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Extraction of virgin coconut oil from the Testa free Albuminous endosperm through yeast mediated aqueous fermentation system: Fourier transform-Infrared Spectra (FT- IR)

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Abstract: Virgin coconut oil has been claimed to have numerous beneficial health effects. The present work is an improvised yeast fermentation method of oil extraction mediated through cellulase and pectinase enzyme. The protein profile analysis of skimmed milk was conducted for explaining nitrogen utilization through 1-D SDS PAGE analysis. The commercial oil and virgin coconut oil were subjected to Fourier Transform –Infra red Spectra analysis for examining the uniqueness of these oils. The present study results showed sound difference in the oil yield in the different combination of the enzyme (cellulase and pectinase) and yeast. The yeast inoculation facilitate 8 times oil yield than the control and similarly for cellulase and pectinase 7.5 and 7.3 respectively. In the combination of cellulase and pectinase the oil yield was 14 times higher than the control and 1.69 times higher than endosperm fermentation process supported with yeast. The 1-D SDS PAGE analysis of skimmed milk shows very high protein utilization in the combination of cellulase and pectinase. FT-IR spectra for virgin coconut oil and commercially available coconut oil extracted through conventional process showed sound difference between samples.

Keywords: FTIR spectra, virgin coconut oil, fermentation, yeast, enzymes, SDS -PAGE

I. INTRODUCTION

The coconut palm is a versatile plant with a variety of uses. Every part of it is useful to mankind. Traditionally coconut oil is extracted from the dried copra (endosperm or meat) of matured coconut through powerful hydraulic press with added heat. Virgin coconut oil (VCO) made from fresh coconuts, no copra. Since high temperatures and chemicals solvents are not used, the oil retains its naturally occurring state and phyto-chemicals which produce a distinctive taste and smell. Coconut oil is a vegetable oil obtained from the endosperm and abundant in lauric acid (Hui, 1996). German and Dillard (2004) cite the virtues of lauric acid of having antiviral, antibacterial, antiplaque and antiprotozoal functions. Nolasco, et al., (1994), found that coconut oil, trilaurin and tripalmitin inhibited the promotion stage of carcinogenesis. Aside from lauric acid, VCO contains a considerable amount of short-chain fatty acids such as capric, caproic and caprylic which were also investigated to have antimicrobial and antiviral effects (Bergasson et al., 1998). VCOs were produced using enzymatic, chilling and thawing, centrifugation, natural fermentation and induced-fermentation processes. Antioxidant activity of Refined Coconut Oil was significantly ($p < 0.5$) lower than those of VCO samples, with induced-fermentation having the highest antioxidant activity of 28.3% and enzymatic extraction resulted in higher quantity of short chain triglycerides (Nurah Tijani Oseni et al.,). The oil extracted through fermentation process is being practicing in a few countries including India for the production of high quality virgin coconut oil (VCO). The present work is an improvised method of oil extraction mediated through cellulase and pectinase enzyme.

II. MATERIALS AND METHODS

Healthy mature brown coloured coconut was freshly collected and de husked and brought to the laboratory for the present work. The whole endosperm was collected from the shell by scraping and care was taken not to include the testa, which has known antimicrobial activity. The weight of shell, whole grated coconut meat (endosperm), whole coconut water was taken separately for the further analysis. The whole coconut scrap and coconut water were divided into half by weight. The volume of coconut water was also measured. The two halves of coconut endosperm were scraped and pooled and half weight coconut meat was used for the milk extraction.

A. Preparation of enzyme solutions

- 1) *Pectinase*: Food grade pectinase enzyme 3,00,000 unit activity /gm was imported and 100mg pectinase solution was prepared in 100 ml distilled water.
- 2) *Cellulase*: Food grade cellulase enzyme 1,50,000 unit activity /gm was imported and 100mg cellulase solution was prepared in 100 ml distilled water.
- 3) *Yeast*: Dissolve 10 gm fresh baker's yeast in 100ml distilled lukewarm water

III.BATTERY EXPERIMENTAL SET UP

The scraped endosperm meat was grinded in a cleaned sterilized mixer grinder for 3 minutes with 250 ml of lukewarm water. This crushed coconut kernel was squeezed through sterilized muslin cloth for the extraction of high quality coconut milk. The collected milk was equally divided into 5 × 2 pre sterilized conical flasks (100ml). The first set of conical flask was provided with coconut milk and kept as control and the second set with coconut milk and inoculated with 10ml yeast extract and the third setup was inoculated 10 ml yeast and 0.1ml cellulase enzyme solution. To the fourth setup 10 ml yeast inoculum and 0.1 ml pectinase enzyme and to the 5th setup 10 ml yeast, 0.1ml cellulase and 0.1 ml pectinase enzyme respectively. The entire setups were plugged with sterilized cotton and transferred to CO₂ incubator. The temperature was set at 34°C and CO₂ tension was provided throughout the experiment.

