



## Ozone technology to enhance quality of Nipa palm (*Nypa fruticans* Wrumb.) products for community enterprise

Noppadon Podkumnerd<sup>1\*</sup>  Kosin Teeparuksapun<sup>1</sup>  Supraanee Wunsri<sup>1</sup>   
Nicha Prasongchan<sup>1</sup>  Vakin Nunumvong<sup>1</sup>  Soemsak Sunyano<sup>2</sup> 

<sup>1</sup>Department of General Education, Faculty of Liberal Arts, Rajamangala University of Technology Srivijaya, 90000, Songkhla, Thailand. E-mail: Noppadon.p@rmutsv.ac.th. \*Corresponding author.

<sup>2</sup>Department of Architecture and Urban Planning, Faculty of Architecture, Rajamangala University of Technology Srivijaya, Songkhla, Thailand.

**ABSTRACT:** The application of ozone as a treatment system for reducing microbial contaminant in Nipa bowls which are local products of the Palian river basin community, Trang province in Southern Thailand, was presented in this research. The ozone treatment system was designed and investigated for its performance to reduce microbial contaminant in nipa bowl products. Parameters affecting the performance of the system were optimized as well as ozone amount and treatment time. Under optimum condition (600 mg/h ozone and treatment time of 4 hours), the microbial decontamination was 4 log reduction, and the products could be stored for one month. The moisture content and the brightness of the ozone-treated products were significantly different from the untreated products when statistically tested at 95% confidence level. The moisture contents were  $9.46 \pm 0.10$  and  $10.54 \pm 0.31$  %, and the brightness ( $L^*$  value) were  $74.93 \pm 0.49$  and  $70.47 \pm 0.65$  for the ozone-treated and untreated products, respectively. Furthermore, the residual heavy metals were investigated, and no trace of metals was reported in nipa bowl samples. As a result, the nipa bowl products had met the standards as regards safety of food containers required by the Department of Medical Sciences Ministry of Public Health, Thailand. Following this collaboration between the university and the community, over 251,564 pieces of nipa bowls were sold which generated a total income of more than 84,000 USD to the Palian river basin community.

**Key words:** ozone treatment, nipa palm, nipa bowl product, food container, Palian river basin.

## Emprego de ozônio para melhorar a qualidade da palmeira Nipa (*Nypa fruticans* Wrumb.) e sua comercialização

**RESUMO:** A aplicação do ozônio como um sistema de tratamento para reduzir o contaminante microbiano em Nipa bowl-a produtos locais da comunidade da bacia do rio Palian, província de Trang no sul da Tailândia, é apresentada neste trabalho. O sistema de tratamento de ozônio foi projetado e investigado por seu desempenho na redução de contaminantes microbianos em produtos de palmeira. Os parâmetros que afetam o desempenho do sistema foram otimizados, incluindo a quantidade de ozônio e o tempo de tratamento. Em condições ótimas (600 mg de ozônio e tempo de tratamento de 4 horas), a descontaminação microbiana foi de 100% e os produtos puderam ser armazenados por um mês. As propriedades físicas dos produtos tratados com ozônio foram significativamente diferentes dos produtos não tratados quando testados estatisticamente no nível de confiança de 95%. Os conteúdos de umidade foram  $9,46 \pm 0,10$  e  $10,54 \pm 0,31\%$ , e o brilho (valor  $L^*$ ) foram  $74,93 \pm 0,49$  e  $70,47 \pm 0,65$  para produtos tratados e não tratados com ozônio, respectivamente. Além disso, os metais pesados residuais foram investigados e nenhum traço de metais foi encontrado nas amostras da palmeira Nipa. Como resultado, os produtos da palmeira Nipa atenderam aos requisitos de segurança de recipientes de alimentos do Departamento de Ciências Médicas do Ministério da Saúde Pública da Tailândia. Após esta colaboração entre a universidade e a comunidade, mais de 251.564 peças de taças de palmeira nipa foram vendidas, gerando uma renda total de mais de US \$ 84.000 para a comunidade da bacia do rio Palian.

**Palavras-chave:** tratamento com ozônio, palma nipa, produto nipa bowl, recipiente para alimentos, Bacia do rio Palian.

## INTRODUCTION

Increasing awareness of environmental pollution has led to the development of new forms of food packaging to replace the unsustainable consumption of plastic materials (CHISENGA et al., 2020; IVANKOVIĆ et al., 2017; POPA et al., 2011).

