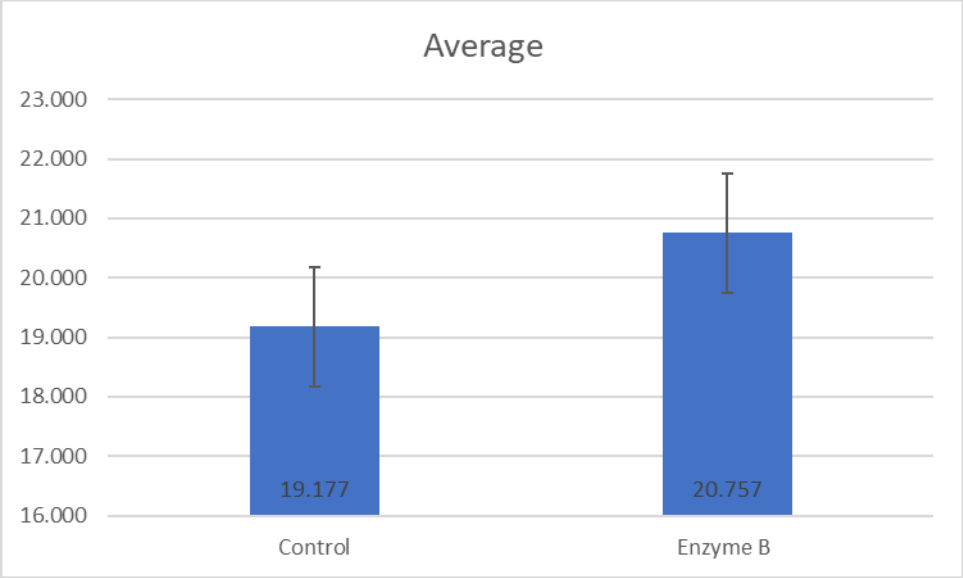
Average oil yield using proprietary Enzyme B.

The oil yield is given in grams and compared against control.



Average oil yield in grams.

Fig 13 : Oil yield (Control Vs proprietary Enzyme A)



Average oil yield in grams.

Fig 14 : Oil yield (Control Vs proprietary Enzyme B)

Activity of Enzymes A and B were analysed. Graph 1 depicts oil yield in substrate treated with enzyme is more in comparison to the control. The error bars do not overlap showing the yield in case of Enzyme A is significantly higher than the control sample. Graph 2 depicts oil yield in substrate treated with enzyme 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.

4.2 Oil loss in sludge and fruit fibre

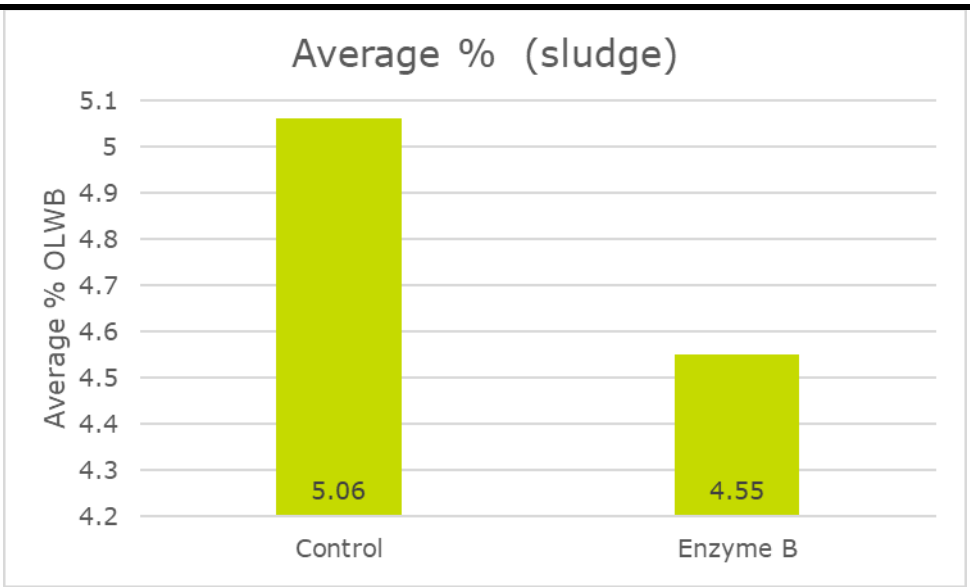
Soxhlet apparatus was used to determine the oil content of the fibre and sludge obtained after the process where Enzyme A and Control was used. The table given below gives the value of the oil loss from the sample. The results show that oil loss in both fibre and sludge was reduced in sample where enzyme was used.

Table 2. Oil loss from Sludge

S. No.	Sample details	Petri Dish wt. (g)	PD+Sample wt. (wet) (g)	Sample wt. (wet) (g)	PD+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	%Oil OLWB	Average %OLWB
1	Control	23.7	61.52	37.82	27.054	3.354	124.5	126.3	1.803	103	103	0.008	1.811	4.79%	
2	Control	22.215	55.345	33.13	25.168	2.953	102.2	103.9	1.665	129	129	0.004	1.669	5.04%	5.06%
3	Control	22.48	50.917	28.437	25.193	2.713	103.6	105.1	1.516	96.04	96.04	0.003	1.519	5.34%	
4	Enzyme B	22.741	54.038	31.297	25.386	2.645	115.2	116.7	1.454	124.5	124.5	0.005	1.459	4.66%	
5	Enzyme B	23.506	55.722	32.216	26.227	2.721	103.6	105	1.459	95.37	95.38	0.009	1.468	4.56%	4.55%
6	Enzyme B	23.55	61.868	38.318	26.791	3.241	122.6	124.3	1.699	102.4	102.4	0.004	1.703	4.44%	

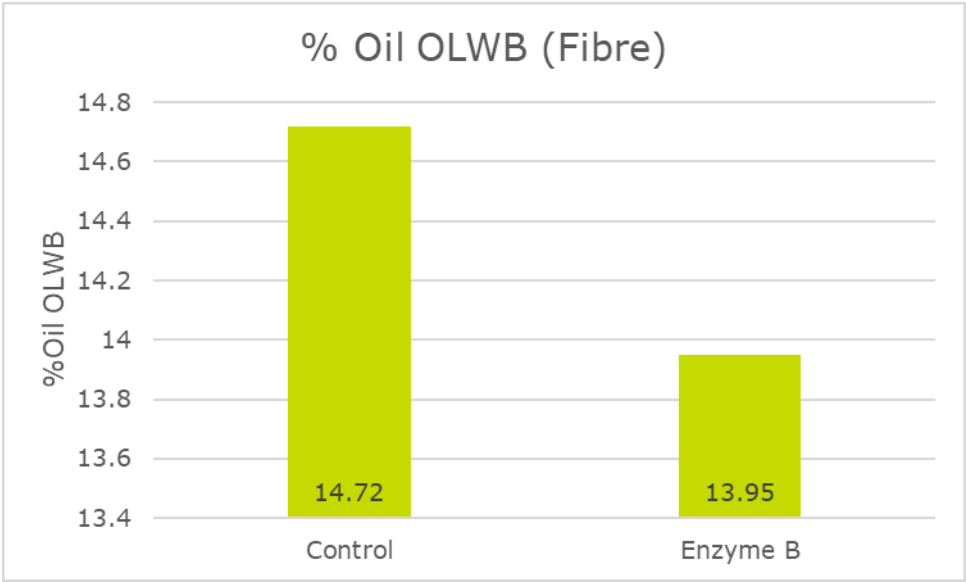
Table 3. Oil loss in Fiber

S. No.	Sample details	Petri Dish wt. (g)	PD+Sample wt. (wet) (g)	Sample wt. (wet) (g)	PD+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	%Oil OLWB
1	Control	45.506	54.963	9.457	50.366	4.86	107.5	108.9	1.38	101.6	101.6	0.007	104.5	104.5	0.005	1.392	14.72%
2	Enzyme B	39.973	50.184	10.211	45.306	5.333	102.5	103.9	1.408	102.6	102.6	0.008	102.6	102.6	0.008	1.424	13.95%



Average loss of oil in sludge

Fig 15 : Oil loss (Enzyme Vs Control) for sludge sample



Average oil loss in fibre.