The entire battery setups were allowed to incubate at 34°C for 2 days. The liquid portion (skimmed milk) is collected by siphoning into a beaker and used for the protein estimation by Lowry method and protein utilization by the yeast was determined by protein analysis (10% 1D – PAGE). The cream remained in the bottom of the conical flask was melted in the optimum temperature 60°C and the oil yield was estimated.

A. Protein Profile Analysis

100µl of coconut skimmed milk was transferred into eppendorf tubes contained 75µl chilled homogenization Tris buffer (pH 7.8) and homogenized using electric homogenizer. Homogenized sample was centrifuged at 10000 rpm for 20 minutes and subsequently 100µl volumes of supernatant sample was digested in 150µl loading buffer and digested samples were stored in deep freezer for SDS- PAGE analysis.

B. 1d Sds -Page

The digested samples were subjected to 1D SDS – PAGE analysis using promega protocols USA. Equal volume (50µl) digested samples along with standards (Sigma USA) were loaded in each well. Electrophoresis was performed at constant current of 15mA in stacking gel and 30mA in separating gel. After electrophoresis, gels were fixed and stained using Coomassie brilliant blue R 250 and subsequently destained till the complete removal of background stains. Destained gels were stored in 12% glycerol still documentation. Stained gels were documented using BIO – RAD molecular image Gel doc (GS 800), USA. Acquired gel image were subjected to further analysis such as molecular weight calculation comparing of tract with control profile were done using quantity one software.

C. Oil Estimation By Incubation Method

After removing the skimmed milk, cream was transferred to oven set at a temperature 60°C for 2 hours and quantity of oil yield were measured.

D. Infrared Spectral Analysis

The virgin coconut oil (VCO) prepared through aqueous fermentation processes and commercially available coconut oil extracted through the conventional methods were subjected to the infrared spectral analysis using Fourier Transform- Infrared Spectra (FT-IR), FT/IT-4100 JASCO UK Limited accredited to ISO 9000:2000. The oil samples were grinded with a quantity of specially purified salt (potassium bromide) finely. This powder mixture is then pressed in a mechanical press to form a translucent pellet through which the beam of the spectrometer can pass. The FTIR transmission fixed for the present work ranging from 349-7800 cm⁻¹. Spectral property were examined in the samples including the strength of stretching absorption due to aldehyde (C=O), esters (C-O), bending absorption (methylene (CH₂) and methyl (CH₃) groups and double bond absorptions (C=C) and hydration level were estimated.

IV.RESULTS

The present study results showed sound difference in the oil yield was noticed in the different combination of the enzyme (cellulase and pectinase) and yeast. The yeast inoculation facilitate 8 times oil yield than the control and similarly for cellulase and pectinase 7.5 and 7.3 respectively. In the combination of cellulase and pectinase the oil yield was 14 times higher than the control and 1.69 times higher than endosperm fermentation process supported with yeast.

The results are included in the Table 1& 2 and Fig .1

TABLE I
TABLE SHOWING THE VIRGIN COCONUT OIL YIELD PRODUCTION

Total weight of the mature coconut	523.59 gm
Weight of the coconut water	56.28 gm
Volume of the coconut water	52 ml
Weight of the coconut albuminous endosperm (meat)	377.31 gm
Weight of the shell	90 gm
Density of coconut water gm/cm ³	1.08231
Total weight of the coconut scrap used for the milk extraction	94.32 gm
Total percentage of milk yield in wet condition by weight in gm	89.06 %
Average density of coconut oil extracted through the fermentation process 0.83765gm/cm ³	
Average density of commercially available coconut oil extracted through conventional process 0.2953gm/cm ³	

The average density difference between virgin coconut oil and the commercially available coconut oil extracted through conventional process were studied in the present work and the results showed sound difference 0.83765 and 0.2955 gm/cm³ respectively. The average density was 2.84 times higher in virgin coconut oil over commercially available coconut oil extracted through conventional process. This may be due to the presence of all the sugars, vitamins, minerals, amino acids and phyto hormones present in the fresh testa free albuminous endosperm.