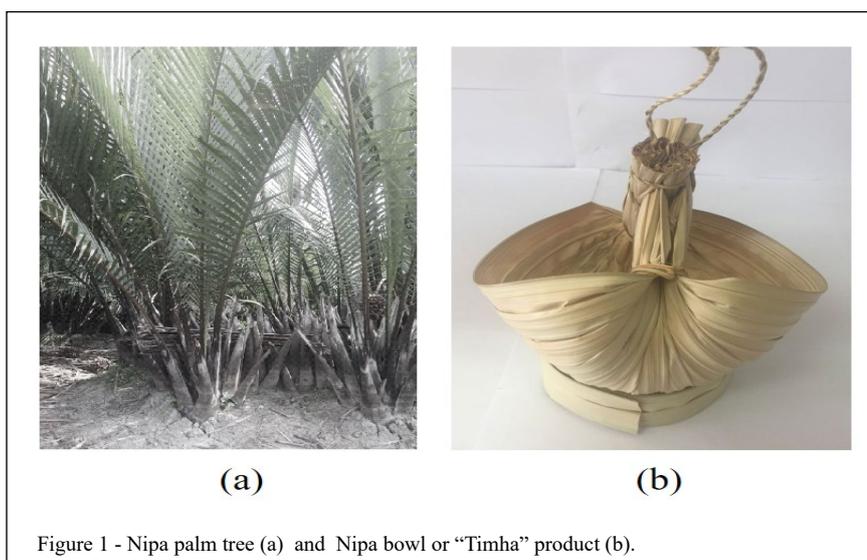
Plastics is currently one of the biggest challenges the world is facing and which is a “critical” issue for retailers and shoppers (MUSA et al., 2013; NIELSEN et al., 2020). People are now highly concerned about plastic packaging. Thus, food and drink manufacturers should prioritize making all their packaging recyclable and environmental-friendly.

One alternative food container is natural leaves food bowl products such as nipa bowls which can replace plastic or foam containers. Nipa bowl is locally named “Timha”. It is a product of the Palian river basin community in Trang province located in the south of Thailand (PODKUMNERD et al., 2019). This bowl is made from leaves of nipa palm (*Nypa fructicans* Wurm.), a tree that grows in the mangrove forest (CARANDANG et al., 2009; HOSSAIN et al., 2015; PODKUMNERD et al., 2019; RONALDO et al., 2014) as shown in figure 1-a. The nipa bowl is the unique traditional wisdom of the Palian river basin community. It is made from nipa leaves waste from the production of cigarette wrappers (PODKUMNERD et al., 2019). Figure 1-b shows the nipa bowl or “Timha”.

During the last few years, Timha products from nipa palm have gained significant interest from food retailers, shops, and restaurants in Thailand. They are used as a container for both foods and beverages. PODKUMNERD et al. (2019) reported the successful work on the production process development of Timha by using solar dryer cabinets. The use of solar dryer technology can increase the production of Timha by 3.5 times resulting in mass production of Timha product. In 2019, Timha was promoted as a green food container. This bowl has become famous, and about 170,000 pieces of Timha were sold in Thailand. In addition, community-based tourism (CBT),

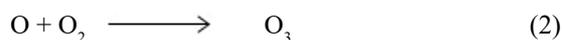
launched by the Tourism Authority of Thailand (TAT), become a national tourism strategy. The CBT has significantly impacted the local economy to benefit local communities directly and to responsibly promote Thailand’s unique regional culture and heritage (BLACKSTOCK, 2005). This strategy has enhanced the demand for Timha in the food markets.

Although, the Palian river basin community produced a massive amount of Timha product, there is still a challenge in facing contamination of foodborne pathogens. The contamination of bacteria is the bottleneck of Timha production. In each production cycle, many Timha products were contaminated with bacteria, yeasts, or molds, leading to the loss of confidence of the customers. The contamination of microorganisms on food container surfaces also affects food quality, shelf-life, and safety (BAE et al., 2012). Several works in literature have reported that various foodborne pathogens such as *Escherichia coli* and *Listeria monocytogenes* can survive on utensils and equipment surfaces for hours or days (KUSUMANINGRUM et al., 2003; MARTINON et al., 2012; WILKS et al., 2006). Cross-contamination of foodborne pathogens is a significant concern since it increases the health risk for humans due to the intake of contaminated food. Thus, as similar to other food containers, the Nipa bowl must be investigated for microorganism contamination. A process to prevent the contamination of these foodborne pathogens is needed during the production process.



