Isolation of microorganism from the fermented coconut milk and comparison of quality parameters of virgin coconut oil recovered by induced fermentation.

T. T. Thanuja, G. S. Sreekala and K.S. Meenakumari.
Department of Plantation Crops and Spices, College of Agriculture, Vellayani, Kerala Agricultural University, Thiruvananthapuram - 695522, Kerala, India.

Abstract
Investigations were carried out at College of Agriculture, Vellayani, Kerala Agricultural University,Kerala, India to produce virgin coconut oil through induced fermentation method during the period 2013-2015. Coconut milk was extracted from grated coconut with coconut water in the ratio 1:1 and was kept for 24 and 36 hours for fermentation and the microorganisms were isolated and screened. Lactobacillus isolated at 24 hours showed significantly superior protease and amylase activity compared to other microorganisms. Lactobacillus was multiplied and one percent of the culture was used to induce fermentation of coconut milk. The virgin coconut oil recovered from induced fermentation was then compared with the natural fermentation method and was found to be significantly superior compared to natural fermentation method. Coconut milk extracted from grated coconut with water in the ratio 1:1, incubated with one percent Lactobacillus and kept for 36 hours showed significantly superior oil recovery compared to other treatments. Quality parameters like free fatty acid content, total phenolic content and moisture content did not differ significantly between treatments and was within the limits of APCC Standards. Thus induced fermentation can be taken as a method for the hygienic production of water clear virgin coconut oil.

Introduction:
Virgin coconut oil is a traditional product of India and enhanced utilization world wide was witnessed in the recent years due to its multifunctional uses. Virgin coconut oil (VCO) is obtained from fresh and mature kernel (12 months old from pollination) of the coconut (Cocosnucifera L.) by mechanical or natural means with or without the application of heat, which does not lead to alteration of the nature of the oil(APCC, 2003). Virgin coconut oil can be extracted from the fresh and mature kernel of the coconut meat by several methods (Bawalan and Chapman, 2006). These methods can be divided into wet and dry methods. In wet method, the coconut meat does not undergo drying process while in dry method, the kernel were heated under specific conditions to remove the moisture in it while preventing scorching and microbial invasion. Wet method can be further divided into chilling and thawing, fermentation, enzymatic method or any of these in combination as the main aim is to destabilize the coconut milk emulsion (Raghavendra and Raghavaraao, 2010). In dry method, the kernel was dried using controlled heating and subsequently pressed mechanically to obtain the oil. Coconut milk is the natural oil in water emulsion extracted from the endosperm of mature coconut. If properly diluted coconut milk is allowed to stand under favourable conditions for several hours, the oil naturally separates from the water and protein that binds them together as coconut milk emulsion. The coconut milk when kept for some time result in fermentation. The natural enzymes produced by the microorganismsare responsible for fermentation of coconut milk. The technique if not hygienically practiced can cause contamination. In traditional natural fermentation, settling and subsequent of coconut milk lasted for 36-48 hours( Bawalan and Chapman, 2006). To overcome this, induced fermentation could be done using probiotic microorganisms having proteolytic, amylolytic and lipolytic capacities which could hydrolyse protein,
carbohydrate and lipid components contained in the coconut kernel. Riniet et al. (2009) observed that the strain of Lactobacillus bulgaricus could effectively extract more virgin coconut oil than the other microbial strains like Saccharomyces cerevisiae, Candida rugosa and Aspergillus oryzae when it was introduced into coconut cream under enzymatic condition at pH 5.0, 45°C and at 5 percent starter concentration. Kumalaningsih and Masdiana (2012) reported the proteolytic and amylolytic enzyme activity of Lactobacillus and the potential of using the culture for the production of high quality virgin coconut oil. Satheesh and Prasad (2013) concluded that Lactobacillus plantarum NDRI-184 as the best choice for induced fermentative production of virgin coconut oil.

The quality standards for virgin coconut oil has been put forward by Asian and Pacific Coconut Community Standards (APCC, 2003). The various factors which determine the quality of oil include moisture content, free fatty acids, peroxide content as well as the total plate count. Hydrolytic rancidity in coconut oil had been attributed to the presence of free fatty acids (Fernandez, 1988). A high acid value may indicate a higher tendency to become rancid (Karim, 1997). Moisture content of oil should be kept low as it will increase the shelf life by Preventing oxidation and rancidity processes (Mansore et al., 2012).