Table Ii
Battery Experimental Setup Showing The Enzyme Combination And Oil Yield

Battery experimental setup	Cost of virgin oil/coconut in Indian				
	Oil yield /mature coconut				
	Oil yield/kg wet endosperm in ml				
	Oil yield in ml				
	60	1.20	3.18	25.44	480
				24.12	455
				23.32	440
				16.21	810

Sl No	Trial condition	Net weight of meat in gm	Amount of water	Yeast inoculum in 100mg/ml	Cellulase enzyme 150units/ml	Pectinase enzyme 300units/ml
1	Coconut milk (control)	18.864	30	0	0	0
2	Coconut milk	18.864	30	1	0	0
3	Coconut milk	18.864	30	1	1	0
4	Coconut milk	18.864	30	1	0	1
5	Coconut milk	18.864	30	1	1	1

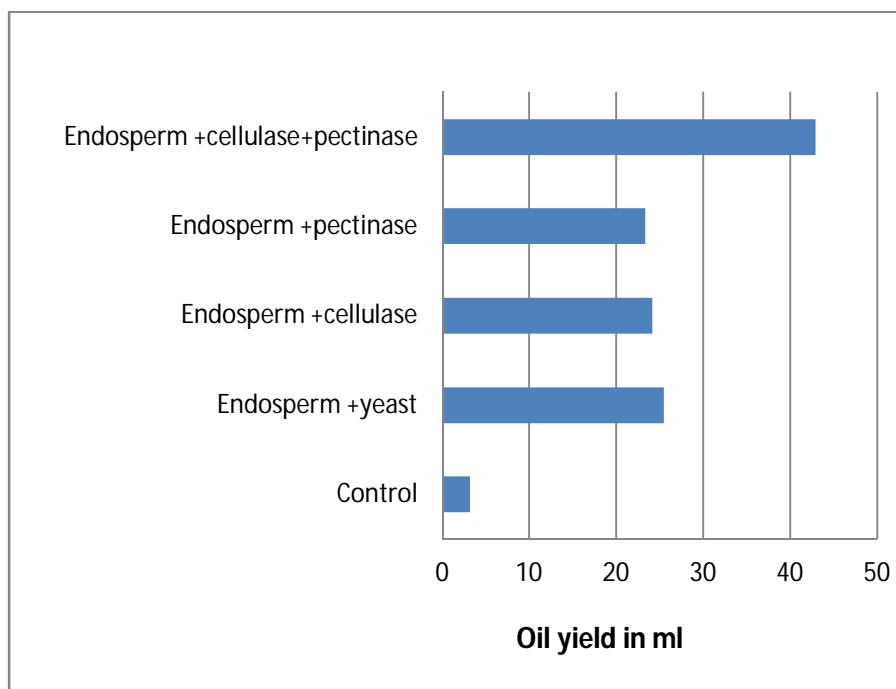


Fig. 1. Battery experimental setup showing the enzyme combination and oil yield

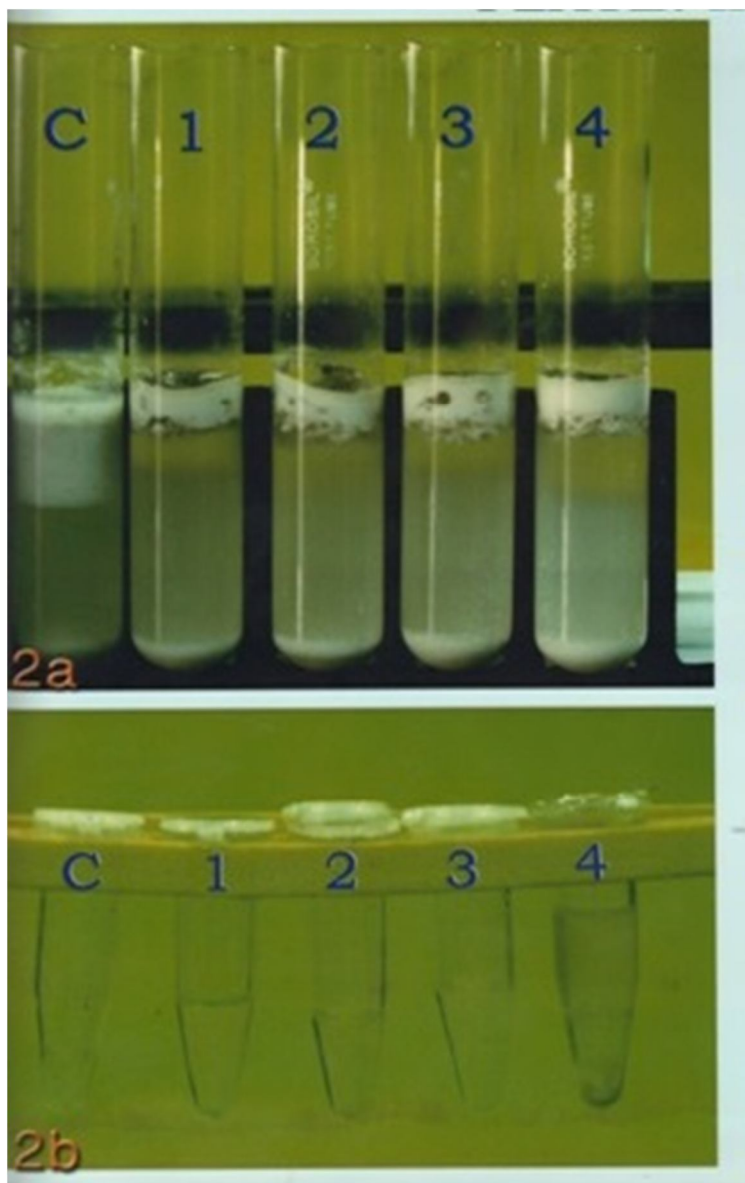


Fig.2a Showing experimental setup of fermentation process

A. Control