One of the technologies to solve the abovementioned problem is ozone treatment.

Ozone ( $O_3$ ) is a colorless gas with a strong irritating smell, and it is a potent oxidant and reactive to biomolecules. Nowadays, ozone is widely accepted and used in areas such as wastewater treatment, food processing, and various environmental applications (NAITOU & TAKAHARA, 2008; NATH et al., 2014; PRABHA et al., 2015). Several researches reported that ozone can destroy pests, fungi, bacteria, and spores of microbes (MOHAMMAD et al., 2019; SHARMA et al., 2008; SUBRAMANYAM et al., 2017; XINYI et al. 2017). Ozone is originated from the incorporation of three oxygen atoms (triatomic oxygen) which are caused by oxygen molecules ( $O_2$ ) and broken down into oxygen atoms (O) as illustrated in equations 1 and 2.



The most common method used to produce ozone is the corona discharge method where oxygen molecules ( $O_2$ ) pass through the strait between two high-voltage electrodes. Some oxygen molecules break down into oxygen atoms (O) and free radical oxygen by colliding with electrons. The oxygen atoms then combine with the oxygen molecules ( $O_2$ ) into ozone

( $O_3$ ). It has been reported that this Corona discharge method is effective in removing microbes and contaminants such as corn microbes, carrot pesticides, and insect pests in well-preserved products (ISIKBER & ATHANASSIOU, 2015; KARACA & VELIOGLU, 2007, WHITE, 2007). Ozone can exert its biological action by oxidative destruction of biomolecules by two main mechanisms. First is the oxidation of polyunsaturated fatty acids to acid peroxides. The later mechanism is the oxidation of sulfhydryl groups and amino acids of enzymes, proteins, and peptides (VICTORIN, 1992; BOCCI, 2010). Thus, ozone has well-documented bactericidal properties in the decontamination of various products (ISIKBER & ATHANASSIOU, 2015; WHITE, 2007).

In this research, an ozone treatment system is used to reduce microorganism contamination in Timha products. The optimization of parameters that affected the performance of the system was investigated and reported.

## MATERIALS AND METHODS

### *Ozone treatment system*

The design of the ozone treatment system is shown in figure 2. The ozone treatment system was designed as a cabinet and hence called an ozone treatment cabinet (OTC). The OTC has a square

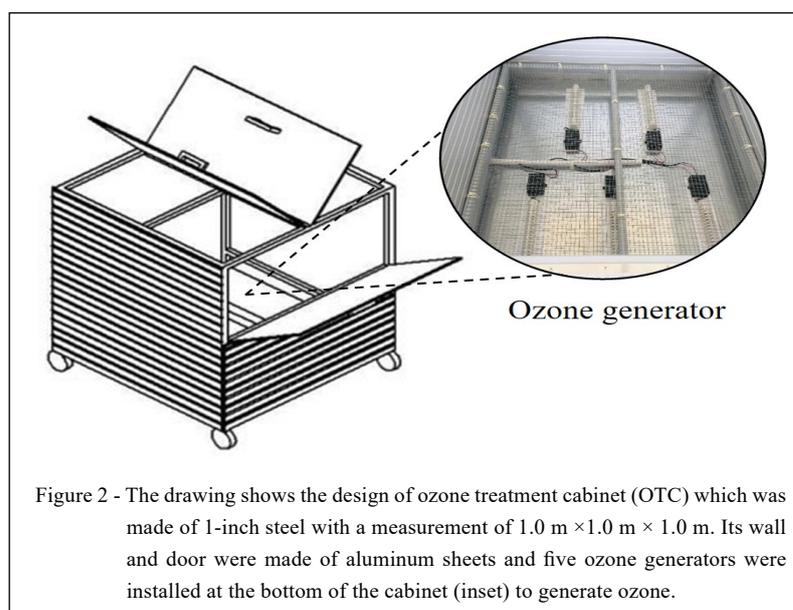


Figure 2 - The drawing shows the design of ozone treatment cabinet (OTC) which was made of 1-inch steel with a measurement of 1.0 m × 1.0 m × 1.0 m. Its wall and door were made of aluminum sheets and five ozone generators were installed at the bottom of the cabinet (inset) to generate ozone.

shape with a dimension of 1.0 m × 1.0 m × 1.0 m. The structure was made of 1-inch steel. The wall and door were made of aluminum sheets. The sieves were equipped inside the cabinet for placing the products, and 5 ozone generators (150 mg/h, Armorn electronic, Thailand) were installed at the bottom, as shown in the inset of figure 2. The ozone generator control system was installed outside the cabinet and used to adjust the amount of ozone.