Phenolic compounds plays an important role providing protection against pathogens and predators (Bravo, 1998). The beneficial effects derived from phenolic compounds had been attributed to their antioxidant activity (Heim et al., 2002). The traditional method of virgin coconut oil in India is by boiling method. The coconut milk extracted is heated to remove the moisture and the solid particles can be separated by sieving. The temperature involved in traditional method is often so high that it will decrease the antioxidant activity of the virgin coconut oil (Marina et al., 2009). More over the peroxide value of cold extracted virgin coconut oil was lower than the hot extracted virgin coconut oil and was observed as 4.95 and 5.65 meq O₂/kg oil, respectively which increased as a function of storage time in all samples up to 12 months of storage (Srivastava et al., 2013).

Hence a study was conducted to isolate microorganisms responsible for inducing fermentation and to compare the physico chemical properties of the virgin coconut oil produced by induced fermentation with the standards specified by APCC.

Materials and methods:
Isolation of microorganisms:
Mature nuts of coconut cultivar, West Coast Tall (WCT) of 12 month were harvested from Trivandrum district of Kerala, India stored for two months and were dehusked and shelled. The solid endosperm was grated and ground in the grinder and coconut milk was extracted from the ground mass with water in the ratio 1:1. The ground mass was then transferred to cheese cloth and pressed manually for coconut milk extraction. The coconut milk extracted was kept for 24 and 36 hours for fermentation and microorganisms were isolated by serial dilution and spread plate method. Isolated microorganisms were tested for clear zone formation and quantitatively for amylase (Sadasiyam and Manikam, 1996) and protease (Enyard, 2008) activity. The microorganism having the highest protease and amylase activity was selected and kept in slants. Mother culture of the selected microorganism was multiplied in broth. To study the multiplication efficiency of the best isolate, it was inoculated in sterilized coconut milk.

Colony count:
The coconut milk extracted with grated coconut and water in the ratio 1:1 was sterilized at 70°C for 30 minutes. 1ml of mother culture of the best isolate was inoculated into the sterilized coconut milk and colony count was taken.

Induction of fermentation:
One ml of mother culture of the best isolate was inoculated into sterilized 100 ml of coconut milk extracted from grated coconut with coconut water in the ratio 1:1, water in the ratio 1:1 and 1:2 and kept for varying hours viz., 18, 24 and 36 hours and the oil recovery of the induced fermentation was compared with the fermentation method and the quality parameters were analysed.

Quality parameters:
The oil recovery was calculated according to the initial weight of coconut milk to the oil extracted after 24, 36 and 48 hours of fermentation and dried at 50°C (AOAC, 1997). Moisture percentage was determined by hot air oven method (AOAC, 1997) with slight alteration. Samples were heated at 50°C in a heated and weighed crucible and cooled to a room temperature and re-weighed with the samples inside until constant readings were obtained. Free fatty acid value, peroxide value, iodine value and saponification value were determined following the methods of...
Sadasivam and Manikam (1992). Total phenolic content of the oil samples was determined according to the procedure of Sadasivam and Manikam (1992) with some modifications by Ramma et al., 2002 using catechin as standard. Refractive index (Ariponnammal, 2012), relative density (ISO/FDS 6883, 2000), specific gravity (AOAC, 2000) and unsaponifiable matter (AOAC, 2000) were also found out. Polenske value of the oil was determined as per ISI (1984). Statistical analysis was carried out using analysis of variance technique (Gomez and Gomez, 1984).

Results and discussion:-

Isolation and screening of microorganisms:-

The microorganisms were isolated from the fermented coconut milk extracted from coconut gratings and water in the ratio 1:1 and kept at 24 and 36 hours. There was a significant difference in the population of isolated microorganisms from coconut milk extracted with coconut gratings and water in the ratio 1:1 and kept for fermentation at 24 and 36 hours after fermentation. The maximum bacterial population of $4.46 \times 10^6$ per ml was observed at 24 hours after fermentation compared to 36 hours. However, there was no significant difference in colony forming units of Lactobacillus and yeast isolated. Soeka et al. (2008) reported that the fermentation of coconut cream occurred when the enzymatic starter had been employed for processing. Crude coconut oil was formed due to a phenomenon of protein digestion that played a role to stabilize emulsion of the coconut cream into a soluble material. The enzymatic starter with high capacity of amylolytic and proteolytic activity could hydrolyse carbohydrate and protein which contained in the coconut cream as its substrate into soluble sugar and amino acid and peptide.