- 1) Coconut milk + Endosperm + Yeast
- 2) Coconut milk + Endosperm + Yeast + cellulase
- 3) Coconut milk + Endosperm + Yeast + pectinase
- 4) Coconut milk + Endosperm + Yeast + cellulase + pectinase

Fig.2b Showing oil yield

B. Control

- 1) Endosperm + Yeast
- 2) Endosperm + Yeast + cellulase
- 3) Endosperm + Yeast + pectinase
- 4) Endosperm + Yeast + cellulase + pectinase

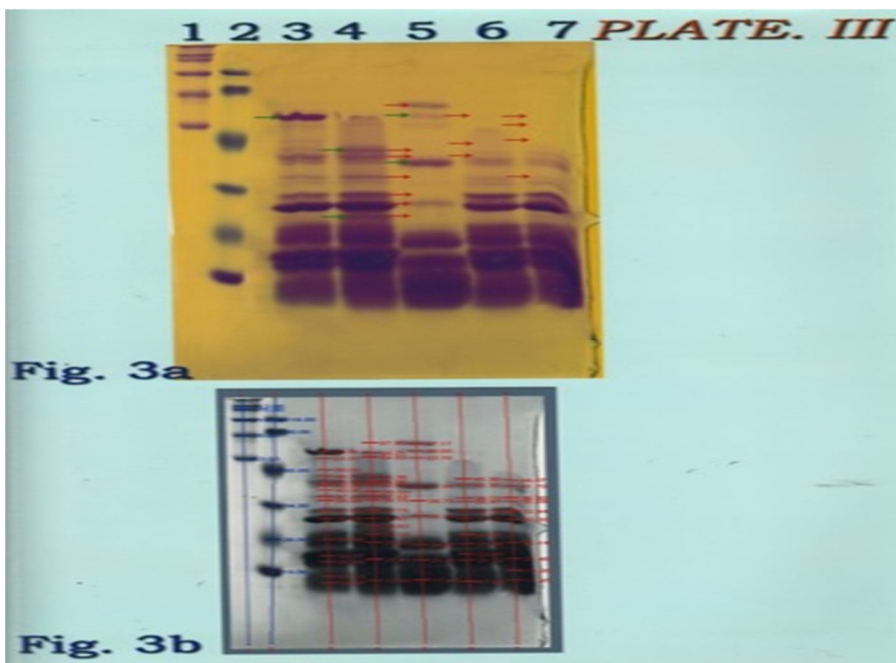


Fig .3a Electropherogram of protein profile of coconut skimmed milk

- C. Molecular standard protein I (weight in kDa)
- 1) Molecular standard protein II (weight in kDa)
- 2) Control
- 3) Endosperm + Yeast
- 4) Endosperm + Yeast + cellulase
- 5) Endosperm + Yeast +pectinase
- 6) Endosperm + Yeast + cellulase + pectinase

Fig .3b Electropherogram of protein profile of coconut skimmed milk showing the molecular weight in kDa , Quantity One data