#### *Optimization of ozone amount and treatment time*

To evaluate the efficacy of ozone treatment for reducing bacterial contamination, the amount of ozone and treatment times were investigated. Timha products obtained from the Palian river basin community basketry group were sampled using a complete random design (CRD) method. The amounts of ozone were varied at 150, 300, 450, 600, and 750 mg/h. The treatment time was investigated at 1, 2, 3, 4, and 5 h. The ozone-treated Timha products were analyzed for total bacterial, yeast, and mold by using Plate Count Agar (PCA) and Rose Bengal Chloramphenicol Agar (RBCA) methods, respectively (AOAC, 2005). Five Timha products were randomly selected and utilized as samples. For each sample, the product was swabbed with cotton bud. The swabbed sample was then immersed in 0.1 % w/v NaCl and then diluted to 5 dilutions ( $10^{-1}$ ,  $10^{-2}$ ,  $10^{-3}$ ,  $10^{-4}$  and  $10^{-5}$ ). Inoculums were spread evenly over the surface of agar plate. The plates were incubated at 35 °C for 48 h. After incubation, the number of colonies was counted on the plate with 25-250 colonies. The amount of bacteria was reported as log CFU/piece taking dilution factors into account.

#### *Storage time of ozone-treated Timha product*

To investigate the storage time of Timha products after ozone treatment, the treated products at optimum condition were kept in a polyethylene bag at a room temperature (28 °C) for one month and then analyzed for total bacterial, yeast, and mold.

#### *Physical characteristics of Timha products after ozone treatment*

The moisture was tested with a moisture analyzer (Sartorius MA150; Germany), and the color was analyzed by using Hunter Colorimeter (Konica; Japan). The untreated Timha products were also tested for comparison.

#### *Heavy metal contamination in Timha products*

Heavy metals are among the most critical sorts of contaminants in the environment. This metal

can be uptaken by plants and can be contaminated in a specific part of plants like leaf. Thus, to ensure that heavy metals were not residual in the leaf of Nipa palm, three heavy metals, namely, Cadmium (Cd), Lead (Pb), and Arsenic (As) in nipa palm leaf were investigated by using inductively-coupled plasma optical emission spectrometry (ICP-OES). This experiment was performed at Central Equipment Division, Faculty of Science, Prince of Songkla University, Songkhla, Thailand.

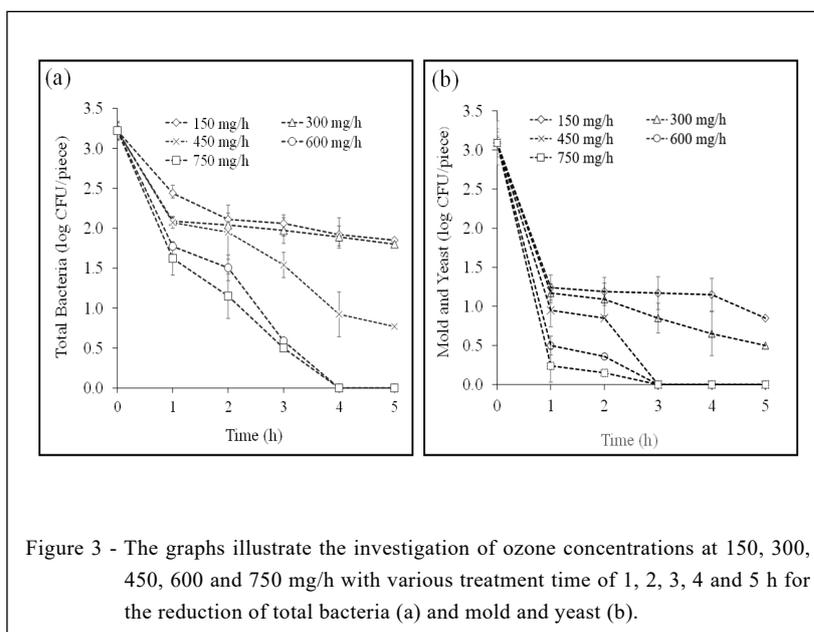
#### *Technology transfer and evaluation*

The OTC technology was transferred to the nearby communities for creating networks in Timha production. The satisfaction of technology was evaluated by using questionnaires.