Fig 16 : Oil loss (Enzyme Vs Control) for Fibre sample

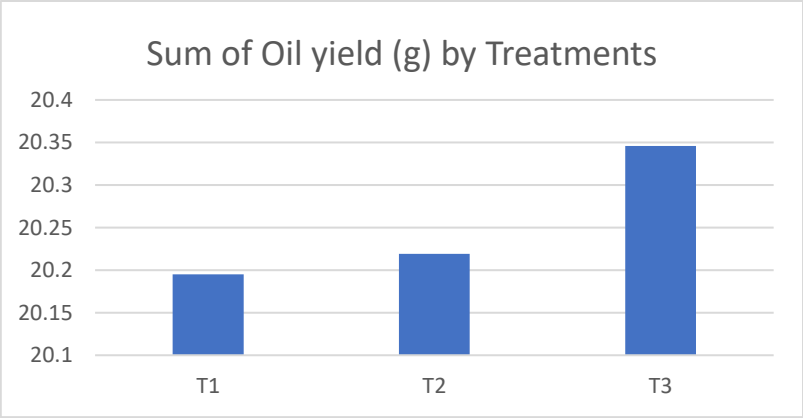
4.3. Rotor Test

Swinging bucket rotor and fixed angle rotor were used in the centrifugation process of the trial. Higher oil yield was observed in fixed angle rotor and oil recovery was easier. The pellet was strong and did not displace easily thereby facilitating easy removal of oil. Therefore, for downscale trial, fixed angle rotors were used. The sample amount used for the test was 50g per sample

Table 4. oil yield using swing bucket rotor

	Treatments	Oil yield (g)
Swing out rotor	T1	3.241
	T2	3.277
	T3	3.331
	T1	3.362
	T2	3.43
	T3	3.456
	T1	3.412
	T2	3.325
	T3	3.367
	T1	3.235
	T2	3.393
	T3	3.438
	T1	3.337
	T2	3.363
	T3	3.378
	T1	3.621
	T2	3.456
	T3	3.374

(T1- Control, T2-Proprietary enzyme A, T3-Proprietary enzyme B)



Graph 17. Oil yield using swing bucket rotor. (Wt. of oil in grams)

Table 5. oil yield using fixed angle rotor

	Treatments	Oil yield (g)
Fixed angle rotor	T1	3.308
	T2	3.543
	T3	3.4
	T1	3.436
	T2	3.387
	T3	3.398
	T1	3.274
	T2	3.417
	T3	3.312
	T1	3.337
	T2	3.383
	T3	3.39
	T1	3.337
	T2	3.391
	T3	3.321
	T1	3.371
	T2	3.401
	T3	3.283

(T1- Control, T2-Proprietary enzyme A, T3-Proprietary enzyme B)

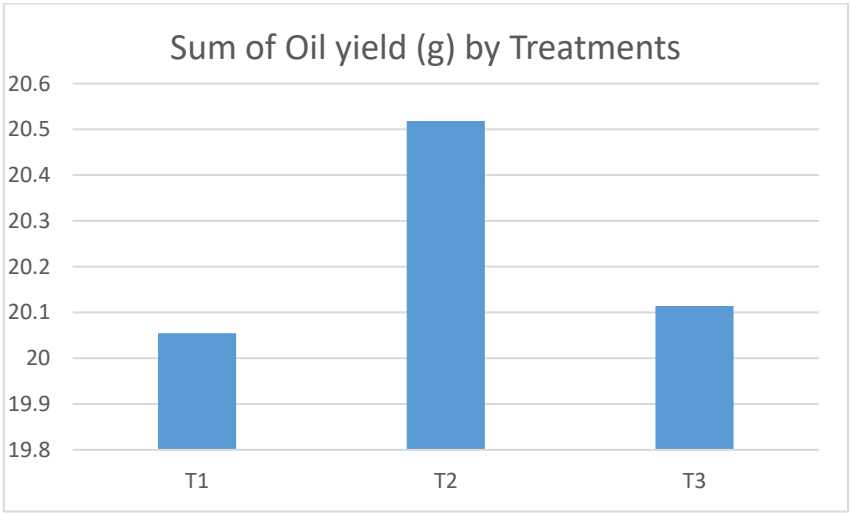


Fig 18. Oil yield using fixed angle rotor. (Wt. of oil in grams)

Table 6. Oil yield with respect to different G-force. (yield of oil given in grams)

Treatments	**Oil yield (g)**	**g force rcf (Centrifugation)**
T1	3.122	3200
T2	3.239	3200
T3	3.317	3200
T1	3.143	3200
T2	3.198	3200
T3	3.174	3200
T1	3.432	3200
T2	3.514	3200
T3	3.379	3200
T1	3.356	3200
T2	3.397	3200
T3	3.362	3200
T1	3.387	3200
T2	3.4	3200
T3	3.328	3200
T1	3.292	3200
T2	3.31	3200
T3	3.192	3200
T1	3.55	6300
T2	3.601	6300
T3	3.554	6300
T1	3.634	6300
T2	3.542	6300
T3	3.557	6300
T1	3.663	6300
T2	3.392	6300
T3	3.421	6300
T1	3.445	6300

T2	3.496	6300
T3	3.501	6300
T1	3.453	6300
T3	3.795	6300
T1	3.688	6300
T2	3.701	6300
T3	3.738	6300
T1	3.748	6300
T2	3.756	6300
T3	3.879	6300
T1	3.924	6300
T2	3.892	6300
T3	3.754	6300
T1	3.874	6300
T2	3.819	6300
T3	3.673	6300
T1	3.791	6300
T2	3.872	6300
T3	3.796	6300
T1	4.137	9600
T2	4.062	9600
T3	3.893	9600
T1	3.881	9600
T2	4.035	9600
T3	3.683	9600
T1	4.152	9600
T2	4.143	9600
T3	3.947	9600
T1	3.739	9600
T2	3.851	9600
T3	3.826	9600

(T1- Control, T2-Proprietary enzyme A, T3-Proprietary enzyme B)