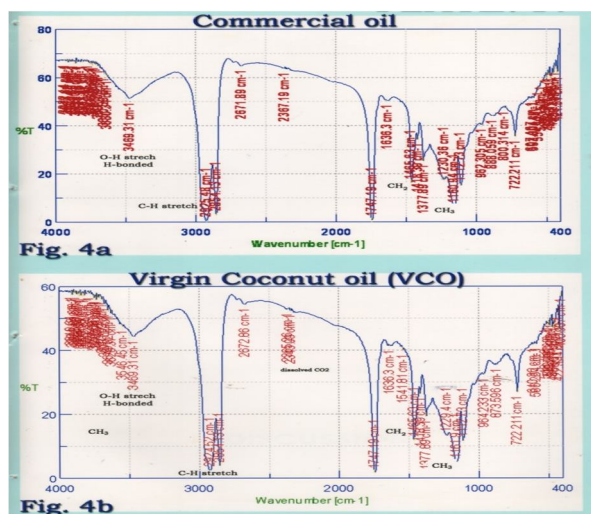


Fig. 4a Fourier Transform Infrared Spectra of Commercially Available Oil

The electropherogram of 1-D protein profile of skimmed milk showed elicitation of new proteins and disappearance of higher molecular proteins from the yeast and enzyme mediated battery fermentation system, Fig .2. The profile of yeast treated set up showed expression of 3 new proteins with apparent molecular weights 67kDa, 28kDa and 27kDa and over expression of 46kDa, Fig.2. These proteins may be yeast contributed profile and the disappearance of high molecular protein 59kDa may due to the yeast

cells utilization. The battery experiment set up equipped with yeast and cellulase showed expression of a few new proteins like 67,41, and 31kDa. This combination also caused the disappearance of many proteins (50,46,43,40,37,32,25,22,18kDa). Similarly in yeast and pectinase combination caused very sound difference between other profiles. The appearance of 26,24kDas and disappearance of proteins (50,53,50,46,40,25,18kDas) were noticed in this set up. The yeast , cellulase and pectinase combination caused heavy protein utilization with disappearance of many proteins (50,53,50,46,40,22,18kDas). The increased utilization of protein as nitrogen source for the growth of yeast cells and produce better oil yield i.e., 14 times difference than the control.

C. Fourier Transform-Infrared Spectra

FT-IR spectra for virgin coconut oil and commercially available coconut oil extracted through conventional process showed sound difference between samples .Generally FT IR spectra show five important peaks explaining the stretching ,bending and double bond absorptions of the oil samples. We observe the stretching absorption of (O-H) at $3680-3450\text{cm}^{-1}$ shows water content of the coconut oil sample. This stretching (O-H) absorption is intermolecular hydrogen bonding of water. Both sample showed the presence of absorption between $3680-3450\text{cm}^{-1}$ which indicate the presence of water in both sample. The observation of C-H stretching absorption occurs at 2925cm^{-1} methylene aliphatics. Two alkanes peaks which is attributed the bending absorption of methylene and methyl groups appears at 1465 and 1375cm^{-1} respectively. Two peaks observed at 1740 and 1160cm^{-1} are due to stretching absorption of aldehyde (C=O) and esters (C-O) respectively . The presence of very unique peak observed at 2343cm^{-1} in virgin coconut oil extracted through fermentation process shows the presence of dissolved CO_2 , a promising chemical marker for the evaluation of degree of fermentation.