## RESULTS AND DISCUSSION

#### *Optimization of ozone amount and treatment time*

The efficacy of ozone treatment for microbial decontamination in Timha products was investigated. The ozone amounts were tested at 150, 300, 450, 600, and 750 mg/h, and the treatment time was varied at 1, 2, 3, 4, and 5 h. Figure 3 (a) shows the number of total bacteria in Timha products after ozone treatment. As shown in figure 3 (a), at the start, the total bacteria of the Timha product were  $3.2 \pm 0.04$  log CFU/piece. When treated with ozone, the total bacteria were decreased. Ozone can destroy bacteria via the damage and deformity of the bacteria's surface structure. The whole body of ozone-treated bacteria collapsed and shrank due to bacteria cell lysis and bacteria death (SUBRAMANYAM et al., 2017). After ozone exposure, the numbers of bacteria decreased in a time-independent manner. For 150 and 300 mg/h ozone. The total bacteria were rapidly decreased after treated with ozone for 2 h and then gradually decreased. At 5 h, the total bacteria concentrations were  $1.9 \pm 0.21$  and  $1.8 \pm 0.14$  log CFU/piece for 150 and 300 mg/h ozone, respectively. When the ozone concentration was further increased to 450 mg/h, the total bacteria was further decreased. The total bacteria concentration was  $0.8 \pm 0.28$  log CFU/piece after 5 h treatment. The most effective concentrations of ozone were 600 and 750 mg/h. As shown in figure 3 (a), When treating with 600 and 750 mg/h ozone, the total bacteria decreased to more than 0.3 log reduction after 2 h. At 4 hours, the amount of bacteria was less than the limit of detection of the method which indicated that in such concentrations of ozone, the bacteria can be efficiently reduced. Thus, the treatment conditions using 600 and 750 mg/h of



ozone at 4 h were selected to investigate the storage ability of Timha products.

For yeast and mold, the use of ozone at every amount could reduce yeast and mold by more than 50 % at a treatment time of 1 h (Figure 3 (b)). However, at 150 and 300 mg/h and 5 h, the amount of yeast and mold still detectable ( $0.9 \pm 0.21$  and  $0.5 \pm 0.3$  log CFU/piece). Reduction of yeast and mold contamination was efficiency obtained after an ozone treatment for 3 h with ozone amount of 450, 600, and 750 mg/h. These concentrations were then further investigated for the storage ability of Timha product.

#### Storage time of ozone-treated Timha product

To investigate the storage ability of Timha products after ozone treatment, Timha products were kept for one month in a polyethylene bag and then analyzed for total bacteria, yeast, and mold. Results are shown in figure 4. For the Timha products treated with 600 mg/h ozone and kept for one month as shown in figure 4 (a), the total bacteria were  $2.2 \pm 0.07$ ,  $2.1 \pm 0.08$  and  $1.2 \pm 0.09$  log CFU/piece, respectively for the products treated with ozone for 1, 2 and 3 h. At 4 and 5 h, the total bacteria was  $< 1.2$  log CFU/piece for a 1:10 dilution. Similar results were observed for Timha products treated with 750 mg/h ozone as illustrated in figure 4 (b). These results indicated that the use of ozone at

600 and 750 mg/h with the treatment time at least 4 hours were effectively decontaminated the bacteria, and the product could be stored for up to one month. Figure 4 (c) and (d) show the results of yeast and mold investigation of Timha products treated with ozone 600 and 750 mg/h from 1-5 h. As expected, the amount of yeast and mold was increased when treated with ozone for 1-3 h, indicating that 3 h treatment was insufficient. Yeast and mold can grow during the storage period. The effective treatment times were at 4 and 5 h. As shown in figure 4 (c) and (d), the yeast and mold were lower than the limit of detection in the products treated with ozone at 600 and 750 mg/h under a treatment time of 4 and 5 h.