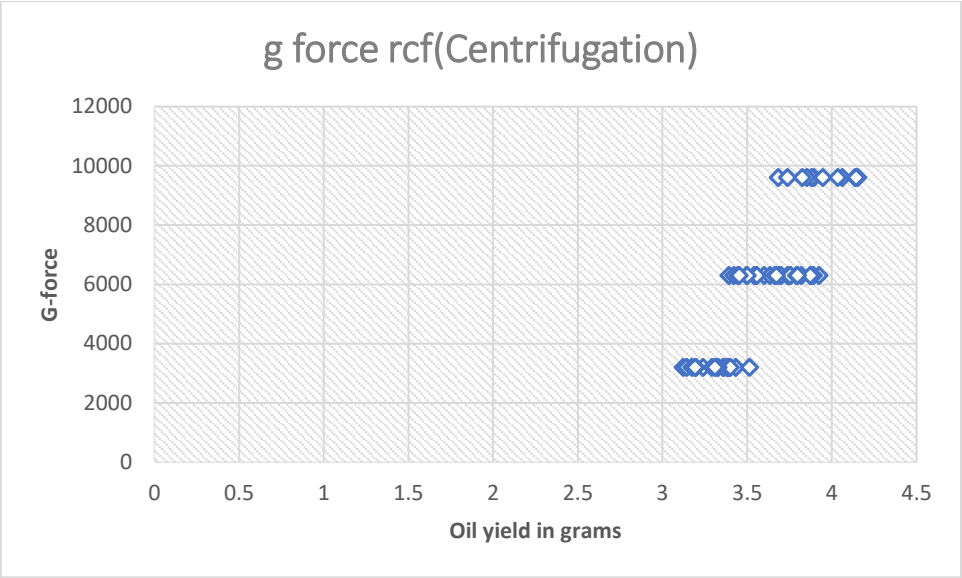


Fig 19. Oil yield with respect to different RCF

The oil yield suggests that the fixed angle rotor setup is giving higher yields at 50g scale of tests. The g-force analysis with respect to oil yield suggest that at 9600 g force the maximum yield was observed. Contrary to the above given result at higher amount of sample the ideal configuration is the swing bucket configuration. At the industrial scale the normal centrifuges are replaced with decanter centrifuge.

4.4. Microscopy

Microscopic examination of substrate before and after enzyme treatment was done.

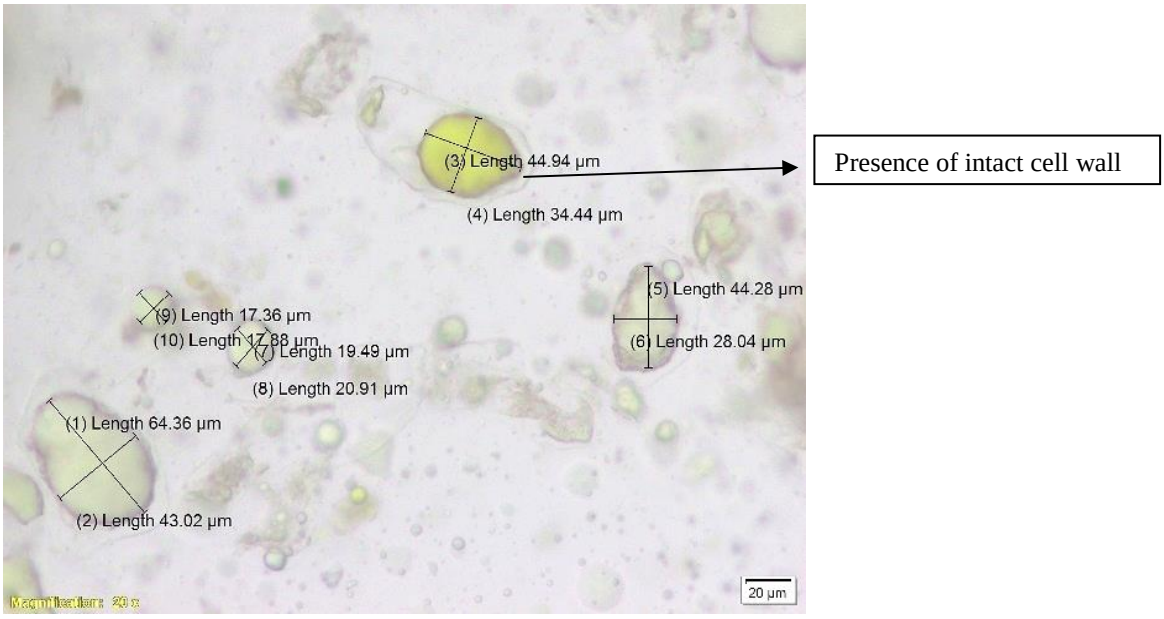


Fig 20. Control sample with intact cell wall.

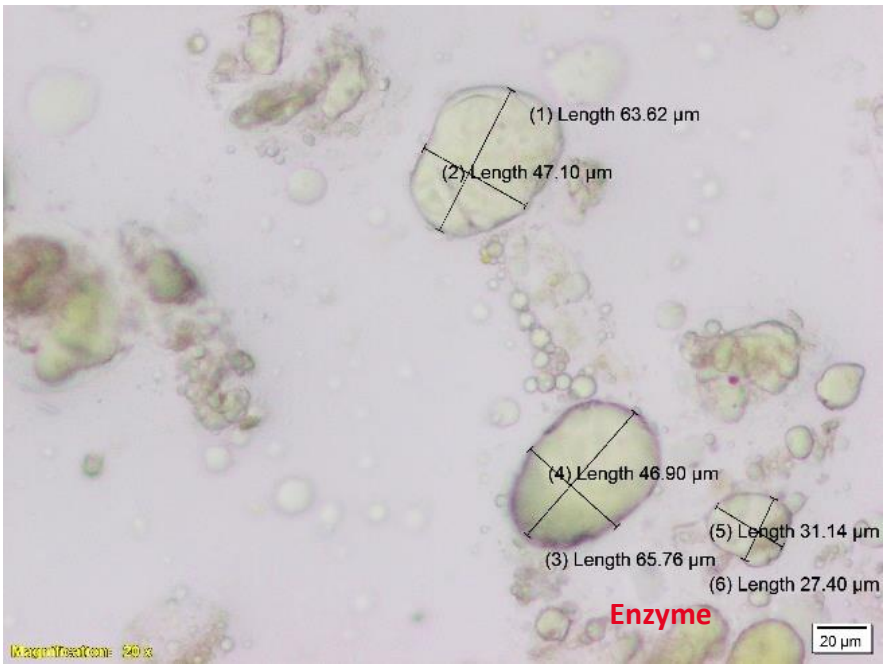


Fig 21. Enzyme treated sample which shows permeated cell wall.

The microscopic analysis shows the effects of enzyme degradation in the sample treated with enzyme. The cell walls appear to be permeated in the enzyme treated sample.

4.5 Vegetable oil mill effluent analysis for reducing sugar conc.

4.5.1 DNS assay

Table 7. standard solution conc. and O.D at 540 nm

STD. CONC of Glucose mg/ml	STD. O.D at 540 nm
0.2	0.103
0.6	0.313

INITIAL REDUCING SUGAR CONC. mg/ml
1.744

Table 8. Reducing sugar conc. for the first hour

First Hour	
Enzyme Conc. (w/w% of sample)	CONC. OF SUGAR (mg/ml)
0	1.725
0.01	2.256
0.1	4.505
1	15.693