V.DISCUSSION

Fermentation as a method of facilitating oil extraction has been reported by several workers (Alexander. 1921a,b;Beckman.1930; Horovitz-Vlasova and Novotelnov,1935). These scientists observed that if the emulsion is allowed to ferment, the oil and protein fractions separate. However, they did not specify whether the enzymes or acid produced or the combination of these along with, perhaps, other factors elaborated by the microorganisms are responsible for breaking the emulsion. Production of virgin coconut oil by induced fermentation with *Lactobacillus plantarum* NDRI strain 184 was studied (Neela satheesh N.B.L Prasad). Y.C. Wong and H. Hartina studied Virgin Coconut Oil production by centrifugation method. The purpose of the fermentation or enzymatic processes is to make the coconut emulsion into unstable condition and therefore easily to separate into oil phase on upper layer and carbohydrate , protein and water phase on below layer . The strain of *Lactobacillus bulgaricus* could effectively extract the virgin coconut oil higher than the other tested microbial strains when it was employed into the coconut cream under the enzymatic fermentation condition at pH 5.0 , 45°C and 5% starter concentration (Rini handayani et al ., 2008). The protein utilization during fermentation process is promising tool for the making of low caloric edible oil. The electrophoretic profile analysis of skimmed milk showed considerable incidence of protein and can be add with livestock food enhancement and value addition. The average density of virgin coconut oil and commercially available coconut oil extracted through the traditional process were estimated in the present . The results were 0.83765 and 0.2953 gm/cm^3 respectively. Similar results were already reported VCO and Virgin Olive oil extracted through the fermentation process i.e., 0.893 and 0.863 gm/cm^3 (Mahmood et al .,2009). Infrared spectroscopy has been widely used as an analytical technique in investigations of edible oils. FT- IR spectra of virgin coconut oil were estimated in the present work and showed very promising results with many future scopes. The contamination and adulteration of oil can be detected at commercial angle. The spectrum of virgin coconut oil and virgin olive oil shows very unique peak profile (Mahmood et al .,2009). About 6 peak were identified both in virgin coconut oil and virgin olive oil, which represented explaining the stretching absorption due to aldehyde (C=O) and esters (C-O), bending absorption (methylene and methyl groups and double bond absorptions (C=O).The presence of water shows a peak between $3680-3450\text{cm}^{-1}$ (Mahmood et al .,2009). However in the present work the FTIR spectra of virgin coconut oil and commercially available coconut oil extracted through the traditional process showed sound variation in the peak profile. The presence of very unique peak observed at 2343cm^{-1} in virgin oil extracted through fementation process was totally absent in the commercially available coconut oil. The presence of this peak is may be due to the presence of dissolved CO_2 . The peak observed at 2343cm^{-1} is a promising chemical marker for the estimation of degree of fermentation.

VI. SUMMARY AND CONCLUSION

Virgin coconut oil (VCO) is made from the fresh endosperm of mature coconut through cold extraction process like fermentation , centrifugation etc, and no sun exposure and totally devoid of chemical solvents. Since high temperatures and chemicals solvents are not used , the oil retains its naturlly occurring state and phytochemicals which produce a distinctive taste , smell and quality. The

present work is an attempt made to improvise oil extraction mediated through cellulase and pectinase enzymes. High quality virgin coconut oil was prepared from the testa free albuminous endosperm of mature coconut through aqueous fermentation process. This is “green technology” demands the least expenditure of power and space, very minimum infra structure and maintenance cost, less skill and overall process completed with very brief time. The present study results showed sound difference in the oil yield between different combinations of the enzyme (cellulase and pectinase) and yeast. The yeast inoculation facilitate 8 times oil yield than the control and similarly for cellulase and pectinase 7.5 and 7.3 respectively. In the combination of cellulase and pectinase the oil yield was 14 times higher than the control and 1.69 times higher than endosperm fermentation process supported with yeast. The average density difference between virgin coconut oil and the commercially available coconut oil extracted through conventional process were studied in the present work and the results showed sound difference 0.83765 and 0.2955 gm/cm³ respectively. The 1-D PAGE analysis of skimmed milk shows very high protein utilization in the combination of cellulase and pectinase. This can be adopted for the production of low calorie edible oil production. Many higher molecular proteins 50, 53, 50, 46, 40, 22, 18 kDas were disappeared for this set up. FT-IR spectra for virgin coconut oil and commercially available coconut oil extracted through conventional process showed sound difference between samples. We observe the stretching absorption of (O-H) at 3680-3450 cm⁻¹ shows water content of the coconut oil sample. The observation of C-H stretching absorption occurs at 2925 cm⁻¹ methylene aliphatics. Two alkanes peaks which is attributed the bending absorption of methylene and methyl groups appears at 1465 and 1375 cm⁻¹ respectively. Two peaks observed at 1740 and 1160 cm⁻¹ are due to stretching absorption of aldehyde (C=O) and esters (C-O) respectively. The presence of very unique peak observed at 2343 cm⁻¹ in virgin coconut oil extracted through fermentation process shows the presence of dissolved CO₂, a promising chemical marker for the evaluation of degree of fermentation. It can be concluded from the results of the study that high quality virgin coconut oil (VCO) can be produced through the fermentation with better yield, the endosperm after extraction of milk and skimmed milk after fermentation can be used as feed substitute for livestock, the presence of very unique peak observed at 2343 cm⁻¹ in virgin coconut oil extracted through fermentation process shows the presence of dissolved CO₂, a promising chemical marker for the evaluation of degree of fermentation. The future work aimed to improvise the nutrient status of virgin coconut oil (VCO) by the incorporation of coconut water in the fermentation system.

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