When taking account of the storage ability, the bacteria, yeast, and mold must be reduced to the lowest level. Otherwise, the microorganism will be able to multiply during the storage period. Experimental results also showed that ozone is very effective in inhibiting the growth of microbes. The mechanism of ozone occurs when ozone molecules interact directly with the proteins that encapsulate cells and nourish the microbes, which prevent microbes from continuing to grow. Thus, from experiment results in 3.1 and 3.2, the treatment of ozone at 600 mg/h and treatment time for 4 hours was selected as the optimum condition for ozone treatment of Timha products.

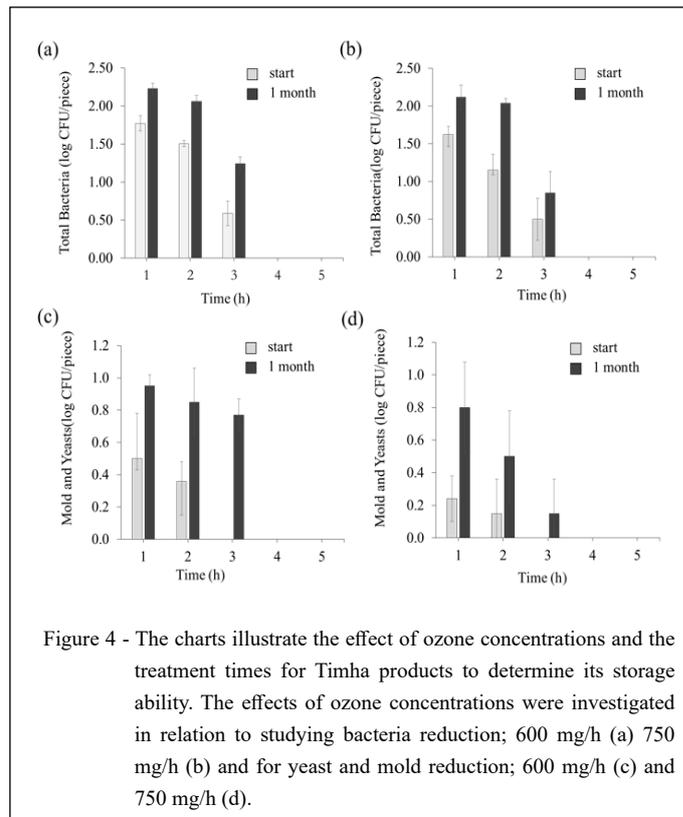


Figure 4 - The charts illustrate the effect of ozone concentrations and the treatment times for Timha products to determine its storage ability. The effects of ozone concentrations were investigated in relation to studying bacteria reduction; 600 mg/h (a) 750 mg/h (b) and for yeast and mold reduction; 600 mg/h (c) and 750 mg/h (d).

The use of ozone for the treatment of Timha products indicated that ozone could effectively reduce the contamination of microorganisms in Timha products. The microbial contamination can be decreased to the standards level set by the Ministry of Medical Sciences, Thailand which regulated that food containers must not be detected of microbial contamination over 3 log CFU/piece (Department of Medical Sciences Ministry of Public Health, 2017). The finding in this research is also consistent with other reports (MOHAMMAD et al., 2009).

#### *Physical characteristics of Timha products after ozone treatment*

Timha products treated with ozone at 600 mg/h for 4 h were investigated for their physical characteristics such as moisture and color. Ideally, the use of ozone should reduce microbial contamination without affecting the physical properties of the Timha product. Table 1 shows the results of the moisture and color value of ozone-treated and controlled

samples. The moisture of the ozone-treated Timha products was  $9.46 \pm 0.10$  % which is a significant difference at the 95% confidence level to the control ( $10.54 \pm 0.31$  %). The lightness ( $L^*$ ) of the ozone-treated samples was significantly different from the controlled samples when the results were statistically tested at 95% confidence.

The  $L^*$  value of ozone-treated and controlled samples were  $74.93 \pm 0.49$  and  $70.47 \pm 0.65$ , respectively. The treated samples show a higher  $L^*$  value indicating the brightening color by ozone treatment.

#### *Heavy metal contamination in Timha product*

Heavy metals are toxic substances. Food container products must be free of heavy metal contamination. For nipa palm, there is a report that this plant can adsorb some heavy metals. The heavy metals can be accumulated in a specific part of the plant. In this research, the Timha product was made from nipa leaves. Thus it is necessary to investigate

Table 1 - Characteristics of ozone-treated nipa palm leaves in the amount of 600 mg/h for 4 hours.