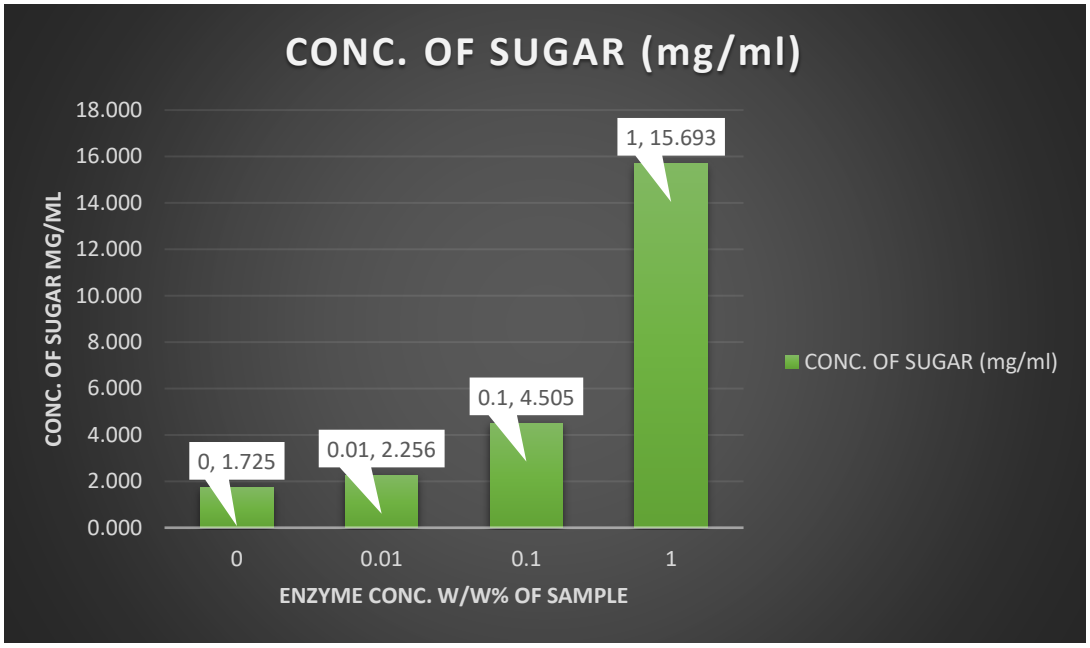


Fig 22. Reducing sugar conc. of First hour after incubation with enzyme.

Table 9. Reducing sugar conc. for the second hour.

Second Hour	
Enzyme Conc.(w/w% of sample)	CONC. OF SUGAR (mg/ml)
0	2.051
0.01	4.415
0.1	7.412
1	16.933

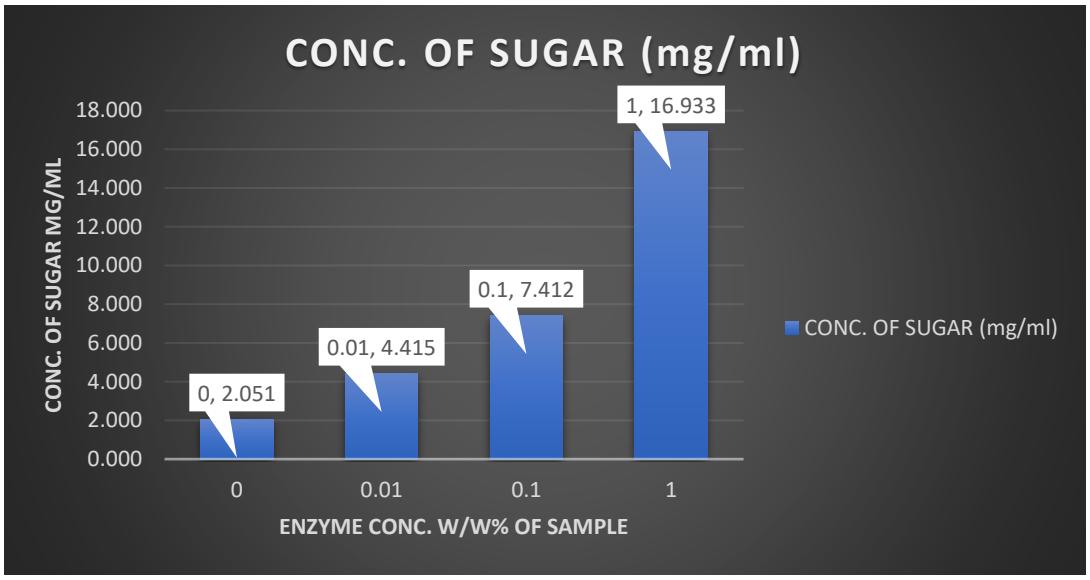


Fig 23. Reducing sugar conc. of second hour after incubation with enzyme.

Fourth Hour	
Enzyme Conc. (w/w% of sample)	CONC. OF SUGAR (mg/ml)
0	1.866
0.01	4.728
0.1	7.700
1	16.550

Table 10. Reducing sugar conc. for the fourth hour

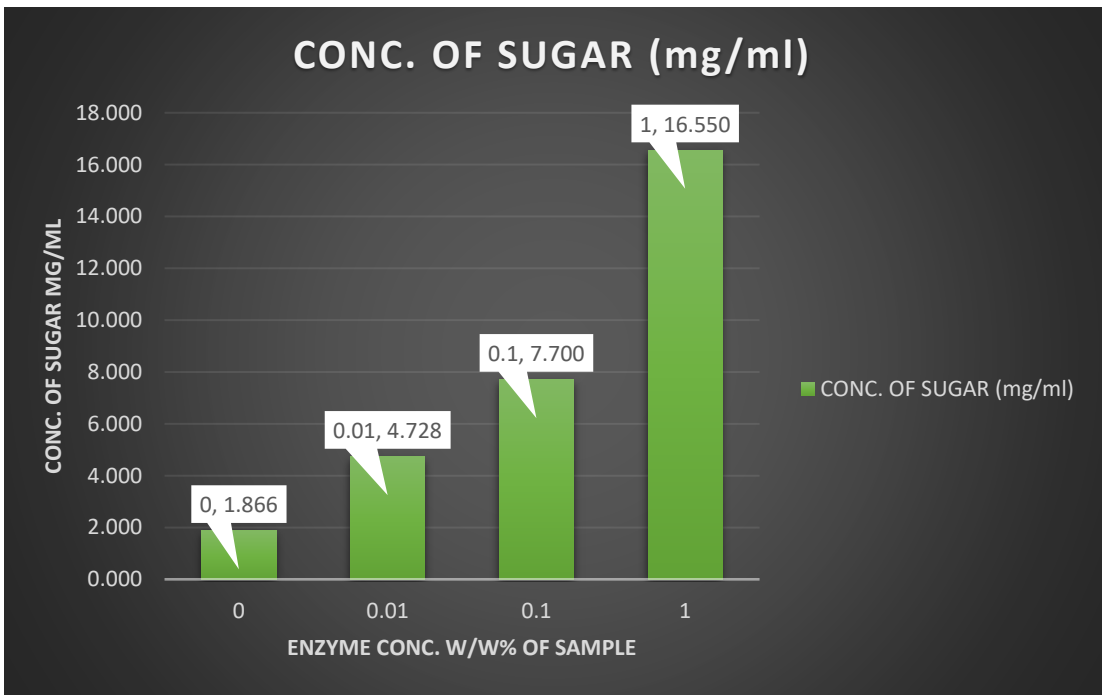


Fig 24. Reducing sugar conc. of fourth hour after incubation with enzyme.