Protease and amylase activity:-

Protease and amylase activity of microorganisms isolated was determined. Lactobacillus isolated at 24 hours showed significantly superior protease (184.200µg tyrosine released in 2 hour) and amylase (26715.000µg of maltose produced during 5 minutes) activity compared to other treatments. The lower protease and amylase activity was observed for yeast isolated at 36 hours. According to Kumalaningsih and Masdiana (2012) lactic acid bacteria have highest proteolytic and amylolytic enzyme activity which indicated that lactic acid bacteria has the potential to be used as pure culture for the production of virgin coconut oil of high quality oil. Lactobacillus isolated at 24 hours was used as the mother culture. According to Satheesh and Prasad (2012) the inoculum concentration of 1, 2 and 5 percent of Lactobacillus plantarum of virgin coconut oil revealed 5 percent as more efficient with an efficiency of 82.91 percent compared to one percent inoculum concentration which showed an efficiency of only 65.67 percent.

Count of Lactobacillus:-

The Lactobacillus count increased with time and maximum population of $9.4 \times 10^4$ cfu per ml was noticed after 36 hours. A higher population at 36 hours showed that the activity was maximum at that period compared to 18 and 24 hours and hence the maximum recovery of oil at that period can be attributed to its higher population at that period.

Quality parameters:-

The effect of induced fermentation and fermentation on oil recovery was analysed. Oil recovery by induced fermentation and fermentation method showed significant difference. The virgin coconut oil recovered from induced fermentation method was significantly superior (17.73 percent) compared to fermentation method (17.3410 percent). Among treatments, the treatment coconut milk extracted from grated coconut with water in the ratio 1:1 and kept for 36 hours (21.780 percent) showed significantly superior oil recovery compared to other treatments.

Free fatty acid content of virgin coconut oil recovered from coconut milk extracted with coconut gratings and coconut water in the ratio 1:1 and water in the ratio 1:1 and 1:2 and inoculated with 1 per cent of Lactobacillus culture and kept for fermentation for 18, 24 and 36 hours and dried at 50°C did not show any significant difference between treatments. High free fatty acid in the oil is an indication of change caused by oxidation resulting in the rancidity of the oil. The fatty acids were in the range of 0.200 to 0.233mg KOH/ g of oil in the treatments. Thus the results were of indication that extraction of virgin coconut oil by any of the methods followed would not alter the free fatty acid content of the virgin coconut oil. Free fatty acid value of virgin coconut oil recovered by adding microbial inoculum (L. bulgaricus) as enzymatic starter was recorded as 0.22 percent (Riniet et al., 2009).
The total phenolic content of virgin coconut oil recovered from coconut milk extracted by different methods and inoculated with one per cent *Lactobacillus* culture did not differ significantly. This might be because of low temperature (50° C) which was used for drying.

The moisture content of virgin coconut oil did not differ significantly among treatments. Moisture content of virgin coconut oil recovered by adding microbial inoculum (*L. bulgaricus*) as enzymatic starter was recorded as 0.30 percent (Rini et al., 2009).

The quality parameters like moisture content, specific gravity, relative density, acid value, peroxide value, iodine value, polenske value, unsaponifiable matter, colour and odour of virgin coconut oil produced by induced fermentation method was found out to be within the range of APCC standards (2003).

**Plate 1. Plate showing growth of yeast and *Lactobacillus***

- a) Yeast
- b) *Lactobacillus*

**Plate 2. Microscopic view of stained *Lactobacillus* and yeast separated from fermented coconut milk**
Fig. 1. Protease activity of microorganisms isolated

![Protease activity chart](chart1)

Fig. 2. Amylase activity of microorganisms isolated

![Amylase activity chart](chart2)
Fig. 3. Count of *Lactobacillus* in coconut milk at different hours of fermentation under sterile conditions

Table 1. Microorganisms isolated from coconut milk fermented at 24 and 36 hours

| Time  | Bacteria \(\times 10^6\text{ cfuml}^{-1}\) | *Lactobacillus* \(\times 10^3\text{ cfuml}^{-1}\) | Yeast \(\times 10^3\text{ cfuml}^{-1}\) |
|-------|------------------------------------------|---------------------------------------------|------------------|
| 24h   | 44.60                                    | 33.12                                       | 5.41             |
| 36h   | 38.10                                    | 37.00                                       | 5.96             |
| SE    | 2.25                                     | 2.24                                        | 0.48             |
| CD(0.05) | 4.74                                     | NS                                           | NS               |