Samples	Properties	
	Moisture (%)	Lightness
ozone treated	9.46 ± 0.10 <sup>a</sup>	74.93 ± 0.49 <sup>a</sup>
control	10.54 ± 0.31 <sup>b</sup>	70.47 ± 0.65 <sup>b</sup>

\*Different characters in each column illustrated average comparison by method of Duncan's multiple range tests at 95% confidence interval, respectively.

the residual of heavy metals in Timha product, namely, Cadmium (Cd), Lead (Pb), and Arsenic (As). The concentrations of Cd, Pb and As in all investigated samples were < 0.003, < 0.003 and < 0.005 ppm. These are less than the limit of detection (LOD) of the analytical method. Results indicated that there was no contamination of these heavy metals. Therefore, Timha product proposed is considered to be safe and can be used as containers for beverages and foods which can make consumers confident in utilizing the products. After launching the products in the markets, Thima has gained significant interest from buyers/consumers which was proven by the numbers of product orders. The product was used for different purpose i.e. container for foods, beverages, ice cream, etc., as shown in figure 5.

#### *Technology transfer and evaluation*

The OTC technology was transferred and implemented for the "Ban Na Yod Tong Nipa palm leaf handicraft group" of Palian river basin community, Trang Province, Thailand, in June 2020. From the technology evaluation, the result shows that the handicraft group can use OTC technology to enhance their production, as shown in figure 6. Every Timha bowl was processed with OTC to reduce bacteria contamination before being packed and delivered to the customers. At present, all Timha products preserved with ozone are guaranteed for their quality. This results in customer confidence. The production amount of Timha can be seen in figure 7.

The highest production of Timha in 2018 was 27,000 pieces per month (PODKUMNERD et al.,

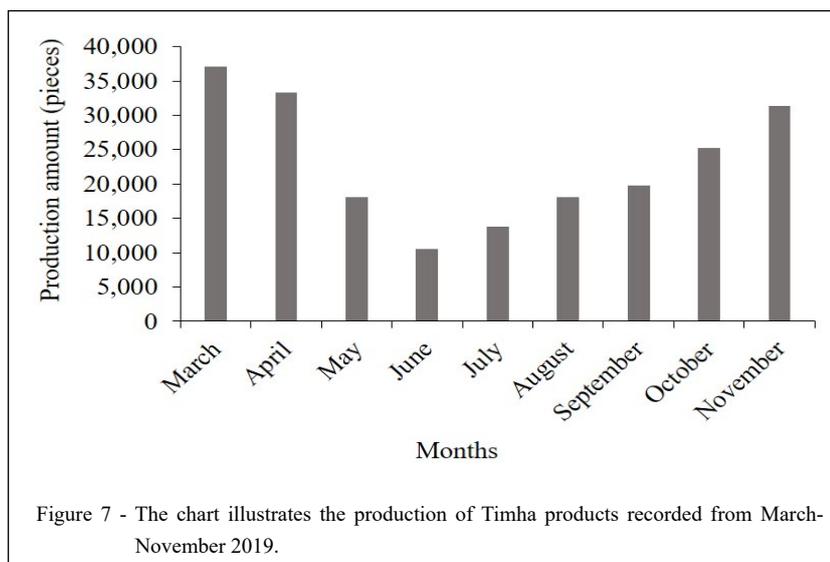


Figure 5 - Application of Timha product as container for food (a), beverage (b) and ice cream (c).



2019). After the OTC technology was implemented in March 2019, the production increased to 37,143 pieces which is 14 % increase compared to the same period. In April, the production declined to 33,320 pieces since the demand for the product decreased due to the low season of the tourism business. The order of Timha was declined continuously to June. However, during the high season of tourist business, the demand for Timha was again increased. A continuously increasing of order was recorded from June-December. The continuous increase in Timha sales is due to the product’s high quality, which

has received confidence from the customers. The markets accept the product for its uniqueness and environmentally friendly. There is also a demand for this product from other businesses, including touring places, hotels and resorts, spa and restaurants from every country’s regions. Timha product has been sold to more than 66 provinces from every part of Thailand, as shown in figure 8. Over 251,564 pieces of Timha have been sold. The average production per month is 25,156 pieces which is a 37.5 % increased. This can generate an income to the group of more than 84,000 USD.