Table 11. Reducing sugar conc. for the sixth hour

Sixth Hour	
Enzyme Conc. (w/w% of sample)	CONC. OF SUGAR (mg/ml)
0	2.032
0.01	5.802
0.1	7.942
1	16.741

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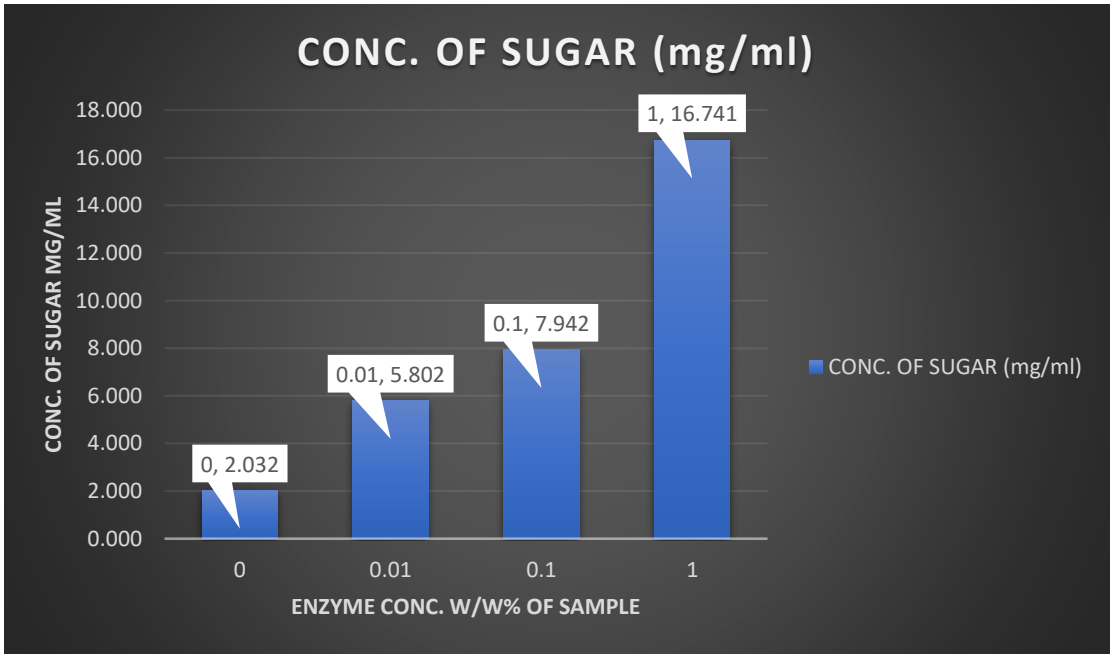


Fig 25. Reducing sugar conc. of sixth hour after incubation with enzyme.

Table 12. Reducing sugar conc. for the eighth hour.

Eighth Hour	
Enzyme Conc.(w/w% of sample)	CONC. OF SUGAR (mg/ml)
0	1.827
0.01	5.987
0.1	8.256
1	16.997

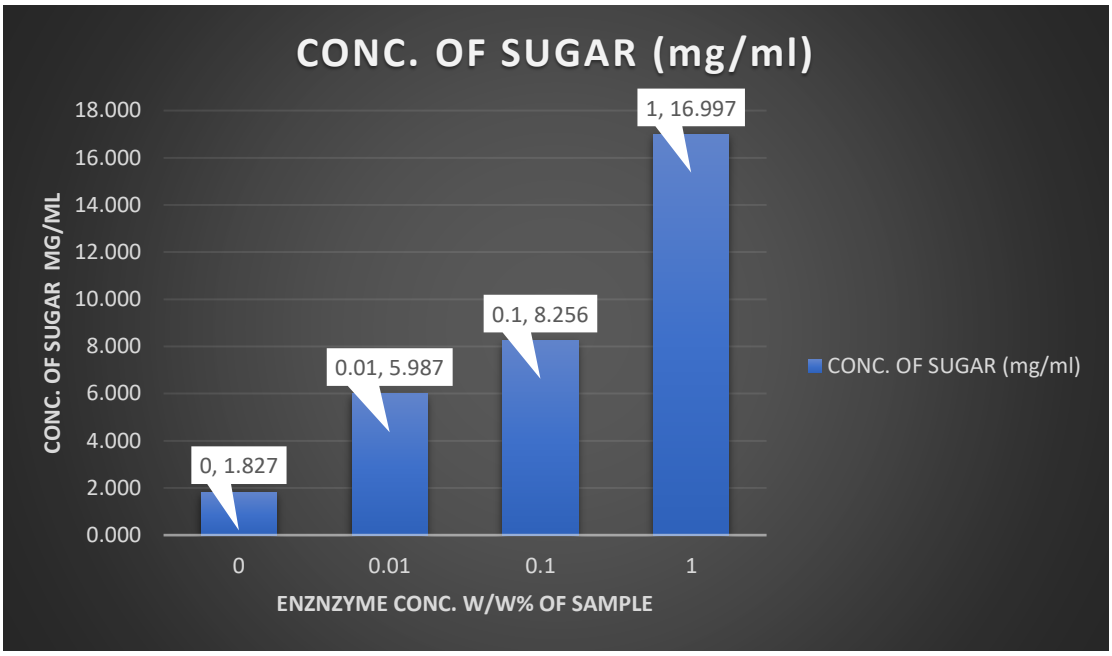


Fig 26. Reducing sugar conc. of eighth hour after incubation with enzyme.

Twenty Fourth Hour	
Enzyme Conc.(w/w% of sample)	CONC. OF SUGAR (mg/ml)
0	2.141
0.01	6.281
0.1	8.262
1	19.553

Table 13. Reducing sugar conc. for the twenty fourth hour

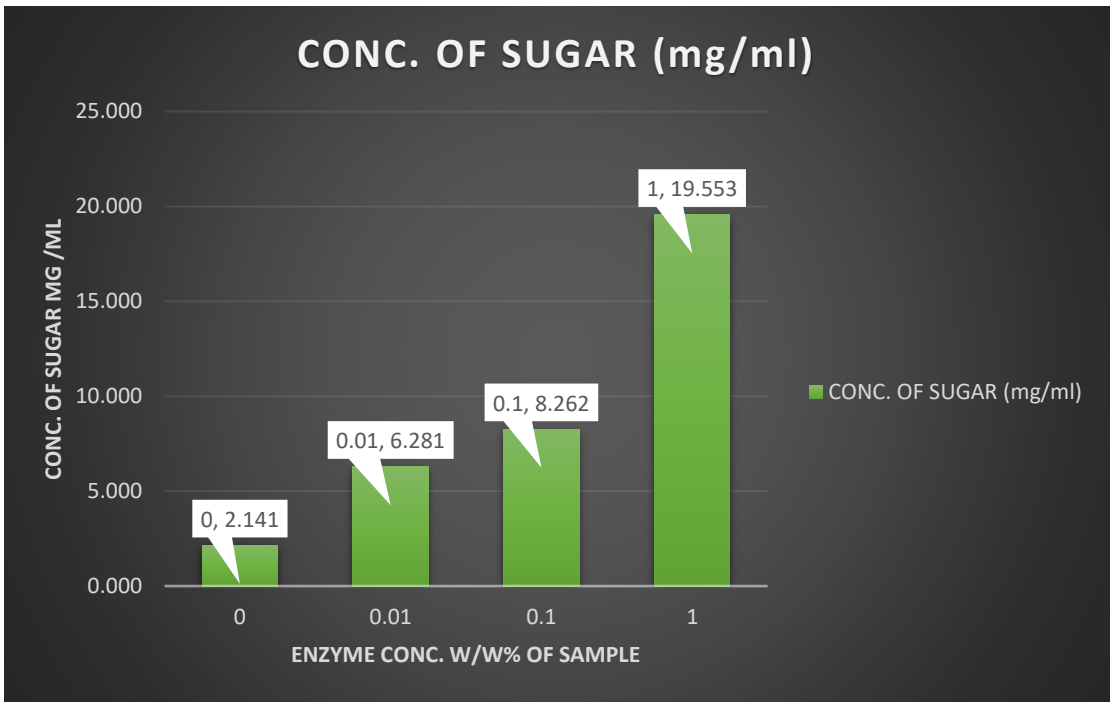


Fig 27. Reducing sugar conc. of twenty fourth hour after incubation with enzyme.