Table 2. Effect of induced fermentation and fermentation on oil recovery (%)  

| Treatments | Oil recovery (percent) | Induced fermentation (Group1) | Fermentation (Group 2, Control) | Mean |
|------------|------------------------|-------------------------------|---------------------------------|------|
| T₁ (CG +CW 1:1 + 18h) | 16.000 | 15.567 | 15.780 |
| T₂ (CG +CW 1:1 + 24h) | 18.833 | 18.467 | 18.650 |
| T₃ (CG+ CW 1:1 + 36h) | 21.300 | 21.067 | 21.180 |
| T₄ (CG +W 1:1 + 18h) | 16.567 | 15.733 | 16.150 |
| T₅ (CG + W 1:1 + 24h) | 19.167 | 18.933 | 19.050 |
| T₆ (CG+ W 1:1 + 36h) | 21.967 | 21.600 | 21.780 |
| T₇ (CG+W 1:2 + 18h) | 13.633 | 12.933 | 13.280 |
| T₈ (CG +W 1:2 + 24h) | 15.200 | 15.400 | 15.300 |
| T₉ (CG +W 1:2 + 36h) | 16.933 | 16.367 | 16.650 |
| Mean | 17.733 | 17.341 | - |

CD (Groups) 0.322
CD (Treatments) 0.152
CD (Induced fermentation Vs fermentation ) 0.456
Table 3. Effect of induced fermentation on quality parameters of virgin coconut oil

| Treatments                  | Free fatty acid (mg KOH/g of oil) | Total phenolic content (mg catechin equivalent /kg of oil) | Moisture content (%) |
|-----------------------------|-----------------------------------|------------------------------------------------------------|----------------------|
| T1 (CG + CW1:1 + 18h)      | 0.220                             | 57.666                                                     | 0.066                |
| T2 (CG + CW1:1 + 24h)      | 0.226                             | 66.000                                                     | 0.080                |
| T3 (CG+ CW1:1 + 36h)       | 0.226                             | 60.000                                                     | 0.080                |
| T4 (CG + W 1:1 + 18h)      | 0.200                             | 65.000                                                     | 0.073                |
| T5 (CG + W 1:1 + 24h)      | 0.226                             | 48.333                                                     | 0.053                |
| T6 (CG+ W 1:1 + 36h)       | 0.200                             | 62.333                                                     | 0.073                |
| T7 (CG+W 1:2 + 18h)        | 0.220                             | 63.666                                                     | 0.073                |
| T8 (CG +W 1:2 + 24h)       | 0.233                             | 71.000                                                     | 0.073                |
| T9 (CG +W 1:2 + 36h)       | 0.226                             | 67.333                                                     | 0.073                |
| SE                          | 0.045                             | 7.816                                                      | 0.025                |
| CD(0.05)                   | NS                                | NS                                                         | NS                   |

Table 4. Comparison of essential composition of virgin coconut oil produced by induced fermentation with APCC standards

| Parameter                             | Desirable Amount (APCC Standard, 2003) | VCO by induced fermentation method |
|---------------------------------------|----------------------------------------|-----------------------------------|
| Moisture (percent)                    | Max 0.1                                | 0.073                             |
| Free Fatty Acid (percent)             | Max 0.2                                | 0.020                             |
| Peroxide Value meq/kg                 | Max 3                                  | 0.220                             |
| Relative density                      | 0.915 – 0.920                          | 0.917                             |
| Refractive index at 40º C             | 1.4480 – 1.4492                        | 1.468                             |
| Saponification Value                  | 250 – 260 milligram                    | 262.428                           |
| Iodine Value                          | 4.1 -11                                | 5.380                             |
| Unsaponifiable matter (percent) by mass, max | 0.2 - 0.5                           | 0.398                             |
| Specific gravity at 30 deg./30 deg. C | 0.915 – 0.920                          | 0.917                             |
| Polenske Value, min                   | 13                                     | 13                                |
| Total Plate Count                     | < 0.5                                  | 0                                 |
| Colour                                | Water clean                            | Water clear                       |
| Odour and Taste                       | Natural fresh coconut scent, free of sediment, free from rancid odour and taste | Natural fresh coconut scent, free of sediment, free from rancid odour and taste |
| Food Additives                        | None permitted                         | Not added                         |

Conclusion:-
In the present study the isolation and screening of microorganisms involved in production of virgin coconut oil from fermented coconut milk was carried out. The *Lactobacillus* culture showed maximum proteolytic and amylolytic activity and hence one percent of the *Lactobacillus* culture was used for inducing fermentation. The oil recovery was significantly superior in the induced fermentation method compared to natural fermentation method and the moisture content, free fatty acid content, peroxide value, iodine value, polenske value, specific gravity, unsaponifiable matter, colour and odour were within the range specified by the APCC standards and hence can be induced fermentation can be recommended as a suitable method for the virgin coconut oil production.

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