The OTC technology was also transferred to the nearby communities to create networks of Timha production such as Yan Sue, Toh Muang, Bang Mhark, Southern Kan Tang, and Ta Praya communities. The network communities were trained to use the technology and produced high-quality and unique Timha products. One of the most successful networks is the group of persons deprived of liberty in Trang Province. This career training can be used for their living after they were received liberty. This strategy will reduce reoffending and, in turn, make safer communities.

Moreover, the technology was also applied to the nipa leaves suppliers who sell the leaves for the handicraft group. The OTC was modified so it can be used to treat Nipa leaves which is a raw material for making the bowl. As a result, the ozone-treated Nipa leaves are free of bacteria, yeast, and mold. Thus, the leaves can be kept in sterilized conditions for a long time. Thus, the production of Timha can be done continuously. The villagers can sell about 170,000 pieces of Nipa leaves to the Timha production group. This could generate extra income for the villagers in the community. Since the technology was transferred to the villagers, each family was able to profit 100

USD/month as an extra income. The use of the technology from this research successfully increased the quality of life of the community. Timha has become a famous product of the Palian river basin community. The traditional way of making Timha is a local wisdom that has attracted significant interest from tourists. A learning center of Timha production was found at Ban Na Yod Tong Nipa palm leaf handicraft. This learning center is a place to pass the value of traditional wisdom from the older to the younger generation.

#### *Timha production and sustainable development*

The production of Timha with OTC can be evaluated for their sustainability from different points of view. Firstly, the technology can preserve traditional wisdom and can be successfully transferred from generation to generation. The production of Timha was long before not so interesting to the young generation since they cannot see its importance. Nowadays, young people can see that the traditional wisdom with the integration of technology can create a unique and saleable product which can create a massive income. Thus, Timha production is now becoming a famous work in the community. Secondly, the production of

Timha products can reduce a massive waste of Nipa leaves from cigarette wrapping production. Upon computation, approximately 1.83 tons of Nipa leaf was reduced. Since Timha can create an income and this can be used as a career for the living, the awareness of Nipa palm value has also increased. Thus, the community has a long-term plan to use Nipa palm. Nipa palm is planted in the community, and this is expected to be used sustainably. In an environmental point of view, Timha is degradable and environment- friendly. Once the bowls were used for food and beverage containers, they can be used for other purposes such as a bio-degradable pot for planting which can be used as a souvenir (Figure 9).

Finally, the production of Timha in the community can connect/unite together people of different ages. Wayback in the early times, Timha production was only made by old-aged people, but young people are now interested in this business. Here we reported that the research successfully reduces the generation gap of old and young people, especially in the family. The production of Timha now becomes a family business run by people of different ages who can use their skills and knowledge to fulfill each other. Moreover, the result from this research is beneficial for aging people. Thailand has begun the challenging process of meeting the needs of an aging society. Here the aging people can be part of

the business. They can meaningfully share their skills and values with younger generations. As a result, they can work and can generate income to support themselves financially. Therefore, they are satisfied with their financial status since they can work and earn a good income.

## CONCLUSION

Here we described the development of an ozone treatment system to reduce microbial contamination in Timha product. The use of ozone 600 mg/h under a treatment time of 4 hours can effectively reduce microbial contamination. The ozone-treated Timha products could be stored for up to one month. The investigation of heavy metals showed no contamination of Cd, Pb, and As in products. The use of ozone could reduce the humidity and create a lighter color. As a result, the quality of Timha products has met the standard requirement of food containers. This product has now been spread in all areas in Thailand, e.g., in “green market”, restaurants, and hotels. The markets of the product expands from “local” to “global”. Moreover, the ozone treatment system can be used to preserve raw materials for Timha production. Thus, it is possible to produce Timha products continuously and can fulfill the requirement of the market. Since the technology



Figure 9 - Nipa bowl is an environment-friendly product. It can be reused as a planting pot after its utilization as food or beverages containers.

was implemented in the community, about 0.25 million pieces of Timha were sold, and this could generate income to the community of approximately 84,000 USD. The production of Timha product can contribute to the preservation of traditional wisdom and to its successfully transfer from generation to generation. It is also an environment-friendly product that can replace plastic or foam. Moreover, it is beneficial from a social point of view, especially for aging adults.

## ACKNOWLEDGEMENTS

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## DECLARATION OF CONFLICT OF INTEREST

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; and in the decision to publish the results.

## AUTHORS' CONTRIBUTIONS

All the authors contributed equally for the conception and writing of the manuscript. All the authors critically revised the manuscript and approved of the final version.

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