From the twenty-four-hour trial it was observed that the treatment with 1% w/w enzyme conc. reached a saturation in reducing sugar conc. by the second hour. The value observed was 16.5 mg/ml of reducing sugar conc. further incubation does not give any substantial rise to the reducing sugar conc. The activity of 1% w/w enzyme conc. was further resolved within 2 hours at 15 min intervals for better understanding the saturation conc.

2 Hourr Assay for 1% enzyme dosage	
Time of Incubation (min)	conc. of sugar mg/ml
0	13.610
15	16.601
30	17.061
45	15.144
60	15.527
75	16.102
90	15.911
105	15.719
120	15.719

Table 14. Reducing sugar conc. for 2 hours at 15 min intervals

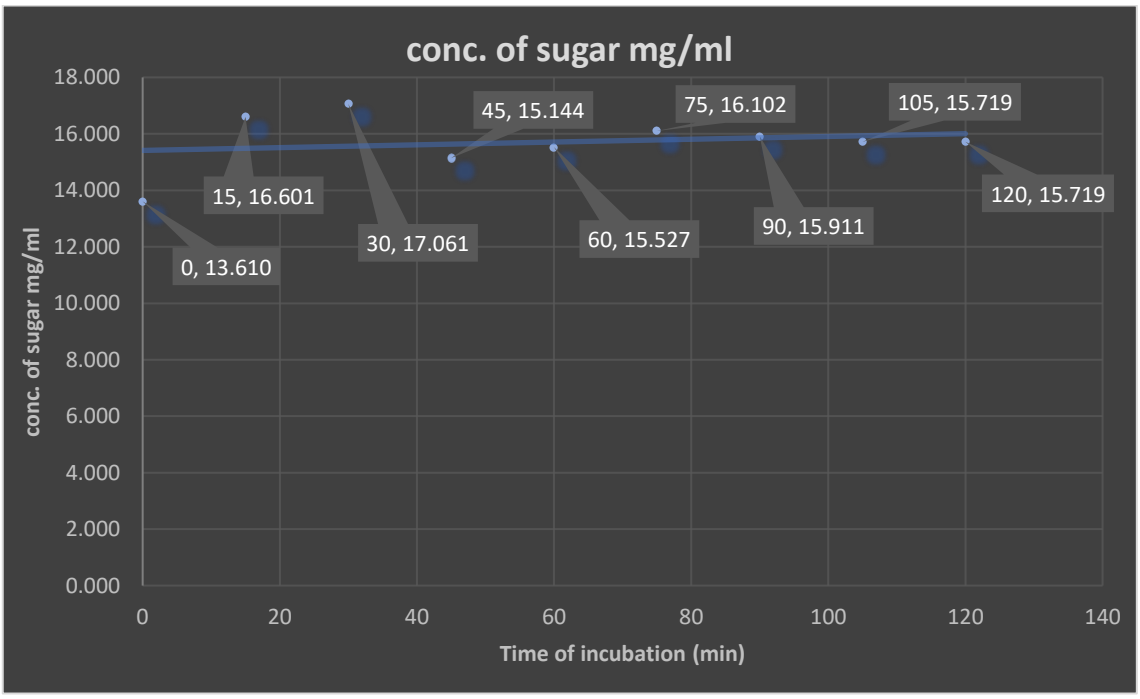
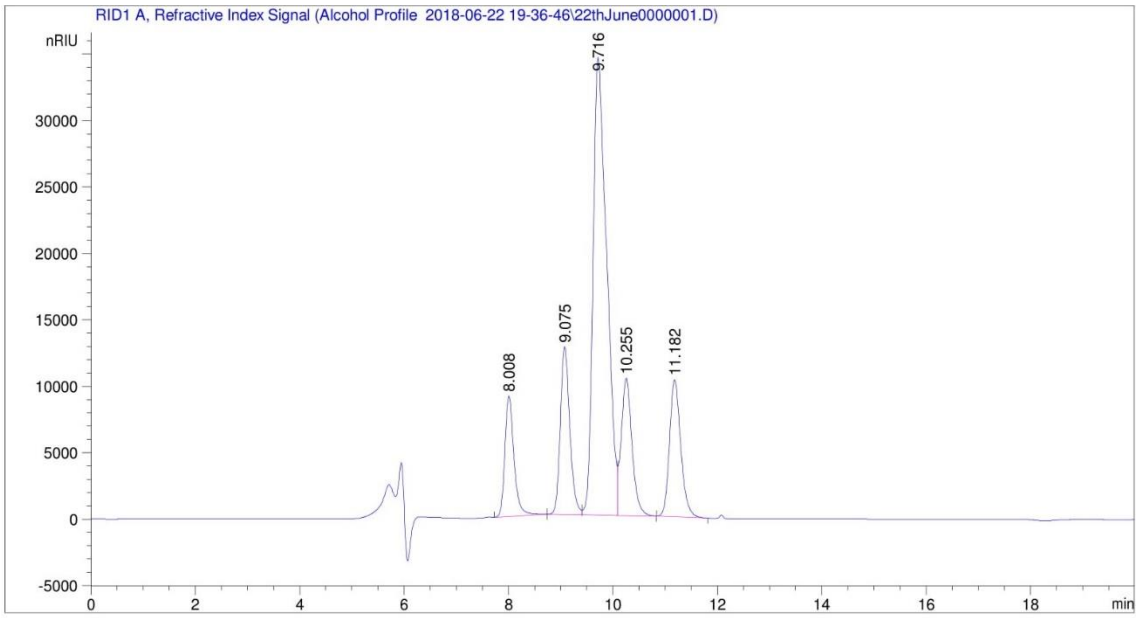


Fig 28. Reducing sugar conc. of two hour with 15 min interval check after incubation with enzyme.

4.5.2. HPLC

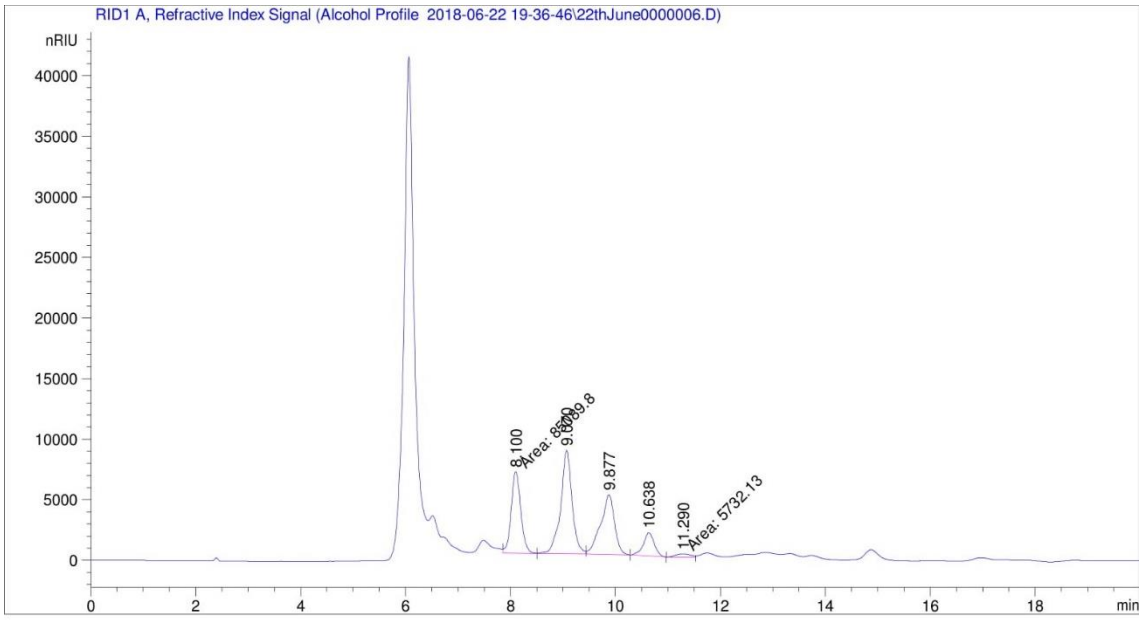
HPLC is done using Agilent infinity series HPLC unit with RID detector. The chromatography is isocratic, and the mobile phase used is 5mM H_2SO_4. The flow rate being 0.6mL/min. The column temperature is set at 60^oc and the RID detector is set at 55^0c temperature. The run time is for 20 min.



X-axis- Retention Time, Y-axis-RIU

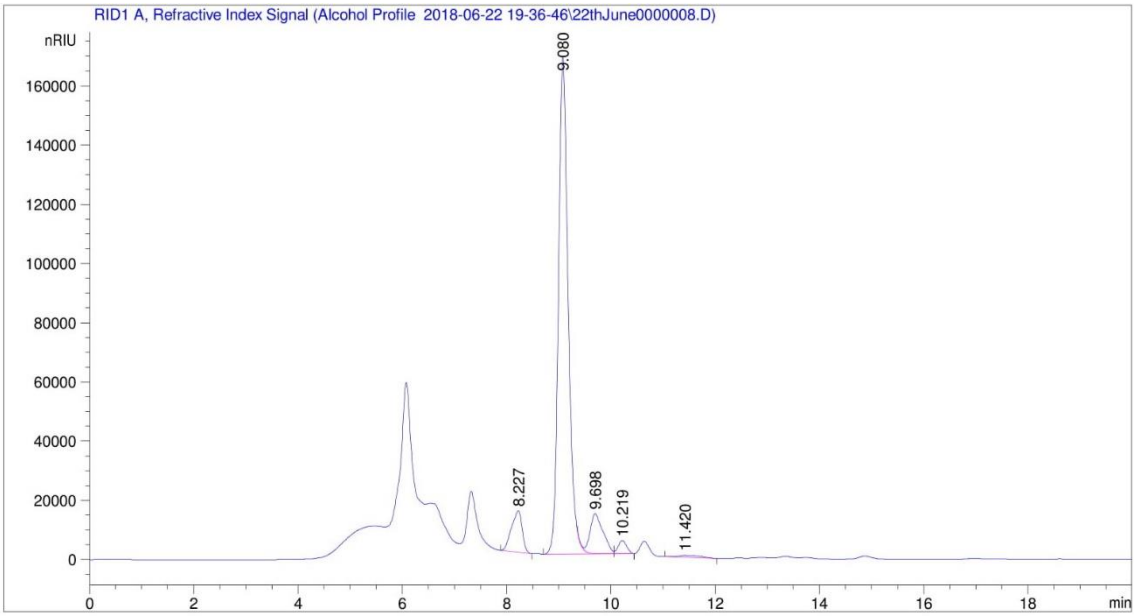
Fig.29 Reducing sugar standard chromatogram.

The peak at 9.075 min retention time represents the glucose peak. This value will be used in further samples to analyse the glucose concentration for that sample.



X-axis- Retention Time, Y-axis-RIU

Fig 30. Chromatogram for VOME sample before enzyme treatment (control).



X-axis- Retention Time, Y-axis-RIU

Fig 31. Chromatogram for VOME sample after enzyme treatment of 1% w/w conc. with an incubation time of 2 hours.

From the analysis of the chromatogram it is learnt that the initial concentration of glucose in the control sample before enzyme inoculation and incubation is less than that of glucose concentration after the treatment with enzyme in the VOME.

The analysis from both DNS assay and HPLC reveals that the glucose conc. (reducing sugar conc.) increases after treatment with enzyme in the VOME. This suggests that the

VOME treated with enzyme has better chance of being converted to biogas by methanogenic microbes due to abundance of easily available simple carbon source.

DISCUSSION

Aqueous enzyme oil extraction is very potent alternative to the conventional oil extraction techniques employed in the vegetable oil industry. The use of proprietary enzymes in degrading the cell wall to access the oil present in the fruits and seeds of oil crops has seen tremendous growth in the current age. Dose response studies on the substrate helps us in understanding the optimum dosage of an enzyme to show a minimum desired activity. It is crucial part of the research and development.

Other than the dose response study and analysing the oil yield microscopic analysis reveal the enzymatic degradation of the vegetable substrate further confirming the enzymatic activity. Even though enzymatic aqueous oil extraction is an effective and environment friendly industrial process, its efficiency is still less than that of solvent extraction. This decrease in efficiency is measured in terms of oil lost in fibre and sludge and this is calculated using Soxhlet process with hexane as solvent. Separation of oil from the extract is also a major step in oil downstreaming and this is done using centrifuges, for lab scale purposes normal centrifuges can handle the quantity but in an industrial scale this is carried out using decanter centrifuge. For experimental purposes at 50g scale of substrate fixed angle rotor configuration was better than swing bucket and at higher substrate amount the trend is opposite.

The effluent produced by the mill poses a serious threat to the environment and thus, it should be treated effectively before being discharged, the effluent is rich in organic matter and has high concentration of polysaccharides. These polymers if made into monomers can be utilized by the microbes easily and if the treatment is further done for Biogas production it becomes a source of renewable energy, that can help in meeting a fraction of the industry’s energy demands. After treatment with enzymes, the effluent is observed to have higher concentration of sugar monomers and this concentration of saturation is achieved within 2 hours if 1 % w/w of enzyme conc. is used.

5. FUTURE PROSPECTS

The use of enzymes in oil extraction dates to 1950's, the rise in demand of oil has pushed the conventional processes of oil extraction to their limit. The present scenario is also pushing the industries to adopt greener alternatives compared to their conventional practices. All these scenarios combined has played in favour of bringing Aqueous enzyme assisted oil extraction a global spotlight. The idea of more from less drives this and it is hypothesized that enzymes can be vital in achieving this goal. The future of enzyme assisted aqueous extraction will focus more on potent enzymes that are effective in very minor concentration, other than this the stability of the enzyme is also a major concern and genetically engineered thermo and pH stable enzymes are also relevant prospects. The use of enzyme in effluent treatment is an interesting new field of enzyme application and this study has focused on the preliminary stages of enzyme treatment of effluent for biogas production. Further studies should be done on mitigating further environmental issues using enzymes. They are also excellent for retrieving oil lost during the production thus, staying true to the idea of more from less.

APPENDIX

INTRODUCTION TO NOVOZYMES

Headquartered at Copenhagen, Denmark, Novozymes is the world leader in bioinnovation. The core business is industrial enzymes, microorganisms, and biopharmaceutical ingredients. Novozymes provide business-to-business biological solutions used in the production of numerous products such as biofuel, detergents, feed, and crops.

With over 700 products used in 130 countries, Novozymes bioinnovations improve industrial performance and safeguard the world's resources by offering superior and sustainable solutions for tomorrow's ever-changing marketplace. Novozymes' natural solutions enhance and promote everything, from removing trans fats in food to advancing biofuels to power the world tomorrow. The never-ending exploration of nature's potential is evidenced by over 6,500 patents, showing what is possible when nature and technology join forces.

RETHINK TOMORROW

A growing global population with a rising need for food, water, energy and other resources is pushing industries to get smarter and produce more with less. Novozymes supports this transformation by replacing conventional chemicals with enzymes and microorganisms, thereby helping reduce the water, energy and raw materials needed in many industrial processes.

In 1925 the brothers Harald and Thorvald Pedersen founded Novo Terapeutisklaboratorium with the aim to produce insulin. In the mid-1930s, Novozymes built the first office in Copenhagen, Denmark. In 1941, Novozymes launched its first enzyme, trypsin, extracted from the pancreas of animals and used to soften leather.

The company was the first to produce enzymes by fermentation using bacteria in 1952. In the late 1980s Novozymes presented the world's first fat-splitting enzyme for detergents manufactured with genetically engineered microorganisms. In the 2000s Novozymes expanded through acquisition of several companies focusing on business outside the core enzyme business. Amongst them were the Brazilian bioagricultural company Turfal and German pharmaceutical, chemical and life science company EMD/Merck Crop BioScience Inc. These acquisitions made Novozymes the largest player in sustainable solutions for the agricultural biological industry.

Food & Beverages

Today's food and beverage manufacturers are facing increasing demand for quality products with fewer additives and preservatives. Novozymes offers attractive possibilities for supporting these trends.

Household Care

Consumers are demanding higher performance and eco-efficiency from their cleaning products. Novozymes helps manufacturers to exceed these expectations with innovative enzymatic and microbial solutions.

Bioenergy

By providing the most advanced, dependable, and well-supported solutions available Novozymes helps customers to lead the way to a future powered by renewable energy.

Agriculture

Novozymes' biofertility, biocontrol and bioyield enhancers help farmers to achieve healthier crops and higher yields. Enzymes for animal nutrition help improve digestion processes and make the most of the feed while keeping feed costs low.

Wastewater Solutions

Novozymes is creating new opportunities for improvements in wastewater treatment with beneficial microorganisms.

Biopharma

Novozymes develops and manufactures high-quality, animal-free, regulatory compliant biological ingredients and technologies to help pharmaceutical and medical device manufacturers deliver improved performance and take products to market faster.

Textiles

Higher production costs, demand for quality textiles, and environmental legislation present challenges for the textile industry. Novozymes helps customers stay ahead of the market by providing revolutionary products and services.

Pulp & Paper

Novozymes' solutions save resources and costs while improving the quality, performance, and appearance of pulp and paper products.

Leather

For over 50 years Novozymes has been producing bioinnovative solutions for the leather industry to deliver high quality, reliability, sustainability, and process optimization.

NOVOZYMES IN INDIA

Over the years, Novozymes has emerged as the largest enzyme supplier in the country, catering to requirements across industries. We work with our customers to optimize their use of raw materials and energy, thereby reducing the environmental impact of their operations and help them make 'more from less'.

Today the region's operations cover India, Sri Lanka, Bangladesh, Nepal and Bhutan. Household care, textiles, food & beverages, oil & fats, starch, beverage alcohol, and leather are some of the key areas of growth for the company. Novozymes is also doing significant work in development of advanced biofuels in India.

Novozymes has a Solid Substrate Fermentation manufacturing plant at Bangalore which supplies pectinases used in the Juice industry for all of our Global and Regional market.

RESEARCH AND DEVELOPMENT IN INDIA

Novozymes started its R&D activities in India in 2006 in Bangalore. The center was created to play an important role in our efforts toward discovering new applications and technologies both globally and locally. Since then, the state-of-the-art center has grown to four departments, namely Strain and Process Development, Protein Engineering, Protein Assay and Technology and Application Technology. The center also serves as an application technology excellence center for Novozymes' global juice and wine industry requirements.

As Indian market is gradually developing, the need for development of specific local applications especially in the food, household care and beverage segment has increased. R&D is working on several unique projects for the Indian markets.

Novozymes India Facility in Bengaluru.

Enzymes used in various industrial segments and their applications:

Industry	Enzyme class	Application
Detergent	Protease	Protein stain removal
	Amylase	Starch stain removal
	Lipase	Lipid stain removal
	Cellulase	Cleaning, color clarification, anti-redeposition (cotton)
	Mannanase	Mannanan stain removal (reappearing stains)
Food & dairy	Protease	Milk clotting, infant formulas, flavour
	Lipase	Cheese flavour
	Lactase	Lactose removal
	Pectin methyl esterase	Firming fruit-based products
	Pectinase	Fruit based products
	Transglutaminase	Modify visco-elastic properties
Starch & fuel	Amylase	Starch liquefaction and saccharification
	Amyloglucosidase	Saccharification
	Pullulanase	Saccharification
	Glucose isomerise	Glucose to fructose conversion
	Xylanase	Viscosity reduction
	Protease	Protein degredation
Baking	Amylase	Bread softness and volume, flour adjustment
	Xylanase	Dough conditioning
	Lipase	Dough stability and conditioning

	Phospholipase	Dough stability and conditioning
	Glucose oxidase	Dough strengthening
	Lipoxygenase	Dough strengthening, bread whitening
	Protease	Biscuits, cookies
	Transglutaminase	Laminated dough strength
Animal feed	Phytase	Phytase digestibility (phosphorous release)
	Xylanase	Digestibility
	β-glucanase	Digestibility
Beverage	Pectinase	De-pectinization, mashing
	Amylase	Juice treatment, low calorie beer
	β-glucanase	Mashing
	Laccase	Clarification (juice), flavour (beer)
Textile	Cellulase	Denim finishing, cotton softening
	Amylase	De-sizing
	Pectate lyases	Scouring
	Catalase	Bleach termination
	Laccase	Bleaching
	Peroxidase	Excess dye removal
Pulp and paper	Lipase	Pitch control, contaminant control
	Protease	Biofilm removal
	Amylase	Starch coating, de-inking, drainage improvement
	Xylanase	Bleach boosting
	Cellulase	

		De-inking, drainage improvement, fibre modification
Fats & oils	Lipase Phospholipase	Transesterification De-gumming, lyso-lecithin production
Organic synthesis	Lipase Acylase Nitrilase	Resolution of chiral alcohols and amides Synthesis of semisynthetic penicillin Synthesis of enantiopure carboxylic acids
Leather	Protease Lipase	Unhearing, bating De-pickling
Personal care	Amyloglucosidase Glucose oxidase Peroxidase	Antimicrobial (combined with glucose oxidase) Bleaching, antimicrobial Antimicrobial

Edible oils are among the most abundant cooking ingredients in the world and form a big sector of the food industry. Production of enzymes for extraction of oils and fats is therefore one of the top priority projects at Novozymes, India. 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. Soybean, sunflower, rape and palm account for more than 70% of vegetable oils. These oils can be used for various cooking (frying, baking, dressing) or non-cooking (cosmetics, bio fuel) purposes and 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.

The solvent extraction method recovers almost all the oils and leaves behind only 0.5% to 0.7% residual oil 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. In the case of mechanical pressing the residual oil left in the oil cake may be anywhere from 6% to 14%. The solvent extraction method can be applied directly to any low oil content raw materials. It can also be used to extract pre-pressed oil cakes obtained from high oil content materials. Because of the high percentage of recovered oil, solvent extraction has become the most popular method of extraction of oils and fats. 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.

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